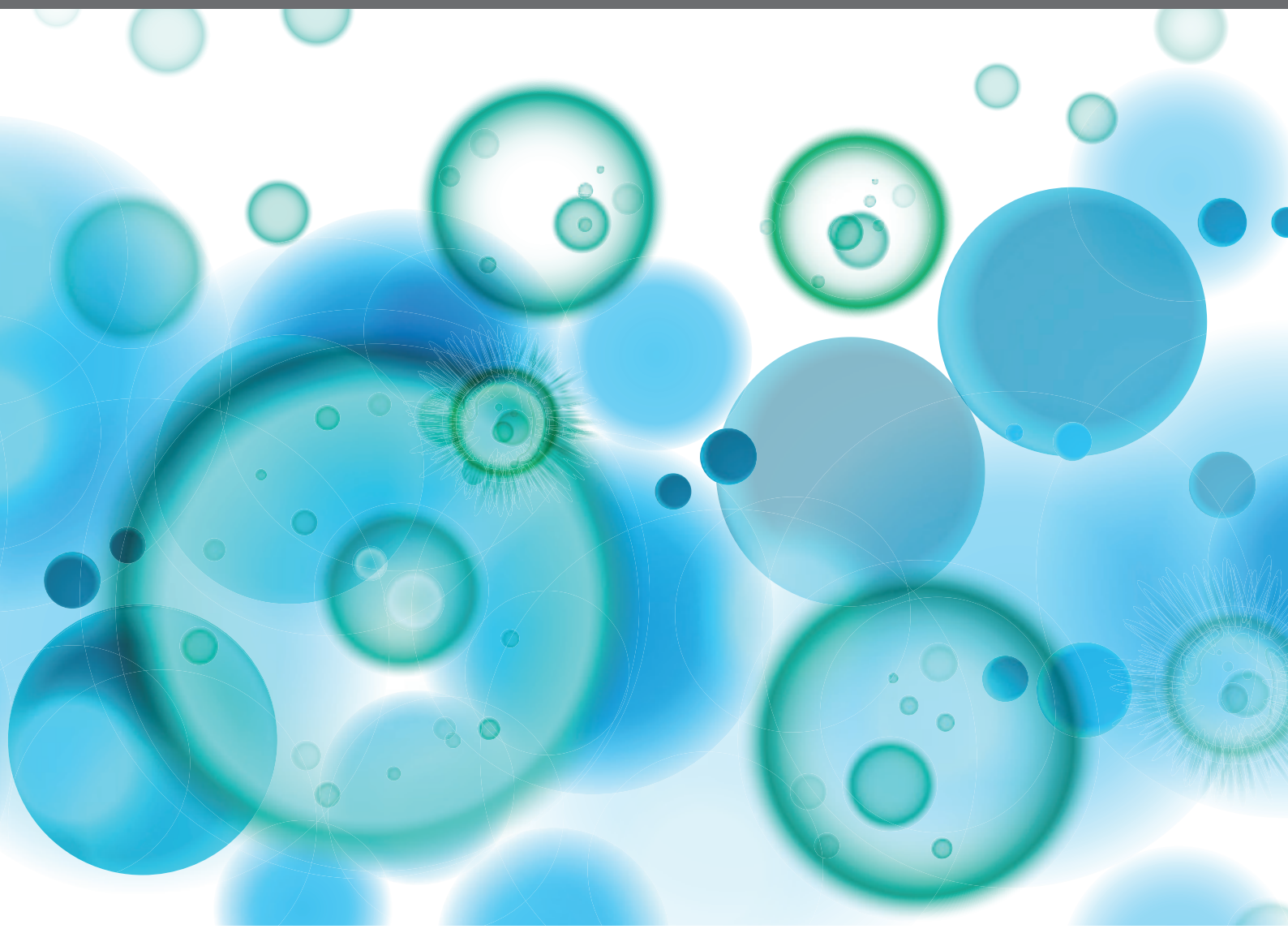
NUCLEIC ACID-ASSOCIATED INFLAMMATION

EDITED BY: Nadine Laguette, Christelle Langevin, David Olagnier, Vincenzo Torraca, Claire Isabelle Vanpouille-Box and Eloi R. Verrier

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frontiers Research Topics

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NUCLEIC ACID-ASSOCIATED INFLAMMATION

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EDITORIAL
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Editorial: Nucleic Acid-Associated Inflammation

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Editorial on the Research Topic

Nucleic Acid-Associated Inflammation

Inflammatory responses are essential to maintain organismal homeostasis and are typically induced when autonomous defenses are impaired. For instance, inflammation can be engaged in response to tissue damage or exposure to a variety of agents, including pathogenic microorganisms such as viruses and bacteria as well as toxins and xenobiotics. Sensing of inflammatory agents largely relies on the recognition of pathogen-associated molecular patterns (PAMPs), including microbe-associated molecular patterns (MAMPs), through pattern recognition receptors (PRRs) (1). Endogenous factors that are present in an aberrant location (*e.g.* cytoplasmic nucleic acid or extracellular ATP), and/or in a disproportionate abundance, can also activate PRRs and are commonly known as damage-associated molecular patterns (DAMPs) (2). Upon PAMPs or DAMPs recognition, PRRs coordinate an early response of the host to endogenous or exogenous threats that prime the host for the activation of adaptive immunity (Okude et al.) (3).

Given the central role of PRRs in the control of invading pathogens and endogenous threats, it is not surprising that genetic or etiological alterations of inflammation foster a wide range of human pathologies. Underscoring this concept, the persistent dysregulation of nucleic acid-associated inflammatory pathways has been associated with the development of chronic liver diseases. The two main etiology agents that are linked with these liver pathologies are hepatitis B virus (HBV) and hepatitis virus C (HPC). These viruses have distinct genomes and viral life cycles but can both repress innate anti-viral defenses through common mechanisms. These strategies are being discussed in our Research Topic "*Nucleic Acid-Associated Inflammation*" by Roca Suarez et al., as well as Xu et al.

Innate immunity largely relies on the recognition of evolutionarily conserved structures that can be identified *via* comparative analyses of innate immune responses, from biological models (zebrafish and mouse) to human. This underscores the existence of tissue- and species- specificities, and is discussed in six reviews of the Research Topic which cover various aspects, ranging from the role of transposable elements to the limitations of *in vivo* models and provides cues towards the development of high content therapeutic strategies in relevant physiological models (Jacquet et al., Jami et al., Magnani et al., Rutherford et al., Sullivan et al., Verrier et al.).

However, despite major advances in the field of innate immunity to identify the pathways involved in the onset of cytokine production in response to immune-stimulatory nucleic acids, there are still many

open questions. Specifically, how these signalling pathways are regulated in respect to various nucleic acid substrates and tissue insults. In this special Research Topic, key opinion leaders in the field offer an overview of (1) the major molecular and cellular aspects of nucleic acid sensing across species; (2) the complexity of innate and adaptive immune responses and their key role in the maintenance of tissue homeostasis; and (3) the intricate connections between deregulated nucleic acid sensing machinery and human disease. For instance, reviews by Santa et al. and by Kumar give a comprehensive overview of the regulatory circuitries of nucleic acid-sensing pathways.

Even though nucleic acid-associated inflammation is the first line of defense of the host, activation of innate immunity is not always guaranteed. Indeed, microbes and malignant cells have developed a variety of strategies to prevent inflammation, in order to counteract the host response or escape the induction of anti-tumor immunity (4). Supporting this concept, evidence demonstrates that nucleic acid sensing is critical to generate radiation-induced anti-tumor immunity (5). Such responses result from the recognition of mitochondrial DNA in irradiated tumor cells by the cyclic GMP-AMP synthase (cGAS) sensor of cytosolic DNA (6). Since then, efforts in uncovering the adjuvanticity of radiation therapy from multiple types of ionizing radiations have pointed towards the role of DNA damage response and cytosolic DNA detection in priming anti-tumor responses. Some of the current views of how genotoxic stress crosstalk with innate immune responses and anti-tumoral immunity are summarized in reviews by Constanzo et al. and by Taffoni et al.

Regulating abnormal nucleic acid sensing is emerging as a potent strategy against inflammatory diseases. Thus, PRRs and their downstream effectors have become attractive targets for the identification of biomarkers and the development of therapeutic agents with broad-range efficacy against inflammatory disorders. Review by Hemphill et al. from this Research Topic is discussing the therapeutic potential of the three-prime repair exonuclease 1 (TREX1) targeting as a novel immunotherapy strategy against cancer.

In conclusion, each one of the reviews and articles presented in *Nucleic Acid-Associated Inflammation* focuses on one specific aspect of nucleic acid sensing, encompassing signaling, regulation, interspecies specificities, and pathology relevance. The Research Topic is equally addressed to expert investigators who may wish to extend their knowledge on inflammation, innate and nucleic acid immunity, and to newcomers to this exciting and quickly progressing field of investigation.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Rapid Evolution of HERC6 and Duplication of a Chimeric HERC5/6 Gene in Rodents and Bats Suggest an Overlooked Role of HERCs in Mammalian Immunity

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Studying the evolutionary diversification of mammalian antiviral defenses is of main importance to better understand our innate immune repertoire. The small HERC proteins are part of a multigene family, including HERC5 and HERC6, which have probably diversified through complex evolutionary history in mammals. Here, we performed mammalian-wide phylogenetic and genomic analyses of HERC5 and HERC6, using 83 orthologous sequences from bats, rodents, primates, artiodactyls, and carnivores—the top five representative groups of mammalian evolution. We found that HERC5 has been under weak and differential positive selection in mammals, with only primate HERC5 showing evidences of pathogen-driven selection. In contrast, HERC6 has been under strong and recurrent adaptive evolution in mammals, suggesting past and widespread genetic arms-races with viral pathogens. Importantly, the rapid evolution of mammalian HERC6 spacer domain suggests that it might be a host-pathogen interface, targeting viral proteins and/or being the target of virus antagonists. Finally, we identified a HERC5/6 chimeric gene that arose from independent duplication in rodent and bat lineages and encodes for a conserved HERC5 N-terminal domain and divergent HERC6 spacer and HECT domains. This duplicated chimeric gene highlights adaptations that potentially contribute to rodent and bat immunity. Our findings open new research avenues on the functions of HERC6 and HERC5/6 in mammals, and on their implication in antiviral innate immunity.

Keywords: HERC, restriction factor, antiviral immunity, gene duplication, genetic conflicts, positive selection, HERC5, HERC6

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INTRODUCTION

As a result of sustained exposure to viral infections, mammals have evolved a sophisticated and diversified immune repertoire against viruses. A hallmark of mammalian antiviral immunity is the induction of type I interferon (IFN) upon viral infection. This cytokine upregulates the transcription of hundreds of interferon-stimulated genes (ISGs) in viral infected cells (1). Many of these ISGs encode proteins with antiviral functions, named restriction factors, which are critical players in the first line of the innate immune defense inhibiting different steps of the viral replication cycle (2).

Viruses have adapted to circumvent, subvert, or antagonize these host restriction factors (3). Reciprocally, restriction factors have rapidly and repeatedly evolved to maintain defenses against evolving viral pathogens, leading to virus-host evolutionary arms-races (3, 4). These dynamics of reciprocal adaptations can leave genetic signatures in the host restriction factors. Significant accumulations of non-synonymous changes over synonymous substitutions—designated as positive selection, as well as codon deletions or insertions that may alter the virus-host interface—are common genetic signatures of such long-term evolutionary arms-races (3–6). At the genomic level, gene duplication and recombination are among the most important mechanisms that can diversify the antiviral immune repertoire. In particular, gene duplication can generate adaptive molecular novelty allowing hosts to escape viral antagonism, evolve new immune functions, or increase the depth of antiviral response (3, 7, 8). The weight of such evolutionary mechanisms in mammalian immunity is highlighted by the extent of multigene families, which encode important ISG-encoded proteins, such as the Tripartite Motif-containing (TRIM) (9–12), Apolipoprotein B Editing Complex (APOBEC3) (13–15), Interferon-induced Protein with Tetratricopeptide Repeats (IFIT) (16–18), Interferon induced Transmembrane protein (IFITM) (19) families. For example, the *APOBEC*3 family has expanded in a lineage-specific manner in primates (20), artiodactyls (21), and bats (15), generating variability in mammalian antiviral response (14). However, the evolutionary and functional diversification of many antiviral families remains poorly characterized in mammals. Deciphering the evolutionary trajectories of multigene family members can provide insights into the genetic mechanisms underlying the diversification of antiviral responses and may allow identifying novel antiviral proteins.

The HECT and RLD domain containing E3-ubiquitin protein ligases, known as HERC proteins, are encoded by a multigene superfamily that is poorly studied in mammals. With six gene members, the HERC family is divided into two subfamilies, the large (*HERC1* and *HERC2*) and the small (*HERC3–6*) *HERCs* (22). The small HERC proteins are structurally characterized by a N-terminal RCC1-like domain (RLD), a spacer region, and a C-terminal HECT (Homologous E6-AP Carboxyl Terminus) ubiquitin E3-ligase domain, while the large HERCs possess at least two RLD domains in addition to a HECT domain (23). This structural difference between large and small HERCs reflects their independent evolutionary history (24). In the antiviral immune context, much attention has been devoted to the small HERCs, in particular to HERC5 - an ISG-encoded antiviral effector - and HERC6 its closest relative (24, 25). In humans, HERC5 acts as a HECT ubiquitin and E3-ligase (26–28). HERC5 notably conjugates the ubiquitin-like protein ISG15 to different protein targets, a process termed ISGylation (29–31). The protein targets may be non-specific newly synthetized viral proteins, specific viral proteins, or specific host proteins (29–31). ISGylated proteins are modified, functionally disrupted, or altered in their localization within the cells. Through this ISGylation activity, HERC5 has an antiviral function against highly divergent viruses, including retrovirus (Human and Simian immunodeficiency viruses, HIV and SIV), papillomavirus, and influenza virus (25, 31–33). For example, HERC5 targets the early stage of HIV assembly by catalyzing the ISGylation of the viral Gag protein (30), while it reduces influenza A viral replication through the conjugation of ISG15 to the viral NS1 protein (31). Besides, HERC5 appears to further interfere with HIV replication in an ISGylation-independent manner by impacting the nuclear export of Rev/RRE-dependent viral RNA, most likely through determinants in the RLD domain (33). In contrast, although HERC6 is the most closely-related protein of HERC5, little is known about its functional implication in mammalian antiviral immunity (25). The antiviral role of HERC6 has mainly been described in mouse, in which the *HERC5* gene has been lost and functionally substituted by HERC6, the main murine E3-ligase of ISG15 (28, 34, 35). In humans, although the HERC6 protein possesses a HECT E3-ligase domain, it is devoid of ISGylation function (25).

These evolutionary and functional differences between mammals suggest lineage-specific adaptive changes in *HERC5* and *HERC6*. Two previous studies showed that *HERC5* and *HERC6* genes have evolved under positive selection during vertebrate evolution (25, 33). They further showed that the RLD domain plays an important role in the antiviral activity of HERC5 and HERC6 proteins. While these studies have provided important insights into the diversification of *HERCs*, the evolutionary analyses have certain limitations: (i) the scarcity of species analyzed (only 12 species, *versus* 81 species with at least 10 species per order in this current study), (ii) the overrepresentation of primates compared to other mammalian species (seven primates, two to three carnivores, two artiodactyls, and one perissodactyl), (iii) the integration of highly divergent species, which may bias the genetic inferences by increasing the number of false positives (36). Moreover, a recent study in primates have shown differences in *HERC5* and *HERC6* selective pressures (37). Therefore, how *HERC5* and HERC6 genes have evolved within mammalian orders has not been fully deciphered. Nor is the evolutionary dynamic of *HERC5* and *HERC6* expansions and contractions across mammals.

Here, we decipher the evolutionary history of mammalian *HERC5* and *HERC6 via* mammalian-wide and lineage-specific phylogenetic and genomic analyses. We analyzed the orthologous sequences of *HERC5* and *HERC6* from bats, rodents, primates, artiodactyls, and carnivores the top five mammalian orders in terms of zoonotic viral diversity they host (38, 39). First, we show that *HERC6*—and to a much lesser extent *HERC5*—has been under strong positive selection.

Second, we stressed the HERC6 spacer region as a potential pathogen-mammal interface, targeting viral proteins and/or being the target of virus antagonists. Finally, we identified independent gene duplications through recombination between *HERC5* and *HERC6* in some bat and rodent lineages, which have led to the fixation of a chimeric *HERC5/6* gene in both mammalian orders. Taken together, our results suggest that HERC6 may be an important antiviral protein in mammals and identified a novel chimeric HERC member in bats and rodents that may contribute to unique antiviral responses in these species.

MATERIALS AND METHODS

Collection of Mammalian *HERC5* and *HERC6* Orthologous Sequences

Full-length *HERC5* and *HERC6* coding sequences were analyzed in bats, rodents, primates, artiodactyls, and carnivores. *HERC5* and *HERC6* coding sequences from each group were obtained using the Little Brown bat (*Myotis lucifigus*), mouse (*Mus musculus*), human (*Homo sapiens*), cow (*Bos taurus*), and dog (*Canis lupus familiaris*) Refseq proteins as queries, respectively, through tBLASTn searches of the "Nucleotide" database in GenBank (40, 41). The species and accession numbers are presented in **Supplementary Table 1**.

Characterizing the Evolution of *HERC5* and *HERC6* Synteny in Mammals

The genomic locus of *HERC5* and *HERC6* genes in Little Brown bat, mouse, human, dog, and cow were obtained from Ensembl (http://www.ensembl.org/index.html), and GenBank Refseq genome database (41). Their coding sequences were used as queries for BLASTn searches against a total of 110 whole genome assemblies from bats, rodents, primates, carnivores, and artiodactyls (**Supplementary Table 1**) available in GenBank database (41). We analyzed eight additional mammalian genomes from Proboscidea, Lagomorpha, Scandentia, Perissodactyla, Sirenia, Soricomorpha, Eulipotyphla, and Tubulidentata orders (*Orycteropus afer*, *Loxodonta Africana*, *Trichechus manatus*, *Tupaia chinensis*, *Condylura cristata*, *Ceratotherium simum*, *Equus przewalskii*, and *Sorex araneus*, respectively) (**Supplementary Table 1**). The synteny of *HERC5* and *HERC6* genes was analyzed and visualized through BLAST searches against the 110 annotated genomes in GenBank database (41). Newly identified HERC-like paralogs (see *Results*) were confirmed by blasting and aligning their whole sequence (intron and exon regions) to the genomic region containing *HERC5* and *HERC6* genes in three bat species (*Myotis lucifigus*, *Rhinolophus ferrumequinum*, and *Phyllostomus discolor*), and three rodent species (*Chinchilla lanigera*, *Mastomys coucha*, *Cavia porcellus*), which are representative genomes with good assembling quality (based on the N50, the number of scaffolds, and sequencing coverage).

HERC5 and *HERC6* orthologs as well as *HERC-like* paralogous sequences were aligned for each mammalian order separately using the program MACSE (42), and the alignments were manually curated. A phylogenetic tree was then built for each gene and mammalian order, and for a combined dataset of *HERC5* and *HERC6* genes (rooted with *HERC3* as an outgroup, which is the most closely related gene to *HERC5* and *HERC6*), using the maximum likelihood method implemented in the ATGC-PhyML Web server (43). Each phylogenetic tree was based on the best substitution model (GTR+G+I), as determined by the Smart Model Selection (SMS) program in PhyML (44) and node statistical support was computed through 1,000 bootstrap replicates.

Assessing Recombination Events in *HERC5* and *HERC6* Paralogs and Orthologs

To test whether recombination has occurred in *HERC5*, *HERC6*, and *HERC-like* genes, we ran the GARD (Genetic Algorithm for Recombination Detection) method (45) implemented in the HyPhy package (46, 47), using a general discrete site-to-site rate variation with three rate classes. The program uses a genetic algorithm to screen multiple-sequence alignment for putative recombination breakpoints and provides the probability of support for each breakpoint. GARD analyses were run for each mammalian order and each gene separately, including the newly identified HERC-like paralog.

Positive Selection Analyses of *HERC5* and *HERC6* Coding Sequences in Mammals

To determine whether *HERC5* and *HERC6* have been targets of natural selection during mammalian evolution, we carried out positive selection analyses on orthologous coding sequences from bats (n = 10 and 13, respectively), rodents (n = 11 and 16), primates (n = 19 and 20), carnivores (n = 20 and 23), and artiodactyls (n = 21 and 11). As combining highly divergent sequences for positive selection analyses can lead to misleading results, we performed positive selection analyses on separate dataset for each mammalian order. For Artiodactyls, three different datasets were analyzed: the first dataset included all the available species, the second was restricted to cetacean species, and the third excluded the cetaceans. Analyzing each mammalian order separately allowed us to qualitatively compare the evolutionary profile of both genes in each mammalian order. We first tested for positive selection at the gene level using two different methods available in the Codeml program, which is implemented in the PAML package (48). This program allows both gene- and site-specific detection of positive selection by comparing constrained models that disallow positive selection (models M1 and M7) to unconstrained models allowing for positive selection (M2 and M8). We ran the different models with the codon frequencies of F61 and F3x4 with a starting omega ω (*dN*/*dS* ratio) of 0.4. Likelihood ratio tests were computed to compare the models (M1 *vs* M2 and M7 *vs* M8), and codons evolving under significant positive selection (*dN*/*dS* >1) were identified using the Bayes Empirical Bayes (BEB) with a posterior probability ≥0.95. The residues under positive selection were further assessed using two other methods, the Fast, Unconstrained

Bayesian AppRoximation for Inferring Selection (FUBAR) (49) and the Mixed Effects Model of Evolution (MEME) (50), both implemented in the HYPHY package. To increase the specificity of our results, we only kept the sites that were identified as being under significant positive selection by at least two of the four methods used. When significant recombination breakpoints were detected, positive selection analyses were carried out for each fragment identified by GARD. Similarly, we sought for signatures of adaptive selection in the *HERC*-like paralogs of rodents (see *Results*), for which five orthologous coding sequences were available. Furthermore, we tested if the three HERC domains (RLD, spacer region, and HECT) have similarly been subjects of positive selection, by analyzing each domain separately.

Finally, to determine if *HERC5* and *HERC6* have experienced episodic selection within mammalian orders, we carried out the branch-specific analysis aBSREL (51, 52), implemented in the HYPHY package. This program allows testing the significance of positive selection and quantifying the *dN*/*dS* ratio for each branch independently.

RESULTS

Lineage-Specific Changes in *HERC5* and *HERC6* Copy Number

To determine the genomic evolutionary history of *HERC5* and *HERC6* in mammals, we performed a complete synteny analysis for 14 chiropteran, 25 rodent, 25 primate, 23 artiodactyl, and 23 carnivore species, and eight more species from different orders. We first found that *HERC5* and *HERC6* synteny is mostly conserved throughout eutherian mammals (**Figure 1**, **Supplementary Figure 1**).

However, we also detected that gene erosion has repeatedly shaped the evolution of *HERC5* and *HERC6* in mammals. While primate and carnivore genomes encode both genes, the artiodactyls and the rodents have experienced gene loss or pseudogenization, generating *HERC* gene copy number variation in each group. Indeed, although all the studied artiodactyl species encode an intact *HERC5* gene, the cetacean genomes showed *HERC6* pseudogenization, through nucleotide deletions that impacted frameshift, as well as early stop codons

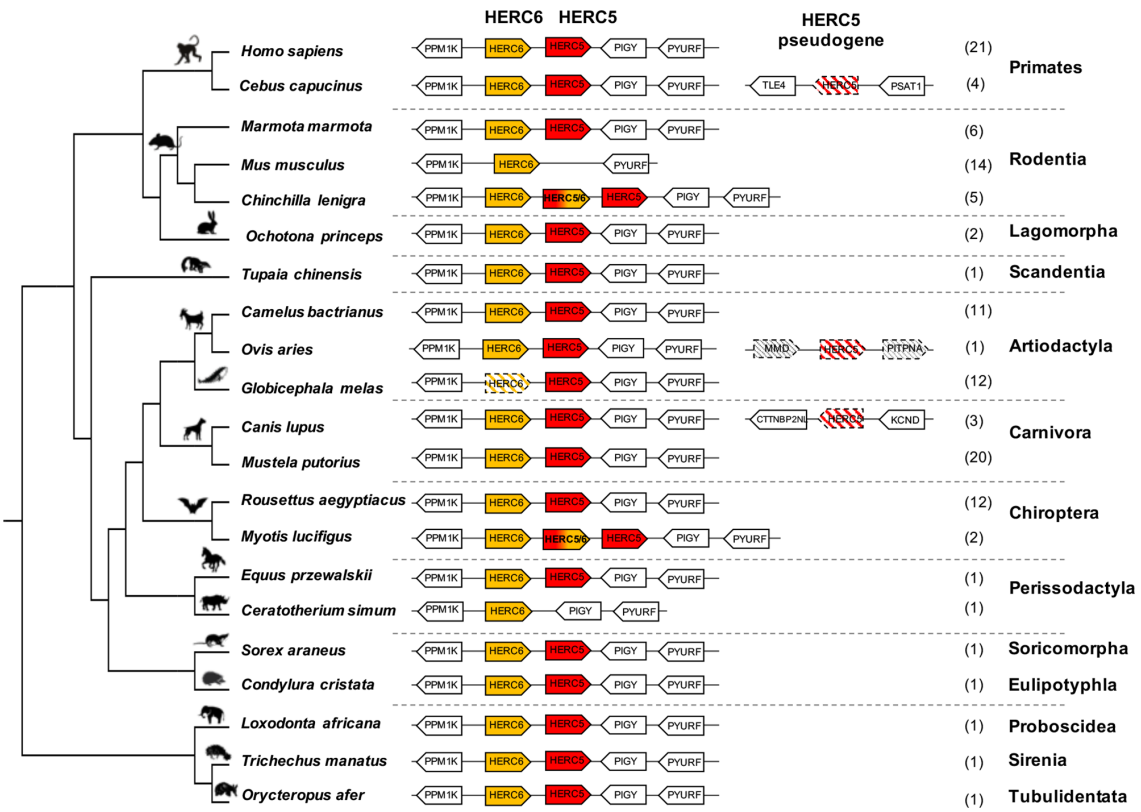


FIGURE 1 | Evolutionary dynamics of mammalian *HERC5* and *HERC6* gene loci. Representation of the *HERC5* and *HERC6* gene loci from mammalian genomes. Plain colored arrows represent intact *HERC5* and *HERC6* genes, striped colored arrows indicate *HERC5* or *HERC6* pseudogenes, and white arrows are adjacent syntenic genes. The newly identified chimeric *HERC5/6* genes are bicolored. The numbers in brackets indicate the total number of genomes analyzed which contain the corresponding genomic organization in each mammalian order. In primates and carnivores, the *HERC5* and *HERC6* genes are well conserved. In the cetacean and rhinoceros' species, the *HERC6* has been pseudogenized and lost, respectively, while rodent *HERC5* has been lost in the Muridae and Cricetidae families. A duplicated *HERC5/6* fused gene is independently fixed in several rodent species and in the *Myotis* genus in bats. Duplication followed by pseudogenization of *HERC5* in primates, carnivores and artiodactyls is presented.

(**Figure 1**, **Supplementary Figure 2**). Moreover, we confirmed the erosion of *HERC5* in rodents (25), and we further showed that this loss has most probably occurred in the common ancestor of the Cricetidae and Muridae. Interestingly, we also did not find the *HERC5* gene in the rhinoceros genome, suggesting at least two independent losses of *HERC5* in mammals, specifically in the Rodent and Perissodactyl orders (**Figure 1**).

Finally, in addition to these independent losses of *HERC5* or *HERC6* in mammals, we found multiple evidences of *HERC5* duplication followed by pseudogenization in primate (*Sapajus apella*, *Cebus capucinus*, *Aotus nancymaae*, and *Saimiri boliviensis*), carnivore (*Canis lupus*, *Vulpes Vulpes*, *leptonychotes weddellii*), and artiodactyl (*Ovis aries*) species, highlighting a strong dynamic of gene gain and loss in mammalian *HERC5* and *HERC6*.

Ancient and Recent Recombinations Have Shaped the Evolution of a Duplicated Chimeric *HERC5/6* Gene in Rodents and Bats

While most mammalian species possess one or both *HERC* genes, we found independent duplications of *HERC5* in the chiropteran *Myotis* genus and the rodent Hystricognathi infra-order (**Figure 1**, **Supplementary Figure 3**). These duplicated genes were identified in three bat *Myotis* species (*M. lucifigus*, *M. brandtii*, and *M. davidii*) and five rodent species (*Fukomys damarensis*, *Heterocephalus glaber*, *Cavia porcellus*, *Chinchilla lanigera*, and *Octodon degus*) (**Table S1**). This dates these duplication events to at least 30 MYA (million years ago) and 44 MYA, respectively.

Surprisingly, alignments of HERC5, HERC-like, and *HERC6* proteins in each mammalian group revealed 96–99% amino acid identity between the *HERC5* and HERC-like N-terminals, and 74–84% amino acid identity between the HERC-like and *HERC6* C-terminals (**Figure 2A**, **Supplementary Figure 4**). To test whether this may be reminiscent of recombination in rodents and bats, we used the GARD program. We identified a significant recombination breakpoint located upstream from the spacer region at 1,103 bp in bats and 1,118 bp in rodents (**Figures 2A, B**). The phylogenetic analyses of the resulting fragments (identified by GARD) confirmed the recombination in both bats and rodents. Specifically, we found that the HERC-like 5'-fragment clustered with the *HERC5* gene (clade supported by a significant bootstrap), while the 3'-fragment grouped with the *HERC6* gene (**Figures 2C–F**). Taken together, our findings reveal that a similar ancient mechanism has independently led to the fixation of a *HERC*-like gene, which in fact is a chimeric HERC5/6 gene containing the *HERC5* RLD domain and the *HERC6* spacer and HECT domains in bats and rodents.

Moreover, by analyzing the phylogenetic trees of *HERCs* in bats and rodents (**Figures 2C–F**), we found that the 5' fragment of *HERC5/6* was genetically closer to *HERC5* from the same species. This was not the case for the 3' end, where all 3' fragments of the *HERC5/6* genes significantly grouped together. Combined with several GARD analyses, this supports that recent recombinations further occurred between the RLD domains of *HERC5* and *HERC5/6* genes (**Figure 2G**).

HERC6, but Not *HERC5*, Has Been Under Strong Positive Selection During Mammalian Evolution

We next investigated whether *HERC5* and *HERC6* have been under selective pressure in mammals.

Our results revealed some signatures of positive selection in artiodactyl, primate, and bat *HERC5* (p-values $<10^{-3}$), but none in rodent and carnivore species (p-value >0.5) (**Table 1**). At the codon level, the signal was overall very weak, with less than three positively selected codons assigned per order (posterior probability threshold fixed at 0.95, and p-value <0.05) (**Table 2**). In primates, two significant positively selected sites were identified in the RLD domain (**Figure 3**). Similarly, less than two codons evolved under positive selection in bats and artiodactyls (except for the MEME method which identified multiple positively selected sites in the artiodactyls), and none of the codons were common between methods (**Table 2**). Interestingly, the separate analysis of cetacean species revealed stronger signatures of positive selection at the gene (p-value = 6.10^{-6}) and the codon levels (five sites identified by at least two methods), suggesting a lineage-specific adaptation.

In contrast, we found very strong signatures of ancient and recurrent positive selection in *HERC6*, at both the gene and the codon levels. All five mammalian orders exhibited a significant excess of non-synonymous rate along the *HERC6* coding sequences, specifically in bats, carnivores, and rodents (p-value $<10^{-43}$ in bats, carnivores, and rodents; and p-value $<10^{-6}$ in artiodactyls and primates). Positive selection was observed in each of the three domains of HERC6—the RLD domain, the spacer region, and the HECT domain—in bat, carnivore, and rodent species, while many of the signatures were concentrated in the spacer region and the HECT domains in primates and artiodactyls (**Table 1**, **Figure 3**). Remarkably, the fastest-evolving codons mapped into the *HERC6* spacer region of all five mammalian orders (p-value $<10^{-26}$ in bats, carnivores, and rodents, p-value $<10^{-5}$ in artiodactyls and primates). More than 14 sites were identified by at least two methods in bats, carnivores, and rodents, thereby constituting a hotspot of highly variable sites in mammalian *HERC6* (**Table 2** and **Figure 3**). In line with this finding, alignment of the spacer region revealed that it is an extremely divergent domain, characterized by multiple amino acid changes and indels between and within groups, in particular in rodents and bats (**Figure 4**).

Therefore, although *HERC5* presents low evidence of positive selection in mammals, *HERC6* has experienced very strong adaptive evolution. Both genes showed differential evolutionary profiles across/between mammals, with lineage-specific and domain-specific adaptations.

The Rodent Chimeric *HERC5/6* Paralog Has Evolved Under Strong Positive Selection

We then addressed whether the newly identified chimeric *HERC5/6* gene, which contains the *HERC5* RLD domain and the *HERC6* spacer and HECT domains, has also experienced positive selection. As coding sequences from five rodent species were available, we assessed the inter-species evolutionary history

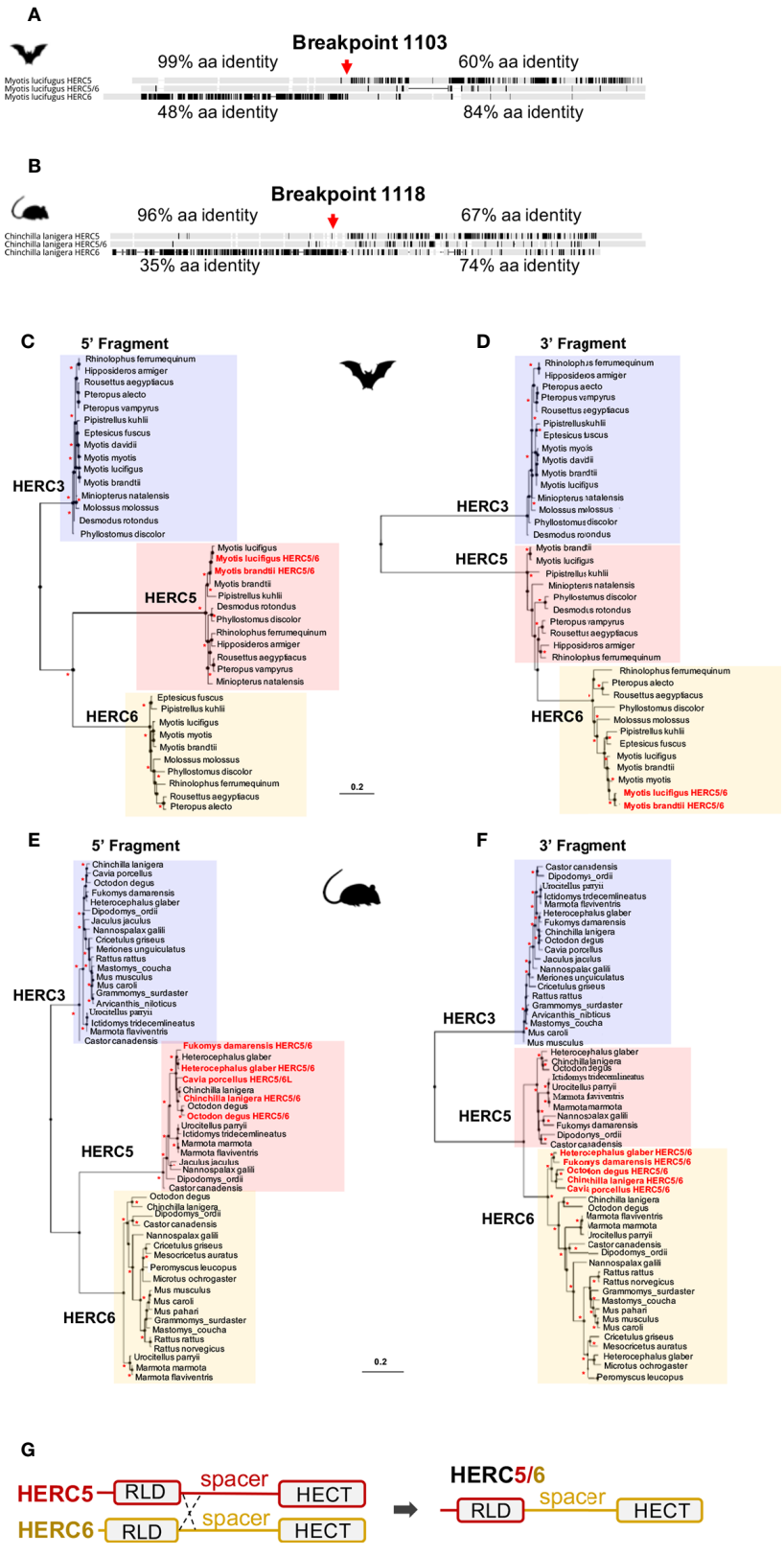


FIGURE 2 | Continued

FIGURE 2 | Independent duplication of a chimeric *HERC5/6* gene through recombination in bats and rodents **(A, B)**. Alignment of the protein sequence of HERC5, HERC5/6, and HERC6 genes from bats and rodents, respectively. Additional sequence alignments are shown in Supplementary Figure 4. The percentages of pairwise amino acid identities between the N-terminals of HERC5/6 and HERC5 or HERC6, as well as the C-terminals of HERC5/6 and HERC5 or HERC6 are indicated. The significant recombination breakpoints (red arrows, p-value <0.05) assigned by the GARD program are shown for the rodent and bat HERC5/6 gene **(C–F)**. Maximum likelihood phylogenetic tree generated with the 5' (at the left) and 3' (at the right) of the *HERC5*, *HERC6*, *HERC5/6*, and *HERC3* (as an outgroup) nucleotide gene alignment based on GARD recombination results (corresponding to the breakpoint 1103 in *Myotis lucifigus* in bats, and 1118 in *Chinchilla lanigera* in rodents). The duplicated chimeric *HERC5/6* genes are shown in red. Asterisks indicate bootstrap values greater than 80%. The scale bar represents genetic variation of a 0.2 (20%) for the length of the scale **(G)**. A linear representation of *HERC5* and *HERC6* structures showing a chromosomal crossover between the 5' regions, upstream of the spacer region. This mechanism has led to a duplicated recombined *HERC5/6* gene containing the HERC5 RLD domain and HERC6 spacer region and HECT domain.

of the chimeric *HERC5/6* gene within this group. Of note, there were insufficient bat sequences/species to perform the corresponding analyses. The likelihood ratio tests revealed significant positive selection in rodent *HERC5/6* (p-value <0.0003, **Table 2**). Importantly, all the positively selected codons mapped in the spacer region, and were concentrated between the amino acids 409 and 660 (**Figure 3**), suggesting that the spacer domain has been the target of strong positive selection as observed in HERC6.

Rodent and Bat *HERC6* Genes Have Been Under Stronger Positive Selection Compared to Other Mammals

Finally, we tested whether positive selection has differentially impacted the evolution of *HERC5* and *HERC6* across mammals. We found that the chiropteran and rodent *HERC6* genes have experienced intensive episodic positive selection compared to the other groups (**Figure 5**). In particular, five chiropteran lineages distributed along bat phylogeny (*Rhinolophus ferrumequinum*,

TABLE 1 | Positive selection analyses of mammalian *HERC5* and *HERC6* genes.

	Codeml M1 *vs* M2			Codeml M7 *vs* M8		
	p-value[a]	% of PSS[b]	M2 ω[c]	p-value[a]	% of PSS[b]	M8 ω[c]
HERC5						
Artiodactyls	0.0045	4.2	2.43	$9.2E^{-05}$	10.3	1.9
Artiodactyls without cetaceans	0.0035	0.3	14.3	0.0024	0.3	13.6
Cetacea	$4.4E^{-06}$	2.8	6.9	$3.2E^{-06}$	2.9	6.9
Bats (1–192)	0.8423	–	–	0.3658	–	–
Bats (193–3,115)	0.0007	1	5.6	0.0002	1.3	4.8
Carnivores	0.6554	–	–	0.2083	–	–
Primates	0.0015	5.1	3.5	0.0009	5.6	3.4
Rodents (1–288)	1	–	–	1	–	–
Rodents (289–3,069)	1	–	–	0.2361	–	–
HERC6						
Artiodactyls whole gene	$3.1E^{-06}$	6.1	3.7	$2.1\ E^{-06}$	7.4	3.4
Artiodactyls RLD domain	0.1071	–	–	0.0552	–	–
Artiodactyls Spacer region	$3.9E^{-05}$	6.9	5	$3.9E^{-05}$	7.8	4.6
Artiodactyls HECT domain	0.0029	1.1	3.6	0.0022	1. 3	3.2
Bats whole gene	$1.4E^{-40}$	7	4.4	$1.1\ E^{-44}$	8	4.2
Bats RLD domain	$3.4E^{-06}$	5.7	3.8	$2.2E^{-07}$	7.2	3.4
Bats Spacer region	$2.9E^{-25}$	10.1	4.5	$2.4E^{-27}$	10.3	4.6
Bats HECT domain	$1.6E^{-10}$	7.7	4.2	$4.1E^{-11}$	9.4	3.9
Carnivores whole gene	$3.2E^{-43}$	7	5.2	$3E^{-46}$	7.5	5.1
Carnivores RLD domain	$2.7E^{-05}$	8	3.3	$1.5E^{-05}$	9.5	3.1
Carnivores Spacer region	$1.5E^{-33}$	9.8	6.1	$5.5E^{-36}$	10	6.1
Carnivores HECT domain	$6E^{-05}$	4.7	4.7	$2.4E^{-05}$	5.3	4.3
Primates whole gene	$4.3E^{-08}$	4.9	4.2	$1.8E^{-08}$	4.5	4.3
Primates RLD domain	0.9476	–	–	0.9016	–	–
Primates Spacer region	$1.2E^{-08}$	8.4	5.4	$1.3E^{-08}$	9.2	5.2
Primates HECT domain	$1.1E^{-06}$	5.1	7.6	$2E^{-06}$	5.2	7.6
Rodents whole gene	$7.2E^{-53}$	9.1	3.7	$1.3E^{-60}$	10.3	3.3
Rodents RLD domain	$6.6E^{-11}$	5.1	3.9	$4.2E^{-13}$	6.7	3.4
Rodents Spacer region	$2.8E^{-39}$	14.1	4.2	$2.7E^{-41}$	15	3.9
Rodents HECT domain	$7.9E^{-14}$	6.8	4.5	$7.6E^{-16}$	7.9	3.8
HERC5/6 paralog						
Rodents HECT domain	0.0003	20	2.1	0.0002	20	2.1

Results of positive selection analyses comparing models that disallow positive selection (M1 and M7) to models allowing positive selection (M2 and M8). [a]p-values generated from maximum likelihood ratio tests indicate whether the model that allows positive selection (models M2 and M8) better fits the data than the nearly neutral one (M1 and M7). [b]Percentage of codons evolving under positive selection (dN/dS ratio > 1 over the alignment). - not significant. cAverage dN/dS ratio associated with the classes K3 and K11, in the Codeml models M2 and M8, respectively, which allow positive selection.

TABLE 2 | Positively selected codons in mammalian *HERC5*, *HERC6* and *HERC5/6* genes.

	MEME	FUBAR	Codeml M2	Codeml M8
HERC5				
Artiodactyls	6, 42, 63, 263, 408, 539, 565, 672, 696, 745, 992	39	–	189
Artiodactyls without Cetacea	–	39, 42, **552**, 555, 1034	–	**552**
Cetacea	**6**, 664, 666, **683**, **712**	**6**, 175, 347, **663**, **676**, **683**, **712**, 732, **782**	**663**, **676**	**6**, 662, **663**, **676**, **712**, **782**
Bats (193–2,913 bp)	22, 39,	550	–	654
Carnivores	89, 90, 91, 93, 250, 265, 298, 323, 568, 573	189, 401, 439, 840	–	–
Primates	–	**12**, **19**, **43**, 233, 296, **480**, **483**	**12**, **19**	**12**, **19**
Rodents (289–2,773 bp)	59, 76, 87, 98, **191**, 363, 462, 658, 870	**191**	–	–
HERC6				
Artiodactyls	56, 62, 112, **116**, 164, 167, 568, 661, 708, 723, 797	15, **116**, 599, 603, 655, 886		
Bats	**6**, 57, 58, 163, 382, 396, **549**, 552, 597, 600, **603**, **608**, 633, **646**, 650, **663**, 665, 671, **690**, **704**, **706**, 716, 814, 910, **911**, 995, 1014, 1015	**6**, **549**, **603**, **608**, **642**, **646**, **647**, **648, 663**, **690**, **704**, **706**, **911**	**6**, **18, 74**, **454, 546**, **549**, **591, 595**, **603**, **608**, **642**, **646**, **647**, **648, 650, 651**, **663**, **690**, **706**, **911**, **964**	**6, 18, 74, 454, 471,546, 549, 591, 595, 603, 608, 642, 643, 646, 647, 648, 650, 651, 663, 690, 706, 911**, **964**, 974
Carnivores	30, 112, **166**, **216**, 225, **398**, **447**, 515, 517, **550**, 574, **593**, **596**, **600**, **602**, **603**, **659**, **666**, **669**, 753, 794, 846, 1023	**166**, **216**, 397, **398**, **447**, 549, **550**, **593**, 595, **600**, **602**, **603**, **659**, **666**, **669**, 713, 968	**9**, **23**, **63**, **70**, **166, 216,** 395, **396, 517, 547, 548, 591, 593, 594, 596, 598, 600, 601, 657, 699, 711, 917, 966**	**9, 23, 63, 70, 166, 216, 396, 517, 547, 548, 591, 593, 594, 596, 598, 600, 601, 657,** 664, 667**, 699, 711, 917, 966**
Primates	401, 472, 556, 647, 759, **828**, 1025	76, **597**, 631, 651, 700, **828**, 950, 989, 1021	**597**, **641**, **710**, **826**	370, 596, **597**, **641**, 649, 698, **710**, **826**, 987, 1020
Rodents	**10**, **11**, **16**, 59, 66, 123, 209, 268, 275, **317**, 328, 366, 450, 474, **546**, **548**, **550,** 554, **602, 606,** 609, **611**, **638, 642**, **643**, 644, 649, 677, **686, 688**, 697, 700, 743, 756, 797, 838, 842, 872	**10**, **11**, **16, 287**, **546, 548, 550, 551**, **557, 593, 594, 597**, **611, 642, 643**, **688**	**10, 16, 22, 125, 166, 287, 317, 513, 517, 547, 550, 551, 552, 553, 557, 593, 594, 597, 601, 602, 603, 605, 606, 610, 611, 613, 614, 635, 638, 642, 643, 645, 656, 657, 686, 688, 901, 952**	**10, 16, 22, 125, 166,** 219**, 287, 317, 513, 517, 547, 550, 551, 552, 553, 557, 593, 594, 597, 601, 602, 602, 605, 606, 610, 611, 613, 614, 635, 638, 642, 643, 645, 656, 657, 686, 688, 901, 952**
HERC5-6 paralogs				
Rodents	69, 138, 261, **409**, **467**, 774, 926	29, 120, 361, **467**, 506, 507, **518**, 556, **566, 660**	**467**	22, **409, 467**, **518**, **566**, **660**

Results from site-specific positive selection analyses, with a posterior probability (PP) of BEB >0.95 for the models M2 and M8 from Codeml, >0.9 for FUBAR, and a p-value <0.05 for MEME. Codons in bold are those that were assigned by at least two different methods. Codon numbering is based on HERC5 sequences from Bos taurus, Tursiops truncates, Phyllostomus discolor, Felis catus, Homo sapiens, and Dipodomys ordii, HERC6 sequences from Bos taurus, Rhinolophus ferrumequinum, Felis catus, Homo sapiens, and Mus musculus, HERC5/6 sequence from Fukomys damarensis.

Pteropus ancestral branch, *Phylllostomus discolor*, *Molossus molossus*, and *Pipistrellus kuhlii)* and five rodent branches (two ancestral branches of mouse related clade, *Cricetulus griseus*, *Mesocricetus auratus*, and *Urocitellus parryii*) have undergone a significant excess of amino acid changes, with a ratio ω >3.8. Differential selective pressure in *HERC5* was also evidenced across mammalian lineages, but to a lesser extent: only two branches were found under significant positive selection, in bats (*Hipposideros armiger*, ω = 189) and carnivores (*Ailuoropoda melanoleuca*, ω = 78).

DISCUSSION

Deciphering the evolutionary and functional diversification of the antiviral innate immunity in mammals is of primary importance to better understand modern viral pathogens, virus-host interfaces, and identify novel antiviral strategies. The functional significance of HERC5 and HERC6 is underlined by their ancient origin and conserved expression in vertebrates (25). However, their evolutionary history in mammals has remained unclear. Here, we have carried out in-depth phylogenetic and genomic analyses to address how mammalian *HERC5* and *HERC6* have evolved over millions of years of divergence.

Differential Evolutionary Fate of *HERC5* Across Mammals

Although mammalian *HERC5* was previously reported as a rapidly evolving gene (33), we only found strong evidences of recurrent positive selection in primates. In particular, two codons in primate *HERC5* have rapidly evolved in the RLD domain, possibly reminiscent of pathogen-exerted pressure (3, 53–55). In line with this, blade 1 of the primate RLD domain was recently reported to be an important functional region for HERC5 anti-HIV antiviral activity (25). Thus, retroviruses may have played a role in the diversification of *HERC5* during primate evolution. Such patterns have been reported in many primate restriction

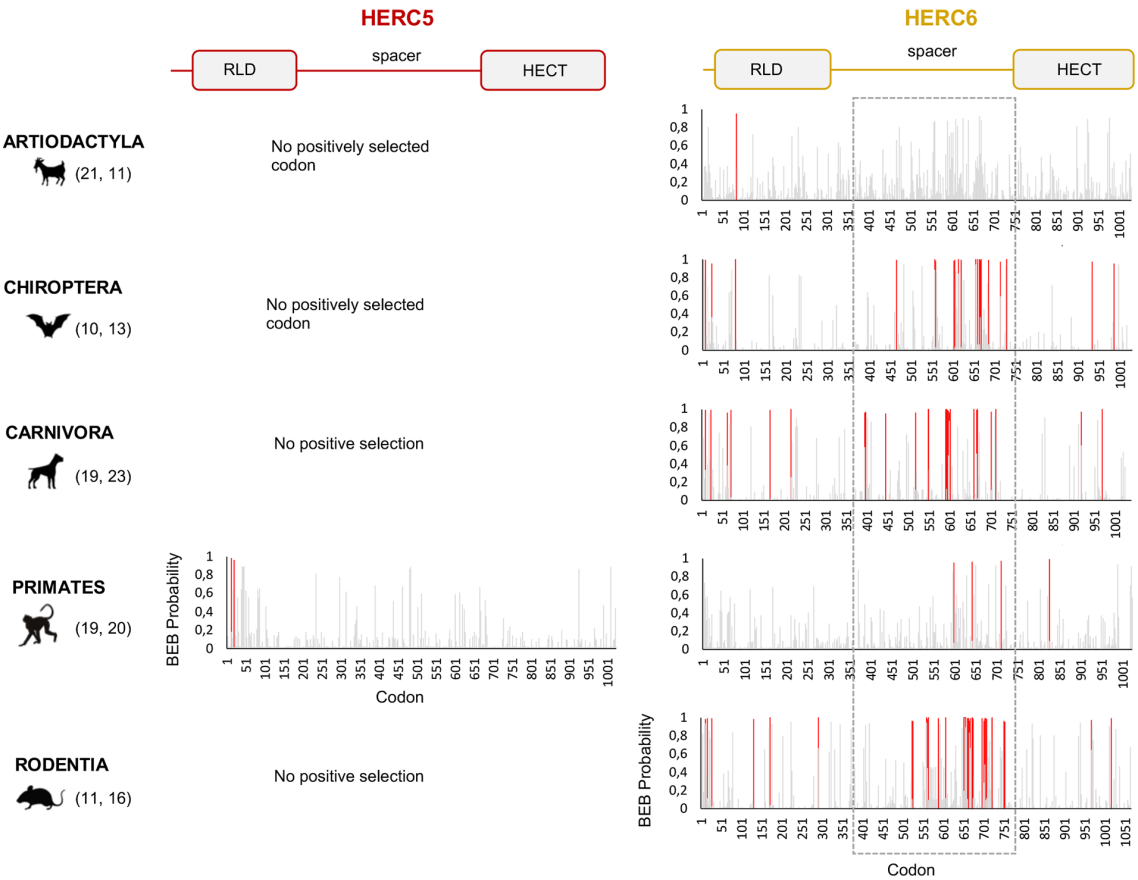


FIGURE 3 | *HERC6*, and not *HERC5*, has experienced strong and mammalian-wide positive selection. Graphic panels represent the posterior probabilities of positive selection (Bayes empirical Bayes, BEB) (y axis) in the M2 Codeml model (allowing for positive selection, ω >1) for each codon (x axis) in *HERC5* (left) and *HERC6* (right) alignments. Red bars indicate the sites identified by both models, M2 and M8, with a BEB posterior probability greater than 0.95. Numbers in brackets are total species analyzed in each mammalian order for each gene. Site numbering is based on HERC5 protein sequences from *Homo sapiens*, HERC6 sequences from *Bos taurus*, *Rhinolophus ferrumequinum*, *Felis catus*, *Homo sapiens*, and *Mus musculus*. Above is a linear representation of HERC5 and HERC6 showing the structural domains, the RLD, spacer region, and HECT domains.

factors, including *BST2* (56–58), *TRIM5* (10, 59–61), and *APOBEC3* (14, 62–64). Because the role of HERC5 is not limited to host defense against retroviruses, its evolution in primates may also reflect past selection against other viral pathogens such as influenza viruses and papillomaviruses.

In contrast, positive selection was solely evident at the gene level for artiodactyl and bat *HERC5*, and absent in rodents and carnivores. This pattern may be a result of lineage-specific selective drivers: differential viral exposure history, distinct mechanisms of viral antagonism, and/or may reflect overall pressure to maintain efficient cellular functions of HERC5. For example, apart from its antiviral role, some evidences suggest that HERC5 might be functionally involved in other pathways, such as spermatogenesis and cell cycle (22), as well as cancer (65). HERC5 may have thus evolved to maintain effective cellular functions rather than to escape viral antagonisms or to target viral pathogens in bats and artiodactyls. In mammals not exhibiting positive selection, viruses may have evolved indirect mechanisms of antagonism to counteract HERC5 function. In line with this, many viruses encode proteins that interfere with the ISGylation activity of HERC5, through direct interaction with the ISG15 protein (66–69). For example, the NS1B protein encoded by influenza virus antagonizes ISG15 conjugation through direct interaction (66).

Accelerated Evolution of *HERC6* in Mammals

HERC6 is the only gene from the small *HERC* family exhibiting such high levels of adaptive changes with an extremely divergent spacer region in all mammals, except artiodactyls. Such rapid evolution of *HERC6*, with accumulated mutations replacing the amino acids and multiple amino acid insertions/deletions, most likely mirrors pathogen-driven adaptations as a result of past evolutionary arms-races (3, 53–55). This highlights a fundamental antiviral role for HERC6 in mammals. Previous functional evidences support that HERC6 is involved in

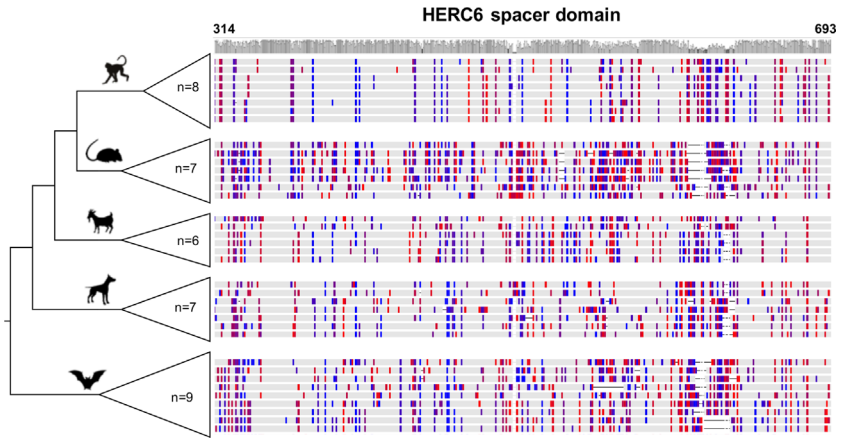


FIGURE 4 | Rapid evolution of mammalian HERC6 spacer region is characterized by multiple amino acid changes and major indels. Multiple alignment and comparison of the HERC6 spacer region between and within mammalian orders. Left, cladogram with the number of sequences used for each clade (n = 6 to 9). Right, colors indicate site variations between the sequences as compared to the consensus sequence with a threshold of 25% (Geneious, Biomatters; blue/red, hydrophilic/hydrophobic residues), while gray represents similarity with the consensus. The average pairwise percentage of identity is graphically represented above in gray. The codon numbers are based on human HERC6 sequence.

mammalian antiviral immune response (28, 34, 35, 66). However, available studies have only focused on HERC6 anti-retroviral activity and ISGylation function. For example, HERC6 has been shown to be an IFN-inducible E3-ligase of ISG15 conjugation in mouse (28, 34, 35). In contrast, human HERC6 lacks the ISGylation activity, but it potently inhibits the primate lentivirus SIVmac viral production (25).

Specifically, we identified the spacer region as a potential pathogen—HERC6 interface, involved in the recognition of viral proteins and/or being the target of viral antagonists. Up to now, most studies have been devoted to the functional characterization of the RLD and HECT domains of HERC proteins, as they belong to the well-characterized protein families, RCC1 and E3-ligases, respectively. In contrast, the structural characteristic of the spacer region has not been related to any known protein, which hinders its description and functional role. However, its propensity for amino acid insertions/deletions and accumulated non-synonymous mutations, including changes with strong chemicophysical differences highlights a strong evolutionary plasticity. Such a hotspot of variability in unstructured regions has been reported for several restriction factors, such as MX1, in which the highly variable L4 loop has led to differential virus-host interfaces and plasticity (70, 71).

It is noteworthy that the RLD and HECT domains of *HERC6* were also subjected to positive selection in bats, carnivores, and rodents. These signatures may reflect different virus-host interfaces. This pattern was reported in the primate PKR protein, in which signatures of pathogen-driven selection are scattered along the protein as a result of interactions with multiple viral antagonists (72, 73). However, it is also possible that all positively selected sites cluster in a same spatial region of the protein. A 3D structural analysis of the *HERC6* protein would help assessing how the rapidly evolving sites are located in the protein, and allow determining whether *HERC6* presents multiple or unique host-pathogen interface(s). Up to now, the 3D structure has only been solved for the HECT domain and RLD domain separately [e.g (74, 75)]. Therefore, how the spacer region connects the HECT and RLD domains in a 3D structural dimension is currently unknown. Further studies on small HERC protein structure would help to better understand how the high variability in *HERC6* impacts its structure and function.

We found differential genetic profiles of *HERC6* across mammalian orders. The strongest signal was found in bats, carnivores, and rodents, suggesting that different strength/intensity of selective pressures have shaped the evolution of *HERC6* in mammals. This was confirmed by the branch-specific analyses, in which rodents and bats exhibit significant lineage-specific adaptive changes. Rodents and bats are the two most diverse mammalian orders, and host the highest viral richness among mammals (38). Both orders have thus been exposed to a greater viral diversity, compared to primates and artiodactyls. This may have increased the strength and extent of selective pressure exerted on the HERC6 protein.

Unequal Recombination Has Led to the Duplication of a Chimeric *HERC5/6* in Rodents and Bats

Lineage-specific expansions of multigene families have shaped and complexified the mammalian antiviral repertoire over million years of evolution. Consequently, many unrecognized genes encoding for antiviral proteins are yet to be discovered. Here, we have identified duplications of a *HERC* paralog in the rodent Hystricognathi infra-order and the chiropteran *Myotis* genus, which has occurred around 30 MYA (76, 77) and 44 MYA (78–80), respectively.

Interestingly, these paralogs are chimeric *HERC5/6* genes coding for the HERC5 RLD fused to the *HERC6* spacer region and HECT domain. This suggests that an independent

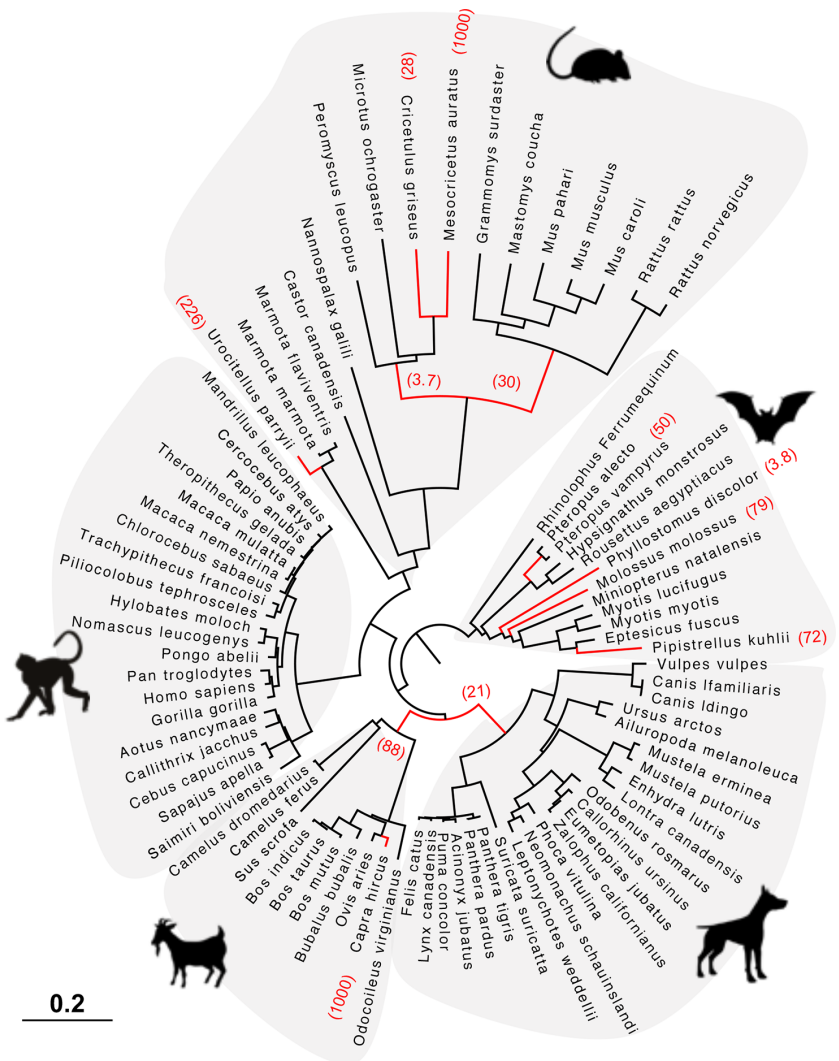


FIGURE 5 | *HERC6* has been under strong positive selection during rodent and bat evolution. Maximum likelihood phylogenetic tree of mammalian HERC6 gene showing the branches under significant positive selection (p-value <0.05, in red) assigned by aBSREL from the HYPHY package. The numbers in brackets indicate the estimated values of the ω at the branch. The scale bar indicates the proportion of genetic variation.

duplication has occurred through a similar mechanism in bats and rodents. Gene duplication can occur by different modes, mainly including unequal crossing over, retroposition, or chromosomal duplication (81, 82). In the former case, duplicated genes are physically linked in the chromosome, and can contain a fragment of a gene, a whole gene, or several genes (81). In contrast, retroposition engenders a retrotranscribed complementary DNA incorporated into the genome, which generally lacks intronic regions and regulatory sequences (81). Given that the *HERC5/6* are located in the canonical locus of *HERC5* and *HERC6* and contain the parental intronic regions, they have most likely resulted from an unequal crossing over between *HERC5* and *HERC6*.

This hypothesis is supported by the recombination analyses, which detected a significant breakpoint upstream of the spacer region in both mammalian orders. The fact that the recombination occurred at the same genetic location can be explained by two different, but not necessarily mutually exclusive, hypotheses. First, the recombination event can only occur at this location because of genomic structural constraints (i.e. genomic homology between paralogs). Second, the HERC6 spacer region and HECT domains are required for functional HERC5/6 proteins. The best example of such tandem duplication with domain fusion is the lineage-specific expansion of the *APOBEC3* family in mammals. Originating from an ancestral *APOBEC3* gene, tandem duplications as well as retrocopying events have radically expanded the repertoire of mammalian *APOBEC3* genes (14, 20). In primates, several of the *APOBEC3* genes have most likely resulted from the fusion of A3 domains, while the murine genome encodes a unique *APOBEC3Z2-*

APOBEC3Z3 fused gene (83), highlighting lineage specific functional adaptations.

Such gene duplications accompanied with gene fusion are major genetic innovations that functionally diversify the antiviral arsenal (3, 4, 7). For example, the expansion of the *APOBEC3* family has functionally diversified the antiviral activities and specificity of targeted viruses in primates (20, 62, 84). Given the antiviral role of HERC5 and the potential implication of HERC6 in antiviral immunity, we hypothesize that the HERC5/6 paralog provides a functional advantage against pathogenic viruses. This is supported by both the extremely rapid episodic evolution of *HERC6* in rodents and bats, and the signatures of positive selection in rodent *HERC5/6*. The fused *HERC5/6* gene may have evolved combined functional features of *HERC5* and *HERC6*. Alternatively, it may have retained the functional implication of the HERC5 RLD domain, but has functionally diverged from HERC6.

This latter hypothesis is more likely as we found that the RLD domain of HERC5 and of HERC5/6 are highly similar and cluster together within species (more than 95% pairwise amino acid identity). This pattern may reflect ongoing gene conversions between the RLD domains of *HERC5* and *HERC5/6*, thereby maintaining a N-terminal similar to the parental HERC5 protein.

Whether the independent fixation of the *HERC5/6* paralog in rodents and bats is a functional/phenotypic convergent evolutionary event has to be investigated. Moreover, it raises the question why both lineages have undergone such genetic innovation. While this duplication could be hazardous, it is possible that the rodent Hystricognathi infra-order and the bat *Myotis* genus share a common selective pressure, such as a viral pathogen family. Other forces such as ecological and/or environmental factors may have played a role, such as life-history traits or biodiversity changes.

Perspectives

Studying the genetic adaptations of host innate immunity can provide insights into the evolutionary and functional determinants of host antiviral response. This approach has been a powerful tool for assessing the functional diversification of virus-host interfaces in many systems [e.g (59, 62, 85, 86)]. In this present study, we identified *HERC6* and *HERC5/6* as potential unrecognized restriction factors in mammals. Further functional investigations are now required to (i) decipher the antiviral function of *HERC6* and HERC5/6, (ii) determine how the accumulated variability in the spacer domain may impact their structure, function, and stability, (iii) unravel HERC6 binding interface with potential viral antagonists or targeted viral proteins, and (iv) determine on the other side which pathogens are targeted by those proteins. It will also be interesting to investigate whether and how the evolution of *HERCs* may impact the cellular partners involved in the ISGylation functions. For example, although we found that ISG15 is mostly conserved in mammals, it bears important variations at the C-terminal (data not shown). This could reflect host protein-protein co-evolutions or indirect viral antagonism of HERC restriction. Moreover, because the *HERCs* have been mostly studied in primates and rodents, it is possible that other proteins than ISG15 are involved in the ISGylation function in other mammals. In addition, deciphering whether and how the *HERC5/6* paralogs afford a selective advantage against pathogens in rodent and bat lineages is of main interest in virology and immunology fields, as both orders are important reservoirs of zoonotic pathogens. Finally, studying the functional implications of *HERC6* adaptation in mammals will not only allow to better understand how pathogens have shaped host immunity, but will provide important insights into the overlooked role of small HERC proteins in mammalian antiviral response.

Altogether, our results represent avenues for future functional studies of importance in mammalian innate immunity.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: NCBI (https://www.ncbi.nlm.nih.gov/) and accession numbers are available in **Supplementary Table 1**.

AUTHOR CONTRIBUTIONS

SJ carried out the analyses of the data. SJ, LE, and DP performed the investigations. SJ, LE, and DP wrote the original draft of the paper. LE and DP acquired the funding, and coordinated the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.605270/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Maximum likelihood phylogenetic tree generated with the whole coding sequences of *HERC5*, *HERC6*, and *HERC3* nucleotide alignment from artiodactyl, carnivore, rodent, bat, and primate species. Asterisks indicate bootstrap values greater than 80%. The scale bar represents the proportion of genetic variation (0.2 for the scale), and is indicated at the bottom. Sequences are collapsed in each order for better readability.

SUPPLEMENTARY FIGURE 2 | Pseudogenization of cetacean *HERC6* **(A)**. Multiple amino acid alignment of six cetacean *HERC6* sequences showing multiple substitutions, insertions, and deletions **(B).** Multiple nucleotide alignment with corresponding amino acids of cetacean, rodent, bat, ruminant, primate, and carnivore *HERC6*, highlighting a conserved stop codon in the cetacean species (codon 174 in *Balaenoptera acutorostrata*). Nucleotide and amino acid sequences are shown using Geneious.

SUPPLEMENTARY FIGURE 3 | Maximum likelihood phylogenetic tree generated with the whole coding sequences of *HERC5*, *HERC6*, *HERC5/6*, and *HERC3* nucleotide alignment in bats (left) and rodents (right). The chimeric duplicated *HERC5/6* genes are shown in red. Asterisks indicate bootstrap values greater than 80%. The scale bar at 0.2 is indicated below.

SUPPLEMENTARY FIGURE 4 | Alignment of the protein sequence of HERC5, HERC5/6, and *HERC6* from bats and rodents. The percentages of pairwise amino acid identities between the N-terminals of HERC5/6 and HERC5 or HERC6, as well as the C-terminals of HERC5/6 and HERC5 or HERC6 are indicated. The significant recombination breakpoints (red arrows, p-value <0.05) assigned by GARD program are shown for bat and rodent *HERC5/6* gene. Because the coding sequence of HERC6 gene from *Heterocephalus glaber* was incomplete with many missing data, it was not included in the figure. Likewise, some portions of the N-terminal of HERC5, as well as the C-terminals, from HERC5/6 and HERC6 are missing in the protein alignment of the chiropteran species, *Myotis brandtii* (top).

SUPPLEMENTARY TABLE 1 | Information on publicly available datasets analyzed in this study. Accession numbers are available in NCBI (https://www.ncbi.nlm.nih.gov/).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immunostimulatory Endogenous Nucleic Acids Perpetuate Interface Dermatitis—Translation of Pathogenic Fundamentals Into an *In Vitro* Model

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Interface dermatitis is a histopathological pattern mirroring a distinct cytotoxic immune response shared by a number of clinically diverse inflammatory skin diseases amongst which lichen planus and cutaneous lupus erythematosus are considered prototypic. Interface dermatitis is characterized by pronounced cytotoxic immune cell infiltration and necroptotic keratinocytes at the dermoepidermal junction. The initial inflammatory reaction is established by cytotoxic immune cells that express CXC chemokine receptor 3 and lesional keratinocytes that produce corresponding ligands, CXC motif ligands 9/10/11, recruiting the effector cells to the site of inflammation. During the resulting anti-epithelial attack, endogenous immune complexes and nucleic acids are released from perishing keratinocytes, which are then perceived by the innate immune system as danger signals. Keratinocytes express a distinct signature of pattern recognition receptors and binding of endogenous nucleic acid motifs to these receptors results in interferon-mediated immune responses and further enhancement of CXC chemokine receptor 3 ligand production. In this perspective article, we will discuss the role of innate nucleic acid sensing as a common mechanism in the perpetuation of clinically heterogeneous diseases featuring interface dermatitis based on own data and a review of the literature. Furthermore, we will introduce a keratinocyte-specific *in vitro* model of interface dermatitis as follows: Stimulation of human keratinocytes with endogenous nucleic acids alone and in combination with interferon gamma leads to pronounced production of distinct cytokines, which are essential in the pathogenesis of interface dermatitis. This experimental approach bears the capability to investigate potential therapeutics in this group of diseases with unmet medical need.

Keywords: interface dermatitis/lichenoid tissue reaction, nucleic acid sensing, damage associated molecular patterns (DAMPs), type I immunity, lupus erythematosus, lichen planus, dermatomyositis, *in vitro* model

INTRODUCTION

Interface dermatitis (ID), also referred to as lichenoid tissue reaction, describes a histopathological pattern defined by morphological anomalies of the epidermal basal cell layer characterized by perishing keratinocytes labeled vacuolar or hydropic colloid bodies. Anti-epithelial activity of autoreactive cytotoxic lymphocytes is causative (1). The distinct pattern is observed in clinically heterogeneous skin diseases including autoimmune skin disorders [lichen planus (LP), lichen sclerosus (LS), cutaneous lupus erythematosus (CLE), dermatomyositis (DM)] and immunologic reactions against viruses, drugs and specific tumors ("lichenoid" keratosis) (2, 3). In 1995, for the first time, Fäh et al. detected MxA expression not only in virally infected tissue but also in dermatoses featuring ID (4). These findings are explained by MxA expression being directly induced by type-I and type-III IFNs (5). Today, it is accepted that activation of the interferon system resulting in a cellular immune response is a pathogenic key feature of the histologic "look-alikes" sharing ID (1).

Amongst the autoimmune skin disorders featuring ID, LP, and CLE are considered prototypic (6): LP may affect the skin including its appendages and both oral and genital mucosa (7). Classical LP presents with violaceous papules generally accompanied by extensive pruritus. When affecting the nails, thinning, scarring, and even complete loss of the nail is possible. Lichen planopilaris affecting the hair follicles may cause scarring and permanent baldness. Affected mucosa usually presents as erosive (8). The clinical spectrum of lupus erythematosus is broad ranging from systemic manifestations [systemic lupus erythematosus (SLE)] to manifestations solely affecting the skin. Cutaneous lupus erythematosus (CLE) may present itself as one symptom of SLE or may occur as an isolated skin disease (9, 10). CLE manifestation can be further subdivided into four main subsets (acute, subacute, intermittent, or chronic). Acute CLE may present either with a localized facial indurated erythematous lesion (malar rash) or with a widespread erythematous maculopapular rash. Subacute CLE is characterized by either annular/polycyclic or by papulosquamous skin lesions. Intermittent CLE shows non-scaling and non-scarring skin lesions. The last subset is chronic CLE which may be further subdivided into chronic discoid lupus erythematosus, chilblain lupus and lupus erythematosus profundus. Chronic discoid lupus erythematosus constitutes the largest group and features scarring erythrosquamous plaques in a disc-like shape. Chilblain lupus is a rare acral variant of chronic CLE whereas lupus erythematosus profundus affects the subcutaneous fat (11).

Despite clinical heterogeneity and although the initial stimulus may differ between diseases featuring ID, final common path is a cytotoxic anti-epithelial directed attack by autoreactive T lymphocytes (12–14) that are recruited to the site of inflammation by keratinocytes producing large amounts of C-X-C Motif Chemokine Ligands 9/10/11 (CXCL9/10/11) (15).

We herein summarize etiopathological mechanisms involved in ID and particularly outline the role of innate nucleic acid sensing in keratinocytes as a hallmark of perpetuation of the proceeding "pro-inflammatory vicious circle". We, furthermore, present a human *in vitro* model that functions as a tool to evaluate potential therapeutic interventions and thus facilitate prediction of therapeutic response to novel treatment strategies in diseases featuring ID.

INTERFACE DERMATITIS—THE PATHOGENIC BACKGROUND

Interferon Signaling and Cellular Response in Interface Dermatitis

A multitude of genes is differentially expressed similarly in both LP and CLE skin when compared to healthy skin. In particular, distinct associations have been described for genes concerning interferon signaling as well as associated downstream cascades (16–18). The type-I [IFNalpha(a)/beta(b)/kappa(k)] (1, 19–21) and type-III interferon system [IFNlambda(λ)] (22) do not only participate in antiviral immune defense, but also play an important pathophysiological role in ID. Particularly, they are expressed by respective lesional keratinocytes. Via autocrine loops, IFNs bind to their corresponding receptor on keratinocytes and unleash pro-inflammatory downstream cascades *via* activation of the JAK-STAT pathway (23–25). Finally, inflamed keratinocytes express CXCL9 (22), CXCL10 (26, 27) and CXCL11 (28). The corresponding CXCR3 receptors are expressed on activated pDCs (29), T cells [Th1-type CD4+ T cells (30) and effector CD8+ T cells (31–33)] and macrophages (34, 35). Hereby attracted pDCs contribute to the inflammation *via* further type-I interferon production (36, 37) and Th1 lymphocytes create a specific inflammatory milieu *via* secretion of distinct cytokines. T cells isolated from lesional skin of LP and CLE patients revealed high frequency of IFNy (IFN gamma) and TNFa, two key cytokines of Th1 lymphocytes (16). The type-II interferon, IFNy, sparks downstream cascades which partly overlap with those of type-I interferons (38): It induces CXCR3 ligands and the differentiation of naïve T cells into Th1 cells and it activates macrophages (39). In response to stimulation with IFNy and TLR (Toll Like Receptor) ligands macrophages undergo classical "M1" activation (40): This pro-inflammatory M1 phenotype is prevalently seen in rheumatic diseases (41) and has specifically been described in lichen planus (42) and lupus skin (43). Cytotoxic lymphocytes represent the

Abbreviations: AIM2, Absent In Melanoma 2; cGAS, Cyclic GMP-AMP synthase; CLE, Cutaneous lupus erythematosus; DAMP, Damage associated molecular patterns; DM, Dermatomyositis; ds, double stranded; eNA, endogenous nucleic acids; ID, Interface dermatitis; IFI16, Gamma-interferon-inducible protein 16; IFNa, Interferon alpha; IFNb, Interferon beta; IFNy, Interferon gamma; IFNk, Interferon kappa; IFN λ, Interferon lambda; IRF1/2/3, Interferon Regulatory Factor 1/2/3; ISREs, Interferon-sensitive response elements; LGP2, (Laboratory of Genetics and Physiology 2); LP, Lichen planus; LS, Lichen sclerosus; MDA5, Melanoma differentiation-associated protein 5; MxA, MX Dynamin Like GTPase A; PAMP, Pathogen associated molecular pattern; pDC, plasmacytoid dendritic cell; PRR, Pattern recognition receptors; RLR, RIG-I-like receptors; SLE, Systemic lupus erythematosus; ss, single stranded; STING, Stimulator of IFN genes; TBK1, TANK-binding kinase 1; TLR, Toll Like Receptor; ZBP1, Z-DNA Binding Protein 1.

last group of CXCR3 receptor carrying immune cells and execute their anti-epidermal attack *via* cytotoxic granules and the perforine/granzyme pathway (44). Apart from upregulation of genes mediating direct cytotoxicity, enhanced expression of markers of apoptosis (FASL) and necroptosis (RIP3) have been detected in ID (16).

Nucleic Acid Sensing Induces Interferon Response and Mediates Inflammasome Activation as Well as Cell Death Cascades

Inflammatory cell death upon cytotoxic attack inevitably results in release of intracellular components, amongst them are endogenous nucleic acids (eNA). Nucleic acid sensing by the innate immune system functions *via* pattern recognition receptors (PRRs), that are activated by pathogen associated molecular patterns (PAMPs) or host molecules (damage associated molecular patterns, DAMPs) (45). Physiologically, PRRs enable sufficient immune response to either an invading pathogen or damage of host cells (46, 47). Sensing of self-RNA and self-DNA, however, also holds the potential to contribute to autoimmunity (45, 48, 49). In ID, the pro-inflammatory capacity of released nucleic acids is supposedly supported by the cathelicidin LL37 which has been shown to be overexpressed in CLE (50, 51), LP (52), and DM (53). Its complex formation with nucleic acids has been proven to enable transport of extracellular nucleic acid fragments into intracellular compartments (54). Specifically, our working group has previously shown that addition of LL37 enhances immunogenicity of nucleic acids in keratinocytes *in vitro* (50). Key features of downstream signaling of PRRs include induction of interferons (45, 46, 55) and inflammasome activation (56) as well as programmed cell death cascades (46).

The respective downstream mechanisms of important PRRs are as follows:

AIM2 (Absent In Melanoma 2) activates the inflammasome upon double stranded (ds) DNA sensing (57–59) which leads to Caspase 1 cleavage and finally maturation of the pro-inflammatory interleukins IL18 and IL1ß (60). Furthermore, activated Caspase 1 cleaves Gasdermin D which executes pyroptosis *via* pore formation in affected cell membranes (61). AIM2 is upregulated in skin samples of lichen planus patients (62). Inflammasome activity is enhanced in lupus erythematosus (63) and the inflammasome-activated cytokine IL18 is highly upregulated in the epidermis of CLE patients (64). The discovery of its dysregulation in autoimmunity suggests inhibition of inflammasome components as an interesting therapeutic approach, as postulated by Kahlenberg et al. (65).

Upon DNA binding to cGAS (Cyclic GMP-AMP synthase) an IFN response is unleashed (66): CGAS activates STING (Stimulator of IFN genes) (67, 68) which, in turn, interacts with TBK1 (TANK-binding kinase) resulting in phosphorylation of IRF3 (Interferon Regulatory Factor) and finally type-I interferon gene transcription (69). Furthermore, the cGAS-STING pathway has multiple functions in mediating cell death that are not fully elucidated, yet (46). Its activation by self-DNA is described as an important mechanism in autoimmunity which might constitute another promising target for therapeutic intervention (49).

IFI16 (Gamma-interferon-inducible protein 16) is a further key DNA sensor in human keratinocytes. It cooperates with cGAS in the activation of STING (70). Excessive IFI16-dependent production of IFN-I is considered an important mediator of autoimmune inflammation (71, 72) and has specifically been shown to contribute to SLE (73) and to cytokine induction in keratinocytes (74). Apart from STING-dependent type-I IFN production, IFI16 enables direct inflammasome activation (75, 76).

ZBP1 (Z-DNA Binding Protein) binds to ds nucleic acids when presenting in the unusual Z-conformation (77). Activated ZBP1 recruits TBK1 and IRF3 (78) and triggers RIP3-dependent necroptosis (79) as well as NLRP3-dependent inflammasome activation (80). Specifically, aberrant sensing of endogenous nucleic acids by ZBP1 has been shown to induce inflammation in murine skin (81). Guo et al. have shown that ZBP1 activation also induces necroptosis in human cells (82) and sera from some SLE patients exhibit anti-Z-DNA autoantibodies (83). Thus, ZBP1 has been suggested as a potential therapeutic target that requires further research (84).

RIG-I-like receptors (RLRs) comprise three important sensors: RIG-I, MDA5 (Melanoma differentiation-associated protein), and LGP2 (Laboratory of Genetics and Physiology 2) (85) with the latter being considered a regulator of the others. RIG-I and MDA5 experience conformational changes upon cytosolic RNA sensing that result in exposure of their CARD domain and consecutive activation of IRF3 *via* phosphorylation of TBK1 and NFkB activation (85, 86). RLRs are also implicated in apoptosis and RIP3-mediated necroptosis (87, 88). Human keratinocytes constitutively express RIG-I and MDA5 (89). Challenge with IFNy or TNFa has induced RIG-I in a human keratinocyte cell line (90) and both RIG-I and MDA5 expression is increased in psoriatic skin (90). A specific single nucleotide polymorphism in the gene encoding MDA5 has been identified in autoimmune diseases, including SLE (91, 92).

Nucleic-acid-sensing TLRs are mainly expressed in the endosomes (93) and comprise TLR3 [recognizes dsRNA (94)], TLR7 and TLR8 [recognize ssRNA (95)] and TLR9 [recognizes unmethylated CpG-containing DNA motifs (96)]. Activation of these TLRs, with the exception of TLR3, incorporates MyD88 to the respective receptor complex which subsequently interacts with TRAF6 leading to nuclear translocation of NFkB (97) and type-I IFN induction (98). TLR3 alternatively signals *via* the adaptor TRIF which activates TBK1 and subsequently leads to type-I IFN induction *via* phosphorylation of IRF3 (97). Furthermore, TLR3 signaling can activate cell death cascades by engaging RIP1 and RIP3 (99). Keratinocytes, constitutively express TLR 3 and 9 and their stimulation with corresponding ligands results in induction of TNFa and type-I IFN as well as ICAM1 (100). Interestingly, in oral LP, induction of TLR9 has been described (101). Although keratinocytes do not constitutively express TLR7 or 8, several case reports describe individuals who have developed LP and LS upon use of Imiquimod, an agonist of TLR7/8 (102–104) which is possibly explained by keratinocytes expressing TLR7 under specific conditions (105).

AN *IN VITRO* MODEL TO STUDY INTERFACE DERMATITIS

Background

In 2016, our working group has first established an *in vitro* model that mirrors our understanding of ID as being fueled by endogenous nucleic acids (106) and further characterized it within a study from 2017 (50). Stimulation with eNA results in a pronounced expression of typical ID-associated cytokines within different keratinocyte models. IFNy, mainly produced by lymphocytes, is known to play a pivotal role in the pathogenesis of diseases featuring ID (107–109), and has been shown to induce typical morphological changes in human epidermis equivalents, *in vitro* (110). Herein, we aim to deliver an in-depth analysis of differentially regulated genes in our ID model and furthermore present synergistic effects of endogenous nucleic acids in addition to IFNy on human keratinocytes.

Results

Cytosolic Localization of DNA Motifs in Interface Dermatitis Keratinocytes

DNA motifs in extranuclear compartments were significantly more present in ID than in healthy control samples (**Figure 1A**).

CXCL10 and MxA are Expressed by Keratinocytes in Interface Dermatitis and the Majority of Infiltrating Immune Cells Express CXCR3 Receptors

Figure 1A depicts findings within a LP skin specimen that are representative for all examined samples: MxA (MX Dynamin Like GTPase A) and CXCL10 are expressed by keratinocytes and the majority of infiltrating immune cells carries CXCR3 receptors.

Stimulation With Endogenous Nucleic Acids Induces a Molecular Signature in Keratinocytes Resembling Interface Dermatitis

In normal human epidermal keratinocytes (HEK), stimulation with eNA significantly induces expression of genes encoding key drivers (111–117) of innate inflammatory pathways (IRFs, IFNs, STAT2, RELA, NFkB, CXCL9/10/11, Mx1, OASL), inflammasome activation (AIM2), cell death (RIP3) and factors mediating interaction between keratinocytes and T cells (ICAM1) as well as the adaptive immune system (BLyS) (**Figure 2A**).

Stimulation With IFNy and Endogenous Nucleic Acids Induce CXCL10 and MxA Expression

Stimulation with IFNy or eNA respectively leads to significant expression of CXCL10 in HaCaT cells. Combined administration of IFNy and eNA results in an over-additive effect concerning CXCL10 release (**Figure 2B**). Strong CXCL10 expression upon concomitant stimulation could be confirmed in normal human epidermal keratinocytes (HEK, **Figure 2C**) and in reconstructed human epidermis equivalents (epiCS, **Figure 2D**). Furthermore, MxA expression is induced upon combined stimulation in epiCS whose staining resembles the pattern detected in ID (compare **Figure 1A**).

Concomitant Stimulation with IFNy and Endogenous Nucleic Acids Has a Direct Cytotoxic Effect on Keratinocytes

Cytotoxic effects in our approach were not significant upon stimulation with eNA or IFNy alone. Upon concomitant stimulation, however, the ability to reduce MTT reagent into its insoluble formazan was significantly impaired in both HaCaT (**Figure 2B**) and HEK (**Figure 2C**) serving as a marker for cell viability.

Methods

Please find a description of applied methods as a supplement to this main text.

DISCUSSION

Our working group has previously described extranuclear DNA motifs being significantly more present in keratinocytes of CLE patients than in healthy skin (50). We herein present analogous findings in LP patients. *In vitro*, stimulation with endogenous nucleic acids induces a gene expression pattern in human keratinocytes which resembles key features of ID: We present IFNλ induction upon stimulation with eNA in keratinocytes, which is known to be significantly elevated in skin diseases featuring ID but neither in healthy controls nor other inflammatory skin diseases (22). The type-I IFNs, IFNb and IFNk, are both induced in keratinocytes upon stimulation with eNA. IFNb has been described to be expressed in basal epidermal layers of LP (118) skin. IFNk has been shown to be highly expressed in CLE (19) and LP skin but not in other inflammatory dermatoses (119) and is acknowledged to be a key regulator of IFN response in keratinocytes. Stimulation with eNA did not upregulate expression of IFNa. Although it has been detected in keratinocytes of the whole epidermis in LP skin (118), pDCs are considered to be the main producers of IFNa *in vivo* (17, 36, 120). Via autocrine loops, all type-I IFNs bind to IFNAR (23, 24, 121) and type-III IFNs signal *via* their receptor IFNLR (24). Activation of both receptors causes phosphorylation of JAK1 and TYK2 (25). Receptor bound STATs (STAT1 and STAT2) are subsequently phosphorylated leading to heterodimerization and formation of ISGF3 together with IRF9 (122). This complex translocates to the nucleus and induces expression of genes that exhibit specific ISREs (Interferon-sensitive response elements) to which the complex binds. Amongst such genes are OAS, MxA and multiple transcription factors, including IRFs (24, 38) which are induced upon eNA-stimulation in our experiments. While IFNAR is expressed on nearly all cell types and IFNLR is mainly restricted to epithelial cells, their downstream signaling is quite congruent (24). Type-I IFN dependency (24), however, is described for ISGF3-like complex formation, which consists of

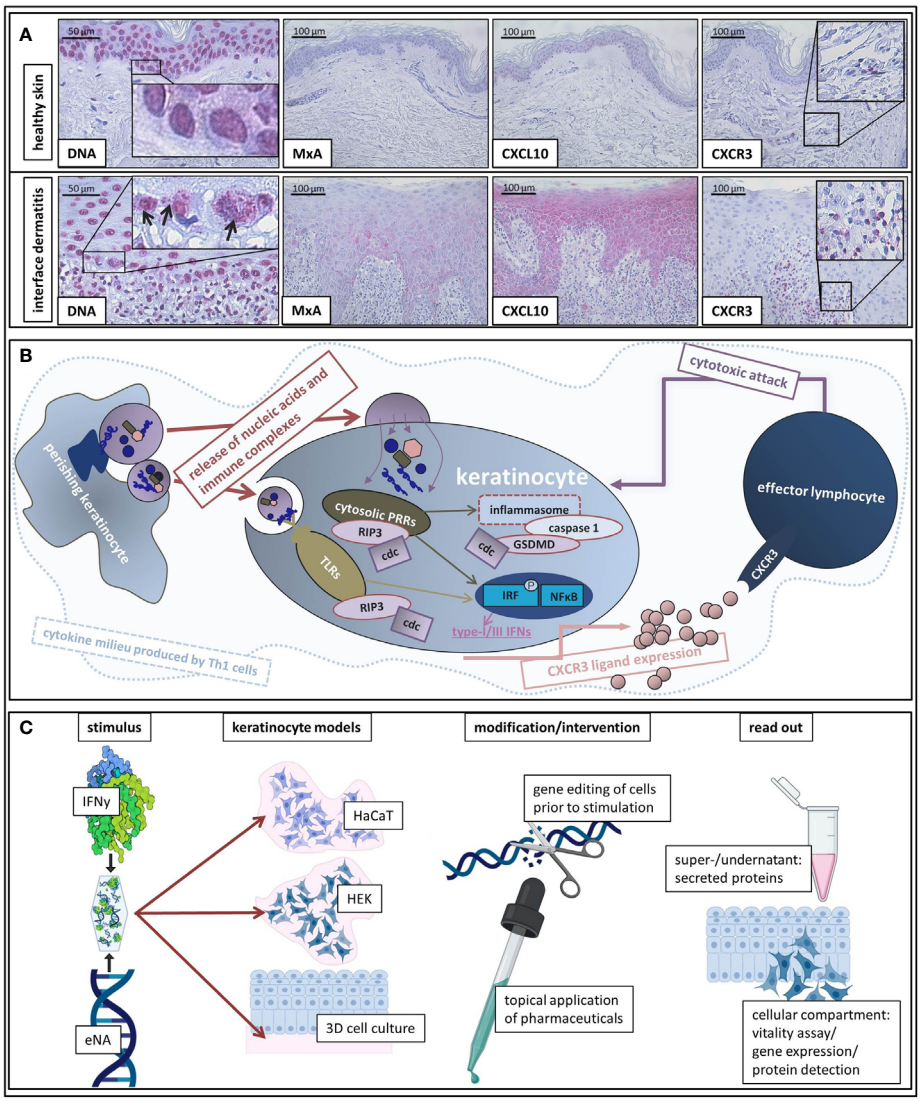


FIGURE 1 | **(A)** Representative histological findings in interface dermatitis and healthy skin. Representative findings of DNA, MxA, CXCL10, and CXCR3 immunostaining in healthy skin and interface dermatitis (lichen planus). Original magnification x200 (x400 concerning DNA). Black arrows highlight extranuclear localization of DNA motifs. Boxes highlight digitally enlarged aspects. **(B)** Schematic presentation of assumed etiopathological mechanisms of interface dermatitis (as reviewed above). ID is characterized by a Th1 cytokine milieu in which endogenous nucleic acids activate PRRs. Downstream signaling unleashes cell death cascades (cdc) and leads to production of type-I and -III IFNs and pro-inflammatory cytokines as well as inflammasome activation. Interferon-inducible chemokines (produced by keratinocytes upon autocrine IFN-stimulation) recruit CXCR3 + effector cells into lesional skin, which induce keratinocyte perishing and thus release of pro-inflammatory cell components. **(C)** Schematic presentation of our *in vitro* model of interface dermatitis. Nucleic acids extracted from keratinocytes and IFNy are administered to different keratinocyte models (HaCaT, HEK, epiCS) as an ID-like stimulus. Genetic modification of the cells of interest can be made prior to stimulation in order to evaluate specific components of ID pathogenesis. Furthermore, the effect of innovative pharmaceuticals on ID-like stimulation can be analyzed. Super-/undernatants and the cellular compartment are available to read out methods.

IRF9 and STAT2 homodimers and can reinstate inflammatory cascades in the absence of STAT1 (123).

In our approach, type-II IFN was not induced in keratinocytes upon eNA-stimulation, which is in accordance with the view that the pronounced presence of type-II IFN (IFNy) in ID skin derives from other sources than keratinocytes. Specifically, it is predominantly produced by natural killer cells, group 1 innate lymphoid cells, yδ T cells and CD8+ cytotoxic T cells as well as CD4+ Th1 cells [as reviewed in (124)]. Its receptor (IFNyR) signals *via* the JAK1/JAK2 and STAT1/STAT2 pathway (107, 125). Shao et al. describe that, *in vitro*, priming of keratinocytes with type-I IFNs, and to an even greater extent type-II IFNs, increases their susceptibility to MHC I-dependent, T-cell mediated cytotoxicity (107). Knock out of JAK2 or STAT1 inhibited this induction of MHC I in keratinocytes upon IFNy-stimulation whereas only minimal suppression was detected in JAK1 or STAT2 KO cells (107). The potential of human keratinocytes as nonprofessional antigen-presenting cells has recently been further

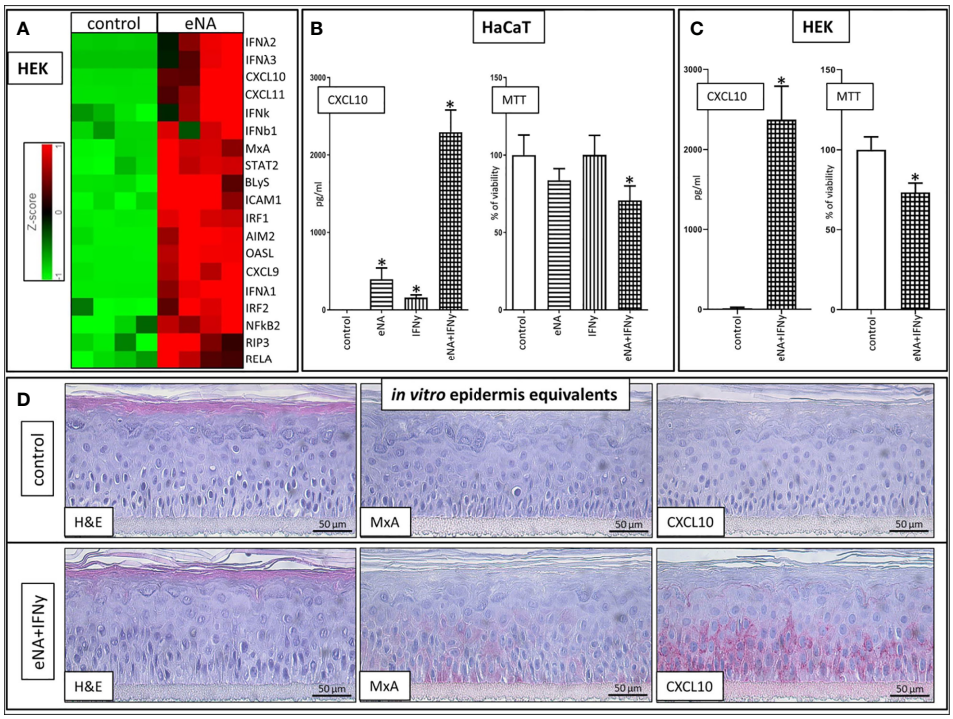


FIGURE 2 | **(A)** Expression of upregulated genes involved in ID pathogenesis in HEK cells stimulated with eNA (12,5 µg/ml) for 24 h compared to HEK cells solely exposed to medium (control), (n = 4, fold change > 2, p < 0.01, Partek® Flow®). **(B)** CXCL10 levels within the supernatant of HaCaT cells stimulated with eNA (5 µg/ml) or IFNγ (1 x 10^3 U/ml) or the combination of both for 20 h compared to HaCaT cells solely exposed to medium (control), MTT assay executed on the cells corresponding with the respective supernatant, mean of controls defined as 100%. Given are respective means with standard deviations indicated by error bars (n = 4, * indicates significance (p < 0.05), Mann Whitney test). **(C)** CXCL10 levels within the supernatant of HEK cells stimulated with the combination of eNA (5 µg/ml) and IFNγ (1 x 10^3 U/ml) for 6.5 h compared to HEK cells solely exposed to medium (control), MTT assay executed on the cells corresponding with the respective supernatant, mean of controls defined as 100%. Given are respective means with standard deviations indicated by error bars (n = 4, * indicates significance (p < 0.05), Mann Whitney test). **(D)** Representative findings in 3D epidermis equivalents upon control (medium) settings and stimulation with eNA (5 µg/ml) and IFNγ (1 x 10^3 U/ml) for 22 h. Hematoxylin and eosin stain, MxA, CXCL10. Original magnification ×400. (n = 3).

underlined by Orlik et al. who have demonstrated the capacity of IFNγ-pretreated keratinocytes to activate co-cultured naïve T-cells (126). ICAM1 is a further mediator supporting interaction between T lymphocytes and keratinocytes that is inducible by IFNγ (127) and is overexpressed in diseases featuring ID (128). Our data, in turn, shows that ICAM1 is also induced upon stimulation with eNA. Furthermore, cell death cannot only be induced *via* activation of infiltrating immune cells but also *via* induction of keratinocytic apoptosis (cleaved caspase 3) and necroptosis (RIP3) as both factors have been shown to be overexpressed in keratinocytes of LP and CLE patients (16, 107). These markers can be induced by IFNγ (107) and although stimulation with eNAs alone does not result in a significant reduction of cell viability as measured by vitality assay, cell death cascades are activated upon stimulation with eNA that mimic the ones described in ID as we detected induction of RIP3 and the inflammasome component AIM2. Furthermore, significant cytotoxicity is detectable upon concomitant stimulation of keratinocytes with eNA and IFNγ. A cross-talk by keratinocytes to the adaptive immune system is mediated by BLyS, a B lymphocyte survival factor (129) which has been described to be overexpressed in CLE and LP (130). We herein show that it is induced upon stimulation with eNA. Another mediator implicated in immune and inflammatory responses is NFkB that has been shown to be among the top regulated genes shared by LP and CLE (16). This crucial transcriptional factor family comprises NFkB1, NFkB2, RELA, RELB, and C-REL (131): Stimulation with eNA induces this important mediator in keratinocytes. As reviewed above, expression of CXCR3 ligands by lesional keratinocytes is decisive for attraction of effector cytotoxic T cells. CXCL10 (26, 27) and CXCL11 (28) have been shown to be inducible by type-I interferons. CXCL9, on the other hand, has repetitively been described as truly dependent on IFNγ (26, 132). Our group, however, has demonstrated CXCL9 induction in keratinocytes upon stimulation with the type-I interferon IFNk earlier (22). Furthermore, a recent study has detected CXCL9 expression in keratinocytes as a result of inflammasome activation (133). As outlined above, inflammasome activation is another major pathway upon sensing of nucleic acids that might explain expression of all three CXCR3 ligands by stimulation with eNA in the absence of externally administered IFNγ.

Stimulation of keratinocytes with endogenous nucleic acids induces key mediators of ID. According to the mechanisms discussed above, we are convinced that addition of IFNγ to the interface dermatitis model promotes an even more realistic

imitation of *in vivo* scenarios. Concomitant stimulation of keratinocytes with endogenous nucleic acids and IFNγ not only promoted direct cytotoxicity but also caused an overadditive effect on CXCL10 level elevation.

OUTLOOK

Lichen planus as well as cutaneous lupus erythematosus go along with a high disease burden and are considered therapeutically challenging because current treatments often fail to achieve disease control (134–136). We are convinced that preclinical studies and clinical trials evaluating innovative future therapeutic approaches should not focus on one particular condition but rather on clusters of diseases featuring common immune response patterns. Our working group has recently successfully employed the here described model to elucidate the influence of JAK inhibition on keratinocytes in an interface-dermatitis-like context (121). In the herein described refined version of the model IFNγ mimics the presence of a T-helper cell mediated cytokine milieu and together with eNA synergistically intensifies the resulting pro-inflammatory signature. Our model represents pathomechanistic key features of ID and thus enables evaluation of potential future pharmaceuticals. It might aid in predicting therapeutic response to novel treatment strategies in therapeutically challenging diseases featuring ID.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Bonn, Venusberg-Campus 1, 53127 Bonn. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CB designed the study. CB, LD, and TF performed the experiments. CB and JW analyzed the data. DN and TB contributed essential resources during manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.622511/full#supplementary-material

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REVIEW
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Dirty Fish Versus Squeaky Clean Mice: Dissecting Interspecies Differences Between Animal Models of Interferonopathy

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Autoimmune and autoinflammatory diseases are rare but often devastating disorders, underpinned by abnormal immune function. While some autoimmune disorders are thought to be triggered by a burden of infection throughout life, others are thought to be genetic in origin. Among these heritable disorders are the type I interferonopathies, including the rare Mendelian childhood-onset encephalitis Aicardi-Goutières syndrome. Patients with Aicardi Goutières syndrome are born with defects in enzymes responsible for nucleic acid metabolism and develop devastating white matter abnormalities resembling congenital cytomegalovirus brain infection. In some cases, common infections preceded the onset of the disease, suggesting immune stimulation as a potential trigger. Thus, the antiviral immune response has been actively studied in an attempt to provide clues on the pathological mechanisms and inform on the development of therapies. Animal models have been fundamental in deciphering biological mechanisms in human health and disease. Multiple rodent and zebrafish models are available to study type I interferonopathies, which have advanced our understanding of the human disease by identifying key pathological pathways and cellular drivers. However, striking differences in phenotype have also emerged between these vertebrate models, with zebrafish models recapitulating key features of the human neuropathology often lacking in rodents. In this review, we compare rodent and zebrafish models, and summarize how they have advanced our understanding of the pathological mechanisms in Aicardi Goutières syndrome and similar disorders. We highlight recent discoveries on the impact of laboratory environments on immune stimulation and how this may inform the differences in pathological severity between mouse and zebrafish models of type I interferonopathies. Understanding how these differences arise will inform the

improvement of animal disease modeling to accelerate progress in the development of therapies for these devastating childhood disorders.

Keywords: type I interferonopathy, Aicardi-Goutières syndrome, autoimmune disease, RNASET2, zebrafish, mouse, white matter disorders, leukodystrophy

INTRODUCTION

Type I interferons (IFNs) play an essential role in the antiviral innate immune response—protecting the host from productive viral infection before the development of adaptive immune response to pathogens (1, 2). Upon detection of foreign nucleotides in the host, canonical type I IFN signaling activates a number of pathways—ultimately leading to upregulation of interferon-stimulated genes (ISGs) and wide-ranging effects comprising host defense (2, 3). However, while type I IFN signaling is protective in response to active viral infection, aberrant activation of this pathway has been suggested to occur in autoinflammatory disease, triggered by genetic mutations in the host (1, 4).

The association between upregulation of type I IFN and autoimmune/autoinflammatory disease was first proposed following the observation of overlapping phenotypes between such disorders and congenital HIV-1 infection (5). Following subsequent genetic characterization, a distinct grouping of disorders has emerged, in which disturbance of the homeostatic control of type I IFN response—and subsequent upregulation of ISGs—due to Mendelian mutations is central to pathogenesis (4, 6, 7). Now collectively referred to as the type I interferonopathies, this group includes the chronic autoimmune disease systemic lupus erythematosus (SLE), the inherited encephalopathy Aicardi-Goutières syndrome (AGS) and a range of often rare but devastating conditions (4).

In this review, we focus specifically on AGS and the closely related RNaseT2-deficient leukodystrophy. Alongside the prominent inflammatory phenotype typical of type I interferonopathies, these disorders present with devastating neurological phenotypes which are not only debilitating to patients but have proven particularly difficult to recapitulate in animal models (8). Accurate, valid animal models are essential for the development of novel therapies: thus far, the translational impact of animal models of interferonopathies has been vastly limited by the lack of neuropathology in preclinical settings. Here, we summarize the human phenotype of AGS and RNaseT2-deficient leukodystrophy and provide a brief overview of the human genetics involved in these disorders. For each of these interferonopathy-linked genes, we analyze the relevance of existing animal models to the human condition, comparing and contrasting models of different species. Finally, we propose that key environmental modulators—namely, early life viral exposure—may account for the differences in phenotype across species and suggest how this theory could be tested to inform our understanding of the human condition.

Abbreviations: AGS, Aicardi Goutières syndrome; CRISPR, clustered regularly interspaced short palindromic repeats; DNA, deoxyribonucleic acid; HIV, human immunodeficiency virus 1; IFN, interferon; ISG, interferon stimulated gene; LINE-1, long interspersed nuclear element 1; MRI, magnetic resonance imaging; RNA, ribonucleic acid; RTI, reverse transcriptase inhibitor; SLE, systemic lupus erythematosus.

THE GENETICS OF AICARDI–GOUTIÈRES SYNDROME AND RELATED INTERFERONOPATHIES

Of all the conditions now recognized as type I interferonopathies, AGS is perhaps among the most extensively characterized. Although rare, patients with this progressive encephalopathy present with severe intellectual, speech and motor disability in infancy—often mimicking aspects of congenital viral infection (7, 9). Clinical phenotypes become apparent within the first year of life for most patients, with disease onset thought to occur *in utero* in up to one in five patients (10). Although symptoms and severity vary, most individuals with AGS present with one of several "classical" clinical presentations—most commonly including white matter disease, intracranial calcification and microcephaly—although additional genetic subtype-specific pathological hallmarks have also been characterized (**Table 1**) (7). Regardless of mutation, patients with AGS show consistent and significant upregulation of type I IFN and ISG expression—supporting their classification as a type I interferonopathy.

To date, seven genes have been identified as the genetic trigger for different subtypes of AGS (AGS1–7), each of which encode proteins involved in detecting or metabolizing nucleic acids and particularly in restricting reverse transcription (see **Figure 1**) (7). Along with the viral-like phenotype of AGS patients, this has led to the hypothesis that type I IFN is triggered by the accumulation of self-derived nucleotides from endogenous retroelements in some AGS patients (44). In support of this, preclinical and initial clinical studies have suggested that reverse transcriptase inhibitors (RTIs) may have clinical benefits in AGS (7, 45, 46). However, the effects of RTIs on neurological phenotype remains unclear: firstly, because the animal models utilized in these preclinical studies do not develop neuroinflammation even before treatment and, secondly, because the patients enrolled in clinical trials had significant impairments at baseline, such that improvement was not to be expected trials (7, 46). Arguably the core component of disease, much remains to be understood about the neuropathology of AGS: how it develops, why it varies between patients and, ultimately, how it can be treated.

In addition to AGS1–7, mutations in *RNASET2* have been linked to a closely related interferonopathy in human patients, with a similar neurological and inflammatory phenotype: RNaseT2-deficient leukoencephalopathy. Much like in AGS,

TABLE 1 | Summary of animal model phenotypes in interferonopathy research.

	Human	Mouse	Zebrafish
TREX1 *(AGS 1)* [loss-of-function]			
Immunological	Upregulation of ISG transcripts (10, 11)	Severe multiorgan inflammation; inflammatory myocarditis; IFN-dependent pathology (12–14)	*[n/a]*
Neurological	White matter abnormalities and intracranial calcification; abnormal sensorimotor development (7)	None reported (14)	*[n/a]*
RNASEH2A, -B and -C (AGS2, -3 and -4) [loss-of-function]			
Immunological	Upregulation of ISG transcripts in some patients (10, 11)	Evidence of upregulated ISG expression (15–17)	*[n/a]*
Neurological	White matter abnormalities and intracranial calcification; abnormal sensorimotor development; non-syndromic spastic paraparesis (7, 18, 19)	None reported (16)	*[n/a]*
SAMHD1 (AGS5) [loss-of-function]			
Immunological	Upregulation of ISG transcripts (10, 11)	Upregulation of ISG transcripts, not reflected at protein level (20–22)	Upregulation of type I IFN, ISGs, and other genes involved in innate immunity (23)
Neurological	White matter abnormalities and intracranial calcification; abnormal sensorimotor development; intracerebral, large vessel disease (intracerebral hemorrhage and infarcts) (7, 24)	None reported (20–22)	Cerebral hemorrhage; cerebral oedema (23)
ADAR1 (AGS6) [loss-of-function]			
Immunological	Upregulation of ISG transcripts (10, 11)	Upregulation of ISG transcripts; embryonic lethal (25–30)	Increased expression of ISGs and other genes involved in innate immunity (23)
Neurological	White matter abnormalities and intracranial calcification; abnormal sensorimotor development; bilateral striatal necrosis; non-syndromic spastic paraparesis (7, 19, 31)	None reported; embryonic lethal (25–27)	Severe developmental defects (23)
IFIH1 (AGS7) [gain-of-function]			
Immunological	Upregulation of ISG transcripts (10)	Severe multiorgan inflammation; upregulated ISG expression (32)	No published gain-of-function mutation; loss-of-function mutation restores expression of immune-regulated genes to wild type levels in mutants with upregulated interferon response (33)
Neurological	White matter abnormalities and intracranial calcification; abnormal sensorimotor development; non-syndromic spastic paraparesis (7, 19)	None reported (32)	None reported (33)
RNASET2 [loss-of-function]			
Immunological	Phenotype mimicking cytomegalovirus infection (34) Upregulation of ISGs in some patients (18, 35)	Neuroinflammation [see below]; no evidence of systemic inflammation (36)	Upregulation of ISG transcripts including isg15 (37, 38)
Neurological	White matter abnormalities, intracranial calcification; subcortical cysts (34)	Enlarged hippocampus and prefrontal cortex; increased reactive astrocytes in hippocampus (36)	White matter abnormalities beginning during embryogenesis (microglial dysfunction); locomotor defects (37, 38)

patients with mutations in *RNASET2* present with psychomotor impairments, micro- or normocephaly and spasticity—mimicking congenital cytomegalovirus infection (34, 35). As with AGS-associated genes, RNaseT2 is involved in the sensing of nucleic acids—either endogenous or virally derived. Thus, we believe discussion of RNaseT2-deficient leukodystrophy alongside AGS in the context of interferonopathy here is warranted.

Recapitulating AGS and RNaseT2-deficient leukoencephalopathy phenotypes in animal models could provide crucial insights into neuropathology and invaluable preclinical therapy development. However, as mentioned above, the translational impact of these models remains minimal—largely as many of these models do not develop neurological abnormalities. Understanding why preclinical models have failed to fully recapitulate the human phenotype is essential to furthering our understanding of interferonopathy progression.

ANIMAL MODELS OF INTERFERONOPATHIES

With such distinct and well-documented genetic underpinnings, it is unsurprising that preclinical research in AGS and related interferonopathies has focused on monogenic animal models. Much of this research has been conducted in rodent and zebrafish models of disease—with mouse models largely dominating the field until recent years. The overall merits of these model organisms in interferonopathy and, specifically, leukodystrophy research have been reviewed elsewhere (8).

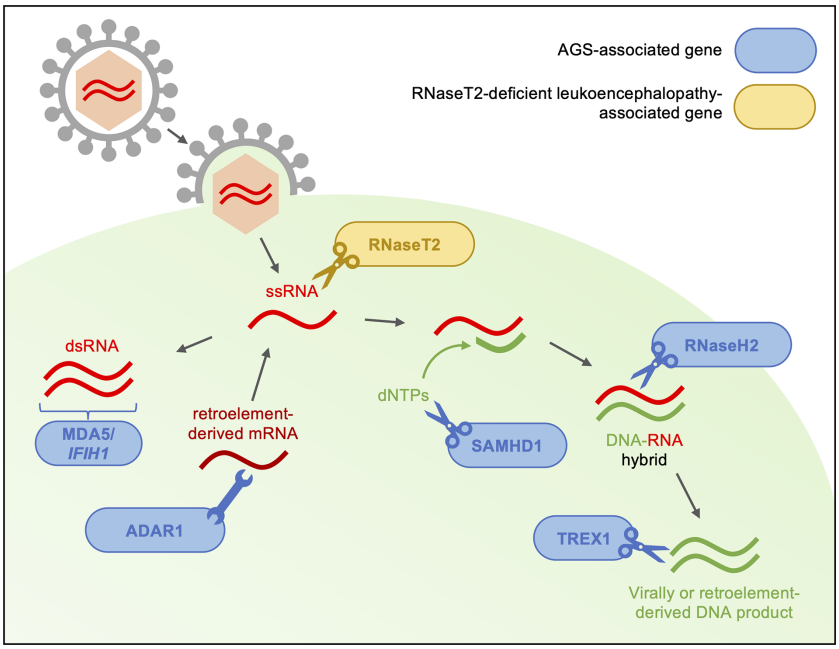


FIGURE 1 | Type I interferonopathy-associated genes are involved in the sensing and metabolism of viral RNA. Genes linked to AGS and RNaseT2-deficient leukoencephalopathy are thought to encode proteins involved in the restriction of reverse transcription of both viral- and endogenous retroelement-derived DNA. The *IFIH1* gene product, MDA5, is involved in the antiviral response through the recognition of dsRNA and subsequent production of type I interferon. With interferon-inducible expression, ADAR1 acts as a suppressor of type I IFN signaling through its RNA editing activity. RNaseT2 is a lysosomal hydrolase involved in RNA metabolism. SAMHD1 limits reverse transcription though degradation of deoxynucleotides necessary for complementary DNA strand formation. Among other roles in DNA synthesis and repair, RNaseH2 is thought to degrade the RNA component of DNA-RNA hybrids formed during reverse transcription. Finally, TREX1 is involved in the regulation of the interferon-stimulatory DNA response after viral infection through metabolism of virally derived nucleotides. In the absence of functioning AGS or RNaseT2 proteins, accumulation of immunostimulatory deoxyribo- and ribonucleotides may trigger upregulation of type I interferon pathway (6, 7, 12, 25, 32, 37, 39–43).

Here, we provide an overview of the phenotypes of currently available mouse and zebrafish models by gene, focusing on their relevance to the human clinical presentation (**Table 1**).

TREX1 (AGS1)

With roles in antiviral response and metabolism of intracellular RNA, the gene encoding 3' repair exonuclease, TREX1, was the first to be linked to AGS (12, 39). Accordingly, the phenotype of Trex1-deficient mice is perhaps the most extensively characterized of all AGS-associated models. *Trex1*$^{-/-}$ mice develop multiorgan inflammation—predominated by inflammatory myocarditis—and limited survival due to circulatory failure (13, 14). Crucially, however, *Trex1*$^{-/-}$ mice do not display any evidence of neuropathology—in fact, the brain appears to be one of the few tissues which does not develop an inflammatory phenotype (14). It is unclear why the brain appears to be protected from pathology in this manner—limiting the utility of Trex1-deficient mice as a preclinical model of AGS.

In addition to their links with AGS, mutations in *TREX1* (and *SAMHD1*, discussed below) have been associated with other autoimmune disorders which are characterized by a more widespread inflammatory phenotype and less prominent neurological involvement (4, 47–50). Although clinical overlap between SLE and AGS has been reported in some patients, it remains unclear why some patients with *TREX1* mutations develop a phenotype dominated by neuroinflammation and others do not (5, 51–53). Therefore, *Trex1*$^{-/-}$ mice may better reflect the SLE phenotype and should therefore be considered a more useful model of this disorder, rather than AGS.

Perhaps the development of complementary zebrafish models could further elucidate the role of TREX1 in AGS neuropathology. Human *TREX1* and *TREX2* are co-orthologous with zebrafish genes *trex3* and *trex4*. Interestingly, *trex3* expression is upregulated in zebrafish injected with type I IFN, suggesting this gene is an ISG and may act as a functional orthologue to TREX1 (54). Hence, experimental manipulation of *trex3* expression may be informative about the role of its human equivalent in AGS.

RNase H2A, -B, and C (AGS 2, 3, 4)

Composed of three subunits, the ribonuclease H enzyme (RNaseH2) complex has roles in DNA synthesis and repair, including LINE-1 retrotransposition (44). Together, mutations in *RNaseH2A, -B* and *-C* account for over 50% of cases of AGS—demonstrating a clear role for this gene in interferon-induced pathogenesis (55). While characterization of an *rnaseh2* zebrafish model is yet to be published, several mouse models have been generated to dissect the role of RNaseH2 in the neurological and inflammatory phenotype of AGS—yet, none have fully

recapitulated the human disease (15–17, 56, 57). While hypomorphic models (with point mutations in single subunits) have demonstrated some upregulation of ISG expression, perhaps the model with the greatest face validity is the RNaseH2$^{\Delta GFAP}$ mouse—a brain-specific knockout, lacking RNase H2 specifically in astrocytes and neurons (15–17). Astrocytes cultures from these animals demonstrated increased ISG transcript levels, along with signs of DNA damage and premature senescence—consistent with a type I interferon response (16). However, this was not accompanied by any evidence of neuroinflammation or overt neurological phenotype in the whole animal—failing to recapitulate the key components of human disease.

SAMHD1 (AGS5)

In accordance with the reverse transcription theory of AGS pathogenesis, *SAMHD1* encodes a protein involved in restricting viral DNA synthesis—degrading the intracellular deoxynucleotides needed for reverse strand formation and therefore limiting both viral DNA replication and retrotransposon transcription (7, 58, 59). Mutations in *SAMHD1* are thought to account for around 13% of AGS mutations and have been linked to several other interferonopathies—demonstrating a clear link between SAMHD1 dysfunction and autoimmunity (4, 10, 60).

While there is considerable variation in the clinical phenotypes of AGS patients—regardless of their genotype—patients with *SAMHD1* mutations present with a somewhat distinct phenotype, with intracerebral large vessel disease being a hallmark of pathology which can present as cerebral arterial stenosis, intracerebral hemorrhage or other cerebrovascular abnormalities such as moyamoya presentation (7). Cerebral hemorrhage has been recapitulated by zebrafish models, following knockdown of *samhd1* using antisense morpholinos (23). These animals present with swelling of the hindbrain ventricle and cerebral hemorrhage during embryogenesis. This neurophenotype is accompanied by upregulated expression of a panel of genes known to be involved in IFN-mediated antiviral response—including *isg15* (an interferon-stimulated gene known to be involved in the zebrafish immune response)—suggesting knockdown of *samhd1* induces an interferon response in zebrafish models mimicking the human state (61).

In contrast, SAMHD1 knockout mice fail to develop both the neurological and immunological components of the human AGS neurological phenotype—remaining healthy into adulthood with no evidence of autoinflammatory pathology (20–22). While ISG transcripts are upregulated in these animals, this is not mirrored at a translational level—with no observable difference in ISG products or IFN proteins across multiple tissues, alongside a lack of inflammatory pathology in the heart and skin.

It is curious that reduced (but not abolished) expression of *samhd1* in zebrafish leads to a more extreme neurological phenotype than complete knockout in mouse models. It has been suggested that the function of murine Samhd1 may differ from that of the human and zebrafish orthologue—perhaps with lesser involvement in the innate immune response to nucleic acids in mice than the other species (22). Conversely, it is possible that a compensatory mechanism exists in the mouse that is not present in humans or zebrafish, suppressing the IFN response and preventing the formation of a neurological phenotype as might be expected in knockout mice (23). Nonetheless, the finding that zebrafish models of AGS better recapitulate the human SAMHD1-linked neurological phenotype relative to their murine counterparts raises interesting questions about the use of these species in interferonopathy modeling.

ADAR1 (AGS6)

Like SAMHD1, ADAR1 has been proposed to be involved in the restriction of reverse transcription due to its intrinsic RNA editing activity (7). Unlike other ADAR isoforms, *ADAR1* expression is interferon-inducible, with a prominent role as a suppressor of type I IFN signaling (6, 25, 40). Both mouse and zebrafish models have been generated to dissect the role of ADAR1 in interferonopathy pathology, with limited success.

Several *Adar1* knockout and mutant lines have been investigated in mice, many of which die during embryogenesis or early life (25–30). Characterization of embryonic lethal *Adar* null mutants revealed upregulation of ISG expression, which could be partially rescued through mutation of *Ifnar1* (IFN-α and -β receptor 1) and fully rescued by mutation of *MAVS*—a key adaptor protein involved in antiviral response—suggesting knockout of *Adar* induces a type I IFN response (25, 28).

A similar immunological phenotype has been reported in zebrafish with impaired expression of the zebrafish orthologue of ADAR1, through the use of antisense morpholinos (23). Although not fully characterized, *adar* ATG and splice morphants display increased expression of a panel of innate immune genes, including the ISGs *isg15*, *irf7*, and *stat1b*.

In contrast to animal models focusing on other AGS-associated genes, it seems that zebrafish and mouse models of ADAR1 dysfunction present with phenotypes that are, in many instances, arguably more severe than the human condition. It is notable that, in mammals, three proteins exist within the ADAR gene family: two of which are thought to have roles in A-to-I editing within the central nervous system (ADAR1 and ADAR2), while the other is thought to have no intrinsic enzymatic activity (26, 27, 62, 63). While each of these expresses discrete functions and ADAR1 is thought to be responsible for most of the editing activity, it has been suggested that ADAR2 may be able to partially compensate in human patients with *ADAR1* mutations—preventing the severe phenotypes and embryonic mortality observed in zebrafish and mouse models (63, 64). Although mice and zebrafish also possess three ADAR genes, it is possible that the distribution of enzymatic activity across these three isoforms differs across species, such that the remaining proteins are less able to compensate for the loss of functioning ADAR1/adar in the models discussed above than in humans (65). Any differences in ADAR function across species in the context of interferonopathies remain speculative at this stage—nonetheless, the disparity between zebrafish, mouse and human phenotypes here highlights an extra layer of complexity when modeling even monogenic disorders.

IFIH1 (AGS7)

Of all of the AGS-associated genes, mutations in *IFIH1* were most recently identified in AGS patients—with *IFIH1* being the only AGS-associated gene to present with gain-of-function mutations in patients. *IFIH1* encodes the RIG-I-like receptor MDA5, which has a prominent role in antiviral defense through the detection of double stranded RNA and downstream activation of type I interferon response (32, 41). Patients with *IFIH1* mutations develop phenotypes typical of AGS, including severe developmental delays, progressive microcephaly and upregulation of ISG transcription (**Table 1**) (41).

The role of MDA5 in activation of the innate immune response is supported by published work with zebrafish loss-of-function crispants (33). While lack of functioning mda5 alone did not lead to significant changes in innate immunity-associated genes (including *irf7* and *stat1b*), mutation of *mda5* was sufficient to restore expression of these genes to wildtype levels in animals with an already upregulated interferon response (*zbtb24* mutants) (33). The immune phenotype of these *zbtb24* mutants is thought to be triggered by increased levels of double stranded RNA transcripts in the cytoplasm—supporting the role of Mda5 as an essential mediator of the innate immune activation in response to RNA. However, to our knowledge, no zebrafish models of *mda5* gain-of-function—the genotype of greatest relevance to AGS—have been published thus far.

In contrast, MDA5 gain-of-function rodent models have been characterized. In accordance with the autoimmune phenotype of patients, *Ifih1* mutant mice develop severe multiorgan inflammation—including nephritis and calcification of the liver—alongside reduced survival and upregulated expression of IFN and ISG transcripts (32). However, despite such a severe systemic inflammatory response, an overt neuroinflammatory phenotype has not been reported in *Ifih1* rodent models. Thus, until a gain-of-function zebrafish model is generated with a view to recapitulating AGS, much remains to be understood regarding the role of *IFIH1* in interferonopathies, particularly in relation to neuropathology.

RNASET2

Much like the monogenic mutations linked to AGS, the association of mutations in *RNaseT2* with a similar interferonopathy has led to the generation of animal models exploring the function of this gene. As previously discussed, patients with mutations in *RNaseT2* present with clinical and radiological phenotypes closely mimicking those seen in AGS—suggesting the possibility of shared pathogenesis (35). Indeed, similar to AGS-linked genes, the lysosomal enzyme RNaseT2 is involved in restriction of reverse transcription through the metabolism of virally- or endogenously-derived single-stranded RNA (**Figure 1**) (42).

While no RNaseT2 mouse models have been published, both zebrafish and rat models have variably recapitulated the human phenotype. *RNaseT2* knockout rats develop a robust neuroinflammatory phenotype—with enlarged prefrontal cortex and hippocampus, accompanied by increased numbers of reactive astrocytes in the hippocampus (36). Accordingly, these animals show impaired object recognition, but are otherwise viable—with normal life expectancy and motor function. However, the overall inflammatory phenotype of these animals remains unclear—no evidence of systemic inflammation has been reported in *RNaseT2*$^{-/-}$ rats. Crucially, these animals also fail to recapitulate the key hallmark of RNaseT2-deficient leukodystrophy pathology: white matter abnormalities.

White matter lesions, subcortical cysts and calcification are central to the pathogenesis of RNaseT2-deficient leukodystrophy, contributing to the devastating psychomotor impairments observed in the clinic (34). Use of magnetic resonance imaging (MRI) has demonstrated that adult *rnaset2* mutant zebrafish develop robust white matter lesions, with further work suggesting white matter abnormalities begin during zebrafish embryogenesis, as reflected in microglial dysfunction just five days post-fertilization (37, 38). Similar to patients, *rnaset2* mutant zebrafish display locomotor defects from early development into adulthood and significantly reduced survival (38). Beyond the neurological phenotype, *rnaset2* mutants display increased expression of ISG transcripts—including *isg15*—mimicking the viral-like phenotype of patients (37).

Thus, while only three of the genes discussed above have been modelled in zebrafish to date, it would seem that fish models are able to recapitulate neurological phenotypes of type I interferonopathies, while their rodent counterparts are somewhat spared from neuropathology. Of each of the rodent models utilized above, only the *RNaseT2* knockout rat develops evidence of neuroinflammation, and even this appears to be limited to the hippocampus—with overall white matter integrity and sensorimotor function preserved. It is notable that rats possess only a single-copy of *RNaseT2*, while mice possess an additional copy of the RNaseT2-encoding gene—highlighting the importance of assessing the genetic background of the model system before considering its relevance to the human phenotype (36). Nonetheless, the consistent differences between zebrafish and murine models pose interesting challenges for interferonopathy modeling in these species.

WHAT CAN WE LEARN FROM INTERSPECIES DIFFERENCES IN ANIMAL MODELS OF INTERFERONOPATHY?

Despite the crisis in translation of preclinical research into therapeutic advances, rodent models have remained at the forefront of immunological research for decades (66–68). Mice have long since been considered of sufficient evolutionary similarity to humans to act as a relevant model of research. Yet, in the field of interferonopathy modeling, it seems the zebrafish—a species more evolutionarily distant from humans—arguably better recapitulates clinical phenotypes, with particular relevance to the neurological symptoms at the core of AGS and RNaseT2-deficient leukodystrophy. What, then, is the missing link between zebrafish and mice in interferonopathy research?

Age of Assessment

One crucial consideration when assessing the face validity of preclinical models—particularly those which model diseases which manifest almost consistently during early life—is the age at which the animals are screened for pathology (10). In patients with AGS, clinical phenotypes frequently emerge during the first year of life, with prenatal disease onset thought to occur in up to one fifth of patients, suggesting analysis of disease phenotypes may be most relevant during early development (10). However, it should be noted that, for the models discussed above, mouse phenotypes were assessed postnatally or in early adulthood, while zebrafish were often screened during embryogenesis or larval stages. This is, in part, due to the intrinsic features of the species used: due to their *ex utero* development and transparency during embryogenesis, zebrafish can provide unique insights into developmental pathology. In contrast, mice are often raised into adulthood before culling, in order to allow for more comprehensive assessment of relevant phenotypes.

It is possible, therefore, that the mouse models discussed develop fetal phenotypes just as the zebrafish do, but these are compensated for at later stages and therefore missed during postnatal screening. Indeed, in human patients, AGS is often characterized by a period of pronounced symptomatic deterioration followed by stabilization and—in rare cases—small improvements (7, 69). However, patients rarely make a complete functional recovery, with the neuropathology and white matter lesions which first presented during early development observable throughout life. Likewise, longitudinal characterization of the *rnaset2* mutant zebrafish revealed white matter lesions and behavioral abnormalities which persisted into adulthood (37, 38). It is, therefore, unlikely that any fetal neuropathology in mice would fully rectify throughout development such that adults appeared neurologically normal at screening. Nonetheless, the discrepancy between mouse and zebrafish phenotypes highlights an important consideration when modeling disorders with such a prominent neurodevelopmental component.

Methodological Considerations When Generating Animal Models

When assessing the validity of any animal model in recapitulating clinical phenotypes, it is important to consider the relevance of the model organism to patient genetics. Like many other interferonopathies, AGS and RNaseT2-deficient leukoencephalopathy are monogenetic disorders—as such, each of the animal models previously discussed disrupt the function or expression of a single gene linked to the human condition.

However, across mice and zebrafish, a range of genetic strategies have been utilized to generate disease models. It is notable that the mouse models discussed here have employed knockout approaches to mimic the loss-of-function mutations seen in many patients (excluding *IFIH1*)—resulting in animals with little-to-no expression of the relevant gene. As discussed elsewhere, these models have little relevance to the human genotype—with most patient mutations resulting in reduced expression of functioning or malfunctioning protein. Crucially, such extreme genotypes may limit the translational impact of these models in the development of therapies—particularly those which aim to reintroduce target proteins, such as enzyme-replacement or gene therapy. Against a constitutive knockout background, the reintroduced protein may initiate an immune response after being recognized as foreign—as has been reported in preclinical models of a closely related leukodystrophy, Alexander's disease (70). In contrast, patients with some endogenous expression of these genes are perhaps less likely to develop an immune response to reintroduced proteins—making it difficult to predict the efficacy of such treatments based on these preclinical mouse models (8). Unlike their murine counterparts, many of the genetic tools used to generate zebrafish models of interferonopathies—such as antisense morpholino oligonucleotides and CRISPR/Cas9 gene editing—result in genotypes frequently more relevant to the human condition, by knocking down gene expression or generating mutated protein (rather than a constitutive knockout) (23, 38).

One might expect animals with a complete lack of relevant gene expression to present with an arguably more severe phenotype than those retaining some level of protein (whether this be reduced levels of functioning protein or dysfunctional enzyme). Indeed, this seems to be the case when considering *ADAR* mouse models—with animals with point mutations in the ADAR gene surviving slightly longer than complete knockouts (25–30). However, the same seems not to apply to animal models of *SAMHD1* and *RNaseT2* dysfunction. For each of these genes, constitutive knockout rodents fail to fully recapitulate the immune phenotypes reported in human conditions—with overtly normal development and survival (20–22, 36). In contrast, *samhd1* and *rnaset2* defective zebrafish develop robust neurological phenotypes relevant to the human condition—with *samhd1* models developing cerebral hemorrhage, while *rnaset2* mutants acquire white matter abnormalities and locomotor dysfunction (23, 37, 38). For each of these models, the genetic strategies utilized preserve some level of protein expression and, yet, their phenotypes are more severe—and arguably more relevant to the human condition—than their murine counterparts.

However, it should be noted that several studies in zebrafish have reported poor correlation between the phenotypes of mutants (i.e. those generated using CRISPR/Cas9) and morphants (those generated by morpholino)—with morphants often presenting with more severe phenotypes than mutants, even in the absence of any observable off-target effects (71, 72). In addition, subsequent research has suggested that the use of morpholinos themselves may induce an interferon-like response, with upregulation of ISGs reported across multiple published morphants (73). As such, it is possible that intrinsic limitations of morpholino-induced knockdown may account for the more severe phenotypes observed in *samhd1* zebrafish models relative to their murine counterparts (20–23). However, these findings still cannot account for the phenotypic differences between *RNaseT2* knockout rats and *rnaset2* mutant zebrafish—the latter of which has been validated using both ENU mutagenesis and CRISPR/Cas9 gene editing and bred to produce stable lines with comparable phenotypes (37, 38). As such,

differences in methodology cannot entirely account for the differences in neurophenotypes reported in rodent and zebrafish models of type I interferonopathies.

In addition to the genetic modifications utilized to generate *in vivo* models, it should also be noted that there are substantial differences in the genetic backgrounds of zebrafish and mice used in experimental settings. For example, laboratory mice are highly inbred to reduce variability—particularly when characterizing phenotypes associated with single gene knockout—resulting in a single line which does not reflect the substantial genetic variability seen in human populations. In contrast, zebrafish are relatively outbred, leading to an accumulation of polymorphisms that vary from one animal to the next and perhaps more closely mimic the complex genetic make-up of humans than mice. The combined effect of these mutations may well act as a phenotypic modifier—resulting in intraspecies variability in pathology, as is seen in human AGS patients with mutations in the same gene (18). However, any increased variation in zebrafish models relative to mice still cannot account for the general trend towards greater neurological involvement in the fish compared to rodents. Thus, perhaps factors beyond genetics also serve to manipulate phenotypes in interferonopathy modeling.

Mind the Microbiome—The Role of the Experimental Environment

When developing animal models of genetic disorders, often little attention is paid to the impact of the laboratory environment. Compared to their wild counterparts, lab mice and zebrafish live in a controlled environment in an effort to simplify our understanding of the relationship between genotype and phenotype. However, there are notable differences in the husbandry of zebrafish and mice—leading to arguably very distinct environment and pathogen exposure.

While zebrafish facilities around the world undoubtedly take great care in optimizing water quality in their aquaria, there is some evidence that pathogens are present in water across a large number of centers (74). A recent study reported evidence of a novel picornavirus-like pathogen transmitted *via* the environment—leading to spontaneous activation of interferon responses in otherwise healthy animals. Infection was associated with no overt phenotype, but rather was identified using an *isg15* transgenic reporter line. Intriguingly, evidence of widespread viral infection was identified in RNAseq datasets from 92 facilities across the world (74). It would therefore seem that "asymptomatic" zebrafish infection may be relatively commonplace in zebrafish research and act as somewhat of a confounding—although not necessarily unhelpful—factor in studying immune responses in these animals.

In contrast, mice live in a relatively "clean" environment compared to their zebrafish counterparts—with sterilization of bedding, food, and water being commonplace in murine husbandry. It is unsurprising, therefore, that lab mice are exposed to significantly fewer pathogens—including viruses—compared with their wild counterparts, contributing to notable differences in immune composition and antiviral response (75). This is in stark contrast to zebrafish and, of course, humans—for whom exposure to low virulence pathogens is commonplace throughout life and may even begin *in utero* (76–79), Perhaps, then, it is the sterile environment of laboratory mice—in which pathogen exposure is extremely low—which might explain immune phenotypes that are notably removed from the human condition.

Possible viral exposure is particularly relevant when modeling interferonopathies—a collection of disorders that have for so long been thought to mimic congenital viral infection and associated with genes involved in the human antiviral response. While active viral infection is usually excluded before a diagnosis of AGS or RNaseT2-deficient leukodystrophy can be made, it is possible that exposure to commonplace, low-virulence viruses could serve as a risk factor—or even a trigger—for activation of type I interferon response in patients that are already genetically predisposed to interferonopathies. It has been suggested that such viruses may be broadly linked to neurological pathologies in a manner that is complex and temporarily removed—this, too, may be the case for interferonopathies (80). Such viral infections may resolve without the development of overt phenotypes at the time of infection—instead, triggering the autoimmune response and resulting in downstream disability.

Perhaps, this previously unappreciated role of viral infection as a trigger for interferonopathy can explain why mice, in general, develop somewhat milder phenotypes, while zebrafish —with virus exposure even during larval stages—go on to develop similar pathology to that which we see in humans.

While viral exposure may be particularly relevant to the interferonopathies—with type I IFNs primarily considered for their role in antiviral response—bacterial infection is also known to trigger type I interferon response (81). In mice, deletion of IFNAR (the type I IFN receptor) has been shown to both protect against and exacerbate infection with different bacterial species—demonstrating a clear, albeit complex, role of bacteria in triggering type I IFN (82, 83). Likewise, in zebrafish, colonization of germ-free larvae with bacteria has also been shown to upregulate the interferon-stimulated genes, among other innate immunity-associated transcripts (84). It is conceivable, therefore, that environmental exposure to bacteria could also act as a trigger for interferonopathy pathology in zebrafish and humans in a similar manner to viruses—further exacerbating differences between murine and zebrafish pathology.

There have been numerous calls for mice to be raised in pathogen-rich environments in order to increase the impact of immune research following a crisis in translation that extends beyond inferonopathy modeling (66–68, 85). Indeed, research has suggested that exposing lab mice to a greater number of environmental pathogens may result in immune responses that better mimic human phenotypes (68). So-called wildling mice—mice born to wild mothers but with the same genetic background as conventional laboratory animals—better predicted patient response to immune-related therapies in clinical trials compared to conventional lab animals (68). While the precise viral exposure of these wildling mice was not assessed, these animals were maternally exposed to a more diverse microbial

population than lab mice—suggesting life-long exposure to pathogens increases the face validity of mouse models in recapitulating human disease.

One might therefore expect that raising interferonopathy mouse models with greater pathogen exposure—or inducing viral infection—in early life may result in a more relevant neuroinflammatory phenotype. It should be noted that preliminary experiments inducing immune challenge in both *RNaseH2* mutant and *SAMHD1* knockout mice failed to find any difference in response compared to wildtype animals. *SAMHD1*$^{-/-}$ mice produced normal levels of IFNα and IFN response following encephalomyocarditis viral infection, while *RNaseH2* mutant mice developed a similar clinical phenotype as their wildtype counterparts following induction of experimental autoimmune encephalomyelitis (16, 21). However, it should be noted that both of these immune challenges were induced in adult—rather than developing—animals, and that long-term downstream effects were not observed. In humans, congenital infection by HIV-1 is characterized by upregulation of interferon α alongside intracranial calcification and white matter abnormalities—a phenotype remarkably similar to that of AGS—suggesting the timing of infection may well modulate the severity of pathology (4, 86–88). Thus, it is still entirely possible similar immune challenges could trigger a downstream inflammatory phenotype in these mouse models if performed during embryonic development or in early postnatal stages.

If asymptomatic, low-virulence viral infection does in fact trigger interferonopathy in humans, this too may provoke the type I interferon response observed in zebrafish models. Indeed, upregulated transcription of *isg15*—an interferon-stimulated gene known to be involved in the zebrafish immune response and the very transgenic reporter line used to identify the novel picornavirus-like pathogen endemic to zebrafish facilities across the world—has been reported in *samhd1*, *adar* and *rnaset2* defective zebrafish models throughout development (23, 38, 74). After hatching (around 2 days post fertilization), zebrafish larvae may be particularly susceptible to viral infection of the brain due to lack of a functional blood brain barrier (BBB) (89). As in mammals, the zebrafish BBB is thought to develop and become functional in a spatiotemporal manner, with the hindbrain BBB becoming functional around four days post fertilization and the midbrain a day later (89). As such, it is feasible that viruses—or, at least, mediators of the antiviral response—are able to enter the larval brain and trigger interferon response. This mechanism could also trigger IFN in human patients—however, our understanding of human BBB formation is less well characterized. Although embryonic BBB is thought to develop and become functional *in utero*, there is some suggestion that full maturation (including inclusion of mature cell types in the neurovascular unit) does not occur until after birth and, even after maturation, pro-inflammatory cytokines are able to cross the BBB with possible deleterious effects (90, 91). Thus, even if the human brain is protected from direct viral infection, it is conceivable that patients with mutations in AGS-associated genes are already susceptible to activation of the interferon response such that the antiviral response initiated by systemic infection may be sufficient trigger neuropathology by infiltration of cytokines into the developing brain.

The role of virus exposure in the zebrafish interferon response could be further dissected by exploiting the *ex utero* development of zebrafish embryos to raise animals in a sterile environment. Bleaching zebrafish eggs at 24 h post-fertilization has been shown to prevent productive viral infection throughout embryogenesis and is a strategy commonly used to raise embryos in a pathogen-free environment (74, 92). If bleached zebrafish mutants were to show an improved inflammatory phenotype compared to their conventionally reared counterparts, this would suggest a role for viral infection in triggering type I interferon response. Thus, careful modulation of the zebrafish microenvironment could be informative about the role of viral infection in triggering type I interferon response in autoimmune interferonopathy.

Recent publications in AGS have suggested that the autoimmune response observed in these patients is triggered by retroelement-derived nucleotides (7). If this is the case, manipulating viral exposure in animal models may well not alter their phenotypes at all. However, we believe that the reliably more severe neurological phenotypes present in the zebrafish compared to the mouse—despite similar genotypes and arguably greater evolutionary similarity between mice and humans than the zebrafish—suggest a prominent role for the environment in modulating pathogenesis of these disorders. These two schools of thought are by no means mutually exclusive: it is possible that viral infection and the presence of foreign nucleotides may provide the first trigger for a breakdown in self-tolerance, whereby individuals develop downstream autoimmune response to endogenous retroelements-derived nucleic acids which further drives pathology. Patients with AGS typically present with severe deterioration during the initial encephalopathic phase, but then stabilize and—in some cases—even show some small improvements (7, 69). Similarly, it has also been reported that some patients with *RNaseH2* and *RNaseT2* mutations may show normalization of interferon response over time: initially showing a positive interferon signature that later becomes negative at follow-up (18). If viral infection is a trigger for pathology, the initial flurry of antiviral response could explain this rapid deterioration and upregulation of ISGs, followed by subsequent stabilization as autoimmunity resolves. In contrast, if the trigger for pathology is truly endogenous in cause, one might expect a continued autoimmune response with consistent deterioration beyond the first year of life. Nonetheless, the points highlighted above suggest an additional layer of complexity in the pathogenesis of interferonopathies—or at least, their animal models—beyond simply genetics.

SUMMARY AND FUTURE PERSPECTIVES

Type I interferonopathies are a group of severe, life-limiting disorders—characterized by a disturbance of the homeostatic control of the interferon response and a range of downstream inflammatory phenotypes. With such profound effect on development and survival, interferonopathies with neurological

involvement—including AGS and RNaseT2-deficient leukoencephalopathy—are particularly debilitating. Yet, despite their devastating effects, much remains to be understood about these disorders and, crucially, how to treat them.

Our understanding of these conditions and the development of novel therapies has thus far been limited by a lack of valid animal models (8). In this review, we have demonstrated consistent limitations in animal models across both species in mimicking the human disease state in AGS. However, mouse models in particular are limited in their recapitulation of the human neurological phenotype.

While there are several key differences between these species specifically relating to each of the AGS-associated genes, we propose that the disparity between rodent and fish models reflects the differing laboratory environments in which these animals are raised, and the corresponding effects on the immune system. Laboratory mice live in relatively sterile environments, and as such have an immune system largely removed from their wildtype counterparts. In contrast, both zebrafish and humans are exposed to a number of pathogens—including viruses—throughout early development: we believe this exposure is essential in modulating the development of interferonopathy neuropathology.

We propose that an initial viral stimulus may serve as the trigger for type I interferon response in AGS and RNaseT2-deficient leukoencephalopathy in human patients and corresponding zebrafish models, leading to subsequent autoimmune pathology due to a compromised genetic background. The absence of viral triggers in lab mice could explain why these animals do not develop the neuroinflammation central to AGS pathology, while the zebrafish—exposed to viruses throughout embryogenesis—develop somewhat more robust neurological pathology. Subsequent work may further explore the effects of viral stimuli in AGS models across both species.

Nonetheless, the vastly different phenotypes between zebrafish and rodent models with mutations of the same gene highlight the importance of model choice, methodological considerations and, perhaps most importantly, pathogen exposure when modeling disorders of the immune system. Future research must carefully consider how these unseen pathogens—or lack thereof—influence pathology if we are to ever understand the complex gene-environment interactions that form human immune response in interferonopathies and beyond.

AUTHOR CONTRIBUTIONS

HR performed the literature search, wrote and revised the manuscript, and designed the graphical figures. PK advised and critically revised the manuscript. NH conceived the study, critically revised and finally approved the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Self-Nucleic Acid Sensing: A Novel Crucial Pathway Involved in Obesity-Mediated Metaflammation and Metabolic Syndrome

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Obesity and overweight are a global health problem affecting almost one third of the world population. There are multiple complications associated with obesity including metabolic syndrome that commonly lead to development of type II diabetes and non-alcoholic fatty liver disease. The development of metabolic syndrome and severe complications associated with obesity is attributed to the chronic low-grade inflammation that occurs in metabolic tissues such as the liver and the white adipose tissue. In recent years, nucleic acids (mostly DNA), which accumulate systemically in obese individuals, were shown to aberrantly activate innate immune responses and thus to contribute to metabolic tissue inflammation. This minireview will focus on (i) the main sources and forms of nucleic acids that accumulate during obesity, (ii) the sensing pathways required for their detection, and (iii) the key cellular players involved in this process. Fully elucidating the role of nucleic acids in the induction of inflammation induced by obesity would promote the identification of new and long-awaited therapeutic approaches to limit obesity-mediated complications.

Keywords: nucleic acids, obesity, metabolic syndrome, metainflammation, inflammation, nucleic acid sensing, non-alcoholic steatohepatitis

Abbreviations: WAT, White adipose tissue; NA, Nucleic Acids; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steatohepatitis; HFD, High Fat Diet; PRR, pathogen recognition receptors; PAMP, Pathogen-associated molecular pattern; DAMP, Danger-associated molecular pattern; NETosis, cell death by Neutrophil Extracellular Traps; gDNA, Genomic Desoxyribonucleic Acid; mtDNA, mitochondrial DNA; CfDNA, Cell-free DNA; MP, Microparticles; HMGB1, high–mobility group box 1; RNA, Ribonucleic Acid; TLR, Toll-Like-Receptor; AIM2, absent in melanoma 2; cGAS, cyclic GMP–AMP synthase; STING, stimulator of interferon genes; NLRP3, NLR family pyrin domain containing 3; TRIF, TIR-domain-containing adapter-inducing interferon-β; MyD88, myeloid differentiation protein 88; NF-κB, nuclear factor-kappa B; IRFs, Interferon Regulatory Factors; IL-, Interleukin; TNF, Tumor Necrosis Factor; IFN, Interferon; IFNAR, IFN α/β receptor; DC, Dendritic Cells; pDC, Plasmacytoid dendritic cells; Mφ, Macrophages.

OBESITY-ASSOCIATED METABOLIC SYNDROME: A GLOBAL EPIDEMIC WITH AN INFLAMMATORY ORIGIN

Over the past 4 decades the prevalence of overweight and obese individuals has continuously and substantially increased, affecting almost one-third of the world population (1). The main cause of obesity is an imbalance between consumed and burned calories (2). Obesity is associated with the development of metabolic syndrome, which is commonly defined by hypertension, hyperglycemia, excess abdominal fat, and abnormal lipidemia (1). Metabolic syndrome frequently has a "domino effect" as it leads to the development of severe diseases such as type II diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), atherosclerosis, and ischemic cardiovascular disease (2). Therefore, obesity has far-reaching consequences for life expectancy, quality of life and healthcare costs (3). Treatment options for obesity are limited and include lifestyle changes that generally do not induce marked and/or sustainable weight loss and bariatric surgery, which effectively induces weight loss and reduces comorbidities but increases perioperative mortality, surgical complications and is associated with relapse (4). Furthermore, specific therapeutic targeting of either interleukin (IL-)1β (5) or tumor necrosis factor (TNF-)α (6) have shown limited success. It is therefore essential to develop new therapeutic avenues to ameliorate and prevent obesity-associated complications (7).

The main tissue affected by obesity is the white adipose tissue (WAT), which becomes hypertrophied and heavily infiltrated by immune cells that adopt a pro-inflammatory profile in response to endogenous signals (8). The resulting chronic low-grade inflammation state, also called metabolic inflammation, or "metaflammation", plays a crucial role in the development of obesity-associated metabolic syndrome and further complications (8). In particular, macrophages (Mφ) that originate mostly from circulatory monocytes and to lesser extent from tissue resident Mφ, accumulate in the adipose tissue of obese individuals (9) and adopt an M1 pro-inflammatory phenotype (8). This switch from anti-inflammatory M2 Mφ, that are dominant in the adipose tissue of lean individuals, to M1 Mφ during obesity promotes the production of pro-inflammatory cytokines (e.g.,TNF-α and IL-1β) that can directly inhibit insulin signaling and lead to cardiovascular and metabolic complications related to obesity (8). However, targeting these cytokines has shown marginal clinical benefits for obese patients (5, 6). Furthermore, recent single-cell RNA sequencing studies have revealed a higher complexity beyond the classic M1/M2 distinction of Mφ in the WAT of obese individuals and mice (10). Therefore, the cellular and molecular mechanisms that are responsible for obesity-triggered metaflammation are not yet fully understood.

The activation of inflammatory pathways is mediated by pathogen recognition receptors (PRRs) upon sensing of exogenous pathogen associated molecular patterns (PAMPs) and endogenous damage associated molecular patterns (DAMPs). Excess nutrient intake causes an accumulation of DAMPs such as free fatty acids and cholesterol crystals but also of PAMPs such as lipopolysaccharides (LPS) originating from the intestinal microbiota in response to obesity-promoted intestinal permeability (11). These DAMPs and PAMPs were shown to contribute to obesity-mediated metaflammation by activating multiple PRRs including Toll-like receptors (TLR)2 and TLR4 and the NLR family pyrin domain containing 3 (NLRP3) inflammasome (12). It is becoming clear that obesity also induces the accumulation of nucleic acids (NA), which function similarly to DAMPs and thus activate innate immune responses (13). The source and the form of these NA are diverse and their recognition by NA sensing PRRs expressed by dendritic cells (DCs) or MΦ seems to be a key initiating event in the pathogenesis of obesity (14–16). Here, we will focus on (i) the main sources and forms of these NA, (ii) the sensing pathways involved in their detection, and (iii) the key cellular players involved in this process.

SOURCES AND FORMS OF NUCLEIC ACIDS THAT ACCUMULATE DURING OBESITY

Multiple recent studies have reported that obese individuals or mice exposed to a high-fat diet (HFD) show elevated levels of circulatory cell-free DNA (cfDNa) (14, 15). Sources of cfDNA vary among obese patients and mouse models of obesity (**Figure 1**). Murine hepatocytes from livers affected by NAFLD were shown to acquire the potential to release mitochondrial DNA (mtDNA) in microparticles (MPs) (15). This MP-associated mtDNA was significantly increased in the plasma of obese patients, particularly in those who had active NAFLD (15). Obesity was also reported to induce neutrophils and MΦ to release extracellular traps (ET), which are composed of NA and antimicrobial peptides (16). Such ET were more abundant in the WAT of obese mice compared to lean mice and showed potent inflammatory properties *in vitro* (16). Importantly, bone marrow-derived myeloid cells from obese mice fed HFD were more susceptible to extrude ET containing DNA upon *in vitro* stimulation, indicating that obesity may systemically boost the potential of myeloid cells to release ET (16). Finally, oxidative stress, hypoxia, inflammation and aberrant adipogenesis that occur during obesity lead to heightened cell death of adipocytes that release both their genomic (g)-DNA and mtDNA, and thus contribute to the systemic accumulation of cfDNA (17). Accordingly, Nishimoto et al. observed *in vitro* that epididymal fat from mice fed HFD constitutively released more cfDNA than the fat from animals on normal diet (14). Furthermore, explant of WAT from obese individuals released elevated levels of self-DNA in culture supernatants (18). The DNA released by WAT explants was associated with high mobility group box 1 (HMGB1), a nuclear protein that was previously shown to be an endogenous DAMP and increases the inflammatory potential of self-DNA (19). Importantly, systemic levels of these cfDNA and HMGB1 positively correlated with the severity of metabolic syndrome

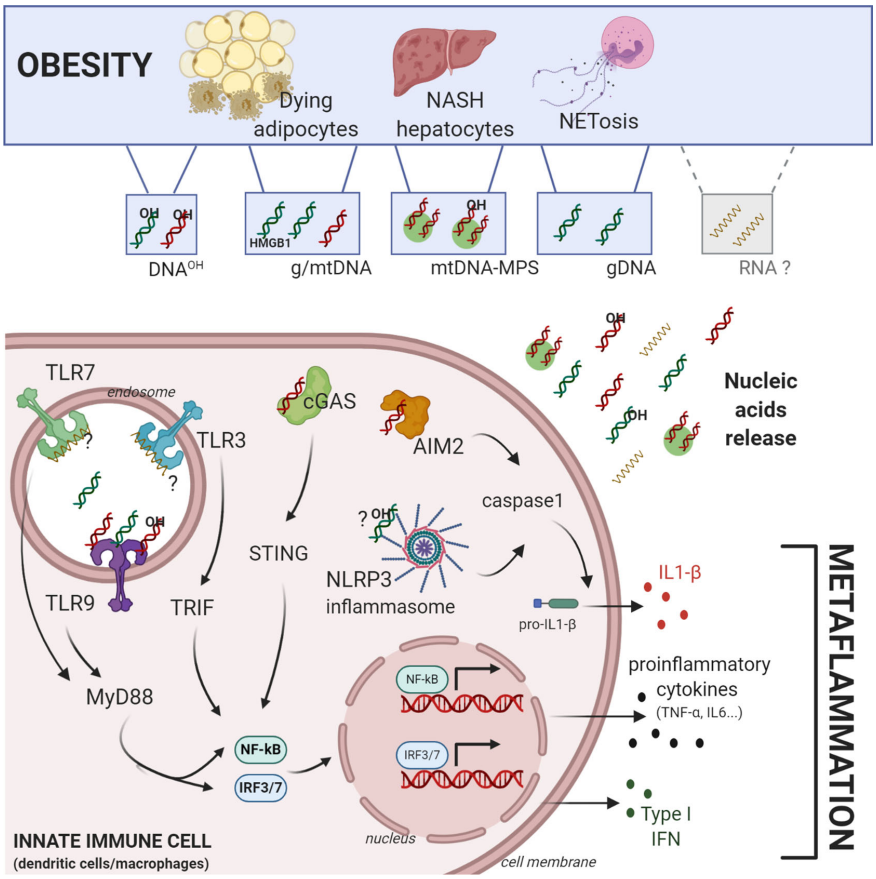


FIGURE 1 | Central role of self-nucleic acids in obesity-mediated metaflammation. Obesity induces an accumulation of different sources and forms of self-nucleic acids (NA). Genomic-, mitochondrial, oxidized-DNA (OH-DNA) and potentially RNA can be released in aberrant amounts during obesity by dying adipocytes, NASH hepatocytes, or NETosis. Self-NA can activate cytosolic or endolysosomal pathogen recognition receptors in macrophages or dendritic cells such as Toll-Like-Receptor 3/7/9, cGAS-STING pathway, AIM2 and NLRP3 inflammasomes. Their activation induces *in situ* production of i) proinflammatory cytokines (IL6, TNF-α…) through the activation of NF-KB, ii) type I interferon through the transcription factors IRF3/7, and iii) IL1-β, through the caspase 1 activation, all of which contribute directly to obesity mediated-metaflammation. Figure was created with biorender.com.

induced by obesity including the extent of visceral fat tissue, insulin resistance, and liver injury (14, 15, 20, 21).

In addition to their elevated quantity, the quality of cfDNA is also affected by obesity. Obesity-mediated inflammation induces an accrual of reactive oxygen species (ROS) that cause DNA oxidation (DNA-OH) (22). Guanosines are the targets of ROS-mediated oxidation and the 8 hydroxydeoxyguanine (8-OHdG) represent the main DNA oxidation marker. Garcia-Martinez et al. showed that the vast majority of mtDNA contained in the circulatory MPs of obese individuals affected by NAFLD contain 8-OHdG reflecting its oxidized nature (15). Such oxidized forms of mtDNA from obese individuals exhibit an elevated potential to stimulate DNA sensing PRR (15) as previously reported in autoimmune contexts (23, 24), likely due to its protection form the degradation by nucleases (25).

Obesity not only modulates systemic levels of self-DNA, but also causes bacterial DNA leakage from the gastrointestinal tract. HFD was shown to modulate intestinal permeability and to contribute *via* the portal circulation to the transport of bacterial DNA to the liver where it ultimately activates inflammatory responses and promotes NAFLD development (26). However, the impact of bacterial DNA to NAFLD pathogenesis was only observed in inflammasome-deficient animals, which present a major intestinal dysbiosis (26). Thus, these observations may explain the susceptibility of certain individuals to obesity-mediated pathogenesis rather than represent a general mechanism of action of obesity.

Overall, obesity appears to induce the accumulation of NA originating from various sources and such NA participate in obesity-mediated pathogenesis (**Figure 1**). Not only obesity increases the quantity of NA, but it also affects their overall quality. Various forms of cfDNA with an enhanced immunogenic potential are preferentially detected in obese individuals, including MPs associated-DNA, ET associated-

DNA, HMGB1 bound-DNA and oxidized-DNA. While different sources and forms of DNA were reported to accumulate during obesity, their individual contribution to complications induced by obesity remain unknown. Besides, whether self-RNA accumulate in obese patients and in which forms, require further investigation.

Multiple safeguard mechanisms are involved in the disposal of dying cells and their NA, preventing NA ability to activate inflammatory immune responses. These protective mechanisms include the clearance of apoptotic cells by phagocytes (efferocytosis) (27) and the digestion of extracellular DNA by circulatory deoxyribonucleases (DNASEs) (28). It is becoming clear that obesity impairs phagocytes ability to clear apoptotic cells by affecting lipid composition of cell membranes and their expression of scavenger receptors (29, 30). Furthermore, HFD in mice was reported to reduce the overall circulatory DNASE activity (16). However, treatment of obese mice with recombinant DNASE1 did not affect the development of metabolic syndrome (16). This absence of therapeutic potential of DNASE1 may be due to soluble mediators present in obese mice that block DNASE1 function and/or to its inability to clear all sources of pathogenic DNA that accumulate during obesity. Indeed, DNASE1 is only capable of digesting "naked" DNA in the extracellular space, so exploring the role of other circulatory DNASES in the regulation of obesity-mediated pathogenesis is essential. It will be particularly relevant to address the function of DNASE1L3 in this context, since it is specifically expressed in immune cells infiltrating metabolic tissues and digest both MP-associated DNA (31, 32) and neutrophil ET-associated DNA (33), the two main forms of DNA that accumulate during obesity. Overall, in addition to an accrual endogenous DNA release, obesity also impair safeguard mechanisms that are involved in their disposal, and these processes together participate in the accumulation of self-DNA in obese individuals.

NUCLEIC ACID SENSING PATHWAYS INVOLVED IN OBESITY-MEDIATED METAFLAMMATION

The sensing of NA is operated by two major classes of PRRs, including endolysosomal and cytosolic NA sensors (**Figure 1**), which in response to stimulation trigger the production of inflammatory cytokines such as type I interferons (IFN-I), IL-1β, and TNF-α, playing a crucial role in obesity-mediated metaflammation (13).

TLRs comprise all-known NA sensing endolysosomal PRR. Among them, TLR9 is specialized in the recognition of DNA, and its deficiency was recently reported to protect from the development of metabolic syndrome induced by HFD. Indeed, *Tlr9*-deficient mice displayed a reduction in WAT and liver inflammation and improved insulin sensitivity compared to wild-type mice upon HFD (14, 16). Injection of the TLR9 ligand CpG DNA (ODN2395) in mice increased fasting glucose levels and immune cell infiltration in WAT and liver, indicating that even in the absence of obesity TLR9 activation leads to metabolic deregulations (16). Conversely, administration of TLR9 antagonist (iODN2088) to HFD-fed mice attenuated metabolic tissue inflammation, improved glucose metabolism (14), and ameliorated manifestation of liver steatosis (15), confirming the crucial role of TLR9 in obesity-mediated pathogenesis. This aberrant activation of TLR9 during obesity was shown to be mediated by the recognition of self-DNA released by dying adipocytes, MPs loaded with mtDNA as well as ET (14–16, 18). TLR3 and TLR7 are also endolysosomal PRRs, but they are involved in the recognition of ds and ssRNA, respectively. The contribution of TLR3 and TLR7 to obesity-mediated pathogenesis was established when their deficiencies were shown to restore glucose tolerance, decrease metabolic inflammation and ameliorate NAFLD in HFD fed animals (16, 34, 35). TLR8, which is also a sensor of ssRNA, showed an increased expression in MΦ infiltrating the WAT of obese patients with or without T2D, and TLR8 expression significantly correlated with disease severity and metabolic tissue inflammation (36). Although TLR8 is unresponsive to ssRNA in mice, its deficiency induced mild metabolic abnormalities and increased the liver inflammation in HFD fed mice (37). This observation was mainly due to increased TLR7 expression in TLR8 knock-out mice (37). The net contribution of TLR8 in obesity thus requires further investigation particularly in transgenic mice expressing human TLR8 (38). While RNA-sensing TLRs seem to play a role in obesity, the origins of the pathogenic RNA, its form and its regulation during obesity remain unknown. Most TLRs signal through the adaptor molecule myeloid differentiation primary response (MyD)-88 with the exception of TLR3, which transduces signaling *via* TIR-domain-containing adapter-inducing interferon-β (TRIF). MyD88 activation leads to the production of inflammatory cytokines through the activation of nuclear factor kappa-B (NF-KB) which was shown to play a critical role in obesity-mediated inflammation (12). Alternatively, endolysosomal TLR3 signaling activates the transcription factor interferon regulatory factor (IRF)-3 *via* TRIF and TLR7-9 activate IRF7 *via* MyD88 (**Figure 1**). Both IRF3 and IRF7 are involved in the induction of IFN-I production (39), which has also been reported to play an important role in obesity-mediated metabolic syndromes. Specific deletion of *Irf7* (40) or IFN-I receptor (*Ifnar*) improved obesity-mediated inflammation and insulin resistance (41). In contrast, the role of IRF3 in obesity is more controversial. *Irf3* deficiency was shown to alleviate adipose tissue inflammation and insulin resistance in HFD-fed mice (42), yet it also exacerbated the development of NAFLD induced by HFD (43). Therefore, IRF3-mediated IFN-I production may have tissue-specific functions and play a protective role in liver pathology induced by obesity.

Cytosolic NA sensing PRRs include Rig-I like receptors (RLR) that are specialized in the sensing of RNA, the DNA-sensor cyclic GMP-AMP Synthase (cGAS), and the absent in melanoma (AIM)-2 inflammasome that specifically detects dsDNA. While RLR function in obesity-mediated inflammation is poorly studied, a role for cytosolic DNA sensing pathways is

being defined (44). The DNA sensor cGAS signals through stimulator of IFN genes (STING) to induce the production of IFN-I in an IRF3-dependent manner and the production of inflammatory cytokines through NF-KB (45) (**Figure 1**). STING expression was reported to be upregulated in the livers of NASH patients compared to healthy controls (46). Furthermore, liver inflammation and steatosis was significantly improved in mice deficient for *Tmem173* (gene encoding STING) that were fed a HFD (46, 47). Despite the effect on liver pathology, *Tmem173* deficiency did not show any impact on glucose metabolism in obese mice (47). The activation of cGAS-STING pathway upon HFD was shown to be mediated by mtDNA released by hepatocytes, which *via* NF-KB leads to preferential production of TNF-α and IL-6 (47). IFN-I was not detected in the liver of HFD mice or in supernatants of liver MΦ (Kupffer cells) stimulated with mtDNA isolated from hepatocytes (46, 47), suggesting that the cGAS pathway contributes to NASH independently of IFN-I. These observations are in accordance with the previously discussed literature indicating that IRF3 may protect against NASH development induced by obesity (43). More recently, obesity induced either by HFD or genetically by the deficiency of the leptin receptor (*db/db* mice) was shown to promote the accumulation of mtDNA and the activation of the cGAS pathway in adipocytes (48). The accumulation of mtDNA into the cytosol of adipocytes was suggested to be due to the inhibition of the disulfide-bond A oxidoreductase-like protein (DsbA-L), which is a mitochondrial matrix chaperone. Accordingly, fat-specific deficiency of DsbA-L aggravated the weight gain and glucose intolerance of HFD-fed mice while WAT-specific overexpression of DsbA-L protected mice from obesity-induced inflammation and insulin resistance (48). These results suggest that beyond NASH cGAS may contribute to obesity-induced metabolic syndromes. However, it remains to be explored whether the impact of DsbA-L deficiency is dependent on the cGAS-STING pathway *in vivo*.

Finally, various members of the inflammasome play a crucial role in obesity-mediated pathogenesis (11). Among inflammasomes, DNA is primarily detected by the AIM-2 inflammasome. AIM-2 triggers caspase-1 activation which is essential for the cleavage of pro-IL-1β and pro-IL-18 into their mature and biologically active forms. Recently circulatory mtDNA isolated from patients with T2D was reported to contribute to AIM-2 inflammasome-dependent caspase-1 activation and IL-1β and IL-18 secretion by MΦ (49) (**Figure 1**). However, the *in vivo* role of AIM-2 in obesity remains controversial. Gong et al. have observed spontaneous obesity, impaired glucose metabolism, and increased WAT inflammation in *Aim-2* deficient mice (50). These results are not only intriguing but also consistent with the observation that the inflammasome has a dual role in obesity, contributing to obesity-mediated inflammation *via* IL-1β (51) and preventing its negative impact by the production of IL-18 (52). Finally, the NLRP3 inflammasome was previously reported to promote obesity-mediated pathogenesis upon its activation by intracellular ceramide (53). Interestingly, oxidized mtDNA (54) and oxidized DNA originating from NETs (55) were also shown to activate the NLRP3 inflammasome (**Figure 1**). Therefore, such DNA that accumulates during obesity is likely to contribute to obesity-mediated pathogenesis though the activation of NLRP3 as well.

CELLULAR PLAYERS INVOLVED IN NUCLEIC ACID DETECTION DURING METAFLAMMATION

Innate immune cells, in particular DC and MΦ, play a crucial role in the recognition of NA and the ensuing production of inflammatory cytokines. While innate immune cells have been shown to participate in obesity-associated inflammation, the contribution of their ability to recognize NA to this process in only beginning to be understood.

It was recently proposed that NA originating from ETs, which accumulate in obese mice, directly activate the production of proinflammatory cytokines by MΦ *via* TLR-7 and TLR-9 (16). Targeted deletion of *Tlr9* in MΦ using *LysM*-Cre protected mice from the development of NAFLD induced by HFD (15). Furthermore, the transfer of STING-deficient bone marrow cells ameliorated HFD induced NAFLD in bone marrow chimeras. In view of these results the authors suggested that the activation of the cGAS-STING pathway in Kupffer cells was crucial for NAFLD pathogenesis (46). However, Kupffer cells are radioresistant and the contribution of bone marrow cells for their replenishment is low (56), therefore it is more likely that cGAS-STING pathway is required in another cell type of hematopoietic origin for the development of NAFLD.

DC contribution to obesity-mediated pathogenesis was previously documented (57); however, specific deletion of NA sensing pathway in DCs during obesity has not yet been investigated. TLR9 is broadly expressed among immune cells in mice, but only in plasmacytoid dendritic cells (pDCs) and B cells in humans (18). Importantly, pDCs are a subset of DCs that are specialized in the production of IFN-I upon sensing of microbial and self-NA (58). pDCs were recently shown to be recruited to the WAT of obese mice and individuals (18, 57, 59) and their infiltration into the WAT correlated with obesity-associated insulin resistance (59). Importantly, DNA-complexed with HMGB1 released by human adipocytes stimulated pDC production of IFN-I in a TLR9-dependent manner (59). Moreover, specific ablation of pDCs ameliorated obesity-associated metabolic syndrome and insulin resistance *in vivo* (41). However, whether pDC-specific expression of TLR9 and production of IFN-I are directly involved in obesity-mediated metabolic syndromes *in vivo* require further investigation.

Despite the systemic accumulation of cfDNA in obese individuals, its ability to activate inflammatory responses in circulatory leukocytes remains poorly documented. It appears that cfDNA primarily activates innate immune cells within metabolic tissues (18, 46). Inflammatory cytokines produced in response to such stimulation are then redistributed systematically upon their

release in the circulation. Given the potential of inflammatory cytokines to activate circulatory leukocytes and to further fuel systemic inflammation, it is quite difficult to distinguish the stimulatory impact on circulatory leukocytes of inflammatory cytokines from the one of cfDNA. Nevertheless, *in vitro* stimulation of a monocytic cell line with healthy individuals' plasma was recently shown modulate their innate immune functions, mostly through cfDNA (60). Given that such cfDNA accumulate during obesity, its ability to directly stimulate circulatory leucocytes and ultimately contribute to obesity-mediated metaflammation requires further investigation.

The cGAS-STING pathway is ubiquitously expressed. It was recently reported that obesity induces the accumulation of mtDNA directly in adipocytes, which ultimately activates the cGAS-STING pathway. In response to such stimulation, these adipocytes produce inflammatory cytokines including IFN-I, contributing to the overall metaflammation induced by obesity (48). These observations indicate that obesity-mediated metaflammation is driven by not only immune cells but also adipocytes.

IFN-I that is produced in response to self-DNA sensing, induces the expression of a plethora of IFN-stimulated genes (ISGs) whose expression was reported to be associated with adipose tissue and systemic insulin resistance in obese patients (18). This pathogenic role of IFN-I in obesity is likely due to its ability to (i) polarize adipose tissue MΦ toward a proinflammatory M1 phenotype (18), (ii) activate innate and adaptive immune responses (61, 62) and (iii) to amplify adipocytes' cell-intrinsic inflammatory capacity (63). Apart from the indirect impact on inflammatory processes, IFN-I was also shown to directly interfere with insulin signaling in metabolically active tissues, particularly in adipocytes (64) and hepatocytes (65). Therefore IFN-I induced in response to the aberrant sensing of self-NA by immune and non-immune cells clearly contributes to obesity-mediated pathogenesis.

FUTURE DIRECTIONS AND THERAPEUTIC AVENUES

In addition to their contribution to inflammatory and in autoimmune diseases (13), it is now clear that NA accumulation and its sensing by various PRRs contribute to the development of metabolic syndrome induced by obesity (as summarized in **Figure 1**). The identification of these novel pathways has opened new therapeutic options. Indeed, Hydoxychloroquine, which blocks endosomal acidification and thus endolysosomal TLR function (66), may have beneficial effects on insulin resistance in obese non-diabetic individuals (67) and prevent the onset of diabetes in patients with autoimmune diseases (rheumatoid arthritis, SLE, psoriasis…) (68, 69). Furthermore, specific antagonists of TLR7,9 and STING, which are in early-phase trials for SLE (70) and various interferonopathies (71), respectively, also show promise for obesity treatment given their therapeutic potency in mouse models of the disease. Similarly, monoclonal antibodies targeting IFN-α (sifalimumab) (72) or IFNAR1 (anifrolumab) (73) and specific ablation of pDCs, which are the main IFN-I producing cells (74), may also have therapeutic value in obese individuals in addition to SLE patients in view of the importance of the pDC-IFN-I axis in obesity. Despite these advances, the specific mechanisms through which HFD and obesity modulate the abundance of nucleic acids remain poorly understood. Understanding these mechanisms will provide a better understanding of the initiation and progression of obesity pathogenesis as well as novel potential therapeutic approaches.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. AF and VS wrote the manuscript.

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REVIEW
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Signaling Through Nucleic Acid Sensors and Their Roles in Inflammatory Diseases

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Recognition of pathogen-derived nucleic acids by pattern-recognition receptors (PRRs) is essential for eliciting antiviral immune responses by inducing the production of type I interferons (IFNs) and proinflammatory cytokines. Such responses are a prerequisite for mounting innate and pathogen-specific adaptive immune responses. However, host cells also use nucleic acids as carriers of genetic information, and the aberrant recognition of self-nucleic acids by PRRs is associated with the onset of autoimmune or autoinflammatory diseases. In this review, we describe the mechanisms of nucleic acid sensing by PRRs, including Toll-like receptors, RIG-I-like receptors, and DNA sensor molecules, and their signaling pathways as well as the disorders caused by uncontrolled or unnecessary activation of these PRRs.

Keywords: Toll-like receptor, RIG-I-like receptor, cGAMP synthase, nucleic acid sensing, autoimmune disease, autoinflammatory disease

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INTRODUCTION

The innate immune system is not only the first line of host defense against invading pathogens but is also essential for the biological responses of the host against various harmful stimuli. Furthermore, its activation subsequently contributes to the activation of the adaptive immune system, which eliminates pathogens to restore host homeostasis. The initiation of immune responses occurs *via* germline-encoded pattern-recognition receptors (PRRs), which recognize widely conserved features in pathogens, termed pathogen-associated molecular patterns (PAMPs), as well as "danger signals", host components released in response to cell or tissue injury, termed damage-associated molecular patterns (DAMPs). PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs) and the cytosolic DNA sensor proteins. When PRRs are activated, they activate their corresponding downstream signaling cascades leading to the induction of innate immune and inflammatory responses through the production of proinflammatory cytokines, type I interferons (IFNs), and other key molecules such as major histocompatibility (MHC) proteins and costimulatory molecules by macrophages, dendritic cells (DCs), neutrophils, and other nonprofessional immune cells (1). Although PRRs are indispensable for host defense to combat invading pathogens and maintain homeostasis, consequential inflammation by aberrant PRRs signaling is likely to be harmful to the organism.

Among a wide variety of PAMPs, nucleic acids derived from pathogens are recognized by TLRs, RLRs, and cytosolic DNA sensors, which provoke antiviral and inflammatory responses mediated by

type I IFNs and proinflammatory cytokines, respectively. However, nucleic acids derived from host cells are also recognized by PRRs under certain conditions, which contributes to autoimmunity and autoinflammation (2). Indeed, accumulating evidence suggests that the excessive activation or dysregulation of nucleic acid-sensing systems is responsible for the pathogenesis of many autoimmune and autoinflammatory diseases and cancers. This review focuses on nucleic acid-sensing receptors, their corresponding ligands, downstream signaling pathways, discrimination between self- and non-self-derived nucleic acids, and related diseases.

NUCLEIC ACID-SENSING TLRS

The TLR family recognizes a wide variety of PAMPs, ranging from lipids and lipoproteins to nucleic acids derived from microbial pathogens. Among TLRs, TLR3, TLR7, TLR8, TLR9, and TLR13 are predominantly localized to endosomes and recognize nucleic acids (**Figure 1**). TLR3 recognizes double-stranded (ds) RNA, TLR7 and TLR8 recognize single-stranded (ss) RNA, TLR9 recognizes unmethylated CpG DNA, and murine TLR13 recognizes bacterial 23S rRNA to activate downstream signaling pathways that induce inflammatory responses (3–6). Their localization in intracellular compartments is essential for proper ligand recognition, discrimination between self- and non-self-derived nucleic acids, and the activation of downstream signaling pathways. All these TLRs are synthesized in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and eventually recruited to intracellular compartments such as endosomes; however, mechanisms related to their transport from the ER to endosomes varies between individual TLRs. TLR9 requires the adaptor protein-2 (AP-2) complex to mediate its endocytosis from the cell surface to endosomes, whereas TLR7

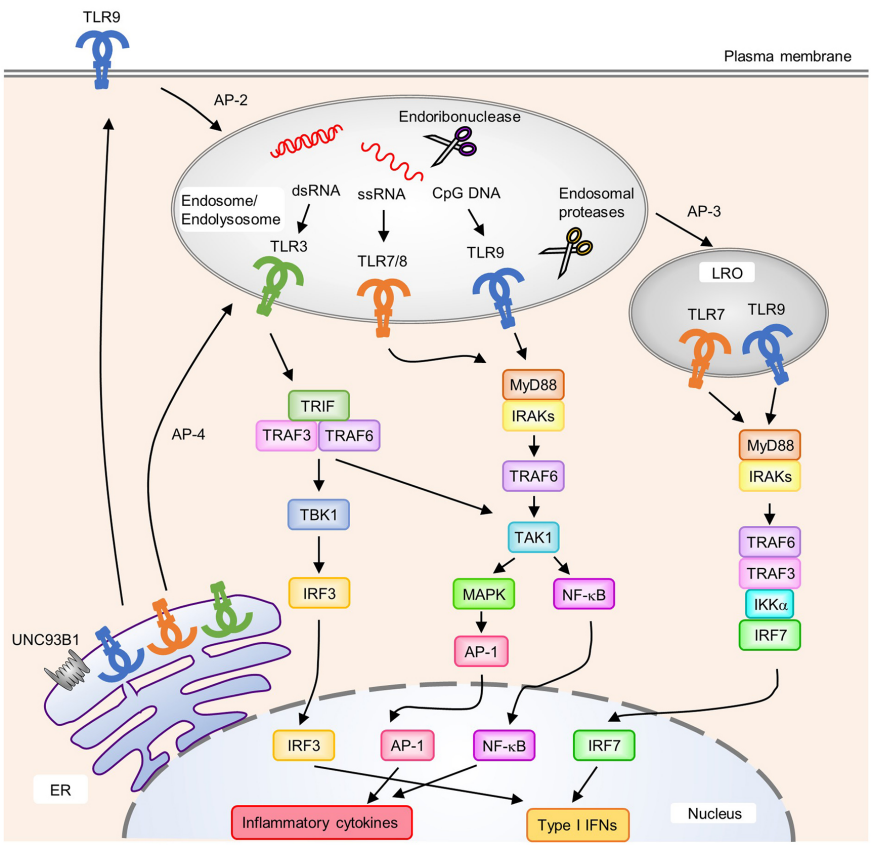


FIGURE 1 | Localization, intracellular trafficking, and signaling pathways of nucleic acid-sensing Toll-like receptors (TLRs). TLR3, TLR7, TLR8, and TLR9 are synthesized in the endoplasmic reticulum (ER) and transported to endosomes *via* UNC93B1. Each TLR is transported to its destination [endosomes, endolysosomes, and lysosome-related organelles (LRO)] by individual mechanisms. TLR9 requires the AP-2 complex to translocate from the cell surface to endosomes, whereas TLR7 interacts with the AP-4 complex for direct trafficking to endosomes. Several endosomal proteases and endoribonucleases in endosomes/endolysosomes process TLRs and nucleic acids, respectively. Upon the recognition of cognate ligands, TLR7, TLR8, and TLR9 recruit MyD88 to activate downstream signaling pathways. MyD88 recruits IRAKs and TRAF6, which subsequently activate TAK1. Activated TAK1 leads to the activation of AP-1 through MAPK to initiate the transcription of proinflammatory cytokines. NF-κB is also activated by TAK1 and induces the production of proinflammatory cytokines. In pDCs, TLR7 and TLR9 in LRO induce the activation of IRF7 by forming a complex with TRAF6, TRAF3, IKKα; and IRF7, which results in the expression of type I IFNs. An AP-3 complex is required for the localization of TLR7 and TLR9 to LRO. TLR3 recruits TRIF to initiate downstream signaling pathway. TRIF recruits TRAF3 and TRAF6 to activate TBK1 and TAK1. Activated TBK1 induces type I IFNs through IRF3, and TAK1 induces proinflammatory cytokine production through NF-κB and AP-1.

interacts with the AP-4 complex to mediate direct trafficking from the trans-Golgi network to endosomes (7). For TLR3, the TRIM3-mediated K63-linked polyubiquitination of TLR3 is required for its trafficking from the Golgi apparatus to endosomes by endosomal sorting complex required for transport (ESCRT) complexes (8). Furthermore, lysosomal trafficking regulator (LYST), which mediates phagosomal maturation, was reported to be important for activation of the TLR3 signaling pathway (9).

Compartmentalization into endosomes is important for the recognition of nucleic acids released from phagocytosed pathogens by TLRs while avoiding contact with self-derived nucleic acids (10). UNC93B1, a 12-transmembrane protein in the ER, is a key molecule that interacts with and transports TLRs from the ER to endosomes (**Figure 1**) (7, 11). Consistently, the loss-of-function of Unc93b1 disrupted the TLR3, TLR7, and TLR9 signaling pathways (12). Moreover, endosomal TLR protein levels were reduced in mice harboring a Unc93b1 loss-of-function mutant that impaired its interaction with TLRs, suggesting a role of UNC93B1 in the stabilization of TLR proteins (13). Furthermore, TLR7 and TLR9 are oppositely regulated—TLR9 is predominantly maintained at a steady state, suppressing TLR7 responsiveness and avoiding TLR7-induced autoinflammatory diseases. This predominance of TLR9 was inhibited by a D34A mutation in Unc93B1, which also exacerbated TLR7 activation and systemic lethal inflammation in mice (14). Recently, Unc93b1 was shown to prevent TLR9 activation in intracellular compartments other than endosomes (15). TLR9 is released from Unc93b1 in endosomes, and this disassociation is required for the activation of signaling pathways. However, TLR7 continues to interact with Unc93b1 in endosomes and can activate signaling pathways without dissociation from Unc93b1. The association of Unc93b1 with TLR7 in endosomes is important for terminating TLR7 signaling. Syntenin-1, a PDZ domain-containing adaptor protein, facilitated sorting of the TLR7-Unc93b1 complex from endosomes into the intralumenal vesicles of multivesicular bodies to terminate receptor signaling (16). After stimulation with TLR7, but not TLR9 or TLR3, the interaction of Syntenin-1 with Unc93b1 is increased. Disruption of its binding to Unc93b1 prevents the sorting of TLR7 into multivesicular bodies and results in exaggerated TLR7 signaling. These findings suggest that UNC93B1 regulates the activities of individual endosomal TLRs *via* different mechanisms. The importance of UNC93B1 in human pathology was also demonstrated in patients with a mutation in *UNC93B1* who developed herpes simplex virus (HSV) encephalitis (17). The pathogenesis of HSV encephalitis in UNC93B1-deficient patients is likely caused by impaired TLR3 signaling in neurons and oligodendrocytes (18). The ectodomains of endosomal TLRs undergo proteolytic processing within endosomal compartments by cathepsins and asparagine endopeptidases to generate functional receptors (**Figure 1**) (19, 20). This proteolytic processing is thought to protect against unwanted interactions with self-derived nucleic acids. Indeed, mice expressing TLR9 mutants that accessed the cell surface, and did not require proteolysis for activation, developed systemic and lethal inflammation (21). The pH of intracellular vesicles might also be important for proteolytic processing and ligand recognition of endosomal TLRs. Blockade of the acidification of intracellular vesicles resulted in impaired innate immune responses mediated by TLR3, TLR7, and TLR9 (22, 23). The localization of nucleic acid-sensing TLRs in cellular compartments is also important for the initiation of cell-type-specific signaling pathways. In plasmacytoid DCs (pDCs), a subset of DCs that produce large amounts of type I IFNs *via* TLR7 and TLR9 signaling, the activation of NF-κB mediated by TLR7 or TLR9 was initiated in endosomes, whereas activation of IRF7 for type I IFN expression requires further transport from endosomes to lysosome-related organelles (LRO) *via* an adaptor protein-3 (AP-3)-dependent mechanism (**Figure 1**) (24).

Upon ligand binding, TLRs form a dimer that promotes the association of their intracellular TIR domains, resulting in the recruitment of TIR-containing adaptor proteins such as MyD88 and TRIF (**Figure 1**) (25). Upon ligand recognition, TLR7 and TLR9 recruit MyD88, IRAKs, and TRAF6. IRAKs and TRAF6 complexes subsequently activate TAK1, leading to the activation of NF-κB and mitogen-activated protein kinases (MAPKs). In pDCs, IRAKs and TRAF6 induce the activation of IRF7 by forming a complex that contains IRAK1, TRAF6, TRAF3, IKKα; and IRF7 (26–30). IRAK1 and IKKα phosphorylate IRF7, leading to the translocation of IRF7 into the nucleus (27, 28). In contrast, TLR3 is the only TLR that signals independently of MyD88 by recruiting TRIF upon ligand binding. TRIF interacts with TRAF3 and TRAF6, which promote the activation of TANK-binding kinase 1 (TBK1) and TAK1, respectively. Subsequently, activated TBK1 phosphorylates the pLxIS motif in TRIF, which in turn recruits and activates the transcription factor IRF3 (31). Finally, activated TAK1 activates NF-κB and MAPKs.

EXPRESSION AND LIGANDS OF NUCLEIC ACID-SENSING TLRS

TLR3 is mainly expressed by DCs, fibroblasts, and intestinal epithelial cells (32–34). TLR3 forms a homodimer and binds to 40–50 bp dsRNA, and multiple dimers bind to long dsRNA (35, 36). Although dsRNA longer than 90 bp can bind to TLR3 in early endosomes (pH 6.0–6.5), dsRNA of 40–50 bp is required to form a complex with TLR3 in late endosomes (lower than pH 5.5) (35). Thus, activation of the TLR3-mediated signaling cascade is thought to be dependent on the length of dsRNA and the localization of TLR3. Furthermore, TLR3 is involved in immune responses to several RNA viruses, such as West Nile virus (WNV), Semliki Forest virus, and encephalomyelitis virus (EMCV). DNA viruses such as mouse cytomegalovirus (MCMV) and HSV-1 also elicit TLR3-mediated immune responses, presumably by recognizing dsRNA intermediates from viruses (2). Accordingly, TLR3 is important for protection against HSV-1 infection of the central nervous system (37–39).

Human TLR7 and TLR8, and mouse TLR7, recognize ssRNA from viruses and bacteria, and imidazoquinoline derivatives,

such as imiquimod (R837) and resiquimod (R848) (5, 40–42). TLR8 in mice does not respond to ssRNA ligands because of the absence of five amino acids corresponding to amino acids in human TLR8 that are required for RNA recognition (43). Compared with other immune cells, pDCs and B cells predominantly express TLR7 (44, 45). In contrast, TLR8 is strongly expressed in immune cells other than pDC, such as monocytes/macrophages and myeloid DCs (46). Structural analysis revealed that TLR7 and TLR8 have two ligand binding sites in their ectodomains through which TLR7 binds to free guanosine molecules and ssRNAs, and TLR8 binds to free uridine molecules and ssRNAs (47, 48). In the presence of ssRNAs, the affinity of these free nucleotides was enhanced and the binding of both ssRNAs and free nucleotides was important for the efficient activation of TLR7 and TLR8. Because the presence of free nucleotides is required for their activation, the degradation of ssRNAs in lysosomes may be important for ssRNA recognition by TLR7 and TLR8. Indeed, activation of the endolysosomal endoribonucleases RNase T2 and RNase 2 is required for the recognition of pathogen-derived RNA by TLR8 (49, 50). In addition to guanosine and uridine, deoxyguanosine and deoxyuridine can also activate TLR7- and TLR8-induced signaling pathways, respectively in the presence of ssRNA (51). Therefore, together with ssRNA, DNA degradation products also synergistically increase the activation of TLR7 and TLR8, and abnormalities in DNA metabolism may trigger the inflammatory response due to increased activation of TLR7 and TLR8, as well as TLR9. Physiologically, TLR7 and TLR8 are involved in host responses against a variety of RNA viruses, including influenza A virus (IAV), human immunodeficiency virus (HIV), and vesicular stomatitis virus (VSV) (2). Although TLR7 and TLR8 are often considered to be similar, a recent report showed that TLR7 and TLR8 in human monocytes elicited different immune responses (52). In that study, activation of TLR7 promoted the expression of cytokines that induced Th17 cell polarization whereas activation of TLR8 induced the expression of Th1-type cytokines and type I IFNs.

TLR9, mainly expressed by pDCs, B cells, and monocytes/macrophages, recognizes DNA with an unmethylated CpG motif from bacteria and viruses (4). TLR9 forms a complex with CpG DNA at a 2:2 ratio (53). This interaction is increased under acidic conditions, and thus localization to lysosomes may allow TLR9 to recognize DNA. In contrast to CpG DNA, TLR9 has a different binding site for DNA with a cytosine at the second position from the 5′ end (5′-xCx DNA) (54). Moreover, binding of this type of DNA with CpG DNA promotes TLR9 dimerization and activation, suggesting that activation of TLR9 is regulated by binding to two types of DNA. Indeed, co-stimulation of mouse bone marrow-derived macrophages and pDCs with CpG DNA and 5′-xCx DNA increased TLR9 activation (54). It remains to be elucidated whether the recognition of 5′-xCx DNA motif has any advantage in inducing immune responses. Studies using TLR9-deficient mice showed that TLR9 was physiologically involved in sensing DNA viruses, including MCMV, HSV-1, HSV-2, and adenovirus (2).

CYTOSOLIC RNA SENSOR: RLRS

Invading RNA viruses release their RNA into the cytoplasm of host cells and force the host cell to synthesize viral components by using the host machinery. The innate immune system can sense cytosolic RNA *via* RLR family members (**Figure 2**). RLRs are composed of retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology2 (LGP2), which are upregulated by type I IFN exposure in various tissues (55–58). RLRs share structural features consisting of a central DExD/H box RNA helicase domain and a C-terminal domain (CTD), which sense RNA. In addition, RIG-I and MDA5 have two caspase activation and recruitment domains (CARDs) at the N-terminus that mediate downstream signaling. In contrast, LGP2 lacks CARD, and its physiological function with regard to RIG-I- or MDA5-mediated signaling remains controversial (59, 60).

RIG-I and MDA5 recognize different dsRNA species. RIG-I recognizes relatively short dsRNA while MDA5 preferentially binds to long dsRNA (>1 kb) (61). In addition to RNA length, RIG-I requires additional properties at the dsRNA 5′-end. Although short dsRNA without a 5′-triphosphate was proposed to activate RIG-I, a 5′-tri- or 5′-di-phosphate end in dsRNA seems to be important for the strong activation of RIG-I (61, 62). Furthermore, a blunt-end at the triphosphate end and unmethylated 5′-terminal nucleotide at the 2′-O position were important for RIG-I activation (63, 64). In addition to dsRNA, RIG-I recognizes ssRNA with a 5′-triphosphate to activate downstream signaling pathways (65, 66). However, the length and the degree of complementarity of dsRNA are considered more important for the activation of MDA5 (**Figure 2**) (61, 67). Because host-derived RNA undergoes 5′-processing, including cap formation by 2′-O-methylation in the nucleus, and long dsRNA is not normally present in host cells, these ligand specificities of RIG-I and MDA5 are critical to avoid the recognition of self-derived RNA.

In the steady state, RIG-I is present in an auto-repressed conformation, which masks its CARDs to prevent signal transduction. Binding of nucleic acids to RIG-I leads to an ATP-dependent conformational change, which results in the release of CARDs from autoinhibition (68). This conformational change allows CARDs to undergo additional modifications such as polyubiquitination (**Figure 2**) (55). Covalent conjugation and non-covalent binding of K63-linked polyubiquitin chains to the CARDs of RIG-I lead to the formation and stabilization of a RIG-I-tetramer that functions as a signaling platform (69, 70). Several E3 ubiquitin ligases positively regulate the RIG-I signaling pathway, including TRIM25, RIPLET, TRIM4, and MEX3C (71–74). All of these E3 ligases are involved in the K63-linked polyubiquitination of RIG-I CARDs, while only RIPLET was reported to mediate the K63-linked polyubiquitination of RIG-I CTD (75). This polyubiquitination of RIG-I CTD promotes the release of RIG-I CARD autoinhibition and is required for TRIM25-mediated RIG-I activation, suggesting RIPLET may be a prerequisite for RIG-I activation (75, 76). Recent studies showed that RIPLET, but not TRIM25, is required for RIG-I signaling (77, 78). These findings support the critical role of RIPLET in the RIG-I signaling pathway, and indicate that other E3 ligases for CARD

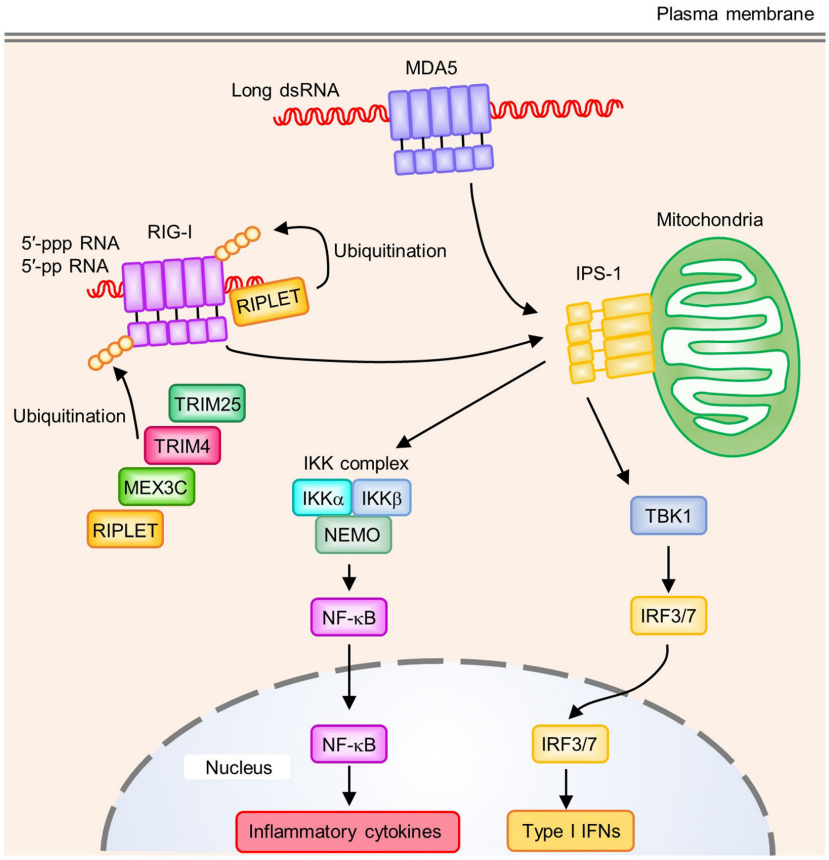


FIGURE 2 | Signaling pathways of RIG-I-like receptors (RLRs). RIG-I recognizes the 5′-tri- or 5′-di-phosphate end of RNA. A blunt-end at the triphosphate end and an unmethylated 5′-terminal nucleotide at the 2′-O position are also required to activate RIG-I. MDA5 binds to long dsRNA, which allows the oligomerization of MDA5 by forming a helical filament-like structure. Polyubiquitination on RIG-I CTD by RIPLET has a critical role in RIG-I activation. In addition to RIPLET, other E3 ubiquitin ligases such as TRIM25, TRIM4, and MEX3C act as positive regulators by mediating K63-linked polyubiquitination on RIG-I CARDs. Oligomerized CARDs of RIG-I or MDA5 interact with IPS-1 on mitochondria, which activates downstream signaling pathways. IPS-1 induces activation of the TBK1 and IKK complex (IKKα, IKKβ, and NEMO), which activates the transcription factors IRF3/7 and NF-κB, respectively. These transcription factors induce the production of type I IFNs and proinflammatory cytokines.

polyubiquitination might be functionally redundant. In addition to ubiquitination, a recent study showed that RIPLET regulated the RIG-I signaling pathway in a ubiquitin-independent manner. RIG-I forms filaments on dsRNA and RIPLET binds to the filamentous oligomers of RIG-I, which induces the cross-bridging of RIG-I filaments and receptor clustering that allows the efficient activation of RIG-I signaling. However, ubiquitination by RIPLET is dispensable for MDA5 activation, which requires the formation of a helical filament along with long dsRNA, allowing the oligomerization of CARDs (79–81). In contrast, ZNF598, another E3 ubiquitin ligase that negatively regulates RIG-I-mediated signaling, interacts with RIG-I to deliver a ubiquitin-like protein FAT10 to RIG-I, which inhibits the K63-linked polyubiquitination of RIG-I and prevents activation of the RIG-I signaling pathway (82).

Oligomerization of the CARDs of RIG-I or MDA5 upon dsRNA recognition induces their interaction with the CARD of adaptor protein interferon-β promotor stimulator 1 (IPS-1, also known as MAVS) (**Figure 2**) (83, 84). In addition to CARD, IPS-1 contains a proline-rich region, TRAF-interacting motifs (TIMs), and a C-terminal transmembrane domain. IPS-1 anchors to the outer mitochondrial membrane (OMM), mitochondrial-associated endoplasmic reticulum membranes (MAMs), and peroxisomes *via* its C-terminal transmembrane domain (85). The binding of IPS-1 to RIG-I or MDA5 leads to the oligomerization of IPS-1 to form prion-like aggregates, which are crucial for activating downstream singling pathways (86). IPS-1 activates TBK1 to induce the IRF3- or IRF7-mediated transcription of type I IFNs, and also the IKK complex (IKKα, IKKβ, NEMO) to induce the NF-κB-mediated transcription of inflammatory cytokines (55, 87).

Although RIG-I and MDA5 sense cytosolic RNA, their responses to RNA viruses are different. RIG-I recognizes Paramyxoviruses, Rhabdoviruses, Orthomyxoviruses, Filoviruses, and Flaviviruses, such as Sendai virus, Newcastle disease virus (NDV), VSV, influenza virus, Ebola, and hepatitis C virus (HCV). In contrast, MDA5 recognizes Picornaviruses, such as EMCV, Theiler's virus, and Mengo virus. Viruses including dengue virus,

WNV, and reovirus are recognized by RIG-I and MDA5 (55). In addition to RNA viruses, several DNA viruses also activate RIG-I and MDA5. Epstein-Barr virus (EBV) and adenovirus stimulate the RIG-I signaling pathway. EBV-encoded small RNAs (EBERs), short noncoding RNAs that are highly abundant viral transcripts in latently EBV-infected cells, are recognized by RIG-I (88). Adenovirus also produces short noncoding RNA called adenovirus-associated RNAs (VA) in infected cells, and VA induce type I IFNs by a RIG-I-dependent mechanism (89). RNA polymerase III (Pol III) is an enzyme that mediates the synthesis of EBERs and VA that contain a 5′-triphosphate from viral DNA. In human primary macrophages, the early induction of type I IFNs against HSV-1 is dependent on MDA5; however, Pol III does not appear to mediate this response (90). MDA5 also induces innate immune signaling against hepatitis B virus (HBV) by associating with HBV-specific nucleic acids (91).

CYTOSOLIC DNA SENSOR: CGAS

Z-DNA binding protein 1 (ZBP1), IFN-gamma inducible protein 16 (IFI16), Pol III, MRE11, and cyclic GMP-AMP (cGAMP) synthase (cGAS) were reported to be cytosolic DNA sensors that induce type I IFNs (55, 92–96). Absent in melanoma 2 (AIM2) is a cytosolic DNA sensor that induces caspase-1-dependent IL-1β production and pyroptotic cell death rather than type I IFNs (discussed below). Among these molecules, cGAS, an enzyme that synthesizes the second messenger cGAMP from ATP and GTP upon its binding to dsDNA, plays a central role in recognizing cytosolic DNA, which induces the production of type I IFNs and proinflammatory cytokines (**Figure 3**). cGAS binds to dsDNA independent of its sequence by forming a 2:2 cGAS-dsDNA complex (97, 98). However, the length or bending of dsDNA seems to be a key factor for cGAS activation. Furthermore, compared to short dsDNA, long dsDNA is a potent activator of cGAS (99, 100). Long and bent dsDNA allows cGAS dimers to form protein-DNA ladder-like structures, which stabilize complexes consisting of two cGASs and two dsDNAs (99). Mitochondrial transcription factor A (TFAM) and high-mobility group box 1 protein (HMGB1) are known as endogenous DNA-interacting proteins that are able to induce U-turns and bends in DNA, which nucleate the formation of cGAS dimers to enhance the activation of cGAS. In addition to its dimerization, the length of dsDNA influences the efficiency of signal transduction (101).

cGAS was reported to localize to plasma membrane, cytosol, and nucleus (95, 102–104). Depending on cell types, the localization of cGAS is different (102). Furthermore, the

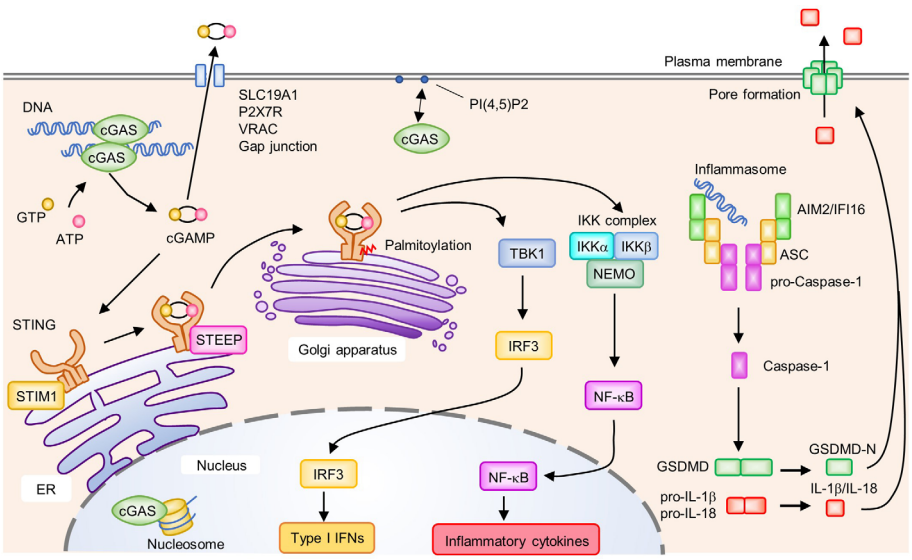


FIGURE 3 | Activation of the cyclic GMP-AMP (cGAMP) synthase (cGAS)-stimulator of IFN genes (STING) signaling pathway and AIM2/IFI16 inflammasome. cGAS recognizes DNA in the cytosol and subsequently synthesizes the second messenger cGAMP from GTP and ATP. In the steady state, STIM1 interacts with STING to retain it on the endoplasmic reticulum (ER) membrane. Binding of cGAMP to STING induces the translocation of STING from the ER to the Golgi apparatus. Upon activation, enhanced interactions between STEEP and STING promote the trafficking of STING from the ER to the Golgi apparatus where it undergoes post-translational modifications such as palmitoylation. Activated STING on the Golgi apparatus recruits and activates TBK1 and the IKK complex (IKKα, IKKβ, and NEMO), which induce the production of type I IFNs and proinflammatory cytokines by IRF3 and NF-κB, respectively. cGAMP activates surrounding cells by being transferred to the extracellular space *via* SLC19A1, P2X7R, VRAC, and gap junctions. cGAS is associated with PI(4,5)P2 on the plasma membrane and is trafficked away from the nucleus to prevent the aberrant activation of cGAS by self-derived DNA. cGAS is also localized in the nucleus where its activity is inhibited by interactions with the nucleosome. Cytosolic DNA is recognized by ALRs, leading to the formation of an inflammasome composed of AIM2 or IFI16, ASC, and pro-Caspase-1. Within the inflammasome, Caspase-1 is activated by proteolytic cleavage from pro-Caspase-1 to Caspase-1. Activated Caspase-1 cleaves GSDMD, pro-IL-1β; and pro-IL-18. The N-terminus of GSDMD (GSDMD-N) forms a pore on the plasma membrane and induces cell death accompanied by the release of biologically active IL-1β and IL-18.

localization of cGAS might change upon DNA stimulation (102). In mouse and human phagocytes, cGAS interacts with PI(4,5)P2, a phosphoinositide in the plasma membrane, to promote its localization to the plasma membrane, which may prevent excessive immune responses to self-derived dsDNA, which is abundant in the nucleus (**Figure 3**) (102). However, recent reports suggested that cGAS is localized in part in the nucleus (103, 104). Although cGAS is expressed as a cytosolic protein, it can bind to self-derived dsDNA when the nuclear envelope undergoes breakdown during the cell cycle and generates daughter cells that contain cGAS in the nucleus. However, the activity of cGAS in response to self-derived dsDNA is less than for exogenous dsDNA although nuclear-localized cGAS can also induce innate immune signaling. Therefore, in addition to the existence of nuclear envelope, there may be an unknown regulatory mechanism which prevents the activation of immune response against self-DNA. Recently, structural analyses of the complex formed between nucleosome core particles (NCPs) and cGAS revealed that the nucleosome inhibits cGAS activation by binding to the DNA binding site of cGAS to prevent its dimerization by steric hindrance with the proximal NCPs (105–109). In the presence of DNAs and nucleosomes, cGAS preferentially binds to nucleosomes, which might be a key regulatory strategy allowing cGAS to localize to the nucleus without persistent activation. Positively charged residues of human and mouse cGAS, such as lysine and arginine, were reported to be important for their specific binding to the acidic part of nucleosomes, and mutations in these positions disrupt the interaction with nucleosomes to abolish the cGAS-suppressive effect of NCPs. Moreover, nuclear cGAS accelerates irradiation-induced genome destabilization and cell death by restraining homologous recombinant-DNA repair. This inhibition is achieved by the compression of dsDNA to a higher order state through its binding to dsDNA and self-oligomerization (103). Thus, cGAS regulates various cellular responses in which nuclear self-derived DNA is involved, in addition to antiviral innate immune responses to foreign DNA.

Upon activation, cGAS produces the second messenger cGAMP from ATP and GTP, which subsequently binds to and activates ER-resident adaptor protein stimulator of IFN genes (STING), resulting in a conformational change and oligomerization of STING (**Figure 3**) (110, 111). Of note, cGAMP can be transferred to surrounding cells *via* SLC19A1, P2X7R, or LRRC8A/LRRC8E-containing volume-regulated anion channels and gap junctions, inducing the STING-dependent production of type I IFNs in neighboring cells (112–116). In addition, cGAMP can be incorporated into viral particles and newly formed viruses transmit antiviral signals to subsequently infected cells (117, 118). Such cell-to-cell transfer of cGAMP promotes the rapid propagation of inflammatory signals and the amplification of inflammatory responses.

Upon DNA stimulation, STING changes its cellular localization from the ER to the Golgi apparatus *via* ER-Golgi intermediate compartments, which is necessary to activate the downstream signaling pathway (**Figure 3**) (119). In the inactivated state, STING is retained on the ER membrane by its association with ER-resident protein stromal interaction molecule 1 (STIM1). The binding of cGAMP to STING reduces the association between STIM1 and STING, and promotes its translocation to the Golgi apparatus (120). TOLLIP, another protein that interacts with resting-state STING, stabilizes STING by preventing its degradation by the lysosome pathway (121). Knockout of TOLLIP ameliorates autoimmune symptoms in Trex1-knockout mice in which cGAS-STING-mediated signaling is activated (121). A recent study showed that CxORF56, also known as STING ER exit protein (STEEP), interacted with STING to promote its trafficking from the ER to the Golgi apparatus. STEEP augments PI(3)P accumulation in the ER and promotes ER membrane curvature, which facilitates COPII-mediated STING ER exit (122). In contrast, myotubularin-related protein 3 (MTMR3) and MTMR4, members of the phosphatase superfamily, negatively regulate STING-mediated innate immune responses by reducing PI(3)P levels. MTMR3 and MTMR4 deficiencies resulted in increased PI(3)P and rapid STING trafficking from the ER to the Golgi apparatus upon DNA stimulation (123). Post-translational modifications such as phosphorylation and ubiquitination are also involved in STING activation (124, 125). In addition, the palmitoylation of STING by palmitoyl transferases (DHHC3, DHHC7, and DHHC15) in the Golgi apparatus is necessary for STING-dependent IFN production (126, 127). Cysteine residues in proteins are the target sites for palmitoylation, and Cys88/91 on STING is thought to be critical for its modification and activation. Activated STING subsequently recruits and activates TBK1 to phosphorylate STING at a pLxIS motif (31, 128). This further induces IRF3 recruitment, and in turn, TBK1 phosphorylates IRF3, leading to type I IFN expression. A small GTPase RAB2B and its effector protein Golgi-associated RAB2B interactor-like 5 (GARIL5) were reported to positively regulate the cGAS-STING signaling pathway (129). The RAB2B-GARIL5 complex colocalizes with STING on the Golgi apparatus to regulate the cGAS-STING signaling pathway by promoting the phosphorylation of IRF3 by TBK1 (129). STING also activates the IKK complex to induce the translocation of NF-κB into the nucleus (**Figure 3**). Notably, cGAS-STING pathway also induces autophagy, which is thought to play a role in mediating the clearance of cytosolic DNA or DNA viruses (130).

A number of studies using cGAS- or STING-deficient mice showed that cGAS is involved in antiviral responses against a wide range of DNA viruses, including HSV, vaccinia virus, and murine gamma herpesvirus 68 (MHV68) (131). Importantly, retroviruses including HIV also activate the cGAS-STING pathway. Following retroviral infection, cGAS recognizes DNA, reverse-transcribed from viral genomic RNA, which is incorporated into the host cell genome (132). In addition to viruses, cGAS is also involved in immune responses against intracellular bacteria, such as *Listeria monocytogenes* and *Neisseria gonorrhoeae (*133, 134). Interestingly, certain bacteria can activate cGAS indirectly by inducing cellular stress. For example, *Mycobacterium tuberculosis* causes the release of mitochondrial DNA (mtDNA) into the cytoplasm from mitochondria, which activates cGAS (135). A recent study

reported that cell fusion induced by bacteria, such as *Burkholderia pseudomallei*, acts as a danger signal in the host and triggers genomic instability and micronuclei formation, resulting in cGAS activation. This activation of cGAS leads to autophagic cell death rather than type I IFN production (136). cGAS can also recognize mtDNA released through Bak- and Bax-mediated pore formation during apoptosis (137). However, the activation of cGAS-STING pathway in apoptotic cells is inhibited by pro-apoptotic caspases which cleave key proteins for production of type I IFNs, including cGAS and IRF3, to prevent inflammation induced by cell death (137–139).

CELL DEATH INDUCED BY NUCLEIC ACID SENSORS

Nucleic acid sensors are shown to trigger cell death such as apoptosis, pyroptosis and necroptosis. RLRs induce apoptosis *via* IRF3 (140). Activated IRF3 interacts with Bax, a pro-apoptotic protein, which induces their co-translocation to mitochondria and triggers Cytochrome c release to cytoplasm (140). This IRF3-mediated apoptosis pathway is not dependent of transcriptional activation of IRF3, but linear polyubiquitination of IRF3 by the protein complex, LUBAC (linear ubiquitin chain assembly complex) (141). IRF3-mediated apoptosis is sufficient for protection against pathogenesis in Sendai virus infection (141). Activation of TLR3-TRIF pathway also induces apoptosis in cancer cells. TRIF interacts with RIPK1 through their RIP homotypic interaction motif (RHIM) domains and forms a complex with caspase-8 to induce apoptosis in the absence of cellular inhibitor of apoptosis proteins (cIAPs) (142).

AIM2-like receptors (ALRs), including AIM2 and IFI16, recognize cytosolic DNA and induce inflammatory responses. The recognition of DNA by ALRs promotes the formation of an inflammasome, a multiprotein complex formed in response to pathogens and endogenous danger signals, leading to a programmed, immunogenic, and lytic type of cell death termed pyroptosis (143). The inflammasome activates Caspase-1 (proteolytic cleavage from pro-Caspase-1 to Caspase-1), resulting in maturation of the inflammatory cytokines IL-1β and IL-18, as well as the cleavage of Gasdermin D (GSDMD). Subsequently, the N-terminus of cleaved GSDMD form a pore at the plasma membrane that leads to pyroptosis accompanied by the release of biologically active cytokines (IL-1β and IL-18) (**Figure 3**). However, in human monocytes, the inflammasome activation by cytosolic DNA is dependent on cGAS-STING-NLRP3 axis, but not AIM2 (144). Mechanistically, STING triggers lysosome membrane permeation, which results in NLRP3 inflammasome activation. Moreover, cGAMP contributes to NLRP3 and AIM2 inflammasomes activation in bone marrow-derived macrophages (145). cGAS-STING-NLRP3 axis is activated upon HSV-1 infection (146). HSV-1 infection promotes the STING-NLRP3 interaction and facilitated the formation NLRP3 inflammasome in ER.

The AIM2 inflammasome is involved in responses against viral and bacterial infections. Several bacterial species were reported to activate AIM2 including *Francisella tularensis*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Brucella abortus*, and *Chlamydia muridarum* (147, 148). Because AIM2 is localized in the cytoplasm, bacterial DNA must be released into the cytoplasm for AIM2 to access it. This is achieved by guanylate-binding proteins (GBPs) that are involved in bacteriolysis and which are important for AIM2 inflammasome activation. Indeed, *Francisella novicida* infection induces the expression of GBP2 and GBP5 in the cytosol, which is dependent on the IRF1-mediated induction of type I IFNs. These proteins associate with *Francisella novicida* in the cytoplasm to trigger bacteriolysis, which allows AIM2 to recognize dsDNA released from bacteria (149, 150). Furthermore, another IFN inducible gene, interferon response gene B10 (IRGB10), is associated with these GBPs, and in combination they induce the membrane rupture of *Francisella novicida* (151). Moreover, AIM2 inflammasome is activated by several DNA viruses, such as MCVM, vaccinia virus, and human papillomavirus (147, 148, 152). In addition to viral DNA, AIM2 recognizes self-derived DNA released from tissues damaged by viral infection. IAV was reported to trigger the release of mitochondrial or nuclear DNA from macrophages and damaged lung tissues, leading to AIM2 inflammasome activation. However, it is debatable whether this activation of AIM2 is protective or harmful (153–155).

IFI16 is localized in the cytosol and nucleus, and is associated with the production of type I IFNs and cell death induced by HSV, HIV-1, Kaposi sarcoma-associated herpesvirus (KSHV), and intracellular bacterium *Listeria monocytogenes* (94, 133, 156–158). Furthermore, in human cells, IFI16 is thought to cooperate with the cGAS-STING pathway to induce robust host anti-viral responses (156, 157). In contrast, STING negatively regulates IFI16 by recruiting an E3 ligase TRIM21 to induce its degradation (159). This STING-mediated negative feedback might prevent excess immune responses mediated by IFI16. IFI16 is localized in the nucleus and acts as a nuclear sensor for nuclear replicating viruses such as KSHV, EBV, and HSV-1 (158). A previous study reported that IFI16 colocalized with virus genomes in the nucleus to form an inflammasome complex, which is then relocated into the cytoplasm to induce inflammasome activation and STING-dependent IFN responses. Furthermore, breast cancer 1 (BRCA1), a DNA damage repair sensor and transcription regulator, was reported to be a positive regulator of IFI16. BRCA1 interacts with IFI16 in the nucleus, and is enhanced upon virus infection. The knockdown of BRCA1 decreased the association of IFI16 with the viral genome and reduced the subsequent activation of inflammasomes and IFN responses (160).

TLR3 and ZBP1 induce necroptosis, a lytic type of cell death which is regulated by receptor-interacting protein kinase 3 (RIPK3) and mixed-lineage kinase domain-like pseudokinase (MLKL). RIPK3 is activated by RIPK1 which is activated by death receptors, such as TNF receptor 1 (TNFR1), CD95, and TRAIL-R, when caspase-8 activation is inhibited (161). Activated RIPK3 phosphorylates MLKL, which in turn triggers MLKL oligomerization, membrane translocation, and membrane disruption. In addition to RIPK1, the activation of RIPK3 is mediated by other RHIM domain-containing molecules. TRIF

and ZBP1, which contain the RHIM domain, also induce necroptosis by interacting with RIPK3 (162, 163).

ROLES OF NUCLEIC ACID SENSORS IN INFLAMMATORY DISEASES

Autoinflammatory Diseases Caused by Aberrant Activation of Nucleic Acid-Sensing Pathways

Although the induction of inflammatory responses through the above mentioned nucleic acid receptors is important for protecting hosts from invading pathogens, autoinflammatory pathology can be caused by aberrant inflammatory responses, specifically abnormalities in receptors, signaling molecules, and nucleic acid metabolism. Mutations in the RNA helicase domains of RIG-I and MDA5 were found in patients with systemic lupus erythematosus (SLE), Aicardi-Goutieres syndrome (AGS), and Singleton-Merten syndrome (SMS), all of which exhibit a type I IFN signature (164–169). A mutation in RIG-I was reported in SMS whereas mutations in MDA5 were associated with the various diseases described above. Mice with an *Ifih1* missense mutation encoding MDA5, developed lupus-like autoimmune symptoms without viral infection (170). TLR7 and TLR9 were reported to be involved in the pathogenesis of SLE. The proportions of B cells and monocytes expressing TLR9 were higher among patients with active SLE than among patients with inactive SLE, and this correlated with the presence of anti-dsDNA antibodies (171). Another study showed that mice overexpressing TLR7 developed SLE-like disease (172). In addition, IFNα production mediated by TLR7 was increased in pDCs derived from SLE patients, and correlated with disease severity. Furthermore, enhanced IFNα production was associated with increased TLR7 expression in the late endosomal/lysosomal compartment in lupus pDCs (173).

Genetic mutations in molecules that function in signaling cascades downstream of nucleic acid sensors also cause autoinflammatory diseases. STING gain-of-function mutations (V147L, N154S, and V155M) are involved in lupus-like syndromes and STING-associated vasculopathy with onset in infancy (SAVI) (174, 175). SAVI is characterized by systemic inflammation, interstitial lung disease, cutaneous vasculitis, and recurrent bacterial infection. The STING mutation, STING-V155M, which is localized mainly in the Golgi apparatus and perinuclear vesicles in fibroblasts independent of the presence of its ligand, interacts with STEEP to a greater degree compared with WT STING (122, 176). Recently, it was reported that C9orf72 is essential for control of immune activation mediated by STING and the loss of C9orf72 promoted the production of type I IFNs (177). Expansion of a hexanucleotide repeat (GGGGCC) in the *C9orf72* gene was shown to be the major cause of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Blood monocyte-derived macrophages from these patients showed an enhanced type I IFN signature (177). Furthermore, IRF5, a downstream mediator of TLR signaling, was also identified as an autoimmune susceptibility gene (178). IRF5 expression is upregulated in SLE patients and this enhanced expression was associated with the risk haplotype of IRF5 (179). IRF5-deficient mice or SLE model mice treated with an IRF5 inhibitor attenuated lupus pathology (180, 181). *Ex vivo* human studies demonstrated that an IRF5 inhibitor blocked SLE serum-induced IRF5 activation in healthy immune cells and significantly reduced basal IRF5 hyper-activation in SLE immune cells (181).

Inflammatory Diseases Caused by Dysregulated Nucleic Acid Homeostasis

Molecules involved in nucleic acid metabolism, such as DNases and RNases, play important roles in avoiding aberrant induction of inflammatory responses against self-derived nucleic acids that lead to autoinflammatory diseases.

TREX1, RNase H2 complex, SAMHD1, DNASE1L3, and DNase II are key enzymes that control the turnover of endogenous DNA, and mutations in these genes cause autoinflammatory diseases (182–184). TREX1 is the major mammalian 3′ to 5′ DNA exonuclease located on the ER membrane. Loss-of-function mutations in the human *TREX1* gene were reported to induce AGS and SLE (185, 186). Mutations in *TREX1* cause the accumulation of cellular nucleic acids, and failure to remove these nucleic acids may result in the excessive activation of immune responses against them. Indeed, single-stranded DNA fragments derived from endogenous retroelements that had accumulated in the heart cells of TREX1-deficient mice might induce type I IFN responses (187). An RNase H2 complex comprised of three proteins encoded by *RNASEH2A*, *RNASEH2B*, and *RNASEH2C*, degrades the RNA strand of the RNA-DNA heteroduplex. Mutations in RNase H2 subunits result in genome instability, which causes AGS (188, 189). SAMHD1, a deoxynucleotide triphosphate (dNTP) triphosphohydrolase, is required to maintain the balance of the dNTP pool in cells. Recently, it was reported that SAMHD1 promoted the degradation of nascent DNA at stalled replication forks by activating MRE11 exonuclease independent of the dNTPase activity of SAMHD1, and that SAMHD1-deficiency caused the accumulation of ssDNA in the cytoplasm (190). Mutations in SAMHD1 caused AGS possibly by the accumulation of ssDNA and a genome instability due to the increase in dNTP pools (190, 191). Importantly, enhanced type I IFN production in AGS caused by TREX1, RNaseH2 complex, or SAMHD1 mutations is mediated by the cGAS-STING pathway (190, 192, 193). Of note, genome instability leads to the formation of micronuclei, small DNA-containing structures that are not incorporated correctly into the nucleus after cell division (194). Micronuclear envelopes are prone to rupture, resulting in the release of damaged DNA to the cytosol, which in turn stimulates the cGAS-STING axis to induce inflammatory responses (195, 196). Genome instability triggers the generation of micronuclei, which are thought to promote excessive cGAS-dependent immune responses in cells carrying these mutations (197). DNASE1L3 is a secreted DNase that digests cell-free DNA and chromatin in microparticles derived from apoptotic cells (198, 199). Loss-of-function mutation in *DNASE1L3* leads to rare form of SLE (183). In Dnase1l3-deficient mice, TLR9 and TLR7, but not cGAS-STING pathway, were redundantly required for autoimmunity (199, 200).

DNase II is localized in lysosome and digests the DNA from apoptotic cells and nuclear DNA expelled from erythroid precursor cells (201, 202). The embryonic lethality of DNase II knockout mice is rescued by lack of *Ifnar1* gene, suggesting that abnormal activation of type I IFN responses is taken place in this mice (203). Mice lacking DNase II and Ifnar1 developed chronic polyarthritis, and loss of DNase II gene in the bone marrow-derived cell was sufficient to induce this arthritis (184, 204).

To prevent excessive inflammation against self-derived RNA, proper RNA-processing systems is also important. Up to 25% of cytosolic Alu RNAs are forming Alu-Alu hybrids which have duplex RNA structures formed by inverted repeat Alu elements (67). These Alu-Alu hybrids are modified by ADAR1, an adenosine-to-inosine editing enzyme of dsRNA, which results in destabilization of RNA duplexes and prevents the recognition by MDA5 (67, 205, 206). Mutations in ADAR1 cause AGS with aberrant type I IFN response (207). mtRNAs also form double-stranded RNA structures, which can activate MDA5 when they escape to the cytoplasm (208). To restrict the levels of mitochondrial dsRNA, mitochondrial RNA helicase SUV3 and polynucleotide phosphorylase PNPase play an important role (208). Knockdown of PNPase, but not SUV3, caused the release of mitochondrial dsRNA into cytoplasm, and increased type I IFN production through the MDA5-IPS1 axis (208). Mutations in PNPT1, which encodes PNPase, cause several disorders including hearing loss and Leigh syndrome (209, 210).

Inflammation Induced by Self-Derived Nucleic Acid Recognition in Other Common Diseases

In addition to autoinflammatory diseases, the pathologies of several common diseases are linked to inflammatory responses induced by self-derived DNA or RNA. The cGAS-STING signaling pathway is activated following myocardial infarction by recognizing self-DNA derived from dead cells in the heart. The genetic or pharmacological disruption of cGAS-STING and type I IFN signaling improved survival and pathological remodeling in a myocardial infarction mouse model (211, 212). Parkinson's disease is also linked to inflammation induced by self-derived DNA. Mutations in Parkin or PINK1, which are involved in mitophagy that removes damaged mitochondria, lead to Parkinson's disease in humans. The accumulation of damaged mitochondria and increased circulating mtDNA in serum were observed in *Prkn*$^{-/-}$ or *Pink1*-knockout mice under mitochondrial stress, and this induced strong inflammation which was rescued by a loss of STING (213). The upregulation of cGAS was observed in the striatum from postmortem Huntington disease (HD) patients, and HD cells showed enhanced inflammatory gene expression and autophagy induction. Numerous micronuclei were found in HD cells indicating they might enhance cGAS activity, which may contribute to HD pathology (214). Psoriasis is another disease in which DNA-induced inflammation is involved. Increased cell-free DNA and mtDNA were detected in the serum of psoriatic patients (215, 216). The topical skin application of imiquimod, a TLR7 ligand, is used to induce psoriasis in mice. In this imiquimod-induced psoriasis model, TLR7 and TLR9, but not TLR7 or TLR9 alone, are required for its pathogenesis, suggesting DNA recognition is important for the development of disease (217). In addition to the activation of TLR7 signaling pathway, imiquimod induces cell death, and thus DNA derived from dead cells might be a trigger of TLR9 signaling in this model (218). LL37, an antimicrobial peptide, has an important role in psoriasis by forming a complex with DNA and delivering cell-free DNA into endosomes, which activates TLR9 (219). Recently, it was reported that topical treatment with cationic nanoparticles, which interfere the DNA-LL37 complex, relieved the symptoms of psoriasis in mice and monkeys (220). Self-RNA-mediated inflammation is also thought to be involved in the pathogenesis of psoriasis. LL37 was reported to form complexes with self-derived RNA as well as self-derived DNA, triggering TLR7 and TLR8 activation in human DCs, which may be associated with psoriasis pathogenesis (221). Another study showed that together with cargo peptides, polyamines form a complex with RNA, promoting endosomal uptake and activation of TLR7 in DCs (222). The decreased expression of protein phosphatase 6 was observed in psoriatic lesions, leading to an increased generation of arginase-1-mediated polyamine. Thus, inflammatory responses induced by self-derived nucleic acids may cause disease as well as contribute to the exacerbation of disease pathogenesis, and the inhibition of nucleic acid-induced inflammation might be a therapeutic target for the treatment of various diseases.

NUCLEIC ACID SENSORS IN CANCER

Role of Nucleic Acid Sensors in Anti-Cancer Treatment

Many studies have demonstrated the involvement of DNA-sensing pathways in antitumor responses as well as tumor development. Cancer cells often contain cytosolic DNA, which may not be present under physiological conditions. The generation of micronuclei and the release of mtDNA from mitochondria, caused by chromosomal instability and mitochondrial damage, respectively, are the main sources of cytoplasmic DNA in cancer cells. Sensing tumor DNA in tumor cells results in type I IFN production, which contributes to the maturation of DCs and the activation of $CD8^+$ T cells that have potent antitumor activity (223). Several reports have suggested the importance of the cGAS-STING axis in antitumor responses, rather than other nucleic acid-sensing receptor-mediated pathways, by bridging innate immune responses and tumor-specific T cell responses *via* the production of type I IFNs. Downregulation of the cGAS-STING pathway in tumors correlates with a poor prognosis in patients with gastric cancer (224). Of note, several human colon cancer cell lines show low or defective STING-mediated signaling, and STING-deficiency in prostate cancer cells increased tumor growth *in vivo* (225, 226). These defects in the STING pathway may be related to epigenetic silencing *via* methylation of the promoter region of cGAS and STING, or the loss-of-function mutations of these genes (227).

In addition to cancer cells, activation of cGAS-STING pathway in immune cells also contributes to antitumor activities. It is well known that antitumor effects are associated with the production of type I IFNs by DNA sensing after

radiotherapy and chemotherapy, which induce DNA damage in cancer cells and the release of DNA into the cytosol (228). The cGAS-STING pathway can be activated by tumor cell-derived DNA, and STING- or IRF3-deficient mice showed defects in priming $CD8^+$ T cells and tumor control (113, 229). Furthermore, cancer cell-derived cGAMP is thought to be transferred to neighboring immune cells, resulting in activation of the STING pathway (113, 230). Moreover, tumor-derived DNA is also thought to be transferred to host immune cells and activate immune responses. Treatment with the anticancer drug topotecan induces the release of exosomes containing DNA, which are then taken up by DCs and presented to activate antitumor immunity *via* the STING pathway (231).

Because of the importance of the cGAS-STING pathway in antitumor activities, cyclic dinucleotide (CDN), a STING agonist structurally related to cGAMP, is thought to be useful for anti-cancer therapy. Indeed, treatment with the STING agonist cGAMP inhibited tumor growth in mice (232, 233). STING-activating nanoparticles containing cGAMP were designed to enhance the efficacy of CDN by protecting it from clearance and increasing its transport to the cytosol, and nanoparticle treatment of mice injected with poorly immunogenic B16.F10 melanoma showed a decreased tumor growth rate and prolonged survival relative to mice treated with pure cGAMP (234). The antitumor efficacy of cGAMP treatment was further enhanced with anti-CTLA4 and PD-1 immune checkpoint blockade (233, 234). Based on their anticancer activities in mice, synthetic CDNs that stimulate STING have been approved for clinical trials to test their anticancer effects in humans (235).

In addition to DNA sensors, RNA sensors also contribute to the elimination of cancer cells. DNA methyltransferase inhibitors have been shown to exert clinical anti-tumor effect by inducing MDA5 and TLR3 signaling pathways (236, 237). The activation of these RNA sensors might be induced by dsRNAs derived from endogenous retroviruses (ERVs) which are normally silenced in cells by DNA methylation (237). Furthermore, ablation of histone H3K4 demethylase LSD1 resulted in upregulation of ERVs and accumulation of dsRNAs that are recognized by MDA5 and TLR3 in cancer cells, which promotes anti-tumor T cell immunity and elicits significant responses to anti-PD-1 therapy in a mouse melanoma model (238). Therefore, inhibition of LSD1 might also be a potent strategy in cancer immunotherapy. Radiation therapy also induces the activation of ERVs, which mediates IPS-1-dependent type I IFN response in A549 and B16F10 cells (239). Another study suggests that RIG-I but not MDA5 is important in inducing IFN signaling and cytotoxic effects in response to radiation therapy in cancer cells, such as human D54 glioblastoma and HCT116 colorectal carcinoma cells (240). In these cells, accumulation of U1 non-coding RNA (ncRNA) in the cytoplasm was observed following radiation, suggesting that this ncRNA may contribute to activate RIG-I signaling pathway (240). These differences in radiation-induced responses may vary according to the strength of radiation or types of cancer cells. Endogenous dsRNAs which are originated from pre-mRNA introns were also reported to induce anti-tumor effects. Heterogeneous nuclear ribonucleoprotein C (HNRNPC) is up-regulated in multiple tumors or tumor cell lines. Knock-down of HNRNPC in breast cancer cells, such as MCF7 and T47D, results in accumulation of endogenous dsRNAs which are largely from Alu introns, and induces tumor-inhibitory effect by activating RIG-I-mediated type I IFN responses (241). Argonaute 1x (AGO1x), a translational read-through isoform of AGO1, is also highly expressed in breast cancer cells and is involved in the responses to endogenous dsRNAs (242). AGO1x interacts with dsRNA-processing proteins such as PNPT1 and this complex prevents the accumulation of dsRNAs in cells. Genetic deletion of AGO1x results in dsRNA accumulation and increased IFN responses (242). Deletion of ADAR1, an adenosine deaminase that limits the sensing of endogenous dsRNA, also induces MDA5-dependent type I IFN production and inflammation, which increase the sensitivity of tumors to radiation therapy and immunotherapy (243). The activation of innate immune cells by ligands of endosomal TLRs is another strategy for antitumor therapy (244, 245). TLR ligands are often studied for their effectiveness as adjuvants to induce antitumor T cell activity. The application of liposomes loaded with tumor-specific synthetic peptides and poly(I:C) induced tumor regression and controlled the outgrowth of melanoma and human papillomavirus-induced tumors (245). Another study showed that the administration of ARNAX, a TLR3-specific adjuvant, with a tumor-specific antigen promoted tumor regression. When in combination with anti-PD-L1, this cocktail led to the relief of anti-PD-1 unresponsiveness (244).

Role of Nucleic Acid Sensors in Tumor Growth

Although there are many reports of cGAS-STING pathway for anti-tumor effects, it was also reported that activation of the cGAS-STING axis in metastatic cancer caused chronic inflammation in tumor tissues, which enhanced cancer cell survival and metastasis. The transfer of cGAMP from cancer cells to astrocytes through gap junctions promoted the production of type I IFNs and proinflammatory cytokines, which in turn supported the brain metastasis of cancer cells (246). While canonical NF-κB signaling is required for antitumor immunity, the noncanonical NF-κB signaling pathway was reported to negatively regulate radiation-induced antitumor immunity (247). In metastatic cells, chromosomal instability is enriched compared with primary tumors, and this leads to activation of the STING-dependent noncanonical NF-κB signaling rather than canonical NF-κB and IRF3 signaling (248). Given that the cGAS-STING pathway can be beneficial and harmful in terms of antitumor immunity, the future direction of therapeutic strategies involving the cGAS-STING pathway should consider the efficiency and safety concerns of the treatment in different stages and type of cancers.

In addition to the cGAS-STING pathway, it was reported that activation of RIG-I signaling pathway in breast cancer cells also enhanced tumor growth, metastasis, and therapy resistance. Cancer cells interact and activate stromal cells to enhance RN7SL1 RNA levels by pol III, which results in secretion of exosomes containing RN7SL1 (249). This exosome activates RIG-I in breast cancer cells and leads to cancer progression.

Endosomal TLRs may also play a role in tumor progression. Mutations in MyD88 (L265P) are frequently (90% of cases) found in Waldenström's macroglobulinemia (WM), a lymphoplasmacytic lymphoma characterized by an excess of IgM-secreting lymphoplasmacytic cells in the bone marrow (250). MyD88-L265P spontaneously activates the Myddosome, resulting in the constitutive production of proinflammatory cytokines. Combined TLR3/7/9 deficiency was reported to induce tumor regression dependent on the activities of $CD4^+$ and $CD8^+$ T cells (251). Moreover, activation of endothelial TLR3 by the detection of extracellular RNA from tumors enhanced metastatic progression. TLR3 activation induced the expression of the axon guidance gene SLIT2 in endothelial cells, which mediated the migration of cancer cells to endothelial cells for intravasation, which was dependent on ROBO1, a SLIT2 receptor (252). The detection of tumor-derived exosomal RNAs by TLR3 in lung epithelial cells might also be involved in tumorigenesis. Activation of lung epithelial TLR3 induced neutrophil recruitment to the lungs and lung metastatic niche formation (253).

CONCLUSIONS

Numerous studies have reported detailed mechanisms for ligand recognition and activation by PRRs. Because all pathogens possess nucleic acids, a defensive barrier network consisting of PRRs recognizing pathogenic DNA or RNA as PAMPs and their downstream signaling pathways is important for host protection against invading pathogens such as viruses and bacteria. However, host cells also possess nucleic acids. Therefore, the recognition system for nucleic acids must be strictly controlled regardless of whether nucleic acids are derived from either the host or the invading pathogen. Indeed, the activation of PRRs and downstream signaling pathways are strictly regulated at multiple steps including cellular localization, post-translational modifications, and binding by inhibitors and activators. Nucleic acid metabolism is also critical for limiting responses to self-derived nucleic acids, and defects in the regulatory mechanism lead to autoinflammatory diseases. Therefore, it is important to investigate how nucleic acid-mediated signaling is activated and terminated. A number of negative regulators have been reported to date, and it will be a topic for future research that should be uncover the detailed mechanisms of how and under what conditions such negative regulators function.

Host immune responses induced by nucleic acids are a double-edged sword for the host. Even if immune responses are physiologically induced in response to invading pathogens or damaged cells as host defense, they can lead to morbid symptoms as side effects, and at worst, death. Therefore, the inhibition of nucleic acid-sensing receptors and their signaling pathways might be a promising treatment for undesired and severe inflammatory conditions. Nevertheless, the activation of nucleic acid-induced signaling pathways, especially the STING pathway, may enhance therapeutic effects in cancer, although the effect of the treatment is dependent on the stage and type of cancer. Coupled with the advances in immune checkpoint blockade therapy, it is expected that CDN-based therapy will be used in combination with such therapies in the future. Because inflammatory responses, even if localized, can affect the immune system throughout the body, investigating the impact of inflammatory responses on surrounding cells and tissues as well as distant locations might be another topic for future research. Elucidating the systematic responses induced by the immune response will contribute to our understanding of the pathogenesis of infectious diseases and autoinflammatory diseases, and developing appropriate treatments.

AUTHOR CONTRIBUTIONS

All authors conceptualized the framework of this review article, corrected, read, and finalized the article, and approved the submitted version.

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Cytokine Storm as a Cellular Response to dsDNA Breaks: A New Proposal

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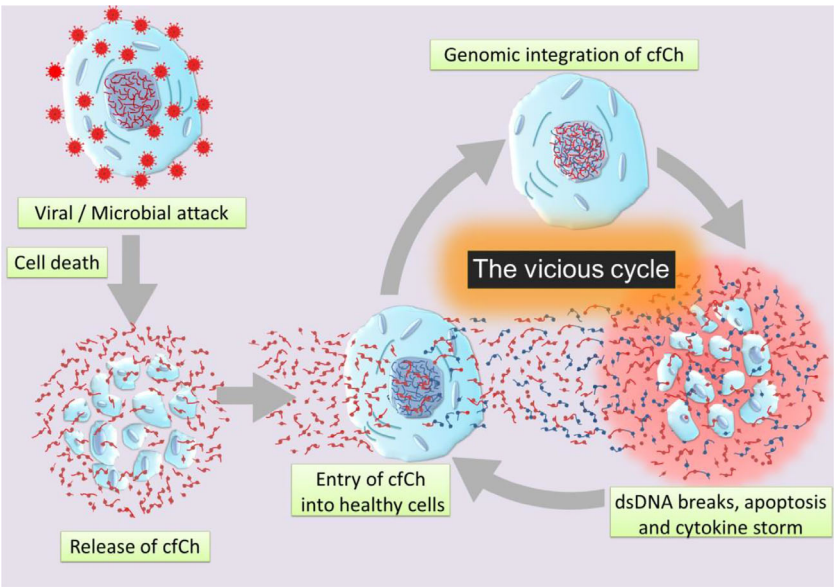
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Pathogenesis of cytokine storm is poorly understood. In this article we propose a new mechanism and suggest innovative therapeutic avenues for its prevention. We have reported that particles of cell-free chromatin (cfCh) that are released from the billions of cells that die in the body everyday can illegitimately integrate into genomes of healthy cells to trigger dsDNA breaks. The latter leads to apoptosis and/or intense activation of inflammatory cytokines in the affected cells. We hypothesise that a similar phenomenon of dsDNA breaks and inflammation is involved in cytokine storm. The abundant cfCh particles that are released from dying host cells following viral/microbial invasion initiate a cascading effect of more cell death resulting in a vicious cycle of further DNA damage, apoptosis and hyper-inflammation which culminate in cytokine storm. We propose that this unrelenting vicious cycle of cellular DNA damage and cytokine storm may be the underlying cause of high mortality from severe COVID-19. We discuss results of our preclinical studies wherein we have shown that endotoxin induced cytokine storm in mice can be reversed by three different agents that have the ability to inactivate cfCh. These agents may be worthy of investigation in clinical trials to reduce mortality from COVID-19.

Keywords: Cytokine storm, dsDNA breaks, COVID-19, inflammation, apoptosis, resveratrol, copper, free radicals



GRAPHICAL ABSTRACT | Schematic illustration of a vicious cycle initiated by genomic incorporation of cfCh resulting in dsDNA breaks, apoptosis, and hyper-inflammation which culminate in cytokine storm.

INTRODUCTION

Cytokine storm is a condition characterized by an overwhelming and uncontrolled inflammation with major implications for global health (1). Cytokine storm is a critical component of the current COVID-19 pandemic, and is associated with severity of the disease and high mortality (2). In the worldwide flu pandemic of 1918, cytokine storm was a major cause of high death rate (3). Cytokine storm has also been described in other pandemics such as H1N1 swine flu (4), H5N1 avian flu (5) and severe acute respiratory syndrome coronavirus (SARS-CoV) (6). It is associated with sepsis in general which affects 48.9 million people worldwide every year of which 11 million die (7). Death from sepsis accounts for 19.7% of all global deaths, especially in poorer countries of the world (7). Several experimental studies and clinical trials have suggested that cytokine storm correlates directly with tissue injury, DNA damage and severity of the disease (1). In spite of intensive research, pathogenesis of the cytokine storm remains poorly understood, hindering development of effective therapies.

CYTOKINE STORM: SUMMARY OF CURRENT KNOWLEDGE

The innate immune response is activated by pattern recognition receptors (PRRs) in response to pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) (8). Activated immune response triggers intracellular signalling cascades in immune cells leading to production of inflammatory cytokines by various cells including macrophages, natural killer (NK) cells, dendritic cells, T cells, mast cells, endothelial and epithelial cells (8–10). The immune response is highly regulated and sequentially and temporally orchestrated (8). However, in certain pathological conditions, a profusion of PAMPs or DAMPs are released in response to cell death and stress (11–15). This causes hyper-stimulation of immune cells leading to intense secretion of inflammatory cytokines which results in the cytokine storm (13, 14). This hyper-inflammation triggering cytokine storm can either be in response to PAMPs which activates pathogen-induced hyper-inflammation, or to DAMPs which are self-molecules derived from host cells itself triggering auto-inflammatory response. Although it is widely accepted that these DAMPs and PAMPs are key molecules that trigger an inflammatory response (11–15), the precise nature of these molecules has not been characterized (16–18).

Recently, there has been a spurt of publications associating genomic stress and DNA damage in activation of inflammation (19–22). According to these reports, DNA that accumulates in the cytoplasm following DNA damage and/or microbial infection, acts as DAMPs and activates the DNA sensing GMP-AMP synthase-stimulator of interferon genes (cGAS-STING)-mediated pathway (19–22). The latter triggers an innate immune response by activating pro-inflammatory cytokines (19–22). In addition to microbial DNA and self-DNA from the nucleus, cGAS-STING pathway is also activated by cytosolic mitochondrial DNA (mtDNA) (23, 24). The latter has the potential to induce inflammatory responses and organ injuries in various diseases including cancer (25), diabetes (26), cardiovascular diseases (27) and trauma (28). Elevated levels of mtDNA in circulation has also been reported to be associated with severity of sepsis (29). Recent reports have also implicated presence of cytoplasmic chromatin fragments (CCF) in immune activation (30–32). CCF that are pinched off from nuclei during

cellular senescence (33) are recognized by cGAS to stimulate an inflammatory response *via* STING (30–32). However, whether presence of DNA and/or CCF in the cytoplasm has the potential to trigger the cytokine storm or whether some other mechanism(s) is involved in triggering hyper-inflammation remains unknown. Thus, although the cytokine storm has been known for more than a century (1, 3), and much has been reported on its pathological consequences (1, 5), the trigger for the cytokine storm continues to remain elusive, hindering the development of effective therapies (34). Herein we put forward the hypothesis that cell-free chromatin (cfCh) particles (nucleosomes) released from dying host cells may contribute to the cytokine storm.

CELL FREE CHROMATIN (cfCh) AS A NOVEL TRIGGER FOR THE CYTOKINE STORM

Origin and Structure of Cell-Free Chromatin (cfCh)

It has been estimated that 10^9–10^{12} cells die in the body, primarily by apoptosis, every day (35). Apoptosis is characterized by nuclear and chromatin condensation followed by fragmentation of DNA by endogenous nucleases, especially caspase-3 and activated DNase (36). Although not demonstrated, it is likely that cfCh particles are also released following other forms of cell death such as necrosis, NETosis, pyroptosis (37). In spite of the body's best efforts to get rid of cfCh (38, 39), a significant amount escapes into the extracellular compartments as well as into the blood circulation (40, 41). cfCh in circulation are cleared by the body by several mechanisms. These include: 1) phagocytosis by macrophages (42); 2) degradation by DNase I present in circulation (43), and 3) liver continuously removing cfCh resulting in a turnover half-life of 10–15 min (38, 39). Low baseline levels of cfCh in healthy individuals play a critical role in maintaining an efficient immune environment. However, elevated cfCh levels as seen in various clinical conditions may lead to runaway inflammation. These conditions have included autoimmune diseases (44), severe infections (45), trauma (46), burns (47), deep vein thrombosis (DVT) (48), cerebral stroke (49), malignancy (50). Increasing cfCh levels positively correlate with age (51).

The Hypothesis

Our hypothesis is based on our recent finding that cfCh particles that are released from the hundreds of millions of cells that die in the body daily to enter into the blood stream can illegitimately integrate into genomes of healthy cells to damage their DNA by inducing dsDNA breaks (52, 53). Such events may also occur locally following cell death in tissues with release of cfCh which integrates into genomes of bystander cells in the neighbourhood (53, 54). Genomic integration of cfCh can have catastrophic consequences, especially since the DNA damage is repaired by the error-prone non-homologous end joining (NHEJ) mechanism, which further accentuates genomic mutations in the form of deletions, insertions, re-arrangements and chromosomal damage which may often cause apoptosis of the cells. The hypothesis also incorporates our recent observation that dsDNA breaks resulting from cfCh integration leads to intense activation of inflammatory cytokines (54, 55). Since cell death is markedly increased following viral or bacterial invasion, we hypothesise that illegitimate genomic integration of cfCh particles that are released from the dying host cells trigger a vicious cycle of more dsDNA breaks, apoptosis and hyper-inflammation which culminate in the cytokine storm (**Graphical Abstract**). We propose that the abundant cfCh that arise following viral/microbial invasion act as DAMPs and activate systemic inflammation. This proposal is supported by reports that circulating levels of cfCh are markedly elevated in patients admitted to ICU with severe sepsis (34). Since the latter is usually associated with the cytokine storm (56), it leads to the possibility that cfCh may be an important factor that contributes to the cytokine storm in sepsis.

Can Cell-Free DNA and/or Free Histones Explain the Cytokine Storm?

Cell-free DNA (cfDNA) and free histones have been shown to have pro-inflammatory properties (57, 58). However, the immune stimulatory effects induced individually by cfDNA and free histones are different when compared to that induced when they are complexed in the form of cfCh (59). Furthermore, the question as to whether naked DNA and/or free histones are indeed present in circulation is in doubt. Apoptotic cell death results in chromosomal condensation and fragmentation with release of chromatin fragments and not of cfDNA or free histones (60). The existence of cfCh in serum and/or plasma can be easily detected by ELISA (61), while the demonstration of cfDNA requires DNA to be extracted from plasma/serum using Proteinase-K treatment. Therefore, the possibility that the isolated cfDNA has, in fact, been derived from circulating cfCh cannot be excluded. Reports of the existence of a direct and strong correlation between circulating cfCh and cfDNA would support such a possibility (62). Similarly, with respect to studies reporting immune-stimulatory effects of free histones (59, 63, 64), the methodologies used to quantify histones did not make a distinction between free histones and cfCh (59). Therefore, whether the latter are present in circulation also remains unclear (59). This uncertainty may have been put to rest by our recent observations made in relation to lipopolysaccharide (LPS) induced sepsis in a mouse model (65). Using confocal microscopy of histological sections of mouse vital organs after staining with fluorescent antibodies against DNA and histone H4, we have shown that it is cfCh, and not free DNA or histones, that are extruded from dying host cells following LPS treatment (65). Therefore, it is likely that cfCh, rather than cfDNA or free histones, is the agent responsible for initiating the cytokine storm in severe infection.

cfCh in Circulation Integrate Into Genomes of Healthy Cells

Although existence of circulating cfCh particles has been known since 1990 (66), whether they have any patho-physiological role to play in the host has only recently been addressed (51, 52). Isolation of cfCh from sera of cancer patients followed by examination under electron microscope revealed particles of

variable sizes (~10 nm >1000 nm) having a beads-on-a-string appearance characteristic of chromatin (52). When cfCh particles isolated from serum where fluorescently labelled and added to cultured mouse fibroblast cells, numerous cfCh particles could be detected in nuclei of recipient cells within 6h (52). The up-taken cfCh rapidly associated themselves with chromosomes of host cells which was followed by activation of an intense DNA damage repair response (DDR) followed by their incorporation into the host cell genomes (52). The activated DDR proteins included H2AX, ATR, ATM, P-p53, P-p21, MDC-1, GADD-34, RAD-50, NIBRIN, MRE-11, DNA-PKcs and DNA ligase IV (52). In addition, apoptotic pathway proteins namely, JC-1, cytochrome-C and caspase 3, were also activated (52) indicating that many of the affected cells were destined to undergo apoptotic cell death. Next generation sequencing detected tens of thousands of DNA reads of human origin in single cell clones developed from the cfCh treated mouse cells; while PCR amplification revealed presence of multiple human Alu sequences (52). cfCh integration resulted in dsDNA breaks as indicated by activation of H2AX which was seen both *in vitro* and *in vivo* (52). A unique mechanism was proposed by which cfCh particles integrate themselves into genomes of healthy cells, and which is facilitated by premature activation of DDR (discussed below).

cfCh Released From Dying Cells Integrates Into Genomes of Bystander Cells

We have reported that cfCh released from dying host cells can also integrate into genomes of surrounding healthy bystander cells (54, 55). When human cancer cells were treated with ionizing radiation and co-cultured with mouse fibroblasts, human DNA (cfCh) signals could be detected in the nuclei of mouse cells by FISH (55). Confirmation that cfCh had actually integrated into the genomes was confirmed by detection of multiple human Alu sequences in the mouse cells (55). Bystander uptake and genomic integration of cfCh released from dying cells was also shown to occur in distant organs (55). When mice were delivered focused mini-beam irradiation (20 Gy) to the umbilical region, intense activation of H2AX, caspase 3, NFκB and IL-6 was detected in brain cells (55).

cfCh Integrates Into the Genome by a Unique Mechanism

How does cfCh enter the cell and integrate themselves into the genome? Our microarray studies have revealed that pathways related to phagocytosis are maximally up-regulated as early as at 6h in mouse fibroblast cells in response to cfCh particles that are released from co-cultured dying Jurkat cells (54). This finding would suggest phagocytosis or pinocytosis to be one of the important mechanisms by which the cell ingests extraneous cfCh. Once inside the cell, cfCh particles integrate themselves into the genome of the host cell by a mechanism which is unique in being the opposite of the classical model of DNA damage and repair (52). According to the classical model of DNA damage, DDR is activated after the DNA damage occurs in response to agents such as ionizing and UV radiation and radiomimetic chemicals (67). According to the proposed new model, entry of cfCh into the cell misleads the cell into perceiving them as broken fragments of its own chromosomes containing dsDNA breaks at both ends (52). This prompts the cell to activate a premature DDR much before any damage to DNA having actually occurred. The activated DDR includes repair proteins such as DNA PKc, DNA ligase IV which link up the multiple heterogenous cfCh fragments into concatamers of different sizes. The latter, containing a mosaic of multiple discontinuous DNA segments in the form of conctamers, now form new substrates for incorporation into the genome of host cells, by non-homologous recombination (NHR). The resulting DNA damage is repaired by the error–prone NHEJ mechanism (68) which creates further mutations in the form of insertions, deletions, genetic rearrangements as well as chromosomal damage (52). Thus, paradoxically, DDR which is supposed to protect the integrity of the genome ends up damaging it by its premature activation. The formation of intracellular concatamers is supported by the argument that since the threshold for detection of FISH signals is of the order of 30–50 kilo bases (69), presence of human DNA signals in mouse cells detected by FISH indicates that relatively long human DNA sequences, rather than discrete cfCh particles, incorporate themselves into the mouse cell genomes. Genomic integration of cfCh concatemers by NHR leads to intense activation of inflammatory cytokines (discussed below).

Genomic Integration of cfCh Leads to Somatic Mosaicism

Illegitimate genomic integration of cfCh, derived from the billions of cells that die in the body every day may result in dsDNA breaks and repair by NHEJ. These damaging events occurring repeatedly throughout life may generate multiple genomic polymorphisms which are likely to increase with age (53). Rapid and cumulative effects of DNA damage may exceed the adaptive capacity of the human genome in aging populations which leads to increased mutagenesis and development of various diseases, including cancer. This would be in accordance with the exploding literature fueled by advances in Next generation sequencing on the discovery of somatic mosaicism in healthy cells (70, 71). Somatic mosaicism is related to aging (72), cardiovascular diseases (73), Alzheimer's disease (74) and cancer (75). The above discussion would suggest that approaches to retard aging would need to take into account accumulating dsDNA breaks that result from life-long and repeated genomic integration of cfCh.

cfCh Integration, dsDNA Breaks, and Activation of Inflammatory Cytokines

The aforementioned co-culture experiment of irradiated dying cancer cells of human origin with mouse fibroblasts, led to activation not only of H2AX but also of multiple inflammatory cytokines (54). The latter included NFκB, IL-6, TNF-α and IFN-γ, all of which were activated simultaneously by 6h (54), and their activation coincided with point of the maximal induction of H2AX (6h) (54). Co-activation of dsDNA breaks and inflammatory cytokines suggested an interrelationship between the two, which was further substantiated by microarray analysis which revealed activation of multiple pathways related to inflammation concurrently with those that accompany DNA damage and cell

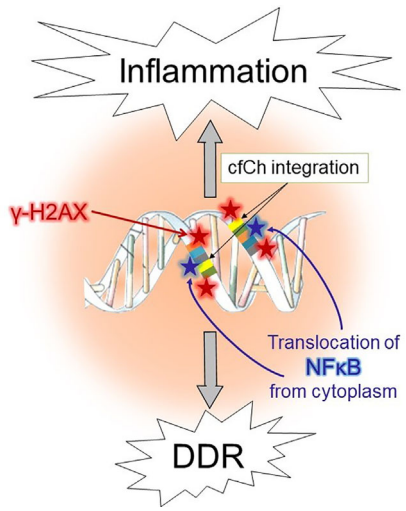


FIGURE 1 | Schematic illustration of activation of DDR and inflammation following illegitimate integration of cfCh into the genome.

cycle at 6h (54). Injection of irradiated dying cancer cells pre-labelled with BrdU intravenously into mice led to uptake and genomic integration of BrdU labelled cfCh particles into nuclei of vital organs accompanied by activation of H2AX (54). Genomic integration of cfCh led to intense activation of multiple inflammatory cytokines to include NFκB, IL-6, TNF-α and IFN-γ. These experiments made the additional novel observation that fluorescent signals of γH2AX co-localized strictly with those of the transcription factor NFκB in the nuclei of vital organs (54). The inactivated form of NFκB normally remains sequestered in the cytoplasm (76) and trans-locates to the nucleus upon activation by stressful stimuli such as DNA damage (77). Although several nuclear translocation sites for NFκB have been described (78), the finding that γH2AX and NFκB fluorescence signals co-localize has led to the proposal that, following the catastrophic event of integration of cfCh into the genome and the consequent dsDNA breaks, NFκB is strongly activated, followed by its translocation from the cytoplasm to the sites of cfCh integration (79, 80). This finding indicated that inflammation might be a direct consequence of dsDNA breaks inflicted by integration of cfCh (80). It also suggests that cfCh acts as a major form of DAMPs. A schematic model to represent the relationship between cfCh induced dsDNA breaks and inflammation is given in **Figure 1**.

Inactivation of cfCh Can Prevent the Cytokine Storm

We have identified several cfCh inactivating agents that can prevent the cytokine storm (55, 65, 81). These have included 1) anti-histone antibody complexed nanoparticles (CNPs) which inactivate cfCh by binding to histones; DNase I which inactivates cfCh by degrading its DNA component; and 3) a newly described pro-oxidant combination of the well-researched nutraceuticals Resveratrol and metallic Copper (R-Cu) which degrades cfCh through the medium of free radicals. We have recently reported that these cfCh inactivating/degrading agents can reverse the cytokine storm following endotoxin sepsis, chemotherapy and radiation therapy in mice. Details of these studies are given in the subsequent paragraphs.

Inactivation of cfCh Can Prevent the Cytokine Storm in Endotoxin Sepsis

The International Sepsis Forum defines sepsis as "a life-threatening condition that arises when the body's response to an infection injures its own tissues and organs" (82). This definition implies that hyper-inflammation and immune suppression in sepsis is a result of body's own response against the pathogen and not due to the pathogen *per se* (83). We have recently shown in an endotoxin induced sepsis model that cfCh particles that are released from dying host cells following viral/microbial infection may be the agents that injure the body's own tissues and organs that leads to sepsis - a finding which would be consistent with the above definition of the International Forum (65).

Several studies have reported that not only PAMPs, but also DAMPs, are recognized by pattern recognizing receptors (PRRs) expressed on immune-reactive cells (84–86). DAMPs are nuclear or cytoplasmic non-microbial molecules, released from the host cells following tissue injury which includes histones, cfDNA, chromatin, HMGB1, etc. (86). Clinical studies have shown a positive correlation of levels of DAMPs, especially of histones and nucleosomes, with sepsis severity (45, 87, 88).

In our study, sepsis was induced in mice by injecting lipopolysaccharide (LPS), a bacterial membrane antigen, which led to extensive cell death and copious release of cfCh particles into extracellular spaces of vital organs and into the circulation (65) (**Figure 2**). cfCh particles thus released followed by their integration into genomes of surviving cells led to extensive dsDNA breaks and apoptosis in cells of multiple organs *viz.*, lung, liver, heart, brain, kidney and small intestine (65), as well as those of immune related organs such as thymus and spleen. cfCh integration and dsDNA breaks led to intense activation of inflammatory cytokines CRP, IL-6, IL-1β, TNF-α, and IFN-γ in multiple organs as well as release of these cytokines in circulation. The extensive DNA damage also led to immune suppression, coagulopathy, fibrinolysis, thrombocytopenia, multi- organ failure and death. All the above pathologies could be abrogated by administration of the cfCh inactivating agents to mice concurrently with LPS. This data provided strong evidence for a relationship between cfCh integration, dsDNA breaks, cytokine storm and sepsis.

Inactivation of cfCh Can Prevent the Cytokine Storm Associated With Chemotherapy and Radiation Therapy

Cancer treatments involving chemotherapy and radiation therapy are known to trigger intense activation of pro-inflammatory cytokines (89, 90). The latter is thought to be activated by unidentified molecules which act as DAMPs and stimulate immune cells to release pro-inflammatory cytokines (91).

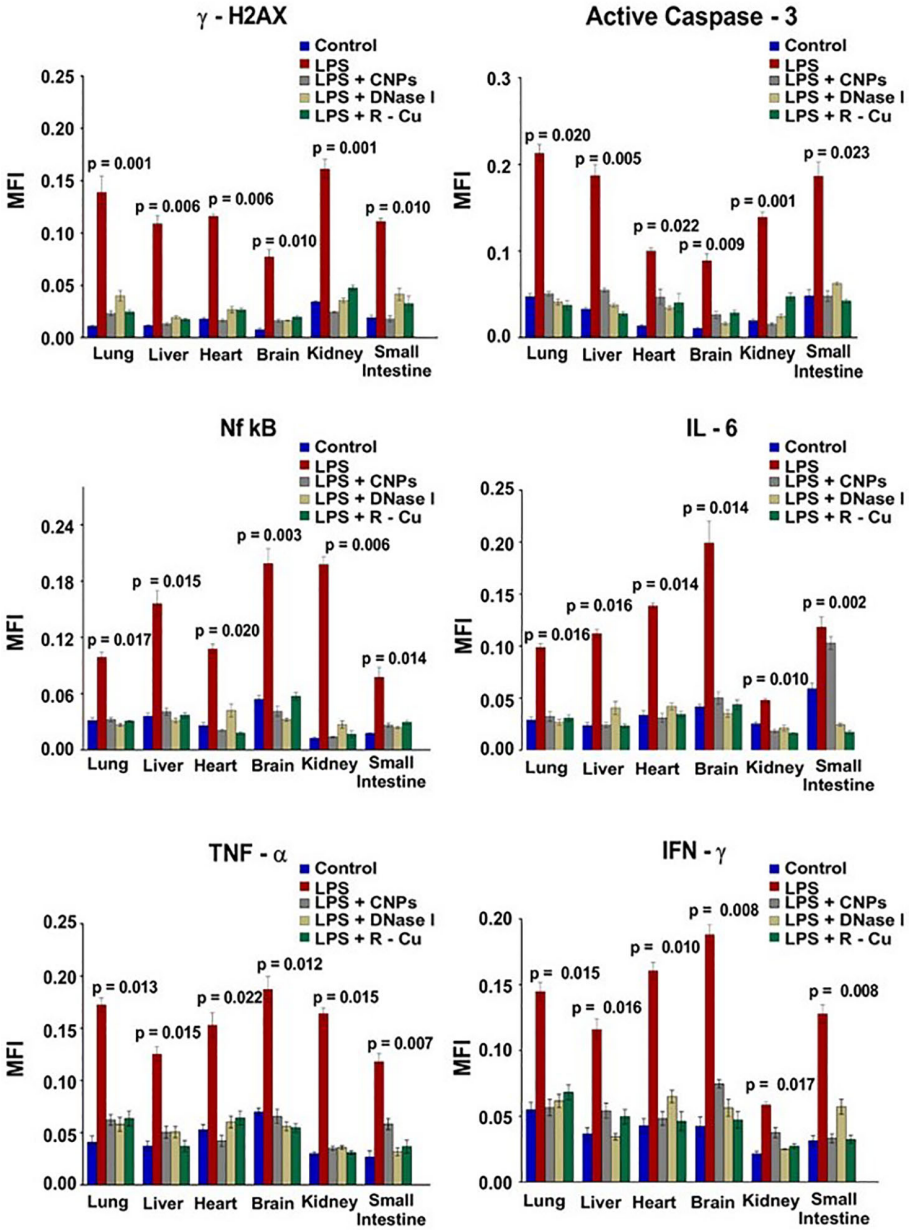


FIGURE 2 | DNA damage, apoptosis and inflammation in multiple organs and tissues induced by LPS can be prevented by concurrent treatment with cfCh inactivating agents. The above parameters were estimated at 72hrs following LPS treatment by indirect immuno-fluorescence. Mean (± SEM) values between groups were compared using non parametric one-way ANOVA (Kurskal—Wallis test) with Dunn's multiple comparison method at the significance and confidence level of p = 0.05. MFI = Mean fluorescence intensity. Reproduced from ref (65).

However, the nature of these DAMPs continues to remain unidentified (17, 92). We have shown that, as in the case of sepsis, cfCh released from chemo- or radio therapy induced dying cells are the elusive DAMPs. Therapy induced cell death and cfCh release triggers a cascading effect of more cell death leading to a vicious cycle of further rounds of DNA damage, apoptosis and inflammation which perpetuate and amplify the toxic effects of these cancer therapies (55, 81). We have further reported that administration of the above three cfCh inactivating agents interrupted this vicious cycle thereby preventing the toxic effects of cancer treatment (55, 81). This reinforces the conclusion reached above, with respect to endotoxin sepsis, that copious release of cfCh particles following cell death resulting from chemotherapy and radiation therapy act as DAMPs to trigger the cytokine storm.

Is Cell Free Chromatin Implicated in Pathogenesis of COVID-19?

Pathogenesis of COVID-19 is not well understood. The disease primarily affects the lungs leading to hypoxemic respiratory failure, secondary bacterial pneumonia and direct tissue damage. The disease is also associated with the cytokine storm with excessive release of inflammatory cytokines which can cause multi-organ damage (93). The other organs that are affected include heart, nerves, brain, vessels, kidneys and skin. We have already alluded to the potential role of cfCh in the cytokine storm, but, currently, literature on direct measurement of cfCh levels in COVID-19 patients is lacking. Elevated levels of cfCh in these patients is to be expected since sepsis forms a major manifestations of the SARS-CoV-2 viral infection (94), and there is abundant literature to show that cfCh levels are elevated in sepsis (45, 87, 88, 95). As the title of current article suggests, and discussed extensively above, the cytokine storm is a likely consequence of DNA and cellular damage inflicted by cfCh. We propose that cfCh induced tissue/organ damage can not only explain the aetiology of the cytokine storm, but also help to explain the multi-organ injury that is associated with COVID-19 as a direct consequence of cfCh induced cellular DNA damage.

CONCLUSION AND FUTURE PROSPECTS

In this article we have proposed that inflammation may be a direct consequence of dsDNA breaks inflicted by genomic integration of cfCh released from dying host cells, and that cfCh may be the key instigators of the cytokine storm (54, 80). cfCh particles released from dying host cells following viral/microbial infection may trigger a cascading effect of more host cell death leading to a vicious cycle of further rounds of DNA damage, apoptosis and inflammation which perpetuate and amplify the pathological effects of the offending agent culminating in the cytokine storm. Although, currently, literature on direct measurement of cfCh levels in COVID-19 patients is lacking, we hypothesise that the high mortality in severe COVID-19 may be due to the cytokine storm related sepsis. The latter being perpetuated by the vicious cycle triggered by profuse release of cfCh particles that result from Corona virus induced cell death. The implication of such a suggestion is that, while eliminating the virus may result in resolution of disease in asymptomatic or early symptomatic COVID-19 patients, once the vicious cycle sets in, elimination of the virus may not prevent death in patients with severe disease. Indeed, a recent study has reported that effects of the cytokine storm can persist for a long time after the virus has been eliminated from the body (96). Furthermore, the observation that elderly patients and those with underlying ageing related co-morbidities such as diabetes (97) and cardio-vascular diseases (98) are more prone to COVID-19 related complications, may be attributable to elevated levels of cfCh in these conditions (51, 99, 100). We propose that treatment of severe COVID-19 should include cfCh inactivating agents to prevent death, and that these agents are worthy of investigation in clinical trials in patients suffering from severe COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IM conceptualized and wrote the article. SS wrote the article and prepared the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cell Surface Expression of Endosomal Toll-Like Receptors—A Necessity or a Superfluous Duplication?

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Timely and precise delivery of the endosomal Toll-like receptors (TLRs) to the ligand recognition site is a critical event in mounting an effective antimicrobial immune response, however, the same TLRs should maintain the delicate balance of avoiding recognition of self-nucleic acids. Such sensing is widely known to start from endosomal compartments, but recently enough evidence has accumulated supporting the idea that TLR-mediated signaling pathways originating in the cell membrane may be engaged in various cells due to differential expression and distribution of the endosomal TLRs. Therefore, the presence of endosomal TLRs on the cell surface could benefit the host responses in certain cell types and/or organs. Although not fully understood why, TLR3, TLR7, and TLR9 may occur both in the cell membrane and intracellularly, and it seems that activation of the immune response can be initiated concurrently from these two sites in the cell. Furthermore, various forms of endosomal TLRs may be transported to the cell membrane, indicating that this may be a normal process orchestrated by cysteine proteases—cathepsins. Among the endosomal TLRs, TLR3 belongs to the evolutionary distinct group and engages a different protein adapter in the signaling cascade. The differently glycosylated forms of TLR3 are transported by UNC93B1 to the cell membrane, unlike TLR7, TLR8, and TLR9. The aim of this review is to reconcile various views on the cell surface positioning of endosomal TLRs and add perspective to the implication of such receptor localization on their function, with special attention to TLR3. Cell membrane-localized TLR3, TLR7, and TLR9 may contribute to endosomal TLR-mediated inflammatory signaling pathways. Dissecting this signaling axis may serve to better understand mechanisms influencing endosomal TLR-mediated inflammation, thus determine whether it is a necessity for immune response or simply a circumstantial superfluous duplication, with other consequences on immune response.

Keywords: TLR3, cell surface expression, nucleic acid-sensing receptors, TLR signaling, innate immune function

INTRODUCTION

TLR3, like all members of the Toll-like receptor family, recognizes pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and plays an essential role in innate immunity. While origins of microbial derivatives of TLR ligands are straightforward, endosomal TLRs can recognize self-nucleic acids emerging, e.g., during tissue damage caused by UV-radiation or from non-apoptotic cell debris (1). The importance of TLR3 in self-RNA recognition was discussed in the work of Takemura et al. (2). where high-dose ionizing radiation severely affected murine epithelial stem cells of small intestine, causing the gastrointestinal syndrome (GIS) Damage of nucleic acids and leakage of cellular RNA from the cells activated TLR3 which proved to be critical to the pathogenesis of the disease as *Tlr3*$^{-/-}$ mice showed significant resistance to GIS. Nevertheless, host-derived TLR ligands may be present in the extracellular environment as well as in endosomes, however, they undergo rapid degradation by nucleases, reducing the risk of autoimmune or autoinflammatory disorders (3). Although mechanisms that control the precise transportation of the endosomal TLRs to the ligand recognition site are strictly regulated, barriers can be overcome and lead to autoimmune diseases such as lupus erythematosus (4), psoriasis (5), or rheumatoid arthritis (6).

TLRs may be classified according to their cellular localization, as they may occur on the cell surface or in the membranes of intracellular vesicles referred to as endosomes. All endosomal TLRs identified in mice and humans: TLR3, TLR7, TLR8, and TLR9, sense nucleic acids or their derivatives, i.e., double-stranded RNA (dsRNA), single-stranded-RNA (ssRNA), uridine-rich or uridine/guanosine-rich ssRNA, and unmethylated CpG DNA respectively (7, 8). The size of human endosomal TLRs is about 1000 amino residues, compared to cell surface-localized TLRs which have approximately 800 amino acids [see **Figure 2** in (5)]. Although TLRs are acknowledged as evolutionarily highly conserved proteins, current studies indicate that TLR3 is the most conserved innate receptor compared to TLR7, TLR8, and TLR9 (9, 10).

Endosomal TLRs are subjected to many elaborate regulations, especially related to transportation and localization in the cell. Recent findings dispute the dogma that TLR3, TLR7, TLR8, and TLR9 are exclusive intracellular receptors. Although the endosomal acidic environment is crucial for the functioning of endosomal TLRs (11, 12), surprisingly, the same receptors may appear on the surface of various cell types and they may trigger signaling pathways (13–16). However, mechanisms leading to and managing such transposition remain obscure. In this review, we sought to reconcile scientific evidence indicating specific conditions that support membrane positioning of endosomal TLRs, particularly TLR3, and outline factors contributing to TLR3 occurrence in the plasma membrane. Insights into TLR biology regarding receptor transportation may permit full comprehension of the impact of receptor localization on its function. Furthermore, highlighting similarities and differences between various cell types may yield valuable knowledge on individual TLRs, regarding therapeutic targets for diseases that may result from receptor localization abnormalities.

Abbreviations: AECs, airway epithelial cells; AEP, asparagine endopeptidase; AP-1, activator protein 1; BM-DM, bone marrow-derived macrophages; BM-MCs, bone marrow myeloid cells; cDCs, classical dendritic cells; CNS, central nervous system; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; dsRNA, double.

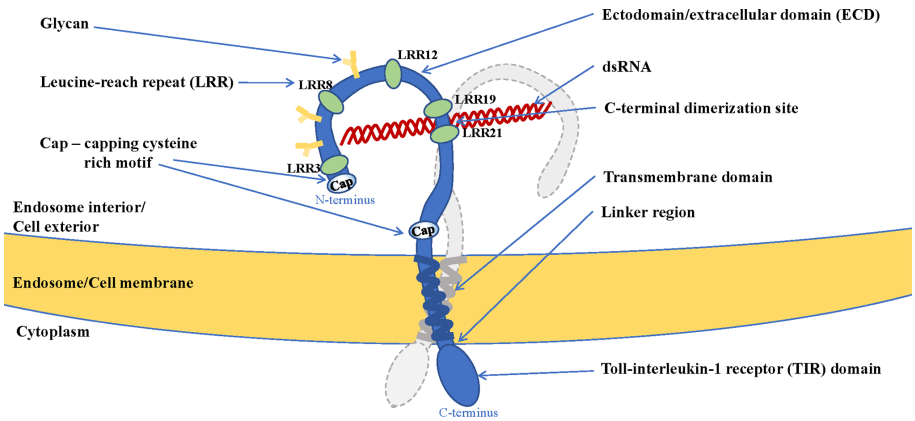
STRUCTURE OF ENDOSOMAL TLRS AND EFFECT ON THE LOCALIZATION IN THE CELL

The type I transmembrane proteins family comprises endosomal TLRs that are characterized by a similar structure. TLR3, TLR7, TLR8, and TLR9 contain N-terminal ectodomain (ECD) with leucine-rich repeats (LRR) involved in TLR-ligand interaction (17), and a cytosolic Toll-interleukin-1 (TIR) domain responsible for enrollment of the signaling pathway components (18). The structure of endosomal TLRs is shown in **Figure 1** and exemplified by TLR3.

Another distinguishing feature of endosomal TLRs is their presence as pre-formed dimers, e.g., human TLR9 are reported to occur in such a manner (29). Following stimulation with the TLR7 ligand, TLR7 forms an m-shaped dimer containing two ligand-binding sites (30). Interestingly, the first site is sufficient for the receptor activation, while the second site enhances the binding affinity of the ligand bound to the first site. Furthermore, each ligand binding site preferentially recognizes different moieties: guanosine or uridine-rich ssRNA, indicating that TLR7 is a dual-receptor. In the case of TLR3, dimerization is necessary for effective ligand attachment (31), and the dimerization interface is located at C-terminal 19-21 LRR components of TLR3 (LRR-CT) (32), contrary to other TLRs in which dimerization may occur in different regions of the C-terminal domain.

Following activation by the ligand, TLR7, TLR8, and TLR9 bind myeloid differentiation primary response (MyD) 88 adaptor protein through the intracellular domain, while TLR3 connects to a different adaptor protein, TIR-domain-containing adapter-inducing interferon-β (TRIF), through the TIR-domain (33). Such interactions initiate signaling cascades that promote nuclear translocation of nuclear factor kappa B (NF-κB), interferon regulatory factor 3 (IRF3), IRF7, and activator protein 1 (AP-1) transcription factors (18, 34). The ultimate goal is aimed at gene transcription and protein expression for cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), IL-6, interferon-inducible protein 10 (IP-10), and type I interferons (IFNs) (IFN-α and IFN-β) that are able to counteract the danger raised by the invading pathogen (33).

Prototypical endosomal TLRs translocate to ligand recognition sites from ER which they populate in resting cells (35–37). Following ER residence, receptors are trafficked to the Golgi apparatus for addition of N-linked glycans, however, they may also reach the endosomes bypassing this organelle (37). TLR9 is an exception in this intracellular transportation route. After the glycosylation, the receptor is transported to the cell surface and recruits AP-2 complex to effectuate endocytosis and



Cap – capping cysteine rich motif	Placed at both ends of LRR region, contains disuphilde bonds which protect hydrophobic core and stabilize domain (Huyton *et al.*, 2007; Botos *et al.*, 2011).
Leucine-reach repeat (LRR)	Ascending part of ECD contains 23 conserved LRRs (Choe *et al.*, 2005).
Glycan	TLR3 contains 15 predicted N-linked glycosylation sites (Sun *et al.*, 2006), TLR3 dimerization and ligand binding sites are nonglycosylated (Bell *et al.*, 2005; Watanabe *et al.*, 2007).
Ectodomain/extracellular domain (ECD)	Governs plasma membrane localization (Pohar *et al.*, 2014).
dsRNA	TLR3 ligand
Linker region	Determines intracellular TLR3 localization (Funami *et al.*, 2004).
Toll-interleukin-1 receptor (TIR) domain	Responsible for adaptor binding and signal transduction (Yamamoto *et al.*, 2002; Imler and Hoffmann, 2003).

FIGURE 1 | Structure of endosomal TLR localized in the endosome/cell membrane, exemplified by TLR3—shown are dimerization site, ECD (ectodomain/extracellular domain), transmembrane helix, and TIR domain (Toll-interleukin-1-receptor domain), as well as functions of the essential elements of the receptor (19–28).

finally anchor in endosomes. In contrast, TLR7 recruits AP-4 complex in the cytoplasm and resettles directly from the Golgi network to endosomes (38).

Endosomal TLRs contain distinctive targeting sequences that direct the receptors to their intracellular location. Endosomal compartmentalization of TLR3 occurs due to the linker region situated between the transmembrane helix and the TIR domain (19) (**Figure 1**), while TLR7 endosomal transposition is determined by the sorting signal from the transmembrane domain (39). Interestingly, murine TLR9 is trafficked owing to the transmembrane domain (40), but human TLR9 transportation to endosomes is mediated by the tyrosine-based motif of the cytoplasmic domain (41). Folding of the adequate structure of the TLR protein may also determine its localization in the cell. For example, cysteines participating in disulfide bond formation play an important role in TLR3 stability and expression. Analysis of TLR3 mutants in the cysteines indicated that some of the modified receptors may exhibit different levels of cell surface expression (42).

INFLUENCE OF UNC93B1 ON CELL SURFACE LOCALIZATION OF ENDOSOMAL TLRS

One of the accessory proteins responsible for transportation of endosomal TLRs from the ER to endosomes which ensures proper localization for effective antimicrobial immune response is UNC93B1 (20, 43). Autosomal recessive deficiency of *UNC93B1* in humans may predispose to herpes simplex encephalitis (HSE) following herpes simplex type I virus (HSV-1) infection through insufficient production of type I (IFN-α and IFN-β) and type III (IFN-γ) interferons (44). In resting cells, UNC93B1 resides in the ER (45), and upon endosomal TLR stimulation interacts with transmembrane segments of the receptors and delivers them to the ligand recognition site (46). Nucleic acids-sensing TLRs such as TLR3, TLR7, and TLR9 of mice with *Unc93B1* loss-of-function mutation are unable to leave the ER (43, 45). Furthermore, these mice are prone to infections with various intracellular pathogens (46). UNC93B1 may stabilize TLR3, TLR7, and TLR9, regulate their maturation at early state and therefore probably is responsible for the correct spatial conformation of these receptors (47). Pelka et al. propose that nucleic acid (NA)-sensing TLRs are most likely misfolded and targeted to the ER-degradation pathway in *Unc93b1*$^{-/-}$ and *Unc93b1*$^{3d/3d}$ mice due to the lack of interaction with the missing/unfunctional chaperone protein. UNC93B1 contributes to the protective role of TLR3 and TLR9 by increasing their half-life, probably through lowering their proteolytic degradation rate (48, 49). However, UNC93B1 upregulation may also increase the responsiveness of TLR3, TLR7, TLR8, and TLR9 to their agonists, and conditions that lead to increased UNC93B1 expression may yield autoimmune disorders (49).

Different proteins from the adaptor protein (AP) family have been proposed to participate in UNC93B1-mediated transition

of individual TLRs to the endosomes. During TLR7 transportation aimed at ligand detection, the receptor is accompanied by UNC93B1 and AP-4, however, direct interaction has been demonstrated between AP-4 and TLR7 but not AP-4 and UNC93B1 (38). UNC93B1 also regulates TLR9 intracellular trafficking by recruiting AP-2 *via* the C-terminal domain (38), which supports clathrin-dependent internalization of TLR9 from the cell membrane (50). Knockdown of AP-2 and the exclusion of the AP-2-dependent sorting pathway of TLR9 increased TLR9 responses and TLR8 activity in HEK cells, indicating the multifaceted role of this adaptor protein (51). Interestingly, TLR3 and TLR9 are subject to regulated release from Unc93b1 in endosomes prior to ligand binding and signaling, contrary to TLR7 which binds ligand and signals while associated with Unc93b1 (52). Furthermore, following Unc93b1 phosphorylation, the Unc93b1-TLR7 complex is able to recruit Syntenin-1 for signaling termination and limiting the receptor reactivity (53).

Kanno et al. (14) observed that the appearance of TLR7 on the surface of splenic DCs occurred in a UNC93B1-dependent manner. The contribution of UNC93B1 in the transportation of TLR9 to the cell surface was reported by Onji et al. (54). Deficiency in *UNC93B1* reduced the abundance of TLR3 on the surface of the human embryonic kidney (HEK293T) cells (55), and no TLR3 was observed on the surface of murine bone marrow myeloid cells (BM-MCs) with the *Unc93B1*$^{3d/3d}$ loss-of-function mutation (56). These results indicate that UNC93B1 may be responsible for the presence or abundance of TLR3 and other endosomal TLRs on the surface of cells.

Stimulation of human umbilical vein endothelial (HUVEC) cell line with TLR3 ligand but not with TLR9 ligand, not only up-regulated *UNC93B1* mRNA expression, but also promoted TLR3 transposition to the cell membrane. Additionally, increased expression of UNC93B1 affected the transportation of TLR3 but not TLR7, TLR8, or TLR9 to the cell membrane in HEK293T cells transfected with *TLR3* and *UNC93B1*. Overexpression of UNC93B1 led to a 13-fold increase in cell surface expression of TLR3, compared to cells with endogenous expression of UNC93B1 (49). The up-regulation of UNC93B1 which increases TLR3 expression on the cell membrane could also imply an increase in intracellular/endosomal TLR3 abundance [see **Figure 4A** in (49)], however, confirmatory studies would be necessary to acknowledge such a phenomenon in cells other than HEK293T and additionally verify whether UNC93B1 can occur together with TLR3 on the cell surface. Such an interaction was revealed for uncleaved TLR9 and UNC93B1 (38). Studies on the role of UNC93B1 in the cell surface localization of TLR5 also show that although UNC93B1 mainly localizes intracellularly, it may be present in the cell membrane [see **Figure 3B** in (57)]. Whether TLR3 requires internalization from the cell surface to endosomes for triggering the signaling pathway is another issue worth investigating. Bioinformatic analyses revealed that the UNC93B1 promoter region may be regulated by poly(I:C)-induced (polyinosinic:polycytidylic acid, synthetic dsRNA) transcription factors such as IRF3, NF-κB or AP-1. Priming of cells with the TLR3 ligand may enhance responses to agonists of other nucleic acids-sensing TLRs through the up-regulation of UNC93B1 (49). These findings shed light on the dependency of TLR3 on UNC93B1 for its surface localization in cells.

N-linked glycosylation is a significant process that arranges localization and assembly and therefore determines proper endosomal TLRs signaling (58). Besides, it may be involved in TLR3 stability (59), since mutations in 2 (N247, N413) of the 15 glycosylation sites gave rise to a non-functional TLR3 (21). The addition of complex glycans to TLR3 takes place primarily in the Golgi apparatus (48), and although TLR3 is one of the most heavily glycosylated TLRs, its lateral face does not contain glycans in order to interact with dsRNA or proteins (60). The endogenous expression or simultaneous overexpression of UNC93B1 and TLR3 generates a differentially glycosylated form of TLR3 on the surface of human cell lines (48, 49), whereas such form of TLR3 was not expressed on the surface of cells with overexpressed murine Unc93b1 (20). Likely, the disparately glycosylated TLR3 may be exclusively destined for the cell membrane, but this requires further examination. Nevertheless, this feature highlights TLR3's uniqueness, since no modified glycosylation pattern has been detected for other endosomal TLRs during UNC93B1 overexpression (49). In the work of Pohar et al. (49), it was considered that such a conservatism constitutes an evolutionary adaptation intended to protect against autoimmune response to self-nucleic acids. Interestingly, another ER resident, the protein associated with TLR4 (PRAT4A), is required for intracellular trafficking of Tlr7 and Tlr9, whose responses were abolished in PRAT4A$^{-/-}$ BM-DCs, BM-macrophages, and splenic B cells. In contrast, Tlr3 responses were not impaired in cells from mice lacking PRAT4A (61).

Taken together, UNC93B1 is a versatile chaperone protein and not only takes part in the escape and transportation of the NA-sensing TLRs from the ER or cell membrane to endosomes, but also remains associated with TLRs for activation or termination of their signaling, and finally contributes to the generation of particular TLR forms on the cell surface. However, little is known about the delivery of cleaved forms of endosomal TLRs to the membranes of particular cell types (56), and whether this may take place in a UNC93B1-dependent manner. Cleavage of endosomal TLRs occurs in endosomes with the participation of cathepsins, important enzymes that may shape the formation of TLR-mediated immune response against pathogens (62–66). More than a dozen cathepsins have been discovered in humans, which belong to aspartic (D, E), serine (A, G), and cysteine (B, C, F, H, K, L, O, S, V, Z/X, W) proteases (67).

CLEAVAGE OF ENDOSOMAL TLRS BY CATHEPSINS

Compartmentalization of TLR3, TLR7, TLR8, and TLR9 is aimed at the delivery of receptors to the ligand location site. However, endosomes not only provide recognition of bacterial or viral nucleic acids but they also prevent TLRs from sensing host nucleic acids and retain the environment necessary for the

activity of cathepsins, which play an important role in receptor performance (54, 64, 66, 68) (**Table 1**). Acidic pH is vital for adequate maturation of endosomes and augments ligand recognition by TLR3 and TLR9 (40, 88), whereas inhibition of acidification likely impedes the immune response (89, 90). Although pH 5.7–6.5 is optimal for TLR3 aggregation and signaling, only pH 7.5 or higher prevented the response of TLR3 to poly(I:C) in human U937 lymphocyte cell line (11). TLR3, TLR7, TLR8, and TLR9 contain individual cleavage sites and are split by various proteases (**Table 1**) into the N-terminal fragment containing part of the ECD and C-terminal fragment consisting of truncated ECD, transmembrane, and TIR domain (65, 77). Noteworthy is the fact that different cell types may have diverse proteolytic specificity and capacity, e.g., in dendritic cells (DCs), apart from "classic" TLR9 processing dependent on the cysteine protease cathepsin B and required for proper signaling, the receptor was subjected to other proteolytic events orchestrated by other enzymes (91). Cathepsin S is active regardless of an acidic environment and may cleave TLR9 between amino acids 441–470 into the 80 kDa form of an active receptor capable of inducing a signaling cascade. Processing of TLR9 between amino acids 724–735 in endosomes leads to the emergence of a soluble form. Such a soluble TLR9 (sTLR9) variant, analogous to sTLR2 and sTLR4, which occur naturally in body fluids and cellular secretions (92–94), inhibits TLR9-dependent signaling, indicating that distinctive proteolytic processes may affect TLR9 responses, an aspect that requires further investigations.

Cleavage of TLR7 or TLR9 by cathepsins is required for signaling, contrary to TLR3, where proteolytic cleavage of the receptor may not determine the activation of the immune response (77). Despite the use of z-FA-FMK cathepsin inhibitor, TLR3 could still be activated in transiently transfected HEK293T, Huh7.5, and BEAS-2B cells in comparison to the control treatment (48), therefore, it is possible that proteolytic cleavage may untie novel functions of the TLR3 derivative forms. Compared to full-length TLR3, both C-terminal and N-terminal forms displayed longer half-life, which may influence the duration of signaling (48). Moreover, mutation of the TLR3 cleavage site or the addition of cathepsin inhibitor reduced the abundance of endosomal TLR3 destined for degradation in lysosomes. Noteworthy is that the cleaved TLR3 forms were more abundant in early endosomes, while the inhibition of cathepsin activity shifted TLR3 localization to recycling endosomes and lysosomes (48). Localization of TLR3 in various types of endosomes may have a significant impact on the signaling, as these dynamic organelles may carry the TLR3 ligand or constitute a site of receptor degradation.

Although the presence of both cleaved fragments may not be indispensable for ligand recognition among endosomal TLRs, C-terminal and N-terminal forms alone have been reported to sense their ligands (**Table 1**). Regarding TLR3, it is suggested that both forms of the receptor contain the ligand-binding domain, however, the ability to bind dsRNA by C-terminal fragment of TLR3 is ambiguous (66, 69). A certain theory postulates that similar to TLR9 (54), it is the association of C- and N-terminal TLR3 fragments which enables response to dsRNA. Cleaved TLR3 fragments are observed during the detection of cellular proteins only under denaturing conditions, which may corroborate the interaction of these forms in murine primary immune cells (56). In HEK293T cells, the deletion of 14 amino acids at the N-terminus of the C-terminal form of TLR3 suppressed immune response, probably due to exclusion of the cleaved fragments association (69). Elongation of the N-terminal receptor form by the same number of amino acids also reduced TLR3 responses (56). These observations were confirmed by experiments in which activation of NF-κB or IFN-β promoter occurred in cells where C- and N-terminal fragments were simultaneously expressed (56). Furthermore, the addition of an antibody stabilizing the interaction between C- and N-terminal forms of the receptor strengthened TLR3 signaling in endosomes. These findings strongly favor the association of cleaved TLR3 fragments, however, we cannot preclude that such cooperation is indirect, e.g., it may occur through the assistance of full-length TLR3 or other proteins. Interestingly, inherent in murine and human TLR7, cysteines of the N-terminal (C98 and C445) and C-terminal (C475 and C722) cleavage forms of the receptor are not only required for the TLR7 proteolytic processing. These unique amino acids also determine the disulphide bonds between TLR7 cleaved molecules and are indispensable for RNA sensing by the cleaved and bound forms of the receptor (78).

Notably, Qi et al. (55). observed that TLR3 mutations in P554S (situated in the region of cleavage and critical for dsRNA binding) and F303S, caused a reduction in TLR3 abundance on the cell surface, compared to wild type HEK293T cells. Earlier, Zhang et al. (95). linked P554S mutation in a patient suffering from HSE with loss of TLR3 function in central nervous system (CNS) cells and increased penetrance of the disease through insufficient antiviral response, as reviewed by Mielcarska et al. (96). Subsequently, F303S mutation was found in a patient with encephalopathy following influenza virus infection, which underlines the pivotal role of TLR3 in the antiviral defense of the brain (55). This highlights the importance of intact TLR3 cleavage site, the influence of the cleavage on ligand recognition and activation of the signaling pathway.

Collectively, different proteases have great importance in the processing of endosomal TLRs through production of active or inhibitory forms, which is continually required for the proper receptor functioning. Receptor proteolysis appears to be conserved across cell types (65), however, a thorough investigation of which enzymes contribute to regulating the TLRs signaling remains to be determined.

OCCURRENCE OF ENDOSOMAL TLRS ON THE CELL SURFACE

Endosomal TLRs may be present in the cell membrane from where they may sense ligands. Ample surface expression of endosomal TLRs is observed in various cell lines and cell membrane-localized receptors are also capable of triggering an immune response. TLR7

TABLE 1 | Comparative presentation of endosomal TLRs and their ligands, enzymes required for cleavage, the importance of cleavage and eventual participation of cleaved fragments in signaling, and the possibility of occurrence on the surface of cells.

Endosomal TLR	Ligand	Enzymes responsible for cleavage	Cleavage required for TLR signaling	The ability of full-length/ cleaved fragments to bind ligand	The requirement of C and N association for signal transduction	Occurrence on the surface of the cell
TLR3	double-stranded RNA	cathepsins B, L, and/or S (48), H (66)	not required (but may modulate the level of antiviral response) (48)/ required (66)	present: FL, N, C (48)	required (56, 69)	yes: $CD8^+$ DCs, MZ B cells, J774 macrophages, BM-MCs (56), HEK293T cells (48), prostate epithelium (70), human conjunctival fibroblasts (HCF) (71), apical and basal plasma membrane of human endocervical cells (72), rat peritoneal mast cells (73, 74), and other
TLR7	single-stranded RNA, guanosine	cathepsin B* (65), asparagine endopeptidase (AEP) (75), furin-like proprotein convertases (76)	not required (64)/ required (30, 75, 77, 78)	present: FL, C (75), C (78), N, C (cooperatively involved) (30)	not required (75)/ required (30, 78)	yes: BM-DM, BM-conventional DCs, BM-plasmacytoid DCs, B cells, peripheral blood mononuclear cells (14), rat peritoneal mast cells (73, 74)
TLR8	uridine-rich or uridine/ guanosine-rich single-stranded RNA	cathepsins*, furin-like proprotein convertase (79)	required** (68, 79)	present**: N, C (68, 79)	required** (68, 79)	unknown
TLR9	unmethylated CpG DNA	cathepsin B (63), F (63), K (62, 77), L (63, 64), S (63, 64, 77), AEP (80)	required (48, 64, 77, 80, 81)	present: FL, N, C (64, 77, 80)	not required (the cleaved 80 kDa form is considered to constitute a mature receptor) (80)/ required (54)	yes: conventional DCs and plasmacytoid DCs (pDCs) (54), rat peritoneal mast cells (73, 74), PBMCs (82), IECs (83), neutrophils (84), human hepatocellular carcinoma (HCC) cell lines (85), B cells (86), splenic monocytes and B cells, RAW 264.7 cells (87)

*The features discussed are presented at the top of each column. FL, full-length; C, C-terminal; N, N-terminal. *studies were carried out indirectly, with the use of cathepsin inhibitor, which can exert broader activity; **result obtained on the basis of structural analysis studies.*

may appear on the surface of cells and become a beneficial target for autoimmune therapy. For instance, in mice suffering from chronic progressive inflammation causing splenomegaly, thrombocytopenia, and chronic active hepatitis due to spontaneous TLR7-dependent systemic inflammation, symptoms were alleviated through the use of anti-surface TLR7 antibodies (14). Administration of an antibody against TLR7 in these mice inhibited the production of cytokines in immune cells such as B cells, macrophages, and DCs. Particularly, the exogenously added anti-TLR7 antibody completely blocked the production of IL-6, CCL5, and TNFα in BM-MCs, and greatly inhibited B-cell proliferation induced by the TLR7 ligand (14). Full-length and N-terminal TLR7 forms were found on the surface of immune cells such as bone marrow-derived macrophages (BM-DM) and macrophage cell lines as well as in BM-conventional DCs, BM-plasmacytoid DCs, B cells, and peripheral blood monocytes, but TLR7 in BM-derived cells was mainly localized in the intracellular compartment. Similarly, TLR7 appeared on the cell surface and intracellularly in connective tissue-type mast cells, however, exhibiting higher expression inside the cells (73). When surface TLR7 was complexed with antibodies, it was detected in lysosomes 24 h later (14). Interestingly, such an internalization process may not be associated with triggering the signaling pathway, but the degradation of the receptor.

Careful investigation of different distribution profiles of endosomal TLRs in cells may yield data on cell type-specific pathways that culminate in antimicrobial response induction. For example, following stimulation of brain cells with let-7b, a TLR7 ligand, the receptor localized to the endosomes in the cortical and hippocampal neurons which underwent apoptosis (97), and to the plasma membrane in the sensory neurons causing stimulation of the cation channel transient receptor potential A1 (TRPA1) (98). The discussed results indicate the localization of TLR7 in different types of neurons as a factor influencing the functional responses of neurons to the stimulation with the TLR7 ligand (13).

TLR8 has not been found on the cell surface thus far, but the receptor may crosstalk with other TLRs. Nucleic acid recognized by TLR8 may be of viral origin or constitute bacterial RNA released within phagosomal vacuoles (99). Total RNA of *Escherichia coli* elicited TLR7 and TLR8 activation in HEK293 cells (100), while stimulation of cell surface TLR2, TLR4, and TLR5 in human primary monocytes down-regulated TLR8-IRF5 signaling, reducing the impact of TLR8-mediated pathogen sensing (101, 102). Interestingly, human TLR8 inhibited activation of TLR7 and TLR9, likewise TLR8 from mice inhibited TLR7 activity (103). Cells from *Tlr8*$^{-/-}$ mice showed increased expression of Tlr7 and were hyperresponsive to various TLR7 ligands, resulting in the animals developing spontaneous autoimmunity (104). Furthermore, *Tlr7*$^{-/-}$ and *Tlr8-/- Tlr7-/-* mice did not show the phenotypes of *Tlr8-/-* animals, emphasizing the significant role for TLR8 control of the

TLR7 expression level and its role in autoantibody production. The functional reverberations of these TLR-TLR dependencies have yet to be thoroughly investigated.

TLR9 may exist on the surface of splenic DCs (54), rat peritoneal mast cells (73), HEK293 cells following stimulation with the TLR9 ligand (36), human peripheral blood mononuclear cells (PBMCs) after the addition of LPS (82), or murine intestine epithelium after stimulation of cells with DNA from pathogenic *Salmonella enterica* (83). Present on the surface of human and murine neutrophils, TLR9 plays an important role in their activation, even after inhibition of endosomal acidification (84). Further, stimulation of TLR9 in human polymorphonuclear leukocytes resulting in their activation, culminated in enhanced expression of the cleaved functional receptor on the surface of cells. On the other hand, the forced occurrence of TLR9 on the cell surface through mutation in the transmembrane region led to inhibition of the receptor proteolysis and lethal inflammation in mice (105). Expression of cell membrane-localized TLR9 was remarkably increased on whole blood B cells of severely mechanically injured patients prone to sepsis compared with healthy controls (106). Also discovered in the plasma membrane of B lymphocytes, surface TLR9 was unable to bind its ligand, however, might negatively regulate endosomal TLR9 responses (86). It remains to be resolved how the non-ligand-binding receptor may signal from the cell surface to influence the intracellular equivalent. Interestingly, TLR9 was expressed on the surface of human HCC cell lines such as HepG2, HLE, Huh7, and SK-Hep1 (85). While full-length TLR9 was mainly expressed on the cell membrane, cleaved forms of TLR9 were abundant in the endosomes. Recently, Murakami et al. (87). confirmed the presence of TLR9 in the plasma membrane of splenic monocytes and B cells. During studies with immunocompetent cells, it was found that TLR9 surface expression varied according to the cell type as well as the status of their differentiation and activation. TLR9, together with TLR7, have been found in human airway epithelial cells (AECs), particularly in the terminal bars and cilia (107). Such an unusual pattern of expression and distribution may favor tissue-specific biological necessities.

Surface TLR3 was first observed on human fibroblast cell line MRC-5 (108). Binding of TLR3 to an antibody inhibited the poly (I:C)-mediated secretion of IFN-β by MRC-5 cells, demonstrating the functional role of the receptor on their surface. In HEK293T cells transfected to express TLR3, full-length, N- and C-terminal forms of the receptor were present on the cell surface. In contrast, the cell surface of BM-MCs subjected to similar experiment abounded in cleaved TLR3 forms rather than full-length receptor (48). These fragments were likely to be transported from endosomes, and motif-containing TLR3 plasma membrane localization dependent on UNC93B1 was assigned to the ECD of the receptor (108). Different monoclonal antibodies binding to TLR3 ECD inhibited the production of cytokines in human lung epithelial cells (109). Surface TLR3 expression was also observed in cell lines such as HUVEC, pigmented retinal epithelium (APRE-19), lung epithelium (A549), human dermal microvascular endothelium (HDMEC), stomach carcinoma (N87), and breast carcinoma (JIMT-1) (110). Recently, surface TLR3 was observed on $CD8^+$ classical dendritic cells (cDCs), BM-MCs, J774 murine macrophages, and marginal zone (MZ) B cells (56). On the other hand, in monocyte-derived immature dendritic cells (MD-iDCs) and $CD11^+$ blood DCs, apart from being on the surface, TLR3 was largely stored intracellularly and upon poly(I:C) stimulation the cells increased cytokine production and maturation (111). Stimulation of rat peritoneal mast cells with LL-37 peptide not only increase TLR9 expression on the cell surface but also contributed to the translocation of TLR3 from the plasma membrane to the cytoplasm (74). The peptide increased intracellular TLR3 abundance while TLR3 expression on the cell membrane decreased.

In the light of the findings discussed in the preceding section, it is justifiable to point that cell surface TLR localization is now an established scientific observation, especially in immunocompetent cells. It remains a matter of thorough investigation to discern whether such a pattern of expression has a beneficial effect for the host. Unlike self-derived ligands of TLR7, TLR8, and TLR9, the endogenous dsRNA in mammalian cells is limited to small amounts in the cytosol formed by complementary ssRNA fragments or microRNAs (miRNAs) (112, 113). The latter, although constantly synthesized by the cells, are unlikely to stimulate antiviral mechanisms. As a consequence, TLR3 present at the surface of cells in distinct organs may pose a lower risk of autoimmune response and function without pathological repercussions comparing to other endosomal TLRs (49).

TLR3 CELL SURFACE EXPRESSION AND ITS POSSIBLE MEANING

Studies over the past several years have reported on the surface occurrence of TLR3, which may facilitate response against pathogens. This pattern of occurrence on the membranes of distinct cells appeared to go in pairs with viral infection. Therefore, a thorough examination of the cell types regarding possible cell type-specific TLR3 regulation is necessary. For instance, the TLR3 shift to the cell surface was observed after respiratory syncytial virus (RSV) infection of airway epithelial A549 cells (114), similar to the epithelium of human bronchi (BEAS-2B cells) after rhinovirus infection (115). During viral infections, dsRNA may be found as an intermediate product of virus replication in the extracellular milieu after the breakdown of the infected cells [(107), see **Figure 7** in (49) and **Figure 1** in (96)]. Abundant surface TLR3 was also detected in primary human corneal epithelial cells (HCECs), where the production of IFN-β and pro-inflammatory cytokines such as IL-6 and IL-8 was initiated after the addition of poly(I:C) (116), which also led to upregulation of surface TLR3 expression. A significant transfer of TLR3 to the surface of alveolar macrophages was observed in mice after lung contusion (LC), in comparison to the uninjured control (117). Suresh et al. demonstrated that such a process was intended to expose TLR3 to extracellular dsRNA released from injured cells. Importantly, the dsRNA-triggered downstream signaling was

independent of NF-κB and type I IFNs, and led to increased macrophage apoptosis and exaggeration of the local inflammatory response which aggravated the degree of lung injury. In such a case, the discovery that the exogenous TLR3 ligand is able to mobilize membrane translocation of the receptor may indicate that ligand-induced cell priming could increase vulnerability to subsequent dsRNA recognition (118).

The issue of TLR3 function, especially, on the airway epithelial cell surface, has attracted scientific curiosity. Poly(I:C) proved to be the most effective epithelial activator in BEAS-2B and primary bronchial epithelial cells stimulated with various TLR ligands (119). Among other genes, it significantly increased the expression of *IL-8*, granulocyte-monocyte colony-stimulating factor (*GM-CSF*), and macrophage inflammatory protein-3α (*MIP-3α*), whose products foster migration and maturation of iDCs. The presence of TLR3 was also confirmed in the apical cell membrane of human tracheal epithelial cells and human AECs (107). These observations confirm the potential significance of TLR3 in defense against inhaled pathogens.

The presence of TLR3 in the cell membrane was also demonstrated in unstimulated BEAS-2B cells (109). The addition of monoclonal antibodies recognizing cell membrane-TLR3 inhibited secretion of cytokines such as IL-6, IL-8, IP-10, MCP-1, RANTES, by up to 60% after stimulation with poly(I:C). Although a significant value, it indicates that other ligand-binding sites existed on the surface TLR3 that were not blocked by the antibody, or induction of the immune response may have occurred due to activation of the endosomal receptor. Contemporaneous signaling through the surface and endosomal TLR would initiate a faster and/or more robust biological outcome, however, this remains to be further explored. Inflammation is crucial for the elimination of infections, however, excessive inflammation may be particularly harmful to the protective functions of the surface of the mucous membranes (72). Therefore, TLR3 has an essential role at the surface of epithelial cells, which constitute essential physical barriers and strengthens the notion that cell surface TLR3 is a propitious target for the regulation of TLR3 responses (56).

In lymphatic endothelial cells (LECs) such as primary human dermal (HD) LECs and lung LECs or transfected h*tert*-HDLECs, TLR3 and TLR9 occurred both intracellularly and on the cell surface (120). Interestingly, all three cell types increased the expression of ICAM-1 and VCAM-1 leukocyte adhesion molecules as well as inflammatory cytokines production in response to TLR3, but not TLR9 ligand. Similarly, primary lung LECs also increased the expression of VCAM-1 following treatment with the TLR3 ligand (120). Furthermore, poly(I:C) up-regulated the expression of ICAM-1 in HT-29 intestinal epithelial cells (IECs) (121). The stimulation effect was diminished when HT-29 cells were treated with an anti-TLR3 antibody, indicating TLR3 functionality on the cell surface. Thus, cell membrane-localized TLR3 may serve as a mediator to promote the trafficking of immune cells through the lymphatic vessels during viral incursion, which reveals a new aspect of the receptor biology.

Healthy epithelial cells of the ileum and colon serve as a defensive line of the intestinal mucosa and also express cell surface TLR3 (122). There was no difference in surface TLR3 expression between non-inflamed mucosa cells and mucosa cells from ulcerative colitis patients. However, a significant reduction of surface TLR3 in mucosa cells was found among patients with Crohn's disease, indicating that such receptor deficiency in the intestinal epithelium may be the disease-related feature.

It still remains difficult to explain the role of the various forms of TLR3 on the surface of cells, although it is postulated that the cleaved receptor localized in such a way may constitute an aim for regulating the antiviral response. Murakami et al. (56). have made significant progress in investigating the possibility of dsRNA recognition and launching of protective immunity by surface TLR3 in their studies on J774 murine macrophages. In these cells, TLR3 present on the surface was mostly cleaved, indicating it may have been modified in endosomes prior to cell surface distribution. The N-terminal fragment occurred on the cell surface as the main TLR3 representative and was able to modulate the antiviral response from this particular setting. However, Murakami et al. (56). argue that surface TLR3 must be internalized in order to become activated by dsRNA. This is very likely due to the acidification which supports TLR3 activation, and because extracellularly present dsRNA, e.g., released from dead cells following viral infection, undergoes endocytosis (123). However, it cannot be precluded that the TLR3 response may be launched directly from the membrane of specific cell types by the ligand prevalent in the extracellular environment (**Figure 2**).

An example of non-beneficial localization of TLR3 on the cell surface was discovered in the metastatic derivative of IECs (15). Stimulation of IECs with poly(I:C) up-regulated UNC93B1 which also increased surface TLR3 expression. Both full-length and cleaved TLR3 forms appeared on the cell membrane, in contrast to non-metastatic cells. The inhibition of acidification in endosomal and lysosomal compartments inhibited the production of CXCL10 following TLR3 stimulation, indicating the significant role of these organelles as well as possible functions of cleaved TLR3 forms in signaling. On the contrary, inhibition of the TLR3 ligand endocytosis only slightly affected TLR3-induced CXCL10 production, however, cells failed to induce IFN-β expression. These results imply that dsRNA does not have to be absorbed into the cells for receptor activation (**Figure 2**). Furthermore, chemokine responses following stimulation of the surface TLR3 in metastatic IECs may induce a conducive environment for tumor progression (15). Although TLR3 promoted invasiveness of IECs in the discussed work, the dsRNA stimulation may entail apoptosis and reduce cell viability in various cancer types in a TLR3-dependent manner (124, 125). Therefore, careful studies of individual cancer types regarding the effects induced by cell-surface expressed TLR3 are indispensable to determine either beneficial or detrimental outcomes.

CONCLUDING REMARKS

Localization of nucleic acid-sensing TLRs in endosomes requires maintenance of a pH suitable for cathepsin cleavage as well as potent ligand affinity and has important implications towards triggering an effective immune response. The presence of the

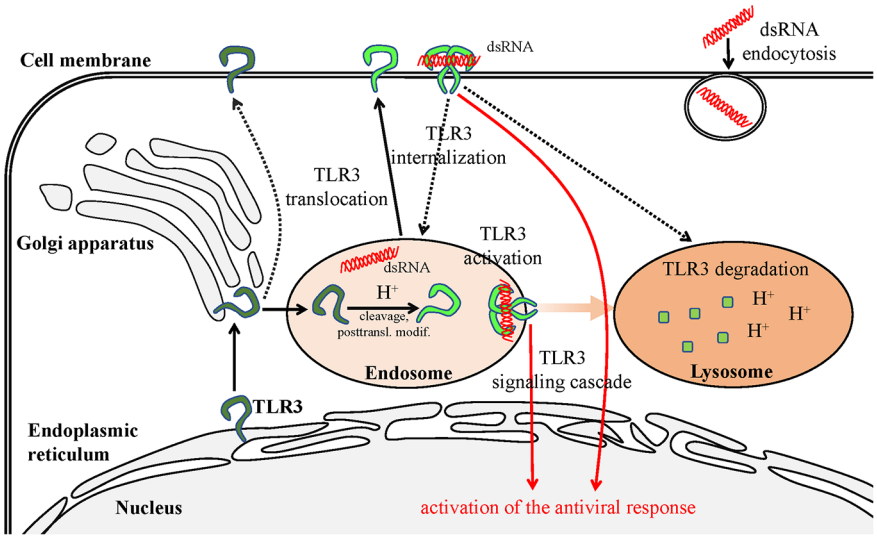


FIGURE 2 | Scheme of TLR3 transportation in the cell. The transportation route is initiated in the ER and terminates in the lysosome—site of degradation. TLR3 present on the cell surface may recognize the dsRNA, which is a viral replication intermediate for some viruses, derived from necrotic infected cells. The dsRNA can also be endocytosed and recognized by TLR3 in the endosomes. Pathways requiring further examination are marked with dashed lines.

endosomal TLRs on the cell surface may indicate an atypical condition or point to abnormal protein segregation and transportation and may affect proper degradation of the receptor. However, endosomal TLRs may also occur on the cell surface in a physiological state, and several studies point out that the localization of TLR3 or other TLRs to the cell membrane may be exploited as a therapeutic target.

TLR3 appears to exist as a functional receptor on the cell membrane more frequently than other endosomal TLRs, probably because endogenous agonists of TLR7, TLR8, and TLR9 are more abundant than dsRNA in uninfected cells, and therefore surface localization of TLR3 poses a lower risk of autoimmunity (49). Although it is believed that TLR3 activation occurs entirely in acidic endosomes (88), perhaps it would also be beneficial for cells to maintain a certain amount of TLR3 in the cell membrane in order to identify the extracellularly present viral dsRNA in case of a viral infection (**Figure 2**) (49). The probability that TLR3 ligand recognition occurs directly on the cell surface should not be disregarded, especially if the pathogen-derived ligand may not be able to reach the endosome. In such a case, it is the presence of a receptor on the cell surface that would allow the immune response to be activated. This statement is consistent with the positive effect of the exogenous dsRNA addition on the elevation of surface TLR3 expression (116) as well as increasing of TLR3 expression on the cell membrane after viral infection (114, 115). Furthermore, it underpins the significance of the surface TLR3 in mediating immune responses to viruses and should be addressed in future studies. TLR3 is engaged in recognizing dsRNA produced during the replication cycle of many viruses (95, 126–136), which may be released after lysis of infected cells.

Additional attention should be directed towards cleaved forms of endosomal TLRs, which also occur on the surface of cells and may play a role in microbial sensing. UNC93B1 is a protein indispensable for proper signaling of endosomal TLRs, however, mechanisms by which it may modulate surface TLR transportation and be involved in trafficking of the cleaved TLR forms await further studies.

The possibility that endosomal TLRs may occur in the cell membrane and act as stable and functional receptors seems particularly interesting. Already, investigations reveal that localized in such a way, these receptors may become disease-conducive or act as salutary immune sensors. Consequently, NA-sensing TLRs present on the plasma membrane may serve as therapeutic targets for functional monoclonal antibodies and might account for the progression of new therapeutical approaches towards rare human diseases that are difficult to treat. Discovering pathways originating at the cell surface may uncover new functions of endosomal TLRs, as well as subserve in better understanding individual aspects of their activation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Nucleic Acid-Induced Signaling in Chronic Viral Liver Disease

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A hallmark for the development and progression of chronic liver diseases is the persistent dysregulation of signaling pathways related to inflammatory responses, which eventually promotes the development of hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The two major etiological agents associated with these complications in immunocompetent patients are hepatitis B virus (HBV) and hepatitis C virus (HCV), accounting for almost 1.4 million liver disease-associated deaths worldwide. Although both differ significantly from the point of their genomes and viral life cycles, they exert not only individual but also common strategies to divert innate antiviral defenses. Multiple virus-modulated pathways implicated in stress and inflammation illustrate how chronic viral hepatitis persistently tweaks host signaling processes with important consequences for liver pathogenesis. The following review aims to summarize the molecular events implicated in the sensing of viral nucleic acids, the mechanisms employed by HBV and HCV to counter these measures and how the dysregulation of these cellular pathways drives the development of chronic liver disease and the progression toward HCC.

Keywords: hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, viral sensing, signaling, inflammation

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INTRODUCTION

The accelerating technological developments in biomedical and genomic research have made us aware of the enormous variety of viruses present in our environment and within ourselves (1, 2). Moreover, this has also given us a glimpse into our own past, as every biologic replication system with appreciable complexity co-evolves with parasites and at the same time develops mechanisms of resistance to them (3, 4). An important evolutionary milestone in vertebrates was the development of interferons (IFNs). IFN types I-III signal *via* the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways, triggering a series of sophisticated antiviral defense mechanisms (5). These consist on the induction of a complex expression pattern comprising a myriad of interferon-stimulated genes (ISGs), which promote an inflammatory state aimed to counter the viral presence or tilt the cellular balance toward apoptosis if the infection is not resolved (6). As this response needs to be transient in order to avoid excessive tissue damage, IFN signaling pathways are tightly regulated by multiple feedback mechanisms, which include members of the suppressor of cytokine signaling (SOCS) family among others (7). IFNs are induced by certain types

of exogenous nucleic acids (8). DNA and RNA are universal molecules in biology and thus any innate immune defense system based on the detection of viral nucleic acids must be capable of differentiating between the ones belonging to the host and ones that are foreign to it (9). Now, more than thirty years after this hypothesis was originally put forward, there has been substantial progress in the identification of these nucleic acid sensors and the understanding of their mechanisms of action (10).

Here, we explore these cellular receptors and their downstream signaling pathways in the context of the two major etiologies of chronic liver disease: hepatitis B (HBV) and hepatitis C virus (HCV). Patients with chronic viral hepatitis present a considerably increased risk of developing hepatic complications such as cirrhosis and hepatocellular carcinoma (HCC), with estimates suggesting that more than 1.4 million deaths each year are associated with these diseases (11, 12). HBV is a small noncytopathic DNA virus from the *Hepadnaviridae* family, and HCV is a single-stranded RNA virus from the *Flaviviridae* family (13, 14). Both exclusively hepatotropic viruses represent a tremendous global health burden with more than 320 million people chronically infected (15, 16). In this review, we introduce this topic with a general description of the cellular components implicated in the sensing of viral nucleic acids. This is followed by exploring the role of such sensors in the context of HBV and HCV infection and the viral strategies aimed to evade innate immune responses. Finally, we address the potential implications arising from the dysregulation of nucleic acid-sensing pathways as a driving component in the development and progression of HCC.

SIGNALING PATHWAYS IMPLICATED IN VIRAL NUCLEIC ACID SENSING

Pattern recognition receptors (PRR) implicated in the sensing of nucleic acids as pathogen-associated molecular patterns (PAMPs) can be divided in two main categories according to their mechanism of action (i.e., indirect or direct antiviral activity). The first category comprises PRRs belonging to the Toll-like receptor (TLR) and RIG-I–like receptor (RLR) families that are involved in RNA sensing, and a series of DNA sensors. These molecules induce the activation of transcription factors that favor the expression of cytokines such as type I IFN, ISGs and chemokines that recruit immune cells to the site of infection (17). In addition, these sensors can induce diverse types of programmed cell death such as apoptosis and pyroptosis in order to limit the spread of the infectious process (18). The second category of receptors includes nucleic acid sensors that possess direct antiviral activity, which is aimed against viral replication, translation or virion assembly (19, 20). Typically, the expression of such sensors is secondary to the production of IFNs or PRR signaling.

RNA sensors of the TLR family include TLR3, TLR7, and TLR8, with the three of them located at the endosomal membrane (21) (**Figure 1A**). In this context, TLR3 is activated

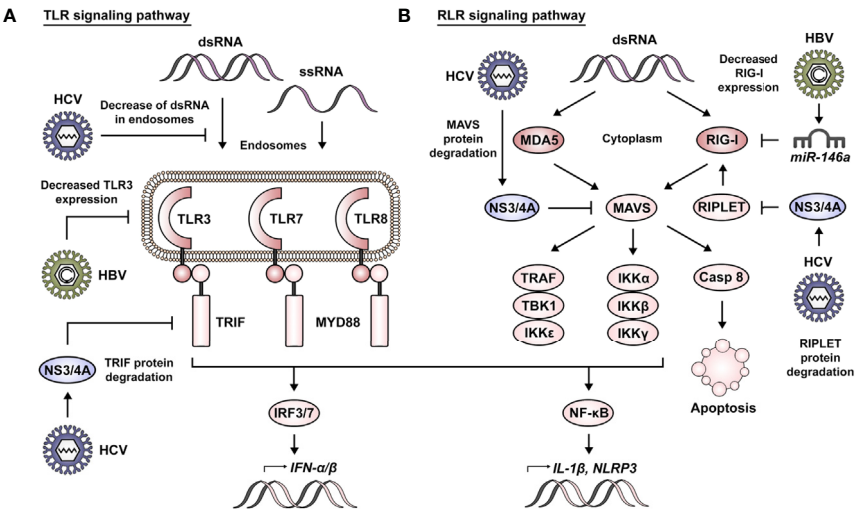


FIGURE 1 | RNA sensing and viral manipulation of TLR and RLR signaling pathways. **(A)** Foreign RNA sensing is mediated by TLR3, 7, and 8 in the endosomal compartment. These signals are transduced *via* TRIF in the case of TLR3 and by intermediary of MYD88 for TLR7 and TLR8. Their activation leads to the production of type I IFNs *via* IRF3/7 and to the expression of other genes such as IL-1β and NLRP3 *via* the NF-κB pathway. HBV has been reported to impair TLR signaling pathways *via* decreased expression of TLR3. Similarly, HCV affects TLR signaling by decreasing the presence of dsRNA in endosomes and *via* NS3/4A-mediated degradation of TRIF. **(B)** Foreign RNA sensing is mediated by MDA5 and RIG-I in the cytoplasmic compartment. K63-linked ubiquitination of RIG-I by RIPLET is necessary for its activation. MDA5 and RIG-I signals converge in the activation of MAVS and are carried *via* TRAF, TBK1 and IKKε for the induction of IFNs, or *via* IKKα, IKKβ, and IKKγ for induction of the NF-κB pathway. Alternatively, MAVS signaling can also induce apoptosis *via* activation of caspase 8. HBV has been reported to impair RLR signaling pathways *via miR-146a*–mediated downregulation of RIG-I. Similarly, HCV affects RLR signaling *via* NS3/4A-mediated degradation of RIPLET and MAVS.

by short double-stranded RNA (dsRNA) of 40 to 50 bp (22). TLR7 and TLR8 detect polyU and GU-rich stretches of nucleic acid, this being mainly dsRNA for TLR7 and exclusively single-stranded RNA (ssRNA) for TLR8 (23–25). These signals are carried downstream *via* TIR domain-containing adaptor-inducing IFN-β (TRIF) in the case of TLR3 (26, 27), while TLR7 and TLR8 do so by intermediary of myeloid differentiation primary response protein 88 (MYD88) (24). Their activation leads to the production of type I IFNs *via* interferon regulatory factor 3 (IRF3)/IRF7, and to the expression of other genes such as interleukin 1 beta (IL-1β) and NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) *via* the nuclear factor-κB (NF-κB) pathway (28, 29).

The RLR family of RNA receptors comprises retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated 5 (MDA5) as main sensors of foreign RNA in the cytoplasm (**Figure 1B**). RIG-I has been described to sense both long and short dsRNA, with the particular context defining if this is dependent or not of 5′ modifications (30–33). Additionally, RIG-I has been reported to recognize cytoplasmic DNA (34). MDA5 is strongly activated by very long dsRNA (30). A third member of the family, laboratory of genetics and physiology 2 (LGP2), functions primarily as a regulator of RIG-I and MDA5 activity (35).

RIG-I- and MDA5-induced signaling converges in the activation of mitochondrial antiviral signaling protein (MAVS), a key component acting as a hub for the induction of an antiviral state in the cell (36). Lysine 63 (K63)-linked ubiquitination of RIG-I by RING finger protein leading to RIG-I activation (RIPLET) is necessary for its interaction with MAVS at the mitochondrial membrane (37, 38). Subsequently, MAVS signaling leads to its association with tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins, TANK binding kinase 1 (TBK1) or IκB kinase ϵ (IKKϵ) in order to trigger activation of the IRF3/IRF7 pathway. Alternatively, this can occur *via* its association with the IKKα, IKKβ, and IKKγ complex for induction of the NF-κB pathway. The transduction of these signals results in the production of type I and III IFNs or the expression of proapoptotic genes such as p53-upregulated modulator of apoptosis (PUMA). Moreover, MAVS signaling can also induce apoptosis *via* activation of caspase 8 (39). As a side note, is worth mentioning that an important tool in the study and characterization of these RNA-sensing pathways has been the use of polyinosinic–polycytidylic acid (poly(I:C)). Structurally, poly(I:C) resembles dsRNA and is able to induce the activation of PRRs, such as TLR3, MDA5, and RIG-I (40, 41).

Foreign DNA sensing is mediated by TLR9 in the endosomal compartment and by absent in melanoma 2 (AIM2), interferon gamma inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) in the cytosol (**Figure 2A**). TLR9 is responsible for the recognition of DNA containing unmethylated CpGs or short DNA/RNA hybrid molecules (42, 43). These signals progress through MYD88, IRF7 and NF-κB, as previously described for the other TLR family members. AIM2 seems to recognize dsDNA with a minimum length of 80 bp (44). cGAS senses dsDNA of approximately 20–40 bp, although these fragments can be shorter (≥12 bp) in the case of G-rich Y-form DNA (45, 46). IFI16 has been reported to recognize longer dsDNA molecules, with an optimal length of 150 bp (47). In the case of cGAS, its activation leads to the production of the dinucleotide cyclic GMP-AMP (cGAMP), which acts as a second messenger to induce downstream signaling *via* stimulator of interferon response cGAMP interactor (STING). The binding of cGAMP to STING induces its dimerization and K63 ubiquitination by the ubiquitin ligases tripartite motif protein 25 (TRIM25) and TRIM56 (48, 49). Activation of STING ultimately leads to its interaction with TBK1 and the transduction of these signals *via* IRF3 in order to produce type I IFNs (50, 51). Similarly, IFI16 binding to dsDNA leads to activation of the STING pathway (52). On the contrary, AIM2 sensing of cytosolic DNA results in its interaction with apoptosis-associated speck-like protein containing a CARD (ASC), a key adaptor protein in the formation of inflammasomes (53). This event leads to the cleavage of caspase 1, the secretion of IL-1β and the induction of pyroptotic cell death (54).

As mentioned before, the second category of nucleic acid receptors is characterized by having a direct antiviral action (**Figure 2B**). Some of the relevant examples of this class of molecules are protein kinase R (PKR), 2′-5′-oligoadenylate synthetase (OAS) and adenosine deaminase RNA specific (ADAR). PKR is activated by recognition of dsRNA with a length larger than 30 bp (55), leading to an inhibition of mRNA translation by interference with eukaryotic translation initiation factor 2 alpha (EIF2A) (56). OAS proteins recognize dsRNA with a similar length as PKR, but in this case, it leads to the synthesis of 2′-5′-oligoadenylate that acts as a second messenger for the activation of RNase L (latent) and the induction of RNA degradation (57). ADAR proteins bind to long dsRNA with complex secondary structures (58), a process that catalyzes the C6 deamination of adenosine to produce inosine (A-to-I editing) leading to the alteration of RNA structure, localization and coding capability (59).

SENSING OF HBV AND HCV NUCLEIC ACIDS IN HEPATOCYTES

Following acute infection, HCV induces a strong inflammatory response characterized by the expression of hundreds of ISGs (60). These transcriptional changes have been shown to correlate with HCV viral load, suggesting that the expression of ISGs is principally mediated by PAMPs that activate innate sensors such as the ones previously mentioned (61). In contrast, HBV has been classically described as a stealth virus due to its capacity not to induce an apparent immune response (62). This might be either due to the replication strategies that allow HBV to escape the innate immune response without being detected or due to direct suppressive action over IFN signaling cascades despite its detection by PRRs. In support of the former notion, it has been shown by the culture of *ex vivo* liver biopsies from HBV-infected

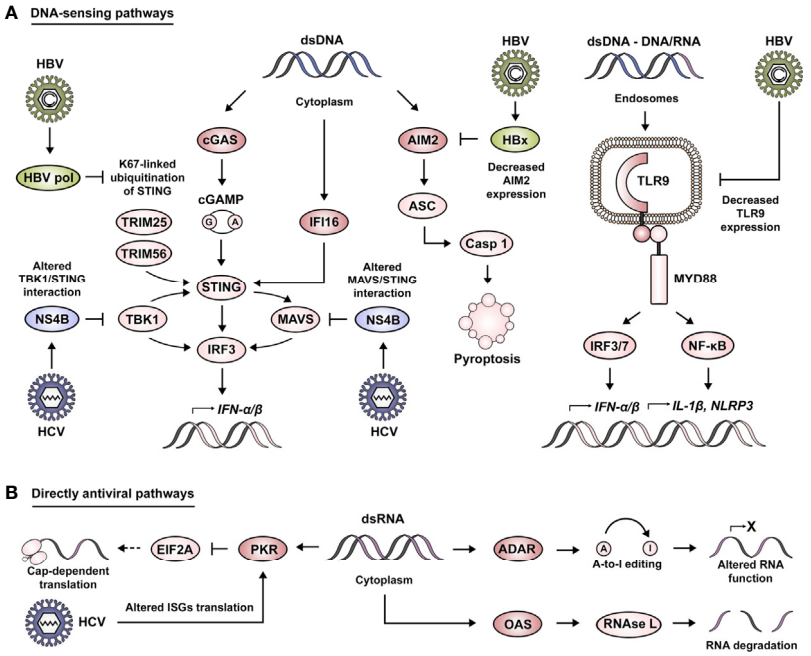


FIGURE 2 | Viral manipulation of DNA-sensing and directly antiviral signaling pathways. **(A)** Foreign DNA sensing is mediated by cGAS, IFI16 and AIM2 in the cytoplasmic compartment. cGAS activation leads to the production of cGAMP, which acts as a second messenger to induce STING activation. cGAMP binding to STING induces its dimerization and K63 ubiquitination by TRIM25 and TRIM56. This leads STING to interact with TBK1 and transduce these signals *via* IRF3 or MAVS in order to produce type I IFNs. Moreover, STING can also be activated by IFI16. AIM2 sensing of cytosolic DNA results in its interaction with ASC, leading to the cleavage of caspase 1, the secretion of IL-1β and the induction of pyroptotic cell death. Foreign DNA sensing is mediated by TLR9 in the endosomal compartment. These signals are transduced by intermediary of MYD88 and IRF3/7 in order to produce type I IFNs, and to the expression of other genes such as IL-1β and NLRP3 *via* the NF-κB pathway. HBV has been described to impair DNA-sensing pathways *via* HBV pol-mediated alteration of K67-linked ubiquitination of STING, HBx-mediated degradation of AIM2 and the decrease of TLR9 expression. Similarly, HCV affects DNA sensing by altering TBK1/STING and MAVS/STING interaction *via* its viral protein NS4B. **(B)** Directly antiviral pathways are mediated by the action of PKR, ADAR, and OAS. PKR activation leads to the inhibition of mRNA translation *via* interference with EIF2A. HCV has been described to exploit PKR activity as a means to alter the translation of ISGs. OAS proteins lead to the activation of RNase L and the induction of RNA degradation. ADAR proteins mediate A-to-I editing, leading to the alteration of RNA structure, localization and coding capability.

patients, that presence of the virus does not hamper RNA-induced TLR and RLR signaling (63). Moreover, although DNA-sensing pathways such as cGAS/STING seem functionally activated, HBV infection suppressed cGAS expression and function in hepatocytes (64), but not in immune mediators such as Kupffer cells (65). However, as we will see in the following paragraphs, an increasing amount of evidence suggests that in spite of being a weak inducer of proinflammatory cytokines, HBV can be recognized by the innate immune system (66).

TLR SIGNALING PATHWAYS

Members of the TLR family, namely TLR3, 7 and 9 have been shown to play key roles mediating immune responses against HBV infection in an IFN-dependent manner (67). Similarly, HCV infection has been reported to induce the expression of TNF-α *via* TLR7/8 signaling. Consequently, TNF-α activates its receptor (i.e., TNFR1) and leads to the suppression of HCV *via* the expression of ISGs (68). The clinical relevance of TLR signaling in the context of these two hepatotropic viruses has been highlighted by the identification of single nucleotide polymorphisms (SNPs) present in the *TLR3* and *TLR9* genes, as they are associated with the clinical course of infection. Indeed, *TLR3* SNPs have been linked to a reduced likelihood of spontaneous HBsAg and HBeAg seroclearance and an increased risk of developing chronic HBV infection (69, 70). These SNPs are also relevant during HCV infection as they are associated with an increased risk of chronic infection and HCV-associated liver disease (70). In particular, *TLR9* SNPs have been associated with spontaneous HCV clearance in women, as the expression of TLR9 in this context was linked to the level of circulating estrogens (71).

RLR SIGNALING PATHWAYS

RIG-I activation during HBV infection results in a weak production of IFN-α/β in contrast to a marked induction of IFN-λ (72). Moreover, the substrate recognized by RIG-I was shown to be a 5′ stem loop in the HBV pregenomic RNA

(pgRNA), a region described to contain the encapsidation (ϵ) sequence. This observation suggests a potential direct antiviral role of RIG-I *via* interference with binding of the HBV polymerase to the viral pgRNA (72). In contrast, HCV-induced RIG-I activation has been shown to produce a strong hepatic IFN-β expression, which is followed by the induction of an antiviral response mediated by ISGs (e.g., ISG54 and ISG56) (73). In this context, it has been observed that cytosolic HCV RNA is sensed by RIG-I, specifically at the HCV 3′ poly-U/UC sequence, the 5′ triphosphate of the uncapped HCV RNA and several short dsRNA regions (73, 74).

MDA5 also mediates the sensing of nucleic acids associated with the HBV infectious process (75). Indeed, HBV replication significantly increases the expression of MDA5 *in vivo*. Moreover, MDA5 overexpression induces a decrease of HBV RNA and encapsidated DNA *in vitro*. In mechanistic studies, the authors demonstrated that overexpression of MDA5 during HBV infection leads to IRF3 activation, NF-κB translocation to the nucleus and the subsequent expression of ISGs (e.g., MxA, OAS1, and CXCL10) (75). There is also evidence for a link between HCV and MDA5 (76). RIG-I and MDA5 activation by HCV occurs in a sequential and MAVS-dependent manner, as the IFN response is mediated by RIG-I at early stages of infection while the action of MDA5 takes place subsequently (77). Just like in the case of TLR components, the clinical relevance of MDA5 in HCV-associated disease has been shown by the observation that SNPs in the *IFIH1* gene (encoding for MDA5) are highly correlated with the resolution of HCV infection. Indeed, expression of these gene variants *in vitro* led to increased secretion of CXCL10 and IFN-λ3, concomitantly with a surge in the expression of other ISGs (e.g., IFN-β, ISG15 and ISG56) (78). Recent evidence suggests that LGP2 plays a role in strengthening MDA5-mediated innate immune responses against HCV infection. Indeed, LGP2 was shown to increase HCV RNA levels in association with MDA5 *via* its ATPase activity, leading to the expression of IFN-β and ISGs (79). On the contrary, other studies have reported that LGP2 negatively regulates these signaling pathways by interacting with TRAF family proteins and interfering with their ubiquitin ligase activity (80).

DNA-SENSING PATHWAYS

The role of cytoplasmic DNA sensors implicated in the induction of antiviral responses has been mostly described for HBV, with very few examples related to HCV infection. In this regard, the impact of two receptors in this category, IFI16 and AIM2, has been studied using woodchuck hepatitis virus (WHV) as a model system since it strongly resembles human HBV infection. In this model, expression levels of IFI16 and AIM2 were reported to be increased following acute infection with WHV. This tendency was consistent in the case of AIM2 following analysis of liver samples from chronically infected animals, but slightly decreased levels were observed for IFI16 (81). Interestingly, a subsequent report described that IFI16 is able to bind the HBV covalently closed circular DNA (cccDNA), particularly after IFN-α stimulation (82). Indeed, IFI16 overexpression was able to hamper the HBV cycle as shown by the decrease in hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), precore (preC)-pgRNA and HBV DNA. The expression level of IFI16 in human liver biopsies was significantly downregulated in patients with chronic HBV infection, negatively correlating between IFI16 and HBV preC-pgRNA. Moreover, taking into account that IFI16 seemed not to affect cccDNA quantity, this observation further suggested its repression at the transcriptional level. Indeed, the authors were able to demonstrate that IFI16 overexpression significantly decreased the levels of active histone marks and promoted the deposition of repressive ones in the HBV cccDNA minichromosome. Furthermore, recruitment of the acetyltransferase CREB-binding protein (CBP) to the minichromosome was impaired. Similarly, cccDNA in association with the deacetylases sirtuin 1 (SIRT1) and histone deacetylase 1 (HDAC1), as well as the lysine methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) was significantly increased. The authors went even further and showed that the interferon stimulating responsive element (ISRE) sequence present in the HBV cccDNA was needed to interact with IFI16 and induce this epigenetic regulation (82).

Although, HBV DNA is protected by the capsid and thus not accessible to DNA sensors in the cytoplasm, cGAS can recognize extracted HBV DNA as foreign (83) and mount an immunostimulatory response *via* the cGAS/STING pathway in hepatic cells (84). A functional role of this pathway beyond sensing was suggested by Eloi Verrier and co-workers, demonstrating that transfection of HBV relaxed circular DNA (rcDNA) induces the expression of ISGs, and importantly, knockout or overexpression of cGAS resulted in a marked increase in HBV infection and impaired HBV cccDNA levels (64). In support of this, the use of a STING agonist in HBV-infected mice decreased viral load and the susceptibility to infection (85). Thus, although STING expression is low in hepatocytes (65), a functional link with HBV seems evident. For HCV, although not a DNA virus, cGAMP stimulation or STING overexpression has an inhibitory effect on viral replication (86), as discussed below in more detail.

DIRECTLY ANTIVIRAL PATHWAYS

In addition to its suppressing role on mRNA translation (87), PKR recognizes the HCV internal ribosome entry site (IRES) and contributes to an antiviral response at early stages of infection by interacting with MAVS and triggering ISG expression (88, 89). Moreover, based on the observation that the activity of PKR is negatively regulated by cyclophilin A (CypA) (90), it was recently reported that PKR signaling determines the potency of cyclophilin inhibitors (CypI) against HCV *via* engagement of IRF1 (91). Other directly antiviral pathways comprise OAS and ADAR1 proteins, which exhibit an intrinsic antiviral activity targeting HCV infection (92, 93). The p100 subunit of OAS3 presents an antiviral role against HCV in an RNase L-dependent manner (92). ADAR1 specifically targets HCV replication *via*

A-to-I editing (94). ADAR1 also targets HBV infection. Guangyan Liu and co-workers have recently shown that *in vitro* overexpression of ADAR1 induces a decrease of HBV RNA and nucleocapsid-associated DNA. The mechanism suggested by the authors involves an ADAR1-mediated upregulation of *miR-122* leading to decreased levels of cyclin G1, a subsequent increase of p53 and ultimately reduced HBV RNA levels (95).

SENSING OF HBV AND HCV NUCLEIC ACIDS IN HEPATIC STROMAL CELLS

Although our view of the liver has been traditionally hepatocyte-centric, recent developments in single-cell omics have opened new insights into the diversity of stromal components and cellular states present in the liver microenvironment (96, 97). This is highly relevant in context of viral liver diseases, as it has been shown for example that HBV and HCV particles or viral components can be detected in association with stromal cells, such as fibroblasts (98), lymphocytes (99), endothelial (100) and dendritic cells (101). Although HBV and HCV may not be able to replicate in these cell types (102, 103), their exposure to viral nucleic acids does induce the activation of pathways implicated in antiviral immune responses (104). In this regard, it has been reported that TLR-mediated antiviral activity is not limited to infected hepatocytes, but is also induced in hepatic stellate cells (HSCs) and peripheral blood mononuclear cells (PBMCs). Indeed, treatment of HepG2 cells with supernatants from poly(I:C)-stimulated Lx-2 cells (HSC-derived cell line) inhibits the release of HBeAg and HBsAg in an IFN-β-dependent manner (105). Likewise, direct contact between HCV-infected hepatocytes and type 2 myeloid dendritic cells (mDC2) leads to the detection of dsRNA by TLR3 and the production of IFN-λ (e.g., IL-28 and IL-29) (106). Moreover, it has been observed that stimulation of PBMCs with HCV RNA leads to the production of TNF-α *via* TLR7/8 and that supernatants from monocytes or plasmacytoid dendritic cells (pDCs) stimulated with HCV RNA are able to reduce viral replication *in vitro* (107). These results are in line with a previous report by Marlène Dreux and co-workers showing how HCV infection in hepatocytes induces the release of exosomes carrying HCV RNA, which is recognized by TLR7 in pDCs (108). In addition to TLR-mediated signaling, the cGAS/STING pathway is also activated in myeloid cells following their stimulation with HBV DNA *in vitro* (84). Although its *in vivo* relevance remains to be established, cGAS/STING signaling in non-parenchymal cells is of potential clinical importance as dysregulation of this pathway is associated with liver pathogenesis (109, 110) (as discussed later).

HBV AND HCV STRATEGIES TO EVADE NUCLEIC ACID-INDUCED ANTIVIRAL RESPONSES

The innate immune system is a result of our co-evolution with pathogens. In response to the evolutionary pressure from the immune system, viruses have developed a wide variety of elaborated evasion mechanisms in order to prevent their elimination by the host (111). As HBV and HCV induce chronic liver infection, both viruses are no exception to this rule.

Immune evasion strategies relay on the ability of the virus to passively go unnoticed or/and actively attenuate downstream signaling events leading to a blunted IFN response. Manipulation of TLR3 signaling by HBV and HCV represents a clear example of both (**Figure 1A**). In this context, a recent report has shown that HCV dsRNA is released in extracellular vesicles, leading to a decreased activation of TLR3 in HCV-infected cells and allowing viral escape from the innate immune system (112). Of interest, HCV also actively interferes with downstream components of this pathway, e.g., by reducing the abundance of TRIF *via* NS3/4A-mediated proteolysis. This results in a delayed expression of ISGs (e.g., ISG15 and ISG56) and the establishment of a persistent infection (113). Although HBV is remarkably proficient in escaping immune detection *via* passive mechanisms, this virus has also been shown to actively drive the suppression of defense mechanisms. Indeed, the observation of a biphasic induction of ISGs following HBV infection suggests a feedback suppression (103). In this regard, HBV impairs TLR signaling by suppressing TLR3 expression in chronically infected patients (114).

The interference of HBV and HCV with RIG-I function is elaborated and complex (**Figure 1B**). Hou *et al.*, revealed that HBV infection induced the downregulation of RIG-I and IFN-β in HepG2 cells and human HBV tissues. Furthermore, the authors uncovered an HBV-induced upregulation of *miR-146a* mediating RIG-I suppression and that inhibition of *miR-146a* accelerated HBV clearance *in vivo* (115). This is consistent with previous observations reporting that HBV can abort cell intrinsic immunity in hepatocytes, with weak, transient IFN-α/β and IL-6 induction. In addition to the HBV-induced repression of RIG-I, this is a consequence of impaired MDA5 and TLR3 pathways during infection (116). Interestingly, HCV inactivates RIG-I signaling *via* the proteolytic activity of its viral protein NS3/4A. NS3/4A inhibits the induction of IFN-β *via* its localization to the mitochondrial membrane, where it is able to cleave MAVS and thus favor HCV evasion from the immune response (117). This is supported by translational studies demonstrating the presence of cleaved MAVS in liver biopsies from HCV- but not HBV-infected patients (118). Indeed, MAVS cleavage is particularly marked in HCV NS3-expressing hepatocytes (119), along with the cytoplasmic, and therefore inactive, form of IRF3 (120). Moreover, the NS3/4A protease complex antagonizes the cellular E3 ubiquitin ligases, TRIM25 and/or RIPLET, thereby also inhibiting RIG-I ubiquitination and thus its activation (121). Finally, the sequential activation of RIG-I and MDA5 can be inhibited by the action of HCV NS5A, as demonstrated both *in vitro* and *in vivo* (77).

Even though the main role attributed to the STING pathway is the sensing of DNA, it has also been implicated during HCV infection, which produces no DNA intermediate (**Figure 2A**). In this regard, HCV protein NS4B impairs IFN-β induction by altering the interaction between STING and TBK1 as a means of

immune evasion (122). Moreover, NS4B competes with MAVS for binding to STING on mitochondria-associated membranes. This interaction results in the displacement of MAVS from the RIG-I/MAVS/STING complex and prevents the downstream transduction of signals *via* IRF3 (123). In agreement with these findings, Guanghui Yi and co-workers demonstrated that the NS4B-mediated STING suppression was more pronounced when using NS4B from HCV genotype 2a (86). For HBV infection, it has been reported that the HBV polymerase inhibits cGAS/STING signaling and the subsequent activation of IRF3 by disrupting K63 ubiquitination of STING, thus preventing the production of IFN-β (124). HBV infection also impairs AIM2 expression *via* HBx. It prolongs the half-life of EZH2, thus favoring its recruitment to the AIM2 promoter, and subsequently blocking its expression (125). Regarding endosomal DNA sensing, it has been demonstrated how the presence of HBV virions and subviral particles specifically downregulates TLR9 at the mRNA and protein level, leading to significantly reduced levels of CpG-induced IFN-α in both pDCs and PBMCs. This is further supported by the observation that TLR9 levels are drastically reduced in chronic HBV patients (126, 127).

As previously mentioned, HCV-induced phosphorylation of PKR leads to the inactivation of EIF2A and ultimately to a halt in protein production as a means of antiviral response (87) (**Figure 2B**). Impaired translation of mRNAs is exploited by HCV in order to overcome the antiviral defense in hepatocytes, mainly by inhibiting the translation of ISGs (128).

DYSREGULATION OF NUCLEIC ACID-SENSING COMPONENTS IMPLICATED IN HCC DEVELOPMENT

An important observation with relevance to virus-associated pathogenesis is that a high number of nucleic acid PAMP receptors are also able to sense host nucleic acids as damage-associated molecular patterns (DAMPs) (129). DAMPs are central players in tissue repair, as they alert the organism about injury, stimulate an inflammatory response and promote the activation of organ regenerative pathways (130). For example, MAVS and STING deficiency results in impaired hepatocyte proliferation and a delayed recovery of liver mass following partial hepatectomy (131). Therefore, it is not surprising that due to continuous viral stimulation or suppression in a chronic inflammatory microenvironment, the dysregulation of innate immune components contributes to liver disease progression and complications including HCC (**Figure 3**).

TLRs activate inflammatory signaling pathways in the liver, which if persistent, can drastically affect the delicate balance between cell death and survival (132). In this context, the proinflammatory and proapoptotic role of TLR3 is evident in poly(I:C)-stimulated cells, which can be rescued by TLR3 silencing (133). Additional details regarding the mechanism behind this observation came from the finding that TLR3 was colocalized with granzyme B (GrB), suggesting an expression predominantly in natural killer (NK) cells. Indeed, analysis of liver samples from an HCC patient cohort (68% of viral etiology) showed that TLR3 expression was correlated with the hepatic infiltration of NK and CD8 T cells. Moreover, *in vitro* stimulation of TLR3 promoted NK cell activation, expression of IFN-γ and GrB and cytotoxicity against HCC cells (133). These observations are in line with the analysis of HCC patient samples, which showed the infiltration of CD8 T cells being positively correlated with cell apoptosis and negatively correlated with cell proliferation. Therefore, the authors suggest that the tumor suppressing effects of TLR3 may come from the induction of hepatocyte death and the attraction/activation of immune cells to the tumor microenvironment. These results could be one of the mechanisms explaining the association between low TLR3 expression and a lower patient survival from HCC (133). Similar results were obtained by Marc Bonnin and co-workers, demonstrating that TLR3 expression was downregulated in HCC lesions as compared to adjacent tissues, and that this was associated with a poor prognosis. Moreover, the study of a TLR3-deficient mouse model allowed to conclude that hepatocarcinogenesis was indeed accelerated in the absence of TLR3. However, an association with immune cell infiltration was not evident in this study (134). The role of TLR7 in HCC is debated. While TLR7 expression is downregulated in tumor lesions of HBV- and HCV-associated HCC as compared to non-viral etiologies (135), another study finds TLR7 overexpressed in HCC lesions as compared to tissues from cirrhosis or viral hepatitis. Moreover, inhibition of TLR7 with a TLR7/9 inhibitor (IRS-954) hampered cell proliferation (136). In agreement with these findings, a positive TLR9 immunostaining had previously been associated with a poor HCC prognosis, which may suggest that TRL7/9 signaling is promoting cancer cell proliferation and survival (137).

The RLRs RIG-I and MDA5 have also been implicated in the pathogenesis of HCC. This has been demonstrated in paired liver biopsies from an HCC cohort (90% HBV-associated) (138). The study concluded that RIG-I was significantly downregulated in HCC lesions as compared to the adjacent tissues. The suggested mechanism involves an epigenetic dysregulation of the *RIG-I* gene, as it presented reduced levels of H3K4me3, but increased H3K9me3 and H3K27me3 marks (138). Taking into account that RIG-I maintains IFN-induced proapoptotic signaling *via* STAT1, the authors revealed that RIG-I prevents STAT1 engagement with its negative regulator Src homology region 2 domain-containing phosphatase-1 (SHP1). Thus, impaired RIG-I function indirectly promotes cell survival. Phenotypically, RIG-I deficiency is associated with the development of HCC, as it favored the incidence, number and size of HCC tumors in a mouse model. Moreover, impaired RIG-I expression was associated with a lower patient survival from HCC (138). These results are in agreement with previous observations, showing that stimulation of RIG-I and MDA5 expression with poly(I:C) in HepG2 cells limited their proliferative capacity *in vivo* (35). Downstream of RLR family members, expression of NLRP3 inflammasome components are impaired in HCC

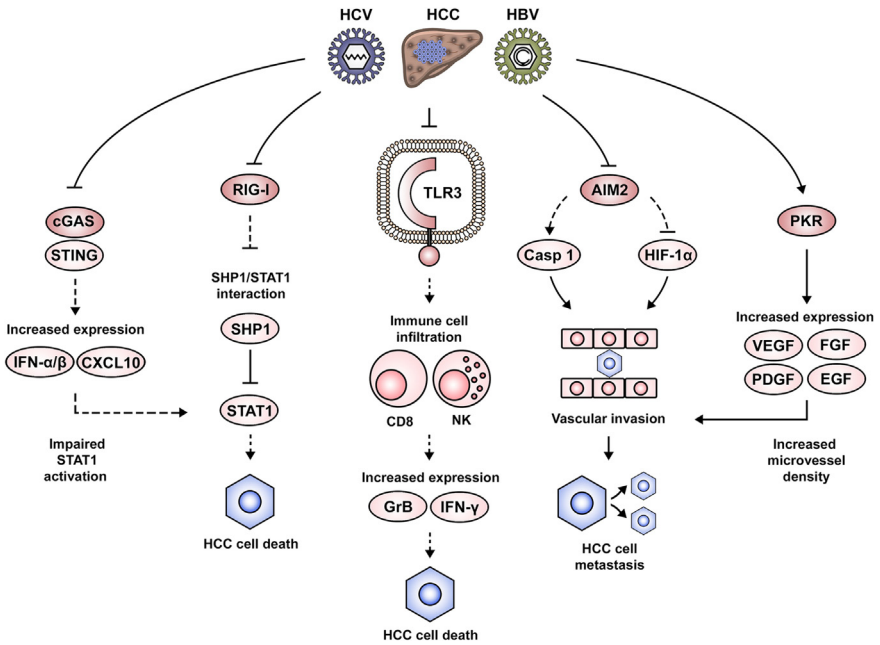


FIGURE 3 | Dysregulation of nucleic acid-induced signaling during HCC development. Altered nucleic acid-sensing pathways associated with HCC pathogenesis include cGAS/STING, RIG-I, TLR3, AIM2 and PKR. Impairment of RIG-I expression favors STAT1 interaction with its negative regulator SHP1, thus altering STAT1-mediated proapoptotic signaling. Similarly, the suppression of IFN signaling *via* the cGAS/STING pathway leads to an altered STAT1 activation. Downregulation of TLR3 in the context of HCC leads to an impaired attraction/activation of immune cells to the tumor microenvironment, the failure to induce a gene expression pattern that mediates cytotoxicity (e.g., GrB, IFN-γ) and the subsequent clearance of cancer cells. AIM2 downregulation alters normal formation of the AIM2 inflammasome and favors expression of HIF-1α, leading to the development of malignant cellular characteristics such as vascular invasion and lymph node metastasis. Activation of PKR in tumor lesions favors the expression of growth factors such as VEGF, PDGF, FGF, and EGF, which favor angiogenesis and potentially the development of vascular invasion and metastasis.

tumor tissues, i.e., ASC, caspase 1 and IL-1β, which inversely correlated with tumor grade and clinical stage (139).

DNA-sensing components linked to HCC development include IFI16, AIM2 and the cGAS/STING pathway. IFI16 protein expression is significantly decreased in HCC tissues (140). *In vitro* experiments suggest that IFI16 may exhibit a tumor-suppressing role, as its overexpression inhibited colony formation and induced cell apoptosis. Mechanistic studies suggested the capacity of IFI16 to induce p53 expression and its activation by promoting serine 15 (S15) phosphorylation (140). *AIM2* transcripts are specifically reduced in tumor tissue of paired liver biopsies from HBV-associated HCC. This impaired *AIM2* expression was associated with higher alpha-fetoprotein (AFP) levels, vascular invasion, poor tumor differentiation and lymph node metastasis. Moreover, patients with low *AIM2* mRNA levels showed shorter overall and disease-free survival times (125). These results are in agreement with the previous findings of Xiaomin Ma and co-workers, demonstrating AIM2 downregulation in HCC tissues as compared to the non-tumoral areas (141). Loss of AIM2 expression correlated with advanced tumor stages and metastasis. *In vitro* experiments demonstrated that AIM2 overexpression hampered cell proliferation, colony formation and invasion, while AIM2 silencing promoted an opposite more aggressive phenotype. The cellular signaling alterations arising from AIM2 dysregulation were also explored, demonstrating that increased AIM2 expression led to a significantly increased caspase 1 activation and IL-1β cleavage, indicating that the AIM2 inflammasome was formed and active. Moreover, blocking the inflammasome formation reversed the malignant characteristics. Additionally, AIM2 silencing enhanced hypoxia-inducible factor 1 alpha (HIF-1α) activity, which raised the possibility of the AIM2/HIF-1α axis contributing to the previously observed malignant cell characteristics (141). An interesting observation related to the involvement of DNA-sensing pathways in HCC development was made by Martin K. Thomsen and colleagues (109). The authors reported that increased STING expression rarely occurs in transformed hepatocytes and the response to STING agonists takes place primarily in non-parenchymal liver cells (e.g., Kupffer cells). Indeed, stimulation of a macrophage-like cell line (i.e., THP-1) with the STING ligand cyclic adenine monophosphate- inosine monophosphate (cAIMP) led to an enhanced expression of T cell-attracting chemokines (e.g., CXCL10), IFN-α/β and NF-κB activation. This molecular expression pattern following STING activation was identified as a driver for the induction of apoptosis, autophagy and the overall immune response. The pathological consequences of impaired hepatic STING function were explored by the authors using a liver cancer mouse model, which showed that STING-deficient animals presented larger tumors as compared

to the wild-type mice. Tumor characterization revealed decreased phospho-STAT1 and phospho-STAT3 levels, suggesting an altered immune response. Additionally, it was demonstrated that treatment with cAIMP led to the development of smaller tumor nodules due to an enhanced apoptotic cell death, as suggested by increased levels of cleaved caspase 3-positive cells (109). The previously discussed report is in agreement with the results obtained by Dou *et al*., showing that STING activity favored hepatic inflammation and a DNA damage-induced senescence-associated secretory phenotype (SASP). Indeed, STING-deficient mice exhibited an impaired expression of SASP genes. Moreover, STING-deficient mice presented NRas-positive intrahepatic tumors, which was not the case in the wild-type animals. These results were confirmed by rescuing STING in the liver of these mice, resulting in the restoration of cytokine levels and immune-mediated clearance (110).

Among the nucleic acid sensors with direct antiviral action, PKR and ADAR have been implicated in HCC pathogenesis (142–144). A PKR inhibitor (C16) reduced cell division in Huh7 cells and markedly decreased liver tumor growth in a mouse model. Moreover, it occurred that microvessel density of such tumors was decreased during PKR inhibition. Indeed, *in vitro* experiments revealed that angiogenesis-relevant growth factor expression—i.e., vascular endothelial growth factor A (VEGF-A), VEGF-B, platelet-derived growth factor A (PDGF-A), PDGF-B, fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), and hepatocyte growth factor (HGF)—was significantly downregulated by PKR inhibition (142). These observations are in line with the results reported following analysis of a human HCC cohort (100% HCV-associated), showing increased activity of PKR in tumor samples as compared to cirrhotic tissues (143). ADAR proteins (ADAR1 and ADAR2) are linked to HCC but seem to exhibit different roles during hepatocarcinogenesis. Immunohistochemistry and quantitative PCR (qPCR) in an HCC patient cohort identified upregulated ADAR1 but downregulated ADAR2 levels in HCC lesions as compared to the corresponding adjacent tissues. This ADAR expression pattern associated with increased incidence of liver cirrhosis and tumor recurrence with shorter disease-free survival times. These results were validated *in vitro* and *in vivo*, showing that overexpression of ADAR1 and ADAR2 was able to accelerate or inhibit tumor growth, respectively (144).

Taken together, the available data suggest that nucleic acid-sensing pathways are not only relevant as antiviral mechanisms against HBV and HCV infection, but—if chronically dysregulated—also contribute to the development and progression of virus-associated hepatic complications.

PERSPECTIVES

As we have explored in this review, nucleic acid-induced inflammation represents a complex signaling pattern designed to push the cell into a transient state of emergency following the detection of viral pathogens. Viruses causing chronic hepatitis like HBV and HCV have developed elaborated strategies to attenuate and divert these antiviral responses contributing to failure of viral clearance and a chronic inflammatory state. Consequently, the chronic dysregulation of the inflammatory response is an important factor in liver disease progression toward HCC. It also emerges that an integrated view on not only infected hepatocytes but also on their complex interplay with stromal cell components will be central to understand viral pathogenesis (145). The microenvironment of chronically-infected livers in single-cell resolution is still poorly characterized. This type of analysis has already been performed in context of nonalcoholic steatohepatitis (NASH), leading to the identification of a cell population tightly linked to disease severity, termed NASH-associated macrophages (NAMs) (146). Therefore, the integrated analysis of multiomics single-cell data could provide a unique opportunity to grasp the impact of nucleic acid inflammatory pathways in each hepatic cell population following viral infection (147). Moreover, such data would be highly relevant for drug discovery and the development of biomarkers for stratification of patients that may benefit from targeted interventions (148). However, access to fresh liver tissues from patients with HCV or HBV viremia, which are required for this kind of analysis, gets more and more challenging as an increasing number of patients currently receive antiviral treatment.

Despite efficient antiviral regiments allowing to control (HBV) and cure (HCV) chronic viral hepatitis in 2020, the risk of HCC cannot be fully eradicated, especially in patients with advanced liver disease. For HCV, accumulating evidence highlights a viral footprint in the host genome maintaining a persistent proinflammatory and prooncogenic environment even after viral cure (149, 150). Thus, the understanding of virus-specific and common evasion mechanisms from the antiviral inflammatory response, genomic imprinting, and the characterization of pro-oncogenic signaling persisting after HCV cure is key to identify targets for future chemopreventive and antifibrotic strategies to help patients at elevated liver cancer risk.

While for HCV, the future challenges are mostly emphasized in the chemoprevention, for HBV, novel antiviral concepts are urgently needed to cure viral infection, potentially, embodied by host-targeting agents (HTAs) aimed to boost innate immunity. This approach is supported by several lines of evidence, including the fact that patients who achieve control of HBV infection do so *via* an efficient immune response. Moreover, the small size of the HBV genome imposes a limit to the possible drugs that can be designed against it and its encoded viral proteins (151). Therefore, HTAs have appeared as a viable option to overcome this and similar issues. In this context, the use of Riboxxol (TLR3 agonist) has been reported to decrease intracellular HBV DNA, as well as extracellular HBeAg and HBsAg *in vitro* (152). Similarly, Selgantolimod (TLR8 agonist) has shown encouraging results in the WHV model, as it was well tolerated, reduced intrahepatic WHV RNA and DNA levels and favored the development of anti-WHsAg antibodies (153). Selgantolimod is currently being evaluated in phase II trials, in

order to assess its safety, tolerability and antiviral activity during chronic HBV infection (NCT03615066 and NCT03491553).

The aforementioned examples represent only a fraction of the potential that this approach has, as future compounds targeting additional nucleic acid sensors may as well be clinically relevant. In this regard, a close collaboration between academic research and industry will be necessary to accelerate drug discovery and evaluate their application as single or combination therapies (154). This will provide us with the possibility to expand our current therapeutic options and ameliorate the unmet medical need that chronic liver disease represents.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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The Trinity of cGAS, TLR9, and ALRs Guardians of the Cellular Galaxy Against Host-Derived Self-DNA

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The immune system has evolved to protect the host from the pathogens and allergens surrounding their environment. The immune system develops in such a way to recognize self and non-self and develops self-tolerance against self-proteins, nucleic acids, and other larger molecules. However, the broken immunological self-tolerance leads to the development of autoimmune or autoinflammatory diseases. Pattern-recognition receptors (PRRs) are expressed by immunological cells on their cell membrane and in the cytosol. Different Toll-like receptors (TLRs), Nod-like receptors (NLRs) and absent in melanoma-2 (AIM-2)-like receptors (ALRs) forming inflammasomes in the cytosol, RIG (retinoic acid-inducible gene)-1-like receptors (RLRs), and C-type lectin receptors (CLRs) are some of the PRRs. The DNA-sensing receptor cyclic GMP–AMP synthase (cGAS) is another PRR present in the cytosol and the nucleus. The present review describes the role of ALRs (AIM2), TLR9, and cGAS in recognizing the host cell DNA as a potent damage/danger-associated molecular pattern (DAMP), which moves out to the cytosol from its housing organelles (nucleus and mitochondria). The introduction opens with the concept that the immune system has evolved to recognize pathogens, the idea of *horror autotoxicus*, and its failure due to the emergence of autoimmune diseases (ADs), and the discovery of PRRs revolutionizing immunology. The second section describes the cGAS-STING signaling pathway mediated cytosolic self-DNA recognition, its evolution, characteristics of self-DNAs activating it, and its role in different inflammatory conditions. The third section describes the role of TLR9 in recognizing self-DNA in the endolysosomes during infections depending on the self-DNA characteristics and various inflammatory diseases. The fourth section discusses about AIM2 (an ALR), which also binds cytosolic self-DNA (with 80–300 base pairs or bp) that inhibits cGAS-STING-dependent type 1 IFN generation but induces inflammation and pyroptosis during different inflammatory conditions. Hence, this trinity of PRRs has evolved to recognize self-DNA as a potential DAMP and comes into action to guard the cellular galaxy. However, their dysregulation proves dangerous to the host and leads to several inflammatory conditions, including sterile-inflammatory conditions autoinflammatory and ADs.

Keywords: absent in melanoma-2-like receptors, absent in melanoma-2, Toll-like receptor 9, cyclic GMP–AMP synthase, stimulator of interferon genes, inflammation, autoimmunity

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INTRODUCTION

The immune system has evolved to protect the host from external pathogens and their microbe or pathogen-associated molecular patterns (MAMPs or PAMPs). The concept of *horror autotoxicus* introduced by the Nobel laureate Paul Ehrlich in 1899 based on experimental studies suggests that the immune system has not developed to self-attack *via* developing self-antibodies (self-Abs) or toxic Abs to endanger the host (1). However, further studies by other researchers showed the autoimmune nature of the disease called paroxysmal cold hemoglobinuria with the evidence of AutoAbs production against self-erythrocytes or red blood cells (RBCs) in 1904 (1). Also, the development of AutoAbs against self-lens protein and the eye lens-induced inflammation in patients with *endophthalmitis phacoanaphylatica* and the incidence of uveitis further strengthened the concept of autoimmunity (2). Hence, the idea of autoimmunity and autoimmune diseases evolved, and now more than 100 autoimmune diseases (ADs) are known. Therefore, the immune system may act against self-proteins and other cellular components, including genetic materials (DNAs and RNAs), once they lose their homeostatic stage at the cellular and organ level, causing a breach in the phenomenon of self-tolerance (3, 4).

The discovery of pattern recognition receptors (PRRs) called toll-like receptor 4 (TLR4) that recognizes the Gram-negative bacterial MAMP/PAMP known as lipopolysaccharide (LPS) in humans in 1997 filled the long-standing gap between the immune system and the pathogen recognition (5–7). To date, 10 TLRs (TLR1-TLR10) in humans and 12 TLRs (TLR1-TLR13) in laboratory mice have been identified, and the TLR10 in mice is a defective pseudogene (5). These TLRs recognize different PAMPs and DAMPs to elicit the NF-κB activation-dependent pro-inflammatory signaling discussed in detail by the author somewhere else (5, 8, 9). However, these TLR signaling pathways have various host-derived endogenous negative regulators, which keep their activation in check through different mechanisms (9). Hence, the TLR signaling activation pathway is a regulated pathway to protect against pathogens, PAMPs, and DAMPs, and any dysregulation causes exaggerated inflammatory signaling affecting different components of immunity causing infection-related or sterile inflammatory conditions. Hence, after TLRs, various other cytosolic PRRs, including NOD-like receptors (NLRs), absent in melanoma-2 (AIM2)-like receptors (ALRs) forming inflammasome, RIG-1 Like receptors (RLRs), cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS), and stimulator of interferon genes (STING) in mammals, including humans have been identified.

The inflammasomes responsible for generating mature IL-1β in response to potent inflammogen called LPS were first described in 2002 (10). Whereas ALRs (AIM2 or p210), have been discovered approximately twelve years ago in 2009 (11–14). The cGAS (a nucleotidyltransferase family member) responsible for the identification of cytosolic DNA and the induction of IRF3-dependent interferon-beta (IFN-β) or type 1 IFNs was discovered in the year 2013 (15, 16). Even the extracellular nucleosomes released due to DNA damage and apoptotic cell death taken up by cells are also recognized by cGAS as they have higher binding capacity than double-stranded DNA (dsDNA) (17). However, this cytosolic exosome recognition by cGAS does not elicit its profound activation hence low quality of pro-inflammatory cytokine and type 1 IFN generation occurs. The involvement of STING (an endoplasmic reticulum (ER) adaptor) in the cGAS signaling pathway-dependent type 1 IFN generation in response to the cytosolic DNA recognition was determined in 2008 and 2009 by the same research group (18, 19). Hence, this trinity of cytosolic PRRs recognizing cytosolic self-DNA as DAMPs is crucial to maintain cell homeostasis and harmony also in addition to recognizing pathogen-derived DNA. The present article discusses the role of the trinity of intracellular PRRs (TLR9, ALRs, and cGAS-STING signaling pathways) guarding the cellular galaxy against cytosolic self-DNAs serving as potent DAMPs to elicit several pro-inflammatory conditions, including autoinflammation, autoimmunity, and cancers.

CGAS-STING-BASED HOST CELL DNA RECOGNITION

The cGAS [C6orf150 or MAB-21 domain containing protein 1 (MB21D1)]-STING signaling molecules have also evolved to serve as intracellular PRRs for the cytosolic dsDNA recognition and comprise a crucial cytosolic innate immune signaling pathway (in different innate immune cells, including fibroblasts, macrophages, and DCs) to induce type 1 IFN production in response to dsDNA viruses, retroviruses (human immunodeficiency virus-1 or HIV-1 and HIV-2), and host-derived self dsDNA (15, 20–26). The cGAS resembles the nv-A7SFB5.1 enzyme of the *Nematostella vectensi* (a sea anemone) from, which humans have evolutionarily diverged around 600 MYA (27, 28). The *N. vectensi* cGAS (nvcGAS or nv-A7SFB5.1) produces 3′,3′CDNs, which is recognized by their STING (nvSTING) through nucleobase-specific contacts absent in humans (27). Of note, nvSTING specifically recognizes the guanine nucleobases of 3′,3′CDNs. The cGAMP or cGMP-AMP (cyclic guanosine monophosphate-adenosine monophosphate) formed upon recognition of cytosolic dsDNA by cGAS (a member of nucleotidyltransferase family) binds to the STING and activates type 1 IFN production through activating interferon regulatory factor 3 (IRF3) transcription factor (TF) (**Figure 1**) (15, 29).

Evolutionary Aspects of cGAS-STING Signaling

The researchers in 2011 first identified human cGAS (hcGAS) as an interferon-stimulated gene (ISGs) (25). The mammalian cGAMP is called 2′3′ cGAMP (contains one noncanonical 2′–5′ phosphodiester bond between G and A, and one canonical 3′–5′ phosphodiester bond between A and G) to distinguish from the bacterial cGAMP that is 3′3′ cGAMP (30–32). STING has ~10 times more affinity for 2′3′cGAMP than other cyclic dinucleotides (CDNs), and ancestral cGAS-STING pathway

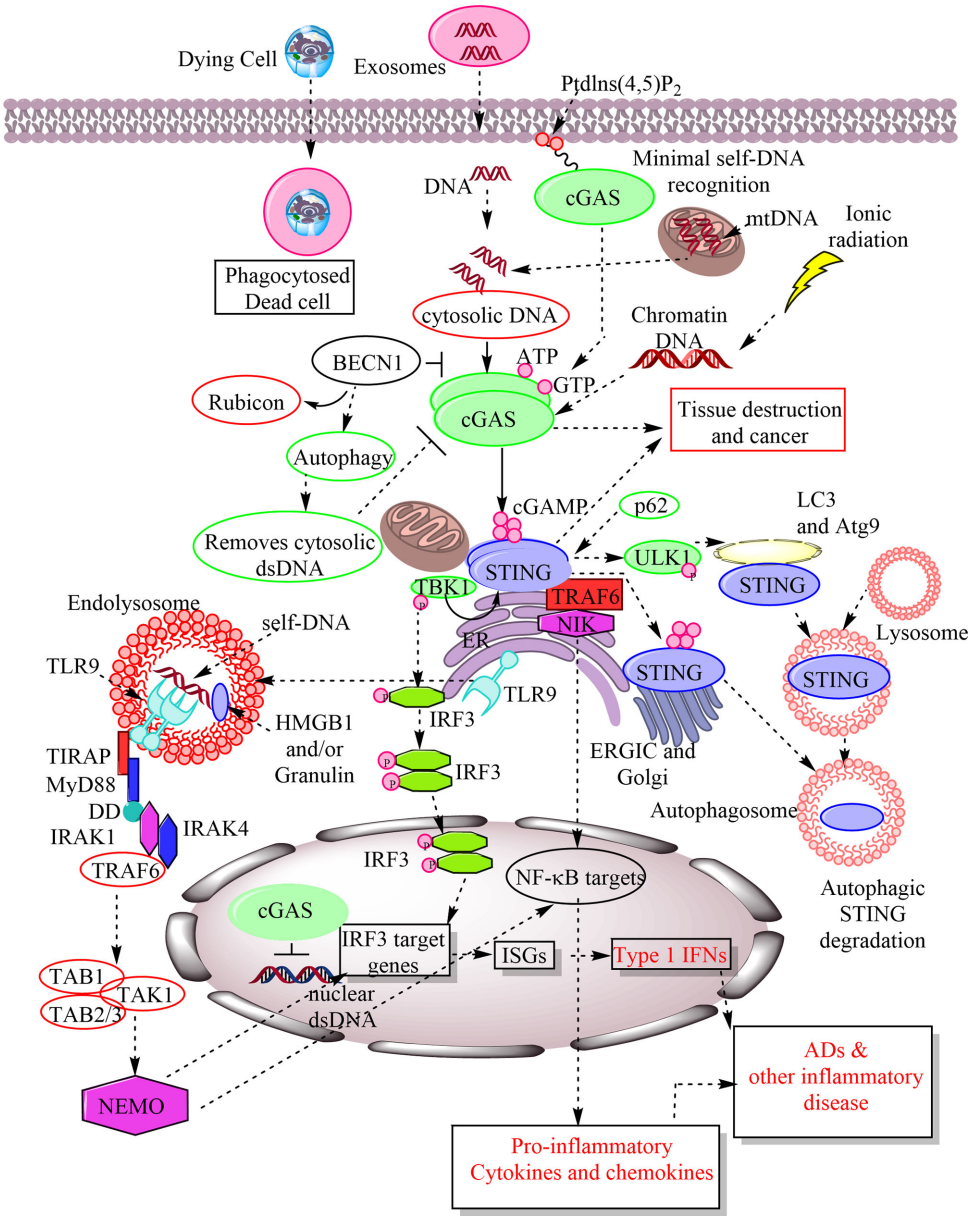


FIGURE 1 | Schematic representation of cGAS-STING and TLR9 signaling in response to the recognition of host-derived self-DNA as a DAMP. The entry of the self-DNA in the cytosol due to mitochondrial damage, nuclear damage, exosome-derived DNA, and the phagocytosis of dead cell does not remain as hidden from cytosolic PRRs. The cGAS identifies them as a potent DAMP in the cytosol and catalyze the cGAMP formation. The cellular exposure to the ionic radiations also induces cytosolic levels of chromatin DNA, which is also recognized by cGAS as a DAMP. The cGAMP is recognized by downstream signaling molecule called STING located in ER. STING activation phosphorylates TBK1 that further activates or phosphorylates IRF3. IRF3 stimulates IRF3 target genes, including ISGs, which also include type 1 IFN. Also, STING activation activates NF-κB target pro-inflammatory genes for cytokines and chemokines. Hence, cGAS-STING signaling plays a crucial role in inflammation, cancer, auto-inflammation and ADs. The cGAMP recognition by STING also induces its autophagosome-mediated degradation. The p62 is an endogenous negative regulator of the STING and induces its autophagic degradation. BECN1 is also an endogenous cGAS inhibitor and their interaction removes Rubicon (an autophagy inhibitor) from the BECN1 that induces autophagy to remove cytosolic DNA. Hence, autophagy serves to remove cytosolic DNA without inducing inflammatory damage. Failure of autophagy increases inflammatory recognition of cytosolic DNAs by different cytosolic PRRs. On the other hand TLR9 is present in the ER during resting stages as soon as cytosolic CpG DNAs or host DNA enter into the endosome or endolysosomes TLR9 also migrates there and recognizes them as a crucial DAMP. TLR9 activation induces MyD88-dependent downstream signaling pathway to activate IRF3-based type 1 IFN production and NF-κB-mediated pro-inflammatory cytokine generation to cause inflammation and inflammatory diseases. MyD88 has a TIR domain and death domain (DD). The TIR domain of MyD88 activates interleukin-1 receptor-associated kinase-4 (IRAK-4) and IRAK-1. IRAK-4 subsequently recruits TRAF6 to activate transforming growth factor-β associated kinase 1 (TAK1). TAK1 is linked to TRAF6 *via* TAB2 adaptor protein, whereas TAB1 adaptor protein interacts constitutively with TAK1 and induces TAK1 kinase activity. TAK1 then phosphorylates IκB kinase (IKK) complex through K63-linked ubiquitination of NEMO (NF-κB essential modulator), an IκB kinase regulatory subunit that is critical for the NF-κB, IRF3, and MAPK signaling.

in *N. vectensis* (starlet sea anemone of the phylum cnidaria diverged from humans around 600 MYA) also binds to 2′ 3cGAMP more preferentially than other forms, including 3′,3′ CDN produced in them (27, 33). The origin of cGAS-STING may date back to the origin of choanoflagellate (closest free-living unicellular and colonial flagellates relatives of metazoans), *Monosiga brevicollis* (34). Hence, the cGAS-STING evolution dates back to the origin of multicellularity, which is approximately 600 MYA (35). Despite, only 29% amino acid (AA) identity the crystal structure of nvSTING is identical to the human STING (hSTING) (36). Also, the STING homologs present in other invertebrates phyla, including *mollusca*, *annelida*, and *cnidarian*, have less than 30% AA identity with hSTING but bind robustly with 2′3′cGAMPs and 3′,3′CDNs (27). Hence, the CDN binding to the STING has remained conserved for more than 600 million years. However, it remains to discover the role of nvSTING in antiviral or antibacterial immune response induction except for autophagy induction that occurs independently of TANK (TRAF(TNFR-associated factor)-associated NF-κB activator)-binding kinase 1 (TBK1) activation (37).

The cGAMP binding translocates STING to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and Golgi in a process depending on the coat protein complex-II (COP-II complex, a set of highly conserved proteins responsible for creating small membrane vesicles originating from the endoplasmic reticulum or ER) and ADP ribosylation factor (ARF) GTPases (37, 38). The heterozygous missense mutations in coatomer protein subunit alpha (COPA, a subunit of coat protein complex-I or COP-I that mediates Golgi to ER transport) cause COPA syndrome (an autosomal dominant autoimmune dysregulatory disease, involving lungs and joints) overlapping clinically with the higher type 1 IFN levels due to the gain of function in the STING, even in the absence of its ligand (39, 40). Furthermore, surfeit locus protein 4 (SURF4) serves as an adaptor molecule to facilitate the COPA-mediated STING retrieval at the Golgi-complex. Thus COPA mediates maintenance of immune homeostasis *via* regulating the STING transport to the Golgi-complex and the dysregulation of COPA overactivates STING causing immune dysregulation in the COPA syndrome (39). Another study has also shown the interaction between COPA and STING, and the mutant COPA is responsible for the accumulation of ER resident STING at the Golgi-complex (41). Hence, ER-Golgi axis also controls autoinflammation and have a potential for therapeutic approaches in the COPA syndrome. The STING with ERGIC induces LC3 lipidation (a key step in the autophagosome formation) through a pathway that depends on the WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) and autophagy protein 5 (ATG5), but does not require Unc-51-like kinase (ULK) and vacuolar protein sorting 34 (VPS34)-beclin kinase complexes (37). The cGAMP-induced autophagy is crucial to clear cytosolic DNA and viruses. Thus nvSTING clears cytosolic self-DNA and viruses through autophagy without producing type 1 IFNs, indicating cGAS-STING signaling-dependent autophagy is a primordial function.

The *Drosophila melanogaster* or the common fruit fly STING called dSTING activates in response to the injected 2′3′-cGAMP and stimulates dSTING-regulated gene expression (42). The activation of immune cell deficiency (Imd) pathway in response to the viral pathogens activates the kinase dIKKβ and the transcription factor Relish, which are required for controlling the viral infection, including picorna-like viruses (43). The dSTING activation upstream of dIKKβ regulates the expression of the antiviral factor called Nazo, means enigma in Japanese (43). Hence, antiviral action of dSTING in *D. melanogaster* occurs independently of type 1 IFN production that indicates its evolutionarily conserved and ancient role in the antiviral immunity. Also, the 2′3′-cGAMP co-injection with a panel of DNA and RNA viruses in *D. melanogaster* results in the substantial decrease in the virus replication and even *D. melanogaster* lacking Atg7 and Argonaute RISC (RNA-induced silencing complex) Catalytic Component 2 (AGO2) genes (encoding autophagy and small interfering RNA pathways) also show protection against viruses upon 2′3′-cGAMP injection (42). However, *D. melanogaster* with mutations in the gene encoding the NF-κB transcription factor Relish does not show any protection against viral infections upon treatment with 2′3′-cGAMP. Also, in silkworm (*Bombyx mori*) cells cGAMP production occurs upon infection with nucleopolyhedrovirus (NPV) that is recognized by the BmSTING (44). The BmSTING deletion inhibits the antiviral immune response in the silkworm larvae due the inhibition of cleavage and nuclear translocation of BmRelish. The caspase-8-like protein (BmCasp8L) interacts with BmSTING and suppresses the BmRelish activation in the absence of cGAMP as cGAMP decreases the BmCasp8L binding to the BmSTING and increases BmRelish activation (44). The death-related ced-3/Nedd2-like caspase (BmDredd) and BmSTING interaction promotes BmRelish cleavage for efficient antiviral immune response to protect the insect cells from viral infection (44). However, upon infection with a spore forming fungus called *Nosema bombycis* the BmSTING induces microtubule-associated protein 1 light chain 3 (LC3)-mediated autophagy to protect the host (45). Hence, dSTING and BmSTING activation regulates NF-κB-mediated antiviral immune response predating the emergence of IFNs in the vertebrates. Also, the dSTING works in the mammalian cells and induces NF-κB activation (46). Of note, dSTING does not require cGAS ortholog to activate innate immune signaling pathway in *D. melanogaster*.

The *Danio rerio* or zebrafish STING (zSTING) is also capable of inducing an antiviral immune response against DNA viruses due to the presence of a conserved serine residue (S373) (47). This recognition is independent of cGAS in the zebra fish but requires zDHX9 (a Zebrafish RNA helicase) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (zDDX41, a member of DExD/H-box helicases superfamily that recognizes cytosolic DNA) to sense DNA viruses, including herpes simplex virus-1 (HSV-1) (47). The zDDX41 also contributes to the zSTING-zSTAT6-mediated chemokine (zCCL20) production *via* its DEADc domain (48). The zDDX41 is a trafficking protein that resides in the nucleus in resting cells and moves to the cytosol upon

stimulation of cells with the cytosolic DNA. The zDDX41 serves as an initiator for the NF-κB and IFN signaling pathways activation in a zSTING-dependent manner through its DEADc domain (48). This signaling pathway protects the zebrafish from bacterial (*Aeromonas hydrophilia* or *Edwardsiella tarda*) and viral infections *via* inducing innate immunity. The C-terminal tail (CTT) of the STING has only evolved in vertebrates that is critical for TBK1 recruitment and IRF3 phosphorylation or activation (49–51). The STING CTT is an unstructured stretch of ∼40 AAs, which has sequence motifs crucial for STING phosphorylation and IRF3 recruitment (52). The human STING residue S366 serves as a primary TBK1 phosphorylation site, which is a part of the *LxIS* motif shared between innate immune adaptor proteins that activates IFN signaling (49, 50, 53). The hSTING CTT also contains a second *PxPLR* motif with a L374 residue crucial for TBK1 binding (50, 54). The *LxIS* and *PxPLR* sequences are highly conserved in all vertebrate STING alleles and serve as IRF3 and TBK1 binding sites/motifs respectively.

The zebrafish STING CTT contains a further extension that is absent in human and other mammalian STING alleles (52). The CTT of the zSTING and *Salmo salar* (Salmon) STING inverts the typical vertebrate signaling response (IRF3-dependent type 1 IFN production) and results in the dramatic (100-fold higher) NF-κB activation and weak IRF3-IFN signaling *via* recruiting TRAF6 (52). Thus, removal of CTT from zSTING prevents NF-κB activation. Hence, zebrafish CTT module is sufficient to reprogram hSTING to activate NF-κB signaling mainly along with immune activation in macrophage cells. The zSTING CTT module can mediate hyperactivation of the IRF3 reporter signaling, only in the presence of hSTING IRF3 binding module (52). This indicates that the cross-talk between individual CTT modules may affect the overall STING signaling. The STING allele from the most primitively diverged vertebrate lineage called *Callorhinchus milii* (Ghost shark) contains humans-like CTT and does not induce heightened NF-κB activation (52). Hence, the STING-dependent IRF3-IFN and NF-κB signaling depends on independent modules in the CTT, which can be gained or lost to balance downstream immune activation. Some amphibians, including *Xenopus tropicalis* (western clawed frog) and *Xenopus laevis,* have lost the CTT domain in the time of evolution (34). However, *X. tropicalis* STING can bind 2′3′cGAMP without inducing any functional response, including immune response (27). The ability of STING to bind CDNs has remained conserved throughout metazoans and antedate the emergence of IFNs and "modern" innate immunity (55). Hence, cGAS-STING signaling pathway has evolved to protect the host *via* different mechanisms (autophagy, type 1 IFN production, and NF-κB activation) about 600 MYA and remained conserved.

cGAS-STING Signaling in Response to the Self-DNA, its Regulation, and Impact on the Immune Response

The cGAS exists in the cells in three forms, including the cell membrane bound cGAS, freely floating in the cytosol, and in the nucleus (56). However, a study has identified that the cGAS exists predominantly as a nuclear protein independently of cell cycle phase or cGAS activation status (57). The nuclear cGAS tightly tethers to the nucleus through a salt-resistant interaction that does not require the domains crucial for the cGAS activation, but needs intact nuclear chromatin (57). The single amino acid (AA) mutation in the tethering surface of the cGAS renders it massively and constitutively active against the self-DNA. Thus the tight nuclear tethering of the cGAS maintains its resting stage and prevents autoinflammatory and autoimmune diseases. The cGAS binds to the cytosolic plasma membrane through its N-terminal phosphoinositide-binding domain that recognizes the phosphatidylinositol 4,5 biphosphate (PtdIns(4,5) P2) or PIP2, a membrane lipid (58). The mutant cGAS lacking N-terminal phosphoinositide-binding domain does not bind to the plasma membrane and moves to the cytosolic and nuclear compartments, and induces a potent immune response in response to the genotoxic stress. However, this mutant cGAS induces a weaker type 1 IFN response to viruses, including modified vaccinia Ankara (a dsDNA virus) (58, 59). The cGAS binding to the plasma membrane serves as a mechanism to prevent cGAS-binding to the cytosolic self-DNA to prevent the generation of self-destructive immune response but enhances the recognition of invading viruses. The cGAS binding to the plasma membrane varies from cell to cell, for example, non-phagocytic cells have larger cytosolic pool of cGAS than phagocytic cells (Macrophages, neutrophils, and DCs) (58, 60). This indicates the context-specific regulation of the cGAS distribution in the cellular environment.

The cGAS optimally recognizes dsDNA equal to or longer than 36 bp to initiate cGAS-STING-mediated signaling to produce type 1 IFNs and other NF-κB-dependent cytokines independent of the sequence (61–63). For example, dsDNAs with 12 bp do not activate mouse cGAS (mcGAS) efficiently (63). Similarly, dsDNAs with 16 bp can also bind to cGAS but do not induce STING activation efficiently. However, dsDNA with 18 bp can induce cGAS-STING activation in comparison to the salmon sperm DNA, a routinely used dsDNA to study the immune response in transfected cells (63). The longer dsDNAs with 20 bp have comparable activity to the salmon sperm DNA. The cGAS-dsDNA interaction involves electrostatic interactions and hydrogen (H) bond formation (63). Most of these interactions occur with bp 2 and 12 of the dsDNA. The two dsDNA binding sites (site A and site B) of cGAS are involved in these interactions, and site B is much more important than site A in this cooperative binding (63). The maximum length of the dsDNA that can stimulate cGAS activation comprises of more than 200 bp in the presence of high mobility group box protein 1 (HMGB1) and mitochondrial transcription factor A (TFAM) through inducing U-turn or curvature (64). ZCCHC3, a CCHC-type zinc-finger protein also serves as a positive regulator of cGAS *via* acting as a co-sensor and directly binding to the dsDNA that enhances cGAS binding to the dsDNA (65).

The cGAMPs or cGMP-AMPs are cyclic dinucleotides (CDNs, which were first described in bacteria), serve as second messengers during cGAS-mediated recognition of cytosolic

dsDNA recognition and type 1 IFN secretion signaling pathway (16, 66). Along with activating IRF3-dependent type 1 IFN production, STING activation is also involved in the NF-κB, MAPK, and STAT6 (signal transducer and activator of transcription 6) activation, and stimulating autophagosome formation through activating LC3 puncta formation due to its co-localization with it and autophagy-related protein 9a (Atg9a, a multi-spanning membrane protein crucial for autophagy) upon recognizing cytosolic dsDNA (**Figure 1**) (49, 67–69). The deficiency or loss of Atg9a impairs innate immune response due to the enhanced assembly of STING and TBK1 (69). Hence, Atg9a also controls the STING-dependent signaling pathway in response to the cytosolic dsDNA. On the other hand, Beclin-1 (BECN1) interacts with cGAS to inhibit cGAMP formation in response to the cytosolic dsDNA *via* blocking their (cGAS and dsDNA) interaction (**Figure 1**) (70). The cGAS-BECN1 interaction releases Rubicon (a negative regulator of autophagy) from BECN1 that activates phosphoinositide 3-kinase (PI3K) class III activity to induce autophagy, which removes cytosolic dsDNA (**Figure 1**).

The phosphorylated p62/SQSTM1 inhibits the STING *via* directing the ubiquitinated STING to the autophagosome (**Figure 1**) (71). Cells deficient in p62 are unable to degrade STING, and overwhelming type 1 IFN production along with other NF-κB-dependent pro-inflammatory cytokines production takes place. Thus STING activation and p62 phosphorylation (responsible for STING degradation) occur to regulate exaggerated cGAS-STING activation in response to the cytosolic dsDNA. It will be interesting to discover factors decreasing or inhibiting p62 levels or phosphorylation in diseases associated with increased cGAS-STING-dependent type 1 IFN production. The STING activation also activates autophagy in a TBK1-independent manner that involves the translocation of the cGAMP bound STING to the ERGIC and the Golgi in a COP-II complex and ARF GTPase-dependent process (37). The ERGIC with STING acts as a source for LC3 lipidation that is a crucial step in autophagosome formation (**Figure 1**). The LC3 lipidation involves cGAMP bound to STING that comprises a WIPI2 and Atg5-dependent pathway without the involvement of ULK and VPS34 (a class III phosphoinositide 3-kinase or PI3K)-Beclin kinase complex (37).

Autophagy-related protein 16 like 1 (ATG16L1) has two distinct membrane-binding regions known as a N-terminal membrane-binding amphipathic helix involved in the LC3B lipidation and C-terminal membrane-binding region dispensable for canonical autophagy but crucial for VPS34-independent LC3B lipidation at perturbed endosome (72). The ATG16L1-C-terminus can compensate WIPI2 deletion to sustain lipidation during starvation (72). However, the C-terminal membrane-binding region is present only in the β-isomer of ATG16L1, indicating that ATG16L1 isoforms mechanistically differentiate between different LC3B lipidation mechanisms (72). The STING-mediated LC3B lipidation occurs onto single-membrane perinuclear vesicles mediated by ATG16L1 through its WD40 domain, which bypasses the requirement of canonical upstream autophagy machinery (73, 74). The WD repeat-containing C-terminal domain (WD40 CTD) of ATG16L1 is crucial for LC3 recruitment to endolysosomal membranes during non-canonical autophagy, but not for canonical autophagy (74) Bafliomycin A1 inhibits the vacuolar ATPase (V-ATPase) through binding to it. A bacterial product SpoF also inhibits V-ATPase *via* catalytically modifying it to prevent LC3B lipidation *via* ATG16L1 (73). Thus cGAS-STING signaling also induces V-ATPase-dependent LC3B lipidation to mediate cell-autonomous host defense that is different from LC3B lipidation onto double-membrane autophagosome (73).

The cGAMP-induced STING stimulation mediating autophagy, but no IFN production has been seen in *N. vectensis*, indicating that the autophagy induction through STING is primordial cGAS-STING signaling (37). However, during *Mycobacterial tuberculosis* infection, ubiquitin-mediated autophagy forms autophagosomes that degrades bacteria in response to the cGAS-mediated recognition of the bacterial DNA *via* STING-induced TBK1 activation (75). Thus the cGAS-STING signaling and autophagy induction impact each other positively and negatively may be depending on the qualities and properties of stimulating DNA or other host factors remaining to identify. The STING-dependent type 1 IFN production involves its translocation from the ER to the endosome that occurs through phosphorylation of the specific tyrosine residue (Y245) in the STING by the epidermal growth factor receptor (EGFR) (76). In the absence of STING phosphorylation through EGFR, it moves to the autophagosome where it degrades and no IRF3 activation dependent type 1 IFN production occurs as seen *in vitro* and in mice (76). Hence, EGFR tyrosine kinase regulates cGAS-STING signaling-dependent type 1 IFN production through STING phosphorylation and promoting its translocation from the ER to the endosome. Failure of this inhibits STING-dependent IFN production.

The extracellular nucleosomes ingested by cells also become a target for cGAS and have a high biding capacity for it but have lower activation potential to produce type 1 IFNs and other cGAS-STING-dependent cytokine production (17). The nucleosome recognition by cGAS may play a role in autoimmune and autoinflammatory diseases along with aggravating other inflammatory conditions. Of note, cGAS can dimerize even in the absence of dsDNA due to its intrinsic capacity to dimerize and behaves like a classic allosteric enzyme (61). Along with the cytosol, cGAS is also present in the nucleus, where chromatin tethering suppresses its activity against the self-DNA (77). The cGAS interacts with the nucleosome core particle with a nanomolar affinity through its two conserved arginine (Arg) molecules to anchor nucleosome acidic patch (comprised of histone 2A (H2A)-H2B dimer and nucleosomal DNA) that is involved in recognizing and binding to the dsDNA (78, 79). The cGAS extensively contacts with both the acidic patch of the H2A-H2B heterodimer and the nucleosomal DNA (80). Also, the cGAS engages the second nucleosome in trans. The cGAS uses two conserved arginines (Arg) to anchor nucleosome acidic patch formed by the H2A-H2B heterodimer *via* dsDNA-

binding site B in both complexes (1:1 and 2;2 cGAS-dsDNA complexes), and could interact with the DNA from the other symmetrically placed nucleosome *via* the dsDNA-binding site C in the 2:2 complex (77, 81). Hence, all the three binding sites of cGAS required for self-dsDNA are not available to form the active 2:2 cGAS-dsDNA state that prevents cGAS dimerization (77, 82). The R236A or R255A mutation of the cGAS impairs its binding to the nucleosome and relives the nucleosome-mediated cGAS inhibition (81). Hence, cGAS is unable to recognize self-dsDNA inside the nucleus due to its interaction with nuclear histones (key constituents of chromatin) that prevents the onset of autoimmune or autoinflammatory diseases in response to the recognition of self-dsDNA inside the nucleus (**Figure 1**) (83). The biallelic mutations in LSM11 (U7 small nuclear RNA associated protein) and RNU7-1 (U7 small nuclear 1) encoding components of the replication-dependent histone pre-mRNA-processing complex have been detected in genetically uncharacterized cases of type I interferonopathy, Aicardi-Goutières syndrome (AGS) (83). These patients also show the altered cGAS distribution and activation in response to the chromatin lacking linker histone. However, cGAS in the nucleus interacts with the replication fork proteins in a DNA-binding manner and slows it down independent of STING to prevent the replication stress (84). Thus cGAS-deficient cells are highly sensitive to radiation and cancer therapeutics. The nuclear cGAS bound to the chromatin promotes tumor growth through suppressing homologous-recombination-mediated repair required for DNA repair (85, 86). Thus under genomic stress nuclear cGAS bound to the chromatin potentiates the genomic destabilization, micronucleus formation, and cell death independent of the STING activation (86). Hence, cGAS targeting may serve as a potential target for anticancer therapies. Thus *via* acting as a decelerator of DNA replication forks, the nuclear cGAS suppresses replication-associated DNA damage that can efficiently target to exploit genomic instability of cancer cells (84). In addition to STING-independent genomic stability, the cGAS-dependent activation of STING/TBK1/IRF3 promotes p21 or cyclin-dependent kinase inhibitor (CDKI) in the nucleus reduces micronucleus formation, delays G2/M transition, and maintains chromosomal stability (87). Hence, cGAS maintains genomic stability through both STING-dependent and independent mechanisms. Also, during mitosis-dependent cell division, the dsDNA moved in the cytosol (due to mitotic nuclear envelope break down or NEBD) escapes cGAS-dependent recognition due to its phosphorylation at S305 (in human cGAS) and S291 (in mouse cGAS) sites in response to the mitotic kinase CDK1-cyclin B complex (88). As soon as mitosis finishes, the phosphorylated cGAS gets dephosphorylated in response to the type 1 phosphatase PP1 to continue its dsDNA sensing function. Further study has shown the absence of cGAS activation along with the STING activation by the vesiculated Golgi in response to the self-DNA during mitosis and the introduction of the foreign DNA (89). However, during HIV-1 infection NONO (Non-POU Domain Containing Octamer Binding) protein binds to its capsid and activates cGAS signaling along with inducing cGAS association with the HIV DNA in the nucleus (90). Hence, nuclear cGAS can recognize viral DNA to initiate type 1 IFN production and pro-inflammatory immune response but is not available for host genomic DNA. Of note, NONO protein directly binds to the HIV-2 (weakly pathogenic) capsid with a higher affinity than the highly pathogenic HIV-1. The DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs) encoded by the missense mutations of protein Kinase, DNA-activated, catalytic Subunit (PRKDC) are associated with autoimmune diseases due the overactivated enzymatic activity of the cGAS (91, 92). However, these patients exert an enhanced antiviral immune response. On the other hand, an acetyltranferase called KAT5 serves as a positive regulator of cGAS *via* catalysing the cGAS acetylation at several lysine residues in its N-terminal domain that promotes its DNA-binding ability (93).

Studies have shown that cGAS-STING signaling in response to the chromatin self-DNA is associated with a senescence phenotype and its (cGAS) deletion in the murine embryonic fibroblasts increases their spontaneous immortalization, and also abrogates associated senescence-associated secretory phenotype (SASP) or that is induced by DNA-damaging agents, including etoposide and radiation (94). The cytoplasmic chromatin-cGAS-STING pathway promotes SASP in primary human cells and in mice (95). The cGAS-mediated SASP production activating STING promotes senescence in a paracrine manner following irradiation and oncogene activation (96). Thus during conditions (exposure to ionic radiations) responsible for cellular senescent, the chromatin DNA in the cytosol induces cGAS-STING signaling to cause short-term inflammation for restraining activated oncogenes that promote tissue destruction and cancer (**Figure 1**) (95). The chromatin DNA recognition by cGAS-STING signaling pathway during senescence promoting conditions occurs due to the defective DNA damage response (DDR) signaling in response to the dysfunctional telomerase activity that creates a preponderance of chromatin fragments in the cytosol (97). This process occurs independently of telomerase shortening through cGAS-mediated recognition of cytosolic chromatin DNA. The cGAS-STING signaling inhibits this premature senescence and progression towards cancer. Hence, the activity of cGAS-STING signaling in both cytosol and nucleus is a highly controlled process, and any impairment may predispose the host to severe autoinflammatory or autoimmune diseases that may also develop different cancers.

The cGAS serves as a potent PRR for the recognition of cytosolic dsDNA in both plasmacytoid DCs (pDCs) and conventional DCs (cDCs), indicating their role in the generation of the potent T-cell-mediated immune response and the B cell-mediated Ab generation (20). In addition to this, STING also directly impacts adaptive immunity as its deficiency promotes the marginal zone B cell development and differentiation *via* activating B cell receptor (BCR) signaling (98). STING positively regulates SHIP-1 (SH2-containing inositol 5′ polyphosphatase-1, that is required for B cell tolerance to self-antigens and dampens naïve and low-dose antigen-primed B cells) activation, but negatively regulates CD19 (a 95 kDa type 1 transmembrane protein of immunoglobulin superfamily that establishes a threshold for intrinsic B cell signaling *via*

modulating BCR-dependent and independent signaling) and Bruton's tyrosine kinase (Btk, essential for B cell development and function of mature B cells downstream to the BCR signaling) (98–101). The BCR activation in the STING$^{-/-}$ B cells increases Wiskott-Aldrich syndrome protein (WASP, which activates downstream to the BCR signaling, links receptor signaling to the actin dynamic through actin-related proteins-2/3 (Arp2/3) complex, and also controls BCR mobility during activation) activation and F-actin accumulation *via* PI3K used by CD19-Btk axis as a central hub (98, 102). Thus, STING regulates B cell function *via* feedback from actin reorganization, indicating the positive impact of the STING on B cell function. On the other hand, STING activation in T cells induces their apoptosis, and its inhibition by Notch signaling prevents it during sepsis as T cells undergo apoptosis during sepsis (103, 104). The notch intracellular domain interacts with STING at its CDN binding site or domain that blocks the binding of the cytosolic pathogenic CDN generated by cGAS. Hence, recognition of cytosolic self-dsDNA by cGAS activates STING in CD4^{+}T cells to induce their apoptosis.

The homeostatic regulation of STING involves TOLLIP (Toll-interacting protein that is an endogenous negative regulator of TLR signaling) as a stabilizer during a resting stage as its deficiency reduces STING levels in non-hematopoietic cells and tissues (105). The removal of TOLLIP from STING upon treatment with polyQ proteins *in vitro* or endogenous polyQ proteins in Huntington's disease (HD) mouse striatum dampens cGAS-STING signaling (105). The TOLLIP deficiency in immune cells makes STING highly unstable, therefore do not produce cGAS-STING dependent type 1 IFNs in response to the cytosolic-dsDNA. On the other hand, inositol-requiring transmembrane kinase/endoribonuclease 1α (IRE1α, transduces signal of the misfolded protein accumulation in the ER called ER stress to nucleus as unfolded protein response or UPR) and lysosomes are responsible for STING degradation (105). Also, the TOLLIP deletion decreases the STING-dependent autoimmune disease in three prime repair exonuclease 1 knockout (KO) or Trex1$^{-/-}$ mice. Thus TOLLIP serves as a STING stabilizer in resting cells that keeps a tug of war fight with its degrader IRE1α-lysosome (105). The STING signaling also plays a crucial role in sepsis, and its severity, including septic shock through different mechanisms, including increased type 1 IFN release, cell death, and impaired autophagy as autophagy has a protective role against sepsis (106, 107). The details of autophagy during sepsis have been discussed somewhere else (107). Furthermore, STING activation increases the severity of abdominal sepsis as its increased levels have been seen in circulating peripheral blood monocytes and intestinal biopsies (108). Even the STING expression in the human intestinal lamina propria of sepsis patients well correlates with the intestinal inflammation, higher circulating intestinal fatty acid-binding protein indicating enterocyte death or damage. The wild type (WT) mice subjected to the cecal-ligation and puncture (CLP)-induced sepsis show increased systemic inflammation, gut permeability, translocation of the bacteria due to the death of enterocytes in response to the recognition of the cytosolic mtDNA as STING$^{-/-}$ mice have alleviated inflammatory immune response and decreased bacterial translocation (108). Another study has shown the protective action of STING inhibition form lethal sepsis (109). Hence, mtDNA-STING signaling inhibition may serve as a novel therapeutic approach for sepsis.

Different Negative Regulators of cGAS-STING Signaling Pathway

Various host-derived endogenous negative regulators of cGAS-STING signaling, including post-translational modifications, have been described somewhere else (20, 59). Protein phosphatase 6 catalytic subunit (PPP6C) of protein phosphatase 6 (PP6) acts as a binding partner of Kaposi's sarcoma-associated herpesvirus (KSHV) open reading frame 48 (ORF48) and also serves as a negative regulator of the cGAS-STING pathway (110). The PPP6C deletion enhances the dsDNA-induced and 5′ppp dsRNA-induced but not poly (I:C)-induced innate immune responses. PPP6C negatively regulates dsDNA-induced IRF3 activation through directly interacting with STING to prevent its phosphorylation but does not affect NF-κB activation (110). The PPP6C deficiency suppresses the HSV-1 and vesicular stomatitis virus (VSV) replication, and the KSHV reactivation, due to increased type I IFN production. PPP6C deficiency may also promote ADs in response to the overactivated cGAS-STING signaling-dependent cytokines and type 1 IFN production. Barrier-to-autointegration factor 1 (BAF) also serves as a natural competitor for cGAS activity for the genomic self-DNA and prevents the interaction of cGAS with the nuclear DNA by displacing the transiently bound cGAS monomers from dsDNA (111). Also, BAF limits the cGAS interaction with chromatin after nuclear envelop (NE) rupture in living cells that is consistent with the competition for DNA binding. BAF serves as a natural inhibitor of cGAS, both in the cytosol and nucleus. The loss of this negative regulation of cGAS may predispose the host to autoinflammatory or autoimmune diseases.

Immunity-related GTPase M (IRGM) also serves as a negative regulator of cGAS-STING signaling *via* interacting with cGAS to facilitate its p62-dependent autophagic degradation (112). IRGM serves as a master regulator of type 1 IFN production as IRGM$^{-/-}$ mice and cells express higher levels of ISGs through over activated nucleic acid sensing pathways (cGAS-STING signaling and retinoic acid-inducible gene 1 (RIG-1)-mitochondrial antiviral signaling protein (MAVS) signaling pathways) and defective mitophagy causing accumulation of defunct leaky mitochondria releasing mitochondrial DNA (mtDNA) and reactive oxygen species (ROS) in the cytosol (112). Hence, IRGM maintains IFN homeostasis and protects the host from autoimmunity. Additionally, human cytomegalovirus (HCMV) tegument protein pp65 or pUL83 also inactivates cGAS without affecting STING to dampen the type 1 IFN response (113). Different mammalian viruses, including DNA virus in the *Poxviridae* family encode poxvirus immune nucleases (Poxins), which cleave 2′3′-cGAMP and serve as cGAMP nucleases to inhibit cGAS-STING signaling pathway

through inhibiting STING activation (114, 115). Poxins cleave 2′ 3′-cGAMP through metal-independent cleavage of the 3′–5′ bond, converting 2′,3′-cGAMP into linear Gp[2′–5′]Ap[3′] (115). Furthermore, poxin homologs with 2′3′-cGAMP cleaving activity are also present in the genomes of moths and butterflies and the baculoviruses, infecting them. Hence, poxins are ancient negative regulators of cGAS-STING signaling pathway.

The Myb-like, SWIRM, and MPN domains 1 protein (MYSM1, a metalloprotease, which deubiquitinates the K119-monoubiquitinated form of the H2A) is another cGAS-STING negative regulator that upregulates after viral infection and intracellular DNA stimulation (116, 117). MYSM1 is also called 2A-deubiquitinase (2A-DUB) or KIAA1915 that is specific for monoubiquitinated H2A (uH2A) (117). MYSM1$^{-/-}$ mice show a hyper-inflammatory immune response, acute tissue damage, and higher mortality than WT mice upon virus infection. The peripheral blood monocytes (PBMCs) isolated from SLE patients show a decreased MYSM1 expression but a higher production of type 1 IFNs. MYSM1 interacts with the STING and cleaves STING K63-linked ubiquitination to suppress cGAS-STING signaling pathway (116). In addition to the cGAS-STING signaling inhibition, MYSM1 also dampens nucleotide-binding oligomerization domain-containing protein 2 (NOD2), or caspase recruitment domain-containing protein 15 (CARD15) or inflammatory bowel disease protein 1 (IBD1)-mediated inflammation through inactivating receptor interacting protein 2 (RIP2, a proximal adaptor protein) complex that prevents the NOD2:RIP2 complex formation crucial for the inflammatory signaling pathway (118). MYSM1 selectively removes K63, K27, and M1 chains from RIP2 to prevent the NOD2:RIP2 complex formation crucial for inflammatory signaling. MYSM1 does not removes K48 polyubiquitin chians from RIP2. The MYSM1$^{-/-}$ mice show unrestrained NOD2-mediated peritonitis, systemic inflammation, and hepatic inflammatory damage (118). Hence, MYSM1-based therapeutics may prove beneficial in cGAS-STING-based autoimmune diseases (SLE and IBD). Thus, these endogenous or different pathogen-derived molecules negatively targeting cGAS-STING signaling have a potential to target these innate immune mechanisms in different inflammatory diseases as described in following sections. However, further studies depending on the race, genetics, and sex of patients, are crucial before using cGAS-STING modulators in different cancers.

cGAS-STING Signaling in Sterile Inflammatory Conditions or Diseases, Including Autoimmunity

The cGAS-STING signaling induced through the self-dsDNA plays a crucial role in various sterile inflammatory diseases, including ataxia-telangiectasia (AT, its patients are more prone to develop cancer), non-alcoholic steatohepatitis (NASH) or fatty liver disease (NAFLD) *via* recognizing mtDNA as a potential DAMP in Kupffer cells of the liver), the chronic exposure of STING activator (5,6-dimethylxanthenone-4-acetic acid or DMXXA) also induces NASH or NAFLD in WT mice (20, 119, 120). Also, the liver tissues of patients with NASH or NAFLD show a higher STING expression than a control group that promotes liver inflammation fibrosis (121). The increased cGAS-STING signaling is also associated with alcohol-related liver disease (ALD) *via* activating IRF3-depending type 1 IFN production (122, 123).

The cGAS-STING signaling is also involved in high-fat diet-induced obesity as deleting STING in mice protects them (124). The cGAS-STING pathway activation in adipocytes in response to the mitochondrial stress-induced mtDNA activates phosphodiesterase PDE3B/PDE4 that decreases cAMP levels and PKA signaling, which reduces thermogenesis. Also, the mtDNA damage in endothelial cells during a high-fat diet containing palmitic acid (PA) activates cGAS-STING signaling that activates IRF3, which induces intercellular adhesion molecule-1 (ICAM-1) expression inducing monocyte endothelial cell interaction/adhesion causing adipose tissue inflammation, obesity, inflammation, glucose intolerance, and insulin resistance (125). Also, PA-induced cGAS-STING activation inhibits Hippo-Yes-associated protein (YAP) activation, upregulates mammalian Sterile 20-like kinases 1 (MST1) that inhibits angiogenesis (126). Hence, cGAS-STING inhibition has the potential to decrease obesity-associated inflammation, type 2 diabetes mellitus (T2DM), NASH/NAFLD, defective wound healing, and angiogenesis. We need further studies in the field. However, obese people have an advantage over lean people in terms of their immune response against 23-valent pneumococcal vaccination due to the STING activation (127).

STING activation also plays a crucial role in bronchopulmonary dysplasia (BPD) in preterm infants (a lung inflammatory conditions induced due to hyperoxia) due to an increase in the long-non coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) that interacts with the cAMP response element-binding protein (CREB) to increase its transcription (128). MALAT1 increases HMGB1 concentration that may further activate cGAS-STING signaling by increasing the curvature of dsDNA that increases its binding potential to cGAS (129). Hence, cGAS-STING pathway inhibition may serve as a new therapeutic approach. However, further studies are required. Activation of STING increases liver perfusion injury, but in aged animals subjected to ischemia-reperfusion, it also activates NLRP3 inflammasome to further enhance the tissue inflammation *via* aggravated IL-1β and IL-18 release along with other pro-inflammatory cytokines (TNF-α and IL-6) (130). Hence, age also affects STING-mediated inflammatory diseases. However, cGAS-induced autophagy independent of STING activation protects from ischemia/reperfusion-induced liver injury (131). Hepatocytes do not express STING under normoxic conditions or after anoxia/reoxygenation. Histone deacetylase 3 (HDAC3) inhibition blocks ischemia-reperfusion-induced brain injury by inhibiting the microglial cGAS-STING signaling pathway (132). The cGAS-STING signaling activates in response to the cytosolic dsDNA, and HDAC3 promotes cGAS transcriptional expression in microglia during ischemia/reperfusion-induced brain injury.

Another study has also shown that inhibiting cGAS activity with its antagonist A151 protects mice from ischemia–reperfusion–induced brain injury or experimental stroke (133). The A151 treatment decreases the cGAS expression, AIM2 inflammasome, and pyroptosis-related molecules, including caspase 1 (CASP-1), gasdermin D (GSDMD), IL-1β, and IL-18. Hence, cGAS-STING signaling also plays a crucial role in ischemia/reperfusion-induced brain injury.

STING also plays a crucial role in systemic lupus erythematosus (SLE) in response to the recognition of self-DNA *via* LYN interaction and phosphorylation to induce conventional DC (cDC) maturation and plasmacytoid DC (pDC) differentiation (134). The oxidized mtDNA released during the process of NETosis (neutrophil extracellular traps or NETs formation) also stimulates cGAS-STING signaling during SLE that further aggravates the disease (20, 135, 136). The transmembrane protein 203 (TMEM203, a conserved transmembrane protein) is an intracellular regulator of STING-mediated signaling, which interacts, cooperates, and co-migrates with STING to activate TBK1 and IRF3-dependent type 1 IFNs (137). Hence, inhibiting TMEM203 can inhibit cGAS-STING mediated cytosolic dsDNA recognition-based type 1 IFN signaling. Of note, TMEM203 is elevated in the T cells isolated from patients of SLE and is associated with the disease severity (137). The author has described the cGAS-STING signaling in response to the self-DNA somewhere else (20). Hence, STING may serve as a potential immunomodulatory target for SLE.

Mice lacking chromosome 9 open reading frame 72 (c9orf72) in their myeloid-cells show age-dependent lymphoid hypertrophy and autoinflammation as indicated by complete lack of c9orf72 gene due to the early activation of type 1 IFN signaling in DCs (138). Myeloid cells without c9orf72 show an increased STING activation upon exposure to the STING activators due to the decreased autolysosomal degradation of the STING in these cells. The STING inhibition in c9orf72$^{-/-}$ mice prevents inflammation, splenomegaly, and increased type 1 IFN production (138). Mice lacking c9orf72 are more susceptibility to the autoimmune disease called experimental autoimmune encephalitis (EAE), an animal model for multiple sclerosis (MS). Patients with familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) commonly have hexanucleotide repeat (GGGGCC) in the C9orf72 gene causing a decreased c9orf72 expression in the brain and peripheral blood cells (138). These patients have higher systemic type 1 IFN levels than sporadic patients of ALS and FTD due to overactive cGAS-STING signaling (in the absence of its negative regulator c9orf72) that can be reversed by the STING inhibitor.

The author has described the details of other autoinflammatory and autoimmune diseases, including type I IFN-dependent autoimmune disease, AGS, STING-associated vasculopathy with the onset of infancy (SAVI), erosive inflammatory arthritis (EIA), and psoriasis) affecting or linked with cGAS-STING signaling somewhere else (20). SAVI causes systemic inflammation characterized by vasculopathy, interstitial lung disease, ulcerative skin lesions, and premature death is an autoinflammatory disease caused by gain-of-function mutations in transmembrane protein 173 (TMEM173) gene that encodes STING (139, 140). The autosomal dominant mutations in the STING trigger IRF3 activation and subsequently upregulate ISGs in SAVI patients. However, the heterozygous STING N153S knock-in mouse model of the SAVI has shown the trigger of IRF-3-independent immune cell dysregulation and interstitial lung disease (141). Also, the mild upregulation of ISGs in STING N153S fibroblasts and splenocytes has been reported along with STING N154S SAVI patient fibroblasts. The STING N154S disrupts calcium (Ca^{2+}) homeostasis in T cells and prime them to become hyper-responsive to T cell receptor (TCR) signaling-induced ER stress and UPR, causing cell death (142). This effect is mediated by the novel region of the STING called the UPR motif. The pharmacological inhibition of the ER stress prevents the cell death among STING N153S positive T cells (142). The crossing between STING N153S positive and OT-1 mice (have MHC class I-restricted, ovalbumin-specific, $CD8^+$ T cells or OT-I cells) fully restores the $CD8^+$T cells and drastically improves STING-induced lung disease (142). Thus STING regulates Ca^{2+} homeostasis, ER stress, and T cell survival independent of IRF3 activation or IFN production.

Another novel gain-of-function G207E STING mutation has been reported with a distinct phenotype causing alopecia, photosensitivity, thyroid dysfunction, and symptoms of SAVI (143). The treatment with the Janus kinase 1 and 2 (JAK1/2) inhibitor baricitinib seems beneficial in these patients. Overactivated STING has been also shown in MHC-matched allogenic hematopoietic stem cell transplantation (aHSCT)-induced graft-versus-host disease (GVHD) (144). This GVHD can be prevented by the early treatment with STING inhibitor soon after aHSCT. However, STING has shown protective effect during in MHC-mismatched aHSCT-induced GVHD and acute intestinal injury (145). Thus STING activation during MHC mismatched aHSCT is protective to the host but becomes harmful during MHC-matched aHSCT. Further studies are warranted in this direction. A recent study has shown the beneficial effect of Lysyl-tRNA synthetase (LysRS) in STING-dependent inflammatory diseases *via* two complementary mechanisms (146). First, LysRS interacts with RNA : DNA hybrids to delay the cGAS-mediated recognition to impede the cGAMP synthesis and secondly, the RNA : DNA hybrids activate LysRS-dependent diadenosine tetraphosphate (Ap_4A) production, which attenuates STING-dependent signaling (146). Thus, LysRS and Ap_4A may serve as pharmacological targets to control STING overactivation and dependent inflammatory conditions.

Trex1$^{D18N/D18N}$ mice show an increased systemic inflammation and recapitulate many characteristics of human AGS and SLE due to the profound activation of cGAS-STING signaling and type 1 IFN production through T cells (147, 148). The cGAS deletion in Trex1$^{D18N/D18N}$ mice prevents the systemic and multiorgan inflammation, ISG production, autoAb production and aberrant T cell activation. The Trex1 is a DNA exonuclease that regulates radiotherapy-induced tumor immunogenicity *via* degrading the cytosolic DNA generated in

response to the radiotherapy (149). Also, patients with Bloom syndrome (BS, an autosomal recessive genetic disorder) lack or have a mutated BLM-RecQ-like helicase crucial for genome integrity or stability. Thus fibroblasts of BS patients have the increased accumulation of micronuclei that induces a constitutive upregulation of ISGs due to the overactivation of the cGAS-STING signaling-dependent IRF3 activation (150). BS patients also have low levels of Trex1 that increases cytosolic self-DNA and the ISG expression in BS fibroblasts. Hence, cGAS-STING signaling also plays a crucial role in the BS pathogenesis. The cGAS-STING signaling also come in action in response to the mtDNA released in the cytosol during the influenza virus infection (151). The mtDNA release in the cytosol during influenza virus infection involves viroporin activity of the influenza virus M2 protein in a MAVS-dependent manner (151, 152). However, the viral non-structural protein 1 (NS1) binds the cytosolic mtDNA and evades the cGAS activation. The cGAS expression increases in the patients with Huntington's disease (HD, a progressive brain disorder) in response to the elevated micronuclei present in the cytoplasm of the neurons, causing their inflammatory damage and altered autophagy (153). Hence, further studies will open new avenues to target cGAS-STING signaling in these inflammatory disease.

cGAS-STING Signaling in Cancer

During normal mitosis nucleosome (a basic repeating structural unit of eukaryotic chromatin, a single nucleosome comprises of 150–200 bp DNA wrapped around eight histone proteins) competitively inhibits cGAS activation in response to the dsDNA, and cGAS-STING signaling does not become fully functional (154). During the mitotic arrest, a low level of cGAS-STING signaling induces IRF3 phosphorylation and its accumulation that does not stimulate the type 1 IFN production but induces apoptotic cell death by alleviating Bcl-xL-dependent mitochondrial outer membrane permeabilization suppression (154). Taxol or paclitaxel (an anti-cancer agent) uses this mechanism for its anti-cancer action in mouse xenograft tumor models (154). Taxane also exerts the same effect on cGAS-STING signaling in non-small cell lung cancer (NSCLC). The cGAS-STING, TBK1, and IRF3 increasingly express in pan-cancer cells, and their gene expression level negatively correlates with their methylation in most cancer types (155). Even the high expression of cGAS-STING in some cancers decreases the inflammatory immune cell infiltration. Hence, their higher expression in some tumors well correlates with the poor prognosis. This study indicates the careful use of cGAS-STING modulators in tumor therapy, including adjuvants in tumor immunotherapies in clinics. We need further studies in the direction. The low cGAS expression in human lung adenocarcinoma patients is associated with high mortality (94). For example, STING activation in NSCLC predicts features of immunotherapy, and cisplatin treatment enhances it (156). The tumor with low STING and immune gene expression shows a high frequency of serine-threonine kinase 11 (STK11) mutation. The treatment with cisplatin increases cGAS-STING signaling and programmed death ligand-1 (PDL-1 or CD274 or B7 homolog 1) expression in different NSCLC preclinical models (156).

The activation of the STING signaling pathway in small cell lung cancer (SCLC) also enhances the protective effects of immunotherapy (157). Another study has indicated that the cGAS-STING signaling pathway inhibition due to upregulated nuclear paraspeckle assembly transcript 1 (NEAT1) in the lung cancer cells and tissues (158). NEAT1 interacts with DNA (cytosine-5)-methyltransferase 1 (DNMT1) to inhibit tumor suppressor p53 and cGAS-STING expression. The NEAT1 inhibition suppresses the lung cancer cell survival, migration, and invasion (158). NEAT1 inhibits the cytotoxic T cell infiltration in the lung cancer microenvironment to promote tumor growth in the syngeneic mouse models. Hence, cGAS-STING signaling plays a crucial role in the immune environment of different tumor microenvironments, including the lung cancer one. The STING activation has also been found beneficial in neuroblastoma *via* increasing the potent tumoricidal T cell-mediated immune response (159). The nanoparticle-based delivery of the STING activator has also increased the antitumor immune response (increased M2 to M1 macrophage polarization, IFN-γ producing T cells, tumor cell apoptosis, and $CD4^+$ and $CD8^+$T cell infiltration in the tumor microenvironment) in the PD-L1-insensitive triple-negative breast cancer (160). Different STING agonists are under phase I and II clinical trials (161). The results will determine their progression to the large phase III clinical trials.

TLR9 RECOGNIZING SELF-DNA

TLRs have evolved more than 500 MYA in eumetazoan ancestors before the divergence of bilaterians and cnidarians, although they were first identified in *Drosophila melanogaster* (*D. melanogaster*) (162, 163). Of the 10 TLRs expressed in human cells, both immune and non-immune cells, only TLR9 recognizes the pathogen-derived DNA (CpG DNA) in the endolysosomes and induces the type 1 IFN production in the plasmacytoid dendritic cells (pDCs) (**Figure 1**) (164). This recognition also induces the polyclonal B cell activation in a MyD88 and interferon regulatory factor 7 (IRF7)-dependent manner (165). TLR9 localizes in the ER membrane of DCs and macrophages in their resting stage, which requires endosome shuttling to initiate pro-inflammatory signaling in response to the CpG DNA binding (**Figure 1**) (166, 167). The CpG DNA moves to the endosomes and subsequently to the tubular lysosomal compartment. Concurrent to the CpG DNA movement, TLR9 also moves from the ER to the CpG DNA containing structures, including endolysosomes, lysosomes, and endosomes (167). The TLR9 trafficking from ER to Golgi is mediated by UNC93B1 (Unc-93 Homolog B1, TLR signaling regulator) that also controls the TLR9 loading to the $COPII^+$ vesicles, which originate from the ER (168, 169). These $COPII^+$ vesicles deliver the TLR9 to the plasma membrane (168, 170). The UNC93B1 deficient mice show a complete loss of intracellular TLRs (TLR3, TLR7, and TLR9) in splenic DCs and macrophages (169). UNC93B1 also controls the plasma membrane localization of TLR5 that recognizes bacterial flagellin (171). Hence, UNC93B1 is

crucial for TLR membrane trafficking. The strength of TLR9 signaling activation in response to the bacterial CpG DNA stimulation depends on its concentration, bacterial species (*Pseudomonas aeruginosa* > *Mycobacterium tuberculosis* > *Klebsiella pneumoniae* > *Escherichia coli* > *Staphylococcus epidermidis*), CG dinucleotide content, and the delivery of the CpG DNA inside the cell (172, 173). Further studies have indicated that the bacterial DNA binding to the TLR9 is sequence-independent and enhanced by the DNA curvature (174). The phosphodiester bond of the binding DNA induces the TLR9 dimerization independent of its sequence. Ligands with phosphorothioate (PS) backbones induce the large TLR9–DNA aggregates formation due to their propensity to self-associate. TLR9 binding site has a strong bias to bind to the phosphodiester backbone over the phosphorothioate backbone of the CpG motif (175). Thus, substituting phosphorothioate linkage for a phosphodiester linkage of just the CpG motif improves the activation potency of a phosphorothioate-based oligonucleotide for human B-cells and pDCs along with mouse bone marrow-derived dendritic cells (BMDCs) and macrophages (175).

Later studies with synthetic oligodeoxyribonucleotides (ODNs) containing CpG (unmethylated deoxycytidylyl-deoxyguanosine dinucleotide) indicate that the nucleotide sequence (nts), length, and dimerization properties of ODNs determine their propensity to bind and activate TLR9 (176). For example, ODNs with lesser than 21 nucleotides (nts), which have adenosine adjacent to their cytidine–guanosine (CG) dinucleotide motif, do not activate TLR9. The minimal ODNs activating human TLR9 comprise 2 CG dinucleotides separated by 6-10 nts, where the first CpG motif precedes through the 5′-thymidine and the poly-thymidine tail at the 3′ end of the ODN (176). However, the presence of short, CpG-containing oligodeoxyribonucleotides (sODNs) as short as two nts can enhance the TLR9 activation despite that they themselves cannot activate TLR9 (177). Hence, sODNs can enhance TLR9 activation in response to the mammalian genomic DNA even at their limiting concentration. The DNA curvature inducing proteins, including HMGB1 and histones H2A and H2C significantly enhance the TLR9 binding of the DNA (174). The cysteine rich protein granulin serving as a co-receptor for CpG DNA also coordinates their delivery to the endosomes or endolysosomes and promotes the interaction between CpG DNA and c-terminal domain of TLR9 to make ensure the TLR9 signaling activation (178, 179). Thus, cytosolic HMGB1 and granulin bind to the CpG DNA and increase their potency to bind to endosomal TLR9 and activation (**Figure 1**). Hence, TLR9 recognizes curved DNA backbones with increased curvature independent of its sequence length. Thus, increase in the curvature of the binding DNA and the presence of shorter ODNs, which themselves do not activate TLR9, further increase the binding tendency and strength of cytosolic DNA with the TLR9.

Earlier studies have indicated that the intracellular localization of TLR9 in different compartments as a strategy to discriminate between self and non-self-DNAs (180). However, in addition to the pathogen-derived CpG DNAs, TLR9 also recognizes self-DNA, including the mtDNA (which also contain CpG motif like bacterial DNA) (181–183). TLR9 has two DNA-binding sites, which functionally cooperate to promote receptor dimerization and activation (176, 184). For example, along with CpG DNA binding site, TLR9 has another DNA-binding site to bind DNA containing cytosine at the second position from the 5′ end (5′-xCx DNA). The binding of 5′-xCx DNA to the TLR9 in the presence of CpG DNA promotes TLR9 dimerization and activation. Hence, TLR9 recognizes two types of DNAs, and their binding increases its dimerization and activation. The human TLR9 (hTLR9) activation requires a pair of closely positioned CpG motifs within ODNs, but an ODN with a single CpG motif present at 4–6 nts from the 5′-end can activate murine TLR9 (mTLR9) effectively (185, 186). The ODNs, which are lesser than 23 nts and greater than 29 nts, lose their tendency to activate DCs through TLR9 activation (186). Thus, ODNs with minimal nts activate Th1 cytokine production in DCs and confirm B cell activation through increasing the expression of cell surface markers (186). Hence, the activation of TLR9 in response to the self-DNA depends on nts length and sequence. For example, due to the double CpG sequence-specificity for hTLR9, their activation decreases in response to the ODNs with a lower frequency of CpG motifs, including mammalian genomic DNA (185). This section will only describe the role of the TLR9 in recognizing self-DNAs under different circumstances or disease conditions.

TLR9 Recognizes Self-DNA During Infections to Modify the Immune Response

Acute and chronic microbial infections, along with emerging infectious diseases (EIDs), including the present COVID-19 pandemics, always remain a threat to human life (187, 188). Although we have made advances in their immunopathogenesis and receptors, recognizing pathogens, we still need to explore the unknowns associated with infection pathogenesis. For example, some groups are resistant, and some are more susceptible to the same infection. For example, TLR9 besides, recognizing pathogen-derived CpG DNA, also recognizes host-derived self-DNA. Enterovirus 71 (EV71, which have a positive-sense single-stranded RNA (ssRNA) as a genome is a non-enveloped virus of the genus *Enterovirus* and family *Picornaviridae*) (189). EV71 (a typical neurotropic virus) is responsible for the head, foot, and mouth disease (HFMD) in children around the world that may also lead to permanent paralysis and even death due to its propensity to cause neurological disease during acute infection (190, 191). However, a recent study has indicated the comparatively increased EV71 replication in pDC isolated from $TLR9^{-/-}$ mice than wild type (WT) mice (189). These WT DCs produce a higher amount of IFN-α, IFN-γ, TNF-α, IL-6, IL-10, and monocyte chemotactic protein 1 (MCP1) than TLR9-deficient DCs due to NF-κB activation. However, EV71 does not directly activate TLR9-dependent NF-κB activation (189). Seven days old $TLR9^{-/-}$ mice infected with EV71 show severe neurological lesion-related symptoms (hind-limb paralysis,

ataxia, and lethargy) of the disease. Hence, TLR9 activation plays a protective role in the EV71 infection, but that TLR9 activation does not involve the recognition of viral genetic material, instead uses the host-derived self-DNA that releases from cells dying due to apoptosis (189). Hence, it will be essential to explore in humans lacking TLR9 genetically and humans with single nucleotide polymorphisms (SNP) in their TLR9 allele that make it inactive and the severity of EV71 infection depending on the self-DNA recognition.

Another study has indicated that the TLR9-mediated recognition of the self-DNA during *Listeria monocytogenes* infection controls cell-mediated immunity (CMI) through a rapid conversion of conventional $CD4^+$T cells to the regulatory T cells (T_{regs}) (192). This process involves the $CD8\alpha^+$ DCs, which through TLR9-dependent recognition of the mtDNA (released from dead neutrophils) release IL-12p70, which generates $FoxP3^+T_{regs}$ from conventional $CD4^+$T cells during a high dose infection, whereas a low dose infection induces $CD8^+$T cell generation (192). Hence, the activation of TLR9 through self-DNA recognition determines the outcome of T cell-mediated immune response, including the generation of T_{regs}, which are potential immunoregulatory T cells, and control the exaggerated inflammation. Furthermore, IL-12p70-dependent highly potent Th1-like T_{regs} inhibit allograft rejection in unmodified patients (193). Hence, TLR9 activation through recognizing self-DNA may help to lower systemic inflammation and inflammatory organ damage depending on the infection.

For example, TLR9 activation in response to the circulating mtDNA induces sepsis-induced acute kidney injury (AKI) and splenic apoptosis during polymicrobial sepsis (194). The TLR9 activation on DCs during polymicrobial sepsis promotes the IL-17A generation from γδ T cells, which induces the sepsis-induced AKI (195). The activation of TLR9 on renal tubular epithelial cells and podocytes promotes ischemic AKI through their apoptotic and necrotic cell death and inflammation as global deficiency of TLR9 does not exert any impact on murine ischemic AKI (196, 197). The activation of p38MAPK and NF-κB downstream to TLR9 signaling plays a crucial role in the podocyte apoptosis (197). Sepsis-induced AKI also involves podocyte apoptosis (198). The generation of circulating mitochondrial DNA in sepsis patients and its recognition by TLR9 also induces adaptive immune cell paralysis through suppressing the $CD8^+$ T cell function to prevent organ damage (199). However, prolonged immunosuppression may predispose them to secondary infections. Thus, depending on the disease stage, activation of TLR9 in response to the self-DNA during different infections, including sepsis, may have therapeutic potential. The TLR9 inhibition during polymicrobial sepsis may protect from sepsis-induced AKI and immunosuppression (200). Senolytics also protect from TLR9 activation-mediated inflamm-aging and age-specific inflammatory responses occurring due to mtDNA recognition and increase life span (201–203). Further studies are required in the direction. Hence, TLR9-mediated self-DNA recognition exerts both protective and destructive effects depending on the pathogen, pathogen load, severity, and extent of TLR9 expression. We need further studies in the field.

TLR9 Recognizing Host-Derived Self-DNA During Sterile Inflammatory Conditions

The chronic beryllium toxicity or exposure (both soluble and crystalline) causes the death of alveolar macrophages (AMs) that releases cellular DNA and IL-1α in the circulation (204). This also increases the $CD80^{hi}$DCs migration in the lung draining lymph nodes (LDLNs), expressing increased TLR9 levels. The TLR9 in DCs recognize phagocytosed self-DNA and induce the expansion of pathogenic $CD4^+$Th1 cells recognizing beryllium-modified HLA-DP2/peptide complex (beryllium-specific $CD4^+$T cells) before the clinical development of pulmonary granulomas characterizing chronic beryllium disease (CBD) (204). Hence, the TLR9 (expressed on mobilized immunogenic DCs)-mediated recognition of self-DNA released from dying AMs plays a crucial role in CBD-induced by soluble or crystalline form. The phosphatase and tensin homolog-induced kinase 1 (PINK-1)-mediated mitophagy induces TLR9 activation in stretch-induced cell injury in response to the mtDNA that further exaggerates the inflammation in patients with mechanical ventilation (205).

TLR9 in Ischemia–Reperfusion Injuries

The role of TLR9 in cerebral and myocardial ischemia needs further studies as some groups have shown its activation has a protective action through activating PI3K/Akt signaling pathway during cerebral-ischemia reperfusion injury and myocardial-ischemia reperfusion injury in mice (206, 207). This protection involves an association between TLR9 and p85 subunit of PI3K, and the inhibition of PI3K/Akt activation abolishes TLR9-mediated protective action. However, a study has shown the activation of the p38MAPK signaling pathway in response to the TLR9 activation aggravates myocardial ischemia-reperfusion injury (208). Further study has shown that the inhibiting TLR9 activation with inhibitory oligodeoxynucleotide (iCpG-ODN) protects from the cerebral-ischemia reperfusion injury (209). However, HMGB1 levels rise in circulation in patients with cerebral and myocardial ischemia (210). A group has shown the protective action of cytosolic HMGB1 released from the nucleus during myocardial infarction (211). They have shown the binding of HMGB1 to the TLR9 exerts the post-myocardial repair effect through decreasing myocardial apoptosis and increasing wound healing and angiogenesis. However, the protective effect of the HMGB1 may be based on its concentration at a particular stage of cardiac injury as it exerts both protective and harmful outcomes (212). However, circulating self-DNA (both nuclear and mtDNA) serves as the marker for the severity of acute ischemic stroke or cerebral damage after acute cerebral infarction and poor outcome at three months (213, 214). The level of cell-free DNA also increases in the circulation in patients with acute myocardial infarction (AMI) (215, 216). Hence, circulating HMGB1 may increase its tendency to bind with TLR9. Further studies are required in the direction of studying the role of TLR9 in human patients of acute cerebral and myocardial infarction.

TLR9 Dysregulation in Early and Later in Life

The TLR9 expression dysregulation during development proves fatal to the neonatal life that depends on the type II IFN signaling driven by macrophages and IFN-γ producing NK cells (217). For example, the expression of TLR9 on transmembrane in mutant mice called TLR9^{TM} in their early life proved detrimental (they suffer severe or lethal hepatitis and pancreatitis, systemic inflammation, and anemia), whereas the same mutation later in life induced only mild inflammation (217, 218). The TLR9^{TM} bypasses the ectodomain proteolysis process before their activation and responds to the extracellular DNA, causing severe systemic inflammation and anemia without the involvement of lymphocytes (T and B cells) (218). Hence, the compartmentalization of TLR9 during embryonic development is necessary to escape from unwanted activation of TLR9 through recognizing self-DNA as a DAMP. Failure to this proves fatal to the neonate due to the ongoing development process involving apoptosis and necroptosis, causing lots of circulating self-DNA.

Both neonatal liver macrophages (Kupffer cells) and circulating Ly6hi monocytes express TLR9, but little or no TLR7 (217). Hence, TLR9^{TM} mutant present on cell membrane breaks the immunologic tolerance mediated by the compartmentalized location of normal TLR9 in endolysosomes, endosomes, lysosomes, and phagosomes. Endolysosomal exonucleases, phospholipase D3 (PLD3), and PLD4 (type II transmembrane proteins) degrade TLR9, and their genetic deficiency causes an enhanced TLR9 expression and TLR9-dependent severe inflammation (lethal hepatitis), causing the death of newborns within two to three weeks after birth (219). PLD4 has a narrow tissue distribution and highly expressed in DCs and myeloid cells, including macrophages and ionized calcium-binding adapter molecule 1 (Iba1)-positive microglia, but PLD3 has a broader tissue distribution than PLD4 (219, 220). The PLD3 localization to endosomes and lysosomes involves an uncommon intracellular biosynthetic route, which depends on the endosomal sorting complex required for transport or ESCRT machinery (221). The newly established anti-TLR9 monoclonal antibody (mAb) called NaR9 has a protective action against fulminant hepatitis developed in response to the over-activated TLR9 upon recognizing self-DNA and inducing systemic cytokine storm (222). Hence, NaR9 mAb has potent therapeutic properties against over-activated TLR9-mediated inflammatory diseases.

TLR9 in Autoimmune or Autoinflammatory Diseases

The autoantibodies (AutoAbs) to self-RNA and DNA are present in SLE patients, and TLR9 signaling engaged with B cell receptor (BCR) signaling helps in the spontaneous generation of AutoAbs against self-DNA in autoreactive B cells (223, 224). However, TLR9 does not impact the development of SLE-associated nephritis in susceptible mice (225). For example, the TLR9 deficiency aggravates the SLE due to the profound activation of lymphocytes and pDCs, and serum levels of IgGs and IFN-α increase (225). Hence, TLR9 is crucial for AutoAbs generation against self-DNA in SLE but not for inflammatory lupus nephritis. A genome-wide association study (GWAS) has also indicated that the three TLR9 polymorphisms (−1486C/T, +1174A/G, and +1635C/T) are not associated with the susceptibility to the SLE in the eastern Asian population (226). Hence, we need further studies in context of TLR9 in SLE. Also, the infants with heterozygous genotypes TLR9—1486T/C and 2848C/T show a higher frequency of cytomegalovirus infection than normal ones (227). Another GWAS has shown the association between TLR9 1174G/A polymorphism with the acute Epstein-Barr virus infection or infectious mononucleosis in children and adolescents (228). Thus, TLR9 polymorphism studies are warranted further in other autoimmune and infectious diseases along with cancers.

TLR9 in Obesity and Obesity-Associated Inflammatory Diseases

The circulating endogenous host-derived ssDNA and dsDNA also increase in obese people, patients with visceral obesity, and high-fat diet (HFD) fed mice (229, 230). The circulating ssDNA levels in obese people well-correlate with the homeostasis model assessment of insulin resistance (HOMA-IR) that serves as an index for insulin resistance. High circulating endogenous host-derived ssDNAs increase the pro-inflammatory M1 macrophage accumulation in adipose tissues through their recognition *via* TLR9 (230). The TLR9 expression level also increases in the adipose tissues in obese people. For example, HFD increases TLR9 expression in vascular adipose tissue (VAT), dominantly in macrophages in mice. These circulating DNAs released from degenerating adipocytes increase monocyte-chemoattractant protein-1 (MCP-1) expression in macrophages upon their recognition by TLR9. The circulating DNAs are engulfed by VAT macrophages of obese mice as indicated by their presence in the macrophage cytoplasm (230). However, macrophages from lean VAT do not increase cytosolic DNA. TLR9$^{-/-}$ mice with HFD do not show obesity-associated inflammation, macrophage accumulation in the adipose tissue and have better insulin sensitivity than WT mice with HFD (230). Hence, TLR9-mediated recognition of the circulating self-DNA plays a crucial role in the obesity-associated inflammation and insulin resistance index.

Blocking TLR9 activity in obese people may reduce obesity-induced adipose inflammation and chances of future development of T2DM. However, the TLR9$^{-/-}$ mice show opposite findings with the increased M1 macrophages and Th1 cells accumulation in the adipose tissue along with increased body weight and fat accumulation fed on HFD (231). Hence, we need further studies in the direction as diet, feeding type, age, sex, and genetic background of the mice may impact these results. Cytosolic HMGB1 increases in obese patients with T2DM only (232). HMGB1 in the islet of beta cells of the pancreas serves as a main stimulatory factor for insulin release. Hence, the HMGB1 bound host cytosolic or circulating DNA should be observed as HMGB1 binding to the host DNA increases its recognition by TLR9, as discussed previously.

The activation of TLR9 releasing type 1 IFNs through self-DNA, including mtDNA, also increases liver inflammation in the NASH or NAFLD through increasing hepatocytes death independent of apoptosis and liver fibrosis (233). TLR9

inhibition through ODN2088 proves beneficial in mice subjected to NASH (233). Attenuation of HMGB1 in NASH inhibits weight gain, and liver inflammation (decrease in TNF-α and MCP-1) in mice (C57BL//6) on HFD indicates that TLR9 recognizes self-DNA bound to HMGB1 (234). The development of atherosclerosis in people with obesity is another problem responsible for heart ailments along with NASH. Animal studies have shown that the angiotensin II (Ang II) infusion increases the plasma concentration of self-DNA that is recognized by the TLR9 expressed on immune cells, including macrophages, which secrete pro-inflammatory cytokines and other molecules promoting atherogenesis in the aortic arch (235). The TLR9 activation in apolipoprotein E KO ($ApoE^{-/-}$) macrophages promotes inflammation partially through the p38 mitogen-activated protein kinase (p38MAPK) pathway activation. This circulating self-DNA in the coronary artery well correlates with the inflammatory features of coronary plaques as indicated by optical coherence tomography (OCT) in patients with acute myocardial infarction or AMI (235).

Even smoking e-cigarette (e-cig) increases the level of circulating self-DNA (mtDNA), TLR9 expression in classical macrophages, and atherosclerotic plaques (including human femoral artery atherosclerotic plaques expressing higher TLR9 levels) and lesions (236). The blockage of TLR9 before the exposure of e-cig vapours (ECVs) decreases atherosclerotic plaques or lesion formation, and the TLR9 expression increases along with lowering pro-inflammatory cytokine levels, $CCR2^{+}$ classical blood monocytes, and the accumulation of lipids and macrophages (236). Hence, the TLR9 activation inducing a pro-inflammatory immune response in plaque macrophages in response to the circulating self-DNA is associated with vascular inflammation and atherogenesis. Circulating self-DNA binding to the TLR9 increases with its binding to the HMGB1. Studies have indicated the higher circulating levels of HMGB1 in patients with coronary artery disease (CAD) and are associated with non-calcified plaque burden with stable CAD patients (237, 238). The circulating HMGB1 levels are also associated with CAD in nondiabetic and T2DM patients (238). Thus targeting HMGB1 may indirectly suppress exaggerated TLR9 activation in these diseases. Hence, TLR9 recognizes cytosolic self-DNA under diverse inflammatory conditions and exerts protective or destruction action depending on several factors, which remain to explore.

ALRS RECOGNIZING SELF-DNA

Different mammals have a different number of ALR genes, for example, the cow has only one ALR, humans have 4 (AIM2, IFI16 (Gamma-interferon-inducible protein Ifi-16 or interferon-inducible myeloid differentiation transcriptional activator), PYHIN1 (Pyrin and HIN (hematopoietic expression, interferon-inducible nature, and nuclear localization) domain-containing protein 1), and MNDA (myeloid cell nuclear differentiation antigen) or PYHIN3 or epididymis secretory sperm-binding protein, present only in the nuclei of cells of the granulocyte-monocyte lineage), and mice have 14 ALR genes (239–241). Of note, no PYHIN genes have been seen in non-mammalian species, along with monotremes (egg-laying predatory mammals, including duckbill platypus and echidnas) (240). The HIN domain comprises of tandem pair of oligonucleotide/oligosaccharide binding (OB) folds that are used by proteins to bind nucleic acids during replication, transcription, and translation (242). The two OB folds of HIN domain are connected by a long linker region and a conserved hydrophobic region between two OB folds holds them together tightly, forming a single compact domain (242). A single HIN domain seems to have evolved in the common ancestors of marsupials and placental mammals, which duplicated in placental mammals to give rise to three distinct forms (HIN-A, -B, and -C) (240). This indicates that they have evolved approximately not more than 200 MYA ago as the common ancestor of marsupials and placental mammals' dates back to approximately 140 to 191 MYA (243, 244). Hence, evolutionarily ALRs are the youngest of both TLRs and cGAS PRRs. HIN-C and pyrin domains (PYD) of AIM2 have diverged from the rest of the PYHIN family, and only PYHIN protein shows orthology across different species. Hence, the defense of the genome against endogenous retrotransposons or retro-elements is an additional evolutionary driver for PYHIN proteins (240). Of note, even within the same gene, the pyrin and HIN domain phylogeny is not compatible, indicating that the recombination may have led not only to species-specific expansions of ALR genes but also scrambled the existing genes into novel combinations of Pyrin and HIN domains (239). This indicates that ALR genes in mammals exhibit remarkable plasticity, and no single ALR gene is preserved among all mammals with a little preserved orthology across species. ALR genes have undergone extensive species-specific diversification that indicates the presence of great evolutionary pressure that has shaped the ALR sequences and function throughout whole mammalian lineages (239).

For instance, the two factors for the dramatic differences in the number and sequences of mouse and rat ALRs include gene expansions in the mouse of three ancestral rodents ALRs, and independent reassortment of the Pyrin and HIN domains, which create an extra diversity. The three human ALRs (aside from AIM2) are not represented within these three rodent-specific clusters. All murine ALRs relocalize when expressed with relevant adaptor proteins (STING and ASC), and co-expression of an ALR with a single adaptor molecule reveals indiscriminate co-localization (239). Murine ALRs show three predominant patterns of localization: (1) AIM2, MNDA, and MNDAL co-localize with ASC adaptor proteins of inflammasome mainly, with a minimal co-localization with STING, (2) PYHIN-B, PYR-A, PYHIN-1, PYHIN-A, IFI204, IFI203, and IFI205 mainly co-localize with STING in a structure called STING-positive-ER-Golgi complex, together with concomitant recruitment of ASC to these areas of ALR-STING co-localization, and (3) The rest three ALRs (PYBLHIN-C, PYR-RV1, and IFI202B) colocalize with the puncta of ASC and not with STING (19, 239). Hence, murine ALRs can recruit ASC, STING, or both depending on their co-localization. AIM2 robustly activates ASC inflammasome to release IL-1β and IL-18 (**Figure 2**).

AIM2 or p210 belongs to the family of p200 or HIN-200 proteins (hematopoietic interferon-inducible nuclear proteins with a 200 amino acid (AA) repeat) or PYHIN (IFI200/HIN-200) protein family (pyrin and HIN-200 domain-containing proteins, which have a DNA-recognizing innate receptors family, including ALRs (245, 246). AIM2 has one HIN domain that binds to the cytosolic dsDNA and one PYD (**Figure 2**) (246). The binding of the HIN domain (contains two oligonucleotide/oligosaccharide binding (OB) folds with great affinity to DNA) to the cytosolic dsDNA promotes the ASC [an adaptor molecule, an apoptosis-associated speck-like protein containing a CARD (Caspase activation and recruitment domain)]-dependent inflammasome assembly through pyrin-pyrin domain interaction to produce mature IL-1β and IL-18 (**Figure 2**) (14, 247–249). AIM2 can be activated by mtDNA, nuclear DNA released in the cytosol due to nuclear death, and self-DNA secreted by exosomes (**Figure 2**) (250, 251). Exosomes are extracellular vesicles (EVs) with a diameter ranging from 40–160 nm (average ~100 nm) and have an endosomal origin (**Figure 2**) (252). Under homeostasis, AIM2 exists as in an autoinhibitory stage due to an interaction between HIN and PYD domain that blocks the availability of PYD for ASC PYD (**Figure 2**) (253).

The binding of cytosolic dsDNA to the HIN domain of the AIM2 does not depend on the DNA sequence and its GC sequence but depends on its length that should have at least 80 bp (13, 254, 255). A dsDNA of ~80 bp may accommodate a maximum of 20 HIN domains of AIM2 (255). However, a dsDNA with ~200 bp allows an optimal AIM2 activation. The dsDNA with ~300 bp induces a significant AIM2 polymerization into filaments than dsDNA with ~72 bp (255, 256). Hence, cytosolic dsDNA binding to the AIM2 depends on its length that further determines its kinetics and magnitude of AIM2 inflammasome activation. Of note, the AIM2 PYD suppresses the HIN: DNA interaction, despite lacking a DNA-binding capacity. Thus, dsDNA binding to the AIM2 HIN domain displaces the PYD from its intermolecular complex to facilitate the PYD downstream signaling to the ASC protein (255). AIM2 HIN domains (consist of two oligonucleotide/oligosaccharide binding (OB, OB1, and OB2) fold and a linker between them) bind both grooves of the dsDNA involving both minor and major grooves indicating their specificity for dsDNA binding or recognition only (255). The DNA interface from the AIM2 OB1 is centered at residues K162 and K163 between β1 and β2' strands and K198 and K204 near the α1 helix. The OB1-OB2 linker contains amphipathic α2-α3 helices, which form hydrogen (H)-bonds and van der Waals (vDW) contacts from R244, K251, or G247, and T249 for different AIM2 HIN domains (255). The OB2 of AIM2 HIN forms salt bridges and vDW contacts with DNA through residues R311 at the β4 strand and residues K335 and I337 at the β5 strand. R311 faces the minor groove of the dsDNA faces R311 and forms bidentate H-bonds with the phosphate backbone of DNA.

AIM2 does not have an oligomerization domain, and electrostatic interaction between the HIN domain and cytosolic dsDNA takes place to activate AIM2 (255). This dsDNA-HIN domain interaction of AIM2 releases the signaling PYD from its intermolecular complex containing the HIN domain. It defines the multivalent ligand dsDNA as the oligomerization platform for forming inflammasome/pyroptosome complex (255) The AIM2 binds to the ASC only after the release of auto-inhibition *via* binding to the cytosolic dsDNA (**Figure 2**) (257). Hence, AIM2, without its bound ligand, is unable to activate ASC-dependent inflammasome and pyroptosome formation to induce pro-inflammatory cytokine release and pyroptosis (**Figure 2**). The PYD of the AIM2 drives the filament formation and dsDNA binding (258). The HIN domain of the AIM2 that comprises the dsDNA binding domain also oligomerizes and assists in the filament formation. Hence, the ability to oligomerize is critical for dsDNA binding that permits the size of the dsDNA to regulate the AIM2 polymers assembly. The AIM2 pyrin oligomers define the filamentous structure (258). The helical symmetry of the AIM2 pyrin filament is consistent with the filament assembled by the PYD of the downstream adaptor ASC. Hence, the AIM2 PYD is not auto-inhibitory, but the generation of the structural template by coupling ligand binding (dsDNA) and oligomerization serves as a crucial signal transduction mechanism in AIM2 inflammasome (258). Thus AIM2 oligomerizes on cytosolic dsDNA that initiates the nucleation of ASC adaptor filament, inducing the pro-caspase-1 (pro-CASP1) filament polymerization activating caspase 1 (CASP1) through auto-proteolysis to produce pro-inflammatory cytokines (IL-1β and IL-18) and induce pyroptosis (**Figure 2**) (12–14, 249, 258). Hence, minimal oligomer assembly requires six protomers, and the optimal one needs ~24 protomers (258).

ALR activation is not essential for inducing type 1 IFN production in response to the cytosolic DNA and also does not contribute to the autoimmune disease in the $Trex1^{-/-}$ mice with AGS (259, 260). Another study has indicated that mouse ALR IFI205 senses cytosolic retrotransposon DNA independently of cGAMP production, and this process does not produce type 1 IFNs as this process prevents its recognition by STING (261). ASC is also called PYCARD (PYD and CARD containing protein)/Target of Methylation-induced Silencing-1 (TMS1) and serves as a central adaptor molecule in the inflammasome complex-dependent pro-inflammatory cytokine release (248, 262). Also, to release mature pro-inflammatory cytokines (IL-1β and IL-18), the interaction between ASC and AIM2 forms ASC pyroptosome that induces pyroptosis in cells containing CASP1, including macrophages (**Figure 2**) (12). The AIM2 inhibition prevents cytosolic DNA recognition and the inflammasome/pyroptosome activation in macrophages. Hence, recognition of the cytosolic DNA by AIM2 induces their oligomerization and AIM2 inflammasome formation to release pro-inflammatory cytokines and induce pyroptosis as an indicator for the cell's internal danger.

Another, dsDNA-binding protein called p202 inhibits the AIM2 signaling in some mouse strains (11). For example, New Zealand Black (NZB) mice express p202 (an endogenous inhibitor of AIM2 activation) and therefore do not secrete a high amount of IL-1β and IL-18 upon stimulation with dsDNA

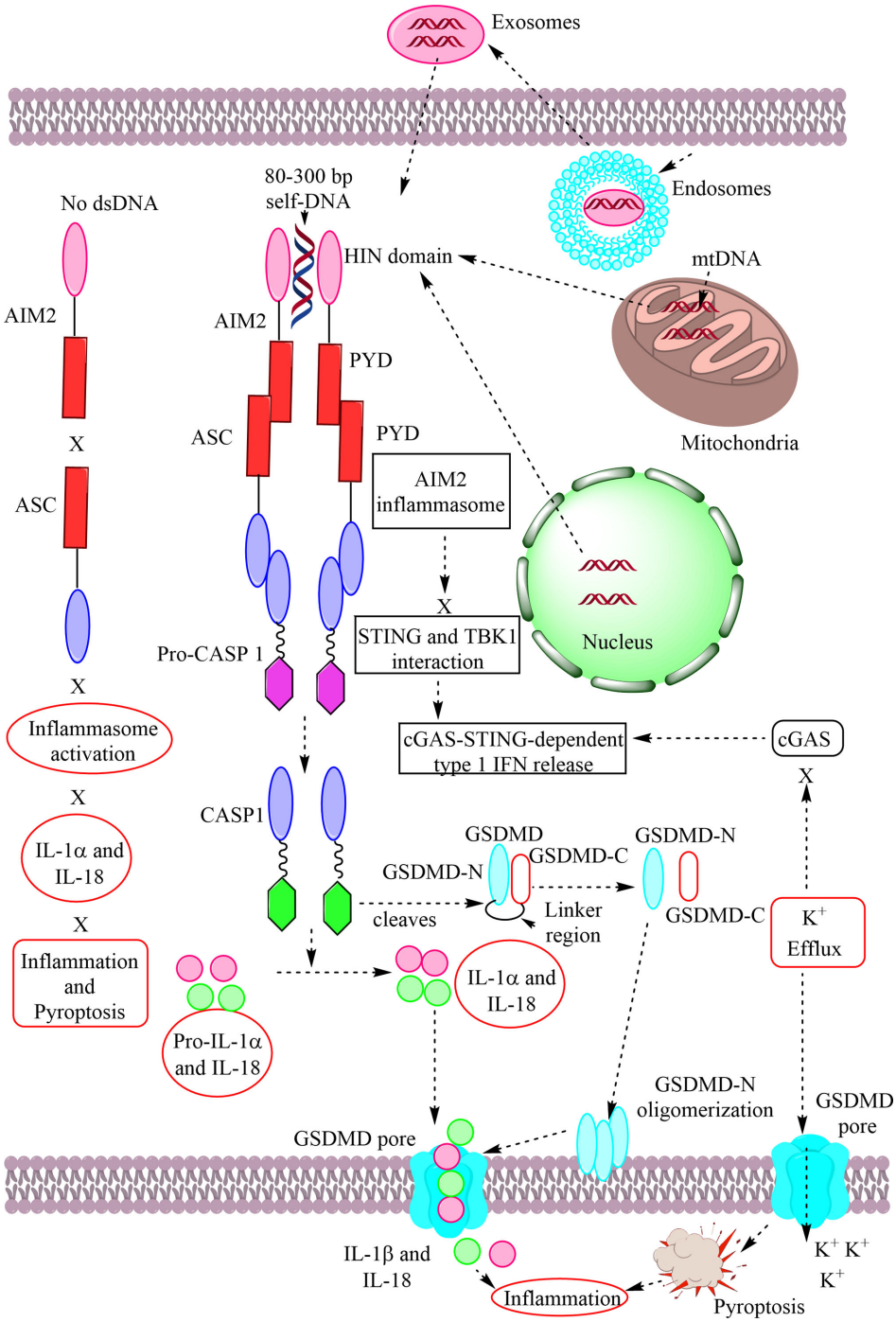


FIGURE 2 | Schematic representation of AIM2 in response to self-DNA. AIM2 is an ALR that becomes activated upon recognizing and binding to the self-DNA coming into cytosol due to cellular damage, including mitochondrial and nuclear damage, and exosome with host DNA. AIM2 activates efficiently in response to self-DNA with 80–300 bp. The HIN domain of the AIM2 recognizes cytosolic DNA and its PYD interacts with the PYD of the ASC to form inflammasome complex that activates procaspase 1 (pro-CASP1) into CASP1. The CASP 1 cleaves pro-IL-1α and -IL-18 into IL-1α and IL-18. CASP1 also cleaves the linker region of the GSDMD and frees GSDMD domains (GSDMD-N and GSDMD-C). The free GSDMD-N terminals interacts with phosphoinositides or other acidic lipids to oligomerize and form GSDMD pore. The GSDMD pore mediates the IL-1α and IL-18 release from the cells. Also, the K^+ efflux from the GSDMD pore inhibits cGAS activity and the cGAS-STING mediated type 1 IFN release along with inducing pyroptosis. The AIM2-induced GSDMD acts as a negative regulator of cGAS-STING-mediated type 1 IFN production. Also, AIM2-ASC inflammasome inhibits STING-TBK1 interaction required for IRF3-dependent type 1 IFN release. The AIM2 remains inactive in the absence of specific cytosolic DNAs.

(263). NZB mice develop anti-erythrocyte Abs and serve as an animal model for autoimmune hemolytic anemia. However, NZB mice also lack another inflammasome protein called the NLR family, PYD containing 3 (NLRP3) due to the point mutation in the NLRP3 gene. The HIN1 domain of the mouse p202 binds to the dsDNA on the opposite site used in AIM2 whereas HIN2 forms a homotetramer that increases its avidity for the dsDNA (264). However, HIN2 of p202 also interacts with the HIN1 of the AIM2 resulting in the spatial separation of the AIM2 PYDs, causing p202-mediated prevention of the dsDNA-dependent clustering of ASC and AIM2 inflammasome activation (264). The 25-hydroxycholesterol (25-HC) production through the activation of cholesterol-25-hydroxylase (Ch25h) that maintains the repression of sterol regulatory element-binding protein 2 (SREBP2, a master regulator of sterol and fatty acid synthesis) activation by macrophages also prevents AIM2 inflammasome activation (250, 265).

Some viral proteins, including HSV-1 tegument protein VP22 and human cytomegalovirus (HCMV) tegument protein pp65 (pUL83) inhibit AIM2 activation *via* direct interaction preventing its oligomerization (an initial step in the AIM2 inflammasome activation) (266, 267). Also, the HCMV immediate early 86-kDa protein (IE86) inhibits the AIM2-mediated release of mature IL-1β *via* associating with the block in the transcription of the pro-IL-1β gene (268). Hepatitis B virus (HBV) hepatitis B e antigen (HBeAg) suppresses the AIM2 inflammasome activation in the chronic hepatitis B (CHB) patients that may induce HBV-induced immunotolerance (269). The post-translational modification plays decisive role in the activation of inflammasome, including AIM2 (270, 271). For example, tripartite motif 11 (TRIM11) inhibits AIM2 inflammasome activation *via* binding to it through its PS domain and undergoes auto-polyubiquitination at K458 to promote TRIM11 and the autophagic cargo receptor p62 association to mediate AIM2 degradation *via* selective autophagy (272). Hence, these proteins or their synthetic homologs have a potential to target AIM2-meditated inflammatory diseases. Additionally, vitamin B_2 or riboflavin also inhibits AIM2 inflammasome activation through preventing the mitochondrial damage and the release of ROS and mtDNA in the cytosol (273).

The AIM2 activation serves as an endogenous negative regulator of cGAS-STING signaling and the type 1 IFN production through GSDMD that depletes intracellular potassium (K^+) *via* forming membrane pores inducing pyroptosis (**Figure 2**) (20, 274–277). GSDMD is a 480-AA protein that contains two defined domains [GSDMD-C (22 kDa) and GSDMD-N (31 kDA)] linked by the linker region (**Figure 2**) (278). The association of GSDMD-N with GSDMD-C through the linker region inhibits the pyroptosis induction (279). The inflammasome-mediated CASP1 activation cleaves the GSDMD linker region and forms a non-covalent complex between the N terminus (GSDMD-N) and C terminus (GSDMD-C) (**Figure 2**). The cleaved N terminus or GSDMD-N auto-oligomerizes on membranes upon encountering phosphoinositides or other acidic lipids to form large circular pores called GSDMD pore (278). The GSDMD pores are essential for IL-1β release from living macrophages exposed to the inflammasome activators, including bacteria and their PAMPs/MAMPs or host-derived oxidized lipids (280). GSDMD pores are required for IL-1β transport across an intact lipid bilayer. Hence, a non-pyroptotic function of the indicates the possibility of GSDMD pores serving as conduits for the secretion of cytosolic cytokines under the condition responsible for cellular hyperactivation (280).

Disrupting the interaction between GSDMD-N and phosphoinositides or other acidic lipids or GSDMD-N oligomerization suppresses the cell killing or death through pyroptosis due to inhibition of GSDMD pore formation (281). Hence, inflammasome activating conditions determine the pyroptosis-mediated cell death through the GSDMD activation or the release of pro-inflammatory cytokines (IL-1β and IL-18) form the living hyperactive cells with intact plasma membrane through GSDMD pore. For example, oxidized phosphorylcholine-derivatives called oxPAPC (produced from dying cells at site of tissue injury and are considered LPS-like DAMPs) are recognized and captured by CD14 expressed on macrophages and DCs (282). CD14 delivers oxPAPC inside the cell that promotes the inflammasomes-mediated DC and macrophage hyperactivation to release IL-1β without their pyroptosis that increases the inflammation without causing death of the cell and experimental animals subjected to sepsis (282). The CD14 null mice are protected from oxPAPC-mediated inflammation. Further studies are required in this direction in context to the AIM2-ASC inflammasome activation. The deficiency of AIM2-ASC signals forming inflammasomes increases the type 1 IFN production and suppresses protective IFN-γ production (283). In addition to the GSDMD activation, AIM2-ASC-dependent inflammasome formation also inhibits cGAS-STING signaling *via* impeding the STING and TBK1 interaction required for IRF3-dependent type 1 IFN production (283). Hence, AIM2-induced ASC-dependent inflammasome formation has been evolved as an endogenous negative regulator of cGAS-STING signaling-dependent type-1 IFN production to prevent exaggerated inflammation during infections (mycobacterial tuberculosis) and other chronic inflammatory diseases that may cause cancer. Further studies are required in the direction.

ALRs Recognizing Self-DNAs During Different Inflammatory Diseases or Conditions

ALRs recognize pathogen-derived DNA (bacterial, viral, and parasite-derived) in the cytosol described somewhere else (251, 284). I will discuss here only its role in recognizing self-DNA during different inflammatory conditions or diseases. The AIM2-mediated inflammasome activation has been observed in the influenza virus infection in macrophages due to the release of mtDNA from the infected macrophages (285). The AIM2 recognizes mtDNA that forms ASC-dependent inflammasome and releases mature IL-1β. The mitochondrial ROS production inhibition by Mito-TEMPO (a well-known mitochondria-specific superoxide scavenger) decreases the AIM2-mediated

IL-1β production. The AIM2 gene polymorphism is associated with severe periodontitis in patients of northern and western European ancestry with haplotype rs1057028 and rs6940 (a missense SNP) (286). Also, the haplotype with IFI16 (rs6940T-rs855873G) is associated with the increased susceptibility to the Behcet disease (BD), a systemic inflammatory disease involving vasculitis and recurrent mucosal (oral and genital) ulcerations due to the lower expression of IFI16 (287). IFI16-β (a novel transcript isoform of IFI16) is a novel endogenous negative regulator of the AIM2 and blocks the AIM2-ASC complex formation *via* interacting with AIM2 (288). IFI16-β also interacts with the dsDNA and decreases its availability to AIM2, and its enforced expression inhibits AIM2 activation-mediated release of pro-inflammatory cytokines and pyroptosis. The cytosolic IFI16 is functionally similar to mouse p202 (a negative regulator of AIM2).

AIM2 activation also plays a crucial role in dietary steatohepatitis that is further aggravated by the TLR9 activation, which further upregulates AIM2 expression and IL-1β production (289). Along with chronic inflammatory liver conditions, AIM2 activation in Kupffer cells in response to the oxidized mtDNA also plays a crucial role in ischemia-reperfusion-induced hepatitis (290). AIM2 activation also induces joint inflammation in patients with chronic polyarthritis *via* recognizing self-DNA as a DAMP (291). The abdominal aortic aneurysm (AAA) can be an inflammatory AAA (accounts for 5–10% of all aortic aneurysm cases and involves inflammatory immune response localized to the blood vessel wall with unknown mechanism) or a typical atherosclerotic AAA (292, 293). Different inflammatory immune mechanisms play crucial roles in AAA (294, 295). However, a recent study has suggested the activation of AIM2 inflammasomes and dependent pro-inflammatory cytokine release in the mouse model of AAA, and its deficiency has decreased the incidence of AAA in $AIM2^{-/-}$ mice by 48.4% (296). The intravenous injection of poly (deoxyadenylic–deoxythymidylic) acid poly (dA: dT), a synthetic dsDNA releases AIM2 inflammasome activation-dependent pro-inflammatory cytokines (IL-β and IL-18), which dysregulate the reendothelialization of the carotid artery and increase the number of circulating endothelial microparticles (EMP) after acute denudation (297). The subcutaneous poly (dA: dT) injection induces atherosclerotic plaque formation, increases ROS production, and EMP release in the $ApoE^{-/-}$ mice due to AIM2 activation (297). Hence, AIM2 activation plays a crucial role in the atherogenesis and we need further studies in this direction.

AIM2 activation also contributes to the chronic cerebral hypoperfusion-induced brain injury and associated vascular dementia (VaD) *via* promoting apoptotic and pyroptotic cell death pathways (298). AIM2 methylation has also been associated with C-reactive protein (C-RP) polymorphism and C-RP levels in people with post-traumatic stress disorder (PTSD) (299). The inflammasome activation, including AIM2 also contributes to the chronic kidney disease (CKD) and ischemia-reperfusion-induce kidney damage (300, 301). The increase in the mtDNA in the peripheral blood and AIM2 in the monocytes/macrophages of T2DM patients predisposes them to the chronic inflammation and diabetic nephropathy (302–305). Hence, the AIM2 activation plays a crucial role in the inflammatory pathogenesis of many diseases. Thus, further studies are required in the direction to explore the source of AIM2 activation in both animal models and human patients of the disease.

AIM2 in Cancer

$AIM2^{-/-}$ mice are more susceptible to develop colon cancer following azoxymethane (AOM)-and dextran sulfate sodium (DSS)-induced colitis-associated carcinogenesis due to the uncontrolled proliferation of intestinal stem cells (ISCs) in response to the aberrant activation of Wnt (Wingless and Int-1) signaling and dysbiosis of the gut bacteria (306). However, the protective action of AIM2 against colon cancer is independent of its inflammasome activation mechanism. Also, more than 50% of patients with small bowel cancer have shown a frameshift mutation in the AIM2 gene in patients with hereditary nonpolyposis colorectal cancer (HNPCC) (307). Also, the mismatch repair-deficient colorectal cancers have frequent inactivation of the AIM2 gene (308). Thus mutation inactivating AIM2 functions has been more frequently associated with colon cancer in humans. AIM2 also promotes NSCLC *via* modulating mitochondrial dynamics to promote mitochondrial ROS that promotes MAPK/ERK signaling required for cancer cell growth and proliferation (309, 310). AIM2 also regulates growth and invasion of squamous cell carcinoma (SCC) of the skin or keratinocyte-derived cutaneous squamous cell carcinoma (c-SCC) through increasing the cancer cell viability and invasion as indicated by the increased production of matrix metalloprotease 13 (MMP13) and MMP1 (two proteases with collagen degrading and invasion promoting properties associated with the invasion of c-SCC cells) and vascularization (311).

AIM2 activation also promotes oral squamous cell carcinoma (OSCC) with inactive tumor suppressor p53 *via* increasing cell proliferation and NF-κB activation (312). AIM2 activation in response to the cytosolic dsDNA also plays a significant role in benign prostate hyperplasia (BPH) independent of androgen receptor status (313). However, clinical tumor samples from prostate cancer patients have low mRNA and protein expression of AIM2. Thus, it may be playing a crucial role in the induction of hyperplasia at the initial stages of prostate cancer, and a decrease in its level at later stages may be aggravating the disease that needs further investigation.

AIM2 activation also plays a crucial role in hepatic cancer induced by diethylnitrosamine (DEN)-induced liver damage (314). Kupffer cells of the liver express AIM2 that further increases in response to the DEN-induced liver injury recognizing damage cellular DNAs to produce high levels of pro-inflammatory IL-1β cytokine (314). The genetic deletion of AIM2 has shown a reduction in DEN-induced liver inflammation and hepatic cell carcinoma (HCC) or hepatoma. Hence, AIM2 activation plays a crucial role in HCC. However, another study has shown the loss of AIM2 activity promotes

HCC due to the mammalian target of rapamycin (mTOR)-S6K1 (ribosomal protein S6 kinase β1 or p70S6K) pathway promoting proliferation, colony formation, and invasion of HCC cells (315). Hence, AIM2 activation may have a protective role in HCC. Thus these two controversial findings suggest that the cause and stage of cancer may play a crucial role in the AIM2-dependent HCC. More studies are warranted. The mTOR-S6K1 signaling is also pivotal for estrogen receptor (ER) positive breast cancer, and S6K1 serves as a biomarker for prognosis and therapeutic target (316). However, AIM2 activation in breast cancer cell line and the orthotopic mouse model of breast cancer exerts a protective action *via* suppressing NF-κB activation and inducing apoptosis among cancer cells (317). Hence, AIM2 suppression may promote breast cancer pathogenesis as mTOR-S6K1 signaling is increased in patients indicating its activity loss. However, further studies are required to establish this in female breast cancer patients.

FUTURE PERSPECTIVES

The trinity of these cytosolic self-DNA recognizing PRRs plays a crucial role in the maintenance of cellular homeostasis. They serve as guardians of the cellular galaxy for dangers entering the cells, including the pathogens. However, under normal conditions host DNA resides in the nucleus and mitochondria that helps in the development of self-tolerance. TLR9 remains bound to the ER under normal conditions and moves to endosomes as soon as self-DNA moves there from the cytosol. However, whenever cells or tissues undergo stressful conditions, including cellular or mitochondrial one, their genetic material comes into cytosol due to mitochondrial or nuclear damage. Thus physical border preventing their recognition by these PRRs is lost and they become a potential threat to maintaining cellular harmony or homeostasis. Along with, self-DNA other DAMPs, including HMGB1, also come out from the nucleus that further enhances the recognition of self-DNA by TLR9 and cGAS-STING signaling pathways. Looking at their evolutionary origin, cGAS and STING have originated before (approximately 600 MYA) TLRs (around 500 MYA). The recognition of the cytosolic DNA by TLR9 depends on its CpG content, whereas in the case of cGAS, it primarily depends on its length (30–200 bp) and its curvature independent of CpG content. The unmethylated CpG motif content is prevalent in the bacterial DNA, and they are absent in vertebrates generally due to their methylation. Hence, the evolution of TLR9 added to the host defense against pathogens based on CpG that escaped the cGAS recognition. However, the CpG islands in mammals, including humans, may avoid their methylation by directly encoding demethylation signals that are an evolutionarily conserved process (318). Hence, mammalian DNA with CpG in the cytosol also becomes a potential target for TLR9-based recognition and the activation of pro-inflammatory and type 1 IFN signaling. On the other hand, AIM2 also recognizes self-DNA (80–300 bp) independent of CpG content along with pathogen-derived DNA.

ALRs, including AIM2, have evolved in the common ancestors from which marsupials and placental mammals have evolved approximately 200 MYA. Thus it will be interesting to explore the evolutionary forces responsible for AIM2 evolution so late after cGAS and TLR9. As activation of AIM2 *via* GSDMD production inhibits cGAS-STING signaling dependent on type 1 IFN production, one can speculate that it has evolved as a negative regulator of exaggerated inflammation in response to the cGAS activation. For example, cGAS activation in response to the pathogen-derived or self-DNA activates the NLRP3 inflammasome in myeloid cells through inducing lysosomal death that increases the K^+ efflux, which is one of the crucial factors stimulating NLRP3 inflammasomes and the release of CASP1-dependent pro-inflammatory cytokines (IL-1β and IL-18) (319). The cGAS-induced lysosomal cell death (LCD) in response to the cytosolic DNA involves the trafficking of the activated STING to the lysosome, inducing membrane permeabilization. Another study has indicated the involvement of STING in activating NLRP3 inflammasome formation through recruiting and facilitating NLRP3 localization in the ER during HSV-1 infection (320). STING also attenuates K48 and K63-linked NLRP3 polyubiquitination during viral infection. More studies in this direction will prove helpful to explore the unknown mechanisms regulating the cytosolic PRRs trinity (cGAS, TLR9, and AIM2). However, the location of cGAS (attached to the inner plasma membrane) inside the cell makes it a primary or first response task force among other cytosolic PRRs for invading pathogens or self-DNA of exosomes. For example, phagocytic cells, including macrophages, DCs, and neutrophils have higher distribution of plasma membrane bound cGAS than non-phagocytic cells, indicating its primary role in recognizing invading pathogens. Of note, membrane-bound cGAS has a minimal recognition for self-DNA generated within the cells. On the hand, non-phagocytic cells have larger pools of free cytosolic cGAS as compared to the phagocytic cells. Further studies are crucial in the direction.

These systems have evolved to protect against both outer and internal dangers. However, their overactivation may lead to different inflammatory diseases. Hence, their controlled or regulated function is crucial for maintaining homeostasis. The trinity of these cytosolic PRRs recognizing self-DNA along with pathogen-derived DNA has the potential to serve as potent innate immune system-based immunomodulators and great adjuvants (cGAS and TLR9 activators, CpG ODNs) for better vaccines and optimal immunotherapy for different infectious diseases and cancers (321, 322). For example, streptavidin (secreted by the soil bacteria called *Streptomyces avidinii* with a high affinity for biotin or vitamin B7) activates the cGAS-STING signaling pathway to secrete type 1 IFNs and clears HSV-1 infection that abrogates this signaling pathway-based antiviral immune response (323–325). Streptavidin has been used as an immunostimulator or an adjuvant in cancer vaccines previously without knowing its exact mode of action on innate immunity (326). Hence, streptavidin can be used with caution with specificity through its stimulatory action on cGAS-STING signaling-mediated type 1 IFN production. The cGAMP-

mediated STING activation has been found effective for cutaneous vaccination as a potent adjuvant without undesired skin irritation (327).

The antitumor effect of the antidiabetic drug metformin occurs through activating the STING/IRF3/IFN-β pathway *via* inhibiting AKT signaling in pancreatic cancer cells, including pancreatic ductal adenocarcinoma (PDAC) (328). Hence, the recognition of this cytosolic trinity of PRRs detecting cytosolic DNA has explained the previously unknown mechanisms of drugs used in clinics, which can be used in the future for other diseases depending on the involvement of these PRRs in the diseases. Also, the STING-based biosensor called BioSTING has been developed to detect CDNs in eukaryotic cells that will prove beneficial in diagnosing different cancers and other inflammatory diseases, including autoimmune or autoinflammatory ones (329). However, caution should be taken to use cGAS and TLR9-based adjuvants as their overactivation is associated with different autoinflammatory or autoimmune diseases (ADs) and other sterile inflammatory conditions. Hence, the homeostasis of the cellular galaxy is maintained till the sleeping status of the trinity of cytosolic PRRs is maintained, their aggressive awakening in response to indigenous DAMPs proves lethal to the cell causing its death (apoptosis, lysosomal cell death, pyroptosis, and necroptosis) and induces different immune-mediated inflammatory diseases.

AUTHOR CONTRIBUTIONS

The author has developed the idea, designed, and wrote the manuscript, along with designing and developing the figures.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GLOSSARY

AA	Amino acid
AAA	Abdominal aortic aneurysm
Ads	Autoimmune or autoinflammatory diseases
AGO2	Argonaute RISC Catalytic Component 2
AGS	Aicardi-Goutières syndrome
aHSCT	allogenic hematopoietic stem cell transplantation
AIM2	Absent in melanoma 2
ALRs	AIM-2-like receptors
ALD	Alcohol-related liver disease
AMP	Adenosine monophosphate
ARF	ADP ribosylation factor
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
Arp2/3 complex	Actin-related proteins-2/3 **complex**
ATG5	Autophagy-related protein 5
Atg9a	Autophagy-related protein 9a
ATG16L1	Autophagy-related protein 16 like 1
BAF	Barrier-to-autointegration factor 1
BCR	B cell receptor
BD	Behcet disease
BECN1	Beclin1
bp	Base pairs
BPD	Bronchopulmonary dysplasia
BS	Bloom syndrome
Btk	Bruton's tyrosine kinase
CARD15	Caspase recruitment domain-containing protein 15
CASP1	Caspase 1
cGAS	cyclic GMP-AMP synthase
CDK	Cyclin-dependent kinase
c9orf72	chromosome 9 open reading frame 72
CLP	Cecal-ligaiton and puncture
CLRs	C-type lectin-like receptors
COPI or II	Coat protein complex-I or II
COPA	Coatomer protein subunit alpha
CREB	cyclic response element-binding protein
CTT	C-terminal tail
DDR	DNA damage response
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
DNA-PK	DNA-dependent protein kinase
DNMT1	DNA (cytosine-5)-methyltransferase 1
EAE	Experimental autoimmune encephalitis
FCVs	E cigarette vapours
EIA	Erosive inflammatory arthritis
ERGIC	Endoplasmic reticulum Golgi-intermediate compartment
ESCRT	Endosomal sorting complex required for transport
EV71	Enterovirus 71
FTD	Frontotemporal dementia
GMP	Guanosine monophosphate
GSDMD	Gasdermin D
GVHD	Graft-versus-host disease
HDAC3	Histone deacetylase 3
HFMD	Head, foot, and mouth disease
HIN	Hematopoietic expression interferon-inducible nature, and nuclear localization
HMGB1	High mobility group box protein 1
HNPCC	Hereditary nonpolyposis colorectal cancer
HOMA-IR	Homeostasis model assessment of insulin resistance
Iba1	Ionized calcium-binding adapter molecule 1
ICAM-1	Intercellular adhesion molecule-1
IFI16	Gamma-interferon-inducible protein Ifi-16
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit β
Imd	Immune cell deficiency
IRAK1	Interleukin-1 receptor-associated kinase-1
IRAK-4	Interleukin-1 receptor-associated kinase-4
IRE1α	Inositol-requiring transmembrane kinase/endoribonuclease 1α
IRF3	Interferon regulatory factor 3
IRGM	Immunity-related GTPase M
LC3	Microtubule-associated protein 1 light chain 3
LSM11	U7 small nuclear RNA associated protein
LysRS	Lysyl-tRNA synthetase
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MAVS	Mitochondrial antiviral signaling protein
MB21D1	MAB-21 domain containing protein 1
MCP1	Monocyte chemotactic protein 1
MNDA	Myeloid cell nuclear differentiation antigen
MS	Multiple Sclerosis
MST1	Mammalian Sterile 20-like kinases 1
MYA	Million years ago
MYSM1	Myb-like, SWIRM, and MPN domains 1 protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEAT1	Nuclear paraspeckle assembly transcript 1
NEMO	NF-κB essential modulator
NET	Neutrophil extracellular trap
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NONO	Non-POU Domain Containing Octamer Binding
NSCLC	Non-small cell lung cancer
nts	nucleotides or nucleotide sequence
OCT	Optical coherence tomography
ODNs	Oligodeoxyribonucleotides
PA	Palmitic acid
PDL-1	Programmed death ligand 1
PI3K	Phosphoinositide 3-kinase
PINK1	Phosphatase and tensin homolog-induced kinase 1
PIP2	Phosphatidylinositol 4,5 biphosphate or PtdIns(4,5)P2
PLD3 and 4	Phospholipase D3/4
PRKDC	Protein Kinase
DNA-Activated	Catalytic Subunit
PYCARD	PYD and CARD containing protein
PYHIN1	Pyrin and HIN domain-containing protein 1
RIG-1	Retinoic acid inducible gene-1
RISC	RNA-induced silencing complex
RLRs	RIG-1-like receptors
RIP2	Receptor interacting protein 2
RNU7-1	U7 small nuclear 1
SASP	Senescence-associated secretory phenotype
SAVI	STING-associated vasculopathy with onset in infancy
SCC	Squamous cell carcinoma
SHIP-1	SH-2 containing inositol 5' polyphosphatase 1
SREBP2	sterol regulatory element-binding protein 2
STING	Stimulating interferon genes
STK11	Serine-threonine kinase 11
TAB 1	TAK1 binding protein 1
TAB2	TAK1 binding protein 2
TAK1	Transforming growth factor-β associated kinase 1
TANK	TRAF-associated NF-κB activator
TBK1	TANK-binding kinase 1
TFAM	Mitochondrial transcription factor A
TMEM203	Transmembrane protein 203
TMS1	Target of Methylation-induced Silencing-1
TOLLIP	Toll-interacting protein
TRAF6	TNFR-associated factor 6
TREX1	Three prime repair exonuclease 1
ULK	Unc-51-like kinase
UNC93B1	Unc-93 Homolog B1

Continued

UPR	Unfolded protein response
VPS34	vacuolar protein sorting 34
WASP	Wiskott-Aldrich syndrome protein

(Continued)

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WD40 CTD	WD repeat-containing C-terminal domain
WIPI2	WD repeat domain phosphoinositide-interacting *protein* 2
WT	Wild type
YAP	Hippo-Yes-associated protein

frontiers in Immunology

MINI REVIEW
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Cellular Metabolites Regulate Central Nucleic Acid Sensing Pathways

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Detection of pathogen-derived DNA or RNA species by cellular nucleic acid sensors prompts release of anti-microbial interferons and cytokines. In contrast to their protective anti-microbial functions, inappropriate or excessive activation of nucleic acid sensors can cause inflammatory diseases. Nucleic acid sensing is therefore tightly controlled by regulatory factors acting through both transcriptional and post-transcriptional mechanisms. Recently, it has become clearer that metabolic pathways—previously thought to be unconnected with immune responses—can influence nucleic acid sensing. This regulation can be observed when immune system cells undergo metabolic reprogramming in response to stimulation with pathogen-associated molecular patterns such as lipopolysaccharide from gram negative bacteria. Metabolic reprogramming leads to accumulation and secretion of metabolites, which have been mostly viewed as end-products of processes providing cellular energy and building blocks. However, metabolites have now been identified as important regulators of nucleic acid sensing. This mini-review aims to outline current knowledge on regulation of central nucleic acid sensing pathways by metabolites during metabolic reprogramming.

Keywords: metabolites, lactate, succinate, itaconate, MAVS, STING, toll-like receptors

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INTRODUCTION

The innate immune response employs a range of pattern recognition receptors (PRRs) to detect danger-associated molecular patterns and conserved microbial pathogen-associated molecular patterns (PAMPs) to trigger defense mechanisms (1). Nucleic acids derived from infectious pathogens are central PAMPs that can be detected by a selection of PRRs—the nucleic acid sensors. Nucleic acid sensing occurs through membrane-associated receptors of the Toll-like receptor (TLR) family and through the sensors of cytosolic nucleic acids RIG-I-like receptors (RLRs), cyclic GMP-AMP synthase (cGAS), and absent in melanoma 2. Ligation of these sensors induces expression of both type I interferons (IFNs) and pro-inflammatory mediators. Although the nucleic acid sensors are critical for protection against infection, they can also be the cause of inflammatory diseases (2). These sensors and their downstream effectors are thus subject to important regulatory mechanisms aimed at preventing excessive pro-inflammatory signals. Regulation has already been described to target nucleic acid sensing through proteasomal degradation of central signaling components, through pre- and post-transcriptional regulation of expression and through enzymatic degradation of the activating nucleic acid agonists (3). Intriguingly, a novel class of underappreciated regulators of nucleic acid sensing has recently emerged (**Figure 1**). These regulators are metabolites derived from metabolic pathways and processes providing energy and building blocks for basic cellular processes. In particular, these metabolites accumulate during a process now known as metabolic reprogramming.

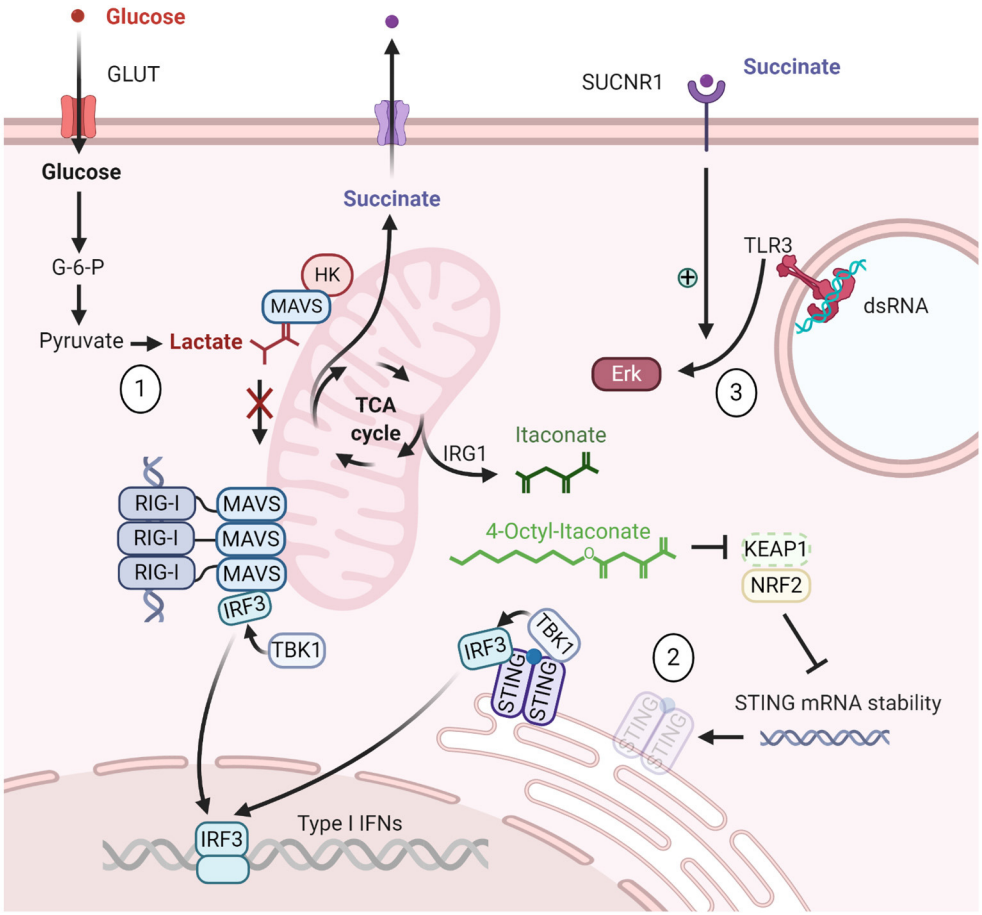


FIGURE 1 | Overview of metabolites regulating nucleic acid sensing pathways. (1) Lactate inhibits the RIG-I-MAVS pathway. (2) 4-Octyl-itaconate inhibits the stability of the STING mRNA. (3) Succinate enhances Erk signaling through TLR3. Created with BioRender.com.

Metabolic reprogramming as a phenomenon was first identified in cancer cells, which display increased consumption of glucose and release of lactate—also known as the Warburg effect. It is now clear that metabolic reprogramming also occurs in other cells. For example, immune cells such as macrophages and dendritic cells (DCs) undergo metabolic reprograming upon stimulation with bacteria-derived ligand lipopolysaccharide (LPS) and ligands mimicking microbial RNA. (4–7). There is evidence that DNA also induces metabolic reprogramming of immune cells. For instance, oxidized DNA from extracellular vesicles formed by the *T. cruzi* parasite causes proinflammatory reprogramming of macrophages (8). Furthermore, TLR9 mediates activation and metabolic reprogramming of plasmacytoid DCs toward glycolysis which is important for their type I IFN production (9).

Metabolic reprogramming is characterized by an elevated consumption of glucose, a disruption of the tricarboxylic acid (TCA) cycle and increased formation of a series of metabolites—most notably lactate, succinate, and itaconate (4–7, 10). Lactate is predominantly formed from pyruvate through the enzymatic control of lactate dehydrogenase (LDHA) (11). Although lactate levels in serum are used as a prognostic tool in many critical medical conditions (10), its potential to affect immune responses has largely been ignored. Intriguingly, studies now highlight lactate as an important cellular regulator of innate and adaptive immunity. Lactate has been shown to support cancer cell proliferation acting as an alternative source to feed the mitochondrial oxidative phosphorylation for efficient ATP production, it can also be metabolized into lipids, or even metabolized by the mitochondrial lactate dehydrogenase B for respiration (12). Also, lactate accumulating in melanomas suppresses T and natural killer (NK) cells function and survival, and thus enables tumors to escape T and NK cell-mediated tumor surveillance (13). Furthermore, tumor-derived lactate promotes tumor growth and M2-directed polarization of tumor-associated macrophages (14). This turns the tumor microenvironment into a more immunosuppressive state and thus enables tumors to evade immune responses.

Succinate is synthesized within the mitochondrial matrix as part of the TCA-cycle. Succinate links the TCA-cycle with the electron transport chain as succinate dehydrogenase (SDH, a.k.a. succinate-ubiquinone oxidoreductase or Complex II) transfers an electron from succinate to ubiquinone generating fumarate from succinate in the process. Succinate accumulates in

LPS-stimulated macrophages (5, 15), but the effects of increased intracellular succinate levels on inflammatory responses remain unclear. While the succinate derivative dimethyl-succinate seems to increase relative expression of the pro-inflammatory cytokine interleukin 1β (IL-1β) (5), the cellular accumulation of endogenous succinate seems to occur after and not before LPS-induced IL-1β (15). Similarly, in experiments by Harber et al., dimethyl-succinate was found to trigger anti-inflammatory responses through a mechanism operating independently of its receptor succinate receptor 1 (SUCNR1) (16). By contrast, unmodified succinate secreted into the extracellular space is now demonstrated to activate pro-inflammatory pathways through the cell surface SUCNR1 which is highly expressed in macrophages (17) and DCs (16). Therefore, it is still unclear to what extend the pro- or anti-inflammatory effects of unmodified endogenous succinate overlap with those demonstrated for modified variants of succinate. Like other metabolites, succinate also accumulates in cancer cells. This accumulation favors cancer progression since it induces epigenetic alterations, alters cancer cell metabolism, promotes epithelial-to mesenchymal transition, migration and invasion, and promotes angiogenesis (18).

Itaconate, is a TCA-cycle-derived metabolite found to accumulate in several models of inflammation including Mycobacterium tuberculosis-infection (19) and LPS-treated macrophages (20). Itaconate is formed from enzymatic conversion of cis-aconitate by the immune responsive gene 1 (IRG1) (21). Expression levels of IRG1 and of itaconate are low to absent in resting macrophages but are highly induced through TLR stimulation with LPS (22). Lampropoulou et al. (20), then described how addition of the itaconate derivative dimethyl-itaconate (DI) inhibited infection-induced inflammatory cytokines and affected macrophage differentiation/activation by downregulating proinflammatory transcripts and inhibiting the inflammasome.

Many advances have been made in the past few years toward recognizing the importance of altered metabolism in health and disease. Metabolic changes affecting multiple cells in pathologies, such as obesity and cancer, confirm that there is a surging focus on improving our understanding of the interphase of metabolism and innate immune responses. Obesity is characterized by metabolic stress due to elevated levels of free fatty acids. In this condition, palmitic acid can provoke leakage of mitochondrial DNA into the cytosol. This activates the cGAS-STING pathway to induce the expression of intercellular adhesion molecule 1 and monocyte-endothelial cell adhesion, which results in endothelial inflammation (23). The link between metabolic changes in obesity and nucleic acid sensing underlines the importance of regulation of nucleic acid sensing by metabolites. Therefore, this mini-review outlines novel discoveries on how cellular metabolites influence nucleic acid sensing.

NUCLEIC ACIDS SENSORS

At steady state, positive and negative regulators secure an appropriate balance between immune activation and suppression. The mechanisms that control nucleic acid sensing include regulation of ligand availability and posttranslational modifications of both the nucleic acid sensors and the signaling adaptor proteins (3). Traditionally, metabolites derived from cellular pathways such as glycolysis and the TCA-cycle were viewed solely as end-products of cellular processes. Interestingly, there is now evidence that metabolites can affect nucleic acid sensing pathways as well and alter the immune response.

TLRs

In the year 2000, the discovery that TLR9 binds and detects DNA marked the first report of nucleic acid sensing by innate cellular PRRs (24). Quickly thereafter, several other nucleic acid sensors were identified among the TLR family members including, TLR3 (25), 7 and 8 (26) which detect pathogen-derived RNA in endosomes. TLR9 and TLR7/8 signal through myeloid differentiation primary response 88 (MyD88) whereas TLR3 signals through TIR-domain-containing adaptor-inducing interferon-b (TRIF) to induce type I IFNs and pro-inflammatory cytokines (2).

As mentioned above, itaconate is strongly induced upon macrophage activation through TLR stimulation. Subsequently, several studies reported strong anti-inflammatory effects of cell permeable derivatives of itaconate including dimethyl-itaconate (DI) and 4-Octyl-itaconate (4-OI) (20, 27). At least to some extent, these effects have been confirmed using unmodified itaconate (15). Mills et al. (27), observed that upon TLR signaling, itaconate is also able to downregulate IL-1β formation through activation of the kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2 (KEAP1/NRF2) anti-oxidant response.

It has also been observed that immune responses upon TLR signaling can be enhanced by interaction of succinate with its receptor SUCNR1. It was previously observed by He et al. (28), that succinate can activate extracellular signal-regulated kinase (Erk), a downstream component of the TLR pathways. Therefore, Rubic et al. (16), investigated whether succinate had an effect directly on the TLRs. They observed that Erk1/2 phosphorylation was slightly induced by the TLR3 stimulator poly I:C alone, but enhanced when succinate was simultaneously present. Consequently, they reported that in combination with some nucleic acid analogs like poly I:C (TLR3 agonist) or imiquimod (TLR7 agonist), succinate potentiates production and secretion (by direct post-transcriptional effect) of pro-inflammatory mediators such as IL-1β and TNFα.

RIG-I—MAVS

The RNA sensors RIG-I and melanoma differentiation-associated protein 5 (MDA5) detect double stranded (ds) RNA to initiate an antiviral response through the signaling adaptor mitochondrial antiviral signaling (MAVS) (29–34). RIG-I is kept in a closed confirmation in absence of RNA. However, upon dsRNA binding, a structural change occurs that enables RIG-I signaling through MAVS (3). The IκB kinase β (IKKβ) or TANK-binding kinase 1 (TBK1) phosphorylate MAVS and this enables IFN-regulatory factor 3 (IRF3) recruitment. TBK1 phosphorylates the recruited IRF3—and phosphorylated IRF3 homodimerizes and translocates to the nucleus where it induces type I IFN

expression (35). Apart from IRF3, MAVS activation also leads to phosphorylation of IκBα (33) and release of nuclear factor κB (NF-κB) that will translocate to the nucleus and mediate expression of proinflammatory genes.

A recent study established a link between this nucleic acid sensing pathway and cellular metabolism. Zhang et al. (36), noted a decrease in the levels of distinct metabolites associated with the glycolytic process—phosphoenolpyruvate, pyruvate, and lactate—during RLR-stimulation, indicating that upon RLR-activation, glucose metabolism is inhibited.

The underlying mechanism is based on the exclusion of hexokinase 2 (HK2) from the mitochondria upon RLR activation. HK2 mediates the conversion of glucose to glucose-6-phosphate and its inhibition thus prevents the first step in glycolysis. In this manner, RLR activation inhibits glycolytic flux through inhibitory displacement of HK2 from the mitochondria. The authors next asked why prevention of glycolysis was necessary for optimal induction of type I IFN by RIG-I. Here, they discovered that lactate, the end product of anaerobic glycolysis, inhibited RLR signaling. Thus, inhibition of glycolysis was necessary to prevent the inhibitory effect of lactate. Co-precipitation experiments and domain mapping showed that lactate directly interacted with the transmembrane domain of MAVS and that this modification of MAVS inhibited its polymerization and subsequently the downstream induction of type I IFNs. Thus, lactate is a metabolite that inhibits RLR-signaling, hereby connecting anaerobic glycolysis to nucleic acid sensing (36).

There is also evidence that lactate suppresses RLR-signaling in a more physiologically relevant context *in vivo*. Using the Hepatitis B virus (HBV) hydrodynamic injection (HI) mouse model, Zhou et al. (37), observed an increase in pyruvate and lactate production by HBV *in vivo*. By administrating sodium oxamate with the aim to reduce lactate production, they measured decreased viral-DNA levels in serum and reduced HBV-replication intermediates in mouse liver tissues. Accordingly, increasing lactate levels by administration of sodium lactate resulted in an opposite effect. In addition, administration of sodium lactate in mice reduced production of IFN-b. By testing the HBV HI mouse model in WT/*Ifnar*$^{+/+}$ and *Ifnar1*$^{-/-}$ mice, they demonstrated that HBV evades the immune response by suppressing RLR-mediated IFN production in a manner dependent on LDHA-mediated lactate production (37). This could be an important mechanism for the regulation of anti-viral responses and an obvious target of human pathogenic viruses to prevent optimal induction of type I IFNs, also a mechanism that could be utilized by cancer cells to avoid IFN-stimulation of immune cells.

cGAS-STING

In 2006, Ishii and colleagues reported that transfection of dsDNA into living cells induced an antiviral response, that required TBK1, but was independent of TLRs. They furthermore observed that the response to dsDNA yielded protection against both DNA and RNA viruses (38). It is now known that cGAS is a central sensor of cytosolic DNA that relays its signal to the signaling adaptor protein stimulator of interferon genes (STING) through the generation of the second messenger cyclic GMP-AMP (cGAMP) (39, 40). Upon cGAMP-binding, STING dimers undergoes major structural changes that allow it to stack side-by-side into tetramer and higher-order oligomer structures—a process that depends on palmitoylation of specific STING residues (41–45). These changes allow TBK1 dimers to activate each other by autophosphorylation (46, 47). Activated TBK1 phosphorylates STING and phosphorylated STING serves as a docking site for IRF3. TBK1, in turn, phosphorylates IRF3. This phosphorylation enables IRF3 to homodimerize and translocate to the nucleus where it activates the transcription of type I IFNs (35).

The cGAS-STING pathway has recently been demonstrated to be suppressed during metabolic reprogramming. This suppression was mediated through decreased expression of STING itself (48). More specifically, activation of the transcription factor NRF2 during metabolic reprogramming suppresses STING expression through a mechanism that could depend on mRNA stability. The NRF2-inhibitor protein, KEAP1, targets NRF2 for proteasomal degradation at steady state conditions. However, several stimuli inactivate KEAP1 allowing NRF2 to translocate to the nucleus. This induces the transcription of NRF2 target genes that protect cells from death and regulate inflammatory responses. Olagnier et al. (48), showed that upon metabolic reprogramming by TLR4/7-activation and in response to the itaconate derivative 4-OI, NRF2 suppresses STING expression. Thus, cellular metabolites that induce NRF2 activation can in this manner indirectly target nucleic acid sensing through decreasing the expression of STING.

Nitro-fatty acids are another group of metabolic by-products that are formed during infection and target the cGAS-STING signaling pathway (**Figure 2**). Nitro-fatty acids are electrophilic elements that react with nucleophilic donors such as cysteines and thiols (49, 50). Mechanistically, the nitro-fatty acids modify STING post-translationally through nitro-alkylation at cysteines 88 and 91. This modification prevents palmitoylation of STING, that is necessary for optimal STING activity, and thus inhibits it signaling. Interestingly, nitro-fatty acids inhibited release of type I IFN in cells from patients with STING-associated vasculopathy with onset in infancy (SAVI)—a genetic disorder caused by gain-of-function mutations in STING (51, 52). Thus, these metabolic by-products could be important regulators of nucleic acid sensing and possibly be utilized as therapeutics in STING-dependent inflammatory conditions.

IMPLICATIONS AND CONCLUSIONS

Our current understanding of immunity to infection is dominated by aggressive and highly inflammatory processes. Although these processes and pathways are effective in eliminating pathogens—they are also highly destructive and result in high degrees of collateral damage to otherwise healthy tissues. Recent advances suggest that basal cellular metabolic processes play a so far underappreciated role in the regulation of such damaging responses. Although great advances have been made in this field over the last handful of year, we have still

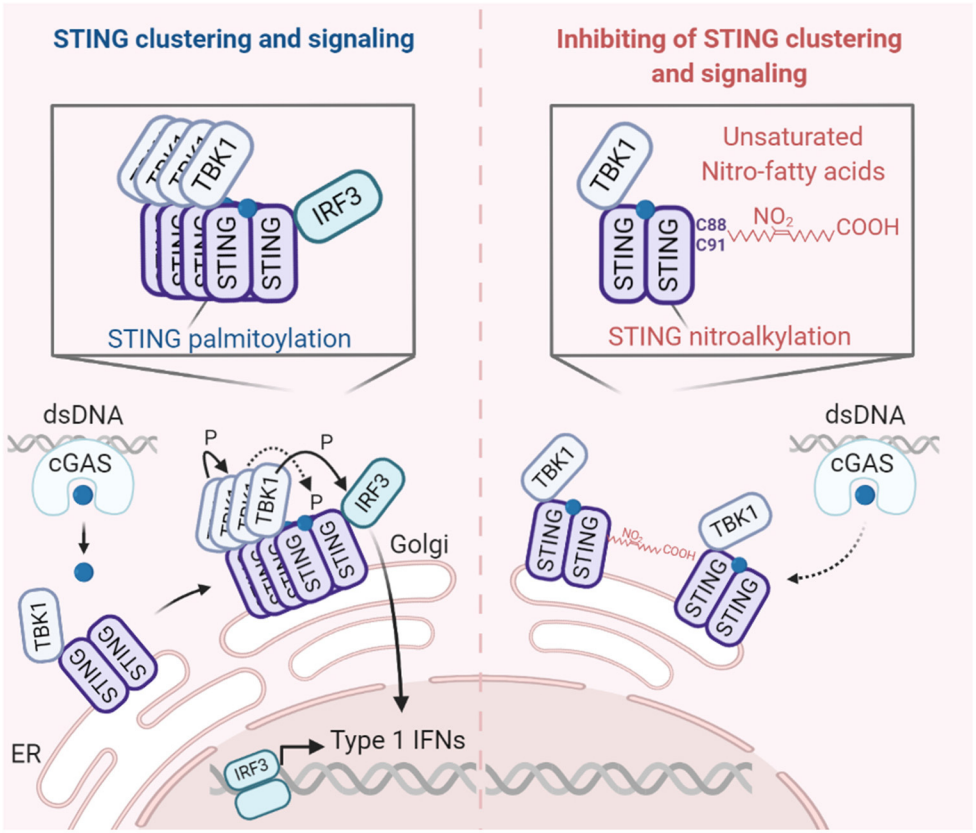


FIGURE 2 | Nitro-fatty acids inhibit STING palmitoylation and signaling. Upon detection of dsDNA, cGAS catalyzes the formation of 2′3-cGAMP. STING resides in the ER as a dimer bound to TBK1. Upon cGAMP-binding, the STING dimers change conformations and stack side-by-side to form oligomers. TBK1 dimers, in turn, autophosphorylate and activate each other. Activated TBK1 phosphorylates adjacent STING dimers. Phosphorylated STING in turn serves as a docking site for IRF3. Upon phosphorylation by TBK1, phosphorylated IRF3 monomers disassociate from STING and homodimerize. The homodimer works as a transcription factor to induce type I IFNs. Palmitoylation of STING in the Golgi is important for STING clustering and signaling. Importantly, nitro-fatty acids inhibit STING palmitoylation and signaling. Created with BioRender.com.

only begun to elucidate the regulatory interphase of cellular metabolism and immunity. Metabolites that were previously thought to not affect immunity are now emerging as important regulators of both innate and adaptive immune responses.

Since many pathologies, such as obesity and cancer, carry metabolic alterations, there is a huge treatment potential in reshaping the metabolism in such conditions. For example, Pankowicz et al. (53) were able to treat hereditary tyrosinemia in mice, a metabolic liver disorder, by targeting the metabolic enzyme hydroxyphenylpyruvate dioxygenase rather than the disease-causing gene. This successfully demonstrates the usefulness of regulating changes in metabolism that could be an effective treatment strategy in other diseases.

In line with this, we have presented studies that identify metabolites as important regulators of nucleic acid sensing pathways pointing to a therapeutic potential of metabolites in inflammation. For instance, in the context of viral infections, it could have a great potential to target virus-induced production of lactate that inhibits the antiviral RLR-MAVS-IFN axis. Many viruses, such as SARS-CoV2 and influenza, cause disease by inducing general inflammation. In these cases, it could be fruitful to limit the disease-causing virus-induced inflammation by targeting nucleic acid sensing with metabolites that dampen the inflammation. Likewise, in inflammatory conditions, it could be beneficial to reduce inflammation by blocking STING signaling using for example nitro-fatty acids.

There is evidence indicating a cross talk between different pattern recognition pathways. For instance, LPS-induced inflammation and metabolic reprogramming through TLR4 lead to accumulation of metabolites, including lactate. Lactate, in turn, inhibits the RLR-MAVS pathway, which results in an anti-inflammatory effect. Such cross talk is important to keep in mind when we aim to modulate immune responses using metabolites.

The future is bound to reveal many more connections between metabolism and nucleic acid sensing that will greatly advance our understanding of how immune responses are kept in check. It is likely that these efforts will also lead to the identification of metabolites and metabolic pathways that can be targeted to either promote or suppress immune responses depending on the context and whether the goal is to increase immunity to cancer and infection or prevent this in the context of inflammatory diseases.

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REVIEW
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Cyclic Guanosine Monophosphate–Adenosine Monophosphate Synthase (cGAS), a Multifaceted Platform of Intracellular DNA Sensing

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Innate immune pathways are the first line of cellular defense against pathogen infections ranging from bacteria to Metazoa. These pathways are activated following the recognition of pathogen associated molecular patterns (PAMPs) by membrane and cytosolic pattern recognition receptors. In addition, some of these cellular sensors can also recognize endogenous danger-associated molecular patterns (DAMPs) arising from damaged or dying cells and triggering innate immune responses. Among the cytosolic nucleic acid sensors, the cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) synthase (cGAS) plays an essential role in the activation of the type I interferon (IFNs) response and the production of pro-inflammatory cytokines. Indeed, upon nucleic acid binding, cGAS synthesizes cGAMP, a second messenger mediating the activation of the STING signaling pathway. The functional conservation of the cGAS-STING pathway during evolution highlights its importance in host cellular surveillance against pathogen infections. Apart from their functions in immunity, cGAS and STING also play major roles in nuclear functions and tumor development. Therefore, cGAS-STING is now considered as an attractive target to identify novel biomarkers and design therapeutics for auto-inflammatory and autoimmune disorders as well as infectious diseases and cancer. Here, we review the current knowledge about the structure of cGAS and the evolution from bacteria to Metazoa and present its main functions in defense against pathogens and cancer, in connection with STING. The advantages and limitations of *in vivo* models relevant for studying the cGAS-STING pathway will be discussed for the notion of species specificity and in the context of their integration into therapeutic screening assays targeting cGAG and/or STING.

Keywords: CGAS, STING, innate immunity, evolution, *in vivo* models

INTRODUCTION

Type I interferons (IFNs) can be secreted by a wide range of immune and non-immune cells in response to various biological stimuli [danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs)] that activate nuclear, cytosolic, or membrane-anchored nucleic acid sensors (1, 2). Discovery and characterization of these specialized receptors, which trigger innate immune responses, started in early 2000 with the description of lipopolysaccharides (LPS) and CpG sensing by TLR4 and TLR9, respectively (3, 4). Since then, extensive investigations have been conducted to identify cytosolic DNA receptors classified as DNA sensor based on DNA binding activity and activation of innate immune responses (5). Recent evidence highlighted their diversity in terms of structure/function, patterns of expression, and signaling pathway (5). This raises important questions on the existence of ligand specificity, the impact of the tissue environment, and the orchestration of overlapping DNA signaling pathways (6). Indeed, numerous DNA sensors have been identified, which belong to PYHIN proteins (HIN200 domain-containing proteins) such as interferon gamma-inducible protein 16 (IFI16) and absent in melanoma 2 (AIM2); to DExH-box helicases (DHX9 and DHX36) or DEAD-box helicase family (DDX41) and to proteins involved in responses to DNA damage (MRE11, or Rad50 and DNA-PK). In addition, DNA-dependent activator of IFN regulatory factors (DAI), RNA polymerase III (RNA pol III), and LRR binding FLII interacting protein 1 (LRRFIP1) were also involved in DNA sensing and type I IFN response [for reviews (5, 7)]. However, the depletion of some of these sensors (DDX41 or DAI) in mouse or cellular models does not always correlate with an impact on DNA-stimulated type I IFN response, which highlights the need for further studies (5). Finally, the cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) synthase (cGAS) has emerged as central to the mounting of nucleic acid-dependent IFN responses *in vivo* (8). It is involved in the detection of a wide range of cytosolic DNA ligands from self and non-self origins. Association of human cGAS (also known as C6orf150 encoded by *MB21D1*) with dsDNA catalyzes the production of cyclic cGAMP. Of note, ssDNA (9) and RNA:DNA hybrids (10) have been shown to activate cGAS leading to cGAMP production. This second messenger triggers the activation of innate immune responses by binding to the adaptor protein STING (also known as MITA, ERIS, or MPYS, encoded by *TMEM173*). STING recruits the TANK-binding kinase 1 (TBK1) and the inhibitor of nuclear factor kappa-B *kinase* subunit epsilon (IKKε) and activates the IRF3 and the nuclear factor kappa (NF-κB)-light-chain-enhancer of activated B cells transcription factors (11, 12). The exact location of STING/TBK1 interaction is still in debate. Induction of the cGAS-STING pathway culminates in the synthesis of type I IFN and pro-inflammatory cytokines (13, 14). Notably, activation of cGAS-STING pathway leads to the establishment of an IFN-based and IFN-independent innate immune response (15–17).

Fine-tuning of the cGAS-STING pathway is necessary to initialize and resolve inflammatory processes, maintain tissue homeostasis, fight against pathogen infections (i.e., bacteria, viruses, and parasites), and modulate the immunity of the tumor microenvironment (toward tumor suppression or tumor and metastasis development in a different context) (18). Therefore, the role of cGAS-STING in auto-inflammatory and autoimmune diseases has been established leading to a chronic activation of the IFN pathway, which can be detrimental (16). This includes inflammatory syndromes such as STING-associated vasculopathy with onset in infancy (SAVI), Aicardi–Goutières syndrome (AGS), and familial chilblain lupus (19–23), but also cGAS related genetic disorders such as TREX1 associated lupus-like autoimmune disorder (24) or Bloom syndrome (19). Systemic inflammation triggers complex pathological phenotypes with multi-organ damages. Although ubiquitously expressed, a growing body of evidence demonstrates the existence of cell- and tissue-related variability in the expression pattern of cGAS-STING (25) as described for IFN and interferon-stimulated genes (ISG) from mammals to zebrafish (26–29). cGAS and STING expressions are IFN-inducible and are involved in the regulation of the type I IFN feedback loops (30). According to the tissue distribution described in the Human Protein Atlas (http://www.proteinatlas.org), *MB21D1* and cGAS protein are ubiquitously expressed with particularly high expression in epithelial cell types in the genital tract or the lungs as well as in hematopoietic cells and dendritic cells, with *TMEM173*/STING presenting a quite comparable distribution pattern (31, 32). In contrast, primary human hepatocytes express low levels of cGAS and STING (33, 34). One putative explanation would be that low cGAS and STING expression would avoid overactivation of this pathway during hepatocyte renewal, which leads to DNA accumulation in the cytoplasm (34, 35). Recent evidence suggests a complex interplay between cGAS and STING in the liver involving multiple cell types, as it has been suggested that cGAMP could be transferred from hepatocytes to liver macrophages (expressing high levels of STING) through gap junctions (36, 37).

In addition, the cGAS-STING pathway has been involved in cancer immunity and the development of immunotherapies. The extensive works carried out to understand the correlation between expression of cGAS/STING and cancer will not be discussed in this review but recently presented in (18, 38).

A better understanding of the cGAS-STING multifaceted platform is required to improve our knowledge of the orchestration of innate immune responses mediated by diverse nucleic acid sensors, activated by self and non-self motifs in a tissue-specific manner. Animal models are critical to predict physiologically relevant functions of the cGAS-STING pathway *in vivo* taking into account the cell and tissue environments in different physiological states (16). Despite an important evolutionary conservation of the cGAS-STING functions in innate immunity, recent data have highlighted certain species specificities, which must be considered when using biomedical models for the identification of biomarkers or therapeutic screening for human health (8, 39, 40). In this review, we depict the evolution and the broad biological functions of the cGAS-STING DNA sensing platform in pathogen recognition, immune activation, and cancer development, as well as its potential for the development of novel therapeutic strategies.

ORIGIN AND EVOLUTION OF THE MOLECULAR MECHANISMS OF THE NUCLEIC ACIDS—cGAS-STING INTERACTIONS

cGAS is composed of a flexible and poorly conserved N-terminal domain and a highly conserved C-terminal catalytic domain composed of nucleotidyltransferase (NTase) core and Mab21 domains [reviewed in (41) and (42)]. The sequence-independent DNA sensing activity contained a positively charged surface and a zinc-ribbon domain. Upon activation, the cGAS dimer exposes a catalytic site formed by a caged tertiary structure composed of typical alpha helices (ligand-binding surface) and the nucleotidyltransferase core domain. Binding of mislocated or infectious cytosolic DNA to cGAS catalyzes the production of 2′-5′/3′-5′ cyclic GMP–AMP, the 2′3′-cGAMP second messenger (**Figure 1**) (43). pppGp(2′-5′)G or 2′,3′-c-di-GMP were also detected as minor products in the absence of ATP (43). Structural homologs of human cGAS have been identified in animals and bacteria. In eukaryotes, it includes metazoans and human proteins such as the antiviral oligo adenylate synthase 1 (OAS1), which produces 2′,5′-oligoadenylate (2–5A) upon sensing of the cytosolic double-stranded RNA. The 2–5A ligand further activates the endoribonuclease RNase L, leading to RNA degradation. In bacteria, the dinucleotide cyclase DnCV of *Vibrio cholerae* is considered to be a founding member of a large family of cGAS homologs, which synthesizes 3′-3′-cGAMPs as well as trinucleotides and oligonucleotides in absence of activation (44). Overall, the structure of the unique catalytic site, which ensures nucleotidyltransferase and dinucleotide cyclase activities in a sequential fashion (43), is an important conserved feature despite low sequence homologies. Is oligomerization necessary for activation? The answer is not really clear, although it clearly contributes to regulate the enzymatic function (42).

Functional characterizations of the nucleotide synthesized by the cGAS-related proteins have been conducted in eukaryotes and bacteria. While cGAMP second messenger triggers innate immunity in mammalian cells by binding to STING, bacterial nucleotides can be recognized not only by phospholipases and riboswitches but also by nucleases, proteases, or pore-forming effectors. Moreover, microbial DNA, cyclic dinucleotides, and host DNA (either mitochondrial- or mislocated self-DNA) were identified as STING ligands capable of inducing the activation

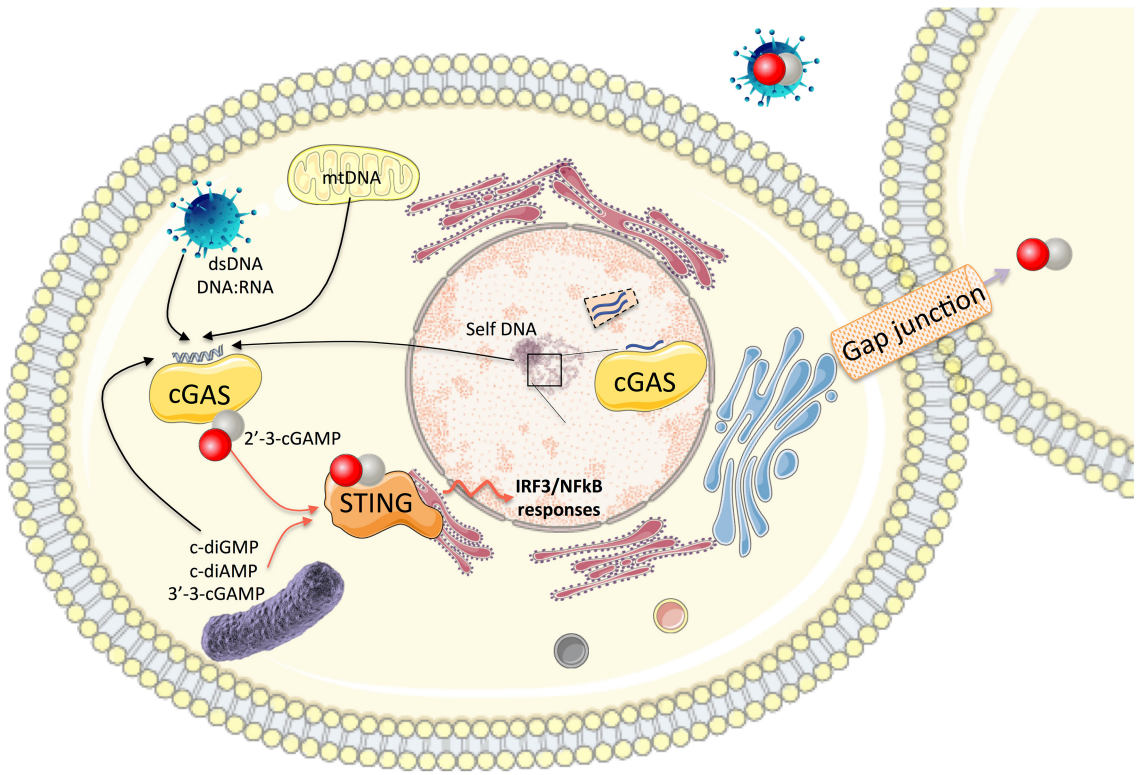


FIGURE 1 | In the cytosol, the association of cGAS with self and non-self cytosolic nucleic acid substrates catalyzes the production of 2′-3′-cGAMP. This second messenger binds to the adaptor protein STING and activates the IRF3 and NF-κB transcription factors for the synthesis of type I IFN and pro-inflammatory cytokines. cGAMP can be transported to neighboring cells through gap junctions or integrated into viral particles, leading to autocrine or paracrine activation of STING. Bacterial dinucleotides (c-diGMP, cdiAMP, and 3′-3′-cGAMP) are also ligands of STING. cGAS also impacts nuclear functions (impairment of DNA repair, genome destabilization, and synthesis of micronuclei). Nuclear cGAS discriminates between self and non-self DNA by binding to chromatin (preventing its activation) or interaction with nuclear proteins such as NONO in response to viral infection to trigger innate immune responses.

of the innate immune response in Metazoa (**Figure 1**) (45). A recent study elegantly dissecting the STING-dependent pathway characterized functional STING homologs in bacteria and demonstrated the conservation of a prokaryotic cGAS-STING-like pathway playing a role in the antiviral defense against bacteriophages (46). STING is an ER protein composed of four transmembrane domains and a cytosolic domain formed by an alpha helix, a cyclic dinucleotide binding domain (CBD), and a C- terminal tail (CTT) carrying the binding sites for TBK1 and IRF3. Phylogenetic comparisons of invertebrate and vertebrate STING versions highlighted that ray-finned fish acquired a signaling module at the extremity of the CTT domain mediating TRAF6 interaction and promoting the transcription of NfκB responsive elements (47), while the sea anemone (*Nematostella vectensis*) lacks the CTT region (11). Functional characterization of STING from diverse animal lineages showed that purified CBD domains from vertebrates could bind 2′-3′-cGAMP. Recognition of 3′,3′-dinucleotides was restricted to mammalian STING orthologs. STING alleles from insects showed no interaction with any of the cyclic dinucleotide (CDN) substrates tested in the assay (11). STING homologs identified in invertebrates (Annelida, Mollusca, and Cnidaria phylum) have conserved the ability to bind 3′-3′-cyclic dinucleotides and 2′-3′-cGAMP despite low sequence identity outside the key residues conserved in divergent STING homologs and implicated in the CDN recognition. Thus, the recognition of the endogenous 2′-3′-cGAMP ligand is a conserved hallmark supported by the unique conformation of the CBD domain of STING receptor in complex with 2′-3′-cGAMP. Comparative studies of human and sea anemone 2′-3′-cGAMP—STING structures showed that they maintained this conservation despite low sequence identity (11). Dinucleotide sensing triggers the activation of type I IFN responses mediated by human STING expression in contrast to the absence of stimulation monitored after the sea anemone STING expression. This has been correlated with the absence of the CTT domain in the sea anemone STING protein since fusion with the human CTT domain is sufficient to restore activation of IFN in response to 2′-3′-cGAMP exposure (11). The STING signaling pathways (IRF3/NFκB) therefore depends on CDN ligand selectivity and conformation of the CBD domain related to the orientation of the b strand lid domain (on the top of the ligand-binding pocket) and of the CTT (regulating the transition from activated to inactivated state) (48). Molecular dynamics simulations comparing human and mouse STING conformations (opened-inactive or closed-active) have been instrumental in describing the species-specificity of STING in an Apo conformation or upon binding to the DMXAA agonist (49). This species-specificity must be taken into account when considering the applicability of the results obtained for STING agonists using mouse models before they enter into clinical trials (50). At the molecular level, the modulation of STING functions occurs through palmitoylation (51), protein multimerization, and translocation from ER to Golgi (52, 53) for the recruitment of downstream signaling partners (13). Auto-inflammatory syndrome related to mutations in coatomer protein subunit α, COPA (that mediates Golgi to ER transport) was recently attributed to the retention of STING dimers in the Golgi in the absence of cGAMP stimulation. This triggers an enhanced and unregulated type I IFN activation similar to STING mutants of the SAVI-associated syndrome localized in the Golgi in the absence of stimulation (53–56). The link between STING multimerization and its activation process was recently re-evaluated by Ergun et al. using structural biology and biochemistry. They showed that the nature of STING polymers (inter-dimer crosslinks) depends on the ligand. Polymers are blocked by the CTT domain and are formed in the RE prior to trafficking to the Golgi (57). In line with this observation, R284S STING mutants (SAVI-associated syndrome) were shown to generate constitutive polymers related to chronic STING activation (57).

However, the molecular mechanisms involved in the control of the cGAS-STING pathway are still poorly understood. The hydrolysis of 2′-3′-cGAMP messenger by the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) constitutes one of these mechanisms (58). Since its discovery, this extracellular enzyme has aroused great interest because of its strong therapeutic potential (59) as inhibitors of ENPP1 could help potentiate cGAS-STING signaling (60). cGAS-STING pathway is also modulated by the epidermal growth factor receptor (EGFR) (61). EGFR is required for the phosphorylation of STING in the ER, leading to its endosomal translocation to activate IRF3 (61). In addition, the lysyl-tRNA synthetase has recently been identified as a potent modulator of the STING-dependent IFN pathway in a two-step mechanism (45). First, it competes with cGAS for the binding of cytosolic nucleic acid ligand, thus impeding the production of cGAMP. Second, its activation leads to the production of diadenosine tetraphosphate (Ap_4A), an endogenous antagonist of STING. Interestingly, the lysyl-tRNA synthetase- Ap_4A axis modulates the IFN pathway *in vitro* and *in vivo* in zebrafish larvae, suggesting an ancestral mode of regulation of cGAS-STING functions conserved across vertebrates (45).

The cGAS family has several features in common with the STING family conserved during the metazoan evolution (42, 46, 48), as they are present early in several simple organisms (42, 46, 48) but were subsequently lost in nematodes and flatworms (11). The study of the molecular evolution of cGAS and STING has shown the important conservation of the catalytic site (cGAS) and cyclic dinucleotide binding domain (STING) despite low sequence homologies (11, 42, 46, 48). It has also revealed the emergence of the zinc-ribbon domain and the N-terminal fragment of cGAS that ensure its ligand specificity and stability (42) as well as the CTT domain of STING carrying the binding sites of signaling molecules (47). The functional conservation of the cGAS-STING pathway highlights its central role in the cellular response to DNA sensing. In the next paragraph, we will present a concise description of the broad cellular functions of human cGAS.

cGAS: A MAIN ACTOR OF CELLULAR RESPONSE TO DNA AND RNA VIRUSES

As described above, cGAS is considered as the main sensor of DNA viruses in the cytoplasm of infected eukaryotic cells. Of note, the bacterial homolog of cGAS belongs to a four-gene operon mediating antiviral defense against a broad variety of phage infection. This relied on cGAMP production and phospholipase activation leading to cell death (62). Recently, analogs of eukaryotic STING presenting a comparable mechanism of cGAMP-mediated activation were characterized, suggesting the conservation of this antiviral system from bacteria to metazoans (46). In this paragraph, we mainly present the interaction between human cGAS and a broad range of DNA viruses. Of note, such viruses usually replicate in the nucleus of infected cells, and their genome is often protected within the capsid in the cytoplasm during infection, limiting their detection by cytosolic sensors.

Initially, cGAS was described as a cGAMP synthase required for IRF3 dimerization following infection of murine fibroblast cells with herpes simplex virus type-1 (HSV-1), a DNA herpesvirus previously known to induce the expression of IFNs through the STING-IRF3 axis (63). The importance of cGAS in mounting the antiviral response against HSV-1 and survival to infection has been further determined *in vivo* (8). Interestingly, it has been shown that HSV-1 capsid was ubiquitinated upon infection of dendritic cells, leading to its degradation by the proteasome and the release of viral DNA in the cytoplasm, triggering its detection by DNA sensors (64). The DNA genome of poxviruses is also recognized by cGAS to induce the innate immune response. Indeed, the knockdown of cGAS inhibits the induction of IFNβ following vaccinia virus (VACV) infection in THP1 cells (63). Notably, cGAS-mediated detection of VACV leads to the production of cGAMP that could be efficiently transferred to bystander cells, triggering the activation of a STING-dependent antiviral immunity in non-infected cells (65). Different models of cell-to-cell transfer of cGAMP have been proposed occurring through extracellular vesicles such as exosomes (66), gap-junctions (67, 68), and incorporation into enveloped viruses (69) in addition to the recently described cGAMP transporters (70–72). Importantly, cGAS was rapidly described as a main sensor of HIV and other retroviruses (73). In the absence of cGAS, HIV, murine leukemia virus, and simian immunodeficiency viral infections do not elicit antiviral response (73). cGAS notably recognizes specific Y-form DNA motifs from HIV-1 in the cytoplasm of infected macrophages (9), and possibly the RNA:DNA hybrids accumulating in the cytoplasm of retrovirus-infected cells (10). Interestingly, by studying the interaction between HIV and cGAS, two independent teams demonstrated the ability of HIV to encapsidate cGAMP within neosynthesized virions, thus leading to paracrine activation of a STING-dependent IFN response in newly infected cells (66, 69). The structure of the capsid is an essential determinant of cGAS-mediated sensing of the cDNA of HIV in dendritic cells, which does not require genome integration (74). Recently, NONO was described as a major actor of HIV capsid detection in the nucleus. NONO directly interacts with HIV capsid in the nucleus of dendritic cells and is required for the presence of cGAS in the nucleus and cGAS-mediated detection of HIV DNA (75). Hence, the detection of HIV capsid by NONO enables the sensing of HIV DNA by the nuclear cGAS, suggesting a novel role of cGAS in the activation of innate immunity in the nucleus and a cellular strategy to distinguish self-DNA from viral DNA in the nucleus of infected cells (75). By redefining cGAS localization patterns, recent studies corroborated this observation, describing cGAS activity in the nucleus [reviewed in (76)], for instance in the context of DNA damage, raising questions regarding the interaction between cGAS and self-genomic DNA (77). Several groups recently demonstrated the importance of extensive binding of cGAS to chromatin in the prevention of cGAS oligomerization and activation, proposing the first clear mechanisms allowing cGAS to discriminate self from non-self-DNA in the nucleus (78–80). These observations were further supported by the role of nuclear histones in suppressing the cGAS mediated immunogenicity of self-DNA (81–83). In line with the importance of cGAS sequestration by histones in limiting its antiviral activity, it was recently described that histone deacetylase 4 restricts DNA viruses such as HSV or VACV through the induction of IFN response (84). Another step to the regulation of cGAS involves the cellular protein barrier-to-autointegration factor 1 (BAF), competing to bind to self-DNA in the context of a breakdown of the nuclear envelope integrity (85). These recent data have important conceptual implications in the interaction between cGAS, cellular components, and viral DNA in the nucleus, even though no direct interaction has been observed apart for HIV capsid so far. In this context, several lines of evidence suggest that the DNA genome of hepatitis B virus (HBV) stimulates cGAS activity and triggers the activation of the cGAS-STING pathway when transfected into hepatocyte-derived cells (33, 86). However, no induction of innate immune pathways is detected upon viral infection (33, 86). The "stealth" pattern of this peculiar virus was initially attributed, in addition to the absence HBV RNAs sensing, to the protection of the genome within the capsid during its transport to the nucleus (87). The recent data confirming the presence of cGAS in the nucleus raise the question of its ability to interact or not with the specific forms of HBV DNA in the nucleus, including the minichromosomal structure covalently closed circular DNA (cccDNA) that serves as a template for the transcription of viral RNAs (88). In this context, the low expression of cGAS and STING in the hepatocytes may also explain the absence of quantifiable induction of IFN response upon infection (34).

Mirroring the central role of cGAS in the innate antiviral response, a high diversity of mechanisms of viral evasion from the cGAS-STING pathway has been described, some of them directly interacting with cGAS [reviewed in (89)], such as KHSV ORF52 that inhibits its enzymatic activity by blocking cGAS DNA binding (90). Virus-induced degradation of cGAMP has also been investigated by performing a biochemical screening of 23 different mammalian poxviruses. It allowed the identification of viral nucleases classified as poxvirus immune nucleases (poxins) (91) for which homologs have been described in insect viruses and bacteriophages. These proteins represent now a broad family of 369 members identified in viral and animal genomes, potent

modulators of the cGAS-STING pathway (92). Of note, viruses that theoretically do not trigger the activation of cGAS are also able to inhibit its activity or expression. For instance, HBV infection leads to a decrease in cGAS and STING expression in infected hepatocytes-derived cells and infected liver, both *in vitro* and *in vivo* (86). Importantly, numerous members of the RNA virus family *Flaviviridae* exhibit an impressive variety of mechanisms regulating the cGAS-STING pathway [reviewed in (93)], such as dengue virus (DENV) protease cofactor NS2B that triggers cGAS degradation in an autophagy-dependent manner (94) and Zika virus NS1 that prevents caspase-1 degradation, leading to cGAS cleavage and modulation of type I IFN signaling (95). At the current stage of our knowledge, no typical genomic or intermediary structures from Flaviviruses are susceptible to be detected by cGAS, raising the question of RNA virus evolution leading to the counteraction of this innate immune pathway in the absence of direct sensing (93). Of note, independently from cGAS, influenza A viral particles have been shown to directly interact the STING through its fusion peptide, thus stimulating IFN response (96). Regarding cGAS, Schoggins et al. observed that cGAS activation led to the development of a broad antiviral response, targeting both RNA and DNA viruses (39). The same study elegantly demonstrated that cGAS-depleted mice were much more susceptible to West Nile virus (WNV) infection, an RNA virus whose detection by the innate immune system does not rely on cGAS (39). These observations suggest a central and broad function for cGAS in the establishment of the innate antiviral response, even in absence of the direct sensing of viral genomic structures. It raises the question of an unknown ligand or crosstalk of signaling pathways that triggers the activation of cGAS to establish a basal antiviral state in the cells, with the ability to control virus infection. In this context, many RNA viruses interact with the cellular DNA repair machinery, leading to DNA damage that may serve as a cGAS ligand upon infection [reviewed in (97)]. Notably, viral oncogenes, such as E7 from the human papillomavirus (HPV), E1A from the adenovirus, and the simian virus 40 (SV40) large T antigen have been shown to modulate the cGAS/STING pathway (98, 99). In the specific case of Flaviviruses, it has been proposed that leaking mitochondrial DNA coming from damaged mitochondria upon DENV infection may trigger cGAS activation in the cytoplasm of infected cells (93). A more comprehensive knowledge of cGAS ligands is still required to understand the complex interaction between cGAS and the diversity of virus infections.

cGAS INTERACTION WITH METAZOAN PARASITES

As intracellular pathogens, several multicellular parasites also trigger the cGAS-STING pathway following the sensing of DNA structures, such as *Toxoplasma gondii*, one of the most common parasites in developed countries and responsible for toxoplasmosis (100), *Trypanosoma cruzi*, a member of euglenoids causing Chagas disease in humans, or *Leishmania* [reviewed in (17)]. In the same vein, genomic DNA from *Plasmodium falciparum*, the causative agent of malaria, is detected by cGAS following infection, leading to type I IFN production and systemic inflammation, with hemozoin, the product from blood digestion by *P. falciparum* playing a key role in the delivery of genomic DNA in the cytosol (101). The importance of cGAS in the control of *P. falciparum* infection was confirmed *in vivo*, as cGAS-depleted mice showed a higher susceptibility to parasitic infection (102). Interestingly, computational analysis from *in silico* screening as well as IFN inhibition assay in a mouse model of AGS syndrome suggested that several antimalarial drugs, such as hydroxychloroquine or X6, could interact with cGAS and inhibit DNA-cGAS interactions, blocking IFN response (103, 104). However, the involvement of this mode of action in the control of *P. falciparum* infection remains to be determined (17).

cGAS AND BACTERIAL DNA: FROM HOST DEFENSE TO INTERACTION WITH MICROBIOTA

cGAS has been also shown to be an important sensor of intracellular bacteria. Three groups simultaneously described the involvement of cGAS in the detection of microbial DNA from *Mycobacterium tuberculosis*, the causative agent of tuberculosis. cGAS and *M. tuberculosis* are notably colocalized in the human tissue from patients with tuberculosis, and cGAS depleted mice are more susceptible to bacterial infection (105). Infection of macrophages revealed a STING-dependent activation of antimicrobial response following direct binding of cytosolic DNA to cGAS, leading to an autophagy-driven elimination of *M. tuberculosis* (106). Notably, *M. tuberculosis* strains isolated from patients with severe tuberculosis do not induce a robust induction of cytokines upon infection of macrophages, including weak induction levels of interleukin-1β (IL-1β) associated with evasion from cGAS sensing (107). Similarly, both cGAS and STING are required for INFβ production following infection of multiple cell types by *Chlamydia trachomatis*, a Gram-negative bacterium mainly causing disease of the genital tract (108). Interestingly, *C. trachomatis* inclusion protein CpoS inhibits the cGAS-STING pathway by targeting STING and limiting apoptosis of the infected cells (109). *Listeria monocytogenes,* a Gram-positive bacterium replicating in myeloid cells, induced IFNβ expression through both IFI16 and cGAS detection upon infection (110). Interestingly, DNA from *L. monocytogenes* can be transferred from infected cells to neighboring naïve cells through extracellular vesicles, leading to the paracrine activation of the cGAS-STING pathway. This was also observed upon infection of both *Francisella tularensis* and *Legionella pneumophila*, suggesting a general pathway of innate immune activation following bacterial infection (111). Independent from the microbial-induced IFN response, STING activation and binding to ITPR1 upon infection play a key role in coagulation and mortality associated with sepsis in animal models infected by *Escherichia coli* or *Streptococcus pneumoniae* through Gasdermin D activation and F3 release (112). In the same vein, the upregulation of STING pathway is also associated with sepsis-associated mortality in patients (112).

In contrast to the above examples for pathogenic bacteria, cGAS-STING also interacts with commensal bacteria and constitutes important regulators of host-commensal microbiota

interactions, which contribute to maintaining gut homeostasis through modulation of the host inflammatory response and function of the gut barrier. Indeed, in this tissue environment, the sensing of genomic DNA from invading pathogens (mediated by cGAS) and of cyclic dinucleotides generated by commensal bacteria (mediated by STING) should be tightly regulated to avoid an exacerbated inflammatory response and preserve intestinal integrity. Studying the role of STING in sepsis pathophysiology in a pilot experiment, Hu et al. sampled human intestine biopsies from patients with sepsis in comparison to healthy control biopsies. Histological analyses have correlated the level of STING expression with tissue injury, apoptosis, and intestinal inflammation (113). This was further investigated in a mouse model of sepsis with STING knock-out (KO) animals, which confirms that the control of the STING-mediated intestinal inflammation allows an improvement of intestinal barrier function and tissue histopathology (113). These results are reminiscent of the elements of clinical diagnosis of human patients with abdominal sepsis and the observations made from other rodent models. In steady-state, $Sting^{-/-}$ KO mice models showed defective intestinal homeostasis functions (altered pattern of villi, decreased number of goblet cells, and mucus vesicles per villi as well as lower levels of secreted IgA) and an immature intestinal immunity similar to the phenotype previously described for germ-free mouse models (114). The composition of the microbiota is also impacted by STING since KO mice presented an increase in pro-inflammatory bacteria (114). Upon intestinal injury (dextran sodium sulfate-induced colitis, T-cell-induced colitis, and enteric *Salmonella typhimurium* infection), STING KO mice develop more severe signs of morbidity and an impaired pro-inflammatory immune response compared to wild-type (WT) mice (114). Therefore, regulation of STING pathway is essential to maintain gut homoeostasis and to activate host innate immune responses.

The influence of cGAS is much less understood but does not seem to directly impact the composition of microbiota or the maintenance of the intestinal homeostasis in a mouse model of dextran sodium sulfate-induced colitis (114). However, cGAS has recently been described as a scaffolding protein, which facilitated the internalization of extracellular cyclic dinucleotides (from self and non-self origin) prior to its binding, which precedes the formation of STING signalosomes and its activation (115).

Hence, the crosstalk between cGAS-STING signaling and pathways activated by an increasing diversity of innate immune sensors, complicates the understanding of host-commensal microbiota interactions and the regulation of intestinal homeostasis (116).

ANALYZING cGAS-STING FUNCTIONS *IN VIVO*: SIMILARITIES AND DIVERGENCES IN MODEL ORGANISMS

Comparative analyses of cGAS-STING pathways in various model organisms have shown the conservation of the activating functions of the type I IFN response despite the diversification of the molecular mechanisms during evolution (6). Ectopic expression of genes encoding vertebrate *Sting* in human cells was used to screen their ability to induce NFκB and IRF3 responding elements. While mammalian STING induced a stronger IRF3 response than NFκB, expression of STING from fish species results in a higher NFκB stimulation compared to IRF3. This phenotype was dependent on the expression of a fish-specific minimal motif in the CTT domain of STING that recruits TRAF6 and promotes NFκB activation (47). Further studies will be needed to demonstrate the role of the STING-TRAF6-NFκB signaling axis in the innate immune responses observed *in vivo*. Interestingly, the activation of the STING-TRAF6-NFκB axis was also reported in different human cell types in response to DNA damage (117). Two studies performed in zebrafish larvae demonstrated the role of zebrafish STING in inducing the expression of type I IFN genes during infection with HSV-1 (118) or detection of hypomethylated DNA (119). In contrast to mammalian species, zebrafish cGAS is dispensable for HSV-1 DNA sensing, which occurs through the alternative DNA sensors DHX9 and DDX41 (118). The recent discovery of another functional cGAS isoform in the zebrafish genome prompted a re-examination of the role of cGAS in the sensing of HSV-1 (120). The possible involvement of pangolins during the emergence of the coronavirus disease-2019 (COVID-19) pandemic puts forward the question of the mechanisms of detection of cytosolic nucleic acid in this species, which has been shown to be infected by viruses closely related to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Comparative genomics of phylogenetical analyses revealed that cGAS and STING have been inactivated in pangolin species by mutations and premature stop codons. This points again to the importance of combining various animal models for the study of innate immune mechanisms and the characterization of alternative mechanisms of nucleic acid sensing (121). In the same vein, as another potential reservoir of SARS-CoV-2-related viruses, an altered IFN response due to a key mutation in the bat version of STING was recently reported (122).

The cGAS-STING pathway was considered as non-dispensable for the detection of DNA viruses *in vivo* (8, 123). However, recent work demonstrated the existence of a STING-independent DNA immune response occurring through the detection of cytosolic dsDNA by the DNA-PK DNA repair pathway. This DNA-PK-dependent IFN production appears to be limited to human cells as it could not be demonstrated in murine cells (124). Alternative *in vivo* models thus contribute to reassessing the impact of other sensing pathways and of the specificities of the species considered (6). Other illustrations of species specificities arise from infectious models for HSV-1 and Zika virus infections. To counter cellular antiviral responses and ensure their replication in the host organism, viruses have developed evasion mechanisms targeting IFN responses and cGAS-STING pathways (89). RNA and DNA viruses inhibit cGAS or STING by inducing their degradation or blocking their interactions with signaling proteins such as TBK1 (89). Interestingly, these processes present cell-(89) and

species-specificities (125). Indeed, STING can promote HSV-1 infection in HEp-2 or HeLa cells (in an ICP0 dependent manner), while it is involved in the antiviral response described in human embryonic lung cells (126). In another study, host susceptibility to Zika virus has been investigated in fibroblasts obtained from human, primate, and murine cells. This comparative analysis showed that the murine fibroblasts are partially resistant to viral infection in contrast to the human and primate cells based on a STING-dependent restriction mechanism. The authors further demonstrate that human STING is targeted for degradation by the NS2B3 viral proteases of four distinct flaviviruses (ZIKV, DENV, WNV virus, and Japanese encephalitis virus) in contrast to murine STING, which does not share the protease cleavage site (125). However, infection of *Sting* KO mice does not recapitulate the *in vitro* observations as the mice have become hypersensitive to Zika infection. This highlights the complexity of co-existing antiviral mechanisms, which co-orchestrate the innate immune response in a cell-and species-specific manner.

DISCUSSION AND PERSPECTIVES: cGAS-STING AS A TARGETABLE PATHWAY IN THERAPY

In addition to its role in anti-pathogenic surveillance and response, accumulating evidence suggests a key role for cGAS in immune activation in cancer cells. Numerous studies reported an antitumor role for the cGAS-STING pathway. This topic has been extensively treated elsewhere (18, 38) and will not be developed in this review. The central role of the cGAS-STING pathway in various human pathologies such as cancer, infections, autoimmune diseases, and inflammatory diseases has prompted the search for therapeutics targeting the cGAS-STING-TBK1 axis (127). The modulation of immune responses remains one of the approaches considered in the treatment of these diseases through the improvement and/or refinement of existing strategies. Indeed, anti-inflammatory [systemic lupus erythematosis (SLE), STING-associated vasculopathy with onset in infancy (SAVI), and Copa syndrome (COPA)], anti-viral (hepatitis and HIV) and anti-tumor treatments target type I IFN signaling. However, significant side effects have been reported resulting from the difficulty in controlling the extent and duration of the IFN response *in vivo* (127). Therefore, extensive studies are being conducted to identify alternative treatments, some of them focusing on agonists and antagonists of the cGAS-STING complex, using *in silico* and high-throughput screening approaches (13, 127, 128). Other approaches target modifying enzymes involved in the synthesis of STING ligands and/or the post-translational modifications of cGAS and STING (129). In addition, targeted approaches are being developed based on the modulators of the cGAS-STING pathway such as the immunosuppressor MYSM1, which may be considered as a therapeutic target for inflammatory and autoimmune diseases (130).

Initial lead candidates are further characterized *in vivo* for stability, pharmacological properties, pharmacodynamics, and toxicity. Nanocarriers (such as nanoparticles, liposomes, or viral particles) have improved the efficacy and delivery of molecules targeting cGAS-STING, used in the treatment of solid tumors, lymphomas or to potentiate influenza vaccine response (131–133). In addition, using a mass spectrometry-based ligand screening technique, Siu et al. successfully generated STING antagonist molecules based on their compatibility with oral administration and efficacy to stabilize human STING dimer in an inactive conformation (134). The development of physiologically relevant biomedical models of cGAS-STING related pathologies is thus essential to validate the efficacy of therapeutic candidates but above all to predict the potential side effects linked to the modulation of the immune system. Modeling the cGAS-STING signaling pathway in distinct environments (infected or inflamed tissues, tumor, immune-privileged organs,...) and pathophysiological contexts (chronic inflammation, immunosuppression, …) constitutes an important challenge to improve the prediction of disease outcomes and reduce the high failure rates of clinical trials.

In this context, STING and cGAS KO mice have been instrumental for the advancement of knowledge and of drug discovery. However, different groups recently highlighted the limitations of such models. Oami and Coopersmith (135) discussed the fact that in these animal models, the gene is invalidated throughout the organism leading to strong phenotypes, which do not recapitulate the endogenous expression of cGAS-STING in various cell subtypes and tissues.

The design of cGAS-STING biosensors has been developed in parallel for example to detect and quantify the 2′-3′-cGAMP second messenger in mammalian cell extracts (136). The high sensitivity of such techniques allowed the measurement of 36 million molecules of 2′-3′-cGAMP produced on average per mammalian cell upon stimulation (136). Other strategies emerged to conduct high-throughput screening (HTS) or measure endogenous cGAMP using a STING-based biosensor (137) or a cGAMP-Luc reporter assay (138). Moreover, several commercial ELISA kits can be used to detect cGAMP in cells and tissues (139). These new tools are suitable for the discovery of cGAS-STING modulators although they are often studied in mouse models, while several studies report the species-specificity of STING ligand detection and activation (49, 140). Thus, further characterization of therapeutic compounds should be carried out with particular attention to the species specificities (6) of the cGAS-STING pathway and crosstalk mechanisms including the recently described STING-independent HSV-1 nucleic acid sensing (124, 141). High-throughput screenings of therapeutic molecules in zebrafish larvae can be considered as a promising approach since this biomedical model is suitable to study human inflammatory pathologies (AGS syndrome, cancer, and infectious diseases) (6, 142–144). Finally, organoids obtained from pluripotent stem cells from patients will soon constitute novels and complementary tools for considering personalized medicine (145). The drug repositioning strategy has also brought promising results (taking advantage of available clinical trials for

toxicity and off-target side effects) while reducing the cost and development time of therapeutic candidates, as demonstrated by the interaction between antimalarial drugs and cGAS activities (103, 104). For instance, epigallocatechin gallate (EGCG) and aspirin were recently suggested as repurposed drugs inhibiting cGAS (146, 147).

Taken together, recent data on cGAS and STING structure and functions revealed the importance of this DNA sensing pathway in regulating the cellular response to pathogens as well as cell cycle and oncogenesis. Although additional studies would be required to get a comprehensive overview of the role of the cGAS platform in health and disease, the understanding of its molecular mode of action will pave the way to the development of urgently needed broad antiviral and anticancer strategies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Role of Nucleases and Nucleic Acid Editing Enzymes in the Regulation of Self-Nucleic Acid Sensing

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Detection of microbial nucleic acids by the innate immune system is mediated by numerous intracellular nucleic acids sensors. Upon the detection of nucleic acids these sensors induce the production of inflammatory cytokines, and thus play a crucial role in the activation of anti-microbial immunity. In addition to microbial genetic material, nucleic acid sensors can also recognize self-nucleic acids exposed extracellularly during turn-over of cells, inefficient efferocytosis, or intracellularly upon mislocalization. Safeguard mechanisms have evolved to dispose of such self-nucleic acids to impede the development of autoinflammatory and autoimmune responses. These safeguard mechanisms involve nucleases that are either specific to DNA (DNases) or RNA (RNases) as well as nucleic acid editing enzymes, whose biochemical properties, expression profiles, functions and mechanisms of action will be detailed in this review. Fully elucidating the role of these enzymes in degrading and/or processing of self-nucleic acids to thwart their immunostimulatory potential is of utmost importance to develop novel therapeutic strategies for patients affected by inflammatory and autoimmune diseases.

Keywords: DNases, RNases, systemic lupus erythematosus, DNA sensing, RNA sensing, interferonopathies, aicardi goutieres syndrome, toll-like receptors

INTRODUCTION

The innate immune system is the first line of defense of an organism against microbial infections. Upon the sensing of microbial components called pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRR), the innate immune system produces inflammatory mediators viz. type-I Interferons (IFN-I) that are critical for the activation of antimicrobial immunity. Multiple PRR have evolved to recognize microbial nucleic acids (NAs) which represent a major PAMP (1). This ability of PRR to detect microbial genetic material confers a great advantage to the host, by enabling the activation of the immune system against a broad range of microbes. However, this specificity of PRR comes with an imminent risk, as PRR specialized in NA sensing do not robustly discriminate between self (endogenous) and foreign NAs (2). To avoid the aberrant immune activation by self-DNA, DNA sensors are strategically located in cellular compartments commonly devoid of self-DNA such as the cytosol and endolysosomes (3). Furthermore, NA

sensors preferentially recognize sequences and/or structures that are enriched in microbial genetic material including unmethylated CpG motifs (abundant in microbial DNAs) and uncapped 5′ tri- and bi-phosphates (abundant in viral RNAs) (2). It was recently reported that histones also prevent aberrant activation of inflammatory responses by genomic DNA (gDNA), through the inhibition of intracellular DNA sensors (4–7). While these mechanisms clearly prevent abnormal activation of NA sensors by endogenous NAs, they are not sufficient given the abundance and availability of self-NAs. Indeed, the natural turnover of cells accounts for millions of dying cells every day that release significant amounts of their genetic material, while cellular stress conditions including genotoxic and oxidative stress, autophagy, etc. can lead to exposure of nuclear and mitochondrial genetic material into the cytosol. Therefore, the relative abundance and antigenicity of self-NAs must also be tightly regulated to limit their immunostimulatory potential and prevent the development of inflammatory and autoimmune disorders (3). This role is ensured by nucleases (DNases and RNases) and NA-editing enzymes, that function extra and intracellularly to prevent self-NA-mediated autoimmunity. After briefly describing the NA sensors and the main sources of potentially immunostimulatory self-NAs, this review will focus on the nucleases and NA-editing enzymes involved in the regulation of self-NA immunogenicity. Particularly we will describe their expression profiles, biochemical properties, functions and mechanisms of action. Moreover, we will simultaneously address how dysregulation and deficiencies in these enzymes contribute to inflammatory and autoimmune diseases. The functional analysis of nucleases' and NA-editing enzymes will be further extended to cancer, another pathological context that involves NA sensing. Finally, the potential therapeutic avenues to overcome pathologies mediated by nucleases and NA-editing enzyme dysfunction will be discussed.

INNATE IMMUNE SENSORS OF NUCLEIC ACIDS

There are two major subtypes of NA sensing PRR that were classified according to their subcellular localization, including cytosolic and endolysosomal NA sensors. Their function, regulation and signaling pathways were recently thoroughly reviewed (2), therefore we will briefly describe them to understand the function of nucleases and NA-editing enzymes in the regulation of NA sensing.

Cytosolic NA sensors are widely expressed across immune and non-immune cells, and recognize cytosolic NAs. Cytosolic double stranded (ds)RNA is sensed by RIG-I-like receptors (RLRs) including RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated protein 5). RIG-I is activated by 5′ tri-phosphorylated, 5′ di-phosphorylated and to lesser extent by 5′-OH short dsRNA (8), while MDA5 recognizes highly branched forms of dsRNA of >1 kbp (9), none of which are found endogenously. RIG-I and MDA5 interact with the mitochondrial antiviral signaling protein (MAVS) on the outer mitochondrial membrane, which activates the MAVS signaling complex leading to IFN-I and proinflammatory cytokine production (3). The principal cytosolic DNA sensor is cGAS (cyclic GMP-AMP synthase), which upon DNA recognition, synthesizes cyclic GMP–AMP (cGAMP), that functions as a second messenger to activate the stimulator of IFN genes (STING) on the endoplasmic reticulum (ER). STING engagement also causes IFN-I production (10). Another cytosolic dsDNA sensor is absent in melanoma 2 (AIM2), which upon activation engages the inflammasome to cause production of interleukin (IL)-1β, IL-18 and other inflammatory cytokines (11). However, all mouse AIM2-like receptors (ALRs) and human IFI16 are dispensable for the IFN-I response to intracellular DNA (12). Most cytosolic RNA sensors mediate responses to various classes of RNA viruses, whereas cytosolic DNA sensors induce antiviral immunity against DNA viruses and retroviruses. Recent reports show that cGAS can also be localized to the nucleus preferentially to centromeric DNA and LINE-DNA repeats upon disruption of nuclear membrane during cell migration and interphase of the cell cycle. Nuclear localization of cGAS has been suggested as a process that might regulate tonic or basal IFN-I signaling (13–15).

Endolysosomal NA sensors consist of the Toll-like receptor (TLR) family members TLR3, −7, −8, −9, and −13. They are mainly expressed by immune cells and identify endocytosed NAs. TLR9 preferentially recognizes unmethylated CpG dinucleotides in dsDNA sequences (16, 17), while TLR3 is activated by 39-48bp of dsRNA (18). TLR8, which is reportedly non-responsive to stimulation by RNA in mice contrary to humans, is expressed in human monocytes, dendritic cells (DCs) and neutrophils, whereas TLR7 is widely expressed in immune cells of both humans and mice (19). Both TLR7 and TLR8 recognize single stranded (ss)RNA and its degradation products. Recent biophysical and biochemical studies have identified critical differences between the two, wherein TLR8 recognizes ssRNA uridine and short oligonucleotides (20), while TLR7 is preferentially activated by guanosine and its derivatives (21). TLR13 is a murine-specific endosomal sensor of bacterial 23S rRNA (22). Ligand binding to TLR7, −8, −9, and 13 initiates signaling via the adaptor protein MyD88 (myeloid differentiation primary response protein 88), while TLR3 signals via the adaptor protein TRIF (TIR domain-containing adaptor inducing interferon-β). Both TRIF and MyD88 pathways lead to NF-κB-mediated inflammatory cytokine production and IFN regulatory factor (IRF)-3/7-mediated IFN-I production, which are both necessary for antimicrobial immune responses (23–25).

SOURCES, FORMS AND IMMUNOGENICITY OF SELF NUCLEIC ACIDS

Most NA sensing PRR do not properly discriminate between microbial and endogenous NAs. Accordingly, endogenous NAs were reported to activate NA sensing pathways and contribute to inflammatory and autoimmune syndromes (2). In this section we will discuss the main sources, forms and properties of

endogenous NAs and how they may gain access to cellular compartments containing NA sensors.

Cell free extracellular NAs were first identified in the circulation of a patient with leukemia in 1931 by Labbe et al. (26) and several years later Mandel and Metais were able to extract both DNA and RNA from the plasma of healthy patients (27). These pioneering studies indicated that endogenous NAs are present systemically and that their quantities are altered in pathological settings. Since then, technological advances in purification, quantification and sequencing has led to better characterization of circulating cell free (cf)-NAs (28).

CfDNA is relatively abundant in the circulation of healthy individuals, ranging from 5 to 10 ng/mL of plasma (29). Studies using gel electrophoresis (30–32) and DNA sequencing (33–35) have indicated a laddering pattern of cfDNA reminiscent of apoptotic DNA products with a dominant DNA species of 167bp corresponding to the length of DNA associated with a single chromatosome. Further examination of cfDNA in sex-mismatched bone marrow (BM) recipients (36) and its methylation profiles (37, 38), have revealed that 80% of cfDNA originates from dying hematopoietic cells, including granulocytes, and lymphocytes. Importantly, these contributions can shift during pregnancy and aging and in pathological contexts (cancer, transplantation, and autoimmune syndromes) (39). In addition, to gDNA, mitochondrial DNA (mtDNA) is readily detected in the circulation of healthy individuals (40), originates from dying cells (41) and was found to be 56-fold more abundant than circulating gDNA (42).

Various forms of gDNA and mtDNA are present in the circulation. They can be free ("naked"), associated with proteins such as histones and HMGB1 (High mobility group box 1) for gDNA and TFAM (Mitochondrial transcription factor A) for mtDNA, and finally they may be associated with microparticles (MPs). MPs are extracellular vesicles typically between 0.1 and 1.0 μm in diameter that originate from the outward budding of the plasma membrane (43). DNA isolated from MPs shows a laddering pattern (31), suggesting that MP-associated DNA is derived, in part, from apoptotic cells. DNA that is associated with MPs can be exposed on their surface and/or remain inside, and usually consists of chromatin fragments containing histones and DNA associated proteins (HMGB-1 and TFAM) (44–46).

While cfDNA derived from apoptotic cells arises naturally, infection, inflammatory conditions, and cancer may lead to release of circulating DNA with different properties. It was reported that necrosis (accidental cell death characterized by a rapid loss of plasma membrane integrity in the absence of nuclear fragmentation) of cancer cells contributes to the accumulation of larger fragments (>1 kb) of cfDNA in the circulation (47). Furthermore, neutrophils undergo a specific cell death process called NETosis, which results in the release of DNA in the form of neutrophil extracellular traps (NETs) (48). These structures facilitate trapping of bacteria, and thus are important for antimicrobial immunity (48). In addition to gDNA, NETs are composed of mtDNA, and both can be associated with the anti-microbial peptide LL37 (48) which protects such DNA from degradation (49). In addition, DNA that is extruded by neutrophils is oxidized, further facilitating its protection from nucleases (50) and enhancing its immunostimulatory potential (51).

Naked forms of gDNA found in the circulation are mostly inert and display relatively low immunostimulatory capacities (52). On the other hand, mtDNA which shares many features with bacterial DNA, harbors elevated levels of unmethylated immunostimulatory CpG motifs (53), and was reported to activate TLR9 (54). The association of cfDNA with HMGB1 and its mitochondrial counterpart TFAM also contributes to the immunogenicity of cfDNA. Indeed, HMGB1 and TFAM were shown to promote cfDNA transport into intracellular compartments and to enhance TLR9 activation by self-DNA particularly in plasmacytoid dendritic cells (pDCs) which are specialized in IFN-I production (55, 56). Moreover, they confer self-DNA-specific secondary structures capable of activating cGAS (57). DNA associated with MPs also carries the potential to activate innate immune responses mainly through TLR9 (58). This ability is likely mediated by the endocytosis of MPs, which grants access to the endolysosomal compartment for further processing but does not facilitate the stimulation of cGAS and other cytosolic DNA sensors. In addition to their forms, it is becoming clear that the size of cfDNA directly impacts its immunogenic potential. Longer DNA fragments stimulate cGAS mediated IFN-I production more efficiently (59) and bind with higher affinity to dsDNA autoantibodies that accumulate in autoimmune syndromes such as systemic lupus erythematosus (SLE) (60). Finally, DNA that is extruded during the process of NETosis is also highly immunogenic and induces IFN-I production in a TLR9- (61, 62) and cGAS-dependent manner (51).

CfRNA levels, sources, physical forms and its potential to become immunostimulatory is less characterized compared to cfDNA. Many attributes of RNA make its presence in circulation unlikely. RNA is a short-lived highly labile molecule susceptible to alkaline pH, heavy metal ions, and RNases abundant in circulation (63). In spite of its instability, several studies have identified numerous RNA species in the plasma, the most prevalent of which include miRNA and piwiRNA and to a lesser extent mRNA, lncRNA, rRNA, and tRNA (64). A study from the Lo group found that "naked" RNA was degraded in human plasma after only 15 s of incubation, suggesting that any RNA normally found in plasma must be protected (65). Indeed, after passing plasma through a 0.2 μm filter there was a 10-fold reduction in the amount of RNA recovered, suggesting a mix of MP-associated and MP-free RNA in circulation (65). Several studies have also shown that cell-free miRNA is particularly resistant to nucleases (66), due to its association to proteins rather than with vesicles, indicating multiple methods of cfRNA stabilization may exist. The immunostimulatory capacity of circulatory MP-associated RNA has been investigated to some degree in the context of RNA specific TLR activation; however this as well as the role of MP-free circulating RNA species remain active areas of research (67, 68).

Intracellular NAs represent a much larger pool of self-NAs residing within cells in the form of gDNA, mtDNA and

RNA. gDNA and mtDNA are segregated from intracellular DNA sensors and post-transcriptional modifications of endogenous RNA restrict its capacity to stimulate cytosolic RNA sensors (2). However, gDNA and mtDNA are known to gain access to the cytosol. Micronuclei are small organelles surrounded by a nuclear envelope containing condensed

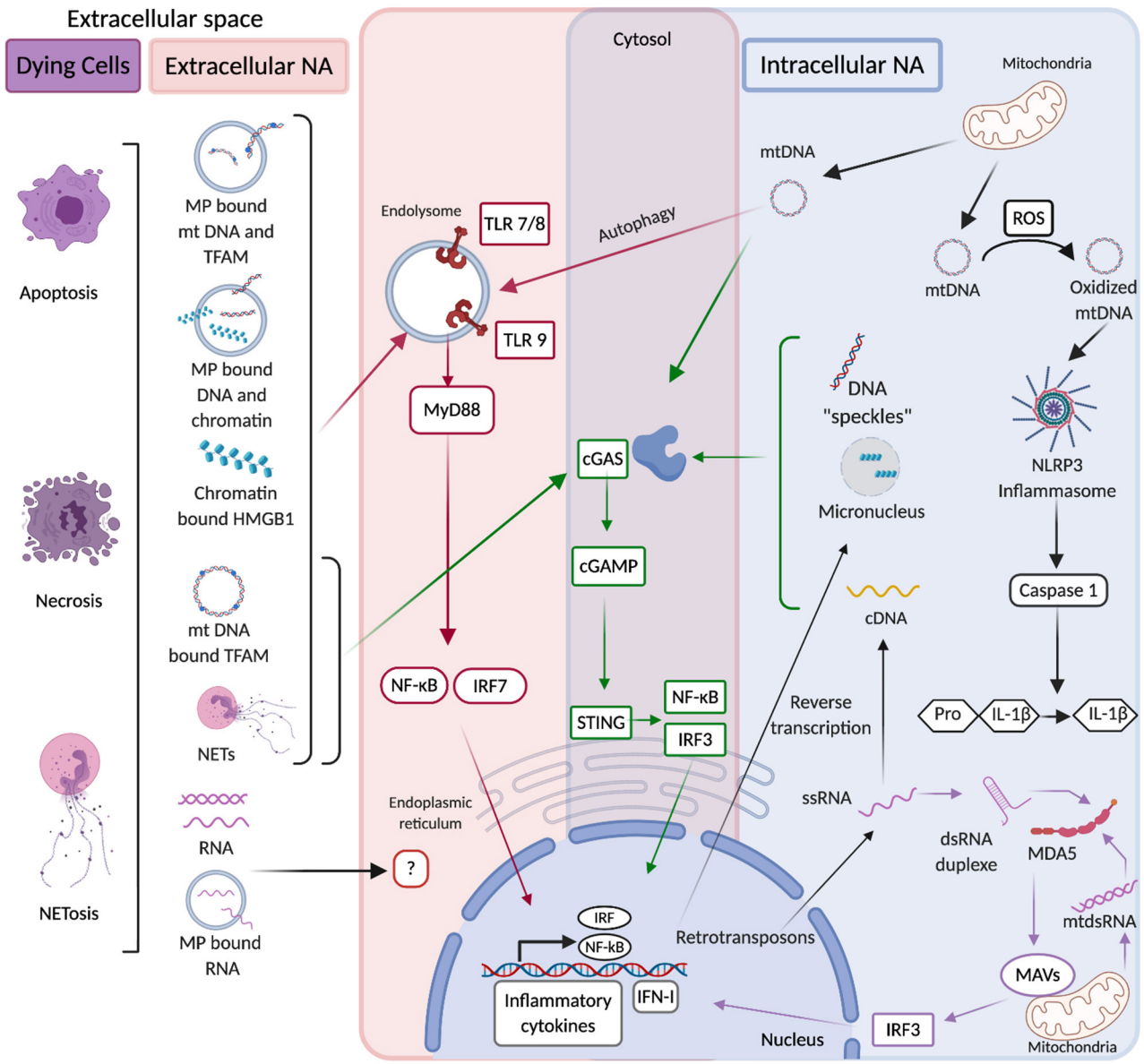


FIGURE 1 | In red are extracellular source of NAs that originate from dying cells in multiple forms including free, microparticle (MP)-associated, Neutrophil Extracellular Trap (NET)-associated, and protein-associated (Histones, HMGB1, and TFAM). Such NAs can be internalized into endolysosomes where they are recognized by Toll like receptors (TLR), which via MyD88 activate NF-kB and IRF7 transcription factors that upon their translocation to the nucleus induce the production of inflammatory cytokines and type I interferons (IFN-I). The detection of extracellular RNA by NA-sensing PRRs is poorly documented. In blue are represented intracellular sources of NAs. They originate from mitochondria and such mtDNA in the presence of ROS (reactive oxygen species) can be oxidized and acquire the ability to activate NLRP3 inflammasome, which triggers caspase 1-mediated cleavage of pro-IL-1β into active IL-1β. mtDNA can also activate cGAS which upon detection of DNA produces cGAMP that is specifically recognized by STING. STING stimulation triggers IRF3-mediated IFN-I production and NF-kB-mediated inflammatory cytokine production. Upon autophagy mtDNA can gain access to endolysosomal compartments and stimulate TLR9 as well. Furthermore mitochondrial double-stranded RNA (mtdsRNA) if not degraded by mitochondrial RNA-degradosome machinery can activate the RNA sensor MDA5 which via the adaptor molecule MAVS activates IRF3-mediated IFN-I production. Endogenous LTR-retrotransposons are also a source of intracellular NAs, they can lead to the production of dsRNA duplexes that activate IFN-I production after their sensing by MDA5. In purple are common pathways of intracellular and extracellular NA sensing. mtDNA and NET-associated DNA from the extracellular space are internalized in the cytosol and activate cGAS. The same pathway can be activated by ssDNA originating from the reverse transcription of endogenous LTR-retrotransposons and by nuclear DNA released into the cytosol during stress conditions in forms of micronuclei or "speckles".

nuclear DNA, and "speckles" are less condensed cytosolic DNA structures (69), which form during mitosis and DNA damage, respectively. Exposure of gDNA in cytosolic "speckles" and/or micronuclei upon rupture of the nuclear envelope (69) stimulate cGAS and induce IFN-I production (70–72). Cell stress and cell death (54) also participate in the release of mtDNA into the cytosol. The proximity of mtDNA to reactive oxygen species (ROS) makes it more susceptible to oxidation, a modification that makes mtDNA more resistant to degradation by nucleases (50), and boosts its immunostimulatory potential. Accordingly, once in the cytosol, unmodified mtDNA stimulates the cGAS-STING pathway (73, 74), while its oxidized counterpart acquires the capacity to activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome (75). As discussed previously mtDNA is rich in un-methylated CpG motifs that constitute the main ligand for TLR9. In addition to the uptake of extracellular mtDNA into endolysosomal compartments containing TLR9 (58), mtDNA can be directly transported to endolysosomes by autophagy and trigger TLR9 activation if mtDNA degradation during this process is incomplete (54).

Endogenous RNAs, as discussed previously, are poor stimulators of intracellular RNA sensors due to their biochemical properties. Nevertheless, non-coding retroelements that make up a large portion of the human genome, are known to produce dsRNA duplexes resembling viral dsRNA. Although not all retroelements are active in this way, the dsRNA products of many have been shown to activate intracellular NA sensors (76). There is some evidence that retroelement-derived dsRNA duplexes, if not degraded, are recognized by intracellular RLRs (77, 78) and trigger inflammatory cytokines and IFN-I production. Recently, mtdsRNA was also shown to signal through MDA5-MAVS to induce IFN-I production if not degraded by mitochondrial RNA-degradosome machinery (79). Finally, endogenous retroelements not only form dsRNA product but also contribute to the generation of complementary DNA (cDNA) upon reverse transcription. These cDNA were also described to be an important source of intracellular DNA, the levels of which if not properly regulated may contribute to the aberrant activation of the cGAS-STING pathway (80, 81).

There are thus multiple sources of endogenous NAs that can be distributed both extra and intra-cellularly (**Figure 1**). They assume various forms that regulate their half-life, distribution and immunostimulatory properties. Endogenous NAs are readily detected extracellularly, but in pathological contexts their abundance is commonly increased, and their physical form is altered. In addition, impairment of the intracellular distribution of NAs, renders them accessible to intracellular NA sensors. According to their source, form, distribution and modifications, endogenous NAs exhibit differential immunostimulatory properties. Nevertheless, most endogenous NAs are capable of activating innate immune receptors and stimulate the secretion of inflammatory cytokines (**Figure 1**). Due to the imminent danger such NAs pose to the host, their availability and immunogenicity must be subjected to stringent regulation.

NUCLEASES: SAFETY NETS THAT PREVENT SELF NUCLEIC ACID IMMUNOGENICITY

There are multiple nucleases and NA-editing enzymes that regulate the abundance and the immunostimulatory potential of self-NAs. They can be subdivided in two classes, extracellular and intracellular NA processing enzymes. Their expression profiles, functions and their contribution to pathologies in both mice and humans are summarized in **Table 1**.

Extracellular Nucleases

Apoptotic cells are rapidly cleared by tissue macrophages. This process, termed efferocytosis, plays a crucial role in the disposal of extracellular self-NAs thus limiting their pathogenic potential (149). Circulatory cfNAs are also eliminated by hepatorenal clearance mechanisms (150, 151); however, both of these regulatory processes are not sufficient and require further help from extracellular nucleases. These enzymes, comprising both DNases and RNases, play a crucial role in the regulation of the abundance, the size, and the immunostimulatory potential of endogenous cfNAs as supported by *in vivo* studies and clinical observations indicating that their deficiencies and dysregulation contribute to the development of autoimmune syndromes.

Extracellular DNases: Key Regulators of Cfdna-Mediated Systemic Autoimmunity

The main extracellular nucleases targeting DNA belong to the deoxyribonuclease (DNase)-1 family and include DNASE1, DNASE1like1 (DNASE1L1), and DNASE1like3 (DNASE1L3). They show a high degree of homology (∼51%) and comparable structures including a DNASE domain preceded by a signal sequence that is required for their trafficking to the ER and intracellular inhibition of their DNASE activity (29). Upon trafficking to the ER the signal sequence is cleaved, allowing the secretion of fully active DNases (152, 153). Contrary to DNASE1, DNASE1L1, and DNASE1L3 have unique C-terminal domains whose function will be discussed below. In addition to their structure these extracellular DNases share common biochemical properties: they function at neutral pH, their enzymatic activity is dependent on divalent cations (Ca^{2+}/Mg^{2+}/Mn^{2+}) and they cleave phosphodiester bonds leaving 3' hydroxy/5' phosphor (3'OH/5'-P) ends (154). Their unique functions in the regulation of endogenous cfDNA abundance and immunogenicity will be further detailed.

DNASE1 is expressed primarily in the kidneys, pancreas, salivary glands, stomach and the small intestine (152). It can also be detected in body fluids such as plasma and urine (155) reflecting its secreted nature. Initially DNASE1 was thought to play an important role in the degradation of DNA from nutrients in the digestive tract but its systemic distribution suggested a broader function in the regulation of extracellular cfDNA levels. DNASE1 present in the plasma is capable of digesting "naked" DNA and nucleosomal DNA in the presence of heparin and/or plasmin (152, 156, 157). Moreover, DNASE1 was shown to digest DNA originating from NETs *in vitro* as well as *in vivo* (158, 159). Its functional characterization and

TABLE 1 | Comparative analysis of murine and human nucleases and NA-processing enzymes.

Nuclease	Main tissue expression (mice and humans)	Main cell types (mice and humans)	Substrates	Mutations in humans and associated pathologies	Deficiency in mice and associated pathologies	Signaling pathway activated upon deficiency
DNASE1	Salivary glands; Kidney; Gut; Plasma	Exocrine cells; Paneth cells	Apoptotic cell-derived DNA; Neutrophil extracellular trap (NET) DNA	Systemic Lupus Erythematosus (SLE)[a] (82)	SLE[a] (83)	Unknown
DNASE1L1	Skeletal and Cardiac muscle	Myocytes	Liposome associated DNA (natural substrate unknown)	Pompe's disease[a]; ANCA-associated vasculitis (84–86)	Muscle weakness and reduced total body mass (87)	Unknown
DNASE1L3	Spleen; Liver; Plasma	Dendritic cells, Macrophages	"Naked" DNA; Nucleosome-bound form; Microparticle (MP)-associated DNA; NET-DNA	Pediatric-onset SLE; Systemic sclerosis (SSc), Rheumatoid Arthritis (RA) (88–99)	SLE (Strong serological features) (45, 100)	TLR7; TLR9; Myd88 dependent (45, 101)
RNASET2	Ubiquitous	Most cells	Extra and intracellular ssRNA	Cystic leukoencephalopathy (102)	Neuropathology and deficits in memory (103)*	Unknown
DNASE2A	Ubiquitous	Macrophages	Apoptotic cell-derived dsDNA and endogenous genomic dsDNA	Rheumatoid arthritis (RA); Anemia, Lupus nephritis (104–106)	Embryonic lethal; Anemia, Rheumatoid arthritis; Nucleic acid specific autoantibodies (107–111)	cGAS/STING, AIM2 and endolysosomal TLRs dependent (111–115)
DNASE2B	Lens	Lens fiber cells	Nuclear dsDNA	Unknown	Cataracts (116)	Unknown
PLD3/PLD4	Most tissue	Macrophages; DC	ssDNA originating from apoptotic cells	Rheumatoid arthritis (RA); Systemic sclerosis (SSc) (117–119)	Chronic immune activation resulting in inflammatory disease (120)	TLR9 dependent (120)
DNASE1L2	Epidermis	Keratinocytes	Nuclear dsDNA	Parakeratosis; Psoriasis. (121)	Hair and nail parakeratosis (122)	Unknown
TREX1	Ubiquitous	Ubiquitous	ssDNA derived from retrotranscribed retroelements	Aicardi Goutières Syndrome (AGS); Familial Chilblain Lupus (FCL), Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL); SLE (123–130)	Lethal autoimmunity, Myocarditis, Lupus like disease; FCL-like disease (81, 128)	STING, cGAS, TBK1, IRF7, IRF3, and IFNAR1 dependent. (80, 81, 113, 128, 131, 132)
RNase H2 complex	Ubiquitous	Ubiquitous	RNA in RNA: DNA hybrid structures	Aicardi Goutières Syndrome (AGS), SLE (133, 134)	Embryonic lethal; IFN-mediated autoimmunity (135, 136)	cGAS/STING dependent (135, 136)
SAMHD1	Most tissue	Immune cells	dNTPs	Aicardi Goutières Syndrome (AGS); Familial Chilblain Lupus (FCL); Arthropathy (137, 138)	Spontaneous type-I IFN production and upregulation of ISGs; No inflammatory disease (139, 140)	Unknown
ADAR1	Ubiquitous	Ubiquitous	dsRNA duplexes	Aicardi Goutières Syndrome (AGS). (141)	Embryonic lethal (142)	MDA5; MAVS-dependent (143, 144)
RNA Exome	Ubiquitous	Ubiquitous	dsRNA	Trichohepatoenteric syndrome (145)	Unknown	RLRs (146)
Endo G	Ubiquitous	Ubiquitous	mtDNA	Unknown	Cardiac hypertrophy (147)	cGAS/STING (148)

Blue are extracellular, Green are endolysosomal and yellow are intracellular nucleases or NA processing enzymes.
*[a]Controversial; *Study in rats.*

relevance in the regulation of extracellular cfDNA abundance and immunogenicity was assessed after the generation of *Dnase1*-deficient (KO) mice (160). These mice spontaneously developed anti-dsDNA and anti-nucleosome autoantibodies, and ultimately glomerulonephritis. With these specific pathological manifestations, *Dnase1* deficiency was suggested to cause SLE development *in vivo* (160). However, these results were obtained in mice that were on a mixed 129sv-C57/Bl6 background which are genetically predisposed to autoimmunity (161), and were not reproduced in *Dnase1* KO mice backcrossed to pure 129Sv and C57/Bl6 backgrounds (162). In addition, genetic targeting of *Dnase1* in these mice caused inactivation of the *Trap1* gene, encoding a mitochondrial chaperone, due to the location of its open reading frame on the opposite DNA strand of *Dnase1* (163). Therefore, the impact of *Dnase1* deficiency on SLE development is dependent on the genetic background and may be confounded by the unintentional *Trap1* inactivation. The levels of circulatory cfDNA and its overall size distribution was also recently shown to be similar between control and *Dnase1* KO mice (164), further supporting a redundant, rather than primary role for DNASE1 in the regulation of cfDNA abundance and immunogenicity. Interestingly, Kenny et al. have generated a new *Dnase1* KO strain on a C57/Bl6 background without affecting the expression of *Trap1*, which shows divergent results. These mice develop SLE features as manifested by elevated levels of anti-dsDNA antibodies and a mild glomerulonephritis (83). It is difficult to estimate the significance of the autoantibody titers detected in these mice in comparison to other SLE-prone mouse strains, therefore further studies are needed to clarify the role of DNASE1 in SLE and its mechanisms of action in mice. Similarly, the involvement of DNASE1 in SLE pathogenesis in humans remains unclear. Genetic studies have identified a heterozygous non-sense mutation in *DNASE1* of SLE patients (82) and single nucleotide polymorphisms (SNP) in *DNASE1* that are associated with susceptibility to SLE (165, 166). Nevertheless, follow up studies failed to shed light on mutations in *DNASE1* in numerous cohorts of SLE patients (167–171). Moreover, SLE patients were reported to exhibit a reduced circulatory DNASE1 activity (172) and such reduced activity was later associated with the development of kidney disease (158). DNASE1 activity in these studies was established by analyzing the ability of SLE patient sera to digest either naked (172) or NET-associated DNA (158), which are substrates shared with other circulatory nucleases such as DNASE1L3 (156). Finally, trials aiming to supplement SLE patients with recombinant human DNASE1 failed to show clinical benefits (173). Hence, in both humans and mice, DNASE1 doesn't seem to be a major safeguard mechanism preventing the break of tolerance to self-DNA. Rather than displaying a systemic function, numerous studies point toward a local role of DNASE1, particularly in kidneys where it may limit the pathogenic properties of immune complexes in SLE patients (174, 175).

DNASE1L3 was initially identified in rat thymocytes (176) and later in the liver and the spleen (177, 178), pointing to a specific expression in hematopoietic cells. Further studies showed that DNASE1L3 is highly expressed in cells of myeloid origin, including DCs and macrophages (45, 179). In addition, inflammatory signals such as IL-4 were recently reported to induce DNASE1L3 expression in human myeloid cells (180). Together with DNASE1, DNASE1L3 was shown to account for most of the DNase activity measured in murine serum (156). DNASE1L3 is capable of digesting "naked" DNA and DNA in NETs, although less efficiently than DNASE1 (156, 158, 159). Besides its shared function with DNASE1, DNASE1L3 possesses a unique ability to degrade nucleosomal DNA (chromatin) without helper proteases (152, 181) and DNA encapsulated in liposomes (179), as reflected by its ability to prevent cell transfection. Given its potential to digest liposome encapsulated DNA, we investigated what may be the natural substrate of DNASE1L3 and identified DNA associated to MPs released by dying cells (45). These unique properties of DNASE1L3 rely on its C-terminal α-helical domain of 21 amino acids that is positively charged and highly hydrophobic. Deletion of DNASE1L3 C-terminus abrogates its potential to digest nucleosomal, liposome-encapsulated and MP-associated DNA without affecting its ability to degrade "naked" DNA (45, 179). The biochemical features of DNASE1L3 C-terminal domain may facilitate lipid membrane binding, penetration as well as the displacement of histones from nucleosomes. Nevertheless, the mechanisms of action of DNASE1L3 C-terminal domain are still speculative and require further investigation. Overall, DNASE1L3 regulates the abundance of numerous sources of extracellular endogenous DNA. The implications of DNASE1L3 in regulating the immunostimulatory potential of endogenous DNA came initially from human studies. Pioneering work by Al-Mayouf et al. has led to the identification of autosomal recessive homozygous null mutation in *DNASE1L3* that caused severe childhood-onset SLE (88). Additional studies followed and identified multiple families with different null mutations in *DNASE1L3* that were associated with the development of early onset SLE and SLE associated diseases (89–92). In addition, SNPs in *DNASE1L3* that cause mutations and functionally impair DNASE1L3 were reported to confer susceptibility to SLE, and related autoimmune diseases such as systemic sclerosis and rheumatoid arthritis (93–99). Altogether these results clearly indicate that DNASE1L3 regulates the potential of endogenous DNA to aberrantly activate autoimmune responses. Similar to humans, *Dnase1l3* KO mice developed anti-DNA and anti-nucleosome antibodies by 5 weeks of age, that accumulated over time and ultimately caused minor kidney pathology (45). The phenotype induced by *Dnase1l3* deficiency in mice was milder than in humans and did not result in lethality. This difference is likely due to the housing of *Dnase1l3* KO mice in specific pathogen free facilities, since their treatment with exogenous IFN-I significantly accelerated the diseases and induced lethality (45). Similar SLE features were reported in an additional strain of *Dnase1l3*-deficient mice and the autoimmune phenotype was further enhanced when associated with Fc gamma receptor IIB (*Fcgr2b*) deficiency (100). Abrogation of DNASE1L3 both in mice and humans caused the accumulation of endogenous cfDNA particularly in MPs (45). Contrary to *Dnase1 KO* mice, *Dnase1l3* KO mice (182), and *DNASE1L3* null humans (183) also display significant modifications of their circulatory cfDNA, including elevated levels of long poly-nucleosomal DNA fragments. These

results clearly demonstrate an important function of DNASE1L3 in reducing the availability of immunogenic cfDNA by restricting DNA length and reducing its exposure on MPs derived from apoptotic cells. The development of SLE features in *Dnase1L3* deficient mice was STING independent but MyD88 dependent supporting the role of endolysosomal TLR in the detection of cfDNA accumulating in these mice (45). TLR7 together with TLR9 were later shown to play a crucial role in SLE development in *Dnase1l3* KO mice (101). The apparent redundancy of TLR7 and 9 in *Dnase1l3* KO may rely on the ability of TLR9 to recognize DNA and TLR7 to broadly recognize NA degradation products such as deoxyguanosines (21). Finally, TLR7/9 activation by endogenous DNA is crucial for stimulating the production of anti-DNA antibodies by B cells and induces production of IFN-I by pDCs which further "boosts" autoreactive B cell responses (101). Therefore, in both mice and humans, DNASE1L3 plays a crucial role in preventing the pathogenic activation of immune responses by endogenous DNA released by dying cells (**Figure 2**).

DNASE1L1 expression is restricted to the skeletal muscle and cardiomyocytes (184, 185). DNASE1L1 contains a glycosylphosphatidylinositol (GPI) anchor located in its C-terminus that prevents its secretion (184) as reflected by its absence in body fluids. DNASE1L1 is anchored to the cell membrane with its DNase domain sticking out in the extracellular space and thus likely functions extracellularly (184). DNASE1L1 is also capable of digesting naked DNA, but its specific function remains poorly understood. Overexpression and siRNA-mediated knockdown in a human rhabdomyosarcoma cell-line demonstrated that DNASE1L1 reduced the transfection of DNA encoding a reporter protein (184). These observations suggest that DNASE1L1, similar to DNASE1L3, may degrade DNA complexed with transfection reagents, but its mechanism of action and natural substrates remain unknown. *Dnase1l1*-deficient mice displayed reduced fatigue tolerance and revealed notable evidence of damage/regeneration in muscle fibers (87), providing further evidence of its specific function in muscle tissues. Myocytes of skeletal muscle contain specialized structures, like T-tubules and caveolae, that have been proposed as entry sites for exogenous DNA and express multiple NA sensing PRR involved in the induction of muscle inflammation (186). Whether DNASE1L1 functions to protect myocytes from extracellular DNA mediated tissue inflammation requires further investigation. Supporting its role in muscle tissue, human studies have identified SNPs in *DNASE1L1* associated with the development of Pompe's disease (84), which is a metabolic disorder characterized by myopathy, respiratory weakness, physical disability and premature death. However, studies involving larger and more diverse cohorts did not validate these observations (85) and the only SNP in *DNASE1L1* that abrogates its endonuclease activity was not associated with Pompe's disease (187). Conversely, a recent analysis suggested that *DNASE1L1* SNPs may be linked to type 1 diabetes, schizophrenia and ANCA-associated vasculitis (86). Therefore, further studies of DNASE1L1 are required to understand its function and whether it also regulates the immunostimulatory potential of cfDNA.

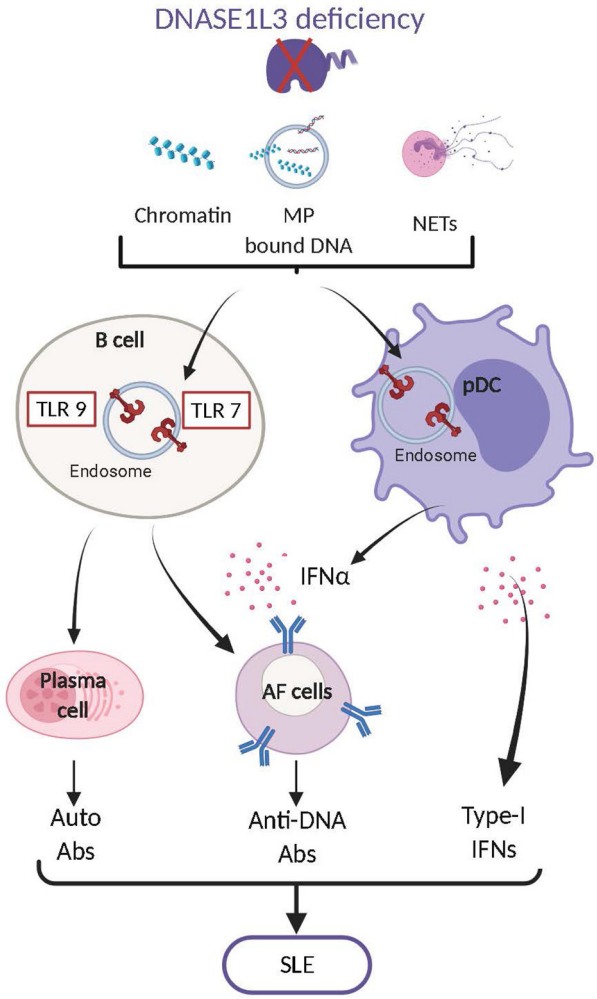


FIGURE 2 | DNASE1L3 deficiency leads to the accumulation of numerous forms of DNA including chromatin, MP associated DNA and NET-associated DNA. Accumulation of such DNA contributes to the aberrant activation of TLR7,9 in B cells and plasmacytoid dendritic cells (pDCs). In B cells TLR7,9 activation leads to their differentiation into plasma cells and antibody forming cells (AFC) that produce autoreactive antibodies mostly directed against dsDNA. In pDCs TLR7,9 activation induces the production of type I interferons (IFN-I) which also play an important role in the transition of B cells into AFC. The production of anti-dsDNA antibodies and of IFN-I will ultimately cause the development of Systemic Lupus Erythematosus (SLE).

Overall, extracellular DNases have distinct tissue distributions and functions (**Table 1**). While DNASE1L1 function is less understood, DNASE1 and DNASE1L3 are required in different ways for the prevention of autoimmune responses induced by cfDNA. DNASE1 deficiency does not cause severe disease nor does it alter the overall length distribution of circulating cfDNA, likely due to the presence of DNASE1L3 and primary role of this enzyme in cfDNA maintenance. It may however display tissue-specific function, i.e., in the kidney where it may alleviate the pathogenic properties of immune complexes. On the

other hand, DNASE1L3 uniquely disposes of poly-nucleosomal DNA and MP-associated DNA, which otherwise contribute to SLE development both in mice and humans (**Figure 2**). Beyond their individual roles, DNASE1 and DNASE1L3 were recently shown to work in concert to prevent vascular occlusion induced by NETs during chronic neutrophilia and sepsis (159).

Extracellular RNases: Unclear Function in the Regulation of Cfrna Abundance and Immunostimulatory Potential

The potential of cfRNA to become immunostimulatory is less characterized compared to cfDNA, and so are the functions of extracellular RNases in the regulation of these processes. There are two main families of extracellular RNases, including RNASEA (also known as RNASE1) and RNASET2.

RNASEA is a vertebrate-specific superfamily of extracellular secreted small cationic ribonucleases expressed mostly within immune cells that share sequence similarities, a disulfide-bonded tertiary structure and the common ability to degrade ssRNA at neutral pH. The family has eight canonical members (RNASE 1-8) that have a conserved RNA-degrading catalytic domain. Additionally, there are 5 reported non-canonical members (RNASE 9-13), that are involved in male-reproductive functions but do not possess ribonuclease activity (188). Although the major function of several RNase A family members is digestion of dietary RNA, several of them have evolved to perform antibacterial, antiviral and immune modulatory functions (189). Importantly, a variety of host defense-related activities attributed to members of the RNase A family are independent of their ribonuclease function (190, 191). A role for RNASEA in the regulation of extracellular cfRNA abundance and immunostimulatory potential was reported in pathological models of hepatic and cardiac ischemia (192, 193). Supplementation of RNASEA prevented the accumulation of circulatory cfRNA released by hypoxic tissues during ischemia and its ability to induce further complications through the activation of inflammatory responses (192, 193). However, there is no clear genetic evidence yet, that RNASEA family members represent important safeguard mechanisms to avoid development of autoimmunity, likely due to their redundant activities.

RNASET2 belongs to a family of ancient RNases whose expression and function are conserved from viruses to humans (194). In vertebrates RNASET2 is broadly expressed, digests ssRNA and functions at acidic pH (194). Although, RNASET2 localizes in lysosomes, where the acidic pH facilitates its RNA digesting activity, it can also be secreted in the extracellular milieu. RNASET2 performs a variety of functions, including modulating host immune responses and serving as extra- or intracellular cytotoxins, reviewed by Luhtala et al. (194). The latter functions of RNASET2 are either dependent or independent of its ribonuclease activity but its role in the degradation of extracellular cfRNA and regulation of RNA-mediated inflammatory responses has only been marginally explored. Loss of function mutation of RNASET2 in humans was shown to lead to the development of a neurological disease called cystic leukoencephalopathy (102). The clinical manifestations induced by *RNASET2* deficiency resulted from aberrant central nervous system inflammation, that may be induced by accumulation of either extra or intracellular immunostimulatory RNA (102). *RnaseT2* deficient rats reproduced neurological clinical features (103) providing an interesting model for the exploration of cellular and molecular mechanisms involved in this process. It would be particularly interesting to understand how the deficiency of a broadly expressed RNase preferentially affects the central nervous system.

Therefore, the function of RNases in the regulation of endogenous RNA abundance, immunostimulatory potential and their involvement in autoimmune and inflammatory disorders await further discoveries.

Intracellular Nucleases and NA-Editing Enzymes

NAs released extracellularly can reach the intracellular space upon uptake by innate immune cells. Intracellular NAs originating from mitochondria and the nucleus can also gain access to these intracellular compartments where they can activate NA sensing PRR and an inflammatory response. Therefore, strategies to regulate the abundance of intracellular NA are crucial for avoiding harmful immune activation. Such safety measures have been selected over the course of evolution and involve intracellular nucleases and NA modifying enzymes. The control of intracellular NA levels and their immunostimulatory potential by these enzymes provides a second line of defense from NAs that escape extracellular control and the first line of defense for NAs derived from intracellular compartments. These nucleases and NA-editing enzymes can be subdivided in two main classes: those which reside in endolysosomes and control NAs internalized from the extracellular space by endocytosis and from the intracellular space by autophagy and those residing in the cytosol which control the levels of intracellular NAs. Their unique properties, functions and their involvement in the induction of tolerance to self-NA will be discussed in detail bellow and are summarized in **Table 1**.

Endolysosomal DNases Prevent Fatal Inflammatory Responses Induced by Self-DNA

DNASE2 family is comprised of 3 members including DNASE2A, DNASE2B and Leucocyte Elastase Inhibitor (LEI) DNASEII (L-DNaseII). While DNASE2A and DNASE2B are conserved between species and share 66% homology, L-DNASEII shows only 29% homology with the two others (29). L-DNASEII is peculiar in that it is derived from post-translational modifications of LEI also called SerpinB1 (195). Due to the inability to dissociate L-DNASEII specific function from LEI, its role in the regulation of endogenous DNA is poorly characterized and thus won't be considered further in this review. On the other hand, DNASE2A and DNASE2B were extensively studied and play an important role in the control of immunogenic self-DNA. They share similar structures with a signal sequence and two phospholipase D (PLD) signature motifs in their catalytic domain. The signal sequence goes

through glycosylation, which is required for their transition into active enzymes (196). The PLD motifs form a single active site containing histidines which are essential for their catalytic function (197, 198). Furthermore, both DNASE2A and 2B harbor conserved mannose phosphorylation motifs involved in their transport into endolysosomes (199). They cleave dsDNA into short oligonucleotides bearing 3'-P rather than 3'-OH ends. The activity of these enzymes is independent of divalent cations and is optimal at acidic pH, found in endolysosomal compartments (195). Although they share many similarities, DNASE2A and DANSE2B have different tissue expression profiles and their dysregulation or deficiency induces different consequences *in vivo*.

DNASE2A is highly expressed in macrophages that are present in most tissues. Initial observations that inhibition of macrophage endolysosomal acidification blocked the fragmentation of engulfed DNA from apoptotic cells, suggested that DNASE2A plays an important role in the disposal of DNA from apoptotic cells (200, 201). This function was confirmed in *Dnase2a* KO mice in which DNA-containing bodies (DCB) originating from apoptotic cells accumulated in multiple organs early in development (107). The highest number of DCB was present in the liver and attributed to the impaired ability of fetal liver macrophages to dispose of extruded nuclei from erythrocytes (107, 108). Consequently, *Dnase2a* KO mice exhibited severe anemia and inflammation caused by the improper clearance erythrocytes' nuclei, that ultimately induced lethality at an early stage of mouse development (E17.5) (108, 202). In addition, thymic development of T cells was severely impaired in *Dnase2a* deficient mice due to poor elimination of apoptotic thymocytes and subsequent inflammation (203). To avoid embryonic lethality and study *Dnase2a* function in adult mice, BM chimeras were established and *Dnase2a*-floxed animals were generated. Mice reconstituted with *Dnase2a* deficient BM cells developed severe chronic polyarthritis, indicating that specific deletion of *Dnase2a* in hematopoietic cells regulates the ability of endogenous DNA to cause arthritis (109). Similar results were obtained in *Dnase2a*-floxed animals crossed with the *Mx1*-cre strain, in which *Dnase2a* was abrogated in all IFN-I responsive cells following poly(I:C) treatment (110). Interestingly, the conditional deletion of *Dnase2a* caused an accumulation of DNA in multiple cell lineages beyond macrophages including T cells, B cells and fibroblasts, suggesting a broader function of DNASE2A (204, 205). In this context DNASE2A was proposed to function in a cell-autonomous manner and to regulate DNA originating from the nucleus and accumulating in autophagosomes (204, 205). Embryonic lethality induced by *Dnase2a* deficiency was attributed to an aberrant production of IFN-I, since *Dnase2a-Ifnar1* double-KO mice were healthy for at least 8 weeks post birth (206). The sensing of DNA causing this IFN-I production in *Dnase2a* deficient mice was shown to be independent of endosomal TLRs (207) but fully dependent on the cGAS-STING pathway (112, 113). These results suggested that DNA from engulfed apoptotic cells and/or that accumulates in a cell autonomous manner, can exit from endolysosomal compartments into the cytosol in the absence of DNASE2A to stimulate cGAS-STING-mediated IFN-I production. Interestingly, while *Dnase2a-Tmem173* (gene encoding STING) double KO mice were healthy (112, 113) *Dnase2a-Ifnar1* double-KO mice develop rheumatoid arthritis that was driven by TNFα (Tumor Necrosis Factor-α) (109, 110). Therefore, aberrant activation of the cGAS-STING pathway in the absence of DNASE2A also contributed to polyarthritis development by inducing the production of TNFα. Furthermore, *Aim2* deletion added to *Dnase2a-Ifnar1* double-deficiency ameliorated the polyarthritis phenotype in these mice, indicating AIM2 inflammasome activation in the absence of DNASE2A contributes as well to this disease (114, 115). *Dnase2a-Ifnar1* double-KO mice develop elevated levels of autoreactive antibodies directed against nuclear material as they age (114). Surprisingly, this production of anti-nuclear antibodies was independent of STING and AIM2 but fully dependent on endolysosomal TLRs (114). Thus, DNA that accumulates in *Dnase2a* deficient mice activates most of the DNA sensing pathways that then differentially contribute to pathological features. These observation in mice were corroborated in human studies by the identification of individuals with null mutations in *DNASE2A* that show severe non-regenerative anemia and deforming arthropathy (104). *DNASE2A* deficiency was accompanied by an up-regulation of interferon-stimulated genes (ISGs) and elevated TNFα levels, suggesting similar pathogenic pathways at play in these patients as in *Dnase2a* KO mice (104). In addition, SNPs in *DNASE2A* were associated with rheumatoid arthritis (105, 110) and revealed weak association with the risk of renal pathology in SLE patients (106). Therefore, in both mice and humans, DNASE2A is critical for eliminating self-DNA and limiting its capacity to induce harmful inflammatory and autoimmune responses (**Figure 3**).

DNASE2B expression is restricted to lens cells where it plays a key role in degrading fiber cell nuclei to regulate their differentiation (116). Accordingly, *Dnase2b* KO mice present undifferentiated fiber cells containing condensed undigested DNA leading to the development of cataracts (116). The expression of *Dnase2b* is regulated by lens-specific heat shock transcription factor 4 (HSF4) (208, 209), the deficiency of which in mice caused cataracts and SNPs in humans were linked to cataractogenesis (210–212). Unlike DNASE2A, DNASE2B is relocated from the endolysosomal compartment to the nucleus of lens cells where it degrades nuclear DNA (213). DNASE2B transport to the nucleus is mediated by cyclin-dependent kinase 1 (CDK1) inhibitor $p27^{kip1}$ whose specific deletion in the lens delays the de-nucleation of lens fiber cells (214). Despite DNA accumulation induced by DNASE2B deficiency, no inflammation was observed in the lens or other tissues of these animals (116). This is likely due to the specific expression of DNASE2B in the eye which is an immune-privileged site that expresses low levels of proteins involved in NA sensing pathways. Thus, DNASE2B displays a cell-autonomous function to allow differentiation of functional lens fiber cells by degrading their nuclear material (209).

Phospholipase D (PLD) are a family of enzymes that are broadly expressed and whose function is conserved from bacteria to mammals. They comprise 4 members (PLD1-4). PLD1 and PLD2 catalyze phosphatidylcholine into choline and phosphatidic acid. In contrast, PLD3 and PLD4 are

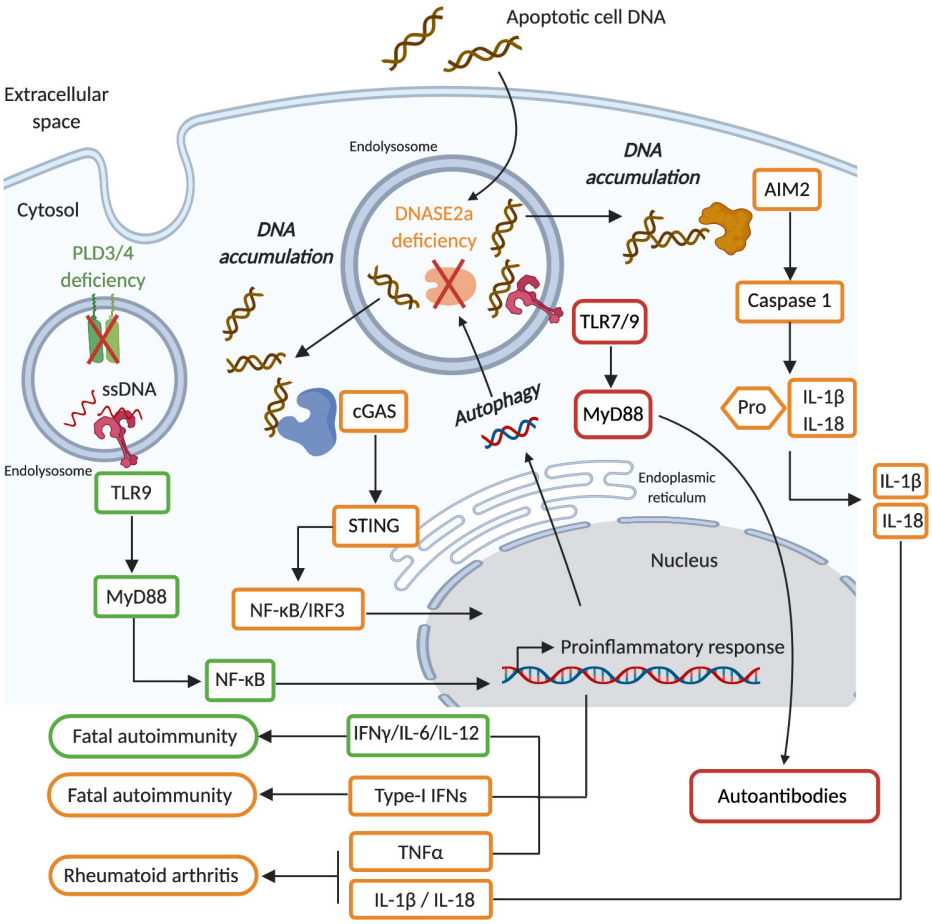


FIGURE 3 | DNASE2A deficiency causes an accumulation of phagocytosed dsDNA as well as nuclear dsDNA transported by autophagy into endolysosomal compartments. Such dsDNA can gain access to the cytosol and activate the cGAS/STING pathway (orange) that leads to the production of type I interferons (IFN-I), which ultimately causes the development of fatal autoimmunity. In the absence of IFN-I signaling DNASE2A deficiency will induce the development of rheumatoid arthritis which is mediated by TNFα, whose production is triggered by the cGAS/STING pathway, but also by IL-1β and IL-18 which are inflammatory cytokines produced upon the activation of the AIM2 inflammasome by cytosolic self-DNA. Finally, DNASE2A deficiency also induces aberrant activation of endolysosomal TLR (red) which contribute via the activation of the MyD88 to the production of autoantibodies. PLD3/4 are novel endolysosomal nucleases (green) involved in the degradation of ssDNA. Their deficiency induces an accumulation of ssDNA in endolysosomes that ultimately activates TLR9-MyD88-NFκB mediated inflammatory cytokine (IL-6 and IL-12) production, causing fatal autoimmunity.

non-classical PLDs that lack phospholipase D activity (215, 216), but contain an N-terminal transmembrane domain allowing their localization in endolysosomes (217, 218). While rare coding variants of *PLD3* were associated with Alzheimer's disease (219, 220) and SNPs in *PLD4* conferred susceptibility to rheumatoid arthritis and systemic sclerosis (117–119), their function remained largely unknown until recently. Gavin et al. have shown that PLD3 and PLD4 are 5' exonucleases that degrade ssDNA at both neutral and acidic pH, which is compatible with their endolysosomal localization (120). Hence *Pld4* KO mice showed signs of chronic inflammation including splenomegaly and aberrant innate immune cell activation which were dependent on IFNγ and TLR9 but independent of T and B cells (120). Conditional deletion of *Pld4* in DCs but not in macrophages showed a similar phenotype, indicating that TLR9 activation by endogenous ssDNA in DCs is mostly responsible for the inflammation induced by *Pld4*-deficiency (120). On the other hand, *Pld3* KO mice did not show inflammatory manifestations of *Pld4* KO mice, but macrophages from *Pld3* KO mice produced elevated levels of proinflammatory cytokines in response to TLR9 stimulation (120). DCs express *Pld4* and macrophages express *Pld3* and they exhibit functional redundancy since *Pld3-Pld4* double deficient animals did not survive longer than 21 days due to severe liver inflammation (120). Thus, PLD3 and PLD4 are novel nucleases working together in the endolysosomes of innate immune cells to prevent endogenous ssDNA sensing by TLR9 and thus the development of inflammatory syndromes (**Figure 3**), but further investigation is needed to address the relevance of PLD3/4 in humans and how they may contribute to specific autoimmune disorders (117–119).

DNASE1L2 belongs to the DNASE1 family and shares similar structure and properties with other family members described above, except that DNASE1L2 functions optimally at acidic

pH (154). The fact that DNASE1L2 degrades its substrates at acidic pH, suggests an endolysosomal and/or autophagosomal localization of this enzyme. DNASE1L2 is specifically expressed in keratinocytes and participates in their differentiation into corneocytes by degrading their nuclei (221). *Dnase1l2* deficient mice, showed a retention of nuclear DNA (parakeratosis) in the hair, nails and other epithelial tissues but did not show any skin defect (122) suggesting that DNASE1L2 function in skin keratinocytes may be compensated. It was indeed the case, and DANSE1L2 was reported to work together with DNASE2A in the disposal of nuclear DNA from skin keratinocytes (222) and with the exonuclease TREX2 in the degradation of DNA from lingual keratinocytes (223). Nevertheless, *Dnase1l2-Dnase2a* and *Dnase1l2-Trex2* keratinocyte specific double deficient animals generated in these studies did not develop signs of epithelial tissue inflammation despite the accumulation of DNA (223). The inability of DNA to aberrantly activate inflammatory responses in this instance is likely due to the low expression of NA sensors in keratinocytes (223) as well as the formation of a cornified envelope that physically prevents DNA from reaching epidermal and dermal compartments rich in innate immune cells. Observations in humans however show that SNPs in *DNASE1L2* are associated with psoriasis (121) and that *DNASE1L2* expression is reduced in the inflamed psoriatic skin (221). These observations suggest that DNASE1L2 in humans may somehow regulate skin inflammation but requires further investigation to understand its mechanism of action.

It appears that endolysosomal DNases expressed by innate immune cells are of paramount importance to prevent the development of severe inflammatory disorders induced by the accumulation of intracellular self-DNA (**Table 1**). Particularly DNASE2A disposes of self-dsDNA and prevents its ability to activate most NA sensing pathways, while PLD3/4 degrade self-ssDNA capable of aberrantly activating TLR9 (**Figure 3**). Conversely, DNASE2B and DNASE1L2 are tissue specific nucleases involved in the degradation of nuclear DNA of lens cells and keratinocytes, respectively. Their primary function is thus to contribute to tissues development, while their role in the regulation of immunostimulatory DNA seems secondary or even non-existent. Finally, endolysosomal RNases are poorly characterized and will not be discussed in greater detail here. RNASET2, which was previously discussed, can be found in endolysosomes (194), but its function in the regulation of RNA immunostimulatory potential remains poorly characterized.

Cytosolic Nucleases and NA-Editing Enzymes Prevent IFN-I-Mediated Syndromes (Interferonopathies)

TREX1 (Three Prime Repair Exonuclease 1), also called DNASE3, is a broadly expressed 3'→ 5' exonuclease that is associated with the ER at steady state, and re-localizes into the nucleus after DNA-damage (224, 225). TREX1 contains an N-terminal domain with an exonuclease activity and a C-terminal transmembrane domain allowing its attachment to the ER and regulating its overall activity (226–228). Its exonuclease domain is key for DNA digestion and it has been shown to dispose of ssDNA and dsDNA (229, 230). Initially TREX1 was described to play a role in DNA repair upon its translocation to the nucleus (132, 226), but observations that *Trex1* deficient mouse embryonic fibroblasts (MEFs) accumulated ssDNA in the ER following induction of DNA damage showed that TREX1 is essential for ssDNA degradation (224, 225). TREX1 was also recently ascribed novel functions independent of DNA degradation that mostly rely on its C-terminal domain. Indeed, TREX1 was reported to control the biogenesis of endolysosomal compartments (131) and, through an interaction with the ER resident oligosaccharyltransferase (OST), to regulate the production immunogenic free glycans (129). The generation of *Trex1* deficient mice demonstrated that its main function is to prevent the development of aberrant inflammatory immune responses. *Trex1* KO mice did not show excessive DNA damage but displayed severe inflammatory myocarditis, systemic inflammation and elevated titers of autoantibodies, causing their premature death (81, 130). *TREX1* loss of function mutations in humans were also associated with the development of Aicardi-Goutières-syndrome (AGS) (123). AGS is an inflammatory syndrome characterized by an encephalitis accompanied by lymphocyte infiltration in the central nervous system and an IFN-I signature (124). Furthermore, different mutations affecting *TREX1* in humans were also shown to be associated with SLE pathogenesis (125, 126) and to cause monogenic familial chilblain lupus (FCL), an inflammatory pathology manifested by ulcerating lesions of the skin (124, 127). Some AGS patients also show characteristic skin lesions similar to those of patients with FLC. Most of the mutations associated with AGS, SLE, and FCL are localized in the N-terminal exonuclease domain of TREX1 (231), suggesting an important function of TREX1 in the disposal of potentially immunostimulatory endogenous DNA. The phenotypes induced by the loss of TREX1 are different between mice and humans in that mice do not show neurological manifestations (80). Nevertheless, *Trex1* KO mice provided an excellent tool to further characterize the mechanisms through which TREX1 may control aberrant immune activation. Fatal autoimmunity in *Trex1* KO was rescued by the deletion of the cGAS-STING pathway (80, 81, 113, 132, 232), indicating that absence of TREX1 results in aberrant activation of DNA sensing pathways. ssDNA that is originating from the reverse transcription of endogenous retroelements was proposed the be the main source of endogenous DNA that accumulates upon *Trex1* deficiency and activates the cGAS-STING pathway (81). In agreement with these observations, fibroblasts from AGS patients with *TREX1* mutations showed elevated cytosolic DNA levels originating from endogenous retroelements (233). However, inhibition of reverse transcriptase in *Trex1* KO mice did not protect them from disease (234), suggesting that additional sources of endogenous DNA may contribute to the pathology observed in these mice. These additional sources of endogenous DNA may include dsDNA (235, 236) and micronuclei (237, 238) that were recently reported as TREX1 substrates. Interestingly, *Trex1*-deficient mice showed elevated levels of free glycan which is reminiscent of the ability of TREX1 C-terminal domain do regulate OST activity. Inhibition of OST ameliorated inflammatory manifestations and prolonged the survival of *Trex1* KO mice, indicating that free glycan contributes to aberrant immune activation in these mice (129). Given that free glycans act independently of the cGAS-STING

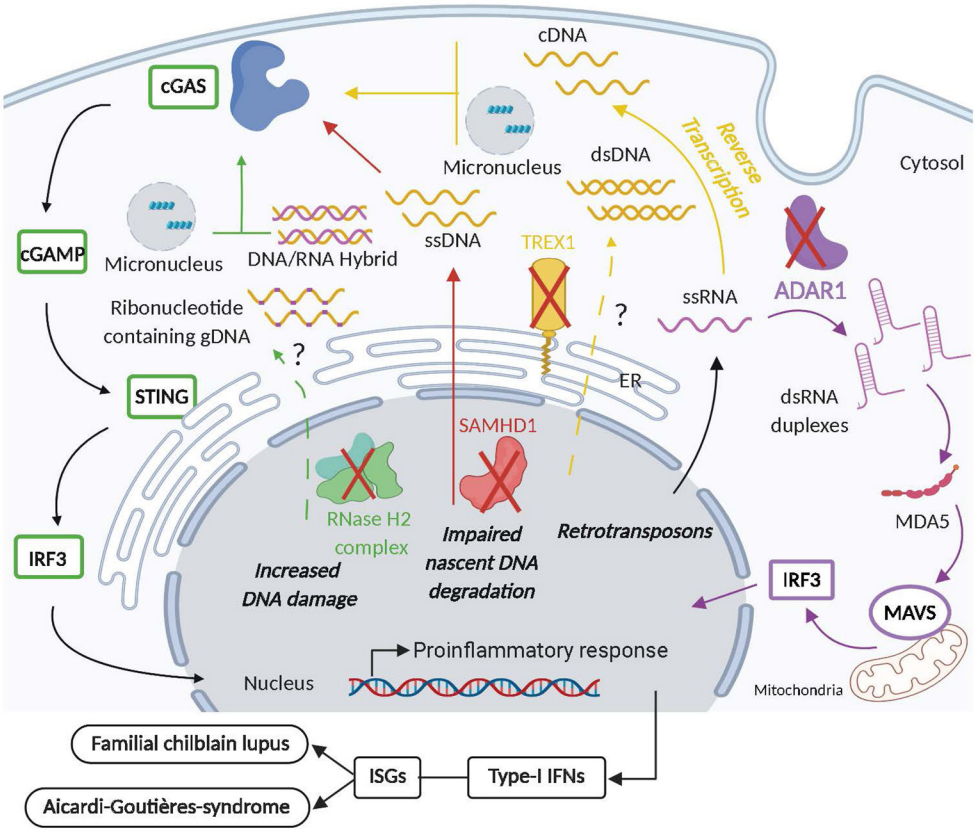


FIGURE 4 | Intracellular nuclease and NA-editing enzyme deficiency aberrantly activates inflammatory and autoimmune responses. Mutations in TREX1 (yellow) that affect its exonuclease activity or cause its deficiency, lead to the cytosolic accumulation of ssDNA originating from the reverse transcription of endogenous LTR-retrotransposons that activate the cGAS/STING pathway causing the secretion of pathogenic type-I interferons (IFN-I) and the development of familial chilblain lupus (FLC) and Aicardi Goutières Syndrome (AGS). Furthermore, dsDNA and micronuclei may be detected in the cytosol of individuals with dysfunctional TREX1 and contribute the aberrant activation of the cGAS/STING pathway as well. Mutation in SAMHD1 (red) causes the release of ssDNA from stalled replication forks that stimulates IFN-I production upon the activation of the cGAS/STING pathway that may ultimately contribute to AGS and FCL. AGS is also associated with mutations in RNase H2 complex (green) that reduces ribonucleotide excision repair (RER) and thus increases DNA damage and possibly the release of DNA:RNA hybrids, micronuclei and ribonucleotide containing gDNA into the cytosol that may activate cGAS/STING-mediated production of deleterious IFN-I. Finally, dysfunction in ADAR1 (purple) leads to the accumulation of dsRNA duplexes originating from endogenous retrotransposon RNA in the cytosol which upon activation of the MDA5 pathway causes the secretion of pathogenic IFN-I responsible for AGS development.

pathway (129), it is difficult to reconcile these results with the observation that cGAS-STING deletion fully protects *Trex1* KO mice from disease (80). Furthermore, *TREX1* C-terminal domain mutations were also identified in humans but were not associated with AGS nor with FCL, but rather with retinal vasculopathy with cerebral leukodystrophy (RVCL) (128). Hence, how TREX1 mediated regulation of free glycans contributes to pathologies associated with TREX1 deficiency requires further investigation. At the cellular level, *Trex1* deficiency in non-hematopoietic cells was proposed to initiate inflammatory pathogenesis as *Trex1* KO mice reconstituted with WT BM cells developed inflammatory disease (80). *Trex1* KO host cells were shown to produce high levels of IFN-I that signals through IFNAR on hematopoietic cells and ultimately causes the disease (80). Nevertheless, *Trex1* KO BM cells were also shown to induce an inflammatory disease when transferred to WT recipients (239, 240), and conditional ablation of *Trex1* in DCs (CD11c-cre) and hematopoietic cells (Cx3CR1-Cre and Tie2-Cre) caused premature death (240). Therefore, both hematopoietic and non-hematopoietic cells are important sources of pathogenic IFN-I induced by *Trex1* deficiency. Finally, T cells and B cells were shown to contribute to the inflammatory pathogenesis mediated by *Trex-1* deficiency, since their individual deletion prolonged survival in *Trex1* KO mice and their combined deficiency fully rescued these mice from mortality (80). Thus, TREX1 is an essential enzyme preventing cytosolic DNA accumulation and its ability to activate cGAS-STING-mediated IFN-I production that ultimately causes fatal inflammatory syndromes (**Figure 4**). TREX1 deficiency differentially affects humans and mice, likely due to species-specific expression profiles of TREX1 and the fact that TREX1 is globally abrogated in mice while human mutations may only lead to partial dysfunction of the enzyme. Accordingly, when the D18N mutation found in FCL patients was knocked into (KI) mice, it induced a milder disease than*Trex1* deficiency

with multiple similarities to human FCL patients including systemic inflammation, production of autoantibodies and kidney disease (235). Interestingly, the FCL-like pathogenesis in *TREX1* D18N KI mice was due to failed degradation of dsDNA (235), rather than of ssDNA as described in *Trex1* KO mice (81). Hence, investigating human TREX1 mutations in murine models *in vivo*, may be of particular relevance to better understand its pathogenic functions.

RNases and NA editing enzymes, within the nucleus and the cytosol are essential for the limiting the immunostimulatory potential of several endogenous NAs, and thus protect the host from the development AGS and IFN-I mediated inflammatory pathologies, as summarized in **Table 1**.

The RNase H2 complex belongs to the ribonuclease H family of ubiquitously expressed enzymes that cleave the RNA in RNA-DNA hybrids that form during replication and repair in a non-sequence-specific manner, or cleave the phosphodiester bond 5' of a single ribonucleotide embedded within a DNA duplex. The RNase H2 complex is comprised of three proteins: the catalytic subunit RNASEH2A, and subunits RNASEH2B and RNASEH2C, which do not harbor catalytic activity but are necessary for the overall function of RNase H2 complex, including its translocation from the cytosol to the nucleus (241, 242). Biallelic loss-of-function mutations in any of the three RNase H2 subunits cause AGS (133). RNase H2 enzyme complex initiates the process of ribonucleotide excision repair (RER) by removing ribonucleotides from gDNA which have been incorrectly incorporated by replicative polymerases, and hence is an integral part of the genome surveillance machinery (243, 244). This function is essential in higher eukaryotes, as loss of RNase H2-mediated RER renders gDNA susceptible to DNA strand breaks. Indeed, RNase H2 deficient mice are embryonic lethal due to a p53-dependent DNA damage response and cell cycle arrest. Not surprisingly, biallelic null mutations of RNase H2 in humans have not yet been reported. Heterozygous mutations in RNase H2 complex were shown to be associated with SLE (134) and hypomorphic *RNASEH2A/B/C* mutations were reported to cause AGS (132), both of which are enough to enhance levels of embedded ribonucleotides in gDNA (242). The ensuing sub-lethal DNA damage induces a chronic DNA damage response characterized by a heightened IFN-I response to UV light-induced thymidine-dimers (134). Using KI mouse models of human *RNASEH2A* and *2B* missense mutants detected in AGS patients, the induction of IFN-I and ISGs was found to be dependent on reduced RER and increased stimulation of the cytosolic cGAS-STING DNA sensing pathway (135, 136). Although RNase H2 mutant mice do not recapitulate the pathological features of human type-I interferonopathies, they provide compelling evidence that in the absence of a functional RNase H2 complex, endogenous NAs accumulate in the cells and are improperly sensed as non-self, leading to induction of IFN-I-mediated immune responses (**Figure 4**). RNase H2 dysfunction causes DNA damage due to failure to remove embedded ribonucleotides from gDNA (135, 243, 245), with micronuclei formation as one of the consequences (72). Micronuclear DNA (72, 246) as well as cytosolic RNA-DNA hybrids (247) have been shown to activate cGAS, however, the exact chemical nature of NAs stimulating the cGAS-STING pathway *in vivo* in absence of functional RNase H2 complex needs further investigation.

SAMHD1 [Sterile alpha motif (SAM) domain and Histidine-aspartate (HD) domain-containing protein 1] is a deoxynucleotide triphosphohydrolase that is mostly expressed within immune cells. It prevents viral infections in macrophages and DCs by hydrolyzing the intracellular pool of deoxynucleotide triphosphates (dNTPs) into 2' deoxynucleoside and inorganic phosphates, thereby blocking reverse transcription of the viral genome (248). Moreover, it is upregulated through IFN-I in a MyD88-independent manner (137). Therefore, it can be surmised that dysfunction of SAMHD1 would lead to an increase in intracellular dNTP pools, thereby promoting viral replication. Interestingly, apart from *TREX1* and RNase H2, a SNP array genome-wide scan of several AGS patients and families revealed homozygous mutations in the *SAMHD1* gene, identifying a monogenic cause of AGS (137). In addition to typical AGS, dominant inheritance of a heterozygous mutation in SAMHD1 causes FCL (138). Indeed, it was subsequently shown that increased dNTP concentrations due to SAMHD1 deficiency cause genome instability, constitutive DNA-damage signaling, cellular senescence and upregulation of ISGs (249). Similarly, in mice, the absence of *Samhd1* triggers spontaneous IFN-I production and upregulation of ISGs in various cells types, however, pathological AGS-like symptoms or any inflammatory features are absent (139, 140). These studies establish SAMHD1 as a negative regulator of IFN-I signaling across species, but IFN-I production induced by *Samhd1* deficiency in mice is not sufficient to induce the development of inflammatory disorders. Although the mechanism(s) of SAMHD1 are still under investigation, it has been postulated that the excessive availability of dNTPs in the absence of SAMHD1 allows for the generation of aberrant immunostimulatory DNA intermediates which potentially trigger IFN-I response through DNA sensors (250). Additionally, recent studies have uncovered a novel function of SAMHD1 as a DNA-ds break repair enzyme working in a complex with C-terminal binding protein 1-interacting protein (CtIP) and the exonuclease meiotic recombination 11 (MRE11) (251). Mutations in SAMHD1 outside of its dNTPase activity site were found to prevent degradation of nascent DNA at stalled replication forks. The ssDNA which accumulated in the cytosol due to this defect caused aberrant activation of IFN-I signaling via the cGAS-STING pathway (252) (**Figure 4**).

ADAR (adenosine deaminases that act on RNA) are a family of broadly expressed post-transcriptional RNA editing enzymes catalyzing adenosine (A) deamination to create inosine (I) in highly structured dsRNA in a non-sequence specific manner. Most polymerases recognize inosine as a guanosine, thus by changing the primary sequence information in an RNA, ADARs generate new protein isoforms. In addition, because inosine base-pairs with cytidine, ADARs can change the structure of an RNA by changing an AU base-pair to an IU mismatch (253). In addition to its role in generating functional protein diversity, ADAR is a crucial negative regulator of IFN responses. ADAR1 deletion in mice causes embryonic lethality at E11.5–E12.5,

accompanied by liver pathology, global upregulation of type I and II IFN–inducible transcripts and rapid apoptosis of hematopoietic cells (142). Not surprisingly, *ADAR1* null humans are unknown, whereas mutations in *ADAR1* cause AGS, with typical IFN-I signature (141). The conclusive evidence affirming the direct negative regulatory role of ADAR1 in the IFN-I pathway came from mouse studies, wherein conditional ADAR1 deficiency in hematopoietic cells caused a global overexpression of ISGs (141). Furthermore, the death of ADAR1 RNA editing-deficient mice (*Adar1*E861A) at embryonic day 13.5, was rescued by concurrent deletion of the cytosolic sensor of dsRNA, MDA5 (143). Similarly, ablation of MAVS, the downstream adaptor of MDA5 and RIG-I, rescued ADAR1-null mice to birth, overall demonstrating a suppressive function of ADAR1 in the RLR pathway (144). It is known that about half of the human and mouse genome is composed of non-coding retroelements such as SINEs and Alu-repeats, which typically form dsRNA duplexes. Importantly, retroelements are known substrates for extensive A-to-I RNA editing (254, 255). Indeed, genome-wide analysis of the *in vivo* substrates of ADAR1 identified clustered hyper-editing of long dsRNA stem loops within 3′ untranslated regions of endogenous transcripts, while in the absence of ADAR1 editing, long dsRNA stem loops formed that activated MDA5 (143). Overall, it is speculated that in the absence of ADAR1, unedited dsRNA transcripts originating from endogenous retroelements accumulate and activate MDA5 to induce IFN-I signaling (**Figure 4**). Thus, the primary physiological function of ADAR1 is to edit endogenous dsRNA to prevent sensing of this substrate as non-self by MDA5 and subsequent IFN-I response.

RNA exosome is a multimeric protein complex that is found in all cells and plays an essential role in the degradation of endogenous RNA. Superkiller viralicidic activity 2-like (SKIV2L) helicase which is part of the RNA exosome was recently proposed to play an important role in preventing the ability of endogenous RNA to activate IFN-I responses. shRNA-mediated knock down of *Skiv2l* in BM-derived macrophages increased RLR mediated IFN-I stimulation. Reduction of *Skiv2l* expression also induced IFN-I production in macrophages once the unfolded protein response was stimulated in a MAVS dependent manner. Furthermore, individuals with hypomorphic *SKIV2L* variants showed an elevated IFN-I signature (146). Therefore, RNA exosome through SKIV2L may negatively control endogenous RNAs to prevent activation of RLR mediated IFN-I response and thus the development of interferonopathies. Nevertheless, further investigation is needed to identify endogenous RNAs regulated by SKIV2L and its relevance in the control of the immunostimulatory potential of endogenous RNA *in vivo,* since *SKIV2L* hypomorphic mutations in humans are associated with tricohepatoenteric syndrome a rare congenital bowel disorder (145) and not severe autoimmunity.

Overall cytosolic TREX1, RNase H2 and NA-editing enzymes regulate the potential of endogenous DNA and RNA to causes inflammatory and autoimmune disorders (**Table 1**, **Figure 4**). Their dysfunction in mice and humans induces severe interferonopathies caused by an aberrant stimulation of intracellular NA sensors by endogenous NAs.

New Players in the Regulation of Mitochondrial Nucleic Acids

Endonuclease G belongs to the family of DNA/RNA-non-specific nucleases that are located in the mitochondrial intermembrane space (256, 257). MEFs deficient for endonuclease G show increased levels of ROS and elevated levels of mtDNA in the cytosol, indicating that this enzyme may regulate mtDNA abundance. In addition, mtDNA that accumulates in endonuclease G deficient MEFs was shown to induce the expression of multiple ISGs in a cGAS-STING dependent manner. mtDNA in the absence of endonuclease G accumulates into the intermembrane space of mitochondria and ultimately reaches the cytosol through voltage-dependent anion channel (VDAC) (148). SLE patients were shown to accumulate mtDNA in their circulation mostly originating from NETs (51, 61) and such accumulation of mtDNA was associated with reduced endonuclease G activity (148). Blockade of mtDNA transport to the cytoplasm using VDAC inhibitors reduced the amount of circulatory mtDNA and ameliorated lupus-like symptoms in SLE prone mice (148). Therefore, endonuclease G is an important regulator of mtDNA abundance and prevents its potential to active IFN-I production.

SUV3 and PNPase: Novel Regulators of mtdsRNA

Cellular RNA degradation is known to be mediated by protein complexes in specific subcellular granules such as processing bodies (P-bodies), found in the cytoplasm of eukaryotes (258). Moreover, it was shown that human mtRNA degradation is mediated by the mitochondrial RNA degradosome comprising an RNA-helicase (hSuV3) and a polynucleotide phosphorylase (PNPase), that occur in distinct foci within the mitochondria. Indeed, silencing of PNPase and/or hSuv3 caused accumulation of undegraded mtRNA decay intermediates (259, 260). More recently, the mtRNA degradosome machinery was characterized *in vivo* and the clinical and physiological relevance of mtdsRNA degradation was presented (79). A hepatocyte-specific PNPase1 deficiency caused modest increases in IFN-β and ISGs. Accordingly, patients carrying hypomorphic mutations in *PNPT1*, which encodes PNPase, displayed mtdsRNA accumulation coupled with upregulation of ISGs and other markers of immune activation, underscoring the importance of preventing cytosolic sensing of mtdsRNA for which the MDA5-MAVS pathway of cytosolic RNA sensing was required but TLR3 signaling was dispensable (79).

ROLE OF NUCLEASES IN PROMOTING INNATE IMMUNE RESPONSES AND NA SENSING

Nucleases not only prevent accumulation of immunogenic ligands but also promote their generation. *Dnase2a* deficiency causes pathogenic activation of the cytosolic cGAS-STING pathway and autoinflammation, whereas it prevents processing

of CpGA-DNA and consequently abrogates TLR9 activation in DCs. Accordingly, CpG-DNA that was pre-processed by DNASE2A was able to stimulate inflammatory cytokine production in *Dnase2a* deficient DCs (261). In addition, *Dnase2a* deficient B cells were also unresponsive to TLR9 activation by DNA complexed immunoglobulins (111). Thus, DNASE2A seems to positively regulate endosomal DNA sensing and negatively regulate cytoplasmic DNA-sensing which may be dependent on the size of DNA fragments generated by DNASE2A-dependent processing. Similarly, in addition to their role in restricting self-RNA/DNA sensing, as discussed above, intracellular RNases are also involved in generating immunogenic RNA ligands necessary for responses against pathogenic RNA. Recently, two endolysosomal enzymes RNASE2 and RNASET2 were shown to cooperatively process RNA to release uridine from RNA ligands which promote stimulation of TLR8 and provide protection against pathogenic microorganisms (262, 263). Interestingly, in addition to processing NA in order to create better ligands for PRR, nucleases may play broader regulatory functions. Particularly DNASE1L3 was reported to regulate the activation of inflammasomes. Indeed, DNASE1L3 inhibition *in vitro*, significantly altered NLRP3 and NLRC4 mediated IL-1β production upon activation (264). However, the importance of this regulatory loop *in vivo* awaits further investigation.

ROLE OF NUCLEASES IN CANCER

It is becoming evident that the activation of anti-tumor immune responses is intimately connected with the activation of NA sensing pathways (265). Ablation of cGAS and STING prevented the activation of spontaneous anti-tumor immune responses (266), and suppressed the immunogenic potential of cytotoxic treatments such as radiotherapy (267) and chemotherapy (268). Similarly, TLR9 mediated IFN-I production was required for the therapeutic activity of multiple chemotherapeutic drugs (269). To activate potent anti-tumor immune responses, endogenous tumor-derived NAs have to escape degradation by nucleases and processing by NA editing enzymes. Upregulation of TREX1 following lethal irradiation of tumor cells was recently reported to prevent cGAS-STING mediated IFN-I production in tumor cells and tumor infiltrating DCs. Sequential delivery of sub-lethal doses of irradiation prevented TREX1 upregulation in tumor cells and allowed the activation of anti-tumor immune responses in an IFN-I-dependent manner, indicating that TREX1 may act as a rheostat to control radiation-induced tumor-derived immunostimulatory DNA (270). Multiple studies have also described the function of ADAR1 in the regulation of anti-tumor immunity. Deletion of ADAR1 in tumor cells delayed tumor growth and increased the therapeutic potential of immune checkpoint blockade therapies. ADAR1 was shown to edit RNA originating from endogenous retroelements in tumor cells and thus inhibited their ability to stimulate MDA5-mediated IFN-I production that is required for the activation of anti-tumor immune responses (271). Furthermore, epigenetic therapies that increase the expression of endogenous retroelements were potent stimulators of anti-tumor immune responses only when ADAR1 was depleted from cancer cells (272). The endolysosomal DNASE2A was also shown to negatively regulate the production of inflammatory cytokines induced upon the uptake of dying tumor cells by macrophages (273). Therefore, in the context of cancer, blocking cell-intrinsic nucleases and NA-editing enzymes that limit the immunostimulatory potential of endogenous NA may be of relevance to enhance anti-tumor immune responses. Alternately, homozygous deletion of *RNASEH2B* has been shown to occur in chronic lymphocytic leukemia (CLL) and other malignancies (274), Similarly, pathogenic mutations in SAMHD1 have been reported in up to 11% of CLL patients (275). These studies suggest tumor suppressor roles for SAMHD1 and RNase H2 and are currently under investigation.

Patients with cancer present elevated levels of circulatory cfDNA, mostly originating from tumors. Such elevation of cancer cfDNA arises from a combination of increased cancer cell abundance and the reduced activity of extracellular DNases in cancer patients' sera (276). In agreement with their overall low DNase activity, patients with cancer also showed increased levels of circulatory NETs (277, 278). The accumulation of NETs in mice and individuals with cancer contributed to metastasis by trapping circulatory cancer cells in target organs. Importantly targeting NETs by the administration of recombinant DNASE1 significantly inhibited metastasis in murine models of cancer (279, 280). DNASE1L3, an important regulator of several sources of cfDNA, was shown to be downregulated in multiple cancers. Importantly, a recent study that stratified patients with hepatocellular carcinoma found a significant correlation between reduced DNASE1L3 expression and poor survival (281). These results suggest that extracellular DNases are impaired in cancer patients and may contribute to tumor growth and metastasis.

CONCLUSION AND THERAPEUTIC AVENUES

Overall, comprehensive studies performed in patients and validated in experimental mouse models certify the prominent role of nucleases and NA-editing enzymes in the prevention of autoimmunity, autoinflammation, and malignancy. Not surprisingly, all of these pathogenic conditions involve irregularities in inflammation because NA degrading and processing enzymes function as sentinels to restrict the activation of PRRs, which are central to all inflammatory pathways. Prudently, a significant amount of research and pharmaceutical effort focuses on finding therapeutics to block PRR-mediated inflammatory pathways in autoimmune inflammatory conditions. However, the greatest challenge in targeting the PRR-pathway of autoinflammation is the unwanted subversion of immune-responses against opportunistic infections. Therefore, to achieve the best therapeutic outcomes, a clear understanding of the genetic aberrations that cause autoinflammatory conditions is essential, and yet, this path also has considerable challenges. For example, it is evident that monogenic aberrations in one or more intracellular

nucleases and NA editing enzymes lead to interferonopathies mediated by the activation of the cytosolic cGAS-STING pathway, which makes these attractive targets for therapeutic intervention in such patients. However, caution needs to be exercised when using STING inhibitors in AGS patients because, as detailed in the previous section, disruption of cGAS and STING suppresses spontaneous anti-tumor immune surveillance (266). Indeed, several STING agonists are being tested as therapeutics in clinical trials for solid tumors (clinicaltrials.gov). Nonetheless, PRRs are key targets for therapeutic interventions. In fact, as a proof of principle, well-known antimalarial drugs—chloroquine and hydroxychloroquine (Plaquenil), are TLR-signaling inhibitors, successfully in clinical use as first line treatments for SLE, rheumatoid arthritis and Sjogren's syndrome (282). Not surprisingly, several small molecule inhibitors, antibodies, oligonucleotides, lipid-A analogs and microRNAs that interfere with TLR signaling are emerging as promising therapeutics for inflammatory autoimmune diseases, reviewed in detail by Gao et al. (283). Other important rational targets of therapeutic intervention include effector cytokines and their signaling components. Indeed, monoclonal antibodies against IFN-I (Sifalimumab) and IFNAR1 (Anifrolumab), and inhibitors of Janus Kinases (Baricitinib, Tofacitinib), have shown promising results in clinical trials for SLE and type-I interferonopathies (284–286).

Another notable methodology to tackle NA-mediated inflammation is to target the immunogenic NAs themselves by: (1) preventing immunogenic NA generation, or (2) promoting immunogenic NA degradation. As noted previously, retroelements constitute about 40% of the human genome, which are reverse transcribed within the cells and potentially generate highly immunogenic NAs due to their microbial origins. The immunogenic potential of these self-NAs is restricted by TREX1 and ADAR1, which play a central role in endogenous retroelement metabolism. Indeed, restriction of reverse transcriptase activity by using inhibitors (RTIs) like abacavir, lamivudine and zidovudine have shown clinical efficacy in reducing inflammatory responses in AGS patients (286). Extracellular nucleases, specifically, DNASE1L3 has emerged as a novel immunosuppressive cell-extrinsic agent that regulates extracellular immunogenic DNA (45, 182). Unlike intracellular nucleases that could be difficult to manipulate, exogenous supplementation of recombinant DNASE1L3 protein offers a viable therapeutic modality to prevent immunogenic DNA-dependent TLR signaling. This process would perhaps involve engineering and modifying DNASE1L3 protein to enhance its nuclease activity and increase its half-life in circulation.

In conclusion, clinical and experimental studies support the fact that regulation of NA-metabolism is at the heart of maintaining self-tolerance. Undoubtedly, nucleases and NA-editing enzymes have emerged as crucial sentinels in preventing autoinflammation and should be explored as viable therapeutic targets for autoimmune and inflammatory disease conditions.

AUTHOR CONTRIBUTIONS

PS wrote the section on extracellular nucleases. AG wrote the section on intracellular DNases. LS wrote the section on endogenous nucleic acid forms and sources. CS wrote the part on nucleic acids sensors and RNases and VS wrote the rest of the manuscript and together with CS compiled the entire manuscript. AF, PS, AG, and VS designed figures and the table and PB edited the manuscript and provided valuable advice throughout the writing process. All authors listed have made a substantial, direct, intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
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Corrigendum: The Role of Nucleases and Nucleic Acid Editing Enzymes in the Regulation of Self-Nucleic Acid Sensing

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Keywords: DNases, RNases, systemic lupus erythematosus, DNA sensing, RNA sensing, interferonopathies, aicardi goutieres syndrome, toll-like receptors

A Corrigendum on

The Role of Nucleases and Nucleic Acid Editing Enzymes in the Regulation of Self-Nucleic Acid Sensing

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In the original article, there was a mistake in the legend for **Figure 2** as published. The correct legend for **Figure 2** was mistakenly omitted and replaced with the legend of figure 3. The correct legend appears below.

DNASE1L3 deficiency leads to the accumulation of numerous forms of DNA including chromatin, MP associated DNA and NET-associated DNA. Accumulation of such DNA contributes to the aberrant activation of TLR7,9 in B cells and plasmacytoid dendritic cells (pDCs). In B cells TLR7,9 activation leads to their differentiation into plasma cells and antibody forming cells (AFC) that produce autoreactive antibodies mostly directed against dsDNA. In pDCs TLR7,9 activation induces the production of type I interferons (IFN-I) which also play an important role in the transition of B cells into AFC. The production of anti-dsDNA antibodies and of IFN-I will ultimately cause the development of Systemic Lupus Erythematosus (SLE).

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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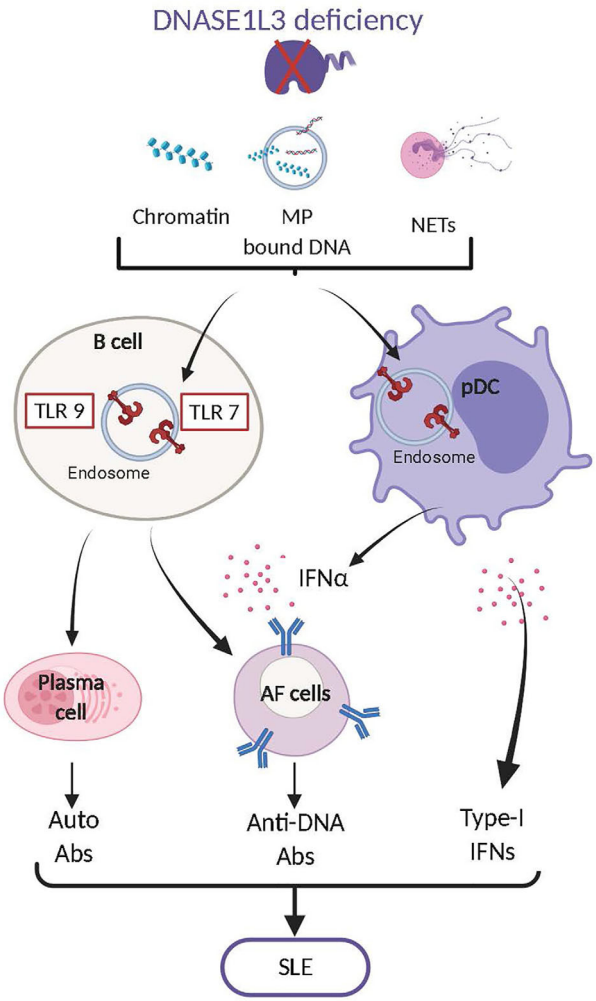


FIGURE 2 | DNASE1L3 deficiency leads to systemic lupus erythematosus (SLE). DNASE1L3 deficiency leads to the accumulation of numerous forms of DNA including chromatin, MP associated DNA and NET-associated DNA. Accumulation of such DNA contributes to the aberrant activation of TLR7,9 in B cells and plasmacytoid dendritic cells (pDCs). In B cells TLR7,9 activation leads to their differentiation into plasma cells and antibody forming cells (AFC) that produce autoreactive antibodies mostly directed against dsDNA. In pDCs TLR7,9 activation induces the production of type I interferons (IFN-I) which also play an important role in the transition of B cells into AFC. The production of anti-dsDNA antibodies and of IFN-I will ultimately cause the development of Systemic Lupus Erythematosus (SLE).

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ORIGINAL RESEARCH
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uhrf1 and *dnmt1* Loss Induces an Immune Response in Zebrafish Livers Due to Viral Mimicry by Transposable Elements

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Activation of transposable elements (TEs) can cause cellular damage. Cytoplasmic nucleic acid sensing pathways evolved to detect pathogens, but can also serve to cull cells with inappropriate TE activation as TEs can be viral mimetics. Epigenetic silencing of TEs is mediated in part by DNA methylation, but it is not clear if TE activation or the immune system contribute to the cellular damage caused by loss of DNA methylation. Here, we provide mechanistic insight into the observation of an activated interferon response in the liver of zebrafish larvae with deletion in critical components of the DNA methylation machinery, *uhrf1* and *dnmt1*. We focus on dissecting the relationship between DNA methylation, TE activation and induction of an immune response through cytoplasmic DNA and double stranded RNA sensing pathways and identify *tnfa* as a mediator of cell death in the liver of these mutants. Integrated RNAseq and methylome analysis identified LTR transposons as the most upregulated in these mutants and also the most methylated in control larvae, indicating a direct role of DNA methylation in suppressing this TE subclass. RNAseq analysis from these same samples revealed expression signatures of a type-I interferon response and of *tnfa* activation, mimicking the pattern of gene expression in virally infected cells. CRISPR/Cas9 mediated depletion of the cellular antiviral sensors *sting* and *mavs* reduced expression of interferon response genes and *tnfa* depletion dramatically reduced cell death in *uhrf1* mutant livers. This suggests that the antiviral response induced by DNA hypomethylation and TE activation in the liver is mediated by the signaling pathways activated by both cytoplasmic double stranded RNA and DNA and that *tnfa* mediates cell death as a potential mechanism to eliminate these damaged cells.

Keywords: *uhrf1*, *dnmt1*, transposable element, interferon, TNFa, zebrafish, DNA methylation

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INTRODUCTION

The primary functions of the immune system are to sense danger and to differentiate non-self from self in order to control the expansion of infectious agents. One way that danger is sensed by immune and non-immune cells alike is the presence of nucleic acids in cellular locations where they are not meant to be. For instance, the presence of DNA or double stranded RNA in the cytoplasm is monitored by cytoplasmic sensors. These sensors trigger signaling and transcriptional

pathways that lead to release of interferons and cytokines that recruit immune cells, shut down viral reproduction, and promote cell death (1). In most cases, these responses clear infected cells and prevent invasion of the infectious agent. If the signal persists, however, chronic activation of anti-viral pathways can lead to pathologies, as exemplified in the hyperinflammation that characterizes autoimmune diseases (2) and, of more recent interest, the exacerbated immune response to SARS-CoV-2 infection which causes severe or lethal COVID-19 (3).

In addition to sensing pathogen invasion, cytoplasmic nucleic acid sensing pathways are also utilized in settings where cells are damaged, such as in response to toxic exposures, oncogenic transformation, genotoxic stress, or epigenetic damage (4). In these cases, damaged or dead cells release nucleic acids into the cytoplasm or extracellular space that are detected as inappropriate by the anti-viral sensing pathways and trigger sterile inflammation. In addition to cell damage, the expression of latent endogenous retroviruses (ERVs) and transposable elements (TEs) can activate the same mechanism to cause sterile inflammation. This can be advantageous, as cells with TE activation can be oncogenic (5), and therefore it is beneficial to have an immunomodulatory mechanism to eliminate potentially pre-cancerous cells.

It is not known how changes to the epigenome contribute to immune activation. One idea is that the chromatin landscape can restrict or potentiate the transcriptional activity of key transcription factors such as STAT1, NF-kB, interferon response factors (IRF) 3 and 7, and others (6) that mediate the response to cytoplasmic nucleic acid sensing pathways (7). Evidence for this model is suggested through studies on the regulation of Tumor Necrosis Factor alpha (Tnfa). This highly pleiotropic cytokine affects nearly all cell types to trigger cellular responses spanning from the induction of inflammatory pathways, stimulation of cellular proliferation and differentiation to the activation of programmed cell death such as apoptosis and necroptosis. Loss of DNA methylation causes robust induction of Tnfa in the gut of zebrafish larvae (8) and in mouse macrophages (9). This is accompanied by modest decrease in the level of DNA methylation in the Tnfa promoter, leading to the conclusion that DNA methylation directly represses the expression of the Tnfa gene. An alternative hypothesis is that widespread changes in the repressive epigenome can derepress TEs, mimicking a viral infection, activating an interferon response, and activation of Tnfa. In this model, DNA methylation loss directly impacts the expression of TEs, and Tnfa is activated by an indirect mechanism. Delineating between these mechanisms is an important step in determining how widespread changes to the methylome contribute to chronic immune activation. In addition, it is important to determine if there are tissue specific immune responses to DNA methylation loss, as most studies in the field are carried out using cell culture models.

We approached this through studying the effect of widespread loss of DNA methylation during development of zebrafish embryos by deleting *uhrf1*, a core component of the DNA methylation machinery. *uhrf1* recognizes hemi-methylated DNA and recruits DNA methyltransferase 1 (*dnmt1*) onto replication foci to maintain the DNA methylation pattern in daughter cells (10, 11). Unlike mouse embryos where *uhrf1* deletion is embryonic lethal (12), *uhrf1* mutant zebrafish embryos survive past the early embryonic stages due to maternal supplies (11–13). Loss of either *uhrf1* or *dnmt1* in zebrafish causes profound developmental defects leading to premature death by 8 days post fertilization (dpf) (13–16). *uhrf1* is essential for cell proliferation and development of the eye (17), intestine (8, 16), and liver (14, 15). Our previous work in *uhrf1* mutant zebrafish larvae showed increased circulation of neutrophils and activation of an interferon response, causes by cytoplasmic nucleic acid sensing pathways triggered by TEs expression (18). This is complemented by studies by others where loss of *uhrf1* is pro-inflammatory: *uhrf1* mutant zebrafish have intestinal inflammation associated with cell death that is dependent on *tnf*a (8), mice with *uhrf1* deficient regulatory T-cells develop spontaneous colitis in part due to a loss of the immunomodulatory function of these cells (19) and a recent study showing that deleting *uhrf1* in mature T-regulatory cells leads to spontaneous inflammation in multiple organs and the acquisition of a pro-inflammatory gene expression pattern, counteracting the immunosuppressive function (20). In addition, loss of *uhrf1* in macrophages makes mice more susceptible to colitis in response to stimuli and *uhrf1* deficient macrophages display an enhanced proinflammatory profile when stimulated (9). Given the implication of *uhrf1* in many immune mediated responses, it is important to identify the mechanism by which alterations in *uhrf1* expression or function are proinflammatory.

Here, we investigate specific outcomes of DNA methylation loss in zebrafish livers. We focus on the liver because inflammation plays a central role in several important liver diseases, including viral and non-viral hepatitis, which, in the setting of fatty liver can progress to a life threatening steatohepatitis (21) and the inflammation during hepatic fibrosis can severely reduce liver function and regenerative capacity (22). Some studies have uncovered inappropriate TE activation as a common feature of liver cancer (23, 24) and in other cancer types, viral mimicry by TEs has been proposed to trigger activation of the interferon response (25–27). In the current study, we test the hypothesis that the interferon response in the liver of zebrafish larvae with loss of DNA methylation is mediated by unleashed TEs that trigger cytoplasmic nucleic acid sensing pathways and we test whether Tnfa, a key downstream target of these pathways, is involved in the cell death phenotype that characterizes the hepatic phenotype of *uhrf1* mutants. We find that LTRs are preferentially induced in these models, and that these same TEs are heavily methylated in wild-type (WT) embryos. We uncover a robust type I interferon response, activation of NF-kB and Tnfa signaling in the liver, which was attenuated upon deletion of cytoplasmic viral sensors suggesting that the nucleic acid sensing pathways, and not direct epigenetic regulation of immune genes, trigger the immune response. Furthermore, we discovered that *tnfa* depletion rescues cell death in *uhrf1* mutant livers. This advances the understanding of how DNA hypomethylation leads to a tissue specific hyperactivation of inflammatory mechanisms and shows that the immune response contributes to the removal of cells with epigenetic damage.

MATERIALS AND METHODS

Zebrafish Husbandry and Genotyping

Adult fish were raised in accordance with the policies of the NYU Abu Dhabi for Animal Care and Use Committee (IACUC) on a 14:10 h light:dark cycle at 28°C. *uhrf1* [hi272 allele; (28)] and *dnmt1* [s904 allele; (29)] mutant embryos were generated by incrossing of heterozygous carriers and were identified based on characteristic phenotypes of small liver, small and defective jaw, small eye, and flat gut as described (14, 15) or by genotyping individual embryos. *uhrf1*$^{-/+}$ adults were genotyped by PCR as described (14) (see **Supplementary Table 1**) and *dnmt1*$^{-/+}$ were identified by outcross to *dnmt1*$^{-/+}$ adults. To be able to use the *Tg(c269*^{off}*; 10XUAS:dsRed)* line to monitor DNA methylation in the liver of live larvae, we generated a line that expresses the Gal4 driver in hepatocytes *Tg(fabp10a:Gal4;cmlc2:EGFP)* (**Supplementary Figure 1**). This serves to activate transcription from an unmethylated 10XUAS:dsRed reporter which is included in this line, but cannot activate the 10XUAS:GFP in the *Tg(c269*^{off}*; 10XUAS:dsRed)* line because that promoter is silenced due to accumulation of 5-methyl cytosine (5-MeC) (30–32). In *Tg(fabp10a:Gal4;cmlc2:EGFP; c269*^{off}*; 10XUAS:dsRed)* larvae, EGFP will only be activated in the in hepatocytes when the promoter is unmethylated (**Supplementary Figure 1**), such as in *uhrf1* and *dnmt1* mutants.

The Tg(*fabp10a:Gal4*; cmlc2:EGFP) line was generated using Gateway cloning (Invitrogen) to produce vectors with *tol2* transposon sites flanking the transgenes (33). Transposase mRNA was produced by using mMessage mMachine kit (Invitrogen) by following manufacturer's instructions. 40 ng of vector containing the Tg(*fabp10a:Gal4*; cmlc2:EGFP) cassette was injected together with 80 ng of transposase mRNA. Larvae with *cmlc2:EGFP* expression were raised and outcrossed to identify founders, and stable transgenics from allele A were crossed to the *c269*^{off} background.

Crispr/Cas9 Generation and T7 Endonuclease Assay

sgRNA for *mavs* was designed by using ChopChop (https://chopchop.cbu.uib.no/). sgRNA for *slc45a2* (gene involved in pigmentation), *sting* and *tnfa* were previously designed and validated (8). Genotyping primers were designed by Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) and validated in USCS Genome Browser (https://genome.ucsc.edu/cgi-bin/hgPcr). sgRNAs were produced by sgRNA IVT kit (Takara Bio) by following the manufacturer's instructions and RNA was isolated by Trizol (Invitrogen). sgRNAs were quantified by Qubit RNA BR kit and diluted at 50 ng/μl and stored as single use aliquots. The efficiency of each sgRNA was assessed by injecting WT embryos with equal volume of previously diluted nls-Cas9 protein (IDT; 0.5 μl of nls-Cas9 added with 9.5 μl of 20 mM HEPES; 150 mM KCI, pH 7.5) and sgRNA, incubated at 37°C for 5 min and then 1 nl was injected in 1–2 cell stage embryos. At 24–72 hpf, 12–16 embryos from each sgRNA were individually collected and genomic DNA was extracted by heat shock denaturation in 50 mM NaOH (95°C for 20 min). For each embryo, PCR was performed on genomic DNA by using Q5 High-Fidelity Taq Polymerase (New England Biolabs) followed by T7 endonuclease I assay (New England Biolabs) to detect mutations. For T7 endonuclease I assay, 10 μl of PCR product was incubated with 0.5 μl of T7e1 enzyme (New England Biolabs) for 30 min at 37°C. Digested and undigested fragments were run in parallel in 2% agarose gel to assess the presence of indels. Efficiency was calculated as the number of embryos that show a positive result based on T7e1 assay divided by the total number of embryos assayed for each sgRNA.

All sgRNAs demonstrated to generate indel mutations were injected into the 1-cell embryos generated by an incross of *uhrf1*$^{-/+}$ adults as previously described. The resulting F_0 larvae were considered crispants. For each clutch and each sgRNA, *uhrf1*$^{-/-}$ mutants were divided from phenotypically WT siblings at 5 dpf based on morphological differences and used for following analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling Assay

Larvae collected at 5 dpf were fixed in 4% Paraformaldehyde for 4 h at room temperature, and gradually dehydrated through a graded series of methanol and stored in 100% methanol at 4°C overnight. Gradual rehydration to PBS through a graded series of methanol/PBS dilutions was carried out at room temperature. Larvae were permeabilized with 10 μg/ml Proteinase K (Macherey-Nagel) in PBS containing 0.1% tween (PBST) and fixed in 4% Paraformaldehyde for 10 min at room temperature. Livers were then dissected out of the larvae and subjected to TUNEL assay according to manufacturer's instructions (*In Situ* Cell Death Detection kit, Fluorescein; Roche). Nuclei were counterstained with Hoechst (Thermo Fisher Scientific) diluted 1:1000 in PBS, mounted on a microscope slide with Vectashield (Vector Laboratories) and covered with a 0.1 mM coverslip for imaging using Leica SP8 confocal microscope. LAS X software (Leica software) was used for quantification from 3 separate optical sections per livers which were then averaged from 3 livers per clutch per condition and 4 clutches per sample were analyzed. Results were plotted in GraphPad Prism 8.

RNA and DNA Extraction

For each sample, 10 to 20 livers were microdissected and RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions with some modifications. Briefly, during precipitation in isopropanol, 10 μg of Glycoblue (Thermo Fisher Scientific) was added and precipitation was performed overnight at −20°C followed by 1 h centrifuge at 12,000 g at 4°C. RNA was resuspended in water and used in the following procedures. Genomic DNA was extracted from 20 to 30 livers by using a DNA extraction buffer (10 mM Tris-HCl pH9, 10 mM EDTA, 200 mM NaCl, 0.5% DSD, 200 μg/ml proteinase K) as previously described (18). DNA was resuspended in water and quantified by Qubit dsDNA High Sensitivity kit.

cDNA Production and qPCR

After RNA extraction, RNA was retrotranscribed without quantification. cDNA was synthetized using Qscript cDNA

synthesis kit (Quanta Bio) following the manufacturer's instructions. cDNA was diluted 12 times and used for qPCR using Maxima® SYBR green/ROX master mix (Thermo Fisher Scientific). *rplp0* was used to normalize expression levels by using the calculations for delta-Ct and WT siblings were used to calculate delta-delta-Ct (DDCt) as previously described (34). To determine changes in expression between control and experimental samples, the fold change was calculated, the $\log_2$ was derived (L2FC) for display. All experiments were performed on samples from at least 3 independent clutches as indicated in the figure legends.

Slot Blot Analysis of 5-MeC

Slot blot was performed as previously described (15). Briefly, 2 ng of genomic DNA was denatured in 400 mM NaOH/10 mM EDTA and blotted onto nitrocellulose membrane in duplicate for dsDNA and 5-MeC using a slot blot apparatus. Membranes were incubated 1 h at 80°C, blocked with 5% skim milk in TBST (37 mM NaCl, 20 mM Tris pH 7.5, 0.1% Tween 20), and incubated overnight at 4°C in either anti-dsDNA (Abcam, 1:8000 in 2% BSA in TBST) or anti-5-methyl-cytosine (m5C–Aviva Biosystem clone 33D3, 1:2000 in 2% BSA in TBST). Membranes were washed in TBST and probed with anti-mouse HRP secondary antibody (Promega; 1:5000 in 2% BSA in TBST) for 1 h at room temperature followed by development in ECL (Thermo Fisher Scientific). ChemiDoc (BioRad) was used to detect and quantify the chemiluminescent signal. Gel Analyzer was used to measure the signals and ratio between 5-MeC and dsDNA was plotted for controls and mutants in each clutch.

RNAseq

Total RNA was extracted from ∼20 livers dissected from 5 dpf zebrafish larvae for each condition. For *uhrf1*$^{-/-}$ mutants and their phenotypically WT siblings, 5 clutches were collected while for *dnmt1*$^{-/-}$ mutants and their phenotypically WT siblings, 3 clutches were used. RNA was treated by DNAse I for 30 min at 37° C followed by RNA purification (RapidOut DNA Removal Kit–Thermo Fisher Scientific). RiboZero was used to remove ribosomal RNA and the remaining sample was used for library preparation according to manufacturer's instructions (Illumina) from 80 to 100 ng of RNA. Libraries were sequenced on NextSeq550 (Illumina) to obtain 150 bp paired-end reads. Sequencing quality was assessed by using MultiQC v1.7 (https://multiqc.info). Adaptor sequences were removed and reads were aligned to the *D. rerio* GRCz10 reference genome using HISTA2 for alignment with default parameters so that only paired reads were aligned and multiple alignments were kept (35). To estimate gene expression, reads that mapped to the exon of each gene that had an annotated Ensembl ID were counted with HTSeq (36). A generalized linear model implemented in DESeq2 in Bioconductor (37) was adopted to test differential gene expression between each mutant compared to their respective sibling controls. Adjusted *p*-value with a false discovery rate of <0.05 was treated as significantly different expression between mutant and controls. Data is available in GEO (GSE160728).

TE quantification was assessed using RepeatMasker based on the annotation of *danRer10* provided by the UCSC Table Browser (https://genome.ucsc.edu/cgi-bin/hgTables) and were counted with HTSeq using union mode which allowed for each read to be counted only once; reads were not designated as strand specific. All TEs were quantified based on families. Statistical analysis was implemented with DESeq2 using the same protocol as the differential gene expression analysis described above for TEs with simple repeats excluded.

Reduced-Representative Bisulfite Sequencing

RRBS was performed on genomic DNA extracted from 10 *uhrf1*$^{-/-}$ mutants and phenotypically WT siblings at 5 dpf. Briefly, 50–250 ng of gDNA was digested with 200 U of MspI (New England Biolabs) for 24 h at 37°C. Digested DNA was used for preparing library as previously described (38), with the exception that the adaptors used for multiplexing were purchased separately (Next Multiplex Methylated Adaptors–New England Biolabs). Libraries were size-selected by dual-step purification with Ampure XP Magnetic Beads (Beckman Coulter, Agencourt) to specifically select a region of fragments from 175 to 670 bp. Bisulfite conversion was performed with Lightning Methylation Kit (ZYMO Research) by following the manufacturer's instructions. Libraries were amplified using KAPA HiFi HotStart Uracil+ Taq polymerase (Roche) and purified with Ampure XP Magnetic Beads (Beckman Coulter, Agencourt) before sequencing. Libraries were sequenced using the Illumina Nextseq550. Fastq files are available in GEO (GSE160728).

Quality control of the RRBS sequencing data was assessed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were quality trimmed using Trimmomatic (39) to remove low quality reads and adapters. Reads passing quality control were aligned to the genome reference GRCz10 using the default parameters in Bismark (40), which adopts Bowtie2 as the aligner (41) and call cytosines methylation at the same time.

CpG methylation levels were detected with the R package "methylKit" (42). CpGs covered at least 10 times in each condition were included in the analysis. CpGs with methylation level below 20% were treated as unmethylated and above 80% were considered as methylated. Genomic element annotation of CpGs was performed with R package "genomation." For plotting and statistical analysis, R package "ggplot" and GraphPad Prism 8 software were used. Transposons were identified using the Repeat Masker table annotation on the reference genome assembly GRCz10 (danRer10). The WashU EpiGenome Browser (http://epigenomegateway.wustl.edu/browser/) was used to display distinct genome locations.

Bioinformatics

RNAseq and RRBS data were analyzed as previously described and visualized in RStudio (version 4.0) using code that is publicly available on Github (https://github.com/zcmit/NYUAD_Sadler-Lab/blob/master/uhrf1%20and%20dnmt1%20loss%20induces%20an%20immune%20response%20in%20zebrafish%20livers

%20due%20to%20viral%20mimicry%20by%20transposable%20elements). For Gene Ontology (GO), zebrafish gene names were converted in human gene names by using Biomart and then used for the GO analysis. GO enrichment analysis was conducted using the GO hypergeometric over-representation test in the "ClusterProfiler" package in R using default parameters, and REVIGO was subsequently used to eliminate redundant enriched terms. An adjusted $p < 0.05$ was treated as significant for all analyses. Specific gene lists of type-I interferon response and Tnfa were collected from IPA database and used to subset specific group of genes. Heatmaps were performed by using R package "pheatmap."

Statistical Analysis

All experiments were carried out on embryos from at least 3 biological replicates and, where appropriate, technical replicates were also included and are indicated in the figure legend of the relevant data. The number of replicates for each experiment is indicated in each figure. Methods to evaluate the statistical significance include Students *t*-test with adjustment for multiple comparisons or Chi square analysis; the tests used are indicated in each graph and table. All the plots were generated in GraphPad Prism 8 and RStudio. Statistical analysis is performed in GraphPad Prism 8.

RESULTS

uhrf1 and *dnmt1* Loss Causes DNA hypomethylation in the Liver

We used a biochemical assay and a novel imaging approach that uses a methylation reporter in live zebrafish to assess the status of DNA methylation in the liver of *uhrf1* and *dnmt1* mutant larvae. First, bulk DNA methylation was assessed using slot blot analysis of 5-MeC on total genomic DNA extracted from the liver of 5 dpf larvae with mutation in *dnmt1* or *uhrf1* (**Figures 1A,B**). In both cases, 5-MeC is decreased by more than 50% compared to levels detected in the liver of phenotypically WT siblings, with equivalent levels of residual methylation in both models (**Figure 1B**). This is comparable to the degree of hypomethylation detected in whole larvae from these two mutants (13, 15).

We next used a novel *in vivo* DNA methylation reporter line based on the GAL4-UAS system [(43); **Supplementary Figure 1**]. In the line termed *c269*^{off}, 10 copies of the UAS promoter lie upstream of GFP, and these have been silenced by DNA methylation over generations of breeding [**Supplementary Figure 1**, (30–32)]. This high level of methylation on the promoter blocks the ability of GAL4 to activate GFP expression in WT animals, but when the 10XUAS is unmethylated due to loss of *uhrf1* or *dnmt1*, GAL4 can access the promoter and GFP is expressed (**Supplementary Figure 1**). As a positive control for GAL4 activity, this line was also crossed to a line containing *Tg(10XUAS:dsRed)* in which the UAS promoter was not silenced by DNA methylation (30, 32), and therefore the expression of dsRed is used as a control for GAL4 activity. To monitor DNA methylation specifically in hepatocytes, we generated a transgenic line expressing GAL4 under the *fabp10a* promoter which utilized the *cmlc2:EGFP* cassette as a reporter of transgenesis *Tg(fabp10a:GAL4; cmlc2:EGFP)*. These transgenes were crossed to the *dnmt1*$^{+/-}$ and *uhrf1*$^{+/-}$ mutants to generate and *Tg(c269*^{off}*; 10XUAS:dsRed; fabp10a:GAL4; cmlc2:EGFP);dnmt1*$^{+/-}$ larvae. In phenotypically WT siblings, GFP was not detected in the liver of any larvae, whereas in the liver of all *uhrf1* and *dnmt1* mutants examined, GFP was detected as early as 80 h post fertilization (hpf), which is the time when their liver bud is easily visible using these transgenes, and GFP expression persisted through 120 hpf (**Figure 1C**). These data indicate that DNA hypomethylation in the liver of *uhrf1* and *dnmt1* mutants is detected as soon as hepatocytes differentiate. Previous studies showed that the small liver phenotype observed in these mutants is correlated with massive cell death (14, 15, 29). We hypothesize that the cell death and small liver phenotypes are due to both a direct effect of DNA methylation on the ability of hepatocytes to appropriately go through DNA replication and also to the activation of the immune system which could serve to induce cell death.

Retrotransposons Are Overexpressed in the Liver of *dnmt1* and *uhrf1* Mutant Larvae

In terminally differentiated tissues, DNA methylation functions primarily in imprinting, maintaining chromosome stability and silencing repetitive elements such as TEs and pericentromeric DNA. More than 50% of zebrafish genome is constituted by repetitive elements, with DNA transposons making up the large majority of these (**Supplementary Figure 2**). Our previous analysis of TE expression in RNAseq generated from the whole *uhrf1* and *dnmt1* mutant larvae uncovered widespread retrotransposon activation (18).

Given that tissue specific expression patterns of both the genes that regulate TE expression and the TEs themselves have been reported (44), we used RNAseq to ask whether there was specificity in the TE expression pattern in the liver of 5 dpf *dnmt1* and *uhrf1* mutants compared to phenotypically WT siblings. A caveat is that the repetitive nature of TEs means that the short-reads generated by next generation sequencing could fail to capture the full spectrum of expression. Regardless, this method uncovered widespread changes in TE expression in both models. LTR transposons predominated as the most affected with 329 and 332 LTRs categorized as upregulated in *dnmt1* and *uhrf1* mutant livers, respectively (**Figures 2A–C**, **Table 1**). This is an overrepresentation, as LTR transposons occupy <10% of the zebrafish genome (**Supplementary Figure 2**). Among the LTRs, members of the Gypsy and Pao families were the most upregulated and the most enriched in the datasets from *uhrf1* and *dnmt1* mutants (**Supplementary Table 2**). In contrast, while the DNA transposons dominate the TE landscape in the zebrafish genome (**Supplementary Figure 2**), only 103 and 113 DNA transposons were upregulated in *dnmt1* and *uhrf1* mutants, respectively (**Figure 2C**; **Table 1**). The changes in the expression of some LTRs was dramatic, with $\log_2$ fold change (L2FC) ranging from >6 to <−2. In contrast, DNA transposons had a L2FC range ± 1 (**Figures 2A,B,D**). TE expression was highly correlated in both mutants, with LTRs showing the strongest linear correlation

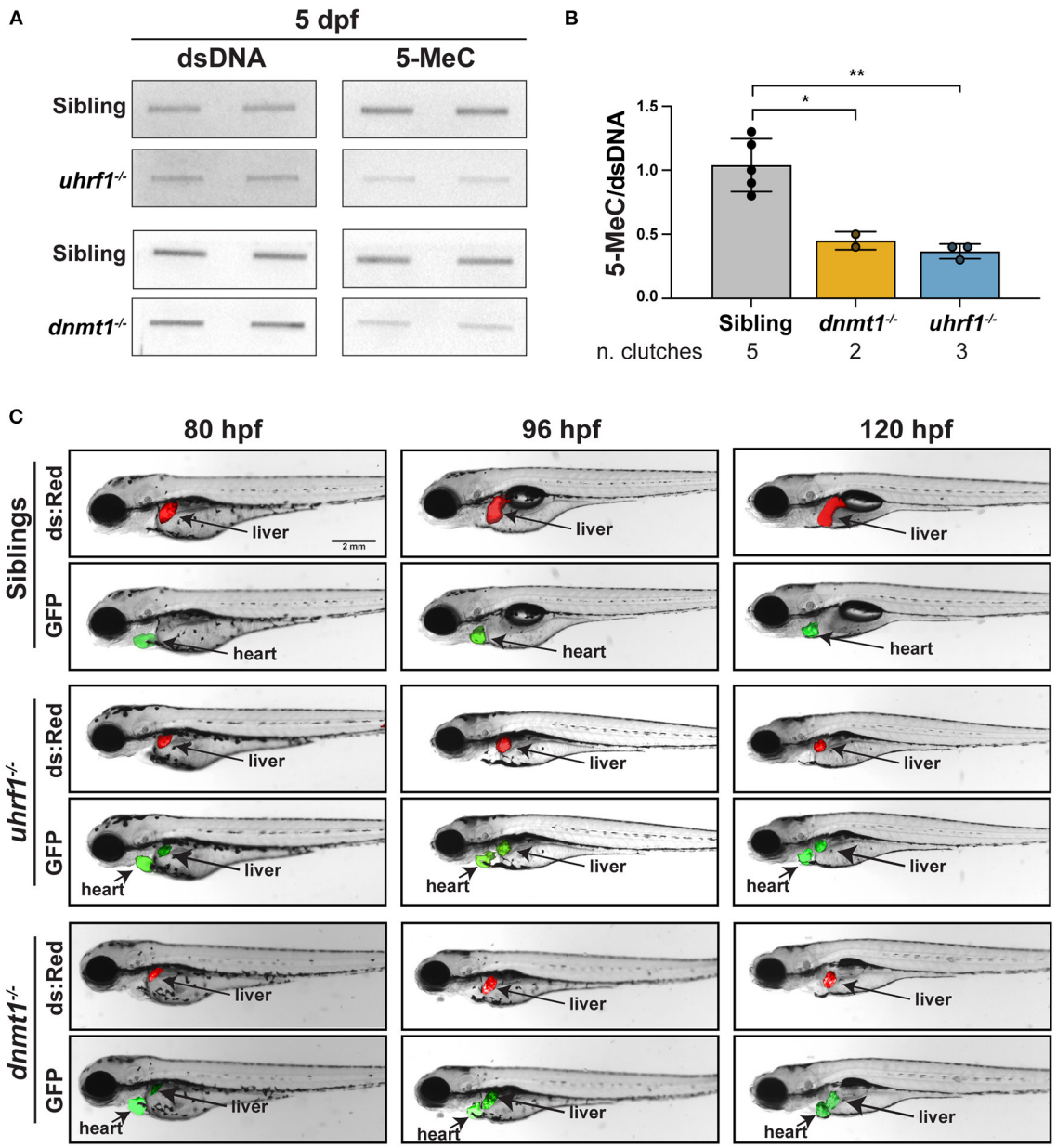


FIGURE 1 | *uhrf1* and *dnmt1* loss causes DNA methylation loss in zebrafish livers. **(A)** Slot blot of genomic DNA extracted from pools of 5 dpf *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant and WT siblings livers. **(B)** Quantification of 5-MeC measured by slot blot normalized to double stranded DNA (dsDNA). Each dot represents one clutch. *$p < 0.05$ and **$p < 0.005$ calculated by unpaired *t*-test. **(C)** Time course imaging of live larvae by fluorescent stereoscope microscopy. *Tg(c269°ff ; 10XUAS:dsRed; fabp10a:GAL4; cmlc2:EGFP);dnmt1−/−* and *Tg(c269°ff; 10XUAS:dsRed; fabp10a:GAL4; cmlc2:EGFP);uhrf1−/−* are shown at each age, showing the liver (dsRed), the heart (GFP) as a marker of transgenesis and, in green, the liver with DNA hypomethylation in mutants only. Images are representative of 100% of larvae from 3 clutches observed for each genotype.

(**Figure 2D**). In addition to the upregulated TEs, there were 502 and 525 repetitive elements which were categorized as down-regulated in *dnmt1* and *uhrf1* mutants, respectively (**Table 1**), albeit modestly compared to the upregulated TEs (**Figures 2A,B**).

One possible explanation for the differential expression of TEs in these models is that they are bystanders which are influenced by the transcriptional changes at neighboring genes. Alternatively, the unmasking of TEs could lead to the aberrant expression of nearby genes. Bioinformatic approaches to analyze short read sequences combined with the intrinsic nature of repetitive elements does not allow us to determine the precise genomic location of all reads derived from TEs. We therefore took an alternative approach to determine whether the upregulation of TEs were

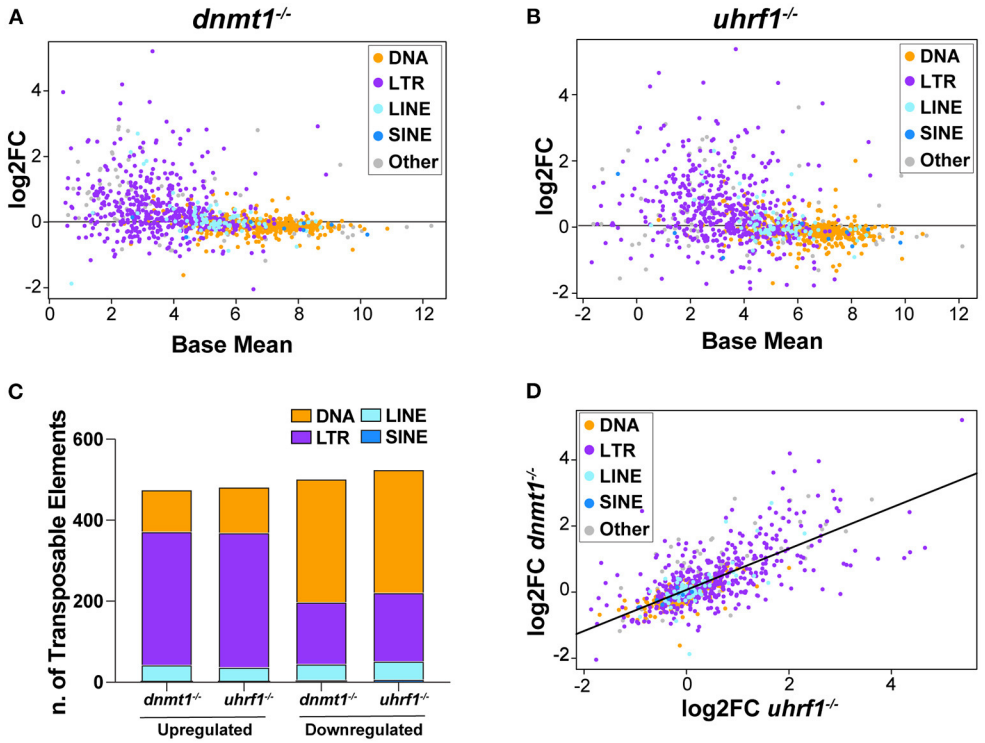


FIGURE 2 | *dnmt1* and *uhrf1* loss causes overexpression of retrotransposons. RNAseq analysis of Transposable Elements in *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant livers. **(A)** MA plot showing $\log_2$ fold change of repetitive elements in *dnmt1*$^{-/-}$ livers calculated on WT siblings and Base Mean in WT siblings. Repetitive elements are divided by Class in DNA transposons (yellow), LTR (purple), LINE (light blue), SINE (blue), and other (gray). **(B)** MA plot showing $\log_2$ fold change of repetitive elements in *uhrf1*$^{-/-}$ livers calculated on WT siblings and Base Mean in WT siblings. Repetitive elements are divided by Class in DNA transposons (yellow), LTR (purple), LINE (light blue), SINE (blue), and other (gray). **(C)** Bar graph of Transposable Elements divided by class in *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant livers. Upregulated TEs have $\log_2$ fold change > 0 and downregulated TEs have $\log_2$ fold change < 0. **(D)** Correlation plot of repetitive elements in *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant livers. Upregulated TEs have adj as pedix < 0.05 and $\log_2$ fold change > 0; downregulated TEs have $p_{adj} < 0.05$ and $\log_2$ fold change < 0. Log_2 fold change is calculated between mutants and their own WT siblings.

TABLE 1 | Transposable elements are differentially expressed in *dnmt1*$^{-/-}$ and *uhrf1*$^{-/-}$ mutant livers at 5 dpf.

	dnmt1^{s904}		*uhrf1*^{hi272}	
	Up	**Down**	**Up**	**Down**
DNA	103*	304*	113*	304*
Retrotransposon				
LTR	329*	153*	332*	169*
LINE	41	39	35	45
SINE	2	6	2	7

*$p < 0.0001$ measured by Chi-square test.

correlated with upregulation of neighboring genes, which could suggest a bystander effect. We selected 10 of the most upregulated TEs in *uhrf1* mutants (**Supplementary Table 2**) and then counted the number of copies of each transposon in the zebrafish genome (**Supplementary Figure 3A**). We then identified the nearest gene, with no distance limit, to each location and determined the expression (log base mean) of that gene in the RNAseq datasets from *uhrf1* mutant and WT sibling livers (**Supplementary Figure 3B**). Note that in some cases, two or more TEs of the same family were in close proximity so that a single gene was assigned as the nearest neighbor of multiple TEs. If these upregulated TEs are coregulated with neighboring genes, then these genes should also be expressed at higher levels in *uhrf1* mutants. We found that only the genes that were detected close to "Gypsy105-I_Dr" and "Gypsy153-I_Dr" to be significantly upregulated in *uhrf1* mutants compared to WT siblings while there was no correlation between expression of TEs and expression of genes in their proximity (**Supplementary Figure 3B**) for all the other analyzed transposons.

Together, these results correlate loss of DNA methylation in zebrafish livers with derepression of expression of TEs, in particular of LTRs. Moreover, these data suggest that DNA methylation does not exert a uniform essential repressive function on all classes of TEs, as not all classes are found to be differentially expressed in these models. Finally, these data indicate

that the expression of most TEs in these datasets is not attributed to a bystander approach, indicating a direct and inverse relationship between LTR expression and DNA methylation.

TEs Derepressed in Hypomethylated Livers Are Heavily Methylated in Controls

In order to further delineate the relationship between DNA methylation and TE expression in this system, we performed Reduced-Representative Bisulfite Sequencing (RRBS) on two biological replicate samples of DNA extracted from whole 5 dpf *uhrf1* mutant and WT sibling larvae. We combined these two replicates to increase genome coverage so that our dataset captured 4.75% of the all CpGs present in the zebrafish genome (**Table 2**). This method enriched for CpGs in the intergenic regions and reduced coverage of CpGs in introns (**Figure 3A**) and therefore largely captures the TEs which are found in the intergenic regions (**Supplementary Figure 4**). To determine the pattern of DNA methylation changes between WT and mutant samples, all CpGs that were common to both datasets were categorized as methylated if >80% of the reads indicated 5-MeC, and unmethylated if this score was <20% of reads. As expected, most of the methylated CpGs were found in the intergenic regions whereas the unmethylated CpGs are enriched in promoters and depleted from introns in WT siblings (**Figure 3A**). Also as expected, there is a bi-modal distribution of CpG methylation in WT larvae, with most of the CpGs in either methylated or non-methylated state (**Figure 3B**; **Table 3**). This is consistent with the finding that in terminally differentiated tissues CpGs can be either in a methylated or unmethylated state and bulk level of DNA methylation is between 75 and 85% (45, 46). In *uhrf1* mutants, this bi-modal pattern is lost (**Figure 3B**), as all the fully methylated CpGs are lost and the average level of methylation shifts to around 35% (**Figure 3B; Table 3**), consistent with the level of bulk DNA methylation loss detected by slot blot [**Figure 1A**, (15)]. We found that the CpGs which were fully methylated in control samples shifted to partial methylation of 45% (**Figure 3C**), while CpGs that were unmethylated in controls remained unmethylated in *uhrf1* mutants. This emergence of a large population of partially methylated CpGs likely reflects the heterogenous cell population analyzed in these samples extracted from whole embryos.

We next evaluated whether the pattern of methylation on TEs correlated with differences of their expression in *uhrf1* mutants by overlapping the RRBS with the RNAseq expression data. The CpGs that were both located in annotated TEs and also covered by our RRBS analysis were mainly present in the intergenic regions (**Figure 3D**), and reflects the distribution of the TEs in the genome (**Supplementary Figure 4**). We next tested the hypothesis that the TEs that are the most upregulated in *uhrf1* mutants would be those that were the most heavily decorated with 5-MeC in controls. Since the analysis of the expression of TEs is based on families, and for each TE there are multiple copies in the genome (**Supplementary Figure 3A**) is not possible to determine the precise genomic location that accounts for each TE read. We therefore analyzed the methylation status of all the possible locations in the genome for each TE family that was upregulated in *uhrf1* mutants and compared them to equal number of randomly selected repetitive elements and then assessed the level of methylation across these two aggregates of genomic loci. This showed that the baseline level of DNA methylation was uniformly high and not significantly different between the TEs that were upregulated in *uhrf1* mutants compared to random selected repetitive elements. In contrast, the upregulated LTRs have higher DNA methylation levels compared to equal number of randomly selected regions (**Figure 3E**). These data suggest that the LTRs which derepressed in *uhrf1* mutants are heavily methylated. This is exemplified by the LTR transposon DIRS1, which is heavily methylated in WT embryos and becomes unmethylated and upregulated in *uhrf1* mutants (**Figure 3F**). On the contrary, several DNA transposons do not change methylation upon *uhrf1* loss: DNA25TWA1_DR is not methylated in controls or mutants and remains silenced as well as TDR13B which is highly methylated in WT samples and, surprisingly, retains DNA methylation in *uhrf1* mutants, and is not expressed in either samples (**Supplementary Figures 5A,B**). These data underscore the pivotal role of DNA methylation in silencing retrotransposons in somatic tissues, and presents a more complex picture of how other TEs are suppressed.

dnmt1 and *uhrf1* Mutation Activates Anti-Viral Response in the Liver

uhrf1 or *dnmt1* deficiency has been shown to be a potent activator of a type I interferon response in tissue culture cells and in whole zebrafish larvae (18, 25, 27, 47). We used RNAseq to

TABLE 2 | RRBS analysis of *uhrf1* whole embryos at 5 dpf.

	Sequenced reads	Mapping rate %	CpG_{10} (coverage >10)	% of CpGs covered %	CpG_{10} common to all samples	#Common methylated CpG_{10}	#Common unmethylated CpG_{10}
Sibling #1	15,468,377	43.6	1,565,271	6.53	1,138,340 (4.75%)	616,305	212,750
Sibling #2	15,662,370	42	1,567,074	6.54			
uhrf1$^{-/-}$mutant #1	16,023,806	36.9	843,023	3.52		33,064	319,740
uhrf1$^{-/-}$ mutant #2	24,087,844	35.6	1,166,438	4.87			

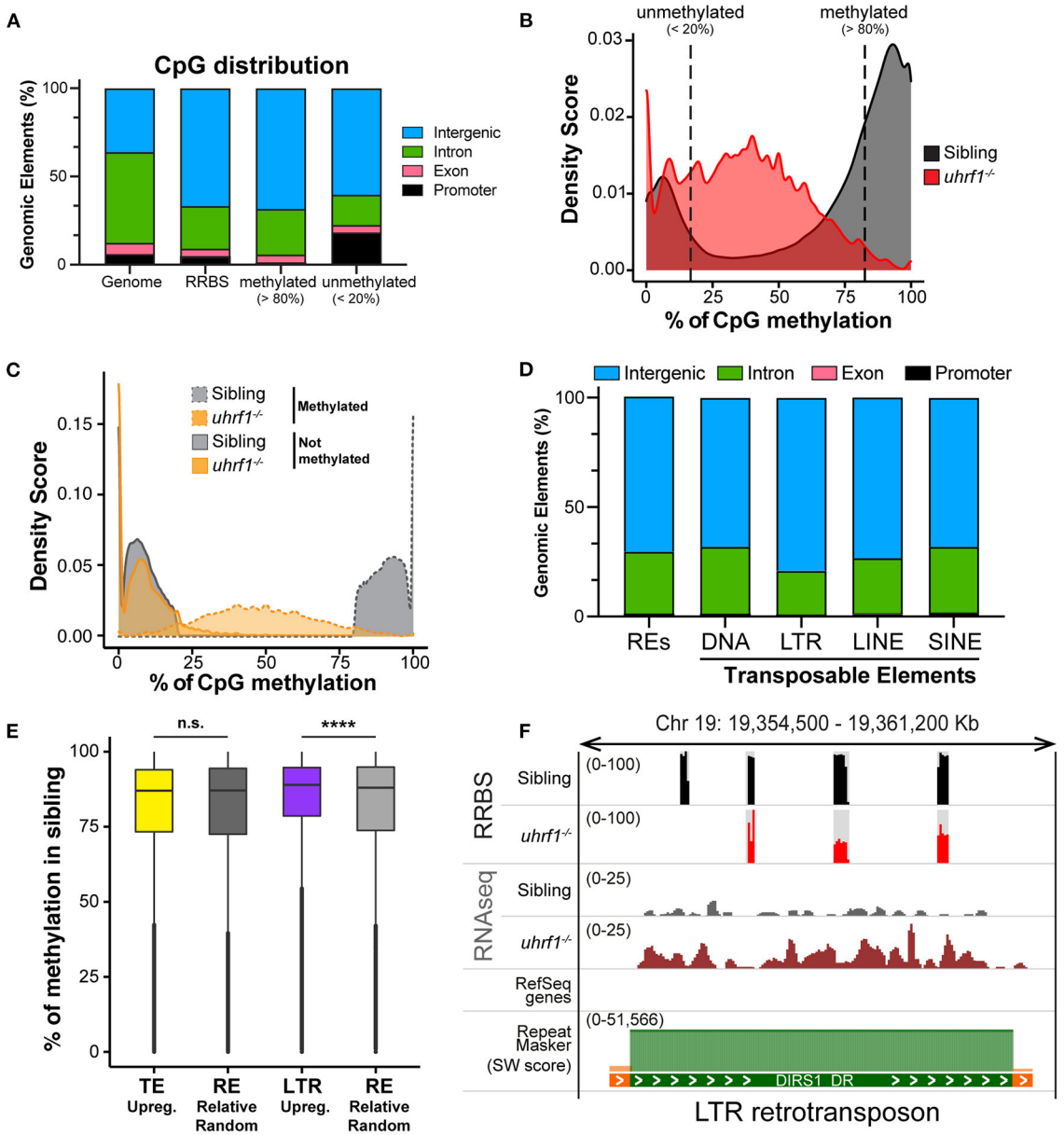


FIGURE 3 | DNA methylation is enriched on TEs that become activated in *uhrf1* mutants. RRBS analysis on genomic DNA of TEs in *uhrf1*$^{-/-}$ mutant larvae. **(A)** Genomic Annotation of all CpGs common to the unified dataset from *uhrf1*$^{-/-}$ mutants and WT siblings are divided by level of methylation in WT siblings in methylated (>80%; 616,305 CpGs) and unmethylated (<20%; 212,750 of CpGs) and were then classified based on their location in annotated genomic element. **(B)** Density plot of percentage of methylation of CpGs in *uhrf1*$^{-/-}$ mutants and wild-type siblings. **(C)** Density plot CpGs in *uhrf1*$^{-/-}$ mutants and wild-type sibling. CpGs were classified based on the percentage of methylation in the sibling in methylated (>80%) (dashed gray line) and not methylated (<20%) (solid gray line). For each group, methylation levels were plotted for both mutants (dashed orange line) and siblings (solid orange line). **(D)** Genomic annotation of CpGs covered in RRBS and overlapping with the TEs annotated in the RNAseq. **(E)** Box plot describing the percentage of methylation of CpGs in WT siblings: from left, CpGs contained in TEs upregulated (p_{adj} < 0.05 and $\log_2$ fold change > 0 in *uhrf1*$^{-/-}$mutants–yellow) and in equal number of REs randomly selected (193,397 regions–dark gray); CpGs contained in LTRs upregulated (p_{adj} < 0.05 and $\log_2$ fold change > 0 in *uhrf1*$^{-/-}$mutants–purple) and in equal number of REs randomly selected (30,353 regions–gray). ****p < 0.0001 calculated by unpaired non-parametric Mann–Whitney test. **(F)** Genome browser screenshot shows an example of RNA transposons (LTR retrotransposon, DIRS1_DR) that is demethylated and expressed in *uhrf1*$^{-/-}$ mutants. SW score is determined by Repeat Masker and it is used as indicator of the age of transposons. High SW score corresponds to highly conserved TEs, indicating younger TE.

determine if this same pattern occurred in the liver of 5 dpf *uhrf1* and *dnmt1* mutants (**Supplementary Figures 6A–D**). In *dnmt1* mutant livers, 5,397 genes were significantly differentially expressed genes (DEGs; p_{adj} < 0.05); of these, 1,578 were upregulated [$\log_2$ fold change (L2FC) > 1.5] and 679 were downregulated (L2FC < −1.5) (**Table 3**). In *uhrf1* mutant livers, 7,587 DEGs were detected (p_{adj} < 0.05), with 2,595 upregulated (L2FC > 1.5) and 756 DEGs downregulated (L2FC

TABLE 3 | RNAseq analysis of *uhrf1* and *dnmt1* mutant livers at 5 dpf.

	***uhrf1*$^{-/-}$ mutant**		***dnmt1*$^{-/-}$ mutant**
Total number of genes detected	19,464		23,413
Significant genes ($p_{adj} < 0.05$)	7,587		5,397
Significant UP ($p_{adj} < 0.05$ & $\log_2FC > 1.5$)	2,595		1,578
Significant DOWN ($p_{adj} < 0.05$ & $\log_2FC < -1.5$)	756		679
Significant in both		3,908	
Significant UP in both		1,166	
Significant DOWN in both		252	

< -1.5) (**Table 3**). This shows that the pattern of DEGs in both samples is toward the upregulation of genes, and also shows that many genes are highly induced in the liver of these mutants (**Supplementary Figures 6A,B**). While there are unique cellular functions of *uhrf1* and *dnmt1* which could induce distinct transcriptional responses, we reasoned that the responses induced by loss of DNA methylation would be shared in both datasets. Comparison of significant DEGs from both samples in a 4-quadrant plot shows a high correlation between the DEGs in these datasets (**Figure 4A**; **Table 3**), with < 8 of genes displaying a discordant expression pattern.

To determine the unique and shared cellular pathways that are differentially affected in the liver due to mutation of *uhrf1* and *dnmt1*, the zebrafish gene name was converted to the human gene name, and we then performed gene ontology (GO; **Figure 4B**) and Ingenuity Pathway Analysis (IPA; **Figure 4C**) on the human gene names, as annotation of human gene function is superior to the annotation of the zebrafish genome. We found that metabolic pathways and liver related processes are most represented among the downregulated pathways in both mutants (**Figure 4B**, in green; **Supplementary Figure 7A**), suggesting that hepatocytes do not achieve their full metabolic function in these mutants. In contrast, the majority of the upregulated pathways were immune related (**Figures 4B,C**). Of particular interest are the upregulation of the protein kinase R pathway and the corona virus pathogenesis pathway, which are induced in response to RNA viruses, NF-kB pathway, interferon signaling and response to virus. All of these are triggered by a nucleic acid sensing pathway that culminates in type I interferon response. One of the type I interferons, *ifnphi1*, is upregulated in both samples, whereas *ifnphi2* and *3* are only induced in *dnmt1* mutant livers (**Supplementary Figure 7B**). To further analyze the immune pathways deregulated in these samples we used IPA. This showed that the most prominent pathways activated the liver of these mutants are the pathways activated in autoimmunity (systemic lupus erythematosus), immune response to infection with an RNA virus (i.e., Coronavirus Pathogenesis Pathway), NF-kB and the interferon response (**Figures 4C–E**) among the most significantly enriched in the upregulated genes. The finding that the pathway activated by SARS-CoV2, an RNA virus which induces a distinct set of genes in the airway epithelial cells in infected patients (48) had significant overlap with the genes activated in *uhrf1* and *dnmt1* mutant livers (**Supplementary Figure 7C**) indicates that loss of DNA methylation induces an immune response similar to infection with an RNA virus (**Figure 4C**, **Supplementary Figure 7C**). ClueGO analysis provided a network view of the deregulated pathways, further showing that immune system processes were at the center of coregulated pathways in *uhrf1* and *dnmt1* mutant livers (**Supplementary Figure 8**).

To investigate the relationship between upregulation of immune genes and loss of DNA methylation on promoters, we analyzed DNA methylation levels around the Transcription Start Site (TSS) of upregulated immune genes in *uhrf1* mutants compared to WT siblings. While this shows that there is a significant loss of DNA methylation at these gene promoters, the same degree of loss is also seen around the TSS of immune genes that do not change expression upon *uhrf1* loss (**Figure 4F**). Since both groups of genes show a similar DNA methylation profile in WT and *uhrf1* mutant larvae, the upregulation of immune genes cannot be solely attributed to DNA methylation loss on their promoters. Since *uhrf1* mutation causes global loss of DNA methylation, this pattern was observed in all genes, and therefore it is not possible to rule out the possibility that, for some genes, DNA hypomethylation of promoters may impact expression. Regardless, these results are not consistent with the hypothesis that the induction of these genes is a direct effect of DNA hypomethylation in *dnmt1* and *uhrf1* mutants, but instead that the robust anti-viral immune response in the liver is attributed to the expression of TEs that are normally silenced by DNA methylation.

Anti-Viral Signaling Components Sting and Tnfa Are Partially Required for the *uhrf1* Phenotype

To determine whether the antiviral response and the hepatic phenotype in *uhrf1* and *dnmt1* mutant larval livers was induced by cytoplasmic viral sensors, we used CRISPR/Cas9 to deplete essential genes regulating these pathways asking if this rescued the activation of immune response using qPCR. We selected a panel of genes involved in the antiviral response and we tested their expression in *dnmt1* and *uhrf1* mutant livers. *ifnphi1*, *tnfa*, and *nfkb2*, and interferon I target genes, such as *irf7*, *irf9*, *isg15*, and *irf1b* (**Figure 5A**) involved in this response were significantly upregulated in both *dnmt1* and *uhrf1* mutant livers (**Figure 5A**). Since the GO and IPA analysis highlighted the response to RNA viruses as activated and our previous studies suggested that the response to cytoplasmic DNA was involved in the immune response in *urhf1* mutants, we assessed key components of both pathways. Mavs is involved in detecting cytoplasmic double stranded RNA and Sting is activated by cytoplasmic DNA. Both sensors lead to activation of a type-I interferon response (49) and to induction of *tnfa*, a central signaling molecule that functions to trigger an anti-viral response.

sgRNAs were first validated for the ability to generate indel mutations when injected into WT and *uhrf1* mutant embryos

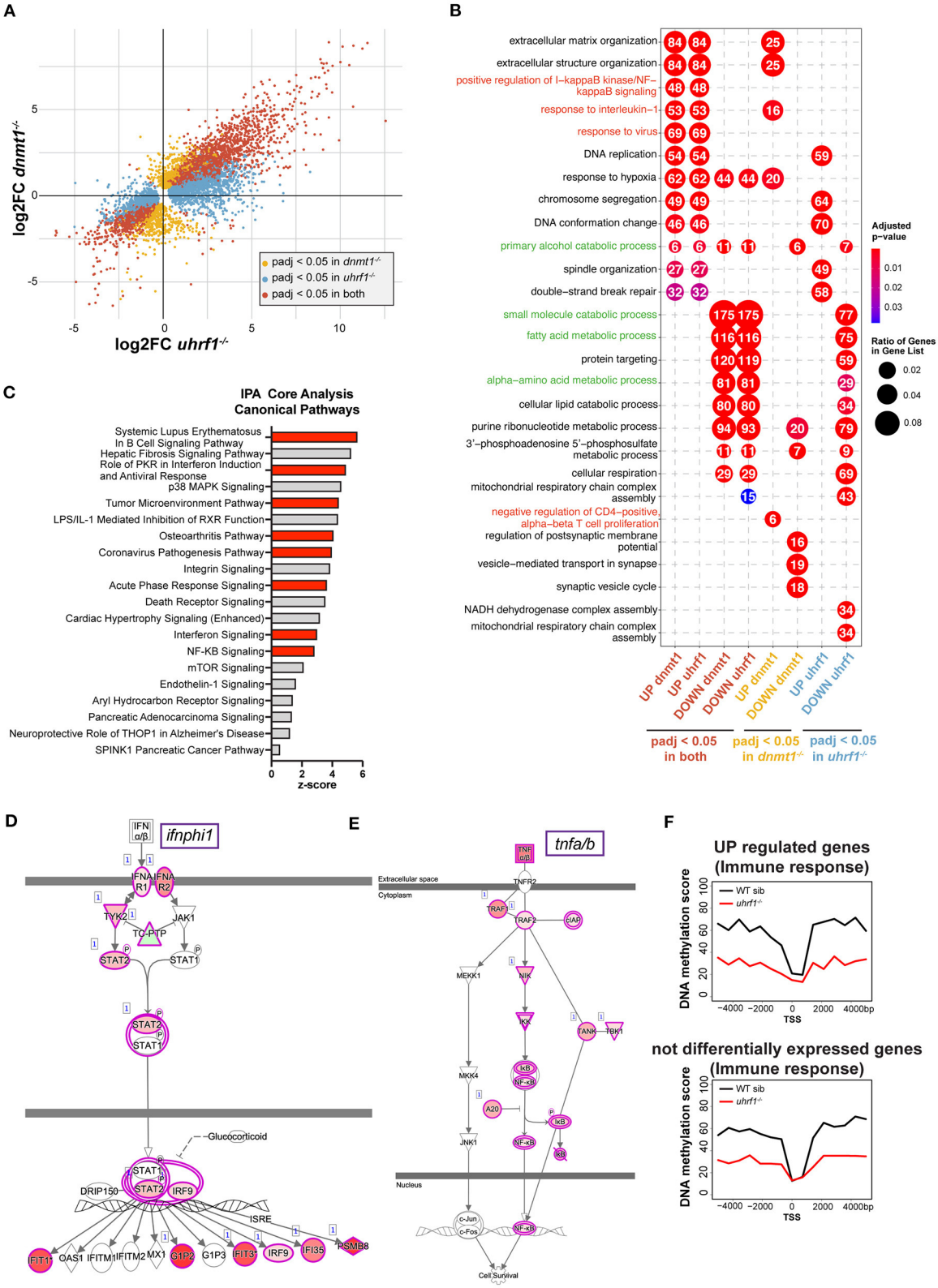


FIGURE 4 | *dnmt1* and *uhrf1* loss activates typel interferon and NF-kB mediated immune response. **(A)** RNAseq analysis of RNA extracted from *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant livers. Four quadrants plot of log_2 fold change of *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutant livers calculated on their own WT siblings. The genes that are

(Continued)

FIGURE 4 | significant are indicated in red. Genes that are significant ($p_{adj} < 0.05$) in *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$, in yellow the genes significant only in *dnmt1*$^{-/-}$ mutants and in blue the one significant only in the *uhrf1*$^{-/-}$ mutant livers. **(B)** Gene Ontology of the upregulated and downregulated genes in each dataset. Significant genes ($p_{adj} < 0.05$) for each category in **(A)**, are divided based on $\log_2$ fold change in upregulated ($\log_2$ Fold Change > 0) and downregulated ($\log_2$ Fold Change < 0). In red the GO terms involved in immune pathways and in green GO terms associated to metabolism and liver specific pathways. **(C)** Bar graph shows the 20 most significant positively induced pathways according identified in IPA. Pathways are ranked ordered based on z-score and it indicates the likelihood of activation based on comparison with a model that assigns random regulation directions. In red, the immune related pathways. **(D)** IPA analysis of Interferon pathway. The color of the circles represents the expected trend of the genes when the pathways is upregulated (pink for upregulated and green for downregulated), the color inside the circles represents the observed trend of that gene in our RNAseq. **(E)** IPA analysis of Tnfa pathway. The color of the circles represents the expected trend of the genes when the pathways is upregulated (pink for upregulated and green for downregulated), the color inside the circles represents the observed trend of that gene in our RNAseq. **(F)** Metaplot of the DNA methylation levels in WT siblings and *uhrf1*$^{-/-}$ mutants of the +/– 4 kb region surrounding the Transcription Start Site of the genes involved in Immune response pathways (GP006955) that are upregulated in the *uhrf1*$^{-/-}$($p_{adj} < 0.05$ and $\log_2$FC > 0, top panel) or not differentially expressed ($p_{adj} > 0.05$, bottom panel).

(**Supplementary Figure 9A**). sgRNAs demonstrated over 90% efficiency when injected with Cas9 protein into 1 cell embryos generated from an incross of *uhrf1*$^{+/-}$ adults. The F_0 "crispants" were assessed for morphological abnormalities from 0 to 5 dpf and, at day 5, the left liver lobe size was measured to test whether the depletion of these genes influenced liver development. There were no significant changes in larvae morphology or liver size of the crispants in WT siblings or *uhrf1* mutants (**Supplementary Figures 9B,C**). At 5 dpf, livers were dissected and the expression of genes involved in the antiviral response were analyzed by qPCR in the liver of *uhrf1* mutant and phenotypically WT siblings. Although the statistical significance was reached only on some of the analyzed genes (*isg15, irf1b*, and *nfkb2*), all genes analyzed showed a decreased expression in the crispants compared to not-injected larvae (**Figure 5B**). This suggests that, overall, both *mavs* and *sting* are required for the expression of immune genes in *uhrf1* mutant livers.

Cytoplasmic nucleic acid sensing pathways lead to activation of the *tnfa* pathway (50). Tnfa can trigger apoptosis and necroptosis of infected cells (51, 52). Since *tnfa* signaling was activated in the liver of *uhrf1* mutants (**Figures 4D,E**) and deletion of *sting* and *mavs* significantly decreases *nfkb2* (**Figure 5B**), one of the main targets of *tnfa* cascade (**Figure 4E**), we hypothesized that Tnfa could mediate the cell death phenotype that characterized *uhrf1* (14, 15). To test this, Tnfa was depleted using CRISPR/Cas9 (**Supplementary Figure 9A**) in *uhrf1* mutants and used the TUNEL assay as a readout of the effect on cell death. While *uhrf1* mutant livers were characterized by high levels TUNEL positivity, in WT siblings TUNEL staining was completely absent (**Figure 5C**). *uhrf1* mutants that were *tnfa* crispants showed a significant decrease in TUNEL positive cells (**Figure 5C**). This indicates that activation of *tnfa* contributes significantly to cell death in *uhrf1* mutant livers.

These findings indicate that both the double stranded RNA sensing arm mediated by *mavs* and the cytoplasmic DNA sensing arm mediated by *sting* are activated upon DNA methylation loss and that they induce apoptosis that can be rescued, at least partially, by the deletion of *tnfa* indicating that the hyperactivation of this pathway is deleterious for the liver leading to cell death. Interestingly, the reduction of cell death did not rescue the small liver size in *uhrf1* mutants/*tnfa* crispants (**Supplementary Figure 9C**), suggesting that the cell cycle block in *uhrf1* deficient hepatocytes (14, 15) is the prominent driver of the small for size liver phenotype. Alternatively, it is possible that the TUNEL positive cells detected in the liver of *uhrf1* mutants could be detecting cell fragments in immune cells or even dying immune cells which infiltrate the liver in this model (18).

DISCUSSION

DNA methylation is a primary epigenetic modification that maintains repetitive regions of the genome in a repressed state and loss of the DNA methylation machinery during cell division leads to DNA hypomethylation. Many studies have demonstrated that DNA hypomethylation leads to cell damage and, in some cases, activation of an antiviral response. However, the relationship between DNA methylation loss, immune activation, and cell damage and death have not been fully investigated in whole animal models. Here, we investigated how DNA hypomethylation in the liver of *dnmt1* and *uhrf1* zebrafish mutant embryos leads to an immune response. Loss of *uhrf1* has been implicated in inflammatory bowel disease, based on findings that *uhrf1* zebrafish mutants develop inflammation in the intestine (8, 9, 16, 20) and in mice, *uhrf1* deletion in macrophages makes them hypersensitive to activation in the intestine (9). We hypothesized that depleting DNA methylation by *uhrf1* or *dnmt1* mutation would activate TE expression and triggering an antiviral immune response. Several studies, including ours, have demonstrated that loss of DNA methylation leads to activation of anti-viral pathways, in part, due to viral mimicry achieved by retrotransposons. We report that *uhrf1* or *dnmt1* mutation causes DNA hypomethylation in the liver and is associated with activation of a specific class of TE, LTR retrotransposons.

The immune response in cells with high level of aberrant expression of repetitive sequences has been coined as Transcription of Repeats Activates Interferons (TRAIN) by one group who reported that loss of DNA methylation and p53 inactivation, features common to most cancer cells, cause robust expression of TEs (47). We reported a similar response in whole zebrafish embryos with DNA hypomethylation due to mutation of *uhrf1* or *dnmt1* (18). Here, we expand on this finding to investigate the relationship between DNA methylation and TE activation in this model and then delineate the immune response in the liver of these mutants. This is particularly relevant to the liver, where inflammation is a critical factor in

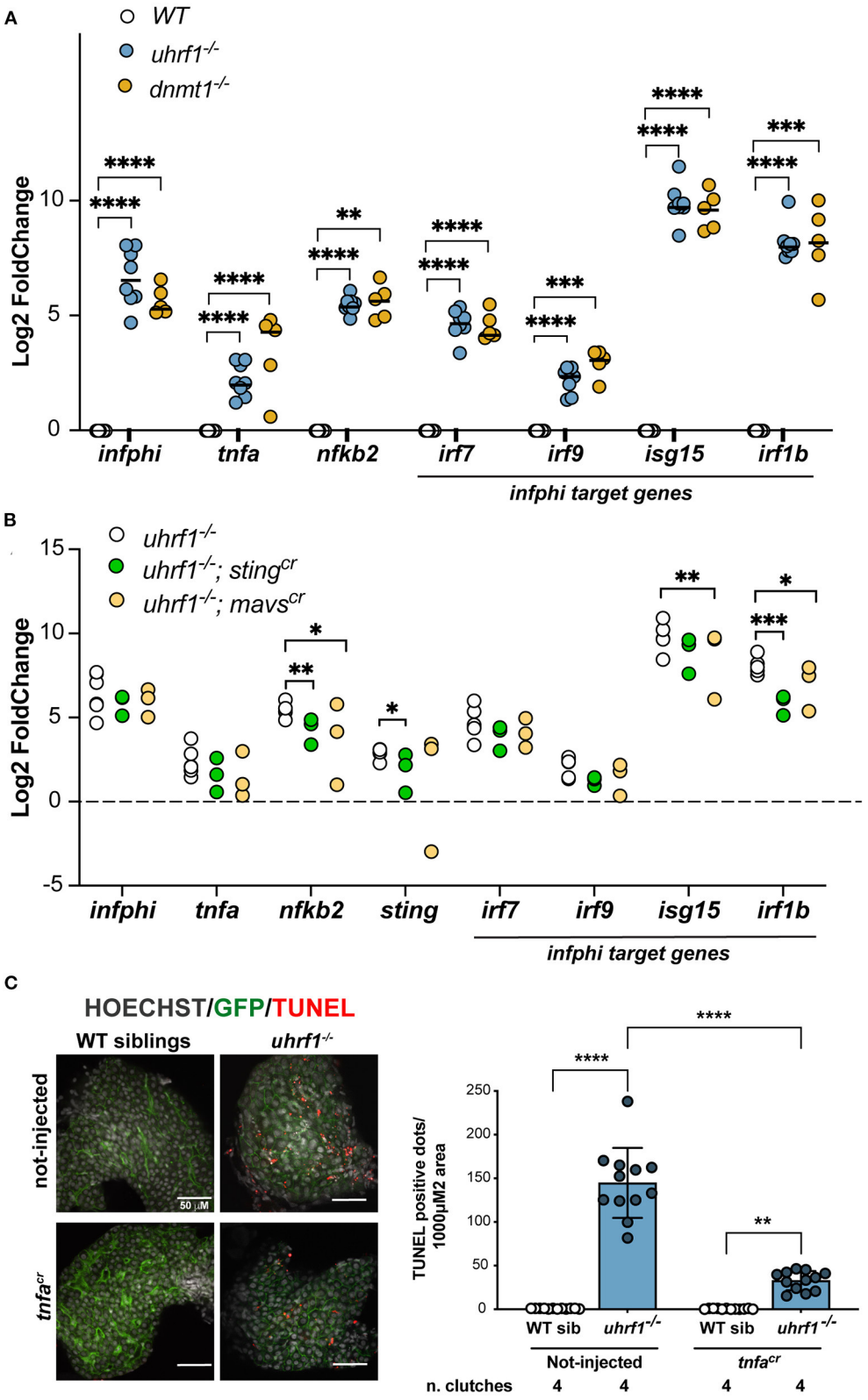


FIGURE 5 | Viral signaling pathways are required for the gene expression and cell death phenotypes in *uhrf1* mutants. (**A**) qPCR analysis of immune genes in *uhrf1*$^{-/-}$ and *dnmt1*$^{-/-}$ mutants compared to their WT siblings. *Rplp0* is used as loading controls and the delta-delta Ct (DDCt) values were calculated by normalization to

(Continued)

FIGURE 5 | *rplp0* and WT sibling controls for each individual clutch. Lines in the graph represents the median. Statistical significance is calculated by paired t-test. $^{**}p < 0.05$, $^{***}p < 0.005$, $^{****}p < 0.001$. **(B)** qPCR performed on the livers from *mavs*^{cr} and *sting*^{cr} F_0 crispants in the *uhrf1* and WT backgrounds to assess immune gene expression. DDCt is calculated after normalization of each gene to *rplp0* and WT sibling controls for each clutch; this was performed for each crispants and for the not-injected embryos. Significance is calculated using 2-way Anova. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0005$. **(C)** TUNEL analysis of *uhrf1*$^{-/-}$ and phenotypically WT sibling livers at 5 dpf in *tnfa*^{cr} F_0 crispants and non-injected controls. Quantification of the number of TUNEL positive foci per total liver area for at least 3 livers per clutch in 4 clutches for each condition. Significance is measured by 2-way Anova test. $^{**}p < 0.05$, $^{****}p < 0.0001$.

the progression of liver injury to liver disease and chronic inflammation is fundamental to the formation of cirrhosis and liver cancer.

Several studies performed in zebrafish, human cells and mice are consistent with our finding that DNA methylation loss activates RNA transposons, ERVs in particular (18, 27, 53–55). We report that TEs with high level of methylation are more prone to be derepressed in *uhrf1* and *dnmt1* mutant livers. Despite the fact that the majority of the TEs were upregulated, we found a group of TEs, mostly DNA transposons, that were downregulated. This surprising finding suggests the existence of compensatory mechanisms that suppress DNA transposons when DNA methylation is removed. This is supported by our previous finding in mouse livers where *uhrf1* loss does not induce TEs expression or cause an immune response, likely due to the relocation H3K27me3 to hypomethylated TEs to compensate for loss of DNA methylation (56). Similar findings were reported in a mouse model of glioblastoma where H3K27 acetylation activates ERVs and that was further enhanced by the global DNA methylation loss in tumors (57). This evidence suggests that repressive histone modifications in collaboration with DNA methylation could control distinct populations of TEs or select those with distinct features, such as their age or CpG content. Further investigation into the repertoire of epigenetic mechanisms that contribute to the distinct patterns of TE expression found here is warranted.

Zebrafish are a powerful and widely used model for studying inflammation and immunity (58). We leveraged these advantages for our studies. In early mouse and zebrafish embryos, *uhrf1* or *dnmt1* is essential for development after gastrulation and depletion or loss of one of these factors leads to early embryonic death (12, 13). Maternal supply of *uhrf1* and *dnmt1* in zebrafish embryos sustains their development through the early stages allowing for examination of embryos at later stages of development. The zebrafish *dnmt1* and *uhrf1* mutants have systemic developmental defects that are revealed at later developmental stages, including reduced size of the digestive organs characterized by an underdeveloped and inflamed gut (8, 16) and a small liver (14, 15, 29). We found that *dnmt1* and *uhrf1* loss induces TEs and activation of anti-viral sensing pathways, culminating in the activation of typeI interferon response and Tnfa signaling. The signaling pathways activated in the liver of these mutants mimic human cells infected with SARS-CoV2 (48), indicating that it is a *bona fide* anti-viral response, including the Tnfa pathway. In hepatocytes, TNFa levels determine the choice between pro-survival or pro-apoptotic signaling as demonstrated in acute liver injury models where TNFa is necessary to protect hepatocytes from apoptosis (59, 60). In other scenarios, Tnfa activation can also promote liver injury (61, 62). Upon DNA methylation loss, we found that *tnfa, tnfr2* and downstream components of the Tnfa signaling pathway were induced (**Figure 4E**). Importantly, *tnfa* crispants rescued the cell death phenotype in the liver of *uhrf1* mutants (**Figure 5C**), similarly to what was found in the intestine (8). This could have implications for leveraging this pathway to target cancer cells which show widespread DNA hypomethylation and aberrant TE expression (5, 63).

Despite the growing body of evidence linking the expression of TEs with the activation of anti-viral responses, it is known if the TEs directly cause immune-mediated responses or whether changing the epigenetic landscape that contributes to TE activation also can contribute to the expression of genes involved in inflammation. Some studies interpret the hypomethylation of the *tnfa* promoter in *uhrf1* mutants in both mouse (9) and zebrafish (8) as a finding demonstrating that promoter methylation has a direct role in controlling *tnfa* expression. However, the promoter of *tnfa* in zebrafish, mouse and human does not contain a CpGs island, and indeed in zebrafish it contains only 7 CpGs in the distal promoter (−999 to −620 bp from the transcription start site), indicating that there are only a few potential sites that render this gene susceptible to regulation by DNA methylation. Unfortunately, the RRBS dataset generated here did not cover the *tnfa* promoter with sufficient depth to enable us to examine this locus directly in our samples. However, the preponderance of evidence suggests that DNA methylation is not likely to be a major mechanism regulating *tnfa* but instead an indirect mechanism, mediated by the activation of antiviral sensing pathways causes Tnfa pathway activation. Indeed, our finding shows that deletion of *mavs* and *sting* reduces expression of *tnfa* and significantly reduces the downstream effector, NF-kB. Since there is no expectation that these sensors have any impact on the epigenetic status of the promoter of these genes, we conclude that the most likely explanation for *tnfa* activation in this system is due to response to TE activation. A caveat to our study is that we cannot exclude other possible causes of Tnfa activation or the immune response: for instance, pericentromeric DNA could have been unmasked, *uhrf1* mutants may acquire a different microbiome that changes their immune response or perhaps the loss of *uhrf1* or *dnmt1* in the immune cells makes them more susceptible to immune signaling. Indeed, we cannot exclude that some variable that we have not detected or controlled for is creating the immune response reported in the liver of *uhrf1* and *dnmt1* mutants.

What is the functional relevance of TE activation and induction of the innate immune response? DNA hypomethylation is a common characteristic of cancer

cells, and global loss of DNA methylation is found prior to malignant transformation, as the pattern of DNA methylation in senescence cells is the same as those in tumors (64). In this scenario, loss of DNA methylation could lead to expression of TEs, and if these become mobile, they could cause genome instability that is the foundation for cancer cell evolution. We propose that TE expression can be a harbinger of a damaged epigenome, and the resulting immune response can serve to eliminate these damaged and potentially dangerous cells. However, the prolonged activation of an antiviral response can be deleterious as this can promote liver damage, enhance fibrosis and be a key factor promoting tumorigenesis in the liver. Understanding how specific TEs are regulated and defining how inappropriate TEs activation can promote inflammation in the liver will inform the design of tailored approaches that can enhance the aspects of the immune system that repair damage and limit those aspects that promote pathology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found at: https://www.ncbi.nlm.nih.gov/, GSE160728.

ETHICS STATEMENT

The animal study was reviewed and approved by NYUAD IACUC Committee.

AUTHOR CONTRIBUTIONS

EM and KCS conceived the study. EM, FM, BPM, and FA performed the experiments. EM, FM, BPM, CZ, FA, and KCS analyzed the data. EM, FM, BPM, CZ, and KCS wrote the manuscript. All the authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.627926/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Erratum: *uhrf1* and *dnmt1* Loss Induces an Immune Response in Zebrafish Livers Due to Viral Mimicry by Transposable Elements

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Due to a production error, there was a mistake in the **Author Contributions** statement. The corrected author contributions statement appears below.

"EM and KCS conceived the study. EM, FM, BPM, and FA performed the experiments. EM, FM, BPM, CZ, FA, and KCS analyzed the data. EM, FM, BPM, CZ, and KCS wrote the manuscript. All the authors revised the manuscript."

The publisher apologizes for this mistake. The original version of this article has been updated.

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TREX1 as a Novel Immunotherapeutic Target

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Mutations in the TREX1 3' → 5' exonuclease are associated with a spectrum of autoimmune disease phenotypes in humans and mice. Failure to degrade DNA activates the cGAS-STING DNA-sensing pathway signaling a type-I interferon (IFN) response that ultimately drives immune system activation. TREX1 and the cGAS-STING DNA-sensing pathway have also been implicated in the tumor microenvironment, where TREX1 is proposed to degrade tumor-derived DNA that would otherwise activate cGAS-STING. If tumor-derived DNA were not degraded, the cGAS-STING pathway would be activated to promote IFN-dependent antitumor immunity. Thus, we hypothesize TREX1 exonuclease inhibition as a novel immunotherapeutic strategy. We present data demonstrating antitumor immunity in the TREX1 D18N mouse model and discuss theory surrounding the best strategy for TREX1 inhibition. Potential complications of TREX1 inhibition as a therapeutic strategy are also discussed.

Keywords: exonuclease, small-molecule, inhibition, immunotherapy, cancer

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A BRIEF HISTORY OF TREX1

Three-prime Repair EXonuclease 1 (TREX1) is a nonprocessive 3' → 5' exonuclease (1). Biochemical investigations of TREX1 established similar degradation activities using ss- and dsDNA substrates, with some preference for dsDNA with 3'-mismatches and 3'-overhangs. TREX1 activity using RNA and RNA-DNA duplexes is approximately 1000-fold less than with DNA, implicating DNA as the endogenous polynucleotide substrate (1–5). TREX1 is a 314 amino acid polypeptide composed of an N-terminal catalytic domain (1-242) containing the exonuclease activity (2), and a C-terminal region (243-314) (6) that facilitates localization of the enzyme to the perinuclear space in cells (7). The TREX1 C-terminal region has also been proposed to interact with the oligosaccharyltransferase (OST) complex (8, 9), and TREX1 has been proposed to function in the SET complex (10). TREX1 is a stable homodimer (**Figure 1**) with the protomers connected by an extended β-sheet core and a highly stable network of hydrogen bonds and hydrophobic interactions, such that the homodimer does not measurably dissociate after initial formation (11). The obligate dimeric structure of TREX1 is unique among exonucleases, and highly relevant to TREX1 catalytic activity. We have demonstrated that residues from one TREX1 protomer communicate across the dimer interface and contribute to catalysis in the opposing protomer, illustrating the requirement of TREX1's dimeric structure for full exonuclease activity (12). These

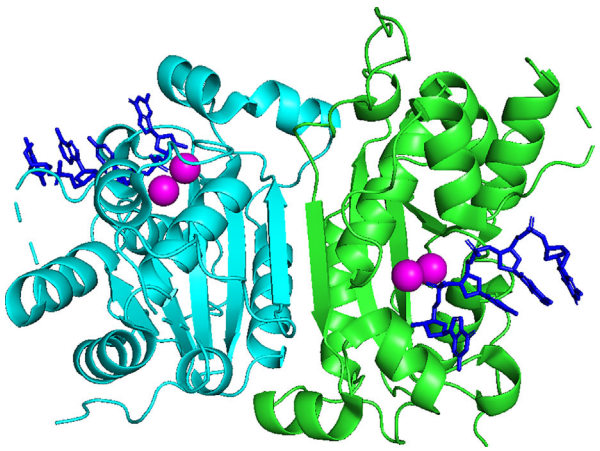


FIGURE 1 | Crystal Structure of the Dimeric Exonuclease mTREX1(1-242). Structure includes only the TREX1 catalytic domain (1-242). Protomers are distinguished by green and cyan, ssDNA by blue sticks, and calcium ions by magenta coloring. Crystal structure was visualized in PyMOL using the PDB structure 2OA8 from ref (6).

studies further suggest a potential mechanism for inter-protomer regulation and/or coordinated catalysis.

TREX1 is a member of the DEDD family of 3' → 5', whose members are defined by a conserved Asp-Glu-Asp-Asp motif that facilitates catalytic activity (13–15) (**Figure 2**). Members of the DEDD nuclease family frequently have a role in DNA replication and/or repair (1, 3, 17), prompting early investigations in this area for TREX1. However, mice lacking TREX1 do not develop a hyper-mutator phenotype, but instead develop an aggressive autoimmune phenotype characterized by

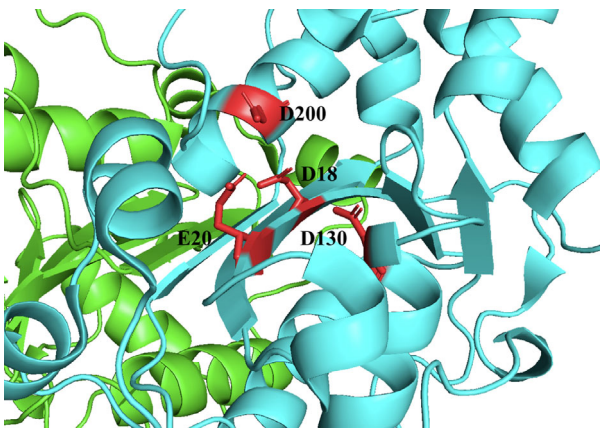


FIGURE 2 | TREX1 is a Member of the DEDD Family of Exonucleases. Structure includes only the TREX1 catalytic domain (1-242). Protomers are distinguished by green and cyan cartoons, and D18-E20-D130-D200 motif residues are shown as red sticks with black labels. Crystal structure was visualized in PyMOL using the PDB structure 3MXJ from ref (16).

inflammatory myocarditis (18). More than sixty TREX1 mutations have now been identified [reviewed in ref (19)] that exhibit dominant and recessive genetics and occur as inherited or *de novo* mutations, dependent upon the specific mutant allele. TREX1 disease alleles include missense mutations, insertions, duplications, and frame shifts that locate to positions throughout the 314-amino acid-coding gene. There is a causal relationship between TREX1 genetic variants and multiple mechanisms of TREX1 enzyme dysfunction that have now been linked to a spectrum of autoimmune diseases in humans (19). There is also some correlation between the positions of TREX1 mutations and the observed clinical phenotype. Most of the TREX1 mutations affecting the catalytic domain are recessive and are largely associated with Aicardi-Goutières Syndrome (AGS) or Familial Chilblains Lupus (FCL) (19). The dominant TREX1 mutations produce enzyme that competitively inhibits wild-type enzyme activity on bulky dsDNA substrates (11, 20–22). TREX1 mutations that cause Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) exhibit dominant inheritance and are exclusively frame-shift mutations in the C-terminal tail region of the enzyme (8, 19, 23). Additional frame-shift mutations in the C-terminal region result in recessive AGS (19). All together the TREX1 mutations indicate a complex relationship between TREX1 structure, function, genetics, and clinical disease.

A hallmark of TREX1 mutation is chronic type-I interferon (IFN) signaling. TREX1 deficient mice are completely rescued from mortality and pathology by introducing IFN receptor (IFNAR) deficiency, demonstrating that TREX1 disease pathology is driven by IFN signaling (24). Similar genetic studies have also demonstrated stimulator of interferon genes (STING) (25), interferon regulatory factor 3 (IRF3) (24), and cyclic GMP-AMP synthase (cGAS) (26–28) as critical components of the pathological mechanism, establishing the cGAS-STING DNA-sensing pathway's role in TREX1 deficiency disease. In the cGAS-STING pathway, binding of dsDNA to cGAS causes synthesis of a 2'-3'-cyclic GMP-AMP (cGAMP) (29), which in turn binds to and activates the endoplasmic reticulum-associated protein STING (30, 31). Upon activation, STING traffics to the Golgi apparatus where it recruits Tank Binding Kinase 1 (TBK1) to phosphorylate it (32). Phosphorylated STING recruits IRF3 for phosphorylation by TBK1, and activated IRF3 then dimerizes and translocates to the nucleus to promote expression of IFN (33). After its expression, binding of IFN to IFNAR induces immune activation by promoting the proliferation and maintenance of natural killer (NK) and memory CD8+ T cells, stimulating dendritic cells (DC), and more broadly by increasing the expression of interferon-stimulated genes (ISGs) (34). The cGAS-STING pathway has been proposed to act as a broad sensing pathway for many sources of DNA (33). Collectively, studies to date support a model where deficiency in TREX1 exonuclease activity leads to accumulation of TREX1 DNA substrate(s), which then stimulate the cGAS-STING pathway and promote pathology *via* subsequent type-I IFN signaling.

TREX1 exonuclease dysfunction and subsequent cGAS-STING signaling raises questions about the source of immune

activating DNA. Multiple sources of DNA have been proposed as potential sources of TREX1 substrate *in vivo*, including ssDNA replication intermediates (35), retroelements (24), and enucleated erythroblast DNA (36). Our lab (37) and others (38) have demonstrated that TREX1 inactivity in bone marrow-derived cells drives any discernable pathology, but other cells can contribute to IFN signaling. Ultimately, the question of TREX1 biological substrate(s) remains an area of active investigation. We have recently published a review of TREX1 (39), which we recommend for further details.

TREX1 & cGAS-STING in the Tumor Microenvironment

TREX1 and the cGAS-STING pathway have been implicated in the tumor microenvironment [reviewed in refs (39–45)]. TREX1 activity has been negatively correlated with outcomes in multiple cancers (46–48). In addition, treatment of cancerous cells *in vitro* with UV-light or various genotoxic anti-cancer drugs is associated with *TREX1* upregulation, and siRNA knockdown of *TREX1* enhances cancer cell death following these treatments (49). A dose-dependent effect of DNA-damaging agents on TREX1 expression has been demonstrated, and showed that TREX1 degrades damaged DNA from drug-treated tumor cells (50). Finally, multiple studies have shown that the dose-dependent efficacy of radiotherapy is at least partially attributable to TREX1 activity (51–53).

IFN-dependent antitumor immunity following radiotherapy is STING-dependent, as demonstrated by its ablation in STING-deficient mice (54, 55). Additionally, cGAS-deficiency in DCs has been reported to be sufficient to abrogate antitumor immunity *in vitro* (54). However, there are additional studies that indicate IFN-production *in vitro* is unaffected by cGAS-knockout in DCs, but is attenuated by cGAS-knockout in tumor cells, by STING-knockout in DCs, or by connexin 43-knockout in tumor cells (56). These data led to the proposal that cGAS-mediated DNA-sensing is tumor-intrinsic, and that cGAMP, produced in tumor cells, is transferred *via* gap junctions to host DCs activating STING and initiating IFN-dependent antitumor immunity (56–59). Yet, additional work indicates that tumor-intrinsic and tumor-extrinsic STING participate in driving antitumor immunity (60). Thus, while current studies support cGAS-STING function in antitumor immunity following radiotherapy and/or chemotherapy, the precise nature of cGAS and STING's roles in tumor and immune-cell function remain unresolved.

TREX1 is the gatekeeper enzyme of the cGAS-STING pathway, and tumor-derived DNA generated spontaneously or induced by radiotherapy or chemotherapy can be degraded by TREX1. DNA that is not degraded by tumor-intrinsic TREX1 can stimulate the cGAS-STING pathway to generate an IFN-response and drive immune cell recruitment to facilitate tumor regression. The initial cGAS-stimulation resulting from undegraded DNA could be tumor-intrinsic or immune cell-intrinsic with the resulting cGAMP signaling molecule transferred to neighboring cells. How tumor-derived DNA locates to the cytosol of immune cells remains unclear. Direct immune cell phagocytosis of tumor cells or exosome shuttling of tumor-derived DNA are possible, and the abundance of tumor-derived DNA correlates with tumor-intrinsic TREX1 expression (61). Thus, it is possible that cGAS-STING stimulation contributes to antitumor immunity in both tumor and host immune cells indicating that TREX1, cGAS, and STING are candidate targets to modulate antitumor immunity. Regardless, studies to date have demonstrated that TREX1, cGAS, and STING can be targeted to modulate antitumor immunity.

cGAS AND STING AS THERAPEUTIC TARGETS

TREX1 dysfunction activates the cGAS-STING DNA-sensing pathway resulting in autoimmunity. Thus, preventing cGAS-STING activation could provide therapeutic benefit to treat TREX1-mediated autoimmune disease. Inhibition of cGAS (62–65) and STING (66) using small molecules and anti-sense oligonucleotides have been shown to ameliorate pathology in mouse models of autoimmunity, and to limit brain injury following ischemic stroke (67). These studies support cGAS and STING as candidate targets for inhibition in autoimmune disease.

Conversely, stimulation of the cGAS-STING pathway is a novel approach to immune activation in cancer immunetherapy. Small-molecule STING agonists have been used to activate the cGAS-STING pathway and promote antitumor immunity (68–76). Additional work indicates STING agonists are effective in combinatorial therapies for infection (77). STING agonists are currently in clinical trials (78–80). DMXAA is a potent STING agonist that initially appeared promising in pre-clinical studies (70, 81, 82), but failed in human trials due to critical amino acid differences between the mouse and human STING proteins (83). These STING agonists indicate the potential in immunotherapy for cGAS-STING pathway activation. Since TREX1 exonuclease inactivity is known to stimulate cGAS-STING signaling, we propose TREX1 inhibition as an anticancer immunotherapeutic strategy.

TREX1 INHIBITION AS AN IMMUNOTHERAPEUTIC STRATEGY

The molecular and cellular properties of TREX1 indicate it has distinct advantages as a molecular target for immune activation. Studies indicating TREX1 expression is induced by genotoxic stress and that TREX1 exonuclease activity protects cancer cells from anticancer drugs and radiation suggest TREX1 inhibition would promote anti-cancer effects (40, 49, 51–53, 84). This concept is supported by studies showing that cells deficient in TREX1 activity show reduced recovery from treatment to DNA damaging agents (49, 84). Thus, inhibition of TREX1 in combination with chemotherapy may increase efficacy. Additionally, TREX1 functions to degrade DNA in dying cells (10, 50) and inhibition of TREX1 in tumor cells should potentiate

the innate immune anti-tumor effect as these cells die during treatment. Thus, small molecules that inhibit TREX1 acting upstream of STING in the pathway could produce the added benefit to amplify the signal producing a more robust IFN-signal relative to the current, direct STING receptor-small molecule agonists. Furthermore, enzyme inhibitors are generally more easily developed and refined than activating molecules. Currently, STING agonists have demonstrated relatively poor pharmacokinetics and biodistribution, restricting their dosing routes primarily to intratumoral injection (70, 72, 80, 81, 85). By contrast, our work has identified several TREX1 inhibitors with good solubility and oral drug-like (86, 87) physicochemical properties (ex. compound discussed in **Figure 6**). Consequently, TREX1-targeted therapeutics have the potential to be administered through more convenient oral dosing routes and promote more robust, systemic antitumor immunity than their STING-targeted counterparts.

There are limited published data directly testing the effect of TREX1 ablation on antitumor immunity. In one study, human T lymphocytes derived from a *TREX1* compound heterozygote (c.262 ins AG het + c.290 g>a R97H het) with exonuclease-deficient enzyme exhibited an increased capacity to inhibit neuroblastoma cell growth *in vitro* (88), indicating the immunotherapeutic potential of TREX1 inhibition. It's important to consider that acute TREX1 inactivation in wild-type organisms might not elicit the same biological response in *TREX1* mutants with chronic TREX1 inactivity. However, another study using microRNA-based TREX1-knockdown successfully demonstrated tumor regression *in vivo* (89), and in two additional studies it was shown that microRNA-based TREX1-knockdown generates an IFN signature in uninfected wild-type cells [see control data in refs (90, 91)]. Interpretation of these data is complicated by the complete loss of TREX1, including the TREX1 C-terminal region not required for exonuclease activity. However, we also observe that WT mice still produce IFN signatures when they receive bone-marrow transplants from mice with catalytically-inactive enzyme (*TREX1*^{D18N} mice), though to a lesser degree than seen in the donors (37). These bone marrow transplants do not perfectly represent an acute induction of TREX1 dysfunction in the recipients, since the donor cells still developed in an environment of chronic TREX1 deficiency. Still, together these studies support the immunotherapeutic potential of acute TREX1 inhibition.

The Perrino lab used allelic replacement to introduce the TREX1 D18N missense mutation into mice and showed that the TREX1 D18N mutation exhibits dysfunctional dsDNA-degrading activity resulting in immune activation in these mice (92). We tested the anti-cancer therapeutic potential of abolishing TREX1 exonuclease activity using the genetically precise TREX1^{D18N} mice (D18N mice), that express the mouse TREX1 D18N allele from its endogenous promoter that controls the level of expression in the appropriate genomic context. In this mouse model, the TREX1^{D18N} enzyme maintains structure, localization, and presumably protein-protein interactions making it an excellent model of specific inhibition of TREX1 exonuclease activity (7, 16, 92). The specific D18N mutation locates to the TREX1 active site in a way that TREX1 inhibitors might also bind and inhibit TREX1 DNA degradation, making the D18N mouse an appropriate model for TREX1 inhibition. We measured tumor resistance in the exonuclease deficient D18N mice by challenging WT and D18N mice with H31m1, a syngeneic, chemically induced sarcoma. When 5x10^6 H31m1 cells were implanted subcutaneously in TREX1 WT mice, the tumor grew until one axis extended past 20 mm and mice were euthanized. In WT mice median survival was 13 days (**Figure 3A**). In sharp contrast, the inactivity of TREX1 exonuclease in the TREX1 D18N mice resulted in a dramatically reduced tumor

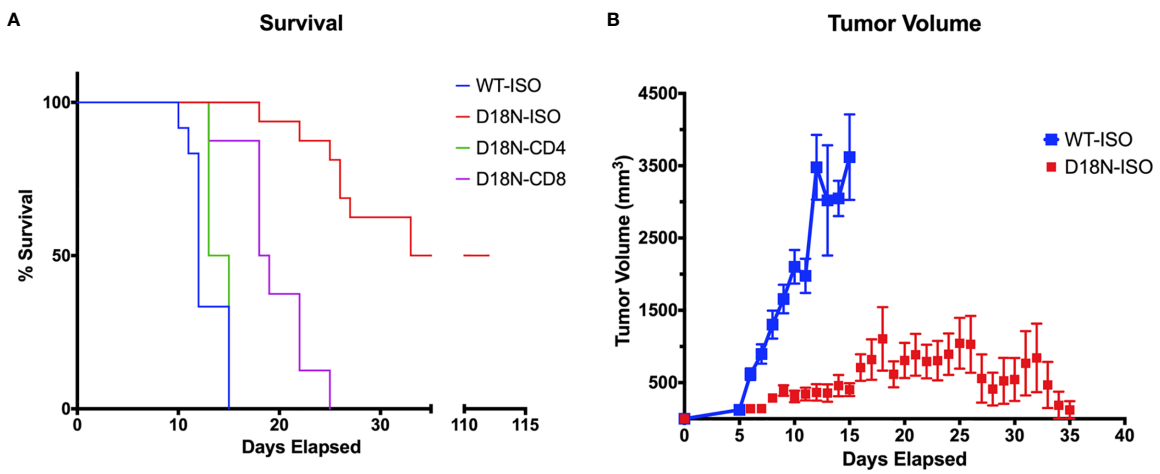


FIGURE 3 | TREX1^{D18N} Mice Display T-cell Dependent Antitumor Immunity. **(A, B)** 5x10^6 H31m1 tumor cells were injected subcutaneously into WT and D18N mice, and survival **(A)** and tumor volume **(B)** tracked daily (see Methods). Mice were treated with αCD4, αCD8, or the respective isotype-control antibodies to test the effects of T-cell depletion (see Methods). Isotype controls are presented together. Tumor volumes are average and standard deviation. Background of mice and tumor cells was 129S1/SvImJ, and each group represents 8-16 mice across 2-4 independent experiments. Data originally submitted for ASBMB 2020 conference (93). Graphs generated with Prism 7.0 (GraphPad).

volume that was always ~10-fold less than WT and an equally dramatic increase in median survival that was extended from 13 to 78 days in long-term survival studies, with ~40% of animals cured of their tumor for at least 120 days (**Figure 3B**).

Further studies were done to identify the mechanisms that control tumor growth. Mice were pretreated with antibody specific for murine CD4 (to eliminate helper T cells) or CD8 (to eliminate cytolytic T cells) or with the isotype controls in a depletion analysis. Isotype-treated TREX1 D18N mice had significantly reduced tumor growth and longer median survival of 78 days sharply contrasting the TREX1 WT mice treated with control antibodies that had rapid tumor growth and a median survival time of only 13 days (**Figure 3A**). We have also examined the immune response in the spleen, contralateral lymph node (CLN), draining brachial and axillary lymph nodes (TDLN), and tumor infiltrating lymphocytes (TIL) using multidimensional flow cytometry for multiple cell types in the adaptive immune response in order to determine how the anti-tumor response is altered in TREX1 D18N mice. **Figure 4A** shows an example of T cells staining in the spleens of WT mice. Similar numbers of activated $CD4^{+}CD44^{high}$ T cells were observed in the TIL (**Figure 4B**), TDLN (**Figure 4B**), CLN (**Figure 4B**), whereas the spleens of TREX1 D18N animals had increased effector/memory $CD4^{+}$ T (**Figure 4B**). When CD8+ T cells similar trends were also observed (**Figure 4C**). The most dramatic difference observed is in the fold induction in PD-1 levels on activated $CD8^{+}CD44^{high}$ T cells in the tumor (compared to $CD8CD44^{low}$ T cells in the spleen). Here we observed that WT $CD8^{+}$ T cell had a 40-fold induction of PD-1 levels (as measured by Mean Fluorescent Intensity (MFI)) whereas in TREX1 D18N mice levels were only increased ~20-fold, consistent with lower T cell exhaustion (**Figure 4C**). Taken together these results argue that the increased long-term survival observed after tumor challenge in TREX1 D18N mice is potentially

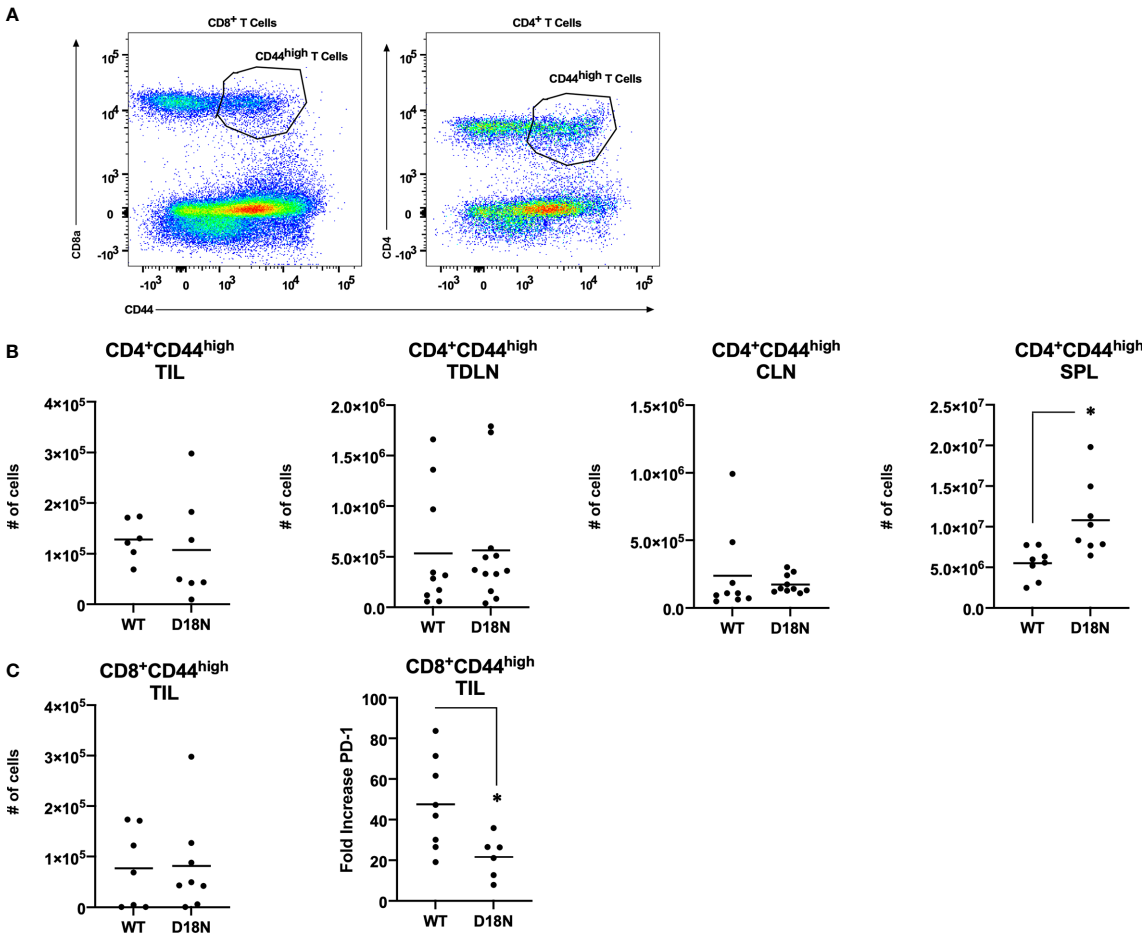


FIGURE 4 | Similar T-Cell Number but Decreased PD-1 Expression in $TREX1^{D18N}$ Mice During Tumor Progression. **(A–C)** WT or D18N mice were challenged with $5x10^{6}$ H31m1 cells, cells were isolated from the indicated tissue on Day 8, and **(A)** activated/memory $CD4^{+}$ and $CD8\alpha^{+}CD44^{high}$ T-cells were measured by flow cytometry (see Methods). Numbers of indicated **(B)** $CD4^{+}$ or **(C)** CD8+ T cells were determined. 'SPL' = spleen, 'CLN' = contralateral lymph nodes, 'TDLN' = tumor draining lymph node, and 'TIL' = tumor infiltrating lymphocytes. **(C)** PD-1 M.F.I. on activated/memory $CD8^{+}CD44^{high}$ T-cells in the tumor were determined, and the fold change compared to naïve T-cells in the spleen was calculated (see Methods). Individual mice (6-9 total, 3 independent experiments) are plotted, with averages represented by horizontal bars. *p-value < 0.05 *via* two-tailed independent Student's t-test. All graphs prepared in Prism 9.0 (GraphPad).

due to altered function of $CD4^+$ and $CD8^+$ T cells. These studies support enhanced tumor immunity in the D18N mice, and TREX1 inhibition as a viable anticancer immunotherapeutic strategy.

METHODS OF TREX1 INHIBITION

Newly developed therapeutics are typically one of three general categories: RNA-based drugs (RBDs), biologics (BLGs), or small molecules (SMs). The advantages and disadvantages of these various molecules as therapeutics have been previously reviewed (94). RBDs are a variety of specifically designed RNA molecules that modulate the activity of a protein target by interfering with its translation (95, 96). It is possible that an RBD strategy targeting TREX1 and resulting in complete ablation of TREX1 protein could generate a successful immune activation anti-cancer effect. Indeed, it has been demonstrated that microRNA targeting of *TREX1* expression successfully promotes tumor regression *in vivo* by modulating the tumor microenvironment (89). However, we, and others, have demonstrated that it is elimination of TREX1 exonuclease activity specifically that leads to cGAS-STING pathway activation (92, 97). The complete removal of TREX1 protein using RBD could potentially impact, unnecessarily, additional TREX1 functions that are independent of the cGAS-STING pathway (8, 9). In fact, mutations that completely abolish TREX1 protein generally produce much more severe phenotypes in mice and humans than those specifically affecting the exonuclease activity (18, 19, 92). BLGs are a diverse category of mostly proteinaceous biologic macromolecules capable of target-binding (94, 98). Currently, there are no BLG inhibitors of TREX1 reported. However, several antibodies for TREX1 are commercially available (99), though not as neutralizing/inhibiting molecules. SMs are organic molecules with molecular weights typically in the 100-1000 Daltons range (86). SMs modulate the activity of a protein target *via* a direct and limited binding interaction, an approach most compatible with attenuation of a specific activity for multifunctional targets. Also, SMs have been quite successful as cancer immunotherapeutics (100), prompting our choice for designing TREX1 inhibitors (93).

DEVELOPING SMALL MOLECULE TREX1 INHIBITORS

A high-throughput screening (HTS) strategy is critical to identifying candidate small molecule TREX1 inhibitors. We have reported methodology to successfully purify large quantities of recombinant TREX1 enzyme (101) to facilitate a scalable biochemical assay. Since the desired therapeutic effect from TREX1 inhibition is cGAS stimulation (26, 27, 97, 102) and because cGAS has specificity for dsDNA (103), TREX1 exonuclease activity on dsDNA is the appropriate biochemical metric for an inhibitor's potential. We have described a fluorescence-based exonuclease assay to measure TREX1's degradation of dsDNA (101) that is scalable to a 384-well microplate HTS assay. Importantly, this assay utilizes substrate concentrations at or below the TREX1 dsDNA K_m of ~15 nM^2, allowing the assay to readily detect small molecules with a broad range of inhibition kinetics (104). In addition, our own work has shown that TREX1 activity is not impacted by concentrations of up to 0.01% Triton X-100, which could be included in a HTS to limit false-positives from promiscuous aggregation-based inhibitors (105–107). Thus, our TREX1 biochemical studies have positioned us well to undertake a HTS endeavor.

Optimal TREX1 drugs developed from initial inhibitor molecules should minimize off-target effects and exhibit a high level of specificity for the target. Counter-screening candidate TREX1 inhibitors against enzymes of varying relatedness (104) provides context for the inhibitors' relative affinities for the target. Cross-activity on a highly unrelated enzyme might suggest significant promiscuity by the candidate molecule, while inactivity against an enzyme likely indicates a level of specificity proportional to the enzyme's relatedness to the target. Three-prime Repair Exonuclease 2 (TREX2) is structurally (6, 108) and biochemically (2) related to TREX1, making it the ideal choice for counter-screens to identify highly specific TREX1 inhibitors. Indeed, the similarities between TREX1 and TREX2 raise concerns about the potential for off-target effects *in vivo*, since TREX2 dysfunction has been linked to skin carcinogenesis in mice (109). However, TREX2 mutant mice exhibit a conditional phenotype requiring genotoxic stress (109, 110), suggesting that some level of TREX2 cross-activity by a TREX1 inhibitor might be tolerable for therapeutic applications. Despite the remarkable structural similarities, TREX1 and TREX2 contain multiple different structural elements and specific residues that could be exploited as TREX1-inhibitor contacts to achieve specificity (**Figure 5**). In addition, the potential for species specificity of small molecules, as evidenced by the STING agonist DMXAA's ability to activate murine but not human protein (83), indicate biochemical analysis of human and mouse TREX1 to be a valuable approach. Our work using human and mouse TREX1 and TREX2 has already led to the identification of a class of small molecules with exceptional specificity for the hTREX1 enzyme (**Figure 6**).

The development of candidate inhibitors identified from initial screening into effective therapeutics requires iterative chemical modification and testing to improve potency and specificity. This process benefits significantly from 'rational' design of the chemical modifications, which relies heavily on structural information about the target-inhibitor interactions. In this capacity, TREX1 is well suited for rational drug design. We have published a detailed protocol for generating large quantities of high purity TREX1 enzyme (101) and multiple structures of the mTREX1 enzyme solved by x-ray diffraction (6, 11, 16, 92), including apoenzyme and co-crystallizations with TREX1 substrates and product. These structures demonstrate the capacity for mTREX1 to be co-crystallized with a variety of molecules and can also be used in computational approaches to model the binding mechanisms of candidate inhibitors (111, 112). Our previously published mTREX1 apoenzyme indicates an active-site readily accessible *via* solvent channels in the crystal (**Figure 7**), suggesting TREX1-inhibitor co-structures could be determined by soaking compounds into existing apoenzyme crystals. We have also solved several structures of the

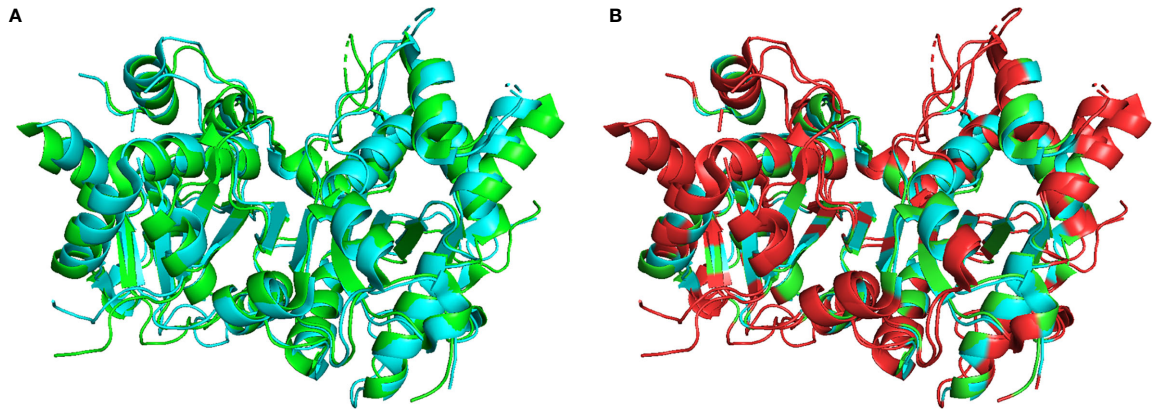


FIGURE 5 | Structural Comparison of TREX1 and TREX2. Graphic **(A)** shows structural alignment of mTREX1(1-242) and hTREX2 in cyan and green, respectively, and graphic **(B)** is the same alignment with discrepant residues colored red. Alignment and graphics were generated in PyMOL using the PDB structures 3MXJ and 1Y97 from refs (16, 108).

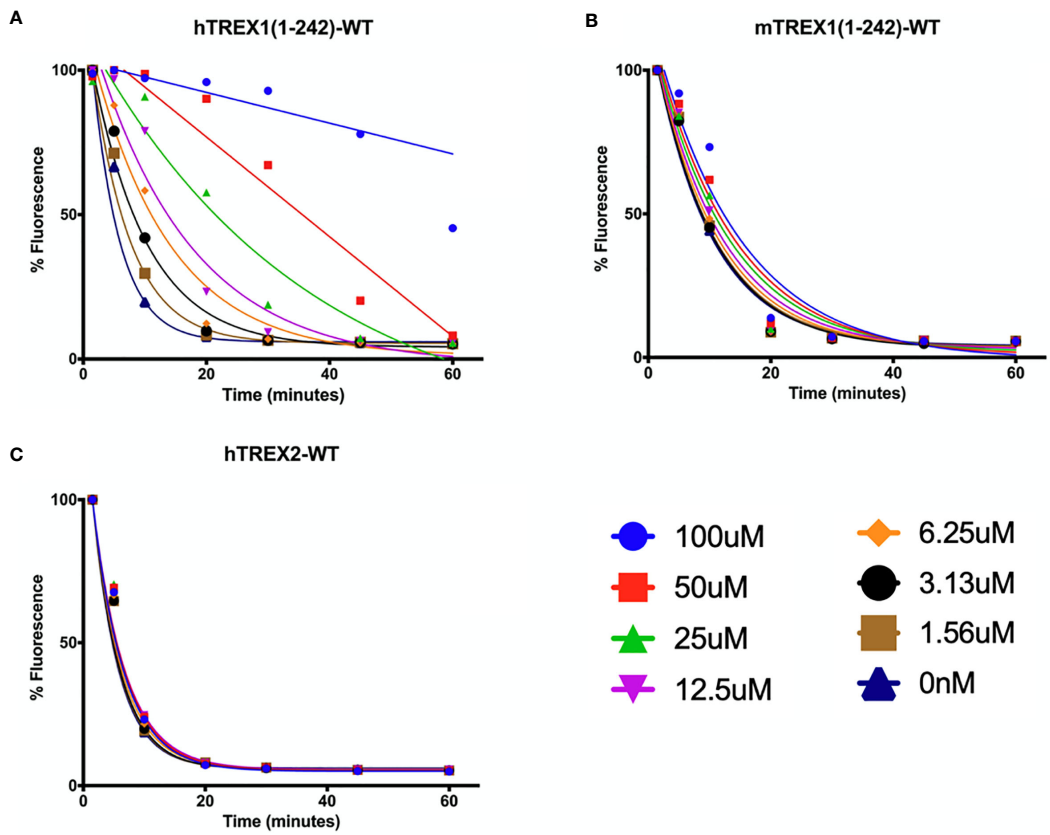


FIGURE 6 | Small Molecule Inhibitor with High Specificity for hTREX1. **(A–C)** Standard time-course reactions were prepared in 150 µL volumes containing vehicle or indicated concentrations of inhibitor, and hTREX1 **(A)**, mTREX1 **(B)**, or hTREX2 **(C)**. Reactions were incubated 1-hr at room temperature, and 20 µL samples of each reaction taken at time-points of 0, 5, 10, 20, 30, 45, & 60 minutes and quenched in 20 µL of 15X SYBR Green. Fluorescence was measured, and fluorescence vs time plots were normalized to maximal initial fluorescence and background fluorescence (see Methods). Plots were fit with nonlinear regression. Plots were generated in Prism (GraphPad) and combined in PowerPoint.

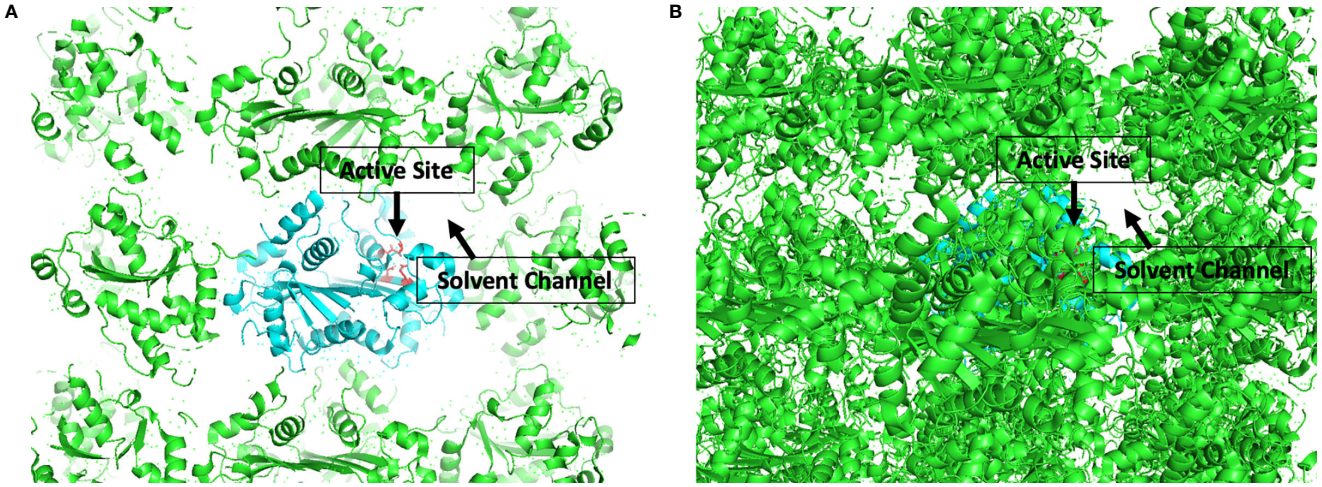


FIGURE 7 | Active Sites are Accessible by Solvent Channel in TREX1 Apoenzyme Crystals. Structural representation of crystal lattice for mTREX1(1-242) apoenzyme crystal. Functional unit of interest is colored cyan with DEDD active site residues for one protomer shown as red sticks; other functional units are colored green. Graphic **(A)** is a slice through the crystal lattice where the active site is visibly facing the solvent channel, and graphic **(B)** looks through the solvent channel into the crystal lattice. Alignment and graphic were generated in PyMOL using the PDB structure 3MXJ from ref (16).

hTREX2 enzyme (108, 113). Thus, crystallographic studies with the TREX2 enzyme present an alternative strategy to deduce target interactions in TREX1 inhibitors exhibiting cross reactivity. Altogether, our structural studies lay the groundwork for rational design modifications that contact residues discrepant between the two enzymes.

SUMMARY

The 3' → 5' exonuclease TREX1 acts *in vivo* to degrade DNA and prevent aberrant nucleic acid sensing. In the absence of TREX1 exonuclease activity, substrate accumulation stimulates the DNA-sensing pathway cGAS-STING, which drives IFN-signaling and autoimmunity. TREX1 and cGAS-STING have been proposed to function in the tumor microenvironment, where TREX1 is believed to degrade tumor-derived DNA that would otherwise stimulate the cGAS-STING pathway and elicit antitumor immunity. Thus, we propose TREX1 as a novel immunotherapeutic target, and provide data demonstrating significant antitumor immunity in TREX1-deficient mice. We propose small molecules as a viable strategy for TREX1 inhibition in the context of past work with other targeting strategies.

MATERIALS AND METHODS

Expression and Purification of TREX Enzymes

Detailed protocols for hT1, mT1, and hT2 enzyme purification have been published (101) and summarized here. For homodimers, pLM303x constructs encoding the recombinant TREX enzyme fused to an N-terminal maltose-binding protein (MBP) are transformed into Rosetta II cells, and the MBP-linker-TREX fusion protein is overexpressed. Cells were pelleted, subjected to pressure-lysis, and the supernatants subjected to amylose column chromatography. Eluent was treated with protease to cleave the fusion protein linker and purified by phosphor-cellulose column chromatography to obtain pure TREX enzyme.

Fluorescence-Based Exonuclease Assay

Our detailed protocol for this assay is published (101). Reaction mixture was prepared containing variable concentrations of a dsDNA substrate, 5 mM $MgCl_2$, 2 mM DTT, 20 mM Tris base (pH 7.5). Compounds were added at various concentrations as DMSO solutions to the reaction mixture prior to enzyme addition, and final DMSO-vehicle concentration is 2.5% in all exonuclease experiments. Enzyme was diluted to 10X the final reaction concentration *via* serial dilutions into 1 mg/mL BSA, and then diluted 10-fold into the reaction to initiate resulting in the appropriate TREX enzyme and BSA at 100 μg/mL. Reactions were at room-temperature for 1hr. Samples (20 μL) were removed at varied time points and quenched in a 384-well black microplate containing 20 μL of 15X SYBR Green solution. Fluorescence of quenched samples was measured using a PolarStar Omega microplate reader (BMG LabTech) at excitation/emission of 497/520.

The DNA substrate was generated by linearizing the ~10-kb pMYC plasmid with the SacI (NEB) restriction enzyme per vendor specifications and included in assays at a concentration of 5 ng/μL. Enzyme concentrations were 15 nM for mT1, 75 nM for hT2, and 15 nM for hT1. Time-course reactions were from 20 μL samples taken at 0, 5, 10, 20, 30, 45, & 60-minute time points. Initial reaction volumes were 150 μL, compound

concentrations were as indicated in the figure, and vehicle control reactions were always included.

Tumor Challenge

Nine week 129 S6/SvEvTac D18N mutant and WT mice were generated as previously described (92). At 10 to 12 weeks, 5×10^6 H31m1 tumor cells were subcutaneously (s.c) injected in 200 µl PBS into the shaved right flanks of recipient mice. Tumor size was measured by a digital caliper every day and presented as the cube of its diameters. Studies included 6-9 mice/group across 3 independent experiments. H31m1 cells were obtained from Robert Schreiber (Washington University). All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Wake Forest University School of Medicine.

Antibody Depletion Experiments

To determine which cells were essential for enhanced clearance in D18N mice, we depleted $CD4^+$ or $CD8^+$ T cells by administering 1500 µg of antibody (BioXCell) for 3 days prior to and during tumor challenge (-2, 0, + 2, i.p.). This resulted in 99% selective depletion as assessed by flow cytometry on PBMCs isolated at day 10. Clones 53-6.7 and GK1.5 were used for $CD8\alpha^+$ and $CD4^+$ T cells, respectively.

Cell Isolation

The spleen was removed from mice after cervical dislocation. Following mechanical disruption of splenocytes on a wire mesh screen, red blood cells were removed by osmotic lysis in ACK buffer (NH4Cl, KHCO3, and EDTA). Splenocytes were then resuspended in complete media containing RPMI 1640 supplemented with 10% fetal calf serum (FCS, HyClone), L-glutamine (HyClone), penicillin-streptomycin (Cellgro), non-essential amino acids (GIBCO), and 2-mercaptoethanol (GIBCO). For $CD8^+$ T cell purification, splenocytes were resuspended in PBS supplemented with FCS and EDTA. $CD8^+$ T cells from splenocytes were then negatively selected by magnetic bead using $CD8^+$ T-Cell Purification Kit (Miltenyi Biotec) according to the manufacturer's instructions.

In tumor studies, the contralateral and draining lymph nodes (brachial and axillary) were isolated. Tumor tissues were dissociated by mechanical disruption and incubated with enzymes in Tumor Dissociation Kit (Miltenyi Biotec) at 37°C for 30 mins. TILs were then washed with RPMI 1640 supplemented with 1% fetal calf serum, and resuspended in complete media. Tumor analyses used whole tumors of approximately equivalent volume that were taken at 8-days post challenge. Cell numbers in **Figure 4** were determined by flow cytometry by gating on either CD8 or CD4, then CD44 (as shown in **Figure 4A**) and the PD-1 mean was calculated for $CD8^+CD44^{high}$ T-cells.

Surface and Intracellular Staining

In this study, the following antibodies were used: rat anti-mouse CD8α-phycoerythrin (PE), rat anti-mouse CD8α-peridinin chlorophyll protein (PerCP), rat anti-mouse CD8α-V500, rat anti-mouse CD90.1 (Thy1.1)-allophycocyanin (APC), rat anti-mouse CD90.1- fluorescein isothiocyanate (FITC), rat anti-mouse CD90.1-eFluor450, rat anti-mouse CD90.2 (Thy1.2)-V500, rat anti-mouse CD4-APC, rat anti-mouse CD4-V500, rat anti-mouse CD44-PerCP, rat anti-mouse CD44-eFluor450, rat anti-mouse CD44-APC-eFluor780, rat anti-mouse CD127-FITC, rat anti-mouse KLRG1-PE, rat anti-mouse CD27-PE-Cyanine7, rat anti-mouse CD62L-APC-eFluor780, rat anti-mouse CD69-PE-Cyanine7, rat anti-mouse PD-1-FITC, rat anti-mouse LAG-3- PerCp-eFluor710, rat anti-mouse BTLA-PE, rat anti-mouse IFN-γ-FITC, rat anti-mouse TNF-α-PE-Cyanine7, rat anti-mouse IL-2-APC, rat anti-mouse CCL3 (MIP-1α)-PE. KLRG1 antibody was purchased from Abcam. CD8-V500, CD8-PerCp, IFN-γ-FITC, TNF-α-PE-Cyanine7 and IL-2-APC were purchased from BD Pharmingen. All other antibodies were purchased from eBioscience. Surface staining was performed by incubation of Abs at a 1:100 dilution in fluorescence-activated cell sorter (FACS) buffer for 30 min on ice. KLRG1 staining was performed at a 1:25 dilution. Tetramer staining was performed at a 1:200 dilution. BTLA staining was performed at a 1:333 dilution. To measure intracellular cytokine levels, cells were incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (ION) for 5 h at 37°C, and then treated with the BD Biosciences Cytofix/Cytoperm kit according to the manufacturer's instructions. Intracellular transcription factor stain was performed by using eBioscience Mouse Regulatory T Cell Staining Kit according to the manufacturer's instructions. After staining, samples were fixed in 1% formaldehyde (Polysciences, lnc., Warrington, PA) and acquired on a BD FACS Canto instrument. Manual gating was performed on FlowJo software (TreeStar, San Francisco, CA).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee at Wake Forest School of Medicine.

AUTHOR CONTRIBUTIONS

WH provided the inhibitor data and prepared the manuscript. SS and ML contributed to the murine tumor data and assisted with manuscript revisions. FS contributed to the inhibitor data and assisted with manuscript revisions. TH contributed to the inhibitor data and assisted with manuscript revisions. JG provided the murine tumor data and assisted with manuscript revisions. FP ideated the proposal and assisted with manuscript

revisions. All authors contributed to the article and approved the submitted version.

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Nucleic Acid Immunity and DNA Damage Response: New Friends and Old Foes

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The maintenance of genomic stability in multicellular organisms relies on the DNA damage response (DDR). The DDR encompasses several interconnected pathways that cooperate to ensure the repair of genomic lesions. Besides their repair functions, several DDR proteins have emerged as involved in the onset of inflammatory responses. In particular, several actors of the DDR have been reported to elicit innate immune activation upon detection of cytosolic pathological nucleic acids. Conversely, pattern recognition receptors (PRRs), initially described as dedicated to the detection of cytosolic immune-stimulatory nucleic acids, have been found to regulate DDR. Thus, although initially described as operating in specific subcellular localizations, actors of the DDR and nucleic acid immune sensors may be involved in interconnected pathways, likely influencing the efficiency of one another. Within this mini review, we discuss evidences for the crosstalk between PRRs and actors of the DDR. For this purpose, we mainly focus on cyclic GMP-AMP (cGAMP) synthetase (cGAS) and Interferon Gamma Inducible Protein 16 (IFI16), as major PRRs involved in the detection of aberrant nucleic acid species, and components of the DNA-dependent protein kinase (DNA-PK) complex, involved in the repair of double strand breaks that were recently described to qualify as potential PRRs. Finally, we discuss how the crosstalk between DDR and nucleic acid-associated Interferon responses cooperate for the fine-tuning of innate immune activation, and therefore dictate pathological outcomes. Understanding the molecular determinants of such cooperation will be paramount to the design of future therapeutic approaches.

Keywords: cytosolic nucleic acids, DNA damage responses, inflammation, cGAS-STING, IFI16, DNA-PK, tumorigenesis

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INTRODUCTION

Innate immunity, the first line of host defense, is classically triggered in response to pathogen infection or local lesions to promote infection clearance or wound-healing processes. The activation of innate immune responses vastly relies on pattern-recognition receptors (PRRs) that detect danger associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). Upon recognition of PAMPs or DAMPs, PRRs trigger signaling cascades leading to the production of

soluble mediators, such as type I Interferons, cytokines and chemokines. Pathogen-derived nucleic acids constitute major PAMPs that are detected by a vast array of PRRs that operate in specific subcellular localizations. In recent years, self-nucleic acids, originating from replication stress (1), DNA or mitochondrial damage (2), and endogenous retroelement activation (3), have been identified as substrates for cytosolic PRRs, and are thus considered as DAMPs. Because nucleic acids are abundant in cells, the activity of PRRs engaged in their detection is regulated and compartmentalized (4). PRRs dedicated to nucleic acid detection also present substrate specificity, with subclasses dedicated to the detection of particular moieties (5).

A plethora of cytosolic nucleic acid sensors have been described to participate in triggering Interferon responses. Such receptors notably include the ubiquitous DNA-dependent activator of Interferon regulatory factors (DAI) (6), AIM2 (7, 8), Interferon gamma-inducible protein 16 (IFI16) (9), melanoma differentiation factor 5 (MDA5) (10) and retinoic acid-inducible gene (RIG-I) (10). An extensive description of the mechanism of action of the above mentioned PRRs can be found in (11). Among pathways involved in cytosolic nucleic acid detection, the Stimulator of Interferon genes (STING) protein constitutes a central signaling hub (12, 13). Initial reports indicated that STING activation requires detection of cytosolic nucleic acid species such as double strand (dsDNA), single strand (ssDNA), or RNA : DNA species (14–16) by the cyclic GMP-AMP (cGAMP) synthase (cGAS) PRR (14). The main signature of activation of this signaling pathway is the production of type I Interferons that in turn promote the production of interferon-stimulated genes (ISGs). This signaling pathway has attracted tremendous biomedical interest in recent years, notably with observation that agonists of STING can boost antitumoral immunity (17).

However, there is emerging evidence for an intricate signaling network beyond the cGAS-STING cascade, which cannot be overlooked in therapeutic strategies aiming to boost STING activation. Of particular importance is the fact that cGAS and STING have been both described as involved in genotoxic stress response and to participate to the maintenance of genomic integrity. Furthermore, the DNA-PK complex, which is best known for its function in non-homologous end-joining (NHEJ)-mediated repair of dsDNA breaks (DSB), has been shown to serve as an alternative route to stimulate type I Interferon production (18–21). In parallel, the Interferon Gamma Inducible Protein 16 (IFI16) was also reported to detect, in concert or not with cGAS, DNA damage-derived nucleic acid species (9, 22, 23), and to cooperate with DDR proteins to promote STING-dependent immune responses following genotoxic stress (22). Furthermore, cGAS and STING have been shown to control genomic stability (24, 25). Thus, the current literature highlights tight connections between DNA repair processes and nucleic acid-associated inflammatory responses. Indeed, proteins involved in the recognition of abnormal DNA, regardless of their origin, appear to possess common roles in the initiation of inflammatory responses and surveillance of genomic integrity.

In this *mini review*, we discuss this interconnection between DNA repair mechanisms and nucleic acid immunity, by focusing on the cGAS and IFI16 receptors and the way in which they control STING activation. While several DNA repair proteins have been involved in the fine tuning of inflammatory responses (22, 26), here we focus on the DNA-PK complex, responsible for NHEJ, for which a role in controlling nucleic acid-dependent inflammatory responses has been reported (26). We discuss how dissecting these signaling networks will deepen our understanding of Interferon responses, which is likely crucial to the design of therapeutic responses to pathological inflammation.

CYTOSOLIC NUCLEIC ACID DETECTION: STING AS A CENTRAL SIGNALING HUB

The cGAS-STING Pathway

The production of type I Interferons, in the presence of cytosolic nucleic acid species, was initially described to rely mostly on cGAS (14). Indeed, cGAS detects dsDNA, ssDNA, or RNA : DNA species (14–16) in the cytosol and catalyzes the synthesis of cGAMP (**Figure 1A**). Although the binding of cGAS to nucleic acid species is sequence-independent, cGAS activation is increased by longer dsDNA fragments (27, 28), suggesting that portions of chromosomes, such as those arising in the micronucleation process, would serve as potent substrates for cGAS. cGAMP interacts with the endoplasmic reticulum (ER)-resident STING adaptor protein (29, 30), promoting conformational changes (29, 31), oligomerization (32) and translocation to perinuclear compartments, including the Golgi apparatus (12, 33). Subsequent recruitment of the TANK-binding kinase 1 (TBK1) (34), together with transcription factors, such as Nuclear Factor Kappa B (NF-κB) and Interferon Response Factor 3 (IRF3), ultimately leads to the transcription of a repertoire of inflammatory cytokines characterized by a type I Interferon signature (12, 35) (**Figure 1A**). NF-κB activation may also be promoted by IKKε, in addition to TBK1, in macrophages (36). The cGAS-STING cascade is triggered upon cytosolic exposure of foreign nucleic acid species, following pathogen infection, but also by nucleus- and mitochondria-derived self-nucleic acids that leak into the cytosol following various types of stress (2, 37–40) and through DNA recombination processes (41).

Yet, the cGAS-cGAMP-STING signaling axis has recently emerged as far more complex than initially anticipated. First, multiple post-translational modifications influence signaling output (42–44). Second, STING can be directly activated by bacterial cyclic di-nucleotides (45, 46), while its activation is skewed by alternative di-nucleotides (16) or other metabolites (47). Third, co-sensors, co-factors and alternative upstream STING activators have been described, that can operate in cell type-specific manners (23, 48–50). Finally, in addition to the cell-autonomous capacity of cGAMP to activate STING-dependent Interferon responses, cGAS-STING signaling may also be amplified through transfer of cGAMP to neighboring cells

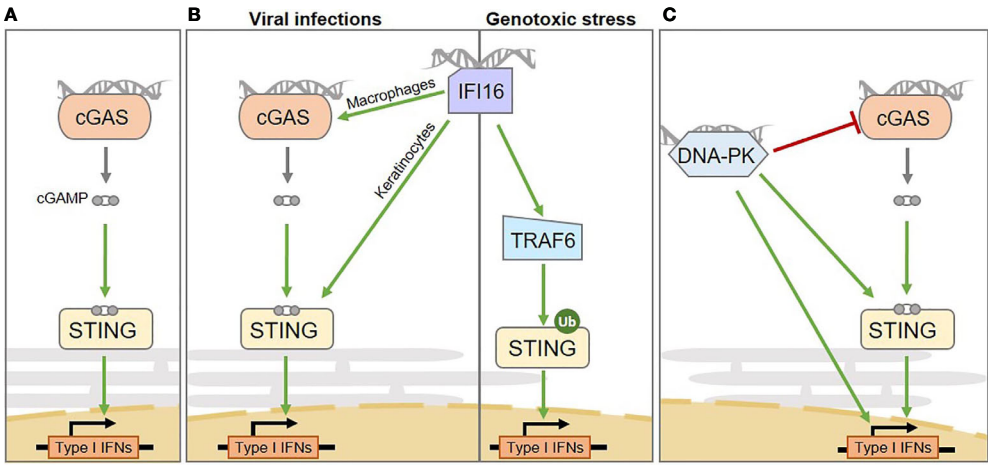


FIGURE 1 | Intertwined cytosolic nucleic acid pathways involved in Interferon and pro-inflammatory cytokine production in human cells. **(A)** The cGAS sensor activates STING *via* the production of the cGAMP second messenger. **(B)** *From left to right:* the IFI16 sensor mediates inflammation through multiple routes: upon viral infection it activates STING in a cell type-specific manner, either enhancing cGAS-dependent cGAMP production in macrophages or by directly activating STING in keratinocytes; upon genotoxic stress it mediates cGAS-independent, but TRAF6-dependent STING activation. **(C)** the DNA-PK DNA repair complex was shown to play a role in inducing type I Interferon production upon cytosolic dsDNA detection. However, multiple downstream mechanisms have been proposed, that require STING activation or not, ultimately leading to the phosphorylation of transcription factors responsible for type I Interferon production. The catalytic subunit of the DNA-PK complex (DNA-PKcs) can also suppress cGAS enzymatic activity, by promoting its phosphorylation. IFNs, Interferons.

through gap junctions (51–53), direct secretion (54) or in vesicles (55).

Below, we focus on IFI16 and DNA-PK as alternative sensors involved in the regulation of STING-dependent Interferon responses.

IFI16: An Alternative Nucleic Acid Sensor

IFI16 is a predominantly nuclear protein that has been described as involved in the induction of innate immune responses upon infection by viruses, including Herpes simplex virus (9), Epstein-Barr virus (56), and Kaposi's sarcoma-associated Herpes virus (57). Indeed, in this context, IFI16 promotes IRF3- and NF-κB-dependent Interferon production *via* STING (9) (**Figure 1B**). Similar to cGAS, IFI16 is capable of detecting self and non-self DNA, and displays a preference for long non-self dsDNA (58). Unlike cGAS, IFI16 operates mostly in a cell-type-dependent manner (23, 50). The interplay between cGAS and IFI16 has been explored, revealing cooperation between IFI16 and cGAS upon infection (**Figure 1B**, left). This cooperation relies on cell-type specific molecular mechanisms. Indeed, in both keratinocytes and macrophages, IFI16 cooperates with cGAS for STING activation upon infection (23, 50). However, in macrophages, IFI16 enhances cGAS-dependent cGAMP production (50), while in keratinocytes, IFI16 does not influence cGAMP production, but rather directly activates STING (23). Additionally, IFI16 has been shown to promote inflammasome activation in the nucleus, leading to production of Interleukin-1β (IL-1β), IL-18, and IL-33 cytokines (56, 59).

In contrast, following genotoxic stress, IFI16 triggers cGAS-independent STING activation (**Figure 1B**, right). Indeed, upon etoposide-induced DNA lesions, IFI16, together with DDR proteins, activates STING, promoting the assembly of a non-canonical STING signalosome (22). Within this complex IFI16 promotes TNF Receptor Associated Factor 6 (TRAF6)-dependent STING ubiquitination and activation ultimately leading to the predominant activation of the transcription factor NFκB, rather than IRF3 (22). Therefore, this signaling cascade results in the expression of a repertoire of cytokines that differs from that triggered upon cGAS-mediated detection of dsDNA, including a specific IL-6 and CCL20 signature (22). Yet, most of the described mechanisms were inferred from the study of keratinocytes or myeloid cell lines, leaving uncertainties concerning the activation of IFI16 in cancer cells.

DNA-PK: Bridging DNA Repair and Nucleic Acid Immunity

The DNA-PK complex has been reported to play a role in controlling nucleic acid-dependent inflammation. The DNA-PK complex is a key holoenzyme, composed of $KU70^{XRCC6}$, $KU80^{XRCC5}$ and the DNA-dependent protein kinase catalytic subunit $DNA\text{-}PKcs^{PRKDC}$, central to the repair of DSBs by NHEJ. NHEJ is involved in the repair of approximately 80% of DSBs and can operate regardless of the cell cycle phase. It promotes relegation of DNA ends without requirement for an intact template (60). KU70/KU80 heterodimers interact directly with damaged DNA ends and are responsible for the recruitment of DNA-PKcs to these lesions. DNA-PKcs bears a kinase activity and promotes both DNA-PKcs autophosphorylation and the phosphorylating-activation of effector proteins required for the NHEJ process. For a complete recent view of NHEJ refer to (61).

Besides its canonical role in NHEJ while it recognizes self dsDNA, there are several reports for a central role of DNA-PK in the detection of exogenous DNA species and interference with

viral life cycles (62). Subunits of the complex have been independently reported to trigger or skew inflammatory responses toward either type I or type III Interferon production in response to non-self dsDNA. Indeed, KU70 triggers DNA-dependent type III Interferon responses through activation of Interferon Regulatory Factor 1 and 7 (IRF1 and IRF7) (19, 63), independently of DNA-PKcs (19).

In contrast, recent reports indicate that the catalytic activity of DNA-PKcs is also crucial for antiviral responses. Indeed, DNA-PKcs promotes IRF3 phosphorylation following infection by DNA and RNA viruses (18, 64) (**Figure 1C**). Interaction between DNA-PKcs and the progenome of the Human Immunodeficiency Virus (HIV) retrovirus has also been shown, although the link to inflammation is unexplored (65). Interestingly, some DNA viruses have evolved proteins that counteract DNA-PKcs-dependent detection (20) while others hijack NHEJ to the benefit of their replication (66), highlighting the tight interplay between viral life cycles and DDR (67). Yet, there is as of today, limited knowledge concerning the ways in which DNA-PK-dependent inflammatory responses, IFI16- and cGAS-dependent STING activation are orchestrated.

Indeed, whether DNA-PK requires STING for production of Interferons remains debated (18, 20, 68) (**Figure 1C**). It was reported that DNA-PKcs is recruited to dsDNA in the cytoplasm of both human and murine cells through KU80, triggering IRF3-dependent inflammatory responses (18). However, while some reports indicate that the catalytic activity of DNA-PKcs is responsible for direct activating phosphorylation of IRF3 (64), others indicate that the measured Interferon production can occur independently of the catalytic activity of DNA-PKcs (18). In this latter scenario, questions remain open concerning what would trigger IRF3 activation. It has also been proposed that DNA-PKcs would act upstream of TBK1 and IRF3 (18) and that KU70 can form a complex with STING prior to (18), or upon (19) DNA transfection. This notion was comforted by Morchikh et al., in 2017, showing that DNA-PK subunits (DNA-PKcs, KU70 and KU80) are associated with a ribonuclear complex that is remodeled by foreign DNA, leading to enhanced recruitment of STING, activated DNA-PKcs, and IRF3 (68). However, a recent study has shown that DNA-PKcs can also operate independently of STING (20) and that DNA-PKcs can phosphorylate cGAS and suppresses its enzymatic activity (21) (**Figure 1C**). Considering the tight link between DNA-PK activation and cell cycle progression (69), and in view of the recent reports linking cGAS activation and cell cycle stage (70), the crosstalk between DNA-PK and cGAS-STING activation would certainly benefit from integrating the temporality of events to the study. In agreement, it was previously reported that inhibition of NHEJ components reduces Interferon signaling, in a cell cycle progression-dependent manner (71).

Adding a layer of complexity, DNA-PKcs immune signaling appears to be species-specific. Indeed DNA-PKcs can activate innate immune responses independently of STING in human cells, but not in murine cells (20). This is consistent with previous reports that in mouse cell lines, where the immune response is largely dependent on STING, DNA-PKcs would signal through STING (18). Furthermore, the current state-of-the-art does not allow determining whether the role of DNA-PK in inducing type I Interferon responses may be subjected to cell type-specific regulatory mechanisms, as was reported for IFI16. In this respect, how IFI16 activation is regulated in contexts where DNA-PKcs activates inflammatory responses remains to be elucidated.

REGULATION OF THE DNA DAMAGE RESPONSE BY INNATE IMMUNE SENSING PATHWAYS

cGAS Suppresses DNA Damage Responses

The cGAS protein was initially identified as the main receptor for cytosolic nucleic acid moieties that promote type I Interferon responses (14). However, it was recently demonstrated that an abundant pool of cGAS is tethered to the chromatin, in absence of inflammatory stimulus (72–74). Active export of cGAS through the Chromosomal Maintenance 1 (CRM1) exportin was recently demonstrated, suggesting that shuttling of cGAS to the cytosol may be a prerequisite for its activation (75). However, the molecular mechanisms triggering cGAS nuclear export and whether cGAS may also be activated in the nucleus, remains to be clarified. There is evidence for a role of cGAS in the inhibition of Homologous Recombination (HR)-mediated repair of DSB. Contrary to NHEJ, that operates in a cell cycle stage-independent manner, HR requires the presence of the sister chromatid for accurate repair of DNA lesions (76) and therefore operates mostly during the G2/M phase of the cell cycle. HR is a complex, multistep process that can be completed through several interconnected pathways, for which a complete overview can be found in (77). Two mechanisms have been proposed for cGAS-dependent HR inhibition (**Figure 2**, left). On one hand, Liu et al. showed that DNA damage triggers cGAS nuclear translocation and interaction with activated DNA damage-dependent Poly [ADP-ribose] polymerase 1 (PARP1), which is a first responder in DNA damage detection. Interaction of cGAS with PARP1 prevents the recruitment of proteins required to proceed through HR process and does not require the cGAS DNA-binding domain (79). However, since the majority of cellular cGAS is nuclear (72–74), one may question why the cytosolic rather than the chromatinian pool of cGAS would be mobilized. This may be linked to the high affinity of cGAS for the acidic patch of histones that renders chromatinian cGAS not easily displaceable (73). On the other hand, Jiang et al. observed that the DNA-binding domain of chromatin-bound cGAS is crucial for cGAS oligomerization on DNA, hindering the formation of displacement loops, which are required for HR to proceed (80). Consequently, upon irradiation, cells expressing cGAS present increased accumulation of DSBs as compared to cells that do not express cGAS. Intriguingly, this function is reportedly independent of cGAS-mediated innate immune sensing (80).

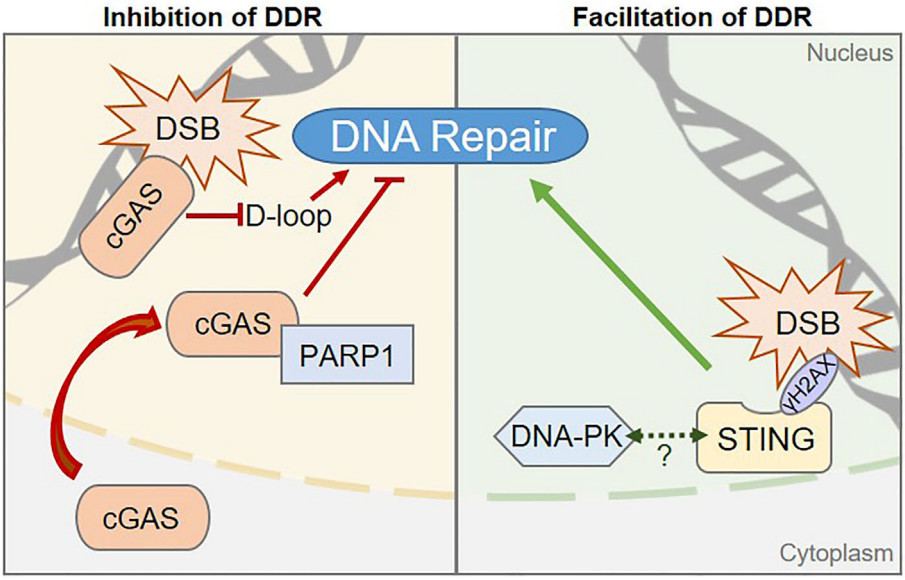


FIGURE 2 | Pattern Recognition Receptors are involved in regulating DNA damage repair processes. *Left*, The cGAS sensor can inhibit DNA Damage Responses (DDR) *via* two distinct mechanisms in human cells: 1) cGAS inhibits the Homologous Recombination (HR) pathway by preventing displacement loop (D-loop) formation. 2) cGAS-PARP1 interaction impedes the formation of a PARP1-based complex required for HR-mediated DNA repair. *Right*, STING promotes DDR in human cells through a yet to be elucidated molecular mechanism, but may rely on the control of components of the Non-Homologous End Joining DNA repair pathway, such as the DNA-PK complex. Dashed arrow between STING and DNA-PK represents the reported interaction between the two proteins (19, 68, 78). Whether this interaction is related to STING-associated DDR control is unknown.

STING as a Promoter of DNA Damage Responses

STING, the major downstream partner of cGAS, has been proposed to promote DDR and to enable cell survival, in an inflammation-independent manner (**Figure 2**, right). An important part of the regulation of STING activation is linked to its subcellular localization, with inactive STING resting in the ER and activation promoting its relocalization to the Golgi apparatus. Interestingly, in certain contexts, such as following chemotherapy regimens, STING colocalizes with γH2AX-positive DNA damage foci, at the inner nuclear membrane (78). In addition, cells knocked-down for STING present accumulation of DNA damage as compared to WT cells (78). No clear molecular mechanism has been proposed yet, although STING has been demonstrated to interact with NHEJ proteins, including DNA-PKcs, KU70 and KU80 (18, 19, 68, 78), suggesting that it may participate directly in the regulation of NHEJ. Moreover, STING overexpression leads to increased binding of DNA-PK to chromatin, suggesting that STING may cooperate with DNA-PK to control NHEJ-mediated DNA repair (78). However, the contribution of STING to NHEJ efficiency was not addressed, calling for further investigation.

Altogether, the subcellular localization of PRRs is central to the regulation of their activity, and determines whether they mediate repair- or immune-related functions. This is similar to what is witnessed for components of DNA-PK that are engaged in DNA repair or innate immune activation, depending on their subcellular localization and interactors. How these pathways cooperate or antagonize each other in given pathological situations, and in particular in the case of genotoxic stress that induces both repair and immune activation, remains to be elucidated.

COOPERATION BETWEEN DDR AND INNATE IMMUNITY IN TUMORIGENESIS

The interplay between innate immune activation and DNA repair pathways is likely to be central to our understanding of several human pathologies. For instance, several cancer susceptibility syndromes, such as Fanconi Anemia, that present with inheritable deficiencies in DNA repair pathways also display hematological disorders, such as bone marrow failure or auto-immunity, together with elevated type I Interferon levels. Mutations in DNA repair proteins are also found in diseases primarily defined as auto-inflammatory as described thoroughly in Ragu et al. (26). Indeed, deficient DDR frequently leads to pathologies, such as Ataxia-Telangiectasia, Werner Syndrome and Bloom Syndrome, in which inflammation plays a great part (81–83). Likewise, chronic inflammation plays an important role in all stages of sporadic cancer, from the onset of neoplastic lesions to metastatic dissemination (84).

Although STING targeting immunotherapies have seen a huge biomedical interest in recent years, the study of the

impact of STING activation on tumorigenesis has revealed an extremely complex relationship with tumor fate. In many cases STING activation has been shown to promote tumor clearance. Nucleic acid substrates for cGAS in tumors can result from the release of chromatin fragments in the cytosol of tumor cells (85), leading to cGAS-STING activation and cell cycle arrest (85–87). In addition, released self-DNA, from dying tumor cells in the tumor microenvironment can be engulfed by intra-tumoral antigen-presenting cells (APCs), such as dendritic cells and macrophages and likely activates the cGAS-STING pathway (88), through mechanisms that are still under debate (89). The resulting cGAS-STING pathway activation promotes maturation and cross-presentation (90), ultimately leading to the recruitment and the infiltration of cytotoxic CD8(+) T cells at the tumor site (91, 92). Moreover, tumor-derived cGAMP promotes immune cells infiltration (52). Importantly, the cGAS-STING pathway was shown to potentiate the response to radiotherapy and chemotherapy (88, 93) and to synergize with checkpoint inhibitors (94, 95). Thus, activating the cGAS-STING axis in combination with chemotherapy and/or immunotherapy appears as a valuable therapeutic strategy.

However, there is evidence that cGAS-STING-dependent inflammation can fuel tumorigenesis (96), promote tolerogenic responses, impair the establishment of long-term immunity (97) and lead to chemoresistance (98, 99). Indeed, transfer of cGAMP from metastatic cells to astrocytes through gap junctions was also shown to support metastatic dissemination and chemoresistance (100). Finally, accumulation of micronuclei in the cytoplasm of cancer cells following ionizing radiation promotes STING-dependent inflammation (40, 71) and metastasis (101). It has been proposed that tumor grade and origin may account for these differential outcomes following cGAS-STING stimulation, calling for stratification strategies to identify patients that would benefit from cGAS-STING targeting immunotherapies.

Moreover, present therapeutic regimens include the use of DDR inhibitors in combination or not with radiotherapy (94, 102–104). Indeed, this approach induces accumulation of inflammatory cytosolic nucleic acids, leading to cGAS-STING pathway activation (95, 105) and promoting T cell infiltration and thus tumor regression (95, 102). Significant tumor regression has also been observed using DNA-PKcs inhibitors in combination with chemotherapy or radiotherapy (69), however the role of inflammation in this process is at present unexplored. Considering the emerging role of DDR proteins in innate immune responses, it is tempting to speculate that upon genotoxic stress, DDR proteins may directly fuel cancer-related inflammatory responses. In addition, numerous tumors down regulate the expression of cGAS and/or STING (106, 107). In these contexts, it would be important to examine if DDR proteins may take over the production of inflammatory cytokines.

Furthermore, STING activation has been shown to promote two distinct transcriptional programs. On one hand, activation of genes under the control IRF-3, leads mostly to the production of type I Interferons that are generally accepted as acting anti-cancer agents (108), while NF-κB activation promotes the production of cytokines that are mostly considered pro-tumorigenic, such as IL-6 (109, 110). Indeed, increased plasma levels of IL-6 generally negatively correlate with patient survival in many cancers (110). It would be crucial to determine whether the differential outcomes of STING activation observed in studies describing STING activation as pro-tumorigenic would result from IL-6 secretion. Ultimately, it would be crucial to determine, in those contexts where alternative receptors to cGAS would potentiate STING-dependent signaling, whether they would lead to skewing of the response toward IL-6 production and promote pathological outcomes.

Reciprocally, regulation of DDR by PRRs is likely to affect tumorigenesis. HR inhibition by chromatin-bound cGAS accelerates genome destabilization and micronuclei generation, leading to cell death both *in vitro* and *in vivo* (80). Thus, cGAS may thereby restrict the propagation of cancer cells. To the contrary, alterations of cGAS shuttling toward the cytosol correlate with poor patient prognosis (79). This suggests that nuclear translocation of cGAS and subsequent HR inhibition may promote tumorigenesis (79), although this may also be linked to defective cGAS-dependent Interferon responses. Furthermore, IFI16 has also been reported to present nuclear functions (57, 111), including a role in regulation of cell cycle arrest (111, 112). Supporting an association between IFI16 and tumorigenesis, IFI16 levels are frequently decreased in breast cancer cell lines (113). Yet, there is as of today no clear implication of IFI16-dependent cytokine production in tumorigenesis. This leaves open the possibility that IFI16 may be mobilized in tumors where cGAS expression is downregulated. Thus, deciphering the molecular cues leading to the mobilization of the different pools of cGAS, or alternative receptors such as IFI16, to detect immune-stimulatory DNA - and the impact of the different PRRs in DNA damage responses - is likely primordial to the understanding of how nucleic acid detection dictates tumor fate.

DISCUSSION

Accumulation of cytosolic nucleic acids, including ssDNA, dsDNA and RNA : DNA hybrids, has been documented in several etiologically distinct human pathologies that present with pathological type I Interferon responses (114). Importantly, the range of symptoms experienced by patients is broad, and as of today not fully understood.

Much attention was brought to the cGAS-STING axis, notably because it was shown that cGAS is non-dispensable for STING activation *in vivo*. Indeed, in cells, including dendritic cells, macrophages or fibroblasts, from cGAS-deficient mice, nucleic acid-dependent STING activation was abolished (115). Yet, recent research has underlined the existence of species-specificities in innate immune detection of nucleic acids (116). Thus, although the cGAS-STING cascade represents a crucial cytosolic dsDNA detection route, a more complex picture is currently emerging. In addition to the many direct regulators of the cGAS-STING pathway, alternative receptors such as IFI16 and DNA-PK, may mediate stimulus-specific Interferon

responses. Therefore, previously overlooked nucleic acid sensors should be re-examined (117). In particular, the recently uncovered cooperation between DDR and nucleic acid immunity can be expected to contribute to the health alterations witnessed in patients presenting with chronic inflammation while feeding cancer susceptibility directly.

Importantly, in inflammatory pathologies, it is generally considered a risky approach to directly act on pathways responsible for Interferon production (118). This is intrinsically linked to the duality of the impacts of Interferons, that can either be beneficial or promote cytopathic effects, depending on multiple parameters that are as of today poorly understood. Several chronic inflammatory pathologies, presenting with type I Interferon overproduction, such as type I Interferonopathies, or Aicardi-Goutières Syndromes are treated with inhibitors of the Janus kinase 1, 2 and 3 (119). This treatment, rather that halting Interferon production, prevents the induction of ISGs following the interaction of Interferons with its cognate receptor. However, such disruption of immune pathways comes at the expense of increased risk of infection (119). Identification of pathways responsible for activation of pathological immune responses and the design of specific targeting strategies may be valuable in these pathologies. Addressing whether DDR proteins are involved in the inflammatory signature present in these diseases is thus important.

Altogether, the current state-of-the-art supports that STING is an attractive target for the treatment of autoimmune, inflammatory diseases and cancer (17, 120). However, emerging regulators, cell type specific or stimulus specific responses, together with alternative functions of STING and its activators, indicate that our understanding of nucleic acid immunity is still in its infancy. Our view of how immune-stimulatory DNAs are detected is likely grow in complexity, notably with the addition of DNA repair proteins to the list of PRRs. Therefore, the regulatory mechanisms and crosstalk between engaged pathways will surely remain an area of intense research in coming years.

AUTHOR CONTRIBUTIONS

CT, AS, IV, HC, JM, and NL drafted and edited the manuscript. Visualization: IV. All authors contributed to the article and approved the submitted version.

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Deciphering the Fine-Tuning of the Retinoic Acid-Inducible Gene-I Pathway in Teleost Fish and Beyond

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Interferons are the first lines of defense against viral pathogen invasion during the early stages of infection. Their synthesis is tightly regulated to prevent excessive immune responses and possible deleterious effects on the host organism itself. The RIG-I-like receptor signaling cascade is one of the major pathways leading to the production of interferons. This pathway amplifies danger signals and mounts an appropriate innate response but also needs to be finely regulated to allow a rapid return to immune homeostasis. Recent advances have characterized different cellular factors involved in the control of the RIG-I pathway. This has been most extensively studied in mammalian species; however, some inconsistencies remain to be resolved. The IFN system is remarkably well conserved in vertebrates and teleost fish possess all functional orthologs of mammalian RIG-I-like receptors as well as most downstream signaling molecules. Orthologs of almost all mammalian regulatory components described to date exist in teleost fish, such as the widely used zebrafish, making fish attractive and powerful models to study in detail the regulation and evolution of the RIG-I pathway.

Keywords: interferon, RIG-I-like receptors (RLRs), innate response, immune homeostasis, fish

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INTRODUCTION

The antiviral innate immune response in vertebrates is mediated by type I interferon (IFN) and its actions as an autocrine signal for the infected cell and as a paracrine "early warning" signal to neighboring cells (1, 2). This host response against virus infection is characterized by the induction of a rapid non-specific antiviral state that blocks virus replication and spread. The IFN system is remarkably well conserved in vertebrates which highlights its critical importance (3). Teleost fish possess functional orthologs of pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) detect pathogens in the extracellular or the endosomal compartments, while retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), and cytoplasmic DNA sensors serve as intracellular PRRs. These sensors are able to detect distinct viral molecular patterns, such as nucleic acids or viral proteins, collectively known as pathogen-associated molecular patterns (PAMPs). They synergistically trigger the activation of multiple signaling cascades that induce the production of IFN and other cytokines, thereby establishing an antiviral state and shaping an appropriate adaptive immune response. Among the PRRs, RLRs play a key role in sensing viral RNA in the cytosol and are essential in the

early induction of IFN (4, 5). The ability of IFNs to restrict virus replication in mammals is largely mediated through the induction of hundreds of interferon-stimulated genes (ISGs), collectively referred as the "interferome" (6). Similarly, up-regulation of ISGs by IFNs in lower vertebrates has been extensively reported. Several studies point to the maintenance of a stable set of core ISGs during evolution (7) and their key functions for fish defense against viruses (3, 8).

RIG-I-LIKE RECEPTORS: FROM RNA SENSING TO IFN INDUCTION

The sensing of non-self-cytosolic RNA is mediated by RLRs which include RIG-I (DDX58) (9), melanoma differentiation-associated gene 5 (MDA5/IFIH1) (10–12), and laboratory of genetics and physiology 2 (LGP2/DHX58) (13, 14). Notably, RIG-I detects viral replication not only in the cytoplasm, but also in the nuclear compartment (15). In mammals, it is now recognized that most if not all viral infections from RNA and DNA viruses can be recognized by RLRs. RIG-I and MDA5 are DExD/H box RNA helicases comprising three domains; two N-terminal caspase recruitment domains (CARDs) in tandem involved in signal transduction, a central helicase domain and a C-terminal domain (CTD) critical for RNA recognition and autoinhibition of CARDs (16). LGP2 contains a helicase domain but lacks CARDs and thus a signal-transducing activity. LGP2 is a regulator with distinct effects on RIG-I and MDA5. While LGP2 clearly upregulates the signaling activity of MDA5, its action on RIG-I-mediated antiviral signaling remains unclear (13, 17, 18). In fact, LGP2 deficiency has different effects depending on the nature of the viral infection (19, 20). Nevertheless, LGP2 can associate with the C-terminus of TNF receptor associated factors (TRAFs) and can regulate TRAF activity downstream of RIG-I and MDA5, indicating that LGP2 can suppress both MDA5-dependent and RIG-I-dependent signal transduction (21). RLRs are remarkably well conserved in vertebrates and teleost fish possess functional orthologs of human RLRs, including RIG-I, MDA5, and LGP2 (4, 22) as well as several downstream molecules (**Figure 1A** and **Table 1**). Although identified in many fish species belonging to Cypriniformes (e.g. carp and zebrafish), Siluriformes (e.g. channel catfish) and Salmoniformes (e.g. salmon and trout), RIG-I has not been reported in certain fish of the superclass Acanthopterygii (e.g. medaka, tetraodon, pufferfish, stickleback, sea bream and sea bass). It is still unclear whether the RIG-I gene has been lost in some fish species as it has been reported for chicken (105) and Chinese tree shrew (106).

RIG-I and MDA5 recognize specific RNA features that are not typically found in most cellular RNAs in the cytoplasm of vertebrate cells (107). RIG-I binds preferentially, but not exclusively, to ssRNAs phosphorylated at the 5' end, whereas MDA5 recognizes long dsRNAs. This difference in ligand preference results in specificity for the recognition of distinct virus species. In the resting state, CARDs are sequestered, while upon binding of RNA to CTD and helicase domains, CARDs are released by a conformational change of the molecule. Exposed CARDs interact with the CARD of the mitochondrial activator of virus signaling (MAVS) protein (IPS-1, VISA or Cardif) (29–32). MAVS is an integral protein of the mitochondrial outer membrane that associates with the mitochondrial membrane *via* its C-terminal domain and acts as a key determinant of the antiviral signaling cascade. Fish MAVS contains similar domains as those found in mammals, with a N-terminal CARD domain and a C-terminal transmembrane (TM) region, both of which are essential for its antiviral function, as well as a central proline-rich region containing TNF receptor-associated factor (TRAF)-binding motifs (4, 25). The interaction between RLRs and MAVS induces the recruitment of adaptor proteins, such as TRAF3 or TRAF6, and the activation by phosphorylation of serine/threonine-protein kinases, TANK-binding kinase 1 (TBK1) and inhibitor-κB kinases. Consequently, IRF3/IRF7 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors are activated, translocate from the cytosol to the nucleus and induce the expression of IFNs and inflammatory cytokines.

REGULATION OF RIG-I-LIKE RECEPTORS

Under homeostatic conditions, IFNs are expressed at very low and often undetectable levels. Given the critical role of the RIG-I-mediated IFN induction pathway, a tight regulation is essential to maintain the immune homeostatic balance and to ensure proper termination of the antiviral response in order to avoid extensive tissue damage, chronic inflammation, and autoimmune diseases. Moreover, since most RIG-I pathway components are ISGs and that their overexpression leads to constitutive IFN production, it is clear that cells must regulate them not only at the transcriptional level, but also at post-transcriptional and post-translational levels. These distinct regulatory mechanisms act on each sensor and downstream molecule to control antiviral signaling. Regulation at the post-transcriptional level includes alternative pre-mRNA splicing leading to functionally distinct proteins (108), long non-coding RNAs (lncRNAs), and micro RNAs (miRNAs) that both serve as important regulators of RLR signal transduction (5). Some lncRNAs have even been shown to directly bind to RLRs. Post-translational modifications (PTMs) involve the covalent linkage of new functional groups to amino acid residues which in turn fine tune protein properties by regulating protein folding, stability, location, and interaction with other molecules. Several regulatory mechanisms mediated by PTMs have been described (109). Among them, phosphorylation and ubiquitination are the best characterized. Other PTMs such as ISGylation (conjugation with the IFN-inducible ubiquitin-like protein ISG15), SUMOylation, methylation, acetylation and deamidation have also been reported to control the RIG-I pathway. In addition, several RLR-binding proteins have been identified as important modulators of RLRs for RNA binding (acting as co-receptors), oligomerization, ubiquitination or affecting subcellular localization (5). In addition, spatiotemporal dynamics of MAVS in mitochondria, in mitochondrial-associated endoplasmic reticulum membranes (MAMs), and in

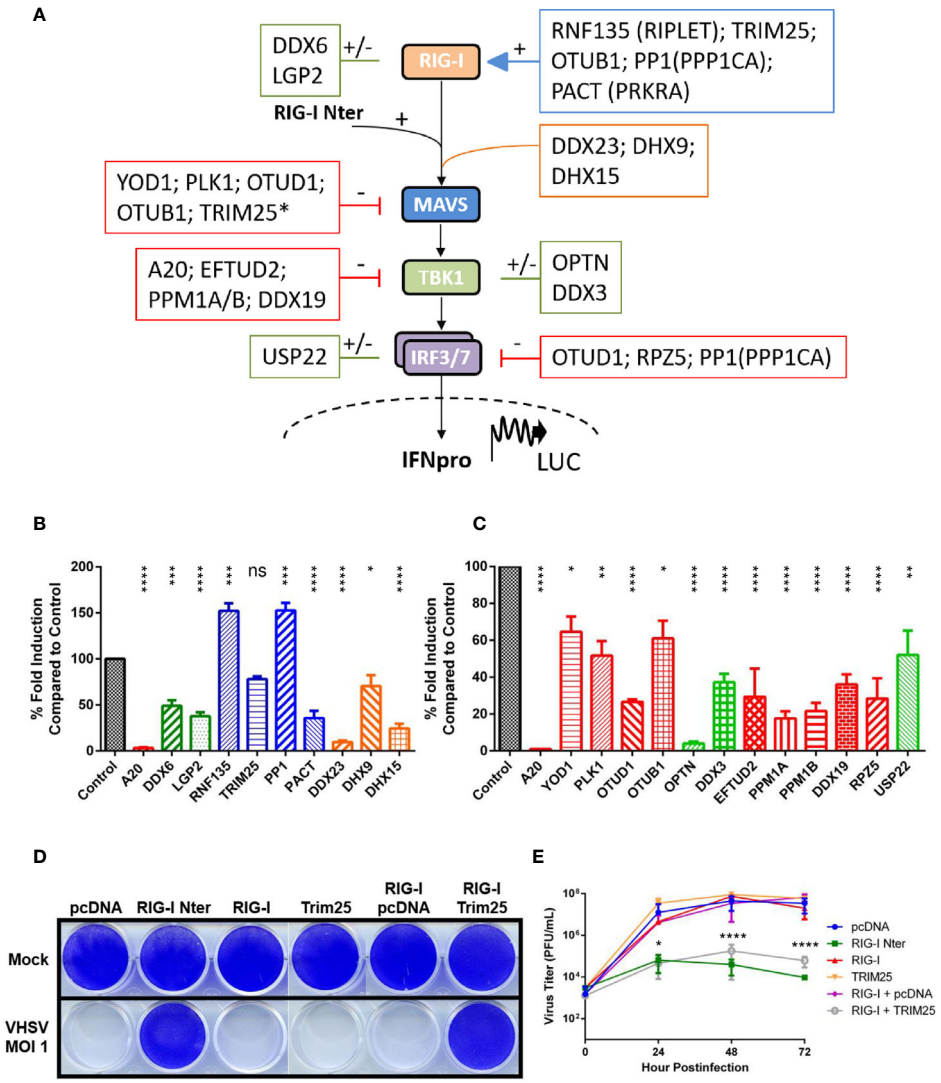


FIGURE 1 | Regulation of RIG-I-mediated signal transduction by a conserved set of cellular proteins in vertebrates. **(A)** Schematic representation of RIG-I pathway including the main downstream components for signal transduction leading to promoter activation and expression of type-I interferon. A set of cellular regulators that are evolutionarily conserved between fish and mammalian species are placed next to their targets. The effect of each regulator is symbolized by (–) for inhibition (in red), by (+) for activation (in blue), or by (+/-) for ambivalent (in green) reported functions on the RIG-I pathway, based on the literature (mainly from studies with mammalian orthologs; see **Table 1**). Other cytosolic sensors or co-receptors involved in the RIG-I pathway are boxed in orange. *Although TRIM25 promotes the degradation of MAVS, this step is required for IRF3 phosphorylation by TBK1 (23). **(B, C)** Fathead minnow orthologs of mammalian regulators were amplified from total RNA extracted from EPC cells, cloned into the eukaryotic expression vectors pcDNA1.1/Amp (Invitrogen) and fully sequenced. Nucleotide sequences of each regulator were deposited in GenBank (see **Table 1** for accession numbers). To test their effect on the RIG-I pathway, EPC cells were transfected with the indicated plasmids (1 μg) together with a luciferase reporter construct driven by the promoter of IFN1 derived from EPC cells (1 μg) and the RIG-I Nter-eGFP inducer and internal transfection control construct 0.5 μg in **(B)** or 1 μg in **(C)**, as previously described (24). Twenty-four hours after transfection, the cells were lysed for luciferase assays. Luciferase activity was measured and normalized to eGFP fluorescence. No significant variation in eGFP expression was observed between each condition. The percentage of fold-induction were calculated as the ratio of stimulated (+ RIG-I Nter) versus unstimulated (– RIG-I Nter) conditions and compared to the induction control (RIG-I Nter + empty vector). Means of at least three independent experiments are shown together with the standard errors. The color coding used for the histograms is the same as the one used in panel **(A)** For statistical analysis, a comparison between groups was performed with a one-way ANOVA and Tukey's multiple comparison tests using GraphPad Prism (GraphPad, San Diego, CA). Groups that are not significantly different from each other are denoted ns ($P > 0.05$), whereas those that are significantly different are denoted *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) or ****($P < 0.0001$). **(D, E)** EPC cells were transfected with the indicated plasmids (2 μg each) or an empty vector (pcDNA1.1/Amp) as a control, as previously described (25). All transfection mixtures were adjusted with an empty vector to contain an equal amount of plasmid DNA. Twenty-four hours after transfection, cells were infected with a fish novirhabdovirus, viral hemorrhagic septicemia virus (VHSV) at an MOI of 1 and incubated at 15°C. Cell monolayers were stained with crystal violet 3 days postinfection **(D)**. The culture supernatants from infected cells were collected at different times postinfection and the viral titer was determined by plaque assay **(E)**. Each time point is represented by two independent experiments, and each virus titration was performed in duplicate. Average values are shown. The standard errors were calculated and the error bars are shown. Asterisks indicate significant difference (*$p < 0.05$; ****$p < 0.0001$) as determined by two-way ANOVA and Tukey's multiple comparison tests. ns, not significant.

TABLE 1 | *Pimephales promelas* RIG-I pathway components and orthologs.

Full Name	Symbol (Synonym)	Target/ Partner	GenBank #	*Homo sapiens* GeneID	*Danio rerio* GeneID	Selected References
RIG-I Pathway Components of *Pimephales promelas*						
DExD/H-box helicase 58	DDX58 (RIG-I)	MAVS	FN394062	23586	100333797	(9, 25, 26)
DDX58 CARD domains	RIG-I Nter	MAVS	FN178456			
Interferon induced with helicase C domain 1	IFIH1 (MDA5)	MAVS	MG799354	64135	565759	(10–12, 27, 28)
Mitochondrial antiviral signaling protein	MAVS (IPS1, CARDIF, VISA)	TRAF3 TBK1	FN178455	57506	562867	(25, 29–33)
TANK binding kinase 1	TBK1	IRF3/7	LT174673	29110	692289	(24, 34–38)
Interferon regulatory factor 3	IRF3	IFN promoter	**MN781134**	3661	564854	(39–44)
Interferon regulatory factor 7	IRF7	IFN promoter	**MN781135**	3665	393650	(34, 45–47)
Interferon 1 promoter region	IFN1 promoter	_	HE856618	IFNα/β promoter	DQ855952[1]	(48–50)
Interferon 1	IFN1	IFN receptor	FN178457	IFNα/β	360134	(2, 25, 51, 52)
RIG-I Pathway Regulators/Co-receptors/Receptors of *Pimephales promelas*						
DExH-box helicase 58	DHX58 (LGP2)	RIG-I	**MW591879**	79132	100148871	(13, 17, 19, 21, 27, 49, 53–55)
DEAD-box helicase 6	DDX6	RIG-I	**MW591868**	1656	564633	(56, 57)
Ring finger protein 135	RNF135 (RIPLET)	RIG-I	**MW591864**	84282	101882927	(58–63)
Tripartite motif containing 25	TRIM25	RIG-I	LT174676	7706	393144	(23, 64–66)
OTU deubiquitinase, ubiquitin aldehyde binding 1	OTUB1	RIG-I TRAF3	**MW591878**	55611	436684	(67–69)
Protein phosphatase 1 catalytic subunit alpha	PPP1CA (PP1A)	RIG-I IRF3/7	**MW591866**	5499	407980	(70, 71)
Protein activator of interferon induced protein kinase A	PRKRA (PACT)	RIG-I	**MW591865**	8575	557370	(18, 72)
DEAD-box helicase 23	DDX23	MAVS	**MW591869**	9416	334283	(73)
DExH-box helicase 9	DHX9	MAVS	**MW591876**	1660	568043	(74–76)
DEAH-box helicase 15	DHX15	MAVS	**MW591867**	1665	321931	(77–79)
YOD1 deubiquitinase	YOD1 (OTUD2)	MAVS	**MW591873**	55432	550411	(80)
Polo like kinase 1	PLK1	MAVS	**MW591874**[2]	5347	280649	(81)
OTU deubiquitinase 1	OTUD1	MAVS IRF3	**MW591870**	220213	100537398	(82, 83)
DEAD-box helicase 3	DDX3	MAVS TBK1	LT174679	1654	566947	(24, 75, 84–88)
TNF alpha induced protein 3	TNFAIP3 (A20)	TBK1	LT984694	7128	564497	(89–91)
Elongation factor Tu GTP binding domain containing 2	EFTUD2	TBK1	LT174678	9343	393480	(24, 92)
Protein phosphatase, Mg2+/Mn2+ dependent 1A	PPM1A	TBK1	LT174675	5494	30704	(24, 93–95)
Protein phosphatase, Mg2+/Mn2+ dependent 1B	PPM1B	TBK1	LT174674	5495	100003481	
DEAD-box helicase 19	DDX19	TBK1	**MW591875**	55308	192339	(96)
Optineurin	OPTN	TBK1	**MW591877**	10133	336159	(97–101)
Rapunzel 5	RPZ5	IRF7	**MW591871**	No ortholog	100003142	(24, 102)
Ubiquitin specific peptidase 22	USP22	IRF3	**MW591872**	23326	692275	(103, 104)

[1] *GenBank accession number;* [2] *from Danio rerio; new sequences deposited in the GenBank database are in bold.*

peroxisomes regulates RLR-mediated signaling (110). Therefore, the integrity of these subcellular compartments together with their own regulation indirectly act on RLR function (111, 112). A few examples of such mechanisms have been described in fish cells, mainly miRNA-mediated regulation and alternative splicing isoforms of RLR components (113, 114).

In order to explore the degree of conservation of these regulatory mechanisms among vertebrates, we cloned and fully sequenced 22 genes of fathead minnow (*Pimephales promelas*) encoding orthologs of human proteins described as important regulators of the RIG-I pathway (**Table 1**). Fathead minnow is a relevant fish species for at least two reasons: 1) EPC cells (*Epithelioma Papulosum Cyprini*; ATCC CRL-2872), the most widely used fish cell line in virology, is derived from this fish species (115), and 2) fathead minnow belongs to the family Cyprinidae together with the zebrafish (*Danio rerio*), an animal model offering great potential for the study of human and fish viral diseases and the development of antiviral drugs (116–118). This list of 22 fish orthologs is far from exhaustive and only represents a small fraction of proteins described as modulating RIG-I-mediated IFN expression (109, 119). Nevertheless, these orthologs are of importance because they correspond to human proteins acting on the RLR pathway *via* three key modes of action: phosphorylation, ubiquitination, and RLR-binding.

REGULATION BY KINASES AND PHOSPHATASES

Phosphorylation is a reversible PTM of proteins in which serine, threonine or tyrosine residues are modified by a kinase by the addition of a covalently bonded phosphate group (109). Phosphorylation results in a structural conformation change of a protein, often modifying its function to become activated or deactivated. The reverse reaction of phosphorylation is called dephosphorylation, and is catalyzed by phosphatases. Phosphorylation regulates almost all components of the RLR pathway. In resting cells, RIG-I is negatively regulated by phosphorylation by several kinases keeping RIG-I in a non-activated state. When viral RNAs are detected, the CARDs of RIG-I are rapidly dephosphorylated by protein phosphatase 1 (PP1A), thus activating the sensor (70). However, PP1A is also able to dephosphorylate IRF3/7 leading to an inhibition of RIG-I-mediated signal transduction at a downstream level (71). Fish PP1A is highly conserved and share 90% sequence identity at the amino acid (aa) level with its human ortholog. To determine fish PP1A action on RIG-I-mediated IFN expression, we tested its ectopic overexpression in a cell-based luciferase reporter system (**Figure 1B**). As previously published (48), the expression of a constitutively active form of RIG-I (RIG-I Nter; in which the C-terminal repressor domain maintaining the protein in an inactive state is deleted) significantly activates the IFN1 promoter of EPC cells. As a control, the co-expression RIG-I Nter with A20, a negative feedback regulator of the RLR signaling (89), drastically reduced the induction. In contrast, co-expression of PP1A with RIG-I Nter significantly increase IFN1 promoter activation, indicating that fish PP1A share a common function with its mammalian orthologs by enhancing RIG-I activity. MAVS activation is also regulated by phosphorylation (120). Polo-like kinase 1 (PLK1) has been reported to negatively regulate MAVS (81). PLK1 does not directly phosphorylate MAVS but, rather, requires phosphorylation of MAVS for docking and disrupting the MAVS–TRAF3 interaction. Fish PLK1 is well conserved (71% aa sequence identity) and also exerts a negative regulatory role on MAVS (**Figure 1C**). The last example is TBK1. As a critical kinase involved in IFN expression, the activity of TBK1 must be tightly regulated. Because TBK1 activation occurs by trans-autophosphorylation, phosphatases play a critical role in the control of TBK1 activity. Two Ser/Thr protein phosphatases, PPM1A and PPM1B, have been reported to target TBK1 and MAVS for dephosphorylation and to down regulate signaling mediated by cytosolic nucleotide sensing in fish and mammalian species (**Figure 1C**) (24, 93–95).

REGULATION BY UBIQUITIN LIGASES AND DEUBIQUITINASES

Ubiquitination is the covalent and reversible addition of ubiquitin to lysine residues on a protein substrate (121). Ubiquitin is itself an 8.5 kDa protein composed of 76 amino acids. Ubiquitination is catalyzed by three distinct classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), on which lies most of the substrate specificity. Lysine residues can be modified with a single ubiquitin (monoubiquitination) or chains of ubiquitin (polyubiquitination). Different types of ubiquitin chains are thus generated based on the seven lysine residues present on ubiquitin. Among them, K48-linked ubiquitin chains target protein for proteasome degradation while K63-linked ubiquitin chains mediate protein-protein interactions. Ubiquitination is a reversible and dynamic event, since the conjugated ubiquitin chains can be cleaved by a family of ubiquitin-specific proteases, termed deubiquitinases (DUBs). More than 600 and 100 genes encoding putative E3 ligases and DUBs, respectively, have been annotated in the human genome, indicating the ubiquitous importance and specificity of these PTMs in the control of cellular processes. In the RLR pathway, most of the sensors, adaptor proteins, and kinases are ubiquitinated to efficiently activate or repress IFN production.

RIG-I is finely regulated by ubiquitination which is critical for its activation and degradation. Tripartite motif containing 25 (TRIM25) was the first identified enzyme to catalyze the conjugation of K63-linked ubiquitin chains to RIG-I CARDs (64, 65). Ring finger protein 135 (RNF135/RIPLET), another ubiquitin ligase, is also involved in K63-linked polyubiquitination at multiple sites in CARDs and CTD leading to the activation of RIG-I (58–60). Whether RNF135 promotes TRIM25 binding on RIG-I in a sequential ubiquitination process or RNF135 by itself, without involvement of TRIM25, is essential for RIG-I activation is still unclear (61, 62). However, TRIM25 is also capable of promoting K48-linked ubiquitination and degradation of MAVS. The proteasomal degradation of MAVS is required to release the signaling complex into the cytosol, allowing IRF3 phosphorylation by TBK1 (23). Zebrafish orthologs of TRIM25 and RIPLET have also been reported as positive regulators of RIG-I (63, 66). **Figure 1B** shows that fish RNF135 has an enhancing effect on the activity of RIG-I CARDs, whereas TRIM25 has no effect. Nevertheless, TRIM25 co-expression with full-length RIG-I is required to protect EPC cells against a viral infection and to inhibit viral production (**Figures 1D, E**), highlighting that fish RNF135 and TRIM25 are both positive regulators of the RLR pathway.

Several DUBs of ovarian tumor proteases (OTUs) and ubiquitin-specific proteases (USPs) families, have been described as important regulators of RLR pathway. Among them, mammalian and fish A20 has been shown to be a strong inhibitor of the RLR signaling (**Figures 1C, D**) (89–91). In addition, the function of OTUB1, OTUD1, YOD1, and USP22 fish orthologs was investigated (**Table 1**). They all have significant inhibitory effects on signal transduction by RIG-I CARDs (**Figure 1C**). Fish OTUD1 has the strongest effect, likely by mediating the targeted degradation of the MAVS/TRAF3/TRAF6 signalosome as well as by reducing the DNA binding capacity of IRF3, as described in mammals (82, 83). YOD1, which

acts at a later step along the pathway to abrogate the formation of prion-like aggregates of MAVS (80), has a limited effect on IFN promotor induction at an early time point post-stimulation. In contrast, mammalian OTUB1 and USP22 were reported with opposite regulatory effects on the RLR pathway (67–69, 103, 104). The inhibitory effect observed after ectopic expression of the fish orthologs may be a result of the inherent bias associated with the overexpression of enzymatically-active protein, mislocalization and inadequate cell type and does not allow to distinguish the opposite functions previously described in mammals.

REGULATION BY RLR-BINDING PROTEINS

The RLR pathway is regulated by multiple host factors. Protein activators of PKR (PACT, also known as protein activator of interferon induced protein kinase A) binds to RIG-I CTD and enhances RIG-I signaling in part by stimulating RIG-I ATPase and helicase activities (72). Moreover, recent studies have indicated that the PACT-LGP2 interaction was necessary to regulate the responses mediated by RIG-I and MDA5 (18, 122). As for mammals, the role of fish LGP2 in RLR signaling is unclear. It appears that depending on the nature of the splicing isoform, LGP2 can have a negative or a positive effect on the RIG-I pathway (27, 49, 53–55). The *dhx58* cDNA amplified from EPC cells encodes LGP2 protein which exerts a strong inhibition on signaling mediated by RIG-I CARDs (**Figure 1B**). Moreover, a similar inhibition is observed during expression of RIG-I CARDs together with PACT (**Figure 1B**). This is in contrast with PACT's enhancing function observed in mammals. However, fish PACT only shares 44% aa sequence identity with human PACT. Another dsRNA-binding protein, TAR-RNA-binding protein (TRBP), which shares 39% protein sequence identity with PACT with a similar structure, has recently been reported as an inhibitor of RIG-I signaling (123). Because fish PACT still retains some degree of relatedness to both human proteins, PACT and TRBP, it cannot be excluded that PACT acts as a negative regulator of RIG-I in fish.

The involvement of multiple RNA helicases in RLR signaling has been demonstrated, as recently reviewed by Taschuk and Cherry (124). For instance, DDX6, DHX9, DDX3, and DHX15 can function as co-sensors of RIG-I or as RLR-independent sensors of nucleic acids through interaction with MAVS (56, 74, 77–79, 84–86). DHX9, DHX15, and DDX23 have been recently described as cytoplasmic viral RNA sensors in the lancelet (amphioxus) (73). However, limitations or contradictions have been reported concerning their role in IFN and ISGs production. DDX6 is also described as a suppressor of ISGs (57). DHX9 is an important viral dsRNA sensor only in myeloid dendritic cells (74). DHX15 contributes to the activation of NF-κB but not IRF3 in response to RNA virus infection (78). DDX3, for which multiple roles as a pro- or antiviral factor were identified (84), has recently been described as an inhibitor of IFN production during arenavirus infection (87). Fish orthologs are highly conserved and share at least 69% aa sequence identity with human proteins. Fish DDX3 and DHX9 bind dsRNA (75) and DHX9 is a potential sensor for DNA virus infection *in vivo* (76). Fish DDX3 is a binding partner for the nonvirion (NV) proteins of two fish novirhabdoviruses, suggesting that DDX3 plays an important role in either enhancing innate immunity or promoting virus replication (24). Moreover, the overexpression of fish DDX3 alone seems to induce the IFN promoter (88). In our cell-based reporter system, a negative effect on RIG-I CARDs-mediated signaling was observed for DDX6, DHX9, DDX3, DHX15, and DDX23 (**Figures 1B, C**), probably through a competition for MAVS adaptor or another mechanism yet to be further investigated. In any case, these RNA helicases are potentially involved in the innate immune system of vertebrates. Finally, another RNA helicase, DDX19, has been shown as a negative regulator of IFN production (96). Mechanistically, DDX19 does not sense viral RNA but inhibits the phosphorylation of IRF3 by TBK1. DDX19 is highly conserved between fish and mammals (sharing 86% aa sequence identity) and share the same inhibitory effect on the RLR pathway (**Figure 1C**).

The optineurin (OPTN) is another regulator of the RLR pathway but its action is controversial. Although OPTN was initially reported to negatively regulate IFN induction (97), other studies indicated that OPTN was necessary for optimal TBK1 and IRF3 activation (98, 101). However, recent studies pointed out a crucial role for OPTN in dampening the IFN response (99, 125). Moreover, chicken OPTN has been reported as an inhibitor of MDA5-mediated IFN production (100). As shown in the **Figure 1C**, fish OPTN has also an important inhibitory effect on RIG-I-mediated induction of the IFN promoter.

The function of the NV proteins of two novirhabdoviruses in the inhibition of the host immune response has been described using an interactome proteomics approach (24). Among the cellular partners of NV, PPM1B was shown to be specifically recruited to terminate RIG-I-mediated IFN induction. In addition to DDX3, two other proteins were identified to be likely involved in the RLR pathway: the elongation factor Tu GTP binding domain containing 2 (EFTUD2) and the rapunzel 5 protein (RPZ5). EFTUD2 was discovered to restrict infection by hepatitis C virus (HCV) through IFN-independent stimulation of the innate immune response (92). EFTUD2 upregulates RIG-I expression by pre-mRNA splicing. Fish EFTUD2 is highly conserved with its mammalian counterpart (89% aa sequence identity) but its overexpression does not protect fish cells against rhabdovirus infection in contrast to its human ortholog that protects human cells against HCV (data not shown). Surprisingly, overexpression of EFTUD2 has a negative effect on RIG-I-mediated IFN expression in fish cells (**Figure 1C**), a finding that requires further investigation. Unlike most of the factors described above, RPZ5 has no mammalian or bird orthologs. Zebrafish RPZ5 has recently been implicated in blocking RLR-mediated IFN induction by mediating the degradation of phosphorylated IRF7 (102). In **Figure 1C**, we confirm the inhibitory effect of fish RPZ5 on the RIG-I pathway and its uniqueness among teleost fish.

CONCLUSIONS AND PERSPECTIVES

The IFN system is remarkably well conserved in vertebrates and it is remarkable that teleost fish possess most post-transcriptional and post-translational regulatory mechanisms of the RLR signaling pathway as described in mammals. Thus, these multi-level regulatory mechanisms were selected very early on and maintained throughout the evolution of vertebrates indicating their crucial role in the control of immune homeostasis for these organisms. Although numerous regulators have been reported in mammals, underlying the complexity and the relative redundancy of these mechanisms, their distinctive roles and functional differences depending on the cell type considered (*e.g.* immune versus epithelial cells), their own regulation, and their sequential chronology required to orchestrate the RLR signaling remain elusive, and in some cases, opposite functions have been reported for a same effector. In teleost fish, characterization of the components of the RLR pathway and factors involved in its fine tuning has begun but the overall picture is still poorly understood and is mainly modeled on the knowledge acquired from studies based on mammalian systems. The experimental approaches to study the innate immune system in fish has long been based on the overexpression in cell lines of identified genes with the known benefits and limitations of a such screening method. However, with the adaptation of the CRISPR/Cas9 genome editing for fish cells (126, 127), gene knock out studies will be greatly improved compared to the low efficiency and biases observed with RNA silencing (128). Moreover, the *in vivo* relevance of these factors in antiviral immunity still needs to be addressed since their description was exclusively done *in vitro* in non-immune cells. Since many decades, zebrafish is an important animal model in biomedical research due to multiple advantages including low maintenance cost, high fecundity, short generation time, small size, optical transparency of embryos, and a relatively high degree of conservation with human genes (**Table 1**) (129, 130). Together with the large available collection of transgenic lines and the relative ease to silence or overexpress specific genes, these advantages make zebrafish a model of choice for studying the spatio-temporal regulatory mechanisms of the RLR pathway. An improved understanding of the precise mechanisms of regulation in different viral and animal species and cell types will enable the development of novel therapeutic strategies against infectious diseases, immunological disorders, and cancer.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MN781134 MN781135 MW591879 MW591868 MW591864 MW591878 MW591866 MW591865 MW591869 MW591876 MW591867 MW591873 MW591874 MW591870 MW591875 MW591877 MW591871 MW591872.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The cGAS-STING Pathway: Novel Perspectives in Liver Diseases

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Liver diseases represent a major global health burden accounting for approximately 2 million deaths per year worldwide. The liver functions as a primary immune organ that is largely enriched with various innate immune cells, including macrophages, dendritic cells, neutrophils, NK cells, and NKT cells. Activation of these cells orchestrates the innate immune response and initiates liver inflammation in response to the danger signal from pathogens or injured cells and tissues. The cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway is a crucial signaling cascade of the innate immune system activated by cytosol DNA. Recognizing DNA as an immune-stimulatory molecule is an evolutionarily preserved mechanism in initiating rapid innate immune responses against microbial pathogens. The cGAS is a cytosolic DNA sensor eliciting robust immunity *via* the production of cyclic GMP-AMPs that bind and activate STING. Although the cGAS-STING pathway has been previously considered to have essential roles in innate immunity and host defense, recent advances have extended the role of the cGAS-STING pathway to liver diseases. Emerging evidence indicates that overactivation of cGAS-STING may contribute to the development of liver disorders, implying that the cGAS-STING pathway is a promising therapeutic target. Here, we review and discuss the role of the cGAS-STING DNA-sensing signaling pathway in a variety of liver diseases, including viral hepatitis, nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), primary hepatocellular cancer (HCC), and hepatic ischemia-reperfusion injury (IRI), with highlights on currently available therapeutic options.

Keywords: DNA sensor, cyclic GMPAMP synthase, stimulator of interferon genes, Innate immunity, inflammation, liver diseases

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Abbreviations: ALD, alcoholic liver disease; cGAMP-AMP, cyclic guanosine monophosphate-adenosine monophosphate; cGAS, cyclic GMP-AMP synthase; DAMPs, damage-associated molecular patterns; dsDNA, double-stranded DNA; ER, endoplasmic reticulum; HCC, hepatocellular cancer; IFNs, type I interferons; IRF3, IFN regulatory factor 3; IRI, ischemia/reperfusion injury; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAMPs, pathogen-associated molecular patterns; RIG-1, retinoic acid-inducible gene I; ROS – reactive oxygen species; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; IKK, IκB kinase; NF-κB, nuclear factor-κB.

INTRODUCTION

Liver disease presents a globally recognized health threat with a mortality rate of 2 million deaths per year worldwide (1). It often occurs in response to hepatocyte injury caused mainly by the hepatitis B and C virus, alcohol abuse, bile duct damage, nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH) (2–4). Hepatic inflammation is a critical player in triggering liver diseases. During the initial event of hepatic inflammation, innate immune cells, such as macrophages, neutrophils, natural killer (NK) cells, and NKT cells recognize cell damage or invading pathogens with intracellular-expressed pattern recognition receptors (PRRs) at the cell surface. PRRs detect distinct evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), and trigger innate inflammatory responses by activating a multitude of intracellular signaling pathways (5). Indeed, the innate immune system depends on PRRs, including the cyclic GMP-AMP (cGAMP) synthase (cGAS) and its downstream effector stimulator of interferon genes (STING), inflammasomes, and Toll-like receptors (TLRs) that recognize PAMPs and coordinate antimicrobial defense (6–9). PRRs also recognize a plethora of damage-associated molecular patterns (DAMPs), such as nucleic acids of uncontrolled death of host cells, to further activate the innate immune system, contributing to inflammatory diseases and cancer (10, 11). Therefore, aberrant nucleic acid recognition has emerged as a critical host defense mechanism mediated by cytosolic nucleic acid sensors.

DNA generally resides within the nucleus and mitochondria of eukaryotic cells. Aberrant presence of DNA in the cytoplasm from cellular damage or infection elicits robust immunity leading to activation of type I interferon-stimulated genes (ISGs) that confer increased susceptibility to the pathogens and promote host survival (12). The most robust response following DNA stimulation is initiated by cyclic GMP-AMP (cGAMP) synthase (cGAS), which is activated upon binding to double-stranded DNA (dsDNA) (13). cGAS is a critical cytosolic DNA sensor that catalyzes the synthesis of cGAMP from ATP and GTP and activates type I interferons (IFNs) through the endoplasmic reticulum (ER)-resident adaptor protein STING (13, 14), which subsequently activates the transcription factors NF-kB and IFN regulatory factor (IRF) 3 *via* the TANK-binding kinase 1 (TBK1) (13, 14). Besides, the binding of cGAS to DNA is irrespective of DNA sequence (15). Thus, self-DNA from the mitochondria or nucleus could also act as the cGAS ligand to activate the cGAS-STING pathway in triggering inflammatory responses (16). Recent studies suggested that endogenous cGAS was tightly tethered in the nucleus and prevented its autoreactivity against self-DNA (17–19). The structural basis for inhibiting cGAS by chromatin was verified *via* cryo-electron microscopy by other studies (20, 21). Moreover, cGAS was reported to inhibit homologous recombination-mediated DNA repair and promote genome destabilization, micronucleus generation, and cell death under conditions of genomic stress *via* a STING-independent manner (18). These findings indicate that activation of the cGAS-STING pathway by exogenous or endogenous DNA may contribute to the development of various human diseases. Here, we provide an overview of the cGAS-STING pathway in immunity. Moreover, we summarize and discuss the role of the cGAS-STING DNA pathway in a variety of liver diseases. Finally, we highlight current or prospective therapeutic strategies targeting the pathway.

ACTIVATION OF THE cGAS-STING PATHWAY

DNA is a crucial DAMP that is recognized by innate immune receptors and triggers intracellular signaling cascades (22). dsDNA is primed by damaged mitochondria, dying cells, DNA damage, genomic instability, bacteria, DNA viruses, and retroviruses (12, 23, 24). DNA viruses can induce type I interferon production through activation of the STING pathway (25). Emerging evidence demonstrated that cGAS was required to trigger innate immune response during HIV and other retrovirus infections (26). The cGAS consists of a critical catalytic domain, C-terminal nucleotidyltransferase (NTase) domain, which is composed of two structural lobes with the active site (7). dsDNA activates cGAS by forming 2:2 cGAS-dsDNA complexes (27, 28). The stabilized structure modulates the catalytic domain's rearrangement to transform GTP and ATP to cGAMP through induction of a conformational change in the C-terminal domain (13, 27, 28). cGAMP is an endogenous second messenger with a high affinity for STING (29). The binding of cGAMP to STING promotes STING translocation to the Golgi apparatus and activates TBK1, which phosphorylates STING and IRF3 transcription factor (13). The activated IRF3 enters the nucleus and triggers the production of type I IFNs, leading to the expression of IFN-stimulated genes (7, 30). STING can also recruit IκB kinase (IKK), which in turn catalyzes the phosphorylation of the nuclear factor-κB (NF-κB) inhibitor IκBα. IκBα phosphorylation accelerates nucleus translocation of NF-κB to promote transcription of target inflammatory cytokines (16). In addition, the N-terminal domain is also responsible for the maintenance of the liquid phase dsDNA and cGAS (31, 32). DNA binding to cGAS promotes the formation of liquid-like droplets, which facilitates cGAS activation *via* augmented cGAS liquid phase separation and enzyme activity (31). These findings demonstrate the multivalent interactions between DNA and the binding domain of cGAS in activating innate immune signaling (**Figure 1**).

THE cGAS-STING PATHWAY IN VIRAL HEPATITIS

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections remains a major public health problem in the 21st century with over 300 million people worldwide affected, despite the implementation of various therapeutics (33, 34). HBV is an enveloped partially double-stranded DNA virus (35). HBV infection of human hepatocytes leads to acute and chronic hepatitis, which remarkably increases the risk of liver cirrhosis and hepatocellular carcinoma (HCC) (33, 36). The role of innate immune response in the HBV natural infection process remains unclear and controversial (37, 38). Accumulating data suggested that HBV can escape from recognition by the innate system (39–42). Lacking

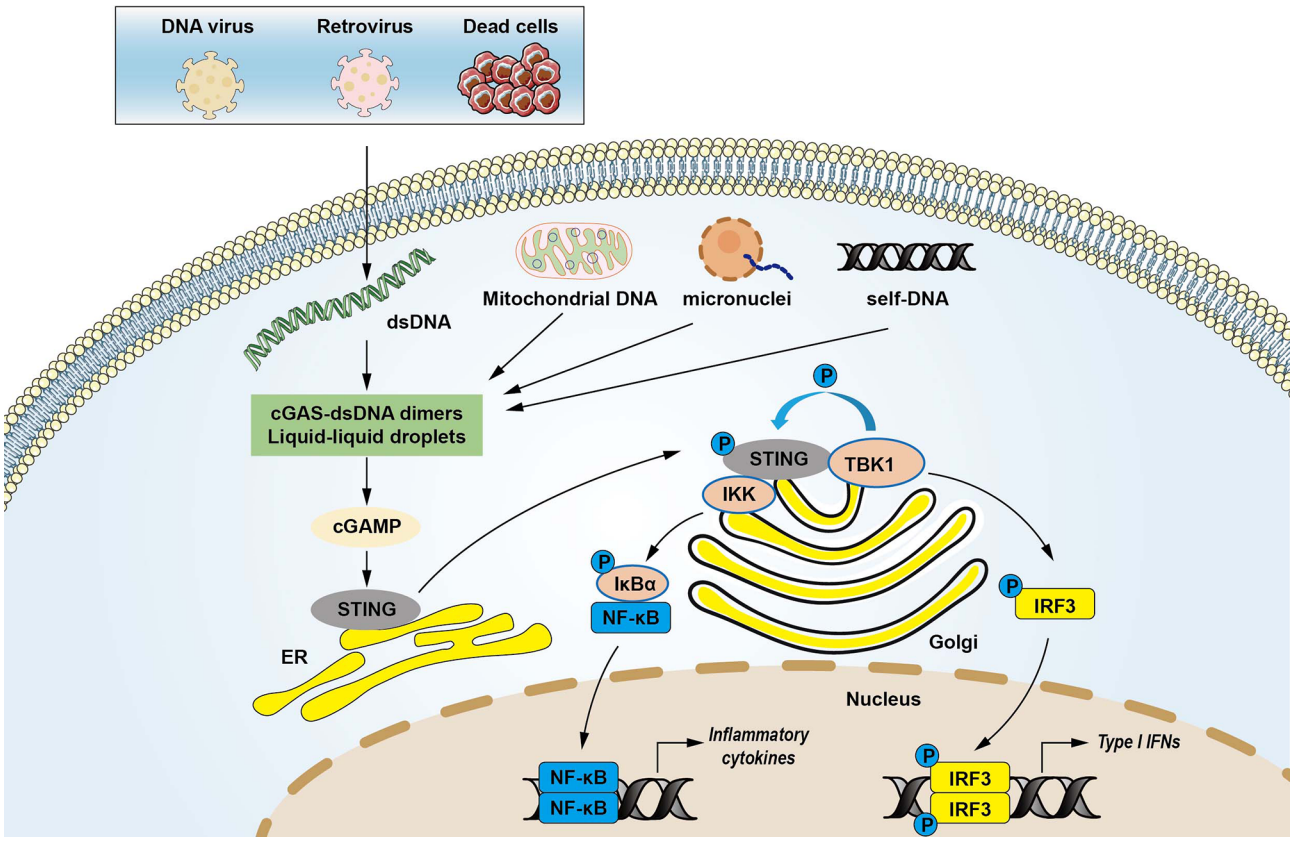


FIGURE 1 | The cytosolic DNA-sensing cGAS-STING pathway in innate immunity. Cyclic GMP-AMP synthase (cGAS) is a protein, which detects various cytosolic dsDNA, including viral DNA, damaged self-DNA released by dying cells, micronuclei, and mitochondrial origins. dsDNA activates cGAS *via* forming cGAS-dsDNA in 2:2 complexes. Mitochondrial damage and the release of mitochondrial DNA (mtDNA) in the cytosol also activates cGAS. The interactions of cGAS with DNA induce the formation of the liquid droplets through a phase transition, in which cGAS exerts its catalytic role to create the second messenger cGAMP that stimulates the stimulator of interferon genes (STING) at the endoplasmic reticulum (ER). STING then translocates from the ER to Golgi compartments and recruits kinases such as TANK-binding kinase 1 (TBK1) and IκB kinase (IKK), which in turn catalyzes the phosphorylation of IFN regulatory factor 3 (IRF3) and the nuclear factor-κB (NF-κB) inhibitor IκBα. Phosphorylated IRF3 translocates to the nucleus to activate transcription of genes encoding type I interferons and other inflammatory genes. IκBα phosphorylation accelerates nucleus translocation of NF-κB to promote transcription of target inflammatory cytokines, leading to activating inflammatory responses.

strong innate immune responses may also account for the convenient transformation of HBV infections to chronic HBV hepatitis (43). Other studies have identified that HBV-derived dsDNA fragments (44), viral genomic relaxed circular (RC) DNA (45), and naked HBV genome (46) could activate the innate antiviral immune responses. As a critical DNA cytosolic DNA sensor, the role of the cGAS-STING pathway during HBV infection has been investigated by several research groups (42, 44, 46–48). Recent studies demonstrated that both primary murine hepatocytes and primary human hepatocytes (PHH) failed to produce type I IFN in response to the foreign DNA in the cytosol or HBV infection due to the lack of STING expression in these hepatocytes (42). However, the hepatoma cell line HepG2 showed an innate immune response after HBV infection since STING expression was observed (42). The lack of DNA-sensing signaling impaired the hepatocytes' ability to control HBV but induction of STING *in vivo* reduced viral gene expression and replication in hepatocytes (42), suggesting that the absence of the intracellular DNA-sensing pathway dampens the innate immune response against HBV infection in hepatocytes. These results were further validated by another *in vitro* study, which showed that increased STING expression exhibited resistance to HBV infection whereas disruption of STING expression depressed IFN response and enhanced HBV transcription activity in human immortalized hepatocyte NKNT-3 cells (49). Thus, the STING pathway is essential for modulating susceptibility to HBV.

Interestingly, another study suggested that the packaged HBV genome evaded recognition by innate immune cells during natural infection, while naked HBV genomic rcDNA was sensed in a cGAS-dependent manner in human hepatoma cell line HepG2-NTCP (46). Moreover, HBV infection could inhibit the cGAS expression and function in cell culture and humanized liver chimeric mice by downregulating the cGAS-related gene MB21D1, a classic member of IFN-stimulated genes (ISGs) (46). However, HBV-derived dsDNA can also induce the innate immune response by expressing high levels of cGAS in human hepatoma Li23 cells (44). Activation of the cGAS-STING

pathway induced ISG56, one of the antiviral genes mediated by type I IFNs, and inhibited HBV assembly (44). Moreover, activation of the cGAS-STING pathway by dsDNA or cGAMP significantly depressed HBV replication *in vitro* and *in vivo* (48). A recent study revealed that HBV DNAs but not RNAs in the viral particles were immunostimulatory and sensed by the cGAS-STING pathway in HepG2 cells (47). HBV rcDNA triggered the hepatocyte response, whereas HBV infection did not suppress the DNA-sensing pathway but can evade the surveillance of the cGAS-STING mediated immune response (47). Indeed, activation of cGAS or STING with pharmaceutical treatment induced IFN response and inhibited viral replication in HBV-infected human hepatoma cells and immortalized mouse hepatocytes (50, 51). As an essential part of the innate immune system, Kupffer cells, which are macrophages residing in the liver, may also contribute to detecting foreign DNA and induction of inflammatory response by phagocytosis during HBV infection. Unlike PHH, the Kupffer cells certainly have intact DNA-sensor signaling, as they exhibit significantly enhanced cGAS-STING pathway levels after HBV infection (41, 42). Pharmaceutical activation of STING by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in macrophages could remarkably inhibit hepatocyte HBV replication in mice (50). Although Kupffer cells are positive regulators of antiviral immunity during HBV infection (37), the HBV core has been known to activate TLR2 on Kupffer cells leading to inhibition of HBV-specific T cell response by producing IL-10 (52). Genetic knocking out of TLR2 or pharmaceutical depletion of Kupffer cells resulted in a stronger antiviral immune response (52). Another study suggested that instead of promoting liver inflammation, Kupffer cells can inhibit immune response by removing apoptotic hepatocytes during HBV infection (53). These conflicting results on the role of the cGAS-STING pathway in hepatocytes and Kupffer cells during HBV infection suggest that more investigation is needed to clarify the underlying mechanism. Thus, it would be interesting to explore the cGAS-STING signaling role in other innate immune cell types in HBV infection.

HCV infection, followed by liver failure, liver cirrhosis, and HCC, is considered one of the most common causes of liver transplantation in Western countries (54). Evading innate and adaptive immune responses is the primary mechanism for HCV to defeat host immune surveillance and responses. The mechanism underlying HCV regulaton of host interferon response has been investigated for years. Several studies revealed that casein kinase II (CK2) was required for HCV core protein-mediated modulation (55) and served as a critical regulator in controlling IFN response. Activation of CK2 inhibited retinoic acid-inducible gene I (RIG-I)-mediated immune response, whereas disruption of CK2 promoted STING-mediated TBK1 activation and triggered IFN-β immune defense against HCV infection (56, 57). In addition, the hepatitis C virus non-structural 4B (HCV-NS4B) protein, an essential component of viral replication, was found to directly and specifically bind to STING and block the STING-Cardif interaction, contributing to potent inhibition of RIG-I-medicated IRF-3 phosphorylation and IFN-β (58). HCV-NS4B was also found to impair the interaction of STING and TBK1 (59, 60). These findings suggest that the STING-mediated immune defense mechanism contributes to host antiviral immune response.

Recently, it was reported that the delivery of synthetic cGAMP agonist for activation of the cGAS-STING pathway remarkably inhibited the HBV replication by inducing IFN production in the HBV-infected mouse model (48). The therapeutic drugs combined with an effective vaccine have shown high efficacy in eliminating viral hepatitis (61). As an HBV or HCV vaccine adjuvant, administration of STING agonists can induce a robust immune response *via* up-regulation of cytokines and chemokines, which may restrain tolerance in patients with chronic viral hepatitis (62, 63). Collectively, the interaction between the cGAS-STING pathway mediated innate immune response and HBV in hepatocytes and macrophages during natural infection is still elusive and controversial. Much more work is needed to investigate the molecular mechanisms underlying the role of the cGAS-STING pathway in HBV and HCV infection. These studies may provide a novel therapeutic approach for viral hepatitis.

THE cGAS-STING PATHWAY IN NONALCOHOLIC FATTY LIVER DISEASE/ NONALCOHOLIC STEATOHEPATITIS, AND ALCOHOLIC LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) is characterized by a series of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), subsequent cirrhosis, and even hepatocellular carcinoma. Currently, NAFLD is increasing globally, and the prevalence of NAFLD is about 25% (64). NAFLD is becoming the most common cause of chronic liver disease and the leading cause of liver failure requiring liver transplantation in western countries (65). However, there is no safe and effective therapy for patients with NASH due to the pathogenesis of NASH not being fully understood.

It is well known that the innate immune system, especially macrophages, plays an essential role in the development of hepatic steatosis to NASH (66). During the past years, numerous reports have identified the vital role of the cGAS-STING signaling pathway in NASH progression by regulating innate immune activation. Metabolic stress, such as a high-fat diet (HFD), could activate cGAS and the STING-IRF3-mediated inflammatory response (67). By contrast, STING deficiency mitigated HFD-induced adipose tissue inflammation, obesity, insulin resistance, and glucose intolerance (67). Disruption of either STING or IRF3 significantly attenuated free fatty acid (FFA)-induced inflammatory response, lipid accumulation, and hepatocellular apoptosis through regulation of the nuclear factor κB (NF-κB) signaling pathway (68). As lipotoxicity appears to be the central driver in NASH progression by oxidative stress and ER stress (69), lipotoxic activation of TBK1, a downstream of

cGAS-STING kinase, is also crucial for the control of the NASH development (70). Recently, mitochondrial DNA (mtDNA) released from injured hepatocytes has been recognized as an endogenous DAMP, which activates the cGAS-STING pathway and promotes hepatic inflammation through release of cytokines in NASH (71), suggesting that cytosolic mtDNA sensed by the cGAS-STING signaling is key to trigger innate immune response in NASH progression. Several studies have indicated that human and murine hepatocytes did not express STING protein (42, 71, 72). However, increased STING expression was observed in Kupffer cells in patients with NASH (72). Myeloid-specific STING induced TGF-β1 and activated hepatic stellate cells (HSCs), which promoted NASH progression, whereas disruption of myeloid STING alleviated hepatic inflammation, steatosis, and liver fibrosis in a mouse model of HFD or methionine and choline-deficient diet (MCD)-induced NASH (72), suggesting that activation of STING regulates macrophage function and augments hepatic lipid accumulation, profibrotic gene expression, and proinflammatory responses in NASH (**Figure 2**). Moreover, a study in liver samples from 98 patients with NAFLD revealed that STING expression in Kupffer cells and monocyte-derived macrophages (MoMFs) was correlated with hepatic inflammation and fibrosis in human NAFLD (73). These findings indicate that activation of the cGAS-STING pathway in macrophages is critical in NASH progression.

The hepatic inflammatory response has a fundamental role in NASH progression. Activation of STING induces the IRF3 and NF-κB pathways, and produces various inflammatory cytokines (74). It was reported that global knockout (KO) of IRF3 were significantly reduced liver injury, steatosis, and inflammation (75). However, another study showed that disrupted IRF3 resulted in increased insulin resistance and liver inflammation in HFD-induced NAFLD (76). Indeed, STING activated the innate immune response and contributed to the NASH progression in an NF-κB dependent manner (71). Moreover, IRF3 KO mice showed higher fasting glycemia and higher body weight (76), which was not consistent with the model of HFD-fed STING-deficient mice (71). STING might regulate glucose levels but not body weight in an IRF3-dependent manner. Further studies are needed to elucidate the underlying mechanism. Therefore, targeting STING to inhibit innate immune activation could provide a novel approach to managing NAFLD and NASH in patients.

Alcohol-related liver disease (ALD) affects more than 150 million people worldwide. It is the second most common indication for liver transplantation due to ALD-induced cirrhosis (77). Liver failure by ALD accounts for approximately half of liver cirrhosis-associated deaths in the United States (78). A previous study found that ER stress-induced IRF3 activation in the liver was associated with ER adaptor protein STING in the acute ALD model (79). IRF3 deficiency ameliorated hepatocyte apoptosis and the inflammatory responses in an ethanol-feeding mouse model (79). Alcohol-feeding remarkably increased cytoplasmic mtDNA release, resulting in activating the cGAS-IRF3 signaling (80). Activation of IRF3 by cGAS drove liver inflammation and injury in both alcohol-exposed hepatocytes and the neighboring parenchyma through a gap junction intracellular communication pathway (80). RNA-seq analysis of ALD patients showed that the cGAS-IRF3 pathway was positively associated with disease severity (80). Thus, cGAS, STING, and IRF3 are crucial determinants in the pathogenesis of ALD and potential therapeutic targets in ALD (**Figure 3**).

THE cGAS-STING PATHWAY IN HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is the second leading cause of cancer-related death in the world (81). Despite the availability of multiple therapeutic approaches for the early stage of HCCs, including surgical liver resection, liver transplantation, and percutaneous ablation, most patients are diagnosed at relatively advanced stages with fewer treatment options and a poor prognosis (82). Recently, cancer immunotherapy has emerged as an effective therapy for various types of cancers (83). Accumulating evidence demonstrates the vital role of the innate immune system in liver cancer immunosurveillance and immunotherapy (84). During tumorigenesis, tumor cell death and genome instability could lead to abnormal localization of genomic DNA in the cytosol and micronuclei formation (16, 85). As a solid tumor, the hypoxic microenvironment inside the HCC tumor can also induce cancer cell necrosis, which promotes the release of mitochondrial DNA (mtDNA) (86, 87). These exogenous and endogenous cytosolic DNA are subsequently recognized by the immune cells, resulting in activation of the innate immune response. Emerging studies show that cGAS also detects tumor-derived DNA, initiating antitumor immunity in some cancers (88). Indeed, the cGAS-STING pathway plays an essential role in HCC progression. It was reported that low levels of STING in tumor tissues were associated with poor prognosis in HCC patients (89). Activation of the cGAS-STING pathway augmented immune cell infiltration in HCC tissues (90). The cGAS-STING pathway members also displayed strong associations with immune markers involved in clinical stages, pathological grades, and overall survival in patients with HCC (90), suggesting that the cGAS-STING pathway members could be used as potential prognostic biomarkers in patients with HCC. In a mouse model of mutagen-induced HCC, STING deficiency reduced phosphorylated-STAT1, autophagy, and cleaved caspase 3 levels but accelerated tumor progression, with increased numbers of large tumors at advanced stages. In contrast, treatment with a cyclic dinucleotide (CDN) STING agonist promoted cell death, autophagy, and IFN responses in HCC (91). Notably, CDN treatment markedly reduced tumor size and the number of HCC in mice (91). These findings indicate STING is a promising therapeutic target for the treatment of HCC.

Immunotherapy has been rapidly expanded as a novel option in the treatment of advanced HCC. Data from the early stage of clinical trials with PD-1/PD-L1 therapy have suggested promising results with encouraging survival and safety data in HCC patients (92). While some therapeutic benefits have been reported with immune checkpoint blockade therapy, the low efficacy of immunotherapy remains a significant challenge in HCC treatment. Several studies have revealed that STING-deficient mice are less responsive to

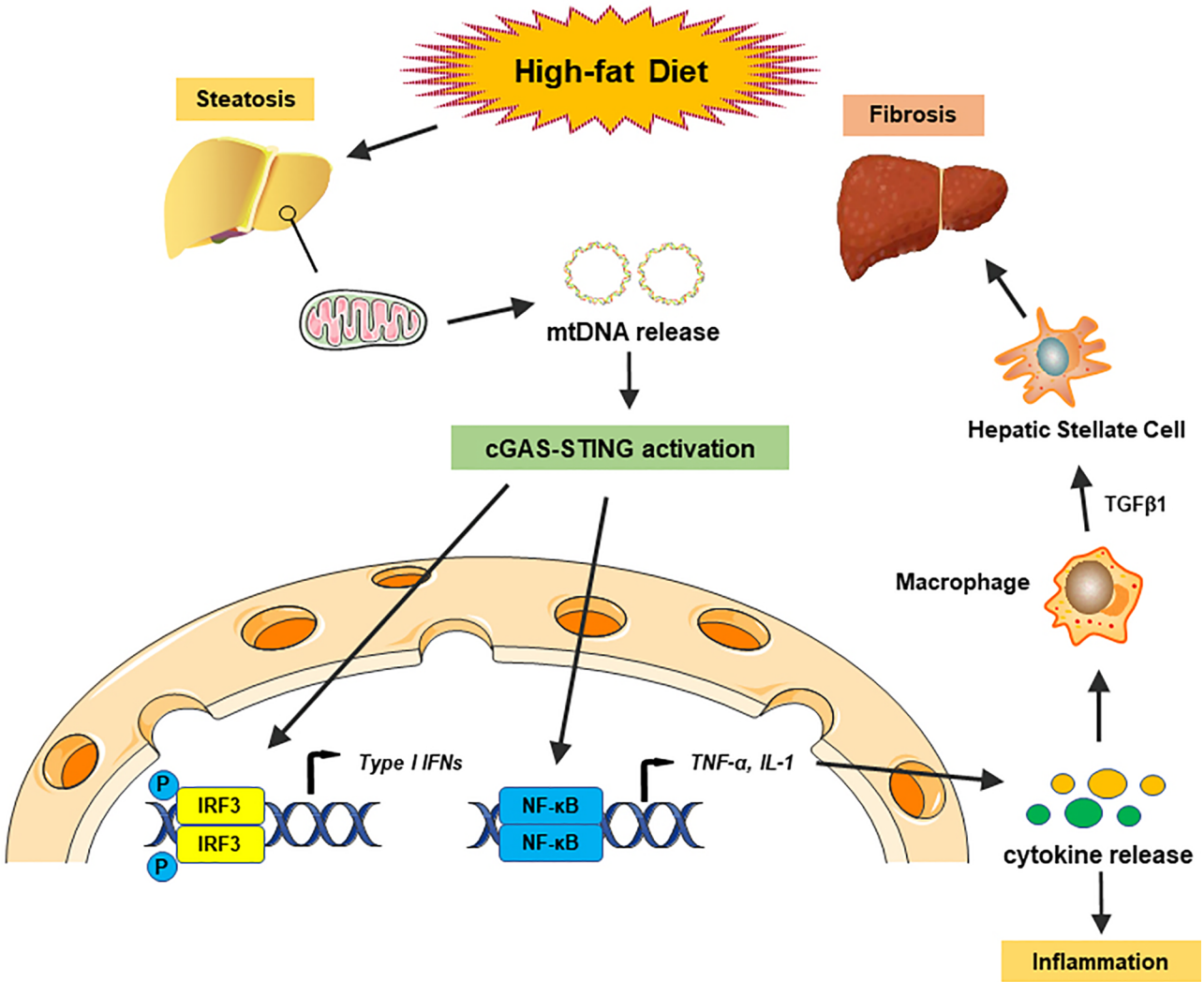


FIGURE 2 | The cGAS-STING pathway in nonalcoholic fatty liver disease. A high-fat diet (HFD) causes steatosis, which induces mitochondrial stress damage in hepatocytes and subsequent releases of mitochondrial DNA (mtDNA) into the cytosol. Cytosolic mtDNA is recognized as an endogenous DAMP, which activates the cGAS-STING pathway and induces the IRF3 signaling to promote transcription of type I IFNs. Activation of the cGAS-STING pathway also induces the NF-κB signaling to produce proinflammatory cytokines, which triggers hepatic inflammatory responses. Moreover, proinflammatory cytokines activate macrophage function and produce TGF-β1, which activates hepatic stellate cells (HSCs) and promotes liver fibrosis in NASH.

immunotherapy (93, 94). A combination treatment of cGAMP with PD-L1 inhibitor has shown a more potent antitumor effect in a xenograft model (95), indicating that stimulation of the cGAS-STING pathway may improve immunotherapeutic efficacy for the treatment of HCC. Further studies are needed to elucidate the crosstalk between the cGAS-STING and PD-1/PD-L1 pathway in antitumor immunity against HCC.

THE cGAS-STING PATHWAY IN LIVER ISCHEMIA AND REPERFUSION INJURY

Liver ischemia-reperfusion injury (IRI), an innate immunity-dominated local sterile inflammatory response (96), is a significant cause of hepatic dysfunction and failure in liver transplantation (97). Oxidative and ER stress are important factors in the pathogenesis of hepatic IRI. IR-induced stress activates liver macrophages (Kupffer cells) to generate reactive oxygen species (ROS), leading to sterile inflammation in the liver (98). ROS, an endogenous 'danger' signal released from necrotic and stressed cells, triggers toll-like receptor 4 (TLR4) or NLRP3 inflammasome-driven innate immune response in ischemic livers (98–101). ROS can induce oxidative mitochondrial damage, resulting in mtDNA leaks into the cytosol (102). The mtDNA is recognized by the DNA sensor cGAS and activates STING, which triggers an innate immune response (103). Recent studies showed that mtDNA release from hepatocytes was significantly increased during liver IRI (104). Increased

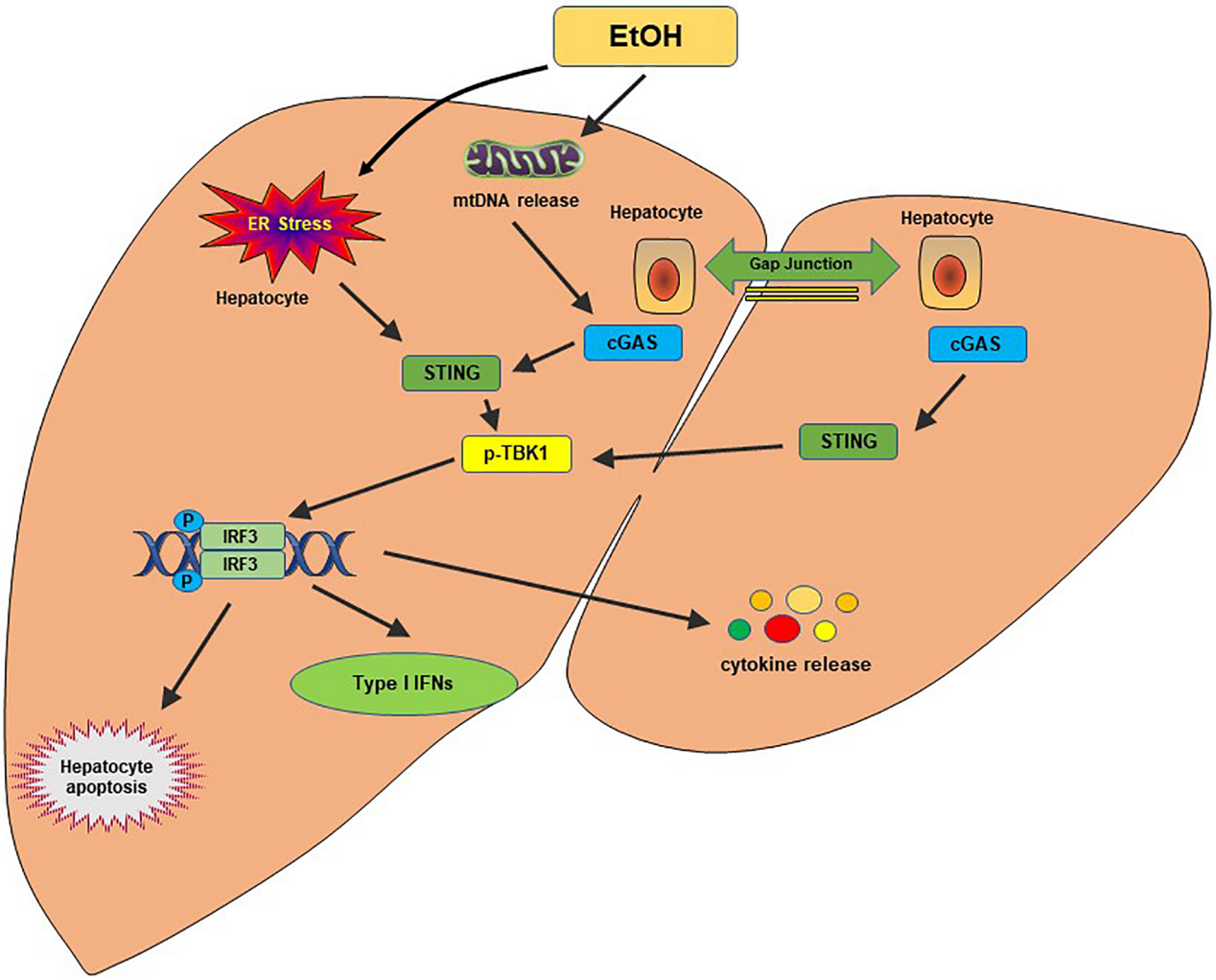


FIGURE 3 | The cGAS-STING pathway in alcohol-related liver disease. Alcohol-induced ER stress and mtDNA release activate the STING pathway. STING facilitates IRF3 phosphorylation by TBK1, which results in the translocation of IRF3 into the nucleus, where it induces transcription of type I IFNs. A gap junction intracellular communication pathway between alcohol-exposed hepatocytes and the neighboring parenchyma also contributes to the IRF3 activation by cGAS. Activation of IRF3 could trigger hepatocyte apoptosis, type I IFN response and produce proinflammatory cytokines, leading to hepatic inflammation and injury.

mtDNA induced STING activation in macrophages after liver IRI, whereas disruption of STING reduced NLRP3 activation and proinflammatory mediators in mtDNA-stimulated macrophages from aged mice (105).

Moreover, another study showed that IR-induced stress in hepatocytes promoted cGAS expression but they did not express STING under oxidative stress conditions (106). Interestingly, cGAS global knockout (KO) mice displayed increased IR-induced liver injury compared to the wild-type or STING-deficient mice. Disruption of cGAS in hepatocytes augmented cell death and apoptosis but reduced autophagy induction in response to oxidative stress (106), suggesting that cGAS regulates hepatic autophagy in a STING-independent manner during liver IRI. Indeed, the tissue-specific roles and regulatory mechanisms of the cGAS-STING pathway remain mostly elusive. As liver macrophages, including resident Kupffer cells and infiltrated bone marrow-derived macrophages, are a major player in innate immune responses in the pathogenesis of liver IRI (98, 99, 107), it is also unclear how the cGAS-STING pathway influences the interplay between hepatocytes and innate immune cells in liver IRI. Further studies will be needed to elucidate the coordination and orchestration of these IR-stressed cells regulated by the cGAS-STING pathway.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It is now clear that innate immunity plays a central role in the pathogenesis of liver diseases. The innate immune response may drive the progression of liver disease and contribute to liver

damage, fibrosis, cirrhosis, and even HCC. The cGAS-STING pathway functions as a direct innate immune sensor of cytosolic DNA. While self-DNA sensor cGAS can recognize cellular or tissue damages, excessive activation of the cGAS-STING pathway triggers liver inflammation and subsequent disease. Studies on the cGAS-STING pathway in liver diseases have led to a better understanding of the role of the innate immune response in the development of liver inflammation and injury. New findings involved in regulating the cGAS–STING pathway will allow us to identify the essential molecules as potential therapeutic targets for liver diseases. Indeed, the cGAS–STING pathway is a dual-edged sword. Transient activation of this pathway shows an antitumor and antiviral effect, but persistent activation may promote inflammation-driven tumorigenesis (108). cGAS-STING dependent DNA-sensing of micronuclei in tumor cells can stimulate tumor metastasis due to chromosomal instability (109). However, tumor-derived cGAMP triggered natural killer (NK) cell response and inhibited tumor growth by activating the STING pathway (110). Although the STING agonists have shown promising results in HBV/HCV infection and HCC therapy in disease models (50, 51, 91, 111), more preclinical studies and early-stage clinical trials are needed to verify these encouraging survival and safety data.

The current research on the cGAS-STING signaling pathway in liver diseases has revealed only 'tip of the iceberg'. Further studies on tissue-specific roles of the cGAS-STING pathway with other DNA sensing pathways in liver inflammation and injury are critical. They may provide new insights into the mechanism of therapy for liver diseases.

AUTHOR CONTRIBUTIONS

BK was responsible for the conception of the review. DX wrote the first draft of the manuscript and YT produced ideas for the figures. QX and BK participated in scientific discussion. All authors contributed to the article and approved the submitted version.

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REVIEW
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Modeling Virus-Induced Inflammation in Zebrafish: A Balance Between Infection Control and Excessive Inflammation

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The inflammatory response to viral infection in humans is a dynamic process with complex cell interactions that are governed by the immune system and influenced by both host and viral factors. Due to this complexity, the relative contributions of the virus and host factors are best studied *in vivo* using animal models. In this review, we describe how the zebrafish (*Danio rerio*) has been used as a powerful model to study host-virus interactions and inflammation by combining robust forward and reverse genetic tools with *in vivo* imaging of transparent embryos and larvae. The innate immune system has an essential role in the initial inflammatory response to viral infection. Focused studies of the innate immune response to viral infection are possible using the zebrafish model as there is a 4-6 week timeframe during development where they have a functional innate immune system dominated by neutrophils and macrophages. During this timeframe, zebrafish lack a functional adaptive immune system, so it is possible to study the innate immune response in isolation. Sequencing of the zebrafish genome has revealed significant genetic conservation with the human genome, and multiple studies have revealed both functional conservation of genes, including those critical to host cell infection and host cell inflammatory response. In addition to studying several fish viruses, zebrafish infection models have been developed for several human viruses, including influenza A, noroviruses, chikungunya, Zika, dengue, herpes simplex virus type 1, Sindbis, and hepatitis C virus. The development of these diverse viral infection models, coupled with the inherent strengths of the zebrafish model, particularly as it relates to our understanding of macrophage and neutrophil biology, offers opportunities for far more intensive studies aimed at understanding conserved host responses to viral infection. In this context, we review aspects relating to the evolution of innate immunity, including the evolution of viral pattern recognition receptors, interferons and interferon receptors, and non-coding RNAs.

Keywords: innate immunity, neutrophils, reactive oxidative species, inflammation, virus infection, zebrafish

INTRODUCTION

Deadly hyperinflammatory responses to diseases like COVID-19 and influenza A result when the immune system overreacts (1–6). Cytokine storms induced by viral infections trigger this hyperinflammatory state, leading to serious consequences, including acute respiratory distress syndrome (ARDS), pulmonary edema, multiple organ failure, and death. The antiviral response encoded in vertebrate genomes incorporates an inflammatory rheostat (7) that is designed to ramp up or tamp down in response to infection. This response provides the host a measure of resilience and promotes its survivability. Under some circumstances, this inflammatory response to viral infection may become dysregulated, at which point an immunological tipping point is reached, leading to increased rates of mortality. This review describes progress in using the zebrafish (*Danio rerio*) as a powerful model system for the study of infection and inflammation, and it is increasingly being used to model human viral infections. Zebrafish possess several inherent characteristics that make them excellent biomedical and biological model systems, including optically clear embryos, high fecundity, a fully sequenced genome, amenability to multiple modes of injection and manipulation, and robust forward and reverse genetics tools. We review recent studies on viral recognition receptors in zebrafish that are homologous to those found on human cells. For example, we have shown that zebrafish possess α2,3- and α2-6-linked sialic acid receptors that are required for infection by certain influenza A virus (IAV) strains, including H1N1 (8). Because human viruses can infect zebrafish cells, it is possible to recapitulate aspects of the human viral disease in zebrafish, including the host inflammatory response. Many elements of the host immune response to human viral infection are retained in zebrafish, and this is owed to significant cellular and molecular conservation between zebrafish and humans. As neutrophils have critical roles in inflammation, we begin our review on neutrophils and their roles in antiviral response pathways that include toll-like receptors (TLRs), interferon (IFN) signaling, and the respiratory burst response. Next, we review zebrafish studies on fish and human viruses and include methodological details about these zebrafish models and functional assays. We also describe recent studies of non-coding RNAs that regulate neutrophil function. It is our view that the zebrafish offers tremendous promise as a model to understand how some of the mechanisms underlying a normal immune response to viral infection in humans become excessive, leading to increasing morbidities and mortalities.

IMMUNE CELL CONSERVATION IN ZEBRAFISH

Definitive Hematopoiesis

In zebrafish, definitive hematopoiesis begins as early as 26 hours post-fertilization (hpf) and gives rise to self-renewing hematopoietic stem cells (HSCs) that can differentiate into cells with myeloid, lymphoid, and erythroid lineages (9). The sites of definitive hematopoiesis differ between zebrafish and humans. For zebrafish, definitive hematopoiesis transitions from the ventral wall of the dorsal aorta (26 hpf) through the caudal hematopoietic tissue (CHT) (~2 days post-fertilization (dpf)) and eventually to the thymus (~3 dpf) or the pronephros/kidney (~4 dpf) (9–11). In mammals, definitive hematopoiesis is transitory as well, moving from the aorta-gonad-mesonephros region in the ventral wall of the dorsal aorta, to the mammalian fetal liver, and finally to the bone marrow (12). The earliest stage of definitive hematopoiesis in both zebrafish and mammals is restricted to analogous ventral dorsal aorta regions. From there, the anatomical sites of hematapoiesis differ (11). Nonetheless, the genetics and molecular signaling underlying definitive hematopoiesis in vertebrates are largely conserved across species. Importantly, the morphology and function of zebrafish neutrophils are conserved with mammalian neutrophils (13). As many studies of neutrophil function in zebrafish are done during embryonic and larval stages, it is worthwhile noting that neutrophils also arise from hematopoietic precursors in the yolk sac (14). As neutrophils are the first immune cells that migrate to the site of inflammation, our review will focus on these phagocytes.

Neutrophils

The first immune cells that migrate to the site of inflammation are neutrophils (15). Zebrafish neutrophils, also known as heterophils, respond to infection and injury in a manner that is similar to human neutrophils. For example, zebrafish neutrophils have been shown to migrate to the sites of bacterial (16), fungal (17–23), and viral (8, 24, 25) infections. Additionally, wounding studies have demonstrated neutrophil migration to the site of injury in zebrafish (26). Like human neutrophils, the response of zebrafish neutrophils to pathogens include phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs). Central to the response of neutrophils is the release of reactive oxidative species (ROS), which is described in detail later in this review. Both azurophillic and non-azurophillic granules are found in zebrafish neutrophils, with azurophillic granules being more abundant (27, 28). Like primary azurophilic granules in mammalian neutrophils, zebrafish neutrophil granules contain the enzyme myeloperoxidase (Mpx) (27). During respiratory burst, Mpx catalyzes the conversion of H_2O_2 and Cl^- to produce cytoxic hypochlorous acid (HOCl) (29). Neutrophils also generate reactive nitrogen species (NO). NETs are released by neutrophils through a cell death process, named NETosis, to inactivate and destroy extracellular viral particles, bacteria, and fungi. In human neutrophils, NETs are composed of a scaffold of decondensed chromatin with at least 24 cytosolic and granule proteins, including myeloidperoxidase (MPO) and neutrophil elastase (ELANE) (30). NETs were observed to be generated by neutrophils found within whole zebrafish kidney tissue *ex vivo* following stimulation with calcium ionophore, phorbol myristate acetate (PMA), and β-glucan (31). Two features associated with NETs have been observed at the sites of localized hindbrain *Candida albicans* infection *in vivo*. First, increased levels of extracellular DNA were detected with neutrophil invasion following hindbrain *C. albicans* infection (32). Second, extrusion of a neutrophil-specific histone 2B-mCherry fusion protein was observed following neutrophil recruitment to *C. albicans* but not

C. auris hindbrain infection (33). The activation and translocation of NETs is initiated by ROS that, in turn, stimulate MPO and ELANE expression in mammalian cells (34). Even though mammalian *ELANE* does not have an obvious homolog in zebrafish (35), elastase activity was associated with zebrafish NETs (31). Given the central role of ROS in the neutrophil response, a major focus in this review will be on ROS.

Neutrophil and Macrophage Reporter Lines

Several zebrafish fluorescent reporter strains have been developed to visualize neutrophils and macrophages *in vivo*, and for fluorescently-activated cell sorting (FACS). Transgenic zebrafish neutrophil reporter lines have used *mpx* and lysozyme (*lyz*) promoters to drive the expression of fluorescent proteins. Frequently used neutrophil reporter lines include the GFP reporters, *Tg(mpx:GFP)^{i114}* (36), *Tg(mpx:GFP)^{uwm1}* (37) and *Tg(lyz:EGFP)^{nz117}* (38), and the red fluorescent protein reporters, *Tg(mpx:mCherry)^{uwm7}* (39) and *Tg(lyz:DsRED2)^{nz50}* (38). Additional reporter lines using the photoconvertible fluorescent reporter, Dendra2 (40), have been developed to study migration of macrophages and neutrophils. Dendra2 protein photoswitches from green to red following exposure to visible blue or UV light. This photoconvertible reporter line enables tracking of neutrophil forward and reverse migration (41). Another photoconvertible protein, Kaede, has also been used to study neutrophil migration when expressed as part of a GAL4/UAS bipartite expression system, such as the *Tg(mpx:Gal4);Tg(UAS:Kaede)^{i222}* line. As many zebrafish macrophage reporter lines have also been developed, it is possible to use double transgenic lines, such as *Tg(mpeg1:Gal4-VP16/UAS : Kaede/mpx:EGFP)*, to allow for *in vivo* imaging of neutrophils and macrophages simultaneously (42). These macrophage reporter lines use a promoter from the membrane attack complex/perforin-domain containing gene, macrophage expressed gene 1, tandem duplicate 1 (*mpeg1.1*) (43), to drive the expression of reporters, such as EGFP (*Tg(mpeg1:eGFP)^{gl22}*) (42), mCherry (*Tg(mpeg1:mCherry)^{gl23}*) (42), and YFP (*Tg(mpeg1:YFP)^{w200}*) (44). Migration of macrophages can also be monitored using the Dendra2 reporter in the *Tg(mpeg1:Dendra2)^{uwm12}* line (45). The promoter for microfibril associated protein 4, tandem duplicate 1 (*mfap4.1*) has also been used for macrophage reporter lines (46) as the expression of *mpeg1* was shown to be attenuated following infection of *Salmonella thyphimurium* and *Mycobacterium marinum* (47). Several of these neutrophil and macrophage reporter lines have been used for FACS for cell-specific functional analysis (38, 48, 49).

OVERVIEW OF ANTIVIRAL RESPONSE

Defense against viral infection is governed by both the innate and adaptive immune systems. Even though the adaptive immune system can provide protection from viral infection through B and T lymphocytes, the innate immune system provides an initial response to viral infection and is the focus of this review. The innate immune system includes physical barriers, phagocytic cells, pattern recognition receptors (PRRs), interferons and interferon-stimulated genes (ISGs), cytokines and chemokines, and the complement system. Physical barriers include the mucus barrier that is composed of polymeric secreted mucins. Phagocytes include neutrophils and macrophages that can kill virus particles and recruit additional phagocytes to sites of infection. An important response of phagocytes is a respiratory burst response that releases ROS to kill virus particles and recruit additional phagocytes. Critical to the activation of immune response are PRRs that bind pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and trigger the expression of interferon and cytokines through NF-κB and interferon response factor (IRF) transcription factors. Interferon elicits a potent response to viral infection that includes the activation of a battery of ISGs. Inflammatory cytokines and chemokines recruit phagocytes at the site of infection. The complement system functions to respond to microbial pathogens by recognizing motifs through three convergent activation pathways that lead to complement-mediated lysis (50). **Figure 1** illustrates components of response to viral infection using IAV as an example. Genes that have shown to respond to the inflammatory and antiviral response using zebrafish models of viral infection are shown in **Tables 1** and **2**, respectively.

The zebrafish model system holds particular promise for understanding the innate immune response to viral infection. Zebrafish lack a fully functional adaptive immune response for the first 4-6 weeks of development (66) and rely upon their innate immune response for defense against all forms of infection. Many aspects of the innate immune system, including those listed below, are functionally conserved in zebrafish, and thus the zebrafish can effectively model how normal inflammatory responses to viral infections can lead to extensive tissue damage and mortality.

Pattern Recognition Receptors (PRRs)

PRRs bind PAMPs and DAMPs, triggering a signal transduction cascade that activates several transcription factors critical to the antiviral and pro-inflammatory immune response. Viral PAMPs include surface glycoproteins, single-stranded RNA, double-stranded RNA, and other RNA and DNA species. DAMPs produced by damaged cells can also activate the immune response. DAMPs include denatured intracellular proteins, such as high-mobility group box protein 1 (HMGB1) (67). PRRs include TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I-like (RIG-I)-like receptors (RLRs), scavenger receptors, and C-type lectin receptors (CLRs).

PAMPs from viral particles that have entered the phagolysosomal degradation pathway are recognized by mammalian endosomal TLRs: TLR3, TLR7, TLR8 and TLR9. These TLRs traffic from the endoplasmic reticulum (ER) to endosomes with the chaperone, UNC93B1 (68). Double-stranded RNA, single-stranded RNA, RNA degradation products, and CpG-deoxynucleotides (CpG-DNA) are recognized by TLR3, TLR7, TLR8 (69) and TLR9, respectively. TLR3, TLR7, TLR8, and TLR9 are conserved in zebrafish as the homologs *tlr3* (65), *tlr7* (70), *tlr8a* (70), *tlr8b* (70), and *tlr9* (70, 71) (**Table 3**). In zebrafish, two additional antiviral TLRs, *tlr21* and *tlr22*, have been described that recognize CpG-DNA (71)

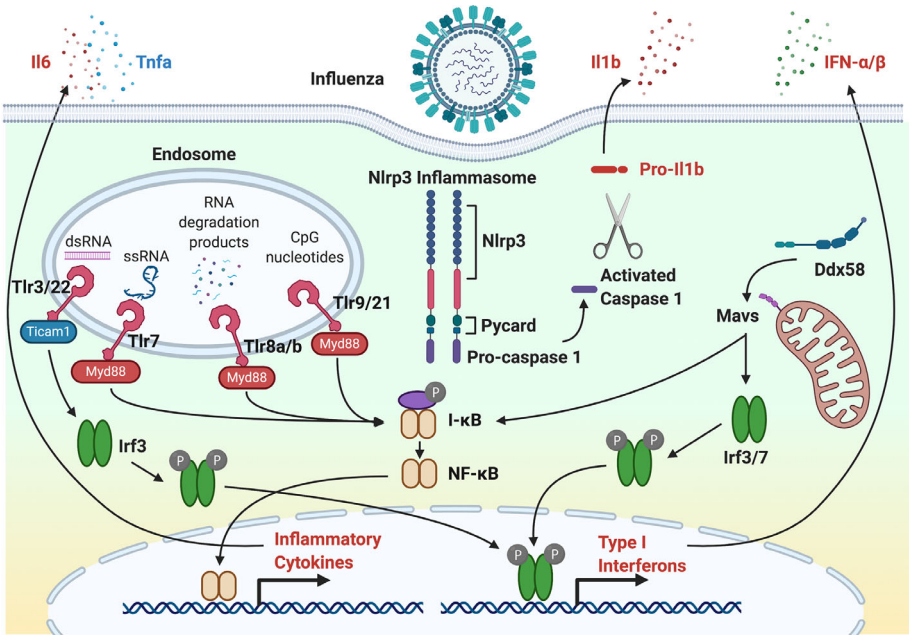


FIGURE 1 | The antiviral response to Influenza A Virus infection. Following IAV entry and infection, single-stranded RNA (ssRNA) and RNA degradation products incorporated into endosomes are recognized by Tlr7 and Tlr8a/b, respectively. In other virus infections, double-stranded RNA by Tlr3 and Tlr22. CpG motifs are recognized and Tlr9 and Tlr21. For Tlr7, Tlr8a/b and Tlr9, the TLR-adaptor, Myd88, activates the NF-κB transcription factor through IkB. NF-κB initiates transcription of inflammatory cytokines, such as Il6, Il1b, and Tnfa. For Tlr3, the TLR-adapter, Ticam1, activates Irf3 that initiates transcription of type I interferons. DAMPs and PAMPs can activate the Nlrp3 inflammasome through activated caspase 1. Activation of RIG-I (Ddx58) by cytosolic viral RNA activates Irf3 and Irf7 transcription factors through Mavs. Irf3 and Irf7 initiate the expression of type 1 interferons that further exacerbates the antiviral innate immune response to infection.

TABLE 1 | Table of proinflammatory genes studied in zebrafish models of viral infection.

Gene Symbol	Example Viruses				
	Group V				
	Spring Viremia of Carp Virus (SVCV)	Tilapia Lake Virus (TLV)	Snakehead Rhabdovirus (SHRV)	Infectious Hematopoietic Necrosis Virus (IHNV)	Influenza A Virus (IAV)
caspa	(51)				
cxcl8a	(52)	(53)			(25)
ifng1	(54)	(53)			
ifnphi1	(51, 55, 56) # (57)	(53)	(58–60)	(61)	(8)
ifnphi2	(51)				
il1b	(51, 52, 54)	(53)			(25)
irf3	(52, 56, 62)	(53)			
irf7	(52, 62)	(53)			
lta	(52, 56)				
sting1	(62)				
pycard	(51)				
rarres3	(51)				
tnfa	(51, 54, 55)	(53)			(25)
tnfb	(55)				

#*Functional study.*

and double-stranded RNA (73, 79), respectively. Homologs of *tlr21* and *tlr22* have not been observed in mammalian genomes, but *tlr21* is conserved in avian species.

The TLR signaling pathway in zebrafish includes the adaptor proteins Myd88, Tirap, Ticam1, and Sarm1 for downstream signaling. The gene encoding the Ticam2 adaptor protein found in mammals is absent in zebrafish (74). In mammals, Myd88 is required for all TLRs except for TLR3 and TLR4 (80). TLR signaling is mediated by tumor necrosis factor receptor associated factor 6 (TRAF6) and interleukin-1

TABLE 2 | Table of antiviral genes studied in zebrafish models of viral infection.

Gene Symbol	Example Viruses					
	Group III	Group V				
	Infectious Pancreatic Necrosis Virus	Spring Viremia of Carp Virus	Tilapia Lake Virus	Snakehead Rhabdovirus	Infectious Hematopoietic Necrosis Virus	Influenza A Virus
defb2		(54)				
foxo3b		(52)				
ifit8		(51)				
ifit14		(51)				
isg15	(61)	(61)			(61)	
mavs		(62, 63)				
ifih1		(63)		(60)		
mxa		(51, 54, 63)	(53)	(59)		(8)
mxb		(51, 54, 55)				
mxc		(52, 54, 56)				
nod2		(63)				
pkz		(56)				
prmt3		(56)				
rela		(64)				
ddx58		(51, 52, 62, 63)	(53)			
ripk2		(63)				
tbk1		(62)				
tlr3		(51)	(53)	(65)		
tlr7		(51)				
tlr8a		(51)				
tlr22		(51)	(53)			
rsad2		(55)				

receptor-associated kinase 4 (IRAK4) that activate the NFκB, IRF, STAT, ATF, and AP-1 families of transcription factors. The expression of *tlr3*, *traf6* and *irak4* was upregulated in embryonic and adult zebrafish following snakehead rhabdovirus (SHRV) infection (65). Beyond these four TLRs, knockdown of two adaptors for TLR signaling, Ly86 and Cd180, found increased susceptibility to spring viremia carp virus (SVCV) in zebrafish larvae (81). In mammals, LY86 and CD180 are adaptors for TLR4, a TLR that responds to lipopolysaccharide (LPS).

We previously described a model for the history of *TLR4* genes in humans and zebrafish that we believe accounts for the functional divergence that has been observed, specifically in regards to the reduced LPS sensitivity seen in fishes (75). We hypothesize that *TLR4* was duplicated in an ancestral genome with the second whole genome duplication event, yielding the *TLR4A* and *TLR4B* genes (75). Our model projects that there was lineage divergence and a reciprocal loss of *TLR4* ohnologs. The ancestral *TLR4A* was retained in the lineage that gave rise to mammals, including humans, and *TLR4B* was lost. The *TLR4A* gene, by convention, is referred to as *TLR4*. In the lineage that gave rise to zebrafish, the ancestral *TLR4B* gene was retained, and the ancestral *TLR4A* gene was lost. The ancestral *TLR4B* gene was subsequently duplicated, giving rise to the *tlr4ba* and *tlr4bb* paralogs observed in the current zebrafish genome.

There are data that indicate that *TLR3*, *TLR7*, *TLR8*, and *TLR9* are, at least to some extent, functionally conserved in zebrafish as the homologs *tlr3, tlr7, tlr8a, tlr8b*, and *tlr9*. To fully exploit the zebrafish model as a means to understand antiviral responses, it is necessary to undertake meticulous gene history studies to support orthology. Indeed, based on data available through Ensembl (82), ZFIN (83), and the Synteny Database (84), there appear in certain instances to be discrepancies in the identification and/or naming of zebrafish *TLR* genes that consequently imply a gene orthology (or lack of orthology) and functional conservation with human *TLR* genes despite sufficient evidence. For example, ZFIN predicts that zebrafish *tlr8a* and *tlr8b* are co-orthologous to human *TLR8*; however, this prediction is not supported by Ensembl or the Synteny Database where they do not list any orthologs for human *TLR8*. According to Ensembl, zebrafish *tlr8b* has a one-to-many orthologous relationship to the spotted gar gene *ENSLOCG00000013826*, which has been annotated as *tlr3*. Due to its evolutionary position as a non-teleost and non-tetrapod, jawed vertebrate model organism, the spotted gar genome serves as an "orthology bridge" to link the gene histories of the zebrafish (and other teleosts) and human genomes (85). The *ENSLOCG00000013826* gene has no human ortholog but does have a one-to-many orthologous relationship to a zebrafish gene annotated as *tlr3*. According to Ensembl and the Synteny Database, this zebrafish *tlr3* gene is an ortholog to human *TLR3*. This brief example demonstrates the inconsistencies present in current zebrafish databases and lends credence to the idea that the *tlr8* paralogs found in zebrafish (and other fishes) have no ortholog in the human genome, and thus are likely misnamed. In addition to these issues related to the evolutionary history of zebrafish *tlr* genes, there are also important concerns about the mechanisms by which the proteins encoded by these genes are engaged. Specifically, there is evidence that zebrafish TLR proteins do not bind PAMPs and other ligands in the same manner as human TLR proteins (69). There is also evidence indicating that the mechanisms by which zebrafish TLR proteins engage TIR

TABLE 3 | TLR genes in zebrafish.

Zebrafish Gene Symbol	Ensembl Zebrafish Gene ID	Zebrafish Chr.	Zebrafish Refs.	Predicted Human Ortholog	Ensembl Human Ortholog Gene ID	Human Chr.	Orthology Resource(s)
tlr1	ENSDARG00000100649	14	(70)	*TLR1**	ENSG00000174125	4	Synteny DB, ZFIN
				*TLR6**	ENSG00000174130	4	Ensembl
tlr2	ENSDARG00000037758	1	(70, 72)	*TLR2*	ENSG00000137462	4	Synteny DB, ZFIN, Ensembl
tlr3	ENSDARG00000016065	1	(65, 73, 74)	*TLR3*	ENSG00000164342	4	Synteny DB, ZFIN
tlr4al	ENSDARG00000075671	13		**			
tlr4ba	ENSDARG00000019742	13	(75)	**			
tlr4bb	ENSDARG00000022048	13	(75)	**			
tlr5a	ENSDARG00000044415	20	(72, 76)	*TLR5*	ENSG00000187554	1	Synteny DB, ZFIN
tlr5b	ENSDARG00000052322	20	(72, 76, 77)	*TLR5*	ENSG00000187554	1	Synteny DB, ZFIN
tlr7	ENSDARG00000068812	9	(70, 78)	*TLR7*	ENSG00000196664	X	Synteny DB, ZFIN
tlr8a	ENSDARG00000090119	KN150362.1	(70)	*TLR7*	ENSG00000196664	X	Synteny DB
CU914164.1 (*tlr8*)	ENSDARG00000104832	9		*TLR8*	ENSG00000101916	X	ZFIN
tlr8b	ENSDARG00000073675	10	(70)	*TLR7*	ENSG00000196664	X	Synteny DB
				TLR8	ENSG00000101916	X	ZFIN
tlr9	ENSDARG00000044490	8	(71)	*TLR9*	ENSG00000239732	3	Synteny DB, ZFIN
tlr18	ENSDARG00000040249	16		–		–	
						–	
tlr19	ENSDARG00000026663	16		–		–	
tlr20.1	ENSDARG00000115923	9		–		–	
tlr20.2	ENSDARG00000088701	9		–		–	
tlr20.3	ENSDARG00000114057	9		–		–	
tlr21	ENSDARG00000058045	16	(71)	–		–	
tlr22	ENSDARG00000104045	21	(73, 79)	–		–	

*Predictions were made using information from Ensembl, ZFIN and SyntenyDB. Ensembl gene IDs for each gene are listed, along with their respective chromosome locations. *Synteny Database and ZFIN predict TLR1 as the human ortholog, while Ensembl predicts TLR6. In the human genome assembly, TLR1, TLR6, and TLR10 are directly adjacent to one another. **Human TLR4 is not orthologous to tlr4ba, tlr4bb, or tlr4al (75).*

Known zebrafish toll-like receptors with predicted human orthologs.

domain containing adaptor proteins may sometimes differ (74). There are also many questions related to where within or on a cell a zebrafish TLR protein is expressed. Taken together, it is clear that assumptions about zebrafish TLR protein function based upon protein similarity and even phylogenetic analyses need further verification through comprehensive gene history analysis and thorough validation through functional assays.

Cytosolic PAMPs and DAMPs are recognized by NLRs and RLRs. After ligand binding, two NLRs, NOD1 and NOD2, can activate NFκB after recruiting the serine/threonine kinase RIPK2 through MAP kinase signaling. Several NLRs, including NLRC4, NARP1 and NARP3, function as PAMP and DAMP receptors for inflammasomes. Inflammasomes are multiprotein complexes that activate inflammatory caspases and pro-inflammatory cytokines through canonical signaling and non-canonical pathways to induce pyroptosis (86). In the canonical NLRP3 inflammasome signaling pathway, ligand binding to NRLs activate caspase 1 (CASP1) that then then activates the pro-inflammatory cytokines, interleukin 1β (IL1B) and interleukin 18 (IL18). Activation of CASP1 is dependent on the adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (PYCARD), which is also part of the inflammasome complex. In the non-canonical NLRP3 inflammasome signaling pathway, activated inflammasomes hydrolyze gasdermin D (GSDMD) leading to a N-terminal fragment that perforates the cell membrane to enable the release cytokines and subsequent cell death through pyroptosis. Inflammasome NLRs recognize ligands from both infection and sterile stressors. NLRP3 recognizes double-stranded RNA and activates CASP1 after binding the adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (PYCARD). Pycard-dependent activation of Il1b by Nlrp3 inflammasomes through caspase 1 (*caspa*) was found to be conserved in zebrafish larvae using morpholino knockdown of Nlrp3 and a *nlrp3* mutant challenged with *Edwardsiella tarda* (87). Li et al. also showed Nlrp3 initiated cell pyroptosis through Caspb activation in a gasdermin E (Gsdmeb/Gsdmea)-dependent, but independent of Pycard-activation (87). While several aspects of inflammasome signaling are conserved in zebrafish, differences do exist. Zebrafish have over 400 NLR genes (88), but only two have been associated with inflammasome function, *nlrp1* (89), and *nlrp3* (87, 90), that were shown to function similar to NLRP1. An additional inflammasome adaptor, *caiap*, was found to regulate inflammasome activation in zebrafish in response to *Salmonella typhimurium* infection (91). While the pro-inflammatory cytokine, *il1b* is conserved with zebrafish, an ortholog to IL18 has not been identified in zebrafish. Homologs to IL18 have been identified in other ray-finned fishes, including the pufferfish (*Takifugu rubripes*) (92) and rainbow trout (*Oncorhynchus mykiss*) (93).

Cytosolic viral RNA can also be detected by RLRs that are a family of DExD/H box RNA helicases consisting of RIG-I (encoded by the gene *DDX58*), melanoma differentiation-associated factor 5 (MDA5; encoded by the gene *IFIH1*), and

laboratory of genetics and physiology 2 (LGP2; encoded by the gene *DHX58*). Activation of RLRs by binding viral RNA leads to activation of the antiviral response and type 1 interferon (IFN) expression through interferon regulatory factor 3 (IRF3), IRF7, and NF-κB transcription factors. Upon binding viral RNA, the CARD domains of RIG-1 and MDA5 interact with the adaptor protein, mitochondrial antiviral signaling (MAVS). The conserved role of Mavs in regulating the IFN antiviral response in zebrafish larvae has been demonstrated through studies of chikungunya virus (CHIKV) infection (94). The IFN response and survival was significantly reduced in Mavs morphants infected with CHIKV. Zebrafish homologs of *DDX58*, *IFIH1* and *DHX58* have been identified as *ddx58*, *ifih1*, and *dhx58*.

Additional PAMP receptors include scavenger receptors and CLRs. In mammalian models, the scavenger receptor, macrophage receptor with collagenous structure (MARCO), has been shown to recognize several viruses, including respiratory syncytial virus and vaccinia virus. In zebrafish, *marco* has been used as a marker of macrophages and dendritic cells in adults. Marco was demonstrated to be required for phagocytosis and the proinflammatory response to *Mycobacterium marinum* and *Salmonella typhimurium* in larvae (95). Increased bacterial burden and decreased proinflammatory signaling was observed in infected Marco morphants. Another scavenger receptor, the expression of cluster differentiation antigen 36 (*cd36*) was upregulated in zebrafish following infection by viral hemorrhagic septicemia virus (VHSV) (96). Knockdown of Cd36 in zebrafish embryos resulted in higher bacterial burden following infection by *Mycobacterium marinum* (97). Several transmembrane CLR proteins function as PRRs on myeloid cells. Two CLRs include mannose-binding lectin 2 (MBL2) and CD209. MBL2 can activate the lectin complement pathway (98) after binding to mannose, fucose and N-acetylglucosamine on microbial pathogens, including viruses. MBL2 was shown to bind to influenza A virus (IAV) and inhibit the hemagglutinating activity of IAV (99). CD209 can also recognize microbial pathogens, including viruses that express mannose-rich oligosaccharides. CD209 was shown to function as an attachment receptor for influenza A virus on mammalian cells and mediate sialic-acid independent attachment and infection (100). While the functions of these specific CLRs have not yet been investigated in the context of viral infection in zebrafish, both *mbl2* and *cd209* are present in the zebrafish genome.

The complement system has important roles in innate immunity and neutralization of viruses. Mechanisms for complement activation include C-reactive protein (CRP), and recognition of PAMPs and DAMPs. The classical, lectin and alternative complement pathways activate C3 convertase that cleaves complement component C3 to produce the C3a and C3b peptides. In the alternative pathway, C5 convertase cleaves C5 to produce C5a and C5b. Both anaphylatoxin, C3a, and C5a have important roles in regulating inflammation (101). C3a inhibits the migration of neutrophils to sites of acute inflammation (102) whereas C5a has the opposite function (103). The complement system is largely conserved in zebrafish, but there are differences (50). For example, there are two groups of paralogs for C3, *c3a* with six paralogs (*c3a.1, c3a.2, c3a.3, c3a.4, c3a.5*, and *c3a.6*), and *c3b* with two paralogs (*c3b.1* and *c3b.2*), however there is only one C5 homolog, *c5*. A zebrafish study of CRP genes and proteins in the response to SVCV and VHSV infection showed that *crp2*/Crp2 and *crp5*/Crp5 had the largest increases in expression (104).

Interferons and Interferon-Responsive Genes

The innate immune response to viral infection is governed by interferon (IFN) and genes induced by interferon. In mammals, there are three classes of interferon genes (IFNs): type I (α, β, ω, ε, and κ), type II (γ) and type III (λ). Both type I and type III IFNs have well established antiviral activities in mammals, whereas the function of type II IFNs is associated with the response to bacterial infection. Type II IFNs do not exclusively respond to bacterial infection, as they have been associated with the response to vesicular stomatitis virus infection in mice (105). Beyond the type I IFN genes discussed in detail below, zebrafish have two paralogs of the type II IFN, *IFNG*, named *ifng1* (interferon gamma 1) and *ifng1r* (interferon gamma 1 related) (106).

Activation of IFN is a conserved response to viral infection across vertebrates, including zebrafish. One of the first studies in zebrafish showed that IFN expression was induced in zebrafish liver cells when infected by SHRV (58). In addition to the IFN gene first characterized in that study (now named *ifnph1*), zebrafish have three additional IFN genes (*ifnphi2*, *ifnphi3*, *ifnphi4*) that are activated in response to viral infection (**Table 4**) (107, 108). Considerable efforts to identify and characterize IFN genes in fishes have been undertaken, and several excellent reviews describing the complexity of IFN signaling in fishes, including zebrafish, have recently been published (110–112). Type I IFN signaling mediated by zebrafish bears many similarities but also significantly differs from that observed in humans. For example, at the gene level, fish type I IFN (including zebrafish) have retained introns, while mammalian type I IFNs do not. It is thought that the absence of mammalian type I IFNs was a result of a retrotransposition event in amniotes (111). In addition, unlike mammalian type I IFNs, which are typically secreted upon viral induction, fish type I IFNs can be alternatively transcribed with or without signal peptides for extracellular expression (57). Zebrafish type I IFNs can be separated into two groups: Group I and Group II (111). Group I IFNs include Ifnphi1 and Ifnphi4, while group II IFNs include Ifnphi2 and Ifnphi3. Group I IFNs are characterized by a pair of conserved cysteine residues that form a disulfide bridge. Group II IFNs are characterized by two pairs of conserved cysteine residues that form two disulfide bridges (113). Group I and group II IFNs engage different receptor complexes, but each receptor complex is thought to include cytokine receptor family member b 5 (Crfb5) (108). Group I IFNs are thought to interact with Crfb1/Crfb5 complexes, and group II IFNs are thought to interact with Crfb2/Crfb5 complexes. Interestingly, knockdown of caveolin 1 (Cav1) in zebrafish disrupted Crfb1 IFN receptor clusters, thereby decreasing antiviral immune responses (114). Activation of the IFN receptor clusters signal through the Jak/STAT pathway to activate IFN-stimulated genes (ISGs) that

TABLE 4 | IFN genes in zebrafish.

Zebrafish Gene						Predicted Human Ortholog	Ensembl Human Gene ID	Orthology Resource(s)
Gene Symbol	Ensembl Gene ID	Chr.	IFN Type	Refs.	Role in Virus Infection			
ifnphi1	ENSDARG00000025607	3	Type I (Group 1)	(58)	SHRV (58–60)SVCV (51, 55, 56, 61)# (57)INHV (61)IAV (8)TLV (53)CHIKV# (94)			–
ifnphi2	ENSDARG00000069012	3	Type I (Group 2)	(107, 108)	SVCV (51)			–
ifnphi3	ENSDARG00000070676	3	Type I (Group 2)	(107, 108)				–
ifnphi4	ENSDARG00000100678	12	Type I (Group 1)	(107, 108)	SVCV (108) INHV (108)			–
ifng1	ENSDARG00000024211	4	Type II	(106)	SVCV (54, 109) TLV (53)	*IFNG*	ENSG00000111537	ZFIN, Ensembl
ifng1r	ENSDARG00000045671	4	Type II	(106)		*IFNG*	ENSG00000111537	ZFIN, Ensembl

#*Functional study.*

share a IFN-stimulated response element (ISRE) (115). Multiple studies have shown a large set of ISGs in response to viral infection in zebrafish, many of which have mammalian orthologs that are ISGs in mammalian models. Among some of these conserved ISGs are *mxa* (116), *rsad2* (57), and *isg15* (61). One study compared ISGs that responded to a poor IFN inducer, infectious hematopoietic necrosis virus (IHNV), to a strong IFN inducer, CHIKV, with and without knockdown of the IFN receptors, Crfb1 and Crfb2 (117). A study of zebrafish infected with SVCV found that 382 and 926 genes were differentially expressed in brain and spleen, respectively (118). Given that ISGs have antiviral effects and, in some cases, also enhance the replication of viruses (115), more studies are needed to understand the complexity of IFN signaling.

In zebrafish, the *ifnphi1* gene can express two transcript isoforms: a longer, constitutively-expressed transcript, which lacks sequence encoding a secretion signal peptide, and thus is likely retained within the cells, and a shorter, virally-induced transcript, which contains a signal peptide that causes the protein to be secreted (57). Transcripts encoded by the *ifnphi1* gene also exhibit discrete spatiotemporal patterns (108). Basal levels of *ifnphi1* are elevated in adult spleens relative to whole larvae. In both adult and larval fish, viral infection could induce increased expression levels. Using the transgenic zebrafish line *Tg(ifnphi1:mCherry)*, Palha et al. (94) showed expression of mCherry fluorescent protein driven by the *ifnphi1* promoter in hepatocytes and neutrophils following infection with CHIKV. Transcripts encoded by the *ifnphi2* gene were below the level of detection in larval zebrafish and were expressed levels comparable to *ifnphi1* in adult spleens (108). In adult fish, splenic expression of *ifnphi2* transcripts could be induced by SVCV infection. Transcripts encoded by the *ifnphi3* gene are expressed at elevated basal levels in both adult spleens and whole larvae and were not induced by SVCV or IHNV infection (108). Interestingly, expression of *ifnphi3* transcripts were not observed in the same cells in a *ifnphi3* promoter reporter transgenic fish, although these data were shared as part of a personal communication and were not yet published (111). Transcripts encoded by the *ifnphi4* gene are expressed at modest basal levels and are mildly induced by SVCV in larvae (108).

RESPIRATORY BURST RESPONSE

One of the important functions of macrophages and neutrophils during infection and injury is a respiratory (also called oxidative) burst response that functions to recruit additional phagocytes and degrade pathogens. Following a respiratory burst response, reactive oxidative species (ROS), hydrogen peroxide (H_2O_2), and superoxide anion O_2^- are produced by the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (PHOX) complex (**Figure 2**). The PHOX complex is conserved between humans and zebrafish (119). The major catalytic component of PHOX, NOX2, is composed of $p91^{phox}$ (encoded by *cybb*) and $p22^{phox}$ (encoded by *cyba*) and is bound to the phagosome membrane. The activity of NOX2 is stabilized and activated by three regulatory subunits, $p47^{phox}$ (encoded by *ncf1*), $p67^{phox}$ (encoded by *ncf2*) and $p40^{phox}$ (encoded by *ncf4*), along with the small GTPase, Rac (encoded by *rac1*). GTP-Rac interacts with NOX2 that, in turn, interacts with $p67^{phox}$ to activate NOX2 at the phagosome membrane. $P47^{phox}$ has major roles in both NOX2 activation and stabilization at the plasma membrane. First, phosphorylation of $p47^{phox}$ exposes two SRC-homology 3 domains that interact with the proline-rich motif of the NOX2 subunit, $p22^{phox}$. Second, additional PHOX homology domains on activated $p47^{phox}$ can bind the phosphoinositide, phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), that is produced by phosphoinositide-3-OH kinase (PI (3)K). Activated PHOX produces superoxide through the reduction of oxygen into superoxide.

Humans with mutations in PHOX subunits may develop chronic granulomatous disease (CGD), which is characterized by inflammatory disorders, granuloma formation, and increased susceptibility to infection. Individuals with mutations $p91^{phox}$ (*CYBB*), $p22^{phox}$ (*CYBA*), $p47^{phox}$ (*NCF1*), $p67^{phox}$ (*NCF2*), or $p40^{phox}$ (*NCF4*) develop CGD. Zebrafish have been used to model CGD in the context of fungal infection by *Aspergillus*

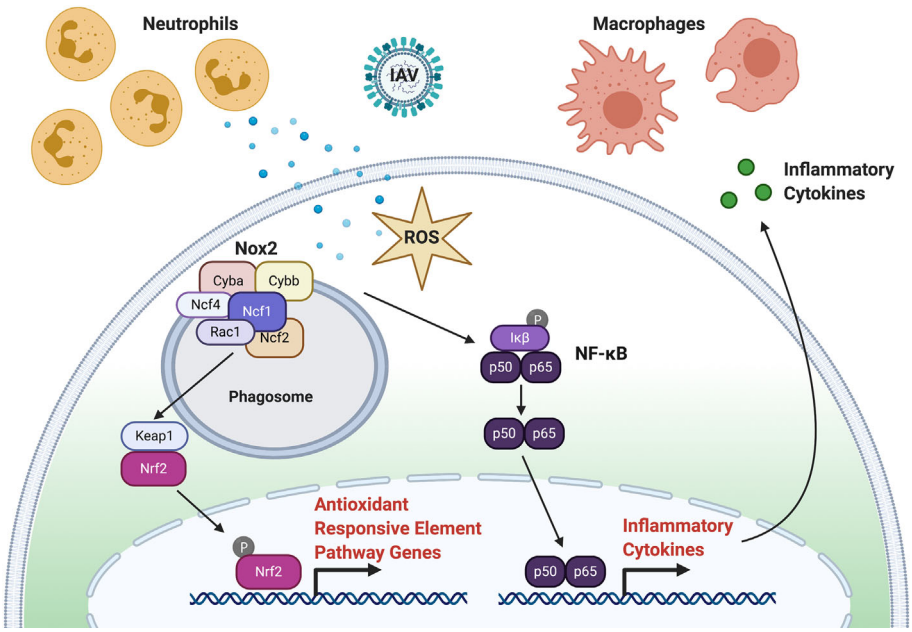


FIGURE 2 | ROS Signaling in Response to Virus Infection. Following infection, production of ROS through the respiratory burst response function to recruit phagocytes (neutrophils and macrophages) to the site of infection and inactivate virus particles. Activation of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (PHOX) complex produces ROS. The PHOX complex is composed to Cyba, Cybb, Ncf1, Ncf2, Ncf4, and Rac1. Activated Nox2 can activate NFκB (p60, p65) that leads to subsequent inflammatory chemokine and cytokine expression. Activated Nox2 can also activate the NRF2 transcription factor through KEAP1 to initiate the expression of antioxidants.

nidulans (120). Zebrafish embryos with a mutation in $p22^{phox}$ (*cyba*^{sa11798}) were observed to have decreased survival to *A. nidulans* infection, similar to what has been observed in CGD patients with fungal infections. Neutrophil migration was disrupted in the homozygous mutants as recruitment that should have peaked at 24 h post-infection (hpi) continued to 96 hpi. Antisense morpholino knockdown of Ncf1 in zebrafish was shown to increase susceptibility to *Candida albicans* infection and decrease the respiratory burst response to infection (17, 18). In other studies PHOX has been inhibited using small molecules, such as diphenyleneiodonium (DPI) (121), VAS-2870, and Phox-I2 (122). DPI was shown to inhibit NOX and the production of superoxide generated by PMA (phorbol 12-myristate 13-acetate)-stimulated macrophages (121). VAS-2870 was first described to inhibit platelet growth factor (PDGF)-dependent NADPH ROS production in vascular smooth muscle cells (123), but has also been shown to inhibit NADPH oxidase activity in regulatory T cells to block the suppression of CD4+ cells (124). Phox-I2 was designed to target the Rac1 GTPase binding site on $p67^{phox}$, and was shown to suppress ROS production in mouse neutrophils (122).

The NADPH oxidase (Nox) gene family in zebrafish is comprised of *nox1*, *cybb*, *nox4*, *nox5*, and the dual oxidases, *duox* and *duox2* (119). While Nox1 and Cybb are part of PHOX and regulated by cytosolic factors, Nox5, Duox and Duox2 are activated by calcium (Ca^{2+}) as they share helix-loop-helix EF-hand domains. Like Cybb, Nox4 is stabilized by $p22^{phox}$, but it is constitutively active. Nox family members also differ by their expression and roles in different tissues. For example, human *NOX1*, *NOX3*, *NOX4*, *NOX5*, and *DUOX2* are expressed in cardiovascular tissues. During the first 2 days of zebrafish embryonic development, the expression of *cybb* was stable during the first 2 days of development with *nox1*, *nox5* and *duox* being more dynamic (125). Zebrafish Duox was shown to be required for the recruitment of neutrophils to fin bud injury by generating a H_2O_2 gradient (26). Duox was also shown to be required for peripheral axon regeneration in zebrafish (126). Several NAPDH oxidase inhibitors have been developed in addition to DPI and VAS-2870, including the general NADPH inhibitor celastrol. Celastrol was shown to have higher inhibitory activity for Nox1 and Nox2 than Nox4 and Nox5 in zebrafish embryos (127). Nox1 inhibitors of human NOX1 include ML171 (128). GKT137831 and GKT136901 were shown to be an inhibitors of mouse NOX1 and NOX4 (129, 130). Specific NOX4 inhibitors include GLX7013114 (131), GKT137928 (132) and ACD084 (133). These and other small molecule inhibitors may be useful to screen for the relative contribution of different NADPH oxidases to inflammatory responses during viral infection.

The amount of ROS production following a respiratory burst response is indicative of the intensity of the immune response and overall health of the organism. A method to assay the respiratory burst response was developed for zebrafish embryos and adult tissues (134–136). This assay measures production of H_2O_2 in response to phorbol myristate acetate (PMA) by detecting the oxidation of dihydrodichlorofluorescein (H_2DCF)

to the fluorescent product, dichlorofluorescein (DCF) to determine the fold induction of the respiratory burst (16). These assays have been used to study how low-dose arsenic reduces the capacity of zebrafish embryos infected with SHRV to mount a respiratory burst response (137). The same assays have been used to measure the respiratory burst response in zebrafish embryos following bacterial (16, 137) and fungal infection (18). A single cell respiratory burst assay has been developed to complement "whole embryo" methods described above (138). Dissociated cells from zebrafish embryos are stimulated with an oxidant, such as rotenone or H_2O_2, incubated with a fluorescent ROS-detecting probe, such as CellROX, and then analyzed using FACS. ROS from specific cell types can be measured by assaying fluorescent reporter lines, such as neutrophils from the *Tg(mpx:EGFP)* line, to measure respiratory burst activity specifically in zebrafish neutrophils. This method has recently been used to study the roles of neutrophils in excessive inflammation following tissue injury in cystic fibrosis transmembrane conductance regulator (*cftr*) zebrafish mutants (139).

Apoptosis of neutrophils at the site of inflammation is one mechanism by which inflammation is resolved. A method to measure neutrophil apoptosis at the site of tailfin injury was developed for *Tg(mpx:GFP)^{i114}* zebrafish embryos using immunohistochemistry to screen for pharmacological agents that could promote neutrophil apoptosis (140). Pyocyanin a phenazine pigment produced by *Pseudomonas aeruginosa*, and roscovitine, an inhibitor of cyclin-dependent kinases, both reduced the number of neutrophils at the site of injury at 24 hours post injury. Agents to delay neutrophil apoptosis and prolong inflammation were also screened. Of the agents tested, the dipeptide pan-caspase inhibitor, benzyloxycarbonyl-Val-Asp-fluoromethylketone (zVD.fmk), decreased neutrophil apoptosis the most. This inhibitor was previously shown to prolong inflammation following tailfin injury in zebrafish embyros (36).

The distribution of ROS in zebrafish embryos has been assayed using high resolution intravital imaging. ROS can be detected using fluorescent imaging of zebrafish embryos treated with the cell-permeable dye, dihydroethdium (DHE), that is sensitive to superoxide (141, 142). DHE has blue fluorescence until it is oxidized by superoxide to form oxyethidium that emits red fluorescence and intercalates with nucleic acids (143). Phan et al. developed a model of bacterial infection that stimulated neutrophil and macrophage activation by injecting *Escherichia coli* into the notocord that was impenetrable by phagocytes (144). The role of neutrophil generated superoxide to clear infection was characterized using this model. Using the DHE assay, superoxide production was observed in neutrophils of infected *Tg(mpx:GFP)* embryos compared to controls. The superoxide response was shown to be neutrophil specific by examining infected embryos treated with Lipo-Clodronate to deplete macrophages, and colony stimulating factor 3 receptor (Csf3r) morphants that had depleted neutrophils.

Intracellular hydrogen peroxide (H_2O_2) production has been visualized in zebrafish using the fluorescent reporter protein, HyPer (26, 145). H_2O_2 production following wounding in the tail bud of zebrafish larvae was visualized *in vivo* in the fluorescent reporter line, *Tg(actb:HyPer)*, that drives the expression of HyPer line using a β-actin (*actb*) promoter (26). This study demonstrated that a gradient of H_2O_2 after wounding was required for neutrophil recruitment to the site of injury. Visualization of H_2O_2 production within neutrophils after wounding was achieved using a zebrafish fluorescent reporter line, *Tg(lyz:HyPer)^{ka4}*, that drives the expression of HyPer line using a *lyz* promoter (145).

Additional Zebrafish Models to Study Neutrophil Function

Several additional zebrafish transgenic and mutant lines have been developed to study neutrophil function. Defects in neutrophil trafficking have been modeled using four different transgenic lines. Humans with Warts, Hypogammaglobulinemia, Infections, and Myelokathexis (WHIM) syndrome have mutations in the chemokine receptor, CXCR4. A zebrafish model of WHIM syndrome, *Tg1(-8mpx:cxcr4b-EGFP)^{uwm3}*, was developed by expressing a truncated Cxcr4b protein tagged with a EGFP reporter in neutrophils using a *mpx* promoter (146). A dominant-negative *rac2* zebrafish line (*Tg(mpx:mCherry, rac2_D57N)^{zf307}*) was used to show that Rac2 was required for neutrophil migration to a tailfin injury (147). As described in the non-coding RNA section of this review, the microRNAs, miR-722 (148, 149) and miR-199 (150), are two additional zebrafish neutrophil trafficking mutants. Defects in Mpx function have been modeled in the "spotless" mutant, *mpx^{NL144}*, which has a premature stop codon in the *mpx* gene (151), and the *durif* mutant, *mpx$^{gl8/gl8}$*, which has cis-acting point mutation in *mpx* (145). Myeloperoxidase activity was absent in these mutants, as assayed using Mpx TSA and anti-nitrotyrosine staining (151). These models are complementary to Csf3r morphants that have depleted neutrophils (144, 152).

ZEBRAFISH MODELS OF VIRAL INFECTION

The zebrafish is a powerful model system for the study of virus infection and host immune response. Initial studies involved using the zebrafish to model fish viruses to develop strategies for mitigation, including fish virus vaccines. These studies often focused on critical factors like temperature and route of infection (immersion and different forms of injection) in order to replicate viral disease observed in other fish species. With time came the recognition that zebrafish viral infection models could also be used to study the host immune responses. These studies have become more sophisticated, moving from the realm of pathology and interferon and interferon-stimulated genes responses to more complex studies examining issues such as immune cell behavior. The zebrafish is uniquely positioned as a model in this regard due to the generation of various transgenic lines that label immune cells such as neutrophils and macrophages. As discussed previously, zebrafish possess numerous inherent advantages that make this type of investigation possible, including near

transparency during the embryonic and larval periods of development, an array of forward and reverse genetics tools, and deeply sequenced genome. These advantages enable directed studies at the host-viral pathogen interface, where it is possible to answer questions about how cells like macrophages and neutrophils work to limit the spread of infection and regulate the inflammatory rheostat. Below is a summary of several viral models that have been developed in zebrafish, including fish viruses, human viruses that infect zebrafish, and xenograft models. Additional information about these and other viruses can be found in **Table 5**.

Fish Viruses for Heterologous Gene Expression

Some of the earliest published virus studies performed in zebrafish used vesicular stomatitis virus (VSV) envelope containing glycoprotein (VSVG) pseudo-typed retroviruses. These efforts demonstrated that it was possible to stably transfer and express

TABLE 5 | Viruses studied in zebrafish.

Virus Family	Virus	Preferred Host	Method(s) of Infection	Zebrafish or Human Receptor
Group I: Double-stranded DNA Viruses				
Herpesviridae	Cytomegalovirus (CMV)	Human	One-cell stage injection with pUL97 plasmid (153)	Human: OR14l1 (154)
	Herpes simplex virus type 1 (HSV-1)	Human	Inoculation by injection in the dorsal telencephalon or olfactory bulb (155–159)	Zebrafish: Hs3st4 (156) Human: HS3ST4
	Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8)	Human	Xenograft (160)	Zebrafish#: Cd209, Itga3b, Itga5 Human: heparin sulfate, CD209, ITGA3, ITGA5
Iridoviridae	European sheatfish virus (ESV)	Fish	Immersion (161)	Unknown
	Infectious spleen and kidney necrosis virus (ISKNV)	Fish	Intraperitoneal injection, natural occurrence (162–164)	Unknown
	Lymphocystis disease virus (LCDV)	Fish	Intraperitoneal injection (165)	Unknown
Group III: Double-stranded RNA Viruses				
Birnaviridae	Infectious pancreatic necrosis virus (IPNV)	Fish	Vertical transfer (female), natural occurrence, immersion, intraperitoneal injection (61, 166, 167)	Unknown
Group IV: Positive Sense Single-stranded RNA Viruses				
Nodaviridae	Betanodavirus (nervous necrosis virus) (NNV)	Fish	Intraperitoneal injection, natural occurrence, immersion (168–171)	Zebrafish#: Hspa8
Caliciviridae	Norovirus (NoV)	Human	Yolk sac injection, immersion (172)	Unknown
Picornaviridae	Cyprivirus	Zebrafish	Natural occurrence (173)	Unknown
Togaviridae	Chikungunya virus (CHIKV)	Mosquito, Human	Caudal vein, aorta (94, 117, 174)	Unknown
	Sindbis virus	Mosquito, Birds	Caudal vein, aorta (175, 176)	Zebrafish#: rpsa Human: heparin sulfate, RPSA
Flaviviridae	Zika virus	Mosquito, Human	Xenograft (177)	Zebrafish#: Axl Human: AXL (178)
Retroviridae	Zebrafish endogenous retrovirus (ZFERV)	Fish	Natural occurrence (179, 180)	Unknown
Group V: Negative Sense Single-stranded RNA Viruses				
Rhabdoviridae	Spring viraemia of carp virus (SVCV)	Fish	Immersion, intraperitoneal injection, duct of Cuvier (51, 57, 61, 108, 181–183)	Unknown
	Snakehead rhabdovirus (SHRV)	Fish	Immersion, intraperitoneal injection (59, 60, 65, 184)	Unknown
	Piscine novirhabdovirus (VHSV)	Fish	Immersion, intraperitoneal injection (61, 185–188)	Unknown
	Infectious hematopoietic necrosis virus (IHNV)	Fish	Intraperitoneal injection, immersion, caudal vein, aorta (61, 108, 117, 166, 189–191)	Unknown
Orthomyxoviridae	Influenza A virus (IAV)	Human	Duct of Cuvier, swimbladder (8, 24, 25)	Zebrafish: Sialic acid (8) Human: Sialic acid
Flaviviridae	Dengue virus (DENV)	Mosquito, Human	Intraperitoneal injection (192)	Zebrafish#: Cd209, Rab5aa, Rab5ab, Hspa5 Human: CD209, RAB5A, HSPA5,
Amnoonviridae	Tilapia lake virus (TiLV)	Fish	Immersion, intraperitoneal injection (53)	Unknown
Group VII: Double-stranded DNA Viruses With an RNA Intermediate in Their Life Cycle				
Hepadnaviridae	Hepatitis b virus (HBV)	Human	One-cell stage injection with transgenic plasmid (193–195)	Zebrafish#: Slc10a1 Human: SLC10A1
	Hepatitis c virus (HCV)	Human	One-cell stage injection with transgenic plasmid (194, 196, 197)	Zebrafish#: Cd81a, Cd81b, Scarb1, Cldn1, Oclna, Oclnb, Npc1l1 Human: CD81, SCARB1, CLDN1, OCLN, NPC1L1

#*Zebrafish ortholog identified using the Zebrafish Information Resource (ZFIN; https://zfin.org).*

genes in zebrafish *via* retroviral vectors (198–200), albeit at efficiencies lower than seen in human cells. Subsequently, the fish rhabdovirus IHNV [also formerly known as Oncorhynchus 1 novirhabdovirus now preferably known as the salmonid novirhabdovirus (201, 202)] and the aquatic birnavirus infectious pancreatic necrosis virus (IPNV) was shown to trigger infections in adult zebrafish following intraperitoneal injection and improve viral infection efficiency (166). In this study, it was noted that the infections particularly affected the head kidney, the principal site of hematopoiesis in the fishes, and that hematopoietic cells were affected. The results supported a role for this approach in complementing VSVG heterologous gene expression studies.

Fish Viruses

Spring Viremia of Carp Virus (SVCV)

The spring viremia of carp virus (SVCV), a species of virus belonging to the genus *Vesiculovirus* of the *Rhabdoviridae* family, is associated with acute infectious dropsy of carp and spring viremia of carp (181). Naturally occurring infections have been detected in numerous cyprinid species, and SVCV has been isolated from Nile tilapia and rainbow trout (203, 204). To better understand the disease process, a model in which adult zebrafish were challenged with SVCV by immersion was developed to mimic a natural route of infection (181). Zebrafish are typically maintained at 28°C-28.5°C to mimic their natural environment. Lethal SVCV infections most often occur at temperatures below 15°C. In order to more closely model a natural infection, zebrafish were acclimated to lower temperatures and exposed by immersion to differing doses of SVCV. Several profound gross pathological changes that resembled natural infections were noted in zebrafish exposed to these lower temperatures; however, many of the histological changes that are typically noted in natural infections (e.g. edema, hemorrhage, inflammation, and necrosis) were not observed. This was attributed to the fact that the zebrafish were not able to mount a robust immune response at 15°C or 20°C as their natural environment is approximately 28°C.

Another larval zebrafish model for SVCV infection was developed in which virus was injected into the systemic circulation *via* the caudal vein (57). Using this model, several ISGs were induced following SVCV infection, including *rsad2*, *mxa*, and *mxb*. Levraud et al (57) further adapted their SVCV model by introducing a morpholino-mediated, loss-of-function approach that knocked down Ifnphi1 expression. Survival to SVCV infection was improved in transgenic embryos that overexpressed *ifnphi1* using beta-actin promoter. In addition, they identified Crfb1 and Crfb5 as subunits of the zebrafish IFN receptor complex, as Crfb1 and Crfb5 morphants lacked an interferon antiviral response to SVCV infection.

Lopez-Munoz et al. (182) developed an immersion model for SVCV infection using zebrafish larvae. They observed that 3 dpf larvae exposed to SVCV at 26°C were susceptible to infection, with 50% survival seen between 3- and 4-days post-infection (dpi). In addition, using their immersion strategy, they observed that SVCV failed to induce a robust antiviral IFN response, although there was evidence of a strong pro-inflammatory response with increased *il1b*, *tnfa*, and *lta* expression. Espín-Palazón et al. (55) applied a larval SVCV immersion model to determine that the pleiotropic pro-inflammatory cytokine Tnfa functioned to inhibit SVCV clearance by blocking autophagy in the host. Using the LC3-GFP autophagy transgenic line [*Tg (CMV : EGFP-map1lc3b)*] (205) and the zebrafish ZF4 fibroblast cell line, the authors found that Tnfa inhibits the formation of autophagosomes during viral infections. Libran-Perez et al. (206) further investigated the importance of autophagy in SVCV infection using the zebrafish larval infection model. They determined that exposure to palmitic acid, an anti-inflammatory compound known to induce autophagy, could increase zebrafish survival and reduce viral load and replication.

There have been three studies aimed at understanding the effects of SVCV infection on the transcriptomes of adult zebrafish (118, 183, 207). Encinas et al. (183) performed a microarray study in an effort to identify genes that participate in multiple pathways in the antiviral response and upon survival and were significantly up-regulated or down-regulated. They argued that specific targeting of these genes with candidate drugs could be an effective strategy in mitigating impacts on fisheries of SVCV. Wang et al. (118) performed a high-throughput RNA sequencing (RNA-Seq) experiment using brain and spleen tissue derived from SVCV-infected and control adult zebrafish. They identified 382 differentially expressed genes in the brain and 926 differentially expressed genes in the spleen. In each study, the authors identified differential expression of genes associated with inflammation and immunity. Valenzuela-Muñoz et al. (207) performed an RNA-Seq experiment comparing the long non-coding RNA (lncRNA) transcriptomes of kidney tissue from control and $rag^{+/-}$ heterozygous adult zebrafish following SVCV infection. As described later in this review, putative functional annotation of candidate lncRNA were assigned using Gene Ontology (GO) terms annotated to protein-coding genes within the proximity of the lncRNA (10 kbp up- or down-stream). Using this approach, the authors identified lncRNA genes associated with adaptive immunity based on their differential expression in the $rag1^{+/-}$ heterozygotes. In addition, they also identified lncRNA genes that could be linked to metabolic processes, including the activation of immune cells, and to positive regulation of TOR signaling, which may lead to the inhibition of autophagy. The authors noted that autophagy has been linked to both pro-viral and anti-viral responses.

Infectious Spleen and Kidney Necrosis Virus (ISKNV)

The infectious spleen and kidney necrosis virus (ISKNV) belongs to the genus *Megalocytivirus* in the family *Iridoviridae*. ISKNV and ISKNV-like viruses infect more than 50 marine fish species and impact fisheries of commercial value (208). In fact, natural infections of laboratory zebrafish have been noted (162). These zebrafish infections exhibited bloating, elevation of scales, and petechial hemorrhaging in adults. Xu et al. (163) developed an ISKNV adult zebrafish infection model using intraperitoneal injections of virus. Zebrafish infected with ISKNV exhibited mortalities and clinical symptoms reminiscent of natural infections, including elevation of scales and petechia. In addition, the virus induced cellular hypertrophy in the kidney and spleen. In a follow-up study comparing the course of ISKNV infection in *Tetraodon nigroviridis* and zebrafish, Xu et al. (209)

showed significant induction of *ifnphi1* and *tnfa* transcription in zebrafish, which is indicative of robust antiviral and pro-inflammatory responses to infection.

Piscine novirhabdovirus (Formerly Oncorhynchus 2 Novirhabdovirus or Viral Hemorrhagic Septicemia Virus [VHSV] or Egtved Virus)

Piscine novirhabdovirus belongs to the *Novirhabdovirus* genus of the *Rhabdoviridae* family and causes a prolific viral disease that afflicts over 50 freshwater and marine species in the northern hemisphere (201, 202). Novoa et al. (185) developed juvenile and adult zebrafish immersion and intraperitoneal injection models for piscine novirhabdovirus infection. They observed that adult zebrafish infected by intraperitoneal injection developed disease similar to that found in nature, with evidence of petechial hemorrhage, exophthalmoses, distended visceral cavities, and erratic swimming behaviors. Further, they observed in the kidney increased expression of gene transcripts associated with antiviral and pro-inflammatory responses, including *tlr3, ifnphi1, mxa, ifng1*, and *tnfa*. Novoa et al. (185) also demonstrated that a recombinant salmonid novirhabdovirus (IHNV) lacking an NV gene, but expressing piscine novirhabdovirus G gene, had dose-dependent protective effects for zebrafish in resisting piscine novirhabdovirus infection, as measured by a significant reduction mortality.

Snakehead Rhabdovirus (SHRV)

Snakehead rhabdovirus (SHRV) belongs to the *Novirhabdovirus* genera of the family *Rhabdoviridae* and is closely related to the other commercially significant viruses IHNV and VHSV. We have previously published a comprehensive characterization of SHRV infection in zebrafish (59). Our laboratory group developed and applied embryonic and adult zebrafish models for SHRV infection to address questions related to the host immune and inflammatory response to infection (59, 60, 65). Zebrafish between 24 hpf and 30 dpf were susceptible to infection by immersion, while adult zebrafish could only be infected by intraperitoneal injection. Infected zebrafish presented with petechia, abdominal redness, and erratic swim behaviors. Histological examination of embryonic and juvenile fish revealed evidence of inflammation, including pharyngeal epithelium and liver necrosis and congestion of the swim bladder by cellular debris. There was also evidence of monocyte accumulation in the infected areas, which is indicative of inflammation. Adult fish infected with SHRV exhibited more localized effects closer to the site of infection, including evidence of inflammation with edema, petechia, and fluid and immune cell accumulation in the abdomen. In addition, SHRV infection by immersion induced expression of antiviral *ifnphi1* and *mxa* transcripts. In another study, Phelan et al. (65) determined that SHRV upregulated expression of the immune genes *traf6* and *tlr3* and slightly downregulated the expression of *irak4* in both embryonic and adult zebrafish. Gabor et al. (60) showed that the overexpression of a full-length Mda5 was protective against SHRV infection, while overexpression of a dominant-negative Mda5 receptor (with a CARD domain deletion) could increase SHRV mortality. Kortum et al. (184) applied the adult SHRV infection model to characterize its effects on polymeric immunoglobulin (Ig) receptor (pIgR) expression. pIgR expression is thought to be regulated by Tlr3 and Tlr4 signaling and to link aspects of the innate immune response to the adaptive immune response (210). Upon SHRV infection, Kortum et al. (184) observed that *pigr* and *pigrl* transcripts were reduced, leading to speculation that SHRV suppresses the immune response, at least in part, through this mechanism.

Zebrafish Picornavirus-1 (ZfPV-1)

Recently, evidence for a natural picornavirus infection in the zebrafish gut was detected in a viral metagenomics analysis of zebrafish gut tissue (173). *In situ* hybridization revealed infection of the apical surfaces of enterocytes, as well as near the mucosal layer and within the lumen of the intestine. While AB zebrafish infected with ZfPV-1 were asymptomatic, the virus appears to be widespread in research facilities, with 56% of the 41 institutions tested exhibiting evidence of infection within the fish populations. The prevalence of ZfPV-1 in wild populations has not been determined. Development of a picornavirus model that can infect zebrafish naturally and not trigger symptoms has the potential to reveal novel insights into the underpinnings of the host-pathogen interaction in a low-level infection. It may be possible to gain an understanding of the role these viruses play in dysregulating immune and inflammatory responses over time, including in the presence of secondary infections, and in affecting embryonic development. In addition, a zebrafish picornavirus model could be applied to test the immune robustness of different zebrafish strains as well as the importance of various immune responsive genes.

As described, there are numerous advantages to modeling fish viruses in the zebrafish. The ability to have an easily maintained, relatively low cost, teleost model to study viral infection makes it possible to study an array of research questions. There are several challenges that need to be overcome in order to model viral disease, including determining the appropriate life stage, potential issues with viral tropism, and especially difficult hurdles related to temperature. Nevertheless, there is now a considerable body of literature demonstrating the usefulness of the zebrafish models in the study of fish viruses and immune response. It is particularly noteworthy that many of these viruses can be modeled during the embryonic and larval periods. This ability to infect embryonic and larval fish enables researchers to ask far more precise questions, particularly in the realm of host-virus interaction and immune response. Future studies should take advantage of these developing models to answer critical questions related to vertebrate immune responses to viruses that are universal and conserved across all species.

Human and Mammalian Viruses

Zebrafish possess many of the same receptors required by human and other mammalian viruses for entry and infection (**Table 5**). The following summarizes some of the human virus research that has been conducted in the zebrafish model. These studies highlight the flexibility of the zebrafish model, particularly with

regard to its ability to acclimate and then be infected by viruses that are typically most virulent in temperature ranges more conducive to humans and mammals.

Chikungunya Virus

Chikungunya virus (CHIKV) is a single-stranded, positive-sense *Alphavirus* that causes acute, febrile illnesses accompanied by severe arthralgia (211). CHIKV is a mosquito-borne virus endemic to Africa, Asia and the Indian subcontinent, although there have been outbreaks in other parts of the world, including in the regions of the Americas (212). Palha et al. (94) developed a larval zebrafish model for CHIKV infection. Using a GFP-labeled CHIKV, the authors observed the development of a systemic infection that largely resolved by 4 days post-infection (dpi). Interestingly, CHIKV infections persisted in the brain parenchyma until at least 7 dpi. CHIKV induced a powerful type I interferon response, as measured by *ifnphi1* expression, that was largely mediated by neutrophils and hepatocytes. The role neutrophils played in producing this antiviral *ifnphi1* response was particularly intriguing because their function in viral infections has not been fully appreciated. These findings were bolstered by experiments that compared the relative importance of macrophages and neutrophils in containing CHIKV infections. Palha et al. (94) observed that reductions in neutrophil populations (induced by morpholino knockdown of Csf3r) made zebrafish more susceptible to CHIKV infection, while macrophage depletion by a drug-inducible cell ablation system led to only a modest increase in disease severity.

Briolat et al. (117) performed microarrays on larval zebrafish that had been infected with either IHNV or CHIKV. Each of these viruses has different disease kinetics and induce differing type I interferon response. While IHNV stimulates a milder type I interferon response, CHIKV induces a far more robust expression. Using the microarray approach, the authors identified a suite of zebrafish ISGs that they could compare to human studies. With this information, Briolat et al. (117) identified ISGs that are conserved across vertebrate species.

Sindbis Virus

Like CHIKV, the Sindbis Virus (SINV) is an *Alphavirus* capable of neuroinvasion. Passoni et al. (175) developed a larval SINV infection model in the zebrafish and observed that the virus could infect multiple organs and replicate throughout the larvae. Further, they established the means by which CHIKV and SINV entered the central nervous system. Based on the data they collected, Passoni et al. (175) speculated that CHIKV enters the CNS by infecting the brain microvasculature endothelial cells at the blood-brain barrier and that SINV enters the CNS through axonal transport *via* the peripheral nerves.

Boucontet et al. (176) observed that larval zebrafish infected with SINV exhibited increased mortality when infected secondarily with the bacterium *Shigella flexneri*. The authors also noted increased bacterial burdens in those animals that were infected with SINV first and *S. flexneri* second. The initial viral infection induced expression of antiviral *ifnphi1*, pro-inflammatory *tnfa* and *il1b*, and anti-inflammatory *Il10* transcripts. It also affected neutrophil populations, function, and behavior. Specifically, Boucontet et al. (176) noted fewer neutrophils and more dying neutrophils in larvae that had been infected with SINV and then *S. flexneri*. Interestingly, they noted an increase in neutrophils by 120 hpi when zebrafish were infected with SINV. The authors speculated that the SINV infection triggers an IFN polarization that renders affected cell populations unable to mount antibacterial responses. They also observed the neutrophils exhibited defects in recruitment to areas of infection, and they attributed this finding to the upregulation of *il10* that was observed. Taken together, these data indicate an important role for neutrophils in containing secondary infections following SINV infections and offer this superinfection model as a means to test these phenomena.

Dengue Virus

Dengue virus (DENV) is a single-stranded, positive-sense, mosquito-borne *Flavivirus* that can induce a broad range of manifestations in infected humans, from asymptomatic to severe flu-like. Recently, Balkrishna et al. (192) described an adult zebrafish model for Dengue virus serotype 3 (DENV-3) infection. The authors collected serum containing DENV-3 from human subjects and then performed intramuscular injections of serum into adult zebrafish that served as carriers to propagate the virus. After 14 days, serum from infected zebrafish was harvested, diluted, and injected intramuscularly into secondary adult zebrafish, which served as the study subjects. Using a qPCR-based approach to measure DENV-3-specific transcripts, Balkrishna et al. (192) observed a viral load that was sustained through 15 days post-injection. Histological analysis of the liver indicated necrosis, increased numbers of inflammatory cells, and increased presence of erythrocytes. Blood smears indicated increasing numbers of leukocytes over the course of infection, decreasing numbers of erythrocytes, and decreased numbers of platelets, which is commonly seen in human DENV infections. Close inspection of caudal fins revealed evidence for DENV-induced hemorrhage that was not seen in control groups. Further, increases in the expression of *ang2*, a pro-angiogenic gene and indicator of inflammation, and *ccl3*, a chemokine, were noted. The ayurvedic herbal drug, Denguenil, was shown to limit the effects of DENV-3 infection in this zebrafish model in a dose-dependent manner, as evidenced by decreased levels of necrosis, reduced numbers of inflammatory cells, and decreased levels of erythrocytes in the liver; decreased number of leukocytes, increased numbers of erythrocytes, and decreased numbers of platelets in blood smears; diminished evidence of hemorrhage in caudal fins; and decreases in the levels of *ang2* and *ccl3* transcripts.

Human Noroviruses

Human noroviruses are single-stranded, positive-sense, non-enveloped RNA viruses belonging to the family *Caliciviridae* and are the primary causes of viral gastroenteritis. Van Dycke et al. (172) recently described a larval zebrafish model for human norovirus infection. Zebrafish at 3 d post-fertilization were subjected to yolk injections of human norovirus collected from the stool of human test subjects. A concurrent set of experiments with mouse norovirus was conducted, but it was determined the mouse noroviruses could not cause infections. The authors

observed that human norovirus replicated in zebrafish, as detected by qPCR assays designed to detect viral RNA copies. These data were supported by ELISA, in which evidence of increased viral antigens was observed. Human norovirus replication was detected by immunohistochemistry in both the intestine and caudal hematopoietic tissue of the larval zebrafish. These findings supported the idea that there is a dual tropism for human noroviruses in zebrafish. Infections with human norovirus also induced antiviral responses in the zebrafish, as evidenced by significant increases in the expression of *ifnphi1*, *mxa*, and *rsad2* transcripts relative to controls. Zebrafish infected with the human norovirus exhibited significant reductions in viral load following exposure by immersion to the antiviral compound 2'-C-methylcytidine (2CMC) (as measured by EIA). These findings demonstrated the utility of this infection model for testing antiviral drugs.

Herpes Simplex Virus – Type 1

Herpes simplex virus – type 1 (HSV-1) is a double-stranded DNA virus that belongs to the *Alphaherpesviridae* subfamily. In humans, HSV-1 may be transmitted by saliva or other bodily secretions. It is most often associated with cold sores, but can also cause an array of other herpetic lesions, including herpetic sycosis, herpes gladiatorum, and herpetic whitlow (213). Burgos et al. (155) developed an adult zebrafish model for HSV-1 infection. Following intraperitoneal injections, zebrafish were monitored for the presence of HSV-1 DNA. Between 1- and 4-days post-infection, zebrafish experienced active infection, as demonstrated by the presence of HSV-1 DNA. In addition, histological examination of zebrafish injected with HSV-1 demonstrated that there was a concomitant inflammatory response, even at sites distal to the site of injection. There were indications of degeneration of secondary oocytes and hemorrhage within the muscle tissue. The authors also noted tropism for neuronal tissue by the HSV-1.

Human heparan sulfate modifying enzyme 3-O-sulfotransferase-3 (3-OST-3) functions as a cellular receptor for HSV-1 infection. Zebrafish express multiple isoforms of (3-OST) (214). Several studies were performed in which the zebrafish 3-OST isoforms 3-OST-2, 3-OST-3, and 3-OST-4 were heterologously expressed in hamster CHO-K1 cells. CHO-K1 cells are normally resistant to HSV-1 infection; however, when the zebrafish 3-OST isoforms 3-OST-2, 3-OST-3, and 3-OST-4, and 3-OST-6 were heterologously expressed, these cells became sensitive to HSV-1 infection (156, 215–217). Interestingly, both zebrafish 3-OST-2 and 3-OST-4 are widely expressed in the central nervous system. Because of this, zebrafish may represent an ideal model in which to study effects of HSV-1 infection on the central nervous system and test potential therapeutics (156).

Ge et al. (157) demonstrated that HSV-1 could infect zebrafish at different larval stages from 48-96 hpf. They noted that HSV-1 infection triggered potent antiviral responses that included the upregulation of IFN and ISGs, including *isg15* and *rsad2*. While they demonstrated that the antiviral response that was generated was mediated through a Sting1-mediated cytosolic DNA sensing pathway initiated by Dhx9 and Ddx41 orthologues, they surprisingly found that cyclic GMP-AMP synthase (*cgas*) was not required for Sting1 signaling. These data support a mechanism by which zebrafish can mount a robust Sting-mediated inflammatory response, as has been demonstrated in other models (218).

Hepatitis Viruses

Similar to DENV, hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus belonging to the *Flaviviridae* family of viruses. In addition to causing hepatitis, or inflammation of the liver, persistent HCV infections can lead to hepatocellular cancer. To date, no vaccine has been developed to prevent HCV infection. *In vitro* HCV studies had proven difficult until the development of subgenomic replicons that replicate autonomously (12, 219). Ding et al. (196) recently adapted a subgenomic replication scheme for use in zebrafish to model HCV replication in a live animal. In their study, the authors demonstrated by the presence of HCV transcripts that replication occurred. In addition, they observed that HCV replication could be inhibited by the drugs ribavirin and oxymatrine. Ding et al. (196) also noted expression of the HCV subgenome transcripts in the zebrafish liver and that this disrupted the expression of homologous genes similarly affected in human HCV-infected liver cells. These data indicated that this zebrafish model effectively recapitulates aspects of HCV infection and may be useful in better understanding the effects of HCV-triggered inflammation on transformation to hepatocellular cancer

Li et al. (220) modified this HCV model to restrict its expression to the zebrafish liver. Using this zebrafish liver-specific HCV subgenomic replication model, the authors observed opposing effects on autophagy when either human ATG10 or ATG10S was overexpressed. Specifically, ATG10 overexpression triggered amplification of the HCV-subgenomic replicons, while ATG10S overexpression caused their degradation. These data, coupled with data from experiments using the autophagy inhibitors 3MA and CQ, provide evidence for how autophagy may influence aspects of HCV replication. Because of the linkages between autophagy and inflammation (221), this model may facilitate studies aimed at understanding these processes in the context of HCV.

Influenza A Virus (IAV)

We have described zebrafish models for IAV infection that resemble human disease (8, 24). We demonstrated that zebrafish possess the α-2,6-linked sialic acid residues on their cells that provide IAV viruses a way to bind, attach, and enter cells. We showed that two different strains of IAV (A/PR/8/34 [H1N] and X-31 A/Aichi/68 [H3N2]) could infect, replicate, and cause mortality when injected into the circulatory system of a larval zebrafish. Using a recombinant IAV strain carrying a GFP reporter (NS1-GFP) (222), we demonstrated the progression of an infection that could be monitored by fluorescence microscopy. In addition to being a model for disseminated infection, we also developed a model for localized IAV infection using the swimbladder (8, 24), which is considered the functional analogue of the human lung in fish (223). Zebrafish infected with IAV produce strong antiviral responses, as measured by increased expression of *ifnphi1* and *mxa*. Zebrafish also exhibit strong pro-inflammatory responses to IAV infection, with increases in the expression of *il1b* and *cxcl8* transcripts observed,

increased NFκB activation as noted in *Tg(6xHsa.NFκB : EGFP)* transgenic fish, and extensive damage to zebrafish muscle fibers, with neutrophils recruited to sites proximal to the unanchored ends of some fibers (25).

Zika Virus (ZIKV)

The Zika virus (ZIKV) is a positive sense, single-stranded, enveloped RNA virus belonging to the *Flaviviridae* family (224). ZIKV is transmitted to humans primarily by some types of *Aedes* mosquitoes (*A. aegypti* and *A. albopictus*), but there are other modes of transmission, including through sexual intercourse, laboratory exposure, blood transfusion, and from mother to fetus during the pre- and peri-natal periods. Most ZIKV infections trigger mild symptoms, including rash, fever, joint pain, and/or non-purulent conjunctivitis; however, ZIKV infections during pregnancy can have profound effects on the developing fetus' nervous system. These may include congenital Zika syndrome (CZS), which is characterized by severe microcephaly accompanying the fetal brain disruption sequence (FBDS), as well as other brain and ocular defects and congenital contractures (225).

Ayala-Nunez et al. (177) developed a xenotypic system in their study aimed at understanding the role infected human monocytes play in disseminating ZIKV to the neural cells. In their model, they labeled human CD14+ monocytes with the dye CellTrace Yellow and injected them *via* the duct of Cuvier into the circulation of 48 hpf zebrafish embryos. By performing live imaging, the authors observed that monocytes infected with ZIKV exhibited increased capacity for transmigration. They also noted that monocytes exposed to ZIKV were more prone to arrest in zebrafish vessels and suggested that this behavior may facilitate attachment to the endothelial cells of the blood vessel. These data support a likely role for the microenvironment in mediating transmigration. We speculate that this zebrafish model could be applied to study the effects an inflammatory microenvironment has on monocyte transmigration when infected with ZIKV. It is worthwhile to note that ZIKV infects human cells that are cultured at temperatures 10°C higher than zebrafish embryos and the cooler temperature may alter function of the ZIKV-infected human monocytes. A follow-up experiment in the same study was performed using a transwell migration assay system in which infected human monocytes were added to a well containing a porous membrane layered with cells mimicking the blood brain barrier. Under the transwell, neural organoids were added. In this experiment, more ZIKV-infected monocytes were observed to migrate across the membrane than control monocytes. Further, the authors noted infection of the neural organoids by ZIKV, which indicated viral dissemination by the monocytes.

These research studies, coupled with the studies conducted with fish viruses, demonstrate the strength of the zebrafish model and highlight several of its attributes, including its fully sequenced genome, which allows for the identification of putative viral receptors that can often be inferred based on homology. The zebrafish model has been aided by the development of a variety of fluorescent reporter lines that label immune and other cells. Regarding host-virus interactions, there has been a wealth of knowledge garnered through the development of alternative vertebrate model systems. Nevertheless, the zebrafish model allows researchers to investigate questions often more difficult to answer in these other models. As an example, alternative vertebrate models for influenza A infection exist, including those in mice, guinea pigs, cotton rats, hamsters, ferrets, and macaques (226, 227). Each have distinct advantages and disadvantages, but none is ideal. For example, the mouse model is limited by the fact that many human influenza A viruses are unable to infect it due to differences in the viral receptors they possess. On the other hand, the ferret model possesses similar viral receptors to humans and mimics the viral kinetics most closely, but it is difficult to use due to its relative size and cost of husbandry, in addition to a lack of reagents and methods. When applied to appropriate research questions, zebrafish can have real advantages over mice, ferrets, and these other vertebrate models, particularly in areas related to neutrophil and macrophage biology. Using the zebrafish model, it is possible to track individual cells and ascertain their role in host defense and host inflammation using the full array of transgenic reporter lines and other reagents available. As described above, in each of the other human viruses tested, the zebrafish model has been utilized to make significant contributions. It is important for researchers interested in modeling virus infections to recognize the strengths and limitations of their respective models. Cross-model approaches have the potential to illuminate areas of host-virus biology that cannot be observed otherwise.

NEUTROPHILS AND HYPERINFLAMMATORY TISSUE DAMAGE

Tissue damage can be caused by neutrophils when they fail to properly resolve inflammation. This can occur when neutrophils become over-activated and/or the number of neutrophils at the site are not reduced. In this uncontrolled response, neutrophils and macrophages recruited by these cytokines can destabilize the vasculature and damage tissues as they migrate to the site of infection as shown in a mouse model of IAV infection (228). Reduction of the number of neutrophils at a site of inflammation can occur by pyroptosis and reverse transendothelial migration. Failure to reduce the number of neutrophils at the site of inflammation can result in tissue damage (229). The inflammatory cytokine, *il1b*, has been shown to have a critical role in prolonged inflammation in the zebrafish notochord that cannot be infiltrated by macrophages and neutrophils during early stages of bacterial infection (230). Knockdown of Il1b was used to demonstrate that Il1b was required for the recruitment of neutrophils to the notochord. The same study also described how neutrophils can degranulate without having direct interaction with a pathogen. A subsequent study identified how neutrophil-generated ROS cleared bacterial infection of the notochord even though neutrophils cannot infiltrate the notochord (144).

Damage to skeletal muscle was observed in a zebrafish model of IAV infection (25). By 24 hours post infection, zebrafish embryos were observed to have mild muscle degeneration with sarcolemma damage and defects in extracellular matrix adhesion.

Confocal imaging of IAV-infected *Tg(mpx:EGFP)* showed that neutrophils localized to sites of fiber damage. Muscular degeneration phenotypes observed in the zebrafish model of Duchenne Muscular Dystrophy, $dmd^{ta222a/ta222a}$, were found to be exacerbated following IAV infection.

TRANSCRIPTIONAL PROFILING TO IDENTIFY STAGES OF HYPERINFLAMMATORY RESPONSE

High-throughput RNA sequencing (RNA-Seq) of bulk tissues has begun to be applied to study zebrafish models of viral infection. The response to SVCV infection in zebrafish was characterized by RNA-Seq in the brain and spleen tissues (118). Levraud et al. (174) used RNA-Seq to characterize the response to CHIKV infection following morpholino-mediated knockdown of Crfb1 and Crfb2. Another important aspect of this study was identifying 97 ISGs that had human orthologs previously identified as ISGs in other studies. Another study of SVCV infection profiled gene expression in kidneys at 24 hpi in six-month old adult zebrafish with and without an impaired adaptive immune system by comparing heterozygous $rag^{+/-}$ and wild-type zebrafish (207). Sixteen proviral insertion sites in Moloney murine leukemia virus (PIM) kinases were recently found to have increased gene expression following SVCV infection in adult zebrafish kidneys at 24 hpi using RNA-Seq, and that three pan-PIM kinase inhibitors blocked viral entry (231). As several zebrafish fluorescent reporter strains have been used for FACS to isolate macrophages or neutrophils for cell-specific functional analysis (38, 48), RNA-Seq could be applied to characterize these FACS sorted cell populations following virus infection. Single cell RNA-Seq (scRNA-Seq) has been applied to study embryonic development (232) and tissue regeneration (233) in the zebrafish. This technology should prove valuable in characterizing the inflammatory response to viral infection and potentially identify genes that differentiate phagocytes between various states of activation.

ROLES OF NON-CODING RNA

Genes function together in complex networks with multiple layers of genetic regulation that include both protein coding and non-protein coding genes. In the Ensembl annotation of the zebrafish genome [Ensembl version 103 annotation of GRCz11 (234)], there are 25,592 protein-coding genes, 3,227 small non-coding, and 3,278 long non-coding genes. These non-coding genes lack long open reading frames, and map to intergenic regions, introns, or antisense to protein-coding genes. Studies of non-coding genes in human and mouse have demonstrated important *cis*- and/or *trans*-regulatory roles in immune function as summarized below.

Long non-coding RNAs (lncRNAs) have transcripts that exceed 200 bp, and are classified based on their genomic location and orientation. Classes of lncRNAs include long intergenic RNA (lincRNA), antisense, bidirectional, intronic, and enhancer-associated RNAs. Diverse functions of lncRNAs have been described. They can function as both positive and negative regulators at the DNA, RNA or protein level in *cis* and *trans*. Some lncRNAs function in the nucleus to interact with chromatin, while others interact with RNAs or proteins in the cytoplasm. An example of a *cis*-regulatory lncRNA is the mouse antisense lncRNA, *Gm14023* (235). *Gm14023*, is antisense to *Il1a* and functions to regulate the recruitment of RNA polymerase II to the *Il1a* promoter following TLR ligand stimulation (235). Examples of *trans*-regulatory lncRNAs include the mouse antisense lncRNA, *Ttc39aos1*, that was originally named, *lncRNA-EPS* (236). A mouse knockout of *Ttc39aos1* and gain-of-function experiments showed that it was required to control the expression of immune response genes in macrophages (236). An example of a lncRNA that has been shown to function in both *cis* and *trans* is the mouse long intergenic RNA, *Ptgs2os2*, that was originally named, *lncRNA-Cox2* (237). Knockdown of *Ptgs2os2* by shRNA showed that the expression of proinflammatory genes (including *Tlr1*, *Il6*, and *Il23a*) was decreased, and chemokines (*Ccl5* and *Cx3cl1*), chemokine receptors (including *Ccr1*), and interferon-stimulated genes (including *Irf7*, *Oas1a*, *Oas1l*, *Oas2*, *Ifi204* and *Isg15*) were upregulated (237).

A study of the role of the adaptive immune system in response to SVCV in zebrafish kidneys found that 12,165 putative lncRNAs were expressed (207). The study examined lncRNA candidates by looking for differentially expressed protein coding genes that mapped to within 10 kbp of the lncRNA and testing for enriched Gene Ontology terms. Among putative lncRNAs investigated were two lncRNAs that map adjacent to *rag1* and *rag2* in the zebrafish genome, suggesting a regulatory role.

MicroRNAs are negative regulators of gene expression that have been shown to be required for zebrafish immune function (49, 148, 149, 238) in addition to embryonic development (239), and tissue regeneration (240, 241). Downregulation of both miR-722 (148, 149) and miR-199 (150) have been shown to be required for neutrophil migration in zebrafish. Studies of zebrafish with systemic *Pseudomonas aeruginosa* PAK strain infection showed that neutrophil expression of miR-722 was required for regulating the inflammatory response through Rac2 (149). Overexpression of miR-722 in the $Tg(lyz:mir722\text{-}Dendra2)^{pu6}$ line had increased survival to lethal inflammation caused by acute *Pseudomonas* infection. A screen of several microRNAs showed that miR-199-3a was required for neutrophil migration (150). Using the neutrophil-specific overexpression line, $Tg(lyz:mir722\text{-}Dendra2)^{pu19}$, it was shown that miR-199 regulates cyclin-dependent kinase 2 (*cdk2*). Hypermaturation of neutrophils and defective interferon signaling was observed in miR-142a and miR-142b double-knockout zebrafish (49). Genes differentially expressed in miR-142 double-knockout included *stat1a* and *irf1b*. The neutrophil inflammatory response to tailfin injury was shown to be regulated by miR-223 by regulating nuclear factor (NFκB) signaling (238). Using miR-223 knockout and multiple miR-223 transgenic lines, it was shown the expression from both neutrophils and the basal and apical epithelium functioned to negatively regulate neutrophil recruitment. NFκB activity, visualized using the $Tg(6xHsa.NF\kappa B : EGFP)^{nc1}$ line, was upregulated following tailfin injury in miR-223 mutants. The contribution of miR-233 expression in neutrophils was studied using the $Tg(lyz:RFP\text{-}mir223)^{pu9}$ along

with a transgenic line that expressed a miR-223 sponge in apical epithelial cells, *Tg(krt4:RFP-bsmir223)*^{pu12}. Specific miR-223 targets identified included *cul1a, cul1b, traf6*, and *tab1*.

MiRNAs are important candidate genes to study in the inflammatory response to virus infection, but miRNAs conserved with humans should be prioritized. MiRNAs are highly conserved across animal taxa in an evolutionary context (242). One of the first miRNAs discovered, let-7, is conserved across metazoa, but other miRNAs, such as miR-722, are only found in teleost fish. MiRNAs are organized into families based on their seed sequence that is used to determine targets. Once a miRNA family evolves, it is rarely lost during evolution. As described in MiRGeneDB (243), the roundworm (*C. elegans*) has 145 miRNAs in 90 families, the zebrafish has 390 miRNAs in 113 families, the mouse has 447 miRNAs in 224, and humans have 556 miRNAs in 267 families. The number of miRNA families correlate with complexity that is estimated by the number of distinct cell types (242). In addition, the complexity of immune systems across metazoa correlates with the number of miRNA families. Studies of miRNAs in the response to viral infection in the zebrafish are promising as a total of 79 families, including miR-199, are conserved between zebrafish and humans (**Figure 3**).

DISCUSSION

Modeling viral infection in the zebrafish and other fishes have provided valuable information about the inflammatory response and other host-virus interactions that are complementary to other model systems. Zebrafish models of viral infection take advantage of the strengths of the model that include genetic tools and reporter lines that allow for *in vivo* imaging. One aspect of the inflammatory response to viral infection that needs additional study is the contribution of neutrophils. As summarized in this review, several existing zebrafish models have been designed to study neutrophil function. Some of these tools have begun to be the applied to study viral infection as the role of the inflammatory response of neutrophils during viral infection is largely unknown.

We hypothesize that there is an immunologic tipping point during viral infection between the beneficial antiviral activity and tissue damaging hyperinflammatory response of neutrophils (**Figure 4**). ROS generated by virus-infected cells may initiate neutrophil chemotaxis during an IAV infection. By recruiting neutrophils to areas of virus-induced tissue damage through the formation of H_2O_2 gradients, these neutrophils may then be retained at the site because the high ROS levels suppress cell motility. ROS play critical roles in the immune response, serving both as indicators of immune dysregulation and as mediators of various immune processes, including neutrophil migration. The roles of ROS in viral infection have not been definitively identified. In addition, type I and type II IFN together reduce neutrophil migration and limit hyperinflammation during IAV infection. The connections linking the effectors of ROS production, however, like the NADPH oxidase and myeloperoxidase, as well as the mechanisms driving the suppression of neutrophil migration by interferon signaling, are unknown. Our hypothesis is that neutrophils, while controlling an IAV infection, trigger excessive inflammation through mechanisms involving ROS production and type I IFN signaling.

The importance of neutrophils in the innate response to viral infection is an ongoing subject of controversy. Zebrafish models of virus infection are uniquely poised to enable characterization of the molecular signals that stimulate neutrophils to migrate *in vivo* and elucidate pathways that lead to generation of ROS and other mediators of inflammation in the antiviral response. Furthermore, studies that model human viruses in zebrafish, such as IAV, have the potential to provide unique insight regulating neutrophil function during the inflammatory and

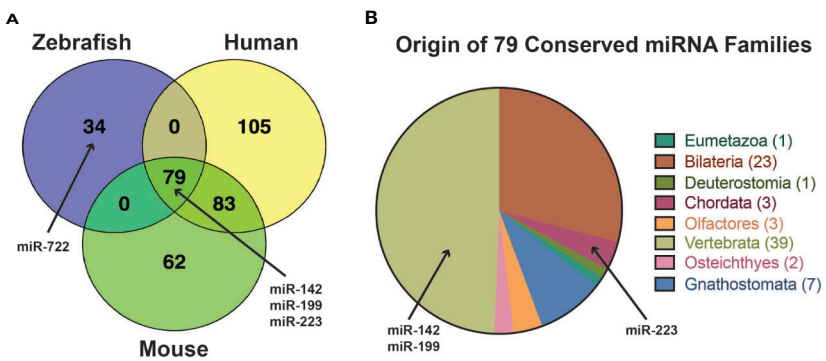


FIGURE 3 | Overlap among miRNA families in zebrafish, mouse, and human genomes. **(A)** 79 miRNA families are conserved among zebrafish, mouse and human genomes, including miR-142, miR-199 and miR-223. 34 miRNA families are found in the zebrafish, but not in the mouse or human genome. One of the 34 miRNA families is miR-722 which was shown to regulate zebrafish neutrophil migration. 62 miRNA families are found in the mouse, but not in the zebrafish or human genome. 105 miRNA families are found in the human genome, but not in the zebrafish or mouse genome. 83 miRNA families are conserved between the mouse and human genomes that are not found in the zebrafish genome. **(B)** The origin of the 79 conserved miRNA families are labeled by the last common ancestor for Eumetazoa, Bilateria, Deuterostomia, Chordata, Olfactores, Vertebrata, Osteichthyes, and Gnathostomata with the number of families shown in parentheses. Two of the 79 miRNAs are miR-199 and miR-223 that have roles in neutrophil function. The node of origin for miR-142 and miR-199 is Vertebrata, and Gnathostomata for miR-223.

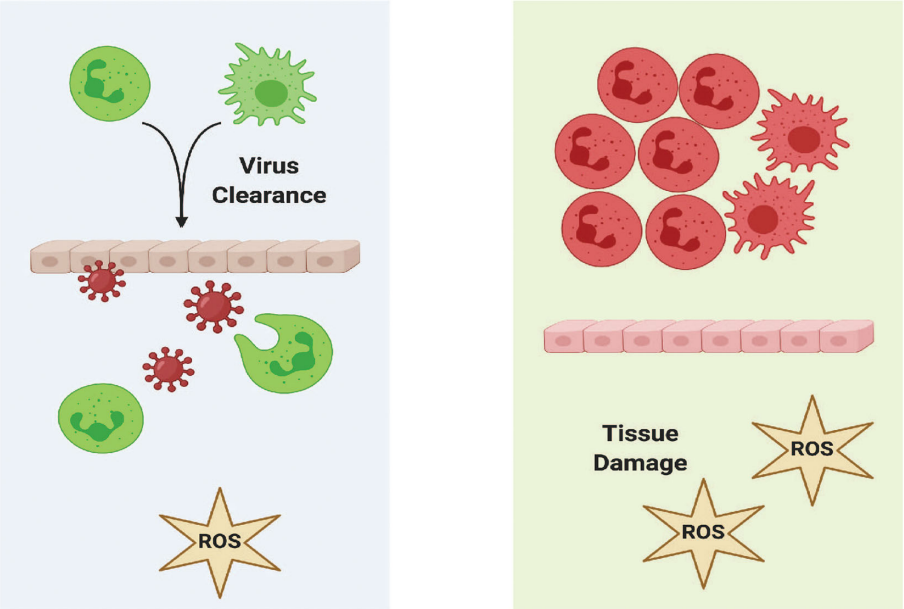


FIGURE 4 | Immunological Tipping Point in The Inflammatory Response to Virus Infection. A balance between the role of antiviral and hyperinflammatory responses by neutrophils must be maintained to avoid tissue damage during infection by IAV or other viruses. We hypothesize that the modulation of ROS is a central factor in regulating the response to virus infection.

antiviral responses. One advantage of the zebrafish model is the potential to screen small molecules to identify potential candidate therapeutics at relatively low cost. One example was demonstrating that the neuraminidase inhibitor, Zanamivir, extended survival in our zebrafish model of IAV infection (8). These advances may inform the development of new treatments that modulate the inflammatory response to viruses like IAV.

AUTHOR CONTRIBUTIONS

CS and BK developed the overall outline of the manuscript. B-LS created all tables and **Figures 1**, **2** and **4**. BK created **Figure 3**. CS, B-LS, CK, PM and BK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Radiation-Induced Immunity and Toxicities: The Versatility of the cGAS-STING Pathway

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In the past decade, radiation therapy (RT) entered the era of personalized medicine, following the striking improvements in radiation delivery and treatment planning optimization, and in the understanding of the cancer response, including the immunological response. The next challenge is to identify the optimal radiation regimen(s) to induce a clinically relevant anti-tumor immunity response. Organs at risks and the tumor microenvironment (e.g. endothelial cells, macrophages and fibroblasts) often limit the radiation regimen effects due to adverse toxicities. Here, we reviewed how RT can modulate the immune response involved in the tumor control and side effects associated with inflammatory processes. Moreover, we discussed the versatile roles of tumor microenvironment components during RT, how the innate immune sensing of RT-induced genotoxicity, through the cGAS-STING pathway, might link the anti-tumor immune response, radiation-induced necrosis and radiation-induced fibrosis, and how a better understanding of the switch between favorable and deleterious events might help to define innovative approaches to increase RT benefits in patients with cancer.

Keywords: radiation, radiotherapy, targeted radionuclide therapy, inflammation, nucleic acids, bystander immunity, cGAS, STING

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INTRODUCTION

In one century, radiation therapy (RT) has become a cornerstone of cancer treatment and is proposed in about 50% of therapeutic schedules. RT goal is to deliver high amounts of energy in cancer cells that will produce unrepairable damage leading to cell death. However, already the first studies on RT reported that healthy tissues, such as skin, are limiting organs showing specific side effects (for instance, erythema and telangiectasia for skin). The amount of energy delivered to tissues was identified as the critical parameter of RT, and the radiation dose in gray units (Gy) was defined for treatment rationalization. It was also observed that tumors and healthy tissues respond differently when the radiation dose is fractionated. Until the 1940s, various dose and dose per fraction were systemically tested to improve RT efficacy and to better protect skin from early and late reactions (1). This led to the standard therapeutic schedule used today: 2 Gy per fraction, 5 fractions per week, and 6-8 weeks of overall treatment time (2). This has been accompanied

by improvements in radiation delivery to the tumor, and the current image-guided radiotherapy systems provide high ballistic precision.

These advances have comforted the target cell theory according to which only tumor cells crossed by radiation will die, ultimately leading to eradication of clonogenic tumor cells and to tumor control. However, exposure of healthy tissues remains a matter of concern (3). Specifically, it has been observed that the response to RT is not the same in all patients, and late radiation toxicities, such as radiation-induced necrosis [RN (4, 5)] and fibrosis (RIF) (6–8), have been described. Besides the intrinsic patient radiosensitivity, total dose, dose per fraction, irradiated volume, and treatment combinations (e.g. endocrine therapy, chemotherapy, history of surgery) (9, 10) could be involved in such side effects.

A new paradigm was established in the 1950s when a possible role for RT-enhanced immune response against cancer cells was suggested. Regression of cancer cells at a distance from the radiation field was reported, leading to the introduction of the *abscopal* effect concept (11). These observations that challenge the target cell theory have been supported by many other studies (12–15), and the immune response role during RT is today strengthened by the benefit observed when combining RT and immunotherapy, which stimulates or suppresses the immune system to help the body fight cancer (e.g. monoclonal antibodies) (14, 16, 17).

Here, we will review how RT modulates the immune response towards a better tumor control or side effects associated with inflammatory processes. After briefly describing the cellular and tissue responses to RT and the different RT modalities, we will discuss how the innate immune sensing of RT-induced genotoxicity might link anti-tumor immune response, RN and RIF, and how a better understanding of the switch between favorable and deleterious events might help to define innovative approaches to increase RT benefit in patients with cancer.

Cellular and Tissue Responses to RT

RT is based on the principle that radiation will produce lethal lesions in exposed cells. This starts with the ionization and excitation of molecules contained in cells, leading to the production of radical species, such as reactive oxygen (ROS) and nitrogen species (NOS) that will damage cell constituents. These damages may be repaired (cells will survive), misrepaired (cells undergo abnormal proliferation), or not repairable (cells will die). Among all the radiation-sensitive targets, nuclear DNA has been the most investigated. Indeed, survival of irradiated cells is closely related to the level of unrepaired DNA double-strand breaks, and the DNA damage response (DDR) plays a major role in the final cellular outcome. Other subcellular targets, such as cell membrane (18–20), mitochondria (21, 22) and lysosomes, also may contribute to the final outcome. It must be noted that cell killing will be more important when the dose and dose-rate increase than when the dose is fractionated or delivered at low dose-rate.

Target cell death upon RT leads to reduction in tissue function (1). As RT delivers high fractionated dose (2 Gy per fraction, 5 fractions per week, total dose between 40 and 70 Gy), the priority is to precisely control the exposure to radiation of tumor cells and healthy tissues. The determinist effects, occurring beyond a certain dose-threshold (>0.5 Gy), are proportional to the dose, according to a *S* shape curve (sigmoid curve), before reaching a plateau at high dose. Therefore, by controlling the dose, it is possible to predict the biological effect, e.g. the tumor control probability. The S curve obtained for healthy tissues (normal tissue complications probability) is quite similar as the one obtained for tumor cells, but the dose threshold is higher. This indicates that the tumor is more sensitive to radiation than healthy tissues when using the previously described fractionated schedule. Therefore, it is possible to define a therapeutic window where tumor growth can be controlled with acceptable side effects. The organs concerned by deterministic effects usually display high proliferation rates (i.e. tumor, skin, bone marrow, digestive tract), but other organs also may be concerned, for instance the nervous system.

RT Modalities and Differential Effects on Tissues

It took more than 50 years of preclinical and clinical data to define the current standard therapeutic schedule of RT. This schedule allows controlling the tumor, while minimizing side effects. At the beginning of RT, the first systems produced low energy X-rays that delivered huge doses to the skin, which was used as the guide for therapeutic schedules. Schedules were progressively improved to deliver the maximum dose not to the skin but to the tumor. This was the beginning of a huge progress in the design/development of technological devices with the final goal of increasing the ballistic accuracy and improving the ratio between disease control and toxicity (23). Three-dimensional conformal radiation therapy (3D-CRT), intensity-modulated radiation therapy (IMRT), stereotactic body radiation therapy (SBRT/SABR) and stereotactic radiation surgery (SRS), proton therapy (and to a lesser extent hadrontherapy with heavy ions) (24), and more recently FLASH RT (25) have progressively been implemented. For example, 3D-CRT aims at delivering radiation to the gross tumor volume with a margin for microscopic tumor extension and a further margin uncertainties for organ in motion, while IMRT allows the oncologist create irregular-shaped radiation doses that conform to the tumor whilst avoiding critical organs. For instance, the optimal radiation technique to treat breast cancer may vary with patient anatomy and laterality of the breast cancer. IMRT provide better conformality of the high dose to the target regions than conventional 3D-CRT, but at the expense of more tissue (contralateral breast and lung) exposed to low radiation doses. Also, due to physical properties, proton therapy improves target coverage and conformality with a high dose volume to the target, and significantly reduces both organs at risks and integral doses. Thus, the more the radiation technique allows a perfect coverage of the tumor shape while avoiding healthy surrounding tissues, the more the dose can be increased (improving the cytotoxic effect of the physical dose), intensificated, or hypofractionated to further improving outcomes.

However, some of conventional RT modalities are not always suitable for the treatment of disseminated or diffuse disease or of tumors located very close to organs at risk because it would lead to an unacceptable exposure of healthy tissues to high radiation doses. Very early, clinical radionuclides were identified as an alternative to RT because they emit radiation and can be used as unsealed sources for intravenous injection. In 1941, iodine 131 (26), which is taken up by the thyroid gland, was the first tested radionuclide for hyperthyroidism treatment, marking the birth of nuclear medicine (27). Recently, Xofigo™ ($^{223}RaCl_2$) has been approved for bone metastasis management in patients with prostate cancer (28). In brachytherapy (also called Curietherapy), radionuclides are locked in a sealed capsule placed close to the tumor (e.g. prostate cancer), and then the radiations cross the capsule and irradiate the localized tumor. In 1951, for the first time, radionuclides were radiolabeled with vehicles, such as monoclonal antibodies against cancer cells (29–31) and later peptides. For instance, Lutathera™ (^{177}Lu-DOTATATE) has been approved for treating neuroendocrine tumors (32–34). However, radionuclide therapy also is associated with side effects due to exposure of healthy tissues. For example, treatment with Lutathera™ strongly increases progression-free survival in patients (32), but whole blood and bone marrow are inevitably exposed to radiation that may lead to long-term toxicities. Subacute hematologic toxicity (grade 3/4) after Lutathera™ has been observed in 11% of patients (35), and long-term safety concerns include myelodysplastic syndrome (MDS) and leukemia (32, 36).

The choice between the different RT modalities depends on the tumor type and its localization. The chosen modality will influence the delivered dose and dose-rate and the nature of the lesions produced in cells. For example SBRT and SRS, which deliver high individual radiation doses with enhanced precision accuracy in only few treatment fractions, can be used to ablate small and well-defined primary tumors anywhere in the body, such as non-small cell lung cancer (NSCLC) (37–39), or brain metastases (SRS) (40, 41). However, these modalities may cause late RIF and RN. RN is a well-characterized effect of SRS and is occasionally associated with serious neurologic sequelae (42). A preclinical study in normal rats whose brain was exposed to a single radiation dose (37 Gy at 30% using a Gamma Knife© device) found vascular disorders and neovascularization (43) with no detectable behavior changes at day 54 post-irradiation. At day 110, rats exhibited large RN surrounded by an increasing gradient (distal to proximal from the RN) of microglia that accumulated near newly sprouted blood vessels, upregulation of Iba1$^+$CD68$^+$ macrophages, and infiltrating CD3$^+$ T cells (44). These effects were accompanied by irreversible neuroinflammation, memory loss and a decrease in anxiety-like behavior (44). In the context of brain RN pathophysiology, there are two main theories whether it is likely that the true cause is multifactorial: i) the vascular injury theory and ii) the glial cell theory. In the first case scenario, radiation disrupts the blood-brain barrier, resulting in increased capillary leakiness and vascular permeability. Radiation, especially in large fraction sizes >8 Gy, activates acid sphingomyelinase and causes upregulation of ceramide, which in turn causes endothelial apoptosis (20, 45). This leads to increased oxygen-free radicals, a pro-inflammatory milieu (through release of tumor-necrosis factor and interleukin-1β) (46), and amongst other increased production of vascular-endothelial growth factor (VEGF). This cascade leads to anarchic vessel sprouting resulting in ischemia and cell death (47). In the second case, radiation can also damage glial cells. Damage to oligodendrocytes and their progenitors result in demyelination (48), accompanied by leaky capillaries, which result in perilesional edema (43, 48). Therefore, it is important to understand the balance between beneficial and deleterious effects of the radiation-induced inflammatory response, and how exposed tumor cells communicate with their microenvironment.

Revisiting the Target Cell Paradigm Accounting for Non-Irradiated Bystander Cell Killing

For about one century, RT has been considered as a ballistic therapeutic approach where radiation is seen as projectiles targeting tumor cells. Accordingly, only cells traversed by radiation will die. There is now a huge body of evidence indicating that irradiated cells communicate with non-irradiated neighboring cells, leading to the so-called bystander response to radiation that includes cytotoxic and genotoxic effects, such as chromatid exchange (49), mutagenic effects (50), micronucleus formation (51) and DNA damage-inducible protein upregulation (52, 53). Besides these short-distance effects, there are long-range effects that involve the immune response activation through the production/release by irradiated cells of pro-immunogenic factors, such as tumor antigens (54), Natural Killer (NK) receptor G2D (NKG2D) ligands that act as danger signals to alert NK cells (55), and through the recruitment of CD8$^+$ T cells and myeloid cells (56) together with the production of type I Interferon (IFN) (57). Simultaneously, RT can lead to immunogenic death of cancer cells (15) that can subsequently favor the immune cell response toward the surveillance and eradication of transformed cells (58). Immunogenic cell death consists in the release of immunostimulatory damage-associated molecular patterns (DAMPs) by dying cells (59), for instance extracellular ATP (60), extracellular DNA (61), nuclear DNA-binding protein high mobility group box 1 (HMGB1) (62), and endoplasmic reticulum chaperones, such as calreticulin (63). Irradiated cells produce also inflammation-related cytokines (e.g. IFNs, IL-1, IL-6, IL-8, VEGF, EGFR, and TNFα) encoded by "early response" genes (64) that are induced within minutes to hours following RT exposure. This is associated with ROS production and cytokine production that will participate in the creation of a DAMP-associated proinflammatory micro-environment. Mediators of systemic effects and DDR/DNA repair components interact also with components of the innate immune response, such as pattern recognition receptors, and with DNA repair proteins (BRCA1, XRCC1, DNA-dependent protein kinases, Ku70/80) (64). For instance, during RT (or chemotherapy), dendritic cells (DCs) require signaling through Toll-like receptor 4 (TLR4) for efficient

processing and cross-presentation of antigen from dying tumor cells (releasing HMGB1). Apetoh et al. demonstrated that *in vivo*, local RT reduced tumor growth on CT26 colon cancers and TS/A breast carcinomas, and prolonged the survival of tumor-bearing immunocompetent wild-type mice, which was less effective in $Tlr4^{-/-}$ and athymic nude mice (65).

Critical Tissues and Cell Response to RT: Bone Marrow and Circulating Blood Cells

Although treatment planning allows delivering most of the radiation dose to the tumor, the surrounding healthy tissues also are exposed to radiation, but at lower doses. Consequently, the surrounding tissues, including the vascular system, also are included in the exposed volume. The tumor and infiltrating immune cells (myeloid cells and lymphocytes), whose number depends on the tumor immune microenvironment (hot, cold, and immune-altered), are also irradiated. Consequently, RT can have detrimental effects on the hematological compartment. Bone marrow aplasia occurs for doses >3 Gy and death due to hematopoietic syndrome occurs upon whole-body exposure to doses that are expected to cause the death of 50% of exposed people (LD50 = 4.5 Gy) (66). When irradiation is not fatal, the number of hematopoietic stem cells returns progressively to normal, but this can take years. Higher intramedullary cytotoxicity due to abnormal hematopoiesis can be observed, although blood formula has returned to normal values. This might be due to RT-linked modifications of the stem cell microenvironment, niches and/or vascularization. Long term effects of irradiation of bone marrow have been reported in patients treated for ankylosing spondylitis (67) or in atomic bomb survivors. They mainly consist of acute leukemia a myelodysplasia occurring between 5 and 10 years after exposure. However their occurrence depend on the dose and have not been observed after RT alone but more after combination with chemotherapy (68, 69).

Bone marrow is a tissue with a hierarchical organization that is involved in the early response to RT. Quiescent or proliferating hematopoietic stem cells are located in bone marrow. Except for T lymphocytes that differentiate in thymus, hematopoietic cells proliferate and differentiate in the bone marrow before entering the blood circulation. During RT, a proportion of stem cells is killed and the negative effect on hematopoiesis is proportional to the irradiation dose. As blood cells have a limited lifespan, blood cell depletion will be detectable after the non-replacement of mature cells by young differentiating cells. The immune cell radiosensitivity depends on the lineage, maturity, and activation status. All bone marrow cells and particularly progenitors are sensitive to RT, and 1 Gy kills about 2/3 of all progenitor cells. Conversely, mature cells, except lymphocytes, are relatively resistant to RT. Lymphocytes are particularly radiation-sensitive, and a decrease in circulating lymphocytes, due to apoptosis, is observed already with 0.3 Gy. At 1 Gy, the decrease becomes significant and occurs within 3 days. B cells and naïve T helper (Th) cells are the most radiation-sensitive, whereas T memory cells, natural killer T cells, and regulatory T cells (Tregs) are more resistant (64).

In recent years, the transcriptional response to radiation exposure has been much studied. This is important because it has been reported that the gene expression signature of blood lymphocytes can help to predict the clinical outcome in human cancers (70). Upon exposure to RT, multiple signal transduction pathways are activated, resulting in complex alterations in gene expression in circulating immune cells [e.g. Kabacik et al. (71)]. For instance, $CD4^+$ and $CD8^+$ T cells produce IFN-γ, contributing to the formation of an inflammatory environment that favors the anti-tumor immunity (72). Irradiated human monocytes and macrophages activate transiently p53- and ATM-dependent mechanisms. The transcriptional factors TP53 and nuclear factor kappa B (NF-κB), which play a central in immune and inflammatory responses by regulating the expression of pro-inflammatory cytokines and chemokines such as TNF-α, induce the expression of inflammatory cytokine-encoding genes, thus establishing a direct link between radiation-induced DNA damage response and radiation-induced inflammation (73).

Circulating leukocytes are only exposed when passing through exposed blood vessels and receive a much lower dose, which is difficult to calculate accurately. Yet, this is a crucial issue because the transcriptional changes observed *in vitro* following exposure of whole blood samples are quantitatively (74) and qualitatively (75) different in function of the dose. High doses induce mainly p53-dependent signaling, and genes involved in the stress response and apoptosis. Their level of expression is dose-dependent down to 10-50 mGy. Low doses predominantly induce the NF-κB pathway and the regulation of genes involved, for instance, in cytosolic DNA sensing and chemokine and cytokine signaling, rather than radiation-induced direct cell killing. NF-κB, p53, breast cancer associated protein 1 (BRCA1) and AP-1 are among the main transcription factors activated by radiation exposure and regulated by the DDR (76), but low doses induce more immune-stimulatory responses (75). Therefore, it can be hypothesized that the dose received by immune cells and consequently the triggered responses are determined by their localization during RT. Interestingly, the influence of the tumor presence on the expression of several stress genes in circulating white blood cells has been investigated, and similar levels of expression in pre-exposure cancer samples and in normal donor samples were observed (77).

Additionally, the type of radiation (X-rays, gamma, proton, beta or alpha particles), the dose rate (around 1 Gy per minute, FLASH irradiation in seconds, or protracted - days - irradiation in targeted radiotherapy, TRT) and the RT type (e.g. IMRT or SABR), which limits the dose to the microenvironment and surrounding organs, can modify the volume of irradiated blood, the dose to circulating leukocytes and consequently the associated transcriptional modifications. This is illustrated by the different modulation of the expression of inflammation genes, such as TGFβ1 (cytokine with anti-inflammatory properties), IL-1β and IL-6 (pro-inflammatory), CCL3 (involved in the recruitment and activation of granulocytes) and IL8 (neutrophil recruitment), in function of the RT type (IMRT and SABR) and total dose (78). For instance, TGFβ may be a major obstacle to the optimal activation of antitumor T-cell

responses by RT. Bouquet et al. demonstrated that TGFβ inhibition prior to radiation attenuated DNA damage responses, increased clonogenic cell death, and promoted tumor growth delay, and thus may be an effective additional therapy in cancer RT (79). Also, in preclinical models of metastatic breast cancer, Vanpouille-Box et al. showed that anti-TGFβ antibodies administered during RT uncovered the ability of RT to induce T-cell responses to endogenous tumor antigens (80). Interestingly, only the combination of RT with anti-TGFβ, but not each treatment alone, induced T-cell-mediated rejection of the irradiated tumor and non-irradiated metastases in mice, indicating that blocking TGFβ unleashes the potential of RT to promote an *in situ* tumor vaccine (80). In addition, TGFβ activation depends on radiation modalities. Vozenin's research team demonstrated that conventional RT (15 Gy) triggered lung fibrosis associated with activation of the TGFβ cascade, whereas no complications have been observed after doses of FLASH below 20 Gy for more than 36 weeks after irradiation (81).

Also, the effects of RT on suppressive immune cells, such as regulatory T cells (Tregs), in the tumor microenvironment (TME) are not fully elucidated. For example, across several tumor models (B16/F10, RENCA, and MC38) Muroyama et al. demonstrated that RT (10 Gy) significantly increased tumor-infiltrating Tregs compared with non-irradiated tumors. The authors found that tumor-infiltrating Tregs from irradiated tumors had equal or improved suppressive capacity compared with non-irradiated tumors, independently of TGFβ (82). Consequently, blocking Tregs infiltration in tumors might be an interesting therapeutic strategy in combination with RT and anti-PD-L1, to overcome RT-induced immunosuppressive Tregs and drive an abscopal effect (83).

In conclusion, there is a direct link between radiation-induced DNA damage-dependent changes in gene expression and radiation-induced inflammation. These changes need to be better investigated to decipher these complex interactions.

Overall, this section showed the complex interaction between ionizing radiation, tumor cells and TME. It also highlighted that not all observed effects are linked to direct radiation damage crossing cancer cells, but also to bystander and systemic effects.

JANUS-FACED TUMOR MICROENVIRONMENT COMPONENTS DURING RT

RT is detrimental for bone marrow and circulating blood cells through their direct irradiation, but it can also *via* its indirect effects, trigger the activation of immune cells, as observed when RT is combined with immunotherapy (84). RT physical parameters, such as dose and dose-rate, are key determinant of the response type. Nevertheless, it must be kept in mind that the immune response can participate in cancer control, but can also contribute to the deleterious inflammatory effects observed in healthy tissues. The balance between radiation-induced immunity and toxicity is influenced by the TME cell composition, architecture and intercellular communications. The role of macrophages, endothelial cells, fibroblasts and mesenchymal stem cells (MSC) in the TME is presented in the following paragraphs.

Macrophages

In macrophages, ionizing radiation induces the pro-inflammatory phenotype that favors their pro-invasive and pro-angiogenic functions *in vitro* (85). This involves the transient activation of p53- and ATM-dependent responses. The transcription factors p53 and NF-κB, which have key roles in the immune and inflammatory responses, regulate the expression of pro-inflammatory cytokines and chemokines, such as TNF-α, and lead to the expression of inflammatory cytokine-encoding genes, thus establishing a direct link between radiation-induced DDR and radiation-induced inflammation (73). Indeed, Mikhalkevich et al. demonstrated macrophages irradiation induced an altered secretory phenotype (through human endogenous retroviruses), characterized by an increase of proinflammatory factors, such as IL-6, IL-1β, TNFα, CCL2, CCL3, CCL8, and CCL20, in addition to an elevated secretion of anti-inflammatory IL-10, which may facilitate their tumorigenic activity (86). In mice xenografted with insulinoma, melanoma or prostate cancer cells and exposed to low radiation doses (2 Gy), macrophages in the TME show increased inducible nitric oxide synthase (iNOS) expression that favors their ability to inhibit abnormal tumor angiogenesis and promote tumor antigen-specific T-cell immunity (87). The activation of a signaling cascade involving NOX2-mediated ROS production, ATM and IRF5 is required in 2 Gy-irradiated macrophages for the acquisition of the RT induced pro-inflammatory phenotype. Moreover, $NOS2^{+}CD68^{+}$ macrophages are enriched in tumor lesions from patients with colorectal cancer showing good response to neoadjuvant RT (88). Interestingly, a study based on the observation that human papillomavirus 16 (HPV16)-positive head and neck cancers are more sensitive to immunotherapy than $HPV16^{-}$ specimens found that IL-6 production by $HPV16^{+}$ cancer cells specifically favors RT-induced macrophage polarization toward an immunostimulatory phenotype, which is linked to the establishment of an effective anti-tumor immunity (89). Furthermore, blockade of IL4/IL14 signaling by inhibiting STAT6 suppresses the induction of the immunosuppressive phenotype in the THP1 human macrophage cell line, thus reducing the radiation resistance of the co-cultured inflammatory breast cancer cell lines (90). Macrophage behavior following radiation appears versatile and influenced by the TME. However, 2 Gy irradiation of mouse macrophages reduces their ability to induce T-cell proliferation *in vitro* (91, 92). The positive impact of macrophages following RT remains largely debated because despite the induction of a pro-inflammatory phenotype, these cells are unfavorable to the establishment of an effective anti-tumor immune response in multiple contexts. In agreement, macrophage depletion upon 10 Gy RT promotes the adaptive immunity and the response to immune checkpoint inhibitors in mice harboring MC38 colorectal cancer cell xenografts (93). Similarly, 25 Gy

irradiation and 4 Gy fractionated irradiation of mice xenografted with TRAMP-C1 prostatic cancer cells drive ARG1, iNOS and COX2 expression in macrophages. Moreover, the transfer of macrophages isolated from 25 Gy-irradiated tumors increases tumor growth *in vivo* (94). Finally, CD163 expression, a marker of immunosuppressive macrophages, is negatively associated with survival in patients with $HPV16^-$ head and neck primary tumors after RT with various radiation modalities (95).

Although macrophage plasticity in response to the radiation modalities and TME might favor the anti-tumor immune response and radiation resistance, this cell type has been constantly associated with RT-induced toxicity. In mice exposed to localized colon irradiation, depletion of monocytes and macrophages using clodronate is associated with a major reduction of colon infiltration by T lymphocytes, an 1.4-fold decrease of colon vascularization and lower collagen deposition in crypts, suggesting a reduction of the fibrotic process (96). In mice, irradiation of the upper region of the right lung (20 Gy as single dose or fractionated) leads rapidly (72 hours) to infiltration by macrophages and neutrophils and later to collagen deposition and fibrosis (week 26) (97). Interestingly, in mice, soy isoflavones increase $Arg1^+$ immunosuppressive macrophage survival, avoid immunostimulatory phenotype activation in interstitial macrophages, and reduce neutrophil recruitment following 10 Gy irradiation to the lung (98). Similarly, treating mice with the anti-inflammatory fucoidan reduces the accumulation of macrophages and neutrophils after 10 Gy irradiation that is associated with decreased expression of CXCL1, TIMP1, MCP1 and MIT2 (99). These modifications in the early response to RT are particularly important because lung fibrosis was strongly decreased in this model. Co-inhibition of PDGF and TGFβ in mice during and after lung irradiation (20 Gy) strongly reduces lung fibrosis and increases mouse survival. Similar results and the concomitant reduction of immunosuppressive macrophage infiltration in lungs were obtained by blocking connective tissue growth factor (CTGF) in mice (100, 101). CTGF blockade might abrogate TGFβ downstream effects (cell mobility and epithelial-to-mesenchymal transition, EMT) on MSCs, fibroblasts and endothelial cells (101), and deeply remodels the lung immune infiltration following RT (102). In a rat model of RT-induced gut toxicity, 25 Gy irradiation of the gut led to increased expression of MMP2, MMP9, VEGF, TGFβ, endostatin and angiostatin. These factors might strongly influence the behavior of endothelial cells (103). In conclusion, most cellular responses associated with lung fibrosis are caused by or linked to infiltration by macrophages with a pro-inflammatory phenotype.

Endothelial Cells

The establishment of an effective anti-tumor immune response depends on the functionality of the tumor vasculature. Yet, ionizing radiation profoundly modifies blood vessel functionality by activating ATM signaling, oxidative stress responses and DAMP signaling in endothelial cells that ultimately drive NRF2, AP-1 and NF-κB activation [for review see Baselet et al. (104)]. Interestingly, genetic engineering allows the specific sensitization to RT of the vasculature or of tumor cells through the conditional knockout of the *Atm* gene in cancer or endothelial cells in a mouse model of lung adenocarcinoma. Strikingly, RT anti-tumor activity is not increased in mice where *Atm* was knocked out specifically in endothelial cells, despite the massive destruction of the tumor vasculature. Conversely, *Atm* knockout specifically in cancer cells strongly increases the response to RT (105). Hence, in some RT modalities, endothelial cells can be killed by radiation, but this does not seems to contribute significantly to the sensitivity to RT. It is noteworthy that regarding ATM signaling in a tumor context, Zhang et al. demonstrated that ATM regulates IFN signaling in pancreatic cancer such that its inhibition induces TBK1 activation and IFN-I production that is further enhanced by RT (106). *In vivo*, the authors showed that ATM silencing increased IFN signaling as well as PD-L1 expression. Consequently, ATM-deficient tumors are sensitized to combination therapy with PD-L1 blockade and RT. The regulation of IFN signaling by ATM represents a connection between the radiation-induced DDR and innate immunity that can be exploited to enhance the efficacy of immune checkpoint blockade therapy.

Exposure of human coronary artery endothelial cells to 10 Gy irradiation (single dose or five fractions of 2 Gy) leads to higher modifications of the DDR, immune response, apoptosis and inflammatory response gene expression profile upon fractionated treatment. DDR and the expression of DNA repair genes were decreased in irradiated cells, while expression of ICAM1, VCAM1, CXCL10, CXCL11, CXCL12, CXCL16, CCL2, CCL5, CCL20, CCL23, IFNE, IFNA4, IL1A, IL1B, IL15, TGFB1, TGFB1, CXC4, CXCR7 and FAS was increased (107). In TNFα pre-activated endothelial cells, exposure to low radiation doses (0.3 to 0.6 Gy) reduces leukocyte adhesion, unlike moderate doses (2-5Gy). This suggests that differences in radiation doses might confer to endothelial cells the capacity to support (<2Gy) or reduce (<0.5Gy) immune cell extravasation (108). Similarly, 2-6 Gy irradiation of endothelial cells increases cancer cell/endothelial cell adhesion *in vitro*, and this effect is enhanced by pre-incubation with TNFα. Furthermore exposure of human umbilical vein endothelial cells (HUVEC) to 2 or 4 Gy photon irradiation increases the endothelial cell monolayer permeability for tumor cells through a mechanism involving ADAM10-mediated degradation of VE-cadherin (109). Thus, through the induction of an inflammatory response, radiation reduces the endothelial barrier permeability and promotes the release of pro-inflammatory factors that orchestrate the architecture of the tumor immune microenvironment. The exact contribution of endothelial cells to the induction or the suppression of an effective anti-tumor immune response upon RT remains unclear. Nevertheless, the implication of these cells in RT-induced cardiac toxicity is well established through the induction of cell death, premature senescence and pro-thrombotic reactions (110, 111). Moreover, deletion of plasminogen activator inhibitor type-1 in endothelial cells protects mice from RT-induced colitis through a reduction of

macrophage accumulation and collagen deposition in the irradiated colon (112). Similarly, inhibition of radiation-induced CCL2 signaling preserves lung endothelial cell function in irradiated mice, reduces macrophage and neutrophil contribution to lung fibrosis, and metastatic colonization (113).

Fibroblasts and Mesenchymal Stem Cells

After cancer cells, fibroblasts are the main cell population in the TME of many solid cancers. They play a crucial role in the TME and cancer progression, and they are usually referred to as cancer-associated fibroblasts (CAFs). CAFs are considered to be extremely resistant to RT, and indeed they are not killed by exposure to high radiation doses (18 Gy) (114, 115). Fibroblasts are normally in a resting state with low transcriptional and metabolic levels, but they can change to a more active phenotype following RT. Once activated, fibroblasts start to produce and secrete many factors, such as cytokines, ROS, nitric oxide (NO) and extracellular matrix components (116), that strongly influence the TME effects on immune and cancer cells. CAFs have been extensively described as suppressor cells for both innate and adaptive immune responses. After a single dose (18 Gy) or fractionate irradiation (3 x 6 Gy), CAFs can inhibit the migratory capacity and pro-inflammatory cytokine secretion of immunostimulatory macrophages, redirecting them toward an immunosuppressive phenotype (117). RT-treated CAFs (1 x 18 Gy or 4 x 2 Gy) also suppress Tcell function and migration through the secretion of soluble factors that inhibit IFNγ and TNFα production by T cells (114).

The CAF secretome after irradiation influences also cancer cells behavior. Upon activation induced by irradiation (1.8, 9, or 18 Gy), CAFs isolated from human colorectal cancers secrete IGF1 that then activates the mTOR pathway in cancer cells, thus promoting their survival and proliferation, especially at high radiation dose (115). Similarly, in a model of pancreatic ductal adenocarcinoma, conditioned medium from irradiated fibroblasts (5 Gy) increases iNOS/NO signaling in cancer cells, activating the production of pro-inflammatory cytokines through NF-kB signaling. The activation of this pathway increases cancer cell aggressiveness, with higher cell growth, migration invasion and metastatic potential (118). CAFs promote cancer cell aggressiveness also by secreting factors that induce EMT. For instance, upon exposure to 4 Gy, CAFs secrete CXCL12 and IL-6 that drive EMT in pancreatic cancer cells, making them more prone to migration and invasion (119). Also, RT-induced-CAF-dependent IL-6 expression plays a crucial role in EMT of esophageal adenocarcinoma cells, as shown by monitoring the effect of conditioned medium of fibroblasts isolated from patients after treatment (chemotherapy and radiotherapy) (120). This CAF-dependent mesenchymal phenotype is also associated with resistance to radiotherapy (120). Most importantly, CAFs influence the TME also by remodeling its structure (121) through the production of collagen, fibronectin and other extracellular matrix (ECM) components (122). Following RT, this process is accompanied by downregulation of metalloproteinase expression and culminates in the accumulation of ECM components. ECM restructuration and the pro-inflammatory and highly oxidative microenvironment created by CAFs can lead to tissue fibrosis (121)

A promising approach to overcome RIF is based on the use of MSCs (123). MSCs migrate to the injured tissue also thanks to the expression of CXCR1 that binds to IL-8 produced by RT-damaged cancer cells (124, 125). There, they can regenerate the damaged tissue through their ability to differentiate into various cell types. Several evidences highlight MSC important contribution to RT-induced vascular injury repair by differentiating into endothelial cells (124, 126). MSC role in RIF repair is also mediated by their immunomodulatory secretome that counteracts inflammation and oxidative stress in fibrotic tissue caused by CAFs and cancer cells (127, 128). Inhibition of RT-derived inflammation by MSCs also decreases the risk of lung metastases after irradiation (124). Moreover, in a mouse model of melanoma, the response to RT (2 Gy) is enhanced by associating local or systemic injection of MSCs (129). Similar results were obtained in a mouse model of irradiated glioblastoma (10 Gy) (125). Hence, MSC administration appear to be a key strategy to counteract RT side effects and improve its outcome.

Altogether, these observations highlight that common mechanisms are involved in RT-induced anti-cancer immunity and side effects. Indeed, the amplification of the anti-tumor immunity and deleterious fibrosis and necrosis are the consequence of bystander transmission of ROS-induced cell stress through macrophages, endothelial cells, fibroblasts and MSC sterile-inflammatory responses. A new component, called STING-mediated innate immune signaling, has recently be added to this complex anti-cancer immunity-side effects cross-talk. Accumulating evidences tend to position this pathway at the interface between RT-induced immunity and toxicity.

THE STING PATHWAY IN RT INDUCED IMMUNITY

To detect pathogens, the mammalian innate immune system has evolved distinct sensing strategies, including extranuclear DNA recognition. Nucleic acid-sensing is based on cytosolic receptors that detect extranuclear DNA or extracellular RNA as DAMP signals. These pathways can trigger cell death in malignant cells and recruit immune cells into the TME, and are investigated as promising adjuvants in cancer immunotherapies (130). To date, one of the major pathways that mediate the immune response to DNA is governed by the DNA-sensing enzyme cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP–AMP) synthase (cGAS) (131, 132). cGAS is activated upon binding to double-stranded DNA (dsDNA). Activated cGAS converts adenosine 5′-triphosphate (ATP) and guanosine 5′-triphosphate (GTP) into cyclic GMP–AMP (cGAMP). Cyclic GAMP acts as a secondary messenger that binds to and activates stimulator of interferon genes (STING), ultimately triggering a variety of inflammatory effector responses (133). In addition, retinoic acid inducible gene-I (RIG-I) and melanoma

differentiation-associated protein 5 (MDA5) might induce growth inhibition or apoptosis of different cancer cell types upon activation by RNA ligands in an IFN-dependent or -independent manner (134). This review focuses only on the cGAS-STING pathway.

Radiation Induces Cytosolic Double-Stranded DNA Accumulation That Is Sensed by the cGAS-STING Pathway

Radiation-induced chromosomal aberrations represent an early marker of late effects, including cell killing and transformation (135). Micronuclei are small nuclei found in the cytoplasm in addition to the primary cell nucleus of mammalian cells and are produced during mitosis by various mechanisms (e.g. acentric fragments, multicentric chromosomes, etc.) (136). When damaged cells go through mitosis, micronuclei may follow four major possible fates: degradation, reincorporation, extrusion, and persistence (137). Micronuclei may be degraded in the cytoplasm after collapse of their nuclear envelope, leading to irreversible loss of compartmentalization during interphase, and are characterized by chromatin compaction (138). Hatch and colleagues observed multiple foci or a single large focus of accumulated γ-H2AX in approximately 60% of disrupted micronuclei located in the cytosol of cancer cells, indicating that DNA damage accumulation is strongly correlated with micronucleus disruption (138). In the context of ionizing radiation, micronucleus production increases in function of the irradiation dose (139) and is correlated with cell killing. Moreover, Piron et al. demonstrated that mis- or un-repaired DNA double strand breaks might lead to micronucleus formation and to mitotic death of damaged cells (140). However, these data suggest that acute cell death associated with low doses and low dose-rate of ^{125}I-labeled antibodies (Auger electron emitters) is not due to defective detection of DNA damage by the cells. Impaired repair of double strand breaks might be involved in the low dose-rate efficacy of TRT using ^{125}I-labeled antibodies in a non-dependent dose-effect relationship (140).

Accumulation of dsDNA in disrupted micronuclei present in the cell cytosol can explain the activation of the cGAS-STING pathway following RT (141). When the nuclear envelope of a micronucleus collapses (138), the DNA content is detected by the cGAS-based surveillance mechanism that links genome instability to innate immune responses (141). Harding et al. showed that cell cycle progression through mitosis following dsDNA breaks induced by 10-20 Gy RT, leads to the formation of micronuclei, which precede activation of inflammatory signaling and are a repository for cGAS (142). For instance, Vanpouille-Box et al. found that cytoplasmic dsDNA was about ten times more abundant in TSA cells exposed to a single dose of 8 Gy or 3 fractions of 8 Gy (X-rays) compared with untreated cells. This was associated with the release of IFN-β and increased expression of IFNAR1 and CXCL10 (143). In addition, it is unclear how cytoplasmic dsDNA is transferred from cancer cells to immune cells, especially to DCs, although transfer *via* exosomes has been suggested (144, 145).

Radiation-induced pro-immunogenic effects in cancer cells are observed in conventional RT with radiation doses from 2 Gy up to 30 Gy or more; however, the optimal radiation regimen to induce a clinically relevant anti-tumor immunity remains to be defined (13, 146). The previous examples about micronucleus and cytosolic dsDNA accumulation suggest a complex relationship between irradiated tumor and host immune system. Vanpouille-Box et al. investigated dsDNA content in the cytosol of cells exposed to radiation (X-rays) doses ranging from 0 Gy to 30 Gy in different murine and human cancer cell lines (143). Surprisingly, they observed that dsDNA accumulates in the cytosol up to a critical threshold when it abruptly decreases at doses between 12 to 18 Gy. The authors demonstrated that doses above this threshold do not confer immunogenicity, mainly due to the dose-dependent upregulation of three-prime repair exonuclease 1 (TREX1). TREX1 is a DNA nuclease with a main role in the degradation of cytoplasmic double- and single-stranded DNA (147). Vanpouille-Box et al. found in TSA cells that upon RT (single doses above 12 Gy), cytosolic dsDNA is cleared by TREX1, precluding the activation of the cGAS pathway to induce type I IFN, therefore abolishing the RT-induced anti-tumor immune response (143, 148).

Cytosolic leakage of mitochondrial DNA (mtDNA) also results in activation of the cGAS–STING pathway (149–151). Mitochondria are sources of ROS that plays a major role in the induction and persistence of oxidative stress following exposure to radiation (152). They are also involved in non-targeted radiation effects (153, 154), suggesting their implication in radiation-induced systemic responses. However, mtDNA is not the primary target of radiation. Friedland et al. used track structure simulations to demonstrate that the probability of DNA double strand breaks induction in mtDNA is about 0.03% at 1 Gy of γ-rays or densely ionizing radiation (155). The involvement of mitochondria in late radiation effects is more likely to be an indirect consequence of ROS generation after irradiation and of the nucleus–mitochondrion signaling pathway. Nevertheless, mtDNA might leak in the cytosol after a direct hit from a charged particle, such as beta particles (e.g. ^{177}Lu, ^{90}Y radionuclides), alpha particles (external α beam or ^{225}Ac/^{213}Bi radionuclides), or Auger electrons (e.g. ^{125}I).

Altogether, these observations highlights the facts that radiation-induced micronuclei and dsDNA are required for anti-tumor immunity induction *via* cGAS sensing and STING activation. However, the radiation regimen (type of particles, dose, fractions, etc.) to obtain these effects in patients is not known yet. In 2014, a phase II clinical trial was started in patients with NSCLC who progressed after chemotherapy and with at least two measurable disease sites to determine whether radiation and immunotherapy with ipilimumab can stimulate the immune system and stop the growth of tumors that are outside the field of radiation (NCT02221739). Patients receive ipilimumab within 24h of local RT initiation (6 Gy × 5 fractions, 3D-CRT or IMRT). In the case of lack of response, a second phase II trial will be performed with a new RT regimen (9.5 Gy × 3 fractions).

cGAMP in Bystander Immunity

The role of cGAS and STING in the bystander communication between tumor and non-tumor cells is linked to the concept of

cGAMP, a second messenger that activates the STING pathway. Deng et al. demonstrated that exogenous cGAMP treatment promotes the antitumor efficacy of radiation (156). In wild-type mice, the cGAMP and radiation combination reduces tumor burden more effectively than cGAMP or radiation alone. Moreover, about 70% of mice in the combination arm showed complete tumor regression at treatment completion (156). All these data indicate that boosting STING signaling activation can enhance tumor growth inhibition after irradiation. Moreover, Liu et al. demonstrated that in mice grafted with B16-OVA melanoma cells (intravenous injection to model lung metastases), nanoparticle-cGAMP inhalation synergizes with fractionated RT (8 Gy × 3 in the right lung) to generate a potent antitumor immunity against melanoma metastases in both irradiated and non-irradiated lungs (157). This combination led to metastasis growth inhibition in the irradiated and non-irradiated lung, and complete regression of lung metastases in some mice, through TME remodeling (157).

Cytoplasmic cGAMP can diffuse to adjacent cells *via* gap junctions (158, 159). Ablasser and colleagues clearly described a unique immune signaling mechanism that comprises cGAMP production by cGAS in the sensing cell, which is transmitted through gap junctions to bystander cells, leading to remote STING activation and subsequent antiviral immunity. Noteworthy, type I IFN-dependent induction of antiviral immunity in bystander cells takes considerably longer, given the requirement of *de novo* transcription and translation. Therefore, cancer cell-derived cGAMP following irradiation could provide a fast antitumor immune response. These data suggest that bystander activation and signal amplification could have a beneficial role in RT; however, cGAMP transfer might at the same time aggravate cancer resistance and the metastatic potential of STING-dependent tumors. For instance, Chen et al. demonstrated that functional CX43-based gap junctions between cells allow cGAMP transfer from cancer cells to astrocytes (159). This leads to the activation of the STING pathway and the release of cytokines, including IFNα and TNF, which provide a growth advantage to brain metastatic cells by protecting them against physiological and chemotherapeutic stresses. Unlike the transfer of cGAMP to bystander cells that intensifies the immune response, cGAMP transfer from brain metastatic cells to neighboring astrocytes triggers downstream signaling that supports metastatic outgrowth.

Schadt et al. proposed that cGAMP, and not cytoplasmic dsDNA, is transferred from cancer cells to DCs in a CX43-dependent manner, thus enabling the production of type I IFN and antitumor immunity priming (160). This connexin-dependent transfer of cGAMP was corroborated by Pepin et al., who observed the potentiation of macrophages cultured with the conditioned medium of cGAMP producing cells (161). Similarly, Marcus et al. showed that cGAMP, and not dsDNA, is transferred from cancer cells to DCs (162). Indeed, experiments using transplantable tumor models in STING- and cGAS-deficient mice revealed that cGAS expression by tumor cells is critical for tumor rejection by NK cells. Conversely, cGAS expression by host immune cells is not necessarily required, suggesting that tumor-derived cGAMP is transferred to non-tumor cells where it activates STING (162). These observations raise questions about the molecular mechanism involved in the fusion of sEVs purified from tumor cells with recipient (bystander) cells. Indeed, it would be important to know what surface molecules allow their fusion with the recipient cell membrane for cGAMP or dsDNA delivery into the cytosol. Alternatively, other mechanisms could also contribute, such as formation of channels between the apposed membranes of a sEV and the recipient cell (163).

Overall, these studies demonstrated that cancer cell irradiation leads to cGAMP release in immune cells and that STING has a major role in immune cells in radiation-induced immunity, while it is not required in tumor cells. Furthermore, Bakhoum et al. showed that the cGAS-STING pathway is activated in human cancer cells with chromosomal instability. Improper segregation of chromosomes during cell division leads to the formation of unstable micronuclei, releasing their DNA into the cytosol. In this study, Bakhoum et al. demonstrated that inflammatory response involves activation of NF-κB signaling and promotes metastasis in a STING-dependent manner (164). Accordingly, our recent data suggests that STING expression in lung cancer cells might contribute to tumor formation and that low STING expression in these cells fails to induce type-I IFN expression and potentially favors the establishment of an immunosuppressive microenvironment (165). **Figure 1** summarize the bystander communication between cancer cells and immune cells.

The STING Pathway in the Induction of the Senescence-Associated Secretory Phenotype and of RT-Induced Adverse Effects

Through DDR activation, ionizing radiation is a potent driver of accelerated cancer cell senescence, a process that involves ATM, ATR, DNA-dependent protein kinases (166), p53, P16INK4a, p21WAF1, CHEK1 and CHEK2 (167), in breast cancer, colon carcinoma, neuroblastoma and fibrosarcoma. Although senescent cells have exited the cell cycle, they can maintain an active metabolic activity and participate in resistance to therapy and disease progression (168). Indeed, senescent cells can secrete many different bioactive molecules, such as cytokines, proteases and growth factors that influence and shape the surrounding microenvironment. This has been described as Senescence-Associated Secretory Phenotype (SASP) (169). Among the many SASP factors, IL-6, CCL5, CXCL12, CCL2 and IL-8 have a particularly important role in supporting cancer cell metastasis formation and the establishment of an immunosuppressive microenvironment, although in some cancer models they can be found in the immune stimulatory secretome (**Figure 2**).

As RT can induce tumor cell senescence, NK cell recruitment by SASP factors could be a general mechanism by which NK cells help to clear tumor cells in response to senescence-inducing therapies (170). Indeed, in a mouse model of radiation-induced osteosarcoma, the retinoblastoma tumor suppressor gene (RB1) is required for SASP expression and infiltration of NK T-cells in

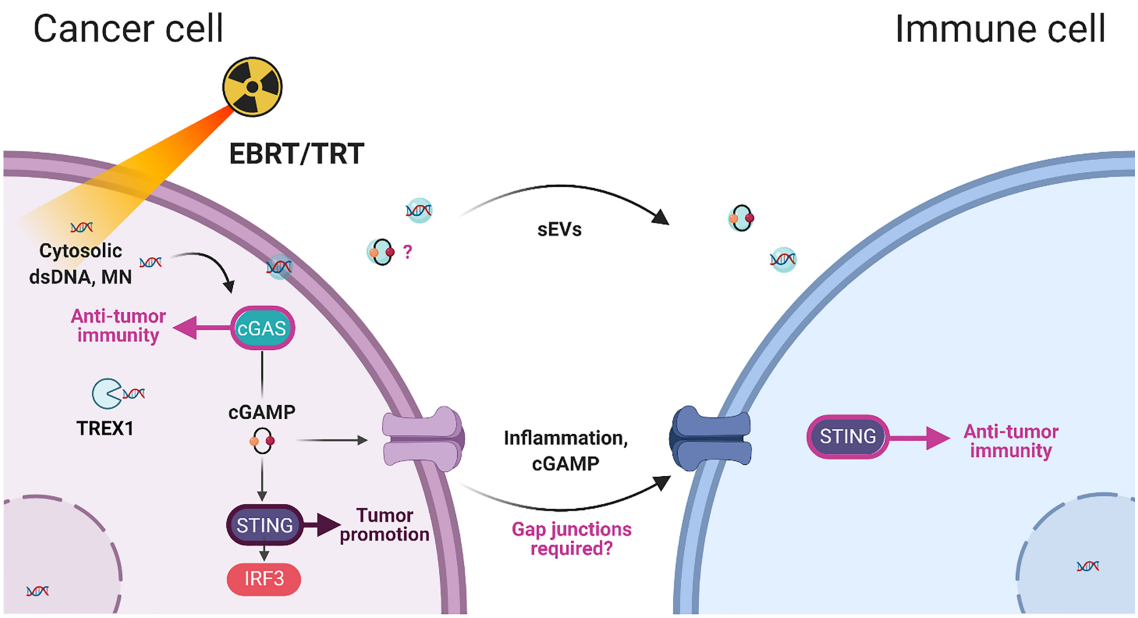


FIGURE 1 | Summary of cancer-immune cell interactions after irradiation (EBRT, external beam radiation therapy; TRT, Targeted Radionuclide Therapy) and the involvement of dsDNA,double-stranded DNA; MN, micronucleus; sEVs, small extracellular vesicles; and cGAMP, cyclic GMP–AMP in bystander immunity.

bones of mice exposed to carcinogenic doses of ^{45}Ca (four postpartum injections; low energy beta-emitting particles) (171). Il-6 and MIP2 (the murine homolog of IL-8) induce neutrophil accumulation *in vivo* (172), and MIP2 is also implicated in NK T-cell recruitment to the spleen (173). Kansara et al. showed that *Cd1*$^{-/-}$ mice, lacking NK T cells, are predisposed to ^{45}Ca-induced osteosarcoma development when crossed with *Trp53*$^{+/-}$ mice, consistent with previous findings that NK T-cells play an important role in sarcoma development (174). Growth inhibition of IL-6-deficient osteosarcoma cell lines in wild type mice is accompanied by NK T-cell infiltration, further supporting a role for these cells in host-dependent tumor suppression *in vivo*. Interestingly, in this model, IL-6 not only recruits NK cells that limit tumor growth, but also reinforces the senescence phenotype through autocrine and paracrine mechanisms (171), indicating that bystander (initially) non-senescent tumor cells can be targeted as well (**Figure 2**, left panel). Better understanding how radiation induces SASP factors (dose, fractions, etc.) production by osteoblasts could be beneficial for the management of patients with bone metastases treated with Xofigo^{TM} (^{223}Ra, alpha emitting particles) among whom some reported jaw osteonecrosis (175).

Extranuclear DNA sensing *via* the cGAS-STING pathway might play a major role in radiation-induced SASP. The involvement of cGAS in senescence induction has been shown in primary human lung cells (IMR90) in which cGAS and STING knockdowns abolish expression of key SASP-related markers (p16INK4a, IL-8, CXCL1,2,3, IL-6 and CCL2) upon senescence induction with HRasV12 or etoposide. Senescence induction is also reduced in STING knockout mice, as indicated by the absence of hair greying three months after sublethal irradiation, and the impaired immunosurveillance against N-Ras (liver tumor formation) (176). Senescence induction in p53-proficient cells is an important protection mechanism against cell transformation upon oncogenic signaling activation (PTEN loss, Ras signaling). Hence, activation of the cGAS-STING pathway in cells during oncogene-induced SASP is also tightly linked to the expression of the cytoplasmic exonuclease MRE11, TREX1 and DNase2 that rapidly degrade cytoplasmic DNA fragments (177, 178). Whereas DNases mediate the clearance of dsDNA, an excessive amount of DNA escaping from DNases is responsible for induction of type I IFN, through the activation of DNA sensors such as the cGAS-STING pathway. Conversely, cGAS, STING, TBK1 and IRF3 knockdowns are characterized by reduced p21 expression in HeLa cells that leads to higher mitotic activity and ultimately chromosomal instability (179). Altogether, these observations demonstrate that the cGAS-STING pathway might play an important role in maintaining chromosome integrity through senescence induction, and that in this context this pathway also contributes to SASP instauration in cancer cells. However, senescence induction and SASP are intrinsically linked to a functional p53 pathway, and the functionality of the STING-IRF3 pathway in cancer cells harboring p53 mutations has not been investigated yet.

On the other hand, SASP induction following ionizing radiation promotes tissue fibrosis (180). For instance, type-II pneumocyte (181) and alveolar stem cell (182) senescence contributes to RIF in lungs. Similarly, endothelial cell senescence induced by RT is causal in the establishment of cardiovascular disease (183). Considering the critical role of STING signaling in the expression of the complete SASP phenotype, STING expression in endothelial cells and

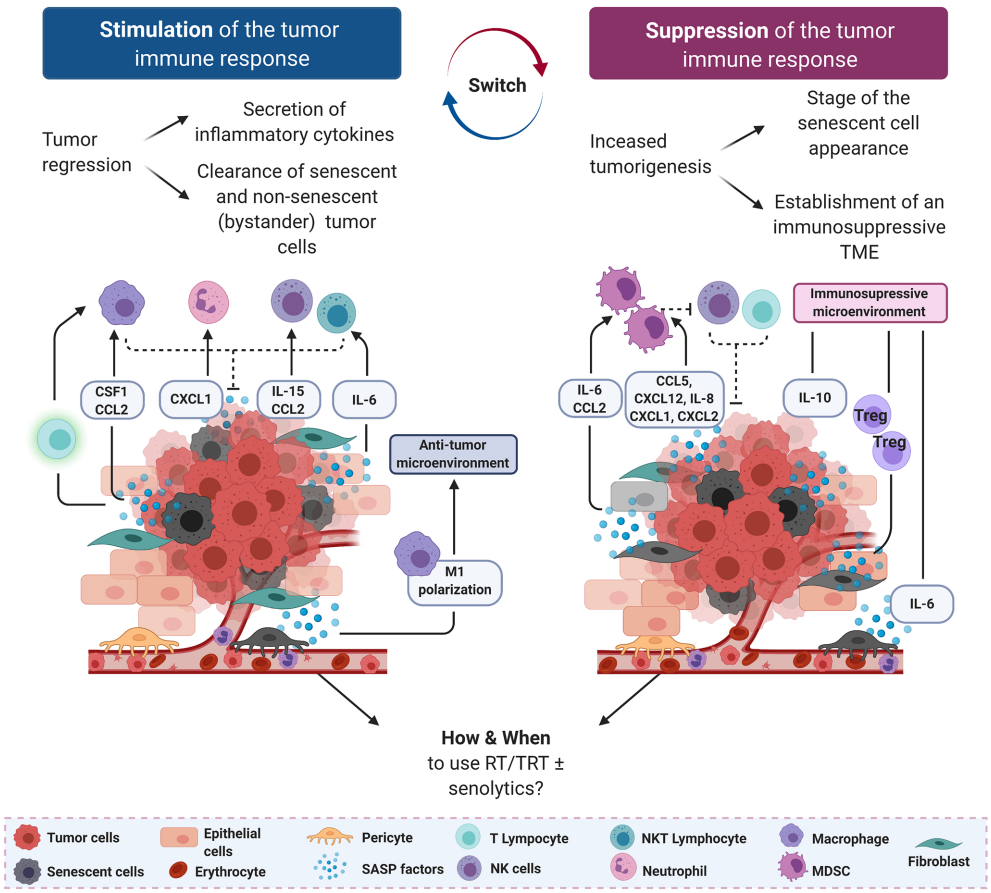


FIGURE 2 | Senescence-associated secretory phenotype (SASP) factors can support or suppress anti-tumor immune responses. On the left, in an immunostimulatory scenario, SASP factors secreted by tumor cells and pericytes drive the recruitment of innate immune cells (macrophages, neutrophils, natural killer (NK) and NK T cells) to mediate the clearance of senescent tumor cells. On the right, in an immunosuppressive scenario, SASP factors secreted mostly by stromal cells recruit immature myeloid cells and myeloid-derived suppressor cells (MDSCs) to dampen the cytotoxic effect of NK cells and CD8+ T lymphocytes. Anti-inflammatory mediators, including IL-6 and IL-8, are also secreted by senescent stromal and tumor cells, further increasing the immunosuppressive environment. Senescent cells are represented by a gray cytoplasm, regardless of their origin. CCL, C–C motif chemokine ligand; CXCL, C–X–C motif chemokine ligand; NK natural killer; NKT, natural killer T lymphocyte.

pneumocytes might directly contribute to these RT-induced deleterious effects. *In vitro*, irradiation (2 Gy) of human coronary artery is sufficient to activate the STING pathway and consequently type-I IFN expression (184). Furthermore, STING contributes to cardiac hypertrophy and fibrosis in a model of pressure-overload cardiac hypertrophy through the recruitment of inflammatory macrophages and the release of angiotensin-II (185). STING might also play an important role in the endothelial cell response to RT. Indeed, tumor-derived cGAMP can drive endothelial cell activation, leading to upregulation of adhesion molecules (V-CAM1, I-CAM1) and T-cell recruitment. Constitutive STING activation (due to a mutation) drives microvessel thrombosis and pulmonary syndrome development in infants through an autoimmune reaction, leading to chronic inflammation and macrophage recruitment (186). This reaction that involves endothelial cell dysfunction and chronic sterile inflammation is reminiscent of RT-induced lung fibrosis and maculopathy. All these data suggest that STING signaling in endothelial cells might contribute to the anti-tumor immunity through recruitment of immune cells. However, most of the observation made *in vivo* and in patients suggest that endothelial cell STING signaling could also be an important player in RT-induced cardiac toxicity (187) and possibly lung fibrosis. The impact of STING expression in fibroblasts on RT response remains to be elucidated. Finally, these studies suggest that because many current standard treatments for cancer can induce senescence, which can have wide-ranging effects, some patients might benefit from the addition of senolytic therapy to inhibit the pro-tumorigenic stroma.

Altogether, these observations highlight the key position of STING signaling following RT where it contributes to cancer immunogenicity, DC activation and anti-tumor T-cell response, while simultaneously playing a central role in SASP induction in many cell types. This might be an initiating event towards the aggravation of RT-induced cytotoxicity.

CONCLUDING REMARKS

In the past decade, RT entered the era of personalized medicine, thanks to the striking improvements in radiation delivery, treatment planning optimization, and better understanding of the cancer response. However, the next challenge is to identify the optimal RT regimen to induce a clinically relevant anti-tumor immune response. Indeed, bystander and abscopal effects have been demonstrated in preclinical studies and in some clinical cases, but the exact dose threshold and range need to be defined in function of the tumor type and characteristics, and the patient's immune status. We hypothesize that radiation could be used as an immunological adjuvant, by lowering the dose per fraction (and/or the total dose) in "hot" tumors, specifically to preserve the viability of intra-tumor lymphocytes. Conversely, the dose could be increased in "cold" tumors. However, healthy organs at risks and the TME often limit the radiation regimen possibilities due to the high risk of adverse toxicities. For instance, radiation reduces the endothelial barrier permeability, facilitating the release of pro-inflammatory factors that orchestrate the architecture of the tumor immune microenvironment. Also, RT-activated macrophages have been repeatedly associated with RT-induced toxicity. Therefore, it is important to find how to modulate macrophage activation to avoid deleterious phenotypes.

RT involves the activation of an anti-tumor response through cytosolic dsDNA sensing by the cGAS-STING pathway. However, a major open question is how to choose the most effective radiation regimen to increase dsDNA accumulation without reaching the critical threshold leading to the activation of DNases, such as TREX1. Interestingly, tumor and immune cells can communicate through the transfer of cGAMP and sEVs, demonstrating that cGAS expression by host immune cells is not necessarily required, while STING is.

Furthermore, there is still an important gap of knowledge on the cGAS-STING pathway role in cancer cell SASP induction upon RT. STING signaling following RT contributes to cancer immunogenicity, DC activation and anti-tumor T-cell response, while simultaneously playing a central role in SASP induction in many cell types. SASP induction is involved and most probably is an initiating event in the aggravation of many RT-induced cytotoxicity events. Radiation dose threshold and SASP are linked through the expression of cytoplasmic DNases, such as TREX1. Once again, the fine-tuning of radiation dose regimens should allow an optimal anti-tumor immune response while limiting adverse effects.

OUTSTANDING QUESTIONS

- What are the optimal radiation dose regimens and fractions?
- What is the best therapeutic window to enhance RT anti-tumor immune response?
- How is the cGAS-STING pathway playing an important role in cancer cell SASP induction upon RT?
- What is the impact of STING expression in fibroblasts exposed to radiation?
- In which conditions inhibitors (e.g. ATM, STING) should be concomitantly administered with RT?
- Whether and when, during cancer development, a senolytic treatment or a drug targeting the SASP should be employed?

AUTHOR CONTRIBUTIONS

The review results from the discussion and the consensus of all authors listed (JC, JF, CU, CB, and J-PP). Literature review on the topic was analyzed, produced and written by JC, JF, CU, CB, and J-PP. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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