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Vascular- and immuno-metabolism as drivers of cardiovascular disease: insights obtained from omics approaches

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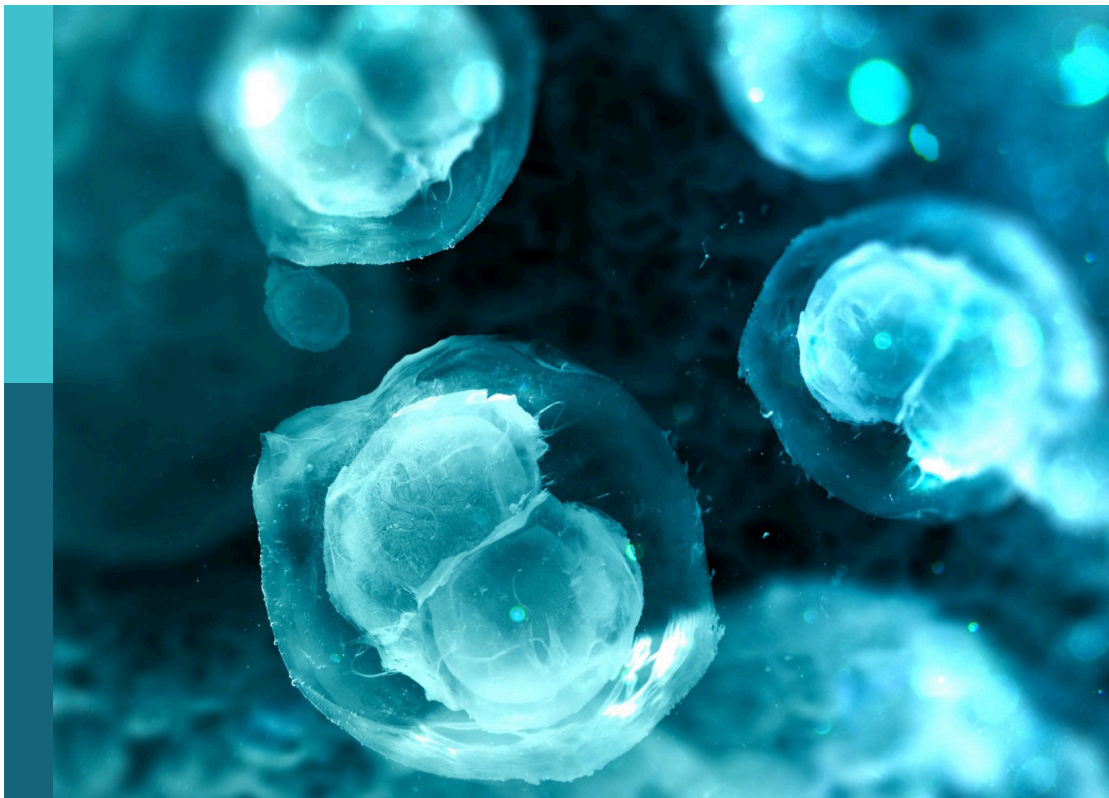

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Vascular- and immuno-metabolism as drivers of cardiovascular disease: insights obtained from omics approaches

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Editorial: Vascular- and immuno-metabolism as drivers of cardiovascular disease: insights obtained from omics approaches

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Editorial on the Research Topic
Vascular- and immuno-metabolism as drivers of cardiovascular disease: insights obtained from omics approaches

Despite previous achievements in the management of cardiovascular disease (CVD), and the fact that the mortality rate from CVD has declined over the last 50 years, atherosclerosis, the chronic condition responsible for the occurrence of a myocardial infarction (MI) and stroke, remains one of the primary causes of global morbidity and mortality. Due to the rising aging population in combination with an increase in cardiometabolic risk factors, primarily driven by the obesity epidemic, the number of individuals affected by CVD is still rising. Therefore, it is of utter importance to develop new strategies aimed at reducing CVD risk and elucidate the molecular mechanisms and important players of atherosclerosis.

Over the past years, it became increasingly clear that atherosclerosis is a multifactorial disease that is not only driven by lipids but also by vascular damage and inflammation (Ajoolabady et al., 2024; Doring et al., 2024; Kong et al., 2022). Compelling evidence that inflammation play a crucial role in atherosclerotic CVD was provided by CANTOS, performed in 2017. Here it was shown that a monoclonal antibody targeting interleukin-1b, termed Canakinumab, effectively reduced CVD risk and mortality, especially in patients characterized with residual inflammation. This

effect was independent of lipid-level lowering (Ridker et al., 2017). In late 2019, the inflammation hypothesis of atherosclerosis was confirmed in COLCOT, using the anti-inflammatory agent colchicine in patients with recent MI (Nidorf et al., 2020). A follow-up randomized clinical trial in 2020 applying colchicine involving patients with chronic coronary disease (LoDoCo), also showed significant risk reduction (Tardif et al., 2019). These landmark studies set the stage for identifying drug targets that block atherosclerosis-specific inflammatory pathways as a highly promising strategy to reduce cardiovascular risk.

It is now undisputed that cellular metabolism is important in fueling many pro-atherosclerotic processes in the plethora of cells involved in the disease progression, ranging from endothelial to smooth muscle cells, neutrophils, T and B-lymphocytes and monocytes (Bories and Leitinger, 2017; Domingo et al., 2024; Zhao et al., 2023). Advancing omics technologies provide unprecedented insights into cellular mechanisms, offering a comprehensive and unbiased view of metabolic and immune functions (de Winther et al., 2023; Zhang and Schmidlin, 2024). The articles in this Research Topic provide crucial insights into the role of both vascular- and immuno-metabolism, as important players and drivers of CVD, which is of utmost importance to be able to offer new therapeutic approaches to combat CVD progression.

One of the main challenges of multi-omics approaches is to obtain material for various omics techniques from the same cell population. In their research article, Del Barrio Calvo and Bindila describe a phenotyping approach in which simultaneous extraction of lipids, metabolites and RNA from single cell populations is employed, enabling multi-omic molecular profiling of very low cell numbers. Furthermore, they phenotype MyD88-knockout macrophages as proof of principle and demonstrating the potency of their approach.

Another original research manuscript by Ma et al. deploys bioinformatic analysis and machine learning approaches to evaluate shared pathogenic mechanisms between atherosclerosis and ankylosing spondylitis. They identified ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 (ST8SIA4), a polysialyltransferase located in the Golgi apparatus as a key diagnostic marker in the progression of both atherosclerosis as well as ankylosing spondylitis, revealing a component of the common pathological mechanism.

Moreover, Rauterberg et al. investigates the impact of Proprotein convertase subtilisin/kexin type 9 (PCSK9) on the heart function after MI, one of the main clinical outcomes due to atherosclerosis development, showing that the lack of *Pcsk9* in mice improves survival post-MI. Interestingly, Alirocumab (PCSK9 inhibitor) treatment did not replicate these beneficial effects in mice, highlighting that there seems to be important mechanistic differences and differential outcomes between PCSK9 pharmacological inhibition and genetic deficiency.

In the context of MI, Peletier et al. summarizes the current state-of-the-art of cardiovascular 3D models in the context of myocardial ischemia-reperfusion injury (IRI). This elaborate review particularly focusses on the key aspect of cell-cell communication and the potential of multi-omics approaches in these models to enhance our understanding of IRI.

Besides these original research articles, the Research Topic also includes several comprehensive reviews. Indeed, Pi et al. provide a detailed overview of the evolution of atherosclerosis and the involvement of innate and adaptive immune cells in this pathology. Particularly, omics studies, especially single-cell RNA-sequencing studies are discussed to highlight the large degree of cellular heterogeneity within the different immune subsets. These insights are further supported by a review by Annink et al. which also highlights the importance of inflammation and innate and adaptive immune cells in atherosclerosis, emphasizing various approaches that are being pursued in order to identify novel therapeutic targets in this context. Another review focusses specifically on type 2 innate lymphoid cells (ILC2s), which have recently emerged as major regulators of the pathogenesis of various cardiometabolic diseases (Kral et al., 2023). Kral et al. provide a comprehensive overview of the current understanding of ILC2s in inflammation and metabolic disorders. In this review, particularly recent omics studies are discussed that provided crucial insights into the molecular and cellular characteristics of ILC2s, which thereby enhance our understanding of the diversity of this cell type and their involvement in metabolic diseases. Moreover, a review by Dai et al. describes the metabolic cellular changes in macrophages, neutrophils, vascular endothelial cells, vascular smooth muscle cells, and lymphocytes in the context of atherosclerosis and comorbidities. An elaborate understanding of such changes is crucial as it could be shown that various diseases can impact the cellular metabolism, while *vice versa* an altered cellular metabolism can also severely impact disease development.

Another key player in atherosclerosis development is the NOD-like receptor protein 3 (NLRP3) inflammasome, which has been studied extensively already in this context. The systematic review by Miao et al. provide a valuable overview regarding the NLRP3 inflammasome research field over the last decade in the context of CVD. Their analysis reveals leading contributors to the field of NLRP3 research and highlights main pathogenic mechanisms of the NLRP3 inflammasome, like oxidative stress, pyroptosis, and inflammation.

In conclusion, this Research Topic aims to provide a series of articles covering all aspects of how vascular- and immuno-metabolism impact CVD and how metabolic modulation could be used to alter disease progression and thereby contribute to improved diagnostic and therapeutic treatment options in the future.

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EV: Writing–original draft. JK: Writing–review and editing. RG-P: Writing–review and editing. YD: Writing–original draft.

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Current research status and future prospects of NLRP3 inflammasome in cardiovascular diseases: a bibliometric and visualization analysis

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Background: Cardiovascular disease (CVD) is a leading cause of global mortality, with atherosclerosis (AS) contributing to its pathological basis. Inflammation plays a critical role in the pathophysiological process of AS, and the NOD-like receptor protein 3 (NLRP3) inflammasome has been extensively studied in this context. This study aimed to analyze the research status of the NLRP3 inflammasome in cardiovascular disease and provide research directions for further exploration in this field.

Methods: Using the "Bibliometrix" and "CiteSpace" software, a total of 516 articles were retrieved from the Web of Science (WoS) database published between 2012 and 2023. The search query used the keywords "["CVD" OR "cardiovascular disease"] AND ["NLRP3 inflammasome "OR "NLRP3"]". Visual analysis was performed on authors, countries, institutions, journal sources, keywords, references, and future trends.

Results: A total of 516 English articles were retrieved, showing an overall upward trend in annual publication volume with slight fluctuations. China, the United States, and Europe were the countries and regions with the highest number of published articles. Among them, China had the highest article count (170), while the United States had the highest citation count (18,664), centrality score (0.43), and h-index (90), indicating its influential role in this research area. These countries also possessed elite institutions, professional researchers, and high-impact journals, making them leading contributors in this field. The main pathogenic mechanisms of the NLRP3 inflammasome in CVD were identified as "oxidative stress", "pyroptosis", and "inflammation". The most frequently studied signaling pathways included "NF-κB", "IL-1", and "C-reactive protein". The most studied disease types were coronary heart disease, atherosclerosis, metabolic syndrome, and myocardial infarction. Additionally, research on the correlation between cholesterol markers and inflammatory indicators associated with NLRP3 inflammasome in CVD risk assessment has gained significant momentum, with the main mechanism being NLRP3/IL-6/hs-CRP and cholesterol lipoproteins emerging as a major keyword in this context.

Conclusion: This study provides valuable insights into the research hotspots and emerging trends of the NLRP3 inflammasome in cardiovascular disease. The findings offer guidance for researchers and scholars in this field and facilitate the exploration of new research directions.

KEYWORDS

cardiovascular diseases (CVDs), bibliometrics, CiteSpace, NLPR3 inflammasome, visualization analysis

1 Introduction

Despite significant advancements in the diagnosis and treatment of cardiovascular disease (CVD), it remains a leading cause of global mortality. The latest statistics released by the American Heart Association (AHA) reveal that CVD is one of the primary causes of death worldwide (1). In 2019, there were a total of 9.6 million male and 8.9 million female deaths attributed to CVD, accounting for approximately 30% of global deaths, which may be attributed to China having the highest number of CVD-related deaths (2). Atherosclerosis (AS) is a chronic inflammatory disease of the vasculature. Mediated by various risk factors (3), it is the pathological basis of CVD and is characterized by a long course with mild early symptoms, often leading to missed diagnoses and delayed treatment. Multiple studies have confirmed that inflammation is one of the main factors in the pathophysiological process of AS. Pro-inflammatory states, associated with different inflammatory mediators, are closely related to endothelial dysfunction and the development of AS. Various cytokines and inflammatory factors interact in the vascular wall to respond to endothelial injury, abnormal lipid metabolism, and hemodynamic disorders, thereby inducing chronic inflammation in the vessel wall (4). Given that excessive inflammation, platelet activation, and endothelial dysfunction increase the risk of thrombosis in atherosclerotic cardiovascular disease (ASCVD) (5), anti-inflammatory therapy is therefore a feasible strategy and a new target for treatment of this patient population.

The NOD-like receptor protein 3 (NLRP3) inflammasome, a crucial component in the pathogenesis of AS, has been extensively studied in recent years as a major causative agent of cardiometabolic diseases (6). The NLRP3 inflammasome regulates the inflammatory response in the body and is composed of NLRP3, CARD, Caspase-1, and cysteine asparaginase recruitment structural domain (ASC). Activated NLRP3 can interact with CARD8 to activate Caspase-1, leading to pyroptosis (7). The landmark study of anti-inflammatory and antithrombotic effects in AS, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), has opened a new era of anti-inflammatory treatment for ASCVD (8). Subsequent trials such as the Colchicine Cardiovascular Outcomes Trial (COLCOT) and the LoDoCo2 trial (9–12) have further confirmed the involvement of specific inflammatory pathways in human ASCVD and emphasized the NOD, LRR, and NLRP3 inflammasome-related pathways as effective therapeutic targets for alleviating ASCVD. Therefore, anti-inflammatory treatment targeting the NLRP3 inflammasome may become one of the most effective therapeutic approaches for cardiovascular disease. This study aimed to use visualization analysis software ("CiteSpace" and "Bibliometrix") to explore the research trends and hotspots related to the NLRP3 inflammasome in cardiovascular disease over the past 12 years.

2 Materials and methods

"Bibliometrix" is a software program developed by Massimo Aria, Corrado Cuccurullo from the University of Naples, Italy, and Luigi Vanvitelli from the University of Campania. Written in R language, it features an intuitive and well-organized interface for conducting comprehensive bibliometric analysis. Functionalities include support for various database sources, performance analysis, and comparative analysis of visualization options (13). "CiteSpace" is a visualization software developed in 2003 by Professor Chaomei Chen of Drexel University. Programmed in Java, CiteSpace measures the literature in a specific field and constructs visual maps to represent it (14). Within CiteSpace software, each node represents an evaluated object. The larger the node diameter, the more entries it represents. Colors differentiate the years of publication. Lines connecting nodes reflect collaborative or co-citation relationships between entries, with the thickness of the lines indicating the degree of closeness in those relationships. Thicker lines signify stronger relationships (15). Centrality is a metric used to assess the importance of an element within the network. Elements with a centrality greater than 0.1 are depicted with a purple ring, highlighting their relative significance (16). When Q is greater than 0.3 and the mean profile value is greater than 0.5, it suggests a sufficiently significant clustering structure with good homogeneity, leading to convincing results (17). This software enhances understanding for relevant practitioners and provides crucial support for analyzing research hotspots and trends in a specific field. Therefore, in this study, we utilized both "Bibliometrix" and "CiteSpace" to visualize and analyze the literature pertaining to the NLRP3 inflammasome in CVD research. Our focus encompassed publication trends, sources, core authors and teams, countries and institutions, keywords, and research trends. Overall, we sought to provide a scientific basis for the systematic promotion of anti-inflammatory therapy in CVD.

2.1 Data sources

This study used the Web of Science (WoS) database, a well-established data source that provides databases and citation data in the life sciences, social sciences, physical sciences, and health sciences (13). WoS is a large and recognized database that contains abstracts and references of high-quality and influential scientific papers (18–21). The time span of this search ranged from 2012 to 01-01 to 2023-12-31. The search terms used were as follows: TS = ["CVD" OR "cardiovascular disease"] AND TS = ["NLRP3 inflammasome "OR" NLRP3 "]. The main types of literature chosen for this study were articles and reviews, limited to the English language, and resulted in 525 documents.

2.2 Data processing

To ensure a comprehensive analysis, this study employed specific inclusion and exclusion criteria for articles retrieved from the WoS database. Included documents were restricted to the English language and full-length publications encompassing either articles or reviews. Conversely, materials such as

proceeding papers, book chapters, and meeting abstracts were excluded. (See Figure 1 for the detailed flowchart).

The retrieved 516 documents from the WoS database were exported in plain text format (*.txt) and then imported into both the Bibliometrix (R4.3.2) software and CiteSpace (6.1. R6) for visualization and analysis.

3 Results

3.1 Descriptive statistics

Table 1 presents the key findings from the visual analysis, which will be discussed in more detail in the following sections.

3.2 Annual distribution of publications

Figure 2 depicts the annual publication volume of CVD-related NLRP3 inflammasome research. The volume steadily increased from 2012 to 2016, followed by a transient decrease in 2017. A subsequent surge emerged from 2017, reaching a peak of 94 publications in 2022. The year 2023 saw a decline in publications. Overall, despite minor fluctuations, the publication trend for this field exhibited a clear upward trajectory. The average number of citations (mean TC) per year also demonstrated an upward trend from 2012 to 2014. However, it fluctuated between 2014 and 2016 before showing a downward trend from 2017 to 2023.

To visualize the collaboration landscape of authors, we used "Bibliometrix" software and configured the following options: number of nodes: 20, local citation score (LCS) 10, global citation score (GCS) 50, and label: short ID (author, year). This resulted in a co-citation network (Figure 3) with 18 publications and 3 clusters, which may shed light on the observed fluctuation in publication numbers. Notably, in 2013, the FREIGANG team (GCS:243, LCS:11) initiated influential research on "CVD and inflammation" (Figure 2). Four years later, in 2017, publications by FUSTER (GCS:820, LCS:22) and VAN DER HEIJDEN (GCS:237, LCS:34) confirmed the pathogenic role of the NLRP3 inflammasome in AS and the potential of its inhibition for mitigating the disease. This likely contributed to the subsequent rise in research on "NLRP3 and CVD".

3.3 Sources and cocited journals

This study analyzed publications on "CVD" and "NLRP3 inflammasome" from 2012 to 2023. Table 2 lists the top 10 journals based on the number of articles published, with the International Journal of Molecular Sciences leading with 26 articles (5.04%). Table 3 showcases the top 10 journals in terms of literature influence, encompassing a total of 269 journals included in the analysis. These influential journals included Frontiers in Cardiovascular Medicine (16 articles, 3.10%),

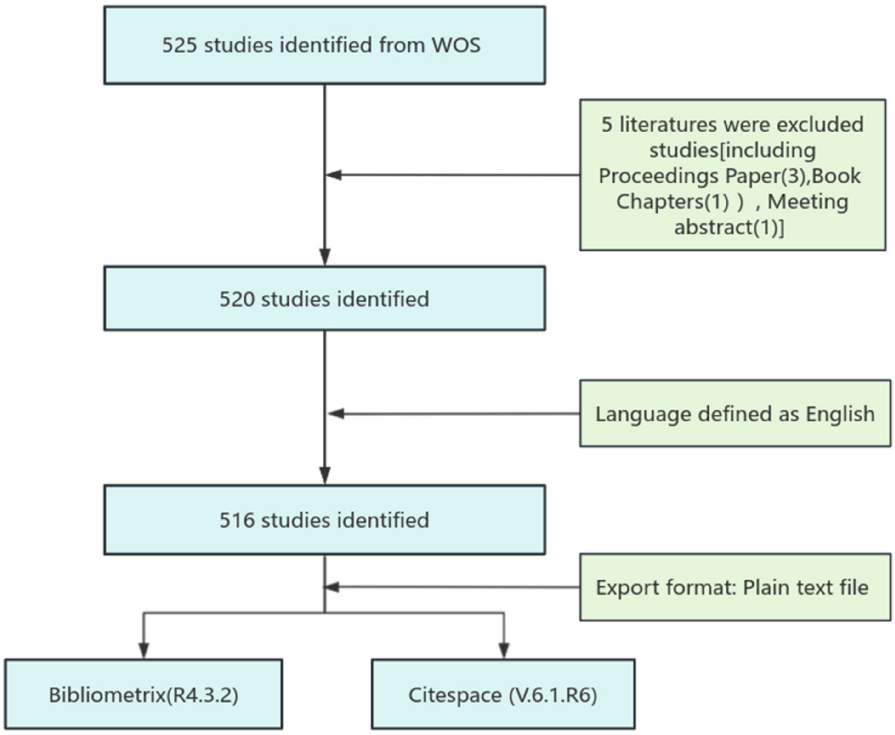

FIGURE 1
Detailed process for literature screening.

TABLE 1 Main information.

Description	Results
Main information about data	
Time span	2012:2023
Sources (Journals, Books, etc.)	269
Documents	516
Annual growth rate %	39.68
Document average age	4.03
Average citations per doc	48.11
References	39,766
Document contents	
Keywords plus (ID)	1,801
Author's keywords (DE)	1,211
Authors	
Authors	3,004
Authors of single-authored docs	11
Authors collaboration	
Single-authored docs	12
Co-authors per Doc	6.78
International coauthorships %	25.58

Frontiers in Pharmacology (13 articles, 2.52%), Frontiers in Immunology (10 articles, 1.94%), Cells (9 articles, 1.74%), Circulation Research (9 articles, 1.74%), Frontiers in Physiology (9 articles 1.74%) Antioxidants & Redox Signaling (8 articles, 1.55%), Biomedicines (8 articles, 1.55%), and Cell Death & Disease (7 articles, 1.36%).

An analysis of the top 10 journals by publication volume (Table 2) revealed that CIRCULATION RESEARCH boasts the highest Impact Factor (IF) score of 20.1, signifying its exceptional influence within the field. Notably, 70% of these top-publishing journals were classified as Q1 journals, indicating their placement in the highest quartile based on citation impact. The remaining 30% fell under the Q2 category. In recent years, the H-index has emerged as a prominent metric for evaluating academic contributions and predicting future scientific output (22). Examining the top 10 journals based on literature influence (Table 3), we observed that the International Journal of Molecular Sciences held the highest H-index value (12). This underlined its significant impact within the field from 2012 to 2023.

An analysis of journal co-citation network centrality revealed that CIRCULATION was the most frequently cited journal with a total of 380 citations, followed by NATURE with 364 citations, and PLOS ONE with 342 citations (Table 4, Figure 4B). Interestingly, among these top 10 most-cited journals (Table 4), the New England Journal of Medicine achieved the highest IF score of 158.5. Additionally, 80% were categorized as Q1 journals, indicating their placement in the highest quartile based on citation impact, with the remaining 20% falling under the Q2 category. The AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE (0.09) had the highest centrality score among the journals analyzed (Table 5). Free Radical Biology and Medicine (0.05) and Biochimica et Biophysica Acta —Molecular Cell Research (0.05) followed closely in centrality scores. These journals with high centrality scores demonstrated a significant influence within the field.

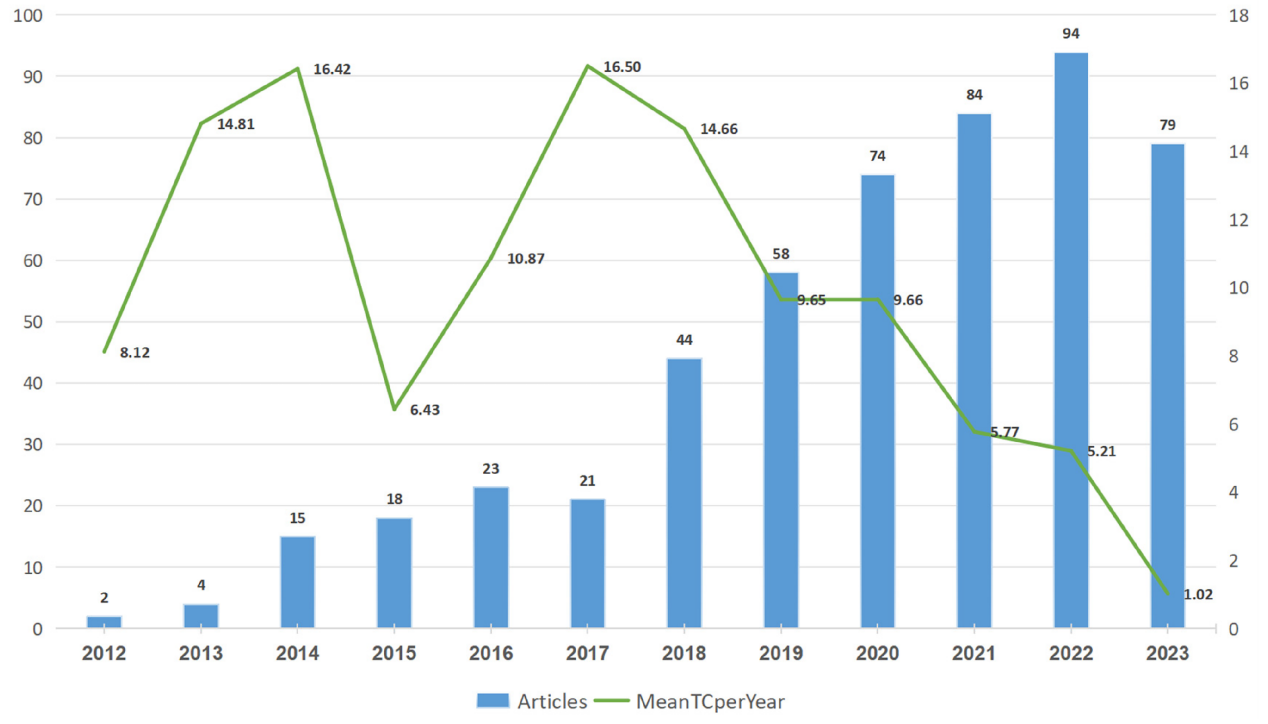

FIGURE 2
Annual distribution and average annual citations of publications from 2012 to 2023.

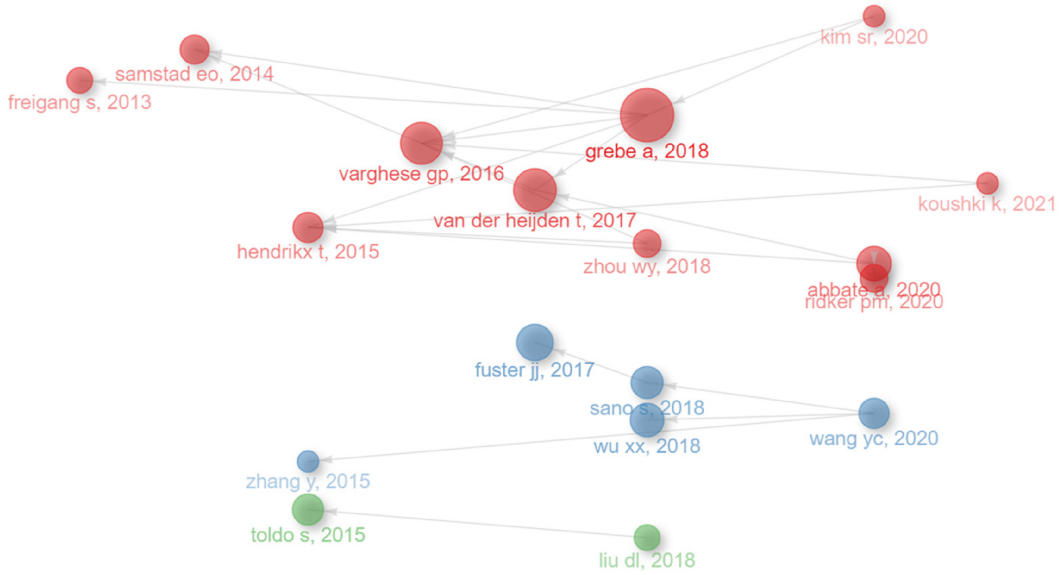

FIGURE 3
Historiograph.

TABLE 2 Top 10 journals with publication volumes on "CVD" and "NLRP3 inflammasome" (2012–2023).

Sources	Articles	JCR	IF (2023)
International Journal of Molecular Sciences	26	Q1	5.6
Frontiers in Cardiovascular Medicine	16	Q2	3.6
Frontiers in Pharmacology	13	Q1	5.6
Frontiers in Immunology	10	Q1	7.3
Cells	9	Q2	6
Circulation Research	9	Q1	20.1
Frontiers in Physiology	9	Q1	4
Antioxidants & Redox Signaling	8	Q1	7
Biomedicines	8	Q2	4.7
Cell Death & Disease	7	Q1	9

TABLE 3 Top 10 influential journals from 2012 to 2023.

Sources	H-index	G-index	M-index
International Journal of Molecular Sciences	12	24	1.714
Circulation Research	8	9	0.8
Frontiers in Cardiovascular Medicine	8	13	1.143
Frontiers in Pharmacology	8	13	1.143
Antioxidants & Redox Signaling	7	8	0.7
Cell Death & Disease	7	7	0.875
Frontiers in Immunology	7	10	1
Frontiers in Cell and Developmental Biology	6	7	1.2
Oxidative Medicine and Cellular Longevity	6	7	0.667
Pharmacological Research	6	7	0.545

TABLE 4 TOP 10 Co-cited journals, 2012–2023.

Co-cited journals	Articles	JCR	IF (2023)
Circulation	380	Q1	37.8
Nature	364	Q1	64.8
Plos one	342	Q2	3.7
Circulation Research	328	Q1	20.1
New England Journal of Medicine	309	Q1	158.5
Proceedings of the National Academy of Sciences of the United States of America	305	Q1	11.1
Journal of Biological Chemistry	293	Q2	4.8
Nature Medicine	289	Q1	82.9
Arteriosclerosis, Thrombosis, and Vascular Biology	283	Q1	8.7
Journal of Clinical Investigation	283	Q1	15.9

3.4 Authors and co-cited authors

Table 6 presents the top 10 most prolific authors and their teams who contributed to publications on "CVD" and the "NLRP3 inflammasome" from 2012 to 2023. This analysis, encompassing 3,004 authors, revealed an average of 6.78 co-authors per paper and an international co-authorship rate of 25.58%. To further explore author collaboration patterns, a co-occurrence analysis was conducted using "CiteSpace" on authors with at least three publications (Figure 5). Abbate Antonio emerged as the most productive author, contributing to seven articles within the timeframe (Figures 5A,B). Following closely were Boini Krishna M, Chen Yang, and Li Pin-Lan, each with four publications. Centrality scores, displayed in Table 5, provide an additional indicator of authorial influence within the field. DINARELLO CA stood out with the highest score (0.11), exceeding the threshold of 0.1. This signified the significant impact of DINARELLO CA's research on the research landscape. Other prominent scholars included DUEWELL P (0.09), BAUERNFEIND FG (0.09), and MENU P (0.08).

Figure 5C provides a visual analysis of the co-cited author network, while Figure 5D showcases the top 10 most-cited

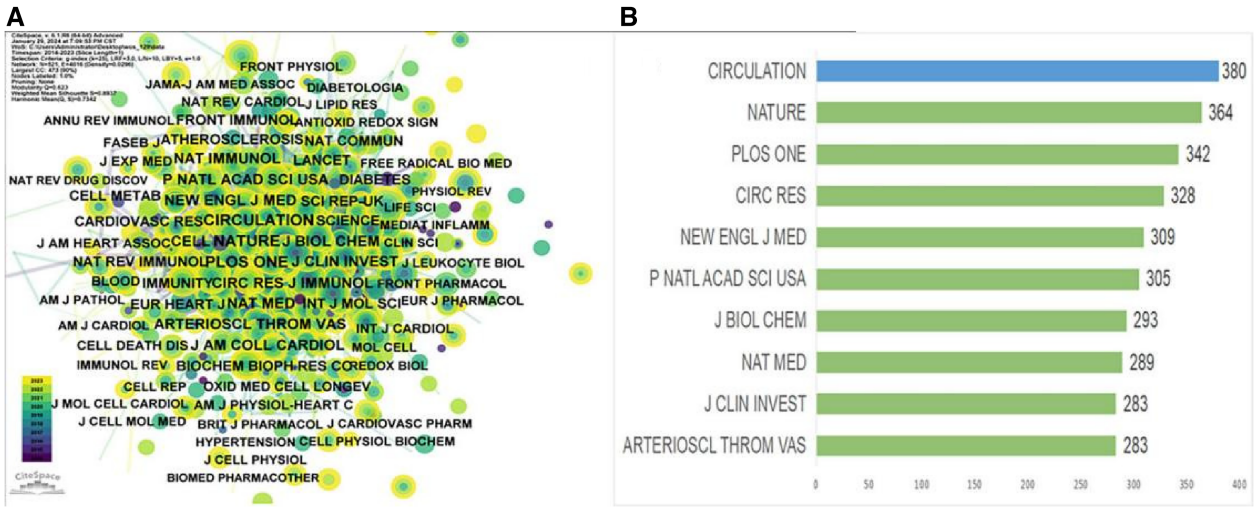

FIGURE 4
Visualization of journals and co-cited journals 2012–2023. (A) Co-citation journal co-publication cooperative network. (B) Top 10 journals in total citations from 2012 to 2023.

TABLE 5 2012–2023 Top 10 journals for centrality.

Sources	Centrality	JCR	IF (2023)
American Journal of Respiratory and Critical Care Medicine	0.09	Q1	5.6
Free Radical Biology and Medicine	0.05	Q2	3.6
Biochimica et Biophysica Acta—Molecular Cell Research	0.05	Q1	5.6
Oxidative Medicine and Cellular Longevity	0.04	Q1	7.3
American Journal of Physiology-Heart and Circulatory Physiology	0.04	Q2	6
American Journal of Cardiology	0.04	Q1	20.1
Journal of Lipid Research	0.04	Q1	4
Cell Death and Differentiation	0.04	Q1	7
Biochemical Pharmacology	0.04	Q2	4.7
Molecular and Cellular Biology	0.04	Q1	9

TABLE 6 Most relevant authors.

Authors	Articles	Articles Fractionalized
Ridker PM	5	2.56
Roche HM	6	2.07
Zhang Y	14	1.84
Chen Y	8	1.54
Li X	7	1.38
Bornfeldt KE	3	1.33
Li PL	7	1.29
Latz E	5	1.29
Abbate A	7	1.20
Schertzer JD	3	1.20

authors based on their reference citations (Table 6). Notably, Ridker PM ranked among the most influential authors in the field of NLRP3 inflammasome research in the context of CVD, as evidenced by their high citation count. The CANTOS trial, led by Ridker PM's team, represents a significant breakthrough in anti-inflammatory treatment for atherosclerosis (8). This landmark study provided hitherto undocumented evidence that anti-inflammatory drugs targeting interleukin-1β (IL-1β) (canazumab) could effectively reduce the incidence of cardiovascular adverse events in patients with myocardial infarction, even when combined with lipid-lowering drugs. This finding ushered in a new era of anti-inflammatory therapy for ASCVD. Subsequent studies, such as COLCOT and LoDoCo2, have further confirmed the efficacy of colchicine in reducing cardiovascular risks among patients with chronic coronary heart disease and recent myocardial infarction who have received standard care (9–12). These studies have also highlighted the involvement of specific inflammatory pathways in ASCVD development. The NOD, LRR, and NLRP3 inflammasome-related pathways have been identified as promising therapeutic targets for achieving remission in ASCVD. In 2010, a groundbreaking discovery by Duewell et al. (23) employed novel observation techniques to reveal the presence of minuscule cholesterol crystals in the early stages of atherosclerotic plaque formation. These crystals were found to coincide with the infiltration of inflammatory cells and could activate the NLRP3 inflammasome, triggering Caspase1 activation, and inducing the release of abundant mature IL-1β. The authors hypothesized that cholesterol crystals function as endogenous molecules, activating the NLRP3 inflammasome and contributing to the progression of AS. Table 6 presents the top authors with the highest number of citations and their "Author Contribution Rate" to the respective articles. This metric reflects the proportional contribution of each author to the publications they are listed on.

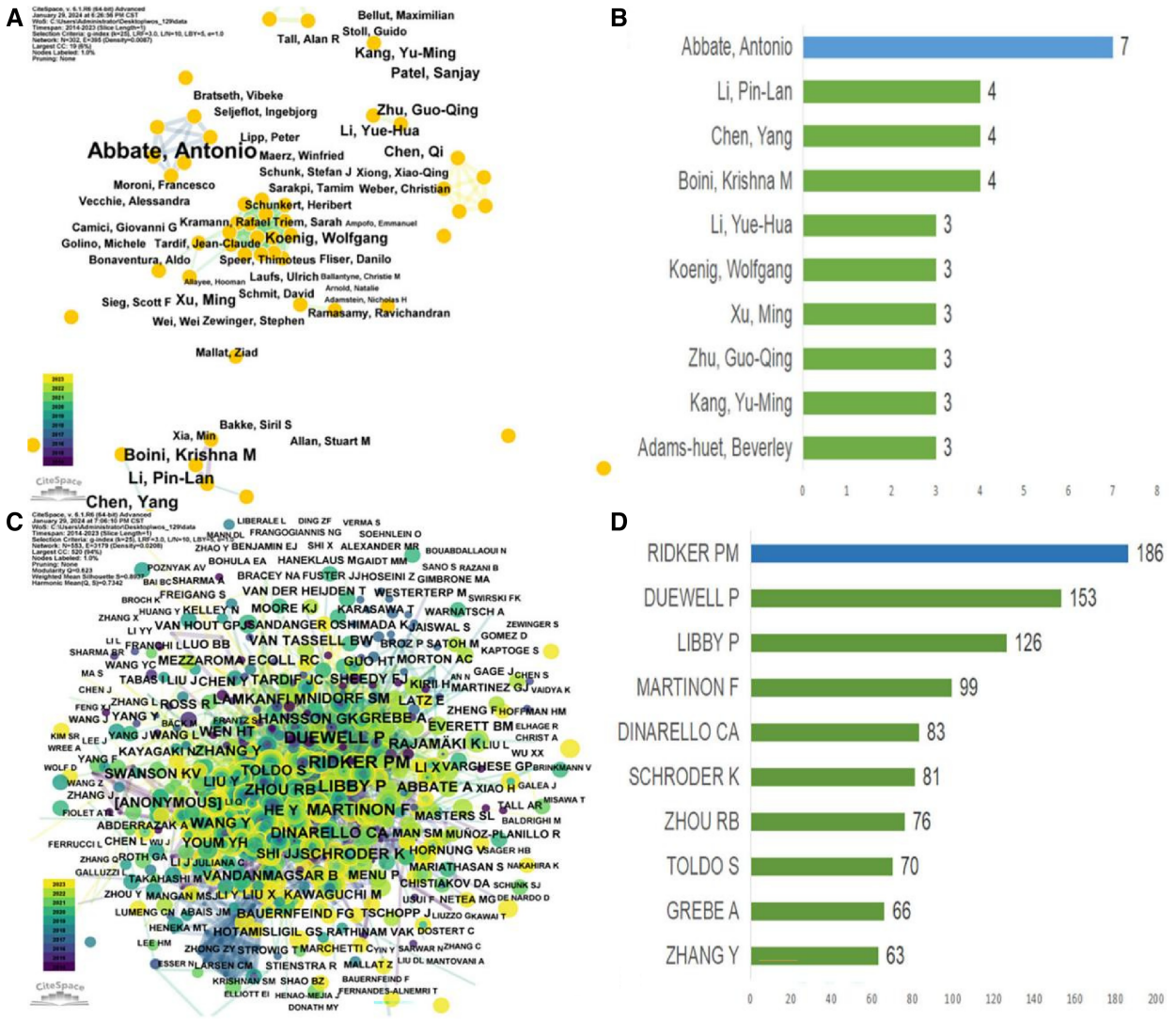

FIGURE 5
Analysis of author and co-cited author visualizations in relevant studies (2012–2023). (A) Co-authors; (B) The number of top 10 authors and their articles; (C) Co-authors co-occurrence; (D) The number of top 10 co-cited authors and their articles.

3.5 Countries and institutions

Figures 6A,B and 7A reveal that research on the NLRP3 inflammasome in cardiovascular diseases has spanned 54 countries over the past 12 years. China led in publication volume with 170 articles (32.95%), followed by the United States (148/28.68%) and Germany (42/8.14%). An analysis of collaboration intensity (Figures 6C,D,G) indicated that China exhibited the strongest collaboration network, with a total collaboration strength of 567. The United States (436) and Germany (138) were next in terms of collaborative publications. The highest collaboration intensity was observed between the United States and China (19 publications), followed by the United States and Germany (16), and the United States and the Netherlands (12). Articles from the United States received the highest total citations (8,664) (Figure 6E), followed by China (6,207) and Germany (1,574). When considering centrality (a measure of a country's influence within the collaboration network), the United States (0.43) ranked highest, followed by India (0.25) and Iran (0.14) (Figure 6H). The H-index (an indicator of a country's research impact and productivity) was highest for the United States (90), followed by China (69) and Germany (48) (Figure 6F). Interestingly, although China had a slight edge in publication volume, the United States held a significantly higher H-index. By considering these publication, citation, and collaboration metrics, it is evident that China, the United States, and Europe have been the primary contributors to this field, with the United States holding a relatively prominent position.

An analysis of institutional contribution reveals that Harvard University led in publication output with 35 articles, followed by Harvard Medical School (25 articles) and the University of Oslo (24 articles) (Table 7). Centrality scores, which indicate an

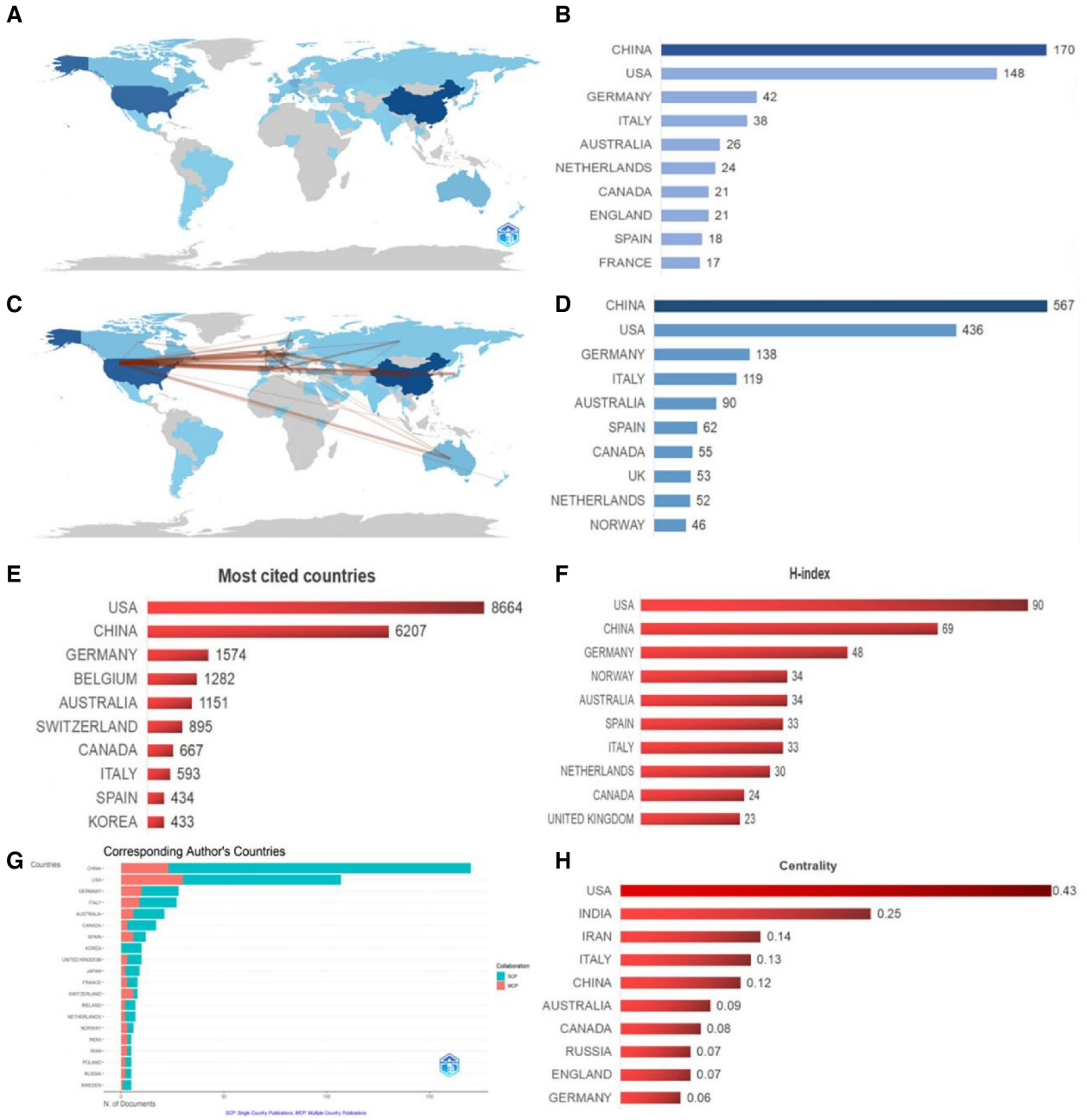

FIGURE 6
Visual analysis of countries involved in relevant studies from 2012 to 2023. (A) Number of publications per country. (B) The top 10 countries with the highest number of publications and the corresponding number of articles. (C) A map illustrating country cooperation, where lines represent collaborations between countries and the line thickness indicates the intensity of cooperation. (D) The top 10 countries with the highest level of cooperation. (E) Top 10 countries ranked by total citation frequency. (F) The top 10 countries in terms of the H-index. (G) A global map illustrating the intensity of cooperation among countries. (H) The top 10 countries with the highest centrality.

institution's influence within the collaboration network, were highest for the Chinese Academy of Sciences (0.34), followed by CIBER-Centro de Investigacion Biomedica en Red (0.16), and Harvard University (0.12) (Table 7). Figure 7B visually depicts the close collaborative ties among these institutions. A comprehensive network visualization is presented in Figure 8, encompassing authors, institutions, and countries involved in NLRP3 inflammasome research within the context of CVD.

3.6 Keywords

This study employed the "Bibliometrix" software to analyze keyword usage within the retrieved publications. Keywords appearing more than ten times were included in the analysis. A total of 1,211 keywords were extracted, with the top 50 most frequent keywords visualized in Figures 9B,E. Furthermore, co-occurrence analysis (Figure 9A) and cluster analysis (Figures 9C,D)

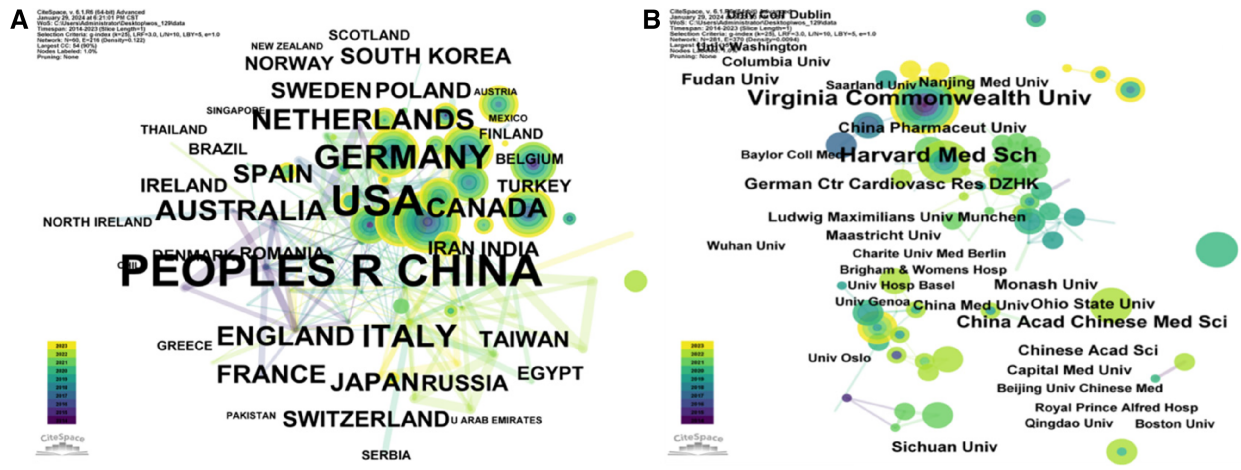

FIGURE 7
Country and agency map. (A) Co-occurrence map of countries. (B) Co-occurrence map of institutions.

TABLE 7 Ranking of the top 10 institutions conducting relevant research based on the number of studies and center status.

Rank	Affiliations	Counts	Rank	Affiliations	Centrality
1	Harvard University	35	1	Chinese Academy of Sciences	0.34
2	Harvard Medical School	25	2	CIBER—Centro de Investigacion Biomedica en Red	0.16
3	University of Oslo	24	3	Harvard University	0.12
4	University System of Ohio	24	4	Boston University	0.12
5	University of Sydney	17	5	Instituto de Salud Carlos III	0.12
6	Virginia Commonwealth University	17	6	German Centre for Cardiovascular Research	0.08
7	Brigham and Women's Hospital	16	7	Virginia Commonwealth University	0.07
8	Ohio State University	16	8	University of Queensland	0.06
9	German Centre for Cardiovascular Research	15	9	Egyptian Knowledge Bank (EKB)	0.06
10	Nanjing Medical University	14	10	Southern Medical University—China	0.06

were conducted on the keywords using "CiteSpace" software. This analysis resulted in the identification of nine distinct research clusters, each representing a specific research direction or field.

This analysis identified nine distinct research clusters based on keyword co-occurrence. Cluster 0, focusing on disease ($n = 64$ keywords), included terms like "acceptor" and "mechanism". Cluster 1 comprised coronary heart disease ($n = 56$ keywords), encompassing keywords such as "IL-1", "inflammation", and "myocardial infarction". Cluster 2 involved pyroptosis ($n = 56$ keywords), with terms like "apoptosis of cells" and "nrf2" appearing frequently. Cluster 3 focused on apoptotic cells ($n = 41$ keywords) with keywords including "reactive oxygen species" and "foam cell formation". Cluster 4 comprised SARS-CoV-2 ($n = 40$ keywords), highlighting risk factors like "Sirt1" and diabetic complications. Cluster 5 examined platelet activation ($n = 38$ keywords), with terms like "homocysteine" and "vascular smooth muscle cells" present. Cluster 6 involved metabolic syndrome ($n = 37$ keywords), featuring keywords like "obesity" and "insulin resistance." Cluster 7 was associated with chronic kidney disease (CKD) ($n = 28$ keywords), with "innate immunity" and "kanakulizumab" emerging as important keywords. Finally, Cluster 8 focused on endothelial cells ($n = 24$ keywords), with "signaling pathways" and "microRNA" being prominent terms.

Clusters 2, 3, 4, and 5 predominantly involved the cellular mechanisms and molecular pathways by which NLRP3 inflammasomes contribute to cardiovascular diseases. Keywords within these clusters highlighted terms like pyroptosis, apoptosis, and platelet activation. In contrast, clusters 0, 1, 6, and 7 primarily represented the major disease types investigated in the context of NLRP3 inflammasomes and cardiovascular disease, encompassing keywords such as coronary heart disease, metabolic syndrome, and chronic kidney disease. An analysis of keyword publication years revealed that obesity, ferroptosis, and inflammation were the most recently appearing keywords. This suggested that these topics represented current research hotspots in the field.

3.7 References

Citespace analysis revealed that four out of the top 10 most cited references (Figure 10, Table 8) focused on the association

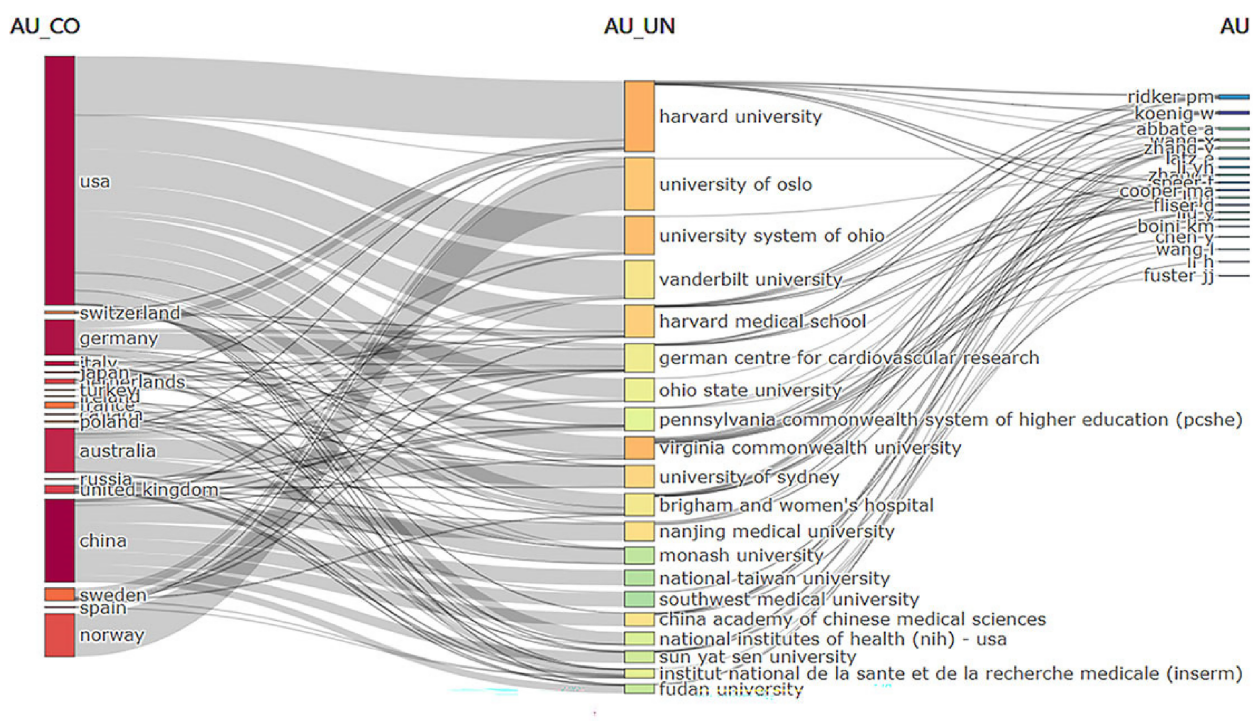

FIGURE 8
Three-region map analyzing national institutions and authors in relevant fields. Middle region: institution; Left region: countries; Right region: author.

between NLRP3 inflammasome and atherosclerosis. Two others were large cohort studies investigating NLRP3 inflammasome-based anti-inflammatory therapy for cardiovascular diseases. Figure 11 illustrates the relationships among references, authors, and keywords in the field of CVD and NLRP3 inflammasome research. Notably, the most cited article by Duewell P (23). reported that endogenous cholesterol crystals activate the NLRP3 inflammasome, promoting the development of atherosclerosis. This finding suggests the potential benefit of combining lipid-lowering and anti-inflammatory therapies. It is now understood that activation of the NLRP3 inflammasome leads to Caspase-1 activation and subsequent secretion of inflammatory factors IL-1β and IL-18. Elevated levels of IL-1β have been linked to the severity of atherosclerotic disease (24). IL-1β stimulates various responses: synthesis and secretion of other cytokines and chemokines, activation of macrophages and lymphocytes, promotion of vascular smooth muscle cell migration and proliferation, enhanced cell-cell interactions, triggering of cell apoptosis, and contribution to extracellular cholesterol accumulation. Additionally, deposited crystals induce lysosomal disruption and trigger reactive oxygen species production in macrophages, further activating the NLRP3 inflammasome. Simultaneously, Caspase-1 activation can induce pyroptosis, a form of programmed cell death triggered by pathogens or endogenous factors, in monocytes and macrophages (25). This process leads to the release of tissue metalloproteinases by macrophages, compromising the stability of atherosclerotic plaques. These mechanisms create a positive feedback loop, enlarging the plaque area and reducing its stability. We then performed a cluster analysis of the citations based on the log-likelihood ratio (LLR), a statistical measure used to assess the association between variables. This analysis identified nine distinct clusters (Q = 0.5888, mean profile value = 0.6987) (Figure 10C). These clusters summarize two main aspects. Firstly, they highlighted the role of the NLRP3 inflammasome in the pathogenesis of cardiovascular disease. This includes the involvement of pyroptosis (#1), ketone bodies (#2), NLRP3 inflammasome (#3), ferroptosis (#4), and thioredoxin-interacting/inhibiting protein (#5). Secondly, the clusters represent the types of diseases studied in this field, such as obesity (#0), nonalcoholic steatohepatitis (#7), and covid-19 (#8). The timeline diagram (Figures 10D,E) revealed that recent research trends in this field focus on cardiovascular disease, targeted therapies, and lipoprotein cholesterol. Additionally, Figure 11 illustrates the interconnected network of authors, references, and keywords within the field.

3.8 Discipline distribution

CiteSpace's double graph superposition function was employed to analyze the distribution of academic journals across disciplines (Figure 12). This analysis revealed two main citation paths within the network. Interestingly, the source articles themselves spanned a broad range of disciplines, including mathematics, medicine, clinical medicine, ecology, molecular biology, and immunology.

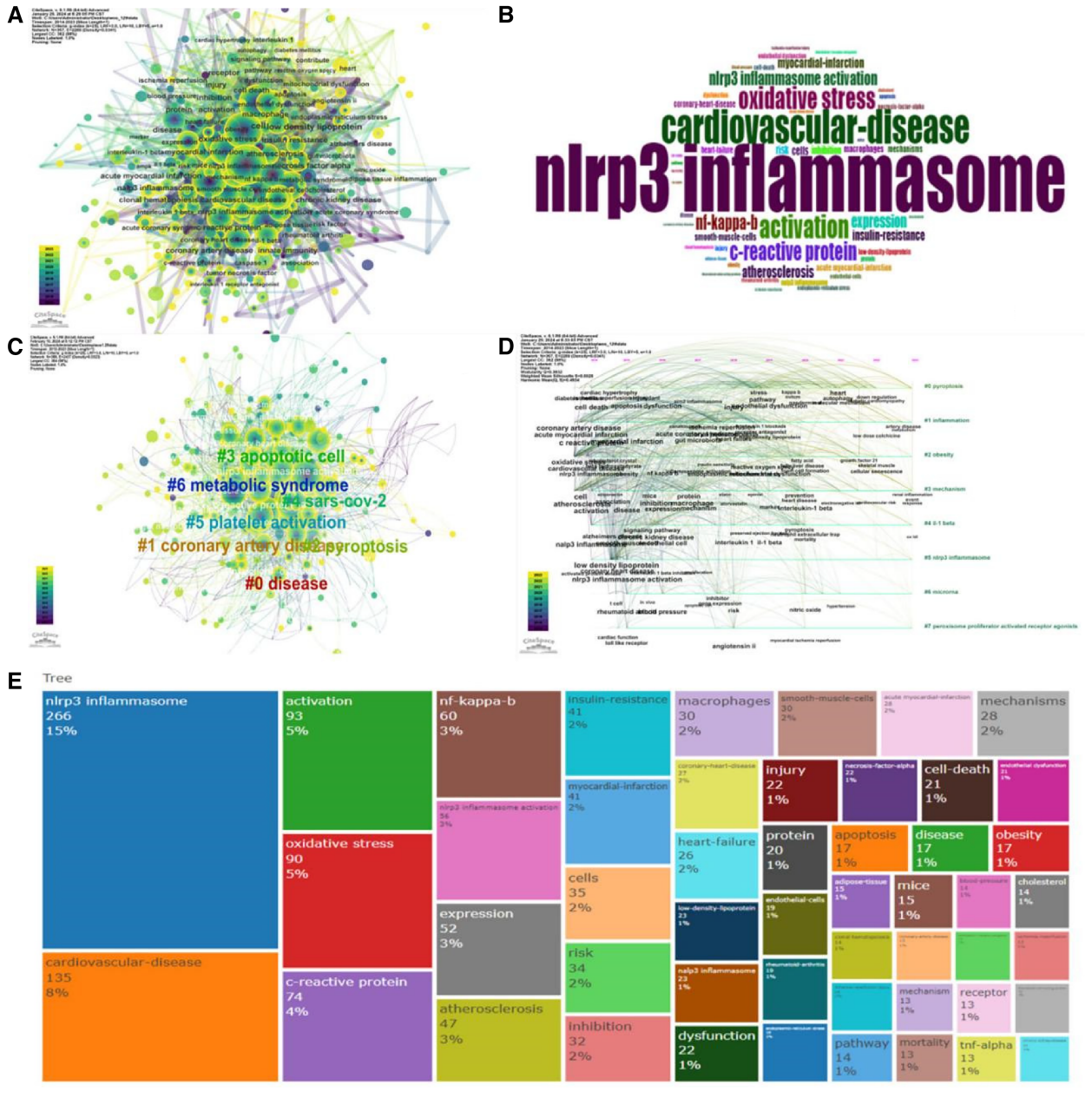

FIGURE 9
Visual analysis of keywords spanning the years 2012 to 2023. (A) Map illustrating the co-occurrence of keywords. (B) Word cloud depicting the keywords. (C) Diagram illustrating the co-aggregation of keywords. (D) Diagram displaying the timeline clusters of keywords. (E) Dendrogram depicting the relationships between keywords.

Conversely, the cited references were primarily published in journals focused on biomedicine, such as biochemistry, biology, pharmacology, pharmaceutics, cardiology, immunology, general medicine, research methods, endocrinology, chemistry, and peripheral vascular disease.

4 Discussion

This study utilized Bibliometrix, CiteSpace, and Microsoft Excel to analyze 516 articles retrieved from the Web of Science Core Collection, focusing on NLRP3 inflammasome research in cardiovascular diseases. The investigation aimed to identify key research areas and emerging trends within this field. The analysis revealed a steady increase in publication volume over the past twelve years, with a particularly notable acceleration after 2017. This upward trajectory suggested that NLRP3 inflammasome research in cardiovascular diseases has reached a relatively mature stage of development.

Our analysis identified the United States, Europe, and China as the leading contributors to research in this field. While China boasted the highest publication output, the United States

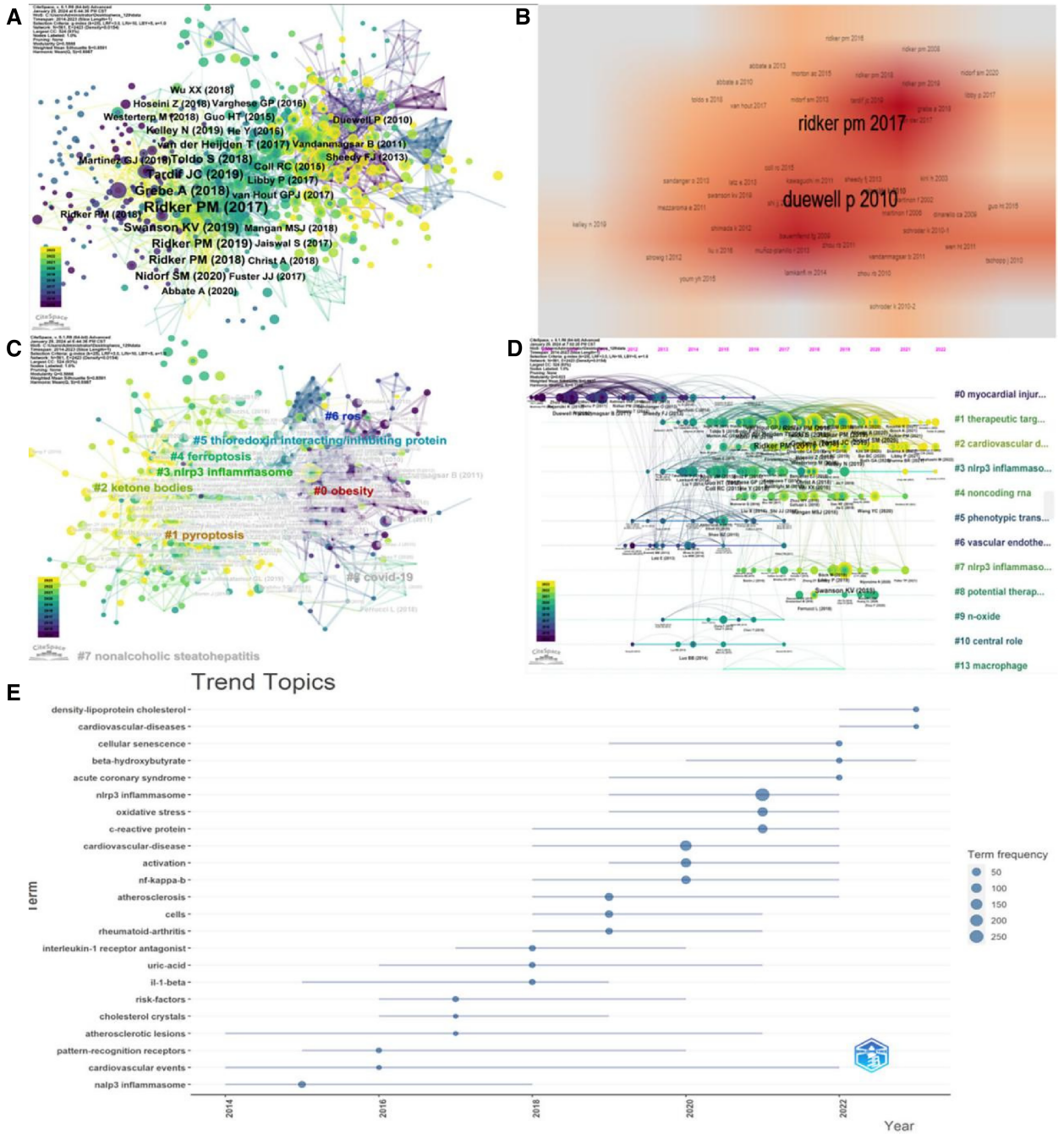

FIGURE 10
Visual analysis of cited references. (A) Co-cited literature network. (B) Density visualization of co-cited references. (C) Cluster analysis of co-cited references. (D) Timeline graph of cluster analysis. (E) Timeline distribution of topics.

emerged as the frontrunner in terms of both citations and H-index. Notably, among the top 10 most prolific authors and institutions, 40% were affiliated with the United States, while the remaining 60% originated from China. This trend continued for institutions, with 90% of the top 10 being US-based, compared to just 10% from China. However, the landscape shifted when considering citations and centrality. Here, the US retained a strong presence with 60% of the top 10 authors, but its institutional dominance lessened, with 30% of the top institutions being US-based. These findings suggest that the United States harbors a robust network of globally recognized research institutions and accomplished scholars, contributing significantly to advancements in this field.

Analysis of co-cited literature and keywords offers valuable insights into the central themes and primary focus of current research. Additionally, examining frequently cited references helps establish the foundational knowledge and background of the field (26). Notably, this study observed a high degree of

TABLE 8 Top 10 co-cited references.

Authors	Journal	Year	Citations
Duewell P	Nature	2010	152
Ridker PM	New Engl J Med	2017	144
Grebe A	Circ Res	2018	64
Vandanmagsar B	Nat Med	2011	59
Martinon F	Nature	2006	56
Rajamäki K	PLoS One	2010	56
Schroder K	Cell	2010	56
Sheedy FJ	Nat Immunol	2013	56
Zhou RB	Nature	2011	50
Tardif JC	New Engl J Me	2019	47

concordance between co-cited literature clusters and keyword clusters. This overlap suggests that the identified topics represent genuine research hotspots within the field.

Since 2017, research has increasingly focused on the role of NLRP3 inflammasome-mediated inflammatory responses in the development and progression of cardiovascular disease. Atherosclerosis serves as the underlying pathology for most CVD cases. Building upon the "inflammation theory" of AS proposed by Russell Ross in 1990, numerous studies have provided evidence that inflammation is a primary driver of AS and its complications (27). This process involves various cell types, including monocytes, macrophages, vascular endothelial cells, vascular smooth muscle cells, and T lymphocytes, as well as inflammatory cytokines such as C-reactive protein (CRP), interleukin-6 (IL-6), and IL-1β. These factors contribute to the activation of inflammatory signaling pathways throughout AS formation and development. At the molecular level, the formation of the NLRP3 inflammasome in macrophages plays a crucial role in propagating inflammation. The NLRP3/IL-1β/IL-6/high-sensitivity C-reactive protein (hs-CRP) classical inflammatory pathway is widely thought to be closely associated with an increased risk of vascular atherosclerosis (28). To validate the role of inflammation in CVD, researchers have conducted large-scale clinical trials targeting this mechanism. Examples include CANTOS, COLCOT, LoDoCo2, Cardiovascular Inflammation Reduction Trial (CIRT), and CLEAR Outcomes (8–12, 29–32).

Keyword analysis further highlighted the pivotal roles of NF-κB, oxidative stress, and CRP in NLRP3 inflammasome-related cardiovascular diseases. The NLRP3 inflammasome is a multi-protein complex requiring the coordinated regulation of two signaling pathways. First, a priming signal triggers the binding of stress molecules to Toll-like receptors on the cell membrane. This activates the NF-κB pathway, leading to increased expression of NLRP3 and pro-IL1β. Subsequently, upon recognition of a pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP), a triggering signal induces the assembly of NLRP3, ASC, and pro-caspase-1 into functional NLRP3 inflammasomes. This results in the release of mature caspase-1, IL-1β, and IL-18. The NLRP3 inflammasome, a key driver of inflammation in atherosclerosis, can be activated through three main mechanisms. The first involves changes in potassium levels: either a decrease inside the

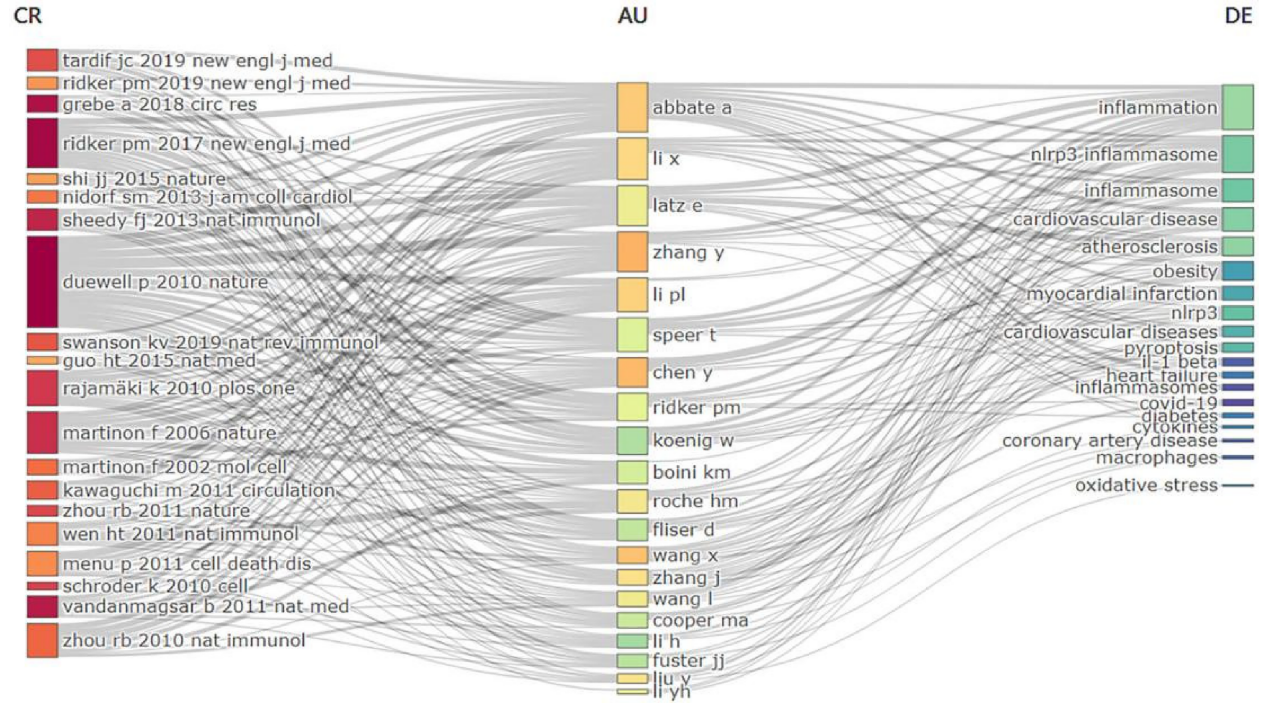

FIGURE 11
Three-field plot of references and keyword analysis in relevant fields. Middle region: author; Left region: references; Right region: keywords.

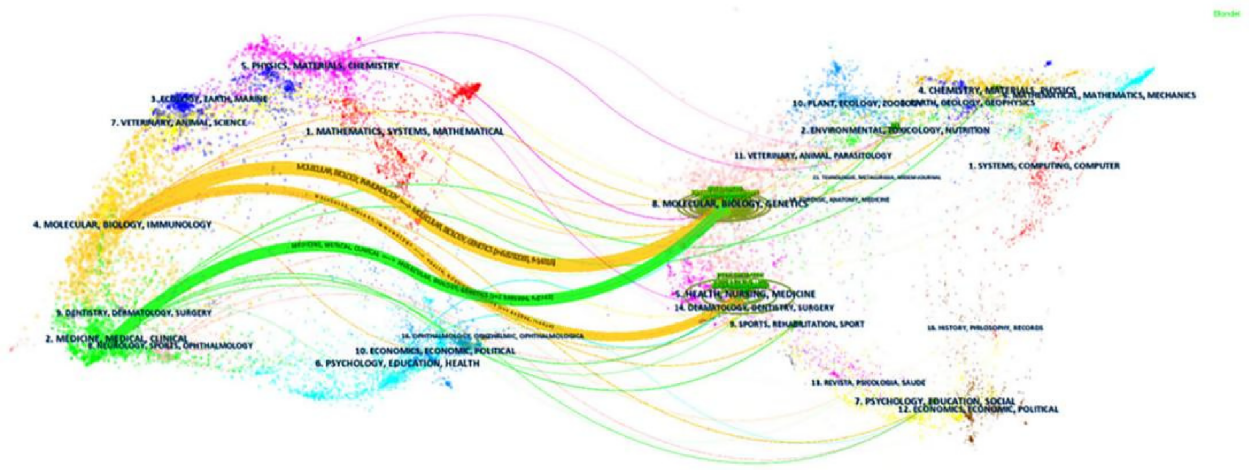

FIGURE 12
Visualization of the distribution of subjects. The left side represents the subject area covered by the citing journal, while the right side represents the subject area covered by the cited journal.

cell, an increase outside, or the binding of ATP to a specific receptor. This disrupts the cell membrane, allowing NLRP3 agonists to enter and trigger inflammasome assembly. Secondly, instability or rupture of lysosomes can also activate the NLRP3 inflammasome. Finally, the production of reactive oxygen species, particularly from mitochondria, is another potential mechanism, although the exact details remain unclear (33). Macrophages exposed to low oxygen or cholesterol crystals experience further stress, leading to the formation of protein complexes. Activation of the NLRP3 inflammasome in these cells then triggers the release of IL-1β and IL-18. These cytokines activate various inflammatory cells and induce IL-6 production, which subsequently stimulates the liver to produce CRP. This process amplifies the inflammatory response within the artery wall (34). In heart failure, activation of the NLRP3 inflammatory mediates the release of pro-inflammatory mediators such as IL-1β and IL-18, considered significant contributors to myocardial fibrosis and cardiac dysfunction in heart failure (35). A study found activation of NLRP3 inflammasomes in a mouse model of myocardial ischemia/reperfusion injury and high levels of NLRP3 further aggravated myocardial injury (36). Toll-like receptors (TLRs) represent an important class of protein molecules involved in innate immunity. TLR4 has been demonstrated to facilitate NLRP3 inflammasome activation through the NF-κB pathway. Inhibition of TLR4 has been shown to suppress the inflammatory response, thereby attenuating myocardial ischemia-reperfusion injury (37). Several NLRP3 inflammatory vesicle inhibitors containing structural domains of the NACHT, LRR, and PYD protein family have been tested in animal models of acute myocardial infarction. Colchicine can act downstream of NLRP3 by inhibiting the polymerization of ASC-containing apoptotic speck-like proteins (38). In arrhythmias, the activation of NLRP3 inflammatory vesicles induces the upregulation of ultrarapid delayed rectifier K^+ channels and shortening, which leads to myocardial potentiostatic inappetence and action potential duration. These two key factors contribute to cardiac electrical remodeling. It was found that increased pro-fibrotic signaling and fibrosis, as well as abnormal Ca^{2+} release from the sarcoplasmic reticulum, were strongly associated with arrhythmogenesis in wild-type mice fed a high-fat diet. In contrast, NLRP3 knockout in high-fat diet-fed mice (NLRP3 $^{-/-}$) prevented the upregulation of K^+ channels and the evolution of electrical remodeling, the upregulation of pro-fibrotic genes, and the aberrant sarcoplasmic reticulum Ca^{2+} release induced by high-fat chow in wild-type mice. This suggests that the activation of NLRP3 inflammatory vesicles may be a key driver for the development of arrhythmias (39).

Furthermore, trend analysis identified lipoprotein cholesterol as a significant keyword with a strong association with NLRP3 inflammasomes in CVD research. The combined analysis of references and keywords suggested a thematic distinction within the field. Disease research appeared to be primarily focused on metabolic disorders, such as obesity and ASCVD. In contrast, mechanistic research seems to be concentrated on oxidative stress and pyroptosis pathways. It is well-established that inflammatory cytokines, mediated by NLRP3 inflammasomes, can exert both autocrine (acting on the same cell) and paracrine (acting on neighboring cells) effects on various cell types within metabolic tissues. This phenomenon is believed to contribute to the development of several metabolic disorders, including diabetes, obesity, atherosclerosis, cardiovascular disease, gout, and neurodegenerative diseases (40).

Since 2017, a new era of anti-inflammatory therapy for ASCVD has been ushered in by the groundbreaking CANTOS trial led by Professor Ridker. This study demonstrated that residual inflammation significantly increases the risk of recurrent events in ASCVD patients (8). Further support for targeting the NLRP3 inflammasome pathway as a therapeutic strategy for ASCVD

comes from the COLCOT and LoDoCo2 studies published in 2021. Professor Ridker's most recent clinical trials (2023) (31, 32) demonstrated that combining anti-inflammatory treatment with statin-based lipid-lowering therapy could effectively reduce residual cardiovascular risk in CVD patients. These studies have established high-sensitivity C-reactive protein as a more reliable predictor of future cardiovascular events and mortality risk compared to low-density lipoprotein cholesterol (LDL-C). Collectively, these landmark clinical trials highlight the critical role of chronic systemic inflammation in ASCVD development and progression. Therefore, managing chronic systemic inflammation holds significant promise for reducing cardiovascular risk events.

Cholesterol has long been viewed as the primary culprit in ASCVD. However, research advancements now recognize ASCVD as a progressive inflammatory response, not just lipid buildup in arteries. In ASCVD patients, inflammation can be triggered by various factors beyond just metabolic conditions (diabetes) and autoimmune diseases. It can also originate from adipose tissue itself. In obesity, the accumulation of visceral fat leads to lower adiponectin levels, higher free fatty acids in the blood, and the release of inflammatory cytokines. This prolonged inflammation can damage the vascular endothelium (lining of blood vessels), alter blood flow dynamics, remodel the heart muscle, and ultimately contribute to atherosclerosis development and progression. These processes significantly impact heart structure and function (41). Studies have shown that a 10% weight loss can reduce hs-CRP levels by 40%, suggesting weight management offers cardiovascular benefits, likely due to improved inflammatory state (42). The SELECT trial, a groundbreaking global study, demonstrated that semaglutide 2.4 mg not only aids weight management but also reduces the risk of cardiovascular events (cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke). This discovery represents a significant advancement in obesity treatment for patients with cardiovascular disease. Data from the SELECT trial revealed a remarkable 39.1% reduction in hs-CRP levels in the semaglutide group, accompanied by an early separation in cardiovascular event incidence compared to the placebo group. These findings suggest that semaglutide 2.4mg's cardiovascular benefits may extend beyond weight loss and might be partly due to its ability to improve the chronic inflammatory state in obese patients with cardiovascular disease (42).

In recent years, inhibitors targeting NLRP3 inflammatory vesicles have demonstrated potential efficacy in clinical trials. For instance, a small molecule drug (MCC950) that selectively targets NLRP3 inflammatory vesicles can effectively block its activation, thereby significantly delaying the progression of atherosclerosis (43). In streptozotocin-induced diabetic mice with aortic atherosclerotic lesions, MCC950 prevented the formation of atherosclerotic lesions, reduced the expression of inflammatory mediators, and improved vessel wall function (44). In addition, another NLRP3 inflammatory vesicle-targeting drug, quercetin, has been demonstrated to effectively prevent and control neuroinflammatory diseases. Furthermore, quercetin has been shown to significantly reduce plaque area and lipid deposition, stabilize plaques, and inhibit macrophage pyroptosis and NLRP3 expression, ultimately leading to an ameliorative effect on AS in ApoE $^{-/-}$ mice (45). In addition, selective NLRP3 inhibitors have been studied in phase I-II clinical trials over the past five years. Colchicine, a non-selective NLRP3 inflammatory vesicle inhibitor, has established efficacy in the treatment of pericarditis and is regarded as a standard of care. It is currently approved by the US Food and Drug Administration for reducing the risk of atherosclerotic thrombosis in patients with coronary artery disease (38).

Effective management of inflammation is essential for mitigating the risk of cardiovascular events. The NLRP3 inflammasome, a key player in the inflammatory response linked to cardiovascular disease, presents a promising target for novel therapeutic approaches. Targeted anti-inflammatory treatments against the NLRP3 inflammasome offer significant potential for the development of highly effective CVD therapies.

5 Limitations

This study utilized bibliometric methods to analyze research on the NLRP3 inflammasome in cardiovascular diseases retrieved from the Web of Science Core Collection. This analysis aimed to identify the current research landscape, emerging trends, and key areas of focus within this field. However, some limitations should be acknowledged. First, the restriction to a single database (Web of Science) for literature screening stemmed from software limitations that currently prevent simultaneous analysis of multiple sources. While the Web of Science is a well-respected academic database, future studies will explore methods to incorporate additional databases for a more comprehensive analysis. Second, the chosen timeframe (2012–2023) might limit the capture of early keyword usage and the evaluation of long-term trends. Notably, significant advancements have occurred within this field since 2017, and the 12-year window may not fully encompass this progress. Additionally, the relatively small sample size of 516 publications suggests substantial room for future exploration. Finally, the combined use of CiteSpace and Bibliometrix for literature analysis might lead to potential information gaps during the analysis process, introducing some bias into the results. Future work will focus on optimizing the selection of bibliometric methods to address these limitations.

6 Conclusions

Bibliometric analysis reveals a promising and rapidly growing field of research investigating the NLRP3 inflammasome in cardiovascular diseases. Notably, the publication volume has reached its peak within the past two years. Examining key contributors, geographic regions, and prominent publications within this field demonstrates a primary focus on coronary heart disease, atherosclerosis, obesity, metabolic syndrome, ASCVD, and related conditions. Recent studies have highlighted the significant roles of NF-κB, oxidative stress, and CRP in the

pathogenesis of NLRP3 inflammasome-associated CVDs. A pivotal shift occurred in 2017, with research momentum growing around anti-inflammatory treatment for cardiovascular diseases. This shift coincided with the recognition of hs-CRP as a more reliable biomarker for predicting heart disease risk compared to cholesterol levels. Consequently, the research focus has pivoted towards developing anti-inflammatory therapies in the context of CVD, with particular interest in their synergy with lipid-lowering therapy, as well as their potential to mitigate NLRP3 inflammasome-induced oxidative stress and pyroptosis.

Author contributions

MM: Data curation, Resources, Software, Visualization, Writing – original draft. YY: Writing – review & editing. HD: Funding acquisition, Writing – review & editing.

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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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Exploring the common mechanisms and biomarker ST8SIA4 of atherosclerosis and ankylosing spondylitis through bioinformatics analysis and machine learning

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Background: Atherosclerosis (AS) is a major contributor to cerebrovascular and cardiovascular events. There is growing evidence that ankylosing spondylitis is closely linked to AS, often co-occurring with it; however, the shared pathogenic mechanisms between the two conditions are not well understood. This study employs bioinformatics approaches to identify common biomarkers and pathways between AS and ankylosing spondylitis.
Methods: Gene expression datasets for AS (GSE100927, GSE28829, GSE155512) and ankylosing spondylitis (GSE73754, GSE25101) were obtained from the Gene Expression Omnibus (GEO). Differential expression genes (DEGs) and module genes for AS and ankylosing spondylitis were identified using the Limma R package and weighted gene co-expression network analysis (WGCNA) techniques, respectively. The machine learning algorithm SVM-RFE was applied to pinpoint promising biomarkers, which were then validated in terms of their expression levels and diagnostic efficacy in AS and ankylosing spondylitis, using two separate GEO datasets. Furthermore, the interaction of the key biomarker with the immune microenvironment was investigated via the CIBERSORT algorithm, single-cell analysis was used to identify the locations of common diagnostic markers.
Results: The dataset GSE100927 contains 524 DEGs associated with AS, whereas dataset GSE73754 includes 1,384 genes categorized into modules specific to ankylosing spondylitis. Analysis of these datasets revealed an overlap of 71 genes between the DEGs of AS and the modular genes of ankylosing spondylitis. Utilizing the SVM-RFE algorithm, 15 and 24 central diagnostic genes were identified in datasets GSE100927 and GSE73754, respectively. Further validation of six key genes using external datasets confirmed ST8SIA4 as a common diagnostic marker for both conditions. Notably, ST8SIA4 is upregulated in samples from both diseases. Additionally, ROC analysis confirmed the robust diagnostic utility of ST8SIA4. Moreover, analysis through CIBERSORT suggested an association of the ST8SIA4 gene with the immune microenvironment in both disease contexts. Single-cell analysis revealed that ST8SIA4 is primarily expressed in Macrophages, Monocytes, T cells, and CMPs.

Conclusion: This study investigates the role of ST8SIA4 as a common diagnostic gene and the involvement of the lysosomal pathway in both AS and ankylosing spondylitis. The findings may yield potential diagnostic biomarkers and offer new insights into the shared pathogenic mechanisms underlying these conditions.

KEYWORDS

atherosclerosis, ankylosing spondylitis, WGCNA, machine learning algorithm, ST8SIA4

1 Introduction

Atherosclerosis (AS) serves as the foundational pathological mechanism for cardiovascular diseases, which rank prominently among the leading causes of disability and mortality globally (1). According to the Global Burden of Disease Study, the incidence of cardiovascular diseases saw a significant escalation rising from 271 million instances in 1990 to 523 million in 2019. Concurrently, fatalities linked to these conditions climbed from 12.1 million to 18.6 million within the same timeframe (2). AS develops from the accumulation of fibro-fatty lesions in arterial walls, accompanied by the infiltration of immune cells such as macrophages, T cells, and mast cells and is characterized as a chronic inflammatory condition with autoimmune features, influenced by complex interactions among immune metabolic alterations and oxidative stress (3). Spondyloarthritis (SPA), encompassing a range of inflammatory diseases affecting the spine, peripheral joints, and synovium, is categorized into peripheral spondyloarthritis and ankylosing spondylitis, with the latter further divided into radiographic axial spondyloarthritis (also known as ankylosing spondylitis) and non-radiographic axial spondyloarthritis (4). Ankylosing spondylitis, the second most prevalent form of spondylitis, predominantly impacts the spine, peripheral joints, ligaments, and tendons, and is associated with a heightened cardiovascular risk (4, 5). Research indicates that ankylosing spondylitis may increase the risk of AS leading to a 50%–100% higher incidence of cardiovascular diseases in these patients (6–9). Inflammation in ankylosing spondylitis directly compromises vascular structures and exacerbates AS by affecting cardiovascular risk factors such as lipid levels, blood pressure, and insulin resistance (6, 10). In one study, 39% of Psoriatic arthritis (PsA) patients exhibited carotid plaques during ultrasound examinations (11). A smaller study using coronary computed tomography angiography revealed a 76% prevalence of coronary artery plaques in PsA patients, compared to 44% in the control group (12). PsA and ankylosing spondylitis, as distinct forms of inflammatory arthritis, share genetic backgrounds, immunological responses, and clinical treatment approaches, thereby demonstrating a significant interconnection (13). Further, Ozdowska et al. reported a higher occurrence of subclinical coronary AS in patients with ankylosing spondylitis (14).

Although the intrinsic connection mechanisms between AS and ankylosing spondylitis are not fully understood, it is evident that inflammatory responses and abnormal activation of the immune system play significant roles in both diseases.The pathogenesis of AS primarily involves the subendothelial deposition of low-density lipoprotein (LDL), which triggers a local inflammatory response. Subsequently, macrophages that ingest LDL transform into foam cells, further intensifying inflammation, leading to endothelial damage and hardening of the arterial walls (15). Ankylosing spondylitis is typically understood as an autoimmune disorder where the immune system erroneously targets the body's own tissues, especially affecting the spine and pelvic joints. Research shows that immune cells, including T cells and macrophages, play a role in this process by targeting these areas and discharging inflammatory mediators, which in turn leads to symptoms such as pain, swelling, and stiffness of the joints (16). Furthermore, the advancement of both AS and ankylosing spondylitis is linked to the synthesis of various cytokines and inflammatory mediators. Notably, increased concentrations of CRP (C-reactive protein), TNF-α (tumor necrosis factor-alpha), and IL-6 (interleukin-6) have been observed in these diseases, playing pivotal roles in the enhancement of inflammatory responses and the progression of disease-related pathologies (17–19). The similar expression patterns of these cytokines reflect the similarity in the immune-inflammatory responses of the two diseases. Thus, the detection of immune infiltration and associated inflammatory molecules could offer diagnostic advantages for patients with AS combine with ankylosing spondylitis, which is essential for preventing severe cardiovascular outcomes.

Currently, genetic studies investigating the association between AS and ankylosing spondylitis are limited. This study aims to identify common biomarkers and elucidate the molecular mechanisms underlying AS and ankylosing spondylitis. We evaluated mRNA expression data from the GEO database. Through employing weighted gene co-expression network analysis (WGCNA) and machine learning technique, ST8SIA4 was pinpointed as a key diagnostic biomarker for both conditions. Furthermore, Gene set enrichment analysis (GSEA) revealed potential involvement of the lysosomal pathway in their pathology. Analysis using CIBERSORT also indicated that ST8SIA4 is implicated in the alterations of the immune microenvironment linked to these disorders (Figure 1). Overall, our findings spotlight ST8SIA4 as a critical diagnostic gene and the lysosomal pathway as a common pathway in AS and ankylosing spondylitis, aiming to foster novel diagnostic and therapeutic approaches for these conditions.

2 Materials and methods

2.1 Data collection and data processing

We obtained gene expression profile datasets along with clinical data from the GEO database by conducting searches with

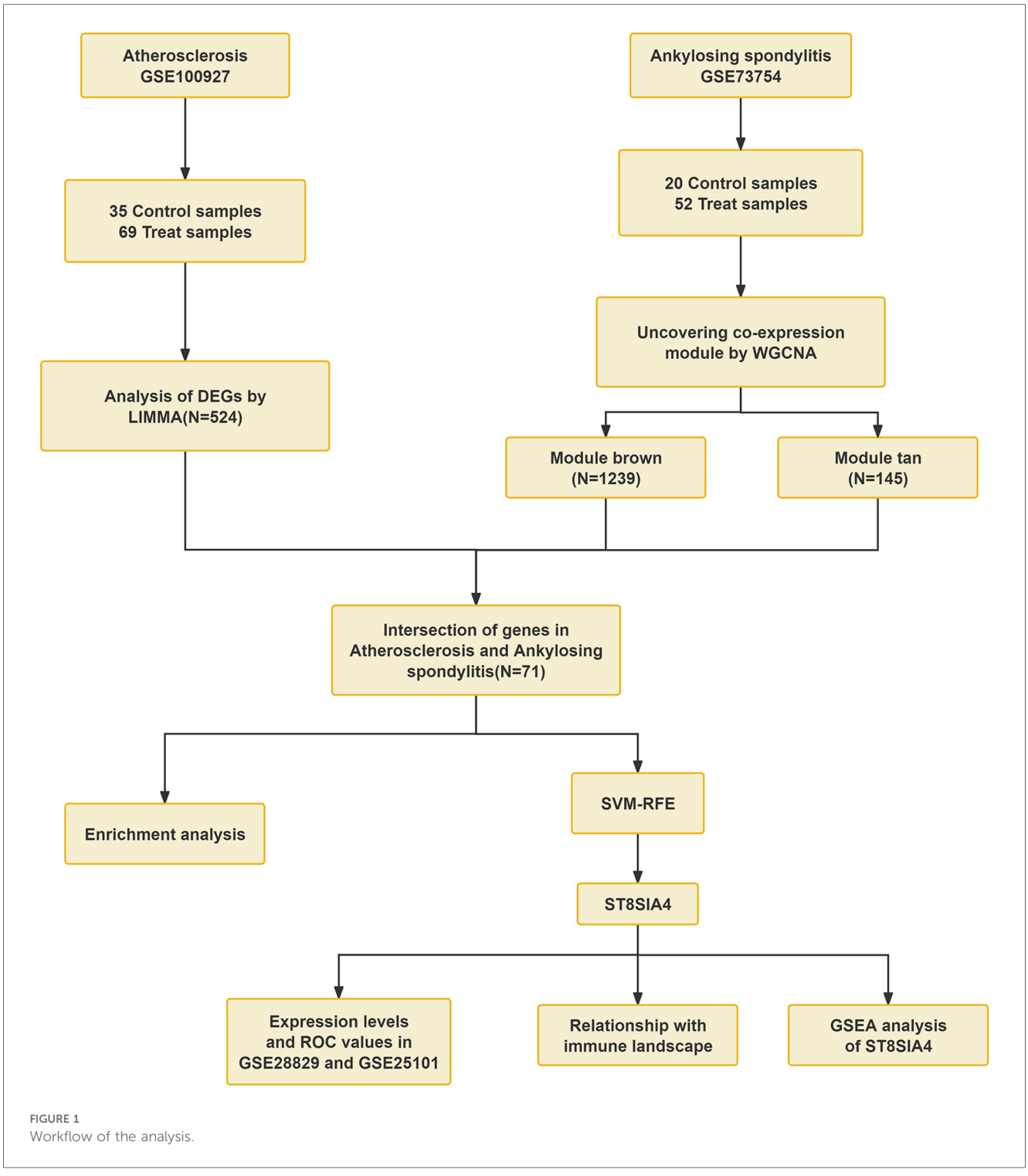

FIGURE 1
Workflow of the analysis.

the terms "atherosclerosis" and "ankylosing spondylitis" for microarray datasets. To ensure the accuracy of WGCNA, we excluded non-human specimens and selected sample groups containing a minimum of 15 samples. The final datasets obtained included GSE100927, GSE73754, GSE28829, GSE25101 and GSE155512. The GSE100927 dataset, utilized on the GPL17077-17467 platform, included tissue samples from 35 healthy controls and 69 patients with AS. The GSE73754 dataset, employed on the GPL10558 platform, consisted of peripheral blood mononuclear cells (PBMCs) from 52 patients with ankylosing spondylitis and 20 healthy controls. Furthermore, the dataset GSE28829, which relies on the GPL570 platform, including carotid artery plaque samples from 16 late-stage and 13 early-stage atherosclerosis patients, served as an external validation set. In a similar manner, the GSE25101 dataset, based on the GPL6947 platform, including gene expression data from whole blood of 16 patients with ankylosing spondylitis and 16 gender and age-matched healthy controls, used as an external validation

set. The GSE155512 dataset includes tissue samples from three individuals who underwent carotid endarterectomy. These samples were procured from atherosclerotic carotid arteries, from which single cells were isolated using the 10× Genomics Chromium Single Cell Gene Expression system. Subsequently, these cells were analyzed through RNA sequencing.

2.2 Differential gene expression screening

Differentially expressed genes (DEGs) in the GSE100927 dataset were identified employing the "Limma" R package. Criteria for screening included |logFC|>1 and adjusted *P*-value <0.05. For visualization, the "ggplot2" package was used to create volcano plots and heatmaps for the top 50 genes with differential expression rankings.

2.3 Weighted gene coexpression network analysis

The analytical framework known as weighted gene co-expression network analysis (WGCNA) was applied to identify significant clusters of co-expressed genes and to elucidate the connections between gene networks and pathological conditions. In our research, the WGCNA R package was harnessed to discern correlations between gene expressions and phenotypic traits by establishing a gene co-expression network (20). Initially, genes displaying the bottom 50% in terms of median absolute deviation (MAD) were omitted. Following this, the Pearson correlation coefficients were computed for all gene pairs, and a weighted adjacency matrix was formulated using average linkage along with weighted correlation coefficients. A soft thresholding power (b) was then applied to compute adjacency, which was further transformed into a Topological Overlap Matrix (TOM). For the purpose of clustering genes that exhibit similar expression patterns, average linkage hierarchical clustering was executed using TOM-based dissimilarity, setting the smallest module size at 60 genes. The process culminated in evaluating the congruence of hub genes across modules, establishing a cutoff for the module dendrogram, and amalgamating multiple modules. This approach was specifically employed to pinpoint crucial modules in the context of ankylosing spondylitis and to produce visual representations of the pertinent gene networks.

2.4 Functional enrichment analysis

To investigate the biological roles of genes, we employed the "clusterProfiler" package in R. Our initial step involved performing Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, recognizing pathways as significant if they presented with a *P*-value <0.05 (21, 22). We then proceeded to ascertain the genes common between the DEGs in AS and the principal module genes in ankylosing spondylitis, conducting additional GO and KEGG analyses on these shared genes. Visualization of the results was facilitated through the use of the "ggplot2" package.

2.5 Identification and verification of diagnostic biomarkers

To pinpoint potential diagnostic markers for AS with ankylosing spondylitis, we utilized the Support Vector Machine Recursive Feature Elimination (SVM-RFE) technique. This method, facilitated by the "e1071" package in R (23), strategically removes feature vectors to isolate key genes that may serve diagnostic purposes. We corroborated the relevance of these genes using datasets GSE28829 and GSE25101. Further, we explored the associations between these identified genes and specific immune cells within the datasets to establish their significant linkages to immune cell activities. For evaluating the diagnostic accuracy of our model, we calculated the area under the receiver operating characteristic (ROC) curve (AUC) using the "pROC" package in R, which measures the effectiveness of the identified core genes.

2.6 Gene set enrichment analysis

We conducted single-gene Gene Set Enrichment Analysis (GSEA) employing the "ClusterProfiler" package in R to discern pathway-level distinctions between disease and control cohorts. The gene set utilized for GSEA, designated as c2.cp.kegg.v7.5.1.symbols.gmt, was sourced from the MSigDB database (24). Only KEGG pathways demonstrating a *P*-value <0.05 were deemed statistically significant. We displayed the outcomes using the "enrichplot" package in R.

2.7 Immune analysis algorithm

To investigate immune cell infiltration in AS and ankylosing spondylitis samples, we applied the CIBERSORT deconvolution algorithm, designed to estimate the proportions of 22 distinct immune cell types using gene expression data. The analysis was conducted on the datasets GSE73754 and GSE100927 (25). We visualized the outcomes employing the R packages "corrplot", "ggplot2", and "ggpubr". Furthermore, we utilized the non-parametric Spearman correlation method to assess the associations between core biomarkers and the expression of immune-infiltrating cells.

2.8 Single-cell sequencing analysis

We downloaded the AS scRNA-seq dataset GSE155512 from GEO. Downstream analysis was performed using the Seurat R package (26). Initially, a Seurat object was created from the single-cell expression data, and quality control was applied, excluding cells with fewer than 50 expressed genes or mitochondrial gene expression exceeding 5%. The data was normalized using the

"LogNormalize" method, and 1,500 highly variable genes were identified with the "FindVariableFeatures" function. Subsequently, principal component analysis (PCA), cluster analysis using Seurat's "FindClusters" function, and t-SNE (t-distributed stochastic neighbor embedding) were conducted for non-linear dimensionality reduction, illustrating the results in t-SNE.

3 Results

3.1 Identification of differentially expressed genes

This analysis compared the gene expression profiles of patients with atherosclerosis and healthy controls in the GSE100927 dataset. DEG analysis revealed 524 genes exhibiting differential expression within the dataset pertaining to AS. Among these, 363 genes were found to be upregulated, while 161 genes were downregulated. Volcano plots for all differentially expressed genes and heatmaps for the top 50 most significantly expressed genes were generated using R (Figures 2A,B).

3.2 Weighted gene co-expression network analysis and identification of key modules

We employed WGCNA to develop a scale-free co-expression network and pinpoint modules strongly associated with ankylosing spondylitis. For the GSE73754 dataset, a "soft" threshold of $b = 13$ was adopted, predicated on achieving scale independence and optimal average connectivity (Figure 3A; Supplementary Data Sheet 1). WGCNA revealed 13 modules. Clinical correlation analysis demonstrated that the "MEbrown" and "MEtan" modules exhibited the highest positive and negative correlations with ankylosing spondylitis, respectively (MEbrown: $r = 0.39$, $p = 8e\text{-}04$; MEtan: $r = -0.66$, $p = 4e\text{-}10$) (Figures 3B,C). Consequently, we selected the brown module, which comprises 1,239 genes, and the tan module, which comprises 145 genes, for further analysis.

3.3 Identification of shared genes and pathways

A total of 71 genes have been discovered that coincide between the DEGs linked to AS and the key modules relevant to ankylosing spondylitis (Figure 4A; Supplementary Data Sheet 2). It is suggested that these genes might play a role in the development of both AS and ankylosing spondylitis. Enrichment analysis conducted on these genes indicated significant associations. GO analysis revealed that the biological process (BP) genes are predominantly involved in leukocyte migration, positive regulation of the defense response, leukocyte cell-cell adhesion, and positive regulation of leukocyte activation (Figures 4B,C). Cellular component (CC) genes are enriched in ficolin-1-rich granules, endocytic vesicles, phagocytic vesicles, and lysosomal membranes. Molecular function (MF) genes are enriched in functions such as integrin binding, Toll-like receptor binding, and actin binding. Furthermore, KEGG pathway analysis shows significant enrichment in pathways related to tuberculosis, neutrophil extracellular trap formation, phagosomes, leishmaniasis, and leukocyte transendothelial migration (Figure 4D).

3.4 Identification and validation of potential shared diagnostic biomarkers

SVM-RFE is a machine learning approach derived from Support Vector Machines, designed to identify optimal core genes by filtering

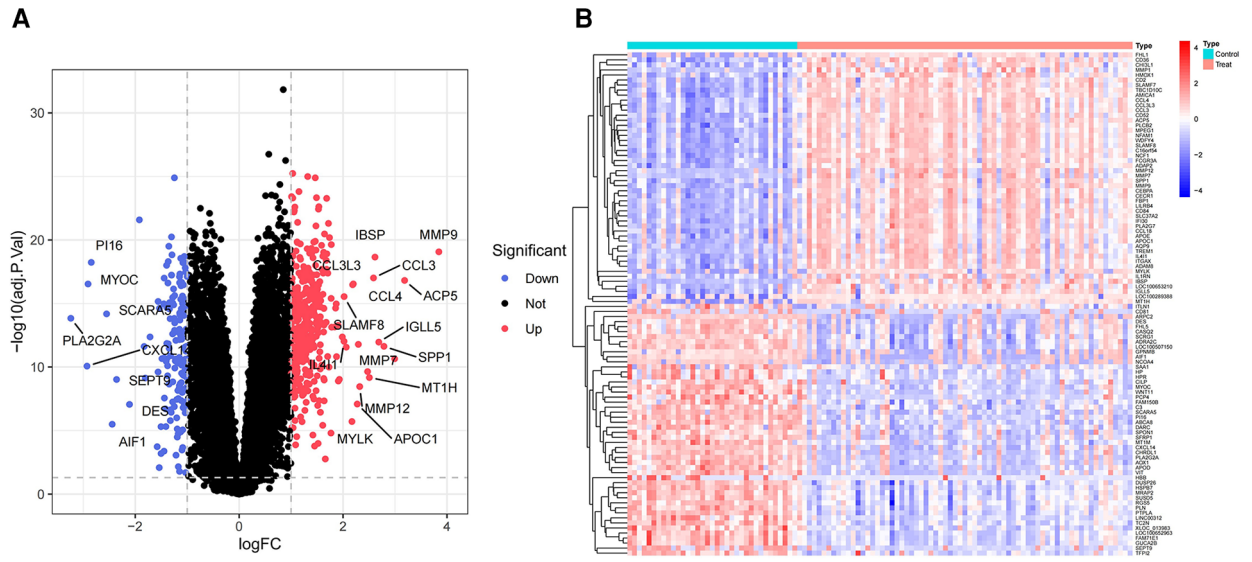

FIGURE 2
Volcano plot and heatmap of the DEGs identified from GSE100927. (A) Volcano map of DEGs from GSE100927. (B) Heatmap of DEGs from GSE100927.

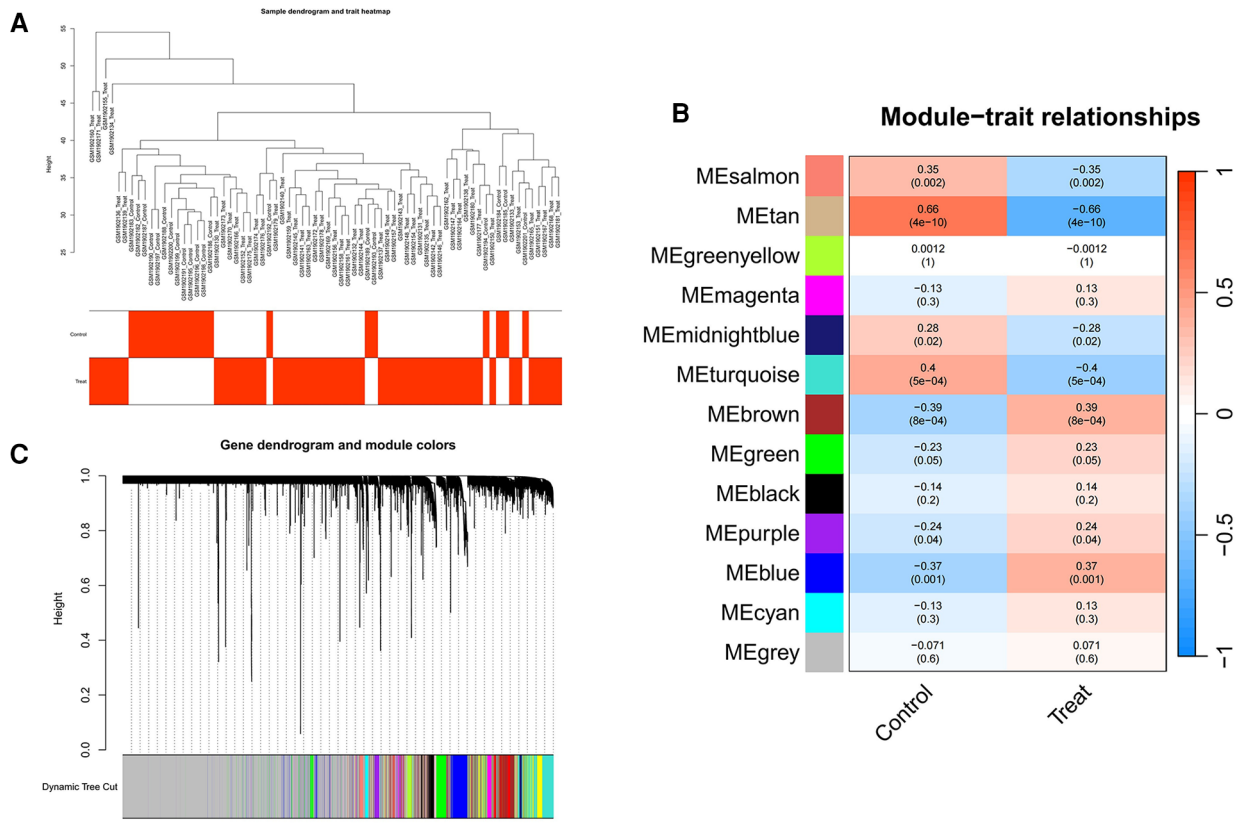

FIGURE 3
Screening of genes in the GSE73754 datasets using the WGCNA algorithm. (A) Clustering dendrogram of samples. (B) The correlation coefficients between gene modules and ankylosing spondylitis. (C) Dendrogram of the gene modules. WGCNA, weighted gene coexpression network analysis.

out feature vectors produced by the SVM. Analysis of 71 common genes revealed 15 potential central diagnostic genes in GSE100927 (Figure 5A), and 24 in GSE73754 (Figure 5B). After intersecting the datasets, six core genes were identified (Figure 5C). Validation using external datasets revealed no significant differences for CSF3R, CTSS, MNDA, and TLR2. Furthermore, it was noted that the CYTH4 gene was not present in the GSE25101 dataset. Ultimately, ST8SIA4 was confirmed as the most likely optimal diagnostic biomarker for AS combine with ankylosing spondylitis (Supplementary Data Sheet 3). Subsequent verification across four datasets showed that ST8SIA4 expression levels were consistently higher in the AS or ankylosing spondylitis groups compared to healthy controls (Figures 6A–D). Furthermore, ROC analysis demonstrated significant diagnostic efficacy of ST8SIA4, with AUC values of 0.976 in GSE100927 and 0.773 in GSE73754 (Figures 6F,H). In further independent analyses, ST8SIA4 demonstrated significant diagnostic effectiveness in datasets GSE28829 and GSE25101, achieving AUC values of 0.846 and 0.812, respectively (Figures 6E,G).

3.5 GSEA analysis

The GSEA results demonstrate that in AS samples, the lysosomal, cytokine receptor interaction, and toll-like receptor signaling pathways are positively enriched (Figure 7A). Furthermore, in samples from ankylosing spondylitis, positive enrichment is also evident in the lysosomal and chemokine signaling pathways, as well as in response to pathogenic Escherichia coli infection (Figure 7B). Notably, the lysosomal pathway is consistently enriched across both conditions.

3.6 Immunocellular infiltration analysis

Our research further delved into the association between the ST8SIA4 gene and the immune system's cellular composition by evaluating the prevalence of 22 distinct immune cell types within samples from AS and ankylosing spondylitis (Figures 8A, 9A). The AS samples displayed increased levels of several immune cells, including B cells memory, T cells follicular helper, T cells regulatory, T cells gamma delta, macrophages M0, and mast cells activated. Conversely, the same samples showed decreased quantities of B cells naive, plasma cells, T cells CD4 memory resting and activated, NK cells resting, monocytes, macrophages M1 and M2, and mast cells resting (Figure 8C). In comparison to healthy controls, samples from ankylosing spondylitis cases notably had higher levels of T cells CD4 naive, T cells regulatory, and neutrophils, but lower levels of T cells CD4 memory activated, T cells gamma delta, and NK cells resting (Figure 9C).

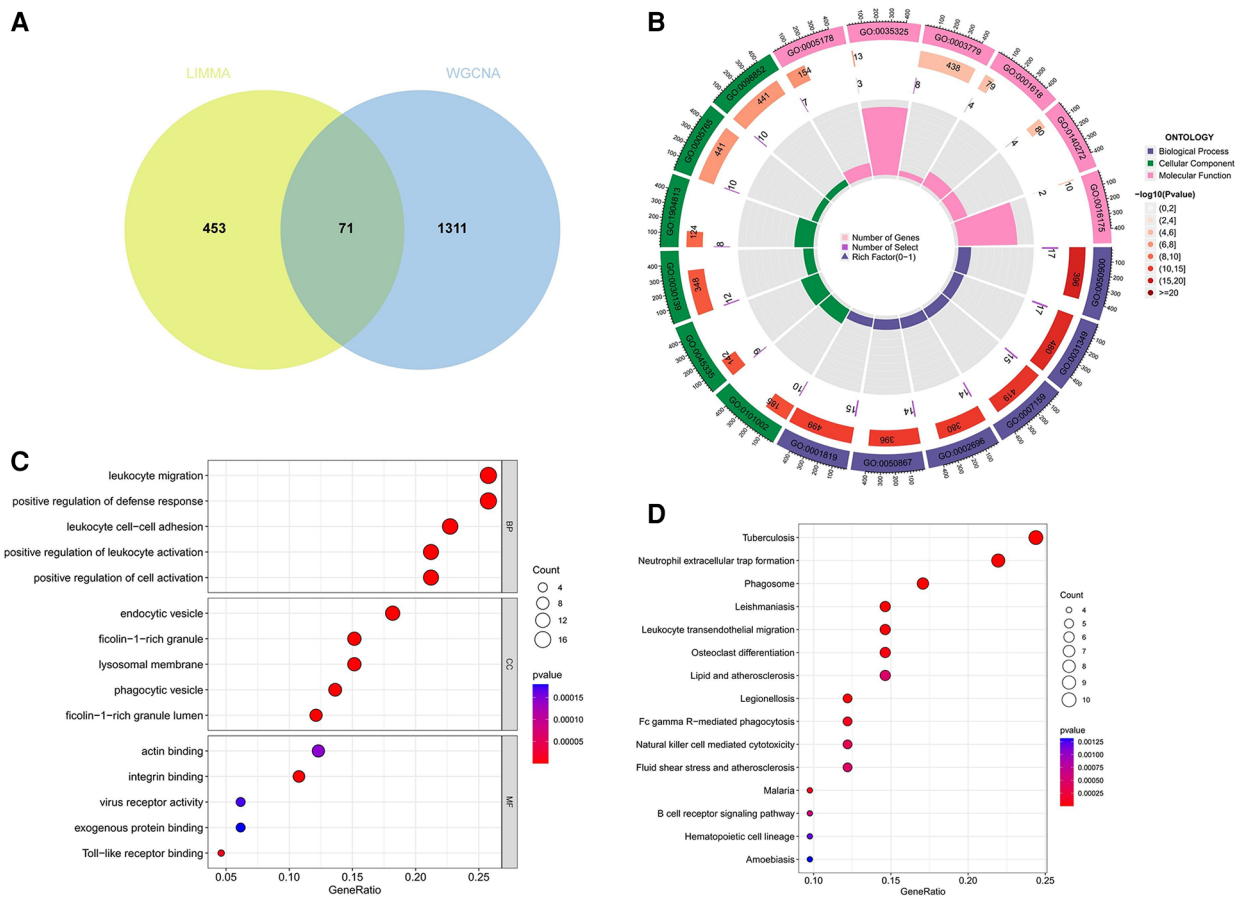

FIGURE 4
Function enrichment analysis of the intersecting genes. (**A**) The intersecting of DEGs via Limma and WGCNA module genes includes 71 genes, which were shown in the Veen diagram. (**B**,**C**) Circle plot and bubble plot of GO enrichment analysis includes biological process, cellular component and molecular function. (**D**) Bubble plot of KEGG enrichment. GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

Furthermore, we observed a positive correlation between the ST8SIA4 gene expression and the presence of T cells CD8 and neutrophils, and a negative correlation with macrophages M2 and T cells CD4 naive in AS samples (Figure 8B). Similarly, in ankylosing spondylitis, there was a positive correlation with neutrophil levels and a negative correlation with T cells CD4 naive and T cells CD4 memory resting (Figure 9B). These findings, which are statistically significant with a *P*-value <0.05, clarify the influence of ST8SIA4 on immune cell dynamics.

3.7 Expression of ST8SIA4 in single cells

We retrieved single-cell data from the GSE155512 dataset and performed analysis using the Seurat toolkit, applying the t-SNE algorithm for clustering cells. After quality control measures, cells that did not meet quality standards were excluded (Figures 10A). Cells from three distinct samples were categorized into seven subgroups: Chondrocytes, Macrophages, Endothelial Cells, T Cells, Monocytes, CMP, and Smooth Muscle Cells (Figure 10B). Further analysis indicated that the genes ST8SIA4 was predominantly expressed in Macrophages, Monocytes, T Cells, and CMP (Figure 10C).

4 Discussion

Both AS and ankylosing spondylitis involve inflammatory responses and abnormal immune system activation. Studies suggest that AS development commences with endothelial cell damage and dysfunction. This damage facilitates the recruitment of inflammatory cells, especially monocytes and T cells, into the subendothelial space. The activated cells contribute to the accumulation of oxidized low-density lipoprotein (LDL) and foam cell formation, which are pivotal in plaque development within vascular walls. Released cytokines and enzymes from these cells during plaque formation accelerate its growth and complexity, eventually leading to vascular narrowing or thrombosis (15, 27). In ankylosing spondylitis, inflammation primarily affects the spine and sacroiliac joints, involving chronic immune cell activation and continuous inflammatory mediator release. A critical pathway in this condition is the activation of the IL-23/IL-17 axis, which

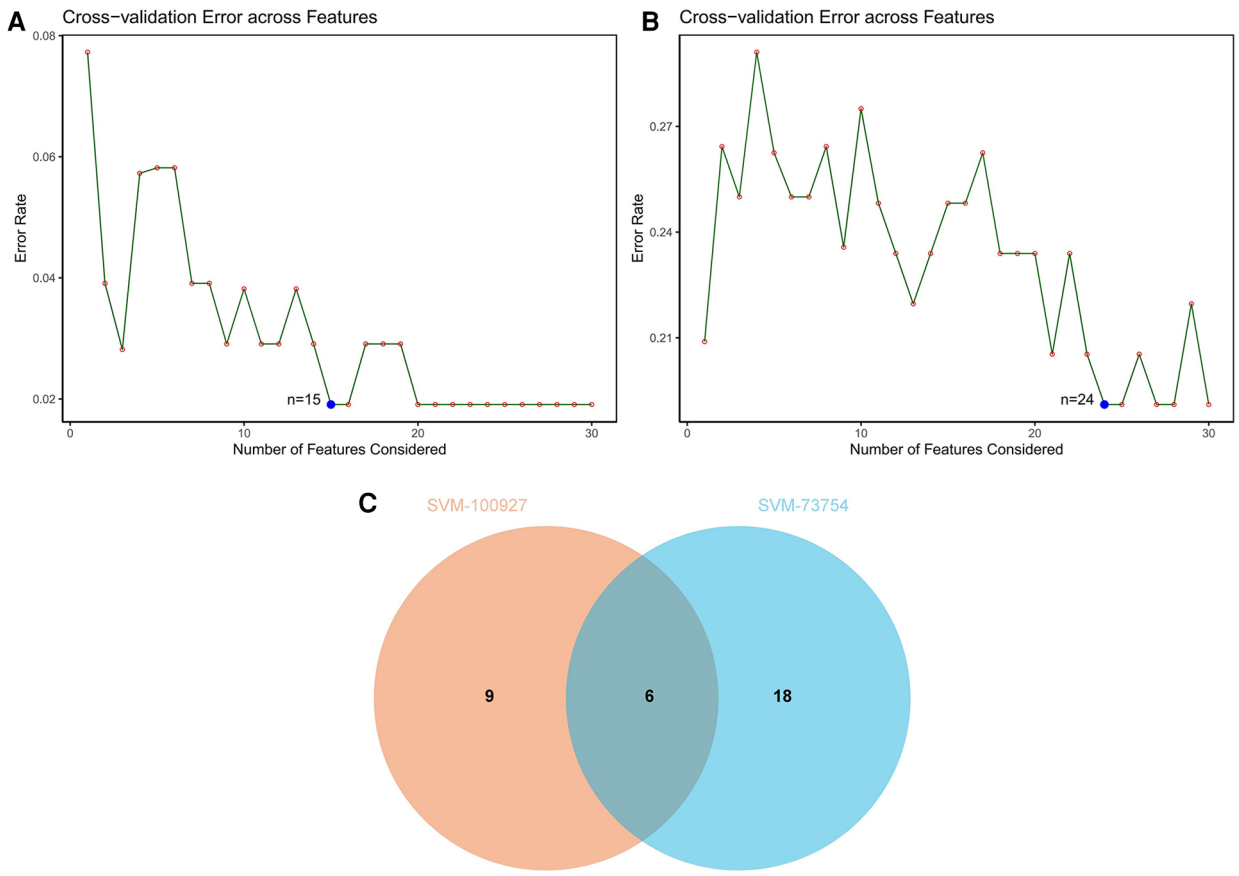

FIGURE 5
Identification of diagnostic genes using SVM-RFE algorithm. (A) Feature genes selection in GSE100927. (B) Feature genes selection in GSE73754. (C) Venn diagram of overlapping feature genes.

promotes the proliferation of Th17 cells and increased expression of IL-17. This cytokine not only stimulates inflammatory cell recruitment and activation but also influences bone metabolism, contributing to pathological bone formation (28–30). Additionally, the IL-23/IL-17 axis in AS is linked to vascular inflammation and diminished plaque stability (31). Moreover, both conditions share key aspects of immune regulation, notably elevated T-cell-mediated responses and inflammatory cytokines like TNFα, IL-6, and IL-1β, reflecting an increased cardiovascular disease risk in ankylosing spondylitis patients (4, 18, 32). The extensive inflammatory state may predispose these individuals to cardiovascular conditions, akin to the chronic inflammation observed in AS. Despite affecting different physiological systems, the similarities in their inflammatory pathways and immune dysregulation underscore potential links between these conditions, providing valuable insights for novel therapeutic approaches focusing on inflammation and immune regulation. Further research is imperative to explore these pathways more comprehensively, aiming to devise new treatment strategies and enhance patient outcomes.

The exact mechanisms underlying both AS and ankylosing spondylitis remain elusive. This study investigates their common pathways and biomarkers through bioinformatics analysis. RNA-seq profiles from the GEO database were analyzed to compare AS and ankylosing spondylitis. Employing the SVM-RFE algorithm, we identified key core genes, notably ST8SIA4, and elucidated a shared lysosomal pathway in both diseases. The potential of ST8SIA4 as a diagnostic biomarker for AS and ankylosing spondylitis was further validated. Analysis of immune infiltration profiles revealed elevated levels of regulatory T cells in the disease cohorts, suggesting a shared immunological basis in the pathogenesis of AS and ankylosing spondylitis.

T cells regulatory (Tregs) comprise a specific group of cells recognized for their ability to modulate immune responses. They achieve this by restraining the proliferation, activation, and secretion of inflammatory cytokines from effector T cells (Th) through mechanisms such as the release of suppressive cytokines (IL-10, TGF-β) and through direct cell-to-cell contact inhibition. This action maintains self-tolerance and mediates anti-inflammatory effects (33). In AS, arterial wall inflammation is driven by adaptive and innate immune responses (34–36). Tregs mitigate this inflammation by curbing pro-inflammatory responses from Th1/Th17 cells and reducing antigen presentation by dendritic cells (37–39). Furthermore, Tregs suppress cytokine secretion, inhibit macrophage-driven inflammation, and regulate cholesterol metabolism and foam cell formation (40). Studies

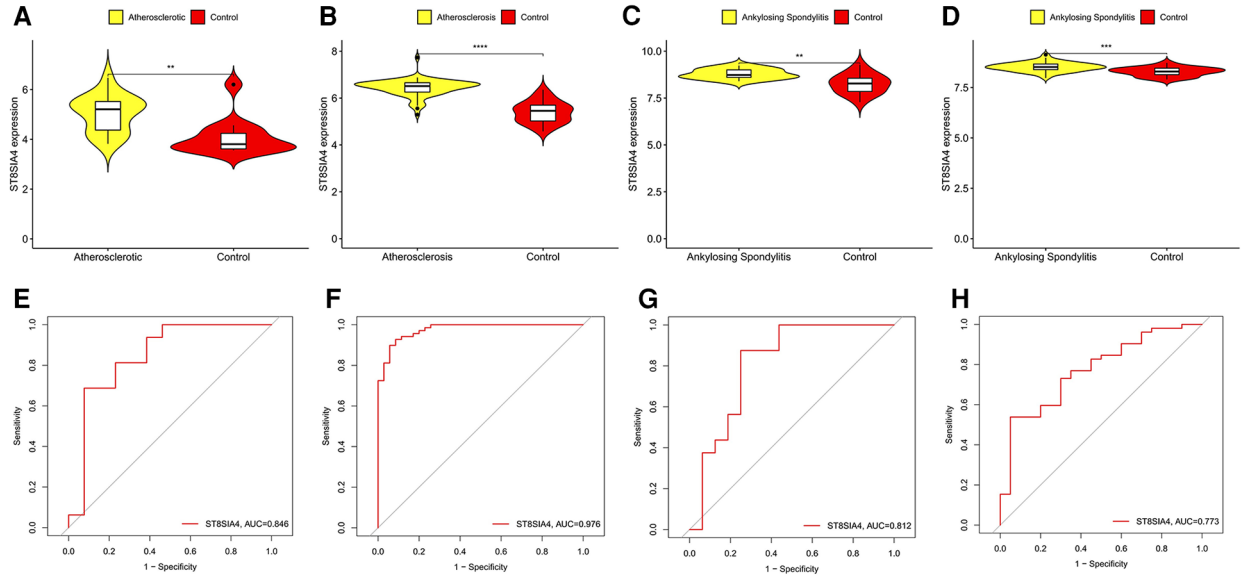

FIGURE 6
Validation of the expression level and diagnostic efficacy of ST8SIA4 gene. The violin plots of ST8SIA4 gene in GSE28829 (**A**), GSE100927 (**B**), GSE25101 (**C**) and GSE73754 (**D**) The ROC curves of ST8SIA4 gene in GSE28829 (**E**), GSE100927 (**F**), GSE25101 (**G**) and GSE73754 (**H**) $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

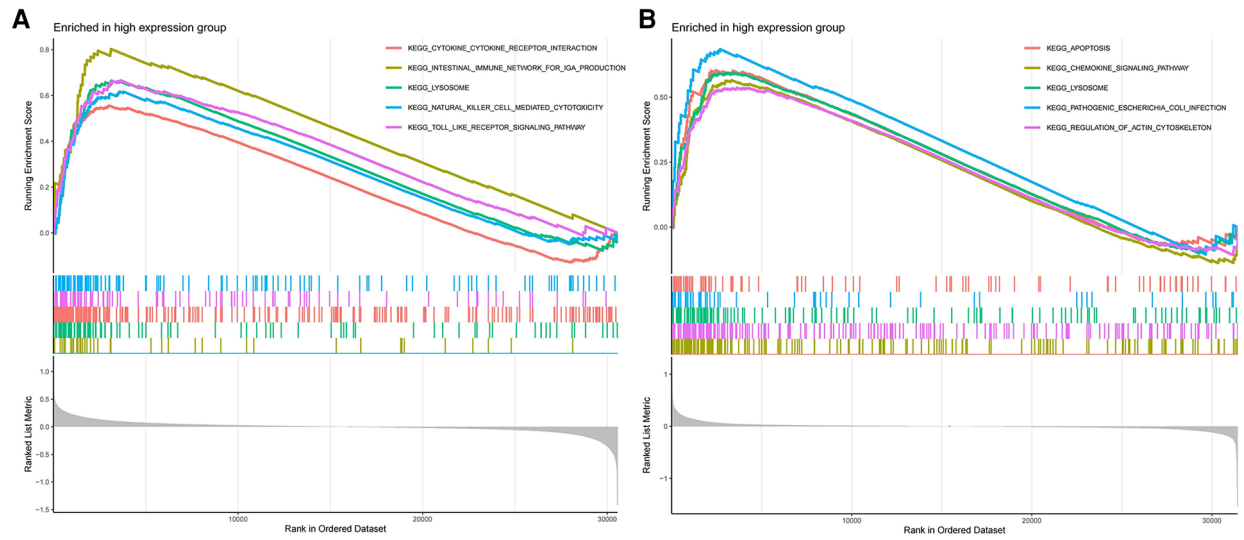

FIGURE 7
(**A**) GSEA analyses results of aS samples. (**B**) GSEA analyses results of ankylosing spondylitis samples.

have shown that reduced Treg numbers within atherosclerotic plaques correlate with increased plaque vulnerability and rupture, whereas increased Treg levels can slow AS progression (41–43). Research by Nilsson J et al. suggests that Tregs control autoreactive T cell activity to prevent AS development (44). Similarly, animal models have demonstrated Treg responsiveness to apolipoprotein B100-derived peptides, reducing AS incidence and progression (45, 46). Conversely, Treg depletion exacerbates AS in hypercholesterolemic mice (38, 39). These findings underscore Tregs' crucial role in managing AS occurrence and progression. Moreover, studies indicate Treg functional impairments in ankylosing spondylitis, potentially due to IL-2 deficiencies, reduced STAT5 phosphorylation, decreased FOXP3 expression, and increased CpG methylation in the CNS2 region

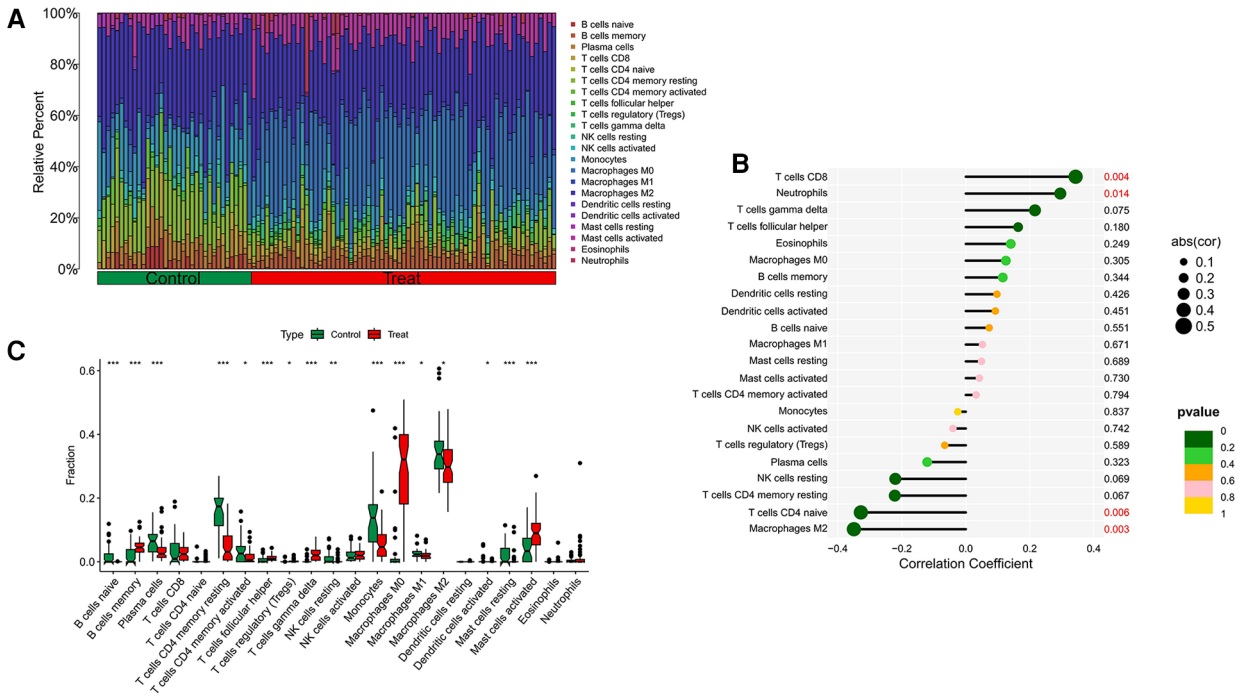

FIGURE 8
Immune infiltration analysis of ST8SIA4 gene in aS. (**A**) Histogram of proportion of immune cells. (**B**) Correlation between ST8SIA4 and immune cells content. (**C**) Proportion of 22 kinds of immune cells in a boxplot diagram. *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

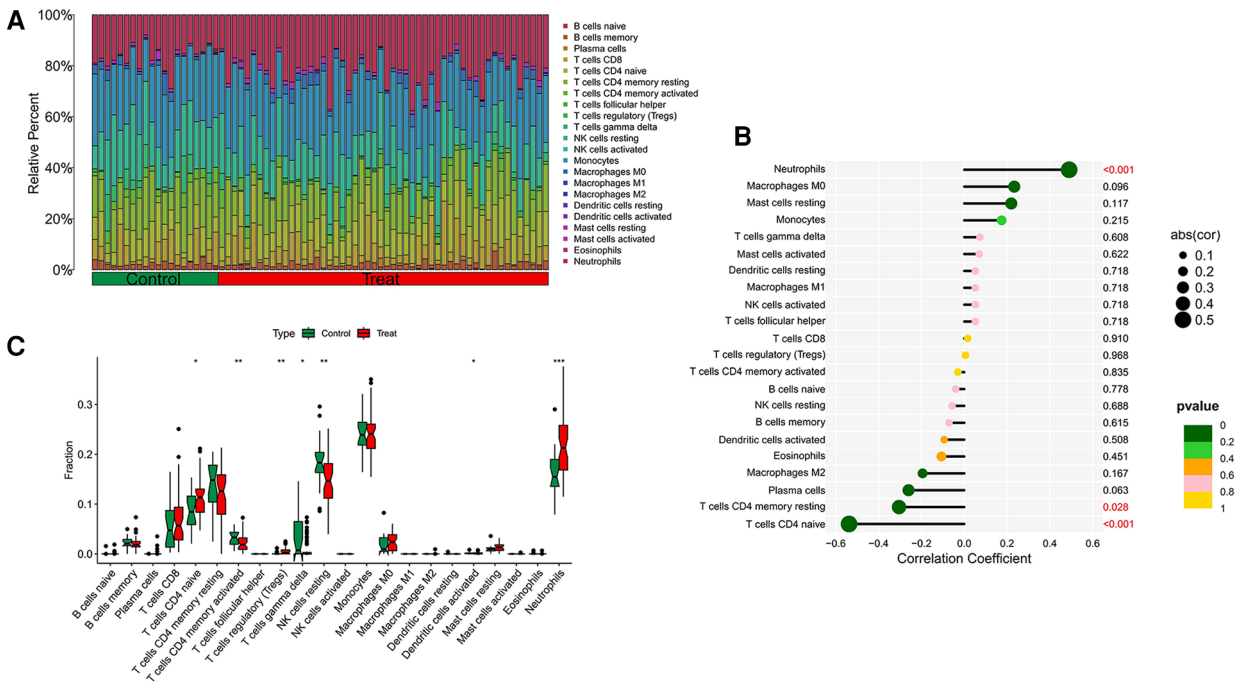

FIGURE 9
Immune infiltration analysis of ST8SIA4 gene in ankylosing spondylitis. (**A**) Histogram of proportion of immune cells. (**B**) Correlation between ST8SIA4 and immune cells content. (**C**) Proportion of 22 kinds of immune cells in a boxplot diagram. *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

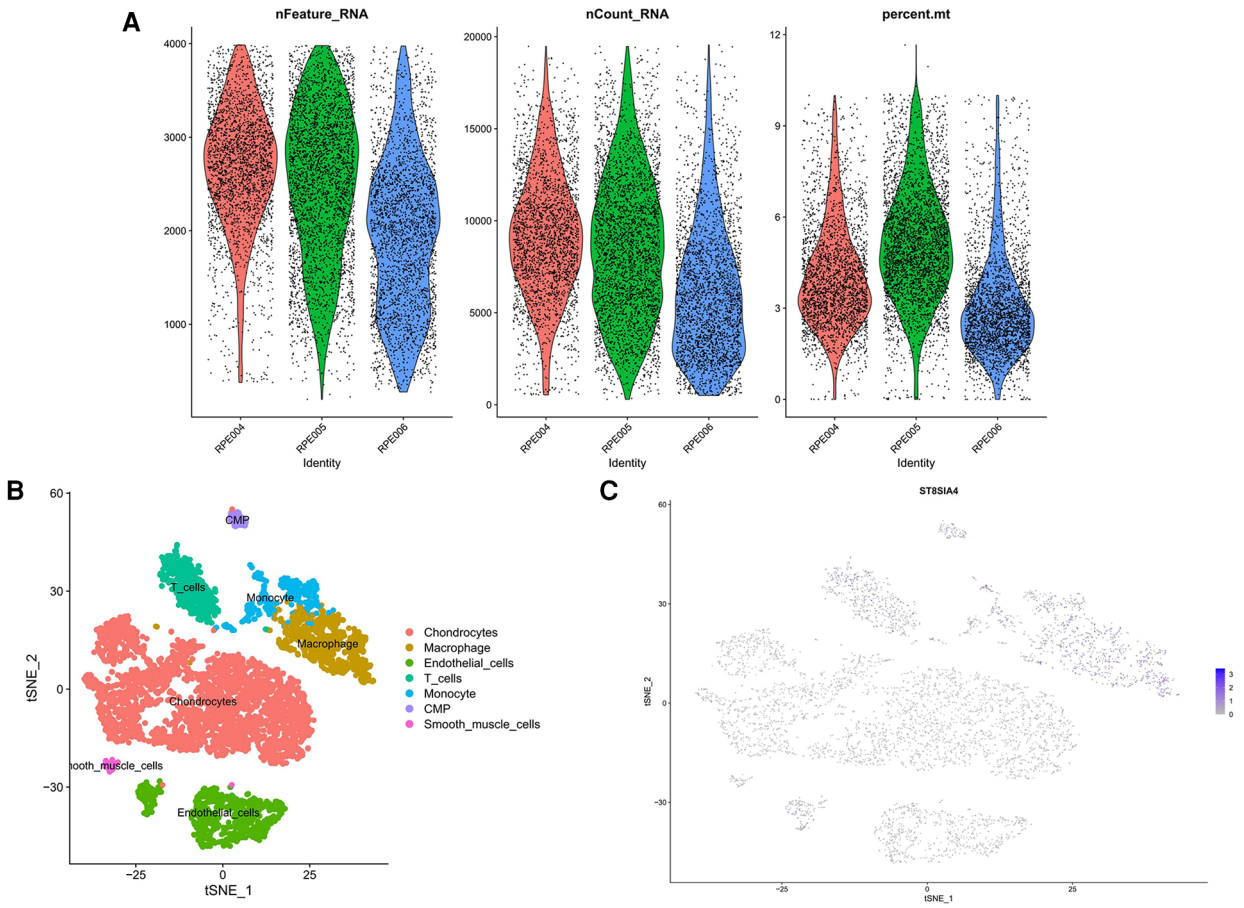

FIGURE 10
Quality control results for single-cell data. The number of genes, the number of gene reads and the proportion of mitochondrial genes were represented in order from top to bottom. (A) The panel was post-processing. (B) Cellular subtypes of AS. (C) Scatter plot of the expression of ST8SIA4.

of the FOXP3 gene. These impairments lead to uncontrolled effector CD4+ T cell proliferation and immune imbalance (47, 48). Liao et al. found that increased Treg numbers in ankylosing spondylitis patients correlate positively with disease activity, supporting a feedback mechanism where inducible Tregs arise from persistent inflammation (49–51). Continued research is essential to elucidate the mechanisms by which Tregs influence AS and ankylosing spondylitis development.

This study identifies ST8SIA4 as a key diagnostic marker in the progression of AS and ankylosing spondylitis, noting a significant increase in its expression in affected patients. ST8SIA2 and ST8SIA4, two polysialyltransferases located in the Golgi apparatus, facilitate the addition of polysialic acid chains to proteins, including themselves via autopolysialylation (52). Notably, these enzymes are significant for polysialylating the Neural Cell Adhesion Molecule (NCAM) specifically at its fifth Ig domain, which leads to the formation of polymers that include 60–90 sialic acid residues (53, 54). The introduction of this extensive, negatively charged side chain is crucial as it interferes with PSA-NCAM binding interactions, subsequently promoting enhanced cell motility (55). Despite these insights, the functional mechanisms of ST8SIA4 remain unclear. Shu et al. have established that increased St8sia4 expression correlates positively with the presence of MDSCs, macrophages, and Treg cells. This association suggests that St8sia4 plays a pivotal role in altering the tumor microenvironment, thereby facilitating tumor growth and metastasis (56). In this research, we observed that in AS patients, ST8SIA4 correlates positively with neutrophil and CD8+ T-cell levels, but negatively with naïve CD4T cells and M2 macrophages. In cases of ankylosing spondylitis, similar positive correlations with neutrophils and negative correlations with both naïve and resting memory CD4T cells were noted. Although direct evidence linking ST8SIA4 to these diseases is lacking, the data suggest its involvement in regulating cell adhesion, immune responses, and inflammation in vascular tissues, thereby potentially influencing the pathogenesis of AS and ankylosing spondylitis through complex immune and inflammatory mechanisms, warranting further investigation (56, 57).

Lysosomes serve as crucial regulators of cellular and organismal homeostasis, mediating essential processes such as signal transduction, cellular metabolism, proliferation, differentiation, secretion, and the quality control of proteins and organelles

(58, 59). Studies have identified a significant role for lysosomes in cholesterol metabolism; dysfunction in these organelles can result in cholesterol accumulation within cells (60, 61), contributing to foam cell formation and atherosclerotic plaque development. Additionally, lysosomes facilitate the removal of cellular debris, including damaged proteins and organelles, via the autophagy pathway (62, 63). Autophagy aids in the hydrolysis of lipid droplets and the efflux of free cholesterol from foam cells, thereby playing a protective role against AS (64). Conversely, lysosomal dysfunction can impair autophagic processes, leading to the buildup of cellular waste, which in turn promotes inflammatory responses and AS progression (60). Furthermore, abnormalities in lysosomes can lead to the activation of the NLRP3 inflammasome, which initiates the secretion of pro-inflammatory cytokines like IL-1β and IL-18 (65–67). These cytokines are instrumental in enhancing the recruitment and activation of inflammatory cells, including macrophages and T cells (58, 68), and in increasing the expression of cytokines, chemokines, and adhesion molecules (69, 70). This cascade amplifies inflammatory responses and exacerbates the progression of AS.

The lysosomal pathway is intricately linked to the pathogenesis of ankylosing spondylitis. Research has demonstrated that the disease-associated HLA-B27 subtype exhibits increased resistance to lysosomal degradation, potentially leading to a prolonged immune response and contributing to AS pathogenesis (71). Additionally, lysosomes play a pivotal role in autoimmune diseases like AS by modulating cell death, autophagy, inflammasome-related cytokines, and various metabolic pathways, including sphingolipid metabolism (58). This investigation additionally recognizes the lysosomal pathway as a plausible shared mechanism contributing to the development of AS, highlighting the necessity for ongoing investigation into these interconnected pathomechanisms.

The focus of this paper is the investigation of shared immune pathways, pivotal genes, and immune infiltration characteristics of AS. This retrospective study recognizes the need for external validation and has validated key genes across multiple datasets. Future research will aim to verify the function of these genes both *in vitro* and *in vivo*. The consistency with previous studies enhances this research's reliability. Subsequent efforts will focus on the collection of peripheral blood specimens, performing RT-qPCR analysis on essential genes, and executing cohort studies to assess the gene expression in patients with combined AS and ankylosing spondylitis, their linkage to cardiovascular incidents, and their prognostic value.

5 Conclusion

In this study, we employed bioinformatics techniques and machine learning algorithms to identify ST8SIA4 as a shared diagnostic gene for AS and ankylosing spondylitis. We additionally confirmed the diagnostic utility of ST8SIA4 through an independent dataset. Furthermore, CIBERSORT analysis showed a correlation between the expression of ST8SIA4 and the infiltration of immune cells. Single-cell sequencing analysis reveals that ST8SIA4 is primarily expressed in macrophages, monocytes, T cells, and CMP. GSEA analysis indicated that the lysosomal pathway is enriched across both examined conditions. This investigation supports the potential of ST8SIA4 as a diagnostic biomarker and aids in exploring the common mechanisms associated with AS and ankylosing spondylitis. Future research is directed towards understanding the function of the lysosomal pathway and the effects of ST8SIA4 via both *in vitro* and *in vivo* studies.

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

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

YM: Methodology, Resources, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Software, Validation, Visualization, Investigation. JL: Methodology, Resources, Writing – original draft, Writing – review & editing. QW: Investigation, Methodology, Writing – original draft, Writing – review & editing. LS: Methodology, Writing – review & editing. YW: Methodology, Writing – review & editing. XL: Formal Analysis, Writing – review & editing. QZ: Data curation, Writing – original draft. JW: Writing – original draft, Writing – review & editing, Funding acquisition, Project administration, Validation.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2024.1421071/full#supplementary-material

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Moving from lipids to leukocytes: inflammation and immune cells in atherosclerosis

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Atherosclerotic cardiovascular disease (ASCVD) is the most important cause of morbidity and mortality worldwide. While it is traditionally attributed to lipid accumulation in the vascular endothelium, recent research has shown that plaque inflammation is an important additional driver of atherogenesis. Though clinical outcome trials utilizing anti-inflammatory agents have proven promising in terms of reducing ASCVD risk, it is imperative to identify novel actionable targets that are more specific to atherosclerosis to mitigate adverse effects associated with systemic immune suppression. To that end, this review explores the contributions of various immune cells from the innate and adaptive immune system in promoting and mitigating atherosclerosis by integrating findings from experimental studies, high-throughput multi-omics technologies, and epidemiological research.

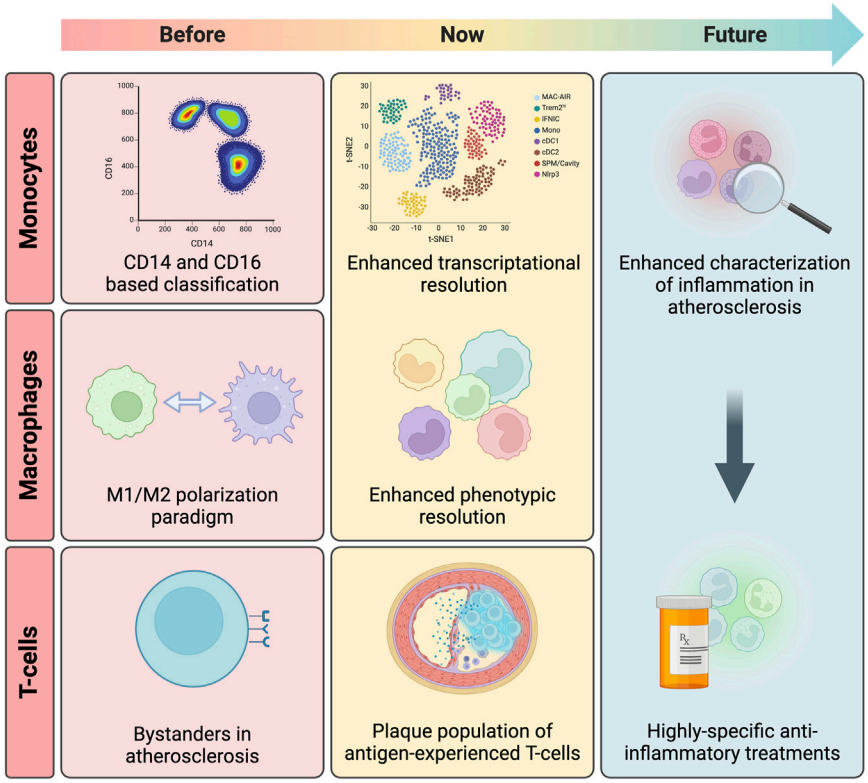
KEYWORDS

vascular medicine, atherosclerosis, inflammation, cardiovascular disease, immune cells

1 Introduction

In recent years, atherosclerotic cardiovascular disease (ASCVD) has grown to be the leading cause of mortality worldwide (Vos et al., 2020). The formation of atherosclerotic plaques, or atherogenesis, is preceded by increased vessel wall activation and increased vascular permeability. Following this, in a process that spans many years, accumulation of low-density lipoprotein (LDL) within the vascular endothelium eventually leads to plaque formation. This process occurs mainly at sites of the vasculature that are characterized by disturbed blood flow, such as branch points and bifurcations. As the plaque grows, sudden occlusion can follow rupture or erosion of the plaque's surface, leading to ischemic events with clinical consequences such as myocardial infarction and stroke (Farb et al., 1996; Libby et al., 2011; Bentzon et al., 2014).

Experiments in human and murine models, including the well-known *Apoe*$^{-/-}$, *apoE***3-Leiden.CETP*, and *Ldlr*$^{-/-}$ mice models, have countered the traditional view that atherosclerosis is a disease of mere passive lipid accumulation. On the contrary, it is now widely accepted that low-grade inflammation is a hallmark of the pathophysiology of atherosclerosis. Leukocytes densely populate the arterial walls both in healthy and affected individuals. Their number and composition, however, differ in health and disease. Plaque



GRAPHICAL ABSTRACT

inflammation is driven by involvement of both the innate and adaptive immune system, which is caused by persistent pro-inflammatory triggers that facilitate both plaque progression and the occurrence of plaque rupture and erosion.

Clinicians have traditionally focused on vigorously lowering plasma cholesterol levels as the predominant approach to stalling plaque development and prevention of cardiovascular events. This paradigm, however, is due for reconsideration. In a considerable proportion of patients that receive optimal lipid-lowering therapy in accordance with current guidelines, a residual inflammatory risk of recurrent cardiovascular complications remains (Sampson et al., 2012). A recent meta-analysis of clinical trials, encompassing over 30.000 patients with a history of ASCVD, showed that residual inflammatory risk, defined as high-sensitivity C-reactive protein ≥2 mg/L, is a larger driver of recurrent cardiovascular events than risk attributed to residual LDL cholesterol (LDL-C) in patients already receiving optimal lipid lowering therapy (Ridker et al., 2023). The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) was the first landmark study to demonstrate that a monoclonal antibody (canakinumab) targeting IL-1β could significantly reduce the recurrence rates of cardiovascular events independent of changes at the lipid level (Ridker et al., 2017). Similar results were obtained using low doses of the anti-inflammatory drug colchicine (Nidorf et al., 2020). It is therefore now widely recognized that mitigating inflammation is of paramount importance to further reduce residual cardiovascular risk. It should be noted however, that trials like CANTOS have shown that broad targeting of inflammation comes with serious adverse side effects. Additionally, in the Cardiovascular Inflammation Reduction Trial (CIRT), administration of a low dose of the broad anti-inflammatory agent methotrexate did not result in a reduction of cardiovascular events, which is indicative of the complexity of inflammatory pathways in atherosclerosis. Research efforts should therefore focus on more specific anti-inflammatory therapies that target, for instance, one particular immune cell type or inflammatory process that is causative for atherosclerosis. They should also recognize that inflammation is a crucial component of host homeostasis (Xu et al., 2022). Effective therapeutic strategies might therefore involve not just the suppression of "bad" inflammation but also the enhancement of "good" inflammation to maintain a balanced immune response in the face of pro-atherosclerotic triggers. In light of this, detailed knowledge about the inflammatory processes governing atherogenesis is indispensable. The introduction of novel high-throughput methodologies, such as single-cell RNA sequencing (scRNA-seq) has greatly accelerated our ability to characterize the immune landscape in atherosclerosis. These techniques will continue to enhance our understanding of the complex cellular interactions and molecular pathways driving disease progression, paving the way for novel personalized and effective anti-inflammatory therapies that are cell- or pathway-specific.

In this review, we will explore the functions of various immune cells and the molecular mechanisms at play in atherosclerosis, emphasizing contributions from high-throughput technologies where relevant. By using these insights to find possible therapeutic targets, it paves the way for novel methods to mitigate inflammatory cardiovascular risk.

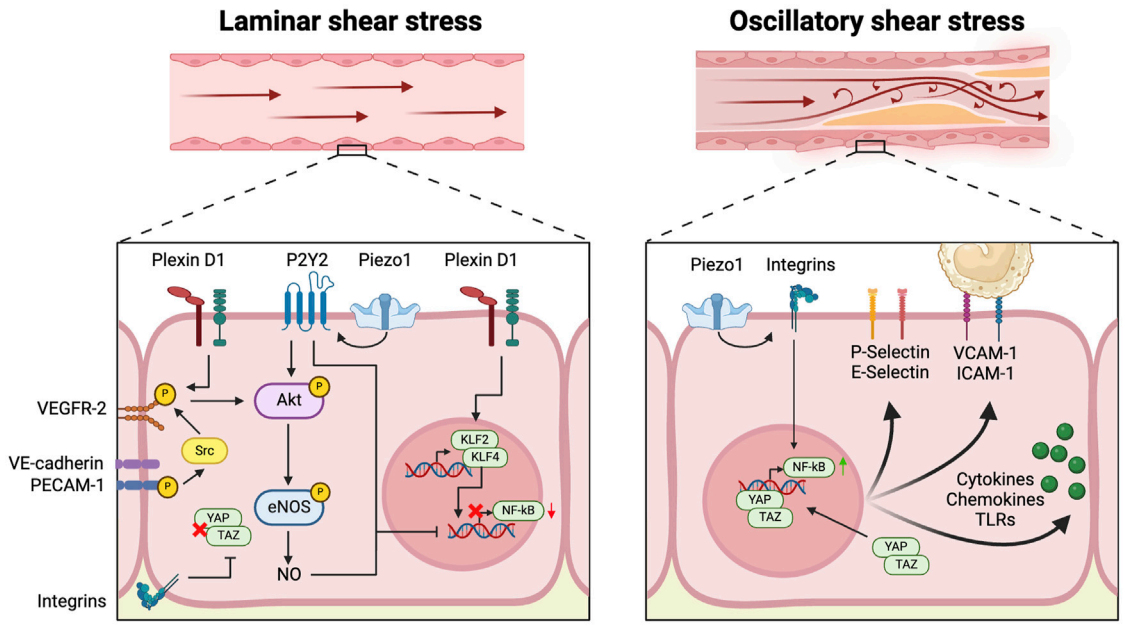


FIGURE 1
Mechanosensing of disturbed flow induces endothelial activation At the apical surface of the vascular endothelial cell, Plexin D1, Piezo1 and P2Y2 act as mechanosensors and sense laminar flow, leading to activation of Akt and endothelial nitric oxide synthase (eNOS), in addition to upregulation of Kruppel-like transcription factors (KLF) 2 and 4 and downregulation of the pro-inflammatory nuclear factor-κB (NF-κB). At cell-cell junctions, vascular endothelial cadherin (VE-cadherin) works in concert with platelet endothelial cell adhesion molecule (PECAM1) and vascular endothelial growth factor receptor 2 (VEGFR2) to induce Akt activation in response to laminar flow. At the basal membrane, integrin signaling downregulates yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) signaling. In contrast, Piezo1 induces expression of NF-κB through integrin signaling in response to disturbed flow. Coupled with unbridled YAP-TAZ signaling, this leads to a variety of pro-inflammatory responses: upregulation of P-Selectin, E-selectin, Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1), and increased production of various cytokines, chemokines and Toll-like receptors (TLRs).

2 Endothelial cell activation primes the vessel wall for an inflammatory response

2.1 Laminar shear stress induces an anti-inflammatory and atheroprotective effect

The arterial wall is lined by a single layer of endothelial cells (EC), which are constantly exposed to variations in shear stress patterns and regulated by a multitude of mechanical and molecular factors that either promote or mitigate inflammation (Luscinskas Francis and Gimbrone, 1996). EC activation serves as a first line of defense against atherogenic stressors and potentiates an increase in interactions between ECs and circulating immune cells. It is largely driven by variations in blood flow-induced shear stress throughout the arterial vasculature (Gimbrone et al., 2000). High laminar shear stress (LSS), occurring in regions characterized by stable flow triggers various downstream anti-inflammatory signals within ECs. These signals are sensed by a complex network of mechanosensitive protein complexes present on cell-cell junctions and the apical and basal endothelial surface of ECs (Figure 1) (Demos et al., 2020).

On the apical surface, the cation channel Piezo1 relays downstream signals through the activation of the P2Y2 and Gαq/11 pathways, which in turn activate Akt, upon mechanical detection of LSS (Iring et al., 2019). At cell-cell junctions, LSS leads to the phosphorylation of the mechanosensory cell-cell adhesion protein Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1). Through its interaction with Vascular Endothelial Cadherin (VE-cadherin), phosphorylated PECAM-1 initiates Src-dependent phosphorylation of VEGFR2 and -3 and subsequent Akt activation (Tzima et al., 2005; Coon et al., 2015). Recent research has shed light on the role of the guidance receptor plexin D1, which has a mechanosensing function on the apical endothelial membrane. This receptor forms a complex with Neuropilin 1 (NRP1) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) to contribute to activation of the latter in response to LSS (Mehta et al., 2020). These mechanosensory pathways, involving Piezo1, plexin D1, and PECAM-1, converge within the endothelial cell to activate various anti-inflammatory signaling pathways (Fleming et al., 2005; Wang et al., 2015; Iring et al., 2019; Mehta et al., 2020). This triggers a series of downstream atheroprotective effects, such as the upregulation of endothelial nitric oxide synthase (eNOS), leading to increased production of endothelial nitric oxide (NO) and thus increasing endothelial barrier integrity and reducing oxidative stress (Fleming et al., 2005; Wang et al., 2015; Iring et al., 2019; Mehta et al., 2020). Even more significantly, LLS-induced mechanosignalling increases transcription of Kruppel-like transcription factors (KLF) 2 and 4, which are recognized as pivotal regulators in the flow-sensitive activation of anti-atherogenic pathways (Novodvorsky et al., 2014). Notably, NO, KLF2 and KLF4, as well as Piezo1-mediated P2Y purinoceptor 2 (P2Y2) and Gαq/11 signaling inhibit the transcription of the pro-inflammatory nuclear factor kappa B (NF-κB) (Gofman et al., 1950;

Albarrán-Juárez et al., 2018). Furthermore, KLF2 directly inhibits transcription of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase-3 (PFKFB3), thereby repressing glycolysis in ECs and promoting a quiescent endothelial state. Doddaballapur et al. (2015) Moreover, exposure of endothelial mechanosensory integrin β to LSS at the basal membrane results in the inhibition of yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) signaling within the pro-inflammatory Hippo pathway (Wang et al., 2016).

2.2 Oscillatory shear stress leads to endothelial cell activation

In contrast to LSS, oscillatory shear stress (OSS), predominantly occurring in branch points and bifurcations of the vasculature incites a pro-inflammatory reaction within the endothelium (Figure 1). In this scenario, attenuation of NF-κB expression and YAP-TAZ signaling by mechanosensors Piezo1, PECAM-1, and integrins is lost (Tzima et al., 2005; Albarrán-Juárez et al., 2018). Activation of Piezo1 in response to disturbed flow further induces expression of NF-κB through integrin activation (Albarrán-Juárez et al., 2018). Increased NF-κB and YAP-TAZ signaling trigger endothelial activation, as evidenced by the upregulation of monocyte adherence molecules including Vascular Cell Adhesion Molecule-1 (VCAM-1), E-Selectin, P-selectin, and Intercellular Adhesion Molecule-1 (ICAM-1), and expression of potent pro-inflammatory mediators including Toll-like receptor (TLR) 2, chemokine (C-C motif) ligand 2 (CCL2), Interleukin (IL)-6, and IL-8 (Tzima et al., 2005; Wang et al., 2016; Bondareva et al., 2019). Substantiating these experimental observations, a recent scRNA-seq study on ECs from human coronary arteries in transplanted human hearts provided compelling *ex vivo* evidence that endothelial activation is essential for atherosclerotic plaque formation. In this study, a distinct EC subpopulation that was characterized by upregulation of genes associated with inflammation and endothelial activation was identified, constituting over 80% of all ECs (Hu et al., 2021). This subpopulation was more abundantly present in atherosclerotic arteries compared to healthy controls. A similar EC phenotype was identified in a subsequent scRNA-seq study of human carotid plaques, where a large majority of intra-plaque ECs showed expression of genes such as *PECAM1* and *VCAM1* that are associated with endothelial activation (Depuydt et al., 2020).

3 The role of the innate immune system in atherosclerosis

3.1 Monocyte subtypes play different roles in atherogenesis

Monocytes are bone marrow derived immune cells capable of differentiating into macrophages and, under certain inflammatory conditions, into monocyte-derived dendritic cells (DCs). As (activated) monocytes predominantly interact with activated endothelium, they have historically played a central role in atherosclerosis research (Kim et al., 2020). Classical cardiovascular risk factors, such as dyslipidemia, lead to monocytosis through upregulation of bone marrow activity in mice (Moore et al., 2013). In humans, a positive history of ASCVD has been linked to increased metabolic activity of hematopoietic tissues of the spleen and bone marrow as well as an enhanced functional status of hematopoietic stem and progenitor cells, indicating increased hematopoietic activity as a potential driver of monocytosis and low-grade inflammation in ASCVD (van der Valk et al., 2016). The relation between cardiovascular health, bone marrow activity and atherogenesis was further studied in the Progression of Early Subclinical Atherosclerosis (PESA) study (Devesa et al., 2022). Here, classical risk factors such as metabolic syndrome, hypertension, dyslipidemia, diabetes and BMI correlated significantly with bone marrow activation on ^{18}F-FDG PET/MRI. Consequently, these subjects showed increased leukocyte counts and elevated markers of inflammation, indicating low-grade systemic inflammation. In turn, bone marrow activation was associated with arterial uptake of ^{18}F-FDG, indicating early plaque formation.

Within atherosclerotic plaques, monocytes transition into macrophages, which have an affinity for the uptake of modified LDL (Zernecke et al., 2020). Flow cytometry and fluorescence-activated cell sorting (FACS) are often used to classify monocyte subsets in humans based on CD14 and CD16 receptor levels: classical monocytes (~90% of circulating monocytes; $CD14^{++}CD16^{-}$), followed by intermediate (~5%; $CD14^{++}CD16^{+}$) and non-classical monocytes (~5%; $CD14^{+}CD16^{++}$). A similar classification is used in mice, where $Ly6^{high}$ monocytes correspond to human classical monocytes, and $Ly6^{low}$ monocytes to non-classical monocytes (Mehta and Reilly, 2012). Generally, non-classical monocytes are ascribed a role in homeostasis and atheroprotection, as depletion of non-classical monocytes in murine models has resulted in aggravation of atherogenesis and increased apoptosis of ECs (Quintar et al., 2017). Intravital microscopy experiments have shown that classical monocytes engage in ICAM-1 and -2-dependent "patrolling" along the endothelial surface of murine atherosclerotic arteries (Quintar et al., 2017). Furthermore, they typically avoid entering the subendothelial space (Quintar et al., 2017). Classical monocytes, on the other hand, are attracted to the atherosclerosis-prone endothelium, targeted by CCL2 on the endothelial surface in a manner reliant on the leukocyte C-C Chemokine Receptor Type 2 (CCR2). Their important role in atherogenesis is confirmed in murine knock-out models of CCR2 and CCL2, which show a significant reduction in atherosclerosis formation compared to wild type mice (Boring et al., 1998; Gu et al., 1998). Aside from CCL2, CCL5 plays a role in chemotaxis of classical monocytes through interaction with leukocyte CCR5 in the atherosclerotic vessel wall. In murine atherosclerosis models, CCL5 expression is significantly enhanced in the vessel wall compared to wildtype mice. CCR5 expression is upregulated in tandem in circulating monocytes. Various separate experiments involving administration of function-blocking antibodies to CCR5, genetic depletion of CCR5, and genetic depletion of CCL2 have all demonstrated a significant reduction in lesion size (Tacke et al., 2007; Combadiè et al., 2008; Jongstra-Bilen et al., 2021). Finally, it is this specific group of classical monocytes that undergoes expansion in reaction to hypercholesterolemia and atherosclerosis, whereas

formation of non-classical monocytes is impaired under these circumstances (Swirski et al., 2007). This finding underscores the largely opposite roles of the two types of monocytes. Nonetheless, the precise role of non-classical monocytes in atherosclerotic plaque formation and inflammation in humans remains subject of further study.

Although monocyte subtypes have traditionally been classified based on CD14 and CD16 surface expression, recent advancements in scRNA-seq and mass cytometry have challenged this triadic categorization, uncovering more monocyte diversity than initially thought. For example, a study using scRNA-seq in human monocytes found that monocytes that had previously been defined as intermediate subtype showed considerable overlap with the classical and nonclassical subtypes. Surprisingly, these monocytes clustered into two additional and previously undefined clusters as well, introducing novel heterogeneity of intermediate monocytes (Villani et al., 2017). Subsequent investigations in a study using machine learning to reclassify monocyte subpopulations based on scRNA-seq and mass cytometry data, proposed that one of these novel monocyte subtypes represented a cluster of Natural Killer (NK) cells (Dutertre et al., 2019). Likewise, other potential novel monocyte subtypes have been proposed following studies using a variety of different high-throughput strategies and analytical approaches, both in humans (Roussel et al., 2017; Thomas et al., 2017) and in mice (Mildner et al., 2017). Even though reaching a consensus on these subpopulations and their distinct functions will require further research, the enhanced subclassification of monocytes through high-throughput techniques and omics approaches are likely to prove essential to atherosclerosis research. In this context, it is advisable to strive for standardization of markers and clustering methods used to ensure reproducible identification of cell clusters. More precise identification of monocyte subsets will enable dissection of their specific pro-inflammatory and pro-atherosclerotic contributions allowing for more focused and hypothesis-driven research.

3.2 Monocytes migrate into the vessel wall and differentiate into foam cells

Once near the activated endothelium, monocytes undergo transient rolling interactions, followed by firm adhesion mediated by integrins and chemokine activation, ultimately leading to their migration across the endothelium barrier into the subendothelial space (Figure 2) (Timmerman et al., 2016). Early experiments in mice models of atherosclerosis involving genetic depletion of P-selectin made clear that this protein facilitates leukocyte rolling, extravasation and by consequence, plaque formation (Mayadas et al., 1993; Johnson et al., 1997; Dong et al., 2000). Subsequent experiments involving murine knockout models of its ligand, leukocyte P-selectin glycoprotein ligand-1 (PSGL-1), confirmed the interaction of these proteins as a major driver of monocyte recruitment in atherosclerosis (An et al., 2008). Moreover, involvement of other adhesion molecules on the activated endothelium, such as ICAM-1, ICAM-2 and VCAM-1, have been associated with progression of atherosclerosis in *Apoe*$^{-/-}$ mice following coronary ligation (Sager et al., 2016).

Upon recruitment to the subendothelial space, monocytes differentiate into macrophages. Aside from monocyte influx, the accumulation of macrophages is driven in large part by the proliferation of pre-existing tissue-residing macrophages, which occurs mainly in advanced atherosclerotic lesions (Robbins et al., 2013). These macrophages take up modified apoB lipoproteins that are retained in the subendothelial space, of which minimally modified LDL (mmLDL) is the most prominent. This uptake occurs in various ways. On the one hand, through phagocytosis mediated by scavenger receptor A1 (SRA1), Lectin-like Oxidized Low-Density Lipoprotein Receptor-1 (LOX-1) and CD-36 (Moore and Freeman, 2006; Poznyak et al., 2021). On the other hand, native LDL is also internalized by macrophages, albeit to a lesser extent, through micropinocytosis. Once internalized by the macrophages, LDL undergoes degradation within macrophagic lysosomes, with degradation byproducts being stored as droplets in the cytoplasm. Microscopic analysis showed that the accumulation of these droplets give macrophages the characteristic appearance of cholesterol-laden foam cells (Brown and Goldstein, 1983). These foam cells contribute significantly to plaque growth and instability and are a hallmark of the initial fatty streak phase of atherosclerosis.

3.3 Persistent hypercholesterolemia overwhelms foam cells, leading to a pro-inflammatory response

The influx and subsequent proliferation of foam cells is regarded as a main driver of fatty streak formation and subsequent plaque growth. Even though monocyte-derived macrophages are well-recognized contributors to foam cell formation in atherosclerosis, scRNA-seq analysis in mouse models of atherosclerosis have indicated a role for vascular smooth muscle cells (VSMCs) that is far from negligible. Stimulated by TGF-β, which is secreted by -amongst others- macrophages, ECs and T-cells, VSMCs express high levels of smooth muscle α-actin and engage in the production of a complex extracellular matrix containing elastin, proteoglycans and collagen (Bobik, 2006; Bentzon et al., 2014). This matrix forms a fibrous cap that surrounds a core of foam cells (Libby, 2000). Interestingly, recent fate-mapping experiments have evidenced that VSMCs within a fibrous cap are derived from proliferation of a single VSMC in the medial layer of the vessel (Misra et al., 2018). The proliferation of VSMCs and the formation of this fibrous cap marks a critical point for the plaque, after which the likelihood of spontaneous regression of the plaque diminishes (Gofman et al., 1950; Bennett et al., 2016). On the other hand, incorporation of VSMC-derived extracellular matrix into the fibrous cap may increase stability, lowering the chances of plaque rupture and subsequent atherothrombotic events (Bennett et al., 2016). As the plaque progresses, VSMCs migrate into the plaque and undergo transdifferentiation into a variety of transcriptionally heterogenic phenotypes as evidenced by several murine scRNA-seq studies (Hutton et al., 2023). Remarkably, a large subset of these VSMCs gain expression of macrophage markers and engage in the uptake of lipoproteins to become the majority of plaque foam cells, over macrophages (Francis, 2023). scRNA-seq studies in human plaques have confirmed that VSMCs in humans form a heterogeneous population as well (Depuydt et al., 2020), but the extent to which phenotypic switching plays a role in human disease remains a matter of ongoing investigation.

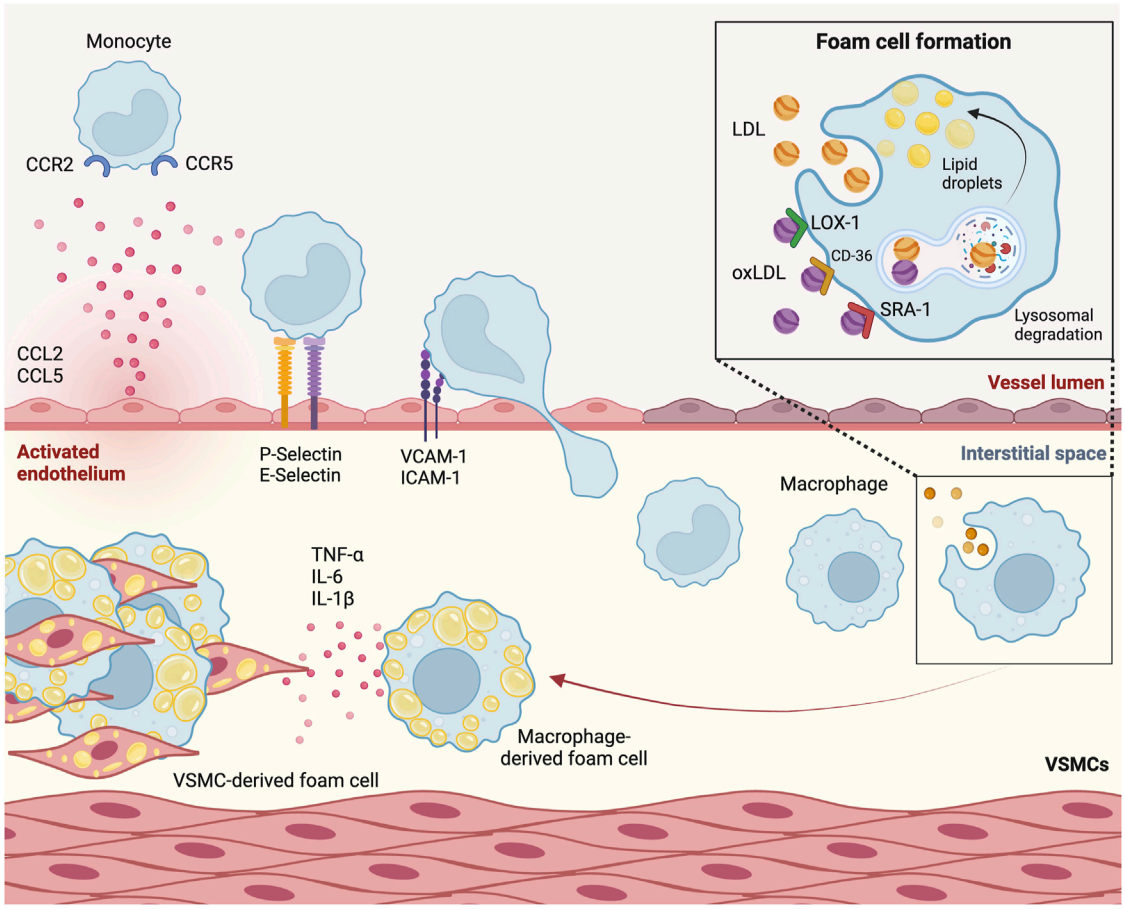


FIGURE 2
Monocytes transmigrate through the activated endothelium to form foam cells. Circulating monocytes are attracted to the activated endothelium by chemokines chemokine (C-C motif) ligand 2 (CCL2) and CCL5. P-selectin and E-selectin on the endothelial membrane are involved in monocyte recruitment. Once in close proximity, molecules such as Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) initiate monocyte transmigration. Once in the subendothelial space, monocytes differentiate into macrophages. Through a process of phagocytosis mediated by scavenger receptor A1 (SRA1), Lectin-like Oxidized Low-Density Lipoprotein Receptor-1 (LOX-1) and CD-36, and through micropinocytosis, macrophages take up (oxidized) low-density lipoprotein [(ox)LDL]. These lipoproteins undergo lysosomal degradation, after which their byproducts are stored as lipid droplets, leading to foam cell formation. Sustained influx of lipoproteins leads to secretion of cytokines such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and IL-1β. VSMC, vascular smooth muscle cell.

Following periods of persistent hypercholesterolemia, sustained influx of lipoproteins can outpace the metabolic capacities of foam cells. When this happens, pro-inflammatory responses are triggered, such as the NF-κB-dependent secretion of cytokines like tumor necrosis factor α (TNF-α), IL-6 and IL-1β (Figure 2) (Yvan-Charvet et al., 2008). This ultimately leads to the infiltration and activation of pro-atherogenic leukocytes, coupled with amplified lipoprotein modification, and aggravated foam cell formation (Williams et al., 2019). Moreover, cholesterol is incorporated into the cell membrane of foam cells, amplifying inflammatory signaling (Tall and Yvan-Charvet, 2015). Endoplasmatic reticulum stress caused by prolonged lipid overload may trigger foam cells to undergo apoptosis or necrosis, after which they are removed by macrophages in a process called efferocytosis (Neels et al., 2023). Initially beneficial to plaque regression, foam cell death and subsequent clearance of cell debris by efferocytosis diminishes the number of cells present in the plaque. Over time however, the ability of efferocytes to efficiently clear apoptotic and necrotic cells diminishes, resulting in accumulation of cell debris and necrotic core formation. This induces increased plaque vulnerability (Gonzalez and Trigatti, 2017; Neels et al., 2023). In this context, it has been shown that efferocytes release their pro-inflammatory cellular and lipid contents, further contributing to leukocyte recruitment (Kojima et al., 2017).

3.4 Inflammasome-mediated inflammation in atherosclerotic plaque formation

As the CANTOS and COLCOT trials generated promising evidence for the anti-atherogenic effects of IL-1β antibodies and colchicine, the inflammasome has amassed attention as the major driving factor of IL-1β-driven inflammation in atherosclerosis. Inflammasomes are located within the cytoplasm of immune cells of the innate immune system. They function as intracellular sensors that respond to damage-associated molecular patterns (DAMPs; released during cellular stress), as well as pathogen-associated molecular patterns (PAMPs; associated with microbes) (Kelley

et al., 2019). These distinct patterns are detected by specialized receptors referred to as pattern recognition receptors (PRRs), which include TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs) (Kelley et al., 2019). These NLRs, the most prominent of which include NLR Family Pyrin Domain Containing 1 (NLRP1), NLRP3, and NLR Family CARD Domain Containing 4 (NLRC4), guide the assembly of inflammasomes upon recognition of DAMPs or PAMPs, ultimately leading to activation of caspase-1. This enzyme then converts the inactive forms of the pro-inflammatory cytokines IL-1β and IL-18 into their active counterparts (Kelley et al., 2019). Within atherosclerotic plaques, the accumulation of modified lipoproteins leads to the creation of cholesterol microcrystals that activate the inflammasome through enhanced signaling of the nuclear receptor subfamily three group C member 2 (NR3C2) (Chen et al., 2023). One experimental study definitively established the inflammasome as a link between cholesterol and atherosclerosis, as peritoneal exposure of atherosclerosis-prone mice to these cholesterol crystals was found to be able to trigger inflammation and atherosclerosis in a NLRP3 inflammasome-dependent manner (Duewell et al., 2010). It is therefore unsurprising that genes associated with the NLRP3 inflammasome exhibit substantially higher expression levels in atherosclerotic plaques compared to non-atherosclerotic areas in human arteries (Paramel et al., 2016). These changes were found to be particularly pronounced in patients with symptomatic lesions in a sub-group analysis of the same experiment (Paramel et al., 2016), and an elevated expression of NLRP3 in the aorta was found to correlate with an increased risk of developing coronary artery disease (Zheng et al., 2013). Experimental lentiviral NLRP3 silencing reduced atherosclerotic plaque area, macrophage count within lesions, lipid accumulation, and heightened plaque stability via increased collagen content in *Apoe*$^{-/-}$ mice (Zheng et al., 2014).

The NLRP3 inflammasome promotes inflammation by facilitating the release of IL-1β and IL-18 (Kelley et al., 2019). Of these, IL-1β has been found to play a significant role in promoting endothelial activation by enhancing the expression of endothelial adhesion molecules such as ICAM-1 and VCAM-1 and monocyte adhesion to ECs (Cejkova et al., 2019). Additionally, NLRP3 inflammasome activation induces the release of other key pro-inflammatory cytokines like CCL2, CXCL2, -3 and 8, IL-6 and matrix metalloproteinases (MMPs), which are enzymes that promote fibrous cap dissolution. Therefore, plaque instability is promoted through an increased risk cap rupture and subsequent thrombus formation (Popa-Fotea et al., 2023). These events also contribute to the influx of leukocytes and the uptake of LDL from the intravascular space, thereby perpetuating the cycle of inflammation (Botts et al., 2021). Interestingly, it has been shown that the phenotypic switch and transdifferentiation of VSMCs towards macrophage-like cells is likely dependent on the activation of the NLRP3 inflammasome in VSMCs (Burger et al., 2021). Corroborating the effectivity of mitigating IL-1β activity in human atherosclerosis, depleting IL-1β genetically in *Apoe*$^{-/-}$ mice has been shown to notably reduce atherosclerosis progression (Kirii et al., 2003). IL-18 similarly holds significance as a driver of inflammation and plaque progression. Similarly to IL-1β, it also increases the expression of adhesion molecules and inflammatory cytokines through NF-κB and MAPK signaling, albeit to a lesser extent (Yasuda et al., 2019). IL-18 acts as an important costimulatory cytokine, essential for the production and secretion of interferon-γ (IFN-γ) from T-helper 1 (Th1) cells and NK cells, macrophages, DCs, and VSMCs (Yasuda et al., 2019). Indeed, experimental genetic depletion and overstimulation of IL-18 in *Apoe*$^{-/-}$ mice has shown that the cytokine consistently demonstrates a direct association with the progression of atherosclerotic lesions, operating through an IFN-γ-dependent mechanism (Whitman et al., 2002; Elhage et al., 2003; Tan et al., 2010).

3.5 Macrophages: beyond the M1-M2 paradigm

Not all macrophages within the plaque exhibit identical pro-inflammatory characteristics. Macrophages have the capacity to undergo polarization, dictated by their microenvironment, leading to phenotypic and functional changes (Wu et al., 2023). Traditionally, macrophage phenotypes have been categorized into two groups: pro-inflammatory M1 and anti-inflammatory M2 macrophages (Moore et al., 2013). The initial categorization was based on their specific *in vitro* stimulation factors. Subsequent research, both *in vitro* and in murine atherosclerosis models, have led to one overarching principle: M1 macrophages are linked to the promotion of plaque inflammation, whereas M2 macrophages are connected to the resolution of plaque inflammation (Zhao et al., 2023). In these studies, M1 macrophages have been detected within atherosclerotic plaques in humans, specifically localized in lipid-enriched regions spatially separate from M2 macrophages (Stöger et al., 2012). Their accumulation and subsequent apoptosis or necrosis leads to expansion of the necrotic core, which causes plaque progression and destabilization. Aside from the pro-inflammatory cytokines TNF-α and IL-1β, M1 macrophages produce MMPs, which, as discussed, dissolve the fibrous cap and promote plaque instability. They also secrete high levels of IL-6 and IL-12, which promote differentiation of naïve T cells into pro-inflammatory Th1 cells (Mosser, 2003). On the contrary, murine atherosclerosis models have suggested that M2 macrophages promote plaque regression (Feig et al., 2012). M2 macrophages secrete high levels of IL-10, which promotes differentiation of naïve T cells into anti-inflammatory Th2 cells. Furthermore, IL-10 promotes plaque stabilization through extracellular matrix formation. Notably, consistent findings from murine models indicate a reduction in macrophage population, at times accompanied by an increased presence of M2 macrophages, correlates with plaque regression (Jinnouchi et al., 2020). Although M2 macrophages have also been identified within human plaques, uncertainty remains regarding their role in plaque development (Stöger et al., 2012). Nevertheless, it's imperative to acknowledge that the translatability of these observations to humans is in some ways limited due to the inherent differences in macrophage subtypes between mice and humans. For instance, while general functional characteristics of macrophage subsets, including the factors that steer their differentiation, show a high degree of conservation between mice and humans, surface markers seem to differ substantially between the species (Chinetti-Gbaguidi et al., 2015).

Today, the significance of the M1-M2 paradigm in atherosclerosis is a major area of debate. scRNA-seq has allowed investigators to identify previously undiscovered macrophage subtypes characterized by distinct gene expression profiles involved in atherosclerosis over recent years. Importantly, these subtypes do not necessarily align with the two subtypes defined by the classical M1-M2 paradigm. Many of these scRNA-seq datasets are publicly available, a meta-analysis of which was recently carried out by Zernecke et al. (2023). They found that in murine models of atherosclerosis, as many as 10 functionally distinct macrophage subpopulations could robustly be identified. These cell clusters appeared to be conserved in human atherosclerosis. To date, efforts to further characterize macrophage subpopulations are increasing. These have recently been reviewed elsewhere (Wieland et al., 2024).

3.6 Neutrophils attract monocytes to the vessel wall and modulate macrophage phenotypes in atherosclerosis

Neutrophils have long been overlooked in atherosclerosis research, possibly due to their short lifespans and phenotypic plasticity, making their *in vivo* detection challenging (Zhang et al., 2023). In recent years, however, experimental findings have shed a new light on these cells in the context of atherosclerosis. Neutrophils are recruited to the activated endothelium by chemokines such as CCL-1 and CXCR2 (Drechsler et al., 2010; Lam et al., 2018). Similarly to monocytes, neutrophils bind to activated ECs in a P- and E-selectin and CCR2-dependent manner (Lam et al., 2018; Zhang et al., 2023). Upon adhering to the endothelium, neutrophils produce reactive oxygen species (ROS), thereby contributing to the oxidation of lipoproteins within the endothelium and the permeability of the vessel wall (Domínguez-Luis et al., 2019; Lian et al., 2019). Neutrophils further increase EC permeability and facilitate the transmigration of both neutrophils and other immune cells through secretion of pro-inflammatory cytokines TNF-α and IL-1β (DiStasi and Ley, 2009; Zhang et al., 2023).

In a landmark study of murine atherosclerosis, researchers examined the aortas of neutropenic mice and their high-fat diet-fed control counterparts. In the aortas of the neutropenic mice, the number of monocytes and macrophages was significantly reduced, as well as the size of atherosclerotic lesions. These findings suggest that neutrophils play a role in the accumulation of monocytes and monocyte-derived macrophages within atherosclerotic lesions (Drechsler et al., 2010). Recent findings from a murine model of advanced atherosclerosis indicate that the pro-atherosclerotic activity of neutrophils depends on signaling by Signal transducer and activator of transcription 4 (STAT4) (Keeter et al., 2023). Several mechanisms have been implicated in the link between neutrophils and atherogenesis. For instance, a recent study used intravital microscopy in *Apoe*$^{-/-}$ mice to demonstrate that when stimulated by activated ECs, neutrophils release neutrophil extracellular traps (NETs) along the arterial wall, consisting mostly of DNA strands, histones and neutrophil granules. This release promoted monocyte adhesion independently of receptor signaling (Schumski et al., 2021). Another study demonstrated that exposure to cholesterol crystals could induce the release of NETs from neutrophils in both a mouse model of atherosclerosis where it induced the release of pro-inflammatory cytokines from macrophages (Warnatsch et al., 2015) and in human neutrophils (Awasthi et al., 2016). *In vitro* studies with human macrophages have demonstrated that citrullinated histones, associated with NETosis, enhance the oxidation of LDL and the formation of foam cells (Haritha et al., 2020). Finally, it has been shown that DNA and neutrophilic granules, such as those present in NETs, facilitate the growth of atherosclerotic plaque in mice in a manner dependent on an increased production of interferon-α.

Furthermore, neutrophils enhance monocyte chemotaxis and adherence by releasing CCL2 and pentraxin 3 (Winter et al., 2018; Popa-Fotea et al., 2023), demonstrating that neutrophils are able to initiate efficient monocyte extravasation into the subendothelial space. Beyond their role in monocyte recruitment, neutrophils have also been implicated in modulating macrophage phenotypes. Within the plaque, neutrophils secrete azurocidin and α-defensins, which induce a shift in macrophages towards a T-helper cell (Th)-17 stimulating M1 phenotype through β2-integrin signaling and subsequent interferon-γ release (Zhang et al., 2023). Furthermore, experimental exposure of lipid crystals to neutrophils has been shown to lead to increased NET release. This in turn triggered M1 polarization in *Apoe*$^{-/-}$ mice (Warnatsch et al., 2015). Moreover, the effect of neutrophils on plaque content and macrophage functionality was evidenced in a study, in which *in vitro* incubation of macrophages with neutrophil-derived defensin increased the expression of CD36, which in turn enhanced the uptake of LDL and promoted foam cell formation (Quinn et al., 2011). These results were corroborated in a recent study, in which human neutrophils exposed to LDL were visualized using fluorescent microscopy. In this study, LDL induced NET release *in vitro*, which in turn promoted LDL oxidation, LDL accumulation and foam cell formation (Haritha et al., 2020). Finally, a recent study has established a role for NET-derived histone H4 in exacerbating plaque instability. Here, histone H4 exerted a deleterious function on the cell membrane of VSMCs, ultimately contributing to an increase in plaque instability in mice and humans (Silvestre-Roig et al., 2019). These findings underline the important role neutrophils play in promoting atherogenesis and plaque inflammation.

4 The role of the adaptive immune system in atherosclerosis

4.1 Auto-antigen-specific T cells modulate atherosclerosis

T cells express the T-cell receptor (TCR) in conjunction with co-receptors that align with their specific function and T cell subtype. CD4 co-receptor-expressing cells differentiate into Th cells following antigen presentation and are the most widely studied T cell subtype in the context of atherosclerosis (Popa-Fotea et al., 2023). Expression of the CD8 co-receptor, on the other hand, is found in naïve and effector cytotoxic T cells (Popa-Fotea et al., 2023). These cells regulate immune responses to antigens presented by antigen-presenting cells (APCs) such as macrophages and DCs on their major histocompatibility complex (MHC) class II, as well as

by all nucleated cells on MHC class I. Concurrent interactions between TCRs and antigens on MHC, coupled with stimulation by co-stimulatory molecules presented on APCs, drive the clonal proliferation of T cells and determine their phenotype (Saigusa et al., 2020).

During the early stages of atherogenesis, T lymphocytes are abundantly present (Saigusa et al., 2020). They are drawn to the activated endothelium by a variety of chemokines, including CCL5, CXCR10, and CXCL16 (Wuttge et al., 2004; Heller et al., 2006; Li et al., 2016). Subsequently, these T cells migrate into the endothelium through the interaction of P-selectin and PSGL-1 *in vivo* (MacRitchie et al., 2019). In *Apoe*$^{-/-}$ mice, a considerable portion of plaque-resident CD4$^+$ T cells exhibit CD44 expression, a T cell activation marker that allows discriminating effector and memory T cells from naïve T cells. This suggests that these lymphocytes have previously encountered and been activated by their corresponding antigen (Koltsova et al., 2012). In recent years, omics approaches have been of great value in exploring this hypothesis. Mass cytometry in human atherosclerotic plaques confirmed that chronically activated and differentiated T cell phenotypes predominated over the naïve population (Fernandez et al., 2019). This finding was corroborated by two separate studies that used single-cell RNA and TCR sequencing to establish that many T cells found in human coronary plaques were antigen-experienced memory cells that had clonally expanded within the plaque (Chowdhury et al., 2022; Depuydt et al., 2023). Comparison of T cell subsets in the plaque and peripheral blood revealed that it is mainly effector CD4$^+$ T cells, and not CD8$^+$ T cells that undergo clonal expansion within the plaque (Depuydt et al., 2023).

Regarding the role these antigen-experienced T cells play in atherogenesis, the depletion of CD44 in a murine model of myocardial infarction resulted in heightened inflammatory leukocyte infiltration and increased cytokine expression (Huebener et al., 2008). Identifying the antigen specificity of T cells *in vivo* has proven technically difficult. ApoB has been identified as an auto-antigen that likely is important in this regard. Immunization with human ApoB reduced atherosclerosis significantly in *Apoe*$^{-/-}$ mice (Fredrikson et al., 2003; Chyu et al., 2022). Additionally, both in murine atherosclerosis models and humans with cardiovascular disease, a population of apoB-specific T-helper cells was identified in experiments that used recombinant MHC (Kimura et al., 2018). Other auto-antigens that have been implicated in atherogenesis include beta2-glycoprotein I (Profumo et al., 2010), cathelicidin (Mihailovic et al., 2017) and collagens (Lio et al., 2020). These findings underscore the important role that antigen-specific T cells assume in modulating inflammation in cardiovascular disease. The diverse range of potential immune-dominant antigens implicated in atherogenesis may help clarify the complex and sometimes contradictory roles of T cells in atherosclerosis, which could vary depending on the disease stage and the specific antigens involved.

4.2 CD4$^+$ T-helper cells: different subtypes have different functions in atherosclerosis

T-helper cells, which are classified into subtypes based on their signature cytokines and transcription factors, play a variety of roles in the context of atherosclerosis (Figure 3). Th1 cells, which express the transcription factor T-bet as a lineage-defining marker, predominate in number among all T cell subtypes in the plaque (Li et al., 2016; Wolf and Ley, 2019). Their role is pro-inflammatory and pro-atherogenic due to the secretion of IFN-γ, IL-2, IL-3, and TNF-α (Popa-Fotea et al., 2023). This is highlighted by genetic depletion experiments of T-bet and IFN-γ, which have been found to inhibit plaque progression and instability in *Ldlr*$^{-/-}$ mice (Buono et al., 2003; Buono et al., 2005). Furthermore, IFN-γ plays a role in plaque progression by promoting LDL oxidation and uptake of modified LDL by foam cells, polarizing macrophages to their M1 subtype, and promoting the proliferation of VSMCs (Rocha et al., 2008; Lee et al., 2021).

On the other hand, the precise role of Th2 cells, which mainly produce IL-4, IL-5, and IL-13, remains a topic of debate (Winkels et al., 2018). Levels of Th2 cells and IL-4 released in peripheral blood are inversely correlated with carotid intima-media thickness in a healthy study population, even after correcting for other cardiovascular disease risk factors (Engelbertsen et al., 2013). However, depletion of IL-4 has shown conflicting effects on plaque growth in mice (King et al., 2002; Mallat et al., 2009). IL-5 and IL-13, on the other hand, have definite anti-inflammatory and anti-atherogenic effects (Saigusa et al., 2020).

The role of Th9 cells in atherosclerosis, too, remains uncertain, but preliminary evidence points to a pro-atherogenic function. The main cytokine produced by this subset is IL-9. Several clinical studies found that IL-9 levels, but not the number of Th9 cells, are higher in patients with atherosclerotic disease (Gregersen et al., 2013; Lin et al., 2013). In murine models of atherosclerosis, IL-9 has been found to have pro-atherogenic effects (Zhang et al., 2015). More research is needed to fully elucidate the role of Th9 cells and IL-9 in the pathogenesis of atherosclerosis.

Th17 cells feature the expression of transcription factor RORγt as their lineage-defining factor. Previous murine *in vitro* studies have elucidated the complex nature of Th17 cells: due to their high level of plasticity, their phenotype (pro-inflammatory or anti-inflammatory) is highly dependent on the cytokines they are polarized with (McGeachy et al., 2007; Ghoreschi et al., 2010; Lee et al., 2012). This heterogenic nature of Th17 cells might be the reason why previous research efforts have found conflicting roles for this cell type in atherosclerosis. Indeed, Th17 cell counts correlated with atherosclerotic plaque size in *Apoe*$^{-/-}$ mice. Neutralizing antibodies against IL-17 could diminish plaque size and leukocyte infiltration (Nordlohne et al., 2018). On the other hand, one study found increased plaque stability in *Ldlr*$^{-/-}$ mice with artificially increased Th17 cell counts (Brauner et al., 2018).

T follicular helper (Tfh) cells are a specialized subset of CD4$^+$ T cells, hallmarked by the expression of B-cell lymphoma 6 (Bcl-6), that play a critical role in the formation and maintenance of germinal centers, where they aid B cells in producing high-affinity antibodies. They facilitate B cell differentiation and antibody class switching through the secretion of cytokines and direct cell-cell interactions (Qi et al., 2023). Evidence from experimental studies point to divergent functions of Tfh cells in atherosclerosis. An experimental study found that Tfh cells isolated from *Apoe*$^{-/-}$ mice had a gene expression profile that was more pro-inflammatory than those isolated from wild-type mice. This effect was mediated by enhanced IL-27 production from dendritic cells

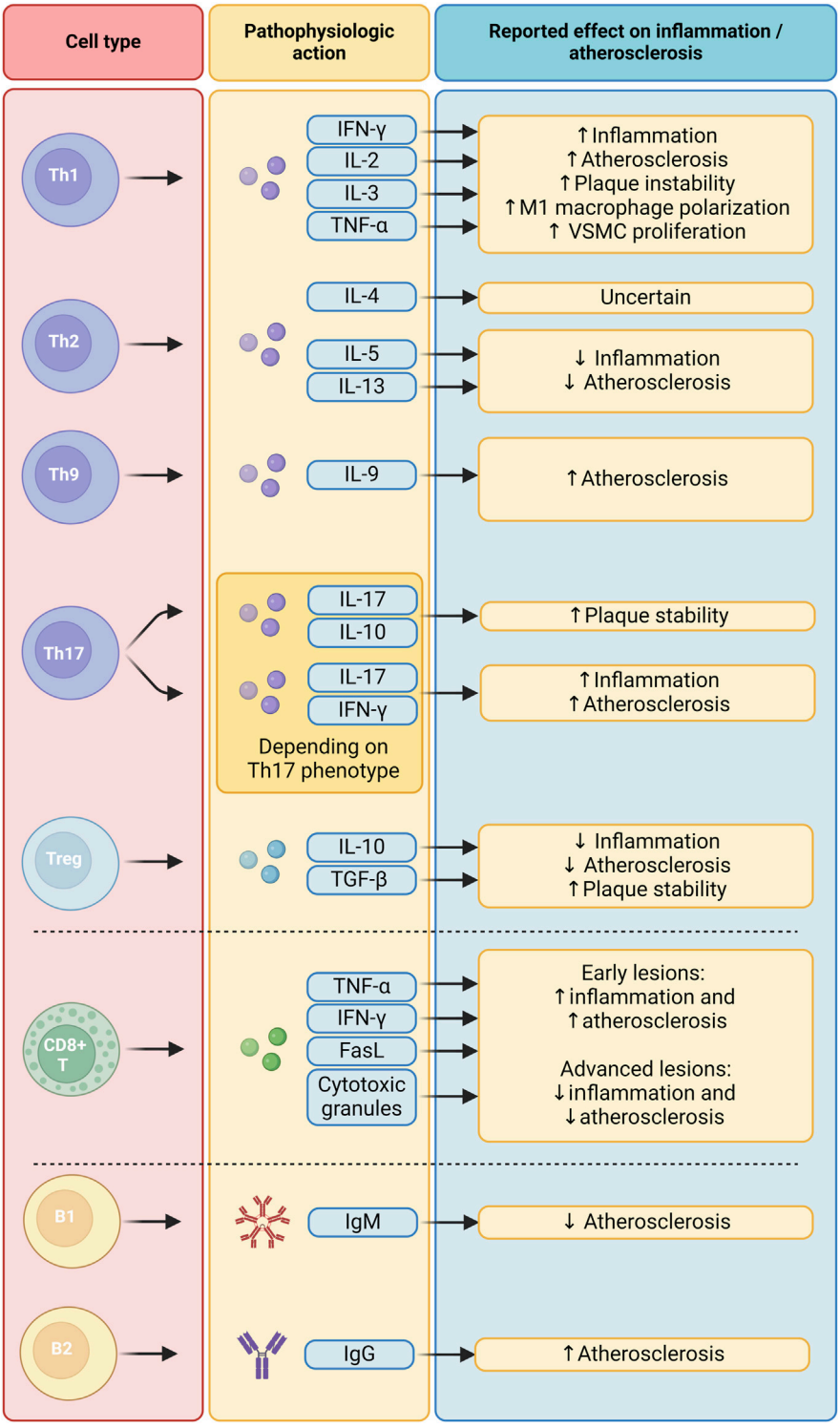


FIGURE 3
Functions of the adaptive immune system in atherosclerosis. B1, B1 cells; B2, B2 cells; CD8⁺ T, CD8⁺ T cells; FasL, Fas Ligand; IFN-γ, Interferon gamma; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IL-10, Interleukin 10; IL-13, Interleukin 13; IL-17, Interleukin 17; IL-2, Interleukin 2; IL-3, Interleukin 3; IL-4, Interleukin 4; IL-5, Interleukin 5; TGF-β, Transforming Growth Factor beta; Th1, T helper 1 cells; Th17, T helper 17 cells; Th2, T helper 2 cells; Th9, T helper 9 cells; TNF-α, Tumor Necrosis Factor alpha; Treg, Regulatory T cells; VSMC, Vascular Smooth Muscle Cell.

(Ryu et al., 2018). Another study showed that marginal zone B cells inhibit pro-atherogenic Tfh cell activity in *Ldlr*$^{-/-}$ mice (Nus et al., 2017). Additionally, genetic depletion of Tfh cells in *Apoe*$^{-/-}$ mice led to a reduction of atherosclerosis (Gaddis et al., 2018). In a recent experiment in an atherosclerotic mouse model, however, genetic depletion of Tfh cells led to an aberrant antibody response of

marginal zone B cells and an associated increase in atherosclerotic plaque formation (Harrison et al., 2024). These conflicting findings might reflect the recently uncovered heterogeneity of Tfh subsets (Seth and Craft, 2019), warranting further delineation of the roles of these subsets in atherosclerosis.

Regulatory T cells (Tregs) express the forkhead box P3 (FoxP3) and have been identified as having anti-inflammatory and atheroprotective effects. Depletion of this population has been found to promote atherosclerosis in *Ldlr*$^{-/-}$ mice (Klingenberg et al., 2013), and a strong negative correlation between Treg cells and atherosclerosis exists in humans (George et al., 2012). Tregs exert their anti-inflammatory function through the secretion of IL-10 and TGF-β (Saigusa et al., 2020). Depletion of either these factors was found to increase atherosclerotic plaque size and instability in murine models (Pinderski et al., 1999; Mallat et al., 2001). The population of apoB-specific Tregs appears to be diminished in the peripheral blood of patients with cardiovascular disease compared to healthy controls (Pinderski et al., 1999). Interestingly, as atherosclerosis progresses, Treg numbers in peripheral blood and the plaque diminishes in favor of effector Th1/Th17 cells in both murine models and in mice and humans. A substantial proportion of Tregs that remain were found to have acquired Th1 and Th17-defining transcription factors while simultaneously losing the expression of FoxP3 (Butcher et al., 2016; Wolf et al., 2020). These findings suggest that a decline in the number of bona fide Treg cells, along with their increasingly pro-inflammatory phenotype, might be an independent driver of disease progression in atherosclerosis.

4.3 The role of CD8$^+$ T cells in plaque inflammation remains to be elucidated

CD8$^+$ T cells activate and differentiate into effector T cells following the interaction of their TCR with an antigen presented on MHC class I molecules. They then undergo clonal expansion and produce TNF-α, IFN-γ, Fas-ligand, and cytotoxic granules. This leads to the induction of apoptosis or necrosis in the targeted cell (Schäfer and Zernecke, 2020). While CD4$^+$ T cell functions have been extensively studied in atherosclerosis, the role of CD8$^+$ cytotoxic T cells in this context is less well known (Figure 3).

Similarly to CD4$^+$ T cells, CD8$^+$ T cells are prominently present within atherosclerotic plaques in both mice and humans (Cochain et al., 2018). The precise contribution of CD8$^+$ T cells to atherosclerosis, however, needs to be studied further. One piece of evidence for their mechanistic role in atherosclerosis was provided by an experiment involving the depletion of CD8$^+$ T cells in *Apoe*$^{-/-}$ mice using anti-CD8α antibodies, which resulted in a significant reduction in plasma CCL2 levels, as well as the accumulation of macrophages and a reduction in atherosclerotic plaque size in the early stages of disease progression. These effects could be mediated by their cytotoxic effects and subsequent growth of the necrotic plaque core (Kyaw et al., 2013). A recent study which employed depletion of the CD8$^+$ T cell line in a murine model of atherosclerosis, found that CD8$^+$ T cells induce VSMC dedifferentiation toward a phenotype associated with plaque calcification (Schäfer et al., 2024). On the other hand, an experiment with a longer follow-up time contradicted these findings and provided evidence for a potential atheroprotective role of CD8$^+$ T cells in advanced lesions (van Duijn et al., 2019). Another study showed that in *Apoe*$^{-/-}$ mice, a subset of regulatory CD8$^+$ T cells is involved in the regulation of Tfh cell activity, thereby reducing atherosclerosis (Clement et al., 2015). These conflicting results illustrate the complexity of CD8$^+$ T cell functions in atherosclerosis and suggest that their impact may vary depending on the stage of the disease. Given these discrepancies, further investigation into the precise role of CD8$^+$ T cells is warranted to better comprehend their influence on atherogenesis.

4.4 B1 and B2 cells appear to have opposing roles in atherosclerosis

The presence of B cells in atherosclerotic plaques has been confirmed in scRNA-seq studies, but it appears they are generally sparse (Winkels et al., 2018; Fernandez et al., 2019). Instead, they predominantly inhabit the lymphoid tissue surrounding the arterial wall and the peritoneal cavity (Mangge et al., 2020). These cells are traditionally categorized into two distinct subtypes (Figure 3). B1 cells, integral to the innate immune response, secrete germline encoded IgM antibodies of low affinity aimed at common pathogens. Conversely, B2 cells necessitate stimulation from T-follicular helper cells (Tfh) to mature into plasma cells within germinal centers, ultimately releasing high-affinity IgG antibodies. In the context of atherosclerosis, B2 cells localize to and interact with T cells and APCs in unique adventitial structures termed artery tertiary lymphoid organs (Mohanta et al., 2014).

Observational studies reveal a dichotomous impact of these subgroups. Titers of IgM antibodies targeting apoB exhibited an inverse correlation with atherosclerosis, while titers of apoB-specific IgG antibodies displayed a positive correlation with disease progression in both murine models and humans (Karvonen et al., 2003; Tsimikas et al., 2007; Bjö et al., 2016). Intriguingly, the depletion of B1 cells through splenectomy in atherosclerosis-prone mice exacerbates the formation of atherosclerotic lesions, whereas artificial expansion of B1 cells attenuates atherosclerosis (Kyaw et al., 2011; Srikakulapu et al., 2017; Hosseini et al., 2018). This effect is hypothesized to result from the adverse effects of apoB-specific IgM antibodies on macrophage-mediated lipoprotein uptake (Kyaw et al., 2011). Conversely, an initial experiment involving the broad antibody-mediated removal of all B2 cells in mouse models of atherosclerosis has shown to mitigate inflammation and atherosclerosis (Ait-Oufella et al., 2010). However, the precise extent and characterization of B cells' involvement in human atherosclerosis remain subjects for further investigation. Again, it is important to consider the antigen-specificity of B cells involved in atherogenesis, as this may significantly impact the role of B cells in this disease.

5 Conclusion

In conclusion, this review describes atherosclerosis as a disease of low-grade vascular inflammation that is driven by a myriad of different pro-inflammatory processes and cellular players. A large body of evidence in this regard is derived from experimental murine

models of atherosclerosis for obvious reasons: it is impossible to capture the complexity of this disease relying solely on *in vitro* experiments. Nevertheless, it is important to note that significant differences exist between the cardiovascular systems of mice and humans that should be considered when translating findings. For instance, shear stress levels are higher in the murine vasculature (Greve et al., 2006). Furthermore, spontaneous plaque rupture is rare in mice, necessitating the use of ligation as a model of plaque rupture (Schwartz et al., 2007). With regards to the murine and human immune system, important differences exist as well. Many cytokines lack a cross-species counterpart (Shay et al., 2013) and the distribution of peripheral leukocytes is different between the two species (Mestas and Hughes, 2004). Consequently, an increasing number of experiments are attempting to more closely replicate the conditions of the human vasculature by employing vasculature-on-a-chip systems or organoids (Abaci and Esch, 2024). However, despite the limitations of mouse models, they continue to play a vital role in research in this field, offering the advantages of a fully developed mammalian immune and cardiovascular system.

The success of several clinical outcome trials has cemented the role of anti-inflammatory therapeutic strategies as means to put plaque inflammation to a halt, thereby reducing the ASCVD event rate in patients with residual inflammatory risk. However, rather than broadly targeting inflammation, which has been shown to lead to an increase in infection rate due to systemic immune suppression, the key to successfully combating inflammation in patients that are at risk of ASCVD in the future will lie in zooming in on more specific pro- and anti-inflammatory processes that are crucial to disease progression and targetable in humans. One example currently under investigation is the use of rituximab in patients who experienced a myocardial infarction in order to alter B cell populations (Zhao et al., 2022a). Another avenue that is currently being explored is the use of low-dose aldesleukin, a recombinant IL-2, in patients with stable ischemic heart disease to specifically stimulate Tregs (Zhao et al., 2022b). Luckily, the toolbox of atherosclerosis research has been enriched over recent years has facilitated this, with high-throughput methodologies such as scRNA-seq as an important means of exploring the innate and adaptive immune system in atherogenesis in more detail.

This review has provided an overview centered around the knowns and the unknowns of the cellular players in atherosclerotic inflammation, thereby providing a basis on which future research efforts to characterize the plaque immune landscape can build.

Author contributions

MA: Conceptualization, Investigation, Methodology, Visualization, Writing–original draft, Writing–review and editing. JoK: Conceptualization, Investigation, Methodology, Supervision, Visualization, Writing–original draft, Writing–review and editing. ES: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. JeK: Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing–original draft, Writing–review and editing.

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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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Cellular metabolism changes in atherosclerosis and the impact of comorbidities

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Cell activation and nutrient dysregulation are common consequences of atherosclerosis and its preceding risk factors, such as hypertension, dyslipidemia, and diabetes. These diseases may also impact cellular metabolism and consequently cell function, and the other way around, altered cellular metabolism can impact disease development and progression through altered cell function. Understanding the contribution of altered cellular metabolism to atherosclerosis and how cellular metabolism may be altered by co-morbidities and atherosclerosis risk factors could support the development of novel strategies to lower the risk of CVD. Therefore, we briefly review disease pathogenesis and the principles of cell metabolic pathways, before detailing changes in cellular metabolism in the context of atherosclerosis and comorbidities. In the hypoxic, inflammatory and hyperlipidemic milieu of the atherosclerotic plaque riddled with oxidative stress, metabolism shifts to increase anaerobic glycolysis, the pentose-phosphate pathway and amino acid use. We elaborate on metabolic changes for macrophages, neutrophils, vascular endothelial cells, vascular smooth muscle cells and lymphocytes in the context of atherosclerosis and its co-morbidities hypertension, dyslipidemia, and diabetes. Since causal relationships of specific key genes in a metabolic pathway can be cell type-specific and comorbidity-dependent, the impact of cell-specific metabolic changes must be thoroughly explored *in vivo*, with a focus on also systemic effects. When cell-specific treatments become feasible, this information will be crucial for determining the best metabolic intervention to improve atherosclerosis and its interplay with co-morbidities.

KEYWORDS

atherosclerosis, cell metabolism, glycolysis, fatty acids, diabetes, comorbidity

1 Introduction

Atherosclerosis describes the accumulation of immune cell-rich, lipid-rich plaques known as atheroma in large and medium-sized arteries (Roy et al., 2022). Around the world, atherosclerosis is a major contributor to cardiovascular diseases (CVDs), which include myocardial infarction and stroke, amongst others (Libby, 2021). CVDs are extremely common and have been steadily rising for decades to become the world's top cause of death (Roth et al., 2020). Currently, CVD accounts for almost four million deaths in Europe annually, or almost 44% of all deaths, with ischemic heart disease accounting for 44% of these CVD deaths and stroke accounting for 25% (Townsend et al., 2022). In 2016, China had around 2.4 million deaths from atherosclerotic CVD (ASCVD), accounting for 61% of CVD deaths and 25% of all deaths. This roughly represents a doubling of the absolute and relative number of deaths (1.4 million deaths from ASCVD, 11% of all deaths) compared to 1990 (Zhao et al., 2019).

Atherosclerosis is the narrowing of arteries caused by endothelial dysfunction, the accumulation of excess lipids in the vessel intima and the associated recruitment of inflammatory cells such as monocytes/macrophages. These undergo foam cell formation by taking up lipids and drive inflammatory processes. Atherosclerotic plaque formation occurs preferentially at artery branch points, which show a disturbed, non-laminar blood flow and are particularly vulnerable to inflammation and oxidative stress caused by hyperlipidemia. During all stages of atherogenesis, endothelial dysfunction, oxidative stress and inflammation contribute to disease development and progression (Hurtubise et al., 2016; Baaten et al., 2023). Also, neutrophils, B- and T-lymphocytes contribute to atherosclerosis (Nus and Mallat, 2016). Furthermore, vascular smooth muscle cells (VSMCs) in the tunica media can migrate into the intima, where they produce extracellular matrix molecules, forming a protective fibrous cap over the developing lesion (Sano et al., 2001; Bennett et al., 2016; Basatemur et al., 2019). On the other hand, VSMCs can also support lesion progression via SMC apoptosis or through differentiation into fibrochondrocyte-like or into macrophage-like cells that contribute to foam cell formation (Bennett et al., 2016; Basatemur et al., 2019). Plaque rupture is a main process leading to thrombus formation. Furthermore, superficial erosion is a rising contributor to the formation of thrombi in the coronary arteries and involves a shedding of the endothelial cell (EC) layer, mostly at plaques that show less lipid and macrophage accumulation, but a higher extracellular matrix content compared to plaques prone to rupture (Franck et al., 2017; Libby et al., 2019).

Patients with hypertension, dyslipidemia, type 2 diabetes mellitus (T2DM) or chronic kidney disease (CKD) have an increased risk of atherosclerosis (Selvin et al., 2005; Speer et al., 2022; Noels and Jankowski, 2023). Increased low-density lipoprotein-cholesterol (LDL-C), non-high-density lipoprotein cholesterol (non-HDL-C) and triglycerides, but a decline in HDL-C all independently increase the risk of ASCVD (Kopin and Lowenstein, 2017; Soppert et al., 2020). LDL and its oxidized form oxLDL play a key role in endothelial damage, macrophage foam cell formation and necrosis, as well as inflammatory signaling, whereas HDL plays an atheroprotective role by driving reverse cholesterol transport and by its anti-inflammatory properties (Soppert et al., 2020). T2DM is characterized by chronic hyperglycemia. Furthermore, diabetes can lead to hyperlipidemia and atherosclerosis due to an increased hepatic synthesis of triglyceride-rich very low-density lipoprotein (VLDL) (Poznyak et al., 2020). Hyperinsulinemia, insulin resistance, hyperglycemia and lipotoxicity all contribute to the creation of advanced glycation end products (AGEs), elevated free fatty acids and oxLDL, and can contribute to endothelial dysfunction, macrophage foam cell formation, inflammation and phenotypic switching of VSMCs, thereby supporting atherosclerosis (Zhao et al., 2024). Hypertension contributes to atherosclerosis through endothelial dysfunction as well as a hyperactivation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS) (Zaheer et al., 2016). CKD - defined as kidney damage or a glomerular filtration rate below 60 mL/min/1.73 m^2 for three or more months - is an independent risk factor for the development of CVD, including atherosclerosis, with CKD patients displaying a higher prevalence and progression of atherosclerotic lesions (Valdivielso et al., 2019; Noels and Jankowski, 2023). CKD contributes to atherosclerosis not only by increasing traditional risk factors like hypertension (Valdivielso et al., 2019), dyslipidemia and inflammation, but also through CKD-specific alterations like albuminuria and the accumulation of uremic retention solutes (Harlacher et al., 2022; Baaten et al., 2023; Vondenhoff et al., 2024).

In the past decade, a dysregulation of cellular metabolism has come into focus, leading to the identification of cell metabolism alterations in atherosclerotic lesions (Tomas et al., 2018) as well as in different cell types involved in atherogenesis in hyperlipidemic or/and pro-inflammatory context (Tomas et al., 2018). Understanding atherosclerosis and the contribution of altered cellular metabolism, as well as the impact of comorbidities on this, could support the design of novel strategies to lower the risk of CVD. Consequently, in this review, we will discuss cellular metabolism changes in the context of atherosclerosis and elaborate on how comorbidities may impact on atherosclerosis through effects on cellular metabolism.

2 Metabolic changes in atherosclerotic plaques

In the last decade, many efforts have been invested in exploring cell metabolism changes in relation to disease, especially in relation to immune cells and inflammatory diseases. Immune cells can make use of different substrates and energy metabolic pathways to produce energy in the form of ATP through processes of glycolysis, fatty acid oxidation (FAO) and amino acid metabolism, coupled to the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) cycle. Such breakdown of substrates to produce energy is referred to as "cellular respiration." Furthermore, metabolites generated during these processes are used to support cellular functions and survival and can modulate cell signaling pathways to influence both pro-inflammatory and anti-inflammatory outcomes (O'Neill et al., 2016; Voss et al., 2021). Depending on the context, alterations in cell metabolism can be observed. For example, upon infection or inflammation, macrophages from the innate immune system, as well as B-cells as cells from the adaptive immune system increase glycolysis and downregulate FAO, which has been linked with cellular activation profiles required to fight inflammation (Rhoads et al., 2017).

However, also in context of diseases, cell metabolism alterations have been observed and over the past years, multiple studies focused on understanding cell metabolism changes in various pathophysiological conditions, including inflammation and atherosclerosis - with the aim to develop new therapeutic strategies.

In 2002, it was shown that symptomatic, unstable plaques demonstrated increased uptake of the glucose analogue [18F]-fluorodeoxyglucose compared to asymptomatic plaques, whereas healthy carotid arteries did not show glucose uptake (Rudd et al., 2002). Furthermore, in an elegant study addressing the relation between the metabolic profile and vulnerability of human atherosclerotic lesions, Tomas et al. described in 2018 two distinct clusters of carotid artery plaques based on metabolite profiling and revealed that the metabolic profile was indicative for both the vulnerability of the lesions as well as future cardiovascular risk (Tomas et al., 2018). Plaques from symptomatic patients and with high vulnerability – characterized by a high lipid and macrophage content, low SMC and collagen content and signs of hemorrhages – showed metabolic alterations in terms of metabolite quantities and transcription levels of metabolic enzymes compared to plaques from asymptomatic patients and with low vulnerability (Tomas et al., 2018). More specifically, the high-risk plaques showed signs of reduced FAO (with a decrease in short-chain acylcarnitines) but increased glycolysis (with reduced levels of glucose but increased lactate) and amino acid use (with reduced substrates of glutamine and serine) (Tomas et al., 2018). In parallel, high-risk plaques showed increased mRNA levels of genes involved in glycolysis and the pentose-phosphate pathway as well as a stronger pro-inflammatory profile and were also associated with a higher risk of cardiovascular events over a seven-year follow-up (Tomas et al., 2018).

Recently, this was confirmed by Seeley et al., which in addition showed the spatial location of metabolites revealing the differential presence of lactic acid in the necrotic core of stable plaques, while pyruvic acid was more prevalent in the fibrous cap. In the fibrous cap of unstable plaques, 5-hydroxyindoleacetic acid was more prevalent (Seeley et al., 2023). Combined, this supported the notion that cellular metabolism may play a crucial role in supporting inflammation and the high-risk phenotype of atherosclerotic plaques (Tomas et al., 2018). Nevertheless, how metabolism of individual cell types changes, remains to be discovered.

Before elaborating on metabolic alterations in different cell types involved in atherosclerosis, important metabolic pathways are first introduced in the next section.

3 Brief description of important metabolic pathways

3.1 The tricarboxylic acid (TCA) cycle (also called Krebs cycle or citric acid cycle)

The TCA cycle takes place within the mitochondrial matrix and is a central regulator of energy production by oxidation of acetyl-coenzyme A (acetyl-CoA) derived from the metabolism of carbohydrates (and more specifically from glucose-derived pyruvate), fatty acids as well as proteins (Alabduladhem and Bordoni, 2024). Acetyl-CoA is used to produce citrate from oxaloacetate in the first step of the TCA cycle. Then, citrate is subsequently converted to other metabolites in eight successive steps, ultimately resulting in the regeneration of citrate. Each complete turn of the TCA cycle generates one GTP molecule as well as three molecules of NADH - the reduced form of nicotinamide adenine diphosphate (NAD)- and one molecule of $FADH_2$ – the reduced form of flavin adenine dinucleotide (FAD) -, which will enter the oxidative phosphorylation pathway to further generate energy in the form of ATP (Arnold and Finley, 2023). Metabolites generated during the TCA cycle - such as acetyl-CoA, citrate, aconitate, succinate and fumarate - can alter the response of both the innate and adaptive immune systems (Martínez-Reyes and Chandel, 2020).

3.2 "Oxidative phosphorylation" (OXPHOS) or "electron transport chain-linked phosphorylation"

OXPHOS follows the TCA cycle and is the final step of cellular respiration. It takes place at the inner mitochondrial membrane. In this process, the molecules NADH and $FADH_2$ - derived from the TCA cycle or from the OXPHOS cycle of fatty acid metabolism - are oxidized and thereby function as electron donors in a series of redox reactions (also referred to as the electron transport chain) with oxygen as ultimate electron acceptor (Mehta et al., 2017; Judge and Dodd, 2020). During this process, electrons are pumped over the inner mitochondrial membrane, generating a proton gradient that in a subsequent step can trigger energy release to power the enzyme ATPase for the production of ATP from ADP.

3.3 The glycolytic pathway or glycolysis

Glucose is an important substrate for cellular energy production and is metabolized by a process called glycolysis, which occurs in the cytoplasm. With the help of the enzymes hexokinase, phosphofructokinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) and pyruvate kinase, glycolysis rapidly converts a single molecule of glucose into two molecules of pyruvate with a net gain of two ATP molecules and two molecules of NADH. Pyruvate can subsequently be transformed to lactate with the help of the enzyme lactate dehydrogenase and is then secreted from the cell. Alternatively, in the presence of sufficient oxygen, pyruvate can be transported into the mitochondria, where it will be converted to acetyl-CoA to fuel the TCA cycle for additional ATP production in the mitochondria (Chandel, 2021a). Overall, this mitochondrial respiration via the TCA cycle and OXPHOS can generate up to 36 ATP molecules per glucose molecule, making it much more efficient in terms of energy production compared to solely glycolysis taking place in the cytoplasm. However, glycolysis also provides the cells with different substrate intermediates that are required for the synthesis of nucleotides (glucose-6-phosphate, for nucleotide production via the pentose phosphate pathway), amino acids (3-phosphoglycerate for serine biosynthesis) and fatty acids (via glyceraldehyde 3-phospate) (Chandel, 2021a). This explains why also in aerobic conditions, glycolysis is heavily used by cells with a high anabolic need (i.e., a need of building blocks for the

biosynthesis of nucleotides, lipids or proteins), even despite that such "aerobic glycolysis" (i.e., the conversion of glucose into pyruvate and subsequently lactate in the cytoplasm) is much less efficient in ATP generation compared to shuttling glucose-derived pyruvate to mitochondrial respiration (i.e., into the TCA/OXPHOS cycle). For example, glycolysis has a crucial role in the metabolism of rapidly proliferating cells (O'Neill et al., 2016) and many proliferation-inducing signaling pathways - including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways - enhance the cellular usage of glycolytic metabolism (Papa et al., 2019). In oncology, this has been referred to as the "Warburg effect" displayed by highly proliferative cancer cells. Similarly, the inflammatory activation of macrophages has been shown to induce increased glucose uptake and a shift from OXPHOS to aerobic glycolysis with enhanced pyruvate to lactate conversion (O'Neill and Hardie, 2013), as discussed in more detail below.

3.4 The pentose phosphate pathway (PPP)

The pentose phosphate pathway (PPP) is another glucose-metabolizing pathway that takes place within the cytoplasm and crucially contributes to *de novo* nucleotide synthesis, amino acid production, as well as the biosynthesis of fatty acids and triacylglycerol, but not to the formation of ATP (Dionisio et al., 2023; TeSlaa et al., 2023). Two branches can be distinguished, dependent on the initial glycolytic substrate that is being used.

The non-oxidative branch of the PPP uses fructose 6-phosphate and glyceraldehyde 3-phosphate as initial substrates; it allows intermediates from the glycolytic pathway to be channeled into the production of nucleotides through the production of ribose 5-phosphate, and can thereby contribute to cell growth and proliferation (O'Neill et al., 2016). The other way around, this branch of the PPP can also convert pentoses back to intermediates of the glycolysis pathway. The oxidative branch of the PPP metabolizes glucose 6-phosphate and is required for the generation of NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate). NADPH plays an important role in maintaining a favorable cellular redox environment, producing reactive oxygen species (ROS) via NADPH-dependent enzymes but also in preventing cellular oxidative stress (TeSlaa et al., 2023). Furthermore, NADPH is required for several cellular biochemical reactions such as the synthesis of fatty acids and triacylglycerol, nucleotides as well as amino acids (O'Neill et al., 2016; Dionisio et al., 2023).

3.5 Fatty acid metabolism/fatty acid β-oxidation (FAO)

Beyond glucose, cells can use fatty acids for energy production, which are metabolized through FAO in the mitochondria. FAO converts fatty acids into the energy-generating products acetyl-CoA, NADH and $FADH_2$, which are subsequently used in the TCA cycle and electron transport chain to generate ATP (Soppert et al., 2020). To be able to enter FAO metabolism, fatty acids are first converted to fatty acid-acyl-CoA in the cytosol (O'Neill et al., 2016). Then, they are transported to the mitochondria by either passive diffusion (for short-chain fatty acids) or with the help of carnitine palmitoyl transferase I (CPT1) and CPT2 (for medium- and long-chain fatty acids) to initiate the FAO process (Mehta et al., 2017). For each step of FAO, fatty acids are gradually shortened (with two carbons per step) with a parallel production of acetyl-CoA and the electron donors NADH and $FADH_2$. Fatty acid oxidation is a strong energy producer and can generate >100 ATP molecules per fatty acid molecule as palmitate (O'Neill et al., 2016).

3.6 Fatty acid synthesis (FAS)

Opposite from FAO, fatty acid synthesis (FAS) is an anabolic process which uses products derived from several other metabolic pathways - being glycolysis, the TCA cycle and the PPP - to synthesize fatty acids in the cytoplasm (O'Neill et al., 2016). The activity of the FAS pathway is intimately linked to mammalian target of rapamycin (mTOR) signaling, which promotes FAS by inducing sterol regulatory element binding protein (SREBP), which in turn activates fatty acid synthase (FASN) and CoA carboxylase (ACC) (Jones and Pearce, 2017). During FAS, acetyl-CoA is converted with the help of malonyl-CoA to a growing fatty acid chain, such as palmitic acid as the most abundant saturated fatty acid in the human body. Other fatty acids can be generated through processes of fatty acid elongation and desaturation. The resulting fatty acids can be esterified to glycerol-3-phosphate or cholesterol to form triglycerides or cholesterol esters, respectively (Vassiliou and Farias-Pereira, 2023). Finally, these fatty acids are packaged into VLDL to enter the bloodstream to be delivered to tissues in our body (Judge and Dodd, 2020). VLDL can be converted to the atherogenic LDL (Soppert et al., 2020).

3.7 Amino acid metabolism

Amino acids (AAs) are the individual monomers that make up proteins. They can be classified into essential AAs (Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine), conditionally essential AAs (Arginine, Cysteine, Glutamic Acid, Tyrosine, Glycine, Ornithine, Proline, Serine) and non-essential AAs (Alanine, Asparagine, Aspartic Acid, Glutamine) (Judge and Dodd, 2020). Furthermore, AAs can play some specific roles in metabolism. For example, glutamate can function as a nitrogen donor and acceptor and thereby can facilitate nitrogen movement among amino acids (Chandel, 2021b). Higher levels of glutamate in blood are linked to elevated estimates of both total and visceral adiposity, along with dyslipidemia and insulin resistance. Additionally, glutamate plasma levels are increased with the occurrence of subclinical atherosclerosis (Lehn-Stefan et al., 2021). Tyrosine serves as the building compound for the synthesis of the catecholamines norepinephrine, epinephrine and dopamine (Chandel, 2021b), hormones that have a closely relationship with hypertension and CVD. Furthermore, tryptophan serves as a precursor for the production of the neurotransmitter serotonin (Chandel, 2021b), which exerts pro-atherosclerotic effects (Shimabukuro, 2022). Moreover, methionine can have a proatherogenic effect by increasing the production of homocysteine, which has been identified as a risk factor for

atherosclerosis (Škovierová et al., 2016). On the other hand, cysteine, glutamate, and glycine contribute to the production of the antioxidant glutathione, which is atheroprotective (Chandel, 2021b; Rom et al., 2022). Also, arginine is required for the synthesis of NO, which is a key protective regulator of vascular homeostasis and immune cell function (Nitz et al., 2019). Furthermore, amino acids are closely linked to key anabolic cell signaling pathways, like the mTOR pathway (O'Neill et al., 2016). mTOR-containing complexes have a function in sensing amino acid levels, and mTOR-driven anabolic growth requires sufficient amino acid availability (Saxton and Sabatini, 2017).

4 Macrophage metabolic alterations in the context of inflammation and atherosclerosis

Macrophages contribute to the development, expansion and rupture of atherosclerotic plaques but also support processes of plaque regression (Tabas and Bornfeldt, 2020; Xue S. et al., 2023). Macrophages are heterogeneous and plastic, and dependent on the micro-environment, can change their phenotype. Although a plethora of macrophage phenotypes has been identified by the advent of single cell biology - amongst others resident macrophages, TNF^+ pro-inflammatory macrophages, $IL1\beta^+$ pro-inflammatory macrophages, $Trem2^+$ macrophages, lipid-associated Trem2 hi-perilipin$^+$ macrophages, and $IL10^+TNFAIP3^+$ macrophages (de Winther et al., 2023; Dib et al., 2023), little is known about the metabolism of these newly identified subsets. Traditionally, two main categories of macrophage polarization were distinguished, being the classically activated, pro-inflammatory M1 macrophages (which are stimulated by lipopolysaccharide (LPS) and interferon (IFN)-γ and trigger a pro-inflammatory response) and alternatively-activated, anti-inflammatory M2 macrophages (which are stimulated by IL-4 and IL-13 and inhibit inflammatory responses) (Xue S. et al., 2023; O'Rourke et al., 2022; Batista-Gonzalez et al., 2019). Macrophages of the M1 pro-inflammatory phenotype, resembling several of the pro-inflammatory transcriptional macrophage subsets, are increasingly accumulating in atherosclerotic lesions upon progression towards more inflammatory lesions (Khallou-Laschet et al., 2010). Instead, macrophages of the M2 phenotype do not have a clear counterpart in the transcriptional subsets, but its protein markers arginase and mannose receptor are found more in the shoulder regions covering the lipid core region and are less prone to develop into foam cells (Chinetti-Gbaguidi et al., 2011).

The metabolic pathways of glycolysis, the TCA and OXPHOS cycle, the PPP, lipid as well as amino acid metabolism play an important role in macrophage polarization and subsequently their inflammatory phenotype and contribution to atherosclerotic plaques (Koelwyn et al., 2018; Tabas and Bornfeldt, 2020; Xue S. et al., 2023).

In general, M1-type macrophages rely on glucose metabolism through aerobic glycolysis and the anabolic PPP, whereas M2-type macrophages have increased FAO, rely extensively on the mitochondrial TCA/OXPHOS cycle and show a decreased dependency on the PPP (Rhoads et al., 2017; Koelwyn et al., 2018) (Table 1) (Figure 1).

4.1 Glycolysis, oxidative phosphorylation, and the pentose-phosphate pathway

In general, pro-inflammatory activation of macrophages – for example by pathogen associated molecular patterns (PAMPs) as Toll-like receptor (TLR) stimulation - induces a profound metabolic reorganization that is characterized by an increase in glucose uptake and glycolysis, a disrupted TCA cycle and (depending on the stimulus) a reduction in OXPHOS (Rodríguez-Prados et al., 2010; Jha et al., 2015).

TABLE 1 Metabolic profiles of M1 vs. M2-polarized macrophages.

	M1 (pro-inflammatory)	M2 (anti-inflammatory)
Triggered by	IFN-γ, LPS	IL-4, IL-13
Cytokine production	TNF, IL-6, IL-1β, IL-18, IL-1α	TGF-β, IL-10
Glycolysis	High (with high pyruvate to lactate conversion)	Low
Pentose-phosphate pathway	High	Low
TCA cycle	Interrupted, with accumulation of TCA cycle substrates (e.g., succinate, citrate) and byproducts (e.g., itaconate)	Intact
OXPHOS	Low (but high in trained monocytes/macrophages)	High
Lipid metabolism	Lipotoxicity is due to increased fatty acid uptake but reduced FAO. Increased fatty acid synthesis	Increased FAO
Amino acid metabolism	Arginine to citrulline and NO Glutamine fuels succinate acid production Tryptophan, Serine, Methionine and Aspartate metabolism stimulate M1 polarization Phenylalanine attenuates M1 polarization	Arginine to ornithine Glutamine conversion to α-KG, with high α-KG/succinate ratio facilitating M2 polarization

PPP, pentose-phosphate pathway; OXPHOSP, oxidative phosphorylation; TCA, tricarboxylic acid cycle; IFN-γ, interferon gamma; α-KG, α-ketoglutarate; IL-1β, interleukin-1β; TNF, tumor necrosis factor; IL-4, interleukin-4; IL-6, interleukin-6; IL-1α, interleukin-1α; IL-18, interleukin-18; IL-10, interleukin-10; IL-13, interleukin-13; NO, nitric oxide; LPS, lipopolysaccharide; TGF-β, transforming growth factor-β; FAO, fatty acid oxidation.

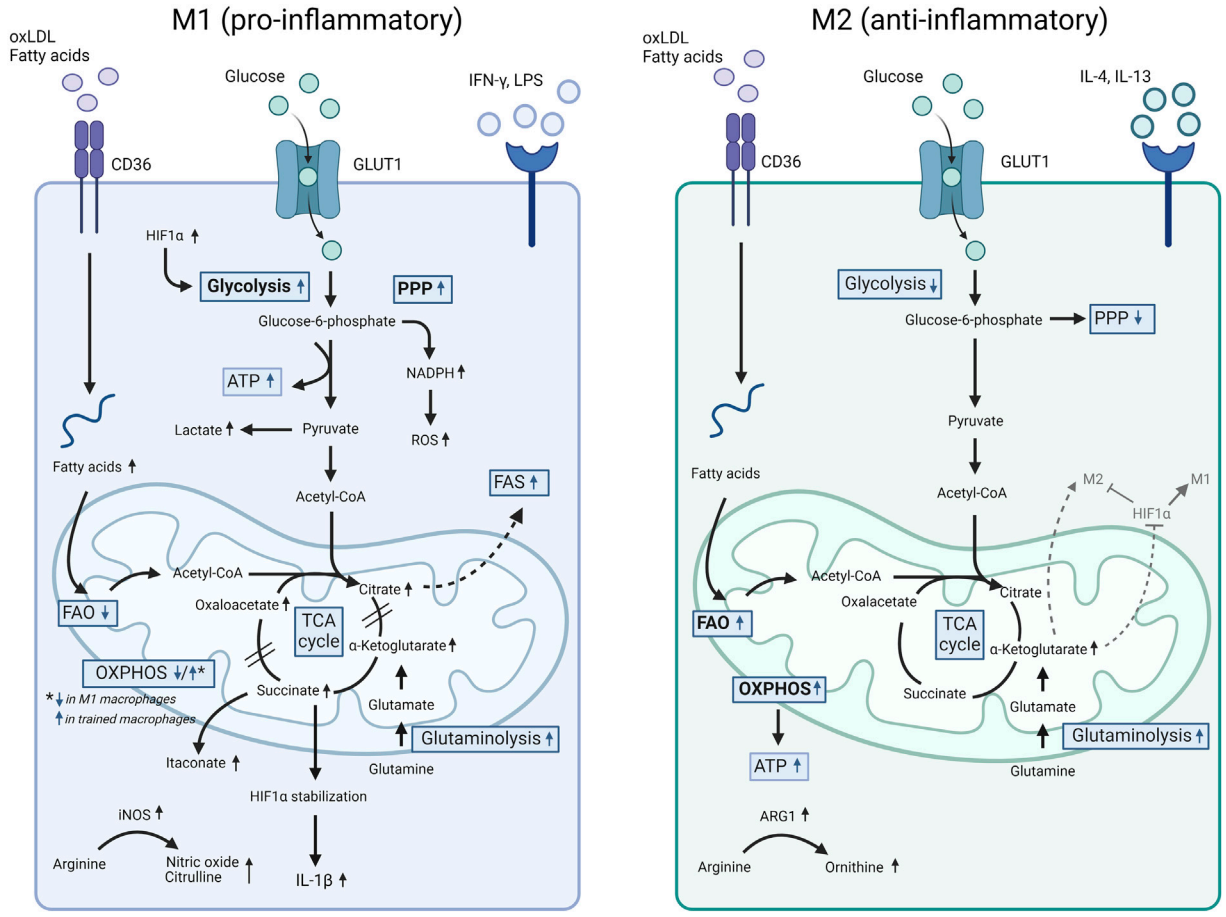

FIGURE 1
Comparison of cell metabolism in M1 versus M2 macrophages. Pro-inflammatory M1 macrophages (e.g., induced by LPS and oxLDL) show increased glycolysis with enhanced pyruvate to lactate conversion, triggered by ROS production and HIF1α accumulation, as well as an activated PPP. Furthermore, LPS downregulates the OXPHOSP cycle and triggers an accumulation of TCA intermediates. Increased glycolysis and an accumulation of TCA intermediates were also observed in trained monocytes induced by oxLDL and LPS. In these conditions, a simultaneous increase in OXPHOSP as well as increased glutaminolysis was also detected. Finally, fatty acid synthesis (FAS) supports the M1 pro-inflammatory phenotype. In comparison to M1, M2 macrophages downregulate glycolysis and PPP whereas upregulate the FAO and OXPHOSP pathways and show an intact TCA cycle. For more details, we refer to the text. ARG1, arginase 1; ATP, adenosine triphosphate; FAO, fatty acid oxidation; FAS, fatty acid synthesis; GLUT1, glucose transporter 1; HIF1α, hypoxia inducible factor 1-alpha; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; oxLDL, oxidized LDL; OXPHOS, oxidative phosphorylation; PPP, pentose-phosphate pathway; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

4.1.1 Increased glycolysis in hypoxia, pro-inflammatory conditions and monocyte/macrophage trained immunity

In hypoxic conditions - such as in atherosclerotic lesions with a high content of proliferating and metabolically active cells -, glycolysis is upregulated through increased hypoxia-inducing factor (HIF)1α mediated expression of glycolysis-regulating genes, such as PFKFB3 (Folco et al., 2011; Folco et al., 2014; Tawakol et al., 2015; van der Valk et al., 2015; Dib et al., 2023). However, while acute, systemic silencing of PFKFB3 ameliorated macrophage inflammation and atherosclerosis, chronic myeloid-specific inhibition of PFKFB3 did not have any effects on atherosclerosis (Tawakol et al., 2015; Tillie et al., 2021). In addition to regulating glycolysis, mild hypoxia increases the levels of the NLRP3 inflammasome and – associated with this – drives the production and secretion of pro-inflammatory IL-1β (Folco et al., 2011; Folco et al., 2014), with proven causality of systemic inhibition on human atherosclerosis (Ridker et al., 2017).

However, also in aerobic conditions, activation of the HIF1α pathway upon pro-inflammatory stimulation of macrophages can drive the upregulation of aerobic glycolysis with increased glucose consumption and a glycolytic flux of pyruvate towards lactate production rather than fueling the TCA cycle and mitochondrial respiration, as for example observed upon stimulation with oxLDL (Lee et al., 2014), LPS (Mills et al., 2016) or β-glucan (Cheng et al., 2014).

OxLDL not only promotes the transformation of macrophages to M1, but also inhibits the polarization into M2 macrophages (He and Liu, 2023). Mechanistically, oxLDL triggered the generation of ROS via NADPH-dependent oxidase (NOX)-2, which supported increased glucose uptake through the accumulation of HIF1α triggered by hypoxia (Lee et al., 2014). Also, LPS increased glycolysis in macrophages, coinciding with

an increased GLUT1 and hexokinase expression and increased absorption of F-fluorodeoxyglucose (Mills et al., 2016). In parallel, LPS downregulated the OXPHOS cycle and triggered the accumulation of succinate, which contributed to the stabilization of HIF1α and–via succinate oxidation by mitochondrial succinate dehydrogenase–to the production of mitochondrial ROS and subsequent pro-inflammatory gene expression with enhanced production of IL-1β (Mills et al., 2016).

Such metabolic rewiring towards increased aerobic glycolysis also formed the basis for β-glucan-induced trained immunity of monocytes and was associated with epigenetic histone modifications and increased expression of genes involved in glycolysis triggered by the AKT-mTOR-HIF1α pathway (Cheng et al., 2014; Arts et al., 2016). β-Glucan-trained monocytes also showed an upregulation of glutamine utilization and – with glutamine metabolism able to replenish the TCA cycle via α-ketoglutarate (α-KG) – an accumulation of different intermediates of the TCA cycle (succinate, fumarate, malate) (Arts et al., 2016). Fumarate itself was able to trigger HIF1α stabilization and the expression of HIF1α-dependent genes, and in parallel increased histone methylation by inhibiting the activity of the histone demethylase KDM5, thereby triggering increased expression of TNF and IL-6 (Arts et al., 2016). In a comparable way, also oxLDL induces a trained innate immunity in monocytes, i.e., an increased secondary response following an initial first stimulus (Sohrabi et al., 2018; Di Gioia et al., 2020; Keating et al., 2020). Mechanistically, this was found to be triggered by mTOR-mediated ROS production, which increased HIF1α-mediated gene expression and – in line with HIF1α known to drive glycolysis (Cheng et al., 2014) - upregulated glycolysis and a glycolytic flux towards lactate production (Sohrabi et al., 2018). A role for both cytoplasmic and mitochondrial ROS was revealed in the regulation of mTOR-HIF1α, metabolic reprogramming and trained immunity (Sohrabi et al., 2018). Furthermore, oxLDL-induced metabolic rewiring complemented epigenetic histone modifications in oxLDL-primed monocytes, which also contributed to a trained immunity and increased pro-inflammatory cytokine production upon secondary stimulation (Bekkering et al., 2014). In parallel to increased glycolysis, also the OXPHOS cycle was found to be upregulated and required for the trained phenotype in oxLDL-trained monocytes with secondary stimulation with LPS (Keating et al., 2020; Groh et al., 2021). A role for both glutamine conversion to glutamate – which can then feed the TCA cycle – and CPT-1 in oxLDL-trained pro-inflammatory cytokine production was shown, suggesting that glutamine and/or fatty acids could serve as initial substrates towards the OXPHOS cycle (Groh et al., 2021). In line, the antihyperglycemic drug metformin – which activates AMP-activated protein kinase (AMPK) but blocks mTOR signaling (and thereby glycolysis) and which also inhibits the OXPHOS cycle by interfering with the electron transport chain – interfered with both β-glucan- and oxLDL-induced trained immunity of monocytes (Arts et al., 2016; Keating et al., 2020). A simultaneous activation of glycolysis and OXPHOS, together with a dependency on glutaminolysis, an accumulation of TCA intermediates and a hyperactive state was also observed in LPS-primed macrophages with secondary stimulation with oxidized phospholipids (Di Gioia et al., 2020) as well as in oxLDL-primed monocytes with secondary LPS stimulation (Keating et al., 2020). Among substrates that feed the TCA cycle preceding the OXPHOS cycle, α-KG (as end-product of glutamine utilization) and oxaloacetate accumulation enabled a strong IL-1β production (Di Gioia et al., 2020). The other way around, inhibiting glutamine utilization or oxaloacetate production protected macrophages from hyperactivation and reduced atherosclerotic lesion formation *in vivo* (Di Gioia et al., 2020).

Of note, while a central role for HIF1 in macrophage glycolysis can be appreciated from aforementioned, silencing of HIF1α or HIF2α, or exaggerated HIF signaling by knockdown of its upstream regulators has many other cellular effects that complicate its use as intervention in atherosclerosis (Marsch et al., 2014; van Kuijk et al., 2022).

4.1.2 Impact of pyruvate kinase and lactate on macrophage phenotype

Pyruvate kinase is a key enzyme in the glycolysis pathway: it catalyzes the dephosphorylation of phosphoenolpyruvate into pyruvate and is responsible for the production of ATP during glycolysis. Increased glucose absorption and glycolytic flux was observed in monocytes and macrophages from patients with coronary artery disease (CAD), along with a high production of mitochondrial ROS. This altered the conformation state of pyruvate kinase M2 (PKM2) from tetrameric to dimeric and thereby favored its nuclear translocation, where PKM2 phosphorylated the transcription factor signal transducer and activator of transcription 3 (STAT3) to increase pro-inflammatory IL-6 and IL-1β production (Shirai et al., 2016). However, lactic acid as glycolysis byproduct derived from pyruvate, can modulate the macrophage phenotype from a pro- towards an anti-inflammatory M2 direction by inducing the lactylation of PKM2 (Wang et al., 2022). Mechanistically, PKM2 lactylation alters its conformation to a preferential tetrameric over its otherwise dimeric/monomeric form, and thereby reduces PKM2 nuclear accumulation, where it would otherwise drive the expression of proglycolytic genes in interaction with HIF1α (Wang et al., 2022) as well as pro-inflammatory genes (Shirai et al., 2016). Also, in the late phase of M1 macrophage polarization, lactate-derived lactylation of histone lysine residues as observed in conditions of hypoxia and bacterial challenges served as an epigenetic modification that directly stimulated the expression of homeostatic genes that have been traditionally associated with M2-like macrophages (Zhang et al., 2019). Furthermore, through its receptor GPR81, lactic acid can decrease the pro-inflammatory response of LPS-stimulated macrophages by reducing Yes associated protein (YAP), thereby blocking also downstream NF-κB activation and the macrophage pro-inflammatory response signaling (Yang et al., 2020).

4.1.3 The PPP triggers an M1 macrophage phenotype

The activated M1 macrophage phenotype is controlled by the PPP. The PPP is activated in response to LPS and IFN-γ, which increases the production of NADPH in macrophages (Umar et al., 2021). On the one hand, this NADPH production is important for maintaining the redox balance in the cells by producing antioxidants that protect immune cells from ROS. But on the other hand, NADPH

supports ROS production via NOX2 and thereby, increased PPP and NADPH contribute to triggering ROS-dependent pro-inflammatory signaling cascades such as NF-κB and MAPK (Dionisio et al., 2023). Furthermore, activated macrophages have increased phagocytic activity, which is supported by their increased PPP activity. Also, PPP-derived NADPH is especially important for FAS and cholesterol metabolism, two processes that are critical to macrophage activity (Koelwyn et al., 2018; Batista-Gonzalez et al., 2019). For example, fatty acids and derived complex lipids are important for membrane remodeling as well as for the production of pro-inflammatory lipid mediators in M1 macrophages (Koelwyn et al., 2018; Batista-Gonzalez et al., 2019). Finally, metabolic reprogramming required for proper M1-vs. M2-like macrophage polarization was also shown to be controlled by CARKL, a sedoheptulose kinase of the PPP: CARKL represses the M1 phenotype and its pro-inflammatory gene expression and ROS production, whereas sensitizing macrophages towards M2 polarization (Haschemi et al., 2012).

4.2 Tricarboxylic acid cycle (TCA)

Whereas M2-type macrophages exhibit an intact TCA cycle along with elevated ATP and OXPHOS levels, M1-type macrophages experience disruptions in the TCA cycle at multiple nodes, with trained innate monocytes/macrophages harboring an increased accumulation of TCA cycle intermediates (e.g., succinate, citrate) and byproducts (e.g., itaconate) (Rhoads et al., 2017; Koelwyn et al., 2018; Xu et al., 2024). An accumulation of TCA intermediates may subsequently have an impact on the macrophage phenotype, e.g., by contributing to a pro-inflammatory phenotype [e.g., as shown for succinate (Tannahill et al., 2013) and oxaloacetate (Di Gioia et al., 2020)], fatty acid synthesis for membrane production or pro-inflammatory fatty acid-derivatives as prostaglandin [for citrate (Infantino et al., 2011)] or by triggering innate immune memory [for fumarate (Arts et al., 2016)].

4.2.1 TCA intermediate accumulation in M1 and trained macrophages

More specifically, increased glutamine uptake in LPS-stimulated macrophages triggered the accumulation of succinate, which stabilized HIF1α and thereby increased the expression of the inflammatory mediator IL-1β (Tannahill et al., 2013). Also, mitochondrial oxidation of accumulated succinate triggered ROS production (Mills et al., 2016). Citrate not only provides a bridge between carbohydrate and fatty acid metabolism, but it can also be used to increase fatty acid biosynthesis, resulting in an increased generation of inflammatory prostaglandins (Williams and O'Neill, 2018). In this context, citrate is transported out of the mitochondria into the cytosol via the mitochondrial citrate carrier SLC25a1 and - with the help of ATP citrate lyase – functions as an acetyl donor to support the generation of acetyl CoA and fatty acid-derived derivatives. Citrate-derived acetyl-CoA can also support histone acetylation, with for the latter both pro- as well as anti-inflammatory effects described in – for example - LPS-stimulated macrophages (Ryan and O'Neill, 2020; Lauterbach et al., 2019). While macrophage-specific knockout of ATP citrate lyase stabilized murine plaques and showed dysregulated cholesterol and fatty acid metabolism, main effects seemed to be exerted via changes in apoptosis (Baardman et al., 2020).

Fumarate accumulation in β-glucan trained monocytes increases histone methylation associated with an increased pro-inflammatory gene transcription of TNF and IL-6 (Arts et al., 2016). On the other hand, increased levels of α-KG are used as a cofactor by the histone demethylase lysine demethylase 6B (KDM6B, also known as JMJD3) to promote anti-inflammatory gene expression reminiscent of the macrophage M2 phenotype (Liu et al., 2017). Also, itaconate is increasingly produced by macrophages with a disrupted TCA cycle - as shown upon LPS stimulation (Strelko et al., 2011) - but triggers subsequently protective effects, e.g., by the inhibition of succinate dehydrogenase (which controls levels of pro-inflammatory succinate), glycolysis and pro-inflammatory NLRP3 inflammasome activation (Strelko et al., 2011), whereas activating the anti-inflammatory transcription factors NRF2 and ATF3 (Peace and O'Neill, 2022). *In vivo*, itaconate could suppress atherogenesis by inducing NRF2-dependent inhibition of pro-inflammatory responses in macrophages (Song et al., 2023).

4.3 Lipid metabolism

4.3.1 Fatty acid oxidation

FAO supplies $FADH_2$ and NADH molecules required for the OXPHOS process, which is considered to be an important process supporting M2 polarization (Van den Bossche et al., 2017). Analyzing the mechanisms of fatty acid metabolism in IL-4-induced murine macrophage polarization, Huang et al. revealed that OXPHOS stimulates the production of genes essential for M2 polarization and raises the spare respiratory capacity of mitochondria (Huang et al., 2014). Besides, IL-4 actives peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1β (PGC-1β) and STAT6, which results in macrophage mitochondrial biogenesis and FAO (Vats et al., 2006). However, opposite results proposed that FAO is not required for M2 polarization (Namgaladze and Brüne, 2014; Nomura et al., 2016), and others also showed a role of glycolysis in fueling the TCA cycle for mitochondrial respiration in M2-polarized macrophages dependent on the mTORC2 and STAT6 pathways towards IRF4 activation (Huang et al., 2016).

4.3.2 Fatty acid synthesis

FAS has been identified to be necessary for M1 induction (Batista-Gonzalez et al., 2019). Mechanistically, FAS is required for macrophage membrane remodeling: a loss of FAS resulted in modifications in the plasma membrane composition and Rho GTPase trafficking, which attenuated macrophage inflammatory signaling (Wei et al., 2016). Furthermore, the pro-inflammatory macrophage response of NLRP3 inflammasome induction and subsequent release of IL-1β and IL-18 in response to an LPS challenge is also mediated by the activation of FAS, driven by mitochondrial uncoupling protein 2 (UCP2) and FASN triggering AKT signaling required for NLRP3 expression (Moon et al., 2023).

4.3.3 Cellular lipotoxicity and fatty acids

One of the main initiators of macrophage foam cell development and atherosclerosis is the disturbance of regular lipid metabolism in macrophages. Excessive lipid uptake leads to the formation of foamy macrophages, inhibits their migratory

capacity and traps them in the intima, ultimately leading to the induction of macrophage apoptosis and amplification of chronic inflammation (Moore et al., 2013). Via the CD36 receptor, oxLDL triggered the cellular accumulation of long-chain fatty acids by increased cellular uptake and mitochondrial import vs. a reduced mitochondrial FAO. This was associated with mitochondrial dysfunction and ROS production as well as pro-inflammatory NF-κB activation and increased cytokine production (Chen et al., 2019).

Lysosomes break down internalized lipoproteins, releasing a lot of free cholesterol and fatty acids (Moore et al., 2013). Transcriptional regulation of cholesterol homeostasis is governed by liver X receptors (LXRα and β) and sterol regulatory element-binding transcription factor 2 (SREBP2). The activation of LXRs, triggered by the accumulation of cholesterol in cells, results in enhanced cholesterol efflux and diminished cholesterol import (Luo et al., 2020). Furthermore, LXR activation raises the generation of lactate and acetyl-CoA, upregulates the expression of SREBP1 and stimulates the mevalonate pathway and IL-1β signaling in human monocytes, thereby contributing to a pro-inflammatory trained immunity phenotype (Sohrabi et al., 2020).

Fatty acids can be taken up by cells and transported to the mitochondria for FAO and ATP production. However, if saturated fatty acids accumulate in excess in cells, they become toxic and induce the generation of ROS, endoplasmic reticulum stress and NLRP3 inflammasome activation, which are all associated with a pro-inflammatory macrophage phenotype (Legrand-Poels et al., 2014). Furthermore, the saturated fatty acid palmitate is highly pro-inflammatory by triggering TLR2- and TLR4 signaling and activating NF-κB, in contrast to the long-chain omega-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid, which was able to counteract this effect (Huang et al., 2012). Overall, saturated fatty acids have been shown to be more pro-inflammatory and cytotoxic compared to unsaturated fatty acids, caused by their lower efficiency of being esterified in triglycerides for storage in lipid droplets (Legrand-Poels et al., 2014; Soppert et al., 2020; Noels et al., 2021). Nonetheless, also unsaturated fatty acids have been associated with pro-inflammatory responses in macrophages: atherosclerotic lesions from hyperlipidemic mice were shown to be highly abundant in the mono-unsaturated fatty acid oleic acid and the omega-6 PUFAs linoleic acid and arachidonic acid, which – in contrast to the saturated fatty acids palmitic acid or stearic acid - could trigger inflammasome-independent IL-1α production in macrophages. Oleic acid triggered foam cell formation and macrophage IL-1α secretion *in vitro* and the formation of atherosclerosis in mice. Mechanistically, a role of intracellular calcium release and uncoupling of mitochondrial respiration in oleic acid-induced IL-1α secretion was revealed (Freigang et al., 2013).

4.4 Amino acid metabolism

In macrophages, amino acid catabolism plays a crucial role in controlling multiple macrophage response pathways - including mTOR signaling and NO generation – and immune characteristics (Kieler et al., 2021).

4.4.1 Glutamine

Glutamine metabolism not only promotes the synthesis of succinic acid by fueling the TCA cycle in M1-type macrophages, but also drives M2 polarization via the conversion to glutamate and then α-KG, with high α-KG levels and a high α-KG/succinate ratio facilitating macrophage reprogramming towards the M2 phenotype (Liu et al., 2017). Mechanistically, α-KG destabilized HIF1α and downregulated inflammation-induced IL-1β production (Tannahill et al., 2013). Also, α-KG downregulated the mTORC1 pathway and instead induced nuclear PPARγ as driver of FAO gene expression (Liu et al., 2017). Furthermore, α-KG triggered metabolic reprogramming to an M2 gene expression profile by promoting JMJD3-dependent histone demethylation in the promoter of M2 marker genes (Liu et al., 2017). Moreover, glutamate can be metabolized to citrate in the TCA cycle. This supports the production of acetyl-CoA, which is then used for the production of cholesterol and fatty acids. Glutamine and glutamate can trigger triglyceride accumulation in macrophages, whereas glycine, alanine, leucine, and cysteine can downregulate triglyceride levels (Rom et al., 2017). Macrophages deficient for glutamate 1 synthase (GLS1) did not show major changes in cell proliferation or cell death, but – surprisingly - induced an M2-like reparative macrophage phenotype. However, the capacity of GLS1-deficient macrophages to clear apoptotic cells (efferocytosis) was dampened, leading to exaggerated atherosclerosis and necrotic core formation (Merlin et al., 2021). Unexpectedly, this was attributed to a non-canonical transaminase pathway, independent from canonical α-KG-dependent immunometabolism (Merlin et al., 2021).

4.4.2 Arginine

Arginine is transformed into citrulline and NO by the M1 marker inducible nitric oxide synthase (iNOS), with NO inhibiting the electron transport chain and thereby mitochondrial respiration (Kieler et al., 2021). Inversely, arginase 1 (ARG1) is constantly expressed by anti-inflammatory M2 macrophages. ARG1 can convert arginine to ornithine, which can enhance macrophage efferocytosis and thereby counteract atherosclerosis (Yurdagul et al., 2020), a process that also involves macrophage autophagy (Liao et al., 2012). Therefore, the M1 vs. M2 phenotype of macrophages is co-determined by the equilibrium in the arginine metabolism by ARG1 vs. iNOS. Of note, this pathway of arginine-induced efferocytosis (with arginine derived from engulfed apoptotic cells) is complemented by efferocytosis-induced glycolysis dependent on activation of the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2), triggering enhanced binding of apoptotic cells to the macrophage cell surface via glycolysis-derived lactate (Schilperoort et al., 2023).

4.4.3 Tryptophan

Tryptophan was shown to promote macrophage M1 polarization *in vitro*, with breast cancer macrophages revealing a high tryptophan metabolism along with a high M1 gene expression score (Xue L. et al., 2023). Besides, the tryptophan metabolites 3-hydroxy-kynurenine, 3-hydroxyanthranilic acid and quinolinic acid regulate oxidative stress processes, such as lipid peroxidation, and may act both in a pro-oxidative and anti-oxidative way (Nitz et al., 2019).

4.4.4 Methionine

Methionine can induce M1 macrophage polarization, which was linked to a pro-inflammatory state of macrophages with increased TNF production and iNOS activity, alterations in extracellular nucleotide metabolism, and an increased hydrolysis of ATP and ADP (Dos Santos et al., 2017; Franceschi et al., 2020).

4.4.5 Phenylalanine

Phenylalanine has been proposed to attenuate the inflammatory profile of M1 macrophages by reprogramming the transcriptomic and metabolic profiles, thereby augmenting oxidative phosphorylation in M1 macrophages. This reprogramming subsequently mitigated the activation of caspase-1, consequently hindering the production of IL-1β and TNF by M1 macrophages (Zhang et al., 2023).

4.4.6 Serine

Serine metabolism is important for LPS-mediated induction of IL-1β mRNA expression and glutathione synthesis in macrophages (Rodriguez et al., 2019). On the other hand, suppressing serine metabolism increased IGF1 expression, which subsequently triggered the activation of the p38-dependent JAK-STAT1 pathway, promoting macrophage M1 polarization while concurrently inhibiting STAT6-mediated macrophage M2 activation (Shan et al., 2022).

4.4.7 Aspartate

Aspartate can enhance the activation of the inflammasome and HIF1α and promote macrophage M1 polarization (Wang et al., 2021).

4.5 Associated effects of atherosclerosis comorbidities on macrophage metabolism

Macrophage polarization changes during many disorders such as obesity, T2DM and hypertension, which are important comorbidities and risk factors for atherosclerosis.

4.5.1 Obesity

Obesity is defined by adipose tissue accumulation by an energy overload (Yao et al., 2022). Numerous M1 macrophages are recruited into the adipose tissue (Yao et al., 2022). Obesity increases adipocyte development and cell hypoxia, which stimulates inflammatory chemokines and cytokines via HIF1α in adipose tissue macrophages (ATMs), inducing their polarization into the M1 phenotype (Fujisaka et al., 2013). Furthermore, increased circulating levels of free fatty acids – as observed in obesity – trigger M1 polarization of ATMs via TLR4 signaling activation (Shi et al., 2006). Increased consumption of free fatty acids by monocytes/macrophages induces a change in fatty acid metabolism from OXPHOS to triglyceride, phospholipid and ceramide production, which in turn enhances lipotoxicity and an M1 phenotype (Namgaladze and Brüne, 2016).

4.5.2 T2DM

In long-term hyperglycemic conditions, macrophages undergo a state of heightened stress, rendering them susceptible to excessive reactivity to external stimuli. This triggers the secretion of excessive inflammatory factors like TNF, IL-6, and CCL2, which are more likely to promote M1 polarization (Wu H. et al., 2021). Matsuura et al. demonstrated in mice that diabetes diminishes the expression of GLUT1 and GAPDH, thereby reducing glucose uptake and glycolysis in peritoneal macrophages (Matsuura et al., 2022). Furthermore, glutamine metabolism is changed in T2DM patients, characterized by a decrease in glutamine and α-KG vs. an increase in succinate, favoring macrophage polarization into the M1 phenotype (Ren et al., 2019). The other way around, M1 macrophages secrete a lot of inflammatory cytokines, such as IL-1β, which lead to insulin resistance in the liver, adipose and musculoskeletal tissues, as well as pancreatic β-cell malfunction (Eguchi and Manabe, 2013).

4.5.3 Hypertension

The activation of the RAAS and the SNS - which are both involved in regulating the salt-water balance and cardiovascular function - is increased in hypertension. Both stimulation of the RAAS (Barhoumi and Todryk, 2023) as well as the SNS (Harwani, 2018) mediates the polarization of macrophages towards an inflammatory phenotype. The other way around, renal denervation - which decreases the activation of SNS and RAAS and thereby attenuates blood pressure - leads to a substantial reduction in M1 macrophages into the medulla of the kidney and an increase in the production of kruppel-like factor-4, which drives macrophage M2 polarization (Liao et al., 2011; Xiao et al., 2015).

5 Neutrophil metabolic alterations in the context of inflammation and atherosclerosis

Neutrophils contribute to the development of atherosclerosis and the destabilization of plaques (Soehnlein, 2012). Specifically, the role of neutrophil extracellular traps (NETs) and ROS in causing tissue damage and fostering atherosclerosis and thrombosis has gained a lot of attention over the last years (Josefs et al., 2020).

Neutrophils can influence macrophage behavior, exhibit innate immune memory, and contribute significantly to the progression of atherosclerosis (Sreejit et al., 2022). Proteins released from distinct neutrophil granule subsets (e.g., IFN-γ, S100A9 and HNP1-3) provide guidance for the recruitment and activation of various other immune cells, including monocytes, macrophages and dendritic cell subsets (Soehnlein et al., 2009). Additionally, neutrophils produce lipid mediators through the oxygenation of arachidonic acid, leading to leukotriene A4 and its further processing into the potent chemoattractant leukotriene B4 (Soehnlein, 2012). Recent findings from mouse models of atherosclerosis reveal that neutrophils accumulate in large arteries shortly after the initiation of a high-fat diet (van Leeuwen et al., 2008; Drechsler et al., 2010). The size of the lesions exhibits a positive correlation with the counts of circulating neutrophils and the depletion of neutrophils significantly decreases atherosclerotic lesion size, supporting a causal involvement of neutrophils in the initial stages of atherosclerosis (Drechsler et al., 2010).

Although in the classical view, neutrophils mainly depend on glycolysis, recent progress in the field of immunometabolism has unveiled that neutrophils engage in diverse metabolic pathways

during inflammatory processes (Kumar and Dikshit, 2019). In contrast to other immune cells, where metabolic reprogramming typically leads to differentiation into distinct subtypes, neutrophils adjust their metabolic pathways to carry out various effector functions (Figure 2). These functions include chemotaxis, ROS generation, NET formation and degranulation.

5.1 Neutrophil basal metabolism

5.1.1 Glycolysis

Neutrophils primarily rely on glycolysis as the dominant metabolic pathway for generating energy in the form of ATP (Jeon et al., 2020) (Figure 2). Resting neutrophils express glucose transporter proteins (e.g., GLUT1, GLUT3 and GLUT4). Upon activation, there is a notable elevation in the surface expression of these glucose transporters concurrent with an increase in glucose uptake (Rodríguez-Espinosa et al., 2015). Depleting glucose significantly impairs most neutrophil functions (Azevedo et al., 2015; Rodríguez-Espinosa et al., 2015). In contrast, neutrophils, which contain fewer mitochondria than other immune cells, do not heavily rely on the OXPHOS cycle for energy production (Chacko et al., 2013). In the absence of glucose, neutrophils turn to glycogenolysis as an alternative energy source, particularly for phagocytosis (Weisdorf et al., 1982). In inflammatory conditions, neutrophils exhibit heightened glycogen accumulation (Robinson et al., 1982), emphasizing the dominance of glycolysis as an essential metabolic pathway.

5.1.2 Fatty acid oxidation

FAO is mostly used by immature/differentiating neutrophils (Rodríguez-Espinosa et al., 2015). Autophagy - a crucial cellular degradation and recycling process that provides free fatty acids - was demonstrated to support mitochondrial respiration and energy production during neutrophil differentiation in the bone marrow (Riffelmacher et al., 2017). Deficiency of Atg7 (Autophagy related 7 protein) resulted in heightened glycolytic activity but impaired mitochondrial respiration, reduced ATP production as well as lipid droplet accumulation in neutrophil progenitor cells, indicating that during neutrophil development autophagy supports a metabolic shift from glycolysis to FAO and mitochondrial respiration (Riffelmacher et al., 2017). Furthermore, inhibition of FAO resulted not only in reduced neutrophil maturation, but also in the accumulation of lipid droplets (Riffelmacher et al., 2017). In contrast, mature neutrophils primarily utilize glycolysis for energy production, making FAO dispensable. Nonetheless, FAO can mediate neutrophil effector functions. For example, tumor infiltrating neutrophils utilize mitochondrial FAO for ROS generation (Rice et al., 2018). In addition, neutrophils from diabetic rats with impaired glucose and glutamine metabolism presented increased FAO (analyzed as palmitic acid oxidation) in a compensatory mechanism of ATP generation (Alba-Loureiro et al., 2006).

5.1.3 Pentose phosphate pathway

Utilizing both oxidative and non-oxidative phases of the PPP, neutrophils utilize glucose-6-phosphate (an intermediate of the glycolytic pathway) as a precursor. The PPP generates NADPH and ribose- 5-phosphate, which are then utilized for the production of nucleotides (Stanton, 2012). In stressed neutrophils, during an oxidative burst, glycolysis is shifted to the PPP, allowing the neutrophils to promptly mount a first line of defense against pathogens, using the generated NADPH for redox signaling (Britt et al., 2022).

5.1.4 Oxidative phosphorylation

Despite possessing intact mitochondria and a functional TCA cycle, mature neutrophils exhibit minimal reliance on mitochondrial respiration for their energy production (Rodríguez-Espinosa et al., 2015), since the inhibition of mitochondrial respiration had no impact on ATP generation in mature neutrophils (Maianski et al., 2004). Instead, immature neutrophils rely on OXPHOS and FAO, with their energy production depending on degradation of lipid droplets through lipophagy with the resultant fatty acids directed to the TCA and OXPHOS (Riffelmacher et al., 2017).

5.2 Neutrophil extracellular trap (NET) formation

5.2.1 NET formation in atherosclerosis and thrombosis

NETs serve a crucial role in the immune system, neutralizing pathogens (Brinkmann et al., 2004). Apart from their function as a host defense mechanism, NETs also play a crucial role in non-infectious contexts, including in CVD [e.g., atherosclerosis (Warnatsch et al., 2015)] and thrombosis (Fuchs et al., 2010) as well as in pathologies enhancing CVD risk, such as diabetes (Wong et al., 2015). In this context, neutrophils expel nuclear DNA associated with histones and proteins, which organize in extracellular web-like structures called traps, in a process called NET formation (Brinkmann et al., 2004). NET-associated granule proteins include MPO (myeloperoxidase), NE (neutrophil elastase), defensins, calprotectin, cathelicidins, cathepsin G, lactoferrin, matrix metalloproteinase-9, peptidoglycan recognition proteins, pentraxin and LL-37. Incorporated histones include H1, H2A, H2B, H3 and H4 (Brinkmann et al., 2004; Hirose et al., 2014; Lim et al., 2018). During NET formation, calcium fluxes activate the enzyme peptidyl arginine deaminase 4 (PAD4), which converts the positively charged amino acid arginine to the neutral citrulline on histones. This impacts the interaction of histones with the negatively charged DNA and triggers DNA unwinding, nuclear expansion and chromatin condensation. In this context, pro-inflammatory cytokines (e.g., TNF, IL-1β, IL-8) can activate NET formation (Brinkmann et al., 2004).

In the context of CVD, NETs not only exert cytotoxic and prothrombotic effects (Warnatsch et al., 2015), but also contribute to plaque formation (Silvestre-Roig et al., 2019). First, NETs have been identified in the luminal area of murine and human atherosclerotic lesions (Megens et al., 2012; Silvestre-Roig et al., 2019). NETs damage the endothelium and induce its activation, leading to an increased expression of adhesion molecules and secretion of tissue factor (TF), which aggravates thrombosis risk. Additionally, NETs interact with von Willebrand Factor (vWF) and factor XII (FXII), promoting thrombus formation and initiating the intrinsic coagulation pathway, respectively (Grässle et al., 2014; Badimon and Vilahur, 2015; Folco et al., 2018).

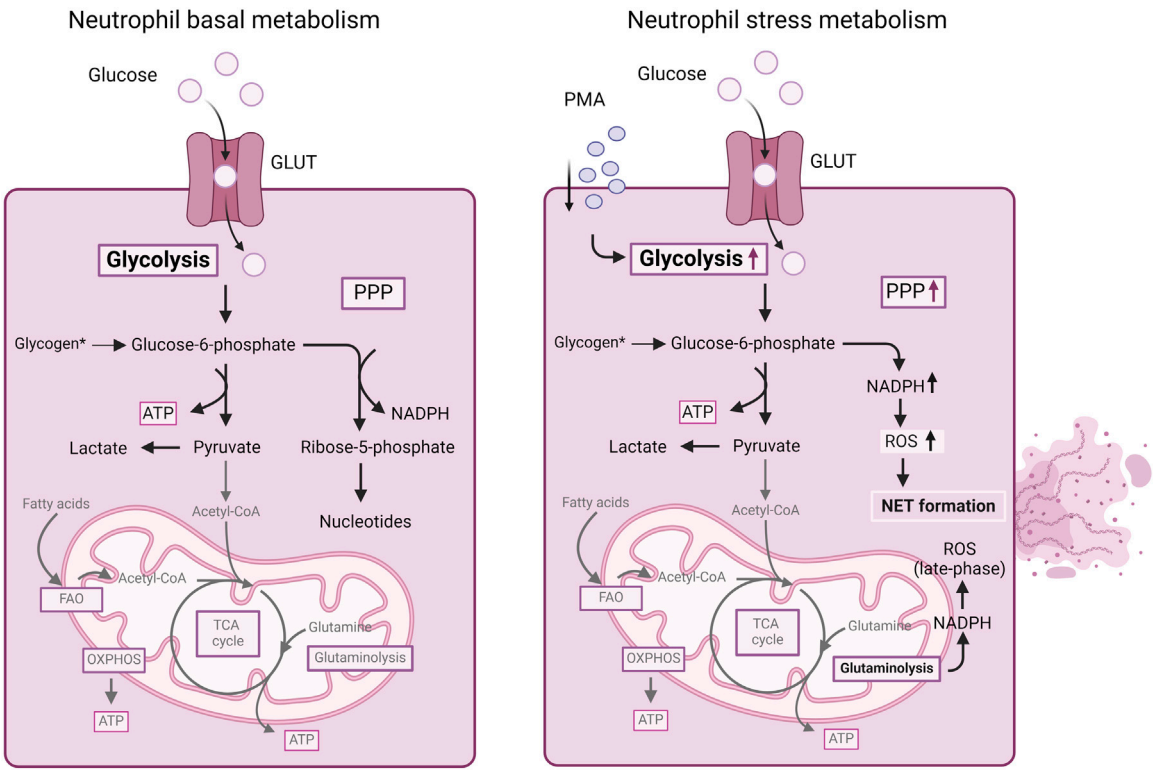

FIGURE 2
Summary of cellular metabolism in neutrophils. Mature neutrophils mainly depend on glycolysis for energy production. Glucose uptake and glycolysis increases in neutrophils stimulated with PMA. Furthermore, in stressed neutrophils, during an oxidative burst, the PPP is upregulated, allowing the neutrophils to generate NAPDH for redox signaling and ROS production as well as NET formation. Also, NADPH obtained from glutaminolysis can contribute to ROS generation in neutrophils. For more details, we refer to the text. ATP, adenosine triphosphate; FAO, fatty acid oxidation; GLUT, glucose transporter; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); OXPHOS, oxidative phosphorylation; PMA, phorbol myristate acetate; PPP, pentose-phosphate pathway; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

Mergens et al. reported adhered neutrophils excreting NETs in 57% of the atherosclerotic lesions of mice fed with a high-fat diet vs. 0% of control mice (Megens et al., 2012). In addition, Pertriwi et al. reported an increased presence of neutrophils and NETs particularly in autopsy-derived plaques exhibiting ruptures, superficial plaque erosions or intraplaque hemorrhages (Pertiwi et al., 2018). Furthermore, intimal-delivered NET-associated histones induce necrosis of SMCs and damage to vascular tissues, ultimately contributing to plaque destabilization (Silvestre-Roig et al., 2019). Moreover, NETs trigger the AIM (absent in melanoma 2) inflammasome in macrophages, facilitating the release of the pro-atherogenic cytokines IL-1β and IL-18, which contributes to the development of unstable atherosclerotic lesions (Paulin et al., 2018). Not only was the depletion of neutrophils shown to attenuate lesion formation (Zernecke et al., 2008), but also the inhibition of NET formation was able to preserve plaque stability in mouse models (Silvestre-Roig et al., 2019).

When observing CAD patients, markers of NET formation (e.g., double stranded DNA (dsDNA), nucleosomes, and MPO–DNA complexes) were increased in plasma (Borissoff et al., 2013). Borissoff et al. observed that nucleosomes can potentially predict severe coronary stenosis; that dsDNA was increased in patients with severe CAD or coronary artery calcification; that circulating dsDNA, nucleosomes and MPO–DNA complexes were positively associated with luminal stenosis, and that these markers of NET formation were predictive indicators for major adverse cardiovascular events (MACE) (Borissoff et al., 2013).

5.2.2 Neutrophil metabolism during NET formation

Glycolysis serves as the primary energy source for neutrophils during NET formation (Rodríguez-Espinosa et al., 2015; Behnen et al., 2017). Rodríguez-Espinosa et al., 2015. observed that the metabolic progression of NET formation could be delineated into two stages: the initial phase, characterized by chromatin decondensation, remains unaffected by exogenous glucose; and the later phase involving NET release is strictly contingent upon exogenous glucose availability and glycolysis (Rodríguez-Espinosa et al., 2015). Increased GLUT-1 surface expression and glucose uptake were detected in healthy human neutrophils stimulated with PMA. Glucose deprivation together with glycolysis inhibition (using 2-deoxy-glucose, 2-DG) resulted in the inhibition of NET formation (compared to untreated PMA-stimulated neutrophils), while the addition of glucose alone maintained NET formation in the same conditions (Rodríguez-Espinosa et al., 2015) (Table 2).

In addition, Azevedo et al. unveiled a metabolic shift towards the PPP during NET formation (Azevedo et al., 2015). When inhibiting the PPP with 6-aminonicotinamide (6-AN), a glucose-6-phosphate dehydrogenase (G6PD) inhibitor or when performing glucose deprivation, PMA-induced NET formation was blocked, mainly by

TABLE 2 Metabolism of neutrophils during NET formation.

References	Cells	Treatment/ condition	Effect on metabolism	Associated effect	NET formation	Readouts	PMID
Rodríguez-Espinosa et al. (2015)	Human healthy neutrophils	PMA	↑ Glycolysis	↑ GLUT-1 ↑ Glucose uptake	↑	NET formation is dependent on glucose. Glucose-free medium inhibited NETs	25545227
		Glucose deprivation, glycolysis inhibitor	↓ Glycolysis		↓*		
Behnen et al. (2017)	Human healthy neutrophils	PMA	↑ Glycolysis		↑	NET formation depends on glycolysis	28293240
Azevedo et al. (2015)	Human healthy neutrophils	PMA	↑ PPP	PPP provides NADPH for ROS generation	↑	Shift to PPP is necessary for NETs release	26198639
		Glucose deprivation, PPP inhibitor	↓ PPP	↓ NOX activity	↓*		
Awasthi et al. (2019)	Human healthy neutrophils	PMA, calcium ionophore, lactate	↑ Glycolysis	↑ Lactate	↑	Lactate induced NET formation, while inhibition of LDH activity significantly reduced NET formation by NOX-dependent and -independent mechanisms	31473341
		Lactate dehydrogenase (LDH) inhibitor		↓ Lactate	↓*		
Awasthi et al. (2023)	Human healthy neutrophils	PMA	↑ PPP; ↑ Glutathione metabolism; ↑ Glutamic acid metabolism	ROS generation and apoptosis	↑	Metabolic reprogramming from aerobic glycolysis to PPP in NETting cells (stimulated with PMA)	36265832
Tambralli et al. (2024)	Human healthy neutrophils	PMA		↑ ECAR and OCR (peak)	↑	Inhibiting either glycolysis or the PPP tempered PMA and APS IgG-induced NET formation, but not NET formation triggered by Ca ionophore	38869951
		Ca ionophore A23187 (Ca iono)		Only direct, short peak in ECAR and OCR	↑		
		Antiphospholipid syndrome (APS) IgG *(vs. control IgG)*	↑ Glycolysis and PPP	Slow, persistent ↑ in ECAR/OCR; ↑ Lactate, G6P (after 1 h), NAPDH (after 1 and 2 h) and intracellular glycogen	↑		
		2-DG (glycolysis inhibitor)	↓ Glycolysis ↓ PPP	↓ Lactate	↓ (for PMA and APS IgG induced); no difference for Ca iono iono		
		G6PDi-1 (G6PD inhibitor)	↓ PPP	↓ G6PD activity			
		DPI (NOX inhibitor)		↓ Total ROS			
		GPI (Glycogenolysis inhibitor); *in glucose-free media*					

* = vs. stimulated cells without inhibition of the metabolic pathway. G6PD, glucose-6-phosphate dehydrogenase; GLUT-1, glucose transporter 1; NET, neutrophil extracellular traps; NOX, NADPH oxidase; PMA, phorbol myristate acetate; PPP, pentose phosphate pathway; ROS, reactive oxygen species.

the reduction of NADPH oxidase activity (Azevedo et al., 2015). Furthermore, Awasthi et al. observed that human neutrophils exposed to lactate presented increased NET formation and that inhibition of lactate dehydrogenase (LDH) inhibited lactate-induced NET formation (Awasthi et al., 2019) (Table 2). More recently, the same group observed a metabolic reprogramming from aerobic glycolysis to PPP in netting neutrophils (when stimulated with PMA) (Awasthi et al., 2023). Fueled by metabolomics data, numerous metabolic pathways were observed to undergo alterations, encompassing intermediates in carbohydrate metabolism, redox-related metabolites, nucleic acid metabolism, and amino acid metabolism. Enrichment analysis of the detected metabolites revealed increased significance of the PPP and glutathione metabolism in PMA-induced netting neutrophils (Awasthi et al., 2023) (Table 2). Overall, mostly PMA-induced metabolic changes have been studied in relation to NET formation, whereas the impact of physiological stimuli on neutrophil metabolism remains mostly uninvestigated. A recent study by Tambralli et al. revealed that antibodies associated with the thromboinflammatory autoimmune disease antiphospholipid syndrome also triggered NET formation via glycolysis, the PPP and NADPH-dependent ROS production (Table 2) (Tambralli et al., 2024). However, further insights into the relation neutrophil metabolism-NET formation upon physiological stimuli relevant for atherosclerosis remain to be revealed.

5.3 ROS production

5.3.1 Neutrophil-derived ROS in atherosclerosis

Within neutrophils, three protein components (MPO, NADPH oxidase, and lactoferrin) participate in redox reactions, capable of generating ROS. The respiratory burst in neutrophils is characterized by an elevated production of the superoxide anion radical ($O_2^{\bullet-}$), a principal ROS generated in response to external stimuli, e.g., inflammation (Chistiakov et al., 2015).

In the context of atherosclerosis, various factors can induce the respiratory burst, including pro-inflammatory cytokines, lipids such as cholesterol, oxLDL, acute phase proteins like CRP, growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), circulating immune complexes, and others (Ferrante et al., 1988; Kannan et al., 2007). Studies revealed that the $O_2^{\cdot-}$ production by neutrophils (and other phagocytes) not only promotes oxidative stress in the phagocytes but also in adjacent cells in the vessel wall (Dinauer et al., 1990; Cahilly et al., 2000).

In addition, activated by atherosclerosis-relevant cytokines, MPO is released by neutrophils into the extracellular matrix (Singh et al., 2009). MPO converts chloride anions and hydrogen peroxide into hypochlorous acid, a potent oxidant and chlorinating species, which modifies plaque proteins, colocalizing with MPO (Hazell et al., 1996). Elevated serum levels of MPO predict future cardiovascular events in patients with acute coronary syndrome (Baldus et al., 2003) as well as development of CAD in healthy individuals (Meuwese et al., 2007).

Moreover, experimental findings suggest that neutrophil ROS and proteases contribute to superficial erosion in vulnerable plaques (Hosokawa et al., 2011). There is a detrimental impact of neutrophil-released ROS on ECs, which contributes to plaque formation and inflammation, inducing e.g., vascular hyperpermeability (Meegan et al., 2017). This mechanism could potentially lead to erosion and the disruption of the endothelial cell layer in the advanced stage of atherosclerosis.

5.3.2 Neutrophil metabolism during ROS production

Glycolysis supplies ATP, while the PPP pathway provides NADPH, essential for ROS generation in neutrophils (Petty et al., 2005; Baillet et al., 2017). Additionally, NADPH generated via mitochondrial glutaminolysis contributes to ROS production (Furukawa et al., 2000). Baillet et al. observed that neutrophils activated by PMA exhibit heightened phosphorylation of PFK-2, a pivotal enzyme in glycolysis. Inhibiting this enzyme resulted in reduced glycolysis rates and diminished NADPH oxidase activity in neutrophils (Baillet et al., 2017) (Table 3).

Moreover, Petty et al., observed that neutrophils under high glucose stimulation mainly use the PPP for ROS generation, with the inhibition of PPP or NOX attenuating high glucose-induced ROS generation in neutrophils (Petty et al., 2005) (Table 3). Similar effects were observed by Britt et al. when cells were stimulated with zymozan, TNF, fMLP or PMA. The treatments increased PPP and glycolysis mediators, and when the PPP pathway and NOX were inhibited, the neutrophils' oxygen consumption rate as readout of the neutrophil oxidative burst was reduced (Britt et al., 2022). A role for the PPP and NOX in PMA-induced ROS formation was confirmed by Tambralli et al., with a similar role for these pathways revealed in neutrophil ROS production triggered by antiphospholipid syndrome-associated antibodies (Tambralli et al., 2024) (Table 3).

Although the PPP provides the majority of cellular NADPH, a substantial amount of NADPH can also be obtained by glutaminolysis, and Furukawa et al. revealed that glutamine supplementation boosted ROS generation in neutrophils from patients that had undergone a major gastrointestinal surgery (Furukawa et al., 2000). Despite being dispensable for energy production, mitochondria in neutrophils remain functionally intact, suggesting that neutrophils might opt for the mitochondrial glutaminolysis pathway as an alternative means of supplementing NADPH during ROS generation (Furukawa et al., 2000) (Table 3).

Rice et al. demonstrated that the inhibition of glycolysis strongly reduced the early phase of the oxygen consumption rate (as readout of the oxidative burst) and ROS release in mouse bone marrow-derived neutrophils stimulated with PMA, while inhibition of mitochondrial respiration impacted to a small degree late-phase ROS release (Furukawa et al., 2000; Rice et al., 2018) (Table 3).

5.4 Associated effects of atherosclerosis comorbidities on neutrophil metabolism

Neutrophils exhibit metabolic reprogramming during metabolic disorders such as obesity and T2DM as important comorbidities and risk factors of atherosclerosis and CVD.

5.4.1 Obesity

Cichon et al. demonstrated that glycolysis and/or the PPP play a role in NETs release by neutrophils from mice on normal diet in both physiological and inflammatory conditions. In contrast, neutrophils from septic mice on high-fat diet utilize these pathways primarily for spontaneous NET release, whereas upon secondary *ex vivo* activation, they exhibit an "exhausted phenotype," characterized by diminished NET release, despite maintaining a high glycolytic potential and flexibility to switch to oxidize fatty acids in specific context (Table 4).

5.4.2 T2DM

Many functions of neutrophils, particularly ROS generation, NET formation, bactericidal activity, and chemotaxis, are dysregulated in patients with T2DM (Delamaire et al., 1997; Omori et al., 2008; Wong et al., 2015). Joshi et al., observed that in patients with T2DM, as well as in healthy donor neutrophils exposed to high glucose levels, neutrophil functions compete for NADPH. This resulted in increased cytosolic ROS production upon high glucose treatment, but insufficient NADPH availability for NET production in response to LPS. Notably, supplementing NADPH and using a pharmacological inhibitor of aldose reductase – an enzyme using NADPH oxidase for the formation of sorbitol in high-glucose conditions - successfully restored the sensitivity to LPS-induced NET formation in high glucose conditions, while reducing high glucose-induced cytosolic ROS production, neutrophil elastase secretion and spontaneous NET release (Joshi et al., 2020) (Table 4). Furthermore, in the context of hyperglycemia, Alba-Loureiro, et al. proposed that neutrophils

TABLE 3 Metabolism of neutrophils during ROS production.

References	Cells	Treatment/ condition	Effect on metabolism	Associated effect	ROS	Readouts	PMID
Baillet et al. (2017)	Human healthy neutrophils and PLB985 cells	PMA, fMLP	↑ Glycolysis	↑ pPFK-2	↑	Stimulation leads to an increase of the glycolysis rate and PFK-2 inhibition prevents both hyperglycolysis, leading to a decrease in ATP concentration and NADPH oxidase activation	27799347
		PFK-2 inhibitor	↓ Glycolysis	↓ NADPH oxidase	↓*		
Petty et al. (2005)	Human healthy neutrophils (pregnant or not)	Glucose	↑ PPP		↑ (and ↑ NO)	Neutrophils mainly use the PPP for ROS generation in high-glucose conditions. The inhibition of PPP and NOX attenuates high glucose-induced ROS generation in neutrophils	16390806
		PPP and NAD(P)H oxidase (NOX) inhibitor	↓ PPP (by PPP inhibitor)		↓*		
Britt et al. (2022)	Human healthy neutrophils and HL-60 cells	Zymozan, TNFα, fMLP and PMA	↑ PPP and glycolysis mediators	↑ NOX-dependent OCR	↑	Neutrophil stimulation causes rapid (10 or 30 min) metabolic changes. Activated neutrophils shift to PPP to increase NADPH production for oxidative burst and other effector functions	35347316
		PPP and NOX inhibitors	↓ PPP (by PPP inhibitor) no effect on glycolysis	↓ NOX-dependent OCR	↓*		
Furukawa et al. (2000)	Neutrophils from patients undergoing major gastrointestinal surgery	Basal	↓ Glutaminolysis	↓ Phagocytosis	↓	Glutamine supplementation enhances phagocytosis and production of reactive oxygen intermediates in patients that underwent major gastrointestinal surgery	10793298
		Glutamine	↑ Glutaminolysis	↑ Phagocytosis	↑		
Rice et al. (2018)	Mouse bone marrow-derived neutrophils	PMA		↑ OCR	↑ (↑ H_2O_2)	ROS production requires two distinct metabolic pathways, with glucose metabolism required for early phase ROS and mitochondrial function only facilitating the late phase. Neutrophils adapt to glucose-limited environments by using mitochondrial FAO for ROS production via NADPH oxidase	30504842
		Glycolysis inhibitor		↓ early-phase OCR	↓ (↓ H_2O_2)*		
		Mitochondrial respiration inhibition		↓ late-phase OCR	↓ (↓ H_2O_2) in late phase*		
		FAO inhibition or mitochondrial respiration inhibition in conditions of glucose usage blockade		↓ OCR	↓ (↓ H_2O_2)*		
Tambralli et al. (2024)	Human healthy neutrophils	PMA			↑	Inhibiting either glycolysis or the PPP tempered PMA and APS IgG-induced ROS production	38869951
		Ca ionophore A23187 (Ca iono)			No effect		
		Antiphospholipid syndrome (APS) IgG *(vs. control IgG)*	↑ Glycolysis and PPP	Slow and persistent ↑ in ECAR/OCR; ↑ Lactate, G6P (after 1 h), NAPDH (after 1 and 2 h) and intracellular glycogen	↑		
		2-DG (glycolysis inhibitor)	↓ Glycolysis ↓ PPP	↓ Lactate	↓ H_2O_2 (for PMA and APS IgG induced)		
		G6PDi-1 (G6PD inhibitor)	↓ PPP	↓ G6PD activity			

(Continued on following page)

TABLE 3 (*Continued*) Metabolism of neutrophils during ROS production.

References	Cells	Treatment/ condition	Effect on metabolism	Associated effect	ROS	Readouts	PMID
		DPI (NOX inhibitor)					
		GPI (Glycogenolysis inhibitor); *in glucose-free media*					

* = vs. stimulated cells without inhibition of the metabolic pathway. FAO, fatty acid oxidation; fMLP, N-Formyl-methionyl-leucyl-phenylalanine; G6PD, glucose-6-phosphate dehydrogenase; OCR, oxygen consumption rate; PFK-2, phosphofructokinase-2; PMA, phorbol myristate acetate; PPP, pentose phosphate pathway; ROS, reactive oxygen species; NOX, NADPH oxidase; TNF, tumor necrosis factor.

may initiate compensatory FAO due to impaired glucose and glutamine metabolism as well as reduced lactate production and diminished PPP activity, which overall was associated with impaired phagocytosis and H_2O_2 production (Alba-Loureiro et al., 2006).

6 Metabolic alterations in endothelial cells in the context of inflammation and atherosclerosis

ECs are the lining between the circulation and the vessel wall and thereby play a crucial role in the regulation of leukocyte recruitment. Activation and dysfunction of ECs is one of the first signs of initial atherosclerosis formation, occurring particularly at bends and branch points in the vasculature (Hajra et al., 2000; Tabas et al., 2015). These dysfunctional ECs are characterized by a pro-inflammatory phenotype with reduced barrier function, leading to the infiltration of LDL into the vessel wall and further activation of the endothelium. Although the endothelium was originally believed to be an inert barrier, it is now viewed as a metabolically active organ playing a crucial role in vascular homeostasis and pathologies (Theodorou and Boon, 2018; Stroope et al., 2024) (Figure 3).

6.1 Endothelial cell metabolism in health

6.1.1 Glycolysis

The main energy source accounting for around 80% of the total ATP production in ECs is glycolysis (De Bock et al., 2013) (Figure 3). Such predominant anaerobic metabolism may safeguard the ECs against ROS production (Li et al., 2019). Further indicating the essential need for glycolysis is the notion that inhibition of glycolysis by 2-deoxy-D-glucose induces cytotoxicity in ECs (Merchan et al., 2010). Slight reductions of glycolysis in ECs have also been shown to reduce its proliferation, migration and sprouting capacity (De Bock et al., 2013; Schoors et al., 2014; Yu et al., 2017), while glycolysis is increased by EC stimulation with vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) inducing cellular proliferation and migration (De Bock et al., 2013; Yu et al., 2017). It could be shown that glycolysis in ECs is mainly regulated by PFKFB3 (De Bock et al., 2013). This could also be validated *in vivo* in an EC-specific *Pfkfb3*-deficient mouse model, demonstrating that the lack of *Pfkfb3* results in decreased branch points and sprouts in a postnatal retina model (De Bock et al., 2013). Additionally, PKM2 seems to play an important role in EC function and angiogenesis as silencing of PKM2 resulted in decreased sprouting (Gómez-Escudero et al., 2019), but also in reduced VE-cadherin expression resulting in destabilization of the cellular junctions (Gómez-Escudero et al., 2019).

6.1.2 Pentose phosphate pathway

Besides the use of glucose as energy source, it can also be phosphorylated by hexokinase 2 to generate glucose-6-phosphate, which subsequently can be stored as glycogen or further processed in the PPP to yield NADPH (Mann et al., 2003; Lunt and Vander Heiden, 2011). Also, this pathway has cellular effects as silencing of the G6PD enzyme–the first and rate-limiting enzyme of the PPP – reduces EC proliferation and migration while increasing intracellular ROS (Leopold et al., 2003). It remains however to be determined what the effect of the glycogen metabolism is on EC function.

6.1.3 Fatty acid metabolism

ECs only have a relatively low mitochondrial content (<2–12% of cellular volume) compared to other more oxidative cell types and therefore also exert a lower mitochondrial respiration (Barth et al., 1992; De Bock et al., 2013). However, ECs have a relatively large spare respiratory capacity, which might enable them to utilize fatty acids and glutamine for example, as alternative energy source in stress conditions (Krützfeldt et al., 1990; Wilhelm et al., 2016). Since ECs only derive around 15% of their ATP via oxidative pathways, the mitochondria in ECs seem to play a more important role in biomass synthesis rather than energy production (Schoors et al., 2015). For example, fatty acids are used for the generation of the amino acid aspartate, which is a nucleotide precursor as well as for deoxynucleotides, required for DNA synthesis (Schoors et al., 2015). Additionally, fatty acid metabolism also plays a role in EC functionality as *Cpt1* deficiency resulted in defects in vascular sprouting due to reduced EC proliferation (Schoors et al., 2015) as well as to increased endothelial permeability (Patella et al., 2015). FAO also plays an important role in maintaining the endothelial identity by maintaining the pool of acetyl-CoA and therefore reducing transforming growth factor β (TGF-β)-induced endothelial-to-mesenchymal transition (EndMT) (Xiong et al., 2018).

In addition to these intracellular effects, ECs also regulate the transport of fatty acids toward metabolically active tissues (Mehrotra et al., 2014). Circulating fatty acids can enter the ECs either by passive diffusion or by transporter proteins and subsequently either be stored in lipid droplets to protect against ER stress or released again to the underlying tissues (Kuo et al., 2017).

6.1.4 Amino acid metabolism

The most highly consumed amino acid in ECs is glutamine, which plays a crucial role in angiogenesis (Huang et al., 2017). The lack of glutamine or inhibition of glutaminase 1, the rate-limiting enzyme in glutaminolysis, reduces EC proliferation, while its role in migration remains controversial (Huang et al., 2017; Kim et al., 2017). Methionine and cysteine have also been shown to be involved in angiogenesis as restrictions of these amino acids promoted VEGF production thereby triggering angiogenesis (Longchamp et al., 2018). Furthermore, arginine can be converted to citrulline and NO, which has a wide range of effects on ECs and plays a key role in maintaining vascular homeostasis, by for example suppressing thrombosis, inflammation, and oxidative stress (Tousoulis et al., 2012; Theodorou and Boon, 2018). Valine is another amino acid that influences ECs as it can be converted into 3-hydroisobutyrate (3-HIB), thereby promoting transendothelial fatty acid transport (Jang et al., 2016).

6.2 Endothelial cell metabolism in atherosclerosis

In physiological conditions, ECs remain rather quiescent (Gimbrone and Garcia-Cardena, 2016). At sites of undisturbed flow, Kruppel-like factor (KLF) 2 and 4 are expressed driven by FAO, driving the expression of endothelial nitric oxide synthase (eNOS) to generate NO (Atkins and Jain, 2007). This in turn suppresses inflammatory pathways intracellularly (Atkins and Jain, 2007) (Figure 3). Furthermore, KLF2 drives autophagy (Dabravolski et al., 2022), including in endothelial cells (Guixé-Muntet et al., 2017) in which autophagy counteracts endothelial apoptosis and inflammation (Vion et al., 2017). Also, KLF2/4 expression induce the production of NADPH, while reducing the expression of PFKFB3 (Doddaballapur et al., 2015).

However, due to stimuli like for example disturbed blood flow dynamics resulting in laminar shear stress, ECs can become activated (Gimbrone and Garcia-Cardena, 2016) (Figure 3). ECs at atheroprone regions in the vessels experiencing this disturbed shear stress demonstrate an activation of pro-inflammatory pathways and increased expression of glycolytic enzymes (Feng et al., 2017). Additionally, disturbed blood flow reduces KLF2/4 expression and thus NO and NADPH production, while PFKFB3 expression increases which is at least partly responsible for the increased glycolysis (Stroope et al., 2024). Furthermore, expressed pro-inflammatory cytokines in ECs increase the glucose uptake and glycolysis in these cells, via NF-κB activation (Cantelmo et al., 2016). Disturbed shear stress as well as pro-inflammatory cytokines also activate YAP, which not only stimulates EC activation and atherosclerosis development, but also EC glycolysis (Wang et al., 2016a). Additionally, disturbed shear stress activates AMPK and thereby induces HIF1α-mediated upregulation of glycolysis-related genes (Wu et al., 2017; Yang et al., 2018). Thereby, EC inflammation enhances glycolysis in these cells but also initiates a vicious cycle as this increased glycolysis again drives inflammation.

Interestingly, YAP also enhances EC glutaminolysis, suggesting a role for glutamine in atherogenesis (Bertero et al., 2016). Glutamine starvation of ECs reduces protein synthesis and thus results in ER stress and increased cellular inflammation (Tabas, 2010; Huang et al., 2017). However, the exact effects on atherosclerosis development remain to be investigated.

While the role of FAO in the inflammatory responses of ECs remains rather elusive, it could already be shown that FAO is important for maintaining the EC barrier function (Patella et al., 2015; Xiong et al., 2018). Furthermore, FAO inhibits EndMT which has a plaque destabilizing outcome (Chen et al., 2015; Xiong et al., 2018; Libby et al., 2019). Thereby endothelial FAO seems to reduce atherosclerosis development, although clear causal evaluations on this aspect are currently still missing.

6.3 Associated effects of atherosclerosis comorbidities on endothelial cell metabolism

Several comorbidities, such as T2DM and obesity, are hallmarked by endothelial dysfunction and a dysregulated EC metabolism, resulting in an accelerated development of atherosclerosis.

6.3.1 T2DM

The high circulating glucose levels caused by T2DM increase endothelial ROS production, leading to DNA damage and subsequent activation of poly (ADP-ribose) polymerase 1 (PARP1) (Du et al., 2003; Forrester et al., 2018). This PARP1 activation causes an inhibition of the glycolytic enzyme GAPDH, resulting in an accumulation of intermediate products that are upstream of this enzyme (Du et al., 2003). This accumulation and further redistribution of glycolysis intermediates results in the generation of AGEs and PKC activation (Du et al., 2003; Shah and Brownlee, 2016). These AGEs induce EC dysfunction and activate pro-inflammatory signaling cascades together with ROS formation (Shah and Brownlee, 2016). Thereby, T2DM influences ECs in a manner that generates a more atherosclerosis-prone cellular phenotype.

6.3.2 Obesity

One of the characteristics of obesity is the elevated circulating saturated fatty acids and triglyceride-rich lipoproteins, which are a major source of fatty acids (Goldberg and Bornfeldt, 2013; Nordestgaard, 2016). These fatty acids can induce EC dysfunction by impairing vasodilatation and increasing vascular permeability, oxidative stress, inflammatory signaling as well as EC apoptosis (Inoguchi et al., 2000; Wang et al., 2016b). This is further supported by the notion that overexpression of PPAR-γ coactivator 1-α, resulting in increased FAO and thus reduced intracellular lipid levels, reduces again the FA-induced EC dysfunction and apoptosis (Won et al., 2010). Intriguingly, it remains to be determined why ECs do not spontaneously increase FAO to compensate for the lipid overload, particularly considering their large spare respiratory capacity.

TABLE 4 Impact of obesity and diabetes on neutrophil metabolism and function.

References	AS comorbidity	Cells	Cells origin	Treatment/ condition	Effect on neutrophil metabolism	Associated effect on neutrophil function	PMID
Cichon et al. (2021)	Obesity	Mouse primary neutrophils	Obese mice	HFD	↓ GLUT1 expression ↓ Glycolysis (tendency)	↓ NET formation after 1 h LPS stimulation ↑ NET formation after 6 h LPS stimulation	34299338
				HFD and sepsis	↑ Glycolysis (tendency)	↑ Spontaneous NET formation ↓ NET formation after LPS stimulation	
Joshi et al. (2020)	Diabetes	Human primary neutrophils	Healthy donors	High glucose	↑ Polyol pathway intermediates (NADPH-dependent formation of 1-anhydrosorbitol via aldose reductase) ↓ Glutathione metabolism (with NADPH required for glutathione synthesis)	↑ Cytosolic ROS ↑ Neutrophil elastase secretion ↑ spontaneous NADPH oxidase-dependent NET formation but ↓ LPS-induced NET formation	32827651
				High glucose + Aldose reductase inhibitor		No high glucose-induced increase in cytosolic ROS, neutrophil elastase secretion or spontaneous NET formation, but restored responsiveness to LPS-stimulated NET formation	
Alba-Loureiro et al. (2006)	Diabetes	Rat primary neutrophils	STZ-treated rats		↓ Metabolism of glucose and glutamine ↓ Lactate production and PPP activity	↓ Phagocytosis ↑ Production of H_2O_2	16461555

AS, atherosclerosis; GLUT-1, glucose transporter 1; HFD, high-fat diet; LPS, lipopolysaccharide; PPP, pentose phosphate pathway; STZ, streptozotocin; ROS, reactive oxygen species.

7 Metabolic alterations in vascular smooth muscle cells in the context of inflammation and atherosclerosis

VSMCs play a key role in the process of atherosclerosis (Grootaert and Bennett, 2021). Although VSMCs have previously mainly been appreciated for their role in fibrous cap formation and thus atheroprotective function, multiple VSMC phenotypes and detrimental VSMC functions have now been identified in the context of atherosclerosis (Grootaert and Bennett, 2021; Chen et al., 2023; Elmarasi et al., 2024). Contractile VSMCs populate the medial layer of the healthy vessel. In the pre-atherosclerotic stage, characterized by a diffuse intimal thickening, an increase in synthetic VSMCs can be detected. With the progression of atherosclerotic lesions, medial VSMCs can migrate from the media to the intima and generate extracellular matrix molecules (including interstitial collagen and elastin, as well as proteoglycans and glycosaminoglycans), forming a protective fibrous cap over the developing lesion (Bennett et al., 2016; Basatemur et al., 2019). On the other hand, synthetic VSMCs can differentiate into cells resembling macrophages (Basatemur et al., 2019; Elmarasi et al., 2024). Increased lipid buildup, inflammation and VSMC death, as well as VSMC differentiation into osteochondrogenic cells (contributing to plaque calcification) are observed in later stages of atherosclerosis progression (Basatemur et al., 2019; Elmarasi et al., 2024). Over the past few decades, a growing body of research has revealed a role of cellular metabolism – particularly glucose uptake, glycolysis, and amino acid metabolism – in the regulation of VSMC phenotype (Stroope et al., 2024).

7.1 VSMC metabolism in health and atherosclerosis

7.1.1 Glycolysis

In healthy VSMCs, cytoplasmic or aerobic glycolysis is central for ATP production and triggers high rates of lactate production under fully oxygenated conditions, rather than relying on glucose oxidation in the mitochondria (Lynch and Paul, 1983; Butler and Siegman, 1985). Overall, 90% of the glucose taken up by VSMCs is converted to lactate (Michelakis and Weir, 2008).

In vitro, platelet-derived growth factor (PDGF)-BB-induced proliferation of VSMCs markedly increased glucose flow and glycolysis (Werle et al., 2005). Blocking glycolysis with 2-deoxy-D-glucose or blocking phosphoinositide 3-kinase (PI3K) prevented both PDGF-stimulated VSMCs proliferation (Figure 4A) as well as the increase in glycolysis and mitochondrial reserve capacity (Perez et al., 2010). In line, deletion of PKM2 – which catalyzes the last step of glycolysis – in VSMCs attenuated injury-induced neointimal hyperplasia by hindering VSMC proliferation and migration, suppressing the synthetic phenotype and diminishing aerobic glycolysis by decreasing signaling through extracellular signal-regulated kinase (ERK), mTOR and STAT3 pathways (Jain et al., 2021). Furthermore, single-cell and bulk RNA-seq analyses of mouse brachiocephalic arteries and *in vitro* models showed that aerobic glycolysis is important for the transition of VSMCs to $ACTA2^+$ myofibroblast-like cells, which play an important role in the formation of the protective fibrous cap in atherosclerotic lesions (Newman et al., 2021) (Figure 4A). Furthermore, increased lactate levels - as observed in anaerobic conditions as ischemia injury (Yang

et al., 2017) as well as in the necrotic core of atherosclerotic lesions (Seeley et al., 2023) - supported a more synthetic phenotype of VSMCs, with a proteomics and pathway profile suggestive of increased extracellular matrix production and VSMC proliferation (Yang et al., 2017) (Figure 4A). Also network analyses based on gene expression studies revealed dysregulations in glycolysis metabolism during human aortic SMCs phenotype plasticity (Perry et al., 2023).

OxLDL as important contributor to atherosclerosis induces the generation of ROS and oxidative stress in VSMCs (Locher et al., 2002). High concentrations of OxLDL can also exacerbate apoptosis in VSMCs, which could support the destabilization of atherosclerotic plaques (Kataoka et al., 2001). In terms of cellular metabolism, oxLDL significantly enhanced the PKM2-dependent glycolytic rate and established glycolysis as the primary energy source in oxLDL-treated VSMCs (Zhao et al., 2020). This metabolic shift was evidenced by increased lactate and ATP production, while mitochondrial OXPHOS remained unchanged (Zhao et al., 2020).

7.1.2 Pentose phosphate pathway

The PPP plays a critical role in maintaining the redox balance and supporting biosynthetic processes through the generation of NADPH, essential for glutathione reduction and counteracting oxidative stress. Accordingly, PPP activation with increased G6PD activity and NADPH production has been linked with increased VSMC viability (Dong et al., 2015) (Figure 4A).

7.1.3 Fatty acid metabolism

Saturated free fatty acids, such as palmitic acid, have been shown to trigger pro-inflammatory responses in a multitude of cell types (Soppert et al., 2020; Noels et al., 2021), including VSMCs (Ma et al., 2011; Quan et al., 2014). Furthermore, administering the monounsaturated free fatty acid oleic acid to VSMCs *in vitro* or elevating its plasma levels upon feeding mice with a diet enriched in oleic acid stimulated VSMC foam cell formation through CD36 and the development of atherosclerotic lesions (Ma et al., 2011). Oleic acid could also trigger SMC proliferation via the free fatty acid receptor 1 (FFAR1) and signaling through PI3K/AKT and MEK/ERK (Matoba et al., 2018).

In relation to energy production, VSMCs can use fatty acid for ATP production, as shown by incubating VSMCs with octanoate in glucose-free medium, which increased oxygen consumption and could cover for the reduction in glucose-dependent ATP production (Barron et al., 1994). FAO was also reported to be increased upon PDGF-BB treatment of VSMCs with a concomitant downregulation of glycolysis (Salabei and Hill, 2013), the latter however in contrast to other studies (Werle et al., 2005).

7.1.4 Amino acid metabolism

Arginine, homocysteine, glutamine, and tryptophan contribute to phenotypic changes of VSMCs.

7.1.4.1 Arginine

Plaque stability and VSMC phenotypic regulation are critically dependent on arginine metabolism. NO can be produced by arginine metabolism (Zhao et al., 2015). While EC-derived NO stimulates vasodilation through cyclic GMP-mediated activation of soluble guanylate cyclase in VSMCs, NO produced by VSMCs functions as a free radical that inhibits proliferation and migration, and induces apoptosis (Ignarro et al., 2001; Tzeng et al., 2017). Furthermore, arginine is converted by ARG1 to ornithine, which is subsequently converted to the polyamines putrescine, spermidine and spermine. Inhibiting ARG1 impeded VSMC proliferation (Ignarro et al., 2001). In line, ARG1 stimulated VSMC proliferation, reduced the expression of inflammatory cytokines in macrophages and improved the stability of atherosclerotic plaque *in vivo* (Wang et al., 2014). Also, treatment with spermidine mitigated necrotic core expansion by stimulating VSMC autophagy and preventing lipid accumulation by triggering cholesterol efflux from VSMCs (Michiels et al., 2016).

7.1.4.2 Homocysteine

Homocysteine triggers VSMC proliferation (Tsai et al., 1994) and VSMC phenotypic switching to a synthetic phenotype via endothelin-1 signaling (Chen et al., 2020). In line, treating patients with folate to reduce homocysteine levels dramatically lowered the rate of restenosis following coronary angioplasty (Schnyder et al., 2001), which is driven by injury-induced VSMC proliferation. Also, homocysteine triggered ROS production in VSMCs (Chang et al., 2004).

7.1.4.3 Glutamine

Blocking glutamine transport and thereby glutamine metabolism inhibited serum- and PDGF-BB-induced VSMC proliferation and migration *in vitro*, as well as injury-induced neointima formation in mice (Park et al., 2021).

7.1.4.4 Tryptophan

In VSMCs, the specific deletion of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1) increased the expression of runt-related transcription factor 2 (RUNX2) and exacerbated vascular calcification, which could be reversed by administering the IDO1 product kynurenine (Ouyang et al., 2022). Also, by activating the aryl hydrocarbon receptor, kynurenine limited the transition of VSMCs to chondrocytes and maintained the integrity of the fibrous cap in atherosclerotic lesions (Kim et al., 2020). The tryptophan metabolite 3-hydroxyanthranilic acid can activate NF-κB and increase MMP2 expression in VSMCs (Wang et al., 2017).

7.2 Associated effects of atherosclerosis comorbidities on VSMC metabolism

Comorbidities as hypertension and T2DM are also associated with an altered VSMC phenotype and/or function.

7.2.1 Hypertension

Abnormal proliferation, migration and apoptosis of VSMCs can lead to vascular remodeling and increased peripheral resistance (Guarner-Lans et al., 2020). VSMCs of spontaneously hypertensive rats (SHR) displayed hypertrophy and produced more extracellular matrix while having reduced cell death and apoptosis rates compared to VSMCs from normotensive rats

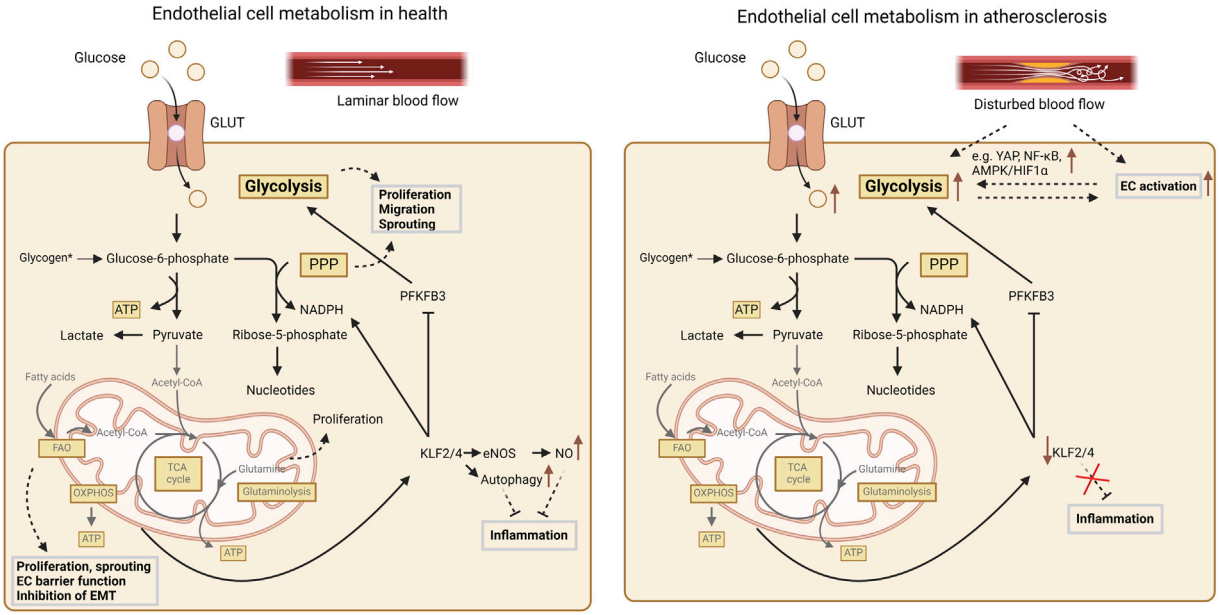

FIGURE 3
Summary of cellular metabolism in endothelial cells. In healthy conditions glycolysis accounts for ~80% of the total energy production in endothelial cells. Furthermore, glycolysis as well as the PPP support EC proliferation, migration and sprouting. Mitochondrial oxidative pathways and FAO are less important for energy production but contribute to biomass synthesis. FAO also supports endothelial proliferation, maintenance of the EC barrier function and EC sprouting and furthermore counteracts endothelial-to-mesenchymal transition (EMT). Additionally, FAO also stimulates KLF2/4 expression, which not only promotes NO production but also NADPH production, while it inhibits PFKFB3. Finally, glutamine contributes to EC proliferation, whereas glutamine starvation triggers endoplasmic reticulum stress and inflammation. Endothelial activation and disturbed flow – as in the context of atherosclerosis – are associated with reduced KLF2/4 expression, which coincides with increased glycolysis, which on its turn further contributes to endothelial inflammation. For more details, we refer to the text. AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CoA, acetyl-coenzyme A; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FAO, fatty acid oxidation; GLUT, glucose transporter; HIF, hypoxia inducible factor; KLF, kruppel-like factor; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; OXPHOS, oxidative phosphorylation; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PPP, pentose-phosphate pathway; TCA, tricarboxylic acid cycle; YAP, yes associated protein.

(Arribas et al., 2010). Furthermore, with endothelial NO production triggering vasodilation in VSMCs, endothelial dysfunction as observed in hypertension contributes to vasoconstriction via decreased NO production (Wu Y. et al., 2021). *In vitro*, VSMCs from SHR rats also showed increased ROS production and NADPH activity along with hyperproliferation compared with normotensive rats, which could be counteracted by increasing intracellular NO levels (Sarkar et al., 2017). In terms of metabolic pathways, reduced glucose uptake and metabolism in VSMCs due to reduced vascular expression of GLUT4 may contribute to the contractile dysfunction observed in hypertension (Atkins et al., 2001).

7.2.2 T2DM

In conditions of high glucose, the pro-inflammatory cytokine IL-1β induced an increase in glucose uptake as well as glucose metabolism over the PPP via upregulated G6PD expression. This triggered enhanced NADPH oxidase activity, resulting in increased ROS production and pro-inflammatory signaling (Peiró et al., 2016). Along with ROS production and inflammatory signaling, VSMCs showed a marked increase in proliferation, migration and monocyte adhesion when exposed to high glucose levels, with a role for RAGE revealed herein (Su et al., 2019). In a mouse model of metabolic syndrome with *Ldlr*$^{-/-}$ mice on high-fat and high-sucrose diet, increased glycolysis through transgenic overexpression of GLUT1 in VSMCs – but not in myeloid cells - accelerated the development of atherosclerotic lesions and promoted monocytosis and the recruitment of pro-inflammatory Ly6C^{high} monocytes to lesions (Wall et al., 2018). Of note, this effect of VSMC-specific overexpression on atherosclerosis was not observed in mice without metabolic syndrome (Wall et al., 2018). *In vitro*, GLUT1 overexpression in VSMCs enhanced the TNFα-induced production of the pro-inflammatory cytokines TNFα and CCL2 (Wall et al., 2018).

In addition to a direct impact of high glucose levels, long-term hyperglycemia conditions can also lead to the accumulation of AGEs, which can stimulate the migration and proliferation of VSMCs as well as the production of extracellular matrix (Hwang et al., 2018). During VSMC calcification, AGEs reduced glycolysis and lactate production, the PPP as well as mitochondrial respiratory capacity, resulting in reduced glucose consumption. In parallel, AGEs increased vascular calcification via HIF-1α and pyruvate dehydrogenase kinase 4 (Zhu et al., 2018).

8 Metabolic alterations in lymphocytes in the context of inflammation and atherosclerosis

Also, lymphocytes impact on atherosclerosis. T helper 1 cells are pro-atherogenic, in contrast to regulatory T-cells (Tregs) exerting an atheroprotective function (Saigusa et al., 2020). B-cells can be either

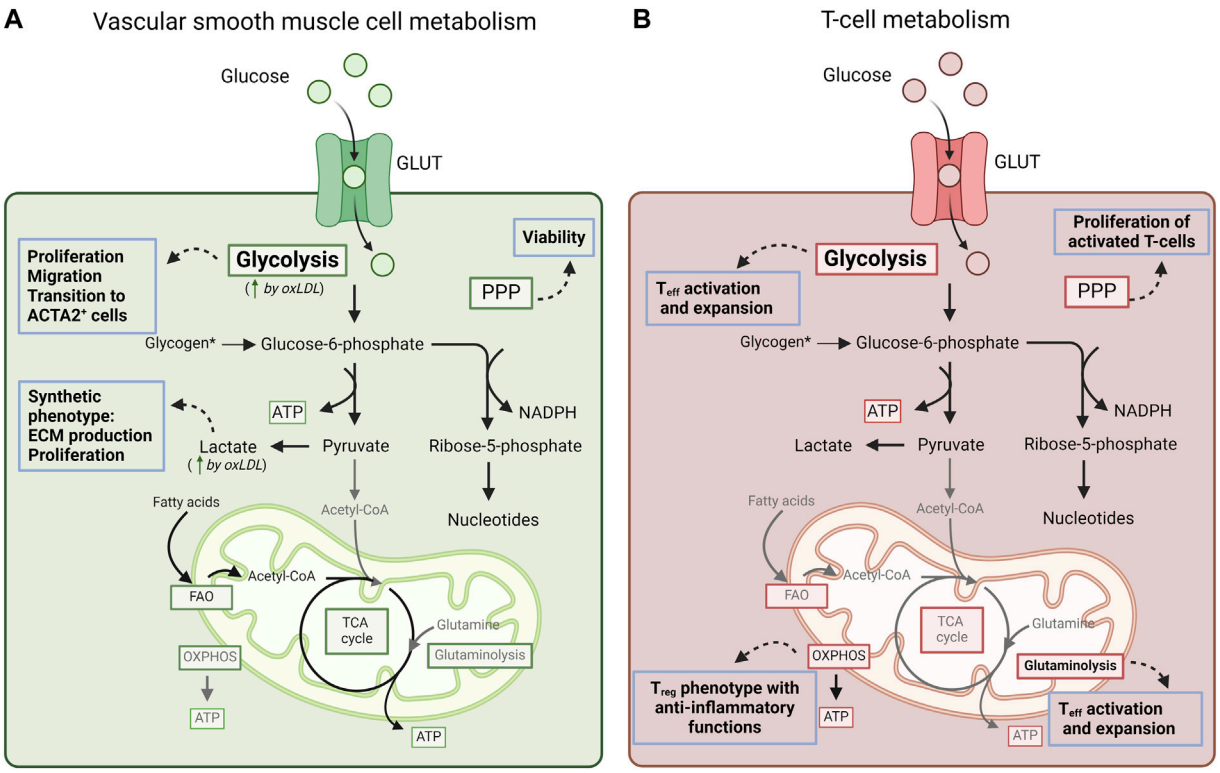

FIGURE 4
Summary of cellular metabolism in vascular smooth muscle cells and T-lymphocytes. **(A)** Overall, 90% of the glucose taken up by VSMCs is converted to lactate by aerobic glycolysis. Glycolysis supports VSMC proliferation and migration, and triggers the transition of VSMCs to ACTA2$^+$ myofibroblast-like cells. Increased lactate levels support a more synthetic phenotype of VSMCs with increased extracellular matrix production and VSMC proliferation. OxLDL increases glycolysis rates and lactate production. Furthermore, PPP activation has been linked with increased VSMC viability. VSMCs can also use fatty acids for ATP production as shown in glucose-free medium. Overall, the impact of metabolic pathway alterations in VSMCs on atherosclerosis outcome remains to be further investigated. **(B)** Activation of effector T-cells (T_{eff}) increases glycolysis and glutaminolysis, which both support lipid biosynthesis (and thus the possibility of cell expansion) via the production of citrate. Also, the PPP supports the proliferation of activated T-cells by providing precursors and NADPH for the biosynthesis of nucleotides, amino acids and fatty acids. Instead, regulatory T-cells (T_{reg}) depend on OXPHOS-dependent metabolism to exert their anti-inflammatory function. ACTA2, Actin alpha 2, smooth muscle; ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; OXPHOS, oxidative phosphorylation; PPP, pentose-phosphate pathway; VSMC, vascular smooth muscle cells.

pro- or anti-atherosclerotic depending on the subset they belong to and their effector response involving antibody production (atheroprotective IgM vs. pro-atherogenic IgG and IgE) and cytokine production (Sage et al., 2019).

Cell metabolism of T- and B-lymphocytes has been studied intensively in the context of inflammation and auto-immune diseases (van der Windt and Pearce, 2012; Rhoads et al., 2017). Auto-immune responses also underlie lymphocyte responses in the context of atherosclerosis (Wolf et al., 2020; Wang et al., 2023) (Figure 4B). Lymphocyte activation triggers a shift to anabolic metabolism with increased glycolysis and glutaminolysis dependent on mTOR signaling, as shown for activated B-cells and effector T-cells (van der Windt and Pearce, 2012; Rhoads et al., 2017). Both metabolic pathways support lipid biosynthesis via the production of citrate, which in the cytosol can be converted to acetyl-coA as basis for lipid synthesis (van der Windt and Pearce, 2012). Also, the PPP supports the proliferation of activated T-cells by providing precursors for nucleotides and amino acids as well as generating NADPH to support lipid and nucleotide biosynthesis (van der Windt and Pearce, 2012). Memory lymphocytes convert back to catabolic cell metabolism with increased fatty acid and pyruvate oxidation via OXPHOS, although with enhanced metabolic flexibility to ensure fast secondary responses (Rhoads et al., 2017). Furthermore, Tregs depend on OXPHOS-dependent metabolism to exert their anti-inflammatory function (Rhoads et al., 2017). In line with these findings, nutrient alterations impact on lymphocyte differentiation and responses. For example, glucose restriction reduces effector T-cell responses and supports a differentiation towards Tregs (Kedia-Mehta and Finlay, 2019). Also, reduced glutamine availability reduces mTOR signaling and thereby shifts CD4$^+$ T-cells towards a Treg phenotype (Klysz et al., 2015). For a more in-depth discussion of cell metabolism in T-lymphocytes in the context of atherosclerosis, we refer to another review (Stroope et al., 2024).

9 Conclusions

In summary, in the context of atherosclerosis, hypoxia and inflammatory triggers generally stimulate glycolysis and the PPP that govern the pro-inflammatory macrophage phenotype. Fatty acid synthesis and many amino acids generally also stimulate a pro-inflammatory phenotype. Macrophages stimulated with IL4/

IL13 – acquiring an anti-inflammatory phenotype - downregulate glycolysis and the PPP, while upregulating OXPHOS and FAO. Immature neutrophils mostly rely on OXPHOS and FAO, while mature neutrophils and those forming NETs mainly depend on glycolysis and the PPP to generate NADPH and exert their function. ECs mainly depend on glycolysis and the PPP, both supporting EC proliferation and sprouting. However, ECs can also use FAO, which supports cell proliferation and counteracts EndMT. Endothelial activation and disturbed flow – as in the context of atherosclerosis – are associated with increased glycolysis, which on its turn further contributes to endothelial inflammation. In VSMCs, increased glycolysis supports cell proliferation as well as the transition to a synthetic phenotype, while the PPP has been linked with increased VSMC viability. Finally, B- and T-lymphocytes upregulate glycolysis and glutaminolysis upon activation to support cell proliferation and cellular effector functions, additionally supported by the PPP. Instead, Tregs depend on OXPHOS-dependent metabolism to exert their anti-inflammatory function.

Thus, in most cell types associated with atherosclerosis including macrophages, neutrophils, ECs and T-lymphocytes, pro-inflammatory conditions are associated with increased glycolysis and – for macrophages and neutrophils – an increase in the PPP. Instead, in VSMCs, additional studies directly addressing cellular metabolism alterations and the direct impact on atherosclerosis are required.

Cardiovascular risk factors as obesity, T2DM and hypertension may impact on cellular metabolism and thereby on inflammatory processes and cardiovascular risk. Obesity, T2DM and hypertension trigger a pro-inflammatory phenotype in macrophages as well as endothelial cell dysfunction, and also ROS generation and NET formation are dysregulated in obesity and T2DM. However, the relation with cellular metabolism changes in cardiovascular comorbidities and risk factors remains to be further elucidated.

Of note, most observations in this review have been derived from *in vitro* experiments. Instead, relatively few studies addressed the *in vivo* impact of altering cell metabolism, and it needs to be taken into account that cell-specific interventions *in vivo* did not always show similar changes in cell state or function. This could be the result of a change in cell metabolism of one cell type that will alter substrate availability for other cells, and hence affect the overall microenvironment. Also, metabolites of cellular metabolism contribute to cellular crosstalk, which impacts disease development and progression. Such cellular crosstalk further complicates unraveling the impact of cell metabolism changes on inflammation and atherosclerosis. Additional complexity is added by the observation that specific metabolic pathways can have both pro- and anti-inflammatory effects even in the same cell type (for example glycolysis driving the macrophage M1 phenotype but also being involved in efferocytosis) or may have differential effects in terms of inflammatory outcome in different cell types. Thus, although interventions in these pathways may seem an attractive therapeutic strategy, the risk of adverse effects of systemic and cell type-specific effects cannot be ignored. Therefore, the impact of cell type-specific changes in metabolism needs to be explored more widely *in vivo*, with special attention to systemic effects. By the time that cell type-specific treatments are achievable by delivering therapeutics in a directed way through nanoparticle-based strategies, this information will be crucial to decide on the most effective metabolic intervention to ameliorate atherosclerosis and its interplay with metabolic co-morbidities.

Author contributions

YD: Investigation, Writing–original draft, Writing–review and editing. CJ: Investigation, Writing–original draft, Writing–review and editing. LS: Investigation, Writing–original draft, Writing–review and editing. JW: Visualization, Writing–review and editing. JS: Writing–review and editing. EV: Funding acquisition, Investigation, Writing–original draft, Writing–review and editing. HN: Conceptualization, Funding acquisition, Investigation, Supervision, Writing–review and editing.

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Conflict of interest

Author HN is founding shareholder of AMICARE Development GmbH.

The other 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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Integrated cellular 4D-TIMS lipidomics and transcriptomics for characterization of anti-inflammatory and anti-atherosclerotic phenotype of MyD88-KO macrophages

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Introduction: Recent progress in cell isolation technologies and high-end omic technologies has allowed investigation of single cell sets across multiple omic domains and a thorough exploration of cellular function and various functional stages. While most multi-omic studies focused on dual RNA and protein analysis of single cell population, it is crucial to include lipid and metabolite profiling to comprehensively elucidate molecular mechanisms and pathways governing cell function, as well as phenotype at different functional stages.

Methods: To address this gap, a cellular lipidomics and transcriptomics phenotyping approach employing simultaneous extraction of lipids, metabolites, and RNA from single cell populations combined with untargeted cellular 4 dimensional (4D)-lipidomics profiling along with RNA sequencing was developed to enable comprehensive multi-omic molecular profiling from the lowest possible number of cells. Reference cell models were utilized to determine the minimum number of cells required for this multi-omics analysis. To demonstrate the feasibility of higher resolution cellular multi-omics in early-stage identification of cellular phenotype changes in pathological and physiological conditions we implemented this approach for phenotyping of macrophages in two different activation stages: MyD88-knockout macrophages as a cellular model for atherosclerosis protection, and wild type macrophages.

Results and Discussion: This multi-omic study enabled the determination of the lipid content remodeling in macrophages with anti-inflammatory and atherosclerotic protective function acquired by MyD88-KO, hence expedites the understanding of the molecular mechanisms behind immune cells effector functionality and of possible molecular targets for therapeutic intervention. An enriched functional role of phosphatidylcholine and plasmenyl/plasmalogens was shown here to accompany genetic changes underlying macrophages acquisition of anti-inflammatory function, finding that can serve as reference for macrophages reprogramming studies and for general immune and inflammation response to diseases.

KEYWORDS

4D-lipidomics, cell lipidomics, cell transcriptomics, multi-omics, ether lipids, MyD88, atherosclerosis

1 Introduction

As minimal functional and structural units organized by complex hierarchical interactions in multicellular organisms cells and cell's functionalities are defined by the overlapping role of genome, epigenome, transcriptome, proteome, and metabolome (Vandereyken et al., 2023). Additionally, cells are embedded in an environment led by autocrine, paracrine and endocrine factors that act through ligand-receptor and other interactions to create networks among cell populations. New findings indicate that individual cells display different metabolic characteristics within a clonal population (Yuan et al., 2017). Thereby, the study of the different omics layers contributing to the phenotype and function of cells, at high cellular population resolution, i.e., single-to-low cell numbers, are imperative to address complex questions in translational biology and to understand the molecular circuits underlying cell function. For instance, the holistic view of single cell multi-omic studies can constitute a powerful tool in systems biology, by correlating specific molecular information from single cells to cell populations, tissues, or even organisms. Multi-extraction protocols are becoming increasingly important for therapeutic target discovery due to their ability to capture a wider range of biomolecules. These protocols employ a combination of techniques to maximize the diversity of biomolecules extracted from a single sample. Biological processes rarely operate in isolation, often involving intricate interactions between various biomolecules, including DNA, RNA, proteins, lipids, and metabolites. Multi-extraction protocols allow researchers to study the correlations, networks, and pathways that are dysregulated in disease, in a more holistic manner. Additionally, the broader molecular coverage offered by these protocols increases the chances of discovering novel biomarkers. Multi-extraction protocols also open the door to personalised medicine, as the understanding of an individual's unique molecular makeup, such as variations in their genes, proteins, and metabolites, can enable treatments tailored to their specific needs, maximizing efficacy and minimizing adverse effects (Boggi et al., 2024). Previously, our lab developed an integrated lipidomics and transcriptomics strategy for low tissue amounts and successfully applied it for investigation of lipidome and transcriptome of functional brain regions and subregions. The dual lipid/RNA extraction was combined with targeted mass spectrometry analysis and qPCR to enable sensitive quantitative profiling of specific lipid and RNA targets relevant to epileptic seizures in mouse models (Lerner et al., 2018; Lerner et al., 2019; Post et al., 2022).

The latest advances in cell isolation together with high-end analytical techniques and new computational tools for data processing and integration, allow multi-omic investigations for molecular profiling of single cells and also the collection and analysis of large-scale data from different omics (Zhu et al., 2020; Watson et al., 2022). Building on this, single cell studies comprising multiple layers of molecular information, unravel cell-to-cell heterogeneity and stochasticity, whereas during bulk cellular analysis, variability is eliminated by averaging, masking molecular signatures of individual cells and leading to biased conclusions. Primary emphasis of single cell multi-omic analysis lies on the integration of DNA and RNA or DNA/RNA and protein data, allowing for disease subtyping based on DNA and/or RNA data, and a more detailed molecular insight provided by the proteomic dimension. Unlike proteome, genome, or transcriptome, which are also governed by regulatory mechanisms involving post-translational modifications of proteins and epigenetic regulation, the capture of cell dynamics, real-time biochemical depiction, and ultimate downstream biochemical products essential for phenotype association can only be accomplished through the integration of metabolomics and lipidomics. This is because, lipidome and metabolome composition varies substantially during various cellular differentiation, proliferation, and reprogramming states and across cell populations. (Lee et al., 2020). Multi-omics analyses at the cellular level are crucial for elucidating the complexities of cellular biology. For instance, in-depth molecular characterization enabled by cellular multi-omics helps define the unique properties of each cellular population, including surface markers, gene expression patterns, protein abundance, and metabolic activity, as well as their functional diversity. This knowledge is essential for advancing our understanding of development, disease, and personalized medicine (Liang et al., 2024). Hence, there is a pressing need for the combined analysis of transcriptomics, lipidomics and metabolomics in single cell and cell subset studies (Capolupo et al., 2022).

Increasing evidence suggests that abnormal cellular metabolism, including lipid dysfunctions, of immune and non-immune cells is connected to abnormalities in the immune response. The immune system plays an important role in inflammation, which is linked to various chronic disorders such as obesity and diabetes, cardiovascular diseases, cancer, neurodegenerative and metabolic diseases (Zhang et al., 2022). The same is true for pro-inflammatory signaling molecules, which interfere in lipid metabolism. Despite the evident crosstalk between lipid metabolism, inflammation and health, the molecular pathways and lipid function underlying these pathological and physiological conditions are still little-known. In this regard, higher resolution cellular multi-omics can be a promising venue to accelerate early-stage identification of cellular phenotype changes in disease conditions and the subsequent determination molecular and pathway targets for therapeutic intervention (Yu et al., 2014).

Modern mass spectrometric technologies coupled with high resolution ion mobility (IMS-MS) enable higher structural resolution and possibly sensitivity allowing in-depth molecular profiling of small biological specimens (Burnum-Johnson et al., 2019). While MS allows for the separation of ions based on their mass-to-charge (m/z), ion mobility enables gas-phase separation of ionized organic molecules by their collisional cross section (CCS), which is inherently dependent on conformation, charge, as well as mass. This extra dimension of ion separation can increase sensitivity of individual species detection and peak sampling capabilities, making IMS-MS a robust analytical tool to elucidate chemical structure and separate complex mixtures (Kanu et al., 2008). The high ion utilization efficiency of trapped ion mobility spectrometry (TIMS) along with a novel MS scan mode called parallel accumulation-serial fragmentation (PASEF) make it an enticing platform for in-depth and sensitive qualitative and quantitative molecular profiling particularly for lipidomics and proteomics. (Meier et al., 2018; Paglia et al., 2022; Bennett et al., 2023; Lerner et al., 2023; Mayer and Karl, 2023; Shapiro and Bassani-Sternberg, 2023; Merciai et al., 2024). Multi-omic analysis for disease marker

identification and better understanding of disease mechanisms has become an essential approach in biomedical research. Through multi-omic cellular approaches, researchers can elucidate the molecular pathways and regulatory networks and identify novel targets and biomarkers for the diagnosis, treatment, and prevention of different chronic diseases (Liang et al., 2024). Accordingly, we set out to develop an integrated lipidomics and transcriptomic protocol for cellular profiling, amenable for high lipidome and transcriptome coverage from cell subsets and single cell populations of low number of cells. We subsequently applied this approach for the characterization of the lipid and RNA changes and pathways associated with an anti-inflammatory and anti-atherosclerosis macrophage phenotype of MYD 88 KO.

Atherosclerosis is a chronic condition that affects the arteries and is linked to systemic inflammation. It is responsible for about fifty percent of all deaths in westernized societies. A thorough understanding of the cell-specific signalling mechanisms that mediate the inflammatory response is crucial for improving anti-inflammatory therapies and reducing mortality and morbidity (Ridker and Thomas, 2014). Atherosclerosis involves the dysregulation of macrophages due to uptake of modified lipids, formation of cholesterol crystals, and lipid and inflammatory mediators that favour foam cell formation. This also affects monocytes and leads to different states of macrophage activation with both pro- and anti-inflammatory phenotypes (Poznyak et al., 2021). MyD88 (myeloid differentiation primary response 88) is an adaptor protein that plays a significant role in initiating and amplifying the immune response in atherosclerosis by inducing signalling from multiple receptors at the plasma membrane and endosomes (Akira 2003; Podrez et al., 2002; Ishii et al., 1996; Ma et al., 2022). MyD88 signalling can trigger production of pro- or anti-inflammatory cytokines as well as the activation of other inflammatory factor such as type I IFNs, NF-kB and AP-1 through various receptors including TLRs and several cytokine receptors that are associated to the ability of macrophages to polarize toward the M1 phenotype. M1 macrophages constitute the most common cell population in lesions of patients with coronary heart disease. While this pathway was initially characterized in innate cells, it has been found that MyD88 is broadly expressed across most cell types of the immune system and cardiovascular systems, often exerting distinct roles specific to certain cell types within cardiovascular disease contexts (Blagov et al., 2023). While its role during pathogenic responses is well understood, new insights into molecular mechanisms underlying inflammatory responses in atherosclerosis are emerging, providing valuable insights for potential therapeutic targets. MyD88 knockout macrophages have demonstrated reduced plaque formation indicating their potential use for studying atherosclerosis protection. These findings highlight targeting MyD88 signalling in macrophages as a promising approach for reducing inflammation and protecting against atherosclerosis (Bayer and Alcaide, 2021). The widespread involvement of these pathways in cardiovascular endurance is the basis for future mechanistic studies which may identify MyD88 as effective target for therapeutic intervention in cardiovascular diseases (Akira, 2003). In this study we set out to characterize in-depth how the lipid composition changes and which lipid pathways are effected in macrophages upon acquiring an anti-inflammatory phenotype due to MyD88 knock-out gene. Additionally, the interplay with the transcriptome changes is expected to expand the window of understanding of the anti-atherosclerotic and anti-inflammatory function of macrophages and to serve as a reference for future studies of macrophages and immune cell reprogramming and function. Specifically, we aimed to uncover potential molecular mechanisms and fingerprints related to atherosclerosis protection of MYD 88 deficient macrophages. To this end, we developed and applied a dual extraction approach of cellular lipidome and transcriptome and combined it with high-end 4D-TIMS lipidomics and RNA sequencing in order to, for the first time due to our knowledge, comprehensively investigate the lipidome, of more than 400 lipids, and the transcriptome of MyD88-KO macrophages. The dual-extraction protocol of lipidome and transcriptome from a single cellular population of low number of cells, presented in this article, combined with the cellular 4D-TIMS lipidomics and RNA sequencing, enable uncovering of lipids, RNA, and their interplay and associations with diseases and identification of new potential disease-specific fingerprints and integrated pathways, not accessible through single-extraction methods.

2 Materials and methods

2.1 Samples

Human Embryonic Kidney (HEK) 293 cells were obtained from the Clinical Lipidomics Unit and Institute of Physiological Chemistry of the University Medical Center of Mainz, Germany. Macrophages MyD88-KO (ENH179-FP) and CT (ENH167-FP) were purchased from Kerafast (Shirley, United States). In-house existing mouse brain tissue was used as a proxy to multicellular biological sample in the first steps of 4-Dimensional (4D) trapped ion mobility mass spectrometry-(tims) development for cellular profiling and in assessment of amenability of dual lipidomics and transcriptomic extraction for subsequent unbiased lipidome analysis at high coverage (Lerner et al., 2018; Post et al., 2022).

2.2 Co-extraction and analysis of lipids, metabolites, and RNA in cells

Lipids and metabolites extraction from reference cells and tissues was carried out using a classical liquid-liquid extraction (LLE) technique, utilizing methyl tert-butyl ether (MTBE)/methanol (MeOH) (10:3; v/v) and 0.1% formic acid (FA) to separate non-polar and polar compounds into distinct phases. This extraction was used to: i) assess and tailor analytical and processing parameters for 4D-cellular lipidomics that are conducive to high coverage of lipidome in complex cellular matrices, e.g., cells and tissue sample as a proxy to multicellular biospecimens. For the latter, in-house available brain tissue was used; ii) evaluate the lipidome coverage following co-extraction of lipids and RNA and compare it with the lipidome coverage obtained after classical lipid LLE extraction, iii) establish an initial spectral library, as well as RT and CCS reference values of cellular lipidome and multicellular lipidome using brain tissue as a proxy. This cellular spectral library and annotation parameters for 4D-TIMS analysis

was curated by manual annotation of lipid identities and structures, collisional cross section values, retention times and fragmentation patterns, using MS-Dial data bases and our previously established 4D-libraries from lipid standards and plasma lipids.

Lerner et al. improved the traditional liquid-liquid extraction method of tissues by integrating RNA co-extraction using the RNeasy® Mini Kit, enabling dual extraction of lipids and RNA from the same tissue sample and subsequent analysis via a targeted lipidomic approach (Lerner et al., 2018; Post et al., 2022). Essentially, chloroform and RNeasy® Micro Kit buffer along with internal standards were added to the sample prior to extraction of RNA and subsequent MTBE-based LLE extraction of lipids. Building upon this prior dual extraction protocol, we further optimized the dual extraction protocol to achieve cell-level resolution using LLE strategy and the RNeasy® Micro Kit for RNA extraction, while also evaluating the effectiveness of the sample preparation and extraction procedure for high coverage lipidome by untargeted 4D-TIMS lipidomics. Internal standards mixture was prepared in 200 µL of MeOH and added to the cell pellet together with the extraction solvent. The cell solution is further homogenized using Precellys® (5,000 rpm, 15 s) to ensure its efficient disruption and subsequently centrifuged at maximum speed for 5 min, to separate the upper aqueous phase and the lower organic phase. Different solvent ratios were tested for 1 million HEK293 cells for the lower organic phase (A, MTBE:MeOH (10:3; v/v)/FA (0.1 M) in a proportion of 8/2; B, MTBE:MeOH (10:3; v/v)/FA (0.1 M) in a proportion of 6/4 and; C, MeOH). The lipid extract was obtained after vortexing (45 min, 4 °C) and centrifugation at (15 min, 1,300 g). Classical extraction of lipids using LLE method from HEK293 cells was used to compare the performance of dual lipidome/transcriptome extraction. For this, 1 million HEK293 cells was homogenized with Precellys® (5,000 rpm, 15 s) and centrifugated at maximum speed for 5 min, after the addition of internal standards and extraction solvent. To evaluate linearity and lowest number of cells from which lipidome and transcriptome is analyzable, 1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250, 15,625, 7,813 and 3,906 cells, respectively, were extracted and analyzed using both extraction protocols. All lipid extracts were evaporated and stored at −20°C till further analysis. The aqueous phases obtained from the dual extraction protocol were immediately processed for RNA extraction using RNeasy® Micro Kit protocol. The lipid extracts were resolubilized in MeOH/H_2O (9:1; v/v) for 4D lipidomics analysis. 4D-TIMS lipidomics and Metaboscape 2021b (Bruker, Bremen Germany) with in-house created 4D-lipid cellular library and/or Metabobase, for metabolite identification, was utilized for the subsequent lipidomic and metabolomic investigation (Figure 1).

2.2.1 Lipid deuterated and non-deuterated internal standards

Deuterated and non-deuterated internal standards (ISTDs) from Avanti Polar Lipids, Inc., USA, were used for relative quantification (see Supplementary Material).

2.2.2 Chemicals and reagents

The following LC-MS grade solvents and reagents used in analytical workflow were purchased from Merck (Germany): chloroform water, methanol (MeOH), 2-propanol, formic acid (FA), triethylamine, ammonium formate, acetonitrile (ACN) and methyl tert-butyl ether (MTBE). Absolute ethanol was purchased from Honeywell (North Carolina, United States). The RNeasy® Micro Kit was purchased from QIAGEN (Venlo, Netherlands).

2.2.3 Untargeted 4-dimensional (4D) trapped ion mobility mass spectrometry (TIMS) cellular lipidomics

For lipid separation, an Elute UHPLC system (Bruker Daltonics, Bremen, Germany) with a C18 Luna Omega column (100 Å × 2.1 mm × 1.6 µm) purchased from Phenomenex (Germany) was used to perform the reversed phase (RP) chromatographic separation of samples. The column was thermostated at 45 °C. The separation solvent and gradient system used for the lipidomic and metabolomic approach in negative and positive ion mode as well as the liquid chromatography (LC) lipidomic gradient is the same as described by Lerner et al. (2023). This gradient was run at a flow rate of 0.2 mL/min. In positive mode, the injection volume onto the column was 10 µL, whereas in negative mode, it was 20 µL. Throughout the analysis, the auto-sampler remained consistently at 4°C. The experiments were conducted in a hybrid TIMS-qToF mass spectrometer coupled to an Elute UHPLC using a Bruker Daltonics TIMS-qToF pro instrument (Bruker Daltonics, Germany) for both negative ion mode and positive ion mode. For fragmentation analysis, the scan mode was set to PASEF with the mass scan range of 100–1,350 Da for both MS and MS2 acquisition. The acquisition cycle consisted of 0.1 s with the mobility scan range of 0.55–1.87 V*s/cm2 for the positive mode and 0.55–1.86 V*s/cm2 for the negative mode. Both the TIMS and mass calibration of the instrument was carried out on a weekly basis with the following peaks from the Agilent ESI LC-MS tuning mix [m/z, 1/K0: (322.0481, 0.7318 Vs. cm−2), (622.0289, 0.9848 Vs. cm−2), (922.0097, 1.1895 Vs. cm−2), (1,221.9906, 1.3820 Vs. cm−2)] in the positive mode, and [m/z, 1/K0: (666.01879, 1.0371 Vs. cm−2), (965.9996, 1.2255 Vs. cm−2), (1,265.9809, 1.3785 Vs. cm−2)] in the negative mode. The parameters utilized in these experiments are consistent with those described by Lerner et al. (2023).

2.2.4 Transcriptomics

RNA validation and quantification was performed using a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Germany). RNA integrity number (RIN) and RNAseq were performed using Agilent 2100 Bioanalyzer System in Starseq facility at Johann-Joachim-Becher-Weg 30a (D-55099) Mainz, Germany.

2.3 Data and statistical analysis and pathway determination

Compass Hystar 6.2 direct the LC instrument, while timsControl 2 (Bruker Daltonics in Bremen, Germany) is used to control and monitors the TIMS-TOF instrument's instrumental calibration and data collection. Compass DataAnalysis and Metaboscape 2021b, both from Bremen, Germany's Bruker Daltonics, were used for processing and extraction of the lipid features, quality control assessment, annotation and curation of lipid data, respectively. Metaboscape 2021b was used for extraction of the 4 dimensional (4D) features, peak area of individual signals, lipid identification and curation, spectral library establishment. Compass DataAnalysis was used to

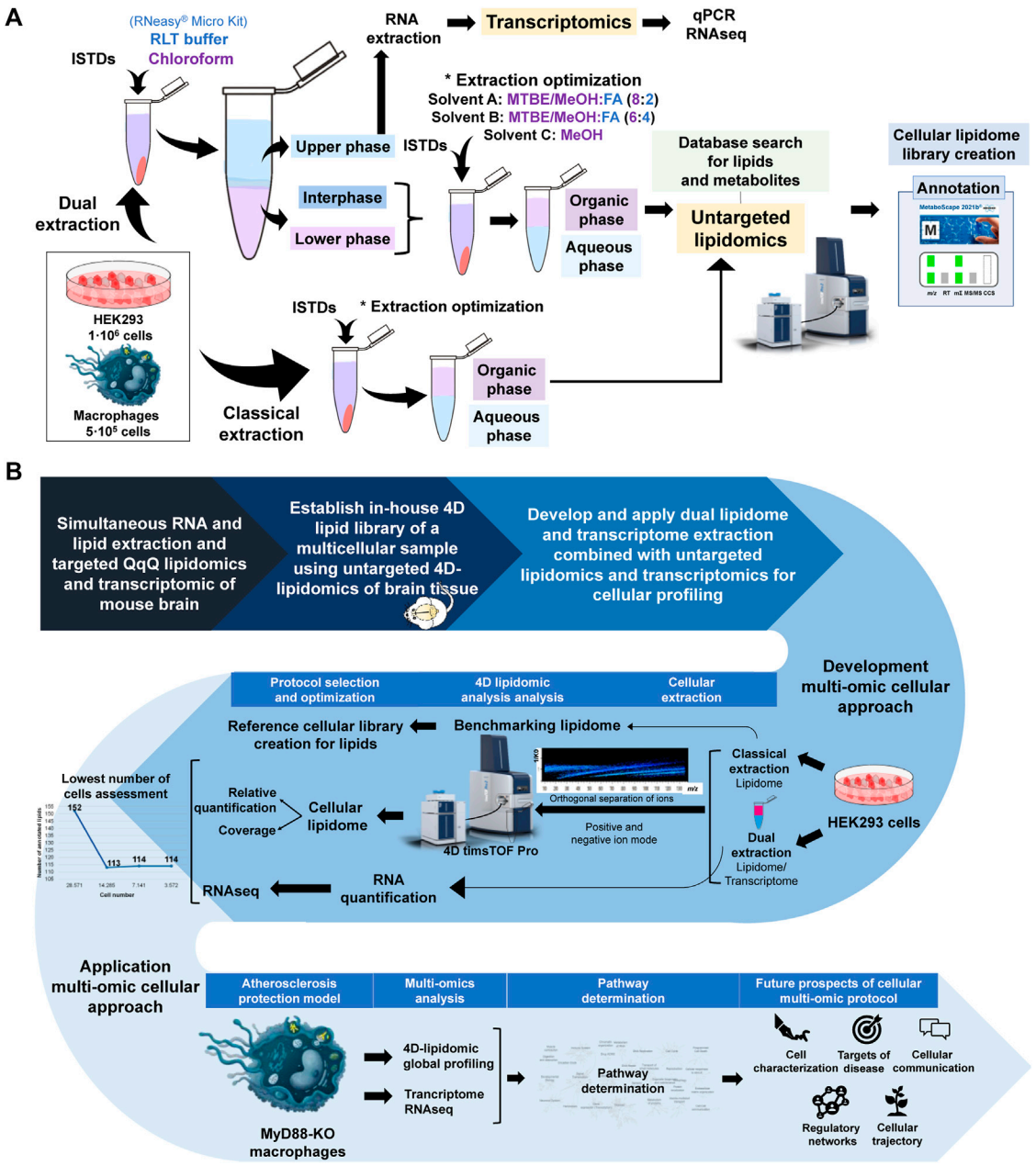


FIGURE 1
(A) Schematic of experimental workflow. UHPLC Elute LC system and TIMSTOF Pro MS are reproduced from Bruker (Bremen, Germany). **(B)** Schematic display of the rationale of study design, analytical workflow and application of the cellular lipidomics and transcriptomics approach for tha characterization of the MYD88 KO macrophages. UHPLC Elute LC system and TIMSTOF Pro MS are reproduced from Bruker (Bremen, Germany).

retrieve ion mobility and extracted ion chromatogram for lipid data curation and verification, fragmentation spectra inspection in individual samples, and general data visualisation for annotation and quality control purposes. (Lerner et al., 2023).

GraphPad Software (Boston, United States) and Origin (OriginLab Corporation, Northampton, United States) were used for statistical analysis. Lipid Pathway Enrichment Analysis (LIPEA, https://hyperlipea.org/home), created by Biomedical Cybernetics Group (Dresden, Germany) and Reactome (http://www.reactome.org), a curated and peer-reviewed resource of human biological processes, were used for pathway discovery.

3 Results

3.1 Development of dual lipidomic and transcriptomic methods for cells

3.1.1 Assessment of 4D-TIMS lipidomics profiling method for cellular lipidome and metabolome

First, we assessed the effectiveness of the untargeted 4D LC-TIMS-PASEF-MS for the comprehensive analysis of cellular lipids and metabolites. To this end, untargeted 4D-TIMS analysis of a brain tissue lipidome, extracted using classical lipid extraction was

performed to ascertain the suitability of 4D-TIMS lipidomics from a complex multicellular matrix and establish an extended panel of 4D lipid library to be used for initial annotation of cellular lipidome. Similarly, lipid extracts of 1 million HEK293 cells and subsequent 4D-TIMS-PASEF analysis was performed. HEK293 cellular lipidome was annotated using the in-house existing 4D-libraries for tissue, lipid standards, MS-DIAL, and manual annotation. (Lerner et al., 2023). Additionally, metabolites were annotated with Bruker Metabobase spectral library. These results laid the foundation for creating an initial 4D-cellular reference lipid library which will aid in identification of lipid structures in subsequent stages of development including for macrophage lipidome annotation.

This approach illustrates the potential of combining mass spectrometry and ion mobility with a UHPLC microliter flow for orthogonal separation of molecules in this cellular matrix (Figure 2A). The advantage of orthogonal lipid separation by microliter flow RP-UHPLC chromatography combined with TIMS-PASEF for cellular lipids analysis is evident in Figure 2B, where mobility-based separation of PC 17:0_14:1 and PS 16:0_18:1 isomers compensates the chromatographic separation and allows delineation of the compositional and/or possible configurational isomers. The EIM frames, as shown in Figure 2B, demonstrates how ions are separated in this additional mobility dimension, enabling discovery, separation, and identification of molecules. Accordingly, the EIM results indicate that ion mobility introduces a new separation dimension facilitating peak-based separation of isomers for both phospholipid species. The MS and MS/MS of each mobility frame are named from 1 to 3, from the lowest to the highest CCS value, respectively. The MS/MS spectra obtained for the second mobility frame in Figure 2B (left) show the diagnostic fragments corresponding to PC 17:0_14:1, whereas in Figure 2B (right) diagnostic fragments corresponding to PS 16:0_18:1, as well as the FA 18:0 corresponding to another lipid specie are detected. Although MS/MS from the precursor ions at *m/z* 762.53428 in Figure 2B were only obtained for the second mobility frame, IM demonstrates its potential in reducing background noise and revealing new characteristic CCS features of each ion, allowing thus identification and discovery of lipids that would not be possible with TIMS off. Figure 2C depicts how the values of *m/z*, RT, and CCS contribute to the spatial separation of lipids within their respective classes (Supplementary Material). It is evident that CCS plays a significant role in determining the spatial distribution of lipids, enhancing peak resolution and confidence of structural identification. Therefore, with this strategy we are able not only to enhance the characterization and differentiation of complex mixtures, but also to provide valuable insights into chemical structure and composition.

3.1.2 Optimization of dual cellular lipidome extraction in HEK293 cells

The previous assessment steps have all contributed to ascertain the advantages of 4D-TIMS- cellular lipidome for facilitating high coverage of lipidome and high-throughput profiling, which are necessary for integrating cellular multi-omic approaches. We, hence focused further on assessing, using reference HEK293 cells, the amenability of dual cellular lipidome and transcriptome extraction for subsequent comprehensive 4D-cellular lipidome profiling and RNA sequencing. Comparative coverage of the lipidome using 4D-TIMS lipidomics was performed to ascertain performance of classical and dual extraction methods for high lipidome coverage and quantification.

Lipids extracted using the previously developed dual lipidomic & transcriptomic protocol and the classical lipid extraction were analyzed in negative mode within a single batch and simultaneously processed (annotation and relative quantification) in Metaboscape to better compare the lipidome coverage using both methods. While 7 lipids were identified specifically using the classical extraction and 1 lipid was present only when the dual extraction was applied, 151 lipids were commonly identified with both extraction methods (Figure 3). The metabolite interrogation using Metabobase annotation in both extracts demonstrates the superior effectiveness of the dual extraction in profiling the metabolome of this cell model. Although only 15 metabolites were detected using dual lipidomic & transcriptomic extraction compared to 6 metabolites using the classical extraction (Figure 3) it suggests that prospective use of an appropriate metabolomic platform can allow the co-investigation of low mass metabolites and provision of additional valuable information on metabolome. Based on the results obtained for the 4D-lipidome of HEK293 cells, two spectral libraries were created for positive and negative ion modes, named: “Multi-omic-derived cellular Lipidome 2023 pos” and “Multi-omic-derived cellular Lipidome 2023 neg”, respectively.

The comparison of lipid levels between the classical and dual extraction methods in negative ion mode (Figure 3) evidence that the dual extraction method yields higher lipid levels for certain lipid classes compared to the classical method Specifically, LPE, PS, and PS-O exhibit higher quantification values when the dual extraction strategy is employed. On the other hand, the classical extraction strategy results in higher levels of the PE-O/P lipid class compared to the dual extraction and PE, PG, and PI are equally represented in both extraction methods. Considering the future prospects of this method in single cell multi-omics, where sensitivity holds significant value, the dual extraction procedure was selected as the preferred approach for further analysis in cells. The ability to enable simultaneous analysis of RNA, lipids, and metabolites from the same sample is particularly advantageous in cellular multi-omics research, where limited sample availability and low volumes of cell suspensions or homogenates are frequently encountered and not readily aliquotable for individual omic extractions. Moreover, dual extraction demonstrated comparable lipid coverage and superior metabolite coverage and lipid quantification values for most lipid classes. Aiming at further identifying the optimal solvent composition and solvent ratios for qualifying and quantifying lipids and metabolites in cells using dual extraction, two experiments were conducted in parallel using the classical and dual extraction protocols, respectively. For each extraction, three distinct solvents were employed for 1 million HEK293 cells. The lipid extracts were analyzed using 4D-TIMS untargeted lipidomics and annotated using the established extended lipidome 4D-library (see above). The primary focus was to assess the suitability of extraction procedure for 4D-TIMS cellular lipidomics and metabolomics using a population of 1 million HEK293 cells, with emphasis on lipid and metabolite coverage as well as lipid quantification. Three different extraction solvents were assessed to enhance the yield of lipidomic and metabolomic analyses. (Each extraction method is designated by a letter corresponding to the type and proportion of solvents used): A, MTBE:MeOH (10:3; v/v)/FA (0.1 M) in a proportion of 8/2; B, MTBE:MeOH (10:3; v/v)/FA

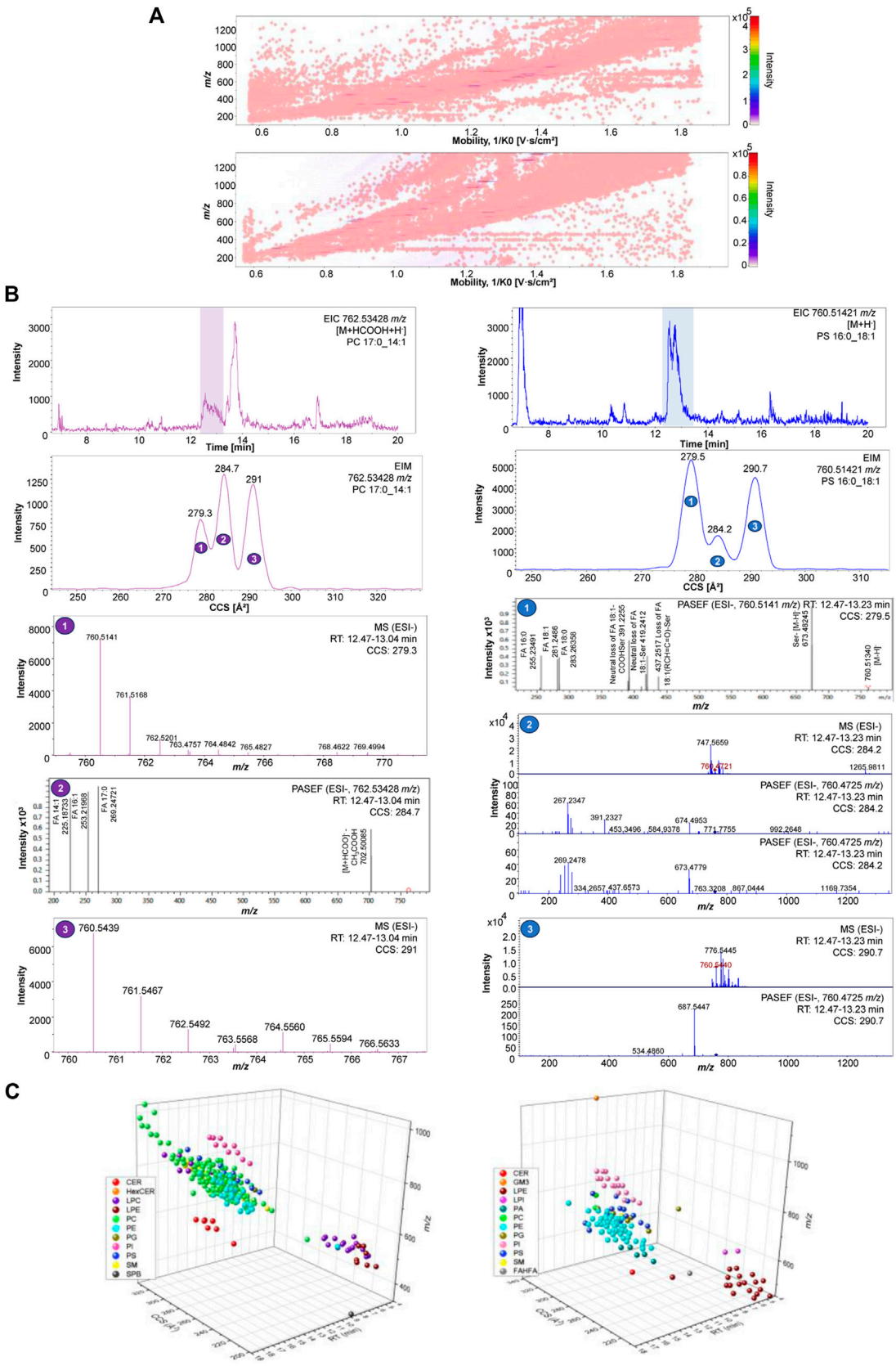


FIGURE 2
(A) 2D-hystogram of cellular lipidome distribution of raw data in 2D mobility and *m/z* dimension of ions using ESI+ (left) and ESI- (right). **(B)** PC 17:0_14:1 762.53428 *m/z* EIC, EIM, MS and MS/MS in ESI- (left); PS 16:0_18:1 760.51421 *m/z* EIC, EIM, MS and MS/MS in ESI- (right). **(C)** 3D-scatter plot cellular lipidome separation across lipid classes using using ESI+ (left) and ESI- (right).

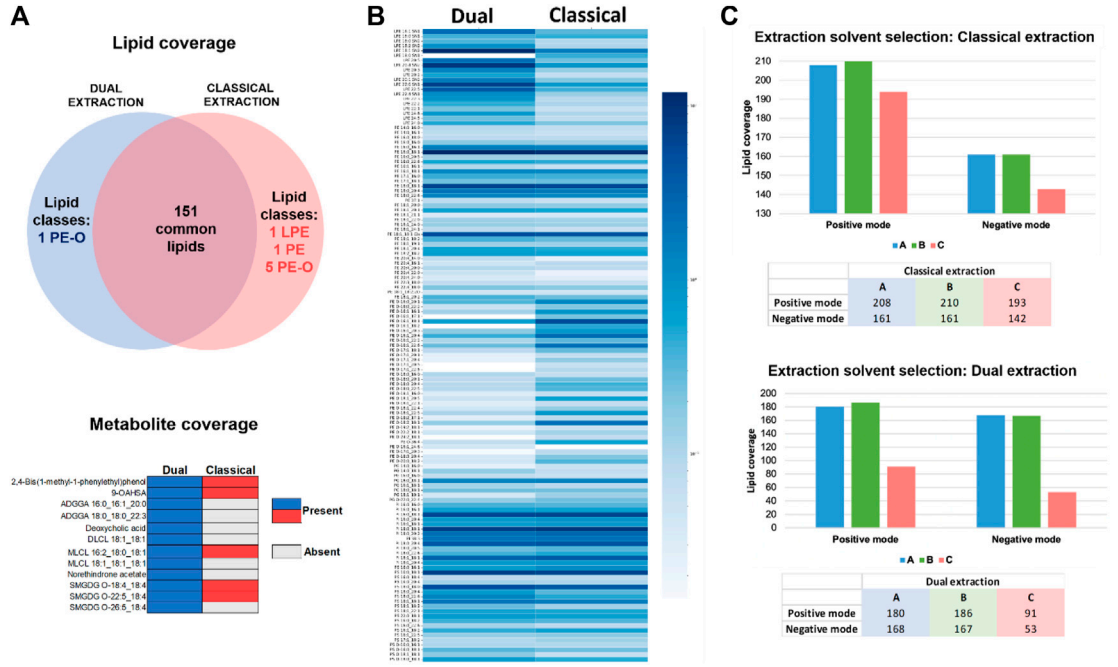


FIGURE 3
(A) Venn diagram representation of specific and common lipids to classical and dual extraction methods in ESI- (up). Metabolites presence and absence in classical versus dual extraction methods in ESI- (down). **(B)** Lipid quantification comparison of dual vs. classical method in ESI-. Log_{10} of the quantified values was used for the visualization. Color grade from dark to light blue indicating each lipid specie abundance. **(C)** Lipid coverage comparison for three different solvents using the classical extraction in ESI+ and ESI- (upper graph) and dual extraction in ESI+ and ESI- (bottom graph).

(0.1 M) in a proportion of 6/4, and; C, MeOH. First, a higher lipid coverage was obtained for solvents A and B compared to C in both negative and positive mode (Figure 3), demonstrating the superiority of MTBE:MeOH/FA over MeOH in terms of lipid coverage. Secondly, upon closer examination of the number of annotated lipids in the MTBE:MeOH/FA fractions, it is observed that B solvent yields slightly higher results in both classical and dual extraction methods (Supplementary Material).

To conclude, similar quantification results were obtained with both classical and dual extractions, demonstrating that the solvents A and B containing MTBE are better suited than methanol as they provide improved quantification values and a higher lipidome coverage, while the HEK293′ characteristic lipidome significantly fades in both experiments and ion modes when MeOH is employed as extraction solvent (Supplementary Figure S1). Hence, it is evident that MTBE-containing solvents render an acceptable lipid coverage using both classical and dual extraction strategies. Solvent B consistently outperforms A solvent in terms of quantitative coverage across various lipid classes. This is evident from the consistently improved quantification coverage of LPE-O and SM, as well as PE, PE-O, PE-P, PC-O, PI-O, and PS species.

3.1.3 Cellular lipidomics, metabolomics and transcriptomics in a low number of cells

In order to develop a sensitive cellular analytical platform using dual-omic extraction and microliter flow 4D-TIMS cellular lipidomic profiling, two experiments were conducted to achieve comprehensive lipidome profiling at high cellular resolution, i.e., in low cell numbers. In a first experiment, the organic phase previously extracted using dual extraction was serially diluted. In another experiment, the cell suspension of 1 million cells was also serially diluted for subsequent extraction using dual and classical protocols for lipid and RNA analysis. During the initial experiment, the organic phase aliquot corresponding to 28,571 cells was sequentially diluted by factors of 1/2, 1/4, and 1/8 resulting in dilutions corresponding to 14,285, 7,141, and finally down to 3,572 cells respectively. The diluted lipid extracts were analyzed in negative ion mode and annotated with the extended 4D-cellular lipidome library. Supplementary Figure S2 depicts the peak area linearity, e.g., R-squared (R^2) for different lipids across dilutions. The analysis reveals that a significant portion of the annotated lipids (78.07%) exhibit an R^2 value ranging from 0.8 to 1, evidencing the alignment of our results with a linear regression across different dilutions. Only 21.92% of the annotated lipids exhibit an R^2 below 0.8. The robustness of 4D-TIMS lipidomics and dual omic extraction for low cell numbers profiling is illustrated in Figure 4, where the lipid coverage across extracts of different cell numbers is represented. When comparing the diluted extracts, a significant decrease, i.e., from 152 to 113 in the number of identified lipids is observed between 28,571 and 14,285 cells, followed by a rather steady coverage with, i.e. 114, and 109 lipids for subsequent dilutions, respectively. It is obvious that the sensitivity of lipid detection reaches a steady threshold from about 14.000 cells downwards. This indicates, however, the robustness of the approach in terms of maintaining consistent the lipid coverage in cellular lipidomics in a low number of cells, but also the lowest threshold of cell number at which with current analytical conditions cellular lipidome can be reliably quantified. We consider, however,

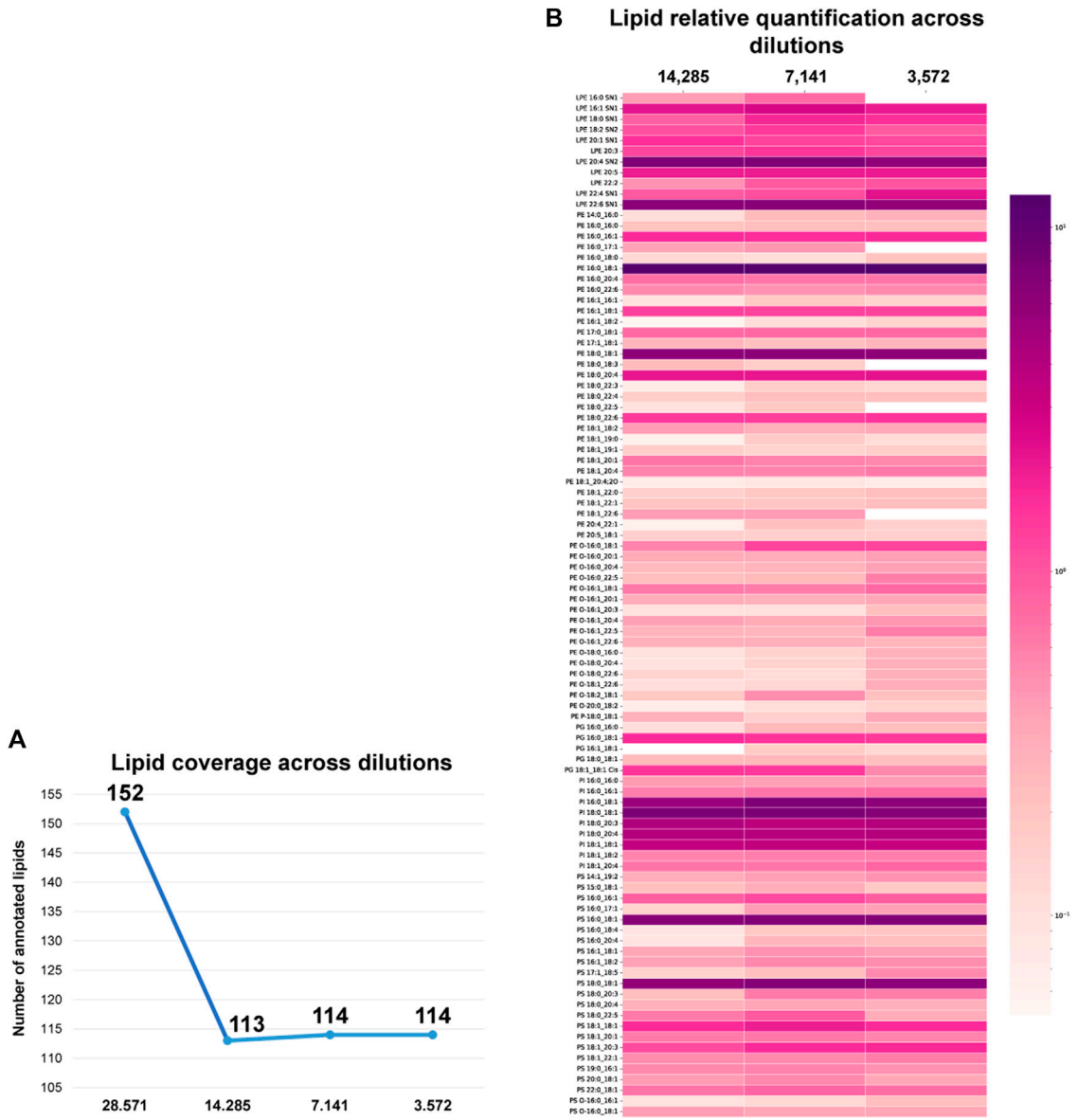


FIGURE 4
(A) Lipid coverage across organic extracts of 28,571, 14,285, 7,141 and 3,572 cells in ESI-. **(B)** Lipid quantification values across different cell numbers in negative ion mode 4D-cellular lipidomics. Log_{10} of the quantified values was used for the visualization. Color grade from dark purple to light pink indicating each lipid specie abundance.

that the 113, 114 and 109 number of identified lipids across the three lowest dilutions points in negative ion mode is sufficiently informative of researching the cellular lipidome content and function as it contains most of the representative and specific species of each lipid class. The method's quantitative performance for low cell numbers is illustrated in Figure 4 where the normalized lipid values (normalized to internal standards) across the different dilutions are compared. Remarkably, the relative quantification values remain consistent across the various cell dilutions, including the highest dilution, highlighting the method's ability to reliably quantify lipids even in limited cell amounts and for low abundant lipid species as well. Only a few lipid species are absent in the extract of 3,572 cells, indicating a good limit of detection and quantification of a specific set of lipids in samples with as low as 3,572 cells.

Finally, we applied the optimized dual extraction and lipidome analysis on 9 different cell dilutions (1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250, 15,625, 7,813 and 3,906 cells). The lipid extracts of the diluted cell samples were analyzed in negative and positive ion mode and annotated using the 4D-cellular lipidome library, MSDIAL and Bruker Metabobase spectral libraries for lipids and metabolites. Figure 5 displays the lipid coverage, data linearity and reproducibility of identified lipids extracted with optimized dual extraction and analyzed by 4D-TIMS cellular lipidomics covering 9 cell number dilutions (1,000,000, 500,000, 250,000, 125,000, 62,500, 31250,15,625, 7,813 and 3,906 cells). The graph demonstrates that this approach enables the coverage of most lipid classes even in the lower cell dilution. Exceptions make lipid species of the GM3, LPA, LPC-O, LPE-O, LPG, LPI, PG-O,

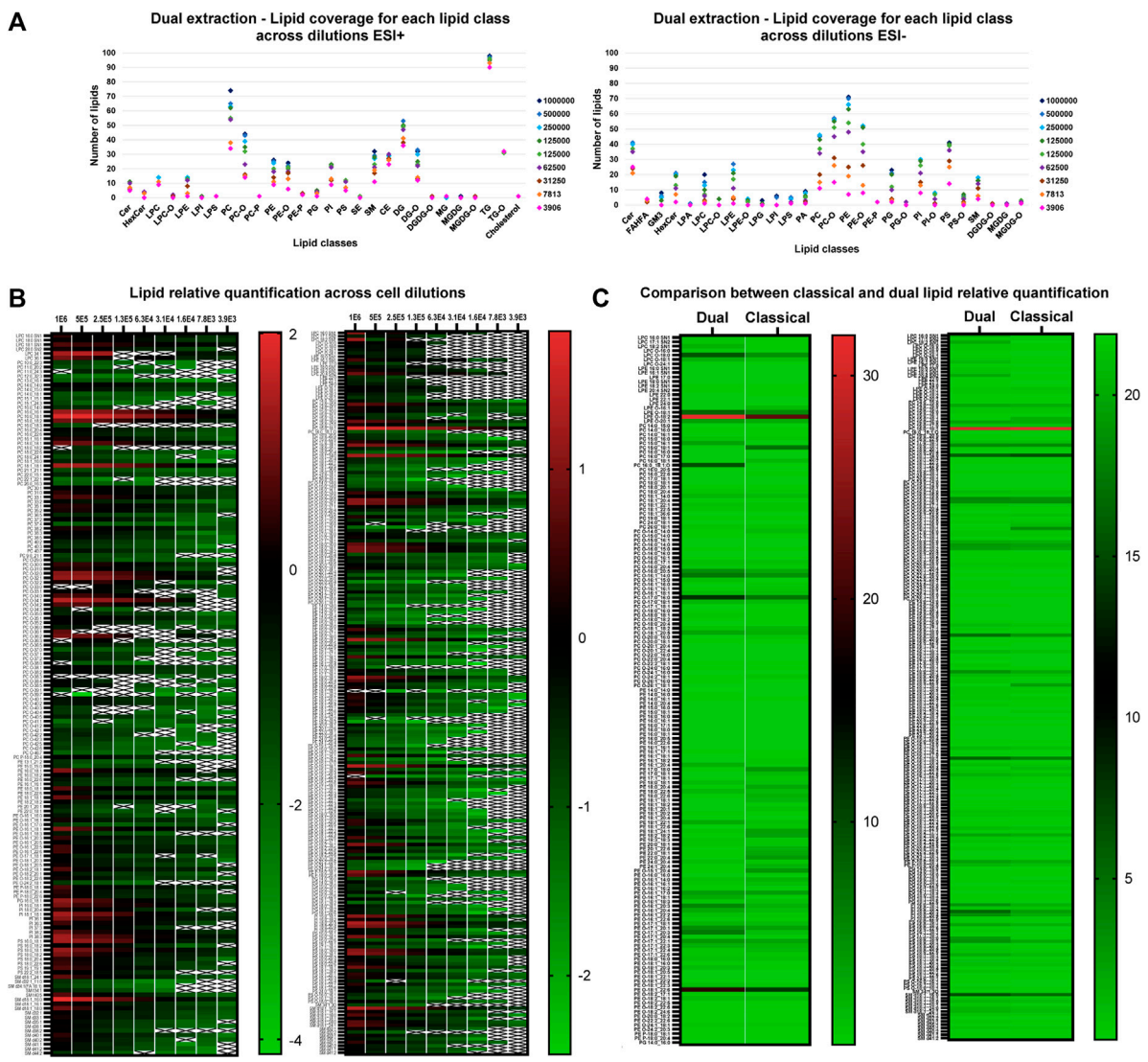


FIGURE 5
(A) Lipid coverage in positive (left) and negative ion mode (right) by 4D-TIMS lipidomics across different cell number dilutions (1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250, 15,625, 7,813 and 3,906 cells) via optimized dual lipidome and transcriptome extraction. **(B)** Lipid relative $\log_{10}$ quantification values across cell dilutions (1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250, 15,625, 7,813 and 3,906 cells in ESI+ (left) and ESI- (right) using dual extraction. With a "X" represented those samples which were not quantifiable. To simplify, TG, DG and CE were discarded from the analysis. **(C)** Lipid quantification values across cell dilutions in ESI+ (right) and ESI- (left) comparing both extraction methods. To simplify, TG, DG and CE were discarded from the analysis.

PI-O, PS-O and DGDG subclasses, which are not detectable in negative ion at the low cell number, e.g., 3,906 cells.

The comparative quantification values of lipids obtained across the various dilutions in both positive and negative ion species mode of lipids extracted with the optimized dual extraction method was carried out to determine the lower limit of quantification of lipid classes and/or species in relation to the number of cells extracted. Supplementary Material shows the relative quantification results in negative and positive ion mode using the dual extraction method across the different dilutions. The peak area linearity was calculated for both ion modes, with a R^2 value superior to 0.8 for 90.51% and 91.11% of the lipids in positive ion mode and negative mode, respectively. Conversely, less abundant lipid species maintain consistent quantification values even at the lowest dilutions. The lowest dilution point corresponds to lipid extracts from 157 cells injected on the column in negative ion mode and 79 in positive ion mode, which substantiates a good sensitivity of the overall workflow. Particularly, considering that no instrumentation specific to single-cell omics was here used approach (Gerichten et al., 2023).

In Figure 5B, the relative quantification values of 9 cell dilutions obtained from the two extraction methods are contrasted. The PE lipid class generally demonstrates higher values with classical extraction than with dual extraction method in positive ion mode, while LPCs, LPEs, and PIs are more prevalent with dual extraction compared to classical extraction in negative ion mode. However, these differences are particularly significant only for specific lipid species within these classes; overall results indicate a similar level of relative quantitation profile under these conditions.

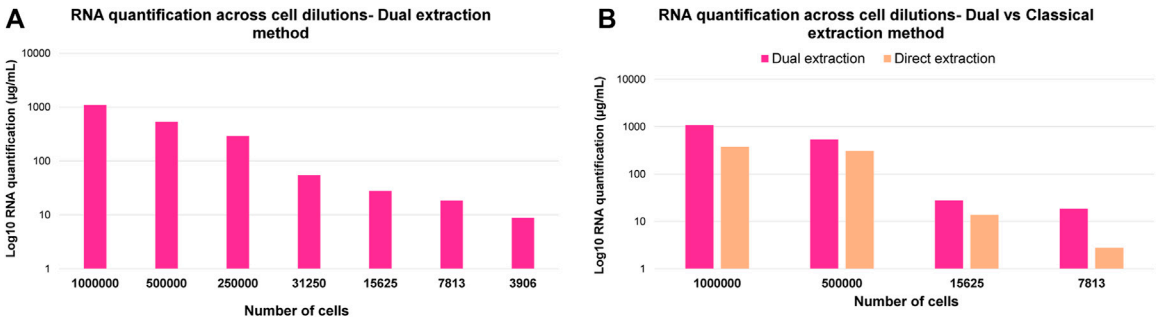


FIGURE 6
(A) Log_{10} RNA quantification results across cell dilutions corresponding to 1,000,000, 500,000, 250,000, 31,250, 15,625, 7,813 and 3,906 cells. **(B)** Comparison of Log_{10} RNA quantification results across cell dilutions corresponding to 1,000,000, 500,000, 15,625 and 7,813 cells after direct and dual extraction.

The dual extraction method appears comparable to classical extractions' quantification values and offers an additional advantage by allowing RNA extraction to add a new omic dimension.

In order to further demonstrate the suitability of this optimized dual extraction protocol for analyzing both omic layers, lipidomics and transcriptomics, RNA was extracted from the aqueous phase using RNeasy® Micro Kit protocol designed for human cells and subsequently quantified. The RNA was in parallel also extracted and quantified directly from the cell pellet to compare and benchmark the performance of dual lipidome/transcriptome extraction from cells. Figure 6A illustrates how the RNA quantification values decrease linearly across cell dilutions, while Figure 6B demonstrates a significant improvement in RNA extraction yield when using dual extraction compared to direct extraction of RNA. Additionally, it shows that this optimized method is adequate for RT-qPCR analysis with as few as 31,250 cells for 0.5 ug of RNA when utilizing the entire volume of extract. RNAseq can be performed on 7,813 cells with a total RNA of approximately 150 ng, meeting the required amount of genetic material for this analysis. Hence, these findings indicate that the optimized multi-extraction method and the selected kit are appropriate for isolating RNA, making it readily amenable for subsequent analysis using RNA-seq or qPCR, depending on the specific objectives of the study.

To conclude, satisfactory global qualitative and quantitative profiling of lipids and RNAs is obtainable from a low number of cells, using combinatorial approach including: i) co-extraction of lipidome, transcriptome and to some extent metabolome, ii) untargeted 4D-TIMS cellular lipidomic, and iii) transcriptome analysis by RNA sequencing and/or qPCR.

3.2 Characterization of MyD88-KO macrophages' phenotype for atherosclerosis protection: application of optimized multi-extraction for lipids, metabolites, and RNA, as well as 4D-TIMS cellular lipidomics and transcriptomics

In order to showcase the applicability of this cellular multi-omic platform and also to characterize the lipidome and transcriptome associated with an atherosclerosis protective phenotype of macrophages, we investigated the specific molecular changes in MYD88-KO macrophages versus control cells.

To investigate this question, wild type (WT) (C57BL/6J) and knock-out MyD88-KO (C57/129) macrophages from mice were chosen. When MyD88 is knocked out or disabled in macrophages it inhibits the typical signal transduction pathways that would lead to the pro-inflammatory M1 phenotype in macrophages. One critical factor in the initiation and progression of atherosclerosis is the release of inflammatory factors and cytokines produced by the MyD88 upon the activation of toll-like receptor 4 (TLR4). MyD88 contributes to the migration and polarization of macrophages to form M1 macrophages that will release more proinflammatory factors and hence, enhance monocyte migration and plaque formation. Therefore, the key role of MyD88 in the initiation and amplification of this cascade leading to formation and growth of the atherosclerotic plaque, makes it an excellent model to study the impact of reduced pro-inflammatory signaling on the lipidome and transcriptome of macrophages in the context of atherosclerosis. This approach would involve the extraction of lipids, RNA, and metabolites from macrophages.

3.2.1 Macrophages lipidome

This study utilized two biological replicates of MyD88-KO and WT, each consisting of 500,000 cells. To simplify, we labeled the biological replicates of wild-type (WT) and knockout (KO) macrophages as 167.1, 167.2 for WT, and 179.1, 179.2 for KO. The lipidome profiling was performed using three technical replicates for each biological group. The bucket list of lipid signals during data processing in Metaboscape was curated utilizing the previously generated HEK293 4D libraries, "Multi-omic-derived cellular Lipidome 2023 pos" and "Multi-omic-derived cellular Lipidome 2023 neg", along with other routine databases. Integration of common and specific lipids to both ionization modes in MyD88-KO and WT macrophages was performed for relative quantification. The results were visualized via principal component analysis (PCA) in Figure 7A, where each data point represents one of the technical replicates of both cell types. The loadings for each principal component analysis axis are included in Supplementary Figure S3. Biological replicates of both WT and KO macrophages shows dispersion in the PC2 space due to sample variability. The

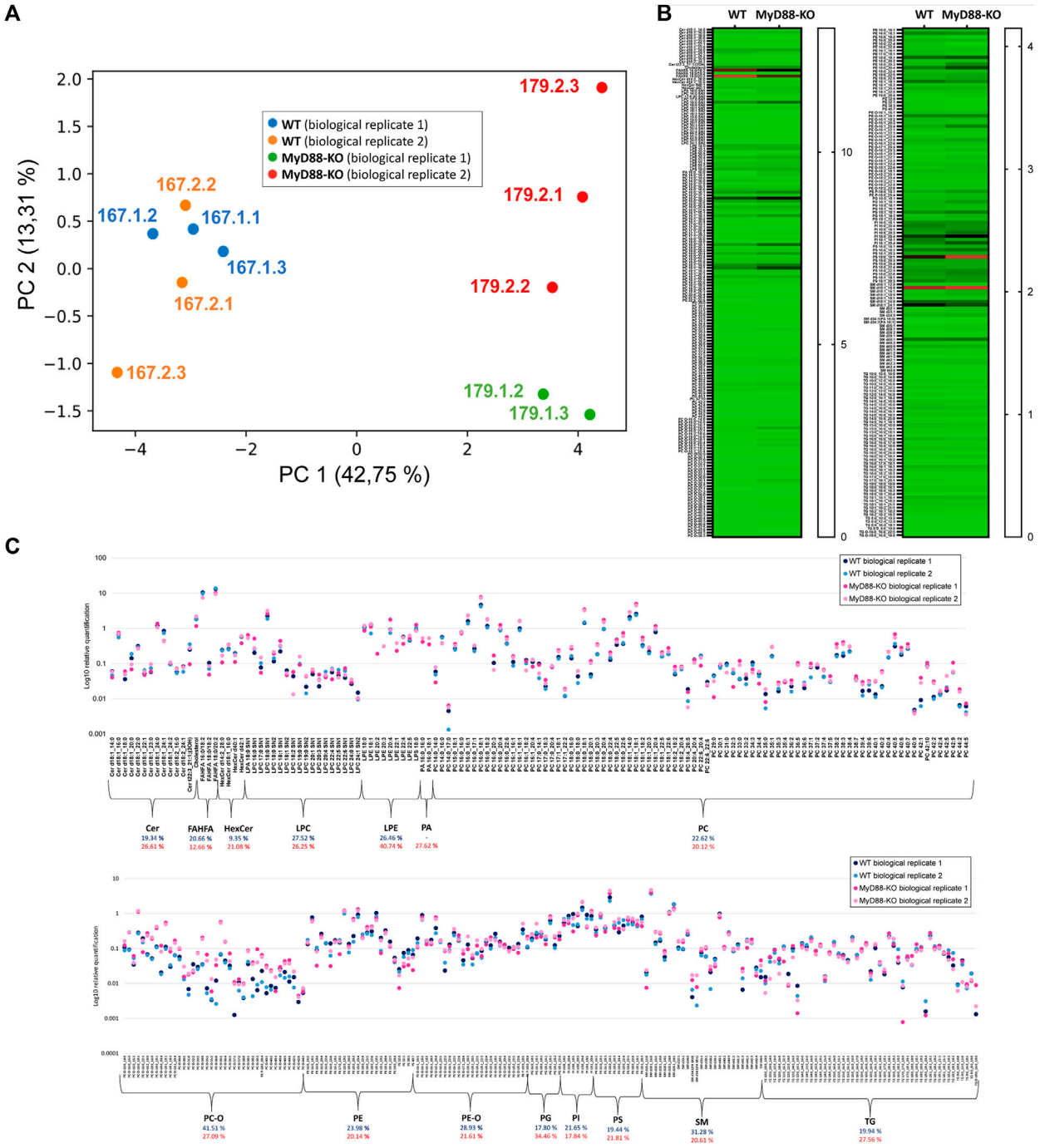
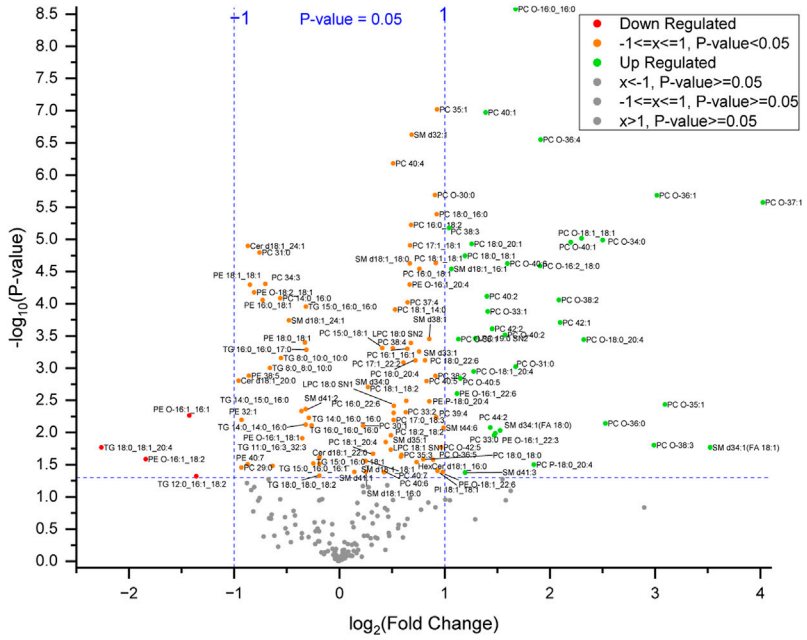


FIGURE 7
(A) PCA of all the quantified lipids using UHPLC (RP)-TIMS-TOF(ESI+) and UHPLC (RP)-TIMS-TOF(ESI-), showing the separation between MyD88-KO and WT macrophages classes. **(B)** Heatmap showing the relative quantification values for lipids comparing two macrophage models (WT and MyD88-KO). **(C)** Scatter plot depicting the logarithmic relative quantification of lipid species and coefficient of variation across lipid classes, with blue representing MyD88-KO and red representing WT macrophages.

dispersion is higher in the case of MyD88 macrophages probably due to the heterogeneity of this experimental group triggered by the inactivation of this gene. However, the two macrophage groups are fully distinguishable by their distribution and separation across PC1 space (Figure 7A). One technical replicate "179.1.1" was excluded from the analysis as an outlier due to its noticeably different distribution compared to the other technical replicates in the PCA space. The distinct lipid profile of MyD88-KO macrophages indicates the direct effect of MyD88 on their lipid composition.

The heatmap in Figures 7B, C depicts the relative quantification values of various lipid species in the KO and WT macrophages. While the relative abundance of most FAHFA species is lower in MyD88-KO compared to WT, ether-linked lipids (PC/PE-O) as well



LIPID classes	DOWN regulated	SIGNIFICANT	UP regulated
Cer		2,88	
FAHFA		1,92	
HexCer		2,88	
LPA		0,96	
LPC		2,88	2,22
LPE	33,33		
PA	33,33		
PC		40,38	20,00
PC-O		2,88	51,11
PC-P			2,22
PE		7,69	4,44
PE-O		2,88	8,89
PE-P		1,92	
PG		2,88	
PI		3,85	2,22
PS		2,88	
SM		11,54	8,89
TG	33,33	11,54	
TOTAL	100,00	100,00	100,00

FIGURE 8
Volcano plot of differentially regulated lipids and percentage contribution of each lipid class to the dysregulation of these lipids in MyD88-KO. Each point represents a lipid species plotted based on fold change and statistical significance. The *x*-axis shows the $\log_2$-fold change (FC) in lipid expression, with positive values indicating upregulation and negative values indicating downregulation. The *y*-axis represents the $-\log_{10}$(*P*-value), reflecting statistical significance. Points above the horizontal threshold line denote lipids with statistically significant changes.

as PE and PC, show remarkably higher values for MyD88-KO compared to WT macrophages. The TG species do differ between both cell types. PS, PG and PI phospholipids are also dysregulated in MyD88-KO. Since MyD88 is linked to inflammatory responses, these results underscore the role of lipid patterns in regulating inflammatory pathways associated with atherosclerosis in macrophages (Blagov et al., 2023). This demonstrates the importance of these lipids in the mechanistic pathways that can be potentially involved in regulating anti-inflammatory mechanisms in cardiovascular diseases, which are remarkably important in the formation of the atheroma plaque. The potential of these lipids as important targets for protecting against atherosclerosis, makes the study of their metabolic and mechanistic functions crucial for future research. The reproducibility of relatively quantified levels of lipids and the average coefficient of variation (CV) for each lipid class indicate a good quantitative reproducibility of the MyD88-KO lipidome phenotype. The average CV for both cell types is 21.58% and 22.87% and demonstrate a similar quantitative reproducibility among phenotypes despite the different molecular matrix of both cell types (Chiu et al., 2010). In conclusion, the multi-omic cellular approach used in this study provides a reliable and consistent lipidomic profile of macrophages anti-inflammatory state helping us to derive valuable insights into molecular mechanisms associated with MyD-88 function in macrophages.

So far, the findings suggest a clear lipid profile difference in the KO cells, potentially linked to the influence of MyD88. To investigate significantly up- or downregulated lipids (*P*-value < 0.05) in this anti-inflammatory macrophage model more closely, a volcano plot was utilized (Figure 8). The percentage contribution of each lipid class to this dysregulation is also visualized. Ether-linked lipids, PC/PE-O and PC/PE-P, constitute the most prominent group of the total upregulated lipids (62.2%) followed by PC and SM. The high representation of the ether-linked lipids and PC lipid class within the upregulated lipids in MyD88-KO macrophages makes them a potential target of the anti-inflammatory mechanism and response. In view of the cell reprogramming events, it will be interesting to explore whether these classes play an essential role in the reprogramming macrophages toward an anti-inflammatory phenotype (Cortés et al., 2023; Kelly and O'Neill, 2015; Pérez and Rius-Pérez, 2022). LPE, TG and PA lipid species contribute equally to the downregulated effect of MyD88 in the lipidome of this macrophage model.

3.2.2 Transcriptomic profiling of WT and MyD88-KO macrophages

RNA obtained via the improved dual-omics extraction technique was analyzed using RNAseq. The RIN are 2.4 for MyD88-KO and 2.3 for WT samples. To facilitate subsequent transcriptome profiling of both WT and MyD88-KO macrophages T, RNAr depletion was carried out. This approach increased specificity in capturing target mRNA molecules by reducing ribosomal RNA presence that could disrupt downstream analysis processes.

The pie chart in Figure 9A depicts 3,381 differentially expressed genes and 6,482 differentially expressed transcripts compared to the total count of expressed and tested genes with MYD-88 KO. It provides firsthand information about the extensive high number of genes and transcripts expressed and tested in MyD88, potentially involved in regulating anti-inflammatory pathways modulated by MyD88 in macrophages. Furthermore, Figure 9B shows a PCA plot

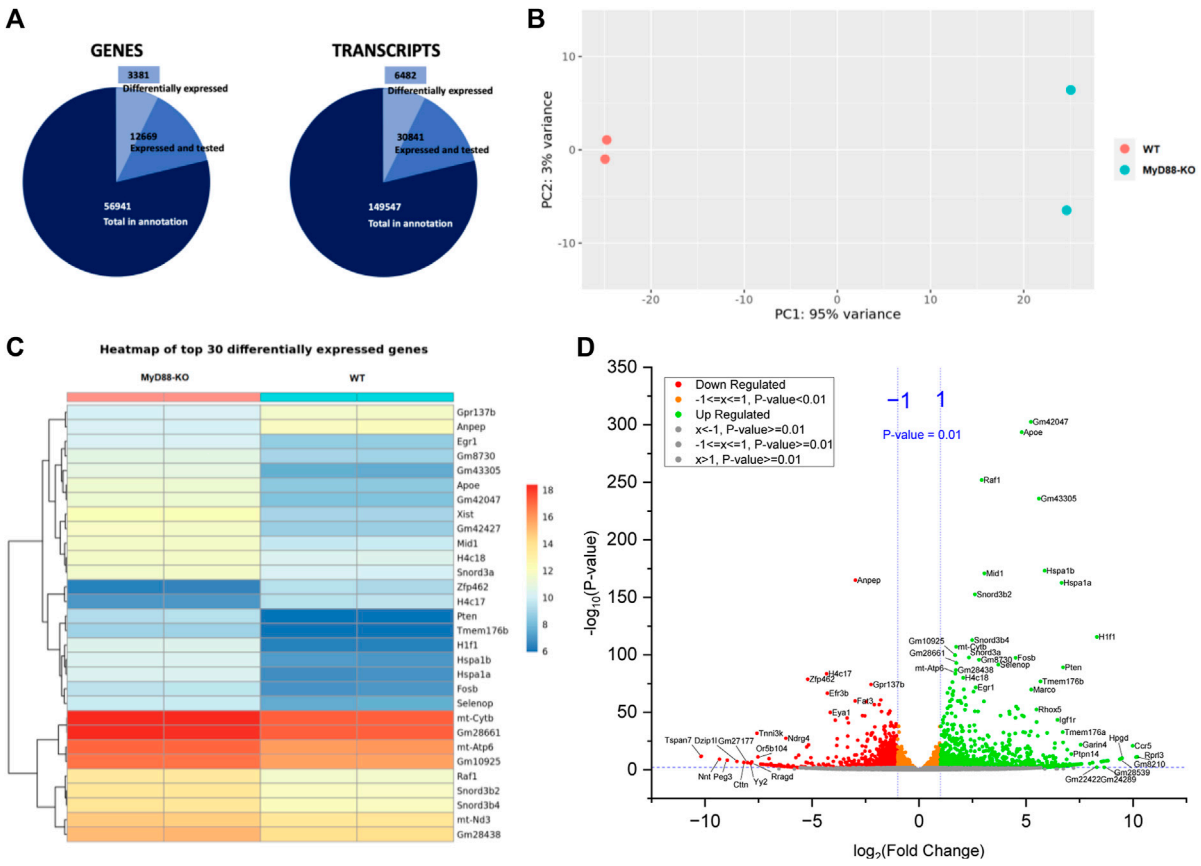


FIGURE 9
(A) Pie chart of genes selected for RNAseq analysis. **(B)** PCA plot of RNAseq sample distribution of WT and KO. **(C)** Heatmap showing the expression of the first 30 differentially expressed genes with adjusted *P*-value < 0.05 for WT and MyD88-KO macrophages. **(D)** Volcano plot of up- and downregulated genes expressed in MyD88. 0.01 was defined as *P*-value threshold as well as a FC of 2.

that illustrate distinct clustering and separation of WT and MyD88-KO macrophages based on their gene expression profiles, with technical replicates of the same cell type clustering together. This group separation fully aligns with the results showed previously in the PCA analysis of the lipidome sample distribution. The genes and transcripts differentially expressed in MyD88-KO compared to WT macrophages primarily contributing to this differentiation are depicted in the heatmap in Figure 9C of the first 30 differentially expressed genes. Selected genes with up- and downregulation trends were based on a *P*-value < 0.01 and fold change (>1 or < -1). Figure 9D illustrates the direction of regulation trends in MyD88-KO macrophages using a volcano plot, displaying the gene expression difference between MyD88-KO and WT macrophages with the significance (*P*-value < 0.01) and a log 2-fold change. The analysis also indicates distinct transcriptome profile differences in this anti-inflammatory macrophage model.

3.2.3 Pathway investigation of MyD88-KO macrophages

Significantly represented lipids and RNA from the globally profiled lipidome and transcriptome in MyD88-KO macrophages compared to WT macrophages, were investigated by Reactome pathway to unveil their mechanistic function in this anti-inflammatory response. The pathway clusters marked in yellow in Figure 10 are those affected by the dysregulation of RNA and lipids in MyD88-KO macrophages. Upon initial examination of these pathways obtained by the comprehensive analysis of the impact of MyD88 deficiency on the transcriptome, there is a significant influence on genes associated with cellular responses to stimuli and signal transduction. The metabolism of RNA, DNA repair, gene expression transcription, and programmed cell death are also impacted, as well as some disease pathways related to cellular responses to stress, DNA repair, transmembrane transporters, signal transduction by growth factor receptors and second messengers and programmed cell death (from lower to higher FDR). Both up- and downregulated lipids impact metabolism, whereby lipid metabolism is predominantly upregulated with only a few downregulated species. An in-depth analysis of the pathways affected by imbalanced lipid levels indicates that both up- and downregulated lipids impact metabolism. Additionally, vesicle-mediated transport, small molecule transport, protein metabolism, immune system function, and disease pathways are specifically affected by the upregulated lipids produced by MyD88 absence. The predominant upregulation of PC-O among the upregulated lipids, substantially influences the dysregulation of the highlighted pathways in Figure 10A, potentially involving their anti-inflammatory effects. The graph in Figure 10B depicts the 25 most significant pathways underlined by the upregulated lipids, and the

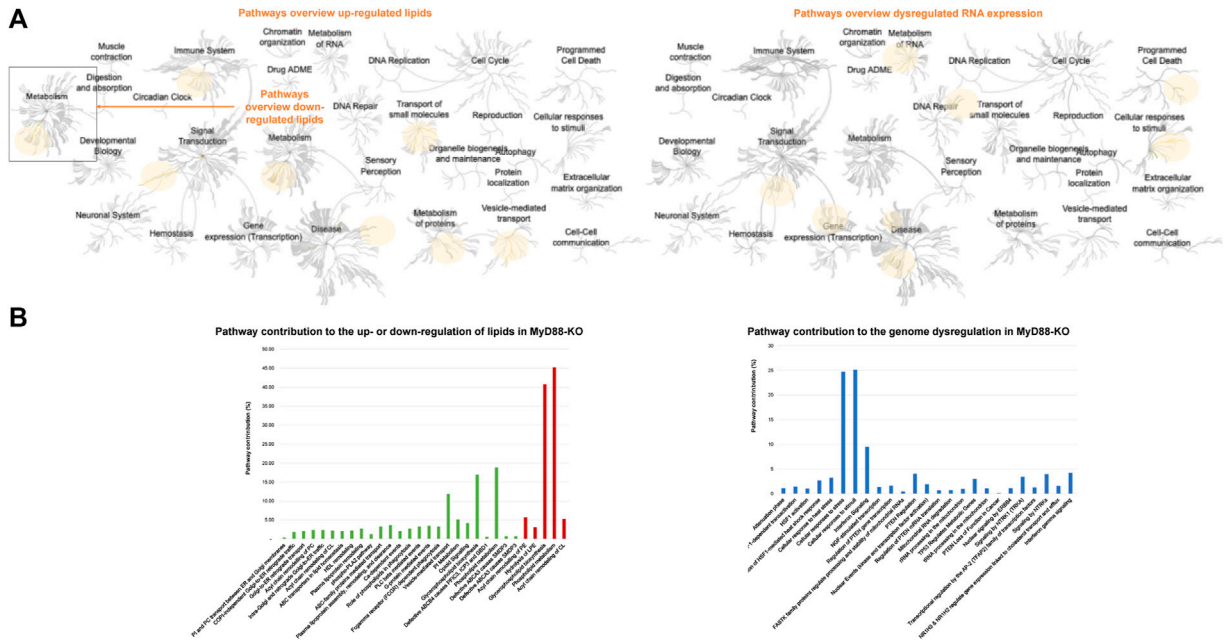


FIGURE 10
(A) General pathways' overview of up- and downregulated lipids, generated by Reactome (left). General pathways' overview, generated by Reactome, affected by the first 30 differentially expressed genes with a 99% confidence and fold change superior to 2 (right). **(B)** Contribution of each pathway to the up- or downregulation of lipids in MyD88-KO. Pathway list generated using Reactome. In green those pathways found for upregulated lipids and in red those discovered for the downregulated lipids (left). Contribution of each pathway to the genome dysregulation in MyD88-KO. Pathway list generated using Reactome (right).

5 significantly identified pathways for the downregulated lipids along with their percentage contribution. Only the pathways with a *P*-value < 0.05 and a false discovery rate (FDR) < 0.2 were chosen for further analysis, prioritizing those with the highest significance and lowest FDR. Similarly, to simplify the analysis of the transcriptome, only the first 30 differentially expressed genes between MyD88-KO and WT were selected for pathway discovery based on their *P*-value < 0.01 and fold change > 1 or < −1 (Figure 10B). In both graphs, the pathways with highest significance values are represented from top to bottom in both graphs. These findings indicate that vesicle-mediated transport and glycerophospholipid biosynthesis exhibit the highest contribution rates in this anti-inflammatory macrophage model. In addition, the highest significantly disrupted pathways by the change in the lipidome are related with the membrane transport and trafficking of molecules between Golgi and endoplasmic reticulum (ER), as well as the remodeling of other lipids and proteins such as: PC, cardiolipins (CL), high density lipoprotein (HDL) and other plasma lipoproteins. Other pathways affected are ATP-binding cassette (ABC) transporters and G-proteins transport, phospholipase C beta (PLCβ) and A2 and (PLA2) metabolism and phagocytosis mediated by phospholipids and by Fc gamma receptor (FCGR). The pathways affected by the transcriptome disruption, cellular response to stress and stimuli as well as interferon (IFN) signaling pathways, have the highest contribution rates and are also the most significant in this anti-inflammatory macrophage model.

The mechanistic function of these pathways is potentially involved in anti-inflammatory protection against the development of macrophages' pathogenic phenotypes. Thus, remodeling of these pathways also by alternative, or adjuvant venues to knocking out the MyD88 could play a crucial role in the re-programming of macrophages to prevent the formation of atheroma plaques and advancement of atherosclerosis disease.

4 Discussion

The suitability of 4D-cellular multi-omics for analyzing cellular lipids and metabolites, from single lipidome extraction or dual extraction of lipids and RNA stemming from a small number of cells was demonstrated using HEK293 as a cell model. In terms of lipids and RNA cellular resolution, the qualitative and relative quantitative analysis of the lipidome and the relative quantitative analysis of the total extracted RNA of the serial dilutions of cell numbers, illustrated the applicability range of this protocol when working with minimal sample amounts. Similar or increased levels of certain lipid classes, such as PI, SM, PE-O/P and LPE was obtained by dual lipid and RNA extraction compared to lipid-only extraction protocol, suggesting a differential dislocation of these lipids from the cell's biomolecular network due to physico-chemical properties these extraction parameters are enabling. A core set of cca 100 lipids mostly covering the glycerophospholipidome are consistently detected and can be quantified in high (cca 1 mil) and lower number cells (cca 3,900). Determination of the lower limit of quantification and detection, in terms of the lowest number of cells required for detection of individual cellular lipid species is valuable in deriving the lipid function knowledge that is preserved with lowering number of cells. Besides, it also allows prospective

informed-study design, including selection of minimum number of cells for a given set of lipid targets, when aiming to investigate specific lipid alteration in cellular populations.

This is particularly advantageous when dealing with limited sample availability. This comprehensive dual extraction method combined with 4D-TIMS cellular lipidomics and RNA analysis is essential for protecting and maximally utilizing valuable scarce samples as well as for improving molecular read-out/per sample. It also reduces errors from single-variable data variability and diverse molecular distribution characteristics, during aliquoting and sampling facilitating effective data combination. Certainly, combining the dual extraction protocol with analytical technologies dedicated to single-cell omic analysis, single cell lipidomics and transcriptomics is expected to increase the lipidome and transcriptome coverage and depth of functional analysis in cells. Moreover, prospective inclusion of metabolite analysis will add a new molecular dimension, making this approach an excellent tool for translational biology. (Chen et al., 2023; Bayer and Alcaide, 2021).

The effects of MyD88 in the immune system activity and its correlation with some disease processes are rather well studied, particularly its high potential as a target for combating inflammatory processes involving important pathways such as NF-kB and AP-1. As an example, a recent study demonstrated that MyD88 deletion decreased macrophage recruitment and affected macrophage function in plaques (Bayer and Alcaide, 2021). Macrophages isolated from MyD88$^{-/-}$ mice exhibited reduced activation, lipid accumulation and foam cell formation in response to ox-LDL treatment, a key factor in atherosclerosis. Additionally, endothelial reactive oxygen species formation, which drives ox-LDL formation, was also decreased (Bayer and Alcaide, 2021). Therefore, the reasons why MyD88 antagonists were largely studied to combat inflammation and associated diseases such as atherosclerosis are evident. Small molecules that mimic the BB-loop in the Toll/IL-1 receptor (TIR) domain of MyD88 were found to inhibit MyD88-mediated pro-inflammatory signaling (Saikh, 2021). Clinical trials using MyD88-targeted therapy for chronic obstructive pulmonary diseases have shown promising results. Dietary supplementation with glycosaminoglycans such as chondroitin sulfate has also been observed to inhibit MyD88-dependent inflammatory signaling in chondrocytes. Medications in the tricyclic family targeting neurotransmitter release and uptake, as well as opioids, have been shown to modulate TLR activity and MyD88 activity respectively (Bayer and Alcaide, 2021). These results reinvigorated the idea that MyD88-targeted therapeutic intervention of pro-inflammatory signaling could be feasible in attenuating severe inflammatory diseases and opens a great opportunity in treating chronic inflammatory diseases. All of these drugs showed varying levels of activity on TLR/MyD88 signalling, and have varying pharmacodynamic properties, therefore could be useful for specific forms of cardiovascular diseases (CVD) depending on the exact contributions of MyD88. However, while safety profiles have already been established, further study would need to be done to demonstrate utility in repurposing them for CVD, since MyD88 antagonists may also impact the protective mechanisms against macrophage infections.

Therefore, it is crucial to thoroughly study the molecular targets for addressing chronic diseases due to the influence of MyD88 cascades on TLR4 signal activation, which significantly contributes to inducing trained immunity (Owen et al., 2022). TLR/MyD88 signalling extends beyond immune cells, and most of the work in preclinical models have used globally deficient mice. However, upregulated TLR/MyD88 signalling has been shown to alter endothelial cell function and contribute to the pathogenesis of vascular disease, expanding the importance of this pathway to other cardiovascular cell types (Guerrini and Gennaro, 2019).

For this reason, the lipidome- and transcriptome-associated anti-inflammatory cascade and the implied partners were studied in this study in the search of good alternatives in the protective mechanism against atherosclerosis in macrophages.

Multi-omics studies involve large and complex datasets. To simplify the functional analysis of RNA and lipids in context of MyD88 depletion, we used two different software tools, LIPEA and Reactome, to filter and visualize the most significant results and focused here our further discussion. on the multi-omic profile of a macrophage model in an anti-inflammatory state for atherosclerosis protection. When looking at each molecular layer independently, i.e., the lipidome dysregulation in MyD88 macrophages, the predominant role of plasmalogens upregulation in this anti-inflammatory response is evident. Ether-linked lipids (PC/PE-O) contribute 60% of the total upregulated lipids constituting the most prominent group. Plasmalogens are unique membrane glycerophospholipids with a vinyl-ether bond at the sn-1 position and enriched in polyunsaturated fatty acids at the sn-2 position. Their physiological roles vary across different tissues, metabolic processes, and developmental stages due to their lability to oxidation and utilization by higher organisms (Braverman and Ann, 2012). The high representation of this lipid class within the upregulated lipids in MyD88-KO macrophages makes them a potential target of the anti-inflammatory mechanism and response. The correlation between plasmalogens and atherosclerosis can be understood in terms of the role plasmalogens play in cellular functions and oxidative stress, whereby antioxidant properties can protect cells from oxidative stress by scavenging reactive oxygen species. Plasmalogens also play a role in anti-inflammatory mechanisms, being present in inflammatory cells are believed to regulate the function of enzymes associated with inflammation. Lower levels of plasmalogens have been associated with multiple inflammation diseases. including in atherosclerosis (Ridker and Thomas, 2014; Wallner et al., 2018). As they are present in endothelial cells, and endothelial dysfunction is an early event in atherosclerosis, changes in their levels might impact endothelial function, which plays a pivotal role in maintaining vascular health. Low plasmalogen levels have been found in individuals with atherosclerosis, suggesting a potential role for plasmalogens in the development or progression of the disease. However, while there are strong associations between plasmalogen levels and atherosclerosis, the exact mechanistic links are not fully defined and further research is required to fully elucidate the precise role plasmalogens play in atherosclerosis and whether modulation of plasmalogen levels could be used as a therapeutic strategy for cardiometabolic diseases, including atherosclerosis (Paul et al., 2019; Braverman and Ann, 2012; Deng and Angelova, 2021).

Studies carried out until the moment, have focus their attention in the influence of dietary changes through increasing plasmalogens levels in inflammation and disease as well as the *in vitro* effects of

changes in the lipid profile in plasmalogens levels. As an example, Wallner et al., 2018 *in vitro* showed the effects of oxidized lipoproteins in plasmalogen levels in human monocyte derived macrophages (Wallner et al., 2018; Paul et al., 2019 demonstrates that phagocytic activity of plasmalogen deficient mutant macrophages was significantly improved when plasmalogen content was restored through supplementation of lysoplasmalogen, highlighting the importance of plasmalogens in regulating macrophage phagocytic activity (Paul et al., 2019). Plasmalogen enrichment via batyl alcohol supplementation attenuated atherosclerosis in ApoE- and ApoE/GPx1-deficient mice (Rasmiena et al., 2015). Lin D. and collaborators also demonstrated that the supplementation with DHA/EPA enriched ethanolamine plasmalogen (EPA-PlsEtn) dramatically reduced atherosclerotic lesions by 78% (Ding et al., 2020). A reduction in arachidonate-containing plasmalogens has been noted in extensively diseased carotid plaque samples compared to minimally diseased ones (Ménégaut et al., 2019). In this context, our findings of upregulated plasmalogens in MyD88 KO macrophages concur well with the above previous findings on their role as anti-inflammatory regulators. It would be interesting to prospectively investigate whether targeted increase of plasmalogens will suffice acquisition of an anti-inflammatory macrophage phenotype (that is without MyD-88 depletion), and also generally how cell-reprogramming into therapeutic functions can be achieved by modulating specific lipid content.

The pathway analysis determination of the transcriptome has rendered important data regarding the potential disrupted pathways during this inflammatory protective model, such as cellular response to stimuli and stress and interferon signaling. From all the significantly dysregulated genes in this model apolipoprotein E (ApoE) is highlighted due to its role in lipid metabolism, and its implications in inflammation, particularly in the context of atherosclerosis. ApoE is 1 out of 5 of the most significant genes, out of the total 3,381 differentially expressed in this model. APoE affects 3 of the 25 significantly identified pathways which are: nuclear signaling by ERBB4, transcriptional regulation by the AP-2 (TFAP2) family of transcription factors and NR1H3 & NR1H2 regulate gene expression linked to cholesterol transport and efflux. ApoE is involved in clearing lipoproteins and cholesterol efflux, potentially reducing the opportunity for foam cell formation and subsequent inflammation contributing to atherogenesis. ApoE's anti-inflammatory properties might also influence the stability of atherosclerotic plaques. A deficiency in ApoE can lead to increased inflammation and oxidative stress, contributing to plaque instability such as inhibiting T cell proliferation and dampening cytokine production by macrophages (Yamazaki et al., 2019). However, different apoE alleles can have distinct effects. Genetic variation in ApoE can affect atherosclerosis risk. The ε4 variant has been associated with higher cholesterol levels and increased risk for cardiovascular disease, whereas the ε2 variant is often associated with lower risk (Ringman et al., 2014). ApoE is significantly overexpressed in MyD88-KO macrophages with a *P*-value of 3.07×10^{-294} and a FC of 4.8 (Supplementary Material). Overall, the correlation between ApoE and atherosclerosis is well-established, with the protein playing a multifaceted role in lipid metabolism, inflammation, and atherogenesis. The presence and function of ApoE are thus critical factors in the overall risk and development of atherosclerotic disease. Due to the expression complexity of this gene, further studies will focus on the identification of the corresponding alleles.

Multi-omics can provide valuable insights into the lipidome alterations occurring together with gene expression patterns in MyD88-KO macrophages during the anti-inflammatory state, shedding light on the underlying mechanisms involved in atherosclerosis protection. Noteworthy here is that the primary lipid changes, (PCs, PC-Os and PE-Os) in MyD88-KO uniquely underscore pathways of immune system, transport of small molecules, vesicle-mediated transport and metabolism of proteins. Complementary, cell-survival and death, cellular response, and metabolism of RNA and transcription are uniquely delineated by gene expression changes. This newly provided knowledge on the specific function of plasmalogens in mediating immune response and metabolism of proteins in anti-inflammatory macrophages is of general importance to understand macrophage reprogramming and strategies to drive this process for therapeutic purpose. In addition, new strategies in multi-omic studies at high-cell resolution are of vital importance to provide new insights in disease mechanisms in limited samples and tissue microenvironments. Low-cell number multi-omic studies present challenges in relation to sensitivity. However, this new approach presents a coverage of 89 lipids in the lowest cell dilution analyzed using the dual extraction protocol in HEK293 cells, corresponding to lipids injected from 157 cells in negative ion mode and 79 cells in positive ion mode. Even though these results can vary depending on the studied cell type, as demonstrated in other studies, the results highlight the sensitivity of this new approach (Gerichten et al., 2023).

It is conceivable that proteins and other water-soluble molecules can be extracted from the aqueous flow-through remaining after RNA extraction, following optimisation based on the existing micro RNEasy QIAGEN protocols (Micro Handbook, 2021). Given that, essentially, the micro RNEasy extraction protocol was only modified by the addition of chloroform, subsequent protein purification, buffer exchange of the aqueous phase and optimization of protein extraction are envisaged to enable also efficient proteome extraction in combination with dual extraction of lipids and RNA. Also, considering the advents in single cell proteomics, the proteome analysis following such an integrated lipidome/transcriptome/proteome analysis is envisaged to be feasible. We expect that the future combination of the extraction of proteins and other metabolites alongside the dual extraction and analysis of RNA and lipids, coupled with high-end proteomics, metabolomics, lipidomics and transcriptomics, will enable the prediction of disease biomarkers and potential therapeutic targets (Åkesson et al., 2023).

Overall, this model for atherosclerosis protection can further enhance our understanding of the specific role of MyD88 signaling in the development and progression of atherosclerosis. Through this multi-omic cellular approach, researchers can elucidate the molecular pathways and regulatory networks involved in the anti-inflammatory state of macrophages and identify novel targets and biomarkers for the diagnosis, treatment, and prevention of atherosclerosis. Enhancing plasmalogen levels could possibly offer a promising and safe therapeutic approach for mitigating atherosclerosis and lowering cardiovascular disease

risk, especially in situations characterized by increased oxidative stress and inflammation. Future studies will be focused on the multi-facetted role of ApoE and prospective analysis of plasmalogens causal relationship and potential therapeutic implications of the macrophages' protective profile against atherosclerosis.

Data availability statement

The transcriptomic data presented in the study are deposited in the NCBI repository (https://www.ncbi.nlm.nih.gov/bioproject), accession number PRJNA1148506. Lipidomic data are available in the Supplementary Material.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

CB: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing–original draft, Writing–review and editing. LB: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing–review and editing, Methodology.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2024.1450971/full#supplementary-material

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Immunological perspectives on atherosclerotic plaque formation and progression

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Atherosclerosis serves as the primary catalyst for numerous cardiovascular diseases. Growing evidence suggests that the immune response is involved in every stage of atherosclerotic plaque evolution. Rapid, but not specific, innate immune arms, including neutrophils, monocytes/macrophages, dendritic cells (DCs) and other innate immune cells, as well as pattern-recognition receptors and various inflammatory mediators, contribute to atherogenesis. The specific adaptive immune response, governed by T cells and B cells, antibodies, and immunomodulatory cytokines potently regulates disease activity and progression. In the inflammatory microenvironment, the heterogeneity of leukocyte subpopulations plays a very important regulatory role in plaque evolution. With advances in experimental techniques, the fine mechanisms of immune system involvement in atherosclerotic plaque evolution are becoming known. In this review, we examine the critical immune responses involved in atherosclerotic plaque evolution, in particular, looking at atherosclerosis from the perspective of evolutionary immunobiology. A comprehensive understanding of the interplay between plaque evolution and plaque immunity provides clues for strategically combating atherosclerosis.

KEYWORDS

atherosclerosis, plaque evolution, immune response, immune cell heterogeneity, inflammatory microenvironment, immunotherapy

1 Introduction

Atherosclerotic cardiovascular disease (ACD) is a prominent global cause of mortality (1). Atherosclerosis is a slow process, characterized by multifocal structural changes in the vascular wall of large and medium- sized arteries, which result in the development of atherosclerotic plaques (2). Atherosclerotic plaques are the pathophysiological basis of almost all arterial vascular diseases. Advanced plaques may rupture, triggering thrombosis

that blocks arteries and disrupts blood flow, leading to an array of life-threatening clinical outcomes called major adverse cardiovascular events (MACEs) (3). Past epidemiologic studies have revealed many risk factors for atherosclerosis, among which traditional risk factors include dyslipidemia, hypertension, hyperhomocysteinemia, hyperfibrinogenemia, diabetes mellitus, smoking, obesity, and genetic predisposition. In recent years, non-traditional drivers such as sleep disorders, lack of exercise, air pollution, environmental stress, as well as inflammation and clonal hematopoiesis associated with the immune system have also received attention (3, 4).

The evolution of atherosclerotic plaques can be broadly categorized into three stages: initiation, progression and complications. It has been shown that fatty streaks are the initial marker of atherosclerosis, which develops in four steps: low-density lipoprotein (LDL) cholesterol uptake, endothelial cell (EC) activation, leukocyte activation and foam cell formation (5). During the development of fibrous plaques, atherosclerotic plaques experience a shift from fatty streaks to intimal growth, and this step is marked by the formation of a lipid-rich necrotic core covered by a fibrous cap. The fibrous cap is composed of vascular smooth muscle cells (VSMCs) which migrate to the side of the arterial lumen and VSMC-derived extracellular matrix (ECM). The atherosclerotic plaques can rupture at the point where the fibrous cap is thinnest exposing the material inside to blood tissue triggering thrombosis. If the lumen is blocked, the narrowing of the diseased artery can also lead to other complications such as heart and brain infarction (6, 7).

Among the many factors that influence the evolution of atherosclerotic plaques, the immune system plays a significant role. During the formation and development of atherosclerotic plaques, the local microenvironment undergoes a series of complex changes, accompanied by the infiltration of multiple immune cells. Evidence suggests that circulating monocytes and resident vascular macrophages are the earliest immune cells recruited into early atherosclerotic plaques (8). Following this, various immune cells such as neutrophils, natural killer (NK) cells, DCs, T cells and B cells gradually infiltrate the plaque and perform their regulatory functions. Subpopulations of leukocytes in the arterial wall are heterogeneous they play a pro-inflammatory or regulatory role in atherosclerotic plaque formation. Most patients with atherosclerosis are immunocompetent individuals (9). The key takeaway about the impact of immune function is that it is complex, and immunomodulation can positively or negatively affect the evolution of atherosclerotic plaques.

The molecular signals that regulate leukocyte recruitment to atherosclerotic lesion sites are complex, and chemokines and their receptors play a key role and have received much attention in the study of atherosclerosis. Chemokines are a class of small, secreted cytokines with chemotactic properties. Depending on the location of their cysteine residues, they can be divided into four subclasses: C, CC, CXC, and CX_3C. They act mainly by binding to specific G-protein-coupled chemokine receptors (10). In atherosclerotic lesions, chemokines and many of their receptors are expressed in endothelial cells, leukocytes, and smooth muscle cells, with highest expression especially in regions near the necrotic core. They are widely involved in all stages of atherosclerosis by promoting immune cell adhesion, migration, infiltration, differentiation, and homing to the lesion site (11).

With the advent and advancement of various experimental techniques, we have gained an understanding of the mechanisms involving the immune system in plaque evolution. Here, we view atherosclerotic plaque lesions from the perspective of evolutionary immunobiology, specifically reviewing the immunologic aspects of plaque evolution, including the immune responses involved, the effects of immune cell heterogeneity, and plaque dynamics in the inflammatory microenvironment. The goal is to provide insight into the search for immunotherapy for atherosclerosis.

2 Understanding atherosclerotic plaque evolution

In 1859, Charles Darwin coined the phrase “natural selection” to describe the process of evolution as observed by the different survival outcomes of individuals under environmental stress owing to phenotypic differences. Natural selection has provided a crucial force in evolution (12). It also happens in the evolution of atherosclerotic plaques, as the same mechanism applies to immune cells infiltrating the plaque. The host immune system is a major source of selection pressure during the evolution of atherosclerotic plaques. The immune system has different cell types, states, and positions. The complex networks, interactions and reactions of immune cells give rise to a cellular ecosystem consisting of numerous cell types accompanied by the genetic diversity of antigen receptors (13). Under selective pressure from the immune system, immune cells exhibit differential phenotypes that distinctively influence the evolution of plaques.

Many believe that atherosclerosis is an inevitable progressive process that develops over time, but current evidence supports a more dynamic and discontinuous evolution of the atherosclerotic plaques (14, 15). Fatty streaks are the earliest form of atherosclerotic plaque lesions. Regardless of the prevalence of coronary artery disease, fatty streaks are observed in the aorta of children in all countries. These early pathologies may progress to more advanced lesions or recede. The fatty streaks progress to fibrous plaques, occurring late in the second decade of life and early in the third decade. Atherosclerotic plaque regression is unlikely to occur during this phase. The progression of fibrous plaques is associated with various complications: calcification, internal bleeding, ulceration, and the release of embolic fragments, as well as the formation of blot clots. Thrombosis causes acute events like myocardial infarction and ischemic stroke (4, 6, 7). Generally, plaques take decades to form and do not develop consistently or steadily. Moreover, once fibrous plaques develop, they are more prone to a variety of complications, which can be life-threatening in severe cases.

3 Immune responses in atherosclerotic plaques

The immune system is categorized into innate and adaptive responses. As the first line of defense against invading pathogens,

innate immunity is characterized by its ability to produce rapid and nonspecific responses. The main types of cells in the innate immune system involve neutrophils, monocytes/macrophages and DCs (16). Innate immune cells are capable of recognizing pathogen-associated and damage-associated molecular patterns by means of pattern recognition receptors, encompassing scavenger receptors (SRs) and toll-like receptors (TLRs) (17). Adaptive immunity is more specific, but slower. Adaptive immunity is mainly composed of T cells and B cells that identify the specific epitopes on pathogens as well as antigens for which they produce a variety of antigen-specific T cell receptors and immunoglobulins (18). The evolution of immune cells plays a key role in the development of atherosclerotic plaques, actively promoting plaque formation and development (Figure 1).

3.1 The arterial wall

The arterial wall is composed of three main layers: the intima, the media, and the adventitia. The intima is the innermost layer in contact with blood flow and is covered by a single layer of ECs on

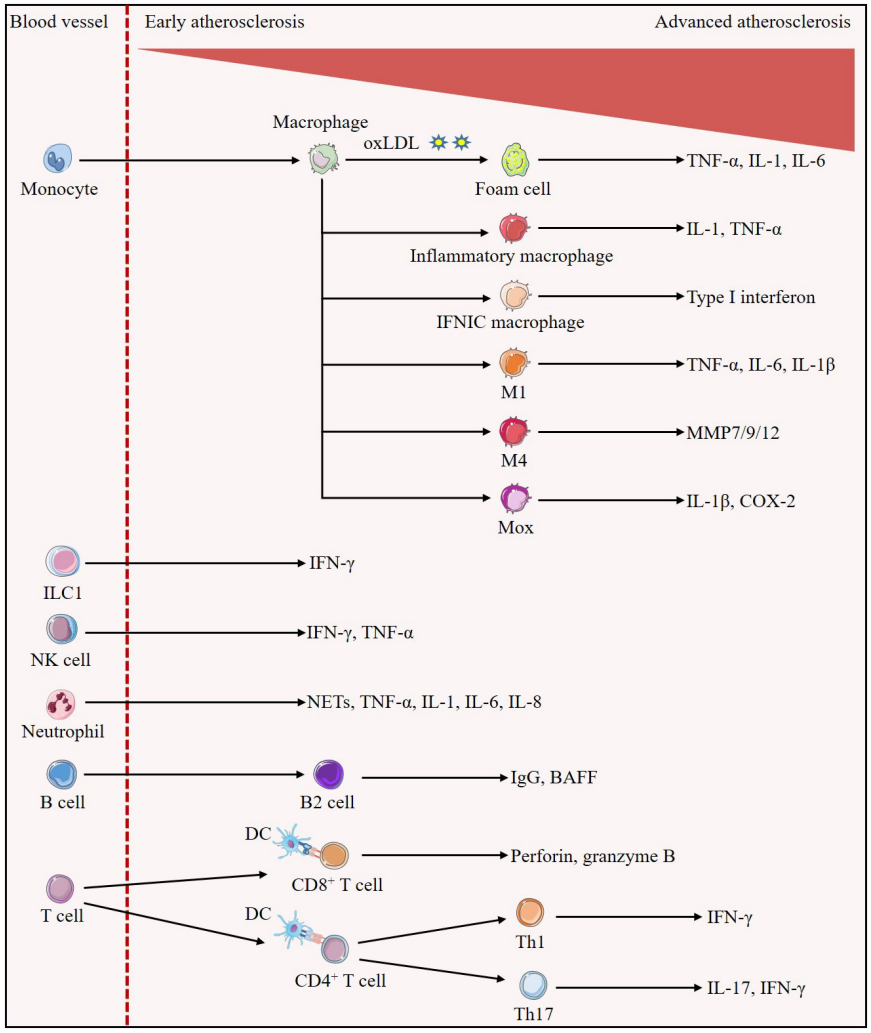

FIGURE 1
Evolution of immune cells in the development of atherosclerotic plaques. Early in the lesion, monocytes/macrophages predominate. Monocytes differentiate into macrophages, which can uptake oxLDL to become foam cells, and also differentiate into inflammatory macrophages and IFNICs macrophages, which secrete IL-1, TNF-α, and type I interferon to exert pro-inflammatory effects, respectively; in addition, they can also be polarized into the M1, M4, and Mox phenotypes, which secrete pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, or MMP, promoting atherosclerosis. In all stages of atherosclerosis, ILC1 is identified as the most abundant subset of ILCs and is involved in reinforcing plaque formation through the production of IFN-γ. Atherosclerotic lesions trigger migration and activation of NK cells as well as release of pro-atherogenic factors IFN-γ and TNF-α. Neutrophils secrete pro-inflammatory mediators such as NETs, TNF-α, IL-1, IL-6 and IL-8, increasing plaque inflammation and susceptibility to rupture. A large number of B cells with a structure similar to tertiary lymphoid organs are found in the adventitial layer of blood vessels, in which B2 cells secrete mediators that aggravate atherosclerosis such as IgG and BAFF. In advanced atherosclerosis, numerous T cells homing to the plaque. Activated DCs present cognate peptides on MHC class I molecules to $CD8^+$T cells and on MHC class II molecules to $CD4^+$T cells. Activated $CD8^+$T cells exert atherogenic effects by secreting perforin and granzyme B and inducing cell death. $CD4^+$T cells are polarized into different subsets, and Th1 cells are able to further stimulate foam cell formation and exacerbate atherosclerosis through the secretion of their major cytokine, IFN-γ. IL-17 secreted by Th17 cells may accompany the increase of IFN-γ to promote plaque inflammatory response and play pro-atherogenic roles.

one side of the lumen (vascular endothelium) and an inner elastic layer on the peripheral side. The media consists mainly of VSMCs surrounded by ECM. The adventitia is composed of fibroblasts and connective tissue (19). The vascular endothelium is the initial obstacle for circulating molecules, cells, or pathogens in the bloodstream (20). Disruption of intravascular homeostatic regulatory mechanisms leads to endothelial dysfunction (21), which facilitates the development and progression of atherosclerotic plaques and can be considered as an independent vascular danger factor.

In addition to endothelial dysfunction, perivascular adipose tissue (PVAT) dysfunction is also a key factor in plaque formation. PVAT, a mixture of brown and white adipose tissue that surrounds most of the vascular system, is thought to be an active component of the vascular wall that regulates vascular homeostasis and influences the pathogenesis of atherosclerosis (22). Under physiological conditions, PVAT exhibits potent anti-atherosclerotic properties through its thermogenic capacity and secretion of several bioactive molecules such as adiponectin and NO. Under the influence of pathological states, such as obesity, diabetes and hypertension, which are metabolic diseases, PVAT becomes dysfunctional and secretes high levels of pro-inflammatory factors such as leptin, resistin, inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL) -6, and chemokines such as monocyte chemotactic protein-1 (MCP-1), which induce endothelial dysfunction, immune cell infiltration and inflammation, and ultimately promote the development of atherosclerosis (23, 24).

Atherosclerotic plaque formation begins with endothelial dysfunction followed by LDL accumulation and its modification in the intima (25). As a result of impaired endothelial barrier function and elevated blood levels of plasma lipids, LDL infiltrates the endothelium and deposits on the arterial wall via endocytosis and is retained in the intima by ECM macromolecules (26). As soon as they enter the subendothelial space, the captured LDL particles are oxidatively modified to activate ECs and VSMCs. Activated ECs induce selective monocytes to be recruited to the intima, rapidly differentiating into tissue macrophages and transforming into foam cells through accumulation of trapped and modified lipoproteins. Notably, a small proportion of foam cells are derived from VSMCs and ECs (27, 28). The accumulation of foam cells is a driving factor in plaque growth. Overall, the combination of endothelial cell injury, oxidative modification of LDL, and macrophage activation lead to increased LDL uptake, which promotes the formation and progression of atherosclerosis.

3.2 Innate immunity

Lipid accumulation stimulates the production of inflammatory mediators and cytokines by ECs. EC activation triggers the release of leukocyte adhesion molecules (AMs) and chemokines that stimulate inflammatory monocyte migration and infiltration. Infiltrated monocytes in the intima are stimulated to mature into macrophages by macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by activated ECs (29). Macrophages express a range of SRs, including CD36, CD68, lectin-type oxidized LDL receptor 1 (LOX-1), SR-A, and SR-B1, which mediate ligand internalization, degradation, and antigen presentation. In contrast, TLRs can directly induce an inflammatory response (17). Many TLR family members such as TLR1, TLR2, TLR4, and TLR5 that are detected in human plaque and atherosclerosis mouse models are mainly expressed by macrophages and ECs (30). Of these, TLR4 is noteworthy as it is highly expressed in human atherosclerotic plaques and its expression is upregulated by oxidized LDLs (oxLDLs) (31). Myeloid differentiation factor 88 (MyD88) is a critical adaptor protein involved in TLR4 signaling. Apolipoprotein E -deficient ($ApoE^{-/-}$) mice lacking both TLR4 or MyD88 show reduced atherosclerosis (32). Overall, SRs and TLRs play a significant role in the evolution of atherosclerotic plaques.

Other innate immune cells such as neutrophils, Innate lymphoid cells (ILCs) and DCs can also influence the evolution of atherosclerotic plaques. Neutrophils are the major responders to tissue damage and infection, releasing pro-inflammatory mediators to neutralize dangers such as toxins (33). Neutrophils aggravate endothelial dysfunction, induce monocytes to move into atherosclerotic lesions, activate macrophages and promote foam cell formation by releasing granule proteins. Analysis of human plaque specimens suggests that neutrophil-derived proteases and reactive oxygen species (ROS) contribute to plaque destabilization (34). In addition, cholesterol crystals trigger neutrophils to release neutrophil extracellular traps (NETs), complex structures composed of nuclear chromatin and proteins sourced from the nucleus, granules, and cytoplasm. NETs cause macrophages to release cytokines that activate Th17 cells, expanding the recruitment of immune cells in atherosclerotic plaques (35, 36). Therefore, neutrophils play a facilitating role in the evolution of plaques.

ILCs are a unique type of lymphocytes that have been newly discovered in recent years. Five distinct subsets have been identified: ILC1s, ILC2s, ILC3s, lymphoid tissue-inducing cells and NK cells. ILCs are mainly localized in barrier tissues such as skin, intestinal mucosa, and lung, and play an important role in innate immune response to infections, lymphangiogenesis, and remodeling of damaged tissues by responding to microenvironmental signals released from surrounding tissue (37, 38). ILC1s are the predominant subset of ILCs in mouse and human atherosclerotic plaques and are involved in enhancing plaque formation by producing IFN-γ in response to stimulation by the transcription factor T-bet; conversely, removal of ILC1 attenuated plaque formation in $ApoE^{-/-}$ mice (39). ILC2s require the transcription factor GATA3 and are protective against atherosclerosis through the production of type 2 cytokines that promote B1 cell proliferation and IgM production as well as modulate diseased macrophages (40). ILC3s are the least abundant lymphocyte subset in atherosclerosis, and its dependence on the transcription factor RORγt for the production of IL-17 and IL-22 on the effects of atherosclerosis has been inconsistently investigated, leading to our lack of clarity on the role of ILC3s in atherosclerosis (41). Because of the overlap in gene expression between ILCs and T cells and the limitations of high-dimensional single-cell techniques, our

information on the role of ILCs in atherosclerosis is limited, and the exact mechanisms require more research.

NK cells are innate immune system effector lymphocytes that sense pathologic alterations or stressed cells by activating receptors and play a cell-mediated cytotoxic role through the release of granzymes and perforin or cytokines, as in the case of interferon-gamma (IFN-γ) (42). NK cells have been detected in both human and mouse atherosclerotic plaque lesions (43, 44). Nevertheless, there is some controversy regarding the role of NK cells in atherosclerotic plaques. An early study of the role of NK cells in atherosclerosis using beige LDL receptor$^{-/-}$ (*Ldlr*$^{-/-}$) mice showed that NK cells have atheroprotective effects (45). Another study investigating the impact of NK cell loss and gain of function in atherosclerosis development demonstrated that NK cells possess a pro-atherogenic role. Their production of interferon-γ, perforin, and granzyme B, as well as the expansion of the necrotic core within the lesion, facilitates the evolution of atherosclerotic plaques (46). Therefore, additional research is necessary to elucidate the precise role of NK cells in atherosclerotic plaques.

DCs are present in healthy arteries and can accumulate in atherosclerotic lesions. They are especially enriched in rupture-prone areas within atherosclerotic plaques, which can lead to plaque destabilization, and are involved in a variety of pathogenic and protective mechanisms during plaque formation. In the earliest stages of plaque formation, DCs may uptake lipids and form a foam-cell-like appearance, as well as regulate lipid metabolism through other mechanisms (47). DCs can also produce many pro-inflammatory cytokines, including TNF, IL-6, and IL-12, which are all pro-atherosclerotic (48). In addition, DCs in the arterial wall also express TLR4, which performs an essential role during the formation and development of atherosclerotic plaques. DCs as specialized antigen-presenting cells link innate and adaptive immunity by presenting antigens to T cells. Thereby, DCs significantly influence the occurrence and advancement of atherosclerotic lesions.

3.3 Adaptive immunity

The antigen-specific adaptive immune responses are triggered after the innate immune response and are mediated by T and B cells (49). The hypothesis that adaptive immunity plays a role in human atherosclerosis has been long studied.

T cells are recruited into atherosclerotic plaques through a mechanism that resembles monocyte recruitment via the receptor C-X-C motif chemokine receptor 3 (CXCR3), which is expressed by all CD4$^+$ T lymphocytes in the same lesion (50). By inhibiting CXCR3 and blocking the migration of T cells from the circulation into the atherosclerotic plaque, plaque formation can be reduced (51). In addition to CXCR3, other chemokine receptors have been found to be responsible for T cell recruitment. Galkina et al. (52) found that CXCR6 expressed on multiple T cell subsets can facilitate T cell aggregation in the aorta and thus promote the development of atherosclerosis. Li et al. (53) demonstrated that CD4$^+$ T cells homing to atherosclerotic plaques specifically required CC chemokine receptor 5 (CCR5), and blocking or knocking down CCR5 significantly prevented T cell recruitment. Similarly, CCR2 deficiency limits T cells entering the arterial intima and suppresses atherosclerosis (54). Recent studies have found that deletion of CCR7 not only leads to reduced plaque content in mice, but also results in disturbed access of T cells to and from the site of plaque inflammation (55).The *ApoE*$^{-/-}$ mice lacking CD4$^+$ T cells show reduced atherosclerosis (56). CD4$^+$ T cells from *ApoE*$^{-/-}$ mice were transferred to *ApoE*$^{-/-}$ mice crossed with severe combined immunodeficiency (SCID) strains of mice, leading to greatly increased fatty streak lesions compared to immunocompetent *ApoE*$^{-/-}$ mice (57). These studies indicate that CD4$^+$ T cells play a pro-atherogenic role in the early stages of atherosclerotic plaque formation.

The CD4$^+$ T cells that play a pro-atherosclerotic role are mainly T helper (Th) cells, especially Th1 cells, while regulatory T cells (Tregs) have been identified to play an anti-atherosclerotic role. CCL1 is a potent leukocyte chemotactic agent that plays an essential role in recruiting Tregs by binding to CCR8. Disruption of the CCL1-CCR8 axis facilitates atherosclerosis by inhibiting IL-10 production and Tregs recruitment and function (58). Likewise, CCL17 acts as a chemoattractant for CCR4$^+$ T cells that inhibits Tregs migration and expansion, further limiting their function. Blocking CCL17 increases Tregs infiltration and reduces atherosclerosis (59). In addition, a study by Shao et al. (60) found that in *ApoE*$^{-/-}$ mice, CCR5 mediated Tregs homing to the aorta in the presence of IL-35, which ultimately inhibited atherosclerosis by maintaining the suppressive functions of Tregs.

Various studies have come to distinct conclusions about the effects of CD8$^+$ T cells on atherosclerosis. In early human atherosclerotic plaques, CD8$^+$ cytotoxic T cells (CTLs) are not as plentiful as CD4$^+$ T cells, but in late human plaques they represent up to 50% of the leukocytes. CTLs are activated during hypercholesterolemia and can facilitate plaque inflammation and instability in lesions (61). Mice lacking antigenic peptide transporter 1 (TAP1) have lower numbers of CD8$^+$ T cells and *ApoE*$^{-/-}$*Tap1*$^{-/-}$ mice exhibit the same atherosclerotic lesions, immune cell infiltration and lipid accumulation as *ApoE*$^{-/-}$ mice (62). This indicates that CD8$^+$ T cells have no or minimal effects on atherosclerotic plaques. However, other studies using the transfer of CD8$^+$ T cells lacking perforin, granzyme B or TNF-α have shown that CD8$^+$ T cells contribute to the evolution of atherosclerotic plaques by perforin- and granzyme B-mediated cytotoxicity and TNF-α-mediated inflammation by promoting apoptosis in macrophages, ECs and VSMCs and the development of the necrotic core (63). Thus, CD8$^+$ T cells may play a secondary role in the early stages of atherosclerotic plaque evolution, but they may be triggered by intracellular infection, which then promotes the accumulation of atherosclerotic plaque or regulates the later stages of atherosclerosis.

Atherosclerotic plaque formation is intensely influenced by diverse arms of the immune system, including B lymphocytes (64). B cells are detected in atherosclerotic lesions, which have a structural organization similar to tertiary lymphoid organs and contribute to atherosclerotic plaque formation (65). Two major subsets of B cells have been found in atherosclerotic plaques. B1 cells spontaneously produce natural IgM antibodies to protect against atherosclerosis. Conventional B2 lymphocytes produce pro-atherogenic IgG, IgA, and IgE antibodies (64). In fact, it was

discovered 40 years ago that atherosclerotic plaques contain immunoglobulins, particularly IgM and IgG, which are present in all stages of lesion development (66, 67). In addition, specific monoclonal antibodies against different epitopes of oxLDL, now referred to as oxidation-specific epitopes, have been discovered both in human and mouse plasma and in atherosclerotic plaques (68). B cells regulate plaque evolution by releasing antigen-specific antibodies that mediate humoral immune responses.

The earliest evidence that B cells are involved in atherosclerosis in mice came from Caligiuri et al. (69) who indicated that increased atherosclerosis in *ApoE*$^{-/-}$ mice after splenectomy was reversed through adoptive transfer of splenocytes, supporting an atheroprotective role. In addition, Major et al. (70) demonstrated that transplantation of B-cell-deficient mouse bone marrow to *Ldlr*$^{-/-}$ mice caused an increase in atherosclerotic plaque formation, clearly demonstrating the protective effect of B cells against atherosclerotic lesions. Further, injection of polyclonal immunoglobulin preparations into *ApoE*$^{-/-}$ mice resulted in a reduction in fatty streaks and fibrous plaques, indicating that atherosclerosis could be inhibited (71). Consistent with these results, the use of bone marrow transplants, whereby *Ldlr*$^{-/-}$ mice were reconstituted with wild-type or IL-5$^{-/-}$ bone marrow, resulted in reduced secretion of T15/EO6 clonotype natural IgM antibodies and accelerated atherosclerosis (72). The above studies suggest that humoral immunity and B cells have properties that inhibit the evolution of atherosclerotic plaques. In contrast, the use of CD20-specific monoclonal antibody-mediated depletion of mature B cells reduced the size of atherosclerotic plaque lesions in *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (73). Furthermore, relay transfer of conventional B2 cells into lymphocyte-deficient *ApoE*$^{-/-}$ mice and B cell-deficient *ApoE*$^{-/-}$ mice effectively promoted the development of atherosclerotic plaques (74). These studies, in turn, show that B cells have a pro-atherosclerotic role. Overall B cells have both a protective and pathogenic role in atherosclerosis depending on the subclass, but more research is required to elucidate the role of each subclass.

4 Evolutionary mechanism for untreated atherosclerotic plaque under immunomodulation

Atherosclerosis is now recognized as a chronic inflammatory disease, with inflammation having an essential role in all stages of the pathogenic process, encompassing plaque formation, progression, and rupture. Inflammation is the response to the existence of exogenous and endogenous antigens by the immune system. In general, driven by the immune system, inflammation profoundly influences the evolutionary trajectory of atherosclerotic plaques.

4.1 Inflammation is the driving factor of atherosclerotic plaque initiation

Inflammation underlies atherosclerotic plaque formation. Plaque inflammation is driven by cytokines, chemokines, AMs, inflammatory signaling pathways, bioactive lipids, and immune cells (75, 76). Nearly all conventional risk factors for atherosclerosis are associated with and actively contribute to inflammatory processes.

Infiltration and retention of oxLDL in the arterial wall serve as critical initiating events that trigger the inflammatory response and facilitate atherosclerotic plaque formation (77). It has been demonstrated that atherosclerotic plaques in both humans and experimental animals contain oxidatively modified LDL (78). OxLDLs contain oxidized lipids, and the products of their degradation make them critical inflammatory components that contribute to the development of atherosclerotic plaques. According to the level of LDL oxidation, oxLDLs are divided into minimally modified LDL (mmLDL) or extensively oxLDL (79). The extensively oxLDL and mmLDL can trigger pro-inflammatory responses in ECs and macrophages, leading to endothelial dysfunction and recruitment of leukocytes to the site of the lesion. Furthermore, mmLDL induces upregulation of TLR2 and TLR4 expression in monocytes/macrophages, and increased TNF-α secretion promotes plaque inflammatory responses (80). In addition, oxLDL and corresponding antibodies bind to form immune complexes (ICs) that have pro-atherogenic and pro-inflammatory properties and ICs activate the inflammasome through signaling at multiple receptors (81, 82). NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) is the most broadly studied of the numerous inflammasomes and an influential regulator in the pathogenesis of cardiovascular disease. A characteristic feature of atherosclerotic plaques is increased expression of the NLRP3 inflammasome component (83), and inhibition of the NLRP3 inflammasome using MCC950 inhibitor reduces the development of atherosclerotic plaques in *ApoE*$^{-/-}$ mice (84). In conclusion, oxLDL promotes plaque inflammatory responses by interacting with immune components.

SRs are distributed on arterial vessel wall cells and vascular ECs, and oxLDL binds to the corresponding receptors to promote the evolution of atherosclerotic plaques through multiple mechanisms. CD36 is a pattern recognition receptor expressed on multiple cell types and is a part of the class B family of SRs. CD36 is not only involved in the uptake of oxLDL and foam cell formation, but also in atherosclerotic plaque formation by interacting with oxLDL and triggering an inflammatory response (85). LOX-1 belongs to the C-type hemagglutinin family and is a specific receptor for oxLDL. LOX-1 is expressed by ECs, macrophages and VSMCs in various stages of atherosclerotic lesions (86). Overexpression of LOX-1 facilitates endothelial dysfunction, vascular inflammation, and plaque formation, and participates in the destabilization of atherosclerotic plaques *in vivo* (87, 88). In contrast, LOX-1 deficiency maintains endothelial function and reduces atherosclerosis development (89). Altogether, oxLDL recognizes the corresponding receptors and promotes the development of plaque inflammation.

4.2 Inflammation accelerates the evolution of atherosclerotic plaque

The accumulation of oxLDL and its binding to receptors initiate plaque inflammation, while the infiltration of immune cells, the

activation of plaque-associated inflammatory signaling pathways, and the response to host tissues and microorganisms maintain the development of inflammation, thereby further accelerating the evolution of plaque.

Infiltration and accumulation of pro-inflammatory and anti-inflammatory leukocytes in the intima of the arterial wall are hallmarks of atherosclerotic plaque formation and progression, as well as drivers of atherosclerotic lesion growth. Immune cell infiltration is triggered by chemokines and AMs (90). Members of the chemokine and AM families mediate the recruitment of immune cells to infiltrate the lesion and accelerate the development of the plaque inflammatory response. In addition, they modulate cellular homeostasis, leading to endothelial dysfunction as well as involvement in thrombosis (91, 92). Many previous studies have focused on P-selectin glycoprotein ligand-1 (PSGL-1), an adhesion ligand which is expressed on leukocytes and ECs. Knockdown of PSGL-1 in an *ApoE*$^{-/-}$ mouse model reduced monocyte infiltration and leukocyte adhesion, decreased atherosclerotic plaque area and was protective against atherosclerosis (93, 94). Furthermore, systemic low-grade chronic inflammation triggered by the common risk factor obesity is also capable of promoting immune cells infiltration, including lymphocytes and macrophages, which impairs systemic metabolism by exacerbating adipose tissue inflammation, leading to cholesterol deposition in the blood vascular wall, with the ensuing immune process supporting the growth of atherosclerotic plaques and contributing to cardiovascular complications (95).Overall, the development and growth of plaques in atherosclerosis cannot occur without the infiltration of immune cells.

Signaling pathways that are mediated by immune, inflammatory mediators are associated with the evolution of atherosclerotic plaques. Nuclear factor-κB (NF-κB) serves as the primary transcription factor in the inflammatory response. The NF-κB pathway is induced and activated by pro-inflammatory cytokines, adhesion molecules, chemokines and growth factors and exerts a pivotal role in atherosclerotic plaque inflammation (96). Activated NF-κB is observed in several cells including macrophages, VSMCs and ECs in atherosclerotic lesions. Studies have shown that activated NF-κB is involved in many features of atherosclerosis, including mediating foam cell formation, enhancing vascular inflammation, stimulating VSMCs proliferation and migration, exacerbating vascular calcification, promoting plaque formation and destruction, and regulating vascular apoptosis (97). In *Ldlr*$^{-/-}$ mice fed an atherogenic diet for a prolonged period, the NF-κB signaling pathway in the endothelium was activated, leading to increased atherosclerotic plaque formation (98). Furthermore, TLRs are expressed in all atherosclerotic plaque immune cells and are engaged in the inflammatory response to plaques. The TLRs signaling pathway can be broadly divided into MyD88-dependent and non-MyD88-dependent pathways. MyD88 activates NF-κB and MAPK signaling pathways and attracts the expression of inflammatory cytokines. The non-MyD88-dependent pathway, TIR-domain-containing adapter protein-inducible interferon (IFN)-β (TRIF)-dependent pathway, activates IFN regulatory factor 3 (IRF3) and induces the expression of type I IFNs (99, 100). Among these, TLR4 signaling plays an important role in activating atherosclerotic plaque inflammation and lipid accumulation. Knockdown of Adenosine triphosphate-binding cassette transport increases TLR4 and MyD88/TRIF signaling in macrophages, enhances expression of inflammatory cytokines and accelerates plaque inflammation (101). In general, TLRs can induce the synthesis of pro-inflammatory mediators as well as promote plaque inflammation through activating complex cell signaling pathways. Next, janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways exert pro-inflammatory effects in atherosclerotic plaques by inducing diverse pro-inflammatory cytokines such as IL-6, TNF-α and IL-1 (102). The JAK/STAT pathway is activated when various cytokine receptors on the cell membrane bind to the corresponding ligands. The JAK family is composed of four members: JAK1, JAK2, JAK3, and TYR2, while the STAT family is composed of seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Gharavi et al. (103) found that activated STAT3 was predominantly present in ECs and inflammatory cells in inflammatory areas of atherosclerotic plaques, with less in non-inflammatory areas. Mice with STAT3 knockout in the endothelium showed reduced fatty streak formation and decreased macrophage content compared to wild-type mice. In addition, there is growing evidence that the Wnt pathway is involved in multiple processes in the evolution of atherosclerotic plaques, including regulation of endothelial dysfunction, VSMC proliferation and migration, regulation of inflammation and foam cell formation, and pathological angiogenesis and calcification, all of which are key processes in plaque formation and stabilization (104). In conclusion, in the plaque inflammation microenvironment, crosstalk between signaling pathways further accelerates inflammation and plays a role in plaque evolution.

Research into atherosclerotic plaque development has also focused on the host tissues and microorganisms that may influence this process. Endothelial-mesenchymal transition (EndMT) is present in atherosclerotic plaques, where ECs can obtain a mesenchymal phenotype, with loss of expression of EC markers and function, and gain of expression of mesenchymal cell markers and function, directly contributing to plaque evolution (105). In addition to this, the brown adipocyte-specific PPARγ knockout mice were crossed with *ApoE*$^{-/-}$ mice and the resulting double knockout showed increased vascular and systemic inflammation and significantly increased atherosclerotic lesions (106). Thus, host cells and tissues can contribute to the development of plaque lesions by increasing inflammation.

Furthermore, the presence of microorganisms in atherosclerotic plaques is well recognized. Pathogenic microorganisms are able to infect arterial wall cells, facilitate local inflammation of the plaque, evoke an adaptive immune response and influence plaque progression. For example, *Chlamydia pneumoniae* infection enhances monocyte adhesion, induces foam cell formation, activates LDL receptors, and triggers inflammation and atherosclerosis (107). *Porphyromonas gingivalis* induces endothelial cell dysfunction, promotes VSMC proliferation, migration, and calcification and the formation of foam cells, which also leads to an imbalance of Tregs and Th cells,

suppresses T cell immunity, facilitates inflammatory responses, and ultimately promotes the evolution of atherosclerotic plaques (108). Moreover, a study showed that gut microbiota (GM) are key environmental factors in regulating inflammation in atherosclerotic plaques (109). GM can influence the evolution of atherosclerotic plaques through direct invasion of plaques and by regulating cholesterol metabolism and the production of harmful metabolites. An animal study showed that transplantation of the pro-inflammatory GM from caspase1$^{-/-}$ mice to *Ldlr*$^{-/-}$ mice enhanced systemic inflammation and increased atherosclerosis (110). In conclusion, microorganisms can regulate plaque progression by influencing inflammatory responses through multiple mechanisms.

In addition to bacteria, some viruses can also influence the evolution of atherosclerotic plaques. For example, cytomegalovirus (CMV) is capable of infecting almost all plaque immune cells, expressing viral cytokines and chemokines to promote an inflammatory environment, and triggering plaque formation through mechanisms such as induction of endothelial damage, increased lipid deposition, and VSMC proliferation and migration (111). *ApoE*$^{-/-}$ mice infected with CMV increases lesion size and IFN-γ levels, contributing to the development of atherosclerotic plaques (112). Other viruses including Epstein-Barr virus, herpes simplex virus type I, hepatitis virus, and human immunodeficiency virus have been reported to be associated with atherosclerosis, but the exact mechanisms need to be further studied. An in-depth study of the effects of pathogenic microorganisms on plaque evolution may also bring insights for the prevention and treatment of atherosclerosis.

5 Immune cell heterogeneity contributes to atherosclerotic plaque evolution

Leukocyte subsets accumulate at different stages of plaques and are involved in the immune response in the atherosclerotic lesion process. Single-cell RNA sequencing (scRNA-Seq) reveals heterogeneity of immune cells in atherosclerotic plaques (113). Studying immune cell heterogeneity could provide new insights into the evolutionary mechanisms of atherosclerotic plaques.

5.1 Monocyte/macrophage heterogeneity

Monocytes are heterogeneous populations and human blood monocytes are classified into three subpopulations according to differences in the expression of the lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16: classical CD14^{++}CD16^{-}, intermediate CD14^{++}CD16^{+} and non-classical CD14^{+}CD16^{++} monocytes (114). In mice, monocytes are categorized into two major subpopulations depending on Ly-6C expression: Ly6C^{hi}CCR2^{+}CX$_3$CR1^{low} and Ly6C^{low}CCR2^{-}CX$_3$CR1^{hi} (115) (Figure 2). Similarly, macrophages are a heterogeneous cell population arising from the heterogeneity of monocytes and the stimulation of the inflammatory microenvironment of plaques. In recent years, the concept of macrophage heterogeneity has been better explored, but in atherosclerosis the traditional classification of M1 and M2 subsets continues to be used (116). A full understanding of the heterogeneous expression of plaque monocytes/macrophages will help to uncover the role of different functional phenotypes on plaque evolution.

Different monocyte/macrophage subsets play different roles in the evolution of atherosclerotic plaques. Classical CD14^{++}CD16^{-} monocytes account for about 85% of total monocytes, intermediate CD14^{++}CD16^{+} monocytes for about 5% and non-classical CD14^{+}CD16^{++} monocytes for about 10% (117). Classical monocytes, as major phagocytes, are capable of producing M1 macrophages and foam cells with strong peroxidase activity, producing ROS and secreting cytokines such as IL-10 in response to LPS and taking up LDL during infection and inflammation. They also exhibit high levels of CCR2 and CD62L, as well as low levels of CX3CR1. Human CD14^{++}CD16^{+} intermediate monocytes are pro-inflammatory. This subset exhibits the highest ROS production and lowest peroxidase activity and secretes IL-1 and TNF-α in response to LPS associated with the evolution of atherosclerotic plaques. It also expresses CXCR1, CCR2, and CCR5 (118). It has been demonstrated that enzymatic degradation of LDL preferentially binds intermediate monocytes and is able to induce foam cell formation, suggesting that this subset has a pro-atherogenic effect (119). Non-classical monocytes are weak phagocytes that preferentially take up oxLDL and have an inflammatory effect, producing numerous inflammatory cytokines for example TNF-α and IL-1β. Additionally, they also express an elevated level of CX3CR1 and have a strong affinity for ECs, acting more like patrolling immune cells (120). Overall, human monocyte subsets differ not only in their proportions and phenotypes, but also significantly in their functions, but all promote plaque development.

Murine monocytes are the most studied. The Ly6C^{hi} subset resembles human classical monocytes and is referred to as "inflammatory" monocytes, while the patrolling Ly6C^{low} subset resembles non-classical monocytes in part and is referred to as "resident" monocytes (121). Ly6C^{hi}CCR2^{+}CX$_3$CR1^{low} monocytes rely on CX3CR1, CCR2, and CCR5 for transport to plaques, while Ly6C^{low}CCR2^{-}CX$_3$CR1^{hi} are recruited to plaques through CCR5. In the arterial intima, Ly6C^{hi} monocytes can differentiate into M1 macrophages, which go on to produce foam cells and produce inflammatory cytokines such as TNF-α, IL-6, ROS, and matrix metalloproteinases (MMPs) that actively contribute to plaque formation and progression (122). Research has demonstrated that the amount of Ly6C^{hi} cells in the blood increases dramatically as atherosclerosis progresses in hypercholesterolemic *ApoE*$^{-/-}$ mice, suggesting that Ly6C^{hi} monocytes have a crucial role in plaque progression (123). Ly6C^{hi} monocytes can be converted into Ly6C^{low} cells. Unlike Ly6C^{hi}, Ly6C^{low} monocytes are recruited less to plaques but preferentially express the DC-associated marker CD11c (124). Ly6C^{low} monocytes rely on CX3CR1 to patrol healthy tissue in a long-range crawl across the endothelium, which allows them to rapidly invade infected tissue, initiate an early immune response and differentiate into macrophages when early inflammation occurs (125). Monocyte heterogeneity is

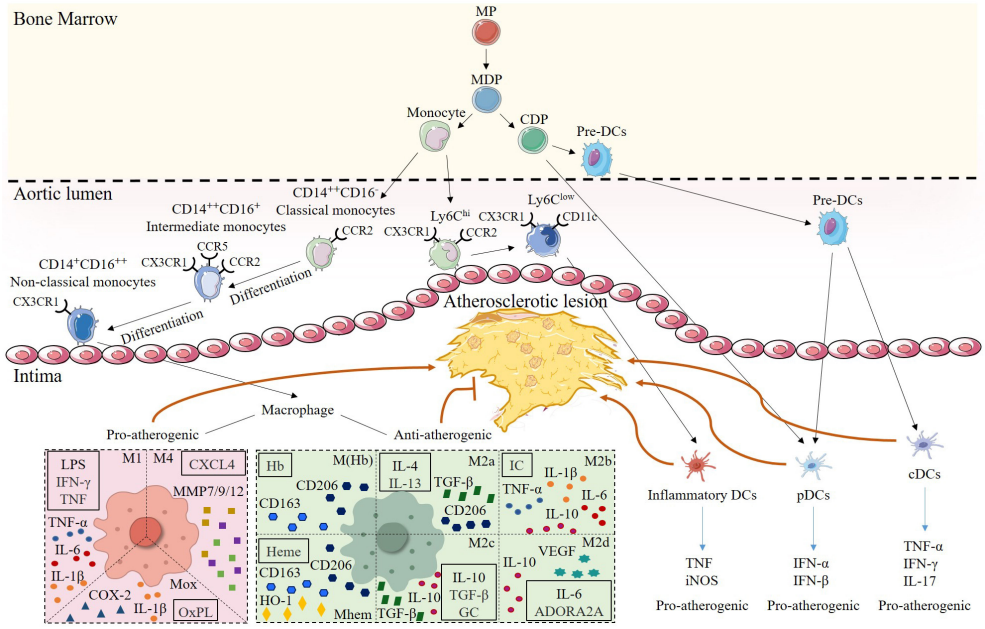

FIGURE 2
Heterogeneity of monocytes/macrophages and DCs in atherosclerotic lesions. Monocytes and DCs share a common origin in myeloid progenitor (MP). Monocyte-DC precursor (MDP) gives rise to monocytes and common DC precursor (CDP). Among them, mouse monocytes are classified into two subsets according to their surface expression of Ly-6C. Ly6C^{hi} subsets have pro-inflammatory functions and highly express CCR2. Ly6C^{low} subsets primarily patrol along the vascular endothelium, are engaged in tissue repair, and can express CD11c, and differentiate into inflammatory DCs to promote plaque progression in the plaque inflammatory microenvironment. Human monocytes are subdivided into three subsets based on the expression of CD14 and CD16.CD14^{++}CD16^{-} classical monocytes, which express high levels of CCR2, can differentiate into CD14^{++}CD16^{+} intermediate monocytes that express CCR5, and then further differentiate into CD14^{+}CD16^{++} nonclassical monocytes, which are highly expressive of CX3CR1. Once monocytes enter the intima, they can mature into macrophages. Differentiation of different macrophage subsets depends on the stimulation of the inflammatory microenvironment within the lesion. Pro-atherogenic macrophages include M1, M4, and Mox, which are activated by Th1 cytokines, CXCL4, and OxPL, respectively, and secrete pro-inflammatory mediators, such as TNF-α, MMP, and IL-1β, to exert pro-inflammatory effects. Anti-atherogenic macrophages include M(Hb), Mhem, and M2. M(Hb) and Mhem are induced by Hb and Heme and secrete CD163 and CD206 to exert anti-inflammatory effects. M2 macrophages are subdivided into M2a, activated by IL-4 and IL-13; M2b, activated by IC; M2c, activated by IL-10, TGF-β and GC, and M2d, activated by IL-6 and ADORA2A. M2 macrophages mainly secrete Th2 cytokines such as IL-10 as well as TGF-β, which inhibit the development in atherosclerosis. CDP can further differentiate into pDCs and pre-DCs, and pre-DCs enter the arterial intima and become pDCs and cDCs, respectively, and play pro-atherogenic roles by secreting TNF or IFN.

important in the evolution of atherosclerotic plaques, but the mechanism of action of some subsets is unclear and much future research is needed.

Indeed, macrophage phenotype is influenced by microenvironmental factors within the atherosclerotic plaques. Both M1 and M2 macrophage populations are present and increasing in number throughout the atherosclerotic process (126). Monocytes are induced by GM-CSF and stimulated by Th1 cytokines, for example TNF-α and IFN-γ, to differentiate into M1 macrophages (127), which express various pro-inflammatory cytokines, including IL-13, IL-6, and TNF-α, leading to the recruitment of inflammatory cells and accelerating the development of plaques. In addition, M1 macrophages can also secrete MMPs, which degrade the ECM and cause plaque rupture. M1 macrophages accumulate in the rupture-prone shoulder area of the plaque, and both M1 and M2 macrophages are expressed at the fibrous cap near the necrotic core (126). Unlike M1 macrophages, M2 macrophages are induced to polarize by different cytokines like M-CSF, IL-4, IL-13, and IL-10 (127). Among these, large amounts of IL-13 are produced mainly from ILC2s.Activation of ILC2s has been shown to be associated with reduced atherosclerotic burden. Engelbertsen et al. (128) found that treating *Ldlr*$^{-/-}$*rag1*$^{-/-}$ mice with IL-2/anti-IL-2 complexes resulted in the expansion of CD25^{+} ILC2s, which lowered very low-density lipoprotein cholesterol and atherosclerosis, and conversely ILC2s depletion led to an acceleration of the atherosclerotic process (129). Mantani et al. (130) also observed that IL-25 treatment of *ApoE*$^{-/-}$ mice inhibited the development of atherosclerosis by a massive expansion of ILC2s, an increase in IL-5 concentration, and greater numbers of B1 cells and IgM antibodies. Meanwhile, the expansion of ILC2s secreted large amounts of IL-13, which enhanced plaque stability and prevented the development of atherosclerotic lesions by increasing collagen deposition and promoting macrophage polarization towards M2 (131–133). According to the stimulation signal, M2 macrophages are further divided into four different subsets. M2a macrophages are induced by IL-4 and IL-13 to secrete anti-inflammatory cytokines including IL-10 and IL-1, which have strong effects on anti-inflammation, as well as high expression of mannose receptor, pro-fibrotic factors, and transforming growth factor involved in the repair of damaged tissues (134, 135). M2b macrophages can be stimulated by immune complexes, TLR agonists in combination with IL-1 receptor agonists, or LPS to produce and express large numbers of the anti-inflammatory cytokine IL-10, which exerts an

immunomodulatory role in atherosclerotic plaques, but also secretes pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (121, 134). M2c macrophages are induced to activate by IL-10, TGF-β or glucocorticoids and they are capable of releasing IL-10 and pentraxin 3 for anti-inflammatory effects, and high expression of Mer receptor kinase for its efficient efferocytosis (136, 137). The process of M2d macrophage polarization is mediated by IL-6 and TLR stimulation of the adenosine A2A receptor, producing large amounts of IL-10 and vascular endothelial growth factor (VEGF) to promote angiogenesis and plaque growth in atherosclerotic plaques (138) (Figure 2). Overall, in addition to M2b macrophages, M2 macrophages produce many anti-inflammatory cytokines that maintain effective efferocytosis in atherosclerotic plaques as well as reducing plaque inflammation.

Recently, many research studies have revealed the presence of other macrophage subsets in atherosclerotic plaques, in addition to M1 and M2 types. M4 macrophages are induced by CXCL4, which is widely expressed in plaques, and exert atherosclerotic effects by producing MMP12, MMP7, and MMP9 leading to increased plaque vulnerability (139–141). The presence of M (Hb) and Mhem-type macrophages in areas of advanced atherosclerotic plaque bleeding is induced by hemoglobin and heme, respectively (142, 143). They characteristically express proteins and receptors such as the SR cysteine-rich type 1 protein M130 and macrophage mannose receptor 1 and activate related signaling pathways to increase liver X-receptor (LXR)-α activity to enhance cholesterol efflux, thereby preventing foam cell formation and exerting anti-atherogenic effects (143–145). Mox macrophages, which lack phagocytosis, account for approximately one-third of all macrophages in mouse advanced atherosclerotic plaques. Their formation is dependent on oxidized phospholipids and the transcription factor Nrf2, and they are capable of secreting pro-inflammatory cytokines such as IL-1β and cyclooxygenase 2, which contribute to plaque evolution and inflammation (146). It follows that macrophages can polarize in the complex microenvironment of the atherosclerotic plaque, and that the great heterogeneity of macrophages has a different impact on plaque evolution.

Single-cell techniques have greatly improved our understanding of macrophage subsets in plaques. Zernecke et al. (147) comprehensively analyzed leukocyte subpopulations identified by scRNA-Seq, and cell counting (CyTOF) and confirmed five macrophage subsets emerging in the aorta of atherosclerotic mice: resident -like macrophages, inflammatory macrophages, Trem2^{hi} foamy macrophages, interferon-inducible cell (IFNIC) macrophages and cavity macrophages. The newly discovered macrophage subsets exhibit heterogeneity in gene expression, with each subset having genes that are more significantly enriched in expression relative to the other subsets. Resident-like macrophages express *Lyve1*, *Timd4*, *Mrc1*, *Pf4* and *Ccr2* (148, 149). Inflammatory macrophages highly express the inflammatory chemokines *Ccl2-4* and *Cxcl2* and are also enriched in a large number of classical pro-inflammatory transcripts, such as *Il1α*, *Il1β*, and *Nlrp3*, which play an important role in regulating the progression of atherosclerosis (150). Trem2^{hi} foamy macrophages possess a gene expression profile that partly overlaps with those of inflammatory macrophages and in addition express *Lgals3*, an atherosclerosis biomarker that promotes the differentiation of monocytes to macrophages and is linked to alternative macrophage activation and plaque evolution (151, 152), as well as expressing the *Cd9* and *Ctsd*, the gene encoding cathepsin D. Cathepsin D is involved in the modification of LDL and facilitates phagocytosis of oxLDL by macrophages, which leads to the formation of foam cells (153). IFNIC macrophages are characterized by type I interferon and express a variety of interferon-inducible genes such as *Ifit3*, *Irf7* and *Isg15*, it is known that type I interferons usually have pro-atherogenic effects (154). Cavity macrophages express MHCII encoding genes as well as *Cd226*, *Itgax* and *Ccr2*, and the function of the cells is unknown (147). In conclusion, resident-like macrophages have anti-inflammatory properties, IFNIC macrophages and inflammatory macrophages are pro-inflammatory, Trem2^{hi} foam macrophages are not pro-inflammatory but function in lipid metabolism and cholesterol efflux (155, 156). Similarly, integrated analysis of scRNA-Seq data from immune cells in human atherosclerosis revealed that macrophages in human lesions exhibit similar transcriptional status to subsets of mouse aortic macrophages (157). The newly discovered subsets add an important dimension to our study of plaque macrophages, but the impact on plaque evolution and the specific mechanisms of action still need to be clarified by in-depth scientific studies and explorations.

5.2 DC heterogeneity

DCs are heterogeneous blood-borne professional antigen-presenting cells with the ability to capture, process, and present antigens to T cells that recognize antigens and induce antigen-specific immune responses (158). The functional heterogeneity of DCs identified in plaques suggests their complicated and multifaceted roles in atherosclerotic disease pathogenesis. The plaque inflammatory microenvironment and exogenous stimuli affect the DCs phenotype and are able to control the switch to an inflammatory or tolerant phenotype. DCs can be categorized into several subsets based on their origin, location, and function (159). In atherosclerotic plaques, the focus is on myeloid DCs (mDCs), plasmacytoid DCs (pDCs), pre-DCs, conventional DCs (cDCs), and inflammatory DCs (Figure 2).

mDCs are characterized by the expression of CD1c, CD11c, and CD33 and they secrete IL-12. In contrast the pDCs express CD123 and produce type I IFN (160). The differences in the surface markers of mDCs and pDCs results in inconsistent quantitative changes exhibited in atherosclerotic disease. For example, in coronary artery disease patients mDCs precursors, but not pDCs precursors, are significantly reduced, which is contrary to other research showing a marked reduction in the amount of pDCs and a remarkable increase in the amount of mDCs in patients with coronary artery disease (161, 162). In atherosclerosis, mDCs and pDCs were confined to the shoulder of lesions to produce IFN-α, demonstrating their association with plaque instability (163). mDC and pDC coexistence synergized TLR4 and TLR9 ligand inflammatory effects as demonstrated by increased production of TNF-α, IL-12, and MMP9 (164). The development and

stereotyping of mDCs are modulated by a variety of transcriptional and hematopoietic growth factors, with *CCr7*, *Zbtb46*, and *Flt3* representing the core genes responsible for the development as well as functional and phenotypic maintenance of mDCs. mDCs have been shown to be associated with the pathogenesis of atherosclerosis. Various subsets of mDCs may have both atherogenic and atheroprotective activities during atherogenesis. The pro-inflammatory effects of mDCs include pro-inflammatory subsets that produce inflammatory molecules and initiate effector T cells. On the contrary, tolerogenic mDCs combat inflammation by inhibiting the activity of pro-inflammatory T cells and macrophages and by inducing immunosuppressive Tregs (165). Deficiency of FMS-like tyrosine kinase 3 ligand in mice leads to lack of $CD103^+CD11c^+$ mDCs and other DC subpopulations as well as advanced atherosclerosis. In $Ldlr^{-/-}$ mice, $CD103^+CD11c^+$ mDCs exert immunosuppressive effects through mechanisms that support $FoxP3^+$ Treg homeostasis (166). These findings suggest that $CD103^+CD11c^+$ mDCs are atheroprotective. Transplantation of MyD88-deficient $CD11c^+$ DCs into $Ldlr^{-/-}$ mice decreases stimulation of peripheral effector T cells, reduces the aggregation of effector T cells and Tregs in plaques, and results in an increase in plaque size owing to accumulation of myeloid-derived pro-inflammatory cells (167). Overall, mDC subsets play diverse roles in atherogenesis.

Several studies have discussed the role of pDCs in atherosclerotic plaque evolution. In a mouse model of atherosclerosis, depletion of pDCs using multiple antibodies against bone marrow stromal cell antigen 2 (BST2) promotes the accumulation of T cells in plaques, leading to enhanced atherosclerosis in $Ldlr^{-/-}$ mice (168); whereas in $ApoE^{-/-}$ mice, atherosclerosis is reduced by decreasing macrophage infiltration in plaques and stabilizing plaques by increasing collagen content (169, 170). The transcription factor E2-2/Tcf4 is an important regulator of pDCs development. Additional studies have found atheroprotective effects with specific deletion of the transcription factor E2-2/Tcf4 or impaired MHCII antigen presentation in pDCs, suggesting that in atherosclerosis, pDCs also promote MHCII-dependent antigen presentation through T-cell responses (171). Therefore, pDCs can drive immune responses in atherosclerosis and their function is influenced by environmental factors.

Pre-DCs are the final precursor stage in the formation of DCs, and they can develop into DCs without dendrites but require further development to obtain dendritic forms and full DC function. Little cell division is involved in their development into DCs, leading to different types of DCs being produced by different pre-DCs, such as some monocytes, pDCs, and cDCs (172). Some studies have reported increased accumulation of pre-DCs in plaques, which may be attributed to increased levels of circulating chemokines of CCL2, CCL5, and CXCL12 enhancing the entry of pre-DCs into plaques (173, 174). cDCs are also classified as migratory DCs, e.g., Langerhans cells and dermal DCs and lymphoid-tissue-resident DCs, which are not able to migrate to the lymph nodes. Recently, based on different developmental pathways, cDCs have been further classified into cDC type 1 (cDC1), including $CD8\alpha^+$ DCs in lymphoid tissues and $CD103^+$ DCs in nonlymphoid tissues, and $CD11b^+$ cDC2s (47). cDCs can be engaged in interactions with T cells and NKT cells, resulting in increased production of IFN-γ, IL-17, and TNF-α by T cells promoting plaque development. Among them, $CD11b^+$ cDC2s were able to participate in lipid accumulation and foam cell formation (48). cDCs can regulate the evolution of atherosclerotic plaques in an antigen-dependent manner in part by modulating T cell activation and adaptive immune responses. Inflammatory DCs do not exist in a stable state, instead they appear as a result of inflammation (160, 172). CX3CR1 deficiency affects the accumulation of DCs in the aortic wall and significantly attenuates the atherosclerotic burden, which could demonstrate that inflammatory DCs may differentiate primarily from $Ly6C^{low}$ monocytes and promote plaque evolution (175). The pro-inflammatory microenvironment at the atherosclerotic plaque provides an excellent option for the predominant differentiation of circulating DC precursors and monocytes toward inflammatory DCs, which in turn can promote the differentiation of naïve T cells into inflammatory and pro-atherosclerotic Th subsets, such as Th1 and Th17 (158). Inflammatory DCs act as an important player in the pro-inflammatory response at plaque lesion sites. In conclusion, pre-DCs develop into different subsets of mature DCs under the influence of different factors and function in the evolution of plaques.

5.3 T cell heterogeneity

In atherosclerotic plaques, T cells are less numerous than monocytes/macrophages, but like monocytes/macrophages that exhibit heterogeneity in markers and function, T cells also exhibit heterogeneity and regulate the adaptive immune response to plaque. $CD4^+$ T cells are commonly found in atherosclerotic plaques, and $CD4^+$ T cell subsets are substantially heterogeneous. In response to different stimulus activations, $CD4^+$ T cells differentiate into different Th subsets, including Th1, Th2, Th17 and Tregs (Table 1).

Th1 is the prominent T cell subtype in atherosclerotic lesions and Th1 cytokines predominate in atherosclerotic mouse models and human plaques. They secrete mainly IFN-γ, which exerts a pro-atherogenic effect (176). IFN-γ is a major inducer of atherosclerotic lesions and promotes plaque development and instability in a number of ways, including endothelial dysfunction, recruitment of inflammatory cells, downregulation of intraplaque cellular cholesterol efflux, promotion of lipid deposition and foam cell formation, and induction of pro-inflammatory cytokine secretion (177, 178). IFN-$\gamma^{-/-}$ mice crossed with $Ldlr^{-/-}$ mice showed significantly fewer atherosclerotic lesions compared to $Ldlr^{-/-}$ mice after 8 weeks of feeding a cholesterol diet (179). Similarly, $ApoE^{-/-}$ and IFN-γ receptor double knockout mice showed a substantial reduction in atherosclerotic lesions, as evidenced by reduced lipid accumulation and reduced numbers of inflammatory cells, compared to $ApoE^{-/-}$ mice after 3 months of feeding a Western type diet (180), whereas exogenous injection of IFN-γ to $ApoE^{-/-}$ mice resulted in increased lesions (181). All the above studies suggest that IFN-γ plays a facilitating role in the evolution of plaques. T-bet is a transcription factor essential for Th1 cell

TABLE 1 Heterogeneity of $CD4^+$ T cell subpopulations.

$CD4^+$ T cell subsets	Transcriptional regulators	Cytokines produced	Role in atherogenesis
Th1	T-bet	IFN-γ, TNF-α, TNF-β	Proatherogenic
Th2	GATA-3, c-Maf	IL-4, IL-5, IL-13	Atheroprotective/ Proatherogenic
Th17	RORγt, RORα, STAT3, AhR, RUNX1	IL-17	Atheroprotective/ Proatherogenic
Treg	FOXP3, RORγt	TNF-β, IL-10	Atheroprotective

differentiation, and *Ldlr*$^{-/-}$ mice lacking T-bet show diminished atherosclerosis (182). This further suggests that Th1 cells promote atherogenesis and also confirms that atherosclerosis is driven by the Th1 response.

Th2 cells are poorly represented in atherosclerotic plaques, and their function in atherosclerosis is still controversial. Th2 cells typically produce cytokines such as IL-4, IL-5, and IL-13. IL-4 can effectively inhibit the Th1 response, Th1 cytokine formation and IFN-γ secretion, and has atheroprotective effects (176, 183). However, other studies have shown that IL-4 deficiency leads to decreased atherosclerotic lesion formation in *Ldlr*$^{-/-}$ mice, suggesting a pro-atherogenic effect of Th2 cells (184). Furthermore, in angiotensin II-induced atherosclerotic *ApoE*$^{-/-}$ mice, IL-4 administration showed no effect on plaque development (185). In summary, IL-4 exerts different effects on the development of atherosclerotic plaques. Unlike IL-4, IL-5 and IL-13 have a defined atheroprotective effect. IL-5 induces B1 cells to produce atheroprotective natural IgM antibodies specific for oxLDL. Second, IL-5 deficiency enhances plaque formation (72). Similarly, plaque development is accelerated by IL-13 deficiency, and IL-13 exerts atheroprotective effects by increasing collagen content, reducing monocyte recruitment, and inducing M2-type macrophage polarization (133). Furthermore, ILC2 cells, which are thought to correspond to Th2 cells, have been found to perform class II MHC restricted antigen presentation and to orchestrate Th2-like immune responses (186). IL-25 as well as IL-33 induced by Th2 cells can promote the proliferation of ILC2 cells, and the activation of ILC2 cells in turn promotes the Th2 response, thereby reducing the burden of atherosclerosis (187). In conclusion, the exact role of Th2 cells in atherosclerosis is still unclear. Possible reasons for this include different research teams have various experimental designs, study methods, and focuses; cytokines produced by Th2 cells, especially IL-4, exhibit complex biological properties; most studies have been conducted in animal models, but animal models of atherosclerosis have certain limitations; and there are also complex interactions between immune cells, and Th2 may synergize with or antagonize other immune cells, thereby affecting its role in atherosclerosis. More in-depth and comprehensive studies are needed to elucidate the role of Th2 in atherosclerosis.

The Th17 subset has been identified in atherosclerosis-prone mouse and human atherosclerotic plaques and its role remains controversial (188). Differentiation of Th17 cells is mediated by retinoid-related orphan nuclear receptor (ROR)γt (189) and other transcription factors such as RORα, STAT3 (190), Aryl hydrocarbon receptor, and runt-related transcription factor 1 (191, 192). Unlike classical Th1 and Th2 cells, Th17 cells exhibit significant heterogeneity due to the unstable expression of RORγt and its property of being influenced by environmental cues. Th17 cells mainly secrete the cytokine IL-17. The effect of IL-17 in atherosclerotic plaques would seem to be complex. IL-17 further exerts pro-atherosclerotic effects by inducing IL-6, CXCL8 and CXCL10, and GM-CSF in VSMCs to promote inflammatory activation (193). On the other hand, the atheroprotective effect of IL-17 is associated with inhibition of the pro-atherogenic factor IFN-γ and production of the anti-atherogenic factor IL-10, as well as suppression of the expression of vascular cell adhesion molecule 1 (VCAM-1) leading to a reduction in leukocyte recruitment within the lesion (188). Due to the complexity of IL-17's action, several related experimental studies have yielded different results. Experiments with functional blockade of IL-17 have demonstrated that IL-17 has pro-atherosclerotic effects, and that inhibition of IL-17 reduces plaque development, decreases plaque vulnerability, and reduces leukocyte infiltration (194). Another study showed that IL-17 exerts atheroprotective effects by promoting collagen cap formation and stabilizing plaques (195). In conclusion, Th17 plays a complex role in atherosclerosis, as demonstrated by its ability to both promote atherosclerosis and inhibit its development.

Tregs are a highly heterogeneous population of $CD4^+$ T cells with immunosuppressive functions. In the pro-inflammatory microenvironment formed by pro-inflammatory factors such as IL-1β, IL-2, and IL-23, $CD4^+FOXP3^+$ T cells co-express FOXP3 and RORγt transcription factors and are able to produce IL-17 upon stimulation (196). IL-1β and IL-2 induce the differentiation of the initial $FOXP3^+$ Tregs into Th17 cells, and similarly IL-23 is able to differentiate Tregs into Th17 cells through inducing a high level of RORγt expression and enhancing the loss of FOXP3 (197, 198). Tregs have been clearly demonstrated to be atheroprotective, with reduced numbers and impaired suppressive function linked to the evolution of atherosclerotic plaques (199). The amount of Tregs was reduced in mice that developed atherosclerosis compared to *ApoE*$^{-/-}$ mice in which no atherosclerotic plaques were detected (200). In a functional study of $CD4^+FOXP3^+$ Tregs, it was found that when Tregs were depleted by anti-CD25 antibodies, plaque vulnerability increased, and lesion progression was accelerated (201). Despite the small number of $CD4^+FOXP3^+$ Tregs found in mouse and human plaques, their secretion of cytokines, such as TGF-β and IL-10, has a profound impact on plaque evolution. In *ApoE*$^{-/-}$ mice, specific blockade of TGF-β signaling promotes atherosclerosis, as evidenced by increased macrophage activation, reduced collagen, increased plaque size, and increasing instability. In contrast, TGF-β overexpression reduced plaque vulnerability and atherosclerosis

(202). Similarly, IL-10-deficient mice were found to have a substantially increased susceptibility to atherosclerosis compared to normal mice, as evidenced by increased T cell infiltration, high IFN-γ expression, and reduced collagen content (203). Overall, Tregs exert atheroprotective effects on atherosclerosis by secreting cytokines to inhibit plaque-associated inflammatory responses.

Using scRNA-seq screening methods, Winkels et al. (204) found five T cell populations in *ApoE*$^{-/-}$ mice; Gu et al. (205) identified three T cell populations; and Cochain et al. (150) detected four T cell populations in *Ldlr*$^{-/-}$ mice. Recently, Winkels et al. (206) synthesised most of the available scRNA-seq studies and outlined four T cell phenotypes that have been more consistently studied: *Cxcr6*-expressing T cells, where CXCR6 is expressed by CD4$^+$ T cells and NKT cells and is able to direct T cell homing. The overall lack of CXCR6 reduces atherosclerosis and T cell accumulation (52). Naive T cells, expressing *Cd28*$^+$*Ccr7*$^+$, increase IL-7 signaling and support T cell survival, differentiation as well as proliferation in more advanced atherosclerosis (207). *Cd8*$^+$ cytotoxic T cells, in scRNA-seq, about 31% of the T cells were identified as cytotoxic T cells based on the genotype and diet of the tested mice. The cells mainly expressed *Cd8a/b*,*Nkg7*, *Ms4a4b*, *Ccl5* and *Gzmk*. Depletion of CD8$^+$ T cells promotes atherosclerosis and reduces plaque stability (204). Another population of T cells that express both CD4 and CD8 or neither may represent thymocyte-like T cells (204), but this is still controversial.

5.4 B cell heterogeneity

B cells are heterogeneous populations of lymphocytes derived from bone marrow and composed of multiple subsets of cells with distinct localization properties, activation requirements, survival characteristics, and immunoglobulin secretion profiles (64). In mouse atherosclerosis, B cells are subdivided into two main subsets, B1 and B2, where B1 cells are divided into CD5$^+$ B1a cells and CD5$^-$ B1b cells according to the expression of the leukocyte differentiation antigen CD5, and B2 cells contain follicular (FO) and marginal zone (MZ) B cells. CyTOF, and scRNA-Seq also confirmed the presence of B1- and B2-like cells in mouse atherosclerotic vessels (204).B1-like cell cluster is enriched for B1 cell genes (*Tppp3*, *S100a6*, and *Cd9*), and the B2-like cell cluster shares gene expression with germinal center and MZ B cells, for example, *Fcer2a* and *Cd23* (147).Various subsets of B cells can contribute to diverse, and sometimes contradictory roles in the development of atherosclerotic plaques.

B1 cells are intrinsic immune cells that are usually found in plasma membrane cavities such as the pleural and peritoneal cavities. Without the need for Th cells, both B1a and B1b cells can secrete IgM under antigenic stimulation, which specifically binds to LDL oxidizing epitopes in atherosclerotic plaques and reduces lipid uptake by macrophages, thus decreases the formation of foam cells. At the same time, IgM specifically binds to apoptotic cells so that inflammatory cells can be eliminated, thus slowing down atherosclerosis progression (64). B1a cells undergo migration to the spleen and differentiate into GM-CSF-producing innate response activator (IRA) B cells stimulated by LPS. In atherosclerotic mice, IRA-B cells congregate in large numbers in secondary lymphoid tissues, activate the Th1 immune response, stimulate extraparenchymal hematopoiesis, and activate DCs to promote atherosclerotic plaque formation (208). Kyaw et al. (209) found that splenectomy in *ApoE*$^{-/-}$ mice resulted in a decrease in peritoneal B1a cells as well as a substantial reduction in plasma IgM levels; however, transfer of B1a cells to splenectomized mice was effective in attenuating atherosclerotic lesions and reducing plaque necrotic cores and apoptotic cells. However, the role of B1b cells in atherosclerosis remains uncertain and necessitates additional investigation. Overall, B1 cells exert a protective effect against atherosclerosis primarily through the secretion of natural IgM antibodies that bind oxLDL and apoptotic cells.

B2 cells are what we commonly refer to as mature B lymphocytes, produced in the bone marrow and differentiated in secondary lymphoid tissues. Preliminary studies on the role of B2 cells in atherosclerosis have demonstrated their pro-atherogenic effect. The use of a CD20-specific monoclonal antibody to selectively remove B2 cells, but not B1a cells, from *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice reduced atherogenesis and progression of atherosclerosis (73, 74). In addition, the fact that B2 cell depletion was correlated with the reduction of activated splenic CD4$^+$ T cells, T cells proliferation, and diseased T cells suggests that B2 cells through a T cell-dependent mechanism exacerbate atherosclerosis (210). Consistent with the effects of B2 cell depletion, Kyaw et al. (74) found that the introduction of splenic B2 cells, but not B1 cells into lymphocyte-deficient recombinase activating gene 2 (*Rag2*)$^{-/-}$*γ-chain*$^{-/-}$*ApoE*$^{-/-}$ or B-cell-deficient/*ApoE*$^{-/-}$ mice through adoptive transfer exacerbated atherosclerosis. The pro-atherogenic effect of B2 cells was further demonstrated by studies of B cell-activating factor receptor (BAFFR)-deficient effects in atherosclerosis-prone mice. Being a member of the tumor necrosis factor receptor family, BAFFR plays a crucial role in the maintenance of mature B2 cells (211). In a separate study, Kyaw et al. (212) showed that selective inhibition of BAFFR with an anti-BAFFR antibody resulted in depletion of B2 lymphocytes and reduction of atherosclerosis in *ApoE*$^{-/-}$ mice. Collectively, these studies provide clear evidence that substantial reduction in B2 cell count mitigates the progression of atherosclerosis. In contrast to Kyaw et al., however, Doran et al. (213) discovered that adoptive transfer of *ApoE*$^{-/-}$ mouse splenic B2 cells into cholesterol-fed μMT/*ApoE*$^{-/-}$ mice significantly reduced the area of atherosclerotic plaques in mice. This discrepancy may be due to differences in the ratio of FO to MZ B cells or in the genetic background of the mice. Further research has confirmed the specific role of FO and MZ B2 cell in atherosclerosis. Namely, FO B cells promote atherosclerosis primarily through IgG production and activation of Th1 cells (214). Similarly, MZ B cells promote atherosclerotic plaque progression by secreting IgG, activating follicular helper T (Tfh) cells, and inducing the expression of inflammatory factors such as IFN-γ (215).

The variety of functions performed by B cells, such as antibody production, cytokine release, and antigen presentation, as well as the unique ways in which B cells adapt to the inflammatory microenvironment of plaques, have led to different B cell subsets exhibiting complex heterogeneity in the evolution of atherosclerotic plaques.

6 Immunotherapy for atherosclerosis

The evolution of atherosclerotic plaques is regulated by both the immune system and inflammatory responses, and the study of immune as well as anti-inflammatory based therapies for atherosclerosis is of great clinical importance. Canakinumab is a human monoclonal antibody (mAb) that binds to IL-1β and blocks the interplay of IL-1β with IL-1R, preventing the inflammatory response from occurring. IL-1β plays multiple roles in the atherosclerotic process, including promoting adhesion of immune cells to vascular endothelial cells, promoting proliferation of VSMCs, and inducing procoagulant activity (216). A large-scale, double blind, randomized clinical trial, the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS), has confirmed for the first time the inflammatory hypothesis of atherosclerosis (217). By dividing 10,061 atherosclerotic patients with prior myocardial infarction and a high-sensitivity C-reactive protein (hs-CRP) ≥2 mg/L in 39 countries into a placebo group and three different doses of canakinumab, administered subcutaneously every three months. The risk of canakinumab on primary endpoint events including nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death was assessed. Ultimately, the 150 mg and 300 mg doses of canakinumab were found to reduce patients' levels of hs-CRP and other inflammatory markers, the risk of cardiovascular events, and the incidence and mortality of selected cancers (218). It is worth noting that the use of canakinumab is accompanied by certain risks, and further studies are needed to investigate its precise mechanism of action as well as its safety and efficacy in different patients. Overall, the CANTOS study opens up new avenues for immunotherapy in cardiovascular disease.

Advances have also been made in the study of antibody immunotherapies targeting other cytokines that play pro-inflammatory roles in plaque evolution, as well as chemokines and their receptors that also have important roles. In patients with psoriatic arthritis, the use of anti-TNF-α antibodies significantly reduced the size of carotid plaques and inhibited plaque development (219). Additionally, adalimumab treatment steadily reduced the levels of E-selectin, hs-CRP, and IL-22, suppressed systemic inflammation, and decreased the risk of atherosclerosis (220). At chronic kidney disease patients, the anti-IL-6 antibody remarkably decreased biomarkers of atherosclerotic inflammation and thrombosis (221). Blocking the binding of CCR2 to its ligand CCL2 with the highly specific MLN1202 in patients with atherosclerotic cardiovascular risk reduced the levels of hs-CRP (222).

Anti-programmed cell death protein 1 (PD-1) mAb has recently been found to exhibit potential therapeutic efficacy in the treatment of atherosclerosis. There are activated subsets of PD-1^+ T cells in the plaques that act as pro-inflammatory (223). PD-1 belongs to the immunoglobulin superfamily and is mainly expressed on the surface of T cells. It usually binds to its ligands PD-L1 and PD-L2, mediating immune suppression signals. It plays an important role in regulating T cell functions such as proliferation, survival, cytokine production and other effector functions and maintaining immune system homeostasis (224). A latest study by Fan et al. (225) found that the use of an anti-PD-1 mAb that binds to Fc was effective in reducing the size of human atherosclerotic plaques. The potential mechanism of action is that the Fc-binding ability of the anti-PD-1 mAb enables it to be trapped by the Fcγ receptors, which then interact with PD-1 expressed on the surface of T cells as an alternative PD-1 ligand to inhibit PD-1^+ T-cell function in atherosclerotic plaques. This finding suggests that T cell- targeting immunotherapy could be a novel strategy to address atherosclerosis. Here we have only reviewed relevant antibody immunotherapies that have been used in human clinical trial studies (Table 2). It is known that quite a number of immune molecules show considerable therapeutic prospects in animal models. We believe that future studies will reveal more targets and translate them into clinical applications, providing more options and possibilities for the treatment of atherosclerosis.

Antigen-antibody reactions play a pivotal role in the evolution of plaques. Therefore, the use of immunomodulatory strategies to activate immune responses against relevant antigens has the potential to alter the natural course of atherosclerosis. The purpose of vaccination is to prevent the progression of atherosclerosis by stimulating the body to produce antibodies that block the target antigen. What has received earlier attention from researchers is LDL-related vaccines. OxLDL is thought to be critical in causing intimal inflammation and foam cell formation in atherosclerosis. One of the most studied is the specific malondialdehyde-modified apolipoprotein B100 (MDA-ApoB100), which is a relatively important oligopeptide fragment of oxLDL molecules with strong antigenicity (226). Fredrikson et al. (227) reported that *ApoE*$^{-/-}$ atherosclerotic mice vaccinated with a peptide vaccine of the MDA-ApoB100 fragment showed a reduction of atherosclerotic plaques by up to 60%, and a significant increase in collagen content in the residual plaques, and a decrease in macrophages, which increased the stability of plaques to some extent and inhibited plaque evolution. In addition to more studies on oxLDL-related vaccines, the results of cholesterol transporter protein (CETP) vaccine studies have been promising. The primary function of CETP in atherosclerosis is to regulate the transport of high-density lipoprotein (HDL) cholesterol to LDL cholesterol (228). CETP-associated vaccines play a role in inhibiting plaque development through mechanisms such as elevating HDL levels, decreasing LDL levels to promote reverse cholesterol transport, and reducing plaque burden (229). Other vaccines such as CD40L and PCSK9 have been found to play an inhibitory role in atherosclerotic plaque evolution (230–233). Plaque evolution is a complex, multifactorial process. From the perspective of developing an anti-atherosclerotic vaccine, not only can we explore the impact of immunotherapy on the mechanism of plaque evolution, but also contribute substantially to the prevention and treatment of atherosclerosis.

Inflammation is a well-studied therapeutic target, and strategies to control inflammation have been successfully applied against many diseases. After many studies it appears that inflammation has also emerged as an important target for the treatment of atherosclerosis. Anti-inflammatory therapeutic agents for atherosclerosis have been extensively studied in recent years. Among the most widely researched and used drugs are statins, which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A

TABLE 2 Antibody immunotherapy for human atherosclerosis.

Antibody Type	Study	Mechanism	Effect on atherosclerosis	Reference
Anti-IL-1β (e.g. canakinumab)	Atherosclerotic patients (prior myocardial infarction and hs-CRP ≥2 mg/L)	Block the interaction of IL-1β with IL-1R; Reduce hs-CRP and IL-6 levels	Reduce atherosclerosis; Decrease risk of primary endpoint events	(218)
Anti-TNF-α (e.g. adalimumab)	Psoriasis patients (atherosclerotic plaques)	Suppress systemic inflammation	Inhibit plaque progression; Lower risk of atherosclerosis	(219, 220)
Anti-IL-6 (e.g. ziltivekimab)	Chronic kidney disease patients (hs-CRP ≥ 2 mg /L)	Reduce hs-CRP levels	Reduce atherosclerotic inflammation; Reduce thrombosis	(221)
CCR2 mAb (e.g. MLN1202)	Patients at high risk for ACD (≥2 risk factors and hs-CRP >3 mg/L)	Block the binding of CCR2 to its ligand CCL2;Reduce serum CRP levels	Decrease the risk of ACD	(222)
Anti-PD-1 mAb	Tumor patients (atherosclerotic plaques)	Inhibit the function of PD-1$^+$ T cells	Reduce atherosclerotic plaque size	(225)

reductase and are usually used to treat atherosclerosis. They are the drugs of choice for clinical lipid-lowering, with commonly used drugs such as rosuvar, atorval, and simvastatin calcium tablets. Statins exert various anti-inflammatory effects in addition to their lipid-lowering properties. Statins can inhibit macrophage growth, reduce the level of pro-inflammatory cytokines, downregulate the expression of adhesion molecules and chemokines, and prevent monocyte recruitment (234), as well as inhibit T cell activation and attenuate inflammatory responses through direct binding to lymphocyte function-associated antigen-1 (235). Furthermore, statins promote the stabilization of plaques by decreasing MMP expression and inhibiting tissue factor to reduce the risk of thrombotic events (236). In summary, statins can prevent, reduce, and even reverse atherosclerotic plaque burden. Reducing inflammation may be a key mechanism by which statins alter plaque biology and slow disease progression.

In addition to statins, there are several common anti-inflammatory drugs that are also used to treat atherosclerosis. Colchicine is utilized for treating inflammatory diseases such as atherosclerosis due to its anti-inflammatory and antifibrotic activity. Its anti-inflammatory mechanism is mainly through downregulation of multiple inflammatory pathways including inhibition of phospholipase A2, reduction of leukotriene B4 and prostaglandin E2 release from monocytes, inhibition of neutrophil function through microtubule proteins, and reduction of endothelial adhesion, which leads to inhibition of inflammation and increase of plaque stabilization (237–239). Methotrexate is a better non-specific anti-inflammatory agent for treating atherosclerosis due to its lesser effect on the levels of atherosclerosis-related cytokines. Methotrexate performs its anti-inflammatory effect by inhibiting folate metabolism and reducing T-cell proliferation (240). In addition, methotrexate can regulate the expression of intercellular cell adhesion molecule-1, E-selectin, and VCAM-1, suppress cyclooxygenase and lipoxygenase, decrease C-reactive protein levels, and regulate the secretion of IL-6, TNF-α, and metalloproteinases, thereby regulating plaque evolution (241). In summary, targeting inflammatory pathways to prevent and treat atherosclerosis is a promising new avenue.

7 Conclusion

Atherosclerosis is defined as an inflammatory disease, with inflammatory responses throughout the disease progression, and inflammatory mediators, cytokines, through inflammatory signaling pathways exerting different roles in the regulation of the plaque inflammatory microenvironment. Many studies have clearly demonstrated that immune responses mediate the entire process of atherosclerotic plaque evolution, including initiation, progression, and thrombotic complications. Crosstalk between innate and adaptive immune pathways strongly regulates plaque activity and progression, while the heterogeneity of immune cells plays a pivotal role in plaque evolution.

The evolution of atherosclerosis involves complex interactions of immune cells as well as phenotypic plasticity. Omics studies, especially scRNA-seq, have highlighted the specific transcriptional profiles of various cell lineages at the site of atherosclerotic lesions. The detailed map of the cells revealed not only enhances our understanding of the heterogeneity of different cells in the plaque environment, but also deepens our comprehension of the mechanisms of disease occurrence and progression and provides a solid scientific basis for the development of novel precision therapeutic strategies. By combining high-throughput omics data with experimental studies to analyze the gene expression profiles and functional properties of specific cell subsets, it is possible to develop targeted drugs against specific cell subsets or the immune molecules they produce, such as immune checkpoint inhibitors against PD1$^+$ T cells (225), antibodies against pro-inflammatory cytokines or chemokines (218–222). Gene expression products and functional markers may also become important biomarkers for assessing atherosclerosis disease, predicting progression and monitoring treatment effects, and the development of these biomarkers could help to achieve early diagnosis and precise treatment of the disease. Furthermore, adjusting the dose and type of therapeutic drugs according to the number of immune cells and the level of cytokines produced, and developing a more personalized treatment plan can improve the effectiveness of treatment and reduce adverse reactions. In addition,

understanding the interactions between different cell subsets and exploring the possibility of combining drugs, such as an integrated treatment strategy combining lipid-lowering and anti-inflammatory drugs, has shown certain efficacy in clinical trials (242, 243), with a view to achieving breakthroughs in reducing plaque formation, stabilizing lesions and preventing MACEs.

However, the vast majority of investigations into the pathophysiologic mechanisms of atherosclerotic plaque pathology have originated from experimental animal models, and there are significant barriers to translating interventions from animal models to the clinic, primarily because mouse models reproduce human atherosclerosis to a very limited extent. It is encouraging that the integration of bioinformatics-, transcriptomics-, and proteomics-based datasets will help us to define the immune profile of human atherosclerosis in a more comprehensive manner. As multidimensional approaches continue to progress, it is expected that the cellular and molecular mediators associated with human atherosclerotic plaques will be characterized in depth, and new potential therapeutic targets will undoubtedly emerge. It is our firm belief that our understanding of immune-mediated processes in atherosclerosis will continue to evolve, and the specific mechanisms of atherosclerotic plaque evolution will be further resolved from an immunologic perspective.

Author contributions

HP: Writing – original draft. GW: Writing – original draft. YW: Writing – original draft. MZ: Writing – original draft. QH: Writing – original draft. XZ: Writing – review & editing. KY: Writing – review & editing. GZ: Writing – review & editing. TJ: Writing – review & editing.

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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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(Multi-) omics studies of ILC2s in inflammation and metabolic diseases

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Type 2 innate lymphoid cells (ILC2s) have emerged as pivotal regulators in the pathogenesis of diseases, with their roles in inflammation, metabolism, and tissue homeostasis becoming increasingly recognized. This review provides an overview of the current understanding of ILC2s in inflammation and metabolic disorders, including their functional contributions. Moreover, we will discuss how these cells adapt their metabolic processes to support their function and survival and how their metabolic requirements change under different physiological and pathological conditions. Lastly, we will review recent omics studies that have provided insights into the molecular and cellular characteristics of ILC2s. This includes transcriptomic, proteomic, and metabolomic analyses that have elucidated the gene expression profiles, protein interactions, and metabolic networks, respectively, associated with ILC2s. These studies have advanced our understanding of the functional diversity of ILC2s and their involvement in metabolic disease.

KEYWORDS

ILC2, metabolism, metabolic diseases, omics, single-cell RNA sequencing

Introduction

Innate lymphoid cells (ILCs) belong to a newly discovered, rare cell population of the innate immune system. In the past years, it has become clear that they exert important functions in host defense against invading pathogens, in guiding immune reactions and in tissue immune homeostasis (Kim et al., 2021; Spits et al., 2013; Walker et al., 2013). Since their discovery in 2010, several subsets have been characterized based on their phenotype and functional properties (Artis and Spits, 2015; Spits and Cupedo, 2012). ILCs develop from a common lymphoid progenitor (CLP) that gives rise to group 1 ILCs (ILC1s), ILC2s, and ILC3s. Due to the similarities of transcription factor and lineage-specific cytokine expression to T helper cells, they are considered to be their innate counterparts (Spits et al., 2013). For example, T-bet and interferon (IFN)-γ are expressed by both ILC1s and Th1 cells. While for Th2 cells, shared GATA binding protein 3 (GATA3) expression and type-2 cytokine production (Interleukin (IL)-4, IL-5, and IL-13) makes ILC2s their innate counterparts, ILC3s require RAR-related orphan receptor gamma t (RORγt) for their

development and are able to produce Th17- and Th22-like cytokines and are therefore thought to be the innate equivalent of Th17 and Th22 cells.

Among ILCs, ILC2s are important mediators of tissue remodeling and immune homeostasis by orchestrating immune crosstalk between cells of the innate and adaptive immune system (reviewed in (Kral et al., 2023)). These cells mainly reside at mucosal barriers or within tissues, and therefore serve as first line of defense for invading pathogens. Here, release of the key activating epithelial cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) leads to the activation of ILC2s. However, the expression of their receptors (IL-25R, ST2, and TSLPR, respectively) varies across different tissues and other activating cytokines have been identified depending on the localization (Ricardo-Gonzalez et al., 2018). For instance, it has been shown that skin ILC2s express low levels of these receptors and are rather activated by IL-18 (Ricardo-Gonzalez et al., 2018). Importantly, ILC2 are also located in metabolically active tissues, such as the adipose tissue, where they are essential in maintaining immune homeostasis in the tissue (Moro et al., 2010; Sasaki et al., 2019). This is ensured by the secretion of IL-5 and IL-13, resulting in the recruitment of anti-inflammatory M2 macrophages and eosinophils.

Dysfunction of ILC2s has been implicated in the development of various diseases. Due to their local availability in metabolic active tissues, several studies pointed towards a role of impaired ILC2 activity in the pathogenesis of metabolic diseases (Brestoff et al., 2015; Michailidou et al., 2022; Molofsky et al., 2013). Such diseases are characterized by disrupted energy metabolism and include Type 2 Diabetes (T2D), hyperthyroidism and hypothyroidism, and the metabolic syndrome, among others. Importantly, patients suffering from the metabolic syndrome have an increased risk of developing atherosclerotic cardiovascular disease (ACVD) (Li et al., 2021). ACVD is characterized by plaque buildup in the artery walls due to accumulation of lipids and immune cells, resulting in enhanced secretion of pro-inflammatory mediators at the site of inflammation (Bullo et al., 2003). As a result, thickening of these vessels can, for example, cause coronary heart disease, cerebrovascular disease, depending on the location. Lifestyle modifications can reduce the risk for this chronic inflammatory condition; however, CVDs are still one of the leading causes of death. Current medical interventions only treat the symptoms but not the origin of the disease. Therefore, a better understanding of the underlying mechanisms leading to this condition could critically contribute to the development of more specific, beneficial preventive and therapeutic approaches.

In recent years, the perivascular adipose tissue (PVAT) surrounding the artery vessel walls, has been recognized as important regulator of vascular biology. In a healthy state, PVAT regulates vascular tone and intravascular thermoregulation (Stanek et al., 2021). However, sustained inflammatory conditions contribute to an inflammatory milieu by the release of pro-inflammatory mediators (e.g., TNF, IL-6) (Stanek et al., 2021; Guzik et al., 2006; Guzik et al., 2007). Moreover, reactive oxygen species (ROS) production in response to increased oxidative stress in PVAT leads to damage of endothelial cells (Barp et al., 2021). In addition, dysregulation of vascular tone under these conditions can cause an increase in blood pressure (Ma et al., 2023). Collectively, PVAT dysfunction has been shown to be linked to cardiovascular and metabolic disorders. Notably, ILC2s are highly abundant in PVAT and due to their anti-inflammatory properties, they might impact vascular health (Newland et al., 2017). Accordingly, increased release of pro-inflammatory cytokines in dysfunctional PVAT critically modulates ILC2 functions by reducing their ability to produce IL-5 and IL-13. Therefore, the interaction of ILC2s and PVAT is an important factor in the development of chronic vascular inflammation leading to CVDs. However, this research area is still emerging, and more studies are necessary to fully understand the mechanisms of ILC2-PVAT interactions in the pathogenesis of CVDs.

Hence, dysregulated functions of ILC2s in regulating metabolic homeostasis in adipose tissue (AT) can critically contribute to obesity and related metabolic disorders. Therefore, the next section will focus on ILC2 metabolism and how its dysregulation impacts inflammation and the progression of diseases.

ILC2 metabolism

In this regard, the field of "immunometabolism" has gained a lot of attention in the past years. Importantly, immune cells have an increased demand of energy supply for their functionality, differentiation, and survival and lack of this supply might impact the development of diseases (Aderinto et al., 2023). It is thus important to gain a better understanding of immune cell metabolism in order to implement these findings for novel therapeutic strategies of immune response modulation.

In order to be functional, ILC2s rely on a high metabolic state to fuel their activity. For most immune cells glucose is the primary source for their activation and effector functions in producing cytokines (Soto-Heredero et al., 2020). Intriguingly, depending on the activation state and the environmental milieu, ILC2s can also use mitochondrial oxidative phosphorylation (OXPHOS) to meet their metabolic demands (Surace et al., 2021). Alternatively, ILC2s can also depend on fatty acid oxidation (FAO) in order to provide a sustained source of ATP required for their survival (Wilhelm et al., 2016) (Figure 1A).

Even though our understanding of immune metabolism of ILC2s is still in its infancy, several studies have already revealed key metabolic pathways for their activity. First studies showed that upon helminth infection ILC2s increase their demand on FAO in order to produce IL-13 (Wilhelm et al., 2016). Conversely, under steady state conditions ILC2s do not rely on FA metabolism for maintaining their numbers (Wilhelm et al., 2016). Moreover, it could be demonstrated that ILC2s use arginine and branched-chain amino acids to fuel their energetic demands upon proliferation and activation (Monticelli et al., 2016). In this regard, a study found evidence that the enzyme arginase-1 (Arg1) regulates ILC2 metabolism during acute and chronic lung inflammation (Karagiannis et al., 2020) (Figure 1B). More specifically, the amino acid transporter SLC7A8 has been identified as important regulator of key metabolic pathways in ILC2s and that its deficiency in lymphocytes reduced type 2 immune responses to helminth infection (Panda et al., 2022; Hodge et al., 2023).

These different energy sources are used depending on the local fluctuations of metabolic nutrients and thus, ILC2s have to adapt to such environmental changes. Moreover, ILC2s reside in various

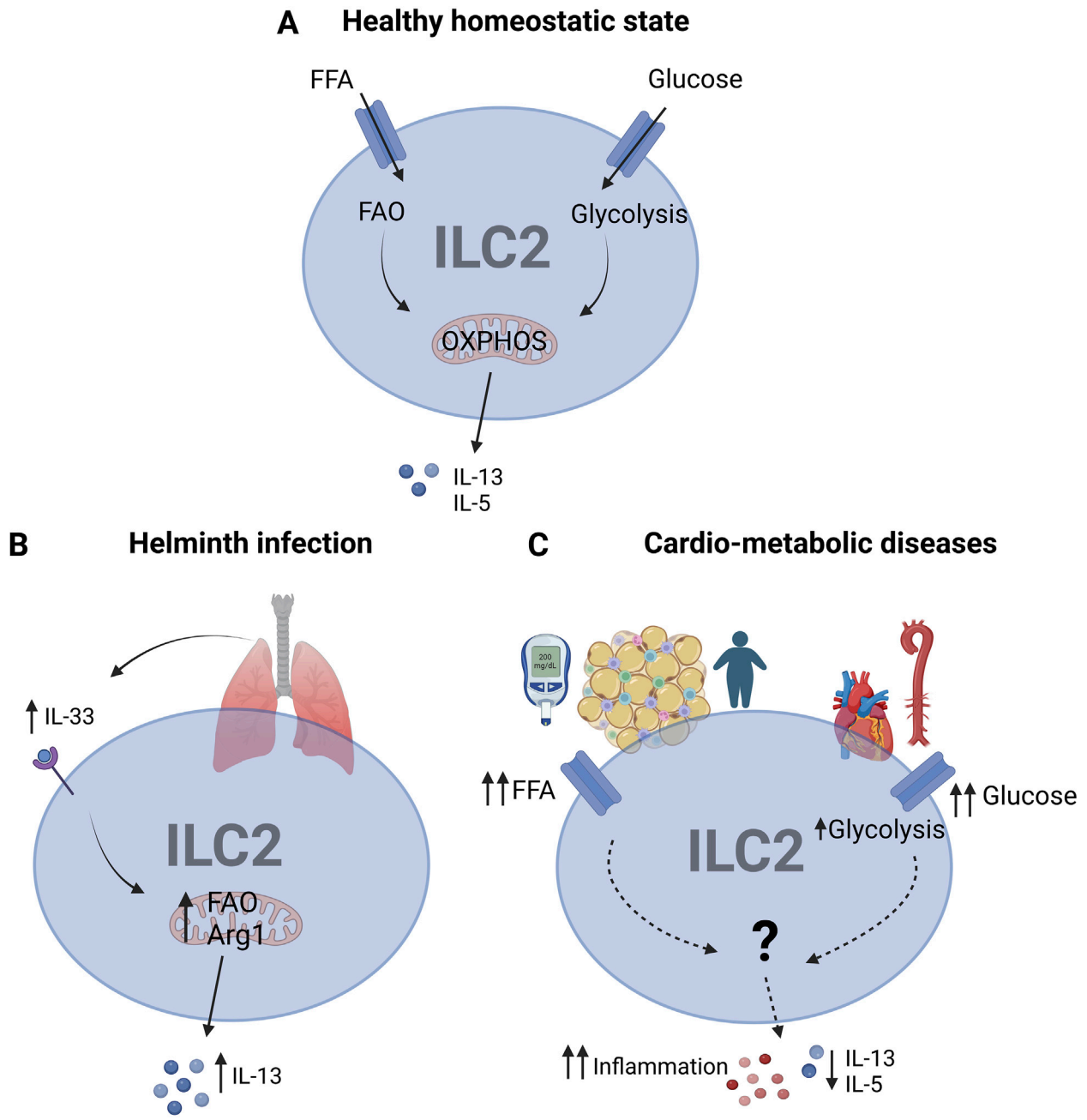

FIGURE 1
ILC2 metabolism in healthy and diseased state **(A)** In healthy homeostatic state, ILC2s use glycolysis and FAO to fuel OXPHOS for the production of IL-5, IL-13 cytokines to maintain their survival. **(B)** Upon helminth infection in the lung, IL-33 is released which in turn activates ILC2s, leading to FAO and Arg1 activity for IL-13 production in order to enhance the anti-inflammatory and tissue-repair functions of ILC2s. **(C)** In metabolic disorders, impaired metabolism due to high glucose and free fatty acids (FFA), ILC2 metabolism is disrupted, partly leading to increased glycolysis to manage the excess of glucose Abbreviations: Arginase-1 (Arg1), Fatty acid oxidation (FAO), Free fatty acids (FFA), Innate lymphoid cells type 2 (ILC2s), Interleukin (IL), oxidative phosphorylation (OXPHOS). Made with biorender.com. The "?" indicates that there is a yet unknown mechanism.

tissues such as skin, lung, and AT which have tissue-specific nutrients available (Yu et al., 2022). This flexibility and adaptive capacity upon metabolic changes of ILC2s makes them an attractive candidate for the development of therapeutic interventions. Previous studies could highlight how ILC2s are able to respond to the availability of different nutrients in a tissue and disease-specific context (Yu et al., 2022). Accordingly, their metabolic state has been implicated in different metabolic diseases as detailed below.

ILC2 metabolism in disease

- Cardio-metabolic disorders

Metabolic disorders are a result of disrupted energy metabolism, leading to diseases such as obesity and T2D. Diet-induced obesity is a complex condition resulting from fat accumulation and its prevalence is rising dramatically, making it a growing epidemic

(Mitchell et al., 2011). Chronic low-grade inflammation that occurs in obesity affects multiple organs in the body, such as the AT, pancreas and the liver (Park et al., 2014). Since ILC2s are present in those tissues in order to maintain immune homeostasis, they have been described to play a role in these metabolic disorders. In 2015, ILC2s have been identified in human white adipose tissue (WAT), where they are involved in maintaining metabolic homeostasis (Odegaard and Chawla, 2015). Importantly, Brestoff *et al.* could link decreased ILC2 responses in WAT with obesity in both mice and men (Brestoff et al., 2015). Specifically, IL-33 induces the recruitment of uncoupling protein 1 (UCP1) positive beige adipocytes in WAT. This process is referred to as "beiging" or "browning" to regulate caloric expenditure (Cohen et al., 2014; Harms and Seale, 2013; Shabalina et al., 2013) and IL-33-induced beiging was dependent on ILC2s (Brestoff et al., 2015). Similarly, one study found that the lack of ST2, the receptor for IL-33, led to a decrease of ILC2s in WAT (Okamura et al., 2021). Intriguingly, ST2 deficiency on ILC2s increased the numbers of ex-ILC2s, resembling ILC1-like features, which is associated with decreased beiging in WAT and concomitant impairments in energy metabolism (Okamura et al., 2021). These studies highlight the importance of ILC2s in controlling metabolic homeostasis in AT.

Furthermore, chronic obesity can cause the development of several obesity-associated diseases. T2D develops as a result of persistently increased blood glucose levels, eventually causing damage to other organs (Galicia-Garcia et al., 2020). Notably, hyperglycemia in patients with T2D can also cause metabolic stress on ILC2s, consequently altering their cellular metabolism (Painter and Akbari, 2021) (Figure 1C). In this regard, one group found that liver ILC2s are involved in regulating glucose homeostasis (Fujimoto et al., 2022). However, studies in the context of CVDs still need to be published. Similarly, alterations in lipid metabolism in T2D, e.g., enhanced levels of free fatty acids (FFA) can modulate ILC2s (Painter and Akbari, 2021) (Figure 1C). Here, the energy sensor mammalian target of rapamycin (mTOR) controls the enzymes peroxisome proliferator activated receptor gamma (PPARγ) and diacylglycerol-O-acyltransferase 1 (DGAT1) in storing FFAs in droplets within the cells (Karagiannis et al., 2020). Consequently, impaired lipid processing leads to accumulation of FFA in adipose ILC2s, resulting in lipotoxicity.

Similar impairments in ILC2 metabolism can be observed in the context of CVDs (Roberts et al., 2022). Importantly, impaired lipid metabolism drives a metabolic shift to increased glucose intake as a compensatory mechanism (Figure 1C). Current understanding suggests that this immunometabolic shift might impact the functionality and survival of ILC2s, thereby exacerbating tissue inflammation in CVDs (Yu et al., 2022). Therefore, tight control of glucose and fatty acid metabolism is essential for ILC2 function and survival. Even though impairments in ILC2s have been linked to CVDs, the mechanisms of their dysfunction remain poorly understood.

Targeting metabolic pathways of different cell types as novel therapeutic approach offers possibilities to fine-tune treatment efficacy and can help to restore normal metabolic functions. Therefore, such therapies hold significant promise for a variety of diseases. Since there are significant differences in energy production pathways in ILC2s depending on the tissue and disease context, a better understanding on how they fuel their metabolic needs is required. Here, data derived from "omics" studies will help to gain critical insights into the specific metabolic dependencies. Even though this research area is still in its infancy, several studies aimed at addressing altered metabolism in immune cells using omics data and will be detailed below.

ILC2 omics

- Transcriptional analysis

Transcriptomics analyses enable the study of gene expression profiles, and it can be applied to distinguish gene expression patterns between healthy and diseased tissues or cells. Here, RNA-sequencing (RNA-seq) offers a powerful tool for immune profiling by identifying specific markers on immune cells in a disease state that can be used as targets for therapeutic approaches. Moreover, this technique aids to identify novel immune cell subsets, including those that are present at low frequencies (Haque et al., 2017). With respect to ILC2s, their identification as well as characterization still remain a major challenge due to their similarities with other immune cells. To overcome this hurdle, one study in 2017 applied a novel multi-class gene expression-based model of single-cell RNA-seq data of natural killer (NK) and ILCs in order to identify ILCs in this mixed population (Suffiotti et al., 2017). For their analysis, the group used mouse gene expression data obtained from Robinette et al. (2015). In their study, they identified several subclusters of NK cells, ILC1s and ILC3s, whereas ILC2s were defined as the most distinguishable class ($CD127^+$, $Sca\text{-}1^+$, and $ST2^+$) isolated from the lamina propria in the small intestine of 6-week-old C57BL/6 male mice (Robinette et al., 2015). Due to that, in the multi-class gene expression-based model from 2017, ILC2s achieved the best predictions and can be studied at the gene expression level even though ILCs are present at a lower frequency as compared to NK cells (Suffiotti et al., 2017). Moreover, they were able to find ILC2s in a pool of bulk single-cell transcriptomics data by applying this new classifier. Even more striking, all of the predicted ILC2s represent actual ILC2s, showing that their model offers a reliable tool to accurately identify this specific cell-type. Thus, this model allows for a computation framework to study ILC2s in published datasets without the use of specific antibodies (Suffiotti et al., 2017).

In a study conducted in 2022, the technologies of RNA-seq and Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) have been combined to obtain information about the three-dimensional (3D) genome organization required for the development and function of murine ILC2s in steady state and during allergic aiway inflammation (Michieletto et al., 2023). With this approach, the authors could show how local spatial configuration of the genome significantly affects ILC2 biology. Specifically, they found that the development of ILC2s and the progression of allergic airway inflammation are governed by a distinctive 3D configuration which contained the ILC-lineage-defining factor *Id2* (Michieletto et al., 2023). Here, multiple interactions between the *Id2* promoter and distal regulatory elements bound by the transcription factors GATA3 and RORα reveal an important mechanism for ILC2 development and

homeostasis. Moreover, they found binding sites for transcription factors that are required for ILC2 function and development, such as ETS proto-oncogene 1, transcription factor (ETS1) and RUNX family transcription factor 1 (RUNX1). In conclusion, both studies provide an in-depth analysis to improve ILC2 identification and functions (Suffiotti et al., 2017; Michieletto et al., 2023).

Since ILC2s show a vast adaptation to environmental cues depending on the tissue, one group aimed at characterizing the impact of these stimuli (e.g., cytokine production, lipid mediators, nutrients or hormones) on ILC2 immunity in different human tissues of healthy donors (blood, tonsil, lung and colon) using single-cell RNA-seq (Mazzurana et al., 2021). The annotation of their identified ILC2 clusters was based on a previous study, which intended to analyze the heterogeneity of ILCs in human tonsils (ILC2: $Lin^{-}CD127^{+}CRTH2^{+}ST2^{+}CD25^{+}KLRG1^{+}GATA3^{+}$) (Bjorklund et al., 2016). In brief, they found tissue-specific signatures of ILCs with respect to migration, activation as well as modified metabolism. Interestingly, they could also reveal differentiation pathways from circulating and tissue-resident naive ILCs to fully differentiated ILC subsets (Mazzurana et al., 2021). Moreover, they were able to identify a novel subcluster of lung-resident ILC2s ($CRTH2^{-}$ ILC2). Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) has been shown to be required for ILC2 activation and cytokine production (Xue et al., 2014). However, in their study they observed a new acquired phenotype by ILC2s upon exposure to tissue-derived alarmins, suggesting that activated ILC2s in the lung do not depend on CRTH2 expression for their functionality (Mazzurana et al., 2021). In turn, pulmonary ILC2s also express CCR8, a receptor recently found to control the homeostasis of Tregs via non-canonical effects involving CCL17 and CCL3 (Döring et al., 2024; Puttur et al., 2019) Overall, this work enhances the potential of single-cell RNA-seq to characterize ILC2s in a tissue-specific manner. Importantly, their single-cell RNA-seq dataset together with two others was used to perform an integrative interference analysis of ILCs in order to assess their transcriptional imprinting across six different human tissues including healthy and diseased tissues (Song et al., 2023). They could identify four ILC2 subsets with different tissue distributions. Two subsets were identified as $c\text{-}Kit^{+}$ ILC2s and only occurred in hepatocellular carcinoma (HCC), resulting from plasticity in the tumor environment. In line with this, another study reported an increased frequency of the $c\text{-}Kit^{+}$ ILC2 population in HCC-derived supernatant with high IL-6 and TGFβ (Heinrich et al., 2022). One Killer cell Lectin-like receptor $G1^{+}$ ($KLRG1^{+}$) ILC2 subset was predominantly found in blood, and ILC2s expressing the tissue-residency marker $CD69^{+}$ showed the highest expression in tonsil. The latter subset also displayed high levels of regulators for an anti-inflammatory immune response (i.e., Dual Specificity Phosphatase 1 (*DUSP1)* and Early growth response protein 1 (*ERG1*)). Intriguingly, circulating ILC2s expressing KLRG1 from patients with allergic rhinitis did not produce the anti-inflammatory cytokine IL-10, which could be restored upon allergen immunotherapy (Song et al., 2023; Golebski et al., 2021). Such analyses are still lacking for metabolic and CVDs but would help to obtain a better understanding of the similarities and differences of ILC subsets in the different tissues and give important insights into their phenotypical characteristics and how they change in a diseased state.

In this regard, Jiang *et al.* conducted a study to reveal the role of infiltrating ILC2s in ischemic myocardium (Jiang et al., 2023). Specifically, they applied single-cell RNA-seq on heart tissues of 8-week-old male C57BL/6J mice that were subjected to myocardial infarction (MI) and myocardial ischemia-reperfusion injury (MIRI) (Jiang et al., 2023). Importantly, they could identify five ILC2 subsets - ILC1, ILC2a, ILC2b, ILCdc and ILCt, from which the last two were unique for heart tissue. ILC2a and ILC2b showed high expression of typical ILC2 markers (i.e., *Klrg1*, *Gata3*, *Rora*, *Il5*, *Amphiregulin (Areg)*). However, ILC2b expressed in addition to that *Stathmin 1 (Stmn1)*, *Baculoviral IAP Repeat Containing 5 (Birc5)* and *PCNA Clamp Associated Factor (Pclaf)*, which represent cell-cycle-associated genes. Moreover, they could find genes related to proliferation, overall suggesting that the ILC2b cluster is required for the proliferation 3 days after MI Interestingly, the ILCdc cluster showed expression of ILC-reg-related genes, such as Id3 (Jiang et al., 2023; Thomas and Peebles, 2022). Additionally, the ILCt cluster expressed signature genes of T cells, suggesting that this subset might act in parallel with T cells. Moreover, they published a ligand–receptor–transcription factor–target gene regulatory network to reveal crosstalk between the different ILC clusters (Jiang et al., 2023). By applying this network analysis, they revealed communications among the clusters and found that the cluster of ILCdc received signals from all other clusters, whereas ILCdc-derived messages only reached to itself and to ILC2b. This communication might be important to dissect the specific roles for each ILC subcluster in myocardial ischemia diseases. Furthermore, the identification of tissue- and disease-specific ILC2 subsets can be used as potential targets for the treatment of myocardial ischemia diseases by allowing a more specific targeting approach for the relevant ILC subset. Overall, such studies help to gain critical new insights into the functional adaptation of ILC2 at different tissue sites and in disease and reveal novel strategies for treatment approaches.

In order to provide in-depth single-cell analysis, mass cytometry, also known as CyTOF (Cytometry by Time-Of-Flight) offers a powerful tool to decipher complex biological systems and to study immune cell diversity, among others. This technology combines flow cytometry principles with mass spectrometry, thereby enabling measurements of multiple parameters simultaneously (Zhang et al., 2020). In this regard, Zernecke *et al.* published a meta-analysis of leukocyte diversity based on published data using either single-cell RNA-seq or CyTOF technologies from atherosclerotic aortas of mice (Zernecke et al., 2020). The data is derived from published studies using different mouse models to obtain atherosclerotic aortas: two genetic knockout models - $Ldlr^{-/-}$ (low-density lipoprotein receptor knockout) (Cochain et al., 2018; Kim et al., 2018) and $Apoe^{-/-}$ (Winkels et al., 2018), or adeno-associated virus PCSK9 (protein convertase subtilisin/kexin type 9) induced lipoprotein changes (Lin et al., 2019). Importantly, in their analysis they could find an ILC2 cluster displaying signature genes such as *Areg* (encoding amphiregulin), *Il1rl1* (encoding the IL-33 receptor ST2), RORA and GATA3 in datasets of Cochain et al. (2018) and Winkels et al. (2018). More importantly, the presence of this ILC2 cluster could be related with data from previous work revealing their atheroprotective

functions (Newland et al., 2017). In this study, they found that ILC2-deficient mice displayed an accelerated progression to atherosclerosis, which was partly due to ILC2-derived IL-5 and IL-13. Moreover, they found profound phenotypical changes of ILC2s in tissues of mice fed a high-fat diet. Specifically, they observed that ILC2s derived from mesenteric and para-aortic lymph nodes of old *Apoe*$^{-/-}$ mice had low ST2 expression and thus, resembled more the inflammatory phenotype of ILC2s as described by Newland et al. (2017), Huang et al. (2015).

Combined, it is clear that transcriptomic approaches can be a valuable tool to further elucidate the complex regulatory mechanisms and functionality of ILC2s, although more applied studies are needed to gain sufficient insights.

- Proteomics

In contrast to genomic or transcriptomic studies, proteomics involves the large-scale study of proteins present in a biological sample. Data derived from proteomics analysis for ILC2s can offer additional information regarding the abundance, post-translational modifications, and interactions with other proteins involved in metabolic pathways, energy production, and signaling cascades (Aslam et al., 2017).

In a recent study, quantitative mass spectrometry-based proteomics was applied to analyze proteins expressed by ILC2s and ILC3s obtained from healthy human skin and blood of donors (Teunissen et al., 2024). Briefly, ILCs were identified as CD45^{+}Lin^{-}CD127^{+}CD161^{+} (Vivier et al., 2018) and ILC2s were further distinguished from ILC3 by the expression of CRTH2 (Teunissen et al., 2024). Their proteomic analysis identified cluster of differentiation marker profiles of the ILC subset and further allowed to study their distribution and abundance of known proteins. Specifically, they could find proteins that were differentially expressed between these two subsets in the skin. For instance, they found prostaglandin D synthase (HPGDS) and CRTH2 to be exclusively expressed by ILC2s. Furthermore, they could find novel subset-specific protein signatures. Importantly, the data set confirmed the expression of ILC2-specific proteins, which are also expressed at the mRNA level, such as GATA3 and CD161 (Vivier et al., 2018). Intriguingly, ST2 and TSLPR cytokine receptors could not be identified in ILC2s in their proteomic study (Teunissen et al., 2024). Accordingly, this finding is in accordance with another study which could also not identify these receptors on human blood-derived ILC2s of healthy individuals (Bal et al., 2016). It is important to note that, most phenotypical characteristics of ILC2s derive from studies using inflamed tonsils, which is therefore most likely the cause of the observed discrepancies (Vivier et al., 2018). These discrepancies in ILC2 phenotypes enhance the importance to study tissue- and disease-specific characterizations of ILC2s.

Therefore, combining transcriptomics and proteomics data would provide a more comprehensive understanding of biological systems by bridging the gap between gene expression and protein activity. While transcriptomic studies reveal mRNA levels for potential protein expression, data derived from proteomics could confirm the actual abundance of a specific protein. Additionally, the combination of these approaches would also enable the identification of novel markers for a more accurate characterization of ILC2s.

- Metabolomics

Similar to proteomics, metabolomics focuses on large-scale analysis of proteins. However, more specifically, metabolomics is the comprehensive study that gives insights into small molecules, or metabolites that are present in a biological sample. With respect to ILC2s, these data can reveal important insights into the metabolic pathways that are required for their activation and function, as well as the abundance of certain metabolites or any metabolic changes.

For instance, Surace *et al.* gave important insights into the dichotomous metabolic networks of proliferation and effector function of human blood ILC2s obtained from healthy donors (Surace et al., 2021). Specifically, the authors could reveal that circulating 'naive' ILC2s exhibit an unanticipated metabolic profile, showing higher levels of OXPHOS as compared to NK cells. Additionally, these circulating ILC2s did not express ST2. Intriguingly, it has been shown that IL-33 induced ILC2 activation and proliferation, led to an increase in glycolysis to produce IL-13 while maintaining OXPHOS for their cellular fitness and proliferative capacity (Surace et al., 2021). Importantly, these different metabolic pathways offer various angles to therapeutically manipulate ILC2s in a diseased state. Accordingly, targeting immunometabolism has emerged as promising approach to control both inflammatory and anti-inflammatory immune responses. Here, specific metabolic events can be used to dampen inflammation (reviewed in (Pålsson-McDermott and O'Neill, 2020)). For instance, metformin is used for the treatment of T2D due to its action in reducing hepatic gluconeogenesis and it has been shown to affect macrophages, T cells and B cells (Kim et al., 2008; Diaz et al., 2017; Kelly et al., 2015; Zarrouk et al., 2014). Modulating key metabolic pathways by inhibiting or activating glycolysis or OXPHOS could give important insights into ILC2 activity. This can be achieved by applying drugs that specifically target metabolic pathways or enzymes involved in ILC2 metabolism. For instance, 2-deoxyglucose (2-DG) is a nonmetabolizable glucose analog and can be used as inhibitor for glycolysis (Nakada and Wick, 1956). Notably, 2-DG is intensively studied as potential anti-cancer agent (Raez et al., 2013; Stein et al., 2010; Xi et al., 2014; Zhang et al., 2014) and it has been approved as emergency drug in 2021 for the treatment of COVID-19 (Aiestaran-Zelaia et al., 2022). However, studies looking at the requirements for modulating ILC2 metabolism remain scarce and thus need to be further explored.

Another study used an unbiased metabolomics analysis of feces derived from mice infected with *N. brasiliensis* (*Nippostrongylus brasiliensis*) to reveal how environmental and metabolic stimuli shape ILC2 immune responses upon helminth infection (Hodge et al., 2023). Interestingly, they found an increase of essential amino acids upon infection. To further scrutinize the importance of the availability of these amino acids for ILC2 function, they fed the mice a low

TABLE 1 Overview of ILC2 focused omics approaches.

Omics	Studies in ILC2s	Relevance to inflammation and metabolic diseases	References
Genomics	Identification of genetic variants and susceptibility loci associated with ILC2 function and regulation	Genetic predisposition to cardiovascular inflammation and atherosclerosis linked to ILC2-related genes	Liu et al. (2022), Xu et al. (2023)
Epigenomics	DNA methylation and histone modification patterns in ILC2s during cardiovascular stress	Epigenetic regulation of ILC differentiation and function affecting cardiovascular disease progression and response to therapy	Michieletto et al. (2023)
Metabolomics	Metabolic profiling of ILCs revealing unique metabolic pathways and metabolites in a disease- and tissue-specific context	Understanding how metabolic states of ILC2s influence their function in cardiovascular pathology, including oxidative stress and impaired lipid metabolism	Surace et al. (2021)
Transcriptomics	Differential expression of key genes in ILC2s under inflammatory conditions	Insights into the phenotypical characteristics of ILC2s in promoting or mitigating atherosclerosis	Jiang et al. (2023), Zernecke et al. (2020)
Proteomics	Characterization of protein expression profiles and signaling pathways in ILC2s	Identification of protein markers and signaling molecules involved in ILC2-mediated vascular inflammation	Teunissen et al. (2024)
Multi-Omics Integration	Combined analysis of genomic, transcriptomic, proteomic, and metabolomic data in ILC2s	Holistic understanding of ILC contribution to CVDs, leading to the identification of novel therapeutic targets and biomarkers	as applied in (Song et al., 2023)

protein diet after infection. Indeed, ILC2 numbers were reduced in those mice fed a low protein diet by day 7 after the infection with *N. brasiliensis*, thereby suggesting that amino acid availability impacts ILC2 immune responses (Hodge et al., 2023).

Summary and conclusion

Overall, the integration of multiple omics approaches, including transcriptomics, proteomics, and metabolomics can provide a comprehensive understanding of the metabolic profile and functional characteristics of ILC2s in health and chronic vascular inflammation. Table 1 provides an overview of the different omics fields that can be applied to reveal the function and characteristics of ILC2s with respect to metabolic and CVDs. It is important to note that the specific experimental conditions, sample preparation methods, and data analysis techniques should be taken into consideration when interpreting omics data for metabolic profiling of immune cells like ILC2s.

Nevertheless, integrating omics data from different immune cell types provides critical insights into the complex interactions between immune cells and vascular tissues in health and disease. Furthermore, dissecting this interplay together with the cytokine networks will be crucial for a better understanding of inflammatory processes driving vascular pathologies. Even further, network analysis based on omics data can reveal molecular players and signaling pathways linking ILC2s to CVDs. In this regard, Fabian Theis' group just recently published a novel method to model intercellular communication in tissues using spatial graphs of cells, the so-called node-centric expression models (NCEM) (Fischer et al., 2023). These graph-based neural networks aim at revealing the interplay between tissue niches and gene expression without losing spatial information.

Such advances in omics data have revolutionized the field of life sciences, allowing for a deeper understanding of the complex interactions within biological systems. Moreover, the integration of multi-omics data will aid in developing new diagnostics, therapeutics, and personalized medicine approaches.

Author contributions

MK: Writing–original draft. EvdV: Writing–review and editing. CW: Writing–review and editing. YD: Writing–review and editing.

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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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Multicellular 3D models to study myocardial ischemia–reperfusion injury

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Coronary heart disease is a major global health threat, with acute myocardial ischemia–reperfusion injury (IRI) being a major contributor to myocardial damage following an ischemic event. IRI occurs when blood flow to ischemic tissues is restored and exacerbates the cellular damage caused by ischemia/hypoxia. Although animal studies investigating IRI have provided valuable insights, their translation into clinical outcomes has been limited, and translation into medical practice remains cumbersome. Recent advancements in engineered three-dimensional human *in vitro* models could offer a promising avenue to bridge the "therapeutic valley of death" from bench to bedside, enhancing the understanding of IRI pathology. This review summarizes the current state-of-the-art cardiovascular 3D models, including spheroids, organoids, engineered cardiac microtissues, and organ-on-a-chip systems. We provide an overview of their advantages and limitations in the context of IRI, with a particular emphasis on the crucial roles of cell–cell communication and the multi-omics approaches to enhance our understanding of the pathophysiological processes involved in IRI and its treatment. Finally, we discuss currently available multicellular human 3D models of IRI.

KEYWORDS

ischemia–reperfusion, 3D models, organoids, cardiac tissue, endothelial cell, cardiomyocyte

Abbreviations: 3D, three-dimensional; ACS, acute coronary syndrome; BPM, beats per minute; CaO, cardiac organoid; cFB, cardiac fibroblast; EC, endothelial cell(s); EC-EVs, endothelial cell-derived extracellular vesicles; ECM, extracellular matrix; EEV, endothelial extracellular vesicle; EHT, engineered heart tissue; EV, extracellular vesicle; FAO, fatty acid oxidation; FBs, fibroblasts; HOC, heart-on-a-chip; hiPSC-CM, human-induced pluripotent stem cell-derived cardiomyocytes; hiPSC-EC, human-induced pluripotent stem cell-derived endothelial cells; hiPSC-FB, human-induced pluripotent stem cell-derived fibroblasts; HUVEC, human umbilical vein endothelial cell; IRI, ischemia–reperfusion injury; MPTP, mitochondrial permeability transition pore; MYH7, myosin heavy chain 7; OXPHOS, oxidative phosphorylation; PDMS, polydimethylsiloxane; PBMC, peripheral blood mononuclear cell; PPARα, peroxisome proliferator-activated receptor alpha; ROS, reactive oxygen species; RYR2, ryanodine receptor 2 (gene); SMCs, smooth muscle cells; TUNEL, terminal deoxynucleotidyl transferase dUTP Nick end labeling; α-SMA, alpha smooth muscle actin.

1 Introduction

Myocardial ischemia–reperfusion injury (IRI) refers to the cellular damage or dysfunction of cardiac tissue that occurs when blood flow and oxygen supply are restored in the myocardium, following acute coronary syndrome (ACS), a period of restricted or blocked cardiac blood flow. IRI is a complex condition that can manifest in various forms, including microvascular obstruction, lethal myocardial reperfusion injury, reperfusion-induced arrhythmias, and myocardial stunning (Fröhlich et al., 2013). This reoxygenation-induced damage can contribute up to 50% of the final infarct size and can lead to chronic heart failure and death (Yellon and Hausenloy, 2007).

The pathological mechanisms underlying IRI are multifaceted. In general, during ischemia, oxygen-deprived cardiac tissue shifts toward anaerobic metabolism, particularly in cardiomyocytes, which normally obtain their energy primarily through oxygen-dependent fatty acid oxidation (FAO). This metabolic shift leads to increased lactic acid production and results in intracellular metabolic acidosis, ion imbalance, and cell swelling. When oxygen levels return upon reperfusion, the rapid influx of calcium into the cardiomyocytes leads to an excessive production of reactive oxygen species (ROS) that can result in local cell damage and microvascular obstruction (Buja and Vander Heide, 2016; Heusch, 2020). Although ROS sensitizes the mitochondrial permeability transition pore (MPTP), the substantial increase in calcium levels in the mitochondrial matrix is responsible for MPTP opening. The opening of the MPTP occurs during the first minutes of reperfusion and is a crucial factor in IRI. It results in the collapse of the mitochondrial membrane potential and uncoupling of oxidative phosphorylation with subsequent ATP depletion, which significantly contribute to cardiomyocyte death and infarct size (Buja and Vander Heide, 2016; Heusch, 2020; Ong et al., 2015).

Despite the significant progress in understanding IRI, many aspects remain unclear and continue to be the focus of ongoing research (Fröhlich et al., 2013; Hausenloy and Yellon, 2013). For example, calcium overload is a crucial factor at the onset of IRI, but how calcium fluxes between different cellular compartments, like the sarcoplasmic reticulum, mitochondria, and cytosol, is not fully mapped yet. Additionally, while the opening of the MPTP plays a key role in aggravating reperfusion injury, the precise role of various triggers remains to be discovered. Although multiple forms of cell death, including apoptosis, necroptosis, and ferroptosis, are all involved in IRI, necrosis appears to be the most prominent, especially closest to the ischemic core of the infarct (De Villiers and Riley, 2020). However, the relative importance and contributions of each form of cell death remain largely unclear. Furthermore, it is currently unknown which form of cell death predominates at different stages of injury or in different cell types within the heart. Finally, the environmental factors and genetic predispositions that render individuals more susceptible to IRI than others require further investigation (Hausenloy and Yellon, 2013; Zhang et al., 2024).

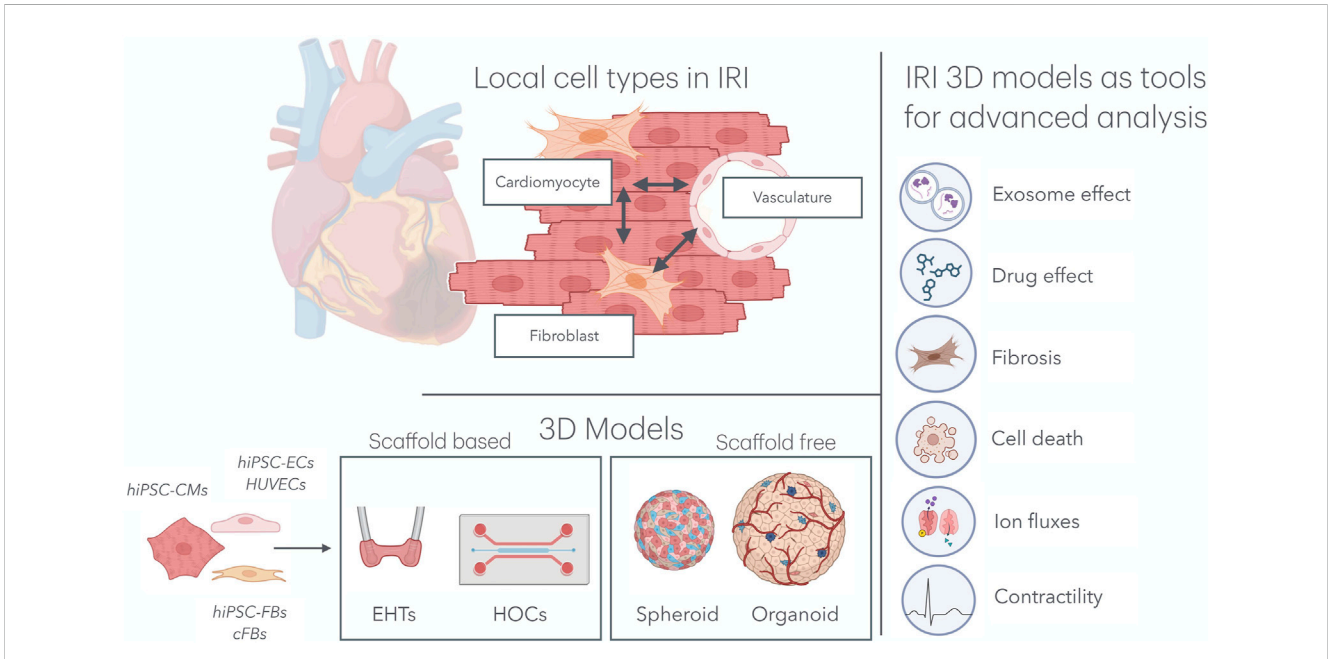

FIGURE 1
Multicellular organ-on-a-chip technology for IRI research. **Top left:** In the cardiac tissue, hiPSC-CMs, hiPSC-FBs, and the cardiac microvasculature interacts directly with one another, mimicking native tissue architecture. **Bottom left:** Various combinations of cell types, including hiPSC-CMs, hiPSC-ECs, HUVECs, hiPSC-FBs, and cFBs, have been used to model IRI in both scaffold-based 3D systems (e.g., EHTs and HOCs) and scaffold-free systems (e.g., spheroids and organoids). **Right:** These models have been subjected to IRI environments, providing key insights into the effects of exosomes and drugs on the progression of IRI, as well as deepening our understanding of cardiomyocyte cell death, fibrosis, ion fluxes, and contractility. IRI, ischemia–reperfusion injury; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; hiPSC-FBs, human induced pluripotent stem cell-derived fibroblasts; hiPSC-ECs, human induced pluripotent stem cell-derived endothelial cells; HUVECs, human umbilical vein endothelial cells; cFBs, cardiac fibroblasts; EHT, engineered heart tissue; HOCs, heart-on-chips.

Despite these still unfulfilled gaps in the knowledge of IRI, significant progress has been made in the medical treatment of ACS, including the polypill, used as the secondary prevention tool after the first event (Castellano et al., 2022). However, mortality rates within the first year after an ACS event remain alarmingly high and vary between 6% (Steen et al., 2022) and 10% (Ulvenstam et al., 2023). Moreover, the prevalence of adverse outcomes and long-term health implications, including infarct size and progressive cardiac dysfunction, which can ultimately result in chronic heart failure, remains substantial. Notably, there are currently no clinically approved, effective treatments available that alleviate short-term complications like myocardial stunning, arrhythmias, no-reflow phenomenon, and IRI-induced cell death (Hausenloy and Yellon, 2013). The low success rate of clinical trials has not only contributed to the already high cost of drug discovery, which could lead to a reduced interest from pharmaceutical companies in pursuing research and development in this area, but it has also been proposed that improved preclinical screening methods could potentially identify around 70% of cardiac toxicities observed in clinical trials (Olson et al., 2000). Preclinical studies in animals have played an important role in advancing our understanding of disease mechanisms and in the development and testing of therapeutic compounds. In rodents, the commonly used technique of ligation of the left arterial descending coronary artery is used to test potential cardioprotective strategies prior to their application in larger animal models (De Villiers and Riley, 2020). Before moving to human clinical trials, studies are conducted in canine, ovine, porcine, and non-human primate models due to their increased physiological similarity and predictive value, as reviewed in detail elsewhere (Rahman et al., 2023). However, despite the success of certain compounds in preclinical trials, such as MTP-131 to target oxidative stress (Gibson et al., 2016), cyclosporin A to target MPTP opening (Ottani et al., 2016), or carperitide to promote vasodilatation (Suwa et al., 2005), they were all tested in rodent and canine models but showed low translatability in clinical trials. Part of this is attributable to genetic, molecular, and cellular variations in the cardiovascular system between humans and animals (De Villiers and Riley, 2020; Milani-Nejad and Janssen, 2014). To overcome part of these limitations of non-human models while mimicking the complexity of multiple cell types in one system, advanced cardiac 3D models, using human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) to study IRI, are currently being developed at a fast pace.

In conclusion, while IRI is currently incurable and its pathology causes considerable damage to the heart after cardiac arrest, its multifactorial nature and multiple unanswered questions require further development of models investigating the disease. Because of its rapid advancement, this review focuses on human multicellular 3D culture systems in the study of IRI.

2 Local interactions in IRI

Although cardiomyocytes constitute the majority of the cardiac volume, they account for only approximately 50% of the total number of cells (Litviňuková et al., 2020). Given the complex and multifaceted nature of IRI, it is essential to consider the role of other local cell types that significantly influence the aggravation and resolution of IRI, particularly endothelial cells (ECs) and cardiac fibroblasts (cFBs) (Figure 1).

2.1 Cardiac fibroblasts

cFBs are geometrically interspersed between cardiomyocytes and, in normal circumstances, lead to extracellular matrix (ECM) homeostasis in the cardiac tissue. Immediately after IRI, activated fibroblasts differentiate into myofibroblasts, triggering an initially protective form of cardiac fibrosis aimed at preserving structural integrity. Fibrotic EMC remodeling and inflammatory activation can lead to long-term cardiac complications due to excessive scarring (Hinz et al., 2012). Myofibroblast differentiation is driven by the expression of contractile genes, such as *ACTA2*, which encode for smooth-muscle α-actin (α-SMA) (Tallquist and Molkentin, 2017). Myofibroblasts can worsen local inflammation by activating the NLRP3 inflammasome response (Sandanger et al., 2013). Once differentiated, myofibroblasts increase the ECM, deposition factors like collagen -I, -III, -IV, -V, and -VI, glycoproteins such as fibronectin and tenascin-C, and proteoglycans, all responsible for the healing and scarring processes, as reviewed in detail elsewhere (Tallquist and Molkentin, 2017; Kanisicak et al., 2016). The scarring process is necessary for maintaining the structural integrity and functionality of the heart. However, excessive fibrosis can result in adverse remodeling, increased stiffness, reduced contractility, and, ultimately, heart failure (Zhang et al., 2024).

2.2 Cardiac vasculature

ECs are more than just a protective barrier controlling the exchange of nutrients and gasses to the cardiac muscle. In a stress and inflammatory setting, ECs regulate the adhesion and transmigration of immune cells, contributing to the inflammatory response within the cardiac tissue. During IRI, ECs play an important role in the aggravation of and protection against IRI. ECs impair vasodilation by reducing NO production, increasing vascular permeability inflammation that could contribute to microvascular obstruction, one of the hallmarks of IRI. Additionally, in the context of IRI, ECs may drive tissue remodeling and hypertrophy after ischemia, as reviewed by Yang et al. (2015) and Singhal et al. (2010). EC-derived extracellular vesicles (EC-EVs) play a crucial role in the alleviation of IRI (Yadid et al., 2020; Li et al., 2023), illustrated by a ~50% increase in cardiomyocyte viability after an IRI stimulus when EC-EVs were added (Yadid et al., 2020). Mechanistically, Liu et al. discovered that exosomes, a small subtype of extracellular vesicles, derived from human-induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) restored the expression and activity of reticulum Ca^{2+} ATPase 2a (SERCA-2a) and ryanodine receptor (RYR2) in cardiomyocytes. Consequently, intracellular Ca^{2+} transient and cardiomyocyte contractions were enhanced after MI (Li et al., 2023).

When developing an appropriate research model to study the pathophysiology of IRI, it is crucial to consider both beneficial and deleterious local interactions. Incorporating these interactions will

enable the creation of an *in vitro* model that more accurately reflects the human *in vivo* environment.

3 Usage of hiPSC-CMs in IRI research

Due to the challenges in obtaining cells from the adult human heart—stemming from limited accessibility, poor proliferation capacity, and reduced viability in culture—alternative approaches are often required. Development of hiPSC-CMs has emerged as a valuable alternative, although their immature nature currently limits translation into clinical practice. Although hiPSC-CMs exhibit inherent genetic and epigenetic variations across different lines to a greater extent than non-iPSC cultures, this allows for easier genetic disease models and opportunities for personalized medicine (Zhan et al., 2023; Stein et al., 2021).

The use of immature hiPSC-CMs in IRI modeling, using a relatively prolonged period of hypoxia, 8–24 h and subsequent reoxygenation of 24 h, has been observed to elicit alterations in several key parameters, including the beating rate, polarization time, field potential, and sarcomere structure (Häkli et al., 2021). Recent work using a 2D culture of hiPSC-CMs revealed the upregulated expression of early growth response 1 (ERG1) in IRI-induced apoptosis, an effect that could be suppressed using miR-124-3p (Yang et al., 2024), revealing the possibility of using 2D hiPSC-CMs for simple IRI modeling.

However, critical for IRI, hiPSC-CMs rely primarily on glucose metabolism, making them more resistant to hypoxia and reperfusion than adult cardiomyocytes, which mainly utilize fatty acid oxidation and can lead to reduced accuracy in modeling IRI (Wu et al., 2021; Vučković et al., 2022). As reviewed by Vučković et al. (2022), hiPSC-CMs can be subjected to different maturation protocols in order to partially overcome these important metabolic differences, reporting up to 2–4-fold increase in fatty acid oxidation and a subsequent 50% reduction in glycolysis, more similar to the metabolic phenotype of primary cardiomyocytes. Metabolic maturation accomplished by the supplementation of the media with fatty acids while removing glucose as a substrate leads to up to ~30% cell death post-hypoxia and reoxygenation, compared to only ~5% in immature iPSC-CMs, highlighting the translational importance of cell maturation (Hidalgo et al., 2018). Although hiPSC-CMs currently represent the only widespread alternative for modeling human cardiomyocytes *in vitro*, metabolic maturation from glucose to oxidative phosphorylation dependency is an important feature for increasing the accuracy of the induced IRI (Wu et al., 2021; Feyen et al., 2020). These more matured hiPSC-CMs are, among others, characterized by increased sarcomere length (approximately 1.6 μm–2.2 μm), increased upstroke velocity (15–50 V/s to 230–400 V/s), the presence of T-tubules, and increased quantity of mitochondria, which are responsible for their metabolic shift (Wu et al., 2021). Although the use of mature hiPSC-CM 2D models allow for the investigation of basic cell behavior, they do not fully recapitulate the *in vivo* dynamic microenvironment structures and different cell types of the heart, hence limiting the ability to accurately reflect the complex pathophysiology of IRI (Ahmed et al., 2020). In the next section, we categorize the current advances in both scaffold-based and scaffold-free 3D culture systems containing hiPSC-CMs.

4 Advances in scaffold-based models

4.1 Engineered heart tissues

Engineered heart tissues (EHTs) are 3D structures typically created by mixing hiPSC-CMs with a hydrogel into a casting mold, providing a highly reproducible tissue structure. CMs in EHTs align along the force lines of the cell or culture they are placed on, such as rings (Katare et al., 2010), sheets—as used by Yadid et al. (2020), measuring 3.2 mm × 4.2 mm—or elastomeric pillars (Ahmed et al., 2020; Arslan et al., 2022; Mastikhina et al., 2020). Mastikhina et al. (2020) included fibroblasts in their cardiac EHT and investigated the fibrotic effects after ischemia using 2.5*10^5 cells placed between two polydimethylsiloxane (PDMS) pillars. The CMs created a coherent beating syncytium that allows for the detailed measurement of contractile function, including force generation and electrophysiological properties. EHTs are generally easy to produce and can be manufactured on a relatively large scale; however, due to the lack of flow, they are prone to the development of necrotic inner tissue due to oxygen deprivation (Stein et al., 2021).

4.2 Heart-on-chips

Heart-on-a-chip models are based on a microfluidic system that enables dynamic culture, perfusion, and the addition of a vascular channel (Arslan et al., 2022; Paz-Artigas et al., 2023). Heart-on-chips utilize a range of biocompatible natural and synthetic materials, including hydrogels, (3D-printed) synthetic polymers such as PDMS, or extracellular matrix, to provide structural support and guide cell alignment and organization. These materials allow for the precise spatial arrangement of cell culture chambers, fluidic channels, and measuring devices, such as electrodes (Liu et al., 2020; Paloschi et al., 2021). Nevertheless, heart-on-a-chip systems require specialized equipment, like microfluidic pumps, and can be equipped with biosensors, like microelectrode arrays or intracellular electrodes, for measuring extracellular field potentials and action potentials (Liu et al., 2020) or mechanical biosensors, like force transducers or cantilevers, to measure contractile forces (Yadid et al., 2020). The chip to be cultured on, the microfluidic pump system, and potential sensors add technical complexity and costs compared to 2D and scaffold-free 3D systems.

4.3 Spheroids

Spheroids belong to the scaffold-free models, together with organoids. Spheroids are simple micro-size 3D aggregates often generated by hanging drop or ultra-low-attachment plate methods (Paz-Artigas et al., 2023; Cho et al., 2021). They are generated from hiPSCs alone or in a mixture with ECs, FBs, or even smooth muscle cells (SMCs). These cells can exhibit synchronized contractions, which may be perceived as beating heart tissue. Regarding their compact nature, they are at an increased risk of O_2-mediated cell death; this feature can be used to recreate differences in oxygenation, which can be translated to gradients of IRI damage (Richards et al., 2020).

TABLE 1 Current multicellular 3 d models to investigate ischemia–reperfusion injury.

Reference	Model type	Cell types used	Ischemic/reperfusion stimulus	Ischemia-reperfusion readout	Main finding/model characteristics
Song et al. (2024)	Organoid	hiPSC-CMs, hiPSC-ECs, and hiPSC-FBs	Ischemia: 50 uM cobalt chloride, no glucose, high calcium ion 75 h	Cell death (TUNEL 2-fold increase, caspase-3 2-fold increase)	IRI in organoids leads to electrophysiological abnormalities, cardiac fibrosis, and disrupted calcium ion handling
Yadid et al. (2020)	EHT	iPSC-CMs and HUVECs (extracted exosomes)	Ischemia: 1% O_2, 3 h. High-acidity, low-glucose medium reperfusion: 3 h	2-fold cell death (EthD-1/Hoechst)	EEVs taken up by CMs are protective against Human IRI—EEVs reduced cell death, partial conservation of the proteome, and normalized contractile IRI stress
Veldhuizen et al. (2022)	Heart-on-chip	hiPSC-CM cFBs	Ischemia: 1% O_2, 24 h Reperfusion: 1 h and 24 h	Increased beating variability, 2-fold lactate increase, and cell death	Fibrosis, cell toxicity, sustained contractile irregularities, sustained lactate levels, and gene expression
Richards et al. (2020)	Organoid	iPSC-CMs, hCFs, HUVECs, and hADSCs	Ischemia: 10% O_2, 10 days + adrenergic stimulation via norepinephrine	TUNEL staining and decreased NADH autofluorescence	Organoid development similar to human ischemia on the transcriptomic level including pathological calcium handling
Ellis et al. (2022)	Heart-on-chip	hiPSC-CMs hiPSC-ECs	Ischemia: 0.1% O_2, 3 h Reperfusion: 3 h	miRNA (miR-208b and miR-499) increase similar to IRI *in vivo*	Revealed the potential of miRNA biomarkers for IRI diagnosis, similar to *in vivo*. Changed exosome surface markers
Sebastião et al. (2020)	Spheroid	iPSC-CMs, HUVECs (conditioned media)	Ischemia: < 0.4% O_2, no glucose, high Na^+ lactate, low pH (6.8) 5 h. Reperfusion: 16 h	Sarcomere filament disruption and apoptosis at core	Increase in the inflammatory, migrational, and angiogenic proteome. Conditioned media on HUVECs lead to increased angiogenesis

4.4 Organoids

Organoids are more complex, self-organizing 3D structures that mimic the structural and functional properties of the heart without the need for external support to maintain mechanical integrity (Kaushik et al., 2018). Organoids are made using cells that grow in a 3D structure that resembles the organ in structure and function; this development is similar to the mesodermal development of the human heart (Song et al., 2024; Hofbauer et al., 2021a). These effects can be enhanced by the addition of BMP, VEGF, FGF, and TGF-beta-containing medium, creating organoids consisting of further differentiated cells into different lineages. Adding these factors led to further development of other cell types of the heart, reflected by a significant increase in endothelial VE-cadherin expression, increasing from 4% to 15%. Additionally, CD90 positivity, a fibroblast marker, increased from 6% to 27% when compared to CaO of a similar age and size (Song et al., 2024). Organoids are more complex structures than spheroids and more closely resemble the human heart (Gunti et al., 2021). However, they are more challenging to generate and may exhibit greater structural and functional heterogeneity.

5 3D systems to investigate ischemia–reperfusion injury

As discussed previously, not only cardiomyocytes but the whole cardiac tissue including the vasculature and cardiac fibroblasts play an important role in the aggravation and resolution of IRI. Only 3D systems containing multiple cell types are discussed further. The initiation of IRI can be achieved by changing local oxygen concentrations (Yadid et al., 2020; Hidalgo et al., 2018) using a chemical ischemic stimulus of time or by establishing a physical oxygen gradient (Richards et al., 2020). Additionally, cardiac cryoinjury can be used to investigate healing and ECM accumulation in an IRI-like setting in organoids consisting of mostly hiPSC-CMs (Voges et al., 2017) or organoids containing hiPSC-CMs, cFBs, and ECs (Hofbauer et al., 2021a). Both multicellular scaffold-free (Mastikhina et al., 2020; Richards et al., 2020; Song et al., 2024; Richards et al., 2017) and scaffold-based (Yadid et al., 2020; Veldhuizen et al., 2022; Veldhuizen et al., 2020) setups are currently being developed to investigate IRI. All models containing multiple human cell types in an IRI setting are summarized in Table 1.

5.1 Scaffold-based models

Although some previous IRI-on-chip models were developed utilizing hiPSC-CMs (Paz-Artigas et al., 2023), Yadid et al. (2020) advanced the field by creating a multicellular model consisting of an EHT assembled on a flexible chip mimicking ventricular-like muscle. Although indirect, this model was designed to investigate IRI on a microfluidic platform (Yadid et al., 2020). A flexible cantilever chip was used to study the effects of exosomes on hiPSC-CM survival and function. The CM cantilever chip consists of micro-patterned films to facilitate hiPSC-CM alignment, creating an organized CM sheet more similar to a naïve myocardium than to a 2D monolayer. Hypoxia was induced by exposing the cells to a 1% O_2 environment for 3 h, combined with glucose-depleted and slightly acidified media (pH 6.4) to simulate the ischemic event. This was followed by 1.5 h under normal culture conditions to mimic the reperfusion phase. Exosome production was approximately 6.5 times higher in hypoxic EC-EXs than in normoxic EC-EXs. Additionally, the heart cantilever receiving EC-EXs 3 h prior to ischemia demonstrated

improved cell survival and enhanced twitch stress recovery compared to non-EX-treated controls (Yadid et al., 2020). Although the uptake was equal, the positive results could not be replicated when neonatal rat cardiomyocytes were stimulated using human EC-EXs. These results could indicate a species-specific effect of human EC-EXs.

The effect of EVs in IRI was exploited further by Ellis et al. (2022). Their heart-on-a-chip model comprising hiPSC-CMs and hiPSC-ECs was used to investigate the potential role of extracellular vesicle-derived miRNAs in the context of IRI. During ischemia, about 3.5 times more EC-EXs were secreted than during reperfusion or non-ischemic controls; these results were validated in clinical plasma samples. During reperfusion, an anion exchange membrane (AEM)-based miRNA sensor was used to investigate the presence of miRNA in chip effluents compared to clinical controls. miRNAs miR-208 and miR-499 were identified in the model effluent, and they were also present in clinical IRI samples, implicating the potential of this IRI-on-a-chip model for biomarker discovery (Ellis et al., 2022).

In mice, the administration of EC-EVs intranasally every day the first 3 days and twice a week up to 3 weeks after IRI has been demonstrated to improve cardiac function in mice after a 28-day period (Wang et al., 2023). The impact of EVs on reperfusion injury depends on their source. EVs obtained not only from ECs but also isolated from the ischemic heart were administered via an intracardiac injection into a second heart just before reperfusion exacerbated IRI in mice. Further analysis revealed increased M1 polarization of macrophages, as well as increased local cytokine expression (Ge et al., 2021). The development of technologies such as IRI-on-a-chip (Yadid et al., 2020; Ellis et al., 2022) and EHT fibrosis models (Mastikhina et al., 2020) could potentially enable a more detailed investigation of the content, secretion, and effects of these EVs in human models. These advancements offer an opportunity to more comprehensively explore potential therapeutic targets or biomarkers associated with EVs.

Veldhuizen et al. (2020) developed a microdevice with a heart-on-a-chip configuration and surface topographical patterning to facilitate two-dimensional cell alignment. This microfluidic chip was constructed from PDMS and filled with hiPSC-CMs and cFBs at a ratio of 4:1 in an ECM comprising a collagen/fibronectin (Veldhuizen et al., 2020) or collagen/matrigel (Veldhuizen et al., 2022) mixture. These cells formed aligned tissues around the embedded microposts. Culturing on the PDMS chip led to the increased maturation of hiPSC-CMs compared to 2D control, as evidenced by the upregulation of genes involved in calcium uptake and release, including *HCN1*, *KCNQ1*, *CAV1.2*, *CAV3.1*, *PLN*, and *RYR2* (Veldhuizen et al., 2020). Placing this model in a hypoxic setting (1% O_2 for 24 h), followed by reperfusion for either 1 or 24 h, revealed no change in cell death during hypoxia. However, a significant increase in cell death was observed following oxygen reperfusion at both the 1-h and 24-h time points, indicating the successful induction of reperfusion-related damage (Veldhuizen et al., 2022). Furthermore, 24 h of hypoxia led to a near 2-fold increase in lactate secretion, which is comparable to human physiological levels during IRI, where ischemia only caused minor fluctuations in the inter-beat interval, while reperfusion led to a notable increase in inter-beat variability. Additionally, reperfusion also led to increased expression of α-SMA (Veldhuizen et al., 2022). This *de novo* expression of α-SMA is a hallmark of myofibroblast activation, allowing for the formation of stress fibers and the production of extracellular matrix, which induces fibrosis and scarring (Hinz et al., 2012). The transcriptomic analysis revealed a notable increase in glycolysis and other metabolic pathways, accompanied by a reduction in oxidative phosphorylation (OXPHOS). Collectively, similar pathways and matching functional readouts compared to human ischemia serve as validation for IRI-on-a-chip models and allow for further developments of these 3D-IRI-on-a-chip models (Veldhuizen et al., 2022).

5.2 Scaffold-free models

Although both scaffold-based and scaffold-free models allow for the co-culture of cardiomyocytes with different cell types like EVs and cFBs, an acute limitation of scaffold-free models, both organoids and spheroids, is the lack of a functional vascular network to facilitate nutrient exchange and waste removal (Homan et al., 2019), especially in modeling the reperfusion phase, where fast nutrient/oxygen exchange and waste removal are warranted. In static circumstances, only spheroids with a diameter of less than 150 μm have been used to study cardiac ischemia (Richards et al., 2017) as in this size, passive oxygen diffusion is still possible. Small organoids or spheroids are usually less complex and exhibit greater variability in their properties and reduced contractile function than their larger (600 μm) counterparts (Hoang et al., 2021).

Richards et al. (2020) used the absence of a vascular network as a means of gradually introducing ischemia. In their study, 300-μm cardiac organoids were subjected to a 10-day, 10% oxygen + adrenergic stimulation via norepinephrine. The quantification of oxygen diffusion in these microtissues revealed a reduction to 1% oxygen at the core of the organoid. Not only did the low-oxygen treatment result in significant cell death, visualized by TUNEL staining, in accordance with metabolic responses in IRI, but a reduction in non-mitochondrial respiration and an increase in glycolysis during ischemia were also observed. A meta-analysis of transcriptomic changes in these human cardiac infarct organoids revealed significant similarities with the transcriptomes of acute post-infarct tissues from animal models and human cardiac samples affected by ischemic cardiomyopathy. Gene Ontology terms between control and infarcted organoids revealed changes in pathways indicative of altered calcium handling, such as ion transport, calcium signaling, and arrhythmogenic right ventricular cardiomyopathy. A reduction in well-studied calcium handling components, including *ATP2A2, RYR2, CACNA1C*, and *SLC8A1*, was observed, and the peak calcium ion concentration, crucial for CM contraction, was reduced in the interior of the infarcted organoid compared to the edge or control CMs. In addition to altered calcium handling, bulk RNA sequencing analysis revealed changes in pathways related to fibrosis, with genetic alterations mirroring those observed in the infarcted mouse heart. Functionally, there was a notable increase in myofibroblast-like structures (α-SMA + fibroblasts) and a significant increase in tissue stiffness, following infarction (Richards et al., 2020). To conclude, this model provides a

valuable opportunity to investigate the mechanisms of fibrosis and calcium fluxes, and it may also serve as a model for exploring druggable targets in ischemia. However, a significant limitation of this model is that due to the distance to the center of the microtissue, even under normoxic conditions, oxygen levels do not exceed 6%, limiting effective reperfusion and potentially affecting the study of reperfusion injury.

As an indirect co-culture containing both iPSC-CMs and human umbilical vein endothelial cells (HUVECs), Sebastião et al. (2020) developed cardiac spheroids, measuring approximately 260 µm, which were cultured for 18 days. The cardiac hiPSC-CM spheroids were subjected to a 5-h-long ischemic protocol, comprising both nutrient and oxygen deprivation with low pH and increased lactic acid supplementation, resulting in sarcomere disturbances and a reduction in viability in the spheroid core. The angiogenic potential of HUVECs was enhanced when stimulated with conditioned media from IRI spheroids, as opposed to hiPSC-CM control media. The present study reveals the indirect, angiogenic effect of the IRI spheroid secretome on the vasculature.

In intestinal organoids, IRI has successfully been modeled to recapitulate properties of *in vivo* IRI responses while reaching a larger size between 200 µm and 400 µm (Kip et al., 2021). One of the defining characteristics of intestinal organoids is their formation of a lumen-enclosed structure, which allows for more effective oxygen differentiation. Although not yet deployed in IRI, the development of cardioids, a specific type of organoid that self-organizes into chamber-like structures (Hofbauer et al., 2021b), could offer the potential to work with scaffold-free models while maintaining proper perfusion.

Recent advantages in the synergic integration of the flow dynamics of the heart-on-a-chip model, together with the self-organizing capacity of cardiac organoids, revealed great advancements over static models and could facilitate the use of larger, more stable and more complex organoids. In a kidney organoid model with fluidic culturing, transcriptomic analysis revealed 229 signaling pathways not identified in the static model (Hiratsuka et al., 2024). Min et al. (2024) revealed the impact of flow EHTs comprising hiPSC-CMs, ECs, and CFs within a 3D heart extracellular matrix hydrogel using a microfluidic chip. Placing these large 1-mm EHTs in a chip system, providing them with nutrient flow, led to increased oxygen concentrations within the cardiac tissues and reduced expression of cleaved caspase-3 in the organoid, compared to no-flow or flowing the organoids in a plate. Functionally, the application of flow on a microfluidic chip resulted in an increase in sarcomere length, the contraction amplitude, both indicators of hiPSC-CM maturation, and a heart rate of 76.59 ± 15.7 beats per minute (BPM) within the frequency range of a healthy human heart. RNA sequencing revealed significant differences between static and plate-flowed EHTs, with an observed increase in genes related to heart development, extracellular matrix organization, and angiogenesis. The relative mRNA expression of CM (*MYH7* and *TNNT2*), EC (*VWF*), and cFB markers (*COL1A1* and *PDGFRA*) increased in the chip-flow model compared to the no- or plate flow models, revealing increased functional differentiation of different cell types (Min et al., 2024). Altogether, the combination of EHTs with chip-based flow enables the functional maturation of diverse cell types within organoids while simultaneously facilitating an increase in size up to 1 mm.

In conclusion, a meta-analysis of transcriptomic changes in human ischemic organoids revealed a strong resemblance to post-infarct murine hearts while still preserving key human-specific characteristics (Richards et al., 2020). The utility of organoids in IRI research is currently constrained by the absence of a vascular network, which is a crucial element in reperfusion studies. However, recent developments in organoid technology have led to the creation of organoids containing chamber-like structures, which have opened new avenues of research in the field of organoid-ischemia–reperfusion studies (Hofbauer et al., 2021b). Moreover, the integration of chip flow, intersecting scaffold-free self-organization and complexity with scaffold-based nutrient exchange (Min et al., 2024), presents promising opportunities for bridging the knowledge and treatment gap in IRI.

6 Limitations and future opportunities

6.1 Maturation of hiPSC-CMs

As the knowledge on maturation is rapidly evolving, mature hiPSC-CMs will come closer to but remain different from adult human CMs (Ahmed et al., 2020; Tu et al., 2018). Relative immaturity of hiPSC-CMs can lead to reduced translatability of IRI findings compared to more established animal models, potentially limiting their predictive accuracy for human outcomes.

Microtissues comprising hiPSC-CMs and cFBs demonstrated enhanced electrophysiology and contractility superior sarcomere structure and augmented mitochondrial respiration, compared to hiPSC-CMs alone (Giacomelli et al., 2020). In addition, the amplitude of intracellular calcium flux during the contraction–relaxation cycle of hiPSC-CMs increased when co-cultured with cFBs (King et al., 2022), indicating enhanced cardiomyocyte maturation. Both fibroblasts and the co-culture of hiPSC-CMs with ECs result in the increased maturation of hiPSC-CMs (Giacomelli et al., 2020; Abecasis et al., 2019; Gisone et al., 2022; Liu et al., 2021), resulting in more organized and longer sarcomeres than the single-culture controls (Gisone et al., 2022), a feature suggesting a more adult-like phenotype and often associated with improved contractile function (Skorska et al., 2022). Additionally, the co-culture resulted in the increased expression of proteins involved in the deposition of several extracellular matrix components such as collagens and fibronectin (Abecasis et al., 2019), increased the contractility and higher expression of the ventricular cardiomyocyte marker *IRX4* (Liu et al., 2021), and reduced the beating frequency (King et al., 2022).

Both the integration of multiple cell types and functional heart-on-a-chip integration of electrical pacing capabilities (Schneider et al., 2022) or mechanical stretch/stress (Mozneb et al., 2024) can significantly increase hiPSC-CM maturation. A recently developed multi-cell heart-on-a-chip model using multi-cell-type hiPSC-CMs and HUVECs could incorporate both dynamic fluid flow (shear stress) and biomechanical cyclic stretch. The combination of maturation strategies led to an improvement in the functional and transcriptional maturity of hiPSC-CMs, an improvement in the alignment of hiPSC-ECs grown on a heart chip and the facilitation of the formation of a tube-like EC network (Mozneb et al., 2024). Although hiPSC-CMs have yet to reach the

maturity or mitochondrial capacity of adult CMs (Wu et al., 2021), it becomes increasingly feasible to combine multiple maturation methods to optimize hiPSC-CMs for modeling IRI in this fast progressing field.

6.2 Addition of 3D immune response

One limitation of current 3D IRI models is the absence of the immune component as an injury-response mediator. Recently, Ze Lu et al. not only developed a heart-on-a-chip system that incorporates HUVECs, CFs, and hiPSC-CMs but also tested the addition of an immune fraction in the form of human peripheral blood mononuclear cells (PBMCs) within the vascular channel that initiates migration through the system under the appropriate conditions. This high-throughput setup allows for the straightforward collection of flow data and the measurement of cardiac redouts, such as beating force (Ze Lu et al., 2024). The introduction of SARS-CoV-2 to the 3D system did not result in significant alterations in the secretion of the cytokines IL-6, IL-8, and MCP-1, beating force, or contraction slope, while the addition of PBMCs caused significant alterations in these readouts. Although it has not been tested in an IRI setting, the incorporation of an immune component could potentially enhance physiological relevance of the 3D culturing system.

6.3 Optimizing IRI protocols for 3D cardiac models

It is important to recognize the differences in *in vitro* protocols modeling IRI as the methods for inducing both ischemia and reperfusion can vary significantly between studies. This variation highlights the complexity of accurately modeling IRI *in vitro* and underscores the need for careful consideration when comparing findings across different experimental approaches. With oxygen modulation varying between 0.1% (Ellis et al., 2022) and 10% (Richards et al., 2020) and the time subjected to ischemia or reperfusion varying between 1 and 24 h, even at similar oxygen concentrations (Yadid et al., 2020; Veldhuizen et al., 2022), the majority of studies seek to achieve a total cell death rate between 20% and 50%.

Although oxygen deprivation is frequently used to induce ischemia, other physiological variables, like the lack of nutrients and the accumulation of cellular waste products, can also be introduced into the models to induce IRI. For instance, the accumulation of lactic acid during ischemic conditions (20 mM sodium lactate) and the corresponding reduction in pH 6.4 to 6.8 (Sebastião et al., 2020), instead of the physiological range of 7.2–7.4, combined with nutrient and oxygen deprivation, can be utilized to induce IRI in spheroids (Sebastião et al., 2020) and EHTs (Yadid et al., 2020; Chen and Vunjak-Novakovic, 2019). Hidalgo et al. (2018) compared oxygen reduction only to oxygen reduction together with physiological changes in pH and glucose availability during the ischemic episode in mature H9-NCX1+ CMs. A reduction in pH to 6.2, in conjunction with a 2-h period of 0% oxygen and 0 mM glucose, resulted in an approximate 60% increase in the death of CMs *in vitro*, measured by LDH concentration, compared to CMs only treated with 0% oxygen (Hidalgo et al., 2018).

To date, caution is still warranted when comparing and interpreting different IRI results as protocols are optimized per model as the field develops. Rapid developments in the field of hiPSC-CM maturation, as well as further development and standardization of IRI models, are expected to improve the consistency and reliability of IRI modeling *in vitro*.

6.4 Technological advancements

Increased sensitivity and opportunities in functional electrophysiological readouts offer great opportunities in IRI development. Integrated live oxygen sensor integration (Schneider et al., 2022) and the integration of patterned intra- and extracellular electrodes on a heart-on-a-chip model (Liu et al., 2020) allow for the measurement of extracellular beating frequency, spatial waveform propagation, and precise action potential measurement, even in an IRI environment (Liu et al., 2020).

Progression of readout techniques has facilitated further progress in this field; Gao et al. pioneered using a multi-omics approach in an IRI setting (Gao et al., 2023). By integrating bulk and single-nucleus RNA sequencing with metabolomics profiling of reperfused rat hearts at various time points post-MI, it was discovered that early reperfusion reduced myocardial IRI by preserving fatty acid metabolism, a process regulated by PPARα. Functionally, pretreatment with the PPARα agonist fenofibrate upregulated genes associated with fatty acid oxidation and TCA pathways and revealed significantly upregulated PPARα expression, indicating that fenofibrate maintains energy metabolism post-infarct. These results were confirmed in animal experiments showing smaller infarct size and reduced fibrosis compared to non-fenofibrate-treated controls (Gao et al., 2023). Although yet to be used in organoid models, spatial transcriptomics combined with single-cell RNA-seq in embryonic hearts has enabled the generation of a three-dimensional cellular map, used to investigate cardiac organoid physiology and behavior in depth (Asp et al., 2019). Omics technology is becoming increasingly extensive and more readily available (Babu and Snyder, 2023) as cellular interactions and specific metabolic changes can be mapped in more detail, paving the way for future scientific advancements and therapeutic strategies.

7 Conclusion

Although these new models are a major leap forward that can help increase our insights into IRI pathology, it remains a challenge to fully recapitulate the complexity of human IRI *in vitro*. Consequently, we remain reliant on animal IRI studies to complement the findings obtained in 3D systems. Nevertheless, in light of the rapid advancement of bioprinting, tissue engineering, and microfluidics, along with the growing use of multi-omics testing, these 3D models offer invaluable tools for drug, biomarker, and discovery research, surpassing conventional 2D systems while maintaining human relevance. As the physiological relevance of the 3D models is enhanced by increasing physiological cues and different cell types, data extraction is facilitated by the use of multi-omics and more complex electrophysiological readouts. Furthermore, 3D models offer

distinctive advantages in terms of human relevance, availability, experimental control, and reproducibility, making them a valuable addition to animal studies in addressing the significant unanswered questions and facilitating a drug-testing platform in IRI.

Author contributions

MP: conceptualization, investigation, methodology, resources, visualization, writing–original draft, and writing–review and editing. XZ: writing–review and editing. SK: writing–review and editing. JK: funding acquisition, resources, supervision, and writing–review and editing.

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Conflict of interest

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PCSK9-antibodies fail to block PCSK9-induced inflammation in macrophages and cannot recapitulate protective effects of PCSK9-deficiency in experimental myocardial infarction

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Background and aims: Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a crucial role in cholesterol homeostasis by regulating low-density lipoprotein (LDL) receptor levels. Despite its known effects on cholesterol metabolism, the role of PCSK9 in cardiac function, especially post-myocardial infarction (MI), remains unclear. This study investigates the impact of PCSK9 on heart function post-MI and evaluates the effects of PCSK9 inhibition via Alirocumab.

Methods: We used PCSK9 knockout (KO) mice and wildtype (WT) mice and *in vivo* treatment with Alirocumab to analyze cardiac function and survival post-MI induced by permanent ligation of the left anterior descending artery. PCSK9 and LDL receptor levels were measured using ELISA and qRT-PCR. Cardiac function was assessed via echocardiography and isolated working heart model experiments. Gene expression changes were evaluated using RNA sequencing, and inflammatory responses in bone marrow-derived macrophages (BMDMs) were analyzed *in vitro*.

Results: PCSK9 was expressed in murine heart tissue at levels comparable to the liver, despite minimal heart RNA expression. PCSK9 KO mice had lower plasma cholesterol levels and showed reduced cardiac functions in the working heart model compared to WT mice. Post-MI, PCSK9 KO mice demonstrated significantly improved survival and reduced ventricular rupture compared to WT mice. Alirocumab treatment, while effective in lowering plasma cholesterol, did not replicate the survival benefits seen in PCSK9 KO mice and even worsened cardiac function post-MI. *In vitro*, PCSK9 induced significant inflammatory responses in macrophages, which were not mitigated by Alirocumab.

Conclusion: PCSK9 accumulation in the heart post-MI contributes to adverse cardiac remodeling and inflammation. Genetic deletion of PCSK9 confers protection against post-infarct mortality, whereas pharmacological inhibition with Alirocumab fails to reproduce these benefits and may exacerbate cardiac dysfunction. These findings highlight the complex role of PCSK9 in cardiac pathology and caution against the assumption that PCSK9 inhibitors will necessarily yield cardiovascular benefits similar to genetic PCSK9 deficiency.

KEYWORDS

PCSK9, PCSK9 inhibitors, alirocumab, PCSK9 deficiency, myocardial infarction, inflammation, macrophages

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proprotein convertase family and plays a pivotal role in cholesterol homeostasis (1, 2). PCSK9 regulates low-density lipoprotein (LDL) cholesterol levels by binding to hepatic LDL receptors (LDLR), promoting their lysosomal degradation, and thus reducing LDLR availability for clearing LDL cholesterol from the bloodstream (3, 4). Gain-of-function (GOF) mutations in the PCSK9 gene have been identified as a genetic cause of familial hypercholesterolemia, with numerous such mutations reported (5, 6), increasing the risk for atherosclerotic cardiovascular diseases such as coronary artery disease (CAD), cerebrovascular disease, and peripheral atherosclerosis.

The critical role of PCSK9 in cholesterol regulation has made it an attractive target for lipid-lowering therapies (7, 8). This has led to the development of PCSK9 inhibitors, including monoclonal antibodies (e.g., Alirocumab and Evolocumab) and small interfering RNAs (siRNAs) (e.g., Inclisiran), which block PCSK9 function or production. Clinical trials have demonstrated the efficacy of these PCSK9-targeting drugs in reducing serum LDL cholesterol levels significantly and preventing major adverse cardiovascular events.

Although the liver is the main source of circulating PCSK9, its expression has also been confirmed in other tissues, including the kidneys, brain, small intestine, colon and various cell types such as vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and macrophages (2). However, the function of PCSK9 in the heart, especially in hearts suffering from ischemic injury, remains poorly understood. In previous studies, PCSK9 deficient mice showed a concentric left ventricular (LV) remodeling and a significant reduction of exercise tolerance without changes in systolic LV function (9). Da Dalt explained this HFpEF-like phenotype by potential cardiac lipotoxicity due to intracardial lipid accumulation in PCSK9 KO mice.

In patients with a history of heart failure and a recent acute coronary syndrome, treatment with the PCSK9-antibody Alirocumab did not lead to a reduction in cardiovascular events despite potent LDL cholesterol reduction. On the contrary, the rate of non-fatal myocardial infarctions was even higher in this subgroup of patients (10). This observation prompted our interest in investigating the role of PCSK9 in the injured heart and with PCSK9 antibody treatment in particular.

In this work, we profiled hearts of PCSK9-KO mice and tested their fate post myocardial infarction. We treated mice with the PCSK9-inhibitor Alirocumab, which increased hepatic LDLR expression and reduced cholesterol levels, and subjected them to experimental myocardial infarction, while antibody-bound PCSK9 accumulated in the heart. Mechanistically, PCSK9 induced, dose-dependent gene expression changes were explored in cardiomyocytes, fibroblasts and macrophages, identifying macrophages as key responder cells to PCSK9 surges. Notably, PCSK9 binding to antibodies did not block its pro-inflammatory effects in macrophages.

Methods

Cholesterol quantification

Total plasma cholesterol was quantified using a colorimetric assay (Diagnostic Systems, Holzheim, Germany).

Western blot

A tissue sample (~30 mg) was homogenized in RIPA lysis buffer (Santa Cruz Biotechnology), shock-frozen in liquid nitrogen and thawed on ice. After removal of cell debris by centrifugation total protein concentration was determined using a BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). 20–30 µg of Protein per sample were loaded for SDS-PAGE and blotted using the Trans-Blot Turbo Transfer System using nitrocellulose membranes (Bio-Rad Laboratories Ltd, Hercules, CA). Stain free blot acquisition was used for total protein normalization. Primary antibodies (Anti-mouse Cd36 1:1.000, Anti-mouse Gapdh 1:10.000, Anti-mouse Ldlr 1:1.000, Anti-mouse Lrp1 1:5.000, Anti-mouse Vldlr 1:1.000, all Abcam, Cambridge, UK) were incubated overnight at 4°C, secondary antibody (Anti-rabbit IgG HRP-linked, Cell signaling Technology, Denver, MA) for 1 h at room temperature. After multiple washing steps chemiluminescent substrate was added for 1 min. Proteins were quantified using ImageLab 3.1 (Bio-Rad Laboratories Ltd, Hercules, CA).

RNA extraction from myocardial tissue

A tissue sample (~30 mg) was excised with a scalpel and transferred to an ice-cold round-bottom FACS tube. One milliliter of Qiagen QIAzol Lysis Reagent was added, and the tissue was homogenized. The samples were incubated at room temperature, then centrifuged to pellet cell fragments. Chloroform was added to the supernatant. After phase separation by centrifugation, the upper phase was transferred to a new tube and mixed with an equal volume of 70% ethanol. RNA was isolated from the lysate using the Qiagen RNeasy Mini Kit following the manufacturer's protocol.

RNA extraction from isolated cells

RLT Lysis Buffer from the Qiagen RNeasy Micro Kit was supplemented with 1% β-mercaptoethanol. The buffer was added to each well, the cells were detached using a cell scraper. The lysate was transferred to a reaction tube, mixed thoroughly, and shock-frozen in liquid nitrogen. After thawing on ice, an equal volume of 70% ethanol was added and Qiagen QIAshredder was used. RNA was isolated from the flow-through using the Qiagen RNeasy Micro Kit according to the manufacturer's protocol.

qPCR

For reverse transcription of RNA, Thermo Fisher Scientific High-Capacity cDNA Reverse Transcription Kit was used according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed for gene expression quantification using Thermo Fisher Scientific TaqMan Gene Expression Assays probes (CD36 Mm01135198_m1, IL-1β Mm00434228_m1, IL-6 Mm00446190_m1, LRP1 Mm00464608_m1, PCSK9 Mm01263610_m1) and the qPCRBIO Probe Mix Lo-Rox buffer system. β-Actin was utilized as an endogenous control for gene expression normalization. The reaction was carried out using the CFX96 Touch Real-Time PCR System. Data analysis was performed using CFX Manager 3.1 software. The ΔΔCt method was used to analyze the data, and genes with a Ct value greater than 40 were considered non-expressed.

RNA sequencing

The isolated RNA of each sample was eluted with 14 µl of water, from which 4 µl of RNA were used for library preparation using the NEBNext Ultra II Directional RNA Library Prep Kit. The library was sequenced on a NextSeq instrument with 75 bp paired-end reads using NextSeq 500 High Output v2 kit (Illumina). At least 45 million reads were acquired from each bulk sample. Fastq files were transformed to gene counts using the Galaxy platform (https://usegalaxy.eu/). Adapters and end bases were trimmed with Cutadapt (Galaxy Version 1.16.5) with Phred score lower than 20. Trimmed reads with length shorter than 20 bp were discarded. The trimmed reads were aligned to the human genome (hg38) using RNA STAR (Galaxy Version 2.7.2b) with default settings. FeatureCounts (Galaxy Version 1.6.4 + galaxy1) was applied to count the features from the forward stranded bam files. The gene count files were downloaded and imported into R (Version 4.3.1) for downstream analyses. We utilized dplyr (Version 2.3.4) to compute the gene count files. Differentially expressed genes (DEGs) were called by DESeq2 (Version 1.40.2). Significantly regulated genes were defined as genes that increased or decreased significantly as opposed to the untreated control. Statistical significance of DEGs were defined as adjusted p-value < 0.05 post-Bonferroni correction. Pathway analyses were conducted using the online tool Enrichr. Further analyses were performed using the EnhancedVolcano package (Version 1.12.0) and the ComplexHeatmap package (Version 2.10.0).

Isolated working heart model

The hearts of wildtype and PCSK9-KO mice were excised and immediately placed in ice-cold Krebs-Henseleit Buffer (KHB), consisting of (in mmol/L): 128 NaCl, 5 KCl, 1 KH2PO4, 1.3 MgSO4, 15 NaHCO3, 2.5 CaCl2, and 5 Glucose. Retrograde Langendorff perfusion was then conducted at 37°C with KHB at a perfusion pressure of 50 mmHg. After cannulation of the left atrium, the perfusion mode was switched to a working mode with a preload of 15 mmHg and afterload of 50 mmHg. Following an initial equilibration period, the hearts were perfused for 60 min with KHB supplemented with 0.4 mmol/L palmitate bound to 3% BSA. Aortic pressure changes were monitored using a Millar Micro-Tip pressure catheter (Millar Instruments, Houston, TX, USA) inserted into the aortic cannula. The aortic developed pressure was determined as the difference between systolic and systemic pressures. The rate-pressure product was calculated by multiplying the aortic developed pressure by the heart rate.

Aortic and coronary flows were measured by collecting flow from the afterload line and the effluent from the heart, respectively, with cardiac output defined as the sum of both flows. Cardiac work (ml*mmHg/min) was calculated as the product of cardiac output and aortic developed pressure per minute. Myocardial oxygen consumption (MVO2) was assessed by measuring the difference in oxygen concentration between pre- (arterial, aO2) and post-heart (venous, vO2) samples using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL, USA). Cardiac efficiency was then calculated as the ratio of hydraulic work to MVO2.

To measure palmitate oxidation within the same perfusion, the amount of ^3H_2O released from [9,10-^{3}H] palmitate (specific activity, 500 GBq/mol) was determined. ^3H_2O was isolated from [9,10-^{3}H] palmitate by mixing 500 µl of perfusate sample with 1.88 ml of chloroform/methanol (1:2 v/v) for a 15-min incubation, followed by adding 625 µl chloroform and another 15-min incubation. A 2 mol/L HCl/KCl solution was then introduced, mixed, and incubated for a minimum of 30 min to form polar and non-polar phases. An aliquot of 1.8 ml from the polar phase was transferred to another tube and mixed

sequentially with 1 ml of chloroform, 1 ml of methanol, and 900 µl of HCl/KCl solution, with a 15-min incubation after each addition. After the final addition and an incubation period of at least 30 min, two 500 µl aliquots were collected from the upper layer for ^{3}H counting. Palmitate oxidation rates were calculated from $^{3}H_2O$ production, with adjustments for dilution during the separation process. The isolated working heart model was performed as previously described (11).

Echocardiography

The echocardiographic examination was conducted under light anesthesia with isoflurane (induction dose 3.5 vol.%, maintenance dose 2 vol.%). Images were acquired using the Vevo 3100 ultrasound system. For the parasternal long-axis view, the transducer and ultrasound table were adjusted to visualize the aortic valve, minimize foreshortening of the heart apex, and maximize the left ventricle's dimensions. The transducer was then rotated 90° for the parasternal short-axis view. The analysis was performed blinded using VevoLab 3.1.0 software. In the parasternal long-axis view, the contours of the left ventricle were traced during systole and diastole to determine the left ventricular areas. Assuming an ellipsoidal ventricle, the ejection fraction (EF) was calculated. The thickness of the left ventricular posterior wall (LVPW) was measured in the parasternal short-axis view at a height of 6 mm from the apex.

Permanent ligation

Myocardial infarction was induced by permanent ligation of the left anterior descending artery (LAD). The experimental animals were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. To compensate for perioperative blood and fluid loss, 10 ml/kg of isotonic 5% glucose solution was administered intraperitoneally. Following orotracheal intubation, pressure-controlled ventilation was performed. Anesthesia was maintained with 2 vol.% isoflurane. A left lateral thoracotomy was performed. The left anterior descending artery was identified under a microscope and ligated using a suture.

Alirocumab treatment

Subcutaneous injections of 3 mg/kg body weight of Alirocumab or human IgG control antibody were administered, diluted in sterile water with a total volume of 100 µl per mouse. WT mice received a single or 4 weekly injections of 3 mg/kg Alirocumab or IgG1 control antibody.

BMDM cultivation

Both murine femurs were extracted. The epiphyses were removed, and the bone marrow was flushed out with PBS. The cell suspension was filtered, and cell concentration was determined. The suspension was centrifuged and the pellet was resuspended in complete medium (RPMI 1640 with 10% FCS, 1% penicillin-streptomycin, 60 ng/ml M-CSF). Cells were plated at 1 million cells per well in 3 ml in a 12-well plate and incubated at 5% CO2 and 37°C. On day 3, the medium was replaced with fresh complete medium containing 30 ng/ml M-CSF. On day 5, the medium was replaced with 1 ml of starvation medium (RPMI 1640 without FCS), and stimulants were added.

Results

PCSK9 is expressed in the heart

To investigate the role of PCSK9 in the heart, the expression of PCSK9 and its binding partner LDLR were analyzed. PCSK9 expression in murine heart tissue was confirmed and quantified using ELISA, revealing comparable PCSK9 concentrations in heart and liver when normalized to total protein content (Figure 1A). However, at the RNA level, Pcsk9 expression was minimally detectable in the heart by qRT-PCR, indicating negligible on-site production (Figure 1B).

LDLR expression, a common binding partner of PCSK9, was detectable in the liver but not in the heart, as shown by qRT-PCR and Western blot analysis (Figures 1C,D). The expression levels of VLDLR, LRP1, and CD36, which have been proposed as alternative binding partners for PCSK9 (12–15), were explored by both Western Blot and qPCR of murine heart and liver tissue lysates. VLDLR and CD36 were predominantly expressed in the heart, while LRP1 was present in both organs (Figure 1C, Supplementary Figure A).

In summary, PCSK9 protein levels in the heart were relatively high despite negligible Pcsk9 RNA expression in the heart, suggesting that PCSK9 of plasmatic origin accumulated in the healthy heart even in the absence of the canonical binding partner LDLR.

PCSK9 influences cardiac function

First, cardiac function was evaluated in healthy PCSK9 knockout (KO) mice compared with wildtype mice (Figure 1E). Genetic deletion was confirmed via ELISA, which showed undetectable plasma PCSK9 levels in KO mice, while wildtype mice exhibited baseline levels around 0.1 µg/ml (Figure 1F). In line with PCSK9 loss of function mutations in humans, PCSK9 KO mice featured significantly lower plasma cholesterol levels in both males and females compared to wildtype counterparts (Figure 1G).

Applying echocardiography under resting conditions, parameters such as ejection fraction, diastolic left ventricular inner diameter (LVIDd), and fractional shortening showed no differences between the genotypes. Likewise, diastolic function assessed by E/A and E/e' ratios was comparable between PCSK9 KO and WT mice (Figure 1H).

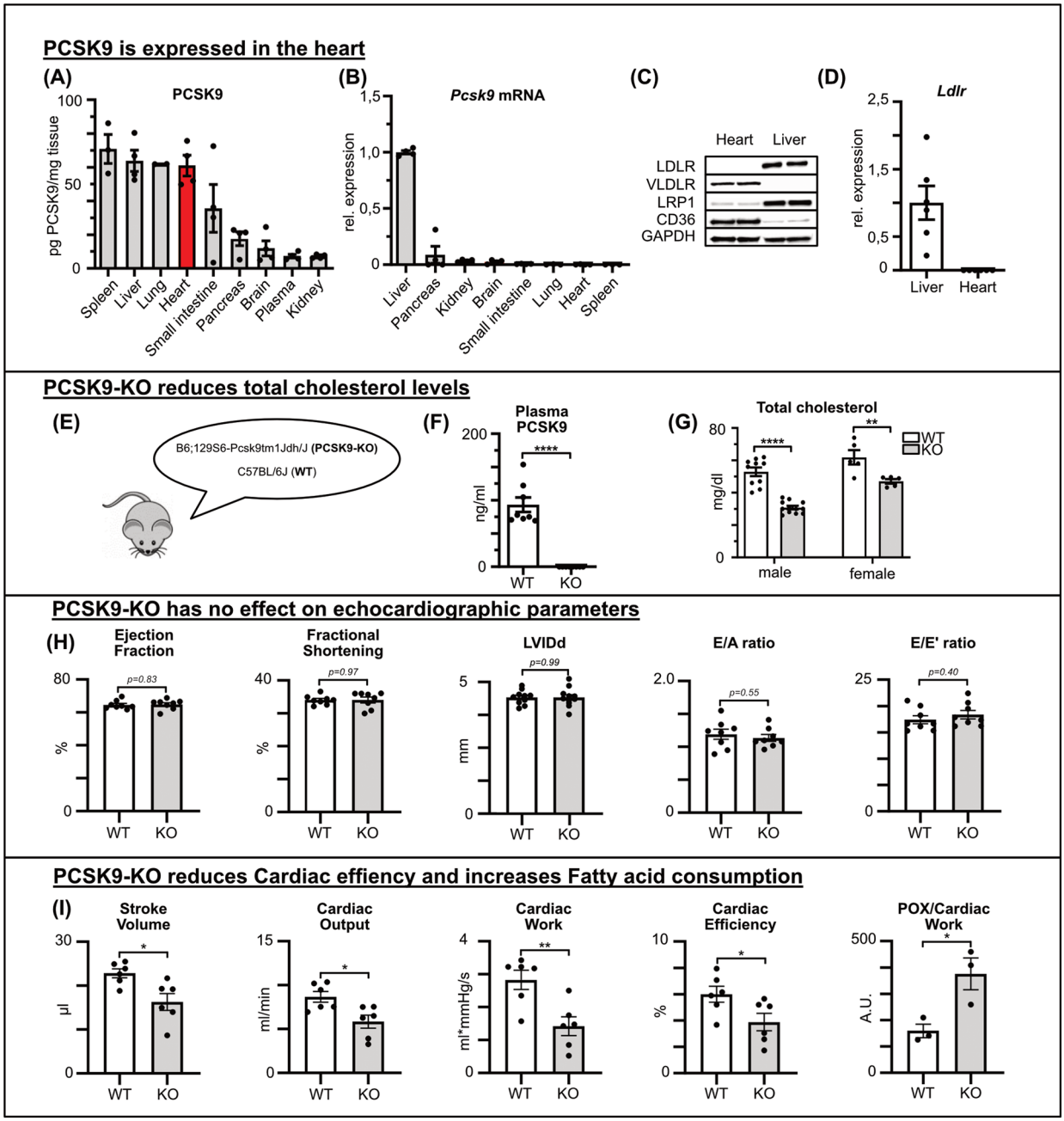

FIGURE 1
PCSK9 is expressed in the heart and influences cardiac functions PCSK9 protein concentration of different tissue and plasma lysates determined by ELISA normalized for total protein concentration determined by BCA assay **(A)** and *Pcsk9* RNA expression relative to hepatic expression **(B)** of C57BL/6 mice ($n = 4$). Using qRT-PCR, the expression of the LDL receptor **(D)** in the liver and heart was quantified ($n = 6$). **(C)** A representative Western blot illustrates potential PCSK9 targets in the heart and liver. PCSK9-ELISA **(F)** and cholesterol assay **(G)** of plasma samples from male and female C57Bl/6J wildtype (WT) and PCSK9-KO mice **(E)**, $n = 8$, $^{**}p < 0.01$, $^{****}p < 0.001$ (*t*-test). No changes in echocardiographic parameters between WT and PCSK9-KO mice **(H)**. Results from Working heart model showing significant reduction in stroke volume, cardiac output, cardiac work, cardiac efficiency and fatty acid consumption/cardiac work. $n = 8$, $^{*}p < 0.05$, $^{**}p < 0.01$ (*t*-test) **(I)**.

To investigate cardiac function in more detail, we used the working heart model *ex vivo* as previously described. In this model, PCSK9 KO mice demonstrated significant differences in key cardiac functional parameters, specifically decreased stroke volume, reduced cardiac efficiency and relatively increased fatty acid oxidation for comparable cardiac work as WT mice (Figure 1I).

To assess the underlying mechanisms of PCSK9's influence on cardiac function, we explored gene expression changes in the hearts of KO and WT mice. RNA sequencing revealed 1,448 differentially expressed genes (DEGs), with approximately half of the DEG being upregulated in KO hearts and the other half being downregulated (Figures 2A,B).

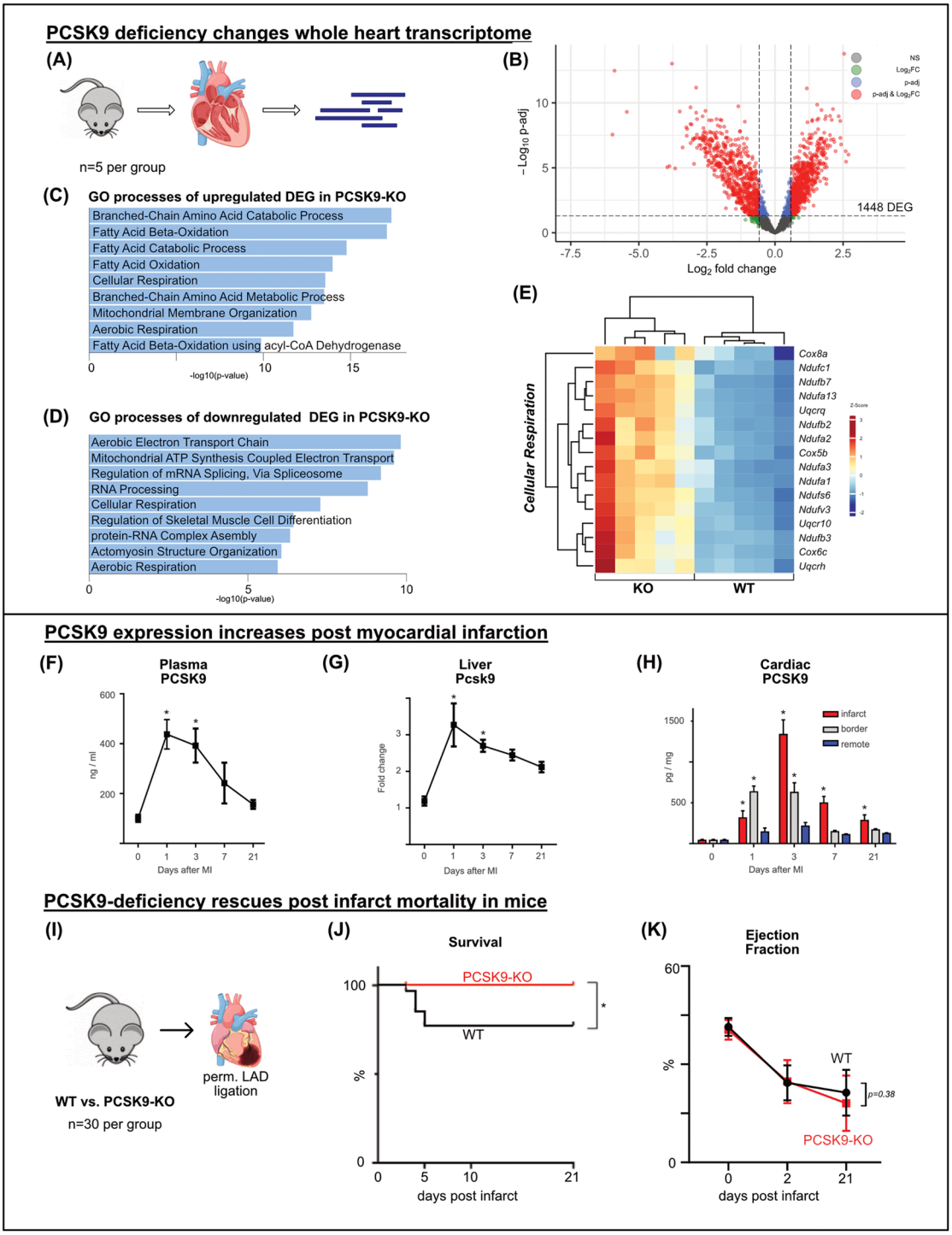

FIGURE 2
PCSK9 deficiency changes whole heart transcriptome and rescues post infarct mortality bulk-RNA sequencing of whole heart tissue was performed comparing the transcriptome of PCSK9-KO mice to WT, $n = 5$ per group **(A)** volcano plot of RNAseq results highlighting differentially expressed genes (DEG) (p-adj <0.05) in red and blue. A total of 1,448 genes were differentially expressed between the groups **(B)** Gene ontology analysis of up- **(C)** and downregulated **(D)** DEG separately using EnrichR webtool showing the top significantly altered gene ontology terms. Heatmap of DEG of the significantly enriched GO-term of cellular respiration **(E)**, $n = 5$ per group. Differential expression analyzed with DESeq2 tool. PCSK9-ELISA of plasma samples **(F)** and cardiac tissue lysates relative to total protein concentration determined by BCA **(H)** and qRT-PCR of liver samples relative to β-Actin expression **(G)** was performed at different timepoints following LAD ligation. Results are presented as mean ± SEM, $n = 4$–9 per timepoint, $^*p < 0.05$ (One-way ANOVA, Dunnet post-test). Kaplan-Meier survival curve after permanent LAD ligation **(I)** showing significant survival benefit in PCSK9-KO mice **(J)**, $n = 30$ per group, $^*p < 0.05$ (Log-rank test). No significant changes in ejection fraction at day 2 or 21 post MI **(K)** (Two-way ANOVA, Bonferroni's post-test).

Gene ontology (GO) analysis using EnrichR was conducted separately for upregulated and downregulated DEGs. In line with the working heart model results, GO terms related to fatty acid consumption featured most prominently among the upregulated DEGs in KO hearts. Matching reduced cardiac work and efficiency in KO hearts, gene expression related to the aerobic electron transport chain and ATP synthesis was downregulated (Figures 2C–E).

PCSK9 appears to support homeostatic and metabolic cardiac function, but its loss can be compensated in the heart *in vivo* under resting conditions.

PCSK9 levels rise post myocardial infarction

Permanent ligation of the left anterior descending artery (LAD) in mice was used as an experimental model for myocardial infarction. Animals were sacrificed before (day 0) and at 1, 3, 7, and 21 days post-surgery. PCSK9 concentration in plasma was determined by ELISA, showing a significant increase peaking at 0.4 µg/ml on day 1 post-infarction. Although the concentration gradually decreased thereafter, PCSK9 levels did not return to baseline for one week (Figure 2F).

In parallel, Pcsk9 gene expression in the liver, the main site of PCSK9 production, was analyzed using qRT-PCR. On day 1 post-infarction, liver Pcsk9 expression increased threefold from baseline, and then gradually decreased within one week mirroring the plasma PCSK9 levels (Figure 2G).

Myocardial tissue post-infarction was separated into three regions: the infarct area, a border zone region, and the non-infarcted remote area. These areas were lysed separately, and PCSK9 concentrations were measured using ELISA. At all post-infarction time points, a significantly increased PCSK9 concentration was detected in the infarct area, peaking on day 3. Elevated PCSK9 concentrations were also measured within the border zone during the first 3 days post infarction while the remote area showed only minor changed in PCSK9 accumulation (Figure 2H).

Taken together, myocardial infarction leads to a surge in PCSK9 concentrations in plasma, liver, and heart, suggesting a relevant role of PCSK9 in particular during the inflammatory phase of post MI cardiac remodeling.

PCSK9-deficiency rescues post infarct mortality in mice

To evaluate the effect of PCSK9 on infarct healing, the permanent ligation model was applied to PCSK9-KO and WT mice. Between days 3 and 5, 20% of WT mice died, whereas all PCSK9-KO mice survived (Figures 2I,J). Autopsy revealed that ventricular rupture was the cause of death in all cases. Echocardiographic assessments were performed before, as well as 2 and 21 days after myocardial infarction. Among the surviving mice, no significant differences in cardiac function were observed between the genotypes as exemplified by drops in ejection fractions between days 2 and 21 post-infarction (Figure 2K). Our results indicate that the absence of PCSK9 has favorable effects on post-infarction survival and protection from ventricular rupture.

PCSK9-inhibitor alirocumab fails to reproduce protective effects of PCSK9 deficiency in experimental myocardial infarction

To evaluate the translational implications of our findings in PCSK9 KO undergoing MI, we utilized the anti-PCSK9 antibody Alirocumab to inhibit PCSK9 *in vivo*. Wildtype mice received subcutaneous injections of 3 mg/kg Alirocumab or human IgG antibody as a control. PCSK9 targets the LDL receptor (LDLR) for degradation. Following a single Alirocumab injection, total murine PCSK9 levels surged in the plasma while being bound to the antibody (Figure 3A). As a result, hepatic LDLR concentrations peaked within three days, and returned to baseline around day 7 post injection (Figure 3B). These findings prompted us to use weekly Alirocumab injections in subsequent *in vivo* experiments. Four weekly injections of Alirocumab 3 mg/kg increased heart tissue concentrations by factor 10 compared to the IgG control group, while significantly decreasing plasma cholesterol levels (Figures 3C,D).

Next, Alirocumab or IgG pretreated mice underwent permanent LAD ligation to assess whether the survival benefit observed in PCSK9-KO mice can be recapitulated (Figure 3E). However, a comparable number of animals died post MI in Alirocumab and IgG treated mice. Echocardiographic evaluation even documented a more pronounced reduction in ventricular ejection fraction and increase in LVIDd in Alirocumab treated mice at day 21 post MI compared to controls (Figures 3 F–H).

In summary, therapy with the PCSK9 inhibitor Alirocumab fails to replicate the beneficial effects observed with PCSK9 knockout. Instead, following myocardial infarction in mice, treatment with Alirocumab even worsens cardiac systolic function.

PCSK9 changes cardiomyocyte and cardiac fibroblast transcriptome only marginally

Given that outcomes post MI diverged between PCSK9-KO mice and Alirocumab treated mice, we hypothesized that the supernaturally increased total PCSK9 levels in the circulation and heart following anti-PCSK9 binding could have deleterious effects on cardiac cells. To test this hypothesis, we isolated and cultured primary cardiomyocytes and cardiac fibroblasts from healthy murine hearts. The cells were divided into three groups and treated with two different doses of PCSK9 *in vitro*. One group received 0.4 µg/ml PCSK9, representing the PCSK9 concentrations measured post-myocardial infarction (MI) in the plasma and in different areas of the heart, while another group received 2.0 µg/ml PCSK9, corresponding to the PCSK9 concentrations measured in plasma following weekly Alirocumab

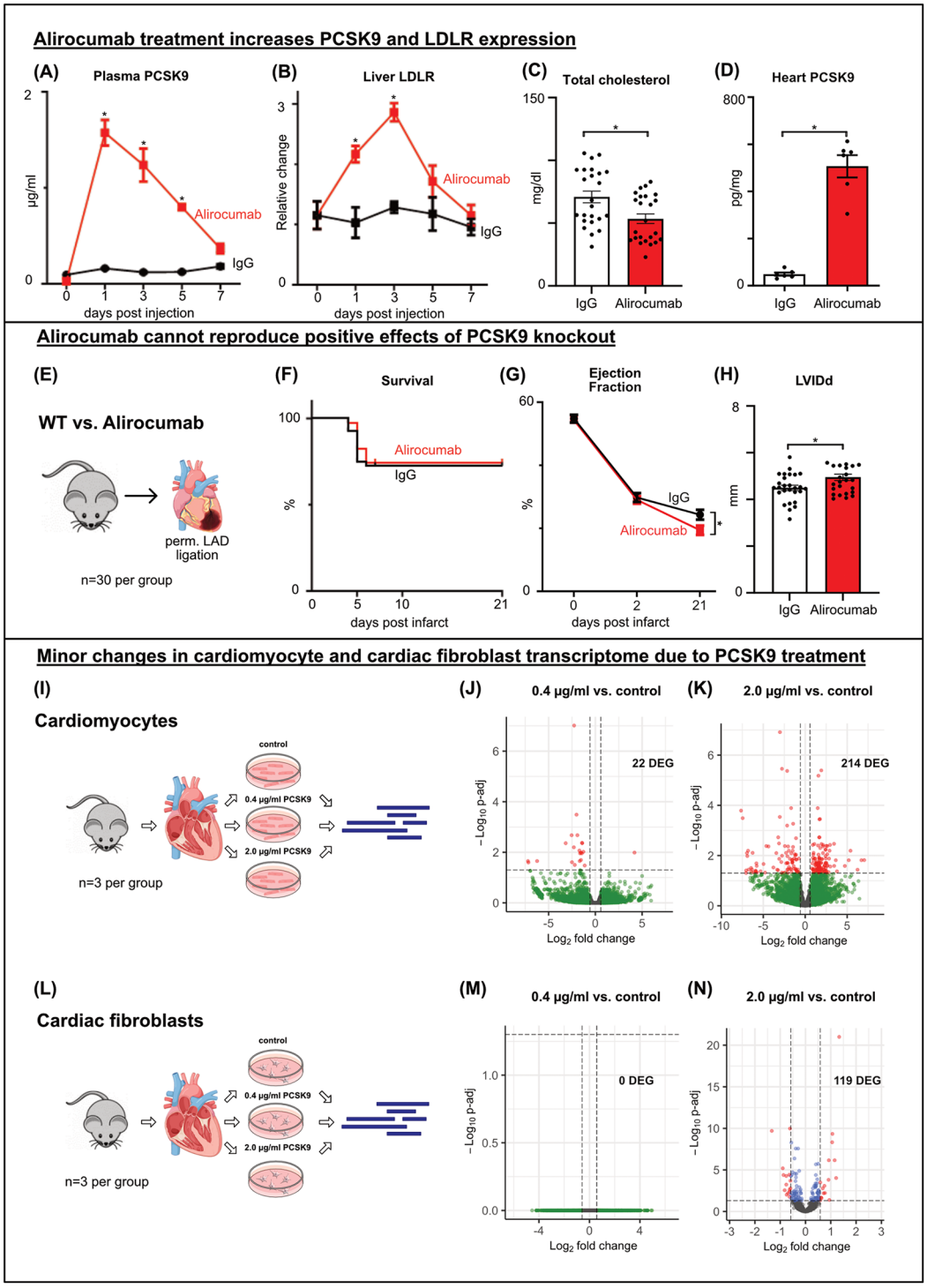

FIGURE 3
Alirocumab increases PCSK9 and LDLR expression and fails to reproduce protective effects of PCSK9 deficiency post MI WT mice received a single **(A, B)** or 4 weekly **(C, D)** injections of 3 mg/kg Alirocumab or IgG1 control antibody. Liver LDLR was analyzed using western blotting normalized for total lane protein **(B)**, plasmatic PCSK9 **(A)** and cardiac tissue lysate **(D)** PCSK9 normalized for total protein concentration using PCSK9-ELISA. Cholesterol assay of total cholesterol levels **(C)** $*p < 0.05$ Two-way ANOVA, Bonferroni post-test (A, B $n = 3$), t-test (C, D $n = 24/6$). WT mice received weekly subcutaneous injections of 3 mg/kg Alirocumab or IgG1 control antibody. LAD ligation **(E)** followed after 4 injections, echocardiography was performed before ligation (day 0) and 2 and 21 days after ligation **(G)**. Kaplan-Meier survival curves showing no differences between Alirocumab and IgG, $n = 30$ per group **(F)**. Left ventricular ejection fraction showing a significantly lower EF post MI in Alirocumab group, $*p < 0.05$, t-test ($n = 25$) **(G)**. LVIDd at day 21 post MI showing increased LV diameter after Alirocumab treatment **(H)**, $*p < 0.05$, t-test ($n = 25$). Results of Bulk RNA sequencing of cardiomyocytes **(I–K)** and cardiac fibroblasts **(L–N)** after *in vitro* stimulation with 0.4 µg/ml **(J, M)** or 2.0 µg/ml **(K, N)** PCSK9 compared to untreated controls ($n = 3$ per group). Volcano plots of DEG comparing PCSK9 treated cells to untreated controls. DEG are highlighted in red and blue (p-adj <0.05). Differential expression analyzed with DESeq2 tool.

injections. The third group served as an untreated control. The cells were stimulated overnight and subsequently processed for RNA isolation and sequencing (Figures 3I,L).

Stimulation with 0.4 μg/ml PCSK9 had negligible effects on gene expression in cardiomyocytes and cardiac fibroblasts. Only 22 DEGs were observed in cardiomyocytes treated with 0.4 μg/ml PCSK9 (Figure 3J, Supplementary Figure C). Stimulation with high-dose PCSK9 (2.0 μg/ml) led to modest transcriptional changes (119 DEG in fibroblasts, 214 DEG in cardiomyocytes (Figure 3K, Supplementary Figures E,G). However, these DEG failed to enrich for distinct GO terms (Supplementary Figures D,F,H).

In summary, gene expression profiling suggests that cardiomyocytes and fibroblasts do not appear as primary target cells for MI-associated or antibody-mediated PCSK9 surges. Therefore, we redirected our attention to monocyte-derived macrophages which feature prominently in the infarct and border zone where PCSK9 accumulates.

PCSK9 induces inflammation in BMDM

To this end, we generated bone marrow-derived macrophages (BMDMs) from WT mice and stimulated these cells *in vitro* with PCSK9 (Figure 4A). In contrast to our observations in cardiomyocytes and fibroblasts, stimulation of BMDMs with 0.4 μg/ml PCSK9 resulted in 734 DEGs, while the higher dose of 2.0 μg/ml PCSK9 yielded 3,760 DEGs (Figures 4B,C). The top 30 differentially regulated genes, ranked by adjusted *p*-value, are presented in heatmaps for the two doses (Figures 4D,G). In addition, GO terms enriched for up- or downregulated genes are presented separately for the two doses (Figures 4E,F,H,I). The 0.4 μg/ml dose induced genes associated with inflammation and cytokine production on the one hand, and suppressed genes related to endocytosis and migration on the other. The 2.0 μg/ml dose also induced many inflammatory genes including Interleukin 6 (IL-6) or tumor necrosis factor alpha (TNF) (Figure 4J). Quantitative RT-PCR analysis confirmed the dose-dependent induction of IL-6 gene expression in BMDMs. When Alirocumab was added to the BMDM culture together with PCSK9 at a dose sufficient to block PCSK9-mediated LDLR degradation in hepatocytes in culture (Supplementary Figure B), the IL-6 induction, however, was not ameliorated (Figure 4K). These results indicate that PCSK9 when bound to Alirocumab cannot facilitate LDLR degradation anymore but still induce inflammation in macrophages.

Discussion

Our study provides significant insights into the role of PCSK9 and its inhibitors in the heart, particularly following myocardial infarction. Initially, we demonstrated the presence of PCSK9 in murine hearts. At the protein level, organ weight-normalized PCSK9 concentrations in the heart were similar to those in the liver, while *Pcsk9* RNA was hardly detectable in the heart, suggesting a dominant plasmatic origin of PCSK9 in the healthy heart. These findings are consistent with transcriptomic data from the human heart, which shows no expression of PCSK9 in various heart cells (16). Epicardial adipose tissue has been proposed as a potential source of PCSK9. In patients undergoing cardiac surgery, PCSK9 was detectable in epicardial adipose tissue at both RNA and protein levels, with its expression correlating positively with various inflammatory cytokines and the thickness of epicardial adipose tissue, but not with plasma PCSK9 concentrations, suggesting a local pro-inflammatory effect from the epicardial adipose tissue (17).The pathway through which PCSK9 exerts its effects in the heart remains unclear. Our study shows that the usual binding partner, LDLR, is not expressed in the heart. However, PCSK9 has been reported to interact with other receptors such as Low Density Lipoprotein Receptor-related Protein 1 (LRP1), Very-Low-Density-Lipoprotein Receptor (VLDLR), Apolipoprotein E Receptor 2 (ApoER2), and Cluster of Differentiation 36 (CD36) (12–15). VLDLR, LRP1, and CD36 were all detectable in murine hearts. These receptors are involved in cholesterol and fatty acid metabolism. For instance, PCSK9-deficiency in mice leads to CD36-dependent accumulation of fatty acids in hepatocytes, which can be cytotoxic (18).

Likewise, increased lipid accumulation was observed in hearts of PCSK9-KO mice (19). Da Dalt et al. described thickened ventricular walls and reduced exercise capacity with preserved left ventricular ejection fraction in 5-month old PCSK9-KO mice (9). In our study, we did not observe significant changes in echocardiographic parameters including diastolic function in PCSK9-KO mice at rest, whereby our mice were 2–3 month old at the time of examination. However, we did observe a reduction in stroke volume and cardiac work in the sensitive *ex vivo* working heart model, alongside inefficient myocardial fatty acid beta oxidation. In line with our observations in the working heart model and with the report by Da Dalt et al., we found genes controlling cellular respiration and mitochondrial ATP synthesis to be downregulated in PCSK9-KO hearts. These data confirm an effect of PCSK9 at physiologic levels on the healthy heart, which can be compensated functionally in young adult mice *in vivo*.

Myocardial infarction triggers a sterile inflammatory response locally and systemically, the balance of which is crucial for long-term heart function (20). Consistent with previous studies, we detected a significant and transient surge in PCSK9 concentrations in the plasma and infarct following permanent LAD ligation, accompanied by elevated expression in the liver (21, 22).

In our study, genetic deletion of PCSK9 in mice prevented ventricular rupture and death following experimental myocardial infarction. All PCSK9 knockout mice survived permanent LAD ligation, while 23% of wild-type animals succumbed between days 3 and 5 post MI. There were no differences in ejection fractions, a key parameter of cardiac function, between PCSK9-KO and WT mice at days 2 (before any WT mice died) and 21 post MI. Previous studies reported improved ejection fractions in PCSK9-KO mice following myocardial infarction but did not

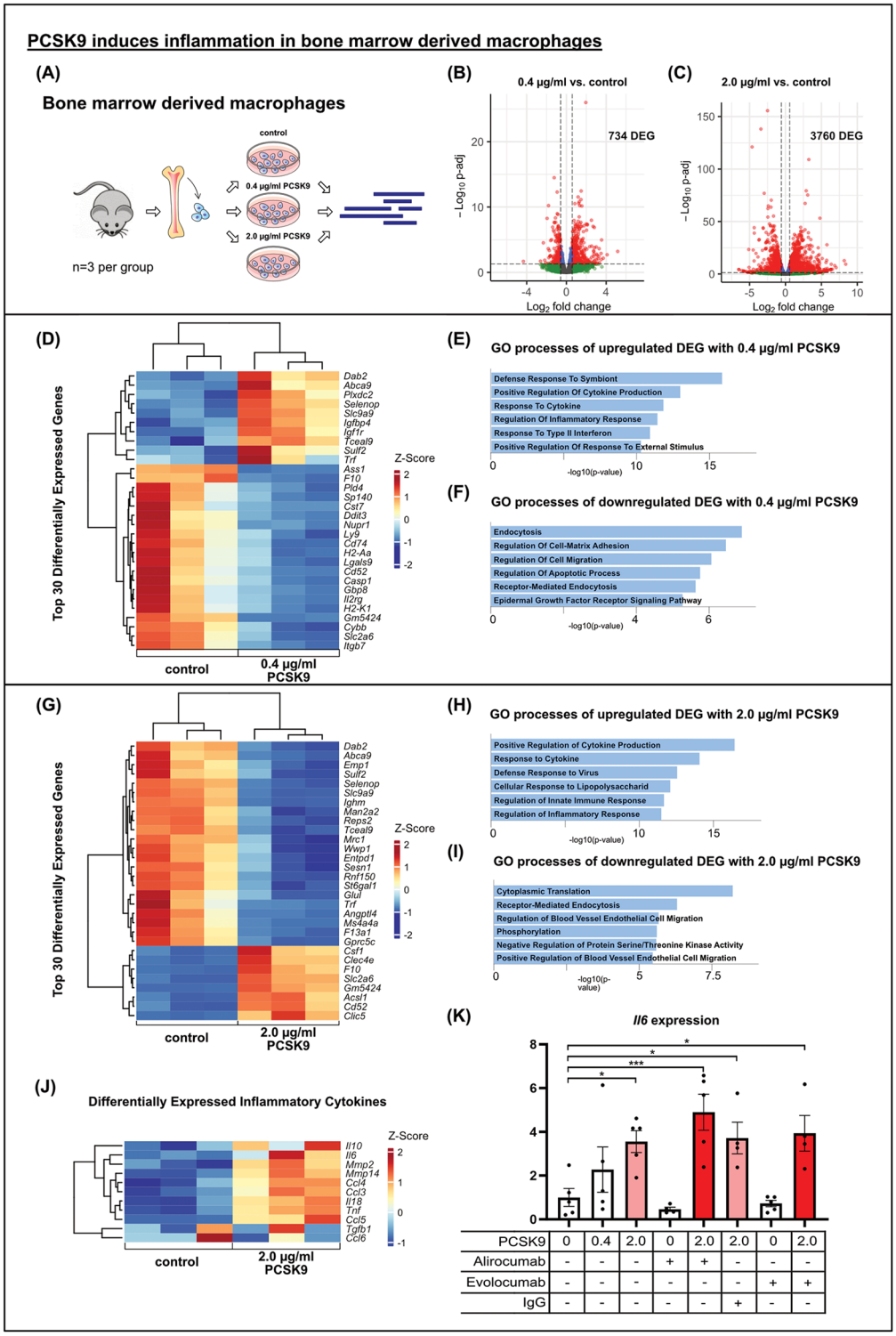

FIGURE 4
PCSK9 induces inflammation in bone marrow derived macrophages results of bulk RNA sequencing of bone marrow derived macrophages (BMDMs) after *in vitro* stimulation with 0.4 µg/ml **(B)** or 2.0 µg/ml **(A, C)** PCSK9 compared to untreated controls ($n = 3$ per group). Volcano plots of DEG comparing PCSK9 treated cells to untreated controls showing major changes in BMDM trancriptome due to PCSK9 stimulation. DEG are highlighted in red and blue (p-adj <0.05). Differential expression analyzed with DESeq2 tool. Heatmaps of the top 30 differentially expressed genes of each comparison (0.4 µg/ml PCSK9 vs. control **(D)** and 2.0 µg/ml PCSK9 vs. control **(G)** and DEG connected to inflammatory processes **(J)**. Gene ontology analysis of up- **(E, H)** and downregulated **(F, I)** DEG separately using EnrichR webtool showing significantly altered gene ontology terms. BMDMs were stimulated with different concentrations of recombinant mouse PCSK9, Alirocumab, Evolocumab and IgG control antibody overnight. IL-6 relative to b-Actin expression in macrophages ($n = 5$) using qRT-PCR **(K)**, $^{*}p < 0.05$, $^{***}p < 0.001$ (One-way ANOVA, Dunnet post-test).

report any mortality (22, 23). The lack of EF differences on day 21 in our study likely reflects selection bias, as WT mice with larger infarcts and adverse remodeling were predisposed to ventricular rupture and thus excluded from late-stage analyses. Ventricular rupture is primarily driven by macrophage-mediated degradation of the interstitial network. Our studies indicate that PCSK9 exerts a strong pro-inflammatory effect in bone marrow-derived macrophages, including increased levels of IL-6, TNF, MMP2 and MMP14. In the absence of PCSK9 the inflammatory response post-MI may be reduced, resulting in lower risk of ventricular rupture and enhanced survival in the PCSK9-KO group. Reduced autophagy has also been proposed as a potential protective mechanism in PCSK9-KO mice post-MI (19, 22). In contrast, increased PCSK9 accumulation in the infarcted heart following PCSK9 antibody treatment will exert the opposite effect, given that PCSK9 remains biologically interactive with macrophages even when antibody-bound.

We observed minimal transcriptional changes in cardiomyocytes and cardiac fibroblasts cultured under normoxic conditions. In contrast, we and others detected an inflammatory response to PCSK9 in macrophages. Ricci et al. were the first to demonstrate that PCSK9 exerts a inflammation in macrophages *in vitro*. Recombinant human PCSK9 led to a dose-dependent increase in the expression of inflammatory cytokines IL-1β, IL-6, TNF-α, MCP-1, and CXCL2 in human macrophages derived from monocytes of healthy donors (24). Wang et al. reported that co-stimulation of RAW264.7 cells with LPS and recombinant murine PCSK9 resulted in higher IL-6 and iNOS expression compared to LPS stimulation alone (25).

In our study, we utilized macrophages derived from murine bone marrow cells (BMDM) as a model for recruited macrophages, stimulated with recombinant murine PCSK9 at different doses. Stimulation with 0.4 μg/ml PCSK9, representing peak plasma levels post MI, induced inflammatory pathways in BMDM, an effect further amplified with a stimulation of 2.0 μg/ml PCSK9. Prominent examples included the inflammatory cytokines IL-6, IL-18, and TNF.

Importantly, our study was the first to demonstrate that the pro-inflammatory effects of PCSK9 in macrophages were not inhibited by the blocking antibodies Alirocumab and Evolocumab. While Alirocumab was able to prevent the degradation of the LDL receptor in PCSK9-stimulated murine hepatocytes, it did not reduce the increase in IL-6 expression upon PCSK9 incubation. These results demonstrate that even antibody-bound PCSK9 can activate macrophages. Since we obtained similar results for both Alirocumab and Evolocumab, we do not attribute these effects to a specific therapeutic agent but rather view it as a class effect. This finding is significant because total PCSK9 levels in plasma, and specifically in the heart, driven by the surge of antibody-bound PSK9, multiply by a factor of 10 and more following repetitive Alirocumab injections in wild-type mice. Schroeder et al. reported similar results in mice expressing human PCSK9, attributing the accumulation of PCSK9 to inhibited degradation by Alirocumab (26). In patients, total plasma PCSK9 also accumulates above physiologic levels with Alirocumab treatment (27). Nakamura et al. administered Evolocumab or placebo to patients with acute myocardial infarction, finding that PCSK9 concentrations multiplied significantly after Evolocumab administration (28). In our study, we observed Pcsk9 accumulation in murine heart tissue following Alirocumab injections capable of exacerbating inflammation and deteriorating cardiac function post MI.

In light of these findings, it is noteworthy that therapeutic PCSK9 antibodies, unlike most cholesterol-lowering drugs, do not lead to a reduction in high-sensitivity C-reactive protein (hsCRP). A meta-analysis found that only the antibody LY3015014 resulted in a—albeit not significant—reduction in hsCRP levels (29). Unlike other antibodies, LY3015014 does not lead to an accumulation of PCSK9 in plasma (26). This finding might indicate a proinflammatory effects of accumulated PCSK9. These potential drug-specific effects are particularly relevant given alternative methods for inhibiting PCSK9. For instance, Inclisiran, which inhibits PCSK9 production via siRNA and reduces LDL cholesterol by 50%, does not lead to accumulation of PCSK9 and did reduce hsCRP inconsistently (30–32).

In conclusion, our study sheds light on a complex role of PCSK9 in the heart. At physiologic levels, mainly originating from the liver, PCSK9 appears to support homeostatic cardiac metabolism and function, although a loss of murine PCSK9 can be functionally compensated *in vivo*. In line, loss-of-function mutations in human PCSK9 were not found to be associated with an increased risk of heart failure in a large UK Biobank case-control study (33). Following myocardial infarction, however, PCSK9 levels exceed baseline levels by factor 4 to 10 in blood, the infarct and border zone, an effect further amplified by PCSK9-antibody mediated accumulation of PCSK9. Although effectively blocking PCSK9-mediated LDLR degradation in mice analogous to its effects in humans, the clinically approved PCSK9 inhibitor Alirocumab did not mitigate PCSK9-induced inflammation in macrophages. On the contrary, anti-PCSK9-mediated accumulation of PCSK9 aggravate adverse cardiac remodeling post MI in our study. This calls for a nuanced approach in clinical applications and a deeper understanding of PCSK9's multifaceted roles in cardiac physiology and pathology. As the landscape of PCSK9 inhibition continues to evolve, our study provides critical insights that could inform the development of more effective and safe therapeutic strategies for patients with cardiovascular disease.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA1209060.

Ethics statement

The animal study was approved by Ethik-Kommission der Albert-Ludwigs-Universität Freiburg. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SR: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. CH: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. JH: Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. PS: Writing – review & editing, Data curation, Investigation, Methodology, Writing – original draft. RP: Writing – original draft, Writing – review & editing. CK: Writing – original draft, Writing – review & editing. TV: Writing – original draft, Writing – review & editing. CE: Writing – original draft, Writing – review & editing. BD: Writing – original draft, Writing – review & editing. DL: Data curation, Methodology, Supervision, Validation, Writing – review & editing. CM: Writing – original draft, Writing – review & editing. DeW: Writing – original draft, Writing – review & editing. DiW: Writing – original draft, Writing – review & editing. IH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. AE: Data curation, Formal Analysis, Funding acquisition, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2024.1463844/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Relative expression of potential PCSK9 binding partners in heart and liver. mRNA levels were determined by qRT-PCR. Results are shown as mean ±SEM **(A)** Primary murine hepatocytes were isolated and stimulated overnight with varying concentrations of Pcsk9 and Alirocumab. The expression of the LDL receptor and β-actin as a control was analyzed via Western blot. Quantification of LDL receptor normalized to β-actin, ($n = 3$). Results are shown as mean ± SEM **(B)** Results of Bulk RNA sequencing of cardiomyocytes **(C-F)** and cardiac fibroblasts **(G, H)** after *in vitro* stimulation with 0.4 µg/ml **(C, D)** or 2.0 µg/ml **(E-H)** PCSK9 compared to untreated controls ($n = 3$ per group). Heatmaps of all **(C)** or the Top 30 DEG of each analysis **(E, G)**. DEG comparing PCSK9 treated cells to untreated controls. Differential expression analyzed with DESeq2 tool. Gene ontology analysis of DEG using EnrichR webtool showing the top significantly altered gene ontology terms **(D, F, H)**.

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