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Exploring macrophage metabolic adaptations to bacterial infection: pathways and immune responses

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Exploring macrophage metabolic adaptations to bacterial infection: pathways and immune responses

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Editorial: Exploring macrophage metabolic adaptations to bacterial infection: pathways and immune responses

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Editorial on the Research Topic

Exploring macrophage metabolic adaptations to bacterial infection: pathways and immune responses

Macrophages undergo profound metabolic changes during bacterial infections. This metabolic reprogramming is recognized as central to their function and is the focus of this Research Topic. It brings together original research articles, reviews, and perspectives that illuminate how intracellular bacterial pathogens manipulate the host metabolism to their advantage.

Peng et al. identified Rv3737 of M. tb., a transmembrane protein and a homolog of threonine transporter, as a novel virulence factor. It promotes macrophage polarization toward an anti-inflammatory state, aiding bacterial survival. Ju et al., performed comprehensive proteomic analyses to distinguish between smear-positive and smear-negative tuberculosis, revealing distinct immune activation and lipid metabolism that could open avenues for precision diagnostics and targeted therapies.

Autophagy is a process by which cells degrade damaged organelles and protein aggregates to maintain metabolic and cellular homeostasis. Hos et al. reveal that the inflammasome complex (ASC specks) degradation through autophagy is regulated by p62, establishing it as a metabolic mediator between autophagy and inflammation. Wang et al. explored foamy macrophages in leprosy, showing that lipid-induced changes enhance immune activation, with CXCL13 playing a central role in lymphocyte recruitment. Perez-Toledo and Llibre compared metabolic responses to M.tb and S.t. infections, noting that, while both pathogens induce glycolysis and lipid synthesis, they exploit these pathways differently: M.tb utilizes lipids as nutrients, while S.t. prefers carbohydrates. Autophagy again emerges as a pivotal process, with pathogen-specific outcomes.

Ting's review on Hypoxia-Inducible Factors (HIFs) and nuclear factor erythroid 2-related factor 2 (NRF2) underscores their key role in macrophage metabolism. HIF-1α drives glycolysis under hypoxia, while NRF2 regulates redox balance and enhances phagocytosis and lysosomal fusion by upregulating the phagocytic receptor Macrophage Receptor with Collagenous Structure (MARCO). These regulators are modulated by metabolites like itaconate and p62, forming a complex network of immune control. Deramaudt et al. reinforces the therapeutic potential of targeting NRF2 by demonstrating that CDDO-Me, a NRF2 activator, lowers bacterial burden both *in vitro* and *in vivo*.

Jiang and Huiang's mini review on extracellular vesicles (EVs) reveal their dual role in bacterial communication and immune modulation. EVs from different bacteria elicit varied macrophage responses, influencing inflammation, cell death, and even antiviral defenses. Engineered EVs hold promise for targeted immunotherapies.

Together, these studies highlight the intricate metabolic choreography between macrophages and pathogens. Understanding these dynamics opens new avenues for precision medicine, offering strategies to harness or modulate immune metabolism in the fight against infectious diseases.

Author contributions

MH: Writing – review & editing, Writing – original draft. AG: Writing – review & editing. RG: Writing – review & editing. NR: Writing – review & editing, Writing – original draft.

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Proteomic analyses of smear-positive/negative tuberculosis patients uncover differential antigen-presenting cell activation and lipid metabolism

Yingjiao Ju[1,2†], Chengji Jin[3†], Shan Chen[3†], Jie Wang[1,2†], Cuidan Li[1†], Xiaotong Wang[1], Peihan Wang[1,2], Liya Yue[1], Xiaoyuan Jiang[1], Bahetibieke Tuohetaerbaike[4], Ying Li[4], Yongjie Sheng[5], Wushou'er Qimanguli[6], Jing Wang[4,3*] and Fei Chen[1,2,4,7*]

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Background: Tuberculosis (TB) remains a major global health concern, ranking as the second most lethal infectious disease following COVID-19. Smear-Negative Pulmonary Tuberculosis (SNPT) and Smear-Positive Pulmonary Tuberculosis (SPPT) are two common types of pulmonary tuberculosis characterized by distinct bacterial loads. To date, the precise molecular mechanisms underlying the differences between SNPT and SPPT patients remain unclear. In this study, we aimed to utilize proteomics analysis for identifying specific protein signatures in the plasma of SPPT and SNPT patients and further elucidate the molecular mechanisms contributing to different disease pathogenesis.

Methods: Plasma samples from 27 SPPT, 37 SNPT patients and 36 controls were collected and subjected to TMT-labeled quantitative proteomic analyses and targeted GC-MS-based lipidomic analysis. Ingenuity Pathway Analysis (IPA) was then performed to uncover enriched pathways and functionals of differentially expressed proteins.

Results: Proteomic analysis uncovered differential protein expression profiles among the SPPT, SNPT, and Ctrl groups, demonstrating dysfunctional immune response and metabolism in both SPPT and SNPT patients. Both groups exhibited activated innate immune responses and inhibited fatty acid metabolism, but SPPT patients displayed stronger innate immune activation and lipid metabolic inhibition compared to SNPT patients. Notably, our analysis uncovered activated

antigen-presenting cells (APCs) in SNPT patients but inhibited APCs in SPPT patients, suggesting their critical role in determining different bacterial loads/phenotypes in SNPT and SPPT. Furthermore, some specific proteins were detected to be involved in the APC activation/acquired immune response, providing some promising therapeutic targets for TB.

Conclusion: Our study provides valuable insights into the differential molecular mechanisms underlying SNPT and SPPT, reveals the critical role of antigen-presenting cell activation in SNPT for effectively clearing the majority of *Mtb* in bodies, and shows the possibility of APC activation as a novel TB treatment strategy.

KEYWORDS

tuberculosis, smear-negative pulmonary tuberculosis, smear-positive pulmonary tuberculosis, proteomics, antigen-presenting cells

Introduction

Tuberculosis (TB) is a significant global health concern and the second most deadly infectious disease after COVID-19, ranking as the 13th leading cause of death worldwide [Huang et al., 2019; World Health Organization, 2022]. In 2022, approximately 10.6 million individuals suffered from TB worldwide, and the disease claimed the lives of 1.6 million individuals [World Health Organization, 2022]. Smear-negative pulmonary tuberculosis (SNPT) and smear-positive pulmonary tuberculosis (SPPT) are two common types of active tuberculosis (Behr et al., 1999; Sossen et al., 2023).They are distinguished through microscopic examination outcomes of sputum samples for acid-fast bacilli (Dutt and Stead, 1994; Sossen et al., 2023). Generally, SPPT and SNPT correspond to relative high and low bacterial loads within the body, respectively (Dutt and Stead, 1994), with SPPT manifesting more severe symptoms and higher infectiousness than SNPT (Murthy et al., 2018; Kendall et al., 2021).

SPPT and SNPT can interconvert along with bacterial load changes. Some SNPT patients may progress to SPPT when the bacterial load increases to a detectable level in sputum smear microscopy (Colebunders and Bastian, 2000; Sossen et al., 2023). Conversely, SPPT can also convert to SNPT after treatment (Xu et al., 2016; Asemahagn, 2021), serving as an essential interim indicator of the effectiveness of the anti-TB treatment regimen and the reduction of infectivity (Calderwood et al., 2021; Yang et al., 2022). This conversion process can be influenced by various factors such as disease severity, host's immune status, and drug treatment effectiveness (Wang et al., 2009; Lee et al., 2012).

Previous studies have also reported some molecular mechanisms of SPPT and SNPT (Mwandumba et al., 2008; Chen et al., 2023; Yang and Feng, 2023). Yang et al. found that SNPT patients exhibited fewer pulmonary cavities and milder inflammatory responses, with lower numbers of immune cells and higher numbers of B-cells (Yang and Feng, 2023). Mwandumba et al. observed higher pro-inflammatory and immunomodulatory cytokine responses in SNPT than in SPPT, based on cellular and cytokine profile analyses in bronchoalveolar lavage (BAL) fluids (Mwandumba et al., 2008). Additionally, Chen et al. unveiled a higher degree of cellular exhaustion in SNPT compared to SPPT by flow cytometry in peripheral blood and bronchoalveolar lavage fluid of SNPT and SPPT patients (Chen et al., 2023).

Overall, although previous studies about SPPT and SNPT reveal some differential molecular mechanisms between the two clinical phenotypes, a comprehensive analysis of the panoramic molecular mechanisms remains absent. An in-depth comprehension of differential molecular mechanisms underlying SNPT and SPPT can provide valuable insights into disease progression, pathogenesis and new treatment strategies for TB.

Proteomics provides a powerful tool for analyzing molecular mechanisms of various disease processes at the protein level (Patterson and Aebersold, 2003). Numerous have reported on molecular mechanism analyses using the sera or plasma proteomic data from active TB patients. Li et al. illuminated the altered apoptosis, blood coagulation, and oxidative phosphorylation in *Mtb* infected macrophages through quantitative proteomics (Li et al., 2017); Arya R et al. demonstrated altered immune responses and lipid metabolism within small extracellular vesicles derived from TB patient serum (Arya et al., 2020); Similarly, Mateos J et al. delineated the up-regulation of complement and immune responses, alongside the down-regulation of lipid transport and iron assimilation in active TB patients (Mateos et al., 2020). Moreover, several studies have focused on the discovery of proteomic biomarkers for TB diagnosis. For instance, Achkar JM et al. identified a set of ten candidates that could distinguish TB from non-TB respiratory diseases with high accuracy (Achkar et al., 2015); Dao TL et al. demonstrated the precise differentiation between TB and non-TB respiratory infections through MALDI-TOFMS analysis of sputum (Dao et al., 2021); Furthermore, Liu J et al. and Liu Q et al. precisely identified SNPT using some characteristic proteins (Liu et al., 2010; Liu et al., 2015).

To date, no studies have utilized proteomics to elucidate different molecular mechanisms in pathogenesis between SNPT

and SPPT. In this study, we investigated proteome-level alterations in the plasma of SNPT, SPPT and control populations, aiming to identify specific protein signatures in the plasma of different TB patients and further elucidate the molecular mechanisms contributing to different disease pathogenesis.

Materials and methods

Participates

The blood samples were collected from 27 SPPT, 37 SNPT patients and 36 individuals without infection (Ctrl), and their demographic and clinical characteristics are shown in Table S1. The tuberculosis patients were recruited from the Tuberculosis Prevention and Treatment Institute of Kashgar, the Second People's Hospital of Aksu, and the Kuqa County Infectious Disease Hospital. The Ctrl group were recruited from the First Affiliated Hospital of Xinjiang Medical University. Here, the inclusion criteria of TB patients included: 1) the people were diagnosed with tuberculosis based on clinical symptoms and microbiological evidence according to Diagnosis for Pulmonary Tuberculosis (WS 288-2017); 2) the patients provided the signed permissions for the use of their clinical data for scientific purpose and informed consent for the anonymous publication of data. The exclusion criteria included: 1) the TB patients in treatment period; 2) the TB patients with other chronic or acute diseases such as pregnancy complications, cardiac dysfunction, renal disease, psychiatric disease, gastrointestinal disease, uncontrolled hypertension, and some severe stress states (including cardiovascular and cerebrovascular events, severe infection, traumatic surgery, and severe wasting diseases). The control group were included according to the following criteria: no prior TB exposure, no specific clinical manifestations of TB, negative results on chest imaging, sputum smear test, and T-SPOT tests (Hu et al., 2022).

Sample preparation

EDTA blood samples were collected from each participant and then centrifuged at 1500 g for 10 minutes at room temperature. The plasma was aliquoted and stored at -80°C. For protein preparation, high-abundance proteins were removed using the Agilent Human 14 Multiple Affinity Removal System according to the manufacturer's instructions. Sample lysis and protein extraction were performed using SDT buffer (4% SDS, 100mM Tris-HCl, 1mM DTT, pH 7.6). The proteins were then digested overnight at 37°C with trypsin (Pierce, Thermo Fisher Scientific). The obtained tryptic peptides were desalted using C18 cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma) and concentrated by vacuum centrifugation. Protein quantification was performed using the BCA Protein Assay Kit (Bio-Rad, USA). Protein integrity was confirmed by SDS-PAGE and silver staining, and samples showing protein degradation were excluded from the proteomic analysis. For metabolite preparation, the plasma samples were thawed on ice, and 150 μL of each sample was transferred to a centrifuge tube. Then, 1 mL of a chloroform-methanol solution was added, followed by ultrasound treatment. The supernatant was collected, and 2 mL of a 1% sulfuric acid-methanol solution was added. The mixture was methylated at 80°C for 30 minutes. Subsequently, 1 mL of n-hexane was added for extraction, and 5 mL of water was used for washing. Before injection, 500 μL of the extracted supernatant was mixed with 25 μL of an internal standard. This study was approved by the Hainan Province Clinical Medical Center.

TMT-labeled quantitative proteomic analyses

The proteomic analyses were conducted by Shanghai Applied protein technology Co., Ltd (Shanghai, China) as previously reported protocol (Wei et al., 2018). The TMT 10plex labeling procedures (Thermo Fisher Scientific, San Jose, CA, US) were performed using the manufacturer's instructions. Within each TMT experiment, three Ctrl samples were labeled with channels 126, 127N, and 127C. Three SNPT samples were labeled with channels 128N, 128C, and 129N. Three SPPT samples were labeled with channels 129C, 130N, and 130C. Additionally, a standard sample was created by mixing equal amounts of proteins from the nine samples and labeled with channel 131. For the labeling, each individual sample (100 μg) was resuspended in 100 μL of 0.1 M TEAB buffer, followed by reduction, alkylation, and trypsin digestion at 37°C for 16 hours. The reagents were dissolved in LC-grade acetonitrile (41 μL per 0.8 mg of reagent). After labeling for 1 hour, the reaction was quenched with 8 μL of 5% hydroxylamine for 15 minutes at room temperature. All channels were then mixed, aliquoted, and dried using a speed-vac. Subsequently, the TMT-labeled peptides were fractionated via high pH RP chromatography using a Zorbax 300 Extend-C18 column (3.5 μm, 4.6 × 250 mm; Agilent). LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60/90 min.

The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm*2 cm, nanoViper C18) connected to the C18-reversed phase analytical column (Thermo scientific EASY column, 10 cm, ID75 μm, 3 μm, C18-A2) in buffer A (0.1% formic acid), and then separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nL/min by Intelli Flow technology. LC–MS/MS analysis was operated in positive ion mode. Full MS scans were acquired in the mass range of 300-1800 m/z. Automatic gain control (AGC) target was set to 1e6, and maximum inject time to 50 ms. The dynamic exclusion duration was set for 60.0 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 35,000 at m/z 200, and isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%. Proteome Discoverer (v.1.4) was used to search all the Q Exactive raw data. False discovery rate (FDR) was set as ≤0.01. The protein ratios were calculated as the median of only

unique peptides of the protein. All peptide ratios were normalized by the median protein ratio, and the median protein ratio should be "1" after the normalization.

Targeted protein quantification by LC-PRM/MS

LC-PRM/MS analysis were performed on 32 DEPs obtained from the TMT quantitative proteomic analysis (Shanghai Applied Protein Technology Co., Ltd, China) using the procedure described previously (Cao et al., 2022). Briefly, peptides were first prepared, with an internal standard reference of a Peptide Retention Time Calibration Mixture (Thermo Scientific). Tryptic peptides were desalted using C18 stage tips and subjected to reversed-phase chromatography on an Easy nLC-1200 system (Thermo Scientific) with 1-hour gradients. PRM analysis was performed on a Q Exactive HF mass spectrometer (Thermo Scientific) using optimized parameters for collision energy, charge state, and retention times. The mass spectrometer was operated in positive ion mode with specific parameters: full MS1 scan resolution of 70,000, AGC target values of 3.0×10^{-6}, and a maximum ion injection time of 200 ms. This was followed by 20 PRM scans at 35,000 resolution (at m/z 200) with AGC set to 3.0×10^{-6} and a maximum injection time of 200 ms. Targeted peptides were isolated with a 1.5 Th window, and ion activation/dissociation was performed at a normalized collision energy of 27 using higher energy dissociation (HCD) in the collision cell. Data analysis was conducted using Skyline (MacCoss Lab, University of Washington), quantifying signal intensities for significantly altered proteins relative to each sample with normalization to a standard reference.

Targeted lipidomics by GC-MS analysis

The metabolic properties of fatty acids obtained from proteomic analyses were then measured by targeted lipidomics using GC-MS analysis as previously described (Wen et al., 2020). In brief, the sample of metabolites were separated on an Agilent DB-WAX capillary column (30 m × 0.25 mm ID × 0.25 μm) gas chromatography system. The temperature programming was as follows: 50°C for 3 min, 220°C for 20 min, and 250°C for 10 min. An Agilent 6890N/5975B gas chromatography-mass spectrometer was used for analysis. The electron bombardment ionization (EI) source, SIM scanning mode, and electron energy were 70 eV.

Functional annotation and statistical analysis of differentially expressed proteins

We analyzed the differentially expressed proteins ($|FC| \geq 1.2$, P-value < 0.05) performing pathway and functional annotation analysis using the Ingenuity Pathway Analysis (IPA, QIAGEN, USA) platform. We conducted core and comparison analyses to obtain canonical pathway and disease and biofunctions, which were evaluated by P-value (P-value < 0.05) and Z-score (Z-score ≠ 0). P-value < 0.05 indicated that the item was significantly enriched; Z-score > 0 indicated that the item was activated and Z-score < 0 indicated that the item was inhibited. Statistical analyses were performed using R software v4.1.0, and P-value < 0.05 indicated statistical significance.

Results

Altered protein expression profiles in SNPT and SPPT

To explore the molecular mechanisms of SPPT and SNPT patients, we performed TMT quantitative proteomic analyses on plasma samples from 27 SPPT, 37 SNPT patients, and 36 Ctrl individuals (Table S1). The results displayed distinct protein expression profiles among the three groups, and 161 differentially expressed proteins (DEPs) were identified ($|FC| > 1.2$, P-value < 0.05) (Figures 1A, B; Table S2: Additional Table 1). Compared with Ctrl, 44 up-regulated and 50 down-regulated DEPs were obtained in SNPT, and 38 up-regulated and 82 down-regulated DEPs were detected in SPPT (Figure 1C); 30 and 55 DEPs were specially expressed in the SNPT/Ctrl and SPPT/Ctrl groups, respectively (Figure 1D). Some of them were further verified by PRM targeted proteomic mass spectrometry (Figure S1), which displayed a consistent pattern obtained from TMT analysis.

Expression panels and patterns of plasma proteins showing increased immune response and lipid metabolic disorder in SNPT and SPPT patients

We then analyzed the top 20 up- and down-regulated DEPs in the SNPT/Ctrl and SPPT/Ctrl groups (Figure 2). Of the top 20 up- and down-regulated DEPs, eight and 11 were shared by the two groups (Figures 2A, B).

For the top 20 up-regulated DEPs (Figure 2A; Table S3), seven of eight shared DEPs were associated with inflammation/immune response (CRP, APMAP, C3, B2RA39, PF4, LBP and IGFBP2), indicating increased inflammatory response in TB patients regardless of SNPT or SPPT. Further analysis about 12 specially up-regulated DEPs showed the stronger immune response in SPPT than in SNPT: seven of the 12 specifically upregulated DEPs in the SPPT/Ctrl group were associated with innate immune response (ORM2, ORM1, P0DOX2, SIRPB1, LILRA2, GOLM1, and MBL2), while five of the specifically upregulated DEPs in the SNPT/Ctrl group were related to anti-inflammation (FGL1, CHIT1, LRG1, SERPINA3, and B4DLL8).

For the top-20 down-regulated DEPs (Figure 2B; Table S3), nine of 11 shared DEPs in the two groups were involved in metabolism and antioxidant stress (Q53H26, B4DF70, CA1, APOA1, S100A4, LTF, AK1, B2RAN2 and S100A6), demonstrating decreased metabolism and antioxidant capacity in TB patients. On the other hand, in the SNPT/Ctrl group, seven out of nine DEPs that were specifically down-regulated were also related to metabolism and

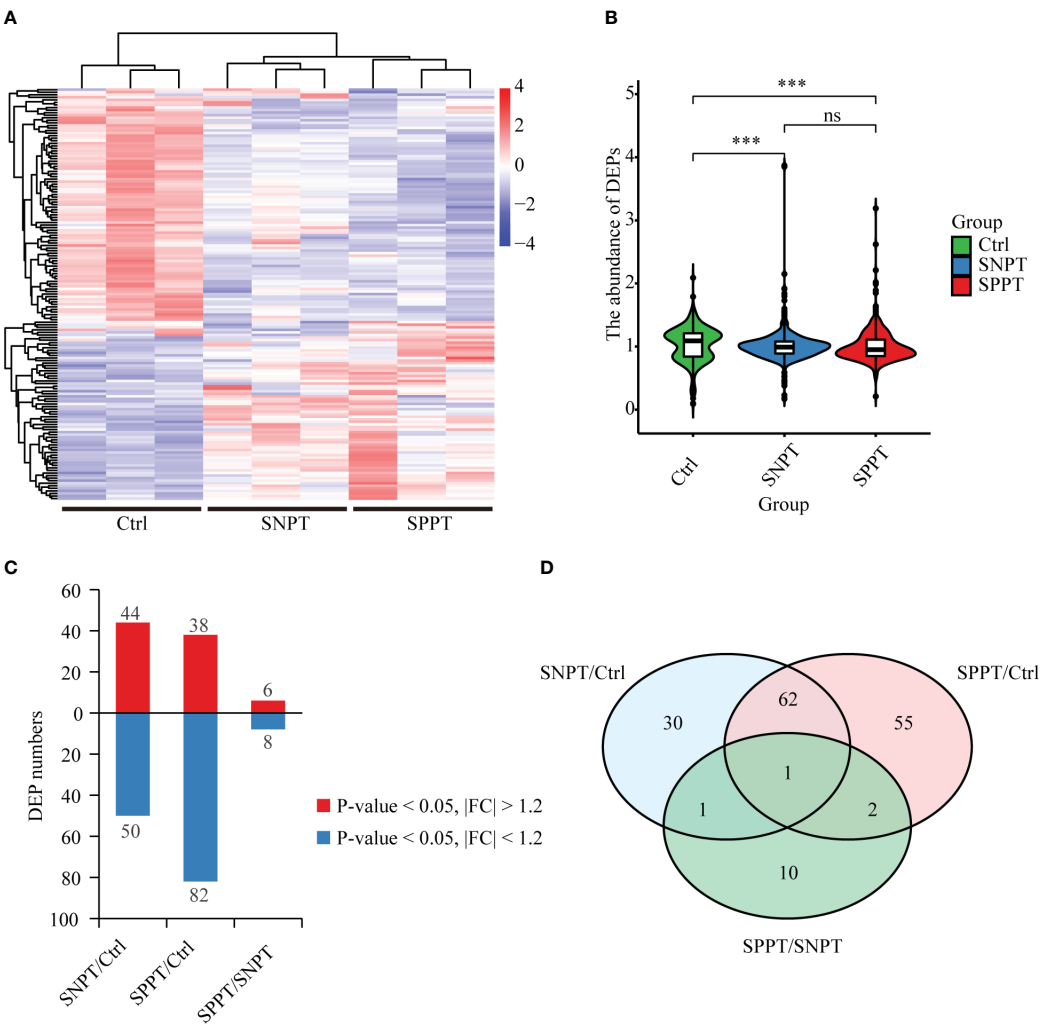

FIGURE 1
Protein expression profiles among SNPT, SPPT and Ctrl samples. **(A)** Heatmap showing protein expression profiles among the three groups. **(B)** Bar plot displaying the up-regulated and down-regulated DEPs in the SNPT/Ctrl, SPPT/Ctrl and SPPT/SNPT groups. **(C)** Violin plot showing the abundance of DEPs with downward trend from Ctrl to SNPT and then to SPPT. **(D)** Venn diagram showing the overlap among the three groups. ***p<0.001.

antioxidant stress (HEL-S-282, BLVRB, B4DM82, CNDP1, APOA4, NME1, and TPI1). This observation suggested a decrease in metabolism and antioxidant capacity in SNPT patients. Among the nine specifically down-regulated DEPs in the SPPT/Ctrl group, four were related to lipid metabolism (KRT17, B2R8I2, ATP8B3, and PI16), and the remaining five were associated with injury/remodeling processes (F6KPG5, CALM2, ITIH1, ZBTB18, and B2R582). These findings indicated an inhibition of lipid metabolism and damage repair processes in SPPT patients.

Additionally, we detected six up-regulated and eight down-regulated DEPs in the SPPT/SNPT group. For the up-regulated DEPs, five were associated with inflammation/immune responses; for the down-regulated DEPs, five were related to metabolism, two of which were associated with lipid metabolism (PGLYRP2 and KNG1) (Table S4). These reflected disordered immune responses and metabolism compared to SNPT.

We then classified all DEPs into six expression patterns based on the protein expression trends among the three groups (Figure 3A; Table S5, S6), and further focused on the top10 proteins in the LMH and HML patterns with continuously up- or down-regulated expression trends from Ctrl to SNPT and then to SPPT (Figure 3B). Of the top10 proteins in the LMH pattern, six were related to innate immune regulation (ORM2, ORM1, CRP, FGL1, P0DOX2, and PF4), indicating continuously enhanced body inflammatory response as increasing bacterial loads. Conversely, among the top 10 proteins of HML pattern, four were related to lipid metabolism, including APOA1, KRT17, B2R8I2, and ATP8B3, demonstrating continuously increased lipid metabolism disorder with the increase of bacterial loads.

For the LHM and MHL patterns (with higher expressed proteins in SNPT compared to SPPT) (Figure S2; Table S6), six out of 13 proteins (~46%), were related to anti-inflammatory responses, indicating their crucial role in protecting the body from excessive inflammatory responses in SNPT. For the 15 DEPs in the HLM and MLH patterns (with lower expressed proteins in SNPT compared to SPPT), four were associated with immune response (B2R6V9, DDT, B2RBZ5, and S6B294), and 10 were related to metabolism and antioxidant stress (Figure S2; Table S6).

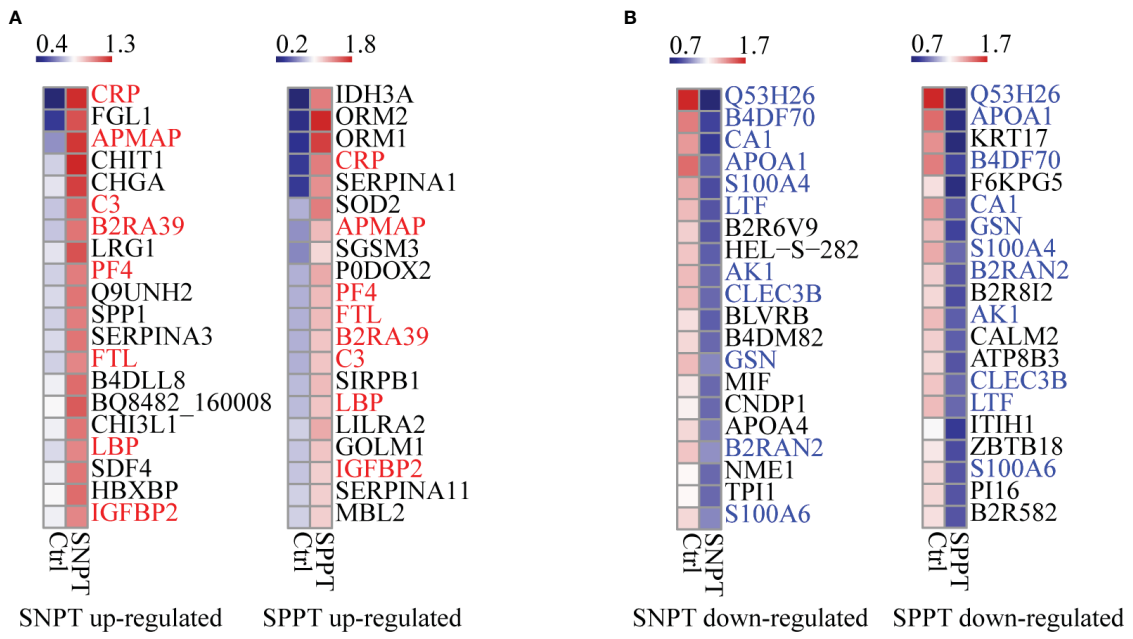

FIGURE 2
Heatmaps showing the top-20 upregulated and downregulated DEPs in the SNPT and SPPT samples. **(A)** The top-20 up-regulated DEPs in the SNPT and SPPT samples. Among them, eight proteins (labeled with red color) are shared in both SNPT and SPPT. **(B)** The top-20 down-regulated DEPs in SNPT and SPPT samples. Among them, 11 proteins (labeled with blue color) are shared in both SNPT and SPPT. The color key indicates the protein expression levels that range from blue (low expression) to red (high expression).

IPA analysis revealing dysfunctional immune response and metabolism in the SNPT and SPPT patients

To investigate the molecular mechanism of SPPT and SNPT, we further performed pathway and functional analyses using IPA. First, 40 significantly enriched pathways (P-value < 0.05) were identified in the SNPT/Ctrl and SPPT/Ctrl groups (Table S7, Table S2: Additional Table 2 and Additional Table 4). Among the top 10 shared significantly enriched pathways in the two groups (Figure S3), five were related to immune response ("Acute Phase Response Signaling", "Production of Nitric Oxide and Reactive Oxygen Species in Macrophages", "IL-12 Signaling and Production in Macrophages", "Clathrin-mediated Endocytosis Signaling", and "Granulocyte Adhesion and Diapedesis"), and three were associated with lipid metabolism ("LXR/RXR Activation", "FXR/RXR Activation", and "Atherosclerosis Signaling"). These indicated dysfunctional immune response and lipid metabolism in the SNPT and SPPT patients.

500 significantly enriched biofunctions (P-value < 0.05) were then identified in the SNPT/Ctrl group and SPPT/Ctrl groups (Table S8, Table S2: Additional Table 3 and Additional Table 5), among which 40 were significantly activated/inhibited in at least one group (|Z-score| ≥ 2, Figure 4A). Herein, 21 of the 40 functions (more than half) belonged to immune related functions (Figure 4A), all of which were significantly activated (Z-score ≥ 2) in at least one group; fatty acid metabolism was significantly inhibited in the SPPT/Ctrl group. IPA biofunction analysis also showed dysfunctional immune and fatty acid metabolism in SNPT and SPPT.

Gradual inhibition of fatty acid metabolism from Ctrl to SNPT then to SPPT

We further explored fatty acid metabolism in SPPT and SNPT patients, and found gradually inhibition of fatty acid metabolism from Ctrl to SNPT then to SPPT (Figure 4A). Among eight enriched lipid metabolism related functions in the two groups (P-value < 0.05), it was worth noting that only one lipid metabolism related function ("Fatty acid metabolism") was significantly inhibited (Z = -2.177) in the SPPT/Ctrl group, while it was inhibited (Z = -1.052) in the SNPT/Ctrl group (Figure 4B). This indicated more serious inhibition of fatty acid metabolism in SPPT than in SNPT, as previously reported in our previous metabolic findings about SPPT and SNPT (Hu et al., 2022).

To verify the above findings, we further performed targeted lipidomics using GC-MS analysis (Wen et al., 2020). The results indeed showed more severe inhibition of lipid metabolism in SPPT than in SNPT, especially for unsaturated fatty acids (Figure S4, Table S2: Additional Table 6). In the SPPT/Ctrl group, eight out of 17 fatty acid molecules showed downregulation, among which six displayed significant downregulation with P-value < 0.05, including two polyunsaturated fatty acids (PUFA); in the SNPT/Ctrl group, most fatty acid molecules (14/17 = 82.4%) showed up-regulation, among which nine displayed significant upregulation with P-value < 0.05, including six unsaturated fatty acid molecules.

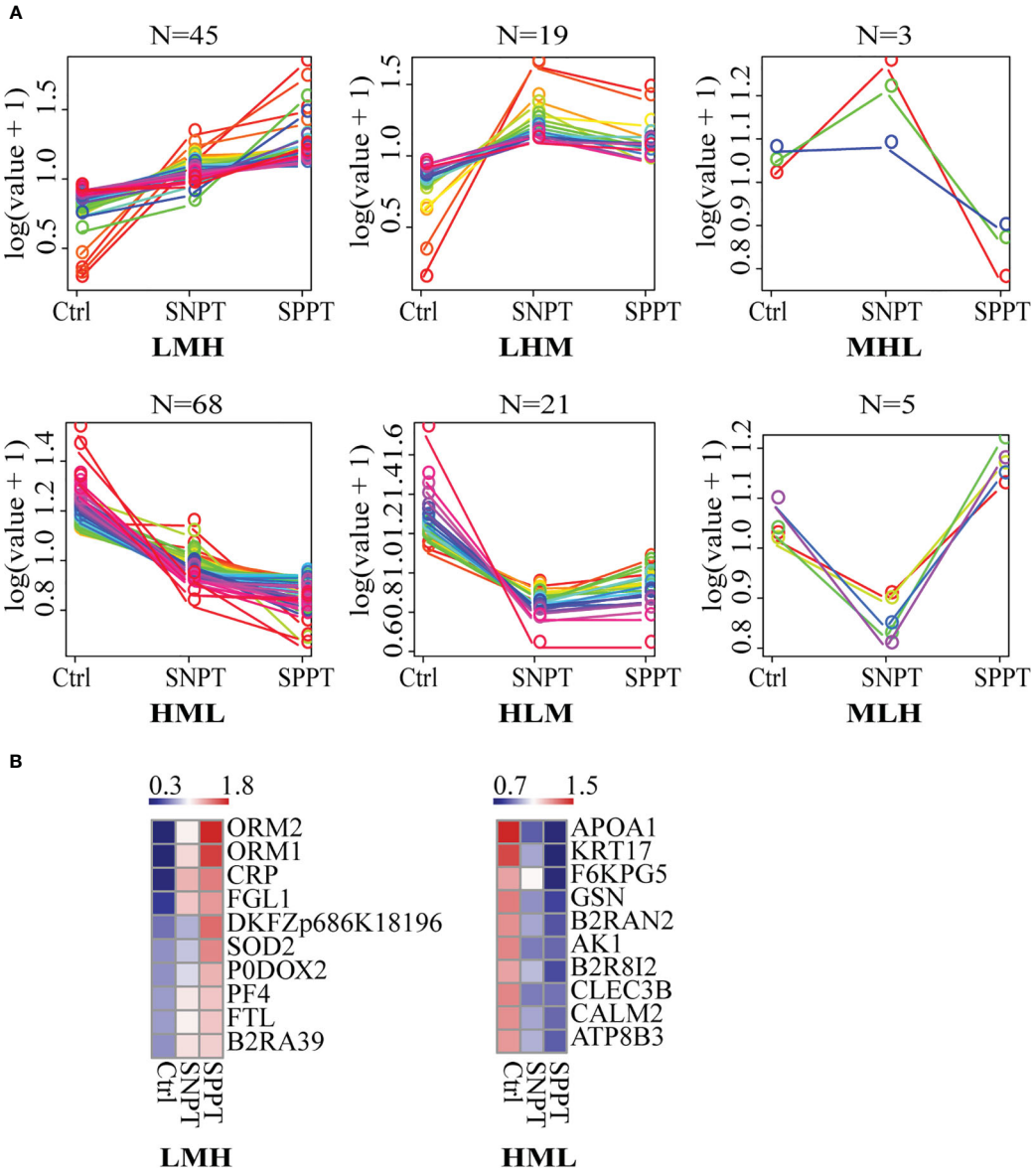

FIGURE 3
The expression patterns of DEPs among the Ctrl, SNPT, and SPPT samples. **(A)** Six protein expression patterns (including LMH, LHM, MHL, HML, MLH, and HLM). L, low expression level; M, medium expression level; H, high expression level; N, the protein numbers of each pattern. **(B)** Heatmaps showing the top 10 continuously upregulated/downregulated DEPs from Ctrl to SNPT and then to SPPT (LMH and HML). Colors indicate the protein expression levels that range from blue (low expression) to red (high expression).

Increased innate immune response but decreased movement of antigen-presenting cells in SPPT patients compared to SNPT patients

To explore the immune mechanism of SPPT and SNPT, we focused on 21 immune-related biofunctions, containing 19 innate- and two acquired-immune related ones (Figure 4A). In the SNPT/Ctrl group, all 11 immune-related biofunctions (including two shared and nine specifically enriched ones) were associated with migration/movement and binding/adhesion of immune cells, among which two specifically enriched ones belonged to acquired immune-related biofunctions and were related to antigen-presenting cell movement. In the SPPT/Ctrl group, five of 12 immune-related functions (including two shared ones) were associated with migration/movement and binding/adhesion of immune cells. The other ten specifically enriched items were related to movement, chemotaxis, infiltration and even death of immune cells, suggesting stronger innate immune response in SPPT patients.

Notably, in the SNPT/Ctrl group, two acquired immune-related biofunctions, "Cell movement of antigen presenting cells" (P-value < 0.05, Z-score = 2.084) and "Migration of antigen presenting cells" (P-value < 0.05, Z-score = 2.179) were significantly activated (Figure 4A), while they were not significantly activated in the SPPT/Ctrl group (Z-score = 0.576 and Z-score = 0.478). In

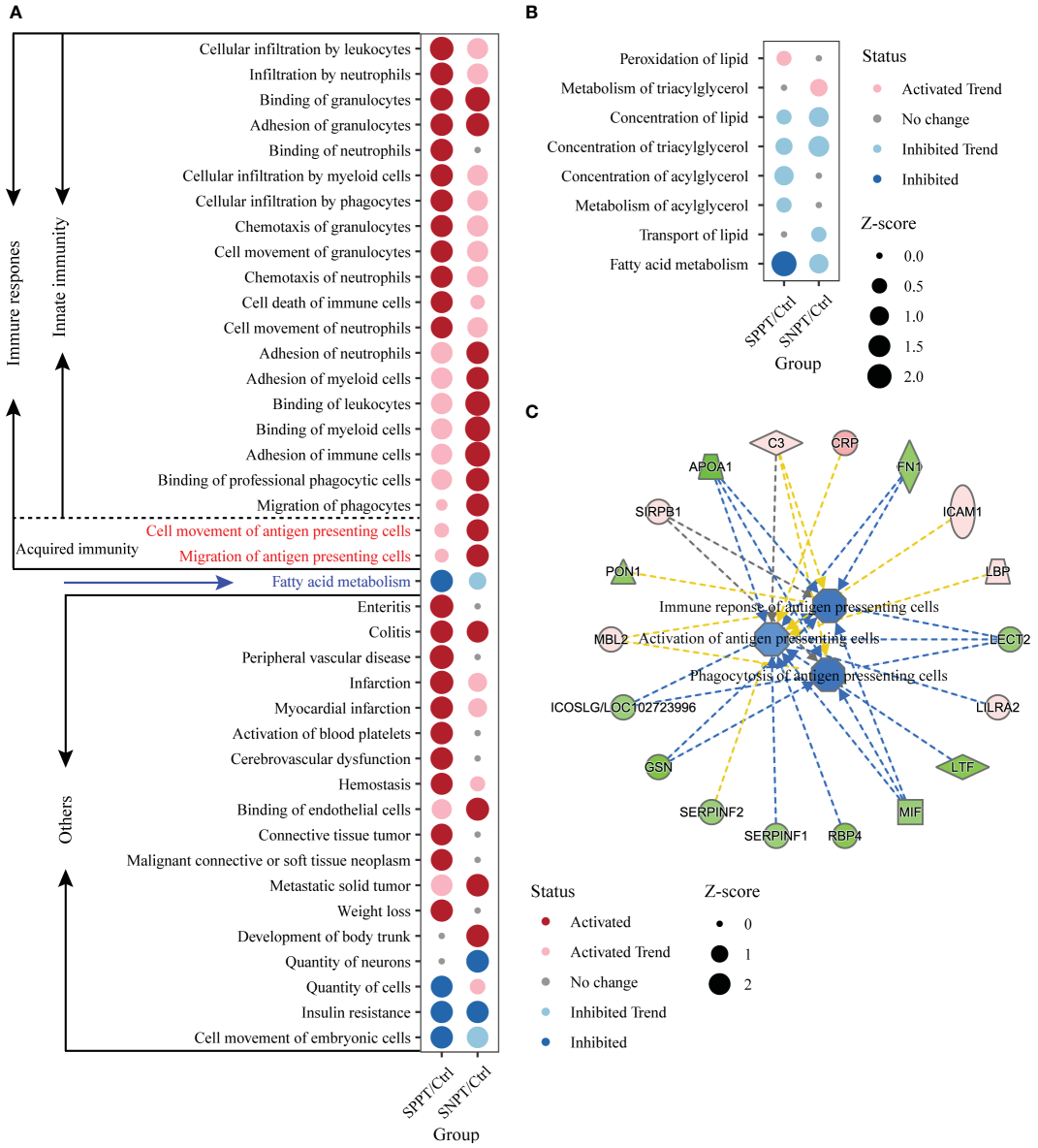

FIGURE 4
IPA function analyses of immune response in the SNPT/Ctrl and SPPT/Ctrl groups. **(A)** Dumbbell chart showing the significantly activated/inhibited functions in two groups. **(B)** Dumbbell chart showing the enriched lipid metabolism related functions in the two groups. The dark red represents significantly upregulated functions (p-value < 0.05, Z-score ≥ 2); the light red represents upregulated functions (p-value < 0.05, 0 < Z-score < 2). The dark blue represents significantly downregulated functions (p-value < 0.05, Z-score ≤ -2); the light blue represents downregulated functions (p-value < 0.05, -2 < Z-score < 0). **(C)** Networks showing the enriched DEPs in the antigen presenting cell related functions in SPPT/Ctrl group. Light red: increased measurement; green: decreased measurement; orange: predicted activation; blue: predicted inhibition; yellow: findings inconsistent with state of downstream molecule; solid line: direct interaction; dashed line: indirect interaction.

addition, we also detected three down-regulated antigen presenting cells related functions in the SPPT/Ctrl group [("Activation of antigen presenting cells" (Z-score = -1.028); "Phagocytosis of antigen presenting cells", Z-score = -1.577; "Immune response of antigen presenting cells", Z-score = -1.482)] (Figure 4C). A significantly down-regulated inducible T cell co-stimulator ligand/key molecule of activating T cells (ICOSLG/LOC102723996) (P-value < 0.05, FC = 0.82) were also detected in SPPT patients. The above findings suggested that antigen-presenting cell activation might be a critical determinant of different bacterial loads/symptoms between SNPT and SPPT.

Stronger activation of antigen presenting/acquired immune related functions/proteins in SNPT than in SPPT

To further investigate antigen-presenting and acquired immunity in SNPT and SPPT, we analyzed all enriched acquired immune-related functions and corresponding DEPs (Figure 5; Table S9). The results showed stronger activation of antigen presenting and acquired immune related functions/proteins in SNPT than in SPPT. Herein, a total of 17 functions related to antigen-presenting and acquired immunity were identified in the

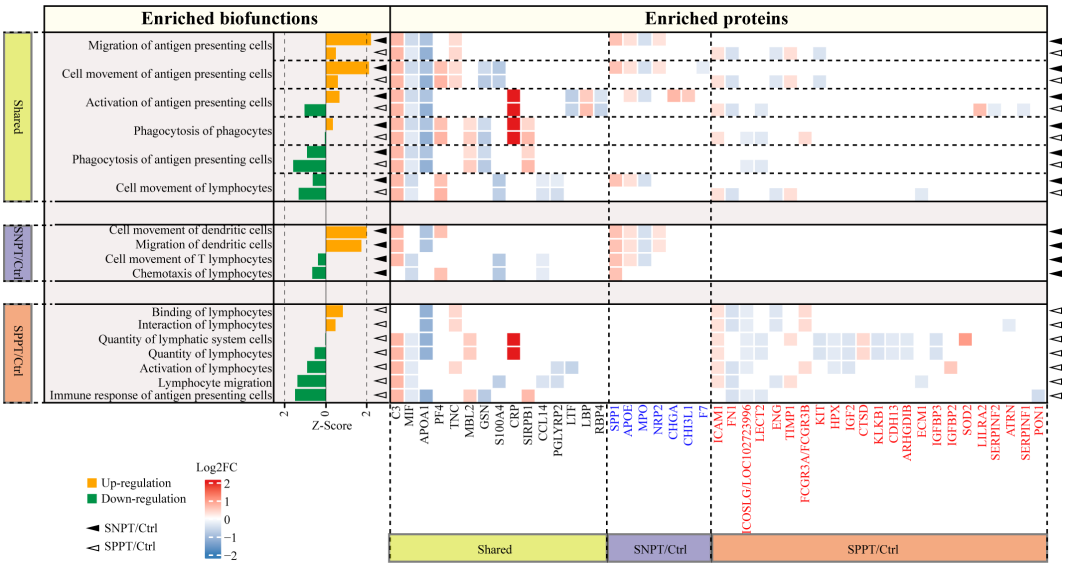

FIGURE 5
Acquired immune-related functions and the corresponding DEPs in the SNPT/Ctrl and SPPT/Ctrl groups. Bar plot showing the acquired immune-related functions in the two groups. Orange and green bars indicate the up- (Z-score > 0, P-value < 0.05) and down-regulation (Z-score < 0, P-value < 0.05), respectively. The heatmap depicts the DEPs and their expression level in each biofunction item. DEPs highlighted in blue and red font correspond to proteins specifically enriched in SNPT/Ctrl and SPPT/Ctrl groups, respectively. The solid triangles and hollow triangles in each row represent the enriched functions and their corresponding DEPs in the SNPT/Ctrl and SPPT/Ctrl groups, respectively. Left and bottom labels: "Shared" indicates the functions and DEPs shared by the SNPT/Ctrl and SPPT/Ctrl groups; "SNPT/Ctrl" and "SPPT/Ctrl" represent the functions and DEPs that are specific to the SNPT/Ctrl and SPPT/Ctrl groups, respectively.

two groups, including six shared ones, four specifically enriched ones in SNPT, and seven specifically enriched ones in SPPT.

First, among the six shared functions, all of them showed stronger activation or weaker inhibition of antigen presenting and acquired immune in SNPT than in SPPT. For antigen-presenting, in addition to the two significantly activated ones in SNPT (described in the previous section), "Activation of antigen-presenting cells" function was activated in SNPT but inhibited in SPPT, and the inhibition degree of "Phagocytosis of antigen-presenting cells" function was lower in SNPT than in SPPT. Secondly, among the four significantly enriched functions in SNPT, half of them were related to antigen-presenting and activated. Thirdly, among the seven significantly enriched functions in SPPT, most of them were inhibited, including "Immune response of antigen-presenting cells". Overall, compared to SNPT, the inhibition of antigen-presenting cells (APCs) activation in SPPT might cause impaired acquired immunity, subsequently resulting in a higher bacterial load in SPPT than in SNPT.

Further analysis revealed that some proteins might be associated with differential antigen-presenting and acquired immune responses between the two groups. First, a total of 45 DEPs were enriched in the aforementioned APC/acquired immune related functions. Among them, 15 were shared in the two groups and exhibited consistent expression trends in both SNPT and SPPT patients, which were therefore not subjected to further analysis. The remaining 30 proteins were specifically enriched in the acquired immunity-related functions of either SNPT or SPPT, suggesting that they might play crucial roles in differential genotypes and phenotypes between SPPT and SNPT.

For seven specifically enriched proteins in SNPT, four of five up-regulated proteins (SPP1, CHI3L1, APOE, and NRP2) have been reported to be involved in promoting T cell activation and facilitating pathogen clearance (van den Elzen et al., 2005; Dela Cruz et al., 2012; Shan et al., 2014; Roy et al., 2017); one of two down-regulated proteins (MPO) has been reported to be associated with inhibition of antigen presenting cell activation (Odobasic et al., 2013).

For 23 specifically enriched proteins in SPPT, six of seven up-regulated proteins (SOD2, LILRA2, IGFBP2, FCGR3A/FCGR3B, TIMP1, and ICAM1) were associated with suppression of antigen presentation and T-cell activation, and upregulation of Treg (Marchesi et al., 2013; O'Mahony et al., 2016; Seo et al., 2019; Keller et al., 2021; Robilliard et al., 2022; Zhang et al., 2022). Here, Treg cells have been reported to play roles in suppressing a variety of immune cell activation, including B cells, CD4+, and CD8+ T cells (Seo et al., 2019). On the other hand, nine of 16 down-regulated proteins (ICOSLG/LOC102723996, ATRN, CDH13, ECM1, ARHGDIB, LECT2, IGF2, FN1, and PON1) have been reported to play roles in antigen presenting and T cell activation (Tang et al., 2000; Chitnis and Khoury, 2003; Zhang et al., 2006; Jodaa Holm et al., 2016; Bai et al., 2018; Bijen et al., 2018; He et al., 2018; Luo et al., 2019; Belfiore et al., 2023).

Discussion

Our proteomic and lipidomic study revealed differential immune and metabolic mechanisms/microenvironments

underling different clinical phenotypes between SPPT and SNPT plasma samples. Although both groups displayed activated innate immune responses and inhibited fatty acid metabolism, SPPT patients showed stronger activation of innate immune responses and inhibition of fatty acid metabolism than SNPT patients. Importantly, our analyses on acquired immune-related functions uncovered activated APCs in SNPT but inhibited APCs in SPPT, which might be a critical determinant of different bacterial loads and phenotypes/symptoms between SNPT and SPPT. Specifically, the activation of APCs in tuberculosis has been reported to stimulate cellular/acquired immune responses and effectively eliminate the majority of *Mtb* strains in bodies (Cooper and Khader, 2008), leading to lower bacterial loads. Conversely, APC activation is inhibited in SPPT, resulting in inhibited acquired immune responses and further losing control of bacterial loads.

The foregoing conclusion were further validated by the analysis for related DEPs enriched in APC/acquired immune related functions (Figure 5; Table S9), also providing some promising therapeutic targets for TB. Among the specifically enriched proteins in SNPT, four specifically up-regulated proteins in SNPT have been reported to be involved in promoting T cell activation and facilitating pathogen clearance (van den Elzen et al., 2005; Dela Cruz et al., 2012; Shan et al., 2014; Roy et al., 2017). SPP1 is required for mDC-dependent activation of T cell subsets (Shan et al., 2014); CHI3L1 facilitates killing bacteria in macrophage by inhibiting caspase-1-dependent macrophage pyroptosis (Dela Cruz et al., 2012); APOE and lipid antigens can be delivered into endosomal compartments of APCs together to activate T-cells activation (van den Elzen et al., 2005); NRP2 is required for T cell activation through DCs migration in response to CCL21 (Roy et al., 2017). On the other hand, one specifically down-regulated protein MPO in SNPT has been reported to inhibit DC activation and antigen uptake/processing (Odobasic et al., 2013).

Among the specifically enriched proteins in SPPT, six up-regulated proteins have been reported to be associated with suppression of antigen presentation and T-cell activation, and upregulation of Treg (Marchesi et al., 2013; O'Mahony et al., 2016; Seo et al., 2019; Keller et al., 2021; Robilliard et al., 2022; Zhang et al., 2022). Overexpression of SOD2 facilitates the expression of FoxP3 (a major transcription factor of Treg cells) (Seo et al., 2019); LILRA2 is an immune modulator by inhibiting DC differentiation or antigen presenting capacity by stimulating an alternative macrophage differentiation pathway (Marchesi et al., 2013); IGFBP2 suppresses the influx of T-cells (Robilliard et al., 2022); FCGR3A (CD16a) plays a role in immature lanDCs, plasmacytoid DCs, or CD1c+ DCs O'Mahony et al., 2016); TIMP1 overexpression leads to Treg upregulation (Keller et al., 2021); ICAM-1-mediated adhesion is a prerequisite for exosome-induced T cell suppression (Zhang et al., 2022).

On the other hand, nine specifically downregulated proteins in SPPT have been reported to play roles in APC and T cell activation (Tang et al., 2000; Chitnis and Khoury, 2003; Zhang et al., 2006; Esensten et al., 2016; Jodaa Holm et al., 2016; Bai et al., 2018; Bijen et al., 2018; He et al., 2018; Luo et al., 2019; Belfiore et al., 2023). ICOSLG/LOC102723996 (an inducible T cell costimulatory molecule) is required for the second signal of T cell activation (Chitnis and Khoury, 2003; Esensten et al., 2016); ATRN can be released by activated T lymphocytes (Tang et al., 2000); CDH13 -derived peptide can activate T cell clone (Bijen et al., 2018); ECM1 is a positive regulator of Tfh differentiation and antibody production (He et al., 2018); ARHGDIB facilitates the connection between antigen-presenting cells and T cells by promoting T cell proliferation (Zhang et al., 2006); LECT2 plays roles in Th1 and Th17-guided immune responses (Jodaa Holm et al., 2016); IGF2 induces dendritic cell maturation (Belfiore et al., 2023); FN1 enhances T cell response in lung cancer cells (Luo et al., 2019); it is worth noting that PON1 is necessary for thymocyte development from double-negative to double-positive transition stages (Bai et al., 2018), therefore its significant downregulation in SPPT patients can inhibit T cell activation (Bai et al., 2018).

Collectively, four specifically upregulated and one specifically downregulated protein in SNPT have been reported to enhance antigen-presenting cell activation and cellular immunity/acquired immune responses, further resulting in more effective clearance of *Mtb* strains in bodies. On the other hand, these SPPT-specific enriched proteins (six upregulated and nine downregulated ones) are able to suppress antigen-presenting cell and cellular immunity in SPPT patients, leading to a higher bacterial load in bodies.

In addition, our expressed pattern analysis revealed differential immune responses between SNPT and SPPT (Figure S2; Table S6). Specifically, six out of the 13 higher expressed proteins in SNPT are related to anti-inflammatory response, indicating weaker immune response and delicate balance of immune responses in SNPT (Table S6): four (CHIT1, LRG1, B7Z4R8, and B4DLL8) have been reported to be involved in macrophage M2 polarization; two (SERPINA1 and B7Z7M2) are identified as acute-phase reactive proteins for protecting the body from excessive inflammatory responses. On the other hand, four out of 15 higher expressed proteins in SPPT (B2R6V9, DDT, B2RBZ5, and S6B294) are associated to immune responses, suggesting stronger immune response in SPPT.

Our expressed pattern and panel analyses also revealed differential lipid metabolism between SNPT and SPPT (Figure 3; Table S5). Notably, four lipid related proteins showed a continuously down-regulation trend from Ctrl to SNPT and then to SPPT (APOA1, KRT17, B2R8I2, and ATP8B3), reflecting gradual inhibition of lipid metabolism from SNPT to SPPT (Table S5): APOA1 can facilitate the excretion of cholesterol and phospholipids in cell; KRT17 has been proved to help to lipid metabolism-related enzyme and antimicrobial peptide expressions; B2R8I2 and ATP8B1 are identified to involve in bile acid and lipid metabolism. In addition, two down-regulated DEPs (PGLYRP2 and KNG1) in the SPPT/SNPT group are associated with lipid metabolism, indicating more dysfunctional lipid metabolism in SPPT (Table S4).

Nevertheless, we have observed consistent expression trends of some shared upregulated/downregulated functions or proteins in both SNPT and SPPT (Figures 3–5), revealing phenotype generality of TB (both SNPT and SPPT). For SNPT patients, there is a delicate balance between immune responses and bacterial loads: on the one hand, some activated innate and acquired immune responses (especially APC activation) are able to clear up the majority of *Mtb* strains in bodies; on the other hand, some immunosuppression-related functions/proteins restrict complete *Mtb* eradication. For

SPPT, since only some innate immune responses are activated while the acquired immune response may be inhibited leading to higher bacterial loads due to broken balance.

Overall, our findings reveal differential immune and metabolic mechanisms/microenvironments underling SPPT and SNPT patients, and highlight the critical role of antigen-presenting cells in SNPT for clearing up the majority of *Mtb* in bodies. This suggests the important role of antigen-presenting cell in promoting acquired immunity for inhibiting *Mtb* infection. Some proteins are further verified to play important roles in the APC activation/acquired immune response process, providing some promising therapeutic promising therapeutic targets for TB. Further studies on these are warranted.

Although our study provides important insights into the pivotal role of APCs in SNPT patients in clearing the infection, it is important to validate the result by experiments. Regrettably, we are unable to conduct such validation experiments due to the revocation of our license to handle *Mtb* infections in animals, a decision made by the Xinjiang government impacting our institution, the First Affiliated Hospital of Xinjiang Medical University. Nonetheless, our findings shed light on the significance of APC-mediated immune responses in countering *Mtb* infections. Further studies on these are warranted by animal experiments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://ngdc.cncb.ac.cn/omix/, OMIX004336.

Ethics statement

This study was approved by the Ethical Committee of First Affiliated Hospital of Xinjiang Medical University (Record number 20171123-06-1908A) and project supported by Hainan Province Clinical Medical Center. All enrolled subjects provided written informed consent. All methods were performed in accordance with the relevant guidelines and regulations. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

FC and JingW conceived the study. YJ, JieW, and CL performed the bioinformatics analyses. CJ, SC, BT, YL, YS, and WQ carried out the experimental analyses. YJ, JieW, CL, XW, PW, LY, and XJ drew the figures. FC and YJ wrote the manuscript. All authors read and approved the final manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1240516/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Verification of 15 proteins using LC-PRM/MS. Fifteen out of 32 selected proteins and verified using PRM targeted proteome mass spectrometry. The expression trends of these proteins were found to be consistent with the TMT results. The colors in the figure represent the expression levels of differentially expressed proteins, ranging from blue (low expression) to red (high expression).

SUPPLEMENTARY FIGURE 2
Heatmaps showing the DEPs in the four patterns (LHM, MHL, HLM and MLH). Colors indicate the protein expression levels that range from blue (low expression) to red (high expression).

SUPPLEMENTARY FIGURE 3
The top 10 significantly enriched items (P-value < 0.05) in both SNPT/Ctrl and SPPT/Ctrl groups. Here most pathways are related to immune response and metabolism (labeled with red and blue, respectively).

SUPPLEMENTARY FIGURE 4
Bar plot showing the abundance change of 17 fatty acid molecules in the SNPT/Ctrl and SPPT/Ctrl groups. Red and blue bars indicate up- and down-regulation, respectively. The left and right panels represent the SNPT/Ctrl and SPPT/Ctrl groups, respectively.

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Influences of bacterial extracellular vesicles on macrophage immune functions

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Bacterial extracellular vesicles (EVs) are crucial mediators of information transfer between bacteria and host cells. Macrophages, as key effector cells in the innate immune system, have garnered widespread attention for their interactions with bacterial EVs. Increasing evidence indicates that bacterial EVs can be internalized by macrophages through multiple pathways, thereby influencing their immune functions. These functions include inflammatory responses, antimicrobial activity, antigen presentation, and programmed cell death. Therefore, this review summarizes current research on the interactions between bacterial EVs and macrophages. This will aid in the deeper understanding of immune modulation mediated by pathogenic microorganisms and provide a basis for developing novel antibacterial therapeutic strategies.

KEYWORDS

extracellular vesicles, bacterial, macrophage, immune, influences

1 Introduction

Intercellular communication is an indispensable process in living systems, with various cells transmitting information and coordinating behavior by releasing extracellular signaling molecules. Bacteria, as single-celled organisms, exchange information with other bacteria and host cells by secreting extracellular vesicles (EVs), thereby participating in the regulation of various physiological and pathological processes (Tiku and Tan, 2021). Bacterial EVs are small membrane vesicles rich in bioactive molecules such as proteins, lipids, and nucleic acids, capable of serving as carriers for signaling molecules and playing crucial roles in biofilm formation, stress responses, quorum sensing, and nutrient acquisition (Kim et al., 2015; Huang et al., 2023).

In recent years, research has revealed that bacterial EVs not only affect the behavior of bacteria themselves but also play significant roles in the interactions between microbes and hosts, especially impacting the host immune system (Kaparakis-Liaskos and Ferrero, 2015). As the main effector cells of the innate immune system, the interactions between macrophages and bacterial EVs have garnered considerable attention. Macrophages recognize, phagocytose, and digest invading pathogens, playing a frontline role in immune defense (Locati et al., 2020). However, how various molecular signals carried by bacterial EVs affect the immune functions of

macrophages remains a hot and challenging field. On one hand, certain pathogenic bacteria can interfere with and evade macrophage immune surveillance through EVs (Kuehn and Kesty, 2005; Bielaszewska et al., 2017). on the other hand, commensal bacteria can enhance macrophage antimicrobial capacity via EVs, playing a role in regulating host-microbe balance (Shen et al., 2012). Additionally, bacterial EVs can influence other physiological processes of macrophages, such as metabolic state and cell death (Dhital et al., 2021). Therefore, fully elucidating the molecular mechanisms of the interactions between bacterial EVs and macrophages is of great significance and value for understanding the immune regulatory networks mediated by microbes.

2 Types and biogenesis of bacterial EVs

First, compared to EVs produced by eukaryotic cells, bacterial EVs exhibit more diverse types and biogenesis mechanisms. Taking Gram-negative bacteria as an example, current research indicates that they can produce EVs through two mechanisms: blebbing and explosive cell lysis (Toyofuku et al., 2023). Specifically, EVs produced through blebbing include outer membrane vesicles (OMVs) and outer-inner membrane vesicles (OIMVs). OMVs are formed due to the disturbance in the Gram-negative bacterial cell envelope, leading to the outer membrane blebbing outward and eventually shedding off (Kadurugamuwa et al., 1993; Kulp and Kuehn, 2010; Schwechheimer and Kuehn, 2015). In contrast, OIMVs are formed when the peptidoglycan layer of Gram-negative bacteria undergoes autolysin-mediated weakening, resulting in the outer and inner membranes blebbing outward together (Perez-Cruz et al., 2013; Perez-Cruz et al., 2015). Additionally, Gram-negative bacteria can produce explosive outer membrane vesicles (EOMVs) and explosive outer-inner membrane vesicles (EOIMVs) through the explosive cell lysis mechanism. This occurs when phage-encoded endolysins in Gram-negative bacteria are activated, degrading the peptidoglycan layer and causing explosive cell lysis, followed by spontaneous assembly and fusion of the fragmented membrane segments (Turnbull et al., 2016; Baeza et al., 2021).

As for Gram-positive bacterial EVs, current research indicates that the peptidoglycan cell wall of Gram-positive bacteria can form pores under the action of endolysins, autolysins, or certain antibiotics that inhibit peptidoglycan synthesis, ultimately leading to the cytoplasmic membrane protruding outward and forming cytoplasmic membrane vesicles (CMVs) (Mitchell et al., 2013; Toyofuku et al., 2017; Andreoni et al., 2019; Abe et al., 2021; Liu et al., 2022). However, throughout this process, Gram-positive bacterial cells do not undergo explosive lysis. Nonetheless, the disruption of cytoplasmic membrane integrity still results in bacterial cell death. Therefore, this mechanism is termed "bubbling cell death" (Jiang et al., 2024).

3 Macrophage internalization of bacterial EVs

Endocytosis is the main pathway through which bacterial EVs enter macrophages. Endocytosis refers to the process by which small molecules cross the bilayer cell membrane and enter the cell (Doherty and McMahon, 2009). Due to the differences in the surface and cargo of bacterial EVs, the pathways of endocytosis can vary. For instance, *Staphylococcus aureus* EVs can be internalized by macrophages through a dynamin-dependent endocytic pathway (Wang et al., 2020). *Klebsiella pneumoniae* EVs can enter macrophages through a lectin-like oxidized low-density lipoprotein receptor-dependent endocytic pathway (Ye et al., 2021). Additionally, clathrin-mediated endocytosis is also closely related to the entry of bacterial EVs into macrophages. Clathrin-mediated endocytosis involves the formation of a clathrin-coated pit, which generates vesicles to absorb target substances (Vercauteren et al., 2010). This mode of endocytosis plays a crucial role in the uptake of external substances by host cells, but it is usually tightly regulated by cell surface receptors, allowing only substances that specifically bind to the receptors to enter the cell (McMahon and Boucrot, 2011; Kaksonen and Roux, 2018; Mettlen et al., 2018). However, most current studies on EV internalization pathways focus on Gram-negative bacteria. Future research could further investigate the pathways of Gram-positive bacteria EV internalization.

4 The impact of bacterial EVs on macrophage immune function

Increasing evidence suggests that bacterial EVs can significantly impact the immune functions of macrophages, including inflammatory responses, antibacterial activity, antigen presentation capacity, and programmed cell death (Ellis et al., 2010; Dhital et al., 2021; Hu et al., 2021) (Figure 1).

4.1 Bacterial EVs can modulate macrophage-mediated inflammatory responses

First, when bacterial EVs come into contact with or are phagocytosed by macrophages, the pathogen-associated molecular patterns (PAMPs) they carry interact with the pattern recognition receptors (PRRs) of macrophages (Asano et al., 2021). This interaction induces macrophages to produce various inflammatory factors (Ellis et al., 2010; Asano et al., 2021; Xu et al., 2022). For example, lipoproteins in *S. aureus* EVs can stimulate macrophages to release IL-6 and TNF-α (Kopparapu et al., 2021). LPS and flagellin in *Pseudomonas aeruginosa* EVs can stimulate macrophages to release MIP-2, TNF-α, and IL-6 (Ellis et al., 2010). Extracellular RNA in *Aggregatibacter actinomycetemcomitans* EVs can promote TNF-α production in macrophages (Han et al., 2019). These examples show that bacterial EVs can significantly promote macrophage-mediated inflammatory responses. However, in addition to their pro-inflammatory effects, EVs from some bacteria, such as *Escherichia coli*, *Francisella tularensis*, and *Clostridium butyricum*, can stimulate macrophages to release the anti-inflammatory cytokine IL-10 (Hu et al., 2020; Pavkova et al., 2021; Guangzhang et al., 2023). This indicates that EVs from certain bacteria have complex dual effects on macrophage-mediated inflammatory responses, capable of inducing the production of both pro-inflammatory and anti-inflammatory

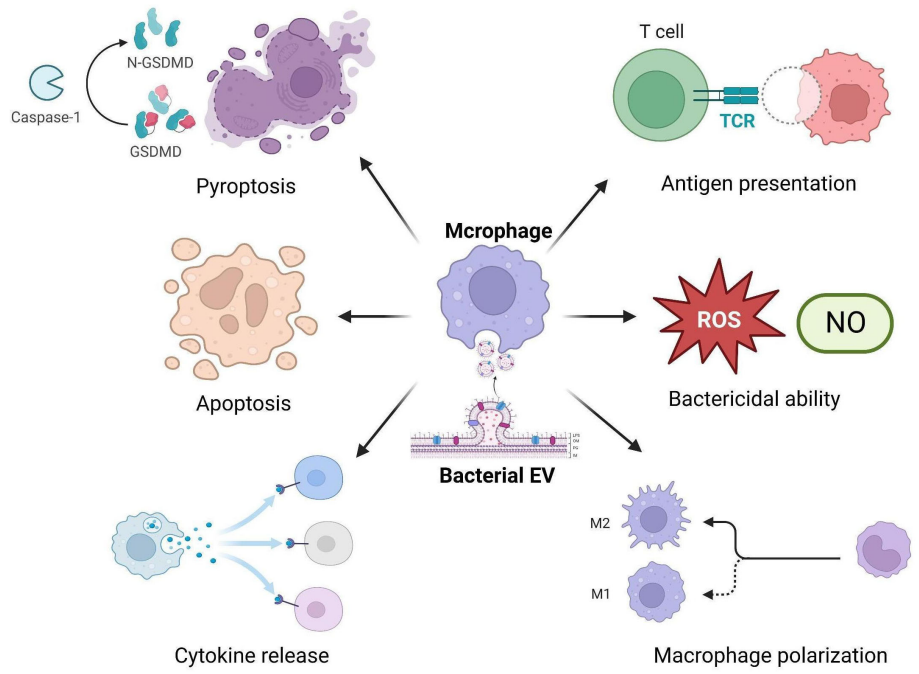

FIGURE 1
Bacterial EVs interact with macrophages and affect their immune functions.

cytokines by macrophages. Moreover, besides directly inducing the production of various cytokines, studies have found that EVs from *E. coli* can induce macrophages to release pro-inflammatory EVs after interacting with them. This implies that EVs derived from *E. coli* have the ability to transmit inflammatory signals to macrophage EVs (Imamiya et al., 2023). Interestingly, although *Porphyromonas gingivalis* EVs can stimulate macrophages to release various inflammatory cytokines, the Arg- and Lys-gingipains enzymes in these EVs can degrade macrophage inflammatory cytokines, thereby achieving fine-tuned regulation of the immune response (Barth and Genco, 2016; Castillo et al., 2022).

Additionally, it is important to note that not all bacterial EVs exhibit pro-inflammatory effects. Current research indicates that EVs produced by some commensal bacteria, such as *Lactobacillus plantarum*, *C. butyricum*, and *Bacteroides thetaiotaomicron*, can induce macrophages to polarize towards the M2 type, leading to the release of the anti-inflammatory cytokine IL-10, thereby inhibiting inflammatory responses (Kim et al., 2020; Muller et al., 2021; Liang et al., 2022). Moreover, studies have found that certain mycobacteria, such as *Mycobacterium avium* and *Mycobacterium kansasii*, also produce EVs with anti-inflammatory properties that can induce macrophages to produce IL-10 (Kamran-Sarkandi et al., 2018; Tavassol et al., 2020). Therefore, these studies demonstrate that EVs from different bacterial sources have varying effects on macrophage-mediated inflammatory responses.

4.2 Bacterial EVs can influence the antibacterial activity of macrophages

Additionally, as immune cells, macrophages possess strong antibacterial capabilities. When pathogens are recognized by macrophages, they form phagosomes on their cell membranes to engulf the pathogens, which then fuse with lysosomes to form phagolysosomes, thereby eliminating the pathogens (Hirayama et al., 2017). However, it is worth noting that bacterial EVs have varying effects on the antibacterial activity of macrophages. For example, Blancá et al (Blanca et al., 2022). investigated the effects of *Bordetella pertussis*-derived EVs on the antimicrobial activity of macrophages. The study found that *B. pertussis* EVs contain various toxins, including adenylate cyclase (CyaA). It was shown that pre-incubating macrophages with EVs significantly weakened their phagocytic and bactericidal activities against *B. pertussis*. This is because the CyaA in *B. pertussis* EVs inhibits the antibacterial capacity of macrophages by reducing the level of complement receptor 3 on their surface. Furthermore, Jung et al. (2016) studied how EVs from *Legionella pneumophila* affect the immune response of macrophages and promote bacterial replication within macrophages. Their research revealed that macrophages pre-treated with *L. pneumophila* EVs responded differently to subsequent Legionella infection. Initially, the EVs enhanced the immune defense response of macrophages and reduced bacterial replication. However, as the infection progressed, this effect gradually shifted, making macrophages more tolerant of bacterial replication. These findings suggest that *L. pneumophila* EVs have a complex dual role in macrophages, initially activating immune defenses and then creating an environment conducive to bacterial survival and replication. Interestingly, studies have shown that while EVs from *Streptococcus pneumoniae* can enhance macrophage phagocytosis of *S. pneumoniae*, this makes macrophages a "reservoir" for the bacteria, negatively impacting their antibacterial activity (Yerneni et al., 2021). On the other hand, bacterial EVs can also promote the antibacterial activity of macrophages. For example, EVs from *P. gingivalis* and *E. coli* can induce macrophages to produce reactive oxygen species (ROS) and nitric oxide (NO) upon interaction

(Imayoshi et al., 2011; Guangzhang et al., 2023). These substances are important antibacterial weapons of macrophages, capable of exerting toxic effects directly on pathogens (Nathan and Hibbs, 1991; Canton et al., 2021; Herb and Schramm, 2021). Additionally, EVs from *E. coli* Nissle can increase the activity of acid phosphatase in macrophages (Hu et al., 2020). This enzyme is located in lysosomes and is closely related to the digestive functions of macrophages (Xu et al., 2012).

4.3 Bacterial EVs can influence the antigen-presentation function of macrophages

Additionally, macrophages possess important antigen-presenting functions. After pathogens are digested by macrophages, they are degraded into multiple small antigen fragments. These antigen fragments bind to major histocompatibility complex (MHC) molecules in macrophages, thereby triggering specific immune responses (Guerriero, 2019). However, it should be noted that some studies have indicated that EVs secreted by certain bacteria have the ability to affect the antigen-presenting function of macrophages. For example, Armstrong et al. (2020) investigated the impact of EVs secreted by *P. aeruginosa* on the expression of MHC-related molecules in human lung macrophages. The study found that *P. aeruginosa* EVs significantly inhibited the expression of 11 different MHC-related molecules in lung macrophages, including several subunits of MHC class II molecules (HLA-DRA, HLA-DPA1, etc.). Additionally, three CpG sites within the CFB gene showed significant hypomethylation under the influence of EVs, which was closely associated with the downregulation of MHC gene expression. These results suggest that *P. aeruginosa* EVs may regulate the antigen-presenting function of macrophages by altering the epigenetic state of their genes. Moreover, another study found that EVs from *E. coli* can promote the expression of the co-stimulatory molecule CD86 on the surface of macrophages (Guangzhang et al., 2023). This molecule plays a crucial role in the activation of T cells induced by macrophages (Smith et al., 2016; Nordlohne et al., 2021). However, current research on the impact of bacterial EVs on the antigen-presenting function of macrophages is still limited, and it has not been clearly identified which components in EVs interfere with this function. This area warrants further exploration in future research.

4.4 Bacterial EVs can induce programmed cell death in macrophages

Programmed cell death is an orderly and controlled process of cell death, which includes apoptosis, pyroptosis, and autophagy (Newton et al., 2024). First, pyroptosis is an inflammation-related form of cell death characterized by the release of cellular contents and the subsequent induction of an inflammatory response (Rao et al., 2022). Current studies indicate that bacterial EVs can deliver a series of bacterial molecules to macrophages to induce pyroptosis. For instance, *E. coli* EVs can transport LPS into the cytoplasm, directly activating caspase-11 to induce pyroptosis in macrophages (Vanaja et al., 2016; Finethy et al., 2017). Similarly, lipoproteins and pore-forming toxins in *S. aureus* EVs can activate caspase-1 through the NLRP3 inflammasome, thereby inducing pyroptosis in macrophages (Wang et al., 2020). Additionally, bacterial EVs containing flagellin can induce pyroptosis in macrophages by activating the NLRC4 inflammasome rather than the NLRP3 inflammasome (Bitto et al., 2018). Unlike the NLRP3 inflammasome, the NLRC4 inflammasome specifically recognizes bacterial flagellin and flagellin-like molecules, leading to a more specific and rapid activation (Gram et al., 2021; Chebly et al., 2022). For example, *Salmonella* EVs containing flagellin can quickly activate the NLRC4 inflammasome in a shorter time compared to flagellin-deficient *E. coli* and *Salmonella* EVs (Yang et al., 2020). Interestingly, *P. gingivalis* itself cannot activate inflammasome formation in macrophages, but its EVs can, as observed in a study by Fleetwood et al. (2017). This might be due to the enrichment of gingipain in *P. gingivalis* EVs, which helps *P. gingivalis* survive within cells (Sheets et al., 2008; Mantri et al., 2015). After being phagocytosed by macrophages, *P. gingivalis* downregulates gingipain levels (Xia et al., 2007). Moreover, the activation of inflammasomes in macrophages by bacterial EVs requires the involvement of guanylate-binding proteins (GBPs) (Finethy et al., 2017; Santos et al., 2018). GBPs are a class of large GTPases that play a crucial role in inflammasome activation (Shenoy et al., 2012).

Additionally, bacterial EVs can also induce apoptosis in macrophages. Apoptosis is a non-inflammatory form of cell death that can occur via intrinsic and extrinsic pathways (Kiraz et al., 2016). Initial studies using *Neisseria gonorrhoeae* EVs to treat bone marrow-derived macrophages found that the toxin protein PorB in N. gonorrhoeae EVs was sufficient to cause mitochondrial dysfunction in macrophages, leading to the release of cytochrome c and inducing apoptosis (Deo et al., 2018). However, recent research has discovered that EVs from *N. gonorrhoeae*, uropathogenic *E. coli*, and *P. aeruginosa* cause mitochondrial dysfunction in macrophages, which inhibits intracellular protein synthesis without inducing rapid apoptosis (Deo et al., 2020). Nonetheless, prolonged inhibition of protein synthesis can regulate members of the BCL-2 family, thereby activating mitochondrial apoptosis (Speir et al., 2016). Consequently, over time, EVs indirectly activate the pro-apoptotic member BAK, leading to apoptosis (Czabotar et al., 2014).

Moreover, autophagy, as a form of programmed cell death, is also an important antibacterial mechanism in macrophages (Wu and Lu, 2019). However, to date, only one study by David et al. has reported on the effects of bacterial EVs on macrophage autophagy. This study found that EVs produced by *E. coli* expressing HlyF can inhibit autophagic flux by preventing the fusion of autophagosomes with lysosomes, thereby hindering the formation of acidic autolysosomes and the clearance of autophagosomes. This suggests that EVs from *E. coli* expressing HlyF can enhance bacterial virulence by inhibiting autophagy in macrophages (David et al., 2022).

4.5 Bacterial EVs can influence the antiviral immune response of macrophages

It is worth noting that, in addition to the aforementioned impacts, current research indicates that bacterial EVs can also affect the antiviral immune response of macrophages. For instance, Bierwagen et al. (2023) explored the interaction between bacterial EVs and human macrophages and how this interaction influences viral replication, particularly concerning influenza virus. This study focused on EVs from different Gram-negative bacteria (such as *Klebsiella*, *E. coli*, and *Salmonella*) and found that these EVs can activate the antiviral immune response in macrophages via the TLR4-TRIF signaling axis. The research demonstrated that these bacterial EVs can induce macrophages to produce type I interferons and other antiviral molecules like Mx1, effectively inhibiting the replication of influenza virus A. Additionally, Bhar et al. (2022) investigated how commensal bacterial EVs influence murine norovirus (MNV) infection by modulating the antiviral immune response of macrophages. The study found that commensal bacterial EVs can bind to MNV and promote the co-inoculation of the virus and vesicles into host cells. This co-inoculation method significantly reduced viral replication in host cells compared to infection with the virus alone. Furthermore, these EVs increased the production of pro-inflammatory cytokines such as IL-6 and TNFα in macrophages during MNV infection, suggesting that EVs control viral infection by enhancing the immune response of host cells.

4.6 Bioengineered bacterial EVs can modulate the anti-tumor effects of macrophages

It is noteworthy that engineered bacterial EVs have also been reported to affect the antitumor immune effects of macrophages. Some tumor cells overexpress the CD47 molecule, which can inhibit the phagocytic function of macrophages, thus avoiding clearance (Kaur et al., 2016). Therefore, effectively activating tumor-associated macrophages is crucial in tumor therapy. In this regard, Feng et al. (2022) developed engineered bacterial OMVs designed to activate the phagocytic function of macrophages in the tumor microenvironment. These engineered vesicles were fused with CD47 nanobodies (CD47nb) on their surface and encapsulated with a layer of selenium-bonded polyethylene glycol (PEG/Se), enabling controlled release in the tumor site triggered by radiation. The results showed that OMV-CD47nb significantly enhanced the phagocytic ability of macrophages against tumor cells, primarily by inducing M1 polarization and blocking the "don't eat me" signal on tumor cells. Additionally, Guo et al. (2021) developed a drug delivery system based on bacterial OMVs for sequential targeting of tumor cells and macrophages. This system utilized OMVs from Gram-negative bacteria loaded with paclitaxel (PTX) and siRNA targeting Redd1 in macrophages, achieving sequential drug release through a pH-sensitive linkage. The system first released PTX in the acidic environment of the tumor site, followed by siRNA delivery to M2 macrophages, enhancing glycolysis and promoting their polarization to the antitumor M1 type. The results showed that this OMV system demonstrated significant antitumor activity both *in vitro* and *in vivo*, effectively regulating macrophage metabolism and inhibiting tumor cell migration and invasion. These studies indicate that engineered bacterial EVs can achieve antitumor effects by influencing macrophages, providing new strategies for future cancer therapies.

5 Conclusion

Bacterial EVs, as crucial information carriers between bacteria and the host, have a profound impact on key immune cells in the host immune system, particularly macrophages. EVs from different bacterial sources can carry various bioactive molecules that regulate macrophage inflammatory responses, antibacterial activity, antigen presentation functions, and induce different forms of programmed cell death, thus reshaping microbe-host interactions at the microscopic level. On one hand, certain pathogenic bacterial EVs can stimulate macrophages to release pro-inflammatory factors, inhibit their bactericidal capabilities, interfere with antigen presentation functions, and induce cell death. On the other hand, some commensal bacterial EVs can induce macrophages to release anti-inflammatory factors, enhance bactericidal activity, and activate antiviral immune responses, thereby providing protective effects for the host. In addition to these impacts, engineered bacterial EVs can also modulate macrophage metabolic states and polarization types, influencing their antitumor immune effects. Moreover, they can serve as novel drug carriers targeting tumors and regulating macrophages, demonstrating significant potential in the field of cancer therapy.

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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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Revisiting the role of hypoxia-inducible factors and nuclear factor erythroid 2-related factor 2 in regulating macrophage inflammation and metabolism

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The recent birth of the immunometabolism field has comprehensively demonstrated how the rewiring of intracellular metabolism is critical for supporting the effector functions of many immune cell types, such as myeloid cells. Among all, the transcriptional regulation mediated by Hypoxia-Inducible Factors (HIFs) and Nuclear factor erythroid 2-related factor 2 (NRF2) have been consistently shown to play critical roles in regulating the glycolytic metabolism, redox homeostasis and inflammatory responses of macrophages (Mφs). Although both of these transcription factors were first discovered back in the 1990s, new advances in understanding their function and regulations have been continuously made in the context of immunometabolism. Therefore, this review attempts to summarize the traditionally and newly identified functions of these transcription factors, including their roles in orchestrating the key events that take place during glycolytic reprogramming in activated myeloid cells, as well as their roles in mediating Mφ inflammatory responses in various bacterial infection models.

KEYWORDS

HIF-1a hypoxia-inducible factor-1a, NRF2, macrophage, LPS, inflammation, NADPH, immunometabolism, redox

Introduction

Immunometabolism is an emerging field of research that studies the metabolic regulation of immune function by integrating both biochemistry and immunology on a cellular and systemic level. Although the term "immunometabolism" first appeared in literature in 2011, the importance of metabolites for immune cell function has been reported many years ago (Alonso and Nungester, 1956; Oren et al., 1963; Newsholme et al., 1986; Fukuzumi et al., 1996; Mathis and Shoelson, 2011). In fact, intracellular metabolism

has always been recognized to play a role more than bioenergetics. For instance, the difference in amino acid metabolism has been traditionally used to define macrophages (Mφs) subsets, with M1 or M[LPS(+IFNγ)] converting arginine into nitrogen oxide (NO) via inducible Nitric Oxide Synthase (iNOS), while M2 or M[IL-4] converting arginine into ornithine via arginase-1 (Corraliza et al., 1995; Modolell et al., 1995; Munder et al., 1998; Murray et al., 2014). Interestingly, in the past, intracellular metabolism was not included in immunological research except for the studies of metabolic diseases, such as obesity or diabetes (O'Neill et al., 2016). This was attributed to a lack of robust and sensitive technologies to directly assess how metabolic pathways are linked to immune cell functions. However, recent advancements made in technologies, such as mass spectrometry-based metabolomics and Seahorse analyzers, now confer possible means to study the contribution of metabolic changes in immune functions.

Among all the immune cell types, the metabolic functions of myeloid cells, such as Mφs and Dendritic cells (DCs), are the most characterized. First discovered by Metchnikoff for their abilities to phagocytose and kill microbes, Mφs are now appreciated as innate immune cells that can detect infection or tissue damage by expressing pattern recognition receptors (PRRs), which are germline-encoded receptors that can bind to conserved compounds derived from microbes or damaged tissues (Medzhitov and Janeway, 2000; Seong and Matzinger, 2004). The successful binding of these ligands to their receptors then activate a series of phosphorylation cascades, which relay this sensing information intracellularly and activate the corresponding transcription factor to transcribe the appropriate set of genes in response to the external stimuli. The classical example of this is the binding of lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR4). LPS is a molecule derived from the outer membranes of gram-negative bacteria, therefore, it is often used as a mimetic of bacterial infection. Upon binding to TLR4, LPS activates a series of phosphorylation cascades, such as Akt and MAPKs, then the subsequent activation of transcription factors, such as NF-κB and AP-1, leading to the transcription of genes related to inflammation, phagocytosis and killing of bacteria. Although the signaling and transcription events post LPS-stimulation has been well characterized, how intracellular metabolic circuits support these events remained a mystery until the metabolites involved were profiled.

In 2010, it was first described in Mφs that LPS stimulation led to the enhancement of glycolytic flux, which can be divided into two time-dependent phases: 0-4hr and 4-12hr (Rodríguez-Prados et al., 2010). The first phase of enhancement, known as the early stage of glycolytic reprogramming, was much slower than the second phase, known as the late stage of glycolytic reprogramming. Transcriptional analyses revealed that during the enhancement of glycolytic flux, the expression of genes related to glycolysis was upregulated while the genes that encode for proteins involved in the oxidative phosphorylation were suppressed. Similar findings were reported in activated DCs whereby TLR agonists induced a metabolic switch to glycolytic metabolism with an impairment in mitochondrial respiration (Krawczyk et al., 2010; Everts et al., 2012). Taken together, these studies have demonstrated that LPS-activated myeloid cells adopt a unique metabolic profile analogous to the Warburg effect, which is a form of cellular metabolism in tumor cells characterized with high levels of glucose uptake, conversion from glucose to lactose, and a decline in oxidative phosphorylation levels (Warburg et al., 1927).

Subsequent studies published after the aforementioned ones have now elucidated the molecular mechanisms behind the induction of glycolysis and its linkage with inflammation, as well as the induction of the resolution phase of inflammation. Notably, the transcriptional regulation mediated by Hypoxia-Inducible Factors (HIFs) and Nuclear factor erythroid 2-related factor 2 (NRF2) orchestrate some of these key mechanisms. Thus, their traditionally well-characterized roles and newly elucidated functions are summarized in this review, followed by a revisit of the key events that take place during the glycolytic reprogramming in myeloid cells.

Hypoxia-inducible factors

Hypoxia-Inducible Factors (HIFs) is a family of transcription factors that are known to activate the transcription of genes in response to decrease in available oxygen, or hypoxia. Although HIFs were first discovered in 1991 for their role in mediating cellular responses against hypoxia (Semenza et al., 1991), it is now revealed that they also plays a vital role in mediating the transcriptional programs related to inflammation, angiogenesis, survival, and glucose metabolism (Ruas and Poellinger, 2005; Tannahill et al., 2013). The importance of discovering this family of transcription factors was eventually recognized by awarding the 2019 Nobel Prize in Physiology or Medicine to Gregg Semenza, Peter Ratcliffe and William Kaelin, the three scientists that significantly contributed to our understanding of how cells sense and adapt to oxygen availabilities.

In general, members of the HIF family are heterodimeric beta helix-loop-helix (HLH) transcription factors composed of an α and a β subunit, in which the α subunit is unstable and oxygen sensitive, while the β subunit is constitutively expressed and insensitive to oxygen. To date, three known α subunits, HIF-1α, HIF-2α and HIF-3α, as well as three β subunits, HIF-1β, HIF-2β and HIF-3β, have been identified (Reisz-Porszasz et al., 1994; Wang and Semenza, 1995; Jiang et al., 1996; Jiang et al., 1997; Pugh et al., 1997; Huang et al., 1998; Ema et al., 1999; O'Rourke et al., 1999; Masson et al., 2001). Among all, HIF-1α and HIF-2α are the most characterized. For HIF-1α specifically, it is structurally characterized with a N-terminal basic HLH followed by Per-ARNT-Sim (PAS) domains, oxygen-dependent-degradation (ODD) domains, and two transcription activation domains in the C-terminus (Figure 1A). The N-terminal basic domain is critical for HIF-1 binding to the consensus sequence of its target genes, which is known as hypoxia-response elements (HRE): 5'-TACGTG-3' (Wang and Semenza, 1993; Melillo et al., 1995). The HLH and PAS domains on the other hand are important for the heterodimerization between the α and β subunits. The ODD domains contain critical conserved proline residues, where they can be hydroxylated by prolyl hydroxylases (PHDs) in the presence

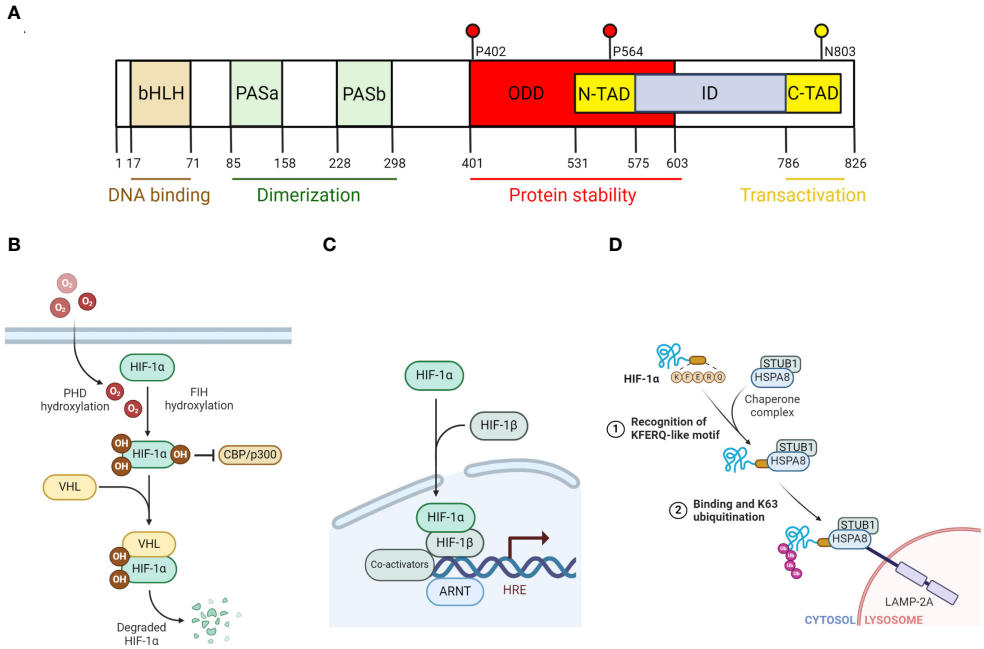

FIGURE 1
The regulation of HIF-1α degradation and function. **(A)** Diagram that illustrates the different domains of HIF-1α. bHLH and PAS domains are important for the formation of heterodimeric complex with HIF-1β and binding to DNA. N-TAD domain contains key proline residues (P402, P564) where prolyl hydroxylase (PHD) hydroxylates. C-TAD domain contains a key asparagine residue (N803) that factor inhibiting HIF (FIH) hydroxylates. **(B)** Diagram that illustrates PHD-VHL-dependent degradation of HIF-1α and the inhibition of HIF-1α transactivation capacity by FIH under normoxic condition. **(C)** Diagram that illustrates HIF-1 transcriptional activation of its targeted genes. Specifically, HIF-1α that escaped from its proteasomal degradation, combines with HIF-1β, to form a heterodimer transcription factor known as HIF-1. Along with its co-activators (such as PKM2, p300/CBP), HIF-1 then binds to its targeted genes, which are characterized with a hypoxia response element (HRE) and transcribes them. **(D)** Diagram that illustrates HIF-1α degradation via the non-canonical chaperone-mediated autophagy pathway. In this model, HSPA8 recognizes the KFERQ-like motif of HIF-1α and binds to it. STUB1, which is associated with HSPA8 as a complex, catalyzes K63 ubiquitination on HIF-1α, targeting it for lysosomal degradation in a LAMP2A-dependent manner. All figures are made by Biorender.com.

of oxygen and target HIF-1α for proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Finally, there are two transcription activation domains in the C-terminus: N terminal transactivation domain (N-TAD) and C terminal transactivation domain (C-TAD). While N-TAD is located within the ODD domain, C-TAD is near the end of the C-terminus and harbors a conserved asparagine residue, in which its hydroxylation can inhibit the transactivation capacity of HIF-1α (Mahon et al., 2001).

The regulation of HIF-1α stability

Given the short half-life of HIF-1α (~5 minutes) (Huang et al., 1998), past efforts in research have been dedicated to elucidating the mechanisms behind the regulation of HIF-1α degradation. In general, HIF-1α can be degraded by the classical ubiquitin proteasome system (UPS), which is dependent on proline hydroxylases (PHDs) and von Hippel-Lindau (VHL) ubiquitin ligase, or alternatively via the autophagy-lysosomal pathway (Hubbi et al., 2013). Other non-canonical pathways that mediate HIF-1α degradation independent of UPS and autophagy have also been reported in past studies (Iommarini et al., 2017). In the UPS model, both oxygen-dependent proline hydroxylation by PHDs and VHL-mediated ubiquitination are required (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000; Bruick and McKnight, 2001; Epstein et al., 2001). Specifically, under normoxic condition, PHDs hydroxylate HIF-1α on two proline residues, Pro402 and Pro564 (for human HIF-1α), within the ODD domain (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). The hydroxylation sites allow VHL to mediate ubiquitination and eventually proteasomal degradation (Figure 1B). However, under hypoxic condition, without oxygen, PHDs activity is inhibited and thus prevent HIF-1α from being degraded. The stabilized HIF-1α can then translocate into the nucleus where it forms a dimer with HIF-1β, which constitutes the HIF-1 complex required for the transcription of its targeted genes (Figure 1C).

As previously mentioned, HIF-1α can also be degraded by the autophagy-lysosomal pathway under certain conditions, specifically, by macroautophagy or chaperone-mediated autophagy (CMA). Macroautophagy involves the sequestration of long-lived proteins and aggregates within the autophagosome and delivering it to the lysosome for degradation. This process is mediated by Sequestosome 1 (SQSTM1) or neighbor of BRCA1 gene 1 (NBR1) (DePavia et al., 2016). Indeed, past studies have shown blocking autophagic inhibition by knocking down SQSTM1 impaired the induction of HIF-1α by Q6, a hypoxia-activated prodrug (Liu et al., 2014). This notion was reinforced by the reduction of HIF-1α levels upon AZD-2014-mediated inhibition of mammalian target of rapamycin complex 1/2 (mTORC1/2),

which activates autophagy (Zheng et al., 2015). Unlike macroautophagy, CMA involves heat-shock cognate protein of 70kDA (HSC70) targeting its substrate, such as HIF-1α (Hubbi et al., 2013), to lysosome-associated membrane protein type 2A (LMAP2A) for degradation. Under this specific condition, E3 ligase STIP1 homology and U-box containing protein 1 mediate HIF-1α K63 ubiquitination instead of VHL (Ferreira et al., 2013; Ferreira et al., 2015) (Figure 1D).

Finally, past studies have reported that under specific conditions, HIF-1α can also be degraded without the involvement of VHL and lysosomes. Specifically, in these studies, authors have shown that WD repeat and SOCS box-containing protein 1 (WSB1) can stabilize HIF-1α by ubiquitinating VHL in cancer cells (Kim et al., 2015). MDM2, another E3-ubqiutin-protein ligase, was also reported to ubiquitinate HIF-1α independent of its hydroxylation by binding to tumor suppressor proteins, such as TAp73 or p53 (Ravi et al., 2000; Amelio et al., 2015). Past studies have also shown that post-translational modifications other than hydroxylation, such as SET7/9-mediated methylation, LSD1-mediated demethylation, p300-mediated acetylation, SIRT2-mediated deacetylation, can all regulate HIF-1α stability (Geng et al., 2012; Seo et al., 2015; Kim et al., 2016). Taken together, these studies have revealed the diverse ways of how HIF-1α stability can be regulated, and such diversity may allow the fine-tuning of HIF-1 transcriptional output in response to a wide range of external stimuli.

The regulation of HIF-1α transactivation capacity

Apart from the proline hydroxylation mediated by PHDs, HIF-1α is also hydroxylated by Factor Inhibiting HIF (FIH) at Asn803 (human HIF-1α) located within the C-TAD domain during normoxic condition (Mahon et al., 2001). This asparagine hydroxylation inhibits the transcriptional activity of HIF-1 by sterically blocking the recruitment of coactivators CBP/p300 (Mahon et al., 2001). Similar to PHDs, FIH catalyzes its hydroxylation reaction via the oxidative decarboxylation of 2-oxoglutarate, producing carbon dioxide and succinate as by products. Like PHDs, the enzymatic activity of FIH is also critically dependent on its catalytic center of iron in its ferrous state (Fe^{+2}), which is maintained by the reducing action of ascorbic acid (Knowles et al., 2003; Pagé et al., 2008). Knockout studies of FIH revealed that it is an essential regulator of metabolism as mice deficient of FIH have decreased body masses with an elevated metabolic rate (Zhang et al., 2010). Moreover, these mice also have improved glucose and lipid homeostasis, thus making them resistant to weight gain induced by high-fat-diet (Zhang et al., 2010).

Although FIH and PHDs are iron (II)-dependent dioxygenases, they have significant structural differences, which underlie their differential regulation of HIF-1 transcriptional output. For instance, FIH has broad functional pockets that allow O_2 and other co-factors to bind tightly, whereas PHDs have a narrow opening of active site (Lee et al., 2003; Flashman et al., 2010). This structural difference enables FIH to work efficiently despite low O_2 availability, while PHDs remain inactive in these situations (Rani et al., 2022). More importantly, this difference in the binding affinity for O2 between FIH and PHDs underlies the graded HIF-1 transcriptional response in response to a wide range of hypoxic conditions. Apart from their differences in binding affinity for O2, FIH and PHDs also exhibit differences in their functional inhibition by TCA cycle metabolites and oxidative stress. For instance, an *in vitro* study has shown that while succinate and fumarate can competitively inhibit PHDs by occupying their active sites, they have no effect on FIH (Koivunen et al., 2007). On the other hand, citrate and oxaloacetate can inhibit both PHDs and FIH (Koivunen et al., 2007). In response to oxidative stress, FIH was found to be more sensitive to its inhibition in comparison to PHDs. Specifically, one *in vitro* study has shown that the activity of FIH was significantly more inhibited by tert-butyl hydroperoxide-induced oxidative stress than PHDs (Masson et al., 2012). Taken together, these studies demonstrate that the differential responses between FIH and PHDs to external stimuli underlie the spectrum of HIF-1 transcriptional reprogramming.

Finally, apart from catalyzing asparagine hydroxylation, an early *in vitro* study has also revealed that FIH could perform secondary functions, such as acting as a corepressor with VHL to block histone deacetylases from binding to DNA (Mahon et al., 2001). Indeed, another recent study has shown that overexpressing FIH can inhibit the mRNA of GLUT1 even in hypoxia condition, in addition to normoxic condition (Wang et al., 2014). This reinforces the possibility that FIH can regulate HIF-1 transcriptional activity independent of its hydroxylation activity.

The inflammatory role of HIF-1α in LPS-activated Mφs

Although the transcriptional role of HIF-1α under hypoxic condition is well established, its role in mediating inflammation in activated Mφs has only been recently elucidated. The investigation first began in 2003 with the initial characterization of myeloid cells that are genetically deficient of *Hif1a* from *Ly2Cre-Hif1a*$^{fl/fl}$ mice (Cramer et al., 2003). In this study, the authors found that myeloid cells that lack HIF-1α have impaired ATP levels, motility, invasiveness, bactericidal activity, and aggregation (Cramer et al., 2003), thereby implicating the role that HIF-1α plays in regulating metabolism and inflammation. This notion was further supported later by studies that show overexpressing HIF-1α leads to the upregulation of M1 signature markers (Takeda et al., 2010; Wang et al., 2017) post LPS and IFNγ stimulation. Taken together, these earlier studies have illustrated a positive role that HIF-1α plays in mediating an inflammatory response in activated M1 Mφs.

To elucidate the mechanisms underlying HIF-1α involvement in inflammatory response in activated Mφs, chromatin immunoprecipitation of HIF-1α was performed and revealed that HIF-1α binds to many glycolytic genes, such as *Slc2a1, Ldha, Hk2, Pfkp*, as well as proinflammatory genes, such as *Il1b* and *Nos2* in LPS-activated Mφs (Melillo et al., 1995; Peyssonnaux et al., 2007; Tannahill et al., 2013). The transcription of glycolysis genes is critical for driving Mφs towards an aerobic glycolytic metabolism, which is essential for fueling many intracellular inflammatory

machineries, such as Pentose Phosphate Pathway (PPP)-derived NADPH production for Nitric Oxide Synthase (NOSes) and NADPH Oxidases (NOXes), TCA cycle-derived citrate for *de novo* lipid synthesis. On the other hand, the transcription of proinflammatory genes directly contributes to the production of inflammatory cytokines. Apart from LPS stimulation, RNA-sequencing analysis also revealed that almost half of Interferon gamma (IFNγ)-induced genes in Mφs are HIF-1α-dependent (Braverman et al., 2016). Taken together, these studies have revealed the direct and indirect roles that HIF-1α play in orchestrating an effective inflammatory response in an activated Mφs.

Understanding how important the transcriptional role of HIF-1α is in activated Mφs, there is now an increasingly growing interest to elucidate the molecular factors that are required for its transcriptional activity. For instance, recent studies have shown that PKM2, which is a pyruvate kinase in the last rate-limiting step in glycolysis, can act as a cofactor for HIF-1α (Luo et al., 2011; Palsson-McDermott et al., 2015) post LPS stimulation. Specifically, PKM2 can transition from its active tetrameric state to its dimeric state, then translocate into the nucleus and interact with HIF-1α to promote its transcriptional regulation. Inhibitors, such as DASA-58 and TEPP-46, that block the dimerization of PKM2 could impair HIF-1 activity and reduced inflammation in *in vivo* pathogenic models (Palsson-McDermott et al., 2015). Overall, these studies have identified the cofactors needed for HIF-1 transcription, and how the manipulation of these cofactors confer possible means to regulate HIF-1 transcriptional output for therapeutic purposes.

Introduction to nuclear factor erythroid 2-related factor 2

Reactive oxygen species (ROS), i.e. super oxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), i.e. nitric oxide (NO)-derived peroxynitrite ($ONOO^-$), are generated as a result of intracellular metabolism and upon exposure to extracellular stimuli (Ma, 2010; Finkel, 2011). Although their uncontrolled production can result in oxidative stress that abrogates cellular function and contributes to the development of many inflammatory diseases, when regulated properly, oxidants are important signaling molecules for a wide range of cellular processes (Finkel, 2011; Sies et al., 2022), as well as mediating the inactivation of pathogens. For instance, the generation of $O_2^{\bullet-}$ from NOX2 is critical for the anti-microbial function of Mφs (Brüne et al., 2013). Similarly, the generation of mitochondrially-derived ROS has been recently shown to be activated by TLR4 signaling and critical for Mφ anti-bacterial responses (West et al., 2011). Finally, the generation of ROS from other sources, including xanthine oxidase (Ives et al., 2015) and peroxisomes (Di Cara et al., 2017) have also been reported to play an important role in mediating the intracellular killing of bacteria in Mφs.

A key transcription factor that regulates the abundance of ROS is known as the nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2). Similar to Nrf1 and Nrf3, NRF2 belongs to the cap "n" collar (CNC) subfamily of basic-region leucine zipper (bZIP) transcription factor (Oyake et al., 1996). First discovered in 1994 for its role in erythropoiesis and platelet development (Moi et al., 1994), it was later revealed that it was essential for mediating the induction of drug-metabolizing and ROS detoxification enzymes, such as NAD(P)H:quinone oxidoreductase 1 (Itoh et al., 1997). All these enzymes share a common DNA sequence, known as the antioxidant response element (ARE), where NRF2 binds to (Nguyen et al., 2003). Specifically, the ARE sequence is 41-base-pair, and contains a conserved sequence, 5'-TGACnnnGC-3', where n represents any base (Rushmore et al., 1991).

Given the importance of NRF2 in regulating cellular responses to oxidative stress, past decades of research have been invested in elucidating the regulation of NRF2 function and found that NRF2 is always degraded under basal conditions by Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (KEAP1)-mediated ubiquitination-proteasomal degradation. KEAP1 was identified as a substrate adaptor of CUL3 (a E3 ubiquitin ligase) via a yeast two-hybrid screen (Itoh et al., 1999). The molecular and structural analysis of NRF2 has revealed that it contains seven NRF2-ECH homology domains (Neh1-7), with each domain serving a distinct function (Hayes and Dinkova-Kostova, 2014) (Figure 2A). For instance, the Neh1 domain includes a CNC-bZIP region that is critical for the DNA binding activity of NRF2, as well as its association with small musculoaponeurotic fibrosarcoma (sMaf) proteins as dimerization partners (Motohashi and Yamamoto, 2004). The Neh2 domain contains the highly conserved DLG and ETGE motifs, which are important for the interaction between KEAP1 and NRF2. This domain also includes the seven lysine residues where they can be ubiquitylated and mediate NRF2 for subsequent proteasomal degradation (Itoh et al., 1999; McMahon et al., 2003; Tong et al., 2006). The Neh3 domain, which contains the transactivation activity, activates the transcription of NRF2 targeted genes, together with Neh4 and Neh5 domains (Katoh et al., 2001; Nioi et al., 2005; Sekine et al., 2016). Finally, Neh6 domain is involved in regulating NRF2 stability independent of KEAP1 (Chowdhury et al., 2013), while Neh7 domain is involved in suppressing the transcriptional activity of NRF2 (Wang et al., 2013). Overall, these studies have demonstrated the intimate link between the structure of NRF2 and the regulation of its function.

The regulation of NRF2 stability

The relatively short half-life of NRF2 (18.5 min) suggests its function is primarily regulated by its stability (Itoh et al., 2003). Indeed, cycloheximide-mediated inhibition of protein synthesis impeded basal and induced expression of NRF2-targeted genes, and that NRF2 can be stabilized with its half-life increased up to 200 min (Ma, 2013; Canning et al., 2015). Since NRF2 is a master transcription factor regulating antioxidative defense mechanisms, its basal levels are always maintained to be very low due to its constant ubiquitination and proteasomal-degradation mediated by the KEAP1/CUL3 complex. As previously mentioned, KEAP1 acts as a

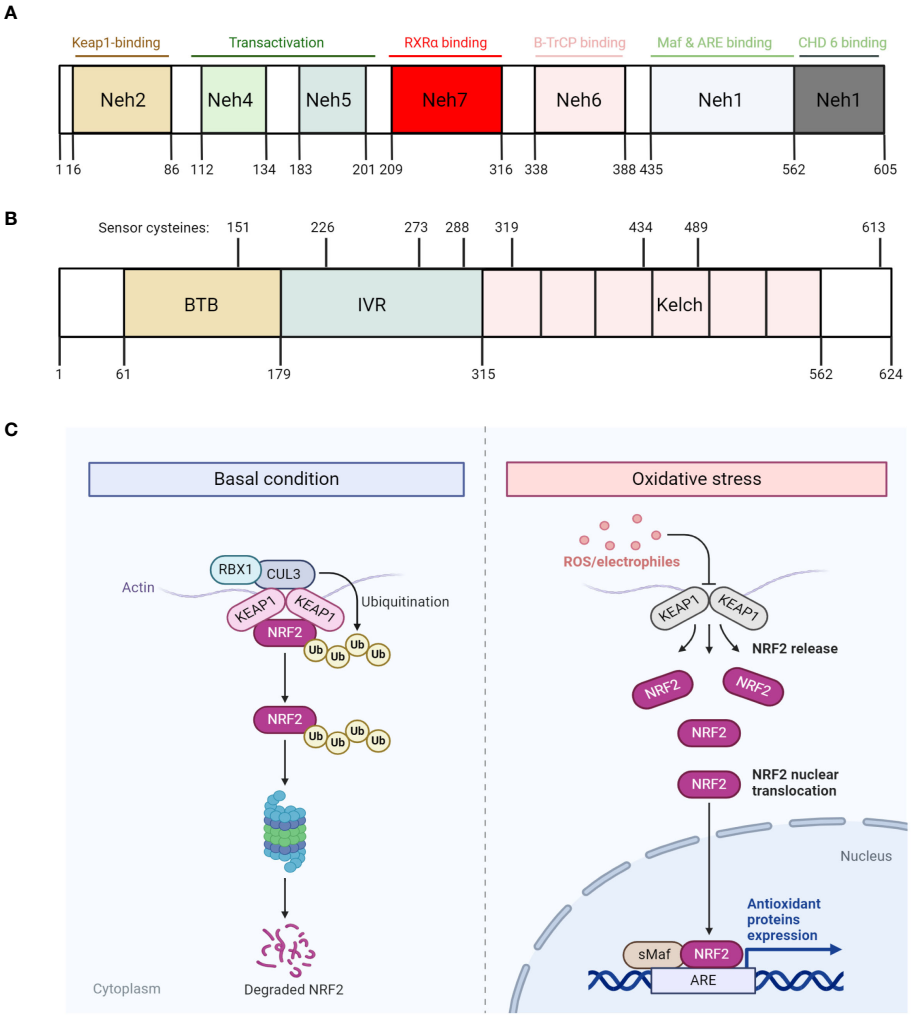

FIGURE 2
The regulation of NRF2 degradation and function. **(A, B)** Diagram that illustrates the different domains of NRF2 **(A)** and KEAP1 **(B)**, with each domain harbors different functions. Specifically, KEAP1 also harbors various critical cysteine residues that readily respond to oxidative stress. **(C)** Diagram that illustrates NRF2 degradation and function under basal condition (left) and oxidative stress (right) respectively. Under basal condition, NRF2 is constitutively degraded by KEAP1 through the proteasome. However, under oxidative stress condition, ROS/electrophiles disrupt KEAP1 conformation by reacting with its critical cysteine residues, thus releasing NRF2 and allowing it to escape from degradation. The escaped NRF2 then translocate into the nucleus, along with sMaf proteins, bind to its targeted genes, which are characterized with antioxidant response element (ARE), and transcribes them. All figures are made by Biorender.com.

substrate adaptor by linking NRF2 to CUL3, thereby mediating the ubiquitination of NRF2 by CUL3. Interestingly, the interaction between KEAP1 and NRF2 is always in a ratio of 1:2, where two KEAP1 proteins interact with one NRF2 as a dimer within the Neh2 domain (Padmanabhan et al., 2006; Tong et al., 2006; Lo et al., 2017). Specifically, structural analyses of KEAP1 have shown that KEAP1 contains two protein-interacting domains, known as the BTB domain in the N-terminal region, and the Kelch repeats in the C-terminal region (Figure 2B). While the BTB domain mediates the binding of KEAP1 to CUL3, the Kelch repeats mediate the binding of KEAP1 to NRF2 (Li et al., 2004; Padmanabhan et al., 2006; Ogura et al., 2010). In between the Kelch repeats and BTB domain lies the linker region, which is rich in cysteine residues that are prone to be oxidized by reactive radicals or electrophilic reagents. Under conditions where redox homeostasis is disrupted (i.e. ROS overproduction), these radicals can readily react with these key cysteine residues, including C151, C273 and C288 (Eggler et al., 2005; Hong et al., 2005; He and Ma, 2010; McMahon et al., 2010; Baird et al., 2013), and presumably induce a conformational change of KEAP1. This enables NRF2 to dissociate from KEAP1 and translocate to the nucleus where it activates the expression of genes that encode antioxidative proteins, such as catalase, glutathione-disulfide reductase and thioredoxin reductase (Figure 2C).

Apart from the classical model of KEAP1/CUL3-mediated NRF2 ubiquitination, past studies have reported that NRF2 stability can be modified by other post-translational modifications, and interactions with other proteins. For instance, NRF2 can be phosphorylated by ERK, JNK, AKT, PKC, CK2 and PERK, in which their phosphorylation increases the stability of NRF2 (Huang et al., 2002; Cullinan et al., 2003; Yuan et al., 2006; Apopa et al., 2008; Hayes et al., 2015). On the other hand, p38 and GSK3-mediated phosphorylation destabilizes NRF2 (Hayes and

Dinkova-Kostova, 2014). Apart from phosphorylation, NRF2 was also shown to be glycated, and its deglycation indirectly induced by fructosamine-3-kinase stabilizes NRF2 (Sanghvi et al., 2019).

In recent years, the regulation of NRF2 by SQSTM1 (also known as p62) is increasingly appreciated. First reported in 2007, Liu et al. found that the overexpression of p62 led to NRF2 nuclear translocation and activation of NRF2-targeted genes (Liu Y. et al., 2007). The underlying mechanism was then elucidated three years later by several groups, where they found that p62 interacts with KEAP1, specifically the NRF2-binding site. Therefore, an ectopic expression of p62 can outcompete NRF2 for KEAP1, sequestering KEAP1 into aggregates or autophagosomes and impedes KEAP1-mediated NRF2 degradation (Copple et al., 2010; Fan et al., 2010; Komatsu et al., 2010; Lau et al., 2010). Interestingly, an ARE element was also found to be in the p62 promoter, thereby supporting the notion that a positive feedback loop exists between the transcription of p62 and inhibition of NRF2 degradation (Jain et al., 2010). Apart from this, another positive feedback loop also exists between p62-mediated KEAP1 degradation and activation of NRF2, as reported by Copple et al. In their study, they found that overexpression of p62 significantly accelerated the degradation of KEAP1 whereas the inhibition of p62 expression increased KEAP1 expression (Copple et al., 2010). Similar results were also reported in liver-specific, *p62*-deficient mice, in which the liver-specific expression of KEAP1 was significantly reduced in comparison to its wild-type counterparts (Taguchi et al., 2012). Overall, it is generally believed that the p62-dependent non-canonical pathway results in prolonged activation of NRF2, in which this condition is found to be favorable for cancer cells to thrive as it confers cryoprotection and antioxidative defense mechanisms (Jiang et al., 2015). Taken together, these studies have demonstrated how NRF2 stability can be diversely regulated, in which this diversity is critical to optimize NRF2 transcriptional output in response to a wide range of oxidative stress conditions.

The anti-inflammatory role of NRF2 in LPS-activated Mφs

Although the role that NRF2 plays in regulating redox homeostasis is well-established, how it regulates intracellular metabolism and how this is linked to antioxidative defense in activated Mφs is not completely understood. Early studies have revealed both redox-dependent and independent roles that NRF2 play in suppressing inflammation in LPS-activated Mφs. For instance, it was discovered that NRF2-activation by itaconate-mediated alkylation of KEAP1 suppressed type I IFN responses in LPS-activated Mφs (Mills et al., 2018). Specifically, authors in this study found that itaconate and 4-octyl itaconate, which is a cell-permeable itaconate derivate, deactivated KEAP1 by alkylation and thereby activated NRF2-dependent antioxidative defense mechanisms (Mills et al., 2018). The upregulation of NRF2 then led to an impaired induction of type I IFN responses, HIF-1α levels, proinflammatory cytokine production and glycolysis (Mills et al., 2018). Overall, the study suggested that NRF2 controls Mφ inflammation by metabolic regulation. In contrast to this study, another study has found that NRF2 could suppress the transcription of LPS-induced *Il1b* and *Il6* by competing with RNA Polymerase II binding (Kobayashi et al., 2016). Specifically, in this study, the authors used ChIP-seq and ChIP-qPCR analyses to demonstrate that in *Ly2Cre: Keap1*$^{fl/fl}$ mice where NRF2 is overexpressed in myeloid cells, NRF2 could bind to the proximity of Il1β and Il6, which blocked the recruitment of RNA Polymerase II binding to their transcriptional start sites (Kobayashi et al., 2016). Overall, these studies have shown that NRF2 can regulate inflammation in ROS-dependent and independent ways.

How NRF2 controls inflammation by metabolic regulation remains unclear. Recent studies have begun to characterize the metabolism of Mφs by manipulating levels of NRF2 via genetic and pharmacological means (Ding et al., 2019; Mornata et al., 2020; Ryan et al., 2022). For instance, a recent study has profiled the proteome, metabolome, and transcriptome of bone marrow-derived Mφs (BMDMφs) isolated from WT, KEAP1-knockout or NRF2-deficient mice (Ryan et al., 2022). In this study, the authors have found that NRF2 significantly altered the proteome post LPS stimulation, including alterations in redox, carbohydrate and lipid metabolism (Ryan et al., 2022). Interestingly, the authors also found that NRF2 can modulate mitochondrial morphology, specifically, promoting their morphological transition from intermediate to fused/elongated forms post LPS stimulation (Ryan et al., 2022). Taken together, the authors found a correlation between these metabolic changes to the suppression of IFNβ responses in LPS-activated Mφs. Apart from BMDMφs, recent research has also identified an enrichment of NRF2-mediated transcriptional signature in tumor-induced myeloid-derived suppressor cells (MDSCs), which are immature myeloid cells that inhibit the activation of T cells (Gabrilovich et al., 2007), the cytotoxic functions of NK cells (Liu C. et al., 2007) and the induction of T regulatory cells (Huang et al., 2006). For instance, Beury et al. have first reported that NRF2 activation in MDSCs protects them from oxidative stress and apoptosis, thereby increasing its suppressive abilities and infiltration in tumors (Beury et al., 2016). Metabolically, Ohl et al. have elucidated that NRF2-induction in MDSCs increased their nutrient uptake through glycolysis, PPP and mitochondrial metabolism, thus supporting their proliferative abilities (Ohl et al., 2018). With respect to the pathways that activate NRF2 in MDSCs, recent research performed by Mohamed et al. have found that PKR-like endoplasmic reticulum (ER) kinase (PERK) signaling leads to NRF2-activation and its regulation on mitochondrial respiratory homeostasis, in which is critical for the immunosuppressive functions of MDSCs (Mohamed et al., 2020). Indeed, the authors found that the suppression of PERK expression abrogated NRF2 signaling in MDSCs, increased mitochondrial DNA content and activated STING-dependent expression of anti-tumor Type I IFN responses. Overall, these studies have demonstrated that NRF2 can exert its anti-inflammatory effects by intrinsically rewiring the metabolism of activated inflammatory immune cells or by supporting the expansion of immunosuppressive cell types.

LPS-induced late phase of glycolytic reprogramming

While the early phase of glycolytic reprogramming is vital to swiftly rewire the metabolic circuits to fuel the immediate bioenergetic needs of inflammatory machineries (0-4hr), the late phase of glycolytic reprogramming is critical for reconfiguring intracellular metabolism to sustain those needs for extended periods of time (6-24hr), and ultimately for the induction of resolution of inflammation (end phase; 48-72hr) (Curtis et al., 2020). Unlike the early phase where the induction is heavily dependent on post-translational modifications, such as phosphorylation signaling cascades, the late stage of glycolytic reprogramming relies on transcriptional regulation, with HIF-1α and NRF2-mediated transcription being the most important in myeloid cells.

As described previously, HIF-1α can be stabilized by TCA cycle metabolites that mediate the inhibition of PHD activity. Indeed, the reconfiguration of TCA cycle during the late stage of glycolytic reprogramming significantly alters the abundance of TCA cycle metabolites in LPS-activated Mφs. Specifically, this is achieved by two metabolic breakpoints in the TCA cycle. The first breakpoint takes place at the isocitrate dehydrogenase (IDH) level due to the suppression of its mRNA expression (Tannahill et al., 2013; Jha et al., 2015), NO-mediated cysteine nitrosation of IDH (Bailey et al., 2019) and autocrine type I interferon signaling (De Souza et al., 2019). As a result of the suppressed IDH activity, citrate then accumulates and is re-directed towards itaconate through *cis*-Aconitate by *cis*-aconitate decarboxylase or exported into the cytoplasm by citrate transport protein. The accumulation of cytosolic citrate can then be converted into acetyl-CoA through ATP citrate synthase, which is critical for supporting *de novo* lipid synthesis (Ryan and O'Neill, 2020) and histone acetylation of inflammatory genes (Lauterbach et al., 2019; Ting et al., 2024b).

On the other hand, the accumulation of itaconate contributes to the second metabolic breakpoint due to its effect on inhibiting the activity of succinate dehydrogenase (SDH) (Cordes et al., 2016; Lampropoulou et al., 2016), although the inhibitory effect of NO on SDH has also been reported (Jha et al., 2015). Together, with the increased influx of succinate derived from glutamine at the level of α-ketoglutarate (anaplerosis) (Tannahill et al., 2013), both pathways significantly increase the abundance of succinate, which inhibits the activity of PHDs and stabilizes HIF-1α. The stabilized HIF-1α then binds to HIF-1β and the HIF-1 complex activates downstream targeted gene expression, including genes involved in glycolysis and inflammation, such as *Il1b* (Tannahill et al., 2013). Apart from stabilizing HIF-1α, dysregulated succinate metabolism can lead to lysine succinylation, a novel post-translational modification that can regulate enzymes involved in remodeling the epigenome (Park et al., 2013; Tannahill et al., 2013). Specifically, succinylation can induce a 100-Da change in mass of targeted proteins and shield the positively charged lysine side chains (Park et al., 2013). Enzymes, such as PKM2, can be hypersuccinylated on lysine 311 which promotes its tetramer-to-dimer transition, thereby inhibiting its pyruvate kinase activity but activates its role as a co-factor for HIF-1α-dependent transcription (Palsson-McDermott et al., 2015; Wang et al., 2017).

Apart from HIF-1α-dependent transcription, NRF2-mediated transcriptional regulation is also critical for regulating the late-phase of glycolytic reprogramming. As previously described, the accumulation of itaconate due to the remodeling of TCA cycle leads to the alkylation of KEAP1 and subsequent activation of NRF2 (Mills et al., 2018). In addition to mediating the transcription of antioxidative genes, NRF2 has been shown to limit LPS-induced inflammatory responses through inhibiting RNA Polymerase II binding to the promoters of *Il1b* and *Il6* (Kobayashi et al., 2016), suppressing HIF-1α-mediated glycolytic reprogramming (Mills et al., 2018) and suppression of Type 1 IFN responses by decreasing the mRNA stability of STING (Olagnier et al., 2018). More recently, it has also been demonstrated that NRF2 can regulate HIF-1α stability and its transcriptional function in a NADPH-dependent manner (Ting et al., 2023b; Ting et al., 2023a; Ting et al., 2024a). Specifically, it has been shown that the enhanced activation of NRF2 led to the increased transcription and translation of NRF2-targeted apoenzymes (Ting et al., 2023b). These pools are proteins then detoxify ROS by consuming the same NADPH pools that is required to synthesize ROS and stabilize HIF-1α, eventually limiting HIF-1-dependent inflammation and fine-tune Mφ inflammatory responses (Figure 3).

Apart from fueling the effector functions of activated Mφs in a sustained manner, the end phase of glycolytic reprogramming is also important for the resolution of inflammation (48-72hr), which is characterized by the subsidence, rather than accumulation, of itaconate and succinate. For instance, a recent study has shown that after the transient accumulation of itaconate and succinate (as a result of the two breakpoints in the TCA cycle), pyruvate dehydrogenase complex and oxoglutarate dehydrogenase complex were inhibited due to the alteration of their lipoylation states (Seim et al., 2019). The inhibition of these complexes led to the normalization of citrate, itaconate and succinate levels, and correlated with a decline of HIF-1α protein levels. Taken together, these studies illustrate that the re-configuration of the TCA cycle is a highly dynamic and time dependent process.

The role of HIF-1α in bacterial infection models

HIF-1 is a master transcription factor that regulates the expression of hypoxic, inflammatory, and glycolytic genes, thus its role in mediating the inflammatory response of Mφs under hypoxic environment has been previously investigated. Indeed, wound and necrotic tissue foci have less oxygen availability (<1% oxygen) than normal healthy tissue (2.5 – 9% oxygen) (Arnold et al., 1987; Vogelberg and König, 1993; Negus et al., 1997), thus suggesting the possibility that the antimicrobial functions performed by Mφs, such as an increased ability to phagocytose, are regulated in part by the hypoxic environment (Anand et al., 2007). In 2008, Michael Karin's group was the first group that characterized the relationship between hypoxia and HIF-1α-mediated immunity, where they have shown that NF-κB could bind to the promoter of *Hif1a* in LPS-stimulated Mφs (Rius et al., 2008). They then showed that HIF-1α accumulation

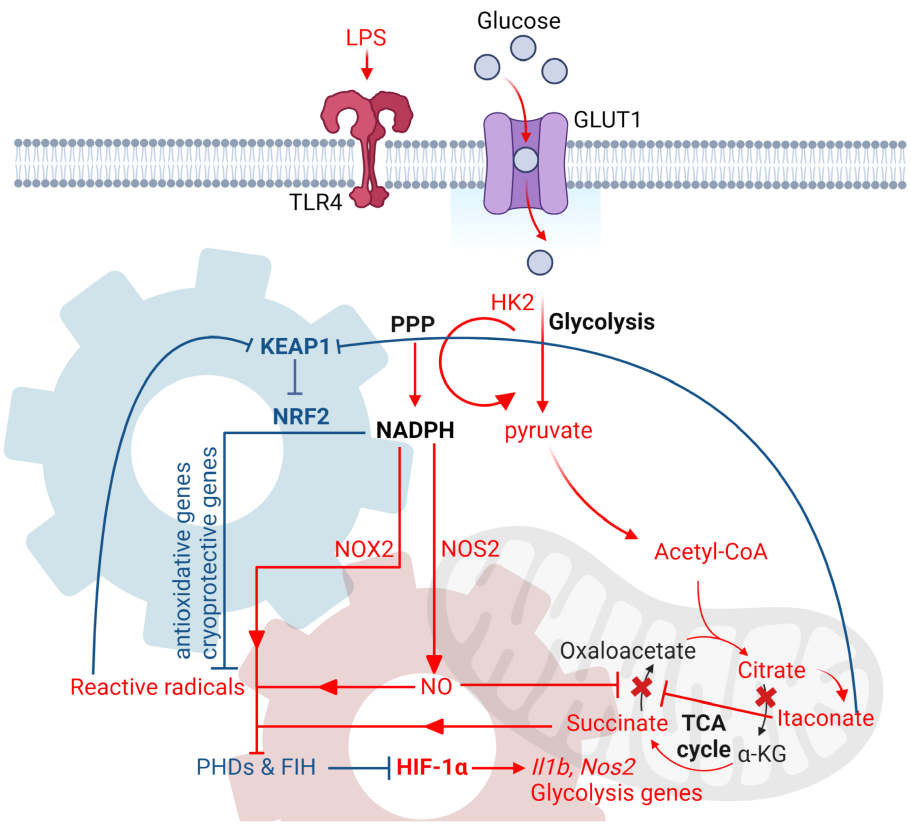

FIGURE 3
LPS-induced glycolytic reprogramming in myeloid cells. Diagram that illustrates the key events during the late phase of glycolytic reprogramming in myeloid cells upon LPS stimulation. During the late phase, the increased rate of glycolysis then feeds into the TCA cycle, where two metabolic breakpoints take place. The first break happens at the level of isocitrate dehydrogenase (IDH), leading to the accumulation of citrate and itaconate. Citrate can then be converted back to acetyl-CoA by ACLY and supports *de novo* lipid synthesis and production of pro-inflammatory cytokines, as well as histone acetylation of inflammatory genes. The second break happens at the level of succinate dehydrogenase due itaconate-mediated inhibition, thus leading to the accumulation of succinate. Succinate, along with the production of reactive radicals derived from NADPH-dependent inflammatory enzymes, such as NOX2 and NOS2, inhibit the activities of PHD and FIH. This subsequently leads to the activation of HIF-1 and transcription of its targeted inflammatory and glycolytic genes. To limit HIF-1-dependent-inflammation, NRF2 is activated in response to oxidative stress and itaconate-mediated alkylation of KEAP1. The enhanced transcription and translation of NRF2-targeted antioxidative proteins then detoxify ROS by consuming the same pool of NADPH that is also required to stabilize HIF-1α. This in turn increases the activity of PHD and FIH, which leads to the reduction of HIF-1 transcription function and ultimately the fine-tuning of Mφ inflammatory response. Red gear denotes HIF-1α transcriptional circuit, while blue gear denotes NRF2 transcriptional circuit. Both circuits consume NADPH as a mechanism of co-regulation. All figures are made by Biorender.com.

in Mφs infected with Group A *Streptococcus* (GAS) and P. *aeruginosa* were dependent on IKK-β, one of the IκB kinases that activates NF-κB by phosphorylating the inhibitor of NF-κB (Häcker and Karin, 2006). Notably, these findings were reproduced in hypoxic conditions where IKK-β could regulate hypoxia-induced HIF-1α expression and activity. Taken together, these results have demonstrated that NF-κB is a hypoxia-regulated transcription factor that activates the transcription of *Hif1a*, providing the first evidence that illustrates how the inflammatory and hypoxic responses of Mφs are integrated.

With respect to how HIF-1α mediates bactericidal activities in Mφs, this was first characterized by Randall Johnson's group back in 2003, where the group has found that Group B *Streptococcus* (GBS) was more viable in HIF-1α-deficient Mφs compared to its wild-type counterparts, which suggests that HIF-1α is important for intracellular killing of bacterial pathogens (Cramer et al., 2003). This was subsequently confirmed in another study performed by the same group, in which they found that HIF-1α-deficiency in Mφs impaired their intracellular killing ability of both GAS and *P. aeruginosa* (Peyssonnaux et al., 2005). On the other hand, the constitutive activation of HIF-1α in VHL-null mice have in turn increased intracellular killing of GAS and *P. aeruginosa.* To assess the role of HIF-1α in regulating myeloid cell bactericidal function *in vivo*, the group then adopted an infection model of GAS and found that mice with HIF-1α genetic deficiency in myeloid cells developed greater necrotic skin lesions. Mechanistically, the group then discovered that HIF-1α is critical for neutrophil-mediated production of granule proteases and antimicrobial peptides, as well as Mφ-mediated secretion of nitric oxide and TNF-α production. Similar findings were also reported by Braverman et al., where the authors have adopted a murine *M. tuberculosis* model and found that HIF-1α is responsible to regulate many IFN-γ-inducible responses in infected Mφs, such as production of nitric oxide and other inflammatory cytokines and chemokines (Braverman et al., 2016). Several years later, Knight et al. took a step further and demonstrated that HIF-1α-dependent transcription of *Hig2* is critical for driving the formation of lipid

droplets in lungs of mice infected with *M. tuberculosis*, an activated immune response from Mφs (Knight et al., 2018). The importance of HIF-1α-mediated antimicrobial activity against M. tuberculosis was also recently shown in human macrophages (Zenk et al., 2021). Finally, the direct binding of HIF-1α to other targeted genes, such as *Il1b*, has also been shown to be critical in limiting *M. marium* infection of zebrafish (Elks et al., 2013; Ogryzko et al., 2019). Specifically, studies have found that *M. marinum* infection of zebrafish induced HIF-1α-dependent transcription of *Il1b*, which then activated neutrophil-mediated production of nitric oxide and protection against infection. Similar to *M. marinum*, mice with genetic deficiency of HIF-1α in their myeloid cells also resulted in increased bacterial burden and necrotic granulomas upon infection by *M. avium* (Cardoso et al., 2015).

Apart from regulating inflammatory responses, HIF-1α is also a central regulator of glycolytic responses, in which its induction serves as the metabolic basis of host immunity. In 2011, Mihai Netea and colleagues proposed the concept of "Trained Immunity" to describe the phenomenon that innate immune cells, such as monocytes and Mφs, can also display immunological memory of past insults (Netea et al., 2011). For instance, Netea's group first demonstrated that β-glucan derived from *C. albicans* could induce trained immunity in monocytes, where the first stimulation primed their enhanced production of pro-inflammatory cytokines upon the second stimulation (Quintin et al., 2012). Next, Netea's group then demonstrated that mTOR/HIF-1α-mediated glycolysis metabolically supports the basis of trained immunity (Cheng et al., 2014). Specifically, the group has found that AKT/mTOR/HIF-1α pathways was downstream of β–glucan stimulation, and that training monocytes with β–glucan against *S. aureus* sepsis was impaired in mice with genetic deficiency of HIF-1α in myeloid cells. Apart from fungi, the importance of HIF-1α-mediated glycolytic response in Mφs was also observed in bacterial infection models, such as *M. tuberculosis* (Braverman et al., 2016) and *L. monocytogenes* (Li et al., 2018). Taken together, these studies have collectively demonstrated that HIF-1α-mediated transcription of inflammatory and glycolytic genes underlie Mφ innate immunity against many different bacteria species.

The role of NRF2 in bacterial infection models

Apart from HIF-1α, NRF2 also plays a central role in regulating Mφ inflammatory responses against bacterial infections, particularly its role in regulating the transcription of its targeted genes. Among all, its transcription of macrophage receptor with collagenous structure (MARCO), an important receptor required for the phagocytosis of bacteria, has been repeatedly shown to be critical in enhancing antibacterial defenses of Mφs. For instance, Harvey et al. have first demonstrated that sulforaphane (SFN)-induced NRF2 activation restored bacteria recognition and phagocytic ability of alveolar Mφs derived from patients with chronic obstructive pulmonary disease (Harvey et al., 2011). Specifically, the authors found that SFN-induced activation of NRF2 significantly enhanced the expression of MARCO and the ability of Mφs to phagocytose *P. aeruginosa*, while the disruption of MARCO or NRF2 impaired their phagocytic abilities. Similar findings were also reported by Pang et al., where the authors found that Early growth response 1 (ERG1)-mediated suppression of phagocytosing *P. aeruginosa* was due to inhibition of NRF2 signaling (Pang et al., 2022). In this study, the authors have found that *P. aeruginosa* induced ERG1 expression and autophagy-related processes during infection of Mφs. The induction of autophagy subsequently enhanced the degradation of p62 and suppressed NRF2 levels. The impaired NRF2 levels then resulted in the reduced transcription of MARCO and Macrophage scavenger receptor 1 (MSR1), which is another scavenger receptor important for phagocytosing bacteria. In addition to *P. aeruginosa*, Wang et al. have also discovered similar results with *E. coli* infection, where they found that IR-61, an inhibitor that disrupts the interaction between KEAP1 and NRF2, augmented Mφs ability to phagocytose bacteria. Mechanically, the authors utilized an *in silico* molecular ligand docking analysis and found that IR-61 bound to KEAP1 and induced NRF2 release and activation (Wang et al., 2023). Finally, Luo et al. have discovered that itaconate is critical in supporting Mφ phagocytic abilities by enhancing NRF2-dependent transcription of *Cd36*, which is another scavenger receptor of Mφs that is involved in internalizing bacteria (Wang et al., 2023). Taken together, these studies have collectively demonstrated that NRF2-dependent transcription of scavenger receptors, such as MARCO, MSR1 and CD36, are critical players in orchestrating Mφ bactericidal responses.

Apart from supporting phagocytosis, NRF2 also regulates Mφ inflammatory responses against bacterial infection through maintaining redox homeostasis, cell survival, the formation of phagolysosomes and iron homeostasis. For instance, Sun et al. showed that activation of NRF2 by oltipraz (OTZ), a synthetic dithiolethione, is critical to protect *M. tuberculosis*-induced oxidative injury and cell death of human Mφs (Sun et al., 2020). Specifically, OTZ-induced NRF2 activation led to the transcription of antioxidative genes and offered cryoprotection against *M. tuberculosis*-induced oxidative stress. Apart from this, Nakajima et al. have found that SFN-induced NRF2 activation increased Mφs control of *M. avium* by promoting the formation and phagolysosome fusion and granuloma formation (Nakajima et al., 2021). This is due to NRF2-dependent transcription of *Slc11a1* (NRAMP1) and *Hmox1* (HO-1) as they are involved in promoting phagosome-lysosome fusion and granuloma formation respectively. Finally, Nairz et al. have shown that nitric oxide production in Mφs is critical for NRF2-dependent transcription of ferroportin-1 (*Fpn1*), an iron exporter that exports iron extracellularly and prevents its acquisition by *S. typhimurium* (Nairz et al., 2013). Specifically, in this study, the authors discovered that Mφs with *Nos2* genetic deficiency have increased intracellular iron storage due to impaired *Fpn1* expression, thus allowing *S. typhimurium* to utilize its iron content. Notably, nitric oxide promoted NRF2 activation and its transcription of *Fpn1*, thus limiting the growth of S. *typhimurium*. Overall, these studies have shown that NRF2 plays a positive role in regulating Mφs responses against bacterial infection by regulating the transcription of its targeted genes that are involved in a variety of cellular processes.

Conclusion

Recent advances in the immunometabolism field have comprehensively demonstrated how the rewiring of intracellular metabolic circuits are linked to the effector functions of Mφs, such as the transcription of inflammatory cytokines and glycolytic genes. Notably, the transcriptional regulation mediated by HIF-1α and NRF2 play significant roles behind these processes. Although these transcription factors were discovered back in the 1990s and their pathways have been since well characterized, the birth of the immunometabolism field has now revealed novel functions that they can play, as well as the possibility that they can co-regulate each other as a mechanism to fine tune Mφs inflammatory responses. Understanding that multiple transcription factors are responsible for relaying complex information of extracellular changes into host cell responses through the activation or inhibition of transcriptional circuits, future research should be warranted to further elucidate the co-regulation between other transcriptional circuits and identify their underlying co-regulators.

HIF-1α-mediated transcriptional responses in Mφs is a general phenomenon against many types of bacteria (Werth et al., 2010). Notably, the transcriptional regulation of HIF-1α on inflammatory and glycolytic genes primarily orchestrate Mφ inflammatory responses, and the oxygen availability (degree of hypoxia) in the environment helps to fine-tune its transcriptional output and the extent of inflammation. While this multi-layered regulation of HIF-1α responses is critical for Mφ immunity and has been well characterized, it also opens the possibility that pathogens can evolve and adapt to this for its survival. Indeed, a recent study has shown that hypoxia-induced HIF-1α-response in Mφs limited the replication of *C. burnetiid* without affecting its viability, thus allowing it to persist chronically and this may be linked to the development of chronic Q fever (Hayek et al., 2019). Future research should thus be warranted to investigate how pathogens have developed mechanisms to resist and adapt to HIF-1α-mediated immunity in Mφs, particularly in the context of chronic bacterial infection or re-infection models.

Similar to HIF-1α, NRF2 also plays an essential role in mediating Mφ responses against bacterial infection, primarily by enhancing its phagocytic abilities through upregulating its targeted scavenger reports, as well as maintaining a favorable redox environment and cell survival. Although many studies have supported a positive role of NRF2 in promoting Mφ bactericidal responses, an interesting study has recently shown that *M. tuberculosis*-induced early NRF2-responses in alveolar Mφs in fact hindered their ability to elicit a robust inflammatory response and its effective control of bacterial growth (Rothchild et al., 2019). This suggests that *M. tuberculosis* may have developed mechanisms over evolutionary time to infect a certain population of Mφs that preferentially upregulate antioxidative defense mechanisms over classical inflammatory responses. This may also explain the reason behind the conflicting effectiveness of pharmacological NRF2 activators in limiting bacterial infection, especially when compared against different types of bacteria species (Ali et al., 2020). Therefore, future research should be warranted to elucidate the mechanisms behind how different types of bacteria adapt to NRF2-mediated responses in Mφs.

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Conflict of interest

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

Publisher's note

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Macrophage variants in laboratory research: most are well done, but some are RAW

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Macrophages play a pivotal role in the innate immune response. While their most characteristic function is phagocytosis, it is important not to solely characterize macrophages by this activity. Their crucial roles in body development, homeostasis, repair, and immune responses against pathogens necessitate a broader understanding. Macrophages exhibit remarkable plasticity, allowing them to modify their functional characteristics in response to the tissue microenvironment (tissue type, presence of pathogens or inflammation, and specific signals from neighboring cells) swiftly. While there is no single defined "macrophage" entity, there is a diverse array of macrophage types because macrophage ontogeny involves the differentiation of progenitor cells into tissue-resident macrophages, as well as the recruitment and differentiation of circulating monocytes in response to tissue-specific cues. In addition, macrophages continuously sense and respond to environmental cues and tissue conditions, adjusting their functional and metabolic states accordingly. Consequently, it is of paramount importance to comprehend the heterogeneous origins and functions of macrophages employed in *in vitro* studies, as each available *in vitro* macrophage model is associated with specific sets of strengths and limitations. This review centers its attention on a comprehensive comparison between immortalized mouse macrophage cell lines and primary mouse macrophages. It provides a detailed analysis of the strengths and weaknesses inherent in these *in vitro* models. Finally, it explores the subtle distinctions between diverse macrophage cell lines, offering insights into numerous factors beyond the model type that can profoundly influence macrophage function.

KEYWORDS

macrophage types, primary macrophages, macrophage-like cell lines, immunity, macrophage polarization (MP)

1 Macrophages

Macrophages represent an ancient cell type in the phylogeny of metazoans (Wittamer et al., 2011; Buchmann, 2014). They are capable of engulfing foreign or endogenous materials and are found in many organisms with multicellular organization, where they have versatile roles in maintaining homeostasis and immunological defense (Mosser and Edwards, 2008; Wynn et al., 2013; Barreda et al., 2016; Mosser et al., 2021). They were named ("makros" = big, "phagein" = to eat, "big eaters") after their most characteristic ability and main function, the active uptake of particles bigger than 0.5 μm, by a process called phagocytosis, firstly discovered by Metchnikoff in the beginning of 1880 (Underhill and Goodridge, 2012; Tauber et al., 2017).

1.1 Phagocytosis: a crucial cellular mechanism in macrophages for immune defense and tissue homeostasis

Phagocytosis is an important cellular mechanism conserved in all multicellular organisms from protozoans to mammals, including humans (Boulais et al., 2010; Gordon, 2016). Macrophages phagocytose endogenous material, like apoptotic cells (Fadok et al., 1998; Liu et al., 2006; Erwig and Henson, 2008; Kono and Rock, 2008; Suzanne and Steller, 2013; Kourtzelis et al., 2020) and cell debris, or foreign objects, such as pathogens (Chen et al., 2007; Gluschko et al., 2018) and toxic substances, like asbestos or silica particles (Murray and Wynn, 2011b; Nakayama, 2018).

Macrophages play a vital role in engulfing and digesting particles through phagocytosis, a function that aids in identifying different subtypes within the macrophage classification based on how efficiently and extensively they perform this process (Ku et al., 1992; Kirkpatrick et al., 1997; Neve et al., 2014; Kresinsky et al., 2016).

For scanning their extracellular surroundings, macrophages express numerous surface and cytoplasmic receptors that detect irregular signals not typically found in the organism's physiological milieu (Uribe-Querol and Rosales, 2020). These include scavenger receptors, which bind apoptotic and necrotic cells, opsonized pathogens, and cell debris; and pattern recognition receptors (PRRs) that detect 'non-self' or 'damaged' signals (Kawai and Akira, 2011), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene 1 (RIG1)-like helicase receptors (RLRs), Fc-receptors, and NOD-like receptors (NLRs) (Elinav et al., 2011; Osorio and Reis e Sousa, 2011; Freeman and Grinstein, 2014; Uribe-Querol and Rosales, 2020).

The interaction of these receptors with ligands recruits actin filaments to internalize the particle, which is then enclosed within a phagosome (Sarantis and Grinstein, 2012; Weiss and Schaible, 2015). Phagosomes fuse with lysosomes to form phago-lysosomes, leading to the degradation of the cargo (Jutras and Desjardins, 2005; Haas, 2007; Levin et al., 2016).

1.2 Efferocytosis: macrophages' strategy for infection control and tissue maintenance

Macrophages can contain the spread of infection through a clearance of infected and dead host cells (Martin et al., 2012). This behavior is called efferocytosis and serves as a protective mechanism to clear dying or dead cells from tissues during growth and remodeling (Boada-Romero et al., 2020). This process is primarily carried out by tissue macrophages and, during the onset of inflammation, by monocyte-derived macrophages (Watanabe et al., 2019).

In instances of infection, a significant number of cells engaged in host defense undergo cell death. It becomes imperative to remove these cells to minimize tissue damage and inflammation (Labbe and Saleh, 2008). Additionally, since these cells are often harboring intracellular pathogens, which lose their habitats due to cell death, containing the infection becomes critical, rendering efferocytosis an indispensable process during the host's response to intracellular bacteria (Weiss and Schaible, 2015; Karaji and Sattentau, 2017). In addition, efferocytosis promotes the transition of macrophages to the anti-inflammatory phenotype, leading to a reduction in pro-inflammatory cytokines and an increase in the release of anti-inflammatory mediators, such as interleukin (IL)-10 and transforming growth factor-beta (TGF-β) and pro-resolving molecules (resolvins and protectins) (Dalli and Serhan, 2012; Angsana et al., 2016; Ge et al., 2022), which aid in the reduction of inflammation during infection (Elliott et al., 2017; Chazaud, 2020).

Moreover, efferocytosis plays a crucial role in tissue restructuring during growth and development, as well as in the process of wound healing (Vaught et al., 2015; Mehrotra and Ravichandran, 2022). Throughout the development of complex organisms, cell death is a natural occurrence that aids in growth and tissue restructuring. Thus, eliminating senescent and deceased cells becomes vital for preserving tissue balance and structure, while also facilitating the healing process (Morioka et al., 2019; Gerlach et al., 2021).

1.3 Optimizing macrophage function: the crucial role of the extracellular matrix and fibroblast interactions

Ensuring a favorable environment for macrophages is important for their optimal function as phagocytes (Chen et al., 2023). The extracellular matrix (ECM) serves as a sophisticated system that offers a structural scaffold supporting immune cells. Particularly, collagen, predominantly synthesized by fibroblasts, plays a key role in facilitating various cellular functions, including the differentiation and adhesion of myeloid cells (Khalaji et al., 2017; Makdissi and Mass, 2021). In addition, the interplay of macrophages and fibroblasts is important for growth factor exchange and reducing abnormal proliferation (Zhou et al., 2018). Finally, modification of ECM can trigger a mechanosensitive

response in macrophages which is critical for tissue regeneration and fibrosis (Meizlish et al., 2024).

As the origin of macrophages, whether isolated from animal or human samples, differentiated with growth factors from precursor cells, isolated as cancer cell lines exhibiting macrophage-like behavior, or generated as *in vitro* cell lines, understanding alterations in macrophage behavior solely based on its origin is futile. Moreover, characterizing macrophages by their phagocytic activity needs to be expanded, as macrophage function during processes such as body development, homeostasis, repair, and immune responses against invading pathogens is of paramount importance.

2 Macrophage development: diverse origins and functional adaptations

As macrophages are pivotal components of the innate immune system, their development is tightly regulated in a series of stages within the hematopoietic system (Wynn et al., 2013). For many years, there was a prevailing belief that macrophages constituted a largely uniform group originating from hematopoietic stem cells (HSCs) in the bone marrow and underwent a defined developmental process known as monopoiesis (Mosser and Edwards, 2008). However, recent findings show that numerous macrophages residing in tissues of adult mice do not depend on HSCs, instead, they originate from yolk-sac progenitors in early embryonic stages (Gomez Perdiguero et al., 2015).

These precursor cells, termed pre-macrophages (pMacs), travel through the bloodstream and settle in various organs. Upon exiting the bloodstream and undergoing differentiation, they develop a unique genetic profile specific to the tissue they inhabit (Mass, 2023; Wang et al., 2023). This maturation process equips macrophages with the ability to adapt to particular tissue contexts (Gordon and Pluddemann, 2017). The resulting macrophages can assume diverse roles, ranging from resident tissue macrophages contributing to homeostasis or to activated macrophages responding dynamically to infections or inflammatory stimuli (Gomez Perdiguero et al., 2015). Tissue-resident macrophages possess the ability to renew themselves and typically do not rely on input from HSCs (Mass et al., 2023).

Furthermore, each organ contains monocyte-derived macrophages (MDMs) with varying lifespans, some persisting long-term while others have shorter lifespans and are continuously replaced by HSCs from the bone marrow. This leads to a complex situation where macrophages originating from fetal and HSC sources coexist in certain tissues (Jenkins and Allen, 2021; Dick et al., 2022).

At the site of inflammation, monocytes are attracted to the tissue and differentiate into macrophages, which cooperate with or replace resident cells for sustaining immunity or promoting resolution of inflammation and tissue regeneration (Italiani and Boraschi, 2015; Muller et al., 2020; Mass et al., 2023).

2.1 Tissue macrophages

Tissue macrophages show remarkable functional diversity and plasticity, which are influenced by the surrounding tissue (Sreejit et al., 2020; Mass et al., 2023). Studies employing fate-mapping techniques involving conditional reporter genes have indicated that most resident tissue macrophage populations are established during embryonic development and sustain themselves in adulthood through self-renewal, with limited contribution from bone marrow progenitors or blood monocytes (van Furth and Cohn, 1968; Hume, 2023). Tissue macrophage progenitors (for skin, spleen, pancreas, liver, brain) are derived from yolk sac and fetal liver (Bertrand et al., 2005; Gomez Perdiguero et al., 2015) or have mixed origins from yolk sac and bone marrow (for lung, kidney) (Hoeffel et al., 2012; Epelman et al., 2014).

Some macrophage populations, that are characterized by low F4/80 receptor expression, can be replenished from bone marrow-derived progenitor cells under certain circumstances, such as tissue infection (Ginhoux et al., 2010; Hoeffel et al., 2012; Schulz et al., 2012). In their basal state, resident tissue macrophages exhibit considerable variation in function, requiring different morphologies, transcriptional responses, and locations (Davies and Taylor, 2015). This functional heterogeneity probably results from the dynamic crosstalk between resident tissue macrophages and client cells that they support (de Sousa et al., 2019).

The roles of tissue resident macrophages have been reviewed (Davies et al., 2013a; Epelman et al., 2014; Davies and Taylor, 2015; Varol et al., 2015; Lazarov et al., 2023). Macrophages are divided into subpopulations based on their anatomical location and functional phenotype (Figure 1). A remarkably large reservoir of tissue resident macrophages lies in the intestine (Gordon et al., 2014).

Interestingly, it is believed that murine intestinal tissue-resident macrophages are regularly replenished by circulating monocytes via a phenomenon referred to as the "monocyte waterfall" (Bsat et al., 2020; Muller et al., 2020). Embryonic precursors colonize the intestinal mucosa and undergo significant proliferation *in situ* during the neonatal phase. However, they are replaced around weaning by Ly6C^{high} monocytes. These monocytes then mature into anti-inflammatory macrophages locally. This process, primarily influenced by the microbiota, needed to persist throughout adulthood to uphold a healthy population of intestinal macrophages (Bain et al., 2014; Desalegn and Pabst, 2019).

Tissue macrophages contribute to the maintenance of healthy tissues by removing dead and dying cells, as well as toxic materials (Murray and Wynn, 2011b). Tissue macrophages also suppress inflammation mediated by inflammatory monocytes, thereby ensuring that tissue homeostasis is restored after infection or injury. Indeed, important homeostatic functions have been assigned to the mononuclear phagocytes in almost every tissue of the body (Murray and Wynn, 2011b; Wynn et al., 2013; Chovatiya and Medzhitov, 2014; Davies and Taylor, 2015; Mosser et al., 2021).

3 Macrophage plasticity and polarization: beyond the M1/M2 paradigm

Macrophages display an incredible plasticity, where their functions can be significantly and specifically altered by cytokine

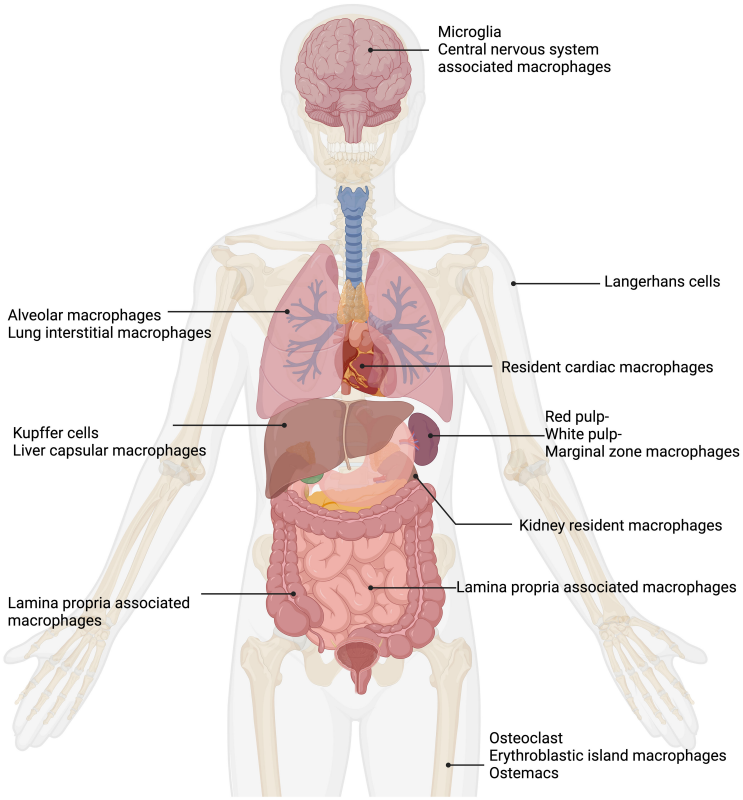

FIGURE 1
Tissue macrophages exhibit remarkable functional diversity depending on their tissue of residence. In the central nervous system (CNS), microglia (Pallares-Moratalla and Bergers, 2024) and CNS-associated macrophages (Prinz et al., 2021) reside. In the skin, tissue-resident macrophages are Langerhans cells (Merad et al., 2002). The lung contains two types of tissue-resident macrophages: alveolar macrophages (Joshi et al., 2018) and lung interstitial macrophages (Schyns et al., 2018). Tissue-resident macrophages of the spleen are divided into red pulp, white pulp, and marginal zone macrophages (Fujiyama et al., 2019). Resident cardiac macrophages critically contribute to heart tissue remodeling (Wong et al., 2021; Suku et al., 2022). Kidney resident macrophages support organ healing (Cheung et al., 2022). The liver comprises two main types of tissue-resident macrophages: Kupffer cells (Dixon et al., 2013) and liver capsular macrophages (Bleriot and Ginhoux, 2019). The gut contains lamina propria-associated macrophages (Nagashima et al., 1996). In the bone, three types of tissue-resident macrophages are present: osteoclasts (Drissi and Sanjay, 2016), erythroblastic island macrophages (Seu et al., 2017), and osteomacs (Batoon et al., 2017). Figure was adapted from the template "Human Internal Organs" by Eunice Huang. Retrieved from https://app.biorender.com/biorender-templates; Biorender.com.

signals (Murray and Wynn, 2011b; Wynn et al., 2013). Due to their ability to change and adapt to various exogenous and endogenous factors, macrophages can rapidly alter their functional profile through a process known as polarization (Mosser et al., 2021). Macrophage polarization is the process by which macrophages respond to incoming stimuli from the local microenvironment (Murray, 2017; Arora et al., 2018). After recognition of the phagocytosed material and/or additional factors, such as secreted pro- or anti-inflammatory cytokines from other cells, several signaling pathways are activated. These pathways ultimately lead to the development of a specific macrophage phenotype tailored to meet the functional requirements (Stout et al., 2005).

Currently, there is a partial consensus among researcher in the macrophage field concerning the classification of various macrophage activation phenotypes (Martinez and Gordon, 2014; Ginhoux et al., 2016; Murray, 2017). Initially, macrophage activation relied on a simplistic binary model, such as classic and alternative activation concepts, also known as M1 and M2. It is now widely acknowledged that this model is overly broad and misinterpreted, hindering the understanding of pathogenesis (Martinez and Gordon, 2014; Muraille et al., 2014).

Classically activated macrophages (or M1-like macrophages) release a plethora of pro-inflammatory cytokines, and produce antimicrobial factors, like reactive nitrogen species (RNS) or reactive oxygen species (ROS) (Nathan et al., 1983; Filipe-Santos et al., 2006; Mosser and Edwards, 2008; O'Shea and Murray, 2008; Orecchioni et al., 2019; Dalby et al., 2020; Herb and Schramm, 2021). Alternatively activated macrophages (or M2-like macrophages) have anti-inflammatory functions and regulate wound healing and tissue repair (Stein et al., 1992; Doyle et al., 1994; Loke et al., 2002; Kreider et al., 2007; Wynn et al., 2011; Sica and Mantovani, 2012; Corliss et al., 2016; Wynn and Vannella, 2016; de Groot and Pienta, 2018). There are in addition many subtypes of M2-like macrophages depending on the anti-inflammatory stimuli: M2a, M2b, M2c, M2d (Mantovani et al., 2004).

The currently prevailing perspective on macrophage activation involves adopting a multidimensional model that incorporates the

diverse signals encountered by macrophages within their unique microenvironments i.e.: M(IL-4), M(IL-13), M(IFN-γ), M(LPS). This system sidesteps the intricacies of classifications, where different laboratories might define activation differently, enabling comparisons and contrasts of new activation conditions with these fundamental examples (Murray et al., 2014; Ginhoux et al., 2016).

Macrophages demonstrate remarkable phenotypic plasticity by rapidly modulating their functional capability in response to exogenous factors, such as infection or injury (Mosser and Edwards, 2008). These immune effectors are extremely dynamic, initially engaging in pro-inflammatory activities and subsequently transitioning to facilitate the resolution phase. This flexibility allows macrophages to regulate immune responses, from inflammation to restoring tissue balance (Mills, 2012; Gordon and Martinez-Pomares, 2017).

Taken together, there is not "the macrophage" per se, but a broad set of different macrophage types that constantly detect and respond to environmental stimuli as well as tissue physiology and change their functional and metabolic state accordingly (Nathan et al., 1983; Pace et al., 1983; Stein et al., 1992; Doyle et al., 1994; Mills and O'Neill, 2016; O'Neill and Pearce, 2016; Formentini et al., 2017). Having said this, it comes with no surprise that there cannot be a one fit all model for studying macrophage biology but that careful consideration is required upfront.

4 Macrophage colony-stimulating factor and its role in macrophage differentiation and cultivation

Macrophage colony-stimulating factor (M-CSF) is a growth factor responsible for the proliferation and differentiation of myeloid progenitors into macrophages in the bodies of humans and mice (Lin et al., 2008; Hamilton and Achuthan, 2013; Roszer, 2018; Hamidzadeh et al., 2020).

In the plasma of mice, approximately 10 ng/mL M-CSF is constantly present, maintained by secretion from various cells in the body, thereby ensuring the recruitment and differentiation of circulating blood monocytes (Hume and MacDonald, 2012; Hamilton and Achuthan, 2013). Under physiological conditions, M-CSF levels are regulated by colony stimulating factor 1 receptor (CSF-1R)-mediated endocytosis, providing feedback control to regulate macrophage production based on the number of mature macrophages (Bartocci et al., 1987). A key characteristic of *in vivo* macrophages is their dependence on M-CSF receptor signaling for differentiation and survival (Ushach and Zlotnik, 2016).

M-CSF is frequently utilized *in vitro* to generate bone marrow-derived macrophages (BMDM) from myeloid progenitor cells in the bone marrow of mice and to differentiate macrophages from peripheral blood monocytes (PBMC) for human-related research (Ushach and Zlotnik, 2016). These cells need M-CSF for both, differentiation and survival *in vitro* (Erbel et al., 2013; Chen et al., 2021b). In contrast, *ex vivo* macrophages, such as peritoneal macrophages, are isolated as mature macrophages and therefore do not need M-CSF for further differentiation. However, they only survive 2-3 days during *in vitro* cultivation without M-CSF supplementation (Wang et al., 2013).

In sharp contrast to *in vitro*-differentiated or *ex vivo*-cultivated macrophages, the macrophage-like cell lines RAW264.7 and J774 do not need M-CSF or any other constantly applied growth factor for cultivation or survival *in vitro* (Ralph and Nakoinz, 1975; Raschke et al., 1978).

Islam et al. reported minimal production of M-CSF in RAW264.7 cells, which was significantly increased due to receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) treatment (Islam et al., 2008). Another study on osteoclast formation from the macrophage-like cell line RAW264.7 stated that the immortalized cell line produces M-CSF (Collin-Osdoby and Osdoby, 2012). Moreover, M-CSF levels in the supernatant of RAW264.7 cells were detected via ELISA in another study (Yang et al., 2014).

Checking available high-throughput RNA-sequencing data, two publications showed the expression of M-CSF in RAW264.7 cells (Chen et al., 2021a; Liu et al., 2023a). The levels of M-CSF significantly increased due to stimulation with lipopolysaccharide (LPS) (Liu et al., 2023a) and decreased due to the infection with *Mycobacterium tuberculosis* (*Mtb*) (Chen et al., 2021a). One publication by Andreu et al. showed that J774 cells express higher levels of the gene for M-CSF compared to BMDM. The level of M-CSF increased over time and in response to infection with *Mtb* in J774 cells (Andreu et al., 2017). The published results of the RNA-sequencing data are summarized in Table 1.

Table 1 shows the total number of aligned reads for the M-CSF gene (raw counts; not shown) for each biological replicate was taken from the published data and used to estimate M-CSF expression by averaging (Avg. counts). The relative M-CSF expression was determined by forming a ratio between J774 and BMDM, RAW264.7 untreated and LPS stimulated or with *Mtb* infected samples. Independent filtering of the DESeq2 R package confirmed that all values have passed the filter threshold for low expressed genes (not shown).

However, only RNA data has been published and no study has reported M-CSF secretion in the macrophage cell line J774. Therefore, it remains unclear whether J774 cells produce their own M-CSF and thus do not require supplementation, or if this cell line simply does not need M-CSF due to their inherent proliferative capabilities as tumor-derived cells.

5 Immortalized and primary mouse macrophage models for *in vitro* studies

In general, immortalized macrophages derived from tumor cells, which exhibit continuous division or cells that have been deliberately modified to proliferate indefinitely, enable their cultivation over numerous generations (Taciak et al., 2018; Lucy et al., 2022; Chalak et al., 2024; Xie et al., 2024). Nonetheless, primary macrophages are the mainstay of study of macrophage function *in vitro* (Murray and Wynn, 2011a).

TABLE 1 M-CSF expression in macrophage-like cell lines and bone marrow-derived macrophages.

Cell Type	Treatment	Avg. counts	Counts relative to LPS	Reference
RAW264.7	untreated (6 h)	103.3	49.3	Liu et al., 2023a
RAW264.7	500 ng/mL LPS (6 h)	5097.3		
			Counts relative to *Mtb* infection	
RAW264.7	untreated (3 h)	142.0	0.4	Chen et al., 2021a
RAW264.7	*Mtb* infection (3 h)	57.0		
			Counts relative to BMDM	
BMDM	untreated (4 h)	14.7	3.4	Andreu et al., 2017
J774	untreated (4 h)	49.3		
BMDM	*Mtb* infection (4 h)	543.7	0.6	
J774	*Mtb* infection (4 h)	309.0		
BMDM	untreated (24 h)	29.3	2.6	
J774	untreated (24 h)	76.3		
BMDM	*Mtb* infection (24 h)	123.3	6.7	
J774	*Mtb* infection (24 h)	827.7		

Each *in vitro* macrophage model has both positive and negative aspects that need to be carefully considered before choosing a model.

5.1 Immortalized mouse macrophage cell lines

The development of tissue culture techniques and establishment of macrophage cell lines has been indispensable for biological research for several decades (Freshney; Ralph et al., 1980; Sumiya et al., 2015; Taciak et al., 2018). Macrophage-like cell lines are important tools to unravel macrophage function, with RAW264.7 and J774 cells as the two most commonly used immortalized macrophage cell lines available from cell banks (Lam et al., 2009; Taciak et al., 2018). Immortalized macrophage cell lines have several advantages: (I) they are easy to handle and self-replicate, thereby representing an unlimited source of cells with various genetic conditions. (II) The cells can be cultivated in almost limitless quantities, (III) they can be stored frozen for a long time, and (IV) they are easily replaced if lost due to e. g. contamination (Kaur and Dufour, 2012; Voloshin et al., 2023; Weiskirchen et al., 2023).

However, there are a couple of limitations if immortalized macrophage cell lines are used. They are derived either from cancerous single cells/tumors or were generated by viral infection. That is why they are susceptible to genotypic and phenotypic drift during culture and passaging. Consequently, macrophage cell lines can lose macrophage-specific functions and acquire a molecular phenotype that is quite different from that of cells *in vivo* or primary isolated cells (Burdall et al., 2003; Pan et al., 2009; Frattini et al., 2015; Bosshart and Heinzelmann, 2016).

For example, RAW264.7 macrophages are lacking apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) which serves as adaptor molecule for various inflammasome receptors (Pelegrin et al., 2008). This hinders RAW264.7 cells to produce and secrete mature IL-1β (while the production of pro-IL-1β is unaffected) after stimulation with nigericin for NLR family pyrin domain containing 3 (NLRP3), double-stranded DNA for absent in myeloma 2 (AIM), and *Clostridioides difficile* toxin b (TcdB) for pyrin inflammasome activation (Zheng et al., 2020). Transfection of RAW264.7 macrophages with ASC subunit restores the ability of pro-caspase 1 cleavage with subsequent production and secretion of mature IL-β (Bryan et al., 2010). In addition, RAW264.7 macrophages have fluctuating expression of several genes and proteins with increasing passage numbers. More than 50 passages increased the gene expression of hypoxia-inducible factor 1-alpha (*Hif1a)*, integrin subunit alpha L (*Itgal)*, cluster of differentiation 86 (*Cd86)*, and others in RAW264.7 macrophages while the expression of arginase 1 (*Arg1)*, transferrin receptor 2 (*Trf2)*, and interferon regulatory factor 8 (*Irf8)* changed already after 15 passages (Taciak et al., 2018).

Moreover, transfection with DNA, which is possible in RAW264.7 cells, but induces cell death in primary macrophages, suggests that this cell line is not comparable to primary macrophages (Hornung et al., 2009; Roberts et al., 2009; Liu and Guan, 2018; Paludan et al., 2019). Notably, J774 cells, similar to primary macrophages but unlike RAW264.7 cells, experience cell death following transfection with plasmid DNA, whereas they do not when transfected with mRNA (Van De Parre et al., 2005).

Validating results from macrophage-like cell lines with studies on primary macrophages is important for characterizing macrophage functions. Moreover, conducting animal experiments could further elucidate the *in vivo* relevance compared with the *in vitro* results of a single cell type.

5.1.1 RAW264.7

The macrophage-like cell line RAW264.7 (Raschke et al., 1978) was isolated from BALB/c mice injected with the Abelson murine leukemia virus (A-MuLV), a replication-impaired virus carrying the v-abl tyrosine kinase oncogene (Hartley et al., 2008). When combined with a suitable type C helper virus, A-MuLV can transform embryo fibroblasts *in vitro* and trigger swift B cell lymphoid leukemia development *in vivo* (Twardzik et al., 1982). RAW264.7 cells are therefore an immortalized cancer cell line with macrophage-like capabilities (Taciak et al., 2018).

A-MuLV is a biosafety level 2 (BSL-2) agent (Feroz et al., 2021) posing a "moderate hazard" to lab personnel and the public,

requiring specific safety protocols (Ta et al., 2019). Although macrophage cell lines originated from A-MuLV-induced tumors, it's unclear if the A-MuLV genome contributed to cell transformation (Raschke et al., 1978). A-MuLV preparations often include a helper virus, but tests for replication-competent virus were negative when initially described (Raschke et al., 1978). RAW264.7 cells, as currently provided by the American Type Culture Collection (ATCC), express both ecotropic MuLV, which exhibits the biological characteristics of the Moloney isolate, and polytropic MuLV (Hartley et al., 2008). Polytropic viruses have a broad host range, allowing them to infect multiple species or various types of cell culture lines (Stoye and Coffin, 1987). The ecotropic virus, on the other hand, is characterized by its integration into cellular DNA and cell surface expression of A-MuLV antigens. Ecotropic refers to a virus with a limited host range, capable of infecting only one or a small number of species or cell culture lines. These findings, warrant caution in experimental design and data interpretation when using RAW264.7 cells (Hartley et al., 2008).

The RAW264.7 cell line is widely used to characterize macrophage phagocytosis (Kassas et al., 2012). The biggest advantage of RAW264.7 cells is that they can be transfected using various methods (electroporation, lipofection) and that they are also relatively easy to use for CRISPR-Cas9 lentiviral dropout screens. This facilitates mechanistic genetic unbiased screening studies (Smale, 2010; Cheung et al., 2015; Napier and Monack, 2017; Scinicariello et al., 2023). However, continuous passaging might even increase the accumulation of mutations and separate the cell line further from the definition of a primary macrophage (Stacey et al., 1993; Thompson et al., 1999; Cemma et al., 2015; Cheung et al., 2015; Paludan et al., 2019). Therefore, although this cell line serves as a practical tool for the initial screening of potentially important factors and functions in macrophages, it is important to consider continued passages of this cell lines could lead to gene depletion and compromise the immune functions of macrophages compared with primary macrophages and *in vivo* studies.

5.1.2 J774

Similar to the RAW264.7 cell line (Raschke et al., 1978) the macrophage-like cell line J774 originally was discovered during cancer research (Ralph and Nakoinz, 1975). The J774 cell line is derived through re-cloning from the primary ascites and solid tumor J774.1 cell line. J774.1 cells were firstly described as cells derived from a murine reticulum sarcoma that exhibited macrophage-like morphology, presence of immunoglobulin receptors, phagocytic capacity and antibody mediated lysis of target cells (Ralph and Nakoinz, 1975). Notably, these original studies never claimed to have isolated macrophages but only described cells that display some typical macrophage properties (Ralph and Nakoinz, 1975; 1977). Nevertheless, similar to the RAW264.7 cell line, J774 cells were further characterized in terms of motility, phagocytosis, and antibacterial activity (Portnoy et al., 1988; Kant et al., 2002; Lam et al., 2009; Kresinsky et al., 2016). Notably, J774 cells, unlike RAW264.7 cells but similar to primary macrophages, undergo cell death after transfection with plasmid DNA, but not with mRNA (Van De Parre et al., 2005).

5.2 Primary mouse macrophages

The cultivation and utilization of primary macrophages offer several advantages over long-term cultured immortalized cell lines (Kaur and Dufour, 2012). They have a shorter lifespan than immortalized cell lines, leading to less accumulation of genetic mutations owing to long-term cultivation (He et al., 2023). Moreover, the origin of the primary macrophages may influence the results (Sreejit et al., 2020). It is of utmost importance to understand which primary macrophages are used in *in vitro* studies, as they differ due to differences in cultivation protocols and cultivation conditions.

5.2.1 Peritoneal macrophages

Peritoneal macrophages (PMs) are a common *in vitro* model used to investigate tissue-resident macrophage response (Liu et al., 2018). Analysis of peritoneal macrophages provides insights into general macrophage biology and *ex vivo* behavior in response to various stimuli and disease models (Cassado Ados et al., 2015).

While working with the PMs, the isolation protocol should be considered. PMs can be obtained as unstimulated resident cells. The cells are isolated through peritoneal lavage (Ray and Dittel, 2010) and represent one of the largest sources of naïve, resident tissue macrophages (Yang et al., 2012b; Cassado Ados et al., 2015; Davies and Taylor, 2015; Davies et al., 2017; Gluschko et al., 2018). However, sorting with a macrophage-specific marker of choice (e.g., CD11b or F4/80) is recommended as the peritoneal lavage contains all cells present in the peritoneal cavity, mainly B cells and to a very small percentage also monocytes and neutrophils (Ray and Dittel, 2010; Gluschko et al., 2018). Relying solely on culture well adherence as the "sorting" step for macrophage purity may not be sufficient to achieve a completely pure peritoneal macrophage culture (Ponte-Sucre et al., 2007; Johnston et al., 2017), which is why authors suggest using the term peritoneal exudate cells for cells that have not been further purified (Johnston et al., 2017).

In this context, however, it must also be mentioned that a further flow cytometric analysis can influence the macrophage biology. Although a higher purity is achieved through purification, this in turn comes at the cost of influencing the biology of the cells. Moreover, extracting naïve peritoneal macrophages from mice has a significant drawback, as only a limited number of cells ($1x10^6$ cells per mouse) are obtained, and only around 40% - 50% are macrophages (Ray and Dittel, 2010; Rios et al., 2017). Therefore, a greater number of mice must be sacrificed to obtain an adequate number of cells for experimental procedures (Zhang et al., 2008).

Consequently, to augment macrophage production, a sterile eliciting agent (such as thioglycollate) can be administered into the peritoneal cavity prior to cell collection (Misharin et al., 2012; Layoun et al., 2015). Thioglycollate consists of an infusion from beef, proteose peptone, sodium chloride, dextrose, sodium thioglycolate, bacto agar, methylene blue (Brewer, 1940; Schleicher and Bogdan, 2009). Composition of thioglycollate was shown to determine killing capacity of intracellular pathogens

compared to naïve PMs. Reduction of agar and methylene blue in the eliciting agent increased killing capacity (Leijh et al., 1984).

Following thioglycollate administration, the macrophage yield per mouse increased by 10-fold (Layoun et al., 2015). Research has indicated that Brewer's thioglycollate elicits significant recruitment of macrophages and leads to increased levels of activation compared to naïve PMs (Pavlou et al., 2017). Despite the increased macrophage yield, Brewer's thioglycollate medium serves as an irritant, triggering an inflammatory response that leads to a swift decrease in overall resident monocyte/macrophage counts. Subsequently, there is an increase of peripheral inflammatory monocytes/macrophages, accompanied by a gradual restoration of resident macrophages (Davies et al., 2011, 2013b). This recruitment may or may not affect gene expression (Layoun et al., 2015). Furthermore, they exhibited an increase in lysosomal enzyme activity (Dy et al., 1978) and in phagocytic uptake (Pavlou et al., 2017).

Two studies have identified multiple leukocyte populations present in both the naïve and thioglycollate-elicited peritoneal cavities (Schleicher et al., 2005; Ghosn et al., 2010). However, maintaining high purity of macrophage culture is crucial for *in vitro* experiments. While freshly isolated thioglycollate-elicited peritoneal cells reportedly contain a high percentage of macrophages (86–95%) (Vodovotz et al., 1993; Schindler et al., 2001), their purity increases to nearly 99% through adherence (Zhang et al., 2008; Schleicher and Bogdan, 2009). It is worth noting that assessing macrophage percentage solely based on antigens like CD11b or F4/80, previously considered "macrophage specific", might be misleading. This is because other myeloid cells such as neutrophils, eosinophils, and dendritic cells also express these antigens. Including markers against dendritic cells (CD11c), eosinophils (Siglec-F) and neutrophils (Ly6G) (Zhang et al., 2004; Connelly et al., 2022; Pfeifhofer-Obermair et al., 2022) might be useful because even minor levels of contaminating cells could impact the results of *in vitro* assays, leading to data misinterpretation (Schleicher et al., 2005; Martinez-Pomares and Gordon, 2008).

A separate study revealed that the proportion of macrophages from the peritoneal cavity was lower than previously reported (Schleicher et al., 2005; Ghosn et al., 2010), with a significant presence of eosinophils (Misharin et al., 2012). Furthermore, there are reports on eosinophil contamination in adherent cultures of peritoneal macrophages (Ruiz-Alcaraz et al., 2020). The contaminating cells affect the functional readouts of standard assays performed on macrophages (Tateyama et al., 2019). Therefore, eosinophils can hinder accurate interpretation of findings from *in vitro* studies using cultured thioglycollate-elicited peritoneal macrophages (Misharin et al., 2012; Tateyama et al., 2019). Eosinophils exhibit several cell surface markers, such as CD45 and CD11b, which are commonly found on other hematopoietic cells typically present in inflammatory sites, such as alveolar macrophages and neutrophils (Stevens et al., 2007). Siglec-F, a member of the sialic acid binding immunoglobulin-like lectin (Siglec) family, has been identified on murine eosinophils' surface (Stevens et al., 2007). Siglec-F is predominantly expressed solely on eosinophils in blood and their precursors in the bone marrow of mice (Zhang et al., 2004). In addition to eosinophils, contamination of natural killer cells from peritoneal exudate cells can markedly influence conclusions regarding macrophage regulatory circuits (Misharin et al., 2012). To exclude the possible influence of NK cells in analyses, peritoneal macrophages, for example, can be induced in recombination activating gene 2 (RAG2)-gamma chain knock-out mice (Schleicher et al., 2005). To minimize the influence of eosinophils, double GATA-site (ΔdblGATA) knock-out mice could be used, for example, in which the development of eosinophils is severely impaired (Yu et al., 2002).

Another important point to consider is the changes in the metabolic activity of macrophages due to differences in the isolation protocols. Resident peritoneal macrophages in their naïve state exhibited lower metabolic activity than the elicited macrophages (Pavlou et al., 2017). Elicited macrophages display elevated levels of glycolysis and oxidative phosphorylation, potentially correlated with their enhanced phagocytic capacity and heightened maturation and activation levels (Pavlou et al., 2017). Gaining deeper insights into the molecular connections between metabolic pathways and cellular function is essential for devising strategies to regulate macrophage function through metabolic reprogramming.

5.2.2 Bone marrow-derived macrophages

Compared to PM, BMDM are not a model for tissue-resident macrophages, as they are differentiated *in vitro* (Fejer et al., 2015). They are generated by flushing myeloid progenitor cells from the bone marrow of the hind legs of mice and stimulating with either recombinant M-CSF or L-929-cell-conditioned media (L929, source of M-CSF) to gain differentiated macrophages. M-CSF induces proliferation and differentiation of the progenitor cells into BMDM via M-CSF receptor-mediated signaling (Becker et al., 1987; Brugger et al., 1991; Klappacher et al., 2002; Stanley and Chitu, 2014; Ushach and Zlotnik, 2016).

The main advantage of using BMDM as a macrophage model is the amount of cells that are generated after isolation and differentiation (4-6 x 10^6 cells per mouse) (Brigo et al., 2022). Differences in culture conditions (medium type, amount of M-CSF, and L929 usage) led to high variability in BMDM generation. However, clear description of the methodology is necessary for a high reproducibility of the results (Murray et al., 2014). In numerous research facilities, the L929 supernatant is favored over the use of recombinant M-CSF due to its cost-effectiveness and ability to produce significantly greater quantities of differentiated macrophages, as fewer animals need to be euthanized (Boltz-Nitulescu et al., 1987; Heap et al., 2021).

Before the availability of recombinant M-CSF, the traditional method, which remains prevalent in many laboratories (Yang et al., 2012b; Bringmann et al., 2013; Johnston et al., 2017; Koster et al., 2017; Nazir et al., 2017; Abuaita et al., 2018; Javmen et al., 2018; McTiernan et al., 2020), involved differentiating BMDM using the supernatant of the L929 immortalized fibroblast cell line, in which M-CSF and a variety of other factors are provided to the developing BMDM precursor (Tomida et al., 1984; Burgess et al., 1985; Portnoy et al., 1988; Pang et al., 2001). While certain strains of mouse L929 cells have the capability to generate significant levels of M-CSF

(Rice et al., 2020), the composition of L929 supernatant can exhibit variability between batches, potentially leading to inconsistencies in experimental results (Warren and Vogel, 1985).

Despite exposure to other substances in the L929 supernatant (Trouplin et al., 2013), L929-derived macrophages exhibit similar phagocytic and pathogen eliminating abilities to M-CSF-derived macrophages (Rice et al., 2020). However, L929-derived macrophages showed distinct cytokine secretion patterns, with lower inflammatory cytokine levels and higher IL-10 secretion (de Brito Monteiro et al., 2020; Heap et al., 2021). They also display heightened metabolic activity and increased accumulation of dysfunctional mitochondria compared with M-CSF-derived macrophages. Although differences in metabolism and cytokine secretion exist, both types of macrophages demonstrate comparable microbicidal effectiveness (de Brito Monteiro et al., 2020).

Using mass spectrometry, the examination of L929 supernatant revealed 2,193 proteins, including notable amounts of M-CSF and other immune-regulating proteins like migration inhibitory factor (MIF), osteopontin, and chemokines such as CC-chemokine ligand (CCL) 2 and CCL 7. When differentiated with L929, macrophages showed a more robust anti-inflammatory M2-like phenotype compared to differentiation with M-CSF. Moreover, macrophages grown in L929 supernatant exhibited reduced responses to oxidative stress, as well as decreased activity in cell division and mitotic machinery (Heap et al., 2021).

In summary, these findings indicate that the choice of differentiation agent influences BMDM phenotypes and proteomes, leading to variations in biological functions. Consequently, it is crucial to recognize the biological implications of different BMDM differentiation methods and the resulting *in vitro* outcomes. Conversely, employing defined concentrations of M-CSF may alleviate experimental variability and improve the standardization of laboratory methodologies (Murray et al., 2014).

5.3 Cell line generation from mouse bone marrow-derived macrophages

Several methods for immortalizing mouse BMDM for generation of macrophage cell lines have been described; however, the low rates of transduction and transfection observed in macrophages pose challenges to these procedures (Zhang et al., 2009; Alamuru-Yellapragada et al., 2017; Warwick and Usachev, 2017; Poltavets et al., 2020). Some potential approaches for immortalizing primary BMDM are outlined in the next section of this review. However, one must carefully consider the advantages and disadvantages of these methods.

Genetic modulation of primary macrophages can lead to changes in their phenotype compared to the initial cell population (Xie et al., 2024). Nevertheless, immortalizing BMDM can lead to a reduction in animal usage (Spera et al., 2021). BMDM from genetically modified mice are frequently employed to analyze mechanisms of the immune system and having a reservoir of these immortalized BMDM readily available facilities the conduction of *in vitro* studies and reduces reliance on live animals (De Nardo et al., 2018; Spera et al., 2021).

5.3.1 Overexpressing oncogenes or a transcription factor in bone marrow-derived macrophages

A method for immortalizing macrophage populations from particular mouse strains by utilizing the Cre-J2 retroviral infection technique (De Nardo et al., 2018). This method of immortalization involves infecting cells with the J2 recombinant retrovirus, which is derived from a replication-defective 3611-Moloney sarcoma virus (MSV) and contains the v-raf and v-myc oncogenes (Spera et al., 2021).

The J2 virus itself lacks the essential viral packaging proteins (gag-pol, env), rendering it replication-deficient. Therefore, a viral packaging helper cell line called Psi-Cre-J2 (derived from NIH 3T3 fibroblasts) is utilized to generate recombinant Cre-J2 retrovirus. The Cre-J2 retroviral infection technique for immortalization has proven effective on various murine macrophage populations, such as those derived from bone marrow, fetal liver, spleen, and microglia (Blasi et al., 1985; Roberson and Walker, 1988; Cox et al., 1989; Blasi et al., 1990).

A commercially available immortalized murine macrophage cell line, designated as BM A3.1A7, originates from adherent macrophages extracted from the bone marrow of adult female C57BL/6 mice. These cells have been rendered immortal by the introduction of elevated levels of raf and myc oncogenes (Kovacsovics-Bankowski and Rock, 1994).

Currently only one study analyzed the immortalized murine macrophage cell line, BM A3.1A7 for their macrophage capabilities. They demonstrated that BM A3.1A7 polarize into either M1-like macrophages, identified by their secretion of inflammatory cytokines such as IL-1β, IL-6, IL-12, and tumor necrosis factor (TNF), along with increased expression of inducible nitric oxide (NO) synthase (iNOS), or M2-like macrophages characterized by their typical elevated ARG1 activity (Banete et al., 2015). Further studies and comparisons with other primary macrophages or other relevant models are needed to better assess the value of the model.

Moreover, hematopoietic precursors are immortalized by retroviral transduction with an estrogen-inducible form of the transcription factor homeobox B8 (Hoxb8) (Wang et al., 2006). Hoxb8 promotes self-renewal and halts differentiation (Leithner et al., 2018). In the presence of elevated levels of the hormone β-estradiol that exceed the physiological range, Hoxb8 is transcriptionally active. By removing β-estradiol, Hoxb8 is inactivated, leading to differentiation of the immortalized progenitor cells depending on the applied cytokine cocktail for the desired myeloid subset (Wang et al., 2006; Odegaard et al., 2007). Therefore, these cells can be grown in cell culture for weeks, providing them with sufficient time to be genetically modified, while still remaining capable of maturing into DCs, macrophages, or granulocytes (Redecke et al., 2013). Macrophages derived from the Hoxb8 lineages exhibited comparable phenotypic and functional attributes and similarly elevated the expression of activation-related genes when stimulated with LPS, as observed in primary macrophages (Wang et al., 2006; Odegaard et al., 2007).

Advantages of these cells are that they can be readily produced from the bone marrow or fetal liver of any mouse strain (Hammerschmidt et al., 2018). Additionally, Hoxb8 cells can be

efficiently modified using viral transduction and CRISPR-mediated genome editing (Di Ceglie et al., 2017; Hammerschmidt et al., 2018). They are a convenient tool for protein overexpression or knockdown experiments (Bromberger et al., 2022). One disadvantage of these cells is the heterogeneity, as the initial bone marrow population can be diverse, potentially impacting the uniformity of the resulting cells (Yu and Scadden, 2016).

5.3.2 Cas9+-immortalized macrophages

Genetic manipulation of macrophages is in general cumbersome (Zhang et al., 2009). There are for instance protocols for RNAi delivery into macrophages (Siegert et al., 2014) or retroviral transduction of bone marrow progenitors (De Veerman et al., 1999). The discovery of the RNA-guided endonuclease Cas9 and its use as gene scissors ('genome editing') has opened new technical possibilities to search genome-wide with CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 for regulators of biological processes starting from the phenotype with so-called 'forward genetic screens' (Joung et al., 2017). Introducing Cas9 protein into cells poses a difficulty within the CRISPR-Cas9 process (Liang et al., 2015). To overcome this challenge Cas9 knock-in mice facilitate the production of various knock-outs in immortalised cells, which was already shown in immortalised DCs of Cas9 expressing mice (Parnas et al., 2015).

As previously mentioned, hematopoietic precursors immortalized by retroviral transduction with Hoxb8 can be cultured for weeks, allowing genetic modifications and maturation into DCs, macrophages, or granulocytes (Wang et al., 2006; Redecke et al., 2013), as Hoxb8 expression facilitates self-renewal and halts differentiation (Leithner et al., 2018). To overcome the inducibility of the Hoxb8 overexpression via removal of β-estradiol, bone marrow of the Cas9-transgenic mice was immortalized by lentiviral transduction, introducing a doxycycline-regulated version of the transcription factor Hoxb8. These cells can be cultured consistently for weeks when doxycycline and puromycin are added. Moreover, these cells facilitate CRISPR/Cas9 technology, as the Cas9 gene is expressed in the cells (Hammerschmidt et al., 2018).

A recent study focused on the notable differences and constraints observed between primary macrophages and immortalized cell lines by creating a new immortalized macrophage cell lines using the CRISP/Cas9 technique (Roberts et al., 2019). Roberts and colleagues introduced ER-HoxB8 by retroviral transduction into hematopoietic stem cells from Cas9 expressing mice, and performed a comprehensive comparison between Cas9+-immortalized macrophages (CIMs) and BMDM, the primary macrophage type. Through a series of meticulously designed experiments, they examined various macrophage functions, including iNOS expression, NO generation, bacterial phagocytosis, and antibacterial activity. CIMs were similar to BMDM, the only significant difference was that killing of *Listeria monocytogenes* was enhanced, which they hypothesize is a result of improved early elimination through the phago-lysosomal pathway. The growth rate of *M. tuberculosis* was similar in CIMs to that observed in BMDM. CIMs and BMMs showed increased expression of iNOS and produced comparable levels of NO when stimulated with the combination of LPS and interferon-γ (Roberts et al., 2019).

6 Immortalized versus primary mouse macrophages: advantages and disadvantages of *in vitro* models

As previously mentioned, numerous studies concentrate exclusively on the primary role of macrophages, which is phagocytosis. Nevertheless, macrophages are not merely scavengers; they respond to diverse environmental cues by generating complex reactions, such as generating reactive oxygen and nitrogen species (ROS and RNS) as well as pro- or anti-inflammatory cytokines and chemokines, which depend on their polarization (Arango Duque and Descoteaux, 2014; Mosser et al., 2021).

It is becoming evident that macrophage-like cell lines differ from primary macrophages, while primary macrophages vary based on isolation and cultivation methods. The subsequent section of this review will illustrate these distinctions.

6.1 Differences between naïve PM and BMDM

Primary mouse macrophages, including BMDM and PM, are commonly employed *in vitro* to investigate various mechanisms such as infection control (Schatz et al., 2016; Brigo et al., 2021), phagocytosis (Herb et al., 2018; Gluschko et al., 2022), and cytokine production (Sasse et al., 2022; Brigo et al., 2023). Interestingly, these two macrophage types exhibit distinct functional differences.

Macrophages in various anatomical locations may exhibit diverse degrees of heterogeneity (Geissmann et al., 2010; Gautier et al., 2012; Gordon et al., 2014; Komohara et al., 2014; Hume et al., 2023). Employing flow cytometry, the observations revealed that BMDM formed tight clusters in both forward scatter and side scatter data, contrasting with naïve PMs, which appeared dispersed among at least two distinct populations (Zajd et al., 2020). These findings suggest that PMs display notable heterogeneity. Conversely, BMDM exhibited a higher degree of uniformity, likely attributable to their induction by M-CSF (Zhao et al., 2017). Moreover, PMs exhibit larger cell sizes compared to BMDM due to containing more cytoplasm and increased lysosomal content (Wang et al., 2013). In addition, proliferation is enhanced in BMDM, cell number increased from day 4 and continued to rise until day 14, reaching a 60-fold increase over baseline compared to PMs, which showed no proliferation during the 14-day culture period (Wang et al., 2013).

BMDM, unlike PM, fail to activate an antimicrobial phagocytosis variant known as LC3-associated Phagocytosis (LAP) (Heckmann and Green, 2019; Herb et al., 2020). Not only do BMDM fail to induce LAP during bacterial infection, but they also exhibit significantly reduced capacities in phagosomal ROS production and intracellular bacteria killing (Gluschko et al., 2022).

ROS production via the NADPH oxidase (Nox) 2 is crucial for LAP induction (Martinez et al., 2015; Koster et al., 2017; Gluschko et al., 2018; Herb et al., 2020). Our investigations have revealed markedly lower protein levels of several Nox2 subunits in BMDM compared to PM, elucidating the pronounced reduction in ROS production in BMDM (Gluschko et al., 2022). Additionally, upstream components essential for Nox2-derived ROS production, namely the integrin Mac-1 and the sphingomyelinase ASMase, are also significantly reduced on the protein level in BMDM compared to PM (Gluschko et al., 2018).

Furthermore, time of cytokine secretion was slower in BMDM (24 hours post-infection) compared to PM (5 hours post-infection) (Herb et al., 2019). Bisgaard et al. conducted a comparative study on the behavior of PM and BMDM in an *in vitro* atherosclerosis model, revealing dramatic differences in chemokine and cytokine expression following cholesterol treatment, with PM exhibiting a tendency towards an M1-like phenotype and BMDM towards an M2-like phenotype (Bisgaard et al., 2016). Wang et al. showed that BMDM exhibited the highest phagocytic ability compared to PMs (Wang et al., 2013). Interestingly, phagocytosis efficiency diminishes in PMs of old mice compared to the younger counterpart. On the other hand, there were no discernible age-related impairments in phagocytosis observed in BMDM, indicating the absence of intrinsic defects within these cell populations (Linehan et al., 2014).

6.2 Differences between elicited PM and BMDM

Several studies have investigated the differences between thioglycollate-elicited PM (TG-PM) and BMDM. Weber and Schilling demonstrated that lysosomes isolated from TG-PM exhibited enhanced lysosomal numbers, more active cathepsin B, and higher levels of pro-cathepsin D and Lysosomal-associated membrane protein 1 (LAMP1) compared to lysosomes from BMDM (Weber and Schilling, 2014). In comparison to the study of Bisgaard et al. (Bisgaard et al., 2016) a recent study by Zajd and colleagues revealed that TG-PM displayed M2 surface markers, while BMDM exhibited M1 surface markers (Zajd et al., 2020). Furthermore, TG-PM showed reduced responsiveness, including decreased phagocytic capacity, weaker response to additional polarization stimuli, and diminished secretion of cytokines and chemokines (Zajd et al., 2020). However, both studies miss a comparison of TG-PM and BMDM to non-elicited naïve PM. This comparison is crucial as the injection of thioglycollate induces sterile inflammation, significantly altering naïve peritoneal macrophages before isolation and the start of experiments (Weber and Schilling, 2014; Zajd et al., 2020).

The study by Zajd et al. (Zajd et al., 2020) showed that both types are morphologically similar in flow cytometry. BMDM, however, exhibit higher phagocytic activity and upregulate chemokine and cytokine expression more robustly in response to polarization. This heightened responsiveness of BMDM reflects intrinsic differences, while TG-PM responses are influenced by their differentiation within the whole animal context. BMDM express higher levels of inflammatory markers such as Ly6C and CD64 and show increased expression of pattern recognition receptors like TLR2 and TLR4 compared to TG-PM. However, their modest expression of MHCII suggests M1-like skewing rather than full activation (Zajd et al., 2020).

Despite the observed differences, these studies (Weber and Schilling, 2014; Bisgaard et al., 2016; Gluschko et al., 2018; Herb et al., 2019; Zajd et al., 2020) suggest that naïve peritoneal macrophages are the preferred tissue-resident model due to their more natural state (Chen et al., 2021c). However, BMDM differentiated with M-CSF demonstrate enhanced sensitivity to polarizing cytokines and improved phagocytic abilities compared to PMs. Consequently, they serve as a favorable model for investigating macrophage plasticity and immune responses to infections (Zajd et al., 2020).

They represent a valuable alternative, particularly in situations where access to a large pool of animals for isolation purposes is limited (Zhang et al., 2008; Layoun et al., 2015). However, the use of TG-PM, with their highly altered phenotype and reduced responsiveness, warrants caution, even though obtaining higher cell numbers for experimental procedures is very tempting.

6.3 Differences between PM and immortalized macrophage cell lines

Comparison of J774 to PM revealed that PM store substantial amounts of cholesterol ester from unmodified low-density lipoprotein (LDL) in contrast to J774 cells (Tabas and Boykow, 1987). Based on these findings, J774 macrophages exhibit a significantly more active acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT) cholesterol esterification pathway in the presence of LDL compared to primary PM. Additionally, there's a notable distinction in the stimulation of ACAT between acetyl-LDL and LDL in mouse PM, even when the lipoproteins are matched for degradation (Tabas et al., 1987).

Infection control to *Cryptococcus neoformans* var. *grubii* strain H99 (serotype A) was different in J774 compared to primary PMs. The J774 macrophage-like cell line demonstrated a tendency to activate caspase-1 and caspase-3 throughout the infection process. Subsequently, primary PMs were examined, revealing activation of caspase-3. Despite increased ROS production, fungal control was diminished in PM compared to J774 cells (Coelho et al., 2015).

Moreover, PMs displayed higher basal NF-κB levels compared to RAW264.7 cells and exhibited faster NF-κB nuclear translocation kinetics upon low-dose LPS activation. Notably, primary mouse PM showed particularly rapid NF-κB translocation kinetics compared to immortalized cell lines (Bagaev et al., 2019).

6.4 Differences between BMDM and immortalized macrophage cell lines

Differences between primary macrophage types primarily arise from variations in isolation and cultivation protocols (Murray et al., 2014). However, macrophage-like cell lines exhibit significant

discrepancies compared to primary macrophages due to their cancerous origin (Ralph and Nakoinz, 1975; Raschke et al., 1978; Tsuchiya et al., 1980).

While some reports found no differences between RAW264.7 cells and BMDM in basic parameters such as antibacterial and antiparasitic activity (Cemma et al., 2015; Frank et al., 2015), other studies highlighted remarkable distinctions. For instance, a study investigating the regulatory role of FAS-associated factor 1 (FAF1) on ROS production (Kim et al., 2019) showed that RAW cells produced significantly less IL-6 and IL-12 but more NO compared to BMDM after infection with *Listeria monocytogenes* (*L.m*). Total cellular ROS production was equal in both cell types (Kim et al., 2019).

Huang et al. detected higher levels of septins in BMDM compared to RAW cells, indicating their importance in phagosome formation (Huang et al., 2008). Furthermore, in another study, a comprehensive proteomics analysis of phagosomes from RAW264.7 and BMDM was conducted (Guo et al., 2015). Over 2,500 phagosomal proteins were quantified, and significant differences in important receptors like mannose receptor 1 and Siglec-1 were analyzed. Additionally, Guo et al. observed that phagosomes in BMDM undergo more rapid maturation through fusion with endosomes and lysosomes, a phenomenon confirmed through fluorogenic phagocytic assays, compared to the immortalized macrophage cell line (Guo et al., 2015). Analysis of the phagosomal proteome of both cell types revealed that 58 proteins were unique for BMDM and 17 were unique for RAW phagosomes, with immune-relevant proteins (TLR3, TLR9, complement receptors, mannose receptor 1 and several integrins and galectins) more abundant in BMDM phagosomes (Guo et al., 2015). This discrepancy between RAW cells and tissue macrophages becomes more evident considering the decreased integrin protein levels in BMDM compared to peritoneal macrophages (Gluschko et al., 2018).

Interestingly, BMDM and RAW cells exhibited comparable phenotypes, characterized by elevated levels of CD11b and F4/80 expression (Berghaus et al., 2010). However, although both populations shared similarities, their phenotypes were not identical; RAW cells displayed notably higher CD14 expression emphasizing their distinct characteristics. Furthermore, both BMDM and RAW cells demonstrated similar responses to TLR 3 stimulation with poly I:C, indicating their shared activation pathway in the monocyte-macrophage differentiation process. This similarity in responsiveness to various stimuli suggests a common functional point in their differentiation (Berghaus et al., 2010).

Additionally, J774 cells showed no antilisterial activity in comparison to BMDM (Portnoy et al., 1988). Andreu et al. observed an increased infection control to *Mycobacterium tuberculosis* in BMDM compared to J774 cells (Andreu et al., 2017). Moreover, infection control with BMDM is faster and stronger (Andreu et al., 2017). The J774 macrophage-like cell line tended to activate caspase-1 and caspase-3 during *C. neoformans* infection, whereas BMDM activated caspase-1, -3, and -8. Protein expression analysis showed upregulation of receptor-interacting protein (RIP) and apoptosis-inducing factor (AIF) in J774 cells early in infection, while BMDM activated AIF and released cytochrome c (Coelho et al., 2015). Additionally, J774 cells displayed increased LDH levels, indicating necrotic cell death, unlike BMDM, which exhibited classical apoptotic features (Coelho et al., 2015).

7 Additional variables that may impact the functionality of macrophages

The origin of macrophages (Gordon et al., 2014; Komohara et al., 2014), duration of culture (Chamberlain et al., 2015), characteristics of biomaterial surfaces (Jones et al., 2007), culture media (Kawakami et al., 2016, 2017) and addition of supplements to the culture conditions (Rinsky et al., 2017; Antonsen et al., 2023) collectively influence the phenotype of macrophages in culture.

7.1 Effects of fetal bovine serum and cell culture media on macrophage culture

Addition of fetal bovine serum (FBS) or fetal calf serum (FCS) to the cell culture medium serves as a vital supplement leading to facilitated cell growth and proliferation (Karnieli et al., 2017). Since the 1950 addition of FBS/FCS into cell culture media is a standard procedure (Puck et al., 1958). FBS consists out of crucial elements necessary for cell growth and upkeep, such as hormones, vitamins, transport proteins, trace elements, and growth factors (Fang et al., 2017). FBS, which is a by-product of cattle husbandry, is acquired from the blood of a bovine fetus during the slaughtering of a pregnant cow (Fang et al., 2017). Although various serum-free medium formulations exist for mammalian and insect cell lines as well as primary cultures, transitioning to serum-free media requires extensive literature review and manufacturer searches for suitable formulations (Brunner et al., 2010; Fang et al., 2017).

FBS is a complex and variable mixture that can contain contaminants and varies significantly due to geographical, seasonal, and environmental factors, contributing to lot to lot differences (Fang et al., 2017).

In a study, various commercially available FBS were analyzed for their effect on epithelial cells. The findings revealed that various FBS samples markedly stimulated IL-8 secretion in the cells, while they did not elicit secretion of TNF and IL-1β. Conversely, some FBS samples had no impact on the secretion of IL-8, TNF, and IL-1β (Liu et al., 2023b).

Moreover, exosomes found in FBS, influenced primary macrophages from Fisher 344 rats, when cultured with LPS. The macrophages demonstrate a dose-dependent decrease in IL-1 compared to macrophages cultured in medium supplemented with exosome-depleted FBS. Furthermore, the inclusion of fetal bovine exosomes also led to reductions in macrophage TNF-α and IL-6 levels (Beninson and Fleshner, 2015).

As exosomes have the potential of being a reliable biomarker as they are stable in body fluids, reflect the physiological state of their parent cells, and facilitate intercellular communication through the transfer of biomolecules (Zhou et al., 2020; Mathew et al., 2021).

The high levels of serum proteins, including exosomes derived from bovine cells, can potentially contaminate exosome samples, leading to significant impurities and artifacts in the yields (Biadglegne et al., 2021). Therefore, a serum free approach is necessary. Abramowicz et al. showed that the presence of high levels of serum proteins contaminating exosomes can cause significant issues in the harvest, isolation, and processing of exosomes (Abramowicz et al., 2018).

Another interesting point to consider is hemolysis. Hemolysis can greatly affect FBS production by releasing free hemoglobin and other intracellular contents into the serum, which can alter its composition and impact its performance and consistency in cell culture applications (Bowen et al., 2010; Arigony et al., 2013; Shah et al., 2016; Chelladurai et al., 2021).

Moreover, trace amounts of endotoxin (lipopolysaccharide: LPS) are believed to contaminate commercially available FBS (Kirikae et al., 1997). Beninson et al. described that endotoxin contamination affects cultured cells by inducing the production of various active mediators, such as TNF, leading to diverse cellular responses (Kirikae et al., 1997). Interestingly, tolerance to endotoxins is a well-known characteristic of macrophages, resulting in a modified macrophage response (Butcher et al., 2018). The innate immune response to infection or injury is shifted from a pro-inflammatory to an anti-inflammatory (Vergadi et al., 2018). Following an initial exposure to LPS in monocytes/macrophages a temporary state of "endotoxin tolerance", characterized by reduced responsiveness to LPS, is observed (Rajaiah et al., 2013).

In animal models, endotoxin tolerance has two phases: an early phase with altered cellular activation and a late phase involving the development of specific antibodies against the polysaccharide side chain of Gram-negative organisms (West and Heagy, 2002). The physiological role of tolerance is to protect host tissue from damage caused by prolonged production of pro-inflammatory cytokines (Rogovskii, 2020). While mostly reversible, LPS tolerance creates a hybrid macrophage activation state that is primarily pro-inflammatory but includes distinct anti-inflammatory regulatory features (Butcher et al., 2018).

Not only FBS may be contaminated by endotoxins, but culture media are also a potential source. Currently, most commercially prepared media are tested for endotoxin and certified to contain less than 0.1 ng/mL. However, reagents added to the medium can also introduce endotoxins (Ryan, 2004). Dumoulin et al. tested five different batches of commercially prepared bovine serum albumin and found endotoxin levels ranging from 0.1 to 12 ng/mL (Dumoulin et al., 1991). Additionally, another study found that some media additives, such as erythropoietin, contained endotoxin levels as high as 50 ng/mL (Case-Gould, 1984). Thus, endotoxin testing is crucial concerning cell culture experiments to ensure the reliability and validity of experimental results (Nomura et al., 2017; Molenaar-de Backer et al., 2021).

Moreover, usage of different cell culture media can affect the phenotype of macrophages. Kawakami et al. presented novel evidence that J774 exhibits varied activated macrophage phenotypes in response to LPS and/or interferon-gamma (IFN-γ) stimulation when cultured in either Ham's F-12 medium (F-12) or Dulbecco's modified Eagle medium (DMEM). Specifically, the production of NO and certain cytokines was notably higher in DMEM compared to F-12 during macrophage activation (Kawakami et al., 2016).

7.2 Influence of biomaterial surfaces on macrophage culture

Macrophages can be distinguished from other cell types based on their capacity to efficiently adhere and proliferate on both glass and plastic surfaces, therefore, the surface of the culture dish is key (Fleit et al., 1984). Normal tissue cells typically do not survive when suspended in a fluid, thus they are considered to be anchorage dependent (Ruoslahti and Pierschbacher, 1987; Discher et al., 2005). These cells must adhere to a solid, which can range from rigid glass to a surface softer than baby skin. The way some cells behave on soft materials is crucial for identifying important phenotypes (Merten, 2015; Discher et al., 2017).

Epithelial cells and fibroblasts were the first to be reported as detecting and responding differently to soft versus stiff substrates (Pelham and Wang, 1997; Deroanne et al., 2001). Although the molecular pathways are still not fully understood, muscle cells, neurons, and various other tissue cells have since been shown to sense substrate stiffness (Wang et al., 2000; Engler et al., 2004). The increasingly clear and affirmative answer to whether cells perceive and respond differently to the rigidity of conventional materials compared to more compliant tissues, gels, or sublayers of cells is significant for its impact not only on standard cell culture but also on understanding disease processes, morphogenesis, and tissue-repair strategies (Discher et al., 2005; Guo et al., 2006; Majhy et al., 2021).

Macrophage cytokine expression is contingent upon both the cell type and the culture surface (Chamberlain et al., 2015). In a study by Chamberlain et al. with primary macrophages or immortalized macrophage cell lines, the cells exhibit a distinct response due to three different solid surfaces of the culture dish. Cell lines demonstrate variability among themselves in terms of adherent morphology, proliferation, cytokine expression, and cell surface marker expression (Chamberlain et al., 2015).

Conversely, another study indicated that the surface chemistry of these four non-cytotoxic biomaterials had only a modest impact on cytokine production (Schutte et al., 2009). Whereas divergences were noted in the capacity of cells to adhere to and subsequently proliferate on polymer surfaces in murine monocyte-macrophages (RAW264.7 and J774), murine macrophage (IC-21) and murine fibroblast (NIH 3T3) cell lines (Godek et al., 2004).

The material surface chemistry influences the phenotypic expression of macrophages. A study found that macrophages on different surfaces showed varying cytokine/chemokine profiles, with hydrophilic/neutral surfaces resulting in fewer but more highly activated cells. Over time, a shift from proinflammatory to anti-inflammatory cytokine production was observed, indicating a resolution of the inflammatory response (Jones et al., 2007).

Collectively, signaling alterations, as well as functional changes in macrophages may be dependent on the culture dish surface, serum addition and cell culture media.

8 Other macrophage models

8.1 Immortalized human macrophage cell lines

8.1.1 THP-1

THP-1 cells were isolated from the peripheral blood of a one-year-old with acute monocyte leukemia (Tsuchiya et al., 1980). Similar to the RAW cell line, THP-1 monocyte-like cells are constantly proliferating and can accumulate several mutations during passaging (Noronha et al., 2020). Despite the fact, that these cells were originally mainly used for leukemia cancer research (Fabian et al., 2006; Yang et al., 2012a; Alves et al., 2018; Chernoryzh et al., 2019), they quickly adapted to a human monocyte/macrophage model cell line (Auwerx, 1991; Chanput et al., 2014; Bosshart and Heinzelmann, 2016; Zhang et al., 2019), which can be achieved by differentiation into macrophage-like cells with phorbol 12-myristate 13-acetate (PMA) (Koster et al., 2017; Sedlyarov et al., 2018) or human rM-CSF treatment (Muczynski et al., 2016). Furthermore, like BMDM, there is a lack of consistent differentiation protocol for THP-1 cells. In a study comparing conditions, researchers found that an optimal PMA concentration enables THP-1 cells to combat intracellular bacteria, while high concentrations lead to faster cell death. Lower concentrations support cell survival and effective defense against intracellular bacteria, like primary human macrophages (Aldo et al., 2013; Starr et al., 2018).

THP-1 cells offer several technical benefits compared to human primary monocytes or macrophages. One key advantage is their uniform genetic background, which reduces variability in cell phenotypes (Chanput et al., 2015). Like the RAW cell line, THP-1-derived macrophages can be easily transfected with plasmid DNA (Maess et al., 2011; Bosshart and Heinzelmann, 2016). Additionally, it is relatively straightforward to genetically modify THP-1 cells using small interfering RNAs (siRNAs) to downregulate specific protein expressions (Chanput et al., 2010).

Several publications have compared the responses of THP-1 monocytes with those of human PBMC-monocytes. The results are well summarized by Chanput et al. (Chanput et al., 2014). Differences have been observed in the levels of gene expression and cytokine secretion, as well as in the baseline gene expression (Chanput et al., 2014).

Thus, a limitation of using THP-1 cells is that their malignant background and cultivation under controlled conditions may result in different sensitivities and responses compared to PBMCs and human monocyte-derived macrophages (Schildberger et al., 2013; Hoppenbrouwers et al., 2022). For instance, compared to THP-1 cells, monocytes are significantly more responsive to LPS. This notable LPS responsiveness in human peripheral blood monocytes is primarily due to the high expression levels of CD14 (Bosshart and Heinzelmann, 2016). THP-1 cells express low levels of CD14, making them a poor model for studying LPS responses compared to primary monocytes (Bosshart and Heinzelmann, 2004). LPS concentrations that can trigger severe, life-threatening reactions in an *in vivo* system are non-toxic to THP-1 cells (Prajitha and Mohanan, 2021)

8.1.2 BLaER1 cell line

The human B-cell precursor leukemia cell line BLaER1 was derived from the transfection of the Burkitt Lymphoma Cell Line Seraphina, an acute lymphoblastic leukemia (ALL) cell line with the CCAAT/enhancer-binding-protein (C/EBPα), the estrogen receptor (ER) coupled to green fluorescent protein (GFP) (Gaidt et al., 2018). Following transfection, cells were sorted based on GFP expression, resulting in the generation of a single subclone (Rapino et al., 2017). The progenitor cell line was derived from the bone marrow of a female patient presenting with a chromosomal translocation t(1;19), trisomy 8 and ALL (Jack et al., 1986). Through tamoxifen or β-estradiol, the transcription factor C/EBPα is activated leading to the conversion of immature/mature B-cells into macrophage-like cells (Xie et al., 2004; Bussmann et al., 2009). BLaER1 cells had a transcriptome that started in a position close to that of peripheral blood B-cells before induction of C/EBPα and ended close to that of normal macrophages after 3-4 days of C/EBPα activation (Bussmann et al., 2009; Rapino et al., 2013). Interestingly, the frequency of induced lymphoid cells converting into macrophages is significantly greater than that observed in the reprogramming of somatic cells into induced pluripotent stem cells by transcription factors associated with embryonic stem cells (Laiosa et al., 2006; Takahashi and Yamanaka, 2006; Bussmann et al., 2009). Infection experiments with *Escherichia coli* and *Candida albicans* showed that the reprogrammed macrophages function as phagocytic cells (Bussmann et al., 2009; Rapino et al., 2013). The capacity of BLaER1 monocytes to support *Leishmania* parasite infection and subsequent activation is comparable to that of primary human macrophages (Volkmar et al., 2024). Moreover, cytokine response due to infection is comparable to M-CSF-derived macrophages and GM-CSF-derived macrophages (Volkmar et al., 2024).

An advantage of the BlaER1 cells is that the genetic modification can be achieved in the undifferentiated B-cell form through the utilization of established CRISPR-Cas9-based methodologies, conferring a distinct advantage over the limited capacity for genetic manipulation observed in other monocytic cells (Gaidt et al., 2016; Schussler et al., 2023; Volkmar et al., 2024).

The selection of a human cell model is a crucial decision that requires careful consideration. For instance, alternative NLRP3 activation has only been documented in BlaER1 cells, highlighting the importance of choosing an appropriate model (Zito et al., 2020). This species-specific NLRP3 inflammasome pathway was identified in human and porcine peripheral blood mononuclear cells, yet was absent from those of murine origin and the THP-1 cell line (Gaidt et al., 2016, 2018).

8.2 Primary human macrophages

8.2.1 Peripheral blood monocytes cells

Differentiated macrophages from human peripheral blood monocytes (PBMC) represent another type of commonly used human macrophage cells. In addition to routine blood collection,

a technique known as apheresis can be employed for the automated isolation of specific blood components. When isolating PBMCs, this automated process is referred to as leukapheresis (Liu et al., 2015).

Protocols to differentiate PBMC into macrophages with human rM-CSF (Stanley et al., 1976; Becker et al., 1987; Brugger et al., 1991; Takahashi et al., 1996; Erbel et al., 2013) or GM-CSF (Herbst et al., 2020) were described, but studies highly differ in their technical approach to date (Mehta et al., 1996; Pilling et al., 2017; Sedlyarov et al., 2018; De et al., 2020; Herbst et al., 2020; Nielsen et al., 2020; Braga et al., 2021). Diverse isolation methods may significantly affect the macrophage outcome, resulting in variations in yield, purity, viability, and cellular phenotype (Nielsen et al., 2020). For instance, leukapheresis yields higher amounts of cells than routine blood collection (Liu et al., 2015).

Factors occurring before or during blood sampling can affect PBMCs and consequent the results of the assay. For instance, induction of stress has been associated with inhibiting cytokine synthesis and the release of immunosuppressive cytokines (Elenkov and Chrousos, 2002).

Another factor impacting PBMC function is nutritional status. This influence, whether due to acute starvation from long-term overall malnutrition or specific nutrient deficiencies, can diminish the organism's immune response capacity to combat pathogens or respond to vaccination (Bourke et al., 2016).

Time of blood sampling is another factor. In humans and mice, the immune system, including lymphocyte movement between blood and tissues, follows circadian rhythms, leading to variations in immune cell counts throughout the day (Scheiermann et al., 2013). Moreover, before isolation, the time and temperature of blood shipping and storage can impact both the isolation process and subsequent assays. Besides reducing cell viability, granulocyte contamination is the primary cause of sample quality degradation over time (McKenna et al., 2009).

Thoughtful evaluation of monocyte isolation techniques is crucial when designing *in vitro* experiments involving PBMCs.

8.2.2 Immortalized pluripotent stem cell-derived macrophages

Induced pluripotent stem cells (iPSCs) provide the opportunity to create various disease-relevant cell types from any genetic background through the processes of cellular reprogramming and directed differentiation (Yamanaka and Blau, 2010; Yanagimachi et al., 2013). iPSCs are undifferentiated pluripotent cells that have the potential to be cultivated for an unlimited period of time (Takahashi et al., 2007; Yamanaka, 2007).

The five-step monocytic lineage differentiation protocol was published by Yanagimachi et al., 2013. This protocol differentiates mature macrophages from human iPSCs through the monocyte stage. In the last step CD14+ monocytic lineage-cells are cultured with M-CSF for one week for macrophage differentiation (Yanagimachi et al., 2013).

The iPSC-derived macrophages showed a notable capacity for phagocytosis of bacteria, although it was somewhat diminished in comparison to blood monocyte-derived macrophages. However, it was observed that the pro-inflammatory responses and transcriptomic profiles were comparable to blood monocyte-derived macrophages (Monkley et al., 2020). It has been suggested that a bias towards a more anti-inflammatory phenotype, perhaps more similar to tissue resident macrophages, may be one reason for the lower phagocytic activity of iPSC-derived macrophages (Buchrieser et al., 2017; Haideri et al., 2017).

The procurement of patient-derived tissue-resident macrophages represents a significant challenge, largely due to their inherent genetic variability and the technical difficulties associated with genetically modifying them (Gastman et al., 2020; Yang et al., 2024). iPSC can be produced from a patient with a specific genetic background and modified by multiple mechanisms, such as lentiviral transduction or CRISPR-Cas9 gene editing (Yamanaka, 2007; Buchrieser et al., 2017; Chehelgerdi et al., 2023). Moreover, the possibility of genetically modifying the original iPSC culture line in order to generate various specific genetic variants in large numbers may represent a valuable approach for large-scale studies, such as those related to drug development (Shi et al., 2017; Huang et al., 2019).

8.3 Comparison of human and mouse macrophages

An area of contention in macrophage biology revolves around the perceived disparities between rodent and human macrophages (Wynn et al., 2013). It has been proposed that human macrophages may be fundamentally different from their rodent counterparts. Especially concerning the expression of ARG1 and iNOS as they play an important role in immune defense, as it is challenging to induce human monocyte-macrophage cell lines to produce iNOS readily (Murray and Wynn, 2011a, 2011b). Moreover, the antimicrobial product, itaconic acid, is two hundred times lower in primary human monocytes compared to mouse cells (Michelucci et al., 2013).

Additionally, microarray analysis comparing human monocyte subsets to mouse subsets revealed gene expression patterns that diverge between species (Ingersoll et al., 2010). A more recent study used in-depth RNA sequencing to assemble a comprehensive dataset of gene expression profiles from 24 unique types of human and mouse lung, lymph node macrophages, human blood and mouse spleen macrophages. Only 130-230 genes of the top 1,000 marker genes, are shared between human and mouse macrophage populations (Leach et al., 2020). Understanding the differences between mouse and human macrophages is detrimental as different results may arise due to the different species.

Figure 2 provides a summary of the advantages and disadvantages of the most commonly used mouse and human macrophage types.

8.4 Macrophages from other species

One species that gives rise to various macrophage cell lines is chicken. Similar to thioglycollate elicited PMs in mice, inflammatory macrophages in the peritoneum of the chicken are recruited with Sephadex as a stimulant (Trembicki et al., 1984; Qureshi et al., 2000). These macrophages can efficiently phagocytose and break down

bacteria through lysosomal acid hydrolysis (Qureshi et al., 2000). Immortalized chicken macrophage cell lines offer an extra resource for macrophage studies.

Chicken macrophage cell lines, like MQ-NCSU (Qureshi et al., 1990) and HD11 (Beug et al., 1979), are widely used in studies. HD11, derived from chicken bone marrow and transformed with avian myelocytomatosis virus type MC29, exhibits macrophage-like characteristics, including enhanced morphology and ROS production (Graf, 1973; Wisner et al., 2011). MQ-NCSU, from a JM/102W strain of Marek's disease virus-infected broiler-type chicken spleen, shows traits of malignancy and mononuclear phagocyte lineage (Qureshi et al., 1990). Even primary monocytes can be isolated from heparinized blood obtained from the wing vein of chickens (Wigley et al., 2002, 2006).

Moreover, macrophages originating from bovine tissue are commonly used for *in vitro* models. Monocyte-derived macrophages from the peripheral blood of cows are used for *in vitro* infection experiments (Liebana et al., 2000; Weiss et al., 2002; Janagama et al., 2006), macrophage polarization studies (Imrie and Williams, 2019) and cytokine production (Werling et al., 2004). Moreover, alveolar macrophages harvested by pulmonary lavage were analyzed for immune response against pathogens (Widdison et al., 2007, 2011). In addition, BMDM generated from the iliac crest of bovines are infected with *Mycobacterium bovis* and *Mycobacterium avium subsp. paratuberculosis* strains to identify potential diagnostic biomarkers by analyzing the cytokine production and gene expression levels (Amato et al., 2024).

9 Conclusion

Macrophages represent one of the most plastic and versatile cell types present in multicellular organisms, characterized by their vast possibilities of signal recognition, cellular adaptations in terms of metabolism, their receptor repertoire, and the array of produced and secreted substances, as well as various forms of phagocytosis and endocytosis. Due to their presence in nearly all body tissues or fluids, macrophages constantly detect and respond to environmental shifts as well as tissue physiology, adapting their functional and metabolic states accordingly. The choice of cell type depends on the specific questions being posed. Consequently, in studies investigating macrophage functions and behaviors, it is crucial to select a macrophage type that closely resembles the *in vivo* setting in order to accurately represent the tissue macrophage type being investigated.

However, this approach is often highly challenging in terms of isolation and cultivation of primary macrophage types. Variables such as cell culture media, serum, and cell culture dishes can affect macrophage function, so a necessary understanding of their potential effects needs to be considered.

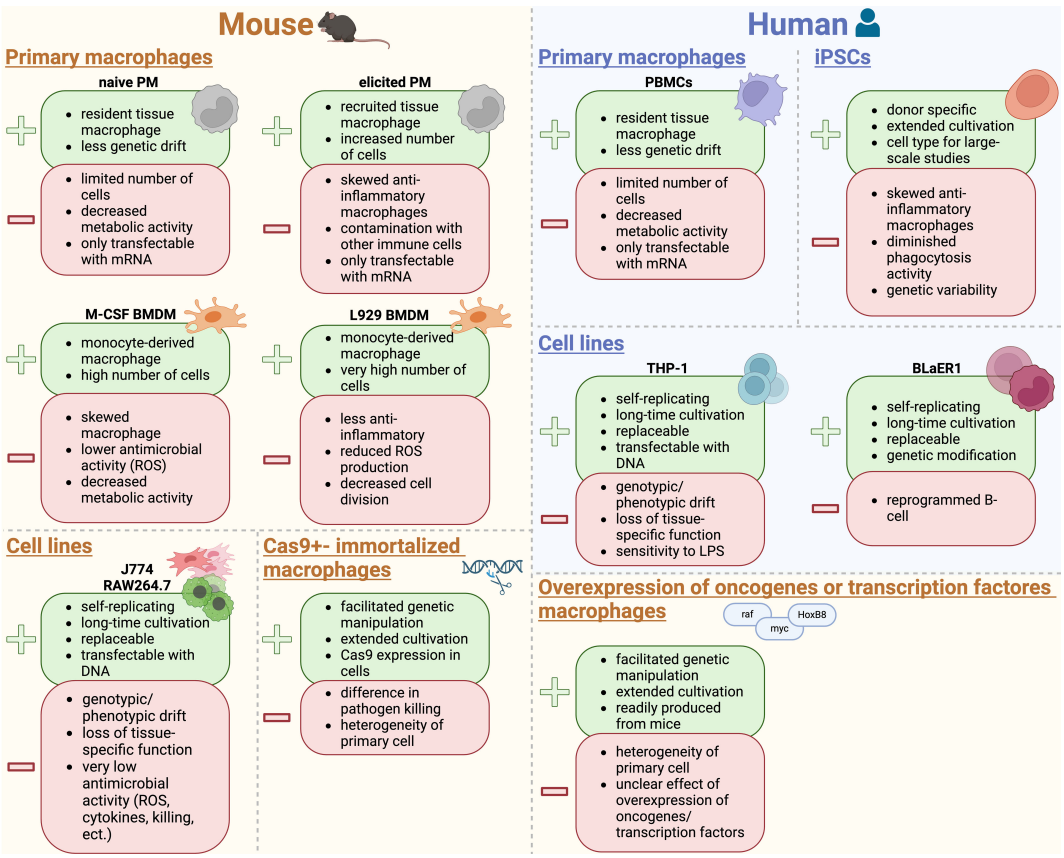

FIGURE 2
Advantages and disadvantages of mouse and human primary cells, commonly used cell lines, as well as, genetically modified cells. Figure created in BioRender.com. PM, peritoneal macrophages; BMDM, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; ROS, reactive oxygen species; PBMCs, peripheral blood monocytes; iPSCs, induced pluripotent stem cells; LPS, lipopolysaccharide.

These observations underscore the need for caution when interpreting *in vitro* studies, as they may not always accurately reflect *in vivo* phenotypes. Therefore, it is essential to validate findings from macrophage-like cell lines with studies involving primary macrophages to accurately characterize macrophage functions. This is especially relevant for results significant to humans, indicating the use of human macrophage cell lines, as human macrophages exhibit specific reactions and interactions that may differ from those of mouse macrophage cell lines.

Author contributions

MH: Conceptualization, Writing – original draft, Writing – review & editing. VS: Writing – review & editing. KH: Writing – review & editing. DH: Writing – review & editing, Funding acquisition. BH: Writing – review & editing. JJ: Writing – review & editing, Funding acquisition. NB: Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

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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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Publisher's note

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Insufficient CXCL13 secretion in leprosy foamy macrophages attenuates lymphocyte recruitment and antimicrobial protein production

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Background: Pathogens trigger metabolic reprogramming, leading to the formation of foamy macrophages (FMs). This process provides a favorable environment for bacterial proliferation and enables bacteria to evade immune killing.

Objective: To elucidate the mechanisms by which pathogens escape immune surveillance and elimination via the formation of FMs.

Methods: We constructed a FM model using monocyte-derived macrophages (MDMs) that were incubated with oxidized low-density lipoprotein (oxLDL). Subsequently, we employed bulk RNA-sequencing (bulk RNA-seq) to comprehensively analyze the immune responses in MDMs and FMs against *Mycobacterium leprae* (*M. leprae*) infection in samples from 10 healthy individuals.

Results: We found that CXCL13, a component of the cytokine-cytokine receptor interaction pathway, was specifically upregulated in *M. leprae* infected MDMs, when compared with *M. leprae* infected FMs. Significantly, further functional analyses revealed that *in vitro* treatment with CXCL13 could enhance the expression of CXCR5, thereby promoting lymphocyte migration and secretion of antimicrobial proteins. Additionally, NLRP12 was found to be specifically and highly expressed in the NOD-like receptor signaling pathway, which was enriched in infected FMs. In macrophages, *M. leprae* infection increased CXCL13 expression via NF-κB signal pathway. Conversely, in FMs, mycobacteria induced upregulation of CXCL13 was suppressed by NLRP12 through the inhibition of p52 factor expression.

Conclusion: In conclusion, the NLRP12/NF-κB/CXCL13 axis is crucial for the immune response of FMs after mycobacterial infection. These findings contribute to a deeper understanding of the pathological mechanisms of mycobacterial infection.

KEYWORDS

foamy macrophage, mycobacterial infection, CXCL13, antimicrobial proteins, NLRP12

Introduction

Macrophages are indispensable cells that play pivotal roles in innate immunity. Their biological functions are intricate and exhibit remarkable plasticity. During the interaction of host and intracellular pathogens, macrophages mediate processes such as recognition, phagocytosis and elimination through autophagy, apoptosis, antigen presentation and cytokine production (1). Nevertheless, pathogens can employ multiple strategies to survive and proliferate within macrophages, including inducing metabolic reprogramming and evading lysosomal degradation (2, 3).

One of the primary consequences of metabolic reprogramming is the accumulation of lipid droplets (LDs) in macrophages, which subsequently gives rise to foamy macrophages (FMs) (4). A diverse array of pathogens, including bacteria, parasites, fungi and viruses, can induce the formation of FMs. Pathogens trigger FMs formation by modulating lipid accumulation in macrophages, such as cholesterol, cholesteryl ester, and triacylglycerides (5–7). Notably, FMs can be formed not only by infected macrophages, but also by surrounding uninfected macrophages (8, 9). Although studies have revealed that FMs biogenesis is disease-specific, it has been observed that FMs not only serve as nutrient sources, but also regulate phagocytosis, cell death and inflammatory reaction in infectious diseases (6, 10, 11). Moreover, FMs can attenuate immune functions and antimicrobial capacities of macrophages, facilitating pathogen survival, and thus emerge as potential therapeutic targets in antimicrobial treatment (12, 13). However, the underlying mechanisms by which pathogens regulate immune responses and evade immune killing in FMs through LD accumulation remain to be thoroughly explored.

M. leprae, the causative agent of leprosy, is an intracellular bacterium that invades macrophages and Schwann cells to ravage skin and peripheral nerves. Leprosy presents a spectrum of clinical forms correlating with the host innate and adaptive immune response, making it a robust model for studying divergent immune responses correlating with the host reaction to the pathogen. Lepromatous leprosy (LL) is a disseminated and severe form of the disease, characterized by diffuse skin lesions and the massive proliferation of *M. leprae* in FMs, which was first described by Virchow in 1863 as one of the classic hallmarks of this clinical type. Modlin et al. (14) discovered that the lipids in FMs were involved in immune response, contributing to the pathogenesis of microbial infections. Furthermore, blocking lipid accumulation reduces the bacterial survival and enhances the mycobactericidal effect of rifampin (15, 16). Additionally, FMs are also evident in lesions infected with pathogenic mycobacteria, such as *M. tuberculosis, M. avium* and *M. marinum*, and support the massive reproduction of pathogens (10, 17). This indicates that FMs are critical for mycobacterial persistence in the host and the pathogenesis of mycobacterial infections. Therefore, in this study, we further elucidated the mechanism by which FMs regulate the host immune response and support the survival of intracellular mycobacteria using leprosy as a model.

To characterize the immune reaction of FMs in response to *M. leprae*, we established an *in vitro* FMs model using monocyte-derived macrophages (MDMs) from healthy individuals. Bulk RNA-sequencing was conducted on FMs and MDMs after *M. leprae* infection. Differential gene and functional enrichment analysis revealed that CXCL13 was specifically upregulated in *M. leprae* infected MDMs. Subsequently, we performed a series of molecular functional tests to investigate the potential pathogenic mechanisms involving CXCL13. We found that CXCL13 treatment could promote the expression of CXCR5 and the secretion of antimicrobial proteins, potentially enhancing intracellular bacterial clearance in macrophages. Correspondingly, in FMs, upregulated NLRP12 inhibited *M. leprae* induced CXCL13 by suppressing the expression of the p52 factor in NF-κB signal pathway. Our results uncovered the pathogen immune evasion mechanisms during mycobacterial infection in FMs, providing potential prospects for the treatment of related diseases.

Materials and methods

Study subjects

A cohort of 10 volunteers were involved in this experiment, including 6 men and 4 women aged 25 to 35 years. These individuals were meticulously screened to exclude those with infectious or immune-related disease. The study protocol was approved by the Ethics Committee of Hospital for Skin Diseases, Shandong First Medical University, and informed consent was obtained from the participants.

Monocyte-derived macrophage differentiation

Forty milliliters of peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors via density gradient. Subsequently, monocytes were purified from the PBMCs using Human CD14+ MicroBeads (130-050-201, Miltenyi Biotec). The isolated monocytes were cultured in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco), and 1% Penicillin-Streptomycin (Gibco). Macrophages were then differentiated by M-CSF (50 ng/ml, Abcam) for seven days at 37°C in 5% CO_2.

Foamy macrophages formation

To induce the transformation of macrophages into FMs, various concentrations of oxLDL (YB-002, Yiyuan Biotechnologies) were added to the culture and incubated for 24 hours. Confocal microscopy and flow cytometry were employed to observe and quantify the lipid content, using Bodipy 493/503 (Thermo Fisher Scientific) for staining. Briefly, black poly-d-lysine coated glass 12-well plates were used in confocal microscopy. The cells were washed twice for 5 minutes in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Subsequently, non-specific binding was blocked at room temperature with 1% BSA for 30 minutes. The cells were incubated with 0.5 μg/mL Bodipy 493/503 for 15 minutes at 37°C and then washed three times rapidly. The cell nuclei were stained with DAPI for 5 minutes at room temperature, and images were captured using an LSM 980 confocal microscope. To determine the optimal concentration of oxLDL for inducing FMs, cells were stained with Bodipy 493/503 as that of confocal microscope mentioned above. They were harvested using 0.25% trypsin-EDTA, centrifugated at 300 × g at 4°C for 5 minutes, and resuspended in PBS. Data were acquired on the FACSAria Fusion flow cytometer (BD Biosciences) and analyzed with the FlowJo software (BD Biosciences).

Phagocytosis of *M. leprae*

M. leprae was kindly provided by Institute of Dermatology, Chinese Academy of Medical Science and was grown in athymic (nu/nu) mouse foot pad as previously described (18). For the analysis of phagocytic capacity, macrophages were incubated with *M. leprae* labeled with PHK26 (Millipore Sigma) at a multiplicity of infection (MOI) of 10:1 for 4 hours at 37°C in 5% CO_2 environment. The cellular lipid was stained with Bodipy 493/503 and the cells were collected for flow cytometry analysis as described above. Data acquisition and analysis were performed using the FACSAria Fusion flow cytometer and FlowJo software, respectively.

Infection of cells with *M. leprae*

Monocytes were seeded in 6-well plates (1×10^6 cells/well) and differentiated into MDMs and FMs. The human MDMs and FMs were then infected with *M. leprae* at a MOI of 10:1 for 24 hours at 37°C in 5% CO2. After collecting the supernatants, the cells were washed twice by PBS and collected in Trizol Reagent (Thermo Fisher Scientific, Carlsbab, CA, USA). Simultaneously, uninfected MDMs and FMs were also harvested as control. Cells were stored at -80°C until RNA sequencing.

RNA isolation and sequencing data analysis

Total RNA was extracted using Trizol and its quality was assessed using Qutbit (Thermo). After rRNA removal, cDNA libraries were generated. Sequencing libraries were generated using VAHTSTM Total RNA-seq (H/M/R) Library Prep Kit for Illumina®. The libraries were sequenced as 151-bp paired-end reads using Illumina Novaseq6000. The clean reads obtained after processing using Skewer version 0.2.2 were aligned to the genome reference genome using STAR version 2.5.3a. The mapped reads were assembled into transcripts and merged using StringTie to obtain a set of unified transcripts. Then, the set of transcripts was compared to Ensembl gene annotations using gffcompare version 0.9.9c. The expression levels of all genes were quantified using StringTie version 1.3.1. Differentially expressed genes (DEGs) were selected according to false discovery rate (FDR) < 0.05 and absolute fold-change > 1.5 ($|\log_2 FC| = 0.585$). Kyoto Encyclopedia of Genes and Genomes (KEGG) was evaluated separately for up- and downregulated DEGs with g: Profiler (http://biit.cs.ut.ee/gprofiler/gost).

Enzyme linked immunosorbent assay

To measure the concentration of CXCL13 in the supernatants, the Human CXCL13 ELISA Kit (EK0739, Boster Biological Technology) was used according to the manufacturer's directions. The absorbance was measured at 450 nm using the GlowMax-Multi detection system (Promega). The concentrations were determined based on the standard curve generated using GraphPad prism version 8.0 (GraphPad Software, Inc.).

Multiple immunohistochemistry

Skin tissues were fixed in neutral buffered formalin and embedded in paraffin. Non-specific binding was blocked by 5% bovine serum albumin (BSA) blocking buffer (Solarbio) after antigen retrieval using TE buffer (pH 9.0). The slides were incubated with primary antibodies overnight at 4°C or for 2 hours at 37°C, followed by incubation with the goat anti-rat secondary antibodies. Color development was performed using a Four-color multiple fluorescent immunohistochemical staining kit (Absin) in accordance with the manufacturer's instructions. The sections were imaged using the EVOSTM FL Auto 2 Imaging System (Thermofisher). Rabbit anti–human ADRP (1:200, 15294-1-AP, proteintech), antibody and CXCL13 (1:200, 10927-1-AP, proteintech) antibody were used.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using total RNA extraction Kit (LS1040, Promega) according to instructions. The isolated RNA was reverse transcribed into cDNA using the Reverse Transcription Kit (A2801, Promega). QPCR was performed using SYBR Green (4368706, Applied Biosystems) as the fluorescent reporter on an Applied Biosystems QuantStudio 5 PCR instrument. The qPCR conditions consisted of an initial holding stage at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C and a melt curve stage of 15 seconds at 95°C, 1 minute at 60°C and 15 seconds at 95°C. The primer sequences were as follows: GAPDH, forward, 5′-GTCTC CTCTGACTTCAACAGCG-3′; GAPDH, reverse, 5′- ACCACCC TGTTGCTGTAGCCAA-3′; CXCL13, forward, 5′- TATCCCTAGA CGCTTCATTGATCG-3′; CXCL13, reverse, 5′- CCATTCAGCT TGAGGGTCCACA-3′; NLRP12, forward, 5′-CAGGCATGAT GCTGCTTTGCGA-3′; NLRP12, reverse, 5′-AGCACAGAAGCCAT CTCCTGAC-3′. GAPDH was used as an internal control. The relative abundance of the gene was calculated by using the equation $2^{-(\Delta\Delta Ct)}$.

Western blot

Cells were homogenized in RIPA lysis buffer (R0020, SolarBio) supplemented with complete protease inhibitor and phosphatase inhibitor (P1049, SolarBio). Equal amounts of protein were separated by 10% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane (0.45 μm) (Amersham Biosciences). After a 1 hour blocking period at room temperature using 5% BSA, the membranes were immunoblotted with antibodies against p100 (3017, CST), NLRP12 (PA5-21027, Invitrogen) and β-actin (HC201, Transgen) overnight at 4°C, followed by incubation with secondary antibodies at room temperature in Tris-buffered saline and Tween 20 (0.5% [v/v]). Immunoreactive proteins were detected using an Amersham Imager 600 with ECL substrate reagent (Millipore).

Flow cytometry

One million PBMCs were cultured in a 12-well plate in 1 ml RPMI 1640 medium with 10% FBS with or without 10ng/mL CXCL13 for 24 hours at 37°C in 5% CO_2. The cells were surface stained with CXCR5-PerCP/Cyanine5.5 (356910, BioLegend), CD3-Brilliant Violet 786 (740961, BD Biosciences), CD4-Alexa Fluor 700 (317426, BioLegend), CD8-Brilliant Violet 605 (344742, BioLegend) and CD19-Brilliant Violet 510 (363024, BioLegend). Intracellular stains were done following Fix/Perm kit (BD Biosciences) and stained with GNLY-Phycoerythrin (PE) (348004, BioLegend), GZMB-FITC (372206, BioLegend), PRF-Pacific Blue (308118, BioLegend) and TNF-α-APC/Cyanine7 (502944, BioLegend). All flow cytometry data were analyzed using FlowJo version 10.8.1. Fixable Viability Stain 780 (BD Biosciences) staining was used to gate out dead cells.

Inhibitor treatment

THP-1 cells were differentiated into macrophages by PMA (200 ng/ml, Abcam). The cells were then pretreated with BAY 11-7085/ BAY 11-7083 (HY-10257, MCE), or p38 MAPK-IN-1 (HY-12839, MCE) for 0.5 hour before *M. leprae* infection. The inhibitors were used at a final concentration of 10 μM. The cells were harvested for RNA and protein extraction at different time points.

Small interfering RNA

Human NLRP12 siRNA (si-NLRP12) and nonspecific control siRNA (si-NC) were synthesized by GenePharma (Shanghai, China). The siRNAs were transfected into cells using Lipofectamine™ RNAiMAX transfection reagent (13778-075, Invitrogen) according to the manufacturer's protocol. RNA extraction and immunoblotting assay were performed 48 hours after transfection.

Statistics

All data were analyzed using Graphpad Prism 8.0 Software. The differences between the means of experimental groups were analyzed using the two-tailed Student *t-test*. Wilcoxon signed rank test was used for flow cytometry results. A $P < 0.05$ was considered statistically significant.

Results

High lipid-laden FMs showed enhanced phagocytic ability

In previous studies of tuberculosis, experimental models of FMs and approaches for dissecting the mechanisms of lipid accumulation and consumption have been developed (19). Moreover, OxLDL, a cholesterol-rich component of lipoprotein, has been detected in LL lesions (15, 20). Consequently, we loaded human MDMs with oxLDL for FMs formation *in vitro*. We incubated MDMs with 10, 30 or 50 μg/ mL oxLDL for 24 hours. Subsequently, the cells were stained with Bodipy 493/503, and confocal fluorescence microscopy and flow cytometry were performed. The results demonstrated a dose-dependent increase in both intracellular lipid levels (Figure 1a) and the percentage of high lipid-loaded cells (Figure 1b) following oxLDL treatment. Further analysis revealed no significant difference in the median fluorescence intensity (MFI) of lipids between the 30 and 50 μg/mL treatment groups (Figures 1c, d). Therefore, 30 μg/mL oxLDL was selected as the optimal concentration for inducing FM formation in our study.

Subsequently, we compared the phagocytic capacity of *M. leprae* in MDMs and FMs. The cells were exposed to PHK26-labelled *M.* leprae for 24 hours, and lipid staining was carried out using Bodipy 493/503. We observed that all cells exhibited fluorescence (Supplementary Figure S1A). OxLDL-treated

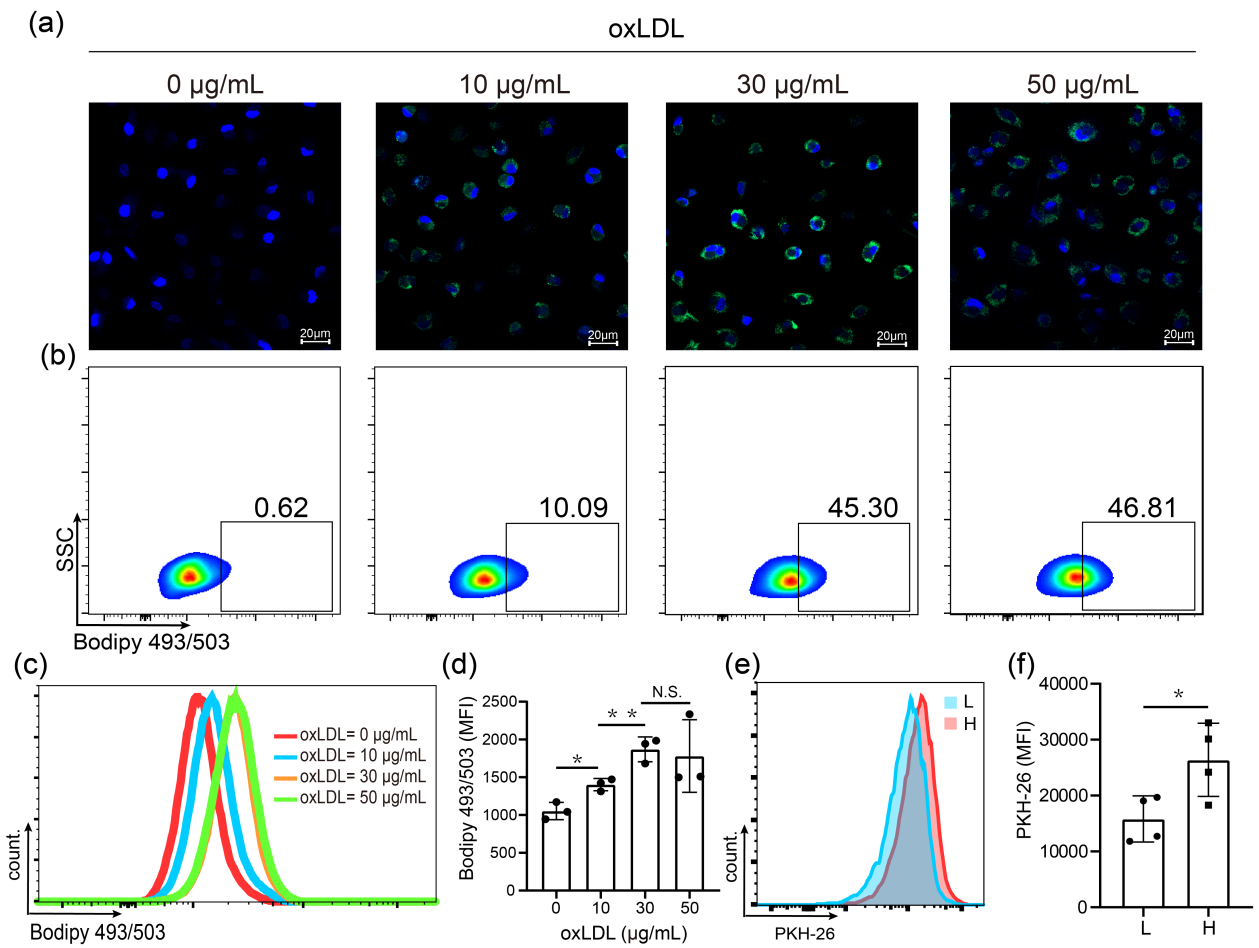

FIGURE 1
OxLDL-induced high lipid loaden foamy macrophages showed enhanced phagocytic ability. **(a)** Visualization of lipid in MDMs treated 24 h with 0, 10, 30 and 50 μg/mL oxLDL by confocal microphage. Nuclei (blue, DAPI); lipids (green, Bodipy 493/503). **(b)** Proportion of high lipid laden macrophages assessed by flow cytometry analysis. **(c)** Histogram plot for BODIPY 493/503 fluorescence. **(d)** MFIs of BODIPY 493/503-labelled lipid. **(e)** Histogram plot for PKH-26-labelled *M. leprae*. **(f)** MFIs of *M. leprae* in low (L) and high (H) lipid-laden macrophages after treated with 30 μg/mL oxLDL. DAPI: 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, MFI: Mean Fluorescence Intensity. Differences between the mean of experimental groups were analyzed using the two-tailed Student *t-test*. *, ** or N.S. indicates p-value <0.05, <0.01 or no statistical significance respectively.

macrophages were then divided into low and high lipid laden FMs (Supplementary Figure S1B). We then assessed the MFI of *M. leprae* in both low lipid laden and high lipid laden FMs. The findings indicated that the MFI of *M. leprae* was significantly higher in high lipid-laden FMs compared to low lipid-laden FMs (Figures 1e, f). In summary, oxLDL effectively induces the formation of FMs and oxLDL-induced high lipid-laden FMs display enhanced phagocytosis of *M. leprae*.

Heterogeneity and similarity of MDMs and FMs after *M. leprae* infection

We generated human MDMs and oxLDL-induced FMs *in vitro*. Subsequently, both cells were separately infected by *M. leprae* for 24 hours *in vitro*, followed by RNA-sequencing (Supplementary Figure S2A). Principal component analysis (PCA) of the sequencing data revealed distinct patterns between uninfected and infected samples. Notably, there was a high degree of overlap between infected MDMs and infected FMs (Supplementary Figure S2B).

To comprehensively analyze the similarities and differences in immune responses between MDMs and FMs, we selected the differential expressed genes (DEGs) (FDR < 0.05 and $|\log_2 FC| > 0.585$) of MDMs and FMs after infection, respectively. 3,344 genes (1,683 upregulated and 1,661 downregulated genes, Figure 2A, Supplementary Table S1) in infected MDMs and 2,297 genes (1,207 upregulated and 1,111 downregulated genes, Figure 2B, Supplementary Table S2) in infected FMs were identified. We observed that the lipid metabolism indicators, such as PLIN2 and CD36 in uninfected FMs, were significantly increased compared to uninfected MDMs, suggesting the successful establishment of foam cell model *in vitro* (Supplementary Figure S3). Functional enrichment analysis of the DEGs from MDMs and FMs after infection were further performed respectively. The upregulated genes were predominantly enriched in cytokine-cytokine receptor interaction pathway (KEGG:04060, Figure 2C). Concurrently, the

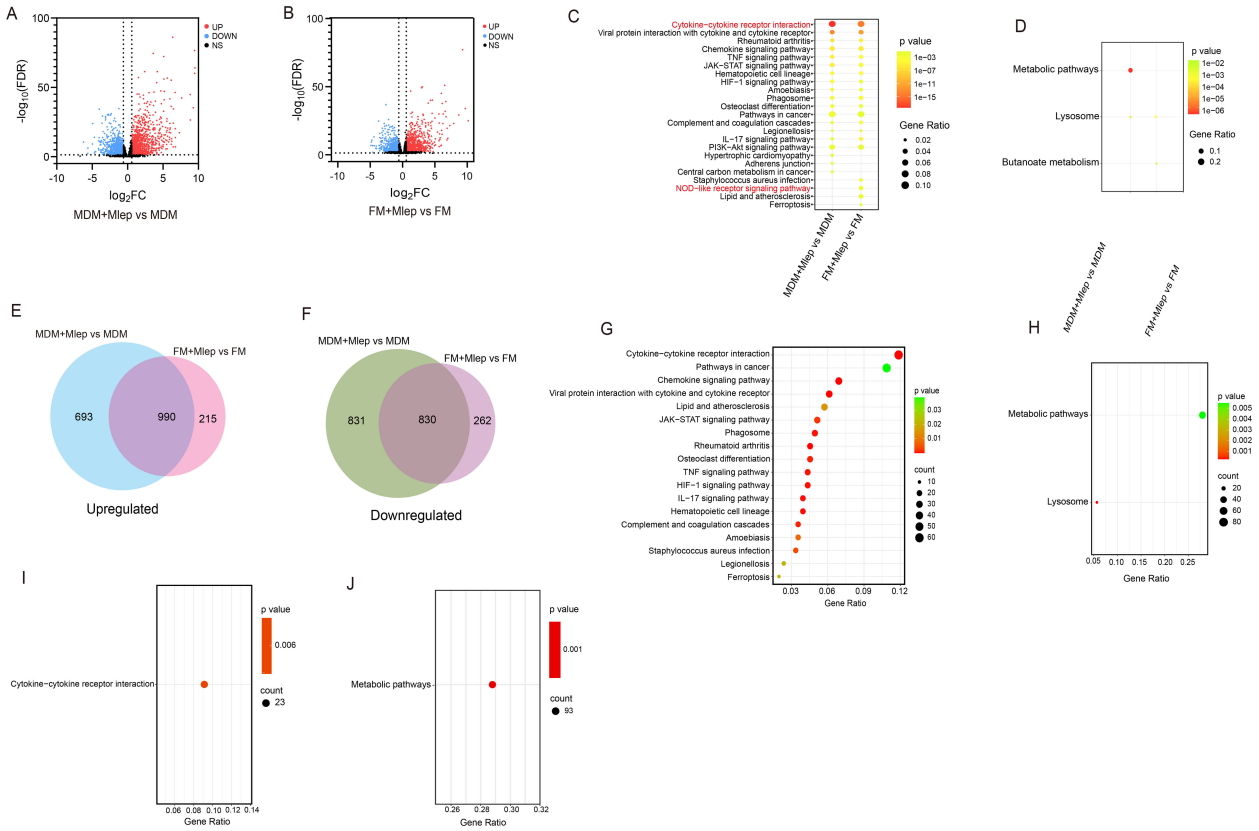

FIGURE 2
RNA-seq analysis of *M. leprae*-infected MDMs and FMs. Volcano plot showing considerably upregulated (red dots) and downregulated genes (blue dots) in *M. leprae*-infected MDMs **(A)** and FMs **(B)**. KEGG enrichment analysis of upregulated **(C)** and downregulated **(D)** DEGs in *M. leprae*-infected MDMs vs uninfected MDMs and *M. leprae*-infected FMs vs uninfected-FMs. Venn diagram of the number of upregulated **(E)** and downregulated **(F)** DEGs in *M. leprae* infected MDMs vs uninfected MDMs and *M. leprae* infected FMs vs uninfected FMs, confirming numerous shared genes (FDR<0.05 and $|\log_2 FC|>0.585$). KEGG enrichment analysis of upregulated **(G)** and downregulated **(H)** DEGs that shared in *M. leprae*-infected MDMs vs uninfected MDMs and *M. leprae*-infected FMs vs uninfected FMs. KEGG enrichment analysis of specific upregulated **(I)** and downregulated **(J)** DEGs in *M. leprae*-infected MDMs vs uninfected MDMs. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, Differential expressed gene.

downregulated genes were mainly enriched in lysosome pathway (KEGG:04142, Figure 2D). Additionally, Venn diagram analysis revealed that 990 upregulated and 830 downregulated genes were shared between *M. leprae*-infected MDMs and FMs (Figures 2E, F). KEGG pathway analysis of these shared DEGs yielded enrichment results that were largely consistent with those of *M. leprae*-infected MDMs and FMs (Figures 2G, H). Collectively, these findings indicated that *M. leprae* may induce similar immune responses in both MDMs and FMs, particularly excessive inflammatory cytokine production.

The difference in bactericidal ability between MDMs and FMs may be attributed to specifically expressed genes in each cell type. Therefore, we further explored the specifically enriched pathways. In *M. leprae*-infected FMs, these pathways included staphylococcus aureus infection (KEGG:05150), NOD-like receptor signaling pathway (KEGG:04621), lipid and atherosclerosis (KEGG:05417) and ferroptosis (KEGG:04216) (Figure 2C). In *M. leprae*-infected MDMs, pathways such as hypertrophic cardiomyopathy (KEGG:05410), adherens junction (KEGG:04520) and central carbon metabolism in cancer (KEGG:05230) were upregulated (Figure 2C). Moreover, Venn diagram analysis identified specific DEGs of *M. leprae*-infected MDMs and FMs (Figures 2E, F). Only cytokine-cytokine receptor interaction (KEGG:04060) was enriched in specific upregulated DEGs from MDMs, and only metabolic pathway (KEGG:01100) was enriched in specific downregulated DEGs from MDMs through the KEGG pathway analysis. Unfortunately, no pathways were enriched in the DEGs from FMs (Figures 2I, J). These results indicate that the specific enrichment pathways from Figures 2C, D were not validated in the specific DEGs obtained from the Venn diagram analysis.

CXCL13 is upregulated in infected MDMs specifically

We continued to directly compare and analyze the transcriptome profiles of MDMs and FMs after infection. Initially, we identified 593 upregulated and 342 downregulated DEGs (FDR < 0.05 and $|\log_2 FC| > 0.585$) (Figure 3A, Supplementary Table S3). KEGG pathway analysis re-confirmed that cytokine-cytokine receptor interaction (KEGG:04060) was still enriched in downregulated DEGs (Figures 3B, C). Among 17 DEGs within this aforementioned pathway, CXCL13 exhibited the most significant difference in

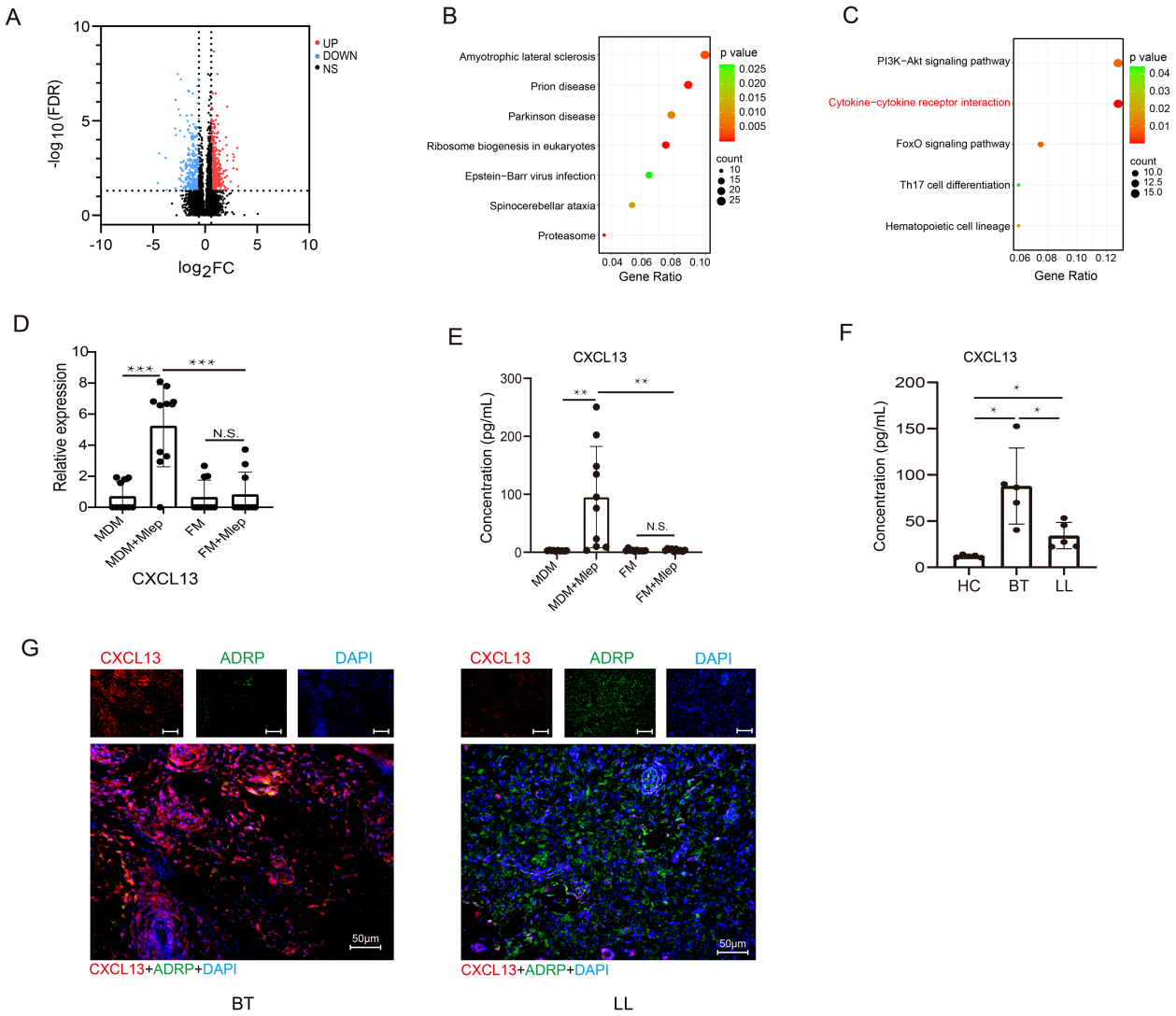

FIGURE 3
CXCL13 is upregulated in infected MDMs specifically. **(A)** Volcano plot showing considerably upregulated (red dots) and downregulated genes (blue dots) in *M. leprae*-infected FMs vs *M.* leprae-infected MDMs. KEGG enrichment analysis of upregulated **(B)** and downregulated **(C)** DEGs in *M. leprae*-infected FMs vs *M. leprae*-infected MDMs. **(D)** Expression of CXCL13 in different groups (n=10 per group). Expression abundance of CXCL13 in the supernatants of MDMs, FMs, *M. leprae*-infected MDMs and FMs (n=10 per group) **(E)**, and in the serum of HCs and leprosy patients (n=5 per group) **(F)**. **(G)** mIHC showing the co-localization of CXCL13 and FMs marker ADRP in leprosy lesions from BT and LL subtypes (n=5 per group). BT, borderline tuberculoid leprosy; LL, lepromatous leprosy; HC, healthy control; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, Differential expressed gene. Differences between the mean of experimental groups were analyzed using the two-tailed Student *t-test*. *, **, *** or N.S. indicates p-value <0.05, <0.01, <0.001 or no statistical significance respectively.

expression based on the bulk-RNA sequencing data (Figure 3D, Supplementary Figure S4). At the protein level, the concentration of CXCL13 was significantly increased in supernatant of MDMs infected by *M. leprae*. In contrast, in supernatant of FMs, *M. leprae* infection did not induce the expression of CXCL13 (Figure 3E).

We also examined CXCL13 levels in the serum and tissues of patients with LL and borderline tuberculoid (BT) leprosy, which is another subtype of leprosy that presents restrict bacillus growth leading to localized disease. As depicted in Figure 3F, compared with healthy controls, there was a significant increase of serum CXCL13 levels in leprosy patients, especially those with BT leprosy. Simultaneously, mIHC revealed that CXCL13 also significantly increased in skin lesions from BT subtype of leprosy. Moreover, in the LL subtype, an inverse correlation was observed between the expression level of CXCL13 and that of ADRP, a well-recognized marker for FMs (Figure 3G). These findings suggest that CXCL13 hyposecretion may be closely related to the clinical manifestations of LL leprosy.

CXCL13 promotes the recruitment of CXCR5+ lymphocytes and secretion of antimicrobial proteins

CXCL13, a chemokine, plays a crucial role in recruiting lymphocytes via CXCR5 to mediate protective immunity (21). Given that the CXCL13/CXCR5 can dictate the homing and

motility of CXCR5+ B and T cells (21–23), in this section, PBMCs from healthy individuals were stimulated using CXCL13 followed by cytometry analysis *in vitro*. The result demonstrated that CXCL13 could upregulate the expression of CXCR5 on both B and T cells (Supplementary Figure S5).

To explore the functional impacts of CXCL13, the expression of bactericidal proteins, such as cytotoxic granule proteins granzyme B (GZMB), perforin (PRF) and granulysin (GNLY) and pro-inflammatory cytokines TNF-α, were examined after stimulation with CXCL13 *in vitro*. We found that CXCL13 could promote the secretion of nearly all bactericidal proteins in lymphocytes. Specifically, GNLY, GZMB, PRF and TNF-α were significantly overexpressed in CD19+ CXCR5+ B cells (Figures 4A, B), GNLY, GZMB, PRF and TNF-α were differentially high expressed in CD4 +CXCR5+ (Figures 4C, D), while GNLY, GZMB and PRF were significantly overexpressed in CD8+ CXCR5+ T cells (Figures 4E, F). Collectively, these findings indicate that CXCL13 could enhance the expression of CXCR5 and bactericidal proteins secretion, which are associated with cytotoxic B- and T-cell responses.

CXCL13 is suppressed by NLRP12 through the regulation of NF-κB signaling

The intrinsic mechanism of CXCL13 hyposecretion in FMs was not explored by previous reports. Given that THP-1 cells are a widely recognized and commonly used macrophage model. Previous studies (24–26) have delved into the functions of FMs derived from THP-1 cells in both infectious and non-infectious diseases. In our current study, we harnessed THP-1 cells to conduct mechanistic investigations. Our findings revealed that CXCL13 expression was significantly increased after infection for 24 hours in THP-1 cells (Figure 5A). It has been previously confirmed that bacterial infection can activate Toll-like receptors (TLRs) and downstream signal pathway, thereby promoting cytokines secretion in macrophages. To investigate the regulatory pathway of CXCL13, we treated THP-1 cells with inhibitors of the NF-κB pathway and the mitogen-activated protein kinase (MAPK) pathway in the presence of *M. leprae in vitro*. We found that only the IκBα kinase inhibitor (BAY 11-7085/BAY 11-7083), an inhibitor of the NF-κB pathway, could suppress the *M. leprae*-induced upregulation of CXCL13 in THP-1 cells (Figure 5B). Previous studies have demonstrated that non-canonical NF-κB pathway regulates the production of CXCL13 (27). The ratio of p52/p100, which are two key proteins in the non-canonical NF-κB signaling pathway, serves as an indicator of the activation state of this pathway. In *M. leprae* infected THP-1-derived macrophages, the p52/p100 ratios were significantly increased. However, in BAY 11-7085/BAY 11-7083 treated macrophages, the p52/p100 ratios at 1 and 2 hours post-infection showed no significant difference compared to those before infection. Nevertheless, when the infection time was extended to 4 hours, the ratio of these two proteins showed a significant upregulation (Figures 5C, D). These results indicated that non-canonical NF-κB pathway could play a key role in *M. leprae*-induced CXCL13 expression.

It has been previously reported that NLRP12 can act as a negative regulator of NF-κB signaling (28, 29). In the present study, we found that in *M. leprae*-infected FMs, NLRP12 is one of the specifically upregulated genes and was involved in the NOD-like receptor signaling pathway (Figure 2C). To confirm the effect of NLRP12 on the non-canonical NF-κB pathway in macrophages, we initially suppressed the expression of NLRP12 in THP-1 cells (Figures 5E–G). Subsequently, *M. leprae* infection can upregulate the p52/p100 ratios. Moreover, the knockdown of NLRP12 exacerbates the aforementioned upregulation (Figures 5H, I). We also observed that the knockdown of NLRP12 promoted the expression of CXCL13 (Figure 5J). Collectively, these findings indicate that NLRP12 inhibits CXCL13 expression by suppressing the activation of p52 in non-canonical NF-κB pathway.

Discussion

Cellular metabolism serves as a critical driving force for macrophage activation during pathogen infection. Among various metabolic processes, lipid homeostasis and metabolism can be dynamically altered in response to physiological stimuli that trigger macrophage activation, thereby modulating macrophage functions (30, 31). In macrophages, the formation of FMs is a direct consequence of abnormal lipid metabolism induced by mycobacterial infections, such as those caused by *M. leprae* and *M. tuberculosis*. FMs play a pivotal role in *M. leprae* infection by facilitating bacterial persistence in the host (32, 33). In our study, we successfully constructed FMs *in vitro* and demonstrated their enhanced phagocytic ability. Subsequently, transcriptional profiles of MDMs and FMs were gained by bulk-RNA sequencing. Our findings revealed that the hyposecretion of CXCL13 in FMs cannot induce the expression of CXCR5 and the secretion of bactericidal proteins by lymphocytes, which in turn promotes the growth of *M. leprae* in LL. The results deepen the understanding of the pathological mechanisms underlying mycobacterial infection, and provide promising prospect for the development of diagnosis and treatment for related diseases.

Tissue-resident macrophages are the first line of defense against pathogens, mediating immunity through recognition of pattern recognition receptors (PRPs) and pathogen-associated molecular patterns (PAMPs) (34). Accompanied by significant reprogramming of lipid metabolism, pathogens can induce the formation of lipid-laden FMs. FMs provide energy and replication sites for pathogens and are involved in immune inflammatory responses (10, 13). Cholesterol, one of the host lipid molecules that accumulate in *M. leprae*-infected macrophages, can recruit TACO to prevent phagosome-lysosome fusion, thereby protecting mycobacteria from degradation in lysosomes (15, 35). Consistent with these findings, our results showed that high lipid-laden FMs can phagocytize more *M. leprae*. However, previous studies have reported conflicting results regarding the phagocytic capacity of FMs. Some studies have shown that the phagocytic capacity remains largely unaffected in oxLDL-treatment macrophages, while others have demonstrated a decrease in phagocytic capacity in oleic-acid-induced FMs (11, 36). These discrepancies may be attributed to the

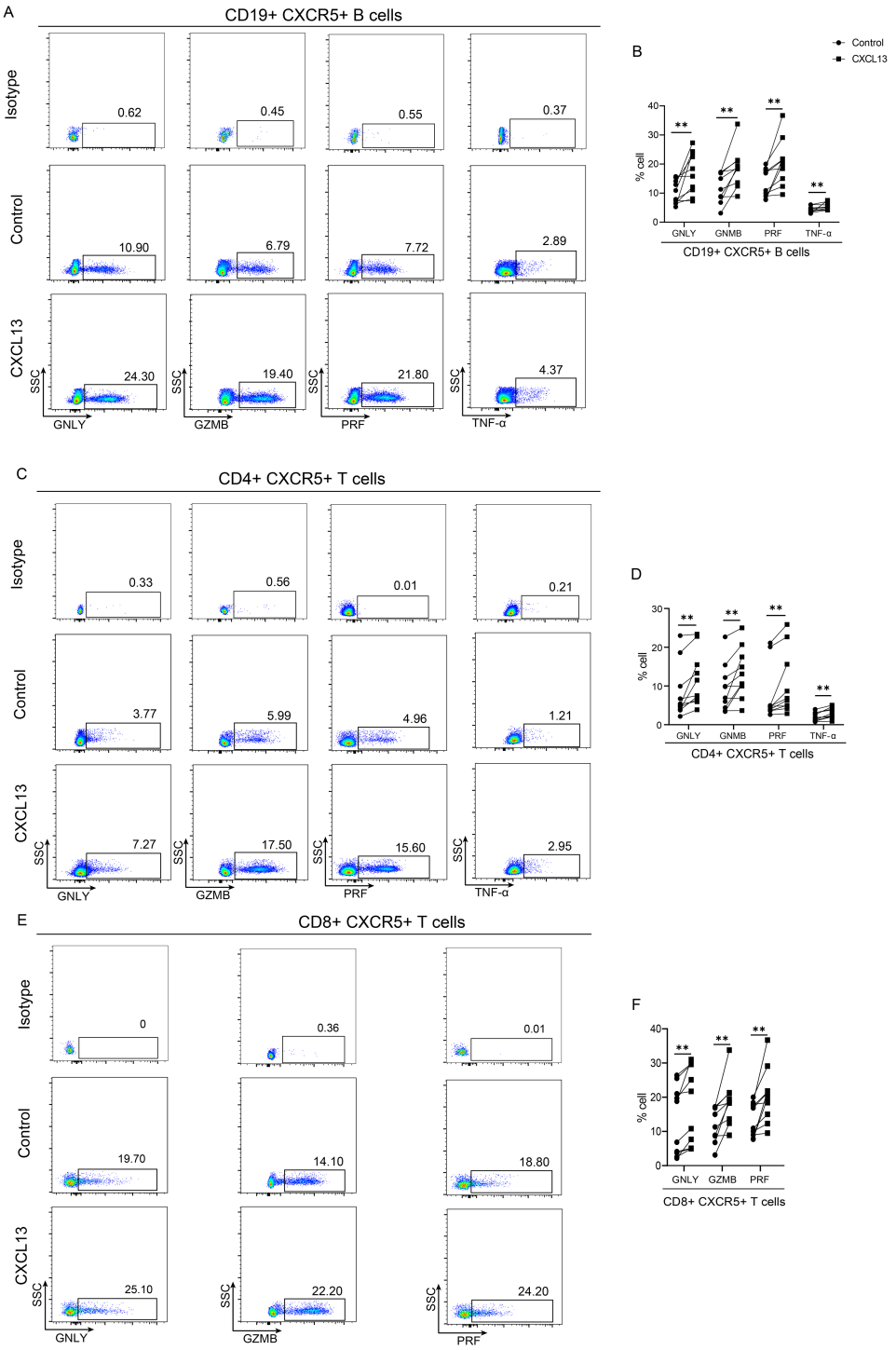

FIGURE 4
CXCL13 promotes the secretion of antimicrobial proteins. PBMCs were collected from healthy volunteers and were stimulated by 10 ng/mL CXCL13 for 24(h) The expressions of GNLY, GZMB, PRF and TNF-α in CD19+CXCR5+ B **(A)** and CD4+ CXCR5+ T cells were detected by flow cytometry **(C)**. The expressions of GNLY, GZMB and PRF in CD8+CXCR5+ T cells were detected by flow cytometry **(E)**. Each connected symbol represents paired samples from one individual donor in CD19+CXCR5+ B **(B)**, CD4+ CXCR5+ **(D)** and CD8+CXCR5+ **(F)** T cells (n=10 per group). PBMC, Peripheral blood mononuclear cell. Wilcoxon signed rank test was performed. ** indicates p-value <0.01.

use of different inducers and varying concentrations of oxLDL in the FMs models.

CXCL13, secreted by monocyte-like and mature macrophages, plays a pathogenic role and regulate lymphoid neogenesis in human inflammatory disease (22, 37). It has been well-established that CXCL13 is a major chemoattractant for B cell and is centrally involved in the recruitment of B cells and certain T cell subsets via CXCR5 (38–40). Our results are consistent with previous reports,

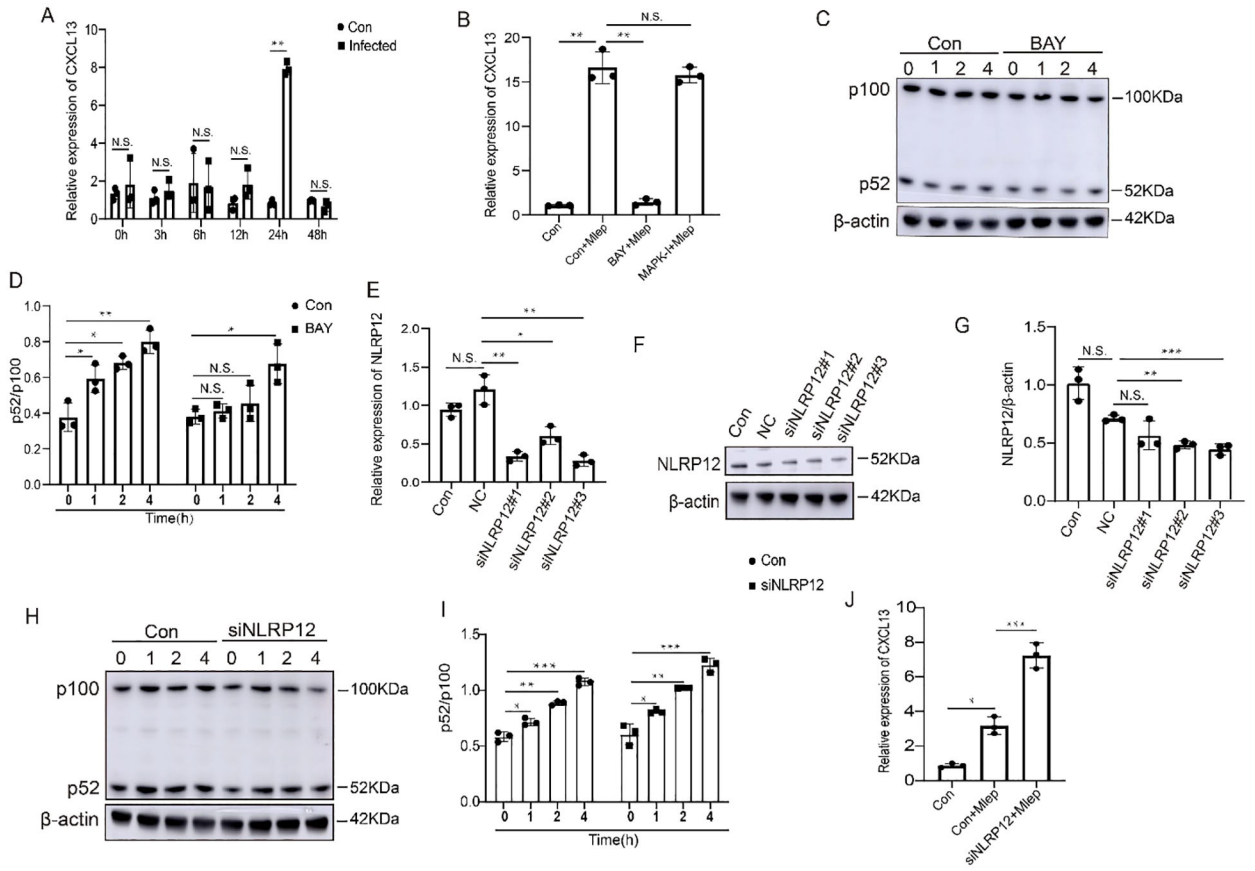

FIGURE 5
NLRP12 regulates expression of CXCL13 by non-canonical NF-κB signaling. **(A)** THP-1-derived macrophages were infected with *M. leprae* for the indicated time and CXCL13 expression was analyzed by qPCR (n=3). **(B)** THP-1-derived macrophages were infected with *M. leprae* for 24 h after pretreatment with BAY 11-7085/BAY 11-7083 or p38 MAPK-IN-1 and CXCL13 expression was analyzed by qPCR (n=3). **(C, D)** THP-1-derived macrophages were infected with *M. leprae* for the indicated time after pretreatment with BAY 11-7085/BAY 11-7083 and the p100, p52 and β-actin protein expression was assessed by WB (n=3). Three siRNAs targeting the human NLRP12 gene were designed and their efficiency was examined by qPCR **(E)** and WB **(F, G)** (n=3). Choosed siNLRP12#3 was transfected into THP-1-derived macrophages and subsequently challenged with *M. leprae*, then the p100, p52 and β-actin protein expression was assessed by WB **(H, I)**, and CXCL13 expression was analyzed by qPCR **(J)** (n=3). qPCR: quantitative polymerase chain reaction, WB: Western blotting. Con: control, NC: negative control. BAY: BAY 11-7085/BAY 11-7083. Differences between the mean of experimental groups were analyzed using the two-tailed Student *t-test*. *, **, *** or N.S. indicates p-value <0.05, <0.01, <0.001 or no statistical significance respectively.

demonstrating that CXCL13 promotes the expression of CXCR5 *in vitro*. Moreover, the CXCL13 not only exhibits chemotactic activity depending on CXCR5 but also acts regulatory roles in chronic inflammation, infectious diseases, autoimmune disorders, neurological conditions and tumors (41, 42). In *M. tuberculosis*-infected mouse lungs, CXCL13 is not required for generation of IFN-γ responses, but is essential for the spatial arrangement of lymphocytes within granulomas, optimal activation of phagocytes, and the subsequent control of mycobacterial growth (43). An increase in CXCL13 expression leads to the accumulation of activated CD4+CXCR5+ T cells, which produce pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2 to combat *M. tuberculosis*. The absence of CXCR5/CXCL13 axis can lead to increased susceptibility to tuberculosis (21, 44). CXCL13 can also mediate the recruitment of CD8+ CXCR5+ T cells to produce IFN-γ and IL-21, thereby controlling HBV infection (23). Cytokine like CXCL13 induce B-cell homing and clonal selection in intraportal lymphoid aggregates, which are correlate with extrahepatic clinical manifestations of HCV infection (45). Furthermore, CXCL13 is also associated with the progression of HIV-infected disease, neuroborreliosis, neurosyphilis, influenza A virus infection and SARS-CoV-2 (46). These studies underscore the significance of CXCL13 in pathogen elimination during infectious diseases. In leprosy, CXCL13 was associated with lipid metabolism, inflammatory response, and cellular immune response. It can distinguish contacts from patients and is down regulated after multidrug therapy (MDT) in multibacillary leprosy (42, 47). In the present study, we found that CXCL13 could induce the secretion of antimicrobial protein by regulating the migration of CXCR5+ lymphocytes during *M. leprae* infection. The role of B lymphocytes is rarely understood in host defense against pathogens (48, 49). Hagn et al. (50) reported that human B cells secrete active GZMB when stimulated *in vitro* by the Epstein-Barr virus (EBV). In our study, we were the first to observe that, apart from GZMB, the expression of GNLY, PRF and TNF-α increase in B cells after CXCL13

stimulation. Furthermore, it has been reported that both CD4+ cytotoxic T lymphocytes (CTLs) and CD8+ CTLs expressing GNLY, GZMB, PRF proteins exhibit antimicrobial activity against intracellular bacteria and in HIV-1 infection (51, 52). Collectively, macrophages-derived CXCL13 may facilitate the accumulation of B, CD4+T and CD8+T cells and induce the secretion of bactericidal proteins, thereby promoting the clearance of mycobacteria.

Macrophages recognize invading *M. leprae* through a series of cell-surface and intracellular PRPs, such as TLRs. This recognition triggers signaling pathways, including the NF-κB and MAPK pathways, leading to upregulation of CXCL13 expression (53, 54). Additionally, prior research has reported that in the absence of IL-23, the establishment of long-term immunity against tuberculosis is impaired. This impairment is attributed to the decreased expression of CXCL13 within B-cell follicles and the diminished capacity of T cells to migrate from blood vessels into the lesion (42). Previously published studies have also indicated that during tuberculosis infection, the expression of CXCL13 is regulated by multiple factors, including IL-17 (55) and the leptin receptor (56). In our study, we added specific pathway inhibitors into cell culture medium and found that *M. leprae* induced CXCL13 expression is dependent on non-canonical NF-κB pathway. In FMs, the inhibition of p52 might be responsible for CXCL13 hyposecretion. Evidence suggests that NLRP12 can negatively regulate noncanonical NF-κB signaling pathway in colon inflammation and tumorigenesis (29). In our study, we found that NLRP12 was specifically increased in FMs after infection. *M. leprae* induced CXCL13 expression was restored in NLRP12$^{-/-}$ macrophages. It has been reported that NLRP12 negatively regulates noncanonical NF-κB signaling by directly interacting with and inducing the proteasomal degradation of NIK (57). We also observed that the expression p52, a downstream factor of NIK in the pathway, was reversed in NLRP12$^{-/-}$ macrophages. In summary, the down-regulation of CXCL13 probably is due to the inhibition of noncanonical NF-κB signaling by highly expressed NLRP12 in FMs.

THP-1 cells were a widely utilized macrophage model. Wichers et al. have reported functional similarity and differences between MDMs and THP-1 macrophages. In an M1 setting, there were more similarities presented between THP-1 cells and MDMs. However, when interested in M2 models, the differences and the objectives should be more considered (58). FMs derived from aforementioned cells exhibited numerous resemblances, particularly in terms of the inflammatory response and the intracellular accumulation of cholesterol-esters (59, 60). Moreover, previous studies have successfully constructed FMs using THP-1 cells and explored their functions in infectious and non-infectious diseases. Suzuki et al. found that in THP-1 derived FMs, *M. leprae* infection could induce PPAR-δ and PPAR-γ expressions in a bacterial load-dependent manner, leading to accumulation of intracellular lipids to accommodate *M. leprae* parasitization (24). The expression of AIM enlarged FMs formation by enhancing intracellular lipid content in THP-1 cells. *M. tuberculosis*-infected AIM-expressing cells upregulated the production of Beclin 1 and LC3II, as well as enhanced mycobacterial phagosomes and LC3 co-localization (25). In THP-1 derived FMs, ox-LDL promoted TRIM64 expression, subsequently enhanced pyroptosis, and inflammation in the development of atherosclerosis (26). In our study, consistent with previous studies, THP-1 cells were used to conduct mechanistic investigations.

Our study is not without its limitations. First and foremost, although we found that CXCL13 hyposecretion is dependent on NLRP12-mediated suppression of non-canonical NF-κB signaling in FMs, the precise mechanism by which intracellular lipid accumulation affects NLRP12 expression remains to be elucidated after *M. leprae* infection. Emerging research has suggested that host-derived metabolites regulate the activation of NOD-like receptor (NLR) members (61). It will be an interesting study to reveal the association between NLRs and lipid metabolism. Moreover, the current study did not incorporate foamy dendritic cells. Salinas-Carmona et al. have reported that *Nocardia brasiliensis* could induce formation of foamy Macrophages and dendritic cells (DCs) both *in vitro* and in murine models (62). Marrow derived DCs (BMDCs) were susceptible to *M. tuberculosis* infection. Such an infection induced delayed lipid droplet accumulation and proinflammatory response (63). Existing evidence shows that dendritic cells were activated during leprosy (64), which holds great promise for future functional investigations into foamy dendritic cells. Last but not least, in our study, considering that pathogen induced FMs are more adept at reflecting the pathophysiological state of the disease, the utilization of oxLDL induced FMs could potentially be another shortcoming.

In conclusion, bulk-RNA sequencing of MDMs and FMs revealed that CXCL13 expression was specifically increased in *M. leprae* infected MDMs. Additionally, CXCL13 promoted the expression of CXCR5 and the secretion of bactericidal proteins by lymphocytes. In FMs, CXCL13 expression was suppressed by NLRP12 by inhibiting p52 factor in the non-canonical NF-κB pathway (Supplementary Figure S6). Overall, NLRP12/NF-κB/CXCL13 axis might serve as a potential target for enhancing host immunity in FMs. Our findings offer promising prospects for the prevention and treatment of leprosy and other mycobacterial infections.

Data availability statement

The data presented in the study are deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix/view/OMIX009614: accession number OMIX009614).

Ethics statement

The studies involving humans were approved by the Ethics Committee of Hospital for Skin Diseases, Shandong First Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CW: Data curation, Formal Analysis, Funding acquisition, Investigation, Validation, Writing – original draft, Writing – review &

editing. YZ: Data curation, Formal Analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. TL: Conceptualization, Methodology, Writing – review & editing. ZM: Conceptualization, Methodology, Writing – review & editing. PS: Conceptualization, Methodology, Writing – review & editing. ZW: Formal Analysis, Writing – review & editing. WL: Formal Analysis, Writing – review & editing. HW: Resources, Writing – review & editing, Funding acquisition. HL: Supervision, Writing – review & editing, Funding acquisition, Project administration. FZ: Funding acquisition, Supervision, Writing – review & editing, Project administration.

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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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1541954/full#supplementary-material

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P62 inhibits IL-1β release during *Salmonella* Typhimurium infection of macrophages

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Macrophages are critical for the innate immune defense against the facultative intracellular Gram-negative bacterium *Salmo\nella enterica* serovar Typhimurium. Following phagocytosis by macrophages, *S*. Typhimurium activates cytoplasmic NLRC3 and NLRP4 inflammasomes, which share the adaptor ASC, resulting in the secretion of the pro-inflammatory cytokine IL-1β. To prevent excessive inflammation and tissue damage, inflammatory signaling pathways are tightly controlled. Recently, autophagy has been suggested to limit inflammation by targeting activated inflammasomes for autophagic degradation. However, the importance of the autophagic adaptor Sequestome-1 (hereafter, p62) for regulating inflammasome activation remains poorly understood. We report here that p62 restricts inflammasome availability and subsequent IL-1β secretion in macrophages infected with *S*. Typhimurium by targeting the inflammasome adaptor ASC for autophagic degradation. Importantly, loss of p62 resulted in impaired autophagy and increased IL-1β secretion, as well as IL-10 and IFN-β release. In summary, our results demonstrate a novel role for p62 in inducing autophagy and balancing major pro- and anti-inflammatory signaling pathways to prevent excessive inflammation during *S*. Typhimurium infection of macrophages.

KEYWORDS

p62, ASC, IL-1β, IL-10, IFN-I, IFN-β, autophagy, inflammasome

Introduction

The Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes gastroenteritis in humans and typhoid fever in mice, which is accompanied by severe intestinal inflammation. After overcoming the intestinal epithelium, *S.* Typhimurium is targeted and phagocytosed by macrophages acting as a first line of defense against invading bacteria (Broz et al., 2012). Macrophages sense *S.* Typhimurium through their toll-like receptors (TLRs), which are activated by multiple pathogen-associated molecular pattern molecules (PAMPs), such as *S.* Typhimurium-derived lipopolysaccharide (LPS). LPS engages TLR4 signaling culminating in the activation of nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3), which control several inflammatory cytokine cascades as well as type I interferon (IFN-I) production (Medzhitov, 2001). While proper activation of inflammatory signaling pathways is critical for innate and adaptive immune responses to *S.* Typhimurium, excessive inflammation causes tissue damage and systemic inflammatory response syndrome (SIRS) associated with increased morbidity and mortality (Ramachandran, 2014). Therefore, balancing pro-and anti-inflammatory responses is important to successfully combat infection.

Inflammasomes are intracellular multi-protein complexes that mediate the release of the pro-inflammatory cytokine interleukin (IL)-1β in response to a broad range of infectious stimuli (Schroder and Tschopp, 2010). Inflammasomes typically consist of a NOD-like receptor (NLR), which senses microbial or endogenous danger signals, and the adaptor Apoptosis-associated Speck-like protein containing a CARD (ASC), which is required for recruitment of pro-caspase-1 (Kelley et al., 2019). Following autocatalytic cleavage, caspase-1 proteolytically processes pro-IL-1β into its mature form, a process termed inflammasome maturation (Lamkanfi and Dixit, 2009). In macrophages, *S.* Typhimurium has been reported to synergistically activate NLRC3 (also known as Ipaf) and NLRP4 (also known as Nalp3) inflammasomes in a virulence-dependent manner (Broz et al., 2010). Inflammasome activation is critical for the innate immune defense against *S.* Typhimurium as mice lacking NLRC3 and NLRP4 or caspase-1 are more susceptible to *S.* Typhimurium infection (Broz et al., 2010).

Macroautophagy (hereafter, autophagy) has recently been suggested to restrict inflammation by targeting inflammasomes for lysosomal degradation (Shi et al., 2012). Autophagy is a highly conserved catabolic process by which protein aggregates or damaged cell organelles are engulfed into LC3 positive double-membrane vesicles, called 'autophagosomes'. Autophagosomes subsequently fuse with lysosomes thereby promoting the proteolytic cleavage of engulfed cargo (Mizushima et al., 2008; Deretic et al., 2013). Proteins selected for autophagy are decorated with a ubiquitin signal that recruits the autophagic adaptor Sequestome-1 (hereafter, p62) (Matsumoto et al., 2011). P62 delivers ubiquitinated proteins for autophagic degradation by bridging ubiquitin chains and autophagosome-associated LC3 via its ubiquitin-associated (UBA) and LC3 interaction region (LIR), respectively (Pankiv et al., 2007; Matsumoto et al., 2011; Albornoz et al., 2019). Although p62 has extensively been studied during autophagy, its importance for regulating inflammasome activation remains poorly understood.

We report here that p62 restricts IL-1β release from *S.* Typhimurium-infected bone marrow-derived macrophages (BMDMs). Mechanistically, p62 was recruited to ASC-containing inflammasomes promoting their autophagic degradation. In addition to its negative impact on IL-1β secretion, p62 also impaired IL-10 and IFN-β release. Taken together, our findings identify p62 as a negative regulator of multiple inflammatory pathways and extend current knowledge on autophagy-dependent inflammasome regulation in macrophages.

Results

S. Typhimurium infection inhibits autophagy and promotes inflammasome activation

We first investigated the expression of the autophagic marker proteins p62 and LC3 following infection of BMDMs with *S.* Typhimurium. In line with our previous work (Ganesan et al., 2017; Hos et al., 2017), p62 levels were markedly decreased at 1 h of *S.* Typhimurium infection compared to uninfected BMDMs (Figure 1A). However, p62 expression was significantly enhanced after 4 h and 6 h of infection (Figure 1A). As p62 is degraded along with autophagic cargo, its protein levels are inversely correlated with autophagic activity. Accordingly, *S.* Typhimurium transiently induced autophagy at 1 h of infection, as assessed by enhanced LC3-I to LC3-II conversion rates (Figure 1B), which resulted in reduced p62 levels (Figure 1A). By contrast, LC3-I to LC3-II conversion was strongly reduced at 4 h and 6 h of infection (Figure 1B), indicative of autophagy inhibition, which promoted p62 accumulation (Figure 1A).

We next assessed *S.* Typhimurium-induced inflammasome activation. As displayed in Figure 1C, *S.* Typhimurium infection caused the formation of large ASC-positive protein complexes (>1 μm), termed ASC 'specks', which have been used as a marker for NLRC3 and NLRP4 inflammasome activation (Stutz et al., 1040). ASC is an essential component of NLRP3 and NLRP4 inflammasomes mediating the downstream activation of caspase-1, which cleaves pro-IL-1β into its active form that is subsequently secreted into the extracellular space (Lamkanfi and Dixit, 2009). Consistently, we found that pro-IL-1β was expressed in BMDMs after 4 h and 6 h of *S.* Typhimurium infection (Figure 1D). After 6 h and 24 h, mature IL-1β was secreted in the supernatants of *S.* Typhimurium-infected BMDMs (Figure 1E). Finally, IL-1 β was significantly reduced in ASC-knockout macrophages (Figure 1F).

These results demonstrate that *S.* Typhimurium inhibits autophagy whereas it promotes inflammasome activation.

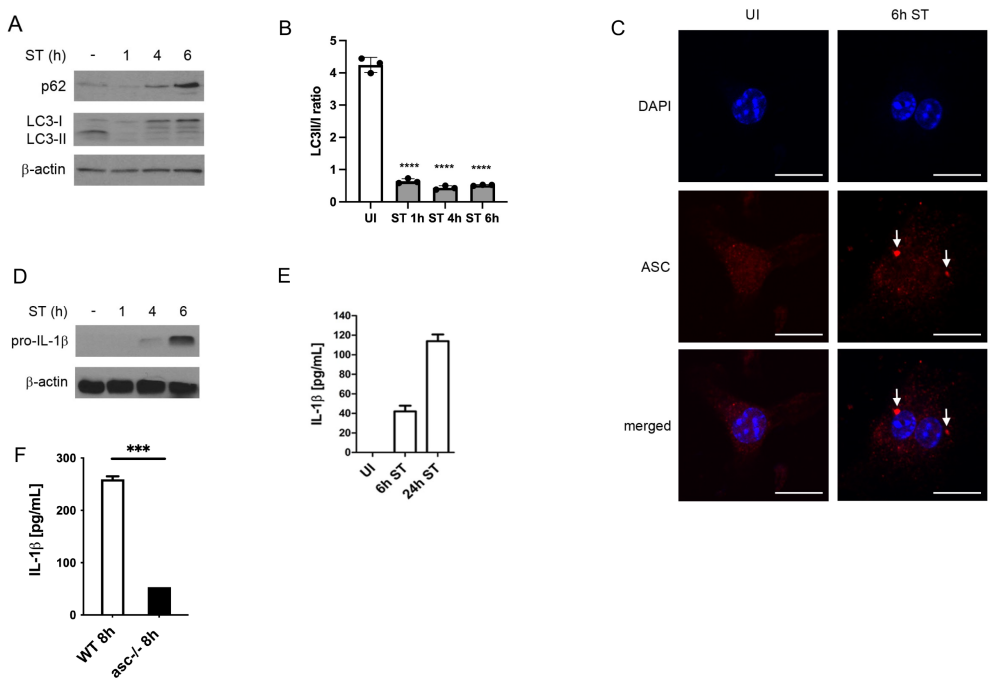

FIGURE 1
S. Typhimurium infection inhibits autophagy and promotes inflammasome activation. **(A)** Immunoblot analysis of autophagy marker proteins. BMDMs were infected with *S.* Typhimurium (ST) for the indicated time and p62 and LC3 expression was determined in total cell lysates by immunoblot. Upon autophagy induction, LC3-I is converted into lipidated LC3-II, which is recruited to the autophagosomal membrane. Both, p62 and LC3-II are degraded along with autophagic cargo and their expression levels are therefore inversely correlated with autophagic activity. **(B)** Quantification of LC3 II/I ratio by densitometric analysis **(C)** Immunofluorescence staining of activated inflammasomes. BMDMs were infected with *S.* Typhimurium (ST) for 6 h, immunostained for ASC (red) and examined by confocal microscopy (scale bars, 10 μm). Arrows point to ASC specks, indicative of activated inflammasomes. UI, uninfected. **(D)** Immunoblot analysis of IL-1β expression. Pro-IL-1β and caspase1 expression was determined by immunoblot in total cell lysates of BMDMs infected with *S.* Typhimurium (ST) for the indicated time. IL-1β is expressed as a pro-protein that requires caspase-1-mediated cleavage before secretion. **(E)** Analysis of IL-1β secretion by ELISA. BMDMs were infected with *S.* Typhimurium (ST) for 6 h and 24 h, respectively, and supernatants were collected and analyzed for IL-1β secretion by ELISA. Following cleavage of pro-IL-1β by caspase-1, mature IL-1β is secreted to the extracellular space. UI, uninfected. **(F)** Analysis of IL-1β secretion by ELISA. WT and ASC-/- BMDMs were infected with *S.* Typhimurium (ST) for 8 h and supernatants were collected and analyzed for IL-1β secretion by ELISA.

P62 restricts inflammasome availability

Recent reports suggested that autophagy and inflammasome activation are tightly co-regulated. Following pharmacological inflammasome activation, ASC-positive inflammasomes have been shown to be targeted for autophagic degradation (Shi et al., 2012). To investigate if inflammasomes are also degraded by autophagy during *S.* Typhimurium infection, we analyzed whether the autophagic adaptor p62 is translocated to ASC-positive inflammasomes. As shown in Figures 2A, B, p62 strongly co-localized with ASC 1 h and 6 h after *S.* Typhimurium infection, indicating that ASC-containing inflammasomes were targeted for p62-mediated selective autophagy.

To assess the role of p62 in regulating autophagy and inflammasome activation in more detail, we transfected BMDMs with non-specific siRNA (siCtrl) or siRNA against *Sqstm1* (the gene encoding p62; sip62) prior to *S.* Typhimurium infection. In line with our previous finding (Figure 1A), LC3-I to LC3-II conversion, indicative of autophagy induction, was transiently triggered at 1 h of infection in siCtrl BMDMs (Figure 2C). After 4 h and 6 h of infection, LC3-I to LC3-II conversion was reduced, indicative of autophagy inhibition (Figures 2C, D). Importantly, knockdown of p62 resulted in reduced LC3-I to LC3-II conversion throughout infection (Figure 2D), demonstrating that p62 deficiency impaired autophagy initiation.

Given that p62 mediates selective autophagy of ASC-containing inflammasomes (Figure 2A), we hypothesized that genetic silencing of *Sqstm1/p62* results in ASC accumulation. Indeed, ASC expression was significantly increased in *S.* Typhimurium-infected sip62 BMDMs, which are defective in autophagy, compared to siCtrl BMDMs (Figure 2E). Likewise, pro-IL-1β expression and caspase 1 were also enhanced in *S.* Typhimurium-infected sip62 BMDMs compared to siCtrl BMDMs at 6h post infection when autophagy is inhibited and p62 accumulates (Figure 2E).

We therefore conclude that p62 inhibits inflammasome availability during *S.* Typhimurium infection by targeting major inflammasome components, ASC, caspase-1 and pro-IL-1β.

P62 negatively regulates major inflammatory pathways

Having shown that p62 limits inflammasome availability through reducing ASC and pro-IL-1β levels, we next analyzed

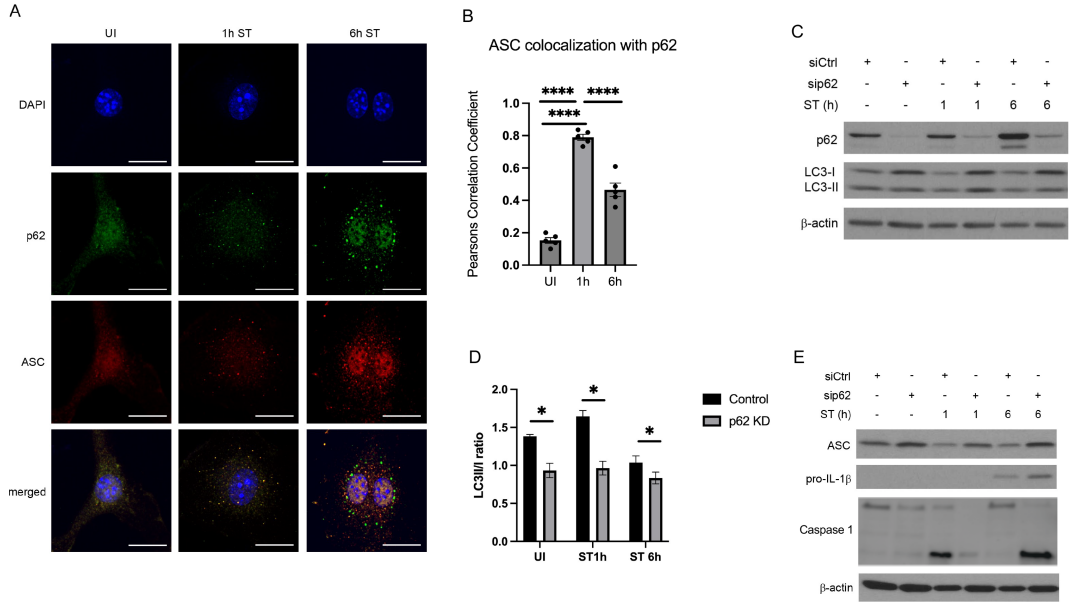

FIGURE 2
P62 restricts inflammasome availability. **(A)** Immunofluorescence staining of p62 and ASC co-localization. After 1h and 6 h of *S*. Typhimurium (ST) infection, BMDMs were immunostained for p62 (green) and ASC (red). P62 and ASC co-localization was examined by confocal microscopy. Scale bars, 10 µm. UI, uninfected. **(B)** quantification of colocalisation of asc and p62 analyzed with Person Correlation Coefficient **(C)** Immunoblot of p62-dependent autophagy initiation. BMDMs were transfected with non-targeting (siCtrl) or siRNA specific for *Sqstm1/p62* (sip62). At 48 h of transfection, BMDMs were infected with *S*. Typhimurium (ST) for the indicated time and p62 and LC3 expression in total cell lysates was determined by immunoblot. **(D)** Quantification of LC3 II/I ratio by densitometric analysis **(E)** Immunoblot of p62-dependent inflammasome availability. BMDMs were transfected with non-targeting (siCtrl) or siRNA specific for *Sqstm1/p62* (sip62) for 48 h. Thereafter, BMDMs were infected with *S*. Typhimurium (ST) for the indicated time and pro-IL-1β, caspase 1 and ASC expression was determined in total cell lysates by immunoblot.

whether p62 also exerted a negative effect on inflammasome maturation. Compared to siCtrl BMDMs, knockdown of p62 significantly enhanced secretion of mature IL-1β after 6 h of *S*. Typhimurium infection (Figure 3A), indicating that p62 additionally impairs inflammasome maturation.

We next assessed the role of p62 in regulating anti-inflammatory signaling pathways. As shown in Figures 3B, C, *S*. Typhimurium induced the secretion of anti-inflammatory IL-10 and immunomodulatory IFN-β from siCtrl BMDMs 6 h after infection. Notably, *S*. Typhimurium-infected sip62 BMDMs secreted approximately 30% more IL-10 than the corresponding controls (Figure 3B). Furthermore, *S*. Typhimurium infection led to 2-fold higher IFN-β secretion from sip62 BMDMs compared to controls (Figure 3C). These results therefore suggest that p62 is a negative regulator of not only IL-1β but also other cytokines such as IL-10 and IFN-β during *S*. Typhimurium infection of macrophages.

Next, we asked if autophagy could restrict the increase in cytokine expression during *S*. Typhimurium infection. We found that IL-1β, IL-10 and were down regulated in Atg7-/- BMDMs infected with *S*. Typhimurium (Figures 3D, E). This suggests that increased cytokine secretion is independent of autophagy.

Discussion

In our present work, we have investigated the role of the autophagic adaptor p62 in regulating inflammasome activation during *S*. Typhimurium infection of macrophages. We report here, that p62 impaired inflammasome activation and subsequent IL-1β secretion independent of autophagic degradation. Beyond restricting IL-1β release, p62 also decreased IL-10 and IFN-β secretion, indicating that p62 plays a critical role in balancing major pro- and anti-inflammatory signaling pathways during *S*. Typhimurium infection of macrophages.

We demonstrate here that p62 controls inflammasome availability to restrain IL-1β secretion from *S*. Typhimurium-infected macrophages. Specifically, we found that p62 was recruited to ASC. Accordingly, Shi et al. reported that p62 was translocated to ubiquitinated ASC on NLRP3 inflammasomes of THP-1-derived macrophages following treatment with LPS and ATP (Shi et al., 2012). While these findings confirm the importance of p62 for acting as an adaptor during selective autophagy of inflammasomes, our results additionally indicate that p62 plays a crucial role in promoting autophagy itself. Knockdown of p62 markedly reduced LC3-I to LC3-II conversion, indicative of impaired autophagy induction, resulting in the accumulation of the inflammasome components ASC and IL-1β. Our finding that p62 initiates autophagy is supported by a previous study showing that knockdown of p62 suppresses TLR4-mediated LC3 conversion in macrophages (Fujita et al., 2011). Together, these findings imply a role for p62 in regulating inflammasome pathway and autophagy during *S*. Typhimurium infection of macrophages.

Autophagy represents a major regulatory pathway for the control of activated inflammasomes in macrophages (Harris et al.,

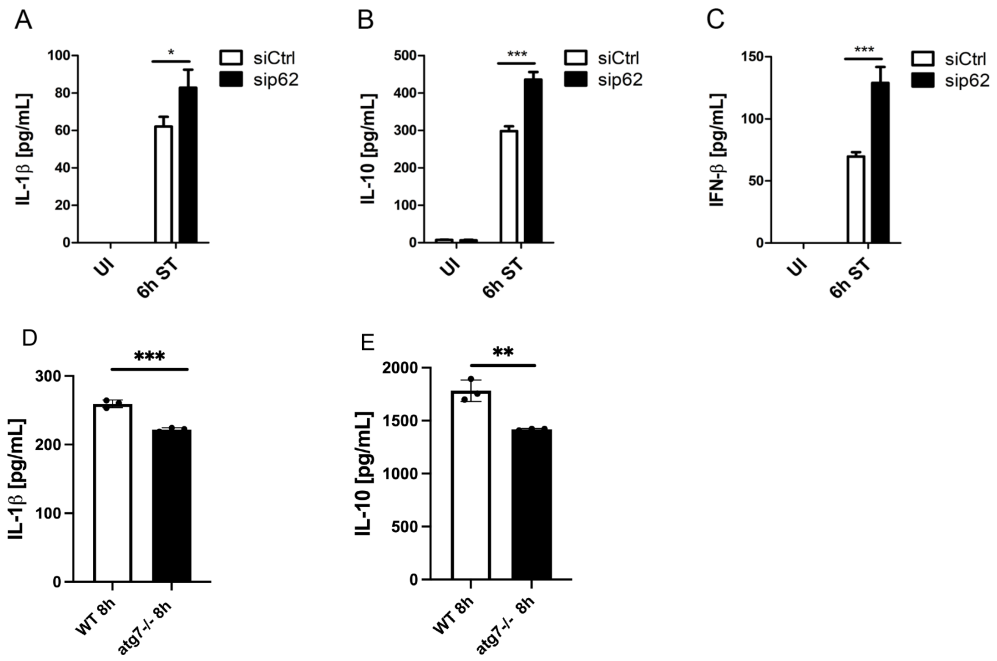

FIGURE 3
P62 negatively regulates major inflammatory pathways independent of autophagy. **(A–C)** P62-dependent IL-1β, IL-10 and IFN-β secretion. Following transfection with non-targeting (siCtrl) or siRNA specific for *Sqstm1/p62* (sip62) for 48 h, BMDMs were infected with *S*. Typhimurium (ST) for 6 **(h)** Thereafter, supernatants were collected and analyzed for **(A)** pro-inflammatory IL-1β, **(B)** anti-inflammatory IL-10 and **(C)** immunomodulatory IFN-β secretion by ELISA. UI, uninfected. **(D)** Analysis of IL-1β secretion by ELISA. WT and Atg7-/- BMDMs were infected with *S*. Typhimurium (ST) for 8 h and supernatants were collected and analyzed for IL-1β secretion by ELISA **(E)** Analysis of IL-10secretion by ELISA. WT and Atg7-/- BMDMs were infected with *S*. Typhimurium (ST) for 8 h and supernatants were collected and analyzed for IL-1β secretion by ELISA.

2011). Defective autophagy has been shown to promote IL-1β release in several inflammatory conditions, including *Mycobacterium tuberculosis* infection and Crohn´s disease (Saitoh et al., 2008; Kleinnijenhuis et al., 2011). In line with this notion, we demonstrate that blockade of autophagy upon knockdown of p62 increases IL-1β expression and secretion from *S*. Typhimurium-infected macrophages. Similarly, Zhong et al. reported that loss of p62 drives inflammasome activation and IL-1β secretion by impairing the autophagic removal of ROS-producing mitochondria from LPS-primed BMDMs treated with NLRP3 agonists (Zhong et al., 2016). Likewise, previous work from Sitoh et al. demonstrated that loss of the essential autophagy protein Atg16L1 impairs autophagy initiation and enhances IL-1β secretion from LPS-treated macrophages (Saitoh et al., 2008). Our findings at a first glance could suggest that autophagy negatively regulates inflammasome availability during *S*. Typhimurium infection of macrophages. However, autophagy has been also shown as a positive regulator of inflammasome activation and IL-1β secretion (Dupont et al., 2011). Furthermore, inflammasome also inhibits autophagy during infection (Suzuki et al., 2007). Thus, inflammasome activation and autophagy can regulate each other (Sun et al., 2017).

Our findings showing increased IL-1β secretion, IFN-β secretion and IL-10 upon loss of p62 and reduction of LC3 conversion could imply that reduced autophagy exacerbates inflammation. However, loss of autophagy during the later time points of infection reduces cytokine secretion. It is possible that accumulation of p62 due to inhibition of autophagy at later time points could be a compensatory mechanism employed by macrophages to limit inflammatory cytokine secretion. This is consistent with increased caspase-1 activation early time point (1h) when p62 levels are lower and autophagy is increased. Furthermore, previous studies have shown that p62-dependent P-bodies regulate inflammasome (Barrow et al., 2024). While our results clearly demonstrate a role for p62 in limiting pro-inflammatory IL-1β production, its importance for regulating anti-inflammatory immune responses remains controversial. Unlike inflammasome-dependent synthesis and secretion of pro-inflammatory IL-1β, are, the anti-inflammatory and immunomodulatory cytokines IL-10 and IFN-β, respectively, are secreted independent of caspase-1-mediated cleavage. In *S*. Typhimurium-infected macrophages, IFN-β is produced in response to TLR4 activation, which leads to nuclear translocation of IRF3 and transcription of *IFN-β*, the predominant IFN-I in mice (Barber, 2015). Upon binding to the transmembranic IFN-I receptor (Ifnar1), IFN-β promotes STAT1 and STAT2 dimerization as well as STAT3 homodimerization, resulting in the transcription of numerous IFN-I stimulated genes (ISGs) (Decker et al., 2005), including *IL-10* (Guarda et al., 2011). IL-10 has recently been reported to limit inflammasome availability through reducing pro-IL-1β expression in a STAT3-dependent manner (Guarda et al., 2011). Accordingly, our previous work demonstrated that IFN-β,

which acts upstream of IL-10, decreases both IL-1β expression and secretion during *S.* Typhimurium infection of macrophages (Hos et al., 2017). Moreover, our present results indicate that IFN-β and IL-10 are additionally regulated by p62, because knockdown of p62 increased IFN-β and IL-10 secretion from *S.* Typhimurium-infected macrophages. It has recently been reported that autophagy limits poly I:C-induced as well as IFN-γ-mediated IFN-I expression by targeting IRF3 for autophagic degradation (Mathew et al., 2014; Kimura et al., 2017).

In summary, this study implies a novel mechanism by which macrophages restrict autophagy and enhance p62 to regulate pro- and anti-inflammatory immune responses during *S.* Typhimurium infection. While p62 is pivotal for controlling pro-inflammatory IL-1β levels, further studies are needed to investigate the significance of p62 for regulating anti-inflammatory pathways. Nonetheless, enhancing p62 function in macrophages may be a novel strategy to control excessive inflammation in various inflammatory disorders.

Materials and methods

Generation of bone marrow-derived macrophages

Bone marrow from 8 to 12 weeks old wildtype C57BL/6 (WT) mice was differentiated into macrophages in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 20% L929 cell culture supernatant for 7 days. Non-adherent cells were removed on days 3 and 5. On day 6, cells were seeded into 6-well or 24-well sterile tissue culture plates, respectively, and adherent bone marrow-derived macrophages (BMDMs) were used for experiments from day 7 to 9. All animal procedures, including bone-marrow extraction from WT mice, were performed according to the institutional guidelines on animal welfare and were approved by the North Rhine-Westphalian State Agency for Nature, Environment, and Consumer Protection (Landesamt für Natur, Umwelt and Verbraucherschutz [LANUV] Nordrhein-Westfalen; File no: 84-02.05.40.14.082 and 84-02.04.2015.A443) and the animal care committee of the University of Cologne.

Salmonella Typhimurium infection

Salmonella enterica serovar Typhimurium (SL1344) was grown to late-exponential phase in Brain Heart Infusion (BHI) broth, resuspended in 10% of sterile glycerol and stored at -80°C until further usage. BMDMs were infected with a multiplicity of infection (MOI) of 10 for 10 min at room temperature (RT) and 30 min at 37°C to allow bacterial invasion. BMDMs were washed with RPMI medium supplemented with 50 µg/mL gentamicin to remove extracellular bacteria and were incubated with RPMI medium containing 10% FBS and 50 µg/mL gentamicin at 37°C. Gentamicin was diluted to 10 µg/mL after 2 h of infection. At the indicated time points, BMDMs were washed once with PBS and samples were collected for experiments.

Immunoblot analysis

After infection with *S.* Typhimurium for the indicated time, BMDMs (1.0-2.0 x 10^6 cells/well) were lysed with radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors and stored at -20°C. Protein concentrations were estimated with the BCA Protein Assay Kit (Pierce, #23227) according to the manufacturer`s instructions. Equal amounts of protein were mixed with SDS-PAGE sample loading buffer, boiled for 10 min, resolved by SDS-PAGE and proteins were transferred to a PVDF membrane. After blocking with 5% milk diluted in tris buffered saline (TBS) containing 0.05% Tween 20 (TBS-Tween), membranes were incubated overnight at 4°C with one of the following antibodies: anti-ASC (Santa Cruz, #sc-22514), anti-IL-1β (R&D, #AF-401-NA), LC3 (Sigma Aldrich, #L7543), SQSTM1/p62 (Cell Signaling, #5114) or β-actin (Santa Cruz, #sc-47778). Next, membranes were treated with the appropriate secondary antibodies (R&D, #HAF008, #HAF007, # HAF109) for 1 h at RT, incubated with an enhanced chemiluminescence (ECL) substrate for 1 min and exposed to X-ray films that were automatically developed with the Curix60 machine (Agfa).

Enzyme-linked immunosorbent assay

BMDMs (0.5-1.0 x 10^6 cells/well) were seeded in 6-well plates and infected with *S.* Typhimurium for 6 h and 24 h. Thereafter, cell culture supernatants from three biological replicates were collected and stored at -80°C until assayed for IL-1β (R&D, #DY401), IL-10 (R&D, #DY417) or IFN-β (pbl, #42410-1) according to manufacturer´s instructions. Absorbance was measured with a PerkinElmer multimode plate reader at 450 nm.

Immunofluorescent staining and confocal microscopy

BMDMs (0.1 x 10^6 cells/well) seeded in 24-well plates containing 12 mm coverslips were infected with *S.* Typhimurium. At the indicated timepoints, BMDMs were fixed with 4% (wt/vol) formaldehyde in PBS for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Coverslips were treated with Image-iT® R FX signal enhancer (Invitrogen, # I36933) for 30 min and blocked with 10% BSA diluted in 0.03% Triton X-100 in PBS for 1 h at RT. Next, BMDMs were incubated overnight at 4°C with primary antibodies against SQSTM1/p62 (Cell Signaling, #5114) and ASC (Santa Cruz, #sc-22514) and appropriate fluorescent secondary antibodies (Invitrogen, #A-11008, #A-11072) for 1 h at RT. Coverslips were mounted on glass slides using ProLong® Gold antifade containing DAPI (Cell Signaling, #8961) and stored at 4°C until image acquisition. Images were acquired with a 60X oil PlanApo objective on an Olympus IX81 inverted confocal microscope. Olympus Fluoview FV10-ASW 4.2 software was used for image acquisition.

Transfection experiments

BMDMs (1.0 x 10^6 cells/well) were incubated with either 50 nM of non-targeting siRNA (Eurogentec, #SR-CL000-005) or 50 nM of siRNA specific for *Sqstm1/p62* (Dharmacon, #L-047628-01-0005) together with the transfection reagent Lipofectamine 3000 (Thermo Fisher, #L3000-008) according to the manufacturer's guidelines. At 48 h post transfection, BMDMs were infected with *S.* Typhimurium for the indicated time and total cell lysates and supernatants were collected for further experiments.

Statistical analyses

Statistical analyses were performed using Prism software (GraphPad, version 5). Differences between groups were assessed by two-tailed unpaired Student´s t test. Data are representative of at least three independent experiments and results are presented as mean ± standard deviation (SD). Differences were considered statistically significant when $p \leq 0.05$ (∗), very significant when $p \leq 0.01$ (∗∗), and highly significant when $p \leq 0.001$ (∗∗∗).

Data availability statement

Requests to access the datasets should be directed to hosnina@yahoo.de.

Ethics statement

The animal study was approved by North Rhine-Westphalian State Agency for Nature, Environment, and Consumer Protection. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. JF: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. AM: Investigation, Writing – review & editing. ZH: Investigation, Writing – original draft, Writing – review & editing. MK: Resources, Writing – original draft, Writing – review & editing. DH: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. NR: Conceptualization, Funding acquisition, Resources, Supervision, 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.

Publisher's note

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Nrf2-dependent effects of CDDO-Me on bactericidal activity in macrophage infection models

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Introduction: Diabetes and chronic kidney disease (CKD) increase susceptibility to bacterial infections, particularly *Staphylococcus aureus*, which is associated with highmortality in CKD patients. Dysregulated macrophage activity and excessive oxidative stress exacerbate immune dysfunction and inflammation in these conditions. Nrf2 (nuclear factor erythroid 2–related factor 2) is a key regulator of antioxidant defenses and macrophage function. CDDO-Me, a synthetic triterpenoid, activates Nrf2, providing antioxidant and anti-inflammatoryeffects. However, its precise role in modulating macrophage activity, polarization, and bacterial clearance remains unclear.

Methods: The effects of CDDO-Me on macrophage function were evaluated *in vitro* (THP-1 and RAW 264.7 macrophages) and an *in vivo* Nrf2 knockout mouse model. Nrf2 activation was assessed via Western blot and luciferase reporter assays, oxidative stress was measured using CellROX reagent, and inflammatory responses were quantified by RT-qPCR. Intracellular *S. aureus* survival and macrophage polarization markers were analyzed to investigate the role of CDDO-Me in enhancing bactericidal activity.

Results: Our results showed that CDDO-Me activated the Nrf2 signaling pathway, reducing oxidative stress and inflammation in macrophages by downregulating pro-inflammatory cytokines (IL-1β, TNF-α). It modulated macrophage polarization, decreasing M1 and M2 marker expression, and significantly enhanced bactericidal activity against *S. aureus*. These effects were Nrf2-dependent, as demonstrated in knockout models.

Conclusion: The ability of CDDO-Me to regulate oxidative stress, inflammation, and bacterial clearance underscores its therapeutic potential for managing inflammatory and infectious diseases indiabetes and CKD.

KEYWORDS

CDDO-Me, macrophages, inflammation, bronchoalveolar lavage, oxidative stress, bactericidal activity, *Staphylococcus aureus*, Nrf2 knockout mice

Introduction

People with diabetes are at an increased risk of bacterial infections, such as foot infections and urinary tract infections, often caused by high sugar and multidrug-resistant bacteria (1).This vulnerability is further exacerbated in individuals with chronic kidney disease (CKD), as CKD weakens the immune system and impairs the body's ability to fight infections. Among the various pathogens, *Staphylococcus aureus* poses a significant threat. Studies on hemodialysis-dependent patients indicate that infections frequently arise from the patients' own *S. aureus* carriage isolates (2). Even in non-dialysis-dependent CKD patients, *S. aureus* bacteremia is associated with markedly increased mortality, highlighting the severe impact of infection in this population (3). Moreover, oxidative stress appears to be an important contributor to the progression of diabetic kidney disease (4, 5).

Inflammation serves as a critical early defense mechanism of the innate immune system, targeting pathogens such as bacteria and viruses, while promoting tissue repair and restoring homeostasis (6). A crucial feature of inflammation is the production of reactive oxygen species (ROS) and nitrogen oxygen species, which are highly toxic to pathogens and prevent their tissue invasion. A hallmark of inflammation is the recruitment of immune cells, such as macrophages and lymphocytes, and the synthesis of signaling molecules, including cytokines and chemokines, which coordinate the immune response (7).

Macrophages are major players in the first line of defense to bacterial infections. They serve a dual role in maintaining immune homeostasis under normal conditions and orchestrating robust immune responses during infections and inflammation. Macrophages exhibit remarkable plasticity, allowing them to adopt distinct functional phenotypes depending on their surrounding environment. These phenotypes range from the pro-inflammatory, classically activated M1 phenotype, essential for microbial killing and initiating immune responses, to the anti-inflammatory, alternatively activated M2 phenotype, which facilitates tissue repair and resolution of inflammation (8). However, dysregulation of macrophage activity or polarization contributes to pathological conditions, including chronic inflammatory diseases and impaired immune responses to infections (8–10). Prolonged oxidative stress and excessive production of ROS can further exacerbate tissue damage and hinder immune function (11).

The transcription factor Nrf2 (nuclear factor erythroid 2–related factor 2) plays a vital role in mitigating oxidative stress and regulating macrophage function (12). Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm by its inhibitor, Keap-1, which targets it for degradation (13, 14). However, during cellular stress, Nrf2 dissociates from Keap-1, translocates to the nucleus, activating antioxidant response elements (ARE) to control the expression of genes involved in detoxification, antioxidation, cytoprotection, and anti-inflammation (15, 16). Activating the Nrf2 signaling pathway is a promising therapeutic strategy for inflammatory and oxidative stress-related diseases, including diabetic complications (17, 18).

CDDO-Me (Methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate), a synthetic triterpenoid derived from oleanolic acid, has gained significant attention due to its ability to activate Nrf2 and exert potent antioxidant, anti-inflammatory, and cytoprotective effects in various cellular and disease models (19–22). CDDO-Me interacts with Keap1, an Nrf2 inhibitor, and modifies its Cys151 residue, disrupting its binding to Cullin 3 and allowing Nrf2 accumulation (23).

CDDO-Me has shown efficacy in various diseases, including diabetic nephropathy, cancer (leukemia, solid tumors), and neurological disorders (4, 19, 24–26) through the modulation of signaling pathways such as Nrf2, NF-κB, PI3K/AKT/mTOR, MAPK, and JAK/STAT pathways (27–30). A Japanese phase II clinical trial demonstrated that CDDO-Me improved renal function in patients with CKD associated with type 2 diabetes by enhancing glomerular filtration rate (31).

Despite its promising therapeutic potential, the precise mechanisms through which CDDO-Me modulates macrophage function and influences polarization, oxidative stress, and bacterial activity, remain unclear. To investigate this, we evaluated the effects of CDDO-Me *in vitro* using THP-1-derived and RAW 264.7 macrophages and an *in vivo* Nrf2 knockout mouse model. Our findings reveal how CDDO-Me influences cell viability, oxidative stress, inflammatory cytokine expression, macrophage polarization, and bactericidal activity, offering new insights into its role as a multifunctional therapeutic agent for inflammatory and infectious diseases.

Materials and methods

Antibodies, reagents, and compounds

Phorbol 12-myristate 13-acetate (PMA), human interferon gamma (IFNγ), lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), anti-GAPDH antibody, β-mercaptoethanol, and bardoxolone methyl (CDDO-Me) were purchased from Sigma (Merck). Dual Luciferase (Firefly-Renilla) Assay System was purchased from BPS Bioscience. DMEM (Dulbecco's Modified Eagle Medium), RPMI (Roswell Park Memorial Institute) 1640 culture medium, phosphate-buffered saline (PBS), and heat-inactivated fetal bovine serum were obtained from Eurobio Scientific. MTT cell viability assay kit (Biotium), Tryptic soy broth and tryptic soy agar (Conda laboratories) were purchased from Dutscher. Live/Dead™ BacLight™ bacterial viability kit, CellROX™ Green Oxidative Stress Reagent, iBind Western blot system, RIPA Lysis and Extraction Buffer, Pierce protease and phosphatase inhibitor cocktail, Maxima First Strand cDNA synthesis kit, Fluoromount-G™ (EMS), Lipofectamine® LTX & PLUS™ Reagent, and Trizol were purchased from ThermoFisher scientific. DC Protein Assay Reagents, 4-20% mini-Protean precast protein gels, and iTaq SYBR green supermix were purchased from Bio-Rad. Anti-Lamin AC antibody, anti-HO-1, and gentamicin were purchased from Abcam. Anti-Nrf2 antibody was obtained

from ProteinTech. IRDye® 680CW goat anti-mouse IgG, and IRDye® 800CW Goat anti-Rabbit IgG secondary antibodies were purchased from LI-COR® Biosciences.

Animals and isolation of primary mouse bronchoalveolar macrophages

C57BL/6J Nrf2 knockout (*Nrf2*$^{-/-}$) mice, provided by Dr. Yamamoto (Tohoku University, Japan) and purchased from Riken BRC (32), were bred at the UFR des Sciences de la Santé Simone Veil-Université de Versailles Saint-Quentin-en-Yvelines under the license APAFIS#28944-2021010815458509v3 approved by the Institutional Animal Care and Use Committee, and the Ministère de l'Enseignement supérieur de la Recherche et de l'Innovation. Nrf2 heterozygote (*Nrf2*$^{+/-}$) littermates were used as control to *Nrf2*$^{-/-}$ mice. Mice were maintained in a standard 12 h light/12 h dark cycle with access to food and water *ad libitum*.

Isolation of bronchoalveolar macrophages was performed via bronchoalveolar lavage (BAL). BAL was conducted following carbon dioxide euthanasia of mice, in accordance with ethical standards. Alveolar macrophages were collected through repeated lavages with 1 mL PBS. For each experimental condition, BAL fluid from a pool of 3 mice was required to obtain a sufficient number of bronchoalveolar macrophages. After cell resuspension, total cell counts were determined using the Countess automated cell counter (Invitrogen, ThermoFisher Scientific),. Primary alveolar macrophages were maintained overnight in complete DMEM supplemented with a penicillin/streptomycin mixture. The antibiotics were subsequently removed by washing with PBS prior to performing *ex vivo* experiments, followed by suspension in antibiotics-free medium.

Cell culture, CDDO-Me preparation, and cell viability assay

RAW 264.7 macrophages (ATCC® TIB-71™) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, in a humidified atmosphere at 37°C and 5% CO_2. Human monocytic THP-1 cell line (ATCC® TIB-202™) was maintained in RPMI 1640 Glutabio medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol in a humidified atmosphere at 37°C and 5% CO_2. Terminal differentiation of THP-1 to macrophages was obtained by washing cells twice with PBS prior to incubation with 50 nM PMA in β-mercaptoethanol-free complete RPMI 1640 medium for 48 h. After differentiation, cells were rinsed twice with PBS to remove PMA and fresh complete medium was added.

For *in vitro* experiments, CDDO-Me was initially resuspended in DMSO at 15 mM then diluted in PBS to the indicated concentrations. For *in vivo* studies, CDDO-Me was administered via intraperitoneal injection at a dose of 2 mg/kg (33). The control group corresponds to DMSO diluted in PBS.

Cell viability was assessed using the MTT assay. THP-1-derived macrophages were seeded at 5 x 10^4 cells/well in a 96-well plate. Cytotoxicity to control, 30% ethanol, or indicated concentrations of CDDO-Me was assessed 24 h after treatment by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay kit to measure cellular metabolic activity following the manufacturer's instructions. Absorbance changes were measured at 550 nm, with background absorbance measured at 600 nm, using the FLUOstar Omega microplate reader (BMG Labtech).

LPS stimulation and LPS/IFNγ-mediated M1 polarization of macrophages

For LPS stimulation, THP-1-derived macrophages cultured in 6-well plates at 1 × 10^6 cells/well were pretreated with control or CDDO-Me (1, 5, 10, 25, 50 nM). After 3 h, low-dose LPS (5 ng/ml) was added to the medium. After 3 h of additional incubation, total RNAs were extracted for analysis.

For M1 polarization, 24 h after PMA treatment, THP-1-derived macrophages were washed twice with PBS, then fresh culture medium supplemented with 100 ng/ml LPS and 20 ng/ml IFNγ were added for 48 h incubation to obtain proinflammatory macrophages (M1). After medium change, M0 and M1 macrophages were treated with control or CDDO-Me for an additional 48 h incubation.

Subcellular fractionation and immunoblotting

For total protein extraction, 1 × 10^6 cells/well were treated with control or CDDO-Me for 24 h before being washed twice with cold PBS and lysed with ice-cold RIPA buffer supplemented with a cocktail of protease inhibitors. After 30 min on ice, cell lysates were centrifuged at 12,000 x g and supernatants containing the protein extracts were collected.

For nuclear fractionation, THP-1-derived macrophages at 3 × 10^6 cells/25-cm^2 flask were treated with control or CDDO-Me for 8 h before protein extraction. Briefly, macrophages were washed twice with PBS and lysed with buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, pH 7.9) supplemented with a cocktail of protease inhibitors. After 10 min incubation on ice, the cell lysates were centrifuged at 900 x g. The cell pellets containing the nuclear fractions were resuspended in buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol, pH 7.9) supplemented with a cocktail of protease inhibitors. After 30 min on ice, followed by centrifugation at 10,000 x g, the supernatants containing the nuclear fractions were collected.

Protein concentrations were quantified using the DC protein assay kit (Bio-Rad) and protein extracts were resolved by SDS-PAGE on 4-20% gradient gels. After protein transfer on polyvinylidene difluoride membranes (Immobilon-FL, Merck), western blots were performed using the iBind Flex western

system (Invitrogen, ThermoFisher scientific) following the manufacturer's instructions. Briefly, primary antibodies targeting Nrf2, HO-1, GAPDH, lamin AC, and secondary antibodies, IRDye680RD and IRDye800RD, were diluted in iBind Flex FD solution. Fluorescence signals were acquired using Odyssey CLx imaging system (LI-COR) and densitometric analysis was achieved using Image Studio Lite v4.0.

Total RNA isolation, reverse transcription, and real-time quantitative PCR analysis

Total RNA was isolated from macrophages treated with either control or CDDO-Me using the Trizol reagent and chloroform extraction technique following the manufacturer's instructions. RNA concentrations were determined using the NanoPhotometer® N120 (Implen; München, Germany). One µg of total RNA was reverse transcribed to cDNA using Maxima First strand cDNA synthesis kit. Quantitative analysis was achieved using real-time quantitative PCR (RT-qPCR), with each cDNA sample done in triplicate. qPCR was realized using the Bio-Rad CFX384 Touch Real-Time PCR Detection system (Bio-Rad) and iTaq SYBRgreen qPCR mix. Table 1 lists the specific primers used for qPCR, IL-6, IL-1β, TNF-α, CCR7, IL-23, PPARγ, CCL22, IL-10, and 18S rRNA, and synthetized by Eurogentec. The cycle threshold (Ct) values of each target genes were first normalized to that of the reference gene 18S rRNA (ΔCt) then the final values (ΔΔCt values) were expressed as folds of control. Data were analyzed on the Bio-Rad CFX manager v3.1 using the ΔΔCt method.

Nrf2 activity assay

Nrf2 activity was quantified using ARE-Reporter kit (BPS Bioscience). Briefly, 7.5×10^4 THP-1 cells per well were seeded in 96-well plates and differentiated with PMA. After 24 h incubation, THP-1-derived macrophages were transfected with ARE-Firefly luciferase reporter vector mixed with constitutively-expressing Renilla luciferase reporter vector using the Lipofectamine® LTX & PLUS™ Reagent (ThermoFisher scientific) following the manufacturer's instructions, and incubated for 24 h. Transfected THP-1-derived macrophages were treated with control or CDDO-Me for an additional 48 h. Chemiluminescence signal from the dual luciferase Firefly-Renilla assay system was measured on the FLUOstar Omega microplate reader (BMG Labtech). Nrf2 activity resulted from the ratio of firefly luminescence signal to the corresponding Renilla luminescence signal.

Quantification of intracellular ROS production

ROS production was measured using the CellROX™ Green Oxidative Stress kit (ThermoFisher scientific). Briefly, 2.5×10^5 cells/well of THP-1 were seeded on coverslips placed in 24-well plates and cells were differentiated with PMA for 48 h. Briefly, macrophages were treated with control or 25 nM CDDO-Me for 3 h prior to addition or not of 5 ng/ml LPS followed by an additional 4 h incubation. Live macrophages were stained with CellROX reagent for 30 min, washed with PBS and fixed with 4% paraformaldehyde solution. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI), and cells were mounted with the Fluoromount-G aqueous mounting medium. Fluorescent images were taken with Leica SP8 confocal microscope (Leica Microsystems) and fluorescent signals were analyzed from selected 7 fields per treatment using Image J v1.53k (National Institutes of Health).

Bacteria strains and growth culture

The gram-positive bacteria, *Staphylococcus aureus* strain (ATCC 25923), was grown aerobically in Trypticase soy broth to the optical density of 1 at 37°C under agitation. Bacterial glycerol stocks were prepared and when required, frozen stocks were thawed and bacteria were diluted in PBS at the appropriate MOI. For intracellular bacterial survival assay, bacteria were seeded on Trypticase soy broth solidified with 1.5% agar.

Bacteria intracellular survival assay

THP-1-derived macrophages and bronchoalveolar macrophages, seeded in 24-well plates at 2.5×10^5 cells/well, were treated with control or 25 nM CDDO-Me for 24 h prior to infection with *S. aureus* at an MOI of 10. Gentamycin was added to the cell culture medium 1 h after infection to kill any extracellular bacteria, and cells were incubated for an additional 24 h before colony forming unit (CFU) assay. Briefly, cells were washed twice with PBS and lysed cells in 1 ml ice-cold sterile water for 20 min. Numeration of intracellular bacteria was obtained by plating 5-fold serial dilutions on Trypticase soy agar plates and incubating at 37°C for 24 h.

Bacteria viability

Viability of *S. aureus* was assessed using the Live/Dead™ BacLight™ bacterial viability kit (ThermoFisher scientific) according to the manufacturer's recommendations. SYTO9 was used to stain bacteria and Propidium Iodide (PI) to stain membrane-damaged bacteria. Stained bacteria were incubated with control or 25 nM CDDO-Me for 90 min. During that incubation time, fluorescent signals were monitored on the FLUOstar Omega microplate reader (BMG Labtech) at 45, 60, and 90 min using 488 nm excitation and measuring emission signals at 530 nm and 630 nm.

TABLE 1 Primers used in qPCR for THP-1-derived macrophages.

Name	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
Human IL-23	GTTCCCATACCAGTGTGG	GAGGCTTGGAATCTGCTGAG
Human CCR7	GATTACATCGGAGACAACACCA	AGTACATGATAGGGAGGAACCAG
Human IL-1β	AATGATGGCTTATTACAGTGGCA	GTCGGAGATTCGTAGCTGGA
Human IL-6	GTAGCCGCCCCACACAGA	CATGTCTCCTTTCTCAGGGCTG
Human TNF-α	GGAGAAGGGTGACCGACTC	TGGGAAGGTTGGATGTTCGT
Human PPARγ	TTCAGAAATGCCTTGCAGTG	CCAACAGCTTCTCCTTCTCG
Human CCL22	ATTACGTCCGTTACCGTCTG	TAGGCTCTTCATTGGCTCAG
Human IL-10	TCAAGGCGCATGTGAACTCC	GATGTCAAACTCACTCATGGCT
Human 18S rRNA	GATAGCTCTTTCTCGATTCCG	CTAGTTAGCATGCCAGAGTC

Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM). All experiments were performed independently and derived from at least 3 independent replicates. Statistical comparisons and graph design were conducted using GraphPad Prism (v8). Student's unpaired *t*-test was used for comparison between two groups, while one-way ANOVA was used for comparisons among multiple groups. A p value <0.05 was considered significant.

Results

CDDO-Me activates the Nrf2 signaling pathway

The toxicity of CDDO-Me was initially tested in THP-1-derived macrophages at concentrations of 10, 25, and 50 nM. Cells were incubated with CDDO-Me for 24 h, and viability was assessed using an MTT assay (Figure 1A). Incubation with 5% ethanol was used as positive control. The results showed no significant cell death at any concentration compared to the control cells. In RAW 264.7 macrophages, CDDO-Me activated the Nrf2 signaling pathway (Figure 1B). Specifically, treatment with 25 nM CDDO-Me significantly increased the expression of Nrf2 and HO-1 proteins.

Similarly, THP-1-derived macrophages responded to 25 nM CDDO-Me stimulation by showing an increase in Nrf2 protein expression (Figure 1C).

CDDO-Me induces Nrf2 nuclear translocation and Nrf2 activity

To determine whether CDDO-Me activates Nrf2, RAW 264.7 macrophages were treated with increasing concentrations of CDDO-Me for 24 h. Nuclear extracts were collected and analyzed by Western blot (Figure 2A). The data showed that CDDO-Me began to stimulate Nrf2 translocation into the nucleus at 10 nM concentration and significantly induced Nrf2 translocation at concentrations of 25 nM and 50 nM. Based on its lack of effect on cell viability and its ability to induce Nrf2 nuclear translocation, 25 nM was selected as the optimal concentration of CDDO-Me for further experiments.

When THP-1-derived macrophages were treated with CDDO-Me, nuclear extracts analysis by Western blot also revealed significant Nrf2 nuclear translocation (Figure 2B). To assess Nrf2 activity, THP-1-derived macrophages were transfected with ARE-reporter, and Firefly-Renilla luciferase assays were performed. The results showed a significant increase in luminescence in CDDO-Me-treated macrophages compared to control macrophages (Figure 2C). These findings suggest that 25 nM CDDO-Me stimulates Nrf2 nuclear translocation and activates the Nrf2 signaling pathway.

Antioxidant effect of CDDO-Me on LPS-stimulated macrophages

To determine whether CDDO-Me activation of Nrf2 impacts oxidative stress in macrophages, THP-1-derived macrophages were treated with CDDO-Me for 3 h prior to stimulation with 5 ng/ml LPS, followed by an additional 3 h incubation. CellROX reagent was added to live cells to quantify of oxidative stress (Figure 3). Macrophages were fixed with 4% PFA, counterstained with DAPI, and imaged using a confocal microscopy.

The data showed a significant increase in ROS levels in LPS-stimulated THP-1-derived macrophages. However, pretreatment with CDDO-Me significantly reduced LPS-induced ROS levels compared to macrophages stimulated with LPS alone. These results suggest that CDDO-Me treatment efficiently suppresses LPS-induced oxidative stress in macrophages.

Anti-inflammatory effect of CDDO-Me in THP-1-derived macrophages

We sought to examine the effect of CDDO-Me on inflammation using RT-qPCR. THP-1-derived macrophages were treated with CDDO-Me for 6 h, after which total RNA was collected (Figure 4A).

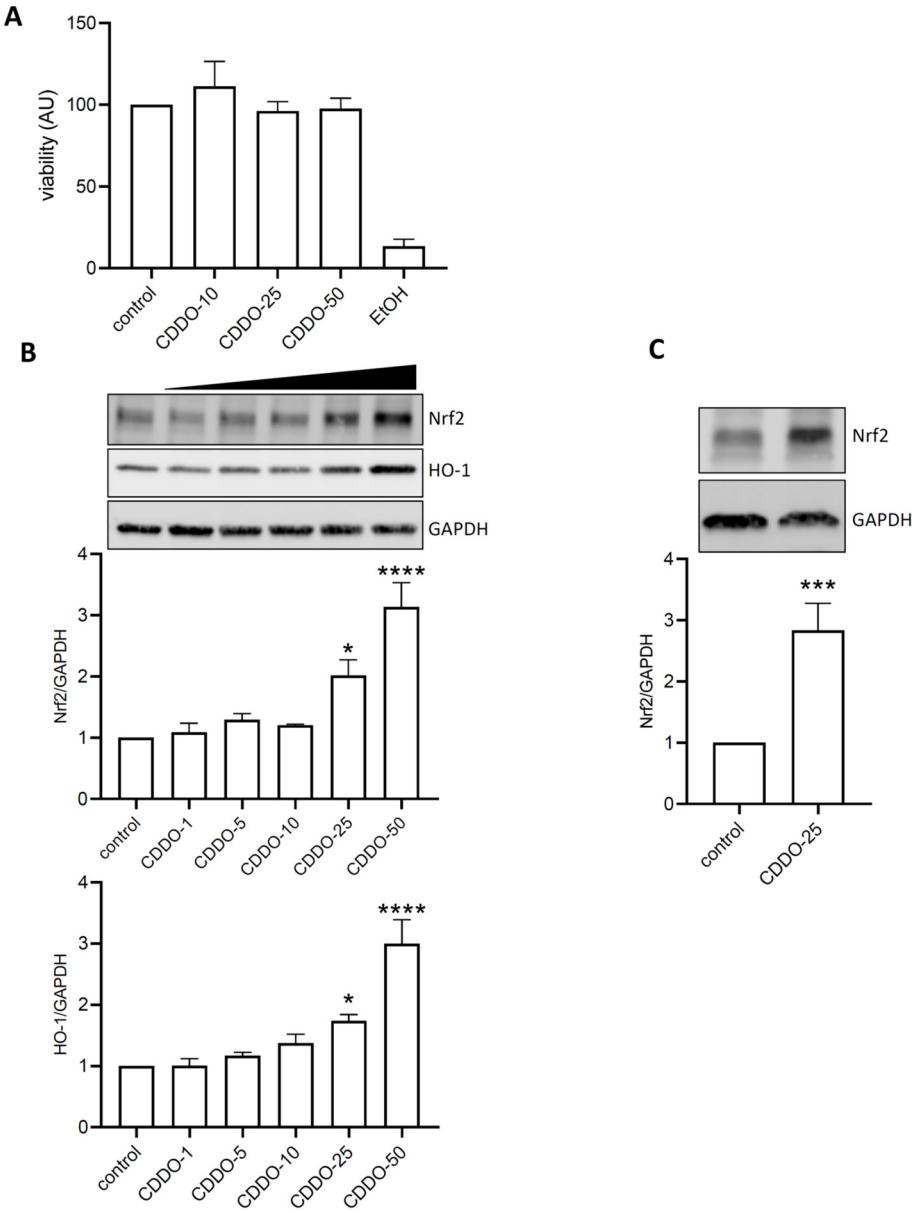

FIGURE 1
CDDO-Me activated Nrf2 signaling pathway. **(A)** THP-1-derived macrophages were treated with 10, 25, or 50 nM CDDO-Me for 24 h before testing for cell viability using MTT assay (n=5). Positive control for cell death was obtained by incubating cells with EtOH. **(B)** Raw 264.7 macrophages were incubated for 24 h in presence of the indicated concentrations of CDDO-Me. Proteins from whole cell lysates were analyzed by Western blot and Nrf2 protein expression levels were detected using specific antibodies for Nrf2 (n=4), HO-1 (n=5), and GAPDH. Immunoblots are representative of 4 independent experiments, Data are presented as mean ± SEM and comparisons were done using one-way ANOVA. **(C)** THP-1-derived macrophages were treated with 25 nM CDDO-Me. Images are representative of 5 independent experiments, Student's t-test. *p<0.05, **p<0.005, and ****p<0.001.

The expression of inflammatory markers (IL-1β, IL-6, and TNF-γ) was assessed by qPCR.

In control THP-1 macrophages, CDDO-Me treatment resulted in a significant decrease in IL-1β and TNF-α, while IL-6 mRNA expression was increased (Figure 4B). In low-dose LPS-stimulated THP-1-derived macrophages, a significant increase in the expression of genes encoding IL-1β, IL-6, and TNF-α was observed (Figure 4C).

When macrophages were pretreated with CDDO-Me prior to LPS stimulation, the expression levels of all three genes (IL-1β, IL-6, TNF-α) were significantly downregulated compared to LPS-stimulated macrophages. These results demonstrate the anti-inflammatory effects of CDDO-Me on macrophages.

CDDO-Me modulates the M1/M2 profile of THP-1-derived macrophages

Next, we examined the effect of CDDO-Me on macrophages phenotypically modified by treatment, specifically M0 and M1

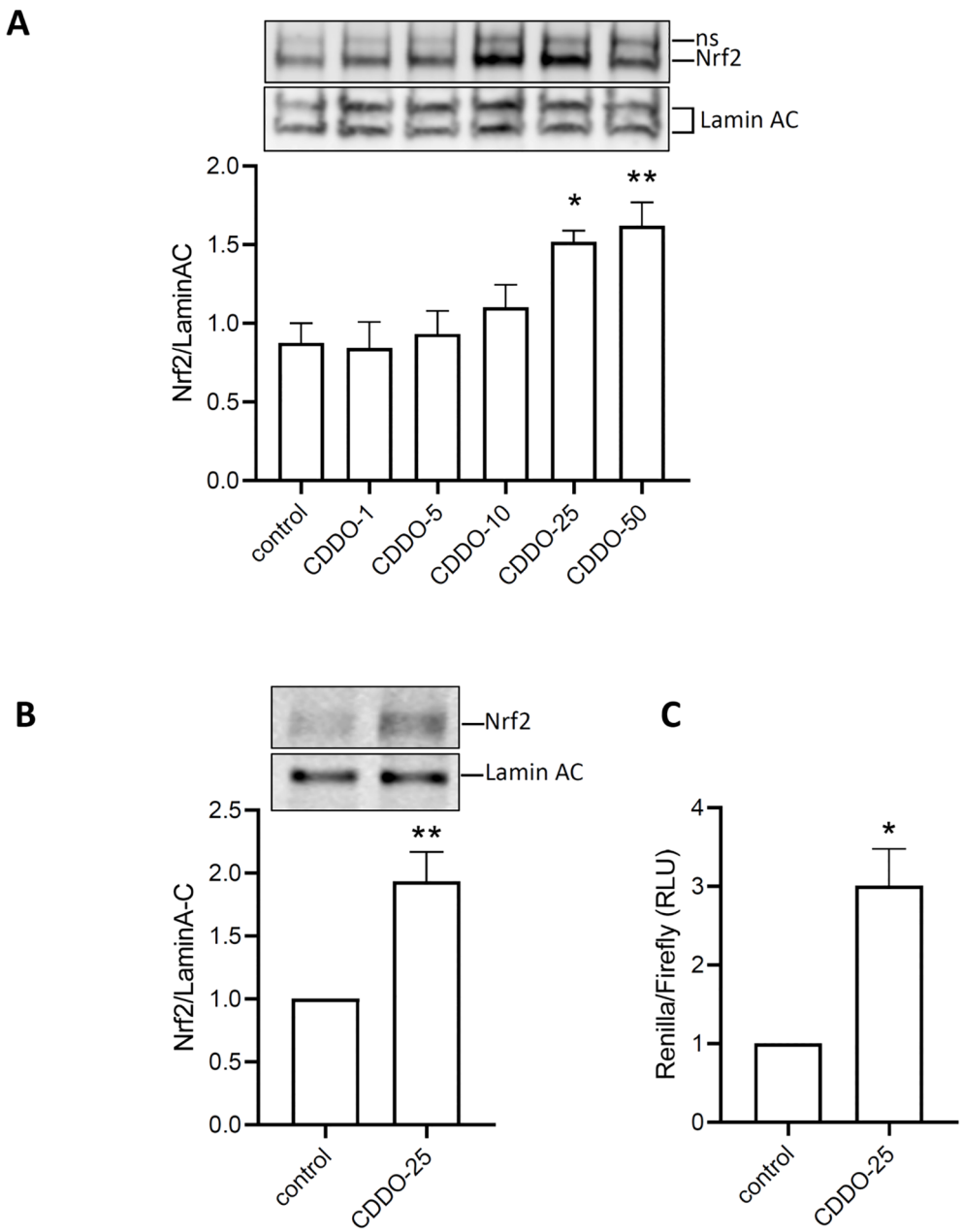

FIGURE 2
Activation of Nrf2 by CDDO-Me. **(A)** nuclear translocation of Nrf2 was observed in protein extracted 6 h after RAW 264.7 was incubated with increasing concentrations of CDDO-Me (n=5; one-way ANOVA). ns: non-specific signal. **(B)** Nuclear translocation of Nrf2 was also observed in THP-1-derived macrophages treated with CDDO-Me 25 nM (n=5, Student's t-test). **(C)** THP-1-derived macrophages, transfected with ARE-Luciferase vector for 24 h, were treated with CDDO-Me 25 nM for an additional 24 **(h)** Expression of ARE-Luciferase reporter was assessed by chemiluminescent assay (n=3 independent experiments done in triplicate, Student's t-test). *p<0.05 and **p<0.01.

macrophages. THP-1-derived macrophages were differentiated with PMA and identified as the M0 phenotype. M0 macrophages were then treated with IFNγ and LPS for 48 h to induce differentiation into M1 macrophages (Figure 5A). The modulation of gene expression for M1 markers (IL-1β, IL-6, TNF-α, IL-23, CCR7) and M2 markers (IL-10, PPARγ, CCL22) was examined by RT-qPCR.

The data showed increased expression of genes encoding M1 markers and the M2 marker IL-10 in M1 macrophages compared to M0 macrophages (Figure 5B). In contrast, the M2 markers PPARγ and CCL22 showed a significant decrease in expression.

In M0 macrophages treated with either control or CDDO-Me, a significant decrease in the expression of the M1 markers CCR7 and IL-1β was observed in CDDO-Me-treated cells compared to control-treated cells, while the expression of IL-23, IL-6, and TNF-α remained unchanged (Figure 5C). IL-10 expression was notably increased in macrophages treated with CDDO-Me compared to those treated with control.

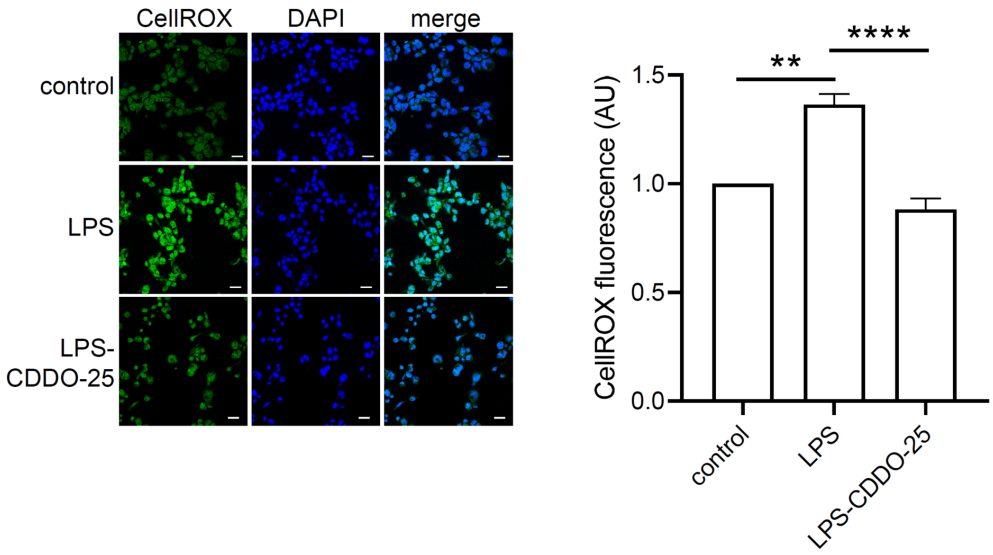

FIGURE 3
Antioxidant effect of CDDO-Me on LPS-stimulated THP-1-derived macrophages. THP-1-derived macrophages were treated with 25 nM CDDO-Me for 3 h prior to stimulation with 5 ng/ml LPS. After 3 h, live cells were stained with CellROX reagent. Stained cells were PFA-fixed, and nuclei counterstained with DAPI. Seven images were taken by confocal microscopy and CellROX fluorescent signals were analyzed using Image J software (n=3 independent experiments; Scale bar = 20 μm). **p<0.01 and ****p<0.001 using one-way ANOVA.

In M1 macrophages treated with either control or CDDO-Me, the expression of IL-23, CCR7, IL-6, and CCL22 was significantly decreased, whereas the expression of IL-1β, TNF-α, PPARγ, and IL-10 was unchanged (Figure 5D).

These results suggest that CDDO-Me treatment reduces the M1 phenotype while having no significant effect on genes encoding M2 markers.

CDDO-Me reduces intracellular survival of *S. aureus*

To determine whether CDDO-Me affects macrophage activity, we performed an intracellular bacterial survival assay. First, we assessed the toxicity of CDDO-Me on *S. aureus* using the Live/Dead bacteria viability kit. Data were collected at 3 different time points using a spectrophotometer, which showed that *S. aureus* viability was not significantly affected by incubation with CDDO-Me (Figure 6A). Next, THP-1-derived macrophages were pretreated with control or CDDO-Me for 24 h before infection with *S. aureus*. One hour after infection, gentamicin was added to the medium to eliminate extracellular bacteria that had not penetrated the macrophages. Intracellular *S. aureus* survival was assessed 24 h post-infection using a colony-forming unit (CFU) assay. The results showed that CDDO-Me significantly decreased intracellular *S. aureus* survival compared to control macrophages (Figure 6B).

These effects observed in THP-1-derived macrophages were also detected in macrophages isolated from bronchoalveolar lavages collected from control *Nrf2*$^{+/-}$ mice intraperitoneally injected with control (Figure 6C). However, in *Nrf2*$^{-/-}$ mice injected with CDDO-Me, there was no change in bactericidal activity compared to control-injected *Nrf2*$^{-/-}$ mice (Figure 6D). These findings suggest that the increase in macrophage bactericidal activity following CDDO-Me treatment is Nrf2-dependent.

Discussion

This study examined the multifaceted roles of CDDO-Me in macrophage modulation, particularly its effects on the Nrf2 signaling pathway, oxidative stress, inflammation, macrophage polarization, and bactericidal activity.

Our findings demonstrate that CDDO-Me is a potent activator of the Nrf2 signaling pathway in RAW 264.7 and THP-1-derived macrophages. Treatment with 25 nM CDDO-Me activates Nrf2, as evidenced by its nuclear translocation, increased expression of HO-1 protein, and enhanced Nrf2-dependent ARE-reporter activity. These observations are consistent with previous studies which underscore the role of CDDO-Me as a pharmacological activator of Nrf2 and its downstream antioxidant defenses in various diseases (17, 19, 30, 34).

CDDO-Me mitigates LPS-induced oxidative stress, as shown by a reduction in ROS levels in THP-1-derived macrophages pretreated with CDDO-Me. This antioxidant effect aligns with Nrf2 activation, which regulates the expression of antioxidant genes such as HO-1. Excessive oxidative stress is a hallmark of inflammation and contributes to macrophage dysfunction in various pathological conditions. Supporting this, Pei et al. reported that bardoxolone ameliorates LPS-induced acute lung injury by reducing oxidative stress and inflammation through Nrf2 activation (35). Interestingly, Moerland et al. demonstrated that CDDO-Me reduced lung tumor burden in a cancer mouse model in an Nrf2-dependent manner, with macrophage

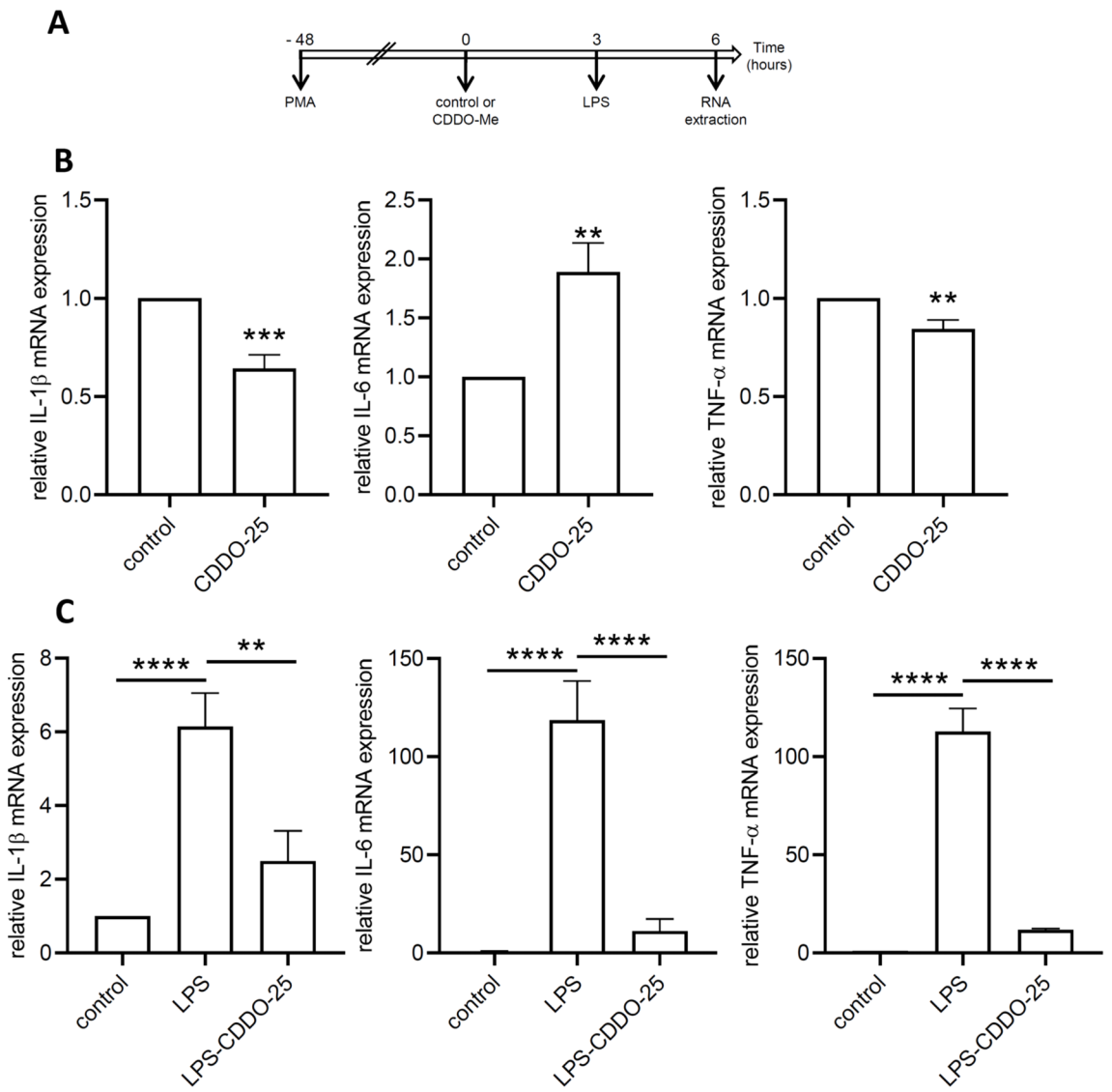

FIGURE 4
Anti-inflammatory effect of CDDO-Me. **(A)** Experimental design. **(B)** THP-1-derived macrophages were treated with 25 nM CDDO-Me or control for 6 (h) Total RNA was extracted and genes coding for IL-1β, IL-6, and TNF-α were quantified by RT-qPCR (n=5). Data are presented as mean ± SEM. **(C)** THP-1-derived macrophages were pretreated with control or CDDO-Me prior to stimulation with low-dose LPS and incubation for an additional 3 (h) Genes coding for IL-1β, IL-6, and TNF-α were quantified by qPCR (n=5 independent experiments done in triplicate, one-way ANOVA). **p<0.01, ***p<0.005, and ****p<0.001. Analysis were performed by comparing two or three groups using Student's t-test or one-way ANOVA, respectively.

polarization shifting from a tumor-promoting to a tumor-inhibiting phenotype (34).

The anti-inflammatory properties of CDDO-Me were observed under both basal and LPS-stimulated conditions 6 hours after treatment. In untreated macrophages, CDDO-Me reduced the expression of pro-inflammatory cytokines such as IL-1β and TNF-α, while modestly increasing IL-6 expression. Under LPS-stimulated conditions, pretreatment with CDDO-Me significantly downregulated IL-1β, TNF-α, and IL-6 expression. These findings suggest that CDDO-Me not only suppresses basal inflammatory signaling but also attenuates LPS-induced hyperinflammatory responses, highlighting its potential therapeutic applications in inflammatory diseases.

CDDO-Me also influenced macrophage polarization by modulating the expression of M1 and M2 markers under different environmental stimuli. The expression of M1 and M2 markers was assessed 4 days after polarization. In M0 macrophages, CDDO-Me reduced the expression of both M1 marker genes (CCR7, IL-1β) and M2 marker genes (PPARγ, CCL22, IL-10), suggesting a general dampening of polarization. Similarly, in M1-polarized macrophages, CDDO-Me reduced the expression of both M1 marker genes (IL-23, CCR7, IL-6) and M2 marker gene (CCL22). These results indicate that CDDO-Me may promote a more balanced and less inflammatory macrophage phenotype.

Furthermore, we observed that CDDO-Me enhances the bactericidal activity of macrophages against intracellular *S.*

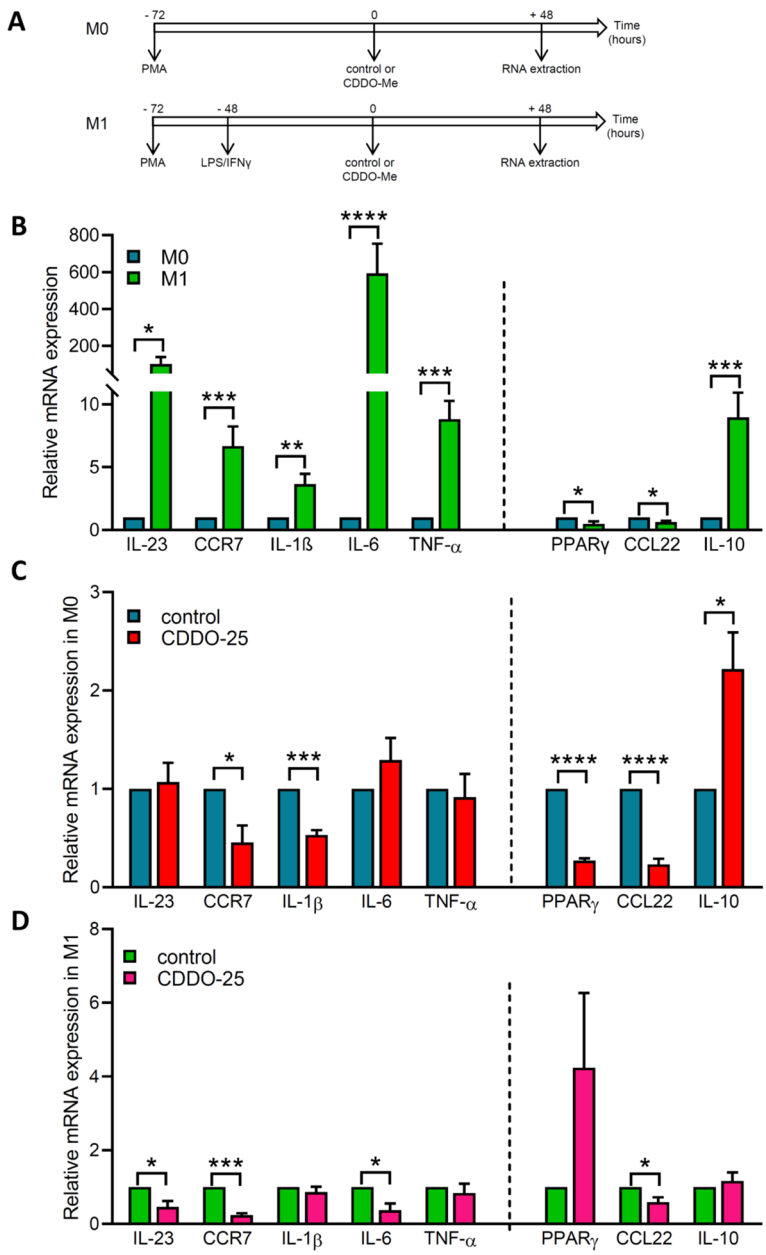

FIGURE 5
Effect of CDDO-Me on genes coding for M1/M2 polarization markers. **(A)** Experimental designs for M0 macrophages and M1 macrophages. M0 macrophages were obtained by differentiation of THP-1 cells into macrophages using PMA. M1 macrophages were obtained by incubating THP-1-derived macrophages with 100 ng/ml LPS and 20 ng/ml IFNγ. Control or 25 nM CDDO-Me were added to macrophages 72 h after PMA differentiation, and incubated for an additional 48 h before RNA extraction and RT-qPCR analysis. Graphs showed on the left side of the dash line M1 marker genes (IL-23, CCR7, IL-1β, IL-6, and TNF-α), and on the right side M2 marker genes (PPARγ, CCL22, and IL-10). **(B)** Fold-change mRNA expression levels in control-treated M1 macrophages and control-treated M0 macrophages. **(C)** Fold-change mRNA expression levels in control-treated M0 macrophages and CDDO-Me-treated M0 macrophages. **(D)** Fold-change mRNA expression levels in control-treated M1 macrophages and CDDO-Me-treated M1 macrophages (n=3 independent experiments done in triplicate). Student's t-test was used to compare genes from CDDO-Me-treated macrophages and control-treated macrophages *p< 0.05, **p<0.01, ***p< 0.005, ****p< 0.0001.

aureus. Pretreatment with CDDO-Me significantly reduced the survival of intracellular bacteria in both THP-1-derived macrophages and bronchoalveolar macrophages. However, this effect was absent in Nrf2 knockout mice, indicating that enhanced bactericidal activity is dependent on Nrf2 activation. These observations corroborate our earlier findings, highlighting the critical role of the Nrf2 signaling pathway in macrophage bactericidal function (17). Supporting evidence from Chi et al. shows that baicalein activates Nrf2 by disrupting the Keap1-Nrf2 connection, thereby reducing oxidative stress and suppressing the M1 phenotype of macrophages (36).

Future studies should investigate whether the MAPK pathways are involved in the ability of CDDO-Me to enhance bacterial clearance. We hypothesize that effects of CDDO-Me may stem from

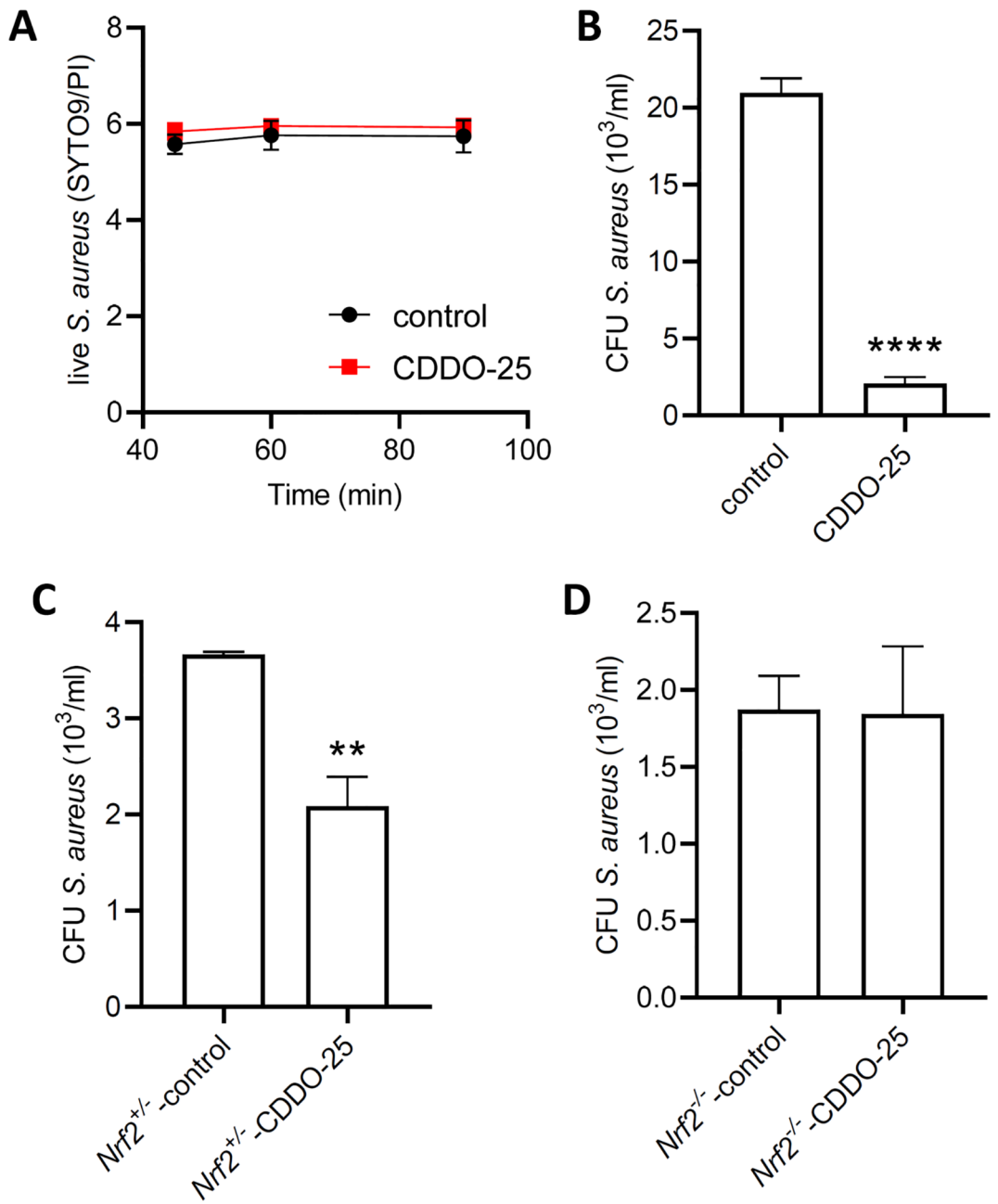

FIGURE 6
Effect of CDDO-Me on the bactericidal activity of macrophages. Bacterial viability was assessed using the BacLight bacterial viability kit. **(A)** *S. aureus* was incubated for 90 minutes in PBS containing 25 nM CDDO-Me or control. Fluorescent signals were measured at 45, 60, and 90 minutes by spectrophotometry (n=4 independent experiments done in triplicate). **(B)** THP-1-derived macrophages were pretreated with control or CDDO-Me for 24 h, infected for 1 h with *S. aureus* at MOI 10, before addition of gentamycin to the medium to eliminate extracellular bacteria. Intracellular survival of *S. aureus* was assessed 24 h after infection by CFU counts (n=4 independent experiments). **(C, D)** Macrophages isolated from BAL of *Nrf2*$^{+/-}$ control littermates **(C)** and *Nrf2*$^{-/-}$ mice **(D)** were submitted to the same treatment and infected with *S. aureus* at MOI 10. CFU counts of *S. aureus* was determined 24 h after infection (n=4 independent experiments). **p<0.01 and ****p<0.001.

its inhibition of LPS- or *S. aureus*-induced inflammation. Our lab has previously shown that Nrf2 activation reduces oxidative stress and inflammation, potentially through the inhibition of *S. aureus*-induced phosphorylation of JNK and p38 MAPK signaling pathways (37). However, this hypothesis requires further investigations.

Several limitations to consider in the use of CDDO-Me as a treatment include the increased risk of heart failure observed in clinical trials of CDDO-Me in patients with type 2 diabetes mellitus and stage 4 chronic kidney disease (38, 39), as well as gender-related differences in oxidation and inflammation, which make males more prone to higher oxidative stress and inflammation, with a weaker response to stimuli (40). Another limitation is the lower incidence of cardiovascular disease in premenopausal women (41). Recently, a phase 3 clinical trial involving patients diagnosed with Alport

syndrome has been conducted, and patients exhibited varying degrees of compromised kidney function at baseline, along with sustained improvements in kidney function and preservation of eGFR (42, 43).

Despite these limitations, the simultaneous activation of Nrf2, suppression of inflammation, reduction of oxidative stress, and enhancement of macrophage bactericidal activity position CDDO-Me as a promising therapeutic candidate for diseases characterized by oxidative stress, inflammation, and immune dysfunction. This is particularly relevant given the increased risk of *S. aureus* infections in patients with CKD, especially those undergoing dialysis, who are highly susceptible to bacterial infections. Future studies should explore its effects *in vivo* in models of chronic inflammatory diseases and infections. Additionally, elucidating the molecular mechanisms underlying its dual effects on macrophage polarization could provide deeper insights into its immunomodulatory properties.

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 studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by UFR des Sciences de la Santé Simone Veil-Université de Versailles Saint-Quentin-en-Yvelines under the license APAFIS#28944-2021010815458509v3 approved by the Institutional Animal Care and Use Committee, and the Ministère de l'Enseignement supérieur de la Recherche et de l'Innovation. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TD: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AC: Methodology, Writing – review & editing. MB: Conceptualization, Funding acquisition, Supervision, Validation, 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.

Generative AI statement

The authors declare that no generative AI was used in the creation of this manuscript.

Publisher's note

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Glossary

CCL22	C-C Motif Chemokine Ligand 22
CCR7	C-C motif chemokine receptor 7
CDDO-Me	bardoxolone methyl
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HO-1	heme oxygenase-1
IL	interleukin
JAK/STAT	Janus kinase/signal transducers and activators of transcription
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B
Nrf2	nuclear factor erythroid 2–related factor 2
PBS	phosphate-buffered saline
PI3K/AKT/mTOR	phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin
PFA	paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
PPARγ	peroxisome proliferator-activated receptor gamma
RAW 264.7	murine macrophage cell line
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
THP-1	human leukemic cell line
TNF-α	tumor necrosis factor-α

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Lessons from cross-pathogen studies: understanding the metabolic rewiring of macrophages upon infection

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Bacterial infections remain a significant cause of morbidity and mortality globally. Compounding the issue is the rise of antimicrobial-resistant strains, which limit treatment options. Macrophages play key roles in the immunity and pathogenicity of intracellular infections, such as those caused by *Mycobacterium tuberculosis* and *Salmonella*. Recent advancements have enabled us to better understand how the host orchestrates immune responses to fight these infections and, specifically how the infected cell rewires its metabolism to face this challenge. The engagement of the host cell in specific metabolic pathways impacts cell function and behaviour, and ultimately, infection outcomes. In this perspective, we summarise key findings regarding the metabolic adaptations in macrophages induced by *Mycobacterium tuberculosis* and *Salmonella* infections. We also explore how cross-pathogen studies can deepen our insights into infection biology to improve therapeutic design.

KEYWORDS

***Mycobacterium tuberculosis*, *Salmonella* typhimurium, macrophage, metabolism rewiring, host-direct therapies**

Introduction

Bacterial infections are significant contributors to deaths worldwide. The most recent report from the World Health Organization highlights that in 2019, approximately 13.7 million deaths were due to 33 bacterial pathogens, collectively making bacterial infections the second leading cause of death globally (Ikuta et al., 2022). Amongst these, infections caused by intracellular bacteria are major contributors, including *Mycobacterium tuberculosis* (*M.tb*), *Chlamydia trachomatis*, *Listeria monocytogenes* and *Salmonella enterica* (World Health Organization, 2024; Kirk et al., 2015). In this review, we focus on *M.tb* and *Salmonella enterica*, since together they are responsible for almost 20 million cases yearly, with nearly 1.5 million deaths (World Health Organization, 2023). Moreover,

both microorganisms share similarities in the immune response they elicit. For example, the induction of IFN-γ^+ CD4 T cells and the formation of granuloma-like structures are hallmark features of infection. An inability to induce these signature responses leads to uncontrolled pathogen growth in both infections (Perez-Toledo et al., 2020; Ramirez-Alejo and Santos-Argumedo, 2014), which highlights the importance of macrophages in the containment of infection.

Elie Metchnikoff discovered macrophages and their phagocytic capacity at the end of the 19th century (Metchnikoff, 1905). Since then, cumulative research has evidenced the vast macrophage heterogeneity. Different macrophage subsets in distinct body locations and at a specific moment will exist within the spectrum of fighters against infection and guardians of homeostasis. Research on how intracellular pathogens infect and survive within macrophages can reveal novel insights into the fundamental biology of such host cells. Lately, the increased interest in understanding the contribution of metabolism in macrophage physiology has also started to shed light on the role of immunometabolism in infection control. Specifically, the process of autophagy enables cell survival and proper cellular function, bridging quality control, cellular metabolism and innate immune defences. Studies in different pathological contexts have started to elucidate how autophagy and glycolysis may modulate each other. In ageing, autophagy can reset glycolysis, boosting the regenerative potential of old hematopoietic stem cells (Dellorusso et al., 2024). Our understanding of the crosstalk between autophagy and metabolic rewiring in infection settings is limited. Studies on viral immunity have reported autophagy positively (Lee et al., 2020) and negatively (Oh et al., 2021) regulating glycolysis. In this paper, we will discuss current advances in metabolism in the context of *Salmonella* and *M.tb* infection to try and complement our understanding of infection biology by comparing two microorganisms that seem so different and are yet so similar in the responses they elicit.

M.tb rewires carbohydrate and lipid metabolism in infected macrophages

The lungs of *M.tb*-infected hosts present high glycolytic activity, evidenced by transcriptomic, proteomic and metabolomic studies quantifying expression of glycolytic enzymes and lactate, the end-product of glycolysis (reviewed in Llibre, Grudzinska et al. (Llibre et al., 2021). Studies using avirulent (HR37Ra, Bacille Calmette-Guerin) or γ-irradiated mycobacteria (e.g. iH37Rv) suggested a shift towards predominantly glycolytic macrophages upon infection (MOI 1-5) (Gleeson et al., 2016; Braverman et al., 2016). This was assessed by bioenergetic profiling (i.e. extracellular flux analysis via the Seahorse platform), and by performing lactate measurements in supernatants as a surrogate of glycolytic activity. More recent studies have shed light on the intricacies and nuances of this macrophage metabolic rewiring (Brown et al., 2025). Glycolysis is critical for effective macrophage TB immunity, and virulent *M.tb* has evolved specific prevention strategies (Cumming et al., 2018; Hackett et al., 2020; Olson et al., 2021). Mechanistically, glycolysis might be essential to produce specific antimycobacterial molecules such as IL1β or nitric oxide (NO) (Tannahill et al., 2013; Gleeson et al., 2016; Huang et al., 2018; Hackett et al., 2020) or to trigger and/or regulate specific immune defence pathways such as autophagy via lactate (O Maoldomhnaigh et al., 2021; Sun et al., 2023). This glycolytic engagement by the infected macrophage helps the host clear the infection, and the strategies employed by *M.tb* to circumvent this metabolic shift might be key for mycobacterial persistence.

Lipid metabolism is profoundly reprogrammed upon *M.tb* infection, inhibiting catabolic pathways and activating *de novo* lipid synthesis and uptake [reviewed in Laval et al. (Laval et al., 2021)]. This causes the development of a foamy, lipid-rich macrophage phenotype, which is a hallmark of TB granulomas (D'Avila et al., 2006; Peyron et al., 2008). There is strong evidence supporting the use of host lipids (cholesterol and fatty acids) by *M.tb* to eat (Pandey and Sassetti, 2008), survive (Daniel et al., 2011) and specifically impair key host immune defence mechanisms (e.g. autophagy, phagosome maturation) (Singh et al., 2012; Chandra and Kumar, 2016) (Figure 1). Therefore, on one hand, the mycobacterial-induced host lipid rewiring enables pathogen survival and immune evasion. On the other, lipid droplets and lipid mediators (e.g. prostaglandin E2, Leukotriene B4) are key in generating effective anti-mycobacterial responses within the macrophage (Mayer-Barber et al., 2014; Mayer-Barber and Sher, 2015; Bosch et al., 2020; Sorgi et al., 2020). Furthermore, cholesterol-derived compounds such as oxysterols and vitamin D can restrict mycobacterial growth (Bah et al., 2017; Varaksa et al., 2021; Foo et al., 2022). Thus, the host lipid metabolic adaptation in response to infection is also essential for effective protective responses. The specific factors that tilt the balance in favour of the host or the pathogen are still not fully understood. Other relevant metabolic adaptations (e.g. amino acids, TCA cycle intermediates, ions) are beyond the scope of this *Perspective* and have been excellently reviewed elsewhere (Neyrolles et al., 2015; Hackett and Sheedy, 2020).

The new macrophage metabolic landscape triggered by infection results in changes regarding the accumulation and availability of specific metabolites. These are the primary sources of post-translational modifications and epigenetic regulation, which regulate essential cellular processes such as autophagy (Sun et al., 2023; Huang et al., 2024; Shu et al., 2023; Nieto-Torres et al., 2023; Park et al., 2022). Thus, the predominance of specific metabolic pathways will dictate gene expression profiles that may impact cell function and behaviour and, ultimately, infection outcome.

Salmonella rewires carbohydrate and lipid metabolism in infected macrophages

Salmonella enterica are important pathogens for human and animal health. Notably, serovars Typhimurium (STm) and

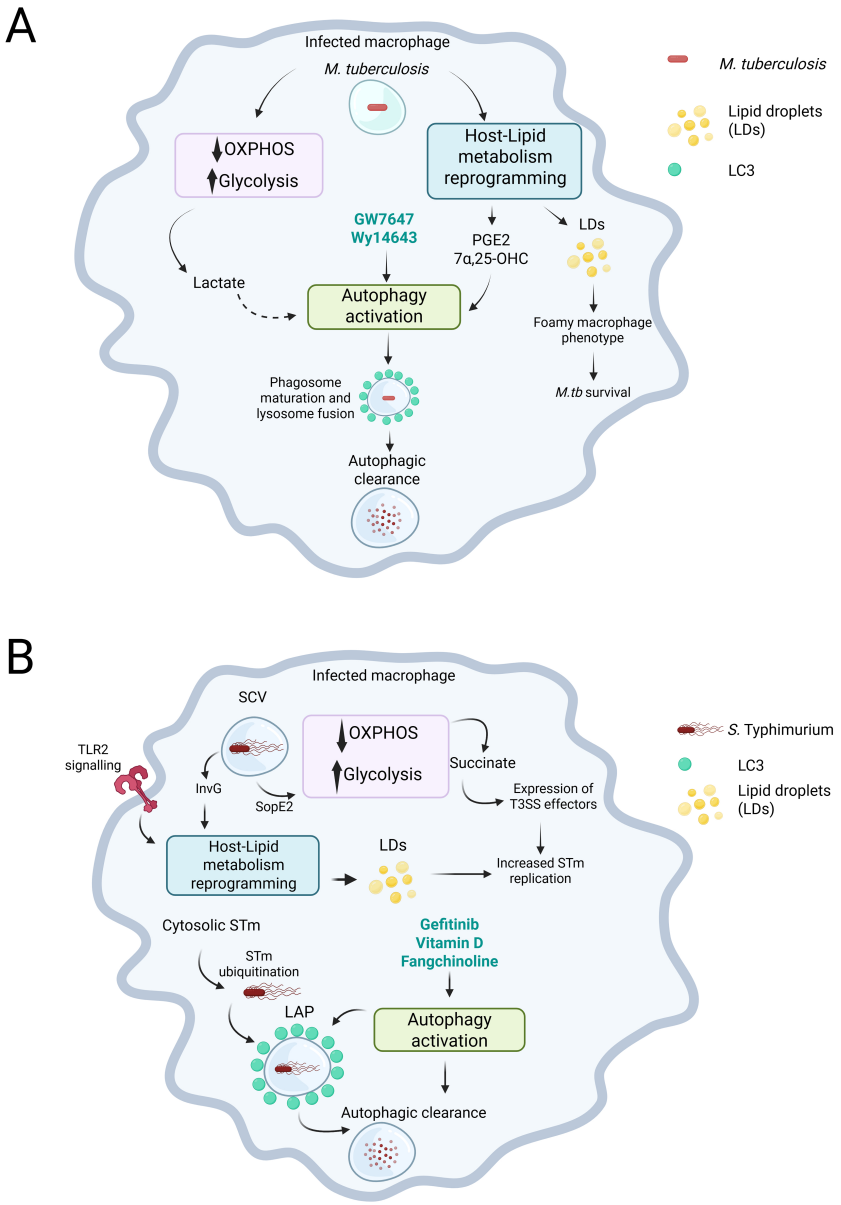

FIGURE 1
M. tuberculosis (*M.tb*) and *S.* Typhimurium (STm) induce metabolic changes in infected macrophages that impact infection outcomes. **(A)** In macrophages, infection with *M.tb* decreases the reliance on oxidative phosphorylation (OXPHOS) while enhancing glycolysis. This metabolic shift leads to increased levels of metabolites, such as lactate. Additionally, *M.tb* infection causes a reprogramming of lipid metabolism in the host. This creates a dichotomy: on one hand, the reprogramming results in the production of lipid droplets, which *M.tb* can exploit to survive within the infected macrophage. On the other hand, it can lead to the production of prostaglandin E2 (PGE2) or the oxysterol 7-α,25-dihydroxycholesterol (7-α,25-OHC), both of which can promote the activation of autophagy. Moreover, agonists of the PPARα receptor, such as GW7647 and Wy14643, can also stimulate autophagy. Although not directly shown in the context of *M.tb* infection, lactate has the potential to activate autophagy, which is an important process for bacterial control. **(B)** In the infected macrophage, STm is located within the *Salmonella*-containing vacuole (SCV). By expressing type III secretion system (T3SS) effectors, such as SopE2, STm alters the macrophage's metabolism, shifting it towards glycolysis, which leads to an accumulation of succinate. STm senses the succinate and respond by promoting the expression of additional T3SS effectors to enhance its replication. Additionally, through TLR2 signalling and the action of another T3SS effector, InvG, STm can reprogram the host's lipid metabolism to exploit it and further boosts its replication. Ultimately, compounds that promote autophagy, such as gefitinib, Vitamin D, and fangchinoline, can promote bacterial killing through the induction of LC3-associated Phagocytosis (LAP). Created in BioRender. Perez toledo, M. (2025) https://BioRender.com/y2oepra.

Enteritidis are linked to non-typhoidal salmonellosis (NTS), which occasionally develops into invasive disease (iNTS), especially in young children and immunocompromised individuals, with mortality rates reaching up to 25% (Feasey et al., 2012). As with *M. tb*, the interaction between STm and macrophages has a profound impact on the successful establishment of infection and the outcome for the host.

Recent research has highlighted how STm affects metabolic reprogramming and the subsequent impacts on infection outcomes. *In vitro*, through the *Salmonella* Pathogenicity Island-I (SPI-I)

effector SopE2, STm represses serine synthesis in peritoneal macrophages. The result is an increase in glycolysis, which is accompanied by a significant accumulation of glycolytic products, such as pyruvate, lactate, and phosphoglycerates, as well as TCA intermediates, such as fumarate, α-ketoglutarate, and succinate (Jiang et al., 2021). The bacteria can then uptake some of these intermediates and use them as carbon sources, which results in increased intracellular replication. Moreover, this effect has also been shown *in vivo*, where mice infected with STm and supplemented with lactate showed increased bacterial burdens in the spleen and liver 3 days post-infection (Wang et al., 2023). The authors attributed this effect to a switch of macrophages towards an M2-like anti-inflammatory phenotype, which has been associated with more permissiveness to STm infection (Pham et al., 2020; Panagi et al., 2020). Recent work by Rosenberg et al. reported that succinate, which accumulates due to the macrophage glycolytic shift and the concomitant truncation of the TCA cycle, is sensed by STm and induces the expression of type 3 secretion system (T3SS) effectors, such as *sseL*, *steC*, *ssrB*, and *pipB2*, resulting in increased bacterial burdens (Rosenberg et al., 2021). However, not everything is bad news for the host. Khatib-Massalha et al. showed that infection with STm increases lactate levels in the bone marrow. This increase leads to increased permeability of the bone marrow, thus increasing neutrophil mobilisation through lactate signalling via the GPR81 receptor (Khatib-Massalha et al., 2020).

Similarly to what has been observed with *M.tb* infection in macrophages, STm can also interact with the mammalian lipid metabolism to induce the production of lipid droplets (LDs). Experiments with monocyte-derived human macrophages supplemented with oleic acid showed that STm infection induced the production of LDs shortly after infection. However, this did not result in better control of bacterial infection (Bosch et al., 2020). A more recent study elegantly dissected the mechanism of LDs formation following STm infection, which required the T3SS effector *invG* and TLR2 signalling (Kiarely Souza et al., 2022). Moreover, this work also showed that the bacteria may benefit from forming LDs, as pharmacologically inhibiting key enzymes involved in forming LDs reduced bacterial burdens (Figure 1) (Kiarely Souza et al., 2022).

So far, most of the evidence shown here suggests a deleterious role for macrophages engaging in glycolysis in the outcome of STm infection, contrasting with the existing evidence in *M.tb* infection. However, this could be the result of an oversimplification of the models used, e.g. Most studies shown here use human or mouse-derived bone marrow macrophages. As not all macrophages are the same (Huang et al., 2018; Gordon and Taylor, 2005; Heieis et al., 2023; Russell et al., 2025), future studies could benefit from considering how the intrinsic differences in macrophage populations can affect their response to metabolic changes induced by pathogens. However, both STm and *M.tb* appear to exploit the changes in lipid metabolism within infected macrophages to their advantage. Further research on this shared pathway will be crucial for identifying potential targets for infection control.

Autophagy in the context of intracellular pathogens such as STm and M.tb

In the previous sections, we have summarised some key immune-metabolic events in host macrophages after *Salmonella* and *M.tb* infection, including changes in glycolysis and lipid metabolism. Immune metabolism comprises the metabolic adaptations of the host cell to face challenges such as infection. Additionally, it covers how cellular engagement on particular metabolic pathways results in the accumulation of specific metabolites (e.g. lactate, succinate, prostaglandins, oxysterols), which can directly act as signalling molecules, fuel, and/or substrates for post-translational and epigenetic modifications. Furthermore, metabolism and immunity converge in essential biological processes to regulate and tackle cellular stress. The process of autophagy perfectly illustrates this. Also known as self-eating, this cellular housekeeping strategy clears the cytoplasm from waste and defective macromolecules and organelles, acting as a quality control for the cell and enabling survival. Autophagy is not only key for metabolic homeostasis but also constitutes an innate immune defence mechanism of paramount relevance for controlling intracellular pathogens, directing them to autophagolysosomal degradation.

The role of autophagy as a key mechanism of *M.tb* defence within macrophages was first described using a combination of RAW cells (Herb et al., 2024) and murine and human monocyte-derived macrophages (Gutierrez et al., 2004). Since then, different studies have assessed the interplay and function of canonical and non-canonical autophagy in the immune responses of macrophages to *M.tb* infection. Evidence for both protective (Golovkine et al., 2023) and non-protective (Kimmey et al., 2015) roles of autophagy in the outcome of *M.tb* infection has been described. Similarly, autophagy has been reported to be an essential mechanism for intracellular control of STm. STm is targeted by autophagy when bacteria escape the *Salmonella*-containing vacuole (SCV) into the cytosol. This triggers the ubiquitination of cytosolic bacteria and the recruitment of LC3 and other autophagy proteins, restricting intracellular bacterial growth (Birmingham et al., 2006). However, STm can also exploit the autophagy machinery to promote SCV maturation, thus inducing the activation of T3SS effectors that supports bacterial replication (Figure 1) (Kreibich et al., 2015).

The macrophage metabolic rewiring that occurs upon *M.tb* and *Stm* infection has the potential to modulate autophagy. For instance, the formation and maturation of autophagosomes, as well as endosome-lysosomal degradation need lactylation of specific core autophagy proteins (e.g. PIK3C3/VPS34, TFEB, a master regulator of autophagy) (Sun et al., 2023; Huang et al., 2024). Although not specifically assessed in the context of infection, lactate has been shown to promote autophagy in different contexts, including retinal degradation (via upregulation of the LCII/II ratio), high intensity interval training (via ERK1/2/mTOR/p70S6K activation, and neurodegeneration (through cytosol acidification)

(Zou et al., 2023; Pirani et al., 2024; Fedotova et al., 2022). Distinct lipid mediators also have the potential to modulate autophagy. For example, LTB4 suppressed autophagy in mouse lung macrophages (Zhang et al., 2019a), and PGE2 induced autophagy in PBMCs from healthy donors and TB patients stimulated with *M.tb* cell lysate (Pellegrini et al., 2021). The oxysterol 7α,25-dihydroxycholesterol (7α,25-OHC) reduced mycobacterial growth through increased autophagy via GPR183 signalling in human monocytes (Bartlett et al., 2020). These examples illustrate how infection triggers host cell's metabolic adaptations, resulting in changes in the intracellular pool of available metabolites which can regulate key biological/immunological processes such as autophagy.

Researchers from both the TB and *Salmonella* fields have exploited the zebrafish model to elucidate both mechanisms of bacterial virulence and host protective responses. The autophagy receptors p62 and Optineurin were shown to be essential for protection against mycobacterial infection and to act independently (Zhang et al., 2019b; Xie and Meijer, 2023). However, the same p62 and Optineurin receptors were shown to be interdependent when promoting autophagy in the context of *Salmonella* infection. Lc3-associated phagocytosis (LAP) was identified as the primary autophagy-related process behind macrophage control for STm infection (Masud et al., 2019a, 2019b). Although the specific role of LAP in the context of mycobacterial infection has not been tested explicitly in the zebrafish model, LAP could not clear *M.tb* infection in a mouse model of TB (Koster et al., 2017). We can learn much about the biological nuances of crucial immune defence mechanisms (i.e., autophagy) by using the same model but different pathogens or distinct models with the same pathogen. Although further research is needed, distinct roles for specific components of the autophagy machinery might be pathogen-specific, knowledge that could be harnessed therapeutically.

Discussion

The threatening increase in antimicrobial resistance has prompted the search for alternative therapeutic strategies, including host-directed therapies (HDTs). In contrast to conventional antibiotic drugs, which target the pathogen, HDTs modulate specific host factors to pursue various aims. For instance, we might want to dampen inflammation to prevent immune-driven tissue pathology, enhance the bactericidal capacity of the infected macrophage, or disrupt the granuloma structure to expose the bacteria to conventional antibiotic treatments (Young et al., 2020). HDTs have dramatically changed the treatment and outcomes of specific cancers (i.e. checkpoint inhibitors such as anti-PD1 or anti-CTLA4 antibodies) (Robert, 2020); unfortunately, their use in infectious diseases has limitedly reached clinical practice.

The first step in designing and using novel HDTs is to have an accurate mechanistic understanding of the host pathway that needs targeting. For instance, anti-mycobacterial autophagy can be promoted via modulation of different host factors, including nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) and estrogen-related receptor (ERR) (Kim et al., 2019), vitamin D (Yuk et al., 2009; Campbell and Spector, 2012) and AMPK (Yang et al., 2014; Singhal et al., 2014; Parihar et al., 2014; Guerra-De-Blas et al., 2019). Specifically, the compounds GW7647 and Wy14643, which are PPARα activators, have been shown to successfully reduce bacterial burdens in *M. tb*-challenged bone marrow derived macrophages, presumably through the activation of autophagy and increased phagosome maturation (Kim et al., 2017). Although these specific PPAR agonists have not been evaluated in the context of STm infection, there is evidence that suggests that PPAR inhibition instead of activation results in better bacterial control and reduced immunopathology (Taddeo et al., 2024). However, the use of an inverse agonist of ERRγ (GSK5182) resulted in lower intracellular burdens in STm infected macrophages, although this was the result of iron metabolism modulation, which is out of the scope of our paper (Kim et al., 2014). Vitamin D induced intestinal epithelial autophagy during *Salmonella* infection (Huang, 2016; Huang and Huang, 2021). The Epidermal growth factor receptor (EGFR) inhibitor Gefitinib reduced *Salmonella* survival in macrophages and mice via host metabolic reprogramming, including autophagy (Sadhu et al., 2021). Also, fangchinoline, a phytochemical with anti-proliferative properties, promoted *Salmonella* killing *in vitro* and *in vivo* through autophagy (He et al., 2025). While most studies were performed *in vitro* using a combination of monocytic cell lines and primary mouse or human macrophages, some of them used *in vivo* models, including mice and zebrafish (reviewed in Adikesavalu et al. and Wu et al. (Wu et al., 2020; Adikesavalu et al., 2021). Furthermore, molecules such as vitamin D and AMPK modulators, such as metformin, have been and continue to be tested in clinical trials. Some (not all) studies show promising results in using these compounds in combination with conventional antibiotic treatment to improve TB outcomes (Salahuddin et al., 2013; Padmapriydarsini et al., 2022; Sutter et al., 2022; Biswal, 2021). Still, more research is needed to find effective HDTs to aid the fight against TB.

The terminology we use in science significantly shapes our understanding of the entities we study. Terms like T cells, B cells, and macrophages can limit our perspective, especially when new cell types are discovered that do not fit into existing classifications. This issue also affects how we approach the study of diseases. In cancer, for instance, concentrating on tumour types based on their tissue location may be less beneficial than focusing on the underlying mechanisms that cause the cancer, such as mutations in the p53 or BRCA genes. Consequently, therapies aimed at addressing the pathological mechanisms driven by mutations in p53 could potentially be applied to various cancer types associated with this same mutation. A comprehensive, mechanistic understanding of the metabolic rewiring undergone by host macrophages upon infection, and how these shape protective responses, is a first key step for the identification of novel host

therapeutic targets. Furthermore, the way we use language regarding pathogens and the host cellular pathways they elicit, could also open new avenues for novel treatments. For instance, instead of referring to infections caused by *Mycobacteria*, *Salmonella*, *Burkholderia*, or *Leishmania*, we could categorise them, as pathogens that promote lipid droplet formation to their advantage, (e.g. STm and *M. tb*), or as pathogens that are LAP-susceptible or LAP-resistant. This shift in how we use language, driven by a deeper understanding of the fundamental biological changes triggered by infection, could revolutionize the development or repurposing of host-directed therapies for infections that continue to claim millions of lives worldwide each year.

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 authors.

Author contributions

MP: Conceptualization, Writing – original draft, Writing – review & editing. AL: Conceptualization, Writing – original draft, Writing – review & editing.

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Depletion of *Mycobacterium tuberculosis* transmembrane protein Rv3737 reduces pathogen survival and induces M1 macrophage polarization against tuberculosis

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Objectives: *Mycobacterium tuberculosis* (Mtb) modulates macrophage polarization to evade host immunity and enhance intracellular survival. Rv3737, a probable conserved transmembrane protein in Mtb, has an unclear biological function. This study investigates the role of Rv3737 in regulating macrophage polarization and Mtb survival within host cells.
Methods: The structure of Rv3737 was predicted using bioinformatics tools. Macrophage polarization markers were assessed by real-time PCR for M1/M2-associated cytokines, and flow cytometry for $CD86^+/CD206^+$ expression. RNA sequencing, along with KEGG and GO analyses, was used to explore underlying regulatory pathways. Western blotting evaluated the phosphorylation status of NF-κB (P65, IκB) and MAPK (ERK, P38, JNK) signaling components. Colony-forming units (CFUs) and inducible nitric oxide synthase (iNOS) levels were examined in H37RvΔRv3737-infected macrophages pretreated with specific inhibitors (JSH-23, U0126-EtOH, SB203580, SP600125).
Results: Rv3737 is predicted to contain 10 transmembrane segments enriched in aliphatic amino acids. Deletion of Rv3737 in H37Rv (H37RvΔRv3737) led to upregulation of M1 markers (TNF-α, IL-1β, IL-6, iNOS, MCP-1, CD86) and downregulation of M2 markers (Arg-1, IL-10, TGF-β, CD206). Conversely, overexpression of Rv3737 (MS_Rv3737) promoted M2 polarization. RNA sequencing indicated NF-κB pathway activation in macrophages infected with H37RvΔRv3737, along with increased phosphorylation of P65, IκB, ERK, and P38. Inhibition of NF-κB (with JSH-23) and P38 MAPK (with SB203580) reduced iNOS levels and partially restored Mtb survival, indicating that Rv3737 deletion enhances the macrophage antimicrobial response.

Conclusions: Rv3737 suppresses M1 macrophage polarization to promote Mtb survival. Its deletion enhances host antimicrobial activity by activating NF-κB and MAPK signaling pathways. Targeting Rv3737 may represent a novel strategy for tuberculosis therapy.

KEYWORDS

***Mycobacterium tuberculosis*, transmembrane protein, Rv3737, macrophage polarization, intracellular survival**

1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a significant global health threat. In 2022, approximately 10.6 million individuals developed TB, and 1.3 million died from the disease—marking the first increase in TB-related mortality among HIV-negative individuals in the past 15 years (WHO, 2023). Macrophages, as the first line of defense, play a central role in the host immune response by phagocytosing Mtb. However, Mtb has evolved sophisticated mechanisms to evade immune surveillance and persist within host cells (Zarin et al., 2024). The complex interaction between Mtb and the host immune system largely determines the course and outcome of infection. Therefore, elucidating the immune mechanisms underlying TB pathogenesis is essential for developing effective treatment strategies (Hmama et al., 2015).

One of the key immune evasion strategies employed by Mtb is the manipulation of macrophage polarization (Pham and Monack, 2023). Upon infection, Mtb modulates macrophage polarization to facilitate its survival and dissemination. Macrophages exhibit plasticity and can polarize into either classically activated M1 or alternatively activated M2 phenotypes (Zheng et al., 2024). M1 macrophages produce pro-inflammatory cytokines such as iNOS, TNF-α, IL-1β, IL-6, and IL-12b, which promote Th1 responses and possess bactericidal activity. In contrast, M2 macrophages secrete anti-inflammatory cytokines like IL-10 and TGF-β and are involved in tissue repair and immune regulation (Zhang et al., 2024). The polarization state of macrophages is dynamic and reversible, depending on environmental cues (Wang et al., 2014). Numerous Mtb-derived factors, including secreted and membrane-associated proteins, contribute to the modulation of macrophage polarization and aid in immune evasion. Several secreted proteins, such as Rv1987 (Liu et al., 2023), PPE36 (Gong et al., 2021), and HSP16.3, are known to influence macrophage phenotypes. For example, ESAT-6 promotes M1 polarization early in infection but later skews macrophages toward an M2-like state (Sun et al., 2024). Rv2882c has been shown to regulate macrophage polarization (Choi et al., 2016). In addition to secreted proteins, transmembrane proteins of Mtb have also been implicated in modulating macrophage polarization. For example, the transmembrane protein Rv0309 may inhibit M1 polarization (Peng et al., 2022), while Rv0426c has been reported to promote Mtb survival by influencing macrophage polarization (Ruan et al., 2020). Understanding the paradoxical roles of macrophage polarization is essential for uncovering the underlying mechanisms. However, whether other Mtb transmembrane proteins are involved in regulating macrophage polarization—and the specific pathways by which they exert their effects—remains largely unclear.

Rv3737, a transmembrane protein of unknown function, has recently garnered attention for its potential role in Mtb pathogenesis. Structural predictions suggest Rv3737 is encoded by a non-essential gene in the H37Rv genome (Minato et al., 2019), and it shares 37.25% sequence homology with the threonine efflux carrier ThrE from *Corynebacterium glutamicum*. It contains three ThrE structural domains and is predicted to function in amino acid transport. Preliminary findings indicate that deletion of Rv3737 disrupts threonine metabolism and upregulates the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Li et al., 2022). Moreover, Rv3737 expression has been linked to amikacin resistance (Li et al., 2024), suggesting a possible association with disease severity in TB patients. Despite these observations, the role of Rv3737 in macrophage polarization and immune regulation remains largely unexplored.

In this study, we investigated the effect of Rv3737 on macrophage polarization and its influence on Mtb intracellular survival. Using an Rv3737-deletion strain (H37RvΔRv3737) and an Rv3737-overexpressing strain (MS_Rv3737), we explored how this protein modulates host immune responses. Our findings demonstrate that Rv3737 is a transmembrane protein whose deletion induces M1-type macrophage polarization, characterized by elevated expression of iNOS, TNF-α, IL-1β, IL-6, and CD86. Mechanistically, Rv3737 depletion activates the NF-κB and p38 MAPK signaling pathways, as evidenced by increased phosphorylation of p65 and p38. Pharmacological inhibition of these pathways reversed the polarization phenotype, reduced iNOS expression, and restored Mtb survival within macrophages. Together, these results reveal that Rv3737 plays a critical role in modulating macrophage polarization and suggest that it may serve as a potential therapeutic target for enhancing host immunity against TB.

2 Materials and methods

2.1 Bacterial strains, cell lines, and reagents

The *Mycobacterium tuberculosis* H37Rv wild-type strain (H37Rv-WT) was generously provided by the Tuberculosis Control Institute of Guizhou CDC. *Mycobacterium smegmatis* MC2155 was obtained from Zunyi Medical University. Recombinant strains—including MS_Vec (vector control), MS_Rv3737 (Rv3737-overexpressing), and H37RvΔRv3737 (Rv3737-deleted)—were constructed in our previous study. All strains were cultured in Middlebrook 7H9 broth (BD Biosciences, USA) supplemented with 10% OADC and the appropriate antibiotics: kanamycin (SolarBio, China) and hygromycin B (Sigma, USA). RAW264.7 murine macrophage cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 Mycobacteria infected macrophages and mice

RAW264.7 cells were seeded in six-well plates and allowed to adhere overnight at 37°C. The following day, cells were infected with either MS_Vec, MS_Rv3737, H37Rv-WT, or H37RvΔRv3737 at a multiplicity of infection (MOI) of 10. At designated time points post-infection, culture supernatants were collected for cytokine analysis. Cells were washed three times with phosphate-buffered saline (PBS) and then lysed for downstream analyses, including gene expression, protein detection, or bacterial load determination.

6–8 weeks C57BL/6 mice were purchased from the Hunan Slack Jingda Experimental Animal Co., Ltd. All animal experiments and protocols were approved by the Ethics Committee of Zunyi Medical University (ZMU22-2302-136). The mice were housed according to the standard humane animal husbandry protocol. Each mouse was intranasally infected with MS_Vec and MS_Rv3737 strains at 2×10^7/ml. After six days of infection, the Lung tissues were harvested from euthanized mice and promptly immersed in cold PBS. The tissues were mechanically homogenized with a tissue homogenizer in RIPA lysis buffer augmented with protease and phosphatase inhibitors. The homogenates underwent centrifugation at 12,000 × g for 10 minutes at 4°C to eliminate tissue debris. The resultant supernatants were collected and preserved at −80 °C until needed. Protein concentrations were quantified via a BCA assay (Thermo Fisher Scientific, 23225). The supernatants were subsequently utilized for Western blot investigation of target proteins. Lung tissues were homogenized and then diluted with PBS. 20 μL of tissue homogenate was added to 7H10 plates with or without kanamycin to analyze the CFU. A few of the lung tissue samples were fixed in 10% formalin and then embedded in paraffin, followed by hematoxylin and eosin staining and acid-fast staining for evaluating the pathological changes in the lung tissue.

2.3 Intracellular survival of H37Rv△Rv3737 and H37Rv-WT strains: colony-forming unit assay

RAW264.7 cells were infected with *M. tuberculosis* H37Rv-WT or H37RvΔRv3737 strains at a multiplicity of infection (MOI) of 10. Four hours post-infection, the culture medium was removed, and cells were washed thoroughly with PBS to eliminate non-internalized bacteria. Cells were then incubated in 10% DMEM containing 200 μg/mL amikacin and 1% antibiotic-antimycotic solution (Gibco) for an additional 24 or 48 hours to kill any remaining extracellular bacteria. To assess intracellular Mtb survival, cells were lysed with 0.01% Triton X-100. The resulting lysates were serially diluted (10^{-1} and 10^{-2}), plated in triplicate on Middlebrook 7H10 agar, and incubated at 37°C for 3–4 weeks. Colony-forming units (CFUs) were then counted to determine bacterial burden.

2.4 Real-time quantitative PCR analysis of M1/M2-associated cytokine mRNA expression

Total RNA was extracted from macrophages using RNAiso PLUS reagent, and cDNA was synthesized using the PrimeScript RT reagent kit, following previously described protocols (Zheng et al., 2024). Quantitative real-time PCR (qRT-PCR) was performed using specific primers (listed in Supplementary Table 1). Gene expression levels were normalized to GAPDH and calculated using the $2^{-\triangle\triangle CT}$ method.

2.5 Flow cytometry analysis of $CD86^+$/$CD206^+$ expression in MS_Rv3737-infected macrophages

RAW264.7 cells (5–10 × 10^5 cells/mL) were seeded in six-well plates and infected with MS_Vec or MS_Rv3737 for 12 or 24 hours. After incubation, cells were blocked with 0.25 μg of Fc receptor blocking buffer on ice for 5–10 minutes in the dark. Surface staining was performed using anti-mouse Percp-CD11b (BioLegend, 101229) and anti-mouse APC-CD86 (BioLegend, 159215) or the corresponding APC isotype control (APC Rat IgG2a, BioLegend, 400511) for 30 minutes in the dark. Cells were then washed three times with PBS and fixed with 500 μL of Fix/Perm Buffer for 30 minutes. After centrifugation at 1000 rpm for 5 minutes and removal of the supernatant, cells were permeabilized with Intracellular Staining Perm/Wash Buffer. Intracellular staining was performed using anti-mouse PE-CD206 (BioLegend, 141705) or its isotype control (PE Rat IgG2a, BioLegend, 400507) for 30 minutes in the dark. Finally, cells were washed 2–3 times and resuspended in 300 μL of 1× PBS. Flow cytometry was performed using an Accuri C6 system (BD Biosciences, San Diego, CA, USA).

2.6 RNA sequencing, coupled with KEGG and GO analyses, explored the mechanisms underlying polarization

RAW264.7 cells were infected with either H37Rv-WT or H37RvΔRv3737 at an MOI of 10 for 24 hours. After infection, cells were washed with PBS, and total RNA was extracted using RNAiso Plus (Takara, 9108Q). RNA sequencing was performed by Novogene Bioinformatics Technology (Beijing, China) using the Illumina HiSeq X Ten platform with 150 bp paired-end reads. Sequencing quality was assessed using FASTQC (v0.11.3) to identify adaptor contamination and low-quality bases. Clean reads were aligned to the mouse reference genome using HISAT2. Differential gene expression analysis was performed using DESeq2 (v1.18.1) in R (v3.4.4), applying a fold-change cutoff >1.2 and p-value <0.05. Functional enrichment analysis of differentially expressed genes was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases.

2.7 Western blot assays of critical signaling molecule expression

RAW264.7 cells were infected with MS or H37Rv strains for 0, 2, or 4 hours. After three PBS washes, cells were lysed using RIPA buffer supplemented with a protease inhibitor cocktail (MCE, HY-K0010) and PMSF (Solarbio, P0100). Lysates were centrifuged at 12,000 rpm for 10 minutes, and the supernatants were collected. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked with 5% non-fat milk for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies targeting: P65 (Proteintech, 66535-1-Ig), p-P65 (CST, 3033S), IκB (CST, 4812S), p-IκB (CST, 2859S), ERK (Proteintech, 67170-1-Ig), p-ERK (CST, 4370S), JNK (Proteintech, 511531-1-AP), p-JNK (Proteintech, 80024-1-RR), P38 (Proteintech, 66234-1-Ig), p-P38 (CST, 4511S), iNOS (CST, 2982S), Arg-1 (CST, 93668S), GAPDH (Proteintech, 60004-1-Ig), and Tubulin (Proteintech, 11224-1-AP). After washing, membranes were incubated with HRP-conjugated secondary antibodies—Goat Anti-Mouse IgG (SA00001-1) or Goat Anti-Rabbit IgG (SA00001-2)—for 2 hours at room temperature. Target proteins were visualized using ECL chemiluminescence (GE Healthcare) and quantified with ImageJ software.

2.8 Pharmacological inhibition assays: CFU and iNOS expression in inhibitor-pretreated macrophages

RAW264.7 cells were pretreated with one of the following inhibitors for 2 hours: 10 μM JNK inhibitor SP600125 (MCE, HY-12041), 10 μM P38 inhibitor SB203580 (MCE, HY-10256), 10 μM ERK inhibitor U0126-ETOH (MCE, HY-12031), or 30 μM P65 inhibitor JSH-23 (MCE, HY-13982). Following pretreatment, cells were infected with H37Rv-WT or H37RvΔRv3737 at an MOI of 10 for 24 hours. Total RNA was extracted as previously described, and iNOS mRNA levels were analyzed via qRT-PCR. For CFU assays, inhibitor-pretreated RAW264.7 cells were infected with H37Rv-WT or H37RvΔRv3737 for 4 hours. Intracellular bacterial survival was assessed via CFU counting as described in Section 2.3.

2.9 Structural prediction and bioinformatics analysis of Rv3737

Bioinformatics analysis of Rv3737 was conducted using several online tools. The FASTA sequence was retrieved from the National Center for Biotechnology Information (NCBI). Transmembrane domains were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), signal peptide regions using SignalP (http://www.cbs.dtu.dk/services/SignalP/), and conserved motifs via MEME Suite (http://meme-suite.org/).

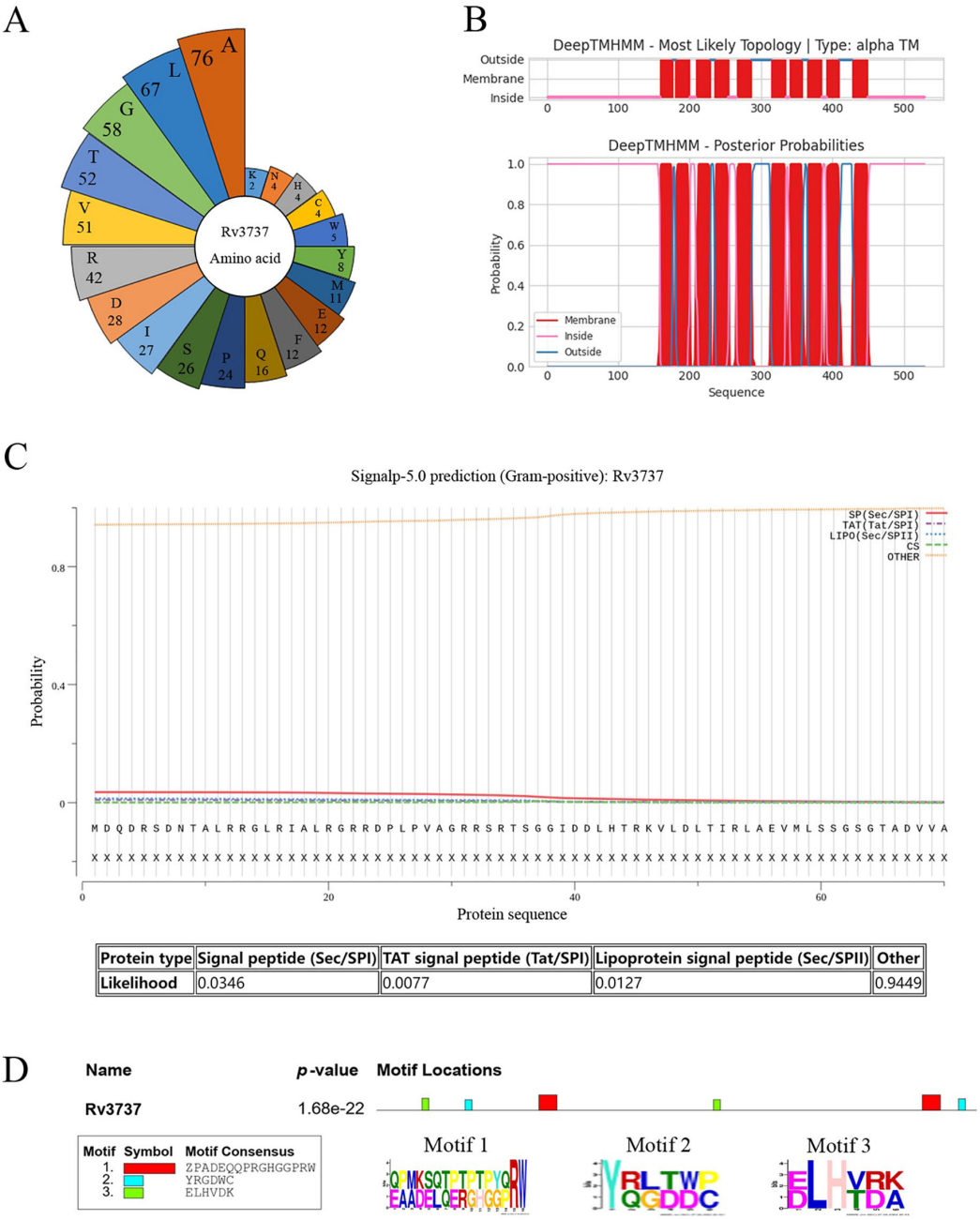
2.10 Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10.3 software. Comparisons between two groups were made using Student's *t*-test, while comparisons across multiple groups were evaluated using two-way ANOVA. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Rv3737 is a transmembrane protein rich in aliphatic amino acids

The structure and function of Rv3737 remain largely uncharacterized. To gain insights into its physicochemical properties, we conducted a comprehensive bioinformatics analysis. The five most abundant amino acids in Rv3737 were found to be aliphatic, contributing to a high aliphatic index of 111.63 (Figure 1A). Additionally, the calculated grand average of hydropathicity (GRAVY) was 0.45, suggesting strong lipophilicity and a propensity for interaction with lipid membranes. Transmembrane domain prediction revealed that Rv3737 contains 10 transmembrane segments (Figure 1B), supporting its classification as a transmembrane protein. Signal peptide prediction using SignalP 5.0 indicated that Rv3737 does not belong to the Sec/SPI, Sec/SPII, or Tat/SPI secretion pathways, implying it is a non-secretory membrane protein (Figure 1C). Sequence conservation analysis showed that Rv3737 is highly conserved in *Mycobacterium bovis* AF2122/97 (Figure 1D), suggesting a potentially important role in bacterial physiology or virulence. Taken together, these findings indicate that Rv3737 is a conserved, lipophilic transmembrane protein likely involved in essential mycobacterial functions.



Protein type	Signal peptide (Sec/SPI)	TAT signal peptide (Tat/SPI)	Lipoprotein signal peptide (Sec/SPII)	Other
Likelihood	0.0346	0.0077	0.0127	0.9449

FIGURE 1
Rv3737 as a transmembrane protein rich in aliphatic amino acids. **(A)** Distribution and abundance of amino acids in the Rv3737 protein. **(B)** Prediction of transmembrane domains using TMHMM. **(C)** Signal peptide prediction of Rv3737. **(D)** Conservation of Rv3737 motifs across four virulent *Mycobacterium* strains, including *M. bovis* AF2122-97.

3.2 Rv3737 suppresses M1 macrophage polarization and pro-inflammatory cytokine production

Macrophage polarization is a dynamic process, and M1-type macrophages play a pivotal role in the immune response against intracellular pathogens (Thomas et al., 2024). To investigate the role of Rv3737 in macrophage polarization, we infected RAW264.7 cells with either H37Rv-WT or H37RvΔRv3737 strains. Deletion of Rv3737 significantly enhanced M1 macrophage polarization, as demonstrated by the upregulated mRNA expression of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, MCP-1, and iNOS in H37RvΔRv3737-infected cells compared to H37Rv-WT-infected controls (Figure 2A). Conversely, overexpression of Rv3737 in MS_Rv3737 significantly suppressed the expression of these cytokines (Figure 2B). In line with these transcriptional changes, iNOS protein levels were markedly elevated in H37RvΔRv3737-infected macrophages (Figure 2C). Furthermore,

the expression of CD86—a surface marker indicative of M1 polarization—was significantly reduced in MS_Rv3737-infected cells at both 12 and 24 hours, as measured by mean fluorescence intensity (MFI) (Figure 2D). To further investigate the Rv3737-mediated macrophage polarization *in vivo*, a mouse model of MS_Vec or Ms_Rv3737 was constructed via intranasal infection. MS_Rv3737 inhibited iNOS mRNA expression levels in the lung tissue compared with the MS_Vec-infected group (Supplementary Figure 1C). The western blotting results indicated that Ms_Rv3737 decreased the expression of iNOS in the lung tissue (Supplementary Figures 1B, E). These findings indicate that Rv3737 negatively regulates M1 macrophage polarization and pro-inflammatory cytokine release.

3.3 Rv3737 promotes M2 macrophage polarization and anti-inflammatory cytokine production

M2 macrophages contribute to an immunosuppressive microenvironment that supports Mtb persistence and latency. To determine whether Rv3737 influences M2 polarization, we assessed the expression of M2-associated markers in infected RAW264.7 cells. Macrophages infected with H37RvΔRv3737 exhibited a significant reduction in the mRNA expression of Arg-1, TGF-β, and IL-10 compared to those infected with H37Rv-WT (Figure 3A). Arg-1 protein expression was also markedly decreased in H37RvΔRv3737-infected cells (Figures 3C, D). In contrast, overexpression of Rv3737 in MS_Rv3737-infected cells led to increased mRNA and protein expression of Arg-1, TGF-β, and IL-10 (Figures 3B, E, F). Consistently, *in vivo* experiments further confirmed that Rv3737 promotes Arg-1 expression, as evidenced by increased Arg-1 levels in lung tissues detected by Real-time PCR and Western blot analysis (Supplementary Figures 1A, D). Moreover, CD206—a surface marker of M2 macrophages—was significantly elevated in MS_Rv3737-infected macrophages at 12- and 24-hours post-infection, as shown by increased MFI levels (Figures 3F, G). These results suggest that Rv3737 promotes M2 macrophage polarization and suppresses the M1-associated pro-inflammatory response.

3.4 Rv3737 facilitated mycobacteria survival and aggravated lung pathology progression *in vivo*

M2 macrophages promote *M. tuberculosis* survival and dampen host resistance. To further investigate the role of Rv3737 in modulating host defense during Mycobacteria infection *in vivo*, we used the MS_Vec and MS_Rv3737 infected C57BL/6 mice. We

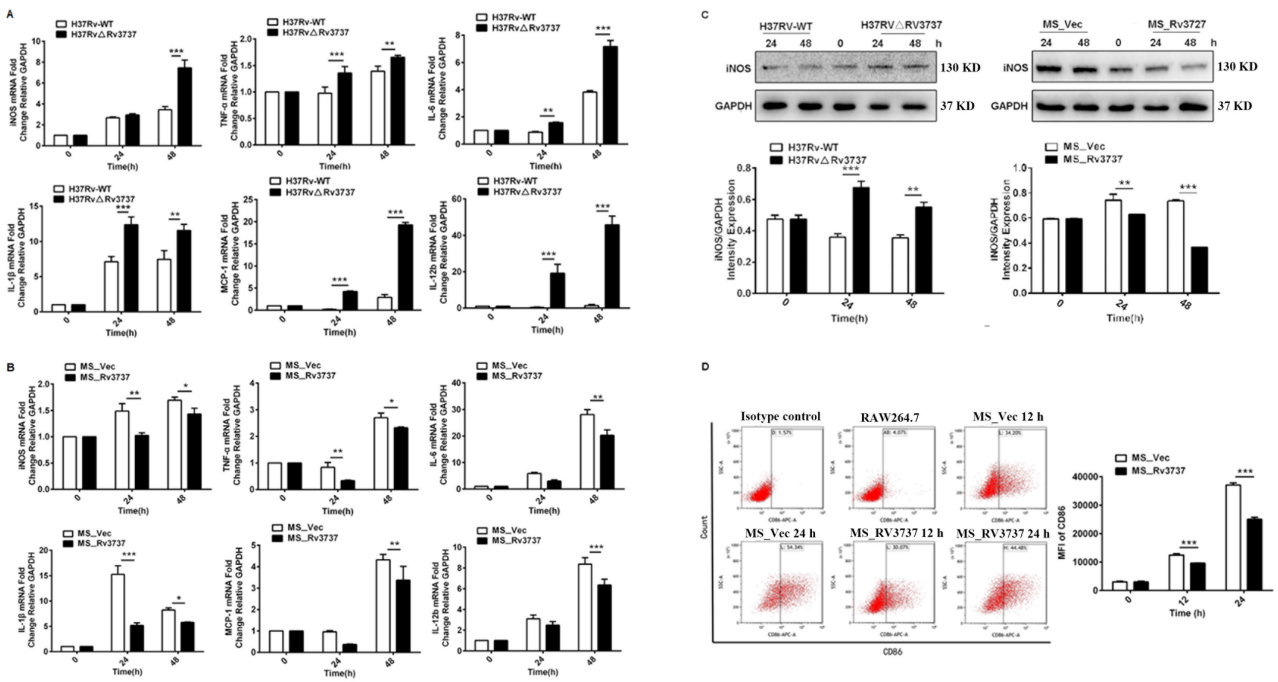


FIGURE 2
Rv3737 inhibits M1 macrophage polarization and pro-inflammatory cytokine expression. RAW264.7 cells were infected with H37Rv-WT, H37Rv△Rv3737, MS_Vec, or MS_Rv3737 at an MOI of 10 for 24 or 48 hours. **(A, B)** mRNA levels of M1 cytokines (TNF-α, IL-1β, IL-6, iNOS, MCP-1) assessed by real-time PCR. **(C)** Intracellular iNOS protein expression analyzed by Western blot. **(D)** Mean fluorescence intensity (MFI) of CD86, an M1 surface marker, measured by flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001.

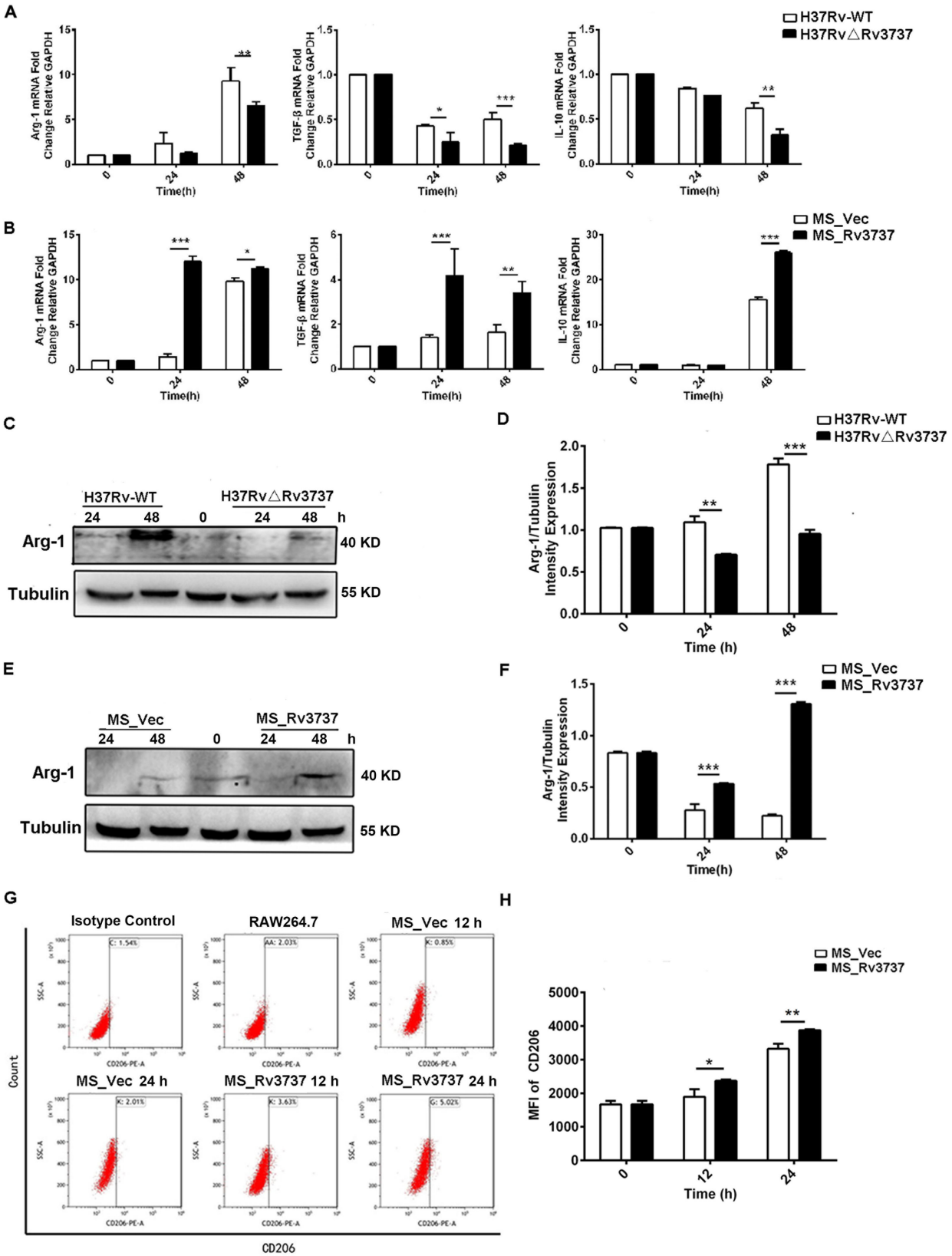


FIGURE 3
Rv3737 promotes M2 macrophage polarization and anti-inflammatory cytokine expression. RAW264.7 cells were infected as above. **(A)** mRNA levels of M2 cytokines (Arg-1, IL-10, TGF-β) in H37Rv△Rv3737- vs. H37Rv-WT-infected cells. **(B)** M2 cytokine expression in MS_Rv3737- vs. MS_Vec-infected cells. **(C, E)** Arg-1 protein expression assessed by Western blot. **(D, F)** Quantification of Arg-1 band intensity. **(G, H)** MFI of the M2 surface marker CD206 detected by flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001. Data are shown as mean ± SD from independent experiments.

established an MS-infected mouse model. On day 6 post-infection, lung bacillary loads were significantly higher in MS_Rv3737-infected mice compared to MS_Vec-infected group (Figure 4A). Consistent with these findings, the acid-fast staining data also demonstrated that Rv3737 promoted the mycobacterial survival *in vivo* (Figure 4B). Hematoxylin and eosin staining results showed that MS_Rv3737-infected mice obviously aggravated lung pathology progression, as evidenced by inflammatory cells infiltration (Figures 4C, D). Overall, these data demonstrated that Rv3737 indeed promoted mycobacterial survival *in vivo* and subsequently exacerbated lung pathology progression *in vivo* by regulating M2-type macrophages polarization.

3.5 Deletion of Rv3737 activates NF-κB and MAPK signaling via enhanced phosphorylation of P65 and P38

To investigate the mechanisms by which Rv3737 modulates macrophage polarization, the NF-κB and MAPK signaling pathways are central to regulating macrophage polarization and inflammatory responses. we performed RNA sequencing (RNA-seq) on RAW264.7 macrophages infected with either H37Rv-WT or H37RvΔRv3737. KEGG pathway enrichment analysis of differentially expressed genes revealed significant enrichment of inflammatory pathways, particularly the NF-κB and TNF signaling pathways (Supplementary Figure 2A). Consistent with this, mRNA levels of AP-1 and NF-κB were upregulated in H37RvΔRv3737-infected macrophages (Supplementary Figure 2B), whereas expression of these transcription factors was significantly suppressed in MS_Rv3737-infected cells (Supplementary Figure 2C). To validate these findings, we assessed the phosphorylation status of key signaling molecules by Western blot. In H37RvΔRv3737-infected macrophages, phosphorylation of P65 and IκBα was markedly increased, particularly at 4 hours post-infection (Figure 5A), indicating activation of the NF-κB pathway. Conversely, MS_Rv3737-infected cells exhibited reduced phosphorylation of these proteins (Figure 5B). We also examined MAPK pathway components. Phosphorylation of P38, JNK, and ERK was significantly elevated in H37RvΔRv3737-infected cells, with P38 showing the most pronounced increase (Figure 5C). In contrast, MS_Rv3737 overexpression led to decreased phosphorylation of these MAPKs compared to vector control (Figure 5D). Collectively, these results indicate that deletion

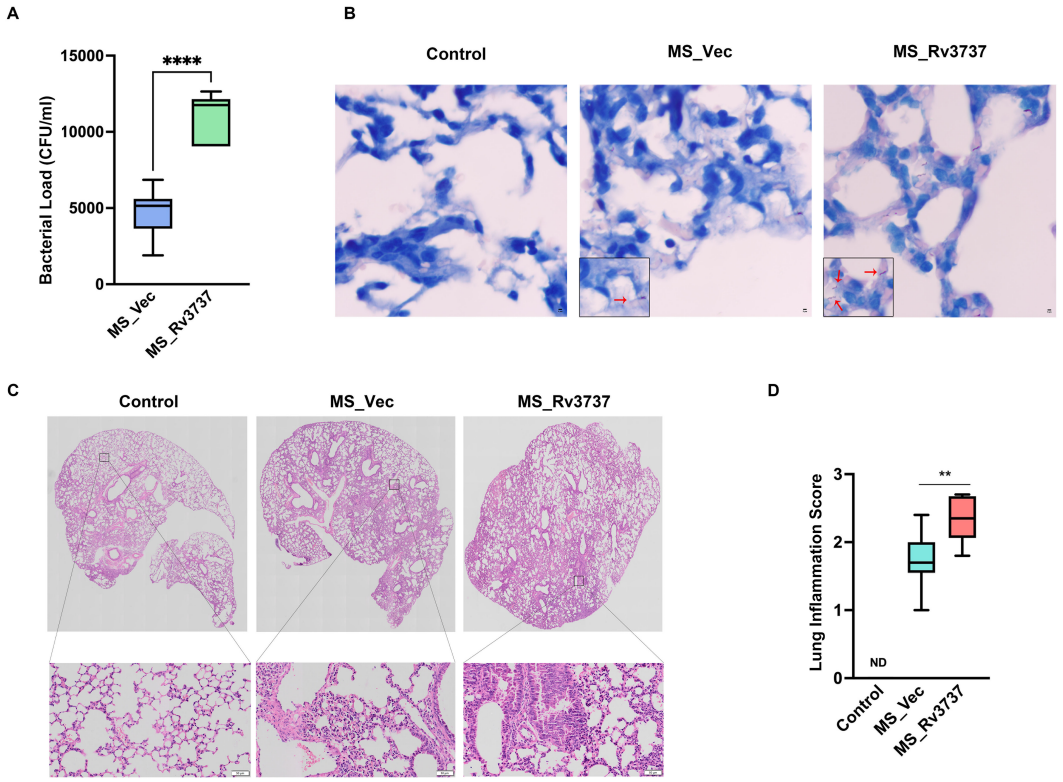


FIGURE 4
Rv3737 facilitated mycobacteria survival and aggravated lung pathology progression in MS-infected mice. 6–8-week C57BL/6 mouse was infected via nasal instillation with Mycobacterium smegmatis at a dose of 2×10^7/mice. The lung tissues were harvested at day 6 post-infection for colony-forming unit (CFU) analysis, Acid-fast staining, and hematoxylin and eosin (HE). **(A)** CFU in the MS_Vec and MS_Rv3737-infected mice lung; **(B)** Acid-Fast staining in the lungs of MS_Vec or MS_Rv3737-infected mice, red arrow represents acid-fast staining bacilli; **(C)** HE staining in lungs of MS_Vec and MS_Rv3737-infected mice; **(D)** Lung injury scores of infected mice at day 6 postinfection. N.D., not detected. The black square indicates cellular infiltration. Scale bar: 50 μm. Statistical analyses were performed in **(A)** using One way-ANOVA. The data presented are 5–95 percentiles. **$p < 0.01$, ****$p < 0.0001$

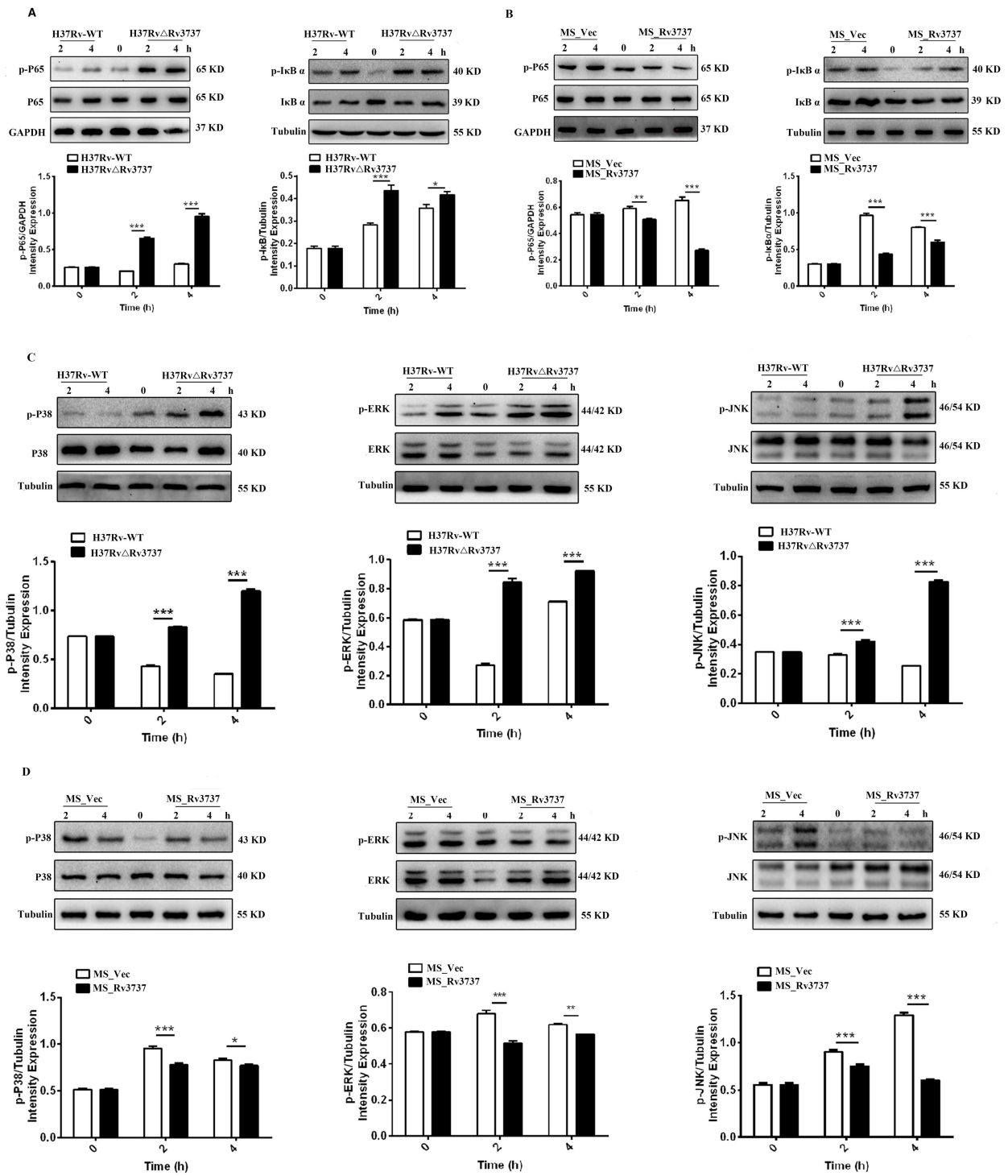


FIGURE 5
Rv3737 knockout activates NF-κB and MAPK pathways via P65 and P38 phosphorylation. RAW264.7 cells were infected at an MOI of 10. Cells were lysed at 2- and 4-hours post-infection. **(A, B)** Western blot analysis of P65 and IκBα phosphorylation in the NF-κB pathway. **(C, D)** Western blot analysis of phosphorylated P38, JNK, and ERK in the MAPK pathway. *p < 0.05, **p < 0.01, ***p < 0.001.

of Rv3737 activates the P65/NF-κB and P38/MAPK pathways, suggesting their involvement in Rv3737-mediated regulation of macrophage polarization.

3.6 Rv3737 deletion promotes M1 polarization and antimicrobial activity via the P65/NF-κB and P38/MAPK pathways

To determine whether the activation of NF-κB and MAPK signaling mediates the enhanced M1 polarization and bactericidal activity observed in Rv3737-deficient macrophages, we pretreated RAW264.7 cells with specific inhibitors targeting JNK (SP600125), P38 (SB203580), ERK (U0126-EtOH), and P65/NF-κB (JSH-23). As expected, iNOS mRNA expression was significantly upregulated in H37RvΔRv3737-infected macrophages. However, this increase was markedly reversed by pretreatment with P38 and P65 inhibitors, whereas inhibition of JNK or ERK had minimal effects (Figure 6A). Furthermore, the enhanced antimicrobial activity associated with H37RvΔRv3737 infection was diminished upon P38 or P65 pathway inhibition, as evidenced by increased intracellular bacterial survival (Figure 6B). These results suggest that Rv3737 knockout promotes M1-type macrophage polarization and enhances the antimicrobial response by activating the P65/NF-κB and P38/MAPK signaling pathways, thereby reducing intracellular Mtb survival (Figure 6B).

4 Discussion

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a major global health concern. Mtb has evolved sophisticated mechanisms to survive within macrophages, primarily by subverting host immune responses, including the regulation of macrophage polarization. During M1 polarization, macrophages mount antimicrobial responses characterized by the production of nitric oxide (NO), enhanced antigen presentation, and secretion of pro-inflammatory cytokines—key processes critical for controlling intracellular pathogens like Mtb (Liu et al., 2022). Several Mtb genes, including those encoding transmembrane proteins such as Rv0309 (Peng et al., 2022), Rv0426c (Ruan et al., 2020), and PknF (Rastogi et al., 2021), have been implicated in modulating macrophage polarization. However, the function of Rv3737, another putative transmembrane protein, has remained unclear. In this study, we explored its role in macrophage polarization and immune signaling, offering new insights into host-pathogen interactions at the molecular level.

Upon infection, Mtb enters macrophages via receptor-ligand interactions, triggering phagocytosis and enabling the bacterium to establish intracellular survival niches (Chen et al., 2023). These interactions are mediated by membrane-associated proteins from both the host and pathogen. The Mtb cell envelope harbors numerous membrane proteins with enzymatic, structural, and virulence-related functions that contribute to immune modulation and survival. Our bioinformatic analysis revealed that Rv3737 is a transmembrane protein with 10 membrane-spanning segments and a high content of aliphatic amino acids—features consistent with roles in host interaction, membrane localization, and immune modulation. These characteristics are reminiscent of other virulence-associated membrane proteins, such as components of the ESX-1 secretion system, which are critical for Mtb pathogenicity (Roy et al., 2020).

Beyond transmembrane proteins, Mtb secretory and cell wall-associated proteins are known to shape macrophage responses. Rv3737 encodes a 55 kDa protein homologous to ThrE, a member of a novel transporter family linked to amino acid export and intracellular survival (Li et al., 2022). In our study, Rv3737 deletion led to increased expression of M1 markers—including TNF-α, IL-6, iNOS, IL-1β, MCP-1, IL-12b, and CD86—while M2 markers were significantly suppressed. This is consistent with previous reports indicating that M1 macrophages drive bactericidal activity through heightened inflammatory responses (Russell et al., 2025). Other Mtb proteins also manipulate macrophage phenotypes. For example, ESAT-6 and CFP-10 promote M2 polarization, while ESAT-6 also exhibits M1-inducing effects during early infection (Deng et al., 2023). Proteins such as HSP16.3 shift macrophages from an M1 to M2 state, and PPE36 promotes Mtb persistence by

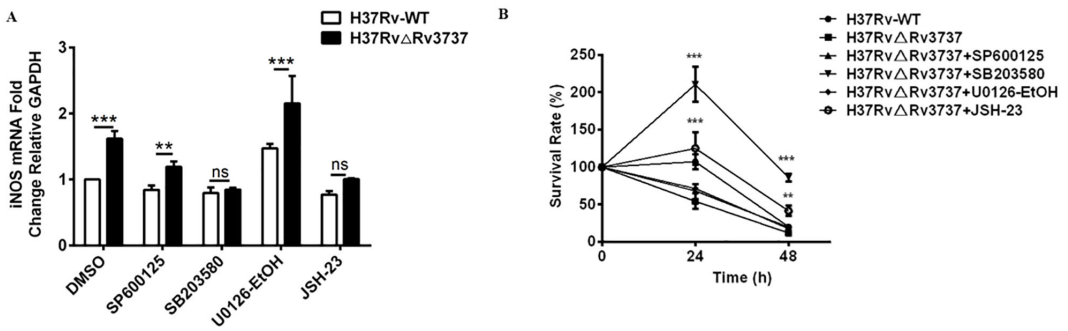


FIGURE 6
Rv3737 deletion enhances M1 polarization and antimicrobial activity via P65/NF-κB and P38/MAPK signaling. RAW264.7 cells were pre-treated with SP600125 (JNK inhibitor), SB203580 (P38 inhibitor), U0126-EtOH (ERK inhibitor), or JSH-23 (P65 inhibitor) before infection with H37Rv-WT or H37Rv△Rv3737. **(A)** iNOS mRNA levels determined by real-time PCR. **(B)** CFU assay for intracellular bacterial survival at 0-, 24-, and 48-hours post-infection; CFUs were counted after incubation on 7H10 agar for 3–4 weeks. **p < 0.01, ***p < 0.001. ns, no significant.

suppressing M1 polarization (Zhang et al., 2020; Gong et al., 2021). Similarly, Rv0426c impairs macrophage apoptosis, facilitating bacterial replication (Ruan et al., 2020). Our findings suggest that Rv3737 deletion disrupts this immune evasion strategy by promoting M1-type polarization and inhibiting M2 responses, potentially enhancing the host's antimicrobial defense.

The mechanism of macrophage polarization is complex and involves multiple signaling pathways. Our RNA sequencing (RNA-seq) analysis revealed that deletion of Rv3737 in *M. tuberculosis* significantly enriched gene expression in the NF-κB and TNF-α signaling pathways. The NF-κB and MAPK signaling cascades are well-established as central regulators of inflammation and M1-type macrophage polarization. Consistent with these findings, our results showed that Rv3737 deletion markedly enhanced the phosphorylation of P65 and P38, key indicators of activation in these pathways. Furthermore, pharmacological inhibition of P65 (via JSH-23) and P38 (via SB203580) reversed the upregulation of the M1 macrophage marker iNOS and led to a significant increase in bacterial load within macrophages. These results support the notion that Rv3737 regulates M1 macrophage polarization via the NF-κB and P38/MAPK signaling pathways. Previous studies reinforce this concept. For example, the Mtb transmembrane protein Rv0309 modulates macrophage polarization by targeting MAPK and NF-κB signaling, thereby suppressing M1 responses and promoting bacterial survival (Peng et al., 2022). Similarly, Rv2626c activates NF-κB signaling through direct interaction with TLR2, leading to downstream effects on macrophage polarization (Kim et al., 2020). Moreover, the Mtb protein PPE36, which also contains a transmembrane domain, has been shown to inhibit M1 polarization through the ERK branch of the MAPK pathway (Gong et al., 2021). Together, these findings suggest that Rv3737, like other Mtb transmembrane proteins, influences immune responses by modulating macrophage polarization to favor intracellular persistence. Although the precise mechanism by which Rv3737 engages host intracellular signaling molecules remains unclear, several lines of evidence point toward possible pathways. Rv3737 is a threonine-rich transmembrane protein, and our previous work demonstrated that deletion of Rv3737 in H37Rv (H37RvΔRv3737) leads to elevated threonine concentrations *in vitro*, whereas overexpression of Rv3737 in *M. smegmatis* (MS_Rv3737) significantly suppresses threonine levels. Emerging studies suggest that threonine/serine metabolism is closely linked to immune cell function and can coordinate multiple pathways involved in macrophage polarization (Huang et al., 2024). Therefore, it is plausible that Rv3737 affects host macrophage responses by altering intracellular threonine/serine metabolism, which in turn may influence inflammatory signaling pathways such as NF-κB and MAPKs. Moreover, whether Rv3737 exerts its effects through upstream serine/threonine kinases, including the PI3K/mTOR axis, which also regulates NF-κB and MAPKs, remains an open question. Further investigation is needed to explore this potential regulatory link. Although we have not yet identified direct molecular interactions between Rv3737 and specific host signaling proteins, our findings lay a foundational framework for future mechanistic studies.

This study also has several limitations. First, due to biosafety constraints, we were unable to perform *in vivo* experiments using H37Rv-infected mouse models. Alternatively, we evaluated the M1 and M2 macrophage polarization markers iNOS and Arg1 in MS_Rv3737 and MS_Vec-infected mice; the results were consistent with *in vitro* findings. These findings further provide *in vivo* support for the immunomodulatory effects of Rv3737 observed *in vitro*. Moreover, the precise mechanisms by which Rv3737 influences the NF-κB and MAPK signaling pathways remain to be elucidated. Future studies will focus on identifying potential interacting partners or investigating whether Rv3737 modulates these pathways through the regulation of threonine metabolism.

In conclusion, our findings demonstrate that deletion of Rv3737 promotes M1 macrophage polarization and reduces *M. tuberculosis* survival in macrophages by activating the P65 and P38 signaling pathways. This study offers new insights into the role of Rv3737 in modulating immune signaling and macrophage polarization, thereby enhancing our understanding of Mtb's immune evasion strategies and highlighting potential targets for therapeutic intervention.

5 Conclusion

Deletion of Rv3737 in *M. tuberculosis* H37Rv markedly increased the expression of M1 macrophage polarization markers (CD86, iNOS, IL-1β, IL-6, and MCP-1), while significantly reducing the expression of M2 markers (Arg-1, IL-10, TGF-β, and CD206). Since M1 macrophages exert potent bactericidal activity through activation of the NF-κB and P38 MAPK pathways, these findings highlight the role of Rv3737 in modulating host immune responses. Overall, this study advances our understanding of macrophage polarization in tuberculosis and provides a potential foundation for the development of host-directed therapies against TB.

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 authors.

Author contributions

ZP: Writing – original draft, Funding acquisition, Conceptualization. CX: Writing – original draft, Formal analysis, Methodology. TY: Writing – original draft, Data curation, Methodology. CS: Writing – original draft, Data curation, Methodology, Software. QL: Writing – original draft, Resources, Methodology. NL: Writing – original draft, Resources, Methodology. RH: Writing – review & editing, Resources, Supervision. LC: Writing – review & editing, Supervision. LX: Writing – review & editing, Supervision, Conceptualization.

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Conflict of interest

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SUPPLEMENTARY FIGURE 1

Rv3737 promoted Arg1 expression *in vivo*. 6–8-week C57BL/6 mouse was infected through nasal instillation with Mycobacterium smegmatis at 2×10^7/mice, the lung tissues were harvested at day 6 post-infection for cytokine measurement, and Western blot analysis. **(A, C)** Real-time PCR detected the Arg1 and iNOS mRNA expression; **(B)** Western blot analysis of Arg1 and iNOS expression in MS_Vec or MS_Rv3737-infected mice; **(D, E)** Arg1 and iNOS intensity relative to Tubulin.

SUPPLEMENTARY FIGURE 2

Rv3737 Deletion Enhances NF-κB and AP-1 Expression, Promoting M1 Polarization. **(A)** KEGG pathway enrichment analysis of top 20 enriched pathways in RAW264.7 cells infected with H37Rv-WT or H37Rv△Rv3737 for 24 hours. **(B, C)** Real-time PCR analysis of macrophage polarization-related signaling molecules (STAT1, AP-1, IRF3, IRF5, JAK1, JAK2, NF-κB, C/EBPβ, CREB, STAT2, STAT5, TAT6, IRF4, JAK3, JMJD3, PI3K, PPARγ) in cells infected with H37Rv-WT, H37Rv△Rv3737, MS_Vec, or MS_Rv3737. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPLEMENTARY TABLE 1

Primer sequences used for detecting M1/M2 polarization-associated cytokines and macrophage transcription factors by real-time PCR.

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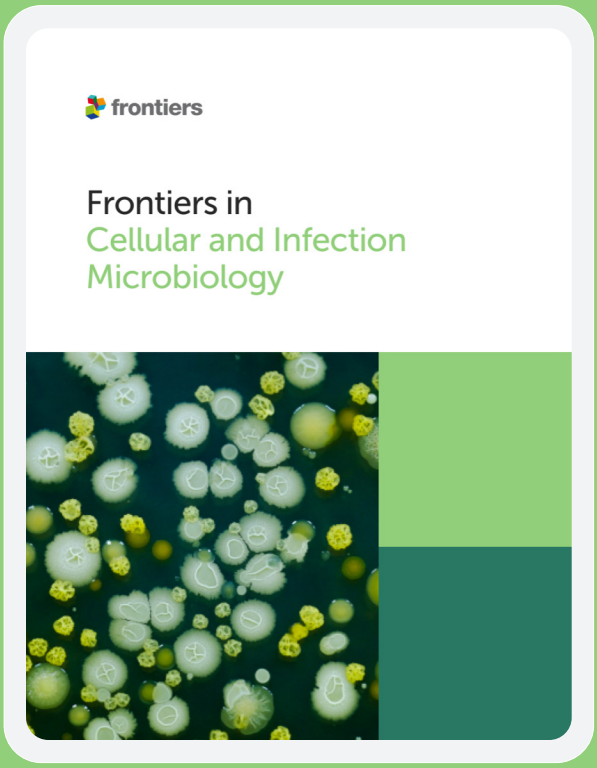

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