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Overcoming challenges in microbial immunology 2022

Edited by
Sylvie Bertholet, Beatrice Omusiro Ondondo and Nathella Pavan Kumar

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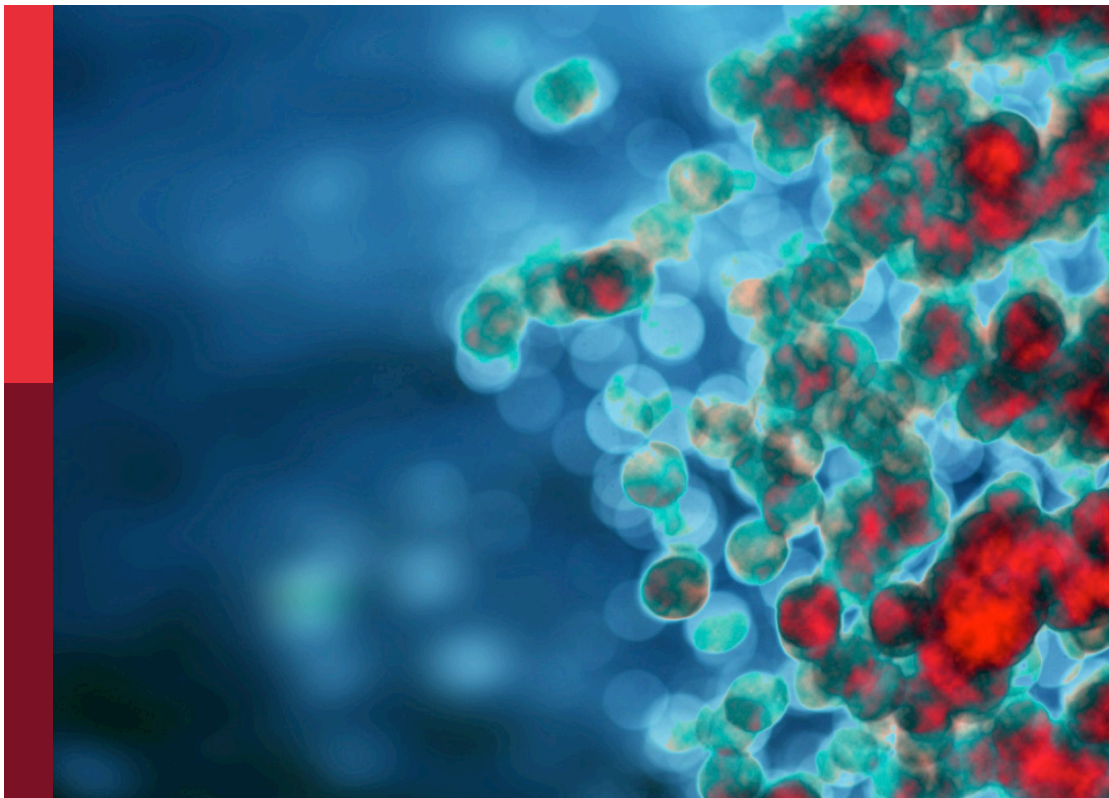

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Overcoming challenges in microbial immunology: 2022

Topic editors

Sylvie Bertholet — GSK Vaccines, United States

Beatrice Omusiro Ondondo — University Hospitals of Leicester NHS Trust, United Kingdom

Nathella Pavan Kumar — National Institute of Research in Tuberculosis (ICMR), India

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Ian Marriott,
University of North Carolina at Charlotte, United States

*CORRESPONDENCE
Beatrice Ondondo
beatrice.ondondo@uhl-tr.nhs.uk

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Editorial: Overcoming challenges in microbial immunology: 2022

Beatrice Ondondo*

Immunology Department, University Hospitals of Leicester National Health Service (NHS) Trust, Leicester, United Kingdom

KEYWORDS

short chain fatty acids (SCFAs), traditional Chinese medicine, rheumatoid arthritis, cardiovascular disease (CVD), Q-fever, sepsis, cutaneous T cell lymphoma (CTCL), phagolysosome pathway

Editorial on the Research Topic
Overcoming challenges in microbial immunology: 2022

As a rapidly expanding field, Microbial immunology faces unprecedented challenges: discovery of new microbes; treatment of drug-resistant microbes; and determining how microbes shape the immune landscape during infection, cancer, and autoimmunity. Furthermore, microbes may indirectly influence the development, progression, and prognosis of chronic non-communicable diseases. This Research Topic highlights the complex interactions of microbes with the immune system and shows that specific targets of such interactions hold promise for novel therapeutic and vaccination strategies. Collectively they point to areas for further development in the field and provide a focus for future research.

Strong links exist between the microbiome and cancer (1–3), and imbalances in the gut microbiome are associated with various chronic diseases including obesity, airway inflammation, colitis, some digestive disorders and cardiovascular disease (CVD) (4, 5). Gut microbiota produce bioactive metabolites including trimethylamine, trimethylamine N-oxide, short-chain fatty acids (SCFAs), and bile acids, which may have a link to the aetiology of CVD (6). Luqman et al. provide an overview of the intricate links between gut microbiota, their metabolites, and the development of CVD. They focus on how intestinal dysbiosis promotes CVD risk factors such as heart failure, hypertension and atherosclerosis, and potential therapeutic interventions using gut microbes and their metabolites. SCFAs regulate the immune system and modulate inflammatory responses (7, 8) through their action on various cell types and can impact the prevention and treatment of disease. Liu et al. summarise the different mechanisms through which SCFAs act in cells with particular emphasis on their regulatory role in innate and adaptive immune systems. They highlight the role of SCFAs in regulating allergic airway inflammation, colitis, and osteoporosis through influencing the immune system, and suggest that metabolic regulation can inform treatment options.

The established relationship between gut microbiota and rheumatoid arthritis (RA) (9) suggests that therapeutic approaches for RA may include the active modulation of gut microbiota. Traditional Chinese medicine (TCM) has been suggested to regulate immunity, reduce inflammation and improve quality of life (10) by exerting its effects on the gut microbiota. Liang et al. explore the complex relationship between TCM and gut microbiota not only in the context of treating RA, but also the role of gut microbiota in its pathogenesis

and prognosis. They further elucidate mechanisms to utilize TCM in the treatment and prevention of RA by regulating gut microbiota and provide an evidence-based rationale for investigating microbiota-targeted intervention by TCM. In recognition of the link between oral health and general well-being, Xu et al. provide a detailed review on how interactions of oral microbiota with the host can lead to alveolar bone resorption. They highlight various mechanisms through which *P. gingivalis*- and *F. nucleatum*, besides causing periodontitis, disrupt the host osteoimmune mechanisms leading to alveolar bone resorption and describe the immunophenotypes observed in host periodontal tissues during pathological conditions.

In contrast to the extensive prior focus on the relationship between gut microbiota and cancer, Wu et al. explore the role of intra-tumoral microbiota in cancer onset, progression, and therapy. They provide insight on how microbiota within the tumour microenvironment exert immunomodulatory effects to promote inflammation and directly compromise anti-tumour immunity. Their review highlights the potential for novel cancer therapies targeted to specific intra-tumoral microbiota and calls for further research to advance this promising field. Using the murine EL4 model, Dey et al. show that phototherapy in conjunction with antibiotic treatment can modulate skin microbiota and alter the course of cutaneous T-cell lymphoma. They demonstrate that the extent of microbial colonisation of the skin correlates with disease severity and tumour growth, and that antibiotics can significantly delay tumour occurrence, leading to increased survival. They found that antibiotics enriched the skin microbiome with commensal Clostridium species while significantly reducing facultative pathogens and *Staphylococcus aureus*. Reduction of pathogenic microbes may curtail the chronic inflammation caused by skin-homing T cells: a prominent characteristic of cutaneous T-cell lymphoma. Their findings may support the development of novel therapeutic agents to modulate the microbial milieu in patients with cutaneous T-cell lymphoma.

Taya et al. highlight the importance of developing new classes of antimicrobial agents that can complement host-directed therapies (HDT) to overcome the significant problem of emerging drug-resistant microbes. If successful, HDT may be extremely useful during overwhelming sepsis before identifying the causative microbes. Taya et al. provide insights on how the phagolysosome pathway, a first line of defence in the innate immune system, can be modulated for HDT despite the myriad of strategies employed by microbes to escape and survive this pathway (11, 12). The abundance of detailed molecular biological analyses of the phagolysosome system (13–15) provide key information for the development of drugs that target various points of action in this pathway including phagocytosis, phagosome maturation, fusion with lysosomes and lysosome acidification. Unlike the stratification of sepsis patients based on genomic and transcriptome data (16, 17), studies utilising immune profiles at protein expression level are scarce. Tang et al. report on the immune landscape of sepsis and a prediction model that classifies patients into three distinct immune endotypes, which are characterised by different survival rates. By comparing signatures of innate and adaptive immune function in sepsis patients to healthy controls, they discover a dysregulation-type immune endotype associated with a lower survival rate owing to significant impairment of innate and adaptive immunity and increased inflammation. Their study suggests that septic immune endotypes could inform future development of personalized therapies.

The concept of trained immunity has been reported in natural infection and following vaccination where it may enhance immunity against microbes or cause aberrant inflammation in certain situations (18). Using flow cytometry to profile *ex vivo* recall responses, Raju Paul et al. demonstrate the occurrence of trained innate immunity following natural exposure to *Coxiella burnetii*, the causative agent of Q fever. Their study reveals long-term persistence of CD14+ monocytes producing elevated levels of IL-6, IL-1β and IL-8 in individuals pre-exposed to *C. burnetii*. If these cells exert sustained protection against Q fever, or significantly alter the course of disease, they may hold useful clues for vaccines against Q fever.

In conclusion, this Research Topic elucidates how microbiota (intestinal, oral, skin, and intra-tumoral) influence disease progression through modulation of innate and adaptive immunity. It provides a glimpse at possible innovative approaches to harness microbe-host-interactions for the treatment of cancer, infections, and chronic diseases. Research that optimises non-conventional therapies such as faecal transplantation, TCM, HDT, and dietary treatments will propel this field forward.

Author contributions

BO: Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

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

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EDITED BY
Beatrice Omusiro Ondondo,
University Hospitals of Leicester NHS Trust, United Kingdom

REVIEWED BY
Som G. Nanjappa,
University of Illinois at Urbana-Champaign, United States

*CORRESPONDENCE
Xian Zhang
zhangxian0772@sina.com
Chao Li
15203216862@163.com

†These authors have contributed equally to this work

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Regulation of short-chain fatty acids in the immune system

Xiao-feng Liu[1†], Jia-hao Shao[1†], Yi-Tao Liao[1], Li-Ning Wang[2], Yuan Jia[1], Peng-jun Dong[1], Zhi-zhong Liu[1], Dan-dan He[1], Chao Li[3*] and Xian Zhang[3*]

[1]Wuxi Affiliated Hospital of Nanjing University of Chinese Medicine, Wuxi, China, [2]School of Chinese Medicine, School of Integrated Chinese and Western Medicine,Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China, [3]Department of Spine, Wuxi Affiliated Hospital of Nanjing University of Chinese Medicine, Wuxi, China

A growing body of research suggests that short-chain fatty acids (SCFAs), metabolites produced by intestinal symbiotic bacteria that ferment dietary fibers (DFs), play a crucial role in the health status of symbiotes. SCFAs act on a variety of cell types to regulate important biological processes, including host metabolism, intestinal function, and immune function. SCFAs also affect the function and fate of immune cells. This finding provides a new concept in immune metabolism and a better understanding of the regulatory role of SCFAs in the immune system, which impacts the prevention and treatment of disease. The mechanism by which SCFAs induce or regulate the immune response is becoming increasingly clear. This review summarizes the different mechanisms through which SCFAs act in cells. According to the latest research, the regulatory role of SCFAs in the innate immune system, including in NLRP3 inflammasomes, receptors of TLR family members, neutrophils, macrophages, natural killer cells, eosinophils, basophils and innate lymphocyte subsets, is emphasized. The regulatory role of SCFAs in the adaptive immune system, including in T-cell subsets, B cells, and plasma cells, is also highlighted. In addition, we discuss the role that SCFAs play in regulating allergic airway inflammation, colitis, and osteoporosis by influencing the immune system. These findings provide evidence for determining treatment options based on metabolic regulation.

KEYWORDS

short-chain fatty acid, innate immunity, adaptive immunity, histone deacetylase, G-protein-coupled receptor

Introduction

Parts of the colon and small intestine contain many microorganisms, mainly bacteria and some fungi. These microorganisms produce short-chain fatty acids (SCFAs) from dietary components in the gut and from biomolecules produced by the host (1). Intestinal SCFAs mainly include acetate (C2), propionate (C3), butyrate (C4) and valerate (C5). Most SCFAs function in the gut, but a small amount of SCFAs reach the peripheral circulation

via the portal vein (2, 3). A growing body of evidence suggests that SCFAs regulate immunity and suppress or promote inflammatory responses in the gut and other organs (4, 5). They play an important role in the regulation of innate and adaptive immunity mediated by a variety of mechanisms, including histone deacetylase (HDAC) inhibition, G-protein-coupled receptor (GPR) signaling, acetyl-CoA production, and metabolic integration. Through a combination of these mechanisms, SCFAs induce or modulate immune responses. However, the mechanism through which SCFAs regulate the immune system is relatively complex, and the mechanism of SCFAs differs among different immune cells; thus, a comprehensive summary is currently lacking. In this review, we more comprehensively introduce the regulatory role of SCFAs in the immune system. In the innate immune system, SCFAs play a role by regulating protein molecules, including the NLRP3 inflammasome and Toll-like receptors (TLRs). SCFAs also play a role by regulating innate immune cells, including neutrophils, macrophages, natural killer cells, eosinophils, basophils, and innate lymphocyte subsets (ILCs). We also highlight the regulatory role of SCFAs in the adaptive immune system, including in T-cell subsets, B cells, and plasma cells.

Synthesis and metabolism of SCFAs

SCFAs are the most abundant microbial metabolites in the colonic lumen and are mainly produced by the microbial fermentation of prebiotics, such as dietary fiber. Among them, the ratio of C2, C3 and C4 is approximately 3:1:1 (6). The differentiation of colon epithelial stem cells and the metabolism of facultative anaerobes in the colon ensure the anaerobic environment of the colon (7–9). Obligate anaerobes in the colon (e.g., *Clostridium* and *Bacteroides*) encode broad-spectrum enzymes that hydrolyze carbohydrates and decompose dietary fibers into sugars (10) (Table 1). The released sugars are then fermented through glycolysis and the pentose phosphate pathway to hydrolyze dietary fibers into SCFAs (16–18). C2 is produced by pyruvates *via* acetyl-CoA or the Wood-Ljungdahl pathway (19). C3 is synthesized from acrylates using lactic acid as a precursor and is produced by the acrylate and propylene glycol pathways or by the succinate pathway that converts succinate to methylmalonyl-CoA (20, 21). C4 is reduced by condensation of two molecules of acetyl-CoA to butyryl-CoA, which can be synthesized through the butyrate kinase and phosphotransbutyrylase pathways (22). Butyryl-CoA can also be converted to C4 *via* the acetate CoA transferase pathway (23). In addition, C4 can be synthesized from proteins *via* the lysine pathway (24). Other nutrients, including proteins and peptides, can be metabolized to produce low levels of SCFAs (1). Among them, the acidic amino acid glutamic acid mainly produces C2 and C4, and aspartic acid fermentation mainly produces C2 and C3. The deamination of the alkaline amino acids lysine, arginine and histidine produces C2 and C4. The neutral amino acid cysteine can produce C2, C3 and C4, and the main products of methionine metabolism are C3 and C4 (25). In this respect, proteins are more likely to be decomposed into small amino acids in pH neutral and weakly alkaline environments and are thus more likely to produce SCFAs in these environments (26). In short, when the pH value in the lumen is 5.5, the bacteria that produce C4 dominate; at a pH of 6.5 in the lumen, C2- and C3-producing bacteria dominate (27).

The concentration of SCFAs in the proximal colon was 9-131 mmol/L, while the concentration of SCFAs in the distal colon was lower (11-80 mmol/L) (2, 28). SCFAs enter cells in the following ways: the dissociated anions bind to MCT1 (SLC16A1), MCT4 (SLC16A3), SMCT1 (SLC5A8), and SMCT2 (SLC5A12)-mediated transporters and GPR receptors in a hydrogen-dependent or sodium-dependent manner (29–35). Most SCFAs are consumed by the epithelial cells of the colon (36). The remaining SCFAs enter the superior mesenteric vein, inferior mesenteric vein and portal vein through passive diffusion and active transport by transporters (C2, C3 and C4 concentrations are 262.8 μM/L, 30.3 μM/L, and 30.1 μM/L, respectively) (2, 3, 37). Some SCFAs are metabolized by

TABLE 1 SCFA Production by Microbes in the Gut.

SCFAs	Receptors that are more likely to activate	Pathways/Reactions	Producers	References
Acetate	GPR43	via acetyl-CoA	*Akkermansia muciniphila, Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.	(11–13)
		Wood-Ljungdahl pathway	*Blautia hydrogenotrophica, Clostridium* spp., *Streptococcus* spp.	
Propionate	GPR43 GPR41	succinate pathway	*Bacteroides* spp., *Phascolarctobacterium succinatutens, Dialister* spp., *Veillonella* spp.	(12–14)
		acrylate pathway	*Megasphaera elsdenii, Coprococcus catus.*	
		propanediol pathway	*Salmonella* spp., *Roseburia inulinivorans, Ruminococcus obeum.*	
Butyrate	GPR41 GPR109A	phosphotransbutyrylase/ butyrate kinase route	*Coprococcus comes, Coprococcus eutactus.*	(12–15)
		butyryl-CoA:acetate CoAtransferase rout	*Anaerostipes* spp.*(A, L), Coprococcus catus (A), Eubacterium rectale (A), Eubacterium hallii (A, L), Faecalibacterium prausnitzii (A), Roseburia* spp. *(A)*	

the liver, and the remaining SCFAs are dispersed to peripheral circulation (the concentrations of C2, C3, and C4 were 172.9 μM/L, 3.6 μM/L, and 7.5 μM/L, respectively) (38, 39). These blood concentrations of SCFAs are thought to be high enough to affect host cells (39), depending on the type and amount of dietary fiber ingested by the host and the health status of the host (e.g., helminthic infection, viral infection, autoinflammation) (40, 41).

The receptors of SCFAs that have been widely reported are GPR41, GPR43, GPR109A, OR51E2 (human) and OLFR78 (mouse) (42–45). SCFAs with different carbon chain lengths have different abilities to activate GPR41, GPR43 and GPR109A receptors. Two to three carbon chains are more likely to activate GPR43 receptors, while 3-5 carbon chains are more likely to activate GPR41 receptors. C4 activates the GPR109A receptor more easily (43, 44). SCFAs are found in high levels in the gut, and most of these receptors are activated in intestinal tissues.

SCFAs are natural inhibitors of HDAC, of which there are 18 types (46, 47). There are four classes of HDAC as follows: Class I (HDAC1-3 and HDAC8), Class II (HDAC4-7 and HDAC9-10), Class III (SIRT1-7) and Class IV (HDAC11) (47). Different types of SCFAs have different inhibition rates of different types of HDAC. For example, C4 can inhibit HDAC up to 80%, C3 can inhibit HDAC up to 60%, and C2 has the lowest inhibition rate (48, 49). SCFAs can affect histone acetylation by regulating the homeostasis between histone acetyltransferase (HAT) and HDAC. HAT transfers acetyl groups to lysine residues in the tail, forming acetylated lysine, which neutralizes the positive charge carried by the histones (50). HDAC deacetylates the acetylated lysine in the histone tail, making the nucleosome compact and making it more difficult to perform gene transcription and expression (51, 52). Therefore, different types of SCFAs affect gene transcription in immune cells by inhibiting the activity of different types of HDACs.

Active functions and signaling pathways of SCFAs

NF-κB signaling pathway

Nuclear factor-κB (NF-κB) mediates the transcription of various cytokines and chemokines, such as the cytokines TNF-α, TNF-β, IL-1β, IL-2, IL-3, IL-5, IL-12, and IL-18 and the chemokines IL-8, MIP-1α, MIP-2, and MCP-1 (53–56). Two subunits of NF-κB, P65 and P50, are acetylated and transferred from the cytoplasm into the nucleus to promote the secretion of proinflammatory cytokines (57). SCFAs produce anti-inflammatory effects by inhibiting NF-κB. The order of inhibition of NF-κB activity was C4>C3>C2 (58).

HDAC can regulate the secretion of inflammatory cytokines by inhibiting the acetylation of NF-KB (59). It was found that the subunits p65 and p50 of NF-κB interact with HDAC to inhibit transcription (59). Deacetylation of p65 by HDAC3 enhances the binding of p65 to IκBα, resulting in the export of the NF-κB complex from the nucleus back into the cytoplasm to inhibit the transcription of proinflammatory factors (60). C3 and C4 are known HDAC inhibitors and have been shown to regulate NF-κB activity. For example, C4 upregulates the production of IL-10 and inhibits the production of the proinflammatory molecules IL-12, TNF-α, IL-1β, and NO by inhibiting NF-κB activity (61–63).

GPR receptors influence the secretion of inflammatory cytokines by regulating the β-arrestin 2 pathway upstream of the NF-κB signaling pathway. The GPR43 receptor reduces the level of NF-κB through the β-arrestin 2 signaling pathway and reduces the amount of the two subunits of NF-κB, p65 and p50, entering the nucleus; thus, the GPR43 receptor inhibits the transcription of proinflammatory cytokines (IL-1β and IL-6) (64, 65) (Figure 1).

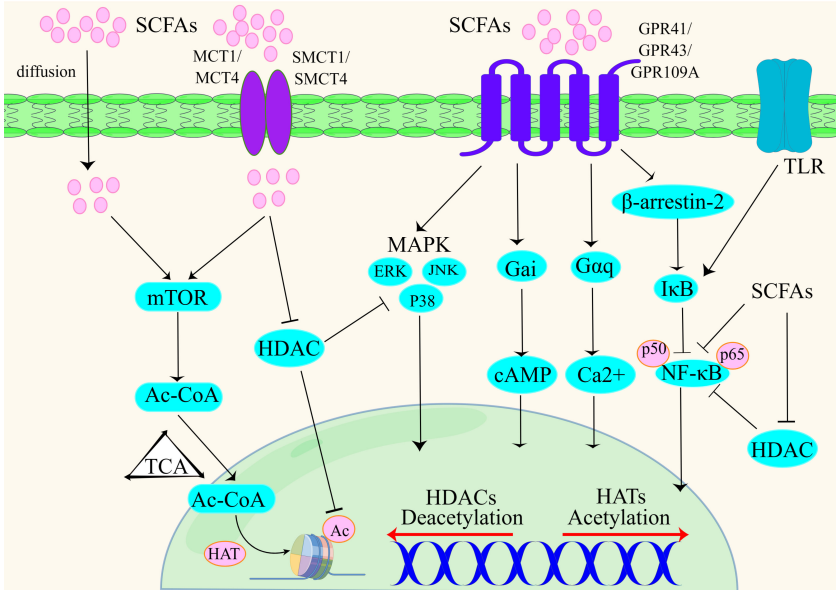

FIGURE 1
SCFAs influence the immune response by a variety of signalling pathways, including epigenetic inheritance in cells (56, 66, 67).

MAPK signaling pathway

Phosphorylated mitogen-activated protein kinase (MAPK) regulates the ERK, JNK and P38 MAPK signaling pathways, gene transcription, and proinflammatory cytokine secretion (68). The acetylated state of MAPK phosphatase-1 (MKP-1) interacts with the MAPK substrate to dephosphorylate MAPK and inhibit the activation of the ERK, JNK and P38 MAPK signaling pathways (69).

SCFAs may regulate MAPK pathways by inhibiting HDAC. HDAC1-3 deacetylates MP-1, and the deacetylation of MP-1 leads to an increase in MAPK signaling and proinflammatory cytokine secretion (70). However, the effect of HDAC may also be independent of MAPK signaling pathways. Treatment of bone marrow-derived macrophages exposed to lipopolysaccharide (LPS) with TSA (an HDAC inhibitor) inhibited TNF-α and IL-6 production in cells in a time- and dose-dependent manner. However, TSA did not inhibit ERK1/2 and p38 phosphorylation in macrophages (71).

SCFAs can participate in proinflammatory effects by activating GPR41 and GPR43 receptors. It has been shown that activation of the GPR41 and GPR43 receptors can induce ERK1/2 phosphorylation, while activation of GPR43 receptors can induce p38 MAPK phosphorylation (72). C2 activates the GPR41 and GPR43 receptors and their downstream ERK2/1 and MAPK signaling pathways and increases the production of proinflammatory factors and chemokines (72, 73) (Figure 1).

mTOR signaling pathway

Rapamycin target (mTOR) is a serine/threonine protein kinase. There are two distinct functional complexes, mTORC1 and mTORC2, that regulate cell growth, proliferation, transcription, mRNA renewal, translation and other important processes (74). Activation of mTOR helps regulate barrier function in the gut and can influence the production of immune cells and cytokines. mTOR increases the acetyl-coA content *via* the glycolysis pathway, and excess acetyl-coA is converted to citrate *via* the tricarboxylic acid cycle (TCA cycle). Citrate, which is involved in the TCA cycle, is converted to acetyl-CoA in the nucleus *via* ATP-citrate lyase. Acetyl-CoA in the nucleus promotes the binding of acetyl groups to histones and increases the acetylation of histones, ultimately regulating gene expression and the production of cytokines such as IL-10 and TNF (75–77). SCFAs enter cells to inhibit HDAC and increase the acetylation of p70 S6 kinase and the phosphorylation of rS6, thereby regulating the mTOR pathway and increasing IL-10 cytokine production (74) (Figure 1).

The activated GPR41 receptor was shown to bind to intracellular Gαi, reducing the level of cAMP. The activated GPR43 receptor conjugates with intracellular Gαq and Gαi to inhibit cAMP levels. Increased intracellular cAMP levels facilitate the entry of intracellular calcium ions into the cytoplasm, a process that regulates gene transcription and translation in immune cells (43) (Figure 1).

SCFAs and immune regulation

Innate immunity

Regulation of the NLRP3 inflammasome by SCFAs

The inflammasome is a multiprotein complex assembled by intracytoplasmic pattern recognition receptors (PRRs) and is an important part of the innate immune system (78, 79). The NLRP3 inflammasome is responsible for the maturation and secretion of the related cytokines IL-1β and IL-18 (80). Studies have shown that SCFAs, after binding with GPR43 and GPR109A in intestinal epithelial cells (IECs), cause intracellular potassium ion outflow and hyperpolarization, calcium ion inflow and activation of the NLRP3 inflammasome (81). In addition, after activating the GPR43 receptor of IEC cells, SCFAs activate the NLRP3 inflammasome by increasing the phosphorylation of ERK (82). However, SCFAs have been shown to inhibit NLRP3 inflammasome activation in other cells. For example, intervention by SCFAs significantly reduced NLRP3 inflammasome activation in astrocytes (83). C4 exerts anti-inflammatory effects by inhibiting the formation and activation of the NLRP3 inflammasome in vascular endothelial cells, but C2 and C3 do not show the same effect; thus, C4 plays an anti-inflammatory role and contributes to the formation of new carotid intima (84). The results discussed above indicate that not only do the same type of SCFAs have different inhibitory or promoting effects on different types of cells, but different types of SCFAs also have different effects on the same types of cells. This reminds us of previous findings that showed that SCFAs have proinflammatory effects on some cell types, such as macrophages and microglia, and anti-inflammatory effects on others (85, 86). Therefore, how SCFAs exert their proinflammatory and anti-inflammatory effects requires further study.

Regulation of TLR family members by SCFAs

The expression of PRRs enables the immune system to distinguish intestinal commensal microorganisms from harmful microorganisms. TLRs, a subtype of PRRs, play an important role in the innate immune response. TLRs can promote the proliferation of intestinal epithelial cells and the expression of antimicrobial peptides (AMPs) (87). Studies have shown that C3 and C4 regulate the response of multiple TLRs and TNF-α by inhibiting the histone acetylation of HDAC (88). Among them, TLR5 is highly expressed in the colon and can recognize the flagellin of gram-negative bacteria by activating a series of pathways within the cell (89). In patients with ulcerative colitis, the concentration of SCFAs in the colon is generally consistent with the expression of TLR5 in the colon. The content of SCFAs decreases gradually from the proximal end of the colon to the distal end, and the expression of TLR5 also decreases gradually from the proximal end of the colon to the distal end, indicating that there may be a certain relationship between the two (90). Further studies showed that the regulation of TLR5 by C4 occurred at the transcriptional level rather than at the translational level. C4 activates PKC isoforms to dephosphorylate and acetylate specific protein 1 (Sp1) by serine and threonine phosphatases,

respectively, and phosphorylates specific protein 3 (Sp3) by ERK-MAPK. This leads to displacement of Sp1 from the promoter and binding to Sp3, which activates the transcription of TLR5 in intestinal epithelial cells (91). This is consistent with a recent study showing that enterobacterial flagellin activates the release of anti-inflammatory factors (IL-10, TGF-β) and reduces inflammation in IECs. C4 is the main metabolite secreted by Enterobacterium, which can initiate TLR5 transcription through Sp3, upregulate TLR5 expression, and inhibit the expression and release of inflammatory factors (IL-6, IFN-γ and TNF-α) (92). In addition, TLR4 can activate innate immune responses by sensing LPS in the cell walls of gram-negative bacteria (93). C4 can promote TLR4 expression and the phosphorylation of MAPKs and NF-κB to regulate the innate immunity of colon cancer cells, but the specific mechanism remains unclear (94). To date, there are relatively few studies on the pathway mechanism of SCFA-TLRs in innate immunity, and the correlation between SCFAs and TLR signaling pathways is not clear. However, existing studies have clearly shown that SCFAs play an anti-inflammatory role by regulating the expression of TLRs, which is important for the regulation of immune homeostasis in the body.

Regulation of neutrophils by SCFAs

Neutrophils are considered the most abundant innate immune cells in the bone marrow and peripheral blood (95). SCFAs affect the recruitment of neutrophils to the site of inflammation and reduce inflammation. SCFAs increase the expression of L-selectin on the surface of neutrophil granulocytes, activate neutrophil chemotactic recruitment to the inflammatory site, and aggravate the inflammatory process without affecting the expression of β2 integrin mRNA (96). SCFAs induce the aggregation of neutrophils to the site of inflammation through the CPR43 receptor. This process is associated with the activation of intracellular protein kinase P38 and the coupling of Gi/o and Gq proteins (97). In a model of gout induced by sodium urate monohydrate (MSU), C2 promoted neutrophil reactive oxygen species (ROS) production in a GPR43-dependent manner, indirectly activated the NLRP3 inflammasome, led to neutrophil recruitment to the inflammatory site, promoted inflammasome activation, and promoted the release of IL-1β and IL-10 (98). C4 significantly inhibits the production of proinflammatory cytokines (e.g., IL-6, TNF-α and IFN-γ) and chemokines (e.g., CCL3, CCL4, CXCL1 and IL-8) secreted by neutrophils in the intestines of patients with colitis to reduce intestinal inflammation, and C4 inhibits the secretion of proinflammatory cytokines in an HCDAC-dependent manner (99). C2 also reduces the infiltration of pancreatic neutrophils and significantly reduces pancreatitis in mice (100).

Macrophages

Macrophages are essential for maintaining homeostasis in the gut (101). Previous studies have shown that the process by which C4 inhibits the production of inflammatory cytokines by intestinal macrophages is related to the inhibition of HDAC activity (102). C4 induces anti-inflammatory properties of macrophages in a GPR109-dependent manner (44). A recent study showed that C4 alters macrophage metabolism and increases their antibacterial activity. Metabolomic analysis of butyrate-treated macrophages revealed a substantial reduction in glycolysis. This was associated with higher amounts of adenosine monophosphate, an inducer of MAPK, which, in turn, inhibits mTOR. As mTOR is a positive regulator of glycolytic enzymes, its inhibition may explain the observed reduction in glycolysis (103–105). Moreover, mTOR is considered a key regulator of autophagy (106). The bacteria-associated autophagy protein microtubule-associated protein 1 light chain 3α (LC3) is a key participant in autophagy, and experiments have shown that the conversion rate of LC3 and ROS production are increased. Further analysis by single-cell RNA sequencing revealed that the C4-induced antibacterial signature is characterized by increased expression of the S100A8 and S100A9 genes, which encode calprotectin, a protein with antibacterial properties. Therefore, C4 helps increase the antibacterial activity of macrophages by inhibiting mTOR (103–105). In addition, in the presence of C4 and pertussis toxin (GPCR inhibitors), macrophages exhibit enhanced antibacterial activity, indicating that C4 enhances the antibacterial activity of macrophages without the involvement of GPCR. Further studies have shown that butyrate increases the expression of the S100A8 mRNA gene through its inhibition of HDAC3. Changes in metabolism enhance the bactericidal function of macrophages (103–105). Similarly, in mouse pancreatitis caused by *Staphylococcus aureus*, C4 enhances the antibacterial program of macrophages by inhibiting HDAC3 (107). Earlier studies have also found that the *in vitro* stimulation of mouse macrophages with butyrate leads to inhibition of inflammatory responses and decreases in nitric oxide levels, a process mediated by HDAC (102). In addition, SCFAs downregulate M2 polarization in human and mouse alveolar macrophages *in vitro* and may activate GPR43 but not GPR41. Butyrate and propionate (but not acetate) increase H3 acetylation and inhibit M2 polarization in part through HDAC inhibition (108).

Natural killer cells

Natural killer cells, which are the first identified ILC subtype, can respond to virus-infected or virus-transformed cells with a variety of effector functions, primarily cell killing and proinflammatory cytokine production (109, 110). The combination of IL-12 and IL-15 activates natural killer cells and promotes metabolic changes needed for increased receptor expression and cytokine secretion (111). In IL-12/IL-15-stimulated natural killer cells, C4 inhibits the expression of the cell surface receptors TRAIL, NKp44, NKp30, and NKG2D and significantly inhibits the production of the proinflammatory cytokines TNF-α, IFN-γ, IL-22, soluble Fas ligand, granzyme A, granzyme B and perforin. C3 inhibits the expression of the receptor NKp30 and the production of the proinflammatory cytokines IFN-γ and granzyme B, but C2 does not have the same effect (112). Researchers have found that C4-treated natural killer cells express higher levels of bromodomain-containing protein 2 (BRD2). BRD2

is an inflammatory cytokine that controls the production of natural killer cells (113). mTORC1 is a key regulator of natural killer cell metabolism (114). c-Myc regulates the secretion of survival cytokines in natural killer cells (115, 116). C4 has been shown to have anti-inflammatory effects by reducing mTOR activity and c-Myc mRNA expression in natural killer cells (113). In addition, C4 inhibits glycolysis and oxidative phosphorylation in natural killer cells by inhibiting the expression of the first enzyme in the glycolysis pathway, HK2 (113).

Eosinophils

Eosinophils promote a variety of complex immunomodulatory functions. Under inflammatory conditions, proinflammatory factors can activate eosinophils and prolong their survival. Activated eosinophils are important participants in the inflammatory process and secrete proinflammatory factors, including IL-3, IL-6, and tumor necrosis factor-α (117). These cells also secrete proinflammatory lipid mediators, including platelet activating factor and cysteine-leukotriene. Anti-inflammatory lipid mediators, including lipoxygenase, lysins and protectors, release reactive oxygen species (118–121). In a mouse model of allergic airway inflammation, a high-fiber diet, probiotics, or direct administration of SCFAs effectively reduced airway eosinophils by altering the gut microbiome and SCFA levels (122–125). Similarly, SCFAs exert the same effect in eosinophilia-related diseases (including asthma, atopic dermatitis, inflammatory bowel diseases, and eosinophilic oesophagitis) (126, 127). A recent study illustrated the mechanism through which SCFAs directly affect eosinophils. Eosinophils treated with C3 and C4 exhibit decreased cell size and number, loss of bilobate nuclei, mitochondrial membrane potential depolarization, and effector caspase activation, which results in the induction of apoptosis by regulating intracellular pathways, a process that may be mediated by inhibition of HDAC independent of the GPR41 and GPR43 receptor pathways. However, C2 does not impair the survival of eosinophils (128). These findings are consistent with a previous study that revealed that C4 alleviates airway hyperresponsiveness and eosinophilic increases in patients with allergic asthma through HDAC inhibition and a process independent of GPR41 and GPR43 receptor activation (125). C2 and C3 bind to GPR43 in human eosinophils, resulting in increased intracellular calcium influx (128). Moreover, C2 and C3 stimulate the production of ROS in human eosinophils in a concentration-dependent manner (128). Researchers have further investigated the potential of SCFAs to regulate the transcription of genes involved in eosinophilic adhesion, migration, and survival. C3 and C4 have been shown to inhibit eosinophilic adhesion and migration to the endothelial monolayer in response to eotaxin-2 (CCL24) (128). In addition, the surface expression of L-selectin on eosinophils is not affected by SCFAs (128, 129).

Basophils

At present, there are many studies on the relationship between SCFAs and eosinophils, but there are relatively few studies on the relationship between SCFAs and basophils. C2-treated basophils resulted in increased intracellular calcium influx, but treatment with C3 and C4 did not have the same effect (130). Activation of basophils is associated with IL-3 induction of CD69 on the cell surface (130, 131). C3 and C4 showed increased IL-3-induced CD69 expression and increased cell IL-13 and IL-4 secretion by inhibiting HDAC (130, 132, 133). In addition, C3 and C4 promote an increase in IgE-mediated basophil degranulation (130, 134). This suggests that SCFAs may be one of the important factors regulating alkaline granulocyte activation, IL-13 expression and degranulation.

Differential regulation of ILC subpopulations by SCFAs

ILCs were divided into groups 1 (NK cells and non-NK cells ILC1), 2 (ILC2) and 3 (ILC3) according to their developmental and functional characteristics (135). It was found that C2, C3, and C4 triggered the P13K, AKT, and mTOR signaling pathways through the excitatory action of GPR43 receptors, thereby promoting the proliferation of intestinal ILC1s and ILC3s but inhibiting the proliferation of intestinal ILC2s (136–138). Similarly, C4 has been shown to inhibit ILC2 proliferation in allergic asthma, but this process may be related to the inhibitory effect of HDAC (125). This suggests that the effect of SCFAs on ILCs is mediated by both GPR receptors and HDAC.

ILC3s are a major producer of intestinal barrier IL-22, which is a member of the IL-10 family and a key cytokine regulating inflammation. It is upregulated in chronic inflammation and achieves anti-inflammatory effects by inducing intestinal epithelial cells to produce AMP and mucin and repair the integrity of the damaged intestinal epithelial barrier (139). In ILC3s, SCFAs differentially activate AKT or ERK signaling and increase IL-22 secretion *via* the AKT and STAT3 axes. Among them, C2 increased the secretion of IL-22 to a greater extent by activating the GPR43 receptor, and C3 increased the secretion of IL-22 to a lesser extent by activating the GPR43 receptor, but C4 had no effect on the secretion of IL-22 (140). The reasons for these findings may be that, on the one hand, C2 and C3 activate the GPR43 receptor in ILCs more easily than C4. On the other hand, it may be that there are other pathways of regulation. For example, C2 enhances the expression of IL-1R in ILC3 cells by activating the GPR43 receptor, and the increased level of IL-1R increases the sensitivity of IL-1β, thereby indirectly inducing the production of IL-22 (141). In addition, SCFAs can promote IL-22 secretion in ILC3s by activating the GPR41 receptor and inhibiting the HDAC pathway (Figure 2). After SCFAs enter cells, they upregulate the expression of hypoxia-inducing factor 1α (HIF1α) and aromatic receptor (AhR), which are differentially regulated by mTOR and STAT3 (141–143). HIF1α directly binds to the IL-22 promoter (144). Finally, histone modification increases the binding of HIF1α to the IL-22 promoter to increase IL-22 secretion (144). In conclusion, different types of SCFAs have different regulatory effects on different types of ILCs, and the mechanism is closely related to the expression of the GPR receptor and the inhibition of HDAC.

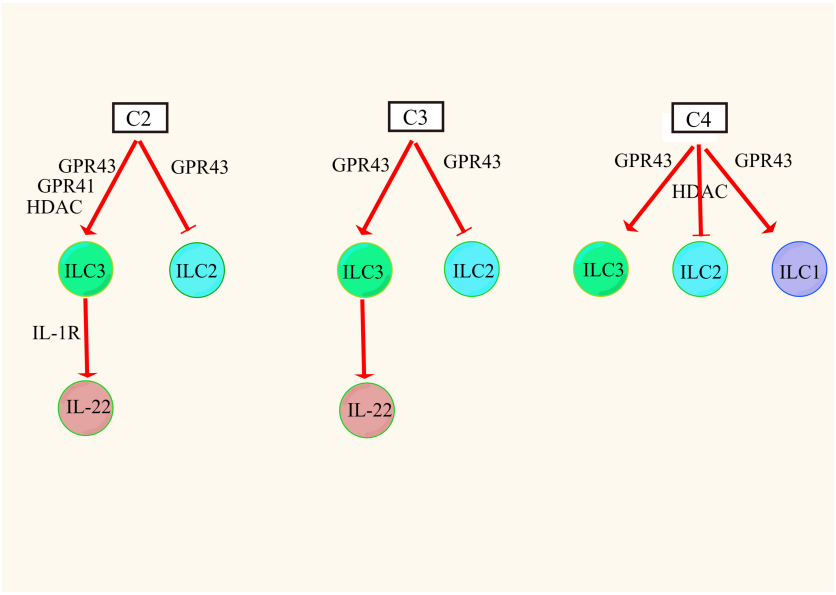

FIGURE 2
Effects of SCFAs on ILCs (125, 136–138, 140, 141).

Adaptive immunity

T cells

Previous studies have shown that 17 strains in the mouse gut microbiota (belonging to clusters IV, XIVa, and XVIII of *Clostridium difficile)* induce the TGF-β response by producing SCFAs, which may contribute to the differentiation and amplification of Tregs in the colons of mice (145). It was later determined that SCFAs promote the proliferation of Tregs (145). Treg cells include FoxP3$^+$ T cells, which prevent inflammatory reactions in the gut by producing IL-10 (146). In the presence of Treg cell polarization, C4 treatment of naive T cells enhanced histone H3 acetylation in the promoter, specifically inducing differentiation of mouse colon Treg cells by upregulating the acetylation of conserved noncoding sequences at FoxP3 (147). SCFAs can also indirectly promote the proliferation of Treg cells and IL-10 production through other cell types. For example, C4 can act on macrophages and DCs in a GPR109A-dependent manner, indirectly inducing FoxP3$^+$ T-cell and IL-10 production (44). However, a recent study found that C5 did not increase the amplification of Treg cells but increased the amount of additional acetyl-CoA in T cells, enhanced glycolysis through the mTOR activation pathway, and increased IL-10 production in lymphocytes by acting as a substrate for HAT to regulate the gene recoding process (76, 148).

SCFAs regulate T-cell metabolism through HDAC inhibition. SCFAs can increase the differentiation of naive T cells into effector T cells, such as Th1 and Th17 effector T cells, which may be related to the inhibitory activity of HDAC. In T cells, SCFAs were found to increase the acetylation of p70 S6 kinase and the phosphorylation of rS6 by inhibiting HDAC activity, thereby increasing mTOR activity to increase the production of Th17 and Th1 cells and IL-10(+) T cells (149, 150).

SCFAs can affect T cells during the antiviral process. It was found that oral SCFAs may affect CD8+ T-cell metabolism in a GPR-dependent manner and by inhibiting HDAC action during active immunity to ensure rapid activation of effector T cells in response to viral infection (151). Further studies later found that the regulation of C4 on CD8+ T cells was mediated by the inhibitory activity of HDAC, independent of the GPR41 and GPR43 receptors (152). SCFAs (specifically C4) also increase the number of CD8+ memory T cells in the spleen and liver and play an important role in establishing an optimal secondary antigen-specific response (153). This process is generally thought to increase glycolysis and mitochondrial mass to promote the survival and activation of CD8 + memory T cells (153). SCFAs have been widely recognized as an energy source for cells. In the tumor microenvironment, SCFAs can enhance the ability of CD8+ T cells to compete with tumor cells for glucose, thus increasing the survival and activation of CD8+ T cells (154). SCFAs enhance the antitumor activity of CD8+ T cells and chimeric antigen receptor (CAR) T cells through metabolic and epigenetic reprogramming (148). Therefore, SCFAs can regulate the metabolism of T cells according to the states of the host.

B cells

B cells require glycolysis, oxidative phosphorylation, and the synthesis of palmitic acid (PA) in the processes of proliferation, differentiation, and secretion of antibodies. Glycolysis and oxidative phosphorylation are essential for the survival of germinal B cells, and fatty acids (FAs) are involved in the process of antibody secretion by regulatory plasma cells (PCs) (155). Previous studies have shown that certain probiotics, such as *Lactobacillus and Bifidobacterium* species, increase fecal and serum IgA and IgG levels but decrease fecal and serum IgA levels in germ-free and antibiotic-treated mice (156). In mice fed prebiotics, SCFA content and IgA levels in feces and the large intestine increased in a dose-

dependent manner, with increased IgA and IgG levels in serum and no change in IgE and IgM levels. These findings suggest that SCFAs produced by prebiotics may promote the differentiation of B cells and the production of antibodies (157). The differentiation of B cells into PCs and the production of antibodies require energy metabolism within the cell to produce sufficient ATP. Previous studies have shown that SCFAs can be converted to acetyl-CoA (which produces ATP in the TCA cycle) *via* acetyl/propionyl/butyryl CoA (158). Acetyl-coA is the main substrate for FA synthesis (159). FA contributes to the differentiation of plasma B cells and stimulates B cells to produce antibodies (160). The contents of acetyl-CoA, ATP, lipids, malonyl CoA and fatty acid synthase (FAS) were increased in B cells treated with SCFAs, and ATP, malonyl CoA and FAS were essential for FA production (161). In addition, SCFAs promote B-cell differentiation and antibody production by increasing glycolysis in B cells (39).

SCFAs affect B-cell differentiation and antibody production through HDAC inhibition and GPR receptor mediation. Studies have found that SCFAs change the expression of B-cell-related genes (IgGs, IgA, Igj, Igk, Igl, Aicda, Xbp1, Irf4, etc.) by inhibiting HDAC. These genes participate in the differentiation of B cells and promote their differentiation into antibody-producing PCs (39, 162–164). B cells can express the GPR receptor, and studies have found that compared with wild-type mice, mice lacking the GPR43 receptor have relatively low IgA levels in the gut (165).

SCFAs regulate B cells through a number of indirect mechanisms. SCFAs increase glucose uptake by T helper cells and follicular helper T-cell (Tfh) induction, which regulates B-cell and antibody production (39, 166). C2 amplifies TLR signals in Tfh cells, and TLR selectively changes the levels of some IgA-producing microorganisms by sensing LPS from different microorganisms (167). In addition, C2 regulates antibody secretion by regulating dendritic cells (DCs), activates B cells by producing BAFF/APRIL and produces retinoic acid (RA) to induce IgA production (165).

SCFAs can regulate the production of B10 cells (regulatory B cells (Bregs) that produce IL-10 to maintain immune homeostasis) by a different mechanism. C2 can be converted to acetyl-CoA synthetase (ACSS), which increases the differentiation of B10 cells in the peritoneal cavity of mice by promoting the acetylation of ATP and lysine produced by the TCA cycle. C3 has no direct effect on the differentiation of B10 cells (168). C4 has been reported to induce an increase in splenic B10 cells by indirectly promoting the production of the serotonin-derived metabolite 5-hydroxyindole-3-acetic acid (169). C4 can also induce IL-10-producing splenic B10 cells by regulating the circadian clock-related genes RAR-associated orphan receptor α and nuclear receptor subfamily 1 group D member 1 (170, 171). A subsequent study showed that SCFAs upregulated peritoneal B10 cells in colitis mice in a manner dependent on their HDAC inhibitory activity. Independent of the GPR receptor pathway, transcriptional analysis showed that the action of C4 on B10 cells was related to the activation of p38 MAPK (172). C5 can not only increase the secretion of IL-10 but also significantly inhibit the apoptosis of Bregs (145). These results indicate that different SCFAs have different effects on B10 cell development, which encourages us to conduct further research.

Association of SCFAs with disease

Allergic asthma

The pathogenesis of allergic asthma is not well understood. Clinical treatment focuses on reducing symptoms by inhalation of corticosteroids and β-2 adrenergic agonists. Recently, mice fed SCFAs showed the protective effects of SCFAs against allergic airway inflammation (122, 173). The inflow of eosinophils into the lung parenchyma is the signature feature of the most common allergic asthma.

During allergic inflammation, IL-5, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF) secreted by Th2 and ILC2 cells promote eosinophil survival (174, 175). Activated eosinophils are major sources of cytokines, growth factors, and cytotoxic granulocytes (such as eosinophil-derived neurotoxins and major basic proteins) that can cause tissue damage and airway remodeling when released (176, 177). Recent *in vitro* studies using human peripheral blood eosinophils have shown that C3 and C4 inhibit eosinophils from adhering to endothelial cells in response to CCL24 flow, and C4 inhibits eosinophil migration and promotes eosinophil apoptosis (128). Surprisingly, these effects may be independent of GPR43 or GPR41 receptors but depend on the flow of these SCFAs into eosinophils *via* monocarboxylate transporters (128). In addition, we found that the biological effects of SCFAs on eosinophils are consistent with epigenetic regulation of gene expression by class IIa DAC, suggesting that these effects of SCFAs on eosinophils may be mediated by HDAC (128, 177). However, a previous study showed that diet-induced C3 prevents airway inflammation, resulting in decreased eosinophilic infiltration in lung tissue and decreased concentrations of the cytokines IL-4, IL-5, and IL-17A. This effect requires the participation of GPR41 but not GPR43 and is attributable to impaired DC activation (178, 179). Therefore, whether the effect of SCFAs on eosinophils depends on GPR43 or GPR41 receptors needs to be further investigated.

ILCs function to coordinate immunity, inflammation and tissue repair and can be present on the mucosal surface of the lung and drive allergic airway inflammatory responses (180, 181). ILC2s are of great concern in the context of asthma and allergic diseases because they promote Th2 immunity. Systemic and local administration of C4 attenuates ILC2-driven airway hyperresponsiveness and eosinophil inflammation. C4 can regulate the expression of ILC2s and inhibit their proliferation at the transcriptional level. C4 inhibits the proliferation of ILC2s and the production of the cytokines IL-5 and IL-13 by inhibiting the activity of HDAC without affecting cell viability and without being mediated by the activation of GPR41 or GPR43 (125, 182). C2 may increase the acetylation of the Foxp3 promoter through the inhibition of HDAC9, thus inhibiting the occurrence of allergic asthma (183). In addition, SCFAs can also affect allergic airway inflammation by affecting lung airway mast cells, Treg cells, Th9 cells, DCs, etc. (166, 173, 184–187). There are multiple mechanisms for the beneficial effects of SCFAs on the human airway immune inflammatory response, and further well-controlled long-term

intervention studies are needed to confirm the beneficial effects of SCFAs in airway immune inflammatory diseases.

Colitis

High dietary fiber intake and increased SCFA levels play an important role in protecting colon immune barrier function and in the colonic secretion of anti-inflammatory factors. SCFA administration can improve the symptoms of various types of colitis and reduce the probability of human colitis. Chronic intestinal inflammation can increase the risk of colon cancer. The concentration of SCFAs in stool is closely related to the incidence of colitis and colon cancer. SCFAs can reduce the risk of chronic colitis developing into colon cancer and promote the apoptosis of cancer cells to play an antitumor role (188). Studies have shown that the number of butyrate-producing bacteria in colon cancer patients is significantly reduced, and the expression of receptors GPR43 and GPR109A is also reduced considerably, indicating that SCFAs have a protective effect on colitis and colon cancer (189). The protective effect of SCFAs on colitis has been discussed extensively. SCFAs can regulate colon inflammation through innate immunity and antigen-specific adaptive immunity. As previously discussed, SCFAs can mediate a natural immune inflammatory response by inhibiting HDAC activity *via* GPR receptors. SCFAs can also affect intestinal IL-10 production and IgA secretion through multiple mechanisms. SCFAs generally show anti-inflammatory effects in the colon depending on the concentration and the immunological environment.

Osteoporosis

Osteohomeostasis is maintained through coordination between the bone formation process managed by osteoblasts and bone resorption managed by osteoclasts. Probiotics prevent bone loss, promote bone formation and increase bone volume in mice (190–192). Previous studies have shown that SCFAs can indirectly stimulate bone formation. C4 increases the proportion of CD4 +/CD8+ T cells and the number of Treg cells in the bone marrow. Treg cells activate NFAT and SMAD signal transduction, which results in indirect induction of Wnt10b production in CD8+ T cells and thus indirect stimulation of bone formation (193). A recent study showed that C3 and C4 directly upregulate osteoblast differentiation. Alkaline phosphatase (ALP) activity is a marker of osteogenic differentiation of mouse embryonic osteoblast progenitor cells (MC3T3-E1 cells). C2 and C3 increase the activity of ALP, and C2 increases the expression of ALP mRNA; however, C4 does not affect the activity of ALP or the expression of ALP mRNA (194). Osteopathic (OPN) is a highly phosphorylated and glycosylated salivary protein that is expressed in osteoblasts and osteoclasts (195). C2, C3 and C4 increase the expression of OPN in MC3T3-E1 cells (194). SCFAs can inhibit osteoclast differentiation (161). The differentiation of precursor cells into mature osteoclasts depends on oxidative phosphorylation, and the bone resorption of mature osteoclasts depends on glycolysis (196, 197). By inducing the metabolic recoding of osteoclasts, C3 and C4 weaken the oxidative phosphorylation of precursor cells through a process dependent on mature osteoclasts, enhance glycolysis, induce a stress response, and prevent osteoclast differentiation. Moreover, C3 and C4 downregulate the expression of the osteoclast genes TRAF6 and NFATC1 to inhibit osteoclast differentiation (192).

Conclusion

Over the past few decades, by sequencing and analyzing different types of human gut microbiota and constructing their metabolic processes, researchers have recognized the important roles of microbial metabolites in health and disease mediated through microbe-host interactions. As one of the most important metabolites of intestinal microorganisms, SCFAs have been shown to regulate host physiology and health through innate and adaptive immunity. For example, SCFAs can affect the inflammatory response of the central nervous system and affect bone diseases (198–204). In addition, it can regulate rheumatic diseases, osteoarthritis, hepatitis, vasculitis and so on (169, 205–209). Although the current review has limitations, it is challenging to decipher all the complexities of the effects of intestinal SCFAs on immune metabolism. This calls for further exploration of the relationship between SCFA pairs and the immune system, which is critical for identifying treatment options based on SCFAs.

Author contributions

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

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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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EDITED BY
Nathella Pavan Kumar,
National Institute of Research in Tuberculosis (ICMR), India

REVIEWED BY
Vincent Kam Wai Wong,
Macau University of Science and Technology, Macao SAR, China
Qingwen Tao,
China-Japan Friendship Hospital, China

*CORRESPONDENCE
Xinchang Wang
ossani@126.com
Weijie Wang
jack1987168@163.com

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Prevention and treatment of rheumatoid arthritis through traditional Chinese medicine: role of the gut microbiota

Yujiao Liang[1], Mengyao Liu[1], Yingxue Cheng[2], Xinchang Wang[3]* and Weijie Wang[3,4]*

[1]The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, China, [2]School of Basic Medical Science, Zhejiang Chinese Medical University, Hangzhou, China, [3]Department of Rheumatology, the Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China, [4]Institute of Basic Theory for Chinese Medicine, China Academy of Chinese Medical Science, Beijing, China

Recently, despite the increasing availability of treatments for Rheumatoid arthritis (RA), the incidence of RA and associated disability-adjusted life years have been on the rise globally in the late decades. At present, accumulating evidence has been advanced that RA is related to the gut microbiota, therefore, the therapeutic approaches for RA by regulating the gut microbiota are anticipated to become a new means of treatment. Traditional Chinese medicine (TCM) can regulate immunity, reduce inflammation and improve quality of life in various ways. Moreover, it can treat diseases by affecting the gut microbiota, which is a good way to treat RA. In this review, we mainly explore the relationship between TCM and gut microbiota regarding the perspective of treating RA. Moreover, we comprehensively summarize the roles of gut microbiota in the onset, development, progression, and prognosis of RA. Additionally, we elucidate the mechanism of TCM prevention and treatment of RA by the role of microbiota. Finally, we provide an evidence-based rationale for further investigation of microbiota-targeted intervention by TCM.

KEYWORDS

Gut Microbiota, Traditional Chinese Medicine, Rheumatoid Arthritis, immunology, rheumatic and immune disease

1 Introduction

RA is an immune-mediated disease, which is characterized by multi-joint, symmetrical, and invasive changes in small joints. RA usually occurs with symmetrical swelling and pain in multiple joints like hands, wrists, and feet. Morning stiffness, joint pain and tenderness, and joint swelling can be seen in the early time, and joint deformity occurs in patients in the later stage. In addition to the articular manifestations, there will also be some extra-articular manifestations such as cutaneous rheumatoid nodules, rheumatoid vasculitis, etc.

Cardiovascular diseases and respiratory diseases are the most usual complications of RA patients and the common causes of death in RA patients (1). The cause and pathogenesis of RA are complicated. Genetic susceptibility and environmental factors are the basis for RA's pathogenesis and development (2). Growing evidence has indicated that changes in gut microbiota are correlated to RA. Gut microbiota disturbance can be discovered in RA patients, and healthy microbiota can be partially recovered after disease-modifying antirheumatic drugs (DMARDs) treatment (3). In addition, the imbalance of certain bacterial lineages and changes in the metabolism of gut microbiota lead to changes in the host immune spectrum, leading to the pathogenesis of RA (4).

Traditional Chinese medicine (TCM) has been used for more than 2000 years in China with its unique theories (5, 6). Some TCM monomers and prescriptions have been used clinically to treat RA. TCM can treat RA in various ways such as immune network regulation and inhibition of inflammatory factors. In addition, TCM can also treat RA by regulating gut microbiota. This review will explore the mechanism and the progress of studies of TCM in treating RA by regulating gut microbiota, and provide new clues in the treatment of RA.

2 Advantages of Traditional Chinese Medicine in treating Rheumatoid Arthritis

RA is characterized by systemic damage and inflammation, affecting joints and extra-articular organs, which can lead to serious injury of joints and disability. Conventional treatments for RA encompass non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressive agents, glucocorticoids, and DMARDs. These drugs can effectively inhibit inflammation. However, long-term use may induce some adverse reactions, such as cardiovascular and gastrointestinal side effects, osteoporosis, etc (7). Fortunately, the types of drugs used in treating RA have steadily increased. The emergence of biological preparation like TNF-a, IL-6, and a small molecule targeted drugs such as JAK can effectively inhibit the progression of RA. But long-term use of biologics also leads to side effects that increase the risk of severe infection (8, 9). Furthermore, RA patients also suffered from great costs of the treatments. A recent meta-analysis estimated an annual direct medical cost in the US for RA of $12,509 for all patients using any treatment regimen and $36,053 for biologics users (10).

The widespread use of DMARD treatment is still hampered by the associated high cost and frequent side effects such as liver damage, cell reduction or increased frequency of infection, and certain cancers (7). Because of the shortcomings of these treatments, there is an active demand to find new drugs with fewer side effects and low costs to treat RA. TCM is rich in a great many chemical constitutions, which not only contain alkaloids, polysaccharides, glycosides, tannins, enzymes, and other active ingredients with therapeutic value but also contain a lot of nutritional active substances (11). TCM acts as an irreplaceable role in the treatment of RA. Some chemical components have been used to treat RA (12). For example, Sinomenine (SIN) has been found to have anti-rheumatic effects and has been ratified by the China Food and Drug Administration (CFDA) for RA treatment (13). The SIN group had a better effect on reducing hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) than the control group (14). Furthermore, some Chinese patent medicines are widely used in clinical treatment. The Zushima tablet (ZT) has been used to treat RA for ten years with good results (15).

Firstly, the combination of TCM and conventional treatments can enhance the therapeutic effect. In a randomized controlled clinical trial (RCT) of 22 RA patients, it was found that the clinical indicators of the Huayu Qiangshen Tongbi formula plus methotrexate (MTX) group improved earlier than those of the leflunomide (LEF) plus MTX group. In addition, bacterial purine degradation decreased, amino acid biosynthesis increased, and 11 and 9 metabolism pathways changed remarkably with time, higher than the 4 and 2 metabolism pathways in LEF plus MTX group (16). Secondly, TCM can reduce the side effects of conventional treatments. In another RCT comparing Tripterygium wilfordii Hook F (TwHF) with MTX in the treatment of RA, adverse events occurred in 49.3% of patients treated with combination therapy, which was much lower than 62.3% of patients treated with MTX alone (17).

3 Relationship between Gut Microbiota and Traditional Chinese Medicine

Gut microbiota is a collection of microorganisms colonized in the intestine (18). It is composed of more than 35,000 bacteria (19). The balance of gut microbiota has the effect of TCM on maintaining immune homeostasis. Both microbial components and microbial metabolites can affect immune regulation. Gut microbiota can affect the response mediated by the pattern recognition receptor encoded by the germ line. The activation of the PRR signaling pathway can lead to the production of antimicrobial peptides, cytokines, etc, and the destruction or change of the signaling pathway can lead to the occurrence of diseases. Monolayer epithelial cells enable microbial metabolites to obtain contact and interaction with host cells, thereby affecting immune response and disease risk (20).Gut microbiota imbalance is the basis of the occurrence and development of many diseases (21). In recent years, it has been indicated that gut microbiota dysbiosis is associated with the development of multiple chronic inflammatory joint diseases, including RA. It may trigger the host's innate immune system and activate the "gut–joint axis", which exacerbates the RA (22).

In a cohort study, *Turicibacter* and some *lactobacillus* species were selectively abundant in the gut microbiota of patients with systemic lupus erythematosus (SLE). Transfer of the fecal microbiota to sterile C57/B6 mice resulted in a series of lupus-like manifestations in the mice, and changes in the amino acid metabolism of the microbiota were observed in SLE mice (23). Moreover, compared with the healthy control group, the gut microbiota of Sjogren's syndrome (SS) patients were enriched in

Lactobacillus salivary, *Bacteroides fragilis*, and *Ruminococcus gnavus* (24).

TCM can reshape the functional components of herbs through trillions of gut microbiota and enzyme activities secreted by host cells (25). TCM has an impact on the gut microbiota, like regulating the structure and proportion of microorganisms (Supplementary Table 1). For example, rhein can reduce uric acid levels by increasing the level of lactobacillus in the intestine of mice (26). The extract of berberine can regulate the gut microbiota of Sprague-Dawley (SD) rats by increasing probiotics like lactic acid bacteria and reducing potential pernicious bacteria such as myxospira (27). Additionally, gut microbiota affects the absorption and metabolism of TCM, which can improve efficacy, reduce toxicity or produce toxic metabolites (28). First of all, gut microbiota can absorb and metabolize TCM. For example, Ellagitanin-containing foods such as strawberries, raspberries, etc, release ellagic acid in the jejunum and metabolize ellagic acid in the gut microbiota, and the resulting metabolites are absorbed (29). Secondly, the lipophilicity of TCM extracts is poor, and the bioavailability is low. The gut microbiota can change its lipophilicity and improve oral bioavailability (30). Therefore, through the role of gut microbiota, the efficacy of TCM may be improved. For instance, mulberry leaves can promote the increase of butyric acid content in the intestine by promoting the multiplication of Prevotella (31). Thirdly, the toxicity of TCM may be reduced. For example, the highly toxic alkaloid diester diterpenoid alkaloids extracted from the roots of Aconitum Carmichael are metabolized by gut microbiota to produce lipid alkaloids or lipo aconitine, and the toxicity is also significantly reduced (32). Finally, toxic metabolites may be produced. Bacteria such as Desulfovibrio can produce high levels of hydrogen sulfide, resulting in loss of colonic epithelial cells and loss of intestinal barrier integrity (33). The compatibility of licorice-kansui in TCM can significantly increase the proportion of Desulfovibrio and increase the concentration of sulfide in feces (34).

Besides, TCM acts on metabolic products of gut microbiota. TCM can regulate the production of Short-chain fatty acids (SCFAs) by acting on the gut microbiota, thus affecting the disease. SCFAs are the main metabolites generated by dietary fiber bacteria during gastrointestinal fermentation (35). The most common are acetic acid, propionic acid, and butyric acid (36). For example, the combined use of licorice (RG) and Beijing Euphorbia (REP) can increase the abundance of *Akkermansia* and *Butyricimonas*, while reducing the content of butyric acid in feces, resulting in adverse reactions to kidney, heart, etc (37). Acetic acid, butyric acid, propionic acid, caproic acid, isobutyric acid, and valeric acid were significantly increased in feces of Collagen-induced arthritis (CIA) rats, while the acetic acid, butyric acid, propionic acid, caproic acid, and valeric acid were significantly decreased after treatment with Angelica Sini Decoction, Among the 6 kinds of gut microbiota improved by Angelica Sini decoction, *g_norank_f_eubacterium_coprostanoligenes_group, g_Romboutsia*, and *g_Lactobacillus* are considered to be the key flora in the treatment of RA by Angelica Sini Decoction (38). Propionic acid and butyric acid can promote the ability of dendritic cells to transform naive T cells into FoxP3 $^{+}$ Treg by inducing IDO1 and Aldh1A2, and also inhibit the ability of naive T cells to convert into IFN-γ^{+} T cells (39). Acetic acid and propionic acid can increase the differentiation of naive T cells into Th17 cells in a dose-dependent manner and the derivation of Th1 cells with the presence of IL-12 (40). In summary, SCFAs can regulate T cells through a variety of pathways, thereby affecting intestinal immunity.

4 The relationship between Rheumatoid Arthritis and Gut Microbiota

Studies demonstrate that gut microbiota and its metabolites play a crucial role in the development of RA. First, the imbalance of gut microbiota can lead to the occurrence of RA. The comparative analysis of feces between RA patients and healthy people showed that the contents of *Klebsiella*, *Escherichia coli*, *Eisenbergia*, and *Flavobacterium* were higher in RA patients, and the contents of *Fusobacterium*, *Pseudomonas*, and *Enterococcus* were higher in healthy people (41). Roles of the gut microbiota in the pathogenesis of RA were also discussed by many studies and reviews through mechanisms including mainly production of proinflammatory metabolites, impairment of the intestinal mucosal barrier, and molecular mimicry of autoantigens (42). Furthermore, Inflammatory responses of some species of microbiota may be also one of the mechanisms influencing RA pathogenesis. *Lactobacillus bifidus* have showed effects on increasing the numbers of IL-17^{+} Th17 cells and activating Th1 cell responses which exacerbate RA (43). *L. plantarum TIFN101* which has effects on mucosal gene transcription, enhanced the intestinal mucosa immunity by increasing percentage of IL-17-producing activated memory Th cells and upregulated MHC-IIα (44).Moreover, the gut microbiota affects the development of RA. In patients with early RA, the abundance of *Pseudomonas aeruginosa* and *intestinal Lactobacillus* increased, *whereas* the fecal microbiota contained *Bifidobacterium*, *B.fragilis*, and *Enterobacter* decreased (42). In the active phase of RA, *Haemophilus* was depleted and the number of *Lactobacillus salivarius* increased (3). *Pseudomonas aeruginosa*, *Haemophilus*, and *Lactobacillus salivarius* are harmful bacteria, which can lead to metabolic disorders and destroy intestinal immunity (45). Intestinal *Lactobacillus*, *Lactobacillus acidophilus*, *Bifidobacterium, and Bacteroides fragilis* are beneficial bacteria with immunomodulatory effects and are closely associated with human health (46–48). In addition, the inducement of RA can be affected by adjusting gut microbiota. When SKG mice (RA model mice) were inoculated with the dominant flora *Prevotella*, the mice showed obvious arthritis, while when the mice were raised under sterile conditions or treated with antibiotics, no arthritis occurred, and the quantity of Th17 cells in the intestine of mice increased after fungal treatment (49) (Figure 1).

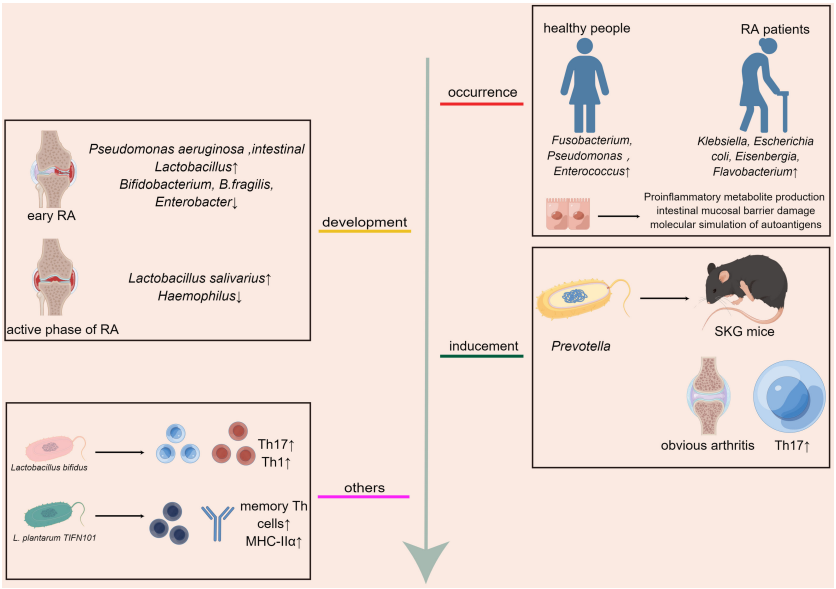

FIGURE 1
Gut microbiota affects the occurrence, development, and treatment of RA,the inflammatory responses of some species of microbiota may be also one of the mechanisms influencing RA pathogenesis.

5 The mechanism of Traditional Chinese Medicine in preventing and treating Rheumatoid Arthritis by regulating Gut Microbiota

TCM treatment can alleviate the symptoms of RA in a variety of ways. Gut microbiota is the largest source of microorganisms, and also the place where microorganisms interact with the human body (50). Dysregulated body state can lead to the imbalance of microbial composition and colonization and other functions, which in turn leads to the occurrence of autoimmune diseases (51). The effect of TCM on RA by acting on gut microbiota is divided into the following aspects. Firstly, the epigenetic modification of related genes could be modulated. After the intervention of Lycium barbarum polysaccharide (LBP), the abundance of *Lachnospiraceae _ NK4A136 _ group* and *uncultured _ bacterium _ f _ Ruminococcaceae* in CIA rat model decreased, and the abundance of *Romboutsia*, *Lactobacillus*, *Dubosiella*, and *Faecalibaculum* increased. LBP can increase the content of S-adenosylmethionine (SAM). The DNA hypermethylation of RA-related genes such as Dpep3 and Gstm6 in the host intestinal epithelium may be caused by increased SAM content (52). Secondly, proinflammatory cytokines were inhibited. Intestinal microflora disorders can trigger the abnormal activation of intestinal innate immune cells, leading to the up-regulation of pro-inflammatory cytokines and the reduction of anti-inflammatory cytokines (42).Atractylodes koreana (Nakai) Kitam can down-regulate inflammatory cytokines by regulating gut microbiota. After the treatment of CIA rats with Atractylodes koreana (Nakai) Kitam, the ratio of *Firmicutes/Bacteroides* increased and *Proteobacteria* and *Verrucomibia* decreased. At the same time,the inflammatory factors like TNF-α, IL-1, and IL-1β in the plasma of CIA rats decreased markedly, indicating that Atractylodes koreana (Nakai) Kitam can inhibit the generation of inflammatory cytokines in CIA rats and play a therapeutic role (53). Moreover, the abundance of specific flora increased in Complete Freund's adjuvant (CFA) rats after LBP intervention. In rats, LBP intervention inhibited the pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, and IL-6, thereby alleviating RA (54). Thirdly, the amino acid disorder could be improved. SCFAs can regulate intestinal endocrine function and play a significant role in host physiology (55). Berberine can reduce the diversity and abundance of intestinal bacteria in CIA rats but can increase the diversity of butyrate-producing bacteria, significantly increase the level of intestinal butyrate, and promote the production of butyrate by regulating gut microbiota as a therapeutic agent for RA (56). The therapeutic mechanism of improving synovial infiltration and vascular proliferation in RA rats after oral administration of Nakai Kitam may be related to the improvement of SCFAs imbalance in addition to the down-regulation of inflammatory factors (53). In addition to acting on SCFAs, TCM can also improve other amino acid metabolism through the gut microbiota. Wu-tou decoction (WTD) can partially inhibit inflammation and regulate gut barrier function by adjusting Bacteroides, Prevotella, Akkermansia and its related SCFAs, cholic acid, and indole propionic acid to improve RA (57) (Figure 2). Fourthly, T lymphocytes were intended to be regulated. Paeonia glycosides (TGP) intervention increased the relative abundance of beneficial symbiotic bacteria *Ruminococcace _ UCG-014*, *Oscillabacter*, and *Paraactoides* in CIA rats. In the meantime, TGP administration down-regulated the levels of Th1 cells and Th17 cells in CIA rats, and up-regulated the levels of Th2 cells and Treg cells. The effect of TGP on the dynamic changes of

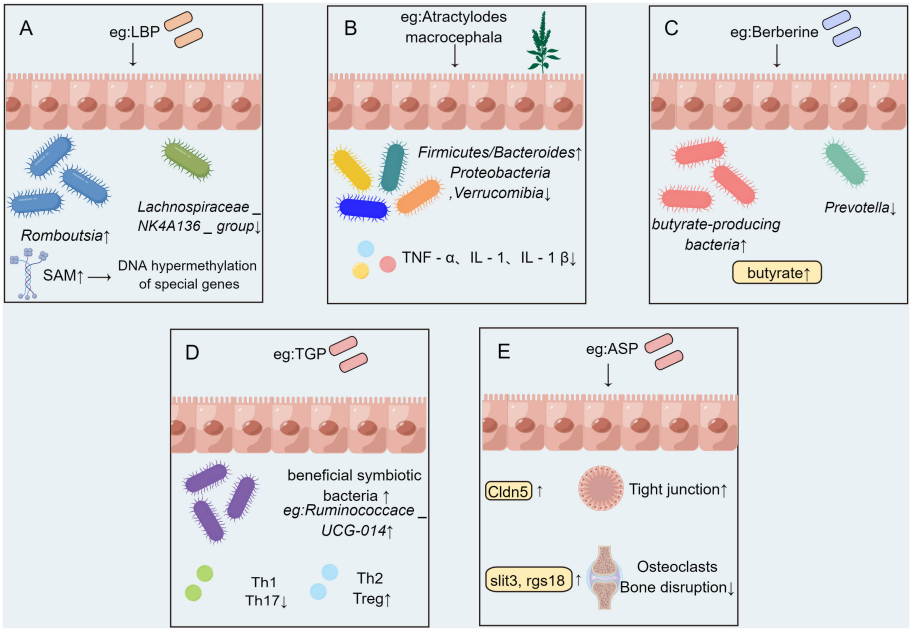

FIGURE 2
The possible mechanism of TCM affecting RA by acting on gut microbiota. **(A)** DNA hypermethylation of host intestinal epithelial related genes induced by Lycium barbarum polysaccharide (LBP) is related to the increase of bacterial metabolite S-adenosylmethionine (SAM) content. **(B)** Atractylodes koreana (Nakai) Kitam regulates gut microbiota and down-regulating the level of inflammatory factors. **(C)** Berberine can increase the abundance of butyric acid-producing bacteria and significantly increase intestinal butyric acid levels. **(D)** Paeonia glycosides (TGP) administration affected the structure of gut microbiota and down-regulated the levels of Th1 cells and Th17 cells, and up-regulated the levels of Th2 cells and Treg cells. **(E)** Angelica sinensis polysaccharide (ASP) shapes the composition of gut microbiota and regulates the expression of intestinal Cldn5, slit3 and rgs18.

gut microbiota supports the hypothesis that the microbiota plays a role in the therapeutic effect of TGP-mediated CIA rats (58). Fifthly, the expression of related gene mRNA changed. Transcriptomics showed that Angelica sinensis polysaccharide (ASP) regulates Cldn5 to improve intestinal dysfunction induced by RA, regulates the expression of Slit3 and rgs18 to regulate the balance of osteoblasts and osteoclasts, which may be related to gut microbiota (59). Taken together, TCM has the characteristics of multi-pathway and multi-target in the treatment of RA, and RA is treated through a variety of pathways (12).

6 Conclusion

The interaction between RA and gut microbiota is mutual. RA can result in changes in gut microbiota, and gut microbiota affect the occurrence, development, and inducement of RA. TCM improves the structure and proportion of gut microbiota and regulates metabolism. Gut microbiota affects the absorption and metabolism of TCM, thereby improving efficacy, reducing toxicity, or producing toxic metabolites. TCM can treat RA by improving gut microbiota structure, adjusting T lymphocytes in the intestine, regulating microbiota metabolites, affecting intestinal immunity and intestinal barrier function, and improving intestinal dysfunction. TCM not only increases the therapeutic effect of conventional treatments but also reduces the side effects of therapeutic drugs.

Early RA usually begins several years to several months before obvious polyarthritis (60). With the continuous development of RA, inflammation aggravates, and organ dysfunction gradually leads to disability. Inhibiting the production of inflammation is the key point for the treatment of RA. However, the TCM to improve the gut microbiota in the treatment of RA has many problems. Firstly, the composition and dosage form of TCM are complex. The ingredients of TCM are complicated, and there are many monomer ingredients. After compatibility, some new effects will be produced, and it is difficult to explain the mechanism through monomer components. Moreover, TCM has different dosage forms, such as pill, powder, and decoction. Therefore, the oral absorption rate could be different, which will have different effects after gut microbiota absorption and metabolism. Secondly, the gut microbiota is complex and affected by many factors. The species and quantity of gut microbiota are numerous, and the dominant species are different at different periods of human growth and development. The composition of gut microbiota is also related to individual physique and state, so it is difficult to unify the conclusion. The gut microbiota is influenced by multi-factor and is numerous, and it is difficult to capture subtle changes.

The treatment of RA through gut microbiota has a broad application prospect, such as the current “bugs as drugs”

bacterial-fecal transplantation therapy. The advantage of this therapy lies in the diversity of microorganisms, including not only bacteria but also viruses, fungi, etc. However, diversity and complexity also limit the reproducibility and measurability of microflora. Therefore, future research should focus on the development of specific microbial combination drugs with standard guarantees in drug purity, identity, and titer, to provide better measurability than fecal transplantation therapy. The beneficial mechanism of TCM in the treatment of RA by regulating gut microbiota is still at the preliminary stage of speculation. Future studies of TCM in the treatment of RA through gut microbiota can be carried out by establishing organoids, that is, by establishing a system highly physiologically related to the intestine of RA patients, to understand the effect of TCM on gut microbiota and the influence on inflammatory cytokines or inflammatory pathways in the intestine. On this basis, it can be verified by the knockout of related genes or the establishment of related transgenic animal models. In addition, after defining the specific gut microbiota, a comparative study of the microbiota can be performed to elucidate the changes in gut microbiota at low, medium, and high TCM doses. By observing the changes in specific gut microbiota structure anterior-posterior treatment with TCM, the structure of gut microbiota can be edited to clarify the pathogenic structure and treatment structure. In clinical research, large-scale sequencing of RA patients can be performed to establish a multi-center study to clarify the influence of TCM on gut microbiota in different RA patients with different ages, stages, and regions. More and more TCM and its active ingredients are needed to be identified and confirmed. TCM treatment of RA by intervening gut microbiota deserves further study.

Author contributions

WW contributed to the conception of the study. YL wrote the first draft of the manuscript. ML and YC performed the literature research. WW and XW supervised the work and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1233994/full#supplementary-material

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EDITED BY
Nathella Pavan Kumar,
National Institute of Research in Tuberculosis (ICMR), India

REVIEWED BY
Nadia Berkova,
Institut National de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), France
Lei Yue,
Chinese Academy of Medical Sciences and Peking Union Medical College, China

*CORRESPONDENCE
Satoshi Gojo
gojos@koto.kpu-m.ac.jp

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Host-directed therapy for bacterial infections -Modulation of the phagolysosome pathway-

Toshihiko Taya[1], Fumiya Teruyama[2,3] and Satoshi Gojo[3]*

[1]Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Pharmacology Research Department, Tokyo New Drug Research Laboratories, Kowa Company, Ltd., Tokyo, Japan, [3]Department of Regenerative Medicine, Graduate School of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Bacterial infections still impose a significant burden on humanity, even though antimicrobial agents have long since been developed. In addition to individual severe infections, the f fatality rate of sepsis remains high, and the threat of antimicrobial-resistant bacteria grows with time, putting us at inferiority. Although tremendous resources have been devoted to the development of antimicrobial agents, we have yet to recover from the lost ground we have been driven into. Looking back at the evolution of treatment for cancer, which, like infectious diseases, has the similarity that host immunity eliminates the lesion, the development of drugs to eliminate the tumor itself has shifted from a single-minded focus on drug development to the establishment of a treatment strategy in which the de-suppression of host immunity is another pillar of treatment. In infectious diseases, on the other hand, the development of therapies that strengthen and support the immune system has only just begun. Among innate immunity, the first line of defense that bacteria encounter after invading the host, the molecular mechanisms of the phagolysosome pathway, which begins with phagocytosis to fusion with lysosome, have been elucidated in detail. Bacteria have a large number of strategies to escape and survive the pathway. Although the full picture is still unfathomable, the molecular mechanisms have been elucidated for some of them, providing sufficient clues for intervention. In this article, we review the host defense mechanisms and bacterial evasion mechanisms and discuss the possibility of host-directed therapy for bacterial infection by intervening in the phagolysosome pathway.

KEYWORDS

bacterial infection, immune evasion, host-directed therapy, sepsis, antimicrobial resistance, phagocytosis, lysosome, V-ATPase

1 Introduction: infection and drug development

1.1 Sepsis

The treatment of bacterial infections has changed dramatically with the development of antibiotics, and many lives have been saved; in the era of the COVID-19 pandemic, tuberculosis, a widespread and deadly disease, has not been conquered, but it has been set aside as an infectious disease. On the other hand, bacterial infections are still strictly a major

threat to people's lives and a major treatment target, one of which is sepsis caused by a rapid and massive bacterial load (1) and another is the emergence of antimicrobial resistance bacteria spurred by antibiotic overuse (2). Infectious diseases other than sepsis, such as tuberculosis, infectious gastroenteritis, bacterial pneumonia, and food poisoning, are limited to localized areas, and dysfunction of the tissues of the infected foci comes to the fore. Sepsis is defined as organ damage due to an inadequate host response to infection (3). In addition to the classic treatment of infusion, removal of infected lesions, and respiratory circulatory support, treatment is aimed at normalizing coagulation abnormalities to maintain organ microcirculation (4). Nevertheless, more than 11 million lives are lost annually to sepsis, making it the cause of nearly 20% of deaths worldwide (5). One of the reasons that current medical treatment for sepsis has been so hampered is that the host's immune system forms a troublesome response to sepsis. That response is the coexistence of an excessive inflammatory response and a prolonged state of immunosuppression (6). The former, also called a cytokine storm, is characterized by an overproduction of inflammatory cytokines as the predominant phenotype (7). Much of the pathology of sepsis is associated with this unhelpfully exuberant reaction of the host, which is thought to be a common end pathway that occurs with viral infections as well as bacterial infections, and suppression of excess cytokines and regulation of their receptors is thought to reduce the disease state (8). However, the major proinflammatory cytokines IL-1 (9) and TNF-α (10) which are major inflammatory cytokines in sepsis, have failed to improve the survival of sepsis. In addition, inhibitors of Toll-like receptor (TLR) 4, which detects many bacteria and transduces intracellular signals that trigger inflammation, have also failed to improve sepsis survival (8). Despite such disappointment, transcriptome analysis of leukocytes from patient blood in sepsis revealed that up to 80% of the pathways of cellular function are altered and that inflammatory and regulatory mechanisms are simultaneously driven in the first few hours after onset (11). The setbacks in these clinical trials and the genetic approach to pathophysiology have led to a major shift in our current understanding of the pathogenesis of sepsis, in which host immunity to sepsis is a conflict between attack and suppression, far from its original goal of eliminating pathogens (12). This understanding of the pathogenesis has led to a search for therapeutic strategies that achieve homeostasis of host immune function.

1.2 Antimicrobial resistant bacteria

For antibiotics, the invention of new drugs in the nearly 30 years since the 1940s, a golden age, has been a wonderful scientific breakthrough that has led to an overly optimistic fantasy that bacterial infections will cease to be a threat to humanity (2). However, in the half-century since the 1970s, only a few new classes of antibiotics have been invented, and in addition, we have been handicapped by the disastrous situation with multidrug-resistant bacteria. Despite advances in understanding the life cycles of bacteria and long-awaited advances in molecular biology and genetics, biology and medicine today are far behind the good old days of the past 30 years in terms of progress in the field of infectious disease treatment. In the past half century, mankind has not only used invented antibiotics in large quantities in medicine but also abused them in livestock in search of economic rewards (13). The result has been a situation in which bacteria that have acquired multidrug resistance, also known as superbugs, have become rampant. Tuberculosis remains an uncontrolled infectious disease worldwide, and is the leading cause of mortality among mono infectious diseases, with 1.4 million deaths per year (14). Currently, it is estimated that 1/4 of the earth's population is infected with *Mycobacterium tuberculosis*, the majority of which is considered to be in the latent stage, but reactivation is a common occurrence (15). Although the current standard of care is to continue the four-drug combination for at least six months (16), reinfection cannon be completely prevented, and 18% of these infections are caused by multidrug-resistant organisms (14, 17). Modern medicine has devoted many resources, and with the struggles of the field, has managed to prevent the global spread of antibiotic-resistant bacteria from becoming a pandemic threat like COVID-19. Given that the doubling time of bacteria is in hours, the speed of their molecular evolution is tremendous. Considering that even if a new drug is invented, it takes a certain amount of time for its clinical development, this weasel-word is extremely at disadvantage for humans. Nevertheless, new antibiotics are being developed to win the battle, and in addition, methods to attenuate bacterial toxins and phage-based methods (18, 19).

1.3 Current microbicidal strategy

Understanding the pathogenesis of sepsis is directly linked to drug development, which is moving toward therapies that can eliminate pathogens while balancing the active and regulatory systems of the immune system (6). One of the molecular basis of sepsis is the transformation of the energy supply system of immune cells, and various compounds related to PGC1α, which activates mitochondrial biosynthesis, are being investigated for their efficacy in the treatment of sepsis (20). In the treatment of antibiotic-resistant bacteria, the use of immune checkpoint inhibitors that block inhibitory signals in T cells, TGF-β to activate T cells, M1-like macrophage adaptive transfer, and strategies such as the administration of gelsolin, an endogenous protein, to enhance the pathogen clearance of macrophages are beginning to be explored (5). Methodologies to intervene in host immunity and promote pathogen elimination are beginning to emerge in the form of specific methods and compounds.

In this review, we focus on therapeutic strategies for infectious diseases through intervention in the host rather than approaches to the pathogens themselves. In the field of cancer therapy, the development of drugs aimed at killing the cancer cells themselves and the intervention of host immunity have made remarkable progress (21). Although immunity plays a major role in infectious diseases, the host approach has been neglected to date. Defense against microorganisms is mediated by the effector mechanisms of innate and adaptive immunity. Innate immunity is mainly responsible for defense in the early stages of infection, whereas

adaptive immunity, together with innate immunity, provides a stronger and more specific response, and establishes a sustained defense posture with immune memory (22). The balance between these host immune responses and the acquisition of microbial resistance determines whether infection is established (23). The initial response of the host to bacterial infection is recognition of the bacteria by cells possessing pattern recognition receptors, release of inflammatory cytokines such as IL-1β, TNF-α, and IL-6, vasodilation, and increased vascular permeability (24). This leads to the accumulation of leukocytes, mainly neutrophils, which are non-specialized phagocytes, and more phagocytes in the infected foci. These cells phagocytose extracellular bacteria and infected cells and serve as the first line of defense in bacterial clearance (25). Inflammatory cytokines activate adaptive immunity, leading to enhanced antibody production by B cells, and opsonized bacteria are subject to phagocytosis by phagocytes, while T cells produce a variety of cytokines, including IFN-γ, to enhance bacterial killing by phagocytosis (26). Antibodies, together with activated complement, cause bacterial neutralization and lysis and play a role in the host defense system. In the early stages of infection, phagocytosis plays a central role in bacterial killing. On the other hand, bacteria that cause intracellular infection ensure their survival and replication by disabling the phagolysosomal system, which is the executor of intracellular disinfection.

Mycobacterium tuberculosis, which causes intracellular infection, can cause delayed-type hypersensitivity and tissue damage. Slow-growing *Mycobacterium tuberculosis* evades the killing of the phagolysosomal system and survives intracellularly, resulting in persistent stimulation of T cells and macrophages and the formation of granulomas (27). This granuloma and the solid tumor microenvironment share common features of immunosuppressive conditions such as lymphocyte exhaustion/elimination, macrophage polarization to M2-like phenotype, hypoxia, immunomodulatory cytokines such as TGF-β/IL-10, and infiltration of myeloid-derived suppressor cells (5). This similarity reminds us that host-directed therapy, which has been successful in anticancer therapy, could bear great fruit in infectious diseases. Among host immune mechanisms, the phagolysosomal system is considered to be at the center of pathogen control and an appropriate target for infection control. In order to examine the possibility of intervention in the phagolysosome system in host-directed therapy, the molecular mechanism of the pathway from phagocytosis to phagosomes reaching lysosomes is discussed from the perspective of host-pathogen interaction. Finally, the current status and future potential of drug discovery targeting the phagolysosome pathway will be discussed.

2 Phagocyte-pathogen interaction

2.1 Intracellular and extracellular microbes

When considering host-directed therapy for bacterial infections, it is important to divide bacteria into those that cause extracellular infections and those that cause intracellular infections. Bacteria that cause extracellular infections include Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Escherichia coli, Vibrio cholerae, Clostridium tetani, Neisseria meningitidis, and Corynebacterium diphtheriae. On the other hand, bacteria that cause intracellular infections include Mycobacterium, Listeria monocytogenes, and Legionella pneumophila (22). Microorganisms can be classified into three types: 1) obligate extracellular growth parasites, which cannot grow inside the cell but only outside, 2) facultative intracellular growth parasites, which can grow both inside and outside the cell, and 3) obligate intracellular growth parasites, which can grow only inside the cell. Obligate extracellular growth parasites are eliminated by phagocytes and have developed resistance mechanisms against phagocytosis (22). S. aureus is classically recognized as a bacterium that causes only extracellular infections such as furuncles, carbuncles, impetigo, abscesses, septicemia, necrotizing pneumonia, and biofilm formation (28). Recent studies have shown that S. aureus can survive and proliferate intracellularly, which is a major factor in pathogenesis, making it a second category of bacteria (29) (Horn). Bacteria belonging to this category have evolved the ability to neutralize the phagolysosome. The third category of bacteria includes rickettsia and chlamydia, which are dependent on the host in terms of membrane structure and metabolism, respectively, but the immune mechanisms against them are beyond the scope of this review and should be referred to the cited review (30).

Innate immunity to extracellular infections is centered on complement activation, phagocyte activation, and inflammatory responses, and the final execution mechanism of bacterial elimination depends on the phagolysosomal system. Phagocytes directly recognize bacteria via mannose and scavenger receptors and enhance phagocytosis (25). In addition, both peptidoglycan, a major membrane component of Gram-positive bacteria, and LPS, an endotoxin of Gram-negative bacteria, activate the alternative complement pathway to opsonize bacteria. Like complement, bacteria opsonized by antibodies enhance phagocytosis (26). Extracellular bacterial protein antigens cause activation of CD4+ T cells, also assisting phagocytosis. Although neutralization and lysis of bacteria by antibodies are important defense systems, phagolysosomes as the final executor of bacterial elimination are central to bactericidal activity. Bacteria that produce intracellular infections have found a microenvironment (niche) within the phagocyte that is isolated from strong adaptive immunity and have acquired mechanisms that allow them to survive and replicate there. These bacteria have evolved mechanisms to disable the phagolysosomal system within the phagocyte and hijack the phagosome to survive (27). Adaptive immunity attempts to execute bacterial clearance through activation of the phagolysosomal system by recruiting phagocytes with the CD40 ligand signal and INFγ by CD4+ T cells. In the process of escape from the phagosome, the host can trigger a mechanism by which CD8+ T cells, upon receiving the signal, eliminate the infected cell itself (22). This section on host-pathogen interactions describes the general effector function of the host's phagolysosomal system on pathogens.

2.2 From recognition to capture: phagocytosis

The immune system quickly detects invading bacteria in the body and timely initiates phagocytosis as the appropriate response to eliminate the threat (Figure 1) (25). Phagocytes are estimated to make up less than 1% of all cells in the body (31). The ability of these cells to adequately patrol and scavenge throughout the body is critical for defense against foreign enemies (32). Although phagocytes form a constant protrusion (33), and signals from the calcium-sensing receptor (CaSR), a G protein coupled receptor, regulate phosphatidylinositide phosphorylation plasma membrane remodeling (34), and polymerization of the branched actin network just below the plasma membrane (35). Pathogen-sensing receptors include the pattern recognition receptors (PRRs) such as TLR4 that directly bind to pathogen surface structures (24), Fcγ receptors, and complement receptors interact with antibodies or complement that opsonize pathogens, and their interactions play a role in signaling to phagosome formation (25). The pattern recognition receptors (PRRs) involved in bacterial infections are Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C- and C- type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs). Ten TLRs have been identified in humans and are present in dimeric form at the plasma membrane or phagosomal membrane (36). In the plasma membrane, TLRs exist as homodimers or heterodimers and

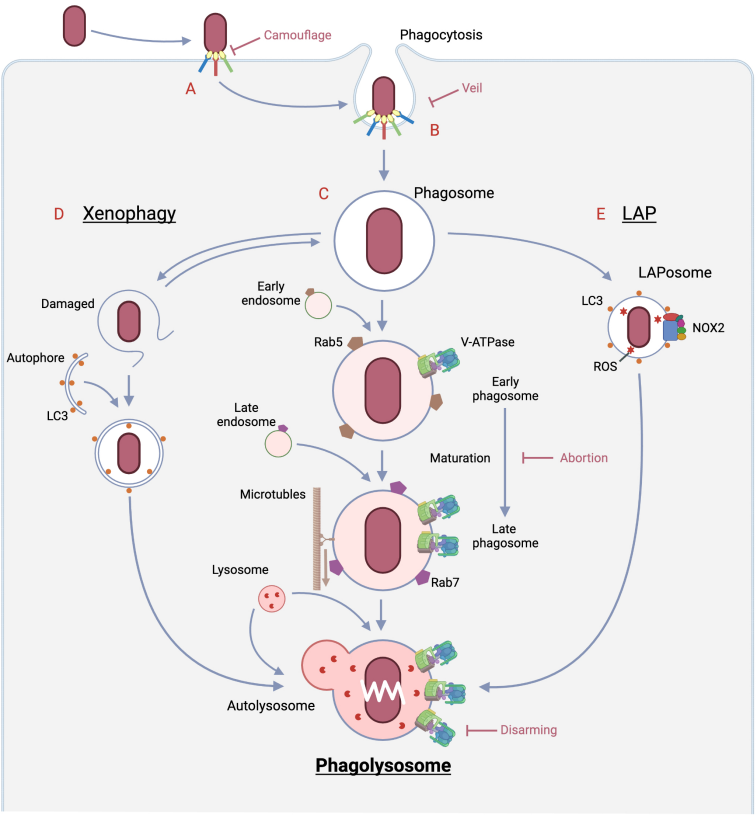

FIGURE 1
Pathways of bacterial eradication by phagocytes and immune evasion strategies by bacteria. A: The encounter between the bacteria and the host phagocyte is the initial site of rivalry that determines whether infection is established or not, and begins with the sensing of the bacteria by the host. Bacteria LPS is sensed by PPRs, and if opsonized by immunoglobulin and complement, by Fc or complement receptors, respectively. In the case of opsonization by complement, some bacteria evade detection by the complement receptor by mimicking the regulatory mechanism of complement. In the case of opsonization by antibodies, some bacteria prevent binding to the Fc receptor by secreting an enzyme that digests the antibody. Some bacteria alter the structure recognized by PRRs by phosphorylation or other means to escape from the PRRs. B: The process from phagocytic ruffle through cup closure and scission to phagosome formation requires dramatic changes in the cytoskeleton and in membrane phosphatidylinositides, which are regulated by many signals such as phosphorylation. Bacteria prevent phagocytosis formation by disrupting these signals through the secretion of dephosphorylases. On the other hand, there are viruses that produce substances that mimic the "Don't eat me" signal as phagocytosis checkpoints. C: Phagolysosome pathway (Middle): Nascent phagosomes undergo fusion with early/late endosomes, hydrolase increases toward full set and V-ATPase also increases. As a result, the phagosome lumen becomes acidified and moves along the cytoskeleton toward the site of lysosome presence, where it fuses with the lysosome. The resulting phagolysosome reaches a pH near 4.6, the optimum pH for many hydrolases, to carry out complete bacterial degradation. D: Xenophagy (Left): When the phagosome is damaged by a bacterial escape mechanism, galectin, which is only exposed in the lumen, is exposed in the cytoplasm, which triggers autophagy initiation. If the cargo is a pathogen such as a bacterium, the autophagy is called xenophagy. The vesicles also eventually fuse with the lysosomes, resulting in complete digestion of the pathogen. E: LAP (Right): LAPosomes, in which LC3, which plays an important role in autophagy, engages the nascent phagosome, recruits NOX2 and produces reactive oxygen species. Reactive oxygen species are formed most efficiently in a neutral environment, and they damage pathogen-forming lipids, proteins, and nucleic acids more rapidly than phagolysosomes. The final disposition of the inclusions of this pathway is also completed by fusion with lysosomes. Created in BioRender.

recognize lipids, proteins, lipoproteins, and other components of microorganisms. On the other hand, in phagosome membranes, they exist as homodimers and recognize microbial nucleic acids (37). TLR1- TLR2 and TLR2-TLR6 are expressed in monocytes, dendritic cells, and are involved in the recognition of triacyl lipopeptide, lipoprotein, lipopeptide, lipoteichoic acid, arabinomannan, peptidoglycan. TLR4 homodimer is expressed in macrophages and dendritic cells and binds to lipopolysaccharide. TLR4 homodimer is expressed in macrophage and dendritic cells and recognizes lipopolysaccharide. TLR5 homodimer is expressed in intestinal epithelial cells and senses flagellin (24). NOD1 in the cytoplasm of intestinal epithelial cells and macrophages recognizes γ-D-glu-meso-diaminopimelic acid in the cell wall of gram-negative bacteria, while NOD2 recognizes muramyl depeptide in the cell wall of all bacteria (38). CLRs are expressed on macrophages and dendritic cells and play a critical role in anti-fungal immunity. They include the mannose receptor, which recognizes mannose units repeated on the surface of bacteria such as mycobacterium and induces phagocytosis, and the Asian glycoprotein receptor family, which includes Dectin-2, which recognizes mannose-capped lipoarabinomannan (39). ALRs are PRRs that recognize intracellular double strand DNA and do not participate in innate immunity but are involved in apoptosis (40).

The protrusion-captured target induce clustering of phagocytic receptors, and the immunoreceptor tyrosine-based activation motif (ITAM) in their intracellular domain (41) is productively phosphorylated by Src-family tyrosine kinases (SFKs), spleen tyrosine kinases (Syk) (42). As soon as the phagocytosis signal begins to amplify and a transient increase in PI (4, 5)P_2 occurs, conversion occurs from PI (4, 5)P_2 to PI (3–5)P_3 by PI3K recruited to adaptor proteins (43). PI (3–5)P_3 surges recruits phospholipase Cγ which breaks down PI (3–5)P_3 to produce diacylglycerol (DAG) and inositol (1, 4, 5)-triphosphate (IP_3) (44). DAG acts as a second messenger for signaling between phagocytic receptors (45), while IP_3 provides calcium spike from the endoplasmic reticulum (ER) into the cytoplasm (46). These two 2nd messengers cooperate to activate small G protein Rap1, which mediates the "inside-out" response of integrin (47). PI3K activates Rho family GTPases that facilitate cytoskeletal remodeling directly and through the GEF. This activation dynamically alters the cytoskeleton to form phagocytic cups. NADPH oxidase 2 (NOX2), which is responsible for the generation of reactive oxygen species (ROS) that cause oxidative bursts, engages in the newly formed phagocytic cups (48).

On the other hand, there are systems that prevent phagocytosis, which phagocytoses pathogens and apoptotic cells, from running amok and eliminating normal cells. In cancer research, immune checkpoints have been identified as entities of T cell regulatory mechanisms (49). Immune checkpoint inhibitors such as anti-programmed cell death protein 1 (PD-1; pembrolizumab and nivolumab) (50), anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4; ipilimumab and tremelimumab) (51), anti-PD-1 ligand 1 (PD-L1: atezolizumab, avelumab and durvalumab) (52) have developed, and demonstrated to significantly improve outcome in patients suffered from devastating cancers (Figure 2A). In innate immunity, phagocytosis checkpoints recognize "Don't eat me" signals during the phagocytosis process, and are beginning to be recognized as important new targets for cancer immunotherapy (53). The discovery was signal-regulatory protein α (SIRPα) expressed in the myeloid lineage (54). Upon the interaction of SIRPα and CD47, the intracellular domain of SIRPα, an immunoreceptor tyrosine-based inhibitory motif (ITIM) recruits SH2-containing protein tyrosine phosphatase 1 (SHP1) or SHP2,

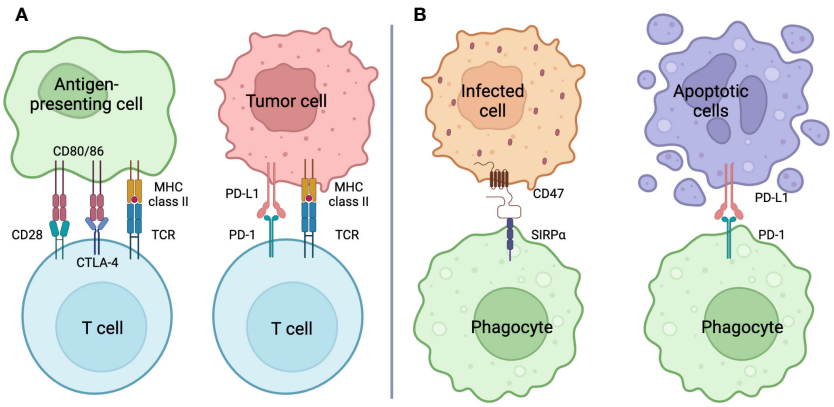

FIGURE 2
Checkpoint Inhibition **(A)** Immune checkpoint: T cells exercise T cell activation, clonal expansion, and effector function by transmitting signals via the TCR and signals from CD28 through binding to CD80/86 as costimulatory signals. On the other hand, inhibitory signals from CTLA-4 through binding to CD80/86 result in T cell deactivation. T cells expressing PD-1, which is also recognized as a T cell exhaustion marker, transmit inhibitory signals by engaging its ligand PD-L1, causing T cell deactivation similar to that of CTLA-4. Antibodies against molecules that comprise this immune checkpoint (immune checkpoint inhibitors: ICIs) shield the target antigen and cause T cell activation by releasing the T cell brake. Host-directed therapy with ICIs for cancer is an important anti-tumor strategy that works in tandem with anticancer drugs that kill the cancer cells themselves. **(B)** Phagocytosis checkpoint: Phagocytes, like T cells, have receptors with intracellular domains that transmit inhibitory signals to control their activity. The lignads, also called "Don't eat me" signals, include CD47 and PD-L1, whose receptors are SIRPα and PD-1, respectively. Cells infected with pathogens show enhanced expression of CD47 as the main inducer of INF-g, while cells in apoptosis express PD-L1 and escape clearance by phagocytes. These phagocytosis checkpoint inhibitors may promote phagosytosis and, in the case of bacterial infection, may promote bacterial killing. Created in BioRender.

preventing myosin IIA dephosphorylation, subsequent rearrangement of the cytoskeleton, and the phagocytosis they form (55) (Figure 2B). Although PD-1 expression is an important marker of T cell exhaustion (56), it also is expressed on various immune cells, including macrophages. The interaction with PD-1 and PD-L1 provides a suppressive signal for the phagocytosis of tumor-associated macrophages (TAMs) (57). Cancer cells can escape macrophage-induced phagocytosis by expressing PD-L1. Sialic acid-binding immunoglobulin-like lectin (SIGLEC), which contains inhibitory receptor motifs (ITIMs) in its intracellular domain, is induced at the surface of macrophages and its expression confers a poor prognosis in cancer patients (58). The ligand for SIGLEC is CD24, and this interaction serves as an entity for anti-phagocytic action (59).

2.3 Non-professional phagocytes

Non-specialized phagocytes involved in bacterial infections include epithelial cells, endothelial cells, osteoblasts, and fibroblasts. Epithelial cells, which play another major role among non-specialized phagocytes, cover the outer and luminal surfaces of the body and organs. Depending on where they are present, epithelial cell functions include absorption in the lungs and intestinal tract, secretion in the kidneys and stomach, and material transport in the trachea and oral-nasal cavity. Regardless of their location, the basic function of epithelial cells is to interact closely with the external environment and, in particular, to serve as the first line of defense in the immune system (60). Epithelial cells possess pattern recognition receptors to detect external hazards, but do not express receptors to capture opsonized pathogens as do specialized phagocytes. Therefore, phagocytosis of pathogens by epithelial cells is initiated by two following main methods. One is a trigger mechanism in which the cytoskeleton is restructured by effector molecules secreted by the bacteria, forming ruffles on the plasma membrane, and the other is a zipper mechanism in which the bacteria attach to proteins involved in cell adhesion, such as integrins and cadherins (61). After internalization, the phagolysosome system takes over for clearance.

2.4 To destination via 3 routes

Bacterial degradation in first line innate immunity is carried out through three main pathways (Figure 1): phagolysosome, xenophagy, and LAP (Microtubule-associated proteins 1A/1B light chain 3B (LC3)-associated phagocytoisis), each of which is followed by a vesicle: phagosome, autophagosomes, and LAPosomes, respectively, through fusion with lysosomes (60). In case of the first pathway, it begins with internalization by phagosomes after pathogen recognition, during which signaling occurs in the cell, leading to phagosome maturation (25) The phagosomes are then translocated to the lysosomes. Subsequently, the pathogen is degraded by phagolysosomes that are generated by fusion with lysosomes (60). On the other hand, autophagy is triggered when the imported bacterium attempts to escape from the phagosome, causing vesicle damage (62). Autophagy to enclose pathogens is called xenophagy, whose efficacy largely depends upon lysosomal function. In the third pathway, LC3, which plays a major role in autophagy, is embedded in the phagosome membrane in the form of LC3 modified by phosphatidylethanolamine (referred to as LC3-II), resulting in the formation of the LAPosome. The LAPosome is partitioned by a single membrane like the phogosome, unlike autophagy, which has a double membrane beginning in the phagophore (63). Its major feature is the quick recruitment of NADPH and the burst of reactive oxygen species that its enzymatic activity leads to, and the maturation of the LAPosome is faster than that of conventional phagocytosis. Review of bacterial killing in LAP and xenophagy are beyond the scope of this review; see other reviews (62, 63).

2.4.1 Phagolysosome pathway: phagosome maturation

Inactivation and decay of phagocytosed pathogens leading to acquired immunity requires dramatic transformation of the formed phagosomes, a process termed phagosome maturation. It is a process that many pathogens target for survival (64). This process leads to two intermediate states: early phagosome and late phagosome, and eventually to the formation of phagolysosomes. Nascent phagosome fuse with early endosomes and are responsible for sorting phagocytosed prey for reusability. The late phagosomes fused with the late endosomes create a more acidic environment in the lumen and migrate along the microtubules toward the lysosomes. The elaborate molecular mechanisms of this process have been detailed, with Rab GTPase and phosphatidylinositide playing major roles.

The newly formed phagosome has a PI (3–5)P3-rich membrane composition, and the recruitment of Rab5 GTPase to it promotes membrane fusion with early endosomes through several pathways (65) Vps34, type III PI3K, is recruited by Rab5 (66) and converts PI to PI3P, which becomes a major component of the membrane and attracts multiple effectors (67). One of them is early endosome antigen 1 (EEA1) (68), which interact with Soluble *N*-ethylmaleimide-Sensitive Factor Attachment Proteins (SNAPs), Syntaxin 6 (69) and Syntaxin 13 (70), to promote membrane fusion of nascent phagosomes and early endosomes (71).

The conversion to the late phagosome begins when the positive feedback loop of Rab5 is severed and replaced by Rab7 (72) PIs that make up the membrane are transformed from PI3P to PI4P by recruitment of 3- phosphatases of the myotubularin family and PtdIns4P kinase 2A (PI4K2A) (73). Rab7 forms homotypic fusion and vacuole protein sorting (HOPS), which mediate a tether between membrane with binding to Rab7, by replacing some of the components of class C core vacuole/endosome tether (CORVET) that mediate Rab5-mediated inter-vesicular tethering (74). GTP-bound active Rab7 recruits two Rab7 effectors: the Rab7-interacting lysosomal protein (RILP) and the long splice variant of the oxysterol-binding protein (OSBP)-related protein 1 (ORP1L) to move toward the microtubule-organizing center (MTOC) for a complete fusion with lysosome (75). They form a scaffold for dynein-dynactin to bridge the microtubule and are transported to the minus end along microtubules (76).

Centripetal movement brings the late phagosome and lysosome into close proximity, and Syntaxin 7 is involved, causing the phagolysosome (77). The two organelles undergo a process of tethering, docking, consolidation, and fusion, in which actin polymerization and calmodulin are involved in the tethering process, protein-protein interaction regulates docking, and Ca spiking leads to consolidation (78). In this stage, the composition of membrane PIs changes dramatically, with PI (3, 5)P2 joining the major PIs components. V-ATPase, which is responsible for intraluminal acidification, is completely incorporated in the phagolysosome membrane (79). Analysis of the immune escape mechanism of *Mycobacterium tuberculosis* revealed that V-ATPase also acts as a major player in membrane fusion and associates with HOPS (80).

2.4.2 Decay

Lysosomes are the defeaters that break down phagocytosed materials into their constituent parts and squeeze out the substances necessary for the host, and they have an arsenal of various weapons for this purpose. Against bacteria, the lysosome attempts to destroy them with reactive radicals, various digestive hydrolases, acidic milieu, and nutrient segregation as its main weapons. However, their regulation differs greatly among cell types, even among professional phagocytes (81). The control of these factors seems to depend on the roles of bactericidal action by reactive oxygen species and nitrogen, followed by complete digestion of the structure and transmission to the acquired immune system by antigen presentation to T cells. Macrophages are highly plastic cells and are classified into two activation states, M1 or classic and M2 or alternative, and the process leading to this state is defined as polarization (82). M1 M2 macrophages are induced by Th1 cytokines such as IFN-γ and TNF-α or LPS and secrete high levels of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α. -13 and secrete abundant levels of anti-inflammatory cytokines such as IL-10 and TGF-β (82). Macrophages have been shown to regulate V-ATPase and NOX2 very oppositely by polarization (83). M1 macrophages and neutrophils have low V-ATPase activity and a near-neutral lysosomal lumen but abundant production of reactive oxygen species (ROS). In contrast, M2 macrophages have high V-ATPase activity, the lysosome lumen is strongly acidic, and reactive oxygen species are not so high. However, the classification of M1/M2 macrophages is an oversimplification, as the two states are flexible and dynamically plastic, with intermediate rather than binary states, and there is a subset of regulatory macrophages in addition to activated and healing macrophages (84). It remains to be seen how polarization and its shift are regulated in infection, when the regulatory subset is committed during infection *in vivo*, and what are the keys that control these processes.

2.4.2.1 V-ATPase

In lysosomes and endosomes, V-ATPase is the only machinery that consumes energy to transfer protons into the lumen, but the acidic milieu produced by V-ATPase provides an optimal pH for the intraluminal hydrolase to perform bacterial killing (Figure 3). V-ATPase also plays an extremely multifaceted role in the phagolysosomal pathway (85). For example, recycling of plasma membrane receptors taken up into the lumen (86), recovery of the mannose 6-phosphate receptor into the trans-Golgi network (87), loading of external antigens into the major histocompatibility complex (88), and endosome tethering in phagosome maturation (80). In addition, acidic milieu plays an essential role in the following processes: neurotransmitter uptake (89), maturation by degradation of prohormone (90), nutrient sensing in association with mTORC1 (91), amino acid supply to the cytoplasm (92), and macroautophagy (93, 94). In signal transduction, WNT and Notch signals require an acidic milieu in the vesicle. In the WNT pathway, the Frizzled/LRP6 complex is in close proximity to V-ATPase via prorenin receptors on the signaling endosome. LRP6 phosphorylation required for the β-catenin destruction inhibition signal is dependent on V-ATPase activity (95). In Notch signaling, the Notch receptor is internalized by ligand binding and transferred onto the signaling endosome, and the acidic environment of the vesicle causes the Notch intracellular domain to be released into the cytoplasm through S3 cleavage by the γ-secretase (96).

Various hydrolases encapsulated in the lysosome have pH-dependent enzymatic activities and their acidity is precisely regulated. The methods reported for its regulation include 1. nutrition, 2. signaling, 3. cofactors, and 4. modification by enzymes. Nutrition was first reported as reversible disassembly in yeast during glucose starvation, and its biological significance is thought to be the limitation of ATP consumption under nutrient-depleted conditions (97). On the other hand, in mammals, V-ATPase assembly was shown to occur at an excess glucose concentration of 25 mM (98). Excess glucose increases glycolysis which leads to acidic environment in the cytoplasm, whereas the promotion of V-ATPase assembly is thought to be responsible for keeping the cytoplasm neutral by accumulating protons in the lysosome. It was reported that reversible disassembly in yeast is mediated by PKA, while regulated assembly in mammals is mediated by PI3K. Initially, the response of V-ATPase to glucose availability in yeast and mammalian cells appeared to be consistent, but the report that glucose starvation also promotes regulated assembly in mammalian cells showed the diversity of the regulatory mechanism (99). Glucose starvation activates AMPK, which is further enhanced by a complex with Regulator and assembled V-ATPase that provides a binding site for AMPK through AXIN (100). This AMPK activation may be directed toward improving energy supply and demand by shifting metabolism toward catabolism, one of which may be autophagy (101). Amino acids also have a significant effect on V-ATPase. In amino acid starvation, Regulator forms a tight complex with V1A of V-ATPase and eliminates mTORC1 while assembling V-ATPase (91). V-ATPase assembly leads to increased activity, and autophagy enhanced by amino acid starvation leads to amino acid acquisition by degradation of proteins brought to the lysosomes, resulting in release of amino acids into the cytoplasm to maintain homeostasis (101). With respect to signals, PI3K and its downstream AKT bring about regulated assembly (102). PI3K inhibitors do not prevent assembly (103), while AKT inhibitors prevent assembly (104). This suggests direct binding of AKT to V-ATPase (105). On the other hand, mTORC1 activity, which is downstream of PI3K/AKT, does

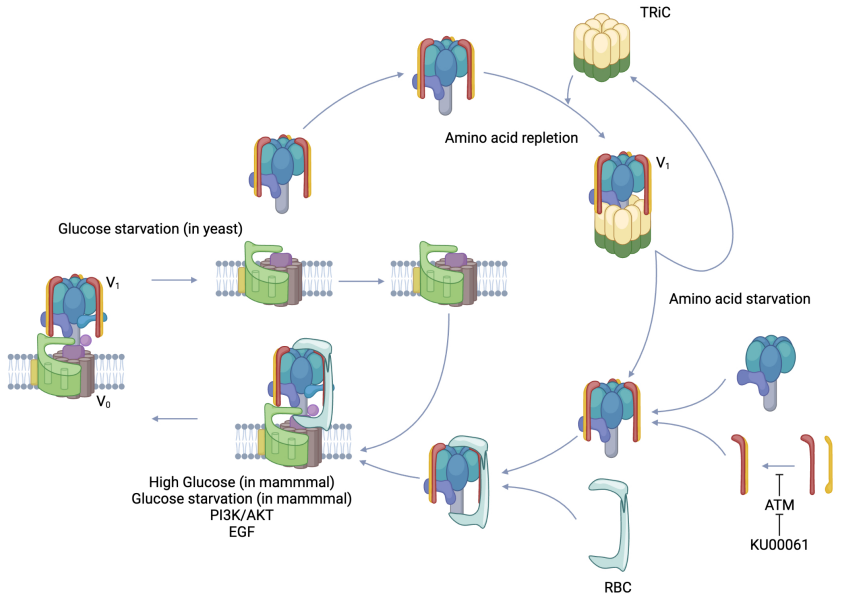

FIGURE 3
Regulated assembly/Reversible disassembly of V-ATPase. V-ATPase is composed of a membrane-integrated V0 complex and a V1 complex that can exist free in the cytoplasm. PI3K/AKT, EGF signal, and in mammals, glucose starvation and high glucose induce regulated assembly. On the other hand, glucose starvation induces reversible disassembly in yeast. In the case of amino acid deficiency, TRiC, which holds the V1 complex in the cytoplasm, releases the V1 complex and leans toward V-ATPase assembly. Rbc3 recruits V1 to V0 as a chaperone molecule. The dimer formation of V1E and V1G, which form the peripheral stalk of the V1 complex, is inhibited by the phosphorylation of V1G by ATM, resulting in inhibition of V-ATPase assembly. KU-60019, which inhibits ATM, promotes V-ATPase assembly. Created in BioRender.

not participate in V-ATPase assembly in the absence of amino acids (103). The AKT-mediated increase in the regulated assembly of V-ATPase, which results in decreased intraluminal pH, enhanced proteolysis, and increased cytoplasmic amino acid content, is also the mechanism by which EGF activates mTORC1 (106).

Cofactors and assembly chaperones such as Rabconnectin3 (Rbc3), TRiC, and mEAK7 and enzymes such as ATM have been reported. In yeast, RAVE (Regulator of H+-ATPase of Vacuolar and Endosomal membranes) regulates luminal acidity of lysosomes and endosomes via V-ATPase assembly, and in mammals Rbc3 is functionally equivalent to RAVE (107). Rbc3 is a heterodimer composed of Rbc3α and Rbcβ (108). The former is composed of either of two isoforms, DMXL1, or DMXL2 (109), and the latter is formed by WDR7. The combination varies among tissues and intracellular organelles (107). Rav1, which is a subunit of the yeast RAVE, recruits free V1C in the cytoplasm and contributes to V-ATPase assembly (110). DMXL1 and DMXL2 are homologs of Rav1, and the amino acid sequence in which Rav1 interacts with V1C is also conserved in DMXL (111). Functionally, silencing of any of the components of Rbc3 reduced the acidity in the vesicles (112). Knockout of WDR7 attenuated V-ATPase assembly (113). The regulation of Rbc3 is still to be elucidated, but one clue is calcium dynamics. CAB2.2, a transmembrane calcium channel, binds to DMXL (114), and CAPS1, which is involved in endoplasmic reticulum acidification through calcium dynamics, also binds to Rbc3 (115). TRiC holds the V1 component in the amino acid-replete cytoplasm, while releasing it for V-ATPase assembly in the presence of amino acid deprivation (116). A regulatory mechanism of TRiC could the phosphorylation of a subunit constituting TRiC. Phosphorylation of CCT2, a component of TRiC, modulates its function (117). mTORC1 signal may modify TRiC components to stabilize the TRiC/V1 component complex. mEAK7 engages V1A, B, and E in the N-terminal domain and binds to V1D in the C-terminal domain, but does not contribute to luminal acidification and affects mTOR signaling (118). Although ataxia telangiectasia mutated (ATM) was initially identified as a protein involved in the DNA damage response, it was recently reported to phosphorylate V1G and prevent the interaction with V1E, resulting in inhibition of the formation of a peripheral stalk (119).

2.4.2.2 Reactive radicals

Among the microbicidal effects induced by bacterial phagocytosis, the production of reactive radicals in the lumen is mainly mediated by NOX2 of the NADPH oxidase (NOX) family and inducible nitric oxide synthase (iNOS), which are triggered most rapidly after pathogen entry. The former produces reactive oxygen species (ROS) most prominently in neutrophils, while the latter produces reactive nitrogen species (RNS) mainly in macrophages (64). The superoxide anion ($O_2^{\cdot-}$) produced by NOX2 leads to the production of ROS represented by hydrogen peroxide (H_2O_2), hydroxyl radical (OH*), and hypochlorous acid (HOCl). This process is called a respiratory or oxidative burst because of the surge in oxygen uptake and glucose consumption unresponsive to cyanide (120).

The activated NOX2 complex transfers electrons from the cytoplasmic NADPH into the lumen of the phagosome, and the resulting charge imbalance is resolved by the voltage-gated proton channel Hv1, indicating that the activity of the NOX2 complex requires this channel to be activated (121). Since this ion channel does not consume energy, it is thought to function only when the pH of the phagosome lumen is near neutral, indicating that the respiratory burst of phagocytes induced by NOX2 complex activation occurs only in a very narrow range near neutral (122). The superoxide anion (O_2^-) generated by the NOX2 complex can utilize electrons from further NOX2 complexes and hydrogen from the phagosome lumen and Hv1 to induce superoxide reductase (SOR) or three types of superoxide dismutases (SODs) to produce hydrogen peroxide, leading to the formation of additional hydroxy radicals (123). These reactive radicals carry out their microbicidal action by disrupting structures containing DNA, Fe-S clusters, hemes, sulfhydryls, thioethers, and alkenes (124). ROS from the NOX2 complex are not prominent in macrophages, but in macrophages that swallowed the pathogen in defense against *S. aureus* infection, mitochondria-derived vesicles, which contain abundant hydrogen peroxide, fuse with phagosomes to provide reactive radicals, which are lacking in bacterial killing (125).

It is mainly the inducible nitric oxide synthase (iNOS) that produces reactive nitrogen species (RNS) that cooperate with ROS in pathogen killing (64). Nitrogen oxide is made of cytoplasmic L-arginine and oxygen, which undergo various catalytic reactions to produce nitrogen dioxide, peroxynitrite, dinitrogen trioxide, and dinitrosyl iron. Unlike NOX2, the regulatory mechanism occurs at the transcriptional regulation, and *de novo* protein synthesis is required for RNS production (126). Activation signals for iNOS include the extracellular proinflammatory cytokine interferon gamma (IFNγ) and the intracellular signaling molecule NF-κB (127).. It had been thought that iNOS is not recruited to the phagosome and remains in the cytoplasm; therefore, the RNS produced reaches the phagosome lumen by diffusion (128). In research on *Mycobacterium* spp., it was revealed that iNOS is recruited to phagosomes through binding with the scaffolding protein EBP50, while the bacillus attenuates the recruitment (129).

2.4.2.3 Nutrients

Iron, alone or incorporated into Fe-S clusters or heme, is essential for respiration, amino acid metabolism, and nucleic acid synthesis, not only in eukaryotes but also in prokaryotes. Excess iron leads to the ROS formation, while catalases and peroxidases that relieve oxidative stress require heme as a cofactor (130). The innate immune system has acquired the tactic of making iron unavailable to pathogens so that they can feed on the pathogens they have taken in (131). Lactoferrin is structurally very similar to transferrin, and it strongly binds to divalent iron ions even in the highly acidic environment of the lumen of the lysosome and exhibits antimicrobial action as an iron chelating agent (132). Iron is absorbed by bacteria via siderophore from the environment. Siderocalin (neutrophil gelatinase-associated lipocalin (GAL)), which inhibits siderophores, has been shown to effectively function, especially in sepsis caused by *E. coli* (133) and *Mycobacterium* spp (134).. Natural resistance-associated macrophage protein-1 (Nramp1/Slc11a1), in the membrane of phagosomes and functions as a divalent metal-proton symporter, has been implicated in the defense of intravesicular pathogens (IPs). Nramp1 starves IPs such as *Mycobacteria, Salmonella typhimurium*, and *Leishmania domovanii by* removing Fe2+, Co2+, and Mn2+ from the phagosome (135).

3 Phagocyte-pathogen interaction: evasion

Pathogens have various strategies to evade immunity and survive. In particular, the pathway from phagocytosis to digestion in the phagocyte, the first line of defense of innate immunity that the pathogen encounters, is the most important site that must be neutralized (64). Numerous molecular mechanisms have been described that allow pathogens to disarm the phagosome pathway and thereby acquire the microenvironment in which to survive and proliferate (Figure 4; Tables 1–3) (136).

3.1 Camouflage/veil

Salmonella typhimurium interferes with TLR4 recognition and signaling through deacylation and palmitoylation of lipid A present on their surfaces (137). *Helicobacter pylori* dephosphorylates lipid A to escape TLR4 (138). *Haemophilus influenzae, H. aegyptius, Streptococcus peumonie*, and *Neisseria gonorrhoeae* secrete proteases that selectively cleave immunoglobulin A, which is responsible for opsonization (139, 140). Complement also plays a role in opsonization, but some bacteria exploit the elaborate activation pathway of complement. *Neisseria mengitidis* is a bacterium that avoids phagocytosis by mimicking the host complement factor H (fH), the regulatory substance of complement, on its own surface (141). *Staphylococcus aureus* also recruits fH to its surface by secreting a substance called SdrE (142). YopH, a protein tyrosine phosphatase produced by *Yersinia* spp., dephosphorylates host phosphotyrosine proteins and prevents phagocytosis (143). *Vibrio parahaemolyticus* secretes an inositol polyphosphate 5-phosphatase, VPA0450, which disrupts host cell membrane integrity and causes blebbing (144). This tactic is *also used by Shigella flexneri, which* secretes IpgD, an inositol 4-phosphatase, as its virulence factor, causing plasma membrane blebbing by converting PI (4, 5)P2 to PI (5)P (145). Although not a bacterium, m128L encoded by *Myxoma virus* has a high homology to CD47, which is known to inhibit host phagocytosis as a "don't eat me" signal (53). The "don't eat me" signal is essential for establishing the lethal infection through inhibition of host phagocytosis (146). Although there are no reports of cases in which the bacteria themselves encode CD47 mimic, enhanced

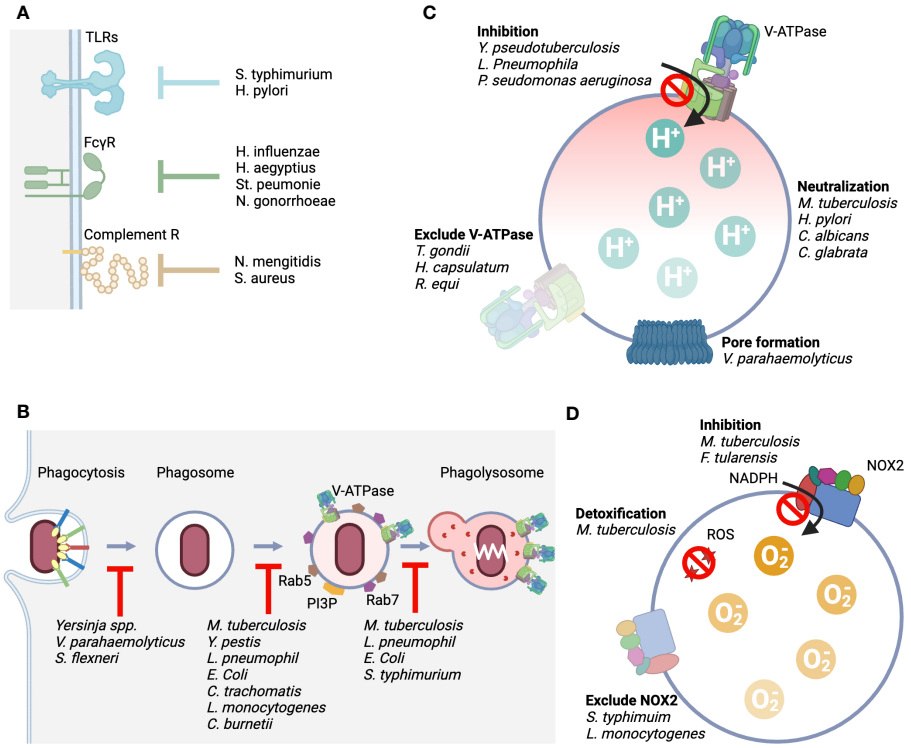

FIGURE 4
Rpresentative mechanisms by which pathogens evade from the host phagolysosomal system. **(A)** Representative mechanisms by which the host senses pathogens include TLRs, FcγRs, and complement receptors, and we show bacteria that possess mechanisms that counteract this sensing. **(B)**. It shows bacteria with an evade mechanism by inhibiting the process of phagocytosis, phagosome maturation, and fusion with lysosomes. **(C)**. The point of action at which the function of V-ATPase is attacked and the bacteria that carry it out. Four typical mechanisms are shown. Direct inhibition of pump function, elimination of the pump from the membrane, loss of proton concentration differences by generating pores, and production of enzymes that alkalinize acidified intralumens. **(D)**. The point of action at which the function of NOX2 is attacked and the bacteria that carry it out. Three typical mechanisms are shown: direct inhibition of the mechanism that produces superoxide, elimination of the complex from the membrane, and detoxification of the produced reactive oxygen species.

expression of CD47 in cells infected with *S. typhi* and *Borrelia burgdorferi* has been reported (147). The upregulation of CD47 expression occurs through signaling from PRRs and is also enhanced by inflammatory cytokines, suggesting that the CD47-SIRPα axis may work to suppress excessive inflammatory responses. Bacteria make good use of this host regulation mechanism to aid immune evasion. On the other hand, *M. tuberculosis*, a phagocytosis-dependent intracellular parasite, is unique in that it does not enhance CD47 expression, unlike other bacteria.

TABLE 1 Immune evasion strategies for processes from sensing to internalization.

Host process	Pathogen	Effectors	Mechanism
Sensing	*Salmonella typhimurium*	Lipid A: Deacylation/Palmitoylation	Escape from TLR4
	Helicobactor pylori	Lipid A: Dephosphorylation	Escape from TLR4
	Haemophilus influenzae	Proteinase for IgA	Inhibit the binding to FcgR
	Haemophilus. aegyptius	Proteinase for IgA	Inhibit the binding to FcgR
	Streptococcous peumonie	Proteinase for IgA	Inhibit the binding to FcgR
	Neisseria gonorrhoeae	Proteinase for IgA	Inhibit the binding to FcgR
	Neisseria mengitidis	Recruit complement factor H (fH)	Inhibit the binding to CR
	Staphylococcus aureus	SdrE to recruit fH	Inhibit the binding to CR
Phagocytosis	*Yersinia* spp.	protein tyrosin phosphatase: YopH	Inhibit the capturing
	Vibrio parahaemolyticus	VPA0450: inositol polyphosphate 5-phosphatase	Blebbing
	Shigella flexneri	IpgD: inositol 4-phosphatase	Blebbing

TABLE 2 Immune evasion strategies to prevent phagosome maturation.

Mycobacterium tuberculosis	NdkA as small GTPase inhibitor	Inhibit RAB5 and RAB7
	SapM as PI3P phosphatase	Inhibit PI3P generation
	MptpB as PI3P, PI4P and PI5P phosphatase	Arrest phagosome maturation
	ManLAM to activate calcium-dependent calmodulin	Inhibit PI3P generation
	phosphatidylinositol mannoside(PIM)	Inhibit RAB7
	trehalose dimycolate (TDM)	Inhibit lysosomal fusion
	sulfoglycolipid-1(SL-1)	Inhibit lysosomal fusion
	protein tyrosine phosphatase A (PtpA)	Inhibit lysosomal fusion
Yersinia pestis	Recruit RAB4a and RAB11b	Deviate the recycle
	Recruit RAB1b	Inhibit maturation
Legionella pneumophil	Dot/Icm type IV secretion system	Over 330 biological process affected
	SidM/DrrA: recrut RABa	Inhibit endosome fusion
Escherichia. coli	K1 capsule (α-2,8-kinked polysialic acid)	Inhibit lysosomal fusion
	Tir as a scaffold to SHIP2	Induce actin pedestal formation
Salmonella typhimurium	SopB as PI (4,5)P2 phosphatase	Inhibit lysosomal fusion
Chlamydia trachomatis	Not determined	Deviate to the secretary path
Listeria monocytogenes	Listeriolysin O	Generate pores in phogosomal membrane, leading to escape to cytosol
	Phospholipase (PlcA)	Purturb phagosomal membrane
	Phospholipase (PlcB)	Purturb phagosomal membrane
Coxiella burnetii	Ank as a type IV secretion system protein	Delayed maturation

3.2 Neutralization of intracellular microbial killing machineries

Although we are far from having a complete picture of the diverse strategies by which bacteria neutralize host immune attack within the cell, learning the molecular mechanisms is the first thing we must do to win the war against bacteria. The strategy of bacteria that acquire permissive niches intracellularly as intracellular pathogens (IPs) (148) provides a great clue for the construction of host-directed therapeutics (HDTs) (27). *M. tuberculosis*, a leading IP, has long been a major scourge to mankind due to its high prevalence and high mortality rate on a global scale (149), and is one of the most carefully investigated (150). *M. tuberculosis* is thought to have evolved in such a way to struggle with the host immune system that an exhaustive list of protein and lipid effectors produced by the bacillus has been compiled (151). In addition to *M. tuberculosis*, other potential IPs include *Rickettsia rickettsia, Chlamydia trachomatis, Legionella peumophila, Coxiella burnetiid, Brucella abortus*, and *Salmonella enterica*; *Cryptococcus neoformans and Aspergillus fumigatus* among fungi. *Candida albicans* uses other intracellular organelles such as mitochondria as a habitat. The mechanisms by which they evade the phagosome pathway have been intensively studied, and detailed molecular mechanisms have been elucidated (152).

3.2.1 Abortions of phagosome

M. tuberculosis has spun out more than a dozen countermeasures in the phagosome maturation stage alone. First, it secretes NdkA to repress the small GTPases RAB5 and RAB7, which are central regulatory molecules in phagosome maturation (153). In addition, SapM produced by the bacillus acts as a PI3P phosphatase and inhibits the formation of PI3P, which is essential for the maturation of membrane composition (154). Glycolipids on the surface of the bacillus also greatly influence this phagosome maturation. Mannose-capped lipoarabinomannane (ManLAM) suppresses PI3P generation via the calcium-dependent calmodulin pathway and prevents phagosome maturation (155). In addition, phosphatidylinositol mannoside (PIM) present in the envelope supports the retention of early endosome RAB proteins such as RAB5, RAB22A, and RAB14 and prevents the recruitment of late endosome RAB proteins such as RAB7 (156). With respect to fusion with the lysosome, trehalose dimycolate (TDM) (157) and sulfoglycolipid-1 (SL-1) (158) as lipid effectors prevent the fusion process between the lysosome and the phagosome. Furthermore, *M. tuberculosis* secretes toxins that inhibit the Ca/Calmodulin-PI3K cascade and attempts to survive as IP through a strategy of inhibiting the fusion of phagosomes and lysosomes (159). In phagosomes harboring *M. tuberculosis*, protein tyrosine

TABLE 3 Immune evasion strategies to neutralize bacterial destruction mechanisms.

Host process	Pathogen	Effectors	Mechanism
V-ATPase	*Mycobacterium tuberculosis*	Protein tyrosine phosphatase A (PtpA)	Inhibit lysosomal fusion
		Antacid 1-tuberculosinyladenosine (1-TbAd)	Neutralization
	Helicobacter pylori	Urease	Neutralization
	Candida albicans	Urease	Neutralization
	Candida glabrata	Urease	Neutralization
	Rhodococcus equi	Virulence-associated protein A (VapA)	Exclude V-ATPae
	Histoplasma capsulatum	Not identified	Exclude V-ATPae
	Yersinia pseudotuberculosis	Not identified	Inhibit proton pump
	L. pneumophila	SidK to bind V1A	Inhibit proton pump
	Pseudomonas aeruginosa	pyocyamin	Inhibit proton pump
	Vibrio parahaemolyticus	VopQ	Neutralization by pore formation
	Toxoplasma gondii	Not identified	Exclude V-ATPase
Reactive radicals	*Salmonella typhimuium*	Salmonella pathogenicity island-2 (SPI2)	Inhibit the accumulation of flavocytochrome b558
	Listeria monocytogenes	Pore-forming cytolysin listeriolysin O	Exclude NOX2
	Fransicella tularensis	fevR	Inhibit NOX2 activity
	Mycobacterium tuberculosis	Iron-dependent enzyme (SodA)	Detoxification
		Copper/zinc-dependent enzyme (SodC)	Detoxification
		KatG: Catalase/Peroxidase/Peroxynitritase	Detoxification
		CpsA	Inhibit NOX2 activity

phosphatase A (PtpA) secreted by the bacillus binds to V-ATPase V1H and inhibits the association of V-ATPase and HOPS, as well as dephosphorylates vacuolar protein sorting 33B (VPS33B), which forms HOPS, and thus loses its function as a fusion machinery (80).

Yersinia pestis targets organelle trafficking and recruits Rab4a early in infection and Rab11b late in infection to prevent phagosome maturation and inhibit acidification in the lumen (160). These small GTPases are involved in endosome recycling, and the *Yersinia*-containing vacuole mimics this process. In addition, *Y. pestis* recruits Rab1b to phagosomes to inhibit phagosome acidification by suppressing lysosome fusion (161). *Legionella pneumophila* has evolved a defect in organelle trafficking: intracellular multiplication (Dot/Icm) type IV secretion system to make the phagosome of alveolar macrophages a proliferative niche (162). This system provides more than 330 effector proteins that interfere with host biological processes to assist in bacterial replication and survival (163). Among them, the system is involved in the recruitment of Rab1 like *Y. pestis* (164) and provides Sid1/DrrA, which is involved in the regulation of Rab1 (165). *E.coli* K1 has a K1 capsule composed of α-2,8-kinked polysialic acid on its surface that inhibits the fusion of phagosomes and lysosomes. *Salmonella*-containing phagosomes also inhibits the fusion of phagosomes and lysosomes (166). *Salmonella* secretes phosphoinositide phosphatase to maintain PI3P levels in the membrane, thereby preventing phagosome maturation and fusion with the lysosome and ensuring its survival (167). *Chlamydia trachomatis* avoids the fusion of its internalized phagosomes with endosomes and directs them to the secretory pathway to avoid an acidic environment (168).

3.2.2 Disarming V-ATPase

It was shown 30 years ago that *M. tuberculosis*, when it reaches the phagolysosome, excludes V-ATPase from its membrane and maintains the lumen at a pH of 6.3 or higher (169). The entity responsible for excluding V-ATPase from the phagosome was PtpA (80). In addition, antacid 1-tuberculosinyladenosine (1-TbAd), which neutralizes acidification of the lumen, is secreted by the bachillus (170). *H. pylori, which* can live in highly acidic stomachs, has evolved various genes to adapt to the acidic environment. One of the effectors is urease, which produces ammonium ions that allow the pathogen survive in the harsh acid environment of the stomach (171). *Candida albicans and C. glabrata* use amino acids in their lysosomes to produce ammonium ions to neutralize the intraluminal pH (172, 173). *Mycobacteria* spp. have the same strategy (174). Research has been conducted to create more effective vaccine that lacks urease (175). a single bacterium has multiple defense mechanisms against the host offense of lysosomal acidification. In addition to *Mycobacterium* spp., *Rhodococcus* spp (176, 177). and *Histoplasma capsulatum* (178) were reported to exclude V-ATPases from phagosomes that contain them.

Some bacteria secrete substances that directly inhibit V-ATPase. *Y. pseudotuberculosis* directly inhibits the activity of the proton pump without affecting the expression level of the protein component of V-ATPase, thereby causing lysosomal deacidification (179). SidK produced by *L. pneumophila* physically binds to V-ATPase V1A and inhibits its proton transport (180). Structural analysis of the binding of SidK to V-ATPase showed that the two α-helical bundles at the N-terminus of SidK bind to V1A and markedly reduce the flexibility of its subunit (181). *Pseudomonas aeruginosa* secretes pyocyamin, which is a potent inhibitor of V-ATPase (182). *Toxoplasma gondii* survives by eliminating all components involved in membrane fusion with endosomes, resulting in non-acidic vacuole (183, 184). *Vibrio parahaemolyticus* secretes VopQ, a type III effector protein, which is incorporated into the lysosome membrane as a channel for the free passage of protons, and the pH in the cytoplasm and lysosome lumen is balanced (185).

3.2.3 Disarming NOX2

Salmonella typhimurium inhibits the accumulation of flavocytochrome b_{558} by releasing Salmonella pathogenicity island-2 (SPI2), a member of the type III secretion system (186). *Listeria monocytogenes* also eliminates the NOX2 membrane component by secreting the pore-forming cytolysin listeriolysin O (187). *Fransicella tularensis* not only excludes flavocytochrome b_{558} but also directly inhibits the activity of the NOX2 complex by releasing a regulatory factor called fevR (188). *M. tuberculosis* has also taken multiple countermeasures against reactive radicals, including two types of SODs that process ROS: iron-dependent enzyme (SodA) (189) and copper/zinc-dependent enzyme (SodC) (190) and both contribute significantly to the virulence of the pathogen. In addition, the bacillus secretes KatG, which serves as a peroxidase and peroxynitritase, to metabolizes reactive radicals produced by the phagocyte oxidative burst (191). When the bacillus is preyed upon by the LAPosome, it secretes CpsA as an effector and inhibits the activity of NOX2 (192).

3.2.4 Securing nutrition

Gram-negative rods *such as E. coli, Salmonella* spp., and *Klebsiella pneumoniae* restore the host-inhibited function of their own siderophores, which are responsible for iron absorption, by producing a protein called iroA (193). The thick waxy cell walls of *Mycobacterium* spp. provide excellent protection against severe environmental and host invasion, but are not conducive to the exchange of nutrients and metabolites necessary for growth and survival with the outside world. *Mycobacterium* attempts to secure iron by producing mycobactin as a siderophore (194). The host interferes with mycobactin, as it did against siderospheres of gram-negative rods, but *Mycobacterium* secretes Esx-3 of the type VII secretion system (Esx-1-5) to support mycobactin and iron absorption (195). *Aspergillus fumigatus* produces HapX in iron deficiency to suppress iron-consuming pathways such as host heme synthesis and respiration, including the TCA circuit, and to increase the production of iron-absorbing siderophores (196).

4 Host-directed therapy for bacterial infections

Against bacterial infections, tremendous resources have been devoted to the development of antimicrobial agents that kill the bacteria themselves. Cancer therapy has long since moved beyond the days when drugs were developed to kill cancer cells themselves, and HDTs have become a major pillar of cancer treatment. The development of HDTs for bacterial infections has just begun, and the development of HDTs for the phagolysosome pathway, the first line of innate immunity, has lagged further behind. The molecular mechanisms of the phagolysosome and bacterial evasion strategies described thus far provide major clues to HDT. In the following part, we would like to describe the current status of drug discovery that intervenes in the phagolysosome pathway.

4.1 Phagocytosis activator

The application of immune checkpoint blockade to infectious diseases has been investigated in the context of the interrelationship between innate and adaptive immunity, rather than between infected cells and innate immunity. Interventions on the PD1-PD-L1 axis are effective in animal studies against infectious diseases such as *malaria, toxoplasma, leishmania, and Listeria* (197). The results of a phase 1/2 trial of nivolumab in sepsis have overcome safety concerns, including the development of autoimmune disease (198). In cancer therapy, although macrophages have been intensively studied as targets for intervention in various aspects, phagocytosis that macrophages execute is recognized as a promising drug discovery (199). The findings of the phagocytosis checkpoint may provide clues to the treatment of infectious diseases (53). The inhibition of the PD-1-PD-L1 axis as a phagocytosis checkpoint enhances phagocytosis in liver Kupffer cells and prevent bacterial infection (200). A study using CD47 KO mice also reported that *E. coli* pneumonia showed better recovery compared to wild type (201).

4.2 V-ATPase activator

It was reported that monocytes from imatinib-treated patients with leukemia showed an increased production of V0a3 and V0c and had more acidic lysosomes. Sera from those patients, which are added to the cell culture of macrophages, enforced more acidic lysosomes and of *M. tuberculosis* (202). One compound was reported to promote regulated assembly of V-ATPase, KU-60119, which was identified as an ATM inhibitor that inhibited assembly of V1E and V1G through phosphorylation of the latter (119). ZLN005 was originally recognized as a peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) activator (203). By administering ZLN005 to the cecum perforation ligation (CPL) model of sepsis and analyzing intraperitoneal cells, ZLN005 was shown to be a transcription factor EB (TFEB) activator involved in lysosome biogenesis as well as a lysosomal acidifier (204). The

compound significantly improved overall survival and drastically reduced intraperitoneal bacterial load in mice at only 2 h after administration of ZLN005 in *in vivo* sepsis model. These results indicate that lysosomal acidification is a therapeutic target for sepsis.

5 Clinical aspects and limitations in host-directed therapy

When considering the pathogenesis of sepsis (205), in most cases the organism of origin is not known at the onset of the disease. When the pathogen is unknown, the choice of treatment, especially antimicrobial agents, is highly dependent on the experience and ability of the clinician. In contrast, the existence of host-directed therapies that are independent of the organisms causing the disease can first reduce the bacterial load. Even if not eradication, significant improvement in survival may be achieved if circulatory collapse and multiorgan damage can be avoided by reducing the excessive bacterial load to a controllable level, which has been beyond the reach of current initial therapy. When addressing infections caused by resistant bacteria (206), countering bacterial interference with lysosomes, which play a crucial role in bacterial elimination, emerges as a significant alternative to traditional disinfection methods.

The disadvantage of augmenting the phagolysosomal system is that overactivation of autophagy is a concern, since the lysosomal system is the final point of autophagy, which forms the basis of cellular function (62). There is also concern that digestion in lysosomes may be problematic by causing energy depletion in the cell, as significant energy expenditure is thought to occur during digestion in lysosomes (20). These are all reasons to believe that if host-directed therapeutics were to be discovered, the method of administration would need to be carefully set up.

6 Perspective

Bacterial infections are a major public health threat no less than malignancies, neurodegenerative diseases, and cardiovascular and metabolic diseases. The development of antibiotics with the goal of disinfection shines as the most significant achievement of 20th century medicine (2). Now, the development of new antibiotics against the emergence of resistant bacteria is taking a backseat to bacterial evolution. Anticancer therapy has seen a breakthrough with the establishment of host-directed therapy in parallel with the development of therapeutic agents aimed at eliminating cancer cells (207). Host immunity plays a major role in the pathogenesis of both cancer and infectious diseases (197). However, host-directed therapy for infectious diseases is still in its infancy (208). In both innate and adaptive immunity, the phagolysosomal system plays a central role in bacterial clearance against bacterial infections (22). Bacteria have evolved to achieve evasion of this phagolysosome system by any means necessary (64, 183). Due to rapid advances in antibiotic technology, the host has not yet evolved to acquire an effector mechanism to counter the evasion mechanism exhibited by resistant bacteria. On the other hand, detailed molecular biological analyses of this system have been performed (26, 101, 209), and the time is ripe for the development of drugs with a point of action in this system. Possible targets of action are numerous, including phagocytosis, phagosome maturation, fusion with lysosomes, lysosome acidification, and lysosome quality control. This system is a fundamental cellular system and is also heavily involved in neurodegenerative diseases (93) and embodies the development strategy of organelle drug discovery rather than the framework of disease-by-disease drug discovery. The development of host-directed therapeutics for bacterial infections has the potential to revolutionize the drug discovery system. It is hoped that the development of HDT together with a new class of antimicrobial agents, will work like two wheels on a cart, dramatically increasing the life-saving rate of sepsis and creating a treatment strategy that is not afraid of superbugs.

Author contributions

SG designed the concept of this review. SG and TT wrote and edited the manuscript and the table. FT prepared figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

FT is an employee of Kowa Corporation, but Kowa Corporation is not involved in any aspect of this study.

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Publisher's note

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EDITED BY
Sylvie Bertholet,
GSK Vaccines, United States

REVIEWED BY
Jayaraman Tharmalingam,
University of Wisconsin-Madison, United States
Dr Beatrice Omusiro Ondondo,
University Hospitals of Leicester NHS Trust, United Kingdom

*CORRESPONDENCE
Ann E. Sluder
asluder@mgh.harvard.edu
Mark C. Poznansky
mpoznansky@mgh.harvard.edu

†PRESENT ADDRESS
Anja Scholzen,
Byondis B.V., Nijmegen, Netherlands
Robert Shepard,
University of Massachusetts Chan Medical School, Worcester, MA, United States
Joshua M. Hess,
Program in Computational Biology &Medicine, Cornell University, New York, NY, United States
Skylar Korek,
Department of General Surgery, Penn State Hershey Medical Center, Hershey, PA, United States

‡These authors have contributed equally to this work and share first authorship

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Cytometry profiling of *ex vivo* recall responses to *Coxiella burnetii* in previously naturally exposed individuals reveals long-term changes in both adaptive and innate immune cellular compartments

Susan Raju Paul[1‡], Anja Scholzen[2†‡], Patrick M. Reeves[1‡], Robert Shepard[1†], Joshua M. Hess[1†], Richard K. Dzeng[1], Skylar Korek[1†], Anja Garritsen[2], Mark C. Poznansky[1*] and Ann E. Sluder[1*]

[1]Vaccine and Immunotherapy Center, Massachusetts General Hospital, Boston, MA, United States, [2]InnatOss Laboratories B.V., Oss, Netherlands

Introduction: Q fever, caused by the intracellular bacterium *Coxiella burnetii*, is considered an occupational and biodefense hazard and can result in debilitating long-term complications. While natural infection and vaccination induce humoral and cellular immune responses, the exact nature of cellular immune responses to *C. burnetii* is incompletely understood. The current study seeks to investigate more deeply the nature of long-term cellular recall responses in naturally exposed individuals by both cytokine release assessment and cytometry profiling.

Methods: Individuals exposed during the 2007-2010 Dutch Q fever outbreak were grouped in 2015, based on a *C. burnetii*-specific IFNγ release assay (IGRA), serological status, and self-reported clinical symptoms during initial infection, into asymptomatic IGRA-negative/seronegative controls, and three IGRA-positive groups (seronegative/asymptomatic; seropositive/asymptomatic and seropositive/symptomatic). Recall responses following *in vitro* re-stimulation with heat-inactivated *C. burnetii* in whole blood, were assessed in 2016/2017 by cytokine release assays (n=55) and flow cytometry (n=36), and in blood mononuclear cells by mass cytometry (n=36).

Results: Cytokine release analysis showed significantly elevated IL-2 responses in all seropositive individuals and elevated IL-1β responses in those recovered from symptomatic infection. Comparative flow cytometry analysis revealed significantly increased IFNγ, TNFα and IL-2 recall responses by CD4 T cells and higher IL-6 production by monocytes from symptomatic, IGRA-positive/seropositive individuals compared to controls. Mass cytometry profiling and unsupervised clustering analysis confirmed recall responses in seropositive

individuals by two activated CD4 T cell subsets, one characterized by a strong Th1 cytokine profile ($IFN\gamma^+IL\text{-}2^+TNF\alpha^+$), and identified *C. burnetii*-specific activation of CD8 T cells in all IGRA-positive groups. Remarkably, increased *C. burnetii*-specific responses in IGRA-positive individuals were also observed in three innate cell subpopulations: one characterized by an $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$ Th1 cytokine profile and lack of canonical marker expression, and two IL-1β-, IL-6- and IL-8-producing $CD14^+$ monocyte subsets that could be the drivers of elevated secretion of innate cytokines in pre-exposed individuals.

Discussion: These data highlight that there are long-term increased responses to *C. burnetii* in both adaptive and innate cellular compartments, the latter being indicative of trained immunity. These findings warrant future studies into the protective role of these innate responses and may inform future Q fever vaccine design.

KEYWORDS

Coxiella burnetii, Q fever, mass cytometry, cytokines, immune profiling, human, innate, cellular immunity

Introduction

Q fever is a zoonotic disease caused by the obligate intracellular gram-negative coccobacillus, *Coxiella burnetii*, and transmitted to humans through aerosols from infected ruminants such as goats, sheep, and cattle (1). *C. burnetii* exhibits significant resilience even in harsh environments (2) and is highly infectious with an ID50 of one bacterium (3). Consequently, *C. burnetii* is classified as a category B bioterrorism agent (4). Acute Q fever is asymptomatic in most infected individuals and, when symptomatic, presents largely with flu-like symptoms (1). Therefore, infection with *C. burnetii* is likely significantly underreported as observed in the largest reported outbreak of Q fever, which occurred in the Netherlands from 2007-2010. Although only 3500 infections were officially reported (5, 6), a survey of serological data suggested that there were an estimated 40,000 infections in the region at the center of the epidemic alone (7). While acute infection is often self-limiting and readily treatable with antibiotics such as tetracyclines, long-term complications of infection are common; 10-20% of patients with acute Q fever later develop Q fever fatigue syndrome, and 1-5% of infected individuals (often with other comorbidities) progress to chronic Q fever, which manifests as endocarditis, aneurysms, or vascular infections (1, 8). The single Q fever vaccine approved for use in humans, Q-VAX®, is an inactivated whole cell vaccine based on phase I *C. burnetii* licensed for use only in Australia amongst high-risk individuals. The vaccine is protective against Q fever (9) but has been reported to cause side effects in previously exposed individuals, particularly at the site of injection (10, 11). Therefore, only unexposed individuals can be vaccinated, and pre-screening is required prior to vaccination using an intradermal skin test and serology to assess for any prior immunity against *C. burnetii*. To eliminate this requirement for pre-screening and the associated cost and time, a number of efforts seek to develop novel vaccines that protect against Q fever whilst having minimal side effects and thus eliminate the need for pre-screening (12–14). These efforts would benefit from a more complete understanding of the cellular components contributing to immune responses to *C. burnetii* (15).

Previous studies characterizing human cellular recall responses in the context of *C. burnetii* exposure have largely focused on cytokine release in response to inactivated or viable *C. burnetii* antigen preparations using whole blood, PBMCs or isolated dendritic cell populations from healthy and exposed, convalescent individuals, patients with chronic *C. burnetii* infection, and those suffering from Q fever fatigue syndrome (16–23). Flow cytometric analysis in humans has been restricted to *ex vivo* phenotypic analysis of circulating innate and adaptive immune cells in individuals with Q fever endocarditis (18, 19, 24, 25). The only studies that have analyzed *ex vivo* recall responses by individual peripheral immune cell populations to *C. burnetii* by cytometry have been conducted in mice pre-sensitized by infection or through vaccination (26, 27).

The goal of the current study was therefore to develop an expanded description of the cell populations contributing to long-term recall responses years after natural exposure to *C. burnetii*. To this end, we performed a comprehensive characterization of innate and adaptive *in vitro* recall responses six to ten years after initial infection in individuals naturally exposed to and convalescent from *C. burnetii* infection during the 2007-2010 Dutch Q fever outbreak. This cohort included four subgroups of individuals based on differing *C. burnetii*-specific IFNγ responses, serological status, and self-reported clinical symptoms during their initial infections. We assessed *in vitro* recall responses to heat-inactivated *C. burnetii* using a combination of bulk cytokine secretion as well as single cell phenotypic analysis by flow cytometry and mass cytometry. Both bulk cytokine release and single cell data showed that both adaptive and innate cellular compartments exhibit long-term increased

responses following natural exposure to *C. burnetii*, which should prompt future studies to determine whether these innate responses also contribute to protection from infection by the pathogen.

Materials and methods

Ethics statement

The human study was reviewed and approved by the Medical Ethical Committee Brabant (Tilburg, Netherlands, NL51305.028.15) and all donors provided written informed consent.

Human study cohort

The human study cohort has been previously described (28). In brief, Q-fever exposed individuals were recruited from a cohort characterized in a previous large Q fever study conducted in the village of Herpen, the Netherlands (29), which experienced a high incidence of *C. burnetii* infection during the 2007-2010 Q fever outbreak (30). In this previous study, 80% of the adult village population was screened in 2014 for evidence of adaptive immunity towards *C. burnetii*. Individuals were invited to participate in this current study following pre-selection based on clinical history and adaptive *C. burnetii*-specific immune responses determined in 2014. All study participants, including immunologically *C. burnetii*-naive controls, resided during and following the Q fever outbreak in the same area and were assigned to study groups based on clinical history and immunological assays performed at the time of study enrollment in 2015. Cellular immunity to *C. burnetii* was assessed with the CE-marked Q-Detect interferon-γ release assay (31) as described previously (28). To maximize the potential to detect *C. burnetii*-specific T cells in this cohort who were predominantly enrolled for epitope-screening (28), preference was given to donors with strong responses to whole heat-killed *C. burnetii* in the IGRA and without potentially confounding immune disorders. In total, 143 participants provided written informed consent. IGRA responses were re-assessed upon enrollment in October 2015, and serological status was additionally assessed by immunoblot. Volunteers were allocated to control Group 1 if they had no history of Q fever disease (29), were negative (32) by IGRA and by immunofluorescence assay (IFA) in spring 2014, and were again negative by IFA and immunoblot when enrolled in the previous study (28) in autumn 2015. The remaining volunteers that were positive by IGRA in spring 2014 were subdivided based on their serostatus (IFA in spring 2014 and immunoblot analysis in autumn 2015) and past Q fever disease (either registered (notified) in the national surveillance system, or self-reported) into Group 2 (seronegative, asymptomatic), Group 3 (seropositive, asymptomatic) and Group 5 (seropositive, symptomatic). A single volunteer fell into Group 4 (seronegative, symptomatic) and was not included in further analysis. At the time of enrolment, all volunteers were convalescent from their original exposure to *C. burnetii*, and none of the volunteers included in this study had known active *C. burnetii* infection or were diagnosed as suffering from acute or chronic Q fever.

All peripheral blood samples for this study were obtained between July 2016 and May 2017, i.e., six to ten years after initial exposure during the 2007-2010 Q fever outbreak in the Netherlands. A total of n=64 individuals contributed to one or more aspects of this immune profiling study (Tables 1, 2).

Whole blood IFNγ release assay (Q-Detect™ IGRA)

The Q-Detect™ assay has previously been described in detail (31). In brief, whole lithium-heparin anti-coagulated blood was stimulated with *C. burnetii* antigen (heat killed Cb02629, a strain isolated during the Dutch Q fever outbreak, lot 14VRIM014 prepared by Wageningen Bioveterinary Research from a master cell bank using a cell-free culture method and quality controlled for protein concentration, functional TLR stimulation, and stimulation of IFNγ in samples from known Q fever-exposed individuals). Assays were performed in 96-well polypropylene plates (Greiner BioOne) by adding 180 µl blood to 20 µl *C. burnetii* antigen diluted in phenol red-free RPMI supplemented with Glutamax (2 mM), Gentamycin (5 µg/ml) and sodium pyruvate (1 mM, all ThermoFisher Scientific). A 1.5% (v/v, final concentration) solution of PHA-M (ThermoFisher Scientific) was added to separate wells for each sample as a positive control. Medium only

TABLE 1 Human study subjects.

Group	N	Age in years (median, range)[1]	Females N (%)	*C. burnetii* IGRA status[1]	*C. burnetii* Sero-status (IFA)[1]	Previous symptomatic Q-fever episode[2]	*C. burnetii*-specific IFNγ response in pg/ml (median, range)[1,3]
G1	21	56 [45–76]	11 (52%)	Neg	Neg	No	3.8 [0-29.4]
G2	10	54 [43–68]	6 (60%)	Pos	Neg	No	75.8 [29–460]
G3	10	56 [44–66]	6 (60%)	Pos	Pos	No	399 [106–1045]
G5	23	50 [32–72]	15 (65%)	Pos	Pos	Yes	384 [64–1048]
All	**64**	**54 [32–76]**	**37 (59%)**				

[1]At inclusion into the study in October 2015.
[2]Either formally notified or self-reported.
[3]Q-detect IGRA ELISA, background subtracted values.
Bold values indicate combined values for all study participants.

TABLE 2 Subject numbers included in each immune profiling assessment.

	Group				
Profiling study	G1	G2	G3	G5	All
Cytokine secretion	20	10	5	20	55
Flow cytometry [1]	17	0	0	19	36
Mass cytometry	9	8	10	9	36

[1]Two rounds of flow cytometry profiling were performed; n=3 subjects from G1 and n=1 subject from G5 were included in both rounds.

was added to the negative control wells for each sample, results from which were used to correct for any background levels of IFNγ in the sample. All stimulations were performed in duplicate. After 22 ± 1 hours whole blood cultures were re-suspended. IFNγ concentrations were assessed in whole blood by ELISA using the IFNγ Pelipair protocol (Sanquin) with minor modifications. The upper detection limit of IGRA under these conditions is 1050 pg/ml. A subject was scored positive by IGRA if the *C. burnetii antigen* induced IFNγ production was ≥16 pg/ml above background and the ratio of the logarithmic value of background-subtracted *C. burnetii* antigen and PHA responses ((log[*C. burnetii*]-log[neg control])/(log[PHA]-log[neg control])) was ≥0.4.

Multiplex cytokine secretion analysis following whole blood stimulation

Whole lithium-heparin anti-coagulated blood was stimulated with *C. burnetii* antigen in 1.5 mL microtubes (Eppendorf) by adding 500 µL blood to 55 µL diluted C. *burnetii* antigen or medium only. After 21-23 hours, plasma supernatants from whole blood cultures were collected and frozen for later multiplex cytokineanalysis. Quantification of secreted IFNγ, IL-1β, IL-2, IL-10 and TNFα was conducted using a sandwich ELISA-based multi-spot electrochemiluminescence detection system (human Proinflammatory Panel 1 V-PLEX kit, Meso-Scale Discovery), following the manufacturer's recommendations. Cytokine levels in plasma supernatants measured following *C. burnetii* antigen stimulation were background corrected before data analysis by subtraction of levels detected in parallel medium-only assays for each sample. Notably, IFNγ levels measured using this V-PLEX assay are approximately 20 times higher than by the Q-Detect clinical IGRA used to assess the cellular immune status for enrolment and group allocation (28). This is because calibrators were differently dose-assigned by the two assay manufacturers. Despite this difference, IFNγ levels measured in the same set of samples by V-PLEX and Q-Detect IGRA directly correlate ($R = 0.87$, $p = 1.44 \times 10^{-9}$).

Flow cytometry analysis following whole blood stimulation

Lithium-heparin anti-coagulated whole blood was stimulated for 24-25 hours with *C. burnetii* antigen in 1.5 mL microtubes (Eppendorf) by adding 500 µL blood to 55 µL diluted C. *burnetii* antigen or medium only. For the final 20 hours of stimulation, Brefeldin A (Sigma) was added to a final concentration of 5 µg/mL. Two tubes were stimulated per donor and condition. Following stimulation, the two replicates of whole blood cultures were pooled, lysed using 10 mL 1x red blood cell (RBC) lysis buffer (eBioscience) and washed prior to staining and flow cytometry analysis. Each sample was evenly divided into three wells of a 96-well v-bottom plate (Sarstedt), washed with PBS and incubated for 30 min at 4°C with 50 µL fixable viability dye eF780 (eBioscience) diluted in PBS. Cells were washed twice with staining buffer (PBS/0.5% BSA), and stained with 25 µL antibody cocktail diluted in staining buffer for 20 min at 4°C. All antibodies used for surface and intracellular labelling are listed in Tables 3, 4. Cells were washed and re-suspended in 50 µL fixation/permeabilization buffer (eBioscience), incubated for 45 min at room temperature, and washed with 150 µL permeabilization buffer (eBioscience). For intracellular staining, cells were incubated for 45 min at room temperature with 25 µL antibody cocktail diluted in permeabilization buffer (eBioscience).

Single stains for compensation were prepared using lysed unstimulated and stimulated whole blood cultures as appropriate. Cells were washed with permeabilization buffer and re-suspended in 200 µL PBS/1% paraformaldehyde. Samples were acquired on a Gallios flow cytometer (BeckmanCoulter) and a FACSCanto II (BD Biosciences). A minimum of 100,000 events per sample was acquired. Data analysis was performed with FlowJo v10 software, using a combination of manual, magnetic and tethered gating (Supplementary Figures S1, S2). Four intracellular markers (CD137, T-bet, FOXP3 and IL-10) had no clearly identifiable positive population and were hence excluded from analysis.

Mass cytometry analysis following PBMC stimulation

Mass cytometry analysis was conducted using stimulated PBMCs rather than whole blood, since initial experiments showed that processing of stimulated whole blood for mass cytometry resulted in cell clumping and massive cell loss during the multiple wash steps required to remove debris after RBC lysis, thereby compromising CyTOF analysis.

PBMCs were isolated from lithium-heparin anti-coagulated blood using Leukosep tubes prefilled with Ficoll (Greiner BioOne) according to the manufacturer's recommendations. Prior to the final wash and counting, erythrocytes were lysed using 1X RBC lysis buffer (eBioscience). Freshly isolated PBMCs were stimulated for 18-20 h at $1x10^6$ cells per well in 96-well U-bottom plates (Corning) in a final volume of 100 µL complete RPMI (phenol red-free RPMI supplemented with 2 mM Glutamax, 5 µg/mL gentamycin, and 1 mM sodium pyruvate; all Thermo Fisher Scientific) with 10% fetal bovine serum (HyClone). For the final 14 hours of stimulation, Brefeldin A (Sigma) was added at a final concentration of 5 µg/mL. Stimulations for each donor were carried out with *C. burnetii* antigen or medium only in five replicate wells each. To accommodate the logistics of sample collection and processing, antigen stimulation and initial staining were performed in four separate runs of nine donors from different combination of two to three groups, for a total of thirty-six donors (Run 1: n=5 Group 3, n=4 Group 5; Run 2: n=3 Group 1, n=1 Group 2, n=5 Group 3; Run 3: n=6 Group 1, n=3 Group 2; Run 4: n=4 Group 2, n=5 Group 5).

TABLE 3 Flow cytometry staining panels Round 1.

Panel	Target	Clone	Label	Source
Fixable viability dye			eF780	Biolegend
T-cell panel (surface)	CD3	UCHT1	BV510	Biolegend
	CD4	RPA-T4	AF488	Biolegend
	CD19[x]	HIB19	APC-eF780	eBioscience
T-cell panel (intracellular)	CD14[x]	63D3	APC-Fire750	Biolegend
	CD16[x]	3G8	APC-Fire750	Biolegend
	CD154	24-31	PE	Biolegend
	IFNγ	4S.B3	PE-Cy7	Biolegend
	TNFα	MAb11	PerCp	Biolegend
	CD137[#]	4B4-1	BV421	Biolegend
Monocyte panel A+B (surface)	CD66b	G10F5	FITC	Biolegend
	CD3[x]	UCHT1	APC-eF780	eBioscience
	CD19[x]	HIB19	APC-eF780	eBioscience
Monocyte panel A (intracellular)	CD14	M5E2	BV421	Biolegend
	CD16[$]	3G8	BV510	Biolegend
	TNFα	MAb11	PerCp	Biolegend
	IL-1β	JK1B-1	AF647	Biolegend
Monocyte panel B (intracellular)	CD14	M5E2	BV421	Biolegend
	CD16[$]	3G8	BV510	Biolegend
	IL-6	MQ2-13A5	PerCp-Cy5.5	Biolegend
	IL-10	JES3-9D7	AF647	Biolegend

[x]Markers were used as part of the dump channel to exclude non-viable cells and those not of interest in this panel.
[$]CD16 expression on monocytes was adversely affected by stimulation; disregarded for monocyte (subpopulation) gating.
[#]Poor staining result with no identifiable positive population; disregarded for analysis.

Cisplatin staining, surface marker staining and barcoding of freshly isolated and stimulated PBMC samples were conducted at Innatoss in the Netherlands, while intracellular staining and mass cytometry analysis were conducted at MGH in the United States. Cryopreserved PBMCs from two donors with no history of Q fever disease were included as reference samples in each stimulation and staining run. Unstimulated cells from one donor were included in each staining run served as a control sample for surface antibody labelling. To establish a control sample for intracellular staining, freshly isolated PBMCs from a second donor were stimulated with a combination of *Staphylococcus aureus* enterotoxin B (final concentration 1 µg/mL, Sigma) and LPS (final concentration 100 ng/mL, eBioscience), cisplatin- and surface-labelled, barcoded, fixed, aliquoted and frozen. A single aliquot of this stimulated sample was included in each run as a control for intracellular staining.

Following stimulation, cells from the five replicate wells per donor and stimulation condition were pooled and washed four times with staining buffer (PBS/0.5% BSA) prior to surface and cisplatin staining. All washes and staining procedures were carried out in 1.5 mL microtubes (Eppendorf). For surface staining of markers sensitive to fixation during the barcoding procedure, cell pellets were resuspended in surface antibody cocktail 1 (anti-CD56 and anti-CD16, Table 5) diluted in staining buffer and cells were incubated for 60 min at 4°C. For the final 10 min of incubation, Cell-ID™ Cisplatin (Fluidigm; final concentration 2.5 µM) was added. Following staining, each sample was washed three times and counted. Up to $3x10^6$ cells per sample were used for barcoding using the Cell-ID™ 20-Plex Pd Barcoding Kit (Fluidigm). For barcoding, samples were first incubated in 400 µL Fix buffer and incubated for 10 min at room temperature, followed by two washes with Barcode Perm buffer (Fluidigm). Each sample was individually barcoded in a final volume of 120 µL barcode in Barcode Perm buffer for 30 min at room temperature. Following two washes with MaxPar cell staining buffer, each cell pellet was resuspended in 100 µL MaxPar cell staining buffer. All unstimulated and *C. burnetii* antigen-stimulated samples from n=9 donors and the unstimulated reference sample were then combined, pelleted and resuspended in 1 µL surface antibody cocktail 2 (Table 5) diluted in cell staining buffer, and incubated for 60 min at 4°C. Cells were then washed once more with cell staining buffer, resuspended in 150 µL 4% paraformaldehyde and incubated for 2 hours at room temperature to inactivate any potential live *C. burnetii* per requirements of the MGH Biosafety Committee (27). Samples were then frozen in a bulk-freezing container (CoolCell) and

TABLE 4 Flow cytometry staining panels Round 2.

Panel	Target	Clone	Label	Source
Fixable viability dye			eF780	Biolegend
T-cell panel A+B (surface)[$]	CD3	UCHT1	BV510	Biolegend
	CD4	RPA-T4	AF488	Biolegend
	CD19[x]	HIB19	APC-eF780	eBioscience
T-cell panel A (intracellular)	CD14[x]	63D3	APC-Fire750	Biolegend
	CD16[x]	3G8	APC-Fire750	Biolegend
	TNFα	MAb11	PerCp	Biolegend
	IL-2	MQ1-17H12	PE	Biolegend
	T-bet[$]	4B10	BV421	Biolegend
T-cell panel B (intracellular)	CD14[x]	63D3	APC-Fire750	Biolegend
	CD16[x]	3G8	APC-Fire750	Biolegend
	CD154	24-31	PE	Biolegend
	IFNγ	4S.B3	BV421	Biolegend
	CD25[#]	M-A251	PECy7	Biolegend
	FOXP3[#]	PCH101	eF660	eBioscience

[x]Markers were used as part of the dump channel to exclude non-viable cells and those not of interest in this panel.
[+]Staining was adversely affected by stimulation and disregarded during gating.
[$]Poor staining with no clearly identifiable T-bet positive population and no differences between stimulated and un-stimulated blood.
Poor staining result with no identifiable CD25+FOXP3+ positive population; excluded from analysis.
[$]T cell panel A also included CD8 PE-Cy7 (clone RPA-T8); disregarded during gating since none of the other T cell panels included CD8.

shipped on dry ice to MGH for intracellular staining and mass cytometry analysis.

At MGH, frozen samples were thawed and labelled with intracellular antibodies for CyTOF; each run was processed on a separate day. One frozen vial of barcoded sample pool and one frozen vial of the stimulated barcoded reference sample were thawed in a 37°C water bath, pelleted and resuspended in 1 mL of 1X eBioscience Fix/Perm buffer for 30 minutes. Cells were then washed twice in 1X perm buffer, resuspended in 500 µL of intracellular antibody cocktail (Table 5) and incubated for 30 minutes at 4°C. Stained cells were washed twice with 1X Perm buffer, resuspended in 150 µL of 4% paraformaldehyde and incubated for 10 minutes at room temperature. Post incubation, cells were pelleted, resuspended in cell staining buffer, and stored at 4°C overnight. The next day the cells were pelleted, resuspended in Cell-ID™ Intercalator Iridium (Fluidigm; final concentration 0.125 µM) and incubated at room temperature for 20 minutes. Cells were then washed twice in cell staining buffer, twice in nanopure water (purified using a Millipore Milli-Q system) and resuspended at 1 million cells per mL in nanopure water containing EQ calibration beads (Fluidigm). The data were acquired on a Helios Mass Cytometer (Fluidigm) at 400-500 events per second.

Mass cytometry data analysis

Data were retrieved from the Helios Mass Cytometer into FCS files. EQ calibration beads were used to normalize all FCS files, to minimize any variation that occurred during data acquisition on the Helios Mass Cytometer. This normalization was performed using the Fluidigm software and sample data were debarcoded using the Fluidigm Debarcoding software. Post-processing, following identification of viable singlet $CD45^+CD66b^-$ mononuclear cells, four major immune populations (CD4 T cells, CD8 T cells, B cells and innate immune cells) were manually gated using FlowJo 10 (Supplementary Figure S3).

Several markers showed strong run-to-run or day-to-day staining variation. For manual gating of $CD14^+$ monocytes and cytokine producing cells, gates were therefore set per run based on the run-specific staining control sample. Prior to unsupervised clustering analysis, marker expression levels had to be additionally normalized across the four runs. This normalization, based on representative concatenate samples (see below), was performed separately for each immune subpopulation using CytoNorm (33) to remove batch-specific variations. Of the 37 antibodies included in the CyTOF panel, four markers (CD45RO, CD45RA, CD197/CCR7, CD19) showed very large batch-to-batch variation outside the range for effective normalization and were excluded from unsupervised clustering analysis. The canonical markers used to identify the four main cell populations (CD45, CD66b, CD3, CD4, CD8, CD20) were not included into the normalization process. The following mass cytometry markers were normalized using CytoNorm: CD16, FOXP3, CD56, CD33, IL-2, CD137, CD154, CD14, IL-6, IFNγ, T-bet, IL-8, HLA-DR, CD69, CD206, CD70, CD27, IL-4, TNFα,

TABLE 5 Mass cytometry staining panel.

Cocktail	Target	Clone	Label	Source
Antibody cocktail 1 (surface)	CD16	3G8	209Bi	Fluidigm Inc.
	CD56	NCAM16.2	162Dy	BWH
Antibody cocktail 2 (surface)	CD11c	Bu15	159Tb	BWH
	CD137 (4-1BB)	4B4-1	166Er	BWH
	CD14	M5E2	151Eu	Fluidigm Inc.
	CD184 (CXCR4)	12G5	173Yb	Fluidigm Inc.
	CD19[#]	HIB19	160Gd	BWH
	CD197 (CCR7)[#]	G043H7	167Er	Fluidigm Inc.
	CD20	2H7	148Nd	BWH
	CD206 (MMR)	15-Feb	145Nd	BWH
	CD27	O323	141Pr	BWH
	CD3	UCHT1	158Gd	BWH
	CD33	WM53	163Dy	BWH
	CD38	HIT2	172Yb	Fluidigm Inc.
	CD4	RPA T4	155Gd	BWH
	CD45	HI30	89Y	Fluidigm Inc.
	CD45RA[#]	HI100	153Eu	BWH
	CD45RO[#]	UCHL1	149Sm	BWH
	CD66b	G10F5	171Yb	BWH
	CD69	FN50	144Nd	Fluidigm Inc.
	CD8a	RPA-T8	146Nd	BWH
	HLA-DR	L243	143Nd	Fluidigm Inc.
Antibody cocktail 3 (intracellular)	CD154 (CD40L)	24-31	168Er	BWH
	CD70 (113-16)	113-16	150Nd	BWH
	FOXP3	PCH101	161Dy	BWH
	GATA3	TWAJ	170Er	Fluidigm Inc.
	IFNγ	4S.B3	165Ho	BWH
	IL-10	JES3-19F1	176Yb	BWH
	IL-17A	BL168	169Tm	BWH
	IL-1β	H1b-27	154Sm	BWH
	IL-2	MQ1-17H12	164Dy	BWH
	IL-4	8D4-8	147Sm	Fluidigm Inc.
	IL-6	MQ2-13A5	156Gd	BWH
	IL-8	BH0814	142Nd	BWH
	Tbet	4B10	175Lu	BWH
	TGFβ	TW4-2F8	174Yb	BWH
	TNFα	Mab11	152Sm	BWH

[#]Markers showed very large batch effects and could not be normalized; excluded from analysis.

IL-1β, CD11c, IL-17, GATA3, CD38, CXCR4, TGFβ, and IL-10. A representative normalization sample was created for each of the four runs by down-sampling each debarcoded sample and concatenating the data. The four representative concatenate samples created in this manner were then used as the normalization reference samples in CytoNorm, to aid normalization of the data across all 4 runs. Since normalization was performed separately for each immune subpopulation, normalized expression levels for a given marker cannot be compared across the major immune populations. To assess if the normalization was accurate, we compared the control unstimulated PBMC sample included in all four runs as a staining control, before and after normalization (Supplementary Figures S4–S11). UMAP dimension reduction was performed on the four control samples prior to normalization, and separately after normalization. Before normalization, the surface and intracellular marker data for this control sample from different runs behaved like different samples. Post-normalization the control sample from each run overlaid the others, indicating that they were properly normalized.

Following normalization, data for all samples from all runs were clustered for each major cell population separately. Clustering was performed using FlowSOM (34), which employs self-organizing maps to identify immune cell clusters. Four major parental cell populations were defined as follows: CD4 T cells ($CD3^+CD4^+CD8^-CD20^-$); CD8 T cells ($CD3^+CD4^-CD8^+CD20^-$); B cells ($CD3^-CD20^+$); innate cells ($CD3^-CD20^-$). The mass cytometry markers used to cluster CD4 T cells, CD8 T cells, B cells and innate cells are listed in Table 6. FlowSOM parameters were initially set to identify 900 clusters for each major cell population. These 900 were subsequently meta-clustered to identify 20 meta-clusters from each major cell population (C1-C20). Using the SpadVizR R package, the median intensity of each marker was plotted in each meta-cluster to enable the phenotypic definition of the cell population via parallel coordinate plots. For each donor, the frequencies of each meta-cluster within each parental population in both control and *C. burnetii* antigen-stimulated conditions were calculated as a percent of the total number of cells clustered from that donor for that cell population. Subsequently, background corrected frequencies for each meta-cluster were calculated to identify whether the respective meta-cluster increased or decreased upon stimulation with *C. burnetii* antigen.

Statistical analysis

Statistical analysis was conducted based on the original group assignments (Groups 1, 2, 3, and 5), as well as combined group assignments, such as Group 2 + 3 + 5 (IGRA-positive). Statistical analysis was performed using GraphPad Prism 9. Spearman's correlation analysis was conducted for all clusters and plasma cytokine secretion levels using R 4.1 and Hmisc. Heatmaps and dendrograms were created using Python 3.10 using Pandas, Pyplot and Seaborn. tSNEs were generated using Python 3.10 and Scikit-Learn.

TABLE 6 CyTOF markers used in FlowSOM clustering.

CD4 T Cell	CD8 T Cell	B Cell	Innate Cell
CD16	CD16	CD16	CD16
FOXP3	CD56	IL-2	CD56
IL-2	IL-2	CD137	CD33
CD137	CD137	CD154	IL-2
CD154	CD154	IL-6	CD137
IL-6	IL-6	IFNγ	CD154
IFNγ	IFNγ	T-bet	CD14
T-bet	T-bet	IL-8	IL-6
IL-8	IL-8	HLA-DR	IFNγ
HLA-DR	HLA-DR	CD69	T-bet
CD69	CD69	CD70	IL-8
CD70	CD70	CD27	HLA-DR
CD27	CD27	IL-4	CD69
IL-4	IL-4	TNFα	CD206
TNFα	TNFα	IL-1β	CD70
IL-1β	IL-1β	CD11c	CD27
CD11c	CD11c	IL-17	IL-4
IL-17	IL-17	TGFβ	TNFα
GATA3	GATA3	IL-10	IL-1β
CD38	CD38		CD11c
TGFβ	TGFβ		IL-17
IL-10	IL-10		TGFβ
			IL-10

Results

In vitro C. burnetii-specific whole blood cytokine release patterns reflect the serological and prior disease status of previously exposed individuals

The individuals included in this study were divided into four groups: Those with no apparent prior exposure based on the absence of humoral and cellular adaptive response (negative IFA and IGRA) and lack of clinical history (Group 1), and previously exposed individuals based on a measurable cellular response by IGRA. The presence of a cellular immune response to *C. burnetii* antigen suggests that the IGRA-positive individuals had experienced prior infection with *C. burnetii* even if the infection did not result in a clinical history of symptomatic Q fever. These IGRA-positive individuals were further subdivided based on serological and prior disease status into Group 2 (seronegative, asymptomatic), Group 3 (seropositive, asymptomatic) and Group 5 (seropositive, symptomatic). No individuals with known active *C. burnetii* infection, as either current acute or chronic Q fever, were included in the study.

We first assessed whether the exposure status of individuals was associated with differential release of cytokines previously reported to be associated with re-stimulation responses to whole-cell *C. burnetii* antigen. We compared bulk cytokine responses as determined by multiplex V-PLEX assay following whole blood stimulation with heat-killed *C. burnetii* antigen in individuals with different states of prior exposure, clinical history, and serological status. Prior exposure (Groups 2, 3 and 5) was associated with significantly elevated release of IFNγ quantified by V-PLEX ($p<0.0001$; Figure 1A), consistent with the positive IGRA results used to define groups at study enrollment. IGRA responses (at enrollment) and IL-2 responses showed a strong correlation (Spearman Rho = 0.86, p= 4.5535E-09), and IGRA positivity was associated with significantly increased release of IL-2 ($p>0.0001$) compared to that observed in cells from unexposed individuals from group 1 (Figure 1A). In contrast, the innate cytokines IL-10, IL-1β and TNFα were released in response to *C. burnetii* antigen-stimulation by cells from both control (Group 1) and pre-exposed individuals (Groups 2, 3 and 5). Median levels of the three innate cytokines were elevated in pre-exposed individuals, although statistical analysis supported a trend toward differential levels only for IL-1β (p=0.062) (Figure 1A). No significant correlation was found between innate and adaptive cytokine responses, or amongst innate cytokines.

Dimensional reduction of whole blood cytokine release data from n=55 individuals by t-SNE followed by k means clustering of t-SNE embeddings identified two main clusters of study participants (Supplementary Figure S12**).** Cluster 1 comprised all control Group 1 individuals, a majority of Group 2 and n=1 donor from Group 3 who showed low IFNγ and IL-2 responses to stimulation with *C. burnetii*. Cluster 2 contained all remaining IGRA-positive individuals showing predominantly high IFNγ and IL-2 responses. TNFα, IL-1β and IL-10 responses to *C. burnetii* antigen did not correlate with the cluster groupings.

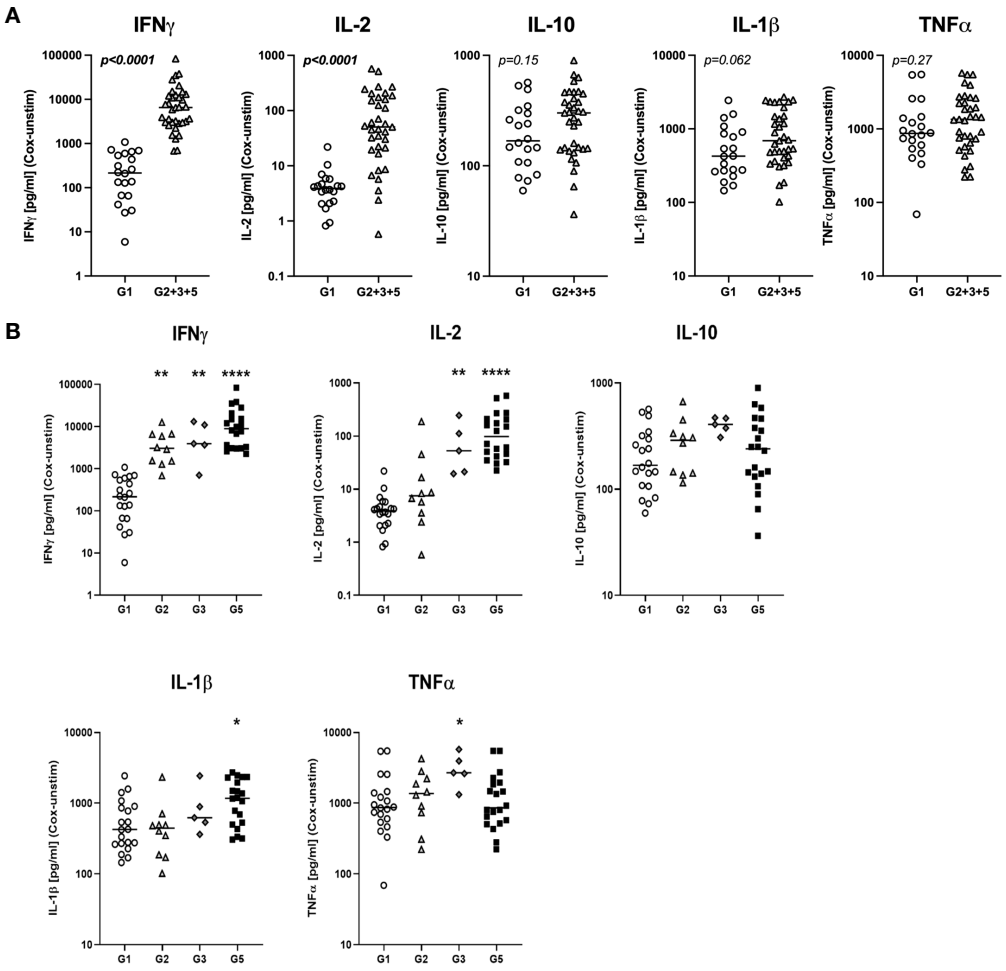

FIGURE 1
C. burnetii-specific whole blood cytokine release patterns. Multiplex cytokine secretion analysis was conducted on supernatants from whole blood stimulations for individuals without immunological evidence of prior exposure to *C. burnetii* (unexposed Group 1; n=20) and those with pre-existing humoral and/or cellular immunity (pre-exposed Groups 2, 3 and 5; n=10, n=5 and n=20, respectively). Background-corrected *C. burnetii*-specific cytokine data are shown for each individual. Data from Groups 2, 3 and 5 are combined in **(A)** and plotted separately in **(B)**. Lines indicate the median. Cytokine responses were compared using the Mann-Whitney U test in **(A)** and Kruskal-Wallis test followed by Dunn's *post-hoc* multiple comparison test for nonparametric data in **(B)**. Asterisks are defined as follows: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.0001$ (****). Background cytokine production levels in unstimulated samples across all groups were as follows (Median with interquartile range (IQR)): IFNγ 6.8 pg/mL (4.6-12.3), IL-2 0.20 pg/mL (0.20-0.20), IL-10 0.27 pg/mL (0.13-0.44), IL-1β 0.33 pg/mL (0.14-0.83), TNFα 2.4 pg/mL (1.7-3.6).

Further analysis of the clinical subgroups defined by clinical and immunological status showed that in contrast to the IFNγ responses used to define prior exposure, IL-2 responses were significantly elevated only in seropositive individuals (Group 3 and 5) regardless of symptoms during infection, while IL-1β release was elevated specifically in seropositive individuals who had convalesced from symptomatic infection (Group 5) (Figure 1B). Finally, TNFα release was significantly elevated only in the small group of pre-exposed individuals with past asymptomatic infection who were analyzed (n=5, Group 3).

Except for IFNγ, released cytokine levels in seronegative Group 2 individuals were indistinguishable from those in the control Group 1 (Figure 1B), and median IGRA responses in Group 2 were lower than in seropositive Group 3 and 5 individuals (Table 1). These lower cellular responses in individuals that fail to show seroconversion has previously already been described in a much larger cohort study in *C. burnetii*-exposed individuals (31) as well as in individuals exposed to HIV (35–37), HBV (38), HCV (39), HSV-2 (40) and SARS-CoV-2 (41, 42).

Overall, of the bulk cytokine responses evaluated, IFNγ and IL-2 were the most informative for inferring prior exposure. Innate cytokines TNFα and IL-1β also showed some association with prior exposure by natural infection, consistent with previous results (43), but elevation was more marginal and restricted to specific subgroups of pre-exposed individuals.

Flow cytometry analysis reveals stronger *C. burnetii*-specific *in vitro* recall responses by both T cells and monocytes from seropositive individuals convalescent from prior symptomatic infection

To explore the cellular source of these cytokines in *C. burnetii* antigen-stimulated whole blood cultures, we first performed flow cytometry analysis for a randomly selected set of n=36 donors from the two groups with the greatest difference in clinical and immunological status: IGRA-negative and IFA-negative individuals with no clinical history of Q fever (Group 1), and IGRA-positive and IFA-positive individuals with past symptomatic infection (Group 5). We focused on the assessment of *C. burnetii*-induced intracellular cytokine production by monocytes and CD4 T cells, effector cells that are known to promote killing of *C. burnetii* and clearance of infection (44, 45).

In *C. burnetii* antigen-stimulated whole blood, a high proportion of monocytes from both controls (Group 1) and IGRA-positive donors with a clinical history of Q Fever (Group 5) produced TNFα (median 10.8% and 17.6%), IL-1β (median 55.8% and 66.6%) and IL-6 (median 43.3% and 55.5%). Only the difference in IL-6$^+$ monocytes between Groups 1 and 5 reached statistical significance ($p = \leq 0.05$) (Figure 2A). However, there was a modest trend toward higher proportions of TNFα, IL-1β and IL-6 producing monocytes in pre-exposed Group 5 individuals.

T cell responses were assessed in two flow cytometry experiments, each including n=10 donors from each of Groups 1 and 5. Initially, a single T cell panel was used to assess IFNγ and TNFα production and the activation marker CD154 (Figure 2B). In a second round IL-2 production was also interrogated using a second T cell panel (Figure 2C). CD4 T cells consistently showed significantly higher proportions of IFNγ$^+$, TNFα$^+$ and CD154$^+$ cells (Figures 2B, C) as well as IL-2$^+$ cells (Figure 2C) in Group 5 individuals with clinical history of the disease compared to Group 1 controls. CD3$^+$ CD4$^-$ T cells, inferred as CD8 T cells, showed no significant difference in *C. burnetii*-induced TNFα or IL-2 production or CD154 expression between control (Group 1) and convalescent (Group 5) individuals (Figures 2B, C). However, amongst the Group 5 individuals assessed in round 1, a small number did show CD4$^-$ IFNγ T cell responses of a similar magnitude as CD4 T cells (Figure 2B). Amongst the individuals in Group 5 assessed in round 2, this IFNγ response in CD4$^-$ T cells was statistically significant and comparable to the response of CD4 T cells (Figure 2C). This suggests that CD8 T cells also contribute to recall IFNγ responses.

Mass cytometry analysis identifies both adaptive and innate cell populations showing *in vitro* recall responses in individuals who were previously naturally exposed

The flow cytometry panels focused on assessing responses by monocytes and CD4 T cells. To characterize the immune cell subsets involved in recall responses to *C. burnetii* antigen more deeply and broadly, we utilized a highly multiplexed CyTOF panel in combination with manual gating and unsupervised clustering analysis. The CyTOF panel included additional lineage markers to investigate responses by CD8 T cells, B cells and innate immune cells, as well as a wider variety of activation markers and cytokines. For this CyTOF analysis we further expanded the selection of subjects: in addition to individuals from Group 1 (controls) and Group 5 (seropositive, symptomatic), we also included Group 2 (seronegative, asymptomatic) and Group 3 (seropositive, asymptomatic) individuals.

Cytokine responses were initially quantified in total CD45$^+$CD66b$^-$ mononuclear cells by manual gating. In *C. burnetii* antigen-stimulated PBMCs, the proportions of cells showing *C. burnetii*-specific production of IFNγ, IL-6, IL-10 and TNFα were significantly higher in pre-exposed IGRA-positive individuals compared to Group 1 controls (Figure 3A), consistent with IGRA responses at study enrollment (Table 1). The median increase with pre-exposure in cells producing these cytokines was two-fold or less, except for the proportion of IL-6$^+$ cells which increased nearly six-fold. In contrast, the proportion of cells producing the innate cytokines IL-1β and IL-8 in response to *C. burnetii*-stimulation was comparable across the two groups and thus independent of prior exposure status. IFNγ levels were specifically elevated in seropositive Group 3 and 5 individuals, and the same was true for IL-10 and TNFα (Figure 3B). Although the proportion of IL-2-producing cells was not significantly different between IGRA-negative Group 1 controls and all IGRA-positive donors (Group 2 + 3 + 5) (Figure 3A),

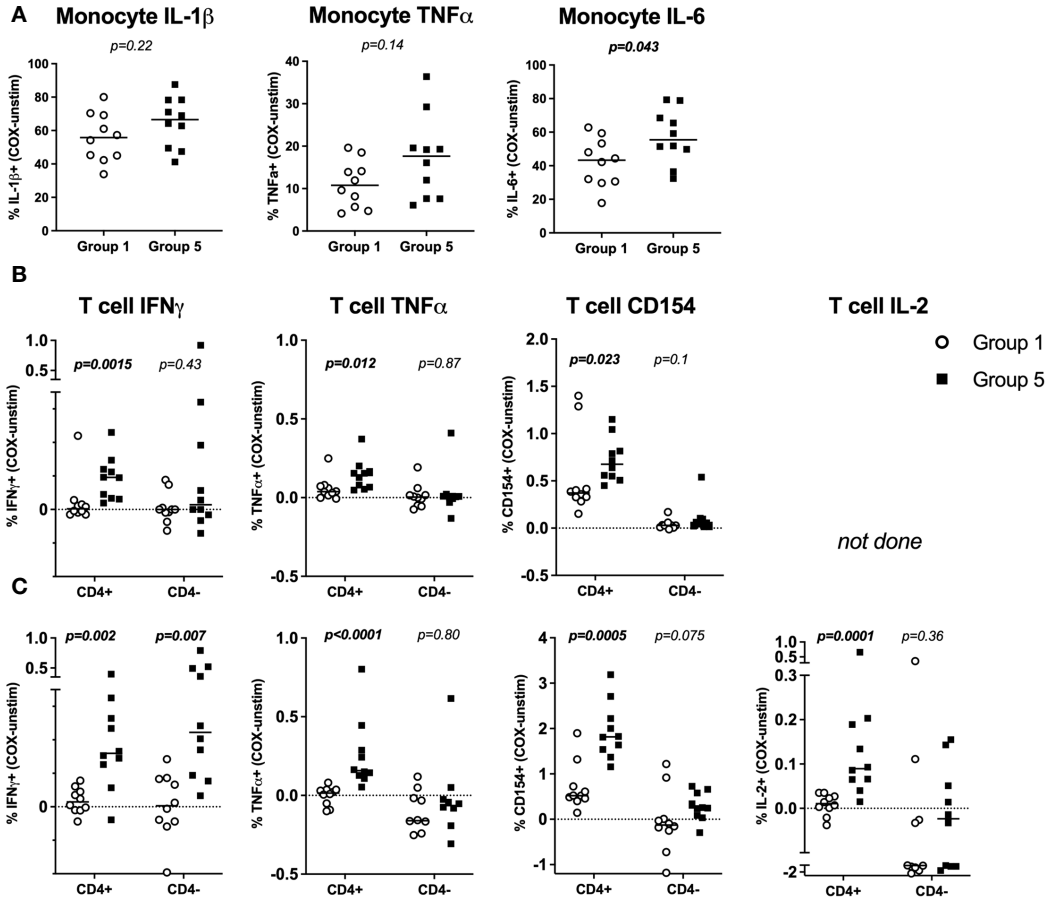

FIGURE 2
Flow cytometry profiles of *C. burnetii*-specific monocyte and T-cell cytokine production in stimulated whole blood. Cytokine production in whole blood following stimulation with *C. burnetii* was compared by flow cytometry between individuals without immunological evidence of prior exposure to *C. burnetii* (unexposed Group 1) and those with pre-existing humoral and cellular immunity as well as past symptomatic infection (exposed Group 5). **(A)** *C. burnetii*-specific cytokine responses by monocytes are depicted as the background corrected percentage of cytokine positive monocytes in unexposed Group 1 (n=10) and pre-exposed Group 5 (n=10) donors. Background proportions of cytokine-positive monocytes in unstimulated samples across the two groups were as follows (Median with IQR): IL-1β 0.53% (0.35-0.86), TNFα 0.14% (0.04-0.25), IL-6 0.43% (0.13-0.52). **(B, C)** *C. burnetii*-specific cytokine responses by T cells were assessed in two independent experimental rounds and using different T cell panels in a total of n=17 Group 1 and n=19 Group 5 donors. In each round, unexposed Group 1 (n=10) and exposed Group 5 (n=10) donors were included. Four donors were analyzed in both rounds. T cell cytokine responses are depicted as background corrected percentage of cytokine positive cells amongst $CD4^+$ or $CD4^-$ T cells. Background proportions of cytokine/activation marker positive T cells in unstimulated samples across the two groups was as follows in round 1 **(B)** (Median with IQR): $CD4^+$ T cells IFNγ 0.01% (0.01-0.02), TNFα 0.04% (0.02-0.05), CD154 0.05% (0.03-0.07). $CD4^-$ T cells IFNγ 0.02% (0-0.03), TNFα 0.03% (0.02-0.07), CD154 0.01% (0-0.02). In round 2 **(C)** the background values were (Median with IQR): $CD4^+$ T cells IFNγ 0.07% (0.06-0.10), TNFα 0.09% (0.03-0.14), CD154 0.12% (0.06-0.17), IL-2 0.01% (0.01-0.03). $CD4^-$ T cells IFNγ 0.11% (0.07-0.16), TNFα 0.45% (0.3-0.6), CD154 1.24% (0.30-3.44), IL-2 0.26% (0.09-0.99). Lines indicate the median. Cytokine responses between Groups 1 and 5 were compared using the Mann-Whitney U test for nonparametric data.

seropositive Group 3 and 5 individuals showed clearly higher IL-2 responses compared to Groups 1 and 2 (Figure 3B), with the difference between Group 2 and 3 individuals reaching statistical significance. *C. burnetii*-specific IL-4 responses were not detectable, and IL-17 responses were only detectable in a very minor proportion of cells (<0.05%) and showed no differences between IGRA-negative and IGRA-positive groups (data not shown).

To assess the cellular source of these cytokines, we separately analyzed $CD45^+$ mononuclear cells positive for each cytokine for individuals who were either IGRA-negative (Group 1) to IGRA-positive (Group 2 + 3 + 5) (Figure 4). IFNγ was broadly produced by all immune cell types evaluated, not solely by CD4 and CD8 T cells, regardless of the status of prior exposure. However, IFNγ expression by CD4 T cells (p = 0.029) and $CD56^-$ innate cells (presumably monocytes and dendritic cells, p = 0.005) increased with prior exposure (higher proportion in Group 2 + 3 + 5 compared to Group 1). The primary sources of IL-2 were CD4 T cells and B cells. While the contribution of CD4 T cells to IL-2 production was slightly increased with prior exposure though this did not reach statistical significance. IL-6, TNFα, IL-1β and IL-8 were produced largely by $CD56^-$ innate cells (presumably monocytes and dendritic cells), consistent with the canonical innate functions of these cytokines, with no difference in this pattern regardless of pre-exposure. CD4 T cells had the greatest contribution to IL-10 production in unexposed individuals (Group 1), but $CD56^-$ innate cells became the predominant source in individuals with prior

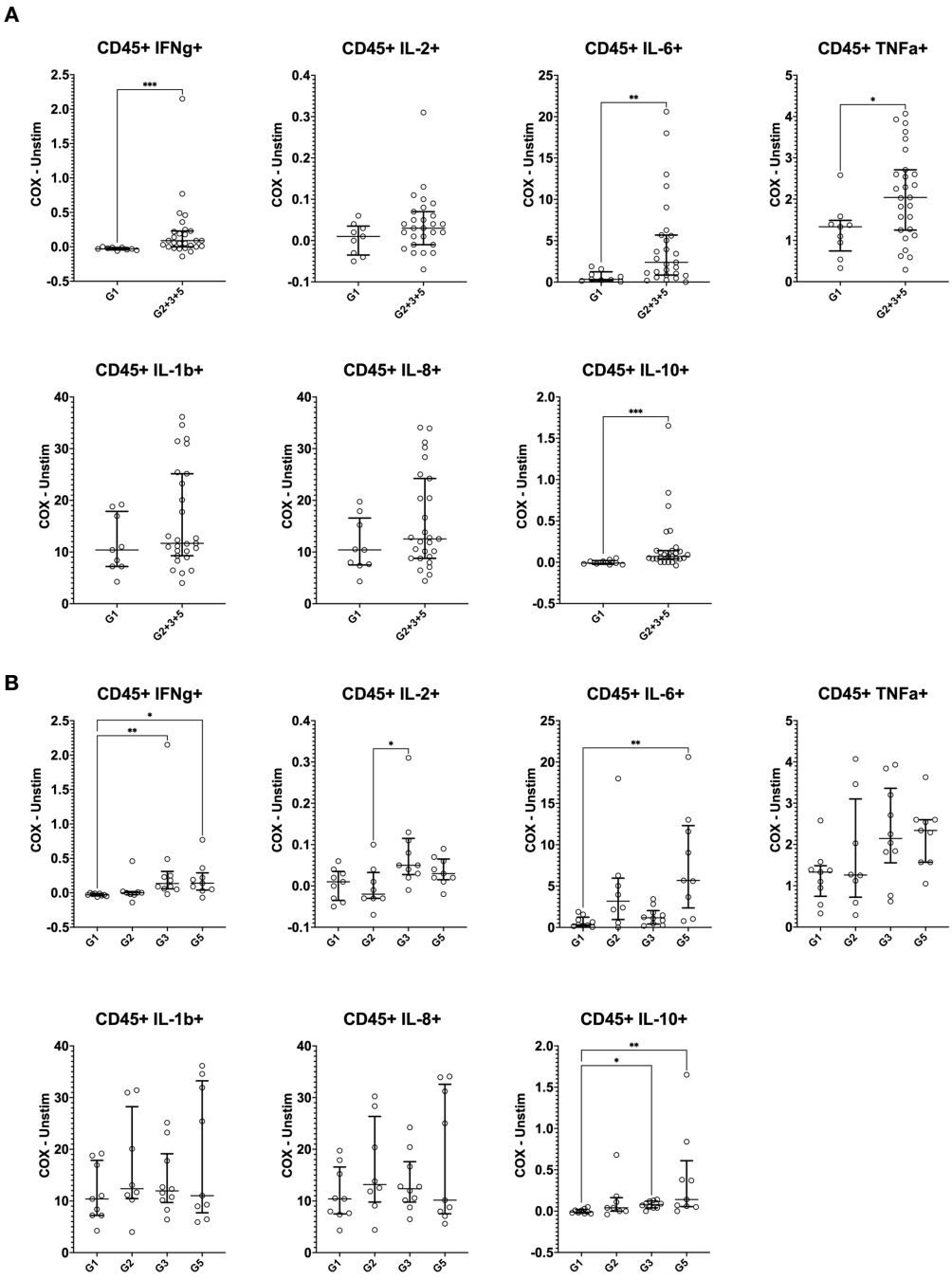

FIGURE 3
Mass cytometry profiles of *C. burnetii*-specific cytokine production in peripheral blood mononuclear cells. Cytokine production in PBMCs following stimulation with *C. burnetii* was assessed by mass cytometry. Scatterplots depict the background corrected percentage of each cytokine (IFNγ, IL-2, IL-6, TNFα, IL-1β, IL-8 and IL-10) in all $CD45^{+}CD66b^{-}$ mononuclear cells in: **(A)** unexposed Group 1 (n=9) and all pre-exposed individuals (n=27) donors (Group 2 + 3 + 5), **(B)** unexposed Group 1 (n=9), seronegative, asymptomatic Group 2 (n=8), seropositive, asymptomatic Group 3 (n=10) and seropositive, symptomatic Group 5 (n=9). Lines indicate the median with interquartile range. Cytokine responses were compared using Mann-Whitney U test in **(A)** and Kruskal-Wallis test followed by Dunn's *post-hoc* multiple comparison test for nonparametric data in **(B)**. Asterisks are defined as follows: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

exposure (higher proportion in Group 2 + 3 + 5 compared to Group 1, $p = 0.005$).

In addition to cytokine production, IGRA-positive individuals also showed enhanced levels of activation markers expression in response to *C. burnetii* antigen stimulation of PBMCs, which was significant for CD69 and CD137 (Figure 5A). A more granular analysis of the different exposure groups showed that relative to the control Group 1, *C. burnetii*-induced CD69 and CD154 expression were significantly elevated in seropositive Group 3 individuals only, while the proportion of cells expressing the activation marker CD137 was significantly increased in both seropositive Groups 3 and 5 (Figure 5B).

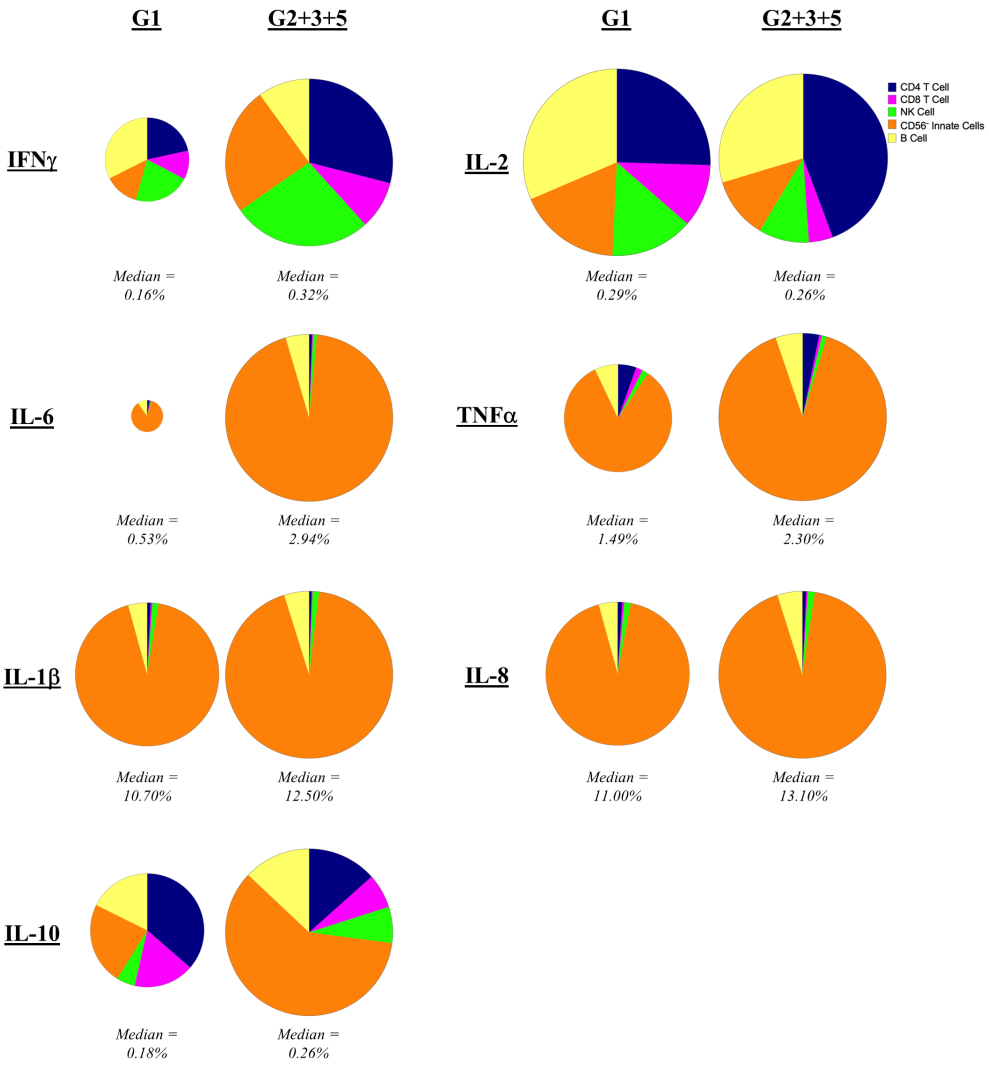

FIGURE 4
Contribution of mononuclear cell populations to *C. burnetii*-specific cytokine production. The contribution of $CD45^+CD66b^-$ cell populations in PBMCs to cytokine production following stimulation with *C. burnetii* was assessed by mass cytometry. Pie charts indicate the median percent contribution of each cell type (CD4 T cells, CD8 T cells, B cells, NK cells and $CD56^-$ innate cells, to the frequency of cytokine-positive (IFNγ, IL-2, IL-6, TNFα, IL-1β, IL-8 or IL-10) cells in *C. burnetii*-stimulated samples (no background correction) in unexposed Group 1 (n=9) and all pre-exposed individuals (n=27) donors. The size of the pie chart for the pre-exposed donors (Group 2 + 3 + 5) is kept constant across panels. The pie chart of the unexposed group is scaled separately for each cytokine relative to the pre-exposed group, to reflect the difference in median frequency of cytokine positive $CD45^+$ mononuclear cells. The median frequency of cytokine positive $CD45^+$ mononuclear cells in *C. burnetii* stimulated samples for each group is displayed below each pie chart.

Finally, we addressed the question of which specific cellular phenotypes characterize the innate and adaptive recall responses induced by *C. burnetii* antigen stimulation of PBMCs from pre-exposed individuals. To this end, FlowSOM clustering (34) identified 20 sub-populations (meta-clusters C1-C20) within each of the four major populations (CD4 T cells, CD8 T cells, B cells and Innate cells) amongst $CD45^+CD66b^-$ cells (Figures 6A, C, 7A, C). We then assessed whether meta-cluster abundance was increased upon stimulation with *C. burnetii* antigen (indicating *C. burnetii*-specific responses, Figures 6B, D, 7B, C) and whether the specific responses (assessed as background-corrected abundances) were higher in IGRA-positive (Group 2 + 3 + 5) individuals compared to Group 1 controls, and thus linked to prior exposure (Figure 8).

Several meta-clusters amongst both CD4 and CD8 T cells showed enhanced abundance upon stimulation with *C. burnetii* antigen. CD4 and CD8 meta-clusters exhibiting *C. burnetii*-specific production of the innate cytokines IL-1β and IL-8 alone (CD4 C2, CD8 C2 and CD8 C3) or in combination with IL-6 and TNFα (CD4 C1 and CD8 C1) did not increase with prior exposure status, or were only found in unexposed individuals (CD4 C3 $IL\text{-}8^+$) (Figure 6). In contrast, the relative abundances of two CD4 T cell meta-clusters producing adaptive cytokines were significantly higher in pre-exposed individuals: CD4 C11 ($CD137^+CD154^+CD69^+IFN\gamma^+IL\text{-}2^+TNF\alpha^+$) and C12 ($CD137^+CD154^+CD69^+$) (Figure 8), consistent with the elevated production of these cytokines by CD4 T cells in Group 5 individuals, as determined by flow cytometry (Figure 2). Amongst CD8 T cells, only C11 ($CD137^+$) showed a higher relative abundance

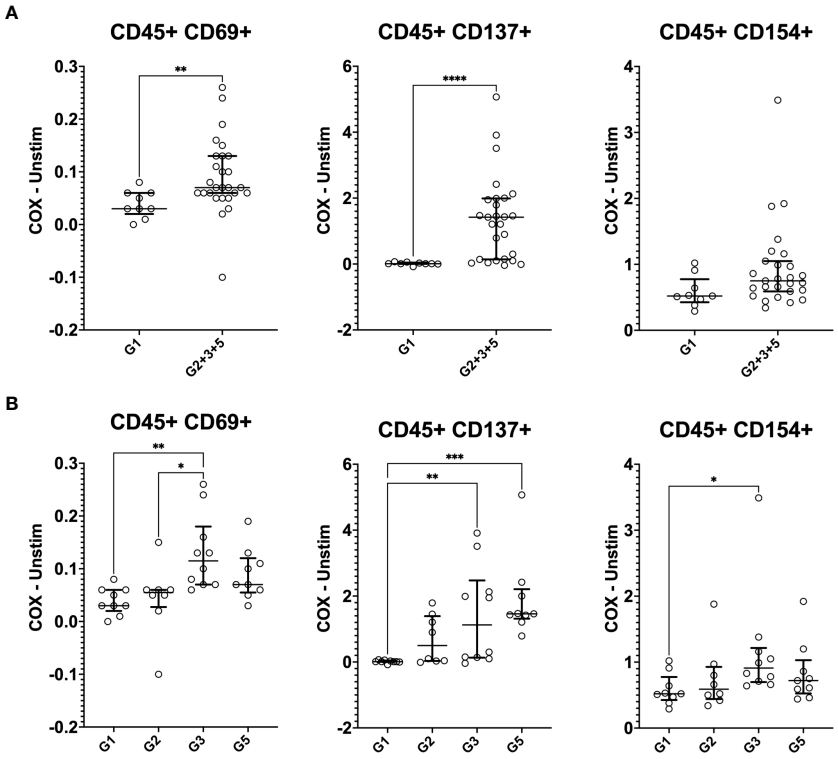

FIGURE 5
Mass cytometry profiles of *C. burnetii*-specific activation in stimulated peripheral blood mononuclear cells. Activation of $CD45^{+}CD66b^{-}$ cells following PBMC stimulation with *C. burnetii* was assessed by mass cytometry. Scatterplots depict the background corrected percentage for each activation marker (CD69, CD137 and CD154) in: **(A)** unexposed Group 1 (n=9) and all exposed individuals (n=27) donors (Group 2 + 3 + 5), **(B)** unexposed Group 1 (n=9), seronegative, asymptomatic Group 2 (n=8), seropositive, asymptomatic Group 3 (n=10) and seropositive, symptomatic Group 5 (n=9). Lines indicate the median with interquartile range. Activation responses were compared using the Mann-Whitney U test in **(A)** and Kruskal-Wallis test followed by Dunn's *post-hoc* multiple comparison test for nonparametric data in **(B)**. Asterisks are defined as follows: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

in pre-exposed individuals. However, this CD8 meta-cluster showed no effector cytokine production, and the difference in abundance between *C. burnetii* antigen-stimulated versus unstimulated samples was very minimal, especially in view of the abundance of this meta-cluster in unstimulated PBMCs (Figure 6B).

In B cells, eight of the 20 meta-clusters (C3, C19, C16, C20, C6, C12, C9 and C15) showed an increased abundance upon stimulation with *C. burnetii* antigen in all individuals. The majority of these *C. burnetii* antigen-stimulation associated B cell meta-clusters was again characterized by production of the innate cytokines IL-1β and IL-8 alone (C19, C6, C12, C9, C15) or in combination with TNFα (C20) or TNFα and IL-6 (C16), and for all relative abundance was unrelated to prior exposure. The same was true for the rare B cell meta-cluster C3 with an activated phenotype but lacking cytokine production (Figures 7A, B). No B cell cluster showed evidence for pre-exposure related *C. burnetii*-specific responses.

Amongst innate cells, there was again an increased abundance upon stimulation with *C. burnetii* antigen in all individuals, regardless of prior exposure, of meta-clusters showing production of IL-8 only (C12 and C13; likely dendritic cells that lack CD14 and CD56 expression and express high levels of HLA-DR and CD11c) or IL-1β and IL-8 (C3, C5, C2 and C4; $CD14^{+}$ monocytes) (Figures 7C, D). Three populations of innate cells, however, showed a significantly higher relative abundance in pre-exposed individuals: the innate meta-clusters C7 ($CD11c^{high}CD14^{+}$HLA-$DR^{+}IL\text{-}1\beta^{+}IL\text{-}6^{+}IL\text{-}8^{+}$) and C11 ($CD11c^{int}CD14^{+}$HLA-$DR^{+}CD69^{+}CD137^{int}CD154^{int}IL\text{-}1\beta^{+}IL\text{-}6^{low}IL\text{-}8^{+}$), both likely representing monocytes, and a meta-cluster lacking expression of any canonical markers, C19 ($CD69^{+}CD137^{int}CD154^{+}IFN\gamma^{+}IL\text{-}2^{+}TNF\alpha^{+}$) (Figure 8).

When we further focused on the separate subgroups of IGRA-positive individuals, CD4 and CD8 T cell clusters C11 and innate cell cluster C7 all were significantly increased in Group 5 (seropositive, convalescent from symptomatic infection), while CD4 T cell cluster C12 and innate cell cluster C19 were highest in Group 3 individuals (seropositive, asymptomatic) (Figure 8). Background-corrected frequencies for all T cell and innate cell meta-clusters correlated with IFNγ levels released following stimulation of whole blood, and CD4 T cell cluster C11 and C12 frequencies additionally correlated with IL-2 levels (Supplementary Table 1).

Discussion

In this study we examined the immune cell subpopulations contributing to long-term *C. burnetii*-specific recall responses

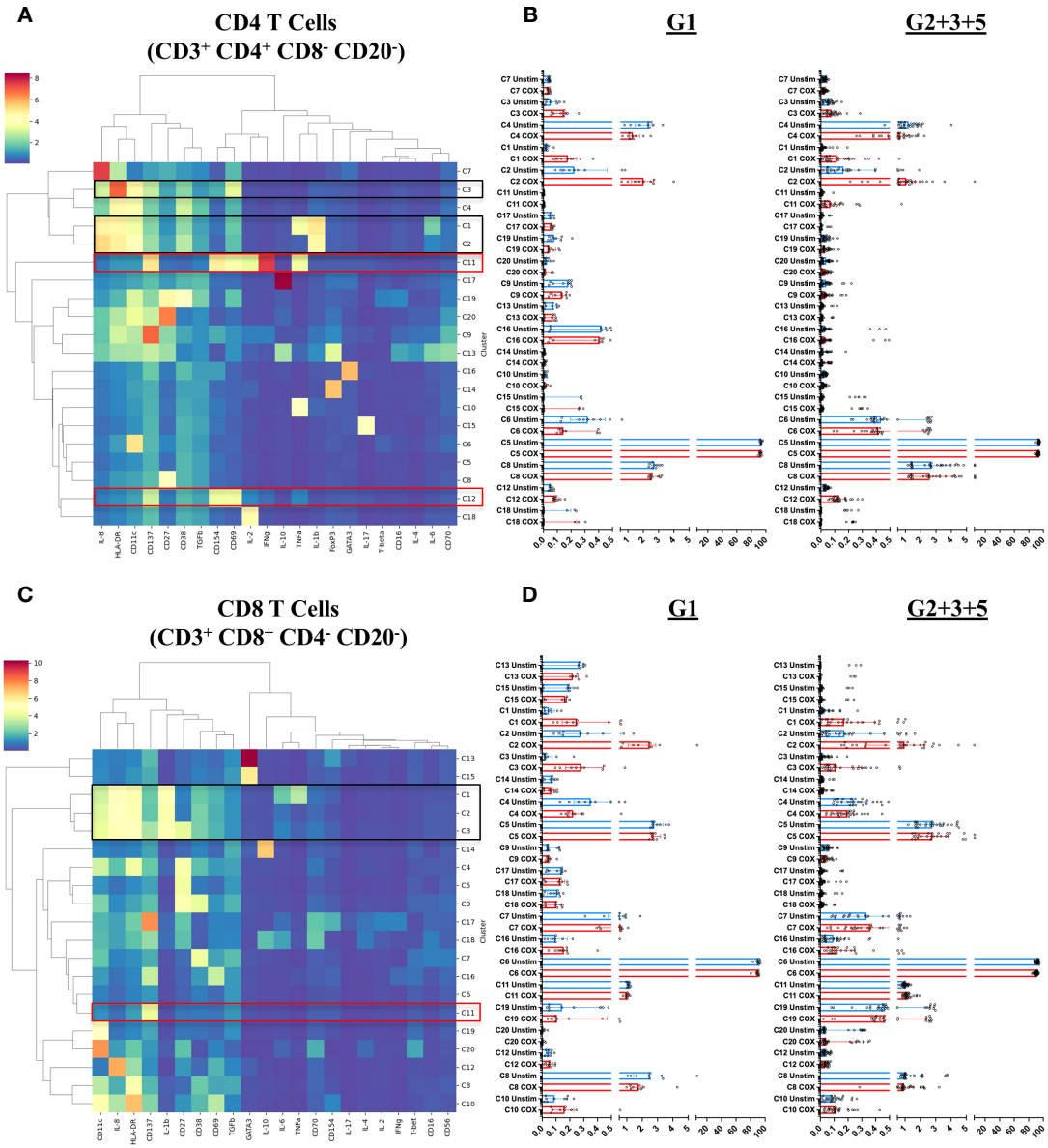

FIGURE 6
T cell subpopulations characterized by mass cytometry in peripheral blood mononuclear cells. Immune cell meta-clusters were identified through FlowSOM-based clustering of mass cytometry labeled samples. The phenotypic profile of each meta-cluster, in **(A)** CD4 T cells and **(C)** CD8 T cells, is depicted in heatmaps. The color gradient on the heatmaps indicates the mean metal intensity for each marker. Dendrograms were generated through hierarchical clustering using Ward linkages. The scatterplots on the right of each heatmap indicate the frequency (abundance) each meta cluster in **(B)** CD4 T cells and **(D)** CD8 T cells in each donor in unstimulated (blue) and *C. burnetii*-stimulated (red) samples. Lines indicate the median with interquartile range. Black boxes in heatmaps **(A)** and **(C)** highlight meta-clusters showing increased absolute abundance upon *C. burnetii* stimulation; red boxes highlight meta-clusters showing higher *C. burnetii*-specific relative abundance (background-corrected) in all pre-exposed individuals (Group 2 + 3 + 5) compared to Group 1.

approximately ten years post exposure in individuals who contracted Q fever during the Dutch Q fever outbreak of 2007 to 2010. These responses were quantified using IGRA, multiplex cytokine secretion assay, flow cytometry analysis of CD4 T cell and monocyte responses, and mass cytometry profiling of diverse cell populations. In general, we found that prior exposure to *C. burnetii* was associated with long-term increased recall responses in not only adaptive but also innate immune cell compartments.

Recall responses in humans have previously been analyzed solely by means of bulk cytokine secretion from peripheral immune cells (16, 17, 19, 21–23). To perform a broader and more in-depth analysis of the peripheral immune system we utilized both classical flow cytometry and mass cytometry (CyTOF), which uses heavy metal ion-labeled antibodies and time-of-flight mass spectrometry. This technology permits a much greater number of markers to be probed in parallel in the same sample and in combination with unsupervised clustering analysis facilitates the identification of novel immune subsets and signatures (46). For instance, CyTOF has been applied to analyses following influenza vaccination (47), during acute Zika virus (48) or SARS-CoV2

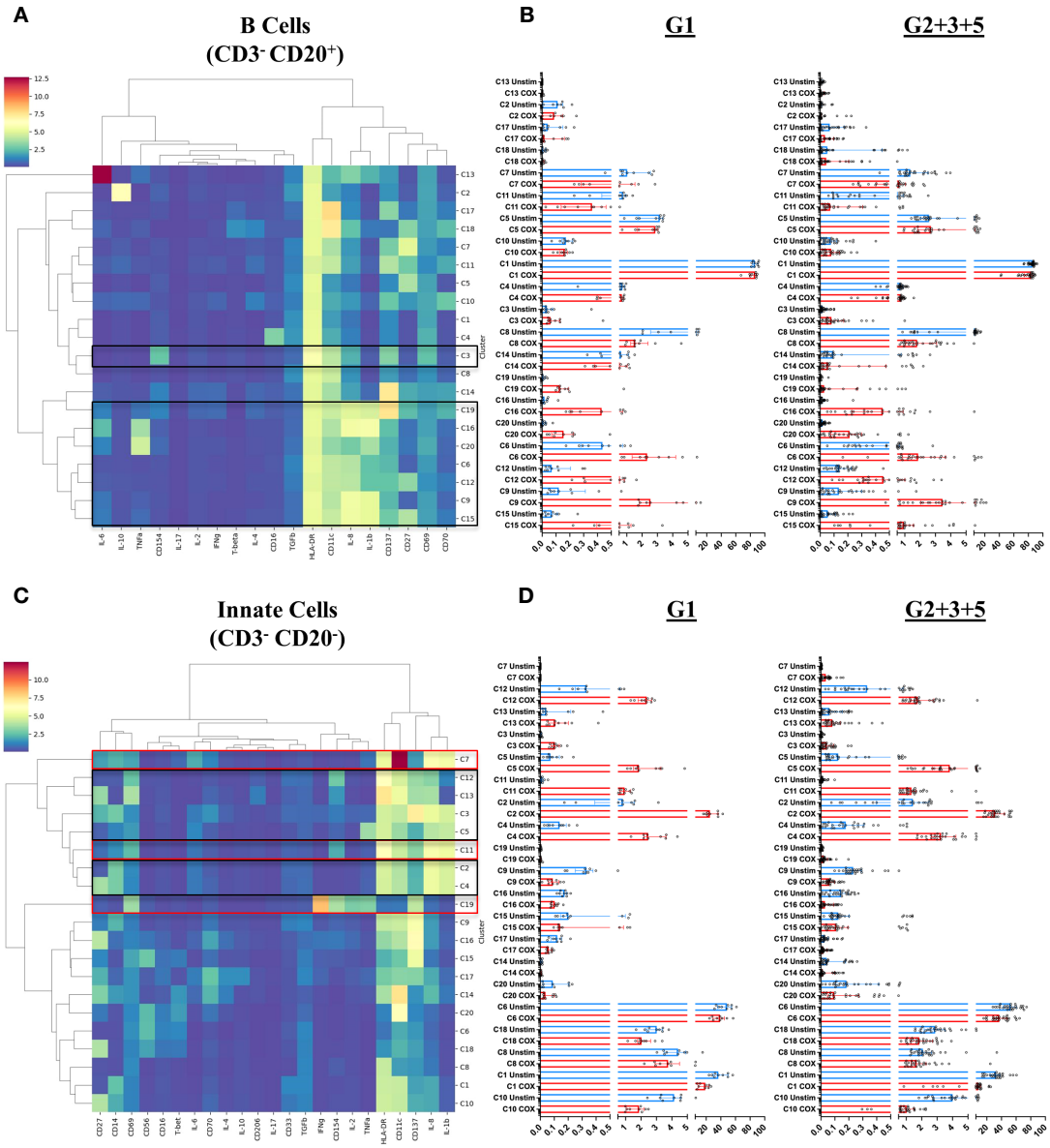

FIGURE 7
B cell and innate cell subpopulations characterized by mass cytometry in peripheral blood mononuclear cells. Immune cell meta-clusters were identified through FlowSOM based clustering of mass cytometry labeled samples. The phenotypic profile of each meta-cluster, in **(A)** $CD3^{-}CD20^{+}$ B cells and **(C)** $CD3^{-}CD20^{-}$ innate cells, is depicted in heatmaps. The color gradient on the heatmaps indicates the mean metal intensity for each marker. Dendrograms were generated through hierarchical clustering using Ward linkages. The scatterplots on the right of each heatmap indicate the frequency (abundance) each meta cluster in **(B)** B Cells and **(D)** Innate cells in each donor in unstimulated (blue) and *C. burnetii*-stimulated (red) samples. Lines indicate the median with interquartile range. Black boxes in heatmaps **(A)** and **(C)** highlight meta-clusters showing increased absolute abundance upon *C. burnetii* stimulation; red boxes highlight meta-clusters showing higher *C. burnetii*-specific relative abundance (background-corrected) in all pre-exposed individuals (Group 2 + 3 + 5) compared to Group 1.

infection (49, 50) as well as following experimental human *Streptococcus pneumoniae* challenge (51).

Our study had several limitations. These included the relatively small number of individuals enrolled for the different subgroups in CyTOF analysis, resulting in limited power to detect statistically significant differences. The division of seropositive individuals into past symptomatic and asymptomatic infection was further based on self-reported data collected in 2015, i.e. five to eight years after the epidemic and may therefore not be fully accurate. Finally, we faced a few technical challenges. In the flow cytometry T cell panels, several markers performed poorly with no clearly identifiable populations (CD137, FOXP3, T-bet, IL-10). CyTOF analysis had to be conducted on PBMCs rather than whole blood, and the limitation of barcoding to 20 samples per CyTOF run made it necessary to assess individuals in four different runs on different days. The batch-to-batch variation between the four rounds precluded normalization of some markers which hence were not carried forward for downstream analysis. As these included the markers to distinguish memory and naïve T cells (CD45RO, CD45RA and CCR7), this precluded analysis of these subsets. Despite these

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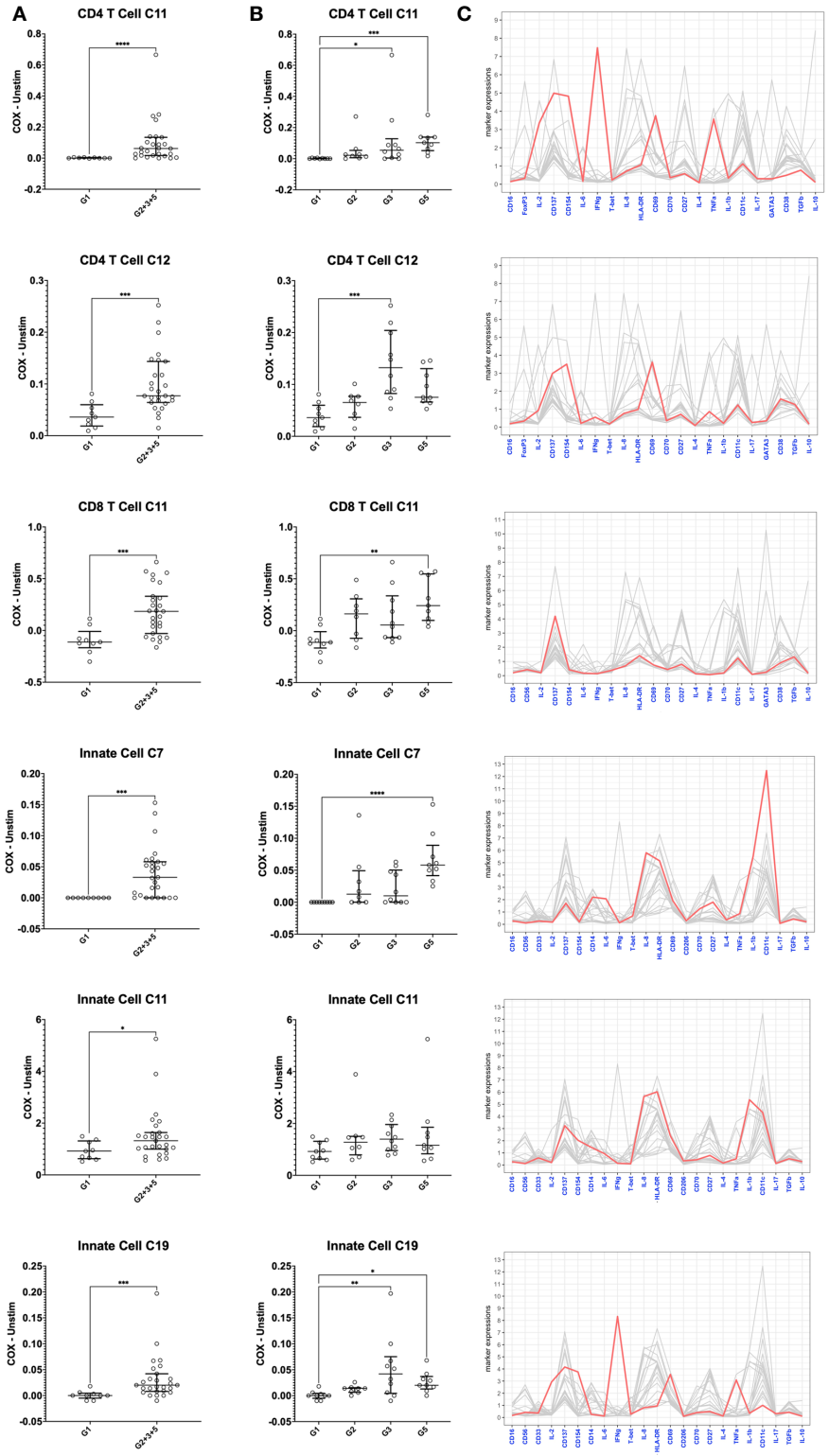

FIGURE 8
Mass cytometry profiles of *C. burnetii*-specific cellular responses in peripheral blood mononuclear cells. Cellular responses following stimulation with *C. burnetii* were assessed by mass cytometry. Scatterplots depict the background corrected percentage of relevant immune cell subpopulations (meta-clusters) in: **(A)** unexposed Group 1 (n=9) and all exposed individuals (n=27) donors (Group 2 + 3 + 5), **(B)** unexposed Group 1 (n=9), seronegative, asymptomatic Group 2 (n=8), seropositive, asymptomatic Group 3 (n=10) and seropositive, symptomatic Group 5 (n=9). Lines indicate the median with interquartile range. Activation responses were compared using the Mann-Whitney U test in **(A)** and Kruskal-Wallis test followed by Dunn's *post-hoc* multiple comparison test for nonparametric data in **(B)**. Asterisks are defined as follows: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). **(C)** Parallel coordinate plots depicting the mean intensity of each marker in the different clusters. Y axis indicates mean metal intensity. Line colored in red depicts the mean metal intensity of markers in that cluster, while lines in gray are depicting the mean metal intensity of markers in all other clusters.

limitations, the results of this study both confirm prior observations and highlight novel aspects of the immune responses to *C. burnetii* that merit future investigation.

The innate and adaptive immune response to *C. burnetii* infection is multi-faceted (52–55). Innate immune responses are the first line of defense during acute infection and direct the induction of adaptive immune responses. However, *C. burnetii* invades and replicates in monocytes and macrophages - key cellular components at the interface of the innate and adaptive immune response - and deploys a range of immune evasion mechanisms to avoid intracellular killing in these innate immune cells (56). Being an intracellular pathogen, the clearance of *C. burnetii* infection largely relies on cellular adaptive immune responses. While antibodies are required to control tissue damage in murine models, they are not sufficient to control infection. Instead, in these animal models T cell responses are critical to controlling early infection, mediating bacterial clearance and conveying vaccination-induced protection (52–55, 57, 58). In addition, there appears to be a greater role for major histocompatibility complex (MHC) class I-restricted CD8 T cell responses during primary infection in murine models (52, 57). For protection from secondary infection, in contrast, there is a greater role for MHC class II-restricted CD4 T cells as well as MHC II-restricted but CD4-independent mechanisms (58). Notably, IFNγ is required for the clearance of primary *C. burnetii* infection (52), but appears to be less critical for clearance during secondary infection in mice (58).

T cells elicit recall responses through IFNγ production (59), which results in *C. burnetii* killing in infected monocytes in a TNFα-dependent manner (44, 45). IFNγ secretion in response to stimulation with *C. burnetii* antigen is known to be enhanced in those having experienced prior infection (31, 59). We detected increased production not only of IFNγ but also of IL-2, IL-6, IL-10 and TNFα following *C. burnetii* antigen stimulation of peripheral immune cells isolated from individuals with prior exposure to *C. burnetii*. Flow cytometry and CyTOF both revealed that CD4 T cells were the most consistent contributors to IFNγ and IL-2 recall responses, while monocytes were the predominant source of IL-6, IL-8, IL-10 and TNFα. Additionally, there were some previously exposed donors who also showed elevated IFNγ response by flow cytometry in CD4-negative T cells (i.e. likely CD8 T cells). In our study, using CyTOF we also identified a population of *C. burnetii*-specific CD4 T cells ($CD137^+CD154^+CD69^+IFN\gamma^+IL\text{-}2^+TNF\alpha^+$) that was significantly increased in IGRA-positive individuals, particularly those who had exhibited clinical symptoms and remained seropositive. Co-production of these cytokines by specific cell subsets is consistent with data from a prior study looking at bulk cytokine responses to *C. burnetii* in individuals with prior exposure to *C. burnetii* (43), which showed that individuals with prior exposure and high IFNγ production also produce high amounts of IL-2. Notably, the $CD137^+CD154^+CD69^+IFN\gamma^+IL2^+TNF\alpha^+$ CD4 T cell cluster C11 and the corresponding lineage-negative innate cell cluster C19 were the only two cell populations showing clear IFNγ production based on the expression-normalized CyTOF data used for unsupervised clustering. The data normalization likely reduced the ability to detect cell populations with lower IFNγ production, such as NK cells and CD8 T cells, which were clearly identifiable by manual gating of CyTOF data or flow cytometry data (for $CD4^-$ T cells). Additionally, at least part of the IFNγ responses observed by flow cytometry in the $CD4^-$ T cell compartment might originate from unconventional γδ T cells, which largely lack CD4 and CD8 expression. Vγ9 Vδ2 T cells have been shown to be activated and expanded during acute human *C. burnetii* infection (60), likely by *C. burnetii*-derived phosphoantigens since Vγ9 Vδ2 T cell activation requires expression of butyrophilin molecules BTN2A and BTN3A on monocytes (61). Since IFNγ is a key cytokine produced by Vγ9 Vδ2 T cells, and these cells have been shown also display the same hallmark ability as adaptive T cells to respond more strongly upon re-exposure (62), it is conceivable that they also contribute to recall responses against *C. burnetii*.

A key finding of our study is that beyond recall responses by adaptive T cells, we also found several cell populations within the innate compartment that showed increased responses to *C. burnetii* antigen in pre-exposed (IGRA-positive and seropositive) individuals compared to controls with no evidence of adaptive immunity to *C. burnetii* (Figures 7C, D, 8). This was particularly evident in individuals with past symptomatic infection (designated Group 5): *C. burnetii* antigen-stimulated whole blood from these individuals showed elevated bulk release of IL-1β as well as increased IL-6 production by monocytes as detected by flow cytometry. We also observed a trend for increased whole blood release of IL-6 and TNFα, which did not reach significance. This is likely due to the small number of individuals assessed in this analysis, since in a previously published study with considerable larger groups from the same village cohort, release of both IL-6 and TNFα from *C. burnetii* antigen-stimulated whole blood was significantly higher in IGRA-positive individuals compared to controls (43). CyTOF analysis of *C. burnetii* antigen-stimulated PBMCs revealed an increased proportion of two innate ($CD3^-$ $CD20^-$) clusters in individuals with prior exposure. Innate cluster C7 with the phenotype $CD11c^{high}CD14^+HLA\text{-}DR^+IL\text{-}1\beta^+IL\text{-}6^+IL\text{-}8^+$ was particularly strongly increased in a subgroup of pre-exposed, past symptomatic individuals (Group 5), while C11 ($CD11c^{int}CD14^+HLA\text{-}DR^+CD69^+CD137^{int}CD154^{int}IL\text{-}1\beta^+IL\text{-}6^{low}IL\text{-}8^+$) was increased in IGRA-positive (combined Group 2 + 3 + 5) donors.

Both innate clusters associated with prior exposure likely represent $CD14^+$ monocytes, and we have previously already hypothesized, based solely on cytokine release data, that increased innate cytokine responses after *in vivo* exposure to viable *C. burnetii* might be due to trained immunity of myeloid cells such as has been described for other pathogens (63). This new cytometry dataset underscores this hypothesis, although it clearly requires further investigation, including experiments evaluating the epigenetic status of monocytes in *C. burnetii*-exposed individuals. Remarkably, these increased monocyte responses were evident six to ten years after primary exposure to *C. burnetii*, a much longer timeframe than the up to one year typically attributed to trained immunity based on the short lifespan of innate immune cells (64). On the other hand, trained immunity following BCG vaccination has been shown to last for up to five years, which may be partially

attributed to the reprograming of hematopoietic progenitor cells (65, 66). Conceivably the long duration of enhanced innate re-call responses in our study might have also been facilitated by (asymptomatic) re-exposure. Indeed, elevated innate responses were most evident in Group 3 and 5 individuals, also showing the strongest adaptive responses. This also raises the question whether these stronger adaptive immune responses in seropositive individuals might have positively influenced training of innate responses. However, the contribution of adaptive responses to trained immunity is controversial, since IFNγ has been shown to both support and hamper the induction of trained immunity (64). Moreover, while stronger adaptive responses in Groups 3 and 5 could theoretically be a result of re-exposure, this in unlikely the case for all individuals in these cohorts since no large outbreaks have been reported after 2010. Of note, trained innate immunity is generally a property of live attenuated whole cell vaccines (67) and indeed we found no evidence for enhanced innate responses following vaccination with the killed whole cell vaccine Q-VAX (43). If such responses indeed are relevant to protection from or resolving future infection, approaches should be considered to combine future *C. burnetii* vaccines with amplifiers of trained immunity, such as BCG (67).

Another cell population that showed an increased abundance in *C. burnetii* antigen-stimulated cultures specifically for IGRA-positive previously exposed individuals was the presumed innate cluster C19 ($CD69^{+}CD137^{mid}CD154^{+}IFN\gamma^{+}IL\text{-}2^{+}TNF\alpha^{+}$). Given the complete lack of lineage marker expression, one possible interpretation of this phenotype is that these cells constitute innate lymphoid cells (ILCs) of ILC1 or ILC3 polarization. IFNγ and TNFα production by these two ILC subsets has been shown to play an important role for the early host defense against a wide range of intracellular pathogens, as reviewed recently (68). Moreover, akin to adaptive lymphocytes, there is accumulating evidence that immunological memory is also a property of innate lymphoid cells and contributes to long-term protection also after vaccination (69). This is likely due to epigenetic modifications, as originally shown for 'trained immunity' in monocytes and recently demonstrated for ILC2 cells in asthma (70). Whether or not ILC1 or ILC3 cells indeed contribute to recall responses to *C. burnetii* requires further work beyond this study, using dedicated flow cytometry or mass cytometry panels (71, 72).

In conclusion, our cytometry data set profiling cellular recall responses in a cohort of individuals up to a decade after natural exposure to *C. burnetii* shows that CD4 T cells are the major driver of previously reported *C. burnetii*-specific production of IFNγ and IL-2. In addition, we find evidence that an innate population possibly resembling ILCs also contributes to this Th1-type re-call response. Finally, our data show that although numerous cell populations in the innate and adaptive compartments produce innate cytokines upon *C. burnetii* antigen stimulation regardless of prior exposure, the release of innate cytokines IL-1β, IL-6 and IL-8 and the proportion of two distinct monocyte populations expressing these cytokines in response to *C. burnetii* was specifically elevated in previously exposed individuals, indicative of trained innate immunity. These findings provide important new insights into the nature of recall responses to *C. burnetii*, warrant future studies to determine whether innate responses contribute to protection from *C. burnetii* infection and have the potential to inform the design of novel vaccines for Q fever.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Medical Ethical Committee Brabant (Tilburg, Netherlands, NL51305.028.15). 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

SRP, AS, PR, AG, AES and MP conceptualized and designed the study and experiments. Experiments were performed by SRP and AS. Data were analyzed by SP, AS, SK, RS, JH, and RD, and interpreted by SRP and AS. AG, AES and MP acquired funding and supervised research activities. SRP and AS wrote the manuscript and PR, AES, AG and MP critically reviewed and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AG is CEO and AS was a senior scientist at Innatoss Laboratories B.V., which provides diagnostic screening for Q fever.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1249581/full#supplementary-material

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EDITED BY
Nathella Pavan Kumar,
National Institute of Research in Tuberculosis (ICMR), India

REVIEWED BY
Beatrice Omusiro Ondondo,
University Hospitals of Leicester NHS Trust, United Kingdom
Abdullah Saeed,
City of Hope National Medical Center, United States

*CORRESPONDENCE
Changchun Zeng
zengchch@glmc.edu.cn

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Intratumoral microbiota: implications for cancer onset, progression, and therapy

Jinmei Wu[1], Pengfei Zhang[1], Wuxuan Mei[2] and Changchun Zeng[1]*

[1]Department of Medical Laboratory, Shenzhen Longhua District Central Hospital, Shenzhen, China, [2]Xianning Medical College, Hubei University of Science and Technology, Xianning, China

Significant advancements have been made in comprehending the interactions between the microbiome and cancer. However, prevailing research predominantly directs its focus toward the gut microbiome, affording limited consideration to the interactions of intratumoral microbiota and tumors. Within the tumor microenvironment (TME), the intratumoral microbiome and its associated products wield regulatory influence, directing the modulation of cancer cell properties and impacting immune system functionality. However, to grasp a more profound insight into the intratumoral microbiota in cancer, further research into its underlying mechanisms is necessary. In this review, we delve into the intricate associations between intratumoral microbiota and cancer, with a specific focus on elucidating the significant contribution of intratumoral microbiota to the onset and advancement of cancer. Notably, we provide a detailed exploration of therapeutic advances facilitated by intratumoral microbiota, offering insights into recent developments in this burgeoning field.

KEYWORDS

intratumoral microbiota, immunotherapy, cancer, treatment, tumor microenvironment

1 Introduction

The presence of numerous microorganisms such as viruses, bacteria, fungi, and other microbes within the human body is vital for human health. These microorganisms exhibit colonization patterns in multiple anatomical sites, encompassing the oral cavity, skin, gastrointestinal tract, respiratory tract, and genitalia. Symbiotic interactions between humans and their microbiome are critical and contribute significantly to human health (1–3). Extensive inquiries into the human microbiome have illuminated variations in the microbial communities among individuals in a state of health and those experiencing pathological conditions. Moreover, the microbiome is closely linked to cancer by influencing the carcinogenesis process in the human body (4). The well-documented link between cancer and specific viruses, such as Epstein-Barr virus and human papillomavirus, underscores their potential to initiate oncogenic activation (5). Oncoviral

infections have been shown to promote tumorigenesis by enabling the incorporation of oncogenes within the human genome structure (6, 7).

Research into host-microbial interactions has notably propelled the comprehension of intratumoral microbiota (8, 9). The advancement of detection technologies and enhanced comprehension of the TME have substantiated the presence of intratumoral bacteria. Tumor tissue presents a significantly reduced presence of microbial and fungal biomass when compared to the abundance observed in the gut environment (10, 11). Recent findings point to exclusive bacterial and fungal patterns characteristic of individual tumor types (12, 13). In comparison to normal tissues, tumor tissues manifested a heightened abundance of bacterial and fungal burdens. Remarkably, a substantial enrichment of multiple bacterial strains was observed specifically within tumor tissues. Intratumoral microbial components, distinguished in several tumor types, manifest meaningful correlations with the onset and advancement of cancer (14, 15). Recent studies underscore the fundamental importance of gut microbiota in governing the immune responses. Additionally, it has been demonstrated that the microbiota present within tumors can significantly shape the local immune responses in the TME, potentially affecting tumor progression (16). Within the TME, intratumoral microbiota conspicuously demonstrate anti-tumorigenic manifestations by orchestrating heightened antigen presentation, activating T and NK cells, executing proficient immunosurveillance, and synthesizing metabolites that suppress tumor progression. Conversely, pro-tumorigenic effects are characterized by elevated levels of reactive oxygen species (ROS), the emergence of driver mutations, the inactivation of T cells, and the induction of immunosuppression (3). The intratumoral microbiota manifests varied roles in anti-tumor immunity, with the potential to either enhance or suppress anti-tumor immune responses (17). Consequently, these roles have implications for the effectiveness of immunotherapy (16, 18). In recent years, there has been a surge in research interest delving into the intricate interplay between gut microbiota and the etiology as well as therapeutic responses in cancer. Nonetheless, increasing attention is being paid to intratumoral microbiota (3).

This review presents a thorough analysis of the burgeoning field of intratumoral microbiota research. We delve into its origins, the rich spectrum of its diversity, the intriguing links between intratumoral and gut microbiota, mechanistic involvement in tumorigenesis, and the exciting potential it holds for innovative tumor therapeutics. This review offers promising avenues for developing innovative therapeutic interventions leveraging intratumoral microbiota toward effective tumor management.

Abbreviations: TME, tumor microenvironment; TCGA, The Cancer Genome Atlas; PR, Progesterone receptor; ER, Estrogen receptor; HER2, Human epidermal growth factor receptor 2; T3SS, Type 3 secretion system; EMT, Epithelial-mesenchymal transition; NFκB, Transcription factor nuclear factor κB; PRRs, Pattern recognition receptors; TLRs, Toll-like receptors; TLR4, Toll-like receptor 4; TNFSF4, Toll-like receptor (TLR) 4 and OX40 ligand; Tregs, Regulatory T cells; MDSCs, Myeloid-derived suppressor cells; TAMs, Tumor-associated macrophages; NK, Natural killer; CEACAM1, Carcinoembryonic antigen-related cell-adhesion molecule 1; BCG, Bacillus Calmette-Guérin; CDDL, Bacterial enzyme cytidine deaminase; dFdU, 2'2-difluorodeoxyuridine; ROS, Reactive oxygen species; STING, Stimulator of interferon genes; FMT, Fecal microbial transplantation; NACI, neoadjuvant chemoimmunotherapy.

2 Intratumoral microbiota: unveiling their features

2.1 Origin of intratumoral microbiota

Despite the significant attention given to intratumoral microbiota, their origins have not been fully elucidated. Recent research has revealed that intratumoral microbiota may arise from distinct sources (Figure 1) (3, 11, 19, 20). The intratumoral microbiota may arise from breaches in mucosal barriers. Intratumoral microbiota is commonly found in cancers originating at mucosal sites, including colorectal, pancreatic, cervical, and lung cancer (21). These organs have externally exposed cavities, and the mucosal destruction that occurs during tumorigenesis can provide a pathway for microorganisms colonizing the mucosa to invade the tumor. Thus, the breach of mucosal barriers, with other factors, may lead to the colonization of microbiota in the TME and facilitate their complex interactions (16, 22). The identified representative bacteria within nasopharyngeal carcinoma tissues exhibit approximately 69% similarity in single-nucleotide variations to bacteria present in the nasopharyngeal microbiota. Subsequently, resemblances are observed with bacteria from the oral cavity (24.1%) and the gut (6.9%). These findings unequivocally establish the nasopharyngeal microbiota as the primary reservoir of intratumoral bacteria within nasopharyngeal carcinoma (23). Although there are abundant microbiomes in human mucosal organs, the idea that intratumoral microbiota can only come from the mucosal site through the mucosal barrier cannot explain all the intratumoral microbiota. A portion of intratumoral bacteria is rare within the mucosal organs of the corresponding tumors, while others are prevalent in non-mucosal origin tumors, such as breast cancer, suggesting other potential sources of intratumoral microbiota (11, 24). Therefore, additional investigation is necessary to clarify the mechanisms that facilitate microbial infiltration from mucosal organs into the TME.

The circulatory system represents another potential origin for intratumoral microbiota (3, 11). The chemotactic gradient of necrotic cell debris within a tumor is a mechanism that attracts microorganisms from different locations into the blood circulation. Malformed blood vessels provide a conducive setting for intratumoral microbiota to colonize the TME through hematogenous spread (9). Hematogenous spread facilitates the recruitment of microorganisms from various sites, including the oral cavity and intestines, to the tumor site, where they can colonize the tumor via infiltration through impaired blood vessels. The circulatory system, including blood, lymphatic fluid, and the internal passages of the alimentary tract, provides a plausible pathway for the transfer of microbiota. Considering the anatomical interconnectedness of the oral cavity, respiratory tract,

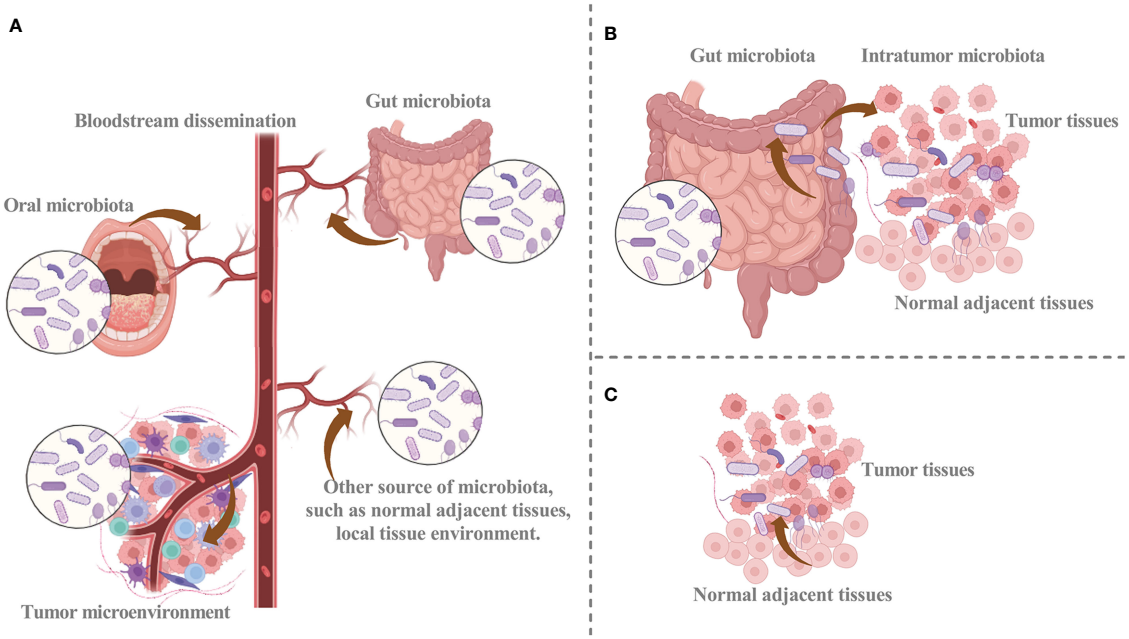

FIGURE 1
The potential sources of intratumoral microbiota. **(A)** Hematogenous spread facilitates the infiltration of intratumor microbes from oral, intestinal, and other sources into tumor sites. **(B)** Microbiota can disrupt the mucosal barrier and infiltrate tumor sites, and intratumoral microbiota of cancer may infiltrate tumor sites via the duct. **(C)** Normal adjacent tissue may provide a source for intratumor microbiota. Graphics created with BioRender.com.

and gastrointestinal tract, it is plausible that oral microbiota can easily migrate to these respective anatomical regions. When the oral microbiota undergoes ecological disruption, they may gain entry into the tumor and convert it into intratumoral microbiota (25).

Bacteria from adjacent normal tissues have been found in organs previously believed to lack microbial presence. Moreover, the bacterial composition within tumor tissues closely resembles that of adjacent normal tissues (3, 11, 26). The significant similarity of microbiota composition between tumor microbiota and normal adjacent tissue microbiota can be explained by the origin of normal adjacent tissue microbiota from TME. Within normal adjacent tissues, microorganisms from blood vessels or mucosal organs may infiltrate the TME stimulated by oxygen and chemotactic gradients (11). In addition, microorganisms in normal tissues may originate from the tumor site. Consequently, it is unclear whether normal adjacent tissues serve as a source of intratumoral microbiota, and further substantiation is necessary to elucidate this matter.

As knowledge of the origin and mechanisms of intratumoral microbiota grows, a more comprehensive understanding of intratumoral microbiota may assist in devising more potent therapeutic approaches. Exploring the various sources of intratumoral microbiota, analyzing their composition, and comparing them with the microbiome of other body sites may facilitate the identification of intratumoral microbiota. Furthermore, investigating the molecular mechanisms that underlie the infiltration of microorganisms into the TME is a compelling area of research.

2.2 Diversity of intratumoral microbiota

Given the possibility of multiple origins of intratumoral microbiota, it is plausible to suggest that the microbiome compositions of various cancer types are heterogeneous (15, 27). Within a variety of prevalent cancer types, there are distinct microbial signatures present in tissue and blood samples, each linked to a specific microbiota. Such microbial signatures have been utilized to differentiate healthy individuals from those with cancer, indicating that these signatures may have diagnostic potential (28). The utilization of a rigorous decontamination pipeline in analyzing The Cancer Genome Atlas (TCGA) database at the whole-genome and whole-transcriptome level has allowed for the discovery of unique microbial signatures present in both blood and tumor tissue that was specific to certain cancer types (15, 27). A recent pan-cancer study investigated the presence of cancer-associated fungi in 17,401 samples from 35 distinct cancer types. The findings indicate that fungal DNA and cells exhibit low abundance in several prevalent human cancers, with diverse community compositions across various cancer types. Distinct fungal species and corresponding cellular compositions were associated with specific types of cancer (15). Tumor microbial communities exhibit a predominance of bacteria, with a lower abundance of fungi. The composition of microbial communities in adjacent normal tissues is similar to that of tumor microbial communities. Some microorganisms have been identified in multiple types of tumors, although their abundance can differ depending on the specific cancer type (26).

Intratumoral bacteria possess some common characteristics. Their prevalence within cancerous tissues is significantly lower when compared to that of the gut, with qPCR and imaging quantification indicating that the bacterial presence is discernible in a fraction of cancer cells, varying from 0.1% to 10%. The microbial diversity is generally diminished in cancerous tissue as opposed to normal tissue, suggesting that tumors may foster a distinct milieu that selects for specific bacterial species. The majority of these bacteria are commensal organisms primarily inhabiting the intracellular compartment. The diverse bacterial ecosystems within cancer tissues could potentially contribute to multifunctional mechanisms when interacting with cancerous cells (14, 29).

The microbiota of colorectal cancer has been investigated, with some bacteria like *Bacteroides fragilis*, *Escherichia coli*, and *Fusobacterium nucleatum* frequently detected within tumor tissues. In addition, fungal species, such as Candida albicans, have been detected in some colorectal cancer samples (30–32). *Helicobacter pylori*, a bacterium responsible for chronic gastritis and peptic ulcers, is linked to the heightened risk of developing gastric cancer. Furthermore, some bacterial species like *Streptococcus anginosus* and *Lactobacillus* have been identified in some gastric cancer samples (33, 34). A pan-cancer analysis of the mycobiome across various anatomical locations revealed the presence of tumor-associated *fungi* and a significant abundance of *Candida* in gastrointestinal malignancies. Mycobiome communities in gastrointestinal tumors exhibit a high prevalence of *Cyberlindnera jadinii*, *Saccharomyces cerevisiae*, and *Candida* species. *Blastomyces* species are prevalent within pulmonary carcinomas, while *Malassezia* species are abundant within mammary tumors (13). *Fusobacterium nucleatum*, associated with colorectal tumors, also exhibited a higher prevalence in pancreatic and breast malignancies. Microbial compositions vary distinctly across different subtypes of tumors. For instance, multiple bacterial taxa exhibited distinct prevalence when comparing various subtypes of breast cancer, characterized by their human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) status. *Granulicatella_Unknown species31* (species) and *Dyadobacter* (genus) exhibit enrichment in HER2+ breast cancer patients. *Corynebacterium* (genus) demonstrates enrichment in ER- breast cancer patients, while *Actinomycetaceae* (family), *Sphingomonas_Unknown species124* (species), *Streptophyta_Unknown genus116* (genus), *Lautropia_Unknown species38* (species), and *Actinomyces odontolyticus* (species) manifest enrichment in ER+ breast cancer patients. *Actinobacteria* (class) displays enrichment in non-triple negative breast cancer. Conversely, *Achromobacter denitrificans* (species), *Bacillus_Unknown species21* (species), *Leptotrichia_Unknown species21* (species), *Streptophyta_Unknown genus116* (genus), *Nocardiopsaceae* (family), and *Achromobacter* (genus) are enriched in triple-negative breast cancer. Moreover, breast tumors exhibited a heightened bacterial abundance in comparison to normal adjacent tissue (14).

2.3 The association between intratumoral and gut microbiota

The current research landscape is witnessing a surge in studies exploring the correlation between intratumoral and gut microbiota. Specific bacterial species within the gut microbiota have the potential to infiltrate the intestinal mucosa, enter the bloodstream, and inhabit neoplastic lesions, thus shaping the composition of the microbiota within tumors. The gut microbiota-tumor interplay has emerged as a critical factor influencing the onset and advancement of diverse forms of cancer. In glioma, intratumoral bacteria can originate not only from the gut microbiota but also from the oral cavity or adjacent brain tissue. Glioma-induced shifts in the local microenvironment, involving the disruption of the blood-brain barrier and immunosuppression, create conducive conditions for bacterial infiltration via either hematogenous or neuronal retrograde pathways. It is plausible that these bacteria existed in the brain tissue before tumorigenesis, with those adapting to the TME demonstrating growth throughout tumor development (35). Nevertheless, the precise mechanisms by which gut bacteria contribute to the intratumoral microbiota remain not completely elucidated and warrant emphasis (3).

The TME is subject to regulatory influences from both intratumoral and gut microbiota, involving modulation of immune responses and modification of cancer cell metabolism (16). Modulation of the TME is achievable through gut microbiota-mediated regulation of intestinal epithelial barrier components, resulting in the activation of lymphoid organs. The gut microbiota may mediate its impact on the TME via metabolites or the immune system, thereby potentially altering the activities of the microbiota within the tumor (24, 36).

Comparable to the gut microbiota, the intratumoral microbiota exhibits the potential to modulate host immune responses. The gut microbiota intricately shapes the effectiveness of immune checkpoint blockade and the ensuing immune responses against tumors (37). Diverse interactions among intratumoral microbiota can trigger unique immune responses, suggesting a potential interplay with gut microbiota (15). Further investigation is warranted to clarify the interplay between intratumoral and gut microbiota.

3 Mechanistic insights into tumorigenesis and intratumoral microbiota

Intratumoral bacteria can regulate cancer cell-intrinsic properties, such as mechanical stress, stem cell flexibility, epithelial-mesenchymal transition (EMT), and adhesion to endothelial cells, which can detrimentally impact the behavior of tumor cells in circulation. Intratumoral bacteria can regulate the extrinsic cancer milieu by releasing exosomes, thereby fostering metastasis, facilitating the breach of the vascular barriers for remote organ colonization, and contributing to the creation of a specialized premetastatic niche. Furthermore, they orchestrate the modulation of the adaptive and innate immune systems, ultimately dictating the resultant immune reaction (38). The intricate interplay between the intratumoral microbiota and cancer manifests in a multifaceted manner, exerting varied influences on cancer progression (Figure 2). These include promoting cancer growth and spread

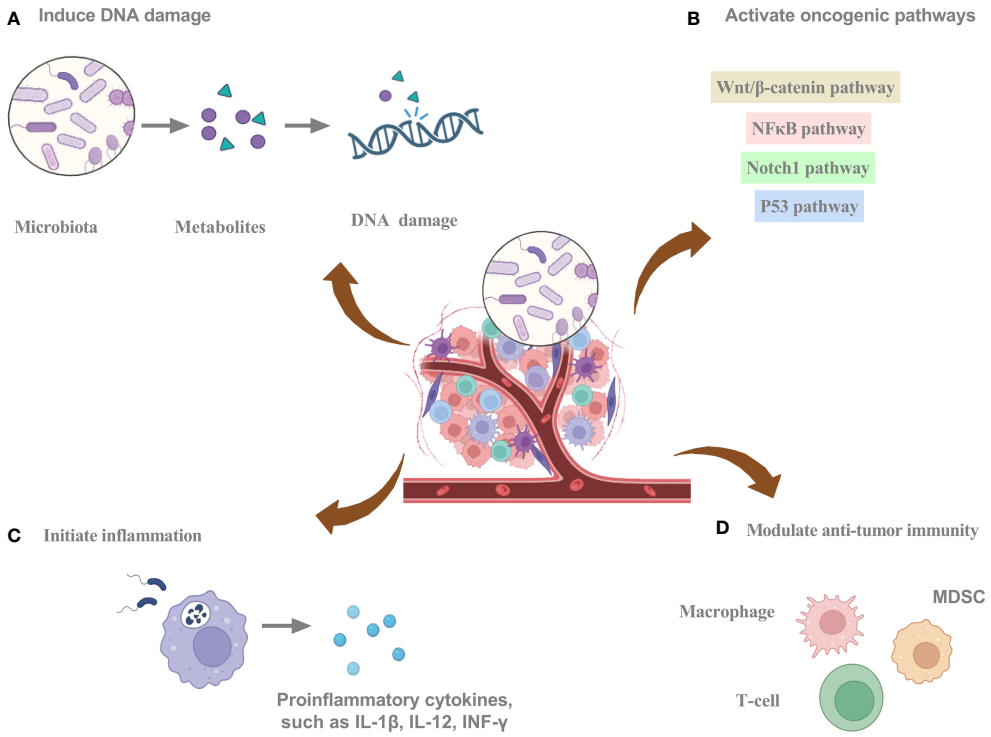

FIGURE 2
The potential mechanisms of intratumoral microbiota promoting tumorigenesis. **(A)** Intratumoral microbiota can secrete metabolites to induce DNA damage. **(B)** Intratumoral microbiota can activate oncogenic pathways. **(C)** Intratumoral microbiota can initiate inflammation. **(D)** Intratumoral microbiota can modulate anti-tumor immunity. Graphics created with BioRender.com.

through increased mutagenesis, epigenetic modifications, modulation of oncogenes or oncogenic pathways, inflammation initiation, and immune response alteration (31, 38–40).

3.1 Induce DNA damage

Several bacterial species have evolved mechanisms to inflict DNA damage, which may instigate mutational events and ultimately promote carcinogenesis (41). Carcinogenic bacteria damage host DNA through a variety of mechanisms involving molecules, proteins, and metabolites. Fragile Bacteroidin exhibits the potential to cause DNA damage, thereby stimulating mutational events (3, 42). Single-cell RNA sequencing enables the identification of bacteria-associated host cells, their interactions, and the dysregulation of transcriptional pathways related to DNA damage repair, cell cycle, and the p53 signaling pathway (9). The production of colibactin by polyketide synthetase (pks)+ *Escherichia coli* can lead to DNA alkylation, provoking DNA damage and facilitating colorectal cancer progression (43). The pathogenic bacteria that adhere to the intestinal epithelium can induce episodes of diarrhea. The type 3 secretion system (T3SS) of these bacterial pathogens plays a crucial role in their interactions with intestinal epithelial cells, through which they can deliver genotoxin-UshA that damages the DNA of the host cells, contributing to the development of carcinogenesis (44). The involvement of microbes in instigating DNA damage through mutational processes is apparent. The mechanisms currently under consideration include *Escherichia coli*-mediated colibactin crosslinking, generating genotoxicity, and *Helicobacter pylori*-mediated aberrant cytidine expression. The exploration of mutational signatures through bioinformatics has opened the door to comprehending the processes underlying genomic alterations that drive oncogenesis. Microbes can elicit DNA damage that impacts the structure of the cancer genome, resulting in alterations to mutational spectra and mutational signatures (42). Additionally, the microbiota can convert numerous dietary metabolites into agents that damage DNA, and under conditions of dysbiosis, certain bacteria can produce toxins that cause DNA damage (3, 9, 45).

3.2 Activate carcinogenic pathways

Intratumor microbiota and their metabolites can influence signaling pathways that contribute to oncogenesis. *Fusobacterium nucleatum* has been implicated in the modulation of pathways and their associated molecules, exerting an influence on the landscape of pancreatic tumor development (46, 47). Through a Fap2-dependent pathway, *Fusobacterium nucleatum* engages with pancreatic cancer cells, inducing cytokine production. Through autocrine and paracrine pathways, cytokines stimulate cancer cell proliferation and enhance migration, ultimately propelling the evolution of the malignancy (47). Infections by bacteria lead to a substantial augmentation of signaling pathways, notably TNF, inflammatory responses, and hypoxia pathways. Furthermore, this fosters cancer cell progression through

EMT and activation of the p53 pathway (9). Microbial metabolites can modulate signaling pathways such as transcription factor nuclear factor κB (NFκB) and Wnt/β-catenin in tumor cells, thereby affecting tumor progression (3). In colorectal cancer, *Fusobacterium nucleatum* is recognized for its ability to trigger the initiation of the E-cadherin/β-catenin signaling cascades via FadA. This initiation eventuates in DNA damage, stimulation of cell growth, and augmentation of chk2 expression (48). CagA, a protein synthesized by *Helicobacter pylori*, can enter the host cell cytoplasm, triggering β-catenin signaling cascades, ultimately promoting the onset of gastric cancer (49). The involvement of Enterotoxigenic *Bacteroides fragilis* in breast cancer initiation is evident through both intraductal and intestinal colonization, emphasizing local and distant impacts. Elicitation of oncogenic effects by the *Bacteroides fragilis* toxin is potentially linked to the stimulation of the β-catenin and Notch1 signaling cascades (50).

3.3 Initiate inflammation

Chronic inflammation can elevate the likelihood of developing particular forms of cancer by activating inflammatory mediators and signaling cascades that promote tumor cell survival, proliferation, and invasion. Inflammatory mediators like ROS, cytokines, chemokines, and nitrogen species can facilitate tumor progression by fostering angiogenesis, elevating growth factor synthesis, and provoking the proliferation of cancerous cells (51, 52). Intratumoral bacteria can aggravate the inflammatory response, leading to the exacerbation of the disease (53). Intratumoral bacteria interacting with pattern recognition receptors (PRRs) can activate inflammatory pathways. Intratumoral bacteria may activate PRRs, leading to the secretion of cytokines and chemokines, the facilitation of angiogenesis, and immune cell recruitment (54, 55). An increased presence of *Fusobacterium* within tissues of head and neck squamous cell cancer has been linked to heightened inflammation and a less favorable prognosis. Moreover, complex interactions between competitive endogenous RNA networks and chromatin accessibility promote the development of microbiome-related inflammatory TME (56). *Fusobacterium nucleatum* can initiate the toll-like receptor 4 (TLR4)-mediated signaling cascade, which activates downstream signaling pathways and NFκB, leading to the induction of genes related to inflammation and the immune response (57). An elevated prevalence of *Enterobacteriaceae* is linked to heightened inflammatory activity, possibly attributed to their metabolizing inflammatory byproducts as an energy source (58). The secretion of virulence factors by *Escherichia coli* exacerbates the inflammatory response (59). The interplay between chronic inflammation and intratumoral bacteria requires further investigation.

3.4 Modulate anti-tumor immunity

Intratumoral microbiota can impact TME through several mechanisms, thus playing a role in tumorigenesis and cancer treatment (Table 1). Bacterial-induced modifications within the TME play a pivotal role in immunotherapy (69). Microbes within the TME elicit recognition by immune and cancer cells by presenting microbial antigens on their cell surfaces, stimulating an immune response and activating immune cells against the tumor (70). Moreover, some microbial antigens display structural resemblance to tumor antigens, activating immune cells that recognize these shared antigens. Consequently, the immune response triggered against microbial antigens can also target tumor cells expressing analogous antigens (71). In addition, some microbes in the TME can trigger immunogenic cell death, characterized by danger signal release and immune system activation, resulting in proinflammatory molecule secretion and tumor antigen presentation, facilitating an immune response against tumor cells (72). Furthermore, microbial component-mediated activation of PRRs boosts the immune response against tumors, eliciting the liberation of proinflammatory cytokines and heightened stimulation of immune cell activity (73, 74). Moreover, microbial-derived metabolites in the TME exert immunomodulatory effects by impacting immune cell behavior and remodeling the TME (75). Additionally, certain microbes in the TME can activate inhibitory checkpoints, diminish immune cell activity, and attenuate the anti-tumor immune response (72). Stimulated by intratumoral microbiota, the initiation of interleukin-17 production is triggered, fostering the infiltration of B cells into the complex microenvironment of tumor tissues. This intricately coordinated response emerges as a substantial factor in contributing to the progression of colon cancer. Within the milieu of colon cancer, polymorphonuclear neutrophils, recognized as highly abundant immune cells, have the potential to ameliorate microbial dysbiosis in colon cancer tissues. This is manifested by a decrease in tumor-associated *Akkermansia* and a concurrent increase in the prevalence of *Proteobacteria* (76). Within microsatellite instability-high colorectal cancers, the Fusobacterium nucleatum-enriched subset exhibits heightened tumor invasion. Furthermore, specific features within the immune microenvironment become evident, highlighting a significant reduction in FoxP3+ T cells spanning the entire tumor and a notable increase in the proportion of M2-polarized macrophages positioned within the tumor (77).

The microbiota may exert a significant impact on an immunosuppressive TME in pancreatic ductal adenocarcinoma (78). By translocating to the pancreas, the gut microbiome can initiate the formation of a TME exhibiting immunosuppressive, promoting tumorigenesis and metastatic spread, consequently impairing the potency of modulators targeting immune checkpoints (78). The increase of immune cells with immunosuppressive properties, such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), along with cytokines, obstruct TILs from penetrating the tumor site (78, 79). In oral cavity tumors, *Fusobacterium nucleatum* load exhibited a negative correlation with immune markers. Elevated *Fusobacterium nucleatum* levels were associated with decreased B lymphocytes, T helper lymphocytes, M2 macrophages, and fibroblasts. In tumors exhibiting a high load of *Fusobacterium nucleatum*, significant reductions were noted in the expressions of Toll-like receptor (TLR) 4 and OX40 ligand (TNFSF4). Significantly, TNFSF9 receptor (TNFRSF9) expression underwent a marked decrease, mirroring an escalation in its ligand (TNFSF9) expression with the mounting *Fusobacterium nucleatum* load. Simultaneously, there was a marked elevation in the levels of the pro-inflammatory cytokine IL-1ß (17). The presence of intratumoral microbiota has been identified as a pivotal factor in fostering an immunosuppressive TME by selectively

TABLE 1 Functional roles of intratumoral microbiota in the modulation of the tumor microenvironment.

Intratumoural microbiota	Mechanism	Cancer	References
Bifidobacterium	The localized delivery of *Bifidobacteria* efficiently triggers STING signaling and enhances the initiation of crossover events in dendritic cells after anti-CD47 treatment	Digestive tract cancer	(60)
Enterococcus faecalis	The pancreatic ductal adenocarcinoma microbiome orchestrates TAM programming through TLR signaling, inducing immune tolerance	Pancreatic cancer	(61)
Fusobacterium and *Treponema*	*Fusobacterium* and *Treponema* species were notably associated with macrophages and aneuploid epithelial cells, resulting in the upregulation of JAK-STAT signaling, interferon, and inflammatory response pathways	Oral squamous cell carcinoma	(62)
Saccharopolyspora, *Pseudoxanthomonas*, and *Streptomyces*	The tumor microbiome's diversity and the inclusion of *Saccharopolyspora*, *Pseudoxanthomonas*, and *Streptomyces* species within tumors could potentially enhance the anti-tumor immune response by aiding in the recruitment and activation of CD8+ T cells	Pancreatic cancer	(63)
Streptococcus	Tissue densities show a positive correlation of GrzB+ and CD8+ T cells with *Streptococcus* and a negative correlation of FOXP3+ and CD4+ T cells with *Streptococcus*	Esophageal squamous cell carcinoma	(64)
Dialister and *Casatella*	*Dialister* and *Casatella* displayed robust associations with MSI. *Dialister* exhibited positive correlations with CD3E and CD8E, indicating overall tumor-infiltrating lymphocytes and cytotoxic T cells	Colorectal cancer	(65)
Fusobacterium nucleatum	Fusobacterium nucleatum is inversely associated with CD3, signifying immunosuppression	Colorectal cancer	(65)
Lactobacillus	*Lactobacillus* prevalence within the tumor may impact local microbiome diversity, leading to elevated PD-L1 expression in ECs and TAMs	Esophageal squamous cell carcinoma	(66)
Lachnospiraceae	*Lachnospiraceae* bacteria within tumors enzymatically degrade lyso-glycerophospholipids, sustaining CD8+ T cell immune surveillance and defending against colorectal carcinogenesis	Colorectal cancer	(67)
Acinetobacter baumannii	*Acinetobacter baumannii* is prominently enriched in the immune-enriched subtype, marked by elevated stromal and immune scores, and a higher presence of CD81 T cells and M1-type macrophages, fostering a proinflammatory microenvironment	Ovarian cancer	(68)
Fusobacterium nucleatum	*Fusobacterium nucleatum*, enriched in immune-deficient patients, drives tumorigenesis through FadA adhesin and outer membrane vesicle, offering tumor protection by binding to inhibitory receptors	Ovarian cancer	(68)

MSI-H, High-level microsatellite instability; TAM, tumor-associated macrophage; TLR, Toll-like receptor.

recruiting specific immunosuppressive cellular populations, including Tregs, MDSCs, and TAMs. Consequently, this orchestrated recruitment acts as a deterrent to the efficacious infiltration of TILs (3, 17, 80). The depletion of CD4+ T cells of the Th1 subtype and CD8 + T cells with cytotoxic activity, accompanied by a shift towards Th2 T cells, as well as the shift of tumor-associated macrophages (TAMs) towards the M2 phenotype associated with immunosuppression, are associated with immune suppression and an unfavorable TME (78, 81, 82). The fibrogenic reprogramming of pancreatic ductal adenocarcinoma stellate cells results in a dense fibrotic stroma, impeding the penetration of therapeutic drugs and immune cells into the tumor locale. Furthermore, the activated pancreatic stellate cells recruit immunosuppressive cells, establishing a TME exhibiting immunosuppressive features, thus facilitating tumor growth and dampening effective immune reactions targeting tumors (78, 83).

Some microorganisms can interface with immune cells in the TME, potentially modulating their activity (11, 24). *Fusobacterium nucleatum* can impede the cytotoxicity exhibited by natural killer (NK) cells against tumors. *Fusobacterium nuclei* strains inhibit the cytotoxicity of NK cells by engaging with the Fap2 protein, leading to subsequent attachment to the inhibitory receptor TIGIT. Tumors exploit the Fap2 protein derived from *Fusobacterium nucleatum* to promote immune escape via TIGIT-mediated inhibition of immune cell function (84). *Fusobacterium nucleatum* can interact with carcinoembryonic antigen-related cell-adhesion molecule 1 (CEACAM1), thereby exerting an inhibitory effect on the function of T and NK cells (85). Commensal microbiota-mediated modulation of γδ T cell functionality impacts immune reactivity. Specifically, the microbiota elicits the activation of T cells, particularly those with the Vγ6+Vδ1+ phenotype, in lung cancer. These γδ T cells facilitate neutrophil penetration and stimulate the growth of tumor cells, thereby influencing the TME and tumor progression (86). Within colorectal carcinoma tissue, an inverse correlation has been observed between the prevalence of *Fusobacterium nucleatum* and the abundance of CD3+ T-cell count. A reduced CD3+ T-cell density can facilitate tumor progression by decreasing immune surveillance and impairing anti-tumor activity (87).

4 The potential of intratumoral microbiota for tumor therapy

Current research has established the considerable contribution of the microbiome to diverse aspects of cancer, such as oncogenesis, therapeutic response, and drug resistance (41). Strategic alteration of the gut microbiota holds promise for mitigation and

management of cancer. However, the therapeutic potential of intratumoral microbiota warrants further investigation (22). Intratumoral microbiota may exert adverse or favorable effects on cancer therapy, depending on the underlying therapeutic mechanism (Figure 3; Table 2) (93). Two principal approaches for microbial-based treatments have progressed to the clinical stage. The first approach employs living or inactivated bacteria to stimulate an immune response via targeting specific antigens. The Bacillus Calmette-Guérin (BCG) vaccine, various bacterial vaccines, and the implementation of live, attenuated, double-deleted Listeria monocytogenes are notable examples of this strategy. The second strategy involves utilizing bacteria as carriers capable of the controlled release of immunostimulants, toxins, and other pharmaceutical agents. Engineered bacteria can elicit an anti-tumor response or serve as carriers for therapeutic applications. Through genetic modifications, engineered bacteria can release products or facilitate specific reactions that impede the progression of tumors. Furthermore, engineered bacteria can function as carriers for the targeted delivery of toxins, immunostimulants, or other therapeutic substances (11).

Intratumoral bacteria have been implicated in altering tumor cell responsiveness to chemotherapy. Specific bacterial enzymes have been noted to mediate the metabolic conversion of gemcitabine into an inactive metabolite. The colonization of pancreatic tumors by *Gammaproteobacteria* has been correlated with their ability to degrade gemcitabine, which subsequently contributes to an enhanced chemoresistance of the tumor (94). In colon cancer, intratumoral *Gammaproteobacteria* facilitated resistance to gemcitabine through the synthesis of bacterial cytidine deaminase (CDDL) enzyme and was subsequently eradicated through the concurrent administration of ciprofloxacin (92). Analysis of taxonomic distributions revealed higher levels of *Gammaproteobacteria* in cholangiocarcinoma tumor tissues resistant to low-dose gemcitabine, low-dose cisplatin, and high-dose gemcitabine, while the abundance of Actinobacteria was lower in low-dose gemcitabine and high-dose gemcitabine resistant groups (95). The intratumoral presence of CDDL-expressing bacteria facilitates the metabolism of gemcitabine into 2'2-difluorodeoxyuridine (dFdU), thus preventing the inhibition of DNA replication within malignant cells. The reduction in bacterial-mediated resistance upon depletion of NupC, the transporter for bacterial nucleosides, in bacteria with active CDDL expression, indicates the involvement of NupC in the internalization of gemcitabine by the bacteria (96). Post

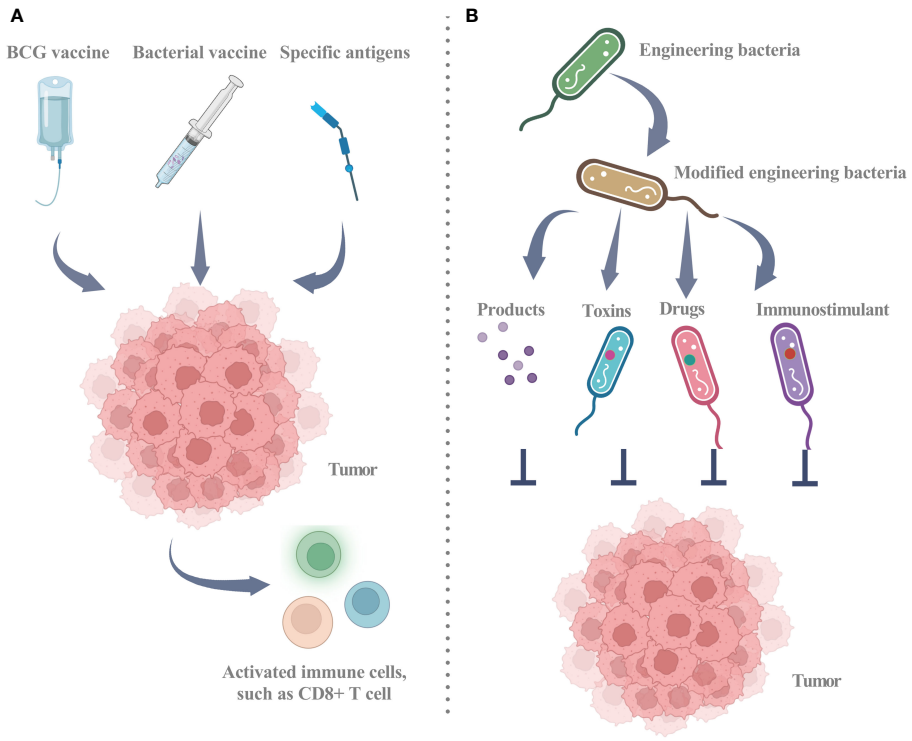

FIGURE 3
Utilizing intratumor microbiota for clinical treatment strategies. **(A)** Utilizing biological agents, such as the BCG vaccine and multiple bacterial vaccines, involves the use of either dead or living bacteria to recruit active immune cells, including CD8+ T cells, thereby triggering an anti-tumor immune response. The strategic utilization of specific antigens stands out as a pivotal mechanism to activate the immune system, fostering a heightened and robust CD8+ T cell response against cancer cells. **(B)** Engineered bacteria as a tool for tumor inhibition through the release of targeted products or reactions and as vehicles for delivering toxins, immunostimulants, or other drugs. Engineered bacteria can be programmatically designed to release targeted products or undergo specific reactions near tumor cells, encompassing toxins for direct cancer cell eradication, anti-angiogenic factors to impede vascular growth within tumors, or other agents impeding tumor progression. Engineered bacteria emerge as promising vehicles for the delivery of therapeutic agents, encompassing toxins, immunostimulants for immune response amplification against cancer, and conventional drugs. This targeted delivery system is designed to heighten treatment specificity and efficacy while mitigating potential harm to healthy tissues. BCG: Bacillus Calmette-Guérin. Graphics created with BioRender.com.

TABLE 2 Exploring therapeutic implications of intratumor microbiota.

Intratumoural microbiota	Therapy	Cancer	References
Dialister and *Prevotella*	Colorectal tumors with MSI-H show higher levels of *Dialister* and *Prevotella*, correlating with increased mutation burden and improved response to anti-PD-1 therapy	Colorectal cancer	(65)
Streptococcus	Increased *Streptococcus* in TME links to an activated tumor immune microenvironment, potentially boosting neoadjuvant chemotherapy with immune checkpoint inhibitor efficacy	Esophageal squamous cell carcinoma	(64)
Bifidobacterium	Accumulation in the tumor microenvironment empowers *Bifidobacterium* to boost local anti-CD47 immunotherapy	Digestive tract cancer	(60)
Fusobacterium nucleatum	The chemotherapeutic 5-fluorouracil serves as a potent inhibitor of *Fusobacterium nucleatum* colorectal cancer isolates	Colorectal cancer	(88)
Acinetobacter jungii	The positive correlation observed between *Acinetobacter jungii* presence and PD-L1 expression	Non-small cell lung cancer	(89)
Haemophilus parainfluenzae	In stage IV patients, the response to targeted therapy or chemotherapy showed a negative correlation with the presence of *Haemophilus parainfluenzae*	Non-small cell lung cancer	(89)
Collinsella, *Alistipes*, *Christensenella*, *Faecalibacterium*, *Ruminococcus*, *Pavimonas*, and *Akkermansia*	*Collinsella*, *Alistipes*, *Christensenella*, *Faecalibacterium*, *Ruminococcus*, *Pavimonas*, and *Akkermansia* showed significant associations with responses to neoadjuvant chemoradiotherapy	Rectal cancer	(90)
Pseudomonas, *Serratia*, and *Streptococcus*	Patients showcasing elevated mitotane levels were notably associated with adrenocortical carcinoma featuring a substantial prevalence of *Pseudomonas* and *Serratia*, or a diminished presence of *Streptococcus*	Adrenocortical cancer	(91)
Gammaproteobacteria	Gemcitabine resistance is linked to intratumoral *Gammaproteobacteria* expressing the bacterial enzyme cytidine deaminase	Pancreatic ductal adenocarcinoma	(92)

MSI-H, High-level microsatellite instability.

neoadjuvant chemotherapy, a substantial augmentation of *Pseudomonas* within breast tumors was witnessed. Moreover, breast malignancies in individuals experiencing distant metastatic spread demonstrated an elevated prevalence of *Staphylococcus* and *Brevundimonas* (97). Variations in intratumoral microbiota signatures distinguish responders from non-responders to neoadjuvant chemoimmunotherapy (NACI) in patients with esophageal squamous cell carcinoma. Responders displayed heightened levels of tumor-resident *Streptococcus*, establishing a positive correlation with the increased infiltration of CD8+ T cells and GrzB+ T cells. Fecal microbial transplantation (FMT) from NACI responders restructured the intratumoral microbiota composition, resulting in *Streptococcus* enrichment in tumor tissues, increased infiltration of CD8+ T cells, and the promotion of positive results with anti-PD-1 therapy (64).

Intratumoral microbiota may exert both immunostimulatory and immunosuppressive effects on anti-tumor immunity, with the potential to promote the advancement of cancer by inducing processes such as heightened production of ROS, fostering an anti-inflammatory milieu, impairing T cell function, and instigating immunosuppressive responses (3). To elucidate the correlation between a specific intratumor microbial signature and the response to immunotherapy, a comparative analysis of metastatic melanomas was carried out. Examination of distinct microbial taxa profiles in patients, including immune checkpoint inhibitor responders (n=29) and non-responders (n=48), unveiled noteworthy distinctions. There were 18 high-abundance taxa and 28 low-abundance taxa among responders compared with non-responders. Notably, responders showed an increased abundance of *Clostridium*, whereas non-responders exhibited a higher *Gardnerella vaginalis* (14). The attenuated vaccine BCG, originating from *Mycobacterium bovis*, has been implemented in clinical therapies for bladder cancer (98). The efficacy of traditional cancer treatments, including radiation and chemotherapy, is diminished in areas with low oxygen levels. *Clostridium novyi-NT* can thrive in this oxygen-deprived environment, facilitating the destruction of hypoxic and necrotic regions within tumors. *Clostridium novyi-NT* bacteria can replicate and selectively target cancer cells. The production of toxins by these bacteria can inflict damage upon tumor cells and incite an immune response leading to the eradication of the tumor (99). In the phase I trial (NCT01924689) involving 24 individuals with solid neoplasms, the intratumoral administration of *Clostridium novyi-NT* initiated the activation of bacterial spores, leading to a 42% incidence of tumor mass breakdown. Among the evaluated cohort of 22 individuals, 41% exhibited a decline in injected tumor dimensions, and 86% showed a stable disease (100). *Bifidobacterium* fosters the effectiveness of anti-CD47 immunotherapy through its accumulation within the TME, mediated by interferon-dependent mechanisms and the activation of the Stimulator of interferon genes (STING) pathway (60). Following bacterial ablation, the pancreatic ductal adenocarcinoma TME underwent immunogenic reprogramming, characterized by diminished MDSCs and heightened M1 macrophage differentiation, facilitating the Th1 polarization in CD4+ T cells and stimulating the induction of CD8+ T-cell. Augmented PD-1 levels following bacterial ablation

were associated with improved efficacy of immunotherapy. An abundant and distinct microbiome triggers the differentiation of suppressive monocytic cells in pancreatic cancer by selectively activating Toll-like receptors (TLRs), ultimately resulting in T-cell anergy (61).

Disruptions in the microbiota contribute to the accumulation of toxic metabolites and the persistence of inflammatory reactions, thus fostering cancer development and the evolution of treatment resistance (2). Remodeling intratumoral microbiota has emerged as a promising avenue for potential therapeutic strategies. Probiotics, antibiotics, and fecal microbiota transplantation are the prevailing techniques utilized for systemic microbiota, offering a feasible avenue for their application in targeting the intratumoral microbiota associated with cancer (31, 101).

5 Conclusions

Amidst the burgeoning interest in unraveling the relationship between gut microbiota and tumors, attention is now directed toward probing the effects of intratumoral microbiota on tumorigenesis and its implications for cancer treatment. Advances in techniques for analyzing the gut and tumor microbiome have enhanced the understanding of the microbiome's impact on human health. Nevertheless, the exploration of intratumoral bacteria is still in its preliminary phase. Recent findings demonstrate the widespread occurrence of intratumor microbiota in various tumor types. The complexity and ambiguity of the host-intratumoral microbiota interplay necessitate future studies to improve the understanding of the intratumor microbiota in carcinogenesis. Intratumoral microbiota exerts immunomodulatory effects within the TME, influencing tumor outcomes by promoting inflammatory responses or regulating anti-tumor activity. Intratumoral microbiota exerts a significant influence on therapeutic effectiveness, offering novel avenues for cancer therapy, diagnostic and prognostic assessment, and potential therapeutic targets (Figure 4). In particular, the complex interactions among intratumoral microbiota, antitumor immunity, and therapeutic efficacy in tumors require further investigation. Comprehensive profiling of distinct intratumor microbiota holds promise for manipulating these bacterial communities to advance

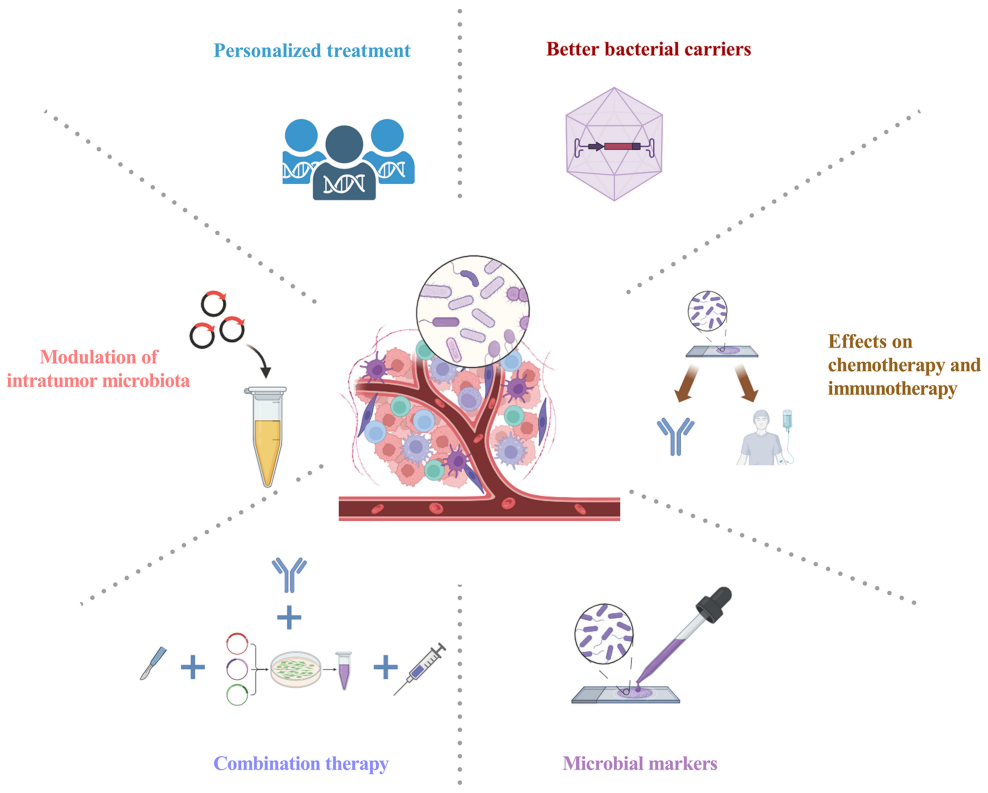

FIGURE 4
Intratumoral microbiota: prospects for clinical application. Personalized treatment: Integrating advanced sequencing techniques allows for the comprehensive analysis of intratumoral microbiota, shedding light on microbial-derived antigens and paving the way for personalized treatment modalities; Modulation of intratumor microbiota: Leveraging probiotics, antibiotics, and targeted interventions stands as a promising strategy for intratumoral microbiota modulation, aiming to reinstate a harmonized microbial community; Combination therapy: Combining antibiotics or bacterial therapies with other anti-tumor treatments, such as chemotherapy or immunotherapy, seeks to optimize cancer therapy by targeting both tumor cells and the intratumor microbiota; Better bacterial carriers: This innovative strategy maximizes specific bacterial attributes, utilizing advanced carriers for precise tumor therapeutics with strong targeting, lower infection risk, and superior payload efficiency; Microbial markers: Utilizing intratumoral microbiota for early cancer diagnosis, prognosis, and monitoring; Effects on chemotherapy and immunotherapy: Impact of intratumoral microbiota on chemotherapy and immunotherapy, evaluating efficacy, tolerability, and toxicity. Graphics created with BioRender.com.

cancer treatment. Further research into the molecular mechanisms of intratumoral microbiota is also necessary. Targeting the intratumoral microbiota presents opportunities for potential universal therapies and synergistic combination approaches with approved chemotherapeutics and immunotherapies. Considering the significant impact of microbial metabolites, integrating microbiome and metabolome profiles may emerge as a pivotal approach for personalized therapies. Undoubtedly, the significance of intratumoral microbiota within tumor biology is poised to assume a pivotal role in forthcoming decades of carcinogenesis investigations.

Author contributions

JW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. PZ: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Writing – original draft. WM: Conceptualization, Supervision, Validation, Writing – review & editing. CZ: Funding acquisition, Supervision, Writing – review & editing, Writing – original draft.

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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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EDITED BY
Nathella Pavan Kumar,
National Institute of Research in Tuberculosis (ICMR), India

REVIEWED BY
Abdullah Saeed,
City of Hope National Medical Center, United States
Farman Ullah Dawar,
Kohat University of Science and Technology, Pakistan
Muhammad Shahid Riaz Rajoka,
University of Alabama at Birmingham, United States
Muhammad Mohsin,
Fujian Agriculture and Forestry University, China

*CORRESPONDENCE
Guixue Wang
wanggx@cqu.edu.cn

†These authors have contributed equally to this work

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Role of the intestinal microbiome and its therapeutic intervention in cardiovascular disorder

Ameer Luqman[1,2†], Adil Hassan[1,2,3†], Mehtab Ullah[1], Sahar Naseem[1], Mehraj Ullah[4], Liyuan Zhang[2], Ahmad Ud Din[5], Kamran Ullah[6], Waqar Ahmad[7] and Guixue Wang[1,2*]

[1]Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing, China, [2]JinFeng Laboratories, Chongqing, China, [3]Chongqing Key Laboratory of Nano/Micro Composite Materials and Devices, Chongqing University of Science and Technology, Chongqing, China, [4]School of Fermentation Engineering Tianjin University of Science and Technology, Tianjin, China, [5]Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Kannapolis, NC, United States, [6]Department of Biology, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan, [7]Basic Medicine Research Innovation Center for Cardiometabolic Diseases, Ministry of Education, Southwest Medical University, Luzhou, China

The gut microbiome is a heterogeneous population of microbes comprising viruses, bacteria, fungi, and protozoa. Such a microbiome is essential for sustaining host equilibrium, and its impact on human health can be altered by a variety of factors such as external variables, social behavior, age, nutrition, and genetics. Gut microbes' imbalances are related to a variety of chronic diseases including cancer, obesity, and digestive disorders. Globally, recent findings show that intestinal microbes have a significant role in the formation of cardiovascular disease (CVD), which is still the primary cause of fatalities. Atherosclerosis, hypertension, diabetes, inflammation, and some inherited variables are all cardiovascular risk variables. However, studies found correlations between metabolism, intestinal flora, and dietary intake. Variations in the diversity of gut microbes and changes in their activity are thought to influence CVD etiology. Furthermore, the gut microbiota acts as an endocrine organ, producing bioactive metabolites such as TMA (trimethylamine)/TMAO (trimethylamine N-oxide), SCFA (short-chain fatty acids), and bile acids, which have a substantial impact on host wellness and disease by multiple mechanisms. The purpose of this overview is to compile current evidence highlighting the intricate links between gut microbiota, metabolites, and the development of CVD. It focuses on how intestinal dysbiosis promotes CVD risk factors such as heart failure, hypertension, and atherosclerosis. This review explores the normal physiology of intestinal microbes and potential techniques for targeting gut bacteria for CVD treatment using various microbial metabolites. It also examines the significance of gut bacteria in disease treatment, including supplements, prebiotics, probiotics, antibiotic therapies, and fecal transplantation, which is an innovative approach to the management of CVD. As a result, gut bacteria and metabolic pathways become increasingly attractive as potential targets for CVD intervention.

KEYWORDS

CVD, HF, HTN, TMAO, SCFAs, FMT

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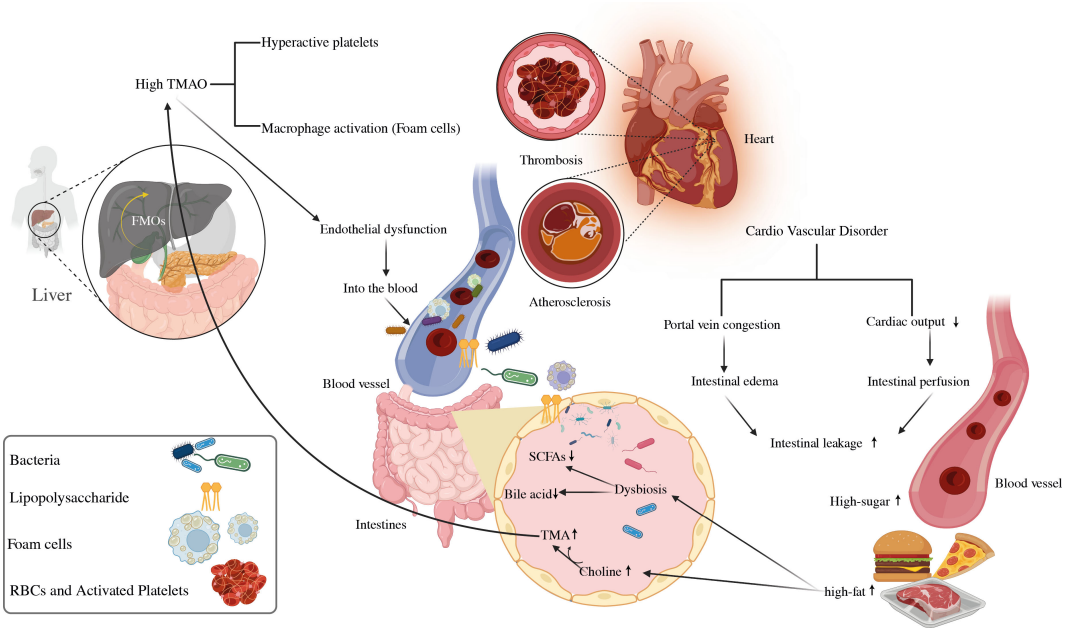

GRAPHICAL ABSTRACT
The significance of microbial-mediated metabolites in the emergence of CVD. CVD, cardiovascular disease; SCFAs, short-chain fatty acids; TMA, trimethylamine; TMAO, trimethylamine N-oxide; LPS, lipopolysaccharides.

Introduction

Understanding the evolution of the gut microbiota and its internal and external impacts on the intestine, as well as the risk factors for cardiovascular diseases (CVDs), such as metabolic syndrome, has attracted a great deal of attention (1, 2). Globally, CVDs are the primary causes of death, encompassing conditions including coronary artery disease (CAD), atherosclerosis, thrombosis, aneurysms, arterial hypertension, and cardiomyopathies that reinforce heart failure and cerebrovascular diseases (3–5). The current studies predict 17.5 million deaths per year by CVD, accounting for around 31% of all overall mortality (6). Among them, heart attack and stroke are directly linked to 85% of the cases (7, 8). Inflammation, dyslipidemia (i.e., elevated serum cholesterol, triglycerides, and low-density lipoproteins), and diabetes mellitus are prevalent pathological mechanisms and risk factors that can impact the progression and emergence of CVD (9, 10). In addition to hereditary factors, environmental factors such as nutrition and gut microbiota composition are going to play a significant influence in the development of CVDs. Furthermore, the rise of obesity and diabetes has been related to intestinal dysbiosis (11, 12), insulin resistance, and sedentary behaviors such as smoking, insufficient exercise, and poor nutrition are all identified risk variables for CVD (13, 14). The research into how the human gut microbiome affects CVD and metabolic diseases has expanded dramatically (15). Gut dysbiosis is a condition defined by changes in intestinal bacteria in adults, can be induced by a range of events such as dietary choices, environmental effects, intestinal infections, or external variables, and it can result in inflammation and metabolic disorders (16). The human gut microbiome comprises an array of over 10 trillion diverse microbes, encompassing bacteria, viruses, protozoa, methanogen archaea, and fungi. This collective term, microbiota, is synonymous with the entirety of these microbial inhabitants residing within the human body (17), while *Actinobacteria, Firmicutes, Proteobacteria*, and *Bacteroides* are the four major bacterial genera that comprise a healthy microbiota (18, 19). Before birth, an infant's gut has very few germs (20), but after birth, the body begins to receive a steady stream of stimulation from the environment. It promotes a gradual rise in the number of bacteria in the colon, eventually leading to the formation of a dynamic and balanced balance in the gut microbiota (21).

The intestinal mucosal surface serves as the interface between the gut microbiota and performs a number of tasks that keep the intestinal epithelial barrier functioning (22). Endotoxins, microbes, and their byproducts can move more easily through the gut wall and into the bloodstream, where they can cause autoimmune disorders. Immune dysregulation and inflammation are at the basis of many CVDs, including atherosclerosis, myocardial infarction (MI), rhythm disorders, pericardial disease, cardiomyopathies, and heart failure (23). Moreover, it is important to highlight that the intestinal tract can be seen as an extensive and diverse ecosystem that produces a significant number of microbial metabolites (24). The host food is broken down by the gut flora into a variety of metabolically active products, including trimethylamine N-Oxide (TMAO), short-chain fatty acids (SCFAs), primary and secondary bile acids, tryptophan and indole derivatives, phenylacetylglutamine (PAGln), and branched-chain amino acids (BCAA), these may contribute to the CVD progression (25). Moreover, N-oxide TMA/TMAO and bile acids are fascinating biomarkers for CVD progression (26), however other gut microbiota components or similar chemicals should be

investigated for use as early CVD markers (11). The loss of SCFA-producing intestinal microbes would disrupt the equilibrium of glucose metabolism, raising the risk of CVD (27). Trimethylamine stimulates macrophage stimulation, producing vascular damage; excessive TMAO levels owing to intestinal dysbiosis promote atherosclerosis, raise the risk of CAD, and hasten arterial plaque development that leads to cardiovascular disease (6). As shown in Figure 1, reducing dietary TMAO precursor intake is a promising strategy for lowering the risk of CVD due to the high amounts of trimethylamine (TMA) and TMAO generation by choline-induced gut flora (28, 29). Microbial sequencing analysis has emerged as a valuable tool for uncovering distinct gut microbiota patterns linked to cardiovascular disease CVD (30–32).

The gut microbiota plays a crucial role in influencing overall health, either through direct mechanisms or indirect pathways. The intricate interactions involving variations in microbiome composition, metabolites, and CVD susceptibility underscore the significance of intestinal microbes as a novel modulator of CVD. The identified association between gut microbes and CVD suggests that modifying the intestinal microbiota could be beneficial in preventing and managing the development of CVD. Nutritional therapy, the use of pre/probiotics and antibiotics, fecal microbiota transplantation, TMAO reduction, and regular exercise are all current ways to manage gut bacteria to improve cardiovascular function (11). The latest study highlights the possible importance of microbial imbalance in CVD disorders. The advent of genomic and metabolomic technologies has allowed for more thorough characterization and molecular research of these microbiota and their metabolites. However, most evidence continues to indicate associations and the particular chemical processes driving a majority of visible events remain unidentified (33). Future studies focusing on microbe-microbe and microbe-host interactions could reveal how specific metabolites influence the disease process. It is also critical to have a better understanding of the bacterial mechanisms involved in the production of CVD-related metabolites, and also their functional roles. These results could provide a solid theoretical basis for the invention of therapeutic methods for CVD individuals. The present paper covers the usual composition and functional significance of intestinal bacteria and also provides new insights into the gut microbiota and its linked metabolites, which are implicated in CVDs. Scientific studies, putative biological explanations, and therapeutic outcomes are of significant interest to researchers. In addition, we discuss studies relating the gut microbiota to inflammatory processes, lipid metabolic disorders, and diabetes, all of which are linked to an elevated risk of cardiovascular disease. As a result, this overview focuses primarily on studying the role of gut microbiota-related metabolites and their therapeutic potential in CVDs, which may eventually provide more insight into the development of CVD prevention.

Gut microbiota and TMAO metabolite

The intestinal *Bacteroidetes* are one of the most significant bacterial colonies in the gastrointestinal microbiota. Despite their wide species composition, these cultures display stability in many gut regions, and some exhibit location-specific differentiation, mainly in the ascending colon (34). A total of around 1,000 different species of intestinal microbes, comprising about 10^{14}, and bacterial-to-human cell ratio varied between 10:1 and 1:1 (35). In cardiovascular patients, more than 90% of these bacteria had an impact on the growth of *Bacteroidetes* and *Firmicutes*, keeping a stable *Firmicutes/Bacteroidetes* (F/B) ratio (36). The emergence of CVD is dependent on a compromised mucosal barrier and decreased intestinal mucosal barrier function, and is mostly caused by gram-negative microbes, such as lipopolysaccharide (LPS), which plays a significant role in the emergence of cardiometabolic diseases (37, 38). A high-fat diet has been shown to reduce gram-positive *Bifidobacteria* levels in the digestive system while increasing the amount of intestinal microbes that hold LPS, both of which contribute to obesity, the primary risk factor for CVD (39). The F/B ratio become a crucial role in the context of obesity, particularly in children (40). This ratio is linked to low-grade inflammation, which increases the probability of diabetes, a known risk factor for CVDs (41).

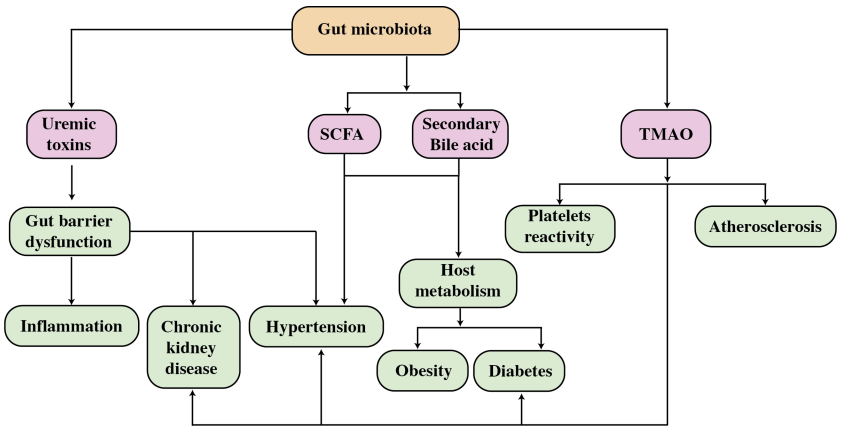

FIGURE 1
A diagram depicting the effect of gut bacteria and metabolites on CVD risk factors. SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide.

Because the gut acts as a bridge between them, the interaction between the host and the gut microbiota is crucial for preserving intestinal integrity. Several microbial metabolites have been linked to CVD (25) including bile acids, SCFAs, branched-chain amino acids, TMAO, tryptophan, and indole derivatives (42). The TMAO is formed when foods rich in choline, lecithin, and L-carnitine, primarily found in animal products, with limited plant-based sources, are ingested. In the gut, lecithin (comprising phosphatidylcholine, a choline source) and dietary choline are metabolized into TMA by the gut microbiota having specialized enzymes TMA lyses transcribed by cutC/cutD genes found in various bacterial strains. Recent data suggests that elevated circulating levels of TMAO are associated with an increased risk of CVD and mortality (43–46). Increased TMAO levels in the bloodstream encourage lipid accumulation in the arteries, which contributes to atherosclerosis. Figure 2 depicts how the inflammatory response influences the development of glucose intolerance, diabetes, and CVD (47–50). A dysbiotic microbe was found to decrease the amount of cholesterol eliminated by feces while increasing absorption and plasma levels of low-density lipoproteins, signaling that dysbiosis may increase the risk of atherosclerosis and CVD (39).

Gut microbiota composition, diversity, and risk factors

The bacterial composition, diversity, and abundance are highly influenced by genetic changes in the host's genome, and by external variables such as the host's lifestyle, diet, sanitation, health, and the use of antibiotics and probiotics (51). The gut microbiota has small genetic differences in various parts of the intestine. Eckburg et al., (24) used metagenomic analysis to discover the gut bacterial community is made up of six phyla: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria*, and *Verrucomicrobia*, with the majority of the organisms in a healthy bacterial community being anaerobic population, as shown in Figure 3 (52). They inhabit unique biological niches on mucosal surfaces and in the gut lumen, where they form sophisticated biochemical interaction networks with both their hosts and with others (53). The synergistic interaction between the host species and the gut microbiome fosters the proliferation of beneficial microbes while inhibiting the growth of harmful bacteria (54). The gut flora regulates many bodily functions, such as providing metabolic fuel to the host, supporting growth and immune system regulation, removing harmful microbes, keeping intestinal wall integrity, and maintaining

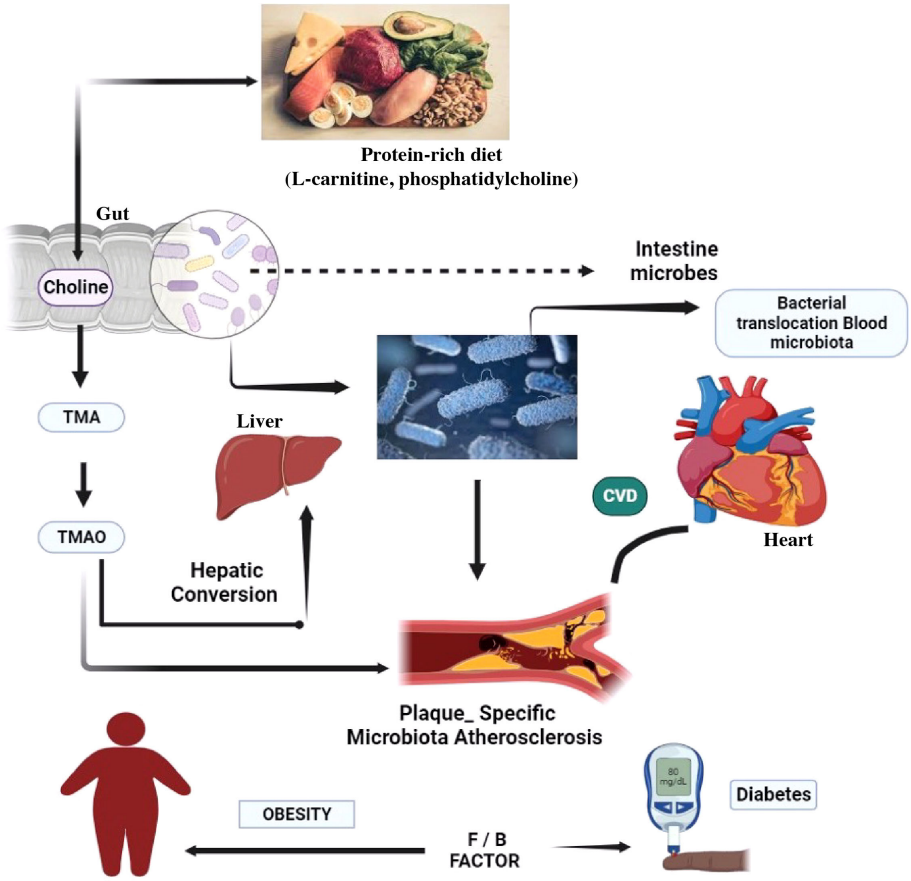

FIGURE 2
The gut microbiota of the target body's functioning mechanisms. A low-fiber diet corresponds with decreased short-chain fatty acid butyrate formation, exacerbating dysbiosis and sustaining local and systemic inflammation via bacterial toxin leaks, most notably LPS. A modern Western diet strong in red meat promotes the synthesis of TMA by bacteria, which is then oxidized in the liver to the pro-atherosclerotic metabolite. CVD, cardiovascular disease; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

overall homeostasis (55). Microbial life has a significant impact on immune function and metabolism, with well-balanced gut microbiota playing an important role in the health of the host. (56). Inadequate dietary intake, excessive stress, significant life events, and antibiotic administration can all impact the diversity of the gut microbiota, leading to a disorder called dysbiosis (20). Elie Metchnikoff, a Russian immunologist and microbiologist renowned for his contributions to the understanding of the immune system, particularly the concept of phagocytosis, is not credited with coining the term "dysbiosis" (57, 58). An imbalance in the typical microbial composition (microbiota) of the colon or other bodily regions is described by this term, which is used in the current discipline of microbiology (59). In particular, Metchnikoff's study has little work with the current concept of dysbiosis, which emerged as knowledge of the function of the human microbiome in health has increased (60). As seen in Figure 3, several risk factors were put out as potential causes of intestinal dysbiosis. There is a lot of literature known about the use of antibiotics, which has been seen to alter the composition of the gut's microbiome and have both short-term and long-term effects (61–64). Obesity and high-fat and sugar meals are all related to persistent variations in the gut microbiota (65–68).

It is believed that external factors at various stages of life influence the formation of gut dysbiosis. The style of delivery, type of feeding, and hospital milieu are all related to the diversity of the bacteria during childhood (69, 70). Further, social stresses and exposure to xenobiotics including pesticides and heavy metals have been related to gut dysbiosis (71, 72). The emergence of a gut microbiome has a genetic basis as opposed to social factors, based on twin studies. Given that identical twins have nearly identical DNA, any changes in their gut microbes must be the result of non-genetic variables like food, medical history, or use of antibiotics (73,

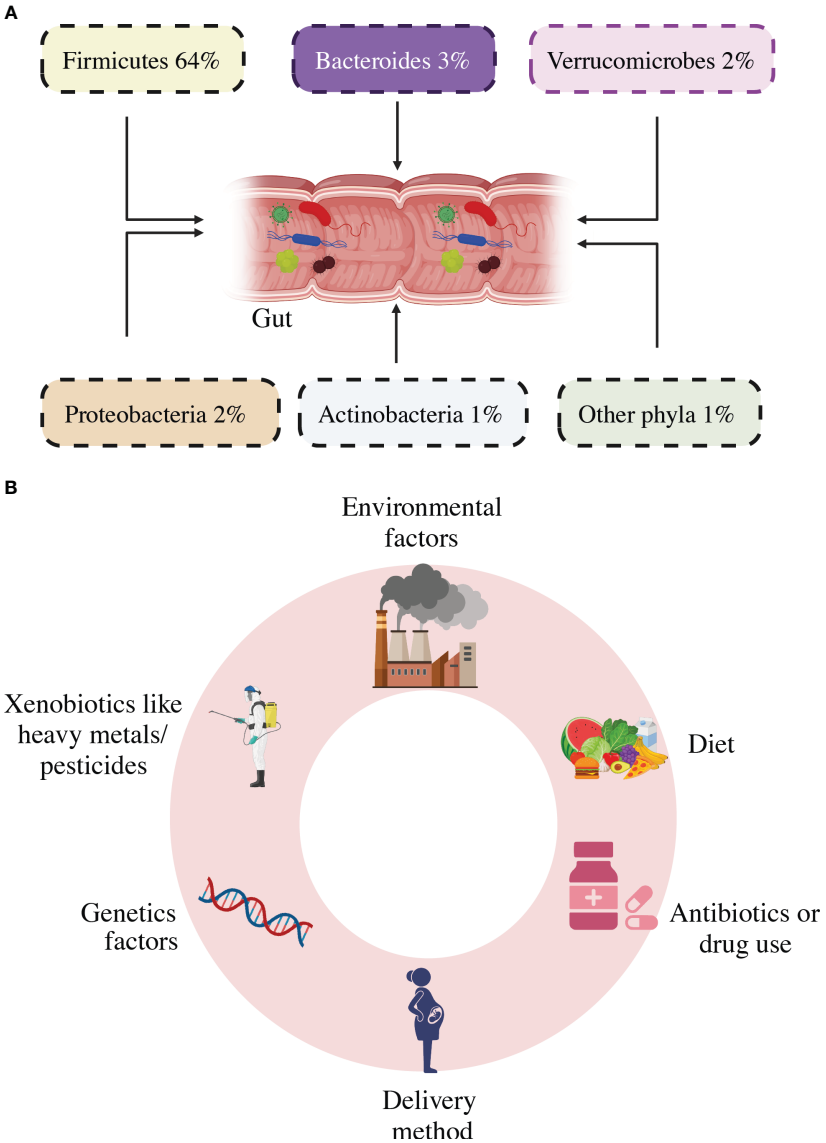

FIGURE 3
Gut microbiota composition **(A)** and diversity and Dysbiosis risk factors **(B)**.

74). The study of bacterial genomes has transformed the field of microbial research. Metagenomic sequencing and 16S rDNA sequencing are two types of sequencing that are frequently utilized to assess the abundance of microbial components. By focusing on the conserved sections that surround the hypervariable regions, the 16S sequencing approach can detect variations in bacterial genomes (75, 76).

Impact of gut microbiota on cardiovascular disease

A broad spectrum of diseases is considered in CVD, including atherosclerosis, aortic valve disease, peripheral artery disease, hypertension, and stroke. Heart failure, hypertension, and atherosclerosis are associated with gut dysbiosis as shown in Table 1. In recent years, significant progress has been made in understanding how the gut microbiome impacts cardiovascular function and the development of these diseases. In this specific section, we intend to highlight several well-supported studies that deliver compelling evidence regarding the role of gut microbiota in the development of cardiovascular disease as depicted in Figure 4 (92).

Gut bacteria; heart failure (HF) with microbial metabolites and current treatments

The disease termed heart failure is defined by the heart's decreased ability to efficiently pump enough blood and oxygen to satisfy the demands of the body (93, 94). It serves as the final stage of multiple CVDs, which are highly prevalent, have significant mortality rates, and pose a significant threat to human well-being (95). While chronic exposure is defined by an altered inflammatory state related to pro-inflammatory aspects that are critical to the beginning of HF, immediate exposure is associated with a variety of inflammation-related symptoms (96). Our understanding of the pathophysiological processes behind HF has greatly increased. The recognition of the vital role of managing neurohumoral processes rather than focusing solely on changes in blood flow is a key shift in this understanding (97, 98). More evidence indicates the stomach is implicated in decreased heart rate and higher systemic congestion, both of which can contribute to intestinal mucosal ischemia and edema. As a result, bacterial translocation may be enhanced, allowing endotoxins into the bloodstream and contributing to the inflammation seen in HF patients (99). Niebauer et al. (100)

TABLE 1 Modifications in the diversity of the intestinal microbes attributed to CVD. CVD-related changes in the diversity of the gut's microbes.

Species	Technique	Modifications in gut microbial diversity attributed to diseases		References
		Decrease	Increase	
Atherosclerosis and coronary artery disease				
Human	Metagenomics sequencing	*Bactericides* and *Prevotella*	*Streptococcus* and *Escherichia*	(77, 78)
Human	Terminal restriction fragment length polymorphism	*Bactericides* and *Prevotella*	Order *Lactobacillales*	(79)
Human	Metagenomics sequencing	*Roseburia* and *Eubacterium*	*Collinsella*	(31, 33)
Human	16S sequencing	*Clostridium, Faecalibacterium*	*Prevotella*	(80)
Human	16S sequencing	*Burkholderia, Corynebacterium* and *Sediminibacterium, Comamonadaceae, Oxalobacteraceae, Rhodospirillaceae, Bradyrhizobiaceae* and *Burkholderiaceae*	*Curvibacter, Burkholderiales, Propionibacterium, Ralstonia*	(33, 81)
Hypertension				
Human	Metagenomic sequencing		*Prevotella* and *Klebsiella*	(82)
Human	Metagenomic sequencing	*Roseburia* spp., *Faecalibacterium prausnitzii*,	*Klebsiella* spp., *Streptococcus* spp., and *Parabacteroides merdae*	(83)
Human	16S sequencing	Butyrate-producing bacteria *Odoribacter*		(84, 85)
Heart failure				
Human	16S sequencing	*Blautia, Collinsella, uncl. Erysipelotrichaceae* and *uncl. Ruminococcaceae*		(86, 87)
Human	Incubation with a selective agar		*Campylobacter, Shigella, Salmonella, Yersinia Enterocolitica,*	(88)

(Continued)

TABLE 1 Continued

Species	Technique	Modifications in gut microbial diversity attributed to diseases		References
		Decrease	Increase	
Human	16S sequencing	*Faecalibacterium*	*Lactobacillus*	(89)
Human	Metagenomic sequencing	*Faecalibacterium prausnitzii*	*Ruminococcusgnavus*	(90)
Atrial fibrillation				
Human	Metagenomic sequencing	*Faecalibacterium, Alistipes, Oscillibacter*, and *Bilophila*	*Ruminococcus, Streptococcus*, and *Enterococcus*,	(91)

conducted a study that revealed a significant association between peripheral edema in HF patients and higher plasma levels of endotoxins and inflammatory cytokines. Specifically, patients experiencing peripheral edema demonstrated elevated concentrations of these markers compared to those without edema. However, the study found a decrease in serum amounts of endotoxins but not cytokines following short-term diuretic treatment. This finding raises the prospect that edema and gut-associated inflammation in cardiac failure may be linked while diuretic medication might be effective (100). Pasini et al. (88) showed a recent comparison of the bacterial and fungal profiles in the feces of heart failure patients and the findings revealed people with chronic heart failure (CHF) had a greater risk of perilous bacterial growth compared with control group.

The presence of *Candida*, *Campylobacter*, and *Shigella* species was linked to 78.3% of CHF disease severity that had significantly greater intestinal permeability. Between TMAO and the risk of atherosclerosis, there was a strong positive correlation between gut permeability and right atrial pressure. An increased TMAO levels have been linked to poor outcomes in people with heart failure (101, 102). Scientists evaluated the levels of TMAO in 2,490 patients with chronic heart disease in the study with a 9.7-year follow-up. The data showed increasing TMAO levels coincided with increased rates of morbidity and mortality, particularly in HFrEF patients. This study suggests that TMAO could be utilized as a biomarker to predict poor outcomes in HfrEF patients (103). A recent meta-analysis offered that some reliable insights into the prognostic importance of TMAO in HF (102). The higher TMAO precursor trimethyl lysine (TML)-derived N, N, N-trimethyl-5-aminovaleric acid (TMAVA) synthesis by the gut microbiota was linked to a progressive reduction in fatty acid oxidation (104). Several studies have consistently reported that patients with HF exhibited a decrease in butyrate-producing bacteria, particularly within the *Lachnospiraceae*and *Ruminococcaceae* families (105). However, the absence of butyrate-producing microbes such *Eubacterium Halli* and *Lachnospiracea* is associated with higher mortality, increased inflammation, and severity of disease. This association implies that the abundance of these beneficial gut bacteria may have a major impact on the progress and results of cardiac failure (106). Dysbiosis has been consistently linked to reduced butyrate production in various heart failure cohorts (105). Additionally, bile acids, particularly secondary bile acids produced through the transformation by gut microbiota, play a crucial role in heart failure. Research has indicated a rise in secondary bile acids among individuals with CHF(97). Indoxyl sulfate produced by gut microbial metabolism, has also been linked to cardiac fibrosis and ventricular remodeling. These findings underscore the importance of the gut microbiota and its metabolites in heart health and offer possible therapeutic targets for heart failure management (107).

Atherosclerosis and therapeutic options

Atherosclerotic cardiovascular disease is a persistent inflammatory state primarily impacting sizeable and intermediate arteries. Numerous firmly established factors are correlated with atherosclerosis, including hypertension, dyslipidemia, advanced age, and smoking (108). This is characterized by the accumulation of low-density lipoprotein within the artery walls, leading to the formation of atheroma and distinct plaques consisting of proliferative fibrous tissue and calcifications (109, 110). Recent decades, there has been a growing interest in finding out how the gut microbiota plays a significant role in the emergence of atherosclerotic lesions (111). Koren et al. did an analysis using shotgun DNA sequencing focused on the gut metagenome that indicated significant changes in the diversity of gut microbial populations between patients with symptomatic atherosclerosis and those assumed to be healthy controls. These findings strongly suggest that the gut microbiota may play a significant role in the atherosclerosis (30, 31). Further, extensive metagenome-wide association research done on a cohort of 218 atherosclerosis patients and 187 healthy controls confirmed a link between the diseases with changed gut microbiome composition. In particular, the study found that people with atherosclerosis had significantly higher concentrations of *Enterobacteriaceae, Ruminococcusgnavus*, and *Eggerthellalenta* (112, 113). Introducing prebiotics and probiotic strains which enhance the production of SCFA and boost the diversity of beneficial microbes might be a valuable strategy in atherosclerosis prevention strategies (114). Various animal studies, including the work by Chan et al., (115) have explored the impact of probiotics and telmisartan on mitigating atherosclerosis induced by a high-fat diet, resulted in an increase in the *Firmicutes* to *Bacteroidetes* ratio. A diet rich in fats was found to decrease the prevalence of *Eubacterium, Anaeroplasma, Oscillospira, Roseburia*, and *Dehalobacterium*, while simultaneously elevating the quantities of *Allobaculum,*

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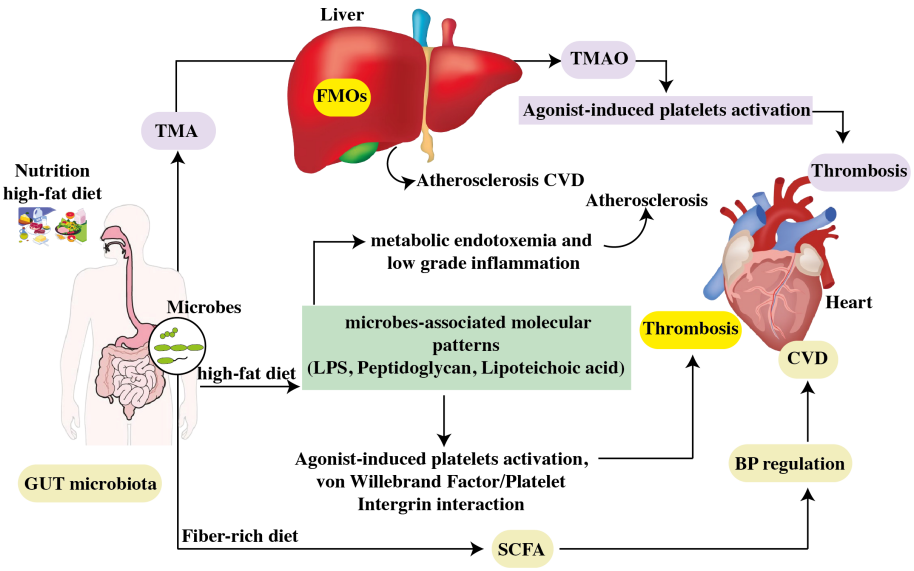

FIGURE 4
The contribution of the gastrointestinal microbiome to CVD. Choline, phosphatidylcholine, and carnitine are all available in high-cholesterol, high-fat diets. Intestinal microbes convert phosphatidylcholine in the diet to choline, which is then turned into trimethylamine. Hepatic flavin monooxygenases convert TMA to TMAO in the liver. By increasing atherosclerosis and generating agonist-induced platelet activation, TMAO promotes thrombosis. High levels of TMAO in the blood are linked to an increased risk of CVD. Furthermore, a high-fat diet raises the levels of microbe-associated molecular patterns like LPS. Increased intestinal uptake of microbe-associated molecular patterns results in metabolic endotoxemia and low-grade inflammation, both of which worsen atherosclerosis. TLR2 induces arterial thrombosis by increasing the interaction between von Willebrand factor and platelet integrin. Furthermore, intestinal bacteria convert carbs to SCFA and produced by gut microbial fermentation regulate blood pressure, a risk factor for CVD progression. FMO: flavin monooxygenases, SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide; TLR2, toll-like receptor-2; LPS, lipopolysaccharide.

Clostridium, *Lactobacillus*, and *Bifidobacteria* (116). The findings show *B. fragilis* can produce extracellular vesicles (EVs), which are lipid bilayer particles. Human research has revealed a relationship between infectious and non-infectious disorders, as well as changes in the systemic levels of EVs derived from gut bacteria (117). It was identified as an essential cell-cell communicator with the potential to increase the knowledge of atherosclerotic disease, ranging from biomarkers to disease pathogenesis (118). Proteomic study has revealed unique protein compositions for EV subtypes, with some indicators assisting in the differentiation of EVs via biogenesis processes. Endosomal sorting complexes required for transport (ESCRT) proteins, Alix, and tetraspanins, for example, are exosome markers, whereas ectosome markers include Annexin A2/A5, ARF6, and Enolase 1 (119). EVs are involved in the immunological response, vascular remodeling, endothelial dysfunction, and apoptosis, which all contribute to atherosclerosis, and EVs in plasma may be useful as atherosclerosis indicators (120).

Scientists used the terminal restriction fragment length polymorphism technique for insight into the gut bacteria contents with coronary artery patients. Their research showed that the microbial diversity in the individuals studied experienced unique alterations. Notably, the levels of *Bacteroides* were decreased while the abundance of *Lactobacillales* and *Clostridium subcluster XIVa* increased in the fecal samples of people. These findings suggest the gut microbiota may contribute to the progression of coronary artery disease. Novel strategies for treating or preventing cardiovascular diseases may be developed as a result of a better understanding of such microbial modification (79). A significant negative association found between the decrease in (1) *Eubacterium* and the rise of inflammatory cytokines like matrix metalloproteinase-9 (MMP-9) and E-selectin; (2) Dehalobacterium and adipocyte fatty acid binding protein (A-FABP); and (3) *Roseburia* and MMP-9. This confirms a connection between imbalanced gut microbiota and the development of atherosclerosis (115). Similarly, Stepankova showed experiments signifying the beneficial impact of gut microbiota in inhibiting the atherosclerotic lesions progression (121).The aortas of the germ-free mice fed a low-cholesterol diet showed atherosclerotic plaques. The results of the study offer strong evidence that microorganisms suppress the progress of atherosclerosis (122).

On the contrary, systems depending on metabolism can be affected by gut dysbiosis, which alters a wide variety of metabolites and may have an effect on the onset and development of atherosclerosis (6). One of the several metabolites produced by the gut bacteria, TMAO, plays a crucial role in the formation of atherosclerosis (123). The accumulation of TMAO in the body has been related to an increase in the risk of atherosclerosis and cardiovascular diseases (124). The blood plasma levels of TMAO in mice with normal gut microbiota grew as they were fed a diet high in choline. In contrast, animals given the same choline-rich diet and antibiotic treatment, which changed their gut microbiota, had minimal TMAO amount (43). Compared to the control TMAO levels, the mice with greater TMAO levels displayed a higher amount of foam cell formation and the development of atherosclerotic plaques. The risk of sudden cardiovascular events

is increased by TMAO linked to plaque vulnerability to its involvement in developing atherosclerosis (28, 47). High levels of TMAO in the bloodstream have been associated with conditions such as obesity, Type 2 diabetes mellitus, chronic kidney disease (CKD), and CVD (125, 126). Moreover, numerous other studies have shown that SCFAs may have a positive impact on atherosclerosis by suppressing inflammation (127, 128). As a result, SCFAs may reduce cholesterol levels and stop the host from developing lipid deposits. Dyslipidemia can result from decreased SCFA production, whereas probiotics (*Lactobicili*) are effective in reducing cholesterol. According to Dieck et al., (129) probiotic anti-cholesterolemic effect can be induced via bile salt hydrolysis (BSH), interference with hepatic *de novo* lipid synthesis by regulation of SCFA, or satiety hormones. It indicates that SCFAs may have a preventive effect by reducing the risk factors for cardiac disease (130).

Association of gut microbiome with hypertension (HTN)

Hypertension is a significant worldwide public health concern and stands as the foremost risk factor for cardiovascular diseases, leading to a substantial economic burden on society. Its epidemiology is defined by a high prevalence, notable levels of disability and mortality, and often insufficient awareness (131, 132). As of 2021, it was estimated that around 330 million people in China were affected by cardiovascular diseases, with approximately 245 million individuals having been diagnosed with hypertension (133). The causes of hypertension involve a combination of factors, including genetic predisposition, lifestyle choices, environmental influences, hormonal imbalances, inflammatory processes, and changes in hemodynamic mechanisms (134). The American College of Cardiology, the American Heart Association, and the European Society of Hypertension have collaboratively formulated behavioral guidelines aimed at maintaining optimal blood pressure levels, with a particular emphasis on non-pharmacological strategies (135). These include using the dietary methods to stop hypertension (DASH) diet, which highlights a high intake of fruits and vegetables while minimizing fat consumption, increasing physical activity through specific aerobic exercises, slicing off salt and alcohol consumption, losing weight, and increasing salt and alcohol consumption (136–138).

A small number of studies mostly in animal models have shown an explicit link between gut microbiota and the control of blood pressure (139–142). For instance, Yang et al. (140) conducted a study where they investigated changes in the fecal microbiota of animal models with hypertension, specifically comparing alterations in the spontaneously hypertensive rat and chronic angiotensin II infusion rat models. They observed a notable gut dysbiosis in hypertensive animals characterized by a decrease in microbial richness, diversity, and consistency (140). Kim (143) also found that among hypertension patients, the presence of butyrate-producing bacteria, such as *Butyricimonas* and anaerobic *Corynebacterium*, had significantly decreased. Studies have revealed a positive correlation between blood pressure and the levels of *Ruminococcaceae*, *Streptococcus*, and *Turicibacter* (140, 144, 145). The metabolite production derived from microbes may also be impacted by changes in gut microbiota. Since they are created by bacterial digestion of dietary fiber and are closely related to good health, SCFAs are of particular significance among these metabolites that are formed from microbes (146), play a vital role in the HTN development. A larger amount of research indicates the potential of SCFAs may effectively decrease the host's blood pressure by interacting with G protein-coupled receptor 41 (147–149). Although TMAO is required for disease start, an animal model was first used to demonstrate the relationship between TMAO and CVD in 2011 (28). Recently, Wang et al. (150) have provided compelling evidence of a causal link between TMAO and its precursors with blood pressure by employing a Mendelian Randomization approach. Moreover, multiple studies have validated the strong association between elevated TMAO levels and an increased prevalence of hypertension (151–153). Ge et al. (153) proved that a rise of 5 and 10 mol/L in TMAO levels corresponded to a 9% and 20% escalation in the risk of hypertension, respectively. Apart from TMAO, other gut microbiota-derived metabolites, including corticosterone, H2S, choline, BAs, indole sulfate, and LPS, are also produced. The SCFAs, TMAO, BAs, H2S, and LPS metabolites have been closely linked to the development of hypertension. So, the intestinal microbiota may have an interconnected role in regulating blood pressure, and any disruptions in their function could be linked to hypertension. Studies have proposed that *Lactobacillus* probiotics might play a beneficial role in blood pressure regulation (154) Additionally, a meta-analysis has shown that probiotics treatment can lead to a significant reduction in blood pressure in patients (155).

Role of microbial derived metabolites and CVD

We will briefly discuss the association between trimethylamine N-Oxide and CVD, and focus on other microbial metabolites in this review as illustrated in Figure 5 (156).

Trimethylamine-N-oxide (TMAO) associated with CVD

The gut microbial digestion of phosphatidylcholine, the main dietary source of choline, was found to produce a proatherogenic metabolite called trimethylamine-N-oxide (157). Among the numerous physiologically active metabolites of microbial metabolism, TMAO is a biologically active molecule that has been linked to an increased risk of adverse cardiovascular events, including acute coronary syndrome (ACS), stroke, and mortality (47, 158, 159). TMAO production occurs secondary to the ingestion of nutrients containing the trimethylamine moiety, such as choline, phosphatidylcholine, and L-carnitine, all of which are found in high concentrations in animal products, including red meat, fish, milk,

and eggs. The metabolism of these nutrients by microbial TMA lyases produces TMA, which enters the portal circulation, is oxidized to TMAO by hepatic flavin monooxygenases, primarily FMO3 (160), and subsequently enters the general circulation (28, 161). It is believed that TMAO may contribute to the development of atherosclerosis, following a proatherogenic pathway. Elevated levels of TMAO in the bloodstream have been positively associated with early atherosclerosis in humans. Moreover, monitoring TMAO levels can be useful in predicting the risk of mortality in patients with stable coronary artery disease and acute coronary syndrome (151, 162). Research has indicated that higher TMAO levels in the bloodstream are linked to the severity of peripheral artery disease and a greater risk of cardiovascular mortality among individuals affected by this condition (163).

In-depth analyses, including meta-analysis and dose-response studies, have further revealed that elevated plasma TMAO levels are associated with a higher occurrence of major adverse cardiovascular events in patients with coronary heart disease (164). Additionally, proinflammatory monocytes and elevated TMAO levels were substantially associated in stroke patients. According to Haghikia et al. (165) higher cardiovascular events such as myocardial infarction, recurrent stroke, and cardiovascular death were also linked to a raised TMAO plasma level. Numerous human investigations have also supported the involvement of TMAO in CVD. Compared to controls, patients with chronic heart failure had higher plasma levels of TMAO, choline, and betaine in a prospective observational analysis of stable CAD and healthy people (166). Similarly, in patients who experienced a myocardial infarction, TMAO was identified as an independent predictor of mortality at the two-year follow-up. The ratio stood at 1.21 (with a 95% confidence interval of 1.03-1.43, P = 0.023), as observed in a study involving 292 events (167). Another study conducted by Tang et al. (48), observed a correlation between elevated TMAO levels and a higher risk of major adverse cardiac events. However, the precise mechanisms by which TMAO affects cardiovascular disease have not been fully investigated.

Short-chain fatty acids and CVD and prevention strategies

The human digestive system cannot break down complex carbohydrates, such as dietary fiber, to support cell activity. Nevertheless, the gut microbiota can utilize fibers by fermenting them, resulting in the production of SCFAs (168). SCFAs are saturated fatty acids composed of carbon chains ranging from one to six carbons. Acetate, propionate, and butyrate are the main types of SCFAs found in the human body (169). The primary bacteria responsible for producing SCFAs are found in the clostridial clusters IV and XIVa within the *Firmicutes* phylum and include various species of bacteria such as *Eubacterium, Roseburia, Faecalibacterium*, and *Coprococcus* (170). It plays crucial roles in regulating anti-inflammatory responses, lipid metabolism, and gluconeogenesis. Notably, butyrate, one of the SCFAs, is considered a significant energy source for intestinal epithelial cells (171). A significant amount of research shows that

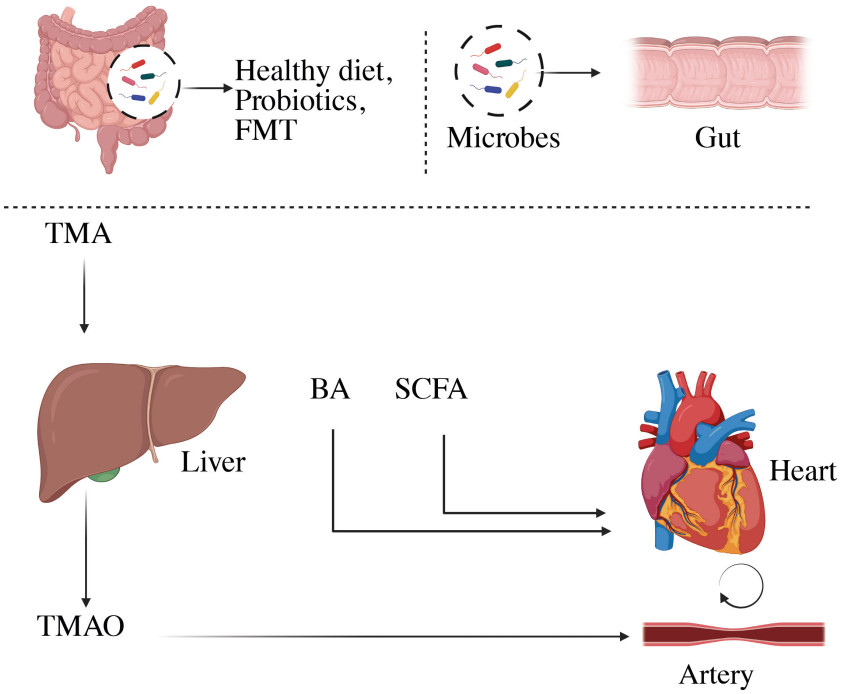

FIGURE 5
Representation of microbial-derived metabolites to CVD. Variations in the composition of the gut microbiome can change the metabolism, allowing bacteria or its fragments and metabolites to enter the circulation more easily. This can aggravate the pro-inflammatory milieu and produce metabolic disturbances, which can lead to CVD. BA, Bile acid; SCFA, short-chain fatty acids; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

SCFAs protect against heart failure and are essential for preserving the integrity of the intestinal barrier by encouraging mucus formation and reducing inflammation (172). The presence of high SCFA levels in fecal samples is linked to markers of hypertension, central obesity, and subclinical indicators of cardiometabolic disorders (173) and to the development of atherosclerosis (174).

Butyric acid in the diet effectively slowed the progression of atherosclerotic plaques in the arterial walls of mice missing apolipoprotein E (Apo-E) in a trial utilizing rodent as a model. The favorable benefits were obtained by slowing macrophage migration, boosting collagen deposition, and improving plaque stability (175). Multiple studies show that the SCFAs contribute to manage blood pressure. For example, when fecal material from hypertension human donors was introduced into germ-free mice vs normotensive donors, researchers saw an increase in blood pressure (176). SCFAs are linked to blood pressure regulation through G-protein coupled receptor (GPCR) pathways, specifically in renin secretion and blood control. SCFA activation of the olfactory receptor (Olfr) 78 and the free fatty acid receptor GPR41 causes an increase in blood pressure and a decrease in blood pressure, respectively. The acetate and propionate have antihypertensive properties due to their ability to reduce systemic inflammation and atherosclerotic lesions, both of which are independent predictors of hypertension (177). However, SCFAs have been implicated in causing damage to the organs affected by hypertension in mice infused with angiotensin II, indicating their role in hypertensive organ damage (178). As a result, a lot of evidence points to the gut microbial community's influence on blood pressure regulation in the host, with SCFAs functioning as one of the microbial components that contribute to vasomotor tone and blood pressure regulation. Recent research has revealed more evidence that SCFAs play a role in a variety of CVD processes, including ischemia-reperfusion injury, heart repair after myocardial infarction, and arterial compliance impairment (179, 180).

Bile acid (BA) association with CVD and therapeutics

Bile acids are produced in the liver through the breakdown of cholesterol, are crucial in controlling the absorption of lipids. Primary and secondary BAs can be identified based on their structural features. BAs can also be classed as bound or free based on whether they are conjugated with glycine or taurine (181). In a healthy adult, the liver produces primary bile acids at a daily rate of 500 mg that constitute approximately 72.5% of the total bile acid pool; chenodeoxycholic acid comprises 35%, while cholic acid constitutes 37.5% (182, 183). The synthesis of bile acids occurs through two distinct pathways: the classic (or neutral) pathway and the alternative (or acidic) pathway, each regulated by a specific enzyme. Cholesterol 7-hydroxylase (CYP7A1) enzyme responsible for the classic pathway, whereas oxysterol 7-hydroxylase (CYP7B1) is involved in the alternative pathway (184). Bile acids are stored in the gallbladder and released during digestion into the small intestine. The primary role of bile acids is to emulsify dietary fats and fat-soluble vitamins, facilitating their absorption and transport in the digestive system. Primary bile acids released into the duodenum have a critical function in emulsifying food components and vitamins that are lipid-soluble, enabling their digestion and absorption (185). Secondary bile acids are created when bacterial enzymes change the primary bile acids, which make up roughly 27.5% of bile acid (186).

However, Deoxycholic acid accounts for 25% of the overall bile acid pool, while lithocholic acid and ursodeoxycholic acid collectively make up 2.5% of these bile acids. In a healthy individual, almost 95% of BAs are efficiently reabsorbed in the distal ileum, primarily due to the process of enterohepatic circulation (187). The bile acids that are reabsorbed in the distal ileum are then transported back to the liver to build an effective recycling process. Surprisingly, bile acids, which make up to 2-4 grams of the body's total weight and play a crucial function, are controlled by a relatively tiny pool (188). This process happens multiple times a day, typically ranging from 5 to 10 cycles daily (189, 190). In order to prevent bile acids from building up to hepatotoxic levels and to limit their impact on cholesterol metabolism, the size of the bile pool is carefully managed through feedback regulation of bile acid synthesis (191). Bile acids also possess strong microbial activity and serve as signaling molecules, acting as ligands for nuclear receptors, thereby impacting various metabolic processes (192). For instance, Farnesoid X-receptor (FXR) activation leads to the suppression of the cholesterol 7a-hydroxylase enzyme. By regulating this enzyme, FXR helps maintain the balance of bile acid synthesis and contributes to the overall control of cholesterol metabolism (193). The gut microbiota plays a significant role in modifying primary bile acids through bacterial salt hydrolase activity. This enzymatic process involves removing the 2OH groups from primary bile acids, transforming them into secondary bile acids (194). Bacteria can lessen BA toxicity by increasing their solubility, giving the gut microbiota a way to defend itself. Additionally, the gut microbiota might change bile acids further before they return to the liver for reconjugation and rejoin the circulation (195). Bile acids serve as a crucial pathway for cholesterol elimination through excretion in feces helping to decrease circulating cholesterol levels and reduce the risk of plaque accumulation (193). However, alterations in the gut microbiome can influence the bile acid synthesis rate, potentially leading to increased plasma levels of LDL cholesterol and an elevated risk of atherosclerosis (196). Thus, maintaining a healthy gut microbiome is essential for regulating bile acid metabolism and its impact on cholesterol levels and cardiovascular health. Additionally, microbial metabolites such as tryptophan and indole have been identified to have significant roles in the development of cardiovascular diseases.

Therapeutic approaches to gut microbiome

The novel research implies that the gut microbiota plays a critical role in the progression of cardiovascular illnesses. Therapeutic techniques for influencing the composition and metabolic activity of the gut microbiota have been developed. As

shown in Figure 6, these options include dietary changes, the use of probiotics and prebiotics, antibiotic treatments, and even fecal transplantation. Notably, these therapies have shown the potential to improve blood pressure control, restore lipid profiles to normal levels, and reduce body weight in people with cardiovascular disease (33). In a vicious cycle, the intricate interaction between dietary components and other variables affects the gut microbiota and pathogenesis of many cardiovascular diseases as shown in Figure 7 (168).

Dietary inventions

Numerous scientific studies have provided persuasive evidence supporting the idea that dietary interventions can significantly decrease the risk of cardiovascular problems (197, 198). Diets that frequently occur in Western industrialized societies that feature high consumption of red meat or animal proteins, saturated fats, and simple carbohydrates have been associated with an increased risk of CVD (199, 200). An increasing mass of evidence refers to the intestinal microbiota as a possible avenue for CVD treatment. Current clinical trials on microbe targeting for CVD therapy are summarized in Table 2 (39). Conversely, the composition of our diet can influence the structure and functioning of the gut microbiome (201). The gut microbiota is greatly influenced by essential food elements such as macronutrients, fiber, polyphenols, prebiotics, and probiotics, which also have an impact on the production and release of major gut microbiome metabolites including SCFAs (202). In a prior study, it was found that diets rich in fiber promote the growth of beneficial symbiotic microbes while preventing the spread of known infectious diseases (203). Moreover, the consumption of a high-fiber diet increased acetate-producing microbiota, which was associated with lower blood pressure and a reduction in cardiac hypertrophy and fibrosis (204). The mediterranean diet, which consists of a high intake of vegetables, fruits, grains, and legumes combined with a low intake of red meat and processed carbohydrates, has been shown to be effective in the prevention of CVD (205). This is mostly due to the high levels of antioxidants, nitrates, and fiber in this diet, and to the low levels of saturated/Trans fatty acids, salt, and phosphate. These elements are expected to reduce inflammation and oxidative stress, promote antioxidant activity, increase nitric oxide bioavailability, and microbiota modulation to improve vascular and cardiac function (206). The Western diet, compared to the Mediterranean

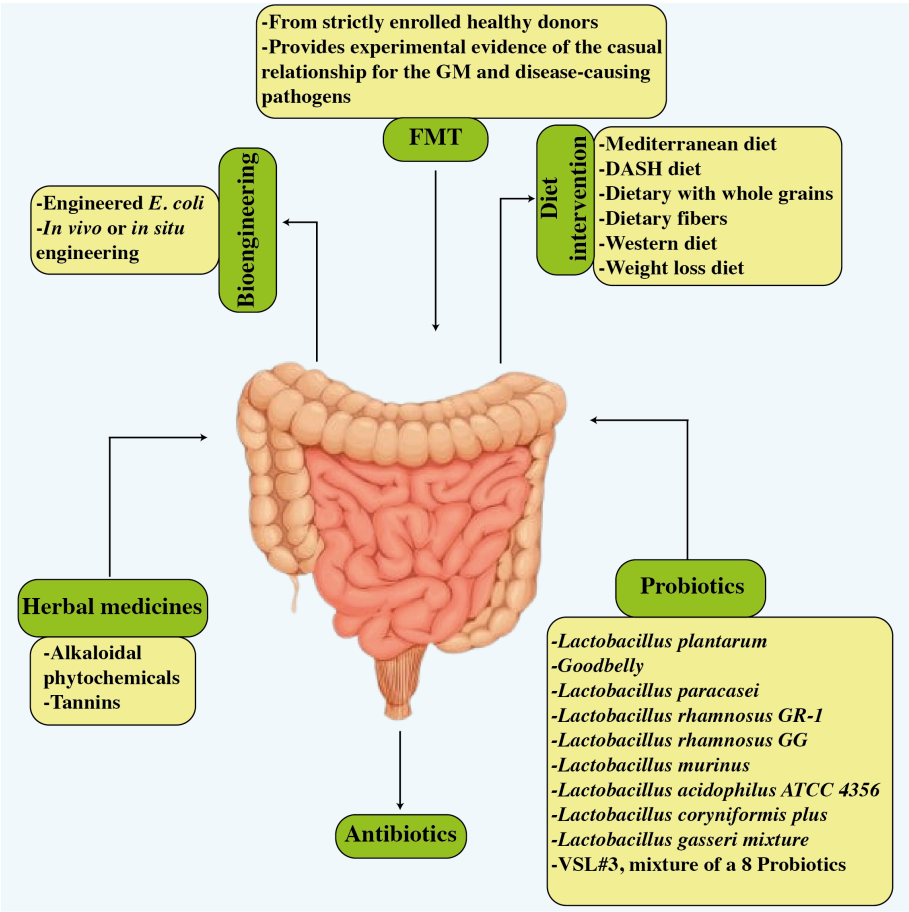

FIGURE 6
Potential treatments related to improved cardiovascular disease results and improved gut microbiota. The figure illustrates six strategies, i.e., dietary modifications, probiotics, antibiotics, FMT, bioengineering, and herbal treatment.

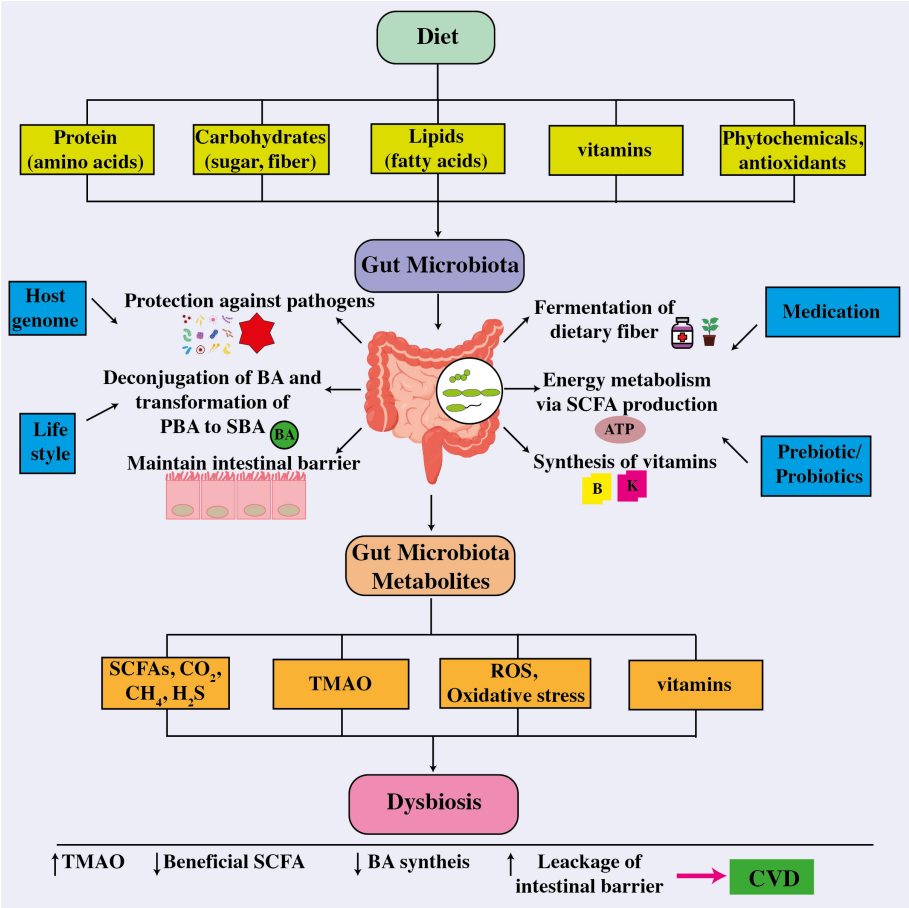

FIGURE 7
The correlation between the intestinal microbiome, metabolites, and cardiovascular disease. The intricate connection between dietary components absorbed and other factors influencing the gut microbiota, whose composition then influences their functionality and metabolite production and release, a disruption which leads to dysbiosis, thereby affecting host health and the onset and cause of different cardiovascular disorders. ATP, adenosine triphosphate; BA, bile acid; CO_2, carbon dioxide; CH_4, methane; CVD, cardiovascular disease; H_2S, hydrogen sulfide; PBA, primary bile acid; ROS, reactive oxygen species; SBA, secondary bile acid; SCFA, short-chain fatty acids; TMAO, trimethylamine-*N*-oxide.

diet, is known to raise CVD risk by lowering gut microbiota diversity and beneficial bacteria such as *Bifidobacterium* (207). A study involving mice fed a Western diet, the findings revealed higher plasma concentrations of TMAO and the development of cardiac dysfunction and heart fibrosis (208). The expression of pro-inflammatory cytokines (IL-10) and tumor necrosis factor (TNF-α) as well as interleukin-1 (IL-1), both of which are indicative of increased inflammation, was shown to be altered (208). In a study involving 153 volunteers from four cities in Italy, researchers found that the consumption of fruits, vegetables, and legumes is consistent with the Mediterranean diet led to an increase in fecal SCFA levels (209). This effect is due to fermentation by a greater abundance of bacteria from the *Firmicutes* and *Bacteroidetes* groups (209).

ATP, adenosine triphosphate; BA, bile acid; CO_2, carbon dioxide; CH_4, methane; CVD, cardiovascular disease; H_2S, hydrogen sulfide; PBA, primary bile acid; ROS, reactive oxygen species; SBA, secondary bile acid; SCFA, short-chain fatty acids; TMAO, trimethylamine-*N*-oxide.

Following a mediterranean diet leads to reduced TMAO levels, thereby helping to prevent cardiovascular issues and heart failure (210, 211). A particular study revealed that incorporating ginger supplements into the diet influenced the gut microbiota composition, resulting in a notable rise in fatty acid metabolism (212). Moreover, the presence of miRNAs within ginger-derived exosome-like nanoparticles has the potential to modify bacterial gene expression, influencing the host genome (213). Currently, there is an obvious association between nutrition and intestinal microbes, resulting in varied gut bacterial communities across various diets and geographic locations. However, there is still a huge gap in our understanding of how our food influences the gut microbiome and the impact of the gut microbiota on general host health. More study is needed since nutrition is a low-cost, easy to manage strategy for the possible prevention, control, and management of cardiovascular disease.

Prebiotics and probiotics

The human colon is filled with probiotics, which are primarily made up of *bifidobacteria* and *lactobacilli*, and are essential for

TABLE 2 Clinical trials targeting the gut bacteria in the treatment of cardiovascular diseases (39).

Models	Intervention	Result	Clinical ID
Diet			
Overweight/obese individuals	Mediterranean diet	Positive	NCT03071718
Patients with CAD	Moderate Alcohol Consumption	Positive	No report
Patients with CAD	Calorie restriction	Positive	IRCT20121028011288N15
Patients with CAD	Lacto-Ovo-Vegetarian Diet	Positive	NCT02942628
Overweight/obese individuals	Dietary fibers	Positive	NCT01719900
Patients with CAD	Vegan Diet or the American Heart Association-Recommended Diet	Positive	NCT 02,135,939
Patients with T2D	Dietary fibers	Positive	No report
Patients with heart failure	DASH diet	Positive	No report
Obese hypertensive patients	hypocaloric diet supplemented with probiotic cheese	Positive	ISRCTN76271778
Probiotics			
Subjects with metabolic syndrome	A. soehngenii	Positive	NTR-NL6630
Patients with heart failure	Saccharomyces boulardii	Negative	NCT02637167
Patients with CAD	Lactobacillus rhamnosus GG (LGG)	Positive	IRCT20121028011288N15
Patients with MI	Lactobacillus Rhamnosus G	Positive	IRCT20121028011288N15
	Inulin		
Patients with MI	Lactobacillus rhamnosus capsules	Positive	IRCT20121028011288N15
Overweight/obese insulin-resistant volunteers	A. muciniphila	Positive	NCT02637115
Patients with stable CAD	Lactobacillus plantarum 299v	Positive	NCT01952834
Patients with heart failure	Saccharomyces boulardii	Positive	NCT01500343
Subjects with high-normal blood pressure and mild hypertension	Lactobacillus helveticus	Positive	No report
Probiotics and Prebiotics			
Patients with CAD	Lactobacillus Rhamnosus G and Inulin	Positive	IRCT20180712040438N4
Healthy overweight or obese individuals	Polydextrose and *Bifidobacterium animalis subsp*	Positive	NCT01978691
Prebiotics			
Overweight to obese men	Inulin	Positive	NCT02009670
Children with overweight or obesity	Oligofructose-enriched inulin	Positive	NCT02125955
Mildly hypercholesterolemic individuals	β-glucan	Positive	NCT01408719
Obese women	Inulin-type fructans©	Positive	NCT00616057
Exercise			
Patients with CAD	Bicycle ergometer	Negative	NCT01495091
Patients with CAD	Exercise stress testing	Negative	NCT01495091
Overweight participants	High-intensity interval training	Positive	ACTRN12617000472370
Drug			
Patients with T2D	Berberine and probiotics	Positive	NCT02861261
Patients undergoing elective coronary angiography	broad-spectrum antibiotics	Positive	No report

(Continued)

TABLE 2 Continued

Models	Intervention	Result	Clinical ID
Patients admitted with acute MI or unstable angina	Amoxicillin, metronidazole	Positive	No report
FMT			
Hypertensive patients	Washed microbiota transplantation	Positive	No report
Obese patients	FMT capsules	Positive	NCT02741518
Patients with metabolic syndrome	Vegan FMT	Positive	NTR 4338
Patients with metabolic syndrome	FMT	Positive	NTR1776

maintaining healthy immune systems, colon microflora, and the production of healthy compounds and also have ability to stop the spread of cancer, lower cholesterol, increase the synthesis of vital cytokines and vitamins, and prevent infections (214, 215). According to Gibson and Roberfroid, prebiotics are defined as non-digestible poly or oligosaccharides that have a positive impact on the host by selectively promoting the growth or activity of specific beneficial bacteria in the colon (216). The *Lactobacillus Plantarum* led to an improvement in the diversity of gut microbial flora, this consumption was linked to a reduction in the incidence of CVD incidents (217). Another study showed by Naruszewicz et al. (218), involving 36 healthy volunteers who were active smokers, revealed that there was a negative correlation between the intake of *Lactobacillus Plantarum* and various health markers, including blood pressure levels, fibrinogen levels, monocyte adhesion, and proinflammatory cytokine levels. These findings suggest that *Lactobacillus Plantarum* may have potential in the primary prevention of atherosclerosis. The normal or moderately elevated cholesterol levels in females experienced low LDL after consuming fermented milk containing *Lactobacillus acidophilus* and *Bifidobacterium longum* (219).

A recent study conducted by Catry et al.(220) revealed a 15-day supplementation of inulin-type fructans (ITFs) had a positive impact on endothelial function in the arteries of n-3 PUFA-depleted ApoE$^{-/-}$ mice. The improvement in endothelial function might be attributed to an increase in bacteria capable of producing nitric oxide, as it helps dilate blood vessels and improve blood flow, ultimately benefiting cardiovascular health. The findings highlight the ITFs potential in promoting cardiovascular well-being, particularly in the context of n-3 PUFA-depleted conditions (221). Similarly, a comprehensive analysis revealed that intake of isolated triterpene fraction yields favorable results on LDL cholesterol levels in the human (222). In addition to ITFs, beta-glucan supplements have also shown the capacity to lower total and LDL cholesterol levels while boosting endothelial vascular reactivity in people in the great health (223). It is crucial to recognize that prebiotics are formed up of a diverse range of chemicals that are controlled by different gut flora (224–227). A fiber-rich diet has been demonstrated to alter the gut microbiota by increasing acetate-producing bacteria, resulting in reduced gut dysbiosis and cardiovascular protection, most notably the transcription factor Egr1, are related to acetate regulation and govern CVD through inflammation, heart fibrosis, and hypertrophy (204).

Another prebiotic, beta-glucan was demonstrated to influence cholesterol levels and glucose homeostasis. A 2-month study that included a beta-glucan dietary plan indicated a significant reduction in LDL and total cholesterol levels. The endothelial function improved in healthy people, showing cardioprotective effects. These effects are mostly due to the production of beneficial SCFA by the gut flora (228). In animal tests, arabinoxylans showed potential as a possible prebiotic. It was discovered that their role in encouraging the growth of *bifidobacteria* and the production of propionate reduces cholesterol and fat deposition (206). Dietary arabinoxylan oligosaccharides raised bacterial populations and butyrate levels in stools in individuals (229). Probiotics help to improve human metabolism by boosting digestive enzyme output, suppressing bacterial enzyme activity, and lowering ammonia generation. *Lactobacillus* and *Bifidobacterium* have beneficial effects on intestinal barrier function and play a protective role in inflammatory diseases by modulating inflammatory and proinflammatory cytokines, which may potentially delay or improve CVD (230, 231). *Akkermansia muciniphila* is also renowned for its probiotic features, and it is related to glucose, insulin, and leptin, all of which have roles in the metabolism of lipids and glucose (232). However, Lactobacillus plantarum efficiently lowered LDL-C and total cholesterol levels while also inhibiting the formation of atherosclerotic plaques in hypercholesterolaemic individuals (233). The preceding research concentrated on the effects of prebiotics and probiotics on cardiovascular risk factors such as inflammation and hypertension, as well as impacts on glucose and lipid metabolism, rather than the direct benefit of atherosclerosis. However, considering their beneficial effect on several CVD risk variables, more research into how these medications affect the onset and progression of CVD is essential.

Antibiotics

In the regulation of host health, the gut microbiome has a huge impact on the host as a result of antibiotic usage. The use of antibiotics may damage the host's health in a variety of methods, both directly and indirectly. This impact can alter a variety of bodily processes, including immunological control, metabolism, and ultimately general health (48, 234–237). A variety of antibiotics have shown evidence of affecting blood pressure and intestinal flora.

A prime example is the drug minocycline, which has been studied for its capacity to alter the nature of the gut microbiota and control blood pressure (BP) in hypertensive rats (140). Using erythromycin, tetracycline, or doxycycline within the previous five years did not reduce the chance of developing a first MI, according to another population-based trial, and its authors disputed their efficacy in avoiding primary coronary heart disease (CHD) (238). Macrolides antibiotics, such as azithromycin, erythromycin, and clarithromycin, comprise a significant class of orally active antibiotics that function as bacteriostatic agents. In 2013, 51.5 million drugs for azithromycin were prescribed in the United States (239). The use of macrolide antibiotics is believed to increase the risk of cardiovascular diseases such as myocardial infarction (MI), ventricular tachyarrhythmias, and sudden cardiac death (SCD) (240, 241). Further, quadruple antibiotic therapy was shown to significantly lower high systolic and diastolic blood pressures in salt-induced hypertensive rats (242). It helps to realize the research on the effect of antibiotics on blood pressure regulation produced varying outcomes (243).

For instance, minocycline and vancomycin medication in rats led to lower *Firmicutes* levels in the gut, resulting in lower blood pressure in hypertensive rats. It's noteworthy to note that identical antibiotic therapy actually raised blood pressure in salt-sensitive rats. This difference underlines the intricacy of the gut microbiota-blood pressure interaction and the significance of taking into account a variety of variables that may affect the results of such mediations (244). In a study by Rune et al., they found that ampicillin had the ability to lower mice's levels of LDL and VLDL cholesterol. Atherosclerosis risk is associated with these forms of cholesterol. As a result, the mice's aortic atherosclerotic lesions were reduced in size (245). Although a few studies provide promising results, indicating possible benefits in this regard, the efficiency of antibiotics for offering preventive benefits against CVD in trials involving patients is yet unknown (246). However, certain analyses have failed to demonstrate a distinct and obvious link between the use of antibiotics and protection against CVD, yielding unclear outcomes. As a result, more research and analysis are needed to determine whether antibiotics can significantly reduce the risk of cardiovascular disease (240). Furthermore, universal antibiotics can have a variety of impacts on the body, making methods of treating CVD with antibiotics contentious. While certain studies have suggested that taking antibiotics to treat CVD may have some benefits, their broad action can have a number of adverse reactions. Thus, any possible benefits of using antibiotics in treating CVD must be carefully balanced against any dangers and adverse effects that could result from their use. Before making antibiotics a common therapy choice, more study is required to better understand their precise mechanisms of action and potential advantages in the management of CVD.

Fecal microbiota transplantation as a prevention strategy

Fecal microbiota transplantation (FMT) is a therapeutic method intended to restore a healthy balance of gut microbiota in a recipient (247), by transferring fecal matter from a donor who is in a healthy condition (248). It gained a lot of attention for its safety and efficacy in therapeutic applications after being extensively studied in an array of mammalian species (249).The more complicated nature of propagating gut bacteria compared to those that inhabit the mouth cavity is one of the difficulties that FMT still challenges (250). The FMT involves transferring fecal matter from an adult donor to a recipient with an unbalanced intestinal microbiota and the fecal matter is rich in various microbial populations such as *Clostridioides difficile* (251, 252).This transplantation has shown promising results in treating several intestinal and other chronic diseases and has been researched as a viable therapeutic alternative in clinical applications (253). Notably, FMT has confirmed effectiveness in treating various conditions, including recurrent *Clostridium difficile* infection (254), inflammatory bowel disease (255), and irritable bowel syndrome (256). New research has explored the potential of FMT as a promising approach for addressing cardiometabolic disorders (257).

In 2013, the US Food and Drug Administration (FDA) granted its initial approval for FMT, specifically for managing recurrent *Clostridium difficile* infection. Since then, FMT has gained recognition as a therapy for a wide range of gastrointestinal as well as non-gastrointestinal conditions. However, there remains limited understanding of its mechanism of action and potential long-term side effects (258). The probable therapeutic effects of FMT have also been demonstrated in a number of animal models involving people with severe multiple sclerosis, autism, multidrug-resistant (MDR) infections, and multiple organ failure in seriously confined people (259–261). Recent findings have indicated a lower abundance of *Clostridia* strains that produce butyrate in the intestines with type 2 diabetes mellitus. Conversely, studies have shown a higher prevalence of non-butyrate-producing *Clostridiales* in these patients by demonstrated that both insulin sensitivity and levels of butyrate-producing intestinal microbiota significantly improved following microbiota transplantation (262). Experiments on mice raised the possibility of a brain-gut-microbiota axis that goes in each direction. Various neurological conditions like anxiety, depression, dementia such as Alzheimer's, and Parkinson's disorder are caused by an imbalance in this axis (263, 264).

Recently, Park et al. (265) from Inho University Hospital in Incheon, South Korea, used FMT to treat a 90-year-old woman who had severe CDI and Alzheimer's dementia. Her fecal microbiota diversity drastically altered after the transplant, and her cognitive abilities significantly improved, according to a comparison of the results from before and after the procedure. The study also demonstrated a strong correlation between gut flora and cognitive function. Segal et al. (266) conducted distinct clinical research at Soroka University Medical Centre in Israel with six individuals suffering from both Parkinson's disease and constipation. These patients were given treatment that included Fecal Microbiota Transplantation (FMT). Moreover, Doll et al.(267) used transplantation of fecal microbiota as add on therapy in two patients with major depression. After 4 weeks, both patient signs of depression improved, and study suggested that FMT be tested

extensively for MDD treatment. It was found that transferring feces microbiota from healthy rats with normal heart rates to rats with naturally elevated levels produced positive results. The results included lower systolic blood pressure, enhanced blood vessel functionality, lower levels of oxidative stress and inflammation within blood vessels, and a more favorable balance between two unique types of immune cells, Th17 and Tregs (126, 268). However, the curative benefits of FMT can be linked to a broader variety of bacteria, viruses, fungi, and archaea that can engraft into the recipient host and increase the functional variety of a microbiota FMT is also being examined in almost 300 clinical trials for a variety of disease indications, including autoimmune diseases, neurological difficulties, cancer, host disease, and metabolic and gastrointestinal disorders. There is currently insufficient data to support the relevance of fecal microbiome transplantation about gut microbiota in human patients with CVD, necessitating more research in this field. Different approaches and processing variations, such as donor selection and testing, fecal microbiome transplantation via the upper gastrointestinal tract, enema, or colonoscopy, as well as short- and long-term patient monitoring for adverse effects and treatment efficacy, introduce new challenges to be investigated.

Exercise

Physical inactivity holds substantial significance as a risk factor for a range of metabolic disorders, and roughly 1/3 of world's population contributes in inadequate levels of physical activity, which has implications for health (269). Statistics indicate that roughly 3.2million deaths annually can be attributed to inadequate levels of physical activity (270), with healthcare expenses amounting to $117 billion yearly, attributed to conditions resulting from a lack of exercise (271). People who adopt a sedentary lifestyle and fail to engage in regular physical activity are more prone to the development of cardiovascular disease (272), and who are less active face a 30-50% higher risk of developing high blood pressure (273). The researchers predicted that lack of exercise was responsible for 12% of myocardial infarctions (MI), a risk proportion that fell within high blood pressure (18%), CVD cases (6%) and diabetic mellitus (10%) recognized risk factors for heart disease whose incidence is also inversely related to physical activity levels (274, 275). The study confirmed that exercise can enrich the microflora diversity; improve the F/M ratio, which may contribute to weight loss, obesity-related pathologies, and gastrointestinal disorders; and stimulate the proliferation of bacteria, which can modulate mucosal immunity and maintain homeostasis (276–278). Research has demonstrated that exercise has the capacity to increase the levels of the bacterial metabolite known as butyrate (279).While human research in this area is limited, data from several laboratories, including our own, indicate that exercise training can exert a noteworthy influence on the gut microbiota in animal models (280–282). Moreover, the modifications in the gut microbiota brought about by exercise are linked to changes in the host's physiology, such changes include metabolic rate modifications (283), immunity (280), and even behavior (281). Certainly, exercise training has been demonstrated to increase the concentrations of short-chain fatty acids derived from the gut microbiota in mouse models (284), comprising of two to six carbon atoms, play a crucial role as an energy source for various tissues and are associated with beneficial effects such as reducing inflammation (285), improving insulin sensitivity (286), and inducing the morphology of the central nervous system (287). Notably, levels of LPS are elevated in cardiovascular disease and specific cardio metabolic disorders (288). However, high-endurance training has been shown to have the potential to decrease plasma LPS levels. This suggests that exercise may have a positive impact on reducing inflammation associated with CVD and related metabolic conditions (289). A crucial observation to highlight is that the advantages provided by the gut microbiota due to exercise training were not enduring. This emphasizes the necessity for consistent and regular exercise to sustain a constructive impact on the gut microbiota and the associated health benefits (279). The findings highlighted the broad-ranging benefits of influencing the gut microbiota via physical exercise, stretching beyond the realm of cardiovascular health. Nonetheless, it's important to recognize that substantial and enduring advantages necessitate prolonged periods and higher-intensity aerobic training. Participating in more extended and intense exercise sessions seems to be pivotal in achieving lasting enhancements in gut microbiota composition and the correlated health advantages for the individual. Consistently adhering to such exercise routines is pivotal for maximizing the influence on the gut microbiota and overall well-being (290). To protect the heart and arteries, physical activity can increase insulin sensitivity, reduce plasma dyslipidemia, proper raise blood pressure, decrease blood viscosity, promote endothelial nitric oxide generation, and improve leptin sensitivity. Furthermore, the preventive impact of exercise on the body involves not only laboratory animal models but also clinical studies, as proven by WHO recommendations (291). Numerous studies have illustrated a clear dose-response correlation between physical activity levels and a decreased incidence of CVD and characterized by reductions in factors such as blood pressure, body weight, oxidized low-density lipoprotein (ox-LDL), and improved glucose tolerance as physical activity increases (292, 293). Although it's known that exercise protects against CVD by reducing sympathetic impulses, arterial pressure, and heart rate, increasing blood flow and endothelial NO production, causing vessel dilation, and decreasing inflammatory cytokines and oxygen radical formation, the precise processes that lead to transcriptional factor modifications are unknown. Future research could focus on the mechanisms of exercise's protective effects on the heart and arteries.

Conclusions

The human intestine is the habitat of the most enormous and varied population of microbes. The main purpose of the gut microbes is to prevent the expansion of potentially lethal germs. However, there is a rising acknowledgment of the intestinal microbiota as a risk variable for developing cardiovascular disease

(CVD). Metabolites derived from the gut microbiota, such as short-chain fatty acids, trimethylamine-N-oxide, bile acids, and polyphenols, are critical in maintaining normal cardiovascular function. When these metabolites are out of balance, it has the potential to contribute to an outbreak of CVD. Variations in the composition and diversity of the gut microbiota, known as dysbiosis, have been associated with disorders such as heart failure, atherosclerosis, hypertension, myocardial fibrosis, myocardial infarction, and coronary artery disease. However, the specific mechanisms behind these relationships are still unknown. As a result, the microbiota and its metabolites have emerged as a novel therapeutic target for both CVD prevention and treatment. Ongoing attempts are being made to widen the application of microbiota therapies not only for CVD but also for a variety of other human disorders. Innovations in genomic and metabolomic technology have enabled improved characterization and molecular research of bacteria and their metabolites. Individual microbiome may be profiled in the future utilizing metabolomic/biomarker analysis to measure individual health, potentially delivering specific guidance on food and lifestyle changes. Dietary treatments, the use of pre/probiotics and antibiotics, FMT, TMAO reduction, and regular exercise are current strategies for regulating gut bacteria to improve cardiac function. Further research on microbe-microbe and microbe-host associations may explain how specific metabolites affect the disease process. Improving our understanding of the complex interplay between gut microbiota, host characteristics, and therapeutic response is critical for developing breakthrough precision therapies for cardiovascular disease.

Author contributions

AL: Writing – original draft. AH: Writing – review & editing. MU: Writing – review & editing. SN: Writing – review & editing. MehrajU: Writing – review & editing. LZ: Writing – review & editing. AU: Writing – review & editing. KU: Writing – review & editing. WA: Writing – review & editing. GW: Writing – review & editing, Funding acquisition, Supervision.

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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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EDITED BY
Sylvie Bertholet,
GSK Vaccines, United States

REVIEWED BY
Fernando Gómez-Chávez,
National Polytechnic Institute (IPN), Mexico
Elopy Sibanda,
National University of Science and Technology, Zimbabwe

*CORRESPONDENCE
Luyi Yang
lyyang@jlu.edu.cn
Xiaoxi Xu
xiaoxi_xu01@163.com

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Oral microbiota–host interaction: the chief culprit of alveolar bone resorption

Jingyu Xu[1], Ling Yu[1], Surong Ye[1], Zitong Ye[1], Luyi Yang[1]* and Xiaoxi Xu[2]*

[1]Department of Orthodontics, Hospital of Stomatology, Jilin University, Changchun, China, [2]Key Laboratory of Dairy Science, Ministry of Education, College of Food Science, Northeast Agricultural University, Harbin, China

There exists a bidirectional relationship between oral health and general well-being, with an imbalance in oral symbiotic flora posing a threat to overall human health. Disruptions in the commensal flora can lead to oral diseases, while systemic illnesses can also impact the oral cavity, resulting in the development of oral diseases and disorders. *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, known as pathogenic bacteria associated with periodontitis, play a crucial role in linking periodontitis to accompanying systemic diseases. In periodontal tissues, these bacteria, along with their virulence factors, can excessively activate the host immune system through local diffusion, lymphatic circulation, and blood transmission. This immune response disruption contributes to an imbalance in osteoimmune mechanisms, alveolar bone resorption, and potential systemic inflammation. To restore local homeostasis, a deeper understanding of microbiota–host interactions and the immune network phenotype in local tissues is imperative. Defining the immune network phenotype in periodontal tissues offers a promising avenue for investigating the complex characteristics of oral plaque biofilms and exploring the potential relationship between periodontitis and associated systemic diseases. This review aims to provide an overview of the mechanisms underlying *Porphyromonas gingivalis*- and *Fusobacterium nucleatum*-induced alveolar bone resorption, as well as the immunophenotypes observed in host periodontal tissues during pathological conditions.

KEYWORDS

oral host-microbial interactome, oral-systemic axis, periodontitis, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, bacterial extracellular vesicles, alveolar bone resorption

Introduction

Oral health is an indispensable element of general health and well-being ensuring the fulfillment of basic daily human functions. However, according to the 2015 Global Burden of Disease (GBD) study, about 3.5 billion people worldwide suffer from oral conditions (1). The pronounced global prevalence and severity of oral diseases have sparked significant concern among the public. These progressive chronic clinical diseases affect the teeth and various tissues within the oral cavity. Dental caries, periodontal diseases, oral mucosal diseases, and oral cancer are the main types of oral diseases, exhibiting high prevalence and severe adverse prognosis for individuals, communities, and society (2).

Beyond their prevalence and public concern, oral diseases are believed to have bidirectional associations with systemic health (3–7). Simultaneous or sequential occurrences of oral diseases and systemic diseases (8–19), such as gastrointestinal, immune, cardiovascular, and nervous system diseases, have been reported. Moreover, the tight relationship between human microbial communities and human health has drawn significant interest from researchers, with the oral microbiome considered to play a vital role in oral diseases and the connection between oral and general well-being.

Oral pathogens colonize the surfaces of different habitats within the oral cavity and form functional groups with pathogenic roles. Typical representatives of these groups include *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. These two periodontal pathogens can disrupt bone homeostasis by excessively activating host immune responses. The resulting microbial–host interaction-induced local inflammation may spread throughout the body, leading to systemic diseases. In this review, we elucidate the mechanisms behind *Porphyromonas gingivalis*-and *Fusobacterium nucleatum*-induced bone resorption, construct the immune defense phenotypes of the human body against the invasion of oral pathogenic microorganisms, and further explore the interaction between oral microbial communities and the host.

Oral microbial ecological guilds and oral diseases

The oral cavity is an open system where microbes are ingested with every breath, meal, and drink, colonizing through close contact with other humans, animals, or the physical environment. It provides a habitat for microbes, with suitable temperature, humidity, and nutrition. Despite there being millions of microbial species on Earth, only approximately 760 have been identified as major oral residents (20). In typical oral ecology, there are only 296 species-level microbial taxa (21), which are collectively referred to as the human oral microbiota (22). Alongside planktonic forms, the oral microbiota tends to assemble into complex spatial structures and form symbiotic communities to adapt to environmental changes and maintain microbial community and host homeostasis.

Oral microbial dysbiosis and its pathogenic pathway

Microbial dysbiosis is generally considered a state that mediates the associations between microbiota patterns and disease states (23). As the oral cavity is an open ecosystem, oral microbial homeostasis is often challenged by many factors, such as genetics, gender, habitat, age, diet, living habits, and environment. Long-term nongenetic factors may cause genetic variation, resulting in dramatic changes in the structure of the bacterial flora (24).

The bidirectional association between oral microbial dysbiosis and general disease states might occur three distinct manners (25). Oral bacteria and their products can be transferred into the circulatory system via open or closed foci, such as inflammatory and ruptured epithelium or infection around the root apices. This transfer can cause transient bacteremia, resulting in systemic inflammation and metabolic and functional disorders (6, 26). Bacterial products, such as gingipains secreted by the typical periodontal pathogen *P. gingivalis*, have the potential to promote such pathological processes by degrading tight junction proteins, not only in periodontal tissues but also in vascular endothelial cells (27).

Oral pathogens can also be disseminated through non-hematogenous processes. Routes such as oro-pharyngeal or oro-digestive pathways may lead to ectopic colonization in the gut, disrupting the local microbial composition, triggering inflammation, compromising the intestinal mucosal barrier function, and inducing systemic diseases (28–31). An imbalance in gut homeostasis can promote the colonization of oral bacteria in the intestines (32–34). In addition, immune cells and factors responsive to oral pathobionts in the gut or other parts of the body can migrate to the oral cavity, exacerbating oral inflammatory conditions like periodontitis (35, 36). These processes illustrate the probability of an oral-systemic axis that regulates human health and disease conditions.

Oral microbial niches and ecological guilds

Microbes in the oral cavity are not uniformly distributed. Only a few dozen species are abundant and constitute the core of the oral microbial community, whereas others are less abundant (37, 38). Heterogeneous colonization of oral microorganisms can be attributed to the uniqueness of oral niches, including the saliva, tongue, oral mucosa, mineralized tooth surfaces, and periodontal tissues (22). The spatial organization of oral microbes is in a state of dynamic equilibrium, maintained by opposing forces such as salivary flow, microbial adhesion, shedding and colonization, and crucially, microbe–microbe and microbe–host interactions (25, 39, 40). The microbiome colonizing the surface of mineralized teeth exists in the form of biofilms. Depending on their composition, nutritional background, ecological site anatomy, and antigen and immune exposure, plaques can be classified as subepithelial or subgingival (41, 42). Microorganisms within plaque biofilms rarely live independently; instead, they interact to form different

functional groups and cooperate as ecological guilds to perform higher physiological functions (43). This applies to periodontal pathogens in subgingival plaques, where dominant species in the periodontal ecological guilds can determine the overall function of the group or play a crucial auxiliary role.

Periodontal pathogens and bone remodeling

The role of periodontal pathogens in amplifying systemic inflammation and organ dysfunction has recently been established in several systemic diseases (44–46), such as inflammatory bowel disease (IBD), stroke, chronic renal diseases, cardiovascular diseases (47–49), diabetes (47), pneumonia, meningitis, rheumatoid arthritis (47, 50), cognitive disorders (51), as well as poor pregnancy outcomes (52, 53) and cancer (54). *Porphyromonas gingivalis*, a keystone periodontal pathogen, can ferment amino acids and grow deep in the glucose-poor periodontal pocket. *P. gingivalis* also invades gingival tissues and epithelial cells, promoting cell proliferation and causing epithelial radicular proliferation, which is a typical manifestation of periodontitis. The interaction between *P. gingivalis* and local host immune responses can have two contrasting outcomes, speculated to be related to the concentration of *P. gingivalis* and its virulence factors, mainly lipopolysaccharide (LPS), fimbriae, and gingipains. The concentration of virulence factors is high in the superficial layer, leading to immune escape, and low in the deep layer, resulting in a pro-inflammatory response that increases nutrient (heme) requirements (55). *P. gingivalis* employs unique and complex pathogenic mechanisms. These include strong invasive properties to allow it to enter the circulatory system, induce cell apoptosis, initiate oxidative stress, influence the host innate immune response by inducing dysfunction in neutrophils and macrophages, and facilitate the expression of acute phase proteins and numerous pro-inflammatory cytokines (52). Furthermore, *P. gingivalis* has the ability to regulate the innate immune response, ensuring the growth, colonization, and invasion of other opportunist and symbiont bacteria such as *F. nucleatum*, *Firmicutes*, *C. rectus*, *Streptococci*, *Staphylococci*, *Enterobacteriaceae*, *Prevotella*, *Hemophilus parainfluenza*, and *Dialister* (56–58). The dysbiotic microbiome induced by *P. gingivalis* is inherently resilient and can be stably transferred and easily restored even after antibiotic therapy is discontinued (59), making the local and systemic disease conditions triggered by *P. gingivalis* difficult to cure.

The obligate anaerobes *Fusobacterium nucleatum*, another core member of dental plaque, is believed to play a significant role in plaque maturation and dental plaque diversity (60). Its ability to co-cluster with various taxa serves as a physical bridge between early and late colonization of dental plaque organisms (60). Other hypotheses suggest that *F. nucleatum* acts as an indicator of establishing an anaerobic microenvironment and promoting plaque maturation (61–63), and has long been considered an initiating factor in periodontal disease. *F. nucleatum* tend to synergistically aggravate periodontitis and other systemic diseases when combined with *P. gingivalis* (52). However, despite being recognized as a periodontal pathogen, recent studies on *F. nucleatum* mostly discuss its role in tumorigenesis and immune evasion, with relatively few studies linking it to periodontal bone destruction.

The involvement of *P. gingivalis* and *F. nucleatum* in bone remodeling has always been a concern because of periodontitis. Periodontitis is a chronic inflammatory disease of the mouth that primarily develops from gingivitis. The accumulation of subgingival biofilm drives the progression from gingivitis to periodontitis, leading to the loss of periodontal supporting tissues. This progression occurs through continuous and complex interactions between the subgingival biofilm and the host's immune response (21, 64, 65). Different clinical phenotypes of periodontitis have been associated with oral flora exhibiting different characteristics (66). While commensal gut microbes also have the capacity to regulate osteoimmune processes in the alveolar bone (67), *P. gingivalis* and *F. nucleatum*, which are oriented toward the commensal oral microbiota, have been shown to independently contribute to alveolar bone remodeling, separate from the systemic microbiome (39).

Pathological mechanisms of alveolar bone resorption induced by periodontal pathogens

Bone homeostasis in periodontal tissues

Pathogenic bacteria flourish in the gingival sulcus owing to their immune resistance, and their secretion of virulence factors or parasitic behavior can stimulate the immune response in the gingival tissues. This immune response effectively transmits virulence signals to the bone marrow cavity, leading to enhanced bone marrow hematopoiesis (39), which is an important pathway for immune cell generation. Under the dual stimulation of dysregulated bacterial flora and an excessive immune response, the homeostasis of alveolar bone tissue is unbalanced. To further demonstrate the roles of *P. gingivalis* and *F. nucleatum* in bone resorption, it is necessary to briefly review the mechanisms of osteoimmunology and the key regulatory axis of bone homeostasis, the receptor activator of nuclear factor-kappa B ligand (RANKL)–receptor activator of nuclear factor-kappa B (RANK)–osteoprotegerin (OPG) axis.

The term 'osteoimmunology,' coined by Arron and Choi in 2000 (68), refers to the field that investigates the interactions between immune cells and bone cells. These interactions mediate skeletal development, modification, and homeostasis under both physiological and pathophysiological conditions. Both innate and adaptive immune cells participate in bone turnover through direct contact or expressing a range of immune molecules, such as cytokines, chemokines, and immunoglobulins.

Recently, a research group provided a cellular atlas of specific oral mucosal positions in health and disease conditions, revealing a distinct stromal–immune responsive axis that dysregulates under inflammatory conditions. This axis may be capable of mediating periodontal osseous tissues homeostasis (69). The major cell types within healthy gingival tissues include epithelial cells, endothelial

cells, fibroblasts, and immune cells. Within healthy gingival tissues, the immune category can be divided into five major clusters: T, NK, B/plasma, granulocyte, and myeloid cells, with T cells being the most numerous. T cells in gingival tissues can be subdivided into αβ $CD4^+$T, TH17, mucosal-associated invariant T (MAIT), αβ $CD8^+$T, γδ T, Treg, and NKT cells. The second largest population was myeloid linages, including neutrophils—which dominated this compartment—macrophages (Mφ), and myeloid dendritic cells (mDC). This result suggests that neutrophil-mediated innate immune responses are activated even when the periodontium is healthy. Sustained and highly coordinated neutrophil chemotaxis from the gingival vessels to the healthy gingival sulcus constitutes one of the major protective mechanisms against colonization by pathogenic microorganisms (65). Proper neutrophil monitoring targeting dental plaque biofilms has a dual benefit, conferring resistance to microbial colonization in periodontal tissues while maintaining an appropriate microbial composition for normal periodontal tissues function (70).

The epithelial and stromal cells present in the oral mucosa exhibit inflammation-related antimicrobial defense functions and can express transcriptional signatures of periodontitis inflammation and recruitment factors for neutrophils (69). This may be one of the reasons for the significantly elevated proportion of neutrophils in the oral mucosa. Stromal and immune cells can interact with each other through the expression of periodontitis susceptibility genes, becoming potential drivers of periodontal inflammation and immune cell over-recruitment, ultimately forming the basis of destructive hyperreactive immune responses (69).

Under healthy conditions, alveolar bone homeostasis is maintained by neutrophil-mediated innate immunity and T cell-mediated adaptive immunity. The cells and molecules involved stimulate bone remodeling cells, such as osteoblasts, osteoclasts, and their precursors, regulating their generation, development, function, and survival, ultimately maintaining bone homeostasis.

Osteoclasts and osteoblasts in bone homeostasis

Bone homeostasis is maintained by the coordinated action of mesenchymal-lineage-derived bone-forming osteoblasts and myeloid-lineage-derived bone-resorbing osteoclasts (71). Osteoclasts resorb osseous tissues by secreting hydrogen ions and lytic enzymes, while osteoblasts support mineralization by secreting unmineralized bone matrix and non-collagenous proteins (72).

Osteoclasts originate from monocyte–macrophage precursor cells, which are originally differentiated from HSCs. Studies have demonstrated that M1 macrophages contribute to osteoclastogenesis (73–75) under pathogenesis, and immature dendritic cells can develop into osteoclasts mediated by RANKL–RANK signaling (76, 77). Macrophage colony-stimulating factor (M-CSF) activates its cognate receptor c-Fms, inducing the expression of RANK on pre-osteoclasts (78), and consequently, induces the expression of NFATc1, a transcription factor that results in osteoclast proliferation and differentiation (79–82). Dendritic cell-specific transmembrane protein (DC-STAMP) (83, 84) and osteoclast stimulatory transmembrane protein (OC-STAMP) (85) are crucial for osteoclast maturation in a RANKL-dependent manner. RANKL-induced expression of the integrin-β3 subunit guarantees the αVβ3-mediated cell adhesion, which can seal certain podosomes, providing a critical microenvironment for osteoclast physiological functions such as motility and bone degradation/resorption (40–42). The secretion of cathepsin K, tartrate-resistant acid phosphatase (TRAP), and proteolytic enzymes occurs via the NFATc1-mediated RANKL signaling pathway (40–42).

Osteoblasts are mesenchymal lineage-originated osteogenic cells that eventually become bone-lining cells or osteocytes. Osteoblast differentiation and function are regulated by the transcription factors osterix and activating transcription factor 4 (ATF4), with the support of WNT, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), insulin-like growth factor (IGF) signaling,. A recent study revealed that RANKL contributes to the osteogenic direction of bone marrow mesenchymal stromal cell (MSC) differentiation (86), indicating that membrane-bound RANKL, as a member of TNF superfamily, possesses the capability to act as a receptor for vesicular RANK derived from mature osteoclasts (87) or apoptotic bodies (88), performing reverse signaling from osteoclasts to osteoblasts and contributing to osteogenesis, and consequently promote the coupling of bone resorption and formation (87). This specific function is regarded as bidirectional signaling transportation, which might be closely related to the intracellular proline-rich motif (87). Given that membrane-bound RANKL is an easy-clustering-featured molecular and the clustering of this type of receptor was proven to induce cell activation (89–93), the accumulation and clustering of RANKL seems to be the critical mechanism triggering RANKL reverse signaling (94). Vesicular RANK binding to RANKL activates osteoblasts and promotes osteogenesis through mammalian target of rapamycin complex 1 (mTORC1) signaling and Runt-related transcription factor 2 (Runx2) activation (87). However, OPG, as a competing receptor for RANKL, cannot stimulate osteoblast activation owing to its characteristic of disturbing RANKL clustering (94).

Thus, the RANKL–RANK–OPG axis produces essential signals that mediate intercellular communication in osteoclast–osteoblast coupling by regulating effector gene expression that drives cell proliferation, differentiation, maturation, function, and survival (Figure 1).

RANKL, RANK, and OPG

The receptor activator of nuclear factor-kappa B ligand (RANKL) and the receptor activator of nuclear factor-kappa B (RANK) were first discovered during the study of T-cell activation, and were found to be essential regulators of T cell and DC activation, thereby influencing T cell-mediated immune responses (95, 96). Subsequently, their critical role in osteoclast differentiation and bone remodeling was revealed (97, 98). RANKL, along with other biological mediators, regulates osteoclast differentiation, and under pathological conditions, it directly upregulates the expression of pro-osteoclastic cytokines and indirectly signals stromal-osteoblastic cells (99–101). Simultaneously, independent research groups identified RANKL as the osteoclast differentiation factor (ODF) from mouse myelo-monocytic cell lines and bone marrow-derived stromal cell lines (102, 103). Similarly, RANK was identified as the osteoclast differentiation factor receptor from mouse macrophage-like cell line

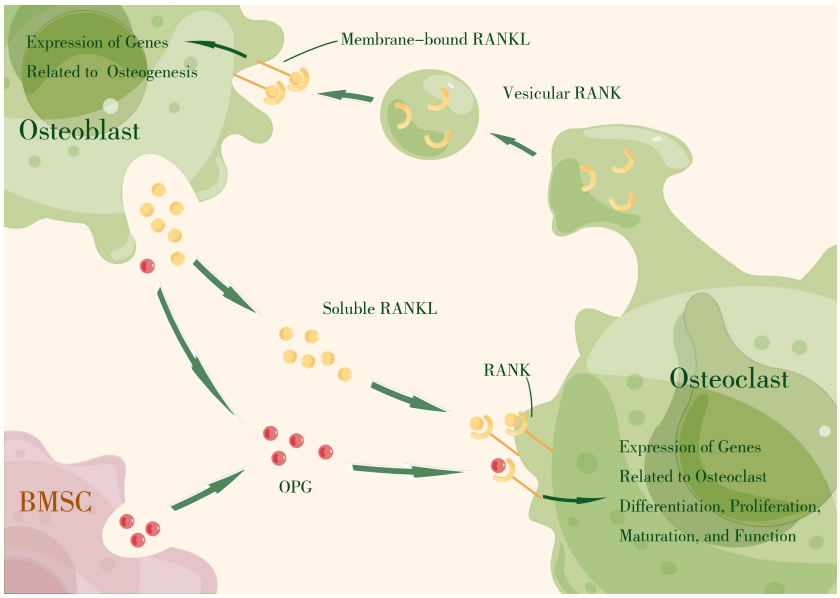

FIGURE 1
The RANKL–RANK axis produces essential signals that mediate intercellular communication in osteoclast–osteoblast coupling by regulating effector gene expression that drives cell proliferation, differentiation, maturation, function, and survival. OPG, primarily expressed by bone marrow stromal cells (BMSCs) and osteoblasts, acts as a decoy receptor, competitively binding RANKL to block RANKL–RANK interaction. (By Figdraw).

(104, 105). Additionally, OPG was discovered to be an inhibitor of osteoclast differentiation (106, 107). These findings laid the foundation for understanding the regulatory effect of the RANKL–RANK–OPG axis in bone homeostasis.

RANKL, encoded by the tumor necrosis factor superfamily member 11 (TNFSF11) gene, is a type II homotrimeric membrane protein. It is produced by a variety of cell types, including osteoblasts, osteocytes, bone stromal cells, and immune cells within skeletal tissues. RANKL exists in three isoforms, with RANKL1 and RANKL2 being membrane-bound forms (108) that can be converted to soluble forms through proteolytic shedding (109, 110). RANKL3 lacks a transmembrane domain and is considered a soluble form (108). The membrane-bound form of RANKL can basically fulfill the function of this protein, but the soluble form contributes to physiological bone remodeling (111).

RANK, encoded by the tumor necrosis factor receptor superfamily member 11a (TNFRSF11A) gene, is a type I membrane receptor mainly expressed by hematopoietic cells, but also by osteoclasts and their precursors (78). It can also be detected on the surface of mesenchymal stem cells (86, 112). The intracellular domain of RANK contains a binding site for TNF receptor-associated factor (TRAFs) (113), which regulates the expression of genes associated with osteoclast function through the TRAF pathway (114).

OPG, encoded by the tumor necrosis factor receptor superfamily member 11b (TNFRSF11B) gene, is a member of the TNFR superfamily. It is primarily expressed by bone marrow stromal cells and osteoblasts, but can also be expressed in B cells, DCs, and follicular DCs. OPG exists only in its secreted molecular form and acts as a decoy receptor, competitively binding RANKL to block RANKL–RANK interaction (106). Local OPG is considered more crucial for skeletal and immune homeostasis compared to circulating OPG (115). In addition to RANK and OPG, LGR4 has been identified as a third competitive receptor that negatively regulates osteoclastogenesis through the GSK3-β signaling pathway by restraining NFATc1 expression (116). However, the binding affinity between RANKL and LGR4 is thought to be lower than that between RANKL and OPG, making OPG the main inhibitor of RANKL–RANK signaling (117).

The RANKL–RANK–OPG axis is a crucial signaling pathway for maintaining bone homeostasis through osteoblast-osteoclast coupling, with the concentration of soluble RANKL playing the key role. Disruptions in this pathway, caused by various stimulatory signals targeting RANKL secretion, can lead to an imbalance in bone homeostasis and contribute to pathogenic bacteria-induced bone resorption. In the following section, we will explore the virulence factors of major periodontal pathogens and their abilities to interfere with RANKL secretion through specific pathways.

Virulence factors of periodontal pathogens and their pathogenic pathways

P. gingivalis and *F. nucleatum* possess various virulence factors that contribute to their pathogenicity. These factors play a significant role in the development and progression of periodontal disease. In recent years, there has been increased research interest in the role of bacterial extracellular vesicles (BEVs) in the pathogenic mechanisms of these microorganisms. We will explore these separately.

The virulence factors of *P. gingivalis*

P. gingivalis, an opportunistic pathogen and member of Socransky's red complex, produces several virulence factors that

induce detrimental effects on the host. The main virulence factors of *P. gingivalis* are LPS, fimbriae, and gingipains, which are crucial for the survival and metabolism of the bacterium.

LPS is an outer membrane component of gram-negative bacteria. It interacts with host cells, triggering a series of intracellular signaling events. LPS molecules consist of core polysaccharides, O-antigens, and lipid A; the latter two in *P. gingivalis* are highly diverse regions that confer antigenic differences and alter the interaction with pattern recognition receptors (PRRs), mainly TLR2, TLR4, and CD14. The disparity in LPS molecules depends on microenvironmental conditions (117) and sometimes leads to opposing immunological actions, immune evasion, or pro-inflammatory responses. This demonstrates that by manipulating the host immune activities, *P. gingivalis* can ensure its adaptation and survival (118, 119).

P. gingivalis LPS stimulates bone resorption in experimental models and activates various cell types, including mono-macrophages, endothelial cells, and epithelial cells, leading to the release of pro-inflammatory mediators and triggering immunoinflammatory reactions in the host tissues (120, 121). In vitro studies have also shown that *P. gingivalis* LPS increases the expression of pro-inflammatory cytokines in monocytes and macrophages, promoting bone resorption. In vivo, *P. gingivalis* LPS can activate mono-macrophages, endothelial cells, and epithelial cells through pathogen-associated molecular pattern (PAMP)-PRR recognition, resulting in the activation of cell signaling pathways like NF-kB and MAPK. These pathways ultimately stimulate the synthesis and release of IL-1, IL-6, TNF-α, NO, and other inflammatory mediators, contributing to a series of immunoinflammatory reactions in host tissues. In vitro, *P. gingivalis* LPS has also been proven to increase the expression of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-α, and IL-18, in monocytes and macrophages (122–125). These pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, have been shown to stimulate bone remodeling cells and influence the RANKL–RANK–OPG axis, thereby promoting bone resorption.

Fimbriae are slender filamentous protrusions on the surface of *P. gingivalis* that that play a role in adherence and have pro-inflammatory capabilities (126–128). These fimbriae can stimulate signal generation through either TLR2 or TLR4, activating two distinct intercellular pathways. This activation leads to the production of pro-inflammatory factors and matrix metalloproteinases (MMPs), including TNF-α, IL-1, IL-6, IL-8, and MMP-9 (129, 130). Fimbriae also promote the expression of cell adhesins such as ICAM-1 (131). Moreover, fimbriae can interact with and activate the binding capacity of Complement Receptor 3 (CR3) through "inside-out" signaling (132, 133), facilitating the internalization of *P. gingivalis* by macrophages and reducing IL-12 production, which may inhibit bacterial clearance (133). Notably, fimbriae play a significant role in inducing bone destruction in experimental periodontitis models (134), and may be a target for immunotherapy aimed at reduce bone resorption (135, 136).

Gingipains, a series of cysteine proteinases generated by *P. gingivalis*, can be categorized into two types: arginine-specific (Arg-X) and lysine-specific (Lys-X) gingipains (137, 138). These gingipains can be present either on the cell surface or secreted in a soluble form. They are considered vital virulence factors of *P. gingivalis* but exhibit contradictory effects on innate immunity. On one hand, gingipains can activate protease-activated receptors (PARs) and act as pro-inflammatory stimulators and enhancers (139, 140) in neutrophils (141), gingival fibroblasts, gingival epithelial cells (142) and T-cells (143). They stimulate the production of IL-6 in oral epithelial cells (142) and IL-8 in gingival fibroblasts (144), and promote the recruitment of polymorphonuclear neutrophils (PMNs) through complement system activation (145, 146). On the other hand, gingipains can hinder the host immunity by cleaving several TCRs (147) and proteolytically inactivating factors such as IFN-γ, IL-4, IL-5, and IL-12 (148–151), even reducing bacterial opsonization (152) to cause increased resistance to bactericidal activity in *P. gingivalis*. Apart from manipulating host immunity, gingipains have also been shown to facilitate the adherence and invasion of fibroblasts and gingival epithelial cells (153–155), as well as increase vascular permeability and hemin availability in periodontal tissues, creating favorable conditions for *P. gingivalis* growth (156).

The virulence factors of *F. nucleatum*

F. nucleatum, a member of the Socransky's orange complex, is a symbiont, opportunistic pathogen, and oncobacterium (157–159). Several virulence factors of *F. nucleatum* have been characterized, including FadA (160–164), which regulates adhesion and invasiveness; the heat-shock protein GroEL, which triggers host inflammatory factors (161); the endotoxin LPS, which activates NLRP3 and induces the release of inflammatory cytokines such as IL-1β (165); the metabolite butyric acid, which promotes the production of reactive oxygen species (ROS) and induces apoptosis of histocytes and immune cells (166); and multiple outer membrane adhesins (167) that can mediate the adhesion and coaggregation with various oral microbiota species, including *Streptococcus gordonii* (168), *Streptococcus sanguis* (169), *Streptococcus mutans* (170, 171), *Staphylococcus aureus* (172), *P. gingivalis* (173–177), and *Candida albicans* (178, 179). These virulence factors contribute to the expression of certain virulence factors, promote the formation and stability of plaque biofilm, and mediate the adhesion to immune cells (167).

F. nucleatum possesses various adhesins, which can be categorized into two types: amino acid inhibitors (e.g., RadD, CmpA, Aid1, FomA) associated with coaggregation with gram-positive bacteria, and lactose inhibitors (e.g., Fap2) associated with gram-negative bacteria. Coaggregation between *F. nucleatum* and *P. gingivalis* is mediated not only by a variety of adhesins but also by the capsular polysaccharide (CPS) and LPS, resulting in increased expression of virulence factors and altered energy metabolism in both species (180).

FadA is the most representative virulence factor of *F. nucleatum*, playing a crucial role in the adhesion and invasion of host cells. FadA exists in two forms: secretory and non-secretory. These two forms work together to regulate the adhesion and invasion of *F. nucleatum*. Through the interaction of the secretory autonomous transporter RadD and membrane occupation and recognition nexus protein 2 (MORN2) (181), *F. nucleatum* can invade gingival epithelial cells by binding to epithelial cadherin (E-cadherin). FadA can also help interact with the intracellular receptor retinoic acid-inducible gene I (RIG-I), activating the NF-

κB signaling pathway to induce inflammatory responses and cause periodontal tissues destruction. Furthermore, *F. nucleatum* can promote epithelial–mesenchymal transition of gingival epithelial cells, up-regulating Snail-1 expression, down-regulating E-cadherin expression, and disrupting the integrity of the gingival epithelium. This promotes the invasion of pathogenic bacteria into deeper periodontal tissues (182). Recent research has discovered that *F. nucleatum* can secrete FadA-containing outer membrane vesicles (OMVs) which stimulate inflammatory bone loss in RA via the FadA–Rab5a–YB-1 axis in macrophages (183), and may have similar effects in periodontitis.

Bacterial extracellular vesicles

BEVs are spherical nanostructures encapsulated in bacterial lipid bilayers. They range in size from 20 to 300 nm and contain various functional active substances secreted by bacteria, including bacterial virulence factors and sRNA (184). Since the first discovery of extracellular vesicles in *Vibrio cholerae* in 1967 (185), BEVs have been considered an important mode of physiological and pathological functions in bacteria. They facilitate bidirectional communication between bacteria–bacteria and bacteria–cells, in addition to direct contact (186), and play crucial roles in bacterial colonization, survival, inflammation, pathogenesis, and regulation of host metabolism and immunity (187–194). At present, the field of cancer-related research believes that BEVs in the tumor microenvironment can be used as a new target for the diagnosis and monitoring of tumors and related diseases (195). Although research on BEVs in the oral pathological microenvironment is limited, these vesicles have the potential to provide valuable insights into the pathogenesis and pathological state of oral diseases, as well as the development of more efficient treatment methods.

Pathogenic pathways of virulence factors

The interaction between the oral microbiome (including living bacteria, virulence factors, and BEVs) and human immunity, known as the oral host–microbial interactome, promotes homeostasis under healthy conditions. The commensal microbiota educates and facilitates the immune system (196), imprinting innate and adaptive immunity memory to mount rapid and effective resistance against massive PAMP invasion. However, this immune memory can lead to overreactions and become a major cause of tissue destruction, including periodontal bone loss (6).

Studies have shown that dental biofilm plaque-induced bone loss in the periodontal tissues has an 'effective radius of action' known as the range of effectiveness. This range typically spans from 0.5 mm to 2.7 mm, with 2.5 mm being the precise measure (197–199). The constant distance between the base of the gingival groove and the alveolar crest, known as the biological width, is approximately 2 mm, falling within the range of effectiveness. This indicates that antigens and virulence factors present in biofilm plaque can traverse the epithelial barrier of the gingival tissues and penetrate the underlying connective tissues. Consequently, this triggers the release of paracrine signaling molecules, thereby affecting the balance of alveolar bone remodeling (65, 200). Research has demonstrated that the stimulation of PAMPs derived from subgingival plaque can elicit characteristic activation signals of bone marrow hematopoiesis, indicating the generation of immune cells derived from the myeloid lineage and the activation of associated immune responses (39). Meanwhile, innate immune cells present in the gums can uptake bacterial antigens from subgingival plaque and migrate to adjacent cervical lymph nodes, where they present antigens to activate the adaptive immune response. As a result, cytokines and immune cells, including T cells and memory T cells, may disseminate to the local gum tissues or even the entire body through the circulatory or lymphatic system (39, 201).

The oral microbial–host interactome can also transmit signals that extend beyond local tissues and contribute to the development of extra-oral comorbidities by initiating systemic inflammation or ectopic colonization in distant parts of the digestive tract (28, 36, 51, 196). Interestingly, a recent study suggested that the majority of healthy individuals do not exhibit detectable microbes in their blood, and even when a few species are detected, the microbial community patterns differ among various samples, with no apparent correlation between microbial species and the phenotype of healthy individuals (202). This implies that local disruption of the mucosal barrier serves as the initial step towards systemic comorbidities. Transient bacteremia facilitates the dissemination of microorganisms, such as oncobacteria, along with their virulence factors, to susceptible sites, thereby initiating or exacerbating disease progression at multiple sites. On a positive note, the microbial profile of gingival tissues in pathological conditions holds potential for aiding the diagnosis and treatment of extra-oral complications through blood microbial detection.

Pathological osteoimmunity: activation of immune cells and cytokines

Under pathophysiological conditions, the subsets of immune cells that exist in a healthy state, such as T/NK, B/plasma, and granulocyte/myeloid cells, do not undergo significant changes in their overall categories. However, there are alterations in their proportions, particularly an increase in neutrophils and plasma cells (69).

The oral mucosal surface constantly faces microbial challenges, and neutrophils play a crucial role in maintaining alveolar bone homeostasis through innate immunity (203). Gingivitis is characterized by decreased neutrophils and bone activation factors, suggesting protective responses of the gingival tissues and bone during inflammation (66). However, as gingivitis progresses to periodontitis, there is an excessive inflammatory response leading to an increase in the number of neutrophils in local tissues. The quantity of neutrophils in the gingival tissues is more closely associated with the health or disease status of the periodontal tissues rather than their bactericidal function, which can be compensated by innate immune cells such as macrophages (204). Numerous studies have shown a positive correlation between the number of neutrophils in gingival tissues and the severity of periodontitis (205–207). In chronic periodontitis, dysfunctions in chemotactic accuracy, increased recruitment, and prolonged survival of neutrophils contribute to their extensive infiltration in periodontal tissues (204, 208, 209). These spontaneous hyperreactive neutrophils release various inflammatory factors (such as TNF, IL-1β, and IL-8), cytotoxic mediators, matrix metalloproteinases, and RANKL, which

worsen periodontal tissues damage and bone resorption (210–213). Neutrophils can also migrate to the lymph nodes, where they interact with DCs to regulate antigen presentation and activate adaptive immunity (214). In the presence of CCL20, neutrophils can induce Th17 recruitment to inflamed tissues (215). They also promote B cell survival, proliferation, and differentiation into plasma cells by secreting B lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) (216, 217). Excessive neutrophils contribute to the progression of periodontitis and skeletal tissues destruction by initiating periodontal tissue lesions, exacerbating immune responses, and secreting local inflammatory factors and osteoclast-related factors. However, neutrophils deficiency in gingival tissues can also lead to periodontitis (218–220). Animal experiments have shown that impaired neutrophils recruitment associated with leukocyte adhesion deficiency Type I leads to increased periodontal inflammation, bone loss, and abnormal expression of IL-17 (221). This phenomenon might be related to a homeostasis mechanism of neutrophils recruitment, clearance, and generation, known as 'neutrostat,' which involves the IL-23–IL-17–granulocyte-colony stimulating factor (G-CSF) negative feedback loop (222). Impaired neutrophils recruitment results in unrestricted expression of IL-23, IL-17, and G-CSF in local tissues, leading to excessive inflammation and tissue damage (221).

Plasma cells are also significantly increased in patients with periodontitis compared to that in healthy individuals. The majority of plasma cells express IgG, while a minority express IgA (69). IgG is the main force in humoral immunity; it undergoes opsonization and antibody-dependent cell-mediated cytotoxicity (ADCC), and can activate the complement system through the classical pathway. These autoimmune responses may be the main factors contributing to periodontal destruction. Plasma cells may play a role in neutrophils recruitment by binding to the IgGFcR on the surface of neutrophils.

B cells have a dual role in periodontitis-related bone loss, which may depend on the activated B cell type. Certain B cell subsets exacerbate the severity of periodontal bone loss. In addition to IgG- and IgA-generated B cells, IgD- and IgM-generated B cells can also be associated with bacteria-induced periodontal bone loss, possibly through RANKL expression (223, 224). Memory B cells can promote osteoclast differentiation and maturation by expressing RANKL and various pro-inflammatory factors, such as TNF, IL-6 and IL-1β, and by increasing Th1 and Th17 production (225–228). Recent studies have highlighted the role of B cell activating factor (BAFF) in promoting periodontitis development by enhancing inflammatory conditions and macrophages activity (229). Conversely, regulatory B cells, also known as B10 cells, can reduce bone loss by upregulating IL-10 expression and downregulating IL-17 and RANKL expression (230–232).

In addition to neutrophils and B cells, T cells can be activated by antigens from *P. gingivalis* and *F. nucleatum* via TCR recognition and can differentiate into various subsets. Under pathologic conditions, T cells can affect bone remodeling by directly increasing the expression of pro-osteoclastic cytokines such as RANKL or indirectly signaling stromal–osteoblastic cells (99–101). Among all T cell subsets, γδ T cells (233); regulatory T cells (Treg) (234, 235); and helper T cells (Th, also known as $CD4^+$T cells), including Th1 and Th2 (236, 237), Th9 and Th22 (238), Th17 (239), may be more closely associated with alveolar bone resorption (237).

Th1 and Th2 cells have been implicated in bone resorption in periodontitis, although the specific mechanisms have not been fully elucidated. The presence or absence of Th1 and Th2 cells may both contribute to bone resorption (240–243). The Th1/Th2 ratio was historically considered an important factor in evaluating the degree of bone resorption in periodontitis, as Th1 cells were believed to mediate the establishment of early periodontitis lesions, while Th2 cells gradually became quantitatively dominant as periodontitis progressed (236, 237). Interestingly, Th2 cells can promote the transformation of B cells into plasma cells by secreting IL-4, which may explain the increased proportion of plasma cells in gingival tissues under pathological conditions.

Current studies on bone remodeling have shifted focus from the Th1/Th2 balance to the Th17/Treg paradigm (67, 244, 245). Th17 has been closely associated with periodontitis and bone loss since their discovery (246, 247), and recent studies have shown that dysbiotic microbiomes activate Th17 cells to mediate oral mucosal immunopathology and periodontitis-induced bone destruction (239). Previous studies have demonstrated increased levels of Th17 and IL-17 in gingivitis and periodontitis (248–252), which are not necessarily related to the active or inactive stage of periodontitis (253–255). IL-23, a cytokine that promotes Th17 differentiation, is also highly expressed along with IL-17 in periodontitis (248, 253). Th17 cells, derived from naive T cells (also called Th0 cells) after stimulation by antigen presentation or pro-inflammatory factors (such as IL-1β, IL-6, and IL-23), are the primary source of IL-17 and can express other pro-inflammatory factors such as IL-21, IL-22, and TNF (256, 257). Although IL-17 may not directly act on the RANKL-macrophage colony-stimulating factor (M-CSF)-osteoclast culture system (258), it can promote osteoclastogenesis through the expression of RANKL mediated by osteoblastic cells (259). Interestingly, Th17-related neutrophil mobilization in gingival tissues can inhibit *P. gingivalis*-induced periodontal bone loss (260, 261), and IL-17 receptor α-deficient mice show reduced cytokine-dependent recruitment of neutrophils and increased bone resorption (262, 263), indicating that Th17 cells also possess bone-protective potential through neutrophil mobilization. Tregs, a subset of $CD4^+CD25^+Foxp3^+$ T cells with anti-inflammatory and homeostatic functions, can secrete IL-10, IL-12, and TGF to achieve negative immune regulation. The presence of Tregs in periodontitis may represent a compensatory mechanism to mitigate excessive tissue damage caused by immune responses (237). Some studies have found that Tregs can improve pathological bone resorption through the CCR4–CCL22 pathway (234, 235). However, Tregs are highly plastic and can lose their immunosuppressive ability in chronic periodontitis (264). They may also differentiate into Th17 cells during the mid-stage of periodontitis (265). Therefore, the Th17/Treg ratio is a reasonable parameter to evaluate the dysbiotic microbiome-mediated periodontal inflammation status to a certain extent.

In addition to T cells, B cells, and neutrophils, other immune cells may also play a role in bone remodeling. NK cells in rheumatoid arthritis can promote osteoclastogenesis by expressing

RANKL and M-CSF (266) and inhibiting osteoblast generation through a pro-apoptotic pathway (267). The degree of mast cell degranulation in chronic periodontitis is proportional to the severity of periodontal disease (268, 269), possibly owing to their ability to secrete IL-17 (270) and indirectly increase RANKL expression through IL-33 secretion (271). DCs synthesize and secrete a series of cytokines to increase RANKL expression (272–274) and immature DCs can differentiate into mature osteoclasts through the RANKL–RANK–M-CSF axis (76, 77, 274). Macrophages are recognized as immune cells that are closely related to osteoclasts. Macrophages are homologous to osteoclasts, as mentioned earlier, and in vivo, macrophages are able to participate in osteoclastogenesis through the RANKL–RANK–OPG axis, with the assistance of M-CSF.

Concluding remarks and future perspectives

Oral health and systemic status are intertwined, with lesions in one affecting the other. Failure to address this cycle can lead to the progression of systemic diseases. The oral microbial community plays a crucial role in oral health, and any disruption in the ecological guilds can contribute to the development of oral diseases, including periodontal disease and the subsequent loss of periodontal hard tissues, which poses a significant threat to oral and systemic health.

The imbalance in osteoblast–osteoclast coupling, mediated by the RANKL–RANK–OPG axis, is at the core of alveolar bone remodeling disruption. Excessive immunity activation triggered by host–microbe interactions appears to be the primary reason for this imbalance. Key members in certain ecological guilds, such as *P. gingivalis* and *F. nucleatum*, drive periodontal inflammation with. Virulence factors from these pathogens activate the host immune system through local diffusion, lymphatic pathways, and blood transmission. Under chronic inflammatory conditions, continuous host–microbe interactions lead to an exaggerated immune response, resulting in periodontal tissues destruction and alveolar bone resorption (Figure 2).

Osteoimmunity involves intricate interactions between immune cells and molecules. The excessive osteoimmune response activated by the major functional microbiota associated with periodontitis cannot be solely attributed to changes in the proportion or function of individual immune cells. The phenotypes of the periodontal immune network must be established by studying the local and systemic immune status in the context of periodontal inflammation. Conversely, periodontal immunophenotypes reflect the characteristics of local ecological guilds. While the human body's immunological characteristics are relatively clear and specific compared to the complexity of symbiotic microbial communities, the application of immunophenotypes holds promise as a straightforward method to evaluate the stability of plaque biofilms and study the symbiotic network of complex ecological guilds.

Although significant progress has been made in understanding the local immunophenotype of periodontitis and the role of pathogenic microorganisms, there are still gaps to be filled. Detailed investigations are needed to interpret the pathogenic effects of periodontal microorganisms. While the role of *P. gingivalis* in promoting alveolar bone resorption is well-established, there is limited research on the role of *F. nucleatum*, which has been recently focused as an oncobacterium in gastrointestinal tumors but not as a periodontal pathogen in alveolar bone resorption. Additionally, as the vital effect of extracellular vesicles gradually come into sight, the contents, secretion characteristics, and roles of *P. gingivalis* and *F. nucleatum* vesicles in bone remodeling are yet to be clarified. Furthermore, the contribution of other members within the periodontal pathogenic ecological guilds to alveolar bone resorption remains to be clarified.

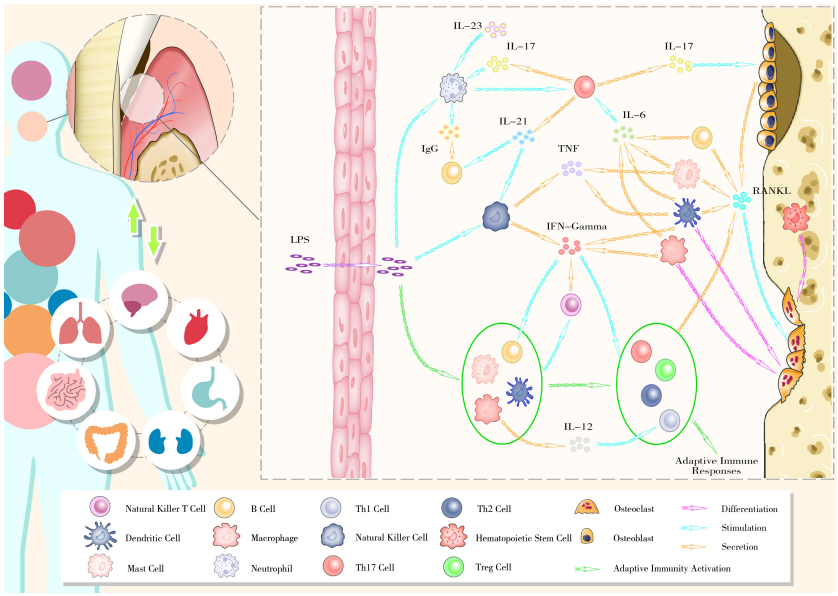

FIGURE 2
The bidirectional relationship between oral health and general well-being and the gingival immunophenotype of periodontitis.

Although the blood of healthy individuals is typically considered sterile, the presence of BEVs is a possibility. These vesicles may participate in immune system education in healthy individuals. However, once susceptible disease sites emerge, BEVs could potentially contribute to disease development even before the mucosal barrier is destroyed. Exploring the existence, content, and functions of vesicles in the blood of healthy individuals is an important area of investigation. Another aspect that remains to be elucidated is the immunophenotype of periodontitis. It is imperative to clarify the interaction network of immune cells and molecules in the disease state, identify the main functional groups, and screen characteristic high-expression cell phenotypes.

Maintenance of periodontal bone homeostasis is crucial in oral treatments that rely on physiological bone remodeling, such as periodontal therapy, orthodontic treatment, and implant restoration. How to block the progression of periodontitis, and restore lost bone, accelerate orthodontic effects by regulating bone remodeling, and how to reduce peri-implantitis to increase the success rate of implant surgery are all research focuses as well as difficulties in stomatology. However, these treatments often introduce various external stimuli to the teeth and periodontal tissues, resulting in oral hygiene challenges and disturbances to the periodontal microenvironment. To achieve optimal therapeutic outcomes, researchers should not simply focus on regulating the function of osteoblasts or osteoclasts, but aim to correct the unbalanced periodontal microenvironment and restore it to a healthy physiological state. By addressing these factors, some of the aforementioned clinical problems may find solutions.

Enabling patients to aesthetics and function healthfully is the fundamental principle of stomatology research. Modern medicine demands that dental practitioners not only control patients' oral health during the short-term treatment and follow-up, but also maintain their lifelong well-being, which aligns with the WHO's '8020' goal, striving for improved oral health for the overall benefit of humanity.

Author contributions

JX: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. LiY: Resources, Writing – review & editing. SY: Resources, Writing – review & editing. ZY: Resources, Writing – review & editing. LuY: Funding acquisition, Supervision, Writing – review & editing. XX: Conceptualization, Supervision, 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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Sylvie Bertholet,
GSK Vaccines, United States

REVIEWED BY
Daniel González Maglio,
University of Buenos Aires, Argentina
Niels Odum,
University of Copenhagen, Denmark
Tej Pratap Singh,
University of Pennsylvania, United States
Emily Avitan-Hersh,
Clinical Research Institute, Israel

*CORRESPONDENCE
Peter Wolf
peter.wolf@medunigraz.at

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Modulation of the skin microbiome in cutaneous T-cell lymphoma delays tumour growth and increases survival in the murine EL4 model

Saptaswa Dey[1], Pablo Augusto Vieyra-Garcia[1], Aaroh Anand Joshi[1], Slave Trajanoski[2] and Peter Wolf[1,3]*

[1]Department of Dermatology and Venereology, Medical University of Graz, Graz, Austria, [2]Core Facility Computational Bioanalytics, Medical University of Graz, Graz, Austria, [3]BioTechMed Graz, Graz, Austria

Cutaneous T-cell lymphomas (CTCL) are a group of lymphoproliferative disorders of skin-homing T cells causing chronic inflammation. These disorders cause impairment of the immune environment, which leads to severe infections and/or sepsis due to dysbiosis. In this study, we elucidated the host-microbial interaction in CTCL that occurs during the phototherapeutic treatment regime and determined whether modulation of the skin microbiota could beneficially affect the course of CTCL. EL4 T-cell lymphoma cells were intradermally grafted on the back of C57BL/6 mice. Animals were treated with conventional therapeutics such as psoralen + UVA (PUVA) or UVB in the presence or absence of topical antibiotic treatment (neomycin, bacitracin, and polymyxin B sulphate) as an adjuvant. Microbial colonisation of the skin was assessed to correlate with disease severity and tumour growth. Triple antibiotic treatment significantly delayed tumour occurrence ($p = 0.026$), which prolonged the survival of the mice ($p = 0.033$). Allocation to phototherapeutic agents PUVA, UVB, or none of these, along with antibiotic intervention, reduced the tumour growth significantly ($p = 0.0327$, $p \leq 0.0001$, $p \leq 0.0001$ respectively). The beta diversity indices calculated using the Bray–Curtis model showed that the microbial population significantly differed after antibiotic treatment ($p = 0.001$). Upon modulating the skin microbiome by antibiotic treatment, we saw an increase in commensal *Clostridium* species, e.g., *Lachnospiraceae* sp. ($p = 0.0008$), *Ruminococcaceae* sp. ($p = 0.0001$)., *Blautia* sp. ($p = 0.007$) and a significant reduction in facultative pathogens *Corynebacterium* sp. ($p = 0.0009$), *Pelomonas* sp. ($p = 0.0306$), *Streptococcus* sp. ($p \geq 0.0001$), *Pseudomonas* sp. ($p = 0.0358$), and *Cutibacterium* sp. ($p = 0.0237$). Intriguingly, we observed a significant decrease in *Staphylococcus aureus* frequency ($p = 0.0001$) but an increase in the overall detection frequency of the *Staphylococcus* genus, indicating that antibiotic treatment helped regain the microbial balance and increased the number of non-pathogenic *Staphylococcus* populations. These study findings show that modulating microbiota by topical antibiotic treatment helps to restore microbial balance by diminishing the

numbers of pathogenic microbes, which, in turn, reduces chronic inflammation, delays tumour growth, and increases survival rates in our CTCL model. These findings support the rationale to modulate the microbial milieu during the disease course of CTCL and indicate its therapeutic potential.

KEYWORDS

CTCL (cutaneous T-cell lymphoma), skin microbiome, PUVA (combination of psoralen and long-wave ultraviolet radiation), UVB 311 nm, topical (local) antibiotics, adjuvant therapy, *Staphylococcus aureus*

Introduction

Cutaneous T-cell lymphoma (CTCL) represents a heterogeneous group of non-Hodgkin lymphomas characterised by the infiltration and expansion of neoplastic mature T cells, primarily in the skin (1). In these lymphoproliferative disorders, an impaired immune system is primarily responsible for more recurrent infections, chronic inflammation, and the suppression of antitumor activity (2, 3). External factors have been proposed as one of the key reasons for the aggravation of the disease (3–5). A recent hypothesis proposes that microbial antigens play a role in promoting chronic inflammation and malignant cell transformation (6); a similar role has been described in several other skin diseases, such as atopic dermatitis, psoriasis, and acne vulgaris (7–10). Advances in modern technologies, and especially in the sequencing methods such as 16s sequencing and whole-genome shotgun sequencing (11), equip researchers with more tools they can use to understand changes in skin-microbial populations up to the species level in different CTCL disease stages and during therapeutic interventions.

Intact human skin provides an effective barrier against environmental effects. The superficial layer of human skin is colonised by a plethora of microorganisms, comprising bacteria, archaea, fungi, and viruses, which form a mutualistic symbiosis. The balance of this heterogenous microbial community is essential for protecting the organism against invading pathogens and the breakdown of natural products (12). The microbiota can modulate the production of various anti-microbial peptides (AMPs), cytokines, and chemokines in the skin (5, 9, 12, 13). Commensal skin microbiota, including bacteria such as *Staphylococcus hominis* (SH) or *S. epidermidis* (SE) (14), ubiquitously colonise human skin and are non-harmful to humans. In contrast, *Staphylococcus aureus* (SA) is associated with various pathogenic skin conditions in humans. SA-derived enterotoxins have evoked particular interest because they belong to a class of "superantigens", which are exceptionally potent activators of T cells. If the skin barrier is breached or the immune system is impaired due to the expansion of malignant T cells in the skin (as in the case of CTCL), this delicate balance between commensal and pathogenic microorganisms is disrupted; this, in turn, triggers chronic inflammation which aggravates the disease phenotype. Furthermore, a chronic pro-inflammatory micro-environment has been shown to promote malignant T cell proliferation (2, 6, 15).

Most patients with early-stage mycosis fungoides (MF), the most common form of CTCL, characteristically present with cutaneous patches and plaques. These patients are treated with phototherapy to clear skin lesions and increase their disease-free survival rates. Narrowband UVB (NB-UVB) and psoralen plus UVA (PUVA) are the two primary forms of phototherapy used to treat these CTCL patients (16–21). Regarding leukemic CTCL (L-CTCL), extracorporeal photopheresis (ECP) is usually prescribed (21, 22) as therapy. UV radiation has been experimentally proven to have profound qualitative and quantitative influences on the composition of the skin microbiome (23, 24). Moreover, a study from our lab showed that the skin microbiome regulates the effect of UV radiation on cellular response and immune function (25). For these reasons, understanding the skin microbiome and host-microbial interactions during the phototherapeutic treatment regime in CTCL is extremely important. For instance, we have recently shown that PUVA induces local type 1 interferon production and antitumor response in CTCL patients, and intriguingly, rescued deficient interferon production may help in fighting infection with *Staphylococcus aureus* (SA), a driving factor in the pathophysiology of the disease (26).

The dysbiosis observed in the microbiome of CTCL patients is considered more than mere coincidence. Studies suggest that alterations in the microbiome can influence immune dysregulation, inflammation, and the progression of CTCL (4, 9, 15). Furthermore, microbial metabolites have been found to modulate T-cell responses and affect tumour microenvironments, potentially impacting disease outcomes (27, 28). The cause of malignant cell transformation in CTCL remains to be elucidated, but multiple factors are associated with the disease progression, such as chromosomal aberrations, oncogenic mutations, environmental factors, and the microbiome. Increased STAT3/5 signalling, resulting from copy number gains on chromosome 17q, and the loss of negative regulators along the JAK/STAT pathway, such as suppressors of STAT1 and SOCS1, are possibly key genetic factors that contribute to increased clonal expansion in leukemic cutaneous T-cell lymphoma. Due to these genetic abnormalities in

the T cell, the immune defence system becomes severely impaired, and patients with advanced forms of the disease often die because of infection rather than lymphoma. Previous studies have demonstrated the crucial role of the microbiome in modulating disease activity in CTCL. Notably, Fanok et al. (29) showed in a lymphoma mouse model comparing germ-free vs. SPF animals that the presence of microbiota can significantly accelerate disease progression.

Their findings indicate that CTCL development is markedly slower under sterile conditions, highlighting the detrimental impact of bacterial presence on disease severity. This pivotal study underscores the interaction between the microbiome and immune response in CTCL, aligning with our current investigation. Our research, employing a distinct model and interventions, further explores this link, providing additional evidence that modulating the skin microbiome through antibiotics and phototherapy can delay tumor growth and improve survival outcomes. These independent yet complementary approaches reinforce the concept that targeting microbial influences offers a promising avenue for therapeutic intervention in CTCL (29). Intriguingly, severe bacterial infections are often seen in CTCL patients because malignant T cells also may induce significant changes in the skin architecture; this, in turn, impairs the skin barrier function, increasing the patient's susceptibility to bacterial infections and their spread. Recent research, has provided insight into this process, showing how primary malignant T cells induce significant changes in the expression of skin barrier proteins in CTCL through cytokine-mediated JAK/STAT signaling, highlighting the intricate relationship between malignant T cell activity and compromised skin barrier integrity in the disease pathology of CTCL (30). SA has also been presumed to play a tumor-promoting role since antibiotic treatment specific to SA has been shown to have an inhibitory effect on the tumor burden in some patients. This observation aligns with the findings by Gluud et al. (31), further supporting the notion of targeted antibiotic therapy as a viable approach to modulate disease progression in CTCL (32).

This study was carried out to elucidate the host-microbial interaction occurring during the phototherapeutic treatment of CTCL, to support the rationale for modulating the microbial milieu during the disease course of CTCL, and to indicate the therapeutic potential of such modulation.

Results

Modulation of the skin microbiome with topical triple antibiotic treatment delays tumour occurrence and increases survival rates in a cutaneous T-cell lymphoma mouse model

We intradermally grafted murine EL4 T-cell lymphoma cells onto the backs of the C57BL/6 mice and treated them with psoralen plus UVA (PUVA) or UVB in the presence or absence of topical triple antibiotic treatment. We then measured the microbial populations and correlated these with the disease severity and tumour growth (Figure 1A). Microbial modulation by topical triple antibiotic treatment reduced the tumour occurrence from 93% in the vehicle group to 60% in the antibiotic-treated group (p = 0.0226) (Figure 1B). Results of a Kaplan–Meier survival analysis of the murine CTCL model showed a significant increase in survival rates, namely 14.3% in the vehicle group as compared to 46.6% in the topical antibiotic-treated group (p = 0.0339) (Figure 1C). Adjuvant therapy of antibiotic intervention along with PUVA, UVB, or neither of these showed reduced tumour occurrence and delayed tumour growth (Figure 2A). The tumour emergence rates in the CTRL, PUVA, and UVB groups were 80%, 60%, and 40%, respectively, in antibiotic-treated mice as compared to 75%, 100%, and 100% in vehicle-treated mice (Figures 2B–D). Tumours were also fast-growing in vehicle-treated mice as compared to mice receiving antibiotic intervention (Figure 2A). A comparison of the tumour growth curve plotted as tumour diameter in (mm) for the vehicle- vs triple antibiotic-treated group with the control (Figure 2E), PUVA (Figure 2F), and UVB (Figure 2G) groups showed a remarkably significant reduction in the tumour diameter in all three groups (CTRL, $p \leq 0.0001$; PUVA, p = 0.0327; UVB, $p \leq 0.0001$). Furthermore, the results of the AUC (Area Under the tumour growth Curve) analysis show that antibiotic application reduced the growth rate of the tumour in all three groups (i.e. Control, PUVA, and UVB) (Supplementary Figures 1A–C). Finally, the antibiotic intervention increased survival rates regardless of the phototherapeutic regime, as shown by the Kaplan-Meier survival analysis results (Supplementary Figures 2A–C) for the control (25%), PUVA (20%), and UVB (0%) subgroups within the vehicle-treated group and the control (40%), PUVA (40%), and UVB (60%) subgroups within the antibiotic-treated group.

Antibiotic intervention of CTCL tumours alters the skin microbial diversity and richness irrespective of the phototherapeutic regime

We analysed the microbial population in the lesional skin of our CTCL model by performing 16s microbial sequencing. Results of a beta diversity analysis performed by non-metric multidimensional scaling (NMDS) with the Bray-Curtis model show a significant difference in microbial population upon antibiotic treatment compared to the vehicle-treated group (p=0.001) (Figure 3A). A comparison of the microbial diversity indices (i.e., Shannon diversity index) shows that microbial diversity was slightly increased in the antibiotic-treated group as compared to the vehicle-treated group; this higher diversity and evenness indicated healthier skin (Figure 3B). We performed a Linear discriminant analysis Effect Size (LefSe) analysis which shows a higher number of facultative pathogens, e.g. *Pelomonas* sp. (p = 0.0306) *Corynebacterium* sp. (p = 0.0009) (Supplementary Data 1). These species have been reported to be associated with pain signatures in CTCL lesional skin (33) (Figures 3C, D). The cladogram shows a taxonomic representation of statistically and biologically consistent differences between the vehicle- and antibiotic-treated groups (Figure 3C).

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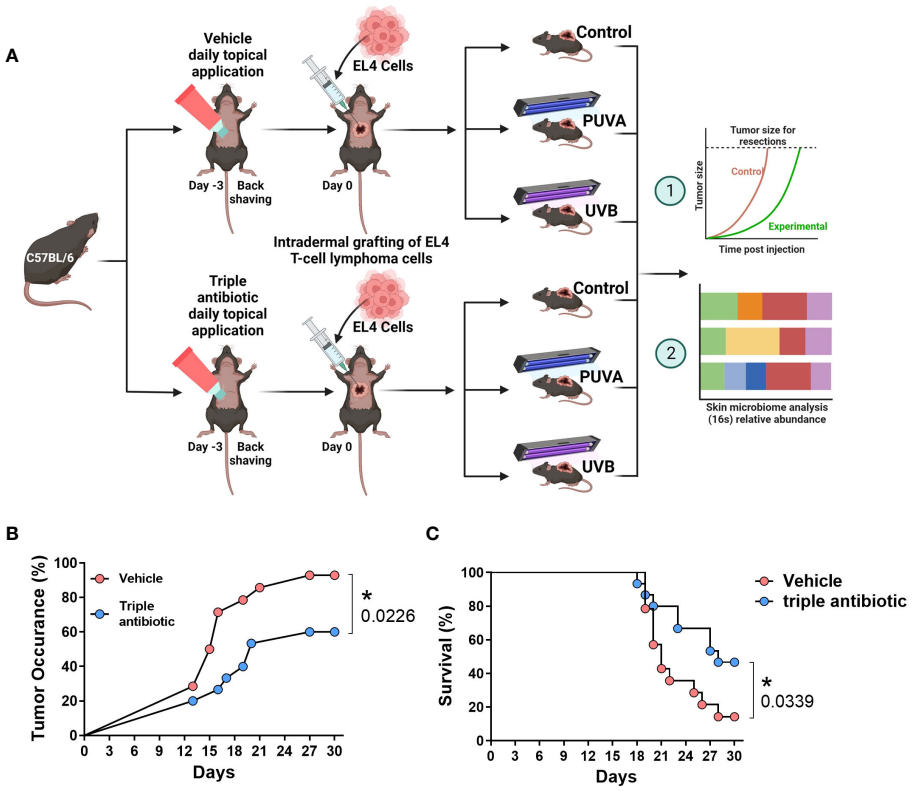

FIGURE 1
Modulation of the skin microbiome with topical triple antibiotic intervention delays the tumour occurrence and increases the survival rate in the cutaneous T-cell lymphoma mouse model. **(A)** Graphical schematic of the mouse experimentation model. **(B)** Tumour occurrence (%) upon topical triple antibiotic intervention as compared with the vehicle-treated control group (*n* = 15). **(C)** Kaplan–Meier survival analysis results comparing the effect of topical triple antibiotic intervention in the antibiotic-treated group and the vehicle-treated control group (*n* = 15). Survival rates were calculated based on the day mice were sacrificed. Individual mice were sacrificed once the tumour reached 10 mm in diameter, as defined in the study protocol.

Results of a beta diversity analysis of (NMDS) 16S microbial sequencing data performed with the Bray–Curtis model show this difference and enable a comparison to be made between the CTRL groups with or without antibiotic intervention (Figure 4A). However, the reduction in beta diversity observed in the PUVA and UVB groups in the presence or absence of antibiotic intervention (Figures 4B, C) was not unexpected, as UV therapy is known to be bactericidal. Furthermore, the microbial richness analysis results show a reduction in microbial richness in all three groups (CTRL, PUVA, and UVB) upon antibiotic intervention (Figure 4D). An analysis of the Shannon diversity indices, however, interestingly shows an increase in the diversity of the antibiotic intervention group when combined with PUVA or UVB (Figure 4E) but in contrast there is decrease in antibiotic treated monotherapy group, which might be due to the low sample size (n=4) in this group. Because the Shannon diversity index is a calculation of the number of species in a community and provides a measure of the evenness, the improved Shannon indices seen in the PUVA and UVB groups indicate that, although the species richness is reduced, the species evenness improved when both phototherapy and antibiotic treatment were used. These findings indicate a lower probability that a single species will dominate the CTCL lesional skin when antibiotic intervention is used with a phototherapeutic treatment regimen. If we examine the relative abundance of the 18 most abundant genera (Figure 4F) (histogram; different genera represented by different colours) based on the 16S sequencing data, an overall decrease in the abundance of *Streptococcus* sp. and an increase in the abundance of *Staphylococcus* sp. is evident in the antibiotic intervention group.

The results of the LefSe analysis of data from the phototherapeutic treatment groups in the presence or absence of antibiotic intervention show a significant difference (LDA score [log 10] 3) in terms of their taxonomic features: *Corynebacterium* sp. is more highly abundant in the vehicle-treated groups of CTRL, PUVA, and UVB (Figures 5A–C) than the respective groups with antibiotic intervention. The cladogram shows the statistically and biologically consistent differences in the taxonomic representation among the control, PUVA, and UVB groups (Figures 5D–F).

Topical triple antibiotic intervention on CTCL lesional skin alters the microbial population by significantly reducing facultative pathogens and increasing skin commensals

A multivariate analysis performed by running a linear models (MaAsLin) analysis on 16s data revealed that antibiotic intervention reduced the detection frequency of *Staphylococcus aureus* in all three

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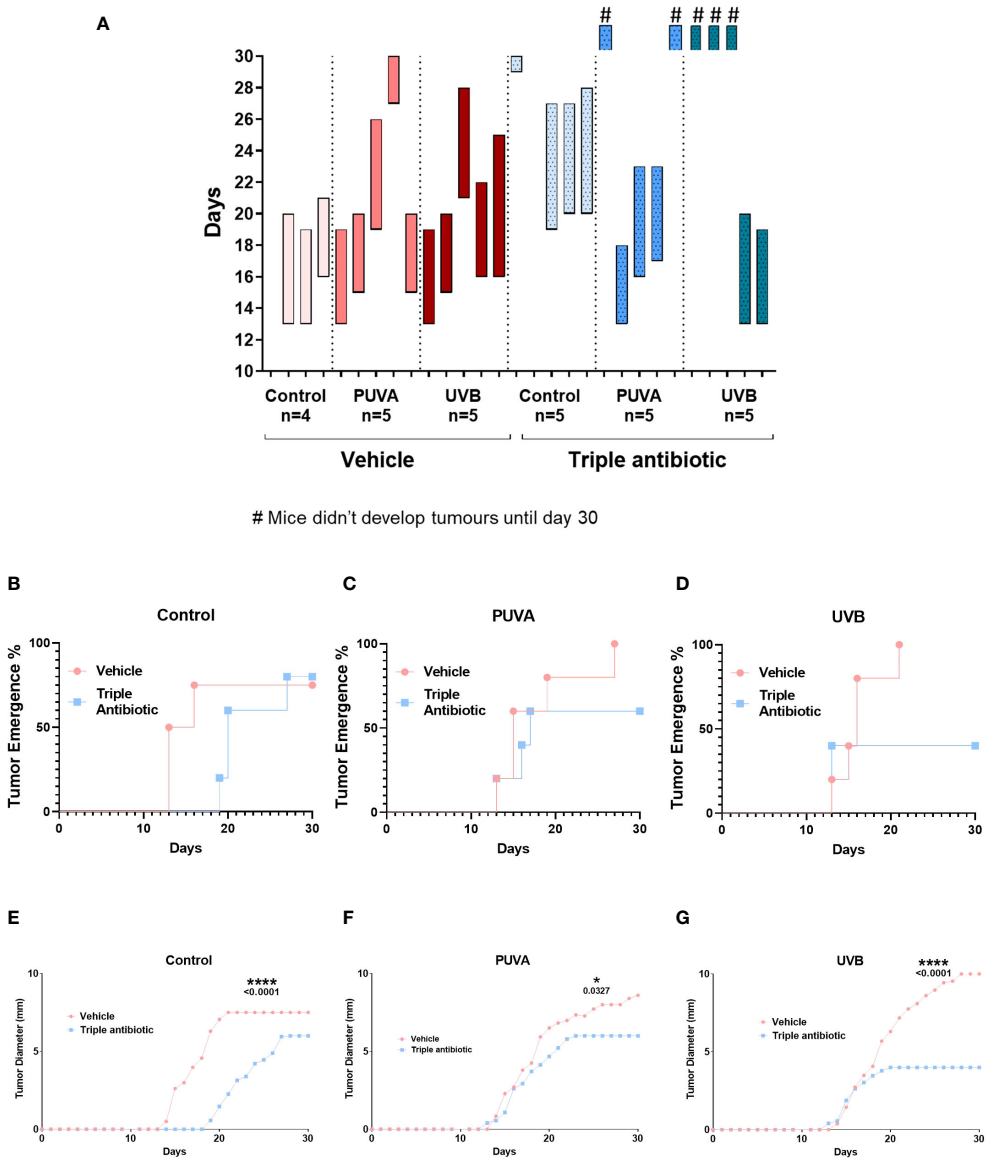

FIGURE 2
Phototherapy and topical triple antibiotic intervention as an adjuvant significantly reduced the tumour occurrence, diameter, and growth rate. **(A)** Tumour timeline: Bars indicate the time from tumour implementation until the death of individual mice, comparing to control, PUVA, and narrowband UVB therapy subgroups in the presence or absence of microbial modulation by triple antibiotic application (n = 4–5 per experimental group). Bars with symbol # indicates the mice which didn't develop visible tumours and were terminated due to end of experiment. Tumour emergence (%) plot of vehicle- vs triple antibiotic-treated mice **(B)** control, **(C)** PUVA, and **(D)** UVB (n = 5 per experimental group). Tumour growth curve plotted as tumour diameter in (mm) for the vehicle- vs triple antibiotic-treated group for **(E)** control, **(F)** PUVA, and **(G)** UVB (n = 5 per experimental group). Statistical significance was calculated using two-way ANOVA.

groups, i.e., CTRL, PUVA, and UVB (p = 0.0001) (Figure 6A). However, an interesting overall increase in the detection frequency of *Staphylococcus* genus upon antibiotic intervention was observed (p = 0.115) (Figure 6B), indicating that this intervention helped the mice to regain microbial balance by diminishing the frequency of pathogenic *Staphylococcus aureus* and increasing the frequency of likely non-pathogenic *Staphylococcus* species. Due to limited depth of our 16s sequencing we were unable to specifically indicate the non-pathogenic *Staphylococcus* species such as *S. hominins* or *S. epidermidis*. Furthermore, the results of a MaAsLin analysis show a significantly high abundance of facultative pathogens, including *Streptococcus* sp. ($p \geq 0.0001$) (Figure 6C), *Pseudomonas* sp. (p = 0.0358) (Figure 6D), and *Cutibacterium* sp. (p = 0.00237) (Figure 6E) in the vehicle group compared to the antibiotic-treated group. In contrast, we saw a higher abundance of *Clostridium species*, e.g., *Lachnospiraceae* sp. *(p = 0.0008)* (Figure 6F) *and Ruminococcaceae* sp. *(p = 0.0001).* (Figure 6G), *Blautia* sp. *(p = 0.007)* (Figure 6H) in the antibiotic-treated group, which is known to be T_{reg}-inducing in inflammatory conditions (34). The abundance of several other microbial species belonging to the facultative skin microbiome, or the gut microbiome, decreased on mouse skin upon topical triple antibiotic application (e.g., *Elizabethkingia* sp., *Undibacterium* sp., *Serratia* sp., *Escherichia*

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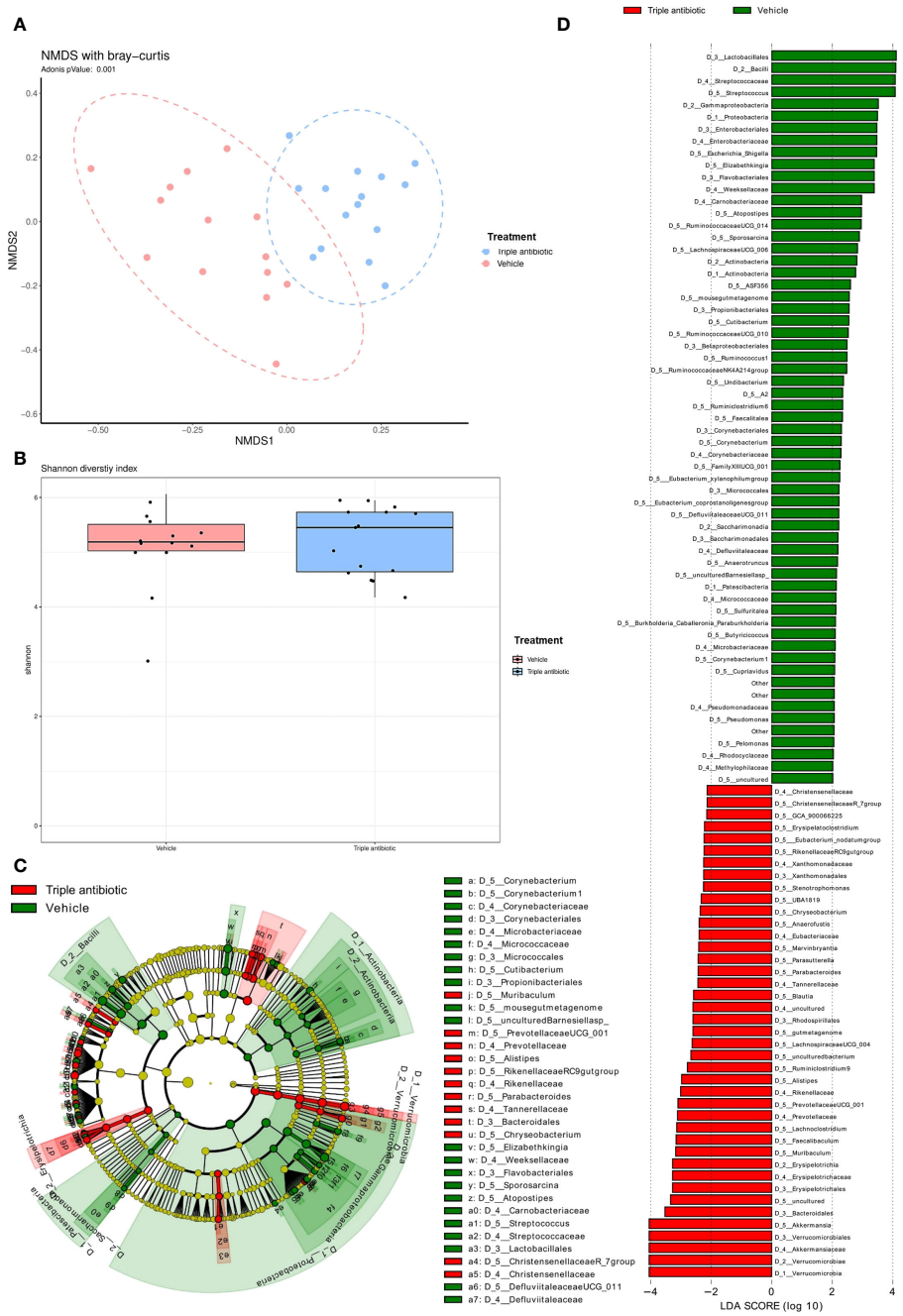

FIGURE 3
Topical triple antibiotic application on the CTCL tumour alters the microbial population on the skin, resulting in a significant increase in skin commensal communities and a reduction in the facultative pathogens. **(A)** Beta diversity analysis results obtained by performing non-metric multidimensional scaling (NMDS) with the Bray–Curtis model and 16S microbial sequencing data show the difference between the groups in the presence or absence of antibiotic intervention. (*n* = 15 per group). **(B)** Comparison of microbial diversity indices (i.e., Shannon diversity index) between the control vs antibiotic-treated group (*n* = 15 per group). **(C, D)** Linear discriminant analysis Effect Size (LefSe) analysis results: **(C)** Cladogram shows the taxonomic representation of statistically and biologically consistent differences between the control (green) and antibiotic-treated (red) groups (*n* = 15 per group). **(D)** Histogram of the Linear Discriminant Analysis (LDA) scores computed for differentially abundant features (LDA score [$\log_{10}$] 2) between the control (green) and antibiotic-treated (red) groups (*n* = 15 per group).

Shigella sp., *Ruminococcus.* sp., and species in the families *Lachnospiraceae*, *Ruminococcaceae*, and *Methylophilaceae*) (Supplementary Figures 3A–H). Furthermore, the abundance of several microbial species increased significantly on mouse skin, indicating that microbial balance was regained upon tropical triple antibiotic application (e.g. *Alistipes* sp., *Akkermansia* sp., *Ruminiclostridium* sp., *Muribaculum* sp., *Rhodospirillales* sp., *Parabacteroides* sp., *Faecalibaculum* sp., *Marvinbryantia* sp., *Lachnoclostridium* sp., *Parasutterella* sp., *Blautia* sp., and species in the families *Erysipelotrichaceae*, *Lachnospiraceae*, Ruminococcaceae, *Prevotellaceae*, and *Rikenellaceae*) (Supplementary Figures 4A–N), (Supplementary Data 2).

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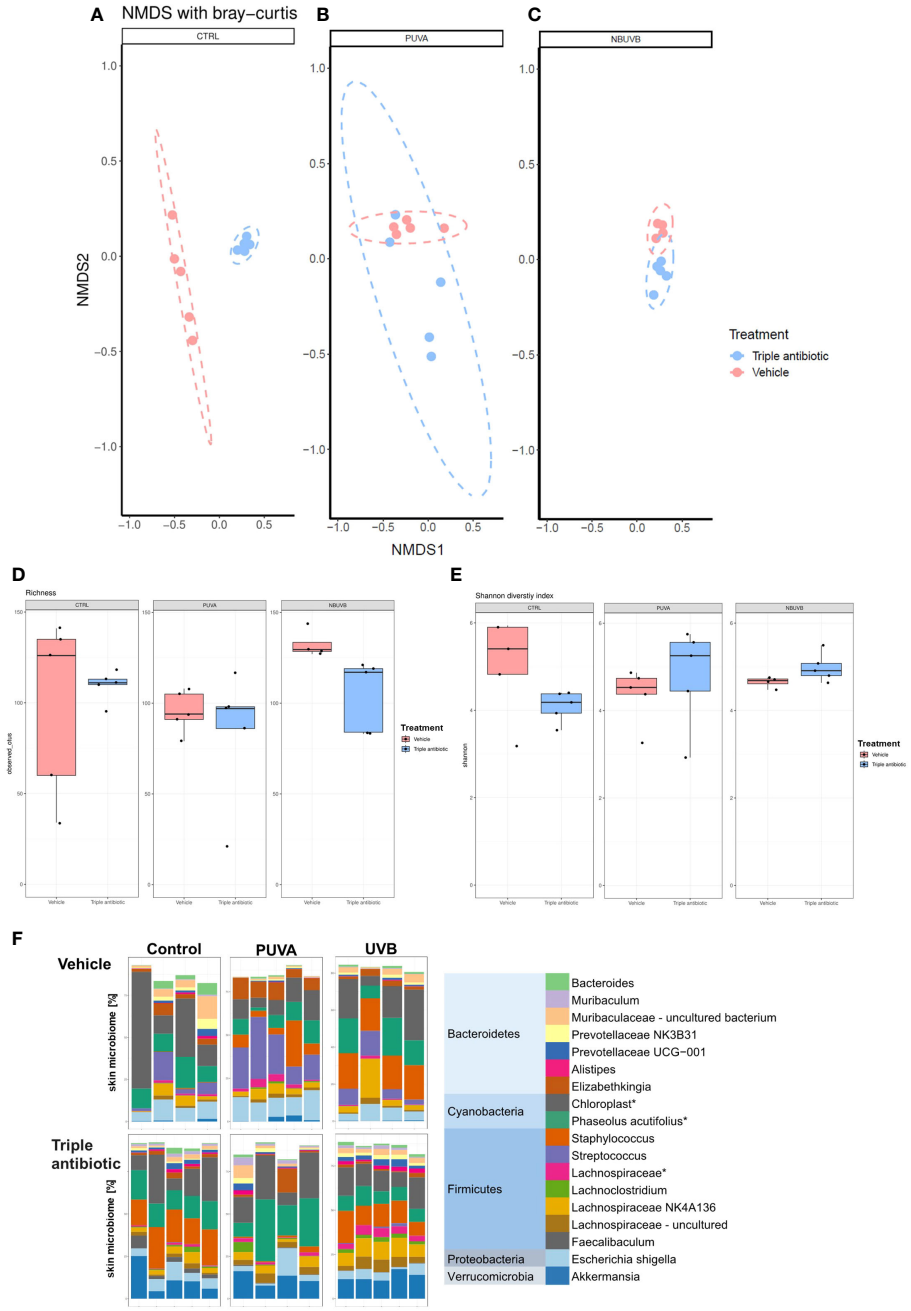

FIGURE 4
Antibiotic intervention of CTCL tumours alters the microbial diversity and richness regardless of the therapeutic regime used. The results of a non-metric multidimensional scaling (NMDS) analysis of 16S microbial sequencing data show the difference between different treatment groups **(A)** CTRL, **(B)** PUVA, and **(C)** UVB) in the presence or absence of antibiotic intervention as adjuvant therapy (n = 5 per experimental group). Box plot showing the differences in terms of **(D)** microbial richness (observed OTUs) and **(E)** microbial diversity indices (i.e., Shannon diversity index) regarding the vehicle-treated and topical triple antibiotic intervention group in the phototherapeutic treatment subgroups (UVB/PUVA/CTRL) (n = 5 per experimental group). **(F)** Relative abundance of the 18 most abundant genera (histogram; different genera represented by different colours) revealed by an analysis of the 16S sequencing data, grouped by presence or absence of antibiotic intervention as adjuvant therapy in the phototherapeutic treatment regime (n = 5 per experimental group).

In summary, chronic inflammation, disruption of skin barrier function, and immune system impairment in CTCL skin lesions disrupt microbial eubiosis and promote the growth of facultative pathogens, e.g., *Staphylococcus* aureus, *Streptococcus* sp., *Pseudomonas* sp., *Cutibacterium* sp. Phototherapeutic treatment along with topical antibiotic intervention as an adjuvant, helps to restore microbial balance by reducing the number of pathogenic microbes and increasing the number of commensals, which in turn reduces chronic inflammation, delays tumour growth, and increases survival rate (Figure 7).

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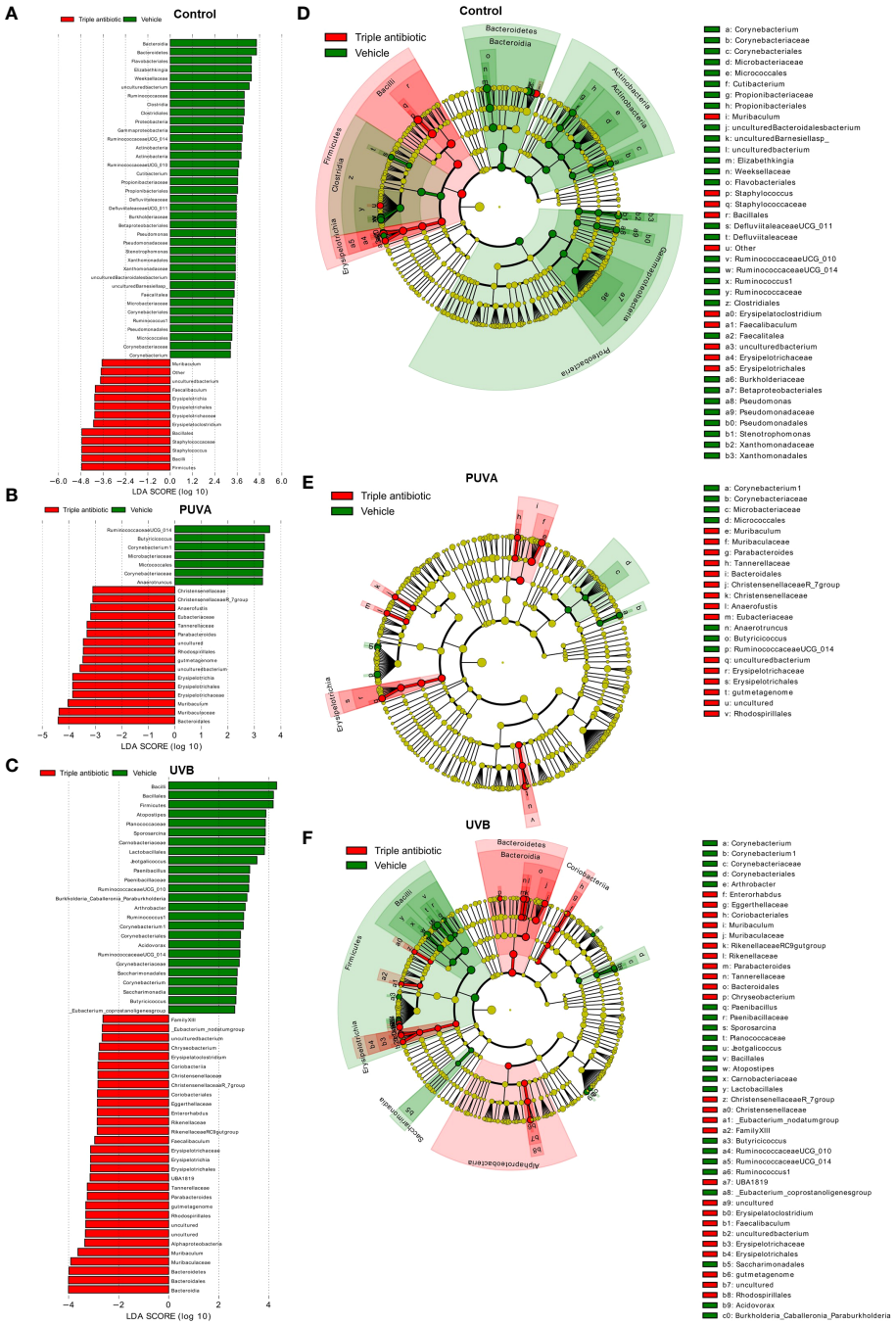

FIGURE 5
Significantly altered taxonomic differences among three phototherapeutic treatment groups (control, PUVA, and UVB) in the presence or absence of antibiotic intervention as an adjuvant. Linear discriminant analysis Effect Size (LefSe) analysis results: Histogram of the Linear Discriminant Analysis (LDA) scores computed for differentially abundant features (LDA score [log 10] 3) between the vehicle- (green) and triple antibiotic-treated (red) groups are plotted for **(A)** control, **(B)** PUVA, and **(C)** UVB (n = 5 per experimental group). The cladogram shows the differences in terms of enriched taxonomic representation between the control (green) and topical triple antibiotic-treated (red) groups in **(D)** control, **(E)** PUVA, and **(F)** UVB (n = 5 per experimental group).

Discussion

New evidence suggests that the skin microbiome in CTCL patients differs significantly from that of healthy individuals. Studies have reported decreased microbial diversity and alterations in the relative abundance of specific bacterial species in CTCL-affected skin (5, 9, 33). Notably, *Staphylococcus aureus* (SA) colonisation (6, 35) was observed in a subset of CTCL patients, exacerbating inflammation and contributing to disease progression. In CTCL, pronounced erythema in the lesional skin was associated with an increase in SA colonisation (6, 15, 35), and severe pain and lesion thickness were associated with the presence of *Corynebacterium* sp. and *Pelomonas* sp (33).

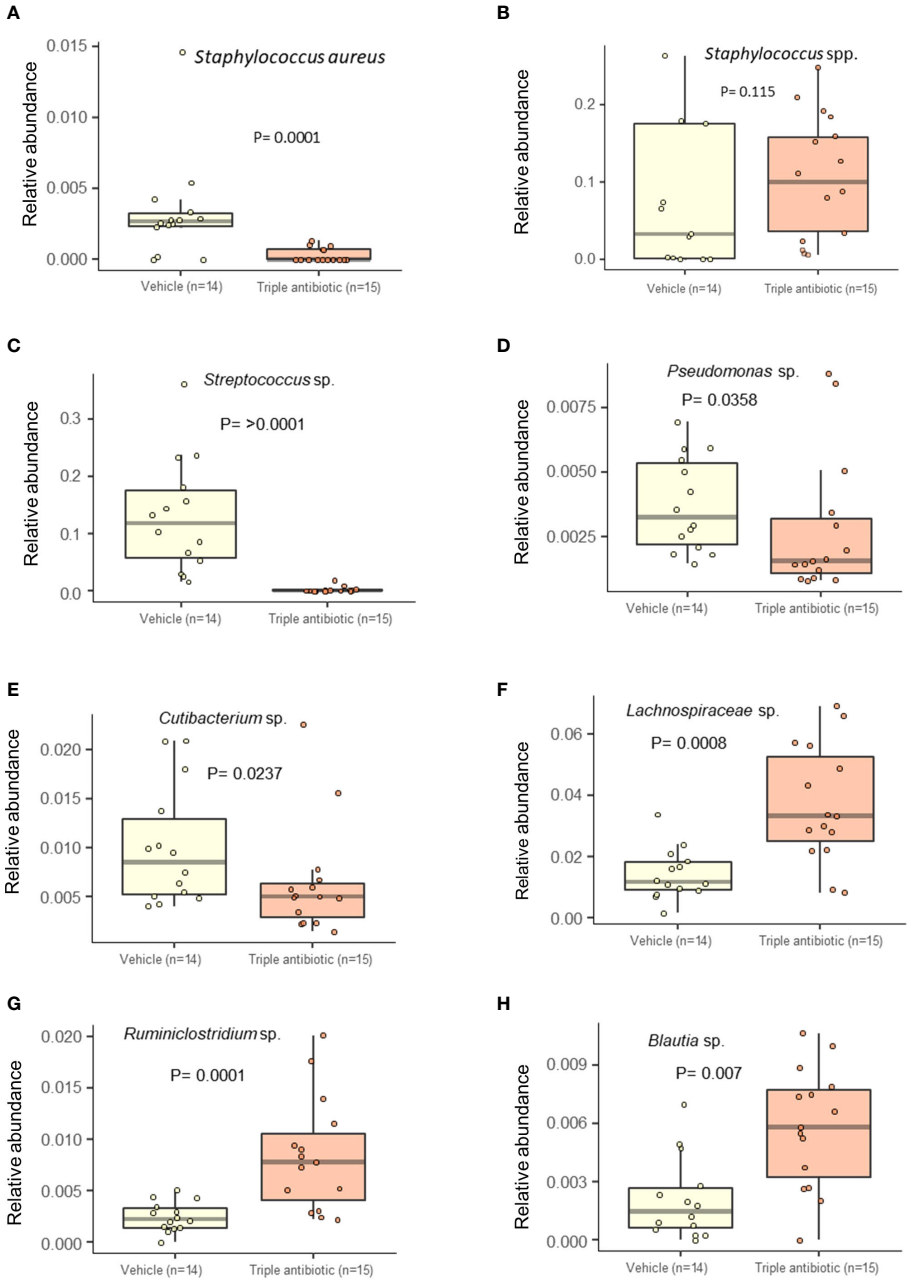

FIGURE 6
Antibiotic intervention significantly decreases the abundance of facultative pathogens and increases the abundance of commensal bacteria. Alteration in the relative abundance of the *Staphylococcus* spp. **(A)** *Staphylococcus aureus* **(B)** *Staphylococcus* genus. Reductions in the relative abundance of the following facultative pathogens were observed: **(C)** *Streptococcus* sp., **(D)** *Pseudomonas* sp., and **(E)** *Cutibacterium* sp. An increase in the abundance of the following commensal *Clostridium* species was observed: **(F)** *Lachnospiraceae* sp., **(G)** *Ruminococcaceae* sp., **(H)** *Blautia* sp. upon antibiotic treatment (n = 14–15 per experimental group).

By using the EL4 T-cell lymphoma cutaneous mouse transplantation model, we could elucidate host-microbial interactions in CTCL during a phototherapeutic treatment regime and study the modulation of these interactions through antibiotic treatment. We observed that triple antibiotic treatment significantly delayed tumour occurrence and growth, which prolonged the survival of mice in the model, irrespective of the allocation to standard therapeutic agents (PUVA, UVB). An analysis of the beta diversity index obtained by applying the Bray–Curtis model showed that the microbial population significantly changed upon antibiotic treatment. This change was linked to an increase in the numbers of T_{reg}-inducing commensal *Clostridium species* (12, 34) and a significant reduction in the numbers of the facultative pathogenic *Corynebacterium*, *Pelomonas*, *Streptococcus*, *Pseudomonas*, and *Cutibacterium* species. Interestingly, we observed a significant decrease in the detection frequency of *Staphylococcus aureus* but an increase in overall number of *Staphylococcus* genus, indicating that antibiotic treatment helped mice to regain microbial balance

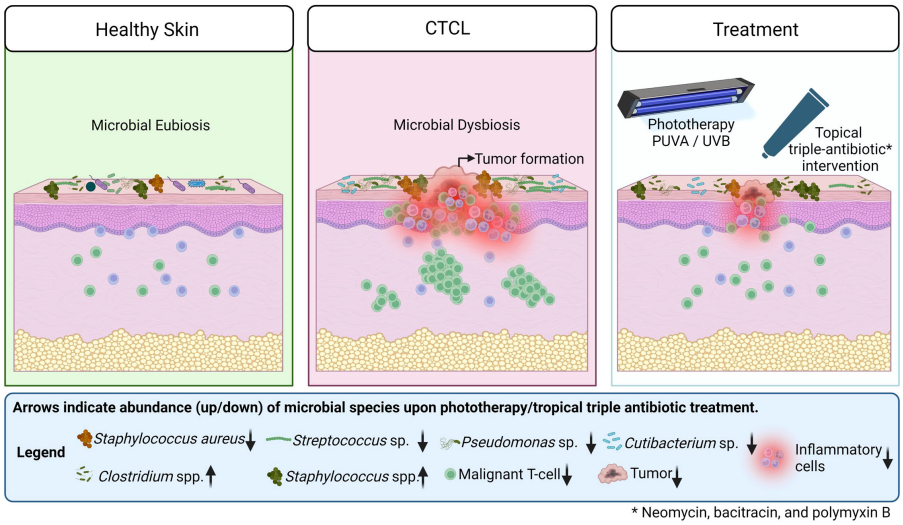

FIGURE 7
A graphical representation of the differences between healthy, CTCL lesional skin and CTCL lesional skin upon treatment. Arrows indicate abundance (up/down) of microbial species upon phototherapy along with topical antibiotic treatment as an adjuvant.

and increased the numbers of non-pathogenic *Staphylococcus* populations, which per se enables the mice to regain microbial eubiosis state (27).

Given the observed dysbiosis and the potential role of the microbiome in CTCL progression, the use of antibiotics may be proposed as a therapeutic approach. Antibiotics could modulate the microbiome, target specific bacterial species, or alter the microbial milieu to favour a more beneficial composition. The complex interplay between the immune system and the microbiome has been recognised by an increasing number of investigators as having a significant impact on human health and disease (35). In recent years, researchers have begun to shed light on the role of the microbiome in CTCL and the potential use of antibiotic intervention to modulate the microbiome in managing this disease (5, 31).

We have explored the impact of the skin microbiome modulation, through antibiotics and phototherapy, on disease progression in CTCL. In light of the groundbreaking findings by Vadivel et al. (36), demonstrating that *S. aureus* can induce drug resistance in malignant T cells, our study gains additional significance. This resistance is mediated through pathways previously implicated in CTCL pathogenesis and treatment resistance, notably TCR, NFκB, and JAK/STAT signaling. Given the established antibacterial effects of phototherapy, its role in CTCL treatment may extend beyond direct antineoplastic activity, potentially also mitigating bacterial-driven drug resistance. Vadivel et al.'s findings underscore the complex interplay between microbial pathogens, immune responses, and therapeutic resistance in CTCL. Therefore, our data suggest that combining phototherapy with targeted antimicrobial strategies could offer a twofold benefit: direct tumor cell cytotoxicity and disruption of bacterial-mediated resistance mechanisms. This approach, opens new avenues for enhancing the efficacy of existing and future therapeutic strategies against CTCL, warranting further investigation (36).

In another recent pivotal study by Liu et al. (37), the characteristics of *S. aureus* colonization in CTCL were extensively examined, revealing significant findings that enhance our current understanding of the microbiome's impact on CTCL. The study included a substantial cohort of over 60 patients, providing a robust dataset for analysis. Liu et al. discovered that S. aureus colonization was present in a significant portion of CTCL patients and that the colonization rates increased with disease progression. Notably, the study also compared lesional and contralateral non-lesional skin sites, finding that *S. aureus* colonization was more prevalent in lesional skin, which may suggest a potential role of bacterial presence in exacerbating disease severity (37). These findings align with our current study's hypothesis on the microbiome's influence on CTCL progression and highlight the importance of considering microbial factors in developing therapeutic strategies. Such insights are invaluable for informing future research and clinical practices, suggesting a potential benefit in targeting microbial colonization as part of comprehensive CTCL treatment plans.

The recent advancements in understanding the interplay between microbial interactions and immune responses have significantly enriched our perspective on CTCL management. The recent study in collaboration with our lab by Yu et al. (26) and the commentary by Goel and Rook (38), have illuminated the promising role of PUVA therapy in enhancing type I IFN responses, a pathway also implicated in the antimicrobial response against Staphylococcus aureus as demonstrated by research highlighting the bacterium's activation of type I IFN signaling through both its Xr domain (39) and via TLR9 in dendritic cells (40). Furthermore, the study on gamma interferon's role in bolstering human endothelial cells against S. aureus infection (41) underscores the critical nature of IFN signaling in mediating resistance to microbial infections. These insights not only underscore the therapeutic potential of targeting microbial interactions and immune pathways in CTCL but also

suggest a broader applicability in enhancing antitumor immunity and patient outcomes through integrated approaches that consider the microbiome's influence on disease progression and treatment response.

In a human study, the bacterial groups *Bacteroides*, *Escherichia/Shigella*, and *Streptococcus* were found to be most prevalent in disease conditions such as polycystic ovary syndrome (PCOS) and obesity (42). In contrast, *Akkermansia* and *Ruminococcaceae* decreased in PCOS and showed opposite results for body weight, sex hormones, and brain-intestinal peptides (42, 43). In our study, also we see a significant decrease in *Escherichia/Shigella* and an increase in the detection frequency of *Akkermansia* and *Ruminococcaceae* upon antibiotic intervention, indicating the role of these microorganisms in disease severity in our model.

The mechanisms underlying the potential efficacy of antibiotics in CTCL are multifaceted. Antibiotics may directly affect bacterial species associated with disease progression, reduce pro-inflammatory stimuli, modulate immune responses, and alter the tumour microenvironment (15, 31). Additionally, antibiotics might influence the production of microbial metabolites that impact T-cell function and immune surveillance (31). For this reason, further host-microbial interaction studies are essential to provide support for the use of specific antibiotic treatment to mitigate CTCL symptoms. Further research should also focus on developing antibiotics or anti-microbial agents with improved specificity and a reduced impact on the commensal microbiome. Targeting specific bacteria associated with CTCL progressions, such as SA, *Corynebacterium* sp., and *Pelomonas* sp. while preserving the beneficial microbiota could provide a more precise approach.

Assessing the long-term effects of antibiotic interventions in CTCL is crucial. Prospective studies are needed to evaluate the extent of microbiome modulation, potential microbiome recovery after antibiotic cessation, and the impact of this treatment on disease progression and overall patient outcomes. The use of precision medicine strategies, including microbial profiling, genomics, and metabolomics (44), can help to identify patients who are more likely to respond to antibiotic interventions. Understanding the individualised characteristics of the microbiome and its interactions with the host immune system could also improve treatment outcomes.

While evidence suggests the clinical benefits of such treatments, further research is needed to elucidate the optimal antibiotic regimens, potential side effects, and long-term implications of antibiotic use. Understanding the complex interplay between the microbiome, antibiotics, and CTCL will contribute to the development of personalised treatment strategies for this challenging disease. We believe that this research describes a rationale for using specific antibiotic interventions to modulate the microbial milieu during the disease course of CTCL and indicates the therapeutic potential of such modulation. In fact, using specific antibiotics may be more effective than eradicating the entire cutaneous microbiome by using other disinfection methods, such as antiseptic whirlpool baths (27), preserve the landscape of commensals and ultimately contribute to a balanced immune response by supporting the production and release of anti-microbial peptides (24, 28, 45, 46).

One limitation of our work is that we did not perform 16s microbiome analysis of healthy untreated mice from our animal housing for baseline control purposes. However, we can refer to existing literature to understand the typical microbial diversity in the skin of normal C57BL/6 mice. Indeed, the gut and skin microbiome of C57BL/6 mice have been extensively studied, revealing that they host a diverse milieu of microorganisms. However, it is important to note that even the 'normal microbiota' can vary based on several factors, including the environment, diet, and genetics of the mice (47–50). For example, it was found that significant differences in microbiome of C57BL/6 mice from different vendors, indicating variability even within the same strain (47, 48). Moreover, it was demonstrated the impact of environmental factors affects the skin microbiome and immune signatures in C57BL/6 mice (49–51). Furthermore, Naik et al. highlighted the dynamic interaction between commensal microbiota and the cutaneous immune system, which is relevant to our study's context of cutaneous T-cell lymphoma (50). Another limitation of our study is that its design did not resemble a treatment schedule once CTCL is diagnosed since it was started before tumor cell inoculation. However, the rationale for initiating antibiotic therapy 3 days prior to the tumoral challenge in our study was twofold: to establish a homogeneous microbial environment at the outset of tumor development and to assess the prophylactic and therapeutic potential of microbiome modulation in CTCL.

The skin microbiota could be modulated in several ways, including antibiotics (31), UV-C lamps (52–54), specific bacterial therapy (14, 55), probiotics (56, 57), or endolysin (58). Our study serves as an indicator that there is an unmet need for modulation of the microbiota along with conventional therapeutic approaches to reduce disease severity and improve survival.

Materials and methods

In vivo intradermal CTCL mouse model

Animal work was done in accordance with institutional guidelines on animal welfare and with the approval of the Austrian Federal Ministry of Science, Research and Economy (BMBWF-66.010/0042-V/3b/2019). Four-week-old C57BL/6 mice (strain Ncrl) were obtained from the Charles River Laboratories (Freiburg, Germany). Mice were maintained under specific pathogen-free (SPF) conditions in individually ventilated cages at the Biomedical Research Facility (BMF) at the Medical University of Graz, Austria. Mice were kept on a 12/12 h light cycle and received standard food and water *ad libitum*. At six weeks of age, mice were shaved on their backs (day -3 of experimental procedures) and randomised into two groups (with or without topical triple antibiotic application), n = 15 per group. Under isoflurane inhalation anaesthesia (1–1.5% in O2, 0.5 L/min), we then intradermally grafted murine $6x10^3$ EL4 T-cell lymphoma cells in the back skin of the mice on day 0. The mice were then further randomised into three subgroups: CTRL (untreated), PUVA, and UVB (n = 5/subgroup).

Antibiotic intervention

Antibiotic intervention by a topical triple antibiotic cream (Neosporin® (neomycin, bacitracin, and polymyxin B sulphate)) or Vaseline® (petroleum jelly). For each application, a precise amount of 50 mg of pre-weighted antibiotic cream or vehicle was administered. The antibiotic cream or vehicle was directly applied to the tumor area and gently spread over the tumor area and adjacent shaved skin. Application was started post-shaving on day -3 and given daily until scarification occurred.

Phototherapy

PUVA or NB-UVB therapy was given every second day at a dose of 1500 mJ/cm^2 (PUVA) or 200 mJ/cm^2 (NB-UVB), starting at day 1 using Waldmann UVA 236 equipment (Waldmann GmbH, Villingen-Schwenningen, Germany). In the case of PUVA, mice were painted on their backs with 200 microliter 8-methoxypsoralen (8-MOP) (Sigma-Aldrich, St. Louis, MO) in ethanol (at a concentration of 0.1 mg/ml), as previously described (59, 60). The mice were then kept for 15 min in individual compartments of standard cages to allow penetration of 8-MOP before UVA irradiation.

Microbiome sampling procedure

Microbiome samples were collected from the skin with sterile swabs 20 days after the EL-4 cell injection. The swab used for sampling was first submerged in a sterile buffer solution (0.15 mol/L NaCl with 0.1% Tween 20) and consequently brushed 20 times in the crosswise direction over the sampled skin site (i.e., tumour and tumour-adjacent non-lesional sites in the murine model).

DNA extraction, library preparation

Microbial DNA was obtained using the QIAamp DNA Microbiome Kit (Qiagen) according to the manufacturer's instructions. The 16s libraries were constructed from the DNA extracted from swab samples using a Nextera XT library prep kit and 1.5 ng as starting material.

16s sequencing, bioinformatics, and statistical analyses

Amplicons were sequenced at the ZMF Core Facility Molecular Biology in Graz, Austria, using an Illumina MiSeq platform. The analysis was performed with the Quantitative Insights into Microbial Ecology QIIME 2 software (Version 2019.7) (61) next-generation microbiome bioinformatics platform integrated into a personal Galaxy server using the Medical University Graz MedBioNode HPC cluster. After initial quality control of the raw sequence data was performed with FastQC and MultiQC, initial data preprocessing was performed with the DADA2 pipeline (62), which included quality filtering and adapter trimming, denoising data, and removing chimeric artefacts. A QIIME2 Naive Bayes classifier trained with the 16S rRNA SILVA 132 database (63) was used to provide taxonomic annotation for representative sequences from the Amplicon Sequence Variants (ASVs) discovered by applying the DADA2 workflow. Alpha diversity indexes (e.g. richness indices and the Shannon and Faith's phylogenetic diversity index), as well as beta diversity distances (e.g. weighted and unweighted UniFrac distance metrics, the Bray-Curtis dissimilarity index, and the Jaccard index), were also calculated with QIIME2, whereas all further statistical downstream analyses and plotting were performed in the R 4.2.2 program (R Core Team, 2022) for statistical computing and graphic illustration. To detect significantly abundant taxa, we used LefSe (Linear discriminant analysis Effect Size) (64) and MaAsLin2 (Microbiome Multivariable Association with Linear Models) (65) tools from the Huttenhower lab (Harvard T.H. Chan School of Public Health, Boston, MA).

Statistical analysis

GraphPad Prism 8 and the R platform were used to perform the statistical analyses. The threshold for statistical significance was set at $p < 0.05$ unless otherwise specified. p-value: <0.05 (*), <0.01 (**), <0.001 (***), <0.0001 (****).

Graphical license

The graphical abstract (Figure 7) and schematics of the mouse experimentation model were created using BioRender.com under the agreement numbers: YU25IH4YPH and SJ25IKLT21.

Data availability statement

The datasets presented in this study can be found in online repositories. Sequence data were deposited in the European Nucleotide Archive (ENA; BioProject No. PRJEB64180).

Ethics statement

The animal study was approved by Austrian Federal Ministry of Science, Research and Economy (approval no. BMBWF-66.010/0042-V/3b/2019). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. PV-G: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – review & editing. AJ: Writing – review & editing. ST: Formal analysis, Software, Writing – review & editing. PW: Conceptualization, Funding acquisition, Project administration, Resources, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1255859/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The topical triple antibiotic application reduces the AUC (Area Under the tumour growth Curve): The area under the tumour growth curve was calculated for individual mice and plotted. **(A)** CTRL (without phototherapy), **(B)** PUVA- or **(C)** UVB-treated group (n = 5 per group).

SUPPLEMENTARY FIGURE 2
Topical triple antibiotic intervention increases survival regardless of phototherapeutic regime: Kaplan-Meier survival analysis of **(A)** CTRL (without phototherapy), **(B)** PUVA, **(C)** UVB subgroups in the presence or absence of antibiotic intervention (n = 5).

SUPPLEMENTARY FIGURE 3
The abundance of several microbial species altered on mouse skin upon topical triple antibiotic application. **(A)** *Lachnospiraceae* sp., **(B)** *Elizabethkingia* sp., **(C)** *Ruminococcaceae* sp., **(D)** *Undibacterium* sp., **(E)** *Serratia* sp., **(F)** *Cupriavidus* sp., **(G)** *Shigella* sp., **(H)** *Ruminococcus* sp., **(I)** *Methylophilaceae* sp.

SUPPLEMENTARY FIGURE 4
Abundance of several commensal microbial species increased significantly on mouse skin upon tropical triple antibiotic application: **(A)** *Alistipes* sp., **(B)** *Akkermansia* sp., **(C)** *Lachnoclostridium* sp., **(D)** *Muribaculum* sp., **(E)** *Rhodospirillales* sp., **(F)** *Erysipelotrichaceae* sp., **(G)** *Parabacteroides* sp., **(H)** *Faecalibaculum* sp., **(I)** *Marvinbryantia* sp., **(J)** *Ruminococcaceae* sp., **(K)**. *Parasutterella* sp., **(L)** *Prevotellaceae* sp.,**(M)** *Erysipelatoclostridium* sp., **(N)** *Rikenellaceae* sp.

SUPPLEMENTARY DATA SHEET 1
A data sheet showing the list of significantly altered bacterial abundance in the vehicle- vs the triple antibiotic-treated groups based on the results of the LefSE analysis. P-value cutoff > 0.05, LDA score [$\log_{10}$] 2 (n = 14-15 per group).

SUPPLEMENTARY DATA SHEET 2
A data sheet showing the list of altered bacterial abundance in the vehicle- vs the triple antibiotic-treated groups according to the MaAsLin2 analysis results. P-value cutoff > 0.05 (n = 14-15 per group).

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EDITED BY
Beatrice Omusiro Ondondo,
University Hospitals of Leicester NHS Trust, United Kingdom

REVIEWED BY
Katrina Traber,
Boston University, United States
Mu Yang,
UESTC, China

*CORRESPONDENCE
Hongyan Hou
houhongyan89@163.com
Feng Wang
fengwang@tjh.tjmu.edu.cn

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The immune landscape of sepsis and using immune clusters for identifying sepsis endotypes

Guoxing Tang, Ying Luo, Huijuan Song, Wei Liu, Yi Huang, Xiaochen Wang, Siyu Zou, Ziyong Sun, Hongyan Hou* and Feng Wang*

Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Background: The dysregulated immune response to sepsis still remains unclear. Stratification of sepsis patients into endotypes based on immune indicators is important for the future development of personalized therapies. We aimed to evaluate the immune landscape of sepsis and the use of immune clusters for identifying sepsis endotypes.

Methods: The indicators involved in innate, cellular, and humoral immune cells, inhibitory immune cells, and cytokines were simultaneously assessed in 90 sepsis patients and 40 healthy controls. Unsupervised k-means cluster analysis of immune indicator data were used to identify patient clusters, and a random forest approach was used to build a prediction model for classifying sepsis endotypes.

Results: We depicted that the impairment of innate and adaptive immunity accompanying increased inflammation was the most prominent feature in patients with sepsis. However, using immune indicators for distinguishing sepsis from bacteremia was difficult, most likely due to the considerable heterogeneity in sepsis patients. Cluster analysis of sepsis patients identified three immune clusters with different survival rates. Cluster 1 (36.7%) could be distinguished from the other clusters as being an "effector-type" cluster, whereas cluster 2 (34.4%) was a "potential-type" cluster, and cluster 3 (28.9%) was a "dysregulation-type" cluster, which showed the lowest survival rate. In addition, we established a prediction model based on immune indicator data, which accurately classified sepsis patients into three immune endotypes.

Conclusion: We depicted the immune landscape of patients with sepsis and identified three distinct immune endotypes with different survival rates. Cluster membership could be predicted with a model based on immune data.

KEYWORDS

sepsis, immune indicators, endotypes, MDSCs, prediction model

Introduction

Sepsis, one of the leading causes of morbidity and mortality in hospitals, was traditionally considered a systemic inflammatory response syndrome due to infection (1–3). Sepsis is now defined as a life-threatening organ dysfunction caused by a dysregulated host response (4, 5). A recent burden of sepsis report highlights nearly 50 million new cases globally per year (3, 6). Although the prognosis of sepsis varies depending on the different organisms, sites of infection, or underlying host conditions, there are an estimated 10 million deaths each year (3, 6). Despite hundreds of clinical trials conducted, there is currently no single treatment that consistently saves lives in sepsis patients (4, 6).

The dysregulated immune response is described as concurrent hyperinflammation and immune suppression, which is related to many protection mechanisms that become detrimental (4, 6). Among the many mediators implicated in sepsis-associated excessive inflammation, neutrophils, macrophages, cytokines, and coagulation systems are prominently featured (7–10). On the other side, immune suppression, which also involves different cell types, is related to enhanced apoptosis of T cells and increased numbers of inhibitory cells, including regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) (11–14). Generally, longitudinal analyses of immune reactions from early pathogen–host interactions to clinically manifested sepsis in humans are lacking, making the concurrent hyperinflammation and immune suppression during the pathophysiological path of sepsis speculative.

Another core challenge in depicting the immune response of sepsis is the considerable heterogeneity in which the extent of proinflammatory and immunosuppressive responses and their relative contribution to sepsis-associated immunopathology varied between patients (15, 16). Heterogeneity is considered a major factor in the failure of immune modulatory trials in patients with sepsis, and it has been proposed that stratification of patients in subgroups with shared features can improve the effect of therapy, in particular if patient classification is based on characteristics of host response (15–17). Recently, attempts have been made to identify sepsis subgroups with different disease outcomes using clinical, laboratory, and transcriptome data and unbiased computational analysis tools (18–23). In spite of the importance of sepsis subgroup classification in understanding the heterogeneity of patients, stratification of sepsis patients into endotypes based on immune indicators is still rare, and the utility of these subgroups in clinical practice needs to be further determined.

In view of the fact that the immune response is complicated and of key importance in the prognosis of sepsis, we systematically investigated the immune indicators involved in innate, cellular, and humoral immune cells, inhibitory immune cells, as well as cytokines and chemokines simultaneously, in the prognosis of patients with sepsis and bacteremia. Furthermore, unsupervised hierarchical clustering was used to identify clusters of patients with sepsis based on similar immune profiles. Notably, we have not only described the immune landscape of patients with sepsis and bacteremia but also identified three clusters of sepsis patients with different survival rates. Additionally, we build a prediction model by using immune data to enable the stratification of patients into three clusters, which might be useful in standard practice as a convenient tool to identify endotypes in the future.

Materials and methods

Study subjects

Between February 2021 and February 2022, patients with positive blood cultures for bacteria who were finally diagnosed with bacteremia or sepsis were recruited from Tongji Hospital (the largest tertiary hospital in central China). Blood culture was performed using an automatic blood culture system, and organisms were identified. Antibiotic susceptibility was carried out using standard microbiological methods. We categorized blood cultures that identified coagulase-negative *Staphylococcus* in only one bottle as contaminated, and consequently, the patients with identified coagulase-negative Staphylococcus were excluded from the study. Another group of healthy controls (HCs) without any clinical symptoms of disease matched for gender and age was randomly selected as the control group. Moreover, another cohort of patients with sepsis enrolled at Sino-French New City Hospital (a branch hospital of Tongji Hospital with 1600 beds) was used to validate the accuracy of the built model. This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (ID: TJ-IRB20211009).

Data collection and patient classification

At the time of notification of a positive blood culture, the physiological indicators (body temperature, heart rate, breathing rate, and sequential organ failure assessment (SOFA) score) and routine laboratory results were collected from electronic medical records. The demographic and clinical information was also recorded. The enrolled patients mainly received appropriate antibiotics and symptomatic treatment. The clinical outcome was 30-day all-cause mortality from the day of the first positive culture. Patients with positive blood cultures were categorized into bacteremia and sepsis groups. Bacteremia was defined as the isolation of bacteria from at least one blood culture with a compatible clinical syndrome during a hospital stay. Sepsis was defined as patients who meet the criteria of bacteremia together with an acute change in SOFA score ≥ 2, according to the sepsis-3 definitions (5, 24).

Flow cytometry analysis

Heparinized blood samples were collected from study participants at the time of notification of a positive blood culture.

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The absolute numbers of T, B, and NK cells were determined by using TruCOUNT tubes and the BD Multitest 6-color TBNK Reagent Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll–Hypaque density gradients. The 10 cell subsets, including $CD4^+$ T cells, $CD8^+$ T cells, Treg cells, T helper (Th) cells, follicular helper T (Tfh) cells, B cells, NK cells, monocytes, dendritic cells (DCs), and MDSCs (Supplementary Table S1), were detected by flow cytometry. All the staining was blocked using an Fc-blocking buffer, and isotype controls with irrelevant specificities were included as negative controls. The pellets were finally analyzed with a FACSCanto flow cytometer (BD Biosciences). The detailed antibody information is presented in Supplementary Table S2. Gating strategies for flow cytometric analysis are shown in Supplementary Figures S1–S8.

Cytokine and chemokine analysis

Peripheral blood samples were collected from study participants, and serum was separated by centrifugation and stored at −80°C until use. The serum concentrations of 24 cytokines and chemokines (CCL2, CCL3, CCL4, CD40L, CXCL10, GM-CSF, granzyme B, IFN-α, IFN-γ, IL-1α, Il-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-33, PD-L1, and TNF-α) (catalog No. LKTM010; R&D Systems, Minneapolis, MN, USA) were measured by microbead array technology using Luminex 200 system (Luminex, Austin, TX, USA).

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) or median (interquartile range), and comparisons were performed by using the Mann–Whitney *U* test or one-way ANOVA test when appropriate. Categorical variables were compared using the Chi-square test or Fisher's exact test. Differences among groups based on immune indicators were also determined by t-distributed stochastic neighbor embedding (t-SNE) analysis with R package "Rtsne". Cluster analysis of the heat map was performed to identify patients with similar immune patterns by using the R package "pheatmap", and represented as a dendrogram. Unsupervised k-means cluster analysis of the immune indicator data was used to identify sepsis patient clusters, and the optimal number of clusters was determined using the elbow method with R package "factoextra" and "cluster". Principal component analysis (PCA) was used to determine major variables between different groups. The prediction model was built using a supervised random forest approach by using the R package "randomForest" and "caret". The importance of each indicator in the classification of patients was estimated by using the mean decrease in accuracy. Kaplan–Meier curves were used for survival analysis and compared by using the log-rank test. Statistical significance was determined as $p < 0.05$. Statistical analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA), GraphPad Prism 8.0 (San Diego, CA, USA), and R 4.0.3 (R Foundation, Vienna, Austria).

Results

The immune landscape of patients with sepsis

A total of 115 patients with positive blood cultures were enrolled, including 25 with bacteremia and 90 with sepsis (24 died, 66 survived). Another 40 healthy individuals were recruited as a control group. Sepsis patients have a median age of 57 years old (IQR: 49–66), with men accounting for 76.67%. The main clinical and demographic characteristics of the participants are presented in Table 1. The results of all immune indicators in enrolled individuals are presented in Supplementary Table S3.

For innate immunity, the percentage of nonclassic monocytes trended higher in bacteremia and sepsis patients versus HCs. Conversely, bacteremia patients displayed lower HLA-DR expression of monocytes than HCs, and this trend was more pronounced in sepsis patients. Accordingly, the frequency of monocytic MDSCs (M-MDSCs) showed a progressive increase from HCs to bacteremia and sepsis patients. The percentage of

TABLE 1 The demographic and clinical characteristics of the participants.

	Healthy controls (*n* = 40)	Bacteremia patients (*n* = 25)	Sepsis patients (*n* = 90)
Age [years, median (25th–75th percentiles)]	54 (47–61)	59 (52–63)	57 (49–66)
Males [*n* (%)]	30 (75.00)	20 (80.00)	69 (76.67)
SOFA	/	1 (0–1)	7 (5–10)
Medical department			
Intensive care unit [*n* (%)]	/	3 (12.00)	39 (43.33)
Department of Infectious Diseases [*n* (%)]	/	9 (36.00)	11 (12.22)
Other departments [*n* (%)]	/	13 (52.00)	40 (44.45)
Species			
Gram-positive bacteria [*n* (%)]	/	8 (32.00)	38 (46.67)
Gram-negative bacteria [*n* (%)]	/	17 (64.00)	48 (53.33)
Underlying condition or illness			
Diabetes mellitus [*n* (%)]	/	4 (16.00)	17 (18.89)
Hypertension [*n* (%)]	/	3 (15.00)	15 (16.67)
Solid tumor [*n* (%)]	/	5 (20.00)	7 (7.78)

Data are presented as number (%) or median (25th–75th percentiles). SOFA, sequential organ failure assessment.

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DCs, especially the subset of myeloid DCs (mDCs), showed a progressive decrease from HCs to bacteremia and sepsis patients. Although NK cell numbers also showed a decreased trend from HCs to bacteremia and sepsis patients, the expressions of functional markers (NKG2A, perforin, and granzyme B) were comparable among them (Figure 1A). For cellular immunity, the numbers of both $CD4^+$ and $CD8^+$ T cells showed a progressive decrease from HCs to bacteremia and sepsis patients, whereas the frequency of activated $HLA\text{-}DR^+CD8^+$ T cells tended to be higher in bacteremia and sepsis patients versus HCs. In addition, sepsis patients demonstrated higher proportions of Th2 and Th17 cells but lower proportions of Th1 and Tfh cells, compared to HCs (Figure 1B). For humoral immunity, bacteremia and sepsis patients had a lower number of B cells compared to HCs. In particular, the frequency of

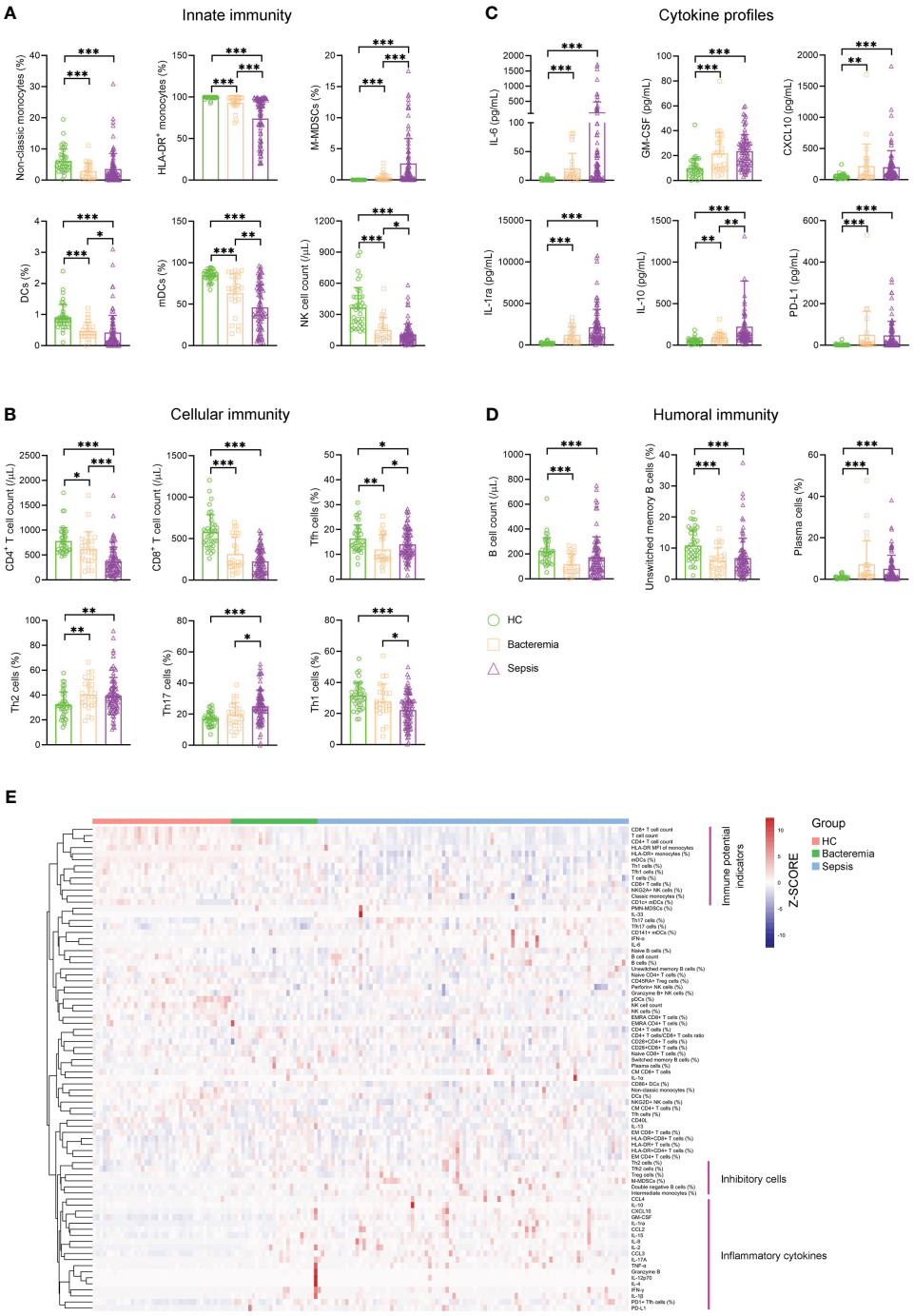

FIGURE 1
The immune landscape of patients with sepsis. Bar graphs showing the results of representative immune indicators involved in **(A)** innate immunity, **(B)** cellular immunity, **(C)** cytokine profiles, and **(D)** humoral immunity in HC and patients with sepsis and bacteremia. Data are presented as mean and SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(E)** Hierarchical cluster analysis of immune indicators in HC ($n = 40$) and patients with sepsis ($n = 90$) and bacteremia ($n = 25$). The pink lines represent typical immune characteristics among different groups. HC, health control.

unswitched memory B cells was decreased while that of plasma cells was conversely increased in bacteremia and sepsis patients compared to HCs (Figure 1D). For cytokine profiles, the levels of both proinflammatory (such as IL-6, GM-CSF, and CXCL10) and anti-inflammatory (such as IL-1ra, IL-10, and PD-L1) cytokines were increased in bacteremia and sepsis patients compared to HCs (Figure 1C).

Remarkably, cluster analysis of the heat map showed that low levels of immune potential indicators (e.g., $CD4^+$ T-cell count, $CD8^+$ T-cell count, and HLA-DR expression on monocytes) coexisted with high levels of inhibitory cells (e.g., Treg cells, M-MDSCs, and Th2 cells), and inflammatory cytokines were the most prominent characteristics in sepsis patients when comparing bacteremia patients or HCs (Figure 1E). These data support the impairment of innate and adaptive immunity accompanying increased inflammation in patients with sepsis.

The differentiation of patients with sepsis and bacteremia

Many indicators displayed efficient performance in distinguishing between sepsis patients and HCs (Figure 2A). Although many indicators like M-MDSCs (%), CD4+ T-cell count, and Tfh17 cells (%) differed significantly between sepsis and bacteremia patients, using a single indicator for distinguishing these two conditions was unsatisfactory. The best indicator was M-MDSCs (%) with an AUC of 0.75 (Figure 2B). Expectedly, the t-SNE analysis based on 80 immune indicators showed that sepsis patients could be well distinguished from HCs. However, patients with sepsis and bacteremia showed much overlap and could not be well separated (Figure 2C). The subsequent principal component analysis showed that the immune potential indicators ($CD4^+$ T-cell count, HLA-DR^+ monocytes (%), and mDCs (%)) and anti-

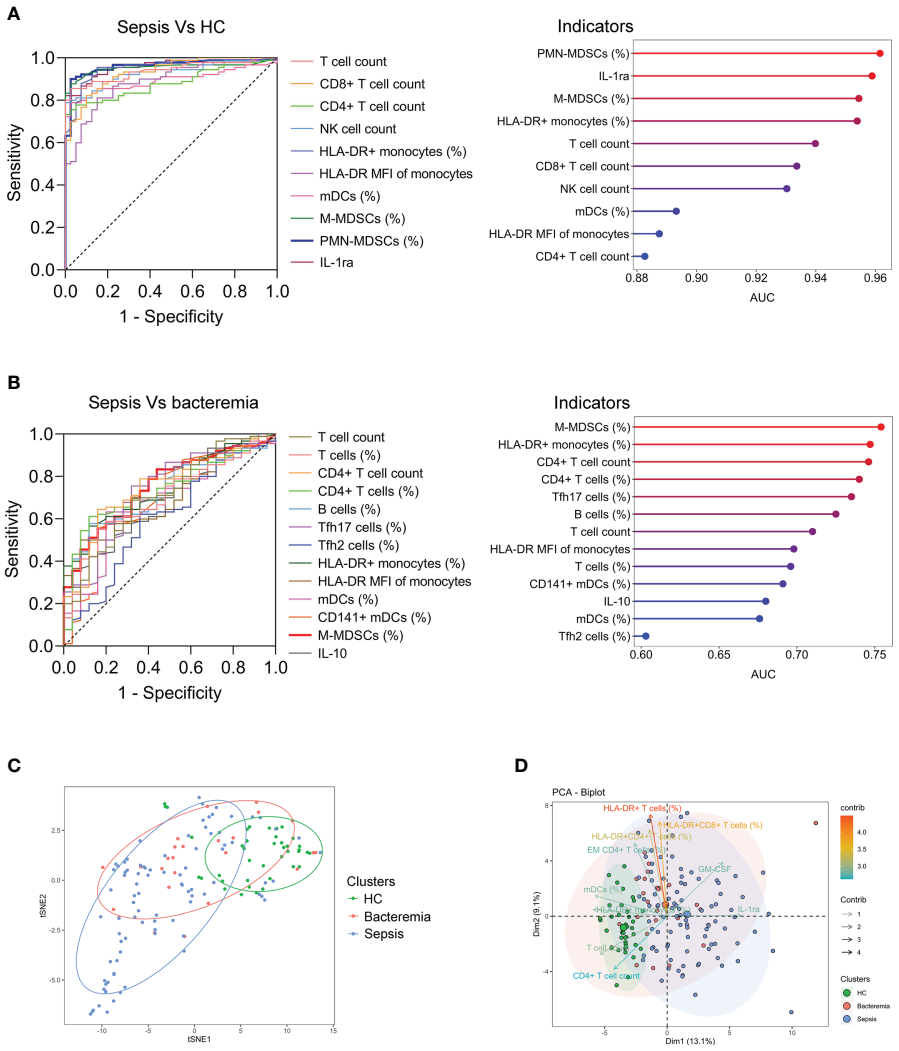

FIGURE 2
The differentiation of patients with sepsis and bacteremia. **(A)** ROC analysis showing the performance of the top ten indicators in discriminating between sepsis patients and HCs. Cleveland dot plots showing the AUCs of these indicators. **(B)** ROC analysis showing the performance of the indicators (with $p < 0.01$) in discriminating between sepsis and bacteremia patients. Cleveland dot plots showing the AUCs of these indicators. **(C)** The t-SNE analysis using 80 immune indicators to clarify the differences among HC, bacteremia, and sepsis patients. **(D)** The PCA showing the most important variables in the differentiation of patients with sepsis and bacteremia. HC, health control; t-SNE, t-distributed stochastic neighbor embedding; AUC, area under the curve; PCA, principal component analysis.

inflammatory cytokine IL-1ra were the most important variables in HCs and sepsis patients, respectively (Figure 2D).

The immune characteristics of sepsis patients with different outcomes

Generally, a few indicators differed significantly between survived and deceased patients with sepsis. Specifically, survived patients displayed higher levels of indicators that represent normal immune potential, such as $CD4^+$ T-cell count, HLA-DR^+ monocytes, and mDCs (%) compared to deceased patients. Conversely, deceased patients demonstrated higher levels of inhibitory cells (M-MDSCs and PMN-MDSCs) and anti-inflammatory cytokines (PD-L1 and IL-1ra) compared to survived patients (Supplementary Table S4). Cluster analysis of the heat map did not reveal any pattern of immune characteristics between deceased and survived patients with sepsis (Figure 3A). Accordingly, the t-SNE analysis did not advocate the possibility of a combination of these immune indicators for distinguishing deceased from survived patients (Figure 3B). Nevertheless, the principal component analysis still showed that the immune potential indicators [$CD4^+$ T-cell count, HLA-DR^+ monocytes (%), and mDCs (%)] and anti-inflammatory cytokine IL-1ra were the most important variables in survived and deceased sepsis patients, respectively (Figure 3C).

Using immune indicators for classifying sepsis endotypes

Considering the difficulty of using immune indicators for either distinguishing sepsis from bacteremia or predicting the outcome of sepsis, which may be attributed to considerable heterogeneity among sepsis patients, we further determined whether sepsis patients could be classified into different clusters based on these indicators. Notably, unsupervised k-means cluster analysis of 80 immune indicators delivered three distinct clusters of patients with sepsis (Figure 4A). Cluster 1 represented 36.7% of the patients with sepsis. Cluster 1 was characterized by an effector phenotype expressed on T cells distinctive from that of the other clusters by virtue of having a high level of HLA-DR on $CD4^+$ and $CD8^+$ T cells, accompanying increased EM $CD4^+$ T-cell frequency (Figures 4B, C).

Cluster 2 represented 34.4% of the patients with sepsis. The patients in this cluster had significantly higher levels of immune potential indicators such as $CD4^+$ T-cell number, naïve $CD8^+$ T-cell percentage, and $CD28^+CD8^+$ T-cell percentage than did the patients

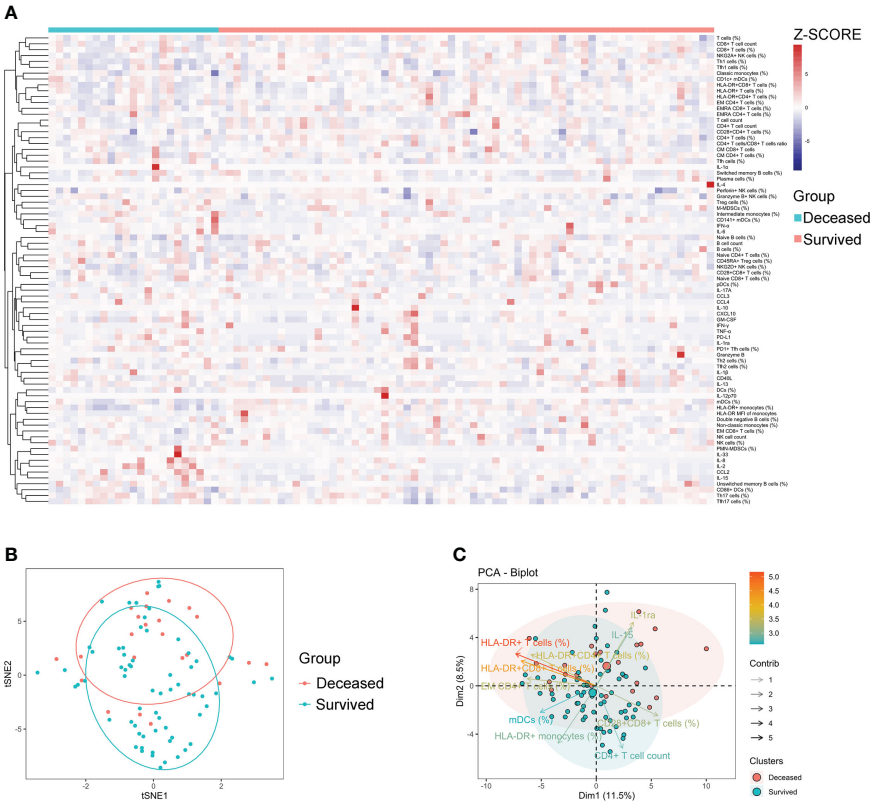

FIGURE 3
The immune characteristics of sepsis patients with different outcomes. **(A)** Hierarchical cluster analysis of immune indicators in deceased and survived sepsis patients. **(B)** The t-SNE analysis using 80 immune indicators to clarify the difference between deceased (n = 24) and survived (n = 66) sepsis patients. **(C)** The PCA showing the most important variables in discriminating between deceased and survived patients with sepsis. t-SNE, t-distributed stochastic neighbor embedding; PCA, principal component analysis.

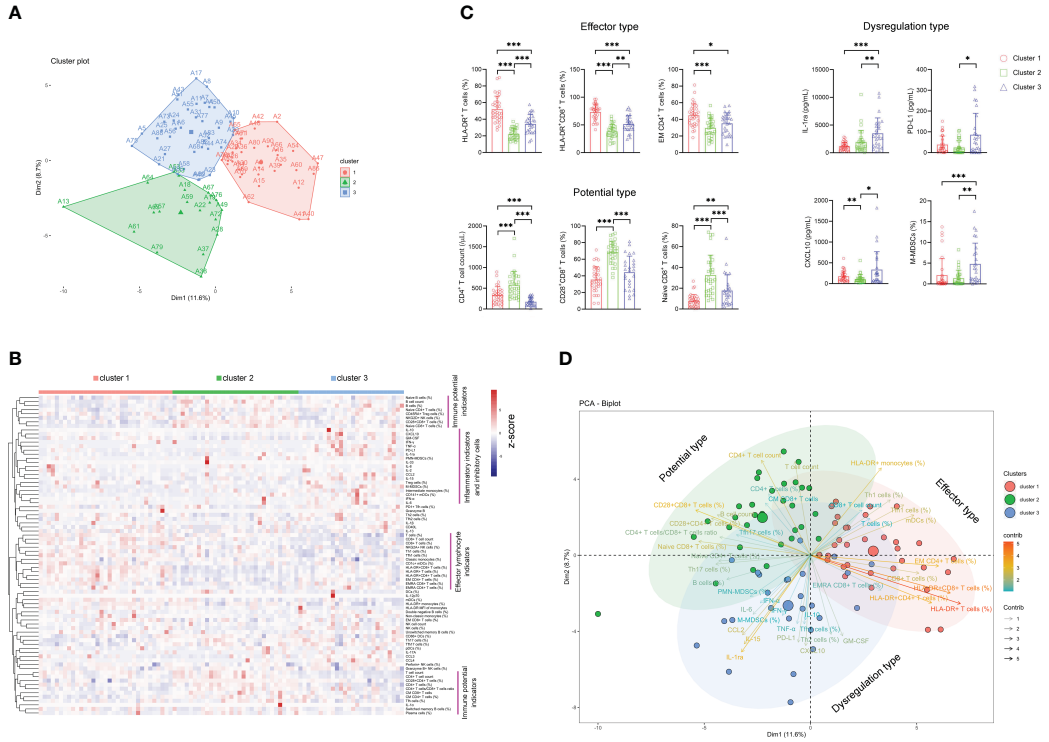

FIGURE 4
The classification of sepsis endotypes based on immune indicators. **(A)** Unsupervised k-means cluster analysis of 80 immune indicators delivering three distinct clusters of patients with sepsis. **(B)** Hierarchical cluster analysis of immune indicators in sepsis patients grouped by immune cluster (cluster 1, n = 33; cluster 2, n = 31; cluster 3, n = 26). The pink lines represent typical immune characteristics among different clusters of sepsis patients. **(C)** Bar graphs showing the results of representative immune indicators in three clusters of sepsis patients. Data are presented as mean and SD. *p < 0.05; **p < 0.01; ***p < 0.001. **(D)** The PCA showing the most important variables in discriminating among three clusters of sepsis patients. PCA, principal component analysis.

in clusters 1 and 3 (Figures 4B, C). Cluster 3 represented 28.9% of the patients with sepsis. Cluster 3 was characterized by a dysregulated immune state distinctive from that of the other clusters by virtue of having the highest levels of proinflammatory cytokines (such as CXCL-10 and GM-CSF), anti-inflammatory cytokine (such as IL-1ra and PD-L1), and inhibitory cells (such as M-MDSCs and Treg cells) simultaneously (Figures 4B, C). Accordingly, we named clusters 1, 2, and 3 as "effector type", "potential type", and "dysregulation type", respectively, in terms of the most important variables by principal component analysis (Figure 4D).

Expectedly, the survival rate of sepsis patients in cluster 2 (potential type) reached 83.87% and was the highest among the three clusters. The survival rates in cluster 1 (effector type) and cluster 3 (dysregulation type) were 75.76% and 57.69%, respectively. Notably, sepsis patients in cluster 2 demonstrated significantly higher survival rates than those in cluster 3 (Figure 5).

Prediction model for classifying sepsis endotypes

Considering the different survival rates in three sepsis endotypes, we further used the random forest approach to build a prediction model for classifying sepsis endotypes (cluster 1, 2, or 3) based on 80 immune indicator data from 90 patients with sepsis. It was found that after 50 trees, the out-of-bag (OOB) error rate tended to be stable (Figure 6A). The top 30 immune indicators were sorted by importance for prediction based on the mean decrease in accuracy (Figure 6B). The top 15 indicators were HLA-DR$^+$CD8$^+$ T cells (%), HLA-DR$^+$ T cells (%), Naïve CD8$^+$ T cells (%), CD28$^+$CD8$^+$ T cells (%), CD4$^+$ T-cell count, B cells (%), T-cell count, T cells (%), CD8$^+$ T-cell count, CD8$^+$ T cells (%), IL-10, Tfh1 cells (%), IL-1ra, B cell count, and mDCs (%).

The confusion matrix shows the accuracy of the model built on the 80 immune indicator data measured in 90 patients with sepsis (Table 2). Except for cluster 3, the other two clusters had a class

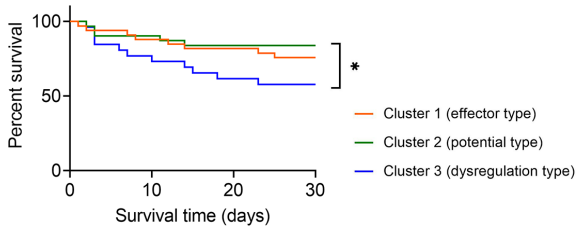

FIGURE 5
Survival analysis of sepsis patients grouped by immune endotypes. Kaplan–Meier survival curves showing the 30-day survival rates for three clusters of sepsis patients. *p < 0.05 (log-rank test).

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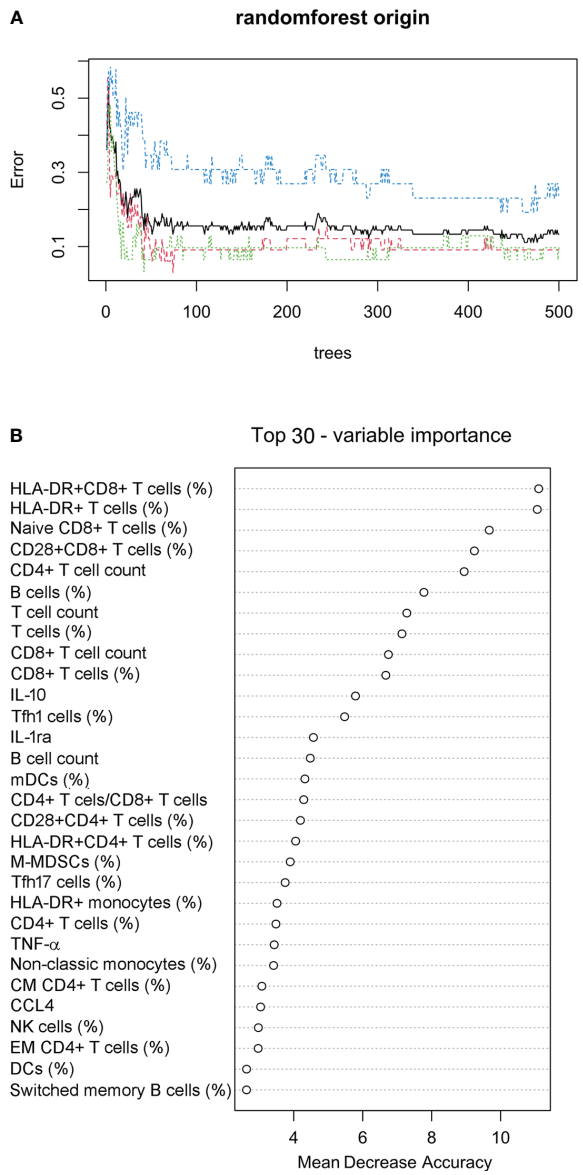

FIGURE 6
Prediction model for classifying sepsis endotypes. **(A)** The random forest approach was used to build a prediction model for classifying sepsis endotypes (cluster 1, 2, or 3) based on 80 immune indicator data from 90 patients with sepsis. The out-of-bag error estimate for the model was 13.33%. **(B)** Cleveland dot plots showing the top 30 immune indicators sorted by importance for prediction based on the mean decrease in accuracy.

error lower than 0.10. Hereafter, the OOB error estimate for the model was 13.33%. Moreover, the built model on the original data set from 90 patients with sepsis was used to predict cluster membership from another cohort of 37 patients with sepsis. The accuracy of the prediction model was 86.5% (95% CI: 71.2%, 95.5%), as shown in Table 3. We also attempted to build the model with the top 30 important immune indicators based on the mean decrease in accuracy, and the performance of the model is shown in Supplementary Table S5. We evaluated the less complex model using our validation cohort, which demonstrated decreased accuracy (Supplementary Table S6).

TABLE 2 The accuracy of the predictive model based on 80 immune indicators measured in 90 patients with sepsis.

Predicted cluster	Original cluster			Class error
	1	2	3	
1	30	2	1	0.09
2	2	28	1	0.09
3	4	2	20	0.23

The out-of-bag error estimate for the model was 13.33%.

Discussion

Currently, sepsis has been defined as life-threatening organ dysfunction caused by the dysregulated or dysfunctional host immune response to infection (25–27). However, the nature and mechanism of immune dysregulation in sepsis still remain ambiguous. In this study, three distinct clusters representing different immune status were identified in sepsis patients, which displayed significantly different survival rates. "Effector type" and "potential type" both signify a normally functioning immune state. In effector-type sepsis patients, T cells exhibit heightened activation, and a larger proportion actively perform their functions, indicating the patient's body is actively and effectively combating the infection. In potential-type sepsis patients, the percentage of T cells is higher, with an increased presence of natural T cells, suggesting the patient's body is actively generating immune cells from the bone marrow to combat the infection. Conversely, the "dysregulation type" is characterized by the simultaneous excessive release of inflammatory and anti-inflammatory factors, coupled with a substantial presence of immunosuppressive cells. This complete immune dysfunction results in a very poor prognosis for the patient. The three immune endotypes we defined all exhibited manifestations of immune dysregulation, with the "dysregulation type" being the most severe and correlated with the highest mortality rate. However, despite experts proposing a new definition of sepsis, a clear definition and explanation of "dysregulation" were not provided (6). The immune characteristics associated with the dysregulation type, as proposed in this study, may serve as a manifestation and explanation of immune response dysregulation within the latest definition. The results of this study may, to some extent, reveal the cause of the heterogeneity of sepsis, and the model we have established may aid clinicians in identifying the potential endotype of sepsis before its onset in patients, which allows for precisely selecting immune modulators for the treatment of the disease.

TABLE 3 The accuracy of the predictive model based on data from another cohort of 37 patients with sepsis.

Predicted cluster	Original cluster		
	1	2	3
1	12	0	0
2	1	12	1
3	2	1	8

The accuracy of the prediction model was 86.5% (95% CI: 71.2%, = 95.5%).

The concept of endotypes has been proposed relatively early in sepsis, with genomics widely applied in defining these endotypes. Brendon et al., utilizing the whole-genome expression profiles of peripheral blood from ICU sepsis patients, employed a combination of unsupervised clustering and machine-learning techniques to categorize sepsis patients into four endotypes. Each endotype corresponds to varying degrees of mortality risk, with new biomarkers defined for each endotype (23). Similarly, Arjun et al., including sepsis patients from both emergency departments and ICUs, conducted transcriptomic sequencing and data mining analysis to classify sepsis patients into five distinct endotypes. They comprehensively characterized the immunological features and mortality risks of these five different endotypes of sepsis patients from an RNA perspective (21). In contrast, our study on sepsis endotypes is based on the protein level. Compared to genomics, protein expression can more accurately reflect the patient's status, and protein detection is more stable. Our approach serves as a complement to endotype research in sepsis.

The complexity of immune cells involved, concurrent hyperinflammation and immune suppression, and heterogeneity of patients are three major challenges to understanding the immunopathology of sepsis. Many immunologic risk factors are involved in the development of sepsis, among them an increase in a variety of inflammatory cytokines, fewer lymphocytes, and an increase in inhibitory cells such as MDSCs and Treg cells, which are prominent characteristics with a poor prognosis (28–30). Consistent with this notion, we found that immune indicators including proinflammatory cells (monocytes, NK cells, DCs, Th1, Th17, and Tfh cells, as well as $CD4^+$ and $CD8^+$ T cells), anti-inflammatory cells (MDSCs, Treg cells, and Th2 cells), and inflammatory cytokines were markedly altered in sepsis. Of note, due to patient heterogeneity, some indicators without significant differences between patient groups may also have the potential to classify the disease. For instance, although the activation marker HLA-DR expression on T cells did not show a significant difference between sepsis and bacteremia patients, it could be an important marker for identifying sepsis cluster 1 in this study. Thus, the indicators with no obvious change in sepsis could be meaningful for the classification of different endotypes of the disease.

Regarding immune suppression in sepsis, despite the increase of inhibitory cells, including Treg and MDSCs, as previously mentioned (14, 30, 31), we observed that some anti-inflammatory cytokines such as IL-1ra, PD-L1, and IL-10 were the key mediators in negative regulation of sepsis. In particular, we observed that IL-1ra was one of the most important variables in clusters of patients with immune suppression, indicating the superior role of IL-1ra in reflecting immune suppression than other anti-inflammatory cytokines. Moreover, given that the most prominent immune characteristic of sepsis is the dysregulated immune response (11, 12, 32), our results are in line with previous reports showing that the concurrent hyperinflammation and immunosuppression (cluster 3) was the most important sepsis endotype with the lowest survival rate.

Several issues deserve mention. First, the interpretation of our findings might be limited by the sample size and specific bacterial organisms. Further validation with a large clinical cohort is necessary; stratified analysis based on the specific pathogen type is necessary; and the results of this study may not extrapolate to patients with virus- or fungus-related sepsis. Second, the longitudinal analysis of patients with sepsis is difficult due to the broad heterogeneity of patients. The endotypes of patients could also be switched. Third, given that blood samples were collected from study participants at the time of notification of a positive blood culture, the previous empirical antibiotic treatment might impact the results of immune indicators. Fourth, we did compare neutrophils among the three groups. However, the performance of neutrophils was found to be inferior to that of PMN-MDSCs. Furthermore, there is a strong correlation between neutrophils and PMN-MDSCs (Supplementary Figure S9). We opted for a ready-made reagent kit for cytokine analysis, which led to the omission of several well-recognized cytokines associated with sepsis progression, such as CXCL-1, IL-18, IL-26, and IL-27.

Taken together, we have described the immune landscape of sepsis patients by systemically analyzing immune cells and their mediators. This study has not only classified sepsis patients into three immune endotypes with different outcomes but also established a prediction model enabling the stratification of patients into different endotypes, which is of potential value in selecting immune modulators for sepsis treatment.

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.

Ethics statement

This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (ID: TJ-IRB20211009). 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

GT: Writing – original draft, Data curation, Visualization, Investigation. YL: Writing – original draft, Visualization. HS: Writing – original draft, Software. WL: Writing – original draft, Investigation. YH, XW and SZ: Writing – original draft, Data curation. ZS: Writing – original draft, Funding acquisition. HH: Writing – original draft, Methodology. FW: Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1287415/full#supplementary-material

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