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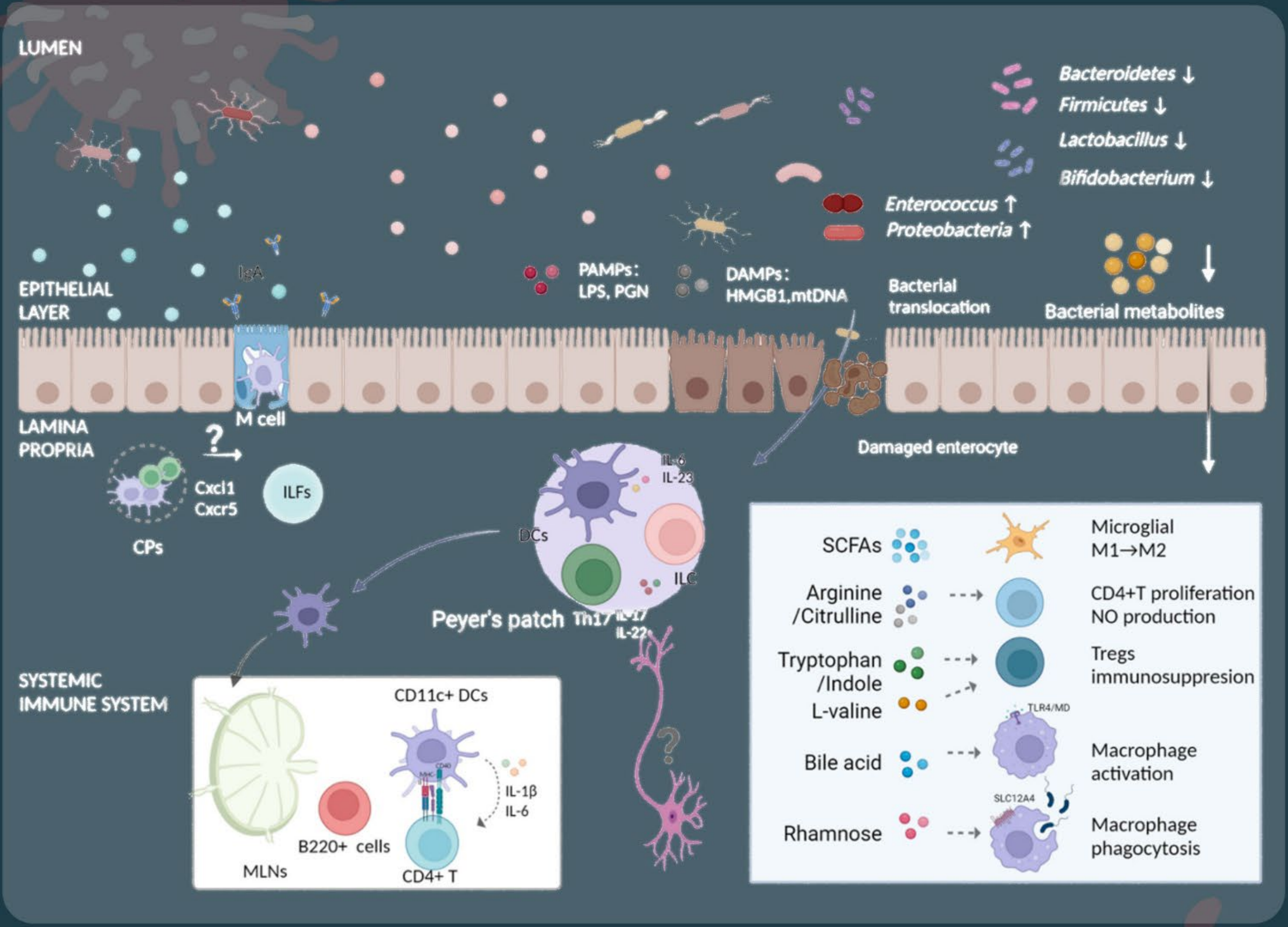

The roles and potential clinical implications of
gut microbiome in sepsis

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EDITORIAL

Immunomodulation and antitumor strategies targeting tumor microenvironment

Xiaobo Wu*

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(This article belongs to the *Special Issue: Immunomodulation and Antitumor Strategies in the Tumor Microenvironment*)

The tumor microenvironment (TME) is the key determinant of cancer initiation, progression, and response to therapy.[1] The TME consists of a heterogeneous network of tumor cells, immune cells, stromal cells, extracellular matrix, and soluble factors that interact dynamically and bidirectionally to shape the tumor phenotype and behavior. The TME also plays a crucial role in modulating the immune system, which can either mount an antitumor response or facilitate tumor evasion and survival. Therefore, understanding the mechanisms of immune regulation in the TME is essential for developing novel immunomodulatory and antitumor strategies that can enhance the efficacy and safety of cancer immunotherapy.

Cancer immunotherapy has emerged as a promising and powerful modality for treating various types of malignancies. Immunotherapy aims to stimulate the immune system to recognize and eliminate tumor cells, either by augmenting the activity of effector cells, such as cytotoxic T lymphocytes and natural killer cells or by blocking the inhibitory signals that dampen the immune response, such as checkpoint molecules.[2,3] However, despite the remarkable clinical success of some immunotherapeutic agents, such as monoclonal antibodies against programmed cell death protein 1 (PD-1) and its ligand PD-L1, only a subset of patients benefit from these treatments, and many develop resistance or adverse events. Moreover, some tumors are inherently immunologically "cold," meaning that they have low immunogenicity and poor infiltration of immune cells, which limits the effectiveness of immunotherapy. Therefore, there is a pressing need to identify new ways to overcome the immunological barriers and challenges posed by the TME and to improve the outcomes of cancer patients receiving immunotherapy.[4]

In this special issue, we have gathered a collection of original research articles and reviews that cover various aspects of immunomodulation and antitumor strategies targeting the TME, with the aim of providing a comprehensive overview of the current state of the art and the future directions in this exciting and rapidly evolving field. The articles span a wide range of topics, including the role of immune cells, cytokines, chemokines, and other mediators in the TME; the molecular and cellular mechanisms of immune evasion and suppression in the TME; the identification and characterization of novel targets and biomarkers for immunomodulation and antitumor therapy in the TME; the development and evaluation of novel immunomodulatory and antitumor agents and approaches that target the TME; and the clinical implications and challenges of TME-targeting immunomodulation and antitumor strategies.

We hope that this special issue will stimulate further research and innovation in the field of immunomodulation and antitumor strategies relevant to the TME and contribute to the advancement of cancer immunotherapy. We would like to thank all

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the authors and reviewers for their valuable contributions and insights, and the editorial staff for their support and assistance. We also invite the readers to peruse articles collected in this special issue and to share with us their feedback and comments.

Conflict of interest

Xiaobo Wu is the editorial board member of this journal and the guest editor of this special issue. The author declared that he has no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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REVIEW ARTICLE

The roles and potential clinical implications of gut microbiome in sepsis

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Abstract

Pronounced dysbiosis in the gut microbiome is common among sepsis patients, resulting in aggravation of the disease. This disturbance not only impacts gut integrity but also initiates localized immune responses that may progress to systemic inflammation. This review explores recent discoveries regarding the dysregulation of the gut microbiome, alterations in gut permeability, and disruptions in intestinal immune responses that occur during sepsis. In addition, we discuss innovative therapeutic strategies, encompassing the impacts of metabolites derived from microbes, the selection of beneficial probiotics, and the utilization of fecal microbiota transplantation in the management of sepsis. Understanding the complexities of the gut microbiome holds the promise of revealing novel strategies that may transform the treatment of sepsis, providing a ray of hope for improved outcomes in critically ill patients.

Keywords: Sepsis; Gut microbiota; Metabolite; Dysbiosis; Immune response

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1. Introduction

Sepsis is a life-threatening disorder caused by the dysregulated host response to infection with a high risk of morbidity and mortality.[1] This syndrome remains a global health concern for the World Health Organization, dramatically influencing resource allocation and causing substantial economic ramifications.[2,3] In the United States, sepsis incurs nearly $24 billion in health-care costs annually, surpassing many other diseases, despite constituting a relatively small portion of hospitalization.[4,5] Recognized through clinical signs and a diverse array of symptoms, sepsis involves a complex interplay of pro- and anti-inflammatory responses, leading to multiorgan dysfunction.[1] Due to the broad spectrum of pathophysiological causes and clinical manifestations, intensivists face challenges pinpointing specific medications for sepsis. Current sepsis treatments mainly consist of antibiotics and supportive care, with limited options for targeted therapies.[6] Therefore, comprehending the underlying mechanisms of sepsis and developing novel therapeutic strategies is crucial.

The gut microbiome is a complex ecosystem that plays a crucial and active role in its host. The microbial inhabitants in our gut consist of approximately 100 trillion cells, outnumbering the cells that make up the human body by 10 folds.[7] The gut microbiome contains about 2 – 4 million genes, showcasing a vast diversity of 100 – 150 times greater than the human genome.[8,9] The advent of 16S rRNA sequencing and metagenomics enables us to delineate the microbial profile effectively.[10] Through these techniques, the gut bacterial species in healthy volunteers primarily comprise three phyla: *Bacteroides*, *Firmicutes*, and *Actinobacteria*.[11] Over the years, evidence has indicated that the gut microbiota may play a crucial role in sepsis.[12] Studies utilizing sequencing methods have elucidated that microbiota imbalance, such as reduced microbial diversity and an abundance of microbial genes, could be impacted by sepsis and *vice versa*.[13,14]

Protein-calorie malnutrition is prevalent among critically ill patients, often resulting from a combination of factors such as anorexia, diarrhea, and decreased body mass, all influenced by the inflammatory response and hypermetabolism.[15] The dysregulated gut microbiota plays a crucial role in the initiation and progression of sepsis. Patients with sepsis commonly exhibit gastrointestinal dysfunction marked by issues such as altered gut motility and permeability, which can severely hinder digestion and absorption, thereby exacerbating inflammation and contributing to multiorgan failure. Given the gut's crucial function in metabolizing dietary compounds into bioactive molecules, gastrointestinal dysfunction during sepsis can significantly impact the production of protective metabolites.[16]

While some aspects of gut dysbiosis have been elucidated, a more comprehensive understanding of how gut microorganisms influence the sepsis process is required. This review aims to deepen our understanding of the intricate relationship between gut microorganisms and sepsis (Figure 1). It specifically focuses on elucidating the changes in gut microorganisms, their functional effects, and the role of microbial-derived metabolites in sepsis, and explores potential therapeutic strategies targeting sepsis through modulation of the gut microbiome.

2. Gut dysbiosis in sepsis

2.1. Sepsis-induced microbiome change

The pathophysiology of sepsis is intricately complex, involving various infection sites and failing organ systems. Reduced intestinal diversity has been linked to unfavorable outcomes in critically ill patients due to its susceptibility to influences such as antibiotic therapy. A study on septic patients in a Chinese cohort revealed that α-diversity was initially similar between septic and non-septic cases on day 1, but within a week, septic patients exhibited a significant drop in diversity compared to the control group.[17] An investigation into intensive care unit (ICU) patients' fecal microbiota discovered a decreased presence of *Faecalibacterium prausnitzii*, known for its anti-inflammatory function, in both septic and non-septic individuals.[18] Furthermore, a multicenter study involving 155 ICU patients found that septic patients had elevated levels of harmful intestinal microbiota such as *Parabacteroides*, *Fusobacterium*, and *Bilophila* species in perirectal swabs.[19] These microbes are associated with endotoxin production, increased mortality risk, and heightened inflammation, further disrupting metabolic and immune homeostasis.[20,21]

2.2. Factors contributing to dysbiosis

Critically ill patients are more prone to experiencing disruptions in their intestinal microbiota. While the precise causal mechanisms remain unclear, various factors can contribute to disturbances in the gut microbiome, encompassing extrinsic influences such as antibiotic treatments and intrinsic factors like disease and systemic inflammation.

In ICUs, antibiotics, particularly broad-spectrum ones, are commonly administered to patients suspected of sepsis before bacteriological results are available. A large epidemiological study spanning 500 hospitals revealed that exposure to high-risk antibiotics during hospitalization could disrupt patients' microbiota, potentially heightening the risk of sepsis.[22] These high-risk exposures comprised third- or fourth-generation cephalosporins, fluoroquinolones, lincosamides, β-lactam/β-lactamase inhibitor combinations, oral vancomycin, and carbapenems. Their findings identified that patients exposed to cephalosporins, vancomycin, and β-lactamase inhibitors exhibited a heightened association with the development of sepsis and septic shock.[22] Long-term antibiotic administration may lead to the development of resistant gut flora such as vancomycin-resistant *Enterococcus faecium* (VRE)[23,24] and *Clostridium difficile* infection.[25] These alterations and disruptions in the gut microbiota can result in bacterial translocation, facilitated by increased intestinal permeability and compromised gut barrier integrity, potentially exacerbating systemic inflammation. Besides, Mu *et al.*[26] described that VRE and *Klebsiella* are predominant in the gut, potentially leading to secondary infections in septic patients following

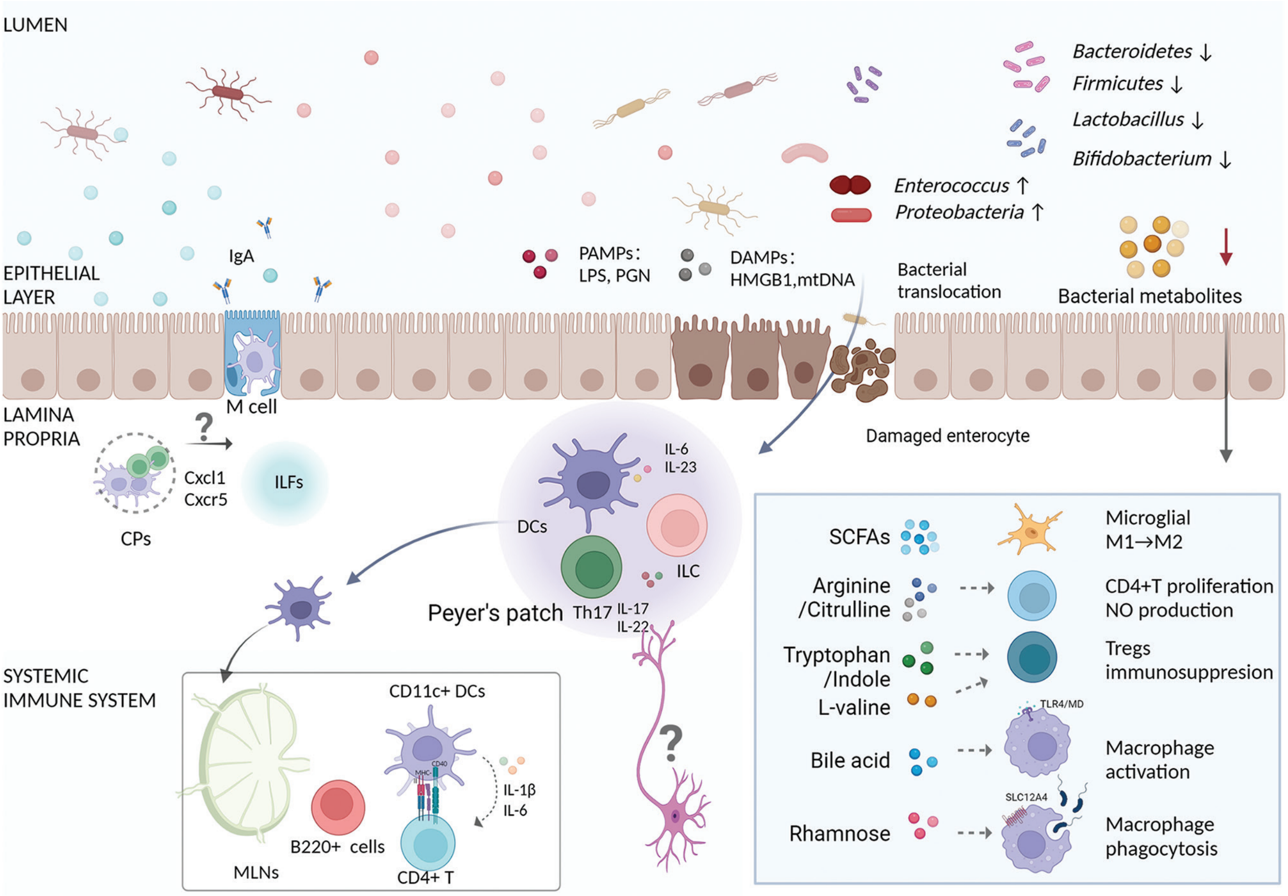

Figure 1. The interplay between gut microbiota, metabolites, and host immune response in sepsis. This schematic illustrates the relationship between the immune response and gut microbiota across the epithelial layer, lamina propria, and systemic immune system. The microbiota is essential for maintaining gut immune function. However, sepsis disrupts the epithelial barrier, leading to an increase in pathogenic bacteria such as Enterococcus and Proteobacteria and a decrease in beneficial probiotics such as Lactobacillus and Bifidobacterium. This disruption facilitates the translocation of bacteria and endotoxins, which trigger immune responses locally and systemically. In addition, alterations in gut-derived metabolites can impact the immune system, with supplementation of these metabolites offering protective effects on immune cells, particularly T cells, and macrophages. Within GALT, the specific roles of isolated ILFs differentiation and PPs function, especially in connection with the nervous system, require further investigation. The schematic diagram was created using BioRender.com.

Abbreviations: CPs: Cryptopatches; DAMPs: Damage-associated molecular patterns; DCs: Dendritic cells; GALT: gut-associated lymphoid tissue; HMGB1: High mobility group box 1; ILC: Innate lymphoid cells; ILFs: Isolated lymphoid follicles; LPS: Lipopolysaccharide; M cell: Microfold cell; MLNs: Mesenteric lymph nodes; PAMPs: Pathogen-associated molecular patterns; PGN: Peptidoglycan; PPs: Peyer's patches; SCFAs: Short-chain fatty acids.

broad-spectrum antibiotic therapy. According to these studies, antibiotic usage is believed to be an essential factor influencing the composition of the intestinal microbiota and elevating the risk of sepsis. However, further research is warranted to elucidate the intricate relationship between antibiotics, the gut microbiome, and sepsis, particularly considering the complexity of factors such as dosage and timing of antibiotic administration before or after ICU admission. In addition to antibiotic use, sedatives and analgesics can also perturb the gut microbiome during sepsis. Sedatives and analgesic medications, such as opioids, benzodiazepines, and propofol, are the mainstay in critical care to enhance patient comfort. Notably, morphine, a commonly used analgesic, has been linked to increased mortality rates in animal models due to the expansion of Gram-positive bacteria such as *Staphylococcus* and *Enterococcus*, leading to bacterial dissemination, upregulation of the proinflammatory cytokine interleukin (IL)-17A, and dysfunction of the gut epithelial barrier.[27]

Nutrition plays a pivotal role in human health and diseases, with its effects partially mediated through the gut microbiota.[28] In contrast to the sepsis samples, the microbial species associated with non-sepsis are frequently observed in non-Westernized populations adhering to traditional lifestyles, characterized by lower fat and phosphatidylcholine intake compared to Western diets. The increased abundance of *Ezakiella*, the butyrate-producing

Megasphaera, and *Prevotella* (especially *Prevotella copri*) could potentially confer a protective effect against sepsis.[19] Similarly, research on a septic animal model treated with cecal ligation and puncture (CLP) indicated that a high-fiber diet could mitigate systemic inflammation and mortality.[29] Conversely, a high-fat diet was found to increase mortality and organ damage in the CLP mouse model of sepsis,[30] corroborating findings from a U.S. cohort study involving 21,404 participants which indicated that a Southern dietary pattern was linked to a higher risk of sepsis.[31] However, the specific impact of a high-fat diet on the gut microbiota remains elusive. Further study to deepen understanding of the relationship between diet and microbiota could unveil novel approaches in formulating dietary interventions for septic patients. The Surviving Sepsis Campaign recommends early enteral nutrition for adult patients with sepsis or septic shock due to its potential impact on reducing gastrointestinal complications and influencing gut permeability, inflammation, and immune responses.[32,33]

3. Host defense mechanisms against sepsis

3.1. Intestine as a barrier in sepsis

The intestinal barrier is a complex structure comprising three essential layers: the luminal layer, mucus layer, and epithelial layer.[34] In the luminal layer, commensal gut microbiomes are crucial in inhibiting opportunistic and pathogenic microbial species from colonizing the intestines, especially in ICU patients.[12] The mucus layer acts as a physical barrier that lubricates the contents passing through the intestine and aids in digesting enzymes. This layer thrives in the presence of the microbiome. In addition, it interacts with secretions from Paneth cells and enterocytes, such as lysozyme and immunoglobulin A (IgA), which collectively exert an antibacterial effect.[35] Wilmore *et al.*[36] revealed the critical role of beneficial microbes in stimulating the production of serum IgA antibodies. These IgA antibodies can help mitigate the severity of polymicrobial sepsis by neutralizing pathogens and impeding their dissemination from the gut into the systemic circulation. Furthermore, the mucus layer is rich in mucins (MUCs) produced by goblet cells. The small intestine has a monolayer of mucus, whereas the large intestine features two substantial layers.[37] Among these mucins, MUC2 stands out as the predominant protein responsible for maintaining mucosal homeostasis by restricting the interaction between pathogens and the underlying epithelial layer. In a study with *Muc2*-deficient (*Muc2*$^{-/-}$) mice, characterized by the absence of an intestinal mucus layer and increased intestinal permeability, it was noted that the efficacy of splenic macrophages in erythrophagocytosis decreased when recognizing senescent red blood cells during lipopolysaccharide-induced inflammation. Furthermore, these mice exhibited reduced immune apoptosis and an elevated presence of fecal IgA+ bacteria, which potentially contribute to intestinal inflammation.[38] This finding illustrated how impaired intestinal permeability can promote bacterial growth and increase the susceptibility to sepsis.

Within the intricate third layer are intestinal epithelial cells, comprising enterocytes, goblet cells, stem cells, neuroendocrine cells, and Paneth cells. Enterocytes, the predominant cell type among intestinal epithelial cells, are responsible for nutrient absorption and immunoglobulin secretion. They facilitate cell–cell communication through structures such as tight junctions, adherent junctions, and gap junctions. Paneth cells, primarily found in the small intestine, can activate toll-like receptors through direct contact with bacteria.[39] Generating microbial cell wall components such as pathogen-associated molecular patterns such as lipopolysaccharide and peptidoglycan can trigger excessive immune activation. In addition, damage-associated molecular patterns originating from apoptotic intestinal epithelial cells, such as HMGB1 and mtDNA, can elevate adhesion molecules on the intestinal endothelium, leading to the recruitment of neutrophils and macrophages.[40] The migration of these immune cells to the gut can induce systemic inflammation characterized by the release of proinflammatory cytokines through MAPK/NF-κB pathways. This cascade can increase intestinal permeability, exacerbating gut barrier dysfunction by modulating tight junction proteins.[41] The redistribution of the tight junction proteins may facilitate the translocation of local bacteria from the gut lumen.[42] One such protein, claudin-2, which forms paracellular cation and water channels, is selectively upregulated in septic patients. Deletion of claudin-2 in mice was found to protect against sepsis-induced pore pathway permeability, reducing IL-17 production, T-cell activation, and intestinal damage. This leads to decreased numbers of neutrophils, macrophages, dendritic cells (DCs), and bacteria in the peritoneal fluid of mice. Consequently, claudin-2 deletion significantly improves survival in sepsis.[43] By targeting claudin-2, interventions could modulate the microbiome composition and function, offering new avenues for sepsis management and treatment strategies.

3.2. Intestinal immune responses in sepsis

While bacteria and endotoxins may not directly enter circulation, they have the potential to trigger immune responses within the local gut-associated lymphoid tissue (GALT), leading to systemic inflammation. GALT, functioning as a secondary lymphatic organ, protects the host from invasions within the gut.[44] Furthermore, mesenteric lymph nodes (MLN), Peyer's patches (PPs), and smaller isolated lymphoid follicles (ILF) are critical components of this complex immune system network.[45] MLNs are the most prominent lymph nodes in the body, comprising both cortex and medulla.[46] During sepsis, circulating lymphocytes migrate to the T-cell zone of MLNs, where DCs then present antigens to the T-cells.[47] Research by Darkwah *et al.*[39] revealed a significant increase in CD4 T-cell proliferation by mucosal MLN DCs in the CLP septic model compared to systemic DCs from the spleen, indicating a gut-derived pathway to systemic circulation triggered by bacterial translocation. In addition, O'Boyle *et al.*[48] identified similarities between organisms in the MLN and the pathogens responsible for sepsis in surgical patients, lending credence to the gut-origin hypothesis for sepsis onset.

PPs are distributed throughout the small intestine, with the highest concentration typically found in the ileum. They comprise lymphoid follicles characterized by a germinal center, subepithelial dome, and follicle-associated epithelium.[49] The germinal center is densely populated with proliferating B lymphocytes, DCs, and macrophages. In contrast, the subepithelial dome contains a mix of B and T lymphocytes, along with DCs and macrophages. PPs have the unique ability to sample luminal antigens by crossing the epithelial barrier through specialized microfold cells that secrete macromolecules.[50] The primary role of PPs lies in their communication with the enteric nervous system, thereby contributing to the microbiota-gut-brain axis.[51] Under normal conditions, DCs within PPs detect mucosa-associated bacteria, triggering the production of IL-6 and IL-23, which in turn regulate IL-17 and IL-22 levels in T cells and innate lymphoid cells. Nonetheless, evidence regarding the immune function of PPs in sepsis remains limited.[52] Schulz *et al.*[53] reported that *Salmonella* infection triggered the hypertrophy of PPs and identified the IFNAR/CD69/S1PR1 axis, which facilitates the lymphocyte egress during infection. Conversely, Fan *et al.*[54] demonstrated reduced PPs cell yield and CD4+T cell count in the CLP model.

ILFs, a specialized type of tertiary lymphoid organs, are notably smaller than PPs and feature a microfold-cell surface epithelium. Despite their significance, there is limited understanding of ILFs in the context of sepsis. These structures typically develop in response to microbial antigens and dietary components in healthy individuals, yet in certain pathological conditions, such as trauma, infection, or other irritations, their formation can be triggered.[55] ILFs are characterized by a sparse population of T cells and lack distinct T-cell zones. In germ-free mice, ILFs can be supplanted by a majority of Lin^- c-kit^+ $\text{IL-7R}\alpha^+$ $\text{ROR}\gamma\text{t}^+$ cells known as cryptopatches, which, upon interaction with commensal bacteria, initiate the development of ILFs.[56] Deficiencies in CXCL13, CXCR5, or RORγt may result in the failure of cryptopatches from maturing into ILFs.[57] Recently, Wu *et al.*[35] shed light on the connection between the complement system and gut immunity by identifying the presence of C3-expressing cells within ILFs. Studies have shown that sepsis can reduce the quantity and size of lymphoid follicles.[58] Notably, patients with sepsis exhibit a decrease in B-cell areas and lymphoid follicle counts compared to trauma patients without sepsis. This depletion is particularly pronounced in patients with prolonged septic episodes.[59] The development of ILFs necessitates lymphoid-inducer cells capable of secreting IL-17 and IL-22, both components of the Th17 signature. In sepsis, IL-17-producing cells such as Th17 and γδ T17 cells are recognized for their pro-inflammatory nature and their potential role in exacerbating sepsis-related conditions. These cells may infiltrate the brain, leading to sepsis-associated encephalopathy,[60] or migrate to the lungs, worsening sepsis-induced acute lung injury.[61] These observations highlight a plausible link between sepsis and the development of ILFs.

4. New strategies for treatment of sepsis

4.1. Probiotics

Recent studies have revealed that specific microbial supplements hold the potential to bolster immune responses and alleviate the severity of sepsis. The PRIMAL clinical trial, for instance, has provided evidence that probiotics *Bifidobacterium* and *Lactobacillus* can effectively ameliorate gut dysbiosis in preterm infants, potentially lowering the risk of severe conditions such as sepsis and necrotizing enterocolitis.[62] In a separate randomized clinical trial investigating probiotic effects on cytokine levels in children with severe sepsis, a notable decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines were observed. This trial involved the administration of a combination of four *Lactobacillus* strains (*Lactobacillus paracasei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii*), three *Bifidobacterium* strains (*Bifidobacterium longum*, *B. infantis*, and *B. breve*), and *Streptococcus*.[63] Furthermore, Xie *et al.*[64] discovered that supplementation with the emerging probiotic *Akkermansia muciniphola*, along with its supernatant, can reduce sepsis-induced mortality in a CLP model. In this study, they identified a novel tripeptide, Arg-Lys-His (RKH), as an endogenous antagonist for Toll-like receptor 4 (TLR4). They also highlighted the increasing significance of *Candida albicans* and its derivative metabolite phenylpyruvate in boosting macrophage bactericidal activity and reducing multiple organ dysfunction syndrome for patients with bacterial sepsis.[65] In addition, another research group revealed that a reduced abundance of *Parabacteroides* during pregnancy could exacerbate inflammation and worsen sepsis outcomes. Treatment with *Parabacteroides merdae* and its metabolites, particularly formononetin, can protect against septic inflammation by inhibiting macrophage pyroptosis.[66] Together, probiotics hold promise as a complementary approach to managing sepsis, offering a novel avenue for therapeutic intervention in this critical condition.

4.2. Prebiotics

Prebiotics are non-digestible compounds that can be selectively metabolized by gut microorganisms, providing significant benefits to the host.[67] While certain non-carbohydrate compounds such as polyphenols and polyunsaturated fatty acids can function as prebiotics, most prebiotics are carbohydrate-based.[68] Low-molecular-weight carbohydrates are efficiently converted by bacteria. Key examples include fructans (such as fructooligosaccharides and inulin [FOS]) and galactans (such as galactooligosaccharides [GOS]), which promote the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*.[69] Clinical studies have demonstrated the potential of prebiotics such as inulin to improve inflammation in conditions such as ulcerative colitis by enhancing the abundance of butyrate-producing *Firmicutes*.[70] In addition, inulin has been shown to modulate gut microbiota, including *Bacteroides* and *Parabacteroides*, to suppress diet-induced non-alcoholic steatohepatitis.[71] FOS and GOS have similarly demonstrated their ability to regulate inflammatory responses, with clinical trials showing that GOS combined with *Bifidobacterium* improves intestinal barrier function.[72,73] In addition, other simple carbohydrates such as lactulose also showed a protective effect on intestinal epithelium against the colonization of *Klebsiella* pneumonia.[74] Despite these promising findings, the use of prebiotics in sepsis remains limited.

Several challenges contribute to the scarcity of research on prebiotics in sepsis. One key issue is impaired gastrointestinal function, including reduced gut motility and disrupted nutrient absorption, which limit the effectiveness of orally administered prebiotics. Furthermore, many ICU patients rely on parenteral or enteral nutrition, where prebiotics may not be well tolerated or easily incorporated. There is also concern that prebiotics stimulating bacterial growth may increase bacterial translocation and worsen systemic infections. While prebiotics show potential in regulating inflammation and improving gut health in various diseases, their application in sepsis and critically ill patients in ICU is underexplored.

4.3. Postbiotics

4.3.1. Short-chain fatty acids (SCFAs)

The gut microbiota metabolites are essential for maintaining the fundamental functions of the host in a healthy state. Disruptions in the production of these metabolites can lead to a range of diseases, including metabolic disorders, cardiovascular issues, and gastrointestinal ailments. SCFAs are metabolites produced by the gut microbiome through the fermentation of dietary fibers. These SCFAs, primarily acetate, propionate, and butyrate, play a crucial role in communicating between the gut and the immune system.[75] Acetate is the most abundant SCFA, produced extensively by bacteria such as *Prevotella* spp., *Bifidobacterium* spp., *and Akkermansia muciniphila*.[76] Acetate has been shown to regulate immune responses in various disease contexts, including colitis[77] and arthritis.[78] Notably, acetate can also modulate the brain's immune system, as demonstrated by its ability to influence microglia during neurodegeneration.[79] The depletion of SCFAs due to antibiotic disruption of the gut microbiome can lead to hyperresponsive macrophages, a condition that disturbs gut immune homeostasis. Significantly, the supplementation of butyrate alters the activation of these macrophages, restoring a more balanced immune response.[80] In addition, broad-spectrum antibiotics have been found to promote the colonization of invasive fungi by decreasing SCFA-producing *Clostridium* species.[81] In the context of sepsis, SCFAs have been shown to affect sepsis-induced encephalopathy[82] by protecting cognitive function and altering the polarization of microglia.[83] These studies highlight the critical role of SCFAs in maintaining gut-immune communication and modulating immune responses.

4.3.2. Amino acids

Bacteria in the gut can produce both essential and non-essential amino acids, including glutamine, arginine, and tryptophan. These amino acids contribute to a variety of physiological processes, such as immune regulation, neurotransmitter synthesis, and gut barrier maintenance. However, the composition and function of these amino acid-producing bacteria can be significantly altered during and after sepsis or other infectious events. These disruptions to the gut microbiome can lead to dysregulation in amino acid metabolism.[84]

(A) Glutamine

Glutamine, produced by gut bacteria such as *Bacteroides* and *Clostridium*, contributes to the proliferation of intestinal cells and maintains the integrity of the gut barrier.[85] Wu *et al.*[86] demonstrated that glutamine is particularly important in preserving the intestinal mucus barrier in a mouse model of burn sepsis. Specifically, the researchers found that glutamine promotes the O-GlcNAcylation of the glucose-6-phosphate dehydrogenase (G6PD). This modification enhances the intestinal cells' antioxidant defenses, helping protect them from oxidative stress. At the same time, glutamine inhibits the S-glutathionylation of the protein AGR2, essential for mucus production. By supporting antioxidant mechanisms and mucus production, these glutamine-mediated effects help preserve the integrity of the gut barrier. This, in turn, reduces the risk of bacterial translocation and the development of systemic complications during sepsis.

(B) Arginine

Arginine is a semi-essential amino acid in various physiological processes, including protein synthesis, nitric oxide production, and immune function.[87] In the gut, arginine is produced by the enterocytes and gut microbiota. During sepsis, the body's demand for arginine increases significantly due to its involvement in immune response modulation, vascular regulation, and tissue repair. However, sepsis-induced dysbiosis can alter the gut microbiota responsible for arginine production, leading to complications in arginine metabolism and availability.[88] Certain gut bacteria, including *Lactobacillus* and *Bifidobacterium* species, decrease in abundance after the onset of sepsis, and these bacteria are involved in the synthesis of arginine.[89] These probiotic bacteria can convert the amino acid ornithine into citrulline, which is further converted into arginine through the urea cycle. Clinical trials have explored the potential benefits of arginine infusion in severe sepsis, focusing on its effects on microcirculation and metabolic function.[90] One randomized controlled trial demonstrated that arginine supplementation could improve microvascular perfusion and support metabolic recovery in septic patients.[91] Furthermore, intravenous administration of arginine has been shown to benefit CD4+ T-cell homeostasis and attenuate liver inflammation in a mouse model of polymicrobial sepsis, thereby boosting T-cell proliferation and function.[90] Arginine's role in enhancing nitric oxide production helps to improve blood flow and reduce lactic acidosis, which is crucial in managing septic shock. However, concerns remain regarding the balance between the beneficial vasodilatory effects of arginine and the potential risk of exacerbating hypotension in unstable septic patients. These studies highlight the need for careful patient selection and dosing to maximize arginine's therapeutic potential in treating sepsis.[92] Investigating how arginine supplementation might restore gut microbiota balance and improve outcomes in septic patients could provide valuable insights into new therapeutic approaches.

(C) Citrulline

Citrulline, a precursor of arginine, has the potential to enhance vasodilation and increase blood flow by stimulating nitric oxide production. Numerous clinical trials have investigated the impact of citrulline on improving the exercise performance of athletes.[93,94] It has also been reported to support gut health by improving intestinal barrier integrity and modulating intestinal inflammation and can be synthesized by beneficial bacteria such as *Lactobacillus*.[95] A study conducted by Wang *et al.*[88] revealed that *Lachnospiraceae* can produce L-citrulline, which, when converted into L-arginine, influences bone mechanical adaptations. During sepsis, especially in ICU patients, citrulline levels often decrease due to gut barrier dysfunction.[96] The damage induced by sepsis compromises the integrity of the gut lining, impairing the ability of enterocytes to generate citrulline. This reduction is closely linked to increased gut permeability, bacterial translocation, and systemic inflammation, all of which exacerbate the severity of sepsis. Therefore, citrulline levels can serve as a biomarker for identifying gut barrier failure and predicting the overall prognosis of septic patients. Citrulline supplementation in sepsis presents both benefits and risks, acting as a double-edged sword. Research by Asgeirsson *et al.*[97] has identified citrulline as an anti-inflammatory agent. Moreover, Reizine *et al.*[98] have demonstrated that enteral citrulline administration can help alleviate sepsis-induced T-cell mitochondrial dysfunction. By restoring arginine levels and promoting nitric oxide production, citrulline boosts T-cell functionality, enhancing immune responses and reducing the severity of sepsis. These findings imply that citrulline may serve as a valuable therapeutic intervention in safeguarding immune function during sepsis. On the contrary, a separate study revealed that citrulline supplementation aggravated sepsis severity in infected preterm piglets.[99] The early administration of citrulline-induced immunosuppression, possibly attributed to excessive nitric oxide production, exacerbates outcomes. This underscores the dangers of immune overmodulation and emphasizes the importance of timing and context in citrulline supplementation. Nevertheless, two clinical trials propose monitoring citrulline levels in septic patients as a potential biomarker for predicting the development of acute respiratory distress syndrome[100] and overall prognosis.[101] A deficiency in citrulline reflects gut barrier dysfunction, which is closely linked to the severity of sepsis and its associated complications. Future investigations could establish citrulline monitoring as a standard protocol for managing septic patients.

(D) Tryptophan

Tryptophan, an aromatic amino acid frequently employed in fluorescence dyes,[102] acts as a precursor for several essential metabolic pathways, particularly the kynurenine pathway, which holds substantial importance in immune regulation during sepsis. Xia *et al.*[103] uncovered the role of tryptophan metabolism in the pathophysiology of melioidosis induced by the Gram-negative bacterium *Burkholderia pseudomallei*, which results in pneumonia and sepsis. Employing a comprehensive metabolic approach, their research unveiled elevated kynurenine levels and increased indoleamine 2,3-dioxygenase activity, resulting in the enhanced conversion of tryptophan into kynurenine. This process hampers T-cell proliferation and function, leading to a state of "immune paralysis" commonly observed in the advanced stages of sepsis. This immune suppression raises the vulnerability to secondary infections, thereby complicating patient outcomes.

4.3.3. Other metabolites

Alongside the metabolites mentioned above, scientists are continuously discovering novel compounds that play pivotal roles in the progression of sepsis. Indole, a bioactive compound produced by various bacteria, including *Clostridium* and *Lactobacillus* species, maintains the integrity of the gut barrier.[104] Through its actions in promoting mucus secretion and tight junction assembly in the intestine, indole protects the gut lining against bacterial translocation. Moreover, indole and its derivatives, such as indole-3-acetic acid and indole-3-aldehyde, function as ligands for the aryl hydrocarbon receptor, a transcription factor involved in modulating immune responses. Activation of aryl hydrocarbon receptors by these metabolites not only dampens the production of proinflammatory cytokines but also suppresses the differentiation of CD4+ regulatory T cells (Tregs).[105] Thus, indole-derived metabolites hold promise for potentially improving outcomes in conditions such as sepsis, where the need to control excessive inflammation while averting immune suppression is paramount.

Li *et al.*[106] unveiled the potential pathophysiological role of gut-derived rhamnose in enhancing macrophage phagocytic activity through its interaction with the SLC12A4 protein, a vital element in the host's defense against polymicrobial sepsis. Other bioactive metabolites such as L-valine have shown promise in preserving intestinal barrier integrity, with findings indicating a negative correlation with APACHE-II and SOFA scores, critical indicators of sepsis severity.[107] Moreover, the secondary bile acid hyodeoxycholic acid (HDCA), derived from primary bile acids by gut microbiota, could mitigate systemic inflammatory responses post-sepsis by curbing the overactivation of inflammatory macrophages.[108] A positive relationship between HDCA levels and the *Eubacterium* abundance was identified, proposing that supplementing this bacterium could enhance HDCA production, potentially aiding in sepsis management.

4.4. Fecal microbiota transplant (FMT)

FMT presents an innovative approach for tackling the gut dysbiosis linked to sepsis, potentially rebalancing the intestinal microbial ecosystem, safeguarding against intestinal damage, and modulating immune responses. A study reported that FMT could mitigate the immunosuppressive effects of pathogens by restoring normal butyrate levels, exerting impacts beyond the intestine.[109] However, several studies have cautioned about severe complications associated with FMT, including the risk of bacteremia. A case report by DeFilipp *et al.*[110] documented instances of *Escherichia coli* bacteremia in two patients post-FMT, which resulted in the unfortunate death of one patient. While FMT holds promise as a therapeutic avenue in sepsis management, comprehensive research is imperative to thoroughly grasp its efficacy and safety profile in clinical settings.

5. Conclusion

Recent advances in metagenomics and metabolomics have markedly enriched our comprehension of the pivotal role the gut microbiome plays in the context of sepsis. This intricate microbial community serves as a cornerstone in regulating host functions, and disruptions within it, known as gut dysbiosis, exert a profound influence on the initiation and progression of sepsis. The intricate interplay between the gut and the immune system presents novel avenues for mitigating organ injury triggered by sepsis, with strategies such as fortifying gut barrier integrity, which demonstrates promise in curtailing bacterial translocation. Following the depletion of gut flora post-sepsis, supplementation with tailored probiotic formulations emerges as a potential avenue for reinstating microbial equilibrium. In addition, the utilization of metabolites synthesized by these beneficial microbes, including SCFAs and essential amino acids, in the formulation of specialized nutritional supplements holds promise in bolstering the recovery of critically ill patients. Interventions aimed at modulating gut microbiomes, such as FMT, exhibit potential for patients grappling with dysbiosis. However, the optimal patient selection, timing of intervention, and administration protocols for FMT warrant further investigation. In summation, a more comprehensive understanding of the gut microbiome harbors the potential to unveil innovative strategies that could revolutionize the management of sepsis, thereby offering a beacon of hope for enhanced outcomes among critically ill individuals.

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Conflict of interest

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Microbial involvement in myalgic encephalomyelitis/chronic fatigue syndrome pathophysiology

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Abstract

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex and disabling disease related to persistent fatigue, exercise intolerance, post-exertional malaise, cognitive dysfunction, and musculoskeletal/joint pain. Gastrointestinal comorbidities, including irritable bowel syndrome, have been reported in affected individuals, indicating a potential role of gut microbiota in disease progression. In this paper, bacterial and metabolomic dysbiosis in subjects with ME/CFS are reviewed, and phenotypic, microbial, and metabolic biomarkers specific to individual cohorts are also identified. Furthermore, microbiome fluctuations or metabolic endotoxemia are proposed as possible disorder biomarkers. Based on the fact that gut microbiota dysbiosis reverts to a state of eubiosis in long-term patients with this condition, it may be hypothesized that disease progression begins with the loss of beneficial gut microorganisms, particularly short-chain fatty acid producers, leading to more widespread gastrointestinal phenotypes that are subsequently reflected in plasma metabolite levels. These alterations, specific of each individual, thereby result in metabolic and phenotypic shifts and in ME/CFS.

Keywords: Myalgic encephalomyelitis/chronic fatigue syndrome; Post-exertional malaise; Gut microbiota; Metabolic biomarkers; Pathophysiological phenotypes

1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex and disabling disease related to persistent fatigue, exercise intolerance, and other symptoms aggravated by physical or cognitive efforts that may manifest immediately or typically be delayed for hours. Prolonged exacerbation of symptoms of ME/CFS, which follows physical activity and, in some cases, cognitive activity, is termed post-exertional malaise (PEM) and may last several days.[1] Due to this symptomatology, the quality of life of people with ME/CFS is significantly restricted in terms of social and occupational participation.[2]

Despite many efforts, the etiology of ME/CFS has yet to be clarified. However, several factors trigger the disease such as genetic predisposition, immune dysfunction, microbial infection, neuroendocrine imbalance, and psychological determinants.[3,4]

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Numerous underlying biological irregularities have been recognized in subjects with ME/CFS, as exemplified by impaired energy metabolism, mitochondrial dysregulation, gastrointestinal and redox imbalances, altered immune processes (including autoimmune response), sleep disturbances, and multiple central and autonomic nervous system abnormalities.[5,6]

Nevertheless, not all patients experience the same clinical manifestations, suggesting the existence of ME/CFS subgroups, according to gastrointestinal disorders linked to microbial gut dysbiosis and metabolic pathways.[7-9] In fact, comorbid conditions such as irritable bowel syndrome (IBS) are often observed in patients with ME/CFS, thereby indicating a potential involvement of the gut microbiota in the progression of the disease.[8] In particular, the involvement of the gut microbiota through gut dysbiosis, the gut-brain axis, gut permeability, and bacterial translocation in ME/CFS has been repeatedly suggested.[5,6,8] It is widely understood that perturbation of the gut microbiota (dysbiosis) and its metabolome can affect several host processes, such as metabolism, inflammation, immunity, and brain function, and also contributes significantly to the development of multiple diseases related to the aforementioned host processes.[6,10]

As ME/CFS prevalence continues to grow globally,[11] establishing the link between the gut microbiota and ME/CFS is a pivotal aspect for delving into the disease's etiology and pathophysiology. Therefore, in the present review, we explore the potential links between the gut microbiota and ME/CFS. First, we review the evidence for bacterial and metabolomic dysbiosis in ME/CFS patients. Second, we identify potential biomarkers particular to patient cohorts across metabolic, microbial, and phenotypic domains.

2. Human gut microbiota and microbial metabolites

The human gut tract is composed of a broad microbial community, with a density of around 10 – 100 trillion of microbial cells.[12] The gut microbiota encompasses the microbial community in the gastrointestinal tract and consists of several microbial taxa, including bacteria, archaea, viruses, protozoa, and fungal species, being the most prevalent the bacteria domain,[13] which form a symbiotic relationship with the human gut.[14] Archaeal genera are present at reduced levels in the healthy human gut microbiota, with *Methanobrevibacter smithii* being the most abundant species.[15] The eukaryotic microorganisms most frequently detected in the gut tract are fungi, such as the genera *Candida* and *Saccharomyces*.[16] Protozoa such as *Blastocystis* have been identified in the human gut microbiota and their presence has been often linked to a decrease in gastrointestinal diseases.[17] The human virome is mainly composed of bacteriophages, and the role that they play in the gut is related to modulating the bacteriome.[18] Typical microorganisms in the human gut are represented by approximately 100 bacterial species that belong to the following eight phyla: Actinomycetota, Bacillota, Bacteroidota, Campylobacterota, Fusobacteriota, Pseudomonadota, Thermodesulfobacteriota, and Verrucomicrobiota.[12,14,19]

Diverse intestinal compartments are characterized by a specific spatial distribution of the microorganisms.[20-22] The human gut microbiota composition differs both functionally and taxonomically based on aspects such as age, antibiotic intake, and diet,[23-25] and the predominant genera within the human gut microbiota are *Bacillus*, *Blautia*, *Clostridium*, *Dorea, Enterococcus*, *Eubacterium, Faecalibacterium*, *Lactobacillus*, *Roseburia*, and *Ruminococcus* (phylum Bacillota); *Bacteroides* and *Prevotella* (phylum Bacteroidota); *Bifidobacterium* (phylum Actinomycetota); and *Escherichia* (phylum Pseudomonadota).[12,14,26]

The gut microbiota exerts beneficial effects for the human host, including metabolic and immunological functions.[12,27] Intestinal microorganisms constitute essential regulators of the digestion process through the absorption of nutrients, and also through the synthesis of diverse metabolites, such as amino acids, lipids, short-chain (2-6 C) fatty acids (SCFAs), and vitamins.[28,29] Furthermore, the gut microbiota exerts a pivotal function in the maintenance of the intestinal epithelium integrity.[27] Moreover, the gut microbiota generates a broad diversity of secreted biologically active compounds from undigested foods,[30] such as histamine, polyamines, SCFAs, and tryptophan catabolites.[31,32] The metabolic products of tryptophan (5-hydroxytryptamine, indole, and kynurenine) have been identified as inhibitory compounds of neuroinflammation.[33] SCFAs, such as acetate, butyrate, and propionate, constitute signaling molecules that locally modulate the gut function via enteroendocrine cells, influencing the metabolism of the brain, liver, and muscle, as well as the host energetic homeostasis.[34,35] In addition, SCFAs exert neuroactive effects via the induction of neuroinflammatory responses.[36]

The state related to the loss of gut homeostasis due to endogenous and exogenous factors is known as dysbiosis, which triggers chronic inflammation and changes in the release of microbial metabolites, including mucin, secondary bile acids, and SCFAs, which are pivotal for the regulation of both the immune and physiological functions of the host.[37] The gut microbiome homeostasis is essential for preserving brain health, such as cognitive function and synaptic plasticity,[38] thereby inhibiting neuroinflammation processes and safeguarding from neurodegenerative diseases by maintaining microglial cells in a healthy mature condition.[33]

3. Gut microbiota dysbiosis and ME/CFS

Alterations in the gut microbiome have been linked to diminished microbial diversity, a reduction of commensal gut microbiota, and an increase in proinflammatory molecules leading to a dysregulated host immune response or adjustment of the gut in individuals with ME/CFS.[39] In addition, several recent studies have reported comorbidity with gastrointestinal disorders, such as IBS.[8]

Diverse studies have shown gut dysbiosis in ME/CFS (Table 1), although the results vary depending on the characteristics of the trials. Wang *et al.*[10], in a retrospective review, reported that the proportions of gut microbial phyla between patients with ME/CFS and healthy controls are distributed as Bacillota (67.1% vs. 78.9%), Bacteroidota (21.2% vs. 10.8%), Actinomycetota (1.8% vs. 2.6%), and other phyla (10.2% vs. 8.9%); therefore, a decrease of the Bacillota/Bacteroidota may be associated with ME/CFS. In addition, the microbial a-diversity (*i.e.*, the observed number in taxa or the relative abundances in those taxa of an average sample within a habitat type) of the gut microbiome was reduced and there was a significant disparity within the general configuration of the gut microbiome b-diversity (*i.e.*, the variability in community composition or the identity in taxa observed among samples within a habitat) in individuals with ME/CFS compared to the non-affected controls. Thus, the role of the gut microbiota in the pathogenesis of ME/CFS is not yet clarified.

Several studies showed differing results regarding the abundance of the bacterial phyla in the gut microbiome. Increased abundance was found in the phyla Bacteroidota (genera *Alistipes*, *Bacteroides*, and *Paraprevotella*),[5,8,40,46] Bacillota (genera *Blautia*, *Clostridium*, *Coprobacillus*, *Enterocloster*, *Eggerthella*, *Erysipelatoclostridium*, *Lachnoclostridium*, *Phascolarctobacterium*, *Ruminococcus*, *Ruminococcaceae_UCG_014*, *Ruthenibacterium*, *Sellimonas*, and *Tyzzerella*),[5,41,44-46] and in the phylum Pseudomonadota.[42] In contrast, a decrease of the abundance was reported in the following phyla: Actinomycetota (genus *Bifidobacterium*),[40,42] Bacteroidota (genera *Bacteroides*, *Coprobacter*, and *Phocaeicola*),[8,41,45] and Bacillota (genera *Anaerostipes*, *Eubacterium*, *Faecalibacterium*, and *Roseburia*).[5,8,40,42,44,45,47]

It has been suggested that gut dysbiosis can cause immunometabolic disturbances (*e.g.*, diminished generation of antimicrobial peptides and SCFAs, perturbed tryptophan/kynurenine pathway metabolism), giving rise to a disrupted gut barrier ("leaky gut"), bacterial translocation, subsequent systemic chronic inflammation, along with neuroinflammation and neuroimmune impairment that may impact the brain and lead to ME/CFS.[6] While the precise mechanism underlying this phenomenon remains unclear, one proposed explanation is that the rise in *Enterobacteriaceae* associated with dysbiosis might drive intestinal inflammation and increased permeability, potentially due to elevated concentrations of lipopolysaccharide (LPS) produced by these bacteria, causing metabolic endotoxemia, which has been reported in ME/CFS.[48] The inquiry into whether a leaky gut also plays a role in ME/CFS has been addressed by Shukla *et al.*,[40] who found greater bacterial translocation and heightened levels of IgA and IgM to LPS in patients with ME/CFS than in healthy controls. Considering that bacterial translocation can induce systemic inflammation, disrupt blood-brain barrier, and lead to neuroinflammation, diverse researchers speculate that this process may account for the development of neurological anomalies in ME/CFS.[49,50] Figure 1 shows the hypothetical pathways regarding the relationship between gut microbiome and ME/CFS.

Other studies reported increased oxidative stress in ME/CFS.[51] Increased oxidative stress and decreased resting antioxidant levels during periods of rest have been noted in patients with ME/CFS when compared to healthy controls.[52] Furthermore, heightened urinary 8-hydroxy-deoxoguanosine (8-OHdG) levels, an indicator of oxidative DNA degradation, have been associated with symptoms of malaise and depression in individuals with ME/CFS.[53] In addition to dysbiosis and metabolic endotoxemia, other factors may be also involved in an oxidant/antioxidant imbalance, such as viral infection, stress, depression, and reduced antioxidants.[54,55]

Gut microbiota also possesses the capacity to generate neurotransmitters and influence their signaling pathways. Dysbiosis can interfere with the synthesis and equilibrium of neurotransmitters, including gamma-aminobutyric acid and serotonin, which are crucial for cognitive function, mood regulation, and other brain activities. Disruptions in neurotransmitter production and signaling processes might play a role in the neurological and psychological manifestations observed in subjects with ME/CFS.[56] Abnormalities in the levels of tryptophan, a neurotransmitter modulated by the microbiome, have been associated with ME/CFS.[57] In addition, the gut microbiome directly impacts vagal nerve stimulation, although this relationship may be bidirectional, as the vagal nerve also innervates the colon.[6]

Finally, dysbiosis can modify the synthesis and accessibility of various metabolites such as SCFAs and

Table 1. Recent studies on gut microbiota dysbiosis of patients with ME/CFS compared to healthy controls

Study/Country	Characteristics	Increased microbial taxa	Decreased microbial taxa
Shukla *et al.*[40]/USA	*N*=10 ME/CFS and *N*=10 HC. Age: 20 – 60 years. Blood and stool samples. 16S rRNA. Pyrosequencing	Blood: Pseudomonadota and Bacillota. Stool: Bacteroidota	Blood: Bacteroidota. Stool: Actinomycetota and Bacillota
Armstrong *et al.*[41]/ Australia	*N*=34 ME/CFS and *N*=25 HC. Mean age: 34.9 years ME/CFS and 33.0 years HC. Stool samples. Culture method. MALDI-TOF	*Clostridium*	*Bacteroides*
Giloteaux *et al.*[42]/USA	*N*=46 ME/CFS and *N*=34 HC. Stool samples. 16S rRNA. Illumina MiSeq	Pseudomonadota	*Faecalibacterium* and *Bifidobacterium*
Nagy-Szakal *et al.*[8]/USA	*N*=50 ME/CFS (21 with IBS and 29 without IBS) and *N*=50 HC (without IBS). Mean age: 51 years. Stool samples. Shotgun metagenomic sequencing. Illumina HiSeq 4000	*Alistipes* in patients with IBS and unclassified *Bacteroides* in patients without IBS	*Faecalibacterium* in patients with IBS and *Phocaeicola* (formerly *Bacteroides*) *vulgatus* in patients without IBS
Mandarano *et al.*[43]/USA	*N*=49 ME/CFS and *N*=39 HC Mean age: 53.0 years ME/CFS and 44.0 years HC. Stool samples. 18S rRNA. Illumina MiSeq	Basidiomycota/Ascomycota fungal phyla ratio	Gut eukaryotic diversity
Kitami *et al.*[44]/Japan	*N*=48 ME/CFS (11 with IBS) and *N*=52 HC. Stool samples. 16S rRNA. Illumina MiSeq	*Blautia*, *Coprobacillus*, and *Eggerthella*	*Faecalibacterium*
Lupo *et al.*[5]/Italy	*N*=35 ME/CFS and *N*=70 HC Mean age: 46.4 years ME/CFS and 55.2 years HC. Stool and saliva samples. 16S rRNA. Illumina MiSeq	Stool: *Bacteroides* and *Phascolarctobacterium.* Oral: *Rothia dentocariosa* and *R. mucilaginosa*	Stool: *Anaerostipes*
Guo *et al.*[45]/USA	*N*=106 ME/CFS (without IBS) and *N*=91 HC. Mean age: 47.4 years. Stool samples. Shotgun metagenomic sequencing. Illumina MiSeq	*Blautia sp., Clostridium scindens, Enterocloster* (formerly *Clostridium) bolteae, Erysipelotoclostridium ramosum, Lachnoclostridium sp., Ruminococcus gnavus, Ruthenibacterium lactatiformans, Sellimonas intestinalis,* and *Tyzzerella nexilis*	*Coprobacter secundus, Eubacterium rectal,* and *Faecalibacterium prausnitzii*
He *et al.*[46]/China	UK Biobank GWAS: *N*=2076 ME/CFS and *N*=460857 HC. Mendelian study of 25 cohorts from populations of European ethnicity in 11 countries	*Paraprevotella* and *Ruminococcaceae_UCG_014*	Not established
Xiong *et al.*[47]/USA	*N*=75 short-term ME/CFS, *N*=79 long-term ME/CFS, and *N*=79 HC. Mean age: 43 years. Stool samples. Shotgun metagenomic sequencing	Short-term patients showed significant microbial dysbiosis, while long-term patients had largely resolved microbial dysbiosis	Microbial diversity: *Roseburia* and *F. prausnitzii*

Abbreviations: IBS: Irritable bowel syndrome; HC: Healthy control; MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight; ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome.

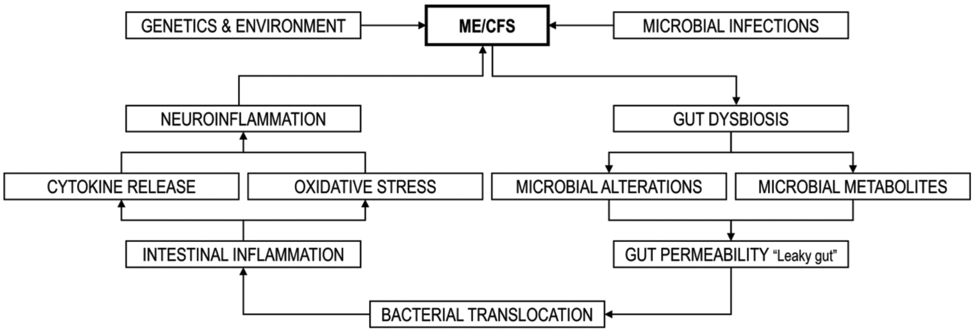

Figure 1. Hypothetical pathways on the relationship between gut microbiome and ME/CFS
Abbreviation: ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome.

cytokines, which may interfere with gut-brain interaction and contribute to the symptoms of ME/CFS.[44,58] An alternative hypothesis suggests that bacterial metabolites could influence ME/CFS by interfering with the pathways of estrogen and Vitamin D receptors. The Vitamin D receptor has been linked to the onset of autoimmune disorders that commonly present alongside ME/CFS. However, this potential connection remains insufficiently explored.[55,59] Several investigations have identified possible variations in metabolite levels between individuals with ME/CFS and controls.[10] In particular, SCFAs (such as butyrate, acetate, or isobutyrate) were significantly reduced in feces, blood, and urine of patients with ME/CFS.[44] A substantial decrease in serum a-tocopherol (vitamin E), an antioxidant that may be metabolized by specific gut microorganisms, was also noted in patients with ME/CFS.[10]

4. Modulation of gut microbiome as a potential therapy for ME/CFS

Based on its lower biodiversity compared to healthy subjects, it is believed that the gut microbiome may be a novel therapeutic target for patients with ME/CFS,[41] leading to several potential therapies, including probiotics, diet, and fecal microbiota transplantation (FMT).[6,55]

Probiotics are live bacteria that are thought to foster health.[60] A pilot study showed that the consumption of five different mixtures of probiotics (Enterelle: *Enterococcus faecium* and *Saccharomyces boulardii*; Bifiselle: *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis*; Rotanelle: *Bifidobacterium longum* strain AR81; Citogenex: *Lactobacillus casei* and *Bifidobacterium lactis*; and Ramnoselle: *Lactobacillus rhamnosus* strain GG and *Lactobacillus acidophilus*) over an 8-week period led to changes in overall welfare, along with alterations in inflammatory and oxidative indices in subjects with ME/CFS, culminating in a decrease of inflammatory indicators.[61] In another study, Sullivan *et al.*[62] investigated the impact of *Lacticaseibacillus* (formerly *Lactobacillus*) *paracasei* subsp. *paracasei* strain F19, *Lactobacillus acidophilus* strain NCFB 1748, and *Bifidobacterium animalis* subsp. *lactis* strain Bb12 on fatigue and physical activity in 15 patients with ME/CFS. After 4-week period, neurocognitive functions showed a significant improvement, while fatigue and physical activity scores did not change. Finally, the administration of *B. longum* subsp. *infantis* strain 35624 to 48 ME/CFS cases demonstrated the capability of probiotics to downregulate levels of systemic proinflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-a), and interleukin 6 (IL-6).[63]

Dietary modification is a quick, consistent, and straightforward approach to modify the gut microbiota.[64] Diet, similar to prebiotics, has been linked to some disease pathophysiology, and is able to alter the balance of microbiota composition and attenuate inflammation.[65] Patients with IBS, obesity, and Crohn's disease have seen positive outcomes with diet therapy, and dietary interventions have also been investigated within the neuropsychiatric field.[23] Intake of omega-3 polyunsaturated fatty acids enhances microbiota diversity and reduces metabolic endotoxemia, while consumption of polyphenols and fibers is also advantageous, due to their protective effects of polyphenols in obesity, neurodegenerative diseases, Type 2 diabetes, and cardiovascular diseases.[65-68] Eicosapentaenoic acid, prevalent in omega-3-rich oil, may mitigate symptoms in patients with ME/CFS.[69] Combining a diet aimed at reducing intestinal permeability with anti-inflammatory and antioxidant agents led to marked improvements in leaky gut, indicating a novel therapeutic strategy for ME/CFS.[70] Similar findings have been observed in individuals with depression, suggesting that gut permeability and the associated heightened immune response might elucidate a connection between major depressive disorder and the cognitive symptoms of ME/CFS.[71]

Recently, FMT has gained attention as a potential treatment for ME/CFS. This approach aims to reestablish a balanced gut microbiota by transferring fecal matter from a healthy donor into the recipient's gastrointestinal tract.[72] About 70% response rate was achieved when 13 non-pathogenic bacteria were delivered through colonoscopy to 60 patients with ME/CFS. After 15 – 20 years of follow-up, 58% of cases were documented to have maintained response without relapse.[73] Kenyon *et al.*[74] conducted an FMT trial in which each patient received 10 FMTs delivered via a rectal catheter into the lower part of the sigmoid colon. Of the 21 patients treated with FMT, 17 reported 65 – 95% improvement, with seven patients describing a normalization of their quality of life. However, a recent study showed that FMT was safe, but did not enhance the health-related quality of life for subjects suffering from CFS.[75]

5. Discussion

While gut microbiome homeostasis has been associated with diverse diseases, its role in the pathogenesis and progression of ME/CFS is still not fully defined. However, several factors implicating the gut-brain axis may explain the connection between gut dysbiosis and ME/CFS.

As changes in the gut microbiome have also been reported in postulated autoimmune diseases such as Crohn's disease, systemic lupus erythematosus 2, and Type 2 diabetes, it would be valuable to explore whether the microbiome could be associated with autoimmune manifestations of ME/CFS.[5,76] In addition, both genetic and environmental factors can affect the gut microbiome, and shifts in local gut microbiota composition should be evaluated in ME/CFS.[77] Although the significance of the gut microbiome in health and disease is becoming increasingly clear,[78] limitations must be addressed regarding studies of the gut microbiota and ME/CFS, such as differing results, small sample sizes, confounding variables, and lack of homogeneity of the microbial gene sequencing platforms.[45,55]

Butyrate and its precursor acetate are two of the most important SCFAs of microbial origin. Both metabolites are reduced in ME/CFS patients likely due to the decrease of *F. prausnitzii, Ruminococcus* spp.*, Eubacterium rectale*, and *C. secundus* in their gut microbiome (Table 1). Thus, a deficiency of these intestinal homeostatic metabolites could contribute to a number of detrimental physiological perturbations, including a weakened epithelial barrier and increased intestinal inflammation, which results in elevated plasma LPS levels in ME/CFS, indicative of microbial translocation.[42] In addition, *F. prausnitzii* and *Lachnospira* (formerly *Lactobacillus*) *rogosae* are the major bacterial species associated with the symptoms of ME/CFS and with individual and total Multidimensional Fatigue Inventory (MFI) scores.[45]

The cognitive problems detected in ME/CFS subjects could be related to alterations in the gut microbiota and also to a substantial decrease in Vitamin E levels. Tryptophan, a precursor for serotonin, can be degraded by determined gut microorganisms, which may exert influence on brain function,[79] and on the pathophysiology of ME/CFS.[80] Thus, studying the changes in gut microbiome composition and function may clarify how tryptophan metabolism affects serotonin concentrations, which may contribute to various symptoms of ME/CFS, such as depression and restless sleep.

Considering the impact of ME/CFS on mental health, the exploration of psychobiotics as a therapeutic option for patients with ME/CFS holds considerable promise. Psychobiotics, which target the gut-brain axis, have demonstrated potential in modulating anxiety, depression, and stress-related disorders through gut microbiome adjustments.[60] Diet also offers possibilities for ME/CFS symptom management. Specifically, vegetarian diets, rich in antioxidant and fiber, are known to enhance gut health, lower inflammatory markers, and reduce oxidative stress,[65] which may be beneficial to mitigate the physiological responses commonly reported by patients with ME/CFS. In addition, vegetarian diets may exert a positive influence on mental health.[81]

Another interesting aspect related to ME/CFS is to delineate the role of microbial infections, both bacterial (*e.g.*, *Coxiella burnetti*) and mainly viral (*e.g.*, Epstein-Barr, Ross River, SARS-CoV-2), in triggering this disease.[82-84] The recent pandemic of COVID-19 caused a serious impairment in quality of life[85] and led to long COVID-19 in numerous patients who "recovered" but did not return to full health.[86] In this regard, long COVID-19 shares many phenotypic abnormalities with ME/CFS following viral infections, including persistent chronic fatigue and myalgia.

6. Conclusion

Research on patients with ME/CFS has revealed variable findings on alterations in gastrointestinal processes that involve microbiota. Microbiome changes or metabolic endotoxemia may be potential biomarkers of ME/CFS. The progression of ME/CFS may begin with the loss of beneficial gut microorganisms, especially SCFA producers that are reflected in plasma metabolite levels. These alterations may result in metabolic and phenotypic shifts and in ME/CFS. Randomized controlled trials in well-identified patients with ME/CFS, using diet, FMT, and other approaches may benefit these patients and establish causality.

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Conflict of interest

The authors declare that they do not have competing interests.

Author contributions

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REVIEW ARTICLE

iPSC-mediated genetic manipulation promotes natural killer cell-centered cancer immunotherapy

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Abstract

Natural killer (NK) cells demonstrate potent cytotoxic activities and the capacity to secrete cytokines. Their distinctive capability to trigger cell death, bypassing the need for major histocompatibility complex recognition, opens promising avenues for their use in clinical settings such as allogeneic transplantation and tumor immunotherapy. Although the ability of NK cells to kill hematological tumors has been widely recognized, their effectiveness in treating solid tumors is not as pronounced. The intricate interplay of NK cells with the tumor microenvironment, specifically in the context of solid malignancies, has been noted to attenuate the anti-cancer prowess of NK cells and foster the ability of malignant cells to elude immune surveillance. Successful NK cell-centered immunotherapy hinges on obtaining a substantial quantity of NK cells with potent tumor-killing capabilities. However, the current challenge lies in the limited *ex vivo* expansion of NK cells and the inefficiency of gene introduction methods. Induced pluripotent stem cells (iPSCs) are multipotent stem cells with relatively easier gene transfection capability and theoretically unlimited proliferation potential. NK cells derived from iPSCs circumvent the challenge of difficult genetic modification in NK cells, offering various potential strategies to counteract the immune suppression induced by the tumor microenvironment.

Keywords: Induced pluripotent stem cells; Natural killer cells; Solid cancer; CAR-NK cells; Immunotherapy

1. Introduction

Innate lymphoid cells (ILCs) are classified into three principal groups: Group 1, Group 2, and Group 3 ILCs. Natural killer (NK) cells are categorized within Group 1 ILCs, along with ILC1.[1] Each of these groups has a corresponding counterpart in adaptive immunity characterized by T helper (Th) cells: Th1 for Group 1, Th2 for Group 2, and Th17 for Group 3, reflecting their roles and functions in the immune response.[2] NK cells, first identified in the 1970s, derive their name from their innate ability to kill target cells without prior antigen stimulation, in contrast to T cells, which require such activation.[3,4] NK cells are primarily involved in directly killing target cells and modulating the activity of other immune cells via cytokine secretion. Their typical targets include virally infected cells and cells that have undergone malignant transformation.[5] Originating from hematopoietic stem cells (HSCs) within the bone marrow, NK cells are not entirely mature upon departure from the bone marrow. They undergo further maturation influenced by specific signals and the microenvironment where they reside.[6,7]

Peripheral blood NK cells can be categorized into two primary subpopulations: $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. The former represents a mere 5 – 10% of the NK cells circulating in peripheral blood and is thought to be at an early stage in NK cell maturation. In contrast, the vast majority of NK cells are $CD56^{dim}CD16^{+}$, which is indicative of a more mature state. Interestingly, the $CD56^{bright}CD16^{-}$ cells, despite their lower abundance, exhibit limited capability for antibody-dependent cell-mediated cytotoxicity (ADCC), a key mechanism through which NK cells mediate their immune response.[8,9] The cytotoxic effect of NK cells is not restricted by the major histocompatibility complex (MHC),[10] making them promising candidates for allogeneic transplantation with favorable clinical prospects. CD16 (Fcγ receptor III), expressed on the surface of NK cells, facilitates ADCC, synergizing with adaptive immunity[11,12] and enhancing the efficacy of therapeutic antibody-mediated targeted therapy.[13] During the late stages of NK cell development, NK cells establish immune tolerance with self-MHC-I molecules[14] through a process known as licensing.[15] The activating and inhibitory receptors, presented on the surface of NK cells are in a delicate state of "dynamic equilibrium".[16,17] During the malignant transformation of normal cells, activating receptor ligands are often upregulated, whereas inhibitory receptor ligands are frequently downregulated, rendering these cells more susceptible to NK cell-mediated targeting.[18] NK cells have shown promising clinical prospects in the treatment of hematological cancer.[19-22] The graft-versus-leukemia (GVL) effect, primarily mediated by NK cells, has garnered increasing clinical attention and is being applied in the context of human leukocyte antigen (HLA) haplotype-mismatched HSC transplantation.[23] However, the efficacy of NK cell-mediated cytotoxicity against solid tumors is not as ideal as expected.[24,25]

The microenvironment of solid tumors is characterized by low nutrient content, high acidity,[26] and low oxygen,[27] which negatively affect the function and activation of NK cells and other immune cells, resulting in poor cytotoxicity of NK cells. The growth of abnormal blood vessels in solid tumors creates hypoxic areas that can cause degranulation of NK cells and mitochondrial damage, leading to NK cell exhaustion.[28] Other tumor-associated cells, such as M2 macrophages or tumor-associated macrophages (TAMs), also weaken NK cytotoxicity.[29,30] Immunosuppressive cytokines in the solid tumor microenvironment, such as transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10), can reduce the surface expression of activating receptors, including NKp30, NKG2D, and CD16, on NK cells (31 – 33), decrease the levels of transcription factors such as T-bet (34), and potentially reprogram NK cells into ILC1 cells, which possess a weaker anti-tumor capacity.[31]

For clinical therapies, NK cells are primarily obtained from peripheral blood,[32] cord blood,[33] or established NK cell lines.[34] Although *ex vivo*-expanded or stimulated NK cells have shown promising safety profiles in clinical applications through autologous or allogeneic transplantation, the full extent of their therapeutic efficacy awaits further definitive validation. Previous research on NK cells has unveiled numerous genes that exert positive or negative influences on NK cell maturation, activation, and their capacity to combat tumors. Certain genes positively influence NK cells by fostering their maturation and boosting their cytotoxic abilities,[35] whereas others act as negative regulators by impeding NK cell development[36] or dampening their activation.[37] These insights significantly enrich our comprehension of NK cell biology and suggest innovative avenues for their therapeutic deployment. Targeting these regulatory genes presents an exciting opportunity to devise novel treatments. Through precise gene editing or molecular modulation, it is possible to improve the anti-tumor potency of NK cells or augment their efficacy within specific immunological niches, enhancing the effectiveness of NK cell-based immunotherapies. Strategies that activate or upregulate genes that positively regulate NK cells could enhance their functional activity and prolong their longevity. On the other hand, the inhibition or deletion of genes that negatively regulate NK cells may alleviate constraints on their functionality, thus bolstering their anti-tumor performance. Due to significant challenges in gene transfection and limited expansion lifespan, both *in vitro* and *in vivo*, NK cells encounter constraints that complicate their genetic engineering and clinical utilization.[38] In comparison, T cells, especially chimeric antigen receptor (CAR)-engineered T (CAR-T) cells, demonstrate superior viral transfection rates, enable substantial *ex vivo* expansion, and have the capability to establish enduring memory T cells within the host.[39] These attributes enable CAR-T cells to persistently identify and eradicate tumor cells that present specific antigens. On the other hand, genetically engineered NK cells encounter difficulties in achieving adequate cell quantities with the desired genetic payload, maintaining effectiveness *in vivo*, and overcoming the immunosuppressive forces of the tumor microenvironment. These challenges significantly limit the utilization of NK cells in the arena of cancer immunotherapy.

Induced pluripotent stem cells (iPSCs), a type of pluripotent stem cells derived from terminally differentiated cells,[40] can be efficiently induced to differentiate into NK cells through specific combinations of cytokines.[41] iPSC-derived NK cells have demonstrated promising anti-tumor activity in pre-clinical and clinical experiments, making them a highly potential therapeutic strategy for NK cell-based cancer treatment.[42] Compared to peripheral blood NK cells, iPSCs are more flexible to gene overexpression, gene silencing, and gene editing. Coupled with the inherent ability of iPSCs for limitless proliferation, this technology supports the feasibility of selecting and purifying genetically modified cells. These advancements not only establish a foundation for precise genetic manipulation of NK cells but also open avenues for further NK cell research and their application in clinical settings. This review explores iPSC-based approaches, particularly genetic modifications, to enhance NK cell-based strategies for combating solid tumors. It covers the biological characteristics of tissue-resident NK cells, challenges related to the resistance of various solid tumors to NK cell-mediated cytotoxicity, and clinical efforts aimed at improving NK cell-mediated killing of solid tumors.

2. Mechanisms employed by solid cancer to evade NK cell attack

NK cells and $CD8^+$ T cells exhibit parallel cytotoxic capabilities, sharing the ability to eliminate target cells through similar mechanisms, such as employing perforin and granzyme B.[43] Despite these shared cytotoxic mechanisms, the activation pathways and tumor cell recognition strategies of NK cells and T cells differ fundamentally, often standing in stark contrast to each other.[44] This distinct divergence in recognition strategies suggests that T cells and NK cells offer complementary approaches to tumor cell identification. In this context, it is critical to delve into the strategies employed by tumor cells to evade NK cell-mediated destruction. These strategies span a broad spectrum, ranging from evading NK cell detection to manipulating the NK cell activation processes. In addition, the tumor immune microenvironment plays a pivotal role in suppressing NK cells, even after they have recognized tumor cells and are primed to execute their cytotoxic function.[45] Understanding these evasion and inhibition mechanisms is vital for the development of targeted therapies that enhance the antitumor efficacy of NK cells. This includes employing genetic modifications or engineering, notably those based on iPSCs, to bolster their tumor-killing capacity.

2.1. Influences on NK cell maturation in peripheral tissues

In human peripheral blood, NK cells can be categorized into two distinct groups based on the intensity of CD56 expression: $CD56^{bright}$ and $CD56^{dim}$. Cells in the $CD56^{dim}$ subset express higher levels of CD16, a crucial Fc receptor necessary for ADCC mediated by NK cells. $CD56^{dim}$ NK cells are generally considered more mature than their $CD56^{bright}$ counterparts. The population of $CD56^{dim}CD16^+$ NK cells, which have cytotoxic function in the peripheral blood, is significantly decreased in hepatocellular carcinoma (HCC) patients. This is accompanied by a reduction in the production of granzyme and interferon (IFN)-γ. Moreover, the number of $CD56^{dim}CD16^+$ cells infiltrating liver tumors is much lower than that in non-tumor regions.[24] In addition, human NK cells can be further stratified into the four-subset model based on the expression levels of CD11b and CD27.[46] Non-small cell lung cancer tumors are infiltrated by a large number of $CD56^+CD11b^-CD27^-$ double negative NK cells. These double-negative NK cells are considered relatively immature, exhibit lower susceptibility to activation, and their presence is positively correlated with tumor malignancy.[47,48]

2.2. Changes in cytokine levels (including chemokines)

TGF-β stands as a pivotal immunosuppressive factor for NK cells, impeding their capacity to eliminate target cells, release cytokines, and attenuate the expression of activating receptors.[49,50] On lung cancer cells, TGF-β downregulates the expression of ligands for NK cell activating receptors, such as NKG2DLs, thereby facilitating cancer cells' evasion from NK cell-mediated destruction.[51,52] Extensive evidence has firmly established that a diverse range of substances contribute to the development, migration, and increased malignancy of lung cancer by modulating the TGF-β signaling pathway.[53-55]

IL-10 represents another cytokine that inhibits the functionality of NK cells. Although NK cells and immature dendritic cells (DCs) have the ability to activate each other, it has been observed that NK cells can also kill DCs that express CD40 or are derived from the activation of IL-10.[56,57] In the lung tumor microenvironment, NK cells not only exhibit reduced cytotoxicity but also play a role in negatively regulating DC maturation, thereby assisting tumors in evading immune surveillance.[58]

A crucial step in the NK cell-mediated eradication of cancer cells is their infiltration into solid tumors. CXCL14, an important chemokine that promotes NK cell migration toward inflammatory or malignant sites,[59] is significantly underexpressed in head and neck squamous cell carcinoma.[60] The presence of hypoxia within the tumor microenvironment can induce the expression of podoplanin, which disrupts the interaction between CCL21 and CCR7, ultimately resulting in diminished NK cell migration toward solid tumors and a consequent reduction in NK cell cytotoxicity.[61]

2.3. Altered expression levels of activating and inhibitory receptors

NKp30 (CD337) collaborates with NKp46 and plays a pivotal role in the activation of NK cells.[62] The reduction of NKp30 expression in NK cells from individuals with acute myeloid leukemia (AML) profoundly affects NK cell function.[63] This reduction in efficacy extends into the realm of solid tumors, including cervical and breast cancers, where the malignant cells bind to NKp30, inhibiting NK cytotoxicity through the release of galactose lectin (Gal-3).[64,65] Moreover, colorectal cancer patients exhibit lower levels of NKp30, NKp44, NKp46, and NKG2D in their peripheral blood NK cells compared to healthy individuals.[66] During malignant transformation, the human BCL2-related protein BAG-6 inhibits NKp30-mediated signal transduction, thereby contributing to immune evasion by tumor cells.[67]

NKG2D, expressed on NK cells, serves as a critical activating receptor.[68,69] However, both virus infections and tumor cells deploy diverse mechanisms to escape NKG2D-mediated cytotoxicity.[70,71] In cases of HCC, the expression of NKG2D ligands is diminished through the action of the β-catenin signaling pathway.[72] The expression of the NK cell activating receptor NKG2D is notably reduced[73,74] in liver cancer. As a consequence, NK cells are less easily activated by tumor cells, leading to diminished cytotoxic effects and a reduced capacity to release cytokines. Similarly, in non-small cell lung cancer, the enzyme indoleamine 2,3-dioxygenase 1 induces NK cell dysfunction by downregulating NKG2D.[75] NK cells exposed to activating signals mediated by NKG2D and NKp46 downregulate the expression of activating receptors and upregulate checkpoint molecules. This results in reduced cytokine production and cytotoxicity. Hypoxia also induces diminished expression of ligands that activate NK cell receptors. This includes the downregulation of MICA/B, the ligand for NKG2D, on breast cancer and pancreatic cancer cells. This downregulation enables these cancer cells to evade NK cell-mediated killing.[76,77]

Inhibitory receptors constitute a pivotal mechanism through which NK cells establish immune tolerance. MHC class I molecules serve as the principal ligands for these receptors. Melanoma cells have evolved various strategies to evade immune system surveillance, with one of the most common being the downregulation of MHC-I expression on their surface, allowing them to evade recognition and elimination by T cells.[78] However, this downregulation also renders melanoma cells susceptible to recognition by NK cells, as they lack the inhibitory signals typically provided by MHC-I. While classical MHC-I molecules such as HLA-B are down-regulated, other non-classical MHC-I molecules, such as HLA-E or HLA-G, exhibit increased expression in melanoma.[79,80] HLA-E is a crucial ligand for the CD94/NKG2A inhibitory receptor. Consequently, the increased expression of HLA-E renders the cancer cells less susceptible to NK cell recognition and attack. In contrast to classical MHC-I molecules, non-classical MHC-I molecules usually do not present tumor antigens to T cells. Therefore, increased expression of HLA-E does not make cancer cells more susceptible to T cell-mediated elimination. The concurrent downregulation of classical MHC-I molecules and upregulation of non-classical MHC-I molecules equip melanoma cells with resistance against T cell-mediated elimination and enable them to escape NK cell-mediated killing simultaneously (Figure 1). Melanoma-induced exhaustion of NK cells is another mechanism that impairs their function. This exhaustion is characterized by impaired cytotoxicity, cytokine secretion, and response, decreased expression of activating receptors, and increased expression of inhibitory receptors.[81]

2.4. Induction of NK cell exhaustion

In the tumor microenvironment, cancer cells and other surrounding cells can induce a state in NK cells similar to T cell exhaustion. Macrophages and monocytes in HCC tissues express high levels of CD48, which leads to early activation of NK cells through binding to 2B4 and subsequent dysfunction. When cocultured with monocytes, NK cells derived from the peripheral blood of HCC patients show increased expression of Ki67, granzyme B (GzmB), CD69, and TRAIL within a short period; however, these NK cells ultimately undergo substantial apoptosis.[82]

Killer cell lectin-like receptor G1 (KLRG1) was initially considered one of the markers for the maturation of NK cells (117). Subsequent research, however, revealed that KLRG1 has an inhibitory effect on the activation of NK cells.[83] Similar to T cells, NK cells also express certain immune checkpoints. The ligands of KLRG1 primarily include molecules related to cell–cell adhesion, such as cadherin.[84] The expression of cadherin in tumor cells often

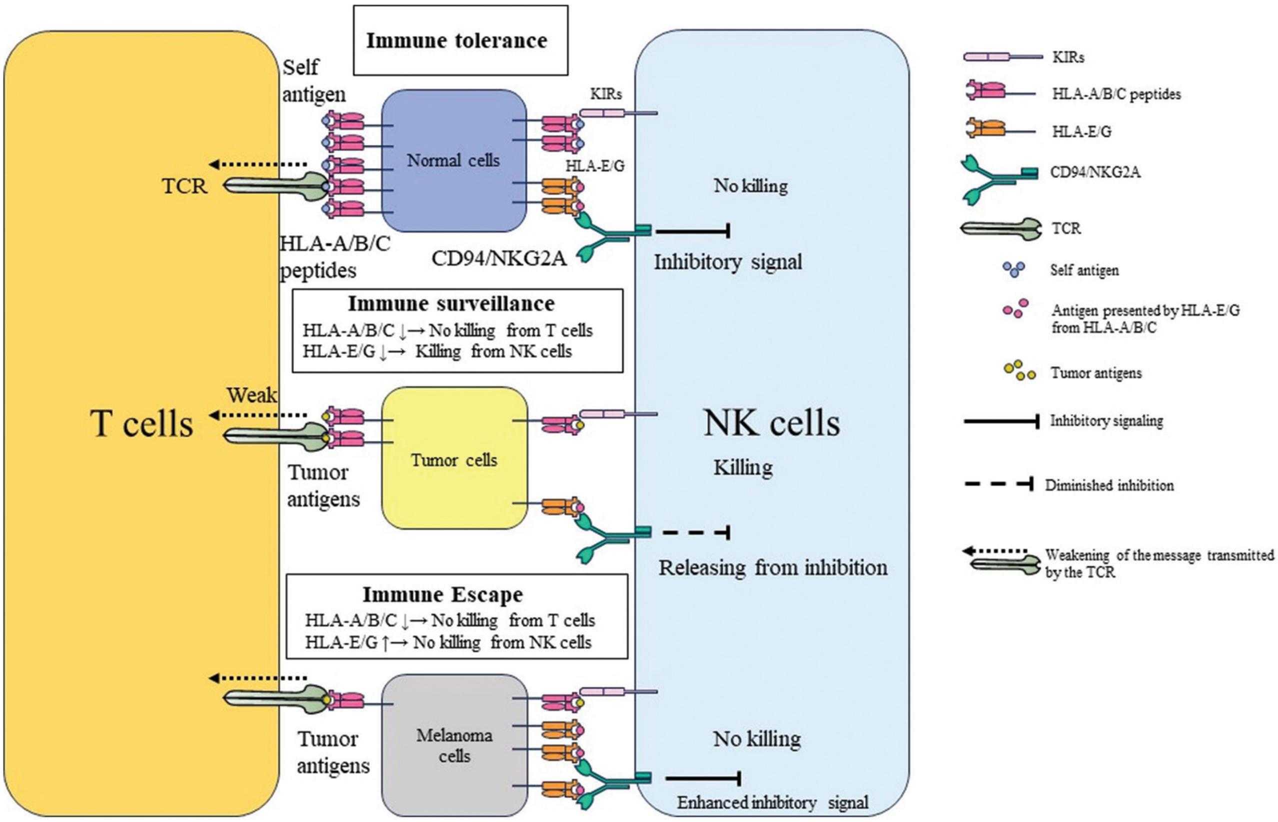

Figure 1. The role of MHC in T cells and NK cell-mediated immune surveillance and escape
Abbreviations: HLA: Human leukocyte antigen; NK: Natural killer; MHC: major histocompatibility complex; KIR: killer-cell immunoglobulin-like receptor; TCR: T cell receptor. Figure created by the authors.

affects the function of NK cells. Elevated expression of neural cadherin (N-cadherin) in oral cancer cells is also believed to induce NK cell exhaustion through KLRG1.[85] In some oncolytic virus studies targeting solid tumors, ectopic expression of epithelial cadherin (E-cadherin) has been employed to help evade NK cell cytotoxicity, leading to prolonged tumor-killing effects.[86]

T cell immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibition motifs (ITIM) domain (TIGIT) is an important immune checkpoint in NK cells and plays an inhibitory role in NK cell function. By binding to CD155 expressed on tumor cells, TIGIT transmits signals of exhaustion into lymphocytes.[87] In patients with multiple myeloma, the levels of CD155 expressed by bone marrow mesenchymal stromal cells are negatively correlated with the levels of effector molecules such as IFN-γ and perforin in NK cells. This exhaustion of NK cells is caused by their interaction with TIGIT.[88] In adaptive NK cells, reducing the expression of TIGIT can confer resistance to immune suppression originating from myeloid-derived suppressor cells (MDSC).[89] Some immune therapies targeting TIGIT have also entered clinical trials for cancer treatment.[90] Interestingly, when inhibitory signals coexist with activating signals, they help mitigate NK cell exhaustion.[91]

3. iPSCs-derived NK cells for cancer therapy

3.1. Overcoming genetic engineering challenges in NK cells with iPSCs

Genetic manipulation of NK cells is challenging. Many gene-editing techniques that are highly efficient in other cell types are difficult to implement in NK cells. Moreover, NK cells have limited capacity for *ex vivo* expansion and, unlike T cells, cannot form long-lasting memory cells *in vivo*, presenting a significant barrier to their clinical application. Compared to NK cells, iPSCs are relatively easier to genetically manipulate and have the capability for extensive *in vitro* expansion. Therefore, iPSC-derived NK cells, especially those genetically modified at the iPSC stage, have a very promising clinical application in cancer therapy.

Differentiation of NK cells from iPSCs typically follows a two-step process. First, iPSCs are differentiated into HSCs. Various methodologies have been established for differentiating pluripotent stem cells, including embryonic stem cells and iPSCs, into HSCs. One such method involves culturing iPSCs on the top of feeder cells, which provides a sufficient microenvironment for the differentiation into HSCs without the need for additional cytokines. For instance, OP9 cells can directly facilitate the differentiation of iPSCs into HSCs. In this process, iPSCs are digested into small aggregates, and the cell suspension containing these iPSC aggregates is then seeded onto OP cells in alpha minimum essential medium supplemented with fetal bovine serum. Within this culture setup, the optimal timeframe for HSC collection is typically between 7 and 8 days.[92] For iPSC-derived NK cells intended for clinical applications, it is preferable to establish a differentiation protocol that operates without the requirement for feeder cells. This need led to the development of the spin embryoid body (EB) method. In this approach, iPSCs are first separated into single cells and seeded at a density of approximately 4,000 cells per well into a low-attachment 96-well plate. The plate is then centrifuged to promote the formation of a cell aggregate at the bottom of each well. The culture medium is based on APEL medium, supplemented with cytokines such as stem cell factor (SCF), bone morphogenetic protein-4, and vascular endothelial growth factor, and includes the addition of a Rho-associated, coiled-coil containing protein kinase inhibitor during the first 2 – 3 days of EB formation. By days 11 – 13 of hematopoietic induction, HSCs can be harvested for analysis or further differentiation.[93] Before differentiating from HSCs into NK cells, the purity of the HSCs is usually assessed. If the percentage of CD43$^+$CD34$^+$ (or CD45$^+$CD34$^+$) cells is around 30%, the process can proceed to NK cell differentiation. It is generally believed that the presence of some non-hematopoietic stromal cells will not impede the differentiation of HSCs into NK cells. If the HSC ratio is too low, magnetic bead purification can be employed to enrich the HSC population before proceeding to NK cell differentiation.[94] The second step initiates the differentiation of HSCs into NK cells. The cytokines necessary for the maturation and differentiation of NK cells have been extensively identified through prior basic research. Therefore, these cytokines are often employed in the process of differentiating HSCs into NK cells. While OP9 cells expressing delta-like 1 (DL1) are utilized in studies of HSC differentiation into NK cells, methods that do not involve feeder cells may hold greater potential for clinical applications. In the feeder-free differentiation system, the EBs formed from HSC differentiation steps are transferred to other cell culture devices, such as 6-well plates, and the medium is replaced with one that facilitates NK cell production, containing IL-3, SCF, IL-7, and IL-15. These cytokines can induce the differentiation of HSCs into NK cells. After approximately 4 weeks of induced differentiation toward NK cells, the majority of the culture system will become CD45$^+$CD56$^+$ NK cells. The differentiated NK cells can be expanded several hundred-fold with the support of IL-2 and artificial antigen-presenting cells, achieving increased purity during the expansion process.[41] In recent reports, 3D culture systems have been introduced in the preparation of NK cells derived from iPSCs, which will further enhance the scalability of NK cell production and improve the efficiency of clinical applications.[95]

3.2. iPSC-derived vs. primary NK cells

The killer-cell immunoglobulin-like receptor (KIR) is not only pivotal for NK cells in establishing immune tolerance but also plays a critical role in providing licensing signals that are essential for NK cell development and maturation.[96] In the clinical context of leukemia treatment through HSC suppression, mismatches in KIR and KIR ligands between donors and recipients can enhance GVL effects and reduce relapse rates, particularly in patients with AML.[23] Although NK cell licensing is modulated by signals from KIR ligands *in vivo*, the expression and functionality of KIR on NK cells following *ex vivo* expansion remain subjects of debate. Some studies have observed that *ex vivo* expansion does not alter the expression state of KIRs on NK cells, thereby preserving the inhibitory impact mediated by their ligands.[97] Conversely, other studies have highlighted that *ex vivo* cytokine stimulation can license previously unlicensed NK cell subsets, which lacked high reactivity *in vivo*, enabling them to become licensed.[98] Once these *ex vivo* activated NK cells are reintroduced into the patients, they might become activated due to KIR and KIR ligand mismatches, thus activating previously tolerant NK cells to target tumor cells more effectively. During the *ex vivo* culturing and expansion process of NK cells, whether derived from iPSCs or peripheral blood, a high cytokine concentration is essential. As a result, the cytotoxic capacity of *ex vivo* expanded NK cells might transcend the limitations imposed by KIRs or KIR ligands, effectively bypassing the KIR-mediated inhibition through KIR ligands. Notably, research indicates that NK cells from different iPSC lines show varied KIR expression patterns. Among iPSC-derived NK cells, there is no significant difference in the levels of activating receptor expression between KIR$^+$ and KIR$^-$ cells, except for CD16, which is expressed at higher levels in KIR$^+$ cells. Importantly, iPSC-derived KIR$^+$ and KIR$^-$ cells do not exhibit significant differences in tumor cytotoxicity, underscoring the nuanced role of KIR in modulating NK cell function.[99] Therefore, maximizing the anti-tumor capability mediated by KIR in iPSC-derived NK cells likely requires further research and more elegant designs.

One notable advantage of NK cells derived from iPSCs is the feasibility of applying genetic modifications. This advantage, combined with the iPSCs' capability for unlimited proliferation, facilitates the screening of positive clones, leading to the production of genetically modified NK cells of exceptional homogeneity. Various nucleic acid delivery methods and gene-editing technologies have shown significant effectiveness in iPSCs. For example, through transcription activator-like effector nucleases (TALEN) gene-editing technology, the introduction of IL-15 and the concurrent deletion of TGFβR2 not only amplify the NK cells' anti-tumor potency but also equip them to counteract the immunosuppressive impact of TGF-β within the tumor microenvironment.[100] Moreover, the potent clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has been employed in iPSC-derived NK cells to remarkable effect. Beyond developing CAR-engineered NK (CAR-NK) cells using this technique, it enables the simultaneous editing of multiple genes in NK cells.[101] It is important to note that editing multiple genes in one cell does not significantly affect the genomic stability of NK cells, highlighting the technique's potential to significantly advance cancer immunotherapy.

3.3. Clinical trials of iPSCs-derived NK cells

NK cells derived from iPSCs have progressively entered clinical trials as their safety and efficacy in treating various diseases, including malignant tumors, are being assessed. It is interesting to note that a wide array of NK cells is currently undergoing clinical trials including both genetically modified and unmodified NK cells, those sourced from peripheral blood and derived from iPSCs, as well as autologous and allogeneic NK cells. However, clinical trials involving iPSC-derived NK cells predominantly utilize genetically modified versions of these cells. In a case report, a 76 years old patient with diffuse large B-cell lymphoma, who had previously undergone eight different treatments including *ex vivo* expanded autologous NK cells, autologous stem cell transplant, and engineered autologous T cells, was treated with iPSC-derived NK cells.[102] These cells were engineered to express a CAR targeting CD19, a non-cleavable CD16, and enhanced cytokine autocrine signaling mediated by IL-15 and its receptor. The treatment not only proved safe but also showed a partial response, with a 50% reduction in tumor size. Another case report involved iPSC-derived CAR-NK cells that also targeted CD19 and augmented the IL-15 signaling pathway.[103] This study additionally engineered the cells to knock out class I MHC molecules and enhance HLA-E expression, aiming to minimize host rejection. The intervention led to an enhanced immune response within the tumor microenvironment and a reduction in tumor size.

In a trial involving 15 patients with advanced solid tumors and lymphomas, including non-small cell lung cancer and classical Hodgkin lymphoma, the therapeutic efficacy of iPSC-derived NK cells was evaluated. During preparation, NK cells were amplified from hematopoietic progenitor stages by over one million-fold.[104] Although the trial is ongoing, initial results indicate a response to treatment in some patients, and the iPSC-derived NK cells were deemed safe at a dose of 3×10^8 cells.[105] In another clinical trial involving 13 subjects with B-cell lymphoma, iPSC-derived NK cells were engineered to express a high-affinity, non-cleavable CD16 Fc receptor, enhancing their capacity for NK cell-mediated ADCC. The trial administered doses of up to 300 million cells, which were well-tolerated without any dose-limiting toxicities. Remarkably, seven of the patients achieved a complete response.[106] Whether *ex vivo* expanded from peripheral blood or derived from iPSCs, and regardless of genetic modification, current clinical trial outcomes primarily provide safety data. The effectiveness of these NK cells in treating tumors still requires further clinical evidence to be substantiated.

4. Boosting anti-tumor efficacy of iPSCs-derived NK cells through genetic engineering

4.1. CAR enhances the targeting of NK cells

Thanks to the success of CAR-T cells in treating hematological disorders and their potential, along with challenges, in solid tumors, CAR-NK cells have emerged as a pivotal focus in the field of genetically modified NK cells. In addition to traditional CAR-T constructs, efforts have been made to invent CAR constructs that are better suited for NK cells. CAR-NK cells based on 2B4,[107] DAP-12,[108] and NKG2D[109] structures have demonstrated superior efficacy, particularly in terms of proliferation, cytokine secretion, and cytotoxicity. These NK cell-specific activation motifs offer a promising avenue for the development of CAR-NK cell therapies and have the potential to significantly enhance the clinical effectiveness of iPSC-derived NK cells, especially in the context of solid tumors.

NK cells sourced from various tissues, including peripheral blood,[110] cord blood,[111] NK-92 cell line,[112] and iPSC-derived NK cells,[110] have all been utilized in the preparation of CAR-NK cells, yielding promising results in pre-clinical studies. Notably, several CAR-NK cell products have progressed to clinical trials.[33] Current CAR-NK cell research predominantly targets CD19, commonly expressed in B-cell leukemia and lymphoma (37), B-cell maturation antigen, found in multiple myeloma (117), and HER2, associated with lung cancer.[113] Importantly, these targets for CAR-NK cells align with the same spectrum of antigens typically targeted by CAR-T cell therapies. However, it is also important to recognize the differences between CAR-NK and CAR-T therapies. Given the distinct activation mechanisms of NK and T cells, the effects of CAR in enhancing T cell-mediated tumor cell killing cannot be expected to elevate NK cell performance to the same extent. In fact, some studies have found that in certain contexts, CAR-NK cells do not demonstrate a significantly stronger ability to kill tumor cells compared to unmodified NK cells.

4.2. Transcription factors are good candidates for optimizing the anti-tumor ability of NK cell

Elevating the expression of key transcription factors is a common strategy to enhance the efficiency of generating immune cells from iPSCs. For example, overexpression of SPI1 and CEBPA enhances the differentiation efficiency of microglia, one of the important immune cells in the neural system.[114] Precise control of the expression of Runx1 and Hoxa9 during T-cell development can significantly improve the efficiency of generating T cells from iPSCs.[115] Numerous transcription factors demonstrate promising potential as targets for optimizing NK cell-based cancer immunotherapies, yet only a select few have been proven in pre-clinical studies to enhance the tumoricidal capabilities of NK cells.

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is widely expressed in immune and non-immune cells. After binding to exogenous or endogenous ligands, AhR translocates to the nucleus and regulates the expression of target genes.[116] Many metabolic products in the tumor microenvironment can serve as ligands for AhR.[117,118] Although resting NK cells do not express AhR at a considerable level, its expression level is greatly increased in response to cytokine stimulation.[119] It is worth noting that co-culturing with IL-21-expressing K562 cells, a common expansion process for iPSC-derived NK cells, significantly increases the expression level of AhR.[120] In human peripheral blood NK cells, CD56^{bright} cells possess higher cytokine secretion capacity, lower cytotoxicity, and higher expression of AhR, compared to CD56^{dim} NK cells. Activation of the AhR signaling pathway increases the cytokine-secreting capacity of CD56^{bright} NK cells.[121] Animal experiments have confirmed that AhR is not only a cell-intrinsic requirement for maintaining the population of liver resident NK cells (possibly ILC1 based on the markers used in flow cytometry assay) but is also necessary for the memory function of liver resident NK cells.[122] Experimental melanoma metastasis also shows that AhR is indispensable for NK cell-mediated cancer surveillance.[119] However, some studies have found different roles of AhR. Activating AhR in NK cells promotes the secretion of IL-10, a suppressive factor for NK cells,[123] which acts on IL-10 receptor-positive NK cells. This mechanism maintains the homeostasis of NK cells in an autocrine and negative feedback manner.[124] Activation of AhR reduces the cytotoxicity of NK cells by affecting metabolism-related signaling pathways.[120] AML can evade NK cell-mediated killing by secreting AhR agonists, which inhibit the development and function of NK cells.[124,125] In some solid tumors, the activation of AhR is believed to inhibit the anti-tumor activity of T cells, DCs, and TAMs.[126] Therefore, although AhR is an easily activatable or inhibitable transcription factor, only a few NK cell-based immunotherapeutic strategies specifically target it. More investigation is needed to realize the therapeutic potential of AhR in cancer immunology.

Eomesodermin (EOMES) and T-box expressed in T cells (T-BET) are two important transcription factors that promote the development, function, and cancer-immune surveillance of NK cells, especially for liver-resident NK cells.[127,128] A recent study showed that remarkable improvements can be achieved in the anti-tumor activity of NK cells by enhancing the expression of T-BET or EOMES through genetic modifications. Notably, overexpression of EOMES has also been found to enhance the ADCC function of NK cells.[129] However, the NK cells in this study are derived from HSCs, and the genetic modifications are also achieved in CD34$^+$ HSCs. If comparable results can be replicated in NK cells derived from iPSCs, it would undoubtedly enhance the potential clinical application of tumor immunotherapy based on NK cells.

4.3. Enhanced chemokine response guides NK cells into solid tumors

As mentioned earlier, one of the factors contributing to the limited efficacy of NK cell-mediated killing in solid tumors is reduced migration. Lymphocyte migration and tissue residency are regulated by the interaction between chemokines and their receptors. Chemokine receptor C-X-C motif Chemokine Receptor 2 (CXCR2) is expressed on many leukocytes but is often lost during the *in vitro* culture of NK cells. NK cells overexpressing CXCR2 are similar to control cells in terms of cytotoxicity and IFN-γ secretion. Furthermore, this overexpression enhances adhesion properties, calcium mobilization specifically in response to CXCR2 ligands, and the ability to migrate down the CXCR2 ligand gradient.[130] CXCR4 is also a vital chemokine receptor expressed in both immune cells and non-hematopoietic cells.[131] The ligand of CXCR4, CXCL12, is highly expressed in multiple types of tumors and plays a stimulatory role in the proliferation of cancer cells.[132] Through the overexpression of the CXCR4 receptor, it is possible to significantly facilitate CAR-NK cells targeting EGFRvIII, enabling them to migrate to CXCL12-secreting glioblastomas. The effectiveness of this approach has been validated through *in vivo* xenograft experiments with solid tumors. Intravenously administration of CXCR4 expressing CAR-modified NK cells further improves survival of tumor-bearing animals compared to NK cells only expressing EGFRvIII-specific CAR.[133] To strengthen the argument for the enhanced anti-tumor effects achieved through ectopic expression of chemokine receptors, acquiring more *in vivo* data and reinforcing the existing preclinical evidence is essential.

4.4. Other methods utilizing iPSCs to enhance anti-tumor functions of NK cell

CD16 (FcγRIIIA) is uniformly expressed in peripheral CD56^{dim} NK cells and plays a crucial role in ADCC.[134] When cells are labeled with antibodies, NK cell cytotoxicity is triggered through the Fc segment of the antibody, representing a notable example of adaptive immunity augmenting innate immunity. In pre-clinical *in vivo* experiments, the combination of anti-GD2 antibodies with NK cells has shown promising therapeutic efficacy.[135] In a clinical trial targeting neuroblastoma, the use of anti-GD2 monoclonal antibodies in conjunction with haploidentical NK cells exhibited anti-cancer activity, particularly at higher doses.[136] Given the unique properties of CD16, it has risen as a key target for genetic engineering in the iPSC-derived NK cell development.[137,138] Although CD16 can mediate ADCC, it is not a high-affinity Fc receptor. CD64 (FcγRI), on the other hand, has a higher affinity for IgG, but it is not expressed on NK cells. Studies have combined CD64 and CD16 to create a new recombinant receptor. This receptor consists of a high-affinity antibody binding to the extracellular region of human CD64, whereas the transmembrane and intracellular regions of human CD16A mediate NK cell signaling.[139] After being equipped with CD64/16A, NK cells demonstrated significantly enhanced abilities in mediating ADCC, increased production of IFN-γ, and improved killing of target cells.[140] CD16, however, is vulnerable to cleavage by metalloproteinases. This cleavage results in a somewhat reduced but still retained sensitivity of NK cells to other activating receptors. For instance, metalloproteinase ADAM17, also known as TNF-α converting enzyme (131), can cause the loss of CD16 when overexpressed on NK cells (132). To mitigate the issue of CD16 cleavage, a non-cleavable version of human CD16 (hnCD16) was engineered.[141] However, it is important to note that CD16, by mediating the break after ADCC, actually helps NK cells to detach from the dying target cells and prepare them to kill the next target cells.[142] Whether this non-cleavable CD16 will have a better tumor-killing effect in the real world compared to the cleavable CD16 remains to be investigated further.

IL-15 is an important cytokine for maintaining NK cell homeostasis and function, increasing the number of CD56^{bright} NK cells and enhancing the cytotoxicity of CD56^{dim} NK cells.[143,144] It is also an important target of NK cell-centered cancer immunotherapy. However, the IL-15 is a short-lived, low-activity cytokine under normal physiological conditions.[145] Previous studies have shown that the IL-15/IL-15R complex can release the function of NK cells from TGF-β inhibition.[146] Activation of the IL-15 signaling pathway in iPSC-derived NK cells can be achieved through the fusion expression of IL-15Rα with IL-15, mimicking transpresentation.[147] Although NK cells overexpressing IL-15 are freed from autocrine cytokine dependence, their true tumor-killing ability to resist immune suppression in solid tumors remains to be further investigated. The combination of IL-15, IL-18, and IL-12 has been used to stimulate NK cells, enhance NK cell proliferation and activation, and produce IFN-γ, as well as to enhance the anti-tumor properties of NK cells.[143,148] CIS protein (cytokine-inducible SH2-containing protein), encoded by the CISH gene, can negatively regulate IL-15 signaling in NK cells, and its deletion can improve the metabolic activity of iPSC-derived NK cells.[149] However, recent studies have revealed that IL-15 may also contribute to the downregulation of certain chemokines, thereby influencing NK cell migration and function. CX3CR1 is present in nearly all CD16^{+}CD56^{bright} NK cells and is crucial for NK cell adhesion, chemotaxis, degranulation, and tumor cell killing. Both the surface expression and mRNA level of CX3CR1 are reduced in the presence of IL-15.[150,151] In proliferating NK cells activated by IL-15, CX3CR1 expression is very low and may not function properly as a chemokine receptor. The reason for this is not clear but it could be to protect normal endothelial cells from NK cell attack.[152] Moreover, the enhancement of NK cell migration and cytotoxic functions through short-term hypoxia in conjunction with IL-15 suggests a potential synergy that could be leveraged to augment anti-tumor responses.[153] This insight underscores the necessity for a more advanced approach to utilizing IL-15 within iPSC-derived NK cell therapies. Such strategies may need to carefully consider the intricate dynamics between IL-15, NK cell biology, and the tumor microenvironment to optimize the therapeutic potential of NK cells against cancer. Methods for augmenting the anti-tumor capabilities of NK cells through genetic modification are summarized in Figure 2.

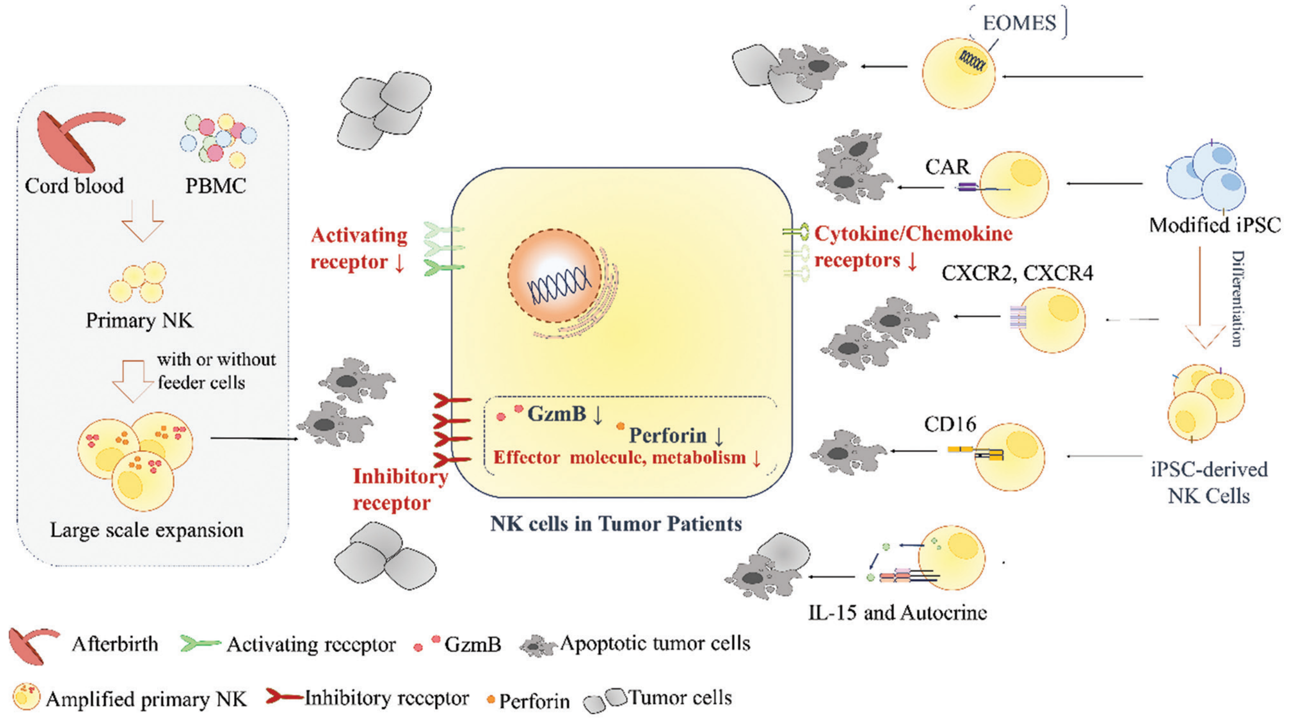

Figure 2. Overcoming microenvironmental barriers and enhancing NK cell antitumor activity through gene editing
Abbreviations: CAR: Chimeric antigen receptor; CXCR: C-X-C motif chemokine receptor; GzmB: granzyme B; IL: Interleukin; iPSC: Induced pluripotent stem cells; NK: Natural killer; KIR: killer-cell immunoglobulin-like receptor; PBMC: peripheral blood mononuclear cells. Figure created by the authors.

5. Concluding remarks

NK cells possess the remarkable ability to target and eliminate both tumor cells and virus-infected cells. Adoptive transplantation of NK cells exhibits exceptional efficacy in combatting hematologic tumors. However, their application in the treatment of solid tumors is limited. Recent years have witnessed the development of numerous strategies aimed at bolstering and adapting NK cells to surmount this limitation. Even though CAR-T cell therapy has yielded promising clinical results, CAR-NK cells have also gained prominence within the realm of cancer immunotherapy. Nonetheless, it is becoming increasingly apparent that CAR-NK cells may not represent the ultimate solution for adoptive NK cell therapy. The imperative lies in tailoring NK cell therapies to fully exploit their distinctive attributes.

NK cells derived from iPSCs have pioneered a new frontier in NK cell-based tumor immunotherapy strategies. These cells have been genetically engineered to incorporate CARs, enhance their ADCC, and enable autocrine secretion of cytokine. However, these cells also face numerous challenges in clinical applications, including issues with allogeneic rejection. While autologous iPSCs could be employed, the reprogramming, selection, establishment, quality control, and differentiation processes are complex and time-consuming, making personalized production prohibitively expensive. Consequently, allogeneic iPSCs-derived NK cells are primarily used, but they may cause potential damage to the recipient's normal cells and face rejection by the recipient. The extended differentiation time of iPSC-derived NK cells leads to prolonged preparation cycles, presenting challenges in maintaining process stability and batch consistency. In addition, variability among iPSCs from different sources affects the comparability of data across various laboratories. Addressing these challenges is crucial, as it would greatly enhance the potential of NK cells in cancer treatment by improving their safety and efficacy, thus offering more effective therapeutic options for patients.

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Conflict of interest

The authors declare no conflicts of interest.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

ABO blood type and cancer susceptibility: Unraveling the complex relationship

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Abstract

Different human diseases have been associated with specific blood groups. Numerous studies have promulgated their findings regarding the probable onset of various cancers based on the type of ABO blood group. However, the findings have been conflicting. This review primarily aims to summarize research findings from around the world to investigate the relationship between the risk of cancer occurrence and ABO blood group. Google Scholar, PubMed, Research4Life, and Web of Science were searched to identify relevant papers published before January 2023. Research papers related to common types of cancers were adequately and critically studied to determine the link between ABO blood groups and the risk of developing cancers. The results were ambiguous, as the findings were inconsistent regarding the relationship between ABO blood groups and cancer development. Therefore, more comprehensive research is needed to validate this relationship. This mini-systematic review emphasizes the need for additional thorough investigations to establish a clear correlation because of the inconsistent results.

Keywords: ABO; Blood group; Cancer risk; Relationship

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1. Introduction

The ABO blood groups are polymorphic, antigenic, and genetic substances and consist of four phenotypes: "A," "B," "O," and "AB."[1] The ABO system was the first genetic polymorphism identified in humans and remains one of the major human blood type systems, with major applications in transfusion medicine and organ or tissue transplantation.[2,3] The term "histoblood group ABO" is used to describe the omnipresent nature of ABO antigens. These blood group antigens are involved in cell signaling, cell recognition, and cell adhesion, which may reflect their potential role in tumorigenesis, metastasis, and prognosis.[4-7] Expanding the clinical significance of the ABO blood system beyond immunohematology, transfusion, and transplant medicine is biologically feasible.[8]

Numerous studies have reported associations between blood types and cancer susceptibility. A relationship between the A blood group and gastric cancer was first demonstrated in 1953.[9] Since then, a higher incidence of the A blood group has been documented in numerous cancers, such as colon, ovarian, and cervical cancers. As cells become malignant, they tend to lose normal blood antigens and gain new tumor-associated antigens.[10] The reduction in normal antigens (A, B, and H) is inversely proportional to the metastatic ability of the tumor.[11] Some tumor antigens are true A antigens, while others are A-like antigens, sharing properties similar to A antigens.[12] Therefore, these antigens can be detected on the tumors of patients with blood type A, as they would be recognized as foreign antigens, triggering an immune attack.[13] However, A or A-like antigens present in tumors are not perceived as foreign in individuals with the A blood group.[14] This may explain why individuals with the A blood group have a higher risk of cancer than those with blood group O.[15,16] This review attempts to decipher the correlation between the frequency of blood groups and the incidence of different cancers.

1.1. Mechanism of the pathology

1.1.1. Glycosyltransferase activity

The ABO blood grouping system involves antigens, including A, B, and H. The ABO system comprises seven exons containing DNA variants that alter the enzymatic activity of the gene (Figure 1). The gene for the ABO blood group is located on chromosome 9q34, which encodes alleles A and B, resulting in specific glycosyltransferases produced from four non-synonymous variants at nucleotides 526, 703, 796, and 803.[17] The A allele encodes α1-3 N-galactosaminyltransferase, which catalyzes the covalent linkage of N-acetylgalactosamine to the non-reducing ends of glycans on the glycoproteins of the H antigen expressed in all red blood cells. The B allele encodes another glycosyltransferase called α1-3-galactosyltransferase, which transfers D-galactose to the H determinant. However, the O-variant allele encodes a non-functional glycosyltransferase and is characterized by a single base pair deletion at base pair 261, resulting in the loss of enzyme translation and unmodified H antigen.[18,19] Thus, the carbohydrate moieties present on the surface of the erythrocyte's membrane define ABO blood group antigens.[20] Besides red blood cells, the expression of ABO antigens is also observed on the surface of various human cells and tissues, such as vascular endothelium, platelets, sensory neurons, mucus secretions, and epithelial tissues.[21]

1.1.2. Immune response

Blood group antigens are considered primarily tissue antigens distributed throughout the body. The rejection of transplanted organs and natural abortions is caused by antibodies produced against these antigens.[22] ABO antigens expressed in normal tissues have been reported to be different from those expressed in cancerous cells.[23,24] This difference is assumed to alter cell motility, apoptosis, and immune escape.[25] The ABO blood type system is linked to several diseases including cancer in humans based on the presence or absence of those antigens throughout the body.[26,27] However, the underlying phenomena associated with the risk of ABO variants with tumor development and progression remain unclear and have become the subject of research.

A possible assumption is that malignancy is induced by the dysregulation of the enzymatic activity of ABO glycosyltransferases that play major roles in intercellular adhesion, cell membrane signaling, and host immune response.[28-31] This mechanism is similar to the process by which ABO glycosyltransferases regulate circulating plasma levels of von Willebrand factor (Figure 2), which was recently discovered as an important regulator of angiogenesis and apoptosis in tumorigenesis.[32-34] Alternation in the host inflammatory system and systemic inflammatory response may also influence blood group

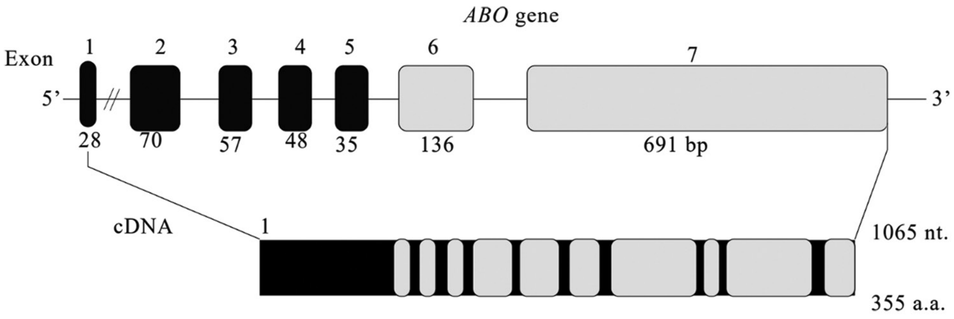

Figure 1. Structure of the ABO gene locus showing the nucleotide sequences of the A, B, and O alleles[17]
Abbreviations: a.a., amino acid; bp, base pair; nt, nucleotide position.

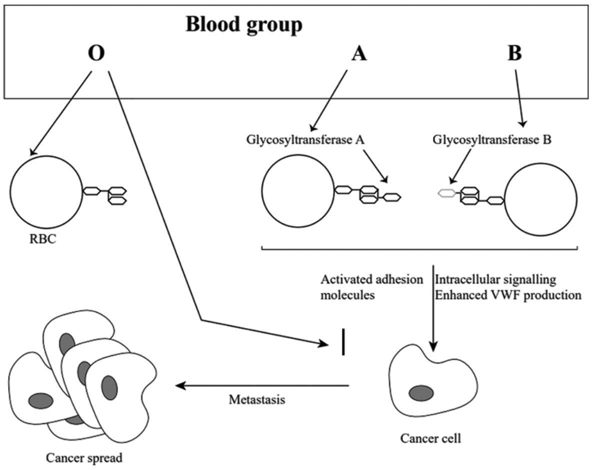

Figure 2. Association between ABO antigens and cancer[8]
Abbreviation: VWF, von Willebrand factor.

antigens to promote the spread and progression of malignancy.[35,36] In addition, glycosylation can direct conformational changes in proteins such as epidermal growth factor receptors or alter the immune surveillance ability of immune cells such as natural killer cells that promote malignancy.[37]

1.1.3. Adhesion molecules

Similarly, the relationship between ABO variants and circulating levels of adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], E-selectin, and P-selectin) has provided a background that supports the influence of ABO in tumorigenesis, as these molecules are pivotal regulators during chronic inflammation and recruitment of immune cells.[8] A reduced level of ICAM-1 in patients with the non-O blood group (specifically the A blood group) than in those with the O blood group is assumed to promote metastasis that causes a decrease in survival in patients with cancer and non-O group and favors prognosis in patients with the blood group O.[38-43] Because the blood group polysaccharides expressed on the cell surface of metastatic cancer cells function as cell adhesion molecules, a correlation was found between blood group antigen expression in tumors and metastasis and prognosis for various malignancies, including colon, breast, and prostate cancer.[44]

2. Methodology

Google Scholar, PubMed, Research 4 Life, and Web of Science were systematically searched to identify relevant research papers published before January 2023. The search strategy was designed to capture peer-reviewed articles and comprehensive reviews that investigated the relationship between the ABO blood group and the risk of developing common cancers. Specific search terms included combinations of keywords such as "ABO blood group," "cancer risk," "oncology," and specific cancer types (e.g., breast, colorectal, lung, and prostate cancer).

Studies that provided original data or meta-analyses linking ABO blood group phenotypes with cancer incidence or susceptibility were included, particularly those presenting significant findings, large sample sizes, or critical insights into the potential mechanisms underlying this association. Studies unrelated to cancer or those that lacked clear ABO blood group data were excluded. Each article was reviewed for quality, relevance, and methodological rigor. Special attention was given to studies that controlled for confounding variables such as age, sex, lifestyle factors, and comorbidities. The results of the selected studies were analyzed thoroughly to assess the strength of the evidence connecting the ABO blood group with cancer risk and to identify patterns or inconsistencies across different types of cancer. This methodology allowed for a comprehensive evaluation of the current understanding of the role of the ABO blood group in cancer development.

3. Some major cancers associated with the ABO blood group

Association of different blood types and its association with the development of different cancers are shown in Table 1.

3.1. Gastric cancer

Gastric cancer comprises 7% of all cancers globally. Studies have shown a stronger association between A and AB blood groups and gastric cancers. For example, the A blood group showed an odds ratio (OR) of 1.13 and a 95% confidence interval (CI) of 1.02 – 1.24, and the AB blood group shared an OR of 1.18 and a 95% CI of 1.02 – 1.36.[45] These ORs indicate a positive association between the A and AB blood groups and the risk of gastric cancer, with these groups having slightly higher odds of developing the disease. The CIs for both blood groups were narrow and >1, meaning that the associations were statistically significant and not likely due to random variations. Recently, the Chi-squared value ($\chi^2 = 4.708$, $P < 0.001$) reported by Yu *et al.*[46] demonstrated a significant relationship between the A blood group and gastric cancer development. Similarly, Rummel and Ellsworth found that the A blood group exhibited a greater risk of gastric cancer.[17] The specific blood type antigen (e.g., Lewis-b antigen), the result of blood type genes at 19q13, was found to mediate the attachment of *Helicobacter pylori* to the human gastric mucosa, a causative agent in chronic active gastritis, in a study on the association between the ABO blood group and gastric cancer.[47,48]

Table 1. Different types of human cancers associated with the ABO blood group

Types of cancer	Association with the ABO blood group	References
Gastric	An increased risk of gastric cancer was observed among individuals with blood group A (incidence rate ratio = 1.20, 95% confidence interval [CI] 1.02 – 1.42)	124
	The risk of gastric cancer was significantly higher in A blood group than in the non-A groups (O, B, and AB) (odds ratio [OR] 1.34; 95% 1.25 – 1.44).	47
	Blood groups A and AB were found to be associated with gastric cancer: Group A, OR 1.13, 95% CI 1.02 – 1.24; group AB, OR1.18, 95% CI 1.02 – 1.36.	45
	The frequency distribution of patients gastric cancer having the A blood group was significantly increased (χ^2=4.708, *P*<0.001), while the frequency distribution patients with gastric cancer having the AB blood group was significantly decreased (χ^2=9.630, *P*<0.01).	46
Pancreatic	Increased risk in blood type A compared with its frequency among regional blood donors (47.63% vs. 39.10%, OR 1.43, *P*<0.01)	52
	Compared with the OO genotype, those with the AO and AA genotypes had ORs of 1.67 (95% CI 1.08 – 2.57) and 1.53 (95% CI 0.80 – 2.91), respectively, whereas individuals with the BO and BB genotypes had ORs of 1.24 (95% CI 0.74 – 2.06) and 3.28 (95% CI 1.38 – 7.80), respectively.	51
	Increased risk in the group with A blood group compared with the control group (OR 1.8, 95% CI (NA), *P*<0.01). A lower rate of risk in the group with AB blood group than in the control group (OR, 0.37; 95% CI (NA), *P*<0.01).	54
	Non-O groups were linked to a higher incidence of pancreatic cancer. Elevated risk found across all non-O blood types (type A, HR 1.45; type B, HR 1.59; and type AB, HR 1.37).	49
	Increased cancer risk of blood type A with the estimated adjusted odds ratios (AORs with 95% confidence intervals [CIs]) of 2.130 (1.409 – 3.220)	55
	Increased risk found in type A compared with the non-O type (HR 0.719, 95% CI 0.521 – 0.974, *P*<0.01)	50
Breast	Higher incidence of blood group A (OR 2.13, 95% CI 1.04 – 2.96, *P*<0.01).	63
	Blood group A was at higher risk (OR 7.444, 95% CI 4.098 – 13.5222).	60
	Blood group A showed a higher incidence of breast cancer (45.88%), whereas a lower incidence was observed in blood group AB (6.27%).	65
	No association was observed.	47,66,67
Prostatic	The non-O blood group was at higher risk than the O group (high-risk percentage: O vs. non-O: 72.5% vs. 85.7%, *P*<0.01)	47
	Blood group B demonstrated an increased risk of cancer (OR 1.23, *P*<0.05).	91
	No significant association was observed.	62
	No significant association was observed (*P*>0.05)	64
Colorectal	No significant association was observed between any blood group and colorectal cancer (*P* interaction = 0.91)	84
	Non-AB blood type was found to be at greater risk with HRs for patients of with A, B, and O of 4.37 (95% CI, 2.65 – 7.20), 2.99 (95% CI 1.81 – 4.96), and 2.78 (95% CI 1.69 – 4.56), respectively, compared with AB blood type.	81
	No significant association was observed (*P*>0.05).	125
	No significant association was found.	49
	Individuals with blood type A had a borderline significant higher risk of colorectal cancer (HR 1.18, 95% CI 0.97 – 1.43, *P*=0.097) than those with blood type O.	47
Kidney	The non-O type was associated with decreased overall survival (HR 1.68, 95% CI 1.18 – 2.39, *P*<0.01).	38
	No significant association was reported.	26,101,102
Urinary bladder	Blood type O had worse recurrence and progression rates than type A (*P*=0.015 and 0.031) or B (*P*<0.01 and 0.075).	107
	Blood type B demonstrated significantly higher risk (OR 1.26, *P*<0.01)	91
Ovarian	Women with blood type A had a greater incidence of ovarian cancer. The incidence ratio was 1:17.	126
	The incidence of ovarian cancer with the B antigen was higher, whereas the A antigen did not share an increasing risk of ovarian cancer.	71

(Cont'd...)

Table 1. ***(Continued)***

Types of cancer	Association with the ABO blood group	References
	The A blood group shared an increased risk of ovarian cancer: (OR 1.09, 95% CI 1.01 – 1.18; $P<0.01$). As per the diplotype analysis, type AO was more linked with increased risk than AA (AO, OR 1.11, 95% CI 1.01 – 1.22, $P<0.01$; AA, OR 1.03, 95% CI 0.87 – 1.21, $P>0.05$). The other diplotype AB and B blood groups had no significant ovarian cancer risk.	108
	A and B antigens shared an increasing risk of ovarian cancer. However, the B blood group (OR 1.48, 95% CI 1.17 – 1.81, $P<0.01$) shared a higher risk of ovarian cancer than the A blood group (OR 1.40; 95% CI 1.13 – 1.73, $P=0.0019$).	109
Cervical	Higher incidence of cervical cancer in patients with the A blood group.	26,114,115
	Higher incidence of cervical cancer (37.9%) in patients with the B blood group.	127
	No significant correlation was observed.	110,111,128,129
Nasopharyngeal	Individuals with blood types A and AB had a higher risk of NPC than those with blood type O.	119
	No significant association was observed between any blood group and NPC.	118
	No significant difference was obtained between patients with the O and non-O blood groups, except for the significantly lower distant metastasis-free survival (DMFS) in patients with the O blood group. The O blood type was associated with an unfavorable DMFS in female patients with NPC.	47
Lung	No significant association was observed between any blood group and non-small-cell lung cancer.	121
	Increased risk of lung cancer in patients with non-O blood type and Rh negative.	62
	Higher risk of lung cancer in those with blood type AB than in those with blood types B and O.	47
Skin	The risks of developing SCC and BCC in participants with the non-O blood group were 14% and 4%, respectively. No significant linkage was observed between the blood type and melanoma.	122
	A significant association was found between patients with BCC and controls in terms of A Rh (–) ($P<0.01$).	123

Note: NA: Not available.
Abbreviations: HR: Hazard ratio; NPC: Nasopharyngeal carcinoma; DMFS: Distant-metastasis-free survival; SCC: Squamous cell carcinoma; BCC: Basal cell carcinoma.

3.2. Pancreatic cancer (PC)

PC is one of the most common cancers and the leading cause of death from cancer worldwide. An overall significant increased risk for PC was found in a Taiwanese cohort study involving 339,432 participants with non-O blood type (hazard ratio [HR] = 1.50, 95% CI 1.04 – 2.19).[49] In a recent comparative study of long-term outcomes of 406 patients with PC between A alleles and non-A alleles according to treatments (resection, $P < 0.05$; chemotherapy, $P = 0.757$; and palliative care, $P = 0.532$), patients with A alleles who underwent resection possessed a greater risk than those with non-A alleles.[50] In a case–control study conducted on 185 patients/1465 controls in the Japanese population, those with the A blood group were at a greater risk of PC than individuals with the O blood group.[51] In the study by Greer *et al*., 274 patients also showed a greater risk for those with the A blood group.[52,53] Another study conducted in the Turkish population among 132 patients/633 controls by Engin *et al.* and in China among 264 patients/687 controls by Li *et al.* also confirmed the increased rate of the risk of the A blood group.[54,55] Therefore, these studies show a possible association between the ABO blood group and the PC risk, with a greater risk for those with the A blood group.[56]

3.3. Breast cancer

Breast cancer is one of the most common cancers in women. According to the Breast Cancer Research Foundation, nearly two million new cases of breast cancer were diagnosed in 2018. Globally, approximately 12% of newly diagnosed cancer is caused by breast cancer.[57-59] Saxena *et al.* reported the maximum occurrence of breast carcinoma in the A blood group and a minimum in the AB blood group. Patients with the A blood group (OR 7.444, 95% CI 4.098 – 13.5222) were observed at higher risk than those with blood group AB (OR 1, 95% CI 0.476 – 2.103) when taking reference to the proportion of breast cancer in the AB blood group.[60] However, no association between the blood group and the risk of breast cancer was observed in a study of 368 patients with cancer in Bhopal, India.[61] Similarly, no relationship was observed between the HER2 status and ABO blood group examined in 294 Turkish female patients with HER2 (+) breast cancer.[62] A study of 160 women with breast cancer during their pre-operative control and follow-up, following mastectomy, in Mansoura University Hospital, Egypt, revealed a higher frequency of breast cancer in those with the A blood group (OR 2.13, 95% CI 1.04 – 2.96, $P < 0.05$) and lower cancer frequency in those with the AB blood group (OR 0.74, 95% CI 0.30 – 1.84, $P = 0.52$) compared with the control participants.[63] However, Flavarjani *et al.* did not find a significant association between blood types and breast cancer ($P > 0.05$) in a study of 549 women including 173 cases and 376 controls.[64] In the study by Meo *et al.*,[65] the incidence of breast cancer was higher in patients with the A blood group (45.88%) than in those with the AB blood group (6.27%).[65] Some studies have not found a relationship between the ABO blood group and breast cancer risk or survival.[66,67]

A meta-analysis of 14 studies conducted by Miao *et al.* did not find an association between the ABO blood group and breast cancer risk but reported that Caucasian people with the A blood group might be at higher risk for this cancer.[68] Two studies observed a significant association of blood type A with a higher risk of breast cancer.[69,70] In contrast, no association was found between the ABO blood group and the risk or survival or mortality of invasive, ductal, or hormone receptor-positive breast cancers.[71,72] Yu *et al.* did not observe an association between the ABO blood group and triple-negative breast cancer.[73] However, Amini *et al.* observed a significant association between the tumor size and axillary lymph node involvement and the ABO blood group.[74] A study reported a positive relationship between the risk of breast cancer and the O blood group.[75] Some studies have suggested that people with blood types B and AB have a significantly increased risk of breast cancer with a poorer overall survival (OS) than those with A and O blood groups.[76,77] Stamatakos *et al.* reported the association of the A antigen with the increased incidence of invasive ductal carcinoma in 166 Greek women.[78] Tryggvadottir *et al.*[79] observed a twofold higher incidence of familial breast cancer in those with the B blood group while studying bilateral breast cancer in familial and sporadic cases of the ABO blood group.[79] In addition, Anderson and Haas showed a positive association between A and B blood groups in women with a family history of breast cancer.[80]

3.4. Colorectal cancer

In comparison with gastric cancer and PC, very few studies have examined the correlation between the ABO blood group and colorectal cancer development. In a study conducted from 1995 to 2002, among 1555 patients, a greater risk was noted in those with the non-AB blood group.[81] In the multivariate analysis, a modest association of the B blood group (HR 1.20, 95% CI 1.00 – 1.45) with colon cancer was observed compared with the O blood group.[82,83] However, in the studies conducted by Khalili *et al.* among 1025 patients and by Li *et al.* among 1314 patients, no significant association was observed between any blood group and colorectal cancer.[84-86] In the Shanghai Cohort Study 1986 – 2013 with 624 patients and 355,797 controls, individuals with the A blood group had a borderline significant higher risk of colorectal cancer (HR 1.18, 95% CI 0.97 – 1.43, $P = 0.097$) than those with the O blood group.[47] Most studies did not show a significant association between the blood group and colorectal cancer. However, some studies have suggested the increased risk of colorectal cancer in the non-AB blood group, and those with the A blood group had a higher risk. In addition, the O blood group was the most prevalent ABO blood group among patients with type I endometrial cancer (EC) and was associated with an increased risk of developing type I EC. However, the ABO blood group was not significantly correlated with the stage or differentiation of type I EC.[87] Patients with hepatocellular carcinoma who have the A blood group may have lower OS and recurrence-free survival rates following hepatectomy compared with those with non-A blood groups.[88]

3.5. Prostate cancer

Prostate cancer accounts for approximately 8% of the global cancer incidence.[58,89] Few studies have reported to date the association between ABO blood groups and prostate cancer. Wang *et al.* demonstrated that the patients with the O blood group have a lower risk of prostate cancer and are less aggressive than non-O blood groups (high-risk percentage: O vs. non-O: 72.5% vs. 85.7%, $P < 0.05$) and suggested that nearly all of the included studies compared patients with prostate cancer with the normal population without revealing an association between the ABO blood groups and the risk of invasiveness in patients with prostate cancer.[90] In a previous study, the B blood group was found to be significantly more frequent in patients with prostate cancer, suggesting that patients with the B blood group were at an increased risk of cancer (OR 1.23, $P < 0.05$).[91] Some studies have not found a relationship between the blood type and prostate cancer risk or survival.[17,47,92-94] A study reported that tumor progression is favored by the loss of antigen expression at the tissue level.[95] However, other studies have suggested that prostate tissue without antigen expression cannot be considered a biomarker of invasion.[96] The loss of blood group A antigen and retention of the H antigens were observed in tumor tissues of the prostate.[97] With tumor progression, the expression of the A antigen has been reported to decrease, which might be associated with the increased risk of biochemical recurrence of prostate cancer in patients with the A blood group.[98,99]

3.6. Kidney and urinary bladder cancer

The expression of ABO antigens is observed on the surface of several normal and cancerous tissues, including kidneys and renal cell carcinoma lines.[100] Several studies have examined the association of the ABO blood group with renal cell carcinoma. A study of 900 patients undergoing surgery for locoregional renal carcinoma reported an association between the ABO blood group (non-O) and OS, indicating the non-O blood group as an independent biomarker of mortality. The study found that the non-O blood group was associated with decreased OS in the multivariate analysis (HR 1.68, 95% CI 1.18 – 2.39, $P < 0.05$).[38] Another study observed that the absence of lymph node metastasis had no suitable effect on the prognosis in patients with the O blood group and found no association between the ABO blood group and survival outcomes.[101,102] Stakišaitis *et al.* did not find any significant relationship between the ABO blood group and kidney cancer survival.[91] While some researchers have identified the ABO blood group as a prognostic predictor, others did not find any association with renal cancer.[20] One study reported no significant association between the ABO blood group and kidney cancer,[26] whereas a cohort study found a higher occurrence of renal cell carcinoma in women with the non-O blood group compared to those with the O blood group.[103]

The prevalence of the A blood group was higher in patients with bladder cancer.[104] In a study of the Lithuanian population, the B blood group was found to be significantly more frequent in people with bladder cancer compared to the control group of blood donors. The B blood group was more common in men (OR 1.27, $P < 0.05$); however, no association was found in women with bladder cancer ($P > 0.05$).[105] Another study found that the B blood group was significantly associated with bladder cancer risk (OR 1.26, $P < 0.05$), whereas no significant association was observed with the O blood group (OR 0.76, $P < 0.05$).[91] Another study suggested that patients with the O blood group exhibited more aggressive tumor behavior in bladder cancer, with higher tumor grade and more relapses than those with the A blood group.[106] Similarly, another study showed that the O blood group was associated with worse recurrence and progression rates compared to the A (P = 0.015 and 0.031) or B (P = 0.004 and 0.075) blood groups.[107]

3.7. Ovarian cancer

Ovarian cancer is the seventh leading cause of death among women globally.[89] It is also linked with certain ABO blood groups.[71,97,108,109] Two studies concluded that carriage of A and B antigens in the ABO blood group increased the risk for ovarian cancer, with the B blood group showing a higher risk.[71] In contrast, another study[108] reported that women with the A blood group were more susceptible to ovarian cancer (OR 1.09, 95% CI 1.01 – 1.18; $P < 0.05$). Similarly, a haplotype analysis revealed that the AO blood group carried a higher risk than the AA blood group.[108] Another study reported a higher risk of ovarian cancer in individuals with the A blood group, with 1 in 17 women affected.[71]

3.8. Cervical cancer

Cervical cancer is a common cancer in women, representing 4% of the global cancer incidence[58,89] Several studies have reported no significant correlation between the ABO blood group and cervical cancer.[109-112] However, a higher incidence of cervical cancer (37.9%) was observed in patients with the B blood group.[113] In a group of Japanese women, most of the women with the A blood group had cervical cancer.[114] A similar result was observed in a study conducted in India.[115] The incidence of cervical cancer was higher in women with the A blood group than in those with the O blood group.[15] However, no association was found between the blood group and the risk of cervical cancer in studies conducted in Nigeria or southeast Siberia.[109,116] In addition, the RhD factor may influence OS in cervical cancer, but current data lack strong significance. Larger studies are needed to confirm its role and explore the impact of blood groups on female cancers.[117]

3.9. Nasopharyngeal carcinoma (NPC)

A study of 2439 patients with NPC from 2001 to 2004 showed no significant difference between patients with O and non-O blood groups, except for a significantly shorter distant-metastasis-free survival (DMFS) in patients with the O blood group (aHR = 1.2268, 95% CI 1.010 – 1.592, $P < 0.05$). In female patients, those with the O blood group had significantly shorter OS ($P < 0.05$) and DMFS ($P < 0.05$) than those with the non-O blood group. However, in male patients, no significant differences were observed ($P > 0.05$).[90] Similarly, Lin *et al.*[118] did not find a significant difference in the distribution of ABO blood groups between the NPC and control groups (P = 0.884). However, a significant difference between the sexes and ABO blood groups was observed, except for the AB blood type (P = 0.246).[118] In contrast, another study found an association between the ABO blood group and NPC risk. The medical data of 1,538 patients with NPC from 2004 to 2011 showed that A and AB blood types were associated ($P < 0.05$ and $P < 0.05$) with an increased risk of NPC when compared with the O blood group. The distant metastasis rate was significantly higher among men with the A blood group than in those with the non-A blood groups ($P < 0.05$).[119] Thus, the correlation between the ABO blood group and NPC remains controversial.

3.10. Lung cancer

In a study conducted among 964 patients with lung cancer in China, those with the AB blood type showed a relatively higher risk of lung cancer (HR 1.01, 95% CI 0.81 – 1.26) than those with the A blood group A.[47] Another study conducted on 417 Turkish patients by Urun *et al.* confirmed the association between the ABO blood group and lung cancer, wherein the non-O blood group (OR 0.864, 95% CI 0.787 – 0.950) was associated with an increased risk of lung cancer compared with the O blood group.[120] In contrast, a study involving 81 patients found no significant effect of the ABO blood group on the prognosis of non-small-cell lung cancer.[121]

3.11. Skin cancer

A study conducted from 1996 to 2006 explored the potential association between the ABO blood group and melanoma, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). The multivariable HRs for the non-O blood groups (A, B, and AB) were 0.91 (95% CI 0.78 – 1.05), 0.86 (95% CI 0.78 – 0.95), and 0.96 (95% CI 0.93 – 0.99) for melanoma, SCC, and BCC, respectively. This suggested a lower risk of developing SCC in participants with the non-O blood group than in those with the O blood group.[122] A study conducted from 2005 to 2012 among 255 patients with skin cancer showed no significant difference in the distribution of O and A blood groups between the SCC and BCC groups ($P = 0.663$).[123] Overall, the results suggest an association between the non-O blood group and a higher risk of non-melanoma skin cancer.

4. Conclusion

The association between blood group and cancer risk was significant only in pancreatic and gastric cancer, with individuals possessing the A blood group being at greater risk. However, results were inconsistent in studies conducted on other types of cancer. This study provides baseline data for understanding the association of blood type with the risk of cancer development.

Further prospective studies with larger patient populations are recommended to better understand the significance of the ABO blood group as a predictive factor in different cancer types. Most recent discoveries have focused on ABO phenotype, making studies on the ABO genotype crucial for identifying new therapeutic targets. Owing to the limited research in this area in Nepal, Nepalese researchers should be encouraged to conduct studies exploring the association between the ABO blood group and cancer risk in the Nepalese population.

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The authors declare no conflict of interest.

Author contributions

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Consent for publication

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REVIEW ARTICLE

The role of copper and core gene network controlling cuproptosis in infection immunity, diagnosis, and treatment

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Abstract

Copper is an essential trace element in living organisms and is involved in a variety of biochemical processes, including cellular respiration, iron metabolism, and nerve function. In recent years, research has shown that copper is not only essential for fundamental physiological functions but also plays an important role in immune response and pathological states. In particular, copper death (cuproptosis), a recently discovered cell death pathway that is strongly associated with copper overload, is emerging as an appealing area of immune research. Excess copper can induce cell death as a result of copper ions directly binding to sulfide proteins in the tricarboxylic acid cycle. In-depth studies of copper metabolism and its related mechanisms will contribute to developing new diagnostic tools and therapeutic strategies and providing new ideas and approaches for tackling infections and other related diseases. This review summarizes the newest understanding of copper death and the latest advancements in disease diagnosis and treatment, providing a valuable reference for the follow-up research on tuberculosis-related vaccines and copper in immunity.

Keywords: Copper toxicity; Cuproptosis; Tricarboxylic acid cycle

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1. Introduction

Bacterial infections can lead to serious health issues, including pneumonia, tuberculosis, diarrhea, hepatitis, and meningitis.[1] They can be transmitted through food, water, air, or contact, and may result in severe diseases, death, or other serious consequences. At present, antibiotics are the most effective means to inhibit bacterial growth, but the problem of antibiotic resistance is a significant concern.[2] Apart from antibiotics, metallic elements possess antibacterial properties that can effectively inhibit bacterial growth and reproduction, thereby preventing deleterious impacts attributed to infections in the human body.[3] Among these metallic elements, copper exhibits antioxidative properties that can deter bacterial toxins from causing harm to the human body.[4]

Copper is an essential nutrient that plays a role in various biological processes involving bacteria, such as redox reactions, protein synthesis, and cell signaling.[5] In addition, copper can promote bacterial growth and development.[6] However, for bacteria, copper is a double-edged sword; while trace amounts of copper can stimulate bacterial growth, an excess of copper can lead to bacterial death.[7] Compared to other forms of programmed cell death, copper-induced cell death, known as cuproptosis, represents a novel mechanism of cell death.[8] Studies have shown that copper ions can induce cell death through mitochondrial sulfurtransferase A. When copper ions bind to the sulfurtransferase A protein in the mitochondria, they inhibit the protein's activity, leading to protein toxicity stress response and ultimately resulting in cell death.[9] Moreover, copper can induce cell death through oxidative stress mediated by lipid acylation proteins.[10] Bacteria experience copper-related cell death when exposed to copper. Copper death, as a type of programmed cell death, may allow bacteria to program a response when faced with pressure from copper ions. Using this mechanism, it is possible to leverage cuproptosis to initiate bacterial cell death under specific therapeutic conditions, to clear the infection. At the same time, appropriate concentrations of copper enhance the response of the host immune system and strengthen the function of macrophages and other immune cells, thereby contributing to infection control. This review primarily summarizes the mechanisms underlying bacterial cuproptosis, aiming to provide novel application insights.

2. Cuproptosis and copper metabolism

Copper-mediated bacterial cell death, also known as cuproptosis, refers to the process of bacterial demise triggered by the toxic effects of copper ions. This unique mechanism of bacterial cell death heavily relies on the toxicity of copper ions to eradicate bacteria.[11] Several studies have elucidated the potential mechanisms underlying copper toxicity observed across various microbial genera. Among these, the most recognized mechanism involves copper ions mediating the Fenton reaction, leading to the generation of oxygen atoms and hydroxyl radicals (reactive oxygen species [ROS]) that inflict oxidative damage on macromolecules such as proteins, lipids, and DNA.[12] While low concentrations of copper ions are essential for bacteria and participate in various biological processes such as cell growth and differentiation, high concentrations can induce cuproptosis in bacteria.[13]

An increase in copper (II) ion concentration results in a sharp rise in ROS levels, leading to decreased survival of mycobacteria.[14] Furthermore, copper has been shown to deplete glutathione, a crucial antioxidant that protects against heavy metal toxicity.[15] It has been suggested that glutathione may bind to copper, disrupting the activity of Fe-S cluster enzymes.[16] In the absence of oxygen atoms, copper replaces iron in Fe-S clusters, forming sulfur bridges with copper instead. Fe-S clusters are organic metal components of iron and sulfur responsible for biological electron transfer, serving as storage reservoirs for iron and sulfur, and playing roles in genome stability and nucleic acid metabolism.[17] Their inactivation leads to the downregulation of several key metabolic enzymes, driving cells into a toxic stress state and ultimately resulting in their demise.

Copper is primarily absorbed in the small intestine, where it is taken up by intestinal cells and transported into the bloodstream.[18] This process relies on specific transporters, such as copper transporter 1 (CTR1).[19] Once in the bloodstream, copper is predominantly carried by proteins such as albumin and ceruloplasmin (plasma ceruloplasmin) to various tissues throughout the body.[20] Within cells, copper is directed to different organelles, including the mitochondria and the endoplasmic reticulum.[21] The liver plays a crucial role in copper storage;[22] liver cells can store copper and release it into the bloodstream as needed. This regulation involves several proteins, such as metallothionein.[23] Excess copper is excreted from the body through bile, and the mechanisms governing copper excretion are influenced by the physiological state and copper levels in the body.[24]

3. Effects of copper metabolism disorders on human

Following the normal physiological processes of copper absorption, transportation, and distribution within the body as described above, any disruption in these finely tuned mechanisms can lead to the onset of copper metabolism disorders. Copper is an essential trace element involved in a variety of physiological functions, including enzyme activity, antioxidant defense, iron metabolism, and nerve conduction.[25] However, an excess or deficiency of copper in the body can cause serious health problems (Table 1). Wilson's disease and Mendelian diseases characterized by copper deficiency can be grouped under the umbrella of copper metabolism disorders. The proper regulation of copper levels is thus of utmost importance for maintaining overall health and homeostasis.

Wilson's disease is a genetic disorder primarily caused by mutations in the *ATP7B* gene, leading to a deficiency in a copper-transporting protein crucial for copper excretion in the liver. This deficiency results in copper accumulation and damage in various tissues, including the liver, brain, kidneys, and eyes.[26] Symptoms of Wilson's disease can be diverse, encompassing liver problems such as hepatitis, cirrhosis, and liver failure;[27] neurological issues such as movement disorders, cognitive impairments, and behavioral changes;[28] and ocular manifestations, notably the Kayser–Fleischer ring – a green or brown deposit at the corneal edge. Additional complications associated with Wilson's disease include kidney injury, anemia, and joint pain.[29]

Menkes disease is a rare X-linked genetic disorder primarily caused by mutations in the *ATP7A* gene, causing disruption to the absorption and transport of copper in the body.[30] This leads to a cellular deficiency of copper, with symptoms often appearing within a few months of birth.[31] Affected individuals may experience growth retardation, neurological issues such as mental retardation, seizures, and poor motor coordination, as well as distinct hair abnormalities characterized by thinning and copper coloration.[32] Additional complications may include abnormal bone development and low immune function.[33,34] Menkes disease is diagnosed by a combination of clinical evaluation, biochemical tests for measuring blood levels of copper and related proteins (such as ceruloplasmin), and genetic testing to confirm *ATP7A* mutations.[35] Current treatment strategies mainly involve copper supplementation, particularly with copper-amino acid complexes, alongside symptomatic management to address neurological symptoms, though the effectiveness of these interventions is limited.[36]

In addition to Wilson's disease and Menkes disease, abnormal copper metabolism can be linked to other conditions. Copper deficiency may arise from malnutrition or absorption disorders, such as Crohn's disease, and can manifest as anemia, immune dysfunction, and osteoporosis.[37] Conversely, while relatively rare, copper overload can occur due to certain liver and kidney diseases or prolonged use of specific medications, such as oral contraceptives, leading to copper accumulation and potential toxicity.[38]

4. The role of copper and cuproptosis in *Mycobacterium tuberculosis* and their broader implications

Copper plays a crucial role in cellular function, but its dysregulation can lead to toxicological effects, particularly in the context of infections such as *M. tuberculosis*. The mechanisms underlying copper-induced cell death primarily involve oxidative stress, mitochondrial damage, and inflammatory responses.[50] A comprehensive understanding of these processes is essential, especially in the context of developing therapeutic strategies targeting copper metabolism disorders, such as Wilson's disease, which is characterized by excessive copper accumulation in the body. Within the copper-related network, several key genes significantly influence cellular responses to varying copper levels. Genes such as *ATP7A* and *ATP7B* are pivotal for maintaining copper homeostasis, they facilitate copper transport and excretion, thereby preventing toxicity.[40] In addition to these transport mechanisms, antioxidant genes such as superoxide dismutase 1 and glutathione peroxidase are critical in mitigating oxidative stress associated with elevated copper levels.[51,52] The regulation of cell fate in response to copper stress is also governed by apoptosis-related genes. For instance, genes such as *Bcl-2* and *Bax* play vital roles in the apoptotic pathways that determine whether a cell survives or undergoes programmed cell death under conditions of copper-induced stress.[51] In addition, inflammatory-related genes involved in the nuclear factor kappa B signaling pathway further complicate the interplay between cell survival and death in environments rich in copper.[53] The activation of this pathway is often linked to the inflammatory response associated with infection and stress, influencing the overall cellular outcome. Recent research has shed light on the protective role of the cuproptosis regulatory factor ferredoxin 1 (FDX1) in clear cell renal cell carcinoma.[54] The

Table 1. Cuproptosis-related immune markers for the diagnosis and treatment of diseases

Disease	Copper-related immune markers	References
Wilson's disease	Plasma ceruloplasmin, non-ceruloplasmin-bound copper	26,39
Menkes disease	Activity and expression level of copper transporter ATP7A	40
Rheumatoid arthritis, etc.	Serum copper level, copper/zinc ratio	41
Breast cancer, lung cancer, etc.	Copper content in tumor tissue and serum ceruloplasmin level	42,43
Primary biliary cholangitis	Serum copper, ceruloplasmin	44
Alzheimer's disease	Urinary copper excretion and hepatic copper content	45
Amyotrophic lateral sclerosis	Copper content in the brain and copper concentration in cerebrospinal fluid	46
Multiple sclerosis	Ceruloplasmin fragments of cerebrospinal fluid	47
Hemochromatosis	Copper/iron ratio	48
Inflammatory bowel disease	Intestinal mucosal copper content, serum copper-related protein	49

expression levels of FDX1 have been correlated with tumor malignancy, suggesting that it may function as a potential therapeutic target.[55] Notably, FDX1 appears to enhance antitumor immune responses, indicating its dual role in both cancer progression and immune modulation.[56] The differential expression and mutation of cuproptosis-related genes across various cancers underscore their potential role in tumor prognosis, immune evasion, and the dynamics of the tumor microenvironment, suggesting that cuproptosis may be instrumental in cancer initiation and progression.[57]

Several cuproptosis-related proteins, such as DBT and SLC31A1, have been shown to correlate significantly with immune cell functions, including those of macrophages, neutrophils, and regulatory T cells.[58] This relationship indicates that cuproptosis may play a dual role in modulating immune responses and influencing the pathological mechanisms underlying diverse conditions, including metabolic disorders such as diabetes. Furthermore, copper exposure has profound implications on mitochondrial function. Elevated copper levels can result in the release of mitochondrial DNA into the cytoplasm, a phenomenon often triggered by deficiencies in transcription factor A.[59] This release activates the cGAS-STING signaling pathway, which is a critical trigger for innate immune responses, particularly in liver cells.

Copper, while an essential trace element, can become toxic at elevated concentrations, necessitating the development of sophisticated detoxification mechanisms to ensure bacterial stability. This is particularly significant for *M. tuberculosis*, which exhibits a complex repertoire of copper resistance genes to mitigate the harmful effects of excess copper (Figure 1). Key genes involved in copper resistance include *copA*, *copB*, *copC*, and *copD*, which contribute directly to the bacteria's ability to withstand copper-induced stress.[60] The copper-inducible transcriptional regulatory factor CopR plays a vital role in orchestrating the expression of these resistance genes, ensuring a coordinated response to copper exposure.[61] In addition, the copper-resistance system comprises essential components such as CopT, a copper-translocating P-type ATPase critical for efficient copper transport, and CopZ, a copper-binding protein that sequesters free copper ions. Another key player is CopY, which functions as a copper tolerance protein, mitigating copper's toxic effects.[62] The interplay among these genes underlies a signaling pathway centered around the regulatory functions of CopR, the transport capabilities of CopT, the binding affinities of CopZ, and the protective roles of CopY.

5. The role of cuproptosis in *M. tuberculosis* immunoevasion

M. tuberculosis has developed intricate strategies to survive and replicate within host macrophages, a feat closely linked to its sophisticated copper homeostasis system. Following the phagocytosis of *M. tuberculosis* by macrophages, the host's immune response actively deploys copper ions as a means to limit bacterial growth. Despite this hostile environment, *M. tuberculosis* has evolved complex regulatory mechanisms to manage copper levels effectively, which enhances its survival and pathogenicity.

A key component of the bacterial response to copper stress is the global regulator sigma factor C (SigC). Acting as a transcriptional activator, SigC facilitates copper acquisition and helps *M. tuberculosis* adapt to conditions of copper scarcity, thus underscoring its vital role in the bacterium's pathogenic strategies during episodes of copper deficiency. This regulatory pathway encapsulates the dual nature of copper, which serves as an essential nutrient for bacterial growth but can also act as a toxin under conditions of excess, particularly through the generation of harmful hydroxyl free radicals. Several copper-related proteins are integral to maintaining copper balance within *M. tuberculosis*. Among these, copper efflux proteins CptV (Rv0969) and MctB (Rv1698) play critical roles in conferring copper resistance by actively transporting surplus copper ions out of the bacterial cytoplasm. In addition, the copper-binding protein MymT (Rv0186A) is involved in sequestering copper, thereby mitigating its toxic effects. The interplay among these proteins is particularly crucial in the host environment, where elevated copper concentrations arise during the immune response against infection.

The dynamics of copper metabolism are further complicated by the action of interferon-gamma (IFN-γ), a cytokine produced by CD4+ T cells, which enhances macrophage activation and boosts their capacity to contain pathogens. IFN-γ upregulates various copper transport mechanisms, including the high-affinity copper uptake protein CTR1. This protein, along with partner proteins such as ATOX1 and ATP7A, is instrumental in facilitating copper transport into phagosomes, thereby amplifying the host's antimicrobial strategies. Notably, the hypoxic conditions associated with granuloma formation during *M. tuberculosis* infection promote the expression of CTR1, highlighting the nuanced relationship between copper metabolism and immune responses.

While copper is essential for bacterial growth, the high concentrations introduced by macrophages can be detrimental, catalyzing the production of reactive hydroxyl radicals. In response, *M. tuberculosis* employs various resistance mechanisms, such as the chelation of copper by MymT and the efflux of copper ions mediated by CptV and MctB. The cooperative action of CptV and MctB is particularly significant, as MctB has been shown to be essential for the virulence of *M. tuberculosis*. In the

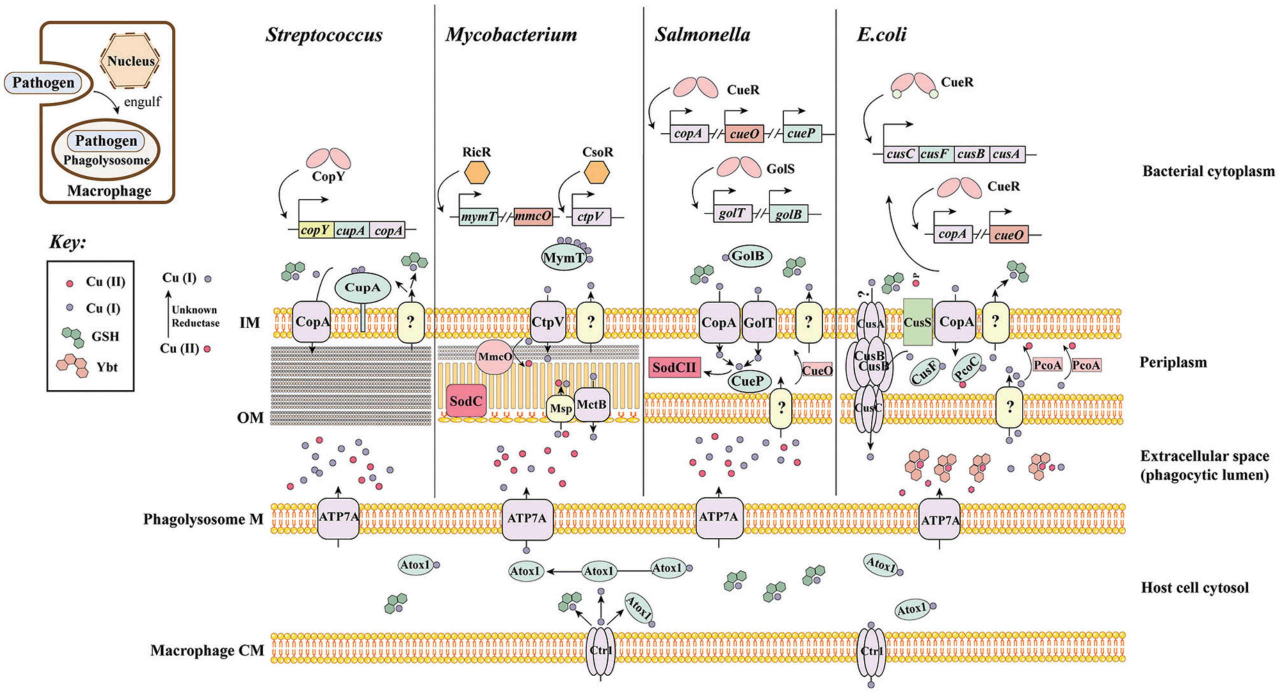

Figure 1. Schematic representation of bacterial pathogens in the context of host-pathogen interaction. This figure illustrates the copper transport, efflux, induction, and resistance pathways within selected bacterial pathogens, namely the Gram-positive *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*, as well as the Gram-negative *Escherichia coli* and *Salmonella*. Notably, whereas the Gram-negative bacteria are characterized by a more prominent periplasmic space, which is integral to their copper handling and other physiological processes, the Gram-positive bacteria, despite having a relatively less conspicuous periplasmic region (as indicated by shading and annotated in the legend), also partake in these mechanisms.

absence of MctB, the bacterium becomes vulnerable to copper overload, which jeopardizes its survival.

The coordination of copper homeostasis in *M. tuberculosis* is further regulated by copper-inducible proteins, including CosR, which modulate intracellular copper concentrations. Under conditions of excess copper, *M. tuberculosis* initiates the expression of MymT to sequester the surplus copper, followed by expulsion through the action of CptV and MctB.[63] This finely tuned regulation of copper ions not only contributes to bacterial resilience against host defenses but also reinforces the pathogen's overall virulence.

6. Similarities and differences in cuproptosis and related gene networks: Infection immunity versus other diseases

The metal's involvement in immune responses is characterized by its ability to modulate the function of immune cells, particularly white blood cells, thereby promoting antimicrobial effects (Figure 2). For instance, copper enhances the bactericidal capacity of macrophages, bolstering the host's defenses against pathogens. However, the relationship between copper, cell death mechanisms, and infection immunity is complex, demonstrating both similarities and distinct differences when compared to other diseases. One of the primary roles of copper in immune function is its influence on antimicrobial activities. During infection, elevated copper levels can stimulate immune responses, aiding in pathogen resistance. This heightened copper concentration is integral to the innate immune response, as it acts as a signaling molecule that regulates the kinase activity of α-kinase 1, enhancing the host's ability to respond to bacterial infections.[64] In addition, copper mediates various cell death pathways that can influence the fate of immune cells, particularly under conditions of infection. For example, copper-induced cell death, referred to as cuproptosis, can lead to immune cell apoptosis in certain contexts, thereby shaping the overall inflammation and immune response.[65]

The metabolic abnormalities associated with copper can lead to the accumulation of ROS, which are critical mediators in many pathological conditions, including infections, cancer, and neurodegenerative diseases. ROS not only influences immune responses but also modulates cellular signaling pathways and gene expression. Notably, the generation of ROS can differ vastly between infectious and non-infectious conditions, impacting cellular outcomes of stress and damage. Despite its essential role in enhancing

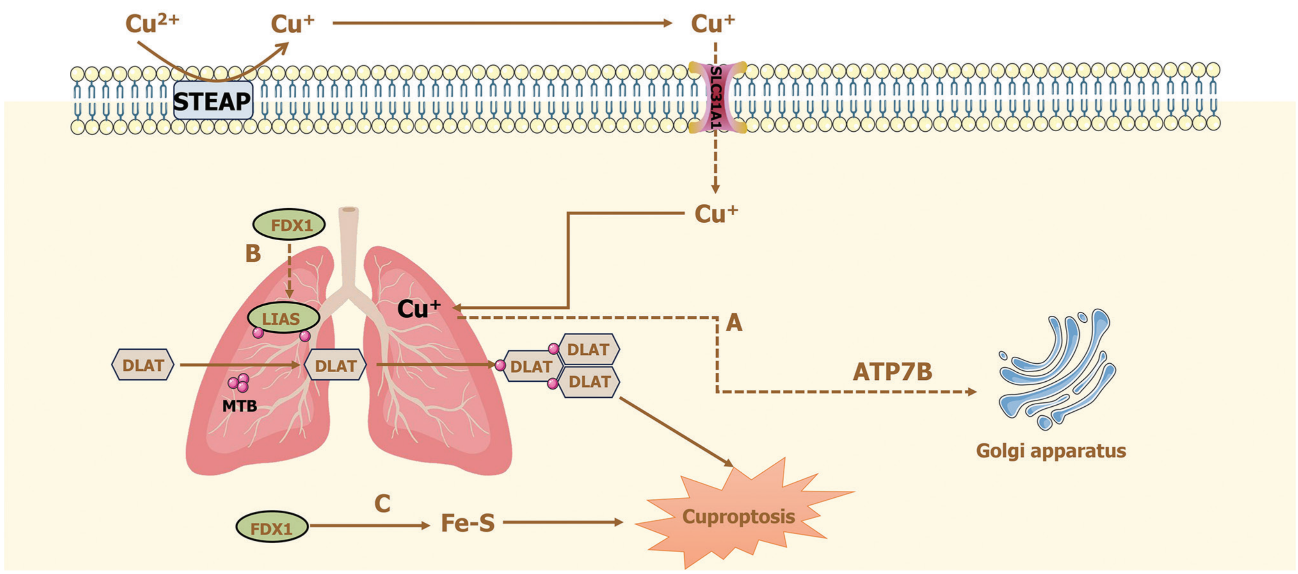

Figure 2. Schematic of copper death-immune network. (A) Copper entry and homeostasis: Cu^{2+} enters cells. ATP7B, under low intracellular copper, transports Cu^{2+} to the Golgi apparatus for ceruloplasmin maturation. (A) Disruption of copper balance, such as excessive intake or expulsion failure, triggers abnormal intracellular copper ion concentration changes, which act as a signal to initiate copper death. (B) Protein lipidation, and copper redox reaction: FDX1 and LIAS regulate protein lipidation. FDX1 reduces Cu^{2+} to Cu^{+}, which then affects the TCA cycle through DLAT. (C) TCA cycle impact and apoptosis: Cu^{+} interaction with TCA cycle components leads to protein aggregation and Fe-S cluster protein destabilization, causing proteotoxic stress and apoptosis. *Mycobacterium tuberculosis* or pathological conditions can alter copper dynamics through proteins such as ATP7B, influencing this network. Abbreviations: DLAT: Dihydrolipoamide S-acetyltransferase; FDX1: Ferredoxin 1; LIAS: Lipoic acid synthase; MTB: *Mycobacterium tuberculosis*; STEAP: Six-Transmembrane Epithelial Antigen of the Prostate family; TCA: Tricarboxylic acid.

immune responses, copper exhibits divergent biological activities depending on the context. While increased copper levels are typically beneficial in pathogen resistance during infections, excessive copper accumulation can be detrimental, promoting cell apoptosis and inflammation in cancer and neurodegenerative diseases.[66] For instance, the copper-dependent transcription factor Mac1 enables the endogenous fungal pathogen *Histoplasma capsulatum* to sense low copper environments within macrophage phagosomes. This adaptation allows the pathogen to modulate its copper acquisition mechanisms and other strategies to counteract the elevated antimicrobial defenses activated by post-immune response.

7. Copper's role in cell death, with analysis and prospects for diagnosis and drug targets

The conservation analysis of copper and the core gene network controlling cuproptosis, involving the conservation and regulatory networks of genes, in different organisms holds huge significance. Across various organisms, copper-related genes and their regulatory factors exhibit high sequence and functional similarities, indicating a crucial role of copper metabolism in the evolutionary process. Copper plays a vital role in cellular signaling, as it induces the expression of alpha-fetoprotein and interacts with C-C chemokine receptor type 5, leading to leukocyte death and subsequent immune suppression, which may explain the phenomenon of recurrent or chronic infections in some patients with mitochondrial disease.[67] These genes and signaling pathways are typically associated with processes such as cellular energy metabolism, redox status, and cell cycle regulation, highlighting the importance of their physiological functions.

In the diagnostic realm, abnormal copper metabolism and cuproptosis-related genes can serve as potential biomarkers for early diagnosis and disease monitoring. For instance, alterations in copper levels and related gene expression in serum or tissues may provide information on pathological conditions such as cancer, neurodegenerative diseases, and others. The potential value of 16 long non-coding RNAs (lncRNAs) associated with cuproptosis in predicting prognosis for lung adenocarcinoma suggests that high-risk patients not only have shorter survival but also face greater risks of immune evasion, indicating that these lncRNAs could become new targets for clinical application and immunotherapy.[68] Cuproptosis also plays a role in Crohn's disease and inflammatory bowel disease, where genes associated with cuproptosis impact immune cell infiltration and metabolic activities in the pathological processes. This suggests that cuproptosis may promote the progression of CD by inducing immune responses and metabolic dysfunction, offering new insights into the disease mechanisms and potential therapeutic targets.[69,70] The detection of these biomarkers altogether can aid in further understanding disease mechanisms and developing personalized treatment strategies.

Copper-related genes and key molecules in the cuproptosis pathway can serve as targets for novel drug development, where inhibitors or activators targeting specific genes or signaling pathways can modulate copper metabolism. For example, inhibiting certain copper transport proteins may help restrict tumor growth. Research has established a cuproptosis-related gene signature that can effectively predict the prognosis of hepatocellular carcinoma patients, revealing that upregulation of pyridoxal kinase promotes the proliferation and metastasis of liver cancer, whereas PDXK deficiency enhances the sensitivity of liver cancer cells to cuproptosis inducers, indicating that PDXK may be a potential diagnostic and therapeutic target for liver cancer.[71] Bioinformatics analysis has identified the significant role of copper metabolism-related genes (CMRGs) in osteosarcoma patient prognosis, immune microenvironment, and drug sensitivity, shedding light on the potential relationship between copper metabolism and osteosarcoma and suggesting that CMRGs could serve as novel prognostic markers and therapeutic targets.[72] Furthermore, therapeutic approaches based on copper-based drugs or copper supplementation have shown potential in anticancer strategies and immunomodulation. These studies present new opportunities for the clinical application of copper-related biology.

Utilizing bacterial cuproptosis mechanisms can lead to the development of novel antibacterial measures. A nano-drug, nitrite-oxidizing nanoparticles embedded in electrodeposited copper (NP@ESCu), combined with copper and elesclomol, has been designed to induce cuproptosis in cancer cells while boosting anti-tumor immune responses, offering a new strategy for future cancer therapies.[73] In bacteria, copper activates the response regulator CopR, which regulates the rearrangement of lipid proteins and the expression of copper resistance genes, thereby reducing the immunostimulatory properties of lipid proteins from high to low. This mechanism may be commonly present in other Firmicutes as well.[74] The following strategies are proposed to combat bacterial copper-related resistance mechanisms: (i) developing inhibitors that specifically target copper resistance genes to disrupt the copper tolerance mechanisms of bacteria. This approach aims to prevent bacteria from effectively handling high copper environments, thereby weakening their survival and growth capabilities; (ii) designing inhibitors against the copper sensor CopR. By blocking CopR, the bacteria's ability to sense changes in copper levels is impaired, which, in turn, disrupts the downstream regulatory pathways related to copper response; (iii) developing inhibitors directed at the copper transport protein CopT. These inhibitors are intended to hinder the process of bacterial copper transport, restricting the uptake or efflux of copper ions and thus disturbing the normal copper homeostasis within the bacteria; (iv) creating inhibitors that target the copper-binding protein CopZ. By disrupting the interaction between CopZ and copper, the bacteria's ability to bind and sequester copper is compromised, leading to abnormal copper metabolism; (v) developing inhibitors against the copper-resistance protein CopY. This can impede the bacteria's overall copper resistance function, making them more vulnerable to the toxic effects of copper in the environment. The importance of copper in human physiological and pathological processes—especially in maintaining immune function, lipid balance, and related diseases such as ischemic heart disease and non-alcoholic fatty liver disease[75]—necessitates further exploration of biomarkers for copper deficiency, dietary recommendations, and its potential impacts on health.

8. Summary and future direction

Copper homeostasis and cuproptosis are correlated with tuberculosis. The cellular metabolic process of cuproptosis mainly disrupts the tricarboxylic acid cycle and may play a regulatory role in the progression of tuberculosis. Cuproptosis is a newly discovered form of regulatory cell death that is closely related to other forms of regulated cell death, suggesting a potential relationship between cuproptosis and tuberculosis. Many researchers are now investigating this relationship with various major diseases. At present, despite existing clinical options, there are no effective treatments for tuberculosis. Treatment efficacy varies among patients due to different factors. Researchers are beginning to explore the link between copper dysregulation and tuberculosis. Studies of tuberculosis have analyzed the connections between specific genes and various aspects of the disease. Some researchers have identified a potentially important role for these genes in the association between cuproptosis and tuberculosis. However, due to insufficient biological evidence and experimental validation, these studies have only indirectly demonstrated a link between cuproptosis and tuberculosis. Whether cuproptosis directly contributes to the pathogenesis of tuberculosis or impacts its progression remains unclear.

The discovery of cuproptosis enhances our understanding of tuberculosis and its underlying molecular mechanisms. Cuproptosis may also hold potential value in drug screening for treating this disease. Future research could focus on developing strategies to lower intracellular copper levels or inhibit copper transport proteins, leveraging the chelating effects of copper chelators. This opens up new avenues for intervention in the treatment of tuberculosis. In addition, copper can be transported into cells using copper ion carriers to increase intracellular copper levels. However, thorough studies of cuproptosis and its associated genes necessitate well-designed pre-clinical experiments and clinical trials, which present significant implementation challenges. Nonetheless, this is a promising research area, and we look forward to future advancements.

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Conflict of interest

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Author contributions

Conceptualization: All authors
Visualization: Junqi Xu
Writing–original draft: All authors
Writing–review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Characteristics and outcomes of pediatric brucellosis cases collected from a tertiary academic hospital in Saudi Arabia

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Abstract

Brucellosis is a zoonotic disease caused by *Brucella* spp., affecting different body systems and leading to multiple complications. Although brucellosis is prevalent in several regions, including Saudi Arabia, limited research has focused on childhood brucellosis. This study aimed to characterize the features and outcomes of brucellosis in pediatric patients. We conducted a retrospective descriptive study involving children (<18 years) with confirmed brucellosis (diagnosed through culture, serology, or both) who received antibiotic therapy. Subjects were stratified into two groups based on age: younger (≤8 years) and older (>8 years). We assessed treatment outcomes, including clinical cure, mortality, and hospital length of stay. A total of 20 patients were included, with 52.3% in the younger group and 47.7% in the older group. The majority were male (65%), with a mean age of 8.9 years, and 45% required hospitalization. Out of all the patients, only 6 (30%) reported consuming dairy products. Serologically, the baseline median antibody titers for *Brucella melitensis* and *Brucella abortus* were 1:1280 and 1:640, respectively. In the younger group, half reported arthralgia and presented with fever. While white blood cell elevation was not significant, C-reactive protein, erythrocyte sedimentation rate, and liver enzymes were elevated at baseline. The administered regimen varied, but about half of the patients received at least three antibiotics. All patients experienced clinical cures, and there were no deaths. This study highlights the characteristics of pediatric brucellosis in a country where the disease is endemic and provides evidence of positive prognosis associated with appropriate antibiotic therapy.

Keywords: Brucellosis; *Brucella*; Pediatric; Childhood; Zoonotic infection; Zoonosis

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1. Introduction

Brucellosis, also known as Mediterranean fever or Malta fever, is a common zoonotic infectious disease caused by *Brucella* spp., a genus within the family *Brucellaceae*, which includes 10 other species. *Brucella* spp. are small, non-sporing, aerobic, non-motile Gram-negative intracellular coccobacilli.[1] The species most commonly implicated in human infections are *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, and *Brucella canis*.[2] The first two species are the most prevalent in Saudi Arabia. The disease mainly affects livestock animals; however, it can be transmitted to humans and causes serious complications if left untreated.

The principal signs and symptoms of brucellosis include fever accompanied by malaise, arthralgia, arthritis, weight loss, hepatosplenomegaly, and lymphadenopathy.[2,3] The two primary risk factors for brucellosis are the consumption of raw, unpasteurized, or non-sterilized dairy products and direct contact with infected animals.[2] In pediatric patients, the main source of infection is the consumption of unpasteurized milk.[3,4] The most frequent complications of brucellosis involve bone and joint tissues, occurring in up to 40% of cases.[2] Numerous uncommon complications of brucellosis have been described, such as cardiac complications (for example, endocarditis and myocarditis), testicular complications in male patients, and neurological complications such as meningitis and encephalitis. Brucellosis may also have ophthalmic complications. However, these are considered rare in pediatrics.[4]

The global annual incidence rate of human brucellosis is estimated at 2.1 million cases.[5] The disease remains a major threat to human health in many developing countries, particularly in North and East Africa, the Mediterranean basin, and the Indian subcontinent.[4] It is also endemic in the Middle East, the Arabian Peninsula, and parts of Central Asia and South America. On the contrary, human brucellosis has been eradicated from several developed countries, including many Northern European countries.[4] In Saudi Arabia, the number of reported cases between 2004 and 2012 was 37,477, with the incidence risk significantly decreasing from 22.9 in 2004 to 12.5 in 2012. The Saudi cities with the highest percentage of cases are Alqassim, followed by Aseer, Hail, and the northern region,[3,6] whereas the western region of Saudi Arabia has a lower incidence.[4] In 2014, the Saudi Pediatric Infectious Diseases Society published clinical practice guidelines for brucellosis in children.[7]

Given that brucellosis is one of the most frequently reported diseases in Saudi Arabia and the limited number of published reports on childhood brucellosis, this study aimed to describe the characteristics, course of therapy, and outcomes of pediatric brucellosis cases in Saudi Arabia.

2. Materials and methods

2.1. Study design and patients

The current retrospective descriptive study was conducted at King Abdulaziz University Hospital, Jeddah, in the western region of Saudi Arabia. Data were collected from pediatric patients over 11 years, from January 2008 to February 2019. Ethical approval was obtained from the Research Committee of the Unit of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (approval reference no. 128-18).

Patients aged 18 years or younger at the time of brucellosis diagnosis, confirmed through blood culture and/or serology, were eligible for inclusion in the study. A list of patients was obtained using test codes for the *Brucella* antibody test and *Brucella* culture. Both paper and electronic medical records were reviewed to identify pediatric inpatient and outpatient patients (age <18 at the time of diagnosis).

2.2. Laboratory diagnosis

Patients included in this study had a confirmed diagnosis of brucellosis, either through a positive blood culture or an antibody titer for *B. abortus*, *B. melitensis*, or both, and received antibiotic treatment. The serological test used in our hospital was the serum agglutination test (SAT), which measured total antibodies (Immunoglobulin [Ig]G and IgM) and provided a semi-quantitative result (titer). Two types of antigens were used in the test – one for *B. abortus* and the other for *B. melitensis*. After mixing the antigen suspension with the patient's serum, the tubes were incubated at 37°C for up to 48 h. The test was validated using positive and negative controls supplied with the kit (different commercial tests were used). The SAT has a reported sensitivity of 95.6 – 100% and a specificity of 96 – 100%.[8,9] In Saudi Arabia, where brucellosis is endemic, an antibody titer of at least 1:640 for either *Brucella* spp. is considered positive for confirming the diagnosis.

Microbiologically, blood samples from patients suspected of having brucellosis were labeled as "Brucella" and incubated at 37°C for up to 14 days in the BD BACTEC™ FX system (Beckton, Dickinson and Company, United States [US]). Positive samples were subcultured onto blood and chocolate agar plates, which were incubated for 24 – 48 h in a 5 – 10% CO_2 environment. If growth was observed on the plate, the bacteria were identified through the morphology of the colonies, Gram staining, and biochemical testing for urease and oxidase (as *Brucella* spp. produces both enzymes). While microbiological culture facilitated the identification of the genus *Brucella*, it does not provide species-level identification. The results from serological testing were used to identify the specific species.

2.3. Data collection

Collected data included demographics, clinical variables (including complications and organ or system involvement), date of admission (for inpatients), duration of hospital stay, antibiotic therapy administered, and the date therapy ended. Clinical cure was defined as the complete resolution of brucellosis signs and symptoms, as documented in progress notes or outpatient visit records. Data were collected up to 3 months post-therapy completion, except for one patient whose data were only available after 8 months post-therapy. Patients were divided into two groups: younger patients (aged 8 years or younger) and older patients (aged older than 8 years). This stratification was based on the contradiction of doxycycline (an essential antibiotic in brucellosis treatment) in patients younger than 8 years and the more frequent occurrence of brucellosis-associated arthritis in the older age group.[10-13]

2.4. Statistical analysis

The collected data and laboratory results were presented descriptively using numbers, percentages, and mean ± standard deviation. Statistical package (Statistical Package for Social Sciences version 24.0) (IBM Corp., US) was used.

3. Results

3.1. Characteristics of the patients

A total of 138 patients were screened for eligibility, of whom 20 met the inclusion criteria and were included in the study. Reasons for exclusions included duplicate records, age older than 18 years, no positive serology, positive blood cultures labeled with "*Brucella*" but did not yield *Brucella* spp., and no documented antibiotic treatment. Table 1 lists the demographics of included patients. The mean age was 8.9 years. About two-thirds were males (65%), and more than half were treated as outpatients (55%). The consumption of a dairy product was reported by 6 patients (30%), whereas the source of infection was unknown in the rest of the cohort. Upon presentation, about half (50%) of the patients complained of arthralgia and were febrile. Six of the 20 patients (30%) had elevated liver enzymes at baseline (three times the upper limit of normal; range 66 – 252 U/L for alanine transaminase [ALT] and 91 – 155 U/L for aspartate transaminase [AST]) that were decreased by the end of therapy by about 55% (range 33 – 64 U/L and 22 – 64 U/L for ALT and AST, respectively). Hepatosplenomegaly was noted in one patient, and splenomegaly was observed in another. Furthermore, two patients had complicated brucellosis (one had neurobrucellosis whereas the other had epididymo-orchitis), whereas the remaining 18 patients had uncomplicated brucellosis. Serological results revealed that 18 patients (90%) had antibodies against both *B. melitensis* and *B. abortus*, whereas the remaining two patients had antibodies against either species. Moreover, four patients had concurrent infections with other pathogens, including two cases of cytomegalovirus infection, one case of rheumatic fever, and one case of urinary tract infection.

3.2. Antibiotic regimens used

Antibiotic regimens varied significantly between the patients; however, most patients (53%) received a combination consisting of at least three antibiotics (Table 2). Most of the regimens in the younger group included rifampin and/or gentamicin with or without trimethoprim/sulfamethoxazole. On the other hand, the older group regimens consisted of rifampin and doxycycline alone or in combination with other agents.

3.3. End of therapy outcomes

Clinical outcomes data were available for 18 patients. All patients were clinically cured with no reported mortality (Table 3). In addition, temperature, white blood cell count, inflammatory markers, and liver enzymes were all normalized. Moreover, the average length of stay of hospitalized patients (n = 9) was 26 days, which was shorter in the older group (14.8 days) than in the younger group (34.4 days). With regards to antibody titers, they remained elevated in some patients but normalized in some, where the median (Interquartile range) was 1:320 (1:80 to 1:1280).

4. Discussion

Although brucellosis is a neglected disease worldwide, it poses a serious threat to public health in developing nations. Likewise, in Saudi Arabia, it continues to be endemic despite efforts to contain its spread. Any child experiencing a fever and having a history of consuming unpasteurized milk or animal contact should be evaluated for brucellosis.[7] The primary objective of our study was to describe the characteristics and the clinical outcomes of childhood brucellosis in patients identified at our institution over 11 years.

In many countries, *Brucella* infection is an occupational disease, which makes it uncommon among children. However, this may not be the case in endemic regions where transmission occurs through non-occupational means. In our study, male patients outnumbered female patients (67% vs. 33%). One possible explanation could be that young male adults are more involved in outdoor activities and animal care. However, these findings disagreed with a national survey that linked female participation in animal milking to a greater infection risk among females.[14] About a third of the patients had a history of consuming dairy products. This observation was consistent with the findings of a previous study conducted in Al-Khafji Joint Operation Hospital between 2011 and 2012, which reported raw animal milk ingestion as the major risk factor for brucellosis reported by up to 83% of pediatric patients.[15] Consuming unpasteurized (or non-boiled) dairy products

Table 1. Patients' demographics

Characteristic	Total (*n*=20)	Younger group (*n*=9)	Older group (*n*=11)
Age (years)	8.9±5.1 (1 – 17)	4.5±1.9 (1 – 7)	10.1±2.9 (8 – 17)
Sex (male)	13 (65)	7 (77.8)	6 (54.5)
Location			
Outpatient	11 (55)	4 (44.4)	7 (63.6)
Inpatient medical ward	9 (45)	5 (55.6)	4 (36.4)
Temperature (°C)	37.9±1.1 (36 – 39.5)	37.9±1.2 (36 – 39)	37.9±0.9 (36.8 – 39.4)
White blood cell count, cells/mm^3	6.7±2.1 (3.3 – 10.6)	6±1.7 (3.3 – 8.9)	7.7±2.7 (5.2 – 10.6)
C-reactive protein (mg/L)	29±38.9 (3 – 169)	11.3±11.7 (3 – 39.7)	42.5±58.1 (3 – 169)
Erythrocyte sedimentation rate (mm/h)	33.8±19.5 (7 – 70)	38.8±23.4 (9 – 70)	28.6±15.5 (7 – 44)
ALT (U/L)	64.4±54.6 (12 – 252)	77.4±73 (19 – 252)	46.4±39.7 (12 – 116)
AST (U/L)	65.8±43.2 (8 – 155)	64.3±28.6 (27 – 102)	58.3±53.8 (8 – 152)
Risk factors for infection			
Consumption of unpasteurized dairy products	6 (30)	3 (33.3)	3 (27.3)
Unknown	14 (70)	6 (66.7)	8 (72.7)
Diagnostic test positivity			
Brucella serology alone	10 (50)	4 (44.4)	6 (54.5)
Both *Brucella* serology and culture	10 (50)	5 (55.6)	5 (45.5)
Brucella spp.			
B. melitensis	1 (5)	1 (11.1)	0 (0)
B. abortus	1 (5)	0 (0)	1 (9.1)
Both	18 (90)	8 (88.9)	10 (90.9)
Presence of co-infection	5 (25)	1 (11.1)	4 (36.4)
Presence of arthralgia	10 (50)	4 (44.4)	6 (54.5)
B. melitensis antibody titer	>1:1280 (1:640 – >1:1280)	>1:1280 (1:480 – >1:1280)	>1:1280 (1:640 – >1:1280)
B. abortus antibody titer	1:640 (1:320 – >1:1280)	1:640 (1:320 – >1:1280)	> 1:1280 (1:640 – >1:1280)

Notes: Data are presented as mean±*SD* (range) or *n* (%), except for the antibody titers, which are presented as median (interquartile range).
Abbreviations: ALT: Alanine transaminase; AST: Aspartate transaminase; *B. abortus: Brucella abortus, B. melitensis: Brucella melitensis.*

is considered the major mode of zoonotic transmission of brucellosis to humans.[2,5]

Brucellosis is a multisystem disease with a broad spectrum of non-specific signs and symptoms. This study found that fever and arthritis or arthralgia were the predominant presenting signs of the disease. This finding was in line with previous studies from endemic areas, such as Iran.[16] Moreover, brucellosis-associated arthritis was previously reported in children older than 8 years.[11-13,17] Although 48% of cases were detected only by the SAT serological tests, doctors are advised to have a high index of suspicion for *Brucella* culture since the symptoms in children might be mistaken for septic arthritis. In areas where brucellosis is widespread, such as Saudi Arabia, persistent exposure to the source of infection increases the titer value in which brucellosis is said to be established as a disease. In such areas, a titer of 1:640 or higher is a good indicator of the presence of the disease, along with signs and symptoms compatible with *Brucella* arthritis in a community where *Brucella* is common.

Brucellosis could also affect any organ or system. Apart from two patients who had organ involvement – one had neurobrucellosis, and the other had testicular involvement – the current study revealed that the majority of patients had uncomplicated brucellosis. Notably, neurobrucellosis is not common among children, with an epidemiological study reporting an incidence of only 1% among children with brucellosis.[11] Although *Brucella* spp. can cause endocarditis and urinary tract infections,[2,18,19] the reported cases of rheumatic fever and urinary tract infections were caused by pathogens other than *Brucella* spp.

Our study reported that 52% of the patients were diagnosed using the SAT along with blood *Brucella* culture. A recent study reported a lack of correlation between

Brucella serology and culture at baseline; therefore, clinicians must base their diagnosis on the clinical picture of the disease and use the culture and/or the serology as a guide to confirm the diagnosis.[20] In our study, leukopenia and thrombocytopenia were the hematological abnormalities that our patient cohort experienced during the active course of brucellosis. Moreover, only 10 of the 20 patients had elevated liver transaminases. These findings are consistent with earlier studies.[21] It should be noted that SAT has a reported high sensitivity rate of 93% – 100%, indicating a low likelihood of reporting false-negative results.[22] However, false-positive results can occur as a result of cross-reactions with antibodies to other Gram-negative bacteria, such as *Salmonella* spp. and *Yersinia* spp.[22] In our hospital, the speciation of *Brucella* is done using serological tests, where all the patients in our current cohort had SAT done for them. Interestingly, however, 18 (90%) of the patients tested positive for both *B. melitensis* and *B. abortus* antibodies compared with two patients who tested positive for either of the two species. Some potential explanations for this observation include co-infection by both species, previous exposure to one of the two species and then developing a new infection by the other since *Brucella* antibodies could persist for years,[23] or cross-reactivity due to overlapping epitopes of *Brucella* antigens leading to a false-positive result for multiple species.[24] The first two explanations could be attributed to the endemicity of the disease in Saudi Arabia.

Table 2. Antibiotic regimens used (in descending order of the number of patients)

Regimen	Age group	*n*
Aminoglycoside+trimethoprim/sulfamethoxazole+rifampin	Younger group	3
Aminoglycoside+trimethoprim/sulfamethoxazole	Younger group	3
Doxycycline+trimethoprim/sulfamethoxazole+rifampin+ceftriaxone	Older group	2
Doxycycline+rifampin+ciprofloxacin	Older group	2
Doxycycline+rifampin	Older group	2
Aminoglycoside+doxycycline+rifampin+ceftriaxone	Older group	1
Aminoglycoside+trimethoprim/sulfamethoxazole+ceftriaxone	Younger group	1
Trimethoprim/sulfamethoxazole+rifampin	Younger group	1
Trimethoprim/sulfamethoxazole+rifampin	Older group	1
Doxycycline	Younger group	1

Notes: The younger group includes those who were ≤8 years old, whereas the older group includes those who were >8 years old. Three patients received treatment as indicated in their medical records, but antibiotics were not identified.

Brucellosis should be treated using a combination therapy; otherwise, patients would be at high risk for relapse or treatment failure.[7,25] In this study, the relapse rate was very low at 1%, and no fatality has been reported. In addition, the patients were successfully treated by the various regimens used. The most commonly prescribed regimens were those comprised of an aminoglycoside, trimethoprim/sulfamethoxazole, and rifampin, as well as a regimen comprised an aminoglycoside with trimethoprim/sulfamethoxazole. The former regimen has been recommended in the Saudi guidelines for patients <8 years.[7] Of note, ciprofloxacin and doxycycline were only

Table 3. End-of-therapy outcome variables

Outcome	Total (*n*=20)	Younger group (*n*=9)	Older group (*n*=11)
Temperature (°C)	36.8±0.2 (36.5 – 37)	36.7±0.3 (36.5 – 37)	36.8±0.2 (36.5 – 37)
White blood cells count (cells/mm^3)	7±2 (3.1 – 9.9)	6.9±2.3 (3.1 – 9.6)	7.1±1.7 (4.9 – 9.9)
C-reactive protein (mg/L)	4.9±2.9 (3 – 13.1)	5±1.8 (3 – 7.8)	4.8±3.5 (3 – 13.1)
Erythrocyte sedimentation rate (mm/h)	14.8±13.3 (3 – 42)	16.7±17.4 (3 – 42)	12.2±4.4 (8 – 17)
ALT (U/L[a])	28.4±11 (12 – 46)	32.1±10.6 (17 – 46)	23.5±10.3 (12 – 39)
AST (U/L[a])	29.7±16.7 (9 – 64)	36.6±17.9 (17 – 64)	20.5±10 (9 – 35)
Brucella melitensis antibody titer (median [IQR])[b]	1:320 [1:80 – >1:1280]	1:240 [1:80 – >1:1280]	1:320 [1:80 – >1:1280]
Brucella abortus antibody titer (median [IQR])[b]	1:320 [1:80 – >1:1280]	1:200 [1:80 – >1:1280]	1:640 [1:80 – >1:1280]
Days of therapy[c]	45.7±32 (7 – 120)	40±22.3 (7 – 72)	51.4±40 (7 – 120)
Length of stay[d]	25.7±19.6 (7 – 67)	34.4±22 (9 – 67)	14.8±9.8 (7 – 29)

Notes: Data are presented as (mean±SD, range) unless otherwise specified. Data are presented as mean±SD (range) or *n* (%), except for the antibody titers, which are presented as median (interquartile range). [a]Data were available from 14 patients (eight in the younger group and six in the older group). [b]Data were available from 15 patients (eight in the younger group and seven in the older group). [c]Data were available from 18 patients (nine in the younger group and nine in the older group). [d]Data were available for the nine admitted patients (five in the younger group and four in the older group).

Abbreviations: ALT: Alanine transaminase; AST: Aspartate transaminase; IQR: Interquartile range; SD: Standard deviation.

prescribed to patients older than 9 years (except for one 4-year-old who was given doxycycline). Recent evidence suggests that doxycycline can be safely given to children and pregnant women.[26] A thorough retrospective analysis of 1843 newborns with prenatal doxycycline exposure revealed no greater risk of birth defects or teratogenic risks when compared to those who were not exposed.[27] These findings suggest that the advantages of doxycycline use in young patients may outweigh the possible risks when treating specific diseases without effective alternatives or when doxycycline is considered the treatment of choice, as in the case of brucellosis.[28]

Ciprofloxacin may be beneficial for brucellosis in cases of drug resistance when used in combination with rifampin.[29,30] The patient who had neurobrucellosis was treated with a combination of doxycycline, trimethoprim/sulfamethoxazole, and ceftriaxone. Per a previous report, doxycycline and trimethoprim/sulfamethoxazole have been found to be effective in neurobrucellosis.[31] Furthermore, third-generation cephalosporins with the ability to diffuse through the central nervous system, such as ceftriaxone, have shown good *in vitro* activity against isolates of *B. melitensis*.[32] The Saudi guidelines recommend a regimen of doxycycline, trimethoprim/sulfamethoxazole, and rifampin to treat neurobrucellosis. They also discussed the advantages of using ceftriaxone in the initial therapy of neurobrucellosis in children older than 8 years.[7]

In our study, even though a clinical cure was achieved, the patient's antibody titers persisted after treatment was completed. The results of an earlier retrospective investigation that found that *Brucella* serology did not correlate with clinical outcomes at the end-of-treatment follow-up provide an explanation for this. Consequently, rather than depending exclusively on serological results during follow-up, clinicians should take into account the full clinical picture of the brucellosis patient and evaluate the attainment of a clinical cure based on the disappearance of baseline signs and symptoms.[20]

This study has a few limitations. As a retrospective study, some data were unavailable. It was also conducted in a single center and included a small sample size; thus, the results might not be generalizable.

5. Conclusion

Our study described the characteristics of children diagnosed with brucellosis in an academic hospital, highlighting typical signs and symptoms of fever, arthralgia, and elevated liver enzymes. We emphasize that using a combination of at least two antibiotics effective against *Brucella* spp. is important for ensuring clinical success.

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Conflict of interest

Abrar K. Thabit is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

Ethical approval was obtained from the Research Committee of the Unit of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (reference no. 128-18), who waived the need for informed consent.

Consent for publication

As this was a retrospective study (old data of patients who have been discharged from the hospital), such consent was not obtained and could not be obtained given the nature of the study.

Availability of data

Data are available from the corresponding author upon request.

Further disclosure

The study abstract was published in the abstract book of the European Congress of Clinical Microbiology and Infectious Diseases 2020. Available at: https://www.researchgate.net/profile/Daire-Cantillon/publication/341164914_ECCMID_2020_abstract_book/links/5eb1c41445851592d6bd3ff0/ECCMID-2020-abstract-book.pdf.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

In vitro evaluation of *Hyphaene thebaica* honey as a multitarget therapeutic product

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Abstract

Hyphaene thebaica honey, commonly known as doum honey (DH), is widely utilized in the Mediterranean region due to its putative health benefits. However, the precise mechanisms underpinning these benefits remain obscure. This study sought to assess the anti-infective, anti-inflammatory, and anticancer properties of DH, and analyze its polyphenolic composition. The antibacterial effects of DH were tested against a range of multidrug-resistant Gram-positive and Gram-negative bacterial strains. In addition, we investigated the anti-inflammatory, antioxidant, and anticancer activities of DH in the MDA-MB-231 human breast cancer cell line. The phenolic compounds in DH were evaluated using quantitative high-performance liquid chromatography (HPLC). The model used to assess the anti-inflammatory properties was lipopolysaccharide (LPS)-activated macrophages. HPLC analysis revealed nine phenolic compounds in DH: Gallic acid, caffeic acid, carvacrol, p-coumaric acid, ellagic acid, kaempferol, pinobanksin, pinocembrin, and galangin. The minimum inhibitory concentration (MIC) values for DH varied between 0.19% and 0.78% w/w for the three Gram-positive strains tested and between 0.024% and 0.39% w/w for the four Gram-negative strains tested. Among all the bacterial strains tested, *Escherichia coli* was found to be the most susceptible, with an MIC of 0.024% w/w. Upon treating LPS-activated THP-1-derived macrophages with DH, the levels of nitric oxide were significantly diminished. Moreover, DH displayed a modest but significant cytostatic effect on the MDA-MB-231 cells. The most noticeable cytostatic impacts were observed at concentrations of 4 mg/mL and 2 mg/mL, resulting in a decrease in cell viability by 25% and 20%, respectively, compared to untreated control cells. A significant decline in the migration rate of MDA-MB-231 cells was observed following DH treatment compared to control cells ($P < 0.05$). Our findings not only corroborate the well-established antibacterial properties of DH but also imply that its recognized anticancer advantages may be partially attributed to its antioxidant, anti-inflammatory, cytostatic, and antimigration effects.

Keywords: *Hyphaene thebaica*; Antibacterial; Antioxidant; Anticancer; Cytostatic; Anti-inflammatory; Antimigration

1. Introduction

Extensive research efforts have concentrated on exploring the therapeutic properties of a diverse array of natural compounds, which have been employed as natural remedies since ancient times and are generally regarded as having low toxicity. Their potential therapeutic benefits have attracted significant interest across various fields, including anti-inflammation, antimicrobial, and anticancer research.[1] A notable type of natural product is honey, a natural substance derived from nectar by honeybees. Honey has played a significant role in traditional medicine for centuries. Drawing from cultural beliefs, theoretical principles, and historical records, subjects from various civilizations, including the Greek, Roman, and Arab-Islamic societies, incorporate honey into their medical practices. In Arab-Islamic medicine, the medical value of honey is highly regarded owing to its therapeutic properties, and thus it is frequently used in wound care.[1-4] Different types of honey, many of which have been scientifically studied, are widely employed as natural remedies for maintaining health and addressing various illnesses.[1,2] Honey as well as its various constituents have been demonstrated to possess antibacterial, anti-inflammatory, antioxidant, antiproliferative, antitumor, antimetastatic, and anticancer properties.[3-5]

Among the traditional therapeutic uses of honey, its antimicrobial effects stand out as a prominent attribute. Antimicrobial resistance is globally acknowledged as a significant challenge to global health. This phenomenon is primarily associated with high morbidity, severe complications, and mortality rates attributed to multidrug-resistant bacteria, a rising public health challenge stemming from the scarcity or complete absence of effective drugs.[6] Thus, immediate measures are imperative to address the looming crisis of antibiotic resistance on both national and global scales. These measures could encompass enhancing public awareness, developing novel antibiotics, implementing antibiotic stewardship to control their usage, and exploring alternative approaches to antibiotics.[7] Alternative strategies to antibiotics may include the use of bacteriophages, antimicrobial peptides, and natural derivatives such as plant extracts, phytochemicals, and honey.[8-13] Antimicrobial efficacy of various forms of natural honey against an extensive array of bacteria has been documented,[10] corroborating their potential in treatment for infections instigated by multidrug-resistant bacteria. While the precise mechanism of natural honey's antimicrobial action remains unclear, it is generally accepted that its antimicrobial activity can be attributed to multiple underlying mechanisms.[10] Honey is believed to eliminate bacteria through one or more of the following mechanisms: Disrupting or damaging bacterial cell membranes, inhibiting bacterial virulence factors, and preventing bacterial adhesion to target cells.[14,15] Contrary to other antimicrobial agents, honey has yet to be thoroughly investigated concerning its microbial resistance.[16]

Besides its antimicrobial properties, honey has been explored for its potential anticancer effects. Cancer, characterized by the uncontrolled proliferation of cells exhibiting potential malignancy, ranks among the primary causes of mortality globally.[17] Breast cancer holds the top position among all cancer types affecting women globally. Its etiology is complex, and influenced by various factors. The occurrence of events, such as pregnancy, breastfeeding, first menstruation, and menopause, along with their duration and the accompanying hormonal imbalances, play a crucial role in potentially inducing carcinogenic changes in the breast microenvironment. In Palestine, the incidence of breast cancer has surged in recent times, attributed partially to heightened awareness and screening efforts, as well as socioeconomic factors affecting lifestyle and dietary patterns. It stands as the most prevalent cancer among Palestinian women, constituting 32% of diagnoses in the West Bank and 18% in the Gaza Strip.[18] Existing cancer management strategies, such as surgery, chemotherapy, and radiotherapy, have significant limitations. In addition, cytotoxic drugs, though essential to treatment, present considerable challenges. Their accessibility and affordability are particularly problematic in developing regions. In addition, the utilization of these drugs often leads to a range of undesirable side effects and adverse reactions.[19] Consequently, a significant segment of the population prefers utilizing natural products. Despite its inherent limitations, natural products present several advantages over synthetic or standard drugs, including affordability, accessibility, and reduced side effects.[3,2,19-21] The escalating public interest in natural products can be attributed to the growing body of research underscoring its health benefits.

In addition, various types of honey have been shown to possess anti-inflammatory and antioxidant properties, which are vital for addressing chronic inflammation and mitigating the accumulation of reactive oxygen species (ROS) generated during inflammatory responses. Inflammation, a natural physiological reaction to harmful stimuli, such as allergens, infections, or injuries, can become a chronic condition due to prolonged exposure to certain factors. Chronic inflammation is linked to a wide range of health conditions, including allergies, metabolic disorders, cardiovascular diseases, cancer, and autoimmune disorders, making it a significant concern for global health.[22] The primary treatments for inflammation encompass anti-inflammatory medications and immunosuppressants, which unfortunately can induce side effects such as gastrointestinal ulcers, cardiovascular toxicity, hormonal imbalances, and disruptions to the body's normal functions.[23,24] Therefore, it is of paramount importance to explore the incorporation of natural anti-inflammatory substances into treatment regimens to enhance pharmacological response and mitigate adverse effects. Recent studies have demonstrated promising results in utilizing natural products such as various types of honey for the treatment of ulcers.[25]

The antioxidant properties of honey play a critical role in lowering the levels of ROS generated during inflammatory processes. Elevated ROS levels are associated with the development of serious health conditions, including cardiovascular, muscular, metabolic, neurodegenerative, and cancer-related diseases. The antioxidant capacity of honey primarily stems from its phenolic compounds, especially flavonoids, which effectively neutralize ROS and interact with metals. Furthermore, flavonoids contribute to regulating enzymes, thereby boosting their antioxidant activity and influencing various biological functions.[26-28]

Hyphaene thebaica and Doum honey (DH) are extensively utilized throughout the Mediterranean region due to its health-enhancing properties, such as anti-infection, anti-inflammatory, and anticancer attributes.[29,30] Scientific evidence on the health advantages of DH remains largely unexplored, given that studies on this specific honey variety are not as comprehensive as those on the more frequently researched types such as Manuka honey. *H. thebaica* L., commonly known as the Doum plant, is a desert tree native to regions such as the Mediterranean region, Sub-Saharan Africa, and West India.[29,30] The precise cellular and molecular mechanisms underlying the traditionally asserted antibacterial, antioxidant, anti-inflammatory, and anticancer properties of the Palestinian DH remain largely unexplored. This study is designed to assess these effects and analyze the polyphenolic composition of DH.

2. Materials and methods

2.1. DH sample collection and preparation

The DH samples, which were collected from the Jordan Valleys in 2021, were obtained from a reputable supplier known as "Honey Spring," situated in Tulkarem in the Northern West Bank. Following their purchase, these samples were sealed in cans for preservation and stored under ambient conditions in a moisture-free environment. They remained in this unopened state until further experimental procedures.

2.2. Assessment of antibacterial activities

The microbial strains used in the current investigation were *Pseudomonas aeruginosa* strain 27853, *Staphylococcus aureus* strain BAA-1026, *Escherichia coli* strain 25922, S*treptococcus* strain 49619, *Klebsiella quasipneumoniae* strain 700603, *Haemophilus influenzae* strain 49247, and *Bacillus subtilis* strain 6633, which were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DH samples in 96-well, flat-bottomed micro-titration plates, were determined as described previously.[31]

2.3. Cell culture

The MDA-MB-231 cell line (European Collection of Authenticated Cell Cultures [ECACC] catalog no. 92020424) is supplied by the ECACC, which is part of the UK Health Security Agency and the THP-1 human monocytic cell line (ATCC: TIB-202) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, Israel) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 μg/mL streptomycin to maintain optimal growth conditions.

2.4. Evaluation of cytotoxic and cytostatic effects

In the cytotoxicity experiments, 20,000 MDA cells per 100 μL of media were seeded in 96-well plates and incubated for 24 h. Subsequently, they were exposed to 0 – 4000 μg/mL of DH for 24 h. The cytotoxic impacts were then evaluated using an MTT assay.

In the cytostatic experiments, 5,000 MDA cells per 100 μL of media were seeded in 96-well plates and incubated for 24 h. These cells were then exposed to a range of honey concentrations, from 0 to 4000 μg/mL, over a period of 72 h. The MTT assay was employed to assess the cytostatic effects, following the methodology outlined by Abu-Farich *et al.*[32]

2.5. Scratch assay

MDA cells were seeded at a density of 400,000 cells per well in a 12-well plate (Corning Costar Corporation, Corning, NY, USA) with 2.5 mL of culture medium and incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. After incubation, monolayer formation in each well was confirmed under a microscope. Scratches were then created across the monolayers using sterile 200 μL pipette tips. The culture medium was removed, and each well was washed 4 times with DMEM devoid of serum and additives. Following this, 4 mL of either 4 mg/mL DH solution or culture medium (for untreated control) was added to the wells in triplicate. Images were taken immediately after the scratches were made and subsequently every 24 h for a total of 48 h.

2.6. Determination of nitric oxide (NO) production

The efficacy of DH samples in inhibiting the formation of NO radicals in lipopolysaccharide (LPS)-stimulated macrophages derived from THP-1 cell line was ascertained utilizing the Griess reagent, in accordance with a previously outlined methodology.[33]

2.7. Quantitative high-performance liquid chromatography (HPLC) determination of phenolic compounds in DH

The presence of polyphenols in DH was analyzed by means of quantitative HPLC, as previously described.[31]

2.8. Determination of total antioxidant capacity

The total antioxidant activity (TAC) was quantified by utilizing the phosphomolybdenum technique, as delineated by Prieto *et al.*[34] The experimental procedure was executed in triplicate, with the resultant values expressed as ascorbic acid equivalents, measured in milligrams per gram of dry weight.

2.9. Determination of total polyphenols content (TPC) and total flavonoids content

TPC and total flavonoid content (TFC) were determined as previously described.[32]

2.10. Evaluation of free radical scavenging activity

The free radical scavenging activity was determined using the microdilution 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with slight modifications, as previously described.[35,36]

2.11. Statistical analysis

The error limits mentioned and depicted error bars indicate basic standard deviations of the average. Typically, numerical outcomes are provided with precision only up to the least significant digit. In comparing various samples, results were deemed statistically significant when $P < 0.05$ (using Student's t-test for unpaired samples).

3. Results and discussion

3.1. Antibacterial properties

Due to the intensifying global challenge of antimicrobial resistance, there exists an exigency to explore alternative methods and substances to supplant antibiotics. This immediate need is especially critical considering the scarce availability of effective antibiotics to tackle multidrug-resistant bacteria,[37] leading to a notable increase in the demand for diverse natural products that demonstrate antibacterial properties and function through mechanisms that are different from traditional drugs. Honey is one such product that has attracted considerable interest.[38,39] Honey has long been valued in traditional medicine for its wound-healing abilities and as a complementary therapy for cancer and other health conditions. Across the globe, many cultures have relied on honey for its medicinal and nutritional properties. It has been used to address a wide range of ailments, such as eye disorders, bronchial asthma, tuberculosis, hepatitis, throat infections, hemorrhoids, eczema, wounds, and ulcers, while also serving as a beneficial dietary supplement.[40] Honey is recognized for its diverse beneficial properties, including antioxidant, antimicrobial, anti-inflammatory, antiproliferative, anticancer, and antimetastatic effects. Research has highlighted its potential in managing various conditions, such as wounds, diabetes, cancer, and disorders affecting the cardiovascular, neurological, and gastrointestinal systems.[5-8] Its antibacterial properties, in particular, have been extensively studied, with findings confirming that natural honey exhibits broad-spectrum antibacterial activity, even against drug-resistant pathogens.[41,42] Hence, honey is recognized as a potential bioactive natural substance demonstrating promising efficacy against pathogenic bacteria in the treatment of various bacterial infections. Given that diverse types of honey exhibit a broad spectrum of antibacterial activity, this activity seems to be unrelated to antibiotic susceptibility or resistance, and it appears improbable that pathogenic bacteria would develop resistance to honey.[43,44]

The antibacterial efficacy of honey is attributed to a multitude of factors. Its substantial viscosity, which is primarily a result of its high sugar concentration and minimal water content, establishes a protective shield that aids in thwarting infections. In addition, its mild acidity coupled with the presence of hydrogen peroxide bolsters its antimicrobial prowess.[45,46] Numerous scientific studies have explored the effects of honey on various bacterial species, revealing that its antibacterial effectiveness can vary widely. Different microorganisms show varying levels of sensitivity to specific types and concentrations of honey. Comprehensive reviews have examined honey's antibacterial properties, tracking the growth patterns of different bacteria under exposure to varying concentrations of honey.[47,48]

This study assessed the antibacterial effects of DH employing the MIC and MBC techniques. Tetracycline and kanamycin against antimicrobial resistance in both Gram-positive and Gram-negative bacteria were taken as positive controls (Table 1).

Table 1. MIC and MBC values of tetracycline and kanamycin

Pathogenic microorganisms	MIC (μg/mL)		MBC (μg/mL)		MBC/MIC ratio	
	Tetracycline	Kanamycin	Tetracycline	Kanamycin	Tetracycline	Kanamycin
Pseudomonas aeruginosa	0.07	0.85	0.07	1.7	1	2
Staphylococcus aureus	0.02	0.11	0.04	0.11	2	1
Escherichia coli	0.03	0.47	0.06	0.94	2	2
Bacillus subtilis	0.78	0.39	1.56	0.78	2	2
Haemophilus influenzae	0.024	0.048	0.024	0.048	1	1
Streptococcus pneumoniae	1.56	0.78	3.12	1.56	2	2
Klebsiella pneumoniae	0.19	0.048	0.19	0.096	1	2

Abbreviations: MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration.

The assessment of MIC and MBC is universally acknowledged as an efficient and relatively cost-effective method for evaluating the efficacy of various antimicrobial substances. DH demonstrated a favorable impact in eradicating all examined bacterial strains. The MIC and MBC values of DH against the tested bacterial strains are stated in Table 2.

The MIC values for the DH fluctuated between 0.19% and 0.78% w/w for the three Gram-positive strains and between 0.024% and 0.39% w/w for the four Gram-negative strains. Among all the bacterial strains examined, *E. coli* was identified as the most susceptible with an MIC of 0.024% w/w, whereas *S. pneumoniae* was the most resistant with an MIC of 0.78% w/w (Table 2).

The ratio of MBC to MIC can offer valuable insights into the nature of an antimicrobial agent, specifically whether it is bacteriostatic (halts bacterial growth) or bactericidal (kills bacteria). If the MBC is not significantly greater than the MIC (typically MBC/MIC ≤ 4), the antimicrobial agent is deemed bactericidal, as it kills bacteria at concentrations near the inhibitory concentration. Conversely, if the MBC is significantly higher than the MIC, the agent is classified as bacteriostatic, as it primarily inhibits bacterial growth rather than killing the bacteria. The DH's ratio of MBC to MIC against all examined bacteria consistently exhibited an approximate increase of up to two-fold over their MIC values. This implies that DH honey exerts a bactericidal effect on all bacteria tested (Table 2).

The MIC values for *E. coli* recorded in this study were lower than the previously documented 0.52 – 1.0% range.[31,47-49] The antimicrobial effects of honey primarily stem from hydrogen peroxide, with additional contributions from non-peroxide components such as phenolic acids and flavonoids, which enhance its antibacterial and antioxidant properties.[50,51] Research indicates that these antibacterial effects can vary depending on the phytogeographical origin of the honey, influencing the production of various compounds.[52,53] Recent studies have also identified other antimicrobial components, such as the bee-derived peptide defensin-1, 5-hydroxymethylfurfural, and methylglyoxal, as well as phenolic compounds, including flavonoids.[54,55]

Table 2. MIC and MBC of DH against various bacterial strains

Pathogenic microorganisms	MIC (% w/w)	MBC (% w/w)	MBC/ MIC ratio
Pseudomonas aeruginosa	0.39	0.78	2
Klebsiella pneumoniae	0.096	0.096	1
Escherichia coli	0.024	0.048	2
Haemophilus influenzae	0.048	0.048	1
Streptococcus pneumoniae	0.78	1.56	2
Bacillus subtilis	0.38	0.76	2
Staphylococcus aureus	0.19	0.19	1

Abbreviations: MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration, DH: Doum honey.

Various varieties of honey from the Eastern Mediterranean region have demonstrated significant inhibitory effects against both Gram-positive and Gram-negative bacteria, including methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* strains.[38] However, compared to other types of honey, studies examining the bioactive properties of DH are limited. This study aimed to address this gap by evaluating the phytochemical composition of DH, as well as its antibacterial, antioxidant, anti-inflammatory, and anticancer properties. The findings revealed broad-spectrum antibacterial activity against all tested Gram-positive and Gram-negative bacteria. Honey has also been shown to be effective against drug-resistant pathogens, including MRSA, drug-resistant hemolytic Streptococci, and vancomycin-resistant Enterococci.[39,55] Furthermore, honey from various global regions has been found to exhibit similar or even greater potency compared to

Manuka honey. Despite this, there are only a few studies exploring the bioactive potential of traditional Palestinian honey.[31,32,47,52]

3.2. Effects of DH on NO levels

NO, a transient bioactive free radical, functions as a signaling molecule in numerous physiological and pathological processes. The overproduction of NO serves as an indicator of the severity and stage of both acute and chronic inflammation.[56] Following the incubation of TPH-1-derived macrophages with LPS, there was an approximately five-fold increase in NO production compared to the control (Figure 1), observed at non-toxic concentrations of DH (Figure 2). Notably, the levels of NO were significantly reduced by DH, with a reversion to the baseline levels observed in untreated cells.

Numerous studies have previously indicated that honey has the capacity to downregulate inducible NO synthase expression and NO production.[57-60] The varying phenolic composition of honey may elucidate the differences in their nitrogen oxides suppression capabilities, as literature has suggested a link between these compounds and NO inhibition.[59,60] A study on Moroccan *Euphorbia* honey revealed a strong negative correlation between the phenolic compounds in honey and the NO scavenging activity of the honey samples.[59] This implies that a higher phenolic content in the honey leads to more effective NO inhibition activity. Another study affirmed that honey possesses anti-inflammatory properties, which could be partially attributed to the inhibition of NO release, indicating that the chemical constituents of honey, including its phenolic compounds, may contribute to this inhibitory effect.[60]

However, it is important to note that the specific chemical constituents of honey that contribute to this effect and the precise mechanisms involved may differ depending on the type of honey, necessitating further research. While these studies suggest a correlation, they do not establish a direct cause-and-effect relationship. The correlation signifies a relationship between the two factors, but it does not definitively prove causality.

3.3. Cytotoxic and cytostatic effects of DH in cells from the MDA cell line

Cancer cells are characterized by uncontrolled growth, a central focus for both traditional chemotherapy and emerging therapeutic strategies. DNA damage can lead to growth arrest at the G0/G1 or G2/M phases or may even initiate apoptosis. Many chemotherapy agents are designed to disrupt the cell cycle, particularly during the S and M phases. Studies have extensively documented honey's ability to induce cell cycle arrest at the G0/G1 phase in various cancer cell lines, such as bladder (T24, 253 J, RT4, and MBT-2), colon (HCT-15 and HT-29), and human melanoma (A375) cells.[61-63] The cytostatic effects of different types of honey have been highlighted in recent research,[64,65] which has focused on identifying components in honey that can inhibit cancer cell proliferation without eliciting cytotoxicity. For instance, an analysis of bioactive honey samples has identified phenolic compounds such as rosmarinic acid, tannic acid, caffeic acid, coumaric acid, gallic acid, ferulic acid, syringic acid, catechin, and pyrogallol.[62]

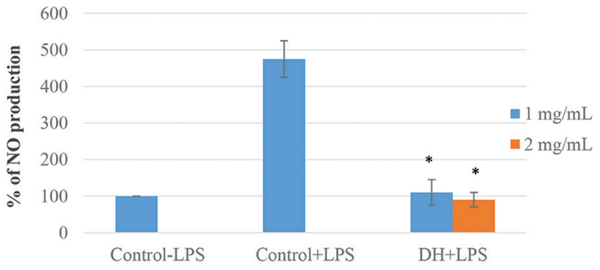

Figure 1. NO production by LPS-activated THP-1-derived macrophages after 72 h of incubation with 1 mg/mL and 2 mg/mL of DH. Data are presented as mean ± SD from three independent experiments performed in triplicate. Comparisons were made between Control + LPS and DH + LPS groups relative to Control - LPS, which was normalized to 100%. $^*P < 0.05$ compared to Control + LPS.

Abbreviations: LPS: Lipopolysaccharide; NO: Nitric oxide; DH: Doum honey.

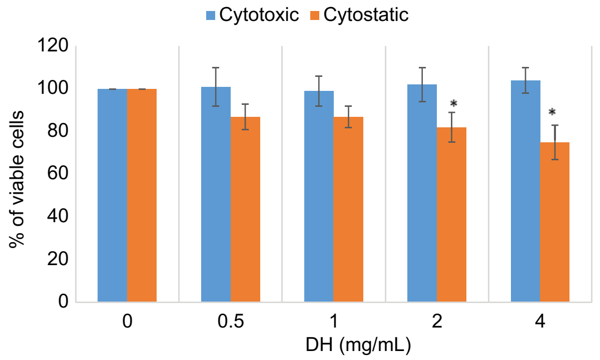

Figure 2. Cytotoxic and cytostatic effects were assessed on MDA cells using MTT Assay following treatment with honey samples ranging from 0 to 4 mg/mL for 24 h (for cytotoxic effects) and 72 h (for cytostatic effects). The absorbance of the MTT formazan was quantified at 570 nm utilizing an ELISA reader. Cell viability was computed as the percentage ratio of absorbance of honey-treated cells relative to untreated cells. Data represent the mean ± SD from three independent experiments conducted in triplicate. $^*P < 0.05$ compared to the untreated control.

Abbreviations: DH: Doum honey; ELISA: Enzyme-linked immunosorbent assay; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.

Figure 2 delineates the cytostatic and cytotoxic effects of DH samples on MDA cells. MDA cells are widely utilized models for breast cancer research, offering a relevant platform to evaluate potential therapeutic interventions. They exhibit characteristics similar to those of aggressive breast cancer phenotypes. Notably, no cytotoxic effects were observed across all tested concentrations. DH exhibited a modest yet significant cytostatic activity on MDA cells. The most pronounced cytostatic effects were noted at concentrations of 4 mg/mL and 2 mg/mL, leading to a reduction in cell viability by 25% and 20%, respectively, compared to untreated control cells. These findings are consistent with prior studies, underscoring that the influence of honey can vary based on its type.[65-68] Furthermore, our results corroborate numerous studies that have highlighted the cytostatic properties of honey on diverse cancer cells, suggesting that these effects are modulated by the phenolic content.[65-68] Recent findings by Imtara *et al.*[66] revealed the cytotoxic effects of various honeys from Morocco and Palestine on human colon adenocarcinoma (HCT-116) and breast cancer (MCF-7) cell lines. Their study highlighted a strong positive correlation between the concentration of antioxidant components such as phenols, flavonoids, and flavonols and the cytostatic effects observed in MCF-7 cells. In addition, an inverse relationship was identified between the levels of syringic and tannic acids and the cytostatic activity in HCT-116 cells. Similarly, research has demonstrated the cytotoxic effects of Sardinian STH honey[51] and Manuka honey from New Zealand[69] on HCT-116 and metastatic colon epithelial adenocarcinoma cells, with reduced cytotoxicity observed in non-cancerous cells. Furthermore, Chinese jujube honey was found to exert cytotoxic effects on HepG2 liver cancer cells.[69,70]

3.4. Effect of honey samples on cell migration of MDA cells

Cancer is characterized not only by the unregulated and accelerated proliferation of cells but also by the invasive and metastatic nature of these proliferating cells. It is well recognized that the tendency of breast cancer cells to metastasize to other tissues significantly increases the mortality risk.[71]

Here, we performed a scratch assay to assess the impact of DH, administered at a dose of 4 mg/mL, on migration of MDA cells. As illustrated in Figure 3, treatment with DH significantly reduced the migration rate of MDA cells compared to the untreated controls ($P < 0.05$). This reduction in tumor cell migration may partly result from the DH's cytostatic properties, as evidenced by its inhibition of cell migration and its cytostatic effects on MDA cells (Figure 2). Notably, the more pronounced suppression of cell migration relative to its cytostatic effects suggests the involvement of additional cellular and molecular mechanisms underlying DH's beneficial effects.

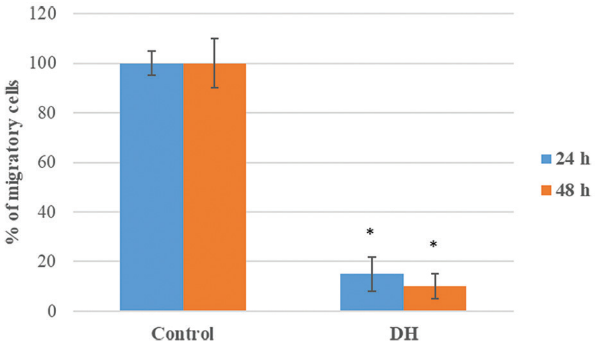

Figure 3. Effect of honey samples on MDA cell migration. The results reflect the extent of wound closure after 24 and 48 h of treatment with honey samples. Each data point was determined based on the initial wound size at time 0 h and was normalized to the untreated control, which was set as 100%. Data represent the mean ± SD from three independent experiments conducted in triplicate. $^{*}P < 0.05$ compared to the untreated control.
Abbreviation: DH: Doum honey.

These findings are consistent with previous studies that have reported the inhibitory effects of various honey types and isolated phytochemicals, such as resveratrol, kaempferol, and epigallocatechin-3-gallate, on the migration of colorectal cancer and oral squamous cell carcinoma cells. To the best of our knowledge, this is the first study to demonstrate the antimetastatic properties of Palestinian honey on MDA breast cancer cell lines.[72-74] Metastasis, the most lethal aspect of cancer, involves intricate processes[75] and a wide array of molecules,[76] including matrix metalloproteinases (MMPs), integrins, cadherins, plasminogen activators, PI3Ks, small GTPases (*e.g.*, Rho, Rac, Cdc42), phospholipase C, and focal adhesion kinases. Although the effects of honey on cancer metastasis remain underexplored, an *in vivo* study using wildflower honey from Croatia revealed a significant reduction in metastasis when administered before tumor cell inoculation in CBA mice and Y59 rats.[77]

Beyond the cytostatic effects of honey samples, the suppression of MMPs is probably a mechanism behind their beneficial properties. MMPs are proteases critical for extracellular matrix degradation and are highly expressed in metastatic cells.[78] Gallic acid, a phenolic compound, has been shown to reduce the gelatinolytic activity of MMP-2 and MMP-9, potentially via modulation of the nuclear factor kappa B (NF-κB) pathway.[63] Furthermore, several studies suggest that honey can inhibit both the expression and nuclear translocation of NF-κB *in vivo* and *in vitro* settings.[79-81] Honey has also been demonstrated to decrease the enzymatic activity of MMP-2 and MMP-9.[82] For example, Fir honey was found to inhibit human keratinocyte migration by downregulating MMP-9 expression.[82,83] Similarly, quercetin, a flavonoid present in honey, has been reported to suppress the expression of MMP-2 and MMP-9 in PC3 prostate cancer cells.[84,85]

3.5. Total polyphenols, flavonoids, and antioxidant capacity

Honey serves as a robust source of natural antioxidants, which play a crucial role in protecting against the effects of oxidizing agents on both food preservation and human health. It contributes to the reduction of health risks such as heart disease and cancer and prevents the deterioration of the immune system, cataracts, and various inflammatory processes.[8,20,25] As presented in Table 3, the TPC values were found to be 101.04 ± 22.46 mg Eq GA/100 g of honey. This value surpasses the TPCs reported in ten Palestinian honey samples from diverse geographical regions,[47] which ranged between 26.96 ± 0.71 mg/100 g and 70.73 ± 0.71 mg/100 g. These findings are akin to those discovered in thyme honey from Morocco.[86] The TFC values were recorded as 4.13 ± 0.31 mg Eq Q/100 g of honey (Table 3), which aligns with the values reported for other Palestinian honey samples.[47]

The ability of DH to neutralize DPPH free radicals, represented as IC_{50}, was evaluated (Table 3). The observed IC_{50} (4.40 ± 0.71 mg/mL) was lower than the values previously reported for other Palestinian samples, indicating a correlation between the IC_{50} of DPPH free radicals and the values of polyphenolic compounds, as well as the TFC.[47,87,88] As mentioned above, the samples from DH exhibited a higher TPC compared to other Palestinian honey samples, which could account for the lower IC_{50} values observed in DH compared to other honey samples.

The determined TAC was found to be 3.05 ± 0.12 g Eq AA/100 g of DH (Table 3). Similar values were found in other Palestinian honey samples that have similar TPC.[47]

3.6. Identification of phenolic compounds of DH by HPLC

A growing body of research, both *in vitro* and *in vivo*, has highlighted the anti-carcinogenic properties of plant-derived polyphenols on tumor cells. These properties include the inhibition of angiogenesis and metastasis, anti-proliferative effects, anti-inflammatory action, and the promotion of apoptosis. Recently, a wide range of novel polyphenolic compounds with potential anticancer activity have been identified globally. Some of these compounds show promise as anticancer agents, capable of treating or preventing cancer growth by targeting various stages of cancer development, such as initiation, promotion, and progression.[89]

Table 3. Free radical scavenging activity, TFC, TPC, and TAC of DH samples

Parameter	Value
Free radical scavenging activity (DPPH IC_{50}; mg/mL)	4.40±0.71
TFC (mg Eq Q/100 g of DH)	4.13±0.31
TPC (mg Eq GA/100 g of DH)	101.04±22.46
TAC (g Eq AA/100 g of DH)	3.05±0.12

Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl; TAC: Total antioxidant activity; TFC: Total flavonoids content; TPC: Total polyphenols content, DH: Doum honey.

We employed HPLC analysis to isolate phenolic compounds with potential anticancer properties from the honey samples under investigation. It is important to note that the polyphenolic composition of honey can vary depending on the source of pollination, as well as geographic and climatic factors.[47,90,91] Studies on the Palestinian honey samples collected from various regions and floral sources revealed phenolic content ranging from 26.96 to 70.73 mg equivalent per gram of honey.[47,66] Table 4 outlines the concentrations (in mg/g) of several phenolic compounds identified in DH, including nine key compounds: Gallic acid, caffeic acid, carvacrol, p-coumaric acid, ellagic acid, kaempferol, pinobanksin, pinocembrin, and galangin. Many of these compounds are known for their potential health benefits, including anticancer effects. For example, caffeic acid, a phenolic compound found in various natural sources such as honey,[47] exhibits a range of biological activities such as antioxidant, anti-inflammatory, anticancer, and antidiabetic properties.[92,93] The therapeutic effects of caffeic acid are thought to be mediated through the repression and inhibition of transcription and growth factors.[94]

P-coumaric acid, a hydroxycinnamic acid found in cereal grains, fruits, and vegetables, has demonstrated a range of health benefits, including antioxidant, antidiabetic,

Table 4. Concentrations of constituent polyphenolic compound and total polyphenols content in DH

Phenolic compounds	Concentration in mg/g DH
Gallic acid	0.37
Caffeic acid	0.68
Carvacrol	0.42
P-coumaric acid	5.03
Ellagic acid	5.22
Kaempferol	11.88
Pinobanksin	5.00
Pinocembrin	2.02
Galangin	4.09
TPC	34.71

Abbreviation: TPC: Total polyphenols content, DH: Doum honey.

anti-inflammatory, antiplatelet, antiulcer, and anticancer effects. It has been shown to inhibit growth and induce apoptosis in certain colon cancer cells and to prevent cancer in a short-term animal model.[95] In addition, this study examined the antibacterial activity of p-coumaric acid, suggesting its potential use in treating microbiome-related inflammation or cancer.[95] P-coumaric acid was found in relatively high quantities in the honey samples (Table 4).

Carvacrol, a compound present in plants such as oregano and thyme, has demonstrated medicinal potential, particularly against various types of cancer cells. Copper complexes, along with other organometallic compounds, are also recognized as potent anticancer agents, effective against a variety of cancer types, including lung and leukemia cells. Copper, being an endogenous metal, is non-toxic to normal cells, contributing to its therapeutic value.[96]

Pinocembrin has been shown to inhibit the viability, migration, and invasiveness of colorectal cancer cells while reducing the expression of MMP-2 and N-cadherin, and promoting the expression of E-cadherin and beta-lactamase-like protein.[97,98]

In a study by Ho *et al*.,[79] the levels of gallic acid, a compound with known antioxidant, anti-inflammatory, antimutagenic, anticancer, and cardioprotective properties, were found to vary in two types of Malaysian honey. Gallic acid, characterized by its trihydroxylated phenolic structure, has been identified in several honey samples.[47,66]

4. Conclusion

This study aimed to evaluate the biological effects of DH, focusing on its antibacterial activity as well as its antioxidant, anti-inflammatory, cytotoxic, cytostatic, and antimigration effects on MDA human breast cancer cells. A quantitative HPLC analysis identified nine phenolic compounds across three honey samples. The results obtained affirm the antibacterial attributes of DH and attribute its potential anticancer advantages to its anti-inflammatory, antioxidant, cytostatic, and antimigration effects. These effects are strongly linked to the presence of specific polyphenols, including carvacrol, pinocembrin, chrysin, protocatechuic acid, rutin, and salicylic acid.

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Conflict of interest

Bashar Saad is the Editorial Board Member of this journal and the Guest Editor of this special issue but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

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Consent for publication

Not applicable.

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Data are available upon reasonable request to the corresponding author.

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ACCSCIENCE PUBLISHING

Microbes & Immunity

MINI-REVIEW

Progress in the development of an Advax-adjuvanted protein capsular matrix vaccine against typhoid fever

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Abstract

Typhoid fever, caused by *Salmonella* Typhi, remains a significant global public health concern, with an estimated 11 – 20 million cases annually. Vaccines are critical to controlling typhoid fever. Widespread vaccination diminishes the emergence of antibiotic-resistant strains of *S.* Typhi. The economic benefits of vaccination are also substantial, as the costs of treating typhoid fever and its complications can be significant. Ty21a®, a killed whole-cell vaccine, and Vivotif®, a live-attenuated vaccine, have been available for decades but have relatively short durations of action and only provide partial protection. Vi polysaccharide-conjugate vaccines have improved the durability of protection, but there is still room for improvement. Typhax™, a novel alternative to traditional conjugate vaccines, utilizes Vi polysaccharide that is non-covalently entrapped in a poly-L-lysine and CRM197 protein matrix crosslinked by glutaraldehyde. When formulated with Advax-CpG™ adjuvant, Typhax demonstrated promising results in a range of animal models including mice, rabbits, and non-human primates in which it induces high and sustained serum anti-Vi immunoglobulin G and serum bactericidal activity, without any safety or reactogenicity issues. This novel vaccine approach offers the potential for a low-cost, more effective, and durable vaccine against typhoid fever, avoiding the need for frequent booster doses.

Keywords: Vaccine; Typhoid; Adjuvant; Immunization; Advax

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1. Introduction

Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), is transmitted to humans through contaminated food and water, and it remains a major cause of pediatric deaths in rural regions of developing countries.[1] According to estimates, there were 14.3 million cases of typhoid and paratyphoid fevers globally in 2017.[2] Immunocompromised people are particularly susceptible to infection with *S.* Typhi and are at increased risk of developing severe disease. *S.* Typhi, a Gram-negative rod, possesses several virulence factors that enable it to survive the host's anti-bacterial response, including toxins and metallophores essential for the bacterium's survival.[3]

Although prompt antibiotic therapy can decrease the severity, duration, complications, and mortality of typhoid fever, *S.* Typhi has acquired resistance to oral antibiotics widely available in recent years.[4] Approximately 1 – 4% of patients chronically harbor *S.* Typhi in their intestinal tract and gall bladder and act as asymptomatic carriers. With increasing antibiotic resistance and slow progress in improving water and sanitation in many developing countries, vaccination against *S.* Typhi is the most effective means of reducing typhoid fever deaths.[5] An episode of typhoid fever typically results in lifelong protective immunity, with both cell-mediated and humoral immunity being elicited following infection.[6] At present, there are three major types of vaccines commercially available for typhoid prevention: oral live-attenuated vaccines, parenterally administered unconjugated Vi polysaccharide, and polysaccharide-protein conjugates (summarized in Table 1).

2. Live-attenuated oral vaccines

The Vivotif® vaccine is an orally administered vaccine that is based on the attenuated S. Typhi Ty21a strain in which multiple pathogenicity-associated genes have been mutated, including those mediating the production of the Vi polysaccharide. Three doses of Ty21a administered in enteric-coated capsules as an every other day regimen were shown in one study to confer 67% protection over 3 years and 62% protection over 7 years.[7] However, this formulation is impractical for infants and toddlers and is only recommended for children over 6 years of age. Of concern, a recent human challenge study revealed no protective efficacy following the live-attenuated oral vaccine regimen with the Ty21a vaccine failing to induce an increase in anti-Vi antibody levels.[8] Pre-existing anti-Vi antibody levels were seen in those study subjects who showed protection, consistent with anti-Vi antibody playing a role in *S.* Typhi protection. Another downside of live-attenuated vaccines is that they are associated with frequent gastrointestinal side effects due to causing an attenuated typhoid-like illness. Live vaccines are contraindicated in those with primary or acquired immunosuppression in whom they could cause severe typhoid disease. Being an older technology, live vaccines also may contain animal-derived products such as bovine collagen which are no longer contained in newer vaccine types.

2.1. Vi polysaccharide subunit vaccines

Vi subunit vaccines (Typhim Vi®, Typherix®, and Typbar®) are single-dose, intramuscularly administered

Table 1. The pros and cons of typhoid vaccine approaches

Vaccine type	Brands	Pros	Cons
Live attenuated *Salmonella Typhi* strain Ty21a	Vivotif oral	• Relatively low cost	• Requires 3 doses • Only indicated in children aged >6 years • Contraindicated in primary and acquired immunodeficiency • Cannot be given at the same time as antibiotics • Frequent gastrointestinal side effects • Contains bovine-derived material • May have weak or no efficacy • Cannot be adjuvanted because it is a live vaccine
Pure Vi polysaccharide vaccine	Typhim Vi®, Typherix®, and Typbar®	• High safety • Only a single dose is required	• Weak efficacy at ~50% • Only indicated in infants aged >2 years of age • Short duration of protection (<2 years) • Unable to boost response • Cannot be adjuvanted as it is T-cell independent
Vi polysaccharide conjugate vaccine	Typbar-TCV™	• High safety • More durable protection • Effective in children aged >3 months • 3- to 6-fold higher peak anti-Vi responses • Can potentially be adjuvanted	• More expensive • Protection would wane after 5 years
Protein capsular matrix vaccine	Typhax/Advax-CpG™	• High safety • Up to 1000-fold higher peak anti-Vi responses, suggesting the possibility of long-term protection • Inclusion of Advax-CpG adjuvant overcomes polysaccharide-associated immune suppression • Anti-Vi antibodies able to be strongly boosted with repeated doses • Induces serum bactericidal antibodies • Low cost	• None

polysaccharide vaccines approved for use in adults and children >2 years of age. Polysaccharide-based vaccines confer variable and short-lived immunity. Estimates of vaccine efficacy of around 50% have been found in areas where typhoid fever is endemic, and evidence of indirect protection of unvaccinated neighbors of vaccinees has been found.[9,10] Immunity wanes within 2-year post-vaccination and there is no evidence that protective efficacy lasts beyond 3 years. Re-vaccination every 2 years is recommended for U.S. travelers to *S.* Typhi endemic areas. Thus, the widely available Vi polysaccharide subunit vaccines confer relatively short-term protection against typhoid in older children and adults and are poorly immunogenic in infants under 2 years of age due to their inability to elicit a T-cell-dependent immune response.[11] This limits the utility of the pure polysaccharide vaccines for *S.* Typhi eradication campaigns.

2.2. Vi polysaccharide-conjugate vaccines

Vi polysaccharide-conjugate vaccines in which the Vi polysaccharide is covalently coupled to a protein antigen have recently been developed and shown to be highly effective in children as young as 3 months of age.[12] Immunization with a 25 µg dose of a Vi-conjugate vaccine using CRM197 as a carrier protein developed by the Novartis Vaccine Institute for Global Health 28 days achieved anti-Vi geometric mean titer (GMT) of 304 EU/mL. Bharat Biotech's Vi-conjugate vaccine (Typbar-TCV™), when administered to human children at a 25 µg dose, achieved anti-Vi GMTs approximately 3-fold higher than those obtained with their equivalent unconjugated polysaccharide vaccine (Typbar™). Two years after vaccination, anti-Vi titers in those receiving the conjugate vaccine remained almost 2-fold higher (GMT 82) than in those that received the polysaccharide vaccine (GMT 46).[13] The International Vaccine Institute reported a phase 2 study of a diphtheria toxoid-conjugated Vi vaccine in children aged 6 – 24 months which achieved anti-Vi GMT of 444.38 EU/mL.[14] In Vietnam, Vi polysaccharide conjugated with recombinant *Pseudomonas aeruginosa* exotoxin A conferred >90% protection against typhoid over the first 27 months and >80% over 46 months.[15] From this study, it was estimated that the protective level of anti-Vi immunoglobulin G (IgG) is 3.5 EU/mL. A phase 1 study of the Vi-diphtheria toxoid conjugate (Vi-DT) conducted in the Philippines enrolled subjects aged 2 – 45 years who received either Vi-DT or Typhim Vi vaccine.[16] The conjugated Vi-DT vaccine generated a 4-fold higher Vi GMT compared to the pure polysaccharide Typhim Vi vaccine. Similarly, in a phase 1 study in European adults, anti-Vi GMT levels 4-week post-vaccination in the Vi-CRM197 group (304 EU/mL) were 6 times higher than in those vaccinated with Typhim Vi (52 EU/mL).[17]

Collectively, Vi-conjugate vaccines elicit approximately 3 – 6-fold higher peak anti-Vi antibody levels than pure polysaccharide vaccines and thereby provide more durable protection. For example, the conjugated typhoid vaccine manufactured in India is said to provide 5 years of protection when used in typhoid-endemic regions. It may be able to achieve this duration of protection because, in endemic regions, periodic re-exposure to *S.* Typhi provides regular boosting to Vi antibody levels, thereby helping maintain titers above protective levels for an extended timeframe. Travelers from countries where typhoid is not endemic would not get the benefit of such periodic endemic re-boosting, meaning that conjugate vaccines may provide a much shorter duration of protection to those living outside of endemic areas.[18] Hence, there remains a need to create more potent and durable typhoid vaccines.

3. Potential next-generation typhoid vaccine approaches

Even with the recent advent of the conjugate vaccines, the unmet need for more effective and durable typhoid vaccines remains. How might this be achieved? One area might be to explore modified polysaccharide antigens better able to present key neutralizing epitopes so as to maximally stimulate memory B cell responses.[19] Consideration could be given to including additional antigens, such as *S.* Typhi lipopolysaccharide (LPS) antigens, in the vaccine.[20] The antigens could be formulated with newer, more potent adjuvants.[21] Finally, vaccine delivery approaches to better stimulate mucosal immunity could be attempted.[22] While mRNA approaches have been touted as a way forward for many other traditional vaccines, these can only encode protein antigens and hence are not currently an option to replace vaccines where non-protein antigens such as polysaccharides are involved.[23] Similarly, while viral-like particles could theoretically be used as the protein carrier on which to conjugate polysaccharide antigens, little work has been done in this area, presumably due to the additional complexity of conjugating polysaccharides to the particles as opposed to conjugating them to soluble proteins.

An example of an alternative to traditional polysaccharide-conjugate approaches is the protein capsular matrix vaccine (PCMV) approach.[19] The PCMV process non-covalently entraps polysaccharide antigens in a crosslinked protein matrix (depicted in Figure 1A). The PCMV process is simpler and cheaper than the manufacture of polysaccharide-conjugate vaccines and allows full-length bacterial polysaccharides to be used, whereas in conjugate vaccines typically only short pieces of

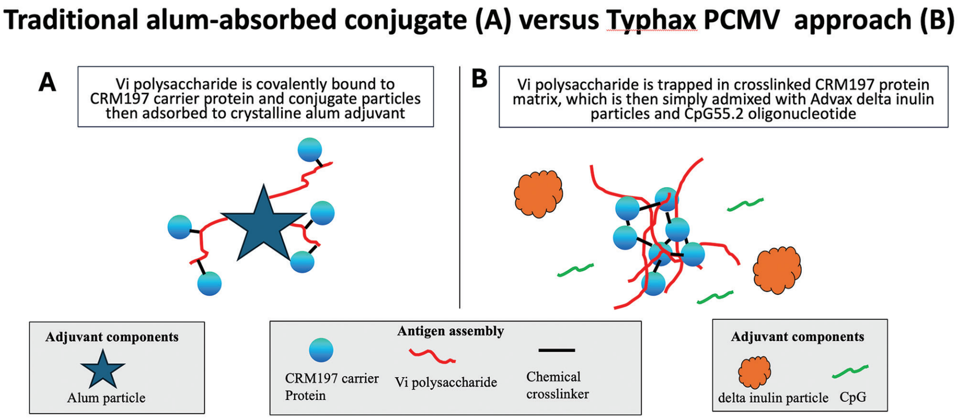

Figure 1. Schematic shows the differences between the traditional polysaccharide conjugation approaches where short segments of polysaccharide are directly crosslinked to the carrier protein and the protein capsular matrix vaccine (PCMV) approach where the carrier protein is crosslinked to itself, thereby trapping the full-length polysaccharide chains in the crosslinked protein matrix. The PCMV antigen can then be formulated with a relevant adjuvant such as Advax-CpG® to further enhance vaccine immunogenicity

the polysaccharides are used. In the PCMV process, the Vi polysaccharide antigen purified from *S.* Typhi is entrapped in a glutaraldehyde-catalyzed matrix of crosslinked α-poly-L-lysine (α-PLL) and CRM197 protein, a genetic toxoid of diphtheria toxin and a common carrier protein used in conjugate vaccines. The non-covalent entrapment of polysaccharide antigens in a crosslinked matrix of protein provides the benefits of conjugated vaccines such as the ability to induce helper T cells and enhance antibody levels, without the complexity and expense of direct multi-step polysaccharide conjugation to a carrier protein.[19] Notably, PCMV was effective in inducing helper T cells for the B cell response to the polysaccharide antigen, showcasing all the benefits of a conjugate vaccine.[24] PCMV technology thereby offers a simpler and cheaper means to manufacture the *S.* Typhi vaccines with similar characteristics as the conjugate vaccine, an important consideration given that the vast majority of *S.* Typhi vaccines are needed in the poorest developing countries where *S.* Typhi is endemic. Such cheap and affordable vaccines, particularly if they provide long-term durable protection, could be extremely important for global *S.* Typhi eradication campaigns. The PCMV approach has already been shown to be safe and effective in a human phase 1 clinical trial of Typhax™.[25]

4. Typhoid vaccine adjuvants

Pure polysaccharide vaccines typically are T-cell independent and hence unlikely to benefit from formulation with traditional adjuvants. By contrast, protein-conjugate vaccines are able to enlist helper T cell responses directed at the carrier protein, with these T cells then able to provide help to polysaccharide-specific memory B cells to become long-lived plasma cells.[12] The PCMV approach allows the use of an adjuvant to further enhance vaccine potency (Table 2). To date, commercial polysaccharide-conjugate vaccines, such as Prevnar, have either been used alone or with aluminum salt adjuvants.[26] Advax® adjuvant (VO_0005324[1]) was developed as part of the NIH Adjuvant Development Program.[27] and is derived from inulin polysaccharide formulated into microcrystalline particles referred to as delta inulin.[28-30] Advax® adjuvants have been demonstrated to enhance immunogenicity and vaccine protection across a diversity of viral, bacterial, and parasitic vaccines.[31-36] Advax® formulations can be complemented by the addition of TLR9-active CpG oligonucleotides to form a combination adjuvant known as Advax-CpG (VO_0005207[2]) that further enhances vaccine potency.[31,32,37] CpG55.2 is a potent human TLR9 agonist that was the first licensed human drug molecule designed by artificial intelligence. Advax® adjuvants were shown to be safe and well-tolerated and enhance immunogenicity in human clinical trials of influenza, hepatitis B, and insect sting allergy vaccines[38-40] and are a key component in SpikoGen® vaccine, a recombinant protein COVID-19 vaccine licensed for use in the Middle East in adults and children aged 5 years and older, with 8 million doses having been safely delivered.[41-44] One of the notable properties of Advax® adjuvants is that they are highly effective in newborns where they uniquely overcome neonatal immune hypo-responsiveness.[45-47] This makes the Advax® family of polysaccharide adjuvants uniquely suited for development with *S.* Typhi vaccines.

5. Advax-CpG adjuvanted Typhax vaccine

Immunizations of mice, rabbits, and non-human primates (NHP) with the Typhax™ vaccine formulated with Advax-

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[1] https://vac.niaid.nih.gov/view?id=38

[2] https://vac.niaid.nih.gov/view?id=37

Table 2. The pros and cons of potential typhoid vaccine adjuvants

Adjuvant	Tradenames	Pros	Cons
Aluminum salts (Alum)	AdjuPhos®, Alhydrogel®	• Low cost • Easy to formulate • Enhances anti-Vi response	• Imparts major Th2 immune bias which may predispose to allergy/anaphylaxis • Does not overcome polysaccharide-associated immune suppression • Only modest enhancement of Vi antibody titers • Low efficacy in children aged <3 months
Delta inulin-CpG oligonucleotide combination adjuvant	Advax-CpG™	• Low cost • Easy to formulate • Overcomes polysaccharide-associated immune suppression • Strong enhancement of anti-Vi response • Induces production of serum bactericidal antibodies • Effectively overcomes neonatal immune hypo-responsiveness in newborns	• None

CpG adjuvant elicited anti-Vi IgG responses up to 1,000-fold higher than those induced by an equivalent dose of the commercial Typhim Vi vaccine.[24] Notably, the Advax-CpG adjuvanted Typhax vaccine did not induce polysaccharide-associated immune suppression[48] with the anti-Vi IgG levels increasing after each booster immunization. Human data showed Vi antibodies induced by Typhim Vi immunization decay back to baseline by 24 months post-immunization.[49] By contrast, in immunized macaques the anti-Vi IgG responses induced by Advax-CpG adjuvanted Typhax vaccine were durable and remained well above baseline levels up to 9 months post-immunization.[24] Based on the estimated protective level of anti-Vi IgG of 3.5 EU/mL,[15] the antibodies induced by Advax-CpG adjuvanted Typhax seem likely to remain well in excess of this level for an extended period that may last many years, thereby avoiding the need for regular boosters every 1 – 2 years. The exceptionally high Vi antibody levels induced by Advax-CpG adjuvanted Typhax vaccine indicate that although the Vi polysaccharide is not conjugated to the CRM197 carrier protein, the Typhax vaccine is working in a T cell-dependent manner. This was confirmed by data showing that the anti-Vi response induced by Advax-CpG adjuvanted Typhax vaccine was markedly attenuated in CD4 T cell-depleted mice, thereby confirming the response to be T cell-dependent.[24] Notably, in NHP, Advax-CpG adjuvanted Typhax vaccine achieved peak anti-Vi responses that were approximately 25 times those achieved by Typhim Vi.[24]

6. Role of serum bactericidal activity (SBA) in typhoid vaccines

SBA has been found to be a strong correlate of protection for bacterial vaccines including those targeted at cholera and pneumococcal and meningococcal diseases.[50,51] SBA has also been shown to be inversely correlated with susceptibility to typhoid fever. Anti-Vi antibody and SBA levels were not correlated in individuals exposed to natural infection with *S.* Typhi in an endemic area.[52] This suggests the main protective *S.* Typhi antibody responses induced by natural infection or oral vaccines may be directed against *S.* Typhi LPS, rather than Vi polysaccharide. Vi-DT contains a small amount of endotoxin (9.65 EU/dose) which is within the acceptable range for human vaccines. Hence, residual *S.* Typhi LPS contaminating Vi and Vi-DT vaccines may act as an antigen and be responsible for inducing antibodies with SBA in vaccinated individuals.[16]

Some studies suggest that anti-Vi IgG contributes to reduced disease symptoms and prevention of *S.* Typhi infection.[8,53] Bactericidal antibody induced by an oral attenuated vaccine reduced typhoid severity but did not protect against clinical disease in a human challenge model.[20] The bactericidal activity was attributed to anti-LPS antibodies rather than anti-Vi antibodies, and the depletion of LPS antibodies significantly reduced bactericidal activity.[20] The Advax-CpG adjuvanted Typhax vaccine group induced high SBA in addition to high anti-Vi IgG.[24] It is possible the SBA was due to anti-LPS antibodies induced by small amounts of bacterial LPS contaminating the *S.* Typhi polysaccharide used to make Typhax. Notably, a human phase 1 study of a Vi-conjugate vaccine showed only a weak correlation between anti-Vi and SBA titer post-immunization with a 6-fold increase post-immunization in SBA in the Vi-conjugate vaccine group and a 4-fold increase in SBA in the Typhim Vi group.[16] In a previous study, Advax-CpG adjuvanted Typhax vaccine drove a 50-fold increase in SBA post-immunization as compared to the Typhim Vi group which induced only a short-lived 2-fold increase in SBA.[24] Human typhoid challenge studies may offer a faster and less expensive way to confirm

Typhax vaccine efficacy as compared to traditional large phase 3 outcome studies that seek to assess vaccine impact on spontaneous natural infections.[54]

7. Future directions

Parenteral typhoid fever vaccines have evolved from pure Vi polysaccharide vaccines to protein-conjugated Vi vaccines that provide better helper T-cell response and result in higher and more durable Vi antibody levels, thereby extending protection. The application of conjugation technology to typhoid vaccines represented a significant advance over unconjugated polysaccharide vaccines, with modest improvements in efficacy and durability, although at an increased cost due to the complexities of polysaccharide conjugation chemistry. A further potential advantage of protein conjugates is that they can benefit from an adjuvant to further increase their potency. In a major step forward, PCMV technology was used to create the Typhax vaccine, a simpler and cheaper approach to polysaccharide conjugation whereby the polysaccharide is trapped in a protein matrix rather than directly conjugated to the carrier protein. This achieves all the benefits of a polysaccharide-conjugate vaccine without involving complex procedures and high costs. To further build on this platform, we were able to show that the PCMV-based Typhax vaccine can be conveniently combined with Advax-CpG adjuvant, thereby delivering a typhoid vaccine of unmatched potency, which for the 1st time has the potential to provide long-term durable protection against *S.* Typhi without the need for regular boosters. This could further raise the bar for typhoid vaccine efficacy and durability. An Advax-CpG adjuvanted Typhax vaccine could make typhoid vaccines more accessible for poor endemic countries such as Nepal by reducing manufacturing costs and reducing the need for regular booster doses. This might thereby help bring the world one-step closer to typhoid eradication. While Typhax PCMV antigen and Advax-CpG55.2 adjuvant have separately been shown to be safe and effective in human testing, what is now needed is a human trial of the combination of these two complementary technologies, with the potential to deliver a typhoid vaccine of unmatched safety, efficacy, and durability.

8. Conclusion

Advax-CpG55.2-adjuvanted Typhax induced high and sustained serum bactericidal activity against S. Typhi in mice, rabbits and non-human primates. It is thereby a highly promising vaccine development candidate to provide robust and durable protection against typhoid fever.

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Conflict of interest

Nikolai Petrovsky is an affiliate of Vaxine Pty Ltd which has proprietary interests in Advax-CpG adjuvant, while Kevin P. Killeen is an affiliate of Matrivax Inc. which has proprietary interests in the Typhax vaccine.

Author contributions

Conceptualization: All authors
Writing–original draft: Nikolai Petrovsky
Writing–review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Further information on adjuvants developed as part of this program is listed on the NIH Adjuvant Database at https://vac.niaid.nih.gov/.

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ACCSCIENCE PUBLISHING

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COMMUNICATION

Establishment of a high-affinity anti-mouse CXCR5 monoclonal antibody for flow cytometry

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Abstract

The CXC chemokine receptor 5 (CXCR5) is a member of the G protein-coupled receptor family that is highly expressed in B cells and a subset of T cells, such as T follicular helper cells. Various types of cancers, including non-small cell lung cancer, breast cancer, and prostate cancer, also express CXCR5. Therefore, antibodies that specifically bind to CXCR5 could be useful for clarification of the mechanisms of cancer progression. In this study, we aimed to develop high-affinity monoclonal antibodies targeting mouse CXCR5 (mCXCR5) for flow cytometry. The established anti-mCXCR5 mAb (Cx_5Mab-3; rat IgG_{2b}, kappa), demonstrated reactivity with mCXCR5-overexpressed Chinese hamster ovary (CHO)-K1 (CHO/mCXCR5) in flow cytometry. Kinetic analyses using flow cytometry indicated that the dissociation constants (K_D) of Cx_5Mab-3 for CHO/mCXCR5 cell is 7.2×10^{-10} M. Furthermore, Cx_5Mab-3 did not cross-react with other mouse CC, CXC, CX3C, and XC chemokine receptors. These results indicate that Cx_5Mab-3 is useful for detecting mCXCR5 in flow cytometry with high affinity and specificity.

Keywords: Mouse CXC chemokine receptor 5; Monoclonal antibody; Peptide immunization; Flow cytometry

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1. Introduction

Chemokines are classified into four different subfamilies: CC, CXC, CX3C, and XC, which depend on the position and number of cysteine residues in their N-terminus.[1-9] The chemokine receptors belong to seven-transmembrane G-protein-coupled receptor families, and trigger intracellular signal transductions through binding to the ligands.[1,8,10-19] They play fundamental roles in development, homeostasis, immune system, cell proliferation, angiogenesis, and lymphocyte differentiation.[20-39]

The CXC chemokine receptor Type 5 (CXCR5) is predominantly expressed on the surface of B cells and a subset of T cells, such as T follicular helper (Tfh) cells.[40] The CXCR5+ Tfh cells interact with germinal center B cells to promote their differentiation into plasma cells or memory B cells formation through somatic hypermutation and class-switch.[41] Therefore, CXCR5+ Tfh cells play an important role in secondary lymphoid tissue orchestration and lymphoid neogenesis in the spleen, lymph nodes, and Peyer's patches.[8,40] CXCL13 is one of the ligands of CXCR5.[42] The CXCL13/CXCR5 axis activates downstream signaling, including PI3K/Akt, MEK/ERK, and Rac pathways, which modulates immune cells to promote lymphocyte infiltration, activation, and differentiation, thereby enhancing the antitumor immune response.[42] Furthermore, CXCR5 is also expressed in cancer cells, which makes pivotal contributions to the development and progression.[8,43-47] Therefore, monoclonal antibodies (mAbs), which specifically target CXCR5 would be useful for cancer therapy and elucidation of the disease progression.

The Cell-Based Immunization and Screening (CBIS) method includes the immunization of antigen-overexpressed cells and high-throughput hybridoma screening using flow cytometry. We have developed specific mAbs against mouse CCR1 (mCCR1; clone C_1Mab-6),[48] mouse CCR3 (mCCR3; clone C_3Mab-3),[49] mouse CCR5 (mCCR5; clone C_5Mab-2),[50] mouse CCR8 (mCCR8; clone C_8Mab-2),[51] mouse CXCR1 (mCXCR1; clone Cx_1Mab-1),[52] mouse CXCR3 (mCXCR3; clone Cx_3Mab-4),[48] and mouse CXCR4 (mCXCR4; clone Cx_4Mab-1)[48] using the CBIS method. Furthermore, we established specific mAbs against mouse CCR2 (mCCR2; clone C_2Mab-6),[53] mCCR3 (clones C_3Mab-6 and C_3Mab-7),[54] mouse CCR4 (mCCR4; clone C_4Mab-1),[55] mouse CCR6 (mCCR6; clone C_6Mab-13),[56] mouse CCR9 (mCCR9; clone C_9Mab-24),[57] and mouse CXCR6 (mCXCR6; clone Cx_6Mab-1)[58] using the N-terminal peptide immunization. In this paper, we report the successful development of a novel anti-mouse CXCR5 (mCXCR5) mAb using the N-terminal peptide immunization method.

2. Materials and methods

2.1. Plasmids, peptides, and cell lines

The synthesized DNA encoding mCXCR5 (Accession No.: NM_007551.3), mouse XCR1 (mXCR1; Accession No.: NM_011798), and mouse CX3CR1 (mCX3CR1, Accession No.: BC012653.1) were purchased from Eurofins Genomics KK (Tokyo, Japan). The complementary DNAs (cDNAs) of mouse CCR10 (mCCR10; Accession No.: NM_007721.4; Catalog No.: MR224922), and mouse CXCR2 (mCXCR2; Accession No.: NM_009909.3; Catalog No.: MR227587) were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The mCXCR5, mXCR1, and mCX3CR1 cDNAs were cloned into the pCAGzeo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The mCCR10 cDNA was cloned into a pCAGzeo_ssnPA16 vector. The mCXCR2 cDNA was cloned into a pCMV6neo vector.

A partial sequence of the N-terminal extracellular region of mCXCR5 ($_1$-MNYPLTLDMGSITYNMDDL-$_{19}$), with a C-terminal cysteine was obtained from Eurofins Genomics KK (Tokyo, Japan). Furthermore, the keyhole limpet hemocyanin (KLH) was conjugated at the C-terminus of the peptide.

Cell lines, including P3X63Ag8U.1 (P3U1), Chinese hamster ovary (CHO)-K1, and LN229 were purchased from the American Type Culture Collection (Manassas, VA, USA). CHO-K1, P3U1, and each chemokine receptor-expressed CHO-K1 were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc., Kyoto, Japan). LN229 and LN229 cells expressed mCXCR5 (LN229/mCXCR5) were cultured in a Dulbecco's Modified Eagle Medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc., Kyoto, Japan).

2.2. Animals

A 5-week-old female Sprague-Dawley rat was purchased from CLEA Japan (Tokyo, Japan). There is no influence of sex on the results of the study. The animal was housed under specific pathogen-free conditions. All animal experiments were performed according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

2.3. Development of transfectants

The plasmids were transfected into LN229 and CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Stable transfectants were established by staining with the following mAbs: anti-mCXCR5 mAb (clone L138D7; BioLegend, San Diego, CA, USA), anti-PA tag mAb (clone NZ-1 for mCCR10), anti-mCXCR2 mAb (clone SA045E1; BioLegend, San Diego, CA, USA), anti-mXCR1 mAb (clone ZET; BioLegend, San Diego, CA, USA), and anti-mCX3CR1 mAb (clone SA011F11; BioLegend, San Diego, CA, USA). The cells were sorted using a cell sorter (SH800; Sony Corp., Tokyo, Japan). After sorting, the cells were cultured in medium supplemented with 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA, USA) or 0.5 mg/ml of G418 (Nacalai Tesque, Inc., Kyoto, Japan). These chemokine receptors-overexpressed CHO-K1 or LN229 (e.g., CHO/mCXCR5) clones were successfully established.[58]

Stable transfectants of the following chemokine receptors were previously established: CHO/mCCR1,[48] CHO/mCCR2,[53] CHO/mCCR3,[49] CHO/PA-mCCR4,[55] CHO/mCCR5,[50] CHO/PA-mCCR6,[56] CHO/mCCR7,[59] CHO/mCCR8,[51] CHO/mCCR9,[57] CHO/mCXCR1,[52] CHO/mCXCR3,[48] CHO/mCXCR4,[48] and CHO/mCXCR6.[58] These transfectants were detected by the following mAbs: anti-CCR1 mAb (clone S15040E; BioLegend, San Diego, CA, USA), anti-CCR2 mAb (clone EPR20844; Abcam, Cambridge, MA, USA), anti-CCR3 mAb (clone J073E5; BioLegend, San Diego, CA, USA), anti-CCR5 mAb (clone C_5Mab-2[50]), anti-CCR7 mAb (clone 4B12; BioLegend, San Diego, CA, USA), anti-CCR8 mAb (clone C_8Mab-2[51]), anti-CCR9 mAb (clone CW-1.2; BioLegend, San Diego, CA, USA), anti-CXCR1 mAb (clone 1122A; R&D Systems Inc., Minneapolis, MN, USA), anti-CXCR2 mAb, anti-CXCR3 mAb (clone CXCR3-173; BioLegend, San Diego, CA, USA), anti-CXCR4 mAb (clone L276F12; BioLegend, San Diego, CA, USA), anti-CXCR6 mAb (clone SA051D1; BioLegend, San Diego, CA, USA), anti-XCR1 mAb, anti-CX3CR1 mAb, and NZ-1.

2.4. Development of mCXCR5-producing hybridomas

A rat was immunized intraperitoneally with 100 µg of KLH-conjugated mCXCR5 peptide (mCXCR5-KLH) with Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA, USA). The procedure included three additional weekly injections (100 µg/rat), followed by a final booster dose (100 µg/rat) 2 days before harvesting spleen cells. The harvested spleen cells were subsequently fused with P3U1 cells, using PEG1500 (Roche Diagnostics, Indianapolis, IN, USA). The resulting hybridomas were grown in RPMI medium supplemented with 10% FBS, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B. For hybridoma selection, hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific Inc., Waltham, MA, USA) were added to the medium.[58] The supernatants were subsequently screened using enzyme-linked immunosorbent assay (ELISA) with the mCXCR5 peptide, followed by flow cytometry using CHO/mCXCR5 and CHO-K1 cells.

2.5. ELISA

The synthesized peptide (MNYPLTLDMGSITYNMDDLC) was immobilized onto immunoplates. After blocking with 1% bovine serum albumin (BSA)-containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), the immunoplates were incubated with the hybridoma supernatants, followed by peroxidase-conjugated anti-rat immunoglobulins (1:20000 dilution; Sigma-Aldrich Corp., St. Louis, MO, USA). The enzymatic reactions were determined by measuring the optical density at 655 nm using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc., Kyoto, Japan).

2.6. Flow cytometry analysis

Cells were washed with PBS containing 0.1% BSA (blocking buffer) and treated with 10, 1, 0.1, or 0.01 µg/mL of Cx_5Mab-3 or L138D7 for 30 min at 4°C. For the peptide inhibition assay, Cx_5Mab-3 (0.1 µg/mL) or L138D7 (0.1 µg/mL) were pre-incubated with 1 µg/mL of mCXCR5 peptide or dimethyl sulfoxide for 30 min at 4°C, and further incubated with CHO/mCXCR5 for 30 min at 4°C. The cells were then treated with anti-rat immunoglobulin G (IgG) conjugated with Alexa Fluor 488 (Cell Signaling Technology, Inc., Danvers, MA, USA). Anti-mouse IgG conjugated with Alexa Fluor 488 and anti-rabbit IgG conjugated with Alexa Fluor 488 (Cell Signaling Technology, Inc., Danvers, MA, USA) were also used to detect the primary mAbs. The fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan). Cells were gated on the dot plot (SSC vs. FSC), and the fluorescence intensity was analyzed using FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

2.7. Determination of dissociation constant (K_D) by flow cytometry

The K_D was determined by fitting saturation binding curves to the built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA). This analysis was performed after the flow cytometry analysis of CHO/mCXCR5 cells treated with serially diluted Cx_5Mab-3 or L138D7, followed by the incubation with anti-rat IgG conjugated with Alexa Fluor 488 (1:200 dilution).

3. Results

3.1. Development of anti-mCXCR5 mAbs using N-terminal peptide immunization

To develop anti-mCXCR5 mAbs, one rat was immunized with the KLH-conjugated mCXCR5 peptide (Figure 1A). Splenocytes from the immunized rat were fused with myeloma P3U1 cells (Figure 1B). Positive wells for the naked mCXCR5 peptide were identified using ELISA; further selection was conducted using flow cytometry to identify supernatants that were reactive to CHO/mCXCR5 cells but non-reactive to CHO-K1 cells (Figure 1C). The ELISA assay identified 74 out of 1342 wells (5.5%), which strongly reacted with the naked mCXCR5 peptide. The flow cytometry analyses identified 18 out of the 74 wells (24.3%), which exhibited strong reactivity to CHO/mCXCR5 cells while showing no reactivity to CHO-K1 cells. After the limiting dilution and several additional

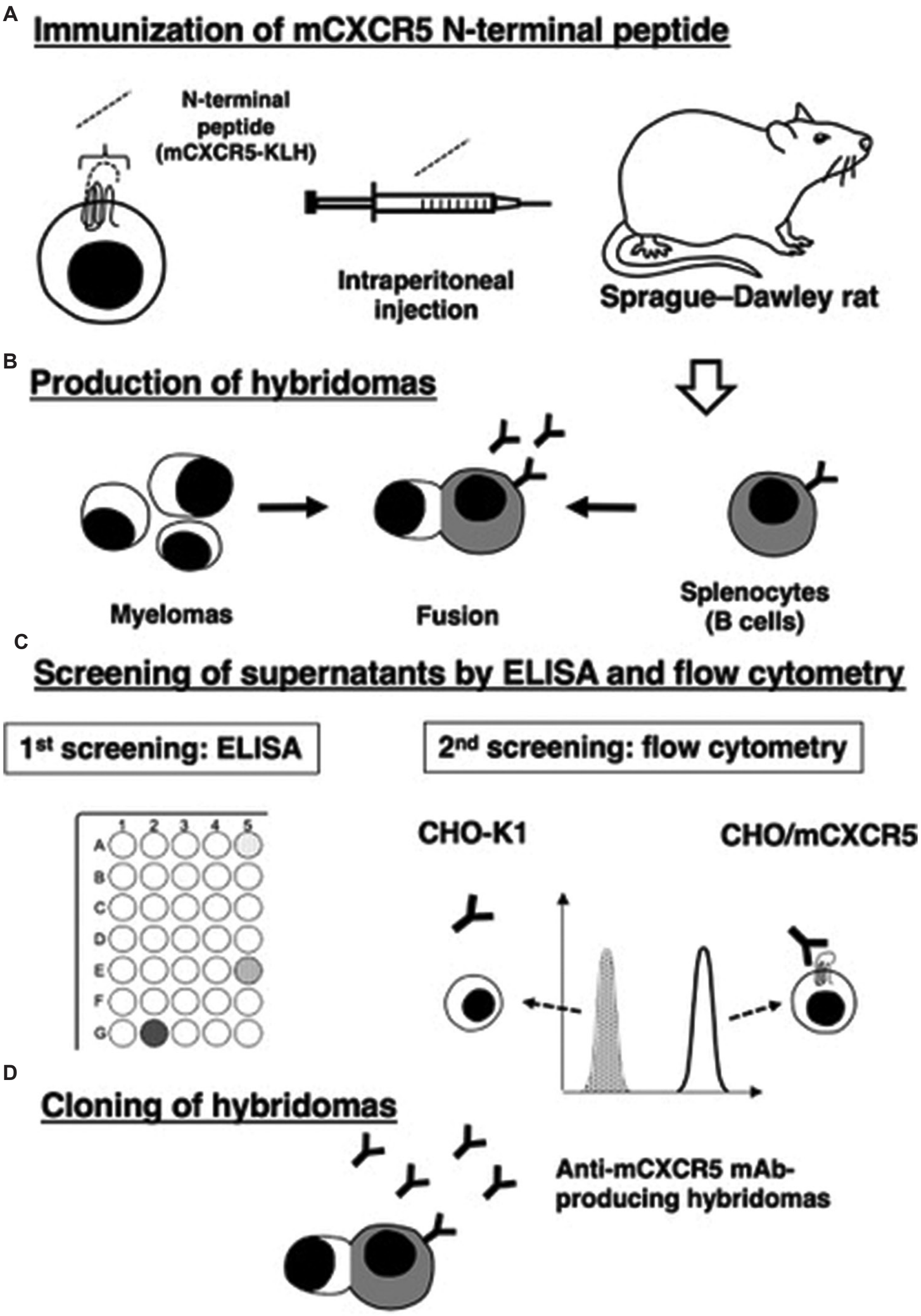

Figure 1. The development of anti-mCXCR5 mAbs. (A) Sprague-Dawley rats were immunized with CHO/mCXCR5 cells. (B) The splenocytes were fused with P3U1 myeloma cells. (C) Hybridomas producing anti-mCXCR5 mAb were screened using ELISA (first screening) and flow cytometry with CHO-K1 and CHO/mCXCR5 cells (second screening). (D) After limiting dilution, the anti-mCXCR5 mAb (Cx_5Mab-3) was successfully established.
Abbreviations: KLH: Keyhole limpet hemocyanin; CHO-K1: Chinese hamster ovary (CHO)-K1; ELISA: Enzyme-linked immunosorbent assay.

screenings, anti-mCXCR5 mAb (Cx_5Mab-3; rat IgG_{2b}, kappa) was successfully established (Figure 1D).

3.2. Flow cytometry analysis using Cx_5Mab-3

To assess the reactivity of Cx_5Mab-3 and L138D7, we conducted flow cytometry analysis on CHO/mCXCR5 and CHO-K1 cells. Cx_5Mab-3 recognized CHO/mCXCR5 cells in a dose-dependent manner at 10, 1, 0.1, and 0.01 µg/mL (Figure 2A). Parental CHO-K1 cells were not recognized even at 10 µg/mL of any mAbs (Figure 2B). The reactivity of Cx_5Mab-3 was also observed in LN229/mCXCR5 cells (Figure 3). The sensitivity against CHO/mCXCR5 or

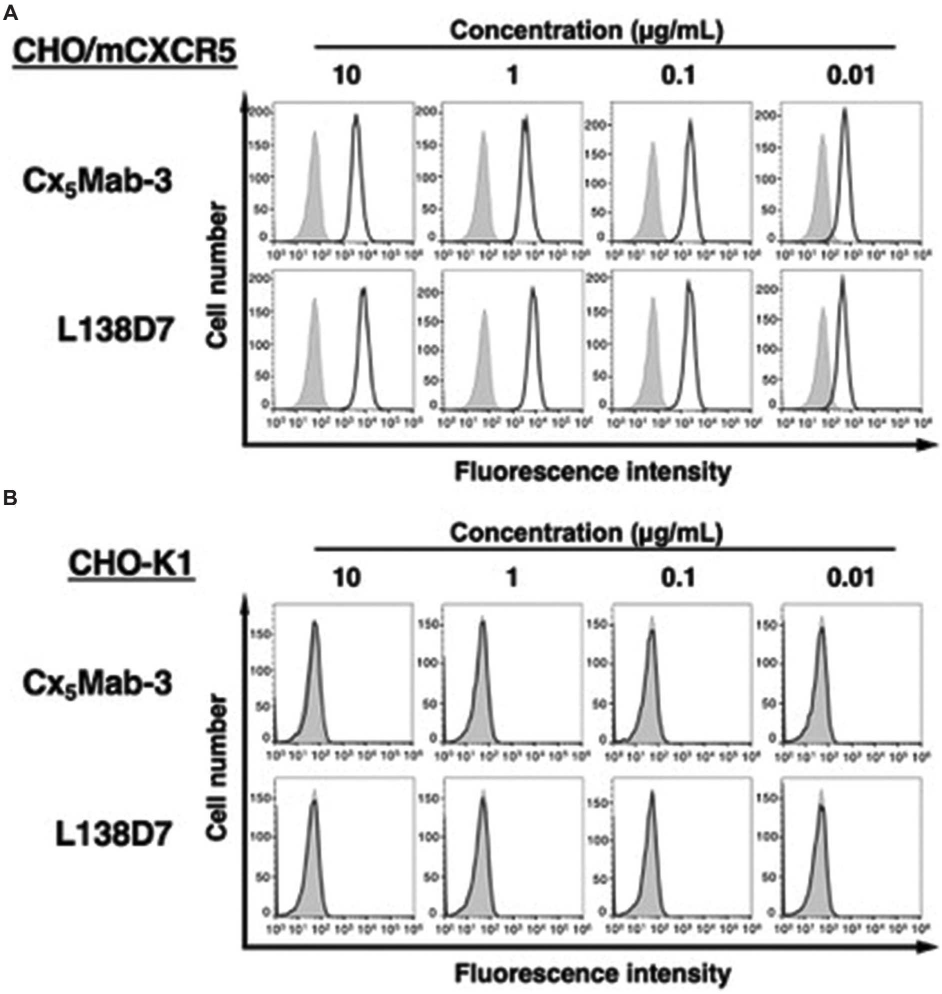

Figure 2. Flow cytometry analysis of mCXCR5-expressing cells using Cx$_5$Mab-3 and L138D7. CHO/mCXCR5 (A) and CHO-K1 (B) cells were treated with 0.01 – 10 μg/mL of Cx$_5$Mab-3 (black line), L138D7 (black line), or control blocking buffer (filled gray). Then, cells were treated with anti-rat IgG conjugated with Alexa Fluor 488. Fluorescence data were collected using the SA3800 Cell Analyzer.
Abbreviations: CHO-K1: Chinese hamster ovary (CHO)-K1; IgG: Immunoglobulin G.

LN229/mCXCR5 cells was similar for both Cx$_5$Mab-3 and L138D7 antibodies (Figures 2 and 3).

We next performed a peptide-blocking assay. As shown in Figure 4, both Cx$_5$Mab-3 and L138D7 reacted with CHO/mCXCR5 cells. The reactivity of Cx$_5$Mab-3 was completely neutralized by the mCXCR5 peptide, indicating that its reaction was mediated by recognition of the N-terminus of mCXCR5. In contrast, the reactivity of L138D7 was not neutralized, suggesting that the epitope recognized by L138D7 differs from that recognized by Cx$_5$Mab-3.

3.3. Determination of dissociation constant of anti-mCXCR$_5$ mAbs against CHO/mCXCR5 cells

We determined the apparent dissociation constant (K_D) of Cx$_5$Mab-3 and L138D7 against mCXCR5 by flow cytometry. The geometric mean fluorescence intensity of CHO/mCXCR5 at each concentration of Cx$_5$Mab-3 and L138D7 was plotted. By fitting one-site binding models, the K_D values of Cx$_5$Mab-3 and L138D7 for CHO/mCXCR5 were determined as 7.2×10^{-10} M and 7.0×10^{-9} M (Figure 5), respectively, indicating that Cx$_5$Mab-3 possesses a higher affinity than L138D7 for CHO/mCXCR5 cells.

3.4. Reactivity of Cx$_5$Mab-3 to CC, CXC, CX3C, and XC chemokine receptor-expressed CHO-K1 cells

We have established anti-mouse CC, CXC, CX3C, and XC chemokine receptor mAbs and evaluated them using these receptors-expressed CHO-K1 cells, as described in the materials and methods. Using these eighteen cell lines, the specificity of Cx$_5$Mab-3 was investigated. As shown in Figure 6A, Cx$_5$Mab-3 recognized only CHO/mCXCR5, but

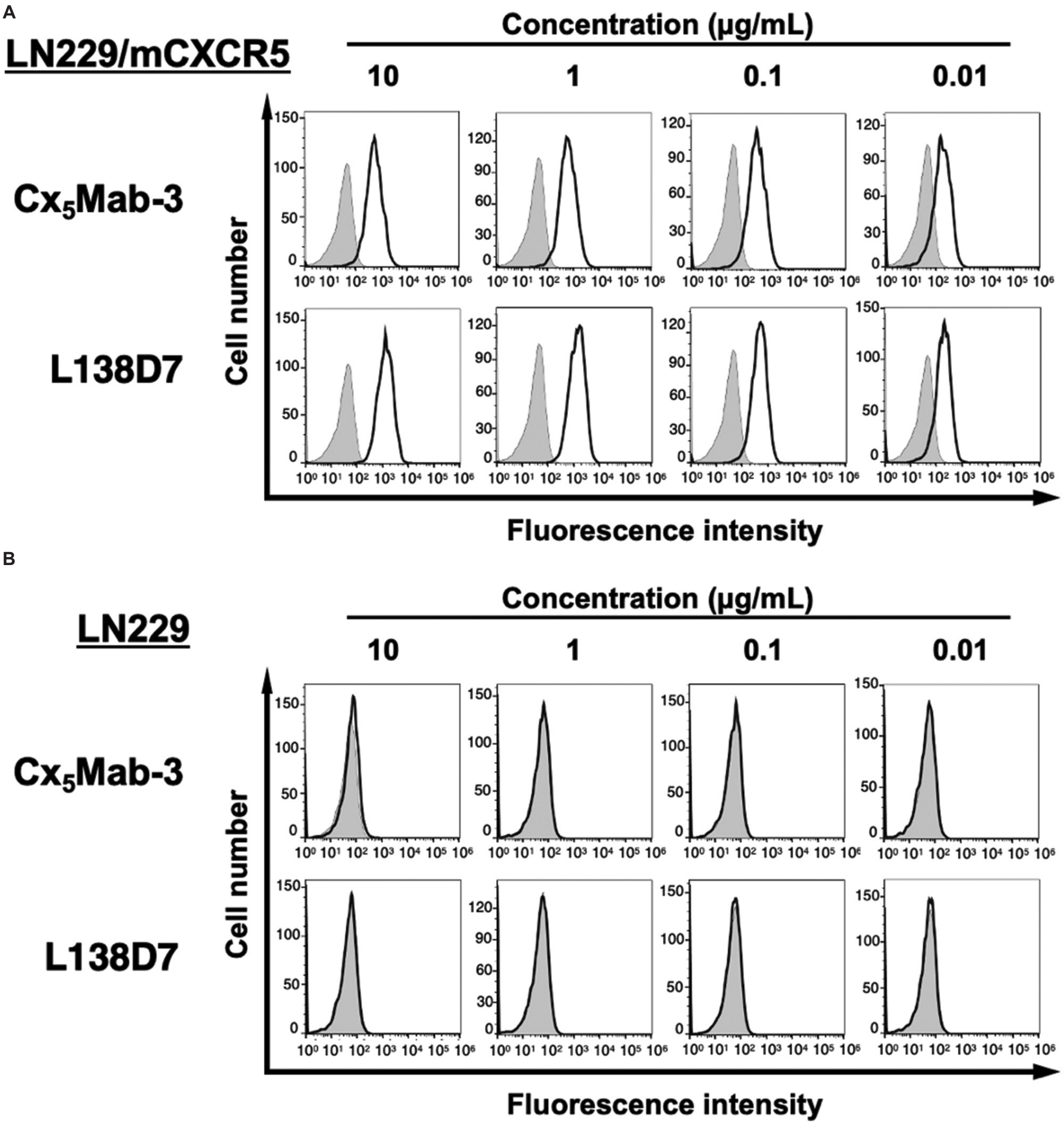

Figure 3. Flow cytometry analysis of mCXCR5-expressing LN229 cells using Cx$_5$Mab-3 and L138D7. LN229/mCXCR5 (A) and LN229 (B) cells were treated with 0.01 – 10 μg/mL of Cx$_5$Mab-3 (black line), L138D7 (black line), or control blocking buffer (filled gray). Then, cells were treated with anti-rat IgG conjugated with Alexa Fluor 488. Fluorescence data were collected using the SA3800 Cell Analyzer.
Abbreviation: IgG: Immunoglobulin G.

not others. We confirmed the expression of each receptor (Figure 6B).

4. Discussion

In this study, we developed a novel anti-mCXCR5 mAb (clone Cx$_5$Mab-3) using N-terminal peptide immunization and investigated its application in flow cytometry to detect mCXCR5 (Figures 2-5). We confirmed the specificity of Cx$_5$Mab-3 among eighteen CC, CXC, CX3C, and XC chemokine receptors (Figure 6). Therefore, Cx$_5$Mab-3 can recognize mCXCR5-expressing cells with high specificity in *in vivo* experiments. We assessed the reactivity of

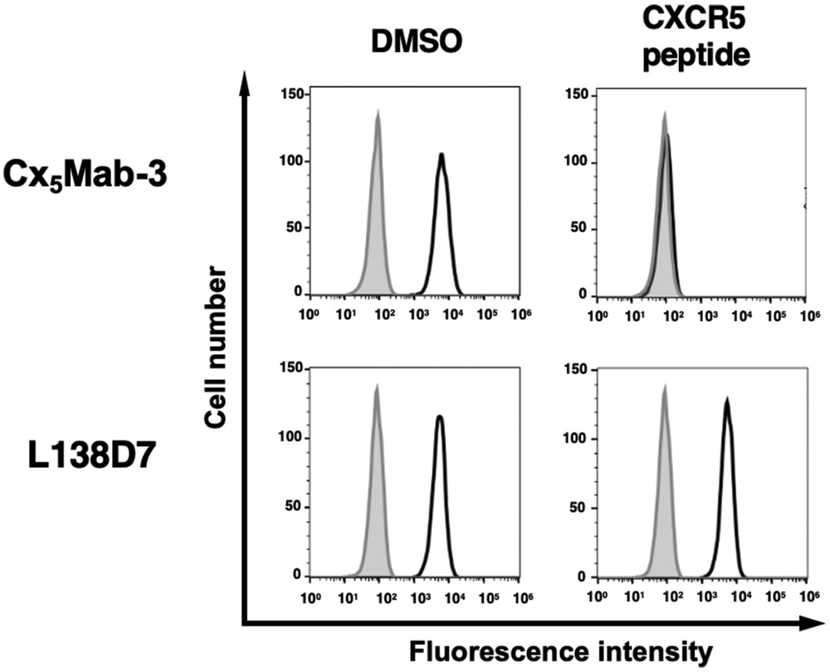

Figure 4. Peptide-blocking assay of Cx$_5$Mab-3 and L138D7 with mCXCR5 peptide. CHO/mCXCR5 cells were incubated with Cx$_5$Mab-3 (0.1 µg/mL) plus control (1% DMSO in blocking buffer), Cx$_5$Mab-3 plus mCCR5 peptide (1 µg/mL), L138D7 (0.1 µg/mL) plus control (1% DMSO in blocking buffer), or L138D7 plus mCCR5 peptide (1 µg/mL) for 30 min at 4°C. Cells were then treated with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The filled gray represents the negative control (blocking buffer).
Abbreviations: DMSO: Dimethyl sulfoxide; IgG: Immunoglobulin G.

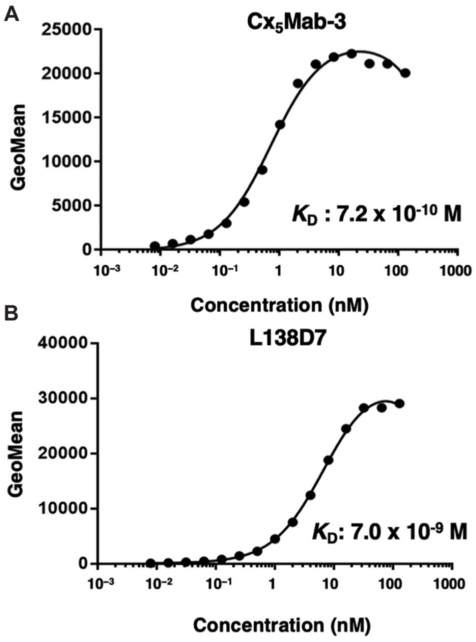

Figure 5. Binding affinity of Cx$_5$Mab-3 and L138D7. CHO/mCXCR5 cells were suspended in serially diluted concentrations of Cx$_5$Mab-3 (A) or L138D7 (B). The cells were treated with anti-rat IgG conjugated with Alexa Fluor 488. The fluorescence data were subsequently collected using the SA3800 Cell Analyzer, followed by the calculation of the K_D using GraphPad PRISM 6.
Abbreviation: IgG: Immunoglobulin G.

Cx$_5$Mab-3 in western blotting; however, the mCXCR5 protein was not detected by Cx$_5$Mab-3 (data not shown), although Cx$_5$Mab-3 detects N-terminal peptide of mCXCR5 in ELISA (Figure 1). This discrepancy suggests that both the cell surface-expressed N-terminal region of CXCR5 and the N-terminal peptide may form specific conformations, which are disrupted by SDS sample buffer in western blotting. A commercially available anti-mCXCR5 mAb (clone L138D7) was developed by immunizing rats with mCXCR5-transfected cells and is useful only for flow cytometry.[60] Since the reaction of L138D7 was not neutralized by the N-terminal peptide (Figure 4), its epitope may be located in other extracellular domains. In the future study, we aim to determine the binding epitope of L138D7.

It has been reported that the development of therapeutic drugs targeting the CXCL13/CXCR5 axis can be effective for treating cancers and inflammatory diseases.[61] CXCR5+ CD4+ Tfh cells mainly contribute to the antibody/B cell receptor class-switching, antibody production, and B cell proliferation during infection, autoimmunity, and cancer.[62] Moreover, CXCR5+ CD8+ T cells not only possess these functions but also maintain cytolytic activity similar to CD8+ T effector cells within tumor microenvironments.[63-65] CXCR5 expression is an important marker of progenitor memory stem-like

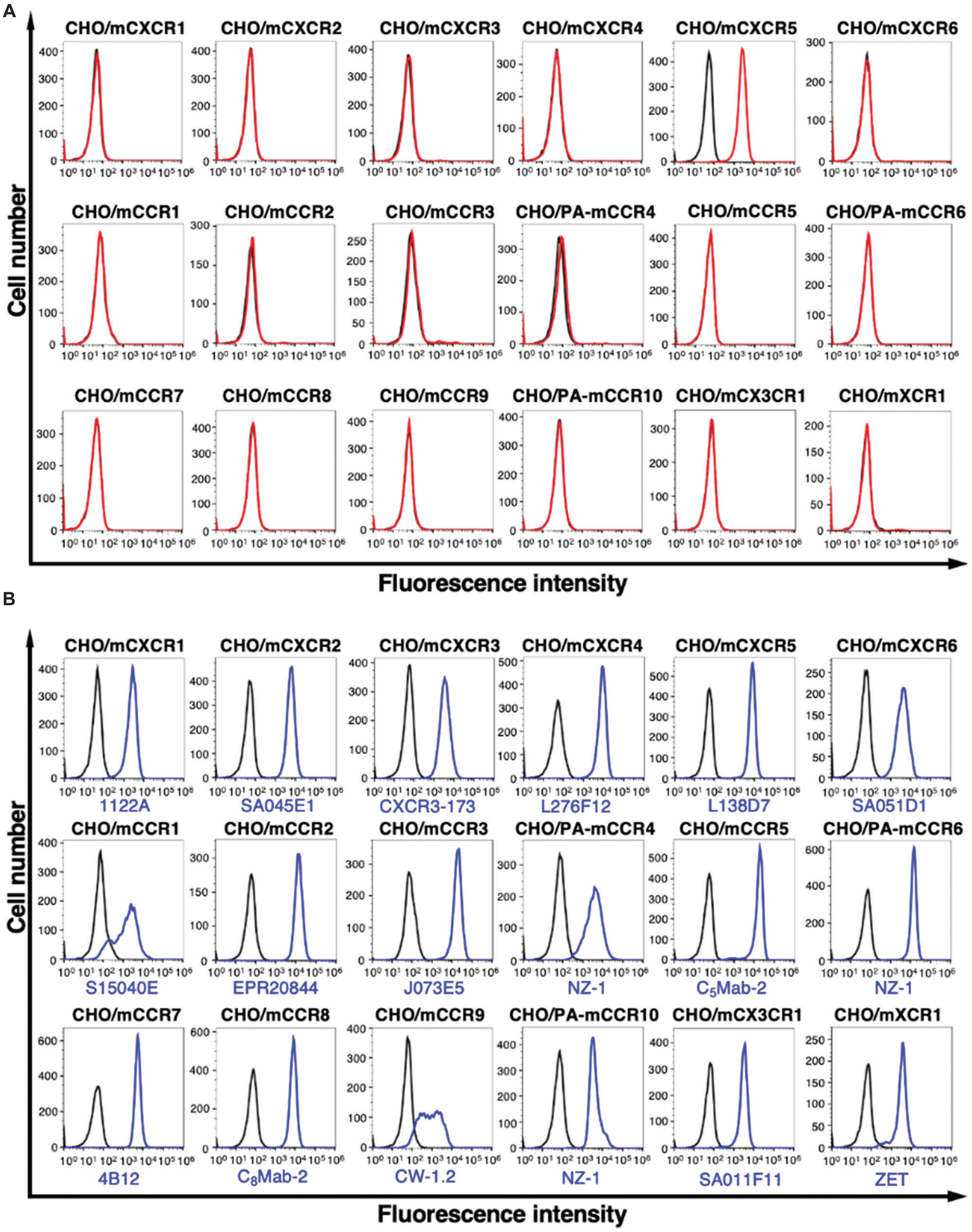

Figure 6. Flow cytometry analysis of Cx$_5$Mab-3 in CC, CXC, CX3C, and XC chemokine receptor-expressed CHO-K1 cells. (A) The eighteen CC, CXC, CX3C, and XC chemokine receptor-expressed CHO-K1 cells were treated with 1 μg/mL of Cx$_5$Mab-3 (red line) or control blocking buffer (black line), followed by the treatment with anti-rat IgG conjugated with Alexa Fluor 488. (B) The cells were treated with 1 μg/mL of corresponding mAbs, including anti-CXCR1 mAb (clone 1122A), anti-CXCR2 mAb (clone SA045E1), anti-CXCR3 mAb (clone CXCR3-173), anti-CXCR4 mAb (clone L276F12), anti-CXCR5 mAb (clone L138D7), anti-CXCR6 mAb (clone SA051D1), anti-CCR1 mAb (clone S15040E), anti-CCR2 mAb (clone EPR20844), anti-CCR3 mAb (clone J073E5), anti-CCR5 mAb (clone C$_5$Mab-2[50]), anti-CCR7 mAb (clone 4B12), anti-CCR8 mAb (clone C$_8$Mab-2[51]), anti-CCR9 mAb (clone CW-1.2), anti-XCR1 mAb (clone ZET), anti-CX3CR1 (clone SA011F11) mAb, or anti-PA tag mAb (clone NZ-1) (blue line). Then, cells were treated with corresponding secondary antibody conjugated with Alexa Fluor 488. Fluorescence data were collected using the SA3800 Cell Analyzer.
Abbreviation: IgG: Immunoglobulin G.

exhausted CD8+ T cells, which can respond to immune checkpoint inhibitor therapy in tumors.[66] Furthermore, CD8+ T cells with high PD-1 expression in tumors secrete high levels of CXCL13.[67] This CXCL13 secretion attracts Tfh cells and B cells to the tumor microenvironment.[67] As a result, CXCL13 can predict the response to immune checkpoint inhibitor therapy that correlates with durable responses and increased overall survival.[67] Therefore, CXCL13 and CXCR5 are novel biomarkers for predicting responses to immune checkpoint inhibitor therapy.[68]

In human breast cancer cell lines, an inverse correlation between the p53 tumor suppressor and CXCR5 expression has been reported.[69] Silencing p53 in MCF7 cells increases CXCR5 expression, which potentiates CXCL13-mediated chemotaxis.[69] A CXCR5 promoter analysis revealed that p53 suppresses the transcriptional activity of NF-κB, which is important for the upregulation of CXCR5.[69] Similarly, related tumor suppressors p63 and p73 regulate CXCR5 through comparable mechanisms.[70] Since CXCL13 is one of the overexpressed chemokines in breast cancer tissues compared with normal breast tissues, mAb therapies targeting CXCR5 could be an important strategy in treating tumors.[70] We previously modified the isotype of mAbs to mouse IgG_{2a} to enable antibody-dependent cellular cytotoxicity (ADCC) and evaluated their antitumor activities in mouse xenograft models.[71-73] Since the isotype of Cx_5Mab-3 is rat IgG_{2b}, an isotype switch to mouse IgG_{2a} will be required in future studies.

The CXCL13/CXCR5 axis is involved in the progression of autoimmune and inflammatory diseases.[65] In inflammatory bowel disease (IBD), CXCL13 levels are elevated in both humans and mouse models.[74] In IBD patients, serum CXCL13 concentrations are significantly higher than that in healthy controls.[74] Similarly, in a mouse model of dextran sodium sulfate-induced colitis, elevated CXCL13 levels were observed in the colon. The CXCL13 deficiency inhibits the occurrence and development of the colitis and restricts CD4+CXCR5+ T cells migration to mesenteric lymph nodes, resulting in an increase of regulatory B cells in the colon.[74] Therefore, antagonizing the CXCL13/CXCR5 axis may work as a potential therapeutic strategy for patients with IBD. Cx_5Mab-3 could contribute to the preclinical study through antagonizing the CXCL13/CXCR5 axis or depleting CXCR5+ T cells. As such, Cx_5Mab-3 may be useful for establishing proof of concept in preclinical studies.[71-73]

5. Conclusion

Cx_5Mab-3 is useful for detecting mCXCR5 by flow cytometry with high affinity and may be useful for establishing proof of concept in preclinical studies.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Writing–original draft: Kenichiro Ishikawa
Writing–review & editing: Hiroyuki Suzuki, Yukinari Kato

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Consent for publication

Not applicable.

Availability of data

The data presented in this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (doi: 10.20944/preprints202410.0497.v1).

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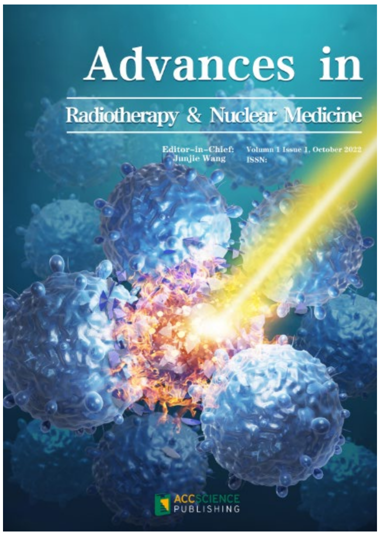
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