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Immunometabolism: Bridging the gap between immunology and nutrition

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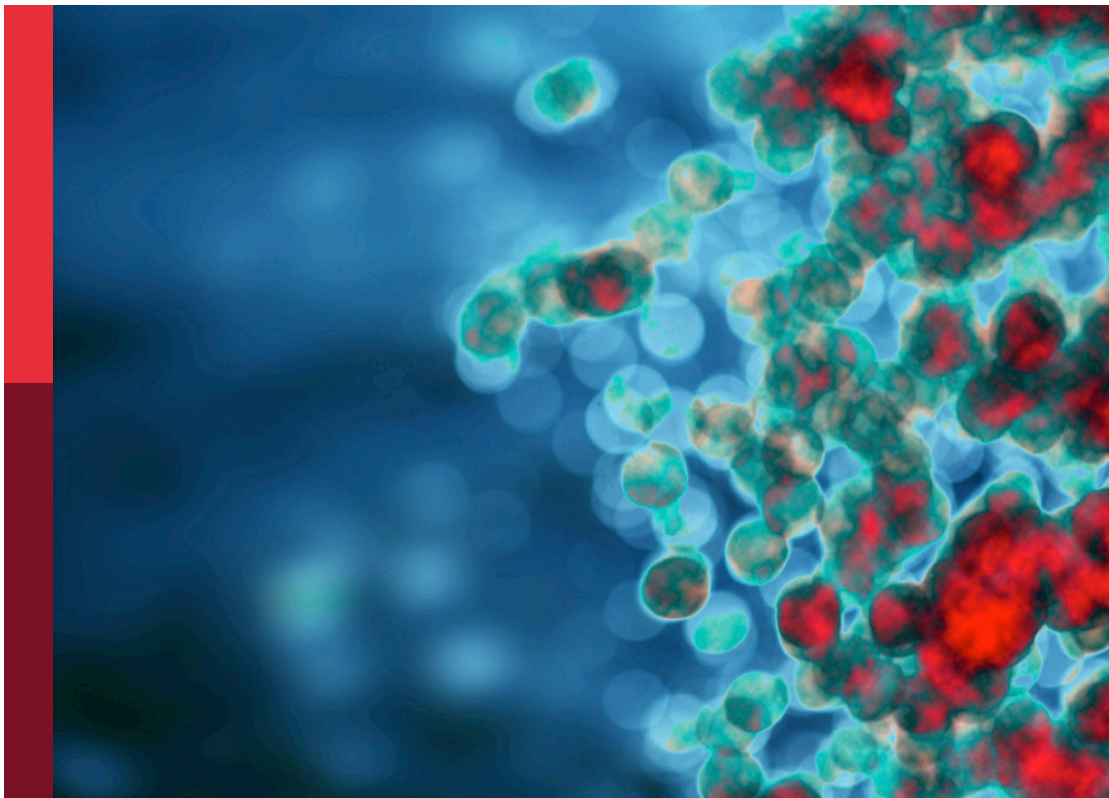

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Immunometabolism: Bridging the gap between immunology and nutrition

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Editorial: Immunometabolism: bridging the gap between immunology and nutrition

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Editorial on the Research Topic
Immunometabolism: bridging the gap between immunology and nutrition

1 Introduction

Over the past 15 years, the field of immunometabolism has seen significant advancements, delving into the complex interactions between the immune system and metabolic processes to modulate immune function. This rapidly expanding field has depicted how metabolic pathways influence different immune cell subsets in both health and disease as well as how genes, transcripts, and proteins interact with these pathways.

A portfolio of evidence indicates that quiescent and semi-quiescent (e.g., memory cells) immune cells primarily rely on oxidative phosphorylation (OXPHOS) to produce relatively high amounts of ATP, to support the maintenance of cellular homeostasis during periods of low activity, and to store energy that can be mobilized for imminent activation. Upon activation, immune cells undergo a metabolic shift to aerobic glycolysis, also known as the Warburg effect (1). This shift allows for rapid but low-efficiency ATP production and provides the necessary biosynthetic precursors to support the increased demands of cell proliferation, cytokine production, and other immune functions essential for an effective response to pathogens or tissue damage. Moreover, additional elements are necessary to sustain cellular anabolism during the activation process. Notably, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), a predominant cytoplasmic co-factor, is an key for anabolic reactions and plays a significant role in both oxidative and anti-oxidative processes. In this edition, Ting et al. elegantly outlined the significance of this co-factor in metabolic reactions within the myeloid cell compartment.

Nevertheless, describing a cell's reliance on aerobic glycolysis or OXPHOS as a "yin-yang" dynamic is overly simplistic, given the complex array of metabolic processes within immune cells: glucose derivatives can be diverted to the pentose phosphate pathway (PPP); OXPHOS can be predominantly fueled by various sources, including glucose, fatty

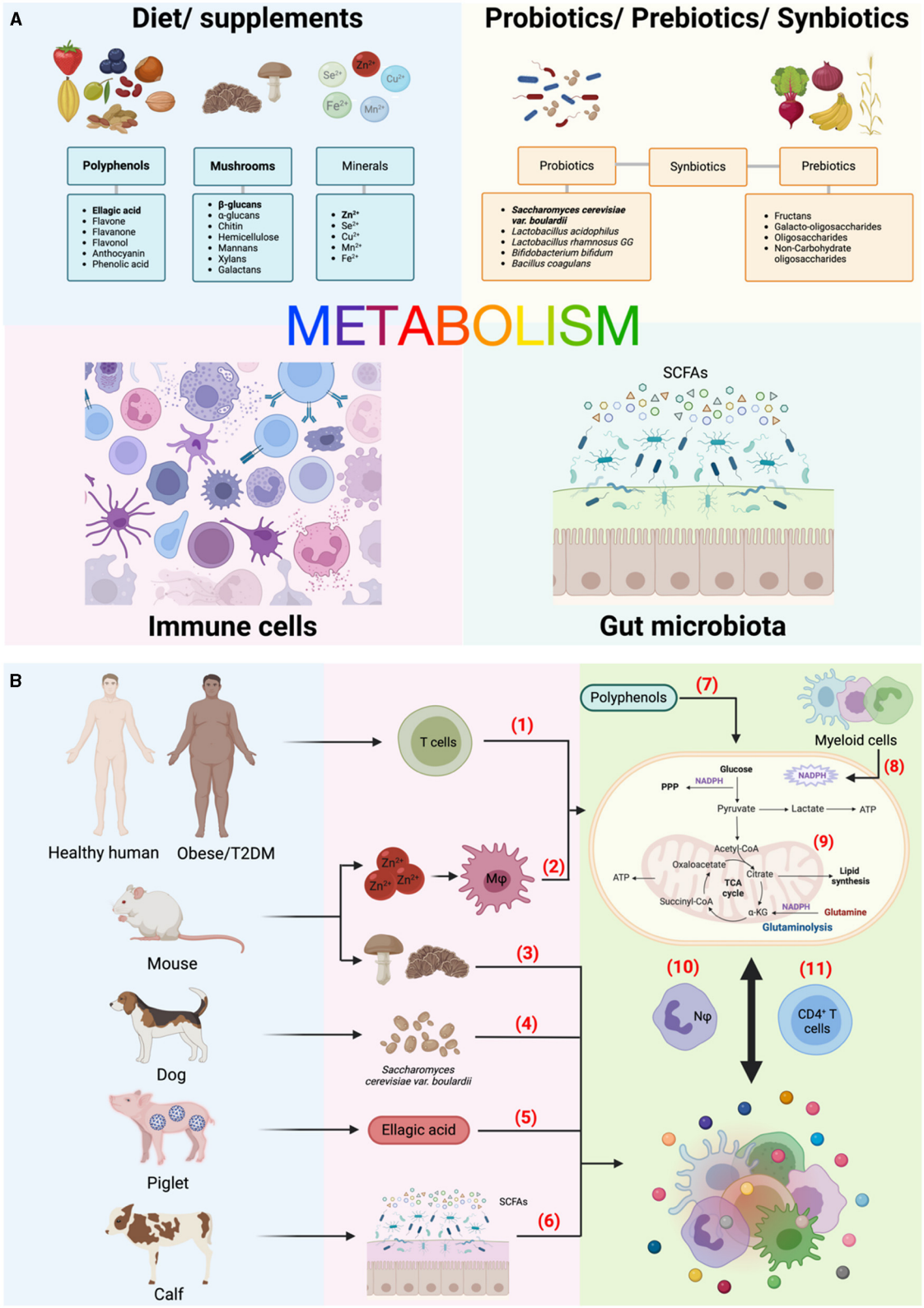

FIGURE 1
An overview of the topic landscape in this edition. **(A)** Diet, microbiota, and immunity are intricately interconnected, with metabolism serving as the pivotal link among these elements. Bold texts indicate some of the specific components investigated in this Research Topic. **(B)** Compilation of studies in this edition. The numbers highlighted in red indicate the brief scope of each study, emphasizing their experimental models and approaches to investigate their impact on immune cell metabolism and function. (1) Tibaes et al., (2) Kijima et al., (3) Case et al., (4) Garrigues et al., (5) Song et al., (6) He and Dong, (7) Ferreira et al., (8) Ting et al., (9) Trinchese et al., (10) Thind et al., (11) Liu et al. a-KG, alpha-ketoglutarate; ATP, adenine triphosphate;
(Continued)

FIGURE 1 (Continued)
Cu^{2+}, copper ion; Fe^{2+}, iron/ferrous ion; Mφ, macrophages; Mn^{2+}, manganese ion; Nφ, neutrophils; NADPH, nicotinamide adenine dinucleotide phosphate; PPP, pentose phosphate pathway; SCFAs; short-chain fatty acids; Se^{2+}, selenium ion; T2DM, type 2 Diabetes mellitus; TCA, tricarboxylic acid (cycle); Zn^{2+}, zinc ion or zinc (II). Created with BioRender.com.

acids (FAs), and other anaplerotic reactions such as glutaminolysis and the citrate-malate shuttle; tricarboxylic acid (TCA) cycle may undergoes "breaks" leading to the accumulation of citrate, succinate and itaconate (2); mitochondrial metabolism may influence and be influenced by other non-metabolic mitochondrial functions such as cell death, autophagy, calcium flow, endoplasmic reticulum stress, superoxide production of the respiratory chain and the resulting cascade of damage induced by reactive oxygen species (ROS), and lipid trafficking through interactions with other organelles via membrane contact sites. Thus, considerable research is still ongoing to fully comprehend how the dynamics of mitochondrial activity and the role each metabolic pathway plays in the diverse functions of immune cells. Here, Liu et al. and Thind et al. provided an in-depth analysis of the latest research on the role of glutaminolysis in $CD4^+$ T cells (e.g., Th1, Th17 and regulatory T cells) and how immunometabolic pathways of neutrophils affects their multiple functions in maintaining host resilience, respectively, while Trinchese et al. explored the myriad of mitochondrial functions, highlighting their primary role as energy producers and their impact on immunometabolism across a wide range of immune cells. These studies elucidate how specific metabolic pathways and mitochondrial functions interact to influence immune responses, and they identify promising new avenues for research to deepen our understanding on these mechanisms.

Current investigations have aimed to understand how external biochemical, metabolic and immune factors are integrated to influence cell fate decisions. This is particularly evident with dietary factors. Different foods not only have unique compositions, but the relative amounts of certain nutrients/micronutrients can significantly alter immunometabolic signals. Indeed, Kijima et al. described that supplementation with zinc ion (Zn^{2+}) can be used to treat spinal cord injury in mice, observing a dose-dependent improvement that affected both macrophages function and neuronal regeneration. Moreover, Case et al. found that pre-treatment with either mushrooms or isolated b-glucans on both murine and human macrophages improved their response to stimulation as well as increased myeloid progenitor cells in the bone marrow of mice, a process called trained immunity. Ferreira et al. and Song et al. used complementary approaches. While the former conducted a comprehensive review of polyphenols - a naturally occurring compound found primarily in plants - and their immunometabolic impact across multiple immune cell subsets, the latter used a specific polyphenol, ellagic acid, to ameliorate intestinal inflammation in piglets infected with porcine epidemic diarrhea virus (PEDV). Common sources of polyphenols include berries, nuts, olives, cocoa, olives, and certain seeds (Figure 1A). Moving in a clinical direction, Tibaes et al. outlined their protocol for the "Nutrition and Immunity (nutrIMM) study" to assess immune changes in healthy vs obese and/or individuals with type 2 diabetes upon a 4-week diet intervention.

Before reaching the bloodstream, nutrients must pass through the gut and interact with the local microbiota, undergoing various pre-processing steps. This process not only influences nutrient absorption but also impacts local and systemic immunity depending on the interspecies balance of the microbial community. Local microbiota produces several metabolites, among them the most well characterized are the short chain fatty acids (SCFAs) such as acetate, propionate and butyrate that are derived from fermentation of dietary fiber. While the interface between SCFAs and immune function has been extensively investigated (3), He and Dong provided an overview of the role of SCFAs in calves, which may have important application for "reverse translational research" in veterinary care due to the complexity of the four-compartment stomach in ruminants. Conversely, dysbiosis, an imbalance in gut microbiota, harms gut health by impairing nutrient absorption and weakening the gut barrier, leading to increased intestinal permeability and inflammation. To counteract dysbiosis, various clinical approaches, such as the use of probiotics, prebiotics, and/or synbiotics, have been employed (4). Garrigues et al. investigated the outcomes of administering *Saccharomyces cerevisae* var. *boullardii* in female dogs, observing positive changes in both microbiota composition and colostrum quality, which in turn, resulted in healthier puppies, with increased circulating levels of anti-inflammatory cytokines independently on the vaccination status.

Last, the studies presented in this Research Topic offer valuable insights into the importance of using different animal models to achieve specific research goals (Figure 1B). The selection of animal models is crucial as it impacts study outcomes due to species-specific genetic and physiological differences, relevance to human diseases, variations in immune responses, and ethical and practical considerations (5). This issue featured original research and reviews involving calves, dogs, and piglets, as well as the commonly used mouse, human, and *in vitro* approaches. This diversity suggests an unprecedented advance in the research community, despite the substantial challenges presented by each animal model.

2 Conclusion and perspectives

The emerging field of immunometabolism reveals how dietary and metabolic interventions can enhance immune health, offering new avenues for therapeutic development. The covered literature in this special edition emphasizes the critical role of nutrients in gut-immune cell axis and highlights the newfound understanding of cellular metabolic adaptability in face of different signal inputs. It particularly underscores the importance of this adaptability in immune cells, which must act swiftly against pathogens and tissue damage. Understanding these complex

interactions is crucial for advancing treatment and preventive personalized medicine.

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Conflict of interest

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

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Glutaminolysis and peripheral CD4$^+$ T cell differentiation: from mechanism to intervention strategy

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To maintain the body's regular immune system, CD4$^+$ T cell homeostasis is crucial, particularly T helper (Th1, Th17) cells and T regulatory (Treg) cells. Abnormally differentiated peripheral CD4$^+$ T cells are responsible for the occurrence and development of numerous diseases, including autoimmune diseases, transplantation rejection, and irritability. Searching for an effective interventional approach to control this abnormal differentiation is therefore especially important. As immunometabolism progressed, the inherent metabolic factors underlying the immune cell differentiation have gradually come to light. Mounting number of studies have revealed that glutaminolysis plays an indelible role in the differentiation of CD4$^+$ T cells. Besides, alterations in the glutaminolysis can also lead to changes in the fate of peripheral CD4$^+$ T cells. All of this indicate that the glutaminolysis pathway has excellent potential for interventional regulation of CD4$^+$ T cells differentiation. Here, we summarized the process by which glutaminolysis regulates the fate of CD4$^+$ T cells during differentiation and further investigated how to reshape abnormal CD4$^+$ T cell differentiation by targeting glutaminolysis.

KEYWORDS

CD4 + T cells, glutaminolysis, T cell differentiation, immunometabolism, intervention strategy

1 Introduction

T cells are derived from pluripotent stem cells of bone marrow, where T cell precursors first develop in the bone marrow, then move to the thymus for further programming and development, and gradually differentiate into T cells with immune activity in the thymus (1). These T cells are transported by the bloodstream to the lymph nodes, peripheral blood, and immune tissues where they colonize and take their final organ-specific characteristics (1). These peripheral naive T cells can be divided into two major subsets, CD4$^+$ and CD8$^+$ T cells, based on the CD cluster (CD) on their surfaces. Naive CD4$^+$ T cells can differentiate

into a variety of forms following antigen stimulation, including T helper (Th1, Th2, Th9, Th17, and Th22) cells, Treg cells, T memory (Tm) cells, and T follicular helper (Tfh) cells (2–4). In general, $CD4^+$ T cells primarily perform the tasks of cytotoxicity, accessory immunity, and immune regulation (5). Each subtype specifically has distinct roles, but the differentiation of Th1, Th17, and Treg cells is of special interest because changes in the balance of these cells have been linked to a variety of illnesses, including autoimmune diseases, transplant rejection, and irritability (6, 7). For example, in systemic lupus erythematosus (SLE), excessive differentiation of Th17 cells and reduced differentiation of Treg cells are the main causes of disease development and tissue damage (8). Besides, Th1 and Th17 cells are closely related to the occurrence of this pathological state in acute cellular rejection induced by organ transplantation (9). Hence, it is crucial to restore the equilibrium of different subtypes of $CD4^+$ T cells in diseases and stabilize it in healthy organisms.

Glutamine (Gln), a kind of immune regulatory nutrient, is frequently used in large amounts to supply cellular energy and to supply intracellular synthesis of genetic material *via* glutaminolysis within rapidly proliferating/dividing cells (10). Therefore, the original focus of studies on the effects of glutaminolysis on cells was tumorigenesis. Since immune cells also require substantial proliferation to function after activation, there has been progressively increasing research focusing on glutaminolysis in immune cells in recent years. Immunometabolism mainly investigates the reciprocal influence of immunity and metabolism in physiology and disease, with the ultimate goal of harnessing the distinct metabolic programs of different immune cell populations to treat disease. With the progress of immunometabolism, the role of Gln, considered as an immunomodulatory nutrient, has been gradually unraveled in immune related diseases. For instance, administration of glutaminolysis enzyme inhibitors can increase the acceptance of allografts in a mouse skin transplant model (11). Similar to this, in a mouse psoriasis model, aberrant glutaminolysis activation can cause lesion aggravation by promoting Th17 cell differentiation (12). These studies suggest that glutaminolysis may play a crucial role in immune related diseases and that the generation of these effects seems to be closely related to $CD4^+$ T cell differentiation. As a result, manipulating glutaminolysis to reshape $CD4^+$ T cell differentiation appears to be an effective intervention for immune-related diseases. In this review, we provide an overview on the role of glutaminolysis in peripheral $CD4^+$ T cell differentiation and on the potential points of intervention in the glutaminolysis pathway for the treatment of various diseases.

2 Glutaminolysis

Glutaminolysis is the process by which cells convert Gln to tricarboxylic acid (TCA) cycle metabolites through the activity of multiple enzymes (Figure 1) (10). To begin with, Gln infiltrates the cytoplasm through amino acid transporters (AATs), which are a type of membrane bound transport proteins that can mediate the transfer of amino acids into and out of cells or organelles. These transporters are mostly sodium ion-dependent neutral AATs, mainly utilizing the concentration gradient of intracellular and extracellular sodium ions to synergistically transport sodium ions and Gln into cells, and then expel excess sodium ions from cells through a sodium ion pump (13). These transporters primarily consist of solute carrier family 1 member 5 (SLC1A5, namely alanine serine and cysteine transporter system 2, ASCT2), SLC38A1 (sodium-coupled neutral amino acid transporters 1, SNAT1) and SLC38A2 (sodium-coupled neutral amino acid transporters 2, SNAT2), with ASCT2 being the most important (14, 15). Subsequently, Gln enters mitochondria *via* SLC1A5 variant (SLC1A5_var), an AAT that locates on the mitochondrial membrane through its N-terminal targeting signal, then is decomposed into glutamate (Glu) and ammonia under the action of mitochondrial glutaminase (GLS), which is also the rate-limiting step of glutaminolysis (14, 16). GLS is the first enzyme in glutaminolysis, mainly including GLS1, GLS2, and GLS1 splicing isomer (Glutaminase C, GAC) (14). On the one hand, in mitochondria, Glu is then transformed into α-Ketoglutaric acid (α-KG) *via* glutamate dehydrogenase 1 (GLUD1), glutamic oxaloacetic transaminase 2 (GOT2) and glutamate pyruvate transaminase 2 (GPT2) (10). Specifically, under the catalysis of these three enzymes, Glu not only produces α-KG, but also produces ammonia, aspartate and alanine, respectively (10, 17). The intramitochondrial α-KG can participate in the TCA cycle, supporting the oxidative phosphorylation (OXPHOS) pathway and ATP generation (14). Glu and α-KG produced in the mitochondria are transported out *via* SLC25A18, SLC25A22 and SLC25A11 on the mitochondrial membrane respectively (14). On the other hand, Glu can also be converted into α-KG in cytoplasm by a group of transaminases, including GOT1, GPT1 and phosphoserine transaminase 1 (PSAT 1) (10). Similarly, GOT1 and GPT1 catalyze Glu to produce aspartate and alanine, respectively (17). PSAT1 is one of the key enzymes in the serine synthesis pathway, and it also produces a portion of α-KG in the pathway of catalyzing serine synthesis (18). Intracellular α-KG can be further catalyzed to generate 2-hydroxyglutarate (2-HG) by isocitrate dehydrogenase (IDH1), a missense mutant metabolizing enzymes (19). In turn, intracytoplasmic α-KG can be regenerated to Glu *via* GOT1 (10). Cytosolic Glu is involved in the biosynthesis of glutathione (GSH) and non-essential amino acids (NEAAs, e.g. alanine, proline, aspartate, asparagine and arginine) (14). Subsequently, Glu is transported out of the cell *via* SLC7A11, exchanging with cystine (Cys) (14). Likewise, excess Gln in the cytoplasm exchanges extracellular branched-chain amino acids (BCAAs, e.g. leucine, valine, and isoleucine) through SLC7A5 (LAT1) (20).

3 Mechanism of glutaminolysis in regulating peripheral naïve $CD4^+$ T cell differentiation

As previously mentioned, peripheral naïve $CD4^+$ T cells can differentiate into different subtypes following antigen stimulation. Concretely, the first signal of cell activation is specifically obtained

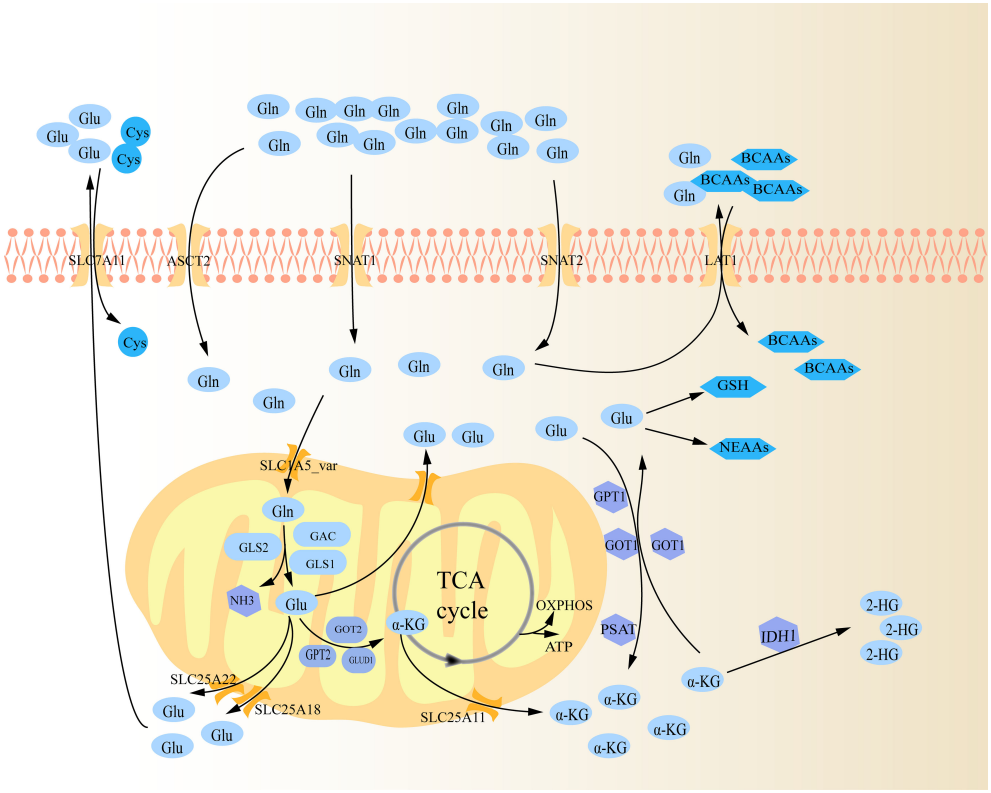

FIGURE 1
Schematic diagram of glutaminolysis. Gln is ingested into cells through several amino acid transporters (ASCT2, SNAT1, and SNAT2), and further transported into mitochondria through carriers on the mitochondrial membrane (SLC1A5_var). It is gradually decomposed within the mitochondria by various metabolic enzymes, and then the metabolites are transported out of the mitochondria to perform their functions respectively.

by naive $CD4^+$ T cells through the interaction of their T cell receptor (TCR) with the antigenic peptide MHC class II (MHC-II) molecular complex that is displayed on the surface of antigen presenting cells (APCs) (21). Then, the second signal of cell activation is produced when these naive $CD4^+$ T cells combine with the corresponding ligand (such as B7) on the surface of APCs and the costimulatory molecule (such as CD28) expressed on its surface (21). In response to dual signals, naïve $CD4^+$ T cells become activated, immediately after which they need to take up large amounts of Gln and glucose to meet the biosynthesis materials and energy required for proliferation/differentiation (21, 22). Further investigation revealed that this shift was brought about by an increase in SNAT1, SNAT2, and ASCT2 expression when TCR was activated (23, 24). Naive $CD4^+$ T cells subsequently differentiate into various subtypes under the influence of various cytokines in the microenvironment. For example, Th0 cells can polarize into Th1 cells when exposed to cytokines like IL-12, whereas Th0 cells can polarize into Th2 cells when exposed to cytokines like IL-4 (25, 26). Studies have shown that even in specific cell differentiation conditions, impairment in glutaminolysis can have a substantial impact on the fate of peripheral naive $CD4^+$ T cells during differentiation (27). The underlying mechanism for how glutaminolysis regulates $CD4^+$ T cell differentiation is discussed below (Figure 2).

3.1 Regulating peripheral $CD4^+$ T cell differentiation *via* epigenetic regulation

As a metabolite of intracellular glutaminolysis, α-KG not only participates in the TCA cycle and the generation of other amino acids, but also participates in the regulation of histone and DNA methylation levels as a cofactor of peroxidase, thus participating in the regulation of gene expression in cells (19, 28). Research revealed that Th17 cells produced more α-KG than Treg cells, suggesting that glutaminolysis may be more active in Th17 cells (29, 30). With studies advancing, 2-HG, the actual molecules behind the role of α-KG, was identified. Xu et al. found that under the condition of Th17 cells, cells can penetrate 2-HG, not α-KG, up-regulate the expression of IL-17A, and down-regulate the expression of Foxp3 in a dose-dependent manner, directly promoting the differentiation of Th17 cells (30). Surprisingly, the addition of 2-HG to naïve $CD4^+$ T cells even inhibited the expression of Foxp3 under the condition of Treg cells (30), suggesting that 2-HG has obvious differentiation regulation. A subsequent study reported that 2-HG can trigger the DNA methylation of Foxp3 to inhibit its transcription, thus suppressing the differentiation of Treg cells and regulating the homeostasis of Th17/Treg cells (30). Mechanistically, this effect depends on the negative regulation of 2-HG on Tet Methylcytosine Dioxygenase 1-3 (TET1-3), a negative regulator of DNA

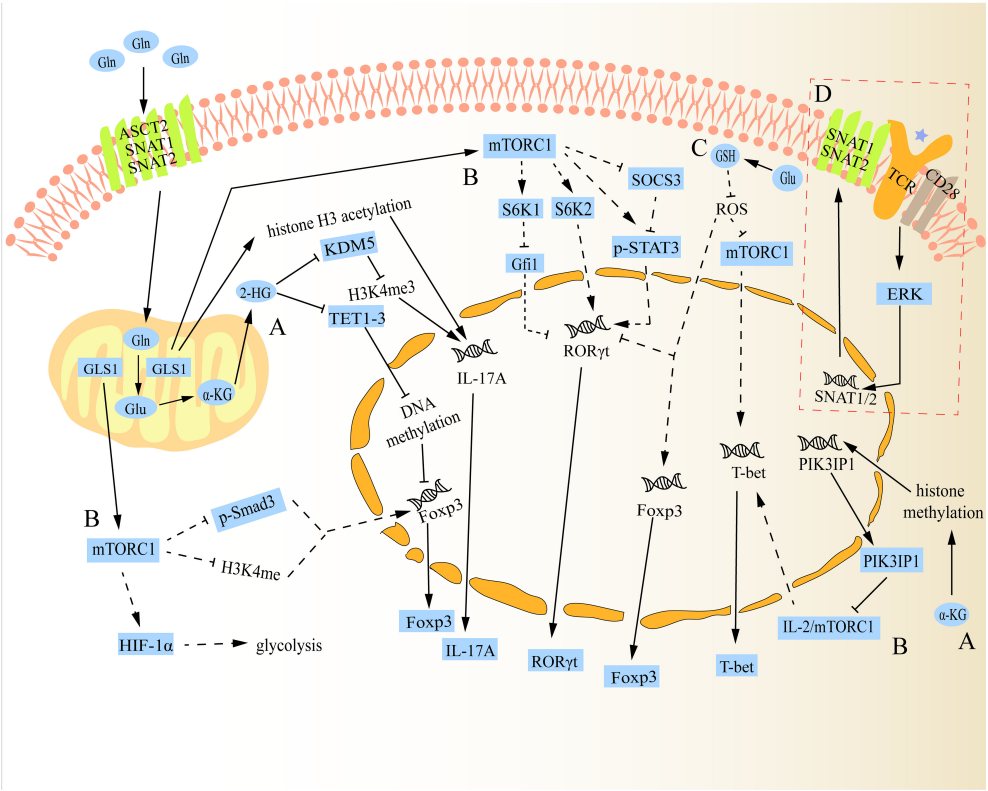

FIGURE 2
Schematic diagram of the mechanism of glutaminolysis regulating the differentiation of naive CD4$^+$ T cells. **(A)** Gln metabolite α-KG can be further catalysed into 2-HG, which can alter the fate of Th17 and Treg cells through regulatory epigenetic regulation. Besides, α-KG can also influence the expression of mTORC1 by epigenetic regulation to change the fate of Th1 cells. **(B)** In addition to regulating the expression of Th1 cells, mTORC1 can also alter the fate of Th17 and Treg cells through various mechanisms. **(C)** Gln metabolite Glu can regulate the differentiation of Th17 and Treg cells *via* Glu-GSH pathway. Additionally, it can also regulate Th1 cell differentiation, but there is a paradox between GSH regulating Th1 cell differentiation and mTORC1 regulating its differentiation. **(D)** After TCR activation, SNAT1/2 expression is up-regulated and promotes its membrane localization. The solid lines in the figure show the direct relationship between glutaminolysis and naive CD4$^+$ T cell differentiation reported in studies. The dotted lines in the figure indicate that no study has reported a direct relationship between glutaminolysis and naive CD4$^+$ T cell differentiation.

methylation (30, 31). Besides, Miao et al. verified that 2-HG can facilitate the differentiation of Th17 cells by forming H3K4me3 (Histone H3, trimethylated lysine 4) modifications in the promoter and CNS2 region of the IL-17A gene locus. This effect is made possible by inhibiting KDM5, a lysine demethylase (32). Another independent study showed that GLS1-mediated glutaminolysis was abnormally activated in psoriasis patients and mouse models, which promoted Th17 cell differentiation by enhancing histone H3 acetylation of IL-17A promoter (12). The function of α-KG or 2-HG in the acetylation of H3 histone, however, was not further investigated in their experiments. As for Th1 cells, another type of inflammatory cells, it was shown that Gln deprivation inhibits the differentiation of naïve CD4$^+$ T cells into Th1 cells and increases the generation of Foxp3$^+$ Treg cells, and this effect can be reversed by the α-KG analogue (33), indicating that it also plays a role in the differentiation of Th1 cells. In line with this, Nakaya et al. found that ASCT2 deficiency hinders Th1 and Th17 cell differentiation, reducing inflammatory T cell responses in a mouse autoimmune model (24). Interestingly, one study claimed that the transient inhibition of GLS1 resulted in an increase in the number of Th1 cells, but it would be exhausted over time (34). In details, after administration of GLS1 blockade, the reduced level of histone methylation in naïve CD4$^+$ T cells led to the reduced expression of PIK3IP1,a negative regulator of mTORC1, further leading to the activation of mTORC1 to promote the differentiation of effector Th1 cells (34). Besides, the inhibition of GLS1 also leads to histone modification, thereby increasing the expression level of IL-2, which is more conducive to Th1 differentiation (34, 35). However, only GLS1 inhibition may cause a reduction in intracellular Glu and an accumulation of Gln. In contrast, an excessive amount of Gln accumulation may have the reverse effect. In order to investigate this distinction, more research should be done in the future on the direction and function of intracellular Gln after GLS1 inhibition.

3.2 Regulating peripheral CD4$^+$ T cell differentiation *via* mTORC1

The activity of mTORC1 plays an important role in integrating the metabolic spectrum and guiding the fate decision of CD4$^+$ T

cells because it senses and integrates multiple signals from the environment to control metabolism (36). Previous research had demonstrated that Rheb (the positive regulatory target of mTORC1) deficient $CD4^+$ T cells suppressed the differentiation of Th1 cells by reducing the response to IL-12 and preventing T-bet transcription (37, 38). On the contrary, Rheb deficient $CD4^+$ T cells showed enhanced phosphorylated STAT6 level in response to IL-4 (Th2 cell polarization factor), which further increased the transcription level of GATA3 in cell nuclear, thus promoting the differentiation of Th2 cells (39). Regarding Th17 cells, to begin with, the activation of mTORC1 leads to an increase in STAT3 phosphorylation at tyrosine 705 in naive $CD4^+$ T cells, which is required for the RORγt genes expression (39). Then, mTORC1 promotes glycolysis by inducing HIF-1α, which in turn supports the differentiation of Th17 cells (40). Besides, mTORC1 enhances the differentiation of Th17 cells in a way that is dependent on S6K1/2, where S6K1 inhibits the down-regulation of Gfi1, a negative regulator of Th17 cell differentiation, and S6K2 enhances the nuclear localization of RORγt (41). Lastly, by blocking SOCS3 (a negative regulator of STAT3), mTORC1 can also promote STAT3 phosphorylation and RORγt expression that are induced by IL-6 (39). However, the function of mTORC1 is reversed during the differentiation of Treg cells. One example is that mTORC1 blocks the development of Treg cells by preventing Smad3 phosphorylation or H3K4 methylation close to the Foxp3 transcription start site, both of which have been shown to encourage Foxp3 transcription (41). Another example is that mTORC1 can increase glycolytic activity *via* inducing HIF-1α, but Treg cells is less dependent on glycolytic metabolic procedure to provide energy compared with Th17 cells, thus leading to a significant difference in the differentiation of Th17 and Treg cells (40, 42).

Amino acids play an important role in the activation of mTORC1 signaling pathway, especially Gln, leucine (Leu), arginine (Arg) and methionine (Met) (24, 43–45). ASCT2, an essential amino acids transporter, is mainly responsible for Gln transporting into cells (46). Additionally, it is in charge of bringing a tiny quantity of Leu into cells (24). ASCT2 has also been identified as necessary for coupling TCR and CD28 signals to activate the mTORC1 pathway (47). Nakaya et al. revealed that a lack of ASCT2 in naive $CD4^+$ T cells reduced the differentiation of Th1 and Th17 cells by attenuating the uptake of Gln and Leu to suppress mTORC1 activation (24). However, they were unable to identify which amino acid (Gln or Leu) intake was reduced as a result of the inhibition of mTORC1 activity brought on by ASCT2 knockout. Recently, Zhang et al. showed that blocking GLS1 promoted Th2 cell differentiation and inhibited Th17 cell differentiation through inactivating the mTORC1 pathway, but they did not notice any changes in Th1 cell differentiation (48). Similar finding was made by Nakaya et al. who discovered that reducing Gln consumption by eliminating ASCT2 could enhance Th2 cell differentiation (24). Taken together, even though the impact of Leu on the activation of mTORC1 has not been completely ruled out in recent studies, glutaminolysis does play a significant role in the differentiation of naive $CD4^+$ T cells by regulating mTORC1.

3.3 Regulating peripheral $CD4^+$ T cell differentiation *via* GSH

Under physiological conditions, glutathione exists mainly in two forms, reduced glutathione (GSH) and oxidized glutathione (GSSG), which can interconvert (49). Intracellular GSH is mainly produced by two pathways, *de novo* synthesis (via glutaminolysis) and recycling process (via the regeneration of GSH from GSSG) (49). The primary cellular antioxidant, GSH, which is primarily made up of Glu, Cys, and glycine (Gly), is responsible for preserving the redox balance in T cells (50). According to a prior research, increasing ROS by inhibiting GSH *de novo* synthesis but not recycling increased intracellular GSH production, which ultimately improved Treg cell differentiation and restricted Th17 cell differentiation (51). Also, they proved that glutaminolysis is the source of Glu, which powers *de novo* GSH production during the differentiation of Th17 cells (51). Furthermore, Miao et al. discovered that inhibiting GLS1-mediated glutaminolysis decreased intracellular GSH, which raised ROS levels to suppress RORγt expression, the key transcription factor for Th17 cell development (32). Subsequent mechanism research revealed that GSH produced from *de novo* synthesis buffers ROS to relieve its inhibition on mTORC1, inducing Th17 cell differentiation (52). As for Th1 cells, studies had shown that administration of GSH supplementation promoted Th1 cell differentiation at the time of viral invasion (53, 54). It has been reported that high levels of GSH can cause APCs to release more IL-12, which can help Th1 cells to differentiate (25). On the contrary, the consumption of GSH led to the decrease of IL-12 secretion, induced the production of IL-4, inhibited the production of Th1-related cytokines and/or promotes Th2-related reactions (55). Nevertheless, the detailed regulatory mechanisms by which GSH regulates Th1/Th2 cells differentiation are still unclear.

4 Intervention strategy to harness glutaminolysis for immunotherapy

The homeostasis of $CD4^+$ T cells is particularly important for the maintenance of organismal health, as several diseases have been linked to aberrant $CD4^+$ T cell differentiation. For example, when Th1 and Th17 cells are differentiated excessively while Treg cell differentiation is insufficient, hyper immunological illnesses such as autoimmune diseases, graft rejection, and irritability result (56, 57). Hence, reshaping the disordered $CD4^+$ T cell subsets is a simple and promising way to achieve immunotherapy. Specifically, intervention is required in humans to prevent the differentiation of peripheral naïve $CD4^+$ T cells into proinflammatory cells in hyper immunological disorders. As previously described, the glutaminolysis pathway is a proper site of intervention to regulate the differentiation of naïve $CD4^+$ T cells. In a nutshell, glutaminolysis is primarily separated into two steps: intracellular Gln uptake and progressive degradation. Therefore, from these features, intervention strategies to harness glutaminolysis for immunotherapy can also be developed (see Table 1 for details).

TABLE 1 Regulatory interventional strategies for glutaminolysis.

Strategy	Target	Regulator/Drug	Mechanism	Ref
Interference with Gln uptake	ASCT2	RNF5	Down-regulating ASCT2 expression by mediating ASCT2 ubiquitination	(58)
		Leptin	Inhibiting ASCT2 function by inhibiting Na ion flow	(59)
		Insulin	Up-regulating ASCT2 expression by activating ERK cascade	(60)
		MiR-137	Down-regulating ASCT2 expression by sponging with its mRNA	(61, 62)
		Benzyl-serine/cysteine/glycine, GPNA	Competitively inhibiting ASCT2 as Gln analog	(63–66)
		1,2,3-dithiazoles	Inhibiting ASCT2 function by forming mixed sulfide with Cys residue of protein	(67)
		TPT, RV, δT	Unknown	(68–70)
		Ab3-8, KM4008, KM4012, KM4018 and KM8094 mAbs	Inhibiting ASCT2 function though targeting cell surface domains of ASCT2	(71–73)
	SNAT1/2	ERK	Up-regulating SNAT1 and SNAT2 expression by activating ERK cascade	(23)
		GPNA	Competitively inhibiting SNAT1 and SNAT2 as Gln analog	(15, 74)
		MeAIB	Competitively inhibiting SNAT1 and SNAT2 as Gln analog	(75)
	SNAT2	Compound 12, V-9302	Unknown	(76)
	CBM complex	CARMA1	Down-regulating CARMA1 expression by its ubiquitination and phosphorylation	(77–79)
		BCL10	Down-regulating BCL10 expression by its ubiquitination and phosphorylation	(79, 80)
		MALT1	Down-regulating MALT1 expression by its ubiquitination and phosphorylation	(79)
		MALT1 inhibitor	Inhibiting CBM complex function by blocking MALT1	(81)
Interference with Gln enzymolysis	GLS1	MiR-145, miR-23a/b, miR-194 and miR-204	Inhibiting GLS1 expression through sponging 3'-UTR of GLS1 mRNA	(82–84)
		PPARγ	Down-regulating GLS1 gene expression by forming heterodimers with retinoid X receptor	(32, 85, 86)
		ICER	Enhancing its activity by binding to the GLS1 promoter directly	(29)
Interference with Gln enzymolysis	GLS1	HIF-1α	Up-regulating GLS1 expression *via* binding to hypoxia-responsive element in the gene	(85)
		DON	Inhibiting GLS1 activity through covalent modification of the ser286 site as Gln analog	(86)
		BPTES, apomorphine	Inhibiting GLS1 activity by stabilizing the inactive tetramer	(87, 88)
		compound 968, CB-839	Allosteric inhibitors of GLS1	(89, 90)
		ebselen	Inhibiting GLS1 activity by forming a selenyl sulfide (–Se–S–) bond with the cys residue of proteins	(88)
		chelerythrine	Inhibiting GLS1 activity by covalent modification of its imine moiety and the thiol group on proteins	(88)
	GOT1	AOA	Unknown	(30)
	GAC	BPTES	Inhibiting GAC activity by stabilizing the inactive tetramer	(87)

(Continued)

TABLE 1 Continued

Strategy	Target	Regulator/Drug	Mechanism	Ref
		compound 968, CB-839, compound 19, UPGL00004	Allosteric inhibitors of GAC	(89–92)
Simultaneous interference with Gln uptake and enzymolysis	ASCT2, GLS1	Rb	Down-regulating mRNA transcription of ASCT2 by inhibiting transcription factor E2F3; Directly inhibiting GLS1 expression	(93)
	ASCT2, SNAT1/2, GLS1	c-Myc	Proteomic finding; Up-regulating target gene expression by acting as a transcription factor probably	(94)

4.1 Interference with Gln uptake

4.1.1 ASCT2

ASCT2 is a homotrimer encoded by SLC1A5 gene, which is the main amino acid carrier for Gln transport into cells (95). Previous studies had revealed that inhibiting Gln uptake *via* targeting ASCT2 could lead to decreased differentiation of Th1 and Th17 cells meanwhile increased differentiation of Treg cells (24, 33). As a result, ASCT2 is a good candidate for intervention. Although the regulatory mechanism of ASCT2 is still unclear, the following methods have been described how to regulate ASCT2 *in vivo*. RNF5, a kind of E3 ubiquitin-protein ligase, can mediate the ubiquitination of ASCT2, leading to the down-regulated expression of ASCT2 (58). White adipocytes secrete a protein called leptin into the bloodstream, which is important for controlling energy homeostasis and can prevent Gln uptake by suppressing ASCT2 expression (59). In addition, it has been noted that insulin activates the ERK cascade to promote ASCT2-mediated Gln transport (60), suggesting that insulin antagonists may have some effect on ASCT2 inhibition. A potent class of non-coding RNAs known as microRNAs (miRNAs) controls gene expression by interacting with target mRNAs to either prevent their translation or promote their destruction (96). Studies have shown that miR-137 can bind to the mRNA of ASCT2, which in turn down-regulates the expression of ASCT2, inhibiting the Gln uptake (61, 62).

Currently, research on the pharmacological intervention of ASCT2 is a focus in addition to the regulatory targets of ASCT2. Benzyl-serine, benzyl-cysteine, and phenyl-glycine have all been reported to inhibit ASCT2 competitively, but they are not specific ASCT2 inhibitors because they also block the other transporters such as LAT1 and ASCT1 (63–65). L-γ-glutamyl-p-nitroanilide (GPNA), an analog of Gln, is a kind of non-specifical blockade of ASCT2 (66). Except for ASCT2 blockade, GPNA can also block SNAT1, SNAT2 and LAT1 (15, 74). A recent study showed that in a mouse asthma model based on ovalbumin, the administration of GPNA significantly alleviated the asthma state and reduced the level of inflammatory cells infiltration in the body (97). The thiol/thiolate groups of Cys are involved in covalent interactions with 1,2,3-dithiazoles, which in turn impede ASCT2 function (67). Besides, topotecan (TPT), resveratrol (RV) and δ-tocotrienol (δT) have also been reported to inhibit ASCT2 (68–70). Monoclonal antibody (mAb) development is another area of study. Studies have demonstrated that the mAbs Ab3-8, KM4008, KM4012, KM4018, and KM8094 can reduce Gln uptake by focusing on ASCT2's cell surface domains (71–73). In a word, since the majority of the currently available ASCT2 inhibitors are non-specific, which may contribute to inhibition of some other AATs, leading to deficiencies in the uptake of some other amino acids, new ASCT2 inhibitory medications must be developed.

4.1.2 SNAT1 and SNAT2

Targeting SNAT1 and SNAT2, encoded by SLC38A1 and SLC38A2 respectively, is the other intervention technique to reduce Gln uptake into naïve $CD4^+$ T cells because they are both capable of mediating Gln transport into cells (98). Even though the regulation mechanisms of SNAT proteins are currently less explored, the following processes also show some precedent significance. Previous research had shown that downstream ERK activation was enhanced after TCR activation, further resulting in the up-regulation of SNAT1 and SNAT2 to promote Gln uptake (23). Hence, targeting ERK cascades seems to be a promising intervention point to achieve the regulation of SNAT1 and SNAT2. Moreover, pharmacological inhibitors, such as GPNA, are also a direction of development. As previously mentioned, GPNA has the ability to non-specifically inhibit SNAT1 and SNAT2 (15, 74). Since SNAT1 and SNAT2 are believed to belong specifically to amino acid transport system A (ATA), N-methyl-aminoisobutyric acid (MeAIB), a substrate of ATA, can bind competitively with Gln to inhibit SNAT1 and SNAT2 (75, 98). Initially thought to be a competitive inhibitor of ASCT2, compound 12 and its isomer V-9302, derived from 2-amino-4-bis (aryloxybenzyl)aminobutanoic acids, were later identified as an inhibitor of SNAT2 as research advanced (76). Combined with the present study, the study of regulation of SNAT proteins is still lacking, and therefore, it will be a potential direction of research.

4.1.3 CBM complex

The CBM complex is composed of the scaffolding protein CARMA1, the adaptor protein BCL10, and the para-caspase enzyme MALT1 (99). It was previously shown that the CBM complex acted as a bridge to transmit the activated TCR signal to the downstream IKK/NF-κB and c-Jun N-terminal kinase (JNK) pathways, thereby causing T cell activation (100). Nakaya et al. found that knockout of any one of the constituent proteins of the CBM complex can result in attenuated Gln uptake and in particular loss of CARMA1 also resulted in down-regulation of ASCT2 mRNA levels both basal and after TCR stimulation (24). As a result, by assisting Gln to enter cells during signal transduction after

TCR stimulation, the CBM complex functions more as an intermediary bridge. The primary regulatory mechanisms for the CBM complex, according to the available reports, are its ubiquitination and phosphorylation (77). For example, CARMA1 may be ubiquitinated by the E3 ubiquitin-protein ligase CBL-b, which will subsequently cause it to be degraded (78). There are several CARMA1-related regulatory mechanisms of phosphorylation and ubiquitination, aside from the CBL-b, that have been well examined (for more information, see refs (77, 79)). Similar to CARMA1, these two methods equally regulate BCL10 and MALT1. For instance, the NEMO/IKKβ complex has the ability to phosphorylate BCL10 at Thr-81 and Ser-85, leading to BCL10 destruction through the lysosomal pathway (80). More relevant regulatory mechanisms about the ubiquitination and phosphorylation of CBM complex can be found in ref (79). Contrary to CARMA1 and BCL10, MALT1 has a strong foundation in pharmaceutical research because it is the only human para-caspase that has received significant attention as an immunomodulatory target for the treatment of autoimmune and inflammatory illnesses. To disturb MALT1, numerous compounds have been created, and drug clinical trials have even started (see (81) for more information). Overall, greater research in this area is worthwhile because the CBM complex appears to be a novel and promising intervention target for reducing the uptake of Gln.

4.2 Interference with Gln enzymolysis

4.2.1 GLS1

GLS1, located in mitochondria, is the first enzyme of glutaminolysis, which plays a role in regulating cell metabolism, maintaining cell redox balance and GSH biosynthesis (14). Previous research has demonstrated that blocking GLS1 could result in increased Treg cell differentiation and decreased Th17 cell differentiation (12, 32). Thus, in order to restore the balance of Th17/Treg cells in some disorders brought on by Th17 over-differentiation, targeting GLS1 may be a promising approach. GLS1 is a type of enzyme that can be regulated *in vivo* through a variety of ways. For example, by sponging the 3'-UTR of GLS1 mRNA, miR-145 and miR-23a/b can decrease the expression of GLS1 (82, 83). In addition, database mining research revealed that miR-194 and miR-204 might specifically target GLS1 and limit its expression (84). By forming heterodimers with the retinoid X receptor (RXR), the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) can regulate the expression of its target genes (101). Miao et al. revealed that PPARγ agonists could remold the balance of Th17/Treg cells *via* down-regulate GLS1 expression in dextran sulfate sodium (DSS)-induced colitis and house dust mite (HDM)/lipopolysaccharide (LPS)-induced asthma mouse models (32). Consistently, Yang et al. used Bergenin, a PPARγ agonist, to block the differentiation of naive $CD4^+$ T cells into Th17 cells by inhibiting GLS1-dependent glutaminolysis under Th17-polarizing condition, thus alleviating asthma in mouse model (102). The transcription factor inducible cAMP early repressor (ICER) had been shown to enhance its activity by binding to the GLS1 promoter directly, promoting the differentiation of Th17 cells (29). HIF-1α can also increase the expression of GLS1 *via* binding to the hypoxia-responsive element (HRE) in the GLS1 gene (85). Thus, inhibiting ICER and HIF-1α by exploring methods is also a reliable strategy for GLS1 blockade. Besides, a series of inhibitors have been developed to target GLS1. 6-diazo-5-oxo-L-norleucine (DON), a common GLS1 inhibitor, competitively inhibits GLS1 by acting as a substrate Gln analogue (86). Specifically, DON binds to the active site of GLS1 by covalently modifying the ser286 site, preventing GLS1 from functioning (86). Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES) is a GLS1 non-selective inhibitor, which can function by stabilizing the inactive tetramer (87). According to research, administering the GLS1 inhibitor BPTES reduced the excessive differentiation of Th17 cells in naïve $CD4^+$ T cells from SLE patients, which is consistent with the results of GLS1 conditional knockout in the experimental autoimmune encephalomyelitis mouse model (103). Besides, 5-[3-Bromo-4-(dimethylamino) phenyl]-2, 3, 5, 6-tetrahydro-2-dimethyl-benzo [a] phenanth-ridin-4 (1H)-one (namely compound 968), telaglenastat (CB-839), ebselen, chelerythrine and apomorphine are also GLS1 inhibitor reportedly (88–90). An example is that after intraperitoneal injection of GLS1 CB-839, the imbalance of Th1/Th2 and Th17/Treg was rectified, alleviating the SLE development (48).

4.2.2 Other enzymes

Except for GLS1, GAC, GLUD1, GOT1, GOT2, GPT2 and IDH1 are other metabolic enzymes of glutaminolysis, implying that they are also potential regulatory targets. For instance, by reshaping the balance between Th17 and Treg cells, selective inhibition of GOT1 with (aminooxy)acetic acid (AOA) ameliorates experimental autoimmune encephalomyelitis in mice (30). Several GLS1 inhibitors (BPTES, compound 968, CB-839), compound 19 and UPGL00004 have also been reported to inhibit GAC (87, 89–92). Besides, IDH1, the primary catalytic enzyme for 2-HG synthesis and a key regulator of naive $CD4^+$ T cell differentiation, has enormous promise as a target for therapeutic intervention. Therefore, more mechanistic research is required to establish the foundation for future target discovery for these downstream metabolic enzymes.

Nevertheless, current studies on the inhibition of Gln metabolizing enzymes mainly focus on Th17 and Treg cells. Inhibition of glutaminolysis enzymes GLS1 alone may cause aberrant Th1 cell differentiation, as revealed by Johnson (34). The reason might be related to the inhibition of GLS1, leading to excessive intracellular Gln accumulation, which might in turn undergo some substance exchange and biological reactions *via* certain amino acid transporters, but regretfully, they could not further design to support this theory. Thus, inhibiting Gln metabolizing enzymes alone may be a good treatment in diseases brought on by abnormalities of Th17/Treg cells, but it still requires additional research in conditions where Th1 cells are predominate.

4.3 Simultaneous interference with Gln uptake and enzymolysis

Both glutamine uptake and metabolic catabolic enzymes can be regulated simultaneously *in vivo* in addition to being targeted separately. According to reports, the RB transcriptional corepressor (Rb) can adversely regulate both ASCT2 and GLS1 expression at the same time. Deletion of Rb can both increase ASCT2 mRNA transcription through an E2F3-dependent mechanism and directly suppress GLS1 expression (93). The function of the c-Myc protein in T cells, which functions as a genetic switch to regulate a number of cellular metabolisms, has been discovered in recent years. Studies had revealed that upon TCR activation, metabolic reprograming of T cells occurred *via* up-regulating c-Myc (94, 104). Moreover, it is found that up-regulated c-Myc in T cells could cause the up-regulated expression of ASCT2, SNAT1, SNAT2 and LAT1 by proteomic analysis (94). Therefore, these data suggest that simultaneous inhibition of Gln uptake and enzymolysis can be achieved by inhibiting c-Myc. Previous studies had shown that immune checkpoints such as CTLA-4, PD-L1 in tumor cell could prevent TCR activation and, therefore further inhibiting downstream metabolic reprogramming (105). In this regard, designing to up-regulate these expressions in immune-excessive non-neoplastic diseases is perhaps also a therapeutic strategy *via* blocking the Gln uptake and enzymolysis in T cells.

5 Conclusion

In conclusion, glutaminolysis plays an irreplaceable role during the differentiation of peripheral $CD4^+$ T cells. Upon TCR activation, naïve $CD4^+$ T cells start taking up Gln in large amounts to promote Th1, Th17 cell differentiation and inhibit Treg cell differentiation through several mechanisms including epigenetic regulation, mTORC1 activation and GSH pathway. After inhibition of glutaminolysis, there was an opposite trend in peripheral naïve $CD4^+$ T cell differentiation. Therefore, regulation of peripheral Th1, Th17, and Treg cell differentiation by intervening glutaminolysis in naïve $CD4^+$ T cells shows great potential to be exploited in immune-excessive diseases. Besides, in other diseases such as tumor, as an infinitely proliferating cell, it requires a large amount of Gln uptake and decomposition, as do anti-tumor $CD4^+$ T cells such as Th1 and Th17 cells. So, although inhibition of Glutaminolysis in tumor cells is effective, it has not completely cleared the tumor lesions, which may be related to the tendency of $CD4^+$ T differentiation changes to Treg after Glutaminolysis inhibition. Meanwhile, although there are many studies focusing on glutaminolysis and naïve $CD4^+$ T cell differentiation, most of these studies focused on mouse cells *in vitro*. Studies on human naïve $CD4^+$ T cell differentiation are still relatively lacking, and this is perhaps an area in which we deserve to explore in-depth. Moreover, at present, there are a few related reports on the regulation of Th2 cell differentiation by glutaminolysis, and how it regulates Th2 cell differentiation is still unknown, thus it is necessary to carry out related researches. Taken together, we summarized the existing studies, concluding several different interventional strategies for glutaminolysis. These strategies are currently mostly used in tumor diseases, and their application in inflammatory diseases still needs to be experimentally confirmed. Therefore, we expect to be able to guide directions on how to appropriately utilize glutaminolysis for future basic research and clinical applications in inflammatory diseases.

Author contributions

TL, SR, CS drafted manuscript, have contributed equally to this work and should be considered co-first authors. PZ helped in literature searching. HW provided administrative and financial support, manuscript revision and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The nutrition and immunity (nutrIMM) study: protocol for a non-randomized, four-arm parallel-group, controlled feeding trial investigating immune function in obesity and type 2 diabetes

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Introduction: Individuals with obesity and/or type 2 diabetes are at higher risk of infection and have worse prognoses compared to healthy individuals. Several factors may influence immune responses in this population, including high adiposity, hyperglycemia, and unhealthy dietary habits. However, there is insufficient data on the independent or clustered contribution of these factors to obesity-related immune dysfunction, especially accounting for dietary intake. This study aims to establish the independent contribution of obesity and hyperglycemia to immune dysfunction independent of diet in adults with and without obesity with or without type 2 diabetes.

Methods: The Nutrition and Immunity (nutrIMM) study is a single-centre, non-randomized, four-arm, parallel-group, controlled feeding trial. It will enroll adults without obesity (Lean-NG) and with obesity and three metabolic phenotypes of normoglycemia, glucose intolerance, and type 2 diabetes. Participants will be assigned to one of four groups and will consume a standard North American-type diet for 4 weeks. The primary outcomes are plasma concentration of C-reactive protein and concentration of *ex-vivo* interleukin-2 secreted upon stimulation of T cells with phytohemagglutinin.

Discussion: This will be the first controlled feeding study examining the contribution of obesity, hyperglycemia, and diet on systemic inflammation, immune cell phenotype, and function in adults of both sexes. Results of this clinical trial can ultimately be used to develop personalized dietary strategies to optimize immune function in individuals with obesity with different immune and metabolic profiles.

Clinical trial registration: ClinicalTrials.gov, identifier NCT04291391.

KEYWORDS

immunology, hyperglycemia, nutrition, obesity, type 2 diabetes, insulin resistance, North American diet

1. Introduction

The immune system is a highly complex network that comprises molecules, cells, organs, and tissues, with the primary function of defending the host against pathogens (1). It consists of two major branches: innate and adaptive immunity, which work together to recognize foreign invaders and mount an effective immune response (2). Several factors can affect the resistance of an individual to infection, such as genetics (3, 4), age (5, 6), sex (7, 8), physical activity (9, 10), stress (11, 12), smoking (13, 14), alcohol consumption (15, 16), medications (17, 18), nutrition (19, 20), and obesity (21, 22) as previously reviewed (23).

Obesity is known to cause chronic low-grade systemic inflammation (24), which is characterized by an increased systemic concentration of acute phase proteins and cytokines, including C-reactive protein (CRP), IL-6, IL-18, and TNF-α (25–27). There is an increased release of pro-inflammatory cytokines and chemokines from adipose tissue in the presence of obesity (28, 29). This state leads to excessive recruitment and infiltration of pro-inflammatory immune cells into adipose tissue, such as M1-like polarized macrophages (30), Th1 (31, 32) and Th17 cells (33), and cytotoxic T cells (34), while reducing the number of immune cells that have anti-inflammatory properties, such as T regulatory cells (35, 36). These changes in the immune system can contribute to the development of chronic diseases, including insulin resistance and type 2 diabetes (T2D) (37, 38).

Studies have shown that obesity and T2D are associated with an increased risk of common infections from both bacterial and viral sources (39, 40). However, the mechanisms responsible for this increased risk are not fully understood. Previous research has focused on alterations in the immune system mediated by adipose tissue in obesity and/or T2D, rather than on immune function (41, 42). Recently, it was discovered that T2D is associated with additional perturbations in immune function, independent of obesity status (43). Obese individuals with T2D exhibit impaired neutrophil function and T cell response upon stimulation compared to BMI-matched normoglycemic (NG) obese individuals, despite having more activated Th cells. However, further studies are needed to determine whether the immune function of the Obese-NG group is comparable to that of lean control healthy subjects (43).

Acute inflammatory responses to dietary challenges, such as the oral glucose tolerance test (OGTT), have been proposed as a sensitive indicator of the impact of glycemia on immune responses (23, 27, 44). The OGTT induces a rise in glucose, insulin, and a transient inflammatory response (45–50), which tends to be stronger and more extended in individuals with obese and T2D (51, 52). *In vivo* studies have demonstrated that hyperglycemia increases the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, by leukocytes (53). Furthermore, after an OGTT, T2D patients had an increased proportion of neutrophils and monocytes over time compared to healthy controls, and the monocyte-AUC correlated positively with the glucose-AUC (54). These findings suggest that glycemia plays a role in immune activation, and further investigation is needed to understand the impact of glycemia on immune responses in individuals with obesity.

Nutrition plays a crucial role in the prevention and treatment of health conditions that have an inflammatory component, such as obesity (55, 56). Saturated fatty acids (SFA) have been shown to have pro-inflammatory properties, while fiber, antioxidants, vitamins, and polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), generally exert anti-inflammatory and/or immunosuppressive effects in the presence of chronic inflammation as previously reviewed (20). Choline is another nutrient that has been shown to be essential for optimal immune function (57), improving the T cell response to immune challenges by increasing IL-2 secretion (58, 59). These findings suggest that specific nutrients in the diet could have a beneficial effect on immune function, particularly in the context of obesity.

Despite the association between obesity, hyperglycemia/T2D, elevated systemic inflammation, and impaired immune cell response, it remains unclear whether these abnormalities are caused by excess adiposity, dysglycemia, or poor food habits associated with obesity. To dissect the impact of excess body fat and glycemia on immune function, it is crucial to account for food intake by performing controlled feeding studies in humans. Therefore, a comprehensive analysis of systemic inflammation and immune function in healthy lean individuals (Lean-NG), individuals with obesity and normoglycemia (Obese-NG), glucose intolerance (Obese-GI) or T2D (Obese-T2D) is required to understand the independent contribution of excess body fat and glycemia on immune function.

2. Methods

2.1. Study objectives and hypothesis

The study objectives are:

1. To establish the independent contribution of obesity and glycemia, independent of diet, on systemic inflammation and immune function outcomes. We hypothesize that systemic inflammation will increase and T cell function, characterized by *ex vivo* IL-2 secretion upon phytohemagglutinin (PHA) stimulation, will decrease progressively from the Lean-NG to Obese-T2D group.
2. To determine the role of glycemia on postprandial inflammatory and immune responses using an OGTT. We hypothesize that a higher glucose AUC will be associated with increased inflammatory responses, a higher proportion of activation markers expressed on immune cells, and a lower proliferation rate by PBMC upon stimulation with anti-CD3/CD28 in Lean-NG, Obese-NG, Obese-GI, and Obese-T2D individuals.
3. To explore the relationship between diet, metabolism, and immune function. We hypothesize that a higher habitual consumption of DHA and choline and lower consumption of SFA, will be associated with enhanced immune function and lower levels of systemic inflammation. Specifically, a higher proportion of DHA in the membranes of RBC will be associated with improved Th1 response (i.e., IFN-γ and TNF-α), despite similar production of IL-2 upon immune challenge, whereas systemic inflammatory markers will be lower. Higher consumption of choline in the form of phosphatidylcholine will improve IL-2 production upon T cell stimulation. Other dietary components (e.g., fiber, antioxidants, and vitamins) will

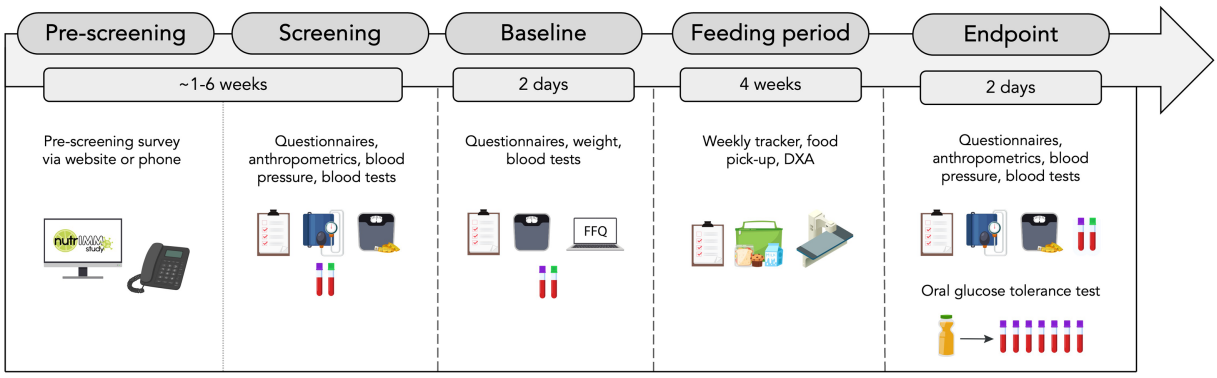

FIGURE 1
Overview of study design. FFQ, food frequency questionnaire and DXA, dual-energy X-ray absorptiometry.

be associated with enhanced immune function in individuals with and without obesity with varying levels of glycemia.

2.2. Outcomes

The primary outcomes of this study are the concentration of IL-2 in the supernatant of *ex vivo* stimulated cells, which is a surrogate marker of T cell proliferation, and circulating concentrations of CRP.

2.3. Study design

The NutrIMM study is a prospective, non-randomized, four-arm, parallel-group, unicentre, controlled-feeding trial conducted at the Human Nutrition Research Unit (HNRU), University of Alberta (Edmonton, AB, Canada). A total of one-hundred and twenty-eight participants aged between 18 and 70 years will be assigned with a 1:1 male to female ratio to one of four groups ($n = 32$ per group): Lean-NG, Obese-NG, Obese-GI, Obese-T2D. All participants will be followed up for a total of 4 weeks from the date of group allocation. It will not be possible to blind participants or researchers due to the inclusion specifications of the groups. An overview of the study design is shown in Figure 1.

2.4. Ethical aspects

The research protocol was approved by the University of Alberta Ethics Board (Pro00085839) and follows the standards proposed by the Canadian Tri-Council Policy statement on the use of human participants in research. This protocol was developed according to the Standard Protocol Items: Recommendation for Interventional Trials (SPIRIT) (60) and the Template for Intervention Description and Replication (TIDieR) (61). All participants will sign an informed consent document approved by the Human Research Ethics Board of the University of Alberta for study participation and to optionally provide stool samples for a sub-study to investigate the role of gut microbiota on immune function (Supplementary File 1). Participants will sign an additional consent form to select if they allow their biological specimens to be stored for up to 20 years for use in ancillary studies.

2.5. Recruitment and enrolment of participants

2.5.1. Recruitment

The trial will recruit males and females from the Edmonton Metropolitan Region via the traditional media (newspaper, radio, and TV), word of mouth, institutional websites, social media advertisements, and posters placed on notice boards at the University of Alberta, obesity clinics, and other facilities from the surrounding area of Edmonton. A website and social media accounts (Instagram and Facebook) with the study information were also developed to support recruitment.[1] Individuals can be included in the study if they meet all inclusion criteria and no exclusion criteria. All participants will be matched by age and sex, if possible, and participants with obesity will be matched for BMI and/or waist circumference. All females in reproductive stage which are not using contraceptive methods will be tested during the same follicular phase of their menstrual cycle (day 2 to 9), to account for immunometabolic changes, considering 4 weeks the mean duration of a menstrual cycle (62, 63). Participants will be allocated to one of four groups according to specific criteria for BMI, waist circumference, blood pressure, fasting glucose, hemoglobin A1c (HbA1c), triglycerides, and high-density lipoprotein-cholesterol (HDL-C) (Table 1). The cut off points used to identify obesity, cardiometabolic risk factors, and T2D were based on the World Health Organization (64), National Cholesterol Education Program-Adults Treatment Panel III criteria for metabolic syndrome (65) and Diabetes Canada (66), respectively.

1 nutrimm.ualberta.ca

TABLE 1 Eligibility criteria per group of adults.

Criteria	Lean-NG	Obese-NG	Obese-GI	Obese-T2D
BMI (kg/m^2)	18.5–24.9 (±0.5)	≥ 30 (±0.5)		
Waist circumference (cm)	Males <102; Females <88	Males ≥102; Females ≥88		
Fasting blood glucose (mmol/L)[a]	<5.6		5.6–6.9	≥ 7.0
HbA1c (%)	<5.5		5.5–6.4	≥ 6.5[b]
BP (SBP/DBP, mmHg)	<130/85		NR	
Triglycerides (mmol/L)	<1.7		NR	
HDL-C (mmol/L)	Males ≥1.03; Females ≥1.29		NR	

BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C, high density lipoprotein-cholesterol; NR, not required in it that could be normal or elevated; SBP, systolic blood pressure; T2D, type 2 diabetes.
[a]If participants are at the upper or lower limits for fasting glucose, HbA1c will be used for group allocation.
[b]Or diagnosis of type 2 diabetes and use of medication.

2.5.2. Inclusion criteria

- age of 18 years to 70 years;
- body weight stable (± 3%) for at least 3 months prior to study commencement;
- BMI (1) between 18.5 and 24.9 (± 0.5) kg/m^2 or (2) between 30 and 50 kg/m^2 (± 0.5) kg/m^2 or waist circumference > 88 cm or > 102 cm for females and males, respectively;
- See Table 1 for specific group allocation criteria based on glucose, HbA1c, HDL-C, and triglycerides.

2.5.3. Exclusion criteria

- current or recent history cardiovascular diseases or events (e.g., ischemic, rheumatic, or congenital heart disease, stroke, peripheral vascular disease, heart failure, familial hypercholesterolemia or other monogenic dyslipidemia), use of cardiac implantable electronic devices;
- current or recent cancer, including remission, during the last 5 years;
- diseases known to affect the immune system, such as infectious, inflammatory, and autoimmune diseases or autoimmune-related or suspected conditions (e.g., T1D, systemic lupus erythematosus, inflammatory bowel disease), except for psoriasis, atopic dermatitis, and rheumatoid arthritis. Continuous use of anti-inflammatory or immunosuppressant drugs and supplements for which washout is not possible, except for medications which participants with obesity could not refrain from (e.g., baby aspirins);
- renal disorders, endocrine disorders other than T2D (e.g., acromegaly, Addison's disease, Cushing's disease);
- untreated or uncontrolled thyroid diseases (e.g., Hashimoto's disease, hypothyroidism, hyperthyroidism);
- known allergy, aversion to any components of the menu, or restricted dietary patterns (e.g., gluten-free diet, vegetarianism, kosher or halal diets) for which accommodations within the menu are not possible;
- participants under titration of their medication or initiating a new treatment or HbA1c >10.5%
- women who are pregnant or plan to become pregnant during the study duration, who are lactating, who have an irregular menstrual cycle or are in perimenopause;
- regular recreational use of cannabis;
- taking part in any other intervention study that might affect the outcomes of the current study.

2.5.4. Enrolment

Potential participants will complete a pre-screening and screening visit before entering the study. The pre-screening consists of a structured questionnaire, which can be completed online or through the telephone (Supplementary File 2). All pre-screening data is stored on the web-based software Research Electronic Data Capture (REDCap) (67). The study coordinator follows up with interested individuals by email and/or telephone to schedule their screening visit or let them know in case they are not eligible. At the screening visit, a trained research staff member (e.g., research coordinator, graduate students, investigators) will obtain written consent from interested individuals to confirm their eligibility by checking every inclusion and exclusion criteria. This visit includes blood tests, anthropometric measurements, and the completion of questionnaires regarding demographics, health, use of medications, and physical activity. During or after the screening visit, participants will not be enrolled in the study if they decline to participate after knowing more study details or if they violate any inclusion criteria. The enrollment period might generally vary from 3 days to 5 weeks depending on the necessity of a washout before study commencement or the availability of participants. The schedule of study visits and procedures are depicted in Figure 2.

2.6. Withdrawal of participants

Participants may be withdrawn from the trial either at their own request or at the discretion of the principal investigator if not compliant with the menu. Participants will be made aware via the information sheet and consent form that data collected up to the withdrawal date may still be used in the final analysis. If participants have consumed the control diet for at least 2 weeks, they will be asked if they would like to provide a blood sample before withdrawal. The reason for and date of withdrawal will be recorded on an electronic spreadsheet.

	STUDY PERIOD						
	Enrolment	Allocation	Post-allocation/ Intervention				Close-out
TIMEPOINT	Screening	Post-screening	Week1	Week 2	Week 3	Week 4	End of week 4
Eligibility screen	X						
Informed consent	X						
Questionnaires	X		X	X	X	X	X
Anthropometry	X		X	X	X	X	X
Blood pressure	X						X
Blood tests	X						X
Allocation		X					
Isocaloric diet			←	—	—	→	
Habitual dietary intake			X				
Physical activity level			X				X
Gastrointestinal symptoms			X				X
Body composition			X				
Metabolic blood markers			X				X
Systemic inflammatory markers			X				X
Immune cell phenotype			X				X
Immune cell function			X				X

FIGURE 2
Schedule of enrolment, interventions, and assessments (SPIRIT figure).

2.7. Intervention

2.7.1. Concomitant care and pre intervention requirements

The administration of vaccines, application of botulinum toxin, use of antibiotics, supplements or other natural products, and blood donation is not permitted throughout the intervention and a four-week washout is necessary prior to study commencement. For recent surgical procedures, approximately 4 months or less, inclusion will be at the discretion of the principal investigator depending on the magnitude of the procedure. Participants should continue to take their regular medications [e.g., antilipemic (fenofibrate and atorvastatin), blood pressure-lowering (amlodipine, enalapril), antidepressants (escitalopram, bupropion, and amitriptyline), and glucose-lowering (metformin, insulin)]. Participants will be asked to refrain from performing vigorous physical activity, taking anti-inflammatory medications, and drinking alcohol 3 days prior to baseline study visits.

2.7.2. Standardized diet

Participants will all consume an isocaloric standardized North American type diet for 4 weeks. All meals will be provided to participants for an optimal control of energy and nutrient intake. A seven-day cyclic menu was designed using The Food Processor Nutrition Analysis Software (v.11.0.3, ESHA Research, Salem, OR, 2015) to reflect as closely as possible current macronutrient intake averages in North America (68–70) with approximately 35% of energy as fat, 12.5% as SFA, 13% as monounsaturated fatty acids, 6% as PUFA, 48% energy as carbohydrates with a significant proportion of these coming from foods containing refined sugars, and 17% as proteins mostly from meat, meat products and dairy products. The breakfast meal represents approximately 20% of the daily energy intake whereas the lunch and dinner meals each provides 40% of daily energy intake (Supplementary File 3). Participants will be instructed to consume their meals entirely but will have the opportunity to spread out their food throughout the day. Participants will have free access to water and other non-caloric beverages during the feeding period according to their habitual consumption (or nearly calorie-free drinks, i.e., black coffee or tea). Adaptations will be made for participants with lactose intolerance, including the use of lactose-free milk, lactase, or fortified vegetable beverage (soy or rice), and for individuals with an aversion to vegetables or other meals of the menu when an adequate substitute based on nutrient composition can be provided.

2.7.3. Estimate of energy requirements

Total energy expenditure of participants will be estimated using the Mifflin St. Jeor equation and metabolic equivalents (MET) to estimate physical activity level using a template spreadsheet by Gerrior et al. (71). If the desired MET was not available in the spreadsheet, they were inserted according to the 2011 Compendium of Physical Activities (72). Energy intake will then be prescribed for each participant according to their individual estimate of total energy expenditure. The energy content of the diet will be revised weekly according to body weight fluctuations throughout the intervention period. If body weight has a significant slope (i.e., increasing or decreasing) at a rate that yields

a change ≥3% during the first 2 weeks, adjustments to the energy content of the diet will be made to offset changes in body weight and keep it constant for the remaining of the study period. Participants will be instructed to maintain their usual physical activity level throughout the intervention period except for the 3 days preceding blood sampling, during which they will be asked to refrain from intense physical exercise.

2.7.4. Food preparation and delivery

All menu items (Supplementary File 4) will be prepared and portioned at the metabolic kitchen of the HNRU in accordance with the food safety standards and guidelines set by the Alberta Health Services. All food handlers have received training on food safety. Meals are prepared weekly and in batches. Batch cooking will be used to optimize weekly meal preparation and delivery to participants. Samples (200 g) of food cooked in batches will be saved and frozen up to 3 months after the last batch is consumed in case food safety analysis is needed. Preparation of high-risk foods (e.g., containing eggs or meat) will be temperature-controlled and recorded in a form. Meals will be prepared from Monday to Thursday and batch cooked meals are thawed overnight at 4°C on the day before portioning (Sunday–Wednesday). Meals will be placed into reusable ready to eat containers that are microwavable and dishwasher safe, sealable plastic bags, or disposable containers. All menu items are labeled, packed into reusable thermal bags, and maintained at 4°C until pick-up time. Food will be individually delivered by a trained study staff, including graduate students or other faculty members of the study team. Participants will come to the research unit to pick up their food every other day and on Friday they receive meals for Saturday, Sunday, and Monday. Participants will be instructed and will sign an agreement to store their food at 4°C within one-hour time frame and to properly re-heat it before consumption. Reusable items will be sanitized using a 200 ppm chlorine solution prepared daily (i.e., thermal bag and ice packs) or at 82° C using a commercial dishwasher (Moyer Diebel Ltd. 501HT-70, Winston Salem, NC, United States) (i.e., food containers).

2.8. Follow-up and extra visits

Participants will be followed up to three times a week during their food-pick up. This visit takes approximately 10 min and includes body weight measurement, follow-up regarding compliance, scheduling of next visits, delivery of thermal bag containing meals and frozen reusable ice packs, and collection of reusable items (i.e., empty containers and ice packs) from participants. Additional site monitoring visits may be scheduled in case participants require additional assistance from the study team to complete the food frequency questionnaire FFQ, which is used to assess habitual dietary intake. Participants will be able to discuss any aspect of the study with the trial manager, including their blood tests, body composition, and dietary assessment results. To help retain participants on the study, they will receive emails or phone calls to support them throughout the duration of the study and will be reminded about their scheduled visits.

2.9. Data collection

2.9.1. Compliance

The participant will complete each week a menu and health tracker (Supplementary File 5), in which they will register their food consumption, medications, and symptoms. Before the study commencement, participants will be instructed to only consume the meals provided by the study team and they will be encouraged to do so during weekly visits. Regardless, the tracker provides a space to indicate unlisted food items that will have been consumed in addition to the formulated diet. Compliance will be determined by calculating the number (and proportion) of food items consumed that were provided and those consumed in addition. Participants who cannot comply to ≥90% of the feeding protocol will be excluded from the study. Participants will also be asked to record any medications (including over the counter medications) that were taken during the study period to account for any changes in inflammation. Participants will be asked to avoid if possible, taking any anti-inflammatory medications, and use acetaminophen instead if able. Regarding gastrointestinal tolerance, participants will complete a gastrointestinal tolerance questionnaire at the beginning and end of the study, to evaluate the effect of the diet on the GI system.

2.9.2. Anthropometric measures, body composition, and blood pressure

Anthropometric measurements including weight, height, waist, and hip circumference will be assessed in duplicate according to standardized procedures (73, 74). Body weight will be measured to the nearest 0.1 kg using a calibrated digital scale (Health o meter® Professional Remote Display, Sunbeam Products Inc., Fla., United States). Body weight will be recorded every other day at food pick-ups throughout the feeding period. Height is measured to the nearest 0.1 cm using a 235 Heightronic Digital Stadiometer (Quick Medical, North Bend, Wash., United States). Waist and hip circumference are measured to the nearest 0.1 cm, using a measuring tape (Hoechstmass®, Sulbzbach, Hesse, Germany).

Body composition is assessed once during the study by dual-energy X-ray absorptiometry (DXA) using a General Electric Lunar iDXA with encore 13.60 software (General Electric Company, Madison, Wis., United States), as described (75). This scan provides compartmentalized and whole-body data on fat mass (FM), lean soft tissue (LST), and bone mineral content (BMC). The coefficients of variation of this device for FM (%), FM (g), LST (g), and BMC (g) are 1.05, 0.99, 0.37, and 0.40%, respectively. The assessment is performed by a certified technologist. All females at premenopausal who are not using contraceptive methods will perform a rapid urine human chorionic gonadotropin immunoassay to confirm non-pregnant status before scanning.

Systolic and diastolic blood pressures will be measured in the left arm after a 10 min rest in the sitting position using a calibrated automatic blood pressure monitor (Spot Vital Signs®, Welch Allyn®, Skaneateles Falls, NY, United States).

2.9.3. Physical activity and habitual diet

Participants will be required to maintain their current physical activity levels throughout the study period. They will complete the self-administered International Physical Activity Questionnaire (IPAQ) – Short Form, at baseline and post-intervention. The IPAQ inquires about time spent sitting, walking (3.3 METs), and doing moderate (4.0 METs) and vigorous-intensity (8.0 METs) activities over the past seven consecutive days. All continuous scores are expressed in MET-minutes/week and used to categorize physical activity level as

low, moderate, or high. The IPAQ is a reliable questionnaire, and it has been validated in different settings across multiple countries (76).

Habitual dietary intake will be assessed at baseline using the Diet History Questionnaire III (DHQIII) (77, 78). The DHQIII is a validated food frequency questionnaire and will be administered to participants before the start of the study to estimate their habitual dietary intake of the past 4 weeks. We have selected the option to inquire about the food intake of the previous month to minimize recall bias as compared to covering the previous year.

2.9.4. Fasting blood collection

Blood will be sampled from participants by venipuncture at five time points, after a 9–12 h overnight fast, by a trained phlebotomist. Blood sample is collected into BD Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, United States), with spray-coated lithium heparin and a polymer gel or spray-coated K_2-EDTA for plasma separation.

At the screening visit (Visit 1), blood samples (~ 8.5 mL) will be sent to the Alberta Precision Labs (Edmonton, AB, Canada) immediately after collection and are analyzed for HbA1c (4 mL K_2-EDTA tube) or fasting glucose, triglycerides, total cholesterol, and HDL-c (4.5 mL lithium heparin tube). These analyses will be repeated at the baseline visit in case it is more than 2 weeks apart from the screening visit.

At baseline (visit 2 and 3) and final visits (visit 4 and 5), one K_2-EDTA tube (~ 4 mL) will be analyzed for complete blood count and differential and HbA1c, and one lithium heparin tube (~ 4.5 mL) will be analyzed for glucose, insulin, lipid panel, and CRP in two consecutive days, to preclude an ongoing infection (i.e., CRP >10 mg/L), by Alberta Precision Labs (as above). Four K_2EDTA tubes (~ 24 mL) will be analyzed in-house. Samples are kept at 4°C until being centrifuged at 1811 g for 10 min at 22°C. Plasma will be aliquoted for storage at -80° C for subsequent analysis. The remaining buffy coat (leukocytes and platelets) and erythrocytes are resuspended with 3 mL of 1% bovine serum albumin (BSA, Sigma-Aldrich, Co., St. Louis, MO, United States) in phosphate-buffered saline (PBS) and layered onto Ficoll-Paque (HISTOPAQUE®-1,077, Sigma-Aldrich, as above), then centrifuged at 1558 g for 30 min with no brake at 20°C for PBMC isolation using density centrifugation. The lymphocyte band at the gradient interface of 1% BSA in PBS and Ficoll-Paque is transferred to a 50 mL conical tube, brought to 30 mL with 1% BSA in PBS, and centrifuged at 478 g for 5 min at 4 ° C to pellet PBMCs. Supernatant is discarded and PBMCs are then washed with 10 mL 1% BSA in PBS and centrifuged as previously. PBMCs are resuspended in a cryopreservation solution containing Roswell Park Memorial Institute (RPMI) 1,640 medium (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 20% (v/v) fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, United States), 25 mmoL/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Corning, Manassas, VA, United States), 2.5 µmol/L 2-mercaptoethanol (Thermo Fisher Scientific, Grand Island, NY, United States), and 1% (v/v) antibiotic/antimycotic solution (AB/AM, Sigma-Aldrich, as above) with 10% (v/v) dimethyl sulfoxide (DMSO, MP Biomedicals, Solon, OH, United States) added before use. PBMCs are then aliquoted to count using the trypan blue (diluted 1:1 with ddH_2O, Corning, as above) membrane dye exclusion method using a 1:1 ratio of cell suspension to trypan blue. The remaining cell suspension is transferred to cryovials in 1 mL aliquots and immediately frozen at -80° C using a freezing container (Mr. Frosty™ Cryo 1°C, Nalgene®, Rochester, NY, United States). PBMCs are transferred to liquid nitrogen the next day for long-term storage. The RBC fraction is washed with 5 mL 0.9% saline and centrifuged at 453 g for 5 min at 4°C. Supernatant is discarded and RBCs are washed and centrifuged as previously. RBCs are lysed by bringing to 3 mL with double-distilled water (ddH_2O), then stored at -80° C for subsequent lipid analysis.

2.9.5. Oral glucose tolerance test

The OGTT will be conducted once at the end of the feeding protocol. After a 9–12 h fast, an intravenous (IV) catheter will be inserted into a forearm vein for blood sampling by an experienced licensed practical nurse. After the IV catheter will be inserted, it will be flushed with saline before the first collection, and following each blood draw to ensure patency and rinse blood out of the catheter. Prior to each blood draw, a discard tube will be filled to a volume of at least 2 mL to clear the saline of the IV line. Blood samples will be collected into BD Vacutainer® tubes (Becton, Dickinson and Company, as above), with spray-coated K_2-EDTA for plasma separation. After the first blood collection (time 0), participants will be given up to 5 min to drink a beverage (296 mL) containing 75 g of dextrose (Trutol® Glucose Tolerance Test Beverage). Then, blood samples will be taken every 30 min (±5 min) up to 180 min. For each time-point, ~ 6 mL of blood will be drawn. The IV site will be regularly monitored for phlebitis according to the Visual Infusion Phlebitis Score. During the test, participants will be allowed to drink water *ad libitum*. Plasma separation and PBMC isolation will be carried as described for fasting samples.

2.9.6. Stool collection

Fecal samples are collected by participants at home or on-site before the start of the study diet and at the end of the study. The collection kit, which includes a collection device (FecesCatcher, TAG HEMI) and a fecal collection tube (DNA/RNA Shield™, Zymo Research Corp, United States) will be provided in a sealed plastic bag along with the instructions for proper handling of samples. Fecal samples will be kept at room temperature until brought to the research unit and will be stored at -80° C for subsequent analysis.

2.9.7. Biochemical analysis

2.9.7.1. Cardiometabolic risk factors/ systemic inflammation

Biochemical analysis performed by Alberta Precision Labs includes (1) enzymatic colorimetric assays for triglycerides, total cholesterol, and HDL-C; (2) UV testing using an enzymatic reference method with hexokinase to quantity glucose; (3) an immunoturbidimetric assay for quantification of CRP, all using an automated photometric analyzer (Roche Cobas c503); (4) an electrochemiluminescence immunoassay to determine insulin using an automated immunology analyzer (Roche Cobas e801); and (5) a turbidimetric inhibition immunoassay for hemolyzed whole blood to determine HbA1c using an analyzer designed specifically for HbA1c quantification (Roche Cobas c513). Low-density lipoprotein-cholesterol (LDL-C) is calculated using the following equation: $Total\ cholesterol - HDLc - \left(Triglycerides \div 2.2\right)$. Non-HDL-C is derived from the calculation of total cholesterol minus HDL-C. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index will be calculated using the equation: fasting insulin (microU/L) × fasting glucose (nmol/L)/22.5 (79).

Fasting and post-OGTT levels of circulating cytokines, chemokines, and soluble adhesion molecules will be measured using multiplex assay kits according to the instructions of the manufacturer. All samples will be run in batches with all timepoints per participant on the same plate to minimize variation. Post-OGTT plasma glucose will be measured using a clinical chemistry analyzer (Abbott ARCHITECT c4000, Canon Medical Systems Corporation, Otawara, Tochigi, Japan). The machine is calibrated and compared to controls based off manufacturer recommendations with a CV <5%.

2.9.7.2. Immune cell phenotype analysis

Fasting complete blood count and differential will be analyzed in whole blood by fluorescent flow cytometry using an automated hematology analyzer (Sysmex XN10) by Alberta Precision Labs. Immune cell subsets from fresh whole blood are identified in-house by direct immunofluorescence assay. Briefly, 96-well V-bottom plates (Costar®, Kennebunk, ME, United States) are pre-conditioned with 200 µL 5% FCS in PBS with AB/AM (IF buffer) for at least 30 min before adding 100 µL of whole blood. The RBCs are lysed using 200 µL of 1X RBC lysis buffer (BioLegend, San Diego, CA, United States). After a 15 min incubation at room temperature, the plate is centrifuged at 402 g for 10 min at 10°C to pellet RBCs. Supernatant is discarded using needle aspiration and RBCs are lysed as previously, incubated for 5–10 min, and plate is centrifuged. If RBC are still evident, a third lyse (5–10 min) will be performed. After RBC lysis, supernatant is discarded and immune cells are washed twice with 200 µL IF buffer and incubated for 30–60 min at 4°C in the dark with a mix of fluorophore conjugated antibodies (except FOXP3) to characterize immune cell phenotypes. The antibodies conjugates, staining reagents, and the corresponding multicolour flow cytometry panels designed to identify immune cell subsets and activation markers [(1) T reg, (2) T cell A, (3) T cell B, (4) B cells, (5) Monocytes, (6) DCs, (7) NK, and (8) T helper] are shown in Supplementary Table S1. After incubation, cells from panels 2–8 are washed twice as previously. Cells are resuspended in 1% paraformaldehyde in PBS for at least 1 hour to fix cells before being transferred to FACS tubes containing 100 µL of IF buffer and stored away from light at 4°C until acquiring. Following the first wash, the Treg cells (panel 1) are resuspended in FoxP3 Fix/Perm solution (Fix/Perm Buffer Set, BioLegend, as above) diluted in PBS, transferred to FACS tubes, and incubated for 20 min at room temperature in the dark for further intracellular FoxP3 staining. Tubes are centrifuged at 428 g for 2 min at 10°C to pellet cells and supernatant is discarded. Cells are washed with FoxP3 Perm buffer solution diluted in PBS and pelleted. After discarding supernatant, cells are resuspended in diluted FoxP3 Perm buffer and incubated, in the dark, at room temperature for 15 min. Cells are pelleted and after discarding supernatant, they are resuspended in 20 µL of anti-FoxP3 antibody mix (diluted with Perm buffer), and incubated at room temperature, in the dark, for 30 min. Cells are washed twice in IF buffer as previously. Cells are resuspended in 300 µL of IF buffer and stored as previously described.

Negative and positive gates are determined using compensation beads (AbC™ Total Antibody Compensation Bead Kit, Thermo Fisher Scientific, as above) to control for fluorochrome spillover. Tandem-specific compensations are employed to account for differences in compensation lot to lot/ vial to vial. Briefly, we label microtubes and FACS tube for each antibody ($n = 13$). Antibodies are diluted in 1:100 µL [BV510, BV421, PerCP, APC, FITC, CD28 (BV711), HLA-DR (BV711)] or 1:200 µL [PE, CD45RO (BV711), CD196 (BV711), CD86 (PECy7), CD185 (PECy7), CD192 (PECy7)] using IF buffer. One drop of positive and one drop of negative beads is added to each FACS tube. 50 µL of previously diluted antibodies are transferred to FACS tubes and mixed with beads. Tubes are incubated at 4°C in the dark for 30 min. IF buffer (200 µL) is added and tubes are centrifuged at 428 g for 2 min at 4°C; 2 min. Supernatant is discarded and wash steps are repeated. Compensations are resuspended in 300–600 µL IF buffer and stored at 4°C await from light. All samples, including compensations, will be acquired within 72 h using a BD LSR-Fortessa X-20 flow cytometer (BD Biosciences, San Jose, CA) and analyzed according to the relative fluorescence intensity using a platform for single-cell flow cytometry analysis (FlowJo 10.8.1, Becton Dickinson & Company). The flow cytometer machine goes through rigorous quality control, including periodical calibration, and is located at the Faculty of Medicine and Dentistry Flow Core at the University of Alberta, which is a recognized laboratory by the International Society for Advancement of Cytometry. Quality control of acquired samples will also be made using a method that adopts algorithms for the detection of anomalous data (flowAI) as previously described (80). Data is processed automatically, with the call of an R function, by optimizing flow rate, signal acquisition, and dynamic range (80). The population of monocytes and lymphocytes (i.e., PMBCs) will be the starting point gate of all immune subsets, which will be determined using established gates based on morphological characteristics of forward and side scatter. Fluorescence minus one (FMO) control will be used to establish positive staining when needed. All analyses are carried out by one individual and gates are reviewed by another to minimize interobserver variability.

2.9.7.3. Immune cell recovery

PBMC cryovials previously stored in liquid nitrogen will be rapidly thawed in a 37°C water bath for 5 min. The cryovials are dried and wiped with 70% ethanol before opening, then PBMCs are transferred to a 9 mL aliquot of 10% FCS RPMI prewarmed at 37°C. PBMCs are spun down and rinsed with 10 mL of 10% FCS RPMI to remove DMSO. PBMCs are resuspended in 5 mL of 10% FCS RPMI and transferred to 12 mL cell culture tubes to rest overnight at 37°C 5% CO_2. (Forma™ Series II Water Jacket CO_2 incubator 3,110, Thermo Fisher Scientific, Asheville, NC, United States). Before using in immune assays, PBMCs are pelleted at 453 g for 5 min and resuspended in 2 mL 10% FCS RPMI and lymphocytes counted using the trypan blue (diluted 1:1 with ddH_2O, Corning, as above) membrane dye exclusion method. All cell counts are carried out by the same person to minimize variation.

2.9.7.4. Mitogen stimulation of PBMC

The quantification of cytokine secretion by PMBCs stimulated with mitogens is used to evaluate the effector function of immune cells. Briefly, PBMCs are cultured in 2 mL 10% FCS RMPI-1640 medium (as above) for 48–72 h at 37°C and 5% CO_2 without mitogen (unstimulated) or with mitogens. The tubes are set up to reach 1.00×10^6 cells/mL. Depending on the cell recovery, in order of priority, we will stimulate PBMCs with (1) phytohemagglutinin (PHA) (25 µg/mL; Sigma-Aldrich, as above), (2) lipopolysaccharide (LPS) (5 µg/mL; Thermo Fisher Scientific, Carlsbad, CA, United States), (3) pokeweed (PWM) (55 µg/mL;), and (4) phorbol 12-myristate 13-acetate plus ionomycin (PMA + I) (2 µg/mL, Thermo Fisher Scientific, Carlsbad, CA, United States). After incubation, PBMCs are centrifuged at 428 g

for 5 min at 18°C to pellet PBMCs. The supernatant is aliquoted and frozen at -80° C for cytokine quantification. The post-pellet fraction is resuspended in 500 µL PBS, transferred to microtubes, and centrifuged at 906 g for 2 min. After discarding the supernatant, the pellet is stored at -80° C for subsequent analysis. All assays are performed with the same number of cells per stimulation and the same concentration of mitogen. For the same participant in both time points we will use mitogens with the same lot number. The incubation time chosen for each mitogen was based off a concentration time course gradient.

2.9.7.5. Quantification of *ex-vivo* cytokine secretion

Concentrations of IL-1β, IL-2, IL-6, IL-10, IFN-γ, and TNF-α in the supernatant are determined in duplicate by commercial ELISA kits (DuoSet®, R&D Systems, Minneapolis, MN, United States) according to the instructions of the manufacturer. The detection limits for the cytokines are as follows: IL-1β (250 to 3.91 pg/mL), IL-2 (1000–15.6 pg/mL), IL-6 (600–9.38 pg/mL), IL-10 (2000–31.3 pg/mL), IFN-γ (600–9.38 pg/mL), and TNF-α (1000–15.6 pg/mL). Cytokine concentrations are quantified using a microplate reader (Biotek® Synergy™ H1, Agilent Technologies, Inc., Santa Clara, CA, United States), with an intra-assay coefficient of variation <10% and R^2 of the standard curve ≥0.99. If samples are above the standard curve, they will be diluted onto the linear portion of the standard curve.

2.9.7.6. Proliferation assay

The proliferation of T cells will be measured at baseline and after the intervention period using the fluorescence of a cell viability reagent (alamarBlue® BUF012A, Bio-Rad Laboratories, Hercules, CA, United States). PBMCs are stimulated with anti-CD3 and anti-CD28 (BioLegend, as above). Briefly, black flat bottom 96-well cell culture plates (NUNC®, Thermo Fisher Scientific, Roskilde, Copenhagen, Denmark) are coated with 100 µL of 5 µg/mL anti-CD3 stock solution and are incubated overnight at 4°C. Then, stock solution is discarded using needle aspiration and rinsed twice with 200 µL sterile PBS. For the unstimulated and stimulated wells, 200 µL and 196 µL of 10% RPMI is added, respectively. The plate is set up to reach 1.00×10^6 cells/mL in 200 µL. Samples are added in triplicate, with 4 µL of 50ug/mL anti-CD28 (previously diluted 10X in 10% RPMI) stimulation of coated CD3 wells. Plates are incubated at 37°C with 5% CO_2 for 68 h. After 68 h, alamar blue is added equal to 10% of total volume (22.2 µL) and cells are incubated for 4 more hours for a total of 72 h. The plate is excited at 560 nm in a fluorescent microplate reader (Biotek® Synergy™ H1, as above) and read at 590 nm. Data is expressed as arbitrary units/OD 590 nm, using individual values or delta.

2.9.7.7. Fatty acids composition of total lipids

In conjunction with dietary information collected via the FFQ, circulating proportions of fatty acids in plasma and RBC will be assessed. Total lipids in plasma and RBCs (200 µL) are extracted using the Folch method with a 4:1 ratio (chloroform: methanol (2:1, 8 mL): 0.1 M potassium chloride (KCl, 1.8 mL) (79). Samples are vortexed then incubated overnight at 4°C. Samples are centrifuged at 906 g for 5 min with maximum brake and total lipids (bottom solvent layer) will be dried down under nitrogen gas and resuspended in boron trifluoride: hexane (1:1, 1.5 mL each) to methylate at 110°C for 1 hour. After cooling, 1 mL of ddH2O are added and samples will be incubated overnight at 4°C. Samples are centrifuged at 906 g for 5 min with maximum brake and total lipids (top layer) are dried down to be resuspended in 100 µL hexane for fatty acid methyl esters (FAME) proportional analysis, using an Agilent 8,890 gas chromatograph coupled with an autosampler (7693A) using a 100 m × 0.25 mm × 0.2um CP-Sil 88 fused capillary column for long chain fatty acids. Identification of fatty acids are based on external GLC 502 and GLC 37 standards. The following fatty acids proportion will be determined in the RBC membrane and plasma at baseline and after the intervention period: decanoic/capric (C10:0), undecanoic/undecylic (C11:0), dodecanoic/lauric (C12:0), tridecanoic/ tridecylic (C13:0), tetradecanoic/myristic (C14:0), tetradecenoic/myristoleic (C14:1 n-9), pentadecanoic/pentadecylic (C15:0), hexadecanoic/palmitic (C16:0), hexadecenoic/palmitoleic (C16:1 n-9), (9Z)-hexadec-9-enoic/palmitoleic (C16:1 n-7), heptadecanoic/margaric (C17:0), 10-heptadecenoic (C17:1), octadecanoic/stearic (C18:0), octadecenoic/elaidic (C18:1 T n-9), octadecenoic/oleic (C18:1 n-9), 11-octadecenoic acid/vaccenic (18:1 T n-7), octadecatrienoic /α-linolenic (C18:3 n-3), octadecadienoic/linoleic (C18:2 n-6), eicosanoic/arachidic (C20:0), 11-eicosenoic/gondoic (C20:1 n-9), 11,14-eicosadienoic (C20:2 n-6), 11,14,17-eicosatrienoic/dihomo-γlinolenic acid (C20:3 n-6), eicosatetraenoic/arachidonic (C20:4 n-6), eicosapentaenoic/EPA (C20:5 n-3), tetracosanoic/lignoceric (C24:0), tetracosenoic/nervonic (C24:1 n-9), docosapentaenoic/DPA (C22:5 n-3), docosahexaenoic/DHA (C22:6 n-3).

2.10. Statistical analysis

Continuous variables characterizing each study group will be reported as means with standard deviations or medians with interquartile ranges. Categorical variables will be represented as frequencies and proportions. Data will be checked for normality using the Shapiro–Wilk test, and logarithmic transformation will be employed for variables not normally distributed. The main comparisons in this study will be (1) between groups at each time point using analysis of variance with groups as the main effect followed by a multiple-comparison *post hoc* test and (2) within groups using paired *T*-test to assess differences between time points (i.e., baseline vs. post-intervention). Correlation analysis and linear regression models will be performed to verify the associations between outcomes (e.g., immune cell phenotypes and function, and systemic inflammation) and exposure (e.g., dietary intake or metabolic parameters, such as glucose, insulin, HOMA-IR, and HbA1c) variables. Mixed model repeated measures will be used to analyze variables of the OGTT to assess the effects of time, group, and time*group interaction. Overall comparisons of postprandial responses among groups will be determined using the incremental AUC with the trapezoid method. The statistical analyses will be adjusted for potential confounders and their interaction with the main group effect in cases where groups are unbalanced regarding factors such as physical activity level, use of medication (e.g., anti-diabetic drugs), and BMI, sex, and age if groups are not matched as initially planned. Sensitivity analyses will be conducted to verify the effect sizes of the primary outcomes of the study to assess the power and validity of our results. Comparative analyses using pertinent variables (e.g., age, sex, diet, metabolic profile, primary outcomes) will be performed between participants that completed the study with those who did not (i.e., participant dropouts or discontinuers).

A p-value <0.05 will be considered to be statistically significant. A detailed analysis plan will be developed before the final analysis with a biostatistician.

2.11. Sample size

A total of 32 participants per group ($n = 128$) will provide adequate power ($\beta = 0.81$, $\alpha = 0.05$) to assess a mean effect size of 30%, which was calculated based on differences in plasma CRP concentrations and *ex vivo* IL-2 secretion by PHA-stimulated PBMCs between Obese-NG and Obese-T2D participants. The sample size calculation was performed using data from a previous study (43) and the software G*Power. We applied a conservative attrition rate of 30% based on previous controlled feeding studies that ranged from 10–35% (81–85), totaling 166 participants.

2.12. Confidentiality, data management/ monitoring and audit

Each participant will be assigned a unique study identifier for use on the case report forms, other trial documents, and the study database. All study records and documents will be treated as confidential and held securely in a locked filing cabinet. These will be retained for at least 5 years. The trial manager will make a separate confidential record of the name, date of birth, unique provincial health care number, phone number, email address, and participant screening number, to permit the identification of all participants enrolled in the study, in case additional follow-up is required. Only the trial manager and principal investigator will have access to the master list of the study that contains identifiable information. Study data are primarily managed using REDCap. All data from clinical visits, excluding data from unredacted source documents, will be uploaded to REDCap and securely maintained by the trial manager in electronic form. Medical information is available through the provincial health information system (Connect Care) and can be assessed by the appropriate medical personnel responsible for the participant's healthcare. All manually entered data will be double-checked by a different member of the research team for accuracy. In addition, further verification of data will occur through the application of range to confirm if entered values are clinically possible values. Computer held data including the trial database will be held securely and password protected. Access to the study information will be limited to the trial staff and investigators. All collected data obtained as a result of this study is considered confidential and disclosure to third parties is only allowed when required by the ethics committee or regulatory authorities. Currently, there is no institutional quality assurance or scheduled audit procedures at the University of Alberta. Monitoring of the data is carried out by the trial manager as the data is collected at the HNRU. The trial manager is responsible to ensure completeness and accuracy of outcomes, adverse events, group allocations, informed consent, and completion/withdrawal. Access to the trial final dataset will be available to study investigators, trial manager, and graduate students. There are no contractual agreements that limit access to data.

2.13. Adverse event reporting

For this study, we followed the reporting requirements of the Health Research Ethics Board Biomedical Panel of the University of Alberta. Adverse events and other unintended effects will be collected weekly throughout the study from a self-monitoring form that participants will be asked to fill out daily. In addition to this method, participants will be advised to contact the study team directly to report any events. Expected events of this study, anticipated in the participants regardless of participation in research, may include unknown food allergies, gastrointestinal symptoms, changes in glycemia, and lipid profile. Although physiological changes may occur during participation in the study due to modifications in dietary patterns, they are not considered an adverse event unless they are unfavourable to the participant. To better understand if events could be related to the study we will evaluate the incident, its duration, and severity of symptoms. If a participant reports an incident of suspected foodborne illness we will investigate it to determine if there are other factors that may have caused it (i.e., improper food handling once food taken from HNRU, other illnesses in the household, food intolerance, etc.). Then we would send the food samples kept on site for testing to confirm if there was the presence of pathogens in the food. Only adverse events that are serious and unanticipated will be reported to the Research Ethics Office electronically through the Alberta Research Information Services (ARISE) System. Care will be provided to participants that suffer intervention-related harms. Participants will be instructed to seek medical attention and will be removed from the study if they are unable to comply with the feeding protocol due to acute illnesses not related to the study that may arise. If participants are willing to continue the feeding protocol after an acute illness (e.g., a cold), they will be asked to remain another week in the study to have their final appointments.

2.14. Protocol amendments

Protocol amendments, once approved by the research ethics board, were disseminated to investigator team by the research coordinator using direct communications and to trial participants, if pertinent. In addition, updates were posted at ClinicalTrials.gov.

2.15. Dissemination policy

The results of this study will be communicated within the scientific community through peer-reviewed publications, presentations at conferences attended by researchers and health professionals, and to the community at large, including coverage of study results in institutional websites and external media. A lay summary will be distributed via email to participants that agreed on communications on their last study appointment. We do not intend to use professional writers and manuscripts will be written by graduate students involved in the project. This trial does not have any publication restrictions. Authorship eligibility will be determined according to the recommendations of the International Committee of Medical Journal Editors guidelines.

3. Discussion

Obesity and T2D are associated with an increased risk of infections and a worse prognosis. Immune dysfunction in these conditions may be caused by abnormalities such as excess visceral adiposity (86), hyperglycemia (87), and unhealthy dietary habits (88). However, there is a lack of clinical trials that have controlled for diet and investigated immune function outcomes in males and females with obesity across different metabolic phenotypes.

To address this gap, the NutrIMM study aims to identify immune markers that are impaired in different health conditions, independent of the effect of diet. This study will also explore the relationship between dietary components, biological sex, and immune function. By understanding these differences, we hope to identify targeted and personalized approaches to improve immunity based on sex and metabolic phenotype.

Our investigation may also lead to the identification of circulating inflammatory markers that predict a lower immune function in individuals with obesity and T2D. This is important for diagnostic purposes as common techniques used to assess immune function are not practical for routine diagnosis. Moreover, our study may identify key nutrients that could improve immune function and guide future targeted and personalized interventions for subsequent trials.

The NutrIMM study is the first to determine the independent contribution of diet, sex, excess body fat, and altered glucose levels on inflammation and immune dysfunction associated with obesity. Other strengths of our protocol include assessing immune function outcomes under controlled feeding conditions, which provides valuable information about the effects of different foods, nutrients, and dietary patterns on health outcomes. This type of study allows for careful monitoring and manipulation of food intake, direct measures of physiological responses to different foods and nutrients, and the development of personalized nutrition recommendations based on an individual's unique physiological response (89–91).

However, the limitations of dietary clinical trials include limited generalizability to larger populations or diverse demographic groups and the inability to accurately reflect real-world dietary patterns that can vary on a daily basis (92). Feeding studies also have a limited application in long-term follow-up to assess the effects of dietary interventions on chronic disease risk or other long-term health outcomes (93). Finally, such studies can be expensive and time-consuming to conduct, which limits the number of participants and studies that can be done and hence the generalizability of findings (94).

Trial status

As of June 16, 2023, the NutrIMM protocol version is 2.1. Enrollment of participants started in October 2019 and will continue until the desired sample size is achieved. Recruitment is expected to be completed in July 2023.

Author's note

Participants will receive a $100 CAD gift card to a grocery store as token of appreciation for their participation upon completion of the study. Parking and/or public transportation expenses will be offered for the costs incurred for study appointments.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Research Ethics Board 3: Health Research Ethics Board-Health Panel/ University of Alberta. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JT: conceptualization, methodology, formal analysis, investigation, writing – original draft, visualization, supervision, and project administration. MS: methodology, formal analysis, writing – review and editing, investigation, visualization, and supervision. AM: methodology, formal analysis, validation, writing – review and editing, investigation, and supervision. PC: investigation, writing – review and editing, supervision, and project administration. CR: conceptualization, methodology, writing – review and editing, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Sponsor

The NutrIMM study is an investigator-initiated trial, therefore the principal investigator (CR) is the trial sponsor. The address of the sponsor is 4-002G Li Ka Shing Centre, Edmonton, Alberta, T6G 2E6, Canada. Telephone: +1 (780) 248–1827. Email: cr5@ualberta.ca. The sponsor-investigator is responsible for designing the study, overseeing data collection, study management, data analysis and interpretation, manuscript writing, dissemination of results, and has ultimate authority over all these activities.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1243359/full#supplementary-material

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Zinc deficiency impairs axonal regeneration and functional recovery after spinal cord injury by modulating macrophage polarization via NF-κB pathway

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Background: Spinal cord injury (SCI) is a devastating disease that results in permanent paralysis. Currently, there is no effective treatment for SCI, and it is important to identify factors that can provide therapeutic intervention during the course of the disease. Zinc, an essential trace element, has attracted attention as a regulator of inflammatory responses. In this study, we investigated the effect of zinc status on the SCI pathology and whether or not zinc could be a potential therapeutic target.

Methods: We created experimental mouse models with three different serum zinc concentration by changing the zinc content of the diet. After inducing contusion injury to the spinal cord of three mouse models, we assessed inflammation, apoptosis, demyelination, axonal regeneration, and the number of nuclear translocations of NF-κB in macrophages by using qPCR and immunostaining. In addition, macrophages in the injured spinal cord of these mouse models were isolated by flow cytometry, and their intracellular zinc concentration level and gene expression were examined. Functional recovery was assessed using the open field motor score, a foot print analysis, and a grid walk test. Statistical analysis was performed using Wilcoxon rank-sum test and ANOVA with the Tukey-Kramer test.

Results: In macrophages after SCI, zinc deficiency promoted nuclear translocation of NF-κB, polarization to pro-inflammatory like phenotype and expression of pro-inflammatory cytokines. The inflammatory response exacerbated by zinc deficiency led to worsening motor function by inducing more apoptosis of oligodendrocytes and demyelination and inhibiting axonal regeneration in the lesion site compared to the normal zinc condition. Furthermore, zinc supplementation after SCI attenuated these zinc-deficiency-induced series of responses and improved motor function.

Conclusion: We demonstrated that zinc affected axonal regeneration and motor functional recovery after SCI by negatively regulating NF-κB activity and the subsequent inflammatory response in macrophages. Our findings suggest that zinc supplementation after SCI may be a novel therapeutic strategy for SCI.

KEYWORDS

spinal cord injury, zinc deficiency, zinc supplementation, NF-κB, pro-inflammatory like macrophage, axonal regeneration

Introduction

Traumatic spinal cord injury (SCI) is a devastating disease that causes severe motor and sensory dysfunction, significantly reducing quality of life (1). Mechanical trauma rapidly causes disruption of the blood-brain barrier, neuronal death, axonal damage and demyelination, followed by a cascade of secondary injuries that expand the inflammatory response, which is characterized by infiltration of circulating cells such as macrophages and neutrophils at the epicenter of the injury (2, 3). Due to the limited endogenous regenerative and reparative capacity of the central nervous system (CNS), it is important to identify SCI exacerbating factors that can be intervened upon (4, 5). Age, blood pressure, and infection are each considered as prognostic factors for SCI, but factors that enable therapeutic intervention remain to be fully elucidated (5–8).

Zinc, an essential trace element, has been widely reported to play a role in regulating inflammation in recent years (9–11). For example, zinc deficiency exacerbates inflammation such as diarrhea and increases mortality from inflammatory diseases such as sepsis (9, 11–14), while zinc supplementation has been reported to improve inflammation and decrease the duration and severity of inflammatory diseases such as respiratory tract infections and sepsis (15–18).

Macrophages, immune cells that infiltrate into CNS, form a line of defense after exposure to invading pathogens and tissue damage (19). After SCI, activated macrophages express cytokines such as tumor necrosis factor-a (TNF-a), interleukin-6 (IL-6) and IL-1b and promote subsequent inflammatory responses (20, 21). Recently, we have shown that macrophage activation is associated with neuropathological outcomes in SCI (22). Although the exact mechanism of macrophage activation is not yet fully understood, several basic studies have reported that zinc is involved in macrophage activation (23). For example, it has been reported that the expression of inflammatory cytokines in macrophage is increased in zinc-deficient rodents, which worsens the prognosis of sepsis (12). In addition, it has been reported that zinc significantly improves the macrophage phagocytic capacity (23, 24). Considering that many elderly people and most chronic disease patients are zinc deficient, zinc may represent a novel therapeutic target to alter macrophage responses and regulate inflammation after SCI (25, 26).

In this study, we investigated the effects of zinc on the pathophysiology and motor function after SCI using an experimental mouse model and *in vitro* experiments. Using physiological and histological analysis and cell type-specific gene expression analysis by flow cytometry, we found that the low zinc status promoted nuclear translocation of NF-κB in macrophages, which altered macrophage phenotype, enhanced inflammatory cytokine expression, inhibited axonal regeneration and worsened motor functional outcome after SCI. Moreover, we showed that zinc supplementation to zinc-deficient mice improved inflammation, axonal regeneration and motor functional recovery after SCI, indicating direct relationship between zinc deficiency and worse outcome. These results suggest that zinc supplementation is an effective treatment for SCI.

Materials and methods

Mice

Adult female C57BL/6 wild-type mice aged 8-10 weeks were used. Mice were kept under constant conditions of a 12-hour light/dark cycle and a room temperature of 23°C ± 2°C, with *ad libitum* access to food and water (5). To create a mouse model of zinc deficiency, mice were fed a zinc-deficient diet (Kyudo company, Saga, Japan). For zinc supplementation, water containing a high volume of zinc (Nacalai Tesque, Kyoto, Japan) was prepared and

provided. Mice were excluded from this study if they died, developed infections resistant to antibiotic treatment, or developed significant autophagy. All animal experiments were approved by our university's Animal Experimentation Ethics Committee and were conducted in compliance with the National Institutes of Health guidelines for the Care and Use of Animals. All efforts were taken to reduce the number of animals used and to minimize animal suffering.

Spinal cord injury

Mice were anesthetized by intraperitoneal injection of mixed anesthesia with midazolam (4 mg/kg), butorphanol tartrate (5 mg/kg), and medetomidine hydrochloride (0-3 mg/kg). After laminectomy at the 10th thoracic level, we exposed the dorsal surface of the dura mater and induced a contusion injury using the Infinite Horizons Impactor (Precision Systems Instrumentation, Lexington, KY, USA) (27). Injury with Impactor was performed at force setting of 70kdyn, its accepted displacement range was 580-620 microns (3). After SCI, the surrounding muscles were sutured, the skin was closed with suture wound clips, and the mice were placed in a temperature-controlled chamber during recovery from anesthesia until thermoregulation was re-established (1). Motor function was assessed using a locomotor open-field rating scale, BMS (4). Footprint analysis was performed as previously reported (6). We dipped the forelimbs and hindlimbs of mice in red and green dyes, respectively. For the grip-walk test, we evaluated each mouse using a 50-cm grid with three patterns: easy (50 steps, 1 cm apart), medium (removed every third step), and hard (removed every other step) (5). The total number of grips for the three patterns was used for analysis (28). To collect cell-free serum, 0.6 ml of blood was collected by cardiac puncture. After standing upright for 30 minutes at room temperature and 6 hours at 4°C, samples were centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was quickly removed and immediately stored at -30°C until further testing (3). Serum zinc concentrations were measured using the Metallo assay Zn LS Kit (ZN02M, Metallogenics, Chiba, Japan) according to the manufacturer's protocol.

Histopathological examination

Mice were reanesthetized and fixed transcardially with 4% paraformaldehyde. The spinal cord was then removed, dehydrated, and embedded in an optimal cutting temperature (OCT) compound. Frozen sections were cut at 16 µm in the sagittal plane (1). Primary antibodies were applied to the sections at 4°C, followed by incubation of the sections with FluoZin3 (F24195, 10 µM; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor-conjugated secondary antibody (1:200; Invitrogen) and Hoechst 33258. THP-1 cells were also fixed and dehydrated, then the antibodies were applied similarly. All images were captured with a BZ-9000 digital microscope system (Keyence, Osaka, Japan) or fluorescence microscope equipped with a digital camera (BX51, Olympus, Tokyo, Japan). For quantitative evaluation, sagittal sections were selected from positions 0.08 mm, 0.16 mm, 0.24 mm, and 0.32 mm to the left and right of the midline and used for analysis. To compare the LFB positive area, sagittal sections of injured spinal cord were selected and measured with the software of BZII-Analyzer (Keyence) to calculate the ratio of LFB positive area to normal sections, as previously described (5, 29, 30). Cell counts and the GAP43-positive area and specific color area were determined using the National Institutes of Health ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Spinal cord samples (6.0 mm long, centered on the lesion) were prepared for flow cytometry as previously described (3, 29). These samples were stained with anti-CD45 (103,131, Biolegend, San Diego, CA, USA), anti-Gr-1 (108,415, Biolegend) and anti-CD11b (101,211, Biolegend). Cells were then counterstained with FluoZin-3 AM (Invitrogen). Intracellular zinc levels were compared based on mean fluorescence intensity (MFI). Samples were analyzed with a FACSAria II flowcytometer (BD Biosciences), San Jose, CA, USA) and analyzed with the FACSDiva software program (BD Biosciences) (3). THP-1 cells were stained with anti-CD68 (Bio-Rad, Hercules, CA, USA) and then counterstained with Alexa Fluor-conjugated secondary antibody (1:200; Invitrogen) and FluoZin-3 AM (Invitrogen) and analysis was performed with Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative reverse transcription-PCR

Total RNA was isolated from spinal cord tissue using the RNeasy Mini Kit (74,004, Qiagen, Hilden, Germany) and from FACS-sorted macrophages and THP-1 cells 4 h after LPS treatment using the RNeasy Micro Kit (74,104, Qiagen) (3). In order to synthesize complementary DNA (cDNA), we performed reverse transcriptase reactions using the PrimeScript first-strand cDNA Synthesis Kit (6110A, Takara Bio, Otsu, Japan). Quantitative real-time PCR (qRT-PCR) was performed by using primers specific for the target gene (see Additional file 1) and SYBR Premix Dimmer-Eraser (RR091A, Takara Bio, Shiga, Japan). Data were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

THP-1 cell culture

The human monocytic cell line THP-1 (Japanese Collection of Research Bioresource, Osaka, Japan) was cultured in RPMI medium 1640 containing 10% fetal bovine serum, 1% penicillin-streptomycin and 2 mM L-glutamine (3). In order to differentiate THP-1 cells into macrophages, THP-1 cells were incubated with phorbol 12-myristate 13-acetate (PMA) (27547-14, Nacalai Tesque) at a concentration of 10 ng/mL for 72 hours (12). The concentration

of zinc in the culture medium was adjusted by adding N,N,N′,N′-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN) (P4413, Sigma-Aldrich, St. Louis, MO, USA) or zinc (Nacalai Tesque). THP-1 macrophages in each culture were treated with LPS at a concentration of 1 μg/ml for 1 hour, changed to the respective culture medium containing LPS and incubated for 3 hours before analysis. Flow cytometry, mRNA extraction, zinc concentration measurements, or immunocytochemical staining were performed as previously reported (12). For cyto-immunofluorescence staining, THP-1 macrophages were incubated with 10 μM FluoZin-3 AM with Pluronic F-127 (P3000MP, Invitrogen) for 60 min at 37°C and then analyzed by confocal microscopy (12).

Statistics

Statistical evaluation between the two groups was performed with Wilcoxon's rank sum test. ANOVA with the Tukey-Kramer *post hoc* test was used for multiple comparisons between groups. For all statistical analyses, the significance level was set at 0.05. Values for each group were presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using the JMP software program (version 15; SAS Institute, SAS Institute, Cary, NC, USA).

Results

Zinc deficiency promotes nuclear translocation of NF-κB in macrophages with change of macrophage phenotype and exacerbates subsequent inflammatory response

In acute SCI, activated macrophages enhance and propagate the subsequent inflammatory response (21). To evaluate the inflammatory response of macrophages under different zinc conditions, we first prepared mediums with different zinc concentrations in which we incubated THP-1 macrophages as described in Methods. The zinc concentrations of zinc-adequate (ZA), zinc-deficient (ZD) and zinc-supplementation (ZS) medium are 81.7 ± 1.2, 29.4 ± 0.6, and 233 ± 1.5 (μg/dl), respectively (Figure 1A). The analysis protocols for macrophages *in vitro* are as follows: for Zinc deficient followed by supplementation (ZDS) analysis, THP-1 macrophages differentiated using PMA were cultured in ZD medium, then LPS was added, and 1 hour later the medium was replaced with ZS medium containing LPS and analyzed 3 hours later. For ZA and ZD analysis, THP-1 macrophages differentiated using PMA were cultured in ZA and ZD medium, respectively, then LPS was added, and 1 hour later the medium was replaced with ZA and ZD medium containing LPS and analyzed 3 hours later as described in Methods (Figure 1B).

In macrophages after LPS addition and macrophages after SCI, it is known that zinc is taken up into intracellular space via the zinc importer SLC39A8 (ZIP8) (3). Therefore, we first evaluated intracellular zinc levels in these three groups by immunocytochemical staining and flow cytometry using the cell-permeable zinc indicator FluoZin-3 (12). As a result, the intracellular zinc levels of ZD macrophages were significantly decreased and those of ZDS macrophages were significantly increased compared to the intracellular zinc levels of ZA macrophages (Figures 1C, E, F). Furthermore, the percentage of zinc-positive population of ZD macrophages was significantly decreased and those of ZDS macrophages were significantly increased compared to the percentage of zinc-positive population of ZA macrophages (Figure 1D). These results indicate that we have successfully developed a protocol for analysis of macrophages in different intracellular zinc status.

Since zinc is reported to negatively regulate the activity of NF-κB p65 (12, 23), we examined whether or not zinc deficiency and zinc supplementation influence the nuclear translocation of NF-κB and the following inflammatory pathology in macrophages. As a result, immunocytochemical staining revealed a significantly increased nuclear translocation of NF-κB in ZD macrophages and a significantly decreased nuclear translocation of NF-κB in ZDS macrophages compared to ZA macrophages, respectively (Figures 1G, H). In addition, qRT-PCR revealed that gene expression of pro-inflammatory cytokines such as *TNF-α*, *IL-1β*, and *IL-6* was significantly increased in ZD macrophages and significantly decreased in ZDS macrophages compared to ZA macrophages (Figure 1I). In contrast, gene expression of anti-inflammatory cytokines such as *IL-10, IL-4* and *TGF-β* did not differ significantly among these three groups (Figure 1J). Moreover, since NF-κB is reported to be involved in the polarization of pro-inflammatory like macrophages (31), we examined whether or not the intracellular zinc level affects the polarization of macrophages in these three groups. As a result, gene expression of the pro-inflammatory like macrophage marker *iNOS* increased significantly in ZD macrophages and decreased significantly in ZDS macrophages compared to ZA macrophages (Figure 1K). In addition, gene expression of pro-inflammatory like macrophage markers such as *CD32* and *CD16* was significantly increased in ZD macrophages compared to ZA macrophages, whereas these changes were cancelled in ZDS macrophages (Figure 1K). In contrast, gene expression of anti-inflammatory like macrophage markers such as *CD206, YM1* and *Arginase-1* did not differ significantly among these three groups (Figure 1L). These results suggest that zinc deficiency promotes nuclear translocation of NF-κB in macrophages, thereby promoting polarization to pro-inflammatory like macrophages and subsequent inflammatory responses, and that zinc supplementation cancels these responses induced by zinc deficiency.

Zinc regulated NF-κB activity and subsequent inflammatory response after SCI

In order to examine the effect of zinc on inflammation *in vivo*, we first observed the injured spinal cords of mice fed a normal diet using immunostaining at 4 days post-injury (dpi). Interestingly, nuclear translocation of NF-κB was increased in macrophages with

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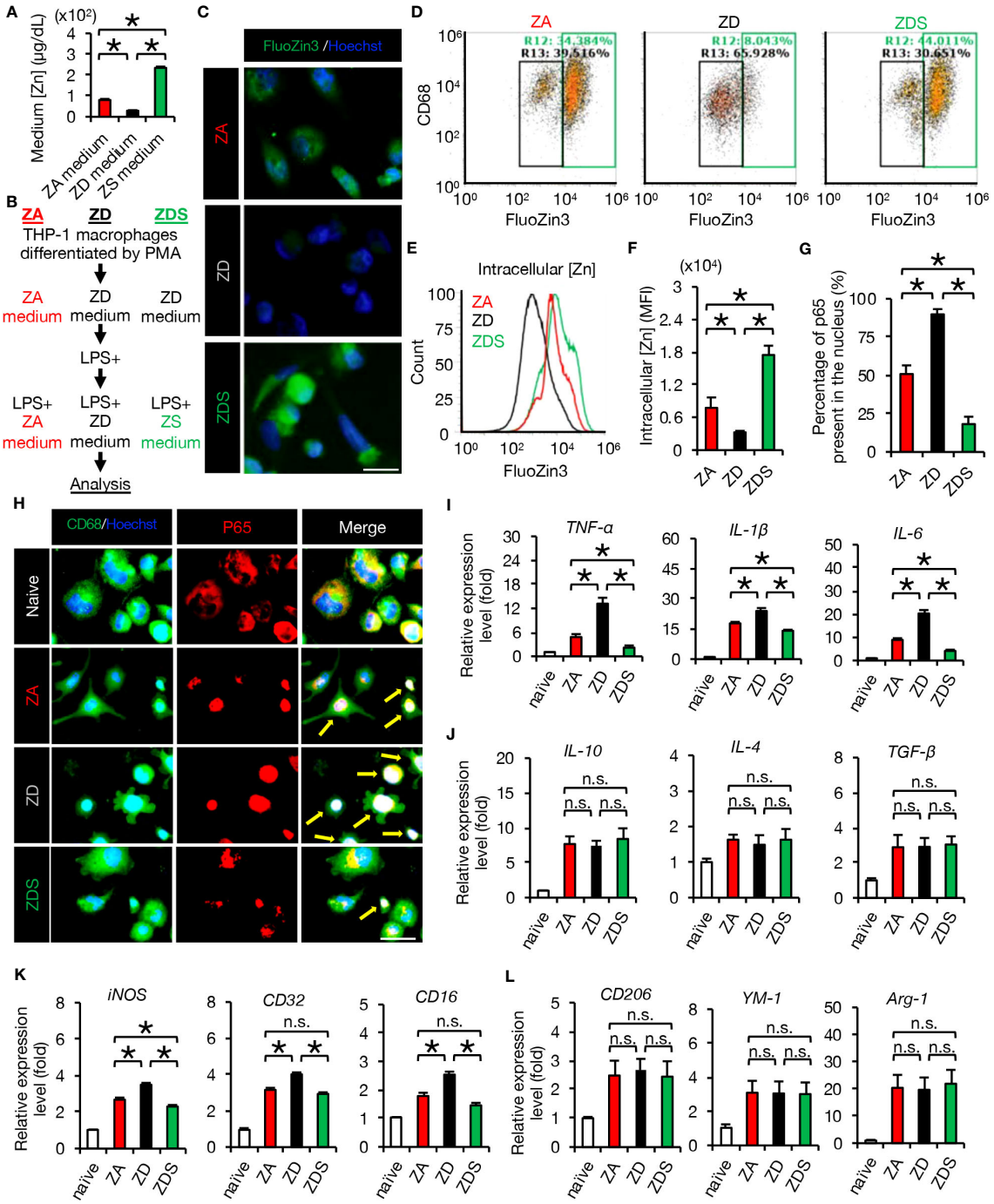

FIGURE 1
Zinc negatively regulates NF-κB activity and inflammatory responses. **(A)** Zinc concentration in culture medium (n = 4 per group). **(B)** Protocol for analysis of THP-1 macrophages *in vitro*. **(C)** Image analysis of macrophages using the cell-permeable zinc indicator FluoZin-3 (green). The nuclei are counterstained with Hoechst 33258 dye (blue). **(D, E)** Flowcytometric analysis and histogram analysis of FluoZin-3-positive macrophages. **(F)** Flowcytometric analysis. The relative levels of intracellular zinc were compared based on the mean fluorescence intensity (MFI) (n = 6 per group). Significant differences in zinc influx into the cells were observed after 4 hours of LPS stimulation. **(G)** To find out how much of all NF-κB p65 is in the nucleus, we measured the area of all p65 (1H, red area in the p65 image) and the area of p65 that has translocated into the nucleus (1H, white area in the Merge image) using the ImageJ function to measure the area of specific colors. The results showed significant differences among the three groups. **(H)** Representative images of immunocytochemical analysis of NF-κB p65 translocation into the nucleus of macrophages after LPS stimulation, stained with CD68 (green), NF-κB p65 (red) and Hoechst (blue). Arrows indicate nuclear translocation of p65 (white). **(I)** Gene expression of pro-inflammatory cytokine of macrophages cultured in each zinc condition. (n = 6 per group). **(J)** Gene expression of anti-inflammatory cytokines of macrophages cultured in each zinc condition. (n = 6 per group). **(K)** Gene expression of pro-inflammatory like macrophage markers (n = 6 per group). **(L)** Gene expression of anti-inflammatory like macrophage markers (n = 6 per group). Images shown in **(C, H)** are representative of 6 sections. Scale bar = 20μm **(C)** and 30μm **(H)**. In qRT-PCR analysis, each group was normalized to GAPDH values. *$P < 0.05$, ANOVA with the Tukey-Kramer *post hoc* test. n.s., not significant. Error bar indicates mean ± SEM. ZA, zinc-adequate; ZD, zinc-deficient; ZDS, zinc deficient followed by supplementation.

low intracellular zinc content compared to those with high intracellular zinc content, indicating that zinc suppresses the nuclear translocation of NF-κB in macrophages after SCI (Figures 2A–C).

For further analyses, we created SCI mouse models with different zinc status. ZA or ZD mice were fed the ZA or ZD diets for 3 weeks respectively, followed by SCI. ZA or ZD mice were then fed the ZA or ZD diet, respectively, for up to 6 weeks until each analysis. ZDS mice were fed a ZD diet for 3 weeks followed by SCI, and injected intraperitoneally high-concentration zinc immediately after SCI. ZDS mice were then received the ZA diet and high-concentration zinc water orally for up to 6 weeks until each analysis (Figure 2D). As a result, a significant decrease in serum zinc concentration was observed in the ZD and ZDS mice before SCI compared to the ZA mice, however, no significant changes in body weight were observed among these mouse groups (Figures 2E–G). The ZDS mouse group also showed a transient increase in serum zinc concentration after intraperitoneal administration of high-concentration zinc water, followed by a gradual increase in serum zinc concentration (Figure 2G). These results indicate that we had successfully developed SCI models with different serum zinc concentration.

Next, we performed gene expression analysis of inflammatory cytokines to assess whether the zinc status altered inflammation after SCI. A qRT-PCR of the injured spinal cord showed that gene expression of pro-inflammatory cytokines significantly increased in ZD mice and significantly decreased in ZDS mice compared to ZA mice (Figure 2H). On the other hand, gene expression of the anti-inflammatory cytokines was not significantly different among these three groups (Figure 2I). To clarify the regulatory mechanism underlying the zinc altered the expression changes of pro-inflammatory cytokines in injured spinal cord, we selectively isolated 5000 infiltrating macrophages from injured spinal cord using a cell sorter and performed gene expression analysis, as described in our earlier studies (3, 5, 29). In brief, macrophages were selectively isolated as a CD11bhigh/Gr-1nega-int/CD45high population after dead cells were removed using DAPI (Figure 2J). Consequently, there was no significant difference in the number of infiltrating macrophages among the three groups (Figure 2K), however, there were significant differences among the three groups in the amount of intracellular zinc concentration and the number of nuclear translocations of NF-κB in macrophages (Figures 2L–O). In addition, consistent with the *in vitro* results, in the isolated macrophages, gene expression of pro-inflammatory cytokines such as *TNF-α*, *IL-1β* and *IL-6* was significantly different among the three groups (Figure 3A), while gene expression of anti-inflammatory cytokines such as *TGF-β*, *IL-10* and *IL-4* was not significantly different among these three groups (Figure 3B). Furthermore, gene expression of the pro-inflammatory like macrophage marker *iNOS* was significantly different among the three groups (Figure 3C), while gene expression of anti-inflammatory like macrophage markers such as *CD206, YM1*, and *Arginase-1* was not significantly different among these three groups (Figure 3D). These results suggest that zinc suppresses the nuclear translocation of NF-κB after SCI, thereby altering macrophage polarization and consequently reducing inflammatory responses.

Zinc supplementation significantly improved axonal regeneration and functional recovery after SCI

Since *TNF-α* has been reported as inducing apoptosis of neurons and oligodendrocytes via the caspase-8/caspase-3 pathway (5, 29), we examined the activation of apoptotic cascade in SCI mice fed a normal diet. As a result, we observed the presence of double immunostained cells of glutathione S-transferase p (GST-p), a marker of mature oligodendrocytes, and cleaved caspase 3/caspase 8 (activated caspase 3/caspase 8) around the lesion at 4 dpi (Figures 4A, B). Furthermore, TUNEL staining revealed that the number of peri-lesional apoptotic cells was significantly increased in ZD mice and significantly decreased in ZDS mice compared to ZA mice (Figures 4C, D). Along with the extrinsic apoptotic pathway mediated by *caspase-8*, another intrinsic apoptotic pathway mediated by *caspase-9* and *Bcl-xL* is known (5). Therefore, the expression of the factors involved in both the extrinsic and the intrinsic apoptosis pathways was assessed. The expression of *caspase-8* and *caspase-3* in the injured spinal cord was significantly increased in ZD mice and significantly decreased in ZDS mice compared to ZA mice, while *caspase-9* and *Bcl-xL* expression was comparable among the three groups (Figures 4E, F). This suggests that after SCI, zinc deficiency promotes neuronal apoptosis not through mitochondrial intrinsic pathway but through extrinsic pathway mediated by *TNF-α*. Oligodendrocyte apoptosis after SCI is known to be associated with demyelination of the injured spinal cord and subsequent impaired functional recovery (5, 32). Here, we observed a greater extent of demyelination in ZD mice and a smaller extent of demyelination in ZDS mice compared to ZA mice (Figures 4G, H). In addition, it has also been reported that *TNF-α* and macrophage inflammation are associated with inhibition of axonal regeneration and subsequent functional recovery (19, 33). With regard to axonal regeneration, immunostaining with an antibody against GAP43 (1), a marker of regenerating axons, revealed that the number of GAP43-positive axons in the caudal area of the lesion was significantly increased in ZDS mice and significantly decreased in ZD mice, compared to ZA mice (Figures 4I, J).

In order to evaluate the clinical application of zinc for the treatment of SCI, we finally assessed the effect of zinc on the recovery of motor function. ZD mice exhibited poorer functional outcomes and ZDS mice exhibited better functional outcomes compared to ZA mice, as measured by the Basso Mouse Scale (BMS) scores, footprint analysis, and the Grip Walk test after SCI (Figures 5A–D). These objective results reinforce the notion that zinc supplementation is a feasible treatment to improve functional recovery after SCI.

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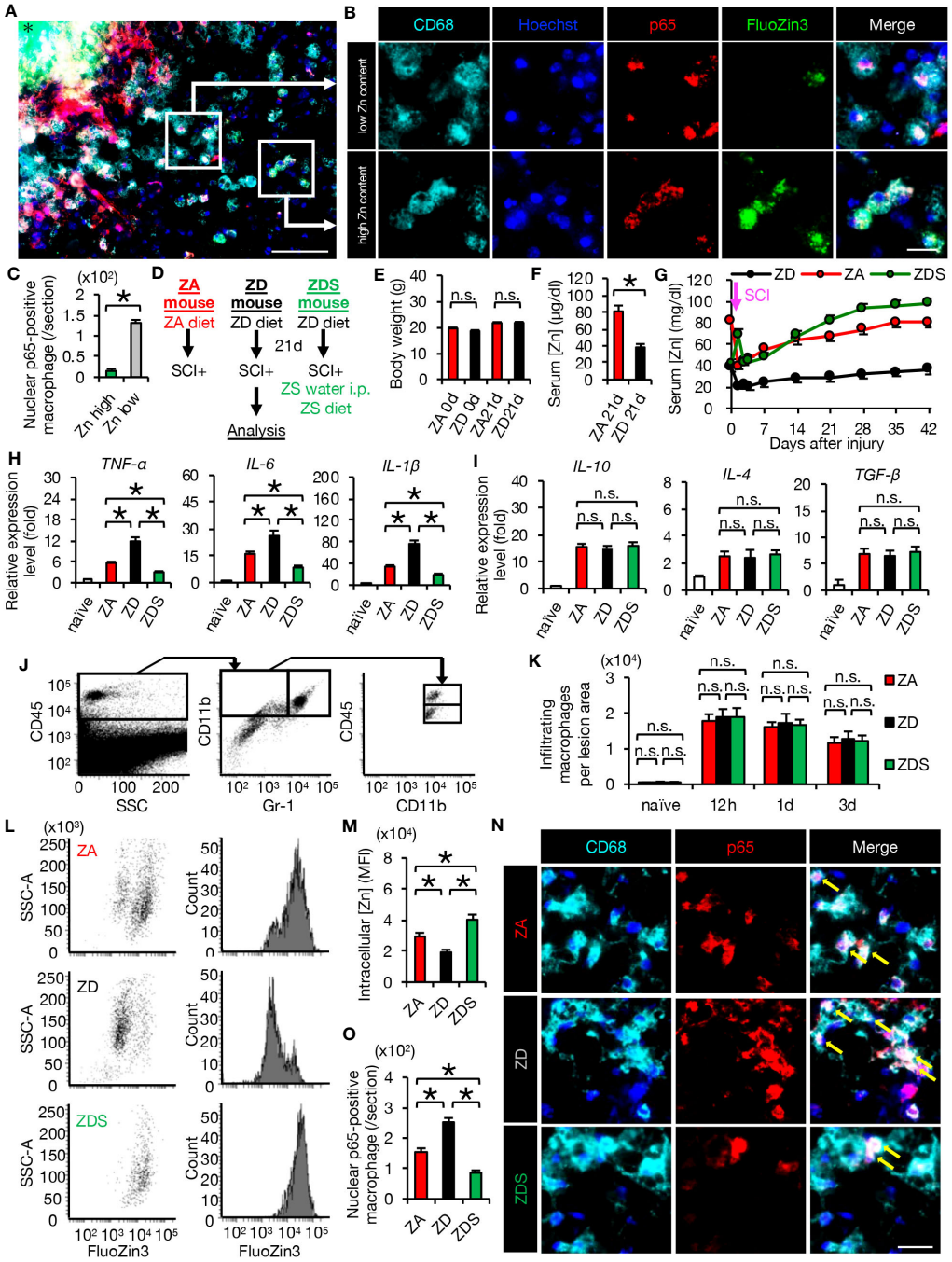

FIGURE 2
Zinc suppressed nuclear translocation of NF-κB in macrophages after SCI **(A, B)** Representative images of immunocytochemical analysis of the perilesional areas in normal-fed mice at 4 dpi, stained with CD68 (cyan), Hoechst (blue), NF-κB p65 (red) and FluoZin3 (green). Nuclear translocation of NF-κB p65 was observed in macrophages with low intracellular zinc, while it was not observed in macrophages with high intracellular zinc. * indicates the lesion epicenter. **(C)** Quantification of nuclear p65-positive numbers in macrophages with low and high intracellular zinc content. **(D)** Animal experiment protocol. **(E)** There were no differences in body weight by diet type (n = 10 per group). **(F)** Serum zinc concentrations before SCI (n = 6 per group). **(G)** Time course of serum zinc concentrations (n = 6 per group at each time point). **(H)** In the spinal cord at 3 dpi, gene expression of pro-inflammatory cytokine, which was increased by zinc deficiency, was decreased by zinc supplementation. (n = 6 per group). **(I)** Gene expression of anti-inflammatory cytokines was not significantly different among the three groups at 3 dpi (n = 6 per group). **(J)** Flowcytometric analysis. The CD11b^{high}/Gr-1$^{nega\text{-}int}$/CD45^{high} macrophage fraction in the injured spinal cord (upper box). **(K)** Changes in the number of macrophages in the lesion (n = 6 per group). **(L, M)** Flowcytometric analysis. Intracellular zinc levels in macrophages at 3 dpi (n = 6 per group). **(N, O)** CD68 (cyan) and p65 (red) double-positive macrophages in the perilesional areas. The nuclei are counterstained with Hoechst 33258 dye (blue). Arrows indicate nuclear translocation of p65. The number of nuclear translocation of NF-κB was increased in ZD mice and decreased in ZDS mice compared to ZA mice at 4 dpi. Images shown in **(A, B, N)** are representative of 8 sections per 6 mice. Scale bar = 150μm **(A)**, 50μm **(B)** and 70μm **(N)**. In qRT-PCR analysis, each group was normalized to GAPDH values. *$P < 0.05$, Wilcoxon's rank sum test, ANOVA with the Tukey-Kramer *post hoc* test. n.s., not significant. Error bar indicates mean ± SEM.

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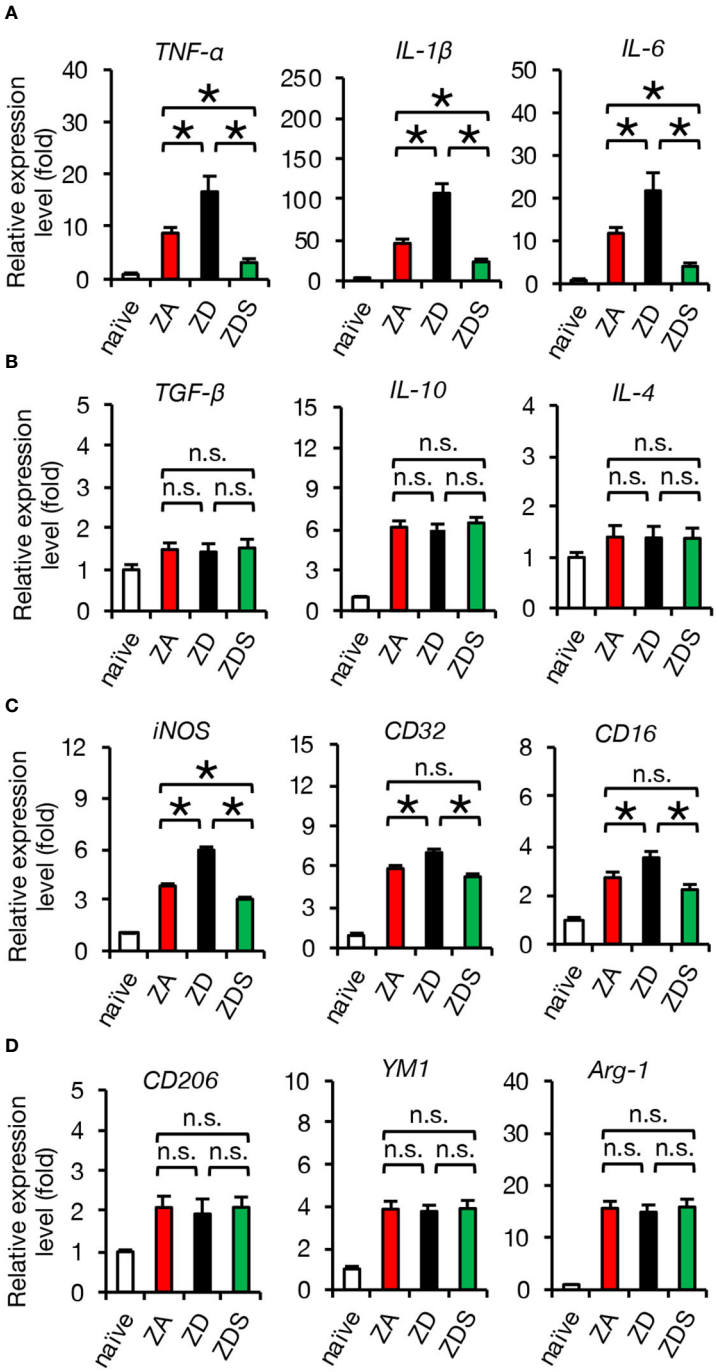

FIGURE 3
Gene expression of isolated macrophages after SCI is altered by systemic zinc status. **(A)** Gene expression of pro-inflammatory cytokine, which was increased by zinc deficiency, was improved by zinc supplementation (n = 6 per group). **(B)** Gene expression of anti-inflammatory cytokines was not significantly different among the three groups at 3 dpi (n = 6 per group). **(C)** Gene expression of pro-inflammatory like macrophage markers iNOS, which was increased by zinc deficiency, was improved by zinc supplementation (n = 6 per group). **(D)** Gene expression of anti-inflammatory like macrophage markers was not significantly different among the three groups at 3 dpi (n = 6 per group). In qRT-PCR analysis, each group was normalized to GAPDH values. *$P < 0.05$, ANOVA with the Tukey-Kramer *post hoc* test. n.s., not significant. Error bar indicates mean ± SEM.

Discussion

In this study, we revealed that zinc deficiency exacerbates the inflammatory response after SCI, thereby inhibiting axonal regeneration and worsening motor function. Conversely, zinc supplementation ameliorated these responses induced by zinc deficiency and improved motor function. Moreover, we investigated the mechanisms underlying the inflammatory regulation by zinc and clarified that zinc inhibits the nuclear translocation of NF-κB, thereby suppressing macrophage polarization to pro-inflammatory like phenotypes and the subsequent expression of pro-inflammatory cytokines. These

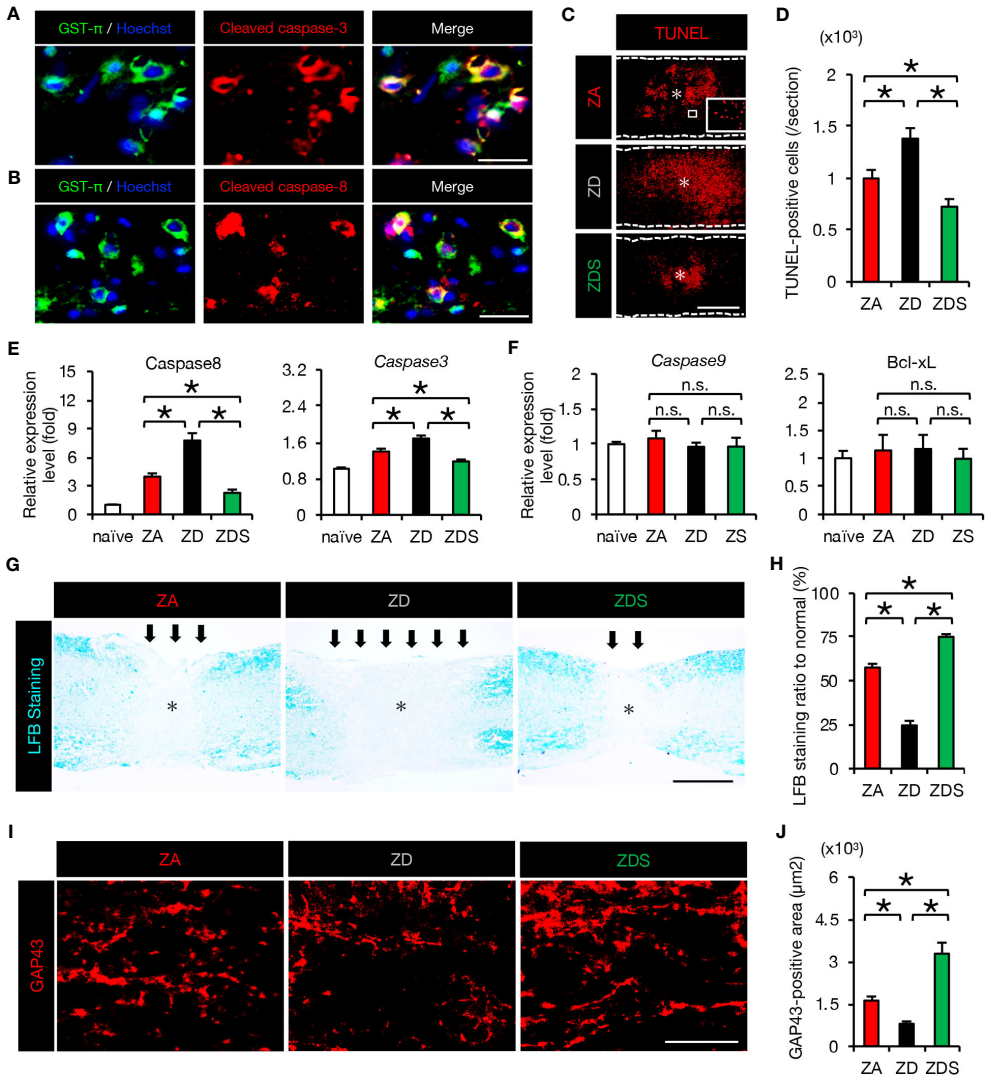

FIGURE 4
The increase in apoptosis and demyelinated areas caused by zinc deficiency attenuated with zinc supplementation. **(A)** GST-π (green) and cleaved caspase-3 (red) double-positive oligodendrocytes in the perilesional areas. at 4 dpi. The nuclei are counterstained with Hoechst 33258 dye (blue). **(B)** GST-π (green) and cleaved caspase-8 (red) double-positive oligodendrocytes in the perilesional areas. at 4 dpi. The nuclei are counterstained with Hoechst 33258 dye (blue). **(C)** TUNEL staining of the section at 4 dpi. Inset: TUNEL-positive cells. * indicates the lesion epicenter. **(D)** Quantification of the TUNEL-positive apoptotic cells in the lesion at 4 dpi (n = 8 per group). **(E, F)** Expression of apoptosis-related genes at 3 dpi (n = 6 per group). **(G, H)** LFB staining at 42 days after SCI showed a greater demyelinated area (arrows) in ZD mice and a smaller demyelinated area in ZDS mice compared to ZA mice. * indicates the lesion epicenter. **(I)** GAP43 staining in the caudal area of the lesion at 42 dpi. **(J)** Quantification of the GAP43-positive area per 1.0 × 10^5 μm^2 (n = 8 per group). The asterisk indicates the epicenter of the lesion. Images shown in **(A, B, G, I)** are representative of 8 sections per 6 mice. Scale bar = 40μm **(A, B)**, 500μm **(C, G)** and 40μm **(I)**. In qRT-PCR analysis, each group was normalized to GAPDH values. *P < 0.05, ANOVA with the Tukey-Kramer *post hoc* test. n.s., not significant. Error bar indicates mean ± SEM.

findings highlight the importance of zinc supplementation to improve motor function after SCI.

The population with zinc deficiency is considered large. According to WHO, in developing countries, 2 billion people are zinc deficient and zinc deficiency is the fifth leading cause of death and disease (11, 18). In developed countries, zinc deficiency in the elderly is considered common, and indeed it has been reported that about 30-40% of the elderly population may be zinc deficient (34). The high prevalence of low zinc concentration in elderly people is well documented (35, 36). Thus, it is estimated that zinc deficiency affects about one-third of the world population (11). In fact, our previous report (Kijima et al., 2019) also confirmed that there are some patients who have low zinc status during the acute phase of SCI (3). Considering the high prevalence of zinc deficiency and chronic disease worldwide and the growing number of elderly patients with SCI, there is potentially a large population of SCI patients who present with low zinc status prior to SCI and require zinc supplementation therapy (3, 35).

In the present study, in inflammatory conditions, low zinc status enhanced the nuclear translocation of NF-κB (Figures 1H, 2N), resulting in macrophage differentiation into pro-inflammatory like phenotypes and increased expression of pro-inflammatory cytokines (Figure 3). It was reported that NF-κB binds to the *TNF-α* promoter, indicating that NF-κB directly modulates *TNF-*

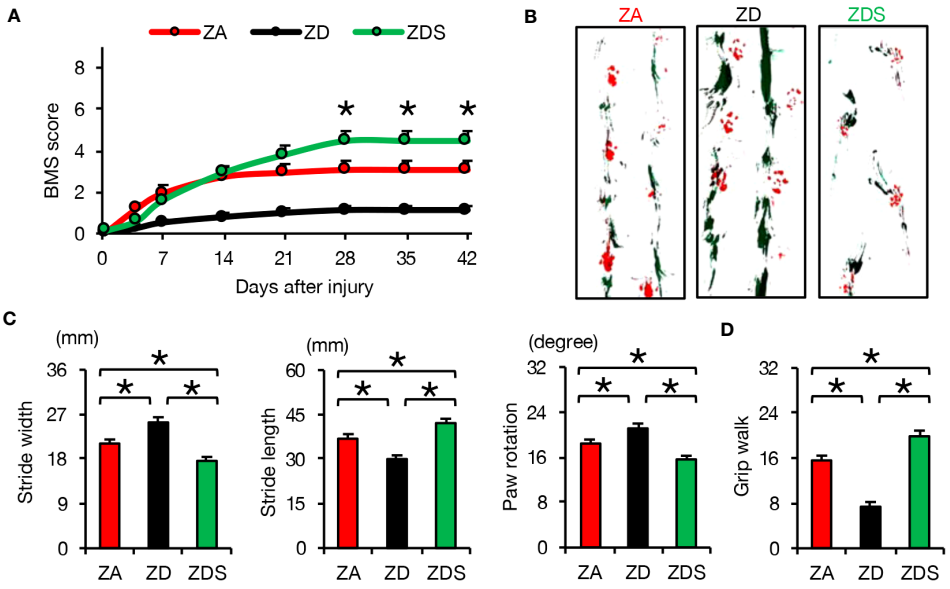

FIGURE 5
Zinc supplementation after SCI improved motor function worsened by zinc deficiency. **(A)** The time course of functional recovery according to the BMS score after SCI (n =15 per group). **(B, C)** The results of the footprint analyses (red, forepaws; green, hindpaws) at 42 dpi (n =12 per group). **(D)** The results of the grid walk test at 42 dpi (n =12 per group). *$P < 0.05$, ANOVA with the Tukey-Kramer *post hoc* test. Error bar indicates mean ± SEM.

α transcription in macrophages (5, 37). *TNF-α* has also been reported to be associated with induction of apoptosis of neural cells, including oligodendrocytes and neurons, and inhibition of axonal regeneration after SCI (2, 19, 33). In addition, the previous report by Bao et al. that *TNF-α* with zinc deficiency increases Caspase-8 activity and that zinc administration prevents this zinc-deficiency-induced apoptosis supports our results (38). Therefore, the overexpression of *TNF-α* observed in macrophages due to the activation of NF-κB in the low zinc state is expected to contribute to poor functional outcome after SCI by increasing apoptosis of neural cells and inhibiting axonal regeneration (Figures 4, 5).

Regarding the mechanism by which macrophages differentiate into pro-inflammatory like phenotypes, TLR4 is considered to possibly play a role. After SCI, necrotic cells release damage-associated molecular patterns (DAMPs) such as heat shock proteins, fibronectin, high mobility group box 1 proteins, and soluble hyaluronan (5, 39), all of which can serve as TLR4 ligands and activate NF-κB signaling (40). In addition, TLR4/NF-κB has been reported to regulate macrophage polarization (41). For example, Ye et al. reported that activation of the TLR4/NF-κB pathway increases polarization toward pro-inflammatory like macrophages (41, 42), and Gong et al. reported that inhibition of the TLR4/NF-κB signaling pathway inhibits polarization toward pro-inflammatory like macrophages (43). Thus, we concluded that differentiation of macrophages into pro-inflammatory like phenotypes after SCI is regulated via the DAMPs/TLR4/NF-κβ pathway. Furthermore, regarding the mechanism by which zinc inhibits the nuclear translocation of NF-κB, it is known that zinc directly inhibits IKK, which is located upstream of NF-κB and phosphorylates the NF-κB dimer to promote nuclear translocation of p65 (44). It has also been widely reported that direct inhibition of the IKK complex with zinc suppresses NF-κB activation and subsequent expression of inflammatory cytokines (12, 15, 45–47). In the present study, zinc deficiency increased the number of nuclear translocations of NF-κB, the number of pro-inflammatory like macrophages, and the subsequent expression of pro-inflammatory cytokines, and zinc supplementation improved these responses. Therefore, zinc would control the DAMPs/TLR4/NF-κβ pathway, which regulates the differentiation of macrophages into pro-inflammatory like phenotypes after SCI, via direct inhibition of IKK.

There are multiple views on the phenotype of macrophages and the issue is still controversial (48). Traditionally, they have been classified as pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, but this classification has been pointed out as an oversimplification (49). It has also been noted that M1 and M2 macrophages are two polar forms of *in vitro* differentiated mononuclear phagocytes with different phenotypic patterns and functions, while *in vivo* there are various phenotypes in between, depending on the microenvironment and natural signals to which the macrophages are exposed (49). In addition, macrophages are reported to be highly plastic in response to microenvironmental stimuli and thus may therefore exhibit a variety of different immune phenotypes with overlapping properties (49, 50). Furthermore, macrophages have been reported not to be clearly divided into M1/M2 categories (51). Indeed, in this study macrophages also show increased anti-inflammatory markers as well as increased pro-inflammatory markers after SCI, suggesting that macrophages may exhibit different phenotypes with overlapping properties. Therefore, in this study we used the terms "pro-inflammatory like macrophage" and "anti-inflammatory like macrophage" to describe macrophage polarization rather than the traditional terms M1/M2 macrophages.

NF-κB is a transcription factor that normally plays a pro-inflammatory role, but is also known to suppress anti-inflammatory macrophage markers (52). In the present study, despite the activation of NF-κB by zinc deficiency, there was no difference in anti-inflammatory markers between the three groups of ZA, ZD, and ZDS (Figure 1J), which could be due to several factors. First, NF-κB activation occurs in the early phase of the inflammatory response, but the expression of anti-inflammatory markers usually increases in the latter phase of the inflammatory response, so it is possible that no difference in anti-inflammatory marker had yet appeared in this study, which was observed in the acute inflammatory phase on day 3 of SCI (53). Second, the regulation of the inflammatory response involves not only NF-κB but also other anti-inflammatory transcription factors and signaling pathways such as PPARγ and STAT3, which may have been affected by these factors (50). Although there were no differences in anti-inflammatory markers between the three groups ZA, ZD and ZDS, the anti-inflammatory markers in the three groups were increased compared to naïve, and when taken together with the increase in inflammatory markers, the results support the idea that there are different phenotypes of macrophages with overlapping properties (49).

Although the existence of zinc has long been known, little was known about how it functions in the body until recently (18, 23). This was due to the difficulty of conventional zinc measurement methods. Conventional measurement methods such as atomic absorption spectrometry or inductively coupled plasma optical emission spectroscopy (ICP-OES) require large amounts of samples and the cost of the equipment was very high (3, 54). However, the newly developed measurement kit can measure zinc concentration easily and inexpensively by applying absorbance measurement (54). In addition, the zinc indicator FluoZin3 allows the visualization of zinc presence and the evaluation of intracellular zinc concentration levels when applied in flow cytometry (3, 12). Due to the establishment of these measurement and visualization methods, zinc research has advanced dramatically in recent years, and zinc is now highlighted as a new therapeutic target in a wide variety of diseases (9, 23, 35).

Although the exacerbating factors that are amenable to treatment for SCI are not fully understood, we have previously reported that acute phase glycemic control improved functional outcome of SCI with attenuated microglial inflammatory response and subsequent demyelination (5). Since we have now demonstrated that low zinc is exacerbating factor and that zinc supplementation is effective in SCI, we expect to further improve functional outcome by regulating not only hyperglycemia but also the low zinc status.

To date, numerous studies have shown that zinc is relatively harmless compared to other heavy metals with similar properties (55). For example, Léonard et al. reported that zinc is not carcinogenic, teratogenic, mutagenic, or cytotoxic (56). In fact, zinc poisoning is reported to be very rare (9). This is because the estimated LD50 for humans, the amount that causes death in half of them, is 27 g zinc/day, which is considerably larger than the recommended dietary intake of zinc (11 mg/day for men and 8 mg/day for women) and the amount emitted (about 225-400 mg) (57, 58). Also, the LD50 of zinc is more than 10 times higher than that of cadmium and 50 times higher than that of mercury, which is quite a large amount, so lethal dose ingestion is highly unlikely (59). Moreover, in addition to acute poisoning, some have reported that long-term high-dose zinc supplementation interferes with copper intake, and that many of its toxic effects are actually due to copper deficiency rather than zinc itself (60). However, this zinc-induced copper deficiency has been reported to be totally reversible when zinc administration is stopped, and the time from zinc administration to the onset of copper deficiency has often been reported to be several months or years (61, 62). Considering that copper deficiency symptoms did not appear after 6 weeks of zinc administration in this study and that 6 weeks after SCI, when motor function mainly improves, is a sufficient time for zinc administration, this zinc administration method is reasonable because zinc administration can be stopped before symptoms appear. Thus, many studies have shown zinc to be a safe essential trace element. Furthermore, considering that zinc is inexpensive, the measurement of serum zinc concentration is simple, and administration methods such as oral and intravascular administration are well established, we believe that zinc is easy to be applied clinically in actual practice as a novel therapeutic agent for SCI.

Conclusion

Zinc deficiency exacerbated motor functional outcome after SCI by promoting nuclear translocation of NF-κB, resulting in macrophage polarization to express increased pro-inflammatory cytokines. Zinc supplementation ameliorated these responses, thereby improving motor function, indicating that zinc supplementation could be a novel treatment after SCI.

Data availability statement

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

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by the Committee of Ethics on Animal Experimentation in the Faculty on Medicine, Kyushu University (A-29-243-0). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KKij: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Software, Writing – review & editing. GO: Formal Analysis, Writing – original draft, Data curation, Investigation, Visualization. KKob: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing, Software, Methodology. HSai: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing, Software, Methodology. MH: Conceptualization, Formal Analysis, Methodology, Supervision, Writing – original draft, Data curation, Investigation. SY: Formal Analysis, Writing – original draft, Data curation. KY: Formal Analysis, Writing – original draft, Methodology. TS: Data curation, Formal Analysis, Writing – original draft. TT: Data curation, Formal Analysis, Writing – original draft. HI: Data curation, Formal Analysis, Writing – original draft. YH: Formal Analysis, Writing – original draft, Data curation. KKit: Formal Analysis, Writing – original draft, Data curation. TU: Conceptualization, Formal Analysis, Supervision, Validation, Writing – review & editing, Methodology. DK: Conceptualization, Data curation, Formal Analysis, Methodology, Project administration, Supervision, Writing – original draft. VE: Conceptualization, Supervision, Validation, Writing – review & editing. CL: Supervision, Validation, Writing – review & editing. HSak: Data curation, Methodology, Resources, Writing – original draft. TM: Data curation, Supervision, Writing – original draft, Resources. KKaw: Data curation, Supervision, Writing – original draft. YM: Supervision, Validation, Writing – review & editing. SO: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing, Formal Analysis, Investigation. YN: Data curation, Project administration, Resources, Supervision, Validation, Writing – review & editing, Software.

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Conflict of interest

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

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The multi-faceted role of NADPH in regulating inflammation in activated myeloid cells

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Recent advances in the immunometabolism field have demonstrated the importance of metabolites in fine-tuning the inflammatory responses in myeloid cells. Cofactors, which are metabolites comprised of inorganic ions and organic molecules, may tightly or loosely bind to distinct sites of enzymes to catalyze a specific reaction. Since many enzymes that mediate inflammatory and anti-inflammatory processes require the same cofactors to function, this raises the possibility that under conditions where the abundance of these cofactors is limited, inflammatory and anti-inflammatory enzymes must compete with each other for the consumption of cofactors. Thus, this competition may reflect a naturally evolved mechanism to efficiently co-regulate inflammatory versus anti-inflammatory pathways, fine-tuning the extent of an inflammatory response. The role of NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$), in mediating inflammatory and anti-inflammatory responses in activated myeloid cells has been well-established in the past decades. However, how the dynamic of NADPH consumption mediates the co-regulation between individual inflammatory and anti-inflammatory pathways is only beginning to be appreciated. In this review, we will summarize the established roles of NADPH in supporting inflammatory and anti-inflammatory pathways, as well as highlight how the competition for NADPH consumption by these opposing pathways fine-tunes the inflammatory response in activated myeloid cells.

KEYWORDS

NADPH, macrophages, LPS (lipopolysaccharide), myeloid cells, immunometabolism, dendritic cells, cofactors, cofactor coordination

1 Introduction

Major milestones in the immunometabolism field have been recently reached to illustrate how intracellular metabolic circuits are rewired to orchestrate a fine-tuned inflammatory response in activated immune cells. For instance, in M1 pro-inflammatory macrophages (Mφs), there is a metabolic break at isocitrate dehydrogenase in the TCA cycle due to suppression of its mRNA expression, leading to the accumulation of citrate and itaconate, which drive lipid synthesis and stabilization of anti-inflammatory transcription factors, such as nuclear factor-erythroid factor 2-related factor 2 (NRF2) and activating transcription factor 3 (1–5). The accumulation of itaconate causes a metabolic break by inhibiting the enzymic activity of succinate dehydrogenase. The consequent increased abundance of succinate leads to the stabilization HIF-1α, which promotes IL-1β transcription (1–3, 6, 7). On the other hand, M2 anti-inflammatory Mφs primarily use fatty acid oxidation and oxidative phosphorylation to support their metabolism, although the upregulation of glycolysis by mTOR complex 2 (mTORC2), IL-4Rα/Stat6 and interferon regulatory factor 4 (IRF4) was also reported to be critical (8). Apart from this, carbohydrate kinase-like protein (CARKL) was also activated in M2 Mφs, which led to the enhancement of the non-oxidative branch of the pentose phosphate pathway (PPP) (9). This subsequently increased the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAC), which is required for the N-glycosylation of many cell surface proteins expressed on M2 Mφs (2). Taken together, these studies revealed that metabolites can regulate both inflammatory and anti-inflammatory processes, in addition to intracellular metabolism and energetics. Although the moonlighting functions of these metabolites, including their roles in signaling, post-translational modification, and epigenetics are being increasingly appreciated (10), how they regulate the extent of inflammation by coregulating inflammatory and anti-inflammatory pathways remain unclear.

Unlike nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) has an additional phosphate on the 2' position of the ribose ring that attaches to an adenine moiety and has a lower intracellular concentration than NAD^+ (11) (Figure 1A). The reduced form of $NADP^+$, known as NADPH, is a well-established, indispensable cofactor required for anabolic reactions, oxidative and antioxidative processes. Although NADPH has multifunctional roles in regulating inflammation, particularly in myeloid cells that have been clearly defined in the past, it remains unclear how NADPH-dependent inflammatory and anti-inflammatory pathways are co-regulated in order to fine-tune the magnitude of an inflammatory response. In this review, we will revisit the traditional roles of NADPH in inflammatory and anti-inflammatory pathways and highlight studies that reveal the competition for its consumption between these opposing pathways as a way to regulate inflammation in myeloid cells.

2 NADPH usage for inflammatory processes

In general, NADPH supports a wide range of inflammatory processes in myeloid cells, including *de novo* lipid biosynthesis and generation of ROS (Figure 1B). For instance, Everts et al. demonstrated that in dendritic cells (DCs), LPS-induced glycolysis is repurposed to replenish citrate, an intermediate metabolite in the TCA cycle that is depleted for the *de novo* synthesis of fatty acids (12). This subsequently increased the synthesis of additional membranes to expand the endoplasmic reticulum (ER) and golgi networks, which is required for the secretion of proinflammatory lipid mediators, such as prostaglandin E_2 (12). Similar to DCs, LPS-induced activation of Mφs also led to an upregulation of fatty acid synthesis (13, 14), which has now been shown to regulate the inflammatory responses of Mφs. For example, Carroll et al. demonstrated that the production of acetoacetyl-CoA by fatty acid synthase (FASN), a key NADPH-dependent enzyme involved in fatty acid synthesis, can regulate TLR signaling as acetoacetyl-CoA is linked to cholesterol synthesis and subsequently involved in modulating the formation of lipid rafts (15). Similar findings were also reported by Wei et al., who found that mice deficient of FASN are protected from diet-induced insulin resistance and inflammation as it altered the composition of plasma membrane and subsequently disrupted Rho GTPase trafficking, a process that is required for the activation of Mφs (16). Apart from lipid raft formation, reports also suggest that LPS-induced *de novo* lipogenesis is important for phagocytosis as it requires ongoing lipid synthesis in the ER for membrane expansion, such that Mφs can surround and capture targeted pathogens for internalization (17, 18). The mechanism that links lipogenesis with phagocytosis was later elucidated by Lee et al., who showed that phagocytosis was impaired in LPS-activated Mφs isolated from mice deficient in sterol regulatory element binding protein 1a (SREBP-1a) (19), which regulates the transcription of genes related to lipogenesis (20). Specifically, the study revealed that SREBP-1a-dependent lipid species mediate the interaction between membrane lipid rafts and the actin cytoskeleton, an association that is critical for the early stages of phagocytosis (19). Finally, SREBP-1a is also known to regulate genes that are related to NADPH synthesis (21), and Mφs with genetic deficiency of SREBP-1a demonstrated decreased cytokine production and inflammasome activation upon challenge by pro-inflammatory stimuli (22). Collectively, these studies have demonstrated the importance of *de novo* lipogenesis, an anabolic process that is NADPH-dependent, in regulating the inflammatory response of myeloid cells.

Apart from lipid biosynthesis, another critical pro-inflammatory process that NADPH supports in myeloid cells is the production of cytotoxic, diffusive reactive radicals, such as reactive oxygen species (ROS) via NADPH oxidases (NOXes), as well as nitric oxide (˙NO) via nitric oxide synthases (NOSes). Indeed, mice with defects of NOX2 or NOS2 failed to restrain bacterial replication (23, 24). The importance of ROS generation by

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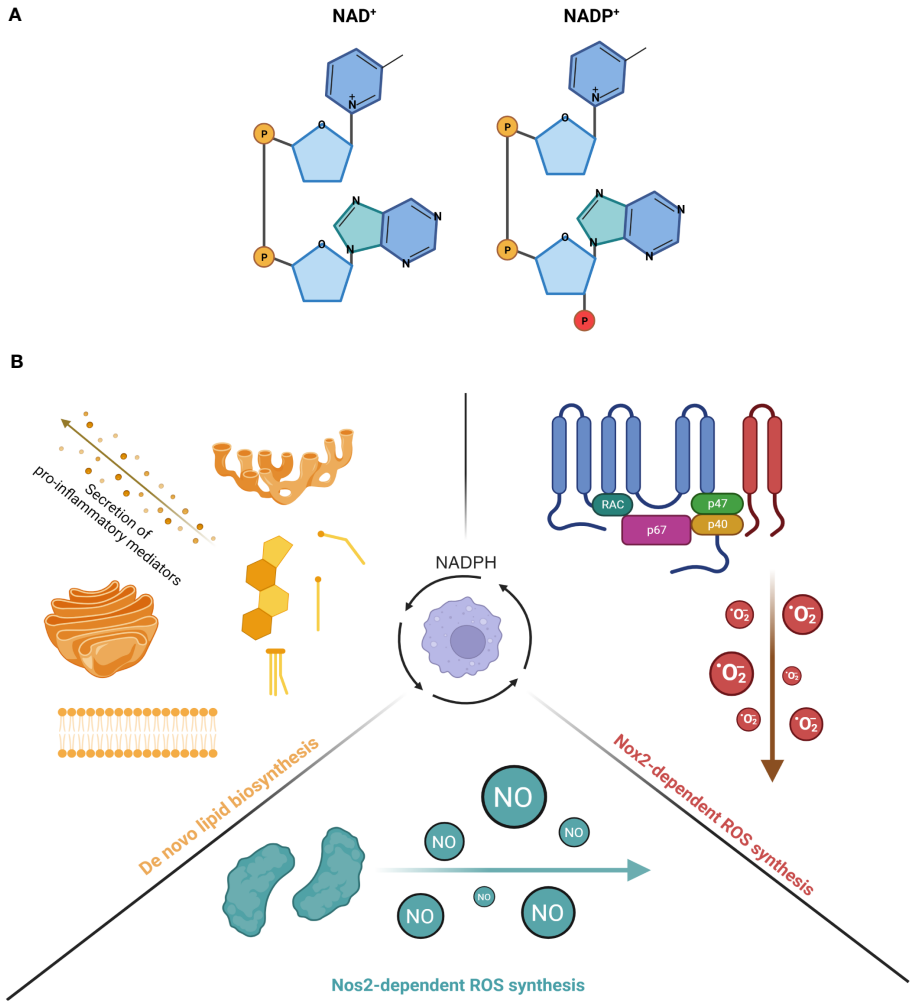

FIGURE 1
(A) The molecular structure of NAD^+ and $NADP^+$. Left diagram shows the structure of NAD^+ while right diagram shows the structure of $NADP^+$. The phosphate in red illustrates the additional phosphate on the 2' position of the ribose ring attached on $NADP^+$. **(B)** The consumption of NADPH in activated myeloid cells for inflammatory processes. (Left) NADPH serves as a co-factor for many enzymes involved in the production of de novo lipid synthesis (steroids, cholesterol and fatty acids). The increased production of these lipid species is important for the membrane expansion of Golgi network and endoplasmic reticulum, a requirement for the secretion of pro-inflammatory cytokines. (Right) NADPH is an important co-factor for NOX2 and its generation of superoxide anions. NOX2 is a 6-subunit complex assembled on the plasma membrane that transfers one electron from NADPH, which is the primary substrate of the reaction, to oxygen, thereby forming O_2^-. Under basal conditions, the components of the complex are localized in different subcellular compartments, with the gp91phox and p22phox subunits localized on the plasma membrane as one heterodimeric complex, known as flavocytochrome b558 (cyt b558). On the other hand, the p47phox/p67phox/p40phox subunits are co-localized in the cytosol, forming another complex. Upon stimulation, p47phox is phosphorylated, which leads the cytosolic complex, together with small GTPase Rac1/Rac2, to bind to the flavocytochrome complex, ultimately forming the final oxidase. (Bottom) NADPH is a critical co-factor for NOS2 and its generation of nitric oxide (NO) in a two-step reaction. The first reaction involves 1 molecule of L-arginine as a substrate being oxidized at a guanidino nitrogen to produce Nω-OH-L-arginine as an intermediate. The second reaction involves Nω-OH-L-arginine being further oxidized to produce 1 molecule of NO and L-citrul-line. During both reactions, a total of 1.5 molecules of NADPH and 2 molecules of dioxygen, which are co-substrates of the reactions, are converted to 1.5 molecules of NADP+ and 2 molecules of water as co-products. Created with BioRender.com.

NOX2, including superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), has been extensively reviewed in the past (25, 26). Apart from Mφs, a new study now revealed that PMA-activated neutrophils repurpose glycolysis and the PPP in order to maximize the yield of NADPH from glucose metabolism (27). This adaptation is required to meet the high demands of NAPDH needed for the oxidative burst mediated by NOXes (27). Taken together, these studies have shown the significance of NADPH-dependent production of ROS via NOXes in regulating the inflammatory response in myeloid cells. Upon activation by pathogenic microorganisms, Mφs produce a burst of ROS and NO that limit bacterial infection in the host (23, 28, 29). Low levels of ROS act as second messengers for activating inflammatory intracellular signaling, such as NF-κB and MAP kinase pathways (30).

Similar to the production of ROS by NOXes, the production of NO by NOSes is also dependent on NADPH. Specifically, three genes encode NOSes in mammals: *Nos1*, *Nos2* and *Nos3*. NOS2 is also known as iNOS ("i" refers to its immunologically inducible nature) and was first cloned in Mφs (31). It is only expressed in cells

that are activated by proinflammatory cytokines or stimuli. NOS2 function is not regulated by the elevation of intracellular Ca^{2+}. The production of NO by NOS2 (in micromolar amounts) is much higher and more sustained than by other NOSes, thereby making NOS2 an important player in regulating inflammation and infection (28, 32). Regardless of the NOS isoform, the biochemical pathway to produce NO is the same and all require cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) and ferrous iron (Fe^{2+}). NADPH is of primary importance as its selective omission mostly impaired the activity of NOS2 in activated Mφs (33).

3 NADPH usage for antioxidative purposes

While cytotoxic reactive radicals produced by myeloid cells are essential for limiting bacterial replication, they can also be harmful to the host (34, 35). It is intriguing that NADPH-dependent detoxification pathways have evolved as defense mechanisms utilized by myeloid cells (Figure 2). In general, three major systems help to protect host cells from oxidative and nitrosative stress: (1) the superoxidase dismutase (SOD) and catalase system, (2) the glutathione system and (3) the thioredoxin system. Briefly, SOD converts superoxide anions to hydrogen peroxide and oxygen, which is then detoxified to water by catalase. After the discovery of SOD as the first line of defense against ROS (36), three SODs have been identified: SOD1 (cytoplasmic and peroxisome), SOD2 (mitochondrial) and SOD3 (extracellular matrix). Like SOD, three types of catalases have also been characterized (37), with the monofunctional heme-containing type being the most common (38). In a two-step reaction, catalase breaks down two hydrogen peroxide molecules, which are derived from the reaction catalyzed by SOD, into one molecule of oxygen and two molecules of water. While the heme group is critical for its activity, past studies have also demonstrated the requirement of a tightly bound NADPH to the active conformation (39, 40). Furthermore, given the important role of catalases in regulating ROS levels, its localization in the peroxisome (41, 42) has been linked to the modulation of innate immune signaling. For instance, the reduction of catalase in peroxisomes from Drosophila-derived Mφs was found to impair actin organization and phagocytic activity in a p38-MAPK-dependent manner (43).

Apart from the SOD and catalase system, the glutathione system, which is composed of glutathione (GSH), γ-glutamyl cysteine synthase (GCS), GSH synthetase, glutathione peroxidase, and glutathione reductase (GSR), also plays an important role in

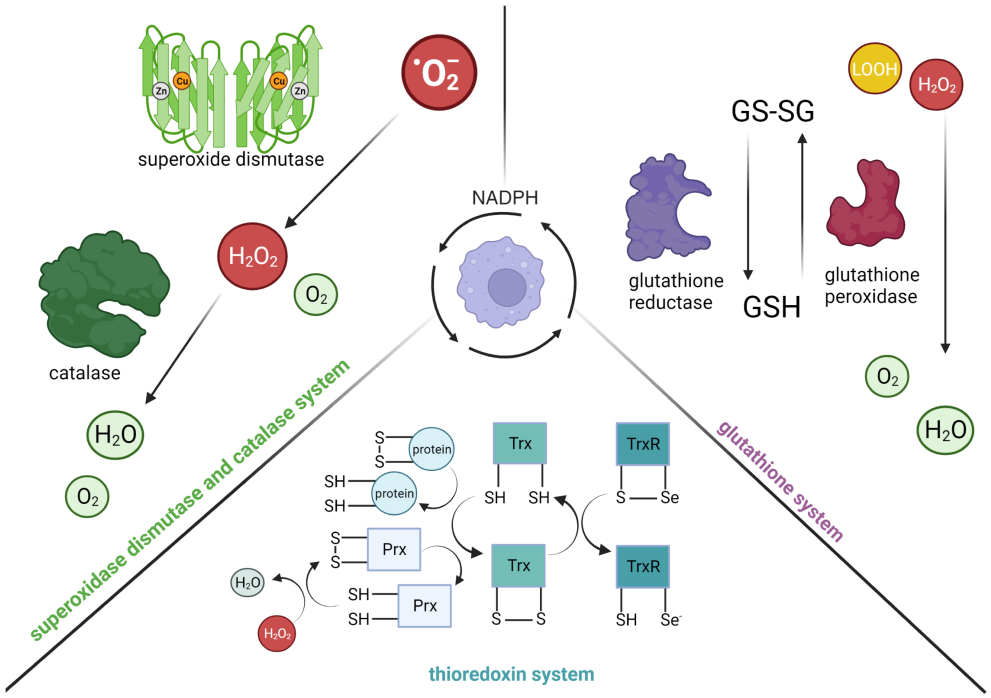

FIGURE 2
The consumption of NADPH in activated myeloid cells for anti-inflammatory processes. (Left) Superoxide dismutase detoxifies superoxide anions ($^{\cdot}O_2^-$) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Binding of NADPH to catalase is critical for activating its enzymatic function, specifically the conversion of hydrogen peroxide to water. (Right) Glutathione reductase reduces glutathione disulfide (GS-SG) to glutathione (GSH) in a NADPH-dependent manner. GSH is an important intracellular antioxidant used by glutathione peroxidase to reduce hydrogen peroxide or lipid hydroperoxide (LOOH) to water and oxygen. (Bottom) Thioredoxin reductase (TrxR) supports the reduction of thioredoxin ($Trx(SH)_2$) proteins and peroxidases ($Prx(SH)_2$) in a NADPH-dependent manner (selenium, Se). The reduction of peroxidases is important for the conversion of hydrogen peroxide to water, as well as reduction of oxidized proteins. Created with BioRender.com.

mediating cellular redox homeostasis. GSH is a tripeptide antioxidant, in which its synthesis is catalyzed by GCS and GSH synthetase from glutamate, cysteine and glycine. Cellular GSH is found mostly in the cytosol, with the remainder spread across organelles (44). Due to the cysteine residues of GSH, it can be readily oxidized to glutathione disulfide (GSSG) by electrophilic species directly (e.g., combine with NO to form less reactive S-nitrosoglutathione), or indirectly through enzymatic reactions (e.g., reduce hydrogen peroxide to water by glutathione peroxidase). GSSG can be reduced back to GSH by GSR in a NADPH-dependent manner.

The thioredoxin system is composed of Trx proteins and Trx reductases (TrxR), in which the reduced form of Trx proteins (Trx $(SH)_2$) are disulfide reductases as they contain dithiol groups in their highly conserved active site (-Cys-Gly-Pro-Cys-) (45, 46). Specifically, the dithiol groups of $Trx(SH)_2$ can directly reduce oxidized proteins or provide electrons to thiol-dependent peroxidases (Prx) to convert hydrogen peroxide to water. The oxidation of the dithiol groups of $Trx(SH)_2$ consequently results in the formation of intra-chain disulfide bridges and hence the oxidized form of Trx proteins ($TrxS_2$). The intra-chain disulfide bridges in $TrxS_2$ can be reversibly reduced back to $Trx(SH)_2$ by TrxR in a NADPH-dependent manner. To date, three isoforms of Trx have been identified in mammalian cells: Trx1 (cytosolic), Trx2 (mitochondrial), and SpTrx (spermatozoa cells) (47). Similar to Trx proteins, TrxR are also oxidoreductases that can catalyze reduction on small-molecule substrates, such as H_2O_2 and lipid hydroperoxide, in addition to $TrxS_2$. Specifically, TrxR are selenocysteine-containing enzymes that utilize the reducing equivalents from NADPH to catalyze reduction reactions.

4 Competition for NADPH between proinflammatory and antioxidative processes

NADPH plays a dual role in regulating both oxidative and antioxidative processes during inflammation; thus, the abundance of NADPH is significantly limited during the activation of myeloid cells. For instance, Everts et al., showed a significant depletion of NADPH pools in LPS-activated DCs, while others also reported similar findings in Mφs stimulated with LPS alone, or LPS with IFN-γ (27, 33, 48). To regenerate more reduced equivalents of NADPH, LPS-activated Mφs upregulate NADPH-generating pathways, including glucose-6-phosphate dehydrogenase (G6PD), which is the rate limiting enzyme of the PPP (2, 49, 50). Specifically, it decarboxylates G6P and forms ribose-5-phosphate (R5P) via three irreversible reactions. During these reactions, two molecules of $NADP^+$ are reduced to NADPH with the simultaneous liberation of one CO_2 molecule. Like enzymes in glycolysis, G6PD is allosterically regulated. Under resting condition where there is a high $NADPH/NADP^+$ ratio, G6PD remains as an inactive monomer since NADPH binds to its allosteric site. However, during inflammatory condition where there is a high demand to consume NADPH, G6PD is released from its inhibition and forms an active homodimer (51, 52). Apart from the PPP, two subtypes of isocitrate dehydrogenase (IDH) isoenzymes can also generate NADPH based on their intracellular localization: mitochondrial $NADP^+$-dependent IDH (*Idh2*), as well as cytosolic and peroxisomal $NADP^+$-dependent ICDH (*Idh1*). Both families of enzymes use $NADP^+$ as cofactors to perform reversible reactions, where isocitrate is oxidatively decarboxylated to alpha-ketoglutarate and generates one NADPH per reaction. In the context of LPS-activated Mφs, the expression of *Idh1* has been conflicting as various studies have reported its expression to be increased (53), decreased (2) or unaffected (50). Finally, folate-mediated one carbon metabolism, in which its activity is induced in LPS-activated Mφs (54) also contributes to the regeneration of reduced NADPH levels, with serine and glycine as the major carbon sources of this pathway. Specifically, methylene tetrahydrofolate (THF) dehydrogenases catalyze the oxidation of 5,10-methylene-THF to form 10-formyl-THF, which is subsequently oxidized to CO_2 with concomitant NADPH production by 10-formyl-THF dehydrogenases (55). Overall, all the studies above have shown that upon inflammatory activation of myeloid cells, there is a high demand for NADPH consumption. This leads to its marked depletion with a concomitant increase of activity in the PPP as the primary mechanism to generate more reducing equivalents of NADPH.

Since the concentration of NADPH in resting Mφs is in the micromolar range (33), which is within the range of the K_m's of NADPH-dependent enzymes, such as NOS2 (33), NOXes (56), glutathione reductase (57), small changes in intracellular NADPH abundance will greatly impact the activity of these enzymes (58). Therefore, under inflammatory conditions where NADPH is even further depleted, these enzymes may compete for reduced NADPH equivalents. Indeed, one study has shown that administrating Kuppfer cells with *t*-butyl hydroperoxide, a substrate for glutathione peroxidase, inhibited the production of superoxide (59), which implied that the increased activity of the antioxidative pathway limits the availability of NADPH for the use by oxidative pathways (58). In addition, recently we showed that NADPH consumption by HIF-1α versus NRF2-dependent apoenzymes is vital for regulating inflammation in Mφs (60). Specifically, the accumulation of oxidized low-density lipoprotein (oxLDL) in Mφs enhanced LPS-induced expression of NRF2-dependent ROS detoxification enzymes (i.e., GSR) and suppressed the expression of HIF-1α-dependent ROS producing enzymes (i.e., NOS2). This subsequently led to a shift of NADPH consumption from oxidative to antioxidative processes, eventually impairing the inflammatory responses in Mφs with accumulated oxLDL.

Apart from this, the competition between NADPH-dependent enzymes in inflammatory versus antioxidative pathways can also be revealed by blocking the function of G6PD under inflammatory conditions as the output of both pathways will be impaired. For instance, for inflammatory pathways, blocking the expression of G6PD impaired pro-inflammatory cytokine expression and lipid accumulation in LPS-activated DCs (12). In addition to *de novo* lipid synthesis, the loss of G6PD function also led to impaired NOS2 activity in Mφs activated by LPS (61) or by IFN-γ and infected by *Trypanosoma cruzi* (62). Human granulocytes that are deficient of G6PD also have impaired production of superoxide, nitric oxide,

and hydrogen peroxide (63). Similar findings were also reported in PMA-stimulated mouse and human neutrophils where inhibition of G6PD significantly impaired their ability to undergo oxidative burst (64).

5 Future perspectives and conclusion

Many studies in the last few decades have identified and characterized the multifunctional roles that NADPH plays in regulating inflammation, redox homeostasis, and anabolic processes. However, how NADPH simultaneously coordinates these disparate functions to regulate the extent of an inflammatory response remains unclear. In this review, we have highlighted studies that demonstrate how NADPH metabolism is altered in activated myeloid cells, and how the competition for NADPH consumption between oxidative and antioxidative pathways reflects a potential way to efficiently regulate the magnitude of inflammation. More importantly, recent technological advancements have enabled the development of tools to quantify and trace NADPH levels in real-time and across subcellular compartments, thereby providing spatial and temporal information that was previously unavailable with traditional methodologies (65–68). For instance, quantitative flux analysis of NADPH, which employs tracking of deuterium incorporation into NADPH, has revealed that folate-dependent methylenetetrahydrofolate dehydrogenase (MTHFD)-mediated NADPH production provides anti-oxidant activity to cells and enables resistance to oxidative stress (68). Not surprisingly, many human cancer cells overexpress genes of the *MTHFD* family (69) and thus are promising targets for anti-cancer therapeutics.

To date, NADPH metabolism has been targeted primarily for cancer therapeutics as cancer cells upregulate NADPH synthesis to support their massive antioxidative and anabolic requirements (55). The differential metabolic requirements between cancerous and non-cancerous cells provides a therapeutic opportunity for regulating selective cellular immune responses. Indeed, inhibitors that target NADPH synthesis enzymes, which aim to manipulate ROS levels and induce cell death selectively in cancerous cells, have been extensively developed (55). Several inhibitors of G6PD and IDH, such as RRx-001, DHEA and AG-881, have demonstrated promising efficacy and have entered Phase III clinical trials (55). However, the synthesis of highly selective or isoform-specific inhibitors that reduce unwanted side effects still remains challenging. Future research is warranted to address these challenges and investigate the possibility of synergizing inhibitors of NADPH synthesis for novel combinatorial therapies with current chemotherapeutics.

Author contributions

KKYT: Writing – original draft, Writing – review & editing. JJ-B: Writing – review & editing. MIC: Writing – review & editing.

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Conflict of interest

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

Publisher's note

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The roles of short-chain fatty acids derived from colonic bacteria fermentation of non-digestible carbohydrates and exogenous forms in ameliorating intestinal mucosal immunity of young ruminants

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Short-chain fatty acids (SCFA) are a class of organic fatty acids that consist of 1 to 6 carbons in length. They are primary end-products which arise from non-digestible carbohydrates (NDC) fermentation of colonic bacteria. They are the fundamental energy sources for post-weaning ruminants. SCFA represent the major carbon flux of diet through the gut microbiota to the host. They also play a vital role in regulating cell expansion and gene expression of the gastrointestinal tract (GIT). Recently, remarkable progresses have been made in understanding the immunomodulatory effects of SCFA and their interactions with the host. The processes involved in this study encompassed inflammasome activation, proliferation of lymphocytes, and maturation of intestinal mucosal immunity maturation. It is important to note that the establishment and maturation of intestinal mucosal immune system are intricately connected to the barrier function of intestinal epithelial cells (IEC) and the homeostasis of gut microbiota. Thus, insights into the role of SCFA in enteric mucosal immunoreaction of calves will enhance our understanding of their various regulatory functions. This review aims to analyze recent evidence on the role of SCFA as essential signaling molecules between gut microbiota and animal health. Additionally, we provide a summary of current literature on SCFA in intestinal mucosal immune responses of dairy calves.

KEYWORDS

dairy calves, gut microbiota, intestinal mucosal immunity, non-digestible carbohydrates, short-chain fatty acids

Introduction

Neonatal calves are primarily protected by their innate immune system, as essential immune components do not become fully functional until they reach four weeks of age or puberty (1). The innate immune responses, which are mediated by neutrophils and macrophages, do not fully develop until late gestation (2). The humoral elements present in calves, such as cytokines and complements, are significantly fewer compared to those found in adults (3). Peripheral blood T cells experience a significant decrease in numbers during the final month prior to birth, as they actively migrate and settle in the lymphoid tissues of fetal calves (4). The number of B cells are much lower in calves than adults (5). The number of dendritic cells is limited in neonates, and their antigen-presenting ability to activate the acquired immune system is delicate (6). The percentage of circulating natural killer cells increases from 3% to 10% of total lymphocytes between 1 to 8 weeks of age (4).

Calves are born with agammaglobulinemia and depend on the absorption of maternal immunoglobulins (Ig), primarily from colostrum, to ensure sufficient passive mucosal immunity after birth (7). The passive transfer of maternal Ig across the small intestine within the first 24 hours of birth serves to protect a calf from common pathogens until its own immature mucosal immune systems becomes fully functional (8). Importantly, the addition of sodium butyrate in milk tended to stimulate the concentration of circulating Ig in piglets (9). Passive immunity poses a dual challenge for unweaned calves. On one hand, it offers direct protection against diseases by providing immunoprotection. On the other hand, it can hinder the development of adaptive immunity establishment post vaccination (10). Multidrug-resistant *Escherichia coli* can also spread via colostrum feeding process among neonatal dairy calves (11). Acquiring immunoprotection through early vaccination is crucial for disease resistance in neonatal calves, especially considering the immense diversity and abundance of enteropathogenic microorganisms present in the environment. These microorganisms include bovine coronavirus, rotavirus, bovine viral diarrhea virus, *Salmonella enterica*, *Escherichia coli*, *Clostridium perfringens*, and Cryptosporidium parvum. Colostrum-fed preruminant calves are capable of generating robust adaptive mucosal and cellular immunity following early vaccination (12, 13).

The endogenous short-chain fatty acids (SCFA) are the primary metabolic end products of the fermentation of non-digestible carbohydrates (NDC), which include acetic acid, butyric acid, and propionic acid. They are widely distributed in colon. Considering that free forms of SCFA present a strong odor and their limiting uses in diet formulation, the beneficial exogeous types of SCFA mainly contained the infusions of both sodium acetate, sodium propionate, and sodium butyrate, which induced ameliorated antioxidant capacity, enhanced expression levels of occludin protein, and increased abundance of rumen bacteria, mainly including *Butyrivibrio*, *Rikenellaceae* RC9, and *Alloprevotella*. Sodium butyrate infusion can also strengthen antioxidant capacity, rumen and gut barrier functions (14). As for colonic digesta, the supplementation of butyrate precursors, such as gluconate, Ca-butyrate, have been shown to increase butyrate production in the GIT. Ca-butyrate increased *in vivo* ruminal acetate absorption and tended to increase *ex vivo* gut barrier function (15). The concentration of SCFA in the GIT, ranging from 20 to 140 mM, is primarily determined by several factors including the presence of microorganisms, transit time of intestinal substrate, metabolic flux of SCFA between the host and microbiota, and the fiber content of in the host diet (16). The production pathways of acetate are widely distributed among gut microbiota, whereas the production pathways of propionate and butyrate seem to be highly conserved and substrate specific in bacteria. Nowadays, metagenomic approaches facilitate characterization of bacteria accounting for SCFA production. Among these organisms, *Akkermansia municiphilla* has been identified as a key propionate producer (17). *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii* and *R. bromii* appear to be the primary organisms responsible for butyrate production (18). More studies are needed to focus on the relationships between dietary intake, gut microbiota diversity, and function, and their significances on calf intestinal health.

As important energy sources, SCFA salvage energy from NDC sources, which contribute 5% to 15% of the total caloric requirements (19). Apart from acting as local substrates for energy production, SCFA play a crucial role in maintaining intestinal mucosal immunity of human beings. They accomplish this by fortifying the barrier function of intestinal epithelial cells (IEC), primarily through enhancing the transcription of mucin genes in the goblet cells (20, 21). SCFA have been demonstrated their effectiveness in restraining the growth of pathogenic bacteria (22, 23). Furthermore, SCFA serve as important regulators of pro-inflammatory mucosal immune responses and the expansion of peripheral T cells (24–26).

The purpose of this review is to summarize recent findings on the functions of SCFA and the underlying mechanisms by which they ameliorate intestinal mucosal immunity in dairy calves. In addition, we aim to provide new insights into the effects of SCFA on immunoregulation and the interactions between gut microbiota, SCFA, and the intestinal mucosal immunity of dairy calves.

The roles and mechanisms of SCFA in intestinal passive mucosal immunity

Regulation of the gastrointestinal barrier function by SCFA

The maturation process of the gastrointestinal tract (GIT) has received significant attention in dairy calves as the first inherent barrier. In fact, the establishment of the innate gastrointestinal epithelial barrier is closely linked to SCFA, tryptophan and its derivatives, and some amino acids. (Figure 1) (27). Short-chain fatty acids, especially butyrate, play a significant role in the postnatal development of the ruminal epithelium in calves (28). A well functioning rumen epithelium could lead to improved calf performance at a younger age.

Previous studies have shown that butyrate supplementation can affect villus growth in pre-ruminant calves (29, 30). In fact, butyrate supplementation and intensive milk feeding stimulate body growth

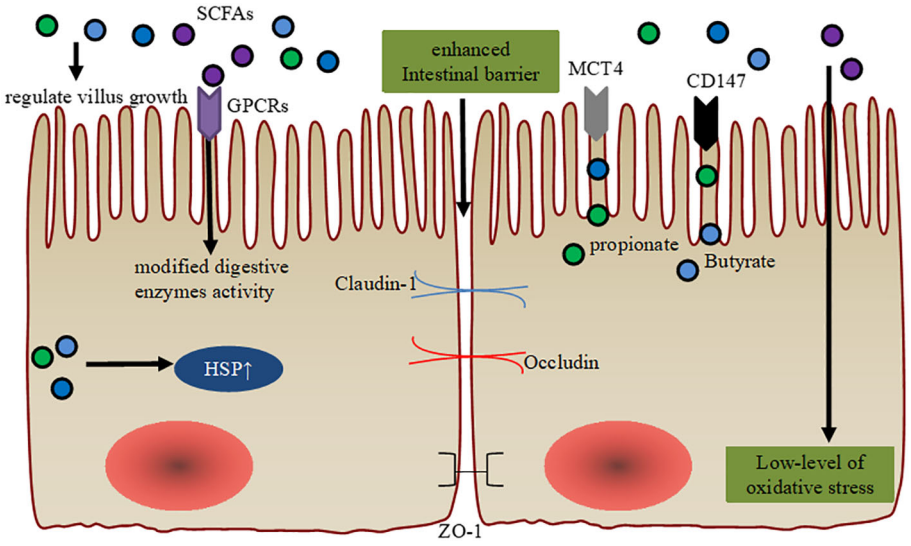

FIGURE 1
Maintenance of intestinal barrier function via SCFA. SCFA regulate intestinal villus growth and modified cellular digestive enzymes activity through GPCR. SCFA orchestrate the expression of the tight junction proteins (ZO-1, Occludin, and Claudin-1) to ameliorate intestinal barrier function. Intracellular SCFA stimulation of the cytoprotection through heat shock protein (HSP) and they have antioxidative stress function. Enhanced gene expression levels of MCT4 and CD147 are observed in the ruminal epithelial cells post-weaning, which are associated with the effects of diet-derived propionate and butyrate.

and affect GIT development in pre-weaning calves. Intensive butyrate supplementation induces an elevated growth of the small intestinal mucosa, but does not affect rumen development during the weaning period (31). Furthermore, extra additions of SCFA, especially acetate, propionate, and butyrate, improve digestibility and feed efficiency by enhancing GIT maturation, modified activity of digestive enzymes, and stimulating cytoprotection through the expression of heat shock protein (HSP) expression in young animals. Importantly, these results indicate an improved gut microbiota structure, including enriched *Butyricicoccus* and *Faecalibacterium* (27, 32, 33). Additionally, increased enterocyte proliferation in the upper jejunum and duodenal villi height were observed post sodium butyrate feeding in pre-weaning calves (29). Interestingly, administration of SCFA have also been found to increase the transcription of mucin genes in human intestinal epithelial goblet cells (20). SCFA or inulin supplementations, induce enriched α-defensin of Paneth cells *in vitro*, and that histone deacetylation (HDAC) and signal transducer and activator of transcription 3 (STAT3) might play a role in butyrate-mediated induction of α-defensins (34). Importantly, extra sodium butyrate addition provided more Na^+ to the ruminal epithelium, which may help stabilize tissue integrity (35).

In other mammals, SCFA-producing microbes or SCFA induce goblet cell differentiation, mucus production, and high IEC integrity, thus maintaining colonic epithelial homeostasis (36, 37). Similarly, butyrate restoration can improve IEC junctional integrity in young mice (38). In weaning calves, sodium butyrate has been used to support expression of tight junction (claudin-1, claudin-4 or occludin) and tight junction-associated proteins (TJP, zonula occludens) in stratified squamous ruminal epithelium (39). Butyrate is also considered the most important regulator of TJP in human IEC cells, upregulating the expressions claudin-1 and zonula occludens-1 (ZO-1) (40).

Unprotected sodium butyrate supplementation (0.3% of dry matter or 45 g/d) has been proven to stimulate growth performance, feed efficiency, and GIT development in pre-weaned calves, and it can be recommended for practical use on dairy farms (30). Importantly, sodium butyrate supplementation increases glutathione peroxidase (GSH-Px) activity and decreases malondialdehyde (MDA) concentration among preweaning calves, helping them cope with the oxidative stress they experience in their young lives (41). Dietary SCFA supplementations in liquid or solid feed can promote GIT development in newborn calves (30). Furthermore, the increased gene expression levels of MCT4 and CD147 are observed in ruminal epithelial cells post-weaning, which are associated with the effects of propionate and butyrate derived from the diet (42). Thus, future studies should focus on comparing the effects of different sources and forms of SCFA on GIT development and calf performance to confirm their beneficial effects on the gastrointestinal barrier. On the other hand, low feed intake during heat stress, transportation, and infectious disease pose significant challenges to the ruminal epithelial barrier. Feed restriction increases the risk of post-restriction subacute ruminal acidosis in weaning calves, as it rapidly and dose-dependently decreases the absorption capacity for SCFA (39). Although oral butyrate is used in weaning calves to support ruminal barrier development, excessive butyrate intervention may promote hyperkeratosis, parakeratosis, and epithelial injury in the fully developed rumen of adult cows (39). Therefore, future research is urgently needed to enhance the understanding of appropriate SCFA concentrations and the maintenance of intestinal barrier function during the early life period of dairy calves.

Facilitation of passive mucosal immunity progression and maintenance of the balance between intestinal immunity and diseases via SCFA

Since Ig are mostly obtained from colostrum, which acts as the only source of antibodies before calves begin to produce its own Ig in sufficient quantities. Proper management and improvement of passive transfer of Ig from dam to newborn calves have been reported to play a vital role in determining the health of these young animals (43). It is known that passive transfer of maternal IgG is facilitated by receptor-mediated endocytosis via the epithelial FcRn receptor and endocytosis using "transport vacuoles" (44, 45). However, as calves mature, epithelial cell endocytosis slowly diminishes. Maternal macromolecule passage over the GIT is largely suppressed in neonatal animals as they grow (46). Currently, the threshold for passive transfer of immunity is a blood IgG concentration of 10 mg/mL or a serum total protein concentration of 5.2 to 5.5 g/dL in the first 2 days of neonatal calves (47). The promotion of passive transfer of innate immunity and the prevention of failure in passive immunity transfer are the underlying reasons for feeding dairy colostrum. In fact, dietary butyrate supplementation has been shown to improve the IgG concentration in porcine colostrum. Butyrate addition in the milk also tends to stimulate the circulating IgA concentration in piglets. This is attributed to the fact that the serum IgG concentration and IgA-positive plasma cell count in the jejunum from pigs fed sodium butyrate were significantly higher than those given the basal diet (9, 48, 49). Importantly, SCFA promote B-cell IgA class switching and intestinal IgA production via the GPR43 of dendritic cells (DCs) in mice (50). However, direct butyrate supplementation, including rumen-protected butyrate and calcium-sodium-butyrate, did not affect serum immunoglobulin concentrations in pre-weaning calves (43, 51). While, feed supplementation with mulberry leaf flavonoids increased the total volatile fatty acid and propionate concentrations in pre-weaning and post-weaning calves, thus inducing enhanced serum concentrations of IgG and IgA (52). Supplementing lambs with Rosmarinus officinalis leaves or Chinese medicine polysaccharides had greater serum IgG and IgA compared to control groups (53, 54). This discrepancies may be attributable to differences in animal species and the assumed fact that SCFA had no direct effect on B-cell IgA or IgG class switching and intestinal IgA or IgG production. Butyrate may reduce IgG absorption by increasing the rate of cell differentiation, thus inducing early maturation of epithelial cells in newborn calves (43). Previous studies also demonstrate that maternal antibody transfer has extra-immunological effects in addition to the classical protective immune effects. These extra effects mainly include direct effects on intestinal growth and other organs in neonatal animals, especially on GIT structure, enteric nervous system, hippocampal development and behavior of animals (55, 56).

In fact, SCFA and IgA supplement each other. The secretory IgA (sIgA) isotype is the most abundant Ig in mucosal secretions and accounts for about 7% of total Ig composition in dairy colostrum (57). Secretory IgA favors the development of commensal bacteria in mice, especially the enriched SCFA-producing bacteria in the gut lumen, and also provides sufficient protection against enteric pathogens through its mucus-binding properties (58, 59). Additionally, the colonization of SCFA-producing bacteria is beneficial in defending against pathogens, stimulating commensal bacteria colonization on the mucosal surface and inducing enriched SCFA in the gut lumen. SCFA-producing bacteria, especially acetate-producing gut bacteria, induced IgA production mainly by the activation of GPR43 (G protein-coupled receptor 43) and cytosolic cGAS-STING pathway (60). While, endogenous IgA cannot reach a functional concentration (1 mg/mL) before 8 days of calf age, and appreciable blood concentration of IgA is only detected 16 days after birth (10, 61). Many schemes have been used to stimulate IgA production. Interestingly, direct Saccharomyces cerevisiae boulardii (SCB) supplementation or the mutual interaction between SCB and bacteria is responsible for IgA production and early bacterial colonization in the GIT of neonatal calves (62). This is attributed to gut microbiota improvement, with reduced *E. coli* and enriched *Fecalibacterium* in the hindgut, thus inducing higher production of SCFA in SCB treatment groups.

As an important part of innate immunity, antimicrobial peptides (AMP) secreted by IEC play a crucial role in regulating intestinal homeostasis by controlling intestinal microflora populations. Butyrate can boost AMP production, such as defensin and regenerative islet derived protein III γ in the IECs of mice through the SCFA receptor GPR43. Furthermore, butyrate also improves the expression of porcine β-defensin-2 and β-defensin-3 (27).

The host's innate responses to pathogens hold the balance between intestinal immunity and diseases. SCFA show promise in the prevention and treatment of intestinal diseases in human health applications and have multi-faceted roles in different metabolic systems (63). It has been demonstrated that SCFA supplementation enhanced IL-4 and immunoglobulin productions in response to challenges, accompanied by enhanced titers for bovine viral diarrhea and respiratory parainfluenza-3. Additionally, supplementation of milk replacer with a blend of butyric acid increases antibody responses and improves growth and feed efficiency in pre-weaning calves. Moreover, alterations in IL-4 mRNA expression levels are closely related to the humoral immune responses of calves (64), highlighting the feasibility of SCFA as novel immunoregulators. This assertion is based on the fact that IL-4 promote the differentiation of T and B cells in Ig synthesis. IL-4 induces IgE and IgG4 secretion by B cells in peripheral blood mononuclear cells (PBMC) of humans (65). Butyrate administration suppresses nuclear factor kappaB (NF-κB) activation in macrophages and also induces the inhibition of histone deacetylase (HDAC) in acute myeloid leukemia in humans (66, 67).

However, to date, the exact mechanisms underlying these immune effects are still unclear. Elucidating the immunomodulatory mechanisms of SCFA in dairy calves will help unravel the advantages of SCFA supplementation and provide clues for preventing and controlling diarrhea and pneumonia.

Maintenance of gut microbiota homeostasis by SCFA

During the first few weeks or months of a calf's life, there is a significant change in their digestive physiology as they transition from being a simple monogastric animal to a fully functional ruminant (68). Unfortunately, calf intestinal diseases have a major impact on productivity and result in substantial economic losses for dairy operations. Out of these diseases, calf diarrhea and other digestive problems are the primary contributors to pre-weaned calf mortality (69, 70).

Proper colonization of microbiota plays a crucial role in the development of the immune system and the establishment of the GIT structure. It also helps neonatal calves develop resistance against pathogenic challenges and creates a functional fermentation environment (71). Additionally, more than 20% of milk solids reach the hindgut during the milk feeding phase, emphasizing the importance of hindgut microbiota in dairy calves (72). The hindgut microbiota's significance on feed fermentation during the pre-weaning period is indicated by the upregulation of predicted microbial genes involved in energy metabolism, amino acids metabolism, and carbohydrate metabolism (68). The close alignment between SCFA, mucosa-attached carbohydrate utilizing microbiota (such as *Coprococcus* 1, *Blautia*, and *Lachnospiraceae* NC2004 group), and pathogenic bacteria (*Escherichia-Shigella* and *Salmonella*) further highlights the importance of hindgut microbiota in fermentation process during the pre-weaning period (73). The presence of *Butyricicoccus*, *Faecalibacterium*, *Collinsella*, and *Coriobacterium*, key commensal bacteria of healthy newborn calves, is positively related to high production of unabsorbed carbohydrates, SCFA, and other prebiotics (74). Tributyrin supplementation significantly increased the abundance of short-chain fatty acid (SCFA)-producing bacteria, including *Ruminococcaceae*, *Lachnospiraceae*, *Prevotella* and *Rikenellaceae*. This increase was negatively associated with TLR2 and IL-1β expressions, but positively linked to intestinal barrier genes expressions (75). Besides, the molar proportion of SCFA have the positive correlation with colon mucosa-associated beneficial bacteria, indicating that SCFA might play an important role in maintaining the gut health of 2-d-old calves (76). Sodium butyrate has also shown the instructive effects on growth and performance occur in tandem with changes in the abundance of health-associated bacteria in the hindgut of milk-fed calves (77).

During the first month of life, milk-fed preruminant calves have a similar number of colonized bacterial species in the rumen and colon. The variation of colonic bacterial composition significantly diminishes by four weeks of age (78). Lactic acid bacteria, such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*, dominate the microbial community in the hindgut. High SCFA concentrations may inhibit the abundance of genus *Bacteroides*, which could be beneficial for intestinal health and survival of the neonatal calves in the early weeks of life (72).

As an important metabolite of gut microbiota, SCFA have great potential as feed additives to ameliorate the gut microbiota species and community of calves. However, there are still many controversies regarding their effects on the early colonizations of microorganisms.

Ameliorations of intestinal inflammatory reaction and protective immunity via SCFA

Previous studies have revealed the regulatory function of SCFA in intestinal immune system (Figure 2). In most cases, they act as signaling molecules that promote tolerogenic and anti-inflammatory cell responses by inhibiting HDAC, which results in inactivated nuclear factor-kappaB (NF-κB) and downregulation of tumor necrosis factor (TNF) production in mammals (24, 25). The inhibition of HDAC by SCFA is a crucial regulator of NF-κB activity and pro-inflammatory immune responses. A cohort study found a higher prevalence of SCFA-producing bacteria belonging to *Ruminococcaceae* and *Lachnospiraceae* in healthy neonatal calves, with an enriched presence of butyric acid compared to the bacterial enteritis group (79). Additionally, in mice, the binding of SCFA to GPR43 and GPR109A in IEC activates inflammasome assembly and enhances the downstream inflammatory cytokine IL-18 (80).

SCFA contain volatile species with short half-lives and rapid metabolism, and only adequate amounts of SCFA are sufficient to trigger HDAC activation in human colonic cells (81). However, their effects may require specific transporters since SCFA can also suppress HDAC through GPCR-dependent mechanisms in mammals (82, 83). Therefore, further studies are needed to investigate the immunoregulatory functions and therapeutic potentials of SCFA in dairy calves.

Previous reports have show that SCFA influence the proliferation of peripheral T cells, especially regulatory T cells (Treg), in the mucosa lamina propria through HDAC inhibition. In mice, inhibition of HDAC9 upregulates the expression of forkhead box P3 (FOXP3) and expands FOXP3 $^{+}$Treg cells (84). Therefore, giving mice a high-fiber or SCFA-supplemented diet not only eliminates colonic inflammation but also suppresses allergic airway diseases by increasing the suppressive activity of FOXP3 $^{+}$Treg cells (85). Orally administered SCFA induced the activation of both effector (Th1 and Th17) and regulatory T cells in ureter and kidney tissues of young mice, leading to T cell-mediated ureteritis and kidney hydronephrosis. Furthermore, systemically administration of SCFA at higher than physiological levels can cause dysregulated T cell responses and tissue inflammation in the renal system of mice (86). Thus, in addition to their immunological effects, the pathological effects of chronically elevated SCFA should also be taken seriously.

Considering that SCFA are ligands for GPCR, many studies have also explored other mechanisms of SCFA-induced GPCR engagement. GPCR is expressed by numerous cell types, including IEC, dendritic cells, and T cells (87). GPR43 expression has been found to be critical for the expansion and suppressive function of Treg cells (26). Additionally, both niacin and butyrate acid can prevent colitis and colon carcinogenesis by upregulating anti-inflammatory molecules secreted by monocytes, promoting differentiation of Treg cells and interleukin-10 (IL-10)-producing

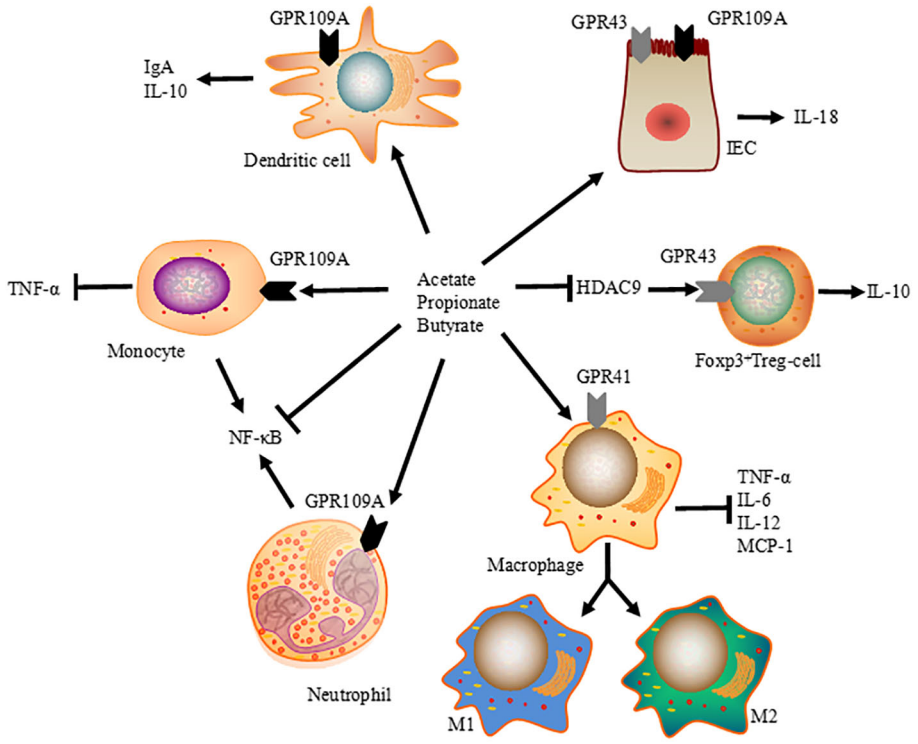

FIGURE 2
Immunomodulatory function of SCFA on intestinal immune system. SCFA modulate tissue inflammation and protective immunity by promoting the differentiation of Foxp3 positive Treg cells, promoting intestinal IgA and IL-10 expressions via dendritic cells, and enhancing IL-18 production in IEC. At the same time, SCFA-induced HDAC inhibition is a crucial regulator of NF-κB activity, reducing the expression of TNF-α, IL-6, IL-12, and MCP-1 in macrophages, and ameliorating TNF-α production in monocytes.

T cells (88). Gallic acid has been shown to mediated colitis attenuation through the upregulation of hindgut acetate and butyrate, with elevated expression of IL-10 and TGF-β in newborn calves (89). Therefore, the immunoregulatory effects of SCFA largely depend on the context and cell types, allowing the host to monitor pro-inflammatory immune responses and maintain mucosal immune homeostasis.

Conclusions and future perspectives

Previous publications have revealed the beneficial effects of SCFA on GIT maturation, the transfer of passive mucosal immunity, microbiota homeostasis, and the moderation of immune responses. SCFA, which provide energy for microbes and strengthen the expansion of IEC, have been found to establish a mutual relationship with the host. in dairy calves. This interaction accelerates the fermentation of undigested complex carbohydrates, leading to the maintenance of microbial communities compositions and homeostasis of host's mucosal immunity.

In fact, SCFA and their derivatives show promise in treating human diseases, particularly inflammatory bowel diseases. Therefore, the application of SCFA as feed additives in calf nutrition is very promising, as they hold potential as replacements for certain antibiotic growth promoters. Further research on SCFA may help in developing valuable supplements and providing alternatives to antibiotics in the dairy industry.

Author contributions

ZH: Funding acquisition, Writing – original draft, Writing – review & editing. HD: Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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A metabolic perspective of the neutrophil life cycle: new avenues in immunometabolism

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Neutrophils are the most abundant innate immune cells. Multiple mechanisms allow them to engage a wide range of metabolic pathways for biosynthesis and bioenergetics for mediating biological processes such as development in the bone marrow and antimicrobial activity such as ROS production and NET formation, inflammation and tissue repair. We first discuss recent work on neutrophil development and functions and the metabolic processes to regulate granulopoiesis, neutrophil migration and trafficking as well as effector functions. We then discuss metabolic syndromes with impaired neutrophil functions that are influenced by genetic and environmental factors of nutrient availability and usage. Here, we particularly focus on the role of specific macronutrients, such as glucose, fatty acids, and protein, as well as micronutrients such as vitamin B3, in regulating neutrophil biology and how this regulation impacts host health. A special section of this review primarily discusses that the ways nutrient deficiencies could impact neutrophil biology and increase infection susceptibility. We emphasize biochemical approaches to explore neutrophil metabolism in relation to development and functions. Lastly, we discuss opportunities and challenges to neutrophil-centered therapeutic approaches in immune-driven diseases and highlight unanswered questions to guide future discoveries.

KEYWORDS

metabolic reprogramming, neutrophil differentiation, glycolysis, immune mediated diseases, autophagy, mitochondrial respiration

1 Introduction

Phagocytic cells have evolved as a key defense line against sterile and microbial insults (1). Neutrophils are the most abundant terminally differentiated effector innate immune cell line in the bone marrow (BM) and peripheral blood. They rapidly localize to sites of infection to implement immediate and effective immune responses for pathogen clearance, and resolution of acute inflammatory responses. In fact, the anti-microbial functions of neutrophils promote an environment that is unfavourable to pathogens at the expense of tissue integrity therefore neutrophil numbers are tightly regulated. Due to their relatively short and variable half-life across tissues (2), neutrophils are constantly replenished from proliferative BM precursors. Differentiation through successive stages maintains their homeostatic levels and ensures their immediate availability to counter invading pathogens (Figure 1). Consequently, most hematopoietic stem cells (HSCs) and hematopoietic stem/progenitor cells (HSPCs) in the BM, that give rise to all blood cells through intermediaries, are committed to the production of neutrophils (3, 4). Neutrophils participate in the capture and destruction of invading microorganisms through chemotaxis, phagocytosis, degranulation, reactive oxygen species (ROS) production, formation of neutrophil extracellular traps (NETs), and production of cytokines and other inflammatory mediators upon pathogen recognition [reviewed in (5, 6)]. In this regard, neutrophil activation and subsequent stimulation of specific cell-surface receptors from a broad receptor repertoire (>30) controls neutrophil antimicrobial functions. Defects in neutrophil development, migration, function and clearance increases susceptibility to infection, inflammation and organ dysfunction in multiple organisms (7–10).

2 Neutrophil biology: the basics

2.1 Neutrophil development from the bone marrow, the circulation and tissues

HSCs and HSPCs in the BM proliferate and differentiate into granulocyte-monocyte progenitors (GMPs). Under the control of granulocyte colony- stimulating factor (G-CSF), produced by macrophages, and endothelial cells (ECs), these GMPs commit to neutrophil generation (granulopoiesis) (11, 12). A pool of hematopoietic progenitors and neutrophil precursors also exists outside the BM, including in the spleen, lung and peripheral circulation, where they differentiate into mature and functionally competent neutrophils to allow acceleration of antimicrobial responses in infected tissues. Notably, neutrophil phenotype reprogramming occurs in tissues regardless of origin (2, 9, 13). During the early phase of an infection and inflammatory response, a rapid increase in *de novo* neutrophil release and production with the appearance of immature neutrophils in the peripheral blood (known as left-shift), termed "emergency" granulopoiesis, helps to meet the enhanced demand for neutrophils as these cells are consumed in large quantities to rapidly counteract bacterial invasion (3, 14–16). This process occurs at the expense of lymphopoiesis and has been reviewed elsewhere in greater detail [reviewed in (14, 17, 18)]. Briefly, conserved microbial elements

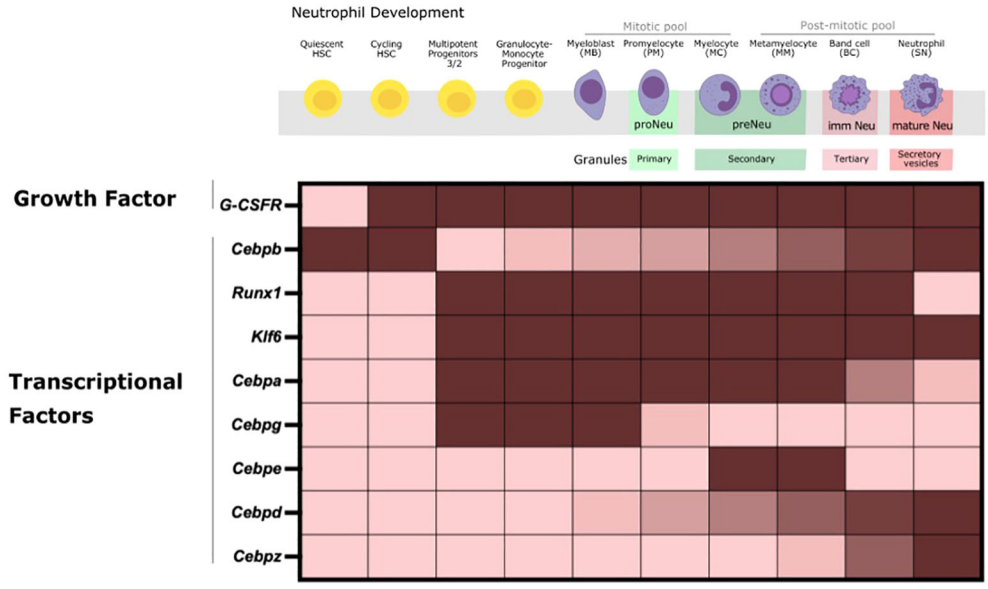

FIGURE 1
Neutrophil Production in the BM during steady-state happens through multiple successive stages. HSCs proliferate and differentiate into oligopotent committed myeloid progenitors (CMP, GMP) and sequentially into unipotent early neutrophil committed and proliferative progenitors that ultimately give rise to mature neutrophils with the full range of effector functions. Cell- intrinsic regulation of the expression of a multitude of TFs, as depicted here, is crucial to tightly regulate the expression of stage-specific granules (primary, secondary, tertiary and secretory vesicles that contain specific effector proteins) and genes for neutrophil commitment, proliferation, differentiation and maturation. Fully differentiated mature neutrophils then exit the BM to enter into the circulation and tissues under steady-state conditions.

known as pathogen-associated molecular patterns and host tissue derived damage-associated molecular patterns are recognized by pattern recognition receptors such as, toll-like receptors (TLRs) which are transmembrane proteins, on the surface of multiple immune and non-immune cell types leading to immune cell activation (19, 20). This causes subsequent cytokine and chemokine production and signalling through a common pathway involving nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation (19). NF-kB, consisting of RelA (p65) and p50, activated downstream of TLRs, which upon phosphorylation by mitogen-activated protein kinases (MAPKs), promote its translocation to the nucleus and leads to robust transcriptional expression of cytokine & chemokine genes including G-CSF, and an array of additional immune genes (19). Dimerization of granulocyte colony-stimulating factor receptor (G-CSFR) by G-CSF, expressed on BM neutrophil precursors (i.e. metamyelocytes and onwards), activates downstream signal transduction pathways [reviewed in (21)]. This regulates HSCs proliferation and differentiation, and directs their commitment and that of early neutrophil progenitors (proNeu1 and proNeu2) (4), and precursors, towards the myeloid lineage (3, 22). This aids in the production of mature neutrophils in order to maintain homeostatic levels under basal and emergency conditions (21, 23). However, G-CSF and G-CSFR independent mechanisms to produce mature granulocytes also exist. This includes IL6-mediated (4) pathways, as well as through intracellular signaling cascades, epigenetic landscapes, and transcriptional networks, such as CCAAT/enhancer-binding proteins (Cebps), to activate specific and tightly regulated gene expression programs for neutrophil differentiation (2). Additionally, there is a role of different nutrient sources, namely glucose, and amino and fatty acids, for HSC lineage specification and commitment (24–26).

As myeloid precursors pass through the various stages of differentiation and maturation to become mature neutrophils in the BM, they undergo morphological changes (nuclear segmentation), increase chemotactic responsiveness and acquire features necessary for microbicidal activity (Figure 1). Neutrophil classification based on nuclear morphology, single-cell RNA sequencing (scRNA-seq), and surface marker expression has allowed assessment of mouse and human neutrophil ontogeny, phenotypic heterogeneity and mapping the developmental continuum of cell fate hierarchies (5, 27, 28). The granulopoietic niches of neutrophils within the BM are divided into proliferative mitotic, post-mitotic and the mature neutrophil pool. Neutrophils are released into systemic circulation as a result of differential expression of C-X-C chemokine receptor type 4 and 2 (CXCR4 and CXCR2, respectively) (14, 29, 30). Directional cues from the chemokine stromal cell-derived factor 1, also known as CXC motif chemokine ligand 12 (CXCL12), produced by BM stromal cells, i.e., CXCL12-abundant reticular cells (CAR cells), regulate the CXCL12/CXCR4 chemokine/surface receptor signaling axis for the retention of neutrophils in the BM (31). In contrast, the upregulation of CXC motif chemokine ligand 2 and 1 (CXCL2 and CXCL1, respectively) and CXCR-2 receptor on BM ECs and neutrophils respectively allows for the recruitment of these cells into the circulation, and into naïve and inflamed tissues through activation of downstream signaling pathways (32).

2.2 Neutrophil interactions in the tissue microenvironment

2.2.1 Neutrophil crosstalk with innate and adaptive immune cells

It is well accepted that macrophages are responsible for the clearance of apoptotic neutrophils at the end of their lifecycle and resolve inflammation (33). In fact, studies report neutrophils that have physiologically "aged" in the circulation ($CD62L^{lo}CXCR4^{hi}$ neutrophils) to be eliminated by macrophages in the BM and tissues. This process also maintains the rhythmic egress of neutrophils into the circulation (30, 34). Similarly, in self-limited lipopolysaccharide (LPS)-induced peritonitis, BM-derived Resolvin D4, a pro-resolving lipid-derived mediator, increases BM-macrophage efferocytosis of apoptotic neutrophils to aid in the resolution infectious inflammation (35). In a mouse model of acetaminophen-induced acute liver injury, neutrophils have crucial functions in liver repair. They do so by promoting the phenotypic conversion of pro-inflammatory $Ly6C^{hi}CX_3CR1^{lo}$ monocytes/macrophages to pro-resolving reparative $Ly6C^{lo}CX_3CR1^{hi}$ macrophages mediated through neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase ROS production (36). Neutrophils also support monocyte and macrophage recovery in blood, BM and spleen following genotoxic injury as well T helper 17 cells and macrophage recruitment and priming in a mouse model of atherosclerosis (2, 37). Importantly here, both macrophage or neutrophil-mediated cytokine production leads to immune cell activation and priming for an exaggerated immune response. Therefore the interaction between both is crucial in infectious and inflammatory disease outcomes. These concepts are reviewed elsewhere in greater details (38–40).

A common phenomenon in chronological ageing known as reverse transendothelial migration (rTEM), where neutrophil exhibit retrograde mobility within ECs junctions and re-enter the vascular lumen. This process is shown to be mast cell-derived CXCL1 dependent (32, 41, 42). Here, the rTEM neutrophils are of noxious phenotype capable of inducing remote organ damage in acutely inflamed aged tissues. In fact, multiple studies also show neutrophil recruitment in physiology to be mast cell dependent (43–46). The impact of neutrophils on T cells in various disease contexts (47–52) is also summarized in excellent reviews (39, 40, 53–55).

2.2.2 Neutrophil crosstalk with non-immune cells

Neutrophils' interaction with non-immune cells is crucial to regulate their biology. In this regard, BM CAR cells, a population of mesenchymal stem cells, produce CXCL12 that mediates the retention of neutrophils in the BM through CXCR4 ligation (31). Platelet-neutrophil interaction are also implicated in homeostasis and inflammation (40, 54, 56–59). Neutrophil and ECs interaction allows for unidirectional migration through venular walls (54, 60,

61). Most importantly, aberrant neutrophil-endothelial interactions are implicated in a wide range of inflammatory diseases that relate to neutrophil influx and tissue damage (62–64). Similarly, neutrophil-epithelial crosstalk is involved in the maintenance of the epithelial-lined organs where uncontrolled neutrophil processes contribute to pathogenesis of diseases (65–68).

2.3 Mechanisms implicated in neutrophil-mediated immunity

Acute and chronic inflammatory and infectious immune responses are marked with heightened immune cell recruitment, and dramatic shifts in tissue and systemic metabolism including nutrient depletion, hypoxia and the generation of large quantities of reactive nitrogen and oxygen intermediates (69). There is an heightened interest in identifying the role of unique metabolites and metabolic pathways in immunoregulation, a field termed immunometabolism, ranging from energy metabolism to the modulation of signalling pathways and post-translational modifications (70–74). Traditionally, neutrophils are thought to be purely glycolytic and the role of mitochondria, a central organelle for energy homeostasis and metabolic control, is believed to be minimal, for neutrophil function (75). However, this view has been challenged, as their critical role as first line defenders requires high metabolic plasticity to respond to environmental cues and regulate innate immune responses (76).

2.3.1 Overview of neutrophil metabolism

Circulating neutrophils primarily rely on glycolysis and the pentose phosphate pathway (PPP), both of which take place within the cytosol, as their preferred metabolic strategy to fuel phagocytosis, ROS production, and NET formation. However, fatty acid oxidation (FAO), tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS) that occur within the mitochondria, are also undoubtedly crucial under both steady-state and inflammatory conditions (77–80). Although immunometabolism (70, 81) and metabolic plasticity is increasingly understood, minimal data is available on neutrophil metabolic reprogramming under diverse nutritional, metabolic and pathologic conditions. This review is therefore focused on key neutrophil functions and their dependence on different key metabolic pathways in experimental animals and human cohorts. We also emphasize approaches used to study immunometabolism and how different nutrient environments contribute to neutrophil biology.

3 Metabolic programmes for granulopoiesis

Neutrophil production is tightly regulated by intrinsic and extrinsic cellular factors via a number of transcription factors (TFs) that regulate subsequent stages of neutrophil development. The hierarchical expression and activation of tightly defined TFs is required for lineage specification of HSPCs and proper commitment and differentiation of myeloid precursors into mature neutrophils in the BM (Figure 1) (9, 31). TFs are largely regulated at transcriptional, translational, and posttranslational levels. Consequently, transcriptional control, isoform usage, phosphorylation, and acetylation of TFs are crucial for the proper activation of gene regulatory mechanisms that orchestrate the differentiation of HSCs into committed cells.

3.1 Transcriptional regulation of granulopoiesis

TF such as Cebps and others work in a combinatorial manner to orchestrate the transcriptional networks for neutrophil lineage commitment, proliferation, differentiation and functional responses. The expression of these TFs is also correlated with stage-specific granule expression across the neutrophil lineage and therefore antimicrobial capacity (4, 23, 82, 83). Changes in its expression can significantly affect downstream genes that impact the neutrophil development and function trajectory (84).

Cepba binds to promoters of myeloid-related genes to activate myeloid-lineage gene expression program and repress non-myeloid lineage genes in hematopoietic progenitors of multi-lineage potential in the BM. The combinatorial expression of Cebpa, Gata1 and low levels of PU.1 are required at the GMP stage for granulopoiesis initiation, and expression of primary granule proteins, and Cebpa deletion is shown to skew progenitors towards lymphopoieses (31, 81, 85, 86). Similarly, Cebpg is a pro-proliferative factor that is particularly required by myeloblasts, promyelocytes and myelocytes. Cebpe further drives differentiation at myelocytes, and metamyelocytes and contributes to the expression of secondary granules (4, 31). Here, Cebpe deletion leads to an accumulation of GMPs (4, 31). Expression of Cebpb, Cebpd, and PU.1 is found in the most mature neutrophil precursors and in peripheral blood granulocytes. However, studies in Cebpb deficient mice do not observe defects in steady-state granulopoiesis, but rather Cebpb is shown to be crucial for "emergency" granulopoiesis (86, 87). On the contrary, Cebpd and PU.1 are involved in the expression of tertiary granules such as matrix metalloproteinase-9 (Mmp9) and CXCL2 at the band cell stage for the terminal differentiation of neutrophils (31, 81). The expression of Cebpz is prominent from band cell onwards with peak levels in the most mature neutrophil precursors and in peripheral blood granulocytes (31, 81). Additionally, Runx1 and Klf6 are shown to modulate neutrophil maturation (9).

Altogether, co- expression of specific TFs within specific subpopulations serves to drive early lineage specification towards distinct progenitors. These TFs also function during the later developmental stages to drive differentiation and maturation for optimal antimicrobial defense. Newer studies focus solely on the global gene expression patterns of these central TFs to drive neutrophil ontogeny through bulk and scRNA-seq to assess transcriptional programs across neutrophil subsets (31, 81, 88). However, the post-transcriptional modifications and mechanisms that enable these transcriptional signals to regulate coordinated neutrophil differentiation remain incompletely understood. Nevertheless, there is some evidence on the role of changes in

genome accessibility, master transcriptional regulators and metabolites for controlling these TFs and subsequently immune responses, some of which are discussed in more detail below (9, 23, 23, 73).

3.2 Metabolic regulation of the transcriptional regulatory networks

An intricate link between cellular metabolism, transcription, and signalling pathways directly and indirectly supports the regulation of genes involved in cellular differentiation and cellular processes (73, 81, 84, 89). It is well-accepted that the mitochondria, through TCA metabolites, control chromatin modifications, DNA methylation, and post-translational modifications of proteins to alter their function. However, how specific metabolites regulate TFs for neutrophil development has not been described previously (73, 90). Enhancing intracellular nicotinamide adenine dinucleotide (NAD^+) levels, through Vitamin B3 supplementation, is found to support sirtuin-1, Cebpa, Cebpb, G-CSF and G-CFSr expression for neutrophilic differentiation and migration in CD34+ hematopoietic progenitor cells, the promyelocytic leukemia cell line HL-60, and in primary bone marrow CD34+ cells from severe congenital neutropenic patients (91). Glucose metabolism has also been shown to regulate Cebpb activity for adipogenic gene expression and differentiation of preadipocytes to adipocytes (92). Glucose can induce nicotinamide mononucleotide adenylyltransferase (NMNAT)-2 expression, an enzyme for NAD^+ biosynthesis, and reduce nuclear poly (ADP-ribose) polymerase (PARP)-1 enzymatic activity with Cebpb. Conversely, glucose deprivation reduces NMNAT-2 levels, increases the enzymatic activity of PARP-1 and reduces the binding of Cebpb to target gene promoters thereby preventing adipogenesis (92). At the same time, activity of mammalian target of rapamycin complex (mTORC)-1 controls Cebpb expression and its activity is inhibited under caloric restriction (93). The mechanism of Cebpb regulation by glucose, NAD+, and mTORC1 remains unknown. The Vitamin D receptor and Retinoic Acid Receptor α in human acute myeloid leukemia cell lines (HL60, and KG1 cells) is also shown to regulate the expression of Cebpa, Cebpb and subsequently Cebpe for granulocytic differentiation (88). While these studies examine the direct and indirect roles of metabolites for Cebp regulation in the adipose, liver tissues and specific cell lines, the metabolic pathways regulating these TFs in the hypoxic BM microenvironment for neutrophil development remains an open avenue for future research. A study of autophagy Atg7 deficient neutrophils demonstrated that the metabolic program does not influence the transcriptional program in neutrophil precursors where these precursors are morphologically and functionally immature (7). Therefore, further studies are needed to identify the parallel roles of transcriptional regulators and metabolic programming, if any, for cellular differentiation. Interestingly, glycolytic and mitochondrial metabolites are shown to control chromatin modifications, and DNA methylation in physiology and disease and may be relevant in this context (73, 94).

3.3 Metabolic programming of cellular differentiation

3.3.1 Metabolism as a key determinant of hematopoietic stem cell fate

Differentiation of HSCs into fully functional mature neutrophils requires considerable energy for extensive cytoplasmic and nuclear remodelling (95) (Figure 2). Autophagy decreases mitochondrial mass in quiescent HSCs for their self-renewal capacity. As a result, quiescent HSCs primarily rely on anaerobic glycolysis to maintain stem cell quiescence and self-renewal in the low oxygen niches of the BM. Increased peroxisome proliferator-activated receptor δ (PPARδ) activation for mitochondrial biogenesis, mitochondrial ROS, and FAO in cycling HSCs demarcates asymmetric division, one daughter with stem cell features and one committed progenitor, and further regulates HSC maintenance (25, 71, 78, 96–100). Therefore, the metabolic programs of HSCs balance self-renewal and commitment. Similarly, a lower mitochondrial mass and mitochondrial activity and increased transcriptional activation of the TF hypoxia-inducible factor 1-alpha (HIF-1a) to drive aerobic glycolysis by Meis1 in quiescent HSCs, allows for a long-term reconstitution capacity of these cells in transplantation experiments. Here, increased mitochondrial biogenesis is associated with exit from quiescence (25, 26, 97, 98, 101). In fact, flow cytometric analysis of the metabolic profile of HSCs using tetramethylrhodamine methyl ester (TMRM) shows only 6%-9% of total BM cells with low mitochondrial potential ($TMRM^{lo}$) but this population contains more than 80% of quiescent HSCs (98). Interestingly, $TMRM^{lo}$ quiescent HSCs cultured for 5 days under differentiation-inducing conditions (with cytokines SCF, Flt3, IL-3 and IL-6) and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) revert to a state of self-renewal. $TMRM^{hi}$ quiescent HSCs are however unable to revert to functional stem cells (25). Additionally, proteomic analyses highlights the role of increased translation of mitochondrial transcription factor A (TFAM) in proerythrocytes and its regulation of downstream genes associated with mitochondrial metabolism for proper erythrocyte differentiation (102). Similarly, to improve the efficacy of hematopoietic cell transplantation, where limited numbers of HSCs are present, transplantation of CD34+ HSCs with peroxisome proliferator-activated receptor γ (PPARG)-specific small hairpin RNA (shRNA) can promote expansion of HSCs and HSPCs. This is through enhanced glycolysis and HSC self-renewal, conversely inhibition of glycolysis and enhancement in PPARG suppresses this expansion (95, 103). These studies highlight the essential role of nutrients and their associated signaling pathways on the self-renewal, differentiation and lineage commitment potential of HSCs.

3.3.2 Metabolism as a master regulator of neutrophil state

The metabolic state also shapes committed progenitor cells. Expression of type III receptor tyrosine kinase ckit, in early committed and immature neutrophil progenitors in the BM requires enhanced mitochondrial function, i.e., oxidative phosphorylation, to promote rapid symmetric differentiation. This further highlights the essential role of increased mitochondrial

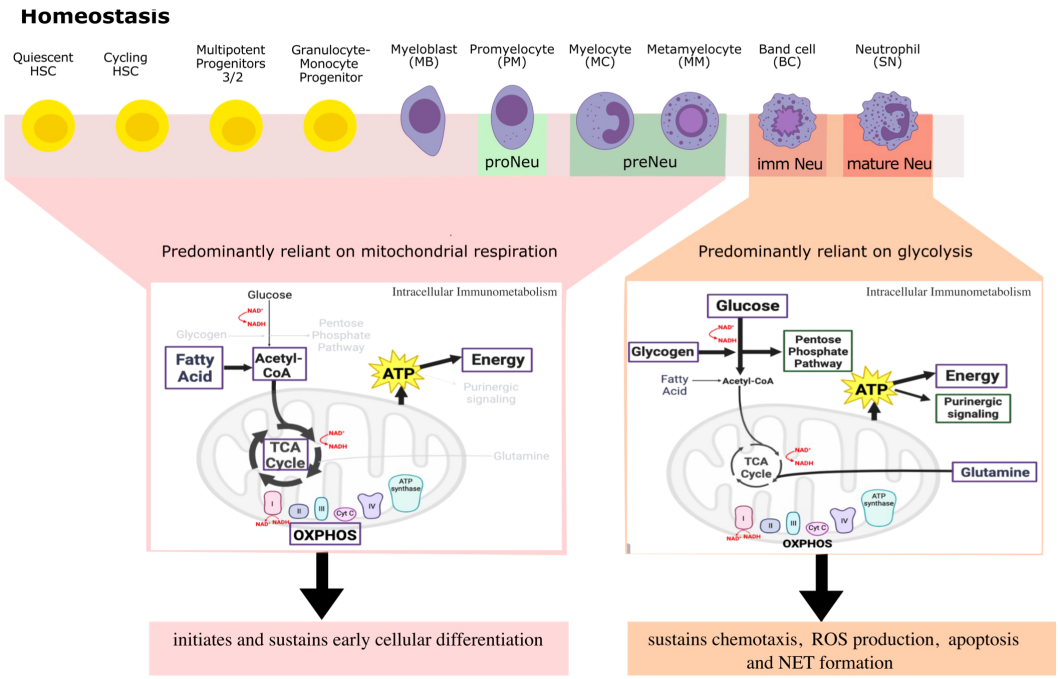

FIGURE 2
A simplified overview of differential metabolites and metabolic pathways that neutrophils engage in throughout differentiation and beyond to regulate their large repertoire of immune responses in steady-state. Here, neutrophil committed progenitors and early precursors engage in fatty acid oxidation, TCA cycle and OXPHOS for ATP generation that sustains neutrophil differentiation programs. Indeed, higher mitochondrial activity is indicative of a more immature neutrophil phenotype. Mature neutrophils on the other hand, rely primarily on glycolysis and little mitochondrial respiration to regulate and sustain ATP production for effector functions and for autocrine purinergic signaling. Metabolic requirements of neutrophils are not 'black and white' in inflammatory conditions, but rather a continuum of these processes works together to regulate an optimal response, a perspective that requires more investigation. Highlighted in purple are metabolites used for energy production and green are pathways used by metabolites to regulate neutrophil functions.

activity for HSC cell fate decisions under steady-state (71, 104). Similarly, transcriptomics and proteomics has shown that mitochondrial function is essential in the early stages of granulopoiesis for lineage commitment and the initiation of neutrophil differentiation in ckit$^+$ neutrophils. Mitochondrial function is key for the differentiation in ckit$^+$ HSCs from free fatty acid utilisation (104–106). Complex III of the electron transport chain may be responsible for ATP generation in early myeloid precursors in the BM in this context (47, 105). Similarly, shRNA-mediated knockdown of alanine, serine, cysteine transporter 2 (ASCT2) glutamine transporter in CD34$^+$progenitors, upregulates CD11b myeloid marker, highlighting the skewness of the progenitors towards the myeloid lineage that happens irrespective of glucose and glutamine metabolism (71). Adenylate kinase 2 (AK2) regulates the homeostasis of mitochondrial adenine nucleotides (ADP, ATP and AMP) by catalyzing the transfer of high-energy phosphate in the mitochondrial intermembrane space, once the HSCs commit to a specific lineage pathway. AK2 deficiency results in impaired proliferation and differentiation in granulocyte precursor cells shown using the HL-60 promyelocytes cell line, through impairment in oxidative phosphorylation for energy metabolism (107). In fact, even though enhancement of glycolysis is evident by accumulation of pyruvate and lactate, neutrophil differentiation is hindered due to incomplete mitochondrial activity by hematopoietic progenitors that is required to meet the greater energy demand. This suggests an indispensable role of mitochondrial metabolism in neutrophil differentiation (106, 107). This is relevant for reticular dysgenesis, an inherited immune deficiency disorder caused by AK2 deficiency. As neutrophils differentiate and become mature neutrophils, there is a decline in mitochondrial number and activity with a change in mitochondrial morphology from a more tubular phenotype to round to regulate spontaneous cell death (108–110).

3.3.3 Role of autophagy for mitochondrial metabolism in neutrophil differentiation

Autophagy and mitochondrial respiration are critical determinants of neutrophil differentiation. Autophagy is a conserved cellular recycling process, involving several conserved autophagy-related genes (*Atg*), that enables the degradation of cytoplasmic content in lysosomes for diverse cellular processes. In fact, autophagy (regulated by *Atg5* or *Atg7*) degrades lipid droplets, providing free fatty acids used by the mitochondria through the TCA cycle and oxidative phosphorylation for ATP production at the myeloblast and myelocyte stage for terminal neutrophil differentiation (Figure 2) (7). As a result, higher oxygen consumption rate (OCR) is evident in BM neutrophils. Here, microfluidic gene expression analysis with the Fluidigm-Biomark array reveals that all 15 glycolytic pathway genes analyzed are downregulated while the mitochondrial content simultaneously

undergoes a 2-fold increase during normal neutrophil differentiation (47, 111). This is further validated by targeted metabolomics. In *Atg7* deficient myeloblast cells, ECAR (measure of glycolysis, extracellular flux assay) and lipid accumulation, assessed through lipidomics, is increased, and mitochondrial ATP production is decreased. As a result, the expression of *Ldha* is also increased to generate lactate and NAD^+ to keep glycolysis going but the ATP levels and differentiation is not restored to wild-type amounts (7). In the same study, administration of either free fatty acids or pyruvate is able to restore energy metabolism and differentiation, supporting that mitochondrial respiration is essential for early neutrophil differentiation where energy requirements are the highest (7). Furthermore, deletion of *Atg7* at the GMP stage also leads to failure of neutrophil differentiation and an accumulation of immature forms which are functionally defective. Deletion of autophagy genes at later development stages results in a different phenotype where mature neutrophil are primarily expressed (7, 112). This reflects the need to carefully consider the developmental stages where autophagy genes are knocked down as it may have differential effect on neutrophil maturation.

Conflicting data indicates that since neutrophil mitochondria have decreased protein expression of some complexes, they may not be fully involved in ATP production but rather in maintaining functional responses through the mitochondrial membrane potential ($\Delta\Psi$mt) (105, 113). In fact, studies show that inhibition of complex III in both glucose-rich and glucose-depleted environments, completely diminishes $\Delta\Psi$mt but without an effect on ATP production. This suggests a role for mitochondria that extends beyond oxidative phosphorylation and ATP production for neutrophil development (105, 109).

4 Metabolism driven regulation of neutrophil migration

4.1 Neutrophil mobilization and trafficking

Neutrophil mobility is essential for cell recruitment to sites of inflammation and infection. Chemotaxis, is the ability of neutrophils to sense gradients, polarize and directionally migrate within a chemotactic gradient field to access invading pathogens [reviewed in (114, 115)]. TFs KLF6 and RUNX1 are shown to be valuable for controlling genes for neutrophil migration into inflamed sites (9). Interestingly, "swarming", tightly regulated by exosomes and lipid leukotriene 4 (LTB4), is also the coordinated feedforward movement of many neutrophils to accumulate in large numbers in inflamed and infected tissues in early stages of infection for optimal bacterial clearance (116–118). This is followed by migration arrest at later stages of infection to avoid uncontrolled aggregation at inflamed sites through G protein-coupled receptor kinase 2 (GRK2)-mediated G protein-coupled receptors (GPCR) desensitization, and prostaglandin E2 (PGE2) synthesis to promote anti-inflammatory programs for tissue repair (119, 120). Resolvin D4 also mediates this migration arrest (35).

In contrast, transendothelial migration (TEM) defines neutrophil migration across the endothelium to both inflamed and naïve sites, albeit at lower levels. This is dependent on receptor ligand interactions of adhesins, integrins and selectins. Adhesion molecules i.e., chemokines, integrin ligands and selectins on the endothelium, bind to G-protein-coupled chemokine receptors, integrins (Mac1-CD11b/CD18), and selectin ligands on the surface of neutrophils. These molecules tightly regulate rolling, adhesion and transmigration, i.e., paracellular (~96% of the time) vs. transcellular into tissues, as has been well defined in excellent reviews [reviewed in (113)]. Additionally, rTEM, re-entry of activated neutrophils from inflamed sites into the circulation, due to overexpression of CXCL1 at endothelial junctions and CXCR2 desensitization in mice also controls inflammatory responses (41, 42).

4.2 Metabolic regulation of neutrophil trafficking

Metabolic stress is a hallmark of several conditions including trauma and infection. Neutrophils isolated from trauma patients have enhanced markers of mitochondrial damage, i.e., mitochondrial-derived damage-associated molecular patterns, inflammasome activation, and reduced expression of CXCR2, thereby impaired chemotaxis (121, 122). Studies further show that upon stimulation of neutrophil-like HL-60 cell line with N-formyl-methionyl-leucyl-phenylalanine (fMLP), which activates formyl peptide receptors (FPRs) expressed on these cells, mitochondria with high $\Delta\Psi$mt localize to cell protrusions and mitochondrial-derived ATP is released into the extracellular spaces. This serves as an autocrine messenger to amplify chemotaxis signals through activation of $P2Y_2R$-mediated mTOR signaling at the leading edge, rather than as a direct energy source (123). In fact, two phases of purinergic signaling are suggested: an initial burst of ATP release that is driven by mitochondrial ATP and a second phase that involves glycolytic ATP production to maintain chemotaxis (Figure 3) (123). In this study, the fluorescent ATP probe 2-2Zn (II) anchored on cell membrane of live neutrophils reveals an increase in extracellular ATP production by the mitochondria immediately upon stimulation of cells with fMLP (123). Similarly, pretreatment with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or FCCP for 2 hours shows a direct relation between loss of $\Delta\Psi$mt, detected by increased green fluorescence of JC-1 probe, rounded cell shape and the inhibition of chemotaxis (75). Studies in zebrafish also show that neutrophils depend on $\Delta\Psi$mt for chemotaxis and that neutrophil-specific disruption of mitochondria *in vivo* is associated with inhibited motility, with an inability to reduce ROS species, and cell apoptosis (Figure 3) (124, 125). Similarly, hyperglycemic conditions in cancer provide microenvironments that promote metastasis of tumor-associated neutrophils and impair mobilization of antitumor neutrophils, resulting in poor prognosis that is seen for these patients. This shows that migration is selective based on the dominant neutrophil subset in the disease state and their preferred fuel of choice (126).

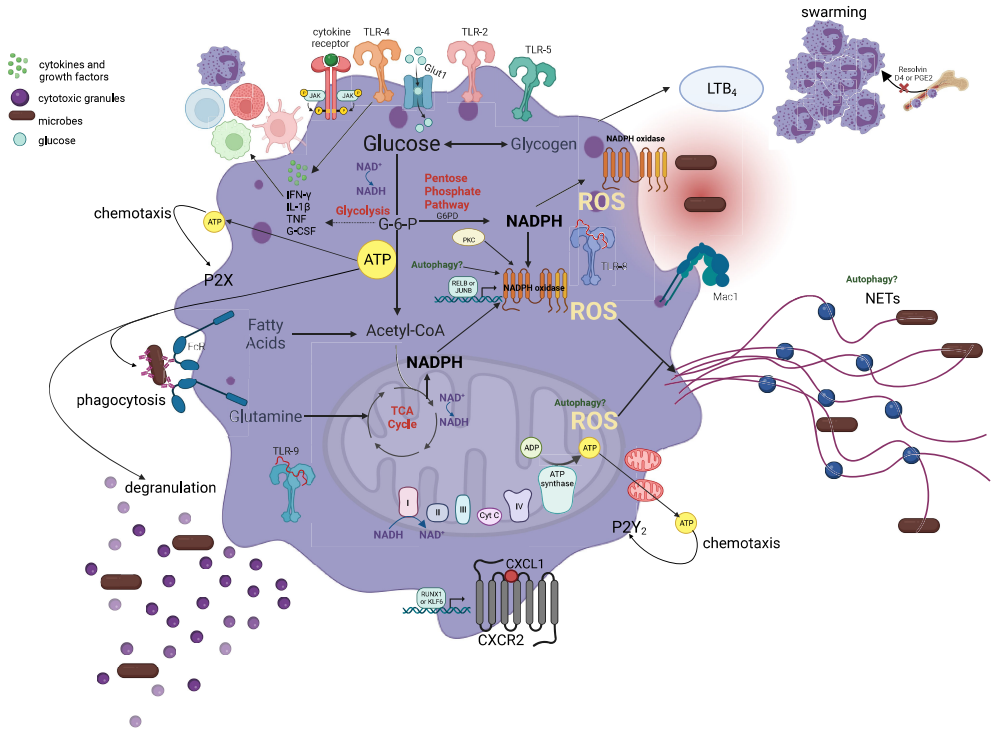

FIGURE 3
Metabolic pathways involved in the maintenance of neutrophil effector functions. Neutrophils rely primarily on glycolysis and very little mitochondrial respiration to regulate and sustain ATP production for chemotaxis, phagocytosis, cytokine expression, ROS production, degranulation, and NET formation in the circulation and peripheral tissues. An initial burst of mitochondrial-derived ATP release and a second phase that involves glycolytic ATP production maintains the autocrine purinergic signaling for chemotaxis. Glucose uptake and glycolysis regulates phagocytosis and degranulation while PPP is indispensable for NADPH generation, which is oxidized by the NADPH oxidase for ROS generation and eventually NETs. Additionally, mitochondrial-derived ROS can also enhance NET production. NADH/NAD ratio is crucial for the maintenance of glycolytic and mitochondrial ATP production and thus functional properties of neutrophils. Multiple metabolites, as depicted in this figure, including glycogen, and glutamine, are also capable of entering these metabolic pathways to regulate and sustain neutrophil responses in tissues. This metabolic flexibility of neutrophils allows them to coordinate optimal effector responses in tissues with diverse substrate availability.

5 Modulation of metabolism regulates NADPH-oxidase dependent functions

5.1 Overview of NADPH-oxidase dependent antimicrobial functions

As myeloid precursors become mature neutrophils, they acquire features necessary for optimal microbicidal activity including stage-specific granule and NADPH oxidase proteins (Figure 1), the most abundant proteins in mouse and human neutrophils and the main complex for neutrophil ROS production, respectively (31, 81, 127, 128). In fact, primary [cathepsin G (CTSG), myeloperoxidase (MPO), elastase (ELANE)], secondary [lactoferrin (LTF), neutrophil gelatinase-associated lipocalin (NGAL), cathelicidin (CAMP)], and tertiary (MMP9, lipocalin 2 (LCN2), CD11b/CD18, CXCL2) granules and secretory vesicles (β2 integrins), are well-defined granules that carry distinct types of cargo as mentioned. These highly toxic granule proteins are released into the phagocytic vacuole or extracellularly in a 'formed-first-released-last' model to help destroy invading pathogens (69, 128). In this context, the strength of the stimulus initiates the release of granules, both intracellularly and extracellularly, with the secretory vesicles being mobilized first and primary granules secreted at the end in response to multiple inflammatory mediators (129).

The NADPH oxidase (NOX) is a multicomponent electron-transfer complex that generates superoxide and other ROS into the phagosome or extracellularly for microbial killing events (23). This complex is comprised of five subunits that are expressed at different stages during neutrophil differentiation and packaged into the granules. More specifically, p22, p40, and Rac1/2 have stable gene expression levels throughout all stages, whereas gp91 has maximal gene expression at the metamyelocyte and band cell stages, and p47 and p67 are upregulated in the metamyelocytes and have the maximal gene expression at the mature stages (23). Consequently, studies show that while all NOX subunits are expressed by the metamyelocyte stage, peak levels of ROS are only reached at the end of differentiation (23). Interestingly, RELB and JUNB are important TFs for the expression of these genes for NADPH ROS generation (9). In steady-state, the membrane-bound (b558 - gp91, p22) and cytosolic components (p47, p67, p40, Rac1/2) do not interact with each other. Upon phagocyte activation in mature neutrophils, consumption of oxygen is increased, and these subunits are phosphorylated by protein kinase C (PKC). The cytosolic components migrate almost instantly to the phagocyte membrane where they assemble with the flavocytochrome b558 to form the active enzyme. In this regard, NADPH-oxidase mediated ROS production accounts for majority of neutrophil ROS production

(127). Excessive ROS production can also be damaging to the host cells therefore, balanced levels of ROS are crucial for pathogen clearance and restoration of tissue homeostasis.

5.2 Metabolic reprogramming for pathogen killing via NADPH oxidase ROS production

In this section, we aim to summarize the literature as it relates to metabolic requirements for neutrophil ROS generation.

5.2.1 Pentose phosphate pathway

When glucose enters cells, it is phosphorylated by hexokinase generating glucose-6-phosphate (G6P). G6P can flow through two different pathways: the glycolytic pathway, generating fructose-6-phosphate that is metabolized to lactate, to produce ATP, or the pentose phosphate pathway (PPP) that converts NADP+ to NADPH (Figure 3) (130–132). The latter is especially required for NADPH oxidase dependent ROS generation upon cell activation, that occurs with infectious and inflammatory stimuli, to enable high NADPH yield (132). Upon neutrophil activation here, PPP intermediates accumulate profoundly while many intermediates in glycolysis increase to a lesser extent, indicative of metabolic adaptation by neutrophils (132). NADPH oxidase, the primary source of neutrophil ROS production, oxidizes cytosolic NADPH to reduce molecular oxygen to the superoxide anion ($O2^-$) that can metabolize into more potent ROS for effective pathogen clearance (133, 134). The role of PPP for NADPH and subsequent NADPH-oxidase ROS production has been validated. In this study, two chemical inhibitors LDC7559, and NA-11, are both shown to activate the glycolytic enzyme phosphofructokinase-1 liver type (PFKL), the main phosphofructokinase-1 in immune cells, and dampen flux through the PPP in human peripheral blood neutrophils. This inhibits NADPH-oxidase ROS production, NETosis, efficient bacterial killing and tissue damage in an *in vitro* model of acute respiratory distress syndrome (ARDS) (130, 132).

5.2.2 Glycolysis and the electron transport chain

Lectins localized on the bacterial surface bind to carbohydrate-containing epitopes (fucose and mannose glycans) on neutrophil surface to induce intracellular ROS production (135). In addition, neutrophils respond to disseminated candidiasis through enhanced glucose uptake and glycolysis. Glycolysis is elevated through an accumulation of glucose transporter 1 (Glut1) on cell membrane to regulate phagocytosis, ROS production, and fungi elimination (136). Here, neutrophil-specific deletion of Glut1 does not affect neutrophil development, but rather antifungal activity and the control of systemic fungal infection through compromised glucose uptake and glycolysis (136). Moreover, BM derived neutrophils ($ckit^+$) appear to be dependent on both glucose metabolism and mitochondrial function for NADPH oxidase dependent ROS production (47, 104). In these studies, inhibition of glycolysis results in a substantial increase in mitochondrial oxygen consumption rate (OCR – measure of ROS production). Inhibition of mitochondria following restricted glucose utilization, completely abolishes OCR (47). On the contrary, inhibition of mitochondrial function in primary murine BM neutrophils with rotenone and antimycin A (complex I and III inhibitors, respectively) alone does not affect early ROS (47). However, reductions in the late phase of the response are seen, suggesting that while the mitochondria is not required for the rapid initiation of neutrophil responses, they may be required to sustain these responses (47). Furthermore, the same study provides evidence that when glycolysis is inhibited by 2- deoxy-D-glucose (2DG) as in cancer, the mitochondria can prioritize ROS production by sustaining levels of NADPH through fatty acid metabolism (Figure 3) in the immature, $ckit^+/CXCR2^+$ BM neutrophil phenotype (31, 47, 134, 135). This study also highlights the role of neutrophil phenotypic and functional heterogeneity for the regulation of disease state outcomes.

Other studies show that mitochondria-derived ROS (mitoROS) by the electron transport chain can indirectly regulate neutrophil activation by promoting degranulation and activation of NADPH oxidase upon cell stimulation with fMLP. This emphasizes that on its own mitoROS is inefficient for neutrophil functional responses and that NADPH oxidase is required for efficient microbial killing (137, 138). Inhibition of complex I or III of the respiratory chain upregulates mitochondria ROS production, diminishes LPS-stimulated neutrophils, and reduces severity of LPS-induced acute lung injury in mice (139, 140).

5.2.3 Metabolic perturbations implicated in NADPH-oxidase dependent ROS production

Mitochondrial dysfunction plays an important role in metabolic diseases such as diabetes. Autophagy is shown to be important for neutrophil function as neutrophils from streptozotocin-induced diabetic rats where high incident of infections is prevalent, show autophagy impairment (141). Consequently, reduced ROS production, depolarization of $\Delta\Psi$mt, low ATP content, and high content of cleaved caspase 3 (apoptosis marker) after phorbol myristate acetate (PMA) stimulation is seen. This is consistent with findings where *Atg7* and *Atg5* knockout mice show impaired degranulation of primary, secondary and tertiary granules and NADPH oxidase ROS (137, 141). Similarly, in another study, phagocytosis and bacterial killing is reduced in *Atg7* and *Atg5* deficient neutrophils (7). This suggests that while mTOR activity, which regulates glycolysis, is enhanced in diabetic rats, optimal neutrophil functions are achieved through mitochondrial metabolism. Here, treatment with metformin and induction of autophagy with AMP-activated protein kinase (AMPK) improves neutrophil responses (111, 125). In acute myocardial infarction (MI), the negative consequences, i.e., tissue damage and heart failure, are attributed in part to upregulation of neutrophil mitochondrial ROS production, and p47 phosphorylation with energy derived from glycolysis (142). However, treatment with arjunolic acid (AA), an antioxidant, reduces ROS via the inhibition of p47 and reduced mitochondrial and glycolytic oxidative burst activity to alleviate the negative consequences of MI (142). *In vivo* models of acute lung injury show that both

metformin (used frequently in the treatment of diabetes) and rotenone, can inhibit complex I of the electron transport chain (ETC). This inhibition increases intracellular ROS production by mitochondria, despite obvious reductions in ATP levels, and diminishes production of pro-inflammatory cytokines to mitigate disease severity (134). The mechanism through which neutrophils can use alternative fuel sources in diverse altered nutrient conditions remains to be explored further.

6 Metabolic requirements for NET formation

6.1 Overview of NETs

Within neutrophils, the ROS released by the NADPH oxidase complex may also induce the formation of neutrophil extracellular traps (NETs). NETs are decondensed chromatin strands of nuclear and mitochondrial DNA (mtDNA), decorated with antimicrobial molecules from primary granules (MPO, NE). These are released into the extracellular space to effectively trap and eliminate large pathogens, such as *Candida albicans* hyphae, *S. aureus*, *K. pneumoniae*, and *P. gingivalis* to name a few, but not small or single microbes, to prevent dissemination of these pathogens to systemic organs (143, 144). NETs generally result in neutrophil death, but NADPH oxidase-independent mechanisms, i.e., mitoROS-dependent, non-lytic form of NET formation also exist, for the degradation of gram-negative and positive bacteria (145). The pathway choice is largely dependent on the specific agonists encountered by the neutrophils (146). Pathogen size sensing by dectin-1 on neutrophils and NE translocation to the phagosome or nucleus, promotes phagocytosis and NET release with small, and large microbes, respectively, for efficient pathogen clearance, minimal tissue damage, and host survival. Lack of dectin-1 or Mpo in mice leads to increased NET formation and local and systemically disseminated *C. albicans* infection (144). However, the origin and mechanism of the DNA scaffold in *in vivo* NETs in bacterial infections remains debated. Transcription factor and kinase (p38 MAPK) activity, peptidylarginine deiminase 4 (PAD4) mediated histone citrullination or ROS-mediated DNA oxidation and repair machinery and resulting chromatin decondensation for NET formation may be important in homeostasis and inflammatory disease states (146–149). In cancer settings, NET formation drives metastases and during sterile conditions, increased NETs are indicative of autoimmune disease states (80, 150). The presence of 'adherent' NETs, extruded DNA adhering to the vasculature, by "fresh" neutrophils enhances vascular damage in mice (10). Therefore, despite their role in host defense, excessive NET formation is associated with pathology. As such, blunted NET formation may help to protect mouse and human lungs from exacerbated injury in pneumonia (10).

6.2 Metabolic requirements for pathogen killing via NETs

NET formation is strictly dependent on glucose through the glycolytic pathway [Figure 3; (136)]. *In vitro* assays show that neutrophils stimulated with 100 nM PMA in glucose-free media exhibit chromatin decondensation within a few hours, resembling the early stages of NET formation, but no NET release. The addition of glucose at a later time point allows for NET formation to occur within 10 minutes (151). Addition of glycolysis inhibitor 2-DG also completely inhibits NET release, while the addition of oligomycin also inhibits the formation of NETs through an undefined mechanism (151). Both NADPH oxidase ROS and autophagy play debated roles in chromatin decondensation for NET formation (152). For example, in trauma patients the increased AMPK activity modulates autophagy and impairs aerobic glycolysis and NETosis causing neutrophil dysfunction and adverse clinical outcomes (121, 153). The role of mTOR and H1F-1a as regulators of NET formation upon LPS stimulation is identified but the mechanism through which H1F-1a regulates NET formation remains to be explored (153). In this case, pharmacological and genetic H1F-1a knockdown reduces NETosis (153). Furthermore, both NADPH-oxidase dependent and independent NET formation by human and murine blood neutrophils are shown to depend on glycolysis dependent lactate formation where inhibition of lactate dehydrogenase (LDH) activity inhibits NETosis (154). The role of glycans, including heparan sulfate, and enhanced glycolysis for NETosis has also been studied extensively (155, 156). The findings presented here suggest that glycolysis, regulated through multiple pathways, plays an undisputed role in regulating maximal neutrophil responses. Here, neutrophil dysfunction following acute trauma is a result of reduced glucose uptake and metabolism. On the contrary, studies show that immature low-density neutrophils (iLDNs; neutrophil precursors) are able to promote breast cancer liver metastasis by executing NETosis under glucose-deprived conditions through glutamate and proline catabolism for mitochondrial-dependent ATP production (80). Furthermore, mitochondrial, optic atrophy type-1 (OPA1), appears indispensable for NOX-independent NET release and bacterial killing through indirect regulation of glycolysis (Figure 3) (81, 113, 153). Other agonists known to induce NET formation such as PMA produced similar effects as NETs of mtDNA origin, but the mechanism is unknown.

7 Expression of inflammatory mediators by neutrophils

7.1 Overview of cytokine and chemokine generation by neutrophils

Expression of cytokines and chemokines by myeloid and non-hematopoietic cells in response to bacterial components via TLR/NF-kB and MyD88 signaling drives neutrophil activation, and inflammation. Neutrophils themselves are capable of generating pro-inflammatory cytokines such as IL1a, IL1β, TNF and IL6 and the chemokines CCL2 and CXCL2, in response to stimulation (2, 9, 128). The TF JUNB is identified as a major regulator of the expression of these mediators (9). The aberrant production of these mediators can also drive pathological destruction of tissues (9, 41, 42).

7.2 Metabolic requirement for cytokine and chemokine generation by neutrophils

Studies examining the metabolic requirements for cytokine and chemokine generation by neutrophils in homeostasis and disease states are currently limited. In sepsis, neutrophils are crucial components of the innate immune response and the cytokine storm that characterizes the acute phase response, and this process requires glycolysis (157–159). Inhibition of glycolysis with metformin and rapamycin diminishes survival of mice with sepsis caused by *C. albicans*, lowers *ex vivo* cytokine production (of TNF and IL-1β) and increases fungal growth. Therefore, pro-inflammatory cytokine production appears to be a glycolysis driven process whereby stimulated mature neutrophils in inflamed tissues directly generate these inflammatory mediators (Figure 3) (9). In cancer, heterogenous neutrophil subsets indirectly influence cytokine production. In fact, a more glycolytic neutrophil ($ckit^{-}CXCR2^{+}$ phenotype) is unable to induce T cell death and inhibit interferon-γ (IFN-γ) production by T-cells, thereby enhancing anti-tumor immunity (47). On the contrary, oxidative neutrophils ($ckit^{+}CXCR2^{-}$ phenotype) in the tumor microenvironment suppress anti-tumor immunity through IFN-γ (47).

8 Inherited metabolic disorders that affect neutrophil function

A total of 485 inborn errors of immunity (IEI) with altered molecular, cellular, and immunological mechanisms contribute to our understanding of the inheritable monogenic defects for immunological disorders. IEI are categorized (160) by the International Union of Immunological Societies Expert Committee. Here, particular gene defects, characterized as combined immunodeficiencies, and congenital defects of phagocyte number or function, relate specially to neutropenia, neutrophil dysfunction and recurrent infections. We do not discuss all the inborn neutropenia and neutrophil disorders in this article. We only focus on those that link inherited metabolic defects with neutrophil dysregulation as metabolic processes are critical for neutrophils' functional fitness. Neutrophils are capable of glycogen cycling through glycogenesis and gluconeogenesis for energy production and maintenance of effector functions (161). Here, the enzyme glucose-6 phosphatase catalytic subunit 3 (G6PC3) hydrolyzes glucose-6-phosphate to generate glucose for glycolysis and downstream metabolic processes. Neutrophils from hypoglycemic patients with glycogen storage disease type Ib (GSD-Ib) and G6PC3 deficiency in severe congenital neutropenia have impaired ROS, killing defects, and recurrent infection (162–164). This emphasizes the indispensable role of glycolysis and PPP for neutrophil effector functions and infection outcomes. Here, dietary glucose and gene therapy holds therapeutic potential to restore neutrophil dysfunction (165). Similarly, deficiency of any of the oxidase components, due to mutations of autosomal or X-linked recessive genes encoding the 5 components, and the resultant inability to oxidize NADPH results in chronic granulomatous disease (CGD). Neutrophils from these patients migrate and phagocytose normally but fail to generate an NAPDH-oxidase dependent oxidative burst with the resultant failure of intracellular killing leading to recurrent bacterial and fungal infections, including pneumonia (134, 145). The literature on these inherited disease states is reviewed more extensively elsewhere (166) which readers are referred to. Mitochondrial respiration is known to maintain neutrophil differentiation and functions. Pearson syndrome is a rare mitochondrial disorder accompanied with large-scale mtDNA deletions, severe defects in erythroid and myeloid precursor cells, neutropenia, and both severe and fatal infections (167, 168). This emphasizes the previously underappreciated role of mitochondria in neutrophil development and control of infection outcomes. Additionally, 3-Methylglutaconic aciduria due to CLPB, an enzyme for leucine degradation, deficiency is also associated with mitochondrial dysfunction and altered neutrophil differentiation (160). Patients with mutated OPA1 develop autosomal dominant optic atrophy (ADOA) and neutrophils from these patients have a reduced ability for extracellular DNA released and impaired ability for pathogen killing due to impaired complex I activity, and limited NAD^{+} for glycolytic ATP production (113).

9 Regulation of neutrophil functions in various nutrient environmental conditions

Nutrition orchestrates cellular metabolism. Fluctuations in nutrient availability, i.e. nutrient excess and deficiency can modulate metabolic processes and consequently cause hyperactivation or immunosuppression of neutrophils associated with diverse inflammatory disease states (Figure 4). Inadequate and inefficacious immune responses are common in more affluent and developed countries with excess nutrient intake (171). Nutrient deficiency is associated with exaggerated inflammation and impaired resolution of inflammation (172, 173).

9.1 Micronutrient-dependent regulation of neutrophils in homeostasis and inflammation

9.1.1 Overview of micronutrients involved in neutrophil biology

Different micronutrients relate to neutrophil biology in homeostasis and disease states (174), in particular, Vitamin C (175–178), iron (179–181), zinc (182–185), Vitamin D (186, 187) and Vitamin A (188, 189). In general, a deficiency in any one of these micronutrients is associated with dysregulated neutrophil functions, particularly neutrophil recruitment, degranulation, ROS formation and NETosis, resulting in higher susceptibility to recurrent and severe infections. We particularly focus on the rapidly evolving field of nicotinamide and nicotinic riboside, which are both forms of Vitamin B3 and central mediators of metabolic processes, for the regulation of neutrophil biology.

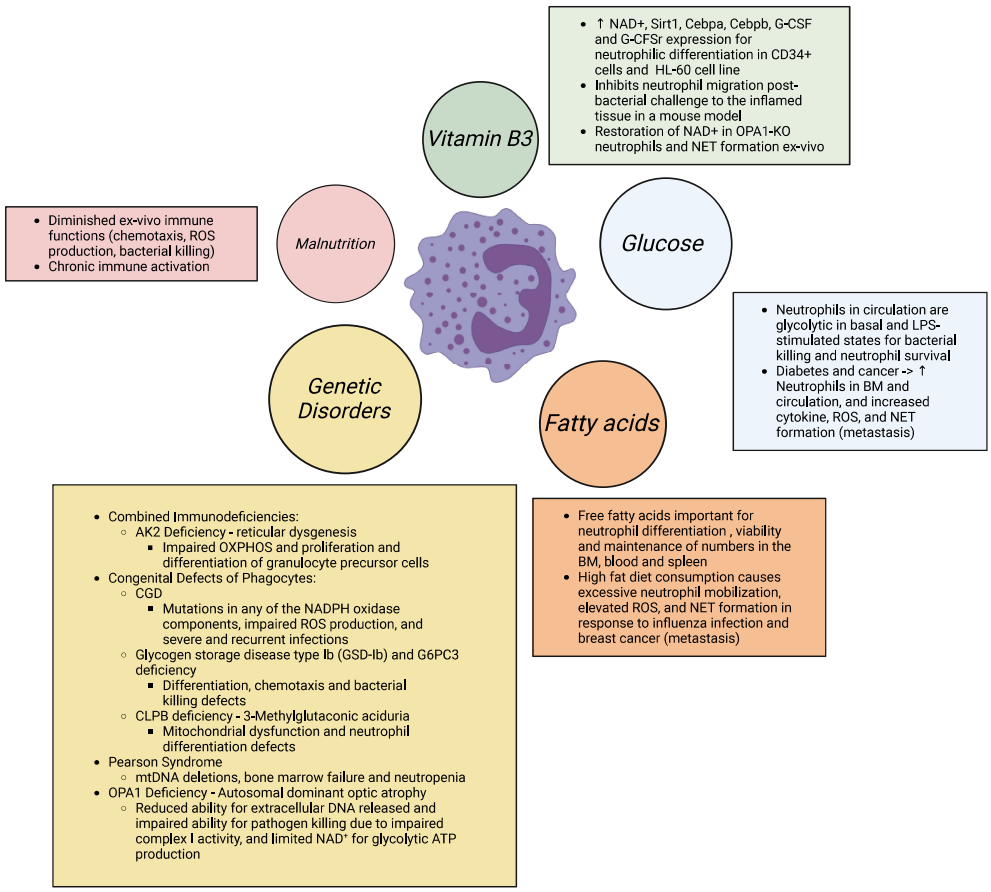

FIGURE 4
Neutrophil differentiation and functions are disrupted with altered nutrient availability, as well altered usage in multiple genetic diseases. In obesity and associated co-morbidities (diabetes, cancer, chronic inflammation), excess nutrient availability, i.e., glucose and free fatty acids, has shown to prime neutrophils for elevated ROS and pro-inflammatory cytokine production, degranulation, and NETosis for elevated tissue damage. Studies further show that elevated NETs and the metabolically flexible neutrophil subset in obese hosts (immature low- density neutrophils; iLDNs) makes the host more prone to breast cancer cell lung and liver metastasis and adverse outcomes in cancer (8, 169, 170). In contrast, in malnutrition, defined with scarce substrate availability, especially of amino acids, neutrophils are unable to kill pathogens and therefore, have a sustained hyperinflammatory response that leads to morbidity and mortality in this vulnerable population. Similarly, in a myriad of inherited immunodeficiency diseases, altered substrate utilization also predisposes the host to severe recurrent and fatal infections. The IEI classification is based on published literature (156). Supplementation with NAD+ precursors may provide therapeutic benefit in these conditions of neutrophil dysfunction.

9.1.2 Overview of NAD and vitamin B3 metabolism

Nicotinamide adenine dinucleotide (NAD^+) is an essential cofactor that plays an indispensable role in key redox reactions and serves as a substrate for PARPs, sirtuins, CD38, ADP-ribosyl-transferases, sterile alpha and TIR-motif-containing protein 1, and RNA polymerases. For its role in oxidation-reduction reactions, it accepts electrons from glycolysis, FAO and TCA intermediates to form NADH and feeds them into complex I of the electron transport chain for OXPHOS. As such, NAD^+/NADH are essential regulators of many cellular processes. Despite its fast and dynamic metabolism, NAD^+ concentration reflects the balance between NAD^+ consumption and synthesis from the *de novo*, Preiss-Handler and salvage pathways. The *de novo* biosynthesis of NAD results from the breakdown of tryptophan mostly in the liver (190). However, the major pathway of NAD^+ biosynthesis is the salvage pathway. Here, NAD^+ is converted to nicotinamide (NAM), a form of vitamin B3, and reconverted to NAD^+ using the enzyme nicotinamide phosphoribosyltransferase (NAMPT). Nicotinamide riboside (NR), another form of vitamin B3, can also enter the salvage pathway through NR kinase (Nrk1/2)-mediated phosphorylation of NR into nicotinamide mononucleotide (NMN) (99). Progressive decline in NAD^+ homeostasis and mitochondrial function are common hallmarks of ageing and disease pathologies including neutrophil dysfunction and hyperinflammation. While some studies report beneficial effects of NAD^+ precursors such as NR and NAM to counter age-related functional decline in murine models, others report no such effects in human subjects [reviewed in (191)]. We focus on the role of NAD metabolism in neutrophil biology.

9.1.3 NAD and vitamin B3 metabolism in neutrophils

The treatment of hematopoietic progenitor ($CD34^+$) cells in healthy individuals with either G-CSF or NAM increases intracellular levels of both NAMPT and NAD^+. Neutrophilic differentiation is also induced through NAD+-dependent sirtuin-1 activation, subsequent binding and activation of Cebpa and Cebpb, and, ultimately,

upregulation of G-CSF synthesis and G-CSF receptor expression (91). NAMPT expression is highest in promyelocytes and NAMPT is specifically localized to the mitochondria (91, 192). This suggests that neutrophil precursors express functional mitochondria for NAD^+ conversion from NAM and therefore neutrophil differentiation regulation via the mechanism that is proposed (91). However, no study has studied this mechanism in closer detail.

NR supplementation is shown to improve HSC and progenitor function by increasing mitochondrial clearance in HSCs. This finding is surprising since multiple publications consistently show an increase in mitochondrial fitness with NR treatment (99, 193, 194). Nevertheless, no study has yet examined how NAD^+ precursor treatment in committed progenitors affects differentiation where mitochondrial function is preferred to sustain increased ATP demands. While an increase in granulocytic differentiation is shown in steady-state through the involvement of NAMPT, NAD^+, G-CSF and G-CSFR upon NAM treatment, the role of NAM on NAD^+ in regulating neutrophil homeostasis in steady-state and emergency granulopoiesis needs clarification. Oral NR supplementation at a dose of 400 mg/kg/day for 8 weeks shows an alleviation of the BM HSC ageing phenotype, that is normally dominated by a significant expansion of the HSC pool and differentiation skewed towards the myeloid lineage and increased metabolic activity of HSCs. NR promotes molecular and mitochondrial changes in the aged BM that resembles the young HSC state including improved NAD/NADH ratio, reduction in the frequency of myeloid and lymphoid progenitors, improvement in age-deregulated HSC genes, and a reduction in the metabolic potential of aged HSCs at the transcriptional level accompanied with increased sirtuin-3, for mitophagy. Here, there is a modest restoration of the ageing HSC phenotype with NR but no effect of NR is observed in the differentiated myeloid cells (26). Moreover, increased extracellular NAD^+ in inflammatory conditions can help delay mitochondrial-dependent neutrophil apoptosis at inflamed sites (169). This is dependent on NAD^+ inhibiting the degradation of Mcl-1 (anti-apoptotic), suppressing Bax translocation to the mitochondria, attenuating the dissipation of mitochondrial membrane potential and cytochrome C release from mitochondria into the cytosol and supressing caspase-9 and -3 activation (169). The role of administrating 1000 mg/kg NAM in attenuating exacerbated neutrophil recruitment to inflamed tissues in murine models has also been studied although the downstream molecules regulating this process has not been determined (195, 196). NAM and nicotinic acid effectively inhibit neutrophil migration when administered twice, i.e., 30 minutes before and 1 hour after bacterial insult while single administration after bacterial challenge fails to prevent neutrophil recruitment into the mouse pleural cavity (195, 196). In mice with OPA1 gene mutations where NAD^+ is reduced, treatment with NMN rescues systemic NAD^+ levels. This in turn helps to indirectly regulate NET formation following neutrophil activation (Figure 3) (113).

9.2 Glucose requirements in neutrophil biology

Neutrophils function in metabolically challenged environments. Human peripheral blood neutrophils are primarily glycolytic in both basal and LPS-stimulated states (161). With glucose deprivation, these cells can maintain their intracellular energy homeostasis provided their glycolytic pathway is active due to increased glycogen stores in these cells (161). Interestingly, neutrophils express enzymes required for active glycogenesis and gluconeogenesis that enables this metabolic flexibility in glucose deprivation and other metabolically challenged environments. Through ^{13}C labeling, glutamine is identified as the substrate in gluconeogenesis, where inhibition of these processes and associated enzymes impairs bacterial killing and neutrophil survival, and promotes systemic spread of bacteria in mice (161).

9.2.1 Role of glucose in obesity-associated comorbidities

Hyperglycemia, as is commonly in diabetic mice and humans, is directly associated with an increase in BM and circulating neutrophils with an increased expression of the calprotectin subunit S100A8, and extracellular ROS production (197). Similarly, incubation of neutrophils from healthy donors with high glucose media or neutrophils from diabetic mice produces more PAD4 and NETs for an impaired wound healing response (198). Additional dysregulated functions of neutrophils in type 1 and 2 diabetes have been reviewed elsewhere (199). The mechanisms by which increased glucose availability alters neutrophil biology, for the heightened inflammatory response and tissue damage has not yet been addressed. Persistent elevation of glucose levels in diabetes and polyol and hexosamine pathway may be involved but this has not been studied mechanistically in the forementioned studies and should be the focus of future studies.

9.3 Fatty acid metabolism in neutrophils

Endogenous fatty acid synthesis and utilization is crucial for the maintenance of mature neutrophils. Dietary lipid intake in neutrophil-specific fatty acid synthase (FAS) knockout mice can partially reverse the aberrant phenotypes of FAS deficiency, reduced neutrophil viability and numbers in the BM, blood and spleen (200). Similarly, in *Atg7* deficient murine myeloblasts, pyruvate treatment or exogenous free fatty acids (linolenic acid or a mix of unsaturated and saturated free fatty acids) alone is sufficient to restore normal glucose metabolism and rescue the defective neutrophil differentiation.

9.3.1 Role of free fatty acids in obesity and associated comorbidities

High fat consumption is linked to poor outcomes of influenza infection in BALB/c mice fed a high-fat diet (HFD) for 18-weeks due to elevated H_2O_2 concentration and NET formation (201). Additionally, mice on the HFD for 15 weeks injected with breast cancer (BC) cells have lung neutrophilia, and increased lung vascular permeability compared to mice fed an isocaloric low-fat diet. This is attributed to higher NADPH-oxidase dependent ROS, granule proteins, pro-inflammatory cytokines and NETosis by lung neutrophils from obese hosts. Therefore, BC cells in the lungs (lung metastasis) of obese mice are increased compared to lean mice. In

this regard, depletion of neutrophils with anti-Ly6G antibody in obese mice reduces lung permeability and BC cell extravasation (8, 202). Similarly, obese human subjects with elevated BMI (35–68 kg/m2) and serum triglycerides have elevated chemotaxis and superoxide generation in unstimulated and stimulated neutrophils compared with lean controls. In this case, phagocytosis is not affected (203).

9.4 Protein and amino acid metabolism for neutrophils' biological processes

The role of key amino acids such as glutamine on neutrophil biology has been discussed above. In this section, we discuss the impact of perturbed amino acid and protein homeostasis for neutrophil biology. In chronological aging, alterations in protein intake and anabolic metabolism, especially of the essential branched-chain amino acid, leucine, is associated with age-related progressive loss of muscle mass, osteoporosis and frailty, and can be overcome with a higher level of protein intake (204–206). In this context, increased neutrophil numbers and degranulation, reduced coordinated neutrophil migration, phagocytosis, ROS production and NET generation, and a heightened pro-inflammatory state contributes to loss of skeletal muscle mass [reviewed in (207–209)]. Particularly, an expansion of neutrophils with a capacity for reverse transmigration and increased remote organ damage has been identified (41). Additionally, various basic leucine zipper TFs constitute the transcriptional network for the neutrophil life cycle of which, leucine content is a major determinant. However, no study to date has yet explored how dysregulation of specific amino acids, such as leucine, influences neutrophil biology in aged tissues. A randomized clinical trial has assessed the effects of whey protein and leucine ingestion post-exercise on neutrophil functions, which are attenuated during intensive and prolonged endurance exercise, during 6 days of intense cycling in 12 male cyclists (210). Leucine-enriched whey protein ingestion improves neutrophil oxidative burst post-exercise, to prime neutrophils for host immunity and tissue repair (210). Additionally, the effects of a hypocaloric Mediterranean diet (MD) and two high protein diets, with (HPW) and without (HP) whey protein supplementation, on body composition, lipid profiles, inflammation and muscle-damage blood indices in overweight, sedentary, young participants has been assessed (211). Neutrophil-mediated inflammation and muscle-damage is increased in HP and HPW compared to the MD group. No other aspects of neutrophil activity or functions are studied here (211). The association between dysregulated tryptophan-kynurenine metabolism and neutrophils in obesity is also summarized extensively (212).

9.4.1 Nutrient deficiencies as contributors to neutrophil dysfunction

Earlier, we focus on excess intake of macronutrients as they relate to co-morbidities of obesity including diabetes, cancer, and chronic inflammation. Multiple excellent articles and reviews have already been published on this in great detail that readers are referred to for additional learning [reviewed in (213–216)]. The section below is focused on inadequate nutrient intake as it relates to undernutrition and neutrophil perturbations, on which very little has been published to date.

In undernutrition, referred to as malnutrition in this section, inadequate intake of protein and calories, and micronutrient deficiencies predisposes children to diminished immune functions and chronic immune activation that increases susceptibility to infections and multiple organ failure [reviewed in (173, 217–219)]. There are limited studies performing functional assays of immune cells in low-and-middle-income countries in children with varying forms of malnutrition. Some longitudinal studies compare immune cell function in children with severe malnutrition at admission, and during recovery with exclusion of HIV-positive children. Even more limited studies in childhood malnutrition suggest some impairments in peripheral blood neutrophil function. Here, *in vitro* assays show reduced chemotaxis in children with severe malnutrition but no infection (220, 221). In another context, Leishmania donovani, a parasite that causes visceral leishmaniasis, in children with malnutrition reduces *in vitro* neutrophil and monocyte TEM after PMA stimulation as determined indirectly by flow cytometry using CD62L (222). The same study also shows reduced ROS production in these cells as determined by Dihydrorhodamine 123, but the study did not stratify by the degree of malnutrition in the sample (222). In children with severe malnutrition but no infection, *in vitro* microbicidal defects are shown to occur with *S. aureus*, *E. coli* and *C. albicans* in mononuclear and polymorphonuclear cells, while the postphagocytic morphological events, including vacuole formation and degranulation, are normal (220, 221). The data on phagocytosis is inconsistent, with some reporting unaffected phagocytosis whist others observing reduced phagocytosis especially when infection is present (217, 219, 223–225). Similarly, consensus points to impaired ROS production determined through nitroblue tetrazolium reduction assay and impaired bacterial killing capacity in children with severe malnutrition with or without infection (217, 224–227). It is unknown from these assays however whether NADPH or mitochondrial ROS production is hindered in malnutrition. These studies are conducted in different geographical sites, and differing disease states which may underlie the inconsistencies in findings. While the data suggests that not all functions are affected to the same extent, these studies only show associations and not the causal pathways linking malnutrition to impaired functions and clinical outcomes. Mice fed a protein-free diet have less neutrophils in infected lungs and this reduction could be related to impaired granulopoiesis, but the mechanism is not elucidated (224, 228). On the contrary, increased immature neutrophils are found in the blood defined through electron microscopy and flow cytometry in human cohorts while others report no change in total blood leukocyte count (220, 226, 229).

9.5 Therapeutic strategies for neutrophil recovery in nutritional deficiencies and excess

Anorexia, characterized by phenotypes and increased susceptibility to bacterial pathogens observed in undernutrition, is also an evolutionary conserved common response to infectious

diseases. Immunological changes reported in anorexia are reviewed elsewhere in greater detail (230). Here, the refeeding syndrome is a serious complication of anorexia treatment that results from excessive nutrient supplementation resulting in rapid hormonal and metabolic disturbances and high morbidity and mortality (170, 231). In fact, diminished ROS production during the initial period of refeeding (11-40 days of hospitalization) in patients with anorexia that return to normal values to that of healthy control subjects with an extended period of refeeding is commonly seen (232). A fiber-rich diet in this refeeding regime may be sufficient to restore efficient immunity (233).

In conditions of nutrient excess, inadequate and inefficacious immune responses also underlie poor infection outcomes, as discussed above. This includes exacerbated neutrophil mobilization, and ROS and NET formation that prolongs inflammation in obesity (8, 216). Here, mice fed a mild calorie restricted diet regimen had an improved pulmonary anti-mycobacterial host response with reduced bacterial load, and lung immunopathology (172). In addition, adiponectin, a hormone that is depleted in obesity, repletion in obese mice and humans regulates neutrophil oxidative burst through its anti-inflammatory properties (234). Clinically approved drugs for type 2 diabetes including pioglitazone (235), a PPARγ agonist and metformin, can induce adiponectin in mice and humans to regulate the exacerbated inflammatory responses in this context. How these therapeutics directly target immune cell metabolism remains to be studied further, but modulation of autophagy and mitochondrial metabolism may be important here (236). Gout is a debilitating chronic inflammatory arthritis, exaggerated by age and diet-induced lipotoxicity. Here, a ketogenic diet ameliorates neutrophil IL-1β secretion by increasing levels of β-hydroxybutyrate and inactivating NLRP3 inflammasome to protect the mice against inflammation (237, 238). Through its histone deacetylase inhibitory activity and therefore gene expression, β-hydroxybutyrate also regulates neutrophil-mediated immunity (233, 239). As a result, this therapeutic strategy is also relevant for other inflammatory diseases related to nutrient excess that are driven by chronic neutrophil activation.

10 Concluding remarks & perspectives

In the last decade, progress has been made to discern the metabolic requirements and flexibility of neutrophils that extends beyond glycolysis using traditional and novel immunometabolism approaches. Importantly, we discuss in this review from evidence in humans and mouse models reviewed here that neutrophils rely on the activity of multiple metabolic pathways to fulfill their energy requirements throughout their life stages. Hindrance in these processes, substrate utilization and breakdown, disrupts optimal neutrophil biology. Here in steady-state, neutrophil committed progenitors rely on fatty acid oxidation and mitochondrial ATP generation for differentiation while mature neutrophils require both mitochondrial and non-mitochondrial sources for energy production. In inflammatory states including obesity, and undernutrition, the ability to sense and reprogram metabolism based on nutrient availability also marks the response in these conditions. In these contexts, heterogenous neutrophils populations with different energy requirements may be recruited to mediate the inflammatory response and clinical outcomes, although this remains to be explored further. By further understanding the unique role of metabolism, although not 'black and white' for neutrophil biology, we will be better able to therapeutically modulate these pathways for better outcomes in inflammatory disease states.

11 Outstanding questions

Despite advancement in the understanding of neutrophil metabolism with newer technologies, much more work needs to be done to uncover the molecular mechanisms coordinating the neutrophil lifecycle and antimicrobial functions before we fully understand neutrophil metabolism for its modulation in different disease contexts. Here, we provide few examples of key areas where additional work is needed.

11.1 HSC and neutrophil development in the BM

Although HSCs are early determinants of neutrophil commitment, we still do not know how the cellular and metabolic components of HSCs interact, either directly or indirectly, to regulate neutrophil development and functions under steady-state and stress conditions. The mechanism linking NAD^+ supplementation with regulation of TFs for neutrophil differentiation and maturation (Cebpa, Cebpb) has not been addressed to date and remains to be studied. Autophagy, mitochondrial respiration, and their link with gene regulation remains to be studied in the human neutrophil developmental trajectory.

11.2 Neutrophil migration

Newer technologies provide a better understanding of the metabolic requirements for efficient neutrophil migration in healthy tissues. Therefore, investigating neutrophil migration behaviour in inflammatory pathology and metabolic challenge may have implications in the resolution of human disease where neutropenia or neutrophilia is common. The metabolic requirements for TEM and swarming also remain unexplored and require further investigation.

11.3 NADPH oxidase ROS production

There is evidence that glycolysis regulates NADPH oxidase activity, but we still do not understand how glycolytic activity regulates NADPH oxidase subunits' activation and the mechanism that connects reduced glucose utilization to enhanced mitochondrial capacity in inflammatory disease states. Here, the role of metabolites in controlling chromatin modifications, DNA

methylation, and post-translational modifications of proteins for the determination of cell fate and function will be relevant, but this has never been explored for neutrophil biology. The advent in the integration of multiple single cell 'omics' enables for such research and might be worth exploring in this context. Additionally, the mechanism of the interplay between neutrophil metabolism, differentiation and ROS-mediated functions requires further investigation in homeostasis and disease states to aid in the development of targeted therapeutics.

11.4 NET formation

It was highlighted in the text that NET formation requires autophagy and while autophagy may play a role in removing damaged mitochondria, mitophagy, the exact mechanism through which autophagy regulates NET formation remains unclear and requires further clarification. Accumulation of lactate and NETs may partially explain disease severity and adverse outcomes associated with sepsis, however how lactate can trigger NETosis remains elusive (154). Although H1F-1a regulates NETosis, optimal NETosis also requires NADPH oxidase ROS, which is decreased under hypoxia due to limited oxygen availability. It would therefore be interesting to explore the extent to which H1F-1a can help recover NETosis in hypoxia where NADPH oxidase ROS is limited. Additionally, the role of mitoROS for NOX-independent vital NET release in this mechanism remains disputed and requires more exploration.

11.5 Neutrophil crosstalk with other cells

Studies are needed to examine metabolic programming involved directly in neutrophil communication with immune and non-immune cells irrespective of the soluble mediators that have often been discussed previously.

11.6 Neutrophils in altered nutritional environments

As decline in NAD^+ has been implicated in many diseases, future studies must investigate the impact of changes in NAD^+ availability on innate immune responses, specifically that of neutrophils and the mechanisms involved in the dysregulation. Additionally, studies must investigate the mechanistic pathway regulating granulopoiesis and neutrophil functions with the supplementation of NAD^+ and its precursors'. Studies have also not yet addressed how free fatty acids regulate epigenetic modifications to drive neutrophil differentiation, irrespective of mitochondrial ATP generation. The molecular mechanism through which excess glucose and metabolic rewiring in diabetes promotes neutrophilia and exacerbates inflammatory disease requires further exploration in mice and humans. This is crucial in the context of diverse tissue microenvironments where excess circulating glucose is accompanied by overt pathologies in multiple organ systems. Similarly, it is not known if correction of circulating glucose levels in these conditions eliminates neutrophil dysfunction and associated pathologies. Additionally, how excess glucose alters neutrophil phenotype and function needs to be studied further. Future research needs to focus on the exploration of novel neutrophil-centered treatments to resolve inflammation and improve patient outcomes in conditions of excess glucose availability. The causative mechanism of fatty acid excess on neutrophil functional diversity and inflammation has not been identified. Studies mentioned in the *Role of Free Fatty Acids in Obesity and associated Comorbidities* section suggest that although free fatty acids are crucial for normal neutrophil functions, excess substrate availability promotes the emergence of functionally distinct neutrophils that can promote aberrant disease states. More mechanistic studies are needed to evaluate the effects of varying quantities of fatty acid consumption on neutrophil biology. The existing studies with the existent pre-clinical models do not accurately represent phenotypic changes associated with severe malnutrition where multiple physiological changes are observed therefore the translational ability of these studies is questioned. This warrants the need to develop pre-clinical models that are representative of real-life in which to modulate metabolism and improve neutrophilic processes. Additionally, most of the studies conducted in children with malnutrition have major limitations: small sample size, lack of appropriate control groups, i.e., adequately nourished children from high-income counties as controls with no overt infection, and several forms of malnutrition. These studies are also limited by ethical constraints and methodological limitations, i.e., tissue sampling and immunological techniques. The mechanisms exacerbating impaired neutrophil functions in response to malnutrition and infection remains unexplained since observational studies can only determine associations, and not causality (145, 217). In this case, genetic approaches to decipher proposed pathways have not been undertaken and therefore causation cannot be determined. It is also impossible to delineate from these studies whether neutrophil dysfunction is a cause or consequence of malnutrition when infection is present. Additionally, the impact of specific metabolites and metabolic perturbations has not been closely explored in neutrophils from children with malnutrition especially since multiple metabolic derangements exist in this context and the role of limited substrate availability in this case also requires further exploration. Impaired autophagic flux and mitochondrial dysfunction has been associated with hepatic steatosis and gut barrier dysfunction in a mouse model of severe malnutrition (240–242). Differences in blood metabolomic profiles of children with severe malnutrition that died, especially an increase in metabolites of mitochondria-related bioenergetic pathways has also been identified (243, 244). Therefore, the role of mitochondria for neutrophil-associated catastrophe in the malnourished host requires further exploration. Additionally, the impact of specific micronutrient deficiencies on neutrophil biology has not been studied in detail in malnutrition. The impact of refeeding in malnutrition on additional neutrophil functions beyond ROS production and how it contributes to the refeeding complications is not well known. The role of changes in specific macro and micronutrient composition with refeeding and their impact on neutrophil biology is unknown and requires further exploration. It is well-accepted that mitochondrial function is lost as neutrophils mature and heterogeneity in neutrophil metabolic programming exists

between different tissues and within neutrophil subsets. As such, uncovering neutrophil metabolic adaptations in inflammatory disease states and across heterogenous neutrophil subsets may help in targeting these pathways to improve long-term outcomes. Similarly, the role that the metabolic switch plays at inflammatory/infectious sites during metabolic disorders for outcome determination requires further investigation. We believe these studies will help novice and expert researchers in the field expand our understanding of the unique role of metabolism in steering neutrophil biology, a field that has largely remained neglected.

Author contributions

MT: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing, Project administration. HU: Writing – review & editing. MG: Writing – review & editing. NP: Writing – review & editing. CB: Writing – review & editing. AG: Writing – review & editing. CL: Writing – review & editing. JB: Writing – review & editing. RB: Writing – review & editing, Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Resources. AF: Conceptualization, Investigation, Project administration, Supervision, Writing – review & editing, Validation, Writing – original draft.

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Conflict of interest

HU received research support or consultancy fees from Janssen, Eli Lilly, UCB Pharma, GSK, BMS/Celgene, MiroBio, OMass, and Mestag. AF is employed by Omega Laboratories Inc.

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Protective effects and mechanisms of ellagic acid on intestinal injury in piglets infected with porcine epidemic diarrhea virus

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The present study was conducted to decipher the protection effects of ellagic acid (EA) on piglets infected with porcine epidemic diarrhea virus (PEDV). Thirty 7-day-old piglets were randomly assigned to three treatment groups: control, PEDV, and EA + PEDV groups. After a 3-day period of adaption, piglets in the EA + PEDV group were orally administered with 20 mg/kg·BW EA during days 4-11 of the trial. On day 8, piglets were orally administered with PEDV at a dose of 10^6 $TCID_{50}$ (50% tissue culture infectious dose) per pig. Additionally, intestinal porcine epithelial (IPEC-1) cells infected with PEDV were used to investigate the anti-PEDV effect of EA *in vitro*. The results showed that EA at a dose of 10-40 μmol/L increased the viability of PEDV-infected IPEC-1 cells, and EA administration mitigated intestinal edema in piglets challenged with PEDV. Further studies indicated that EA treatment significantly increased the proportion of white blood cells in blood and concentrations of IL-6, IL-1β, and IL-10 in the serum, but decreased the TNF-α content and gene expression of *IL-6*, *IL-1β*, *TNF-α*, and *CXCL2* in the jejunum. Moreover, EA intervention considerably elevated the activity of total superoxide dismutase (T-SOD), but decreased the H_2O_2 concentration in the ileum of piglets. Importantly, EA suppressed the increased expression of antiviral-related genes and proteins (including MXI, ISG15, HSP70, and p-IRF7) induced by PEDV challenge in the jejunum. Furthermore, PEDV infection increased the protein abundance of p-JAK2 and p-STAT3, which were further enhanced by EA supplementation. In conclusion, our results revealed that EA could promote the restoration of intestinal homeostasis by regulating the interferon pathway that was interrelated with the activation of JAK2/STAT3 signaling. These findings provide theoretical basis for the use of EA as a therapy targeting PEDV infection in piglets.

KEYWORDS

ellagic acid, interferon, JAK2/STAT3 signaling, piglets, porcine epidemic diarrhea virus

Introduction

Porcine epidemic diarrhea (PED), caused by porcine epidemic diarrhea virus (PEDV), is a highly pathogenic intestinal infectious disease in pigs. PEDV mainly infects and proliferates in villus enterocytes of the small intestine and it can lead to intestinal function destruction, absorption dysfunction, severe diarrhea, and even death of piglets (1). The emergence of porcine epidemic diarrhea was first reported in Britain in 1971 and then rapidly spread to other countries, which had an adverse impact on the pig industry (2, 3). At present, the effective prevention and control measures of PEDV infection mainly depend on vaccines. However, there are still some disadvantages, such as the enhancement of virulence and incomplete inactivation of traditional vaccines, which in turn results in the continuous recombination and mutation of PEDV and makes the prevention and control of PED more difficult (4). In the no-antibiotic era, it is of great practical significance to develop nutritional intervention strategies with anti-PEDV infection and protection of normal intestinal function.

Ellagic acid (EA) is a kind of polyphenol dinolactone, which exists widely in kinds of fruits and nuts and has multi-biological functions, such as anti-oxidation, anti-inflammation, antiviral and antibacterial activities (5). The four phenolic hydroxyl groups of EA are the basis for the reaction with various reactive oxygen species (ROS). The phenolic hydroxyl groups can provide H^+, which can combinate with oxygen free radicals and reduce the content of oxygen free radicals in the body (6, 7). Studies have reported that EA possess the anti-inflammatory function by inhibiting the production of inflammatory cytokines and regulating the intestinal microbial structure (8, 9). Moreover, the antiviral activity of EA is associated with inhibiting virus multiplication, reducing virus titer and preventing virus from binding to host cell receptors. Importantly, it has been demonstrated that EA could improve intestinal health in weaned piglets by increasing gene expression of intestinal tight junction proteins and reducing mRNA levels for inflammatory cytokines (10). Based on the potential benefits of EA, we hypothesized that EA might also be advantageous to protect against PEDV-induced intestinal damage in piglets. Therefore, we conducted the present study to confirm this speculation and seek to reveal the relevant mechanisms by combining *in vitro* and *in vivo* experiments.

Materials and methods

Cell culture and viability detection

Intestinal porcine epithelial (IPEC-1) cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium, and 0.5% epidermal growth factor at 37°C with 5% CO_2. 6×10^3 cells per well were seeded in a 96-well plate. When cells fusion reached 40%, they were incubated with medium added with EA (0, 10, 20, 40, 60, 80 μmol/L) for 72 h. A cell counting kit-8 (Beyotime, Shanghai, China) was used to measure the cell viability according to the manufacturer's instructions. Moreover, another 96-well plate was used to determine the effect of EA on the viability of PEDV-infected cells. When cells fusion reached 100%, IPEC-1 cells were infected with PEDV (MOI=0.1) for 1 h, then the cell supernatant was removed and cells were treated with EA (0, 2.5, 5.0, 10, 20, 40 μmol/L) for 24 h to detect the cell viability by the cell counting kit-8. The detailed timeline of these *in vitro* experiments was shown in Supplementary Table 1.

Monolayer transepithelial electrical resistance determination

Cell monolayer transepithelial electrical resistance (TEER) was determined according to the method of Ji et al. (11). Briefly, IPEC-1 cells were seeded in the apical side of transwell inserts in 12-well plates with a density of 2×10^4 cells per well. Cells were incubated with 10 μmol/L EA for 72 h. The TEER of cells was determined by using a Millicell ERS-2 Volt-Ohm Meter (Millipore, USA) on the indicated times and cells were collected for total protein extraction. In addition, IPEC-1 cells were seeded in the apical side of transwell inserts in another 12-well plates with a density of 2×10^6 cells per well. Cells were challenged with PEDV for 1 h after 100% cell fusion. Then, the TEER of cells at different time points was measured. Once the minimum TEER approached 0, cells were collected to extract total RNA for the detection of *PEDV-M* and *PEDV-N* genes expression. The detailed timeline of the above experiments was shown in Supplementary Table 1.

Animal experimental design

Thirty 7-day-old crossbred (Duroc × Landrace × Large White) healthy piglets were randomly assigned to three treatment groups: control, PEDV, and EA + PEDV groups. Each group contained 10 replicates with one pig per replicate. The entire experiment period was 11 days. The first three days were adaptation period and all piglets were fed with the liquid milk replacer. Nutrient components of the milk replacer were shown in Table 1. During days 4 to 10 of the trial, piglets in the EA + PEDV group were orally administered with 20 mg/kg·BW EA (purchased from Macklin Inc., Shanghai, China; purity ≥ 90%; dissolved in the liquid milk replacer), and piglets in the other two groups were treated with the same volume of milk replacer. On day 8, piglets in the PEDV and the EA + PEDV groups were orally received 1 mL PEDV at a dose of 10^6 $TCID_{50}$

TABLE 1 Nutrient components of the milk replacer (as fed basis), 100%.

Items	Crude protein	Crude ash	Crude fiber	Moisture	Lysine	NaCl	Calcium	Total phosphorus
Milk replacer	≥20.0	≤9.0	≤1.0	≤10.0	≥1.4	0.3-1.5	0.4-1.1	≥0.3

(50% tissue culture infectious dose) per pig, while those in the control group consumed the same volume of sterile saline solution.

Piglets were carefully observed daily throughout the trial period to record their diarrhea occurrence and health status. On day 11 of the trial, after overnight fasting, all piglets were orally administered with 10% D-xylose (1 mL/kg·BW) and anterior vena cava blood was collected 1 h later. Subsequently, all piglets were sacrificed to collect intestinal samples. Intestinal damage scores were evaluated as follows: 0: no visible intestinal or lung damage; 1: sporadic bleeding points in the intestines and lungs; 2: obvious damage in the intestine, lung, and liver can be seen by the naked eye, intestinal ulcers appear, and the intestinal wall becomes thin and permeable; 3: massive lung bleeding, intestinal ulcer, erosion, severe thinning of the intestinal wall and congestion. About 1-cm-long intestine segments were fixed in 4% paraformaldehyde to observe intestinal morphology, and the remaining intestines were rapidly frozen in liquid nitrogen and stored at -80°C until further analysis.

Blood indices

The collected blood was placed at room temperature for 1 h, centrifuged at 3000 g, 4°C for 10 min, and the upper serum was collected. The concentration of D-xylose in the serum were measured using a commercially available kit (Jiancheng Institute of Biological Technology, Nanjing, China) according to the instruction. Blood cell counts were analyzed by the automated hematology analyzer (Siemens ADVIA 2120i, Germany) using the whole blood.

Cytokine level detection

The contents of TNF-α, IL-1β, IL-6, IL-10, TGF-β1, and IFN-α in the serum were determined by using commercial ELISA kits (AAT Bioquest, California, CA, USA) according to the manufacturer's guidelines. The jejunum tissue was accurately weighed and 9 times the volume of normal saline was added for mechanical homogenization under ice bath condition. The homogenate was centrifuged at 3000 g, 4°C for 10 min, and the supernatant was taken to measure jejunal TNF-α, IL-1β, IL-6, IgA, IL-10, and IFN-α concentrations also by using commercial ELISA kits (AAT Bioquest, California, CA, USA) according to the manufacturer's guidelines.

Antioxidant related indices in the jejunum and ileum

Homogenates in the jejunum and ileum were used to determine antioxidant-related indexes. Activities of total superoxide dismutase (T-SOD) and myeloperoxidase (MPO), as well as the concentration of hydrogen peroxide (H_2O_2) were calculated by the colorimetric method and standard curve according to commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Morphological structure analysis in the intestine

The morphological structure of small intestines was observed as previously described (12). Briefly, 4% paraformaldehyde fixed intestine samples were dehydrated and embedded in paraffin. Then, sections of 6-μm thickness were deparaffinized in xylene and dehydrated in ethanol for hematoxylin and eosin (H&E) staining. Villus height, crypt depth, and villus width in each section were quantitatively analyzed as described by Frankel et al. (13). Briefly, villi height is the vertical distance from the tip of the villi to the opening of the crypt; crypt depth is the vertical distance from the opening of the crypt to the base of the crypt; villus surface area is the product of villus height and villus width. 10 villi with the most complete morphological structure were selected, and the intestinal morphological structure of each sample was measured by the Olympus BX41 microscope (Olympus, Tokyo, Japan) with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Ratio of villus height to crypt depth and villus surface area were calculated and recorded.

Total RNA extraction and quantitative real-time PCR

The RNAiso Plus (Takara, Dalian, China) reagent was used to extract total RNA. Sequentially, cDNA was synthesized using the PrimeScript®RT reagent kit with gDNA Eraser (Takara, Dalian, China). Real-time quantitative PCR (qRT-PCR) was performed by using the ABI 7500 real-time PCR system (ABI 7500, Alameda, CA, USA) with SYBR® Premix Ex Taq™ (Tli RNaseHPlus) (Takara, Dalian, China). All the operation steps were completed according to the instruction of the manufacturer. Relative gene expression was determined using the $2^{\Delta\Delta Ct}$ method and the *RPL4* gene was used for normalization. The primer sequences were shown in Supplementary Table 2.

Western blot analysis

The protein of jejunum was extracted by a whole protein extraction kit (KeyGEN, Jiangsu, China), and the protein concentration was quantified by a BCA kit (Beyotime, Shanghai, China). Equal amounts of protein were separated by SDS-PAGE gels, followed by transferring onto PVDF membranes (Millipore, Billerica, MA, USA). The membrane bands were incubated with primary antibodies overnight at 4°C, and then incubated with secondary antibodies for 2 h at the room temperature. Finally, the grayscale value of protein band was determined by using an imaging system (Alpha Innotech FluorChem FC2, CA, USA). Antibodies used in the present study were as follows: MX1 (ab222856, Abcam, 1:1000), ISG15 (ab233071, Abcam, 1:1000), HSP70 (ADI-SPA-810-F, Enzo Life Sciences, 1:1000), IRF7 (QC8422, Sigma, 1:1000), p-IRF7 (PA5-114591, Invitrogen, 1:2000), JAK2 (#3230, Cell Signaling Technology, 1:2000), p-JAK2 (#3776, Cell Signaling

Technology, 1:2000), STAT3 (#30835, Cell Signaling Technology, 1:1000), p-STAT3 (#9145, Cell Signaling Technology, 1:1000), Occludin (TC259714, Invitrogen, 1:2000), ZO-1 (61-7300, Invitrogen, 1:2000), Claudin-1 (RF217968, Invitrogen, 1:2000), E-cadherin (PA5-142828, Invitrogen, 1:2000), and β-actin (PA1–46296, Invitrogen, 1:4000).

Statistical analysis

Data expressed as means ± SEM and all data were analyzed by one-way ANOVA or non-paired t test with SPSS 26.0 statistical software (SPSS, Inc., Chicago, IL, USA). The *Duncan* multiple comparison method was used to determine the differences between means among the treatment groups. Graphs were created by using the GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered significantly different between groups.

Results

EA promoted the viability and barrier function in PEDV-infected IPEC-1 cells

Results showed that 10-60 μmol/L EA significantly promoted the proliferation of IPEC-1 cells by 13.57-15.26%, but the dose of 80 μmol/L had no significant effect (Figure 1A, $P < 0.05$). Moreover, 10-40 μmol/L EA also noticeably improved the viability of PEDV-infected cells by 12.68-24.03% (Figure 1B, $P < 0.05$). Based on these results, therefore, we chose 10 μmol/L EA in the following studies. Interestingly, cells incubated with 10 μmol/L EA exhibited higher TEER value than those in the control group at 48 h without PEDV infection, which suggested that EA had the ability to reinforce the barrier function of IPEC-1 cells (Figure 1C, $P < 0.05$). Moreover, EA treatment notably increased the protein expression of E-cadherin at 48 h (Figure 1D, $P < 0.05$). Nevertheless, EA supplementation didn't affect the membrane resistance of PEDV-infected cells (Figure 1E). The N and M gene encodes the nuclear protein and the membrane protein of PEDV, respectively, both of which are marker genes for the detection of PEDV viral load. In the current study, we found that EA failed to inhibit the replication of PEDV *in vitro*, which was reflected by no obvious changes in the mRNA expression of *PEDV-N* and *PEDV-M* genes (Figure 1F).

Effect of EA on the intestinal injury of PEDV-infected piglets

Compared with the control group, anatomical observation showed that the intestinal wall of PEDV-infected piglets was thinner and intestinal flatulence was exerted (Figure 2A). Although the statistical difference was not significant, supplementation with EA reduced intestinal edema and intestinal injury scores (Figure 2B). Plasma D-xylose concentration is an important indicator for intestinal absorption function. PEDV infection strongly decreased D-xylose concentration compared with the control group, and EA intervention had no significant

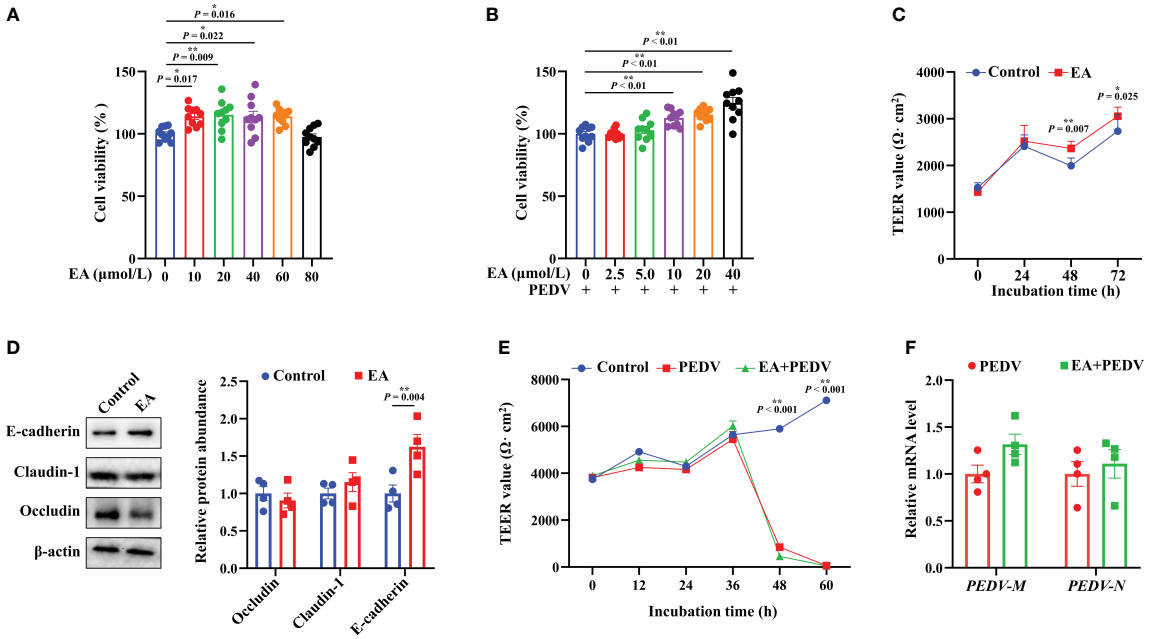

FIGURE 1
Effects of EA treatment on the viability and barrier function of IPEC-1 cells infected with PEDV. **(A)** Concentration-dependent effects of EA on the proliferation of IPEC-1 cells, n = 10; **(B)** Alleviation of EA on the cytotoxicity in PEDV-infected IPEC-1 cells, n = 10; **(C)** TEER values of EA treated-cells, n = 8; **(D)** Expression levels of tight junction proteins at 72 h, n = 4; **(E)** Effect of EA on the TEER values in PEDV-infected cells, n = 4; **(F)** mRNA levels for PEDV marker genes, n = 4. Data are presented as means ± SEMs. *$P < 0.05$, **$P < 0.01$.

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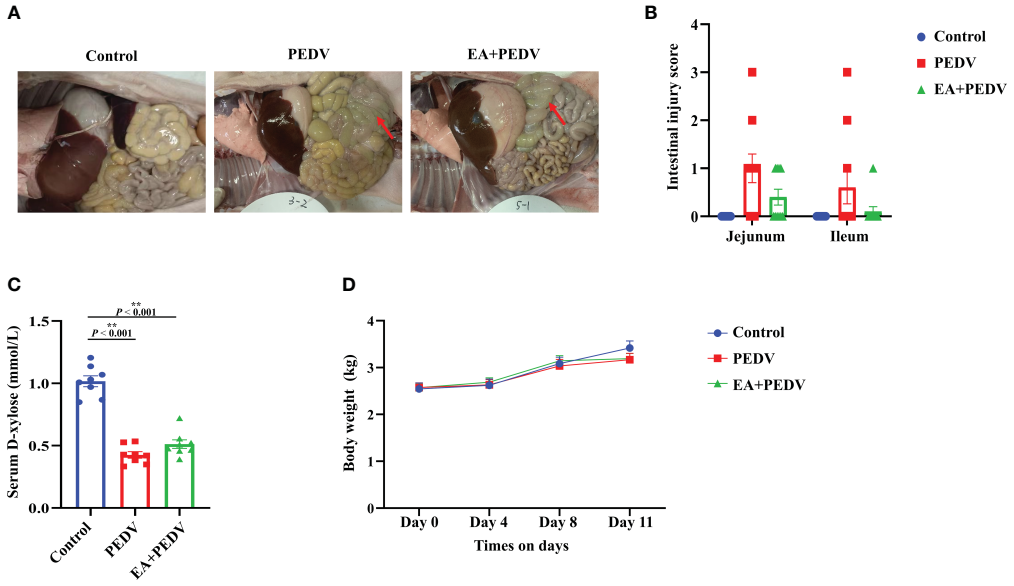

FIGURE 2
Effect of EA administration on the intestinal injury and body weight change in piglets infected with PEDV. **(A)** Representative pictures showing the intestinal edema; **(B)** Intestinal injury scores of the jejunum and ileum; **(C)** D-xylose concentration in the serum; **(D)** Body weight changes. Data are presented as means ± SEMs (n = 10). *$P < 0.05$, **$P < 0.01$. Red arrows show intestinal edema.

influence on D-xylose level in the serum of piglets (Figure 2C). Additionally, EA had no effect on body weight in piglets (Figure 2D).

Effects of EA on the intestinal morphology of PEDV-infected piglets

As presented in Figure 3A, intestinal villi were severely atrophied or deciduous due to PEDV infection, EA had no obvious influence on the villus morphology. Statistical results showed that PEDV infection significantly decreased villus height in all small intestines, as well as crypt depth, and villus surface area in the jejunum and ileum ($P < 0.05$); EA treatment neither restored the intestinal villi height nor crypt depth to the normal value (Figures 3B–M). These results indicated that EA could not alleviate the damage of intestinal villus caused by PEDV infection.

Inflammation-related indicators in blood and the jejunum

As displayed in Figures 4A–F, PEDV infection significantly decreased the proportion of white blood cells, lymphocytes, monocytes, and eosinophils in comparison with the control group ($P < 0.05$), and EA supplementation conversely increased the number of white blood cells and monocytes. Additionally, compared with the control group, PEDV infection considerably increased the serum concentrations of TNF-α and TGF-β1 (Figures 4G, K), and EA treatment decreased the TNF-α level ($P < 0.05$). PEDV infection had no significant effect on IL-6, IL-1β, IL-10, and IFN-α levels in the serum (Figures 4H–J, L), while EA administration highly elevated concentrations of IL-6, IL-1β, and IL-10 when compared with PEDV group ($P < 0.05$).

We also examined the relevant indices of jejunal inflammatory response. As shown in Figures 4M, N, EA significantly reversed the decrease of IgA and IL-10 contents in the jejunum of piglets induced by PEDV infection ($P < 0.05$). Additionally, concentrations of IFN-α, TNF-α, and IL-1β in the jejunum were enhanced by PEDV challenge, whereas EA treatment decreased the TNF-α level (Figures 4O, P, $P < 0.05$). In line with the results regarding cytokine content, we also found that the mRNA levels for *IL-6*, *IL-1β*, *TNF-α*, *IL-8*, chemokine ligand 2 (*CXCL2*), as well as regenerating islet-derived 3 gamma (*REG3g*) were more abundant in the PEDV group than those in the control group, and these gene expressions were blunted by EA receiving (Figure 4Q, $P < 0.05$), except for *IL-8*. These results emphasized that EA can ameliorate the inflammatory response of PEDV-infected piglets.

Effects of EA on the intestinal antioxidant function of PEDV-infected piglets

The antioxidant enzymes and related products of the jejunum and ileum were determined to investigate the antioxidative effect of EA on piglets. Compared with the control group, PEDV infection considerably reduced the activity of T-SOD both in the jejunum and ileum (Figures 5B, E, $P < 0.05$), had no effect on jejunal MPO and H_2O_2 levels (Figures 5A, C) but increased the activity of MPO and the concentration of H_2O_2 in the ileum (Figures 5D, F, $P < 0.05$). EA intervention obviously restored the T-SOD activity and the H_2O_2 content to the normal level in the ileum of pigs ($P < 0.05$).

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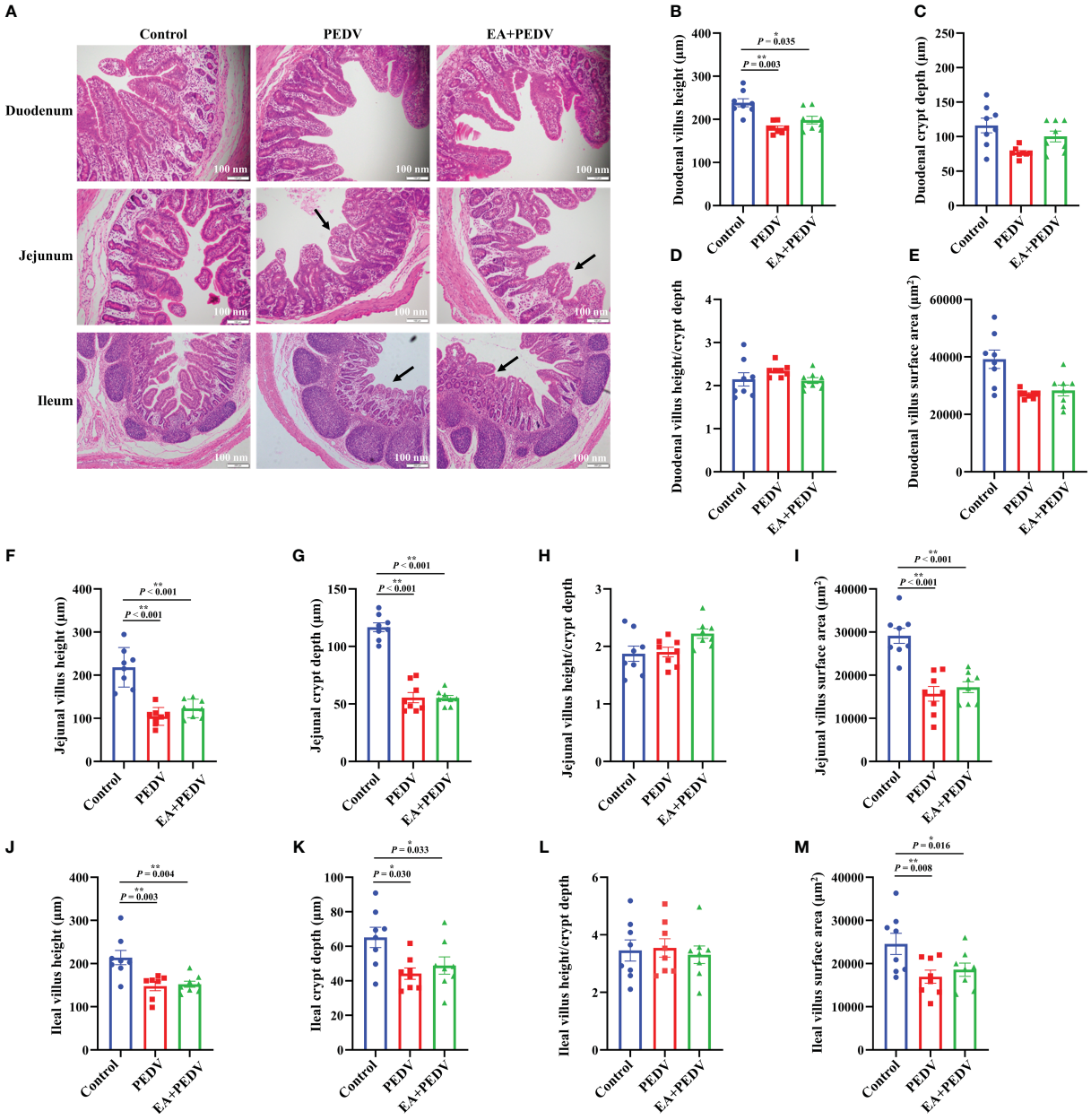

FIGURE 3
Effects of EA administration on the villus structure in piglets infected with PEDV. **(A)** H&E staining. Scale bar: 100 nm; Villus height **(B, F, J)**, crypt depth **(C, G, K)**, villus height/crypt depth **(D, H, L)**, and villus surface area **(E, I, M)**. Data are presented as means ± SEMs (n = 10). *$P < 0.05$, **$P < 0.01$. Black arrows show the atrophy or shedding of intestinal villi.

The antiviral effect of EA in the jejunum of PEDV-infected piglets

Consistent with the results *in vitro*, no significant differences were observed in *PEDV-M* and *PEDV-N* mRNA levels between the PEDV and EA groups in the jejunum of piglets (Figure 6A). Nevertheless, mRNA levels for antiviral-related genes, such as interferon β (*IFN-β*), myxovirus resistant 1(*MX1*), interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*), and interferon induced trans-membrane proteins 3 (*IFITM3*), were generally increased by PEDV infection. Among these genes, EA consumption lowered the *MX1* and *IFIT1* expressions (Figure 6B, $P < 0.05$). Moreover, PEDV infection significantly upregulated the protein abundance of MX1, interferon-stimulated gene 15 (ISG15), heat shock protein 70 (HSP70), and the phosphorylation of interferon regulatory factor 7 (p-IRF7), which was inversely changed by EA supplementation (Figures 6C, D, $P < 0.05$). Further exploration showed that both the protein abundance of p-JAK2 and p-STAT3 were elevated by PEDV challenge, and the addition of EA ulteriorly promoted the expression of p-JAK2 and p-STAT3 (Figure 6E, $P < 0.05$). These results indicated that although EA cannot inhibit PEDV proliferation, it had a certain antiviral potential through immunomodulatory effects.

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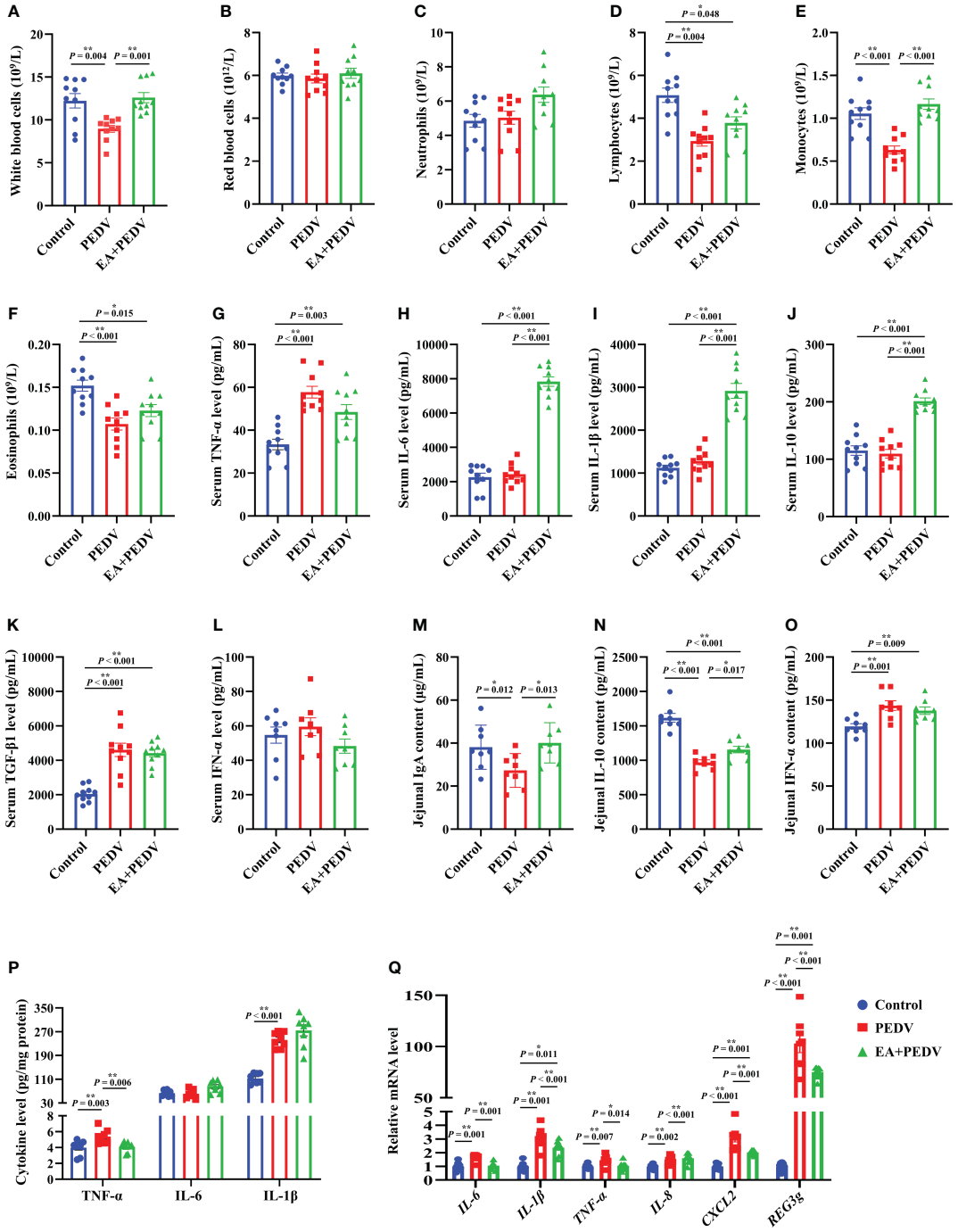

FIGURE 4
Effects of EA administration on inflammatory indices of blood and the jejunum in piglets infected with PEDV. **(A–F)** Blood cell counts; **(G–L)** Concentrations of TNF-α, IL-6, IL-1β, IL-10, TGF-β1, and IFN-α in the serum; **(M–P)** Concentrations of IgA, IFN-α, TNF-α, IL-6, and IL-1β in the jejunum; **(Q)** mRNA levels for inflammation-related genes in the jejunum. Data are presented as means ± SEMs (n = 8). *$P < 0.05$, **$P < 0.01$. TNF-α, tumor necrosis factor α; IL, interleukin; TGF-β1, transforming growth factor β1; CXCL2, chemokine ligand 2; REG3g, regenerating islet-derived 3 gamma.

Effects of EA on jejunal nutrient transport function and barrier function in PEDV-infected piglets

As shown in Figure 7A, compared with the control group, PEDV infection decreased the relative mRNA abundance of ion channel-related proteins, mainly aquaporin (AQP8 and AQP10), potassium inwardly-rectifying channel, subfamily J, member 13 (KCNJ13), Na^+/H^+ exchangers (NHE3), and lipid transporter such as apolipoproteins (APOA1, APOA4, APOC2) and sodium/glucose cotransporter (SGLT1) ($P < 0.05$), but no apparent differences were observed in the expression of these genes between the PEDV and

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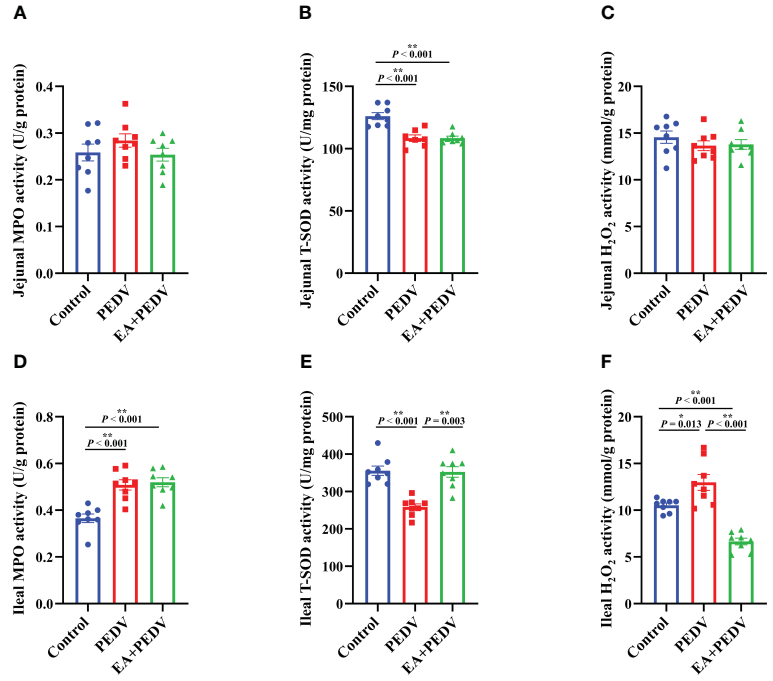

FIGURE 5
Effects of EA administration on the antioxidation function in piglets infected with PEDV. Activities of MPO **(A, D)** and T-SOD **(B, E)**, as well as the concentration of H_2O_2 **(C, F)**. Data are presented as means ± SEMs (n = 8). *$P < 0.05$, **$P < 0.01$. MPO, myeloperoxidase; T-SOD, total superoxide dismutase; H_2O_2, hydrogen peroxide.

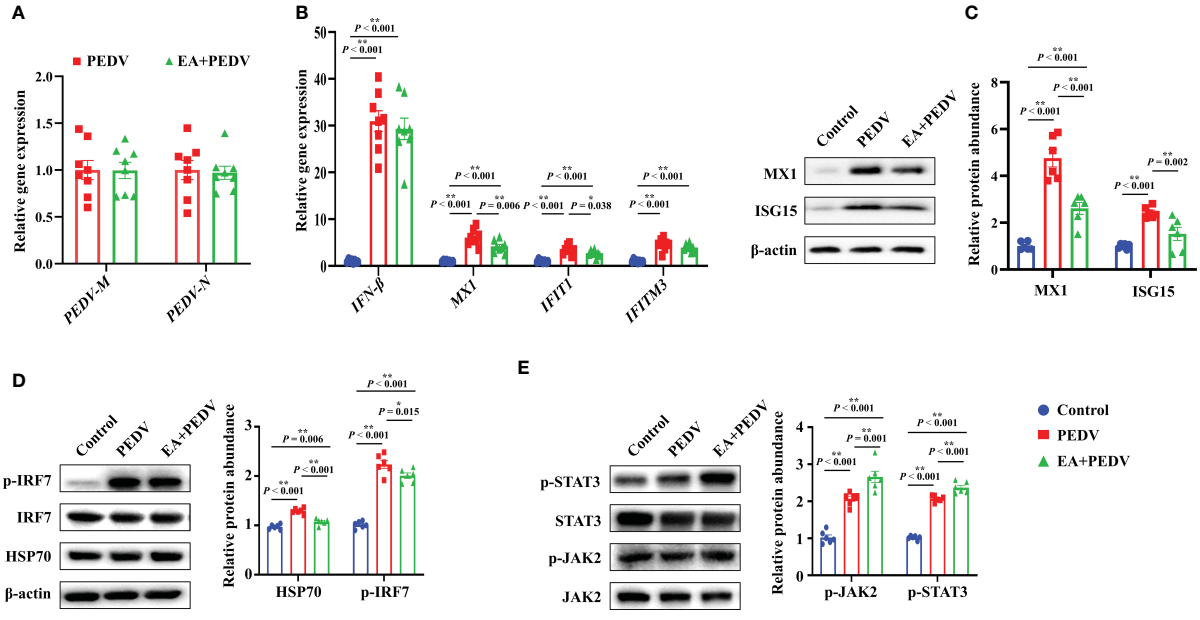

FIGURE 6
Expression of antiviral-related genes and proteins in the jejunum. **(A)** mRNA levels of PEDV marker genes; **(B)** mRNA level for genes involved in the interferon pathway; Protein abundance of MX1 and ISG15 **(C)**, HSP70 and p-IRF7 **(D)**, p-JAK2 and p-STAT3 **(E)**. Data are presented as means ± SEMs (n = 8). *$P < 0.05$, **$P < 0.01$. IFN, interferon β; MX1, myxovirus resistant 1; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IFITM3, interferon induced trans-membrane proteins 3; ISG15, interferon-stimulated gene 15; HSP70, heat shock protein 70; IRF7, interferon regulatory factor 7; JAK2, Janus tyrosine kinase 2; STAT3, signal transducer and activator of transcription 3.

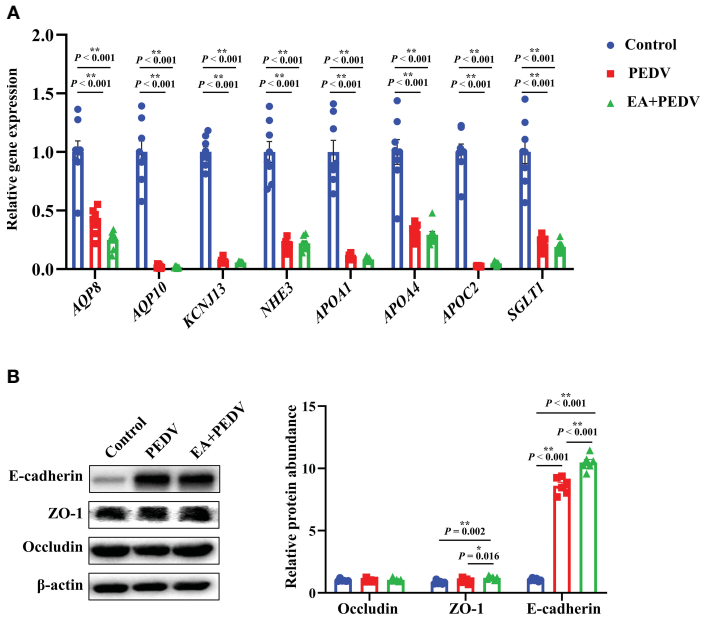

FIGURE 7
Expression of nutrient absorption and transport-related genes **(A)** and tight junction proteins **(B)** in the jejunum. Data are presented as means ± SEMs (n = 8). *$P < 0.05$, **$P < 0.01$. AQP, aquaporin; KCNL13, potassium inwardly-rectifying channel, subfamily J, member 13; NHE3, Na^+/H^+ exchangers; Apo, apolipoproteins; SGLT1, sodium/glucose cotransporter 1; ZO-1, zonula occludens-1.

EA supplementation groups. Results about the expression of jejunal tight junction proteins showed that EA administration elevated the protein abundance of ZO-1 and E-cadherin in comparison with the PEDV group (Figure 7B, $P < 0.05$).

Discussion

Lethal watery diarrhea in piglets, especially in weaned piglets, is still a popular problem in the world and is quite challenging. The continuous variation of virulent strains makes classical vaccines fail to provide effective protection against PEDV infection. It has been proved that EA is a promising feed additive with anti-oxidation, anti-inflammation, and growth-promoting properties. In the present study, we found that EA enhance enterocytes proliferation and barrier function, and prevented PEDV infection-induced intestinal inflammation and oxidative stress. This beneficial effect may be interrelated with the downregulation of inflammatory cytokines and antiviral-related genes, as well as the activation of JAK2/STAT3 signaling.

PEDV mainly proliferates in the small intestine, therefore, the PEDV-challenged IPEC-1 cells model was used to preliminarily evaluate whether EA has a protective effect against PEDV-infection. Our results showed that although EA could not inhibit the replication of PEDV, it enhanced the proliferative activity of PEDV-infected cells and increased the protein expression of E-cadherin and TEER value *in vitro*. E-cadherin is an intercellular adhesion protein that is generally distributed in the junctions between cells on the surface of epithelial cells (14). The enhanced E-cadherin expression suggested that EA may have the capability to improve intestinal barrier function in piglets. Thereafter, we conducted the animal experiment to further explore the protective effects of EA on intestine function of PEDV-infected piglets.

Given that addition of EA alone had no negative effect on the growth performance of piglets (Supplementary Figure 1), we focused on the regulatory effects of EA on the intestine function by using an experimental model of pigs infected with PEDV. In the present study, PEDV infection severely damaged the villus structure of small intestine, and thereby led to intestinal edema, intestinal wall thinning, and diarrhea. EA supplementation alleviated jejunal edema to some extent, but failed to improve the morphological integrity of villus. Intriguingly, Lu et al. found that dietary EA increased the average daily gain and reduce the diarrhea rate in weaned piglets (15). The reason for this inconsistent result may be due to the pigs with different physiological stages have different bioavailability of EA.

It is an important role for blood cell indicators to reflect the status of health and the capacity of body metabolism. Blood cells are divided into red blood cells, white blood cells and platelets (16). Among that, white blood cells are exudative and chemotactic, and their main functions are to phagocyte pathogens and secrete interferon and interleukin mediators. There are five types of white blood cells: neutrophils, eosinophils, basophils, lymphocytes and monocytes, and different cell subsets have their own special roles (16, 17). Viral infections usually impair the function of white blood cells, resulting in a decrease in the number of white blood cells, especially lymphocytes. Importantly, study found that there were significant neutropenia and lymphocytopenia in peripheral blood of pigs 2-3 days after African swine fever virus infection (18). EA treatment noticeably increased the counts of total white blood cells, and monocytes, which argued that EA can alleviate the

immunosuppression caused by PEDV and improve the system immunity of piglets. Consistent with the increase in white blood cell count, EA intervention numerically elevated IL-6, IL-1β, and IL-10 levels in the serum. Note that the body has a function of self-immune regulation and inflammatory mediator are not continuously secreted. Serum cytokine contents in PEDV-infected piglets may have recovered when we sampled at the end of the trial. EA has an immunological intervention effect, which might cause a slower reduction of cytokine concentrations. Therefore, the capacity of self-maintaining homeostasis may ultimately result in higher IL-6, IL-1β, and IL-10 contents in the EA treatment group than in the PEDV infection group. Generally, these results revealed that EA may contribute to alleviating the inflammatory response in piglets induced by PEDV.

Complying with our previous studies (19, 20), a severe inflammatory response was triggered in the jejunum of PEDV-infected piglets, as characterized by the markedly decrease in IgA and IL-10 contents and increase in concentrations of IFN-α,TNF-α, and IL-1β in the jejunum, whereas EA addition conversely increased IgA and IL-10 concentrations and decreased the TNF-α level. Consistently, the increased gene expression of jejunal cytokines (*IL-6*, *IL-1β*, *TNF-α* and *CXCL2*) was also counteracted by EA intervention, which revealed that EA could ameliorate jejunal inflammation induced by PEDV infection. Proteins in the regenerating gene (REG) family have been found to serve as multifunctional molecules with antimicrobial, anti-apoptotic, anti-inflammatory, and probably immuno-regulatory effects (21). Accumulating evidence have uncovered the potential role of the REG3g in the development of inflammation-associated gastrointestinal diseases (22, 23). Our results presented that the relative gene abundance of *REG3g* was lower in EA treated piglets than those in the PEDV-challenged group. In line with our findings, Fan et al. also reported that PEDV infection was accompanied by upregulated *REG3g* expression in the jejunum (23), which highlighted that the REG3g may contribute essential functions for suppressing the PEDV replication.

Usually, the oxidation and antioxidant systems maintain a dynamic balance to prevent against the accumulation of ROS in the body. However, insufficient expression of antioxidant enzymes can result in incomplete clearance of ROS and causing oxidative stress (18). MPO is a specific marker in neutrophils and its elevation indicates a high proportion of neutrophils, which indirectly reflects the inflammatory response (24). SOD is an important antioxidant enzyme that mainly exists in the cytoplasm and it can facilitate the breakdown of superoxide radical into O_2 or H_2O_2 to reduce oxidative stress. Total SOD (T-SOD) includes CuZn-SOD and Mn-SOD. In addition, as an oxidative metabolite, high concentration of H_2O_2 can diminish the integrity of cell membranes and then precipitate cell apoptosis (25). The physiological structure and function of the intestine make it constantly expose to the external environment, food oxides, bacteria and virus. Therefore, the intestine is more likely to produce excessive ROS, evoke intestinal inflammation and the damage of intestinal barrier function. In the present study, the activity of ileal MPO and H_2O_2 concentration were increased by PEDV challenge, while the T-SOD level was strongly lowered both in the jejunum and ileum. The changes in these indicators suggested that PEDV induced oxidative stress in the intestine of piglets, which was in concordance with our previous findings that the activity of antioxidant enzymes could be suppressed by PEDV infection (26). However, EA treatment considerably enhanced the activity of T-SOD, but decreased H_2O_2 concentration in the ileum, which indicated that EA could effectively alleviate oxidative damage in PEDV-infected piglets. Consistent with our findings, Xiao et al. reported that EA relieved paraquat-induced intestinal oxidative stress in weaned piglets (19). Collectively, EA might be a potential additive to prevent oxidative stress-mediated gut diseases.

Existing studies reported that EA possess a potential to inhibit the replication of HIV and SARS-CoV-2 virus *in vitro* (27–29). Considering that EA ameliorated the oxidative stress and inflammation elicited by PEDV infection in piglets, we proposed that EA had an anti-PEDV effect *in vivo*. However, concordant with results in IPEC-1 cells, EA also had no significant influence in mRNA levels for *PEDV-M* and *PEDV-N*, which suggested that EA failed to block the proliferation of PEDV. The body mainly relies on the immune system to fight viruses. After virus invasion, the body's self-protection mechanism will be activated, resulting in increased expression of antiviral-related genes. Expression of genes (mainly *IFN-β*, *MX1*, *IFIT1* and *IFITM3*) involved in antiviral was generally increased in the jejunum of PEDV-infected piglets, and EA supplementation inversely blunted the transcriptional level of *MX1* and *IFIT1*. Importantly, EA treatment also decreased the protein abundance of MX1 and ISG15 in the jejunum. These results again suggested that EA can respond to PEDV infection through immunomodulatory effects. IFN-β is one kind of type I interferon that responses mainly by inducing the expression of IFN stimulate gene (ISGs) and producing a variety of antiviral factors, which not only has a direct antiviral effect, but also has a certain immune enhancement function (30). IFIT1 is a member of the interferon-induced protein with tetratricopeptide repeats family (IFITs) and it has extensive antiviral activity and anti-inflammatory effect. Genes in the IFITs family belong to ISG genes and virus infection can make the expression of them rapidly increased within a short time (31). Additionally, MX1 is also a member of ISGs, which recognizes the nucleocapsid structure of the virus and interferes with the invasion of viral nucleic acid fragments into cells (32). The alterations of these genes and protein expressions suggested that although EA treatment did not directly reduce PEDV proliferation, it conveyed a protection on the immune homeostasis through downregulating these ISG genes and proteins in the gut of PEDV-infected piglets. This phenomenon was consistent with our previously described that nutritional intervention can relieve intestinal damage induced by PEDV through the interferon signaling (26, 33).

Interferon regulatory factors (IRFs) are multifunctional transcription factors, which are critical for the production of type I IFN in regulating cell signal transduction and immune response. Thirty years ago, the JAK/STAT pathway was found to be involved in cells response to IFN (34). The secreted IFN activates JAK-STAT pathway according for inducting ISGs (35). IRFs comprise nine members, ranging from IRF1 to IRF9. Studies showed that IRF7 is mainly expressed in lymphocytes, splenocytes, thymocytes,

dendritic cells and other immune-related cells (36). In the present study, we found that PEDV challenge increased the protein abundance of p-IRF7, p-JAK2 and p-STAT3, which was partly agreement with Li et al., who reported that virus in piglets can induce the transcription of type I interferon and stimulate the activation of JAK/STAT pathway by regulating key signaling molecules such as IRF7 and then trigger the innate immunity (37). Interestingly, EA treatment decreased the phosphorylation of IRF7, but ulteriorly enhanced the protein richness of p-JAK2 and p-STAT3. The JAK/STAT pathway is central to extracellular cytokine activated receptor-mediated signal transduction, which is involved in cell proliferation and differentiation, organ development, and immune homeostasis (34). We hypothesized that the addition of EA promoted the expression of other cytokines or growth factors, which could also activate the JAK2/STAT3 pathway. Additionally, our previous study found that puerarin, another plant extract, exerted anti-PEDV effects by activating STAT1 (38). Therefore, there may be common and specific mechanisms for the regulatory effects of plant extracts on activating the JAK/STAT pathway in anti-PEDV virus, which deserves further investigation. Moreover, as Park et al. described that HSP70 enhanced PEDV replication by interacting with membrane proteins (39). We also found that PEDV infection upregulated the HSP70 protein expression, which was restored by EA ingestion. Heat shock proteins (HSPs) are a class of chaperones that are responsible for maintaining cellular proteostasis and metabolism homeostasis (40). Recently, Lubkowska et al. summarized that HSP70 is essential for the proliferate of a wide range of virus, such as dengue virus, influenza A virus, human enterovirus, and the hepatitis C virus (41). Therefore, our study and Park et al. provided evidence that the HSP70 is of utmost importance for PEDV replication, and EA may be an effective additive to target HSP70 for improving immune response in PEDV-infected piglets.

Diarrhea is associated with abnormal expression of ion channel proteins that influence the digestion and absorption of nutrients. The gut is the main place for the digestion and absorption of nutrients, and ion channels play important role in nutrient transport and absorption. AQPs are water channels and are responsible for transporting the water from lumen to enterocytes (42); KCNJ13 is a member of the inwardly rectifying potassium channel family of proteins and accounting for potassium ions passing into a cell (43); NHE3 is mainly related to the exchange of sodium ions inside and outside the cell (44); APOA1, APOA4, and APOC2 are associated with lipid digestion and absorption in the small intestine (45); SGLT1 is responsible for glucose transport (46). In the present study, EA had no significantly influence on mRNA levels of ion channel-related and nutrient absorption-related genes, which may suggest that it did not promote the nutrient absorption of PEDV-infected piglets. This result may partially provide the explanation for the findings that EA could not exert the positive effects on the intestine morphology and body weight in PEDV-infected piglets.

Generally, the invasion of pathogens damages the integrity of the intestinal epithelium and decreases the expression of tight junction proteins. However, our results showed that PEDV infection increased the protein expression of E-cadherin. E-cadherin is a class of Ca^{2+}-dependent intercellular adhesion molecules expressed in mammalian epithelial cells and it is essential for the maintenance of epithelial tissue formation, organ morphological development, and control of epithelial cell proliferation. The rise in E-cadherin expression is also associated with a recovery in the number of healthy epithelial cells (14, 47). Our seemingly contradictory result may be due to the body's self-

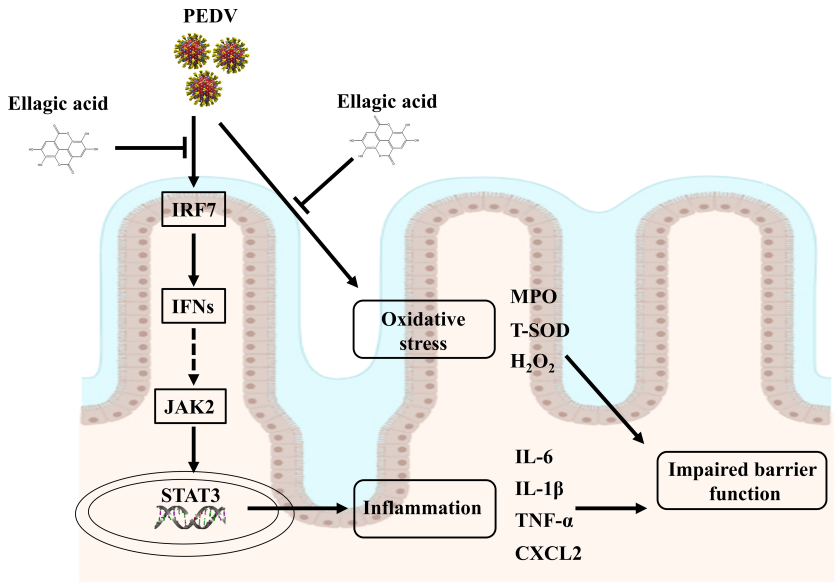

FIGURE 8
Suggested mechanisms of EA action on ameliorated intestinal injury in PEDV-infected piglets.

regulation ability to resist pathogen invasion under certain conditions by promoting the expression of proteins related to the intestinal barrier function. Moreover, partly consistent with results of *in vitro* experiments, EA administration further enhanced the protein abundance of ZO-1 and E-cadherin in the jejunum of PEDV-infected piglets, which indicated that the function of intestinal epithelium was improved by EA treatment and the increased E-cadherin may be contributing to fighting against the invasion of PEDV for piglets. Qin et al. also found that dietary EA supplementation attenuated intestinal damage with the enhancement of tight junction proteins in weanling piglets (8). Therefore, the alleviation of intestinal inflammatory response and oxidative stress by EA administration in PEDV-infected piglets may be benefit from its improving effect on intestinal barrier function.

Conclusions

EA administration could alleviate oxidative stress and intestinal inflammation in PEDV-infected piglets, as manifested by increased T-SOD activity and decreased cytokine expressions. Furthermore, EA administration improved the antiviral function to restore intestinal homeostasis probably by regulating the interferon pathway and that was companied with the trigger of JAK2/STAT3 signaling (Figure 8). Overall, EA may function as a nutritional feed additive to protect against intestinal injury.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by the Animal Care and Use Committee of Wuhan Polytechnic University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZS: Data curation, Formal analysis, Investigation, Writing – original draft. CD: Data curation, Investigation, Methodology, Writing – review & editing. QC: Investigation, Methodology, Writing – review & editing. SZ: Data curation, Investigation, Methodology, Writing – review & editing. PL: Formal analysis, Methodology, Software, Writing – review & editing. TW: Data curation, Methodology, Writing – review & editing. YH: Funding acquisition, Supervision, Conceptualization, Writing – review & editing. DY: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing, Project administration, Supervision.

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Conflict of interest

Author TW is employed by the company Hubei Horwath Biotechnology Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1323866/full#supplementary-material

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Glossary

Apo	apolipoproteins
AQP	aquaporin
CXCL2	chemokine ligand 2
EA	ellagic acid
H&E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HSP70	heat shock protein 70
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFITM3	interferon induced trans-membrane proteins 3
IFN	interferon β
IL	interleukin
IRF7	interferon regulatory factor 7
ISG15	interferon-stimulated gene 15
JAK2	Janus tyrosine kinase 2
KCNL13	potassium inwardly-rectifying channel
subfamily J	member 13
MPO	myeloperoxidase
MX1	myxovirus resistant 1
NHE3	Na^+/H^+ exchangers
PED	porcine epidemic diarrhea
PEDV	porcine epidemic diarrhea virus
REG3g	regenerating islet-derived 3 gamma
SGLT1	sodium/glucose cotransporter 1
STAT3	signal transducer and activator of transcription 3
TEER	transepithelial electrical resistance
TGF-β1	transforming growth factor β1
TNF-α	tumor necrosis factor α
T-SOD	total superoxide dismutase
ZO-1	zonula occludens-1

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β-glucans from *Agaricus bisporus* mushroom products drive Trained Immunity

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Introduction: Macrofungi, such as edible mushrooms, have been used as a valuable medical resource for millennia as a result of their antibacterial and immuno-modulatory components. Mushrooms contain dietary fibers known as β-glucans, a class of polysaccharides previously linked to the induction of Trained Immunity. However, little is known about the ability of mushroom-derived β-glucans to induce Trained Immunity.

Methods & results: Using various powdered forms of the white button mushroom (Agaricus bisporus), we found that mouse macrophages pre-treated with whole mushroom powder (WMP) displayed enhanced responses to restimulation with TLR ligands, being particularly sensitive to Toll-like receptor (TLR)-2 stimulation using synthetic lipopeptides. This trained response was modest compared to training observed with yeast-derived β-glucans and correlated with the amount of available β-glucans in the WMP. Enriching for β-glucans content using either a simulated *in-vitro* digestion or chemical fractionation retained and boosted the trained response with WMP, respectively. Importantly, both WMP and digested-WMP preparations retained β-glucans as identified by nuclear magnetic resonance analysis and both displayed the capacity to train human monocytes and enhanced responses to restimulation. To determine if dietary incorporation of mushroom products can lead to Trained Immunity in myeloid cells in vivo, mice were given a regimen of WMP by oral gavage prior to sacrifice. Flow cytometric analysis of bone-marrow progenitors indicated alterations in hematopoietic stem and progenitor cells population dynamics, with shift toward myeloid-committed multi-potent progenitor cells. Mature bone marrow-derived macrophages derived from these mice displayed enhanced responses to restimulation, again particularly sensitive to TLR2.

Discussion: Taken together, these data demonstrate that β-glucans from common macrofungi can train innate immune cells and could point to novel ways of delivering bio-available β-glucans for education of the innate immune system.

KEYWORDS

mushroom, Trained Immunity, β-glucan, digestion, immunometabolism

Introduction

Memory-like properties have recently been ascribed to cells of the innate immune system (1). The mechanisms underlying these vary with cell type; however, triggering enhanced responsiveness in myeloid cells including macrophages and monocytes has been termed Trained Immunity (2). Exposure to specific Training stimuli leads to functional reprogramming in these cells and their hematopoietic progenitors, which includes metabolic reprogramming and epigenetic priming of inflammatory genes. This trained response is non-specific, meaning trained cells respond more efficiently to a broad range of stimuli which re-activate them (2). Trained Immunity has been proposed to underlie protective effects like heterologous immunity seen in some vaccines and increased resistance to infection, by promoting innate immune function. However, it has also been implicated in various inflammatory diseases where metabolic and disease-associated stimuli train innate immune cells for inappropriate activation underlying pathogenesis (2, 3). Recent evidence suggests that the long-term protective effects of Trained Immunity proceed not through altering the function of peripheral cells like monocytes and macrophages, but rather through altering the fate of central medullary hematopoietic stem-progenitor cells HSPCs, which give rise to mature myeloid cells (2, 4).

One of the earliest training stimuli described were fungal β-glucans (5), structural carbohydrates found in cell walls, which trigger the innate immune receptor Dectin-1 (6, 7). β-glucans are a diverse family of biomacromolecules found across multiple kingdoms of life, which differ in their physical and chemical properties and their interactions with mammalian cells (8, 9). Signaling through Dectin-1 is a key step for the initiation of β-glucan-induced Trained Immunity (5), although fungal β-glucans can trigger other receptors, including CR3 on neutrophils to prime for degranulation in a complement-dependent manner (10), and can co-operate with CR3 to drive pro-inflammatory cytokine IL-1β production and cell death responses in macrophages (11). Crucially, differences in Dectin-1 binding and responses have been described between low MW, soluble β-glucans and larger, particulate β-glucans. Goodridge et al., reported that particulate β-glucans drive the phagocytotic synapse for full anti-microbial activity in myeloid cells, which can be blocked by binding of Dectin-1 by low MW β-glucans (12). Different Dectin-1 isoforms exist which differ in the length of the stalk region. Low MW, soluble β-glucans seem to bind and signal through the less abundant but larger isoform, Dectin-1a, while particulate β-glucans can bind through both isoforms (13, 14). Many of the processes involved in β-glucan driven Trained Immunity have been reported using a β(1→3)-glucan preparation from *Candida albicans* (5, 15–18), although evidence is emerging that baker's yeast *Saccharomyces cerevisiae*-derived β-glucans can also drive this process (19–23). The impact of more common β-glucans on Trained Immunity, particularly those found in foods, like plant/oat and mushroom β-glucans, is less well described (24, 25).

Edible mushrooms represent a diverse class of macrofungi and apart from their nutritional benefits, have long been held as sources of novel medicinal and psychoactive compounds (26). Outside the mushroom cell membrane, β-glucans lie between an inner layer of chitin and outer layer of mannoproteins, the latter of which have been described to have anti-inflammatory properties (27). β-glucans in mushrooms are generally more soluble due to shorter, more linear glucose polymers made up of β(1→3) and/or β(1→6) glycosidic linkages, with less branching than β-glucans from other fungal species like yeasts (28, 29). They can further be differentiated structurally from β-glucans present in oat and cereals, which comprise of β(1→3, 1→4)-glucose units (30). Laminarin from brown seaweed represents another category of β-glucans composed of β(1→3) and β(1→6) linkages, however is distinguishable from mushroom β-glucans due to differences in their glucose-linked backbone. Laminarin has a backbone of β(1→3) with branching at C6, which is converse to mushroom β-glucans encompassing a β(1→6) linked backbone with some branching at C3 and occasionally at C4 (31–33). Because of these chemical differences, it has been speculated that mushroom β-glucans are more immunologically inert and hence, well tolerated by humans. Despite this, β-glucans from edible mushrooms have been promoted for immune benefits in a variety of settings worldwide and there is considerable evidence that mushroom-derived β-glucans and other compounds enhance/augment anti-cancer therapies (26, 34). We thus hypothesize that mushroom β-glucans may support innate immune function through triggering innate immune memory responses via Trained Immunity. To test this, we used powdered mushroom derived from the common white button mushroom species, *Agaricus bisporus* (35) and tested the capacity of this to trigger Trained Immunity using a number of established assays (18, 23). We also included *Saccharomyces cerevisiae*-derived whole glucan particles (WGP) and Laminarin in the study to compare the performance and efficacy of β-glucans from different sources.

Results

Mushroom powders contain modest concentrations of β-glucan with distinct Dectin-1 binding properties

Since fungal compounds have been linked to the induction of Trained Immunity in myeloid cells, we investigated if the powdered

TABLE 1 Megazyme assay glucan content of dry powders/preparations.

Sample	[Total Glucan] w/w (mean ± s.d.)	[α-Glucan] w/w (mean ± s.d.)	[β-Glucan] w/w (mean ± s.d.)
Barley preparation*	43.9 ± 0.6	0.4 ± 0.2	43.5 ± 0.7
Yeast WGP	78.5 ± 4.5	5.5 ± 0.8	73.0 ± 4.2
Filtered WMP	10.2 ± 0.1	1.2 ± 0.1	8.9 ± 0.1
Se-WMP	12.4 ± 0.3	5.0 ± 0.2	7.4 ± 0.5
VitD-WMP	8.7 ± 0.6	1.1 ± 0.2	7.6 ± 0.6
IVD-WMP	14.5 ± 0.3	1.0 ± 0.0	13.5 ± 0.3

*Barley preparation used as positive control for Megazyme assay.

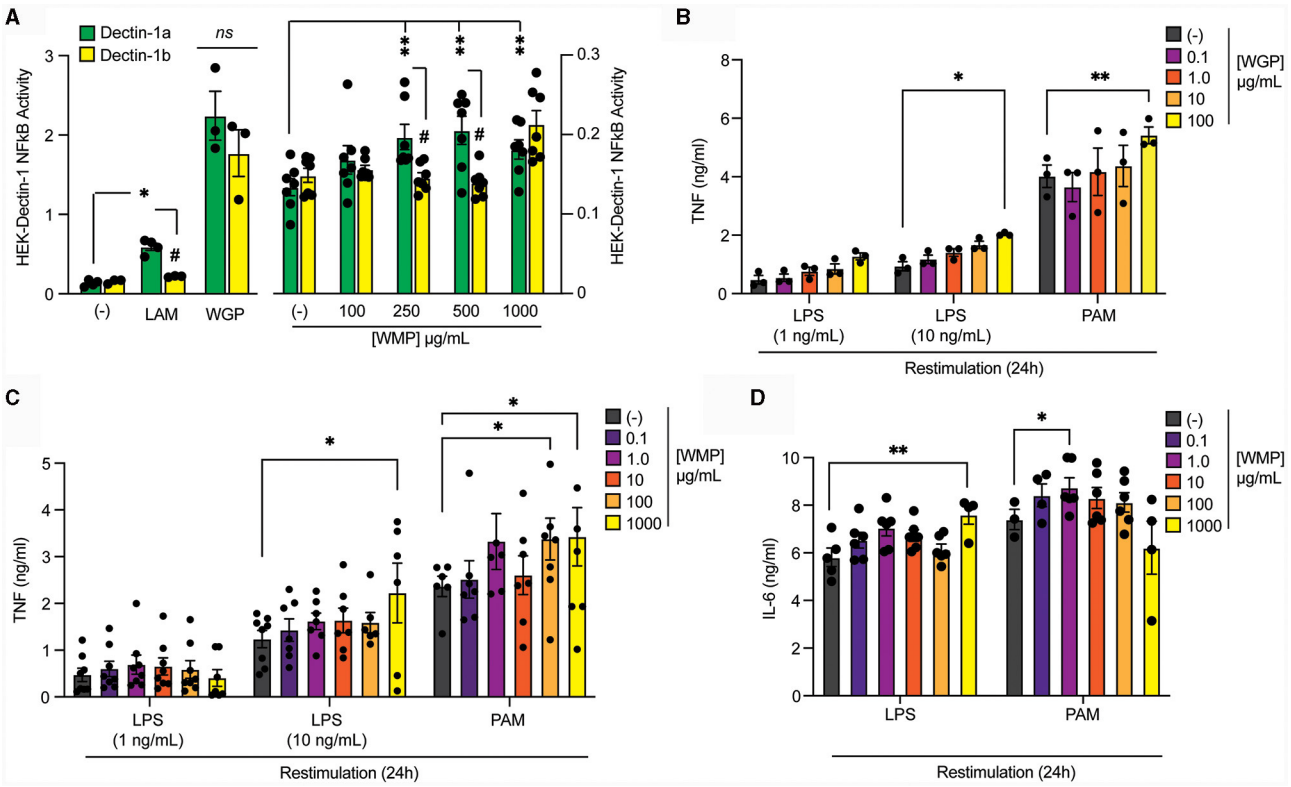

FIGURE 1
Pre-treatment of macrophages with mushroom powders augments long-term responses to restimulation. **(A)** HEK-Dectin-1a or HEK-Dectin-1b-cell lines were treated with the increasing concentrations WMP (0.1–1,000 µg/mL), laminarin (1 µg/mL), WGP (10 µg/mL) or left untreated (-) for 24 h. SEAP activity was measured using QuantiBlue. Data is mean relative induction over untreated ± s.e.m. of $n = 3$–6 replicates. **(B–D)** Mature bone-marrow derived macrophages (BMDMs) were treated with yeast-derived whole-glucan particle [WGP, **(B)**], *A. bisporus* derived whole mushroom powder [WMP, **(C, D)**] at the indicated concentrations (µg/mL) or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with LPS (at indicated concentrations or 10 ng/mL) or Pam3CSK4 (PAM, 10 µg/mL) for 24 h. Supernatant was removed and the indicated cytokines measured by ELISA [TNF, **(B, C)** & IL6, **(D)**]. Data is mean cytokine concentration ± s.e.m. of $n = 3$ **(B)**, $n = 6$ **(C, D)** independent experiments. */#P value < 0.05, **P value < 0.01, determined using 2-way ANOVA with the indicated *post-hoc* multiple comparisons (Fisher's LSD test).

form of edible white button mushroom (*A. bisporus*) could induce similar effects in macrophages. We previously demonstrated that these unprocessed powders contain ~8% β-glucan by dry weight [(35) and Table 1]. This compares to 70%—80% in other commercially available and concentrated yeast-derived β-glucan purifications, notably *Saccharomyces cerevisiae*-derived whole glucan particles (WGP) [(28) and Table 1]. We began by examining the ability of various β-glucan containing preparations to bind and signal through the Dectin-1 receptor using *in-vitro* reporter assays, specifically HEK293-cells overexpressing either the Dectin-1b or Dectin-1a isoform. Ten µg/mL of yeast WGP triggers activation of an NFκB-linked reporter gene to a similar extent across both cell types, while Laminarin [a macroalgal-derived low MW β-glucan (36)] drives Dectin-1a only (Figure 1A left panel). Given the differences in % β-glucan content in WGP and WMP, we tested a range of WMP concentrations in HEK-Dectin-1a/b cells—with 100 µg/mL of WMP containing equivalent total β-glucan content to 10 µg/mL of WGP. WMP drove a dose-dependent response in HEK-Dectin1a cells only and to a much lower extent than seen with Laminarin or WGP, despite controlling for differences in β-glucan content by testing a higher range of concentrations (250–1,000 µg/mL, Figure 1B right panel). The increased binding to Dectin-1a suggest that the β-glucans contained in WMP are structurally different to yeast β-glucan and likely represent more soluble, less branched β-glucans (29) (akin to Laminarin). Since matching for total β-glucan concentrations could not drive similar levels of NFκB-linked Dectin-1 signaling, it is possible that WMP contains polysaccharides (e.g., a higher relative ratio of α-glucans, Table 1) and other molecules that interfere with Dectin-1 binding. Having thus measured activation of Dectin-1a by WMP, we proceeded to assess the impact of these WMPs on innate immune memory responses.

Pre-treatment of macrophages with mushroom powders augments long-term responses to restimulation

To compare the ability of these different β-glucans to impact Trained Immunity, we exposed mature mouse bone-marrow

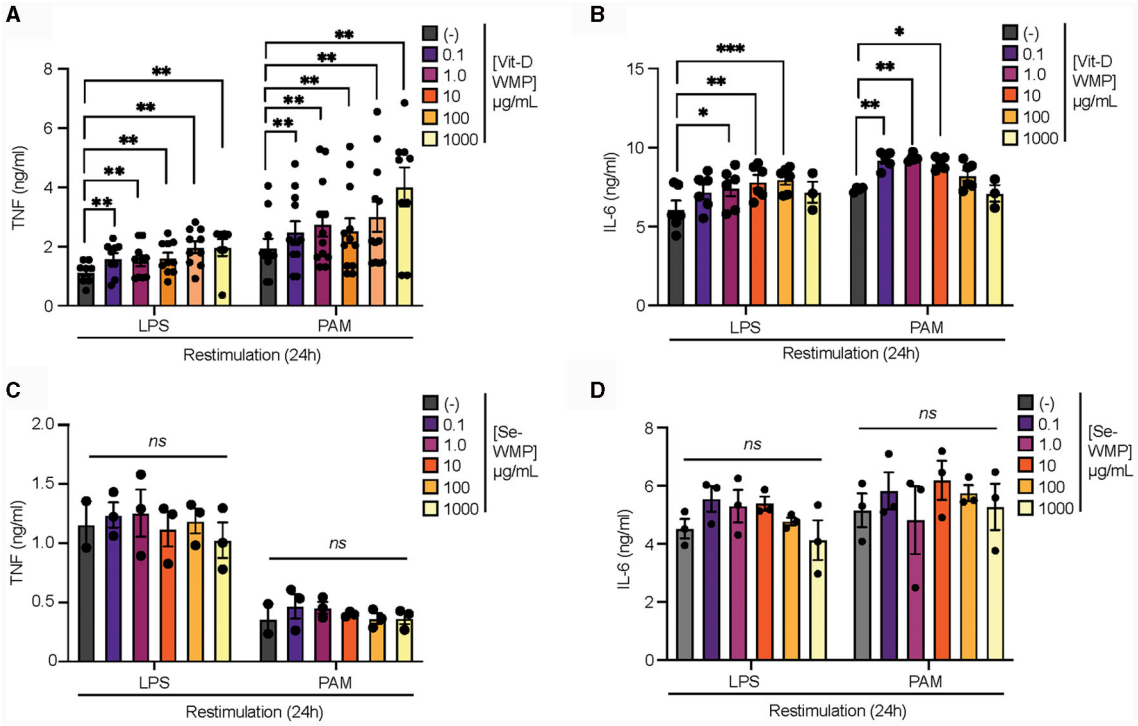

FIGURE 2
Vit-D enriched mushroom powder retains the capacity to train macrophages. **(A–D)** Mature BMDMs were treated with Vitamin-D (Vit-D) enriched or Selenium (Se-) enriched *A. bisporus* derived whole mushroom powder (WMP) at the indicated concentrations (µg/mL) or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with LPS (10 ng/mL) or Pam3CSK4 (PAM, 10 ug/mL) for 24 h. Supernatant was removed and the indicated cytokines measured by ELISA [TNF, **(A, C)** & IL6, **(B, D)**]. Data is mean cytokine concentration ± s.e.m. of $n = 8$ **(A)**, $n = 6$ **(B)** or $n = 3$ **(C, D)** independent experiments. $^{*}P$ value < 0.05, $^{**}P$ value < 0.01, $^{***}P$ value < 0.005, determined using 2-way Mixed Effect ANOVA with the indicated *post-hoc* multiple comparisons (Fisher's LSD test).

derived macrophages (BMDMs) to the β-glucan-containing compounds for 24 h followed by an extensive wash-out phase. Cells were further matured for an additional 7-days before restimulating with TLR ligands, either LPS (TLR4) or Pam3CSK4 (PAM, TLR2). Significantly enhanced TNF production was observed in cells trained with a high concentration of yeast-derived WGP (100 µg/mL) for both LPS and PAM restimulation (Figure 1B). Training BMDM with similar concentrations of WMP (between 0.1–1,000 µg/mL) leads to enhanced TNF responses to LPS and PAM restimulation (Figure 1C), to a similar extent as that seen with an equivalent β-glucan concentration as WGP (1,000 µg/mL WMP; ∼1.5–2.0-fold over untrained controls), albeit with more variation. Interestingly, PAM restimulation is more sensitive to training with WMP than LPS, with enhanced responses observed at lower concentrations of WMP (100–1,000 µg/mL). A similar effect was observed when the production of the pro-inflammatory cytokine IL6 was measured (Figure 1D). Thus, training with WMP can drive similar enhanced long-term responses to restimulation to that seen with equivalent concentrations of yeast-derived β-glucans, albeit with more variation to that observed with more pure preparations.

Vitamin-D2 enriched mushroom powder retains the capacity to train macrophages

Edible mushrooms naturally enriched in micronutrients are being developed to increase bioavailability (35, 37, 38). However, their impact on immune function is unclear. Here, we tested the ability of Vit-D(2) enriched WMP and high Selenium-WMP to impact Trained Immunity in BMDM. Vit-D WMP drove similar responses as previously observed with WMP, particularly enhanced TNF responses to PAM restimulation, even at doses as low as 1 µg/mL (Figure 2A). Similarly, both LPS and PAM responses were significantly enhanced with Vit-D WMP when IL6 production was measured (Figure 2B). Selenium has been linked to anti-inflammatory responses in other cases (35), and accordingly, we did not observe significant Trained responses in BMDM trained with Se-enriched WMP when TNF or IL6 production was measured (Figures 2C, D). Although overall β-glucan concentration is not significantly altered in Se-enriched WMP relative to regular WMP, an increase in α-glucans was measured specifically in Se-enriched WMP (Table 1), which may alter immune training activity.

TABLE 2 Effect of filtration and simulated digestion on total carbohydrate content of powders.

[Carbohydrate Content], % w/w			
		Mean	SD
WMP	Unfiltered	20.8	1.9
	Filtered	6.2	0.6
IVD-WMP	Unfiltered	48.2	2.2
	Filtered	17.6	1.4

Mushroom β-glucans drive enhanced responses to restimulation

Our data thus far suggests that WMP contains the capacity to train myeloid cells in the long-term. However, whether consumption of mushrooms orally drives similar responses *in vivo* is unclear. To begin to address this, we performed simulated *in-vitro* digestion of WMP (39) and examined the impact of the undigested products on BMDM function. As previously reported (35), the simulated digestion process retains and slightly enriches total β-glucan content of WMP, increasing to ∼13% in *in-vitro* digested product of WMP (IVD-WMP, Tables 1, 2). We found that 3 concentrations of IVD-WMP could drive trained responses over control untrained BMDM. However, this was not concentration-dependent and did not match the training seen with a high concentration of WMP (100 μg/mL, Figures 3A, B). Aside from enriching β-glucan content (as measured by Megazyme assay, Table 1), the simulated digestion also alters total carbohydrate concentration (40) (Table 2, going from 6.2 ± 0.6% to 17.6 ± 1.4% w/w) and therefore likely alters the structure and composition and activity of digestion-resistant fiber in the digested product, making direct comparisons to undigested powder more difficult. Despite this, significant enhancement of both TNF and IL6 responses are observed in IVD-WMP trained cells. The alterations in β-glucan content suggest that β-glucan is indeed the active component driving Trained Immunity by WMP. To test this more formally, we analyzed the Trained Immunity properties of 3 fractions of *A. bisporus*, ranging from hot-water extracted (F1) to 2 more basic wash fractions (F2; KOH & F3, NaOH), which should be more enriched in β-glucans than regular water soluble WMP (41). Consistent with this, we observed enhanced responses to LPS restimulation, particularly significant in the F3-trained BMDM (Figure 3C). These data suggest that β-glucan is the bioactive component of WMP, which drives Trained Immunity responses in macrophages.

Mushroom products train human monocytes

To determine if human cells can be modulated by WMP in a similar way, we isolated monocytes from human blood and incubated these with WMP or similar bulk concentrations of the *in-vitro* digested powder (IVD-WMP) for 24 h as before. After washing, cells are matured to human-monocyte derived macrophages (hMDM) for a further 6-days before restimulation (18). We first examined the kinetics of enhanced TNF production after restimulation with LPS (Figure 4A). Consistent with Trained Immunity altering kinetics of inflammatory and immune genes (2), we observed significantly enhanced TNF production as early as 6h post-stimulation with both 2 concentrations of regular WMP and similar doses of IVD-WMP. This was maintained at 24 h in IVD-WMP trained hMDM. Using a wider panel of restimulation signals, we observed enhanced TNF production to LPS and the Dectin-1 fungal ligand Zymosan-A (ZYM). In contrast to BMDMs, responses to TLR2 stimulation via PAM were not significantly enhanced in the hMDM system (Figure 4B). Beyond TNF, IL6 production was also significantly enhanced in response to LPS, PAM and ZYM restimulation (Figure 4C). These results are consistent with the idea that Trained Immunity has broad non-specific responses in human cells (2, 42).

Mushroom products drive metabolic reprogramming in trained myeloid cells

Metabolic reprogramming has emerged as a hallmark of Trained Immunity (15). Rapid phagocytosis of β-glucan limits inflammatory activation (43) and we recently demonstrated that this process also promotes intracellular reprogramming required for trained responses (*in-press*). We now demonstrate that acute stimulation of mouse BMDM with mushroom powders or digested products triggers minimal TNF production [Figure 5A; and (35)], similar to yeast WGP. Whereas LPS, which drives long-term tolerance (44), triggers significant TNF production. We thus examined Lactate production as a surrogate of glycolytic activity in human monocytes after mushroom powder treatment. We detected significant up-regulation of extracellular Lactate production only after stimulation with a high concentration of IVD-WMP (Figure 5B). We thus further analyzed metabolic responses using the more sensitive extracellular flux analysis system in BMDM. Extracellular acidification rate (ECAR) was found to be up-regulated in BMDM after 24 h treatment with higher concentrations (10 μg/mL) of both WMP and IVD-WMP with increased basal ECAR (Figure 5C). Similar levels of ECAR were measured across treatments after 72 h stimulation, with an increase observed in baseline glycolysis in untrained differentiating macrophages. However, although unchanged after 24 h treatment, we found significant up-regulation in oxidative consumption rates (OCR, an indicator of glucose-dependent oxidative phosphorylation) in BMDM treated with WMP and IVD-WMP after 72 h (Figure 5D). This is consistent with recent reports that β-glucan training employs reprogramming of both glycolysis and TCA to fuel the epigenetic changes required for altered responsiveness (17, 45). Accordingly, targeted inhibition of histone demethylases using 5′methylthioadenosine (MTA) pre-treatment prior to WMP stimulation, blocked the enhanced responsiveness to LPS-restimulation in BMDM (Figure 5E).

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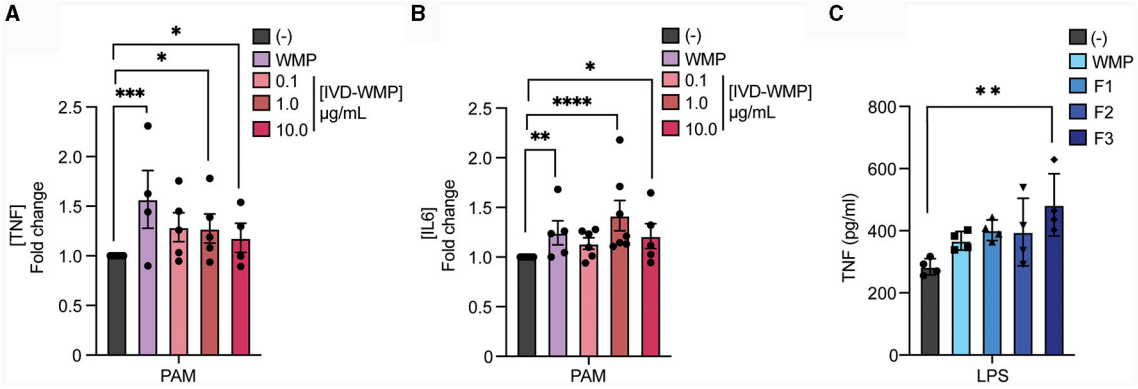

FIGURE 3
Mushroom β-glucans drive enhanced responses to restimulation. **(A, B)** Mature BMDMs were treated with the undigested product of simulated *in-vitro* digestion of *A. bisporus* derived whole mushroom powder (IVD-WMP) at the indicated concentrations (µg/mL), undigested WMP (100 µg/mL) or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with Pam3CSK4 (PAM, 10 ug/mL) for 24 h. Supernatant was removed and the indicated cytokines measured by ELISA [**(A)**, TNF & **(B)**, IL6]. Data is mean fold change in cytokine production normalized to untreated cells (-) ± s.e.m. of $n = 5$ independent experiments. **(C)** Mature BMDMs were treated with glucan-enriched fractions from *A. bisporus* derived whole mushroom powder (F1-F3, 100 µg/mL), unfractionated WMP (100 µg/mL) or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with LPS (10 ng/mL) for 24 h. Supernatant was removed and TNF measured by ELISA. Data is mean cytokine concentration ± s.e.m. of $n = 3$ independent experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.005, **** P value < 0.001 determined using 1-way ANOVA with the indicated *post-hoc* multiple comparisons (Sidak's multiple comparisons test).

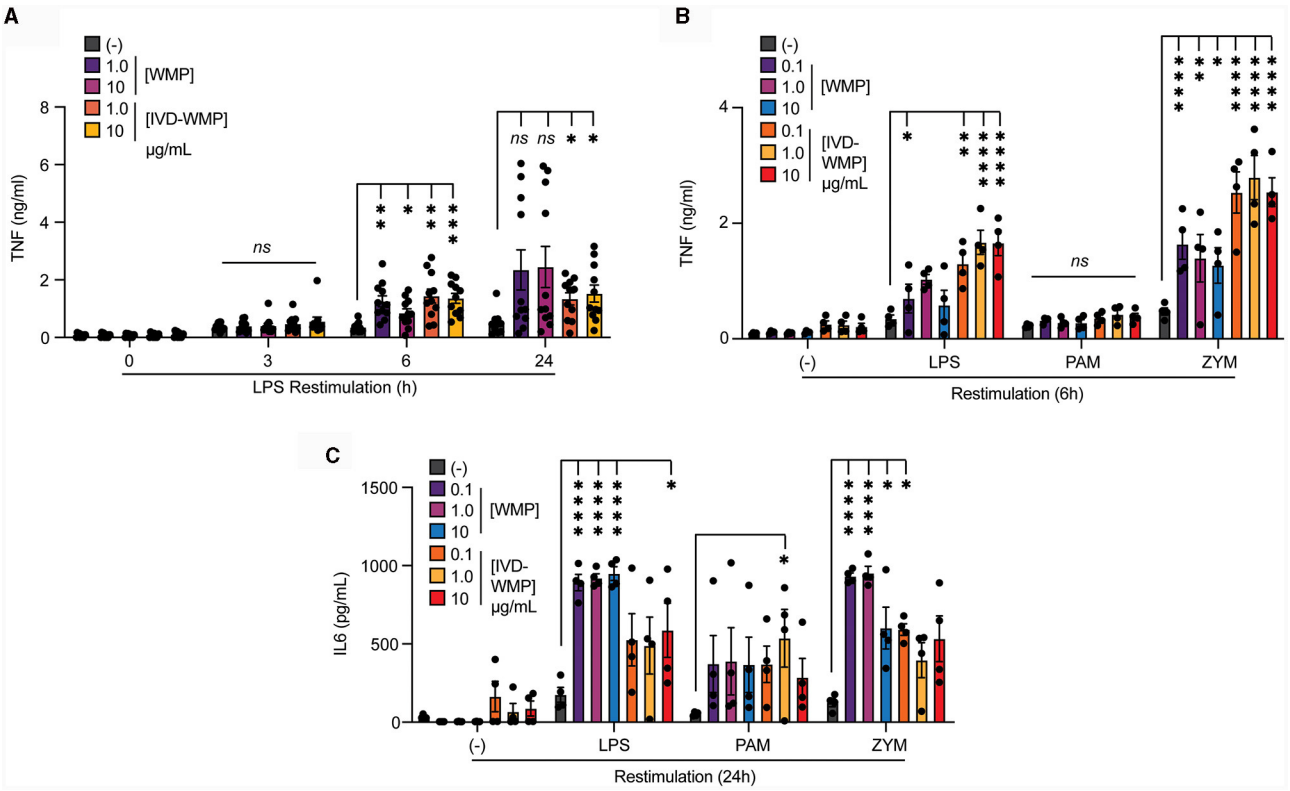

FIGURE 4
Mushroom products train human monocytes. **(A–C)** Monocytes from human PBMCs were isolated and treated with *A. bisporus* derived WMP or the undigested product of simulated *in-vitro* digestion of *A. bisporus* derived whole mushroom powder (IVD-WMP) at the indicated concentrations (µg/mL), or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 6 days prior to restimulation with LPS (10 ng/mL) for the indicated times between 0–24 h **(A)**, or restimulated with LPS (10 ng/mL), (PAM, 10 µg/mL), Zymosan-A (ZYM, 10 µg/mL) or left untreated (-). TNF production was measured after 6 h **(B)** or IL6 production measured after 24 h **(C)**. Data is mean cytokine concentration ± s.e.m. of $n = 11$ **(A)** or $n = 4$ **(B, C)** donors. *P value < 0.05, **P value < 0.01, ***P value < 0.005, ****P value < 0.001 determined using 2-way ANOVA with the indicated *post-hoc* multiple comparisons (Fisher's LSD test).

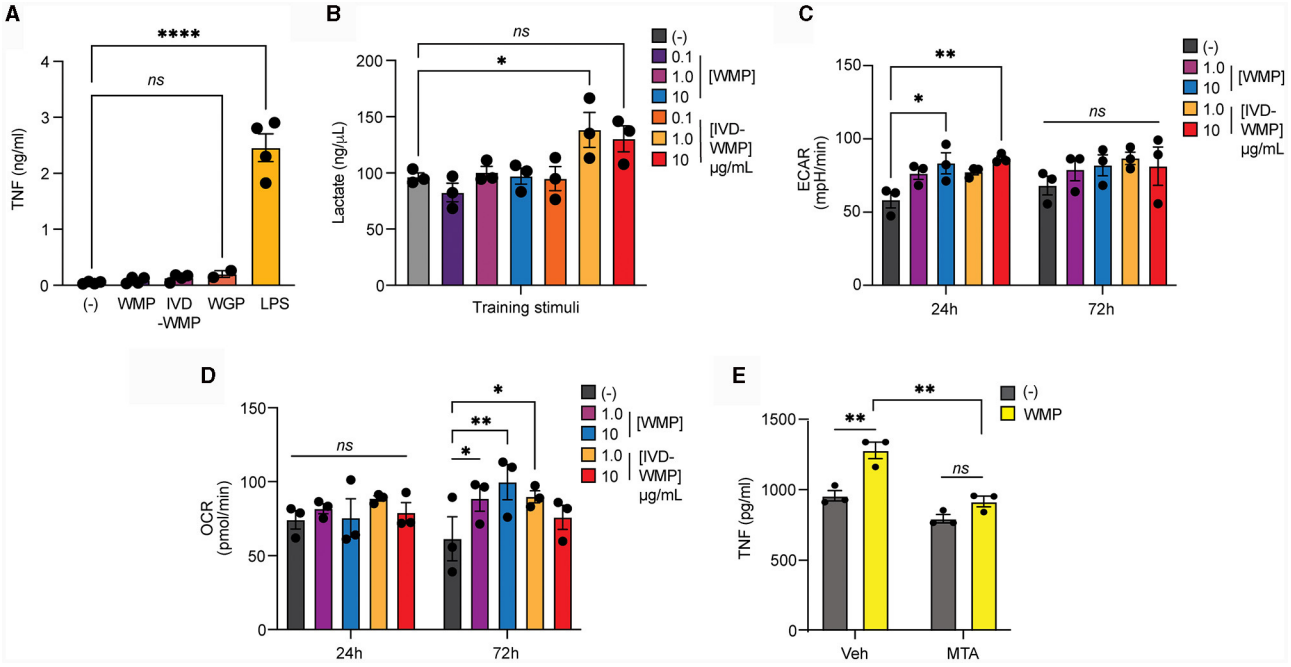

FIGURE 5
Mushroom products drive metabolic reprogramming in myeloid cells. **(A, B)** Monocytes from human PBMCs were isolated and treated with *A. bisporus* derived WMP or the undigested product of simulated *in-vitro* digestion of *A. bisporus* derived whole mushroom powder (IVD-WMP) at the indicated concentrations (µg/mL) or left untreated (-). Extracellular TNF production **(A)** or Lactate accumulation **(B)** was measured after 24h treatment. Data is mean concentration ± s.e.m. of $n = 4$ donors. **(C, D)** Mature BMDM were stimulated with WMP or IVD-WMP as indicated or left untreated (-) and metabolic flux measured on distinct Seahorse plates at 24 h or 72 h. Data is the calculated mean basal extracellular acidification rate [ECAR, **(C)**] or oxygen consumption rate [OCR, **(D)**] for $n = 3$ replicates. **(E)** Mature BMDM were treated with 5-methyladenosine (MTA, 2 mM) or vehicle control for 1 h prior to training with 1,000 µg/mL WMP. After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with Pam3CSK4 (PAM, 10 µg/mL) for 24 h. Supernatant was removed and TNF production measured by ELISA. Data is mean TNF concentration ± s.e.m. of $n = 3$ independent experiments. *P value < 0.05, **P value < 0.01, ****P value < 0.001, determined using 2-way ANOVA with the indicated *post-hoc* multiple comparisons (Fisher's LSD test).

Pre-treatment of bone-marrow cells with mushroom powders drives long-term responses to TLR2 restimulation

Intraperitoneal delivery of *Trametes versicolor* β-glucan was shown to alter the frequency and function of myeloid progenitors in bone-marrow (46). Therefore, we examined whether *ex vivo* culture of bone-marrow cells (BMC) with WMP could alter the fate of mature macrophages derived from these cultures. First, we validated this system using yeast-derived WGP. Culture of BMC with WGP for 24 h followed by washing & 5-day maturation, led to enhanced responses to PAM and higher concentration of LPS restimulation with 100–1,000 µg/mL WGP (Figure 6A). Similar culture of BMCs with WMP led to enhanced PAM restimulation responses at most concentrations used (from 1–1,000 µg/mL, Figure 6B), an effect also observed with Vit-D enriched WMP (Figure 6C). Notably, LPS responses were not enhanced by WMP culture, although we noted that the control level of TNF production after LPS treatment was much lower in these cells. Overall, these results suggest that culture of bone-marrow progenitor cells with WMP can enhance the function of mature macrophages derived from HSPCs in these cultures. Whether this represents a direct effect of WMP on HSPCs or other stromal cells is currently unclear.

Oral delivery of mushroom powder alters bone-marrow cell function and fate

To more directly address if WMP impacts myelopoiesis, WMP was delivered to mice daily by oral gavage for 1-week prior to sacrifice. Bone-marrow was isolated and HSPCs monitored by flow cytometry. Although the overall number of Lineage-, c-Kit+ and Sca1+ (LKS)-cells was not significantly changed by oral gavage of WMP relative to control PBS-treated mice (Figure 7A), some alterations in the relative frequency of specific subsets was observed. This suggests dynamic remodeling of bone-marrow HSPC populations. Notably, long-term (LT)-HSPC's were increased after WMP delivery, while corresponding short-term (ST)-HSPCs were not changed (Figures 7B–D). The more-committed multi-potent progenitors (MPPs) were not significantly altered by WMP delivery either (Figure 7D). However, examining the specific lineages within these we found an increased proportion of the myeloid-committed MPP3 population in WMP-treated mice, with a corresponding decrease in the proportion of lymphoid-committed MPP4 cells (Figures 7E, F). This skew toward myelopoiesis is characteristic of *in-vivo* Trained Immunity (47), therefore we tested the function of BMDM derived from the same bone-marrow. Consistent with increased activity, production of TNF induced by a range of macrophage activating stimuli was

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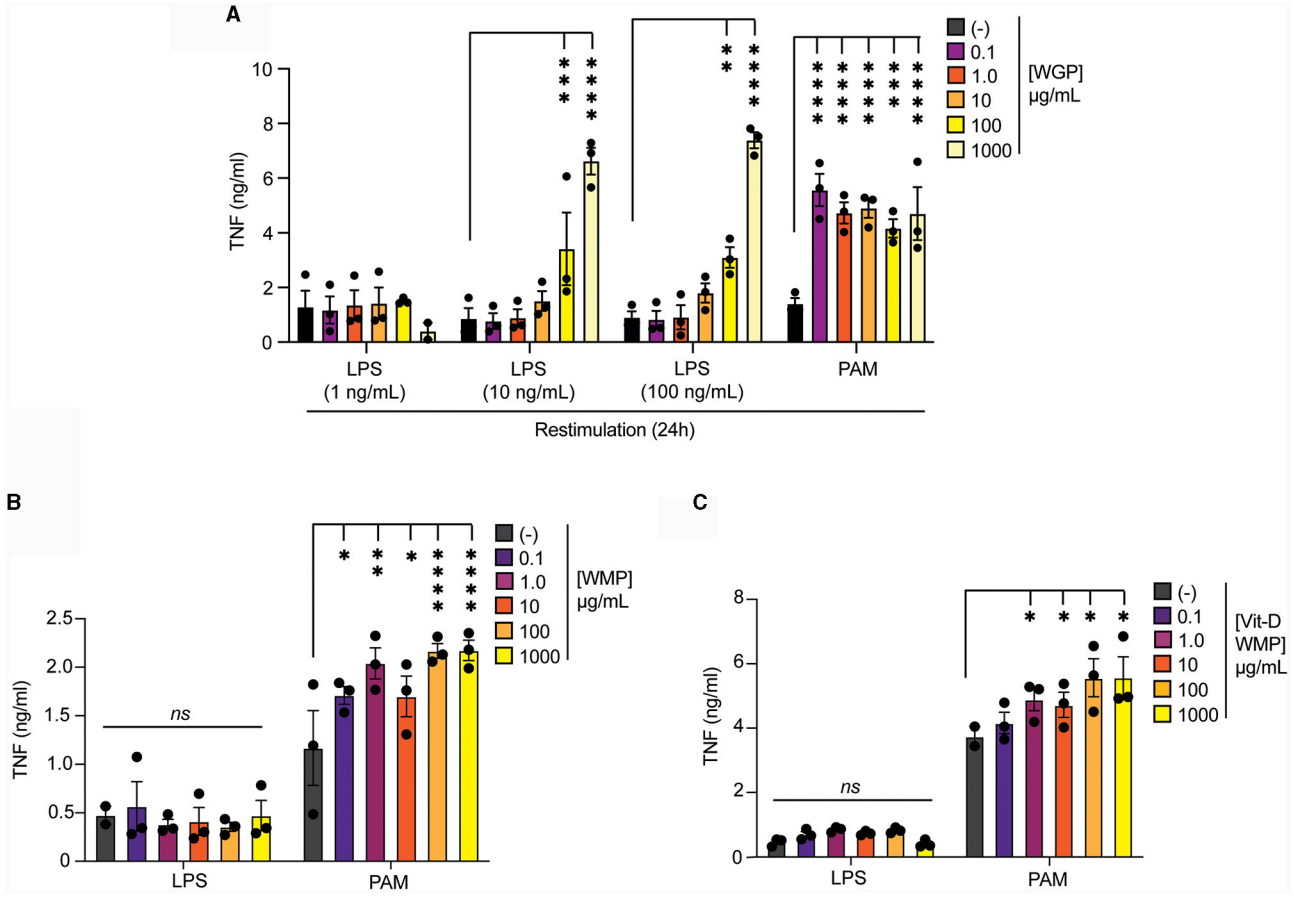

FIGURE 6
Pre-treatment of bone-marrow cells with mushroom powders drives long-term responses to TLR2 restimulation. **(A–C)** Mature BMDMs were treated with yeast-derived WGP **(A)**, *A. bisporus* derived WMP **(B)**, Vit-A-enriched WMP **(C)** at the indicated concentrations (µg/mL) or left untreated (-). After 24 h, media was removed and cells washed and matured for a further 7 days prior to restimulation with LPS (as indicated or at 10 ng/mL) or Pam3CSK4 (PAM, 10 µg/mL) for 24 h. Supernatant was removed and TNF production measured by ELISA. Data is mean cytokine concentration ± s.e.m. of $n = 3$ independent experiments. *P value < 0.05, **P value < 0.01, ***P value < 0.005, ****P value < 0.001, *n.s.* P value > 0.05, determined using 2-way ANOVA with the indicated *post-hoc* multiple comparisons (Fisher's LSD test).

enhanced in WMP-treated mice, from LPS, PAM but also seen in response to ZYM and heat-killed *Mycobacterium tuberculosis* (hk-MTB, Figure 7G). A similar enhancement in IL-10 production was observed (Figure 7H). Curiously, IL-6 production was decreased in BMDM from WMP-treated mice (Figure 7I). Despite this, the increased TNF production and expansion of myeloid progenitors in mice receiving WMP suggests the ability of orally delivered WMP β-glucan to drive Trained Immunity *in-vivo*.

Chemical analysis of *A. bisporus* mushroom powder and it's *in vitro* digested product

Our data suggests that β-glucans in *A. bisporus* mushroom powder drive Trained Immunity in myeloid cells and retain this property after digestion. We thus undertook chemical profiling of WMP and IVD-WMP samples using one-dimensional and two-dimensional NMR analysis to detect and characterize *A. bisporus* β-glucans. The workflow is outlined in Figure 8A. Briefly, partial solubility prevented the generation of accurate ^{13}C spectra by 1D NMR, although ^{1}H NMR spectra were generated and are shown in Figures 8B, C for both WMP and IVD-WMP, respectively. ^{1}H NMR of WMP reveals a complex spectrum with several signals, indicating a mixture of metabolites. As *A. bisporus* mushroom has been reported to contain carbohydrates (glucans), lipids and amino acids as major constituents (33, 48, 49) and our study focuses on immunomodulatory effects of glucans, the spectrum shown is labeled for glucans (middle) and non-carbohydrate residues A & B (to the left and right of glucans). Residue A region ranges from 0.93–2.75 ppm and B ranges from 6.9–7.5 ppm, which covers aliphatic and aromatic protons respectively, that might correspond to lipids and amino acids (49, 50). The region between 3.0 ppm and 4.0 ppm with relatively high peak abundance denotes adequate amounts of glucans as discerned by the cyclic protons of glucosyl moieties. Though a sharp signal occurs at δ1.36 in the aliphatic region, it corresponds neither to methyl group at C6 of deoxy sugars [fucose (δ1.26) or rhamnose (δ1.18)] (51, 52), nor represents -OCH_3 group as in 3-*O*-methylgalactose (δ3.45), a moiety commonly produced by oyster mushroom, *Pleurotus citrinopileatus* (53).

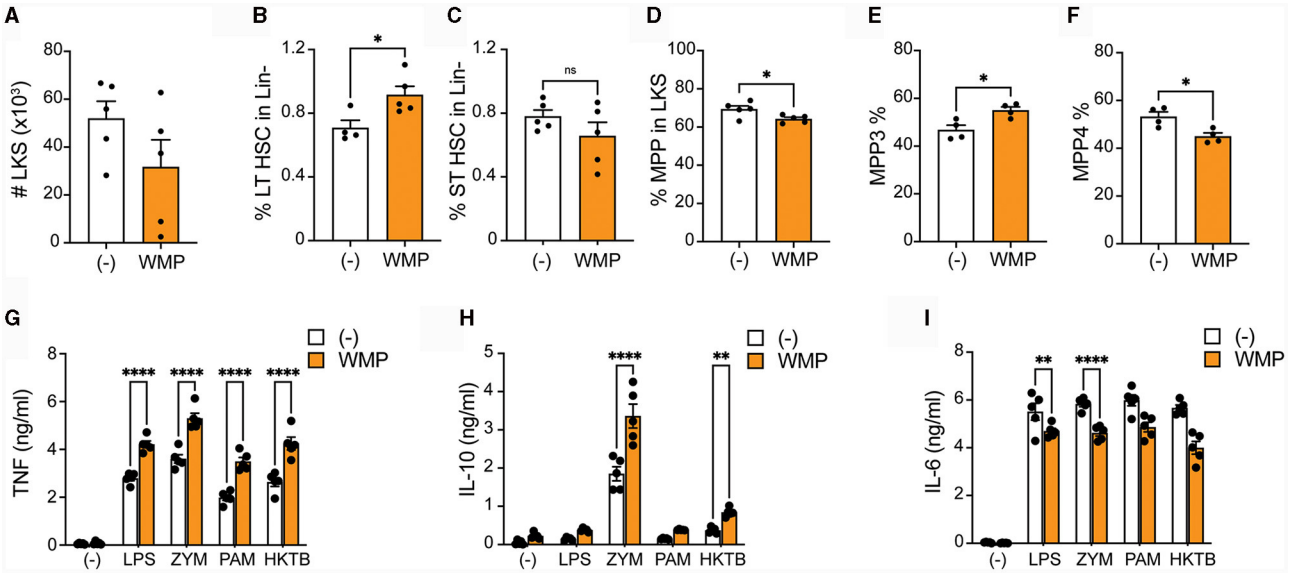

FIGURE 7
Oral delivery of mushroom powder alters bone-marrow cell function and fate. *A. bisporus*-derived WMP (10 mg) was delivered to C57/BL6 mice via oral gavage daily for 7-days prior to sacrifice. Control mice received PBS (-). Bone-marrow cells were isolated and stained for multi-parameter flow cytometry **(A–F)** or used to generate mature **(G–I)**. **(A–F)** Lineage-, c-kit+, and Sla-1 negative (LKS)-cells were enriched and analyzed by flow cytometry. Graphs shown illustrate differences in absolute cell numbers per leg or relative % differences in populations across PBS and WMP-treated mice. **(G–I)** Mature BMDM were stimulated with LPS (10 ng/mL), Zymosan-A (ZYM, X ug/mL), Pam3CSK4 (PAM, 10 ug/mL), or heat-killed *Mycobacterium tuberculosis* (HKMTB, dose) for 24 h. Supernatant was removed and the indicated cytokines measured by ELISA [**(A)**; TNF, **(B)**; Il10, **(C)**; IL6]. Data is mean cytokine concentration ± s.e.m. of $n = 5$ mice per group. *P value < 0.05, **P value < 0.01, ****P value < 0.001, *n.s.* P value > 0.05, determined using Students t-test.

To further characterize the carbohydrates present, ^{1}H NMR (Figures 8B, C) and TOCSY (Total Correlation Spectroscopy; Figures 8E–G) measurements were made on a 400 MHz NMR instrument to capture maximum chemical information about WMP and IVD-WMP. Measurements at 600 MHz NMR were attempted to acquire ^{13}C NMR spectra; however, this was only achievable for WMP. The digestion process which alters total carbohydrate content by enriching for fibers (Table 2) impacts solubility, leading to a lower ^{13}C signal abundance for IVD-WMP. For this reason, HSQC (Heteronuclear Single Quantum Coherence) of WMP sample only was recorded on 600 MHz instrument (Figure 8D). Consequently, ^{1}H/^{13}C spectral assignments were obtained for WMP (Table 3), while for IVD-WMP interpretation was limited to ^{1}H chemical shift values (Table 4). Due to the complexity of signals generated, we have primarily focused on assigning chemical shifts corresponding to glucans (Tables 3, 4). The signals at δ5.24 and δ4.66 correspond to anomeric protons of reducing α-glucopyranosyl (α-Glc*p*, **A**) and β-glucopyranosyl (β-Glc*p*, B) residues, while signals at δ4.66 and δ4.46 correspond to anomeric protons of terminal β-Glc*p* (**C**) and (1→6)-β-Glc*p* (**D**) residues respectively. This assignment was complicated due to the absence of ^{13}C NMR spectrum. Hence for WMP, the ^{1}H/^{13}C assignments were first picked by HSQC data and then correlated with TOCSY data. Since IVD-WMP was obtained by digesting WMP, their ^{1}H NMR and TOCSY spectra were compared (Figures 8B, C, G) and ^{1}H chemical shift values were deduced. For residues **A**, chemical shifts corresponding to H1/C1, H2/C2, H3/C3, H5/C5, and H6a,b/C6 were assigned as 5.24/91.83, 3.57/70.31, 3.74/71.18, 3.80/70.45, and 3.71, 3.97/60.35 respectively. The signals for H4/C4 were not found. Similarly, the signals for residues **B** corresponding to H1/C1, H2/C2, H3/C3, H4/C4, H5/C5, and H6a,b/C6 were assigned as 4.66/96.08, 3.26/74.85, 3.48/75.87, 3.41/-, 3.50/-, 3.68, and 3.87/62.40, respectively. For this moiety, δC4 and δC5 were not found in HSQC and hence δH4 and δH5 were assigned based on TOCSY correlations. Similarly, the chemical shifts corresponding to anomeric signals for residues **C** (δ4.66) and **D** (δ4.46) were not found in HSQC but deduced from TOCSY interactions. The residues **C** and **D** have the same shift values for H2/C2 (3.26/74.85), H3/C3 (3.48/75.87), and H4/C4 (3.44/70.31) but differ for H5 (δ3.50 for **C**; δ3.55 for **D**) and H6a,b/C6 (3.58, 3.87/62.40 for **C**; 3.73, 3.97/69.72 for **D**), due to the linkage at C6 in residue **D,** thereby shifting the proton and ^{13}C signals downfield. For IVD-WMP, the signal intensity of ^{1}H NMR spectra was very low compared to WMP as visible in Figures 8B, C, which made the data interpretation challenging. As a result, complete NMR assignment was not possible, but the signals corresponding to H3, H4, H5, and H6a,b have been labeled for the residues **A**, **B**, **C,** and **D** (Table 4). The peak assignment for WMP and IVD-WMP was found to be in accordance with literature (54, 55). In summary, we identified reducing α- and β-Glc*p*, terminal β-Glc*p* and 1, 6-β-Glc*p* residues and assigned δH/δC chemical shifts for WMP and δH for IVD-WMP. The results establish the presence of α-glucose, β-glucose, and (1→6)-linked β-glucans in mushroom powder. Focusing on β-glucans, we used this data to generate a predicted structure of *A. bisporus* powder β-glucans, shown with a terminal glucopyranosyl unit and a repeating unit of β(1→6)-linked glucopyranosyl units (Figure 8H).

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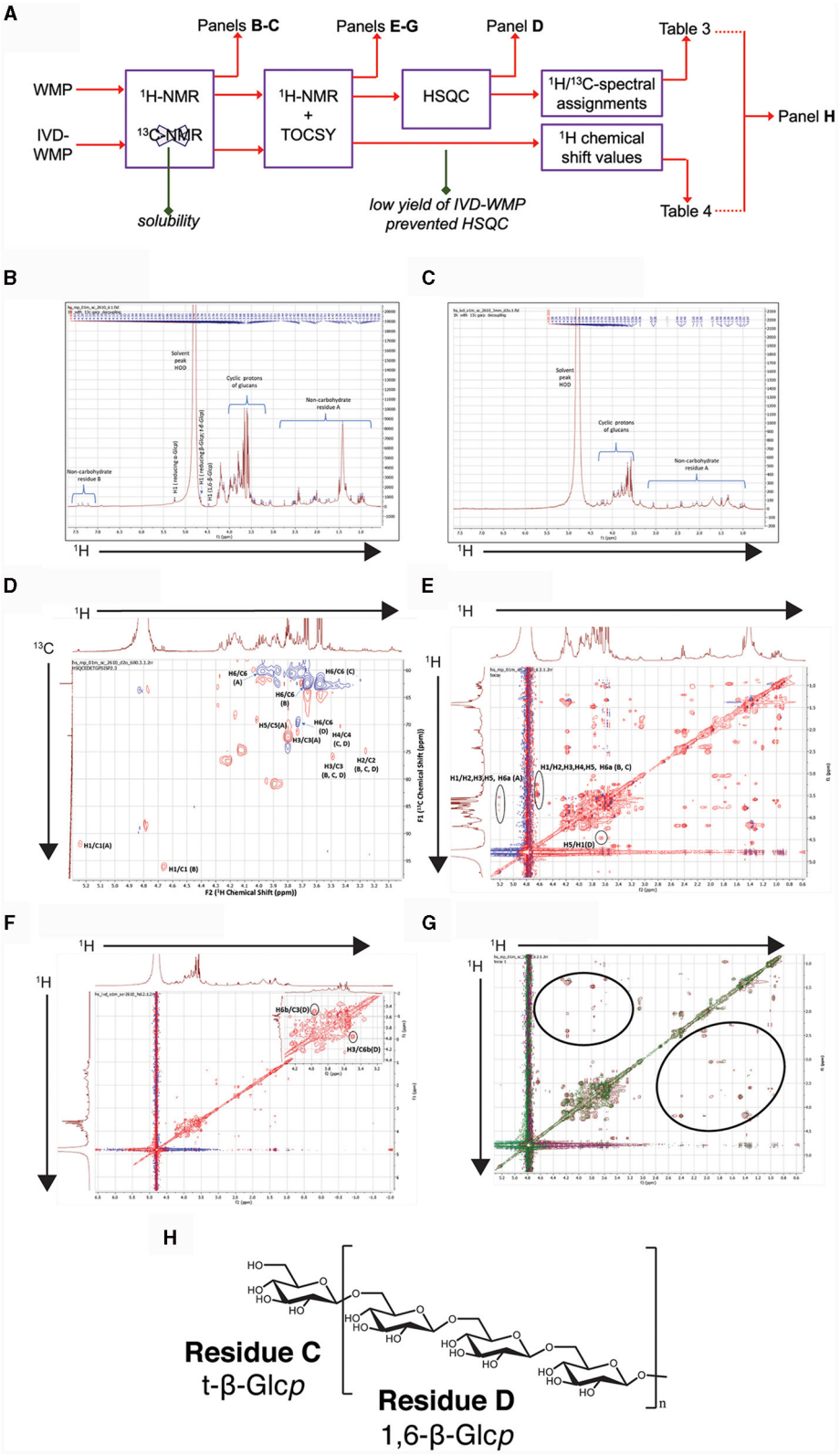

FIGURE 8
Chemical profiling of mushroom powder and IVD-WMP. **(A)** Overview of workflow used for both *A. bisporus* WMP and digested product (IVD-WMP) showing how subsequent plots were derived. **(B, C)** ^{1}HNMR spectrum of WMP **(B)** and IVD-WMP **(C)** dissolved in deuterated water (99.95%) as solvent, with the solvent peak at 4.80 ppm used as a reference peak. Signals in aliphatic (non-carbohydrate residue **(A)** and aromatic (non-carbohydrate residue **(B)** regions are indicated, alongside anomeric protons of glucans. **(D)** 2D-NMR HSQC spectrum of WMP; Residues **(A–D)** represent reducing α-Glc*p*, reducing β-Glc*p*, t-β-Glc*p*, and 1, 6-β-Glc*p*, respectively. **(E)** TOCSY spectrum of WMP; **(F)** TOCSY spectrum of IVD-WMP; **(G)** Superimposed TOCSY spectrum of WMP and IVD-WMP, brown and green signals represent WMP and IVD-WMP respectively. Absence of green signals in regions marked by black ring indicates digestion of WMP carbohydrates and enrichment of β-glucans. **(H)** Predicted structure of *A. bisporus* β(1→6)-glucans based on 2D-NMR and annotations made in Table 3.

TABLE 3 ^{1}H and ^{13}C NMR spectral assignments based on HSQC and TOCSY data for polysaccharide residues present in mushroom powder (WMP).

Assignment	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6a, H6b/C6
'A' Reducing α-Glc*p*	5.24/91.83	3.57/70.31	3.74/71.18	-	3.80/70.45	3.71, 3.97/60.35
TOCSY correlations	3.71(H6a)/5.24(H1)	5.24(H1)/3.57 (H2) 3.74(H3)/3.57 (H2)	5.24(H1)/3.74 (H3) 3.57 (H2)/3.74 (H3)	-	5.24(H1)/3.80 (H5) 3.97(H6b)/3.80 (H5)	5.24(H1)/3.71(H6a) 3.57 (H2)/3.71(H6a) 3.80 (H5)/3.97(H6b)
'B' Reducing β-Glc*p*	4.66/96.08	3.26/74.85	3.48/75.87	3.41/-	3.50/-	3.68, 3.87/62.40
TOCSY correlations	3.26(H2)/4.66(H1) 3.50(H5)/4.66(H1) 3.41(H4)/4.66(H1)	4.66(H1)/3.26(H2) 3.48(H3)/3.26(H2) 3.41(H4)/3.26(H2)	4.66(H1)/3.48(H3) 3.26(H2)/3.48(H3) 3.87(H6b)/3.48(H3)	4.66(H1)/3.41(H4) 3.26(H2)/3.41(H4)	4.66(H1)/3.50(H5) 3.48(H3)/3.50(H5)	4.66(H1)/3.68(H6a)
'C' t-β-Glc*p*	4.66/-	3.26/74.85	3.48/75.87	3.44/70.31	3.50/-	3.58, 3.87/62.40
TOCSY correlations	3.26(H2)/4.66(H1) 3.50(H5)/4.66(H1) 3.48(H3)/4.66(H1) 3.44(H4)/4.66(H1)	4.66(H1)/3.26(H2) 3.48(H3)/3.26(H2) 3.44(H4)/3.26(H2)	4.66(H1)/3.48(H3) 3.26(H2)/3.48(H3) 3.87(H6b)/3.48(H3)	4.66(H1)/3.44(H4) 3.26(H2)/3.44(H4)	4.66(H1)/3.50(H5) 3.48(H3)/3.50(H5)	4.66(H1)/3.68(H6a) 3.58(H6a)/3.87(H6b)
'D' 1, 6-β-Glc*p*	4.46/-	3.26/74.85	3.48/75.87	3.44/70.31	3.55/-	3.73, 3.97/69.72
TOCSY correlations	3.55(H5)/4.46(H1)	3.48(H3)/3.26(H2) 3.44(H4)/3.26(H2)	3.26(H2)/3.48(H3) 3.73(H6a)/3.48(H3)	3.26(H2)/3.44(H4) 3.48(H3)/3.44(H4)	3.48(H3)/3.55(H5) 3.73(H6a)/3.55(H5)	3.26(H2)/3.73(H6a) 3.55(H5)/3.73(H6a) 3.73(H6a)/3.97(H6b) 3.48(H3)/3.97(H6b) 3.97(H6b)/3.73(H6a)

In relation to the effect of simulated digestion, ^{1}H NMR analysis of IVD-WMP (Figure 8C) shows the absence of anomeric and aromatic signals. Further, the strong peak at δ1.38 was drastically reduced. Although the overall signal intensity was reduced relative to WMP, the ratio of the glucan to non-carbohydrate region A peaks is increased in IVD-WMP, suggesting enrichment of glucans. The TOCSY spectra of WMP and IVD-WMP (Figures 8E, F), along with the superimposition of both TOCSY (Figure 8G), also demonstrates a dramatic effect of digestion. The circled regions display signals indicating non-carbohydrate residues present in WMP which are lost in IVD-WMP, while the complex carbohydrate regions shown by cyclic protons signals of glucans are preserved in IVD-WMP (overlapping regions). All these observations establish that *in vitro* digestion of WMP proved to be effective in degrading other compounds and enriching β-glucans and thus explain why in our in-vitro assays (Figures 3–5) and oral delivery *in-vivo*, digested mushroom powders retain the ability to drive Trained Immunity.

Discussion

Although powdered mushroom contains lower overall levels of β-glucan, the data herein demonstrates that delivery of powders to mouse and human innate immune cells can drive functional reprogramming to similar levels as that observed with equivalent amounts of more common β-glucan purifications. That this property and the presence of complex β-glycosidic-linked carbohydrates is conserved after simulated digestion of the powders and coupled with features of Trained Immunity observed in mice after oral delivery of large amounts of the powders, supports the notion that mushroom consumption can support overall innate immune function. These findings may explain in-part some of the positive health benefits previously ascribed to dietary mushrooms or mushroom-derived functional foods, including anti-cancer, anti-oxidant effects, gut health supports and immunomodulatory roles (56–60).

Heterogeneity in β-glucan chemical structure impacts biological activities (8, 9). Mushroom β-glucans are thought to be more linear, β(1→3) linked polymers, with less β(1→6) branching than seen in yeasts and other fungi (eg; *Aspergillus*) (29, 61). As a result of shorter chains, their reported MW is lower (seen in Lentinan and Pleuran β-glucans) and this can affect their solubility (62). Our NMR analysis of *A. bisporus*, although limited, detected predominantly β(1→6) linked glucans with little evidence for branching, although this cannot be discounted. The presence of these β(1→6) linkages confers recognition by Dectin-1 (6) and could be crucial for the induction of Trained Immunity by *A. bisporus* products. Although mushroom β-glucans do not contain long, linear β(1→4) and β(1→3) linkages seen in plants (cereals) and algae (e.g., Laminarin) (63, 64), the binding properties for different Dectin-1 isoforms displayed by *A. bisporus* WMPs reported here, suggests similar biological activity (13). Low MW β-glucans are known to antagonize binding to Dectin-1 by larger particulates (12, 14) and have also been reported to have

TABLE 4 Proton spectral assignments based on ^{1}HNMR and TOCSY data for polysaccharide residues present in *in vitro* digested mushroom powder (IVD-WMP).

Assignment	H1	H2	H3	H4	H5	H6a, H6b
'A'	-	3.58	3.72	-	3.79	3.72, 3.96
Reducing α-Glcp						
TOCSY correlations		3.72(H3)/3.58 (H2)	3.58 (H2)/3.72 (H3)	-	3.96(H6b)/3.79 (H5) 3.58(H2)/3.79 (H5)	3.58 (H2)/3.72(H6a) 3.79 (H5)/3.72(H6a) 3.79 (H5)/3.96(H6b)
'B'	-	-	3.49	3.41	3.51	3.68, 3.86
Reducing β-Glcp						
TOCSY correlations	-	-	3.41(H4)/3.49(H3)	-	-	3.86(H6b)/3.68(H6a)
'C'	-	-	3.49	3.43	3.51	3.58, 3.89
t-β-Glcp						
TOCSY correlations				3.51(H5)/3.43(H4)	3.43(H4)/3.51(H5)	3.58(H6a)/3.89(H6b)
'D'	-	-	3.49	3.43	3.55	3.79, 3.96
1, 6-β-Glcp						
TOCSY correlations	-	-	3.96(H6b)/3.49(H3)	-	-	3.49(H3)/3.96(H6b) 3.79(H6a)/3.96(H6b)

anti-inflammatory properties (36) similar to that reported for *A. bisporus* powders by us and others (35, 65). Despite this, there are reports of larger MW β-glucans, with increased degrees of branching, across different mushroom species (e.g., Schizophylum and Cordyceps) (29, 61) and these may act differently to *A. bisporus* WMP's reported here. A caveat to interpretation of our data is that for *in-vitro* work with immune cells, our WMPs are filtered. This reduces total carbohydrate content (Table 2) and may preclude larger MW β-glucans normally found in edible mushrooms from our *in-vitro* experiments. Thus, our results likely reflect the function of lower MW *A. bisporus* β-glucans and other smaller, less complex polysaccharides (62), as detected by our NMR analysis. Similarly, our *in-vivo* delivery of WMP used WMPs after filtration, to remove potential contamination of endotoxins which may impact immune function *in-vivo*.

As well as using 1D and 2D NMR chemical analysis to characterize the glucan component of *A. bisporus* WMP's and thereby explain differences in biological activities relative to other β-glucan's tested, this also allowed us to determine the effect of simulated digestion of mushroom products (IVD-WMP). Despite the challenge of obtaining clean ^{13}C NMR spectra for the two samples due to their low solubility in deuterated water (D_2O), ^{1}H, HSQC, and TOCSY played a significant role in identifying reducing α- and β- Glc*p*, terminal β-Glc*p* and 1, 6-β-Glc*p* residues and assigning δH/δC chemical shifts for WMP and δH for IVD-WMP. The results establish the presence of α-glucose, β-glucose and β(1→6)-linked glucans in mushroom powder. There have been several reports on α- and β-glucans from *Agaricus bisporus* (33, 66–68), present as a backbone, with other monosaccharides (galactose, fucose, xylose, and mannose) attached as side chain or independently in small amounts (33). We came across only one report on fucogalactan isolated from *A. bisporus* with a (1→6)-linked α-Galp backbone and fucose branching (52). Although more commonly used for storage and energy utilization, less is known about the immunomodulatory effects of α-glucans. Though present in much lower amounts than β-glucans in our *A. bisporus* mushroom powders, there was a striking increase in α-glucans composition in powders from Selenium enriched WMPs, which did not display the ability to drive trained responses in mouse macrophages. It remains to be seen if their increased abundance simply outcompetes mushroom β-glucans or if α-glucans can directly block Dectin-1 binding. The presence of α-glucose, as well as β(1→6) linked glucans was inferred in digested WMP, while other simpler carbohydrate and non-carbohydrate residues seemed to be lost. Although the signal obtained was lower, which impeded complete assignment of residues in IVD-WMP, our results suggest that the simulated digestion process modifies the carbohydrate content and abundance of WMPs. Although the overall amount likely decreases as a result of digestion, the relative abundance of complex, glucan species compared to simpler carbohydrates increases. Thus, digestion *in-vivo* likely preserves dietary fibers like β-glucans which can then mediate biological and immune effects.

In support of this, our *in-vivo* delivery of filtered WMP via oral gavage led to features of Trained Immunity, including bone marrow myelopoiesis and enhanced responses to stimulation in BMDM from trained mice. Many groups have observed expansion in the total frequency and amount of bone marrow hematopoietic stem and progenitor cells (HSPCs), defined as LKS+ cells, after *in-vivo* delivery of training stimuli, generally via intra-peritoneal injection (21, 46). Other studies of oral β-glucan delivery in humans did not find evidence of trained responses in circulating PBMCs (69), which suggests that the route of administration is central to the development of trained responses. Previous work in our lab (*in-press*) revealed that although IP injection of yeast β-glucans leads to a similar expansion in LKS+ cells, oral gavage of equivalent amounts of the same β-glucan did not alter total LKS+ numbers, similar to what was observed here after WMP gavage. Despite this, in both cases—after yeast β-glucan and WMP gavage, the populations of more committed hematopoietic progenitor cells (HSCs), particularly the multi-potent progenitors (MPPs) revealed a skew toward myeloid progenitors (MPP3), indicative of increased myelopoiesis. We hypothesize that oral delivery can

indeed reprogramme myeloid progenitors, as evidenced herein by enhanced functional responses in mouse BMDM. However, this route of administration likely avoids the typical systemic inflammatory response seen with peritoneal delivery, which may contribute overall to the inflammatory milieu suggested to be required to trigger emergency granulopoiesis (70) and thus give rise to the noted expansion in total LKS+ cells after delivery. How these 2 processes may be disentangled in response to specific stimuli and routes of administration and their relative contributions to features of central Trained Immunity remain unclear. Although direct incubation of bone-marrow cells with WMP *in-vitro* led to some trained responses, this population contained both HSPCs and mature bone-marrow resident cells which may mediate the training effect. This, coupled with the lack of expansion in total LKS+ cells after oral delivery, suggests a more indirect route mediating *in-vivo* Trained Immunity after oral delivery of WMPs.

The short-term delivery of β-glucans and WMP employed here precludes a role for major changes in the architecture of gut microbiome and their metabolites (71), although this cannot be completely discounted. Although mammals are not thought to express β-glucanases, previous experiments have demonstrated that some soluble β-glucans are absorbed after oral delivery including laminarin and glucan-phosphate (72). Importantly, these treatments were associated with increased non-specific immune responses (72), analogous to Trained Immunity. While a role for the gut microbial flora as a source of β-glucanases cannot be discounted, our simulated digestion modified β-glucan composition of WMP and preserved the capacity to train macrophages *in-vitro*, independently of the microbiome. Thus, WMP-derived products likely mediate the features of Trained Immunity observed *in-vivo*, although their direct substrate remains unclear. Analysis of RNA and proteomic sequencing databases (e.g., The Human Protein Atlas) and experimental evidence demonstrates functional expression of both Dectin-1(a/b) isoforms in primary human intestinal epithelial cells, which regulate biological responses to β-glucan treatment (73), including cytokine production. Other work has demonstrated that macrophages can phagocytose larger, insoluble β-glucan particulates to release soluble β-glucans and thereby mediate their biological effects (74). Further work will determine if intestinal recognition of dietary β-glucans—either intact particulates or more soluble products of digestion—occurs through epithelial or gut-associated lymphoid tissue (GALT)-mediated Dectin-1 expression and if this mediates Trained Immunity via the bone-marrow, possibly through regulating systemic cytokine responses or other mechanisms.

Our model of *in-vivo* WMP delivery mimicked daily consumption of common white button mushroom containing foods. Analysis after 1 week revealed features of Trained Immunity. However, the longevity of innate immune memory triggered through this pathway remains unclear. Indeed, whether repeated dosing functions similarly to repeat prime/boost immunization strategies or reaches a threshold level—after which tolerance mechanisms or negative regulation kicks in, is currently unclear. Studies of innate memory triggered by the endotoxin component LPS suggest that lower concentrations of stimuli trigger Trained Immunity (75, 76), while large amounts trigger a tolerance phenotype (44). The modest trained response measured here with dietary WMP dosing may be sufficient to augment innate immunity, yet avoid negative regulation feedback despite repeated dosing. At the same time, Trained Immunity has now been described as underlying pathogenesis in many inflammatory diseases including diabetes and arthritis (77, 78), with triggering of innate immune cells by damage-associated molecular patterns (DAMPs) preprogramming for increased activity in disease (3, 79). We hypothesize that training with β-glucans, although it enhances pro-inflammatory cytokine production via epigenetic priming, does so in a non-specific fashion. Although anti-inflammatory cytokines like IL10 have not been reported to be enhanced by β-glucan training (5), their expression is not lost—consistent with a self-limiting nature to the trained innate immune response. Indeed, in our *in-vivo* WMP model increased IL10 was observed. In this way, strategies which target Trained Immunity will not skew toward a generalized pro-inflammatory response, but rather promote enhanced, balanced responses. In this way, Trained Immunity increases early responses to infection, which if delayed, can lead to dissemination of infection and uncontrolled inflammation -exemplified by defects in early viral containment associated with severe COVID-19 (80). Similarly, particulate β-glucans have been shown to restore defective inflammatory responses in models of chronic wound healing (81) and Trained Immunity may underlie the augmented macrophage response observed. Dietary manipulation of innate immune function through the delivery of modest amounts of β-glucan containing foods thus, may in fact represent a beneficial way to fortify the immune system. As well as being traditionally linked with health benefits, and more recently investigated for anti-cancer, anti-inflammatory and anti-oxidant properties (26, 34, 60), mushroom products—specifically the powdered *A. bisporus* products employed here—have been associated with increased animal health—with measurable changes in body weight and lifespan (37). Whether Trained Immunity underlies these changes remains unclear, but warrants future investigation.

In summary, we have demonstrated that powdered products of the common and edible white button mushroom *A. bisporus*, contain β-glucans with the capacity to drive Trained Immunity in innate immune cells *in-vitro*. This powdered form mimics the products of mastication, however simulated digestion preserves this property. Although the overall carbohydrate content is altered by simulated digestion, our NMR analysis suggests that β-glucans are retained. Oral administration of powdered *A. bisporus* products leads to features of Trained Immunity in mouse bone-marrow and derived mature macrophages. The approaches employed here provide both a rationale and mechanism to investigate further whether reprogramming of innate immune cells by mushrooms products enhances effector functions in these circumstances.

Methods

Mushroom powders and fractionation

All powders used in this study were from *A. bisporus* mushroom crops obtained from MBio, part of the Monaghan Mushrooms group as previously described (37). Powders were re-suspended in 1x PBS for cell culture and animal experiments and incubated

overnight rolling at 37°C to dissolve. Solutions were then passed through 20 μM filters and aliquoted for use. For fractionation studies, *A. bisporus* mushroom stalk powder (25 g) was suspended in deionised water (250 mL) and stirred at room temperature for 6 h. Supernatant (Cold Water extract, CW-E) was prepared using centrifugation. The residue from CW-E was re-suspended in water and heated to 60°C for 6 h. Supernatant (Hot Water extract, HW-E) was prepared using centrifugation. Thermal concentration of the HW-E supernatant following three consecutive hot water extractions was condensed by boiling and precipitated by diluting it 1:3 in ethanol. The residue from three hot water extractions was suspended in 1M KOH and heated to 60°C for 20 min. The pH of the extract was adjusted to 7.4 using HCl. Supernatant (KOH-E) was prepared using centrifugation. Supernatant was condensed by boiling. The pH of the extract was adjusted to 7.4 using HCl. The residue from the 1M KOH extraction was initially subjected to an acid hydrolysis using $NaNO_2$ and HCl to break linkages with chitin. The alkali-soluble "free" glucans were captured using 1M NaOH. The pH of the extract was adjusted to 7.4 using HCl (NaOH-E). Extracts were weighed and diluted from 1:1,000 to 1:10 for cell assays.

Simulated *in-vitro* digestion of mushroom powder

Simulated oral, stomach and intestinal digestion fluids were prepared as buffers as outlined in the published INFOGEST protocol (39). Enzyme activity for each digestive enzyme preparation (pepsin, gastric lipase, and pancreatin) was measured and concentrations used adapted according to Minekus et al. (82) such that pepsin (2,000 U/mL), gastric lipase (60 U/mL) and pancreatin measured by trypsin activity was 100 U/mL. One g of mushroom powder was dissolved in simulated oral digestion fluid for 2 h at 37°C without enzymatic digestion, since the mincing procedure mimics mastication. Gastric digestion fluid, buffer and enzymes were added to the oral bolus and digested for a further 2 h at 37°C with shaking. One hour prior to intestinal digestion, bile was added to solubilise. Pancreatin was prepared and the intestinal digestion carried out for 2 h at 37°C with shaking. After digestion, mushroom products were centrifuged at 200 rcf for 5 min. Pelleted residue was collected (*in-vitro* digested mushroom product) and subject to dialysis in deionised water (3–4 water changes). The content from dialysis bags was moved aseptically into petri dishes and subjected to freeze drying at −80°C resulting in the product referred to as *A. bisporus in-vitro* digested whole mushroom powder (IVD-WMP). For cell experiments, IVD-WMP powder was prepared similarly to WMPs described above, by resuspension in PBS, overnight shaking incubation, and filter sterilizing prior to use.

Cell stimulations

All PRR ligands (*E.coli*, ultrapure lipopolysaccharide, yeast Zymosan-A, Pam3CSK4, *L. digitata*-derived Laminarin, and heat-killed) *Mycobacterium tuberculosis* were purchased from Invitrogen. Yeast whole-glucan particle was a gift to FJS from Kerry Group (Ireland).

β-glucan and total carbohydrate quantification

β-glucan content of whole mushroom powder and IVD-WMP was determined using the β-Glucan Assay Kit (Yeast and Mushroom, Product code: K-YBGL). Briefly, total glucan content was measured by solubilizing all glucans after acid denaturation and digesting and oxidizing glucose units. α-glucans were measured after alkali hydrolysis with subsequent glucose oxidation and this value was subtracted from a value obtained for total soluble glucans obtained after acid denaturation, digestion and glucose oxidation. The *Mega-Calc*™ software tool was used for raw data processing and analysis.

Carbohydrate content of WMPs was measured using a resorcinol sulphuric acid method, based on a reported protocol (83). Briefly, 25 μL of sample solutions (250 μg/mL, 500 μg/mL) were pipetted in 96-well microtiter plate, to which 25 μL of a freshly prepared resorcinol solution (10 mg/mL) was added followed by addition of 105 μL of concentrated sulfuric acid with vigorous mixing. The plate was heated for 30 min at 90°C in oven and then cooled to room temp. in dark with regular shaking for another 30 min. Finally, the absorption of the resulting brownish orange color was measured by a BMG microtiter plate reader at $\lambda = 450$ nm, with $\lambda = 690$ nm as a reference wavelength. A calibration curve generated from glucose standards (conc. 10–1,000 μg/mL) was used to quantify the carbohydrate content.

Reporter cell line assays

HEK-Blue hDectin-1a and HEK-Blue hDectin1b ($3–7 \times 10^6$ cells) were cultured in growth medium (GlutaMAX™ DMEM, 10% FBS, 50 μg/ml Penicillin/Streptomycin, 100 μg/ml Normocin) with the addition of selective antibiotics (Puromycin, HEK-Blue™ CLR Selection). For reporter assays, cells were plated in flat-bottomed 96 well plates at 1×10^5 cells/well in 180 μL HEK growth medium, minus selective antibiotics. Cells were treated with 20 μL of PRR ligands/mushroom solutions and incubated at 37°C for 24 h. Cell supernatants were harvested for QuantiBlue SEAP quantification. SEAP activity was assessed by reading the optical density (OD) at 595 nm with a microplate reader.

Mouse bone-marrow derived macrophages culture and training assays

BMDM were isolated as described (84). After isolation and red blood cell lysis (Lysis Buffer Hybri-Max™, Sigma-Aldrich), bone marrow cells were resuspended in DMEM, 10% FBS, 20% L929-conditioned media and seeded to be differentiated into BMDMs. Five days after isolation, mature BMDM were lifted by placing on ice and reseeded at the required density (1×10^6 cell/mL) in DMEM, 10% FBS, 20% L929-conditioned media and allowed to rest

overnight. Six-days post isolation, re-seeded mature BMDM were incubated with training stimuli (WGP or WMPs) for 24 h. Seven-days post-isolation, media was removed and cells washed three times and incubated in fresh media (DMEM, 10% FBS, 5% L929-conditioned media) for a further 5 days, changing the media after 3 days. Six-days post training, BMDM were stimulated with PRR agonists and supernatant sampled at 3 h, 6 h, and 24 h time points for analysis of cytokine production. For inhibition experiments, 5′methylthioadenosine (MTA, 1 mM) was added to mature BMDM 2 h prior to addition of WMP. Control cells were treated with a similar volume of vehicle (DMSO/media).

Human monocyte derived macrophages and training assays

Buffy packs from human blood donations were obtained from the Irish Blood Transfusion Service, St James' Hospital under clinical indemnity. PBMCs were isolated using Lymphoprep and monocytes enriched by centrifugation through a percol gradient (Sigma-Aldrich), as described (18). Monocytes were seeded in 48-well plates at a density of 6×10^5 cells/mL in a volume of 500 μL per well and cultured in RMPI growth media supplemented with 10% human serum (Sigma-Aldrich). Twenty Four hours post isolation the cells were stimulated with training stimuli (WGP/WMPs). After a further 24 h, cells were washed again three times with 1X PBS (+Mg, + Ca) and fresh media was added. On day 6 post isolation, the hMDM's were stimulated with PRR ligands as described. Samples of the supernatants were taken at 3 h, 6 h, and 24 h time points for analysis of cytokine production.

Cytokine quantification

Supernatant cytokine concentration was determined using Invitrogen Uncoated ELISA kits (Thermo-Fisher) for mouse TNF (# 88-7324-88), IL-6 (#88-7064-77), and IL-10 (# 88-7105-88) as per manufacturer's instructions. After the final incubation with streptavidin-horseradish peroxidase conjugates, plates were washed seven times. Fifty μL of TMB substrate reagent was added to each well. The reaction was stopped with 25 μL of 1 M H_2SO_4. The plate was read using a microplate reader set to a wavelength of 450 nm. Microsoft Excel software was used to generate a standard curve from which the cytokine concentration of the samples was determined.

Metabolic analysis

For metabolic analysis of trained cells, lactate concentration was measured in supernatants using the colorimetric Lactate Assay Kit (MAK064, Sigma-Aldrich). Extracellular flux analyses were carried out using an XFe24 Extracellular Flux analyser (Seahorse Biosciences) in Seahorse Media freshly supplemented with 10 mM glucose and 2 mM l-glutamine. An adapted version of the XF cell mito-stress test was used to measure key parameters of both mitochondrial and non-mitochondrial function through the oxygen consumption rate (OCR) as well as analysis of the extracellular acidification rate (ECAR) of the media to investigate glycolytic flux. Cells were plated in the seahorse plate at 100, 000 cells per well for 24 h stimulation assays or 50,000 cells per well for 72 h stimulation assay. Cells were stimulated as previously described. For 72 h measurements, cells were washed with PBS and 200 uL after 24 h of stimulation and fresh cDMEM/5%LCM was added. On the day prior to measurement the calibration cartridge was hydrated with 200 μL of XF Seahorse calibration media and was placed in a non-CO2 incubator overnight at 37°C. On the day of the assay, cells were washed X2 with seahorse medium. Each well was then topped up with 180 μL of seahorse medium and the plate was placed into a non-CO2 incubator at 37°C for 20 min before the beginning of the Seahorse run. Following calibration and the cell culture plate was loaded for real-time analysis. During the run, the following inhibitors (diluted in seahorse media) were injection to interrogate metabolism oligomycin (2 μM), fluoro-carbonyl cyanide phenylhdrazone (FCCP, 1 μM), rotenone/antimycin-A (0.1 μM/4 μM), and 2DG (30 μM). Normalization for cell number was carried out with a Crystal Violet dye assay.

Oral delivery of WMP

For *in-vivo* WMP delivery, an oral bolus equivalent to 500 mg of WMP per kg mouse body weight was delivered resuspended in PBS in a maximum volume of 100 μL. Five hundred mg/kg equates to 10 mg WMP in a 20 g adult mouse. The equivalent human dose is 40 g in an 80 kg adult. Mice were given WMP oral gavage bolus once a day at morning-time for 7 days prior to sacrifice. Oral gavage was chosen as the optimal way to deliver the same amount of WMP to all mice via the gastro-oral route. Control mice received PBS. Bone-marrow cells were isolated and used to generate mature bone marrow derived macrophages. Mature BMDMs were stimulated on day 5 post isolation with LPS (10 ng/mL), Zymosan-A (ZYM, 10 ug/mL), Pam3CSK4 (PAM, 10 ug/mL) or heat-killed *Mycobacterium tuberculosis* (HKMTB, MOI 5) for 24 h. Supernatant was removed and the indicated cytokines measured by ELISA.

Multiparameter flow cytometry analysis of mouse bone marrow cells

To analyse HSPC populations in mouse bone marrow, HSPC cells were enriched using selection for c-Kit before immune staining and characterization flow cytometry. Isolated bone marrow cells from 1 femur per mouse were centrifuged and resuspended in 125μl of cold PBS. Twenty five μl of anti-CD117 (cKit) beads were added to 20 million bone marrow cells. Cells were resuspended and incubated on ice for 5 min. 1.5 μl of anti-CD117 APC flow antibody was added, gently vortexed and incubated on ice for 15 min in the dark. Five ml of cold PBS was added and cells were centrifuged at 450 G for 5 min at 4°. LS MACs column were activated with the addition of 2 ml of PBS. Cells were resuspended in 1 ml of PBS. Using an insulin syringe and a short 25G needle, cells were transferred onto the LS column. The column was then washed with an additional 1 ml of cold PBS. The column was removed from the magnet and 5 ml of cold PBS was added to the column. cKit

positive cells were detached from the column by plunger. cKit+ cells from MACs column were washed with 1 ml PBS. Cells were stained in 100 µl of zombie aqua (Live/Dead stain, concentration 1 in 1,000) for 20 min at 4°. Cells were washed with 1 ml of PBS and centrifuged at 450 G for 5 min. Ten µl of count beads were added to each sample and cells were then incubated with 50 µl of primary antibody master mix (Primary antibody master mix: concentration 1 in 100, biotin conjugated CD11b, B220, CD5, TER119, Ly6G/C, CD8a, and CD4—antibodies made up in PBS) for 30 min at 4°. Cells were then washed with 1 ml of PBS and centrifuged at 450 G for 5 min. Cells were then resuspended in 50 µl of secondary antibody master mix (Secondary antibody master mix: concentration 1 in 100 of CD34 FITC, Flt3 PE, CD48 PerCP-efl710, cKit APC, Streptavidin APC-Cy7, Sca-1 PE-Cy7, and CD150 efl450) for 30 min at 4°. Cells were washed with 1 ml PBS and centrifuged at 450 G for 5 min. Cells were fixed using 100 µl fixation buffer (Fischer Scientific) for 15 min at room temp. Cells were washed with 1 ml PBS and ran on BD FACs Canto. Fluorescence Minus One (FMO) controls were performed using 1×10^6 cells obtained by mixing equivalent volumes of samples coming from the different experimental conditions and stained with the proper antibodies. Compensation controls were obtained after staining UltraComp eBeadsTM Compensation Beads (Invitrogen) with the appropriate antibodies. Cells were acquired on the BD flow cytometer Canto II with FACSDiva software. Data analysis and flow charts were performed using FlowJo software v.7.6 (TreeStar).

NMR analysis

The homonuclear magnetic resonance experiments (^{1}H NMR, TOCSY) were performed on Bruker 400 NMR spectrometer operating at 400.23 MHz, while heteronuclear measurement (HSQC) was performed on Bruker Avance III 600 NMR spectrometer operating at 600.13 MHz and 150.6 MHz. due to low solubility of sample that further reduce the abundance of ^{13}C in soluble portion. A 10–15 mg of WMP and IVD-WMP were dissolved in 0.75 mL of deuterated water (99.95%, Sigma-Aldrich) and filtered through prewashed glass wool. ^{1}H, HSQC and TOCSY NMR data were recorded at 26°C. For ^{1}H NMR, acquisition parameters were as follows: 1,024 scans were recorded with an acquisition time of 2.04 s, relaxation delay of 1 s and spectral width of 8,012.8 Hz. For TOCSY, 128 scans were recorded with an acquisition time of 0.301 s, relaxation delay of 1 s and spectral width of 3,401.4 Hz. For HSQC measurements at 600 MHz instrument, 128 scans were recorded with an acquisition time of 0.095 s, relaxation delay of 1 s and spectral width of 5,411.3 Hz, 22,624.4 Hz. For NMR data processing, all the spectra were referenced to the solvent peak at 4.80 ppm. The spectra were processed and analyzed in MestReNova chemical suite software.

Data analysis and figure generation

All figures shown represent the mean of independent experiments carried out with a variety of mushroom powder batches manufactured between 2022–2023. For experiments with multiple groups, ANOVA was carried out followed by the indicated *post-hoc* individual tests and *p*-values determined for key comparators as indicated. Raw experimental data for each experiment was compiled and analyzed using Microsoft Excel. GraphPad Prism was used to pool results of replicate experiments and generate graphs. Figures were prepared using Adobe Illustrator.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Trinity College Dublin Faculty of STEM Research Ethics Level 2 Committee and Level 1 Sub-Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal studies were approved by Trinity College Dublin Animal Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

SCa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—review & editing. TO'B: Data curation, Formal analysis, Investigation, Writing—review & editing. AL: Formal analysis, Investigation, Methodology, Writing—review & editing. SCh: Investigation, Methodology, Writing—review & editing. CH: Investigation, Writing—review & editing. EH: Investigation, Data curation, Formal analysis, Methodology, Writing—review & editing. MO'S: Formal analysis, Investigation, Methodology, Writing—review & editing. HC-M: Investigation, Methodology, Conceptualization, Writing—review & editing. ED: Investigation, Methodology, Writing—review & editing. SY: Investigation, Methodology, Conceptualization, Formal analysis, Project administration, Resources, Supervision, Writing—review & editing. JW: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Funding acquisition, Validation, Writing—original draft. SCo: Funding acquisition, Methodology, Supervision, Writing—review & editing. SN: Methodology, Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Resources, Writing—original draft. FS: Conceptualization, Data curation, Project administration, Funding acquisition, Supervision, Writing—original draft.

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Conflict of interest

JW and SY are scientists at Monaghan Mushrooms who supplied the powdered mushroom products for this study and employed by MBio.

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

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Mitochondria: the gatekeepers between metabolism and immunity

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Metabolism and immunity are crucial monitors of the whole-body homeodynamics. All cells require energy to perform their basic functions. One of the most important metabolic skills of the cell is the ability to optimally adapt metabolism according to demand or availability, known as metabolic flexibility. The immune cells, first line of host defense that circulate in the body and migrate between tissues, need to function also in environments in which nutrients are not always available. The resilience of immune cells consists precisely in their high adaptive capacity, a challenge that arises especially in the framework of sustained immune responses. Pubmed and Scopus databases were consulted to construct the extensive background explored in this review, from the Kennedy and Lehninger studies on mitochondrial biochemistry of the 1950s to the most recent findings on immunometabolism. In detail, we first focus on how metabolic reconfiguration influences the action steps of the immune system and modulates immune cell fate and function. Then, we highlighted the evidence for considering mitochondria, besides conventional cellular energy suppliers, as the powerhouses of immunometabolism. Finally, we explored the main immunometabolic hubs in the organism emphasizing in them the reciprocal impact between metabolic and immune components in both physiological and pathological conditions.

KEYWORDS

immunometabolism, metabolic flexibility, mitochondrial function, mitochondrial dynamics, metabolic reprogramming

Abbreviations: ATP, Adenosine triphosphate; PGC1, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; STAT6, signal transducer and activator of transcription 6; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid; mTOR, mechanistic target of rapamycin; SREBPs, sterol regulatory binding element proteins; HIF-1α, hypoxia inducible factor-1α; FAO, fatty acid oxidation; DAMPs, damage-associated molecular patterns; TFAM, mitochondrial transcription factor A; mtROS, mitochondrial ROS; TNF-α, tumor necrosis factor-α; MAVS, mitochondrial antiviral signaling protein; MFNs, mitofusins; OPA1, Optic-atrophy-1; Drp1, Dynamin-related protein-1; NET, neutrophil extracellular traps; ETC, electron transport chain; FFAs, free fatty acids; LSECs, liver sinusoidal endothelial cells; IFN-I, I interferon; PBMCs, Peripheral blood mononuclear cells; SCFAs, short chain fatty acids.

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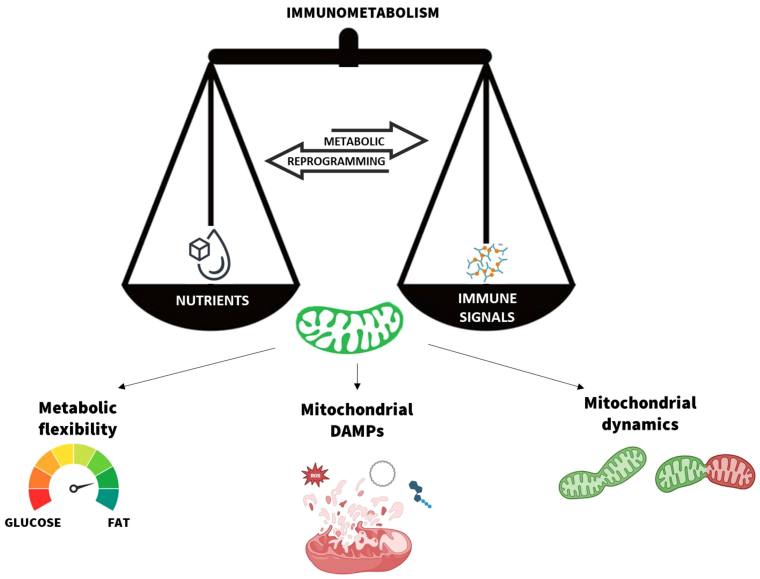

GRAPHICAL ABSTRACT
Mitochondria are the scale needle of the immunometabolism. The metabolic flexibility, mitochondrial damage-associated moleuclar patterns (DAMPs) release, and dynamics are the main processes that modulate the mitochondrial function in response to nutrients and immune signals. Their dynamic balance ensures the body's immune and metabolic homeostasis.

1 Introduction

The rules of human survival teach us that: i) we cannot be unarmed against the pathogen attack and ii) nothing survives without nourishment. The "immunometabolism" defines the portal between immunology and metabolism, two trials which our organism trusts to maintain a state of wellness. These processes are inextricably linked and the interfaces between the immune and metabolic systems mediate the whole-body homeodynamics. The crosstalk between these two major balancers of the body health has multiple facets (1). The immune system continually perceives and reacts to pathogenic or environmental dangers with secretion of cytokines, chemokines, and inflammatory mediators by the innate immune cells, and with the proliferation of adaptive immune cells. These processes are bioenergetically expensive and need an accurate control of cellular metabolic pathways (2). The immune response requires the reallocation of nutrients within immune cells in order to: provide the substrates for ATP production serving to sustain the functions of activated immune cells; and build blocks for the production of necessary macromolecules for the proliferation of immune cells. The cellular metabolic reprogramming, that help to regulate specific immune cell functions, is an aspect of immunometabolic research which has already been to some extent explored (3). Indeed, the concept that metabolism influences cellular functions and fate may seem obvious, but taking this step backwards is the appropriate approach for a detailed understanding of the immunometabolic mechanisms, consequently useful to design effective strategies to ensure the health of the organism. The feature of this review is the establishment of a thread which examines the several facets of the immunometabolism, starting from the intersection mechanisms of the mitochondrial metabolism with the functionality of the immune cells. Then, we highlighted the mitochondrial dynamics processes in the activation phases of immune cells, and the immunometabolic regulation in different organs and tissue in both physiological and pathological conditions.

2 Metabolic adaptations of immune cells

The cells of the immune system are arguably the most dynamic components of our organism, they need to function in different contexts, including those where the availability of nutrients is restricted or compromised (4). Immune cells possess a broad set of skills ranging from being sleeping sentinels to inducing clonal expansion, modulating surface receptor expression and secreting large amounts of effector molecules (5, 6). The performance of these distinct functional activities is tightly dependent by the metabolic flexibility of these cells (7). Indeed, recent findings have demonstrated that peripheral immune cells can adapt to environmental shifts by metabolizing alternative non- glucose substrates, such as amino acids or fatty acids (8–15). A system in which nutritional and energy inputs are properly processed and substrate utilization is properly regulated is defined as a metabolically sensitive and flexible system. The mitochondrial machinery is responsible for switches in the oxidation of substrates, and the choices are orchestrated by an intricate network of cell signaling events. This metabolic flexibility enables peripheral immune cells to perform a multitude of functions in disrupted environments where the availability of carbon sources varies (16).

In the 20th century, Warburg was the first to launch the immunometabolic research describing the metabolic changes and aerobic glycolysis in cancer cells (17). Recent research has highlighted that each subset of immune cells has a different metabolic control and nutrient utilization. Vats et al. highlighted the molecular pathway that directly links mitochondrial oxidative metabolism to the anti-inflammatory program of macrophage activation, identifying peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and signal transducer and activator of transcription 6 (STAT6) as metabolic regulators that can control macrophage activation (18). To date, increasingly detailed experiments have shown that naïve T and B cells dynamically switch their metabolic programs upon activation. Several studies highlighted signaling and transcriptional networks that regulate metabolic state to tune the management of T cells fate (19). The results converge on the increase of the mitochondrial metabolism and oxidative phosphorylation (OXPHOS) (20–22) and on the induction of anabolic metabolism in T cells, specifically, evidencing that this occurs during the period of quiescence exit in parallel with higher glucose uptake and lactate production (23). Glutaminolysis is an important pathway in these cells, given that glutamine replenishes tricarboxylic acid (TCA) cycle intermediates as they are picked up for biosynthesis (15). These events are mediated by the mechanistic target of rapamycin (mTOR)-associated signaling, in part, by upregulating transcriptional programs mediated by MYC (24), sterol regulatory binding element proteins (SREBPs) (25), and hypoxia inducible factor-1α (HIF-1α) (26–28). T_{reg} cells and memory T cells have a dissimilar metabolism from their activated effector T cells counterparts, indeed, for their development and persistence depend on mitochondrial fatty acid oxidation (FAO) rather than aerobic glycolysis (29).

The interaction between nutrients, metabolic programs and signaling pathways has recently well described also in naïve B cell activation, differentiation and fueling of the antibody secretion machinery. Several studies have found that glucose uptake is increased upon B cells activation (30–33). It was also demonstrated that the initial B cell activation induces metabolic reprogramming, with increased glucose uptake without accumulation of glycolytic metabolites, suggesting that glucose is fluxing through the glycolytic pathway and is probably directed to alternative metabolic pathways in activated B cells. Indeed, stimulated B cells increase programs for OXPHOS, the TCA cycle, and nucleotide biosynthesis (34). The signaling and nutrient sensitive mechanisms that mediate B cell activation and differentiation and the function of energetic and biosynthetic pathways were widely reviewed (35). Several experiments have shown that the functional destination (tolerance, effector or regulatory activities) of B lymphocytes dictates the choice of their metabolic programming, also depending on the receptors and the co-activation molecules stimulated (36). Indeed, glucose restriction did not affect B cell functions, whereas OXPHOS inhibition or glutamine restriction significantly impaired B cell growth and differentiation (34). Metabolic restrictions (such as low ATP reserve and mitochondrial mass, or transcriptional repression of glucose transport and limited activity of the pentose phosphate pathway) provide a safeguard against autoreactive or premalignant B cells (37). This may happen through hyperactivation-induced metabolic stress, such as ATP deprivation and oxidative damage. Meanwhile, prolonged exposure to nutrient oversupply subverts B cell gatekeeper functions, promotes malignant B cell transformation and progression of autoimmune disease (38).

Notable, the metabolic availability influences immune cell fate decisions also through its impact on the epigenome (39, 40). Indeed, several metabolic checkpoints exist to limit epigenetic instability and restrain B cell development (7). Compelling evidence demonstrated that metabolic intermediates are ideal signaling mediators. Their levels are in dynamic equilibrium with systemic, microenvironmental and cell-intrinsic cues, whereby fluctuations inform cell fate decisions (41). They can both inform the fitness of extracellular conditions and integrate this sensing into the epigenome, serving as cofactors for chromatin remodeling enzymes (42). Metabolic-dependent epigenetic reprogramming might explain why changes in cellular metabolism are crucial for multi-stage B cell specification. Lastly, the metabolic state influences immune cell function not only through epigenetic remodeling but also through a restructuration of intracellular architecture, this will be discussed in more detail below in a dedicated section.

3 Immunometabolic skills of the mitochondria

In recent years, the scientific research has produced new striking knowledge of mitochondrial function in metabolism, leading to consider the mitochondria as targets for the development of new therapeutic approaches. Alongside the paradigm widely described in biochemistry textbooks, which defines these organelles as ‘energy powerhouses’ of the cell, a new image has emerged of the mitochondria as a ‘Pandora’s box’, an intracellular ‘container’ crucial not only for the life but also for the cell death (43, 44). For this reason, it is essential to consider their expertise in immunometabolic management.

Mitochondria represent the most ancient endomembrane system in eukaryotic cells. They arose around two thousand million years ago and, over the years, mitochondria have continuously demonstrated their autonomy and ability (45). In 1907, they were defined as cellular organelles responsible for the functions of respiration and energy production (46). Around the 1950s, Kennedy and Lehninger discovered that the TCA, FAO and OXPHOS take place in the mitochondria (47). In 1967, Margulis revived the long-forgotten endosymbiont theory on the origin of organelles (48). Since the 1970s, the mechanism of mitochondrial biogenesis has been elucidated and it is recognized that mitochondria are semi-autonomous organelles, capable of synthesizing 5% of the proteins they require and importing the rest from cytoplasmic synthesis (49). Morphologically, like their bacterial ancestor, mitochondria consist of two separate and functionally distinct outer and inner membranes that enclose the intermembrane space and matrix compartments. The architecture of mitochondria is essential for their proper functioning and also for the containment of immunogenic molecules derived from mitochondria (50, 51). They also contain the mitochondrial

DNA (mtDNA), a circular genome, which has been reduced in the course of evolution through gene transfer to the nucleus. The bacterial-like characteristics of mitochondria also reinforce the idea that they are hubs of immunity (52). The proteins present in mitochondria are structurally similar to those in bacteria and allow them to be recognized by the same receptors as the immune system (53). To date, as already hypothesized by Altmann in 1890, the main role of mitochondria is to provide metabolic energy in all eukaryotic cells (54). However, these organelles orchestrate mechanisms which directly impact cell fate and fitness, so to consider them trivially 'powerhouses of cells' would be limiting, to say the least. Indeed, it is well known that the metabolic functions of mitochondria reach far beyond bioenergetics. Additionally to their exclusive ability to carry out the OXPHOS, these organelles participate in intermediary metabolism, regulate programmed cell death, calcium homeostasis, and control the production of reactive oxygen species (ROS) (55–57).

A proper mitochondrial functionality is fundamental for the cellular homeostasis. In this regard, several molecules extruded from mitochondria alert neighboring cells, the immune system (58), and the producing cell itself about mitochondrial dysfunction (59). Several studies demonstrated that mitochondrial ROS also contribute to adaptive stress signaling pathways, such as hypoxia and control cell proliferation and differentiation (60, 61). Likewise, the levels of nitric oxide (NO), another by-products of mitochondrial respiratory activity, act as initiators through which mitochondria modulate signal transduction pathways implicated in the induction of cellular defense mechanisms and adaptive responses (62). Mitochondria are also the source of molecules, including proteins, lipids, metabolites and mtDNA, collectively named damage-associated molecular patterns (DAMPs). These DAMPs are endogenous danger molecules that are released from damaged or dying cells and activate the innate immune system by interacting with pattern recognition receptors. The DAMPs, when imbalanced, employ immunogenic capacity in immune and non-immune cells (63). ATP, succinate, cardiolipin, N-formyl peptides (NFPs), mtDNA and mitochondrial transcription factor A (TFAM), are examples of DAMPs that serve as danger flags for immunological signaling (63). The secretion of succinate triggers pro-inflammatory differentiation of T-lymphocytes (64) and have synergic effects with Toll-like receptors ligands in dendritic cells for the production of cytokines. The succinate is a regulator of inflammation, in M1 macrophages due to a break point of Krebs cycle it was observed its accumulation, and demonstrated a prominent proinflammatory activity and roles in immunity (65). High levels of extracellular ATP signals induces release of pro-inflammatory cytokines, inflammasome activation (66), neutrophils degranulation, apoptosis and ROS release through P2X receptors (67, 68). The exposition of cardiolipin to the extracellular media is associated with increased apoptosis and autophagy: the cardiolipin can bind directly to Nod-like receptor 3 (NLRP3) and activate inflammasome-mediated immune response (69). Moreover, cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in mammalian cells (70), this process is facilitated by the activated Gasdermin D permeabilization of mitochondrial membranes that cause rapid, cardiolipin-dependent mitochondrial destruction (71). N-formyl peptides are extruded by the mitochondria of damaged or dying cells, they act as chemoattractant of neutrophils via formyl-peptides receptors (72). Extra mitochondrial mtDNA has been widely shown to induce a proinflammatory state (73, 74), its binding to TLR9 induces proinflammatory cytokines production, chemotaxis and phagocytic activation via a MyD88-dependent signaling cascade (75). TFAM enhances the immunogenicity of mtDNA (76), TFAM is recognized by the receptor for advanced glycation end products, which guides TFAM-mtDNA complexes to the endosomal pathway, also, TFAM enhances cytokine secretion in combination with NFPs (77). mtDNA may also amplify the activation of NLRP3 by mitochondrial ROS (mtROS) (78). ROS are a consequence of mitochondrial disruption in both M1 macrophages (40) and effector T cells and control adaptive immune-cell activation. It is noteworthy that ROS are also guiding signals in the production of inflammatory cytokines. T and B cells require ROS production to trigger an adequate immune response. T cell activation induces a spike in mtROS production, and blockade this process neutralizes IL-2 production by T cells (79). B cell activation is also managed by mtROS. The activation of B cell-surface receptors stimulates in turn the calcium release into the cytoplasm, which promotes ROS production, this cooperative interaction acts in a feedback manner to amplify the early signal generated (80). An interesting research showed that isolated human monocytes exposure to mtDAMPs generated significantly less interleukins IL-1β, IL-6, IL-12-p70 and tumor necrosis factor-α (TNF-α) upon lipopolysaccharide challenge when compared to their untreated counterparts, leading to speculate to the induction of a transient state in which these cells are refractory to further endotoxin stimulation (81). Further studies will be crucial to understanding the role of this phenomenon, that could be the root of the onset of noncommunicable chronic diseases, typified by mitochondrial dysfunction and disruption of the immune system. Recently, the intermediate role of mitochondria in toll-like receptor-mediated innate immune responses and in the activation of the NLRP3 inflammasome complex has highlighted, supporting the striking functions of mitochondria in innate immunity (82). Indeed, besides being DAMPs producers, mitochondria are also linked to immunity through their role as innate immune platforms that host the mitochondrial antiviral signaling protein (MAVS) as a viral RNA sensor and the inflammasome NLRP3 as a multiple immunogenic receptor (63).

Understanding how non-immune cells respond to DAMPs released following mitochondrial harm and the mechanisms implicated in these responses are among the main targets of recent researches (63). Understanding the conditions under which damage to non-immune cells leads to chronic and systemic inflammatory responses is relevant. This is discussed in a later section.

4 Mitochondrial dynamics drive the immunometabolic pathways

The multifaceted contributions of mitochondria to cell metabolism as bioenergetic powerhouses, biosynthetic centers, ROS production managers and waste management hubs is

undisputed (83). Unequivocally, mitochondria perform a plethora of cellular functions besides energy production (84). It is equally clear that the fate and function of innate and adaptive immune cells depends crucially on mitochondrial bioenergetics (58). Exciting evidence demonstrated that mitochondria constantly change their morphology depending on the cell's metabolic requirements, highlighting reciprocal crosstalk between mitochondrial dynamics and metabolism (85, 86).

Mitochondrial dynamics refers to the formation of a dynamic network involving a continuous alternation of fusion and fission/division processes in order to maintain their cellular abundance, morphology, quality and function control (87). Mitochondrial fusion and division typically counterbalance each other. Three proteins that control mitochondrial fusion and division have identified: i) mitofusins (MFNs) (outer mitochondrial membrane fusion), ii) Optic-atrophy-1 (OPA1)/mitochondria genome maintenance 1 (inner mitochondrial membrane fusion), and iii) Dynamin-related protein-1 (DRP1)/Dynamin-1 (division of outer and inner mitochondrial membranes) (88). Mitochondrial fusion is the physical merging of the mitochondrial membranes of two originally distinct mitochondria. Mitochondrial division is the separation of a single organelle into two or more independent structures. These two active and combined effects originate the mitochondrial networks. Several studies performed in metabolic tissues, such as the liver, the skeletal muscle and the central nervous system demonstrated that the unbalance in mitochondrial fusion/fission dynamics cause cell and tissue dysfunction and altered metabolic homeodynamics (89–92). A decade ago, it was already proven that deletion of any of the dynamics machinery perturbs OXPHOS and glycolytic rates at baseline (93). Tissue-specific deletion of mitofusin-2 (Mfn2) in muscles of mice disrupts glucose homeostasis (94), and Drp1 ablation in the liver results in reduced adiposity and elevated whole-body energy expenditure, protecting mice from diet-induced obesity (95). The evidence that the alterations in fusion/fission machinery alter the mitochondrial function and with it the cell function holds true across various tissues, including the immune system. Indeed, mitochondrial dynamics are a critical control point also for immune cell function (96).

In several immune cells, including neutrophils, macrophages, mast cells, and T- and B-cells, mitochondria adapt specific mitochondrial morphologies according to the cellular activation state (78, 97, 98). In LPS-activated macrophages the inhibition of mitochondrial fission, through the Drp1 inhibitor Mdivi-1, reduce glycolytic reprogramming that these cells implement to achieve polarization into a proinflammatory M1 state (97). The mitochondrial dynamics impact also on the role of the mitochondrial membrane as a signaling platform. Indeed, the depletion of Mfn1/2 or Opa1 reduced MAVS-driven innate antiviral signaling in a mitochondrial membrane potential-dependent manner (99, 100). In neutrophils, the mitochondrial fusion is implicated in the formation of neutrophil extracellular traps (NET), the deletion of OPA lead to a decrease of ATP levels which is fundamental for microtubule network assembly and NET formation (101). Also in human mast cell immune response was investigated the role of mitochondrial dynamics revealing that degranulation processes and secretion of preformed TNF are regulated by Drp1 activation (98). The different roles of T-lymphocytes as T effectors (T_e) and T memory (T_m) cells impose them large changes in ATP demand and nutrient utilization. T_e cells promote aerobic glycolysis to sustain anabolic pathways of metabolism, while T_m cells engage catabolic pathways, like FAO, and these metabolic differences are reflected in mitochondrial morphology (96). Memory T cells have more fused mitochondrial networks suggesting a requirement for mitochondrial fusion in memory T cell metabolism and homeostasis. Indeed, the fusion protein OPA1 is required for tight cristae organization in T_m cells, facilitating efficient electron transport chain (ETC) activity and favorable redox balance, its deletion caused defects in T_m survival (97). While, more fragmented mitochondria (fission process, with low expression of the fusion proteins MFN2 and OPA1 and high levels of active DRP1) were observed in T_e cells, leading to punctate mitochondria, cristae expansion and reduced ETC efficiency which promote aerobic glycolysis (97, 102). In T cells the processes of IL-2 production and immune synapse formation are dependent on mitochondrial fission (103, 104). Indeed, activated T cells show an increase in the production of mtROS, required in the activation of the transcription factor NF-kB, which transcribes IL-2. Inhibition of DRP1 by Mdivi-1 reduced IL-2 mRNA levels and T-cell proliferation (96).

Mitochondrial fission also occurs during B-cell activation, while naïve B cells have predominantly elongated mitochondria. Activated B cells increase glucose uptake, TCA cycle and OXPHOS and have fragmented mitochondria, while naïve B cells maintain a predominance of elongated mitochondria (30, 34). These findings are similar to those noted in T cells; however, it was found that naïve B cells have significantly fewer mitochondria in comparison to naïve T cells (96, 105). It seems that B cells use the mitochondrial remodeling as a key mechanism to control the optimal function of these few mitochondria to compensate this restriction in the number and volume of mitochondria and ATP reserves (106). B cells predominantly favor mitochondria fission and thus house smaller, less functional mitochondria with limited capacity for oxygen consumption and ATP production (34, 107). However, a recent study demonstrated that T cell–dependent activation of murine B cells not only temporarily increased metabolic activity (e.g., glucose uptake and glutamine consumption) but also increased mitochondria number through fission in the absence of mtDNA replication (34).

These exciting evidence highlight that mitochondria are tightly interlaced with metabolic and immune cell homeostasis, it follows that the proper function of mitochondria is crucial to ensuring the health of the organism. From now on, talking about mitochondrial-driven immunometabolic homeostasis would not be a hazard.

5 Immunometabolism in health and disease: the main immunometabolic hubs in the organism

Metabolic homeostasis and immune function are pivotal requirements at the root of systemic health monitoring. The crosstalk between immune and metabolic processes is coordinated

by communication circuits between specialized tissue-resident cells and organs that include messenger molecules such as hormones, neurotrophic peptides, cytokines, and metabolites (108–112). The intervention of these components of systemic immunometabolism underlie the impact of the metabolism on systemic inflammation and vice versa, both in health and in disease (Figure 1).

Nutritional habits are key determinants of body composition and systemic metabolism (113). Meantime, it is well known that malnutrition leads to inflammation and influences systemic immune responses (114, 115). The unbalance between the body energetic management (metabolism) and the ability to defend itself against pathogens (immune response) has critical implications for the occurrence of a wide range of chronic noncommunicable diseases, including obesity, diabetes, cardiovascular pathologies and cancer (108). Indeed, it has been well established that chronic inflammation is the trigger of the above-mentioned diseases (108) and, recently, the immunological adaptations in response to nutritional status have been highlighted. Undernutrition impairs immunity, causing inefficient responses to infections and vaccinations. Conversely, the metabolic overload in obesity can affect immunometabolism favoring chronic activation of both innate and adaptive immune cells, with subsequent low-grade systemic inflammation and altered susceptibility to autoimmune diseases (116). A thorough understanding of the intracellular network and intercellular correlations that regulate immunometabolism systemically is quite complex. For this reason, below we attempt to trace the main immunometabolic hubs of the organism.

5.1 Adipose tissue

The adipose tissue was identified as an important immune cell niche during homeostasis and an important immune-metabolic communication hub in metabolic syndrome (108, 117–121). Indeed, a wide range of immune cells are accumulated in the adipose tissue in the course of diet induced-obesity influencing the systemic metabolism (122, 123). The macrophages accumulate within adipose tissue produce the inflammatory cytokines IL-6 and TNF-α (124, 125). Locally, IL-6 can induce lipolysis in neighboring adipocytes and impair lipoprotein lipase, decreasing adipocyte lipid depot. The high circulating levels of IL-6 and free fatty acids (FFAs)

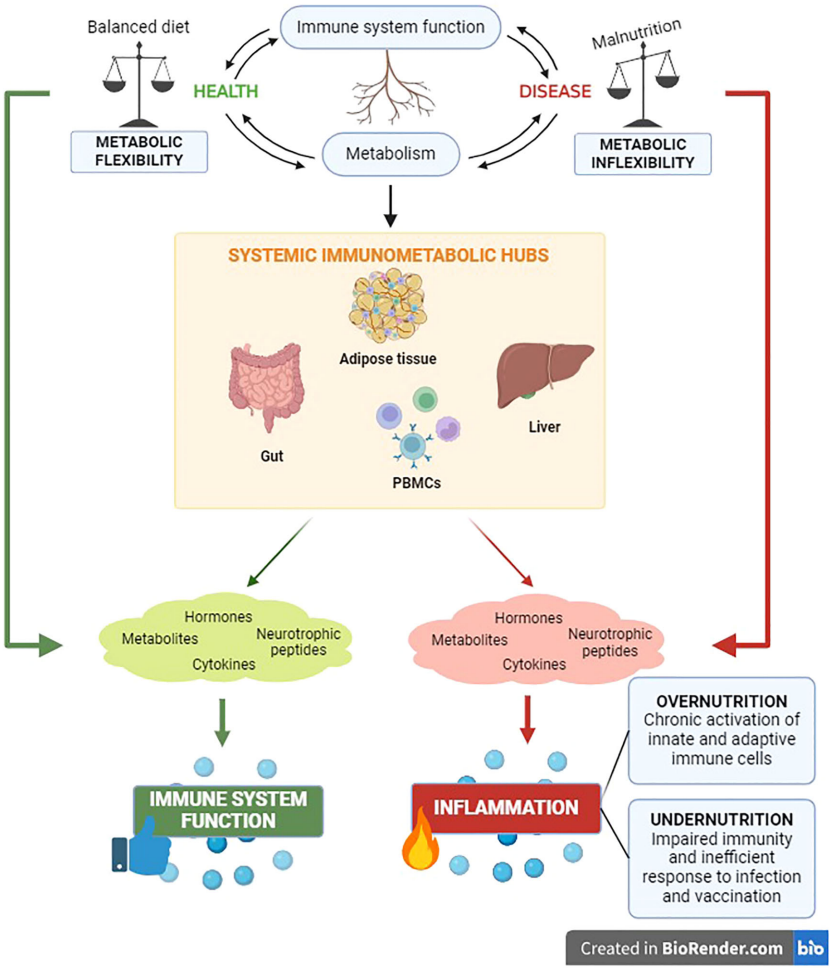

FIGURE 1
The immunometabolic control has significant clinical relevance for the health status of the organism. The reciprocal impact between metabolic and immune components unveils in the main body's immunometabolic hubs in both physiological and pathological conditions.

promote insulin resistance and immune cell function alterations affecting both local and distal tissue microenvironments. Moreover, the accumulation of FFAs promotes ROS production in macrophages, which in turn augments activation of the NLRP3-ASC inflammasome (126), and peripheral insulin resistance, mediated by the secretion of the IL-1β. When inflammation persists, like in obesity and in non-communicable diseases, prolonged disruption of metabolic homeostasis could lead to immune cell dysfunction and dysregulated systemic metabolism. In this context, we are faced with 'sterile metabolic inflammation' that consists of persistent inflammation in the absence of infection (127). Here, the bilateral crosstalk between aberrant metabolism and immune regulation is disrupted and results not only in disease progression but leads also to immune senescence (128). Buck et al. have extensively reviewed the metabolic instruction of immunity and attempted to shed light on how feeding behaviors can also affect host immune fitness, but the relationship between the variety of factors that influence the systemic metabolism and immune cell activity is only beginning to be explored (7).

Recently, several lines of evidence on why the metabolic overload from obesity blunt the immune system and increases the vulnerability to infectious and autoimmune diseases have been reported (116, 129). Firstly, a functional impairment of both the innate and adaptive immune system is attributable to chronic low-grade inflammation that lead to impaired chemotaxis, dysregulated production of pro- and anti-inflammatory factors and altered macrophages differentiation (130). This disrupts the delicate balance of adipose tissue between its function of immunologically active adipocytokines-producing organ, and the action of the latter on affecting adipocyte homeostasis and metabolism (131, 132). Moreover, the altered immunometabolism in obesity could lead to autoimmunity. The immune cell differentiation may be impaired in obese people due to excessive stimulation of nutrient- and energy-sensing pathways (such as increased mTOR activity) in immune cells, with consequent increase of proinflammatory TH1 and TH17 cells and decreases Tregs, which increase the risk of self-tolerance lack (116). Lastly, exciting research demonstrated that the increased adiposity observed in obese people increase also the bone marrow adiposity (133), resulting quickly in a profound hematopoietic insult with reduction of lymphocyte population and compromised immune function (134–136).

5.2 Gut

The gut can be considered the main immunometabolic interface of the organism representing a barrier surface, where a single layer of epithelial cells is the main mediator of crosstalk between gut microbes in the lumen and host cells, including immune cells in the lamina propria (DCs, macrophages, innate lymphoid cells, and T cells). Peyer's patches are scattered along the epithelium which house germinal centers that maturate IgA-secreting B cells with the help of follicular helper T-cell help. B cells augment glycolysis upon activation and depend on pyruvate import for longevity as long-lived plasma cells (7). The epithelial cells and intraepithelial lymphocytes in the gut coordinate the tightly regulated immune responses, implemented for both avoiding detrimental responses to commensals or food antigens and adequately respond to pathogens (137). It is noteworthy the role played by the short-chain fatty acids (SCFAs), metabolites produced by commensal bacteria able to influence B cell metabolism and boost antibody responses in both mouse and human B cells, promoting Ig A secretion (138). The presence of SCFAs and vitamins support the maintenance of barrier function by promoting the development and survival of Tregs and innate lymphoid cells. Noteworthy, homeostatic immune-driven signals secreted by gut resident immune cells (e.g., IL-10) may also mediate their effects through metabolic modulation (7). Indeed, it was demonstrated that the deficiency of the pleotropic anti-inflammatory cytokine IL-10 in macrophages is sufficient to recapitulate the onset of severe colitis in mice (139). Possibly, the metabolic shift towards to aerobic glycolysis during innate immune cell activation is the explanation of the anti-inflammatory activity of the IL-10.

5.3 Liver

The liver can be defined as the immunometabolic controller of the organism. The central role played by the liver in the immune-metabolic homeostasis being well recognized (140). The function of the liver as the main metabolic organ inevitably exposes it to newly produced neo-antigens, enhancing the risk of overactivation of components of the immune system with potentially harmful consequences for hepatic cell homeostasis (141). Several evidence point out the importance of the liver as "regulatory system" where different immune and non-immune cell populations work together in order to protect the host from antigenic overload of dietary components and drugs derived from the gut, facilitating tolerance rather than immunoreactivity (141, 142). Indeed, the immune cells coexist in a close symbiotic manner to support the hepatic metabolic functions (143). In the liver, naïve T cells recirculating within the sinusoids make direct contact with sinusoidal cells, such as liver sinusoidal endothelial cells (LSECs) or Kupffer cells (144). Gut-derived food antigens are picked up by Kupffer cells, LSECs, and liver dendritic cells and presented to naïve T cells, leading to immune tolerance of both CD8+ T cells and CD4+ T cells (145). In addition, compelling evidence demonstrated that virus-induced innate immune responses in hepatocytes are mediated by the antiviral cytokine type I interferon (IFN-I) that apart from inducing an antiviral state, rewires cellular metabolism of innate immune cells to boost the production of immune-modulatory metabolites (146–148) and modulates cellular redox homeostasis and central metabolic pathways in hepatocytes (113, 149–151). Moreover, the portal blood delivers to the liver numerous factors derived from gut and visceral adipose tissue (e.g. pro-inflammatory cytokines, lipids and bacteria-derived factors, such as endotoxins) (152) that seem to be critical in the systemic and central inflammation (153). These endocrine and immune mediators, in turn, can modulate the hepatic metabolism by influencing the bioenergetic regulation of hepatic mitochondria. Conversely,

inflammation-induced metabolic reprogramming of hepatocytes can influence systemic energy metabolism (154). These data highlight the important role of the liver as central modulator of systemic immunometabolism and strengthen the bidirectional cause-effect relationship between mitochondrial metabolic stress and immune regulation.

5.4 Peripheral blood mononuclear cells

PBMCs are immunometabolic sentinels of the organism. PBMCs are circulating cells able to sense and respond to systemic metabolic and inflammatory stressors. They circulate continuously throughout the body in the bloodstream, and are subject to changes in blood composition, including those related to fluctuations in nutrients, substrates and hormones (155, 156). PBMCs can be defined sentinel cells able to respond either to internal signals (such as hormones) or external ones (such as nutrients) and to reflect energy metabolism of internal tissues with which they interact, as well as their gene expression profile. In addition, these cells contain respiring mitochondria and, therefore, are a functional biomarker in translational bioenergetics (157). For this reason, PBMCs represent a suitable system to study changes in cell metabolism and to control the management of immune surveillance (156). Moreover, since several researches in animal models demonstrated that PBMCs can reflect the metabolic framework that cannot or can hardly be sampled in humans, such as liver and brain (158–160). These cells can be used as a surrogate tissue to monitor nutritional responses and provide predictive disease risk markers (161).

6 Discussion and conclusions

This review has highlighted that immunometabolic control has significant clinical relevance to the health status of the organism. Furthermore, it has left no doubt that mitochondria are the main players in this fine-tuning between metabolism and immune function, finding the metabolic flexibility of immune cells and the mitochondrial dynamics processes to be the secrets of appropriate immunometabolic homeostasis (see also graphical abstract). In particular, we underlined the importance of the metabolic flexibility of immune cells, exploring the role of their metabolic pathways and how this regulates the outcome of the immune response. On the one hand, it is well established that initial T and B cells activation, during the period of quiescence exit, lead to increased glucose uptake and promote aerobic glycolysis (23). Then, effector T and B cell subsets display differences in metabolic activities on the basis of their subsequent functional specialization (23). T_{reg} cells and memory T cells revert to a catabolic state and rely mainly on mitochondrial fatty acid oxidation (162). Also metabolic programs of activated B lymphocytes change depending on their functional destination (tolerance, effector or regulatory activities) and on the receptors and co-activation molecules stimulated (36). In addition, we explored the ability of mitochondrial DAMPs to employ the immunogenic capacity in immune and non-immune cells, and the significant relevance of mitochondrial ROS production in the trigger of an adequate immune response (163, 164). We traced the thread that leads from malnutrition to metabolic inflexibility in immune and non-immune cells, with consequent systemic meta-inflammation and disruption of the immune system. At once, a proper metabolic regulation supports immune cell activities in physiological contexts, while dysregulated immunometabolism contributes to pathophysiology. In the last chapter of the review, we explored and discussed the intricate intracellular networks and intercellular correlations in the main immunometabolic hubs of the organism. We highlighted as the interference of mitochondrial dysfunction (unbalanced DAMPs and ROS production and metabolic inflexibility) influence also non-immune components and lead to chronic and systemic inflammatory responses, typical features of non-communicable diseases.

For this reason, it is advisable to pursue a constant and in-depth exploration of immunometabolism, both in the detailed molecular pathways involved and with an interdisciplinary approach. The aim is to identify mitochondrial targets useful for the development of new intervention therapies that could help reduce the global burden of metabolic, inflammatory and autoimmune diseases.

Author contributions

GT: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. FC: Investigation, Writing – review & editing, Writing – original draft, Visualization. AC: Writing – original draft. GC: Writing – original draft. MM: Conceptualization, Project administration, Validation, Writing – review & editing.

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Conflict of interest

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

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Polyphenols: immunonutrients tipping the balance of immunometabolism in chronic diseases

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Mounting evidence progressively appreciates the vital interplay between immunity and metabolism in a wide array of immunometabolic chronic disorders, both autoimmune and non-autoimmune mediated. The immune system regulates the functioning of cellular metabolism within organs like the brain, pancreas and/or adipose tissue by sensing and adapting to fluctuations in the microenvironment's nutrients, thereby reshaping metabolic pathways that greatly impact a pro- or anti-inflammatory immunophenotype. While it is agreed that the immune system relies on an adequate nutritional status to function properly, we are only just starting to understand how the supply of single or combined nutrients, all of them termed immunonutrients, can steer immune cells towards a less inflamed, tolerogenic immunophenotype. Polyphenols, a class of secondary metabolites abundant in Mediterranean foods, are pharmacologically active natural products with outstanding immunomodulatory actions. Upon binding to a range of receptors highly expressed in immune cells (e.g. AhR, RAR, RLR), they act in immunometabolic pathways through a mitochondria-centered multi-modal approach. *First*, polyphenols activate nutrient sensing via stress-response pathways, essential for immune responses. *Second*, they regulate mammalian target of rapamycin (mTOR)/AMP-activated protein kinase (AMPK) balance in immune cells and are well-tolerated caloric restriction mimetics. *Third*, polyphenols interfere with the assembly of NLR family pyrin domain containing 3 (NLRP3) in endoplasmic reticulum-mitochondria contact sites, inhibiting its activation while improving mitochondrial biogenesis and autophagosome-lysosome fusion. *Finally*, polyphenols impact chromatin remodeling and coordinates both epigenetic and metabolic reprogramming. This work moves beyond the well-documented antioxidant properties of polyphenols, offering new insights into the multifaceted nature of these compounds. It proposes a mechanistical appraisal on the regulatory pathways through which polyphenols modulate the immune response, thereby alleviating chronic low-grade

inflammation. Furthermore, it draws parallels between pharmacological interventions and polyphenol-based immunonutrition in their modes of immunomodulation across a wide spectrum of socioeconomically impactful immunometabolic diseases such as Multiple Sclerosis, Diabetes (type 1 and 2) or even Alzheimer's disease. Lastly, it discusses the existing challenges that thwart the translation of polyphenols-based immunonutritional interventions into long-term clinical studies. Overcoming these limitations will undoubtedly pave the way for improving precision nutrition protocols and provide personalized guidance on tailored polyphenol-based immunonutrition plans.

KEYWORDS

polyphenols, Mediterranean phytochemicals, immunometabolism, immunonutrition, pharmacological immunomodulation, senolytics, immunometabolic diseases, precision nutrition

1 Polyphenols: leading-edge immunonutrients

The immune system, a complex interactive network of many different immune cells, mediators, and cellular mechanisms, is highly dynamic in the response to changes in the tissue environment and plays a vital role in the balance between health and disease (1). It generally comprises two lines of defense: the innate (or unspecific) which comprises the physical barriers (e.g. skin, mucosal membranes, commensal microbiota) and several innate immune cells such as neutrophils, macrophages (phagocytes), innate lymphoid cells and nonspecific mediators that rapidly detect antigens, and the adaptive (or specific) immunity that involves B and T cells (2).

Strong evidence links undernutrition to immunosuppression, decreased vaccination efficacy (3, 4) and/or a greater difficulty in recovering from infections, broadly recapitulated during the COVID-19 pandemic (5, 6). On the other hand, overnutrition is closely associated with chronic low-grade inflammation and an increased risk of metabolic diseases (7). Thus, nutritional interventions tagging specific metabolic pathways in immune cells are promising to tackle the increasing prevalence of chronic diseases featuring a dysfunctional immunometabolic status (8), as well as the immunosenescence characterizing the aging process (9).

While it is agreed that immune function relies on an adequate nutritional status to function properly, we are only just starting to understand how the supply of single or combined nutrients, all of them denominated immunonutrients, can redrive the polarization of immune cells towards a tolerogenic or less inflamed immunophenotype (1, 6, 10). Many nutrients fall within the definition of immunonutrients, the most well-known being omega-3 fatty acids, glutamine, arginine, branched-chain amino acids (BCAAs; leucine, isoleucine, valine) and nucleotides (11, 12). Immunonutrition, a branch of precision nutrition, outlines the opportunity to integrate specific nutrients, or foods, in the usual diet (12) and has been drawing the attention of the scientific and medical communities due to its promising health benefits arising from immune system modulation in varied contexts, from individuals undergoing surgical procedures to critically ill patients, subjects with immune-related diseases, the elderly and, in a distinct scope, professional athletes (1, 2). In a multidisciplinary perspective, immunonutrition is defined as the modulation of immune system by nutrients and non-nutritive substances (e.g. antioxidants, prebiotics or probiotics), collectively termed immunonutrients, which are administered in doses above those normally obtained from the diet (1). These molecular compounds display a double function: they act as dietary constituents and, at the same time, may optimize immune responses by improving defense function while maintaining diet and commensal tolerance (1, 12). One may consider immunonutrition as a set of four main mutually dependent concepts: immune system, nutrition, body organ metabolism and the microbiome (1). Besides acting as a physical barrier, the microbiome interacts dynamically with both the innate and adaptive immune system of mucosa-associated lymphoid tissue (MALT) (13). Consequently, it has a chief role in MALT-dependent processes such as oral tolerance induction, cytokine secretion and overall regulation of immune responses. The possibility to reshape microbiota through immunonutrition in the form of functional foods, nutraceuticals and/or dietary supplements, is therefore an exciting approach to switch off oxidative stress and low-grade inflammation present in a plethora of immunometabolic diseases (2, 14).

A wide variety of non-nutritive phytochemicals have shown to benefit immune homeostasis, polyphenols the most-representative ones (12, 15, 16). This group of secondary plant metabolites is a promising class of phytochemicals that hold the potential to simultaneously balance the gut microbiome (14, 17) and the immune system by reprogramming immunometabolic pathways towards the repolarization of immune cells into a tolerogenic, less inflamed phenotype (6). Accordingly, much interest has been

created on their potential use as prophylactic or nutritional interventions targeting immunometabolic diseases.

In this work, we aim to shed light on the immunomodulatory effects of polyphenols, leading-edge immunonutrients, on non-communicable chronic diseases that share immunometabolic impairments, both auto-immune and non-autoimmune mediated. It provides a critical appraisal into their capacity to modulate immunometabolic reprogramming, emphasizing polyphenols' immunomodulatory roles in the maldaptation of organ-specific immune functions as well as their potential use as precise immunonutritional interventions in immunometabolic diseases.

2 Polyphenols: dietary sources, structural diversity and bioactivity

2.1 Dietary sources

The therapeutic potential of plant-based natural compounds and the phytochemicals composing them has been a significant point of interest in the last years. The most abundant and widely distributed bioactive molecules are polyphenolic compounds (PCs) (18). PCs are significantly abundant in a series of foods including olive oil, herbs, vegetables, fruits, seeds, nuts, whole-grain cereals, and wine that are frequently held accountable for the health benefits of the Mediterranean dietary pattern (19). Each of the referred food groups is enriched in specific PCs classes: phenolic acids predominate in cereals and whole-grains such as wheat, oats, rice, corn, and triticale (20); flavones and hydroxycinnamic acids in dried herbs such as oregano and peppermint (21); catechins, hydroxycinnamic acids, anthocyanins, and proanthocyanidins in red wine (22); flavonoids, phenolic acids and dihydrochalcones in fruits such as apples, mangos and pomegranates for instance (23, 24), and anthocyanins in berries, in which they are responsible for their unique pigmentation and aroma (25). In fact, Mediterranean nutritional patterns are associated with the consumption of colorful meals composed of a high variety of plant-based foods whose sensory and nutritional qualities, namely astringency, color and scent partially derive from the PCs composing them (26, 27).

2.2 Chemical structures

Polyphenolic compounds present a phenolic ring as their basic monomer (18). Due to their chemical structure, PCs present strong free radical scavenging capacity which confers them the ability to activate biological antioxidant responses (28, 29). Besides scavenging free radicals, some PCs are also capable of inhibiting the formation and/or activation of their precursors (28, 29). Depending on their chemical structure, origin and biological function, PCs can be divided in different classes, the largest ones being (1) flavonoids and (2) phenolic acids (18). Examples of more narrow classes are (3) tannins, which include pro- and antoanthocyanidins, gallotannins and ellagitannins, (4) coumarins, (5) lignans, (6) quinones, (7) stilbenes, including resveratrol and pterostilbene for instance, and (8) curcuminoids such as curcumin and ginerol analogues (30).

2.2.1 Flavonoids

In plants, flavonoids are responsible for the coloring and aroma of flowers and fruits (24) and the majority are found as glycosides (18). The general structural backbone of flavonoids is C6–C3–C6, the carbon of the C ring on which the B ring is attached to being the determinator of the subgroup the compound belongs to (18, 24). When the link between the B and the C rings is in the position 3, they are isoflavones, and when this link happens in position 4 we stand before neoflavonoids (24). Those in which the B ring is attached to the C one in the position 2 are further classified into different subgroups depending on the structure of the C ring, them being flavanones, flavanonols, flavones, flavonols, flavanols and anthocyanins (24). Figure 1 presents the chemical structure of the most common compounds belonging to flavonoid subclasses.

2.2.2 Phenolic acids

Phenolic acids are PCs that possess one carboxylic acid group, and can be divided into two major subtypes: benzoic acids, which present a skeletal structure C6-C1, and cinnamic acids, whose structure is C6-C3 (31). They are present in innumerous plant-based foods, such as fruits, vegetables, seeds, legumes, cereal and coffee, being mainly in a bound form, such as amides, esters and glycosides (31). The most abundant hydroxycinnamic acid found in food is chlorogenic acid (CGA), which is an ester formed between caffeic and quinic acids (31). On another hand, the most common hydroxybenzoic acids are gallic, vanillic, ellagic, syringic, p-hydroxybenzoic, and protocatechuic acids (31). These compounds might act as neuroprotective agents through radical-scavenging activity, being useful in the context of chronic diseases associated with oxidative stress (31). Figure 2 presents the chemical structure of the most common compounds belonging to phenolic acids subclasses.

2.3 Bioavailability and bioactivity

The overall bioavailability of PCs is determined mainly by their chemical structure, their absorption, distribution, metabolism, excretion (ADME), the form of administration, and food matrix (32). Pharmacokinetic studies show that PCs classes vary in terms of bioavailability and can be placed as follows: phenolic acids > isoflavones > flavonols > catechins > flavanones, proanthocyanidins > anthocyanins (32–34).

The polyphenolic content of several plants and fruits is greatly affected by exogenous factors (e.g. climatic conditions, culture types, the degree of ripeness), storage, cooking methods and processing mechanisms (33). Besides, food related factors such as the presence of specific macro and micronutrients can as well modify PCs bioavailability and bioactivity (35). Interestingly, it has been recently suggested that the association between PCs and fiber delays their absorption through the gastrointestinal (GI) tract, potentially optimizing their assimilation (36).

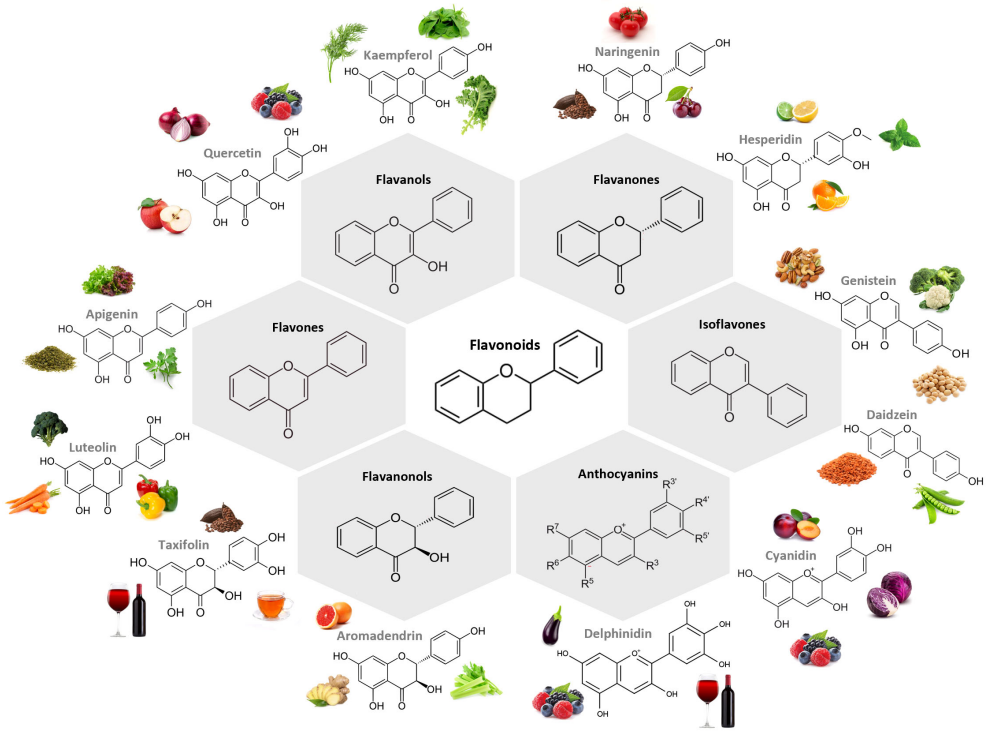

FIGURE 1
Chemical structure of the main subclasses of flavonoids, examples of compounds belonging to each subclass and examples of foodstuff containing them. The largest subgroup of flavonoids are flavanols, in which the hydroxyl group is positioned in the C3 of the C ring. Flavanones and flavones display a hydroxyl group in the C5 of the A ring with the difference between them residing in the double bond formed between positions 2 and 3, which is saturated in flavanones. Isoflavones differ from flavones on the position of the phenyl group, being structurally similar to estrogens. Flavanonols present the hydroxyl group linked to the C ring in the position 3, and no double bound between this and position 2. Anthocyanin hydroxyl groups of the A and C rings is what dictates their color. All subclasses can be found in fruits and vegetables. Figure created in BioRender.com.

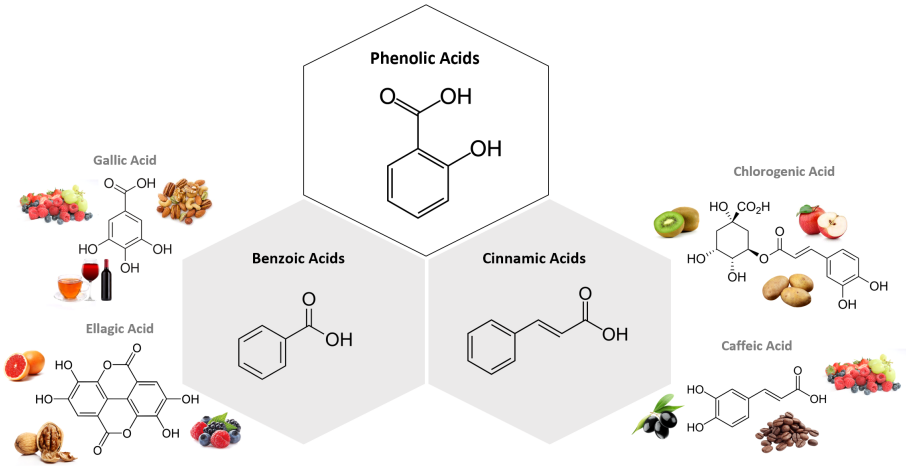

FIGURE 2
Chemical structure of the subclasses of phenolic acids, examples of compounds belonging to each subclass and examples of foodstuff containing them. The most abundant hydroxycinnamic acid found in food is chlorogenic acid (CGA), which is an ester formed between caffeic and quinic acids. On another hand, the most common hydroxybenzoic acids are gallic, vanillic, ellagic, syringic, p-hydroxybenzoic, and protocatechuic acids. Figure created in BioRender.com.

To exert their bioactivity, PCs must be delivered to the GI and absorbed, reach circulation and, posteriorly the target tissues, being subjected to a significant degree of transformation along their journey through the GI tract. As consequence, a single PC is able to generate several different metabolites displaying different activities and properties relatively to the original compound. In general, PCs display low oral bioavailability (5-10%) (37), due to factors such as decreased solubility, the interaction with the food

matrix, difficulties in membrane-crossing, as well as their extensive hepatic and intestinal metabolism and rapid clearance (32). Still, they display a plethora of scientifically proved and extensively documented dose-dependent beneficial effects (38). Accumulating evidence on the health-promoting effects of many PCs make them a topic of interest for scientists, nutritionists, and consumers in general. Their advantageous features, including marked antioxidant, anti-inflammatory, antimicrobial and anti-adipogenic properties grant them great potential to be incorporated in functional foods, nutraceuticals and/or dietary supplements (Table 1). Notably, PCs are closely intertwined to the therapeutic potentiation of the immune system, adding an extra layer of complexity to their pleiotropic actions. Polyphenols immunomodulatory and anti-inflammatory activities correlate to the number, positions and types of substitutions as well as the degree of polymerization based on the chromane ring (53). Moreover, the high degree of hydroxylation in the B-ring of cathecins and anthocyanidins favor metabolic reprogramming and polyphenols' bioactivity (54). In the upcoming section, the impact of polyphenols in immunometabolic reprogramming of both innate and adaptive immune responses will be discussed.

3 Polyphenols and immunometabolic reprogramming: a multi-organelle approach

Immunometabolic reprogramming heavily relies on inter-organelle communication and mitochondria, key organelles for cellular metabolism, act as masters regulators of multi-organelle connections and immune cell-fate determination (55, 56). During the immune response, cells shift from metabolic quiescence to an active phase, and the preferential utilization of specific metabolic pathways can dictate immune cells' differentiation towards a pro- or anti-inflammatory immunophenotype depending on their specialization for mounting protective immunity or tolerance to self or external antigens (57).

The interaction among nutrient signaling networks, adenosine triphosphate (ATP) availability, and immunological cues is crucial to meet the energy demands and functional modifications in immune cell metabolism. AMPK and its downstream target, mTOR, serve as central hubs to nutrient availability by sensing intracellular energy levels (AMP/ADP: ATP ratio). In energy-depleted states, activated AMPK typically inhibits mTOR signaling and promotes mitochondrial biogenesis via the peroxisome proliferator-activated receptor-gamma (PPARγ) co-activator-1 alpha (PGC1α) signaling axis (58). Consequently, cellular metabolism skews towards increased oxidative phosphorylation (OXPHOS) activity and enhanced expression of genes encoding key mitochondrial enzymes. Conversely, in states of overnutrition, mTOR upregulates protein and lipid synthesis to promote immune cell growth and proliferation (59).

Quiescent immune cells, such as naïve T cells, memory T cells (Tmem), Treg or tolerogenic DCs, alongside M2 macrophages, predominantly favor mitochondria-driven catabolic metabolism characterized by OXPHOS and fatty acid oxidation (FAO) to sustain ATP supply for long-term survival (60, 61). Autophagy, a conserved lysosomal degradation pathway that supports immune cell differentiation, is enforced by AMPK activation, thereby restraining glycolysis and maintaining cellular quiescence (7, 62). Contrariwise, activated immune cells, such as M1 macrophages and effector T cells (e.g. Th1, Th17), exit the quiescence state by metabolizing nutrients to ensure an adequate supply of macromolecules for the energy demands associated with cellular growth (55). They shift the balance towards mTOR activation and aerobic glycolysis as a rapid source of ATP, akin to the Warburg effect, to meet the high nutritional and energetic requirements of

TABLE 1 Bioactivity of polyphenolic compounds.

Bioactivity	Polyphenolic compound	Evidence	Reference
Antioxidant	Catechins	Catechins displayed the most favorable results regarding a series of antioxidant activity evaluation assays	(39)
	(-)-Epigallocatechin-3-gallate from Green Tea	Stimulate nuclear factor erythroid 2–related factor 2 (Nrf2) translocation to the nucleus	(40)
	Kaempferol, Gallic Acid, Resveratrol	Potentiate the activity of enzymes belonging to the endogenous biological antioxidant system, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase	(41–43)
Anti-inflammatory	Hesperidin	Reduce interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and nitric oxide levels both *in vitro* and *in vivo*	(44)
	Dehydroxylated Phenolic Acids: 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylacetic acid, 4-hydroxyhippuric acid	Attenuate lipopolysaccharide (LPS)-induced secretion of TNF-α, IL-6 and IL-1β in human peripheral blood mononuclear cells (PBMCs)	(45)
Antimicrobial	Epigallocatechin Gallate, Tea Polyphenols, A-type Proanthocyanidins	Disturbing the cell wall of specific bacteria, their inner cytoplasmatic membrane, or reducing their motility and biofilm-forming ability	(46–48)
Anti-adipogenic	Vanillic Acid, Catechins, Resveratrol	Adipose tissue "browning" Suppress the expression of genes and transcription factors related to adipogenesis	(49–52)

short-term clonal expansion and effector function (4, 10, 60). For example, mTORC1 sustains aerobic glycolysis and upregulates hypoxia-inducible factor 1 alpha (HIF-1α) expression to support Th17 cell differentiation, counteracting Treg expansion (58). Similarly, a significant transition from OXPHOS to aerobic glycolysis occurs in bone marrow-derived DCs upon Toll-like receptor (TRL) activation, resulting in inducible nitric oxide synthase (NOS)-dependent generation of nitric oxide (NO) and blockade of mitochondrial electron transport (61). Metabolic reprograming of activated immune cells also involves glutaminolysis. Glutamine is converted into glutamate and ketoglutarate, two well-known tricarboxylic acid cycle (TCA) intermediates that support the oxidative metabolism of immune cells, particularly macrophages. A high ketoglutarate/succinate ratio promotes alternative (M2) activation and FAO engagement, while a low ratio strengthens the proinflammatory phenotype observed in classically activated (M1) macrophages (63).

Metabolic rewiring entails significant modifications in mitochondrial biogenesis and dynamics, as well as redox signaling pathways, all of which are crucial for immune function. For instance, the immunometabolism of T cells heavily relies on the continuous dynamic reshaping of mitochondria through fusion and fission events to maintain mitochondrial quality. Memory T cells undergo increased mitochondrial fusion to support OXPHOS and fatty acid oxidation (FAO) metabolism. In contrast, activated effector T cells demonstrate heightened rates of mitochondrial fission and reduced cristae, an adaptation to facilitate aerobic glycolysis (64). Membrane-bound organelles such as mitochondria, endoplasmic reticulum (ER) and lysosomes must establish inter-organelle connections through specialized cytosolic microdomains to facilitate the intersection of metabolic signaling and the utilization of products from one pathway efficiently as intermediates for another (65). ER-mitochondria junction signaling provides a regulatory platform for various overarching immune cellular functions. The mitochondria-ER network brings together signaling components to potentiate mitochondria fission and Warburg metabolism, key events for the rapid recall response of newly activated memory CD8+ T cells (66, 67). Similarly, the activation of NLRP3 spatially correlates to mitochondria-derived reactive oxygen species (mtROS) and excessive mitochondrial fission in ER-mitochondria contact sites of macrophages undergoing glycolytic reprogramming (64, 68, 69). In summary, the dynamic behavior of mitochondria and inter-organelle communication, particularly with the ER network and endolysosomal system, is crucial for enabling immune cells to seamlessly adjust to fluctuations in nutrient availability. This aptitude is vital for effectively meeting the functional demands during immune cell remodeling.

Evidence regarding the immunomodulatory effects of PCs have been significantly emerging in the last decades (8, 70, 71). A main reason relies on the fact that different immune cell populations express various kinds of polyphenols' receptors (72). Examples of immune cellular receptors targeted by PCs include the retinoic acid-inducible gene like receptors (RLRs), aryl hydrocarbon receptor (AhR), 67 kDa laminin receptor (67LR), zeta chain-associated 70 kDa protein (ZAP-70), T cell receptor (TCR) αβ, secretory IgM-(sIgM-) B-cell receptor, Toll-like receptor (TLR) 4 (73–79) and Retinoic Acid Receptors (RARs) (80, 81). Upon binding, PCs are able to modulate immune cells metabolism and activity through a multi-modal approach encompassing nutrient-sensing mechanisms, AMPK/mTOR signaling balance, regulation of inter-organellar communication and modulation of metabolism-epigenetic axis (Figure 3).

3.1 Nutrient-sensing mechanisms

Nutrients not only act as building blocks but also activate nutrient sensing via stress-response pathways and growth factors, essential for immune responses (82). Under amino acid starvation conditions`, immune cells activate the amino acid response (AAR)`, a cytoprotective signaling pathway that transiently reduce protein synthesis while enhancing stress-induced gene expression (83). AAR pathway is a potent regulator of inflammatory T cell differentiation. Accordingly, glutamine uptake and glutaminolysis largely cooperate in Th1/Th17 inflammatory T cell response (84). Glutamine, the most abundant amino acid in human plasma, is an important substrate of various ATP generating pathways (e.g. glycolysis, OXPHOS) (85). It is transported across the plasma membrane in mammalian cells by different transporters such as the alanine serine cysteine transporter 2 (ASCT2). Gallate-type procyanidin PCB2 3,3 (PCB2DG) polyphenol, a dimer of epicatechin, interacts directly with ASCT2 glutamine transporter and antagonizes glutamine influx, mTOR/HIF-1 pathway, Th1/Th17 cell production and inflammatory response through interferon gamma (IFNγ) and interleukin-17 (IL-17) production.

Sirtuin-1 (SIRT1) serves as another crucial energy sensor. It is activated by NAD+ in nutrient-deficient states and modulates mitochondrial biogenesis by deacetylating and activating transcription factors such as PCG-1α, signal transducer and activator of transcription 3 (STAT3) or the nuclear factor E2-related factor (NRF)-2. In CD4+ T cells, SIRT1 impedes the process of differentiation of T lymphocytes into Th17 cells through STAT3 deacetylation (86). Therefore, SIRT1 agonists have emerged as promising pharmacological approaches to broaden the array of current therapeutic options focused on reducing the Th17 profile. Among these possibilities, the deacetylase activator resveratrol stands out as particularly promising. In CD4+ T cells, this polyphenol has been observed to encourage Th2 and Treg polarization, immunomodulatory effects that are linked to the diverse beneficial impacts of resveratrol in various pathologies characterized by imbalanced lymphocyte subtypes ratios (87).

3.2 AMPK/mTOR signaling balance

mTOR and AMPK stand out as two additional master regulators of cellular metabolism, enabling adaptation to challenges of nutrient scarcity or excess, ultimately promoting cell survival. They are intricately linked to cell-specific adjustments in response to metabolic stress, and disruptions in these signaling

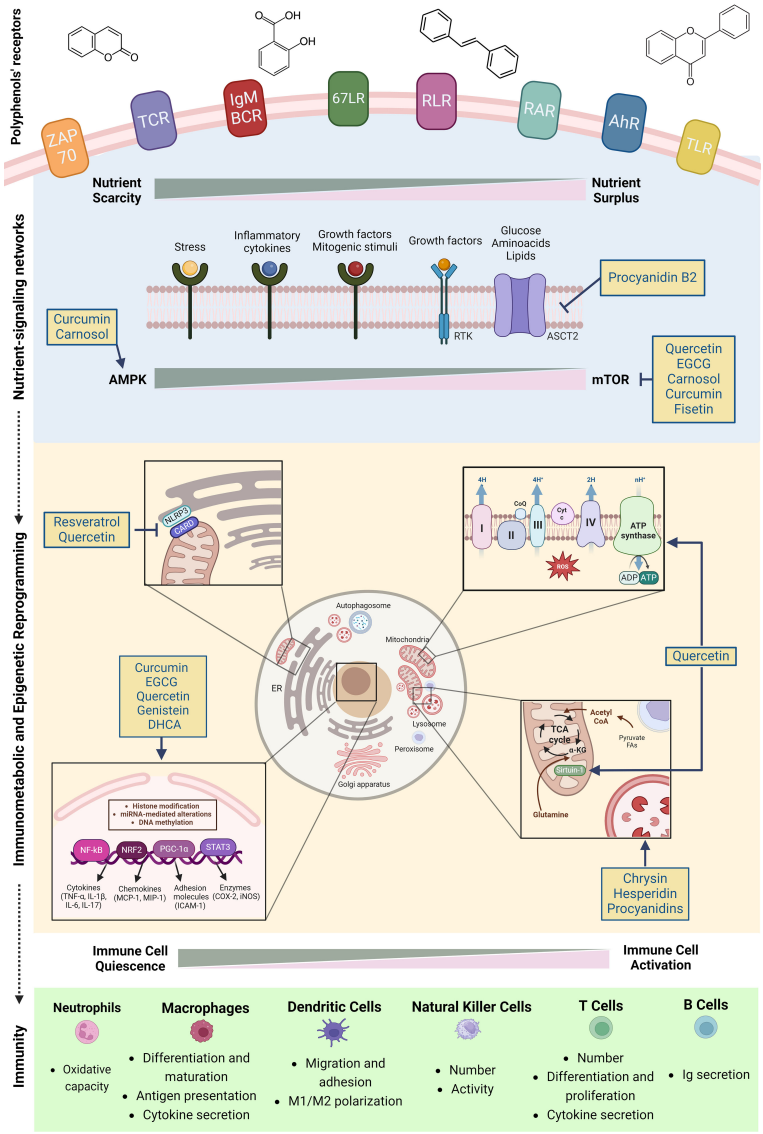

FIGURE 3
Polyphenols and immunomodulation: a mitochondria-centered multi-modal approach. Polyphenols impact immunometabolic reprogramming through four regulatory axes: first, they activate nutrient sensing via stress-response pathways and growth factors, essential for immune responses. Second, polyphenols regulate mTOR/AMPK balance and inflammatory responses in immune cells and serve as well-tolerated caloric restriction mimetics. Third, they interfere with the assembly of NLRP3 in endoplasmic reticulum-mitochondria contact sites, inhibiting its activation while improving mitochondrial biogenesis and autophagosome-lysosome fusion. Finally, polyphenols impact chromatin remodeling through modulation of histone deacetylase/acetyltransferase, thereby coordinating both epigenetic and metabolic reprogramming. Figure created in BioRender.com.

pathways are closely associated with various pathological conditions (88). When nutrients are abundant, organisms prioritize fuel utilization to support cellular growth, with mTOR signaling playing a central role in this process. Conversely, upon nutrient depletion, organisms suppress anabolic pathways and promote autophagy via AMPK signaling to adopt a state geared towards preserving the structural and functional integrity of existing cells. Importantly, SIRT1/PGC1α can exert negative regulation on the phosphoinositide 3-kinase (PI3K)-alpha serine/threonine-protein kinase/(Akt/mTOR) pathway, likely through their influence on the cellular maintenance of autophagy (89). Senolytic drugs can simultaneously upregulate nutrient deprivation signaling (AMPK) and suppress pathways associated with nutrient surplus (mTOR), consequently boosting autophagic flux (90). Accordingly, caloric restriction mimetics are the most extensively studied metabolic interventions and have long been associated to lifespan extension and immunosenescence improvement (91, 92).

PCs are well-tolerated caloric restriction mimetics due to their ability to activate AMPK, a cellular energy sensor, thus improving mitochondrial turnover (93, 94). Quercetin and fisetin, two well-established senolytic drugs, belong to the flavonoid class of polyphenols and are key modulators of immune cell function. In lipopolysaccharide (LPS)-treated macrophages, fisetin inhibited PI3K/AKT/mTOR signaling and inflammatory cytokines secretion (95). In addition, the acetyltransferase inhibitor epigallocatechin-3-gallate (EGCG) was found to downregulate

mTOR-HIF1α signaling, a metabolic checkpoint of Th17/Treg differentiation, leading to the downregulation of glycolysis-associated molecules and inhibition of Th17 differentiation (70). Likewise, carnosol and curcumin effectively inhibit mTOR activation in response to LPS stimulation in human DCs via AMPK-dependent induction of heme oxygenase-1 (HO-1), an important antioxidant enzyme that assist the maintenance of DCs in a tolerogenic state (71).

3.3 Mitochondria and ER-lysosomes inter-organellar communication

The NLRP3 inflammasome, a critical junction between innate and adaptive immunity, relies on ER-mitochondria contact sites to facilitate the association of mitochondria-driven ligands, including dysfunctional mitochondria themselves, as upstream signals for NLRP3 activation. Additionally, self-derived or foreign-derived particulates can be endocytosed by lysosomes, leading to membrane damage and further release of cathepsin B, another common upstream signal for NLRP3 activation (96). In LPS-treated microglia cells, quercetin enhances the mitophagic clearance of damaged mitochondria, countering mtROS accumulation and NLRP3 inflammasome activation during the assembly stage (97). Similarly, resveratrol inhibits the acetylated α-tubulin-mediated spatial arrangement of mitochondria and their ER contacts in macrophages. Consequently, it interferes with the assembly of NLRP3 and its adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), thereby inhibiting NLRP3 inflammasome activation triggered by mitochondrial damage (98).

Interestingly, it has been proposed that polyphenols can be directly endocytosed into lysosomes, regulating key signaling pathways of phagocytic cells such as macrophages and DCs (99). Accordingly, chrysin (a flavone) and hesperidin (a flavonoid) enhanced lysosomal phosphatase activity in a concentration-dependent manner in LPS-stimulated macrophages (100). Similarly, the senolytic drug fisetin facilitates the autophagosome-lysosome fusion and degradation processes in LPS-treated macrophages by regulating a set of genes primarily involved in autophagosome assembly/maturation (95). Comparable effects were observed in LPS-treated DCs, where cocoa procyanidins strongly upregulated gene pathways associated with lysosomal metabolic function and nutrient metabolism, suggesting a significant impact on DC metabolic activity (99).

3.4 Modulation of metabolism-epigenetic axis

Recent research has revealed that alterations in metabolic status can coordinate the function of immune cells by influencing epigenetic changes. This regulatory axis between metabolism and epigenetic enables the microenvironment to mold immune cells, and disruption of this process can contribute to the development of various diseases (101). For example, in LPS-stimulated THP-1 promonocyte cells, TLR4 stimulation triggers glucose-dependent ATP production alongside gene-specific chromatin remodeling. Sirt1 deacetylation activates PGC1α transcriptional activity and orchestrates sequential metabolic reprograming, sensing processes dependent on NAD+, thereby reducing HIF1α-dependent glycolysis and enhancing PGC1α-dependent FAO (102). Notably, quercetin upregulates Sirt1/PGC1α signaling and improves mitochondrial function and morphology (e.g. mtROS, mitochondrial membrane potential, ATP production) in LPS-induced inflammatory macrophages (103).

Chromatin remodeling involves structural changes such as DNA methylation, histone methylation, and acetylation, which greatly impact transcriptional changes of different genes. Several polyphenols have been identified as histone deacetylase (HDAC) inhibitors (EGCG, curcumin, genistein, quercetin), histone acetyltranferase (HAT) activators (genistein) or HAT inhibitors (EGCG, curcumin) (104). For instance, gallic and ellagic acids, along with fisetin, were found to decrease HAT activity in THP-1 cells, resulting in the deacetylation of the p65 subunit of NF-κB and attenuation of pro-inflammatory cytokine release (105, 106). Moreover, EGCG enhances HDAC activity in Treg cells, leading to suppressed nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling and elevated synthesis of the anti-inflammatory IL-10 (107). Finally, treatment with dihydrocaffeic acid (DHCA) led to a decrease in DNA methylation levels in peripheral leukocytes from mice exposed to stressful conditions as well as human and mice peripheral leucocytes exposed to lipopolysaccharide (LPS) *in vitro* (108).

4 Polyphenols and immunomodulation

4.1 Polyphenolic modulation of innate immunity

In a simplified manner, one might consider that innate immunity includes two distinct components: the cellular system and the non-cellular system (70). As its name suggests, the cellular system is composed of a set of different cell populations, such as granulocytes, monocytes, macrophages, natural killer and dendritic cells (70). On another hand, the non-cellular one includes diverse kinds of mechanisms that range from mucous barriers to signaling pathways (70). Both components act in a synergistic manner in order to prevent pathogens' access to the organism or promote their destruction in case the referred barriers have already been broken (70).

4.1.1 Effects of polyphenols on dendritic cells

Due to their antigen-presenting activity, dendritic cells (DCs) are indispensable for initiating and regulating innate immune responses (70). PCs have been showing to influence several aspects related to DCs, including differentiation and maturation, as well as their antigen presentation and cytokine secretion functions. For instance, resveratrol was shown to regulate the

differentiation of healthy human monocytes from the blood into DCs (109). Analogously, both EGCG and quercetin exerted immunosuppressive effects in bone marrow (BM)-derived DCs, impairing their maturation and their expression of major histocompatibility complex (MHC) (110, 111). An *in vitro* study has shown that quercetin's inhibition of DC maturation results from downregulated steroid receptor coactivator (Src)/PI3K-Akt-NF-kB-inflammatory pathways (112). Furthermore, EGCG exposure induced apoptosis of blood monocyte-derived DCs from healthy individuals and modulated developing DCs' phenotype by downregulating MHC II molecules and the surface markers CD11c, CD80 and CD83, which are needed for the process of antigen presentation (113). Interestingly, polyphenols of different natures have been shown to possess immunosuppressive properties towards murine BM-derived DCs stimulated with LPS, including curcumin (114), apigenin (115), daidzein (116), baicalin (117), fisetin (118) and silybin (119). These PCs significantly inhibited the expression of surface markers associated with DC maturation such as CD40, MHC II molecules, as well as costimulatory receptors namely CD80 and CD86, in a dose-dependent manner. As a consequence, they impacted the induction of Th1-mediated immune responses. Additionally, the referred study employing curcumin has also reported a decreased production of IL-1β by DCs, once more repressing their immunostimulant activity (114). Many of these effects seem to derive from the polyphenols' ability to modulate DC metabolism, namely through suppressing mitogen-activated protein kinases (MAPKs) p38, c-Jun-N-terminal kinase (JNK), extracellular regulated kinase (ERK) 1 and 2, and NF-κB activation (111, 114, 115, 119, 120). Analogously, carnosol and curcumin were found to affect AMPK activation and downstream inhibition of the mTOR pathway in lipopolysaccharide-prime DCs (121). The reduced glycolytic flux promoted by the two polyphenols also impacted mitochondria, inhibiting the LPS-induced increase of spare respiratory capacity.

4.1.2 Effects of polyphenols on monocytes and macrophages

Similarly to DCs, macrophages play an important role in antigen presentation mechanisms, as well as tissue inflammation and repair processes (70). Remarkably, the shift between M1 and M2 phenotypes has shown to be influenced by PCs. For instance, *in vitro* culturing of THP-1 macrophages with a cocoa extract resulted in suppressed M1-mediated inflammation and promoted polarization to M2 (122). A similar effect has been observed with resveratrol regarding tumor-associated macrophages (123). Moreover, quercetin, kaempferol, daidzein, genistein (124) and apigenin (125) have exhibited the ability to reduce pro-inflammatory cytokines' secretion by these cells. Quercetin has shown to prevent the secretion of IL-6, IL-1β and tumor necrosis factor alpha (TNF-α) by macrophages by suppressing LPS-induced MAPK and ERK activation (126). Plum polyphenols have also been linked to decreased pro-inflammatory cytokines, ROS and malondialdehyde production by RAW 264.7 macrophages treated with monosodium urate through different signaling pathways involving HIF-1, ErbB and Forkhead box transcription factor O (FoxO) (127). A similar effect has been reported for hesperidin which besides decreasing *ex vivo* IL-12 secretion in LPS-stimulated mouse macrophages also suppressed their migration and adhesion properties *in vitro* (128). An interesting study aiming to evaluate the impact of the flavonoids quercetin, naringenin and naringin on the metabolism of cultured human macrophages has highlighted that the flavonoid-mediated immunomodulation derived from glycolytic downregulation, as well as anti-inflammatory reprogramming of the TCA cycle and antioxidant protection (mainly quercetin), membrane modification (naringenin) and osmoregulation (naringin) (129).

PCs are also able to modulate macrophagic ROS production and iNOS activity, as has been reported for curcumin (130, 131), resveratrol (132, 133), EGCG (134, 135), and genistein (136), to name a few.

Polyphenols further seem to improve macrophages' phagocytic capacity. EGCG and curcumin, for instance, have been shown to trigger murine peritoneal macrophages and RAW 264.7 macrophages' phagocytosis *in vitro* (137, 138). The synergistic effect of these two polyphenols together with resveratrol has been demonstrated against glioblastoma and human papillomavirus (HPV)-infected cells, leading to the repolarization of tumor-associated macrophages and tumor suppression (139).

Interestingly, PCs seem to not only influence macrophages but also their precursors -monocytes – as evidenced by an increase in nitric oxide (NO) production by blood monocytes observed in healthy individuals consuming red wine (72). Moreover, blueberry supplementation has been shown to decrease monocyte expression of monocyte-to-macrophage differentiation-associated (MMD) and C-C motif chemokine receptor 2 (CCR2), reducing inflammation in metabolic syndrome patients (140). EGCG prevented monocyte adhesion to cultured endothelial cells from pig pulmonary aortas by reducing the expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) (141).

4.1.3 Effects of polyphenols on neutrophils

As has been observed for DCs and macrophages, studies highlighting the immunomodulatory effects of PCs on this cell population have been arising, particularly regarding their ability to inhibit *in vitro* neutrophils' oxidative capacity, which correlates with exacerbated neutrophilic inflammation (142, 143). Accordingly, a study performed by Drábiková et al. reported that a series of polyphenols including curcumin, pinosylvin, resveratrol, pterostilbene, piceatannol and N-feruloylserotonin significantly reduced ROS production by human neutrophils *in vitro* (144). Furthermore, human blood cultured neutrophils' exposed to treatment with grape polyphenols exhibit improved chemokinetic accuracy and motility in association with enhanced CD16 shedding and CD66b expression (145). On another hand, a study evaluating the impact of phenolic acids in a mouse model of colitis exalted the ability of ferulic acid to alleviate the disease by suppressing the formation of neutrophil extracellular traps (146).

4.1.4 Effects of polyphenols on natural killer cells

Natural Killer (NK) cells are recognized by their robust cytotoxicity and lytic activity, as well as effector functions (147).

Contrarily to what has been described above regarding the effects of PCs on macrophages, DCs and neutrophils, which are essentially immunosuppressive, their impact on NK cells appears to have a stimulatory nature, increasing their number and activity. As an example, green tea polyphenols and quercetin are able to promote murine NK-mediated cytotoxicity (148) and lytic activity (149), respectively. Similarly, low-dose resveratrol supplementation has promoted NK cell killing capacity in different experimental contexts (150–152), potentially by activating JNK and ERK (152, 153). Nevertheless, this seems to be dose-dependent since high doses of resveratrol exerted the opposite effect. Similarly, a study performed by Oo et al. reported that luteolin, apigenin and quercetin at doses of 12.5 μg/ml and 25μg/ml significantly increased the NK-cell-mediated cytotoxic activity against lung cancer cells (154). Contrastingly, genistein blocks NK cells' activity at low doses (155) but enhances their cytotoxicity at high concentrations (156). These results highlight the dose-dependent behavior displayed by the vast majority of polyphenolic compounds.

In humans, clinical studies showed that blueberry supplementation increases NK cell count in the blood of healthy subjects (157, 158).

4.2 Polyphenolic modulation of adaptive immunity

Alternatively to the innate immune system, the adaptive branch of the immune system involves a unique type of cells - lymphocytes (70). Two primary lymphocyte populations prevail (1): T lymphocytes, which are responsible for cytokines' secretion, cytotoxic destruction of unviable cells and activation of other immune cells, and (2) B lymphocytes, known by their antibody-producing capacity (70).

4.2.1 Effects of polyphenols on T and B cells

The immunomodulatory potential of PCs goes beyond innate immunity, considerably impacting lymphocyte numbers and functionality. For instance, incorporating EGCG in the diet for one week has proven to elevate T regulatory (Treg) cells' number in mice's spleen, mesenteric and pancreatic lymph nodes (159). Furthermore, these cells were able to repress cytotoxic T cell action and proliferation as well as interferon gamma (IFNγ) production (159). A study evaluating EGCG's impact on naïve $CD4^+$ T cell differentiation showed that the green tea polyphenol inhibited Th1, Th9, and Th17 differentiation by downregulating the respective transcription factors T-bet, PU.1, and RORγt, while also preventing IL-6-induced suppression of Treg development. These effects were considered to result from downregulation of Signal transducer and activator of transcription p-STAT1 and p-STAT4 for Th1, and p-STAT3 for Th17 cells, as well as inhibition of IL-6-induced STAT3 phosphorylation, respectively. Analogously, naringenin displayed the potential to induce Treg cells through AhR-mediated pathways (72) and baicalin has shown to inhibit Th17 cell differentiation both *in vitro* and *in vivo* via reducing RAR-related orphan receptor gamma t (RORγt) expression and up-regulating Forkhead box p3 (Foxp3) expression (160). Interestingly, EGCG has also shown to induce Treg cells by repressing DNA methylation, inducing Foxp3 and IL-12 expression both *in vitro* and *in vivo* (159). These outcomes exalt a novel epigenetic mechanism underlying the polyphenol's immunomodulatory activity associated with DNA methyltransferases inhibition. Moreover, Ning et al. provided new evidence for the effectiveness of the green tea flavonoid in vitiligo treatment via Janus kinase 2 (JAK2) kinase activity inhibition, reducing the protein levels of CD11a, CXCR3, and CCR2 receptors in human T lymphocytes, suppressing their adhesion to melanocytes induced by IFN-γ (161). Importantly, EGCG's immunomodulatory properties are not limited to $CD4^+$ T cells. In fact, there are several reports on the flavonoid's competence on increasing $CD8^+$ T cell number and activity in tumorigenic contexts (162, 163). Genistein has exhibited a similar effect, while also enhancing $CD8^+$ T cell IFNγ expression both *ex vivo* and *in vivo*, leading to immune stimulation (156).

A study performed by Ramiro-Puig et al. evaluating the effects of a cocoa-enriched diet in the spleen lymphocyte function of young rats reported that a 10% cocoa intake increased lymphocyte proliferation rate, but down-regulated Th2-associated cytokine levels and decreased immunoglobulin (Ig) secretion (164). Additionally, spleen B cell proportion was raised, and Th cell percentage declined (164).

Similarly, auraptene, a citrus fruit-derived coumarin, was able to suppress the activation of murine inguinal lymph node-derived Th1 cells (165). Finally, genistein has also shown to increase the number of both helper and cytotoxic T cells as well as B lymphocytes in rat spleen (166). Likewise, curcumin administration to Min/+ mice increases mucosal $CD4^+$ T and B cell numbers by modulating CD28, CTLA-4, STAT and NF-kB expression, preventing the formation of intestinal tumors (167). In addition, through inhibiting STAT4 phosphorylation curcumin has also shown to suppress human $CD4^+$ T cells differentiation into the Th1 phenotype (168). Curiously, curcumin's impact appears to depend on the stimulous to which lymphocytes have been exposed, since other studies exalt its immunossupressive activity. For instance, Sharma et al. reported that both resveratrol and curcumin suppressed the activity of concavilin A-stimulated T and B cells by inhibiting their proliferation, antibody production and lymphokine secretion (169). In fact, curcumin's ability to suppress B cell proliferation has also been demonstrated in human Epstein-Barr infected cells (170). Curiously, polyphenol-driven apoptosis of leukemic B cells was shown to correlate with caspase 3 activation, reduced mitochondrial transmembrane potential as well as downregulation of antiapoptotic protein beclin 2 and iNOS expression (171).

5 Polyphenol-based immunonutrition in immunometabolic diseases

Over the past two decades, the pivotal interplay between immunity and metabolism in chronic diseases has become increasingly evident

(172). The burgeoning field of Immunometabolism has progressively illuminated how the immune system orchestrates the functionality of key homeostatic systems within tissues, such as the brain, pancreas, liver and adipose tissue. This modulation occurs through the sensing and adaptation to microenvironmental nutrient fluctuations, driving flexibly reprogramming of metabolic pathways in immune cells that greatly impact their polarization towards a pro- or anti-inflammatory phenotype (172, 173). Accordingly, mounting body of evidence progressively appreciates the mobilization of the innate and adaptive immune systems not only in autoimmune diseases featured by the loss of self-tolerance but also in supposedly non-immune pathologies encompassing neurodegeneration and metabolic disorders (174). Consequently, there's a rising interest in immunonutritional approaches aimed at optimizing immune cells functions to enhance effective defense responses while preserving tolerance.

The following sections delve into the characteristics of immunological disturbances within the spectrum of both autoimmune and non-autoimmune metabolic disorders (175). Additionally, it sheds light on the immunomodulatory roles of polyphenols and draws a mechanistical parallel between their effects and the pleiotropic immunomodulatory actions of drugs currently integrated into corresponding therapeutic algorithms (Figure 4).

5.1 Autoimmune immunometabolic diseases

5.1.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is currently accepted as being a T cell-mediated disease (176). Nevertheless, other adaptive immune cells as well as elements from innate immunity are believed to be involved in T1DM physiopathology.

Namely, T1DM patients display an impaired complement system function (177) and monocytes from these patients display decreased chemotaxis and phagocytic activity (178). Furthermore, infiltration of macrophages (179),neutrophils, and NK cells (180) in the pancreatic Langerhans islands has been detected in NOD mice and human patients. Moreover, hyperglycemia has shown to impair macrophages' autophagic mechanisms (181, 182). Interestingly, insulin has shown to reestablish the normal phenotype in diabetogenic macrophages through Akt and ERK signaling (183), as well as to repress TLRs and CD14 transcription (184). Furthermore, Yu et al. reported insulin's ability to promote phenotype transition of macrophages from M1 to M2 through PI3K/Akt pathways, and PPAR-γ signaling during diabetic wound healing (185).

Nonetheless, cellular infiltrates found on the pancreas of diabetic subjects are also composed of adaptive immune cells, such as $CD4^+$ and $CD8^+$ T as well as B lymphocytes (176). Remarkably, diabetogenic $CD4^+/CD8^+$ T lymphocytes are more dependent on aerobic glycolysis and rely less on OXPHOS (186). Accordingly, glycolysis inhibition induced terminal $CD4^+$ T cell exhaustion in an animal model of T1DM, delaying disease onset (187). Treg cells are also found to be dysfunctional in the pancreatic lymph nodes of T1DM patients (188) and an increase in IL-17-producing T cells has also been detected (188, 189). Diabetogenic T cells are further characterized by mitochondrial membrane hyperpolarization and dysfunction, resulting in increased ROS levels and diminished ATP production (190).

PCs supplementation has been emerging as a potential therapeutic strategy for alleviating the immune dysfunction characterizing T1DM, in part by improving mitochondrial function. In fact, several kinds of polyphenols have shown to improve mitochondrial function, namely through the activation of the key mitochondrial biogenesis' PGC-1α, including ursolic acid (191), resveratrol (192), quercetin (193, 194) and olive hydroxytyrosol (195).

Nevertheless, PCs' effects are not limited to mitochondrial function. For instance, a pomegranate peel extract was able to inhibit immune cell infiltration into pancreatic islets (196). Similarly, oral administration of capsaicin to several mice strains showed to attenuate the proliferation and activation of autoreactive T cells in pancreatic lymph nodes, protecting them from disease development (197). The authors considered these effects to be mediated by capsaicin-mediated enhancement of a discreet population of $CD11b^+/F4/80^+$ macrophages in the pancreatic lymph nodes, which express the anti-inflammatory factors interleukin IL -10 and programmed death-ligand 1 (PD-L1). Moreover, procyanidin B2 gallate has been revealed as a suppressor of TNF-α production by activated $CD4^+$ T cells by inhibiting their glycolytic function via mTOR- HIF-1α interaction (198). Lastly, a study evaluating the impact of black seeds and garlic intake in diabetic rats demonstrated a significant increase in the blood levels of monocytes and granulocytes, while lymphocyte proliferation was suppressed (199). A similar output was verified when administering fenugreek oil to a rat model of T1DM, which blunted the diabetes-induced increase of pancreatic lymphocyte counts (200).

5.1.2 Inflammatory bowel disease

Inflammatory Bowel Disease (IBD) presents defects in peripheral and intestinal immune function (201). A deep analysis of the peripheral immune system of IBD patients has found decreased numbers of NK cells and B lymphocytes opposing to increased counts of neutrophils and memory $CD8^+$ T cells in the blood (202). Besides displaying elevated phagocytosis and cytokine production (201), IBD-associated macrophages also go through the Warburg effect by HIF-1α stabilization and subsequent increased expression of glycolytic enzymes, a process that is modulated by pyruvate kinase 2 (PKM2) (203). The disease further entails gut DCs overactivation, resulting in increased levels of IL-6in the serum and intestine of IBD patients. Decreased numbers of Treg cells in mice peripheral blood and patients' intestinal mucosa, alongside the expansion of Th17 cells and increased production of IL-17 and IL-23 in the intestinal mucosa and lamina propria (LP) have also been detected (201). Interestingly, the expression of pro-inflammatory cytokines by Th17 cells was found to be epigenetically controlled by the glucose transporter GLUT3, which is upregulated in models of IBD (204). Collectively, evidence points to a relevant role of glycolysis in the immunologic dysfunction characterizing IBD.

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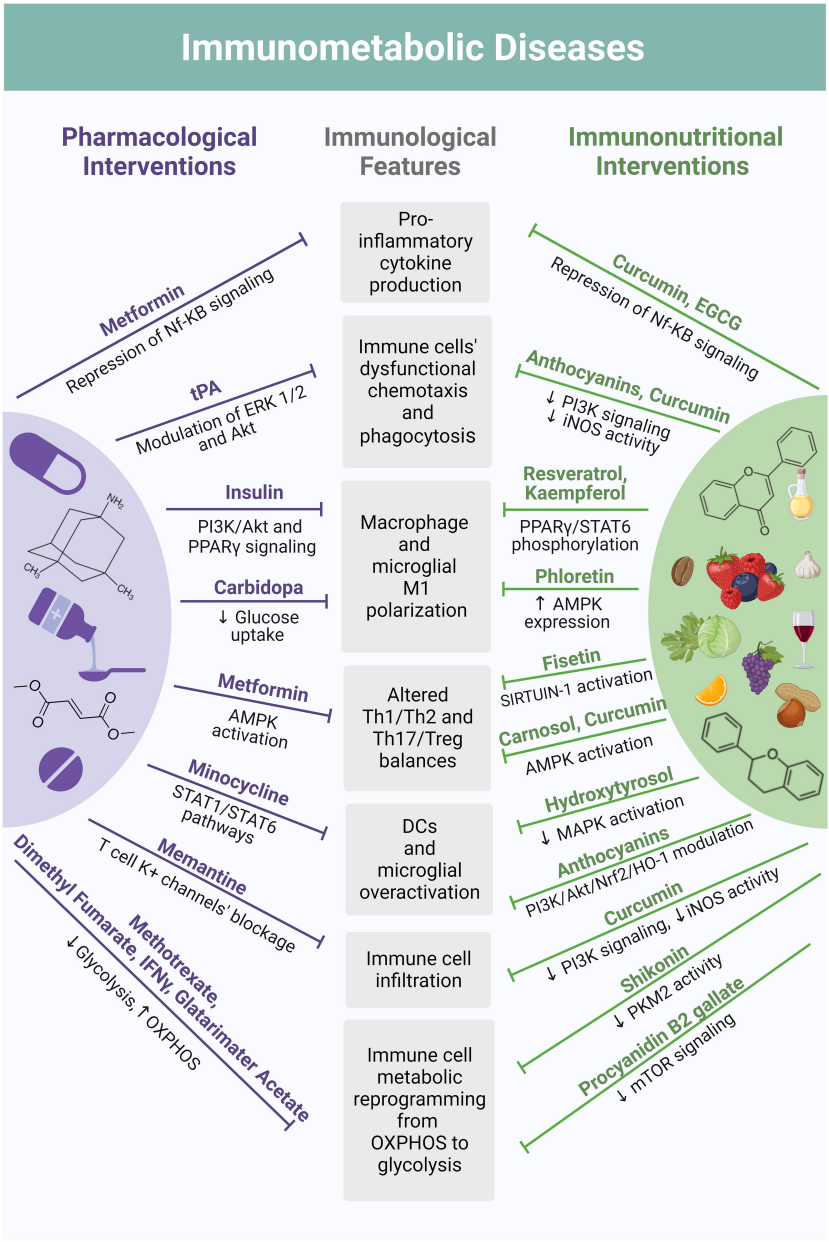

FIGURE 4
Mechanistical parallelism between pharmacological interventions and polyphenol-based immunonutrition in their modes of immunomodulation. Current therapeutic algorithms of immunometabolic disorders include drugs with pleiotropic immunomodulatory actions shared by a plethora of polyphenol-based immunonutritional approaches. For instance, the anti-diabetic metformin suppresses the production of pro-inflammatory cytokines by immune cells through Nf-KB signaling modulation, the same effect being reported for curcumin and the flavonoid EGCG. The anti-diabetic has also displayed the ability to counteract unbalances in T cell subpopulations by activating AMPK, an effect once again shared by the polyphenol's curcumin and carnosol. Tissue plasminogen activator (tPA), a serine protease used in stroke therapy, was found to improve chemotaxis and phagocytic ability of immune cells through metabolic pathways' modulation, including ERK 1/2 and Akt signaling. An equivalent effect has been reported for curcumin and anthocyanins, due to suppression of PI3K signaling and iNOS enzymatic activity. Through these same mechanisms, curcumin also attenuates immune cell infiltration, exerting an effect similar to the NMDA receptor antagonist memantine, an anti-dementia drug widely used in Alzheimer's disease, which blocks T cells' potassium channels. Minocycline, a tetracycline antibiotic currently being studied as a therapeutic strategy for stroke, prevents DCs and microglia cells from excessive activation by modulating the JAK/STAT signaling pathway. Likewise, anthocyanins are suggested to modulate the PI3K/Akt/Nrf2/HO-1 axis in DCs and microglia cells, suppressing their overactivation. This is also achieved with the polyphenol hydroxytyrosol, which reduces MAPK activation. A similar mechanism has been observed for polyphenols belonging to different classes, such as resveratrol and kaempferol, which also regulate PPARγ, inhibiting macrophage and microglia polarization towards a pro-inflammatory (M1) phenotype. These polarization-shifting properties are likewise reported for the flavonoid phloretin and are considered to be mediated by an increased AMPK expression. These effects are analogous to the ones of insulin, which is directed to T1DM patients and interferes with PI3K/Akt and PPARγ signaling, and to the decarboxylase inhibitor carbidopa, which promotes M2 macrophage polarization in the context of Parkinson's Disease by suppressing glucose uptake. Under environmental stimuli, immune cell activation occurs accompanied by metabolic reprogramming. In most cases, this primarily consists of a transition from mitochondrial OXPHOS to aerobic glycolysis. Drugs purposed for Multiple Sclerosis treatment, such as dimethyl fumarate, IFNγ and glatarimater acetate, among others, are known to modulate this shift, suppressing glycolysis and promoting OXPHOS in T cells. The same is observed for methotrexate – an antimetabolite used to treat IBD. Analogously, by interfering with mTOR signaling and PKM2 activity, flavonoids such as shikonin and procyanidin B2 gallate, respectively, also modulate immune cell metabolic reprogramming in the context of immunometabolic dysfunctions. Figure created in BioRender.com.

Accordingly, therapy with methotrexate, which is used for different autoimmune conditions including IBD, appears to suppress glycolytic mechanisms in varied immune cell populations (205), counteracting the metabolic reprogramming associated with disease pathophysiology.

Furthermore, intestinal barrier function is also impaired in the context of the disease, presenting less mucus secretion by goblet cells, reduced antimicrobial peptides (AMPs)' production by Paneth cells and several mutations in genes coding tight junction proteins, resulting in their dysfunction and consequent loss of barrier integrity (201). Defects in mucosa mitochondrial function are also a feature of IBD, including reduced complex I activity, membrane potential, biogenesis, OXPHOS, TCA cycle and fatty acid metabolism alongside increased mitochondrial fragmentation due to fission (206, 207).

Furthermore, the disease is characterized by increased susceptibility to dysfunctional autophagy of macrophages, DCs, Paneth cells and GCs (208). Particularly, Autophagy Related 16 Like 1 (ATG16L1) gene deficiency in macrophages increases the risk of Chron's Disease development (209) and suppresses DCs' ability to induce Treg cells in contexts of intestinal inflammation (210).

Remarkably, a part of PCs' beneficial effects regarding IBD is related to their impact on autophagy. As an example, the flavonoid galangin has shown to alleviate DSS-induced colitis' symptoms in mice by increasing the expression of autophagy-related proteins and promoting colonic autophagosome formation (211). On another hand, resveratrol displayed autophagy-promoting properties in cultured macrophages through sirtuin modulation (212, 213), highlighting its potential to counteract the macrophagic autophagy dysfunction underlying IBD.

PCs further display relevant potential to maintain intestinal homeostasis by protecting the intestinal barrier. Several studies evaluating the impact of polyphenolic supplementation in experimental models of the disease have revealed improved gut barrier function, consequently limiting inflammatory cell infiltration. This has proved to be true for grape seed PCs which increase colonic goblet cell density and mucin 2 mRNA expression (214); anthocyanins by enhancing tight junction molecules (zonulin-1, claudin-1, occludin) and Muc 1/2 expressions (215), just to mention a few. Interestingly, resveratrol and resveratrol-related PCs (e.g. pterostilbene) have further demonstrated to alleviate intestinal inflammation in mice with colitis by regulating the Th17/Treg balance and control the levels of plasmatic and intestinal mucosal cytokines such as transforming growth factor beta (TGF-β), IL-6, IL-10 and IL-17 (216, 217), restoring the percentage of $CD4^+$ T cells in mesenteric lymph nodes (MLNs) and decrease their number in the intestinal LP, as well as reducing the percentage of macrophages in both regions (218). Similarly, curcumin appears to promote colonic Treg cell expansion while decreasing the counts of inflammatory DCs; inhibiting pro-inflammatory cytokines' secretion, T cell infiltration and NF-kB activation (219), as well as to suppress macrophage activation and regulate M1/M2 polarization (220). Chlorogenic acid has also shown to mitigate DSS-induced colitis in mice by inhibiting M1 macrophage polarization through suppressing PKM2-dependent glycolysis and Nod-like receptor protein 3 activation (146).

Moreover, a study performed by Wu et al. reported that the lignan arctigenin inhibits Th17 and Th1 differentiation *in vitro* by repressing STAT3 and STAT4 phosphorylation respectively through mTORC1 downregulation, ameliorating DSS-induced colitis in mice (221).

On another hand, shikonin – a polyphenol widely used in Chinese traditional medicine – has shown to suppress glucose consumption and lactate production as well as inhibit the nuclear translocation and enzymatic activity of PKM2, which is responsible for stimulating the Warburg effect in macrophages, in a DSS-induced colitis mouse model (222).

Furthermore, PCs are known for inducing short-chain fatty acids (SCFAs) production by the gut microbiota, namely butyrate (223, 224), which displays several gut health-promoting properties: it promotes colonic mucus production (225); potentiates the extrathymic conversion of CD4-positive T lymphocytes into Treg cells; is able to reduce mTOR activation and glycolysis in intestinal macrophages, while simultaneously promoting their metabolic reprogramming to OXPHOS and lipid metabolism (203) as well as downregulating their expression of pro-inflammatory cytokines (226). Remarkably, some of the aforementioned effects equally emerge from methotrexate therapy for CD, which elevates OXPHOS in T cells by activating AMPK and blocking mTORC1 (227).

In humans, consumption of mango by IBD patients significantly improved Simple Clinical Colitis Activity Index (SCCAI) score and decreased the plasma levels of pro-inflammatory cytokines related to neutrophil-induced inflammation (228).

5.1.3 Multiple sclerosis

Similarly to T1DM and IBD, Multiple Sclerosis (MS)' pathophysiology is characterized by a series of immunological alterations, the most well-known pathophysiological components of the disease (229). Although the primary events leading to the autoimmune attack characterizing MS are not yet established, a possible explanation is based on molecular mimicry consisting of the activation of autoreactive T lymphocytes through cross-reactivity by viral and/or bacterial antigens structurally similar to central nervous system (CNS) proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) (229). These cells migrate to the CNS fueling neuroinflammatory events that promote BBB opening allow a second wave of immune cells to access the CNS, namely $CD8^+$ T cells, B lymphocytes and macrophages (230). Macrophages within the perivascular cuff of post-capillary venules of animals with EAE display altered metabolism featured by increased expression of the glycolytic enzyme lactate dehydrogenase (LDHA) as well as monocarboxylate transporter-4 (MCT-4), specialized in secreting lactate from glycolytic cells, potentially inducing macrophage infiltration in the CNS (231). Analogously to what is described for T1DM, activated $CD4^+$ T cells from MS patients display an up-regulation of aerobic glycolysis and down-regulation of OXPHOS (232), as well as altered mitochondrial structure, mitochondrial DNA (mtDNA) levels and membrane potential (233). Furthermore, abnormal expression of autophagy-related markers and genes has been found in T cells from MS patients and EAE animals (234). It is worth noting that the role of autophagy in MS pathophysiology is controversial since there is evidence of both protective and deleterious effects of autophagy

induction in immune cells in the context of the disease (234), but there seems to be a consensus regarding the fact that autophagy contributes to MS pathology in macrophages, DCs, T and B cells while having a protective role in neurons and glial cells (235).

On another hand, there is a decreased count of circulating Treg cells in MS patients (236), which also seem to play an important role in EAE development (237). In fact, the immunometabolism of T cells is extremely relevant in the pathophysiology of MS, as evidenced by the fact that several of its therapeutic strategies modulate T cell metabolic features: Dimethyl Fumarate suppresses glycolysis; IFNγ decreases ATP levels, mitochondrial membrane potential and modulates OXPHOS; Teriflunomide limits T cell activation by blocking mitochondrial respiratory chain's complex III; and Glatiramer Acetate promotes OXPHOS and represses glycolysis in $CD4^+$ T cells (238). Likewise, a study evaluating the effects of cinnamic acid in EAE reported that the polyphenol acted as an MCT-4 inhibitor, attenuating immune cell infiltration into the CNS, suppressing glycolysis and lactate production by macrophages and ultimately reducing disease severity (231). The EAE-associated inflammatory phenotype of macrophages has also been reduced by the dihydrochalcone phloretin, which activated Nrf2 by stimulating AMPK-dependent autophagy (239). Additionally, a large number of PCs have shown to attenuate EAE clinical severity or inhibit its development by reducing immune cell infiltration, referring to EGCG (240), curcumin (241) and hesperidin (242). The latter two also seem to impact the Th17/Treg balance in EAE animals, promoting Treg cell expansion and Th17 suppression in the spleen (241), lymph nodes (240, 242) and the CNS, accompanied by repressed pro-inflammatory cytokine secretion (240, 241). Analogously, EGCG as well as naringenin for instance are known to impact the Th1-mediated immune response associated with EAE (240, 243). The described outcomes are potentially attributed to the impact of PCs on the expression of transcription factors associated with each of the referred T cell subsets: Foxp3 for Tregs (242, 243), RORγt for Th17 cells (241–243), and T-bet for Th1 ones (243). Inhibition of Th17 cells' differentiation by curcumin further entails down-regulating IL-6 and IL-21 as well as STAT3 phosphorylation (241). Considering that MS is a T cell-mediated disorder, inhibition of $CD4^+$ T cells' activation might comprise a promising therapeutical strategy. Interestingly, curcumin has shown to induce human T cell death through increased expression of ER stress-related transcriptional factors (244). Analogously, resveratrol inhibits $CD4^+$ T cells' activation and cytokine production by promoting SIRT1 expression and activity both *in vitro* and *in vivo* (245).The stilbene has further been highlighted as able to counteract the decline in brain mitochondrial function characterizing the cuprizone-induced demyelination model by enhancing cytochrome oxidase activity and elevating ATP levels (245).

5.2 Non-autoimmune immunometabolic diseases

5.2.1 Obesity and type 2 diabetes mellitus

Findings arising from pre-clinical and clinical research have been elucidating the mechanisms of immunological dysfunction associated with obesity and type 2 diabetes mellitus (T2DM). Regarding innate immunity, metabolic dysfunctions are characterized by an altered neutrophil functionality, increased M1 macrophage and inflammatory DCs numbers, and abnormal NK phenotypes (246). In obese individuals, neutrophils display augmented chemotaxis and non-directed migration, as well as increased basal levels of superoxide, while neutrophils from diabetic subjects lose a variety of their functions, including migration capacity, phagocytosis and ROS production (246). By increasing leptin levels, obesity alters adipose tissue macrophages' (ATMs) metabolism through Janus kinase 3 (JAK3) and STAT3, and PI3K-Akt-mTOR pathways, increasing glycolytic enzymes' activity and glucose uptake as well as inducing mitochondrial dysfunctions (247). PI3K-Akt-mTOR activation in brain macrophages of diabetic rats has further been implicated in autophagy impairment (248), which originates protein aggregates and fosters damaged mitochondria due to defective mitophagy (249). Dysfunctional mitochondria accumulation leads to increased ROS production and consequent NLRP3 dependent-inflammation by macrophages in both T2DM (250) and obesity (251). Mitochondrial dynamics are likewise affected in the context of both disorders, as evidenced by induced activation of the fission regulator dynamin-related protein 1 (Drp1) by a high-fat diet (252) as well as increased mitochondrial fission and decreased fusion in leukocytes from T2DM patients (253).

NK cells are also dysfunctional in contexts of obesity and T2DM, displaying increased proliferation rates and IFNγ secretion, and impaired degranulation, respectively (246). A dysfunctional mTOR function has also been observed in NK cells from obese patients (254). Furthermore, DCs activation and maturation is promoted in cases of diabetes, and obesity-associated DCs present an inflammatory phenotype triggering Th17 cells' activation (246).

Adaptive immunity is likewise affected by metabolic impairments, resulting in increased numbers of γδ T, Th17 and Th22 cells and a reduction in Tregs (246). B cells display altered functionality, promoting an abnormal antibody response (246). In obesity, $CD4^+$ T cells also reveal a distinct metabolic profile characterized by the activation of glycolysis and OXPHOS (255). Furthermore, mitochondria from T2DM patients' $CD8^+$ T cells display higher oxidative capacity together with elevated ROS levels and fatty acid uptake as well as decreased FAO and AMPK activity (256). In fact, metformin, a widely used oral antidiabetic, has shown to facilitate T cells' shift from a glucose-dependent anabolic state to a catabolic one through mTOR signaling blockage and by restoring mitochondrial FAO (257). Furthermore, it shuts down glycolysis and promotes OXPHOS by activating pathways involving carnitine palmitoyltransferase (CPT)-1 alpha and PGC-1α (258).

The impact of PCs in immune system dysfunctions associated with obesity and T2DM have been consistently highlighted in both *in vitro* and *in vivo* experiments. Analogously to metformin, a variety of polyphenol formulations as well as isolated compounds are described as PGC-1α inducers in the context of T2DM and obesity, such as ginger polyphenols 6-gingerol and 6-chrysophanol (259), epicatechin-enriched cocoa (260), sudachitin (261), and EGCG (262). The latter has also shown to inhibit T2DM-

associated mitochondrial deficiency and dysfunction in diabetic Goto-Kakizaki rats by suppressing enhanced autophagy in muscle cells (263, 264). Furthermore, resveratrol administration to older adult diabetics showed to improve mitochondrial biogenesis and function through SIRT1 upregulation, alleviating the oxidative damage and promoting insulin sensitivity (265).

On another hand, PCs that include capsaicin, curcumin, and anthocyanins for instance, have shown to attenuate macrophage migration (266–268), in part by suppressing MCP-1 expression (268, 269). Curcumin has also displayed relevant suppressive effects on NF-kB signaling in immune cells, leading to a reduction in iNOS expression by macrophages and DCs (270), as well as neutrophils (271). Neutrophils chemotaxis is also apparently impacted by curcumin's ability to suppress PI3K activity and Akt phosphorylation (271). On another hand, evidence suggests that PCs present in the small fruit lingonberry promote macrophage polarization to an anti-inflammatory (M2) phenotype by upregulating PPARγ and STAT6 phosphorylation in experimentally induced obesity (272). Quercetin has also shown to abolish NLRP3 inflammasome activation in macrophages by upregulating Akt signaling, reducing insulin resistance in mice with particulate matter-induced metabolic disorder (273). A similar effect has been reported for red raspberry polyphenols (274).

Regarding adaptive immunity, studies employing cafeteria diet-induced obesity as well as the alloxan-induced model of diabetes in rats have reported that PCs intake lowered the production of pro-inflammatory mediators including ILs, TNFα, IFNγ and TGF-β by MLN and splenic lymphocytes (275, 276). Contrasting to what is observed for macrophages, PCs seem to promote Treg cell recruitment, namely through elevation of Foxp3 gene expression (277). A study employing EGCG in the context of murine diet-induced obesity has reported an increased Treg/Th17 cell balance by decreasing the ratio of STAT3/STAT5 expression (278).

5.2.2 Neurological diseases

Evidence from genome-wide association studies highlight the association between immune cells-mediated inflammation and increased risk of neurodegeneration (279). Most neurodegenerative diseases involve deposition of misfolded proteins, leading to aggregate formation and consequent neuronal loss (279). The initial phases of the referred disorders are characterized by the activation of the immune system and neuroinflammation, partially mediated by a CNS resident macrophage cell population – microglia – that are activated in virtually all neurodegenerative conditions (279). Despite the pivotal role of microglia cells, infiltrations composed of astrocytes, monocytes and/or lymphocytes are also frequent in these contexts (279). Immune cell dysfunctions in the mitochondrial respiratory chain are likewise preponderant features of neurodegenerative diseases, evidence suggesting that leucocytes, neutrophils, monocytes/macrophages, and T cells display increased levels of ROS and NO accompanied by elevated mitochondrial membrane potential and decreased complex activity (238).

Considering the above, previously mentioned reports of PCs ability to limit immune cell activation and cellular infiltration point to these natural substances as promising therapeutic agents in the context of neurological disturbances.

5.2.2.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most common neurodegenerative disease worldwide (279, 280). Its main features include β-amyloid protein (Aβ) deposition and tau protein hyperphosphorylation, originating senile plaques and neurofibrillary tangles (NTFs), respectively (279, 280). The referred aggregates promote microglial activation, which surprisingly appears to play a dual role in disease pathophysiology (280). Initially, activated microglia seem to have a beneficial effect by phagocytizing excessive Aβ, but as the disease progresses, they may lose this ability and acquire a dysfunctional senescent phenotype or become neurotoxic by remaining chronically activated (280). The inflammatory mediators produced by these cells stimulate an analogous response on astrocytes, resulting in neuronal death (280). Furthermore, brain parenchyma infiltration by neutrophils and NK cells also seems to contribute to the neuroinflammatory changes reported in AD (279, 280). On another hand, the role of adaptive immune system in the disease is controversial, since there are studies supporting a neuroprotective role for the adaptive immune cells in AD animal models (281) while others exalt its requirement for disease progression (282). Noticeably, numerous lines of evidence on the impact of metabolic perturbations in microglia mediated-neuroinflammation in AD have been arising. Actually, the age-related decline in glucose metabolism in the brain is associated with cognitive dysfunctions in AD patients (283). Aβ deposition seems to induce mTOR phosphorylation and HIF-1α expression by microglia, originating inflammatory cascades (283). Microglial cells adopt a neuroimmunomodulator phenotype, exhibiting ineffective glycolysis, and TCA cycle accompanied by impaired chemotaxis and phagocytic ability (283). Furthermore, AD features a great degree of mitochondrial dysfunction with concomitant cardiolipin exposure, leading to increased microglial phagocytosis and synthesis of inflammatory mediators, fostering neuroinflammation (284). Moreover, damaged mitochondria release mtDNA which can induce the NLRP3 inflammasome and the NF-kB pathway, exacerbating inflammation (284). AD-associated microglia further display a suppressed autophagic flux due to a reduced expression of key regulatory proteins such as Beclin-1 (285).

Interestingly, Chen et al. has reported that microglial cells mediate T cell infiltration in experimental models of AD as well as human brains, driving neuroinflammation (286). Accordingly, memantine – a drug approved for the treatment of advanced AD – acts on T cell metabolism by blocking potassium channels, normalizing these cells' response (287).

Likewise, PCs exhibit vast potential as therapeutic agents in neurological pathologies by acting on different strands of their etiology, among which are immune dysfunctions. Interestingly, oleuropein aglycone – a polyphenol abundantly present in extra virgin olive oil – is reported to induce autophagy in AD mouse models by modulating AMPK signaling (288, 289) as well as sirtuin activity and histone acetylation (288). Likewise, curcumin has shown to downregulate PI3K, phosphorylated Akt and mTOR protein levels, inducing autophagy in brain tissue of APP/PS1 double transgenic AD mice (290).

Resveratrol, which is currently under investigation in several clinical trials for AD, has demonstrated to possess remarkable

immunomodulatory properties in immune cell populations highly relevant in the context of the disease. It inhibited microglia activation, proliferation and cytokine production (IL-6 and TNF-α) (291) and promoted its polarization towards an anti-inflammatory phenotype in animal models (292), suppressing neuroinflammation. Furthermore, a retrospective study with AD patients demonstrated that treatment with resveratrol induced adaptive immune responses, increasing IL-4, fibroblast growth factor (FGF) 2 and macrophage-derived chemokine (MDC) secretion by macrophages (293). Still regarding microglia cells, anthocyanins were also able to mitigate oxidative stress and neurodegeneration in a mouse model of AD by modulating the PI3K/Akt/Nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) axis (294). Additionally, supplementation of cultured microglia exposed to Aβ with a polyphenol abundantly found in extra virgin olive oil named hydroxytyrosol attenuated mitogen-activated protein kinases (MAPKs) activation as well as ROS generation (295). The flavonoid baicalein has also shown to inhibit microglia-induced neuroinflammation in a mouse model of AD by suppressing NLRP3 activation and the TLR4/NF-kB pathway (296). Similarly, a study performed by Kim et al. described gallic acid's profile as a histone acetyltransferase inhibitor, highlighting its ability to inhibit NF-kB acetylation and reducing cytokine production by cultured microglia (297).

5.2.2.2 Parkinson's Disease

On the frequency ranking for neurodegenerative diseases, Parkinson's Disease (PD) follows AD at second place and is characterized by the accumulation of α-synuclein in neurons, glial cells, and nerve fibers (279). The histopathological hallmarks of PD include loss of dopaminergic neurons in the *substancia nigra pars compacta* (SNpc), presence of activated microglia, astrogliosis and lymphocytic infiltration (279).

Similarly to what happens in AD, the accumulated protein aggregates promote microglia activation, which proceed to release excessive amounts of neurotoxic factors generating a self-amplifying cycle that contributes to progressive neuronal degeneration (279). PD-associated microglia also display impaired mitochondrial function associated with mutations in genes involved in mitophagy and oxidative stress such as Pink1 and Parkin, resulting in inflammasome activation that fosters dopaminergic neurodegeneration (298, 299). Interestingly, research in PD experimental models has shown that NLRP3 inflammasome activation is exacerbated in microglia cells deficient in autophagy related protein 5 (ATG5) (300), highlighting autophagy's relevance in suppressing inflammation. Nevertheless, the role of other immune cells such as astrocytes and NK cells in this inflammatory cascade remains to be enlightened (279, 280). On another hand, the expansion of dysfunctional monocytes appears to be an essential element in PD pathogenesis and might be related to the secretion of inflammatory mediators by microglia cells as well as pro-inflammatory monocytes' recruitment to the brain, fomenting neuroinflammation (279, 280). Furthermore, evidence suggest that M1 macrophages' activation is linked to disease susceptibility and progression (301). Accordingly, the decarboxylase inhibitor carbidopa that is used for PD management has been shown to favor macrophage differentiation to an M2 phenotype (302).

Contrasting to the occurring in AD, the role of the adaptive immune system in PD's pathophysiology is becoming clearer. In fact, numerous authors have found adaptive immune populations, namely Th17 cells, in PD patients' brain samples (303). In parallel to what is observed for AD, glucose hypometabolism has been implicated in disease pathophysiology, being associated with the development of dementia occurring in the brain cortex (283). Furthermore, PD is characterized by deregulation of several glycolytic enzymes and transporters such as pyruvate dehydrogenase kinase 1 (PDK1), PKM2, LDHA, GLUT1, MCT-1 and MCT-4, as well as increased mitochondrial respiratory activity and oxidative damage in neurons (283).

Recently, PD supplementation with PCs has been drawing attention. Once more, resveratrol exposes its neuroimmunomodulatory properties, being able to suppress microglia activation and decrease the levels of TNF-α, IL-1β and IL-6 and their receptors' expression in the SNpc of mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD (304). Analogously, curcumin administration has shown to inhibit microglial morphological alterations in an *in vitro* model (305). Curcumin has further demonstrated protective effects against neurodegeneration in the A57Tα-synuclein model of PD by downregulating mTOR/p70S6 kinase (P70S6K) signaling and recovering macro autophagy (306). Accordingly, researchers employing a nanoformulation of α-mangostin discovered that the polyphenol reprogrammes microglia metabolism from glycolysis to OXPHOS and promotes its autophagic capacity, increasing microglial Aβ clearance (307).

Regarding the mitochondrial dysfunction featuring the disease, morin and mangiferin displayed the ability to attenuate membrane potential loss in neurons (308), as well as quercetin which also enhanced mitophagy by upregulating Pink and Parkin gene expression (309).

On another hand, a study evaluating the impact of genistein in dopaminergic neurodegeneration reported a dose-dependent inhibition of neuronal loss in rats' glial cells (310). The same authors reported the soybean isoflavone's ability to suppress microglia cell activation as well as NO and superoxide production by these cells (310).

It is worth noting that evidence regarding the importance of the gut-brain and spleen-brain axes in PD has been emerging, suggesting an involvement of the intestinal and splenic immune systems in this disease development (311). Wang et al. evaluated the impact of chicory acid in mice with MPTP-induced PD and verified that this PC prevented dopaminergic brain lesions and glial activation, simultaneously reverting the disease-induced alterations in IL-17, IFN-γ and TGF-β levels in both the spleen and colon (311).

5.2.2.3 Stroke and stroke-induced neurodegeneration

Stroke is one of the global leading causes of disability and mortality (312, 313). It is currently acknowledged that the immune system is an active player in stroke's pathogenesis, possibly causing subsequent damage which are collectively designated as stroke-induced secondary neurodegeneration (SND), a condition that shares numerous features with AD, namely Aβ accumulation (314).

Similarly to AD and PD, stroke-associated neuroinflammatory events include microglial activation and consequent release of neurotoxic mediators, as well as stimulation of macrophages and DCs (315). Stroke-associated microglial cells display dysfunctional phagocytosis and chemotaxis, severely compromising neuroinflammation resolution and neurorestoration (316). Furthermore, after stroke events activated M1 microglia cells display enhanced mitochondrial fission, leading to NF-kB and MAPK activation which induces pro-inflammatory mediators' expression (317). These cells have also shown to release damaged mitochondria to neurons where they fuse with neuronal mitochondria, damaging them and promoting mitochondria-mediated neuronal death (318). Additionally, stroke-associated microglia feature increased autophagy in associated with an enhanced inflammatory response (319–321). In agreement, treatment of permanent middle cerebral artery occlusion (pMCAO) mice with an autophagy inhibitor alleviated the inflammatory response, while an autophagy inducer exerted the opposite effect (320).

NK cells and $CD4^+$, $CD8^+$ and γδ T lymphocytes have likewise revealed to be involved in stroke's initial stages, with B cell-mediated neurodegeneration becoming prominent later on in the disease course (315). Therefore, components of immunoreactivity can be found in each phase of stroke pathology. Another similarity between AD, PD and stroke is the metabolic reprogramming of microglia cells shifting from OXPHOS to glycolysis (322). In fact, stroke brains display increased concentrations of numerous glycolytic intermediates, including glucose-6-phosphate, fructose-6-phosphate, LDHA, PKM2, pyruvate, and lactate (322). Furthermore, blocking microglial hexokinase-2, the enzyme responsible for glucose phosphorylation into glucose-6-phosphate, has shown to suppress their activation and reduce the infarct area in male Sprague-Dawley rats subjected to transient middle cerebral artery occlusion (323), highlighting the role of glucose metabolism in microglia-mediated neuroinflammation characterizing stroke. Interestingly, the ability of already existing drugs to modulate macrophage and microglia metabolism in the context of stroke is being studied (324). Minocycline, which has exhibited neuroprotective activity in the context of stroke (325), has shown to promote microglia polarization from an M1 to an M2 phenotype through STAT1 and STAT6 pathways (326). Moreover, studies suggest that tissue plasminogen activator (tPA), a widely employed fibrinolytic agent in stroke therapy, is able to normalize microglial chemotaxis and phagocytosis through metabolic pathways' modulation, including Akt and ERK 1/2 signaling (327, 328).

Likewise, PCs immunomodulatory effects have been demonstrated in situations of stroke and SND as well. For instance, a study evaluating fisetin effects in a mouse model of ischemic stroke highlighted the flavanol's ability to inhibit post-ischemic infiltration of macrophages and DCs as well as repress the intracerebral activation of immune cells (329). In addition, fisetin shown to suppress TNF-α production by macrophages and microglia cells *in vitro* (329). Regarding PCs' impact on microglia, a study performed by Lan et al. showed that the flavanone pinocembrin was able to suppress microglia activation and consequent production of IL-6, IL-1β and TNF-α (330), while also decreasing the expression of TLR4 and its downstream target proteins TRIF and myeloid differentiation primary response 88 (MyD88) (330). Similarly, both curcumin (331) and baicalein (332) appeared to ameliorate ischemic brain damage a by modulating microglia polarization and suppressing TLR4 and NF-kB signaling. Gallic acid has also shown to induce microglia M2 polarization in a MCAO mouse model (333).

PCs further seem to impact stroke-associated microglia mitochondria dysfunction, as evidenced by the reduced post-ischemia neuronal mitochondrial damage resulting from kaempferol administration to rat PC12 cells (334). This effect was considered to derive from an upregulated SIRT1 expression alongside to inhibited gene acetylation of the pro-apoptotic protein P66shc as well as Drp1 recruitment.

Remarkably, recent research has demonstrated that consumption of resveratrol after stroke events might exert neuroprotection through gut-brain-axis modulation (335). In fact, the authors determined that the polyphenolic supplementation promoted a polarization shift of Th cells from Th1 to Th2, reducing intestinal inflammation and vascular permeability, which culminated in mitigation of inflammatory brain lesions (335).

6 Conclusion and future directions

In recent years, the term "immunometabolism" has gained traction within the scientific and research communities as a descriptor of the interface between the immune system and metabolism. The disruption of such complex interactions is increasingly recognized as a common denominator of a wide range of socioeconomically impactful diseases of both autoimmune and non-autoimmune nature (172). The escalating prevalence of immunometabolic disorders and the intricate interplay between metabolic irregularities and scenarios of chronic inflammation underscore the imperative to unravel the mechanisms that dictate the programming of immune cell metabolism. In fact, cells from the immune system display unique energy requirements depending on their activation state, anabolic and catabolic mechanisms, being associated with pro- and anti-inflammatory responses, respectively (336). Therefore, modulating immune cells' metabolic pathways through their respective energetic substrates may significantly impact disease outcome. From this perspective, nutritional interventions emerge as promising tools within the realm of preventive and/or adjunct therapeutic approaches, framing the concept of immunonutrition, a branch of precision nutrition aimed to fine tune pro- or anti-inflammatory immunophenotypes through personalized protocols tailored to individual requirements, health status and metabolic variability (57, 337).

Within the context of immunometabolic disorders, polyphenols stand out due to a wealth of evidence supporting their potential health benefits. Remarkably, varied polyphenols have shown to exert immunomodulatory effects, such as curtailing immune cell hyperactivity and rebalancing pro- and anti-inflammatory T cell subsets, naming a few (70). Particularly, these bioactive compounds are reported to influence immune cell metabolic reprogramming, driving a tolerogenic phenotype and mitigating inflammation, thus showcasing significant potential as key immunonutrients. Such outcomes stem from their influence on nutrient-sensing pathways

primarily involved in processes like glycolysis, mitochondrial biogenesis and dynamics as well as mitochondria-ER-lysosome inter-organelle connections, leading to epigenetic and metabolic reprogramming that yield diverse immunomodulatory effects across different cell populations as depicted in Figure 3 (338). It is worth emphasizing that a substantial portion of these regulatory pathways is similarly influenced by the pleiotropic effects of drugs included in current therapeutic algorithms for the aforementioned diseases (as illustrated in Figure 4), encouraging further exploration on how to best leverage polyphenols as immunonutrients, including optimal dosing, administration routes and potential drug-nutrients interactions requiring clarification. Likewise, precision nutrition practices must account for the metabolic and immunological changes occurring in various life stages, particularly focusing on aging and associated immunosenescence, an imperative yet unmet need.

Additional gaps surface when one considers clinical trial's experimental design and the selection of immune biomarkers in studying the efficacy of immunonutrition approaches in the scope of chronic diseases. The intricate nature of nutritional interventions, their multi-target profile, as well as defining control groups, blinding, randomization, and insufficient adherence pose substantial hurdles to study design, results interpretation, and implementation. Overcoming these limitations will undoubtedly improve the level of precision in the clinical application of polyphenols-based immunonutrition and attenuate the massive burden of immunometabolic disorders currently compose. Multi-omics models and the integration of multi-dimensional datasets comprising nutritional genomics, phenotypes and lifestyles are paramount to understand the metabolic variability between individuals and achieve personalized guidance for tailored polyphenol-based immunonutritional plans.

Author contributions

CF: Writing – review & editing, Writing – original draft, Software, Investigation, Conceptualization. PV: Writing – review & editing. HS: Writing – review & editing. JM: Writing – review & editing, Funding acquisition. MC: Writing – review & editing, Supervision. FR: Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis. SV: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Conflict of interest

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The supplementation of female dogs with live yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 acts as gut stabilizer at whelping and modulates immunometabolic phenotype of the puppies

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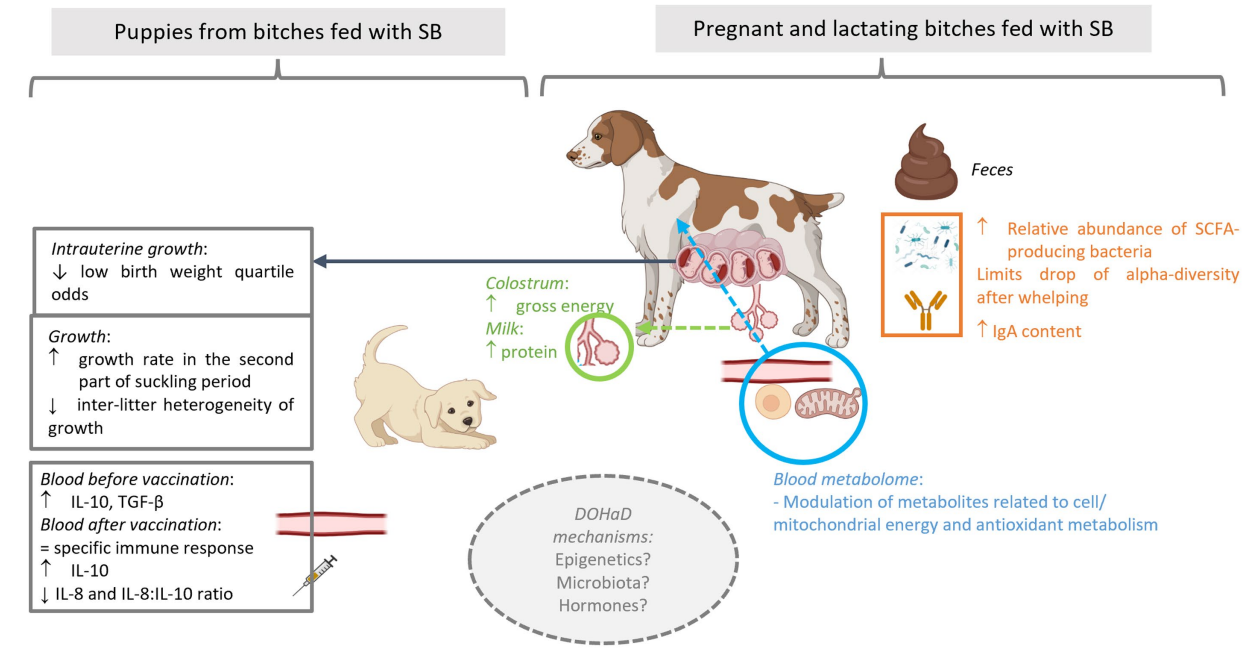
Time around parturition is a stressful period for both bitches and their puppies. The use of probiotics has been proposed, e.g., in pigs, to improve health status of sows, their reproductive performances and in turn, the health and performance of their progeny. The objective of the present study was to evaluate the impact, on both dams and puppies, of a supplementation of bitches with the live yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (SB-1079) during the second part of the gestation and the lactation period. A total of 36 bitches of medium and large-sized breeds were enrolled. They were divided into two groups, one of which received 1.3×10^9 colony forming units of live yeast per day. At dam's level, SB-1079 yeast shaped a different microbiota structure between the two groups just after whelping, impacted alpha diversity and some plasma metabolites related to energy metabolism. Regarding reproductive performances, SB-1079 improved gross energy of the colostrum (1.4 vs. 1.2kcal of ME/g) as well as the concentration of protein in milk at Day 7 after parturition (10.4 vs. 7.6%). SB-1079 also reduced the odds of having low birth weight in the litter. At puppy's level, a modulation of immunometabolic phenotype is suggested by the observation of increased growth rates during the early pediatric period (i.e., between 21 and 56days of life, 225 vs. 190%) and a decrease of the IL-8:IL-10 ratio after vaccination against rabies (4.2 vs. 16.9). Our findings suggest that SB-1079 supplementation during gestation and lactation has the potential to enhance health of bitches and in turn health of puppies through maternal programming.

KEYWORDS

yeast, maternal programming, dog, puppy, immunometabolic phenotype, *Saccharomyces cerevisiae boulardii*



1 Introduction

Gestation, *peripartum* period and lactation are challenging times for dogs. Bitches must cope with rapid changes in their physiological state (1, 2) and puppies must adapt quickly from a protected intrauterine life to an extrauterine life. How the mother manages these periods is essential to ensure her good health but also to optimally support survival, health and well-being of the puppies (3–5). For example, adequate maternal behavior and sufficient colostrum/milk production are essential, not only for puppy survival but also for their growth until weaning. In addition, it was suggested that, as in humans, early events during the fetal and neonatal life of a dog may have long-term impacts on its health. In humans, this concept of the Developmental Origins of Health and Disease (DOHaD) was supported by a large body of epidemiological and experimental data since the first hypothesis in the 1990s (6). More recently some evidence regarding the long-term effects of early life exposures was also described in dogs (7) with, e.g., an increased risk of overweight among low birthweight (LBW) puppies (8).

Given the link between reproduction and nutrition, an appropriate nutritional strategy contributes to improve the health status of dams, their reproductive performances and in turn, the health of their litters at short and long-term (9–12). Probiotics, and particularly *Saccharomyces cerevisiae* var. *boulardii* (SB), have gained extensive attention in animal nutrition and have been demonstrated to modulate digestive microbiota and support the digestive processes (13, 14). More precisely, SB exerts a trophic effect on the intestinal microbiota, resulting in beneficial effects on the microbial community diversity, structure, and support of beneficial microbes and microbial functions. Mechanisms at play are likely to combine oxygen consumption, pathogen binding, production of short-chain fatty acids (SCFA, including acetic acid), production of polyamines, B-vitamins and production of specific proteases (15). This trophic effect on the microbiota ("gut microbiota stabilizer" effect) is expected to be responsible for most of the subsequent benefits measured in the intestine and at systemic level, even if a direct crosstalk between the yeast and the host's cells cannot be ruled out. Those beneficial effects could, in turn, have a positive impact on welfare and performance of the animals. Recent scientific evidence on the use of SB demonstrated the involvement of maternal programming in sows and piglets through the dietary use of live microbial strains administrated during gestation and lactation (13, 16, 17). More precisely, feeding SB during gestation and lactation improves digestive health of the sows through an impact on intestinal transit and a stabilization of gut microbiota at farrowing, by promoting the relative abundance of fiber degrader bacteria while decreasing the presence of undesirable bacteria such as *Campylobacter*. Furthermore, SB is demonstrated as benefiting sows' performances as well as immune status and performances of their piglets (16–18).

Like in swine, canine species are polytocous, and the female (bitch) can give birth to multiple puppies in the same litter. Another similarity with swine is that puppies are born hypogammaglobulinemic, with limited glucose reserves and hypothermic, making the mother and her nursing capacities pivotal for their survival (19). Moreover, just like in piglets, birth weight is critical for the newborn dog, as low birth weight puppies present considerably increased risk of death during neonatal period (20, 21). Despite the growing interest of the scientific community regarding dog's microbiome, literature regarding the impact of live yeast supplementation on reproductive performance in canine species is scarce. The objective of this study was thus to evaluate the effect of live yeast *S. boulardii* CNCM I-1079 supplementation of the pregnant and lactating bitches on their microbiota, health, and reproductive performance and on growth and immune parameters of puppies. The hypothesis is that the "maternal programming" mediated effect observed in sows and their progeny could be extended to the canine species.

2 Materials and methods

2.1 Ethics statement

The animal study was reviewed and approved by the local ethical committee (Comité d'Éthique en Expérimentation Animale, Science et Santé Animale n°115; reference number: SSA_2020-004, Toulouse, France). The experimental protocol followed current applicable guidelines for the care and use of animals. Written informed consent was obtained from the owner of the kennel for the participation of its animals in this study.

2.2 Experimental design

2.2.1 Animals and housing

This study was conducted in a single breeding facility. Thirty-six bitches from medium (> 15 and ≤ 25 kg) or large-sized (> 25 kg) breeds (Supplementary Table S5) were selected on day 28 of gestation (G28) after a positive pregnancy diagnosis performed by the veterinary practitioner with a portable ultrasound machine (G0 = day of ovulation determined by blood progesterone assay). They were randomly divided into two groups according to the breed size format, the age at mating, parity, body condition score and faecal score. From the time of inclusion, one group was supplemented with placebo capsules (Control, $n = 18$) and the other group with capsules containing the live yeast (Yeast, $n = 18$). Then, the follow-up of the dams and their puppies after whelping lasted until separation of the puppies, around 56 days *post-partum* (DPP56). All puppies included were born by natural delivery (i.e., no caesarean section) and remained with their mothers during the entire experiment allowing them to suckle freely.

Dogs were subjected to the same housing conditions with wood shaving as bedding material all the time. From ovulation to 8 days before whelping, bitches were kept in individual outdoor pens. Then, until 35 days *post-partum* (DPP35), they were housed in individual pens in the maternity building equipped with a floor heating system and heating lamps. Finally, dams and litters were moved to individual pens in the pre-weaning building of the breeding facility until the end of the trial. All animals were vaccinated and dewormed following the veterinary protocol in place at the breeding facility (Supplementary Table S1).

2.2.2 Diets and probiotic supplementation

In order to fulfill the recommendations of the National Research Council (NRC) (22) linked to physiological stages of the bitches, two petfood manufactured by CRUSTY FOOD SAS (Montardit, Verteuil d'Agenais, France) were used. From G0 until G28, bitches received Diet 1, a complete extruded food containing wheat, peas, dehydrated poultry proteins, poultry fat, corn gluten, poultry protein hydrolysate, rice bran, meat hydrolysate, dehydrated lamb proteins, beet pulp, minerals and fish oil as feed materials; and trace minerals, vitamin A, vitamin D3 and vitamin E as nutritional feed additives. Then, from G28 to the end of the experiment (DPP56), Diet 2 was fed. It contained dehydrated poultry proteins, rice, wheat, corn, animal fat, rice bran, meat hydrolysate, corn gluten, beet pulp, minerals and fish oil as feed materials; and trace minerals, vitamin A, vitamin D3 and vitamin E as nutritional feed additives. Nutritional values of both diets are available in Table 1. Diet 2 was made available to puppies once they reached 3 weeks of age, after a transition of around one week with the same diet mixed with water. During the experiment, all dogs had *ad libitum* access to water.

The additive tested was *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (Levucell SB®, Lallemand SAS, Blagnac, France; SB-1079) given in 400-mg vegetal capsules (hydroxypropylmethylcellullose capsule size 1 clear, Suheung Co, South Korea) containing 6.25% yeast (measured at 6.4×10^8 colony-forming unit (CFU) of yeast/capsule), 92.75% potato starch and 1% silicic acid. Each dam in the Yeast group received two capsules per day (i.e., 1.3×10^9 CFU of yeast/day), one in the morning and one in the evening, in a bullet of dry food softened in hot water then turned into a bullet, made from the diet at the time (Diet 2 from G28) to ensure full consumption. The Control group was fed the same capsules in a bullet of wet petfood but without the yeast.

2.3 Sampling and measurements

To evaluate the impact of live yeast supplementation, various parameters were measured on the dams and their puppies (Figure 1).

2.3.1 Parameters evaluated at dam level

2.3.1.1 Zootechnical performances of dams

Individual body weight (BW) and Body Condition Score [BCS, 9-points scale (23)] of dams were recorded at G28, G56, DPP1, DPP28 and DPP56. The quantity of kibbles distributed before and remaining after a meal was recorded daily from G28 to the end of the experiment. Individual food intake (FI) was obtained by calculating the difference between the quantity of kibbles distributed daily and the quantity of kibbles remaining at the end of each day.

2.3.1.2 Gut health related parameters

Feces were collected for three consecutive days at four time points: G28, G56, DPP1 and DPP56, pooled for each animal and stored at −20°C before further analysis to evaluate faecal dry matter (DM) and

TABLE 1 Analysis of nutritional values of the feeds used in the study.

Parameter	Feed	
	Diet 1	Diet 2
Moisture, %	5.7	6.3
Crude ash, % as fed	8.7	9.3
Crude protein, % as fed	30.8	28.4
Ether extract[a], % as fed	15.1	16.8
Crude fibre, % as fed	2.7	<2
Carbohydrates[b], %	37.0	37.7
Vitamin E, mg/kg as fed	124	114
Vitamin A, IU/kg as fed	7,670	7,330
Vitamin D, IU/kg as fed	1,080	1,000
Metabolizable energy[c], kcal/100 g	385.8	408.6

[a]Crude fat.
[b]Carbohydrate was calculated from equation: %Dry Matter−(%Ether extract + %Crude Protein + %Crude Ash + %Crude fibre).
[c]Metabolizable energy was calculated from equation (22, 24).

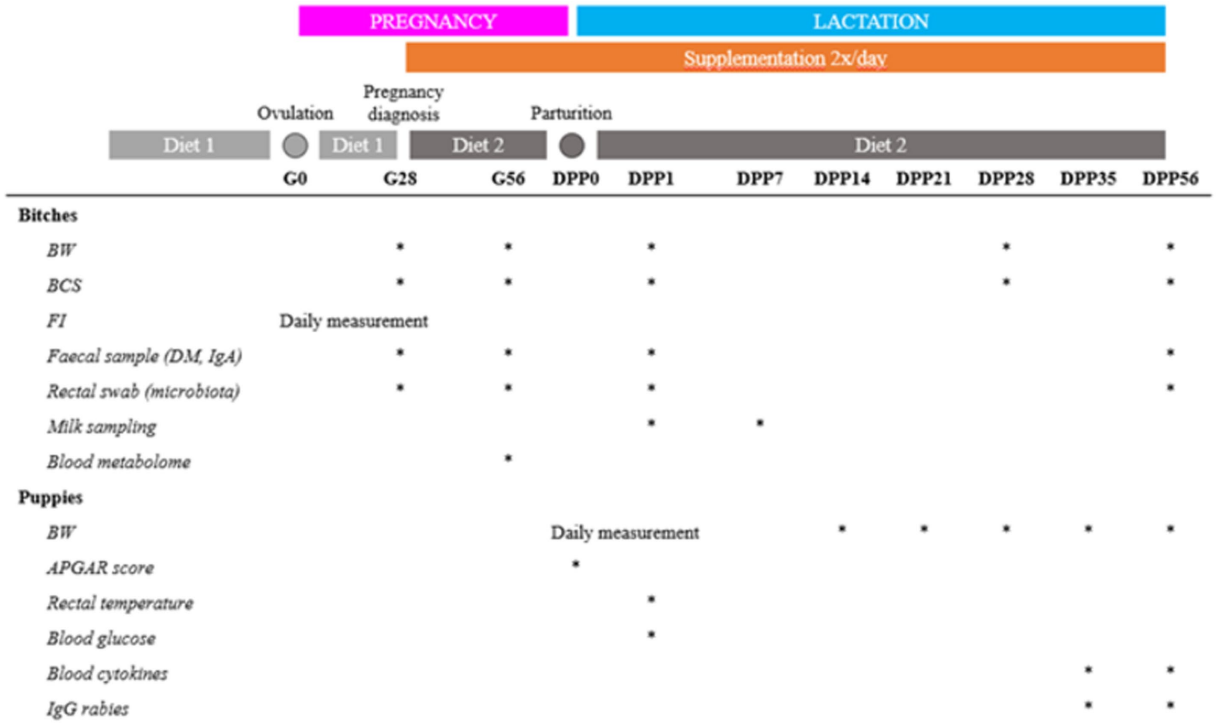


FIGURE 1
Schematic of the trial design. BW = body weight (kg), BCS = body condition score, FI = food intake, DM = dry matter, Gx = day x of the gestation, DPPy = day y post-partum.

immunoglobulin A concentration (IgA). In addition, at the same time points, a faecal swab was collected and stored at −80°C to perform 16S rRNA amplicon sequencing and determine faecal microbiota composition.

2.3.1.3 Blood metabolome

Blood was collected from the cephalic vein before whelping at G56 on a heparinized tube. The blood was centrifugated (16,000 RCF for 10 min) to isolate the plasma, and two aliquots of 250 µL each were put in the Eppendorf tubes and stored at −80°C for further analysis.

2.3.1.4 Reproductive performances

For each dam, reproductive performances were evaluated through litter composition (number of born alive and stillborn puppies), losses (number of puppies dying from birth to DPP56) and colostrum/milk quality. Colostrum and milk were collected at DPP1 and DPP7, respectively, to determine nutritional content, IgG (for colostrum) and IgA concentrations. Before collecting the milk, an intramuscular administration of 1 or 2 UI of ocytocin was performed.

2.3.2 Parameters evaluated at puppy level

Each of the 254 puppies from the 36 litters born alive were identified at birth using a colored collar.

2.3.2.1 Newborn viability

APGAR score was recorded immediately after delivery except for puppies born between 9 p.m. and 4 a.m. A score of 7–10 means no distress, 4–6 means moderate distress and 0–3 means severe distress (25). Bodyweight at birth (BWb) was measured in the first 12 h after birth using a digital scale (EB3 Series, Ohaus, Parsippany, NJ, United States, maximum capacity of 5 kg, precision ±0.1 g). To deal with the variability of BWb depending on the breed (20, 26), puppies were categorized into quartile groups based on their breed and BWb (20, 21). Quartile 1 (Q1) included the 25% puppies with the lightest BWb, while quartile 4 (Q4) included the 25% heaviest. Threshold values for the targeted breeds (Supplementary Table S2) were defined based on the 4,010 BWb registered in the breeding facility during the last seven years. At DPP1, rectal temperature and blood concentration of glucose were measured.

2.3.2.2 Growth of puppies

In addition to weights as such, two growth rates (GR) were calculated [GR 0–21 and GR 21–56, Eq. (1)].

$$GR\ x-y\,(in\%) = \left[\begin{pmatrix} weight\ at \\ Day\ y- \\ weight \\ at\ Day\ x \end{pmatrix}\right] / \ weight\ at\ Day\ x \times 100 \quad (1)$$

2.3.2.3 Vaccination, serum specific IgG and cytokines

57 puppies selected according to their health status and BWb were included for cytokines and immune response analyses. At DPP35, puppies were vaccinated against rabies (Rabigen mono, Virbac). Just before vaccination (DPP35) and at the end of the trial (DPP56) blood samples were collected from jugular vein in dry tubes. The blood was centrifugated (16,000 RCF for 10 min) to isolate the serum, and two

aliquots of 500-750 µL each were put in the microcentrifuge tubes and stored at −80°C for further analysis.

2.4 Chemical analyses and DNA extraction

2.4.1 Chemical analyses of diets

The experimental diets were subjected to Weende (proximate) analysis. They were dried to a constant weight at 103°C to determine dry matter (DM, ISO 1442, 1997). Crude ash was determined by combustion at 550°C (ISO 936, 1998). Crude protein was calculated from Kjeldahl nitrogen (6.25 × N, ISO 5983-1, 2005). Crude fibre was analysed by acid-alkali digestion (ISO 5498, 1981), and crude fat was analysed using acid-hydrolysis followed by Soxhlet extraction (ISO 1443, 1973). Analysis was performed by an external lab (Upscience, Vannes, France).

2.4.2 Faecal dry matter and IgA

200 g samples of pooled faeces were used to determine faecal DM using freeze-dried method (University of Bologna, Italy, internal protocol). IgA content in lyophilized faecal samples was carried out by using a commercial kit (dog IgA ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery, TX, United States; assay range: 15.6–1,000 ng/mL), following the procedure described by Zannoni et al. (27).

2.4.3 Faecal microbiota

DNA was extracted from rectal swabs using Quick-DNA Faecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, United States) following the manufacturer's instructions. Quantification of extracted DNA was checked using a fluorometric method with Quant-iT™ PicoGreen® dsDNA assay kit (Life Technologies, Carlsbad, CA, United States) measured via QuantStudio™3 Real Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, United States). The V3-V4 region of the 16S rRNA gene was amplified by PCR using universal primers 341F (CCTACGGGAGGCAGCAG) and 806R (GGACTACNVGGGTWTCTAAT). PCR amplicons were purified with HighPrep PCR system (MAGBIO GENOMICS, Gaithersburg, MD, United States) and used for library construction with the Illumina NEXTflex PCR-Free DNA sequencing kit (Bioo Scientific corp., Austin, TX, United States). Amplicon libraries were sequenced on an Illumina MiSeq 2,500 platform (Illumina, San Diego, CA, United States) at GeT-PlaGe INRAE Platform (Toulouse, France) for paired-end fragment sizes of 250 bp. All reagents used were molecular grade.

2.4.4 Milk and colostrum composition

Protein, sugar and lipid assays from milk and colostrum samples were carried out in collaboration with the Department of Nutrition at the Smithsonian Institute in Washington using standard colostrum and milk analysis methods developed by Hood et al. (28). For the determination of dry matter, the colostrum and milk samples were weighed, dried in a forced convection oven for 3.5 to 4 h at 100°C and then reweighed. Total nitrogen was determined using the Kjeldahl method with a Dumas nitrogen gas analyser. The total amount of nitrogen was then multiplied by 6.38 to determine total protein (29). Sugars were determined by phenol-sulphuric acid colorimetric procedure using a standard range of lactose monohydrate (30) and read at 490 nm on a microplate reader (Model ELX808; BioTek, Winooski, VT). Fat content was determined using the Röse-Gottlieb method (28), which involves three sequential extractions with diethyl ether and petroleum ether after disaggregation of milk fat globules with ammonium hydroxide and ethyl alcohol. All above nutrient contents were expressed as the percentage of nutrient content per 100 g of colostrum or milk. The gross energy (GE) content of colostrum and milk were calculated from the values of protein, sugar and lipid content using Eq. (2) with fat, protein and sugar expressed as percentage (31).

$$GE = 9.11 \times fat + 5.86 \times protein + 3.95 \times sugar \qquad (2)$$

This equation is likely to slightly overestimate GE because it does not correct for non-protein nitrogen. However, it has been verified against GE values measured by calorimetry (32). Finally, milk was stored at −20°C until IgA and IgG assays using a previously described and validated ELISA method (Dog IgG and Dog IgA ELISA Kits, Bethyl Laboratories, Montgomery, United States) (33, 34).

2.4.5 Serum cytokines determination

The IL-8, IL-10, IFN-α, IFN-γ TNF-α and TGF-β concentrations in serum were tested by ELISA. The range of minimum detection, sensitivity and type of sample that could be used for each cytokine are listed in Supplementary Table S3. Once performed according to manufacturer instructions, the results were read in a spectrophotometer (Biochrom Anthos 2010 Microplate Reader, Biochrom LTD, Cambridge, UK) at the wavelength indicated for the dye used (450 nm). The quantification was made by means of a standard curve. In all cases will be included positive and negative controls (blanks).

2.4.6 Specific IgG against rabies

Quantification of anti-rabies antibodies was assessed using fluorescent antibody virus neutralization (FAVN) test, as recommended in the OIE Terrestrial Manual 2018 (35). Prior to the test, 80 µL of puppies' serum were diluted with 160 µL of Dulbecco's Modified Eagle Medium (DMEM) with 10% of fetal calf serum. All puppies with levels of antibody equal to or above 0.5 UI/mL were considered protected.

2.4.7 Metabolome

Polar and semi-polar metabolites were extracted from the plasma of the bitches by protein precipitation using methanol. 50 µL of plasma sample was homogenized in 200 µL of −20°C cold methanol. After shaking and one-hour incubation at −20°C, samples were vortex homogenized and centrifuged for 15 min at 4°C and 16,000 relative centrifugal force (RCF). Supernatants were than filtered through a 10 kDa centrifugal filter (VWR®, Rosny-sous-Bois, France, 10 kDa), dried under the gentle nitrogen stream and resuspended in 125 µL of water/acetonitrile/formic acid (90/10/0.1, v/v/v). Samples were transferred in to the 0.45 µm centrifugal filters (VWR®, 0.45 µm) and filtered for 15 min at 16000 RCF and 4°C. 50 µL of sample was transferred into the vials and stored at −80°C before the analysis. Quality control samples, pool samples were prepared by assembling an equal volume of each analyzed plasma sample. Blank samples were prepared following the same extraction protocol but for water instead of plasma.

All samples were analyzed using high performance liquid chromatography (Dionex UltiMate 3,000, Thermo Scientific, Bremen, Germany) hyphenated to a high-resolution mass spectrometry (Q-Exactive Plus hybrid mass spectrometer, Thermo Scientific). Samples were analyzed randomly, and pool samples were injected every five biological samples. Reverse and normal phases were used for the chromatographic separation. For reverse phase, a Hypersil Gold C18 (100 mm × 2.1 mm × 1.9 µm) (Thermo Scientific) column was used. Oven temperature was set at 40°C. The flow rate was maintained at 0.4 mL/min, and 0.1% formic acid solutions in water and acetonitrile were used in mobile phases A and B, respectively. A first minute at 0% of B in the isocratic elution was followed by ten minutes on a linear gradient to 100% B, which was then maintained in isocratic mode for two minutes. Gaining initial conditions in one minute was followed by two minutes column equilibration. For normal phase separation, a HILIC (Se-Quant, ZIC-HILIC Peek Coated 150 × 2.1 mm × 5 um, Merck) column was used. The oven temperature was kept at 25°C, and the flow rate at 0.25 mL/min. 16 mM ammonium formate in water was a mobile phase A, and 0.1% formic acid was mobile phase B. Chromatographic separation was done as follows. For first two minutes phase B was kept at 97%, then for 8 min decreased to 70%, for 5 min decreased to 10% and stayed isocratic for 2 min. After this gradient, the initial conditions were achieved in one minute and the column equilibration was achieved in nine minutes. Data acquisitions were obtained in a switching ion polarities mode in the *m/z* 80–100 range and with resolving power 35,000 FWHM (for *m/z* 200). Electrospray needle was kept at ±3.5 kV, S-lens RF level of 55 and a capillary temperature of 320°C. Sheath gas, auxiliary gas and sweep gas flow rates were maintained at 30, 8, and 0 arbitrary units, respectively. MS/MS spectra were acquired on a pool sample using High Collision energy Dissociation (HCD) and Data Dependent Analysis (DDA) method to obtain structural information for the large palette of metabolites.

Raw data were converted to mzXML files and processed using XCMS library in the R environment. Parameters applied for the data processing in each analysis mode are described in Supplementary Table S4.

Tables of each analyzing mode, containing the *m/z,* retention times and intensities of the signals detected in all analyzed samples, were than filtered (coefficient of variation <30% in the repeated QC samples) and normalized (Van-der-Kloet algorithm), and only stable peaks were submitted to the annotation using our in-house database containing the possible adducts *m/z* and retention times of around 1,300 endogenous metabolites. Attributed annotations were verified by comparing the reference and pool MS/MS spectra.

2.5 Statistical analyses

Statistical analyses were performed using R software (version 4.1.0) (36) for all parameters except microbiota for which QIIME2 (version 2020.2) (37) was used.

2.5.1 Models used

The quality of the randomization based on the chosen criteria (age, BCS, faecal score and parity) was assessed using a nonparametric Wilcoxon-Mann–Whitney (WMW) rank test between Yeast and Control groups.

2.5.1.1 Food intake, body weight, body condition score, faecal DM

The effect of the supplementation with SB-1079 on FI (total, during gestation and during lactation) was analysed using linear model with treatment, breed size, litter size and age of the dam as fixed effects (38). BW and BCS were also analyzed using linear mixed models with the same fixed effects plus time, interaction "time × treatment" and "dam" as random effect using lme4 and lmerTest packages (39, 40).

2.5.1.2 Colostrum and milk composition

A linear model was used with treatment, breed size, litter size and dam's age as fixed effects.

2.5.1.3 Litter size and health related endpoints

Count data (i.e., number of puppies born, stillborn, number of weaned puppies) were analysed with generalized linear model (GLM) with a Poisson distribution. Treatment, breed size, litter size (except for number of puppies born) and age of the dam were used as fixed effects.

2.5.1.4 Quartiles of birthweight

First, a Chi-Square analysis was performed to evaluate the repartition of puppies within quartiles between the treatment groups. Afterwards, a multinomial regression was performed using the treatment, the litter size, the age of the dam as fixed factors and individual/female dog as random effect with the ordinal package (note: breed size was not included in the models as this effect was considered in the variable construction).

2.5.1.5 Growth parameters

The effect of the supplementation with SB-1079 on growth rate was analysed using a linear mixed model with treatment, breed size, litter size, age of the dam, quartile of puppies' BWb as fixed effects, dam as random effect. This model allowed to handle correlation among puppies of the same litter and thus to avoid the pseudo-replication that using dogs of the same litter would cause (20, 41).

To adjust multivariate models, litter size was classified into three classes depending on the number of puppies born: less than 7 (Class 1), 7 or 8 (Class 2) and more than 8 (Class 3). Age of bitch at mating is an important factor associated to the reproductive performance (42, 43). It was included using three classes: ≤ 3 years (Class 1),[3; 4 years] (Class 2) and > 4 years (Class 3).

2.5.1.6 Faecal IgA in dams, serum cytokines and specific IgG in puppies

For all those parameters, the yeast effect was evaluated using nonparametric WMW rank tests.

2.5.1.7 Faecal microbiota

All bioinformatics analysis were done according to Garrigues et al. (44) in QIIME2 (version 2020.2) with Greengenes (gg-13-8-99-nb-classifier) using sklearn classifier method according to Bokulich et al. (45). To compare paired differences in alpha-diversity (e.g., the microbial diversity within the faecal ecosystem – i.e. intra-individual diversity of the faecal microbiota, evaluating using the Shannon index), a longitudinal analysis was done using a non-parametric Prentice signed-rank test within treatment and nonparametric WMW

rank test between treatments. PERMANOVA on the Aitchison distance matrix was used to assess the benefits of the yeast on the beta-diversity (i.e., the interindividual diversity of the faecal microbiota) in QIIME2 (version 2020.2). Finally, linear discriminant analysis effect size (LEfSe) (46) from Galaxy was used to identify differences in taxonomy data between the two groups just after whelping and the average composition during the whole study.

2.5.1.8 Metabolomics

Collected, filtered and annotated data from C18 and HILIC chromatographic separation and from positive and negative ionization modes of 83 plasma samples were merged in one non-redundant table containing 228 putatively identified molecules. When a metabolite was detected in both ionization modes or in both chromatographic modes (C18 and HILIC), the one chosen corresponded to the least coefficient of variation in the QC sample and to the highest raw intensity measured. A partial least-squares discriminant analysis (PLS-DA) was used to observe and discriminate bitches fed or not with SB-1079.

2.5.2 Models' construction and validation

Fixed effects and covariates were chosen for their biological relevance. Collinearity was assessed graphically to build the statistical model while a score called the variance inflation factor [VIF, (47)] was calculated to validate the final model. This score measures how much the variance of a regression coefficient is inflated due to multicollinearity in the model. The smallest possible value of VIF is 1 (absence of multicollinearity). As a rule of thumb, a VIF value that exceeds 5 indicates a problematic amount of collinearity (48).

For linear models and quantitative outcomes, adjusted means and standard errors (SE) obtained in the Control and Yeast groups, respectively, were reported, as well as the *p*-values for fixed effects. When required, Tukey *post-hoc* tests were performed (49). Assumptions of normality and homoscedasticity of the residuals were verified with the visual observation of studentized residuals' plots, and with statistical tests of Shapiro and Bartlett for normality and homoscedasticity of the residuals, respectively. If assumptions were not fulfilled, a non-parametric Wilcoxon rank sum test was performed.

For all generalized linear models using a Poisson distribution (number of puppies), the appropriateness of the model to the data was assessed by Pearson residuals test. The dispersion of the residuals was evaluated by the ratio between the residual deviance and the number of degrees of freedom and by a dispersion test (50), testing the null hypothesis of equidispersion in Poisson GLM against the alternative of overdispersion. If surdispersion was detected, a model using a quasipoisson law was performed. *p*-value, incidence ratio, and 95% confidence interval are reported for each regression.

For multinomial regression (51), the appropriateness of the model to the data was assessed by Pearson residuals test. To confirm the importance of the yeast treatment (and other fixed effects) in the model, a likelihood ratio test was performed using nested models, one with and the other without the treatment (or the targeted fixed effect), with a comparison between different models to check the significant of each model tested. Dispersion parameter was also checked by the ratio between the residual deviance and the number of degrees of freedom which should be close to 1. *p*-value of comparison between Control and Yeast groups, odds ratio, and 95% confidence interval are reported.

For all the statistical procedures, an effect was considered as statistically significant with $p < 0.05$, a biological trend was noticed for $p < 0.10$.

3 Results

3.1 Description of the population studied

The final bitches' cohort was composed of 4 and 5 medium-size and 14 and 13 large-size breeds enrolled in the Control and Yeast group, respectively (Supplementary Table S5). No statistical difference was detected for all the parameters considered for the randomization, confirming that the animals were homogeneously balanced between the two groups based on the chosen criteria (Supplementary Table S6).

3.2 Impact of the supplementation at dam's level

3.2.1 Bodyweight, body condition and food intake

No difference was observed between experimental groups at any time for BW, BCS and FI of the dams (all $p > 0.10$, Table 2). As anticipated due to their physiological status, a time effect was observed for BW, which was higher at the end of gestation (G56), lower at DPP1 and intermediate at G28, DPP28 and DPP56 (Table 2). BCS was stable all along the study except just after parturition (DPP1), where it transiently decreased in both groups.

3.2.2 Gut health of the dams

For faecal DM, the interaction between time and treatment tended to be significant ($p < 0.1$). Indeed, a reduction of faecal DM over the course of the experiment was detected in the Control group while it remained stable in the Yeast group (−2% vs. 0%).

Faecal IgA concentrations averaged between G56 and DPP56 tended to be higher in the Yeast group compared to the Control group (23.3 ± 3.17 vs. 14.5 ± 1.98 mg/g, $p < 0.1$) with a significant difference at DPP1 (11.8 ± 2.83 and 23.0 ± 4.16 mg/g DM for Control and Yeast group respectively, $p < 0.05$). No time effect was observed on faecal IgA concentration (data not shown).

The evolution of the Shannon index as a marker of alpha diversity in the faecal microbiota differed between treatments across the experimental period (Figure 2A). In the Yeast group, the alpha diversity did not significantly change during the study period even if a gradual numerical increase was described during lactation. In particular, there was no marked reduction in the alpha diversity around whelping (G56) contrary to what was observed in the Control group. Indeed, significant differences were identified in the Control group between G28 and G56 and then G56 and DPP1.

Whatever the treatment and the time of sampling, the microbiota of the bitches was mostly composed of taxa belonging to the phylum Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria and Proteobacteria, and family Corynebacteriaceae, Fusobacteriaceae, Bacteroidaceae, Clostridiaceae, Veillonellaceae, Lactobacillaceae, Lachnospiraceae, Paraprevotellaceae and Streptococcaceae (Figure 2B). A PERMANOVA analysis showed that the beta-diversity significantly differed at DPP1 between Control and Yeast groups (Figure 2C;

TABLE 2 Average body weight, body condition score, gestation and lactation durations and food intake of bitches supplemented or not with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079.

Parameters	LS means ± SE		*p*-value (fixed effect)					
	Control	Yeast	Treatment	Time	Treatment × Time	Age	Breed size	Litter size
BW, kg	29.3 ± 1.12	26.9 ± 1.11	0.117	<0.001	0.758	0.346	<0.001	0.684
BCS	4.96 ± 0.31	4.78 ± 0.31	0.667	<0.001	0.647	0.422	0.274	0.703
Gestation duration, d	61.6 ± 0.24	61.9 ± 0.23	0.355	NR	NR	0.122	0.017	0.702
Lactation duration[a], d	58.6 ± 0.61	57.8 ± 1.53	0.650	NR	NR			
FI gestation, g/d	524 ± 25.7	529 ± 25.3	0.784	NR	NR	**0**.068	0.005	0.626
FI lactation, g/d	936 ± 75.0	838 ± 73.8	0.409	NR	NR	0.124	0.173	0.893
FI overall, g/d	784 ± 50.3	729 ± 49.5	0.523	NR	NR	0.077	0.071	0.823

[a]Wilcoxon-Mann–Whitney rank test (conditions for a linear model not fulfilled). Raw means are given in this case.
LS means = adjusted least square means, SE = standard error, BW = bodyweight, BCS = body condition score, FI = food intake, NR = non relevant (time effect was not tested), d = day.

Table 3), where relative abundances of *Clostridium hiranonis*, *Faecalibacterium prausnitzii* and *Megamonas* were higher in the Yeast group compared with Control group (Figure 2D), together with *Dorea* and *Bacteroides plebeius* identified by LEfSe discriminant analysis (Supplementary Figure S1). These taxa were also detected across the entire study, suggesting a lasting effect of the yeast on the microbiota of dogs. It is also interesting to note that along the study, the microbiota of bitches in the Control group was characterized by higher relative abundances of *Proteus, Mycoplasma, Tissierellaceae*, Pasteurellaceae or *Vagococcus* compared to the Yeast group (Figure 2E).

3.2.3 Plasma metabolome

In order to identify relevant metabolites allowing to discriminate, at G56, bitches fed or not with SB-1079, two successive PLS-DA were used. The first was used as a basis for fitting a more robust model by selecting the most discriminant metabolites, i.e., those with a variance importance in projection (VIP) score greater than 1.48. The final PLS-DA (R2Y = 0.562, Q2Y = 0.466) was adjusted with 1 latent variable and based on 23 metabolites. The result of cross-validated analysis of variance (CV-ANOVA; $p < 0.01$) indicated that the final PLS-DA model was valid and was able to discriminate between bitches of Control or Yeast group. It highlighted 23 discriminant metabolites (Supplementary Figure S2): xanthosine, N-acetylneuraminate, 2-aminopyridine-3-carboxylic acid, 2-hydroxybutyrate, L-alanine, 16:0-lysophosphatidylcholine, 18:0-lysophosphatidylcholine, indole-3-acetate, itaconate, 16:0-lysophosphatidylinositol, kynurenic acid, 4-methyl-L-glutamate, 2-hydroxyisocaproic acid, butyryl- and octanoyl-L carnitines, 3-hydroxybutanoate, 4-coumarate, 5-methylthioribose, O-acetylserine, ureidopropionate, creatine, creatinine and trans 4-hydroxy-L-proline.

3.2.4 Reproductive performances

3.2.4.1 Colostrum and milk composition

As shown in Table 4, gross energy of the colostrum was significantly higher in the Yeast group compared with the Control ($p < 0.05$) and a tendency of increased glucose concentration was noted ($p < 0.1$). In addition, the concentration of protein in milk was found significantly higher in Yeast than those in Control group ($p < 0.05$), while a trend of higher gross energy was also observed ($p < 0.1$).

3.2.4.2 Litter composition and puppy viability

In total 284 puppies were born during the study (142 in each group) with 254 puppies born alive. As shown in Table 5, no difference in the number of puppies born or in the number of stillborn puppies was evidenced between the two groups. However, the multinomial regression demonstrated that the odds of having puppies with BWb from Q1 and Q2 was lower in Yeast compared with Control group (Table 6; Figure 3A). A litter effect was also observed, with the largest litters having greater odds to have LBW puppies (Q1).

Concerning puppy's general health, no effect of yeast was obtained on APGAR score, rectal temperature or glycaemia (all $p > 0.10$) which averaged 8.2 ± 0.2, 36.1 ± 0.2°C, and 125.5 ± 5.4 mg/dL, respectively (data not shown). A total of 221 puppies were weaned resulting in a mortality rate from birth to DPP56 of 21.8%, with no difference between the two groups (Table 5).

3.3 Impact of supplementation on immunometabolic phenotype of puppies

3.3.1 Postnatal growth of puppies

The supplementation of the bitches with the live yeast did not change the GR 0–21, while increasing the GR 21–56 ($p < 0.05$, Table 6). Interestingly, a significant effect of BWb on GR 0–21 was observed, with higher values in LBW categories ($p < 0.01$, Table 6 and Figure 3B). In addition, a tendency towards a greater GR 0–21 was observed in large-sized puppies compared with the other sizes ($p < 0.1$, Table 7).

In addition, the variation coefficient of litters' average body weight from DPP4 to DPP56 was significantly reduced in puppies from bitches of the Yeast group compared with those of the Control group (Figure 3C; asymptotic test for the equality of coefficients and modified signed-likelihood ratio test (SLRT) for equality of coefficients, $p < 0.05$). At the end of the trial (DPP56) variation coefficients of litters' average BW were 33.2 and 14.2% for Control and Yeast group, respectively.

3.3.2 Immune parameters

Before vaccination (DPP35), puppies from bitches of the Yeast group had higher serum concentrations of anti-inflammatory cytokine IL-10 and of TGFβ, while the 3 other pro-inflammatory cytokines were unaffected (Table 8). After vaccination (DPP56), serum

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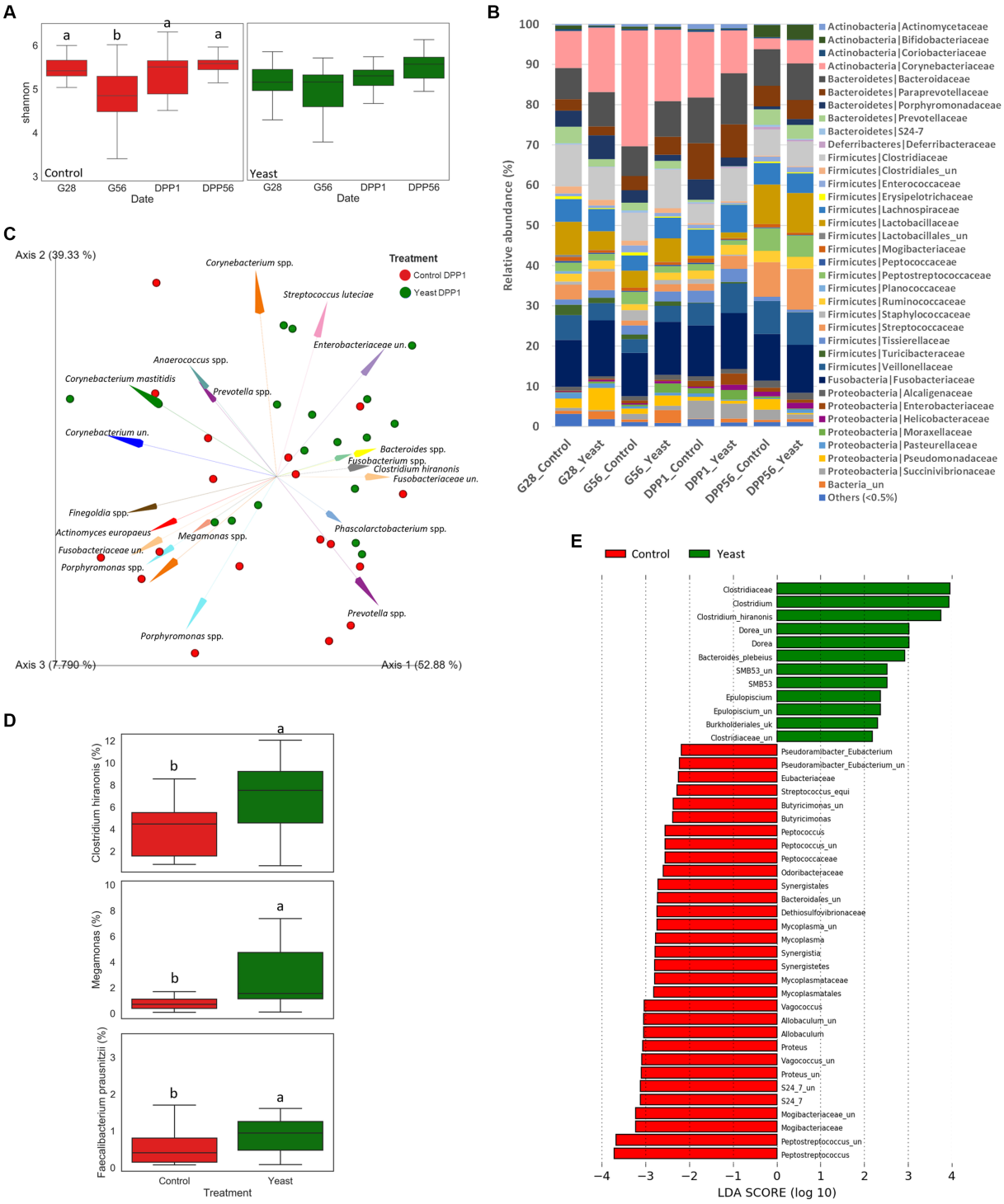

FIGURE 2
Impact of the supplementation with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on gut health of bitches during the second part of the gestation and the lactation period. **(A)** Box and whiskers plots of the Shannon index evolution per treatment during the trial. Significant differences (in control group) are shown with different letters at the top of the boxplots ($p < 0.05$). **(B)** Phylum and family relative abundances (%) at the different time points for each group during the trial. **(C)** Aitchison distances characterizing the supplemented and the control groups at one day after parturition (DPP1) ($p < 0.05$) (un.: unknown genus or species). **(D)** Box plots of 3 key taxa relative abundances at one day after parturition for each group. Significant differences are shown with different letters et the top of the boxplots ($p < 0.05$). **(E)** Linear discriminant analysis effect size (LEfSe) for the overall period.

concentrations of IL-8 tended to decrease while serum contents of IL-10 tended to increase in puppies from yeast-fed mothers, resulting in decreasing the IL-8: IL-10 ratio (Table 8). The evaluation of the specific immune response after vaccination measured by anti-rabies IgG titration revealed that whatever the experimental group all puppies were protected against the virus (Table 8).

TABLE 3 Effect of a supplementation with the additive on the beta-diversity using PERMANOVA on the Aitchison distance matrix at day 1 *post-partum* (DPP1)[a].

Group comparison	Aitchison distance	Pseudo-*F*-value	*P*-value
Aitchinson distance, DPP1			
Control–control (within group)	1.945 ± 0.880	4.076	0.02
Yeast–yeast (within group)	1.634 ± 0.789		
Control–yeast (between groups)	1.984 ± 0.909*		
Aitchinson distance, from G56 to DPP56			
Control–control (within group)	1.953 ± 0.024	1.079121	0.338
Yeast–yeast (within group)	1.860 ± 0.016		
Control–yeast (between groups)	1.826 ± 0.013		

[a]$P < 0.05$ in the PERMANOVA test means that the distances between the two groups are significantly different.

TABLE 4 Impact of the supplementation with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on composition of colostrum and milk ($n = 18$ per group).

	LS means ± SE		*P*-value			
	Control	Yeast	Treatment	Age	Breed size	Litter size
Colostrum (DPP1)						
Dry matter, %	21.0 ± 0.87	22.4 ± 0.89	0.375	0.669	0.022	0.909
Crude protein, %	8.96 ± 0.74	9.46 ± 0.76	0.396	0.912	0.507	0.013
Sugars, %	2.64 ± 0.19	3.08 ± 0.20	*0.059*	0.550	0.186	0.396
Fat, %	6.80 ± 0.61	7.78 ± 0.60	0.117	0.005	0.563	0.998
Gross energy, kcal/g	1.24 ± 0.60	1.39 ± 0.62	0.015	0.032	0.337	0.056
IgA, g/L	12.3 ± 3.05	16.3 ± 3.00	0.512	*0.058*	*0.063*	0.848
IgG, g/L	41.8 ± 7.39	44.0 ± 7.27	0.776	0.031	*0.062*	0.737
Milk (DPP7)						
Dry matter, %	20.8 ± 0.72	22.2 ± 0.65	0.306	0.456	0.043	0.551
Crude protein, %	7.64 ± 0.83	10.4 ± 0.76	0.042	0.003	0.316	0.592
Sugars, %	3.56 ± 0.02	3.46 ± 0.02	0.966	0.398	0.428	0.699
Fat, %	8.98 ± 0.71	9.53 ± 0.65	0.416	0.198	0.473	0.863
Gross energy, kcal/g	1.41 ± 0.09	1.61 ± 0.08	*0.093*	0.215	0.330	0.706
IgA, g/L	13.0 ± 2.60	18.1 ± 2.52	0.297	0.003	*0.083*	0.830

LS means = adjusted least square means, SE = standard error, DPP = day post-partum.

4 Discussion

The gestation, peripartum and neonatal periods are challenging for bitches and their puppies. The aim of the present study was to evaluate whether supplementing the dams with live yeast from mid-gestation until separation of the puppies could support the animals during these periods. The effect of supplementation was investigated both in the dams and in their offspring.

Disruption of the maternal microbiota around birth could compromise normal bacterial colonization of the infant's gastrointestinal tract, and, in turn, increase susceptibility to inflammation and reduce gut barrier function (52–54). Measuring the microbiota resilience is a good way to evaluate the impact of the *peri-partum* period, which is a physiological stress, on the health status of the bitches and their offspring (55). The diversity index has been described as a good indicator of gut resilience in humans (56, 57). Previous work in humans has shown that the diversity of bacterial species within an individual typically declines with gestation and this may contribute to microbiota unbalance (58), probably because of hormonal storm. In our study, in agreement with this observation, significant differences were identified in the Control group between G28 and G56 and then G56 and DPP1; the Shannon index being the lowest at G56. Thus, the supplementation with SB-1079 seemed to limit the variation in the microbiota diversity around parturition. Interestingly, same evolution of alpha-diversity is described in sows, for which microbiota richness is reported to be the lowest on day 1 before and day 3 after parturition, and such result has also been reported in sows (59, 60). Thus, adding SB-1079 before a physiological challenge like parturition can be an interesting strategy to improve microbiota resistance and/or recovery, both components of the resilience.

Overall, the microbiota of the bitches was characterized by ASVs typical of healthy adult dogs like Fusobacteriaceae, *Enterobacteriaceae*,

TABLE 5 Impact of the supplementation of the dam with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on litter size, number of puppies born alive, and number of puppies weaned and mortality at weaning (n = 36 litters and 254 born alive puppies).

Item	Control	Yeast	IRR ± SE	95% CI	*P*-value
Litter size					
Intercept			7.86 ± 1.13	6.19–9.85	<0.001
Treatment (control as reference)	142	142	1.01 ± 1.13	0.80–1.28	0.935
Age (class 1 as reference; ≤3 y)					
Class 2 (>3 ≤ 4 years)	124	78	0.99 ± 1.16	0.74–1.32	0.966
Class 3 (>4 ≤ 6 years)	124	82	1.06 ± 1.15	0.80–1.40	0.690
Breed size (large as reference)	216	68	0.94 ± 1.15	0.71–1.23	0.656
Stillborn puppies					
Intercept			0.30 ± 2.17	0.05–1.17	0.125
Treatment (control as reference)	17	13	0.78 ± 1.47	0.36–1.65	0.509
Age (class 1 as reference; ≤3 y)					
Class 2 (>3 ≤ 4 years)	5	8	2.36 ± 1.78	0.78–7.86	0.136
Class 3 (>4 ≤ 6 years)	5	17	4.89 ± 1.67	1.92–14.9	0.002
Breed size (large as reference)	27	3	0.41 ± 1.92	0.09–1.28	0.174
Litter size (class 1 as reference, <7 puppies born)					
Class 2 (7–8 puppies born)	3	8	1.08 ± 2.03	0.29–5.16	0.918
Class 3 (> 8 puppies born)	3	19	1.87 ± 1.96	0.56–8.56	0.352
Number of weaned puppies					
Intercept			4.33 ± 1.258	2.71–6.66	<0.001
Treatment (control as reference)	111	110	0.97 ± 1.148	0.74–1.27	0.823
Age (class 1 as reference; ≤3 y)					
Class 2 (>3 ≤ 4 years)	108	61	0.95 ± 1.178	0.68–1.30	0.746
Class 3 (>4 ≤ 6 years)	108	53	0.79 ± 1.186	0.56–1.10	0.168
Breed size (large as reference)	161	61	1.22 ± 1.169	0.90–1.65	0.194
Litter size (class 1 as reference, <7 puppies born)					
Class 2 (7–8 puppies born)	30	82	1.50 ± 1.242	0.99–2.33	0.062
Class 3 (> 8 puppies born)	30	110	1.68 ± 1.232	1.13–2.57	0.013

IRR = Incidence Rate Ratio, SE = standard error, CI = confidence interval.

Clostridium hiranonis (recently renamed as *Peptacetobacter hiranonis*), *Bacteroides*, *Phascolarctobacterium* (61), but also by other ASVs that are more typical of gestation and lactation, like *Lactobacillaceae*, *Porphyromonas* and *Corynebacterium*. Pregnancy and lactation periods in bitches are most probably accompanied by changes on the gut microbiota as noticed in other species. In humans and swine, these changes include increased abundance of *Proteobacteria*, *Actinobacteria*, and opportunistic pathogens and a decrease in species richness as well as in SCFA producers bacteria, all occurring as pregnancy progresses (60, 62). Interestingly, the beta-diversity was overall not different between the 2 groups but differed significantly one day *post-partum*.

At day 1 *post-partum* (Figures 2C,D; Supplementary Figure S2), feeding SB-1079 *pre-partum* promoted a higher relative abundance of bacteria known as beneficial and linked to SCFAs production, notably *C. hiranonis*, *F. prausnitzii*, *Dorea*, *Megamonas* and *Bacteroides plebeius*. *F. prausnitzii* is a pH and oxygen sensitive bacteria, which is a well described butyrate producer while *Fusobacterium* spp. are among the "canine heatlhy microbiota bacteria" and can produce butyrate from protein (61, 63). Increased abundance of *Megamonas* spp., an important propionate and acetate producer, has been found in healthy compared to diarrheic cats (64) and in dogs consuming inulin-rich diets (65). Interestingly in sows, a better persistence of relative abundance of fibrolytic bacteria, oxygen and pH sensitive strains, was reported in SB-fed sows (13, 59), suggesting that the live yeast can shape the microenvironment of the gut to favor growth of strict anaerobes. Besides the observed increase in beneficial bacteria, the LEfSe analysis at DPP1 also indicated a higher relative abundance of Enterobacteriaceae in the Yeast group compared to the Control group (Supplementary Figure S1). To deeply explore this difference, we conducted qPCR to evaluate the number of 16S DNA copies belonging to *E. coli* and found no difference between the two groups (data not reported here). All along the study, the microbiota of bitches fed the control diet was characterized by higher relative abundances of diverse bacteria (facultative anaerobes for most of them) known as opportunistic and potentially pathogenic like *Proteus* spp., *Mycoplasma* spp., *Tissierellaceae.ph2.ph2_un* and/or *Vagococcus* spp. compared with Yeast group. Another potential pathogen, *Tissierella*

TABLE 6 Impact of the supplementation of the dam with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on the probability of low birth weight puppies ($n = 36$ litters and 254 born alive puppies).

	Number of Q1 BWb		Multinomial regression	
	Control	Yeast	OR ± SE (95% CI)	*P*-value
Treatment (control as reference)	41	19	3.5 ± 1.77 (1.14–10.69)	0.028
Age (class 1 as reference; ≤3 y)				
Class 2 (>3 ≤ 4 years)	26	17	1.14 ± 1.95 (0.31–4.21)	0.844
Class 3 (>4 ≤ 6 years)	26	17	1.16 ± 1.97 (0.38–5.52)	0.580
Litter size (class 1 as reference, <7 puppies born)				
Class 2 (7–8 puppies born)	4	17	0.59 ± 2.24 (0.12–2.85)	0.508
Class 3 (> 8 puppies born)	4	39	0.21 ± 2.15 (0.05–0.94)	0.041

Q1 BWb = birthweight in the lowest 25%, OR = Odds Ratio, SE = standard error, CI = confidence interval.

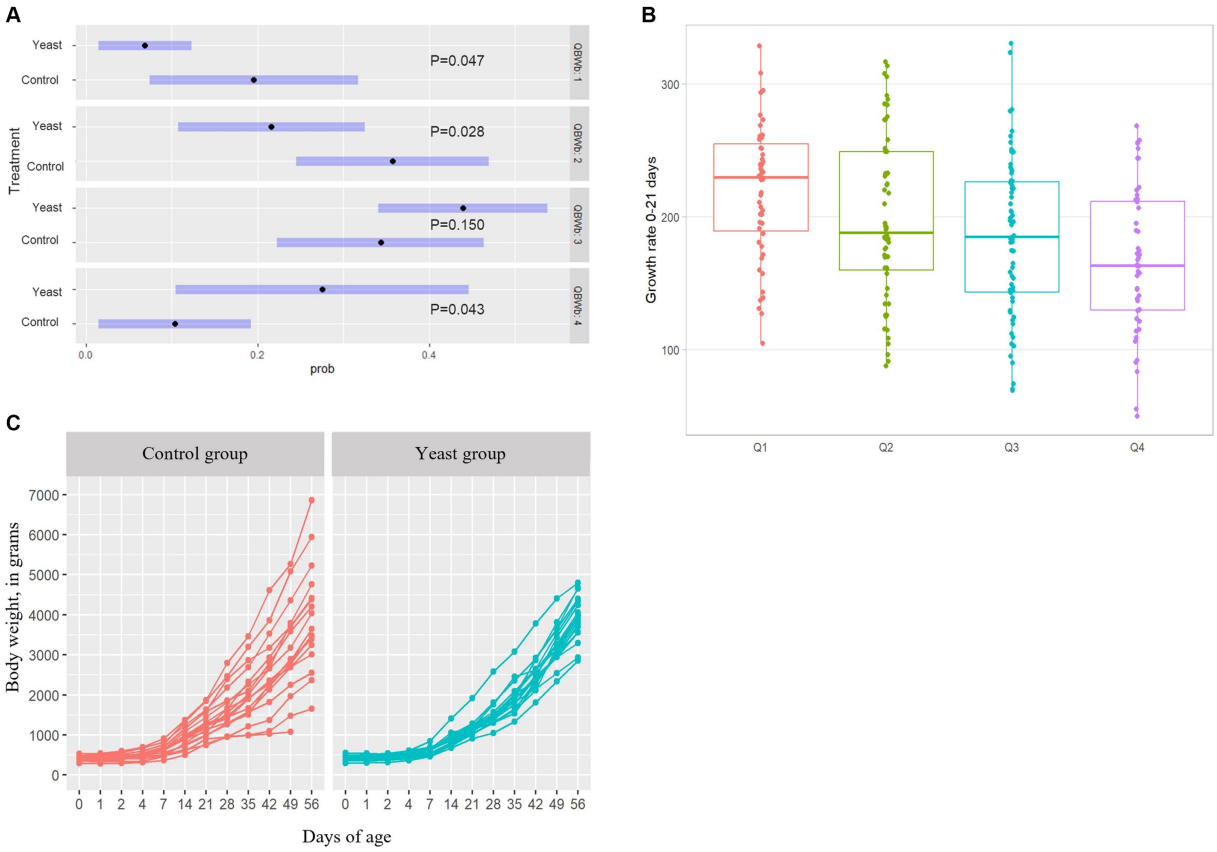

FIGURE 3
Impact of the supplementation of dams with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on puppy's growth. **(A)** Results of the multinomial regression showing that the odds of having puppies' birth weight in other quartiles than Q1 and Q2 was higher in Yeast group compared with Control group. **(B)** Comparison of growth rates during the first three weeks of life depending on the birth weight quartile. **(C)** Evolution of average body weight per litter between birth and two months of age according to treatment groups.

sp. was found in higher relative abundance in LBW puppies (66). Interestingly, Bendahmane et al. demonstrated that the supplementation of bitches with SB-1079 resulted in a decrease of opportunistic pathogenic bacteria relative abundances, especially in LBW puppies (67).

Secretory IgA (sIgA) are used as an intestinal biomarker because they play an important role in maintenance of gastrointestinal homeostasis by coating the bacteria, favoring a tolerant, non-inflammatory relationship with the host and the homeostatic control of the intestinal redox environment. Faecal sIgA concentration is well-correlated with the sIgA concentration in supernatants taken from duodenal explant cultures in dogs, thus measuring faecal sIgA provides a good indicator of mucosal immunoglobulin levels, reflecting what happens in the gut. In our study, SB-1079 supplementation of bitches resulted in the increase in faecal sIgA concentration (23.3 ± 3.17 vs. 14.5 ± 1.98 mg/g in average during all the

TABLE 7 Impact of supplementation of the dam with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on postnatal growth of puppies ($n = 36$ litters and 254 born alive puppies).

	LS means ± SE		*P*-value (fixed effects)				
	Control	Yeast	Treatment	Age	Breed size	Litter size	QBWb
GR 0–21, %	196 ± 12.2	178 ± 12.3	0.247	0.153	*0.054*	0.323	0.005
GR 21–56, %	190 ± 11.0	225 ± 11.2	0.022	0.334	0.393	0.393	0.742

GR x-y = growth rate between Day x and Day y, calculated as [(weight at Day y – weight at Day x) ÷ weight at Day x] × 100, LS means = adjusted least square means, SE = standard error, QBWb = quartile group of birthweight.

TABLE 8 Impact of the supplementation of the dam with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on cytokine levels (medians) of their puppies before and after vaccination against rabies ($n = 57$ puppies).

	DPP35, before vaccination			DPP56, after vaccination		
	Control ($n = 31$)	Yeast ($n = 26$)	*P*-value	Control ($n = 31$)	Yeast ($n = 26$)	*P*-value
Serum cytokines, pg/mL						
IL-8	4,401	3,828	0.975	2,768	2,128	*0.064*
IFNα	708.7	297.8	0.669	1,157	326.7	0.559
IFNγ	0	0	0.598	0	0	0.314
IL-10	247.4	531.3	0.043	168.9	407.6	*0.068*
TGFβ	1,300	1,640	0.001	1,421	1,327	0.754
IL-8:IL-10	14.7	7.77	0.241	16.9	4.22	0.032
Specific IgG anti-rabies, UI/mL						
IgG	0.13	0.13	0.200	3.46	5.29	0.936

study period), especially at DPP1. In agreement with these results, calves supplemented with SB have been shown to exhibit higher concentrations of ileal and colonic sIgA, along with a greater relative abundance of jejunal *F. prausnitzii* (68, 69). On the contrary, Meineri et al. (70) reported a decrease in faecal sIgA in adult dogs supplemented with SB. The sIgA production is still difficult to interpret, as it is intimately connected with stress hormones release, immune cells and microbiota. Nevertheless, decrease in faecal sIgA has been identified in case of stress challenge in rodent models, while higher concentrations have been described as providing a better protection against colonization of *Clostridium difficile* in infants (71). To go further and to fully understand the effect observed in our study, an evaluation of the concentration of butyrate produced as well as a characterization of the faecal IgA-binding bacteria would have been required. Taken all together, the higher faecal IgA concentrations and the positive microbiota changes could mean that SB may favor better colon homeostasis during the challenging period around whelping.

Bitches receiving SB-1079 in their food exhibited subtle modulation in their plasmatic metabolome just before parturition (Supplementary Figure S2). Interestingly, a part of the discriminant metabolites (glycerophospholipids, acylcarnitines, ketone bodies) between Yeast and Control groups seems to be associated to lipid and energy metabolisms. Two glycerophospholipids are found as discriminant between the 2 groups. Interestingly, Alassane-Kpembi et al. (72) reported that the supplementation of piglets with SB resulted in a modulation of blood and liver glycerophospholipid pathway. Acyl-carnitines derivatives are involved in fatty-acids β-oxidation and essential to meet the fetal energy needs and to fuel the placenta. When fatty acid oxidation is defective or diminished an increase in plasma acylcarnitine levels can be observed (73). In humans, several studies tried to find biomarkers of LBW or preeclampsia. The concentration of certain lysophosphatidylcholines in cord blood has been shown to positively correlate with BWb (74). Besides, the increase in serum levels of propionyl-carnitine, malonyl-carnitine, isovaleryl-carnitine, palmitoyl-carnitine and linoleoyl-carnitine were associated with gestational diabetes mellitus risk (75). Finally, ketone bodies are considered as markers of gestational diabetes during pregnancy in women (76). We could thus hypothesize that the supplementation with SB allow a better lipid/energy metabolic status, leading to better perinatal outcomes, i.e., the decreased odd of birth of LBW puppies or a higher gross energy in colostrum. However, those results are preliminary in canine species, and further research would be required to better measure the importance of those potential biomarkers of energy metabolism during gestation.

Another metabolite which raises interest during pregnancy is creatine, which has been found increased and discriminant in the Yeast group. Its homeostasis changes during pregnancy and alterations to maternal creatine homeostasis throughout gestation could impact the growth and well-being of the offspring. There is an increased requirement for maternal creatine due to the rapid growth and increased metabolic requirements of the fetus in the third trimester of pregnancy in humans. Interestingly, maternal urinary creatine excretion across pregnancy was positively correlated with birthweight centile and birth length, suggesting a relationship between maternal creatine status and fetal growth (77). In a retrospective case-controlled study (78), a 18% reduction in maternal serum creatine concentration during the third trimester of pregnancy was associated with a greater incidence of poor perinatal outcomes. Our study suggests for the first time and as in humans, a link between creatine metabolism during gestation and perinatal outcomes in canine species. Whether alterations in maternal circulating creatine concentrations are indicative of other poor perinatal outcomes is still to be ascertained. Thus, while links are beginning to emerge between maternal creatine

homeostasis throughout pregnancy and infant outcomes, further studies are required to understand adaptations to maternal creatine homeostasis throughout gestation and their association with pregnancy outcomes. Supplementation with SB could offer a nutritional strategy for modulating creatine metabolism with the aim of improving perinatal outcomes.

Our study highlights that, in pregnant bitches like in women, there is an interdependence between the gut microbiota and the host. A better faecal microbiota resilience of the alpha-diversity occurs with a consecutive promotion of SCFA-producing bacteria in bitches fed with SB-1079. It would have been interesting to evaluate SCFA production in order to correlate it with the results obtained from microbiota and metabolomic analyses. However, data obtained constitute microbial indications suggesting a relationship between microbiota diversity, SCFAs and energetic metabolism. Coumarate, found as a discriminant metabolite, is a phenolic compound which has been found to be present in high quantity in the extracellular fraction of SB (79) produces through shikimate pathway, an active metabolic pathway in SB (80), and directly absorbed in the upper digestive tract. This compound possesses antioxidant activity, protects against generation of reactive oxygen species and has shown potential therapeutic benefit in experimental diabetes and hyperlipidemia (79, 81). Interestingly, supplementation of pregnant women with polyphenols have been reported to decrease the risk of intrauterine growth restriction (82). Another interesting metabolite that links microbiota and host-metabolism is the itaconate which has been recently discovered to be a mammalian antimicrobial metabolite. Itaconic acid has great potential to be an antimicrobial compound against viruses and antibiotic-resistant bacteria and exhibits immunomodulatory effects (83). Its production occurs after lipopolysaccharide (LPS) activation of macrophages (83) to compete against succinate in the tricarboxylic acid (TCA) cycle. A decrease in the plasmatic concentration of this compound could be related to a positive effect of SB in the gut, resulting in less LPS stimulation through toll-like receptor 2 (TLR2) (84). The discriminant metabolite indole-3-acetate is consistent with a positive effect on gut barrier, as indole and its derivatives may promote the upregulation of adhesion-related genes and enhance the intestinal barrier to prevent enteritis. Of note, the pathway of indolacetaldehyde production from tryptophan is an active pathway in SB (80). Also indole-3 acetate occurs from tryptophan breakdown by the gut bacterial activity [*Clostridium* (85) but also *Bifidobacterium* (86)].

Throughout gestation and then via mammary secretions, female provides her puppies with the energy they need to survive and develop. During the first days after birth, colostrum, providing energy and immunity, is indispensable for newborn puppies to deal with the challenging transition from intrauterine to extrauterine life (19, 87). Many studies in pigs support that live yeast supplementation during gestation and/or lactation improves the quality of colostrum (16, 88–91). In the current study, the SB-1079 supplementation had no effect on the immune composition of the colostrum. Results of other studies using live yeast are controversial. No change in the immune composition was described in dairy cows (92) or in sows (13) but another study in sows demonstrated an increase of the IgG concentration in sow colostrum (93). Additional studies on the impact of SB supplementation on the immune composition of the colostrum in the canine species are required due to its great variation among bitches. Indeed, the IgG and IgA concentrations may vary greatly depending on time elapsed since the onset of lactation and sampling, and depending on the number of puppies born within a litter and their suckling behaviour (33, 94). However, the supplementation of bitches with SB-1079 increased colostrum gross energy (Table 4). It could be beneficial considering the high energy requirements of puppies just after birth, their limited energy reserves and their immature thermoregulation system (95). Interestingly, in bitches, plasma N-acetylneuraminate (also known as sialic acid) was identified as a discriminant metabolite, suggesting a modulation of amino-sugar metabolism (96, 97). Sialic acid has been described as increasing in gestating women, being largely involved in the milk oligosaccharides synthesis, and crossing the placenta to participate to *in utero* body growth and brain development (98). Dunière et al. (91) reported that a supplementation of gestating ewes with a live yeast resulted in significantly higher levels of colostral N-glycolylneuraminic acid. In our study, the decrease in number of LBW puppies as well as the trend to have more sugars in the colostrum are consistent with a modulation of the amino-sugar metabolism. In addition, we previously observed that 2-day old puppies from bitches supplemented with SB had a faecal microbiota richer in Lactibacillaceae which are well-described to use milk oligosaccharides (67). Finally, at day 7 *post-partum*, SB-fed bitches had a higher concentration of milk proteins and a trend of higher energy content. It was described that in the canine species, proteins provide a significant portion of the calories in colostrum and milk (99). The modulation of the plasma metabolome just before parturition could relate to the increase in energy and protein content in the mammary gland secretions, although further research is required for a better understanding of the mechanisms involved. Alternatively, it is possible that supplementation with SB improved overall nutrient digestibility in the bitches (92).

The concept of immunometabolism has emerged recently whereby the repolarizing of inflammatory immune cells toward anti-inflammatory profiles by manipulating cellular metabolism represents a new potential approach to control inflammation. It is of great interest to study it during the early life. Interestingly, main mechanisms described to sustain the DOHaD hypothesis (6) are related to epigenetics, for which mitochondrial metabolites and cofactors play a critical role and for which microbiota has been demonstrated to be a histone methylation and acetylation driver (100). B vitamins group (including nicotinic acids) and SCFAs are for example sources of acetyl-CoA or nicotinamide adenine dinucleotide (NAD) which affect histone acetylation while butyrate induced histone acetylation (100). Thus, supplementing bitches with live yeast SB-1079 could affect the immunometabolic profile of their offsprings, via different mechanisms. In the current study, this profile was evaluated through the body weight at birth, the growth rate, the specific immune response as well as plasma pro- and anti-inflammatory cytokines before and after vaccination against rabies. Further studies are warranted to deeply explore the persistence of metabolic changes observed in puppies, through for example modification of the epigenome (101).

One parameter of litter composition, the repartition of puppies among BWb quartiles, was significantly influenced by the SB-1079 supplementation. Reducing the number of LBW puppies is of great interest for breeders considering their lower energy reserves, lower motility, higher risks of hypoxia, hypothermia and thus, their higher risk of neonatal mortality compared to normal BWb (20, 21, 102). Apart from this major short-term consequence, LBW was also suggested to increase odds to become obese in later life in the canine species (8). Further studies are needed to understand how

supplementation could influence intra-uterine growth, especially as this result has not been found in other studies on pigs. One hypothesis could be that SB supplementation improved overall nutrient digestibility by modulation of dam microbiota and improvement of metabolism (103). The potential antioxidant effect of SB through its metabolites, as well as the effect on energy metabolism suggested in our study could also allow an increase of nutrients dedicated to fetus growth or a better placental function or efficiency (103). Future studies should therefore include an assessment of antioxidant status as well as an evaluation of nutrient digestibility in bitches to support the hypotheses suggested by the results of the current study.

Beside the effect on BWb quartiles, an impact in the growth rate pattern was detected, with notably an improved growth during the second part of the suckling period. As in humans, monitoring the growth of puppies is recommended to identify abnormal patterns. This should be used as a proxy to evaluate short- and long-term health (104). To our knowledge, those results are the first ones reporting such an effect of live yeast supplementation to bitches on the growth pattern of puppies. A rapid growth at the beginning of life is not desired as it may favor obesity in later life (105–107). In dogs, the link between early postnatal growth and later overweight as well as the sensitivity window in which rapid growth should be avoided remain to be explored. Interestingly, our study confirmed that puppies belonging to the LBW quartile (Q1) exhibited a quicker, also called "catch-up," growth compared to the other puppies, already described in rodents or humans (108, 109). Therefore, further studies are required to evaluate the effect of the improved growth detected in the yeast group during the second half of the suckling period on long-term health. Besides the impact on the pattern, yeast supplementation allowed a better homogeneity of postnatal growth between puppies and thus avoided extreme trajectories. Having a homogenous growth pattern in the kennel should also facilitate litter's management for breeders. In our study, puppies were not directly fed with SB, leading to speculate an indirect effect of SB. In swine, SB given to the sows has also been demonstrated to have a beneficial effect on the postnatal growth of piglets. This could be explained by the improved immune status, assessed by the serum IgG concentration, observed in piglets born to supplemented dams (16–18).

In parallel to the growth patterns, inflammatory and immunological status of the puppies was assessed before and after vaccination against rabies virus. This antigen was chosen to make sure that all the dogs, including bitches, were naïve. The aim was to avoid interference with maternal antibodies, but also to ensure that the reaction to the vaccine was not biased by a reaction to the pathogen linked to spontaneous infection. Just before vaccination, the plasma concentrations in IL-10 and in TGF-β were significantly increased when bitches received SB-1079. After vaccination, while the specific immune response was efficient in all puppies, blood IL-8 tended to decrease; blood IL-10 to increase, leading to a significant lower IL-8: IL-10 ratio. The supplementation of the bitches with SB-1079 attenuated the inflammatory response after a vaccination while maintaining appropriate levels of specific IgG. In humans fed SB and suffering from diarrhea-dominant irritable bowel syndrome blood IL-10 increased; while blood IL-8 and TNF-α decreased (110) whereas in sheep, a direct supplementation induced higher seroconversion after vaccination (73). The IL-8 plays a critical role in acute inflammation and its concentration increases in blood of infants suffering from necrotizing enterocolitis (111). More generally, circulating IL-8 is proposed as a promising biomarker of gut permeability in humans (112). Besides, IL-10 and TGF-β are associated with immune tolerance and promotion of Treg cells and are thus of paramount importance during early life.

5 Conclusion

Our study highlighted an effect of SB-1079 on bitches' outcomes and in turn on maternal programming of puppies, confirming that like in other species, maternal nutrition can impact the immunometabolic phenotype of the offsprings. Indeed, in dams, it resulted in modulating gut homeostasis and microbiota while modifying cell metabolism. The inter-talk between microbiota and host seems to be an interesting area to be further investigated, in this physiological condition, but also in others like obesity, insulin resistance or in elderly populations. Feeding bitches with SB-1079 is a promising approach to optimize the health and nutrition of the vulnerable mother–infant dyad, but continued work in nutritional immunology is required to further enhance our understanding of the power of nutrition and diet to improve early-life health of companion animals.

Interestingly, a lot of similarities between humans and dogs have been highlighted, the canine species appearing as a good model to understand the DOHaD in humans and to test the effect of some nutritional strategies. More research on gut microbiota and blood or urinary metabolism of mothers and puppies is required to better understand the maternal programming effects allowed by the supplementation of live yeast to the bitches. To compare with what happens in humans, it could be of great interest to monitor the impact of the SB supplementation of the bitches on the long-term health of puppies, and notably on the obesity and diabetes risk assessment which is a major issue in pets as well as in human population.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1063569/.

Ethics statement

The animal study was approved by Comité d'Éthique en Expérimentation Animale, Science et Santé Animale n°115; reference number: SSA_2020-004, Toulouse, France. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. AM: Formal analysis, Writing – original draft, Writing – review & editing. SC: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. FS: Formal analysis, Investigation, Visualization, Writing – review & editing. J-CM: Formal analysis, Investigation, Visualization, Writing – review & editing. LS: Formal analysis, Investigation, Visualization, Writing

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Conflict of interest

AM, AR, MC, and EA were employed by the company Lallemand SAS.

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1366256/full#supplementary-material

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