REVIEW

Metabolites: deciphering the molecular language between DCs and their environment

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Abstract Dendritic cells (DCs) determine the outcome of the immune response based on signals they receive from the environment. Presentation of antigen under various contexts can lead to activation and differentiation of T cells for immunity or dampening of immune responses by establishing tolerance, primarily through the priming of regulatory T cells. Infections, inflammation and normal cellular interactions shape DC responses through direct contact or via cytokine signaling. Although it is widely accepted that DCs sense microbial components through pattern recognition receptors (PRRs), increasing evidence advocates for the existence of a set of signals that can profoundly shape DC function via PRR-independent pathways. This diverse group of host- or commensal-derived metabolites represents a newly appreciated code from which DCs can interpret environmental cues. In this review, we discuss the existing information on the effect of some of the most studied metabolites on DC function, together with the implications this may have in immune-mediated diseases.

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Introduction

Dendritic cells (DCs) constitute a bridge between innate and adaptive immunity. They are able to receive and interpret signals either from invading pathogens or dying cells, and to respond to them through the acquisition of a mature phenotype. Mature DCs gain the ability to migrate to the lymph nodes (LNs) where they initiate and shape adaptive responses via antigen presentation, the expression of costimulatory molecules and cytokine secretion. These properties enable them to interact with and activate naïve T cells, inducing protective immune responses against non-self-antigens. In addition, DCs participate in a series of processes that act at different levels to ensure the maintenance of immunological tolerance (reviewed in [1]).

One of the major tolerogenic mechanisms accomplished by DCs involves their interaction with regulatory T cells (Tregs), which are crucial in controlling excessive immune responses and preventing autoimmunity [2–4]. These two cell populations are in close contact and regulate one another. DCs participate in maintaining Treg homeostasis [5, 6]. Moreover, DCs can produce soluble factors, such as TGF-β, that are essential for the generation of induced Tregs [7, 8]. Therefore, it has been hypothesized that disruption of DC homeostasis may result in Treg imbalance and autoimmunity. This view is supported by studies evaluating the consequences of DC depletion, in which ablation of CD11c-expressing cells using various genetic mouse models led to increased Th1 and Th17 responses and concomitant lower Treg frequencies [9, 10]. DC deficiency in humans is also related to lower Treg numbers [11, 12] and mice constitutively lacking DCs spontaneously

develop severe autoimmunity, characterized by autoantibody production and tissue infiltration of autoreactive CD4^{+} T cells [10]. Newer studies using transgenic mice with improved DC targeting specificity show that defined DC subsets are required for the generation of peripheral Tregs and oral tolerance, confirming the idea that DCs are essential for establishing and maintaining immune homeostasis [13]. Still, the precise contribution of DCs to immune-mediated diseases remains unclear, though it is conceivable that changes in their phenotype, including their activation state, migratory capacity and production of immunomodulatory factors, could affect the state of tolerance. Indeed, studies from patients with different autoimmune diseases, including Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), Psoriasis, and Inflammatory Bowel Disease (IBD) have revealed several abnormalities in DC activation or function as factors mediating inflammatory sequelae (reviewed in [14]).

The microenvironment where DCs reside and become activated can dramatically modulate their function. Several small metabolites have been shown to exert profound changes on DCs [15]; some have even proved to be essential for DC homeostasis and function. This diverse group includes diet-derived and endogenously produced compounds, the latter being synthesized by neighboring cells or the host microflora. In the human gastrointestinal tract, 10^{13}–10^{14} commensal microorganisms contribute to the breakdown, absorption and synthesis of nutrients and metabolites [16]. Diet can further influence the concentration of these products by direct and indirect mechanisms. For instance, the amount of daily fiber intake can promote colonization by specific bacterial families [17], suggesting that the composition of the microbiota and their products heavily relies on diet [18]. In addition, the intestinal flora produces a vast array of diet-independent metabolites. These products can alter the homeostasis and development of the mucosal immune system. Therefore, metabolites are emerging as a new type of signal shaping the immune system. Interestingly, increasing evidence suggests that the drastic changes in Western diet over the past few decades could account for the rise in autoimmune diseases observed in industrialized countries [19]. It would therefore be intriguing to speculate that changes in diet impairing the tolerogenic capacity of DCs may enhance predisposition to immune-mediated disorders.

Further supporting this view, it is now clear that autoimmunity is not only caused by genetic factors; studies of identical twins have shown that the environment has a major influence on development of autoimmune diseases [20]. Underdeveloped countries present lower a incidence of autoimmune diseases, arguing in favor of a link between lifestyle and immune status. The widely accepted hygiene hypothesis contends that increased exposure to certain pathogens, particularly helminths, reprograms the immune system rendering it less prone to autoimmunity [21]. Recent reports from experiments in mice demonstrate that helminths can alter the composition of the gut microbiota, promoting the expansion of particular bacterial families [22–26]. In addition, human studies also showed a correlation between helminth infection and altered microbiota composition [27, 28].

In this review, we will focus on some of the most studied metabolites, their effect on DCs, and their implication for immune-mediated diseases. Finally, we will also discuss their potential use in immune therapy.

Different regions, different folks: DC subsets and their localization

Because of their functional specialization and broad tissue localization, DCs comprise a highly heterogeneous cell population whose classification and study have been challenging and controversial. Another confounding factor is that some of the markers historically used for identifying DCs, such as CD11c [29] are also expressed by other cell types [30–35]. Recent advances have allowed for the identification of DC precursors and crucial transcription factors required for initiating the DC differentiation programs [36, 37]. This key information has made it possible to pinpoint which of the previously described subpopulations truly classify as DCs, and to determine defined lineages within the total DC population.

In human and mouse, DCs can be broadly subdivided into classical DCs (cDCs) and plasmacytoid DCs (pDCs) [38]. In this review we will focus on murine cDCs unless stated otherwise. cDCs can be found in most lymphoid and non-lymphoid tissues. Lymphoid tissue-resident cDCs remain in the lymphoid organ where they originated for their entire life, whereas non-lymphoid tissue cDCs usually have the ability to migrate to the draining LNs to present the antigens they acquired at their site of origin [39]. Within the cDC subtype, two distinct lineages have been identified, cDC1 and cDC2 [38]. cDC1 DCs in lymphoid tissues express CD8α and represent around 20% of splenic DCs and 70% of thymic DCs. In humans, this DC subset is characterized by the expression of BDCA-3 (CD141) [40–45]. Splenic CD8α^{+} DCs are more efficient at cross-presenting cell-bound and soluble antigens on MHC class I than other DC subsets [46, 47]. They also express receptors such as CD36 and Clec9A that enable them to carry out phagocytosis of dead cells [48, 49]. These unique characteristics make them essential to prime CD8^{+} T cell responses against tumors and intracellular pathogens [50–52], but may also permit the attenuation or exacerbation of autoimmune reactions (reviewed in [53]). In addition, cDC1 DCs are good inducers of Th1 responses due to their ability to secrete considerable amounts of IL-12 upon stimulation [52, 54]. In non-lymphoid tissues the CD103^{+}CD11b^{-} cDC1 subset is the counterpart to the CD8α^{+} cDC1 population and their ontogeny and functional characteristics are closely related [55].

The cDC2 subset, characterized by CD11b expression, is the most abundant in lymphoid organs excluding the thymus. The human counterpart of this subset is defined by the expression of BDCA-1 (CD1c) [56, 43]. In contrast to cDC1 DCs, this subset is poorly characterized, partly because of its heterogeneity. DCs belonging to this subtype are thought to be specialized in driving CD4^{+} T cell responses through antigen presentation via MHC class II [57, 58]. Non-lymphoid tissue CD11b^{+} DCs, historically considered as *bona fide* DCs, are now described as a heterogeneous subpopulation including DCs but also macrophages. This generalization has hindered our understanding of the contribution of the CD11b^{+} compartment to tissue immunity [56]. The latest identification of new markers for DCs and macrophages has enabled an efficient discrimination between these different subsets [59, 60]. Therefore, while the precise study of cDC2 DCs is still in progress, current evidence suggests they may play a role in the induction of Th2 and Th17 responses [56, 60].

Peripheral lymphoid organs also comprise a non-lymphoid tissue DC population that has arrived to the lymph node through the afferent lymphatics. Known as migratory cDCs, they are characterized by higher MHC class II and lower CD11c expression than resident DCs in the steady state. This subset is responsible for delivering tissue-derived antigens in order to reinforce tolerance or initiate adaptive responses [61, 62].

DCs are also localized in most non-lymphoid tissues, especially in those which are in close contact with the environment. In the intestine, cDCs are present in the lamina propria (LP), gut-associated lymphoid tissue (i.e. Peyer's Patches) and mesenteric lymph nodes (mLNs). cDCs in these locations have a dual role, eliciting robust immune responses to pathogens and at the same time, inducing tolerance to food antigens and the commensal flora. In the LP, which constitutes the main mucosal immune effector site, most cDCs express CD103 and can be classified according to their expression of CD11b into CD11b^{−} (cDC1) and CD11b^{+} (cDC2). There is also a CD103^{−} CD11b^{+} population which expresses intermediate levels of CX3CR1. These subsets can also be found in the mLNs, where DCs migrate to prime naïve T cells in a CCR7-dependent manner (reviewed in [63]). Lung DC populations share similarities with LP DCs, though cDC1 are generally CD103^{+}CD11b^{−} and cDC2 are CD103^{−}CD11b^{+} in this organ (reviewed in [64]). In the skin, cDCs are found in the dermis and can also be categorized by their expression of CD11b. CD11b^{−} DCs are developmentally related to the splenic CD8α^{+} subpopulation, whereas CD11b^{+} DCs belong to the cDC2 subset and can be distinguished from CD11b^{+} monocytes by their lack of CD64 expression [65]. The epidermis is colonized by Langerhans Cells (LCs); this subset cannot be classified as *bona fide* DCs, since they do not arise from blood-circulating DC precursors but from hematopoietic progenitors that populate the skin before birth and are not dependent on FLT3L [66, 67]. These characteristics strongly resemble those of tissue resident macrophages. However, unlike this cell subset, LCs have the ability to migrate to LNs and present antigen, thus functionally resembling DCs [68]. Overall, this broad tropism suggests that DCs are exposed to different environments based on their tissues; consequently, various sets of cues may impart distinct functions to DCs in specific sites.

In contrast to other immune cells, which can be easily obtained *ex vivo* in large numbers, DCs are very rare (1–5% in non-lymphoid organs) [64]. This renders assays that require high cell numbers and purity, labor intensive and expensive. As a result, researchers commonly employ *in vitro* culture systems to yield high numbers of DCs for functional studies. The GM-CSF-based protocol for the generation of bone marrow-derived DCs is the most widely used [69, 70]. This culture is predominantly composed by CD11c^{+} cells with different levels of MHC II expression; traditionally it has been assumed that this was due to different degrees of maturation but careful investigation has revealed that it might not consist of a single population. A recent study showed that the GM-CSF method gives rise to a CD11c^{+} MHCII^{+} heterogeneous population of myeloid cells that comprises both macrophages and DCs [71]. Although clustering analysis indicates that the DC fraction is not closely related to the cDC1 or cDC2 subsets found *in vivo*, it shares some signatures with migratory DCs. Worth mentioning, this subpopulation represents only around 30% of the CD11c^{+} MHCII^{+} cells present in the culture. Conversely, bone marrow culture with FLT3L allows for the differentiation of different DC subsets (pDCs, cDC1 and cDC2) equivalent to the populations present in the spleen under steady-state conditions [72]. Therefore, this culture system closely resembles DC differentiation from bone marrow precursors *in vivo*. However, due to its high complexity, if the study of a single population is intended, FACS sorting may be required. Furthermore, a new method for the generation of large numbers of cDC1 DCs with high purity is now available [73]. This protocol requires the addition of GM-CSF and FLT3L and yields a 90% pure population of CD103^{+} DCs, thus eliminating the need for further purification steps for certain applications. Human cDC1 can be obtained following a similar procedure [45, 74]. DC subsets obtained from these cultures are functionally and phenotypically similar to those found *in vivo*, making them particularly useful for studying populations that exist in very low numbers in the body, such as CD103^{+} DCs. Furthermore, this may be potentially relevant for new immunotherapeutic approaches since these DCs display improved migratory capacity which results in enhanced antigen delivery to the LNs [73]. Advances in methods to isolate DCs *ex vivo* and genetic models to conditionally target genes in DC subsets will be instrumental in helping understand DC mechanisms.

Endogenous metabolites and metabolic processes in DCs

In the past few years, the idea of a link between immune cell function and metabolism has gained momentum, giving rise to the field of immunometabolism. Immune cells can respond to changes in the environment, such as oxygen and nutrient availability, by changing their core metabolic program. Interestingly, it seems that this metabolic reprogramming can be accompanied by or even required for changes in DC cellular function.

Under resting conditions, DCs mainly rely on oxidative phosphorylation (OXPHOS) for energy [75, 76]. This process occurs within mitochondria, whereby cells utilize NADH generated from the catabolism of nutrients (e.g. glucose, amino acids and fatty acids) to produce ATP through a series of reduction-oxidation reactions. Engagement of Toll-like receptor (TLR) on GM-CSF derived DCs leads to upregulation of aerobic glycolysis. This process – known as the "Warburg effect" – involves the conversion of glucose into lactate [76–78] and is dependent on the PI3K/Akt pathway. In cancer cells, the Warburg effect accompanies rapid proliferation, whereas DCs are generally non-proliferative and do not divide further upon TLR ligation. Notably, the rise in aerobic glycolysis observed in GM-CSF derived DCs is accompanied by a decrease in mitochondrial oxygen consumption and ATP levels [76, 78]. Expression of Hypoxia-inducible factor 1-alpha (HIF-1α) is also required to trigger these metabolic changes; TLR ligands can induce the expression of this transcription factor through an as yet unknown mechanism [79]. Analysis of $Hif1a^{-/-}$ DCs revealed that these cells do not upregulate glycolysis in response to TLR ligation and are unable to induce T cells responses, highlighting the importance of this signaling pathway for DC immunogenicity [79, 80, 75].

Initial reports on DC metabolism argued that DCs induce glycolysis in order to counteract the inhibition of mitochondrial respiration caused by nitric oxide (NO), a product of nitric oxide synthase (iNOS) [78]. Thus, DCs would upregulate their glycolytic rate to maintain the ATP levels needed for their function and survival. However, this observation is restricted to GM-CSF-derived cells, since *bona fide* DCs do not express iNOS in response to TLR stimulation [71, 75]. In fact, according to Helft et al., not even the DC subset within GM-CSF cultures expresses iNOS upon LPS activation, indicating that the NO produced in this system likely comes from the accompanying macrophage subpopulation [71]. Thus, it would be interesting to determine if *in vivo*, NO generated by other cells during the inflammatory response could indirectly induce changes in the metabolic program of adjacent DCs. More recent studies claim that TLR-driven activation triggers an early induction of glycolysis required for providing biosynthetic precursors for fatty acid synthesis (FAS) [77]. Glycolysis-derived pyruvate can enter the tricarboxylic acid (TCA) cycle in the mitochondria and yield citrate which can

be transported back to the cytosol where it serves as a precursor for FAS. This process sustains the expansion of the ER and Golgi membranes, thus promoting the translation and transport of new proteins involved in DC activation [77]. Nevertheless, since these studies were performed using pharmacological inhibitors which often have unwanted off-target effects, it will be crucial to make use of genetic models to study the contribution of these metabolic pathways to DC function.

The high cell numbers and purity required to conduct most metabolic analyses render it difficult, if not impossible, to study the metabolic features of DCs *in vivo*. The only information available comes from *ex vivo*-isolated DCs. Pantel et al. showed that *in vivo* activation of splenic cDCs via polyinosinic:polycytidylic acid (poly I:C) administration results in type I interferon-dependent upregulation of glycolysis and decreased mitochondrial respiration. [75]. A new study argues that upon activation, both pDCs and cDCs upregulate OXPHOS via an induction of fatty acid oxidation in a type I interferon-dependent manner [81, 82]. Since these findings contradict previous reports [82] this process needs to be studied in further detail. It is possible that interaction of DCs with neighboring cells via the production of metabolites (e.g. NO) could influence their metabolic program, thus accounting for the differences observed.

Interestingly, products of metabolic processes can act as environmental cues on DCs, triggering changes in their core metabolic program and/or their functional characteristics. For instance, DCs can sense the presence of succinate (reviewed in [83]) through the receptor GPR91, which acts as an activating stimulus [84]. Moreover, reactive oxygen species (ROS) which are constitutively produced by the mitochondrial respiratory chain can exert signaling functions by inducing post-translational modifications on proteins [85]. By-products of microbial metabolism also induce changes in DC function; these signals can come either from invading pathogens or the commensal microflora [86, 87]. We are only beginning to understand the metabolic processes that follow DC activation and their importance for DC function. Nonetheless, the information available suggests that modulation of metabolic core pathways could become the next generation therapy for the treatment of immune-mediated diseases. In the following sections we will discuss some of the best studied metabolites influencing DC metabolism and function.

ATP and adenosine

Because of its central role in driving virtually every cellular process, ATP is often referred to as the energy currency of the cell. However, ATP can also be released to the extracellular environment by necrotic and apoptotic cells and in response to different types of stress, or even as part of physiological processes [88–90]. Once in the extracellular space, ATP can be

degraded to ADP, AMP and adenosine by ectonucleotidases such as CD39 and CD73 (reviewed in [91]). Adenosine can be further metabolized by adenosine deaminase (ADA), yielding inosine, which comparatively has no activity [92]. Adenosine can also be taken back into cells through nucleoside transporters (reviewed in [93, 94]).

Released ATP, as well as its degradation products, can be sensed by purinergic receptors. These can be classified into two families: P1, composed of G protein-coupled adenosine receptors (ARs), and P2, which includes receptors that bind to ATP, ADP and other nucleotides. The P2 family can be further subdivided in P2Y, which comprises metabotropic G protein-coupled receptors and P2X, formed by oligomeric ion channels [95].

Adenosine receptor signaling

Early reports show that human immature DCs express the ARs A_1R, $A_{2A}R$ and A_3R, whereas LPS-stimulated DCs downregulate the expression of A_1R and A_3R, displaying only detectable mRNA levels of $A_{2A}R$ [96]. Activation of $A_{2A}R$ inhibits IL-12 and TNF-α production and enhances IL-10 secretion on mature DCs but has no influence on basal levels of secretion of these cytokines [97]. In this way adenosine treatment of mature DCs renders them less efficient at priming Th1 responses [97]. In line with these results, activation of ARs in human DCs also inhibited their capacity to induce $CD8^+$ T cell responses [98]. Similar effects of adenosine receptor activation were observed in murine DCs, although they seem to be dependent on $A_{2B}R$ rather than $A_{2A}R$ [99, 100].

Adenosine also has chemotactic properties on DCs [96, 101]; a recent study showed that Tregs can degrade extracellular ATP to generate adenosine through their expression of CD39 and CD73 [101]. Adenosine then attracts DCs to remove apoptotic cells and promotes their interaction with Tregs to ensure tolerance. This process may be relevant for clearance of dying cells under physiological conditions.

DCs can also express CD39, which allows them to directly dampen immune responses (reviewed in [102]). CD39 expression is dependent on IL-27, a cytokine belonging to the IL-12 family secreted by DCs upon TLR ligation [103] that can act directly on T cells, preventing Th17 responses while promoting the development of IL-10-producing Tregs [104–107]. IL-27 acts in an autocrine or paracrine manner, inhibiting the maturation of DCs [108, 109]. CD39 upregulation leads to a decrease in extracellular ATP levels, which results in lower ATP-dependent activation of the NLRP3 inflammasome. This process may be important in the context of the experimental auto-immune encephalitis model (EAE), the murine model for MS, since treatment of mice with IL-27-conditioned DCs reverses the chronic status after the disease is already established [110].

Conversely, growing evidence supports the notion that DCs may also have mechanisms to deplete adenosine from their microenvironment, implying they could release themselves and other cells in their vicinity from the immuno-modulatory effects of this nucleoside [111–113]. DCs express surface ADA, capable of eliminating the signaling actions of adenosine [113]. In line with these findings, blockade of ADA led to a more pronounced effect of adenosine and synthetic AR agonists on DC maturation [113]. Direct *in vitro* addition of ADA to human immature DCs resulted in upregulation of costimulatory molecules (CD80, CD83, CD86) and higher secretion of Th1-polarizing cytokines, further supporting the concept of a negative regulatory mechanism of adenosine signaling in DCs [114]. This mechanism is likely to be relevant during inflammation, where a significant rise in adenosine levels is expected; in this context, expression of ADA by DCs would enhance their maturation status, thus boosting their immunogenicity. However, it could have a detrimental effect in immune-mediated diseases. For instance, non-obese diabetic (NOD) mice DCs display elevated levels of ADA in comparison to other mouse strains [111]. Furthermore, DCs from NOD mice lacking ADA expression fail to trigger auto-immunity when adoptively transferred, confirming the importance of ADA expression on DCs in the T cell priming phase, as well as in the regulation of the magnitude of the T cell response [111].

In the intestine, ARs are essential for keeping inflammation in check. In a chemically-induced colitis model, genetic ablation or pharmacological inhibition of $A_{2B}R$ resulted in higher severity [115]. In the context of the same model, CD39-deficient mice showed more severe symptoms than wildtype mice, further underscoring the immunomodulatory role of adenosine in the gut [116].

Another setting where immunomodulation by adenosine seems to play a relevant role is cancer. The tumor microenvironment displays high concentrations of adenosine [117, 118], which promotes secretion of VEGF by DCs and inhibits their maturation, leading to increased angiogenesis, inefficient antigen presentation and lower activation of effector T cells [119]. Transfer of AR-stimulated DCs into tumor-bearing mice promoted tumor growth [119]. In addition, treatment with AR antagonists inhibited tumor growth and metastasis formation, due to improved T cell activation and recruitment to the tumor site [120]. This seems to be a consequence of adenosine signaling blockade in the DC compartment, more specifically in the $CD8^+CD11b^-$ subset, which showed higher activation levels after treatment. The use of adenosine receptor antagonists, particularly for $A_{2A}R$, has been put forward as a next-generation checkpoint blockade therapy [121].

P2XR and P2YR signaling

ATP and other nucleotides can be released into the extracellular space by apoptotic cells through different types of channels, e.g. pannexins, or they can also be released by cell damage or lysis [122–124]. These molecules have chemoattractant

properties and have been described to act as a "find me" signal that recruits phagocytes in a P2XR/P2YR-dependent manner in order to ensure clearance of dying cells [88]. DCs express both types of receptors; interestingly, ATP has chemotactic activity on immature DCs but not on mature DCs [125, 126].

Regarding the effect of ATP on DC maturation, there seems to be inconsistencies on what has been reported so far, but that is likely due to different ATP concentrations being used. High ATP concentrations (mM range) induce secretion of TNF-α and IL-1β by LPS-stimulated DCs, but are also highly cytotoxic [127–129]. This is connected to the ability of ATP to activate the NLRP3 inflammasome in a P2X$_7$R-dependent fashion [130–132]. These effects require high ATP concentrations since, compared to other members of the P2XR family, P2X$_7$R has low affinity for ATP [133]. In DCs, this process may be important for inducing anti-tumor immunity: ATP released by dying tumor cells following chemotherapy can trigger inflammasome activation, resulting in proteolytic processing and secretion of IL-1β, which promotes the generation of IFNγ-producing tumor antigen-specific CD8$^+$ T cells [134]. On the other hand, μM concentrations of ATP induce the expression of costimulatory molecules but at the same time inhibit the production of IL-1β, TNF-α, IL-6 and IL-12. The reduced IL-12 production by ATP-treated DCs results in their impaired ability to prime Th1 responses [135]. However, since these effects are very similar to the ones observed for adenosine, and as DCs can express CD39, it would be important to rule out the contribution of ATP degradation.

Commensal intestinal bacteria release large amounts of ATP into the extracellular space [136–138]. Germ-free mice have considerably lower ATP content in their feces, and antibiotic treatment of specific pathogen-free mice reduces fecal concentration of ATP [139]. ATP levels in the intestinal lumen strongly correlate with the frequency of LP Th17 cells, implying an association between bacterial ATP and Th17 cell differentiation. A subpopulation of antigen-presenting cells in the LP (CD70^{high}CD11c^{low}CD11b$^+$CD103$^-$CX3CR1$^+$) expresses higher levels of P2XR/P2YR than other DC subsets and is able to drive Th17 differentiation of naïve CD4$^+$ T cells through the production of IL-6, IL-23 and TGF-β [139]. In addition, their ability to induce the Th17 subset is significantly enhanced by ATP supplementation, indicating a role for ATP in the generation of naturally occurring Th17 cells in the LP. This could have important implications in the context of IBD, where variations in the composition of the microbiota might lead to altered extraluminal ATP concentrations and thus influence disease severity. Indeed, in the context of a T cell-mediated colitis model, ATP administration resulted in higher Th17 cell numbers and worsening of clinical symptoms [139]. Furthermore, intestinal biopsies from patients suffering from Crohn's disease displayed higher expression of the P2X$_7$R, which mainly co-localized with DCs and macrophages [140]. In summary, ATP levels can directly and indirectly influence inflammation.

Short-chain fatty acids

Although for many years it was unclear how gut-colonizing bacteria modulate cell function, current studies indicate a role for bacterial-derived metabolites as emissaries linking the gut flora and the host (reviewed in [141]). Short-chain fatty acids (SCFAs), defined as C1–6 organic fatty acids, represent the main metabolic product of anaerobic bacterial fermentation in the gut [142]. They are produced through the breakdown of dietary fiber, carbohydrates and proteins and serve as an energy source for both commensal bacteria and the colonic epithelium [143]. Acetate is the most abundant SCFA found in the colon, followed by propionate and butyrate in an approximate constant ratio of 60:20:20 [144, 145]. Their concentration is highest in the caecum and decreases towards the distal colon, reflecting the availability of substrate for fermentation throughout the gut [146]. The majority of SCFAs is taken up by colonic epithelial cells through passive diffusion or active transport by solute carriers [147], where they can regulate different cellular processes such as epithelial cell proliferation and differentiation [148]. While butyrate is mostly metabolized by colonocytes, acetate and propionate can enter the circulation, serving as a substrate for gluconeogenesis or *de novo* lipid synthesis [149]. SCFAs influence a wide range of physiological functions including electrolyte and water absorption, regulation of gut motility, as well as leptin and peptide YY secretion (reviewed in [150]). These actions occur by directly acting on target cells or indirectly through a gut-brain neuronal circuit [151].

Accumulating evidence suggests that SCFAs also have profound effects on the immune system where they act via two major pathways. The best characterized effect is the modulation of gene expression through inhibition of class I and IIa histone deacetylases (HDACs) [152]. More recently, SCFAs were found to be the natural ligands for the orphan G-protein coupled receptors GPR41 (free fatty acid receptor 3 – FFAR3) and GPR43 (free fatty acid receptor 2 – FFAR2) [153]. Butyrate also acts on the niacin receptor GPR109a (also known as hydroxycarboxylic acid receptor 2) [154]. FFARs for SCFAs are coupled to pertussis toxin-sensitive G$\alpha_{i/o}$ subunits. Activation of these receptors results in inhibition of adenylate cyclase and decreased cAMP [155]. FFAR2 also couples to Gα_q and promotes calcium release, a prerequisite to induce cell migration [156].

It is currently accepted that within the intestinal microbiota, specific bacterial species can positively influence health status and improve disease resistance against inflammatory diseases such as colitis, arthritis or asthma [125]. For example, IBD, a chronic inflammatory disorder of the gastrointestinal tract, is associated with an imbalance in healthy microbiota especially from the genera *Bifidobacterium* and *Bacteroides* [157], and a parallel reduction in SCFAs [158]. Furthermore, germ-free mice recolonized with *Bacteroides fragilis* [159] present less severe signs of colonic inflammation in the DSS colitis model,

a well-established murine model of human IBD. Although the beneficial effects of *B. fragilis* colonization have been ascribed to Polysaccharide A (PSA), as PSA alone is able to protect mice from experimental colitis by suppressing IL-17 production and promoting the development of IL-10-producing Tregs in the gut [160], feeding of germ-free mice with acetate can mimic the protective effect [161]. In 2009, Sina et al. demonstrated that mice lacking the FFAR2 receptor are more susceptible to acute DSS colitis [162]. In this model, SCFA-induced neutrophil recruitment through FFAR2 contributed to contain systemic bacterial dissemination and was therefore crucial to prevent mortality. Yet, $Ffar2^{-/-}$ mice were protected from chronic DSS colitis [162]. $Ffar2^{-/-}$ mice presented lesser signs of colonic inflammation including less tissue damage and neutrophil infiltration in both models. Strong mucosal neutrophil infiltration and over-activation is a hallmark of IBD (reviewed in [163]). Along the same line, several groups have shown that SCFAs promote neutrophil migration and ROS production in a FFAR2-dependent manner [155, 164–166, 161]. However, using a different transgenic mouse strain, Maslowski and colleagues found that absence of FFAR2 results in more severe inflammation as well as higher levels of neutrophilic infiltration and activation during the acute phase of DSS treatment [161]. The protective effect of FFAR2 was attributed to its expression on immune cells as revealed using bone marrow chimeras. In the same study, greater morbidity and impaired weight recovery was observed in $Ffar2^{-/-}$ mice during chronic DSS colitis [161]. The reasons for these discrepancies between studies are not yet clear. Since FFAR2 deficiency in neutrophils does not seem to affect their ability to migrate to other chemoattractants [166], increased neutrophilic recruitment in the latter model might be due to the presence of other potent chemokines at the site of inflammation. Besides, one should take into account the strong inter-laboratory variability of the DSS colitis models, mainly due to the different colitogenic potential of the DSS batches, but also to the mouse strain used, as well as environmental factors such as the hygiene conditions of the mouse facility [167] which in turn would impact the microbiota. In this sense, while Bacteroidetes produce preferentially acetate and propionate, Firmicutes generate mainly butyrate. Acetate and propionate are highly potent at activating FFAR2 whereas butyrate has less activity. Conversely, FFAR3 has higher affinity for butyrate than propionate and marginally binds acetate. Thus, the net effect of deleting one receptor may be subjected to several variables, including the presence or absence of specific commensals as well as diet composition. A further limitation to define the role of FFAR2 in SCFA activity *in vivo* relates to the wide expression pattern of this receptor in immune cells. ImmGen gene expression data suggests that in addition to neutrophils, *Ffar2* is also expressed in eosinophils, monocytes, splenic, mesenteric and small intestine DCs, as well as in resident macrophages from the lung [33].

In macrophages, SCFAs suppress inflammatory cytokine production through the attenuation of HDAC activity and concomitant modulation of gene expression [168]. Along the same line, a study using immature and LPS-matured human monocyte-derived DCs revealed that both butyrate and propionate strongly reduced the expression of several pro-inflammatory chemokines and cytokines at the transcriptional level [169]. Similar observations were made with murine bone marrow-derived DCs, where acetate, butyrate and propionate inhibited the LPS-induced expression of costimulatory molecules CD80, CD86 and CD40 and production of pro-inflammatory cytokines [170, 171]. DCs exposed to SCFAs also displayed a strong Treg-inducing capacity [172]. Analysis of the gene expression profile of murine *in vivo* FLT3L-expanded splenic DCs exposed to butyrate or the HDAC inhibitor Trichostatin A showed repression of LPS-responsive genes, particularly *Il12a*, *Il6* and *Relb*, a member of the NF-κB family mediating DC maturation [172]. Thus, in contrast to neutrophil migration and activation, which are dependent on GPCR activation, the anti-inflammatory effects of SCFAs on macrophages and DCs are linked to the modulation of gene expression, an effect that might be related to HDAC inhibition. Accordingly, the effect of butyrate on Treg induction by splenic DCs was independent of GPR109a expression, yet the contribution of FFAR2 to these effects was not investigated [172]. Conversely, a later study reported that $Gpr109a^{-/-}$ colonic DCs and macrophages express less RALDH1 and IL-10 and as a consequence fail to induce Tregs [173]. Furthermore, $Gpr109a^{-/-}$ mice showed lower Treg numbers and frequencies in colonic LP, as well as enhanced susceptibility to acute DSS colitis and colonic inflammation [173]. Of note, while both colonic and splenic macrophages exhibit similar amounts of GPR109a, colonic DCs express more GPR109a than splenic DCs, possibly explaining the contradictory results. Further studies using conditional knockout mice will be required to better understand the effects of SCFAs on specific cell populations *in vivo*.

SCFAs have also been reported to act directly on T cells promoting either immunity or tolerance depending on the cytokine milieu. Using the TNBS T cell-dependent colitis model, more severe symptoms were observed in $Ffar2^{-/-}$ mice, accompanied by exacerbated intestinal Th17 responses [161]. The same mice were also more susceptible in the models of K/BxN serum-induced inflammatory arthritis and OVA-induced allergic airway inflammation [161]. Furusawa et al. showed that a high fiber diet increases caecal levels of SCFAs, which results in enhanced differentiation of colonic IL-10-producing Tregs (cTregs) and ameliorates the development of adoptive transfer colitis [174]. This effect was mediated by commensal fermenting bacteria, since no expansion of Foxp3+ Tregs could be observed under germ-free conditions [174]. In the same vein, microbiota-deficient broad spectrum antibiotic-treated mice displayed reduced SCFA

concentrations in the stool and less cTregs [172, 175]. Butyrate administration to mice which selectively lack peripherally-derived Tregs but display intact thymic Treg differentiation, due to deletion of the conserved non-coding DNA sequence (CNS) 1 in the *Foxp3* gene, did not result in Treg expansion. These findings suggest that butyrate is only able to act on extrathymic Tregs. Mechanistically, the authors demonstrated that these effects were not due to improved proliferation or survival of Tregs, but enhanced histone acetylation in the promoter and conserved non-coding regions of the *Foxp3* locus, thereby enhancing the accessibility of other transcription factors to enhancer elements [174]. These results are supported by previous studies showing the inhibitory effect of Class IIa HDAC during Treg induction [176–179]. In addition, butyrate promotes the acetylation of Foxp3 itself increasing its stability [174]. Nevertheless, HDAC inhibition might not be the only mechanism involved in cTreg induction by SCFAs. *Ffar2*$^{-/-}$ mice failed to expand their cTreg compartment after propionate administration, advocating for an involvement of this receptor [175].

While these findings link SCFAs and fermenting commensals with the induction of cTregs, thus providing a new molecular mechanism for their anti-inflammatory effect, the effect of SCFAs on other T helper (Th) cell subsets is less well understood. Several groups reported no major effects of SCFAs on Th subsets [175, 174, 172]. In contrast, other studies indicate that propionate and butyrate potentiate IFNγ and IL-17 production during Th1 and Th17 differentiation, although these cells produce more IL-10 and have suppressive capacity [180]. These effects were not mediated by FFAR2 or FFAR3 as T cells do not express FFAR2 or FFAR3 at functionally relevant levels [180]. Instead, SCFAs act by inhibition of HDAC activity and subsequent enhancement of the mTOR-S6K pathway [180].

Given their ability to be transported to the systemic circulation, SCFAs can also exert functions in other organs besides the intestine. An interesting study linked fiber consumption to lung homeostasis, showing that low fiber intake renders mice more susceptible to allergic airway inflammation (AAI). This effect is mediated by SCFAs, since a high fiber diet promoted the growth of SCFA-producing bacteria, resulting in increased systemic SCFA levels, and administration of propionate in a model of AAI ameliorated the disease symptoms in a FFAR3-dependent manner [17]. Although no SCFAs were detected in the lungs, activation levels of DCs from lung-draining lymph nodes negatively correlated with fiber intake. Indeed, propionate can directly act on DC precursors in the bone marrow, promoting the differentiation of cells with lower Th2-inducing potential [17]. Another study reported that SCFAs are able to reach the brain, where they can influence microglial function [181]. Absence of microbiota leads to disruption of microglial homeostasis under steady state conditions and stunted microglial activation in response to inflammatory stimuli.

SCFA administration was able to revert these effects, though no expression of FFAR2 was detected in microglia. Therefore, SCFAs might either modulate microglia through a FFAR2-independent mechanism and/or act in an indirect way, targeting an intermediary cell subset that will respond by producing factors that affect microglial function. Analysis of the microglial compartment in *Ffar2*$^{-/-}$ mice advocates for a FFAR2-dependent mode of action, but the specific population which participates in this dialog remains elusive.

Collectively, this evidence points to a tight relationship between microbiota and tolerance. Yet, studies attempting to use SCFAs as a therapeutic approach in inflammatory situations have rendered contradictory results (reviewed in [182]). Thus, further investigation will be required to better understand their mechanism of action on specific cell types and to prove their anti-inflammatory potential as new therapeutic tools.

Medium-, long-chain and polyunsaturated fatty acids

In addition to the above mentioned GPCR recognizing SCFAs, interest has been put on other receptors recognizing medium-chain (C6-C12) and long-chain fatty acids (C10-C18) as well as polyunsaturated fatty acids (PUFAs), usually acquired from the diet. For example, GPR120 (FFAR4), which recognizes long-chain unsaturated fatty acids such as docosahexaenoic acid (DHA), possesses potent anti-inflammatory actions effects by preventing the secretion of pro-inflammatory cytokines in macrophages [183]. The effects mediated by GPR120 may explain, at least in part, the beneficial effects of omega-3 fatty acids contained in the Mediterranean diet, long recognized for its positive effects on health. Still, PUFAs can also exert their function through peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptors that act in an anti-inflammatory manner, both by directly regulating gene expression as well as through interference with the prototypic inflammatory mediator NF-kB (reviewed in [184]). GPR40 and GPR84 represent two other examples of previously considered orphan receptors that are now jumping to fame because of their recently described effects on the immune system and the identification of their endogenous ligands [185, 186]. Studies targeting these receptors in specific cells types, and particularly in DCs, as well as investigating their role in immune-mediated diseases will contribute to our understanding of their function and their potential therapeutic applications.

Vitamin A

Vitamin A (all-*trans* retinol, VitA) is a fat-soluble vitamin that has many essential functions for the life of all vertebrates and its deficiency has several detrimental effects on human health. Since animals lack the required machinery for *de novo* VitA

synthesis, they must obtain it from their diet, either from plants and microorganisms in the form of carotenoids, or by ingesting animal-derived products that contain retinyl esters. After absorption in the intestine, both sources are transported to the liver in the form of retinyl esters. The majority travels incorporated into chylomicrons and is taken up by hepatocytes, where they are hydrolyzed. Free retinol can then associate with retinol-binding protein (RBP), which allows the secretion of the retinol-RBP complex into plasma. Another fate of free non-esterified retinol is storage within lipid droplets in hepatic perisinusoidal stellate cells, which occurs especially under VitA-sufficient conditions (reviewed in [187]). Although the main site of VitA depot is the liver (>90%), its presence has been detected in other organs such as skeletal muscle and bone marrow. Plasmatic concentrations of retinol are tightly regulated and maintained around 2 µM regardless of fluctuations in daily VitA intake (reviewed in [187]).

Target cells are responsible for carrying out the conversion of all-*trans* retinol to all-*trans* retinoic acid (ATRA), which is the active form of VitA. This two-step process involves oxidation of all-*trans* retinol to all-*trans* retinal followed by oxidation of retinal to ATRA. The first step can be performed by cytosolic alcohol dehydrogenases (ADH) and membrane bound short-chain dehydrogenases/reductases (SDR). The second step can be executed by at least three different enzymes, known as retinal dehydrogenases 1, 2 and 3 (RALDH1–3) [187].

ATRA exerts most of its functions through the activation of Retinoic Acid Receptors (RARs), which are ligand-activated nuclear receptors [188, 189]. There are three different subtypes of RARs (α, β and γ), which display higher homology between different species than among themselves, implying they have specific roles in retinoic acid signaling. RARs function as heterodimers, associated with another nuclear receptor, Retinoid X Receptor, which also exists in three different forms (α, β and γ). Upon ligand binding, RAR-RXR heterodimers associate with specific DNA sequences known as RAR elements (RARE) and retinoid X response elements (RXRE) located in promoter regions of target genes. RAR-RXR heterodimers can then modulate gene transcription by recruiting negative or positive regulatory proteins. More than 500 genes have been suggested to be regulated by ATRA, either by direct or indirect mechanisms, which accounts for the pleiotropic effects of this metabolite [190].

ATRA participates in maintaining the homeostasis of cDC subsets. The fate commitment of pre-cDCs into different DC subpopulations is dependent on the concentration of this metabolite. In particular, the development of splenic CD11b$^+$CD8α^- and small intestine LP CD11b$^+$CD103$^+$ DCs is severely impaired in absence of VitA [191, 192], whereas other DC subsets and progenitors are able to develop normally. ATRA can also guide the differentiation of *in vitro*-generated CD103$^+$ DCs [73] into the cDC1 and cDC2 subsets found in the intestinal LP, CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ [193]. Addition of ATRA to human monocyte-derived DCs during their development imprints them with mucosal-like properties. ATRA-conditioned DCs express CD103 [194, 195] and constitutively produce the anti-inflammatory cytokine IL-10. They also display higher levels of CCR7 than their non-treated counterparts, suggesting that these DCs would exhibit enhanced migration to the draining LNs [194]. Some studies also reported the induction of RALDH2 after ATRA administration [195].

In the gut, a specific subset of intestinal DCs expresses RALDHs which allow them to convert retinol into ATRA [196]. This subpopulation of DCs is characterized by the expression of CD103 and is present in Peyer's Patches, mLNs and LP. Regarding the signals required to induce ATRA-synthesizing ability in gut-associated DCs, the available information suggests it is mediated by a mechanism dependent on TLR1/2-MyD88 signaling. Expression of ATRA-synthesizing enzymes can be induced in DCs by TLR2 ligation [197, 198]. Furthermore, DCs isolated from *Tlr2*$^{-/-}$ mice express lower levels of ATRA-producing enzymes and show an impaired capacity to imprint gut-homing molecules in T cells. The same was observed for *Myd88*$^{-/-}$ DCs [198].

ATRA exerts several functions in the intestine; first of all, it is essential for maintaining homeostasis of the mucosal immune system, by inducing the gut homing receptors α4β7 integrin and CCR9 in T cells and B cells. Depletion of VitA in mice caused a significant reduction of α4β7 integrin$^+$ CD4$^+$ T cells in secondary lymphoid organs and complete absence of T cells in the LP, indicating that this metabolite is essential for T cell migration to gut tissues [196]. Moreover, ATRA is required by CD103$^+$ DCs in the gut to induce the generation of intestinal Foxp3$^+$ Tregs via a mechanism which depends on TGF-β [199, 7, 200, 201]. Induction of Tregs by intestinal CD103$^+$ DCs may play an important role in maintaining tolerance to dietary antigens and commensal flora. ATRA-conditioned DCs can induce gut homing IL-10 producing Tregs which is dependent on their ability to produce ATRA [195]. Furthermore, ATRA seems to regulate the balance between Th17 and Treg differentiation. Despite their opposing roles, both of these subsets require TGF-β for their development. Notwithstanding, the additional presence of ATRA is able to inhibit Th17 differentiation *in vitro*. This mechanism could be particularly relevant in mucosal tissues, where tight regulation of immune responses by ATRA would be crucial for maintaining integrity of the intestinal barrier [202–204]. However, the ATRA concentration used in this work appears to be 1000-fold higher than physiological levels in plasma (µM versus nM) [205], suggesting that these results should be interpreted with caution. Later studies have shown that nM concentrations of ATRA do not impair Th17 differentiation [206–208]. In fact, addition of an ATRA receptor antagonist inhibited the differentiation of Th17 cells *in vitro* [206], while VitA-deficient mice showed a dramatic decrease of Th17

cells in the gut [207, 208], which could be explained by altered numbers and composition of the microbiota. Therefore, low levels of ATRA may be required for the development of intestinal Th17 cells.

Interestingly, the study of a mouse reporter strain that expresses luciferase upon RA signaling revealed high levels of RAR activity in CD4^{+} T cells upon activation. Furthermore, RA signaling proved to be essential to mediate rejection in a skin allograft model [209]. Ablation of RA signaling caused a shift from Th1/Th17 to a Th2 phenotype [209]. Furthermore, Th1 and Th17 mucosal and systemic responses were severely impaired in VitA-deficient mice, further underscoring the role of RA signaling in adaptive immunity [210]. These findings could explain the considerable amount of data showing a correlation between VitA deficiency and impaired responses to several pathogens. Several studies have revealed the importance of VitA in fighting infections. VitA deficiency in children from developed and developing countries has been linked to greater mortality caused by infectious diseases [211, 212]. Clinical trials showed that VitA supplementation in neonates and children reduced mortality by 12 and 25%, respectively [213, 214]. Mucosal IgA responses in the gut and respiratory tract are impaired in VitA-deficient individuals, possibly due to the essential role of ATRA in imprinting homing receptors to lymphocytes and in the induction of IgA responses [215]. Insufficient levels of VitA cause deficient immune responses to vaccines and respiratory and gastrointestinal pathogens. It has been reported that VitA deficiency in mice leads to decreased numbers of antigen-specific CD8^{+} T cells in the lower respiratory tract which express unusually high levels of CD103 [216]. This high CD103 expression has been suggested to interfere with T cell migration to the lower respiratory tract, thus accounting for the lower numbers observed. VitA-deficient mice also showed an altered IgA/IgG production ratio in response to intranasal inoculation of a Sendai virus vaccine [217]. Co-administration of VitA with the vaccine was able to improve the mucosal IgA response in VitA-deficient mice [218, 219]. Further work is needed to determine how the interplay between DCs and T cells can be tuned by VitA levels to regulate mucosal immunity.

Vitamin D

Vitamin D (VitD) is mostly produced in the skin, where a cholesterol derivative, 7-dehydrocholesterol, is converted upon exposure to sunlight via a UVB-dependent reaction. VitD can also be acquired from the diet in limited amounts. Once it enters systemic circulation, VitD must undergo 2 hydroxylation steps to be transformed into the metabolically active form, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$). The first hydroxylation occurs mostly in the liver and it is catalyzed by 25-hydroxylases. The resulting product, 25-hydroxyvitamin

D3, is the main circulating VitD metabolite and has a half-life of approximately 15 days. 25-hydroxyvitamin D3 is then further hydroxylated by 1-α hydroxylases to yield the final active metabolite of VitD, $1,25(OH)_2D_3$. This reaction mainly takes place in the kidneys and is tightly regulated by parathyroid hormone levels and serum calcium and phosphorous levels (reviewed in [220, 221]).

VitD has various described immunomodulatory effects. The first reports of the effect of $1,25(OH)_2D_3$ on immune cells [222–224], as well as the expression of a high-affinity receptor for this metabolite in different immune subpopulations [225, 226] were made over two decades ago. In line with these studies, VitD administration showed a therapeutic effect in different mouse models of autoimmune disease, including encephalomyelitis [227, 228] and type 1 diabetes [229].

$1,25(OH)_2D_3$ exerts its effects on immune cells by binding to the Vitamin D Receptor (VDR), a ligand-activated nuclear receptor that acts as a transcription factor upon binding of $1,25(OH)_2D_3$. When this metabolite binds to the receptor, it induces its heterodimerization with RXRs. The heterodimer binds to VitD response elements in the promoter of VitD target genes, inducing or inhibiting their transcription [230]. Analysis of $Vdr^{-/-}$ mice revealed lymph node hypertrophy and elevated numbers of mature DCs in the subcutaneous lymph nodes, highlighting the importance of the VitD-VDR signaling axis in maintaining DC homeostasis [231].

Studies of in vitro-generated human [232–234] and murine [231, 235] DCs conditioned with $1,25(OH)_2D_3$ or VitD analogs revealed that these cells are resistant to maturation induced by inflammatory stimuli. The involvement of VDR signaling in the effect mediated by $1,25(OH)_2D_3$ on DCs was confirmed in experiments with $Vdr^{-/-}$ DCs, where the immunomodulatory effects of this metabolite were lost upon deletion of the receptor [231]. Although there are some contradicting reports regarding their phenotype under steady state conditions, they unanimously show that $1,25(OH)_2D_3$-conditioned DCs fail to acquire a fully mature phenotype in response to inflammatory stimuli such as LPS, TNF-α and CD40 ligation. Treatment with $1,25(OH)_2D_3$ prevents the upregulation of MHC II and costimulatory molecule (CD40, CD80, CD86) expression [236, 232, 233, 231, 237]. $1,25(OH)_2D_3$-conditioned DCs produce significantly lower levels of IL-12 [232, 233, 231, 237], but higher amounts of the anti-inflammatory cytokine IL-10 [232, 234]. This results in a decreased capacity to induce T cell responses in a mixed-lymphocyte reaction setting [236, 234, 235, 232, 233, 237]. In addition, $1,25(OH)_2D_3$-conditioned DCs are able to induce IL-10-producing Tregs that display potent immunosuppressive activity [237]. In conclusion, these studies showed that $1,25(OH)_2D_3$ treatment induces a tolerogenic phenotype in DCs and introduced the idea of using $1,25(OH)_2D_3$-conditioned DCs for immunotherapy. Although tolerogenic DCs

can also be obtained by administering other compounds such as glucocorticoids, $1,25(OH)_2D_3$-treated DCs present the advantage of inducing Tregs with antigen specificity [237]. As a whole, this opens the door to using $1,25(OH)_2D_3$-treated DCs loaded with antigen as immunotherapy, excluding the possibility of unwanted off-target effects. Indeed, in a model of allogeneic pancreatic islet transplantation, adoptive transfer of antigen-loaded DCs only prevented graft rejection if DCs were pre-treated with $1,25(OH)_2D_3$ [238]. Furthermore, treatment with $1,25(OH)_2D_3$ enabled antigen-loaded DCs to promote prolonged survival of skin grafts expressing the same antigens [231].

Regarding the mechanism through which $1,25(OH)_2D_3$ exerts its effects on DCs, there are several reports implicating inhibition of NF-κB by this metabolite [239–242]. A recent study showed that VDR is able to interact directly with IKKβ, preventing assembly of the IKK complex and consequently inhibiting phosphorylation and degradation of IκBα. This results in impaired translocation of NF-κB to the nucleus and hence lower transcriptional activity [243]. In addition, VDR activation could induce metabolic reprogramming in DCs. Proteomic analysis revealed that $1,25(OH)_2D_3$ treatment induces dramatic changes in DC glucose metabolism, including proteins involved in glycolysis, the TCA cycle and pentose phosphate pathway [244]. Lipid metabolism was also modulated by this metabolite, in particular fatty acid oxidation and elongation in the mitochondria, glycerophospholipid metabolism and phospholipid degradation [244]. Later studies confirmed these findings, showing that $1,25(OH)_2D_3$ upregulates oxidative metabolism and aerobic glycolysis through the PI3K/Akt/mTOR pathway. Interestingly, $1,25(OH)_2D_3$-treated DCs rely on glycolysis to induce and maintain their tolerogenic phenotype [245, 246].

Serum levels of the active form of VitD $1,25(OH)_2D_3$ are in the picomolar range, which is around 100 times lower than the dose used in the studies described above. Conversely, the concentration of the monohydroxylated precursor is approximately 1000 fold higher [247], implying that the effect of VitD on DCs could be influenced by its local conversion by neighboring cells or even DCs themselves. This local production could lead to accumulation of this metabolite in the microenvironment, thus reaching effective concentrations to act on its target cells. Although the liver and kidneys are the major sites of VitD modification, the expression of VitD-metabolizing enzymes in immune cells [248, 249], including DCs [250] has been reported. In studies conducted by several groups, human in vitro-generated DCs and freshly isolated DCs from blood were capable of producing $1,25(OH)_2D_3$ when an external source of 25-hydroxyvitamin D3 was added [250–252]. $1,25(OH)_2D_3$ production was further increased when LPS was added to the culture. This observation was accompanied by the discovery of 1-α hydroxylase expression in these cell subsets, which was inducible by LPS stimulation [250]. Nevertheless, the VitD-metabolizing ability of the different DC subsets in vivo remains

unknown. It is important to consider that $1,25(OH)_2D_3$ generated by DCs could also act on other cells that express the VDR, such as T cells [225]. This would have important implications during the adaptive immune response by downregulating T cell activation and promoting generation of Tregs. In addition, it could be important for maintaining tolerance. $1,25(OH)_2D_3$ has a direct effect on T cells in vitro, characterized by an upregulation of Foxp3 and CTLA-4 and inhibition of pro-inflammatory cytokine production [253]. In contrast, the addition of 25-hydroxyvitamin D3, the precursor of the active metabolite, only had an effect on T cell activation when DCs were present, supporting the idea that DCs can regulate the activation of nearby T cells by metabolizing the VitD precursor into its active form [252]. A study by Sigmundsdottir et al. claimed that VitD conversion by DCs might play an important role in the skin. $1,25(OH)_2D_3$ induced the chemokine receptor CCR10 in T cells, thus promoting their migration and retention in the epidermis once they enter the skin [254]. Furthermore, this effect was further enhanced by IL-12. These findings led the authors to hypothesize that T cell recruitment to the epidermis could have emerged as a response to the epidermal damage caused by sun exposure. Nevertheless, the implications of this and whether this process functions as a tissue-repair mechanism need to be further investigated. Interestingly, the ImmGen database shows high VDR expression in LCs [33], suggesting that $1,25(OH)_2D_3$ synthesized in the skin could have an effect on this cell subset. Analysis of human ex vivo dermal DCs and LCs revealed that $1,25(OH)_2D_3$ diminished the immunogenicity of both subpopulations, but it only endowed LCs with Treg-inducing ability [255].

Current studies estimate that 1 billion people worldwide suffer from VitD deficiency [256]. A growing body of evidence supports the notion that low VitD levels correlate with higher occurrence of autoimmune diseases, including MS, type 1 diabetes, SLE and RA. In many of these cases, the information available suggests that VitD supplementation could prevent/delay the onset of disease or ameliorate its outcome. Epidemiological studies point towards a connection between geographical latitude and prevalence of MS; regions located closer to the equator have a lower incidence of the disease [257]. This gives room for speculation regarding the role of sun exposure, and therefore, VitD, in the development of MS. Supporting this hypothesis, the amount of VitD in serum from MS patients negatively correlate with disease severity [258]. Moreover, a recent clinical trial revealed that high-dose VitD supplementation in MS patients is able to downregulate IL-17 production by $CD4^+$ T cells, as well as the frequencies of effector $CD4^+$ T cells [259]. Another link between VitD and MS incidence was provided by several genome-wide association studies which implicated the genes coding for the VitD-metabolizing enzymes (*CYP27B1, CYP24A1*) in the pathogenesis of MS, showing that specific alleles correlate with greater risk of developing the disease

[260, 261]. Exon sequencing of *CYP27B1* from individuals belonging to families with history of MS identified a number of loss-of-function variants which were associated with higher incidence of MS [262]. There is also evidence of an association between specific polymorphisms in the VDR gene and predisposition to the disease [263, 264]. Although the polymorphisms implicated do not result in changes to the protein structure, they might affect the stability of the mRNA or its translation efficiency.

VitD has also been implicated in IBD. IBD patients display deficient VitD levels [265, 266], while $Vdr^{-/-}$ mice display higher susceptibility to different models of colitis [267, 268, 269]. These findings suggest a link between VitD levels and gut homeostasis. A study of the gut microbiome of $Vdr^{-/-}$ and $Cyp27b1^{-/-}$ mice, which cannot synthesize $1,25(OH)_2D_3$, revealed that these mutations result in alteration of the composition of intestinal flora, supporting a role for VitD in regulating colonization by different bacterial families. Together, it appears that reduced VitD levels can give rise to reduced tolerogenic DC frequencies, leading to insufficient Treg numbers and break of tolerance.

Concluding remarks

The field of metabolites exerting effects on DCs, as well as other immune cell populations, is ever expanding. Other classes of metabolites which were not included in this review due to space limitations include tryptophan derivatives and aryl hydrocarbons (reviewed in [270]), cholesterol derivatives and bile acids (reviewed in [271]), lipoxins and resolvins (reviewed in [272]), and others (reviewed in [141]).

Although the first studies ascribing immunomodulatory properties to the metabolites listed in this review were published over three decades ago, their role as a means of communication between DCs and the environment has only recently begun to be deciphered. This is due in part to major advances in the development of tools to study DC biology and ontogeny that took place in the last few years. A significant proportion of the information available so far derives from studies using GM-CSF-derived DCs which present several limitations. Therefore, readdressing some of the initial questions with more physiological culture systems is highly important. Together with the use of mice that allow targeting specific DC subsets *in vivo*, this will shed more light on how metabolites can influence DC function in the context of immune-mediated diseases.

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