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Vitamin D and microbiota: Two sides of the same coin in the immunomodulatory aspects

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ABSTRACT

The gut microbiota is crucial for host immune response, vitamin synthesis, short chain fatty acids (SCFAs) production, intestinal permeability, nutrient digestion energy metabolism and protection from pathogens. Therefore, gut microbiota guarantees the host's predisposition to gastrointestinal diseases. Intestinal microbiota may be damaged by environmental components with negative health conditions. Dysbiosis consisting in alteration in the gut microbiota has been involved in several disorders including inflammation, allergic reactions, autoimmune diseases, heart diseases, obesity, and metabolic syndrome and even in the state of malignant carcinogenesis existing in humans. Several epidemiological studies have shown that inadequate solar exposure results in vitamin D insufficiency/deficiency which has a strong impact on different immune responses and the occurrence of a wide range of pathological conditions. Additionally, new evidence indicates that the vitamin D pathway plays a key role in gut homeostasis. Due to the strong connection between vitamin D and microbiota, herein we focus on the new findings about intestinal bacteria–immune crosstalk and the impact of vitamin D in gut microbiota regulation, in order to offer new clarifications on their interaction. Understanding the mechanism by which vitamin D can affect the gut microbiota composition and its dynamic activities, as well as the innate and adaptive state of the immune system, is not only a fundamental research but also an opportunity to improve health status.

1. Introduction

The gastrointestinal (GI) tract covers a large area and interacts with many foodborne and microbial antigens. The microbiota embraces an enormous number of commensal microorganisms that colonize the GI. The microbiota residing in the human intestinal tract includes a population of about 100 trillion active and different microorganisms (including 500 and 1,000 different species) [1]. As a whole, the gut microbiota embraces almost 100 times more genes than the human genome [1]. The intestinal microbiota is one of the main environmental elements influencing the disease's onset. Gut microbiota achieves different structural and histological functions, inducing essential metabolic processes able to preserve health, including the digestion of complex carbohydrates and fats, amino-acid synthesis, the uptake of vitamins, and withdrawals of supplementary energy [e.g., short chain fatty acids) from non-digestible dietary substrates [2]. This

Abbreviations: AJs, adherens junctions; AMPs, antimicrobial peptides; AP-1, activator protein-1; APCs, antigen-presenting cells; ASC, apoptosis-associated speck-like protein; Bregs, regulatory B cells; CAMP, cathelicidin antimicrobial peptide; CCL, C–C motif chemokine ligand; CSGG, cell surface β-glucan/galactan; CD206, mannose receptor; CTLA-4 or CD152, cytotoxic T lymphocyte antigen; Cyp24, 25-hydroxyvitamin D 24-hydroxylase; CYPR1, cytochrome P450; CYP27B1, cytochrome P450 family 27 subfamily B member; 1, 25(OH)2D3, 1,25-dihydroxyvitamin D3; DCs, dendritic cells; DEFB4, defensin beta 4 gene; DSS, dextran sulfate sodium; FGF23, fibroblast-like growth factor-23; GI, gastrointestinal; HBD-2, defensin ??2; hCAP18, cathelicidin; HDAC, histone deacetylase; IgA, immunoglobulin A; IL, interleukin; ILCs, innate lymphoid cells; INF-γ, interferon gamma; IBD, irritable bowel disease; IEC, intestinal epithelial cells; IRAK 4, IL-1 receptor-associated kinase 4; JAM-A, junctional adhesion molecule; KO, knockout; LL-37, Cathelicidin leucin-leucin-37; LPS, lipopolysaccharide; LP, lamina propria; M1, classically activated macrophages; M2, alternatively activated macrophages; MAMPs, Microbe-Associated Molecular Patterns; MDP, muramyl dipeptide; MLCK, myosin light chain kinase; MLNs, mesenteric lymph nodes; MyD88, Myeloid differentiation factor 88; NFAT, nuclear factor for activated T cells; NF-κB, nuclear factor kappa light chain enhancer of B cells; Nod2, nucleotide binding oligomerization domain 2; NLRs, NOD-like receptors; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; sIgA, secretory IgA; SCFA, short chain fatty acids; PTH, parathyroid hormone; RORγT, retinoid-related orphan nuclear receptor; SFB, segmented filamentous bacteria; TER, transepithelial resistance; TGF-β, transforming growth factor beta; T, helper 1; Th2, T helper 2; Th17, T helper 17; TJs, tight junctions; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfuric acid; TNF-α, tumor necrosis factor alpha; Treg, regulatory T cell; VDBP, vitamin D binding protein; VDR, vitamin D receptor; VDREs, vitamin D responsive elements; D3, 1,25(OH)2D3; ZO-1, zonulin occluden-1; ZO-2, zonulin occluden-2

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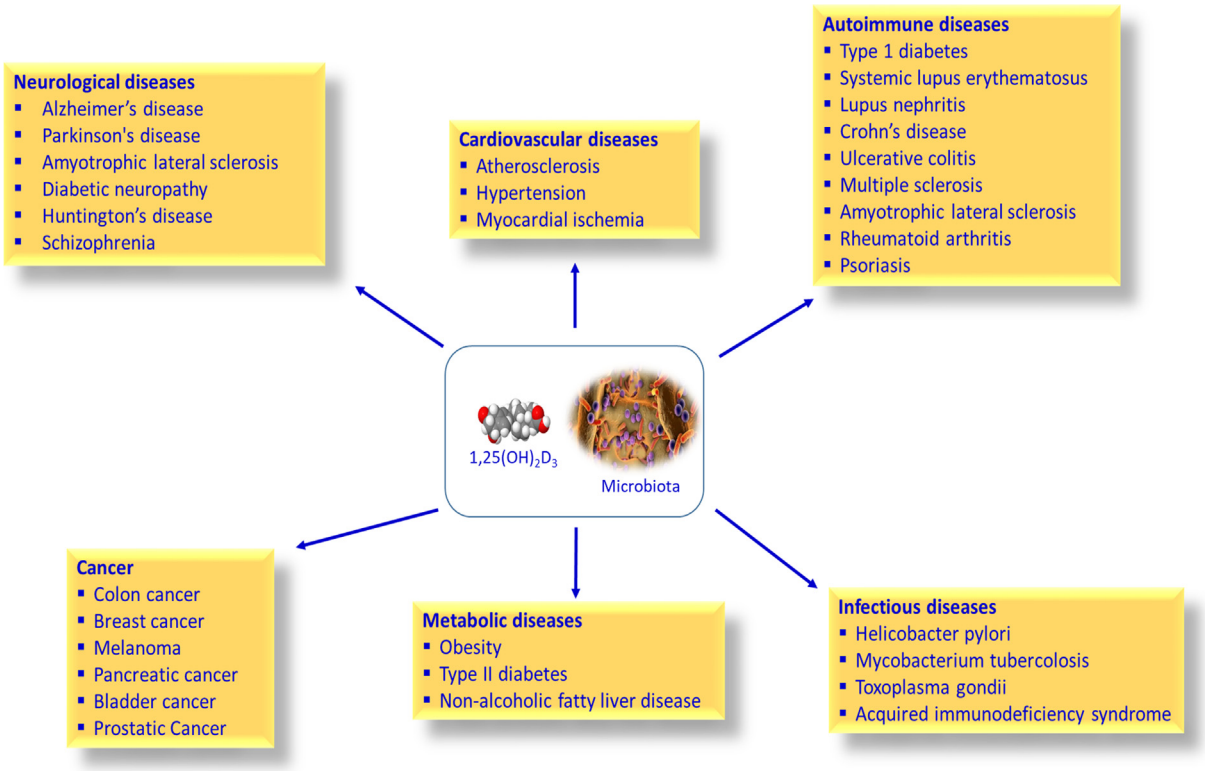

Fig. 1. The most frequent effects of both Vitamin D 3 deficiency and gut microbiota modification are systemic and chronic inflammation. The alteration of the mucosal immune system enhances the susceptibility to infectious diseases and increases the risk of various pathogenic conditions, including metabolic, autoimmune and degenerative disorders and neoplastic process.

sophisticated bionetwork determines the host's susceptibility to GI infections, avoids inflammatory responses, recovers metabolism and protecting against entero-pathogens guarantees resistance to infection [3]. A healthy gut microbiota, being indispensable for host immune function safeguards from autoimmune disease [4,5] (Fig. 1). The composition of the intestinal microbiota constitutes a dynamic element that in each person is predisposed by route of delivery [6] breast-feeding, age, sex, genetic background, environmental factors, lifestyle, nutrients, hygiene, antioxidants, medications and antibiotics intake [6,7]. Alteration of the microbial colonization in the GI tract since birth, at the time of dynamic changes in the newborn gut, may have long-term effects on health status [7]. The majority of the microbial populations, in the adult subject, belong to the phyla *Actinobacteria* and *Proteobacteria*, and approximately 90% to the *Bacteroidetes* and *Firmicutes phyla* [8]. These phyla are differentially disseminated throughout the gut and regulate the microbial ecosystems [9]. Among *Firmicutes phylum*, the *Clostridium coccoides* family is the principal residents in the gut microbiota. The sophistication of the human gut microbiota is demonstrated by the spatial distribution and alternation of microorganisms all through the GI tract. Diverse bacteria populate distinct segments of the intestine and the different layers of the gut [9]. Therefore, the diversity of the microbial population in each intestinal niche would be the most suitable to protect local tissue homeostasis [10]. Since, the autochthonous microbiota has been modified to adapt to new functional specializations, the various environmental factors and dietary variations influence, progressively over time, the human colonic microbiota conferring countless health benefits to the host [11]. So, gut microbiota offers beneficial functions to the host, in return, the host offers an environment rich in nutrient that promotes grow of microbiota and helps the host homeostasis [10]. This symbiotic synergism between the intestinal microbiota with the host, named eubiosis, affects significantly both the immune and neuroimmune system development and, in that way, defends the host from colonization of pathogenic microbes and controls the inflammatory processes [10,11]. On the contrary, the inflammation in the GI causes an alteration in the microbiota, as a result more pathogenic organisms, named *Proteobacteria*, compete with commensals microorganisms initiating alterations in the gut microbiota, namely dysbiosis. Therefore, the variation in the composition of the bacterial microbiota is an intensified reaction of the host to injury. A large number of dietary elements interact with enzymes, transcription factors, and nuclear receptors of human cells stimulating the selection of microbial populations and affecting the shift from eubiosis to dysbiosis [12]. The most frequent effect of dysbiosis are systemic inflammation and chronic inflammatory diseases, the modification of the mucosal immune system and the risk of various diseases, including metabolic, autoimmune and degenerative disorders, and even cancer [13] (Fig. 1). The relevance of microbiota homeostasis in the etiology of diseases underscores the need to recognize regulating factors able to influence the intestinal bacterial composition. Vitamin D deficiency or insufficiency mainly due to insufficient sunlight exposure, altered dietary composition, air pollution, and indoor lifestyles are prevalent in the world and is often associated with various diseases such as autoimmune diseases, hepatitis, and cancer [14–16] (Fig. 1). Vitamin D3 acts as immune modulator able to prevent inflammation and infections [14] and consequently may be important in affecting the early gut microbiota. In this review, we will provide an insight of the interactions between Vitamin D3 and microbiota and their impact in the immune responses in order to disclose the potential association between vitamin D deficiency and dysbiosis.

2. Vitamin D metabolism

Vitamin D is predominantly synthesized in the skin via a photolysis reaction. The nutrients are insufficient to provide adequate levels of vitamin D3. Vitamin D3 produced in the skin by sunlight exposure or by nutrients is inactive. The metabolic processes that transform the

biologically inactive form of vitamin D3 into active metabolites initiates when vitamin D3 is photosynthesized in the skin or once is ingested. In the course of photosynthesis in the skin, the 7-dehyrocholesterol is transformed into pre-vitamin D3, which is then isomerized into cholecalciferol or D3 [17]. The vitamin D3 synthesis is influenced by skin pigmentation, air pollution, sunscreen use, latitude, season, and time of the day [17]. Once vitamin D3 binds Vitamin D-binding protein (VDBP) pours into the vessels where it is transported from the bloodstream to the liver [17]. Alternatively, vitamin D2 or ergocalciferol deriving from nutrient crosses the small intestines and binding to chylomicrons, reaches the lymphatic system, and then in the bloodstream where they are poured into the liver. In the liver, both vitamin D2 and vitamin D3, are hydroxylated by the enzyme cytochrome P450 (CYP R1) to 25-hydroxyvitamin D3 [25(OH) D_3]. The 25(OH)D3 is then transformed to 1α,25-dihydroxyvitamin D3 [1α,25$(OH)_2D_3$] by the 1-α-hydroxylase enzyme cytochrome P450 family 27 subfamily B member 1 (CYP27B1) mainly in the kidneys [17]. The active metabolite 1α,25$(OH)_2D_3$ (or calcitriol), is a powerful steroid hormone [17]. Feedback mechanisms control the synthesis of 1,25(OH)2D in the kidneys via serum levels of parathyroid hormone (PTH), fibroblast-like growth factor-23 (FGF23) calcium, and phosphate [17]. Additionally, 1α,25$(OH)_2D_3$ is synthetized in numerous tissues (e.g., blood vessels, macrophages, colon, pancreas, etc.) by enzymatic processes [17]. 1,25(OH)2D3 binds to the vitamin D receptor (VDR), which can be found in around 30 different tissues [17] and is able to regulate the expression of more than 1000 genes in the genome [18]. The VDR is member of the nuclear receptor family of ligand-regulated transcription factors. The complex1,25(OH) 2D bound to VDR facilitates the transcription of DNA binding to the DNA responsive elements of the controlled gene acting with another nuclear receptor, retinoid X receptor. These elements are located not only in the promotor region, but are disseminated over the gene DNA. The gene expression embraces several nuclear transcription factors which interact with the responsive elements [19].

3. Intestinal microbiota and intestinal barrier function

The GI tract is a selective barrier allowing liquids and nutrients passage while act as the first line of defense against external insults [20]. The intestinal epithelial cells are bound each other by apical junctional complexes including tight junctions (TJs), adherens junctions (AJs), desmosomes and gap junctions, which along with mucus, antimicrobial proteins, and immunoglobulin A (IgA) control paracellular permeability and form an obstacle against toxins and enteric pathogens [21]. AJs are mostly constituted by cadherins linked to the actin cytoskeleton through a member of catenins, while TJs are the product of interactions of occludin, claudin and junctional adhesion molecule (JAM)-A, connected to the actin cytoskeleton via zonula occludens proteins (ZO-1, ZO-2) and α-catenin [22]. Epithelial cells create a physical obstacle, inhibiting the passage of the luminal contents of the internal tissues. Myosin phosphorylation and contraction of the actin–myosin complex control the absorbency of the epithelial barrier [23]. The intestinal epithelium is essential for the absorption of nutrients, and ensures crosstalk with the external surface of the body as well as among gut microbes. Microbiota induces TJ protein expressions and support the host reduction paracellular permeability [24,25]. Variation in the structure of the intestinal microbiota causes epithelial barrier dysfunction [26] (Table 1), which consents the translocation of endoluminal molecules into the deeper layers which, in turn, deteriorates the intercellular connexion initiating inflammatory processes, generating colitis and bacterial infection of the intestinal tissue [27]. The activation of the inflammatory response induces the synthesis of proinflammatory cytokines, such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), which both regulate the expression of several TJ proteins, such as ZO-1, JAM-A, occludin, claudin-1 and claudin-4 [28]. The compromised intestinal barrier function triggered by mucosal ulceration decreases the number of TJ strands and modifies TJ protein expression and allocation. Furthermore, the intensification of the apoptotic process results in an intensified impairment, which makes the host vulnerable to luminal pathogens and dietary antigens that can generate mucosal inflammation [29]. Recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) of epithelial cells is essential for this balance. Through a mechanism mediated by PRR the gut microbiota by its metabolites is able to induce AMPs production by Paneth cells [30] (Table 1). Interestingly, Myeloid differentiation factor 88 (MyD88) is a PRR located at the crossing point of the interaction between microorganisms and the host and is the major adaptor molecule for the majority of the Toll-like receptors (TLRs) [31] (Fig. 2). In the intestinal epithelial cells, it acts as a sensor affecting the gut microbiota structure, energy metabolism and the development of obesity and connected diseases [31].

4. Vitamin D 3 and intestinal barrier function

Vitamin D3 exerts extraordinary regulatory role in protecting the GI epithelium and mucosal barrier homeostasis. Several studies have underscored the relevance of vitamin D for intestinal mucosal immunity, for preserving TJ and function of colonic epithelium. In contrast, vitamin D deficiency induces enhanced gut permeability, colon mucosa bacterial infiltration, and translocation of intestinal pathogens in the gut of the host causing subclinical inflammation and metabolic endotoxemia [32]. Vitamin D considerably reduces the TJ injury-related increase in intestinal mucosa barrier permeability. Additionally, 25 $(OH)_2D_3$ treatments significantly decrease MyD88 expression and zonulin release levels [33], controls intestinal barrier by the up-regulation of TJ proteins including occludin, ZO1, claudin 2 and E-cadherin [34] (Table 2A) preserving in this manner the adhesive phenotype of intestinal epithelial cells. Several studies in VDR gene–knockout (KO) mice with experimental colitis induced using dextran sulfate sodium (DSS) showed serious incidence of colitis [35] demonstrating the deleterious consequences of vitamin D deficiency on inflammatory disease severity. The VDR KO mice displayed an amplified vulnerability to lipopolysaccharides (LPS), expressed significant levels of inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-10, IL-21, and IFN-γ, and were predisposed to weight loss, bleeding, ulceration, septic shock, and death compared to wild-type mice [36] (Table 2B). Increased production of inflammatory cytokines such as TNF-α contributed to the failure of GI barrier integrity in vitamin D deficient and VDR KO mice [36]. Also, 1α,25$(OH)_2D_3$ rises transepithelial resistance (TER) in cultured cells [35], while the absence of VDR significantly decreases TER in experimental colitis mouse models [36], supporting that vitamin D is essential in preserving mucosal epithelial barrier integrity [36]. The permeability of the gut enhanced along with the injury of TJ proteins both in VDR KO mice and vitamin D deficient mice [37] (Table 2B). In *vitro* studies confirmed that vitamin D protects the integrity of intercellular TJ against DSS-induced disruption [35] through the regulation of colonic antimicrobial activity [37]. Additionally, it was shown that in GI epithelial cells 1,25D alone or in combination with calcium stimulates the E-cadherin transcription as a result could reduce the risk for colorectal neoplasms [38]. Interestingly, an experimental study executed in Vitamin D-deficient mice challenged with *C. rodentium* showed colonic epithelial crypt hyperplasia, enhanced intestinal paracellular permeability, synthesis of proinflammatory cytokines and weight loss, compared to vitamin D–sufficient animals [39]. Both vitamin D-deficient study groups without and with infection exhibited an altered composition of the fecal microbiome [39]. *C. rodentium* infection leads to TJ impairment and augmented colonic permeability to macromolecules [40]. TNF-α regulates TJs and subsequently controls intestinal epithelial barrier. It enhances the permeability of human colon carcinoma cell monolayers by triggering the long isoform of myosin light chain kinase (MLCK) and myosin II regulatory light chain phosphorylation. 1α,25(OH)2D3 counteract the damage of the intestinal barrier induced by TNF-α by inhibiting the MLCK pathway in cultured

Table 1A
Mechanisms of microbiota action on GI mucosal immuno-system.

Subjects	Action /effect	Ref.
Human enterocyte cell line HCT-8 and BALB/c mice	Induces TJ expression	[24,25]
Biopsies from Crohn's disease and ulcerative colitis	Preserves mucosal epithelial barrier integrity	[26]
Host tissues	Induces AMPs production by its metabolites	[30]
Intestinal epithelial cells	Influences Nod2 receptors	[70]
T cells:	Affects differentiation of T cell subsets	[74]
	Induces CD4 to produces IL-10 and inhibits IL-17	[112]
Th17	Controls Th17 cells development and function	[107,108,110,114]
Tregs	Enhances Foxp3^{+} Treg cells number	[135]
	Induces the transcription factor RORγt in Treg cells via an MyD88-dependent mechanism	[140]
	Regulate Th17 /Tregs equilibrium	[144]
Bregs	Supports IL-10 producing B-cell differentiation	[155]
Monocytes/macrophages:	Induces AMPs production by its metabolites	[146,147]
M2 subset	Contributes to the M2 differentiation	[171]
	Promotes the anti-inflammatory properties of M2	[176]
Dendritic cells	Supports the development of both tolerogenic and inflammatory moDC	[188]
	Induces tolerogenic state	[187,189]

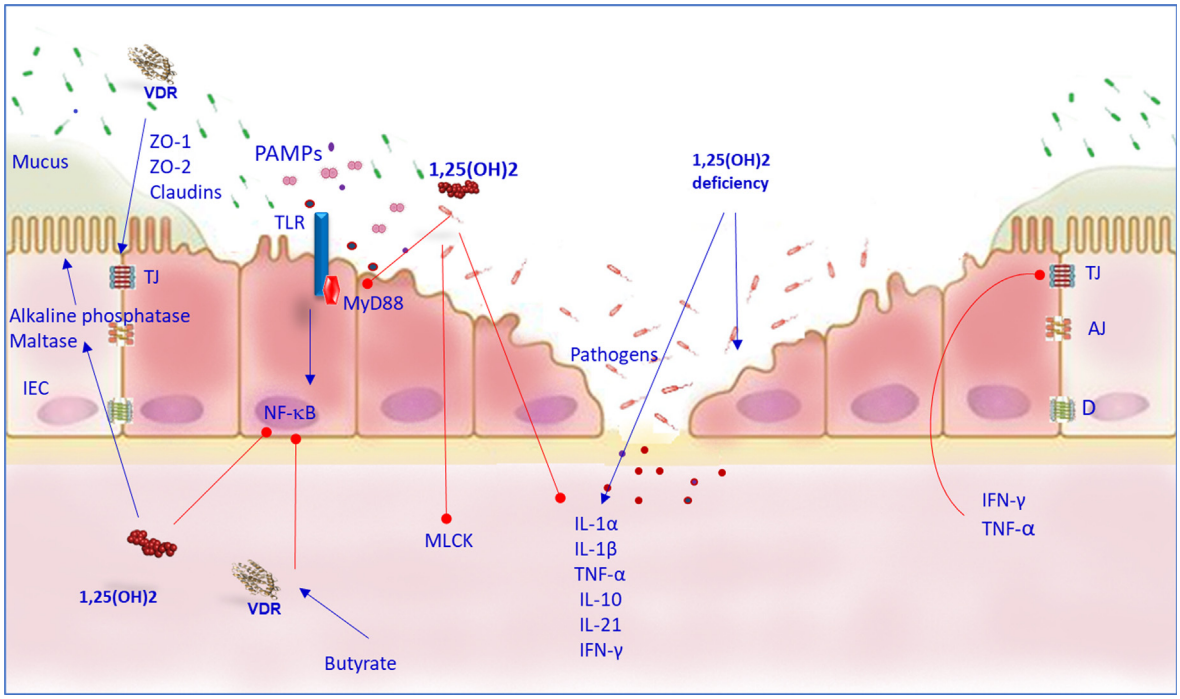

Fig. 2. Vitamin D3, Microbiota and Intestinal Barrier Function. 1,25(OH)2D3 reduces the TJ injury-related increase in intestinal mucosa barrier permeability and decreasing MyD88 expression controls intestinal barrier. Vitamin D deficiency enhances IL-1α, IL-1β, TNF-α, IL-10, IL-21, and IFN-γ. TNF-α induces the loss of GI barrier integrity and colonic inflammation. 1,25(OH)2D3 protects against TNF-α induced injury of the intestinal barrier by suppressing the myosin light chain kinase (MLCK) by inhibiting nuclear factor kappa B (NF-κB) binding to the promoter of the MLCK gene. 1,25(OH)2D3 enhances alkaline phosphatase and maltase expression which increases the formation of microvilli. VDR plays part in butyrate-mediated inhibition of inducible NFκB activation and regulates the expression of the TJ proteins ZO-1, ZO-2. MyD88 influences the role of microbiota-induced pathways that may regulate expression and function of intestinal epithelial TLR adaptors and thus impact on the expression of bacteria sensing TLRs.

HCT116, Caco-2 and SW480 cells [41]. This process is induced by the suppression of nuclear factor kappa B (NF-κB) binding to the promoter of the MLCK gene and is restored by 1α,25(OH)$_2$D$_3$ in a mouse model of colitis [41]. Moreover, 1α,25(OH)$_2$D$_3$ repairs TJ upon injury elicited by bacterial lipopolysaccharide in Caco-2 cells [42]. 1α,25(OH)$_2$D$_3$ maintains mucosal barrier integrity preventing intestinal cell apoptosis triggered by TNF-α-NFκB-PUMA during the inflammatory process [43]. 1α,25(OH)$_2$D$_3$ increases the expression and activity of alkaline phosphatase and maltase which improving the formation of microvilli act as brush border enzymes and are employed as differentiation markers in the small intestine [44] (Fig. 1). The action on alkaline phosphatase is facilitated by the transcription factor activator protein-1 (AP-1) [44]. In CRC cells 1α,25(OH)$_2$D$_3$ rises the expression of a plasma membrane calcium ATPase isoform connected with differentiation [45], while the short-chain fatty acid butyrate increases VDR expression and the pro-differentiative effect of 1α,25(OH)$_2$D$_3$ [46]. Moreover, VDR participates in butyrate-mediated inhibition of inducible NFκB activation in human HT-29 colon carcinoma cells and prevents inflammation in a colitis model [47]. Also, VDRs safeguards intestinal barrier function by circumventing the enhanced intestinal permeability, dysbiosis, inflammation, and a deficiency of immune response in the gut [48]. Interestingly, VDR regulates the expression of the TJ proteins ZO-1, ZO-2 through the up-regulation of claudin 2 and 12 and downregulation of cadherin-17 in the GI [46] (Fig. 2). As compared with wild-type mice, VDR mice treated with DSS exhibit a significant decrease expression of E-cadherin, claudin-1, ZO-1, and occluding proteins [37] (Table 2A). All these proteins are indispensable for maintaining intestinal barrier function and consequently immune homeostasis and preserve from

Table 2A
Mechanisms of Vitamin D3 action on GI mucosal immuno-system.

Subjects	Action /effect	Ref.
Prediabetes and type 2 diabetes mellitus; Intestinal epithelial cells; VDR KO mice; Caco-2 cell lines	Preserves TJ intestinal barrier integrity and function	[32,34,36,42]
Caco-2 cell lines and gluten-sensitized mouse model	Decreases MyD88 expression	[33]
Colorectal adenoma patients	Stimulates E-cadherin transcription	[38]
Human macrophages; VA10 Cell lines	Induces antimicrobial peptides	[52-54,147]
HEK293 cell lines	Induces Nod2 expression	[56,69]
T cell subsets:		
Th17	Suppresses inflammation and Th17-mediated autoimmunity.	[117]
	Alleviates autoimmune diseases by reducing IL-17 production.	[118,119]
	Inhibits the expression of RORγt/IL-17 and NF-kB.	[120]
	Modulates the Th17/iTreg axis	[122]
	Downregulates IL-17 and IL-23 production	[123]
Tregs	Enhances Foxp3$^+$ Treg cells number	[132-134]
	Stimulates IL-10 production	[131,135]
Bregs	Regulates Breg cells and stimulates IL-10 production	[162,163]
Monocytes/macrophages	Promotes the antimicrobial activities	[15,172]
	Prevents the expression of TLR-2, TLR-4, and TLR9. Modifies the TLR9-dependent production of IL-6	[173]
M2 subset	Increases M2 number	[174,175]
	Inducing IL-10 prevent M1 subset differentiation	[176]
Dendritic cells	Inhibits DC differentiation and IL-12 secretion	[183]
	Induces tolerogenic state	[185]

Table 2B
VDR action on GI mucosal immuno-system.

Subjects	Action /effect	Ref.
VDR KO mice	Failure of GI barrier integrity	[36]
	Decreased transepithelial resistance TER	[36]
	Enhanced injury of TJ proteins	[37]
In human HT-29 colon cancer cells	Participates in butyrate-mediated inhibition of inducible NFκB activation	[47]
	Reduces intestinal permeability	[39,43]
Caco-2 and epithelial cells	VDR/RXR complex activates the transcription AMPs	[51,59]
Colonic cells	Increases IL-22-producing innate lymphoid cells triggering dysbiosis	[84]
VDR KO mice	Absence of iTregs induction	[138]
	T cells differentiate in Th17	[132]

autoimmune diseases such as irritable bowel disease (IBD). Moreover, VDR expression enhances *trans*-epithelial electrical resistance between the TJs and reduced LPS levels in Caco-2 cells both treated or not with DSS inducing less intestinal permeability [43]. Likewise, VDR avoids the decline in transepithelial electrical resistance by the pathogenic *Escherichia coli* O157:H7 which, consequently, reduces intestinal permeability in epithelial cells [39] (Table 2B).

5. Vitamin D, gut microbiota and the immune system

In the last decades, one of the most extraordinary discoveries has been that 1α,25(OH)2D$_3$ influences significantly the innate and adaptive immune response [14]. It modulates the immune response through VDR which is expressed in immune cells, including activated or naïve CD4$^+$ and CD8$^+$ T cells, B cells, neutrophils and antigen-presenting cells (APCs) such as monocytes, macrophages and dendritic cells [14] (Fig. 3). Therefore, a viable immune response is significantly influenced by the vitamin D endocrine system which balances inflammation versus anti-inflammation [49]. When CD4$^+$ T cells differentiate into various subsets exert both pro- and anti-inflammatory properties. Several T cells subsets and their cytokines are essential for the physiological function and the pathological change of intestinal mucosa regulating continuously the gut homeostasis and inflammation [50]. VDR functions are crucial in immune response and gut homeostasis [34]. The 1,25/VDR/RXR complex increases in the innate immune cells chemotactic and phagocytic capabilities and meanwhile activates the transcription of anti-microbial peptides (AMPs) in several cell types, including colonic cells [51] (Fig. 3) (Table 2B). AMPs including, cathelicidin (hCAP18) and defensin ??2 (HBD-2) exert numerous functions such as chemotaxis of inflammatory immune cells [6] and microbicidal action [14]. In *vitro* studies, in human macrophages, revealed that toll-like receptor (TLR) activation elicits cathelicidin antimicrobial peptide (CAMP) expression via a vitamin D-dependent pathway [52]. The induction of CAMP vitamin D-mediated intensifies antimicrobial activity against pathogens specifically by direct killing cathelicidin leucin-leucin-37 (LL-37) and increasing phagosome formation [53]. In human primary monocytes 1α,25(OH)2D3 elicits the maturation of autophagosomes and autophagolysosomes by means a hCAP18/LL-37-mediated pathway [54]. In addition, in monocytes TLR activation of generates expression of the defensin beta 4 gene (DEFB4) through the synergistic action of IL-1β and VDR pathways [55]. The stimulation of intracellular pattern recognition receptor nucleotide-binding oligomerization domain protein 2 (NOD2) by its ligand muramyl dipeptide (MDP), a lysosomal breakdown product of peptidoglycan from both gram-negative and gram-positive bacteria, triggers the expression of the HBD-2 gene [56]. 1α,25(OH)2D3 powerfully induces NOD2/CARD15/IBD1 in primary human monocytic and epithelial cells [57]. In particular pre-treatment with 1α,25(OH)2D3 followed by MDP causes a vigorous, synergistic expression of the HBD-2 gene [56]. Therefore, AMPs effect the composition of the intestinal microbiota. Interestingly, AMPs in association with IgA protect mucus layers outside of epithelial cells [58]. In epithelial cells of the luminal lining of the digestive tract AMP expression is also modulated by the bio-products of gut metabolism, thus creating a mucosal barrier and inhibiting the interaction of microbes and pathogens with the intestinal epithelium [59] (Fig. 3). As evidenced by the observation that the short-chain fatty acids such as sodium butyrate, which results from the fermentation process of indigestible polysaccharides (fibres) from microbes in the colon, could stimulate cathelicidin expression [60]. The vitamin D pathway is involved in this stimulation [56], in fact, the synthesis of secondary bile acids by microbes modulates cathelicidin expression in the colon through VDR action [59] (Table 2B).

6. Microbiota and immune system

Continuous cross talk happens among intestinal epithelial cells, gut microbiota, and the gut-associated lymphoid tissue, which is predominantly composed of Peyer's patches, lymphoid nodules surrounded

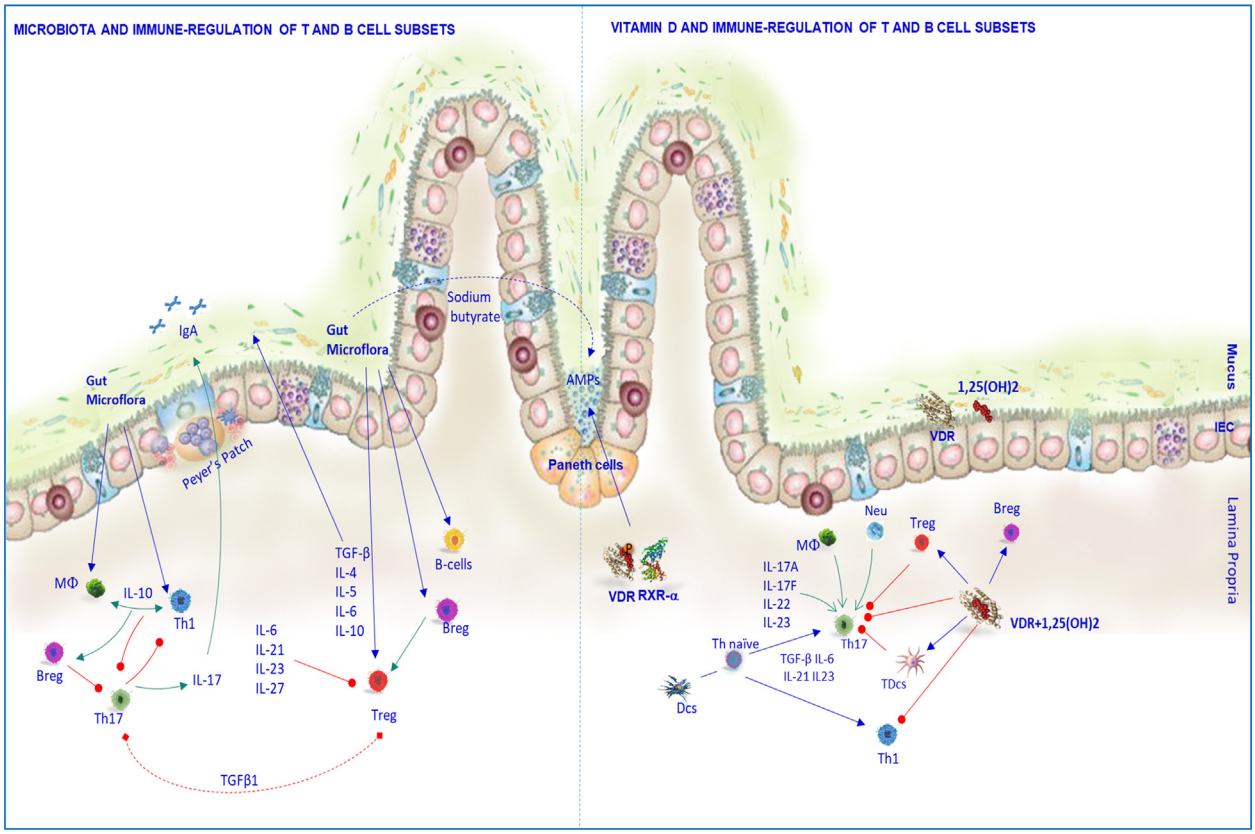

Fig. 3. Vitamin 3 and Immune-regulation of T and B Cell Subsets:1α,25(OH)2D3 regulates the immune system via the VDR present in activated or naïve $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, macrophages and dendritic cells. The 1,25/VDR/RXR complex increases in the innate immune cells chemotactic and phagocytic capabilities and activates the transcription of anti-microbial peptides (AMPs). AMP expression is modulated by sodium butyrate. 1α,25(OH)2D3 and VDR regulate T cells function promoting Tregs development, prevents inflammation turning off the Th1 and Th17 cells function. 1α,25(OH)2D3 and VDR regulate of the T_h17/iTreg axis. 1α,25(OH)2D3 downregulates IL-17 and IL-23 production. 1α,25(OH)2D3 directly influences Treg cell growth and promotes IL-10 production. 1α,25(OH)2D3 regulates Breg cells. 1,25(OH)2D3 induces higher levels of IL-10 in human B cells. **Microbiota and Immune-regulation of T and B Cell Subsets**: Microbiota stimulates the maturation and differentiation of T and B cells. IL-1, IL-6, IL-12, IL-18, and IL-23 regulate the Th17/ Tregs balance toward Th17 cells. Microbiota affects the differentiation of T cell populations into different types of Th. TGF-β, IL-4, IL-10, IL-5, and IL-6 stimulate IgA production. IL-10 and TGF-β sustain the mucosal tolerance. Microbiota regulates Th17/iTreg axis stimulating Tregs. Butyrate downregulate the production of nitric oxide and of IL-6, IL-12. The gut microbiota promotes IL-10-producing B-cells.

in the submucosa of the small intestine, and lymphocytes disseminated through the lamina propria (LP) [61] (Fig. 3). Therefore, the gut microbiota is fundamental for the maturation and differentiation of immune cells, for a suitable host immune response and plays a crucial role in the development of the host's tolerance against foreign antigens [62,63]. The mucosal immune system is extremely specialized and acts autonomously than the systemic immune system [9,63], preserving and activating immune tolerance within the intestine. The major modifications of the mucosal immune system occur after bacterial colonization of the intestinal tract [63]. It removes pathogens preventing pathologies onset. Following the colonization of the mucosal entrance sites of pathogens, microbiota not only eludes the attack by external microbes by rivalling pathogenic bacteria in the gut for nutrients and adhesion factors, but also by producing toxic molecules to neutralise colonization of pathogens, a process named colonization resistance. Epithelial cells, macrophages inside the LP and dendritic cells (DCs) sited in Peyer's patches present PRRs such as TLRs and Nod2 receptors, which control the different immune responses to avoid dysbiosis [64]. TLRs of the membrane of the epithelial and lymphoid cells of the small intestine are engaged in this identification and coordinate the regular development of the intestinal mucosal immune system. They prevent inflammation and stimulate immunological tolerance of normal microbiota constituents. TLRs stimulate the innate intestinal immunity identifying a wide spectrum of Microbe-Associated Molecular Patterns (MAMPs), encompassing various bacterial antigens such as flagellin, muramic acid, and unmethylated bacterial DNA CpG motifs, polysaccharides LPS and peptidoglycan components [64]. TLRs trigger a sophisticated cascade of signals, producing the release of NF-κ light-chain-enhancer of activated B cells, which in turn, stimulates a large quantity of genes coding effectors of the humoral immune response such as acute phase proteins, cytokines, chemokines and antibacterial products [65]. Additionally, TLRs activation by antigens of the normal intestinal microbiota prevents inflammatory response, crucial to preserve intestinal homeostasis [66]. TLRs activity declines in the course of the first weeks of life, enabling the development of a definitive gut bacterial community. Further, NOD-like receptors (NLRs) discriminate numerous microbial specific molecules and activate the inflammasomes assembly, which are cytoplasmic multi-protein complexes constituted by one of NLR proteins, such as NLRP1, NLRP3, and NLRC4, that act as sensors of exogenous or endogenous stress or damage-associated molecular patterns [67]. Following the detection of proper signal, they collect together with the adaptor protein, apoptosis-associated speck-like protein (ASC), into a multi-protein complex that manages caspase-1 activation and successive cleavage of effector pro-inflammatory cytokines including IL-1, IL-6, IL-12, IL-18, and IL-23, which then regulate the Th17/ regulatory T cells (Tregs) balance toward Th17 cells [68] (Fig. 2). It has been demonstrated that NLPRP6 deficiency is connected with reduced levels of IL-18, altered immune response, dysbiosis, and intestinal hyperplasia [68]. It has been proved that Vitamin D is an inducer of NOD2 gene expression and exerts different effects on the

expression of NOD2 and TLRinduced cytokines in Crohn's disease [69]. Intestinal microbiota and its metabolites act on both Nod1 and Nod2 receptors. Their defective signaling lead to inflammatory bowel disease (IBD) onset [70] (Table 1). High-fat feeding enhances plasma LPS-containing microbiota at a dose sufficient to increase inflammation [9]. LPS inhibits IL-1 receptor-associated kinase 4 (IRAK 4), a modulator of IRAK1 required for NF-κB activation [71]. Supplementation with Vitamin D prevents inflammatory cytokine synthesis in LPS-induced acute lung injury [72] and reduces LPS-induced renal oxidative stress by modulating oxidant and antioxidant enzyme genes [73]. GI tract microbiota affects both neutrophil migration and function and the differentiation of T cell populations into various types of helper cells (Th), such as Th1, Th2, and Th17 or into Tregs [74] (Table 1). Moreover, the colonic surroundings arouse *de novo* differentiation and development of peripherally derived Tregs from naïve CD4+ T cells, their dysfunction induces autoimmune disorders [75]. Consequently, initiated by bacterial recognition, the differentiation of effector Th1, Th2 and Th17 cells, the development of Tregs and the synthesis of secretory IgA (sIgA) occurs (Fig. 2). sIgA production specific to different mucosal antigens arises as a result of their capture by Peyer's patches M cells, transformation by primary dendritic cells (DCs), activation of T cells, and B cell class switch recombination in mesenteric lymph nodes (MLNs) and gut-associated lymphoid tissue. Specifically, a set of cytokines, including transforming growth factor beta (TGF-β), IL-4, IL-10, IL-5, and IL-6 stimulates IgA synthesis (Fig. 3). Some of these cytokines, particularly IL-10 and TGF-β are critical in sustaining the mucosal tolerance, thus demonstrating the link among sIgA synthesis, immunity, and intestinal homeostasis [76].

7. Vitamin D and microbiota

Studies assessing the effect of vitamin D deficiency on the microbiota are still few. Vitamin D not only controls the immune response of the microbiota but also influences the gut microbiota leading to dysbiosis [77]. It has been demonstrated that in new-born mice vitamin D deficiency causes a reduction of colonic *Bacteroides* and *Prevotella* later in life. Treatment with $1\alpha,25(OH)_2D_3$ increases *Citrobacter rodentium* load in the colon of mice through impairment of the Th17 response [78]. Instead, after infection with bacteria, vitamin D-deficiency exacerbates barrier dysfunction, inflammation and dysbiosis [77]. In a mouse model of induced colitis, vitamin D deficient mice show reduced colonic antimicrobial activity of angiogenin-4 protein (Ang4), bacterial infiltration and increased expression of 16S rDNA in colonic tissue compared to vitamin D sufficient mice [79]. Moreover, there is an active interaction between bacteria and VDR expression in the gut. In fact, the absence of Vdr in mice generates dysbiosis, with reduction of *Lactobacillus* and augmentation of *Clostridium* and Bacteroides in the faecal matter and decrease of *Alistipes* and *Odoribacter* and enhancement of *Eggerthella* in the cecal section, also induces alterations in crucial pathways of the intestinal microbiota. Overall these manifestations affect detoxification, infection, cancer and other diseases [80]. In particular, the reduction of bacteria producing lactic acid in the fecal microbiota in $Vdr^{-/-}$ mice may influence the intestinal homeostasis altering pH, unbalancing microflora predisposing to inflammation and colon cancer [81]. The increment of *Clostridium* species may increase the risk for gut disorders such as colitis, enteritis and food poisoning [82]. The *Eggerthella* enrichment seems to be correlated with an enhanced risk for IBD, bacteraemia and abdominal sepsis. These findings implied that the faecal microbiota is more deeply influenced by VDR status than the cecal microbiota at the taxonomic level. Other studies showed that mice lacking Vdr show depletion of bacteria from the *Firmicutes* phylum and enhancement of bacteria from the *Bacteroides* and the *Proteobacteria* phyla in the faecal matter [37]. Modifications in numerous bacterial genus members including *Eubacterium*, *Bacteroides* and *Salmonella* have been detected between $Vdr^{-/-}$ and wild-type mice [83]. Further, the absence of Vdr increased IL-22-producing innate lymphoid cells triggering resistance to colonization by *Citrobacter rodentium* and dysbiosis [84]. $Vdr^{-/-}$ mice show inflammation, decreased expression of IkBα, increased basal and bacteria-induced NF-κB activity, along with scarcer Paneth cells in the ileum and higher bacterial load in the intestinal mucosa [84] (Table 2B). Immune modifications caused by Vdr deficiency in mice result in IBD-like inflammation in response to usually non-pathogenic bacteria and intensified susceptibility to Salmonella-induced colitis [85]. Several studies showed that certain pathogenic bacteria and microorganisms downregulate Vdr expression [85]. Nevertheless, pathogenic bacteria can also cause increased expression and relocation of Vdr in the colon [85]. Additionally, probiotic treatment with *Lactobacillus Plantarum* and *Lactobacillus Rhamnosus* strain GG enhances the levels of VDR protein and the expression of target genes in human and mouse intestinal epithelial cells, and prevent Salmonella-induced colitis in wild-type mice but not in $Vdr^{-/-}$ mice [86]. There are still few experimental evidences in humans. In a multicentric study, double-blind, placebo-controlled, randomized, parallel-arm, oral supplementation with probiotic *Lactobacillus reuteri* NCIMB 30,242 improved circulating 25(OH)D levels relative to placebo [87]. Remarkably, in an investigation on dietary variation and microbial composition of the intestinal tract between 52 African Americans and 46 Caucasian Americans were observed contradictory data on vitamin D supplementation and variances between the counts of *Bacteroidetes* in the fecal samples among the two groups [88]. In subjects with higher 25(OH)D concentration it was observed a reduction in the relative amount of *Blautia*, *Roseburia*, *Ruminococcus* and *Dorea* (*Firmicutes* phylum, *Clostridia* class) [89]. Oral vitamin D3 supplementation in healthy volunteers decreased the relative amount of Gammaproteobacteria including *Escherichia*/Shigella, spp., Helicobacter spp. and Pseudomonas spp. [90]. These data confirmed the finding that in CYP27B1 KO mice a calcitriol supplementation efficaciously reduced *Helicobacteriaceae* levels [37]. The important role of vitamin D3 in Helicobacter pylori infections is also confirmed by the finding that Helicobacter pylori itself enhances VDR expression [90].

A large scale observational study showed a correlation between prenatal vitamin D supplementation and the abundance of some key bacterial taxa in the infant intestinal microbiota. The prenatal vitamin D exposure measured in mothers supplemented with vitamin D, or 25(OH)D quintiles, showed a statistically significant negative linear trend with the abundance of Bifidobacterium *spp*. [91]. Whereas, a positive linear trend was observed with the amounts of *B. fragilis* and 25(OH)D quintiles. Minor abundance of *C. difficile* was detected in breastfed newborns whose mothers were more incline to follow different dietary habits [91]. Hence, the different effects of vitamin D supplementation on the child varied according to maternal dietary habits indicate that lifestyle modulated the association between vitamin D and the intestinal bacterial taxa [91]. Additionally, a genome-wide association analysis using two cohorts of 1812 individuals recognized the VDR gene as a host element affecting the gut microbiota [92]. *Bifidobacterium bifidum* is a robust inducer of forkhead box P3 (Foxp3) $^{+}$ Treg cells with different T cell receptor specificity to dietary antigens, commensal bacteria, and *B. bifidum* itself. In fact, cell surface β-glucan/galactan (CSGG) polysaccharides of *B. bifidum* have been recognized as an important component accountable for Treg stimulation. Treg cells induced by *B. bifidum* or purified CSGG exhibit a robust suppressive action against experimental colitis. Moreover, it was observed that CSGG stimulated regulatory dendritic cells through a moderately TLR 2-mediated mechanism and was able to amplify the activity of whole bacteria [93]. Overall, these studies indicate that vitamin D plays an important role in influencing the intestinal microbiota.

7.1. Relationship of calcium induced intestinal inflammation and its regulation through vitamin-D

It is hard to dissociate vitamin D and calcium roles, as 1,25(OH)2D3 maintains normal calcium homeostasis for cellular processes and

stimulates intestinal absorption calcium, for suitable skeleton mineralization [94]. Impaired intestinal calcium absorption predispose to osteoporosis, inflammation state and Crohn's disease (CD) [95,96]. The 1,25 (OH)2D/VDR binding in the enterocytes causes in the intestine the increased expression of the calcium transporter proteins, such as calbindin 9 K, NCX1, PMCA1, and TRPV6, which elicit gut calcium absorption [97]. Interestingly, TRPV6 belongs to Transient receptor potential (TRP) ion channel superfamily which are widely expressed in several tissues throughout the mammalian organism. TRP channels preserve intracellular calcium homeostasis ensuring in the cells the regulation of different functions such as production and release of inflammatory mediators, cell migration and phagocytosis. It has been shown that functional extra-neuronal TRP channel expression in immune and epithelial cells plays important effects for mucosal immunology [98]. Several evidences suggest that vitamin D supplementation promotes calcium absorption by increasing gut calcium transporter proteins, decreasing mucosal inflammation and removing intestinal resistance to vitamin D. In many diseases calcium absorption can be impaired by vitamin D deficiency [99]. Experimental studies in animal models suggest that high-calcium diets have a beneficial impact in gut microbiota composition, favoring the growth of lactobacilli [100]. In dietary obese mice calcium supplementation regulates gut microbiota, enhancing the levels of *Bifidobacterium spp*. and *Bacteroides/Prevotella* and decreasing the levels of *Clostridium coccoides* and *Clostridiumleptum* [101]. Moreover, in mice supplementation of calcium in drinking water allows the modification of gut microbiota composition and specific bacterial abundance in feces [102]. Therefore, also dietary calcium might interfere with gut microbiota. However, at the moment the influence of calcium on human gut microbiota need further investigations.

8. Immune-regulatory cell subsets, vitamin D and microbiota

Many immune-regulatory cell subsets, specialized with exclusive suppressive functions including T cells, B cell, dendritic cells (DCs) and macrophage especially M2 phenotype are crucial to achieving constant immune tolerance in the gut microenvironment inhibiting innate and adaptive immune responses [103]. CD4 T cells producing IL-17 or IL-10 are common in the gut, where their balance is essential to retain tolerance and immunity to the resident microbiota all together [104]. Vitamin D and VDR regulate T cells function promoting regulatory T cell development, turning off the Th1 and Th17 cells function and thus controlling inflammatory response in the gut. $CD4^+$ T cells possess dual opposite roles in immune responses, acting either as Thelper/effector or Tregs. Particularly Th1, Th2, and Th17 cells cause mucosal inflammation and tissue injury, while Tregs, which are important intermediaries of immune tolerance, play anti-inflammatory functions and mitigate mucosal inflammation and stimulate tissue repair.

8.1. T helper 17 cells vitamin D and microbiota

Th 17 cells are a CD4 + T subset, their growth is influenced by signals mediated by IL-6 and TGF-β, IL-21, and IL-23 and by stimulation of the lineage-specifying transcription factor, retinoic acid- related orphan nuclear receptor (RORγT). Th17, which secrete cytokines such as IL-22, IL-17A, and IL-17F, are essential in the gut immune homeostasis and inflammation [105] (Fig. 3). Unlike Th1 and Th2 cells, which after differentiation are secretory cells, Th17 cells retain the ability to produce different cytokine expression and function following their commitment to Th17 [106]. This feature is related to the maintenance of their stem cell–like properties, which consents them to keep on for a long time while retaining the capacity to originate functionally different descendants when stimulated by antigen [106]. Th17 cells are more abundant in the LP of the colon. Segmented filamentous bacteria (SFB) richness in the gut positively regulates Th17 cell expansion [107]. SFB injection in germ-free mice was able to induce Th17 cells in LP [108]. It has been observed that a high percentage of Th17 cells in the gut express TCRs specific for SFB antigens. The specificities of these TCRs are capable to induce Th17 polarization [109]. Th17 differentiation arises on epithelial cells nearness to SFB colonization [110] (Table 1). Th17 cells are important promoters of inflammatory responses in gut mucosal surfaces. Th17 by recruiting neutrophils and macrophages to infected tissues and by their production of IL-17A and IL-17F protect effectively the host against infection to extracellular pathogens. Nevertheless, their expression alters mucosal healing and leads to mucosal destruction. A further cytokine produced by Th17 is IL23, which regulates survival and preservation of the Th17 phenotype and is responsible for the crosstalk between innate and adaptive immunity in the gut [111]. Often, abnormal expression and activity of the IL-17/IL-23 axis plays an important role in the pathogenesis of inflammatory bowel diseases [112] (Table 1). Additionally, Th17 cells synthetizes IL-22 which is responsible in host defense at the mucosal layer as well as in tissue repair [113]. Similarly, to IL-17, IL-22 is helpful to the host in numerous infectious and inflammatory diseases. Nevertheless, synergistically with IL-17, it becomes pathological on account of its proinflammatory properties [103]. Th17 cells are potent stimulators of chronic inflammatory responses in pathological conditions of the gut. The presence of microbiota controls Th17 cells development and increases their function [114]. Th17 cells are absent in the gut of germ-free mice [115]. Particularly, segmented filamentous bacteria are essential for inducing Th17 cells [108]. Interestingly, the administration of capsular polysaccharide from the commensal bacterium *B. fragilis* induces $TCD4^+$ lymphocytes to synthesize IL-10 and inhibits the synthesis of IL-17 and preserves the colonic mucosa against inflammatory responses triggered by bacterial antigens [112]. The strain of *Clostridium butyricum* prevent DSS-induced acute colitis in mice inducing IL-10-producing macrophage [116], instead segmented filamentous bacteria increase the Th17 response to preserve the gut from infection (e.g., *Citrobacter rodentium*) in mice [111]. Likewise, $1\alpha,25(OH)_2D_3$ addition prevents IL-17 production, suppresses inflammation and Th17-mediated autoimmunity [117] (Fig. 3). $1\alpha,25(OH)_2D_3$ could inhibit and partially reverse experimental autoimmune uveitis by reducing IL-17 production [118]. Additionally, it was proved that 1α,25(OH)2D3 suppress IL-17A induction in mouse models of 2,4,6-trinitrobenzene sulfuric acid (TNBS) colitis and early rheumatoid arthritis [118,119]. An effect that could be due to the repression of IL-17A at the transcriptional level by VDR through mechanisms including nuclear factor for activated T cells (NFAT) inhibition, histone deacetylase (HDAC) recruitment, capture of Runx1 and direct induction of Foxp3 [120]. VDR cooperates with P105/P50, P100/P52 and P65 NF-κB family proteins. Th17 cells differentiate in the presence of TGF-β1 and IL-6 by preventing Foxp3 induction and upregulating two other Th17 transcription factors retinoid-related orphan receptor (ROR) γt and RORα [121]. In the spleen tissues of VDR-deficient mice $1,25(OH)_2D_3$ suppressed the inflammatory infiltrates and inhibits the expression of RORγt/IL-17 by avoiding p65 transcription factor translocating to the nucleus [120]. An investigation in VDR KO mice supported that Vitamin D and the VDR are crucial modulators of the Th17/iTreg axis. VDR is important for iTreg activation and Th17 cells inhibition. The absence of the VDR causes Th17 cells overproduction at the detriment of iTregs. In VDR KO the expression of antibacterial peptides, regulated by vitamin D and the VDR, disturb the composition of the microbiota, compared to WT mice, causing the presence of a greater number of Th17 cells [122]. Moreover, VDR is crucial for the increase of tolerogenic DCs that in turn influence the amount of Th17 cells. Since microbial flora indirectly regulates Th17/iTreg axis, this finding suggested that a connection of pathways results in higher amounts of Th17 cells, fewer iTregs and more serious symptoms of IBD in the absence of the VDR [122]. Furthermore, it has been described that maxacalcitol an analogue of vitamin D3 was able to downregulate IL-17 and IL-23 production [123] (Table 2A). Previously it was proved that microbiota-specific Th17 protects the intestinal

mucosa from microbiota and pathogens by its capability to counter-attack infection and stimulate the production of IgA through IL-17-dependent intestinal polymeric Ig receptor expression, IL-21-dependent IgA^+ cell differentiation and IgA class switch recombination. IgA secretion limits microbiota adherence to the intestinal epithelial surface [124].

8.2. Regulatory T Cells, vitamin D and microbiota

In the gut, Tregs are the principal mediator in preserving the immune homeostasis. A small percentage of Treg cells reside within lymphoid organs and peripheral tissues, they suppress immune responses. Tregs operate by inducing apoptosis of effector cells [125]. Tregs, encompass many subtypes including Tregs produced in the thymus via IL-2 signaling and $CD8^+CD25^{High+}FOXP3^+$ T cell, which mostly express FoxP3 the master-switch lineage-specific the main regulatory gene for the development and function of Tregs subset and coordinates the transcriptional process [126]. Instead, $Foxp3^-CD25^-CD4^+$ cells develop from in peripheral organs/tissues following induction with TGF-β and all-trans retinoic acid and are recognised as peripherally derived Tregs [127]. $FoxP3^+$ Tregs are important modulators of immune tolerance by regulating innate and adaptive immune responses against self and non-self-antigens [128]. Their contribution to immune tolerance is so significant that deficiencies in Treg cells function cause severe immune disorders. In intestinal LP, Tregs safeguard homeostasis and by negative regulation of effector T cells are essential in avoiding intestinal inflammation synthesising IL-10 or TGF-β and expressing cytotoxic T lymphocyte antigen (CTLA-4 or CD152) [129]. In Tregs, Foxp3 protein expression is crucial for their inhibitory function. The absence of Foxp3 gene enhance responses to pathogens, induces tumours and autoimmune diseases, such as enteropathy, polyendocrinopathy, and X-linked autoimmunity allergic dysregulation syndrome [130]. Treg cells express vitamin D receptors and are more susceptible to the suppressive action of $1\alpha,25(OH)_2D_3$ than conventional T cells [131] (Fig. 2). Both in vitro and in vivo evidence indicate that 1,25D3 supplementation enhance the numbers of $Foxp3^+$ Treg cells [132]. Studies in animal models and ex *vivo* data demonstrate that vitamin D influences positively the amount of CD4+ Foxp3+ Treg cells [133]. Different concentrations of $1,25(OH)_2D_3$ have the capacity to stimulate Foxp3+ Treg cells depending on the cytokine milieu. It has been suggested that the cytokines IL-2 and TGF-β cooperate in enhancing the differentiation of 1α,25(OH) 2D3 -induced $Foxp3^+$ Treg cells [134]. TGF-β is crucial for the generation of $Foxp3^+$ Treg cells from naive $CD4^+$ T cells, and IL-2 is an important component in TGF-β-mediated induction of $Foxp3^+$ Treg cells. 1,25(OH)2D3 directly influences Treg cell growth and stimulate IL-10 (Fig. 3) production without deteriorating of the activation status and suppressive phenotype [131]. I*n vivo* studies indicate a strong positive correlation among vitamin D sufficiency and $Foxp3^+$ Treg cell amounts and proliferation [131]. 1α,25(OH)2D3 is involved in several stages of the evolution of these T-cell responses. Others findings demonstrating the importance of the cytokine milieu displayed that IL-10 exerts a negative impact on the differentiation of 1α,25(OH)2D3 -induced $Foxp3^+$ Treg cells enhancing $IL\text{-}10^+$ Treg cells [135] (Table 1). VDR is an important regulator of the Th17/iTreg axis (Fig. 3). The FoxP3 promoter is controlled by numerous transcription factors including NFAT, cRel, Creb and STAT5 to FoxP3 promoter [136]. Without interactivity with the VDR, FoxP3 stimulation results ineffective. Moreover, FoxP3 expression is unbalanced and is influenced by the environment. VDR cooperates with NFAT and cRel to regulate transcription [137]. Several findings indicate that the absence of the VDR leads a lack in the induction of iTregs by TGF-β1. Vitamin D-deficient or VDR KO mice are predisposed to IBD [138]. The absence of VDR causes the inability of CD4 T cells to home the GI tract, to express CD8αα, and produce IL-10. Then, in absence of the VDR, T cells preferentially differentiate into Th17 cells to the detriment of iTreg and experimental IBD is more severe [132] (Table 2B). Likewise, the increased differentiation of VDR KO T cells into Th17 cells arises under Treg-inducing conditions. Treating mice with 1,25 dihydroxyvitamin D_3, suppresses IBD [139]. In addition, microbial flora regulates indirectly the Th17/iTreg axis (Fig. 3). Various mechanisms are implicated in microbiota regulation of Treg differentiation and function. Intestinal microbiota plays an important role in the induction of Tregs [135] (Table 2A). For example, *Bacteroides fragilis* and *Clostridia* species avoid the inflammatory responses in the intestine by stimulating Tregs. It has been reported that members of the Clostridiales and Bacteroidales induce the transcription factor RORγt in nascent Treg cells via an upstream MyD88-dependent mechanism to promote tolerance to dietary antigens. Activation of this axis is damaged by the dysbiosis, and can be restored by treatment with therapeutic microbiota [140] (Table 1). Polysaccharide A of *B. fragilis* support the suppressive activity of inducible $Foxp3^+$ Tregs via Toll-like receptor 2 signaling and preserve the TNBS (2,4,6-trinitrobenzenesulfonic acid)-induced colitis in mice [141]. IL-6, IL-21, IL-23, and IL-27 avoid Treg differentiation, but support the induction of proinflammatory Th1/Th17 cells [142,143] (Fig. 3). Various bacterial species control the cytokine milieu affecting T-cell differentiation and activation. Essentially, the same commensal bacteria in diverse circumstances could induce different immune responses. In normal conditions of the intestine *B. fragilis,* unstable *Clostridia flora* and transformed *Schaedler* flora species are capable to fulfil a suppressive function by activating Tregs, while they increase Th1/Th17 cell development in lack of Tregs [108]. Recently, it has been reported that *Lactobacillus acidophilus* rises intestinal inflammation in an acute colitis mouse model by regulation of Th17 and Treg cell equilibrium [144] (Table 1). Several investigations indicated that also the bacterial metabolites regulate the gut immune response. In fact, butyrate downregulates the synthesis of nitric oxide and of proinflammatory mediators of LM macrophages such as IL-6, IL-12, by preventing histone deacetylases [145]. Additionally, butyrate and its end products upregulate cathelicidin in human colon epithelial cells [146] and synergistically with 1α,25(OH)2D3 induces human CAMP mRNA levels in lung epithelial and myeloid cells [147] (Table 1). Butyrate induces the development of Tregs in the colonic LP (Fig. 3) and improves T cell-dependent experimental colitis in mice [148]. Furthermore, it has been shown that butyrate through activation of Gpr109a signalling provides anti-inflammatory properties on colonic macrophages and DCs inducing differentiation of Tregs and IL-10-producing T cells [149]. An imbalance of Tregs and Th17 occurs in IBD. But also the altered cytokine milieus of the intestine contribute to the impairment of differentiation and function of Tregs in IBD. $CD25^+$ regulatory T cells from IBD patients exert a compromised suppressive function, compared with healthy controls, that is connected with the co-expression of RORγt and Foxp3 in$IL\text{-}17^+Foxp3^+$ T cells. Therefore, the regulation of inflammation in the gut depend on the equilibrium between the Th17 cells and inducible regulatory T (iTreg) cells [150]. CD4 cells treated with TGF-β1 express Foxp3 and produce IL-10 [146] (Table 1). Hence, Th17 and iTreg differentiate in response to TGF-β1. In vivo, a proliferation of Th17 cells in the gut is related with a mutual reduction in Treg cells and vice versa [151], so they could operate in part by direct repression of each other [150]. It can be conceivable that Treg and Th17 cells could represent alternative cell subtypes of the same precursor.

8.3. Regulatory B cells vitamin D and microbiota

B cells retain the peculiarity to synthetize antibodies. Additionally, they produce cytokines and act as secondary APCs. B cells differentiate in different subpopulations fulfilling both regulatory and pathogenic functions. A lesser amount of B cell (about 10% of total B-cells in circulation) are represented by Regulatory B cells (Bregs). They exert regulatory/suppressor functions and are necessary for the peripheral tolerance mechanisms. Their regulatory action is usually achieved by

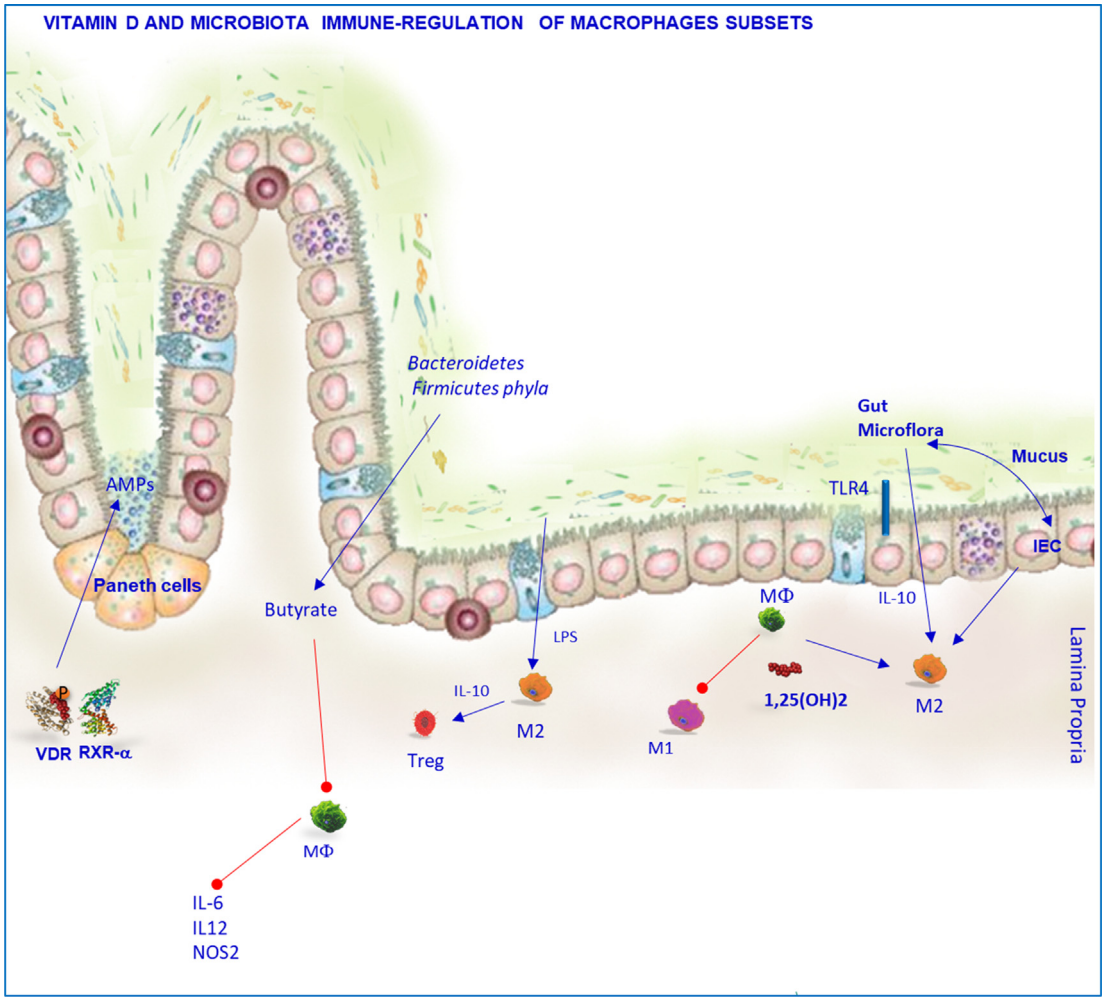

Fig. 4. Vitamin D3 and Microbiota Immune-regulation of Macrophage Subsets. Colonic macrophages show an anti-inflammatory M2-like phenotype, they produce IL-10, promote Tregs proliferation and induce epithelial cell regeneration and proliferation. M2 generate from the interactions with intestinal epithelial cells (IECs) and the gut microflora, and a combination of TLR and IL10 signaling. IL-10 contributes to the development of an anti-inflammatory phenotype in colonic macrophages. $1\alpha,25(OH)_2D_3$ promotes the antimicrobial activities inhibiting TLR-2 and TLR-4 expression in monocytes. M2 regulates intestinal inflammation. 1α,25(OH)2D3 influences macrophage polarization towards M2 phenotype, through the inhibition of IFN-γ. M2 macrophages produce IL-10 which inhibits the differentiation of M1 macrophages. M2 interacts with Butyrate, which is secreted mainly by Bacteroidetes and Firmicutes phyla bacteria and downregulate IL-6, IL-12, and NOS2.

IL-10 production. Less than 20% of these cells are IL-10 producers after stimulation [152]. Inflammation leads vigorous Bregs development and differentiation. A variety of molecules including TLRs, CD40, the B cell receptor, CD80, CD86, and cytokines are necessary to activate Bregs [152]. Based on the activation pathways three different types for Breg cells have been characterized: innate Breg cells requiring signalling via innate receptors, such as Toll-like receptors; immature Breg cells requiring CD40 stimulation; antigen-specific Breg cells requiring both B-cell receptor and CD40 signalling. Bregs hamper inflammation inhibiting Th1 cells activation, Th17 differentiation and preservation of the Treg cell population [153]. It has been reported that IL-33 induces a subset of IL-10-producing B cells ($Breg^{IL\text{-}33}$) in mice, and adoptive transfer of these $Breg^{IL\text{-}33}$ into $IL\text{-}10^{-/-}$ mice efficiently reduced mucosal inflammatory responses in the gut [154]. Interestingly, the gut microbiota could support IL-10-producing B-cell differentiation in the spleen and in mesenteric lymph nodes enhancing IL-1β and IL-6 synthesis in mice [155] (Table 1). IL-10-producing B cells in mesenteric lymph nodes prevent the progression of chronic colitis of TCRα KO mice downregulating directly inflammatory cascades connected with IL-1β synthesis and signal transducer and activator of transcription 3 activation [156]. In addition, Bregs play a regulatory role in some autoimmune disorders. In humans, Bregs have been investigated largely in systemic lupus erythematosus and multiple sclerosis. In these diseases Bregs regulate the autoimmune responses [157]. Studies in chronic intestinal inflammation demonstrated that the percentage of $CD19^{high}CD1d^{high}$ IL-10-producing B cells decreased in patients with Crohn's disease and that this subset of B cells alleviated intestinal inflammation in colitis in a Treg-independent manner [158]. Therefore, Bregs or plasma cells have been suggested as an innovative therapy for IBD [159]. $CD19^{+}CD5^{+}$ $Foxp3^{+}$ B cells, a recently identified type of Bregs, also express Foxp3, but their immunologic function still remains unknown [160]. Notably, $CD138^{+}$ plasma cells, another subset of activated B cells, produce IL-35 and could avoid intestinal inflammation after salmonella infection [161]. Whether or not Breg cells, are responsive to $1\alpha,25(OH)_2D_3$ has not yet been well established. It has been observed that Vitamin D could regulate Breg cells [162] (Fig. 3). Human B cells cultured and stimulated with BCR cross-linking, anti-CD40 and IL-4 for 2 days, in the absence or presence of $1\alpha,25(OH)_2D_3$ secreted higher levels of IL-10. Furthermore, higher percentage of $IL\text{-}10^{+}$ Breg cells, as well as higher mean fluorescence intensities for IL-10 meaning more IL-10 production per cell, were found in presence of 1,25(OH)2D3 [163] (Table 2A).

8.4. Monocytes/ macrophages subsets, vitamin D and microbiota

Among the immune cells present in the colon, the macrophages are the most abundant [164]. Macrophages are well-known for their phenotypic heterogeneity and for the wide range of biological activities that they exert depending on signals received from the different cytokines and from the tissue microenvironment stimulation [164]. Some of these functions seem to be opposing: immunogenic versus tolerogenic activities, anti- versus pro- inflammatory response, tissue-repair versus tissue destruction [164]. Macrophages can be classically activated (M1), by IFN-γ and LPS induction, whereas by IL-4 and IL-13 or indirectly through Th2 cells induction differentiate in alternatively activated macrophages (M2). Macrophage polarization strongly modifies their immune asset. For instance, M1 macrophages exert potent antimicrobial properties, whereas M2 macrophages show marked tissue repair properties [165]. The M1 macrophages are activated by proinflammatory cytokines and PAMPs that result in stimulation of PRRs including the family of TLRs. The M1 macrophages stimulate inflammation and/or type 1/Th1/Th17 immune responses and produce a plethora of proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α, and various cytotoxic molecules that stimulate the acquired immune response and promote the clearance of invading pathogens [166]. During the inflammation, M1 macrophages express the cell surface CCR7 important for their own activation and the exodus of T lymphocytes from blood to inflamed tissues. CCR7 mediated signals promotes the polarization and activation of uncommitted T cells to Th1 cells [167]. M2 macrophages exhibit a strong scavenging activity in the form of increased mannosylated-BSA degradation and express an array of cell surface receptors such as CD163, CD206, MRC1, DECTIN-1, chemokines (CCL16, CCL17, CCL18, CCL22, CCL24), and chemokine receptors (CCR2, CXCR1, CXCR2). Activated M2 macrophages significantly express mannose receptor (CD206) [168]. CD206 expression is induced by IL-10, which recruiting more M2 macrophages decreases the inflammatory response [167,168]. M2 macrophages inhibit inflammation and/or elicit type 2/Th2 immune responses. Colonic macrophages crowding the GI arise from circulating monocytes [169]. Human colonic macrophages include $CD68^+CD14^+HLA\text{-}DR^+CD64^+CD45^+CD163^+$ [170]. In the steady state, colonic macrophages display an anti-inflammatory M2-like phenotype expressing CD206 and CD163, exerting an anti-inflammatory activity under LPS stimulation, producing IL-10, promoting Tregs proliferation and induce epithelial cell regeneration and proliferation [169] (Fig. 4). The exact mechanism inducing colonic macrophages to achieve the M2/anti-inflammatory phenotype is not yet fully known. Likely they generate from the interactions with both gut microbiota and intestinal epithelial cells, and a combination of TLR-signaling regulation and of IL-10 signaling. Along with IL-10-producing T cells, IECs supply IL-10 in the human colon, thus contributing to the improvement of an anti-inflammatory phenotype in colonic macrophages. In fact, it has been demonstrated that in co-cultures of human secondary colonic IECs (SW840, Caco-2 cell lines) with mouse peritoneal macrophages TLR-4 mediate the increased IEC secretion of IL-10 [171] (Table 1). An important antimicrobial pathway in monocytes and macrophages includes the activation of TLRs. The regulation of TLRs appears to be a therapeutic tool to activate the immune responses [52]. Previously it was showed that in human macrophages, $1\alpha,25(OH)_2D_3$ triggers innate immunity inducing the synthesis of antimicrobial peptides in response to TLR-2/1 activation [14]. $1\alpha,25(OH)_2D_3$ promotes the antimicrobial activities also inhibiting TLR-2 and TLR-4 expression in monocytes and promoting a state of hypo-responsiveness to PAMPs [172]. The expression of the co-receptor of TLR-4, CD14, is up- regulated by $1\alpha,25(OH)_2D_3$ in human cells [14]. Furthermore, $1\alpha,25(OH)_2D_3$ prevents the expression of the innate-immunity receptors TLR-2, TLR-4, and TLR9 and modifies the TLR9-dependent production of IL-6 in human monocyte [173]. Accumulating evidence reported that M2 could regulate intestinal inflammation [166]. Vitamin D influences macrophage polarization towards M2 phenotype [174] (Fig. 3). As supported by a study showing that Vitamin D sufficiency enhances the percentage of M2 macrophages and ameliorates IBD [174,175] (Table 2A). M2 macrophages are the greatest producers of IL-10 which prevent the differentiation of M1 macrophages [176] (Table 1). The modification in macrophage phenotype occurs through the inhibition of IFN-γ by $1\alpha,25(OH)_2D_3$ and the failure of M1 macrophage differentiation caused by IL10. In addition, the anti-inflammatory properties of colonic macrophages (M2) could be caused by the interaction with bacterial products. For example, butyrate, which is produced mostly by Bacteroidetes and Firmicutes phyla bacteria, is present at high concentrations in the colon and downregulate the proinflammatory factors including IL-6, IL-12, and NOS2 produced by colonic macrophage in vitro and in vivo [176] (Fig. 4) (Table 2A).

8.5. Dendritic cell subsets, vitamin D and microbiota

Dendritic cells (DCs) are antigen-presenting cells (APCs) and have been regarded as the most efficient APCs initiating innate immune responses and as a bridge connecting innate and adaptive immunity. Once DCs meet pathogens they present antigens to T cells activating them and inducing the immune response. Recently, studies on DC function have recognized the important role of DCs in triggering inflammatory responses against pathogens and tolerance to commensal microflora in the gut [177]. Notably, DCs promote the differentiation of Tregs in the gut from naive T cells. Depending on the expression of the surface marker CD103 have been recognised numerous DC subpopulations in the intestinal mucosa playing important roles in the preservation of the gut homeostasis. Accordingly, DCs have been divided into two principal subsets: $CD11c^{high}CD103^+$ and $CD11c^{high}$ $CD103^-$ DCs [177]. $CD11c^{high}CD103^-$ DCs have the capability to stimulate Th1 and Th17 cell development [177]. Whereas, $CD11c^{high}CD103^+$ DCs derived from the LP of the small intestine aid the differentiation of Tregs in a retinoic acid- and TGF-β-dependent manner [178]. The intestinal DCs population $CX3CR^{high}CD11b^+CD11c^+$ prevent the proliferation of $CD4^+$ T cells avoiding intestinal inflammation [179]. $CD11b^+$ DCs produce IL-23 and are crucial for ILC3 activation. Moreover, by producing IL-22 and inhibiting commensal bacteria-specific $CD4^+$ T-cell proliferation via MHC II-mediated antigen presentation they act as immune regulatory cells [180]. An additional subpopulation of DCs has been phenotyped, termed Tr1-like cells, which secreting IL-10 show suppressive action [181]. Therefore, DCs induce T cell differentiation into an effector cell with both pro- or anti inflammatory functions. Therefore, regulation of APCs is essential in starting and preserving adaptive immune response and self-tolerance [182]. $1\alpha,25(OH)_2D_3$ is a potent inhibitor of DC differentiation and IL-12 secretion [183]. The inhibition of IL-12 expression is accomplished by a direct interaction between DCs and $1\alpha,25(OH)_2D_3$ bound to the VDR, which blocks NF-κB-induced transcription of IL-12 [184]. Since IL-12 is an important cytokine inducing Th1 development this is a significant immune-regulatory activity. Dendritic cells cultured with VDR agonists maintain the monocyte marker CD14 but are unable to upregulate CD1a and fail to express their MHC and co-stimulatory molecules including CD40, CD80, CD86. This results in IL-12 and IFN-γ downregulation, IL-10 and TGF β upregulation with the total consequence of the inhibition of T cell activation [183] (Fig. 5). Dendritic cells treated with $1\alpha,25(OH)_2D_3$ showed a “tolerogenic state” characterized by reduction of inflammatory cytokines, such as IL-12 and TNF-α, and enhanced expression of the anti-inflammatory IL-10. These DCs promote apoptosis in the autoreactive T cells and the differentiation of Treg cells [185] (Fig. 5) (Table 2A). DCs interacting with additional factors mutually retain gut homeostasis [186]. Once DCs interact with microbiota, they increase their immune regulatory functions. Interestingly, MUC2, the building block of gut mucus secreted by goblet cells, imprint DCs with anti-inflammatory DCs indicating that intestinal mucosa not only constitutes a physical barrier but also limits the immunogenicity of gut

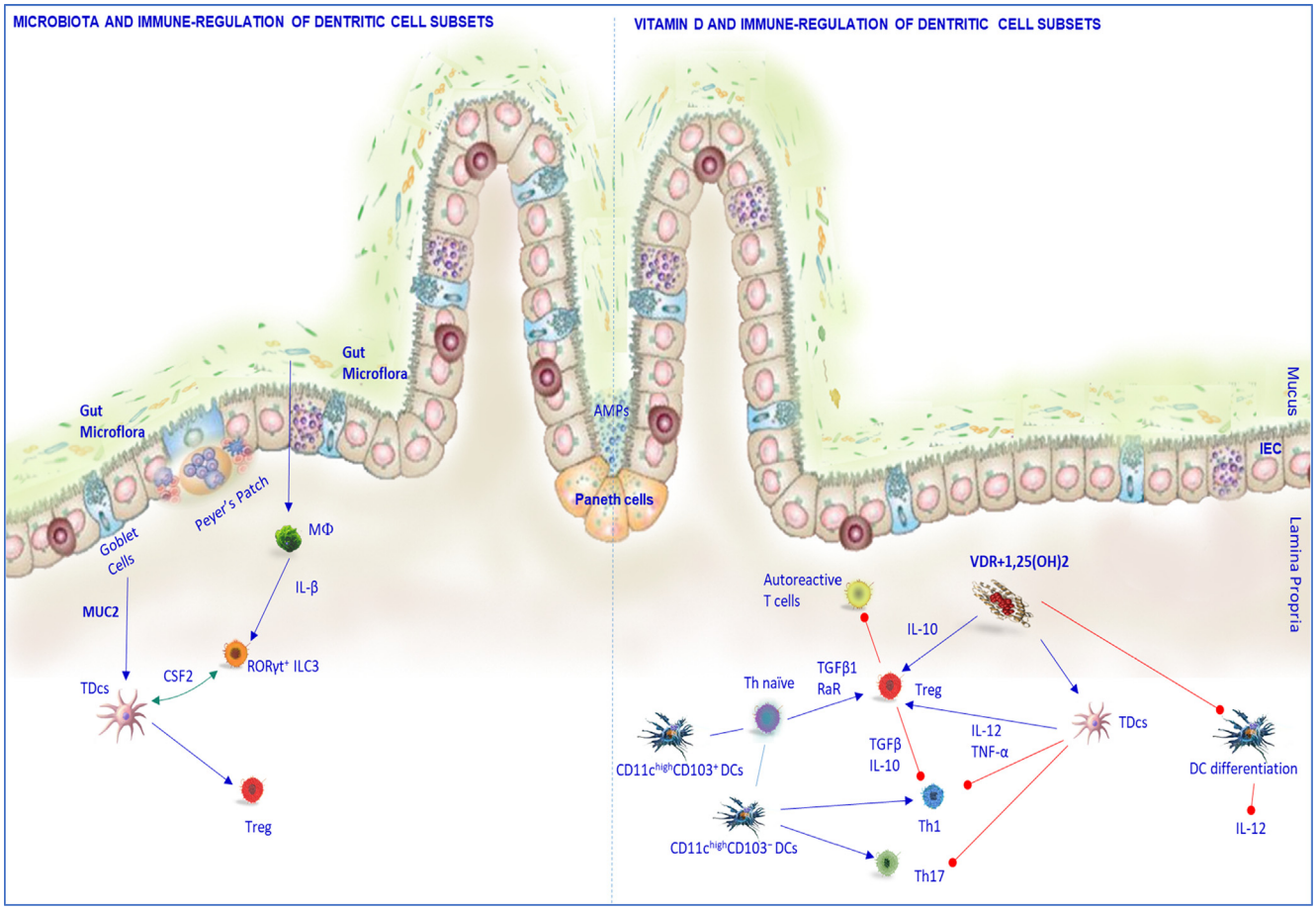

Fig. 5. Vitamin D and immune-regulation in DCs:1α,25(OH)$_2$D$_3$ bound to the VDR inhibits DC differentiation and downregulates IL-12 and IFN-γ, and upregulates IL-10 and TGF-*β*. Dendritic cells treated with 1α,25(OH)2D3 achieve "tolerogenic state". TDCs induce the differentiation of Treg cells and provoke apoptosis in the autoreactive T cells. **Microbiota and immune-regulation in DCs**: DCs interacting with commensal flora amplify their immune regulatory functions. MUC2, secreted by goblet cells, imprint DCs as anti-inflammatory cells limiting the immunogenicity of gut antigens by delivering tolerogenic signals. Commensal microbiota, tissue-resident mononuclear phagocytes, and ILCs form an axis which regulate intestinal homeostasis. Macrophages secrete IL-1β to stimulate RORγt$^+$ ILC3 to produce colony-stimulating factor 2 that further promotes DCs to maintain Tregs.

antigens by supplying tolerogenic signals [179] (Fig. 5). Furthermore, the interaction between DCs and subsets of innate lymphoid cells (ILCs) is essential in intestinal homeostasis regulation. In the steady state of the GI, after recognising and up taking microbiota or their products, macrophages produce IL-1β to stimulate RORγt$^+$ ILC3 to synthetize colony-stimulating factor 2 that in turn induces DCs to support Tregs [187] (Fig. 5). Selected commensal bacterial strains are able to drive strong effector immune responses by moDCs, while in the presence of ATRA, they support the development of both tolerogenic and inflammatory moDC in a RARα-dependent manner [188] (Table 1). Moreover, bacterial metabolites including short-chain fatty acids, produced by bacteria after digestion of dietary fibers are able to enhance dendritic cell regulatory activity, leading to the induction of Treg cells and IL-10-secreting T cells [189] (Table 1). Therefore, microbiota, tissue-resident macrophages and ILCs constitute a network which regulates intestinal homeostasis.

9. Gut microbiota and VDR polymorphisms

In view of the above, VDR influences both microbiota and immune system. VDR expression protects the host from invasive pathogens and maintains intestinal homeostasis also enteric bacteria activate VDR signaling [190]. VDR is a ligand-activated transcription factor which through heterodimerization with the RXR exerts a wide range of biological effects. Both vitamin D and microbial metabolites act through VDR-RXR heterodimer. Bile acids act as both key VDR ligands and regulators of VDR expression [191]. Genetic variation in VDR significantly influences GI mucosal immune-system (Table 2B) and microbial co-metabolism. Wang et al. found that VDR affects individual bacterial taxa such as *Parabacteroides* [192]. These bacteria contain pathways involved in secondary bile acid metabolism [192]. It is conceivable that VDR genetic polymorphisms, including BsmI, ApaI, TaqI, and FokI which have been associated with susceptibility to autoimmune diseases, obesity, metabolic disturbances and cancer [193–195], influencing the intestinal microbiota could be predisposing factor for these pathogenetic manifestations. Future research in this area, with genome-wide association studies may provide further knowledge on the association between vitamin D deficiency, dysbiosis and diseases.

10. Conclusion

The gut is one of the most important target organs of vitamin D, as demonstrated by the local synthesis of 1α,25(OH)2D3 and of VDR expression in most cell types of GI tract. An optimal 1α,25(OH)2D3 status plays an important role in maintaining the gut homeostasis via many regulatory activities such as calcium and phosphate absorption, protection against infection, preservation of the epithelial barrier function, anti-inflammatory action and modulation of the gut microbiota. On the other hand, a number of data have demonstrated a complex connection of the gut microbiota with host metabolism, neuroendocrine and

immune homeostasis, and the potential impairments or disorders of the gut microbiota. Therefore, vitamin D3 and gut microbiota display many similarities in the modulation of the immune system in counteracting the inflammatory responses including inhibition of excessive ROS synthesis, downregulation of the proinflammatory NF-κB pathway, and downregulation of inflammatory markers and induction of anti-inflammatory cytokines synthesis. These similarities could be due, at least in part, to the interaction and the synergistic effect between vitamin D and microbiota metabolites including SCFAs (e.g. butyrate). In fact, we have seen that both exert anti-inflammatory activity by promoting regulatory Tregs function, increasing the levels of the anti-inflammatory cytokine IL-10, affecting the maturation of DC [196]. Both are able to modulate NFκB signalling and to inhibit expression of pro-inflammatory cytokines [46]. Interestingly, it has been reported that the expression and activity of VDR is under the control of butyrate [47]. Butyrate is potent health-promoting effects. It maintains an intestinal barrier function, decreases inflammation, prevents metabolic disorders, protects from autoimmune disease and carcinogenesis [197]. Therefore the association of both vitamin D3 status and commensal microbiota composition with the host in health and disease states reflects a much more complex interactive network, rather than a simple unidirectional "cause and effect." Still, the human studies are very limited [198]. Therefore, new experimental investigations should be reviewed and designer suitably to carefully examine at which step of the disease (for example disease onset, early disease stage, disease progression, active or latent disease) in order to understand how vitamin D3 status, intestinal VDR expression and human microbiota composition may influence pathogenetic manifestations. Moreover, different valuations on vitamin D status, vitamin D genomics, dysbiotic features in specific diseases to be correlated with host immune responses. For example, confounding/modifying variables, such as subject-specific factors such as age, gender, ethnicity, lifestyle, diet, infection, genetic, disease and postnatal exposure to maternal and environmental microflora could aid to clarify the interactions between Vitamin D and microbiota and their impact in the immune responses. Finally, it is necessary highlight that excessive vitamin D supplementation lead to hypercalcemia as novel risk factors which promotes worsening of CNS demyelinating disease [199]. In addition, high dose vitamin D worsens the severity of murine colitis induced by DSS, and is associated with diverse modifications in microbial composition that may be a direct dietary effect or as a result of dysregulation of the gut mucosal immune response [200]. Nevertheless, up to date there are few investigations detecting the effect of high vitamin D levels on immune regulation. Therefore, is indispensable further detect the effects of high levels of vitamin D on gut mucosal immunity to better understand if high as well as low vitamin D levels lead to a dysregulation. Collectively, these considerations indicate that it would be stimulating to investigate the benefits of new combination supplements with vitamin D or vitamin D analogues and probiotic and/or prebiotics as alternative disease management options that may affect the outcome of conventional therapies.

Declaration of Competing Interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.106112.

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