IMMUNE-MODULATORY EFFECTS OF VITAMIN D

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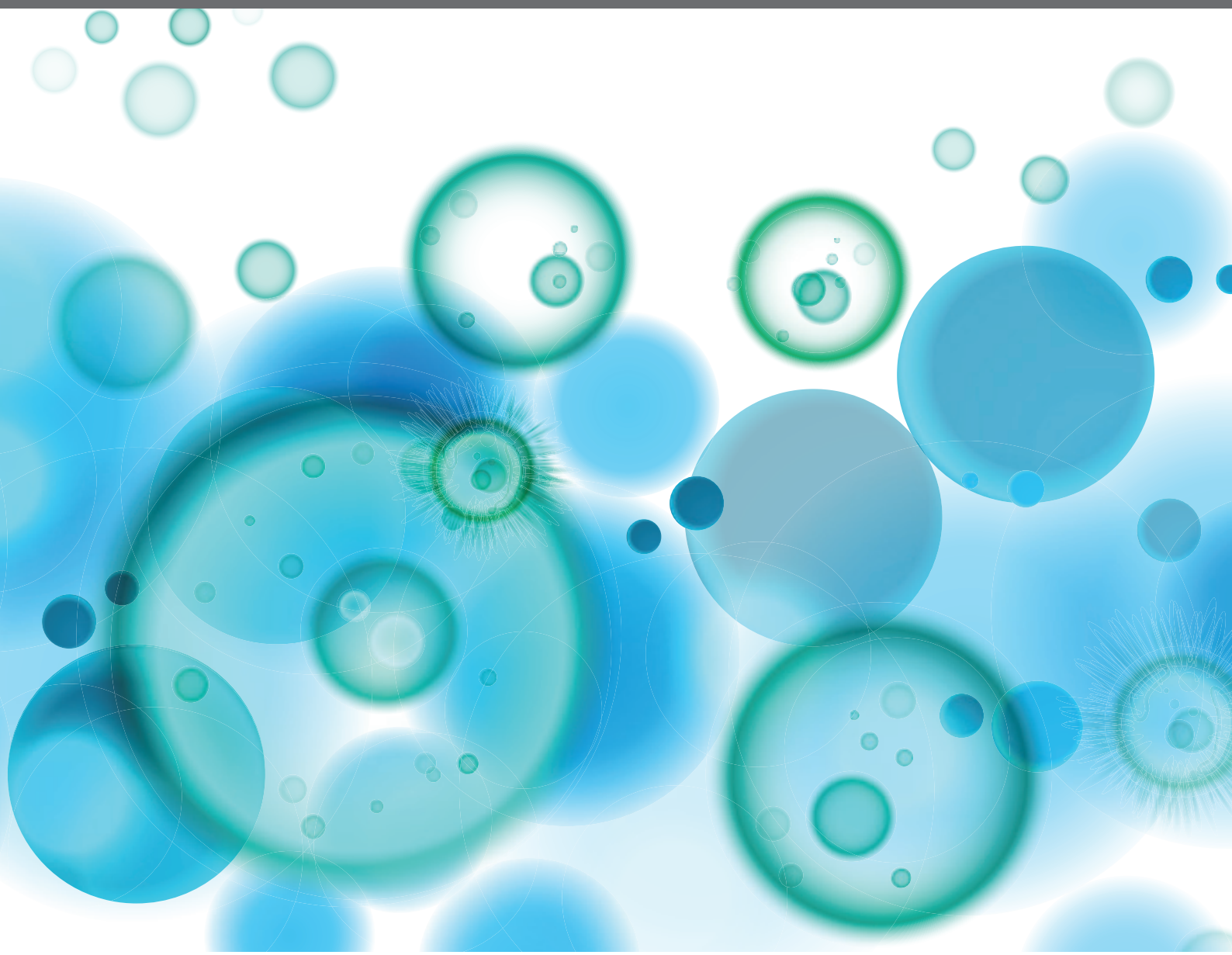

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IMMUNE-MODULATORY EFFECTS OF VITAMIN D

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EDITORIAL
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Editorial: Immune-Modulatory Effects of Vitamin D

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Editorial on the Research Topic

Immune-Modulatory Effects of Vitamin D

Vitamin D plays an essential role in bone development. However, recent studies are beginning to uncover its role as a modulator of the immune system (1). Several reports have shown associations between vitamin D deficiency (2) and the incidence as well as the severity of chronic inflammatory diseases such as cardiovascular disease (3, 4), inflammatory bowel disease (5), asthma (6), and chronic obstructive pulmonary disease (COPD) (7). Consistent with this, vitamin D supplementations have been shown to reduce the severity of and inflammation markers in chronic inflammatory diseases (8). At the molecular level, the hormonally active form of vitamin D (α1,25 dihydroxyvitamin D_3) regulates the expression of vitamin D responsive genes that can lead to differential regulation of signaling pathways in immune cells. For example, vitamin D positively regulates iron homeostasis and erythropoiesis via the iron-hepcidin-ferroportin axis (9). Vitamin D deficiency is highly prevalent world-wide including countries with abundance of sunshine (10). Singh et al. reviewed the causes of vitamin D deficiency where they dissected the complex impact of genetic predisposition, gut microbiota, and immune system. In this review, authors examined GWAS database (11) and listed genes variants with SNPs that associate with risk of vitamin D deficiency. These alleles are common in vitamin D receptor (VDR) and vitamin D binding protein (VDPB). Since gut microbiota plays a crucial role in nutrients and vitamins production, absorption and degradation, authors also highlighted the role of vitamin D metabolism and VDR is regulating host-gut microbiota interactions.

At the cellular level, vitamin D exerts anti-inflammatory effects on immune cells that express the vitamin D receptor (VDR) such as monocytes, macrophages, and T lymphocytes, which in turn shapes the immune response during the onset of inflammation and infection and following vaccination. Vitamin D exerts anti-inflammatory effects by reducing pro-inflammatory cytokines production from macrophages and T cells (12, 13). Carlberg eloquently demonstrated that vitamin D/VDR signaling impacts chromatin modeling leading to significant modification of human monocytes epigenome during perturbation, consequently reducing cytokine release and modulating trained innate immunity. The immune modulatory effects of vitamin D include reduction in inflammatory cytokines release such as IL-1β, which is induced via inflammasome activation. Rao et al. showed that VDR inhibited NALP3-inflammasome activation leading to reduction in IL-1β release. To confirm the inhibitory effect of VDR on NLP3 inflammasome activation, authors used VDR-deficient mice and showed that IL-1β release was significantly reduced *in vivo* confirming that VDR inhibited NALP3 inflammasome activation. Vitamin D deficiency is associated with bone pain in particular and chronic disease pain in general (12, 14). The proposed mechanisms by which Vitamin D/VDR signaling modulate pain sensation is reviewed by Habib et al.. Authors delineate vitamin D/VDR interactions with pain sensing

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pathways including dorsal root ganglion (DRG) neurons, nerve growth factor (NGF), epidermal growth factor receptor (EGFR), glial-derived neurotrophic factor (GDNF), and opioid receptors.

Sufficient levels of vitamin D have been shown to regulate T cell proliferation by controlling T cell antigen receptor and T cell activation as well as enhancing the phagocytic activity of macrophages (12, 14). The immune suppressive effects of regulatory T cells (T-reg) in controlling inflammation and preventing autoimmunity is well-established. Maternal immune tolerance to fetus is also integral to successful pregnancy. Vitamin D deficiency is quite common in pregnancy which has been associated with adverse outcomes. Cyprian et al. reviewed the consequences of vitamin D deficiency during pregnancy and detailed the role of T-regs in mitigating pregnancy loss and adverse events such as preeclampsia. Further, Vitamin D is shown to play an important role in modulating graft-vs-host disease (GvHD) by balancing immune responses. Flamann et al. described the role of vitamin D in inducing immune suppressive T regs and balancing immune responses in allogeneic hematopoietic stem cell transplantation. The immune modulatory effects of vitamin D increased immune tolerance and enhanced anti-tumor activity.

In cases of autoimmune diseases, low vitamin D levels are associated with increased B cell proliferation and autoantibody production. Low vitamin D levels have also been associated with the incidence of autoimmune diseases such as Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), and Systemic Lupus Erythematosus (SLE) (15). A systematic review by Islam et al. on the immunomodulatory effects of diet and nutrients in Systemic Lupus Erythematosus (SLE) presented the beneficial effect of vitamin D supplementation such as alleviating fatigue. Meta-analysis studies showed that SLE patients have significantly lower vitamin D levels compared to healthy participants. In human and animal studies of SLE, vitamin D is shown to inhibit cytokines such as IL-10, IL-17, IFN-γ, and modulate cellular proliferation of B-cells, Th1, Th17, CD4+ T cells. Therefore, vitamin D exerts various immune modulatory effects during autoimmune diseases. Another example of autoimmune diseases is Type 1 diabetes mellitus (T1DM) where insulin producing pancreatic β-cells are attacked by T cell-mediated autoimmunity (12, 16). Stem cell transplantation has emerged as regenerative treatment for T1DM as it possess immune suppressive potential. A clinical trial (NCT03920397) conducted by Araujo et al. on the effect of vitamin D treatment with allogenic adipose tissue-derived stromal/stem cells (ASCs) in 13 patients with recent onset of T1DM. One group received ASC and vitamin D (cholecalciferol 2,000 UI/day) for 3 months while the second group received standard insulin therapy. They observed that allogenic ASC and vitamin D therapy lead to reduced insulin requirement and more stable C peptide.

Low vitamin D status has been shown to be a risk factor for infectious diseases (17, 18). During infections such as Tuberculosis (TB), vitamin D induces the expression of cathelicidin (LL-37), a host defense peptide that enhances the bactericidal activity of immune cells like macrophages, thereby limiting the growth of mycobacteria that causes TB (18). Furthermore, LL-37 exerts anti-inflammatory effects via its ability to neutralize bacterial molecules like endotoxins and capsular polysaccharides that activate TLR signaling pathways, consequently inhibiting the release of pro-inflammatory mediators from macrophages. In this special topic several research papers investigated the role of vitamin D in infection outcomes. Muvva et al. investigated the effect of vitamin D on controlling intracellular *Mycobacterium tuberculosis* (Mtb) infection in human monocytes-derived macrophages. They showed that vitamin D treatment resulted in an appropriate macrophage polarization M1/M2 phenotype with enhanced expression of antimicrobial LL-37 and anti-inflammatory cytokine IL-10 but reduced expression of the immune suppressive enzyme IDO. This *in vitro* study provides evidence to the immune modulatory effects of vitamin D in controlling Mtb infection. Similarly, LL-37 is shown to control Leishmania parasitic infections in human. Crauwels et al. observed increased expression of LL-37 in skin biopsies from cutaneous leishmaniasis. They demonstrated that recombinant LL-37 reduced Leishmania parasite viability in a dose-dependent manner. Accordingly, they investigated the effect of vitamin D treatment on human monocytic-derived macrophages and documented that LL-37 mediated Leishmania restriction in macrophages. Vitamin D role in controlling infection is also demonstrated in acute enterocolitis. Mousavi et al. used an animal model to investigate the beneficial effects of vitamin D on preserving intestinal barrier and exerting anti-pathogenic effects during *Campylobacter jejuni* infection. They reported that preclinical administration of vitamin D ameliorated acute campylobacteriosis in mice model by reducing bacterial colonization and translocation, restoring intestinal epithelial cells regeneration and dampening inflammatory responses.

Vitamin D deficiency increases the risk of bacterial and viral infections (17). The immune modulatory effects of vitamin D during bacterial infections seem to vary from those effects during viral infections. In this regards, Anderson et al. observed the differential effects of vitamin D on induced inflammatory responses in primary human peripheral mononuclear cells (PBMC) during bacterial pneumococcal infection alone, respiratory syncytial viral (RSV) infection or co-infection. They showed that vitamin D exerted anti-inflammatory effects by reducing Th17 inflammatory cell expression and cytokines during pneumococcal infection alone and RSV alone but not during co-infection with RSV.

Genetic predisposition plays a role in host susceptibility to infections (19). Similarly, polymorphism in genes involved in vitamin D metabolism and function such as VDR impacts host susceptibility to infections. Pepineli et al. evaluated four genetic variants of VDR in the Leprosy immune pathogenesis in Brazil. They investigated a cohort of 404 leprosy patients in comparison to 432 control individuals without disease. Although no association is identified in VDR polymorphism genetic frequency between patients with active clinical leprosy and controls, they observed bAt haplotype to confer protection from leprosy. Taken together, data from original research paper,

clinical trial, systematic analysis, and reviews included herein provide compelling evidence to the immune modulatory effects of vitamin D. Therefore, vitamin D deficiency should be treated to gain the extra skeletal beneficial effects on the immune system in health and disease.

AUTHOR CONTRIBUTIONS

SZ wrote the editorial. EL and AB reveiwed the editorial. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Charoenngam N, Holick MF. Immunologic effects of vitamin D on human health and disease. *Nutrients*. (2020) 12:2097. doi: 10.3390/nu12072097
2. Amrein K, Scherkl M, Hoffmann M, Neuwersch-Sommeregger S, Kostenberger M, Tmava Berisha A, et al. Vitamin D deficiency 2.0: an update on the current status worldwide. *Eur J Clin Nutr*. (2020). doi: 10.1038/s41430-020-0558-y. [Epub ahead of print].
3. Archontogeorgis K, Nena E, Papanas N, Steiropoulos P. The role of vitamin D in obstructive sleep apnoea syndrome. *Breathe*. (2018) 14:206–15. doi: 10.1183/20734735.000618
4. Zhu X, Tu Y, Chen H, Jackson AO, Patel V, Yin K. Micro-environment and intracellular metabolism modulation of adipose tissue macrophage polarization in relation to chronic inflammatory diseases. *Diabetes Metab Res Rev*. (2018) 34:e2993. doi: 10.1002/dmrr.2993
5. Fakhoury HMA, Kvietys PR, AlKattan W, Anouti FA, Elahi MA, Karras SN, et al. Vitamin D and intestinal homeostasis: barrier, microbiota, immune modulation. *J Steroid Biochem Mol Biol*. (2020) 200:105663. doi: 10.1016/j.jsbmb.2020.105663
6. Bener A, Ehlayel MS, Tulic MK, Hamid Q. Vitamin D deficiency as a strong predictor of asthma in children. *Int Arch Allergy Immunol*. (2012) 157:168–75. doi: 10.1159/000323941
7. Zhu M, Wang T, Wang C, Ji Y. The association between vitamin D and COPD risk, severity, and exacerbation: an updated systematic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis*. (2016) 11:2597–607. doi: 10.2147/COPD.S101382
8. Jiang WL, Gu HB, Zhang YF, Xia QQ, Qi J, Chen JC. Vitamin D supplementation in the treatment of chronic heart failure: a meta-analysis of randomized controlled trials. *Clin Cardiol*. (2016) 39:56–61. doi: 10.1002/clc.22473
9. Zughaier SM, Alvarez JA, Sloan JH, Konrad RJ, Tangpricha V. The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes. *J Clin Transl Endocrinol*. (2014) 1:e19–25. doi: 10.1016/j.jcte.2014.01.003
10. Hassan-Smith ZK, Hewison M, Gittoes NJ. Effect of vitamin D deficiency in developed countries. *Br Med Bull*. (2017) 122:79–89. doi: 10.1093/bmb/ldx005
11. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, et al. Genome-wide association study of circulating vitamin D levels. *Hum Mol Genet*. (2010) 19:2739–45. doi: 10.1093/hmg/ddq155
12. Harrison SR, Li D, Jeffery LE, Raza K, Hewison M. Vitamin D, autoimmune disease and rheumatoid arthritis. *Calcif Tissue Int*. (2020) 106:58–75. doi: 10.1007/s00223-019-00577-2
13. Dankers W, Gonzalez-Leal C, Davelaar N, Asmawidjaja PS, Mus AMC, Hazes JMW, et al. 1,25(OH)2D3 and dexamethasone additively suppress synovial fibroblast activation by CCR6(+) T helper memory cells and enhance the effect of tumor necrosis factor alpha blockade. *Arthritis Res Ther*. (2018) 20:212. doi: 10.1186/s13075-018-1706-9
14. Wasnik S, Sharma I, Baylink DJ, Tang X. Vitamin D as a potential therapy for multiple sclerosis: where are we? *Int J Mol Sci*. (2020) 21:3102. doi: 10.3390/ijms21093102
15. Dall'Ara F, Cutolo M, Andreoli L, Tincani A, Paolino S. Vitamin D and systemic lupus erythematous: a review of immunological and clinical aspects. *Clin Exp Rheumatol*. (2018) 36:153–62. Available online at: https://www.clinexprheumatol.org/abstract.asp?a=11741
16. Shao F, Zheng P, Yu D, Zhou Z, Jia L. Follicular helper T cells in type 1 diabetes. *FASEB J*. (2020) 34:30–40. doi: 10.1096/fj.201901637R
17. Feketea G, Bocsan CI, Stanciu LA, Buzoianu AD, Zdrenghea MT. The role of vitamin D deficiency in children with recurrent wheezing-clinical significance. *Front Pediatr*. (2020) 8:344. doi: 10.3389/fped.2020.00344
18. Ayelign B, Workneh M, Molla MD, Dessie G. Role of vitamin-D supplementation in TB/HIV co-infected patients. *Infect Drug Resist*. (2020) 13:111–8. doi: 10.2147/IDR.S228336
19. La Vignera S, Cannarella R, Condorelli RA, Torre F, Aversa A, Calogero AE. Sex-specific SARS-CoV-2 mortality: among hormone-modulated ACE2 expression, risk of venous thromboembolism and hypovitaminosis D. *Int J Mol Sci*. (2020) 21:2948. doi: 10.3390/ijms21082948

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REVIEW
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Vitamin D Deficiency in the Gulf Cooperation Council: Exploring the Triad of Genetic Predisposition, the Gut Microbiome and the Immune System

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Vitamin D is a fat soluble secosteroid that is primarily synthesized in the skin upon exposure to Ultraviolet B (UVB) sun rays. Vitamin D is essential for the growth and development of bones and helps in reducing inflammation by strengthening muscles and the immune system. Despite the endless supply of sunlight in the Gulf Cooperation Council (GCC) countries which includes United Arab Emirates, Qatar, Kuwait, Bahrain, Saudi Arabia, and Oman, Vitamin D deficiency in the (GCC) general population at various age groups remains alarmingly high. In parallel runs the increasing prevalence of acute and chronic illnesses including, autoimmune diseases, cancer, type 1 diabetes mellitus, cardiovascular disease and Inflammatory bowel disease in the adult as well as the pediatric population of these countries. The exact association between Vitamin D deficiency and chronic disease conditions remains unclear; however, studies have focused on the mechanism of Vitamin D regulation by assessing the role of the Vitamin D associated genes/proteins such as VDR (Vitamin D receptor), VDBP (Vitamin D Binding protein), CYP27B1 as these are integral parts of the Vitamin D signaling pathway. VDR is known to regulate the expression of more than 200 genes across a wide array of tissues in the human body and may play a role in controlling the Vitamin D levels. Moreover, reduced Vitamin D level and downregulation of VDR have been linked to gut dysbiosis, highlighting an intriguing role for the gut microbiome in the Vitamin D metabolism. However, this role is not fully described yet. In this review, we aim to expand our understanding of the causes of Vitamin D deficiency in the GCC countries and explore the potential relationship between the genetic predisposition, Vitamin D levels, immune system and the gut microbiome composition. Trying to unravel this complex interaction may aid in understanding the mechanism by which Vitamin D contributes to various disease conditions and will pave the way toward new therapeutics treatments for Vitamin D deficiency and its associated outcomes.

Keywords: hypovitaminosis D, microbial dysbiosis, VDR, VDBP, CYP27B1, GCC

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INTRODUCTION

Vitamin D is well-known for its many health benefits. The role of Vitamin D in the elimination of rickets remains one of the most notable discoveries in medicine (1). Calcium and phosphate are two minerals that are required for normal bone formation. Upon demand, Vitamin D stimulates the intestines, bones, and kidneys to maintain calcium and phosphorus levels in the blood, and thus promotes the mineralization of the bone matrix and osteoclasts differentiation (2). Hypovitaminosis D or severe Vitamin D deficiency results in poor mineralization and bone loss, leading to osteoporosis, fractures, muscle weakness, and frank hypocalcemia (3). Vitamin D mediates its biological function via metabolizing into its active steroid form 1α,25-dihydroxyvitamin D3 [1α,25$(OH)_2D_3$ or simply 1,25$(OH)_2$D], often referred to as a hormone, influencing many genes across various tissues in the human body, such as kidneys (4–6), intestines (7), bones (8–11), as well as cancer and immune cells (12–14).

Vitamin D is also known to interact with cells from both the innate and adaptive immune system, where it plays an important role in antigen presentation, immune regulation and antibacterial response (15). Historical Vitamin D supplementation has been used to treat lupus and mycobacterial infections such as tuberculosis and leprosy (16, 17). Studies examining the immunomodulatory properties of Vitamin D have linked its deficiency to a higher incidence of autoimmune diseases, such as type I diabetes (T1D), multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) and also to an increased risk of developing various types of cancers including breast, ovarian, colon, and prostate cancer (18–21). Vitamin D is also a potent effector of vascular endothelial cells and thus has a role in cardio-protection (22), as it regulates blood pressure by stimulating the renin angiotensin system, vascular calcification and smooth muscle cell proliferation (23). Hypovitaminosis D is directly linked to the development of hypertension, and a reduction in blood pressure has been noted to be a consequence of Vitamin D supplementation (24, 25). Evidence also supports the role of Vitamin D in neuroprotection, brain development and maintenance of cognitive functions via immunomodulation, neuronal calcium regulation, antioxidative mechanisms, enhanced nerve conduction, and detoxification mechanisms (26). There have been some reports linking decreased Vitamin D levels with depression (27), cognitive delays and an increased risk of Alzheimer's disease (28). Cerebrovascular events such as the risk of vessel thrombosis, cerebrovascular infracts and strokes have also been associated with deficiency of Vitamin D (29).

Insufficient levels of Vitamin D may also be a contributing factor for other abnormalities such as poor diet, short stature, liver disease, and diabetes (30–32). The role of Vitamin D during pregnancy is also of great consideration since maternal nutritional status determines the health of fetus and newborn. Maternal Vitamin D deficiency has been associated with increased risk of preeclampsia, calcium malabsorption, bone loss, and other myopathies (33). It is suggested that the development of the fetus in a state of hypovitaminosis D can have significant impact on innate immune functions. *In vitro* study conducted with Monocytes cultured in Vitamin D deficient plasma, showed significantly decreased in a TLR-dependent expression of cathelicidin compared to the control (34) Vitamin D status in the cord blood was found to be associated with the risk of lower respiratory tract infection in the first year of life consistent with the *in vitro* study results (35). Severe deficiency can also contribute toward abnormalities such as small stature for gestational age, neonatal hypocalcemia, hypocalcemic seizures, infantile heart failure, enamel defects, large fontanelle, congenital rickets, among others (36, 37). The role of Vitamin D goes above and beyond the traditionally ascribed ones and its significance in human physiology is undeniable. In the most recent years, several papers have addressed the importance of Vitamin D and its intracellular receptor VDR in regulation of gut hemostasis and immune response (38–41), here we aim to go further and present a comprehensive review examining the epidemic of Vitamin D deficiency in Gulf Cooperation Council (GCC) which is an alliance of six Middle Eastern countries—Saudi Arabia, Qatar, Kuwait, the United Arab Emirates, Bahrain, and Oman. We would also like to study the possible role of specific genes in predisposing the gulf population to Vitamin D deficiency, and how this increasing epidemic leads to disturbed microbial balance in the intestines and manifestation of various immune mediated diseases such as IBD.

METHODS USED TO REVIEW THE LITERATURE IN THE FIELD

A comprehensive literature search was carried out in PubMed, ScienceDirect, Google Scholar and SpringerLink databases using keywords like "Vitamin D," "Human microbiome," "Vitamin D metabolism," "Vitamin D and Gut microbiome," "Vitamin D deficiency in Gulf countries," "VDR and Immune regulation," "Immune related disease in Gulf countries," "25-hydroxy vitamin D." Only articles published in English and related to the study topic were included in this review. The search was not restricted to the type of study i.e., species, meta-analysis, case-control, randomized control trials, cohort studies, reviews, sample size, or year of publication. Bibliographies and citation of the included reviews were scanned for additional studies that may have been missed by the database searches. The exclusion criteria comprised the following: unpublished data, conference publications, articles available only in the abstract form, and doctoral or master's thesis. Endnote software (Thomas Reuters, Philadelphia, PA) was used to create library and manage the findings of the search as recognized by the above-mentioned strategies. The selected articles were read and organized under the following headings: (1) Vitamin D, importance, metabolism, status and supplementation (2) Prevalence of Vitamin D deficiency or insufficiency and immune related disorders in GCC countries (3). Possible relationship between disturbances of the gut microbiota and/or Vitamin D deficiency, VDR dysfunction, and role of Vitamin D in immune system and related disorders.

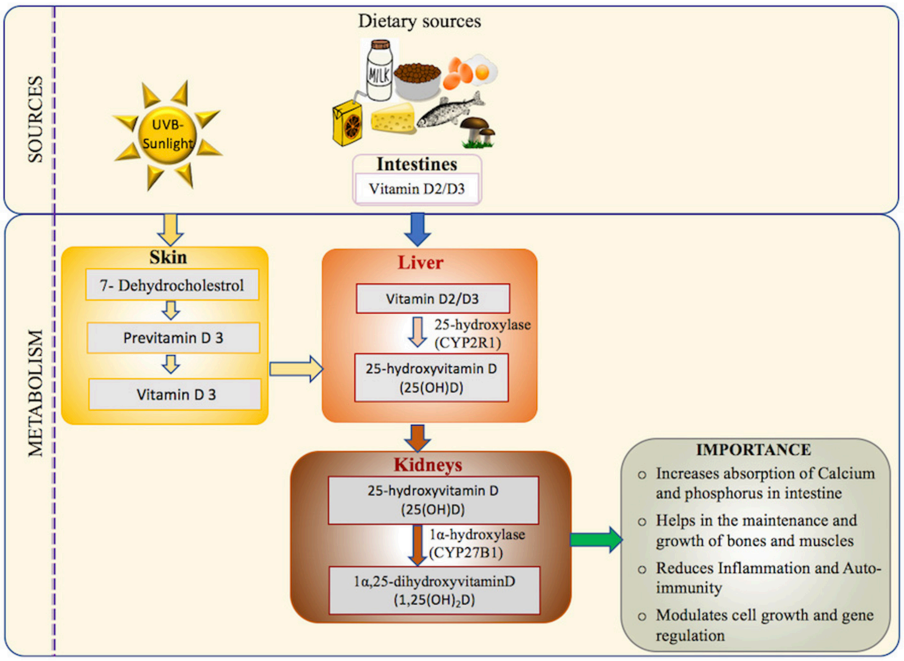

FIGURE 1 | Vitamin D: sources, activation pathway, and importance in the human body: the major source of Vitamin D (UVB-sunlight) and the minor source (Dietary Vitamin D) are transported to liver and metabolized to its main circulating form (25-hydroxyvitamin D) which is measured in the serum in most assays (18, 42, 43). The activated form of Vitamin D is then synthesized in the kidney via hydroxylation to form 1,25-dihydroxyvitamin D, also known as calcitriol (44, 45). Calcitriol has many functions including mineralization of bone matrix, enhancing absorption of calcium and phosphorous from small intestines, reducing autoimmunity and inflammation, and gene regulation (20, 46).

VITAMIN D METABOLISM

In humans, Vitamin D originates from three potential sources: Ultraviolet B (UVB) dependent exogenous synthesis, nutritional sources and supplements (18, 42, 43). However, it is primarily synthesized in the skin by the action of sunlight (UV dependent) as very few naturally occurring food sources have adequate amounts of Vitamin D (18, 42, 43). Dietary sources such as fish, milk, orange juice, and cereals contain one of the two forms, cholecalciferol (Vitamin D_3) or ergocalciferol (Vitamin D_2) (18, 42, 43). Regardless of the source, the Vitamin D synthesis pathway follows several common steps as detailed in **Figure 1**.

Dermal synthesis starts with the conversion of cutaneously derived cholesterol precursor 7-dehydroxycholestrol to previtamin D_3 by the action of UV sunlight (44, 47). Previtamin D undergoes a temperature dependent isomerization to Vitamin D_3. Vitamin D-binding protein (VDBP) carries the Vitamin D_3 synthesized in the skin and Vitamin D_2/Vitamin D_3 absorbed via intestine from the dietary sources to the liver (45). In the liver, Vitamin D_2/Vitamin D_3 is hydrolyzed to 25-hydroxyvitamin D[25(OH)D] by the action of enzyme 25-hydroxylase (CYP2R1) (44). The final step of activation occurs in the kidney with the conversion of 25-hydroxyvitamin D[25(OH)D] to $1,25(OH)_2D$ by the 1α-hydroxylase enzyme (CYP27B1) (48). Finally, $1,25(OH)_2D$ binds to its principal receptor VDR through which it regulates the expression of a large number of genes across various tissues in the human body (46). VDR plays a central role in mediating the biological functions of Vitamin D, via both genomic and non-genomic pathways as elucidated in **Figure 2**.

VITAMIN D STATUS AND DOSAGE

Levels of Vitamin D are measured in the serum as 25(OH)D, reflecting the Vitamin D status. The results of this blood test help with the clinical decision as to whether to take Vitamin D supplement or expose the skin to the sun. However, there is no international guidelines for reading the Vitamin D levels, as different organizations interpret the levels differently. Levels indicated as normal by one method may be interpreted differently by the other. The Vitamin D Council guidelines recommends 40–80 ng/ml as the ideal level, with levels of 0–30 ng/ml being considered deficient and 31–39 ng/ml considered insufficient (50). According to the Endocrine Society (51) the recommended preferred range falls between 40 and 60 ng/ml, levels below 20 ng/ml should indicate deficiency, 20–29 ng/ml defines insufficiency and ≥30 ng/ml is considered sufficient, all these guidelines along with the ones from the Food and Nutrition Board (52) are summarized in **Figure 3**.

Measurement of 25(OH)D can be performed with a number of different analytical techniques. Automated immunoassay's is used by most pathology lab to measure total serum 25(OH) D (25(OH) D_2 + 25(OH) D_3). Because of the clinical importance of Vitamin D testing it is important to note the existing

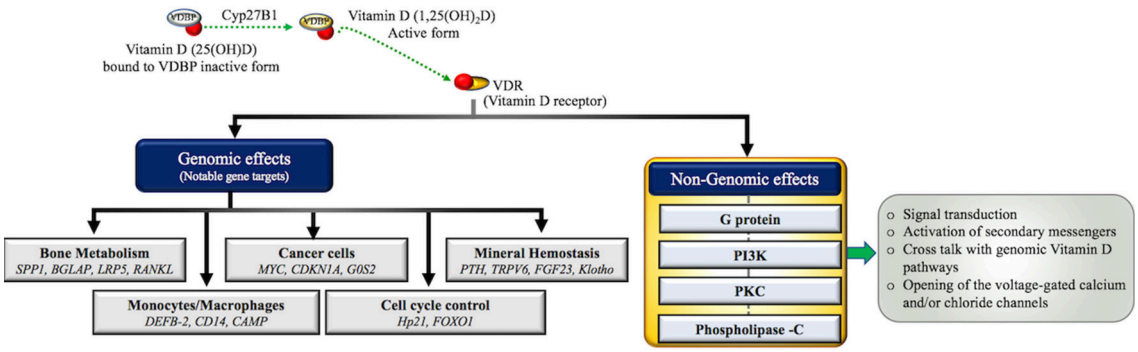

FIGURE 2 | Molecular actions of the Vitamin D/VDR axis. Inactive Vitamin D (25(OH)D) circulates in the blood stream bound to Vitamin D binding protein (VDBP) and undergoes hydroxylation by the renal mitochondrial 1-hydroxylase (CYP27B1) enzyme to convert into active form ($1\alpha,25(OH)_2D$). Active Vitamin D binds to its primary receptor VDR to modulate the expression of more than 200 genes in human body. Some notable ones are grouped into various biological processes including (1) bone metabolism (8–11) (2) immune cell regulation (14, 49) (3) cancer (12) (4) cell cycle (8) (5) metabolism (4–7) demonstrating the wide range of VDR dependent genomic actions. Non-genomic actions include the activation of one or more intracellular signaling molecules, such as G protein-coupled receptors (G-protein), Protein kinase C (PKC), phosphatidylinositol-3′-kinase (PI3K), and Phospholipase C (PLC) resulting in opening of the voltage-gated channels, generation of the specific second messengers and cross talk with genomic pathways (8).

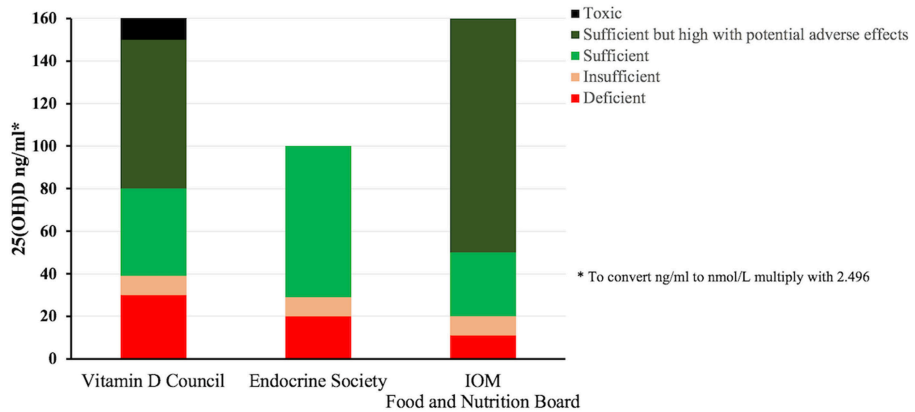

FIGURE 3 | Vitamin D status classification according to different internationally recognized organizations. The lack of a consensus about the ranges for deficiency and sufficiency among various international groups is apparent, Vitamin D council (1) defines Vitamin D deficiency in the range of 0–30 ng/ml whereas Endocrine Society (2) and Institute of Medicine (3) defines it as 0–20 and 0–11 ng/ml, respectively. Similarly, sufficiency is defined as 40–80, 30–100, and >20 ng/ml by (1), (2), (3), respectively. Vitamin D serum levels measured above 150 ng/ml is considered toxic by (1) where as there is no such interpretation by (2) and (3) (50–52).

discrepancies in Vitamin D total immunoassays as reported by several studies (53, 54). Various causes of the discrepancies have been noted (55) including the cross-reactivities of various Vitamin D metabolites (56). Therefore, the assays for Vitamin D testing need to be standardized so there is less variability in the results, the Vitamin D Standardization Program (VDSP), was launched in 2010 in collaboration with the National Institutes of Health, the Centers for Disease Control and Prevention (CDC), the National Institute for Standards and Technology (NIST), and Ghent University in Belgium to correct the disparity and ensure reliable Vitamin D measurement. However, we are still a long way to go to attain the goal of standardization of assay and results, till then we have to rely on the judgement of physicians and experts in the field. The researchers who publish in this area will likely continue to point toward such disparities.

To overcome the deficiency, Vitamin D supplementation is usually advised, however there is no consensus on the optimal dose to be prescribed, as many factors such as age and serum levels of 25(OH)D must be taken into account before recommending the best possible dose for instance for obese people (BMI $>$ 30 kg/m^2), a daily Vitamin D dose was set as "three times" greater than the recommended dose for subjects with normal body weight (51). The most common form of Vitamin D supplementation is Cholecalciferol and Ergocalciferol with both considered highly effective and safe (57). Vitamin D toxicity generally results from having serum levels of 25(OH)D $>$ 150 ng/ml, which in most cases is attributed to prolonged and unintended daily intakes of >40,000 IU of supplementation (58). **Table 1** details the group-wise recommendations for daily dose of Vitamin D supplementation from different organizations.

TABLE 1 | Group-wise recommendations for daily dose of Vitamin D supplementation from different organizations.

	Vitamin D council (IU/day)	Endocrine society (IU/day)	Food and nutrition board (IU/day)
Adult	5,000	1,500–2,000	600
Infant	1,000	400–1,000	400
Children	1,000	600–1,000	600
Pregnant women	4,000–6,000	1,500–1,000	600

Disparity in Vitamin D dosage recommendations by the Vitamin D council (1), Endocrine society (2), IOM food and nutrition board (3) are shown. Generally, however, pregnant or breastfeeding women and the elderly as well as children and adults lacking enough exposure to sunlight are in need of Vitamin D supplementation (50–52).

VITAMIN D DEFICIENCY: A HEALTH PROBLEM WORLDWIDE AND IN THE GCC COUNTRIES

Vitamin D deficiency is a major public health problem worldwide, affecting more than a billion people across the globe from both the developing and industrialized countries (59, 60). Studies have suggested that more than 70% of USA and 50% of UK adults may have insufficient Vitamin D levels (61, 62). Among other European populations, 1 in 8 adults have low circulating levels of 25-hydroxyvitamin D (63) and a similar pattern has also been reported in India, Australia and New Zealand (64, 65).

Despite the ample amount of year-round sunlight in the GCC region, pandemic levels of Vitamin D deficiency have been observed in recent years as represented in **Figure 4**. The 2016–2017 annual report from the Qatar Biobank highlights that close to 86% of the total Qatar biobank population (comprising of adults above 18–85 year-old, 80% of them Qatari national and the rest were long-term residents) suffers from Vitamin D inadequacy and more interestingly, 14% (more women 65% than men 35%) remained deficient despite taking supplementation, 70% of the participants were also obese (71). A review examining mixed population in Saudi Arabia (e.g., pregnant/ lactating women, children, adults) found that in 81% of all the groups the levels of 25(OH) D was $<$20 ng/mL ($<$50 nmol/L) (67). The numbers run high in UAE as well, a study conducted with a large cohort of patients (60,979) including UAE nationals and visitors of other nationalities, showed that up to 82.5% of the population suffers from deficiency or insufficiency of Vitamin D. Around 86.1% UAE nationals and 78.9% visitors had serum levels of 25(OH)D $<$ 75 nmol/L. The study also showed that the extreme cases of deficiency were higher in females (26.4%) than males (18.4%) (68). Comparable percentages have also been reported in different study cohorts' in other countries in the gulf peninsula (69, 70).

Recently many studies have associated high levels of Vitamin D deficiency with immune-mediated and inflammatory diseases. IBD is the term used to describe disorders that involve chronic inflammation of the GI tract. The main types of IBD include: ulcerative colitis and Crohn's disease. Recently the Epi-IBD study reported high prevalence of low Vitamin D levels in treatment-naive European IBD populations, with 79% of the patients showing either insufficient or deficient levels of Vitamin D (72). Vitamin D supplementation was associated with reduced intestinal inflammation in patients with active UC (73) and also in controlling the relapse rate of IBD (74).

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease which involves the connective tissue effecting multiple organs such as the brain, lungs, kidneys, heart, blood vessels, muscles, skin etc., and is more common among women (75). Vitamin D deficiency was significantly higher in SLE patients when compared to healthy controls in a study conducted with a cohort of Bahraini patients (76). Similar results were observed in a Saudi cohort (71) with high prevalence of Vitamin D inadequacy was observed in Saudi patients with SLE.

T1DM prevalence/incidence is increasing worldwide (77) the rise seems much steeper in gulf countries. A study conducted to determine the association between Vitamin D status and T1DM along with other factors in the young population of the State of Qatar found out that the incidences of severe Vitamin D deficiency was considerably higher in T1DM (28.8%) compared with healthy children (17.1%) (78), similarly in the Saudi cohort 84% of the T1DM children, and 59% of the healthy children were Vitamin D deficient (79). Low serum Vitamin D status was found to be associated with high prevalence and early onset of type-1 diabetes mellitus in Kuwaiti children as well (80). In a case control study done with Multiple sclerosis patient in Kuwait, VDR variants *Taq*I and *Bsm*I were found to be associated with MS risk (81).The therapeutic effect of Vitamin D in immune diseases should be further assessed in interventional studies.

A comparative associative study between Vitamin D deficiency and IBS (Irritable bowel syndrome) was conducted with patients visiting the gastroenterology clinic in Saudi Arabia, Vitamin D deficiency was detected in 82% of the patients in the IBS group and 31% in the control group (82).

The potential limitation to the findings of this review should be considered, relatively small number of studies were available to be included in the review and also there is limited data to prove causal relationship between Vitamin D deficiency and risk of Immune system related illness. The studies included employ various definitions of hypovitaminosis D, and these variations may result in the over or underestimation of Vitamin D deficiency in GCC population. Thus, development of global or local standard and guidelines will help in better screening to define the candidates and treating those who are at most risk for Vitamin D deficiency.

As we previously discussed, synthesis of Vitamin D occurs in the skin and it depends on several factors such as time of sun exposure, season, latitude, altitude, clothing, veiling, use of sunscreen, old age, and skin color (83). It was previously shown that limited exposure to sunlight results in poor Vitamin D synthesis (84), thus even in sunny climates like the GCC countries, high rates of Vitamin D deficiency is found because of cultural and social habits that limit exposure to sunlight (85). The association between reduced 25D concentrations and obesity is also well-established with several large-scale studies

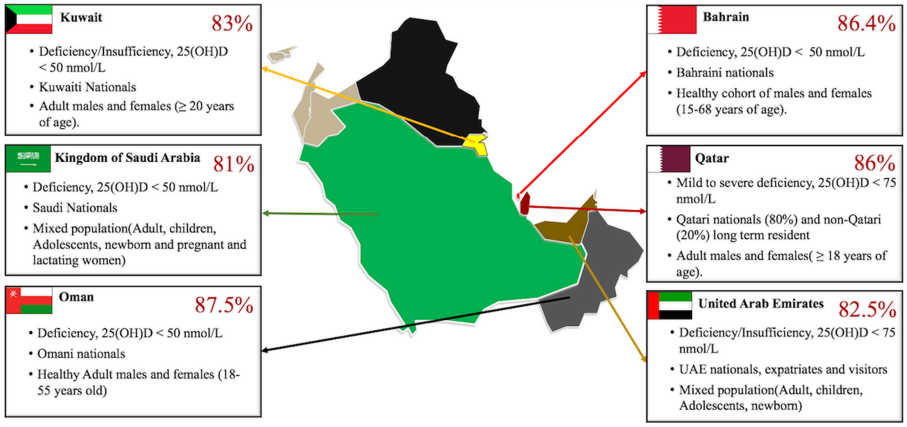

FIGURE 4 | Vitamin D deficiency reported in GCC countries. Gulf Cooperation Council (GCC), alliance of six Middle Eastern countries—Saudi Arabia (KSA), Kuwait, the United Arab Emirates, Qatar, Bahrain, and Oman. The Vitamin D deficiency runs high in the multi-ethnic population of these countries with percentages as high as 86% in Qatar (66) other countries such as KSA (67), UAE (68), Oman (69), and Kuwait (70) follow closely similar alarmingly high rates of Vitamin D deficiency/Insufficiency. The GCC map used was obtained with copyright approval from: https://yourfreetemplates.com/free-middle-east-map/.

that found obesity to be associated with lower 25D, 1,25D concentrations (86, 87). A bi-directional genetic study revealed that though the effect of lower 25D on BMI may not be significant but higher BMI leads to lower 25D (88) various explanations have been proposed to define this association, such as reduced cutaneous synthesis (89) and altered metabolism (90). Dietary sources make up for <10% of the total Vitamin D synthesized in the body (91), so even with a varied balanced diet, individuals will not be able to achieve the recommended Vitamin D levels.

However, the insufficiency of Vitamin D may not be solely due to diet or lack of sunlight. Several studies have suggested that various genes can define the Vitamin D status, thus large-scale genome-wide association studies have identified selected genes mainly those involved in the synthesis, metabolism or transport of Vitamin D to be associated with a variation in the Vitamin D status (92, 93). The SNPs associated with these genes were initially singled out in a European population (92) and were later replicated in the African American (94) and Asian populations (95). We utilized the GWAS catalog to generate the list of 49 gene variants or risk allele associated with Vitamin D Levels as shown in **Table 2** below. After literature review we identified two gene-association studies conducted in the GCC region, one in the Saudi populations (104) and the other in the Kuwaiti population (105). The study performed in the Saudi population identified a significant risk of Vitamin D deficiency and insufficiency associated with the SNP rs2228570 [Chromosome: 12, Position: 47879112, Gene: Vitamin D Receptor (VDR)], rs4588 [Chromosome: 4, Position: 71752606, Gene: GC] and rs10741657[Chromosome: 11, Position: 14,893,332, Gene: Vitamin D 25-hydroxylase (CYP2R1)] (104), while the study conducted in Kuwait showed that the polymorphism in the GC gene coding for the Vitamin D Binding protein (VDBP) may play a major role in determining Vitamin D levels in this population (105).

VITAMIN D AND THE GUT MICROBIOME: IS IT A BIDIRECTIONAL RELATIONSHIP

The human gut microbiota (the microbial taxa associated with humans) is home to an estimated 10^{14} microorganisms, with around 500–1,000 species of bacteria (106). The vastness of the human microbiome (the catalog of human microbiota and their genes) can be imagined in terms that it contains 10 times more cells and 100 times more genes than the human (107). These microorganisms create a "mini-ecosystem" inside our bodies, and they work together as biochemical factories performing a wide array of activities such as acquisition of nutrients, Vitamins production, degradation of toxins and enhancing host immune responses by functioning as a barrier from pathogenic microorganisms (108).

Gut microbiota exists in a symbiotic relationship with the host. The composition of the gut microbiome plays a crucial role in maintaining host homeostasis, as the wrong combination of microbes contribute to an array of chronic digestive or immune disorders such as IBD, obesity, diabetes mellitus, metabolic syndrome, atherosclerosis, alcoholic liver disease, nonalcoholic fatty liver disease, cirrhosis, and hepatocellular carcinoma (109, 110).

The compositions of the gut microbiota is determined by several factors as summarized in **Figure 5**, which include age, host genomics, immune health, exercise, intake of medication specifically antibiotics, and dietary habits (111). This imbalance or maladaptation of microbial communities is often referred to as Dysbiosis (also called dysbacteriosis) which has been categorized into three different types: (1) loss of beneficial organisms, (2) excessive growth of potentially harmful organisms, and (3) loss of overall microbial diversity. These types can occur in combination and are not mutually exclusive (112, 113). In such cases, the normally dominant species become underrepresented and the resulting void is

TABLE 2 | List of key variants or risk allele associated with Vitamin D level as reported by the GWAS catalog.

Number	SNP	Reported gene	Region	Location	Functional class	Reported trait	References
1	rs3755967-T	*GC (Group Specific Component)	4q13.3	4:71743681	Intron variant	Vitamin D levels	(96)
2	rs4588-T	GC (Group Specific Component)	4q13.3	4:71752606	Missense_variant	Vitamin D levels	(97)
3	rs2282679-T	GC (Group Specific Component)	4q13.3	4:71742666	Intron_variant	Vitamin D levels (dietary vitamin D intake interaction)	(96)
4	rs705117-G	GC (Group Specific Component)	4q13.3	4:71742398	Intron_variant	Serum vitamin D-binding protein levels	(98)
5	rs7041-T	GC (Group Specific Component)	4q13.3	4:71752617	Missense_variant	Serum vitamin D binding protein levels	(98)
6	rs2282679	GC (Group Specific Component)	4q13.3	4:71742666	Intron_variant	Vitamin D insufficiency	(92)
7	rs1607741-C	GC (Group Specific Component)	4q13.3	4:71853316	Intergenic_variant	Vitamin D levels	(97)
8	rs2282679-C	GC (Group Specific Component)	4q13.3	4:71742666	Intron_variant	Vitamin D levels	(99)
9	rs79761689-C	GC (Group Specific Component)	4q13.3	4:72005565	Intergenic_variant	Vitamin D levels	(97)
10	rs2282679-?	GC (Group Specific Component)	4q13.3	4:71742666	Intron_variant	Vitamin D levels	(100)
11	rs17467825-A	GC (Group Specific Component)	4q13.3	4:71739800	Downstream_gene_variant	Vitamin D levels	(101)
12	rs1155563-C	GC (Group Specific Component)	4q13.3	4:71777771	Intron_variant	Vitamin D levels	(101)
13	rs4588-A	GC (Group Specific Component)	4q13.3	4:71752606	Missense_variant	Serum 25-Hydroxyvitamin D levels	(102)
14	rs116970203-A	CYP2R1 (Vitamin D 25-hydroxylase)	11p15.2	11:14855172	Intron_variant	Vitamin D levels	(97)
15	rs10741657-A	CYP2R1 (Vitamin D 25-hydroxylase)	11p15.2	11:14893332	Upstream_gene_variant	Vitamin D levels	(96)
16	rs10741657-?	CYP2R1 (Vitamin D 25-hydroxylase)	11p15.2	11:14893332	Upstream_gene_variant	Vitamin D insufficiency	(92)
17	rs2060793-A	CYP2R1(Vitamin D 25-hydroxylase)	11p15.2	11:14893764	Upstream_gene_variant	Vitamin D levels	(99)
18	rs117913124-A	CYP2R1 (Vitamin D 25-hydroxylase)	11p15.2	11:14879385	Synonymous_variant	Serum 25-Hydroxyvitamin D levels	(102)
19	rs17216707-T	CYP24A1 (1,25-dihydroxyvitamin D(3) 24-hydroxylase)	20q13.2	20:54115823	Regulatory_region_variant	Vitamin D levels	(96)
20	rs6127099-T	CYP24A1 (1,25-dihydroxyvitamin D(3) 24-hydroxylase)	20q13.2	20:54114863	Intergenic_variant	Vitamin D levels	(97)
21	rs12785878-T	NADSYN1/DHCR7 (7-dehydrocholestrol reductase)	11q13.4	11:71456403	Intron_variant	Vitamin D levels	(96)
22	rs12785878-?	NADSYN1 (7-dehydrocholestrol reductase)	11q13.4	11:71456403	Intron_variant	Vitamin D insufficiency	(92)
23	rs4423214-T	NADSYN1(7-dehydrocholestrol reductase)	11q13.4	11:71462208	Intron_variant	Vitamin D levels	(97)
24	rs4944062-T	NADSYN1(7-dehydrocholestrol reductase)	11q13.4	11:71476248	3_prime_UTR_variant	Vitamin D levels (dietary vitamin D intake interaction)	(96)
25	rs3829251-A	NADSYN1 (7-dehydrocholestrol reductase)	11q13.4	11:71483513	Intron_variant	Vitamin D levels	(99)
26	rs182244780-A	RRAS2 (RAS related 2)	11p15.2	11:14363985	Intron_variant	Vitamin D levels	(97)
27	rs12287212-A	RRAS2 (RAS related 2)	11p15.2	11:14428315	Intergenic_variant	Vitamin D levels	(101)
28	rs11023332-C	PDE3B (phosphodiesterase 3B)	11p15.2	11:14762564	Intron_variant	Vitamin D levels	(101)

(Continued)

TABLE 2 | Continued

Number	SNP	Reported gene	Region	Location	Functional class	Reported trait	References
29	rs1007392-A	PDE3B (Phosphodiesterase 3B)	11p15.2	11:14753045	Intron_variant	Vitamin D levels	(101)
30	rs117300835-A	CALCB/INSC (Calcitonin Related Polypeptide Beta)	11p15.2	11:15097429	Intergenic_variant	Vitamin D levels	(97)
31	rs55665837-T	COPB1(Coatomer Protein Complex Subunit Beta 1)	11p15.2	11:14473503	Intron_variant	Vitamin D levels	(97)
32	rs148189294-A	SLC4A4(Sodium bicarbonate cotransporter 1)	4q13.3	4:71575200	Downstream_gene_variant	Vitamin D levels	(97)
33	rs117865811-G	SPON1(Spondin 1)	11p15.2	11:14180763	Intron_variant	Vitamin D levels	(97)
34	rs138485827-T	NPFFR2/ADAMTS3 (Neuropeptide FF Receptor2)/(A disintegrin and metalloproteinase with thrombospondin motifs 3)	4q13.3	4:72166226	Intergenic_variant	Vitamin D levels	(97)
35	rs78862524-A	ADAMTS3 (A disintegrin and metalloproteinase with thrombospondin motifs 3)	4q13.3	4:72305473	Intron_variant	Vitamin D levels	(97)
36	rs8018720-C	SEC23A	14q21.1	14:39086981	Missense_variant	Vitamin D levels	(96)
37	rs3819817-T	HAL (Histidine ammonia-lyase)	12q23.1	12:95984993	Intron_variant	Vitamin D levels	(97)
38	rs185378533-G	FLJ42102 (Uncharacterized LOC399923)	11q13.4	11:71422087	Intron_variant	Vitamin D levels	(97)
39	rs2207173-G	CYB5AP4(Cytochrome B5 Type A Pseudogene 4)	20p11.21	20:22824423	Intergenic_variant	Vitamin D levels	(103)
40	rs2277458-G	GEMIN2 (Gem Nuclear Organelle Associated Protein 2)	14q21.1	14:39114277	5_prime_UTR_variant	Vitamin D levels	(97)
41	rs10745742-T	AMDHD1(Amidohydrolase Domain Containing 1)	12q23.1	12:95964751	Intron_variant	Vitamin D levels (dietary vitamin D intake interaction)	(96)
42	rs12868495-A	VDAC1P12(Voltage dependent anion channel 1 pseudogene 12)	13q13.2	13:34067425	Intergenic_variant	Vitamin D levels	(101)
43	rs12144344-T	ST6GALNAC3(ST6 N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 3)	1p31.1	1:76373851	Intron_variant	Serum Vitamin D-binding protein levels	(98)
44	rs11586313-G	IVL (Involucrin)	1q21.3	1:152917994	TF_binding_site_variant	Vitamin D levels	(103)
45	rs6730714-A	PAX3 (Paired box gene 3)	2q36.1	2:222184302	Intergenic_variant	Vitamin D levels	(101)
46	rs156299-G	NPY (Neuropeptide Y)	7p15.3	7:24185113	Intergenic_variant	Vitamin D levels	(101)
47	rs2302190-C	MTMR4(Myotubularin Related Protein 4)	17q22	17:58507147	Missense_variant	Vitamin D levels	(101)
48	rs10508196-A	FAM155A (Family with sequence similarity 155 member A)	13q33.3	13:107827618	Intron_variant	Vitamin D levels	(101)
49	rs4751058-A	MKLN1 (Muskelin 1)	10q26.3	10:129075861	Intergenic_variant	Vitamin D levels	(101)

**GC gene codes for the Vitamin D binding protein (VDBP).*

filled by increasing numbers of opportunistic or pathogenic species resulting in overall decrease in overall bacterial diversity (112). Dysbiosis is commonly reported in the gastrointestinal tract (112) and is associated with a loss of the integrity of the intestinal mucosa, which may result in gut impairment and inflammation (114–116).

Several studies have demonstrated the role of Vitamin D and its receptor VDR in regulating host-microbial interactions. The active and inactive forms of Vitamin D circulate in the bloodstream bound to VDBP. The active form known as Calcitriol 1,25$(OH)_2$D is known to bind to the calcitriol receptor, commonly known as the VDR (117). Calcitriol binds to the VDR, which then forms a heterodimer with the retinoid-X receptor and other co-activators. This transcriptional complex then binds to discrete sites on DNA known as Vitamin D responsive elements (VDREs) resulting in the expression or repression of specific gene products (summarized in **Figure 6**) (131). It is also known that Vitamin D plays a role in various microRNA-directed post-transcriptional mechanisms (132).

The Interplay between Vitamin D, VDBP, Cyp27B1, and VDR not only regulate the transcriptional and post-transcriptional responses, but a growing evidence supports the concept that Vitamin D metabolism impacts the intestinal microbial balance

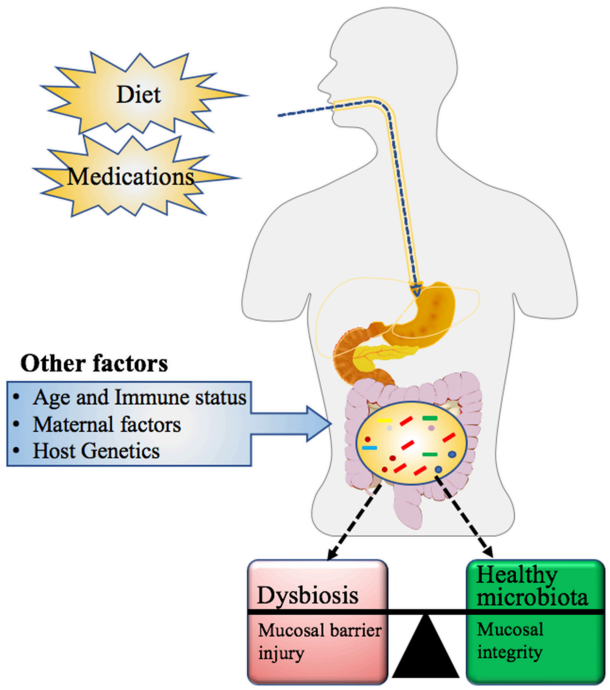

FIGURE 5 | Factors influencing the microbial community composition in Humans. Maternal factors such as mother's nutritional status (use of pre or probiotics), mode of delivery (vaginal versus Cesarean section), gestation age at birth (full or preterm), feeding type (formula feeding or breastfeeding), and other exogenous factors such as diet and medication history, influence not only the gut microbial community but also its metabolic capacity. Other determinants of microbial composition include the age and immune health of the individual along with their genetic makeup (111).

and gut homeostasis (132, 133). As previously mentioned, VDR is expressed in a variety of cell types such as kidney, muscles, prostate, immune cells; and high levels of expression in the cells of the gastrointestinal tract (GI) have been shown (134). Moreover, the Vitamin D activating enzyme Cyp27B1 is also expressed in various immune cells, as well as intestinal epithelial cells (135, 136). These two vital players of Vitamin D metabolism have also been colocalized in different cells of the GI tract suggesting the need for active Vitamin D in those cells (137).

In addition, the type of bacterial communities in the gut have been shown to regulate the expression of both VDR and Cyp27B1 (138, 139). VDR expression is inversely related to the presence of pathogenic bacteria, and correlate with probiotic bacteria with the former decreasing (138) and the latter increasing VDR expression (139). Animal studies done with germ-free and antibiotics-treated mice have reported reduced levels of Cyp27B1 expression in those animal models (140). This suggests that the intestinal bacteria regulate Vitamin D metabolism and, as a result, they regulate the innate immune response. The synthesis of active Vitamin D is modulated according to the local needs by Cyp27B1 and VDR enzymes whose expression is dependent on the "nature of local microbiota."

The VDR, Cyp27B1, and VDBP genes have also been studied for their association with autoimmune diseases such as IBD and its forms Crohn's disease (CD) and Ulcerative colitis (UC) (72, 141, 142). Several VDR polymorphisms such as *ApaI*, *TaqI*, and *FokI* have been associated with the development of UC (143, 144). VDR and Cyp27B1 gene knockout mice have shown increased susceptibility to the development of intestinal colitis, elevated expression of proinflammatory cytokines, and microbial dysbiosis, with more *Proteobacteria* and less *firmicutes*, a condition commonly observed in patients with IBD (145). Vitamin D supplementation in human and 1,25D treatment of CYP27B1 knockout mice decreased *Proteobacteria* and increased beneficial organism including members of the *Firmicutes* phyla (132, 146).

Gut mucosal integrity is a crucial barrier, playing a vital role in protection against pathogenic microorganisms, as disruption in mucosal barrier function and hyperpermeability can predispose to various diseases such as asthma, tuberculosis, cystic fibrosis and intestinal lung diseases (147–149). Vitamin D and its associated molecules provide protection for the epithelial barriers in various tissues including the gut mucosa, by increasing the expression of several tight and adherent junction proteins (150–152). VDR signaling specifically has been known to regulate the gut mucosal cell inflammation by suppressing intestinal epithelial cell apoptosis (153). The increasing awareness of the role of Vitamin D as an immunomodulator was prompted by the discovery of VDR and production of active Vitamin D ($1,25(OH)_2$ D) in almost all immune cells, including activated CD4+ and CD8+ T cells, B cells, neutrophils and antigen-presenting cells (APCs) such as macrophages and dendritic cells. The details of this regulation are described in the section below.

VITAMIN D IN INNATE AND ADAPTIVE IMMUNE SYSTEM

The innate immune system is the first and immediate line of the defense against invading pathogens and is an alliance of components both from the host and resident microbiota. The host defense comprises of diverse components such as physical defense (such as epithelial cells of the skin, mucous membrane, and microbiome), cellular defense (as mast cells, dendritic cells, macrophages, neutrophils, natural killer cells etc.), cell receptors that recognize pathogens (as Toll-like Receptors), antimicrobial peptides and proteins (as defensins, cathelicidins).

Vitamin D is able to modulate many of the above listed components of the innate immune system. It is known to reinforce the physical barrier function of epithelial cells. The active Vitamin D and VDR are important in regulating the genes of proteins required for the tight, adheres, and gap junctions such as zonulin occluden-1, zonulin occluden-2 through the up regulation of claudin 2 and 12 (154). Studies in transgenic mice demonstrated that over-expression of VDR in the intestinal epithelium decreases mucosal inflammation suppressing epithelial cell apoptosis and boosting the function of tight junction (119, 155). The importance of active Vitamin D in maintaining healthy gut microbiota has been discussed in the earlier section.

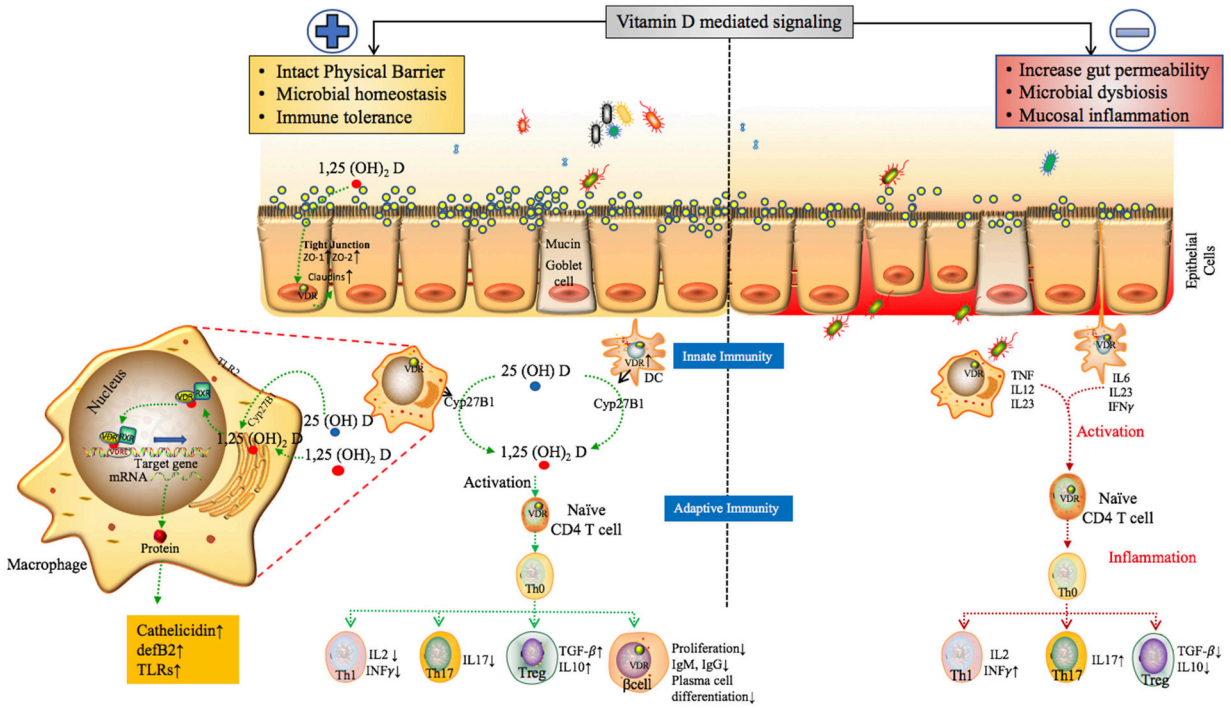

FIGURE 6 | The proposed role of Vitamin D mediated signaling in epithelial cell barrier function, Gut microbial hemostasis and modulation of Innate and adaptive immune responses. VDR regulates the level of tight junction proteins ZO1, ZO2 via claudin2 and 12 (39, 118) and helps maintain the structural integrity of epithelial barrier. Vitamin D deficiency have also been shown to disrupt gut homeostasis leading to systemic inflammation (119). Effects of Vitamin D on different players of the innate and adaptive immune system. In response to an antigenic challenge, macrophages, dendritic cells, and lymphocytes express the Vitamin D receptor (VDR), thereby becoming targets for the active Vitamin D metabolite, 1,25(OH)$_2$D (120). Macrophages and dendritic cells also express the CYP27B1 that synthesizes 1,25(OH)$_2$D leading to intra and paracrine responses such as stimulates enhancing the phagocytotic responses of macrophages and production of antimicrobial proteins such as cathelicidin (49, 121, 122). Active Vitamin D also modulates adaptive immunity. At the level of the APCs (like DCs), 1,25(OH)$_2$D inhibits the surface expression of MHC-II-complexed antigen. In addition, 1,25(OH)$_2$D directly affects T cell responses, by inhibiting the production of Th1 cytokines (IL-2 and IFN-γ), Th17 cytokines (IL-17 and IL-21), and by stimulating Th2 cytokine production (IL-4) and Treg cell development (123–129). Finally, 1,25(OH)$_2$D blocks plasma-cell differentiation, IgG and IgM production and B-cell proliferation (130). The above factors can trigger inflammatory immune responses such as TNF-α and IFN-γ leading to intestinal permeability and susceptibility to pathogenic infections, microbial dysbiosis and manifestation of immune related inflammatory diseases (19).

Monocytes and macrophages are crucial members of the innate immunity, Vitamin D stimulates the differentiation of precursor monocytes to mature phagocytic macrophage, the high expression of VDR by monocytes ensures sensitivity of these cells to the differentiating effects of active Vitamin D (120). Mature macrophages sense pathogen-associated molecular patterns (PAMPs) by means of pattern-recognition receptors, such as Toll-like receptors (TLRs). The presence of CYP27B1 (Vitamin D activating enzyme) in macrophages is important for the physiological action of host defense against infection, activation of 25(OH) D to 1, 25(OH)$_2$ D via CYP27B1 in macrophages leads to regulation of TLR2 (121), enhanced production of defensin β2, cathelicidin antimicrobial peptide (CAMP) (49) leading to induction of autophagy (122). The ability of 1,25(OH)$_2$D to increase the production of other antimicrobial peptides, has been demonstrated both *in vitro* by monocytes stimulation (156) and *in vivo* in pediatric patients' blood (157).

At the same time, the active Vitamin D can inhibit the dendritic cell (DCs) differentiation and maturation. In human and murine DC cultures, the activation of VDR signaling pathways inhibited DC-maturation as shown by downregulation of DC markers, MHC-class II, co-stimulatory molecules (CD40, CD80, and CD86), and other maturation molecules (e.g., CD1a, CD83), chemokine (CXCL10) which is involved in the recruitment of T helper 1 (Th1) cells. Furthermore, active Vitamin D also modulates DC-derived cytokine and chemokine expression, by inhibiting the production of pro-inflammatory cytokine (IL-12 and IL-23) and enhancing the release of anti-inflammatory cytokine (IL-10) and the chemokine [CCL22, involved in the recruitment regulatory T cells (Tregs)] (123–125).

However, this interaction between active Vitamin D and cells from innate immunity also have downstream effects on cells from the adaptive immune system. The adaptive immune system or acquired immune system is the second line of defense against infection. It comes into action upon exposure to pathogens and uses immunological memory to learn about the pathogens and enhance the immune response accordingly. The adaptive immune system is composed of T and B cells and is also responsible for autoimmune reaction. 1,25(OH)$_2$ D suppresses the immune responses mediated by Type 1 T helper (Th1) cells, by inhibiting the inflammatory cytokines IL-2 and interferon gamma (IFNγ) (125, 126) On the other hand, it has been

reported to enhance cytokines associated with Th2 cells (127). The $1,25(OH)_2$ D also regulate Treg cells (known for their role in the inhibition of inflammation) by induction of Foxp3 (the transcription factor involved in the development and function of Treg cells) (128). In addition to Th1, Th2, and Treg cells (subsets from the CD4 + T cell lineage) there are distinct subset termed Th17 cells that produce IL-17, which has been implicated in the pathogenesis of a number of autoimmune diseases. Th17 is inhibited by $1,25(OH)_2$ D (129). $1,25(OH)_2$ D prevents the activation of nuclear factor kappa-B (NF-κB), a component of proinflammatory signaling pathway at the level of transcription [VDR/RXR has been reported to bind to NF-κB promotor (IL-12p20) thus preventing its activation] (158).

The proliferation of VDR-expressing B-cells is also suppressed by active Vitamin D negatively impacting Ig production and also inhibiting the differentiation of plasma cells and class-switched memory cells highlighting a potential role for Vitamin D in B-cell-related disorders such as SLE (130). **Figure 6** details the proposed model of the overall impact of Vitamin D in the regulation of the epithelial barrier function, gut microbiota, and the immune system.

The above data elucidate the role of Vitamin D/VDR signaling in not only maintaining the microbial landscape in the gut in check and keeping the gut mucosal integrity intact but also as a potent immunomodulator. However, to fully understand the extent to which Vitamin D impact these complex systems, the multidirectional interaction between them needs to be considered. The importance of the interplay presents an encouraging field for further research with potential clinical implications.

DISCUSSION

Vitamin D deficiency is a major health problem in GCC countries including Qatar, indicating its importance as a regional and national health problem (67–71). Vitamin D deficiency affects various age groups and threatens the GCC populations with increased prevalence for chronic diseases such as IBD, cancer, diabetes among others. The reasons for the deficiency include limited exposure to sunlight, full body covering and low consumption of food rich in Vitamin D (83–85). The deficiency is treated with Vitamin D supplementation or intramuscular injections, however, despite being treated in a similar manner; there are interracial and interindividual variations in response to Vitamin D intake (159). The differences in serum Vitamin D levels among diverse ethnic groups have been previously reported in GWAS studies that have identified SNPs associated with genes from the Vitamin D pathway (92–95). These studies have indicated that certain ethnic groups may be predisposed to lower serum Vitamin D levels. We believe the same may be true for the population residing in GCC countries. These genetic variations may also impact the response to Vitamin D supplementation in these populations.

Originally known to cure rickets, our understanding on how Vitamin D impacts human health has broadened. The discovery of VDRs and Vitamin D associated enzymes in a variety of cells including cells of the innate and adaptive immune system was crucial in appreciating its role as a potent immunomodulator. Immune cells are not only a target for Vitamin D, but are also able to activate the hormone locally, thus postulating for an autocrine and paracrine role for the active Vitamin D (160). Inadequate amounts of Vitamin D could be linked to defective functioning of the associated autocrine and paracrine circuits eventually leading to various immune abnormalities.

Among other extra-skeletal effects, we have also come to realize its role in maintaining a healthy gut microbiota. The GI tract is home to a plethora of microorganisms that program the immune system preventing colonization by harmful bacteria and viruses. Several studies referenced in this review have suggested that Vitamin D signaling influences the microbial load and composition and ensures adequate innate and adaptive immune responses to pathogenic threats; though the questions still linger about the bidirectional and multidirectional interaction between these intricate systems. Vitamin D deficiency may result in abnormal immune functions causing inflammation, which, when unresolved, may lead to chronic conditions.

In conclusion, Vitamin D signaling mediated by key players, such as VDR, VDBP, and Cyp27B1, has a diverse physiological impact, enhancing the intestinal barrier function and contributing to enteric homeostasis. Studies have shown that low serum Vitamin D levels might be linked to several diseases and given the fact that the Vitamin D status is a modifiable factor, the potential therapeutic benefits from Vitamin D supplementation in the prevention of various diseases and maintenance of a healthy microbiota are undeniable. Therefore, the widespread Vitamin D deficiency in the GCC countries demands discussion about including Vitamin D testing as part of routine clinical care practices.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

1. Steenbock H. The induction of growth promoting and calcifying properties in a ration by exposure to light. *Science.* (1924) 60:224–5. doi: 10.1126/science.60.1549.224
2. Lieben L, Carmeliet G. Vitamin D signaling in osteocytes: effects on bone and mineral homeostasis. *Bone.* (2013) 54:237–43. doi: 10.1016/j.bone.2012.10.007
3. Hewison M. An update on vitamin D and human immunity. *Clin Endocrinol.* (2012) 76:315–25. doi: 10.1111/j.1365–2265.2011.04261.x

4. Haussler MR, Whitfield GK, Kaneko I, Forster R, Saini R, Hsieh JC, et al. The role of vitamin D in the FGF23, klotho, and phosphate bone-kidney endocrine axis. *Rev Endocr Metab Disord.* (2012) 13:57–69. doi: 10.1007/s11154-011-9199-8
5. Forster RE, Jurutka PW, Hsieh JC, Haussler CA, Lowmiller CL, Kaneko I, et al. Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. *Biochem Biophys Res Commun.* (2011) 414:557–62. doi: 10.1016/j.bbrc.2011.09.117
6. Liu SM, Koszewski N, Lupez M, Malluche HH, Olivera A, Russell J. Characterization of a response element in the 5'-flanking region of the avian (chicken) PTH gene that mediates negative regulation of gene transcription by 1,25-dihydroxyvitamin D3 and binds the vitamin D3 receptor. *Mol Endocrinol.* (1996) 10:206–15. doi: 10.1210/mend.10.2.8825560
7. Meyer MB, Watanuki M, Kim S, Shevde NK, Pike JW. The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. *Mol Endocrinol.* (2006) 20:1447–61. doi: 10.1210/me.2006-0031
8. Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms. *Best Pract Res Clin Endocrinol Metab.* (2011) 25:543–59. doi: 10.1016/j.beem.2011.05.010
9. Barthel TK, Mathern DR, Whitfield GK, Haussler CA, Hopper HAt, Hsieh JC, et al. 1,25-Dihydroxyvitamin D3/VDR-mediated induction of FGF23 as well as transcriptional control of other bone anabolic and catabolic genes that orchestrate the regulation of phosphate and calcium mineral metabolism. *J Steroid Biochem Mol Biol.* (2007) 103:381–8. doi: 10.1016/j.jsbmb.2006.12.054
10. Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, et al. The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate healthful aging. *J Steroid Biochem Mol Biol.* (2010) 121:88–97. doi: 10.1016/j.jsbmb.2010.03.019
11. Kim S, Yamazaki M, Zella LA, Shevde NK, Pike JW. Activation of receptor activator of NF-kappaB ligand gene expression by 1,25-dihydroxyvitamin D3 is mediated through multiple long-range enhancers. *Mol Cell Biol.* (2006) 26:6469–86. doi: 10.1128/MCB.00353-06
12. Ryynanen J, Seuter S, Campbell MJ, Carlberg C. Gene regulatory scenarios of primary 1,25-dihydroxyvitamin d3 target genes in a human myeloid leukemia cell line. *Cancers.* (2013) 5:1221–41. doi: 10.3390/cancers5041221
13. Heikkinen S, Vaisanen S, Pehkonen P, Seuter S, Benes V, Carlberg C. Nuclear hormone 1alpha,25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res.* (2011) 39:9181–93. doi: 10.1093/nar/gkr654
14. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
15. Skrobot A, Demkow U, Wachowska M. Immunomodulatory role of vitamin D: a review. *Adv Exp Med Biol.* (2018) 1108:13–23. doi: 10.1007/5584_2018_246
16. Airey FS. Vitamin D as a remedy for lupus vulgaris. *Med World.* (1946) 64:807–10.
17. Herrera G. Vitamin D in massive doses as an adjuvant to the sulfones in the treatment of tuberculoid leprosy. *Int J Lepr.* (1949) 17:35–42.
18. Holick MF. Vitamin D deficiency. *N Engl J Med.* (2007) 357:266–81. doi: 10.1056/NEJMra070553
19. Rosen CJ, Adams JS, Bikle DD, Black DM, Demay MB, Manson JE, et al. The nonskeletal effects of vitamin D: an endocrine society scientific statement. *Endocr Rev.* (2012) 33:456–92. doi: 10.1210/er.2012-1000
20. Giovannucci E. Expanding roles of vitamin D. *J Clin Endocrinol Metab.* (2009) 94:418–20. doi: 10.1210/jc.2008-2695
21. Liu W, Zhang L, Xu HJ, Li Y, Hu CM, Yang JY, et al. The anti-inflammatory effects of vitamin D in tumorigenesis. *Int J Mol Sci.* (2018) 19:E2736. doi: 10.3390/ijms19092736
22. Perez-Hernandez N, Aptilon-Duque G, Nostroza-Hernandez MC, Vargas-Alarcon G, Rodriguez-Perez JM, Blachman-Braun R. Vitamin D and its effects on cardiovascular diseases: a comprehensive review. *Korean J Intern Med.* (2016) 31:1018–29. doi: 10.3904/kjim.2015.224
23. Zittermann A, Schleithoff SS, Koerfer R. Putting cardiovascular disease and vitamin D insufficiency into perspective. *Br J Nutr.* (2005) 94:483–92. doi: 10.1079/BJN20051544
24. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. *Circulation.* (2008) 117:503–11. doi: 10.1161/CIRCULATIONAHA.107.706127
25. Schwarz N, Nicholls SJ, Psaltis PJ. Vitamin D and cardiovascular disease. *Heart Lung Circ.* (2018) 27:903–6. doi: 10.1016/j.hlc.2018.05.098
26. Buell JS, Dawson-Hughes B. Vitamin D and neurocognitive dysfunction: preventing "D"ecline? *Mol Aspects Med.* (2008) 29:415–22. doi: 10.1016/j.mam.2008.05.001
27. Fond G, Godin O, Schurhoff F, Berna F, Bulzacka E, Andrianarisoa M, et al. Hypovitaminosis D is associated with depression and anxiety in schizophrenia: Results from the national FACE-SZ cohort. *Psychiatry Res.* (2018) 270:104–10. doi: 10.1016/j.psychres.2018.09.024
28. Balion C, Griffith LE, Strifler L, Henderson M, Patterson C, Heckman G, et al. Vitamin D, cognition, and dementia: a systematic review and meta-analysis. *Neurology.* (2012) 79:1397–405. doi: 10.1212/WNL.0b013e31826c197f
29. Judd SE, Morgan CJ, Panwar B, Howard VJ, Wadley VG, Jenny NS, et al. Vitamin D deficiency and incident stroke risk in community-living black and white adults. *Int J Stroke.* (2016) 11:93–102. doi: 10.1177/1747493015607515
30. Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, et al. Hypovitaminosis D in medical inpatients. *N Engl J Med.* (1998) 338:777–83. doi: 10.1056/NEJM199803193381201
31. Jawa A, Riaz SH, Khan Assir MZ, Afreen B, Riaz A, Akram J. Causes of short stature in Pakistani children found at an Endocrine Center. *Pak J Med Sci.* (2016) 32:1321–5. doi: 10.12669/pjms.326.11077
32. Kim HS, Rotundo L, Kothari N, Kim SH, Pyrsopoulos N. Vitamin D is associated with severity and mortality of non-alcoholic fatty liver disease: a US population-based study. *J Clin Transl Hepatol.* (2017) 5:185–92. doi: 10.14218/JCTH.2017.00025
33. Hollis BW, Wagner CL. Nutritional vitamin D status during pregnancy: reasons for concern. *CMAJ.* (2006) 174:1287–90. doi: 10.1503/cmaj.060149
34. Walker VP, Zhang X, Rastegar I, Liu PT, Hollis BW, Adams JS, et al. Cord blood vitamin D status impacts innate immune responses. *J Clin Endocrinol Metab.* (2011) 96:1835–43. doi: 10.1210/jc.2010-1559
35. Belderbos ME, Houben ML, Wilbrink B, Lentjes E, Bloemen EM, Kimpen JL, et al. Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis. *Pediatrics.* (2011) 127:e1513–20. doi: 10.1542/peds.2010-3054
36. Mulligan ML, Felton SK, Riek AE, Bernal-Mizrachi C. Implications of vitamin D deficiency in pregnancy and lactation. *Am J Obstet Gynecol.* (2010) 202:429 e1–9. doi: 10.1016/j.ajog.2009.09.002
37. Javaid MK, Crozier SR, Harvey NC, Gale CR, Dennison EM, Boucher BJ, et al. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. *Lancet.* (2006) 367:36–43. doi: 10.1016/S0140-6736(06)67922-1
38. Wang J, Thingholm LB, Skiecevičienė J, Rausch P, Kummen M, Hov JR, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat Genet.* (2016) 48:1396–406. doi: 10.1038/ng.3695
39. Clark A, Mach N. Role of vitamin D in the hygiene hypothesis: the interplay between vitamin D, Vitamin D receptors, gut microbiota, and immune response. *Front Immunol.* (2016) 7:627. doi: 10.3389/fimmu.2016.00627
40. Jin D, Wu S, Zhang YG, Lu R, Xia Y, Dong H, et al. Lack of vitamin D receptor causes dysbiosis and changes the functions of the murine intestinal microbiome. *Clin Ther.* (2015) 37:996–1009 e7. doi: 10.1016/j.clinthera.2015.04.004
41. Chirumbolo S, Bjorklund G, Sboarina A, Vella A. The role of vitamin D in the immune system as a pro-survival molecule. *Clin Ther.* (2017) 39:894–916. doi: 10.1016/j.clinthera.2017.03.021
42. Lamberg-Allardt C. Vitamin D in foods and as supplements. *Prog Biophys Mol Biol.* (2006) 92:33–8. doi: 10.1016/j.pbiomolbio.2006.02.017
43. Wacker M, Holick MF. Vitamin D—effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients.* (2013) 5:111–48. doi: 10.3390/nu5010111

44. Deluca HF. History of the discovery of vitamin D and its active metabolites. *Bonekey Rep*. (2014) 3:479. doi: 10.1038/bonekey.2013.213
45. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol.* (2005) 289:F8–28. doi: 10.1152/ajprenal.00336.2004
46. Gombart AF. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiol.* (2009) 4:1151–65. doi: 10.2217/fmb.09.87
47. Holick MF, Frommer JE, McNeill SC, Richtand NM, Henley JW, Potts JTJr. Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. *Biochem Biophys Res Commun.* (1977) 76:107–14. doi: 10.1016/0006-291X(77)91674-6
48. Fraser DR, Kodicek E. Unique biosynthesis by kidney of a biological active vitamin D metabolite. *Nature.* (1970) 228:764–6. doi: 10.1038/228764a0
49. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* (2004) 173:2909–12. doi: 10.4049/jimmunol.173.5.2909
50. Aloia JF. Clinical review: the 2011 report on dietary reference intake for vitamin D: where do we go from here? *J Clin Endocrinol Metab.* (2011) 96:2987–96. doi: 10.1210/jc.2011-0090
51. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. (2011) 96:1911–30. doi: 10.1210/jc.2011-0385
52. Ross AC, Taylor CL, Yaktine AL, Del Valle HB. *Dietary Reference Intakes for Calcium and Vitamin D*. Institute of Medicine report. Washington, DC: The National Academies Press (2011). p. 1–1132.
53. Carter GD. Accuracy of 25-hydroxyvitamin D assays: confronting the issues. *Curr Drug Targets.* (2011) 12:19–28. doi: 10.2174/138945011793591608
54. Farrell C, Soldo J, Williams P, Herrmann M. 25-Hydroxyvitamin D testing: challenging the performance of current automated immunoassays. *Clin Chem Lab Med.* (2012) 50:1953–63. doi: 10.1515/cclm-2012-0522
55. Fuleihan Gel H, Bouillon R, Clarke B, Chakhtoura M, Cooper C, McClung M, et al. Serum 25-hydroxyvitamin D Levels: variability, knowledge gaps, and the concept of a desirable range. *J Bone Miner Res.* (2015) 30:1119–33. doi: 10.1002/jbmr.2536
56. Lee JH, Choi JH, Kweon OJ, Park AJ. Discrepancy between vitamin D total immunoassays due to various cross-reactivities. *J Bone Metab.* (2015) 22:107–12. doi: 10.11005/jbm.2015.22.3.107
57. Zittermann A, Prokop S, Gummert JF, Borgermann J. Safety issues of vitamin D supplementation. *Anticancer Agents Med Chem.* (2013) 13:4–10. doi: 10.2174/187152013804487290
58. Lowe H, Cusano NE, Binkley N, Blaner WS, Bilezikian JP. Vitamin D toxicity due to a commonly available "over the counter" remedy from the Dominican Republic. *J Clin Endocrinol Metab.* (2011) 96:291–5. doi: 10.1210/jc.2010-1999
59. Papandreou D, Malindretos P, Karabouta Z, Rousso I. Possible health implications and low vitamin D Status during childhood and adolescence: an updated mini review. *Int J Endocrinol.* (2010) 2010:472173. doi: 10.1155/2010/472173
60. Hassan-Smith ZK, Hewison M, Gittoes NJ. Effect of vitamin D deficiency in developed countries. *Br Med Bull.* (2017) 122:79–89. doi: 10.1093/bmb/ldx005
61. Kennel KA, Drake MT, Hurley DL. Vitamin D deficiency in adults: when to test and how to treat. *Mayo Clin Proc.* (2010) 85:752–7; quiz 757–8. doi: 10.4065/mcp.2010.0138
62. Pearce SH, Cheetham TD. Diagnosis and management of vitamin D deficiency. *BMJ.* (2010) 340:b5664. doi: 10.1136/bmj.b5664
63. Hayes A, Cashman KD. Food-based solutions for vitamin D deficiency: putting policy into practice and the key role for research. *Proc Nutr Soc.* (2017) 76:54–63. doi: 10.1017/S0029665116000756
64. Marwaha RK, Tandon N, Reddy DR, Aggarwal R, Singh R, Sawhney RC, et al. Vitamin D and bone mineral density status of healthy schoolchildren in northern India. *Am J Clin Nutr.* (2005) 82:477–82. doi: 10.1093/ajcn.82.2.477
65. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, et al. Vitamin D and health in adults in Australia and New Zealand: a position statement. *Med J Aust.* (2012) 196:686–7. doi: 10.5694/mja11.10301
66. Anonymous. *Qatar Biobank Report 2016/2017*. Qatar Biobank, Doha (2016).
67. Al-Daghri NM. Vitamin D in Saudi Arabia: prevalence,distribution and disease associations. *J Steroid Biochem Mol Biol.* (2018) 175:102–7. doi: 10.1016/j.jsbmb.2016.12.017
68. Haq A, Svobodova J, Imran S, Stanford C, Razzaque MS. Vitamin D deficiency: a single centre analysis of patients from 136 countries. *J Steroid Biochem Mol Biol.* (2016) 164:209–13. doi: 10.1016/j.jsbmb.2016.02.007
69. Abiaka C, Delghandi M, Kaur M, Al-Saleh M. Vitamin D status and anthropometric indices of an omani study population. *Sultan Qaboos Univ Med J.* (2013) 13:224–31. doi: 10.12816/0003227
70. Zhang FF, Al Hooti S, Al Zenki S, Alomirah H, Jamil KM, Rao A, et al. Vitamin D deficiency is associated with high prevalence of diabetes in Kuwaiti adults: results from a national survey. *BMC Public Health.* (2016) 16:100. doi: 10.1186/s12889-016-2758-x
71. Damanhouri LH. Vitamin D deficiency in Saudi patients with systemic lupus erythematosus. *Saudi Med J.* (2009) 30:1291–5. doi: 10.5001/omj.2013.10
72. Chetcuti Zammit S, Ellul P, Girardin G, Valpiani D, Nielsen KR, Olsen J, et al. Vitamin D deficiency in a European inflammatory bowel disease inception cohort: an Epi-IBD study. *Eur J Gastroenterol Hepatol.* (2018) 30:1297–303. doi: 10.1097/MEG.0000000000001238
73. Garg M, Hendy P, Ding JN, Shaw S, Hold G, Hart A. The effect of vitamin D on intestinal inflammation and faecal microbiota in patients with ulcerative colitis. *J Crohns Colitis.* (2018) 12:963–72. doi: 10.1093/ecco-jcc/jjy052
74. Li J, Chen N, Wang D, Zhang J, Gong X. Efficacy of vitamin D in treatment of inflammatory bowel disease: a meta-analysis. *Medicine*. (2018) 97:e12662. doi: 10.1097/MD.0000000000012662
75. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med.* (2011) 365:2110–21. doi: 10.1056/NEJMra1100359
76. Farid E, Jaradat AA, Al-Segai O, Hassan AB. Prevalence of vitamin D deficiency in adult patients with systemic lupus erythematosus in kingdom of Bahrain. *Egypt J Immunol*. (2017) 24:1–8. doi: 10.4172/2153-0645-C1-016
77. Emerging Risk Factors Collaboration, Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet*. (2010) 375:2215–22. doi: 10.1016/S0140-6736(10)60484-9
78. Bener A, Alsaied A, Al-Ali M, Hassan AS, Basha B, Al-Kubaisi A, et al. Impact of lifestyle and dietary habits on hypovitaminosis D in type 1 diabetes mellitus and healthy children from Qatar, a sun-rich country. *Ann Nutr Metab.* (2008) 53:215–22. doi: 10.1159/000184439
79. Bin-Abbas BS, Jabari MA, Issa SD, Al-Fares AH, Al-Muhsen S. Vitamin D levels in Saudi children with type 1 diabetes. *Saudi Med J*. (2011) 32:589–92.
80. Rasoul MA, Al-Mahdi M, Al-Kandari H, Dhaunsi GS, Haider MZ. Low serum vitamin-D status is associated with high prevalence and early onset of type-1 diabetes mellitus in Kuwaiti children. *BMC Pediatr.* (2016) 16:95. doi: 10.1186/s12887-016-0629-3
81. Al-Temaimi RA, Al-Enezi A, Al-Serri A, Alroughani R, Al-Mulla F. The association of vitamin D receptor polymorphisms with multiple sclerosis in a case-control study from Kuwait. *PLoS ONE*. (2015) 10:e0142265. doi: 10.1371/journal.pone.0142265
82. Khayyat Y, Attar S. Vitamin D deficiency in patients with irritable bowel syndrome: does it exist? *Oman Med J.* (2015) 30:115–8. doi: 10.5001/omj.2015.25
83. Plotnikoff GA. Weather or not: the importance of vitamin D status monitoring and supplementation. *Minn Med.* (2009) 92:43–6.
84. Glerup H, Mikkelsen K, Poulsen L, Hass E, Overbeck S, Thomsen J, et al. Commonly recommended daily intake of vitamin D is not sufficient if sunlight exposure is limited. *J Intern Med.* (2000) 247:260–8. doi: 10.1046/j.1365–2796.2000.00595.x
85. Al-Mogbel ES. Vitamin D status among Adult Saudi females visiting primary health care clinics. *Int J Health Sci*. (2012) 6:116–26. doi: 10.12816/0005987
86. Parikh SJ, Edelman M, Uwaifo GI, Freedman RJ, Semega-Janneh M, Reynolds J, et al. The relationship between obesity and serum 1,25-dihydroxy vitamin D concentrations in healthy adults. *J Clin Endocrinol Metab*. (2004) 89:1196–9. doi: 10.1210/jc.2003-031398
87. Lagunova Z, Porojnicu AC, Lindberg F, Hexeberg S, Moan J. The dependency of vitamin D status on body mass index, gender, age and season. *Anticancer Res.* (2009) 29:3713–20. doi: 10.14341/2071-8713-4886
88. Vimaleswaran KS, Berry DJ, Lu C, Tikkanen E, Pilz S, Hiraki LT, et al. Causal relationship between obesity and vitamin D status: bi-directional Mendelian

randomization analysis of multiple cohorts. *PLoS Med.* (2013) 10:e1001383. doi: 10.1371/journal.pmed.1001383
89. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr.* (2000) 72:690–3. doi: 10.1093/ajcn/72.3.690
90. Kong J, Li YC. Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. *Am J Physiol Endocrinol Metab.* (2006) 290:E916–24. doi: 10.1152/ajpendo.00410.2005
91. Holick MF. Vitamin D: a millenium perspective. *J Cell Biochem.* (2003) 88:296–307. doi: 10.1002/jcb.10338
92. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet.* (2010) 376:180–8. doi: 10.1016/S0140-6736(10)60588-0
93. Bouillon R. Genetic and environmental determinants of vitamin D status. *Lancet.* (2010) 376:148–9. doi: 10.1016/S0140-6736(10)60635-6
94. Engelman CD, Fingerlin TE, Langefeld CD, Hicks PJ, Rich SS, Wagenknecht LE, et al. Genetic and environmental determinants of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels in Hispanic and African Americans. *J Clin Endocrinol Metab.* (2008) 93:3381–8. doi: 10.1210/jc.2007-2702
95. Zhang Z, He JW, Fu WZ, Zhang CQ, Zhang ZL. An analysis of the association between the vitamin D pathway and serum 25-hydroxyvitamin D levels in a healthy Chinese population. *J Bone Miner Res.* (2013) 28:1784–92. doi: 10.1002/jbmr.1926
96. Jiang X, O'Reilly PF, Aschard H, Hsu YH, Richards JB, Dupuis J, et al. Genome-wide association study in 79,366 European-ancestry individuals informs the genetic architecture of 25-hydroxyvitamin D levels. *Nat Commun.* (2018) 9:260. doi: 10.1038/s41467-017-02662-2
97. Manousaki D, Dudding T, Haworth S, Hsu YH, Liu CT, Medina-Gomez C, et al. Low-frequency synonymous coding variation in CYP2R1 has large effects on vitamin D levels and risk of multiple sclerosis. *Am J Hum Genet.* (2017) 101:227–38. doi: 10.1016/j.ajhg.2017.06.014
98. Moy KA, Mondul AM, Zhang H, Weinstein SJ, Wheeler W, Chung CC, et al. Genome-wide association study of circulating vitamin D-binding protein. *Am J Clin Nutr.* (2014) 99:1424–31. doi: 10.3945/ajcn.113.080309
99. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, et al. Genome-wide association study of circulating vitamin D levels. *Hum Mol Genet.* (2010) 19:2739–45. doi: 10.1093/hmg/ddq155
100. Lasky-Su J, Lange N, Brehm JM, Damask A, Soto-Quiros M, Avila L, et al. Genome-wide association analysis of circulating vitamin D levels in children with asthma. *Hum Genet.* (2012) 131:1495–505. doi: 10.1007/s00439-012-1185-z
101. Anderson D, Holt BJ, Pennell CE, Holt PG, Hart PH, Blackwell JM. Genome-wide association study of vitamin D levels in children: replication in the Western Australian Pregnancy Cohort (Raine) study. *Genes Immun.* (2014) 15:578–83. doi: 10.1038/gene.2014.52
102. O'Brien KM, Sandler DP, Shi M, Harmon QE, Taylor JA, Weinberg CR. Genome-wide association study of serum 25-hydroxyvitamin D in US women. *Front Genet.* (2018) 9:67. doi: 10.3389/fgene.2018.00067
103. Sapkota BR, Hopkins R, Bjonnes A, Ralhan S, Wander GS, Mehra NK, et al. Genome-wide association study of 25(OH) vitamin D concentrations in Punjabi Sikhs: results of the Asian Indian diabetic heart study. *J Steroid Biochem Mol Biol.* (2016) 158:149–56. doi: 10.1016/j.jsbmb.2015.12.014
104. Sadat-Ali M, Al-Turki HA, Azam MQ, Al-Elq AH. Genetic influence on circulating vitamin D among Saudi Arabians. *Saudi Med J.* (2016) 37:996–1001. doi: 10.15537/smj.2016.9.14700
105. Mezzavilla M, Tomei S, Alkayal F, Melhem M, Ali MM, Al-Arouj M, et al. Investigation of genetic variation and lifestyle determinants in vitamin D levels in Arab individuals. *J Transl Med.* (2018) 16:20. doi: 10.1186/s12967-018-1396-8
106. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature.* (2008) 453:620–5. doi: 10.1038/nature07008
107. Sommer F, Backhed F. The gut microbiota–masters of host development and physiology. *Nat Rev Microbiol.* (2013) 11:227–38. doi: 10.1038/nrmicro2974
108. Ubeda C, Pamer EG. Antibiotics, microbiota, and immune defense. *Trends Immunol.* (2012) 33:459–66. doi: 10.1016/j.it.2012.05.003
109. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature.* (2006) 444:1022–3. doi: 10.1038/4441022a
110. Wang B, Jiang X, Cao M, Ge J, Bao Q, Tang L, et al. Altered fecal microbiota correlates with liver biochemistry in nonobese patients with non-alcoholic fatty liver disease. *Sci Rep.* (2016) 6:32002. doi: 10.1038/srep32002
111. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. *Science.* (2012) 336:1262–7. doi: 10.1126/science.1223813
112. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut.* (2004) 53:1–4. doi: 10.1136/gut.53.1.1
113. Moos WH, Faller DV, Harpp DN, Kanara I, Pernokas J, Powers WR, et al. Microbiota and neurological disorders: a gut feeling. *Biores Open Access.* (2016) 5:137–45. doi: 10.1089/biores.2016.0010
114. Jang SE, Lim SM, Jeong JJ, Jang HM, Lee HJ, Han MJ, et al. Gastrointestinal inflammation by gut microbiota disturbance induces memory impairment in mice. *Mucosal Immunol.* (2018) 11:369–79. doi: 10.1038/mi.2017.49
115. Saito T, Hayashida H, Furugen R. Comment on: Cani et al. (2007) Metabolic endotoxemia initiates obesity and insulin resistance: diabetes. (2007) 56:1761–1772. *Diabetes* 56:e20; author reply e21. doi: 10.2337/db07-1181
116. Creely SJ, McTernan PG, Kusminski CM, Fisher FM, Da Silva NF, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab.* (2007) 292:E740–7. doi: 10.1152/ajpendo.00302.2006
117. Moore DD, Kato S, Xie W, Mangelsdorf DJ, Schmidt DR, Xiao R, et al. International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. *Pharmacol Rev.* (2006) 58:742–59. doi: 10.1124/pr.58.4.6
118. Zhang YG, Wu S, Sun J. Vitamin D, vitamin D receptor, and tissue barriers. *Tissue Barriers.* (2013) 1:e23118. doi: 10.4161/tisb.23118
119. He L, Liu T, Shi Y, Tian F, Hu H, Deb DK, et al. Gut epithelial vitamin D receptor regulates microbiota-dependent mucosal inflammation by suppressing intestinal epithelial cell apoptosis. *Endocrinology.* (2018) 159:967–79. doi: 10.1210/en.2017-00748
120. Reichel H, Koeffler HP, Bishop JE, Norman AW. 25-Hydroxyvitamin D3 metabolism by lipopolysaccharide-stimulated normal human macrophages. *J Clin Endocrinol Metab.* (1987) 64:1–9. doi: 10.1210/jcem-64-1-1
121. Proal AD, Albert PJ, Marshall TG. The human microbiome and autoimmunity. *Curr Opin Rheumatol.* (2013) 25:234–40. doi: 10.1097/BOR.0b013e32835cedbf
122. Campbell GR, Spector SA. Vitamin D inhibits human immunodeficiency virus type 1 and *Mycobacterium tuberculosis* infection in macrophages through the induction of autophagy. *PLoS Pathog.* (2012) 8:e1002689. doi: 10.1371/journal.ppat.1002689
123. von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Odum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat Immunol.* (2010) 11:344–9. doi: 10.1038/ni.1851
124. Almerighi C, Sinistro A, Cavazza A, Ciaprini C, Rocchi G, Bergamini A. 1Alpha,25-dihydroxyvitamin D3 inhibits CD40L-induced pro-inflammatory and immunomodulatory activity in human monocytes. *Cytokine.* (2009) 45:190–7. doi: 10.1016/j.cyto.2008.12.009
125. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. *Curr Opin Pharmacol.* (2010) 10:482–96. doi: 10.1016/j.coph.2010.04.001
126. Lemire JM, Adams JS, Kermani-Arab V, Bakke AC, Sakai R, Jordan SC. 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity *in vitro*. *J Immunol.* (1985) 134:3032–5.
127. Cantorna MT, Snyder L, Lin YD, Yang L. Vitamin D and 1,25(OH)2D regulation of T cells. *Nutrients.* (2015) 7:3011–21. doi: 10.3390/nu7043011
128. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol.* (2009) 183:5458–67. doi: 10.4049/jimmunol.0803217

129. Joshi S, Pantalena LC, Liu XK, Gaffen SL, Liu H, Rohowsky-Kochan C, et al. 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol Cell Biol.* (2011) 31:3653–69. doi: 10.1128/MCB.05020-11
130. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol.* (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
131. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev.* (2016) 96:365–408. doi: 10.1152/physrev.00014.2015
132. Ooi JH, Li Y, Rogers CJ, Cantorna MT. Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis. *J Nutr.* (2013) 143:1679–86. doi: 10.3945/jn.113.180794
133. Proal AD, Albert PJ, Marshall T. Autoimmune disease in the era of the metagenome. *Autoimmun Rev.* (2009) 8:677–81. doi: 10.1016/j.autrev.2009.02.016
134. Shang M, Sun J. Vitamin D/VDR, probiotics, and gastrointestinal diseases. *Curr Med Chem.* (2017) 24:876–87. doi: 10.2174/0929867323666161202150008
135. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch Biochem Biophys.* (2000) 374:334–8. doi: 10.1006/abbi.1999.1605
136. Battault S, Whiting SJ, Peltier SL, Sadrin S, Gerber G, Maixent JM. Vitamin D metabolism, functions and needs: from science to health claims. *Eur J Nutr.* (2013) 52:429–41. doi: 10.1007/s00394-012-0430-5
137. Chun RF, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol.* (2014) 5:151. doi: 10.3389/fphys.2014.00151
138. Waterhouse JC, Perez TH, Albert PJ. Reversing bacteria-induced vitamin D receptor dysfunction is key to autoimmune disease. *Ann N Y Acad Sci.* (2009) 1173:757–65. doi: 10.1111/j.1749–6632.2009.04637.x
139. Appleyard CB, Cruz ML, Isidro AA, Arthur JC, Jobin C, De Simone C. Pretreatment with the probiotic VSL#3 delays transition from inflammation to dysplasia in a rat model of colitis-associated cancer. *Am J Physiol Gastrointest Liver Physiol.* (2011) 301:G1004–13. doi: 10.1152/ajpgi.00167.2011
140. Mukherji A, Kobiita A, Ye T, Chambon P. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell.* (2013) 153:812–27. doi: 10.1016/j.cell.2013.04.020
141. Cantorna MT. IBD: vitamin D and IBD: moving towards clinical trials. *Nat Rev Gastroenterol Hepatol.* (2016) 13:322–3. doi: 10.1038/nrgastro.2016.72
142. Abdo J, Rai V, Agrawal DK. Interplay of immunity and vitamin D: interactions and implications with current IBD therapy. *Curr Med Chem.* (2017) 24:852–67. doi: 10.2174/0929867323666161026124951
143. Wang L, Wang ZT, Hu JJ, Fan R, Zhou J, Zhong J. Polymorphisms of the vitamin D receptor gene and the risk of inflammatory bowel disease: a meta-analysis. *Genet Mol Res.* (2014) 13:2598–610. doi: 10.4238/2014.April.8.2
144. Xue LN, Xu KQ, Zhang W, Wang Q, Wu J, Wang XY. Associations between vitamin D receptor polymorphisms and susceptibility to ulcerative colitis and Crohn's disease: a meta-analysis. *Inflamm Bowel Dis.* (2013) 19:54–60. doi: 10.1002/ibd.22966
145. Kim JH, Yamaori S, Tanabe T, Johnson CH, Krausz KW, Kato S, et al. Implication of intestinal VDR deficiency in inflammatory bowel disease. *Biochim Biophys Acta.* (2013) 1830:2118–28. doi: 10.1016/j.bbagen.2012.09.020
146. Bashir M, Prietl B, Tauschmann M, Mautner SI, Kump PK, Treiber G, et al. Effects of high doses of vitamin D3 on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. *Eur J Nutr.* (2016) 55:1479–89. doi: 10.1007/s00394-015-0966-2
147. Finklea JD, Grossmann RE, Tangpricha V. Vitamin D and chronic lung disease: a review of molecular mechanisms and clinical studies. *Adv Nutr.* (2011) 2:244–53. doi: 10.3945/an.111.000398
148. Agrawal T, Gupta GK, Agrawal DK. Vitamin D deficiency decreases the expression of VDR and prohibitin in the lungs of mice with allergic airway inflammation. *Exp Mol Pathol.* (2012) 93:74–81. doi: 10.1016/j.yexmp.2012.04.004
149. Mak G, Hanania NA. Vitamin D and asthma. *Curr Opin Pulm Med.* (2011) 17:1–5. doi: 10.1097/MCP.0b013e3283411440
150. Li YC, Chen Y, Du J. Critical roles of intestinal epithelial vitamin D receptor signaling in controlling gut mucosal inflammation. *J Steroid Biochem Mol Biol.* (2015) 148:179–83. doi: 10.1016/j.jsbmb.2015.01.011
151. Barbachano A, Fernandez-Barral A, Ferrer-Mayorga G, Costales-Carrera A, Larriba MJ, Munoz A. The endocrine vitamin D system in the gut. *Mol Cell Endocrinol.* (2017) 453:79–87. doi: 10.1016/j.mce.2016.11.028
152. Stio M, Retico L, Annese V, Bonanomi AG. Vitamin D regulates the tight-junction protein expression in active ulcerative colitis. *Scand J Gastroenterol.* (2016) 51:1193–9. doi: 10.1080/00365521.2016.1185463
153. Liu W, Chen Y, Golan MA, Annunziata ML, Du J, Dougherty U, et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest.* (2013) 123:3983–96. doi: 10.1172/JCI65842
154. Lagishetty V, Liu NQ, Hewison M. Vitamin D metabolism and innate immunity. *Mol Cell Endocrinol.* (2011) 347:97–105. doi: 10.1016/j.mce.2011.04.015
155. Du J, Chen Y, Shi Y, Liu T, Cao Y, Tang Y, et al. 1,25-dihydroxyvitamin D protects intestinal epithelial barrier by regulating the myosin light chain kinase signaling pathway. *Inflamm Bowel Dis.* (2015) 21:2495–506. doi: 10.1097/MIB.0000000000000526
156. Liu PT, Schenk M, Walker VP, Dempsey PW, Kanchanapoomi M, Wheelwright M, et al. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS ONE.* (2009) 4:e5810. doi: 10.1371/journal.pone.0005810
157. Georgieva V, Kamolvit W, Herthelius M, Luthje P, Brauner A, Chromek M. Association between vitamin D, antimicrobial peptides and urinary tract infection in infants and young children. *Acta Paediatr.* (2018). doi: 10.1111/apa.14499
158. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest.* (1998) 101:252–62. doi: 10.1172/JCI1050
159. Aloia JF, Patel M, Dimaano R, Li-Ng M, Talwar SA, Mikhail M, et al. Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration. *Am J Clin Nutr.* (2008) 87:1952–8. doi: 10.1093/ajcn/87.6.1952
160. Morris HA, Anderson PH. Autocrine and paracrine actions of vitamin D. *Clin Biochem Rev.* (2010) 31:129–38.

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Vitamin D in Acute Campylobacteriosis–Results From an Intervention Study Applying a Clinical *Campylobacter jejuni* Induced Enterocolitis Model

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Human *Campylobacter* infections are progressively rising and of high socioeconomic impact. In the present preclinical intervention study we investigated anti-pathogenic, immuno-modulatory, and intestinal epithelial barrier preserving properties of vitamin D applying an acute campylobacteriosis model. Therefore, secondary abiotic IL-10$^{-/-}$ mice were perorally treated with synthetic 25-OH-cholecalciferol starting 4 days before peroral *Campylobacter jejuni* infection. Whereas, 25-OH-cholecalciferol application did not affect gastrointestinal pathogen loads, 25-OH-cholecalciferol treated mice suffered less frequently from diarrhea in the midst of infection as compared to placebo control mice. Moreover, 25-OH-cholecalciferol application dampened *C. jejuni* induced apoptotic cell responses in colonic epithelia and promoted cell-regenerative measures. At day 6 post-infection, 25-OH-cholecalciferol treated mice displayed lower numbers of colonic innate and adaptive immune cell populations as compared to placebo controls that were accompanied by lower intestinal concentrations of pro-inflammatory mediators including IL-6, MCP1, and IFN-γ. Remarkably, as compared to placebo application synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice resulted in lower cumulative translocation rates of viable pathogens from the inflamed intestines to extra-intestinal including systemic compartments such as the kidneys and spleen, respectively, which was accompanied by less compromised colonic epithelial barrier function in the 25-OH-cholecalciferol as compared to the placebo cohort. In conclusion, our preclinical intervention study provides evidence that peroral synthetic 25-OH-cholecalciferol application exerts inflammation-dampening effects during acute campylobacteriosis.

Keywords: vitamin D, *Campylobacter jejuni*, campylobacteriosis model, intervention study, host-pathogen interaction, acute enterocolitis, intestinal epithelial barrier function

INTRODUCTION

Campylobacter jejuni constitute major infectious bacterial agents of zoonotic enteric morbidities with increasing prevalences worldwide (1). Humans become infected via the food chain by consumption of raw or undercooked meat derived from contaminated livestock animals or by ingestion of *C. jejuni* containing surface water (2–4). Infected individuals present with symptoms of varying degree depending on the virulence of the acquired bacterial strain on one side and the host immune status on the other (1, 5–7). Some patients display rather mild symptoms including watery diarrhea, whereas others develop acute campylobacteriosis (8, 9). These severely compromised individuals complain about abdominal cramps, fever, and inflammatory bloody diarrhea (8, 9). During infection intestinal tissues are destroyed by innate immune responses and display profound histopathological inflammatory changes such as ulcerations, crypt abscesses, and increased numbers of innate and adaptive immune cells in the colonic mucosa and lamina propria (5, 8, 10, 11). The vast majority of human infections are usually self-limiting and treated (if at all) symptomatically. Only severely compromised patients with immuno-suppressive comorbidities, for instance, require hospitalization and receive antimicrobial treatment (6, 8, 9). In rare cases, however, post-infectious sequelae such as Guillain-Barré syndrome, Miller Fisher syndrome, Reiter's syndrome, and reactive arthritis might arise with a latency of weeks to months (8, 9, 12).

Despite the progressively increasing prevalences of human campylobacteriosis, cellular, and molecular events that are involved in disease development are not yet fully understood. However, previous clinical studies revealed that in humans acute *C. jejuni* induced disease courses and post-infectious sequelae such as Guillain-Barré syndrome are triggered by the pathogenic surface molecule lipooligosaccharide (LOS) causing hyper-activation of the innate immune system in the sialylated form (13). For quite a long time *in vivo* studies have been hampered by the scarcity of appropriate animal models. This is mainly because the gastrointestinal microbiota of mice mediates a strong colconization resistance to *C. jejuni* and mice are *per se* about 10,000-fold more resistant to LOS and lipopolysaccharide (LPS) as compared to humans (14). Our group has recently shown that secondary abiotic IL-10$^{-/-}$ mice in which the gut microbiota had been depleted by broad-spectrum antibiotic treatment can not only be effectively colonized by the pathogen upon peroral infection, but also develop key features of acute campylobacteriosis such as wasting and bloody diarrhea within 1 week (15). One major reason for these severe immunopathological responses mounting in acute ulcerative enterocolitis is the absence of colonization resistance and the lack of interleukin-10 (IL-10) providing murine resistance to *C. jejuni* LOS (16, 17). In consequence, *C. jejuni* infected IL-10$^{-/-}$ mice display pronounced LOS induced and Toll-like receptor-4 (TLR-4) dependent innate and adaptive immune responses that are not restricted to the intestinal tract, but can also be observed in extra-intestinal including systemic compartments (15, 18–25).

Vitamin D has primarily been known for its regulatory properties in bone metabolism due to the tight control of calcium reabsortion in the intestinal tract and in bone remodeling (26). After exposure to ultraviolet (UV) B light the steroid hormone is produced in the skin from 7-dehydroxy-cholesterol followed by hydroxylation steps in the liver and the kidneys to the biologically active forms 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D, respectively (27). After ingestion of food or supplements, circulating 25-hydroxy-vitamin D can be utilized by many cells including immune cells and intestinal intraepithelial cells expressing the 1α-hydroxylase enzyme CYP27B, whereas 24-hydroxylase CYP24A exerts counter-regulatory properties subsequently providing local 1,25-dihydroxy-vitamin D sources in a well-balanced fashion (27).

The identification of the vitamin D receptor (VDR) on peripheral blood mononuclear cells in the 1980s first pointed to immune-related functions of vitamin D (28, 29). In fact, vitamin D has been shown to be involved in modulating both, innate and adaptive immune responses (30–33) and to exert anti-inflammatory effects (34). Furthermore, several reports underline the anti-microbial properties of vitamin D (33). For instance, vitamin D could effectively inhibit the growth of Gram-positive bacterial strains such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus mutans*, but also of Gram-negative species including *Klebsiella pneumoniae* and *Escherichia coli* (35–37). In addition, the production of antimicrobial peptides such as cathelicidin and defensins are stimulated by vitamin D (38–40). Both, immune-modulatory and antimicrobial effects might be responsible for the beneficial effects of exogenous vitamin D observed in infectious morbidities caused by *Helicobacter pylori* (41) and in respiratory tract infections (42). Moreover, vitamin D has been shown to be involved in maintenance of the intestinal epithelial barrier integrity (43).

This prompted us in our present preclinical intervention study to investigate potential pathogen-lowering, immuno-modulatory, intestinal epithelial barrier preserving and hence, disease-alleviating effects of synthetic 25-OH-cholecalciferol applying a clincial model of acute campylobacterosis.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted in accordance with the European Guidelines for animal welfare (2010/63/EU) following approval by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0172/16 and G0247/16). Twice a day clinical conditions of mice were assessed.

Generation of Secondary Abiotic Mice

Female and male IL-10$^{-/-}$ mice (all in C57BL/6j background) were bred and reared under specific pathogen free (SPF) conditions in the same unit of the Forschungseinrichtungen

Abbreviations: CBA, Cytometric Bead Array; CFU, colony-forming units; HPF, High power fields; IFN, interferon; IL, interleukin; LOS, Lipo-oligosaccharide; LPS, Lipo-polysaccharide; MCP-1, monocyte chemoattractant protein 1; MLN, mesenteric lymph nodes; PBS, phosphate buffered saline; p.i., post-infection; PLC, Placebo; Rt, Transmural electrical resistance; SPF, specific pathogen free; Th, T helper cell; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cells; UV, ultraviolet; VDR, vitamin D receptor; VitD, Vitamin D.

für Experimentelle Medizin (FEM, Charité–University Medicine Berlin). Three to five mice were maintained in one cage including filter tops within an experimental semi-barrier (accessible only with lab coat, overshoes, caps, and sterile gloves) under standard conditions (22–24°C room temperature, 55 ± 15% humidity, 12 h light/12 dark cycle) and had free access to autoclaved standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany).

In order to assure stable gastrointestinal *C. jejuni* colonization and to override physiological colonization resistance (44), microbiota-depleted (i.e., secondary abiotic) mice were generated (44, 45). In brief, immediately post-weaning 3-week old mice were subjected to a 10-week course of broad-spectrum antibiotic treatment by adding ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany) and metronidazole (1 g/L; Fresenius, Germany) to the autoclaved drinking water (*ad libitum*) as described elsewhere (45). To assure antibiotic washout, the antibiotic cocktail was withdrawn 4 days prior infection and thus immediately before start of the vitamin D treatment.

Vitamin D Treatment

Vitamin D treatment started 4 days before *C. jejuni* infection. Therefore, synthetic 25-OH-cholecalciferol (purchased from Sigma-Aldrich, München, Germany) was dissolved in Tween 80 (0.2% v/v) and administered to mice via the autoclaved tap water (*ad libitum*). Considering a body weight of ∼25 g per mouse and a daily drinking volume of ∼5 mL, the final concentration of the synthetic 25-OH-cholecalciferol solution was 2.5 μg/mL resulting in a daily treatment dosage of 500 μg per kg body weight (equivalent to 20,000 IU per kg) (46). Hence, the applied daily vitamin D dose was far beyond the toxic doses defined for rodents (i.e., 42 mg/kg/day) (47, 48) and humans (i.e., 150 mg/kg/day) (49). Age and sex matched placebo (PLC) control mice received vehicle (i.e., Tween 80) via the drinking water (*ad libitum*).

C. jejuni Infection, Gastrointestinal Colonization, and Translocation

For infection, a stock solution of *C. jejuni* 81-176 strain that had been stored at −80°C was thawed, aliquots streaked onto karmali agar (Oxoid, Wesel, Germany) and incubated in a microaerophilic atmosphere at 37°C for 48 h. Immediately before peroral infection of mice, bacteria were harvested in sterile PBS (Oxoid) to a final inoculum of 10^9 bacterial cells. Mice (3 months of age) were perorally infected on two consecutive days (i.e., days 0 and 1). Animals were continuously maintained in a sterile environment (autoclaved food and drinking water) and handled under strict aseptic conditions to prevent from contaminations.

In order to assess gastrointestinal colonization and translocation, *C. jejuni* were quantitatively assessed in fecal samples over time post-infection (p.i.) and furthermore, in luminal samples derived from distinct parts of the gastrointestinal tract (i.e., from the stomach, duodenum, ileum, and colon) and in organ homogenates at day 6 p.i. by culture as stated elsewhere (44, 50). The detection limit of viable pathogens was ≈100 CFU per g (CFU/g). To assess *C. jejuni* bacteremia, thioglycollate enrichment broths (BD Bioscience, Germany) were inoculated with ∼200 μL cardiac blood of individual mice, incubated for 7 days at 37°C, and streaked onto respective media for further identification as described (44).

Clinical Conditions

Before and after *C. jejuni* infection the clinical conditions of mice were assessed on a daily basis by using a standardized cumulative clinical score (maximum 12 points), addressing the clinical aspect/wasting (0: normal; 1: ruffled fur; 2: less locomotion; 3: isolation; 4: severely compromised locomotion, pre-final aspect), the abundance of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), and diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces) as described earlier (19).

Sampling Procedures

At day 6 p.i., mice were sacrificed by isofluran inhalation (Abbott, Germany). Luminal gastrointestinal samples (from stomach, duodenum, ileum, and colon) and *ex vivo* biopsies from colon, ileum, mesenteric lymph nodes (MLN), spleen, liver, kidneys, and lungs were taken under sterile conditions. For serum cytokine measurements cardiac blood was taken. Colonic and extra-intestinal samples were collected from each mouse in parallel for microbiological, immunohistopathological, and immunological analyses. The absolute colonic and small intestinal lengths were measured with a ruler (in cm).

Immunohistochemistry

In situ immunohistochemical analyses were performed in colonic *ex vivo* biopsies that had been immediately fixed in 5% formalin and embedded in paraffin as described earlier (51–54). In brief, in order to detect apoptotic epithelial cells, proliferation epithelial cells, macrophages/monocytes, T lymphocytes, and regulatory T cells (Tregs), 5 μm thin paraffin sections of *ex vivo* biopsies were stained with primary antibodies directed against cleaved caspase 3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), F4/80 (# 14-4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50), CD3 (#N1580, Dako, 1:10), and FOXP3 (clone FJK-165, #14-5773, eBioscience, 1:100), respectively. Positively stained cells were then examined by light microscopy (magnification 100× and 400×), and for each mouse the average number of respective positively stained cells was determined within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) by a blinded independent investigator.

Inflammatory Mediator Detection in Supernatants of Intestinal and Extra-Intestinal *ex vivo* Biopsies

Colonic *ex vivo* biopsies were cut longitudinally, washed in phosphate buffered saline (PBS; Gibco, Life Technologies, UK), and strips of ∼1 cm^2 tissue and *ex vivo* biopsies derived from MLN (3–4 lymph nodes), liver, and spleen (one half) were placed in 24-flat-bottom well-culture plates (Nunc, Germany)

containing 500 µL serum-free RPMI 1640 medium (Gibco, life technologies, UK) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL; PAA Laboratories, Germany). After 18 h at 37°C, respective culture supernatants as well as serum samples were tested for IL-6, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences). Systemic pro-inflammatory cytokine concentrations were measured in serum samples.

Electrophysiological Measurements

Distal colonic *ex vivo* biopsies were mounted unstripped in Ussing chambers (0.049 cm^2 area). Transmural electrical resistance (Rt) was recorded under voltage clamp conditions by an automatic clamp device (CVC6, Fiebig Hard and Software, Berlin, Germany) at 37°C over 1 h. The bathing solution was composed of NaCl (113.6 mmol/L), $NaHCO_3$ (21.0 mmol/L), KCl (5.4 mmol/L), Na_2HPO_4 (2.4 mmol/L), $MgCl_2$ (1.2 mmol/L), $CaCl_2$ (1.2 mmol/L), NaH_2PO_4 (0.6 mmol/L), D(+)-glucose (10.0 mmol/L), D(+)-mannose (10.0 mmol/L), beta-hydroxybutyric acid (0.5 mmol/L), and L-glutamine (2.5 mmol/L) equilibrated with carbogen gas (pH 7.4).

Statistical Analysis

Medians and levels of significance were determined using Mann-Whitney test (GraphPad Prism v7, USA) for pairwise comparisons of not normally distributed data, and using the one-sided ANOVA with Tukey post-correction or the Kruskal-Wallis test with Dunn's post-correction for multiple comparisons as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were performed in a blinded fashion and reproduced three times.

RESULTS

Intestinal Pathogen Loads Over Time Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Secondary abiotic IL-$10^{-/-}$ mice were subjected to synthetic 25-OH-cholecalciferol treatment via the drinking water starting 4 days before *C. jejuni* infection. On two consecutive days, namely days 0 and 1, mice were then perorally challenged with 10^9 viable pathogens by gavage. Daily cultural analyses of fecal samples revealed that 25-OH-cholecalciferol application did not affect pathogenic intestinal colonization properties as indicated by stable median fecal *C. jejuni* loads of 10^9 CFU/g over time p.i. that did not differ between both cohorts at respective time points (n.s.; **Figure S1**). Upon necropsy, luminal gastrointestinal *C. jejuni* densities did not differ between 25-OH-cholecalciferol and placebo treated mice as determined in stomach, duodenum, ileum and colon at day 6 post-infection (n.s.; **Figure 1**). Hence, synthetic 25-OH-cholecalciferol treatment did not affect gastrointestinal *C. jejuni* loads.

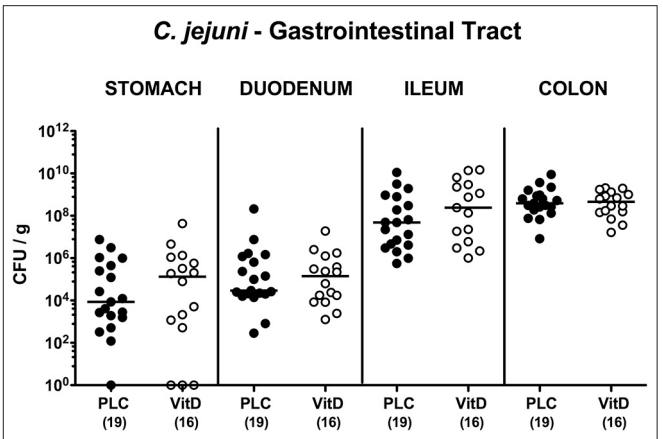

FIGURE 1 | Gastrointestinal *C. jejuni* loads following vitamin D treatment of infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), luminal *C. jejuni* loads were quantitatively assessed from each mouse in distinct gastrointestinal compartments as indicated by culture and expressed in colony forming units per g (CFU/g). Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Comprehensive Survey of Clinical Conditions Over Time Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Within 6 days following *C. jejuni* infection mice from either cohort developed comparably severe symptoms of acute enterocolitis as daily quantitated applying a standardized cumulative clinical scoring system (**Figure S2**) assessing wasting symptoms, abundance of fecal blood, and the severity of diarrhea. Whereas overall pathogen-induced clinical symptoms were comparable between the two cohorts over time (n.s.; **Figure S2**), cumulate relative frequencies of diarrhea were lower in 25-OH-cholecalciferol treated mice as compared to placebo controls as early as 24 h following the latest infection (i.e., day 2 p.i.) until 4 p.i. (**Figure 2**). Hence, synthetic 25-OH-cholecalciferol treatment results in less frequent *C. jejuni* induced diarrhea in the midst of infection.

Macroscopic and Microscopic Inflammatory Sequelae Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Given that intestinal inflammation is association with a significant shortening of the affected part of the intestinal tract (15, 45), we measured the lengths of both, the small and large intestines upon necropsy. In fact, *C. jejuni* infection was accompanied with shorter colons of placebo as well as of 25-OH-cholecalciferol treated mice ($p < 0.001$; **Figure S3A**), whereas the small intestinal lengths were virtually unaffected at day 6 p.i. (n.s.;

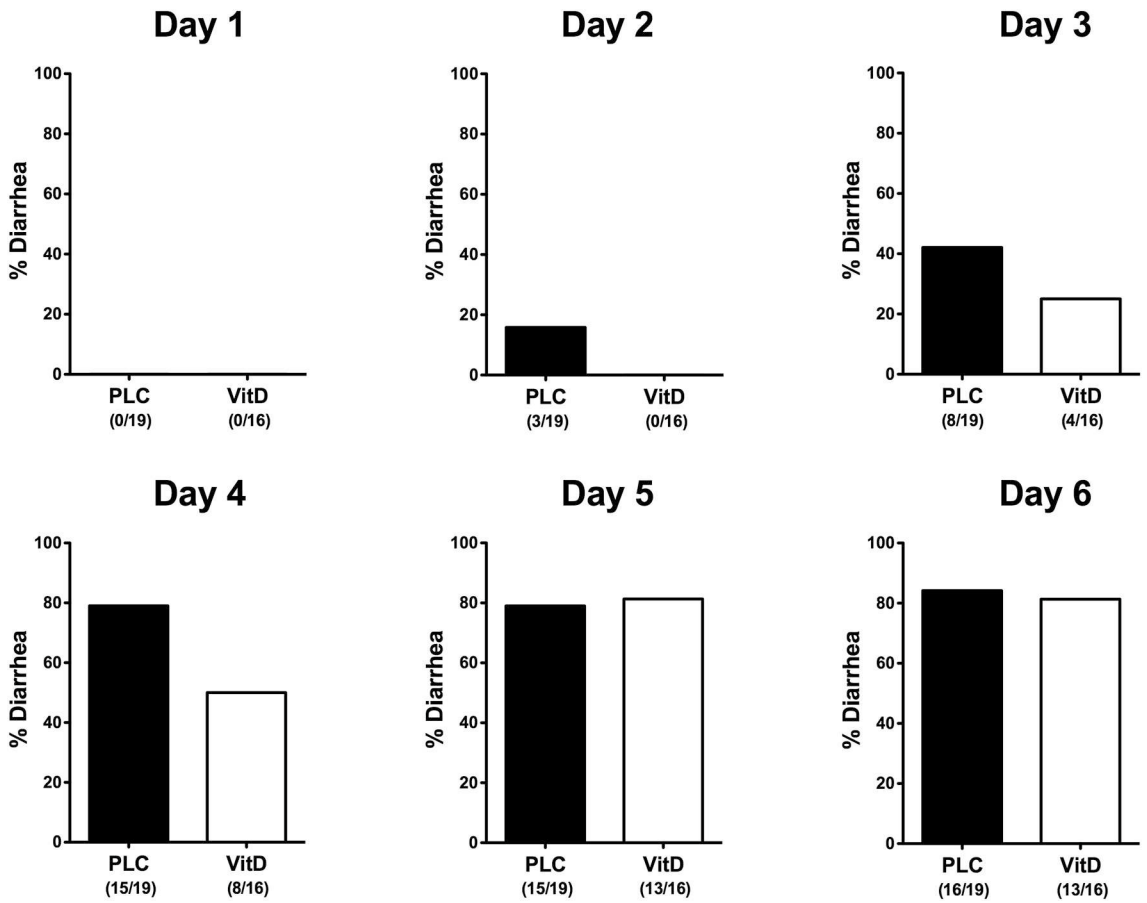

FIGURE 2 | Diarrhea frequencies over time following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, white bars) or placebo (PLC, black bars) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Occurrence of diarrhea was assessed in each mouse from day 0 until day 6 post-infection as indicated applying a standardized clinical scoring system (see Materials and Methods). Bars indicate the cumulative frequencies of diarrhea (in %). Numbers of diarrheal mice out of the total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Figure S3B). Hence, synthetic 25-OH-cholecalciferol treatment does not ameliorate *C. jejuni* induced macroscopic disease.

Since apoptosis is regarded a reliable parameter for the grading of intestinal inflammation (44), we further quantitatively assessed caspase3$^+$ apoptotic epithelial cells in large intestinal *ex vivo* biopsies applying *in situ* immunohistochemistry. At day 6 p.i., *C. jejuni* infected mice exhibited multifold increased numbers of apoptotic cells in their colonic epithelia ($p < 0.001$), that were, however, more than 60% lower in 25-OH-cholecalciferol as compared to placebo treated mice ($p < 0.05$; **Figure 3A**, **Figure S4A**). Conversely, numbers of Ki67$^+$ colonic epithelial cells indicative for cell proliferation and regeneration increased upon *C. jejuni* infection ($p < 0.001$), but more distinctly following 25-OH-cholecalciferol as compared to placebo treatment ($p < 0.05$; **Figure 3B**, **Figure S4B**). Hence, synthetic 25-OH-cholecalciferol treatment dampens *C. jejuni* induced apoptotic cell responses and promotes cell regenerative measures counteracting intestinal cell damage upon pathogenic exposure.

Intestinal Immune Cell Responses Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We further quantitatively surveyed both, innate and adaptive immune cell responses in the large intestinal tract following synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice by immunohistochemical staining of colonic paraffin sections. As early as 6 days upon *C. jejuni* infection, numbers of F4/80$^+$ innate immune cell subsets including macrophages and monocytes had increased in the large intestinal mucosa and lamina propria ($p < 0.001$), but less distinctly in 25-OH-cholecalciferol as compared to placebo challenged mice ($p < 0.01$; **Figure 4A**, **Figure S4C**). Similarly, *C. jejuni* induced increases in adaptive immune cells such as CD3$^+$ lymphocytes, were less pronounced in the 25-OH-cholecalciferol vs. placebo cohort at day 6 p.i. ($p < 0.05$, VitD vs. PLC; **Figure 4B**, **Figure S4D**). Interestingly, numbers of FOXP3$^+$ regulatory T cells (Treg) were slightly higher following vitamin D as

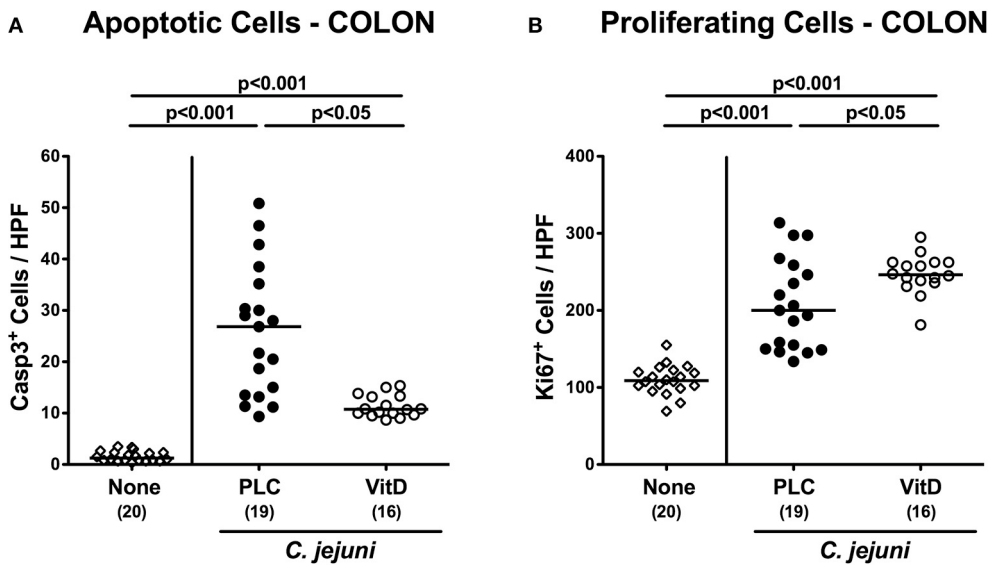

FIGURE 3 | Colonic epithelial cell apoptosis and cell proliferation/regeneration following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the average numbers of colonic epithelial **(A)** apoptotic ($Casp3^+$) and **(B)** proliferating ($Ki67^+$) cells were assessed microscopically from six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained colonic paraffin sections. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction or the one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

compared to placebo treated *C. jejuni* infected mice ($p < 0.01$; **Figure 4C**, **Figure S4E**). Hence, synthetic 25-OH-cholecalciferol treatment results in less pronounced *C. jejuni* induced intestinal responses of distinct innate and adaptive immune cell populations.

Intestinal Pro-inflammatory Mediator Secretion Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We next measured pro-inflammatory mediators in intestinal *ex vivo* biopsies. At day 6 following *C. jejuni* infection increased IL-6 and MCP-1 concentrations could be assessed in the colon of placebo ($p < 0.01$ and $p < 0.05$, respectively), but not 25-OH-cholecalciferol treated mice (**Figures 5A,B**). *C. jejuni* induced increases in large intestinal TNF and IFN-γ concentrations ($p < 0.05$–0.001 vs. none), however, were unaffected by 25-OH-cholecalciferol challenge (n.s. vs. PLC; **Figures 5C,D**). In line, ileal IL-6 and MCP-1 as well as IFN-γ levels were elevated upon *C. jejuni* infection of mice from the placebo ($p < 0.05$–0.01), but not from the 25-OH-cholecalciferol cohort (**Figures 6A,B,D**), whereas like in the colon, ileal TNF concentrations were comparably elevated at day 6 post-infection of either cohort ($p < 0.001$; **Figure 6C**). Hence, synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice results in less pronounced secretion of distinct pro-inflammatory mediators in the intestinal tract.

Extra-Intestinal Inflammatory Immune Responses Following Vitamin D Treatment of *C. jejuni* Infected Mice

We further asked whether the 25-OH-cholecalciferol mediated anti-inflammatory effects were restricted to the intestinal tract or also effective in extra-intestinal compartments. In fact, IFN-γ concentrations were lower in MLN and liver of 25-OH-cholecalciferol as compared to placebo treated mice at day 6 p.i. ($p < 0.05$; **Figures 7A,B**). Interestingly, *C. jejuni* infection resulted in decreased IFN-γ secretion in splenic *ex vivo* biopsies irrespective of the treatment regimen ($p < 0.001$; **Figure 7C**). Hence, synthetic 25-OH-cholecalciferol treatment of *C. jejuni* infected mice resulted in less distinct IFN-γ secretion in MLN and liver.

Systemic Pro-inflammatory Mediator Secretion Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We next addressed whether synthetic 25-OH-cholecalciferol treatment might alleviate systemic *C. jejuni* induced pro-inflammatory immune responses. At day 6 p.i., mice from either cohort exhibited comparably elevated IL-6, MCP1, TNF, and IFN-γ serum concentrations ($p < 0.001$ vs. none; **Figure S5**). Hence, synthetic 25-OH-cholecalciferol treatment does not affect *C. jejuni* induced systemic pro-inflammatory mediator secretion.

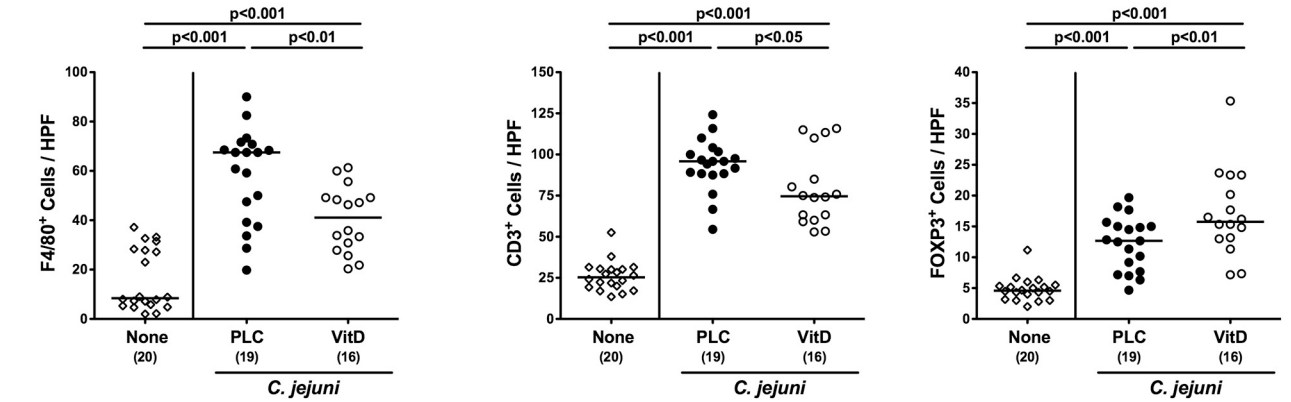

FIGURE 4 | Colonic immune cell responses following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the average numbers of **(A)** macrophages and monocytes (F4/80^{+}), **(B)** T lymphocytes (CD3^{+}) and **(C)** regulatory T cells (FOXP3+) were assessed microscopically from six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained colonic paraffin sections. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Bacterial Translocation Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

We further asked whether synthetic 25-OH-cholecalciferol treatment had an impact of the translocation rates of viable pathogens from the infected intestines to extra-intestinal including systemic tissue sites. Whereas, *C. jejuni* could be cultured at similar frequencies from MLN, liver and lungs derived from 25-OH-cholecalciferol and placebo treated mice (**Figures 8A–C**), cumulative pathogenic translocation rates were lower in the kidneys (12.5 vs. 31.6%) and the spleen (12.5 vs. 26.3%) taken from the former as compared to the latter at day 6 p.i. (**Figures 8D,E**). Notably, all blood cultures remained *C. jejuni* negative (**Figure 8F**). Hence, synthetic 25-OH-cholecalciferol treatment was associated with lower cumulative translocation rates of *C. jejuni* originating from the inflamed intestines to the kidneys and the spleen.

Colonic Epithelial Barrier Changes Following Vitamin D Treatment of *C. jejuni* Infected Mice With Acute Enterocolitis

Given the lower cumulative pathogenic translocation rates we assessed whether synthetic 25-OH-cholecalciferol treatment resulted in a less compromised colonic epithelial barrier function in *C. jejuni* infected mice. Therefore, we performed electrophysiological resistance measurements of colonic *ex vivo* biopsies in the Ussing chamber. In fact, transmural resistances were lower in the large intestines derived from placebo, but not 25-OH-cholecalciferol treated mice at day 6 p.i. as compared to uninfected and untreated control animals ($p < 0.05$; **Figure 9**). Hence, synthetic 25-OH-cholecalciferol treatment results in uncompromised colonic epithelial barrier function following *C. jejuni* infection.

DISCUSSION

Due to the pleiotropic beneficial effects of vitamin D in health and disease, the application of vitamin D as safe dietary supplement is currently discussed as promising option for the adjunct treatment and prophylaxis of various immunopathological morbidities including infectious diseases, intestinal inflammatory conditions, and cancer, for instance (33, 55). In our present vitamin D intervention study applying a clinical acute campylobacteriosis model, prophylactic synthetic 25-OH-cholecalciferol application starting 4 days prior murine infection resulted in dampened *C. jejuni* induced intestinal and extra-intestinal inflammatory sequalae, but could not lower the high intestinal pathogen loads of more than 10^9 viable *C. jejuni* per g feces. In support, recent reports revealed that the beneficial effects of vitamin D during gastrointestinal infection with distinct bacterial species such as *Salmonella* (56) or *Listeria monocytogenes* (57) are rather due to the pleiotropic immuno-modulatory than direct anti-microbial properties of the steroid hormone. In addition, one needs to take into consideration, that, in contrary to humans, the expression of the antimicrobial peptide cathelicidin in mice is not regulated by vitamin D, given that in the murine cathelicidin gene promoter the vitamin D response element is missing (58, 59). This could explain our observation that external 25-OH-cholecalciferol application, even in high doses, did not reduce intestinal *C. jejuni* burdens. However, it is tempting to speculate that this could be the case in humans.

Despite the high intestinal pathogenic burdens, 25-OH-cholecalciferol treated mice suffered less frequently from diarrhea

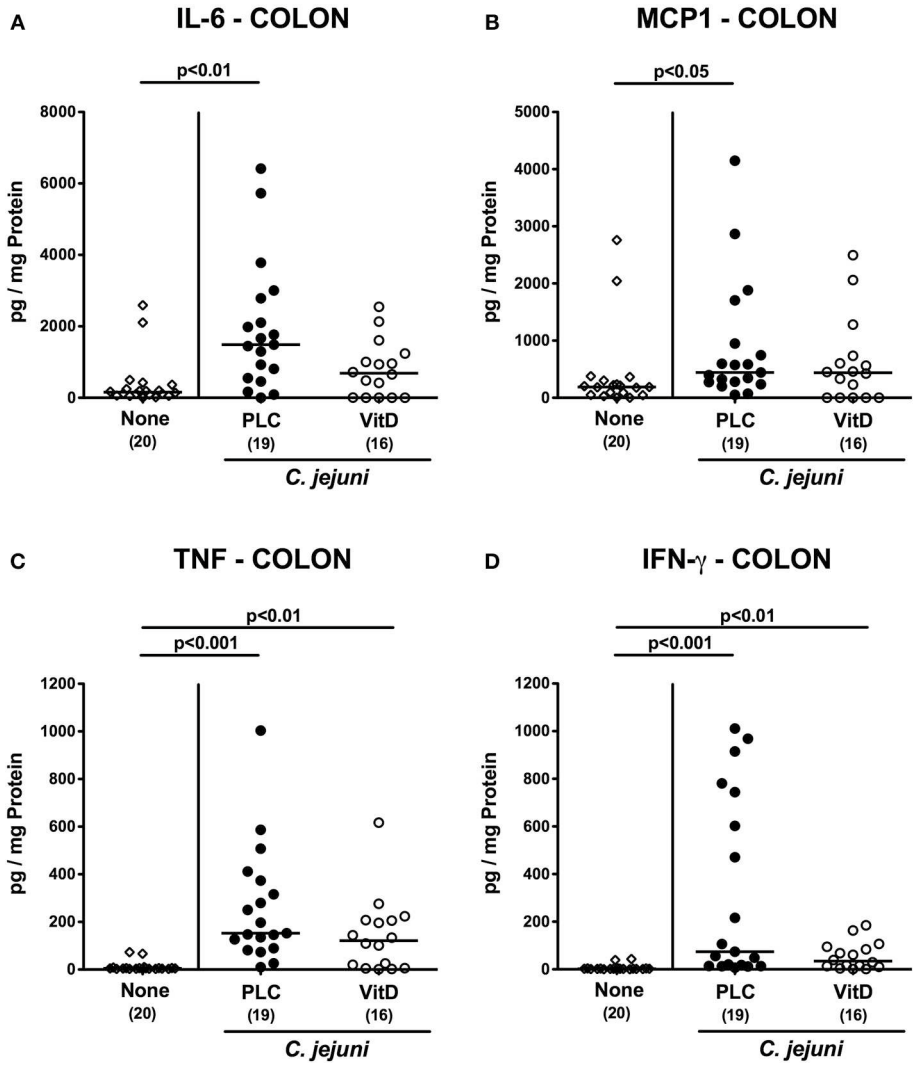

FIGURE 5 | Colonic secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in supernatants derived from colonic *ex vivo* biopsies. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

in the midst of campylobacteriosis development as compared to placebo controls, but exhibited comparable macroscopic disease at the end of the observation period. Notably, the macroscopic outcome particularly in such a non-selflimiting detrimental intestinal infection and inflammation model is due to the sum effect of many different intestinal, extra-intestinal and systemic events within this hyper-inflammatory scenario (24). It is therefore remarkable, that less distinct *C. jejuni* induced apoptosis of colonic epithelial cells, whereas, conversely, large intestinal cell regenerative properties counteracting pathogen-induced cell damage were promoted upon 25-OH-cholecalciferol application in mice suffering from acute enterocolitis. In support, the intestinal epithelial vitamin D receptor has been shown to regulate mucosal inflammation by suppressing intestinal epithelial cell apoptosis (60). Less severe colonic apoptosis upon 25-OH-cholecalciferol treatment was accompanied by less distinct immune cell responses upon *C. jejuni* infection, which is supported by several studies showing that vitamin D regulates both, innate and adaptive immunity (61–63). In our study, lower numbers of innate immune cell populations such as macrophages and monocytes could be assessed in the colonic mucosa and lamina propria of *C. jejuni* infected mice that had been pretreated with synthetic 25-OH-cholecalciferol. In line, recent reports revealed that vitamin D stimulation of antigen presenting cells including macrophages and dendritic cells resulted in decreased pro-inflammatory mediator secretion

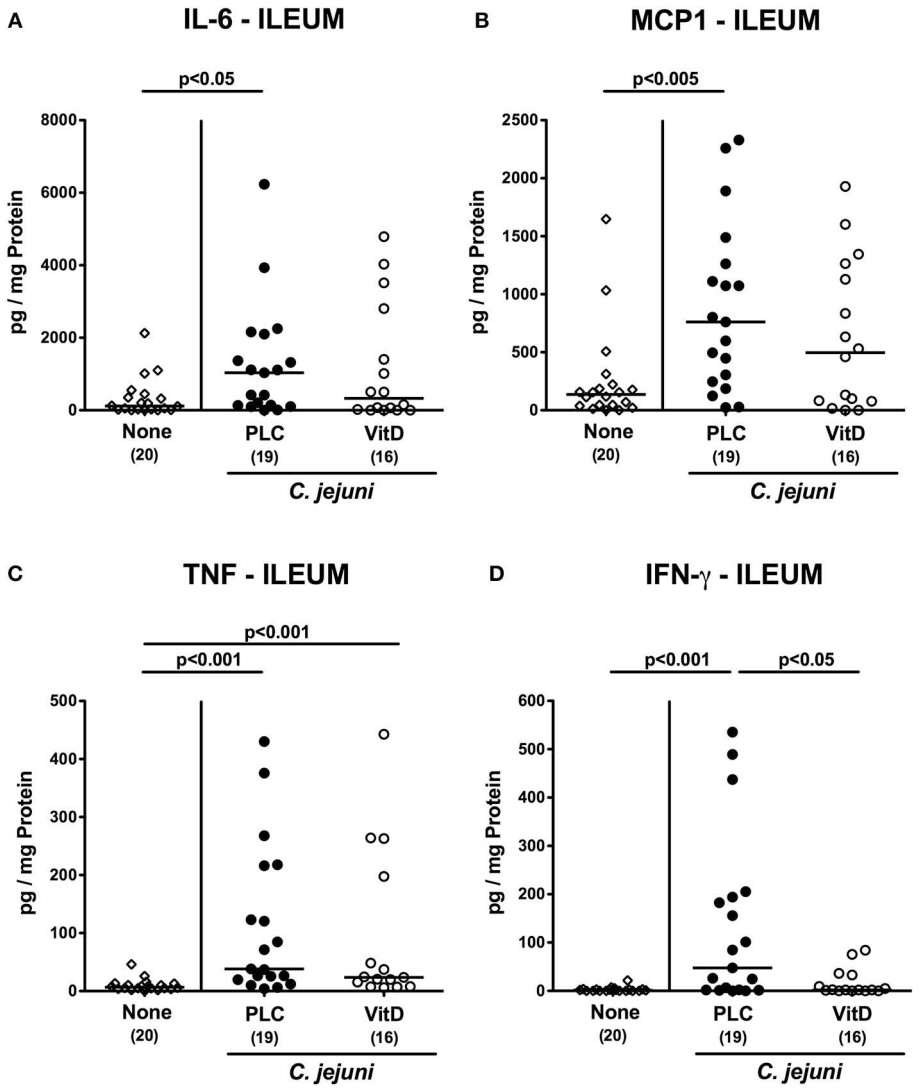

FIGURE 6 | Ileal secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in supernatants derived from ileal *ex vivo* biopsies. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

(59, 64). In addition, colonic mucosal numbers of T lymphocytes were lower in 25-OH-cholecalciferol as compared to placebo treated mice with *C. jejuni* induced enterocolitis. In fact, T cells have been shown to be direct and indirect targets of vitamin D (65, 66). Previous *in vitro*, *ex vivo,* and *in vivo* studies revealed that vitamin D treatment of T cells and of mice resulted in less distinct T cell proliferation and in decreased T helper cell (Th)−1 dependent secretion of pro-inflammatory cytokines and subsequently in ameliorated inflammation (66, 67). In our present study, the colonic concentrations of pro-inflammatory mediators including IL-6 and MPC-1 measured in 25-OH-cholecalciferol pretreated, *C. jejuni* infected mice were comparable to those obtained from naive controls. In support, vitamin D was shown to reduce recruitment of innate immune cells such as monocytes and to decrease IL-6 and MCP-1 releases upon *in vitro* stimulation (68). Notably, the 25-OH-cholecalciferol associated decreased pro-inflammatry mediator secretion was not restricted to the large intestines, the major predilection site of *C. jejuni* induced enterocolitis (15, 69). In fact, *C. jejuni* induced increased secretion of IL-6, MCP-1, and additionally of IFN-γ could be observed in the terminal ileum of mice from the placebo, but not from the 25-OH-cholecalciferol treatment cohort. Interestingly, as opposed to 25-OH-cholecalciferol related decreases in large intestinal T cell

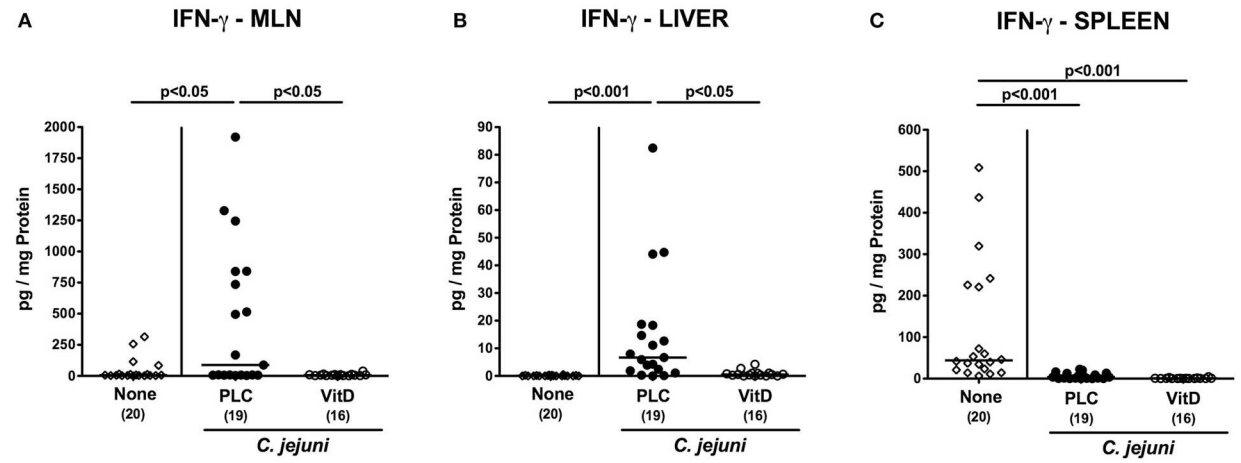

FIGURE 7 | Extra-intestinal IFN-γ secretion following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), IFN-γ concentrations were determined in supernatants of *ex vivo* biopsies derived from **(A)** mesenteric lymph nodes (MLN), **(B)** liver, and **(C)** spleen. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

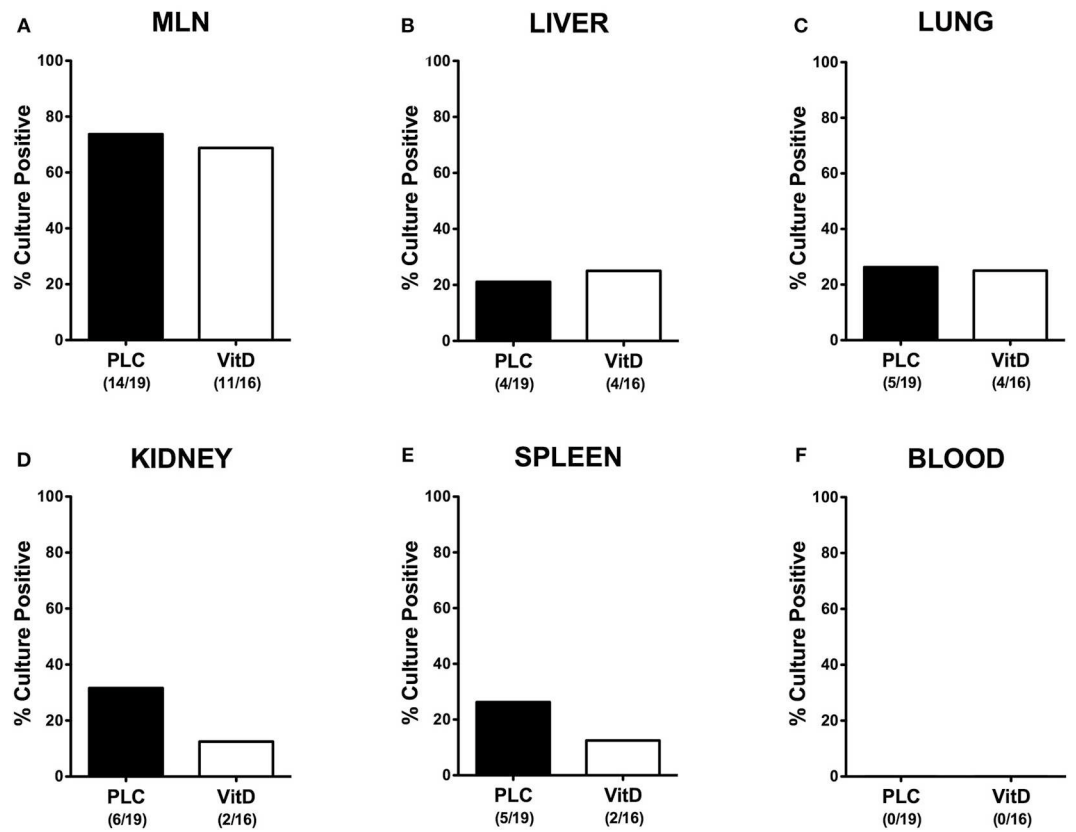

FIGURE 8 | Bacterial translocation following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, white bars) or placebo (PLC, black bars) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Upon necropsy (at day 6 post-infection), the abundance of viable pathogens was assessed in *ex vivo* biopsies taken from **(A)** mesenteric lymph nodes (MLN), **(B)** liver, **(C)** lung, **(D)** kidney, **(E)** spleen, and **(F)** cardiac blood by culture. The cumulative relative translocation rates of *C. jejuni* into the respective compartment out of four independent experiments are indicated in %. The numbers of culture-positive mice out of the total numbers of analyzed animals are given in parentheses.

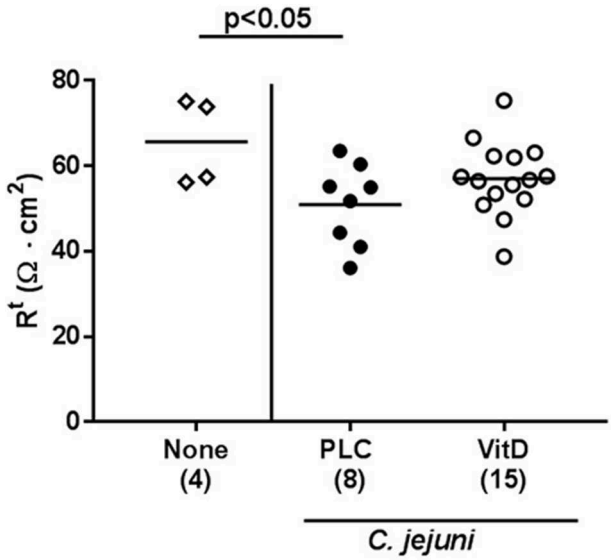

FIGURE 9 | Colonic transmural electrical resistance following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-$10^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the transmural electrical resistance of distal colon was measured in Ussing chambers as described in Materials and Methods. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from two independent experiments.

numbers, higher numbers of (potentially anti-inflammatory) $FOXP3^+$ regulatory T cells could be assessed in the colonic mucosa and lamina propria of 25-OH-cholecalciferol vs. placebo treated mice with enterocolitis. In support, recent studies reported that vitamin D results in enhanced recruitment of regulatory T cells to inflamed tissue sites (70–72). Given that we did not perform co-staining analyses in our present study, however, we can not answer which specific immune cell subset was expressing FOXP3.

Remarkably, the pro-inflammatory immune response-dampening effects of exogenous 25-OH-cholecalciferol were not restricted to the intestinal tract, but were also effective in extra-intestinal compartments given that *C. jejuni* induced IFN-γ secretion was less pronounced in MLN draining the inflamed intestines and in the liver upon 25-OH-cholecalciferol treatment. In line, previous studies provide evidence that vitamin D application or even skin exposure to UV light could ameliorate or prevent from liver inflammation due to vitamin D mediated dampening of immune cellular responses and inhibition of liver apoptosis, for instance (73, 74).

At the first glance unexpectedly, *C. jejuni* infection was associated with decreases in splenic IFN-γ concentrations in either cohort. One possible explanation might be that upon pathogenic infection leukocytes were recruited from the spleen to the site of infection in order to limit pathogenic spread. One could have expected an even more prominent effect following synthetic 25-OH-cholecalciferol application due to the known immune cell recruiting properties of vitamin D (75).

C. jejuni infection results in impaired epithelial barrier function *in vitro* (76) and campylobacteriosis is characterized by a leaky gut syndrome facilitating pathogenic translocation from the inflamed intestines to extra-intestinal including systemic compartments (15, 69). Given that vitamin D has been shown to preserve epithelial barrier function (75), we assessed potential 25-OH-cholecalciferol mediated effects on pathogenic translocation frequencies in our preclinical survey. In fact, when taking results of the four independent experiment together, *C. jejuni* could be cultured less frequently from the kidneys and the spleen of infected mice following 25-OH-cholecalciferol as compared to placebo treatment, whereas cumulative relative *C. jejuni* translocation rates to MLN, liver and lungs were comparable. Of note, all blood cultures remained *C. jejuni* negative, irrespective of the treatment regimen. One needs to take into consideration, however, that soluble bacterial molecules including LOS and others might have been transported via the circulation contributing to the observed extra-intestinal collateral damages of *C. jejuni* infection. Nevertheless, the observed inflammation-alleviating effects upon 25-OH-cholecalciferol application were further accompanied by a less compromised colonic epithelial barrier function in 25-OH-cholecalciferol as compared to placebo treated, *C. jejuni* infected mice. This in turn very likely reduced the risk of spread of both, viable bacteria and soluble bacterial molecules in the former vs. the latter. We therefore hypothesize that the 25-OH-cholecalciferol associated anti-inflammatory effects in particular prevent from further bacteria-induced damages in this acute *C. jejuni* induced inflammation model.

CONCLUSION

Our preclinical intervention study provides evidence that prophylactic peroral synthetic 25-OH-cholecalciferol application dampens intestinal and extra-intestinal inflammatory responses during acute campylobacteriosis in the clinical mouse model applied here. Further studies are needed in order to define appropriate vitamin D doses for the prevention and combat of distinct gastrointestinal infectious morbidities in humans.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

SM performed experiments, analyzed data, and co-wrote paper. FL and RB performed experiments, analyzed data, and co-edited paper. J-DS and SB provided advice in experimental design, critically discussed results, and co-edited paper. MH designed and performed experiments, analyzed data, and wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02094/full#supplementary-material

Figure S1 | Fecal *C. jejuni* loads over time following vitamin D treatment of infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with **(A)** placebo (closed circles) or **(B)** synthetic 25-OH-cholecalciferol (vitamin D, open circles) via the drinking water starting four days before peroral *C. jejuni* 81-176 strain infection on day (d) 0 and d1. Fecal *C. jejuni* loads were quantitatively assessed from each mouse on a daily basis post-infection (p.i.) by culture and expressed in colony forming units per g (CFU/g). Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S2 | Kinetic survey of overall clinical conditions following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with **(A)** placebo (closed circles) or **(B)** synthetic 25-OH-cholecalciferol (vitamin D, open circles) via the drinking water starting four days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Clinical symptoms were quantitatively assessed applying a standardized clinical scoring system from d0 until d6 post-infection (see Materials and Methods). Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S3 | Intestinal lengths following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), the absolute lengths of the **(A)** colon and **(B)** small intestines were measured with a ruler. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by one-sided ANOVA test with Tukey post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

Figure S4 | Representative photomicrographs illustrating apoptotic and proliferating epithelial as well as immune cells responses in large intestines following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D) or placebo via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. Naive mice served as uninfected and untreated controls. Photomicrographs reepresentative for four independent experiments illustrate the average numbers of **(A)** apoptotic epithelial cells (Casp3+), **(B)** proliferating epithelial cells, **(C)** macrophages and monocytes (F4/80+), **(D)** T lymphocytes (CD3+) and **(E)** regulatory T cell (Treg, FOXP3+) in at least six high power fields (HPF) as quantitatively assessed in ileal paraffin sections applying *in situ* immunohistochemistry at day 6 post-infection (100× magnification, scale bar 100 μm).

Figure S5 | Systemic secretion of pro-inflammatory mediators following vitamin D treatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were treated with synthetic 25-OH-cholecalciferol (vitamin D, VitD, open circles) or placebo (PLC, closed circles) via the drinking water starting 4 days before peroral *C. jejuni* 81-176 strain infection on days 0 and 1. At necropsy (i.e., day 6 post-infection), **(A)** IL-6, **(B)** MCP1, **(C)** TNF, and **(D)** IFN-γ concentrations were determined in serum samples. Uninfected and untreated mice (none, open diamonds) served as negative control animals. Medians (black bars), levels of significance (*p*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments.

REFERENCES

1. Young KT, Davis LM, Dirita VJ. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol*. (2007) 5:665–79. doi: 10.1038/nrmicro1718
2. Guerry P, Szymanski CM. *Campylobacter sugars* sticking out. *Trends Microbiol*. (2008) 16:428–35. doi: 10.1016/j.tim.2008.07.002
3. Lane JA, Mehra RK, Carrington SD, Hickey RM. The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol*. (2010) 142:1–13. doi: 10.1016/j.ijfoodmicro.2010.05.027
4. Hermans D, Pasmans F, Messens W, Martel A, Van Immerseel F, Rasschaert G, et al. Poultry as a host for the zoonotic pathogen *Campylobacter jejuni*. *Vector Borne Zoonotic Dis*. (2012) 12:89–98. doi: 10.1089/vbz.2011.0676
5. Janssen R, Krogfelt KA, Cawthraw SA, van Pelt W, Wagenaar JA, Owen RJ. Host-pathogen interactions in *Campylobacter* infections: the host perspective. *Clin Microbiol Rev*. (2008) 21:505–18. doi: 10.1128/CMR.00055-07
6. Havelaar AH, van Pelt W, Ang CW, Wagenaar JA, van Putten JP, Gross U, et al. Immunity to *Campylobacter*: its role in risk assessment and epidemiology. *Crit Rev Microbiol*. (2009) 35:1–22. doi: 10.1080/10408410802636017
7. O'Croinin T, Backert S. Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front Cell Infect Microbiol*. (2012) 2:25. doi: 10.3389/fcimb.2012.00025
8. Kist M, Bereswill S. *Campylobacter jejuni*. *Contrib Microbiol*. (2001) 8:150–65.
9. Backert S, Tegtmeyer N, Cróinín TÓ, Boehm M, Heimesaat MM. Chapter 1–Human campylobacteriosis. In Klein G, editor. *Campylobacter*. London: Academic Press (2017). p. 1–25.
10. van Spreeuwel JP, Duursma GC, Meijer CJ, Bax R, Rosekrans PC, Lindeman J. *Campylobacter colitis*: histological immunohistochemical and ultrastructural findings. *Gut*. (1985) 26:945–51. doi: 10.1136/gut.26.9.945
11. Walker RI, Caldwell MB, Lee EC, Guerry P, Trust TJ, Ruiz-Palacios GM. Pathophysiology of *Campylobacter enteritis*. *Microbiol Rev*. (1986) 50:81–94.
12. Allos BM. Association between *Campylobacter* infection and Guillain-Barre syndrome. *J Infect Dis.* (1997) 176(Suppl 2):S125–8. doi: 10.1086/513783
13. Mortensen NP, Kuijf ML, Ang CW, Schiellerup P, Krogfelt KA, Jacobs BC, et al. Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect*. (2009) 11:988–94. doi: 10.1016/j.micinf.2009.07.004
14. da Silva AMT, Kaulbach HC, Chuidian FS, Lambert DR, Suffredini AF, Danner RL. Shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. *N Engl J Med*. (1993) 328:1457–60.
15. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, et al. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10$^{-/-}$

mice via Toll-like-receptor-2 and−4 signaling. *PLoS ONE*. (2012) 7:e40761. doi: 10.1371/journal.pone.0040761
16. Robertson SA, Care AS, Skinner RJ. Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. *Biol Reprod*. (2007) 76:738–48. doi: 10.1095/biolreprod.106.056143
17. Warren HS, Fitting C, Hoff E, Adib-Conquy M, Beasley-Topliffe L, Tesini B, et al. Resilience to bacterial infection: difference between species could be due to proteins in serum. *J Infect Dis*. (2010) 201:223–32. doi: 10.1086/649557
18. Alutis ME, Grundmann U, Fischer A, Kuhl AA, Bereswill S, Heimesaat MM. Selective gelatinase inhibition reduces apoptosis and pro-inflammatory immune cell responses in *Campylobacter jejuni*-infected gnotobiotic IL-10 deficient mice. *Eur J Microbiol Immunol*. (2014) 4:213–22. doi: 10.1556/EUJMI-D-14-00031
19. Heimesaat MM, Alutis M, Grundmann U, Fischer A, Tegtmeyer N, Böhm M, et al. The role of serine protease HtrA in acute ulcerative enterocolitis and extra-intestinal immune responses during *Campylobacter jejuni* infection of gnotobiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2014) 4:77. doi: 10.3389/fcimb.2014.00077
20. Bereswill S, Ekmekciu I, Escher U, Fiebiger U, Stingl K, Heimesaat MM. *Lactobacillus johnsonii* ameliorates intestinal, extra-intestinal and systemic pro-inflammatory immune responses following murine *Campylobacter jejuni* infection. *Sci Rep*. (2017) 7:2138. doi: 10.1038/s41598-017-02436-2
21. Bereswill S, Grundmann U, Alutis ME, Fischer A, Heimesaat MM. *Campylobacter jejuni* infection of conventionally colonized mice lacking nucleotide-oligomerization-domain-2. *Gut Pathog*. (2017) 9:5. doi: 10.1186/s13099-017-0155-3
22. Ekmekciu I, Fiebiger U, Stingl K, Bereswill S, Heimesaat MM. Amelioration of intestinal and systemic sequelae of murine *Campylobacter jejuni* infection by probiotic VSL#3 treatment. *Gut Pathog*. (2017) 9:17. doi: 10.1186/s13099-017-0168-y
23. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Absence of nucleotide-oligomerization-domain-2 is associated with less distinct disease in *Campylobacter jejuni* infected secondary abiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2017) 7:322. doi: 10.3389/fcimb.2017.00322
24. Schmidt AM, Escher U, Mousavi S, Boehm M, Backert S, Bereswill S, et al. Protease activity of *Campylobacter jejuni* HtrA modulates distinct intestinal and systemic immune responses in infected secondary abiotic IL-10 deficient mice. *Front Cell Infect Microbiol*. (2019) 9:79. doi: 10.3389/fcimb.2019.00079
25. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Microbiota composition and immune responses during *Campylobacter jejuni* infection in conventionally colonized IL-10($^{-/-}$) mice lacking nucleotide oligomerization domain 2. *Eur J Microbiol Immunol*. (2017) 7:1–14. doi: 10.1556/1886.2016.00038
26. Holick MF. Vitamin D deficiency. *N Engl J Med*. (2007) 357:266–81. doi: 10.1056/NEJMra070553
27. Norman AW. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am J Clin Nutr*. (2008) 88:491s−9s. doi: 10.1093/ajcn/88.2.491S
28. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab*. (1983) 57:1308–10. doi: 10.1210/jcem-57-6-1308
29. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science*. (1983) 221:1181–3.
30. Cantorna MT. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. *Prog Biophys Mol Biol*. (2006) 92:60–4. doi: 10.1016/j.pbiomolbio.2006.02.020
31. White JH. Vitamin D signaling, infectious diseases, and regulation of innate immunity. *Infect Immun*. (2008) 76:3837–43. doi: 10.1128/IAI.00353-08
32. Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat Rev Immunol*. (2011) 11:584–96. doi: 10.1038/nri3045
33. Huang FC. Vitamin D differentially regulates Salmonella-induced intestine epithelial autophagy and interleukin-1beta expression. *World J Gastroenterol*. (2016) 22:10353–63. doi: 10.3748/wjg.v22.i47.10353
34. Dauletbaev N, Herscovitch K, Das M, Chen H, Bernier J, Matouk E, et al. Down-regulation of IL-8 by high-dose vitamin D is specific to hyperinflammatory macrophages and involves mechanisms beyond up-regulation of DUSP1. *Br J Pharmacol*. (2015) 172:4757–71. doi: 10.1111/bph.13249
35. Feindt E, Stroder J. Studies on the antimicrobial effect of vitamin D. *Klin Wochenschr*. (1977) 55:507–8.
36. Youssef DA, Miller CW, El-Abbassi AM, Cutchins DC, Cutchins C, Grant WB, et al. Antimicrobial implications of vitamin D. *Dermatoendocrinol*. (2011) 3:220–9. doi: 10.4161/derm.3.4.15027
37. Saputo S, Faustoferri RC, Quivey RG Jr. Vitamin D compounds are bactericidal against streptococcus mutans and target the bacitracin-associated efflux system. *Antimicrob Agents Chemother*. (2018) 62:e01675–17. doi: 10.1128/AAC.01675-17
38. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol*. (2004) 75:39–48. doi: 10.1189/jlb.0403147
39. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. (2006) 311:1770–3. doi: 10.1126/science.1123933
40. Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab*. (2008) 4:80–90. doi: 10.1038/ncpendmet0716
41. Kawaura A, Takeda E, Tanida N, Nakagawa K, Yamamoto H, Sawada K, et al. Inhibitory effect of long term 1α-hydroxyvitamin D3 administration on Helicobacter pylori infection. *J Clin Biochem Nutr*. (2006) 38:103–6. doi: 10.3164/jcbn.38.103
42. Juzeniene A, Ma LW, Kwitniewski M, Polev GA, Lagunova Z, Dahlback A, et al. The seasonality of pandemic and non-pandemic influenzas: the roles of solar radiation and vitamin D. *Int J Infect Dis*. (2010) 14:e1099–105. doi: 10.1016/j.ijid.2010.09.002
43. Barbachano A, Fernandez-Barral A, Ferrer-Mayorga G, Costales-Carrera A, Larriba MJ, Munoz A. The endocrine vitamin D system in the gut. *Mol Cell Endocrinol*. (2017) 453:79–87. doi: 10.1016/j.mce.2016.11.028
44. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PLoS ONE*. (2011) 6:e20953. doi: 10.1371/journal.pone.0020953
45. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J Immunol*. (2006) 177:8785–95. doi: 10.4049/jimmunol.177.12.8785
46. Williamson L, Hayes A, Hanson ED, Pivonka P, Sims NA, Gooi JH. High dose dietary vitamin D3 increases bone mass and strength in mice. *Bone Rep*. (2017) 6:44–50. doi: 10.1016/j.bonr.2017.02.001
47. Gascon-Barre M, Cote MG. Effects of phenobarbital and diphenylhydantoin on acute vitamin D3 toxicity in the rat. *Toxicol Appl Pharmacol*. (1978) 43:125–35.
48. Hatch RC, Laflamme DP. Acute intraperitoneal cholecalciferol (vitamin D3) toxicosis in mice: its nature and treatment with diverse substances. *Vet Hum Toxicol*. (1989) 31:105–12.
49. Alshahrani F, Aljohani N. Vitamin D: deficiency, sufficiency and toxicity. *Nutrients*. (2013) 5:3605–16. doi: 10.3390/nu5093605
50. Heimesaat MM, Haag LM, Fischer A, Otto B, Kuhl AA, Gobel UB, et al. Survey of extra-intestinal immune responses in asymptomatic long-term *Campylobacter jejuni*-infected mice. *Eur J Microbiol Immunol*. (2013) 3:174–82. doi: 10.1556/EuJMI.3.2013.3.4
51. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut*. (2010) 59:1079–87. doi: 10.1136/gut.2009.197434
52. Heimesaat MM, Lugert R, Fischer A, Alutis M, Kuhl AA, Zautner AE, et al. Impact of *Campylobacter jejuni* cj0268c knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. *PLoS ONE*. (2014) 9:e90148. doi: 10.1371/journal.pone.0090148
53. Alutis ME, Grundmann U, Fischer A, Hagen U, Kuhl AA, Gobel UB, et al. The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. *Eur J Microbiol Immunol*. (2015) 5:256–67. doi: 10.1556/1886.2015.00033

54. Alutis ME, Grundmann U, Hagen U, Fischer A, Kuhl AA, Gobel UB, et al. Matrix metalloproteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. *Eur J Microbiol Immunol.* (2015) 5:188–98. doi: 10.1556/1886.2015.00020
55. Nicholson I, Dalzell AM, El-Matary W. Vitamin D as a therapy for colitis: a systematic review. *J Crohns Colitis*. (2012) 6:405–11. doi: 10.1016/j.crohns.2012.01.007
56. Huang FC. The differential effects of 1,25-dihydroxyvitamin D3 on Salmonella-induced interleukin-8 and human beta-defensin-2 in intestinal epithelial cells. *Clin Exp Immunol*. (2016) 185:98–106. doi: 10.1111/cei.12792
57. Bruce D, Ooi JH, Yu S, Cantorna MT. Vitamin D and host resistance to infection? Putting the cart in front of the horse. *Exp Biol Med.* (2010) 235:921–7. doi: 10.1258/ebm.2010.010061
58. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide. (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J*. (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
59. Ooi JH, Li Y, Rogers CJ, Cantorna MT. Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis. *J Nutr*. (2013) 143:1679–86. doi: 10.3945/jn.113.180794
60. He L, Liu T, Shi Y, Tian F, Hu H, Deb DK, et al. Gut epithelial vitamin D receptor regulates microbiota-dependent mucosal inflammation by suppressing intestinal epithelial cell apoptosis. *Endocrinology*. (2018) 159:967–79. doi: 10.1210/en.2017-00748
61. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. *Curr Opin Pharmacol*. (2010) 10:482–96. doi: 10.1016/j.coph.2010.04.001
62. Cantorna MT. Mechanisms underlying the effect of vitamin D on the immune system. *Proc Nutr Soc*. (2010) 69:286–9. doi: 10.1017/S0029665110001722
63. Chun RF, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol*. (2014) 5:151. doi: 10.3389/fphys.2014.00151
64. Bartels LE, Bendix M, Hvas CL, Jorgensen SP, Agnholt J, Agger R, et al. Oral vitamin D3 supplementation reduces monocyte-derived dendritic cell maturation and cytokine production in Crohn's disease patients. *Inflammopharmacology*. (2014) 22:95–103. doi: 10.1007/s10787-013-0197-1
65. Cantorna MT, McDaniel K, Bora S, Chen J, James J. Vitamin D, immune regulation, the microbiota, and inflammatory bowel disease. *Exp Biol Med*. (2014) 239:1524–30. doi: 10.1177/1535370214523890
66. Cantorna MT, Waddell A. The vitamin D receptor turns off chronically activated T cells. *Ann N Y Acad Sci*. (2014) 1317:70–5. doi: 10.1111/nyas.12408
67. Kongsbak M, Levring TB, Geisler C, von Essen MR. The vitamin d receptor and T cell function. *Front Immunol*. (2013) 4:148. doi: 10.3389/fimmu.2013.00148
68. Gao D, Trayhurn P, Bing C. 1,25-Dihydroxyvitamin D3 inhibits the cytokine-induced secretion of MCP-1 and reduces monocyte recruitment by human preadipocytes. *Int J Obes.* (2013) 37:357–65. doi: 10.1038/ijo.2012.53
69. Haag LM, Fischer A, Otto B, Grundmann U, Kuhl AA, Gobel UB, et al. *Campylobacter jejuni* infection of infant mice: acute enterocolitis is followed by asymptomatic intestinal and extra-intestinal immune responses. *Eur J Microbiol Immunol.* (2012) 2:2–11. doi: 10.1556/EuJMI.2.2012.1.2
70. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol*. (2009) 39:3147–59. doi: 10.1002/eji.200839103
71. Van Belle TL, Vanherwegen AS, Feyaerts D, De Clercq P, Verstuyf A, Korf H, et al. 1,25-Dihydroxyvitamin D3 and its analog TX527 promote a stable regulatory T cell phenotype in T cells from type 1 diabetes patients. *PLoS ONE*. (2014) 9:e109194. doi: 10.1371/journal.pone.0109194
72. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev*. (2016) 96:365–408. doi: 10.1152/physrev.00014.2015
73. Gorman S, Black LJ, Feelisch M, Hart PH, Weller R. Can skin exposure to sunlight prevent liver inflammation? *Nutrients*. (2015) 7:3219–39. doi: 10.3390/nu7053219
74. Jahn D, Dorbath D, Kircher S, Nier A, Bergheim I, Lenaerts K, et al. Beneficial effects of vitamin D treatment in an obese mouse model of non-alcoholic steatohepatitis. *Nutrients.* (2019) 11:77. doi: 10.3390/nu11010077
75. Schwalfenberg GK. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol Nutr Food Res*. (2011) 55:96–108. doi: 10.1002/mnfr.201000174
76. Bucker R, Krug SM, Moos V, Bojarski C, Schweiger MR, Kerick M, et al. *Campylobacter jejuni* impairs sodium transport and epithelial barrier function via cytokine release in human colon. *Mucosal Immunol*. (2018) 11:575–7. doi: 10.1038/mi.2017.78

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Vitamin D Signaling in the Context of Innate Immunity: Focus on Human Monocytes

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The vitamin D_3 metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) activates at sub-nanomolar concentrations the transcription factor vitamin D receptor (VDR). VDR is primarily involved in the control of cellular metabolism but in addition modulates processes important for immunity, such as anti-microbial defense and the induction of T cell tolerance. Monocytes and their differentiated phenotypes, macrophages and dendritic cells, are key cell types of the innate immune system, in which vitamin D signaling was most comprehensively investigated *via* the use of next generation sequencing technologies. These investigations provided genome-wide maps illustrating significant effects of $1,25(OH)_2D_3$ on the binding of VDR, the pioneer transcription factors purine-rich box 1 (PU.1) and CCAAT/enhancer binding protein α (CEBPA) and the chromatin modifier CCCTC-binding factor (CTCF) as well as on chromatin accessibility and histone markers of promoter and enhancer regions, H3K4me3 and H3K27ac. Thus, the epigenome of human monocytes is at multiple levels sensitive to vitamin D. These data served as the basis for the chromatin model of vitamin D signaling, which mechanistically explains the activation of a few hundred primary vitamin D target genes. Comparable epigenome- and transcriptome-wide effects of vitamin D were also described in peripheral blood mononuclear cells isolated from individuals before and after supplementation with a vitamin D_3 bolus. This review will conclude with the hypothesis that vitamin D modulates the epigenome of immune cells during perturbations by antigens and other immunological challenges suggesting that an optimal vitamin D status may be essential for an effective epigenetic learning process, in particular of the innate immune system.

Keywords: vitamin D, VDR, epigenome, transcriptome, gene regulation, vitamin D target genes, monocytes, PBMCs

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INTRODUCTION

Vitamin D_3 is an evolutionary very old molecule that is produced from the direct cholesterol precursor 7-dehydrocholesterol in a non-enzymatic reaction using energy provided by the UV-B component of sunlight (1). Thus, every species that exerts cholesterol biosynthesis and is exposed to sunlight should be able to synthesize vitamin D_3. The molecule itself is biologically inert, but when it is converted to 25-hydroxyvitamin D_3 ($25(OH)D_3$) and then to 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), it acts as a nuclear hormone. The jawless fish lamprey is the oldest known species that some 550 million years ago evolved with the transcription factor VDR a nuclear receptor

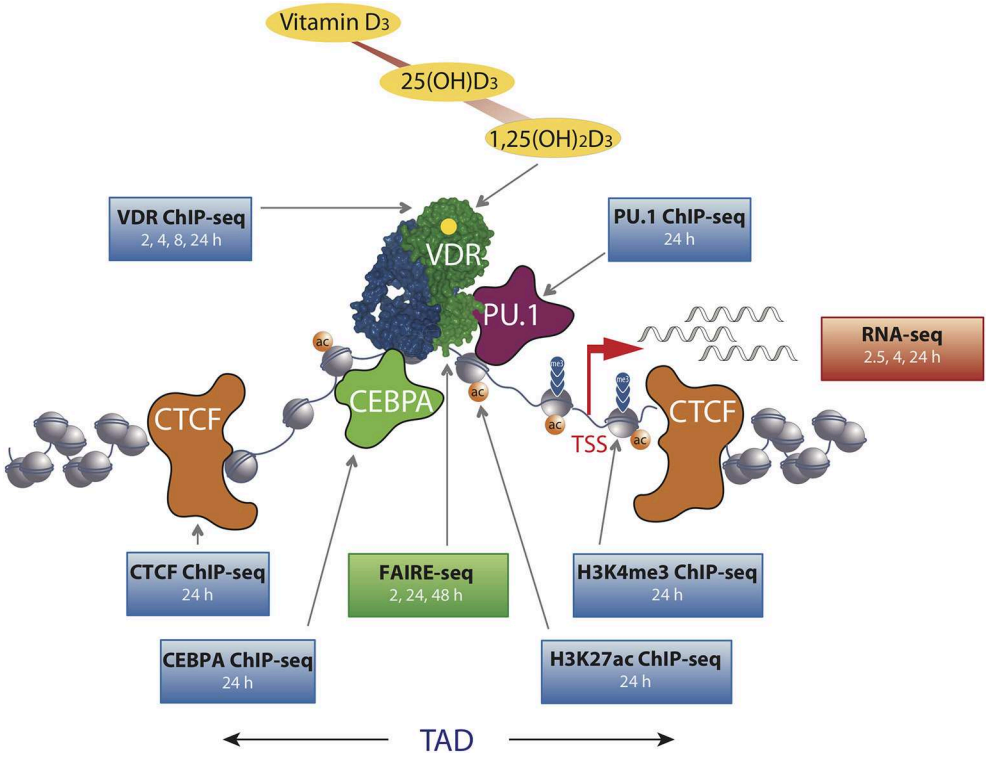

FIGURE 1 | The chromatin model of vitamin D signaling. The model was defined by epigenome- and transcriptome-wide data obtained in THP-1 cells. CTCF proteins define left and right TAD borders, in which vitamin D target genes (red arrow, measured by RNA-seq) are activated by VDR [activated by $1,25(OH)_2D_3$] binding to enhancer regions. The pioneer transcription factors CEBPα and PU.1 help VDR in binding to accessible genomic DNA (measured by FAIRE-seq). This paralleled with changes in markers of active TSS regions (H3K4me3) and active chromatin (H3K27ac). The genome-wide binding of VDR, CTCF, PU.1, CEBPα, and histone markers were determined by ChIP-seq in three biological repeats. The time of $1,25(OH)_2D_3$ stimulation is indicated for each dataset.

that gets activated by $1,25(OH)_2D_3$ at sub-nanomolar concentrations (2). After the manifestation of VDR, vitamin D turned from a product of UV-B absorption, i.e., the output of a radiation protection pathway as found in plankton, to an endocrine molecule in higher species (3). Thus, vitamin D has via its metabolite $1,25(OH)_2D_3$ direct effects on gene regulation (**Figure 1**). In human, the main sites of $1,25(OH)_2D_3$ production for endocrine purposes are proximal tubule cells of the kidneys, but for para- and autocrine use also monocytes, macrophages, and dendritic cells of the innate immune system and other cell types in skin and bone are able to produce the nuclear hormone (4).

Since vitamin D_3 can be synthesized endogenously in human skin (5), the term "vitamin" seems not to be appropriate. However, compared to the past, humans spend far more time indoors, largely cover their skin by textile when being outdoors and often live at latitudes where during winter UV-B radiation is too low for many months, there is insufficient endogenous vitamin D_3 production, i.e., under these conditions vitamin D_3 is an essential micronutrient (6). Average human diet is low in vitamin D, so that dietary products, such as milk, margarine and juices, are fortified and direct vitamin D supplementation via pills is recommended in winter months (7). Interestingly, already more than 100 years ago cod-liver oil as well as UV-B exposure had been proposed for the protection against rickets (an infant bone malformation disease) as well as for the treatment of tuberculosis (an infectious disease caused by intra-cellular bacteria) (8, 9). Thus, vitamin D deficiency causes not only bone disorders (10) but also affects the protective roles of the molecule against a large number of other diseases (11). The autoimmune disease multiple sclerosis is the most prominent example, which may be largely preventable by a sufficient vitamin D status (12). This status is defined via the serum concentrations of the most stable vitamin D metabolite, $25(OH)D_3$, which for

Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; BRD7, bromodomain containing 7; CAMP, cathelicidin antimicrobial peptide; CD14, CD14 molecule; CDKN1C, cyclin dependent kinase inhibitor 1C; CEBP, CCAAT/enhancer binding protein; ChIP-seq, chromatin immunoprecipitation sequencing; CTCF, CCCTC-binding factor; CYP, cytochrome P450; DENND6B, DENN domain containing 6B; FAIRE-seq, formaldehyde-assisted isolation of regulatory elements sequencing; FBP1, fructose-bisphosphatase 1; GABPA, GA binding protein transcription factor α; HLA, human leukocyte antigen; KDM6B, lysine demethylase 6B; MYO1G, myosin IG; NF-AT, nuclear factor activated T cells; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PBMCs, peripheral blood mononuclear cells; PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; PU.1, purine-rich box 1, Spi-1 proto-oncogene (official gene symbol: SPI1); qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing; TAD, topologically associated domain; TNFSF11, TNF superfamily member 11; TSS, transcription start site; VDR, vitamin D receptor; ZMIZ1, zinc finger MIZ-type containing 1.

good bone health should be 50 nM (13), but also levels of 75 nM or more are suggested (14). Accordingly, instructions for daily supplementation with vitamin D_3 range from 10 to 50 μg (400–2,000 IU). However, these population-wide recommendations do not take inter-individual variations into account, such as a different molecular response to vitamin D, which are expressed by the vitamin D response index (15). As discussed below in more detail, this index can be determined *via* the genome-wide response of peripheral blood mononuclear cells (PBMCs) to an *in vivo* challenge with vitamin D_3 (16).

In extension to a recent summary on the nutrigenomic role of vitamin D (17), the aim of this short review is to present the epigenome-wide impact of the nuclear hormone in relation to immunity. Special attention is given to human monocytes and PBMCs serving as *in vitro* and *in vivo* model systems for vitamin D signaling.

VITAMIN D AND THE EPIGENOME

Chromatin is a complex of histone proteins and genomic DNA (18, 19) that by default largely restricts the access of RNA polymerases to promoter regions and of transcription factors to enhancer regions. Therefore, in a differentiated cell only some 200,000 genomic loci are accessible (20). The epigenome comprises genome-wide information represented by covalent and structural modifications of chromatin, such as cytosine methylation, post-translational modifications of histone proteins and 3D structure of the nucleus, that do not involve any alterations in the sequence of genomic DNA (21). Epigenetic programming is a memory creating event that during embryogenesis and cellular differentiation, such as of monocytes after immune challenges (**Figure 2C**), determines the specialized roles of terminally differentiated cells *via* changes of their epigenome (25). In these cases epigenetic programming is irreversible and leads to static outcomes, in order to keep the identity of tissues and cell types. Thus, the epigenome largely determines gene expression and the functional profile of a cell; i.e., alternations of the epigenome precede those of the transcriptome.

A number of diet-derived metabolites, such as resveratrol, genistein, curcumin and polyphenols from fruits, vegetables, spices, teas and medicinal herbs, can affect the activity of chromatin modifiers and transcription factors (17, 26). Chromatin modifiers are nuclear enzymes that catalyze epigenetic modifications, such as DNA methylation as well as histone acetylation and methylation, while chromatin remodelers are another class of nuclear enzymes that change the position and composition of nucleosomes. VDR communicates in a ligand-dependent fashion both with chromatin modifiers, such as lysine demethylase 6B (KDM6B) (27), as well as with chromatin remodelers, such as bromodomain containing 7 (BRD7) (28). This explains how vitamin D can significantly change the intensity of histone markers for active chromatin, H3K27ac, as well as those for active transcription start sites (TSSs), H3K4me3, as observed by chromatin immunoprecipitation sequencing (ChIP-seq) in THP-1 human monocytic leukemia cells (29, 30) (**Figure 1**). The epigenome of these cells responds to a stimulation with $1,25(OH)_2D_3$ at the loci of more than 500 promoters and 2,500 enhancers. Moreover, the method of formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) monitored in the same cellular system that vitamin D changes the accessibility of some 4,500 chromatin loci (out of some 100,000 in total) at a given time point (22). Thus, a stimulation with vitamin D represents a cellular perturbation that results in changes of the epigenome and in this way affects the epigenetic memory of the cell (24). However, in contrast to epigenetic programming during cellular differentiation, many of these epigenetic memorizing events are dynamic; i.e., they persist only for a shorter time period and are reversible.

In human cells, the cistrome of VDR, i.e., the genome-wide binding pattern of the transcription factor, was determined by the ChIP-seq in lymphocytes (31), colorectal cancer cells (32), hepatic stellate cells (33), prostate cells (34), macrophage-like cells (35) and most comprehensively in monocytes (36, 37). In all these *in vitro* cell culture models stimulation with ligand resulted in a 2- to 10-fold increase in VDR binding sites; i.e., the significantly enhanced VDR cistrome represents the most eminent response of the human epigenome to a perturbation with vitamin D. In monocytes the VDR cistrome comprises more than 10,000 loci, of which a subgroup of a few hundred persistent sites is always occupied (37). These sites seem to be the primary contact points of the human genome with vitamin D and may coordinate the genome's spatio-temporal response to the nuclear hormone.

In THP-1 cells, statistically significant epigenome-wide effects of vitamin D were also described for the binding of the pioneer factors PU.1 (38), CEBPA (30) and GA binding protein transcription factor α (GABPA) (39) as well as for the chromatin organizer CTCF (40). The pioneer factors contribute to the increase in VDR loci after ligand stimulation by helping the receptor accessing its genomic binding sites (**Figure 1**). Since CTCF majorly contributes to DNA loop formation of the genome into topologically associated domains (TADs) (41), the vitamin D sensitivity of the protein implies that some 500 TADs are triggered by VDR and its ligand. Thus, vitamin D affects the epigenome on multiple levels, such as the binding of VDR and pioneer factors, histone markers, chromatin accessibility, and 3D organization of the nucleus.

CHROMATIN MODEL OF VITAMIN D SIGNALING

The chromatin model of vitamin D signaling (24, 42) (**Figure 1**) was developed on the basis of above described epigenomic data, which had been primarily obtained in THP-1 cells after a stimulation with $1,25(OH)_2D_3$ for 24 h. The model suggests that a primary vitamin D target gene is modulated in its expression, when the TAD, in which the gene is localized, contains a prominent VDR binding site. This applies to 425 vitamin D sensitive TADs comprising 90% of all target genes in THP-1 cells (40). An additional condition for effective gene regulation is, that

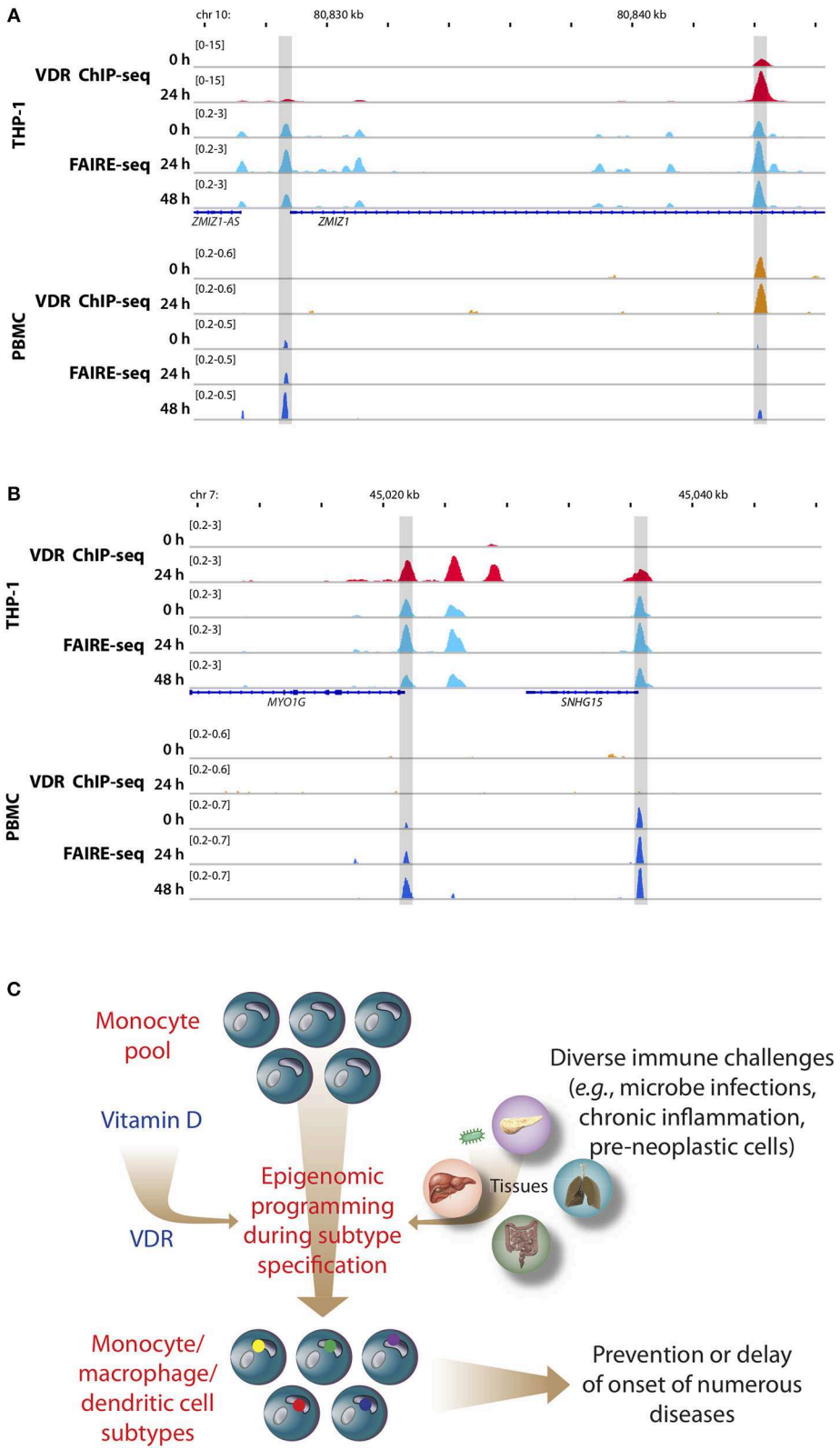

FIGURE 2 | Memory hypothesis. VDR binding and chromatin opening of the loci of the genes *ZMIZ1*, **(A)** and *MYO1G* **(B)** *in vitro* (THP-1) as well as *in vivo* (PBMCs). THP-1 cells were stimulated for 0, 24, and 48 h with $1,25(OH)_2D_3$ and VDR ChIP-seq and FAIRE-seq were performed (22). In a comparable *in vivo* experiment an

(Continued)

FIGURE 2 | individual was challenged with a vitamin D_3 bolus (2,000 μg) and PBMCs were isolated before (day 0) and at days 1 (24 h) and 2 (48 h) (23). The peak tracks represent merger of each three biological repeats. Gene structures are shown in blue. Different types of immune challenges program the epigenome of the pool of human monocytes, which "memorize" these encounters in form of differently programmed epigenomes leading to subtype differentiation (bottom, differently colored dots of in nuclei of monocyte subpopulations, **C**). The recently discovered epigenome modulating effect of vitamin D [via the VDR (24)] modulates on multiple levels this epigenetic programming process. The stabilization of the epigenomes of the subtypes of monocytes, macrophages and dendritic cells by vitamin D can prevent or delay the onset of common age-related diseases.

the TSS of the target gene as well as the vitamin D-sensitive enhancer have to be located within accessible chromatin (43). DNA looping between the enhancer binding ligand-activated VDR supported by pioneer factors, such as CEBPA and PU.1, and the TSS of a vitamin D target genes changes at both genomic regions H3K27ac and H3K4me3 histone marks as well as chromatin accessibility (**Figure 1**). Thus, many epigenetic events are required before RNA polymerase II on the TSSs is activated and mRNA synthesis can start.

A meta-analysis of four independent transcriptome-wide datasets of $1,25(OH)_2D_3$-stimulated undifferentiated THP-1 cells (22, 30, 36, 44) revealed 126 common genes, 72% of which are primary vitamin D targets (45). Nearly all (97%) of these vitamin D target genes are up-regulated and primarily encode for enzymes, receptors and transporters, half of which are located in membranes. Gene ontology analysis indicated the modulation of innate immunity as the most prominent common function of these genes, although this covers <25% of all. Four classes of gene regulatory scenarios, which are based on differential VDR, PU.1, and CEBPA binding to promoter and enhancer regions, can explain the regulation of most (85%) primary vitamin D target genes (45). Interestingly, immune system-related genes are often prominently up-regulated by vitamin D, while genes involved in cellular metabolism are less sensitive to the nuclear hormone. This was confirmed by an independent analysis of vitamin D-triggered TAD classes, where genes that are important for immune function are regulated in a tightly controlled "on/off" modus (37).

VITAMIN D AND IMMUNITY

Based on the evolutionary history of nuclear receptors (46, 47), VDR's original function was the regulation of cellular metabolism. This role specialized into the control of calcium homeostasis, when some 400 million years ago species left the ocean and had to improve their calcium-based skeleton, in order to resist to gravitation (48). Although there are no direct effects of vitamin D on bone mineralization, bone-resorbing osteoclasts derive from monocytes, the differentiation of which is controlled by the vitamin D target gene TNF superfamily member 11 (*TNFSF11*, encoding the cytokine RANKL) (49). VDR's tasks in the control of metabolism involves regulating genes mediating energy metabolism, like the glycolytic enzymes fructose-bisphosphatase 1 (*FBP1*) (36) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (*PFKFB4*) (50), as well as in the catabolism of lipophilic intra-cellular molecules, like those encoding for the cytochrome P450 (CYP) enzymes CYP26B1, CYP19A1, and CYP24A1 (51). Both functions supported and enhanced the expansion of the energy demanding immune system as suggested by the concept of immuno-metabolism (52). Moreover, VDR became a critical transcription factor in regulating the expression of genes involved in inflammation and anti-bacterial defense, such as *CD14* (53) and cathelicidin anti-microbial peptide (*CAMP*) (54). Thus, the immune-modulating function of vitamin D is probably evolutionary older than its role in calcium homeostasis.

During hematopoiesis VDR acts together with the pioneer transcription factors PU.1 and CEBPA as a key regulator of myeloid differentiation toward key cells in innate immunity, such as monocytes and granulocytes (55). Furthermore, vitamin D can inhibit the maturation, differentiation and the stimulatory capacity of dendritic cells, which derive from monocytes (56). A profile change of dendritic cells induces the production of regulatory T cells and leads to immunological tolerance. In parallel, vitamin D and its receptor are able to antagonize the pro-inflammatory actions of the transcription factors nuclear factor activated T cells (NF-AT) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in T cells (57). In this way, vitamin D reduces autoimmunity, such as the onset and progression of multiple sclerosis (58), as well as chronic inflammation, such as in inflammatory bowel disease (59).

Most cells of the immune system have a rapid turnover, which enables them to respond more flexible to environmental changes than other cell types of the human body. For example, monocytes coordinate not only inflammatory pathways, but also control *via* in their differentiated forms, macrophages and dendritic cells, metabolic pathways, and general stress responses. Cellular perturbations, such as an encounter of immune cells with an antigen, affect *via* signal transduction cascades the epigenome. For example, most inflammatory lesions are initiated by monocyte-derived macrophages, the altered gene expression profile of which is based on changes of their epigenome in response to extra-cellular signals (**Figure 2C**). Moreover, the differentiation process of monocytes to macrophages (or dendritic cells) is based on epigenome changes in response to contacts with antigens. Such a subtype specification is also referred to as trained immunity, as demonstrated by studies of the BLUEPRINT consortium (www.blueprint-epigenome.eu) (60, 61). This rather short-term epigenetic memory monitors the close relationship between immune challenges and effects on chromatin. Epigenetic memory prepares innate immune cells for a possible next microbe encounter (62). In the context of these immunological processes, high affinity receptors for lipophilic signaling molecules, such as VDR and other members of the nuclear receptor superfamily, are in a prime position sensing environmental changes and other signals with a potential of creating cellular stress. Thus, VDR and its ligand are predestined for modulating the process of recording epigenetic memory in innate immunity (63) (**Figure 2C**).

IN VIVO RESPONSE OF IMMUNE CELLS TO VITAMIN D

The chromatin model and the suggested regulatory scenarios of primary vitamin D target genes had been previously developed based on the THP-1 *in vitro* cell system, but are supposed to apply also to other VDR expressing tissue and cell types. This should include *in vivo* situations, such as PBMCs obtained from vitamin D_3 treated individuals (23). Human supplementation studies allowed the assessment of vitamin D's molecular action under *in vivo* conditions. The studies were carried on PBMCs isolated from participants before and after the long-term (5 months) trial VitDmet (64–67) [NCT01479933, which applied daily vitamin D_3 supplementation (0–80 μg)] and the short-term (2 days) trial VitDbol (16, 23, 68, 69) [NCT02063334, which used a single vitamin D_3 bolus (2,000 μg)]. Chromatin and RNA had been immediately isolated from PBMCs, i.e., without any *in vitro* culture, for the assessment of chromatin accessibility [using FAIRE-quantitative polymerase chain reaction (qPCR) and FAIRE-seq] and mRNA expression [using qPCR and RNA sequencing (RNA-seq)]. The changes of molecular parameters, such as the expression of vitamin D target genes or the accessibility of vitamin D-triggered chromatin regions, were related to fold changes in 25(OH)D_3 serum levels, in order to rank the individuals based on their vitamin D responsiveness (64, 67). The vitamin D response index (15) is proportional to this ranking and segregates the study participants into high, mid, and low responders. Interestingly, the vitamin D response index is a parameter that is independent of the vitamin D status, i.e., of 25(OH)D_3 serum levels. The vitamin D status is a dynamic parameter and depends on season, diet and supplementation, while the vitamin D index is static, i.e., it is an intrinsic property that is assumed not to change during a person's lifetime. Accordingly, both VitDmet (pre-diabetic elderly participants) and VitDbol (healthy young subjects) agreed on that some 25% of the analyzed cohorts are low responders. These individuals should be supplemented with higher daily vitamin D_3 doses than high responders. Thus, instead of population-based recommendations for vitamin D_3 supplementation there should be personalized recommendations in order to reach a vitamin D status that is optimized for an individual's health protection.

PBMCs are a mixture of monocytes, T and B cells, of which monocytes seem to be the most vitamin D-responsive component (6). Based on transcriptome-wide investigations performed with PBMC samples of five participants of the VitDbol study, a vitamin D_3 bolus significantly changed within 24 h the expression of 702 genes (16, 17). Importantly, 181 (26%) of these genes (such as *CDKN1C*, *CEBPB, CD14*, and *DENND6B*) were already known in THP-1 cells as vitamin D targets (36); i.e., the *in vivo* response of PBMCs (<10% monocytes) to a vitamin D_3 bolus resembles to a larger extent the *in vitro* treatment of THP-1 cells with 1,25$(OH)_2D_3$ than expected from the relative cell counts. On the level of the epigenome the overlap between PBMCs and THP-1 cells is even larger, since a vitamin D_3 bolus significantly affected accessibility of chromatin at 853 genomic loci (23), 87% of which had already been described in THP-1 cells (22). This is exemplified by VDR binding and chromatin opening of the loci of the vitamin D target genes zinc finger MIZ-type containing 1 (*ZMIZ1*, **Figure 2A**) and myosin IG (*MYO1G*, **Figure 2B**) under *in vitro* (THP-1) and *in vivo* (PBMC) conditions, respectively. However, the comparison of both cellular systems also indicates that not all genomic regions respond in the same way to vitamin D stimulation. Nevertheless, PBMCs and THP-1 cells show a better overlap on the level of the epigenome than on the transcriptome. Thus, the principles of the chromatin model of vitamin D signaling, which were formulated on the basis of *in vitro* cultured THP-1 cells, may be extrapolated to PBMCs and the *in vivo* situation. Interestingly, data from *in vivo* challenged PBMCs highlighted the human leukocyte antigen (HLA) cluster in chromosome 6 to have a high density of vitamin D-sensitive chromatin regions (23) as well as the genes *HLA-A* and *HLA-C* as vitamin D targets encoding for class I major histocompatibility complex proteins (16). Thus, the HLA cluster serves as a "hotspot" of vitamin D's physiological activity.

CONCLUDING HYPOTHESIS

Vitamin D is a molecule that is able to modulate *in vitro* as well as *in vivo* the epigenome of immune cells, in particular of monocytes and their differentiated subtypes. In parallel, the rather recently discovered process of trained immunity (70) implies that immune cells memorize challenges, to which they are exposed in their rather short lifespan, in form of changes of their epigenome leading to subtype specification (**Figure 2C**). By combining these two observations, it is tempting to hypothesize that a large part of the immune-related effects of vitamin D are due to a modulation of the epigenomic programing of monocytes, macrophages, and dendritic cells during their differentiation into subtypes. For example, the HLA cluster, which comprises the highest density of immunologically important genes (71), may be programmed differently in the presence of vitamin D than in its absence. Thus, the efficiency of the epigenetic memory effect of trained immunity should be best at an optimized vitamin D status when vitamin D signaling functions best. Thus, personalized vitamin D_3 supplementation may support proper epigenetic programming of immune cells throughout hematopoiesis as well as during antigen encounter. In conclusion, the recently discovered epigenome modulation function of vitamin D (24) is essential for understanding the physiological impact of the nuclear hormone.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

1. Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D_3 photosynthesis in man: skin pigment is not an essential regulator. *Science*. (1981) 211:590–3. doi: 10.1126/science.6256855
2. Whitfield GK, Dang HT, Schluter SF, Bernstein RM, Bunag T, Manzon LA, et al. Cloning of a functional vitamin D receptor from the lamprey. (Petromyzon marinus), an ancient vertebrate lacking a calcified skeleton and teeth. *Endocrinology*. (2003) 144:2704–16. doi: 10.1210/en.2002-221101
3. Holick MF. Vitamin D: a millenium perspective. *J Cell Biochem*. (2003) 88:296–307. doi: 10.1002/jcb.10338
4. Hewison M. An update on vitamin D and human immunity. *Clin Endocrinol*. (2012) 76:315–25. doi: 10.1111/j.1365-2265.2011.04261.x
5. Holick MF. *Photobiology of Vitamin D. Vitamin D,* 3rd ed. (2011). p. 13–22.
6. Carlberg C. Molecular approaches for optimizing vitamin D supplementation. *Vitam Horm*. (2016) 100:255–71. doi: 10.1016/bs.vh.2015.10.001
7. Bendik I, Friedel A, Roos FF, Weber P, Eggersdorfer M. Vitamin D: a critical and essential micronutrient for human health. *Front Physiol*. (2014) 5:248. doi: 10.3389/fphys.2014.00248
8. Holick MF. The cutaneous photosynthesis of previtamin D_3: a unique photoendocrine system. *J Invest Dermatol*. (1981) 77:51–8. doi: 10.1111/1523-1747.ep12479237
9. Grad R. Cod and the consumptive: a brief history of cod-liver oil in the treatment of pulmonary tuberculosis. *Pharm Hist*. (2004) 46:106–20.
10. Carlberg C. The physiology of vitamin D-far more than calcium and bone. *Front Physiol*. (2014) 5:335. doi: 10.3389/fphys.2014.00335
11. Holick MF. Vitamin D deficiency. *N Engl J Med*. (2007) 357:266–81. doi: 10.1056/NEJMra070553
12. Schwartz GG. Multiple sclerosis and prostate cancer: what do their similar geographies suggest? *Neuroepidemiology*. (1992) 11:244–54. doi: 10.1159/000110937
13. Institute-of-Medicine. *Dietary Reference Intakes for Calcium and Vitamin D*. Washington, DC: National Academies Press (2011).
14. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. (2011) 96:1911–30. doi: 10.1210/jc.2011-0385
15. Carlberg C, Haq A. The concept of the personal vitamin D response index. *J Steroid Biochem Mol Biol*. (2018) 175:12–7. doi: 10.1016/j.jsbmb.2016.12.011
16. Neme A, Seuter S, Malinen M, Nurmi T, Tuomainen TP, Virtanen JK, et al. *In vivo* transcriptome changes of human white blood cells in response to vitamin D. *J Steroid Biochem Mol Biol*. (2019) 188:71–6. doi: 10.1016/j.jsbmb.2018.11.019
17. Carlberg C. Nutrigenomics of Vitamin D. *Nutrients*. (2019) 11:676. doi: 10.3390/nu11030676
18. Carlberg C, Molnár F editors. The impact of chromatin. In: *Mechanisms of Gene Regulation.* 2nd ed. Dordrecht: Springer (2016). p. 17–34. doi: 10.1007/978-94-017-7741-4
19. Wu CT, Morris JR. Genes, genetics, and epigenetics: a correspondence. *Science*. (2001) 293:1103–5. doi: 10.1126/science.293.5532.1103
20. ENCODE-Project-Consortium, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, et al. An integrated encyclopedia of DNA elements in the human genome. *Nature*. (2012) 489:57–74. doi: 10.1038/nature11247
21. Beisel C, Paro R. Silencing chromatin: comparing modes and mechanisms. *Nat Rev Genet*. (2011) 12:123–35. doi: 10.1038/nrg2932
22. Seuter S, Neme A, Carlberg C. Epigenome-wide effects of vitamin D and their impact on the transcriptome of human monocytes involve CTCF. *Nucleic Acids Res*. (2016) 44:4090–104. doi: 10.1093/nar/gkv1519
23. Carlberg C, Seuter S, Nurmi T, Tuomainen TP, Virtanen JK, Neme A. *In vivo* response of the human epigenome to vitamin D: a proof-of-principle study. *J Steroid Biochem Mol Biol.* (2018) 180:142–8. doi: 10.1016/j.jsbmb.2018.01.002
24. Carlberg C. Molecular endocrinology of vitamin D on the epigenome level. *Mol Cell Endocrinol*. (2017) 453:14–21. doi: 10.1016/j.mce.2017.03.016
25. Perino M, Veenstra GJ. Chromatin control of developmental dynamics and plasticity. *Dev Cell*. (2016) 38:610–20. doi: 10.1016/j.devcel.2016.08.004
26. Vanden Berghe W. Epigenetic impact of dietary polyphenols in cancer chemoprevention: lifelong remodeling of our epigenomes. *Pharmacol Res*. (2012) 65:565–76. doi: 10.1016/j.phrs.2012.03.007
27. Pereira F, Barbachano A, Silva J, Bonilla F, Campbell MJ, Munoz A, et al. KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. *Hum Mol Genet*. (2011) 20:4655–65. doi: 10.1093/hmg/ddr399
28. Wei Z, Yoshihara E, He N, Hah N, Fan W, Pinto AFM, et al. Vitamin D switches BAF complexes to protect β cells. *Cell*. (2018) 173:1135–49.e15. doi: 10.1016/j.cell.2018.04.013
29. Nurminen V, Neme A, Seuter S, Carlberg C. The impact of the vitamin D-modulated epigenome on VDR target gene regulation. *Biochim Biophys Acta*. (2018) 1861:697–705. doi: 10.1016/j.bbagrm.2018.05.006
30. Nurminen V, Neme A, Seuter S, Carlberg C. Modulation of vitamin D signaling by the pioneer factor CEBPA. *Biochim Biophys Acta*. (2019) 1862:96–106. doi: 10.1016/j.bbagrm.2018.12.004
31. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res*. (2010) 20:1352–60. doi: 10.1101/gr.107920.110
32. Meyer MB, Goetsch PD, Pike JW. VDR/RXR and TCF4/β-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol Endocrinol*. (2012) 26:37–51. doi: 10.1210/me.2011-1109
33. Ding N, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R, et al. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell*. (2013) 153:601–13. doi: 10.1016/j.cell.2013.03.028
34. Fleet JC, Kovalenko PL, Li Y, Smolinski J, Spees C, Yu JG, et al. Vitamin D signaling suppresses early prostate carcinogenesis in $TgAPT_{121}$ mice. *Cancer Prev Res*. (2019) 12:343–56. doi: 10.1158/1940-6207.CAPR-18-0401
35. Tuoresmäki P, Väisänen S, Neme A, Heikkinen S, Carlberg C. Patterns of genome-wide VDR locations. *PLoS ONE*. (2014) 9:e96105. doi: 10.1371/journal.pone.0096105
36. Heikkinen S, Väisänen S, Pehkonen P, Seuter S, Benes V, Carlberg C. Nuclear hormone 1α,25-dihydroxyvitamin D_3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. *Nucleic Acids Res*. (2011) 39:9181–93. doi: 10.1093/nar/gkr654
37. Neme A, Seuter S, Carlberg C. Selective regulation of biological processes by vitamin D based on the spatio-temporal cistrome of its receptor. *Biochim Biophys Acta*. (2017) 1860:952–61. doi: 10.1016/j.bbagrm.2017.07.002
38. Seuter S, Neme A, Carlberg C. Epigenomic PU.1-VDR crosstalk modulates vitamin D signaling. *Biochim Biophys Acta*. (2017) 1860:405–15. doi: 10.1016/j.bbagrm.2017.02.005
39. Seuter S, Neme A, Carlberg C. ETS transcription factor family member GABPA contributes to vitamin D receptor target gene regulation. *J Steroid Biochem Mol Biol*. (2018) 177:46–52. doi: 10.1016/j.jsbmb.2017.08.006
40. Neme A, Seuter S, Carlberg C. Vitamin D-dependent chromatin association of CTCF in human monocytes. *Biochim Biophys Acta*. (2016) 1859:1380–8. doi: 10.1016/j.bbagrm.2016.08.008
41. Dixon JR, Selvaraj S, Yue F, Kim A, Li Y, Shen Y, et al. Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature*. (2012) 485:376–80. doi: 10.1038/nature11082
42. Carlberg C, Neme A. Machine learning approaches infer vitamin D signaling: critical impact of vitamin D receptor binding within topologically associated domains. *J Steroid Biochem Mol Biol*. (2019) 185:103–9. doi: 10.1016/j.jsbmb.2018.07.015
43. Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor. *Steroids*. (2013) 78:127–36. doi: 10.1016/j.steroids.2012.10.019
44. Verway M, Bouttier M, Wang TT, Carrier M, Calderon M, An BS, et al. Vitamin D induces interleukin-1β expression: paracrine macrophage epithelial signaling controls M. tuberculosis infection. *PLoS Pathog.* (2013) 9:e1003407. doi: 10.1371/journal.ppat.1003407
45. Nurminen V, Seuter S, Carlberg C. Primary vitamin D target genes of human monocytes. *Front Physiol*. (2019) 10:194. doi: 10.3389/fphys.2019.00194
46. Escriva H, Bertrand S, Laudet V. The evolution of the nuclear receptor superfamily. *Essays Biochem.* (2004) 40:11–26. doi: 10.1042/bse0400011

47. Krasowski MD, Ni A, Hagey LR, Ekins S. Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR. *Mol Cell Endocrinol*. (2011) 334:39–48. doi: 10.1016/j.mce.2010.06.016
48. Bouillon R, Suda T. Vitamin D: calcium and bone homeostasis during evolution. *BoneKEy Reports*. (2014) 3:480. doi: 10.1038/bonekey.2013.214
49. Kim S, Yamazaki M, Zella LA, Meyer MB, Fretz JA, Shevde NK, et al. Multiple enhancer regions located at significant distances upstream of the transcriptional start site mediate RANKL gene expression in response to 1,25-dihydroxyvitamin D_3. *J Steroid Biochem Mol Biol.* (2007) 103:430–4. doi: 10.1016/j.jsbmb.2006.12.020
50. Vanherwegen AS, Eelen G, Ferreira GB, Ghesquiere B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J Steroid Biochem Mol Biol.* (2019) 187:134–45. doi: 10.1016/j.jsbmb.2018.11.011
51. Newmark H, Dantoft W, Ghazal P. Evolutionary origin of the interferon-immune metabolic axis: the sterol-vitamin D link. *Front Immunol.* (2017) 8:62. doi: 10.3389/fimmu.2017.00062
52. Vanherwegen AS, Gysemans C, Mathieu C. Vitamin D endocrinology on the cross-road between immunity and metabolism. *Mol Cell Endocrinol*. (2017) 453:52–67. doi: 10.1016/j.mce.2017.04.018
53. Carlberg C. Genome-wide. (over)view on the actions of vitamin D. *Front Physiol*. (2014) 5:167. doi: 10.3389/fphys.2014.00167
54. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide. (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D_3. *Faseb J*. (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
55. Novershtern N, Subramanian A, Lawton LN, Mak RH, Haining WN, McConkey ME, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell*. (2011) 144:296–309. doi: 10.1016/j.cell.2011.01.004
56. Barragan M, Good M, Kolls JK. Regulation of dendritic cell function by vitamin D. *Nutrients*. (2015) 7:8127–51. doi: 10.3390/nu7095383
57. Zeitelhofer M, Adzemovic MZ, Gomez-Cabrero D, Bergman P, Hochmeister S, N'Diaye M, et al. Functional genomics analysis of vitamin D effects on $CD4^+$ T cells *in vivo* in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA*. (2017) 114:E1678–E87. doi: 10.1073/pnas.1615783114
58. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA*. (2006) 296:2832–8. doi: 10.1001/jama.296.23.2832
59. Limketkai BN, Mullin GE, Limsui D, Parian AM. Role of vitamin D in inflammatory bowel disease. *Nutr Clin Pract*. (2017) 32:337–45. doi: 10.1177/0884533616674492
60. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: a program of innate immune memory in health and disease. *Science*. (2016) 352:aaf1098. doi: 10.1126/science.aaf1098
61. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*. (2014) 345:1251086. doi: 10.1126/science.1251086
62. Novakovic B, Habibi E, Wang S-Y, Arts Rob JW, Davar R, Megchelenbrink W, et al. β-Glucan reverses the epigenetic state of LPS-Induced immunological tolerance. *Cell*. (2016) 167:1354–68.e14. doi: 10.1016/j.cell.2016.09.034
63. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients*. (2013) 5:2502–21. doi: 10.3390/nu5072502
64. Carlberg C, Seuter S, de Mello VD, Schwab U, Voutilainen S, Pulkki K, et al. Primary vitamin D target genes allow a categorization of possible benefits of vitamin D_3 supplementation. *PLoS ONE*. (2013) 8:e71042. doi: 10.1371/journal.pone.0071042
65. Wilfinger J, Seuter S, Tuomainen T-P, Virtanen JK, Voutilainen S, Nurmi T, et al. Primary vitamin D receptor target genes as biomarkers for the vitamin D_3 status in the hematopoietic system. *J Nutr Biochem*. (2014) 25:875–84. doi: 10.1016/j.jnutbio.2014.04.002
66. Ryynänen J, Neme A, Tuomainen TP, Virtanen JK, Voutilainen S, Nurmi T, et al. Changes in vitamin D target gene expression in adipose tissue monitor the vitamin D response of human individuals. *Mol Nutr Food Res*. (2014) 58:2036–45. doi: 10.1002/mnfr.201400291
67. Saksa N, Neme A, Ryynänen J, Uusitupa M, de Mello VD, Voutilainen S, et al. Dissecting high from low responders in a vitamin D_3 intervention study. *J Steroid Biochem Mol Biol*. (2015) 148:275–82. doi: 10.1016/j.jsbmb.2014.11.012
68. Vukic M, Neme A, Seuter S, Saksa N, de Mello VD, Nurmi T, et al. Relevance of vitamin D receptor target genes for monitoring the vitamin D responsiveness of primary human cells. *PLoS ONE*. (2015) 10:e0124339. doi: 10.1371/journal.pone.0124339
69. Seuter S, Virtanen JK, Nurmi T, Pihlajamäki J, Mursu J, Voutilainen S, et al. Molecular evaluation of vitamin D responsiveness of healthy young adults. *J Steroid Biochem Mol Biol*. (2017) 174:314–21. doi: 10.1016/j.jsbmb.2016.06.003
70. Logie C, Stunnenberg HG. Epigenetic memory: a macrophage perspective. *Semin Immunol*. (2016) 28:359–67. doi: 10.1016/j.smim.2016.06.003
71. Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. *Annu Rev Genomics Hum Genet*. (2013) 14:301–23. doi: 10.1146/annurev-genom-091212-153455

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Vitamin D Receptor Gene Polymorphisms Are Associated With Leprosy in Southern Brazil

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Vitamin D, together with its nuclear receptor (VDR), plays an important role in modulating the immune response, decreasing the inflammatory process. Some polymorphisms of the *VDR* gene, such as *Bsm*I (G>A rs1544410), *Apa*I (G>T rs7975232), and *Taq*I (T>C rs731236) could affect its stability and mRNA transcription activity, while *Fok*I T>C (rs2228570) gives a truncated protein with three fewer amino acids and more efficiency in binding vitamin D. This study evaluated these four polymorphisms in the immunopathogenesis of leprosy in 404 patients and 432 control individuals without chronic or infectious disease in southern Brazil. When analyzing differences in the allele and genotype frequency of polymorphisms between patients (leprosy *per se*, multibacillary, and paucibacillary clinical forms) and controls, we found no statistically significant association. Regarding haplotype analysis, the bAt haplotype was associated with protection from leprosy *per se* ($P = 0.004$, OR = 0.34, CI = 0.16–0.71) and from the multibacillary clinical form ($P = 0.005$, OR = 0.30, CI = 0.13–0.70). In individuals aged 40 or more years, this haplotype has also showed protection against leprosy *per se* and multibacillary (OR = 0.26, CI = 0.09–0.76; OR = 0.26, CI = 0.07–0.78, respectively), while the BAt haplotype was a risk factor for leprosy *per se* in the same age group (OR = 1.34, CI = 1.04–1.73). In conclusion, despite having found no associations between the *VDR* gene polymorphisms with the development of leprosy, the haplotypes formed by the *Bsm*I, *Apa*I, and *Taq*I polymorphisms were associated with leprosy *per se* and the multibacillary clinical form.

Keywords: multibacillary, paucibacillary, genetic polymorphism, case-control studies, gene frequencies, biological markers

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INTRODUCTION

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*, which mainly affects macrophages of the skin, Schwann cells of the peripheral nerve, and eventually other organs and systems (1). Leprosy is among the three neglected diseases with the highest prevalence worldwide. According to the World Health Organization (WHO), in 2017 its incidence reached 210,671 new cases of the disease worldwide. In Brazil, the number of new cases registered in that year was more than 26,000. This sets Brazil at the second place in the ranking of countries with the largest number of leprosy cases in the world (2).

There is considerable clinical variability among leprosy patients once *M. leprae* infection evokes distinct T cell responses in humans. The classification of leprosy according to immunity includes the following clinical forms: tuberculoid (TT), lepromatous (LL), borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL) (3, 4). However, based on the number of skin lesions, the patients can be classified as paucibacillary (PB) and multibacillary (MB) leprosy cases, according to WHO (5, 6). The type of immune response may determine the clinical form, as well as the resistance or susceptibility to the disease. The TT form (also classified as paucibacillary) is characterized by a small number of hypopigmented, well-bordered, anesthetic skin lesions with a low bacillary load, early peripheral nerve impairment, and a T-helper 1 (Th1)-mediated immune response. On the other hand, in form LL (therefore referred to as multibacillary), there is a prevalence of the Th2-mediated immune response, which leads to numerous infiltrated skin lesions displaying high bacillary loads, impaired peripheral nerves, and possible involvement of internal organs (7, 8). However, the predominance of one type of immune response does not mean that cytokines from the other response profiles are not being produced (9).

Many factors can modulate the type of immune response developed by the leprosy patient, such as vaccination with BCG, nutritional status, degree of exposure to *M. leprae*, and infections by other microorganisms. Besides, leprosy susceptibility can also be influenced by genetic factors (10). As vitamin D metabolizing enzymes and vitamin D receptors are present in many cell types, including various immune cells such as antigen-presenting cells, T cells, B cells, and monocytes, these molecules are important targets in the study of polymorphisms that can modulate the immune response against pathogens (11). Among the immunomodulatory roles of vitamin D is the inhibition of MHC class II, CD40, CD80, and CD86, which leads to the blocking of the Th1 response and activation of regulatory T cells (12). Nuclear vitamin D receptor (VDR) is an intracellular polypeptide that binds to the active vitamin D metabolite, 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3), and then interacts with the chromatin, producing a variety of genomic effects (13, 14), such as pleiotropic regulation of human physiology, protection of the cardiac system, cancer prevention, and modulation of the immune system (15, 16). Vitamin D is a direct and indirect regulator of the immune system. The VDR are expressed in T and B cells, dendritic cells, and cells of the monocyte/macrophage lineage (17, 18). Vitamin D acts in suppressing the development of several autoimmune diseases and tissue damage (19–22). The effects of vitamin D on murines and humans include: the development of dendritic cells and T regulatory cells; inhibition of T cell proliferation; inhibition of IFN-γ and IL-17 production; and the induction of IL-4 expression (23, 24). Furthermore, the VDR activation leads to the inhibition of both maturation and proliferation of activated B cells and limits antibody production (25).

The *VDR* locus is at chromosome 12q13.1 and spans over 75 kb of genomic DNA. The human gene has three transcript variants which encode the Vitamin D3 receptor isoforms VDRA and VDRB1. The first exons of the *VDR* gene make up the leader sequence and the exons 2–9 encode the structural portion of the gene product (26–29). There are some polymorphisms located near the 3'UTR region of the *VDR* gene that may affect mRNA stability and translation. These polymorphisms are: *Bsm*I G>A (rs1544410, G allele designated "b" and A allele designated "B"); *Apa*I G>T (rs7975232, G allele designated "a" and T allele designated "A"), and *Taq*I T>C (rs731236, T allele designated "T" and C allele designated "t") (30). Another polymorphism known as *Fok*I T>C (rs2228570, T allele designated "f" and C allele designated "F"), located within the start codon in exon 2 of *VDR*, gives a truncated protein with three fewer amino acids. The F allele gives rise to the variant protein, which is more efficient in mediating vitamin D action (31, 32). Therefore, considering the role that the active metabolite of VDR exert in the mechanisms of immunity, this study evaluated the association of SNPs (single nucleotide polymorphisms) of the *VDR* gene (*Fok*I, *Bsm*I, *Apa*l, and *Taq*I) with the immunopathogenesis and clinical forms of leprosy.

MATERIALS AND METHODS

Study Population

A total of 404 patients (230 men and 174 women) with leprosy from the northwestern region of Paraná, southern Brazil (22° 29′ 30″-26° 42′ 59″ S and 48° 02′ 24″-54° 37′ 38″ W), aged 10–93 years (55.00 ± 13.95), and diagnosed by clinical examination, bacilloscopy, and biopsy were evaluated. Written informed consent was obtained from the participants in this study, including the parents of participants under the age of 16.

In accordance with previous studies, investigations should not be restricted to a sub-analysis of overall leprosy, but should instead contrast multibacillary (MB) and paucibacillary (PB) individuals (6). Thus, patients were reclassified to MB (n = 310) and PB (n = 86). The control group consisted of 432 non-consanguineous individuals from the same region as the patients, and they declared that they did not present any chronic or infectious diseases. Of these, 231 were female and 201 were male, and the age of the controls ranged from 16 to 105 years (50.69 ± 18.07). The characteristics of patients and controls are described in **Table 1**. All participants were classified as a mixed population from southern Brazil, according to the distribution already described in populations of Paraná (33): predominantly of European origin (80.6%), with a smaller contribution of African (12.5%) and Amerindian (7.0%).

Genotyping

The genotyping of the samples with respect to the *VDR* gene polymorphisms: *Fok*I T>C (rs2228570), *Bsm*I G>A (rs1544410), *Apa*I G>T (rs7975232), and *Taq*I T>C (rs731236) was performed by PCR-RFLP (polymerase chain reaction) (34, 35) with modifications. The technique was validated by direct sequencing of 15 samples for each variant for the SNPs, using BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher). Restriction enzymes were used according to the manufacturer's recommendations: *Fok*I # R0109S (BioLabs® Inc), *Taq*I # ER0671 (ThermoFisher), *Apa*I # ER1411 (ThermoFisher), and *Mva*1269I # ER0961 (*Bsm*I) (ThermoFisher). After restriction enzyme

TABLE 1 | Profile of leprosy patients and controls.

Variables		Leprosy *per se* *N* = 404 *n* (%)	PB *N* = 86 *n* (%)	MB *N* = 310 *n* (%)	Controls *N* = 432 *n* (%)
Age	≥40	343 (84.90)	74 (85.05)	263 (84.84)	373 (86.34)
	<40	61 (15.10)	12 (13.95)	47 (15.16)	59 (13.66)
Gender*	Male	230 (56.93)	34 (39.53)	190 (61.29)	201 (46.53)
	Female	174 (43.07)	52 (60.47)	120 (38.71)	231 (53.47)

**Statistically significant difference between leprosy per se patients and controls (P = 0.003, OR = 1.52, 95% CI = 1.15–2.02); PB, Paucibacillary; MB, Multibacillary; N, population size; n, number of individuals; %, percentage.*

digestion, the amplification products were subjected to 2% agarose gel electrophoresis.

Statistical Analysis

The sample size was calculated using the QUANTO software (www.biostats.usc.edu/software), aiming to reach a power of 80%. The SNPStats software (https://www.snpstats.net/start.htm) and OpenEpi program Version 3.01 (https://www.openepi.com/Menu/OE_Menu.htm) were used to determine the allelic, genotypic, and haplotypic frequencies of the *VDR* gene polymorphisms, and to verify the statistical differences between the groups. The association tests were performed for codominant, dominant, recessive, over dominant, and log-additive genetic inheritance models (36). Haplotype frequency estimates were carried out using expectation–maximization algorithms. Odds ratios with 95% confidence intervals were deemed necessary only for significant *P*-values. All tests were carried out using a significance level of 5%. Genotype frequency distributions were evaluated to ensure Hardy-Weinberg equilibrium for all genes in the populations.

RESULTS

In this case-control study, allele and genotype frequency distributions of *Fok*I T>C (rs2228570), *Bsm*I G>A (rs1544410), *Apa*I G>T (rs7975232), and *Taq*I T>C (rs731236) SNPs were analyzed in a total of 404 patients with leprosy *per se* (of these, 310 were classified as multibacillary, 86 as paucibacillary, and 8 individuals were not classified) and 432 controls. The distribution of the genotype frequencies for all analyzed genes was consistent with the Hardy-Weinberg equilibrium ($P > 0.05$). To avoid bias, the gender was used as an adjustment covariate between leprosy *per se* and the control because of the non-pairing between the groups. Differences in the allele and genotype frequency distributions were not observed between patients (leprosy *per se*, MB, and PB clinical forms) and controls in linear analyses in the recessive, dominant or codominant inheritance models. There was also no statistically significant difference when comparing the PB and MB clinical forms. The *Fok*I, *Bsm*I, *Apa*I, and *Taq*I genotype and allele frequency distributions are summarized in **Table 2**.

TABLE 2 | Genotype and allele frequency distributions for *Fok*I, *Bsm*I, *Apa*I, and *Taq*I polymorphisms in leprosy *per se*, PB, and MB clinical forms patients and controls.

Genotype and alleles	Leprosy *per se* *N* = 404 *n* (%)	PB *N* = 86 *n* (%)	MB *N* = 310 *n* (%)	Controls *N* = 432 *n* (%)
***Fok*I (rs2228570)**				
F/F	176 (43.6)	42 (48.8)	127 (41)	191 (44.2)
F/f	168 (41.6)	32 (37.2)	135 (43.5)	195 (45.1)
f/f	60 (14.8)	12 (13.9)	48 (15.5)	46 (10.7)
F	520 (64.4)	116 (67.0)	389 (63.0)	577 (66.8)
f	288 (35.6)	56 (33.0)	231 (37.0)	287 (33.2)
***Bsm*I (rs1544410)**				
B/B	57 (14.1)	12 (13.9)	44 (14.2)	52 (12.0)
B/b	199 (49.3)	44 (51.2)	153 (49.4)	202 (46.8)
b/b	148 (36.6)	30 (34.9)	113 (36.5)	178 (41.2)
B	313 (38.7)	68 (40.0)	241 (39.0)	306 (35.4)
b	495 (61.3)	104 (60.0)	379 (61.0)	558 (64.6)
***Apa*I (rs7975232)**				
A/A	129 (31.9)	30 (34.9)	97 (31.3)	131 (30.3)
A/a	199 (49.3)	43 (50)	151 (48.7)	214 (49.5)
a/a	76 (18.8)	13 (15.1)	62 (20.0)	87 (20.1)
A	457 (56.6)	103 (60.0)	345 (56.0)	476 (55.1)
a	351 (43.4)	69 (40.0)	275 (44.0)	388 (44.9)
***Taq*I (rs731236)**				
T/T	153 (37.9)	32 (37.2)	115 (37.1)	184 (42.6)
T/t	203 (50.2)	44 (51.2)	158 (51)	198 (45.8)
t/t	48 (11.9)	10 (11.6)	37 (11.9)	50 (11.6)
T	509 (63.0)	108 (63.0)	388 (63.0)	566 (65.5)
t	299 (37.0)	64 (37.0)	232 (37.0)	298 (34.5)

PB, Paucibacillary; MB, Multibacillary; N, population size; n, number of individuals; %, percentage.

The *Bsm*I, *Apa*I, and *Taq*I polymorphisms were in linkage disequilibrium (D = 0.93, 0.83, and 0.92, respectively). When we evaluated the influence of the haplotypes formed by the *Bsm*I, *Apa*I, and *Taq*I polymorphisms of the *VDR* gene, the haplotype bAt was associated with protection against leprosy *per se* and the MB clinical form ($P = 0.004$, OR = 0.34, CI = 0.16–0.71; $P = 0.005$, OR = 0.30, CI = 0.13–0.70, respectively), as shown in **Table 3**. When the haplotype was associated with age, the bAt haplotype showed protection against leprosy *per se* and MB in individuals aged 40 or more years (OR = 0.26, CI = 0.09–0.76; OR = 0.24, CI = 0.07–0.78, respectively). Whereas, the BAt haplotype was a risk factor for leprosy *per se* in the same age group (OR = 1.34, CI = 1.04–1.73). The haplotype and age cross-classification interaction are shown in **Table 4**.

DISCUSSION

This case-control study investigated the genotypic and allelic frequencies of certain *VDR* gene polymorphisms in leprosy patients and controls without the disease, in order to evaluate whether these polymorphisms could act as factors of

TABLE 3 | Haplotypes formed by the *Bsm*I, *Apa*I, and *Taq*I polymorphisms of the *VDR* gene evaluated in all individuals (leprosy *per se* patients and controls) and MB clinical form (MB patients and controls).

***Bsm*I, *Apa*I, and *Taq*I**	**All individuals frequencies**	***P***	**OR (95% CI)**	**MB and controls frequencies**	***P***	**OR (95% CI)**
Haplotypes	***N* = 836**			***N* = 742**		
baT	0.4207	Ref.		0.4229	Ref.	
BAt	0.3167	0.05		0.3137	0.09	
bAT	0.1719	0.6		0.1686	0.38	
BAT	0.0416	0.06		0.0414	0.036	0.53 (0.29–0.96)*
bAt	0.0279	0.004	0.34 (0.16–0.71)	0.0294	0.005	0.30 (0.13–0.70)
Rare	0.0213	0.001	0.13 (0.04–0.45)	0.0238		

*MB, Multibacillary; N, population size; P, P value; OR, odds ratio; CI, confidence interval. *This result was disregarded because the CI is so near to one.*

TABLE 4 | Haplotypes formed by the *Bsm*I, *Apa*I, and *Taq*I polymorphisms of the *VDR* gene evaluated in all individuals (leprosy *per se* patients and controls) and MB clinical form (MB patients and controls) and age cross-classification interaction.

	All individuals frequencies			**MB and controls frequencies**		
***Bsm*I, *Apa*I, and *Taq*I**		**Age < 40**	**Age ≥ 40**		**Age < 40**	**Age ≥ 40**
Haplotypes	**(*N* = 836)**	**OR (95% CI)**	**OR (95% CI)**	**(*N* = 742)**	**OR (95% CI)**	**OR (95% CI)**
baT	0.4207	Ref.		0.4229	Ref.	
BAt	0.3167			0.3138		1.34 (1.04–1.73)
bAT	0.1719			0.1686		
TAB	0.0416			0.0414		
bAt	0.0279		0.26 (0.09–0.76)	0.0294		0.24 (0.07–0.78)
rare	0.0213			0.0238		

MB, Multibacillary; N, population size; P, P value; OR, odds ratio; CI, confidence interval.

susceptibility or resistance to the disease or to a specific clinical form. When analyzing differences in the allele and genotype frequency distributions between patients (leprosy *per se*, MB, and PB clinical forms) and controls, we found no statistically significant association.

Consistent with our results, other studies have also found no association between the *Apa*I polymorphism and leprosy (37). The *Bsm*I polymorphism has also showed no statistically significant association with leprosy in a couple of studies (37, 38). A recent meta-analysis of this polymorphism and tuberculosis showed that the b-allele was a risk factor for disease development, but this was only observed in the Asian population (39). Regarding *Bsm*I, *Apa*I, and *Taq*I polymorphisms, it is not clear whether they have an individual effect on the expression or function of the VDR. It is possible that the associations found for these polymorphisms in the various diseases studied, if they actually exist, occurred due to a linkage disequilibrium with a polymorphism that has a functional effect on these diseases (37). A recent systematic review of VDR and leprosy suggests that such a large diversity of results would be a consequence of ethnic heterogeneity, sample size used, design of each study, and also influences from other regions of the gene that have not been studied yet, as well as the likelihood of bacillus virulence being distinct in the different geographic regions where the studies took place (40).

The results concerning haplotypes are in agreement with the hypothesis that the *Bsm*I, *Apa*I, and *Taq*I polymorphisms are not directly related to leprosy. However, it is a significant result because it shows that these SNPs may be in linkage disequilibrium with another functional polymorphism. We have analyzed five haplotype alleles in our study, of which haplotypes 1 (baT; 42%), 2 (BAt; 32%), and 3 (bAT; 17%) were the most frequent and were similar to the ones identified by Uitterlinden et al. (41), haplotype 1 (baT; 48%), 2 (BAt; 39%), and 3 (bAT; 11%). Despite our finding of an association of the haplotype bAt with protection against leprosy *per se* and the MB clinical form, there is no evidence in the literature about the influence of this haplotype in any disease. Moreover, this association could be due to the low frequency of this haplotype in our individuals.

In contrast, the haplotype 2 (BAt) was associated with risk to leprosy *per se* when the patients were divided by age. Although studies do not agree on which VDR haplotypes are related to susceptibility or protection for various diseases, the fact that BAt shows susceptibility to leprosy can be explained due to a strong linkage disequilibrium between the *Bsm*I, *Apa*I, and *Taq*I haplotypes and the poly(A) variable number of tandem repeats (VNTRs) in the 3′UTR of the *VDR* gene. The poly(A) VNTR polymorphism can be characterized as bi-allelic, and subjects can be classified as having alleles with short or long poly(A) stretches. There is a strong linkage between the haplotype 1 (baT)

and the long poly(A) stretch (n = 18–24, long or L alleles), while the haplotype 2 (BAt) is in linkage to the short poly(A) stretch (n = 13–17, short or S alleles). There seems to be a trend for the BAt haplotype to display overall somewhat higher levels of mRNA expression than the baT haplotype. This could be due to a slightly higher mRNA stability and half-life, which would result in higher numbers of VDRs being present in the target cell and better response to vitamin D. Although we can assume that mRNA stability differences might be related to allelic differences, the results are not consistent among the studies (30, 41–43).

Low levels of expression, VDR stability and vitamin D3-VDR interaction were related to leprosy and its complications (44). It has been shown that some individuals with normal levels of vitamin D3, but with low levels of VDR protein, had a high bacilloscopic index and type 2 reaction (45). This fact reinforces the results obtained in the present study, which relate both the polymorphism that changes the interaction vitamin D3-VDR (*Fok*I) and the haplotype formed by the *Bsm*I, *Apa*I, and *Taq*I polymorphisms. Although, the *FokI* polymorphism has a higher consistency of results related to VDR and leprosy, the *Bsm*I, *Apa*I, and *Taq*I polymorphisms, especially the haplotype formed by them, should be further investigated in the immunopathogenesis of leprosy.

DATA AVAILABILITY

This manuscript contains previously unpublished data. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This work was approved by the Permanent Committee of Ethics in Research with Human Beings of the State University of Maringá (COPEP—UEM) n° 2.424.046/2017.

AUTHOR CONTRIBUTIONS

AP performed the experiments and drafted the manuscript. HA, BT, and LM analyzed the data and drafted the manuscript. LV participated in the critical revision of the manuscript. QL and JZ analyzed the data, drafted the manuscript, and participated in the critical revision. AS provided materials and participated in the experimental design. JV designed the study and finalized the manuscript.

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REFERENCES

1. Eichelmann K, González SE, Salas-Alanis JC, Ocampo-Candiani J. Leprosy. An update: definition, pathogenesis, classification, diagnosis, and treatment. *Actas Dermosifiliogr*. (2013) 104:554–63. doi: 10.1016/j.adengl.2012.03.028
2. World Health Organization. Global leprosy update, 2017: reducing the disease burden due to leprosy. *Week Epidemiol Rec*. (2018) 93:445–56.
3. Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis*. (1966) 34:255–73.
4. Britton WJ, Lockwood DN. Leprosy. *Lancet*. (2004) 363:1209–19. doi: 10.1016/S0140-6736(04)15952-7
5. Souza CS. Hanseníase: formas clínicas e diagnóstico. *Rev Med Ribeirão Preto*. (1997) 30:325–34. doi: 10.11606/issn.2176-7262.v30i3p325-334
6. Gaschignard J, Grant AV, Thuc NV, Orlova M, Cobat A, Huong NT, et al. Pauci- and multibacillary leprosy: two distinct, genetically neglected diseases. *Plos Negl Trop Dis*. (2016) 10:e0004345. doi: 10.1371/journal.pntd.0004345
7. Modlin RL. Th1-Th2 paradigm: insights from leprosy. *J Invest Dermatol*. (1994) 102:828–32. doi: 10.1111/1523-1747.ep12381958
8. Sadhu S, Mitra DK. Emerging concepts of adaptive immunity in leprosy. *Front Immunol*. (2018) 9:604. doi: 10.3389/fimmu.2018.00604
9. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL. The continuing challenges of leprosy. *Clin Microbiol Rev*. (2006) 19:338–81. doi: 10.1128/CMR.19.2.338-381.2006
10. Mazini PS, Alves HV, Reis PG, Lopes AP, Sell AM, Santos-Rosa M, et al. Gene association with leprosy: a review of published data. *Front Immunol*. (2016) 6:658. doi: 10.3389/fimmu.2015.00658
11. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients*. (2013) 5:2502–21. doi: 10.3390/nu5072502
12. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system. *Nat Rev Immunol*. (2008) 8:685–98. doi: 10.1038/nri2378
13. Pike JW. Intracellular receptors mediate the biologic action of 1,25-dihydroxyvitamin D3. *Nutr Rev*. (1985) 43:161–8. doi: 10.1111/j.1753-4887.1985.tb02406.x
14. Haussler MR. Vitamin D receptors: nature and function. *Annu Rev Nutr*. (1986) 6:527–62. doi: 10.1146/annurev.nu.06.070186.002523
15. DeLuca HF. Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr*. (2004) 80:1689S—96S. doi: 10.1093/ajcn/80.6.1689S
16. Karatay S, Yildirim K, Karakuzu A, Kiziltunc A, Engin RI, Eren YB, et al. Vitamin D status in patients with Behcet's disease. *Clinics*. (2011) 66:721–3. doi: 10.1590/s1807-59322011000500002
17. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin d3 receptor in the immune system. *Arch Biochem Biophys*. (2000) 374:334–8. doi: 10.1006/abbi.1999.1605
18. Mocanu V, Oboroceanu T, Zugun-Eloae F. Current status in vitamin D and regulatory T cells immunological implications. *Rev Med Chir Soc Med Nat*. (2013) 117:965–73.
19. Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J Nutr*. (1995) 125:1704S—8S. doi: 10.1016/0960-0760(95)00106-A
20. Penna G, Amuchastegui S, Cossetti C, Aquilano F, Mariani R, Sanvito F, et al. Treatment of experimental autoimmune prostatitis in nonobese diabetic mice by the vitamin D receptor agonist elocalcitol. *J Immunol*. (2006) 177:8504–11. doi: 10.4049/jimmunol.177.12.8504

21. Joshi S, Pantalena LC, Liu XK, Gaffen SL, Liu H, Rohowsky-Kochan C, et al. 1,25-Dihydroxyvitamin D3 ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol Cell Biol*. (2011) 31:3653–69. doi: 10.1128/MCB.05020-11
22. Dankers W, Colin EM, van Hamburg JP, Lubberts E. Vitamin D in autoimmunity: molecular mechanisms and therapeutic potential. *Front Immunol*. (2017) 7:697. doi: 10.3389/fimmu.2016.00697
23. Cantorna MT, Snyder L, Lin YD, Yang L. Vitamin D and $1,25(OH)_2D$ regulation of T cells. *Nutrients*. (2015) 7:3011–21. doi: 10.3390/nu7043011
24. Saul L, Mair I, Ivens A, Brown P, Samuel K, Campbell JDM, et al. 1,25-Dihydroxyvitamin D3 restrains CD4+ T cell priming ability of CD11c+ dendritic cells by upregulating expression of CD31. *Front Immunol*. (2019) 10:600. doi: 10.3389/fimmu.2019.00600
25. Røsjø E, Lossius A, Abdelmagid N, Lindstrøm JC, Kampman MT, Jørgensen L, et al. Effect of high-dose vitamin D3 supplementation on antibody responses against Epstein-Barr virus in relapsing-remitting multiple sclerosis. *Mult Scler*. (2017) 23:395–402. doi: 10.1177/1352458516654310
26. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, et al. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci USA*. (1988) 85:3294–8. doi: 10.1073/pnas.85.10.3294
27. Miyamoto K, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, et al. Structural organization of the human vitamin D receptor chromosomal gene and its promoter. *Mol Endocrinol*. (1997) 11:1165–79. doi: 10.1210/mend.11.8.9951
28. Crofts LA, Hancock MS, Morrison NA, Eisman JA. Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci USA*. (1998) 95:10529–34. doi: 10.1073/pnas.95.18.10529
29. Yang CY, Leung PS, Adamopoulos IE, Gershwin ME. The implication of vitamin D and autoimmunity: a comprehensive review. *Clin Rev Allergy Immunol*. (2013) 45:217–26. doi: 10.1007/s12016-013-8361-3
30. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, et al. Prediction of bone density from vitamin D receptor alleles. *Nature*. (1994) 367:284–7. doi: 10.1038/367284a0
31. Alimirah F, Peng X, Murillo G, Mehta RG. Functional significance of vitamin D receptor FokI polymorphism in human breast cancer cells. *PLoS ONE*. (2011) 6:e16024. doi: 10.1371/journal.pone.0016024
32. Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, et al. A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *J Bone Miner Res*. (1997) 12:915–21. doi: 10.1359/jbmr.1997.12.6.915
33. Probst CM, Bompeixe EP, Pereira NF, de O Dalalio MM, Visentainer JE, Tsuneto LT, et al. HLA polymorphism and evaluation of European, African, and Amerindian contribution to the white and mulatto populations from Parana, Brazil. *Hum Biol*. (2000) 72:597–617. Available online at: https://www.jstor.org/stable/41465861
34. Nemenqani DM, Karam RA, Amer MG, Abd El Rahman TM. Vitamin D receptor gene polymorphisms and steroid receptor status among Saudi women with breast cancer. *Gene*. (2015) 558:215–9. doi: 10.1016/j.gene.2014.12.065
35. Papadopoulou A, Kouis P, Middleton N, Kolokotroni O, Karpathios T, Nicolaidou P, et al. Association of vitaminD receptor gene polymorphisms and vitamin D levels with asthma and atopy in Cypriot adolescents: a case–control study. *Multidiscip Respir Med*. (2015) 10:26. doi: 10.1186/s40248-015-0025-0
36. Sole X, Guino E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. *Bioinformatics*. (2006) 22:1928–9. doi: 10.1093/bioinformatics/btl268
37. Fitness J, Floyd S, Warndorff DK, Sichali L, Mwaungulu L, Crampin AC, et al. Large-scale candidate gene study of leprosy susceptibility in the Karonga district of northern Malawi. *Am J Trop Med Hyg*. (2004) 71:330–40. doi: 10.4269/ajtmh.2004.71.330
38. Sapkota BR, Macdonald M, Berrington WR, Misch EA, Ranjit C, Siddiqui MR, et al. Association of TNF, MBL, and VDR polymorphisms with leprosy phenotypes. *Hum Immunol*. (2010) 71:992–8. doi: 10.1016/j.humimm.2010.07.001
39. Areeshi MY, Mandal RK, Dar SA, Alshahrani AM, Ahmad A, Jawed A, et al. A reappraised meta-analysis of the genetic association between vitamin D receptor *Bsm*I (rs1544410) polymorphism and pulmonary tuberculosis risk. *Biosci Rep*. (2017) 37:BSR20170247. doi: 10.1042/BSR20170247
40. Oliveira ALG, Chaves AT, Menezes CAS, Guimarães NS, Bueno LL, Fujiwara RT, et al. Vitamin D receptor expression and hepcidin levels in the protection or severity of leprosy: a systematic review. *Microbes Infect*. (2017) 19:311–22. doi: 10.1016/j.micinf.2017.03.001
41. Uitterlinden AG, Fang Y, Meurs JBJV, Pols HAP. Chapter 68 - Genetic vitamin D receptor polymorphisms and risk of disease. In: Feldman D, Wesley Pike J, Glorieux F, editors. *Vitamin D*. 2nd ed. London, UK: Academic Press (2005). p. 1121–57. doi: 10.1016/B978-012252687-9/50071-1
42. Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP. Genetics and biology of vitamin D receptor polymorphisms. *Gene*. (2004) 338:143–56. doi: 10.1016/j.gene.2004.05.014
43. Durrin LK, Haile RW, Ingles SA, Coetzee GA. Vitamin D receptor 3′-untranslated region polymorphisms: lack of effect on mRNA stability. *Biochim Biophys Acta*. (1999) 1453:311–20. doi: 10.1016/S0925-4439(99)00007-1
44. Singh I, Lavania M, Pathak VK, Ahuja M, Turankar RP, Singh V, et al. VDR polymorphism, gene expression and vitamin D levels in leprosy patients from North Indian population. *PLoS Negl Trop Dis*. (2018) 12:e0006823. doi: 10.1371/journal.pntd.0006823
45. Mandal D, Reja AH, Biswas N, Bhattacharyya P, Patra PK, Bhattacharya B. Vitamin D receptor expression levels determine the severity and complexity of disease progression among leprosy reaction patients. *New Microbes New Infect*. (2015) 6:35–9. doi: 10.1016/j.nmni.2015.04.001

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MINI REVIEW
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Regulation of the Immune Balance During Allogeneic Hematopoietic Stem Cell Transplantation by Vitamin D

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One of the most promising therapeutic approaches for numerous hematological malignancies represents the allogeneic hematopoietic stem cell transplantation (allo-HSCT). One major complication is the development of the life-threatening graft-vs.-host disease (GvHD) which limits beneficial effects of graft-vs.-leukemia (GvL) responses during allo-HSCT. Strengthening GvL effects without induction of severe GvHD is essential to decrease the relapse rate after allo-HSCT. An interesting player in this context is vitamin D_3 since it has modulatory capacity in both preventing GvHD and boosting GvL responses. Current studies claim that vitamin D_3 induces an immunosuppressive environment by dendritic cell (DC)-dependent generation of regulatory T cells (Tregs). Since vitamin D_3 is known to support the antimicrobial defense by re-establishing the physical barrier as well as releasing defensins and antimicrobial peptides, it might also improve graft-vs.-infection (GvI) effects in patients. Beyond that, alloreactive T cells might be attenuated by vitamin D_3-mediated inhibition of proliferation and activation. Despite the inhibitory effects of vitamin D_3 on T cells, anti-tumor responses of GvL might be reinforced by vitamin D_3-triggered phagocytic activity and antibody-based immunotherapy. Therefore, vitamin D_3 treatment does not only lead to a shift from a pro-inflammatory toward a tolerogenic state but also promotes tumoricidal activity of immune cells. In this review we focus on vitamin D_3 and its immunomodulatory effects by enhancing anti-tumor activity while alleviating harmful allogeneic responses in order to restore the immune balance.

Keywords: vitamin D, GvH, GvL, immune balance, macrophages, T cells, infection

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INTRODUCTION

The most promising curative therapeutic strategy for a broad spectrum of hematological malignancies remains the allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1). Its efficacy is mainly mediated by alloreactive donor-derived immune cells eliminating malignant host cells, a process known as graft-vs.-leukemia (GvL) effect (2). However, infused donor cells can also attack healthy host tissues due to histocompatibility mismatches, which leads to graft-vs.-host disease (GvHD). This life-threatening complication limits the beneficial effects mediated by GvL. Restoring the host's immune balance during and after transplantation is one of the major

challenging obstacles in clinical research (3). Alleviating GvHD responses while boosting anti-leukemia activities could be the key to successful treatment in allo-HSCT. Since both processes underlie more or less the same T cell activity, it is very demanding to dissect GvHD from GvL effects (4). The current standard treatment consists mainly of corticosteroids and calcineurin inhibitors such as cyclosporine and tacrolimus (5). Since these immunosuppressive drugs attenuate T-cell-mediated inflammation (6) and the allo-stimulatory capacity of DCs (7), they lead to alleviation of GvHD symptoms. However, these immunosuppressive mechanisms might reduce GvL effects as well. Recent studies have established promising strategies for strengthening GvL responses without exacerbating GvHD. Infusion of donor lymphocytes, CAR-T cells and checkpoint inhibitors have gained pivotal interest in clinical studies over the past years (8). However, none of these therapeutic approaches target both GvHD and GvL.

Though vitamin D_3 has been discovered as an important regulator of calcium homeostasis in the early Twentieth century, its putative immunoregulatory role remained undiscovered until recently (9). Contrary to initial assumptions that vitamin D_3 is mainly produced in kidney and liver, vitamin D_3 receptor (VDR) and vitamin D_3 metabolizing enzymes are also expressed in various types of immune cells (10–12). The novel role of vitamin D_3 in regulating effector functions of human macrophages is closely linked to the expression of the vitamin D-1-hydroxylase CYP27B1. The precursor form of vitamin D_3 is produced in the epidermis upon ultraviolet B (UVB) irradiation or obtained from dietary intake (13). Vitamin D_3-binding protein (VDBP) binds pre-vitamin D_3 and is responsible for its transport into the liver. Upon entering the cell, CYP27B1 catalyzes the conversion of 25-hydroxy-vitamin D_3 ($25(OH)D_3$) into its bioactive form 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$, calcitriol) (14). Levels of $1,25(OH)_2D_3$ are regulated by the inactivating $1,25(OH)_2D_3$ 24-hydroxylase (CYP24A1). $1,25(OH)_2D_3$ binds intracellularly to VDR and induces as a transcription factor the expression of a broad variety of target genes which contain vitamin D_3 response elements (VDRE) within their promoters (15) (**Figure 1**).

Since vitamin D_3 is well-known for exerting both anti-tumoricidal and anti-inflammatory functions, it might be an attractive target for preservation of the immune balance in patients undergoing allo-HSCT (16). In this review, we seek to elucidate mechanisms by which vitamin D_3 might act as potential immune regulator in GvL as well as GvHD while highlighting its effects on both innate and adaptive immune system.

VITAMIN D_3 AND GvHD

At the beginning of the Twenty-first century, vitamin D_3 has gained more attention in the field of allo-HSCT. Given that vitamin D_3 exerts "non-classical" actions besides sustaining bone metabolism and calcium homeostasis, paved the way for pioneering studies which proved that vitamin D_3 deficiency correlates directly with immune diseases such as multiple sclerosis (MS) (17), systemic lupus erythematosus (18), inflammatory bowel disease (IBD) (19), rheumatoid arthritis (20), and autoimmune thyroid disease (21). Vitamin D_3 supplementation has been shown to reduce severity and incidence of such diseases not only in animal models but also in clinical studies (22). Based on studies which show that application of several vitamin D_3 analogs has been effective in some solid organ transplantations (23–25), Pakkala and colleagues successfully achieved prevention of GvHD symptoms in a rat transplantation model by a $1,25(OH)_2D_3$ analog (MC1288) (26). Further investigations proved that certain VDR polymorphisms are associated with higher risk of severe GvHD (27–29). Since patients receiving HSCT are malnourished, less exposed to sunlight and have an altered vitamin D_3 metabolism due to medications and impaired organ function, they are predestined for vitamin D_3 deficiency (30). In fact, Kreutz et al. demonstrated that conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ is impaired in GvHD patients and that $25(OH)D_3$ serum levels were lower in grade III-IV than in grade I-II GvHD patients (31). The high prevalence of low vitamin D3 levels in patients undergoing HSCT is reported in other studies as well and might also be associated with a higher incidence of GvHD (32–34). These findings suggest a pivotal protective role of vitamin D_3 in GvHD pathogenesis. Recently, Chen and Mayne reviewed the immunomodulatory effects of vitamin A and D in the context of GvHD (35). In the following, vitamin D_3 will be analyzed briefly as an important modulator of both innate and adaptive immune system.

Molecular Actions of Vitamin D_3 in the Innate Immune System of GvHD Patients

Antimicrobial Activities

Although the precise mechanisms of vitamin D_3 remained unclear for a long time, patients infected with *Mycobacterium tuberculosis* (*Mtb*) have been treated with UVB irradation and cod liver oil in the pre-antibiotic era (36, 37). In 1980, Rook and colleagues could evidence that growth of *Mtb* was impeded *in vitro* by $1,25(OH)_2D_3$ in human monocytes and macrophages (38, 39). Since then, it became increasingly clear that vitamin D_3 exerts anti-microbial effects (40). Subsequent studies demonstrated that $1,25(OH)_2D_3$ leads to release of anti-microbial peptides such as LL-37 and β-defensin (41–43). LL-37 is the cleavage product of human cathelicidin antimicrobial peptide (hCAP18, CAMP) and is known for its antibacterial function by inducing bacterial lysis and death (44). Upon infection, lung epithelial cells locally produce $1,25(OH)_2D_3$ which in turn enhances LL-37 expression (45). Cathelicidin-deficient mice have been shown to be more susceptible to infections with *Streptococcus*, *Pseudomonas,* and *E. coli* (46). Cathelicidin does not only increase phagocytic activity of macrophages (47) but also promotes reactive oxygen species (ROS) production (48, 49), leading to direct antimicrobial effects. Moreover, cathelicidin triggers autophagy and reactivates phagolysosomal fusion in macrophages, which enhances degradation of intracellular pathogens such as *Mtb*, *Salmonella,* and *Coxiella* (14, 50). Even viral infections with influenza A (51) or fungal infections with *Candida albicans* (52) in mice are reduced by cathelicidin. Accumulating data

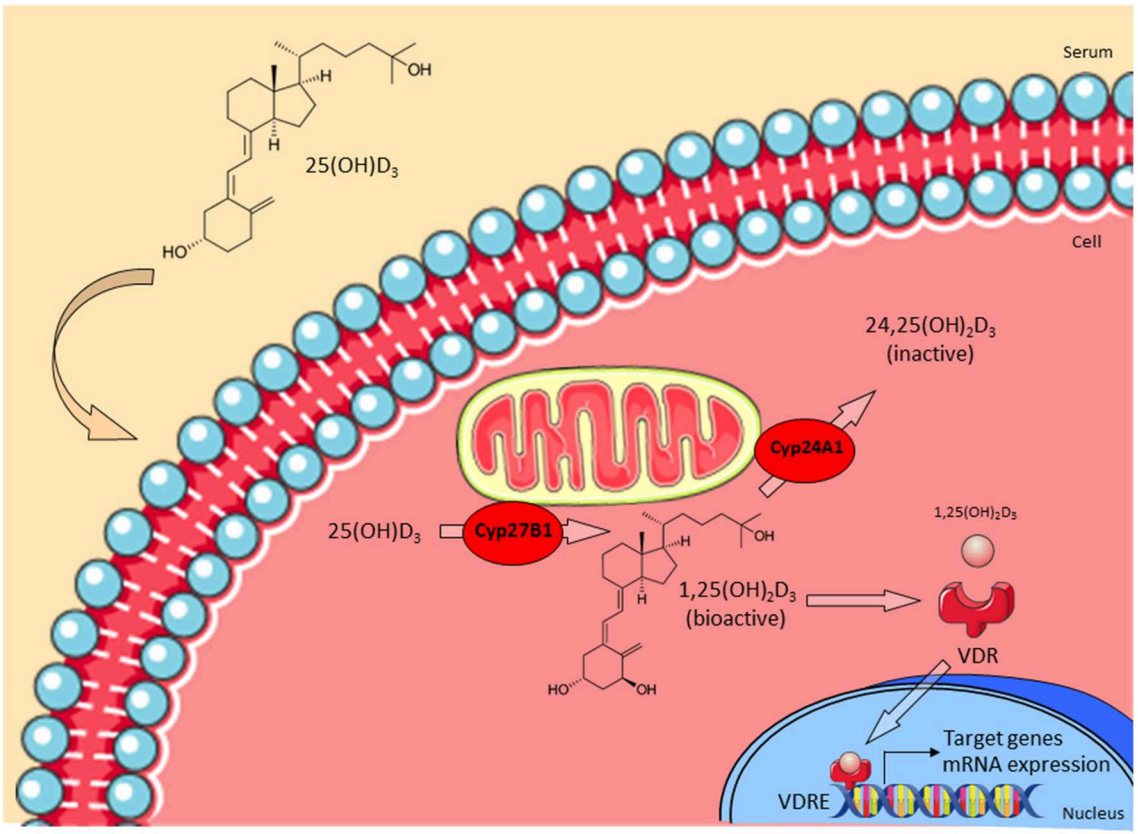

FIGURE 1 | Vitamin D_3 metabolism. The precursor form of vitamin D_3 25(OH)D_3 enters the cell and is then converted into the bioactive form 1,25$(OH)_2D_3$ by CYP27B1. CYP24A1 regulates levels of 1,25$(OH)_2D_3$ by converting it into the inactive 24,25$(OH)_2D_3$. Active 1,25$(OH)_2D_3$ binds to the vitamin D_3 receptor (VDR) in the cytoplasm and this complex translocates into the nucleus. Finally, VDR binds to appropriate vitamin D response elements (VDRE) and triggers transcription of target genes (e.g., LL-37). Adapted from Bruns and Stenger (14).

have revealed that the intestinal barrier is supported by vitamin D_3-dependent upregulation of tight junction proteins (53, 54), which is a fundamental requirement for efficient defense against pathogens. The loss of intestinal barrier function is also considered to be a driving factor for GvHD development (55). Thus, vitamin D_3-dependent release of cathelicidin and the protection of epithelial barriers might improve graft- vs.-infection (GVI) effects in allo-HSCT patients.

Recent studies have now discovered a novel role of LL-37 in cancer (56) and inflammatory diseases (57). Strikingly, LL-37 does not only possess anti-microbial but also anti-inflammatory features, since it has been shown to inhibit the release of pro-inflammatory mediators such as TNF-α, IL-6, and IL-8 by neutrophils (48). Additionally, cathelicidin reduces mortality in mice infected with *P. aeruginosa* by neutralizing endotoxin-mediated inflammation (58).

Hence, vitamin D_3-triggered activity of cathelicidin links anti-microbial and anti-inflammatory effects in the innate immune system. Since GvHD patients have an increased risk for severe infections due to immunosuppressive drugs (59), vitamin D_3-mediated enhancement of antimicrobial defense mechanisms might reduce co-morbidity by infectious diseases. Therefore, it is conceivable that vitamin D_3 might play an important and yet unrecognized role in GVI.

Anti-inflammatory Effects

As already mentioned, vitamin D_3 elicits not only antimicrobial but also anti-inflammatory responses. Even though vitamin D_3 enhances the maturation of human macrophages and their function as phagocytes (60), their capacity of antigen presentation and consequently also the priming of T cells is limited due to reduction of MHC-II expression (30, 61). Instead, 1,25$(OH)_2D_3$ polarizes macrophages toward an anti-inflammatory M2 subtype, which restrains colitis in mice (62). In humans and mice, vitamin D_3 generates a tolerogenic phenotype and alters the cytokine and chemokine profile of mature DCs (mDCs) *in vivo* and *in vitro*, which are inhibited in differentiation, maturation and proliferation (63–65). In mixed lymphocyte reactions, proliferation of T cells, co-cultured with these 1,25$(OH)_2D_3$-induced tolerogenic DCs, was indirectly inhibited. Apart from preventing DCs to home into the lymph node by reducing CCR7-expression, vitamin D_3 also decreases expression of the co-stimulatory molecules CD40, CD80 and

CD86 and secretion of cytokines such as IL-6, IL-12, and TNF-α (66). Recently, Saul and colleagues revealed that CD31 is increasingly expressed on DCs, leading to impairment of cell-cell contact, which is essential for T cell priming (67). Moreover, secretion of immunosuppressive IL-10 is enhanced while IL-12 secretion by DCs is impaired, which leads to a weaker T helper Th1- and Th17- cell immune response (68). As a result, activation and differentiation of alloreactive $CD4^+$ T cells is reduced *in vitro* (65). Furthermore, vitamin D_3-treated DCs increase the frequency of suppressive $CD4^+CD25^+FoxP_3^+$ regulatory T cells (Treg) (69), which fosters peripheral tolerance to allografts (70). One study indicated that vitamin D_3-mediated increase of $CD4^+FoxP_3^+Nrp\text{-}1^+$ cells ameliorates collagen-induced arthritis (71). Recently, Xu and colleagues established engineered DCs to *de novo* produce calcitriol in order to generate more gut-homing Tregs for efficient mitigation of intestinal inflammation (72). These results proved that $1,25(OH)_2D_3$-induced tolerogenic DCs modulate T cells toward a regulatory and anti-inflammatory immune response *in vivo* and ameliorate acute GvHD (aGvHD) in mice (64, 73). Coussens and colleagues suggest that vitamin D_3 supplementation in tuberculosis patients helps to restrict inflammatory responses by reducing circulating concentrations of chemokines such as CXCL9, CXCL10, and MMP-9 (74, 75). Additionally, upregulation of chemokine receptor CXCR3 fosters DC migration to inflammation spots (69). *In vitro* studies showed that Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling and inflammatory cytokines, such as IFN-γ, TNF-α, and Flt-3L, are significantly reduced in NK-cells upon vitamin D_3-treatment or its analog seocalcitol (EB1089) (76). Interestingly, JAK1/2 have already been identified as potential therapeutic targets in GvHD since it was shown to reduce GvHD in mice while GvT could be preserved (77). Clinical trials verified that the JAK1/2 inhibitor Jakafi® (Ruxolitinib) reduces efficiently steroid-refractory GvHD (78, 79) and has recently been approved by the U.S. Food and Drug Administration (FDA).

Altogether, vitamin D_3 modifies the innate immune system by exerting not only anti-microbial but also anti-inflammatory functions. Since GvHD patients often show co-morbidity of fungal, viral and bacterial infections (80, 81), increased infection rate as well as exaggerated inflammation are key issues needed to be combatted in this disease. Since persistence of APCs despite the conditioning regimen is the major cause of generation of alloreactive lymphocyte, manipulation of the innate immune system toward tolerogenic host-DCs by vitamin D_3 in order to reduce their allo-stimulatory potential might help to prevent GvHD (82).

Effects of Vitamin D_3 on the Adaptive Immune System

Apart from the above discussed indirect effects on T cells by vitamin D_3-dependent modulation of innate immune cells, the hormone has also direct impacts on the adaptive immune system since T cells are known to express VDR, which enables them to respond to $1,25(OH)_2D_3$ (83). Although the VDR appeared to be upregulated in activated alloreactive T cells indicating a role of vitamin D_3 in T cell activation (30), studies proved that $1,25(OH)_2D_3$ directly inhibits proliferation and IL-2 production of $CD4^+$ T cells (84, 85). Similar to its effect on APCs, $1,25(OH)_2D_3$ reduces expression of homing receptors such as CCR10 as well as secretion of IFN-γ and IL-10 by T cells (86). Especially Th1 cell proliferation is inhibited via the JAK/STAT signaling pathway (87), while Th2 cells are increased directly (88, 89). Therefore, vitamin D_3 alters the T cell immunity by transforming Th1- and Th17-responses toward an anti-inflammatory Th2-activity. This mechanism is even amplified since expression of CYP27B1 is also enhanced in activated lymphocytes (30). $CD8^+$ T cells are inhibited in proliferation *in vitro* and *in vivo* by vitamin D_3 (90). It is documented that vitamin D_3 inhibits pro-inflammatory T cells in IBD patients (91). Since IBD pathogenesis is driven by loss of intestinal barrier function, clinical manifestations of IBD resemble GvHD symptoms in the gastrointestinal tract (55). Such parallels suggest that vitamin D_3 might achieve similar effects in allo-HSCT. However, $1,25(OH)_2D_3$ does not only affect T cells but also modulates differentiation and antibody-production of B cells (92). In addition, it induces apoptosis and cell cycle arrest of proliferating B cells resulting in impaired plasma cell differentiation and less autoantibody expression (93).

Altogether, vitamin D_3 has an overall anti-microbial and anti-inflammatory effect on both innate and adaptive immune system. Therefore, vitamin D_3 could be a potent supplementary agent in GvHD patients which might improve the patient's life quality by decreasing infectious- and inflammation-mediated co-morbidity.

POTENTIAL GvL-EFFECTS MEDIATED BY VITAMIN D_3

As mentioned earlier, it is pivotal to preserve the immune balance by avoiding alloreactivity of donor T cells against healthy tissue while still maintaining their anti-tumorigenic effect. Interestingly, vitamin D_3 does not only reduce harmful GvHD effects but also exerts anti-tumor activity. So far, scientific literature supporting this assumption in the transplantation setting remains sparse. However, a few studies provide indications for its hypothetical anti-cancer effects. In their retrospective study, Radujkovic et al. could demonstrate that pre-transplant vitamin D_3 deficiency in patients diagnosed with myeloid malignancies correlates with a higher risk of relapse mortality (94). To our knowledge, only three other studies have also investigated this association (90, 95, 96). So far, only one study included few patients which underwent autologous transplantation (97). However, they only figured out that sufficient vitamin D_3 levels are hard to achieve. In summary, these data suggest that prospective randomized trials have to prove whether vitamin D_3 supplementation during stem cell transplantation could enhance GvL effects.

Vitamin D_3 and Cancer

The first correlation of solar radiation and cancer was initially suggested by Apperly in 1941, who attributed sunlight radiation a protective role against many types of cancer except skin

cancer (98). Decades later, Colston et al. were the first ones to show a dose-dependent inhibitory effect of $1,25(OH)_2D_3$ on melanoma cells (99, 100). Epidemiological studies provide evidence that poor sunlight exposure and vitamin D_3 deficiency correlate directly with incidence as well as mortality rate of several cancer types. These findings suggest a protective role of vitamin D_3 in carcinogenesis (101). Accumulating studies have revealed that $1,25(OH)_2D_3$ suppresses tumor growth (102–104) and exhibits anti-proliferative activities in squamous cell carcinoma (105), prostate (106), breast (107, 108), lung (109), head and neck cancer (110) and hematologic malignancies such as Hodgkin's lymphoma (111) or chronic lymphocytic leukemia (CLL) (112). In colorectal cancer, a clinical trial provides evidence that $1,25(OH)_2D_3$ supplementation can efficiently reduce the risk of tumor development (113). However, other epidemiological studies report contradictory results (114–116), which might be a result of using supra-physiological concentrations of calcitriol (117), VDR gene polymorphisms (118), lack of control groups or inappropriate dosage and administration of vitamin D_3.

Apart from the discovery that sufficient vitamin D_3 supplementation could help to prevent cancer pathogenesis, numerous *in vitro* and *in vivo* studies provide evidence that $1,25(OH)_2D_3$ and its analogs could reduce tumor growth and might be used as potential anticancer agent (15, 119–121). Supporting this, animal studies report that VDR-deletion in mice makes them more susceptible to chemical induced carcinogenesis in epidermis, lymphoid and mammary tissue (122). Interestingly, life expectancy of leukemic mice could be prolonged by treatment with a $1,25(OH)_2D_3$ analog (123). A chemoprevention study revealed that $1,25(OH)_2D_3$-treated *Nkx3.1;Pten* mutant mice show retarded development of neoplasias when it was administered during early-stage carcinogenesis (124).

There is clear evidence that cancer cells exploit and dysregulate the vitamin D_3 metabolism enabling them to escape its cancer protective role (15). CYP24A1 has been shown to be overexpressed in cancer cells while activity of CYP27B1 is reduced in human prostate cancer cells (125, 126). Furthermore, CYP24A1 was identified as potential oncogene in breast cancer and elevated expression of VDR in tissues of breast and prostate cancer correlates with better prognosis of survival (127).

Mechanisms of Anti-tumorigenic Actions

Although the precise mechanisms of vitamin D_3-mediated anti-tumorigenic action are not yet fully understood, it has been postulated that vitamin D_3 modulates gene expression involved in apoptosis, cell cycle and autophagy in tumor cells (128). Apoptosis is initiated due to downregulation of anti-apoptotic protein Bcl2 while expression of pro-apoptotic proteins increases (129). Jiang et al. suggest that $1,25(OH)_2D_3$ induces cell death by degrading telomerase reverse transcriptase (TERT) mRNA and thus reduces telomerase activity (130). $1,25(OH)_2D_3$-induced upregulation of p21 and p27, which are cyclin-dependent kinase (CDK) inhibitors, induces cell cycle arrest (121, 129, 131). Furthermore, vitamin D_3 mediates anti-proliferative activity by enhancing expression of Dickkopf-1 (DKK-1), which is an antagonist in the Wnt/β-catenin signaling pathway (132). *In vitro* as well as *in vivo* studies report inhibition of proliferation and angiogenesis by vitamin D_3. It suppresses hypoxia-inducible factor 1-alpha (HIF1A) leading to reduced expression of vascular endothelial growth factor (VEGF) and thereby inhibition of angiogenesis (133, 134). Autophagy is not only triggered in infected macrophages, but also in tumor cells such as breast cancer. Since autophagy appears to protect healthy tissue from cancer initiation, vitamin D_3-treatment might contribute to suppression of carcinogenesis (135). It also increases activity of antioxidant enzymes such as superoxide dismutase 1/2 (SOD1/2) and therefore protects DNA from ROS-induced damage (129). Upon vitamin D_3 administration, DNA damage repair proteins, such as p53, are upregulated *in vitro* (15).

Strikingly, anti-tumor activity of tumor-associated macrophages (TAMs) against lymphomas has been shown to be enhanced by vitamin D_3-triggered increase of antibody-dependent cellular toxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (136). Current observations of Busch et al. reveal that combination of vitamin D_3 with immunomodulatory drugs (IMiDs), such as lenalidomide, helps to restore the defective vitamin D_3 metabolism in myeloma-associated macrophages and improves cytotoxicity against multiple myeloma cells mediated by specific anti-CD38 antibodies such as MOR202 (137, 138). Furthermore, exosomal transfer of microRNAs, which induce tumor-promoting myeloid-derived suppressor cells, was impeded by vitamin D_3 (139). The previously mentioned cathelicidin, which is secreted by human macrophages, has also been shown to mediate direct anti-tumor efficacy against high-grade B cell lymphoma by increasing ADCC (136). In summary, there is strong evidence that vitamin D_3 exerts direct anti-tumorigenic functions which might be applicable in allo-HSCT patients in order to boost GvL effects.

Mediation of Anti-inflammation to Antagonize Carcinogenesis

In 1863, Virchow postulated for the first time that tissue proliferation and hence tumor progression might be provoked by an inflammatory microenvironment connecting cancer with inflammation (140, 141). Since inflammatory tissue provides ideal conditions for genetic mutations, it seems obvious that tumor progression occurs more frequently in inflammatory environment than in healthy tissue. Clinical studies proved that localized persistent inflammation is a risk factor for the development of cancer in adjacent organs, e.g., patients with ulcerative colitis have a higher incidence of colorectal cancer (142). Given that inflammation promotes carcinogenesis, vitamin D_3-dependent anti-inflammatory activity could reduce tumor progression. In esophageal squamous cell carcinoma, $1,25(OH)_2D_3$ impedes tumor growth by inhibition of IL-6 signaling (117). Accumulating data report that $1,25(OH)_2D_3$ inhibits prostaglandin (PG) (143), p38 MAPK (144) and nuclear factor kappa B (NFκB) signaling pathways (15). Although there is increasing evidence for inflammation-driven carcinogenesis, not every type of chronic inflammation evokes tumor development, which appears to be contradictory.

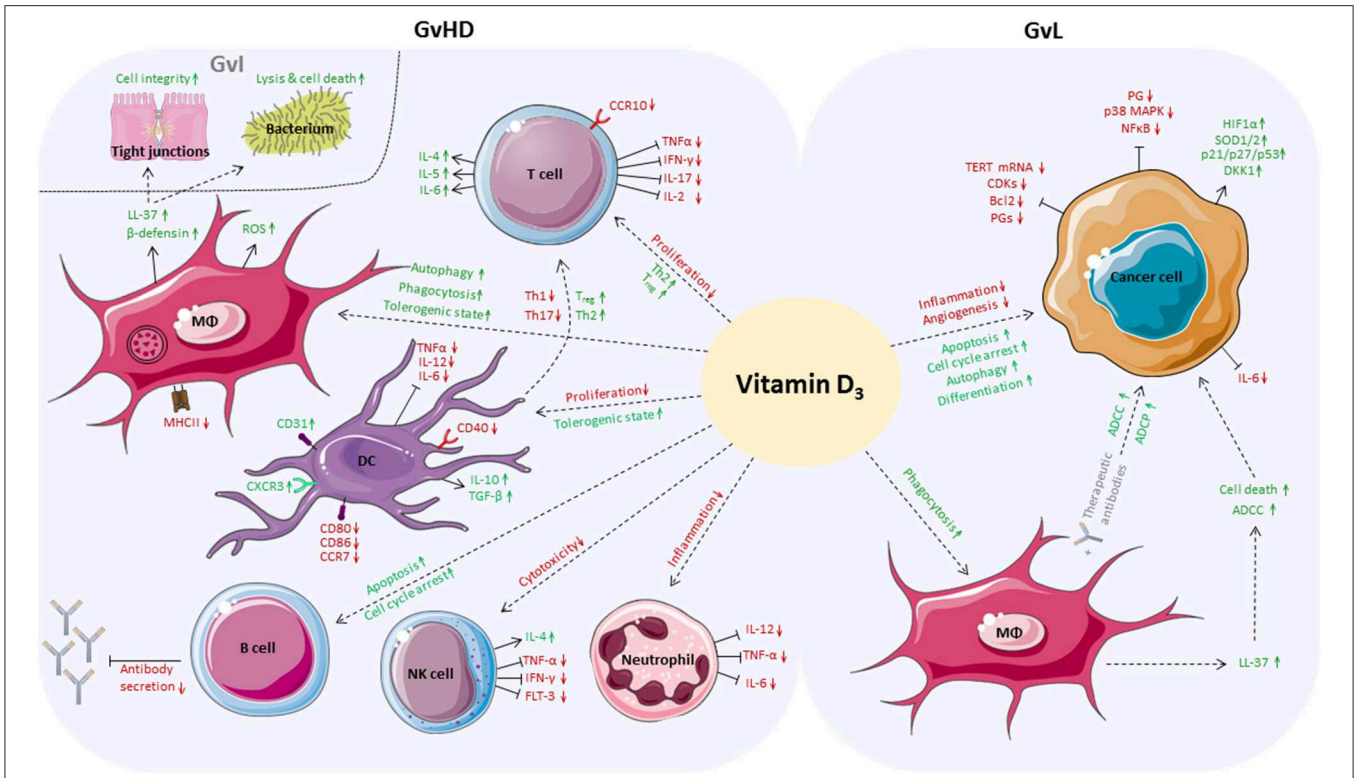

FIGURE 2 | Positive effects of vitamin D_3 in the allo-HSCT setting. Vitamin D_3 affects a broad variety of cells of the adaptive and innate immune system which play an important role in boosting GvL responses while alleviating GvHD. On side of GvHD, proliferation of T cells is inhibited and anti-inflammatory differentiation is favored. Vitamin D_3 enhances autophagy, phagocytic activity and tolerogenic capacity of macrophages. Vitamin D_3-dependent release of antimicrobial peptides by macrophages fortifies GvI effects, increases cell integrity, and induces cell death of bacteria. DCs are inhibited in proliferation and reduce Th1 and Th17 responses while the T_{reg} population increases. Vitamin D_3 triggers apoptosis and cell cycle arrest of B cells and inhibits antibody secretion. Vitamin D_3 reduces cytotoxic activity of NK cells and impedes neutrophils to secrete pro-inflammatory cytokines. In case of GvL, apoptosis, autophagy and differentiation of cancer cells are directly enhanced by vitamin D_3, whereas inflammation and angiogenesis are reduced. Vitamin D_3 enhances ADCC and ADCP of cancer cells by macrophages directly or by release of LL-37.

Despite the well-founded evidence of anti-tumorigenic effects of vitamin D_3 in solid tumors, studies on hematological malignancies remain elusive. Given that vitamin D_3 deficiency correlates with worse relapse-free survival (94–96) and the known anti-tumorigenic effects of vitamin D_3, one might think that it could also enhance GvL. By using mice fed with low and high vitamin D_3 doses or by performing clinical trials with vitamin D_3 supplementation, the actual effect on GvL could be investigated.

CONCLUSION/PERSPECTIVES

In summary, we assume that vitamin D_3 could be a potential immune modulating agent for supplementation before and during allo-HSCT. It is conceivable that vitamin D_3 might be able to maintain and improve the patient's immune balance and epithelial barrier function. Mounting evidence indicates that vitamin D_3 could alleviate GvHD by enhancing anti-inflammatory responses while it might coincidently ameliorate GvI effects due to its anti-microbial activities. Moreover, GvL might be boosted because vitamin D_3 could at least reinforce anti-tumorigenic responses of myeloid cells (**Figure 2**).

Besides its easy availability, economy and role in preserving the intestinal barrier integrity (53), vitamin D_3 helps to maintain calcium and bone homeostasis and hence prevents osteoporosis. Given that allo-HSCT patients often suffer from bone loss upon conditioning regimens, immunosuppressive treatment and immobilization, it might also improve GvHD by preventing osteoporosis (34). Cholecalciferol can usually be administered safely in high doses without occurrence of abnormal calcium metabolism (145). However, sufficient vitamin D_3 levels cannot be achieved in all patients despite high-dose supplementation (97). Therefore, treatment with $1,25(OH)_2D_3$ might be the more efficient version. However, the probably greatest restraining factor of $1,25(OH)_2D_3$ is its dose-limiting toxicity causing hypercalcemia and hypercalciuria. One possible solution might be the administration of $1,25(OH)_2D_3$ analogs, some of which have already been shown to be less calcemic (146, 147).

Until now, only few clinical trials with vitamin D_3 in the allo-HSCT setting have been conducted and have shown effective outcomes (90). The most recent study of Carillo-Cruz et al. suggests that universal vitamin D_3 medication remains challenging due to VDR polymorphisms (29). Our

hypothesis that vitamin D_3 could improve GvL might seem controversial due to its known anti-inflammatory activities on T cells. However, it was shown by Essen et al. that TCR signaling in naïve human T cells induces VDR expression (148). This in turn results in upregulated PLC-γ1 expression and thus higher activation and priming of naïve T cells. Although there is evidence that vitamin D_3 attenuates IL-6 signaling in human esophageal squamous cell carcinoma (SCC) cell lines (117), the *in vivo* study of Bendix-Struve and colleagues demonstrated that T cells of vitamin D_3-supplemented Crohn's disease (CD) patients produced more IL-6 (149). Proliferation of CD4+ T cells was higher in vitamin D_3-treated patients compared to the placebo group. Additionally, VDR was shown to be important for the development of CD8αα+ TCRαβ+ cells, which help to maintain tolerance in the gut and suppress intestinal inflammation (150). Expression of the gut-homing receptor CCR9 is suppressed in T cells upon vitamin D_3 stimulation, which might prevent homing of potential alloreactive T cells into the gut. However, these data provide only indications that vitamin D_3 might promote GvL despite its anti-inflammatory properties.

In conclusion, prospective *in vivo* studies in humans are inevitable to investigate the efficacy of vitamin D_3 supplementation and to achieve approved clinical application.

AUTHOR CONTRIBUTIONS

CF wrote the manuscript and created the figures. HB took the lead in writing the manuscript. MK and KP provided critical feedback, contributed to the final version of the manuscript, and approved it for publication.

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REFERENCES

1. Majhail NS, Farnia SH, Carpenter PA, Champlin RE, Crawford S, Marks DI, et al. Indications for autologous and allogeneic hematopoietic cell transplantation: guidelines from the American Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant.* (2015) 21:1863–9. doi: 10.1016/j.bbmt.2015.07.032
2. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood.* (2008) 112:4371–83. doi: 10.1182/blood-2008-03-077974
3. Ogonek J, Kralj Juric M, Ghimire S, Varanasi PR, Holler E, Greinix H, et al. Immune reconstitution after allogeneic hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:507. doi: 10.3389/fimmu.2016.00507
4. Li JM, Giver CR, Lu Y, Hossain MS, Akhtari M, Waller EK. Separating graft-versus-leukemia from graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Immunotherapy.* (2009) 1:599–621.
5. Martin PJ, Rizzo JD, Wingard JR, Ballen K, Curtin PT, Cutler C, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant.* (2012) 18:1150–63. doi: 10.1016/j.bbmt.2012.04.005
6. Datta A, David R, Glennie S, Scott D, Cernuda-Morollon E, Lechler RI, et al. Differential effects of immunosuppressive drugs on T-cell motility. *Am J Transplant.* (2006) 6:2871–83. doi: 10.1111/j.1600-6143.2006.01553.x
7. Adorini L, Giarratana N, Penna G. Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. *Semin Immunol.* (2004) 16:127–34. doi: 10.1016/j.smim.2003.12.008
8. Chang YJ, Zhao XY, Huang XJ. Strategies for enhancing and preserving anti-leukemia effects without aggravating graft-versus-host disease. *Front Immunol.* (2018) 9:3041. doi: 10.3389/fimmu.2018.03041
9. Deluca HF. History of the discovery of vitamin D and its active metabolites. *Bonekey Rep.* (2014) 3:479. doi: 10.1038/bonekey.2013.213
10. Kreutz M, Andreesen R, Krause SW, Szabo A, Ritz E, Reichel H. 1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. *Blood.* (1993) 82:1300–7. doi: 10.1182/blood.V82.4.1300.1300
11. Fritsche J, Mondal K, Ehrnsperger A, Andreesen R, Kreutz M. Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells. *Blood.* (2003) 102:3314–6. doi: 10.1182/blood-2002-11-3521
12. Gottfried E, Rehli M, Hahn J, Holler E, Andreesen R, Kreutz M. Monocyte-derived cells express CYP27A1 and convert vitamin D3 into its active metabolite. *Biochem Biophys Res Commun.* (2006) 349:209–13. doi: 10.1016/j.bbrc.2006.08.034
13. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients.* (2013) 5:2502–21. doi: 10.3390/nu5072502
14. Bruns H, Stenger S. New insights into the interaction of *Mycobacterium tuberculosis* and human macrophages. *Future Microbiol.* (2014) 9:327–41. doi: 10.2217/fmb.13.164
15. Jeon SM, Shin EA. Exploring vitamin D metabolism and function in cancer. *Exp Mol Med.* (2018) 50:20. doi: 10.1038/s12276-018-0038-9
16. Kamen DL, Tangpricha V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. *J Mol Med.* (2010) 88:441–50. doi: 10.1007/s00109-010-0590-9
17. Munger KL, Ascherio A. Prevention and treatment of MS: studying the effects of vitamin *Mult Scler* D. (2011) 17:1405–11. doi: 10.1177/1352458511425366
18. Hassanalilou T, Khalili L, Ghavamzadeh S, Shokri A, Payahoo L, Bishak YK. Role of vitamin D deficiency in systemic lupus erythematosus incidence and aggravation. *Auto Immun Highlights.* (2017) 9:1. doi: 10.1007/s13317-017-0101-x
19. Cantorna MT, Mahon BD. Mounting evidence for vitamin D as an environmental factor affecting autoimmune disease prevalence. *Exp Biol Med.* (2004) 229:1136–42. doi: 10.1177/153537020422901108
20. Yang J, Liu L, Zhang Q, Li M, Wang J. Effect of vitamin D on the recurrence rate of rheumatoid arthritis. *Exp Ther Med.* (2015) 10:1812–6. doi: 10.3892/etm.2015.2747
21. Kim D. The role of vitamin D in thyroid diseases. *Int J Mol Sci.* (2017) 18:1949. doi: 10.3390/ijms18091949
22. Czaja AJ, Montano-Loza AJ. Evolving role of vitamin D in immune-mediated disease and its implications in autoimmune hepatitis. *Dig Dis Sci.* (2019) 64:324–44. doi: 10.1007/s10620-018-5351-6
23. Johnsson C, Tufveson G. MC 1288–a vitamin D analogue with immunosuppressive effects on heart and small bowel grafts. *Transpl Int.* (1994) 7:392–7. doi: 10.1007/BF00346032

24. Lewin E, Olgaard K. The *in vivo* effect of a new, *in vitro*, extremely potent vitamin D3 analog KH1060 on the suppression of renal allograft rejection in the rat. *Calcif Tissue Int.* (1994) 54:150–4. doi: 10.1007/BF00296066
25. Hullett DA, Cantorna MT, Redaelli C, Humpal-Winter J, Hayes CE, Sollinger HW, et al. Prolongation of allograft survival by 1,25-dihydroxyvitamin D3. *Transplantation.* (1998) 66:824–8. doi: 10.1097/00007890-199810150-00002
26. Pakkala I, Taskinen E, Pakkala S, Raisanen-Sokolowski A. MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. *Bone Marrow Transplant.* (2001) 27:863–7. doi: 10.1038/sj.bmt.1702873
27. Middleton PG, Cullup H, Dickinson AM, Norden J, Jackson GH, Taylor PR, et al. Vitamin D receptor gene polymorphism associates with graft-versus-host disease and survival in HLA-matched sibling allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (2002) 30:223–8. doi: 10.1038/sj.bmt.1703629
28. Rocha V, Porcher R, Fernandes JF, Filion A, Bittencourt H, Silva W Jr, et al. Association of drug metabolism gene polymorphisms with toxicities, graft-versus-host disease and survival after HLA-identical sibling hematopoietic stem cell transplantation for patients with leukemia. *Leukemia.* (2009) 23:545–56. doi: 10.1038/leu.2008.323
29. Carrillo-Cruz E, Garcia-Lozano JR, Marquez-Malaver FJ, Sanchez-Guijo FM, Montero Cuadrado I, Ferra ICC, et al. Vitamin D modifies the incidence of graft-versus-host disease after allogeneic stem cell transplantation depending on the vitamin D receptor (VDR) polymorphisms. *Clin Cancer Res.* (2019) 25:4616–23. doi: 10.1158/1078-0432.CCR-18-3875
30. Ros-Soto J, Anthias C, Madrigal A, Snowden JA. Vitamin D: is it important in haematopoietic stem cell transplantation? A review. *Bone Marrow Transplant.* (2018) 54:810–20. doi: 10.1038/s41409-018-0377-0
31. Kreutz M, Eissner G, Hahn J, Andreesen R, Drobnik W, Holler E. Variations in 1 alpha,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 serum levels during allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (2004) 33:871–3. doi: 10.1038/sj.bmt.1704448
32. Beebe K, Magee K, McNulty A, Stahlecker J, Salzberg D, Miller H, et al. Vitamin D deficiency and outcomes in pediatric hematopoietic stem cell transplantation. *Pediatr Blood Cancer.* (2018) 65:e26817. doi: 10.1002/pbc.26817
33. Glotzbecker B, Ho VT, Aldridge J, Kim HT, Horowitz G, Ritz J, et al. Low levels of 25-hydroxyvitamin D before allogeneic hematopoietic SCT correlate with the development of chronic GVHD. *Bone Marrow Transplant.* (2013) 48:593–7. doi: 10.1038/bmt.2012.177
34. Sproat L, Bolwell B, Rybicki L, Dean R, Sobecks R, Pohlman B, et al. Vitamin D level after allogeneic hematopoietic stem cell transplant. *Biol Blood Marrow Transplant.* (2011) 17:1079–83. doi: 10.1016/j.bbmt.2010.12.704
35. Chen X, Mayne CG. The role of micronutrients in graft-vs.-host disease: immunomodulatory effects of vitamins A and D. *Front Immunol.* (2018) 9:2853. doi: 10.3389/fimmu.2018.02853
36. Brighenti S, Bergman P, Martineau AR. Vitamin D and tuberculosis: where next? *J Intern Med.* (2018) 284:145–62. doi: 10.1111/joim.12777
37. Green M. Cod liver oil and tuberculosis. *BMJ.* (2011) 343:d7505. doi: 10.1136/bmj.d7505
38. Rook GA, Steele J, Fraher L, Barker S, Karmali R, O'Riordan J, et al. Vitamin D3, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes. *Immunology.* (1986) 57:159–63.
39. Crowle AJ, Ross EJ, May MH. Inhibition by 1,25(OH)2-vitamin D3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. *Infect Immun.* (1987) 55:2945–50. doi: 10.1016/0041-3879(88)90036-0
40. Fabri M, Stenger S, Shin DM, Yuk JM, Liu PT, Realegeno S, et al. Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. *Sci Transl Med.* (2011) 3:104ra102. doi: 10.1126/scitranslmed.3003045
41. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* (2004) 173:2909–12. doi: 10.4049/jimmunol.173.5.2909
42. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
43. Martineau AR, Wilkinson KA, Newton SM, Floto RA, Norman AW, Skolimowska K, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. *J Immunol.* (2007) 178:7190–8. doi: 10.4049/jimmunol.178.11.7190
44. Leszczynska K, Namiot D, Byfield FJ, Cruz K, Zendzian-Piotrowska M, Fein DE, et al. Antibacterial activity of the human host defence peptide LL-37 and selected synthetic cationic lipids against bacteria associated with oral and upper respiratory tract infections. *J Antimicrob Chemother.* (2013) 68:610–8. doi: 10.1093/jac/dks434
45. Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol.* (2008) 181:7090–9. doi: 10.4049/jimmunol.181.10.7090
46. van der Does AM, Bergman P, Agerberth B, Lindbom L. Induction of the human cathelicidin LL-37 as a novel treatment against bacterial infections. *J Leukoc Biol.* (2012) 92:735–42. doi: 10.1189/jlb.0412178
47. Wan M, van der Does AM, Tang X, Lindbom L, Agerberth B, Haeggstrom JZ. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. *J Leukoc Biol.* (2014) 95:971–81. doi: 10.1189/jlb.0513304
48. Alalwani SM, Sierigk J, Herr C, Pinkenburg O, Gallo R, Vogelmeier C, et al. The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. *Eur J Immunol.* (2010) 40:1118–26. doi: 10.1002/eji.200939275
49. Choi H, Yang Z, Weisshaar JC. Oxidative stress induced in E. coli by the human antimicrobial peptide LL-37. *PLoS Pathog.* (2017) 13:e1006481. doi: 10.1371/journal.ppat.1006481
50. Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, et al. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe.* (2009) 6:231–43. doi: 10.1016/j.chom.2009.08.004
51. Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, et al. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE.* (2011) 6:e25333. doi: 10.1371/journal.pone.0025333
52. Lopez-Garcia B, Lee PH, Yamasaki K, Gallo RL. Anti-fungal activity of cathelicidins and their potential role in *Candida albicans* skin infection. *J Invest Dermatol.* (2005) 125:108–15. doi: 10.1111/j.0022-202X.2005.23713.x
53. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, et al. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol.* (2008) 294:G208–16. doi: 10.1152/ajpgi.00398.2007
54. Zhang YG, Wu S, Lu R, Zhou D, Zhou J, Carmeliet G, et al. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. *Sci Rep.* (2015) 5:10642. doi: 10.1038/srep10642
55. Nalle SC, Turner JR. Intestinal barrier loss as a critical pathogenic link between inflammatory bowel disease and graft-versus-host disease. *Mucosal Immunol.* (2015) 8:720–30. doi: 10.1038/mi.2015.40
56. Chen X, Zou X, Qi G, Tang Y, Guo Y, Si J, et al. Roles and mechanisms of human cathelicidin LL-37 in cancer. *Cell Physiol Biochem.* (2018) 47:1060–73. doi: 10.1159/000490183
57. Sun L, Wang W, Xiao W, Yang H. The roles of cathelicidin LL-37 in inflammatory bowel disease. *Inflamm Bowel Dis.* (2016) 22:1986–91. doi: 10.1097/MIB.0000000000000804
58. Kirikae T, Hirata M, Yamasu H, Kirikae F, Tamura H, Kayama F, et al. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. *Infect Immun.* (1998) 66:1861–8.
59. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet.* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
60. Kreutz M, Andreesen R. Induction of human monocyte to macrophage maturation *in vitro* by 1,25-dihydroxyvitamin D3. *Blood.* (1990) 76:2457–61. doi: 10.1182/blood.V76.12.2457.bloodjournal76122457
61. Rigby WF, Waugh M, Graziano RF. Regulation of human monocyte HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-dihydroxyvitamin D3. *Blood.* (1990) 76:189–97. doi: 10.1182/blood.V76.1.189.bloodjournal761189
62. Zhu X, Zhu Y, Li C, Yu J, Ren D, Qiu S, et al. 1,25Dihydroxyvitamin D regulates macrophage polarization and ameliorates experimental

inflammatory bowel disease by suppressing miR-125b. *Int Immunopharmacol.* (2019) 67:106–18. doi: 10.1016/j.intimp.2018.12.015
63. Berer A, Stockl J, Majdic O, Wagner T, Kollars M, Lechner K, et al. 1,25-Dihydroxyvitamin D(3) inhibits dendritic cell differentiation and maturation *in vitro*. *Exp Hematol.* (2000) 28:575–83. doi: 10.1016/S0301-472X(00)00143-0
64. Ferreira GB, Gysemans CA, Demengeot J, da Cunha JP, Vanherwegen AS, Overbergh L, et al. 1,25-Dihydroxyvitamin D3 promotes tolerogenic dendritic cells with functional migratory properties in NOD mice. *J Immunol.* (2014) 192:4210–20. doi: 10.4049/jimmunol.1302350
65. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol.* (2000) 164:2405–11. doi: 10.4049/jimmunol.164.5.2405
66. Almerighi C, Sinistro A, Cavazza A, Ciaprini C, Rocchi G, Bergamini A. 1Alpha,25-dihydroxyvitamin D3 inhibits CD40L-induced pro-inflammatory and immunomodulatory activity in human monocytes. *Cytokine.* (2009) 45:190–7. doi: 10.1016/j.cyto.2008.12.009
67. Saul L, Mair I, Ivens A, Brown P, Samuel K, Campbell JDM, et al. 1,25-Dihydroxyvitamin D3 Restrains CD4(+) T Cell Priming Ability of CD11c(+) Dendritic Cells by Upregulating Expression of CD31. *Front Immunol.* (2019) 10:600. doi: 10.3389/fimmu.2019.00600
68. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest.* (1998) 101:252–62. doi: 10.1172/JCI1050
69. Vanherwegen AS, Cook DP, Ferreira GB, Gysemans C, Mathieu C. Vitamin D-modulated dendritic cells delay lethal graft-versus-host disease through induction of regulatory T cells. *J Steroid Biochem Mol Biol.* (2019) 188:03–10. doi: 10.1016/j.jsbmb.2018.12.013
70. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol.* (2001) 167:1945–53. doi: 10.4049/jimmunol.167.4.1945
71. Zhou L, Wang J, Li J, Li T, Chen Y, June RR, et al. 1,25-Dihydroxyvitamin D3 ameliorates collagen-induced arthritis via suppression of Th17 cells through miR-124 mediated inhibition of IL-6 signaling. *Front Immunol.* (2019) 10:178. doi: 10.3389/fimmu.2019.00178
72. Xu Y, Cheng Y, Baylink DJ, Wasnik S, Goel G, Huang M, et al. *In vivo* generation of gut-homing regulatory T cells for the suppression of colitis. *J Immunol.* (2019) 202:3447–57. doi: 10.4049/jimmunol.1800018
73. Vanherwegen AS, Eelen G, Ferreira GB, Ghesquiere B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J Steroid Biochem Mol Biol.* (2019) 187:134–45. doi: 10.1016/j.jsbmb.2018.11.011
74. Coussens A, Timms PM, Boucher BJ, Venton TR, Ashcroft AT, Skolimowska KH, et al. 1alpha,25-dihydroxyvitamin D3 inhibits matrix metalloproteinases induced by *Mycobacterium tuberculosis* infection. *Immunology.* (2009) 127:539–48. doi: 10.1111/j.1365-2567.2008.03024.x
75. Coussens AK, Wilkinson RJ, Hanifa Y, Nikolayevskyy V, Elkington PT, Islam K, et al. Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc Natl Acad Sci USA.* (2012) 109:15449–54. doi: 10.1073/pnas.1200072109
76. Olson KC, Kulling Larkin PM, Signorelli R, Hamele CE, Olson TL, Conaway MR, et al. Vitamin D pathway activation selectively deactivates signal transducer and activator of transcription (STAT) proteins and inflammatory cytokine production in natural killer leukemic large granular lymphocytes. *Cytokine.* (2018) 111:551–62. doi: 10.1016/j.cyto.2018.09.016
77. Carniti C, Gimondi S, Vendramin A, Recordati C, Confalonieri D, Bermema A, et al. Pharmacologic inhibition of JAK1/JAK2 signaling reduces experimental murine acute GVHD while preserving GVT effects. *Clin Cancer Res.* (2015) 21:3740–9. doi: 10.1158/1078-0432.CCR-14-2758
78. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in corticosteroid-refractory graft-versus-host disease after allogeneic stem cell transplantation: a multicenter survey. *Leukemia.* (2015) 29:2062–8. doi: 10.1038/leu.2015.212
79. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of therapeutic JAK 1/2 blockade in graft-versus-host disease. *Blood.* (2014) 123:3832–42. doi: 10.1182/blood-2013-12-543736
80. Young JA. Infectious complications of acute and chronic GVHD. *Best Pract Res Clin Haematol.* (2008) 21:343–56. doi: 10.1016/j.beha.2008.02.017
81. Fuji S, Kapp M, Einsele H. Possible implication of bacterial infection in acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Front Oncol.* (2014) 4:89. doi: 10.3389/fonc.2014.00089
82. Rosenblatt J, Bissonnette A, Ahmad R, Wu Z, Vasir B, Stevenson K, et al. Immunomodulatory effects of vitamin D: implications for GVHD. *Bone Marrow Transplant.* (2010) 45:1463–8. doi: 10.1038/bmt.2009.366
83. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science.* (1983) 221:1181–3. doi: 10.1126/science.6310748
84. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol.* (2009) 183:5458–67. doi: 10.4049/jimmunol.0803217
85. Vanham G, Ceuppens JL, Bouillon R. T lymphocytes and their CD4 subset are direct targets for the inhibitory effect of calcitriol. *Cell Immunol.* (1989) 124:320–33. doi: 10.1016/0008-8749(89)90134-2
86. Baeke F, Korf H, Overbergh L, van Etten E, Verstuyf A, Gysemans C, et al. Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D3 in the immune system. *J Steroid Biochem Mol Biol.* (2010) 121:221–7. doi: 10.1016/j.jsbmb.2010.03.037
87. Zhang Z, Chen F, Li J, Luo F, Hou T, Xu J, et al. 1,25(OH)2D3 suppresses proinflammatory responses by inhibiting Th1 cell differentiation and cytokine production through the JAK/STAT pathway. *Am J Transl Res.* (2018) 10:2737–46.
88. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol.* (2001) 167:4974–80. doi: 10.4049/jimmunol.167.9.4974
89. Hayes CE, Nashold FE, Spach KM, Pedersen LB. The immunological functions of the vitamin D endocrine system. *Cell Mol Biol.* (2003) 49:277–300.
90. Caballero-Velazquez T, Montero I, Sanchez-Guijo F, Parody R, Saldana R, Valcarcel D, et al. Immunomodulatory effect of vitamin D after allogeneic stem cell transplantation: results of a prospective multicenter clinical trial. *Clin Cancer Res.* (2016) 22:5673–81. doi: 10.1158/1078-0432.CCR-16-0238
91. Schardey J, Globig AM, Janssen C, Hofmann M, Manegold P, Thimme R, et al. Vitamin D inhibits pro-inflammatory T cell function in patients with inflammatory bowel disease. *J Crohns Colitis.* (2019). doi: 10.1093/ecco-jcc/jjz090. [Epub ahead of print].
92. Iho S, Takahashi T, Kura F, Sugiyama H, Hoshino T. The effect of 1,25-dihydroxyvitamin D3 on *in vitro* immunoglobulin production in human B cells. *J Immunol.* (1986) 136:4427–31.
93. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol.* (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
94. Radujkovic A, Kordelas L, Krzykalla J, Beelen DW, Benner A, Lehners N, et al. Pretransplant vitamin D deficiency is associated with higher relapse rates in patients allografted for myeloid malignancies. *J Clin Oncol.* (2017) 35:3143–52. doi: 10.1200/JCO.2017.73.0085
95. Hansson ME, Norlin AC, Omazic B, Wikstrom AC, Bergman P, Winiarski J, et al. Vitamin d levels affect outcome in pediatric hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2014) 20:1537–43. doi: 10.1016/j.bbmt.2014.05.030
96. Lee HJ, Muindi JR, Tan W, Hu Q, Wang D, Liu S, et al. Low 25(OH) vitamin D3 levels are associated with adverse outcome in newly diagnosed, intensively treated adult acute myeloid leukemia. *Cancer.* (2014) 120:521–9. doi: 10.1002/cncr.28368
97. Wallace G, Jodele S, Myers KC, Dandoy CE, El-Bietar J, Nelson A, et al. Vitamin D deficiency in pediatric hematopoietic stem cell transplantation patients despite both standard and aggressive supplementation. *Biol Blood Marrow Transplant.* (2016) 22:1271–4. doi: 10.1016/j.bbmt.2016.03.026

98. Bertino JR. Landmark study: the relation of solar radiation to cancer mortality in North America. *Cancer Res.* (2016) 76:185. doi: 10.1158/0008-5472.CAN-15-3169
99. Colston K, Colston MJ, Feldman D. 1,25-dihydroxyvitamin D3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology.* (1981) 108:1083–6. doi: 10.1210/endo-108-3-1083
100. Giammanco M, Di Majo D, La Guardia M, Aiello S, Crescimannno M, Flandina C, et al. Vitamin D in cancer chemoprevention. *Pharm Biol.* (2015) 53:1399–434. doi: 10.3109/13880209.2014.988274
101. Garland CF, Gorham ED, Mohr SB, Garland FC. Vitamin D for cancer prevention: global perspective. *Ann Epidemiol.* (2009) 19:468–83. doi: 10.1016/j.annepidem.2009.03.021
102. Kasiappan R, Shen Z, Tse AK, Jinwal U, Tang J, Lungchukiet P, et al. 1,25-Dihydroxyvitamin D3 suppresses telomerase expression and human cancer growth through microRNA-498. *J Biol Chem.* (2012) 287:41297–309. doi: 10.1074/jbc.M112.407189
103. Frampton RJ, Omond SA, Eisman JA. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D3 metabolites. *Cancer Res.* (1983) 43:4443–7.
104. Honma Y, Hozumi M, Abe E, Konno K, Fukushima M, Hata S, et al. 1 alpha,25-Dihydroxyvitamin D3 and 1 alpha-hydroxyvitamin D3 prolong survival time of mice inoculated with myeloid leukemia cells. *Proc Natl Acad Sci USA.* (1983) 80:201–4. doi: 10.1073/pnas.80.1.201
105. Bernardi RJ, Johnson CS, Modzelewski RA, Trump DL. Antiproliferative effects of 1alpha,25-dihydroxyvitamin D(3) and vitamin D analogs on tumor-derived endothelial cells. *Endocrinology.* (2002) 143:2508–14. doi: 10.1210/endo.143.7.8887
106. Getzenberg RH, Light BW, Lapco PE, Konety BR, Nangia AK, Acierno JS, et al. Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. *Urology.* (1997) 50:999–1006. doi: 10.1016/S0090-4295(97)00408-1
107. Colston KW, Chander SK, Mackay AG, Coombes RC. Effects of synthetic vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem Pharmacol.* (1992) 44:693–702. doi: 10.1016/0006-2952(92)90405-8
108. Ooi LL, Zhou H, Kalak R, Zheng Y, Conigrave AD, Seibel MJ, et al. Vitamin D deficiency promotes human breast cancer growth in a murine model of bone metastasis. *Cancer Res.* (2010) 70:1835–44. doi: 10.1158/0008-5472.CAN-09-3194
109. Higashimoto Y, Ohata M, Nishio K, Iwamoto Y, Fujimoto H, Uetani K, et al. 1 alpha, 25-dihydroxyvitamin D3 and all-trans-retinoic acid inhibit the growth of a lung cancer cell line. *Anticancer Res*. (1996) 16:2653–9.
110. Kornfehl J, Formanek M, Temmel A, Knerer B, Willheim M. Antiproliferative effects of the biologically active metabolite of vitamin D3 (1,25 [OH]2 D3) on head and neck squamous cell carcinoma cell lines. *Eur Arch Otorhinolaryngol.* (1996) 253:341–4. doi: 10.1007/BF00178289
111. Gharbaran R, Zhang B, Valerio L, Onwumere O, Wong M, Mighty J, et al. Effects of vitamin D3 and its chemical analogs on the growth of Hodgkin's lymphoma, *in vitro*. *BMC Res Notes.* (2019) 12:216. doi: 10.1186/s13104-019-4241-0
112. Shanafelt TD, Drake MT, Maurer MJ, Allmer C, Rabe KG, Slager SL, et al. Vitamin D insufficiency and prognosis in chronic lymphocytic leukemia. *Blood.* (2011) 117:1492–8. doi: 10.1182/blood-2010-07-295683
113. Ding EL, Mehta S, Fawzi WW, Giovannucci EL. Interaction of estrogen therapy with calcium and vitamin D supplementation on colorectal cancer risk: reanalysis of Women's Health Initiative randomized trial. *Int J Cancer.* (2008) 122:1690–4. doi: 10.1002/ijc.23311
114. Antunac Golubic Z, Barsic I, Librenjak N, Plestina S. Vitamin D supplementation and survival in metastatic colorectal cancer. *Nutr Cancer.* (2018) 70:413–7. doi: 10.1080/01635581.2018.1445766
115. Brandstedt J, Almquist M, Manjer J, Malm J. Vitamin D, PTH, and calcium and the risk of prostate cancer: a prospective nested case-control study. *Cancer Causes Control.* (2012) 23:1377–85. doi: 10.1007/s10552-012-9948-3
116. Chlebowski RT, Johnson KC, Kooperberg C, Pettinger M, Wactawski-Wende J, Rohan T, et al. Calcium plus vitamin D supplementation and the risk of breast cancer. *J Natl Cancer Inst.* (2008) 100:1581–91. doi: 10.1093/jnci/djn360
117. Chen PT, Hsieh CC, Wu CT, Yen TC, Lin PY, Chen WC, et al. 1alpha,25-dihydroxyvitamin D3 inhibits esophageal squamous cell carcinoma progression by reducing IL6 signaling. *Mol Cancer Ther.* (2015) 14:1365–75. doi: 10.1158/1535-7163.MCT-14-0952
118. Barry EL, Peacock JL, Rees JR, Bostick RM, Robertson DJ, Bresalier RS, et al. Vitamin D receptor genotype, vitamin D3 supplementation, and risk of colorectal adenomas: a randomized clinical trial. *JAMA Oncol.* (2017) 3:628–35. doi: 10.1001/jamaoncol.2016.5917
119. Huerta S, Irwin RW, Heber D, Go VL, Koeffler HP, Uskokovic MR, et al. 1alpha,25-(OH)(2)-D(3) and its synthetic analogue decrease tumor load in the Apc(min) Mouse. *Cancer Res.* (2002) 62:741–6.
120. Otoshi T, Iwata H, Kitano M, Nishizawa Y, Morii H, Yano Y, et al. Inhibition of intestinal tumor development in rat multi-organ carcinogenesis and aberrant crypt foci in rat colon carcinogenesis by 22-oxa-calcitriol, a synthetic analogue of 1 alpha, 25-dihydroxyvitamin D3. *Carcinogenesis.* (1995) 16:2091–7. doi: 10.1093/carcin/16.9.2091
121. Chiang KC, Yeh CN, Hsu JT, Chen LW, Kuo SF, Sun CC, et al. MART-10, a novel vitamin D analog, inhibits head and neck squamous carcinoma cells growth through cell cycle arrest at G0/G1 with upregulation of p21 and p27 and downregulation of telomerase. *J Steroid Biochem Mol Biol.* (2013) 138:427–34. doi: 10.1016/j.jsbmb.2013.09.002
122. Zinser GM, Suckow M, Welsh J. Vitamin D receptor (VDR) ablation alters carcinogen-induced tumorigenesis in mammary gland, epidermis and lymphoid tissues. *J Steroid Biochem Mol Biol.* (2005) 97:153–64. doi: 10.1016/j.jsbmb.2005.06.024
123. Zhou JY, Norman AW, Chen DL, Sun GW, Uskokovic M, Koeffler HP. 1,25-Dihydroxy-16-ene-23-yne-vitamin D3 prolongs survival time of leukemic mice. *Proc Natl Acad Sci USA.* (1990) 87:3929–32. doi: 10.1073/pnas.87.10.3929
124. Banach-Petrosky W, Ouyang X, Gao H, Nader K, Ji Y, Suh N, et al. Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1;Pten mutant mice. *Clin Cancer Res.* (2006) 12:5895–901. doi: 10.1158/1078-0432.CCR-06-1039
125. Whitlatch LW, Young MV, Schwartz GG, Flanagan JN, Burnstein KL, Lokeshwar BL, et al. 25-Hydroxyvitamin D-1alpha-hydroxylase activity is diminished in human prostate cancer cells and is enhanced by gene transfer. *J Steroid Biochem Mol Biol.* (2002) 81:135–40. doi: 10.1016/S0960-0760(02)00053-5
126. Hsu JY, Feldman D, McNeal JE, Peehl DM. Reduced 1alpha-hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D3-induced growth inhibition. *Cancer Res.* (2001) 61:2852–6.
127. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer.* (2014) 14:342–57. doi: 10.1038/nrc3691
128. Hoyer-Hansen M, Bastholm L, Mathiasen IS, Elling F, Jaattela M. Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. *Cell Death Differ.* (2005) 12:1297–309. doi: 10.1038/sj.cdd.4401651
129. Fleet JC, DeSmet M, Johnson R, Li Y. Vitamin D and cancer: a review of molecular mechanisms. *Biochem J.* (2012) 441:61–76. doi: 10.1042/BJ20110744
130. Jiang F, Bao J, Li P, Nicosia SV, Bai W. Induction of ovarian cancer cell apoptosis by 1,25-dihydroxyvitamin D3 through the down-regulation of telomerase. *J Biol Chem.* (2004) 279:53213–21. doi: 10.1074/jbc.M410395200
131. Wang QM, Jones JB, Studzinski GP. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. *Cancer Res.* (1996) 56:264–7.
132. Pendas-Franco N, Garcia JM, Pena C, Valle N, Palmer HG, Heinaniemi M, et al. DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1alpha,25-dihydroxyvitamin D3. *Oncogene.* (2008) 27:4467–77. doi: 10.1038/onc.2008.88
133. Ben-Shoshan M, Amir S, Dang DT, Dang LH, Weisman Y, Mabjeesh NJ. 1alpha,25-dihydroxyvitamin D3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells. *Mol Cancer Ther.* (2007) 6:1433–9. doi: 10.1158/1535-7163.MCT-06-0677
134. Iseki K, Tatsuta M, Uehara H, Iishi H, Yano H, Sakai N, et al. Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 of colon carcinogenesis induced by

azoxymethane in Wistar rats. *Int J Cancer*. (1999) 81:730–3. doi: 10.1002/(SICI)1097-0215(19990531)81:5<730::AID-IJC11>3.3.CO;2-H
135. Tavera-Mendoza LE, Westerling T, Libby E, Marusyk A, Cato L, Cassani R, et al. Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells. *Proc Natl Acad Sci USA*. (2017) 114:E2186–94. doi: 10.1073/pnas.1615015114
136. Bruns H, Buttner M, Fabri M, Mougiakakos D, Bittenbring JT, Hoffmann MH, et al. Vitamin D-dependent induction of cathelicidin in human macrophages results in cytotoxicity against high-grade B cell lymphoma. *Sci Transl Med*. (2015) 7:282ra47. doi: 10.1126/scitranslmed.aaa3230
137. Busch L, Mougiakakos D, Buttner-Herold M, Muller MJ, Volmer DA, Bach C, et al. Lenalidomide enhances MOR202-dependent macrophage-mediated effector functions via the vitamin D pathway. *Leukemia*. (2018) 32:2445–58. doi: 10.1038/s41375-018-0114-0
138. Flamann C, Busch L, Mackensen A, Bruns H. Combination of lenalidomide and vitamin D enhances MOR202-mediated cytotoxicity of macrophages: it takes three to tango. *Oncotarget*. (2019) 10:10–2. doi: 10.18632/oncotarget.26531
139. Bruns H, Bottcher M, Qorraj M, Fabri M, Jitschin S, Dindorf J, et al. CLL-cell-mediated MDSC induction by exosomal miR-155 transfer is disrupted by vitamin *Leukemia* D. (2017) 31:985–8. doi: 10.1038/leu.2016.378
140. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. (2001) 357:539–45. doi: 10.1016/S0140-6736(00)04046-0
141. Menkin V. Role of inflammation in carcinogenesis. *Br Med J*. (1960) 1:1585–94. doi: 10.1136/bmj.1.5186.1585
142. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut*. (2001) 48:526–35. doi: 10.1136/gut.48.4.526
143. Krishnan AV, Feldman D. Molecular pathways mediating the anti-inflammatory effects of calcitriol: implications for prostate cancer chemoprevention and treatment. *Endocr Relat Cancer*. (2010) 17:R19–38. doi: 10.1677/ERC-09-0139
144. Nonn L, Peng L, Feldman D, Peehl DM. Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. *Cancer Res*. (2006) 66:4516–24. doi: 10.1158/0008-5472.CAN-05-3796
145. Wallace G, Jodele S, Myers KC, Dandoy CE, El-Bietar J, Nelson A, et al. Single ultra-high-dose cholecalciferol to prevent vitamin D deficiency in pediatric hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2018) 24:1856–60. doi: 10.1016/j.bbmt.2018.05.019
146. Leyssens C, Verlinden L, Verstuyf A. The future of vitamin D analogs. *Front Physiol*. (2014) 5:122. doi: 10.3389/fphys.2014.00122
147. Alagbala AA, Johnson CS, Trump DL, Posner GH, Foster BA. Antitumor effects of two less-calcemic vitamin D analogs (Paricalcitol and QW-1624F2-2) in squamous cell carcinoma cells. *Oncology*. (2006) 70:483–92. doi: 10.1159/000098813
148. von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Odum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat Immunol*. (2010) 11:344–9. doi: 10.1038/ni.1851
149. Bendix-Struve M, Bartels LE, Agnholt J, Dige A, Jorgensen SP, Dahlerup JF. Vitamin D3 treatment of Crohn's disease patients increases stimulated T cell IL-6 production and proliferation. *Aliment Pharmacol Ther*. (2010) 32:1364–72. doi: 10.1111/j.1365-2036.2010.04463.x
150. Bruce D, Cantorna MT. Intrinsic requirement for the vitamin D receptor in the development of CD8alphaalpha-expressing T cells. *J Immunol*. (2011) 186:2819–25. doi: 10.4049/jimmunol.1003444

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Immunomodulatory Effects of Vitamin D in Pregnancy and Beyond

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In addition to its role in calcium homeostasis and bone formation, a modulatory role of the active form of vitamin D on cells of the immune system, particularly T lymphocytes, has been described. The effects of vitamin D on the production and action of several cytokines has been intensively investigated in recent years. In this connection, deficiency of vitamin D has been associated with several autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), Hashimoto Thyroiditis (HT), and multiple sclerosis (MS). In a successful pregnancy, the maternal immune response needs to adapt to accommodate the semiallogeneic fetus. Disturbances in maternal tolerance are implicated in infertility and pregnancy complications such as miscarriages (RM) and preeclampsia (PE). It is well-known that a subset of T lymphocytes, regulatory T cells (Tregs) exhibit potent suppressive activity, and have a crucial role in curtailing the destructive response of the immune system during pregnancy, and preventing autoimmune diseases. Interestingly, vitamin D deficiency is common in pregnant women, despite the widespread use of prenatal vitamins, and adverse pregnancy outcomes such as RM, PE, intrauterine growth restriction have been linked to hypovitaminosis D during pregnancy. Research has shown that autoimmune diseases have a significant prevalence within the female population, and women with autoimmune disorders are at higher risk for adverse pregnancy outcomes. Provocatively, dysregulation of T cells plays a crucial role in the pathogenesis of autoimmunity, and adverse pregnancy outcomes where these pathologies are also associated with vitamin D deficiency. This article reviews the immunomodulatory role of vitamin D in autoimmune diseases and pregnancy. In particular, we will describe the role of vitamin D from conception until delivery, including the health of the offspring. This review highlights an observational study where hypovitaminosis D was correlated with decreased fertility, increased disease activity, placental insufficiency, and preeclampsia in women with APS.

Keywords: vitamin D, autoimmunity, antiphospholipid antibodies, pregnancy, placenta, fetal origin of adult disease

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INTRODUCTION

First, we will briefly summarize the enzymes and precursors involved in the synthesis of the active form of vitamin D (**Figure 1**). Vitamin D_3 (cholecalciferol) is taken in the diet or is synthesized in the epidermis from 7-dehydrocholesterol by exposure to ultraviolet irradiation (UV) (1). In order to be biologically active, vitamin D must be converted to its active form.

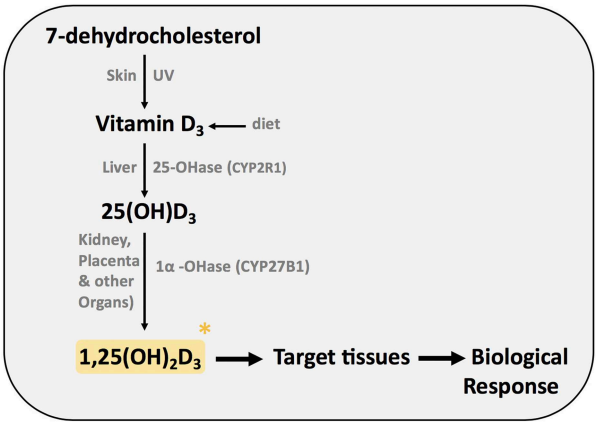

FIGURE 1 | Synthesis of active form of vitamin D ($1,25(OH)_2D_3$).

Vitamin D is transported in the blood by the vitamin D binding protein (DBP). In the liver vitamin D is hydroxylated at C-25 by cytochrome P450 vitamin D 25 hydroxylases, resulting in the formation of 25-hydroxyvitamin D_3 ($25(OH)D_3$). CYP2R1 is the key enzyme required for 25 hydroxylation of vitamin D (1). $25(OH)D_3$ is then hydroxylated in the A ring at carbon 1, resulting in the biological active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The cytochrome P450 monooxygenase 25(OH)D 1α hydroxylase (CYP27B1; 1α(OH)ase) is present in the kidney and other extrarenal sites such as the placenta, macrophages, lungs, and brain. Despite normal dietary vitamin D intake, mice with mutations in the 1α(OH)ase gene develop vitamin D dependency rickets (VDDR) type 1, highlighting the importance of this enzyme. In this review, we will use the term vitamin D to describe the active molecule $1,25\ (OH)_2D_3$ unless we specify the vitamin D metabolite or precursor particularly investigated.

EFFECTS OF VITAMIN D ON THE IMMUNE SYSTEM

Historical Evidence of the Role of Vitamin D on the Immune System

A hundred years ago, the observations of Mellanby suggested a relationship between vitamin D and the immune system. An increase incidence of respiratory infections in rachitic children and dogs was reported in his study (2). Interestingly, vitamin D has been empirically used to treat infections such as tuberculosis (TB) before the discovery of antibiotics. Sunlight exposure and being outdoors was recommended for patients with TB based on the ability of UV to kill bacteria (3). Vitamin D-rich fish liver oil has also been used to treat TB patients. At the time, these observations were attributed to vitamin D deficiency leading to weakness and malnutrition instead of the effect of vitamin D on the immune system. The mechanism of action of vitamin D on the immune system was better understood with the help of molecular biology. We now know that the protective role of vitamin D on the immune system played an important role behind these old therapies to treat TB (4). In this line, present data favor ultraviolet (UV) irradiation and consequent suppression of local and systemic immune responses to reduce the severity of some inflammatory and immune diseases such as psoriasis, multiple sclerosis and asthma (5–7).

Interestingly, recent data demonstrate a link between vitamin D and TB. In this line, patients suffering from TB have shown either vitamin deficiency or vitamin D receptor (VDR) polymorphisms. Furthermore, vitamin D can suppress intracellular growth of *M. tuberculosis in vitro* (8, 9). In addition, the vitamin D-stimulated expression of antimicrobial peptides such as cathelicidin, involved in the first line of defense in TB patients, might be responsible for its protective effect in TB (10).

Immunoregulatory Effects of Vitamin D

The expression of vitamin D receptor (VDR) in immune cells has highlighted an interesting role of vitamin D in immunity. Today a compelling body of experimental evidence indicates that vitamin D plays a fundamental role in regulating both innate and adaptive immune systems (11). Vitamin D displays a local immune effect via intracellular vitamin D receptors (VDR), that are known to be present in monocytes/macrophages, T cells, B cells, natural killer cells (NK), and dendritic cells (DCs). After binding to its receptor VDR (a member of nuclear receptor superfamily), vitamin D forms a heterodimer with retinoid X receptor (RXR). This complex engages vitamin D Response Element (VDRE) and recruits activators and enzymes with histone acetylation activity. Therefore, the structural changes in chromatin induced by this complex results in the regulation of targeted gene (12).

Vitamin D and Innate Immunity

The innate immune system is differentially regulated by vitamin D signaling, where it modulates the synthesis of antimicrobial peptides (AMPs) including, cathelicidin and defensins (13). In this line, promoters of the human genes for cathelicidin, and defensin β2 contain VDRE. NKT cells are thymically derived cells of the innate immune system that produce high amounts of cytokines including IL-4 and IFN-γ. Vitamin D through its interaction with VDR regulates the normal development and function of NKT cells. In this line, NKT cells isolated from VDR knock out mice exhibited diminished secretion of IL-4 and IFN-γ. In addition, vitamin D induced activation in NK cells (14). Recently, Chen et al. studied the effect of vitamin D supplementation on innate immune cells. They observed an enhanced production of IL-1beta and IL-8 by both neutrophils and macrophages, whereas the phagocytic capacity was suppressed in these cells (15) (**Figure 2**). Other studies have similarly revealed that vitamin D suppresses the activation of macrophages resulting in an anti-inflammatory M2 macrophage phenotype (16). Notably, activation of human monocytes using CD40 ligand and interferon gamma (IFN-γ) have been found to induce VDR and CYP27B1-hydroxylase expression, resulting in enhanced autophagy and antimicrobial peptide synthesis (17). Whereas, vitamin D increases phagocytosis and bactericidal activity of pathogens such as *M. tuberculosis* and *P.*

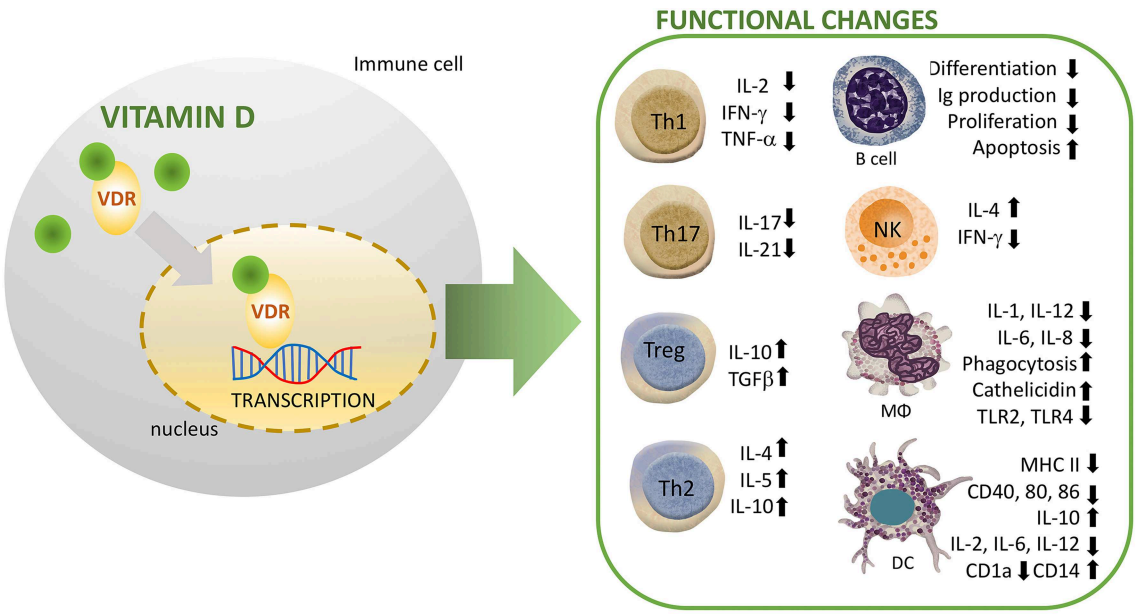

FIGURE 2 | Immunomodulatory effects of vitamin D ($1,25(OH)_2D_3$) on multiple immune cell lineages. NK, natural killer; DC, dendritic cell; R, receptor; VDR, vitamin D Receptor; MΦ, macrophage.

aeruginosa by macrophages (8, 18). Furthermore, the immune-modulating effects of vitamin D and its analogs have been well-characterized in dendritic cells (DCs), which are known to stimulate lymphocytes through antigen presentation. Recent research showed a robust vitamin D-dependent inhibition of maturation, differentiation, and survival of DCs (19). Several *in vitro* and *in vivo* studies have demonstrated a decreased expression level of costimulatory molecules (CD80, CD40, CD86), major histocompatibility complex (MHC) class II, and other maturation-induced surface markers, resulting in impaired maturation of DCs (20) (**Figure 2**). In response to inflammatory signals, vitamin D strongly impairs the migration and maturation of DCs, which culminates in reduced antigen presentation capacity and activation of T cells. Furthermore, cytokine shift with reduced interleukin-2 (IL-2) production, and increased IL-10 expression, leads to suppression of T helper 1 (Th1) phenotype (**Figure 2**). Therefore, by maintaining DCs in an immature phenotype, vitamin D and its analogs contribute to an induction of a tolerogenic state (21, 22).

Vitamin D and Adaptive Immunity

Early studies have shown that the VDR is highly expressed post-activation in both B and T lymphocytes (23). Among the main functions of vitamin D in the adaptive immune system, the effects of vitamin D on T cells deserve special attention. By binding to the VDR on T cells, vitamin D modulates the differentiation and activation of $CD4^+$ lymphocytes (24).

Tregs, a subset of $CD4^+$ lymphocytes suppress the immune response and mediate immune tolerance. Several studies have shown that vitamin D promotes proliferation and effector functions of immunosuppressive $Foxp3^+$ Tregs (25–27). In humans, vitamin D mediates regulation of suppressive T cells in complicated pregnancies (28). In addition, vitamin D signaling enhances the numbers of Tregs both in patients with inflammatory diseases and healthy controls (29). Interestingly, Vitamin D suppresses T lymphocytes proliferation by reducing IL-2 gene transcription, and inhibiting the production of pro-inflammatory Th-cytokines including, IFN-γ, IL-2, and IL-17 (30) (**Figure 2**). In agreement with prior studies, immunophenotyping of naïve and memory T lymphocytes in children has revealed an association between vitamin D and risk of infections. In this line, higher vitamin D levels were associated with protection due to increased number of memory T lymphocytes (31). Similarly, a recent study has demonstrated that reduced levels of vitamin D were associated with altered activation of T-lymphocytes in neonates. In particular, measurement of neonates and mothers' cord blood had revealed lower levels of naïve $CD4^+$ T cells, $CD4^+$ T-helper, and $CD8^+$ cytotoxic T lymphocyte in the vitamin D deficient group. In addition, one out of every six infant that presented with sepsis was deficient in vitamin D, suggesting a higher risk of infection in this group (32). Additionally, single-nucleotide polymorphism (SNP) analysis has identified T cell activation RhoGTPase activating protein (TAGAP) and IL-2RA as vitamin D responsive genes of CD4? T cells in patients with multiple sclerosis (33).

It also appears that vitamin D suppresses proliferation and immunoglobulin production in B cells. In addition, it also suppresses the differentiation of B cells into plasma cells (34, 35). Naïve B cells express very low levels of VDR. However, following activation VDR expression in B cells is increased. Vitamin D signaling potentiates apoptosis of activated B cells in presence of relevant stimuli. Moreover, vitamin D inhibits memory B cell

formation and secretion of immunoglobulins IgG and IgM in activated B cells (36).

Vitamin D and Autoimmunity

Autoimmunity

In view of the immunomodulatory effects of vitamin D on the adaptive immune response, we will discuss next the significance of vitamin D levels in autoimmune disorders. Autoimmune diseases are characterized by self-tissue destruction via the adaptive immune responses which evade immune regulation. As described above, vitamin D has been defined as a natural immune modulator. Vitamin D regulates the differentiation and activity of $CD4^+$ T cells, resulting in a more balanced Th1/Th2 response that limits development of self-reactive T cells preventing inflammation and autoimmunity (37–39). Therefore, a role for vitamin D deficiency in the pathogenesis of autoimmune diseases has been proposed. Several population-based and molecular studies reinforced this observation (34, 40, 41).

As previously described (**Figure 2**), vitamin D modulates adaptive immune cell functions explaining the significant association between vitamin D deficiency and autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), Hashimoto's thyroiditis (HT), and multiple sclerosis (MS) (42–49).

In animal models for MS and SLE, administration of vitamin D either prevented or improved autoimmunity (50). Furthermore, studies performed in mouse models with abrogated vitamin D signaling (dietary or genetic manipulation) demonstrated increased susceptibility to autoimmunity (51–53).

Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is an autoimmune disorder with a very complex pathophysiology. It is believed to be initiated by a Th1 type response resulting in joint destruction by immune cells (54). The presence of 1α hydroxylase and VDR on macrophages, chondrocytes, and synovial cells in the joints suggest that vitamin D might have a role in RA pathogenesis (55). Accordingly, it has been shown that vitamin D downregulates the production of proinflammatory cytokines IL-1β, IL-6, and TNF-α in macrophages in synovial tissue (42). Therefore, it has been suggested that vitamin D deficiency may increase the risk for the development of RA (56, 57). Consistent with this hypothesis, an inverse correlation between the risk to develop RA and vitamin D levels was described in a large population-based study comprising of almost 30 thousand women (58). Furthermore, evidence continues to accumulate supporting a role of VDR polymorphisms in the pathogenesis of RA (59). *TaqI and FokI* vitamin D receptor polymorphisms have been associated with an increase RA risk (60).

Systemic lupus erythematosus (SLE)

Among patients with autoimmune diseases, a higher prevalence of vitamin D deficiency was observed in systemic lupus erythematosus (SLE) (61). Patients with SLE have multiple risk factors for vitamin D deficiency. Increased photosensitivity, responsible for lower sun exposure, leads to a diminished production of vitamin D in the skin. In patients with lupus nephritis, the affected kidney may fail to carry out effective hydroxylation step of 25(OH)D. On the other hand, vitamin D's ability to modulate the immune suggests that hypovitaminosis D might lead to loss of tolerance and production of autoantibodies by B cells (62). In addition, vitamin D insufficient levels exacerbate autoantibody production and disease activity in SLE (63).

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis and obstetric complications. Thirty to forty percent of patients with SLE develop antiphospholipid antibodies. These antibodies may activate a cross talk between inflammation and thrombosis leading to adverse clinical events (64). An active crosstalk between inflammation and coagulation involving the complement system and tissue factor (TF), showed to be directly involved in APS-associated pregnancy complications in both mice and women (65–67). Vitamin D has shown not only immunomodulatory but also anti-thrombotic properties. In a study by Agmon-Levine et al., vitamin D deficiency was documented in almost 50% of APS patients vs. one third of controls and was significantly associated with thrombosis (68). In *in vitro* studies, vitamin D inhibited the expression of TF induced by antiphospholipid antibodies. A recent *in vitro* study in vascular smooth muscle cells demonstrated that vitamin D modulates tissue factor and protease-activated receptor 2 (PAR-2) expression (69). Provocatively, TF/PAR-2 signaling has been involved in the pathogenesis of adverse pregnancy outcomes in a murine model of APS (65).

As previously mentioned, complement activation plays a crucial role in adverse pregnancy outcomes in APS in mice and women (70–75). Interestingly vitamin D showed to increase expression of complement inhibitor CD55 (decay accelerating factor) in human monocytes and the associated inhibition of complement activation led to the prevention of preterm birth, adverse pregnancy outcome observed in APS (76). Reinforcing the role of vitamin D in pregnancy in APS, pravastatin that prevented pregnancy complications in APS in mice and women (65, 77) was shown to increase plasma concentrations of 25(OH)D and vitamin D in a rat model (78). Therefore, indicating that vitamin D might also contribute to the protective effects of pravastatin in obstetric APS (OAPS). Vitamin D deficiency is common among APS patients (52) and is also associated with placental dysfunction and adverse pregnancy outcomes (79). Therefore, vitamin D deficiency might contribute to the abnormal placental development and to the adverse pregnancy outcomes observed in OAPS [see Observational Study: Vitamin D levels in Women With Obstetric Antiphospholipid Syndrome (OAPS)].

Autoimmune thryroiditis—hashimoto thyroiditis (HT)

Vitamin D serum levels has been associated with the onset and progression of several autoimmune diseases including HT (80). In this line, higher serum 25OHD levels were associated with decreased risk of Hashimoto thyroiditis (HT). In this

study, the authors found that vitamin D supplementation leads to a significant decrease in the risk of developing HT (81). Interestingly, a meta-analysis showed a significant correlation between certain VDR gene polymorphisms and HT (82). Animal studies have shown a protective role of vitamin D in the development of experimental autoimmune thyroiditis (83). Vitamin D supplementation, improved inflammation of the thyroid gland by suppressing autoantibodies and proinflammatory cytokines production in mice (83, 84). Interestingly, several studies reported a significant association between vitamin D deficiency with the risk of HT (85, 86). On the other hand, a few studies did not find any link between vitamin D deficiency and the risk of Hashimoto thyroiditis (87, 88).

Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating autoimmune condition targeting the central nervous system (CNS) (89). There is a large body of evidence suggesting an association between lack of vitamin D early in life and development of MS (90). Furthermore, a nearly two-fold increased risk of MS was reported in the offspring of mothers that were vitamin D deficient (<30 nmol/L) during early pregnancy (90). Interestingly, UVB-induced vitamin D has shown protective effects in MS patients by upregulating Tregs and tolerogenic DCs (91). Similarly, these effects have also been observed in the experimental autoimmune encephalitis (EAE) mouse model of MS in which vitamin D induces tolerance via Tregs and IDO+ dendritic cells leading to reduced disease severity (92). Notably, vitamin D showed protective effects in a mouse model through the modulation of tight junction proteins in the BBB and nuclear factor kappa B (NFκB) activation (93). The anti-inflammatory effects of vitamin D toward a Th2 immune response may also contribute to its protection of the CNS (37). It is still debatable if the immunomodulatory effects of vitamin D can be used for clinical benefit in MS.

ROLE OF T CELLS IN PREGNANCY AND ITS COMPLICATIONS

In the last decade, an integrated mechanism, acknowledging both the innate and adaptive immune systems have been described to explain the maternal immune tolerance required to avoid rejection of the conceptus (94). It is established that during implantation, an active immune suppression is required to prevent an immune response against developing embryo. In this context, Tregs play a central role by repressing cytotoxic T cells, Th1 cells, macrophages, DC and NK cells leading to immune quiescence (95). Hence, both a maternal and fetal immune symbiotic relationship is created to allow a conducive environment for fetal growth and development. Several mechanisms support the maternal immune tolerance at the fetal–maternal interface. First, the adaptive immune response is curtailed by immune suppressive pathways or skewed toward immune tolerance. Second, the immune system contributes to the tissue remodeling necessary for placental development and function. In this context, uterine NK cells (uNK) have a special role facilitating trophoblast migration and the consequent development of the spiral arteries in contrast with peripheral cytotoxic NK cells (96). These uNK cells developed under the influence of IL-15 signaling that is expressed widely in the decidua and placenta (97). Furthermore, macrophages and DCs contribute to the immune tolerance in the gravid uterus. This unique immunological environment, important in maintaining a tolerant maternal–fetal interface is sustained by soluble molecules such as cytokines, chemokines, hormones, and prostaglandins (97). Crucial cell surface proteins, involved in induction of tolerance, are immune checkpoint inhibitors such as programmed death-1 (PD-1) and PD1 ligand (PD-L1). The importance of PD-1/PD-L1 has been demonstrated with an augmentation of this immunosuppressive pathway in normal pregnancies (98). The cytotoxic capacity of $CD8^+$ T subset in the decidua is significantly lower compared to peripheral $CD8^+$ T cells (99). In line with the importance of immune checkpoint inhibitors in favorable pregnancy outcomes, increased Tregs, have been observed in mouse models (100). In addition to expression of immune checkpoint inhibitors, Tregs secrete immuno-suppressive cytokines TGF-β and IL-10 (101). Complete abrogation of TGFβ signaling leading to Tregs deficiency results in non-viable mice (102). Restoring TGFβ signaling rescues this phenotype (103). In addition, partial TGFβ signaling leads to recurrent pregnancy loss (102). Decidual cytotoxic $CD8^+$ T cells are regulated in part via Tregs and relative expression of immune checkpoints (98). The loss of these regulatory mechanisms lead to enhanced $CD8^+$ T cell responses and adverse pregnancy outcomes (104).

THE ROLE OF VITAMIN D IN PREGNANCY: FROM CONCEPTION TO PARTURITION

In recent years, "pleiotropic" effects of vitamin D beyond its skeletal regulator functions have been demonstrated. Vitamin D autocrine, paracrine and endocrine functions have been described in numerous organs and systems, in particular the reproductive system. Several studies underscore the role of vitamin D in conception, placentation, progression of pregnancy and pregnancy outcomes including the offspring's health.

Vitamin D deficiency is common in women of reproductive-age (105). In a recent cohort study performed in Norway pregnant women from different ethnic groups showed hypovitaminosis D. Circulating vitamin D levels (<25 nmol/L) were found during pregnancy in women from South Asia (45%), Middle East (40%) and Sub-Saharan Africa (26%) (106). Hypovitaminosis D is a risk factor for infertility and several adverse pregnancy outcomes (107, 108). Furthermore, pre-pregnancy vitamin D levels higher than 75 nmol/L were associated with increased likelihood of pregnancy, reduced pregnancy loss and increased number of livebirths (109).

For many decades it was thought that metabolism of $25(OH)D_3$ only took place in the kidney. However, metabolism of $25(OH)D_3$ was demonstrated in many other organs including the reproductive tract. $25(OH)D_3$ and VDR are present in a variety of female reproductive organs such as pituitary glands,

hypothalamus, uterus, oviducts, ovaries, mammary glands, and the placenta (110). In this line, α-hydroxylase expressed in the decidua and placenta highlights the role of vitamin D synthesis in the fetomaternal interface (111, 112). Altogether, vitamin D supports placental development and function by regulating placental calcium transport, and by exerting immunomodulatory effects, critical for pregnancy maintenance (113, 114).

Role of Vitamin D in Fertility

Vitamin D in Female Fertility

Reduced mating success and fertility was observed in female rats with vitamin D deficiency. Vitamin D-deficient diet caused a reduction of up to 70% in the ability to conceive and a significant reduction in the number of viable pups (115). In agreement with the role of vitamin D in mammalian fertility, synergistic effects of vitamin D and progesterone have been observed in ovum implantation in rabbits (116).

While the diminished fertility in mice can be attributed to inadequate calcium levels to induce oocyte maturation, direct effects of vitamin D on the ovaries and hypothalamic–pituitary axis, including brain neurotransmitters such as serotonin, dopamine, and endogenous opioids should be acknowledged (108). In this line, vitamin D biosynthesis and signaling systems were demonstrated in primate ovarian follicles (117). A recent study demonstrated that vitamin D supplementation promoted survival and growth of antral follicles as well as oocyte maturation (117). Correlations between fertility, seasonal variations and geographical regions, have also been observed. It is now clear that these variations are due to changes in vitamin D-levels dependent on UV exposure (118).

Therefore, it is tempting to speculate that vitamin D deficiency might play a role in infertility, a common and distressing issue that affects around 10% of all couples. Consistent with this, a recent systematic review showed an association between serum vitamin D levels and the number of live births in women undergoing assisted reproductive technology (ART) (119). This study suggests that deficiency and insufficiency of vitamin D could be important factors to treat, particularly in women with compromised fertility to improve ART outcomes.

Vitamin D and Male Reproductive Physiology

The male reproductive tract is among the widespread systems affected by vitamin D. Expression of VDR, activating enzymes (CYP2R1, CYP27A1, CYP27B1), and inactivating enzymes (CYP24A1) have been demonstrated in the spermatozoa, seminal vesicle, prostate, epididymis including the human testis (120, 121). In addition, vitamin D deficiency has been associated with abnormal spermatogenesis and fertility in animal studies (122). In rats, a significant diminution (73%) was observed in the pregnancy rate when wild type females were mated with diet-induced vitamin D deficient males compared to females mated with vitamin D sufficient-males (122). In support of the observation that vitamin D is required for male fertility, oligoasthenospermia was described in α-hydroxylase CYP27B1 null mice. In this connection, men with vitamin D deficiency also exhibited altered sperm motility (123). Moreover, these α-hydroxylase CYP27B1 null mice also showed hypergonadotrophic hypogonadism suggesting a modulatory role for vitamin D signaling in gonadal function (124). Hypocalcemia has been shown to compromise capacitation and acrosomal reactions, crucial steps in fertilization (125). Therefore, hypocalcemia and hypophosphatemia secondary to vitamin D deficiency may also play an important role in male infertility. Furthermore, diets rich in calcium and phosphorous rescue male fertility in VDR knock-out mice and in male rats on a vitamin D deficient diet (126).

Role of Vitamin D in Conception

The rapid induction of VDR and α-hydroxylase CYP27B1 in decidua and placenta early in pregnancy highlights a fundamental role of vitamin D in conception, including implantation and the development of the placenta itself (110, 127).

It has been demonstrated that vitamin D binding to VDR upregulates key target genes, such as *HoxA10* crucial for endometrial development, uterine receptivity and implantation (128). The importance of vitamin D in the process of implantation has been further highlighted by studies using knockout mouse models. Both VDR and α-hydroxylase CYP27B1 knock out female mice present with uterine hypoplasia and infertility (129, 130). Injection of vitamin D has been shown to increase uterine weight and promote decidualization of the endometrium in pseudo-pregnant rats suggesting that vitamin D contributes to a crucial step in blastocyst implantation (131).

In addition to its direct role in the decidualization and placentation, vitamin D may also influence implantation and placentation indirectly via its immunomodulatory actions. The immunosuppressive effects of vitamin D during pregnancy and in particular during implantation were postulated many year ago and might contribute to preventing a maternal immune response against the paternal genes-carrying embryo (127). Therefore, throughout pregnancy, decidual synthesis of vitamin D has the potential to modulate uNK cells, DCs, macrophages and T-cells leading to immune tolerance (132, 133). It is well-established that vitamin D inhibits Th1 cytokines while promoting Th2 cytokines, therefore it may favor the process of implantation (133).

Pregnancy Complications and Vitamin D

Preeclampsia (PE)

Fetal cytotrophoblast and differentiated extravillous trophoblasts (EVT) invasion of the maternal decidua and myometrium in the first trimester of pregnancy is key for placentation and successful pregnancy. Interactions between trophoblasts, decidual stromal, and immune cells facilitate implantation and maintenance of pregnancy. Importantly, defective invasion of EVT can cause abnormal placentation and important pregnancy disorders such as miscarriage, PE, intrauterine growth restriction (IUGR), preterm birth (PTB) and stillbirth. Vitamin D deficiency has been associated with increased incidence of pregnancy complications (134). A recent meta-analysis demonstrated an increased risk of PE in women with hypovitaminosis D (135).

Abnormal expression of 1α-hydroxylase has been observed in syncytiotrophoblastic cells from preeclamptic pregnancies (136). Even more, low levels of vitamin D have been found in women

that developed severe early onset preeclampsia and vitamin D supplementation showed a protective effect against recurrent PE (137, 138). That PE is characterized by defective placentation at early stages of pregnancy and that hypovitaminosis D is frequently found in women with PE, suggest a potential role for vitamin D as a crucial molecule in normal placentation. An association between VDR *FokI* polymorphism and the risk of PE has also been reported, suggesting that the interaction of vitamin D with its receptor is required for placenta development and function (139). It has also been suggested that low levels of vitamin D may disrupt the immune balance leading to overexpression of Th1 cytokines and failure of immunological tolerance toward embryo implantation (133). In this line, higher expression of Th1 cytokines have been described in placentas of preeclamptic pregnancies, suggesting a protective role of vitamin D at the feto-maternal interface (140).

That both abnormal trophoblast invasion and maternal hypovitaminosis D are associated with abnormal placentation and adverse pregnancy outcomes, suggests a link between vitamin D and EVT migration. Interestingly, vitamin D has been described as a modulator of cellular motility and invasion in cancerous cells (141). In this line, *ex vivo* studies have shown that vitamin D promotes migration and invasion of human EVT isolated from first trimester pregnancies, through enhanced expression of matrix metalloproteinases MMP2 and MMP9 (142).

The molecular mechanisms behind the regulatory effects of vitamin D on cell migration and invasion are not completely understood. Vitamin D has been shown to regulate the actin cytoskeleton in numerous cell types, including trophoblasts (143). In addition, vitamin D restored mobility in umbilical vein endothelial cells (HUVEC) derived from pregnancies affected by PE and gestational diabetes (144). Vitamin D may also exert indirect effects on trophoblast invasion by stimulating the secretion of human chorionic gonadotrophin (hCG) and progesterone (145).

In addition to the detrimental effects in placentation and potential causative effects in PE development, vitamin D deficiency might also contribute to hypertension, a characteristic sign of PE. It is known that suboptimal levels of vitamin D are associated with unfavorable effects on the cardiovascular system. Vitamin D deficiency has been shown to activate the renin-angiotensin-aldosterone system (RAAS) and to induce endothelial dysfunction, both contributing to hypertension (146). In support of the role of vitamin D on the RAAS system, VDR knockout mice displayed disrupted renin expression and angiotensin II production (147). Vitamin D deficiency might also play a role in endothelial dysfunction, a crucial feature in the pathogenesis of PE. 1α-hydroxylase is present in the endothelial and vascular smooth muscle cells protecting the vascular walls through generation of vitamin D (148). Furthermore, vitamin D inhibits endothelial cell activation by cytokines as well as adhesion molecules expression that involves TNF-α (149, 150). Therefore, it has been hypothesized that vitamin D supplementation might help protect endothelial function and control blood pressure in preeclamptic patients (151).

Vitamin D in Obesity in Pregnancy

Obesity is a major contributing factor to vitamin D status in pregnancy. While there is no difference between non-obese and obese individuals regarding the synthesis of vitamin D in the skin, the vitamin D concentration in plasma is 57% less in the obese than in the non-obese subjects (152). In this line, it has been demonstrated that excessive adipose tissue causes a decrease in the release of endogenously synthesized vitamin D into the circulation (152). Obesity, a health issue with serious cardiovascular risk also results in higher incidence pregnancy complications associated with increased maternal and fetal morbidity. Studies performed in a large cohort of Chinese couples of reproductive age showed that increases in pre-pregnancy maternal and paternal body mass index (BMI), both independently and combined, increases the risk of adverse pregnancy outcomes such as PTB, low weight birth, and stillbirth (153). The likelihood of conception decreases in a linear fashion with increases in BMI (4% decrease per 1 kg/m^2 weight gain, starting from a BMI of 29 kg/m^2) (154). Diminished bioavailability of vitamin D in obese pregnant women leading to reduced immunomodulatory effects at the fetal–maternal interface might explain the adverse pregnancy outcomes in these women. Limited sunlight exposure and nutrient-poor but hypercaloric diets might exacerbate the vitamin D deficiency observed in obese pregnant women, affecting both the mother and the developing fetus. It has been suggested that supplementation with vitamin D might be beneficial in obese patients (155).

Preterm Birth (PTB)

PTB, is a major public health concern as it is the main cause of neonatal morbidity and mortality, with an estimated prevalence of 10.6% of live births (156). Epidemiologic studies suggested an association between maternal hypovitaminosis D during pregnancy and PTB (157). It has been suggested that low levels of maternal circulating vitamin D could increase PTB risk and that vitamin D supplementation during pregnancy might help reduce this risk (158).

The onset of labor is caused by an inflammatory response that not only involves the resident immune cells but also the recruitment of inflammatory cells into the reproductive tissues. Of note, there is a significant amount of cytokines/chemokines released at the feto-maternal interface (159). Untimely, premature activation of these inflammatory pathways leads to preterm labor, which can result in PTB. It has been suggested that T cell activation participates in these proinflammatory responses at the fetomaternal interface and cervix during preterm labor. Moreover, T cells with a Th1 phenotype were found in the cord blood of preterm but not in term infants (160). That lower levels of vitamin D are observed in women that delivered preterm, suggests that vitamin D may play an important role in suppressing the maternal immune response and Th1-mediated inflammatory pathways that lead to the onset of labor. In line with vitamin D effects on Tregs, a significant correlation between Tregs and blood vitamin D levels was observed in term and preterm parturition (161). A recent meta-analysis that included 15 trials and 2,833 pregnant women, concluded that

supplementation with vitamin D reduced the risk of PTB by 65%, PE by 48% and low birthweight (lower than 2,500 g) by 60% compared with no intervention or placebo (162).

MATERNAL VITAMIN D DEFICIENCY ASSOCIATION WITH FETAL ORIGIN OF ADULT DISEASE (FOAD)

The surrounding environment affects our health in countless ways. Provocatively, the effects of the environment begin early in life; the maternal womb being the first environment to which the organism is exposed. During the intra-uterine life, the developing fetus is particularly vulnerable to insults, not limited to malnutrition (163).

The placenta is formed at gestational week 4 allowing nutrients to reach the developing fetus. From this time until delivery $25(OH)D_3$ easily crosses the placenta reaching concentrations in fetal cord blood equivalent to 87% of the maternal blood levels (164). The biological active vitamin D does not cross the placenta (**Figure 3**). Interestingly, the placenta and fetal tissues express 1α-hydroxylase leading to bioactive vitamin D in the fetal circulation. Therefore, the fetus depends fully on maternal $25(OH)D_3$ supply and hypovitaminosis D during pregnancy may affect fetal development and future health of the offspring in agreement with the concept of fetal origins of adult disease (FOAD), that Dr. David Barker first popularized (165). The FOAD hypothesis proposes that "events during early development have a profound impact on one's risk for development of future adult disease." Low birth weight resulting from poor fetal growth and nutrition, is associated to several adult diseases such as, hypertension, obesity, coronary artery disease and insulin resistance (163). It is now well-recognized that the phenotype of an individual can be determined by the nutritional status of the mother. Poor nutrition can lead to hypovitaminosis D. In this context, during "developmental programming" lack of vitamin D during a critical window of development can lead to permanent alterations in physiological processes. In addition, obesity, that is also characterized by diminished vitamin D availability has been associated with adverse health effects not only in the mother but the developing child and offspring later in life (166). Several prospective birth cohort studies followed long-term health outcomes after complicated pregnancies (167).

Epigenetic modification, defined as non-heritable changes in gene expression that are not mediated by alterations in DNA sequence may occur *in utero* (168). *In utero* epigenetic fetal programming may activate specific genes that control fetal development increasing disease risk. Recent studies demonstrated that epigenetic changes of vitamin D catabolism play an important role in increasing vitamin D bioavailability at the fetomaternal interface (169). In addition, it has been shown that maternal vitamin D modifies the expression of the genes encoding placental calcium transporters, influencing bone mineral accrual in the neonate (170). Maternal supplementation with vitamin D during pregnancy significantly reduces the risk of infantile rickets and hypocalcemia (171).

Vitamin D Deficiency During Pregnancy and the Health of the Offspring—Does Fetal Vitamin D Compromise the Offspring's Immune Function?

Vitamin D and Asthma

According to the World Health Organization (WHO), "asthma is the most common chronic disease among children" (172). Several studies demonstrated that prenatal vitamin D status plays a role in the offspring's susceptibility to develop asthma later in life (173, 174). Recent data has suggested a crucial role for vitamin D in reprogramming $CD8^+$ T-cells to induce an IL-13-secreting signature, suggesting vitamin D as a promising regulator in asthma (175). That VDRs are present in immune cells and the airways, support this hypothesis (176). Interestingly, polymorphisms in VDR and vitamin D metabolism genes are associated with childhood asthma susceptibility (177).

An association between reduced risk of wheeze in the offspring and high dietary vitamin D intake during pregnancy have been shown by two meta-analyses (178, 179). However, the conclusions of these observational studies are still controversial and randomized control clinical trials are necessary to determine the appropriate levels of vitamin D supplementation during pregnancy on maternal, fetal and perinatal outcomes.

Vitamin D, Fetal Neurodevelopment, and Neurocognitive Function

VDR and 1α-hydroxylase have been identified in the fetal brain highlighting the role of vitamin D in brain development (180). In the fetus, serum $25(OH)D_3$ and vitamin D can cross the BBB (**Figure 3**) bind to VDR and stimulate a wide range of responses, genomic and non-genomic. Furthermore, activated microglial cells *in vitro* have been shown to actively synthesize the active metabolite, $1,25(OH)_2D_3$ (181). In addition, *in vitro* studies demonstrated that activated microglia increased the expression of the VDR and 1α-hydroxylase, enhancing their responsiveness to vitamin D. Furthermore, activated microglia incubated with vitamin D showed a reduced expression of pro-inflammatory cytokines, IL-6, IL-12, and TNFα, and increased expression of IL-10, indicative of a immunosuppressive effects of vitamin D in the CNS (182).

Vitamin D has also been shown to regulate neurotrophic signaling, including glial derived neurotrophic factor (GDNF) and nerve growth factor (NGF), critical for the survival and migration of developing neurons in the brain (183). Low concentrations of $25(OH)D_3$ during critical stages of development have the potential to affect the reprogramming of the brain tissue structure and function. It has been shown that vitamin D-deficiency leads to fetal mouse brain abnormal morphology and expression of genes related to neuronal survival (184). The ability of vitamin D to regulate neurotrophic factors and modulate inflammation has led to the suggestion that vitamin D is indeed neuroprotective (185). Furthermore, pre-treatment with vitamin D can decrease glutamate-mediated cell death in cultures of cortical, hippocampal and mesencephalic neurons (186). These neuroprotective effects have been recently highlighted in a study showing the inhibitory effect of vitamin

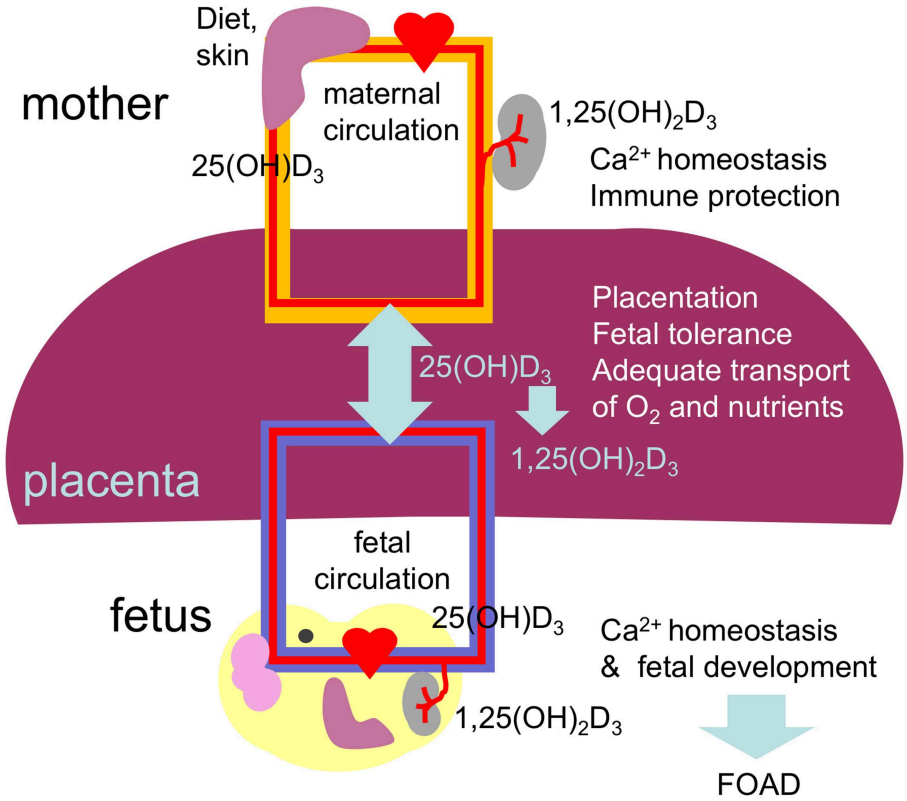

FIGURE 3 | Diagram summarizing the placental transport and role of 25(OH)D3 and 1,25$(OH)_2D_3$ on the placental function and fetal development. Vitamin D during pregnancy is important for placentation (trophoblast migration and remodeling of spiral arteries), immune tolerance, maintaining maternal calcium homeostasis and therefore for fetal development, including the skeletal system and the brain. Low levels of vitamin D during pregnancy can result in abnormal placentation, placental insufficiency and abnormal fetal development leading to compromised health after birth, in agreement with the FOAD concept.

D on reactive oxygen species (ROS) toxicity by increasing the synthesis of antioxidant molecules in both glia and neurons (187).

In line with the neuroprotective effects of vitamin D, hypovitaminosis D during the fetal life was associated with greater susceptibility to MS and greater severity of MS symptoms later in life (89). *In vitro* studies in cell culture demonstrated that vitamin D protects neurons from injury induced by modulating T cell activity (188).

On a tissue level, maternal hypovitaminosis D in rats alters the fetal brain morphology leading to psychological disorders in the developing offspring (189). The changes in brain morphology observed in the offspring born to vitamin D-deficient mothers, thinning of neocortex, and ventricle overgrowth, are similar to the ones observed in brains of schizophrenic children suggesting that maternal hypovitaminosis D may be a risk factor for schizophrenia (190). Epidemiological evidence supporting the association between vitamin D exposure in early life and schizophrenia has also been described (191).

Recent studies suggest that maternal vitamin D insufficiency during early pregnancy is also associated with attention-deficit / hyperactive disorder (ADHD)-like symptoms in offspring at age 4 (192). An association between lower first trimester maternal circulating concentration of 25$(OH)D_3$ and an increased risk of developing autism in offspring at age 3–7 has been reported in the Chinese population (193). A positive association between lower levels of serum 25$(OH)D_3$ (<25 nmol/L) and risk of autism spectrum disorder (ASD) in children was also described in studies performed in Sweden and Iran (194, 195). Moreover, a recent study demonstrated a correlation between mid-gestation vitamin D deficiency and the risk of developing clinical ASD with severe intellectual impairment (196). While results from the latest epidemiological studies support the concept that prenatal vitamin D status impacts the neuropsychological development of children, further research is needed to confirm these observations.

Taking into consideration the important protective effects of vitamin D in fetal development and the future health of the offspring, screening of vitamin D levels during the preconception period and the first trimester of pregnancy should be recommended in women with high risk of hypovitaminosis D, such as women with high body max index, dark skin or autoimmune diseases in order to implement appropriate treatment to prevent adverse pregnancy outcomes, fetal developmental abnormalities and future compromise of the offspring's health in general.

OBSERVATIONAL STUDY: VITAMIN D LEVELS IN WOMEN WITH OBSTETRIC ANTIPHOSPHOLIPID SYNDROME (OAPS)

The association between hypovitaminosis D and dysregulation of the immune system, in particular T cells, leading to autoimmunity and adverse pregnancy outcomes prompted us to investigate the levels of vitamin D in women with OAPS. Knowing that T cells play a crucial role in conception and maintenance of pregnancy, we hypothesized that there is a correlation between vitamin D levels and fertility and pregnancy outcomes in women with OAPS. We also determined the association between vitamin D levels and markers of disease activity (presence of flares and complement C3 consumption) in women with OAPS. Co-presence of autoimmune disease Hashimoto thyroiditis (HT) was also investigated.

Patients and Laboratories Determinations

This observational study, was performed at the Perigenesis, Institute of Obstetric Hematology, Thessaloniki, Greece. All studies in women were performed in strict agreement with the Greece National Bioethics Commission. All patients gave written informed consent.

Antiphospholipid syndrome (APS) was defined by the presence of clinical and laboratory criteria described in the "International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome" (APS) (197). Clinical criteria included one or more clinical episodes of thrombosis and pregnancy morbidity. Pregnancy morbidity was defined as one or more unexplained fetal deaths at or beyond the 10th week of gestation or one or more premature births before the 34th week of gestation due to placental insufficiency such as PE or three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. The laboratory criteria for APS includes the presence of lupus anticoagulant (LA) and/or anticardiolipin (aCL) and/or β2 glycoprotein-I IgG or IgM antibody in plasma or serum on two or more occasions, at least 12 weeks apart (197).

Seventy-six women met the criteria for OAPS before the current pregnancy. All women received conventional low dose aspirin plus low molecular weight heparin (LDA+LMWH) treatment since the beginning of pregnancy (198). Median age for the patients was 37.5 years (IQR 33–40.5).

Vitamin D levels were measured in all women during the first trimester of pregnancy using ELISA tests. Testing for vitamin D is part of standard patient care in Greece. Vitamin D levels were classified as normal (>30 ng/mL) and hypovitaminosis D (<30 ng/mL). Hypovitaminosis D was further classified as deficiency (20.1–29.9 ng/mL) and insufficiency (<20 ng/mL). None of the 76 women received vitamin D supplementation.

Complement activation plays a key role in the pathophysiology of OAPS (70–75). In addition, measuring complement C3 serum levels is a routine practice to monitor disease activity in patients with autoimmune diseases. Therefore, levels of C3 in OAPS women were determined by ELISA. The values of complement component C3 were grouped in three categories (normal: 60–150 mg/dl, low and high). All laboratory tests were performed in the first trimester of the current pregnancy.

Pregnancy complications were classified as follows:

Preeclampsia (PE) was classically defined as a systemic syndrome characterized by new-onset of hypertension and proteinuria in pregnancy. Early onset PE was defined as PE that develops before 34 weeks of gestation. Preterm birth was defined as any birth before 37 completed weeks of gestation. Placental insufficiency refers to placental dysfunction characterized by increased resistance of uteroplacental blood vessels resulting in increased uterine arteries pulsatility index (>95thcentile).

Implantation failure was defined as the inability to achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles.

Flares were defined as the relapse of symptoms that can compromise the skin, the joints, or any other compromised organ.

Hashimoto's disease diagnosis was based on blood tests showing lower serum T3 (triiodothyronine) and T4 (thyroxine) levels (<10% of the reference values) with normal thyroid-stimulating hormone levels and the presence of antithyroid antibodies [anti-TPO (anti-thyroid peroxidase) and anti-Tg (anti-thyroglobulin) antibodies].

Statistical Analysis

All analysis were conducted with GraphPad Prism statistical software (GraphPad Software Inc.). Absolute and relative frequencies were calculated. Fisher's exact test was performed. Two tailed p-values were calculated. $P < 0.05$ was considered statistically significant.

Exploratory Data Analysis

Data from seventy-six pregnant women with OAPS were analyzed. In agreement with the literature (45, 84, 199, 200), a high percentage (77.6%) of these patients showed hypovitaminosis D (**Table 1**). Within this group, 64.4% of the women were vitamin D deficient and 35.6% vitamin D insufficient in the first trimester of pregnancy. Only 17 out of the 76 patients (22.4%) showed vitamin D levels within the normal range. Of note, wearing sunscreen, limited exposure to sun light, dark skin and dairy products not supplemented with vitamin D in Greece might contribute to hypovitaminosis D in this geographic area.

Around 50% of women with hypovitaminosis D conceived after IVF (57.1% in the deficient group and 39.5 in the insufficient group) and a higher incidence, though not statistically significant, of implantation failure was also observed in this group, suggesting an association between lower levels of vitamin D and compromised fertility in OAPS patients.

In accordance with previous studies, low levels of complement C3 were observed in 28% of all OAPS patients (45, 199, 200). Interestingly, 39.5% of the patients in the vitamin D deficient and 57.1% in the vitamin D insufficient presented low levels of C3, suggesting an association between lower levels of vitamin D and lower levels of C3, indicative

TABLE 1 | Vitamin D levels, fertility and pregnancy outcomes, complement levels, disease activity and co-presence of Hashimoto Thyroiditis in OAPS patients.

	OAPS patients ($N = 76$)		
Vitamin D levels	**Normal $N = 17$ (24.3%)**	**Hypovitaminosis D $N = 59$ (77.6%)**	
		Deficiency	**Insufficiency**
		$N = 38$ (64.4%)	**$N = 21$ (35.6%)**
Implantation failure	1 (5.9%)	8 (21%) $p = 0.2469$	6 (28.6%) $p = 0.2204$
		14 (23.7%), $p = 0.1669$	
IVF	2 (11.8%)	15 (39.5%) $p = 0.0584$	12 (57.1%)*$p = 0.0063$
		27 (45.8%)*, $p = 0.0116$	
Low C3 levels	1 (5.8%)	12 (31.6%)* $p = 0.0452$	8 (38%)*$p = 0.0263$
		20 (33.9%)* $p = 0.0297$	
Flares	0	8 (21%)*$p = 0.0479$	14 (66.7%)*$p = 0.001$ **$p = 0.0008$
		22 (37.3%)*, $p = 0.0020$	
Hashimoto thyroiditis	0	17 (44.7%)*$p = 0.0005$	10 (47.6%)*$p = 0.0008$
		27 (45.8%)* $p = 0.003$	
Placental insufficiency	0	6 (15.8%) $p = 0.1615$	8 (38.1%) *$p = 0.0047$
		14 (23.7%), *$p = 0.0308$	
PE (%)	0	6 (15.8%) $p = 0.1615$	6 (28.6%)*$p = 0.0241$
		12 (20.3%), $p = 0.0531$	
PTB (%)	0	3 (7.9%) $p = 1.000$	2 (13.3%) $p = 0.4922$
		5 (8.5%), $p = 0.5812$	

Conception after IVF and the presence of implantation failure (inability to achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles) were investigated as signs of compromised fertility. Placental insufficiency, preeclampsia and preterm birth were used to evaluate pregnancy outcomes. Presence of flares, defined as the relapse of symptoms that can compromise the skin, the joints, or any other compromised organ and complement C3 consumption were used to evaluate disease activity.

N, number of patients; %, percentage of patients; IVF, in vitro fertilization; PE, preeclampsia; PTB, preterm birth.

**Different from patients with normal levels of vitamin D.*

***Different from patients with deficient levels of vitamin D. $P < 0.05$ is considered statistically significant.*

of complement consumption/activation by autoantibodies. While lower C3 levels could be caused by complement C3 deficiency, none of these patients showed increased susceptibility to infection, characteristic of the rare, genetic C3 deficiency.

Monitoring serum levels of C3 to assess for disease activity is recommended in patients with autoimmune diseases, in particular APS. In this study, we found a correlation between decreased levels of C3 and flares in pregnant women with antiphospholipid antibodies. Thirty-seven percent of the OAPS patients with hypovitaminosis D showed disease flares in contrast to none in the group with normal vitamin D levels, emphasizing the link between hypovitaminosis D and immune dysregulation previously described.

Strikingly, autoimmune hypothyroidism (Hashimoto disease, HT) associated with anti-TPO and anti-Tg antibodies was detected in almost 50% of the patients with hypovitaminosis D (44.7% in the vitamin D deficient group and 47.6% of the vitamin D insufficient women). The 17 OAPS patients with normal vitamin D values were euthyroid. While several studies have shown a correlation between vitamin D deficiency and thyroid autoimmunity (84, 201). It is still unclear whether the hypovitaminosis D is the result of HT disease or a part of its cause. One patient in the vitamin D deficient group and one in the vitamin D insufficient group were also diagnosed with autoimmune disorder Sjogren syndrome.

Provocatively, knowing the role of the immune system, in placentation and placental development, the number of cases of placental insufficiency, determined by decreased uterine artery flow, was significantly higher in the OAPS women with hypovitaminosis D compared to vitamin D sufficient OAPS-women. A bigger number of PE cases was observed in the vitamin D insufficient group. Abnormal placentation and pregnancy complications such as PE were not observed in the OAPS patients with normal vitamin D values. 8.5% of OAPS women with hypovitaminosis D delivered preterm in contrast with 0% of the OAPS women with normal vitamin D levels. However, the difference did not reach statistical significance. There were no significant associations between lower levels of vitamin D and other variables such as age, parity and type of aPL autoantibodies.

In conclusion, hypovitaminosis D (<30 ng/mL) was documented in almost 80% of OAPS patients during the first trimester of pregnancy and was associated with complement activation, increased incidence of flares, presence of autoimmune thyroiditis, placental insufficiency, and a higher incidence of preeclampsia. If hypovitaminosis is the cause or the consequence of autoimmunity and adverse pregnancy outcomes needs to be addressed in further studies.

CONCLUSIONS OF THE OBSERVATIONAL STUDIES

While numerous clinical and experimental evidence suggest that vitamin D deficiency is an important factor in the pathogenesis of adverse pregnancy outcomes in APS and the proven immunomodulatory effects of vitamin D and its analogs, as previously described, the effects of vitamin D supplementation in the prevention and treatment of pregnancy disorders are not completely understood. Only small and non-controlled studies have been performed in humans; however, they seem to indicate there is a potential beneficial effect of vitamin D supplementation in modulating the immune system, preventing inflammation and protecting maternal and fetal health. Our small observational study suggests that subnormal vitamin D levels is another contributing factor to adverse pregnancy outcomes in women with APS. The cause-consequence effects and the risks and benefits of vitamin D supplementation in autoimmunity, in

particular APS and HT, and high-risk pregnancies needs to be further investigated.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FC and GG reviewed the literature, interpreted the observational studies, performed statistical analysis, and wrote the review article. EL and KV were responsible for the supervision of the patients and data collection. GG created graphs.

REFERENCES

1. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol*. (2014) 21:319–29. doi: 10.1016/j.chembiol.2013.12.016
2. Mellanby E. Experimental rickets. *Med Res Coun Spec Rep Ser*. (1921) 61:1–78.
3. Weinzirl J. The action of sunlight upon bacteria with special reference to *B. tuberculosis*. *J Infect Dis*. (1907) 4:128–153. doi: 10.1093/infdis/4.Supplement_3.128
4. Kearns MD, Tangpricha V. The role of vitamin D in tuberculosis. *J Clin Transl Endocrinol*. (2014)1:167–9. doi: 10.1016/j.jcte.2014.08.002
5. Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat Rev Immunol*. (2011) 11:584–96. doi: 10.1038/nri3045
6. Johnson-Huang LM, Suárez-Fariñas M, Sullivan-Whalen M, Gilleaudeau P, Krueger JG, Lowes MA. Effective narrow-band UVB radiation therapy suppresses the IL-23/IL-17 axis in normalized psoriasis plaques. *J Invest Dermatol*. (2010) 130:2654–63. doi: 10.1038/jid.2010.166
7. Vieyra-Garcia PA, Wolf P. From early immunomodulatory triggers to immunosuppressive outcome: therapeutic implications of the complex interplay between the wavebands of sunlight and the skin. *Front Med*. (2018) 5:232. doi: 10.3389/fmed.2018.00232
8. Martineau AR, Honecker FU, Wilkinson RJ, Griffiths CJ. Vitamin D in the treatment of pulmonary tuberculosis. *J Steroid Biochem Mol Biol*. (2007) 103:793–8. doi: 10.1016/j.jsbmb.2006.12.052
9. Hewison M. Antimicrobial effects of vitamin D. *Nat Rev Endocrinol*. (2011) 7:337–45. doi: 10.1038/nrendo.2010.226
10. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. (2006) 311:1770–73. doi: 10.1126/science.1123933
11. Chun RF, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol*. (2014) 5:151. doi: 10.3389/fphys.2014.00151
12. Sasaki H, Harada H, Handa Y, Morino H, Suzawa M, Shimpo E, et al. Transcriptional activity of a fluorinated vitamin D analog on VDR-RXR-mediated gene expression. *Biochemistry*. (1995) 34:370–7. doi: 10.1021/bi00001a045
13. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol*. (2004) 173:2909–12. doi: 10.4049/jimmunol.173.5.2909
14. Balogh G, de Boland AR, Boland R, Barja P. Effect of 1,25(OH)(2)-vitamin D(3) on the activation of natural killer cells: role of protein kinase C and extracellular calcium. *Exp Mol Pathol*. (1999) 67:63–74. doi: 10.1006/exmp.1999.2264
15. Chen L, Eapen MS, Zosky GR. Vitamin D both facilitates and attenuates the cellular response to lipopolysaccharide. *Sci Rep*. (2017) 7:45172. doi: 10.1038/srep45172
16. Liang S, Cai J, Li Y, Yang R. 1,25-Dihydroxy-Vitamin D3 induces macrophage polarization to M2 by upregulating T-cell Ig-mucin-3 expression. *Mol Med Rep*. (2019) 19:3707–13. doi: 10.3892/mmr.2019.10047
17. Klug-Micu GM, Stenger S, Sommer A, Liu PT, Krutzik SR, Modlin RL, et al. CD40 ligand and interferon-γ induce an antimicrobial response against *Mycobacterium tuberculosis* in human monocytes. *Immunology*. (2013) 139:121–8. doi: 10.1111/imm.12062
18. Nouari W, Ysmail-Dahlouk L, Aribi M. Vitamin D3 enhances bactericidal activity of macrophage against *Pseudomonas aeruginosa*. *Int Immunopharmacol*. (2016) 30:94–101. doi: 10.1016/j.intimp.2015.11.033
19. Griffin MD, Lutz WH, Phan VA, Bachman LA, McKean DJ, Kumar R. Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs. *Biochem Biophys Res Commun*. (2000) 270:701–8. doi: 10.1006/bbrc.2000.2490
20. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol*. (2000) 164:2405–11. doi: 10.4049/jimmunol.164.5.2405
21. Bartels LE, Hvas CL, Agnholt J, Dahlerup JF, Agger R. Human dendritic cell antigen presentation and chemotaxis are inhibited by intrinsic 25-hydroxy vitamin D activation. *Int Immunopharmacol*. (2010) 10:922–8. doi: 10.1016/j.intimp.2010.05.003
22. Dam TN, Møller B, Hindkjaer J, Kragballe K. The vitamin D3 analog calcipotriol suppresses the number and antigen-presenting function of Langerhans cells in normal human skin. *J Investig Dermatol Symp Proc*. (1996) 1:72–7.
23. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch Biochem Biophys*. (2000) 374:334–8. doi: 10.1006/abbi.1999.1605
24. Palmer MT, Lee YK, Maynard CL, Oliver JR, Bikle DD, Jetten AM, et al. Lineage-specific effects of 1,25-dihydroxyvitamin D(3) on the development of effector CD4 T cells. *J Biol Chem*. (2011) 286:997–1004. doi: 10.1074/jbc.M110.163790
25. Chambers ES, Hawrylowicz CM. The impact of vitamin D on regulatory T cells. *Curr Allergy Asthma Rep*. (2011) 11:29–36. doi: 10.1007/s11882-010-0161-8
26. Mocanu V, Oboroceanu T, Zugun-Eloae F. Current status in vitamin D and regulatory T cells-immunological implications. *Rev Med Chir Soc Med Nat Iasi*. (2013) 117:965–73. Available online at: https://www.revmedchir.ro/index.php/revmedchir/article/view/810
27. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol*. (2009) 183:5458–67. doi: 10.4049/jimmunol.0803217
28. Ji J, Zhai H, Zhou H, Song S, Mor G, Liao A. The role and mechanism of vitamin D-mediated regulation of Treg/Th17 balance in recurrent pregnancy loss. *Am J Reprod Immunol*. (2019) 81:e13112. doi: 10.1111/aji.13112
29. Fisher SA, Rahimzadeh M, Brierley C, Gration B, Doree C, Kimber CE, et al. The role of vitamin D in increasing circulating T regulatory cell numbers and modulating T regulatory cell phenotypes in patients with inflammatory disease or in healthy volunteers: a systematic review. *PLoS ONE*. (2019) 14:e0222313. doi: 10.1371/journal.pone.0222313
30. Bemiss CJ, Mahon BD, Henry A, Weaver V, Cantorna MT. Interleukin-2 is one of the targets of 1,25-dihydroxyvitamin D3 in the immune system. *Arch Biochem Biophys*. (2002) 402:249–54. doi: 10.1016/S0003-9861(02)00082-6
31. Looman KIM, Jansen MAE, Voortman T, van den Heuvel D, Jaddoe VWV, Franco OH, et al. The role of vitamin D on circulating memory T cells in children: the generation R study. *Pediatr Allergy Immunol*. (2017) 28:579–87. doi: 10.1111/pai.12754
32. Youssef MAM, Zahran AM, Hussien AM, Elsayh KI, Askar EA, Farghaly HS. In neonates with vitamin D deficiency, low lymphocyte activation markers are risk factors for infection. *Paediatr Int Child Health*. (2019) 39:111–8. doi: 10.1080/20469047.2018.1528755

33. Berge T, Leikfoss IS, Brorson IS, Bos SD, Page CM, Gustavsen MW, et al. The multiple sclerosis susceptibility genes TAGAP and IL2RA are regulated by vitamin D in CD4+ T cells. *Genes Immun*. (2016) 17:118–27. doi: 10.1038/gene.2015.61
34. Rolf L, Muris AH, Hupperts R, Damoiseaux J. Vitamin D effects on B cell function in autoimmunity. *Ann N Y Acad Sci*. (2014) 1317:84–91. doi: 10.1111/nyas.12440
35. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol*. (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
36. Morgan JW, Kouttab N, Ford D, Maizel AL. Vitamin D-mediated gene regulation in phenotypically defined human B cell subpopulations. *Endocrinology*. (2000) 141:3225–34. doi: 10.1210/endo.141.9.7666
37. Cantorna MT, Snyder L, Lin YD, Yang L. Vitamin D and 1,25(OH)2D regulation of T cells. *Nutrients*. (2015) 7:3011–21. doi: 10.3390/nu7043011
38. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab*. (1983) 57:1308–10 doi: 10.1210/jcem-57-6-1308
39. Yang CY, Leung PS, Adamopoulos IE, Gershwin ME. The implication of vitamin D and autoimmunity: a comprehensive review. *Clin Rev Allergy Immunol*. (2013) 45:217–26. doi: 10.1007/s12016-013-8361-3
40. Iruretagoyena M, Hirigoyen D, Naves R, Burgos PI. Response modulation by vitamin D: role in systemic lupus erythematosus. *Front Immunol.* (2015) 6:513. doi: 10.3389/fimmu.2015.00513
41. Rolf L, Muris AH, Hupperts R, Damoiseaux J. Illuminating vitamin D effects on B cells-the multiple sclerosis perspective. *Immunology*. (2016) 147:275–84. doi: 10.1111/imm.12572
42. Tetlow LC, Smith SJ, Mawer EB, Woolley DE. Vitamin D receptors in the rheumatoid lesion: expression by chondrocytes, macrophages, and synoviocytes. *Ann Rheum Dis*. (1999) 58:118–21. doi: 10.1136/ard.58.2.118
43. Piantoni S, Andreoli L, Scarsi M, Zanola A, Dall'Ara F, Pizzorni C. Phenotype modifications of T-cells and their shift toward a Th2 response in patients with systemic lupus erythematosus supplemented with different monthly regimens of vitamin D. *Lupus*. (2015) 24:490–8. doi: 10.1177/0961203314559090
44. Zhao M, Duan XH, Wu ZZ, Gao CC, Wang N, Zheng ZH. Severe vitamin D deficiency affects the expression of autophagy related genes in PBMCs and T-cell subsets in active systemic lupus erythematosus. *Am J Clin Exp Immunol*. (2017) 6:43–51. doi: 10.1016/B978-0-12-801917-7.00007-3
45. Andreoli L, Piantoni S, Dall'Ara F, Allegri F, Meroni PL, Tincani A. Vitamin D and antiphospholipid syndrome. *Lupus*. (2012) 21:736–40. doi: 10.1177/0961203312446386
46. Tokić S, Štefanić M, Karner I, Glavaš-Obrovac L. Altered expression of CTLA-4, CD28, VDR, and CD45 mRNA in T cells of patients with Hashimoto's thyroiditis – a pilot study. *Endokrynol Pol*. (2017) 68:274–8. doi: 10.5603/EP.2017.0020
47. Munger, KL, Levin, LI, Hollis, BW, Howard, NS, Ascherio, A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA*. (2006) 296:2832–8. doi: 10.1001/jama.296.23.2832
48. Åivo J, Hänninen A, Ilonen J, Soilu-Hänninen M. Vitamin D3 administration to MS patients leads to increased serum levels of latency activated peptide (LAP) of TGF-beta. *J Neuroimmunol*. (2015) 280:12–5. doi: 10.1016/j.jneuroim.2015.01.005
49. Grau-López L, Granada ML, Raïch-Regué D, Naranjo-Gómez M, Borràs-Serres FE. Regulatory role of vitamin D in T-cell reactivity against myelin peptides in relapsing-remitting multiple sclerosis patients. *BMC Neurol*. (2012) 12:103. doi: 10.1186/1471-2377-12-103
50. Arnson Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. *Ann Rheum Dis*. (2007) 66:1137–42. doi: 10.1136/ard.2007.069831
51. Dankers W, Colin EM, van Hamburg JP, Lubberts E. Vitamin D in autoimmunity: molecular mechanisms and therapeutic potential. *Front Immunol.* (2017) 7:697. doi: 10.3389/fimmu.2016.00697
52. Agmon-Levin N, Theodor E, Segal RM, Shoenfeld Y. Vitamin D in systemic and organ-specific autoimmune diseases. *Clin Rev Allergy Immunol*. (2013) 45:256–66. doi: 10.1007/s12016-012-8342-y
53. Mathieu C, Van Etten E, Gysemans C, Decallonnee B, Kato S, Laureys J. *In vitro* and *in vivo* analysis of the immune system of vitamin D receptor knockout mice. *J Bone Miner Res*. (2001) 16:2057–65. doi: 10.1359/jbmr.2001.16.11.2057
54. Müller-Ladner U, Ospelt C, Gay S, Distler O, Pap T. Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts. *Arthritis Res Ther*. (2007) 9:223. doi: 10.1186/ar2337
55. Laragione T, Shah A, Gulko PS. The vitamin D receptor regulates rheumatoid arthritis synovial fibroblast invasion and morphology. *Mol Med*. (2012) 18:194–200. doi: 10.2119/molmed.2011.00410
56. Kerr GS, Sabahi I, Richards JS, Caplan L, Cannon GW, Reimold A. Prevalence of vitamin D insufficiency/deficiency in rheumatoid arthritis and associations with disease severity and activity. *J Rheumatol*. (2011) 38:53–9 doi: 10.3899/jrheum.100516
57. Grazio S, Naglić łB, Anić B, Grubišić F, Bobek D, Bakula M. Vitamin D serum level, disease activity and functional ability in different rheumatic patients. *Am J Med Sci*. (2015) 349:46–9. doi: 10.1097/MAJ.0000000000000340
58. Merlino L, Curtis J, Mikuls T, Cerhan J, Criswell L, Saag K, et al. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. *Arthritis Rheum*. (2004) 50:72–7. doi: 10.1002/art.11434
59. Lee YH, Bae SC, Choi SJ, Ji JD, Song GG. Associations between vitamin D receptor polymorphisms and susceptibility to rheumatoid arthritis and systemic lupus erythematosus: a meta-analysis. *Mol Biol Rep*. (2011) 38:3643–51. doi: 10.1007/s11033-010-0477-4
60. Tizaoui K, Hamzaoui K. Association between VDR polymorphisms and rheumatoid arthritis disease: systematic review and updated meta-analysis of case control studies. *Immunobiology*. (2015) 220:807–16. doi: 10.1016/j.imbio.2014.12.013
61. Müller K, Kriegbaum N, Baslund B, Sørensen O, Thymann M, Bentzen K. Vitamin D3 metabolism in patients with rheumatic diseases: low serum levels of 25-hydroxyvitamin D3 in patients with systemic lupus erythematosus. *Clin Rheumatol*. (1995) 14:397–400. doi: 10.1007/BF02207671
62. Mok C, Birmingham D, Ho L, Hebert L, Song H, Rovin B. Vitamin D deficiency as marker for disease activity and damage in systemic lupus erythematosus: a comparison with anti-dsDNA and anti-C1q. *Lupus*. (2012) 21:36–42. doi: 10.1177/0961203311422094
63. Hassanalilou T, Khalili L, Ghavamzadeh S, Shokri A, Payahoo L, Bishak YK. Role of vitamin D deficiency in systemic lupus erythematosus incidence and aggravation. *Auto Immun Highlights*. (2017) 9:1. doi: 10.1007/s13317-017-0101-x
64. Girardi G. Role of tissue factor in the maternal immunological attack of the embryo in the antiphospholipid syndrome. *Clin Rev Allergy Immunol*. (2010) 39:160–5. doi: 10.1007/s12016-009-8187-1
65. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest*. (2008) 118:3453–61. doi: 10.1172/JCI36089
66. Tobaldini LQ, Arantes FT, Saraiva SDS, Mazetto BM, Colella MP, de Paula EV,. Circulating levels of tissue factor and the risk of thrombosis associated with antiphospholipid syndrome. *Thromb Res*. (2018) 171:114–20. doi: 10.1016/j.thromres.2018.09.058
67. Tedesco F, Borghi MO, Gerosa M, Chighizola CB, Macor P, Lonati PA. Pathogenic role of complement in antiphospholipid syndrome and therapeutic implications. *Front Immunol*. (2018) 9:1388. doi: 10.3389/fimmu.2018.01388
68. Agmon-Levin N, Blank M, Zandman-Goddard G, Orbach H, Meroni PL, Tincani A, et al. Vitamin D: an instrumental factor in the anti-phospholipid syndrome by inhibition of tissue factor expression. *Ann Rheum Dis*. (2011) 70:145–50. doi: 10.1136/ard.2010.134817
69. Martinez-Moreno JM, Herencia C, Montes de Oca A, Muñoz-Castañeda JR, Rodríguez-Ortiz ME, Díaz-Tocados JM, et al. Vitamin D modulates tissue factor and protease-activated receptor 2 expression in vascular smooth muscle cells. *FASEB J*. (2016) 30:1367–76. doi: 10.1096/fj.15-272872
70. Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T. Complement activation in patients with primary antiphospholipid syndrome. *Ann Rheum Dis*. (2009) 68:1030–5. doi: 10.1136/ard.2008.090670

71. Breen KA, Seed P, Parmar K, Moore GW, Stuart-Smith SE, Hunt BJ. Complement activation in patients with isolated antiphospholipid antibodies or primary antiphospholipid syndrome. *Thromb Haemost*. (2012) 107:423–9. doi: 10.1160/TH11-08-0554
72. De Carolis S, Botta A, Santucci S, Salvi S, Moresi S, Di Pasquo E. Complementemia and obstetric outcome in pregnancy with antiphospholipid syndrome. *Lupus*. (2012) 21:776–8. doi: 10.1177/0961203312444172
73. Reggia R, Ziglioli T, Andreoli L, Bellisai F, Iuliano A, Gerosa M. Primary anti-phospholipid syndrome: any role for serum complement levels in predicting pregnancy complications? *Rheumatology*. (2012) 51:2186–90. doi: 10.1093/rheumatology/kes225
74. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest*. (2003) 112:1644–54. doi: 10.1172/JCI18817
75. Redecha P, Tilley R, Tencati M, Salmon JE, Kirchhofer D, Mackman N, et al. Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. *Blood*. (2007) 110:2423–31. doi: 10.1182/blood-2007-01-070631
76. Izban MG, Nowicki BJ, Nowicki S. 1,25-Dihydroxyvitamin D3 promotes sustained LPS-induced NF-κB-dependent expression of CD55 in human monocytic THP-1 cells. *PLoS ONE*. (2012) 7:e49318. doi: 10.1371/journal.pone.0049318
77. Lefkou E, Mamopoulos A, Dagklis T, Vosnakis C, Rousso D, Girardi G. Pravastatin improves pregnancy outcomes in obstetric antiphospholipid syndrome refractory to antithrombotic therapy. *J Clin Invest*. (2016) 126:2933–40. doi: 10.1172/JCI86957
78. Bełtowski J, Atanassova P, Chaldakov GN, Jamroz-Wiśniewska A, Kula W, Rusek M. Opposite effects of pravastatin and atorvastatin on insulin sensitivity in the rat: role of vitamin D metabolites. *Atherosclerosis*. (2011) 219:526–31. doi: 10.1016/j.atherosclerosis.2011.08.009
79. Yates N, Crew RC, Wyrwoll CS. Vitamin D deficiency and impaired placental function: potential regulation by glucocorticoids? *Reproduction*. (2017) 153:R163–71. doi: 10.1530/REP-16-0647
80. Mansournia N, Mansournia MA, Saeedi S, Deghan J. The association between serum 25OHD levels and hypothyroid Hashimoto's thyroiditis. *J Endocrinol Invest*. (2014) 37:473–6. doi: 10.1007/s40618-014-0064-y
81. Talaei A, Ghorbani F, Asemi Z. The effects of vitamin D supplementation on thyroid function in hypothyroid patients: a randomized, double-blind, placebo-controlled trial. *Indian J Endocrinol Metab*. (2018) 22:584–8. doi: 10.4103/ijem.IJEM_603_17
82. Feng M, Li H, Chen S.F, Li W.F, Zhang F.B. Polymorphism in the vitamin D receptor gene and risk of autoimmune thyroid disease: a meta analysis. *Endocrine*. (2013) 43:318–26 doi: 10.1007/s12020-012-9812-y
83. Chen W, Lin H, Wang M. Immune intervention effects on the induction of experimental autoimmune thyroiditis. *J. Huazhong Univ. Sci. Technol. Med. Sci*. (2002) 22:343–5 doi: 10.1007/BF02896782
84. Kim D. The role of vitamin D in thyroid diseases. *Int J Mol Sci*. (2017) 18:1949. doi: 10.3390/ijms18091949
85. Wang J, Lv S, Chen G, Gao C, He J, Zhong H, et al. Meta-analysis of the association between vitamin D and autoimmune thyroid disease. *Nutrients*. (2015) 7:2485–98 doi: 10.3390/nu7042485
86. Wang X, Zynat J, Guo Y, Osiman R, Tuhuti A, Zhao H, et al. Low serum vitamin D is associated with anti-thyroid-globulin antibody in female individuals. *Int. J. Endocrinol*. (2015) 2015:285290. doi: 10.1155/2015/285290
87. Effraimidis G, Badenhoop K, Tijssen JG, Wiersinga WM. Vitamin D deficiency is not associated with early stages of thyroid autoimmunity. *Eur. J. Endocrinol*. (2012) 167:43–8. doi: 10.1530/EJE-12-0048
88. Yasmeh J, Farpour F, Rizzo V, Kheradnam S, Sachmechi I. Hashimoto thyroiditis not associated with vitamin D deficiency. *Endocr. Pract*. (2016) 22:809–81. doi: 10.4158/EP15934.OR
89. Compston A, Coles A. Multiple sclerosis. *Lancet*. (2008) 372:1502–17. doi: 10.1016/S0140-6736(08)61620-7
90. Munger KL, Åivo J, Hongell K, Soilu-Hänninen M, Surcel HM, Ascherio A. Vitamin D status during pregnancy and risk of multiple sclerosis in offspring of women in the Finnish maternity cohort. *JAMA Neurol*. (2016) 73:515–19. doi: 10.1001/jamaneurol.2015.4800
91. Schwab N, Schneider-Hohendorf T, Marziniak M, Mohan H, Bhatia U, Gross CC. Ultraviolet B light attenuates the systemic immune response in central nervous system autoimmunity. *Ann Neurol*. (2014) 75:739–58. doi: 10.1002/ana.24165
92. Farias AS, Spagnol GS, Bordeaux-Rego P, Oliveira CO, Fontana AG, de Paula RF. Vitamin D3 induces IDO+ tolerogenic DCs and enhances Treg, reducing the severity of EAE. *CNS Neurosci Ther*. (2013) 19:269–77. doi: 10.1111/cns.12071
93. Won S, Sayeed I, Peterson BL, Wali B, Kahn JS, Stein DG. Vitamin D prevents hypoxia/reoxygenation-induced blood-brain barrier disruption via vitamin D receptor-mediated NF-kB signaling pathways. *PLoS ONE*. (2015) 10:e0122821. doi: 10.1371/journal.pone.0122821
94. Zenclussen AC. Adaptive immune responses during pregnancy. *Am J Reprod Immunol*. (2013) 69:291–303. doi: 10.1111/aji.12097
95. Robertson SA, Moldenhauer LM. Immunological determinants of implantation success. *Int J Dev Biol*. (2014) 58:205–17. doi: 10.1387/ijdb.140096sr
96. Du MR, Wang SC, Li DJ. The integrative roles of chemokines at the maternal-fetal interface in early pregnancy. *Cell Mol Immunol*. (2014) 11:438–48. doi: 10.1038/cmi.2014.68
97. Gaynor LM, Colucci F. Uterine natural killer cells: functional distinctions and influence on pregnancy in humans and mice. *Front Immunol*. (2017) 8:467. doi: 10.3389/fimmu.2017.00467
98. Veras E, Kurman RJ, Wang TL, Shih IM. PD-L1 expression in human placentas and gestational trophoblastic diseases. *Int J Gynecol Pathol*. (2017) 36:146–53. doi: 10.1097/PGP.0000000000000305
99. Tilburgs T, Strominger JL. CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity. *Am J Reprod Immunol*. (2013) 69:395–407. doi: 10.1111/aji.12094
100. Saito S, Sasaki Y, Sakai M. CD4(+)CD25high regulatory T cells in human pregnancy. *J Reprod Immunol*. (2005) 65:111–20. doi: 10.1016/j.jri.2005.01.004
101. Guerin LR, Prins JR, Robertson SA. Regulatory T-cells and immune tolerance in pregnancy: a new target for infertility treatment? *Hum Reprod Update*. (2009) 15:517–35. doi: 10.1093/humupd/dmp004
102. Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity*. (2006) 25:441–54. doi: 10.1016/j.immuni.2006.07.012
103. Bartholin L, Cyprian FS, Vincent D, Garcia CN, Martel S, Horvat B. Generation of mice with conditionally activated transforming growth factor Beta signaling through TbetaRI/ALK5 receptor. *Genesis*. (2008) 46:724–31. doi: 10.1002/dvg.20425
104. Kahn DA, Baltimore D. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc Natl Acad Sci USA*. (2010) 107:9299–304. doi: 10.1073/pnas.1003909107
105. Mithal, A, Wahl, DA, Bonjour, JP. Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos Int*. (2009) 20:1807–20. doi: 10.1007/s00198-009-0954-6
106. Eggemoen ÅR, Falk RS, Knutsen KV, Lagerløv P, Setner L, Birkeland KI. Vitamin D deficiency and supplementation in pregnancy in a multiethnic population-based cohort. *BMC Pregnancy Childbirth*. (2016) 16:7. doi: 10.1186/s12884-016-0796-0
107. Kiely ME, Zhang JY, Kinsella M, Khashan, AS, Kenny LC. Vitamin D status is associated with uteroplacental dysfunction indicated by pre-eclampsia and small-for-gestational-age birth in a large prospective pregnancy cohort in Ireland with low vitamin D status. *Am J Clin Nutr*. (2016) 104:354–61. doi: 10.3945/ajcn.116.130419
108. Lerchbaum E, Obermayer-Pietsch B. Vitamin D and fertility: a systematic review. *Eur J Endocrinol*. (2012) 166:765–78. doi: 10.1530/EJE-11-0984
109. Mumford SL, Garbose RA, Kim K, Kissell K, Kuhr DL, Omosigho UR. Association between preconception serum 25-hydroxyvitamin D concentrations with livebirths and pregnancy loss: a prospective cohort study. *Lancet Diabetes Endocrinol*. (2018) 6:725–32. doi: 10.1016/S2213-8587(18)30153-0
110. Lambert PW, Stern PH, Avioli RC, Brackett NC, Turner RT, Greene A. Evidence for extrarenal production of 1α,25-dihydroxyvitamin D in man. *J Clin Invest*. (1982) 69:722–25. doi: 10.1172/JCI110501

111. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, et al. Extrarenal expression of 25-hydroxyvitamin D_3-1a-hydroxylase. *J Clin Endocrinol Metab*. (2001) 86:888–94. doi: 10.1210/jc.86.2.888
112. Gray TK, Lester GE, Lorenc RS. Evidence for extra-renal 1 alphahydroxylation of 25-hydroxyvitamin D3 in pregnancy. *Science*. (1979) 204:13. doi: 10.1126/science.451538
113. Bruns ME, Bruns DE. Vitamin D metabolism and function during pregnancy and the neonatal period. *Ann Clin Lab Sci.* (1983) 13:521–30.
114. Tamblyn JA, Hewison M, Wagner CL, Bulmer JN, Kilby MD. Immunological role of vitamin D at the maternal–fetal interface. *J Endocrinol.* (2015) 224:R114–17. doi: 10.1530/JOE-14-0642
115. Kwiecinksi GG, Petrie GI, DeLuca HF. 1,25-Dihydroxyvitamin D3 restores fertility of vitamin D-deficient female rats. *Am J Physiol*. (1989) 256:E483–7. doi: 10.1152/ajpendo.1989.256.4.E483
116. Chambon Y. Synergistic action of vitamin D and of progesterone in obtaining ovum implantation in the castrated pregnant rabbit. *Soc Biol Fil*. (1951) 145:955–9.
117. Xu J, Lawson MS, Xu F, Du Y, Tkachenko OY, Bishop CV, et al. Vitamin D3 regulates follicular development and intrafollicular vitamin D biosynthesis and signaling in the primate ovary. *Front Physiol*. (2018) 9:1600. doi: 10.3389/fphys.2018.01600
118. Rojansky N, Brzezinski A, Schenker JG. Seasonality in human reproduction: an update. *Hum Reprod*. (1992) 7:735–45. doi: 10.1093/oxfordjournals.humrep.a137729
119. Chu J, Gallos I, Tobias A, Tan B, Eapen A, Coomarasamy A. Vitamin D and assisted reproductive treatment outcome: a systematic review and meta-analysis. *Hum Reprod*. (2018) 33:65–80. doi: 10.1093/humrep/dex326
120. Stumpf WE, Sar M, Chen K, Morin J, DeLuca HF. Sertoli cells in the testis and epithelium of the ductuli efferentes are targets for 3H 1,25 (OH)2 vitamin D3. An autoradiographic study. *Cell Tissue Res*. (1987) 247:453–5. doi: 10.1007/BF00218327
121. Osmundsen BC, Huang HF, Anderson MB, Christakos S, Walters MR. Multiple sites of action of the vitamin D endocrine system: FSH stimulation of testis 1,25-dihydroxyvitamin D3 receptors. *J Steroid Biochem*. (1989) 34:339–43. doi: 10.1016/0022-4731(89)90105-2
122. Kwiecinski GG, Petrie GI, DeLuca HF. Vitamin D is necessary for reproductive functions of the male rat. *J Nutr*. (1989) 119:741–44. doi: 10.1093/jn/119.5.741
123. Alzoubi A, Mahdi H, Al Bashir S, Halalsheh O, Al Ebbini M, Alzarir M, et al. Normalization of serum vitamin D improves semen motility parameters in patients with idiopathic male infertility. *Acta Endocrinol*. (2017) 13:180–7. doi: 10.4183/aeb.2017.180
124. Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. *Endocrinology*. (2000) 141:1317–24. doi: 10.1210/en.141.4.1317
125. Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell Endocrinol.* (2002) 187:139–44. doi: 10.1016/S0303-7207(01)00704-3
126. Uhland AM, Kwiecinski GG, DeLuca HF. Normalization of serum calcium restores fertility in vitamin D-deficient male rats. *J Nutr*. (1992) 122:1338–44. doi: 10.1093/jn/122.6.1338
127. Shin JS, Choi MY, Longtine MS, Nelson DM. Vitamin D effects on pregnancy and the placenta. *Placenta*. (2010) 31:1027–34. doi: 10.1016/j.placenta.2010.08.015
128. Du H, Daftary GS, Lalwani SI, Taylor HS. Direct regulation of HOXA10 by 1,25-(OH)2D3 in human myelomonocytic cells and human endometrial stromal cells. *Mol Endocrinol*. (2005) 19:2222–33. doi: 10.1210/me.2004-0336
129. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet*. (1997) 16:391–96. doi: 10.1038/ng0897-391
130. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN. Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci USA*. (2001) 98:7498–503. doi: 10.1073/pnas.131029498
131. Halhali A, Acker GM, Garabedian M. 1,25-Dihydroxyvitamin D3 induces *in vivo* the decidualization of rat endometrial cells. *J Reprod Fert*. (1991) 91:59–64. doi: 10.1530/jrf.0.0910059
132. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol*. (2001) 167:1945–53. doi: 10.4049/jimmunol.167.4.1945
133. Piccinni MP, Scaletti C, Maggi E, Romagnani S. Role of hormone controlled Th1. and Th2-type cytokines in successful pregnancy. *J Neuroimmunol*. (2000) 109:30–3 doi: 10.1016/S0165-5728(00)00299-X
134. Aghajafari F, Nagulesapillai T, Ronksley PE, Tough SC, O'Beirne M, Rabi DM. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. *BMJ*. (2013) 201:f1169. doi: 10.1136/bmj.f1169
135. Akbari S, Khodadadi B, Ahmadi SAY, Abbaszadeh S, Shahsavar F. Association of vitamin D level and vitamin D deficiency with risk of preeclampsia: a systematic review and updated meta-analysis. *Taiwan J Obstet Gynecol*. (2018) 57:241–247. doi: 10.1016/j.tjog.2018.02.013
136. Diaz L, Arranz C, Avila E, Halhali A, Vilchis F, Larrea F. Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies. *J Clin Endocrinol Metab*. (2002) 87:3876–82. doi: 10.1210/jcem.87.8.8730
137. Gala P, Dwarkanath P, Larkin HM, Kurpad A, Mehta S. The role of vitamin D in pre-eclampsia: a systematic review. *BMC Pregnancy Childbirth*. (2017) 17:231–8. doi: 10.1186/s12884-017-1408-3
138. Robinson CJ, Wagner CL, Hollis BW, Baatz JE, Johnson DD. Plasma 25-hydroxyvitamin D levels in early-onset severe preeclampsia. *Am J Obstet Gynecol*. (2010) 203:366–72. doi: 10.1016/j.ajog.2010.06.036
139. Rezavand N, Tabarok S, Rahimi Z, Vaisi-Raygani A, Mohammadi E, Rahimi Z. The effect of VDR gene polymorphisms and vitamin D level on blood pressure, risk of preeclampsia, gestational age and body mass index. *J Cell Biochem*. (2019) 120:6441–8. doi: 10.1002/jcb.27934
140. Dong M, He J, Wang Z, Xie X, Wand H. Placental imbalance of Th1- and Th2-type cytokines in preeclampsia. *Acta Obstet Gynecol Scand*. (2005) 84:788–93. doi: 10.1111/j.0001-6349.2005.00714.x
141. Ma Y, Johnson CS, Trump DL. Mechanistic insights of vitamin D anticancer effects. *Vitamins Hormones*. (2016) 100:395–431. doi: 10.1016/bs.vh.2015.11.003
142. Ganguly A, Tamblyn JA, Finn-Sell S, Chan SY, Westwood M, Gupta J. Vitamin D, the placenta and early pregnancy: effects on trophoblast function. *J Endocrinol*. (2018) 236:R93–103. doi: 10.1530/JOE-17-0491
143. Chan SY, Susarla R, Canovas D, Vasilopoulou E, Ohizua O, McCabe CJ. Vitamin D promotes human extravillous trophoblast invasion *in vitro*. *Placenta*. (2015) 36:403–9. doi: 10.1016/j.placenta.2014.12.021
144. von Versen-Hoynck F, Brodowski L, Dechend R, Myerski AC, Hubel CA. Vitamin D antagonizes negative effects of preeclampsia on fetal endothelial colony forming cell number and function. *PLoS ONE*. (2014) 9:e98990. doi: 10.1371/journal.pone.0098990
145. Barrera D, Avila E, Hernández G. Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts. *Reprod Biol Endocrinol*. (2008) 6:3. doi: 10.1186/1477-7827-6-3
146. Ajabshir S, Asif A, Nayer A. The effects of vitamin D on the renin-angiotensin system. *J Nephropathol.* (2014) 3:41–3. doi: 10.12860/jnp.2014.09
147. Chandel N, Ayasolla K, Wen H, Lan X, Haque S, Saleem MA, et al. Vitamin D receptor deficit induces activation of renin angiotensin system via SIRT1 modulation in podocytes. *Exp Mol Pathol*. (2017) 102:97–105. doi: 10.1016/j.yexmp.2017.01.001
148. Min B. Effects of vitamin D on blood pressure and endothelial function. *Korean J Physiol Pharmacol.* (2013) 17:385–92. doi: 10.4196/kjpp.2013.17.5.385
149. Reynolds J, Ray D, Alexander MY, Bruce I. Role of vitamin D in endothelial function and endothelial repair in clinically stable systemic lupus erythematosus. *Lancet*. (2015) 385(Suppl. 1):S83. doi: 10.1016/S0140-6736(15)60398-1
150. Kanikarla-Marie P, Jain SK. 1,25(OH)2D3 inhibits oxidative stress and monocyte adhesion by mediating the upregulation of GCLC and GSH in endothelial cells treated with acetoacetate (ketosis). *J Steroid Biochem Mol Biol*. (2016) 159:94–101. doi: 10.1016/j.jsbmb.2016.03.002
151. Behjat Sasan S, Zandvakili F, Soufizadeh N, Baybordi E. The effects of vitamin D supplement on prevention of recurrence of preeclampsia in

pregnant women with a history of preeclampsia. *Obstet Gynecol Int*. (2017) 2017:8249264. doi: 10.1155/2017/8249264
152. Danield D, Hardigan P, Bray N, Penzell D, Savu C. The incidence of vitamin D deficiency in the obese: a retrospective chart review. *J Community Hosp Intern Med Perspect*. (2015) 5:26069. doi: 10.3402/jchimp.v5.26069
153. Liu L, Ma Y, Wang N, Lin W, Liu Y, Wen D. Maternal body mass index and risk of neonatal adverse outcomes in China: a systematic review and meta-analysis. *BMC Pregnancy Childbirth*. (2019) 19:105. doi: 10.1186/s12884-019-2249-z
154. van der Steeg JW, Steures P, Eijkemans MJ, Habbema JD, Hompes PG, Burggraaff JM. Obesity affects spontaneous pregnancy chances in subfertile, ovulatory women. *Hum Reprod*. (2008) 23:324–8. doi: 10.1093/humrep/dem371
155. McAree T. Obesity and vitamin D deficiency - current concepts on their impact on pregnancy. *Eur Endocrinol*. (2013) 9:125–7. doi: 10.17925/EE.2013.09.02.125
156. Chawanpaiboon S, Vogel JP, Moller AB, Lumbiganon P, Petzold M, Hogan D, et al. Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. *Lancet Glob Health*. (2019) 7:e37–46. doi: 10.1016/S2214-109X(18)30451-0
157. Bodnar LM, Platt RW, Simhan HN. Early-pregnancy vitamin D deficiency and risk of preterm birth subtypes. *Obstet Gynecol*. (2015) 125:439–47 doi: 10.1097/AOG.0000000000000621
158. Zhou SS, Tao YH, Huang K, Zhu BB, Tao FB. Vitamin D and risk of preterm birth: Up-to-date meta-analysis of randomized controlled trials and observational studies. *J Obst Gynecol Res*. (2017) 43:247–56. doi: 10.1111/jog.13239
159. Gomez-Lopez N, St Louis D, Lehr MA, Sanchez-Rodriguez EN, Arenas-Hernandez M. Immune cells in term and preterm labor. *Cell Mol Immunol*. (2014) 11:571–81. doi: 10.1038/cmi.2014.46
160. Sykes L, MacIntyre DA, Yap XJ, Teoh TG, Bennett PR. The Th1:Th2 dichotomy of pregnancy and preterm labour. *Mediators Inflamm*. (2012) 2012:967629. doi: 10.1155/2012/967629
161. Zahran AM, Zharan KM, Hetta HF. Significant correlation between regulatory T cells and vitamin D in term and preterm labor. *J Reprod Immunol*. (2018) 129:15–22. doi: 10.1016/j.jri.2018.07.004
162. Palacios C, Lombardo LK, Peña-Rosas JP. Vitamin D supplementation for women during pregnancy. *Cochrane Database Sustain Rev*. (2016) 1:CD008873. doi: 10.1002/14651858.CD008873.pub2
163. Calkins K, Devaskar SU. Fetal origins of adult disease. *Curr Probl Pediatr Adolesc Health Care*. (2011) 41:158–76. doi: 10.1016/j.cppeds.2011.01.001
164. Wierzejska R, Jarosz M, Sawicki W, Bachanek M, Siuba-Strzelinska M. Vitamin D concentration in maternal and umbilical cord blood by season. *Int J Environ Res Public Health*. (2017) 14:1121–31. doi: 10.3390/ijerph 14101121
165. Barker DJP. The developmental origins of adult disease. *J Am Coll Nutr*. (2004) 23:588S−95S. doi: 10.1080/07315724.2004.10719428
166. Leddy MA, Power ML, Schulkin J. The impact of maternal obesity on maternal and fetal health. *Rev Obstet Gynecol*. (2008) 1:170–8. Available online at: http://medreviews.com/journal/reviews-in-obstetrics-gynecology/vol/1/no/4/impact-maternal-obesity-maternal-and-fetal-health
167. Power C, Kuh D, Morton S. From developmental origins of adult disease to life course research on adult disease and aging: insights from birth cohort studies. *Annu Rev Public Health*. (2013) 34:7–28. doi: 10.1146/annurev-publhealth-031912-114423
168. Kundakovic M, Jaric I. The epigenetic link between prenatal adverse environments and neurodevelopmental disorders. *Genes*. (2017) 8:104. doi: 10.3390/genes8030104
169. Novakovic B, Sibson M, Ng HK, Manuelpillai U, Rakyan V, Down T, et al. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem*. (2009) 284:14838–48. doi: 10.1074/jbc.M809542200
170. Martin R, Harvey NC, Crozier SR, Poole JR, Javaid MK, Dennison EM, et al. Placental calcium transporter (PMCA3) gene expression predicts intrauterine bone mineral accrual. *Bone*. (2007) 40:1203–8. doi: 10.1016/j.bone.2006.12.060
171. Zhu K, Whitehouse AJ, Hart PH, Kusel M, Mountain J, Lye S. Maternal vitamin D status during pregnancy and bone mass in offspring at 20 years of age: a prospective cohort study. *J Bone Miner Res*. (2014) 29:1088–95. doi: 10.1002/jbmr.2138
172. Available online at: http://www.who.int/mediacentre/factsheets/fs307/en/index.html
173. Wagner CL, Hollis BW. The implications of vitamin D status during pregnancy on mother and her developing child. *Front Endocrinol*. (2018) 9:500. doi: 10.3389/fendo.2018.00500
174. Wolsk HM, Chawes BL, Litonjua AA. Prenatal vitamin D supplementation reduces risk of asthma/recurrent wheeze in early childhood: a combined analysis of two randomized controlled trials. *PLoS ONE*. (2017) 12:e0186657. doi: 10.1371/journal.pone.0186657
175. Schedel M, Jia Y, Michel S, Takeda K, Domenico J, Joetham A, et al. 1,25D3 prevents CD8(+)Tc2 skewing and asthma development through VDR binding changes to the Cyp11a1 promoter. *Nat Commun*. (2016) 7:10213. doi: 10.1038/ncomms10213
176. Wittke A, Chang A, Froicu M. Vitamin D receptor expression by the lung micro-environment is required for maximal induction of lung inflammation. *Arch Biochem Biophys*. (2007) 460:306–13. doi: 10.1016/j.abb.2006.12.011
177. Raby BA, Lazarus R, Silverman EK, Lake S, Lange C, Wjst M, et al. Association of vitamin D receptor gene polymorphisms with childhood and adult asthma. *Am J Respir Crit Care Med*. (2004) 170:1057–65. doi: 10.1164/rccm.200404-447OC
178. Nurmatov U, Devereux G, Sheikh A. Nutrients and foods for the primary prevention of asthma and allergy: systematic review and meta-analysis. *J Allergy Clin Immunol*. (2011) 127:724–33. doi: 10.1016/j.jaci.2010.11.001
179. Beckhaus AA, Garcia-Marcos L, Forno E, Pacheco-Gonzalez RM, Celedón JC, Castro-Rodriguez JA. Maternal nutrition during pregnancy and risk of asthma, wheeze, and atopic diseases during childhood: a systematic review and meta-analysis. *Allergy*. (2015) 70:1588–604. doi: 10.1111/all.12729
180. Kesby JP, Eyles DW, Burne TH, McGrath JJ. The effects of vitamin D on brain development and adult brain function. *Mol Cel Endocrinol*. (2011) 347:121–7. doi: 10.1016/j.mce.2011.05.014
181. Neveu P, Naveilhan P, Menaa C, Wion D, Brachet P, Garabedian M. Synthesis of 1,25-dihydroxyvitamin D3 by rat brain macrophages *in vitro*. *J Neurosci Res*. (1994) 38:214–20. doi: 10.1002/jnr.490380212
182. Boontantart M, Hall SD, Spanier JA, Hayes CE, Olson JK. Vitamin D3 alter microglia immune activation by an IL-10 dependent SOCS3 mechanism. *J Neuroimmunol*. (2016) 292:126–36. doi: 10.1016/j.jneuroim.2016.01.015
183. Pertile RAN, Cui X, Hammond L, Eyles DW. Vitamin D regulation of GDNF/Ret signaling in dopaminergic neurons. *FASEB J*. (2018) 32:819–28 doi: 10.1096/fj.201700713R
184. Hawes J, Tesic D, Whitehouse A, Zosky G, Smith J, Wyrwoll C. Maternal vitamin D deficiency alters fetal brain development in the BALB/c mouse. *Behav Brain Res*. (2015) 286:192–200. doi: 10.1016/j.bbr.2015.03.008
185. Anjum I, Jaffery SS, Fayyaz M, Samoo Z, Anjum S. The role of vitamin D in brain health: a mini literature review. *Cureus*. (2018) 10:e2960. doi: 10.7759/cureus.2960
186. Taniura H, Ito M, Sanada N, Kuramoto N, Ohno Y, Nakamichi N. Chronic vitamin D3 treatment protects against neurotoxicity by glutamate in association with upregulation of vitamin D receptor mRNA expression in cultured rat cortical neurons. *J Neurosci Res*. (2006) 83:1179–89. doi: 10.1002/jnr.20824
187. AlJohri R, AlOkail M, Haq SH. Neuroprotective role of vitamin D in primary neuronal cortical culture. *eNeurologicalSci*. (2018) 14:43–8. doi: 10.1016/j.ensci.2018.12.004
188. Sloka S, Silva C, Wang J, Yong VW. Predominance of Th2 polarization by vitamin D through a STAT6-dependent mechanism. *J Neuroinflammation*. (2011) 8:56. doi: 10.1186/1742-2094-8-56
189. Pet MA, Brouwer-Brolsma EM. The impact of maternal vitamin D status on offspring brain development and function: a systematic review. *Adv Nutr*. (2016) 7:665–78. doi: 10.3945/an.115.010330
190. Baribeau DA, Anagnostou E. A comparison of neuroimaging findings in childhood onset schizophrenia and autism spectrum disorder: a review of the literature. *Front Psychiatry*. (2013) 4:175. doi: 10.3389/fpsyt.2013.00175

191. McGrath M, Eyles DW, Pedersen CB, Anderson C, Ko P, Burne TH, et al. Neonatal vitamin D status and risk of schizophrenia: a population-based case-control study. *Arch Gen Psychiatr*. (2010) 67:889–94. doi: 10.1001/archgenpsychiatry.2010.110
192. Morales E, Julvez J, Torrent M, Ballester F, Rodríguez-Bernal CL, Andiarena A. Vitamin D in pregnancy and attention deficit hyperactivity disorder-like symptoms in childhood. *Epidemiology*. (2015) 26:458–65. doi: 10.1097/EDE.0000000000000292
193. Guo M, Li L, Zhang Q, Chen L, Dai Y, Liu L, et al. Vitamin and mineral status of children with autism spectrum disorder in Hainan Province of China: associations with symptoms. *Nutr Neurosci*. (2018) 20:1–8. doi: 10.1080/1028415X.2018.1558762
194. Magnusson C, Lundberg M, Lee BK, Rai D, Karlsson, Gardner R. Maternal vitamin D deficiency and the risk of autism spectrum disorders: population-based study. *B J Psych Open*. (2016) 2:170–2 doi: 10.1192/bjpo.bp.116.002675
195. Arastoo AA, Khojastehkia H, Rahimi Z. Evaluation of serum 25-Hydroxy vitamin D levels in children with autism Spectrum disorder. *Ital J Pediatr*. (2018) 44:150. doi: 10.1186/s13052-018-0587-5
196. Vinkhuyzen AAE, Eyles DW, Burne THJ, Blanken LME, Kruithof CJ, Verhulst F, et al. Gestational vitamin D deficiency and autism spectrum disorder. *B J Psych Open*. (2017) 3:85–90. doi: 10.1192/bjpo.bp.116.004077
197. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. (2006) 4:295–306. doi: 10.1111/j.1538-7836.2006.01753.x
198. Arachchillage DR, Machin SJ, Mackie IJ, Cohen H. Diagnosis and management of non-criteria obstetric antiphospholipid syndrome. *Thromb Haemost*. (2015) 113:13–9. doi: 10.1160/TH14-05-0416
199. Carrasco M, Jiménez EL, Gálvez-Romero J, Mendoza-Pinto C, Martínez S, Etchegaray-Morales I. The anti-thrombotic effects of vitamin D and their possible relationship with antiphospholipid syndrome. *Lupus*. (2018) 27:2181–89. doi: 10.1177/0961203318801520
200. Gualteriotti R, diGiacomo A, Raschi O, Borghi MA, Meroni PL. Vitamin D and anti-phospholipid antibody syndrome: a comprehensive review. *Open Rheumatol J*. (2018) 12:248–60. doi: 10.2174/1874312901812010248
201. Kim D. Low vitamin D status is associated with Hashimoto's thyroiditis. *Hormones*. (2016) 15:385–93. doi: 10.14310/horm.2002.1681

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Cathelicidin Contributes to the Restriction of *Leishmania* in Human Host Macrophages

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In cutaneous Leishmaniasis the parasitic control in human host macrophages is still poorly understood. We found an increased expression of the human cathelicidin *CAMP* in skin lesions of Ethiopian patients with cutaneous leishmaniasis. Vitamin D driven, Cathelicidin-type antimicrobial peptides (CAMP) play an important role in the elimination of invading microorganisms. Recombinant cathelicidin was able to induce cell-death characteristics in *Leishmania* in a dose dependent manner. Using human primary macrophages, we demonstrated pro-inflammatory macrophages (hMDM1) to express a higher level of human cathelicidin, both on gene and protein level, compared to anti-inflammatory macrophages (hMDM2). Activating the CAMP pathway using Vitamin D in hMDM1 resulted in a cathelicidin-mediated-*Leishmania* restriction. Finally, a reduction of cathelicidin in hMDM1, using a RNA interference (RNAi) approach, increased *Leishmania* parasite survival. In all, these data show the human cathelicidin to contribute to the innate immune response against Leishmaniasis in a human primary cell model.

Keywords: *Leishmania*, human macrophages, vitamin D, cathelicidin (LL-37), human primary immune cells, antimicrobial activity

INTRODUCTION

The disease Leishmaniasis is still affecting 12 million people worldwide, of which up to 30,000 cases die yearly (1, 2). Up to date, no vaccine is available and treatment is not always evident due to the socioeconomic conditions in the affected countries (3, 4). Our knowledge regarding the interaction of *Leishmania* with its human host cell, the macrophage, is still fragmentary, as little is known with respect to antimicrobial mechanisms restricting *Leishmania* growth in human primary macrophages. Moreover, few data is available demonstrating which macrophage phenotype is the most superior for *Leishmania* survival or killing. The human body comprises a broad spectrum of different macrophage phenotypes, related to distinct functional properties (5). Herein, the

M1/M2 polarization has been the main framework for years in the field of immunology. In the murine system, "alternatively activated" type 2 macrophages are shown to support *Leishmania* parasite replication and persistence via an increased arginase I activity, which negatively correlates to the expression of nitric oxide synthase II (6–10). In contrast, "classically activated" M1 inflammatory macrophages enhance the production of free nitric oxide (NO) radicals, hereby eliminating intracellular parasites (11, 12). In human macrophages however, NO-mediated killing of *Leishmania* is still under debate, underlying the controversy of extrapolating immunological aspects from mouse to man (13–16). Nevertheless, antimicrobial peptides (AMPs), comprising defensins and cathelicidins, are key players in the human host's immune defense. In humans, only the cathelicidin antimicrobial protein hCAP18, encoded by the gene *CAMP*, has been identified. The *CAMP* gene product is cleaved to form the amphipathic, active LL37 peptide. LL37 can be found in various cell types, body fluids and tissues, such as the skin, where an increased production has been described to correlate with disease pathologies (17, 18). As a key molecule in host defense, LL37 exerts antimicrobial properties toward bacteria (*Staphylococcus* spp., *Pseudomonas* spp., *Mycobacteria* spp.), viruses, fungi, as well as parasites (19–26). Dos Santos et al. could demonstrate cathelicidin to exert anti-leishmanial activity in *L. donovani* infected macrophages, in line with data of Dos Santos et al. showing an IL-32/cathelicidin-mediated control of *L. braziliensis* in THP-1 cells (27). This AMP, LL37, able to create pores, hereby disrupting membranes. Although the exact mode of action is unknown, two models have been widely accepted being the "carpet" and "toroidal" model (17, 28). The toroidal model defines a pore architecture, formed by peptide channels, whereas the carpet model describes a more severe membrane perturbation, as seen for detergent-induced membrane destruction (29). In this study, we aimed to identify a role for the human cathelicidin during *Leishmania* infection. We could demonstrate *CAMP* to be upregulated in lesion material from Ethiopian individuals suffering from cutaneous Leishmaniasis. Using a human primary macrophage *in vitro* model, we identified *CAMP* to be upregulated specifically in pro-inflammatory macrophages and rLL37 was demonstrated to kill *Leishmania* in a dose dependent manner. By modulating the vitamin D pathway, we demonstrated *CAMP* expression to be upregulated, enhancing the macrophage's parasite killing capacity. In contrast, using a RNA interference (RNAi) approach in human primary macrophages targeting *CAMP* mRNA, the expression of hCAP18 was strongly reduced, enabling *Leishmania* parasites to survive better. In all, these data suggest an anti-parasitic activity of cathelicidin in a human primary *in vitro* cell model for cutaneous leishmaniasis and patient skin lesions.

RESULTS

An Increased Expression of Cathelicidin in Skin Biopsies of African Patients With Cutaneous Leishmaniasis

In search for antimicrobial mechanisms in self-healing cutaneous Leishmaniasis (CL), we investigated the expression of human cathelicidin hCAP18. In Addis Ababa, Ethiopia, clinical samples from patients with CL and controls were collected and tested using RT-PCR. Patients varied in age, ethnicity, disease duration, and wound location, as depicted (**Figures 1A,B**). All patients were tested positive for the presence of *Leishmania aethiopica* by PCR. Interestingly, a significantly higher transcript abundance of the human cathelicidin hCAP18 was detected in skin biopsies of patients with CL, compared to control samples (**Figure 1C**).

Dose Dependent Killing of *Leishmania* Parasites by Recombinant LL37

LL-37 and its precursor, hCAP18, are found in different tissue and cell types, playing an important role in innate immunity against diverse pathogens, e.g., *S. aureus*, *Mycobacterium tuberculosis*, *L. monocytogenes* (17). To define whether the hCAP18-derived peptide, LL37, is contributing to clearance of *Leishmania* parasites, we treated the promastigote and amastigote life stage of both *Leishmania major* (*Lm*) and *L. aethiopica* (*Lae*) with human recombinant LL37 (hrLL37). After treatment with hrLL37, DNA fragmentation and phosphatidylserine (PS) exposure, two hallmarks of apoptosis, were assessed. Treatment with increasing concentrations of hrLL37 resulted in a dose-dependent increase in TUNEL positivity for both *Lae* (15.5 $\pm$ 8.3%; 21.1 $\pm$ 2.3%) and *Lm* (20.2 $\pm$ 0.7%; 22.9 $\pm$ 3.1%), compared to untreated *Lae* (13.3 $\pm$ 1.2%) and *Lm* (6.24 $\pm$ 1.9%) promastigotes (**Figures 2A,B**). In addition, hrLL37 treatment induced a round-shaped morphology, as was described for apoptotic parasites (**Figure 2C**) (30). In line, hrLL37 treatment of promastigotes resulted in significant increase in AnnexinA5-binding parasites, in a dose-dependent manner, for *Lae* (27.1 $\pm$ 14.3%; 31.3 $\pm$ 26%) and *Lm* (51.5 $\pm$ 20.5%; 74.8 $\pm$ 23.2%), compared to untreated *Lae* (8.0 $\pm$ 4.5%) and *Lm* (14.7 $\pm$ 11.1%) (**Figures 2D,E**).

Treatment of *Lae* amastigotes also resulted in a significant increase in AnnexinA5 binding (71.3 $\pm$ 12.6%) and TUNEL positivity (87.0 $\pm$ 6.1%) compared to the untreated controls (18.7 $\pm$ 6.1%; 32.3 $\pm$ 13.3%) (**Figures 2F–H**). Interestingly, treatment of *Lm* amastigotes with rhLL37, resulted in only a minor but significant increase of AnnexinA5 binding (8.3 $\pm$ 3.0%), however TUNEL positivity (12.1 $\pm$ 2.1%) did not significantly increase, compared to the respective controls (3.3 $\pm$ 2.3%; 12.0 $\pm$ 6.6%) (**Figures 2G–I**). In all, we demonstrated hrLL37 to induce cell death characteristics, restricting parasites viability in a dose-dependent manner.

Expression of Cathelicidin Is More Prominent in Pro-inflammatory Than in Anti-inflammatory Human Macrophages

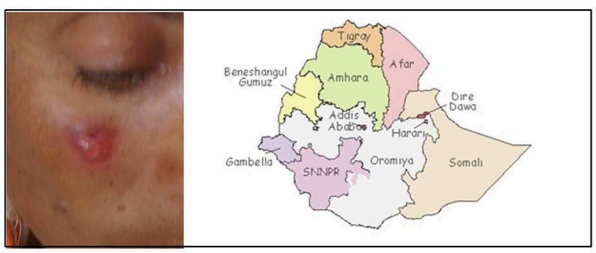
Human macrophages are key players during *Leishmania* infection. In a next step, the suitability of both human primary monocyte derived macrophages type 1 (hMDM1) and type 2 (hMDM2) as host for *Leishmania* parasites was assessed. From human blood, monocytes were isolated and differentiated using rhGM-CSF (10 ng/ml) or rhM-CSF (30 ng/ml), to generate hMDM1 or hMDM2, respectively. The hMDM1 were characterized by their fried-egg shaped morphology and $CD14^{+}MHCII^{+}CD163^{-}$ phenotype (**Figure 3A,** upper lane).

A

Code	Sex	Age	Reason for surgery	Location of skin biopsy	Duration of problem before surgery	Other systemic diseases
H1	F	24	post burn contracture release	Groin area	3 years	None
H2	M	60	post burn contracture release	Posterior to ear near to mastoid bone	several years	None
H3	M	15	post burn contracture release	Groin area	5 years	None
H4	M	20	post traumatic contracture release	Groin area	2 years	None
H5	F	16	Syndactyly release/cleft hand	Groin area	congential/inborn	None
H6	F	50	post burn contracture release	Groin area	6 years	None
H7	F	18	post burn contracture release	Groin area	since child hood	None
H8	M	12	post burn contracture release	Groin area	5 years	None
H9	F	60	post burn contracture release	Groin area	3 years	None
H10	F	15	post burn contracture release	Groin area	2 years	None

Code	Age	Sex	Ethnicity	Type and # of lesion	Duration	Location
P1	19	M	Amhara	CL-1	12 months	Tip of nose
P2	25	F	Oromo	CL-1	6 months	Tip of nose
P3	33	M	Oromo	CL-1	2 months	Ear lobe
P4	23	M	SNNP	CL-1	12 months	Forehead
P5	58	M	Awi	CL-3	12 months	Hand & nose
P6	13	M	Awi	CL-2	4 months	Right cheek & forehead
P7	57	M	Awi	CL-1	3 months	Tip of nose
P8	60	F	Awi	CL-2	12 months	Left cheek
P9	35	F	Awi	CL-1	19 months	Right cheek
P10	12	F	Awi	CL-1	09 months	Left side of nose

B



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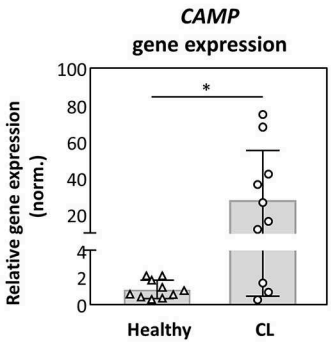

FIGURE 1 | Increased expression of cathelicidin in skin biopsy of patients with cutaneous Leishmaniasis (CL). **(A,B)** Samples were collected from CL patients, with cutaneous lesions **(B)**, derived from people with a different ethnicity **(A)**. **(C)** Skin biopsies, from healthy ($n = 10$) or CL patients ($n = 10$), were collected from which *CAMP* gene expression was assessed by qRT-PCR. Relative gene expression was normalized against GAPDH and presented as mean ± SD. Statistical analysis (Mann–Whitney test) was used to compare groups using GraphPad statistical software (*$p < 0.05$).

In contrast, anti-inflammatory hMDM2 have more elongated cell bodies and were phenotyped as $CD14^+MHCII^+CD163^+$ (**Figure 3A**, lower lane). Interestingly, gene expression analysis demonstrated the gene *CAMP*, which encodes cathelicidin, to be significantly higher expressed in hMDM1, compared to hMDM2 (**Figure 3B**). Also a significant elevated cathelicidin protein amount was present in hMDM1 (0.25 ± 0.85) compared to hMDM2 (0.01 ± 0.04) (**Figure 3C**). In a next step, both hMDM phenotypes were infected with transgenic *Lm* promastigotes or axenic amastigotes, after which parasite infection rate (*Lm*dsRed$^+$ hMDM) was assessed. Of note, the dsRed protein is constitutively expressed, in viable *Leishmania* parasites, as described previously (31). At early (24–48 hpi) and late time points (6–7 dpi) after promastigote infection, a significantly higher infection rate was observed in hMDM2 (52.7 ± 15.6%; 57.4 ± 16.4%), compared to hMDM1 (40.8 ± 11.5%; 50.4 ± 13.4%) (**Figures 3D,E**). Infection with amastigotes resulted in a high infection rate, which however did not differ significantly between early and late time points in hMDM1 (83.2 ± 19.5%; 75.0 ± 22.4%) and hMDM2 (82.9 ± 10.8%; 74.2 ± 16.6%), respectively (**Figure 3F**). Of note, the transgenic *Lm* promastigotes transformed into amastigotes *in vitro*, as the expression of dsRed increased (increase in mean fluorescent intensity, MFI) (**Figure 3G**). Altogether, pro-inflammatory human macrophages were demonstrated to express cathelicidin to a higher extent, which may contribute to an impaired parasite survival.

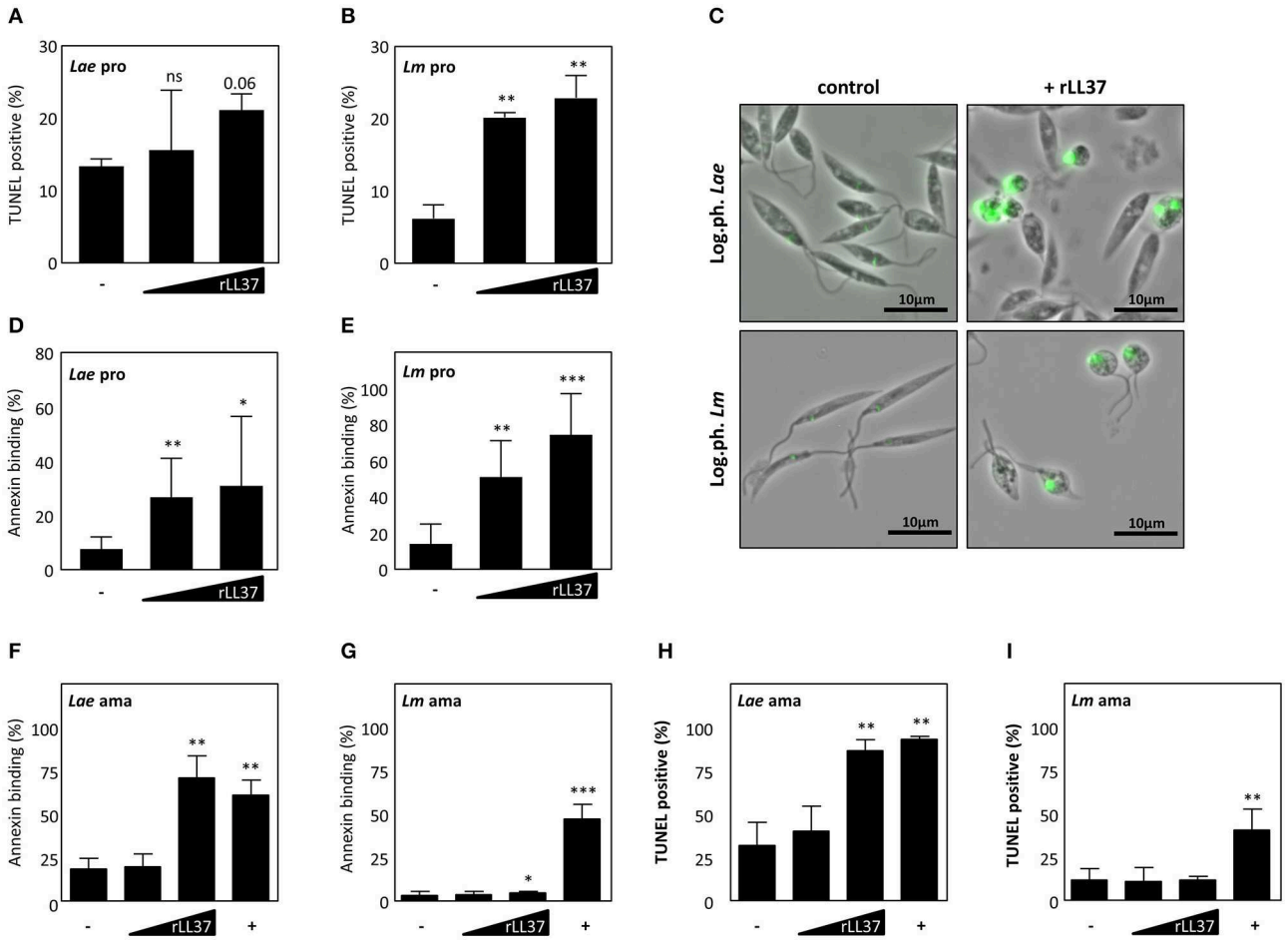

FIGURE 2 | Dose dependent killing of *Leishmania* by human recombinant LL-37. Both logarithmic phase *Lm* and *Lae* promastigotes and axenic amastigotes were treated with different concentrations of rLL-37 or 25 µM staurosporine (+). After 24–72 h, DNA fragmentation was assessed by flow cytometry **(A,B)** for *Lae* and *Lm* promastigotes, respectively, and microscopy **(C)** and for *Lae* and *Lm* amastigotes **(H,I)**. Using flow cytometry Annexin binding was assessed by promastigotes **(D,E)** and axenic amastigotes **(F,G)** from *Lae* and *Lm,* respectively. Data are presented as mean ± SD of at least 3 independent experiments (paired *t*-test; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, not significant; pro, promastigotes; ama, amastigotes).

Vitamin-D Derivatives Induce Cathelicidin-Mediated-*Leishmania* Restriction in Human Primary Macrophages

To investigate the role of cathelicidin in *Leishmania* restriction further, we increased expression of cathelicidin using Vitamin D derivatives. Upon activating the Vitamin-D pathway in hMDM1, using 1α, 25-dihydroxyvitamin D3 (calcitriol, CCT, 100 nM) and calcipotriol (CPT, 100 nM), a synthetic VitD3 analog, we assessed *Leishmania* parasite survival (32). Already 24 h post infection (early), assessment of *CAMP* gene expression showed *Lm*-infected hMDM1 to have a slightly increased *CAMP* expression (2.1 ± 1.8-fold), compared to the uninfected control (normalized to 1; dashed line) (**Figure 4A**). Treatment with CCT and CPT resulted in a 199- (± 41.1) fold and 181- (± 164) fold increase of *CAMP*. After 6 days (late), an even higher expression of *CAMP* was detectable after treatment with CCT (587 ± 173-fold), CPT (282-fold) or during *Lm* infection (3.2 ± 2.1-fold) (**Figure 4A**). In line, protein expression was assessed by Western blot, demonstrating an increased cathelicidin protein expression during *Leishmania* infection (early: 1.8 ± 1.0-fold; late: 4.8 ± 4.0-fold). Furthermore, both CCT and CPT induced a significant increase in cathelicidin protein expression at early (25.5 ± 22.2-fold; 101.9 ± 125.4-fold) and at late time points (152.5 ± 149.6-fold; 101.9 ± 125.4-fold), respectively (**Figure 4B**). In a next step, hMDM1 were pretreated with CCT or CPT, followed by infection with transgenic dsRed-expressing *L. major* or *L. aethiopica*. Using flow cytometry, infection rates and parasite survival, as mean fluorescent intensity (MFI), were assessed. Remarkably, treatment with CCT and CPT significantly reduced *L. major* parasite survival (0.60 ± 0.25; 0.74 ± 0.23) compared to the control (1.00 ± 0.46) (**Figures 4C,D**). However, the percentage of hMDM1 infected cells, indicated as infection rate, did not change significantly (**Figure 4E**). Regarding *L. aethiopica*, similar findings were acquired (data not

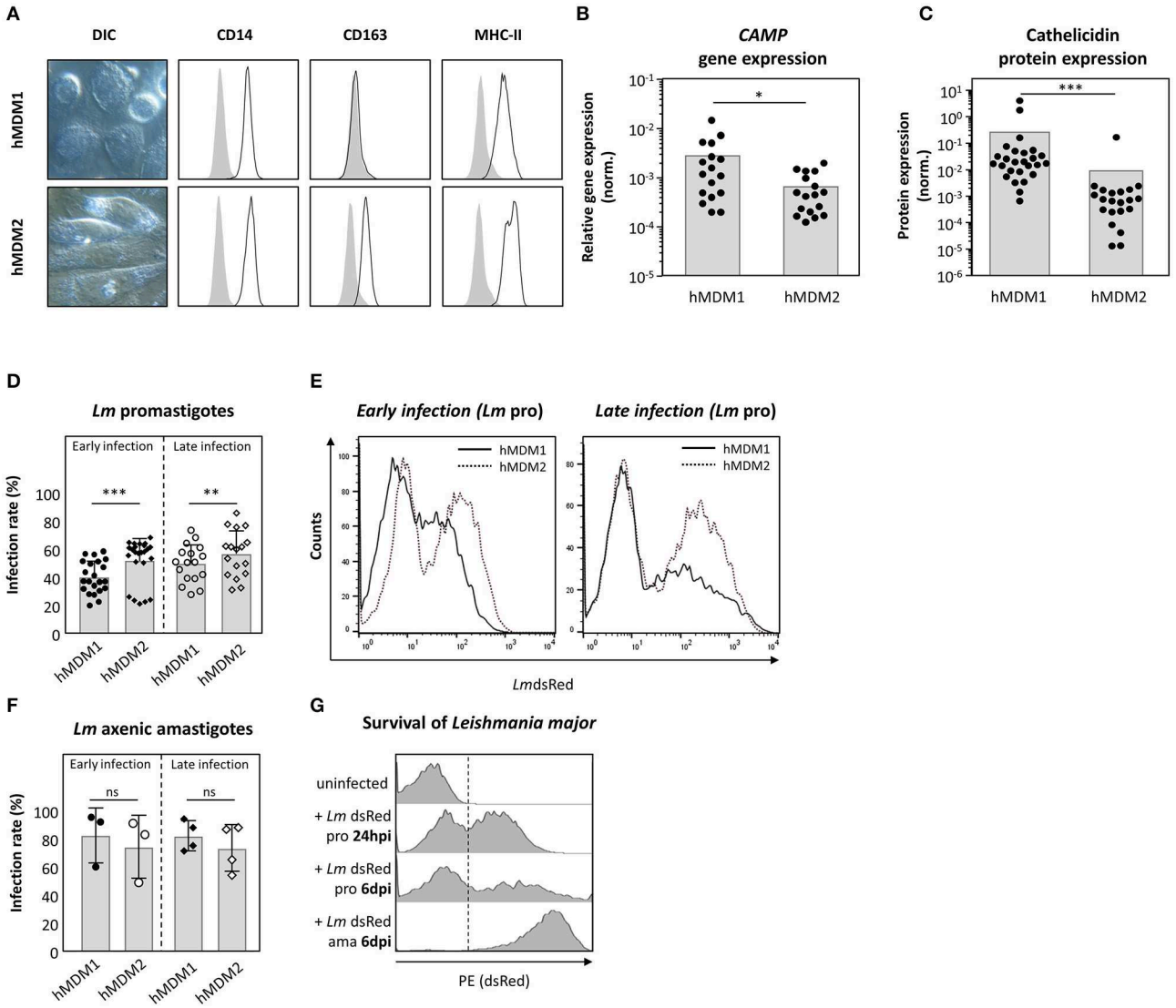

FIGURE 3 | Expression of cathelicidin is more prominent in pro-inflammatory hMDM1 then in anti-inflammatory hMDM2. **(A)** Live cell imaging DIC micrographs of inflammatory hMDM1 and anti-inflammatory hMDM2, characterized by flow cytometry for CD14, CD163, and MHC-II surface expression (black line) in line with the isotype controls (gray). **(B,C)** *CAMP* gene expression ($n = 17$) **(B)** and cathelicidin protein expression ($n = 21$) **(C)** were assessed in hMDM1 and hMDM2 by qRT-PCR and western blot. *CAMP* expression was normalized against the house keeping gene *GAPHD*. Western blots were analyzed by densitometry (ImageJ analysis), normalizing cathelicidin against ß-actin protein expression. **(D–F)** Both hMDM1 and hMDM2 were infected with either transgenic *Lm* pro ($n = 22$–24) **(D,E)** or ama ($n = 3$–4) **(F)** (MOI of 10). After 3 h, extracellular parasites were removed by washing following incubation at 37°C, 5% CO_2. After 24–48 hpi (early infection) or 6–7 dpi (late infection) infection rate was assessed by flow cytometry. **(G)** *Lm* dsRed pro transform into ama over time, indicated by an increased fluorescent intensity. Micrographs, histograms and data, presented as mean ± SD, are representative for at least 3 independent experiments (Wilcoxon matched-pairs signed rank test; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant; pro, promastigotes; ama, amastigotes).

shown). These data demonstrate that targeting the Vitamin D pathway strongly compromises *Leishmania* parasite survival, as cathelicidin is strongly upregulated.

Reduction of Endogenous Cathelicidin in Human Primary Macrophages Promotes *Leishmania* Infection

The human cathelicidin is expressed by monocytes, macrophages as well as neutrophils (17, 33). We already demonstrated (i) cathelicidin to be higher expressed in skin biopsies of patients with the self-healing cutaneous leishmaniasis compared to healthy controls, (ii) hrLL37 to facilitate apoptosis among promastigotes and amastigotes and (iii) hMDM1 to express cathelicidin to a higher extent comparted to hMDM2. To demonstrate a role for the intracellular, endogenous cathelicidin of macrophages in the elimination of *Leishmania* parasites, knockdown (KD) experiments were performed. Using an RNAi approach, we could significantly reduce *CAMP* gene expression (0.15 ± 0.13), compared to the control (1.0 ± 0.0) and non-sense

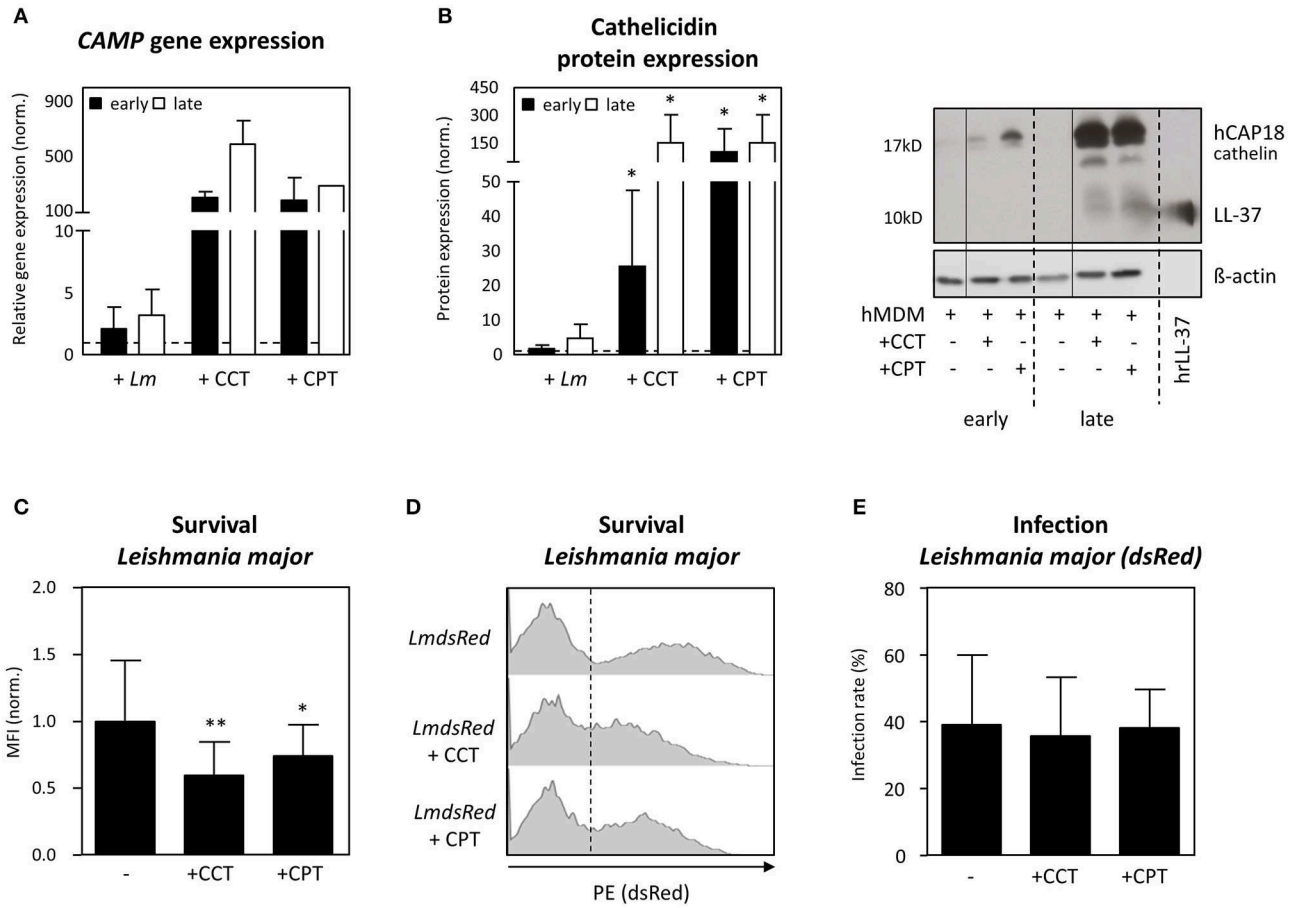

FIGURE 4 | Vitamin-D derivatives induce LL37 mediated Leishmania restriction in human primary macrophages. hMDM-1 were generated and treated with calcitriol (CCT) or calcipotriol (CPT) for 24 h, or left untreated. Subsequently, hMDM-1 were infected with transgenic dsRed expressing *Lm* or *Lae* (MOI10). After 3 h, extracellular parasites were removed by washing, following incubation at 37°C, 5% CO_2. After 24 h (early; black bars) or 6 d (late; white bars) *CAMP* gene ($n = 1$–5) **(A)** and cathelicidin protein expression ($n = 5$–7) **(B)** were by qRT-PCR and western blot analysis. *CAMP* expression was normalized against the house keeping gene *GAPHD*. Western blots were analyzed by densitometry (ImageJ analysis), normalizing cathelicidin against ß-actin protein expression. Gene and protein expression were normalized to the untreated control, which was set to 1 (dashed line). In addition, parasite survival **(C,D)** and infection rates of *Lm* dsRed **(E)**, analyzed by the mean fluorescent intensity (MFI) of the dsRed protein, was assessed by flow cytometry. Western blots, FACS histograms and data, presented as mean ± SD, are representative for at least 3 independent experiments (Wilcoxon matched-pairs signed rank test; $^{*}p < 0.05$; $^{**}p < 0.01$).

siRNA control (1.04 ± 0.74) (**Figure 5A**). We were not able to show a clear cathelicidin protein decrease in siRNA treated cells by western blot (data not shown), as cathelicidin is expressed at low levels under steady state conditions. Therefore, we assessed cathelicidin protein expression in knockdown cells by triggering the Vitamin-D pathway first with CPT and CCT, including β-actin as loading control, demonstrating a strong reduction of cathelicidin protein amount, upon CCT and CPT treatment, in the KD cells compared to the control cells (**Figure 5B**). Next, KD and control cells were infected with *Leishmania* promastigotes, after which intracellular survival was investigated using an end-point titration assay. We could demonstrate that the number of viable *Leishmania* in control (284 ± 228 *Lm*) and non-sense siRNA treated cells (421 ± 303 *Lm*) did not significantly differ. However, KD of *CAMP* resulted in a higher parasite survival (544 ± 388 *Lm*) (**Figure 5C**), although the level of significance was not reached. In all, these data show cathelicidin to play a role in the restriction of *Leishmania* promastigote survival.

DISCUSSION

In the present study, we were able to define a role for the human cathelicidin in human leishmaniasis, based on data from clinical samples. In addition, a human primary *in vitro* cell model was designed to better mimic the *in vivo* interaction between *Leishmania* parasites and their host cell, the human macrophage. The two phenotypes of macrophages were demonstrated to interact differently with *Leishmania* parasites, as in anti-inflammatory macrophages are more susceptible compared to pro-inflammatory human macrophages. Furthermore, the latter pro-inflammatory phenotype expressed the cathelicidin *CAMP* gene transcript and protein more

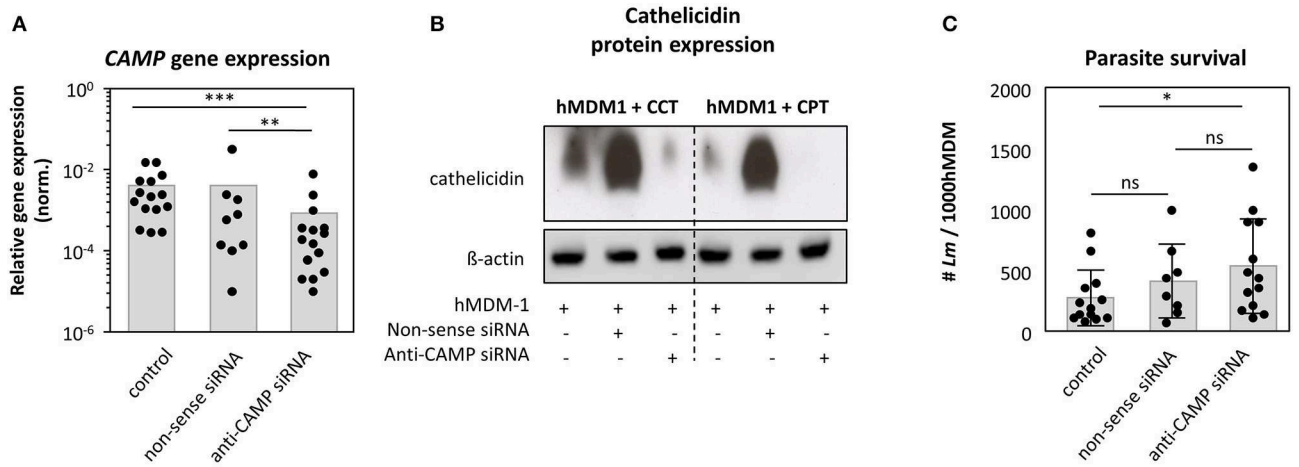

FIGURE 5 | Knockdown of CAMP in human primary macrophages promotes *Leishmania* infection. Human pro-inflammatory macrophages, hMDM-1, were treated with non-sense or anti-CAMP siRNA for 7 h. After 2 d cells were harvested and *CAMP* gene expression and protein expression was assessed by qRT-PCR ($n = 9$–15) **(A)** and western blot upon inducing cathelicidin expression using calcitriol (CCT) or calcipotriol (CPT) **(B)**. *CAMP* expression was normalized against the house keeping gene *GAPHD*. Western blots were analyzed by densitometry (ImageJ analysis), normalizing cathelicidin against ß-actin protein expression. **(C)** Subsequently, cells were infected with *Lm* (MOI10) for 48 h followed by an end-point titration assay (see methods) to assess parasite survival ($n = 8$–13). Data are presented as mean ± SD and are representative for at least 3 independent experiments (Wilcoxon matched-pairs signed rank test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant).

strongly, which we demonstrated to contribute in controlling *Leishmania* infection.

Cathelicidin's Antimicrobial Activity

Cathelicidins have already been described to play a role during infection with e.g., *M. tuberculosis*, *Candida albicans,* and *Cryptosporidum parvum* (34). For *Leishmania* infection animal studies, numerous reports are present. Ramos et al. demonstrated a reduced disease spreading in *Leishmania mexicana* infected BALB/c mice, which were supplemented with calcitriol (35). Ehrchen et al. reported vitamin D receptor KO mice to be more susceptible to infection then control mice (36). In line, CAMP was demonstrated to be crucial for the local control of cutaneous lesion development and parasite growth, using CAMP KO mice (25). Furthermore, progression of visceral Leishmaniasis was demonstrated to be associated with vitamin D deficiency in dogs (37). Few data however, evaluate the effect of cathelicidin and/or the vitamin D pathway in human patients and/or a human cell model. Das et al. could show cathelicidin to augment anti-leishmanial macrophage activating properties of Amphotericin B (38). In line, we identified a strong upregulation of the human CAMP mRNA transcript in clinical samples from African patients with cutaneous Leishmaniasis, suggesting cathelicidin to play a role in human CL *in vivo*.

Cathelicidin-Induced Apoptotic Death of *Leishmania*

We could demonstrate the human cathelicidin to induce an apoptosis-like phenotype in *Leishmania* parasites, in a dose dependent manner in both *L. major* and *L. aethiopica* promastigotes as well as in *L. aethiopica* amastigotes. Although the underlying mode of action remains elusive, rLL37 was demonstrated to induce phosphatidylserine exposure, a round shaped cell morphology and DNA fragmentation, all characteristics of apoptosis (39). Presumably, the amphipathic α-helical peptide LL37 interacts with the negatively charged phospholipids within the parasitic membrane by electrostatic forces, as described for the carpet and toroidal-pore model (17). Surprisingly, recombinant LL37 did not exert apoptosis-inducing effect on the *L. major* amastigote life stage, when looking at TUNEL positivity and DNA degradation. Of note, Kulkarni et al. could show antimicrobial peptides to differently induce parasitic cell death, by means of non-apoptotic (class I) or apoptotic (class II) mediated killing (26). One could speculate these mechanisms to be also applicable in our model, which will be the focus of future research.

Amastigotes also differ in their surface charge compared to promastigotes, as Pimenta et al. could show transformation of *Leishmania mexicana amazonensis* promastigotes to amastigotes to be associated with a shift in the electrophoretic mobility (40). Of general acceptance, is the fact that cationic antimicrobial peptides strongly bind negatively charged phospholipid moieties. Due to the different surface charge between *Leishmania* life stages, we speculate LL37 to only bind *Lm* promastigotes resulting in killing, whereas LL37 to be ineffective in binding *Lm* amastigotes. Overall, our data indicate rLL37 to induce cell death in *Leishmania* promastigotes.

Cathelicidin in Mammalian Innate Immune Defense

Cathelicidins have gained increasing attention, as being an important mediator during innate immunity. Although cathelicidins are primarily present in human neutrophils, also keratinocytes, monocytes and macrophages harbor this antimicrobial peptide (17, 33, 41–43). These cells may indicate

where the cathelicidin is originating from upon *Leishmania* infection. Whether macrophages and keratinocytes exert synergistic effects with regard to cathelicidin production and *Leishmania* elimination is yet to be defined. Focusing on human primary macrophages, we could demonstrate different macrophage phenotypes to express cathelicidin to a different extent. The human cathelicidin is more abundant in pro-inflammatory macrophages, which may not be surprising as it drives macrophages polarization to a pro-inflammatory phenotype (44). The anti-inflammatory macrophages were more susceptible for infection, a finding in agreement with previous data and studies (45, 46). To define an active role for cathelicidin during *Leishmania* infection, we modulated its expression. In concordance with previous studies, we could show *CAMP* expression to be highly enhanced upon activating the vitamin D pathway, using calcitriol or calcipotriol (47–50). Interestingly, the intracellular survival of *Leishmania* parasites was significantly impaired. The group of Agerberth could show LL37 induced expression to be associated with the control of *M. tuberculosis* in human macrophages (51). Furthermore, phenyl butyrate/vitamin D3 treatment, induced LL37-mediated elimination of *M. tuberculosis* by macrophages, strengthening the data of the Modlin's group, showing cathelicidin to be required for the 1,25D(3)-triggered antimicrobial activity against intracellular *M. tuberculosis* (52, 53). In all, triggering the vitamin D pathway in human macrophages, hereby inducing cathelicidin expression, restricts *Leishmania* survival.

Cathelicidin Contributes to a Reduced Parasite Survival

Vitamin D derivatives induce expression of diverse immune modulators, such as cathelicidin, IL-1ß, etc. (54). To target the *CAMP* gene more specifically, a RNA interference (RNAi) approach was chosen. Our data showed *Leishmania* parasite survival to be enhanced. Of note, no significant difference was observed between non-target and anti-CAMP siRNA treatment. One should keep in mind, that all human macrophages were derived from human blood donors, which may differ in gender, immune status, etc., having an impact on host pathogen interactions (55). Furthermore, also transfection as a treatment, may result in RNAi associated immune stimulation through activation of IFN signaling cascades (31). Both aspects, might "bias" our results, with regard to the comparison to the untreated control, as type I interferons have been demonstrated to increase superoxide dismutase (SOD) expression in macrophages, favoring parasite survival (56). Besides the restrictions of the employed methodology, a stronger tendency toward parasite survival, upon *CAMP* RNAi was observed, suggesting cathelicidin to contribute in restricting *Leishmania* parasite survival. Indeed, McGwire's group could show the corresponding murine cathelicidin (*CRAMP*) to control *Leishmania* parasite infection in a mouse infection model (25). Knockout mice for *CRAMP* were reported to develop exacerbated lesions combined with a higher parasites distribution upon *L. major* infection as compared to wild type mice (25). Of note, Gombart et al. showed a vitamin D response element (VDRE) to be conserved in the *CAMP* promotor of primates. The absence of the VDRE region in the genomes of mouse, rat and canine makes the expression of *CRAMP* not tunable by the vitamin D pathway (57). In humans, the great potential of cathelicidin is also highlighted in other disease pathologies. A deficiency of cathelicidin may impede the outcome of inflammation in the lungs of patients with severe sarcoidosis (58). Furthermore, Searing et al. propose an increased production of LL37 to prevent patients with atopic dermatitis from herpes infection (59).

CONCLUSION

In the current study, we revealed the *CAMP* transcript to be strongly upregulated in skin lesion material from cutaneous leishmaniasis patients. Using an *in vitro* model, we demonstrated pro-inflammatory human macrophages to be able to control *Leishmania* infection more efficiently compared to anti-inflammatory macrophages, to which cathelicidin expression is contributing. In addition to the NO-based anti-leishmanial mouse effector mechanism, we propose that vitamin D-inducible cathelicidin expression in combination with GM-CSF polarized macrophages to be a unique mechanism, which contributes to the restriction of *Leishmania* in human macrophages.

MATERIALS AND METHODS

Leishmania Strains

L. major (*Lm*, MHOM_IL_81_FEBNI), *L. aethiopica* (*Lae*, MHOM/ET/72/L100 Z14), and the transgenic *Lm* dsRed (construct pSSUint-DsRed was a kind gift from Dr. Toni Aebischer, Robert Koch Institute, Berlin, Germany) and *Lae* dsRed promastigotes were cultured at 27°C in biphasic Novy-Nicolle-McNeal blood agar medium as described (60). Of note, all viable promastigotes express the transgenic dsRed protein. Upon transformation into the amastigote life stage, a 1 log-scale higher dsRed fluorescence is present, due to the high-level expression in the amastigote life stage (31). Logarithmic-phase or stationary-phase promastigotes were obtained after 2 (log-phase) or 7 (stat-phase) days of culture, respectively. *Lm* and *Lae* axenic amastigotes were generated by incubating log-phase promastigotes in pH 5.5 at 33°C and isolated using a discontinuous Histopaque® 1119 (Sigma Aldrich, Germany) density gradient as described (61).

Assessing Apoptosis

To assess apoptosis, *Lm* and *Lae* promastigotes, which resided in a logarithmic growth phase, were treated with 30 and 60 ng/ml hrLL37 (PeptaNova GmbH, Sandhausen, Germany) for 72 h. *Leishmania* axenic amastigotes were treated with 10 and 100 ng/ml hrLL37 or 25 μM staurosporine for 24 h. Subsequently, DNA fragmentation was assessed by flow cytometry or immunofluorescence imaging using an *in situ* cell-death detection kit, based on terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), as described (30). Exposure of phosphatidylserine (PS) was assessed by AnnexinA5 binding using flow cytometry.

Ethics Approval and Consent to Participate

The study was approved by National Ethical Clearance Committee at Federal Democratic Republic of Ethiopia Ministry of Science and Technology with ethical approval No. 310/227/2007, approved on 30/05/2011. The ethical approval was renewed by the National Ethical Clearance Committee at Federal Democratic Republic of Ethiopia Ministry of Science and Technology on 26/03/2016 with Ref. number 3.10/003/2015. Written informed consent was obtained from study participants.

Sample Collection

Clinically suspected CL patients, who visited the Ankesha and Kela health centers consented to participate in the study, were clinically examined for CL. Ankesha and Kela health centers are found in the leishmaniasis endemic regions in East Gojam Zones of Amhara region and in Gurage zone of Southern regional state of Ethiopia, respectively. Patients, diagnosed for active CL, were recruited to this study prior to treatment. Diagnosis was confirmed by microscopy or culture from skin lesion scraping. After the skin lesion was cleaned, the boarder of lesion was collected for microscopy analysis. Healthy controls were recruited from patients admitted for minor surgery ALERT hospital. All study participants were seronegative for HIV. Skin biopsies from CL patients were taken from the border site of the lesion, using a disposable punch (3 mm in diameter). Local anesthesia with 2% lidocaine was applied. Control skin biopsies were obtained from the leftover samples taken for skin graft of selected individuals (without infection or immunological disorder) visiting the ALERT hospital surgery department.

In addition, from skin samples *Leishmania* promastigotes were cultivated. DNA was extracted from culture and biopsy samples using QIAamp® DNA Mini Kit according to manufacturer's procedure. PCR amplification was performed with 100 ng template and the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) using the primers *Lae* species-specific primers V5F 5′-GGTGATGTGCCCGAGTGCA-3′ and V10R 5′-CGTGCACATCAGCACATGGG-3′.

Generation of Human Monocyte-Derived Macrophages

Human peripheral mononuclear cells (PBMCs) were isolated from buffy coats (DRK-Blutspendedienst Hessen GmbH) by passage over a Leukocyte Separation Medium gradient as described previously (30). Monocytes, obtained by plastic adherence or CD14 selection were incubated either with 10 ng/ml rhGM-CSF (Leukine® Sanofi-Aventis, Bridgewater, US) or 30 ng/ml rhM-CSF (R&D Systems, Abingdon, UK) for a period of 5 to 7 d at 37°C, 5% CO_2 to generate hMDM1 or hMDM2, respectively. Cells were generated in 6 w plates or in 25 cm^2 culture flasks and were detached by cooling cells down on ice, following detachment with a cell scraper. Experimental data, conducted with monocytes obtained from human donors, are depicted as dot plots, in which each dot presents data from a single donor.

Infection of hMDM With *Leishmania* Parasites

HMDM were harvested and transferred into 1.5 ml micro-centrifuge tubes, to which cells do not attach. The cells were co-incubated with stationary phase *Lm* or *Lae* promastigotes or axenic amastigotes at a MOI ratio of 1:10 in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 50 μM β-mercaptoethanol (all from Sigma Aldrich), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 10 mM HEPES (all from Biochrom) for 3 h at 37°C in a humidified atmosphere in a CO_2 incubator. Extracellular parasites were removed by centrifugation and washing the cells. During infection experiments, cathelicidin expression was induced by incubation of hMDM with 100 nM calcitriol or calcipotriol for 24 h. For transgenic *Lm*dsRed flow cytometry was used to analyze infection rates. These transgenic parasites can also be used as a model to follow the parasite propagation, which is based on the development and replication of amastigotes. The *Lm*dsRed promastigotes increase their fluorescence intensity when transforming into amastigotes, which enables the quantification of the parasite propagation by measuring the dsRed mean fluorescence intensity using FACS (62, 63).

Flow Cytometry

(i) FACS analysis of *Leishmania* parasites was performed as described Wenzel et al. (61). Apoptosis, among parasites, was assessed by staining with AnnexinA5-Alexa Fluor 647 using a Ca^{2+} rich buffer.

(ii) For phenotyping hMDM by flow cytometry, hMDM were washed in FACS-Buffer (PBS supplemented with 1% FCS, 1% human serum and 1% BSA) and incubated with anti-CD14-FITC (1:100, IgG2b), anti-CD163-PE (1:50, IgG1, GHI/61), or anti-MHC II-PerCP (1:100, IgG2a, L243) for 30 min on ice in the dark. Corresponding isotype controls were used in the same dilution (all antibodies and isotype controls were from BD Pharmingen, Heidelberg, Germany). The cells were washed in FACS-Buffer and analyzed by flow cytometry.

(iii) To assess parasite survival, Leishmania ($dsRed^+$) infected hMDM were washed in FACS-buffer and infection rate (% of $dsRed^+$ hMDM) and the parasite load (mean fluorescent intensity) were assessed by flow cytometry. Upon analyzing, at least 10,000 events (human cells) or 20,000 (parasites) were recorded using a BD LSR II flow cytometer (BD Bioscience, Heidelberg, Germany). Data were analyzed by BD FACS Diva or FlowJo software (Treestar).

Transfection of Primary Human Cells With siRNA

CD14 selected monocytes were differentiated into hMDM1 by addition of hrGM-CSF over a period of 6 days. On day 3, the medium was refreshed with new growth factors. At day 6, cells were washed with RPMI, without supplements, and 1 ml prewarmed RPMI was given to the hMDM. For transfection 80 pmole of 20 μM siRNA (LL-37-siRNA: ON-TARGET plus SMART pool Human CAMP (LL-37) from Thermo Scientific

Dharmacon, Bonn, Germany; nonsense siRNA: Stealth RNAi siRNA Negative Control from Invitrogen, Darmstadt, Germany) were mixed with 20 µl of Stemfect Buffer and 4.6 µl of Stemfect Reagent were mixed with 20 µl Stemfect Buffer (Stemfect RNA Transfection kit from Stemgent, San Diego, USA). Within 5 min, both compounds were mixed and subsequently incubated for 20 min at room temperature. The transfection mixture was added to the cells for 7 h at 37°C. Cells were subsequently washed and further incubated 2 days at 37°C in Complete Medium before harvesting.

RNA Isolation, Reverse-Transcription PCR, and qRT-PCR

RNA extraction and reverse-transcription, of either human primary cells or skin tissue, were performed using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and the ImProm-II Reverse Transcription system (Promega, Mannheim, Germany), respectively, according to the manufacturers' instructions. Differential gene expression in primary macrophages was analyzed by quantitative real-time PCR using the LightCycler® 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany) and LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Differential gene expression in skin tissue was analyzed by quantitative real-time PCR using the Rotor Gene-3000 system and Rotor-Gene SYBR Green I kit (Qiagen) according to the manufacturer's instructions. The LL-37 gene (5′-GGA CCC AGA CAC GCC AAA-3′; 3′-GCA CAC TGT CTC CTT CAC TGT GA-5′) expression was normalized to the housekeeping gene GAPDH (5′-GAG TCA ACG GAT TTG GTC GT-3′; 3′-TTG ATT TTG GAG GGA TCT CG-5′) using the ΔΔCT–method.

Western Blot

A total number of 0.5×10^6 hMDM were lysed in Lämmli-Buffer (A. bidest supplemented with 0.7 M Glycerol, 1.7% SDS, 0.1 M DTT and 30 µM Bromphenol blue), denaturated at 95°C for 10 min and loaded onto a 15% SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose transfer membrane at 145 mA constant voltage for 1 h. The membrane was blocked with WB-Block-Solution (GE Healthcare, Buckinghamshire, United Kingdom) washed with WB-Wash-Buffer (A. bidest supplemented with 0.5% Tween, 0.14 M NaCl, 10 mM Tris, 1 mM NaN3; pH 8) and subsequently incubated with an anti-LL37 primary antibody (kindly provided by Prof. B. Agerberth) overnight at 4°C. After extensive washing, the membrane was incubated with a HRP-conjugated secondary antibody (1:1000, from Cell Signaling, Danvers, USA) for 1 h at room temperature. The membrane was washed once more and the protein bands were detected using an ECL substrate (GE Healthcare, Buckinghamshire, United Kingdom). Using ImageJ, densitometry analysis was performed to quantify the intensities of the protein bands.

End-Point Titration Assay

The amount of viable intracellular parasites inside hMDM1 (control, non-sense siRNA or anti-CAMP treated) was determined. After 3 h of infection, MOI10, cells were washed to remove extracellular parasites. After 48 h of infection, the end-point titration assay was carried out. Therefore, cell scraper detached hMDM1 were counted and 2000 hMDM1 were seeded, in quadruplicates, in a 96 w plate containing biphasic Novy-Nicolle-McNeal blood agar medium. Wells were serial diluted (factor 1.5) for 24 times. Plates were incubated for 7–10 days at 27°C. By microscopical analysis, plates were analyzed to assess at which dilution growth was seen. Based on the dilution factor and the amount of hMDM1 that were seeded in the first well, the amount of parasites per 1,000 hMDM1 was calculated (63). The formula applied to calculate the amount of parasites per 1,000 macrophages is $1.5^x/2$ were x is the dilution step in which still parasite growth was observed.

Statistical Analysis

Numerical data are presented as the mean ± standard deviation (SD). For statistical analysis, data were tested for their normal distribution, using the D'Agostino and Pearson omnibus normality test. If passed, statistical analysis was determined by a paired Student *t*-test. If data were not normally distributed or in case to few biological replicates were present to test normal distribution, a non-parametric test (Mann–Whitney test or Wilcoxon matched-pairs signed rank test) was used. The software Graph-Pad Prism version 4 was used, by which * indicates statistically difference at $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The study was approved by National Ethical Clearance Committee at Federal Democratic Republic of Ethiopia Ministry of Science and Technology with ethical approval No. 310/227/2007, approved on 30/05/2011. The ethical approval was renewed by the National Ethical Clearance Committee at Federal Democratic Republic of Ethiopia Ministry of Science and Technology on 26/03/2016 with Ref. number 3.10/003/2015. Written informed consent was obtained from study participants.

AUTHOR CONTRIBUTIONS

GZ, PC, EB, BW, UW, MC, and MA helped with substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data for the work. GZ, PC, BA, RK, NR, and UR helped drafting the work or revising it critically for important intellectual content.

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REFERENCES

1. Dietze R, Carvalho SF, Valli LC, Berman J, Brewer T, Milhous W, et al. Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by Leishmania chagasi. *Am J Trop Med Hyg.* (2001) 65:685–9. doi: 10.4269/ajtmh.2001.65.685
2. WHO. *WHO 2014 Leishmaniasis. Fact sheet N°375*. (2014). Available online at: http://www.who.int/mediacentre/factsheets/fs375/en/
3. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. *Clin Transl Immunol.* (2014) 3:e13. doi: 10.1038/cti.2014.4
4. Boelaert M, Meheus F, Robays J, Lutumba P. Socio–economic aspects of neglected diseases: sleeping sickness and visceral leishmaniasis. *Ann Trop Med Parasitol.* (2010) 104:535–42. doi: 10.1179/136485910X12786389891641
5. Benoit M, Desnues B, Mege J-L. Macrophage polarization in bacterial infections. *J Immunol.* (2008) 181:3733–9. doi: 10.4049/jimmunol.181.6.3733
6. Green SJ, Meltzer MS, Hibbs JB, Nacy CA. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J Immunol.* (1990) 144:278–83.
7. Iniesta V, Carcelén J, Molano I, Peixoto PM, Redondo E, Parra P, et al. Arginase I induction during *Leishmania major* infection mediates the development of disease. *Infect Immun.* (2005) 73:6085–90. doi: 10.1128/IAI.73.9.6085-6090.2005
8. Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against Leishmania infection. *Immunol Res.* (2008) 41:15–25. doi: 10.1007/s12026-007-8012-y
9. El-Gayar S, Thüring-Nahler H, Pfeilschifter J, Röllinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. *J Immunol.* (2003) 171:4561–8. doi: 10.4049/jimmunol.171.9.4561
10. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J Immunol.* (2001) 166:2173–7. doi: 10.4049/jimmunol.166.4.2173
11. Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S. Macrophage killing of Leishmania parasite *in vivo* is mediated by nitric oxide from L-arginine. *J Immunol.* (1990) 144:4794–7.
12. Green SJ, Nacy CA, Meltzer MS. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. *J Leukoc Biol.* (1991) 50:93–103. doi: 10.1002/jlb.50.1.93
13. Mestas J, Hughes CCW. Of mice and not men: differences between mouse and human immunology. *J Immunol.* (2004) 172:2731–8. doi: 10.4049/jimmunol.172.5.2731
14. Mossalayi MD, Arock M, Mazier D, Vincendeau P, Vouldoukis I. The human immune response during cutaneous leishmaniasis: NO problem. *Parasitol Tdy.* (1999) 15:342–5. doi: 10.1016/S0169-4758(99)01477-5
15. Amorim FM, Rodrigues YK, Barbosa TP, Néris PL, Caldas JP, Sousa SC, et al. Morita-Baylis-Hillman adduct shows *in vitro* activity against *Leishmania* (Viannia) *braziliensis* associated with a reduction in IL-6 and IL-10 but independent of nitric oxide. *Parasitology.* (2013) 140:29–38. doi: 10.1017/S0031182012001291
16. Lima-Junior DS, Costa DL, Carregaro V, Cunha LD, Silva AL, Mineo TW, et al. Inflammasome-derived IL-1β production induces nitric oxide–mediated resistance to Leishmania. *Nat Med.* (2013) 19:909–915. doi: 10.1038/nm.3221
17. Dürr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta.* (2006) 1758:1408–25. doi: 10.1016/j.bbamem.2006.03.030
18. Schittek B, Paulmann M, Senyürek I, Steffen H. The role of antimicrobial peptides in human skin and in skin infectious diseases. *Infect Disord Drug Targets.* (2008) 8:135–43. doi: 10.2174/1871526510808030135
19. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci USA.* (1998) 95:9541–6. doi: 10.1073/pnas.95.16.9541
20. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* (1998) 16:82–8. doi: 10.1016/S0167-7799(97)01156-6
21. Travis SM, Anderson NN, Forsyth WR, Espiritu C, Conway BD, Greenberg EP, et al. Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect Immun.* (2000) 68:2748–55. doi: 10.1128/IAI.68.5.2748-2755.2000
22. Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol.* (2001) 117:91–7. doi: 10.1046/j.1523-1747.2001.01340.x
23. Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol.* (2004) 172:1763–7. doi: 10.4049/jimmunol.172.3.1763
24. Sonawane A, Santos JC, Mishra BB, Jena P, Progida C, Sorensen OE, et al. Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages. *Cell Microbiol.* (2011) 13:1601–17. doi: 10.1111/j.1462-5822.2011.01644.x
25. Kulkarni MM, Barbi J, McMaster WR, Gallo RL, Satoskar AR, McGwire BS. Mammalian antimicrobial peptide influences control of cutaneous Leishmania infection. *Cell Microbiol.* (2011) 13:913–23. doi: 10.1111/j.1462-5822.2011.01589.x
26. Kulkarni MM, McMaster WR, Kamysz W, McGwire BS. Antimicrobial peptide-induced apoptotic death of leishmania results from calcium-dependent, caspase-independent mitochondrial toxicity. *J Biol Chem.* (2009) 284:15496–504. doi: 10.1074/jbc.M809079200
27. Dos Santos JC, Heinhuis B, Gomes RS, Damen MS, Real F, Mortara RA, et al. Cytokines and microbicidal molecules regulated by IL-32 in THP-1-derived human macrophages infected with New World Leishmania species. *PLoS Negl Trop Dis.* (2017) 11:e0005413. doi: 10.1371/journal.pntd.0005413
28. Ramos R, Domingues L, Gama M. LL37, a human antimicrobial peptide with immunomodulatory properties. In: *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances.* (2011). p. 915–25.
29. Zeth K, Sancho-Vaello E. The human antimicrobial peptides dermcidin and LL-37 show novel distinct pathways in membrane interactions. *Front Chem.* (2017) 5:86. doi: 10.3389/fchem.2017.00086
30. Crauwels P, Bohn R, Thomas M, Gottwalt S, Jäckel F, Krämer S, et al. Apoptotic-like Leishmania exploit the host's autophagy machinery to reduce T-cell-mediated parasite elimination. *Autophagy.* (2015) 11:285–97. doi: 10.1080/15548627.2014.998904
31. Meng Z, Lu M. RNA Interference-induced innate immunity, off-target effect, or immune adjuvant? *Front Immunol.* (2017) 8:331. doi: 10.3389/fimmu.2017.00331
32. Kulkarni NN, Yi Z, Huehnken C, Agerberth B, Gudmundsson GH. Phenylbutyrate induces cathelicidin expression via the vitamin D receptor: linkage to inflammatory and growth factor cytokines pathways. *Mol Immunol.* (2015) 63:530–9. doi: 10.1016/j.molimm.2014.10.007
33. Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A. The human cathelicidin LL-37–A pore-forming antibacterial peptide and host-cell modulator. *Biochim Biophys Acta.* (2016) 1858:546–66. doi: 10.1016/j.bbamem.2015.11.003
34. Nizet V, Gallo RL. Cathelicidins and innate defense against invasive bacterial infection. *Scand J Infect Dis.* (2003) 35:670–6. doi: 10.1080/00365540310015629
35. Ramos-Martínez E, Villaseñor-Cardoso MI, López-Vancell MR, García-Vázquez FJ, Pérez-Torres A, Salaiza-Suazo N, et al. Effect of 1,25(OH)2D3

on BALB/c mice infected with Leishmania mexicana. *Exp Parasitol.* (2013) 134:413–21. doi: 10.1016/j.exppara.2013.05.009
36. Ehrchen J, Helming L, Varga G, Pasche B, Loser K, Gunzer M, et al. Vitamin D receptor signaling contributes to susceptibility to infection with *Leishmania major*. *FASEB J.* (2007) 21:3208–18. doi: 10.1096/fj.06-7261com
37. Rodriguez-Cortes A, Martori C, Martinez-Florez A, Clop A, Amills M, Kubejko J, et al. Canine leishmaniasis progression is associated with vitamin D deficiency. *Sci Rep.* (2017) 7:3346. doi: 10.1038/s41598-017-03662-4
38. Das S, Sardar AH, Abhishek K, Kumar A, Rabidas VN, Das P. Cathelicidin augments VDR-dependent anti-leishmanial immune response in Indian Post-Kala-Azar Dermal Leishmaniasis. *Int Immunopharmacol.* (2017) 50:130–8. doi: 10.1016/j.intimp.2017.06.010
39. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* (2007) 35:495–516. doi: 10.1080/01926230701320337
40. Pimenta PF, de Souza W. Leishmania mexicana amazonensis: surface charge of amastigote and promastigote forms. *Exp Parasitol.* (1983) 56:194–206. doi: 10.1016/0014-4894(83)90063-2
41. Braff MH, Zaiou M, Fierer J, Nizet V, Gallo RL. Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens. *Infect Immun.* (2005) 73:6771–81. doi: 10.1128/IAI.73.10.6771-6781.2005
42. Braff MH, Di Nardo A, Gallo RL. Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. *J Invest Dermatol.* (2005) 124:394–400. doi: 10.1111/j.0022-202X.2004.23443.x
43. Agier J, Efenberger M, Brzezinska-Błaszczyk E. Cathelicidin impact on inflammatory cells. *Cent J Immunol.* (2015) 40:225–35. doi: 10.5114/ceji.2015.51359
44. van der Does AM, Beekhuizen H, Ravensbergen B, Vos T, Ottenhoff TH, van Dissel JT, et al. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J Immunol.* (2010) 185:1442–9. doi: 10.4049/jimmunol.1000376
45. Neu C, Sedlag A, Bayer C, Förster S, Crauwels P, Niess JH, et al. CD14-dependent monocyte isolation enhances phagocytosis of listeria monocytogenes by proinflammatory, GM-CSF-derived macrophages. *PLoS ONE.* (2013) 8:e66898. doi: 10.1371/journal.pone.0066898
46. Bayer C, Varani S, Wang L, Walther P, Zhou S, Straschewski S, et al. Human cytomegalovirus infection of M1 and M2 macrophages triggers inflammation and autologous T-cell proliferation. *J Virol.* (2013) 87:67–79. doi: 10.1128/JVI.01585-12
47. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* (2004) 173:2909–12. doi: 10.4049/jimmunol.173.5.2909
48. Segaert S. Vitamin D regulation of cathelicidin in the skin: toward a renaissance of vitamin D in dermatology? *J Invest Dermatol.* (2008) 128:773–5. doi: 10.1038/jid.2008.35
49. Gombart AF, O'Kelly J, Saito T, Koeffler HP. Regulation of the CAMP gene by 1,25(OH)2D3 in various tissues. *J Steroid Biochem Mol Biol.* (2007) 103:552–7. doi: 10.1016/j.jsbmb.2006.12.095
50. Gombart AF. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiol.* (2009) 4:1151–65. doi: 10.2217/fmb.09.87
51. Bowdish DM, Davidson DJ, Scott MG, Hancock RE. Immunomodulatory activities of small host defense peptides. *Antimicrob Agents Chemother.* (2005) 49:1727–32. doi: 10.1128/AAC.49.5.1727-1732.2005
52. Mily A, Rekha RS, Kamal SM, Akhtar E, Sarker P, Rahim Z, et al. Oral intake of phenylbutyrate with or without vitamin D3upregulates the cathelicidin LL-37 in human macrophages: a dose finding study for treatment of tuberculosis. *BMC Pulm Med.* (2013) 13:23. doi: 10.1186/1471-2466-13-23
53. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against *Mycobacterium tuberculosis* is dependent on the induction of cathelicidin. *J Immunol.* (2007) 179:2060–3. doi: 10.4049/jimmunol.179.4.2060
54. Verway M, Bouttier M, Wang TT, Carrier M, Calderon M, An BS, et al. Vitamin D induces interleukin-1β expression: paracrine macrophage epithelial signaling controls *M. tuberculosis* infection. *PLoS Pathog.* (2013) 9:e1003407. doi: 10.1371/journal.ppat.1003407
55. Garelnabi M, Taylor-Smith LM, Bielska E, Hall RA, Stones D, May RC. Quantifying donor-to-donor variation in macrophage responses to the human fungal pathogen *Cryptococcus neoformans*. *PLoS ONE.* (2018) 13:e0194615. doi: 10.1371/journal.pone.0194615
56. Rossi M, Fasel N. How to master the host immune system? Leishmania parasites have the solutions! *Int Immunol.* (2018) 30:103–11. doi: 10.1093/intimm/dxx075
57. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* (2005) 19:1067–77. doi: 10.1096/fj.04-3284com
58. Barna BP, Culver DA, Kanchwala A, Singh RJ, Huizar I, Abraham S, et al. Alveolar macrophage cathelicidin deficiency in severe sarcoidosis. *J Innate Immun.* (2012) 4:569–78. doi: 10.1159/000339149
59. Searing DA, Leung DY. Vitamin D in atopic dermatitis, asthma and allergic diseases. *Immunol Allergy Clin North Am.* (2010) 30:397–409. doi: 10.1016/j.iac.2010.05.005
60. van Zandbergen G, Bollinger A, Wenzel A, Kamhawi S, Voll R, Klinger M, et al. Leishmania disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. *Proc Natl Acad Sci USA.* (2006) 103:13837–42. doi: 10.1073/pnas.0600843103
61. Wenzel UA, Bank E, Florian C, Förster S, Zimara N, Steinacker J, et al. *Leishmania major* parasite stage-dependent host cell invasion and immune evasion. *FASEB J.* (2012) 26:29–39. doi: 10.1096/fj.11-184895
62. Misslitz A, Mottram JC, Overath P, Aebischer T. Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in Leishmania amastigotes. *Mol Biochem Parasitol.* (2000) 107:251–61. doi: 10.1016/S0166-6851(00)00195-X
63. Stenger S, van Zandbergen G. Measuring the killing of intracellular pathogens: Leishmania. *Curr Protoc Essent Lab Tech.* (2011) 2011:Chapter 14:Unit14.23. doi: 10.1002/0471142735.im1423s93

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Vitamin D Receptor Inhibits NLRP3 Activation by Impeding Its BRCC3-Mediated Deubiquitination

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The NLRP3 inflammasome is a multiprotein oligomer responsible for activation of the inflammatory response by promoting the maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18. Dysregulation of this inflammasome has been linked to several autoimmune diseases, indicating that NLRP3 is tightly regulated to prevent aberrant activation. The regulation of NLRP3 activation remains unclear. Here, we report the identification of vitamin D receptor (VDR) as a negative regulator of NLRP3 oligomerization and activation. VDR can physically bind NLRP3 and block the association of NLRP3 with BRCC3. When BRCC3-mediated deubiquitination of NLRP3 is inhibited by VDR, NLRP3 activation is subsequently inhibited. In the absence of VDR, caspase-1 activation and IL-1β release are increased in response to LPS-induced inflammation or alum-induced peritoneal inflammation, indicating that VDR is a negative regulator of NLRP3 inflammasome activation *in vivo*. In addition, vitamin D negatively regulates the NLRP3 inflammasome via VDR signaling to effectively inhibit IL-1β secretion. These studies demonstrate that VDR signaling constrains NLRP3 inflammasome activation and might be a potential treatment target for NLRP3 inflammasome-related diseases.

Keywords: VDR, NLRP3 inflammasome, BRCC3, deubiquitinating, cytokines

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INTRODUCTION

Pyroptosis is a highly inflammatory form of programmed cell death that promotes the rapid clearance of various bacterial and virus infections. The inflammasome is a multiprotein oligomer that serves as a platform for caspase-1-dependent activation of the maturation of the pro-inflammatory cytokines IL-1β and IL-18; the secretion of these cytokines results in pyroptosis (1). The NLRP3 inflammasome can be triggered by many different stimuli, such as amyloid-β, extracellular ATP, alum, nigericin (an antibiotic from *Streptomyces hygroscopicus*), and crystals (2, 3). Upon activation, NLRP3 assembles a typical multimeric inflammasome complex comprising the adaptor ASC and the effector pro-caspase-1; this complex mediates the proteolytic cleavage of pro-caspase-1 into active caspase-1 and converts the cytokine precursors pro-IL-1β and pro-IL-18 into biologically active IL-1β and IL-18 (4). Dysregulation of the inflammasome has been linked to several autoimmune diseases, such as types I and II diabetes, inflammatory bowel disease

(IBD), gouty arthritis, multiple sclerosis, and vitiligo, as well as auto-inflammatory disorders (5–8). These diseases and disorders have been connected to the increased or decreased secretion of pro-inflammatory cytokines regulated by the inflammasome, indicating that NLRP3 inflammasome activation is tightly controlled in the normal state.

The regulation of NLRP3 inflammasome activation has been extensively investigated. Accumulating evidence indicates that the modification of NLRP3 occurs at the transcriptional and post-translational levels, with particular focus on ubiquitination, and phosphorylation (9). NLRP3 is poly-ubiquitinated with mixed Lys-48 and Lys-63 ubiquitin chains in resting macrophages (10, 11). A decrease in ubiquitinated NLRP3 can be induced by inflammasome activation signals (10). Inhibition of NLRP3 deubiquitination almost completely blocks NLRP3 activation in both mouse and human cells (10). BRCC3, a deubiquitinase, is crucial for NLRP3 activation at the post-transcriptional level due to its role in NLRP3 deubiquitination (10, 11). Although many studies have examined the regulation of NLRP3, the regulatory mechanism of NLRP3 activation remains unclear and requires further investigation.

To understand the signaling mechanism of NLRP3 inflammasome activation, we sought to identify proteins that interact with NLRP3. Using protein mass spectrometry analysis, we identified that NLRP3 can interact with vitamin D receptor (VDR). Beyond its well-established role in calcium–phosphorus homeostasis and bone metabolism, VDR plays anti-inflammatory roles in both innate and adaptive immunity (12, 13). VDR deficiency is associated with increased inflammation and deregulation in several inflammatory diseases, such as inflammatory bowel disease, sepsis, diabetes and asthma (14, 15). Vitamin D and VDR have anti-inflammatory effects and play an immunosuppressive role in autoimmunity. Together, they increase the phagocytic ability of monocytes to modulate the innate immune system (16, 17) and promote the ability of dendritic cells to modulate regulatory T cell differentiation (12, 14, 18, 19). Recent studies on vitamin D and VDR in inflammation-related diseases have received increasing attention.

Here, we demonstrate that VDR acts as an endogenous suppressor of NLRP3 inflammasome assembly to modulate NLRP3 activation. VDR directly interacts with NLRP3 and disturbs the association of NLRP3 with BRCC3, thereby inhibiting the deubiquitination of NLRP3 by BRCC3 and subsequently blocking activation of the NLRP3 inflammasome.

RESULTS

VDR Interacts With NLRP3

To understand the signaling mechanism underlying NLRP3 inflammasome activation, we sought to identify proteins that interact with NLRP3. We expressed Flag-NLRP3 in HEK293T cells and performed Flag immunoprecipitation pull down NLRP3-associated proteins, which were evaluated using liquid chromatography–mass spectrometry. The analysis revealed VDR as a major interacting partner of NLRP3 (**Figure 1A**, **Figure S1A**). To investigate whether VDR binds other components of the NLRP3 complex, HA-VDR was co-transfected with NLRP3, ASC, or caspase-1 into HEK293T cells, and lysates were examined by co-immunoprecipitation. We found that overexpressed VDR interacted with NLRP3 (**Figure 1B**) but not with ASC or pro-caspase-1 (**Figures S1B,C**). The NLRP3–VDR interaction was also detected by endogenous immunoassay (**Figure 1C**), and the co-localization of these proteins in cells was observed in the immunofluorescence assay (**Figure 1D**, **Figure S1E**). To test whether the interaction is direct, we next performed a GST pull-down assay. NLRP3 was further confirmed to directly interact with VDR (**Figure 1E**). To identify the region of NLRP3 that associates with VDR, Myc-tagged wild-type, and mutant NLRP3 were co-expressed with HA-VDR in HEK293T cells, and immunoprecipitation experiments were performed. Interactions were observed between HA-VDR and full-length NLRP3 and NLRP3 mutated in the carboxy-terminal leucine-rich repeat (LRR) domain and nucleotide-binding domain (NACHT), while NLRP3 mutated in the amino-terminal pyrin domain (PYD) showed no interaction with HA-VDR (**Figure 1F**). On the other hand, the ligand binding domain (LBD), not the DNA binding domain (DBD), of VDR was required for the association with NLRP3 (**Figure 1G**). The PYD domain cannot bind to the LBD domain alone (**Figure S1D**). Our data indicated that VDR is a novel binding partner of NLRP3.

VDR Inhibits NLRP3 Inflammasome-Mediated Caspase-1 Activation and IL-1β Secretion in Macrophages

To examine the effect of VDR on inflammasome activation, we first checked the expression of components related to the NLRP3 complex in the absence of VDR. The results showed no significant differences in the proteins, including NLRP3, ASC, pro-IL-1β, and pro-caspase-1, in VDR-KO cells (**Figure S2A**). Next, BMDMs (bone marrow-derived macrophages) isolated from WT and $Vdr^{-/-}$ mice were primed with LPS and then activated by NLRP3 stimuli, such as extracellular ATP, nigericin and alum, to evaluate NLRP3 activation. BMDMs from $Vdr^{-/-}$ mice showed clear increases in caspase-1 cleavage and IL-1β (**Figure 2A**), and the secretion of IL-1β and IL-18 was significantly increased (**Figures 2B,C**). As a control for inflammasome-independent cytokines, TNF-α production was not affected (**Figure 2D**). Similar results were obtained in mouse peripheral macrophages (PMs) (**Figure 2E**). To confirm the ability of VDR to inhibit NLRP3 inflammasome activation, we restored VDR expression in VDR-KO BMDMs by lentivirus-mediated transduction. When NLRP3 inflammasomes were activated by nigericin, VDR overexpression decreased caspase-1 cleavage and IL-1β secretion (**Figures 2F,G**). Considering that non-canonical inflammasome activation is dependent on NLRP3 for IL-1β secretion, we induced non-canonical inflammasome activation with LPS in Pam3CSK4-primed BMDMs and found that IL-1β secretion was increased in $Vdr^{-/-}$ BMDMs (**Figure 2H**). Taken

A

ACC #	Peptides Count		%COV	Protein Name
	Ctrl	NLRP3		
Q96P20	0	49	46.72	NACHT, LRR and PYD domains-containing protein 3
P11473	0	4	10.54	Vitamin D3 receptor

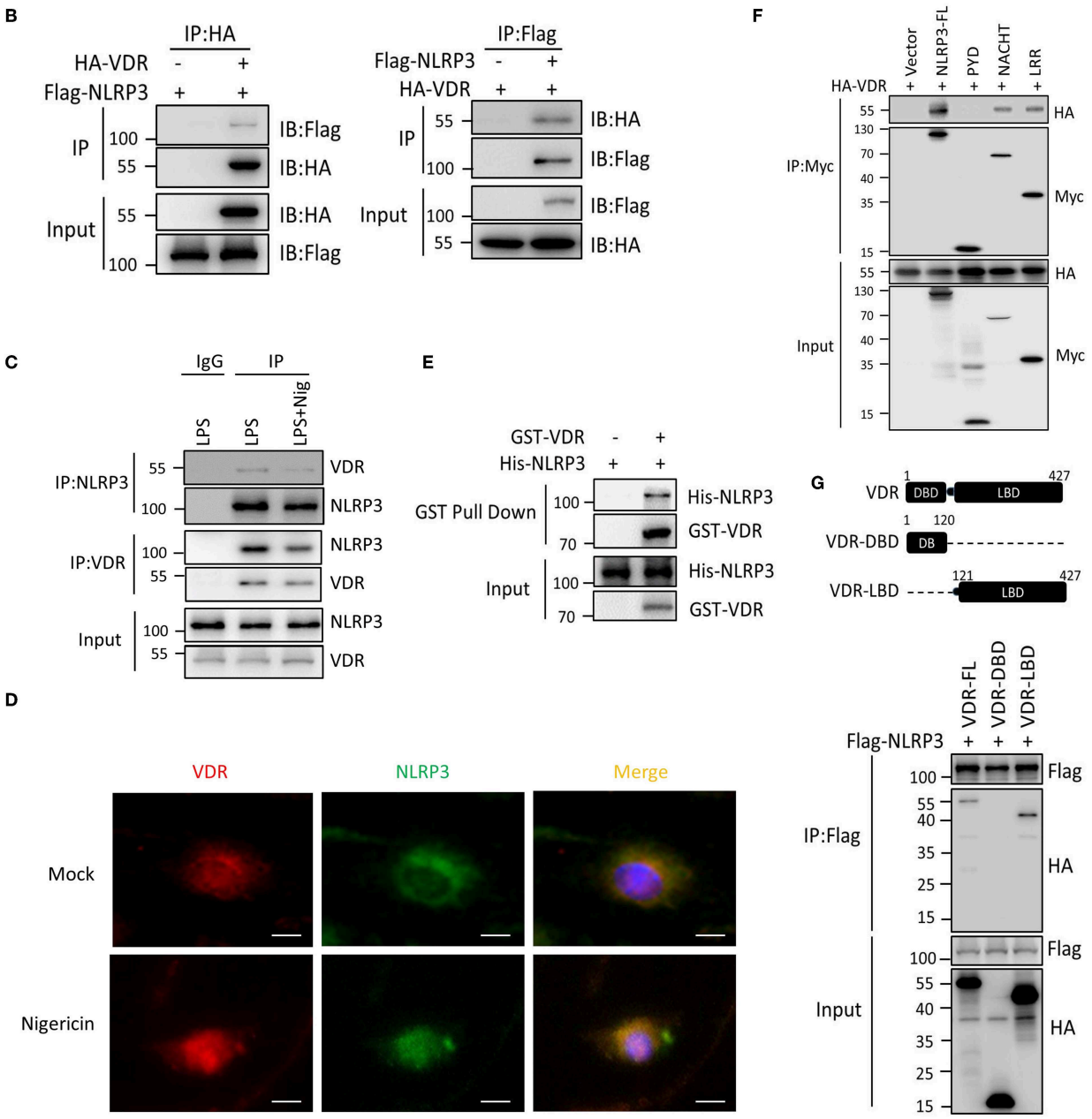

FIGURE 1 | VDR interacts with NLRP3. **(A)** Mass spectrometry analysis of NLRP3 and VDR peptides after immunoprecipitation with Flag to pull down NLRP3-associated proteins in Flag-NLRP3-overexpressing HEK293T cells. **(B)** HA-VDR was co-expressed with Flag-tagged NLRP3 in HEK293T cells; proteins were

(Continued)

FIGURE 1 | immunoprecipitated and analyzed by immunoblotting. Whole-cell lysates are shown as the input. **(C)** LPS-primed BMDMs were unstimulated or stimulated with nigericin for 30 min. Cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies. **(D)** Immunofluorescent staining for VDR and NLRP3 in LPS-primed BMDMs treated with or without nigericin. Scale bar, 10 μm. **(E)** Purified GST-VDR was incubated with purified His-NLRP3 for 2 h. His-NLRP3-Flag bound to GST-VDR was pulled down by glutathione beads and subjected to immunoblot analysis. **(F)** Wild-type or mutant NLRP3 (PYD, NACHT, or LRR) and HA-VDR were expressed in HEK293T cells, immunoprecipitated, and analyzed by immunoblotting. **(G)** Wild-type or mutant VDR (DBD or LBD) and Flag-NLRP3 were expressed in HEK293T cells, immunoprecipitated, and analyzed by immunoblotting.

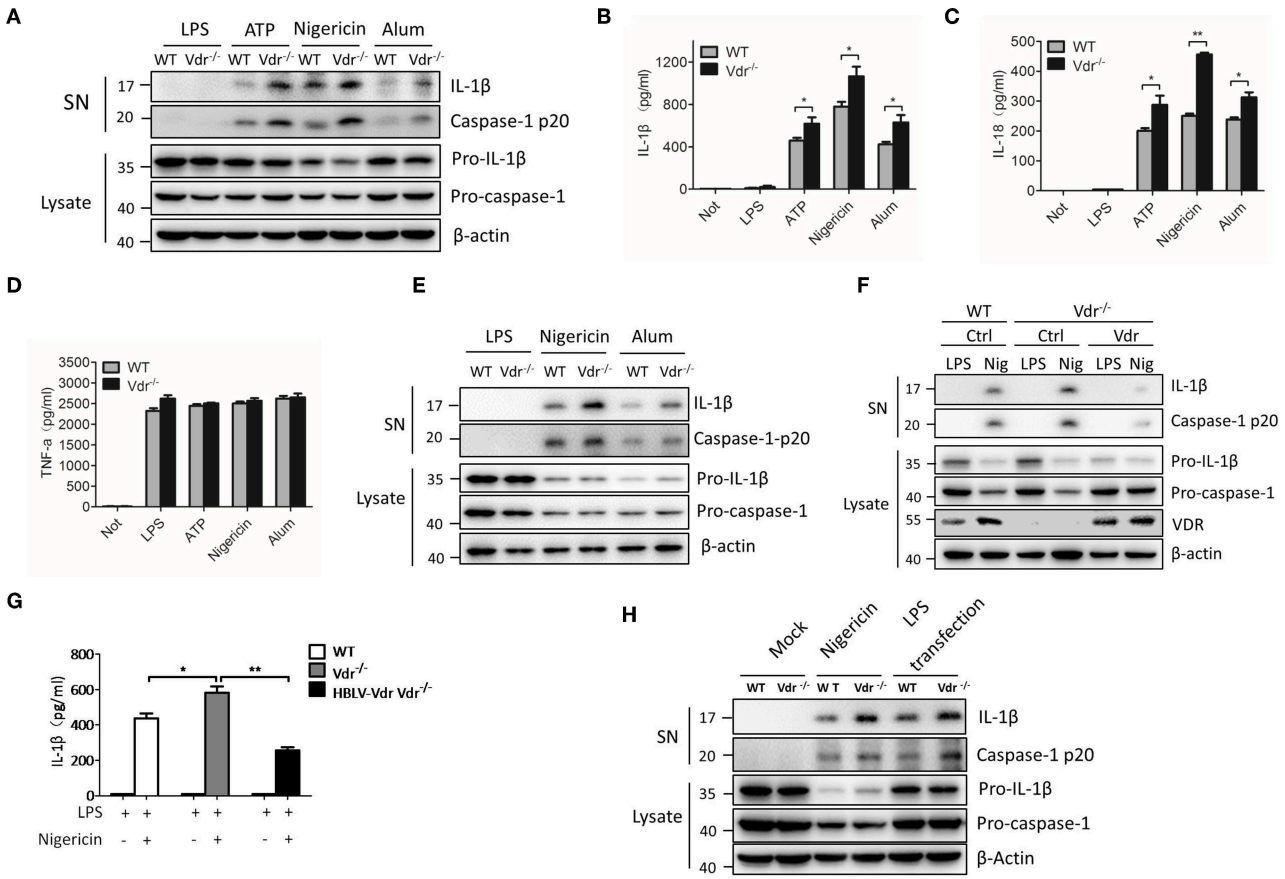

FIGURE 2 | VDR inhibits NLRP3 inflammasome activation. **(A)** Immunoblot analysis of IL-1β and cleaved caspase-1 (p20) in culture supernatants (SN) of LPS-primed BMDMs (wild-type and Vdr$^{-/-}$) treated for 4 h and then stimulated with ATP, nigericin, or alum. Immunoblot analysis of NLRP3, ASC, pro-IL-1β and pro-caspase-1 in cell lysates (Lysate). **(B–D)** IL-1β **(B)**, IL-18 **(C)**, and TNF-α **(D)** ELISAs using supernatants from LPS-primed BMDMs (wild-type and Vdr$^{-/-}$) treated for 4 h and then stimulated with ATP, nigericin, or alum. **(E)** Immunoblot analysis of IL-1β and cleaved caspase-1 (p20) in culture supernatants (SN) of LPS-primed PMs (wild-type and Vdr$^{-/-}$) treated for 4 h and then stimulated with nigericin or alum. Immunoblot analysis of pro-IL-1β and pro-caspase-1 in cell lysates (Lysate). **(F,G)** Immunoblot analysis of IL-1β and cleaved caspase-1 (p20) in culture supernatants (SN) of LPS-primed BMDMs **(F)**. Supernatants were analyzed by IL-1β ELISA **(G)**. **(H)** Pam3CSK4-primed BMDMs (wild-type and Vdr$^{-/-}$) were stimulated by LPS transfection. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblotting. Data are presented as the mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data in **(B–D,G)** are representative of three independent experiments.

together, these data suggested that the VDR inhibits NLRP3 inflammasome activation.

VDR Blocks NLRP3-ASC Speck Formation

NLRP3 activators can induce the rapid formation of large intracellular ASC aggregates called ASC specks (20). In Vdr$^{-/-}$ BMDMs, there was increased formation of ASC specks in the cytosol (**Figures 3A,B**). High-molecular-weight multiprotein complexes are assembled in activated inflammasomes (21), so we resolved cell lysates from WT and Vdr$^{-/-}$ BMDMs by native polyacrylamide gel electrophoresis. In the stimulation time course experiment, more ASC oligomeric complexes were induced in Vdr$^{-/-}$ BMDMs than in control BMDMs (**Figure 3C**), indicating that VDR is involved in the process of NLRP3 inflammasome assembly.

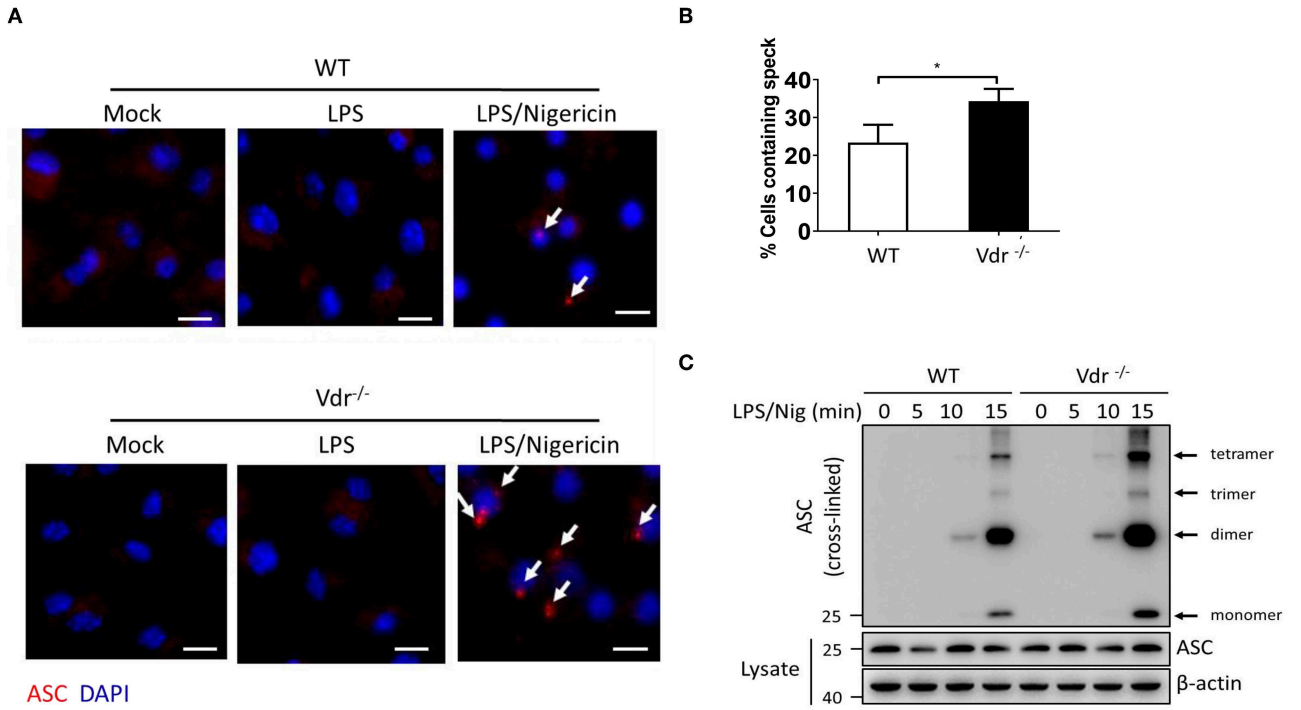

FIGURE 3 | Vitamin D receptor blocks NLRP3 oligomerization and ASC speck formation. **(A,B)** Representative immunofluorescence images and quantification of endogenous ASC specks (arrows). The data show representative results from three combined independent experiments. Scale bar, 10 μm. **(C)** ASC oligomerization induced by the indicated stimuli at 0, 5, 10, and 15 min in WT and $Vdr^{-/-}$ macrophages primed with LPS. Data are presented as the mean ± SEM; $^{*}p < 0.05$. Data in panel B is representative of three independent experiments.

VDR Interferes With the Association Between NLRP3 and BRCC3

NLRP3 ubiquitination is a key inhibitor of NLRP3 inflammasome activation (10). In LPS-treated $Vdr^{-/-}$ BMDMs, the ubiquitinated NLRP3 was decreased (**Figure 4A**), suggesting that VDR might be involved in the NLRP3 ubiquitination. Meanwhile, we found that VDR had no effect on the mRNA expressions of NLRP3-related deubiquitinase and ubiquitinase (**Figures S3A–E**), such as BRCC3, March7, Fbxl2, Trim31, and Pellino2 (22). BRCC3 is a deubiquitinating enzyme that critically deubiquitinates NLRP3 for NLRP3 inflammasome activation. To test whether VDR affects the association between NLRP3 and BRCC3, we analyzed this association in the presence of VDR. The results showed that VDR attenuated the binding of BRCC3 to NLRP3 (**Figures 4B,C**). Similarly, VDR-LBD also attenuated the interaction between BRCC3 and NLRP3, since this VDR domain was required for binding to NLRP3 (**Figure 4D**). To confirm the important role of the NLRP3–BRCC3 association in the VDR-mediated inhibition of NLRP3 inflammasome activation, we knocked down BRCC3 with siRNA and found that the increased caspase-1 cleavage and IL-1β secretion in $Vdr^{-/-}$ BMDMs were eliminated (**Figures 4E,F**). NEK7 and PP2A interact with NLRP3 (23, 24). We found that VDR overexpression had no effect on the association of NEK7 or PP2A with NLRP3 (**Figures S4A,B**). Therefore, VDR affects the NLRP3 inflammasome by specifically blocking the association of NLRP3 with BRCC3. Therefore, we conclude that VDR interferes with the association between NLRP3 and BRCC3.

VDR Inhibits NLRP3 Deubiquitination Mediated by BRCC3

To clarify that NLRP3 ubiquitination is regulated by VDR, we examined the effect of VDR on the BRCC3-mediated deubiquitination of NLRP3. Ubiquitin overexpression triggered the appearance of high apparent molecular weight NLRP3; however, the ubiquitination of Flag-NLRP3 was reduced upon BRCC3 addition (**Figure 5A**), which is consistent with the published report that BRCC3 promotes the deubiquitination of NLRP3. VDR overexpression recovered the level of NLRP3 ubiquitination, suggesting that the BRCC3-mediated deubiquitination of NLRP3 is inhibited by VDR (**Figure 5A**). We further examined the effects of VDR on the ubiquitination of different domains of NLRP3. Individual truncation mutants of NLRP3 (PYD, NACHT, or LRR) were overexpressed with ubiquitin in 293T cells. Only the ubiquitination of the LRR domain was downregulated by BRCC3 (**Figure 5C**); it was reported previously that BRCC3 mediates the deubiquitination of the LRR domain of NLRP3 (11). Consistently, VDR overexpression increased only LRR ubiquitination, not PYD or NACHT ubiquitination, demonstrating the key role of VDR in the BRCC3-mediated deubiquitination of NLRP3

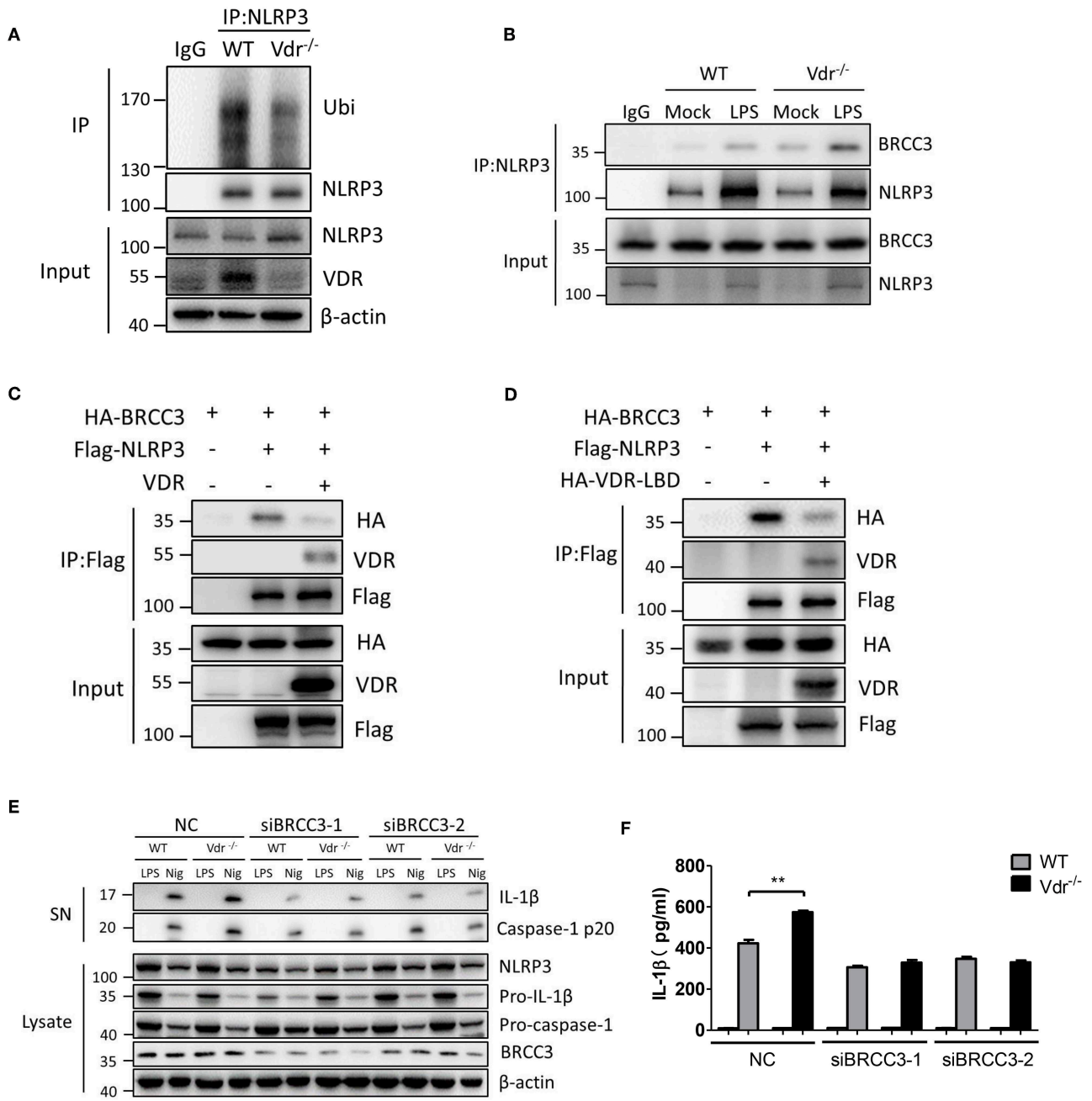

FIGURE 4 | Vitamin D receptor interferes with the BRCC3–NLRP3 interaction. **(A)** Both WT and Vdr$^{-/-}$ BMDMs were treated with LPS for 4 h. NLRP3 ubiquitination was analyzed. **(B)** Immunoblot analysis of BRCC3 protein in mock or LPS-primed WT and Vdr$^{-/-}$ BMDMs lysates immunoprecipitated with the anti-NLRP3 antibody. **(C,D)** HEK293T cells were transfected with the indicated vectors. Samples were immunoprecipitated with the anti-Flag antibody and analyzed by immunoblotting. **(E)** LPS-primed BMDMs (wild-type and Vdr$^{-/-}$) transfected with the indicated non-targeting or BRCC3-specific siRNA were unstimulated or stimulated with nigericin for 30 min. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblotting. IL-1β ELISA **(F)**. Data are presented as the mean ± SEM; $^{**}p < 0.01$. Data in **(F)** is representative of three independent experiments.

(**Figure 5C**). In general, ubiquitination promotes protein degradation. Here, VDR deficiency had no effect on NLRP3 degradation in LPS-primed BMDMs (**Figure 5B**), suggesting that VDR-mediated inhibition of NLRP3 deubiquitination does not affect K48 ubiquitin chains. Consistent with this possibility, VDR overexpression markedly induced the modification of NLRP3 with K63 ubiquitin chains, not K48 ubiquitin chains (**Figure 5D**). Taken together, the data showed that VDR can inhibit BRCC3-mediated NLRP3 deubiquitination.

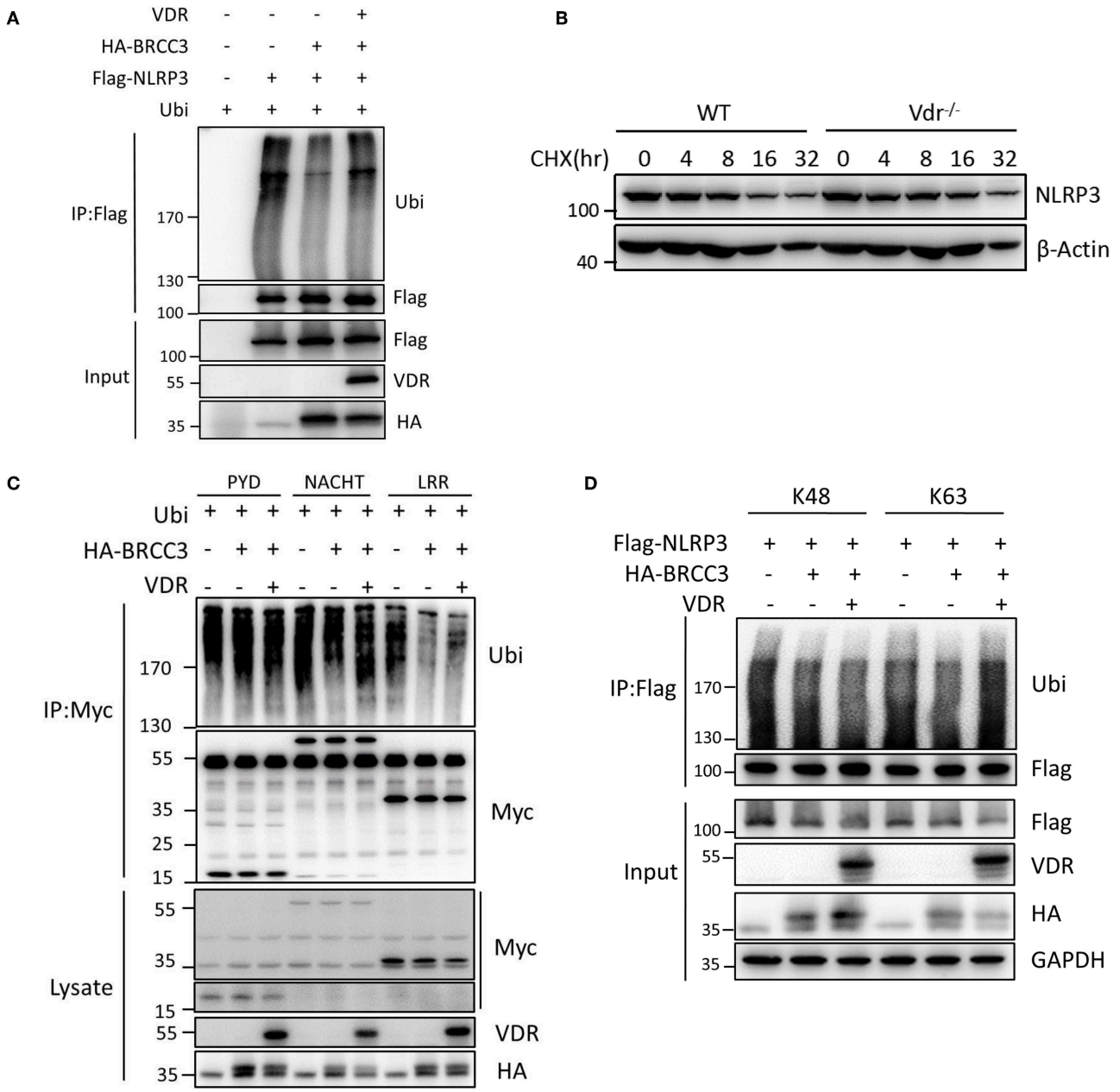

FIGURE 5 | Vitamin D receptor inhibits NLRP3 deubiquitination. **(A)** HEK293T cells were transfected with the indicated vectors. NLRP3 ubiquitination was analyzed. **(B)** Immunoblot analysis of NLRP3 after treatment of LPS-primed BMDMs (wild-type and $Vdr^{-/-}$) with CHX (1 μM) for the indicated time. **(C)** HEK293T cells expressing HA-BRCC3 and Myc-NLRP3 PYD, NACHT, or LRR were co-transfected with VDR as indicated. Myc-NLRP3 immunoprecipitants were analyzed for ubiquitination. **(D)** HEK293T cells expressing Flag-NLRP3, HA-BRCC3, and HA-Ubiquitin K63 or K48 were collected, and the cell lysates were immunoprecipitated with the anti-Flag antibody to detect ubiquitination.

VDR Deficiency Promotes NLRP3-Mediated Inflammation *in vivo*

To address the cross-talk between VDR and NLRP3 *in vivo*, we next induced sepsis in $Vdr^{-/-}$, $Nlrp3^{-/-}$, and $Vdr^{-/-}/Nlrp3^{-/-}$ mice by intraperitoneal injection of LPS (8 mg/kg). With this dose of LPS, there was no significant difference in survival between $Nlrp3^{-/-}$ mice and WT. However, there was a marked reduction in survival in $Vdr^{-/-}$ mice at 36 h (20%, compared to 90% in WT, p-value = 0.0019) that was partly rescued by Nlrp3 deficiency, as $Vdr^{-/-}/Nlrp3^{-/-}$ mice showed 70% survival at the same time point (**Figure 6A**). Our findings suggest that the role of VDR signaling in

sepsis is largely dependent on NLRP3-induced inflammation. Moreover, we found that serum IL-1β and IL-18 levels were significantly increased in $Vdr^{-/-}$ mice but not in $NLRP3^{-/-}$ or $Vdr^{-/-}NLRP3^{-/-}$ mice (**Figures 6B,C**). As a control, serum TNF-α production showed no significant difference among groups (**Figure 6D**). We next addressed the negative role of VDR in NLRP3 inflammasome activation using an alum-induced peritonitis model. Peritoneal neutrophils in lavage samples are an indicator of NLRP3-induced inflammation in response to the intraperitoneal injection of alum, so we examined the recruitment of peritoneal neutrophils. $CD11b^{+}$ $Ly6G^{+}$ cells (neutrophils) were increased in $Vdr^{-/-}$ mice but decreased in $NLRP3^{-/-}$ and $Vdr^{-/-}NLRP3^{-/-}$ mice compared to WT mice (**Figures 6E,F**). These results demonstrated that VDR inhibits NLRP3-induced inflammation *in vivo*.

Vitamin D Enhances the VDR-Mediated Inhibition of NLRP3 Inflammasome Activation

Vitamin D is a VDR ligand, and its active metabolite is 1,25(OH)2D3. In general, vitamin D binds to VDR to activate VDR signaling. When BMDMs were treated with 1,25(OH)2D3, NLRP3 inflammasome activation by LPS and nigericin was gradually inhibited, and IL-1β and caspase-1 cleavage decreased in a dose-dependent manner (**Figures 7A–C**). TNF-α production was not affected by 1,25(OH)2D3 (**Figure 7D**). In human THP-1-derived macrophages, we reconfirmed that vitamin D significantly attenuated NLRP3 inflammasome activation (**Figure 7E**). However, 1,25(OH)2D3 treatment had no effect on NLRP3 inflammasome activation or ASC oligomeric complex formation in $Vdr^{-/-}$ BMDMs (**Figures 7F,G**), suggesting that vitamin D is dependent on VDR to regulate NLRP3 inflammasome activation. Furthermore, 1,25(OH)2D3 dramatically inhibited ASC oligomeric complex formation (**Figure 7G**) and increased the ubiquitination of NLRP3 in LPS-primed BMDMs (**Figure 7H**). These results suggest that 1,25(OH)2D3 enhances the VDR-mediated inhibition of NLRP3 inflammasome activation.

DISCUSSION

The NLRP3 inflammasome is activated by numerous PAMPs and DAMPs and plays a key role in host defense (25–28). NLRP3 inflammasome activation can be regulated at the post-translational and transcriptional levels by several molecules, such as bile acids, dopamine, nitric oxide, type I IFNs, SHP, and A20 (29–34). Here, we report a novel role for VDR as a negative regulator of NLRP3 inflammasome activation in macrophages. Our data demonstrate that VDR directly interacts with NLRP3, inhibits NLRP3 inflammasome assembly, and decreases the NLRP3-mediated secretion of IL-1β and IL-18, suggesting that VDR might be a potential anti-inflammatory factor.

Recently, a two-step model of NLRP3 activation has been well documented, which is triggered by two sequential signals. The first step, NLRP3 needs to be "primed" by Toll-like receptor (TLR) agonists such as LPS. Activation of TLR signaling not only transcriptionally upregulates NLRP3 expression, but also post-transcriptionally activates NLRP3 by phosphorylation and deubiquitination. The second step, defined as "activation," can be induced by several potent stimuli such as nigericin, leading to the oligomerization of NLRP3, and the subsequent assembly of inflammasome (35, 36). The post-translational modification of NLRP3 is essential for inflammasome assembly and activation. Studies demonstrate that NLRP3 is both K48 and K63 ubiquitinated, suggesting that regulation may be more complex (11, 30, 37, 38). Increasing evidence has shown that BRCC3-mediated NLRP3 deubiquitination is critical for inflammasome activation (10, 11). In this study, we demonstrate that BRCC3–NLRP3 complex formation is interrupted by VDR via competition with BRCC3 for NLRP3 binding. The association of NEK or PP2A with NLRP3 is not affected by VDR, so VDR has a special role in regulating BRCC3-mediated NLRP3 activation. BRCC3 promotes inflammasome activation by deubiquitinating NLRP3. Notably, the decreases in IL-1β secretion and NLRP3 ubiquitination induced by VDR deficiency were almost completely eliminated in BRCC3 knockdown cells, which suggests that VDR-mediated inhibition of NLRP3 activation is mainly due to disruption of BRCC3–NLRP3 complex formation. Thus, our findings provide a novel mechanism of regulating NLRP3 activation by controlling BRCC3-mediated deubiquitination.

Notably, several nuclear receptors have been reported to selectively or cooperatively regulate NLRP3 inflammasome activation, including transmembrane G protein coupled receptor-5 (TGR5), farnesoid X receptor (FXR), and the orphan nuclear receptor SHP (30, 32, 39). SHP inhibits NLRP3 inflammasome assembly and NLRP3-dependent IL-1β maturation in the presence of a variety of inflammasome-activating stimuli. TGR5 signaling prevents metabolic disorder by inhibiting NLRP3 inflammasome in a manner dependent on cAMP-PKA. Consistently, VDR is another nuclear receptor that has been shown to be a post-translational regulator of NLRP3 inflammatory responses. Collectively, all these nuclear receptors function as an adaptor associated with the NLRP3 complex. These data suggest the possibility that nuclear receptors might respond to cellular stress in a transcription-independent manner. VDR, an endogenous regulator of NLRP3, mitigates inflammasome-related inflammation *in vivo*, such as LPS-induced systemic inflammation and alum-induced peritoneal inflammation.

Vitamin D initiates biological responses by binding to VDR (40). In this study, we also found that vitamin D blocked NLRP3 deubiquitination and activation dependent on VDR (**Figure 7F**). Vitamin D/VDR system enhances intestinal transcellular transport of calcium and high levels of calcium stimulate inflammasome activation (41, 42). But VDR-deficient mice are hypocalcemic, and the inflammasome activation is increased in VDR-deficient mice, suggesting that VDR inhibits the inflammasome activation in a calcium-independent manner. Collectively, we demonstrate that vitamin D can inhibit NLRP3 inflammasome activation via VDR signaling, suggesting that

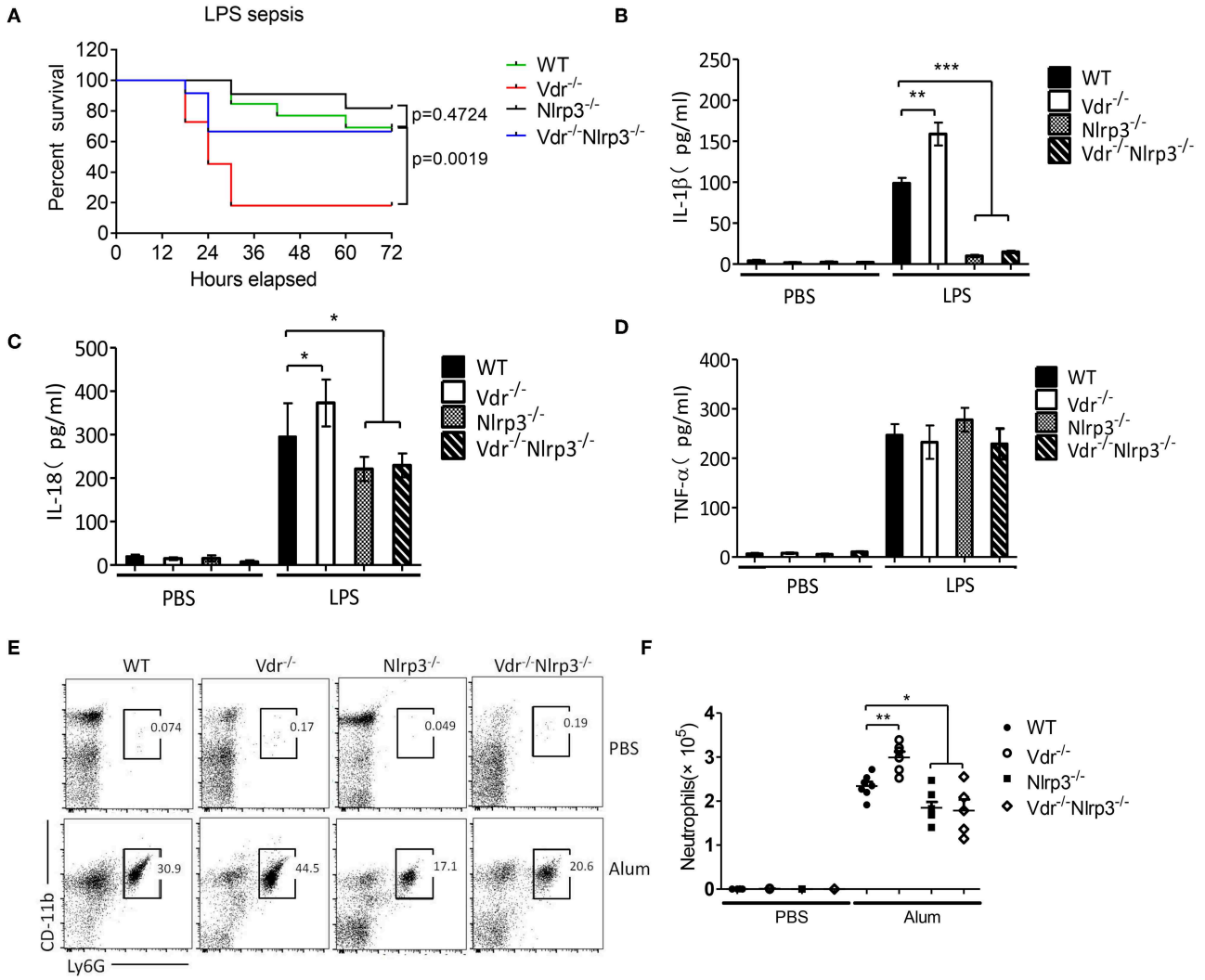

FIGURE 6 | Vitamin D receptor deficiency promotes LPS-induced systemic inflammation and Alum-induced peritoneal inflammation via suppression of the NLRP3 inflammasome. **(A)** Survival of $VDR^{+/+}NLRP3^{+/+}$, $VDR^{-/-}NLRP3^{+/+}$, $VDR^{+/+}NLRP3^{-/-}$, and $VDR^{-/-}NLRP3^{-/-}$ mice ($n = 10$ mice/group) intraperitoneally injected with LPS (8 mg/kg body weight) over a period of 72 h. **(B–D)** Serum IL-1β, IL-18, and TNFα ELISAs at 6 h after intraperitoneal injection of LPS (8 mg/kg body weight) into $VDR^{+/+}NLRP3^{+/+}$, $VDR^{-/-}NLRP3^{+/+}$, $VDR^{+/+}NLRP3^{-/-}$, and $VDR^{-/-}NLRP3^{-/-}$ mice. **(E,F)** Representative FACS plots of peritoneal CD11b+ Ly6G+ cells (neutrophils) from $VDR^{+/+}NLRP3^{+/+}$, $VDR^{-/-}NLRP3^{+/+}$, $VDR^{+/+}NLRP3^{-/-}$, and $VDR^{-/-}NLRP3^{-/-}$ mice at 12 h after an intraperitoneal injection with alum ($n = 5$ mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS $p > 0.05$. Values are the mean ± SEM of five mice per group. Both male and female mice were randomly assigned. Data in **(B–D,F)** are representative of three independent experiments.

vitamin D treatment is a possible strategy for the treatment of NLRP3-related inflammatory diseases.

METHODS

Mice

Vdr$^{-/-}$ were obtained from The Jackson Laboratory. *Nlrp3*$^{-/-i}$ mice were a kind gift from Dr. Vishva M. Dixit (Genentech). Mice were housed in a specific pathogen-free environment in the Animal Core Facility of Nanjing Medical University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Mice were used at 6–8 weeks of age.

Reagents

LPS (O111:B4, L2630), ATP (A7699), disuccinimidyl suberate (S1885), nigericin (N7143), and phorbol myristate acetate (PMA, P1585) were from Sigma-Aldrich; alum (77161) and the c-Myc tag (MA1-980) were from Thermo Fisher Scientific; and poly(dA:dT) was from InvivoGen. The following antibodies were used: anti-Flag (F1804) (Sigma); anti-mouse caspase-1 p20 (AG-20B-0042) and anti-mouse NLRP3 (AG-20B-0014) (AdipoGen); anti-human caspase-1 (ab108362) and anti-BRCC36 (ab108411) (Abcam); anti-mouse IL-1β (AF-401-NA) (R&D); anti-HA-tag (3724) (Cell Signaling Technology); and anti-VDR (sc-13133), anti-ASC (sc-514414), and anti-β-actin (sc-47778) (Santa Cruz). The Mouse TNF ELISA Kit (558534), Mouse IL-1β ELISA Kit

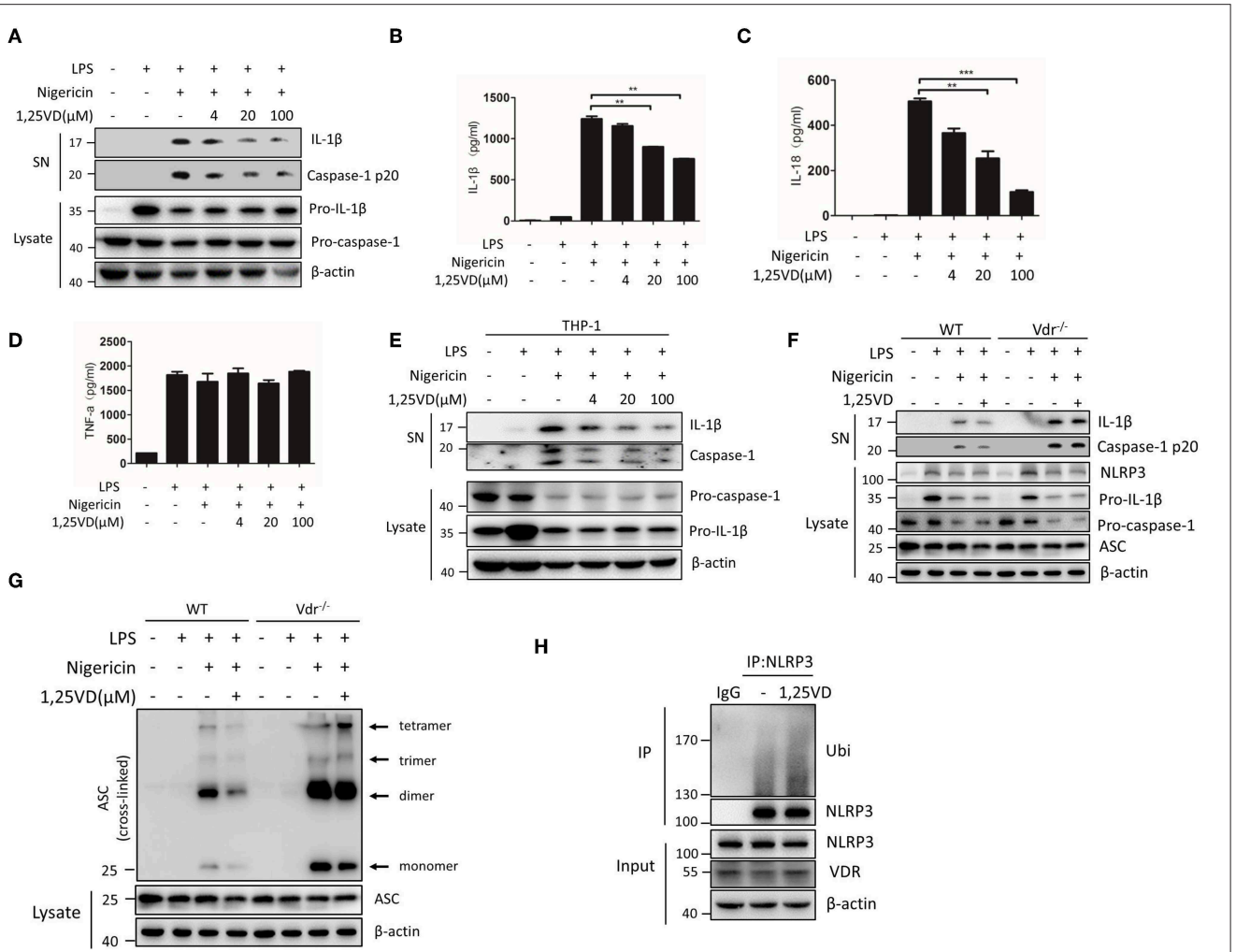

FIGURE 7 | Vitamin D enhances VDR-mediated inhibition of NLRP3 inflammasome activation. **(A)** Immunoblot analysis of IL-1β and cleaved caspase-1 (p20) in culture supernatants (SN) of LPS-primed BMDMs treated for 3 h with various doses (upper lanes) of 1,25 VD and then stimulated with nigericin. Immunoblot analysis of NLRP3, ASC, pro-IL-1β and pro-caspase-1 in cell lysates (Lysate). **(B–D)** IL-1β **(B)**, IL-18 **(C)**, and TNF-α **(D)** in supernatants. **(E)** LPS-primed BMDMs (wild type and $Vdr^{-/-}$) were treated with 1,25 vitamin D (1,25 VD, 20 μM) and then stimulated with nigericin. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblotting. **(F)** LPS-primed THP-1 cells were treated with different doses of 1,25 vitamin D (1,25VD) as indicated and then stimulated with nigericin. Supernatants (SN) and cell extracts (Lysate) were analyzed by immunoblotting. **(G)** The induction of ASC oligomerization by the indicated stimuli in WT and $Vdr^{-/-}$ LPS-primed macrophages. Nigericin stimulation lasted 15 min. **(H)** LPS-primed BMDMs were treated with or without 20 μM 1,25VD for 3 h. NLRP3 ubiquitination was analyzed. Data are presented as the mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data in **(B–D)** are representative of three independent experiments.

(559603), and TMB Substrate Reagent Set (555214) were from BD Biosciences. The mouse IL-18 ELISA set (EMC06) was from ExCell Biotech (**Table 1**).

Cell Culture

Primary BMDMs were generated as described previously (43). The mouse tibia and femur were isolated and flushed with cold PBS through a 25-G needle in a sterile environment. Cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 10% (v/v) conditioned medium from L929 mouse fibroblasts for 6 days. The medium was replaced every 2 days. Peritoneal macrophages were harvested by injecting 5 ml of sterile PBS into the peritoneal cavity. Then, the cells were centrifuged at 1,000×g for 5 min, resuspended in RPMI containing 10% (v/v) fetal bovine serum, and cultured at 37°C. For the inflammasome activation assay, 1×10^6 cells were plated in 12-well plates overnight. Then, the cells were primed with LPS (200 ng/ml) for 4 h and then stimulated with PBS (mock), 10 mM ATP (30 min), 10 μM nigericin (1 h), alum (250 μg/ml, 6 h). For non-canonical inflammasome activation, cells were primed with 100 ng/ml Pam3CSK4 (InvivoGen) for 4 h, after which 2 μg/ml LPS was added with Lipofectamine 2000 (Invitrogen) for 16 h (30). Cell lysates and supernatants were analyzed by Western blot analysis.

Plasmid Construction and Transfection

The PCMV-HA-VDR plasmid was reported previously (44). Flag-NLRP3, HA-ASC, HA-ubiquitin, and Myc-pp2a were provided by Professor Paul N. Moynagh (National University of Ireland, Maynooth, Ireland). Recombinant vectors encoding

TABLE 1 | Reagents.

Reagent or resource	Source	Identifier
Antibodies		
IL-1β	R&D Systems	Cat# AF-401-NA
Caspase-1(mouse)	Adipogen	Cat# AG-20B-0044
NLRP3	Adipogen	Cat# AG-20B-0014
Caspase-1(human)	Abcam	Cat# ab108362
BRCC36	Abcam	Cat# ab108411
HA	Cell Signaling Technology	Cat#3724
VDR	Santa Cruz	Cat# sc-13133
ASC	Santa Cruz	Cat# sc-514414
β-actin	Santa Cruz	Cat# sc-47778
Flag	Sigma	Cat# F1804
c-Myc	Thermo Fisher Scientific	Cat# MA1-980
Chemicals, peptides, and recombinant proteins		
ATP	Sigma	Cat# A7699
LPS	Sigma	Cat# L2630
Alum	Thermo Fisher Scientific	Cat#77161
Nigericin	Sigma	Cat# N7143
PMA	Sigma	Cat# P1585
Pam3CSK4	Invivogen	
Critical Commercial Assays		
TNF ELISA kit	BD Biosciences	Cat#558534
Mouse IL-1β ELISA kit	BD Biosciences	Cat#559603
TMB Substrate Reagent Set	BD Biosciences	Cat#555214
Mouse IL-18 ELISA kit	ExCell Biotech	EMC06
Experimental models: cell lines		
HEK293T cells	ATCC	CRL-11268
THP1 cells	ATCC	TIB-202
Experimental models: organisms/strains		
Mouse: Nlrp3-/-	Dr. Vishva M. Dixit labs(Genetech)	N/A
Mouse: Vdr-/-	Jackson Laboratory	JAX:017969
Oligonucleotides		
MouseBRCC3siRNA1(sense) (Sequence: GGCAGAAAGGUUGGCUGAATT)	This paper	N/A
MouseBRCC3siRNA1(antisense) (Sequence: UUCAGCCAACCUUUCUGCCTT)	This paper	N/A
MouseBRCC3siRNA2(sense) (Sequence: GGAAGAACAGGAUGCAUAUTT)	This paper	N/A
MouseBRCC3siRNA2(sense) (Sequence: AUAUGCAUCCUGUUCUUCCTT)	This paper	N/A
Recombinant DNA		
PCMV-HA-VDR	(44)	N/A
Flag-NLRP3	Professor Paul N. Moynagh (National University of Ireland Maynooth, Ireland)	N/A
HA-ASC	Professor Paul N. Moynagh (National University of Ireland Maynooth, Ireland)	N/A
HA-ubiquitin	Professor Paul N. Moynagh (National University of Ireland Maynooth, Ireland)	N/A
Myc-pp2a	Professor Paul N. Moynagh (National University of Ireland Maynooth, Ireland)	N/A
pCMV-HA-BRCC3	This paper	N/A
pCMV-HA-NEK7	This paper	N/A
Pgex6p-1-GST-VDR	This paper	N/A
Pet28a-His-NLRP3	This paper	N/A

human NEK7 and human BRCC3 were constructed by PCR-based amplification of complementary DNA from THP-1 cells and then cloned into the pCMV-HA eukaryotic expression vector. pGEX6p-1-GST-VDR, pET28a-His-NLRP3, and truncated mutants of NLRP3 and VDR were generated by the Original TA Cloning Kit (Vazyme Biotech). After plasmid construction, the mutation in the gene encoding VDR was introduced into each expression vector by the Fast Mutagenesis Kit V2 (Vazyme Biotech). All plasmid constructs were confirmed by DNA sequencing. Plasmids were transiently transfected into HEK293T cells with PolyJet reagents (SignaGen) according to the manufacturer's instructions.

Mass Spectrometry Analysis of NLRP3-Associated Proteins

Empty Flag constructs or Flag-NLRP3 were transfected into HEK293T cells for 24 h, and the cells were collected and resuspended in lysis buffer [50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, and 100 mM DTT (dithiothreitol)]. Extracts were immunoprecipitated with anti-Flag antibody and Protein A/G-Agarose beads and then dissolved in sample buffer. IP-enriched protein complexes were separated by SDS-PAGE, visualized by Coomassie blue staining, and then excised for in-gel digestion with trypsin. The peptides were extracted from gel bands and subjected to LC-MS/MS analysis. Tryptic peptides were separated on a C18 column and analyzed by an LTQ Orbitrap Velos mass spectrometer (Thermo). The resulting MS/MS data were processed using ProteinPilot^{TM} software 4.5 (AB Sciex). The original MS/MS data were submitted to ProteinPilot (version 4.5, AB Sciex) for data analysis and searched against *Homo sapiens* in the UniProt database (http://www.uniprot.org/proteomes/UP000005640).

ELISA

The concentrations of mouse IL-1β and mouse TNF-α were measured using ELISA kits (BD Systems), and the concentration of mouse IL-18 was measured using ExCell Biotech kits according to the manufacturer's instructions.

ASC Oligomerization Assay

Cells were plated on 12-well plates and stimulated as indicated. Cells were washed three times with PBS and lysed in PBS containing 0.5% Triton X-100 for 30 min at 4°C. The cell lysates were centrifuged at 8000×*g* for 15 min at 4°C (24). Triton X-100-insoluble pellets were washed twice with PBS and suspended in 200 μl of PBS. The pellets were then cross-linked at room temperature for 30 min by adding fresh disuccinimidyl suberate (2 mM). The cross-linked pellets were centrifuged at 8000×*g* for 15 min and lysed in 2× sample buffer for Western blot analysis of ASC oligomers.

RNA Quantitation

Total RNA was extracted from BMDMs using TRIzol reagent (Life Technologies). cDNA was generated from total extracted RNA using HiScript II Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol. Quantitative PCR was performed with ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd) using a Real-Time PCR System (StepOne, Applied Biosystems). The sequences of the PCR primers are listed in **Table 2**. Data were normalized to β-actin expression in each sample.

Immunoprecipitation and Immunoblot Analysis

For whole-cell lysate analysis, cells were lysed in SDS lysis buffer [50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, and 100 mM DTT]. For co-immunoprecipitation, cells were treated as indicated and then collected in 500 μl of RIPA Lysis Buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate], followed by incubation at 4°C for 30 min. After centrifugation for 12 min at 12,000×*g*, the supernatants were collected and incubated with the appropriate antibody overnight. The immunocomplexes were captured by the addition of 30 μl of Protein A/G-Agarose slurry and gentle rotation for 90 min at 4°C. The agarose beads were collected by centrifugation for 3 min at 100 rpm. The supernatant was discarded, and the beads were washed three times with 800 μl of ice-cold RIPA buffer and twice with PBS. The agarose beads were resuspended in

TABLE 2 | Primers.

Nlrp3	Forward 5′-TGGATGGGTTTGCTGGGAT-3′ Reverse 5′-CTGCGTGTAGCGACTGTTGAG-3′
Trim31	Forward 5′-CCAGAGTCAAACCGTGAGCG–3′ Reverse 5′- GGCAACTTGGAGCCCGAA-3′
March7	Forward 5′-GACAGTACCAAGTTCTAGGGACT-3′ Reverse 5′-AGTTGTACGCCTACCTTCATTG-3′
Fbxl2	Forward 5′-CAGTGATGATGGCCTTATCAACA-3′ Reverse 5′-TGGAAGTTAAAAAGATCCACCCG-3′
Pellino2	Forward 5′-ACCAACGGTGTCCTGGTGATG-3′ Reverse 5′-CCTGGTCTCTCGCAAGGTGTA-3′
Brcc3	Forward 5′-GTGCAGGCGGTTCATCTTGA-3′ Reverse 5′-AACTCCCCTATACACAGACCC-3′
β-actin	Forward 5′-TGTTACCAACTGGGACGACA-3′ Reverse 5′-CTGGGTCATCTTTTCACGGT-3′

50 μl of SDS loading buffer and mixed gently. The samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and analyzed by immunoblot. Immunoreactivity was visualized by the Tanon Imaging System. For the NLRP3 ubiquitination assay, cells were collected in 300 μl of RIPA buffer. After centrifugation for 10 min, the supernatants were treated with 1% (w/v) SDS, heated to 95°C for 5 min to dissociate NLRP3 from any associated proteins, and then diluted 10-fold in RIPA buffer before immunoprecipitation.

Immunofluorescence Staining and Confocal Analysis

For ASC speck analysis, BMDMs were plated on coverslips. The cells were fixed for 20 min with 4% paraformaldehyde and then permeabilized with 0.2% NP-40/PBS for 10 min. The cells were incubated with anti-rabbit ASC antibody (1:200) overnight, followed by incubation with anti-rabbit Cy3-conjugated AffiniPure (Jackson ImmunoResearch). Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole hydrochloride; Sigma-Aldrich). HEK293T cells transiently transfected with plasmids encoding Flag-NLRP3, HA-ASC, and GFP-VDR were cultured for 24 h. The cells were incubated overnight with anti-Flag and anti-HA antibodies (1:100) and then incubated with fluorescent secondary antibody. The cells were examined with confocal laser microscopy (LSM710, Carl Zeiss, Germany).

GST Pull-Down Assay

sBL21(DE3) cells were transformed with pGEX-6P1 bacterial expression plasmids encoding GST or GST-human VDR. Recombinant protein expression was induced by 0.1 mM IPTG for 8 h at 20°C. Human NLRP3 was cloned into pET28a bacterial expression plasmids. Recombinant protein expression was induced by 0.1 mM IPTG for 8 h at 26°C. Bacteria were pelleted and lysed with PBS containing 1 mM PMSF, 1 mM DTT, and 1% Triton X-100. Recombinant His-NLRP3 protein was incubated on a rotator at 4°C overnight with GST and GST-VDR. GST proteins were pulled down using Glutathione MagBeads (GenScript). Beads were washed three times with pull-down buffer and twice with PBS before being analyzed by immunoblot.

Retroviral Rescue Assay

Murine Vdr was sub-cloned into the pHBLV-CMV-MCS-3XFlag-GFP-PURO lentivirus vector. Lentivirus-containing medium was obtained from Hanbio. On the first day, WT and Vdr-deficient BMDMs were plated in 12-well plates. On the second day, lentivirus containing the Vdr construct or empty vector was incubated with BMDMs (MOI = 50) at 37°C for 4 h. After 4 h, the medium was replaced with fresh medium, and the cells were incubated for another 48 h. The lysates were analyzed by immunoblot.

siRNA

siRNAs were ordered from GenePharma. The siRNA sequences were as follows: BRCC3-1 (sense), GGCAGAAAGGUUGGC UGAATT; BRCC3-1 (antisense), UUCAGCCAACCUUUC UGCCTT; BRCC3-2 (sense), GGAAGAACAGGAUGCAUA UTT; BRCC3-2 (antisense), AUAUGCAUCCUGUUCUUC CTT; Stealth RNAi negative control (sense), UUCUCCGAA CGUGUCACGUTT; and negative control (antisense), ACG UGACACGUUCGGAGAATT. BMDMs were plated in 12-well plates and transfected with 100 nM siRNA using 5 μl of Lipofectamine RNAiMAX (Invitrogen) in OPTI-MEM. The medium was changed 6 h later, and the cells were cultured for 48 h in RPMI1640 medium supplemented with 10% FBS and 10% L929 medium.

In vivo LPS Challenge

For the survival analysis, C57BL/6J mice aged 6–8 weeks were administered 8 mg/kg LPS by intraperitoneal injection and monitored for 72 h. The moribund state, defined by (1) hypothermia, (2) inability to roll over from side to chest, or (3) dyspnea/labored breathing, was used as humane endpoint. For cytokine analysis, serum samples were collected after 6 h, and cytokines were measured by ELISA.

Intraperitoneal Leukocyte Recruitment

C57BL/6J mice aged 6–8 weeks were intraperitoneally injected with 700 mg of alum and evaluated 12 h later. The peritoneal cavity was washed with 5 ml of PBS. Peritoneal exudate cells (PECs) were analyzed by flow cytometry. The recruitment of neutrophils was visualized with CD11b-PE (M1/70; BD Biosciences) and Ly-6G-APC (1A8; eBioscience). PI (Sigma) was used to exclude dead cells. Neutrophil ($CD11b^+Ly6G^+$) recruitment was analyzed on a CytoFLEX (Beckman Coulter) (45).

Ethics Statement

This study was carried out in accordance with the recommendations of the guidelines of the Animal Care Committee of Nanjing Medical University, Jiangsu, China.

Statistical Analyses

The results are expressed as the mean ± SEM. Statistical analyses were carried out using Student's *t*-test. Data were considered significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

YC and ZR designed the research, analyzed data, and wrote the paper. XC, JW, MX, JZ, BW, and LF provided research reagents and technical assistance. HZ, XW, and SY assisted in data analysis and manuscript preparation. YC was responsible for the overall research design, data analysis, and paper preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02783/full#supplementary-material

REFERENCES

1. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol.* (2016) 16:407–20. doi: 10.1038/nri.2016.58
2. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci.* (2016) 41:1012–21. doi: 10.1016/j.tibs.2016.09.002
3. Baroja-Mazo A, Martin-Sanchez F, Gomez AI, Martinez CM, Amores-Iniesta J, Compan V, et al. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol.* (2014) 15:738–48. doi: 10.1038/ni.2919
4. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell.* (2014) 157:1013–22. doi: 10.1016/j.cell.2014.04.007
5. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, et al. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature.* (2013) 493:674–8. doi: 10.1038/nature11729
6. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature.* (2010) 464:1357–61. doi: 10.1038/nature08938
7. Mortimer L, Moreau F, MacDonald JA, Chadee K. NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations. *Nat Immunol.* (2016) 17:1176–86. doi: 10.1038/ni.3538
8. Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell.* (2015) 160:816–27. doi: 10.1016/j.cell.2015.02.010
9. Rathinam VA, Fitzgerald KA. Inflammasome complexes: emerging mechanisms and effector functions. *Cell.* (2016) 165:792–800. doi: 10.1016/j.cell.2016.03.046
10. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, Alnemri ES. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem.* (2012) 287:36617–22. doi: 10.1074/jbc.M112.407130
11. Py BF, Kim MS, Vakifahmetoglu-Norberg H, Yuan J. Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol Cell.* (2013) 49:331–8. doi: 10.1016/j.molcel.2012.11.009
12. Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids.* (2013) 78:127–36. doi: 10.1016/j.steroids.2012.10.019
13. Sassi F, Tamone C, D'Amelio P. Vitamin D: nutrient, hormone, and immunomodulator. *Nutrients.* (2018) 10:E1656. doi: 10.3390/nu10111656
14. Dimitrov V, White JH. Vitamin D signaling in intestinal innate immunity and homeostasis. *Mol Cell Endocrinol.* (2017) 453:68–78. doi: 10.1016/j.mce.2017.04.010
15. Landel V, Millet P, Baranger K, Loriod B, Feron F. Vitamin D interacts with Esr1 and Igf1 to regulate molecular pathways relevant to Alzheimer's disease. *Mol Neurodegener.* (2016) 11:22. doi: 10.1186/s13024-016-0087-2
16. Sly LM, Lopez M, Nauseef WM, Reiner NE. 1alpha,25-Dihydroxyvitamin D3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. *J Biol Chem.* (2001) 276:35482–93. doi: 10.1074/jbc.M102876200
17. Shin DM, Yuk JM, Lee HM, Lee SH, Son JW, Harding CV, et al. Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cell Microbiol.* (2010) 12:1648–65. doi: 10.1111/j.1462-5822.2010.01497.x
18. Bellastella G, Maiorino MI, Petrizzo M, De Bellis A, Capuano A, Esposito K, et al. Vitamin D and autoimmunity: what happens in autoimmune polyendocrine syndromes? *J Endocrinol Invest.* (2015) 38:629–33. doi: 10.1007/s40618-014-0233-z
19. Vanherwegen AS, Eelen G, Ferreira GB, Ghesquiere B, Cook DP, Nikolic T, et al. Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J Steroid Biochem Mol Biol.* (2018) 187:134–45. doi: 10.1016/j.jsbmb.2018.11.011
20. Bryan NB, Dorfleutner A, Kramer SJ, Yun C, Rojanasakul Y, Stehlik C. Differential splicing of the apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) regulates inflammasomes. *J Inflamm.* (2010) 7:23. doi: 10.1186/1476-9255-7-23
21. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, et al. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol.* (2014) 15:727–37. doi: 10.1038/ni.2913
22. Shim DW, Lee KH. Posttranslational regulation of the NLR family pyrin domain-containing 3 inflammasome. *Front Immunol.* (2018) 9:1054. doi: 10.3389/fimmu.2018.01054
23. Stutz A, Kolbe CC, Stahl R, Horvath GL, Franklin BS, van Ray O, et al. NLRP3 inflammasome assembly is regulated by phosphorylation of the pyrin domain. *J Exp Med.* (2017) 214:1725–36. doi: 10.1084/jem.20160933
24. He Y, Zeng MY, Yang D, Motro B, Nunez G. NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature.* (2016) 530:354–7. doi: 10.1038/nature16959
25. Bullon P, Navarro JM. Inflammasome as a key pathogenic mechanism in endometriosis. *Curr Drug Targets.* (2017) 18:997–1002. doi: 10.2174/1389450117666160709013850
26. Abdul-Sater AA, Said-Sadier N, Ojcius DM, Yilmaz O, Kelly KA. Inflammasomes bridge signaling between pathogen identification and the immune response. *Drugs Today.* (2009) 45(Suppl. B):105–12.
27. Goncalves AC, Ferreira LS, Manente FA, de Faria C, Polesi MC, de Andrade CR, et al. The NLRP3 inflammasome contributes to host protection during Sporothrix schenckii infection. *Immunology.* (2017) 151:154–66. doi: 10.1111/imm.12719
28. Savage CD, Lopez-Castejon G, Denes A, Brough D. NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury. *Front Immunol.* (2012) 3:288. doi: 10.3389/fimmu.2012.00288
29. Voet S, Mc Guire C, Hagemeyer N, Martens A, Schroeder A, Wieghofer P, et al. A20 critically controls microglia activation and inhibits inflammasome-dependent neuroinflammation. *Nat Commun.* (2018) 9:2036. doi: 10.1038/s41467-018-04376-5
30. Guo C, Xie S, Chi Z, Zhang J, Liu Y, Zhang L, et al. Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. *Immunity.* (2016) 45:802–816. doi: 10.1016/j.immuni.2016.09.008
31. Yan Y, Jiang W, Liu L, Wang X, Ding C, Tian Z, et al. Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. *Cell.* (2015) 160:62–73. doi: 10.1016/j.cell.2014.11.047
32. Yang CS, Kim JJ, Kim TS, Lee PY, Kim SY, Lee HM, et al. Small heterodimer partner interacts with NLRP3 and negatively regulates

activation of the NLRP3 inflammasome. *Nat Commun.* (2015) 6:6115. doi: 10.1038/ncomms7115
33. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol.* (2009) 27:519–50. doi: 10.1146/annurev.immunol.021908.132612
34. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Forster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity.* (2011) 34:213–23. doi: 10.1016/j.immuni.2011.02.006
35. Bauernfeind F, Hornung V. Of inflammasomes and pathogens–sensing of microbes by the inflammasome. *EMBO Mol Med.* (2013) 5:814–26. doi: 10.1002/emmm.201201771
36. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol.* (2009) 183:787–91. doi: 10.4049/jimmunol.0901363
37. Song H, Liu B, Huai W, Yu Z, Wang W, Zhao J, et al. The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3. *Nat Commun.* (2016) 7:13727. doi: 10.1038/ncomms13727
38. Humphries F, Bergin R, Jackson R, Delagic N, Wang B, Yang S, et al. The E3 ubiquitin ligase Pellino2 mediates priming of the NLRP3 inflammasome. *Nat Commun.* (2018) 9:1560. doi: 10.1038/s41467-018-03669-z
39. Han CY, Rho HS, Kim A, Kim TH, Jang K, Jun DW, et al. FXR inhibits endoplasmic reticulum stress-induced NLRP3 inflammasome in hepatocytes and ameliorates liver injury. *Cell Rep.* (2018) 24:2985–99. doi: 10.1016/j.celrep.2018.07.068
40. Pike JW, Meyer MB. Fundamentals of vitamin D hormone-regulated gene expression. *J Steroid Biochem Mol Biol.* (2013) 144(Pt A):5–11. doi: 10.1016/j.jsbmb.2013.11.004
41. Hendy GN, Canaff L. Calicium-sensing receptor,proinflammatory cytokines and calcium homestasis. *Semin Cell Dev Biol.* (2016) 49:37–43 doi: 10.1016/j.semcdb.2015.11.006
42. Deng M, Guo H, Tam JW, Johnson BM, Brickey WJ, New JS, et al. Platelet-activating factor (PAF) mediates NLRP3-NEK7 inflammasome induction independently of PAFR. *J Exp Med.* (2019). doi: 10.1084/jem.20190111. [Epub ahead of print].
43. Rao Z, Zhang N, Xu N, Pan Y, Xiao M, Wu J, et al. 1,25-Dihydroxyvitamin D inhibits LPS-Induced High-Mobility Group Box 1 (HMGB1) secretion via Targeting the NF-E2-related factor 2-hemeoxygenase-1-HMGB1 pathway in macrophages. *Front Immunol.* (2017) 8:1308. doi: 10.3389/fimmu.2017.01308
44. Liu W, Chen Y, Golan MA, Annunziata ML, Du J, Dougherty U, et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest.* (2013) 123:3983–96. doi: 10.1172/JCI65842
45. Guo C, Chi Z, Jiang D, Xu T, Yu W, Wang Z, et al. Cholesterol homeostatic regulator SCAP-SREBP2 integrates NLRP3 inflammasome activation and cholesterol biosynthetic signaling in macrophages. *Immunity.* (2018) 49:842–56 e847. doi: 10.1016/j.immuni.2018.08.021

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ORIGINAL RESEARCH
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Polarization of Human Monocyte-Derived Cells With Vitamin D Promotes Control of *Mycobacterium tuberculosis* Infection

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Background: Understanding macrophage behavior is key to decipher *Mycobacterium tuberculosis* (Mtb) pathogenesis. We studied the phenotype and ability of human monocyte-derived cells polarized with active vitamin D [1,25$(OH)_2D_3$] to control intracellular Mtb infection compared with polarization of conventional subsets, classical M1 or alternative M2.

Methods: Human blood-derived monocytes were treated with active vitamin D or different cytokines to obtain 1,25$(OH)_2D_3$-polarized as well as M1- and M2-like cells or fully polarized M1 and M2 subsets. We used an *in vitro* macrophage Mtb infection model to assess both phenotype and functional markers i.e., inhibitory and scavenger receptors, costimulatory molecules, cytokines, chemokines, and effector molecules using flow cytometry and quantitative mRNA analysis. Intracellular uptake of bacilli and Mtb growth was monitored using flow cytometry and colony forming units.

Results: Uninfected M1 subsets typically expressed higher levels of CCR7, TLR2, and CD86, while M2 subsets expressed higher CD163, CD200R, and CD206. Most of the investigated markers were up-regulated in all subsets after Mtb infection, generating a mixed M1/M2 phenotype, while the expression of CD206, HLADR, and CD80 was specifically up-regulated ($P < 0.05$) on 1,25$(OH)_2D_3$-polarized macrophages. Consistent with the pro-inflammatory features of M1 cells, Mtb uptake and intracellular Mtb growth was significantly ($P < 0.01$–0.001 and $P < 0.05$–0.01) lower in the M1 (19.3%) compared with the M2 (82.7%) subsets 4 h post-infection. However, infectivity rapidly and gradually increased in M1 cells at 24–72 h. 1,25$(OH)_2D_3$-polarized monocyte-derived cells was the most potent subset to inhibit Mtb growth at both 4 and 72 h ($P < 0.05$–0.01) post-Mtb infection. This ability was associated with high mRNA levels of pro-inflammatory cytokines and the antimicrobial peptide LL-37 but also anti-inflammatory IL-10, while expression of the immunosuppressive enzyme IDO (indoleamine 2,3-dioxygenase) remained low in Mtb-infected 1,25$(OH)_2D_3$-polarized cells compared with the other subsets.

Conclusions: Mtb infection promoted a mixed M1/M2 macrophage activation, and $1,25(OH)_2D_3$-polarized monocyte-derived cells expressing LL-37 but not IDO, were most effective to control intracellular Mtb growth. Macrophage polarization in the presence of vitamin D may provide the capacity to mount an antimicrobial response against Mtb and simultaneously prevent expression of inhibitory molecules that could accelerate local immunosuppression in the microenvironment of infected tissue.

Keywords: *Mycobacterium tuberculosis*, tuberculosis, macrophages, vitamin D3, immune polarization

INTRODUCTION

As primary reservoirs for intracellular growth and persistence of *Mycobacterium tuberculosis* (Mtb), macrophages play a critical role in the pathogenesis of tuberculosis (TB) disease. Macrophages comprise a heterogeneous population of cells that are involved in the induction of innate as well as adaptive TB immunity and also contribute to tissue remodeling (1). Polarization of macrophage-like cells from monocytes into phenotypically and functionally different cells occur in response to microenvironmental signals (2, 3) that may affect the ability of the cells to control Mtb infection. Although macrophage activation is complex involving a non-linear range of functional states without clear boundaries (4), an operationally useful but simplified concept describes macrophage polarization into classically (M1) and alternatively (M2) activated groups (5, 6). M1 macrophages produce inflammatory cytokines and reactive nitrogen or oxygen species that contribute to host defense, whereas M2 macrophages are poorly microbicidal and instead attenuate inflammation and participate in tissue remodeling (7). M1 macrophages are typically induced by granulocyte macrophage colony-stimulating factor (GM-CSF) together with IFN-γ and a microbial stimuli such as lipopolysaccharide (LPS), whereas macrophage colony-stimulating factor (M-CSF) and the Th2 cytokines, IL-4 and IL-13, have been shown to induce alternative M2 polarization providing a niche for survival of intracellular microbes (8, 9). Accordingly, mycobacterial virulence factors may interfere with M1 polarization and instead promote a phenotype switch toward alternatively activated M2 macrophages, which facilitate mycobacterial growth and intracellular persistence (10–12).

Alveolar macrophages are the resident macrophage population in the lung, and have been classified as alternatively activated M2-like cells due to their receptor expression and their role to regulate tissue homeostasis (13). Since TB is primarily a lung infection, the alveolar M2 macrophages may be the first cells to encounter and phagocytose mycobacteria (14). The local tissue environment is rich in MCSF that is produced by stromal cells in the steady state, favoring M2 activation of macrophages (15), while an infection could trigger GM-CSF as well as the other Th1 cytokines required for the induction of classically activated M1 macrophages (16). Along the spectrum of macrophage polarization, the immunomodulatory hormone vitamin D3 (vitD) may have significant effects on macrophage polarization and function. It has been shown that vitamin D is a potent inducer of the antimicrobial peptide LL-37 (17, 18) as well as autophagy (18, 19) in M-CSF polarized macrophages that correlates to enhanced intracellular inhibition of Mtb growth. Vitamin D is produced in the skin or ingested in the diet and converted in tissue by cells including macrophages to the active form $1,25(OH)_2D_3$ (20). Active vitamin D signals via the intracellular vitD receptor (VDR) that act as a transcription factor complex to induce different host cell genes such as LL-37, and genes associated with autophagy. Mtb-induced toll-like receptor (TLR)-activation of human macrophages has been shown to trigger an up-regulation of the VDR as well as the vitamin D converting enzyme that results in a specific up-regulation of LL-37 and intracellular killing of Mtb (17). This way, vitamin D can induce VDR-dependent regulation of antimicrobial host responses in human macrophages.

In this study, we investigated the ability of $1,25(OH)_2D_3$-polarized monocyte-derived cells to control intracellular Mtb infection compared with conventional M1 and M2 macrophage subsets, and the phenotypic as well as functional alterations associated with immune polarization *in vitro*. We demonstrate that $1,25(OH)_2D_3$-polarized macrophage-like cells exhibit a superior effect on Mtb growth control compared with M1 and M2 macrophage subsets. Mtb infection of $1,25(OH)_2D_3$-polarized cells resulted in a potent up-regulation of pro-inflammatory cytokines, LL-37 but also IL-10, while mRNA expression the IL-1β receptor antagonist, IL-1RA, and the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO), was down-regulated as compared with the other subsets. In conclusion, macrophage polarization in an environment rich in vitamin D may significantly enhance intracellular Mtb growth control, perhaps by inducing innate effector molecules and simultaneously regulating the anti-inflammatory response of the cell.

MATERIALS AND METHODS

Monocyte Isolation and Differentiation

Peripheral blood mononuclear cells (PBMCs) from healthy donors (Karolinska Hospital Blood Bank, Stockholm, Sweden) were isolated from buffy coat blood by density sedimentation over Lymphoprep (GE Healthcare Life Sciences, Logan, UT). Cells were allowed to adhere in 6-well culture plates (Nunc, NY, Rochester, USA) for 2 h at 37°C in serum free media (RPMI 1640, supplemented with 2 mM L-glutamine and 5 mM HEPES (Hyclone, GE Healthcare Life Sciences). The non-adherent cells were removed by washing with phosphate buffered saline (PBS) and thereafter adherent monocytes were differentiated

TABLE 1 | Polarization of monocyte-derived cells.

Stimulation	Nomenclature[a]
Unstimulated	M0 (Control)
Active vitamin D_3	$1,25(OH)_2D_3$-polarized
GMCSF	M1-like
GMCSF + IFNγ + LPS	M1 polarized
MCSF	M2-like
MCSF + IL-4	M2 polarized

[a]*Nomenclature used in this study to describe differentially polarized monocyte-derived cell subsets.*

for 6 days in cell culture media containing 10% of fetal calf sera (FCS) (Sigma-Aldrich, St. Louis, MO). To establish an unbiased culture system that could assess the individual effects of $1,25(OH)_2D_3$ compared to conventional M1 and M2 polarization methods, unstimulated control (M0) and $1,25(OH)_2D_3$-polarized monocytes were differentiated in the absence of external growth factors using similar protocols as previously described (21, 22). As the maturation and activation status of macrophages differs depending on the *in vitro* stimulant (23), the cells obtained with the differentiation protocols used are referred to as monocyte-derived cells. Briefly, cells were left unstimulated or conditioned with 50 ng/mL human M-CSF (Life technologies, Carlsbad, CA) or 50 ng/mL human GM-CSF (ImmunoTools, Germany) for 3 days, after which the media was replaced with fresh media with or without M-CSF or GM-CSF (100 g/ml) for another 3 days of culture. To obtain $1,25(OH)_2D_3$-polarized monocyte-derived cells or fully polarized M1 or M2 subsets, unstimulated monocyte cultures were treated with 10 nmol vitamin D [$1,25(OH)_2D_3$, Sigma-Aldrich], while GM-CSF differentiated monocytes were treated with 50 ng/mL interferon-gamma (IFN-γ) and 10 ng/mL lipopolysaccharide (LPS), and M-CSF differentiated monocytes were treated with 20 ng/mL IL-4 (ImmunoTools, Germany), 18–20 h prior to infection with Mtb. The macrophage polarization protocols (8) and nomenclature used in this study is summarized in **Table 1**. The morphology of monocyte-derived cell cultures was monitored with light microscopy and the viability of adherent cells after 7 days of culture was estimated to >95% using Trypan Blue (Sigma-Aldrich) staining.

We used a fixed dose of $1,25(OH)_2D_3$ (10 nmol) that was in a similar range as the doses previously used by us (18, 24) and other groups (17, 19) to study the *in vitro* effects of $1,25(OH)_2D_3$ on human immune cells. The circulating $25(OH)D_3$ proform (10–100 nmol/L in serum) is converted to equivalent concentrations of the active $1,25(OH)_2D_3$ form inside cells upon uptake of $25(OH)D_3$ (20), which is the basis for using the active form in the nanomolar range in cellular experiments, corresponding to a physiological concentration of active vitamin D.

Mtb Cultures and Infection of Monocyte-Derived Cells

For infection of monocyte-derived cells, the standard laboratory Mtb strain, H37Rv, carrying the green fluorescent protein (GFP)-encoding pFPV2 plasmid (Mtb-GFP) was used (ATCC, Rockville, MD). A multidrug-resistant (MDR)-TB clinical isolate was obtained from the Mtb strain collection from the Public Health Agency of Sweden. The bacteria were stored at −80°C in Middlebrook 7H9 media supplemented with 10% glycerol (Karolinska University Hospital Solna, Sweden). Bacterial aliquots were thawed and cultured in Middlebrook 7H9 media, supplemented with 0.2% (v/v) glycerol, 10% OADC (Oleic Albumin Dextrose Catalase) enrichment and 0.05% (v/v) Tween-80 (Karolinska University Hospital Solna, Sweden) for 2–3 days. Bacteria were then sub-cultured in 50 ml tubes (dilution of bacterial stock 1:20 in new Middlebrook 7H9 media) and incubated at 37°C on a shaker for 7–10 days. Bacterial suspensions were pelleted and washed with phosphate buffer saline pH 7.4 (PBS) containing 0.05% (v/v) Tween-80 and then resuspended in 5 ml of RPMI complete media before transfer into a sterile 15 ml falcon tube. After 10 min pulse-sonication of inoculi, the optical density was measured at 600 nm. The final bacterial concentration was adjusted to $\sim 2.5 \times 10^6$ CFU/ml by adding RPMI complete media without antibiotics.

Primary monocytes were plated in 6-well plates at a concentration of 10^6 cells per well and allowed to differentiate for 7 days before infection with Mtb for 4 h in 37°C at a multiplicity of infection (MOI) of 5. After infection, the cells were washed vigorously three times with PBS-0.05%Tween to remove extracellular bacteria and thereafter resuspended in cell culture media without antibiotics for further experiments and analyses.

Flow Cytometry

Adherent Mtb-infected monocyte-derived cells were detached at 4, 24, or 72 h post-infection using 1.5 mM EDTA (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) for 10–15 min at room temperature. Detached cells (viability < 90–95%) were washed with FACS buffer [PBS containing 0.5% (v/v) FCS and 0.5 mM EDTA] and $0.5–1 \times 10^6$ cells were stained for 30 min at 4°C with fluorochrome-conjugated anti-human antibodies: CD68 (PE-Cy7), TLR2 (FITC), CD200R (PE), CD163 (BV605), CD206 (APC-Cy7), CD64 (PE-Dazzle 594), HLADR (PE-Cy5), CCR7 (BV711), CD80 (BV650), and CD86 (BV421) (Biolegend, San Diego, CA). Cells were washed twice with FACS buffer and fixed with 4% formaldehyde (Sigma-Aldrich) at room temperature for 10 min. Fifty thousand cells were acquired from each well by using BD LSR Fortessa (BD Biosciences, San Jose, CA) and analyzed with FlowJo v.9. H37Rv-GFP expression in CD68-positive macrophages was visualized in the FITC channel.

Colony Forming Units (CFU)

On day 3 post-infection, monocyte-derived cell cultures were lysed with 0.036% SDS-lysis buffer (Sodium dodecyl sulfate from Sigma dissolved in water) for 5 min to release intracellular bacteria. Cell lysis was confirmed by fast-contrast microscopy. Standard CFU counts were determined by diluting samples 1/10 to 1/10,000 by mixing 100 µl of bacterial suspension in 900 µl PBS containing 0.05% (v/v) Tween-80 and finally plating a volume of 100 µl on Middlebrook 7H11 agar plates with 10% OADC (Karolinska University Hospital Solna, Sweden). Each dilution was performed in duplicates. The plates were incubated at 37°C for a minimum of 21 days before colonies were manually counted. As a negative control, supernatants from Mtb-infected cell cultures were collected before cell lysis and plated for CFU

counts, which confirmed the absence of bacterial growth i.e., no extracellular Mtb bacilli remaining in Mtb-infected cell cultures.

mRNA Extraction and Quantitative Real-Time PCR

Human lung tissue biopsies (TB lesion and distal sites), obtained from patients with non-cavitary TB ($n = 5$) as previously described (25, 26), were freeze-fixed in OCT (Tissue-TEK, Sakura) before cutting two 50 μm cryosections that were homogenized in 1 ml of TRI-reagent and processed for mRNA extraction. Similarly, RNA was extracted from uninfected or Mtb-infected monocyte-derived cells 24 h post-infection using Ribopure RNA extraction kit as described by the manufacturer (Ambion, Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using Super Script Vilo™ cDNA Synthesis Kit (Applied Biosystems, Foster City, CA). Transcripts of Arg1, Arg2, CAMP, NOS2A, CCL2, CCL22, TNFα, TGFβ, IL-10, TLR2, IL-6, IL-1β, IL-1RA, and IDO (Indoleamine 2,3-dioxygenase) (Applied Biosystems) were measured in duplicates relative to the reference gene, housekeeping 18S rRNA (18S rRNA-housekeeping gene kit, Applied Biosystems), using quantitative real-time PCT (RT-PCR) (QuantStudio 5 Real-Time PCR System, Thermo Fisher Scientific, MA, USA). The results were analyzed by using the relative standard method. Briefly, the relative expression of the target genes was calculated by relating the Ct-value for Mtb-infected to uninfected M0 monocyte-derived cells. Data are presented as fold change of mRNA.

Statistical Analyses

Data are presented as the mean and standard deviation (SD) or median and interquartile range (IQR) from two or more individual experiments including monocyte-derived cells from a minimum of six healthy donors. The analyses used to calculate indicated *P*-values include one-way Anova, Kruskal–Wallis and Dunn's post-test as well as the Mann–Whitney or Wilcoxon-singed rank test. A $P < 0.05^*$ was considered statistically significant. The statistical analyses were done in GraphPad Prism-6.

RESULTS

Phenotype and Morphology of *in vitro* Polarized Monocyte-Derived Cells

Initially, surface expression of selected M1 (CCR7, CD64, TLR2) or M2 (CD163, CD200R, CD206) markers as well as antigen-presenting (HLADR) and co-stimulatory (CD86, CD80) molecules was investigated on uninfected monocyte-derived cells after immune polarization *in vitro*. CCR7, CD64, TLR2, and CD86 were all significantly ($P < 0.05$–0.0001) up-regulated on M1-polarized cells, and the expression of these markers were also relatively higher on M1-polarized compared with M1-like cells (**Figures 1A–E**). M2-polarized cell subsets had a down-regulated expression of CCR7, CD64, and HLA-DR ($P < 0.05$–0.001) compared with unstimulated M0 cells (**Figures 1A,B,E**) and also a significantly ($P < 0.01$) lower expression of CD80 compared to M1-polarized cells (**Figure 1I**). Instead, M2-like and M2-polarized cells showed a significant up-regulation of the M2 markers CD163 ($P < 0.05$), CD200R ($P < 0.0001$), and CD206 ($P < 0.05$) (**Figures 1F–H**). $1,25(OH)_2D_3$-polarized cells up-regulated TLR2, CD64 and HLADR ($P < 0.05$–0.01) compared with the M2 subsets (**Figures 1B,C,E**) and simultaneously showed a lower expression of CD163 and CD200R ($P < 0.05$–0.0001) (**Figures 1F,G**). In comparison with the M1 subsets, $1,25(OH)_2D_3$-polarization resulted in a lower expression of CCR7, CD64, CD86 ($P < 0.05$–0.0001) (**Figures 1A,B,D**). Similar to M0 cells, $1,25(OH)_2D_3$-polarized cells had a relatively higher expression of CD68 compared with M1 as well as M2 polarized cell subsets (**Figure 1J**). Thus, polarization of uninfected monocyte-derived cells toward M1 or M2 resulted in up- or down-regulation of typical M1/M2 surface markers, while $1,25(OH)_2D_3$-polarization did not promote either M1 or M2 differentiation.

Next, light microscopy was used to study the morphology of immune polarized monocyte cultures (**Figure 2**). Unstimulated $1,25(OH)_2D_3$-polarized cells showed a more circular morphology compared with M1 and M2 cells (**Figures 2A–F**). As expected, M1-like and M1-polarized cells had a more elongated and stretched or dendritic-like morphology compared with the other subsets (**Figures 2C,D**). M2 subsets were confluent but with a rounded shape, and M2-polarized cells were more circular compared with M2-like cells (**Figures 2E,F**). Overall, these results confirmed that M1 and M2 subsets had a phenotype and morphology consistent with classically and alternatively activated macrophages, respectively. Instead, $1,25(OH)_2D_3$-polarized cells maintained the morphology of less mature macrophages, but a high surface expression of CD68, CD64, and HLADR were consistent with differentiation of monocyte-derived macrophage-like cells.

Uptake and Intracellular Growth of Mtb in *in vitro* Polarized Monocyte-Derived Cells

To explore if vitamin D could affect Mtb uptake and subsequently a productive infection of *in vitro* polarized monocyte-derived cells, expression of GFP-labeled Mtb (H37Rv) was assessed after 4 h (uptake), 24 and 72 h (productive infection) post-infection of the different subsets. At 4 h, we observed a relatively lower percentage of M1-like and M1-polarized cells expressing H37Rv-GFP compared with the other subsets (**Figures 3A,B**), and this difference was significant comparing the M1 subsets with the M2 subsets ($P < 0.01$–0.001) (**Figure 3B**). Mtb uptake was evident in 18–19% of M1 cells, while ∼30% of unstimulated M0 as well as $1,25(OH)_2D_3$-polarized cells, and about 82% of M2 cells, were H37Rv-GFP-postive at 4 h (**Figures 3A,B**). However, at 24 and 72 h post-Mtb infection, the level of GFP-expressing cells rapidly and gradually increased in the M1 subsets ($P < 0.05$), while Mtb infectivity declined in M2-like cells ($P < 0.05$) and remained unchanged in the other monocyte-derived cell subsets (**Figure 3B**). At 24 h, the level of productive Mtb infection was significantly lower in $1,25(OH)_2D_3$-polarized cells compared with the M2-like and M2-polarized cells ($P < 0.05$) (**Figure 3B**).

Next, intracellular growth of Mtb was assessed at 4 and 72 h post-infection with H37Rv (**Figure 3C**) or a clinical MDR-TB isolate (**Figure 3D**) using CFU counts. At 4 h post-infection, we observed substantially elevated CFU counts in the M2 subsets compared with the $1,25(OH)_2D_3$ and M1 groups ($P < 0.05$–0.01) (**Figures 3C,D**), indicating a reduced colony

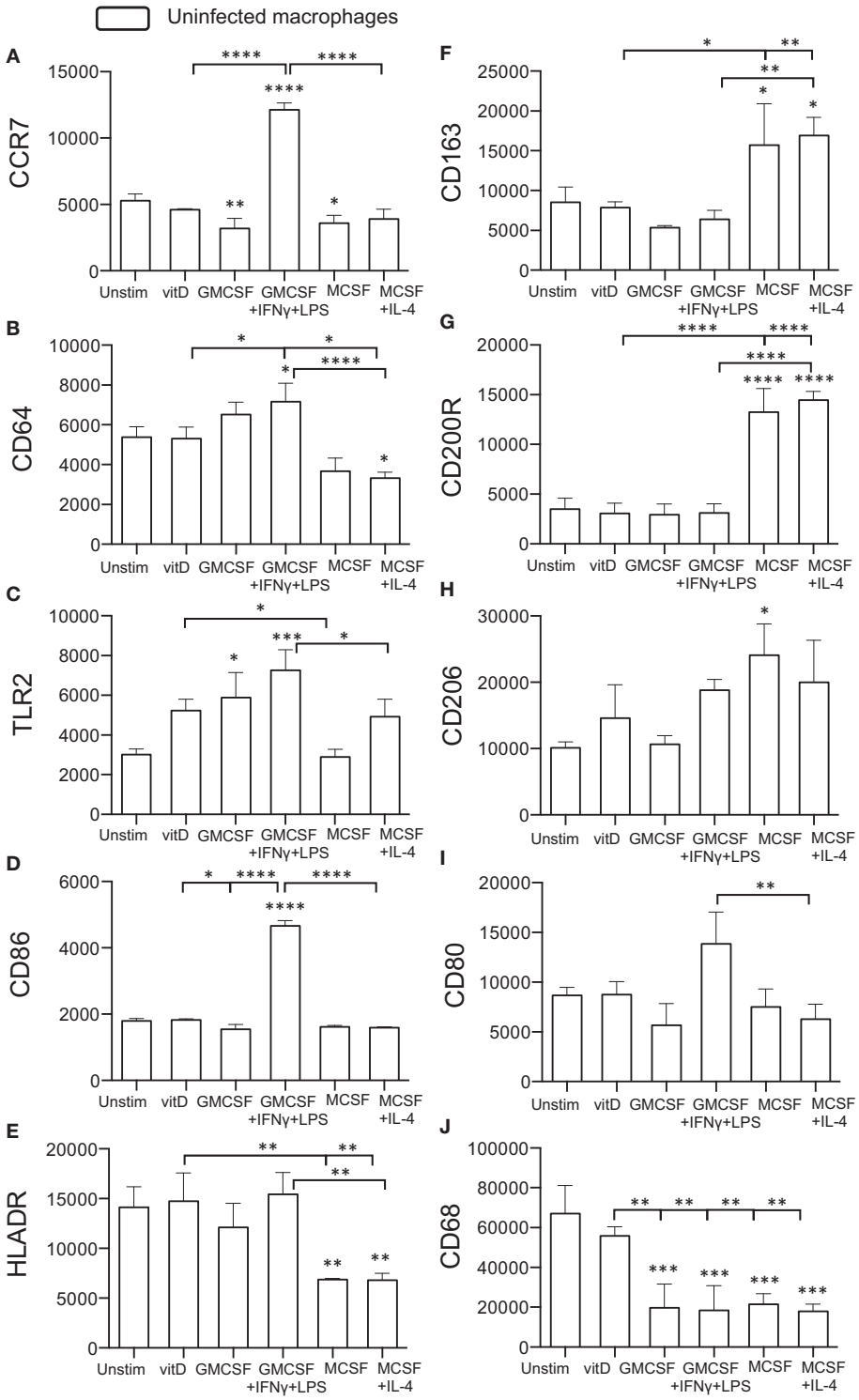

FIGURE 1 | Surface expression of M1 and M2 markers on uninfected *in vitro* polarized macrophages was determined using flow cytometry. The left panel shows typical M1 markers **(A)** CCR7, **(B)** CD64, **(C)** TLR2, **(D)** CD86, and **(E)** HLADR, while the right panel shows typical M2 markers **(F)** CD163, **(G)** CD200R, **(H)** CD206, **(I)** CD80, or macrophage phenotype **(J)** CD68. Results were obtained from $n = 6$ donors. Data (mean fluorescence intensity, MFI) is presented as mean ± SD and was analyzed using one-way ANOVA, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, $p < 0.0001$****. For clarity, selected statistical analyses are shown including comparisons of the indicated group to the M0 control (no bars), or other relevant between group comparisons (half tick down lines).

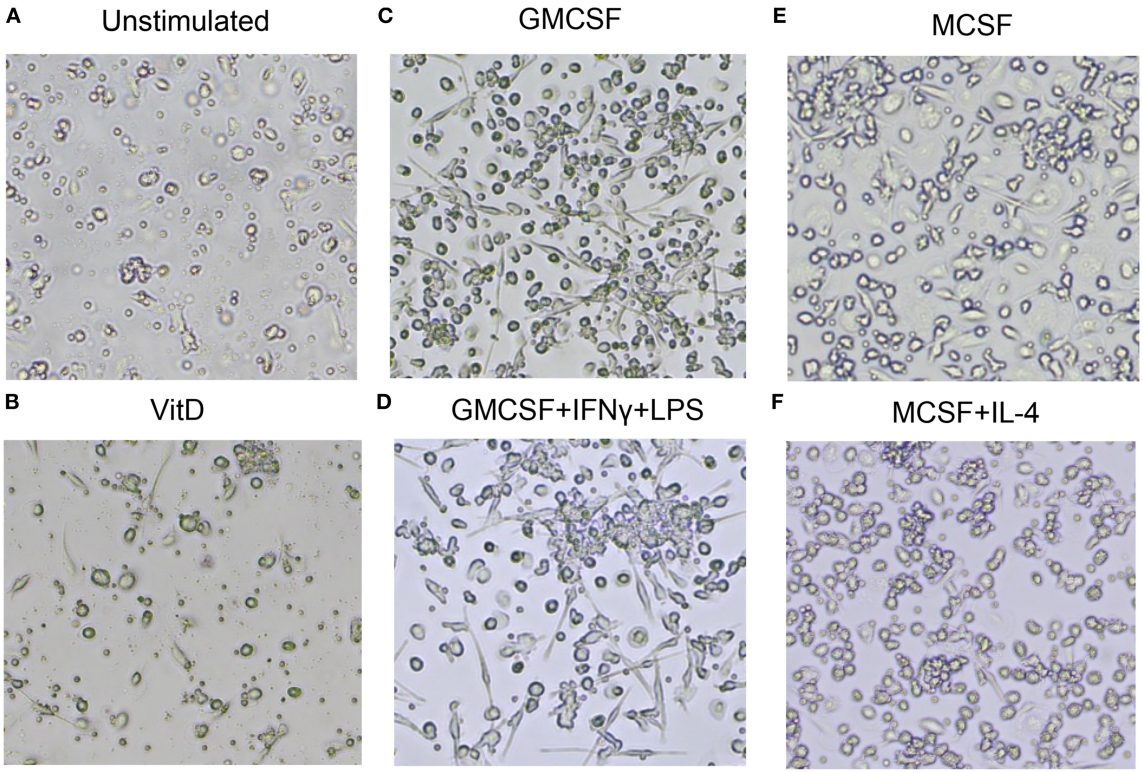

FIGURE 2 | Morphology of uninfected macrophage subsets after *in vitro* polarization. **(A)** Unstimulated M0 cells, **(B)** $1,25(OH)_2D_3$-polarized cells, **(C)** GMCSF-polarized M1-like macrophages, **(D)** GMCSF + IFNγ + LPS M1-polarized macrophages, **(E)** MCSF-polarized M2-like macrophages, **(F)** MCSF+IL-4 M2-polarized macrophages. Magnification 20×.

forming capacity of Mtb bacilli that has been taken up by $1,25(OH)_2D_3$-polarized or M1 cells. This could result from enhanced bacterial killing or reduced metabolic activity (i.e., latency) of the bacteria. Despite a lower Mtb uptake in M1 cells compared with $1,25(OH)_2D_3$-polarized cells (**Figure 3B**), the colony forming capacity was similar in these monocyte-derived cell subsets at 4 h (**Figures 3C,D**). Consistent with the FACS-data, significantly ($P < 0.05$–0.01) enhanced intracellular bacterial growth was detected in the M1- as well as M2-polarized subsets compared with $1,25(OH)_2D_3$-polarized cells, 72 h post-infection (**Figures 3C,D**). Notably, Mtb growth in the $1,25(OH)_2D_3$-polarized subset was around 40% lower compared with unstimulated M0 cells ($P < 0.05$), while Mtb growth was around 100–200% higher in the M1 and M2 subsets at 72 h (**Figures 3C,D**). Accordingly, intracellular Mtb growth was maintained at relatively lower levels in $1,25(OH)_2D_3$-polarized compared with unstimulated M0 cells, while there was substantial growth in both M1 and M2 cells (**Figures 3C,D**).

Alterations of M1/M2-Specific Markers Expressed on *in vitro* Polarized Monocyte-Derived Cell Subsets After Mtb Infection

Next, we assessed surface expression of the tested panel of M1/M2-specific markers on *in vitro* polarized monocyte-derived cells 4 h after infection with H37Rv-GFP. Cells in the same sample were divided into GFP-positive (Mtb-infected) and GFP-negative (uninfected) expression. Interestingly, Mtb infection altered the expression of typical M1 phenotype markers (**Figures 1A–D**), which were all elevated on M2 compared with M1 cell subsets (**Figures 4A–D**). Instead, the M2 marker CD163 was significantly up-regulated on GFP-positive M1-like ($P < 0.05$) and M1-polarized ($P < 0.01$) cells, respectively (**Figure 4E**). All H37Rv-GFP-positive subsets but M2, exhibited a relative up-regulation of all markers except CD200R (**Figure 4**). Furthermore, CD86 was significantly ($P < 0.05$–0.001) up-regulated on all GFP-positive subsets but unstimulated M0 cells (**Figure 4D**). $1,25(OH)_2D_3$-polarized cells also displayed a significant ($P < 0.05$) up-regulation of HLA-DR (**Figure 4C**), the mannose receptor CD206 (**Figure 4G**) and CD80 (**Figure 4H**). Overall, these results suggest that Mtb-infected monocyte-derived cell subsets experience phenotypical alterations suggestive of a mixed M1/M2 profile.

Assessment of kinetic changes of M1/M2-specific markers on M2-like macrophages, revealed a rapid up-regulation of surface markers at 4–12 h post-Mtb infection that declined already at 24–36 h after infection (**Supplementary Figure 1**). At 24 and 72 h post-Mtb infection, there were no significant differences in the expression of M1 or M2 markers between GFP-positive and GFP-negative cells or comparing the different subsets (data not shown).

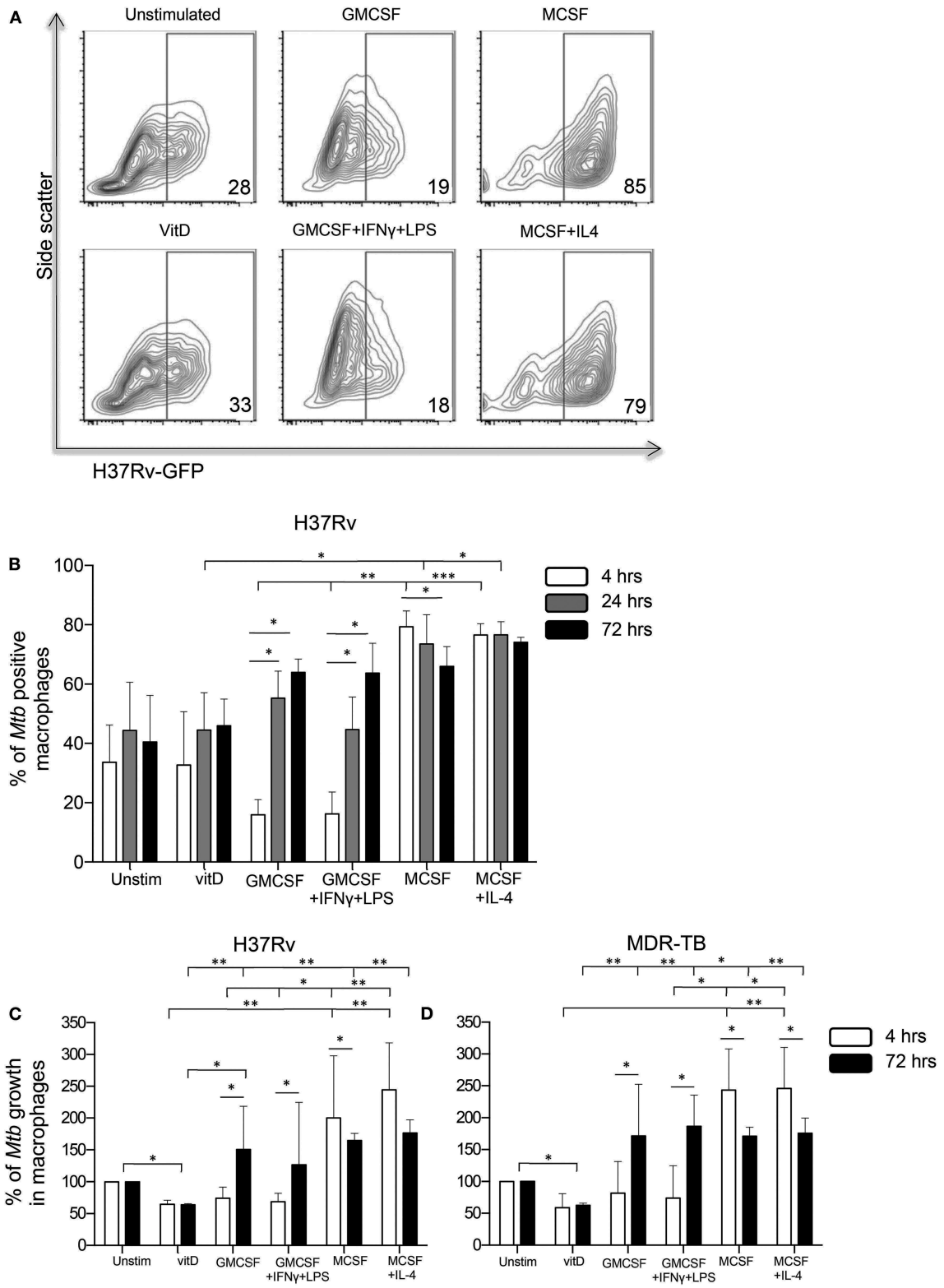

FIGURE 3 | Uptake, infectivity and intracellular growth of Mtb in *in vitro* polarized macrophages after Mtb infection. Flow cytometry was used to assess intracellular GFP-labeled H37Rv in *in vitro* polarized macrophage subsets at 4, 24, and 72 h post-infection. **(A)** Representative contour plots show the expression of H37Rv-GFP in the FITC-channel at 4 h post-infection. The percentage GFP-positive cells are indicated in the corner of each plot. **(B)** Mtb uptake in *in vitro* polarized macrophages

(Continued)

FIGURE 3 | at 4 h (white bars) compared to productive infection at 24 h (gray bars) and at 72 h (black bars) post-infection with H37Rv-GFP. Bacterial uptake and infection are presented as % of Mtb positive macrophages (% H37Rv-GFP positive cells). Intracellular growth of **(C)** H37Rv and **(D)** an MDR-TB clinical isolate in *in vitro* polarized macrophage subsets determined at 4 h (white bars) and 72 h (black bars) post-infection using CFU counts. Intracellular growth is presented as % of Mtb growth in macrophages (unstimulated M0 control = 100% growth). Results were obtained from $n = 6$ donors. Data is presented as median ± IQR and was analyzed using the Kruskal-Wallis and Dunn's post-test (comparing different polarization conditions), Friedman's test (comparing 4, 24, and 72 h time-points) and Wilcoxon signed-rank test (comparing 4 h to 72 h), $p < 0.05$*, $p < 0.01$ **, $p < 0.001$***.

Transcriptional Profiling of *in vitro* Polarized Monocyte-Derived Cells After Mtb Infection

To obtain additional information of the functional properties of *in vitro* polarized monocyte-derived cell subsets, quantitative mRNA analyses were performed on Mtb-infected cells 24 h post-infection and compared with uninfected cells. Mtb infection induced production of pro-inflammatory cytokines, IL-1β and TNF-α (**Figures 5A,B**) or IL-6 (data not shown), in the different subsets ($P < 0.05$), although both IL-1β and TNF-α was relatively higher in $1,25(OH)_2D_3$-polarized cells (**Figures 5A,B**). Moreover, Mtb enhanced expression of the monocyte chemoattractant CCL2 in $1,25(OH)_2D_3$-polarized cells, but also in the M1-like subset ($P < 0.05$) (**Figure 5C**). Instead, the IL-1β antagonist, IL-1RA, was significantly induced in Mtb-infected M2-polarized subset ($P < 0.0001$) compared with the unstimulated control (**Figure 5D**). All other Mtb-infected monocyte-derived cell subsets, apart from $1,25(OH)_2D_3$-polarized cells, also had a significant up-regulation of IL-1RA ($P < 0.05$) (**Figure 5D**). Similarly, Mtb infection induced a high mRNA expression of the immunosuppressive enzyme IDO, in M0, M1 as well as the M2 subsets ($P < 0.05$) (**Figure 5E**). Contrary, IDO mRNA was very low in $1,25(OH)_2D_3$-polarized cells (**Figure 5E**). IDO mRNA was also induced in uninfected M1-polarized cells, which is consistent with the finding that IFN-γ up-regulates IDO (27). IL-10 mRNA was induced in both $1,25(OH)_2D_3$ and M2-polarized uninfected cells ($P < 0.001$) and increased in M1-like as well as M2-like cells ($P < 0.05$) after Mtb infection (**Figure 5F**). mRNA expression of the intracellular enzymes arginase, Arg-1 (**Figure 5G**) and Arg-2 (data not shown), was primarily observed in M1- as well as M2-polarized subsets post-Mtb infection ($P < 0.05$), whereas inducible nitric oxide (iNOS) was relatively higher in uninfected as well as Mtb-infected M1 cells ($P < 0.05$) (**Figure 5H**). Mtb also induced iNOS in M2-polarized cells and to a lesser extent in $1,25(OH)_2D_3$-polarized cells (**Figure 5H**). As expected, mRNA induction of the antimicrobial peptide, LL-37, was superior in $1,25(OH)_2D_3$-polarized monocyte-derived cells ($P < 0.001$). Mtb infection resulted in a potent down-regulation of LL-37 expression in all subsets ($P < 0.05$) (**Figure 5I**), but was still significantly higher in $1,25(OH)_2D_3$-stimulated compared with M1-like ($P < 0.05$) and M2-polarized ($P < 0.0001$) subsets (**Figure 5I**). LL-37 was not induced in unstimulated M0 cells (**Figure 5I**). Likewise, the autophagy markers LC3B as well as Atg5 and beclin-1 (data not shown) were selectively up-regulated in $1,25(OH)_2D_3$-polarized cells. mRNA expression profiles in monocyte-derived cells infected with H37Rv or the clinical MDR-TB isolate were very similar and revealed no significant differences between these mycobacterial strains (data not shown).

Overall, Mtb infection resulted in enhanced or unchanged transcription of the different molecules investigated, with the exception from LL-37 mRNA expression that was significantly suppressed by Mtb infection. Corresponding mRNA profiling of lung tissue samples obtained from patients with non-cavitary TB, confirmed that most of the investigated markers were up-regulated in the TB lesions (site of Mtb infection) compared with unaffected lung parenchyma, supporting the presence of a mixed M1/M2 macrophage polarization *in vivo* (**Supplementary Figure 2**).

DISCUSSION

Immune polarization of macrophages in the local tissue environment may have significant effects on the ability of the cells to control intracellular Mtb infection. We demonstrate that monocyte-derived cells polarized with the active form of vitamin D have a superior effect on Mtb growth inhibition in comparison to cell subsets polarized using conventional M1 and M2 stimuli. While uninfected M1 and M2 subsets expressed typical phenotype markers, Mtb infection altered this expression and generated a mixed M1/M2 activation profile. Mtb infection of M1 cells was initially low but rapidly progressed into a productive infection and enhanced bacterial growth, while Mtb growth remained low in $1,25(OH)_2D_3$-polarized cells. Contrary, infectivity of and Mtb growth in the M2 subsets was high 4 h post-infection but subsequently decreased to levels comparable to M1 cells supporting the mixed M1/M2 model. Mtb growth inhibition in $1,25(OH)_2D_3$-polarized cells was associated with an induction of the antimicrobial peptide LL-37 as well as pro-inflammatory cytokines but also IL-10 mRNA, while expression of IL-1RA and IDO was low in comparison to the other monocyte-derived cell subsets. Our data demonstrates that polarization of monocyte-derived cells in the presence of vitamin D provides the capacity to mount an antimicrobial response against Mtb that is superior to conventional M1 and M2 activation, and that simultaneously prevent expression of inhibitory molecules that could promote immunosuppression in the microenvironment of Mtb-infected tissues.

We have previously conducted human studies to investigate the relevance of vitamin D status in the progression of clinical TB (26, 28), the *in vitro* effects of vitamin D stimulation on monocyte-derived macrophages (MDMs) (18, 24) and the clinical benefit from *in vivo* supplementation of vitamin D together with similar immunomodulatory compounds to patients with pulmonary TB (29, 30). Our findings showed

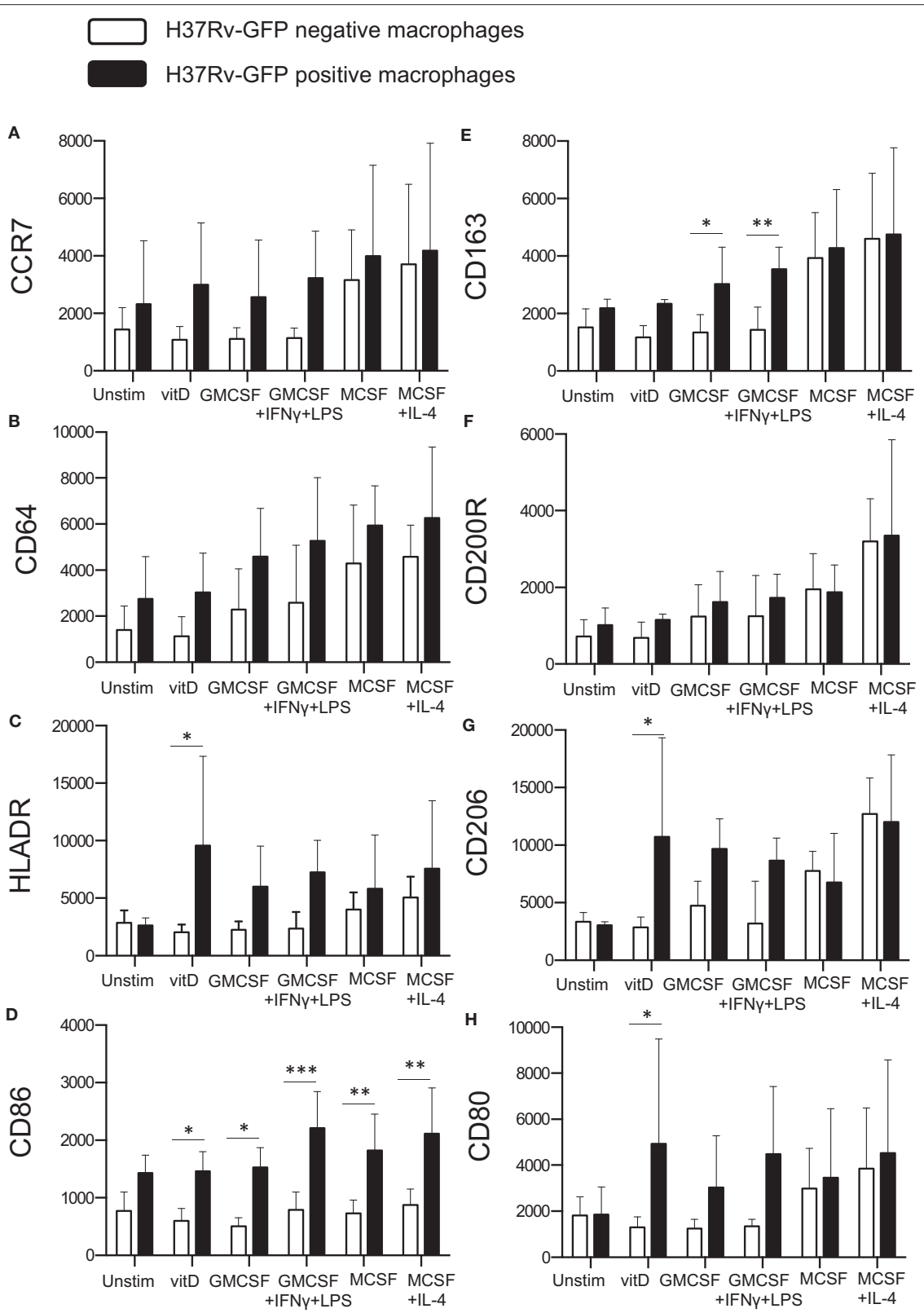

FIGURE 4 | Surface expression of M1 and M2 markers on Mtb-infected *in vitro* polarized macrophages was determined using flow cytometry. H37Rv-GFP positive (black bars) and H37Rv-GFP negative (white bars) cells were assessed in the sample. The left panel shows M1 markers **(A)** CCR7, **(B)** CD64, **(C)** HLA-DR, and **(D)** CD86, while the right panel shows M2 markers **(E)** CD163, **(F)** CD200R, **(G)** CD206, and **(H)** CD80. Results were obtained from $n = 6$ donors. Data (mean fluorescence intensity, MFI) is presented as mean ± SD and was analyzed using two-way ANOVA, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***.

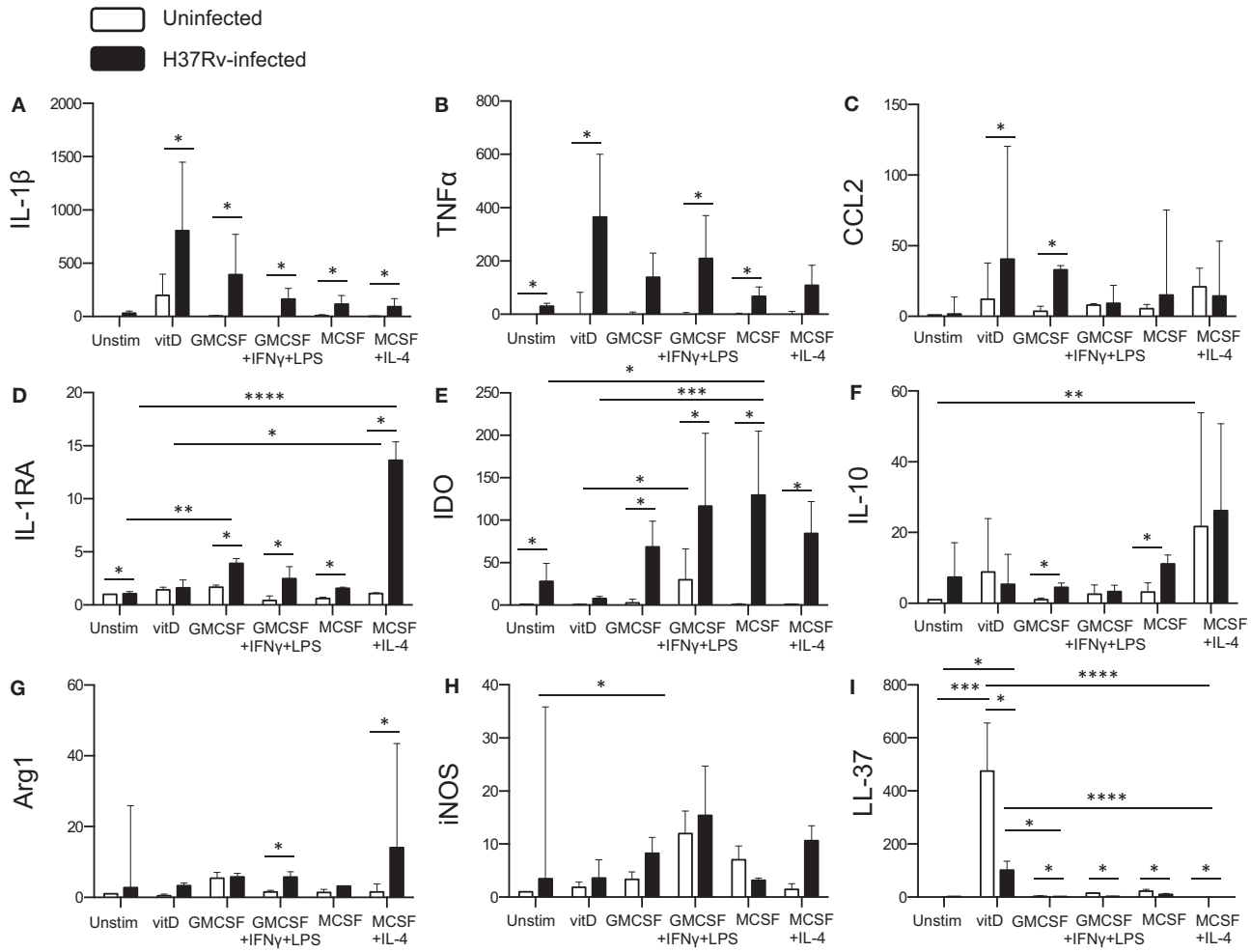

FIGURE 5 | Quantitative mRNA expression in Mtb-infected *in vitro* polarized macrophages was determined at 24 h post-infection. Different effector molecules including pro- or anti-inflammatory cytokines, chemokines, inhibitory receptors, and antimicrobial effector molecules were assessed in uninfected compared to Mtb-infected macrophage subsets. **(A)** IL-1β, **(B)** TNF-α, **(C)** CCL2, **(D)** IL-1RA, **(E)** IDO, **(F)** IL-10, **(G)** Arg-1, **(H)** iNOS, and **(I)** LL-37. mRNA expression was determined using RT-PCR and presented as the fold change of each target gene in the different Mtb-infected subsets compared to unstimulated M0 macrophages. Results were obtained from $n = 9$ donors. Data is presented as median ± interquartile range and analyzed using the Kruskal-Wallis and Dunn's post-test (comparing different polarization conditions), or Wilcoxon signed-rank test (comparing H37Rv-infected to uninfected cells), $p < 0.05$*, $p < 0.01$ **, $p < 0.001$***, $p < 0.0001$****.

that MDMs obtained from TB patients produced increased amounts of IL-1β, TNF-α as well as IL-10 upon stimulation with $1,25(OH)_2D_3$, which were associated to reduced growth of intracellular Mtb (24). Furthermore, $1,25(OH)_2D_3$, in the presence or absence of phenylbutyrate (PBA), had the ability to counteract Mtb-induced down-regulation of LL-37 in MCSF-differentiated macrophages and could also induce autophagy via a LL-37-dependent pathway that promoted intracellular growth control of Mtb (18). Vitamin D deficiency is common in patients with TB, although the cause and effect of low vitamin D status and the development of active TB is not known (20). Nonetheless, several studies provide evidence that LL-37 mRNA and protein expression in different macrophage subsets is dependent on vitamin D status (17, 31, 32). We have shown that a low expression of LL-37 in granulomatous TB lesions from patients with active TB correlates with low plasma levels of vitamin D $[25(OH)D_3]$ (26, 28). In clinical trials (29, 30), we have also observed positive clinical effects using 2–4 months adjunct treatment with vitD + PBA that resulted in improved vitamin D status and enhanced sputum-culture conversion in patients with pulmonary TB. Patient recovery was associated with up-regulated levels of LL-37 in MDMs and PBMCs (29) as well as induction of autophagy and reduced endoplasmic reticulum (ER)-stress in *ex vivo* Mtb-infected macrophages from vitamin D-supplemented TB patients (33). Accordingly, serum from vitamin D deficient individuals failed to support induction of LL-37 in human monocytes, while *in vitro* supplementation with $25(OH)D_3$ promoted LL-37 expression significantly (17). Similarly, vitamin D deficient serum prevented IFN-γ induced activation of LL-37 or β-defensin-4 mRNA in monocytes or macrophages that was

restored by addition of 25(OH)D_3 to the cell cultures (32). As a consequence, IFN-γ induced autophagy and phagolysosome fusion in monocytes as well as Mtb killing in MDMs were dependent on adequate levels of vitamin D (32). Another report revealed that Mtb and its secreted components can prevent the IFNγ signaling pathway and Mtb killing in infected macrophages by inhibition of IFNγ transcriptional responses (34). Notably, one of IFN-γ transcriptional targets is the vitamin D converting enzyme, 1α-hydroxylase (32). Altogether, these data suggest that vitamin D status including sufficient vitamin D concentrations locally in the tissue microenvironment may be of fundamental importance to trigger anti-TB defense pathways in human macrophages and can also contribute to the regulation of IFN-γ signaling.

Similar to our results, it has previously been demonstrated that M1-like macrophages were less susceptible to uptake and infection with avirulent *M. bovis* BCG compared with M2-like macrophages (35). However, BCG-infected M1 cells maintained enhanced intracellular growth control compared with M2 cells up to 6 days post-infection (35), suggesting that the elevated infectivity and Mtb growth observed in M1 cells rapidly after infection is a trait of virulent mycobacteria. A recent study determined that M2 macrophages displayed slightly higher phagocytic activity than M1 cells, and phagocytosis was not changed by treatment with 1,25$(OH)_2D_3$ (36). Furthermore, MDMs treated with low-dose 1,25$(OH)_2D_3$ for 6 days were less infected with dengue virus compared with untreated MDMs, which was proposed to be due to reduced viral uptake via CD206 that was down-regulated on 1,25$(OH)_2D_3$-tretated MDMs (37). In contrast, we found that CD206 was up-regulated on Mtb-infected monocyte-derived cells that were polarized with a higher dose of 1,25$(OH)_2D_3$ the last 20 h of 6 days differentiation. An enhanced expression of CD206 together with HLADR, CD86, and CD80 on 1,25$(OH)_2D_3$-polarized cells could suggest that antigen-presentation and co-stimulation is improved by vitamin D. Interestingly, also Chlamydia infection of polarized MDMs (8) or murine bone-marrow derived macrophages (38) was considerably lower in M1 compared with M2 cells. However, despite microbicidal activity, M1 macrophages failed to eliminate intracellular infection, but suppressed Chlamydia growth correlated with the induction of a bacterial gene expression profile characteristic of persistence (38). This is consistent with our results that Mtb-infected M1 cells were initially very potent to control infection but fail to completely eradicate intracellular mycobacteria that instead can persist in M1 as well as M2 macrophages.

The strength of our results is that we have used virulent Mtb and compared infectivity and intracellular Mtb survival over time in differentially polarized human monocyte-derived cell subsets. We also observed the plasticity of polarized cells, as Mtb infection generated a mixed M1/M2 phenotype. While *in vitro* studies suggest that human monocytes can polarize into M1 macrophages and subsequently switch to the M2 phenotype upon exposure to sequential changes in the microenvironmental conditions (39), different tissues *in vivo* i.e., the lung, may contain varying mixtures of M1- and M2-type macrophages (40). As such, macrophages in skin (41) or brain (41) tissue from patients with cancer or multiples sclerosis, respectively, present heterogeneous activation states more consistent with poly-activated macrophages. Mixed M1 and M2 activation phenotypes was recently shown to be induced by melanoma exosomes, suggesting that subcellular fractions and soluble factors can promote mixed M1/M2 activation (42). This could explain the altered phenotype we observed in Mtb-GFP-positive as well as Mtb-GFP-negative polarized monocyte-derived cells. Altogether, instead of shifting M1 toward M2 activation in Mtb infection, our findings support the mixed M1/M2 model that may facilitate intracellular persistence of Mtb.

The ability of macrophages to acquire different functional phenotypes enables them to execute innate effector functions but also to control the induction of specific T cell responses. After exposure to *M. bovis* BCG and subsequent antigen presentation to $CD4^+$ T cell clones, M1 macrophages were more effective than M2 cells to stimulate proliferation and IFN-γ production (35). Expression of the immunosuppressive enzyme IDO in Mtb-infected cells effectively diminish activation of Th1 cells by degradation of the essential amino acid tryptophan, and may instead produce metabolites that induce $FoxP3^+$ regulatory T cells (Treg) (43, 44). Our observations suggest that IDO was strongly up-regulated by Mtb infection in all conventionally polarized cell subsets, but to a much lesser extent in 1,25$(OH)_2D_3$-polarized monocyte-derived cells. Increased levels of IDO have been observed in TB patients (45, 46) and IDO can be expressed in Mtb-infected macrophages *in vitro* (46). Interestingly, IDO activity has been implicated in the differentiation of monocytes into M2 macrophages (47). Accordingly, ectopic IDO increased the expression of M2 markers but decreased M1 markers, while knock-down of IDO had the opposite effects (27). This suggests that IDO-expressing macrophages may skew differentiation toward the M2 phenotype. Accordingly, studies in non-human primates have shown that *in vivo* inhibition of IDO expression promoted local recruitment of effector T cells to the site of Mtb infection in lung granulomas and reduced bacterial burden significantly (48).

A limitation of this study is the use of an *in vitro* system based on Mtb infection of monocyte-derived cells that may only partially reproduce the complexity of macrophage activation found *in vivo*. However, it is likely that monocytes are recruited from the blood into the lung upon Mtb infection and develop into inflammatory MDMs including the M1 subsets that support tissue-resident macrophages in the combat against the bacteria (40). CCL2 is one of the main chemokines involved in inflammation-dependent recruitment of monocytes to sites of infection (49). Vitamin D-mediated induction of IL-10 could, similarly to IL-10 produced by M2 macrophages, be involved in resolution of inflammation and prevent overt immunopathology in Mtb-infected tissue, as has been described in vitamin D-supplemented TB patients (50).

This study provides new information about the potential local effects of vitamin D in the microenvironment of Mtb-infected tissues e.g., in the lung. Vitamin D is multifunctional molecule that has a broad range of effects on the immune response, and thus more research is required to obtain a comprehensive understanding of the how vitamin D status and vitamin D supplementation could contribute to TB control in patients. While we and others have tested the therapeutic capacity of vitamin D (29, 30), large clinical trials is currently ongoing in an attempt to study the prophylactic effects of vitamin D in preventing development of active TB infection [reviewed in (20)]. Overall, additional *in vitro* and *in vivo* studies will shed light on the role of vitamin D in macrophage polarization and if improved vitamin D status could enhance antimicrobial effector responses and simultaneously ameliorate inflammation without inducing immunosuppression in human TB.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies on immune cells in blood obtained from healthy individuals was reviewed and approved by the ethical review board (EPN) in Stockholm, Sweden (2010/603-31/4). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SB, ML, and JR designed the research. JR and VP performed the experiments. JR, VP, ML, MS, and SB contributed to data analyses and interpretation. SB and JR wrote the manuscript. JR, VP, ML, MS, and SB critically reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03157/full#supplementary-material

REFERENCES

1. Brighenti S, Joosten SA. Friends and foes of tuberculosis: modulation of protective immunity. *J Intern Med.* (2018) 284:125–44. doi: 10.1111/joim.12778
2. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* (2014) 6:13. doi: 10.12703/P6-13
3. Labonte AC, Tosello-Trampont AC, Hahn YS. The role of macrophage polarization in infectious and inflammatory diseases. *Mol Cells.* (2014) 37:275–85. doi: 10.14348/molcells.2014.2374
4. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* (2008) 8:958–69. doi: 10.1038/nri2448
5. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* (2004) 25:677–86. doi: 10.1016/j.it.2004.09.015
6. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity.* (2005) 23:344–6. doi: 10.1016/j.immuni.2005.10.001
7. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* (2000) 164:6166–73. doi: 10.4049/jimmunol.164.12.6166
8. Buchacher T, Ohradanova-Repic A, Stockinger H, Fischer MB, Weber V. M2 polarization of human macrophages favors survival of the intracellular pathogen chlamydia pneumoniae. *PLoS ONE.* (2015) 10:e0143593. doi: 10.1371/journal.pone.0143593
9. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* (2003) 3:23–35. doi: 10.1038/nri978
10. Redente EF, Higgins DM, Dwyer-Nield LD, Orme IM, Gonzalez-Juarrero M, Malkinson AM. Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation. *J Leukoc Biol.* (2010) 88:159–68. doi: 10.1189/jlb.0609378
11. Refai A, Gritli S, Barbouche MR, Essafi M. *Mycobacterium tuberculosis* virulent factor ESAT-6 drives macrophage differentiation toward the pro-inflammatory M1 phenotype and subsequently switches it to the anti-inflammatory M2 phenotype. *Front Cell Infect Microbiol.* (2018) 8:327. doi: 10.3389/fcimb.2018.00327
12. Kahnert A, Seiler P, Stein M, Bandermann S, Hahnke K, Mollenkopf H, et al. Alternative activation deprives macrophages of a coordinated defense program to *Mycobacterium tuberculosis*. *Eur J Immunol.* (2006) 36:631–47. doi: 10.1002/eji.200535496
13. Fels AO, Cohn ZA. The alveolar macrophage. *J Appl Physiol.* (1986) 60:353–69. doi: 10.1152/jappl.1986.60.2.353
14. Day J, Friedman A, Schlesinger LS. Modeling the immune rheostat of macrophages in the lung in response to infection. *Proc Natl Acad Sci USA.* (2009) 106:11246–51. doi: 10.1073/pnas.0904846106
15. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol.* (2008) 8:533–44. doi: 10.1038/nri2356
16. Shibata Y, Berclaz PY, Chroneos ZC, Yoshida M, Whitsett JA, Trapnell BC. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity.* (2001) 15:557–67. doi: 10.1016/S1074-7613(01)00218-7

17. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* (2006) 311:1770–3. doi: 10.1126/science.1123933
18. Rekha RS, Rao Muvva SS, Wan M, Raqib R, Bergman P, Brighenti S, et al. Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages. *Autophagy.* (2015) 11:1688–99. doi: 10.1080/15548627.2015.1075110
19. Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, et al. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe.* (2009) 6:231–43. doi: 10.1016/j.chom.2009.08.004
20. Brighenti S, Bergman P, Martineau AR. Vitamin D and tuberculosis: where next? *J Intern Med.* (2018) 284:145–62. doi: 10.1111/joim.12777
21. Bennett S, Por SB, Stanley ER, Breit SN. Monocyte proliferation in a cytokine-free, serum-free system. *J Immunol Methods.* (1992) 153:201–12. doi: 10.1016/0022-1759(92)90323-L
22. Kreutz M, Krause SW, Hennemann B, Rehm A, Andreesen R. Macrophage heterogeneity and differentiation: defined serum-free culture conditions induce different types of macrophages *in vitro*. *Res Immunol.* (1992) 143:107–15. doi: 10.1016/0923-2494(92)80087-2
23. Vincent F, Eischen A, Bergerat JP, Faradji A, Bohbot A, Oberling F. Human blood-derived macrophages: differentiation *in vitro* of a large quantity of cells in serum-free medium. *Exp Hematol.* (1992) 20:17–23.
24. Eklund D, Persson HL, Larsson M, Welin A, Idh J, Paues J, et al. Vitamin D enhances IL-1beta secretion and restricts growth of *Mycobacterium tuberculosis* in macrophages from TB patients. *Int J Mycobacteriol.* (2013) 2:18–25. doi: 10.1016/j.ijmyco.2012.11.001
25. Andersson J, Samarina A, Fink J, Rahman S, Grundstrom S. Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infect Immun.* (2007) 75:5210–22. doi: 10.1128/IAI.00624-07
26. Rahman S, Rehn A, Rahman J, Andersson J, Svensson M, Brighenti S. Pulmonary tuberculosis patients with a vitamin D deficiency demonstrate low local expression of the antimicrobial peptide LL-37 but enhanced FoxP3+ regulatory T cells and IgG-secreting cells. *Clin Immunol.* (2015) 156:85–97. doi: 10.1016/j.clim.2014.12.003
27. Wang XF, Wang HS, Wang H, Zhang F, Wang KF, Guo Q, et al. The role of indoleamine 2,3-dioxygenase (IDO) in immune tolerance: focus on macrophage polarization of THP-1 cells. *Cell Immunol.* (2014) 289:42–8. doi: 10.1016/j.cellimm.2014.02.005
28. Ashenafi S, Mazurek J, Rehn A, Lemma B, Aderaye G, Bekele A, et al. Vitamin D(3) status and the association with human cathelicidin expression in patients with different clinical forms of active tuberculosis. *Nutrients.* (2018) 10:721. doi: 10.3390/nu10060721
29. Mily A, Rekha RS, Kamal SM, Arifuzzaman AS, Rahim Z, Khan L, et al. Significant effects of oral phenylbutyrate and vitamin D3 adjunctive therapy in pulmonary tuberculosis: a randomized controlled trial. *PLoS ONE.* (2015) 10:e0138340. doi: 10.1371/journal.pone.0138340
30. Bekele A, Gebreselassie N, Ashenafi S, Kassa E, Aseffa G, Amogne W, et al. Daily adjunctive therapy with vitamin D3 and phenylbutyrate supports clinical recovery from pulmonary tuberculosis: a randomized controlled trial in Ethiopia. *J Intern Med.* (2018) 284:292–306. doi: 10.1111/joim.12767
31. Kim EW, Teles RMB, Haile S, Liu PT, Modlin RL. Vitamin D status contributes to the antimicrobial activity of macrophages against Mycobacterium leprae. *PLoS Negl Trop Dis.* (2018) 12:e0006608. doi: 10.1371/journal.pntd.0006608
32. Fabri M, Stenger S, Shin DM, Yuk JM, Liu PT, Realegeno S, et al. Vitamin D is required for IFN-γ-mediated antimicrobial activity of human macrophages. *Sci Transl Med.* (2011) 3:104ra2. doi: 10.1126/scitranslmed.3003045
33. Rekha RS, Mily A, Sultana T, Haq A, Ahmed S, Mostafa Kamal SM, et al. Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy. *BMC Infect Dis.* (2018) 18:303. doi: 10.1186/s12879-018-3203-9
34. Ting LM, Kim AC, Cattamanchi A, Ernst JD. *Mycobacterium tuberculosis* inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. *J Immunol.* (1999) 163:3898–906.
35. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci USA.* (2004) 101:4560–5. doi: 10.1073/pnas.0400983101
36. Dionne S, Duchatelier CF, Seidman EG. The influence of vitamin D on M1 and M2 macrophages in patients with Crohn's disease. *Innate Immun.* (2017) 23:557–65. doi: 10.1177/1753425917721965
37. Arboleda Alzate JF, Rodenhuis-Zybert IA, Hernandez JC, Smit JM, Urcuqui-Inchima S. Human macrophages differentiated in the presence of vitamin D3 restrict dengue virus infection and innate responses by downregulating mannose receptor expression. *PLoS Negl Trop Dis.* (2017) 11:e0005904. doi: 10.1371/journal.pntd.0005904
38. Gracey E, Lin A, Akram A, Chiu B, Inman RD. Intracellular survival and persistence of Chlamydia muridarum is determined by macrophage polarization. *PLoS ONE.* (2013) 8:e69421. doi: 10.1371/journal.pone.0069421
39. Italiani P, Mazza EM, Lucchesi D, Cifola I, Gemelli C, Grande A, et al. Transcriptomic profiling of the development of the inflammatory response in human monocytes *in vitro*. *PLoS ONE.* (2014) 9:e87680. doi: 10.1371/journal.pone.0087680
40. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front Immunol.* (2014) 5:514. doi: 10.3389/fimmu.2014.00514
41. Vogel DY, Vereyken EJ, Glim JE, Heijnen PD, Moeton M, van der Valk P, et al. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J Neuroinflammation.* (2013) 10:35. doi: 10.1186/1742-2094-10-35
42. Bardi GT, Smith MA, Hood JL. Melanoma exosomes promote mixed M1 and M2 macrophage polarization. *Cytokine.* (2018) 105:63–72. doi: 10.1016/j.cyto.2018.02.002
43. Harden JL, Egilmez NK. Indoleamine 2,3-dioxygenase and dendritic cell tolerogenicity. *Immunol Invest.* (2012) 41:738–64. doi: 10.3109/08820139.2012.676122
44. Hirsch CS, Rojas R, Wu M, Toossi Z. *Mycobacterium tuberculosis* induces expansion of Foxp3 positive CD4 T-cells with a regulatory profile in tuberculin non-sensitized healthy subjects: implications for effective immunization against TB. *J Clin Cell Immunol.* (2016) 7:428. doi: 10.4172/2155-9899.1000428
45. Suzuki Y, Miwa S, Akamatsu T, Suzuki M, Fujie M, Nakamura Y, et al. Indoleamine 2,3-dioxygenase in the pathogenesis of tuberculous pleurisy. *Int J Tuberc Lung Dis.* (2013) 17:1501–6. doi: 10.5588/ijtld.13.0082
46. Weiner J III, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, et al. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. *PLoS ONE.* (2012) 7:e40221. doi: 10.1371/annotation/b7f554bc-ad78-4745-9cd6-e14954d6a01d
47. Francois M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther.* (2012) 20:187–95. doi: 10.1038/mt.2011.189
48. Gautam US, Foreman TW, Bucsan AN, Veatch AV, Alvarez X, Adekambi T, et al. *In vivo* inhibition of tryptophan catabolism reorganizes the tuberculoma and augments immune-mediated control of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA.* (2018) 115:E62–71. doi: 10.1073/pnas.1711373114
49. Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, et al. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest.* (2007) 117:902–9. doi: 10.1172/JCI29919
50. Coussens AK, Wilkinson RJ, Hanifa Y, Nikolayevskyy V, Elkington PT, Islam K, et al. Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proc Natl Acad Sci USA.* (2012) 109:15449–54. doi: 10.1073/pnas.1200072109

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frontiers in Immunology

ORIGINAL RESEARCH
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Vitamin D Induces Differential Effects on Inflammatory Responses During Bacterial and/or Viral Stimulation of Human Peripheral Blood Mononuclear Cells

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Streptococcus pneumoniae (pneumococcus) and respiratory syncytial virus (RSV) are the leading causes of respiratory infections amongst children <5 years of age. Co-infection with these pathogens is common during early life and often associated with increased disease severity. Epidemiological studies have shown that low levels of Vitamin D_3 ($VitD_3$) are associated with increased susceptibility to respiratory pathogens. However, the role of $VitD_3$ in the context of pneumococcal and RSV exposure are poorly understood. We found that $VitD_3$ significantly reduced Th17 cell expression and IL-17A and IL-22 secretion in peripheral blood mononuclear cells (PBMCs) when stimulated with a pneumococcal whole cell antigen (WCA). Levels of IFN-γ were also decreased whilst IL-10 and IL-1β were increased. Effects of $VitD_3$ on innate responses following RSV stimulation was limited, only reducing IL-6. $VitD_3$ also reduced the number of TLR2+CD14+ monocytes, whilst increasing TLR7+CD14+ monocytes and TLR4+CD56+ NK cells. In WCA-stimulated PBMCs, $VitD_3$ increased IL-1β levels but reduced TLR2+CD14+ monocytes. For pneumococcal WCA-RSV co-stimulation, $VitD_3$ only had a limited effect, mainly through increased IL-1β and RANTES as well as TLR4+CD56+ NK cells. Our results suggest that $VitD_3$ can modulate the inflammatory response to pneumococci but has limited effects during viral or bacterial-viral exposure. This is the first study to examine the effects of $VitD_3$ in the context of pneumococcal-RSV co-stimulation, with important implications on the potential role of $VitD_3$ in the control of excessive inflammatory responses during pneumococcal and RSV infections.

Keywords: pneumococcal, respiratory syncytial virus, vitamin D, inflammation, peripheral blood mononuclear cells

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INTRODUCTION

Streptococcus pneumoniae (pneumococcus) and respiratory syncytial virus (RSV) are the leading causes of lower respiratory tract infections (LRTIs) amongst children and older adults (1, 2). Importantly, co-infections with these pathogens are becoming increasingly recognized as a major contributor to severe LRTIs requiring hospitalizations (3, 4). Innate responses to RSV or

pneumococcal bacteria can prime the host for secondary infection by activating inflammatory cells such as macrophages and neutrophils (5, 6). T-helper cell 17 (Th17) responses are important in the control of pneumococcal colonization, which is a pre-requisite step in the development of invasive disease (7). However, chronic exposure can lead to dysregulated inflammatory responses and pathology. Balancing the inflammatory response during co-infection may be a strategy to reduce severe morbidity (8).

The discovery of the Vitamin D receptor (VDR) and cytochrome P450 27B1 (CYP27B1) enzyme expression on immune cells has driven exploration into whether the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] ($VitD_3$), has immunoprotective properties in the context of infection (9–12). Numerous observational data and clinical trials suggest that insufficient $VitD_3$ levels are associated with increased susceptibility to respiratory pathogens, and that $VitD_3$ supplementation in high disease burden settings may be beneficial (13–15). Interactions between the VDR and $VitD_3$ mediates anti-inflammatory effects on both the innate and adaptive immune systems, thereby regulating immunity in the context of bacterial and/or viral inflammation. Innate cell subsets including neutrophils, macrophages, and dendritic cells (DCs) all express VDR and respond to $VitD_3$. Following contact with pathogens through binding to their toll-like receptors (TLRs), genes that encode up-regulation of the VDR and production of CYP27B1 become expressed (16). Stimulation of the VDR in these cells enhances their bactericidal, anti-microbial, chemotactic, and phagocytic capabilities (17). Similarly, $VitD_3$ also influences the adaptive immune responses either directly or indirectly through DCs, altering their cytokine production. This influences Th17 activation and function, through increasing IL-10 and decreasing IL-17A secretion, which are associated with RSV and pneumococcal infections (18).

In this study, we examined the effect of $VitD_3$ on inflammatory responses in the context of pneumococcal and RSV co-stimulation. We treated peripheral blood mononuclear cells (PBMCs) isolated from healthy adults with $VitD_3$ and stimulated with either pneumococcal whole cell antigen (WCA), RSV or WCA-RSV together to study host cytokine responses and the frequency of key immune cell populations important during pneumococcal and RSV infections, focusing on Th17 (for pneumococcus) and innate (for RSV alone and pneumococcal-RSV co-infection) inflammatory responses. Our results provide evidence that $VitD_3$ reduces pneumococcal Th17 responses, but had limited effects in modulating the inflammatory response during pneumococcal-RSV co-stimulation. These findings are important in the context of novel strategies such as $VitD_3$ supplementation to reduce the severity and incidence of both pneumococcal and RSV infections in $VitD_3$ in high risk populations.

METHODS

Study Samples

Twelve healthy adults aged from 19 to 64 years old were enrolled into the study. A single blood sample (∼20 mLs) was collected from each individual into a sodium heparin tube. All subjects gave their informed consent and the study was approved by the Royal Children's Hospital Human Research Ethics Committee (HREC).

Materials

The active metabolite of $VitD_3$ was purchased from Tocris Bioscience (Bristol, UK). The pneumococcal whole cell antigen (WCA) was kindly provided by PATH under a Materials Transfer Agreement. Live RSV-A2 strain and A549 cell line was purchased from American Type Culture Collection (ATCC; Virginia, USA).

RSV Preparation

For RSV stock preparation, RSV A2 strain was grown in A549 cells and purified by centrifugation through 30% sucrose layer as described previously (19). The harvested virus was collected in DMEM culture medium containing 20% sucrose and aliquoted, then snap-frozen and stored at−80°C until subsequent experiments. The titre of purified virus stocks were determined by plaque assay according to a previous method (20).

PBMC Culture

The PBMCs were isolated immediately after blood collection by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) at 400 × g for 30 min without brake at room temperature (RT). Isolated PBMCs were then washed twice in RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS, 1,000 IU penicillin-streptomycin and 200 nM L-glutamine (RPMI-FBS) at 500 × g for 10 min at RT. The cells were resuspended in 1 mL RPMI-FBS and the cell concentration was determined using the Trypan blue exclusion dye (Sigma-Aldrich, St. Louis, USA) method where a 1:1 mixture of PBMCs and Trypan Blue dye was added to a haemocytometer (Neubauer chamber) and counted under a microscope. For pneumococcal assays, 1×10^6 PBMCs/mL were pre-treated with 100 nmol/L $VitD_3$ for 24 h then stimulated with 1 μg/mL WCA for 5 days. Based on a model adapted from previous studies (21), 1×10^6 PBMCs/mL in our co-stimulation experiments were incubated simultaneously with live RSV (MOI = 1) and 1 μg/mL WCA and 100 nmol/L $VitD_3$ for 24 h. Supernatants were then harvested and stored at−30°C until use.

Cytokine Measurement

Levels of IFN-γ, IL-8, IL-10, TNF-α, MCP-1, and RANTES in cultured supernatants from PBMCs were measured using a human cytokine multiplex bead array method as per the manufacturer's instructions (Milliplex; Millipore Corporation, Billerica, MA, USA). IL-6, IL-17A, and IL-22 were measured using commercial ELISA kits according to the manufacturer's instructions (R&D Systems; Minneapolis, Minnesota, USA).

Flow Cytometry

To identify specific immune cell subsets in PBMCs following $VitD_3$ treatment, cells were stained with fluorescently-conjugated monoclonal antibodies; CD4-BUV737, CD45RO-APC,

CD161-FITC, CD194-V450, CD196-PE, CD14-BV605, CD19-APC-H7, CD56-BV421, CD282-AF647 (TLR2), CD284-BV786 (TLR4; all from BD Bioscience; San Diego, CA, USA), and anti-TLR7-PE (Gibco Life Technologies, Carlsbad, USA), Zombie Aqua™ Fixable Viability Kit (BioLegend, San Diego, USA). Compensation bead particles were used to account for spectral overlap (BD Bioscience, San Diego, CA, USA) and analyzed using the BD LSRII flow cytometer. Unstained PBMCs and fluorescence minus one (FMO) were used as controls and a minimum of 100,000 events were analyzed per sample gated on live, single cell lymphocyte gate based on FSC and SSC, where the expression of the cell surface molecules was evaluated using FlowJo, LLC v10.4.2 software. Refer to **Supplementary Figures 1–4** for gating strategies.

Statistical Analysis

Data is presented as median ± IQR for cytokine and flow cytometry results. Comparison of $VitD_3$ treated and untreated cytokine responses and cell populations were determined using a paired non-parametric, Wilcoxon sign-rank test. The data was graphically represented and statistically analyzed using Graphpad prism 6 software (Graphpad Software Inc, California, USA). All tests performed were two-tailed and a $p < 0.05$ was considered significant.

RESULTS

$VitD_3$ Reduces Inflammatory Cytokines and Th17 Frequency in PBMCs Following Pneumococcal Stimulation

We examined the potential for $VitD_3$ to modify Th17 responses as this is important in the control of pneumococcal colonization. Pneumococcal WCA was used as this has previously been shown to specifically induce Th17 responses in mice and humans (22, 23). Stimulation of PBMCs with pneumococcal WCA significantly enhanced the proportion of Th17 cells and levels of Th17-related cytokines IL-17A and IL-22 (**Figures 1A–C**). $VitD_3$ significantly reduced the Th17 frequency in both unstimulated and WCA-stimulated PBMCs (both $p = 0.016$; **Figure 1A**), as well as IL-17A and IL-22 in WCA-stimulated cells compared with untreated cells stimulated with WCA (both $p < 0.01$; **Figures 1B,C**). In contrast, $VitD_3$ increased IL-10 in WCA-stimulated PBMCs ($p = 0.001$; **Figure 1D**). Consistent with these findings, the IL-17A/IL-10 and IL-22/IL-10 ratios (both $p = 0.001$) were also significantly decreased by $VitD_3$ (**Figures 1E,F**).

$VitD_3$ also significantly reduced the level of IFN-γ, IL-8, and TNF-α in unstimulated cells (**Figures 2A,B,D**), while in WCA-stimulated cells, $VitD_3$ significantly reduced IFN-γ while increasing IL-1β (**Figures 2A,C**) but did not alter IL-8 or TNF-α levels (**Figures 2B,D**).

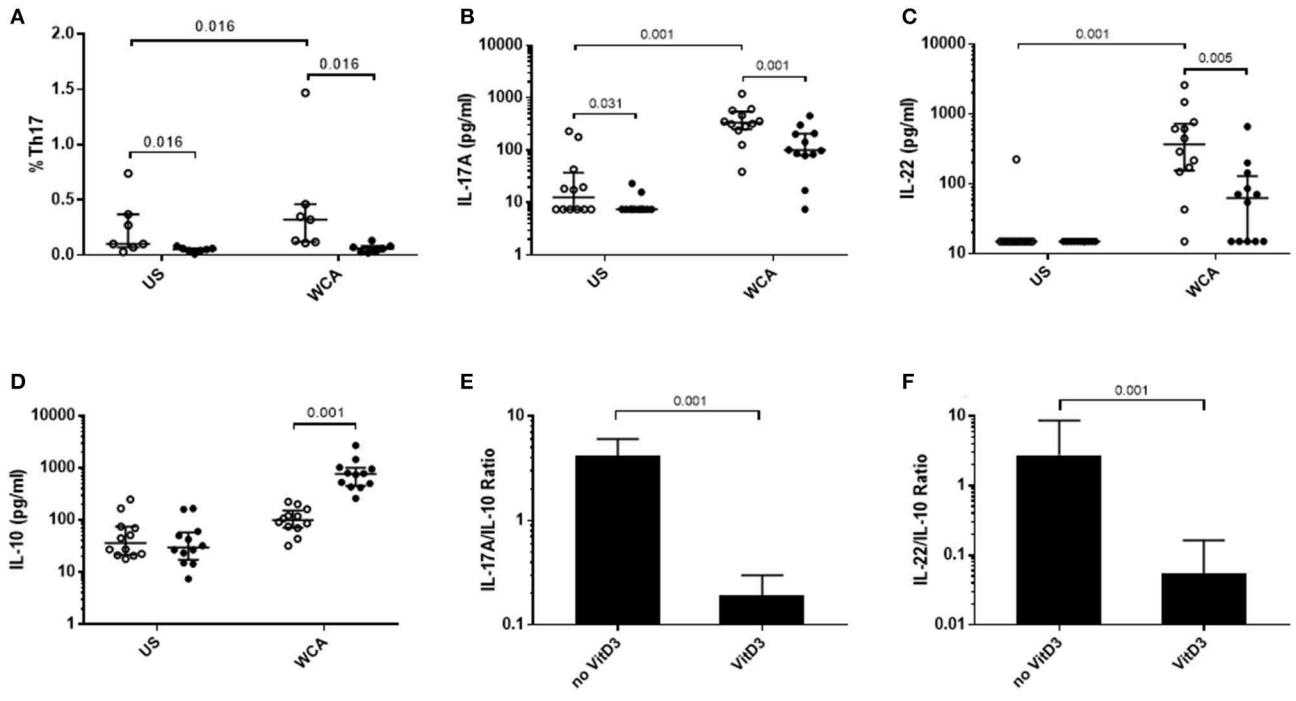

FIGURE 1 | *$VitD_3$ decreases pneumococcal Th17 responses*. 1×10^6 PBMCs/mL were pre-treated with 100 nmol/L $VitD_3$ for 24 h, prior to stimulation with 1 μg/mL WCA for 5 days. Th17 frequency **(A)** was measured by flow cytometry. Th17 populations were determined by positive staining for cell surface markers obtained from live single lymphocytes. These were considered CD4+CD45+CD161+CD194+CD196+. IL-17A **(B)**, IL-22 **(C)**, IL-10 **(D)**, IL-17A-IL-10 ratio **(E)**, IL-22-IL-10 ratio and **(F)**, concentrations were measured by an ELISA in pg/mL. Open circles represent untreated PBMCs, whilst closed circles represent $VitD_3$ treated PBMCs. Data shown represents median ± IQR; $n = 12$ per group for cytokine analyses and $n = 8$ per group for flow cytometry.

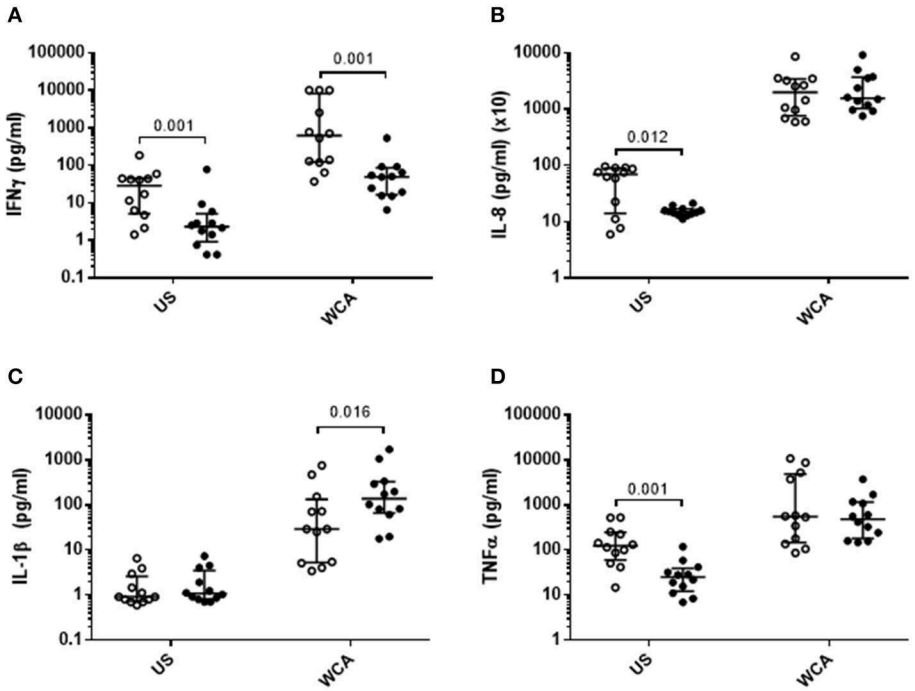

FIGURE 2 | $VitD_3$ reduces pneumococcal pro-inflammatory cytokines and increases anti-inflammatory cytokines. 1×10^6 PBMCs/mL were pre-treated with $VitD_3$ (100nmol/L) for 24 h, prior to stimulation with 1 μg/mL WCA for 5 days. **(A)** IFN-γ, **(B)** IL-8, **(C)** IL-1β, and **(D)**. TNF-α concentrations were measured by a multiplex assay in pg/mL. Open circles represent untreated PBMCs, whilst closed circles represent $VitD_3$ treated PBMCs. Data shown represents median ± IQR; $n = 12$ per group.

$VitD_3$ Does Not Modulate Inflammatory Responses During Pneumococcal-RSV Co-stimulation

To determine the effect of $VitD_3$ in the context of pneumococcal and RSV co-stimulation, we undertook studies of the innate response using a model adapted from previous studies (21). Co-stimulation of PBMCs with WCA and RSV (WCA-RSV) resulted in increased cytokine responses for IL-6, IL-10, IL-1β, and TNF-α compared to RSV alone (**Figures 3B–E**). Compared to WCA stimulation, WCA-RSV significantly increased the level of all cytokines measured (**Figures 3A–G**). $VitD_3$ treatment of WCA-RSV stimulated cells did not affect most cytokines, but increased the levels of IL-1β and RANTES (both $p < 0.05$; **Figures 3D,G**).

For RSV stimulation alone, $VitD_3$ significantly reduced IL-6 only ($p = 0.023$; **Figure 3B**), but had no effect on any of the other cytokines measured. For WCA alone, VitD3 increased IL-1β only ($p = 0.016$; **Figure 3E**), consistent with our earlier observation (see **Figure 1**).

Effect of $VitD_3$ on Innate and Adaptive Cell Subsets and TLR Expression Following Pneumococcal-RSV Co-stimulation

To understand how $VitD_3$ mediates these anti-inflammatory effects, we examined the phenotypic expression of key immune cell markers. We found that $VitD_3$ treatment in the absence of any stimulation increased the frequency of CD3+ T cells ($p = 0.008$) while decreasing CD14+ monocyte populations ($p = 0.039$; **Figures 3H–I**) but had no effect on CD19+ or CD56+ cells (**Figures 3J–K**). When stimulated with WCA alone, $VitD_3$ treatment significantly increased CD3+ cells (**Figure 3H**) but reduced CD19+ and CD14+ cell numbers compared with untreated PBMCs stimulated with WCA alone (**Figures 3I,J**), while for RSV alone, $VitD_3$ only decreased CD14+ cells (**Figure 3I**). The CD14+ cell frequency was significantly decreased following WCA or WCA-RSV compared with unstimulated cells ($p = 0.008$ for both; **Figure 3I**).

$VitD_3$ did not modulate the frequency of any of the immune cell populations in the context of WCA-RSV co-stimulation (**Figures 3H–K**). In the absence of $VitD_3$, WCA-RSV co-stimulation significantly increased the percentage of CD3+ cells compared to WCA alone but reduced CD19+ cells compared to RSV alone (**Figures 3H,J**). Furthermore, CD56+ cells were reduced following stimulation with RSV alone compared with unstimulated cells while WCA-RSV reduced CD56+ cells compared to unstimulated and WCA alone-stimulated cells ($p = 0.008$ for both; **Figure 3K**).

We next examined TLR expression on PBMCs and specific cell populations to determine whether the effects of $VitD_3$ is pathogen or ligand-specific (**Figure 4**). In unstimulated PBMCs, $VitD_3$ significantly reduced the frequency of TLR2+ and TLR4+ cells but only TLR2+ cells following RSV stimulation (**Figures 4A,B**). TLR7 expression was not modulated by $VitD_3$ for any of the conditions tested (**Figure 4C**). For WCA alone, $VitD_3$ did not affect any TLRs. Co-stimulation with WCA-RSV significantly reduced the frequency of TLR2+ cells compared with WCA (p

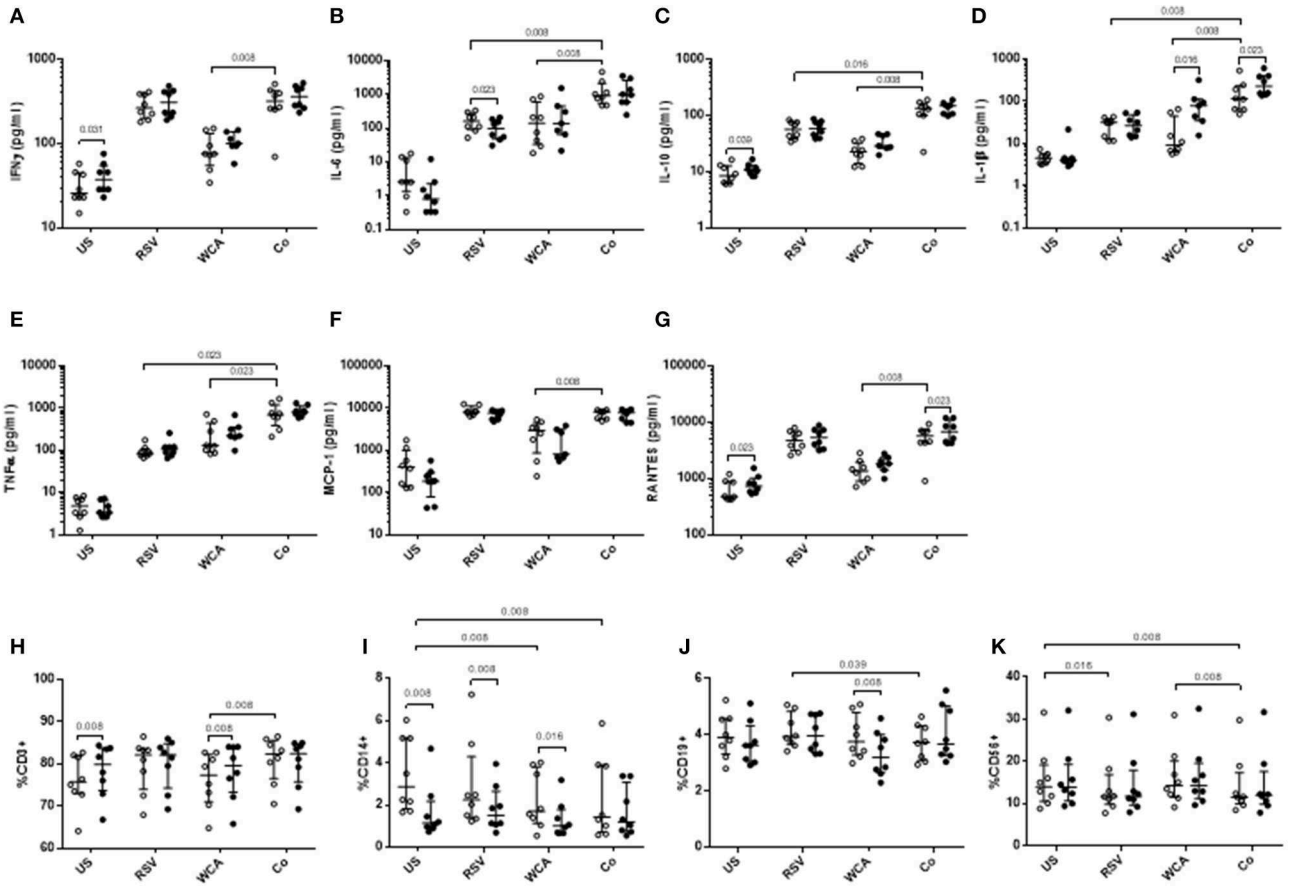

FIGURE 3 | The effect of $VitD_3$ on pro-inflammatory cytokines, chemokines, and immune cell subsets during bacterial-viral co-stimulation. 1×10^6 PBMCs/mL were treated with $VitD_3$ (100 nmol/L) at the time of stimulation. PBMCs were stimulated with live RSV at an MOI = 1, WCA (1 μg/mL) or WCA-RSV (designated "co"). **(A)** IFN-γ, **(B)** IL-6, **(C)** IL-10, **(D)** IL-1β, **(E)** TNF-α, **(F)** MCP-1, and **(G)** RANTES. For immune cell subsets cell populations were determined by positive cell surface staining from live single lymphocytes. **(H)** CD3+, **(I)** CD14+, **(J)** CD19+, **(K)** CD56+. Concentrations were measured by a multiplex assay in pg/mL. Open circles represent untreated PBMCs, whilst closed circles represent $VitD_3$ treated PBMCs. Data shown represents median ± IQR; $n = 8$ per group.

= 0.008) or RSV ($p = 0.016$) alone (**Figure 4A**). However, $VitD_3$ only increased the percentage of TLR4+ cells when co-stimulated with WCA-RSV ($p = 0.039$; **Figure 4B**).

Co-stimulation with WCA-RSV significantly reduced the percentage of CD14+TLR2+ compared to RSV or WCA alone (**Figure 4D**). WCA-RSV had no effect on CD14+TLR4+ cells but significantly increased the frequency of CD14+TLR7+ cells compared with RSV or WCA alone or unstimulated cells (**Figures 4E–F**). However, $VitD_3$ treatment significantly reduced CD14+TLR2+ frequency for RSV ($p = 0.008$) or WCA ($p = 0.016$) stimulation compared with either one alone but not WCA-RSV co-stimulation (**Figure 4D**). For CD14+TLR4+ cells, $VitD_3$ only reduced the frequency in unstimulated cells (**Figure 4E**), while $VitD_3$ significantly increased the percentage of CD14+TLR7+ cells for RSV alone ($p = 0.039$), with non-significant increases for both WCA alone and WCA-RSV conditions (**Figure 4F**). For CD56+ cells, RSV stimulation increased the percentage of TLR2 ($p = 0.008$) and TLR7 ($p = 0.016$; **Figures 4G,I**), $VitD_3$ however only increased TLR4 frequency for RSV alone stimulation and WCA-RSV co-stimulation ($p = 0.039$) but did not alter TLR2 or TLR7 for any other condition tested (**Figures 4G–I**).

DISCUSSION

Co-infection with *S. pneumoniae* and RSV increases host inflammatory responses that often leads to severe respiratory disease requiring hospitalization. The anti-inflammatory effects of $VitD_3$ are well-documented however their ability to modulate responses associated with bacterial or viral exposure are not well understood. This study extends our earlier observations of $VitD_3$ effects on pneumococcal innate responses (24) to examine Th17 immunity and inflammatory responses following co-stimulation with pneumococcus and RSV. We found that $VitD_3$ reduced pneumococcal Th17 inflammation but only had a limited effect on responses to WCA-RSV co-stimulation.

Pneumococcal exposure induces Th17 responses that protect against subsequent pneumococcal acquisition by enhancing recruitment of neutrophils and increasing anti-microbial peptide release through IL-17A and IL-22 secretion (25, 26). However, in

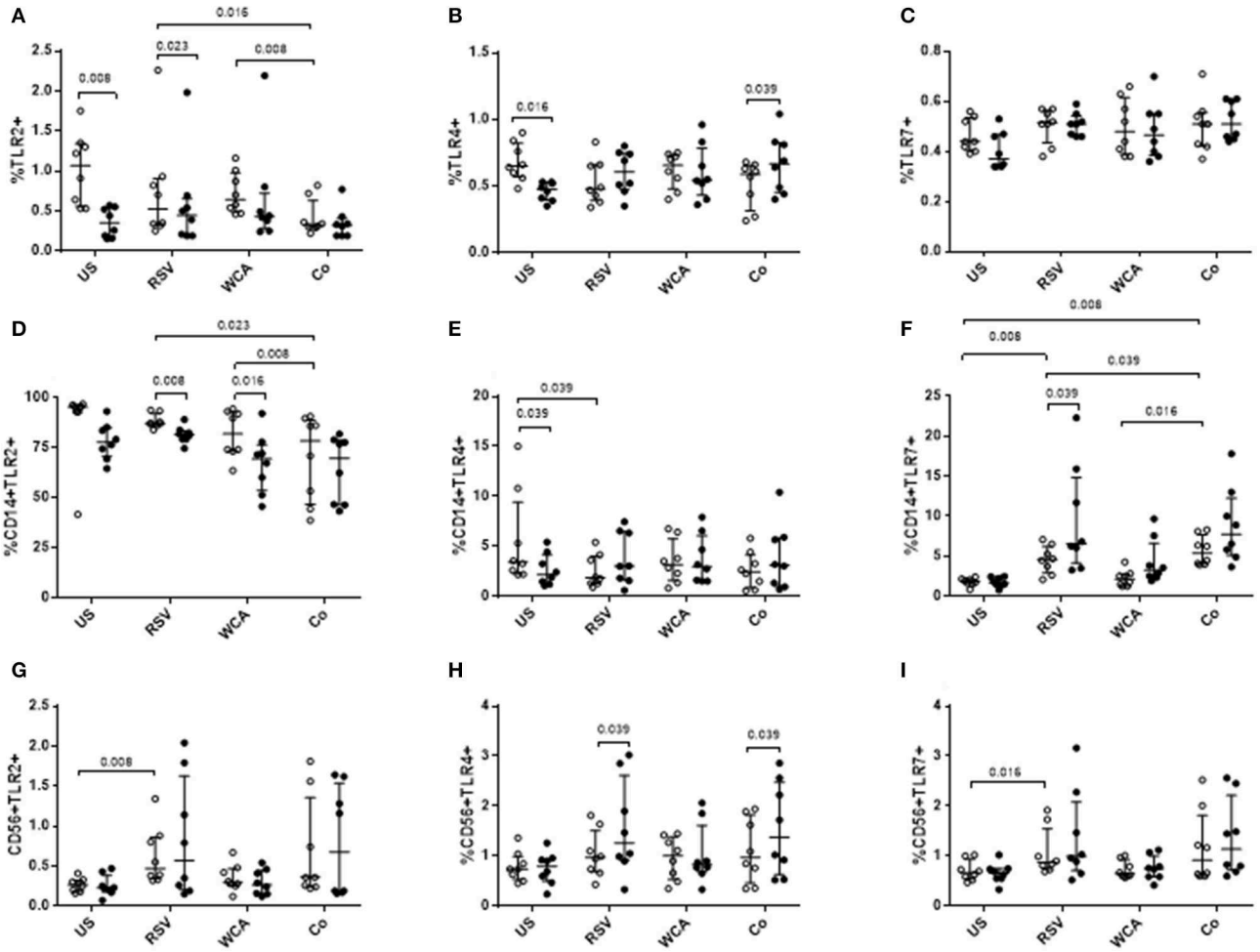

FIGURE 4 | The effect of $VitD_3$ on TLR expression in PBMCs. 1×10^6 PBMCs/mL were treated with $VitD_3$ (100 nmol/L) at the time of stimulation. PBMCs were stimulated with live RSV at an MOI = 1, WCA (1 μg/mL) or WCA-RSV (designated "co") prior to phenotyping by flow cytometry. Cell populations were determined by positive staining for cell surface obtained from live single lymphocytes. **(A)** TLR2+, **(B)** TLR4+, **(C)** TLR7+, **(D)** CD14+TLR2+, **(E)** CD14+TLR4+, **(F)** CD14+TLR7+, **(G)** CD56+TLR2+, **(H)** CD56+TLR4+, **(I)** CD56+TLR7+. Open circles represent untreated PBMCs, whilst closed circles represent $VitD_3$ treated PBMCs. Data shown represents median ± IQR; $n = 8$ per group.

low-and middle-income countries where pneumococcal carriage is high, a persistent IL-17A response occurs. The high level of exposure to pneumococcus in these settings limits the ability of IL-17A to reduce colonization, persisting to chronic inflammation through continued recruitment of neutrophils and macrophages (27). We used pneumococcal WCA as this is a well-characterized inducer of Th17 responses, and was a good model to examine the effects of $VitD_3$ (7). We defined Th17 responses on the basis of CCR4 and CCR6 chemokine receptor expression as this has previously been described as a functional Th17 subset (28, 29). This was confirmed by the increased activation of Th17 cells and their related cytokines by WCA. $VitD_3$ reduced the Th17 cell frequency and level of IL-17A and IL-22, suggesting a potent anti-Th17 effect. VitD3 also enhanced IL-10, lending support for the Th17-Treg axis (30, 31). This is important since unregulated Th17 responses can lead to significant inflammation and pathology. In view of our findings, $VitD_3$ may have a critical role in maintaining the balance between these responses, particularly in populations where there is substantial exposure to pneumococcus during early life.

Increased levels of TNF-α and IL-6 during pneumococcal infection are associated with severity of disease (32). Pre-treatment with $VitD_3$ decreased these cytokines while IL-1β were increased. This is consistent with a recent study by Sommer and Fabri (33), suggesting that $VitD_3$ increases IL-1β to prime the innate response by influencing IL-1β gene transcription as no IL-1β is released prior to infection. This priming of the innate response by $VitD_3$ is important to enhance anti-microbial activity through increased defensin-4 secretion (34–36). Further, studies to examine the production of antimicrobial peptides such as cathelicidins in response to $VitD_3$ would be worthwhile.

To determine the role of $VitD_3$ on the response to WCA-RSV co-stimulation, we used a different model to the Th17 studies to determine innate effects, based on a

previous study (21). We observed increased cytokine responses in PBMCs following co-stimulation with WCA and RSV, recapitulating the effect seen during co-infection. Similarly, children given the live attenuated influenza vaccine had increased pneumococcal carriage, possibly due to increased CCR2 inflammatory monocytes which upregulate bacterial adherence receptors (37). Additionally, pneumococcal carriage also enhances secondary RSV infection. It has been shown that mice carrying pneumococcus prior to RSV infection exhibit much higher viral loads (38). Thus, the increase in IL-1β and RANTES levels by $VitD_3$ is important as this enhances clearance of viral infections such as RSV by recruiting T cells and monocytes to the site of infection (39).

Monocytes and NK cells are crucial in the clearance of RSV, both contributing to type-1 interferon release to reduce pathology mediated by RSV (40, 41). We found that, $VitD_3$ significantly reduced the number of CD14+ monocyte cells in response to WCA or RSV but not for WCA-RSV although a trend toward lower numbers was observed. In contrast, we found $VitD_3$ had increased overall TLR4 expression as well as on CD56+TLR4+ cells following co-stimulation. TLR4 on innate cells such as monocytes and NK cells bind LPS which initiates pro-inflammatory responses to pneumococcus and may also interact with the F-protein on RSV, respectively. As macrophage depletion in secondary pneumococcal infection increases pneumococcal dissemination, increased TLR4 expression may be beneficial in reducing its capacity to spread (42–44). In CD14+ monocytes, both WCA and RSV stimulation alone had decreased TLR2 expression by $VitD_3$, while total TLR2 and TLR4 populations were reduced by $VitD_3$ in unstimulated cells. The expression of TLR2 on epithelial cells is upregulated following RSV infection and plays an important role in innate activation. TLR2 deficient mice show impaired neutrophil migration and pro-inflammatory cytokine production by macrophages, alongside uncontrolled RSV replication (45). Therefore, $VitD_3$ appears to restore TLR2 responsiveness by upregulating VDR transcription factors to influence macrophage and neutrophil activity (42). $VitD_3$ also increased CD3 expression on T cells while lowering CD19 expression on B cells for WCA only suggesting that $VitD_3$ may also have important regulatory roles in terms of T cell differentiation and antibody production. Prior studies have demonstrated that $VitD_3$ can modulate certain T-helper cell populations in the context of pneumococcal stimulation (18), but the implications on B cell function require further investigation. As we did not measure cell proliferation, we cannot rule out the possibility that the anti-inflammatory effect of $VitD_3$ may also involve effects on proliferation.

Interestingly, we found that TLR7 on CD14+ monocytes and TLR4 on CD56+ cells was significantly upregulated by $VitD_3$ for RSV and WCA-RSV, while TLR2 was reduced, suggesting that in response to pathogen encounter, $VitD_3$ may differentially effect bacterial and viral pattern recognition receptors. RSV has a number of surface proteins that can bind directly with TLR4 and/or TLR7, as well as intracellular receptors such as RIG-I (46), suggesting that $VitD_3$ may be important in regulating viral or viral-bacterial co-infection. Indeed, $VitD_3$ has been shown to activate antiviral RIG-I pathways during rotavirus infection of pigs (47). While TLR7 is mainly expressed intracellularly, it has also been shown to be expressed on the surface of immune cells (48). Moreover, previous studies have shown that TLR7 responses are impaired in otherwise healthy individuals with low vitamin D levels (49). Recent evidence has shown that airway neutrophil influx following RSV infection mediates anti-bacterial effects in relation to pneumococcus (50). We previously found that $VitD_3$ was able to reduce neutrophil migration (24), but how $VitD_3$ might regulate this response at the respiratory mucosa during RSV-pneumococcal infection is an important question that remains unanswered.

Respiratory infections are most prevalent during winter when $VitD_3$ status is lowest in individuals. Multiple epidemiological studies have suggested $VitD_3$ deficiency to be an associated risk factor for susceptibility to respiratory diseases (51, 52). Novel strategies to prevent and/or reduce pneumococcal inflammatory responses are important in the context of secondary viral infections and disease (53). The effect of $VitD_3$ in co-infection models is unknown, and further research into the potential benefits are required (54, 55).

Our study has several limitations, the main one being the small sample size. Despite this, we were still able to demonstrate the anti-inflammatory effects of $VitD_3$, similar to other studies (21, 56). While we did not measure $VitD_3$ status in our cohort, we have previously shown that most adults in our setting are $VitD_3$ insufficient (24) which may resemble to some extent the $VitD_3$ status in other geographical settings. Our results also need to be interpreted with caution as we examined $VitD_3$ effects in adults which may not directly translate into a pediatric population (57). Further, studies in high burden settings or high risk groups [e.g., preterm infants; (58)] are required to fully investigate the role of $VitD_3$ to protect against severe respiratory infection during early life.

CONCLUSION

Our results suggest that $VitD_3$ has important biological effects in the context of bacterial stimulation but was less effective for bacterial-viral co-stimulation, through modulation of innate and adaptive responses important for protection. This effect of $VitD_3$ was associated with its effects on Th17 cells as well as expression of TLR responses on key innate cells. Populations most at risk from respiratory infection are generally $VitD_3$ deficient, and this is associated with increased cytokine responses that promote disease severity. Future studies should aim to examine the effect of $VitD_3$ during co-infection, using PBMCs from pediatric cohorts to better determine the potential efficacy of $VitD_3$ trials in high-risk populations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee, Royal Children's Hospital, Melbourne, Australia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JA, LD, and PL conceived the study design and prepared the first draft of the manuscript. JA, ZT, EH, and AR performed the experiments. KM provided advice on study and edited the manuscript. All authors edited and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00602/full#supplementary-material

REFERENCES

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet*. (2009) 374:893–902. doi: 10.1016/S0140-6736(09)61204-6
2. Shi T, McAllister DA, O'Brien KL, Simoes EAF, Madhi SA, Gessner BD, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. *Lancet*. (2017) 390:946–58. doi: 10.1016/S0140-6736(17)30938-8
3. Cebey-Lopez M, Pardo-Seco J, Gomez-Carballa A, Martinon-Torres N, Martinon-Sanchez JM, Justicia-Grande A, et al. Bacteremia in children hospitalized with respiratory syncytial virus infection. *PLoS ONE*. (2016) 11:e0146599. doi: 10.1371/journal.pone.0146599
4. Weinberger DM, Klugman KP, Steiner CA, Simonsen L, Viboud C. Association between respiratory syncytial virus activity and pneumococcal disease in infants: a time series analysis of US hospitalization data. *PLoS Med*. (2015) 12:e1001776. doi: 10.1371/journal.pmed.1001776
5. de Steenhuijsen Piters WA, Heinonen S, Hasrat R, Bunsow E, Smith B, Suarez-Arrabal MC, et al. Nasopharyngeal microbiota, host transcriptome and disease severity in children with respiratory syncytial virus infection. *Am J Respir Crit Care Med*. (2016) 194:1104–15. doi: 10.1164/rccm.201602-0220OC
6. Stark JM, Stark MA, Colasurdo GN, LeVine AM. Decreased bacterial clearance from the lungs of mice following primary respiratory syncytial virus infection. *J Med Virol*. (2006) 78:829–38. doi: 10.1002/jmv.20631
7. Lundgren A, Bhuiyan TR, Novak D, Kaim J, Reske A, Lu YJ, et al. Characterization of Th17 responses to *Streptococcus pneumoniae* in humans: comparisons between adults and children in a developed and a developing country. *Vaccine*. (2012) 30:3897–907. doi: 10.1016/j.vaccine.2012.03.082
8. Ampofo K, Bender J, Sheng X, Korgenski K, Daly J, Pavia AT, et al. Seasonal invasive pneumococcal disease in children: role of preceding respiratory viral infection. *Pediatrics*. (2008) 122:229–37. doi: 10.1542/peds.2007-3192
9. Lang PO, Aspinall R. Can we translate vitamin D immunomodulating effect on innate and adaptive immunity to vaccine response? *Nutrients*. (2015) 7:2044–60. doi: 10.3390/nu7032044
10. Sundaram ME, Coleman LA. Vitamin D and influenza. *Adv Nutr*. (2012) 3:517–25. doi: 10.3945/an.112.002162
11. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol*. (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
12. Chun RF, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol*. (2014) 5:151. doi: 10.3389/fphys.2014.00151
13. Camargo CA Jr, Ganmaa D, Frazier AL, Kirchberg FF, Stuart JJ, et al. Randomized trial of vitamin D supplementation and risk of acute respiratory infection in Mongolia. *Pediatrics*. (2012) 130:e561–7. doi: 10.1542/peds.2011-3029
14. Laaksi I, Ruohola JP, Mattila V, Auvinen A, Ylikomi T, Pihlajamaki H. Vitamin D supplementation for the prevention of acute respiratory tract infection: a randomized, double-blinded trial among young Finnish men. *J Infect Dis*. (2010) 202:809–14. doi: 10.1086/654881
15. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, et al. Vitamin D and health in adults in Australia and New Zealand: a position statement. *Med J Aust*. (2012) 196:686–7. doi: 10.5694/mja11.10301
16. Wei R, Christakos S. Mechanisms Underlying the regulation of innate and adaptive immunity by vitamin D. *Nutrients*. (2015) 7:8251–60. doi: 10.3390/nu7105392
17. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients*. (2013) 5:2502–21. doi: 10.3390/nu5072502
18. Olliver M, Spelmink L, Hiew J, Meyer-Hoffert U, Henriques-Normark B, Bergman P. Immunomodulatory effects of vitamin D on innate and adaptive immune responses to *Streptococcus pneumoniae*. *J Infect Dis*. (2013) 208:1474–81. doi: 10.1093/infdis/jit355
19. Vissers M, Habets MN, Ahout IM, Jans J, de Jonge MI, Diavatopoulos DA, et al. An in vitro model to study immune responses of human peripheral blood mononuclear cells to human respiratory syncytial virus infection. *J Vis Exp*. (2013) 82:e50766. doi: 10.3791/50766
20. Do LAH, Tse R, Nathanielsz J, Anderson J, Ong DS, Chappell K, et al. An improved and high throughput respiratory syncytial virus (RSV) micro-neutralization assay. *J Vis Exp*. (2019) doi: 10.3791/59025
21. Fitch N, Becker AB, HayGlass KT. Vitamin D [1,25(OH)2D3] Differentially regulates human innate cytokine responses to bacterial versus viral pattern recognition receptor stimuli. *J Immunol*. (2016) 196:2965–72. doi: 10.4049/jimmunol.1500460
22. Moffitt KL, Gierahn TM, Lu YJ, Gouveia P, Alderson M, Flechtner JB, et al. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. *Cell Host Microbe*. (2011) 9:158–65. doi: 10.1016/j.chom.2011.01.007
23. Moffitt KL, Malley R, Lu YJ. Identification of protective pneumococcal T(H)17 antigens from the soluble fraction of a killed whole cell vaccine. *PLoS ONE*. (2012) 7:e43445. doi: 10.1371/journal.pone.0043445
24. Hoe E, Nathanielsz J, Toh ZQ, Spry L, Marimla R, Balloch A, et al. Anti-inflammatory effects of vitamin D on human immune cells in the context of bacterial infection. *Nutrients*. (2016) 8:806. doi: 10.3390/nu8120806
25. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*. (2006) 203:2271–9. doi: 10.1084/jem.20061308
26. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med*. (2008) 14:275–81. doi: 10.1038/nm1710
27. Hoe E, Boelsen LK, Toh ZQ, Sun GW, Koo GC, Balloch A, et al. Reduced IL-17A secretion is associated with high levels of pneumococcal nasopharyngeal carriage in fijian children. *PLoS ONE*. (2015) 10:e0129199. doi: 10.1371/journal.pone.0129199

28. Zhao F, Hoechst B, Gamrekelashvili J, Ormandy LA, Voigtlander T, Wedemeyer H, et al. Human CCR4+ CCR6+ Th17 cells suppress autologous CD8+ T cell responses. *J Immunol.* (2012) 188:6055–62. doi: 10.4049/jimmunol.1102918
29. Brucklacher-Waldert V, Steinbach K, Lioznov M, Kolster M, Holscher C, Tolosa E. Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. *J Immunol.* (2009) 183:5494–501. doi: 10.4049/jimmunol.0901000
30. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol.* (2003) 3:253–7. doi: 10.1038/nri1032
31. Sehrawat S, Rouse BT. Interplay of regulatory T cell and Th17 cells during infectious diseases in humans and animals. *Front Immunol.* (2017) 8:341. doi: 10.3389/fimmu.2017.00341
32. Antunes G, Evans SA, Lordan JL, Frew AJ. Systemic cytokine levels in community-acquired pneumonia and their association with disease severity. *Eur Respir J.* (2002) 20:990–5. doi: 10.1183/09031936.02.00295102
33. Sommer A, Fabri M. Vitamin D regulates cytokine patterns secreted by dendritic cells to promote differentiation of IL-22-producing T cells. *PLoS ONE.* (2015) 10:e0130395. doi: 10.1371/journal.pone.0130395
34. Eklund D, Persson HL, Larsson M, Welin A, Idh J, Paues J, et al. Vitamin D enhances IL-1beta secretion and restricts growth of *Mycobacterium tuberculosis* in macrophages from TB patients. *Int J Mycobacteriol.* (2013) 2:18–25. doi: 10.1016/j.ijmyco.2012.11.001
35. Liu PT, Schenk M, Walker VP, Dempsey PW, Kanchanapoomi M, Wheelwright M, et al. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS ONE.* (2009) 4:e5810. doi: 10.1371/journal.pone.0005810
36. Marriott HM, Gascoyne KA, Gowda R, Geary I, Nicklin MJ, Iannelli F, et al. Interleukin-1beta regulates CXCL8 release and influences disease outcome in response to *Streptococcus pneumoniae*, defining intercellular cooperation between pulmonary epithelial cells and macrophages. *Infect Immun.* (2012) 80:1140–9. doi: 10.1128/IAI.05697-11
37. Lin KL, Sweeney S, Kang BD, Ramsburg E, Gunn MD. CCR2-antagonist prophylaxis reduces pulmonary immune pathology and markedly improves survival during influenza infection. *J Immunol.* (2011) 186:508–15. doi: 10.4049/jimmunol.1001002
38. Nguyen DT, Louwen R, Elberse K, van Amerongen G, Yuksel S, Luijendijk A, et al. *Streptococcus pneumoniae* enhances human respiratory syncytial virus infection in vitro and in vivo. *PLoS ONE.* (2015) 10:e0127098. doi: 10.1371/journal.pone.0127098
39. Culley FJ, Pennycook AM, Tregoning JS, Dodd JS, Walzl G, Wells TN, et al. Role of CCL5 (RANTES) in viral lung disease. *J Virol.* (2006) 80:8151–7. doi: 10.1128/JVI.00496-06
40. Klein M, Obermaier B, Angele B, Pfister HW, Wagner H, Koedel U, et al. Innate immunity to pneumococcal infection of the central nervous system depends on toll-like receptor (TLR) 2 and TLR4. *J Infect Dis.* (2008) 198:1028–36. doi: 10.1086/591626
41. Goritzka M, Makris S, Kausar F, Durant LR, Pereira C, Kumagai Y, et al. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *J Exp Med.* (2015) 212:699–714. doi: 10.1084/jem.20140825
42. Sadeghi K, Wessner B, Laggner U, Ploder M, Tamandl D, Friedl J, et al. Vitamin D3 down-regulates monocyte TLR expression and triggers hyporesponsiveness to pathogen-associated molecular patterns. *Eur J Immunol.* (2006) 36:361–70. doi: 10.1002/eji.200425995
43. Yuan FF, Marks K, Wong M, Watson S, de Leon E, McIntyre PB, et al. Clinical relevance of TLR2, TLR4, CD14 and FcγRIIA gene polymorphisms in *Streptococcus pneumoniae* infection. *Immunol Cell Biol.* (2008) 86:268–70. doi: 10.1038/sj.icb.7100155
44. Verma R, Jung JH, Kim JY. 1,25-Dihydroxyvitamin D3 up-regulates TLR10 while down-regulating TLR2, 4, and 5 in human monocyte THP-1. *J Steroid Biochem Mol Biol.* (2014) 141:1–6. doi: 10.1016/j.jsbmb.2013.12.012
45. Kim TH, Lee HK. Innate immune recognition of respiratory syncytial virus infection. *BMB Rep.* (2014) 47:184–91. doi: 10.5483/BMBRep.2014.47.4.050
46. Klein Klouwenberg P, Tan L, Werkman W, van Bleek GM, Coenjaerts F. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. *Crit Rev Immunol.* (2009) 29:531–50. doi: 10.1615/CritRevImmunol.v29.i6.40
47. Zhao Y, Yu B, Mao X, He J, Huang Z, Zheng P, et al. Dietary vitamin D supplementation attenuates immune responses of pigs challenged with rotavirus potentially through the retinoic acid-inducible gene I signalling pathway. *Br J Nutr.* (2014) 112:381–9. doi: 10.1017/S000711451400097X
48. Kanno A, Tanimura N, Ishizaki M, Ohko K, Motoi Y, Onji M, et al. Targeting cell surface TLR7 for therapeutic intervention in autoimmune diseases. *Nat Commun.* (2015) 6:6119. doi: 10.1038/ncomms7119
49. Alvarez-Rodriguez L, Lopez-Hoyos M, Garcia-Unzueta M, Amado JA, Cacho PM, Martinez-Taboada VM. Age and low levels of circulating vitamin D are associated with impaired innate immune function. *J Leukoc Biol.* (2012) 91:829–38. doi: 10.1189/jlb.1011523
50. Sande CJ, Njunge JM. Airway response to respiratory syncytial virus has incidental antibacterial effects. *Nat Commun.* (2019) 10:2218. doi: 10.1038/s41467-019-10222-z
51. Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, et al. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. *Am J Respir Crit Care Med.* (2011) 184:1342–9. doi: 10.1164/rccm.201107-1239OC
52. Pletz MW, Terkamp C, Schumacher U, Rohde G, Schutte H, Welte T, et al. Vitamin D deficiency in community-acquired pneumonia: low levels of 1,25(OH)2 D are associated with disease severity. *Respir Res.* (2014) 15:53. doi: 10.1186/1465-9921-15-53
53. Thorburn K, Harigopal S, Reddy V, Taylor N, van Saene HK. High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis. *Thorax.* (2006) 61:611–5. doi: 10.1136/thx.2005.048397
54. Beadling C, Slifka MK. How do viral infections predispose patients to bacterial infections? *Curr Opin Infect Dis.* (2004) 17:185–91. doi: 10.1097/00001432-200406000-00003
55. Madhi SA, Klugman KP. A role for *Streptococcus pneumoniae* in virus-associated pneumonia. *Nat Med.* (2004) 10:811–3. doi: 10.1038/nm1077
56. Dickie LJ, Church LD, Coulthard LR, Mathews RJ, Emery P, McDermott MF. Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes. *Rheumatology.* (2010) 49:1466–71. doi: 10.1093/rheumatology/keq124
57. Shen E, Wang M, Xie H, Zou R, Lin Q, Lai L, et al. Existence of Th22 in children and evaluation of IL-22 + CD4 + T, Th17, and other T cell effector subsets from healthy children compared to adults. *BMC Immunol.* (2016) 17:20. doi: 10.1186/s12865-016-0158-8
58. Onwuneme C, Molloy EJ. Question 2: vitamin D intake for preterm infants: how much do they really need? *Arch Dis Child.* (2018) 103:808–11. doi: 10.1136/archdischild-2018-315363

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REVIEW
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Vitamin D and Its Potential Interplay With Pain Signaling Pathways

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About 50 million of the U.S. adult population suffer from chronic pain. It is a complex disease in its own right for which currently available analgesics have been deemed woefully inadequate since ∼20% of the sufferers derive no benefit. Vitamin D, known for its role in calcium homeostasis and bone metabolism, is thought to be of clinical benefit in treating chronic pain without the side-effects of currently available analgesics. A strong correlation between hypovitaminosis D and incidence of bone pain is known. However, the potential underlying mechanisms by which vitamin D might exert its analgesic effects are poorly understood. In this review, we discuss pathways involved in pain sensing and processing primarily at the level of dorsal root ganglion (DRG) neurons and the potential interplay between vitamin D, its receptor (VDR) and known specific pain signaling pathways including nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), epidermal growth factor receptor (EGFR), and opioid receptors. We also discuss how vitamin D/VDR might influence immune cells and pain sensitization as well as review the increasingly important topic of vitamin D toxicity. Further *in vitro* and *in vivo* experimental studies will be required to study these potential interactions specifically in pain models. Such studies could highlight the potential usefulness of vitamin D either alone or in combination with existing analgesics to better treat chronic pain.

Keywords: nociception, DRG, VDR, NGF, EGFR, GDNF, opioids, vitamin D toxicity

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INTRODUCTION

The newly proposed definition of pain by the International Association for the Study of Pain states—"An aversive sensory and emotional experience typically caused by, or resembling that caused by, actual or potential tissue injury[1]". Pain represents the body's alarm system and serves as an alert to danger. Chronic pain is a complex disease in its own right for which currently available analgesics have been deemed woefully inadequate since ∼20% of the sufferers derive no benefit. This is thought to be due to the complexity and plasticity of chronic pain, and the underpinning mechanisms are not clear as yet. About 50 million of the U.S. adult population suffer from chronic pain, and regardless of the reason, the current treatments primarily manage the condition rather than provide a cure (1).

Vitamin D, commonly identified as a fat-soluble vitamin, is known for its role in calcium homeostasis and bone metabolism. Recent studies have linked vitamin D status and its receptor activity with several health conditions, including development of chronic pain,

[1]www.iasp.org (accessed March 20, 2020).

the leading cause of disability and disease burden globally (2). Studies on the association between vitamin D status and incidence of chronic pain have been contradictory (3, 4). There are potentially many reasons for the variations in the clinical trial studies. However, a recent well-controlled study in Europeans has shown that reduced vitamin D levels were significantly associated with painful diabetic peripheral neuropathy (5). A strong correlation is also shown for hypovitaminosis D and bone pain (6). Indeed, several other studies have now reported a progressive exacerbation of pain with decreasing serum vitamin D levels and conversely, by increasing serum vitamin D levels through appropriate vitamin D supplementation, especially in vitamin D deficient patients, leads to an improvement in pain-relief (see **Table 1**). However, the potential underlying mechanisms by which vitamin D might exert its analgesic effects are poorly understood. In this review, we discuss pathways involved in pain sensing and processing primarily at the level of DRG neurons and the potential interplay between vitamin D/VDR and known specific pain signaling pathways including nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), epidermal growth factor receptor (EGFR), and opioid receptors. We also discuss how vitamin D might influence immune cells and pain sensitization as well as include a section on the increasingly important topic of vitamin D toxicity.

VITAMIN D ABSORPTION, BIOSYNTHESIS AND TISSUE DISTRIBUTION

Vitamin D, although identified as a fat-soluble vitamin, is increasingly being recognized as a prohormone. Traditionally, vitamin D was considered to be essential for calcium and phosphate homeostasis and thereby, bone health, and its deficiency causes rickets and osteomalacia. Recent evidence, however, advocates a role for vitamin D that extends beyond bone metabolism (6). Our body obtains vitamin D both directly from the diet, albeit from a narrow range of food sources, and through biosynthesis in the skin. Vitamin D3 is the natural form of vitamin D and is produced from 7-dehydrocholesterol in the skin. UVB irradiation helps convert 7-dehydrocholesterol, the precursor in the skin, into pre-vitamin D3 and then to vitamin D3. Synthesis in skin serves as an essential source of vitamin D3 and depends crucially on season and geographical latitude (17). Studies involving human and pig models indicate that vitamin D3 is predominantly distributed in fat tissue (about three quarters) and in smaller amounts in muscles, liver, and skin. Distribution of 25-(OH)D3 was somewhat similar within compartments (serum, 30%; muscle, 20%; fat, 35% and 15% in other tissues) (18). The body fat content has also been reported to inversely correlate with serum levels of 25-(OH)D3, which could indicate that those with high body fat content (obese populations) might be at risk of vitamin D3 deficiency (19, 20).

VITAMIN D METABOLISM

Vitamin D3, by itself, is not biologically active. 1,25-dihydroxy vitamin D3 (1,25-(OH)2D3) is the biologically active form of vitamin D and is produced by hydroxylation of vitamin D3 in two distinct steps and locations. Once synthesized (or absorbed from diet), vitamin D and its metabolites are bound to vitamin D-binding proteins (DBPs), which allow their transport in the blood. DBPs transport vitamin D3 to the liver where the first hydroxylation reaction occurs at C-25 position to produce 25-(OH)D3. 25-(OH)D3 is the major circulating form of vitamin D and hence is used as a biomarker of vitamin D status in the body. CYP2R1 is the key 25-hydroxylase of vitamin D in the liver (21). 25-(OH)D3 is then transported to the kidney where it is filtered and taken up into proximal renal tubule epithelial cells by the action of cell surface receptors for DBP, and megalin/cubulin complex (22, 23). In proximal renal tubules, 25-(OH)D3 is hydroxylated at the C-1 position by renal 25-(OH)D3 1α hydroxylase (CYP27B1), to produce 1,25-(OH)2D3, which is also known as calcitriol and is biologically active (24, 25). The circulating levels of 1,25-(OH)2D3 is regulated by the action of CYP24A1, another hydroxylase of C-24, to generate 24,25-(OH)2D3, which can allow its excretion or reduce the pool of 1,25-(OH)2D3 available for C-1 hydroxylation (26). CYP24A1 also catalyzes conversion of 1,25-(OH)2D3 and 25-(OH)D3 into 1,25-(OH)2D3-26,23 lactone and 25-(OH)D3-26,23 lactone, respectively. Presence of CYP24A1 in most cells expressing vitamin D receptors (VDR), suggests that CYP24A1 can regulate not only the circulating concentrations of 1,25-(OH)2D3 but also modulate its cellular levels locally (27).

VITAMIN D RECEPTOR (VDR) IS A NUCLEAR TRANSCRIPTION FACTOR

Vitamin D mediates gene expression through interaction with its receptor, VDR. The VDR has been shown to recognize the sequence for vitamin D binding response element (VDRE) in the DNA and also forms a heterodimer with retinoid X receptor molecule. The effect of VDR is dependent on several other, as yet unknown, factors including enzymatic activities such as, demethyltransferase and RNA polymerase that mediate the promotion or inhibition of gene expression. Interestingly some of these target genes include those that bind 1,25(OH)2D3 to activate it or that mark it for its degradation, suggesting a self-regulating mechanism (28) and perhaps restricting the possibility for vitamin D toxicity due to over-exposure to sunlight or dietary intake. Other implicated target genes are those encoding for proteins involved in pain signaling pathways, such as growth factor receptors, ion channels, neurotrophic factors, and proteins involved in modulating neuronal axon growth, thereby implicating that vitamin D has a role in pain sensing and processing (see below).

DOES VITAMIN D PLAY A ROLE IN SENSING PAIN (NOCICEPTION)?

Pain is a subjective sensory experience that involves multiple signaling pathways (29–31). However, there are several categories of pain. In the proceeding sections we will discuss the interplay between vitamin D and different types of pain.

TABLE 1 | Examples of clinical studies showing benefit in pain-relief following vitamin D supplementation.

		Descriptors for population				Vitamin D treatment if any					
Type of pain	**Vitamin D status**	**Age (year)**	**Geographic location**	**Gender M/F**	**Study size *(n)***	**Type**	**Duration**	**Dose**	**Route**	**Main findings/conclusion**	**References**
Back pain in overweight/obese	Deficient	31.8 ± 8.9	Australia	31M, 18F	54	Cholecalciferol (D3)	16 weeks	100,000 IU—Bolus followed by 4,000 IU per day for 16 weeks	Oral	No change in backpain intensity was noted; however, in markedly vitamin D deficient subjects (<30 nmol/L), back pain disability score was significantly improved	(7)
Diabetic neuropathic pain	Insufficient	43–78	Turkey	34F, 23M	57	Cholecalciferol (D3)	12 weeks	300,000 IU	im	Improvement in pain associated with small diameter c-fiber neurons (electric shock pain and burning pain) was reported after a single dose of IM vitamin D3	(8)
Menstrual pain in dysmenorrhea patients	Insufficient; deficient; severely deficient	18–30	Turkey	F 100	100	Cholecalciferol (D3)	3 months	Up to 1,040 IU (insufficient); up to 1,950 IU (deficient); up to 2,990 IU (severely deficient) for 2 months. In the third month all patients received 780 IU	Oral	The vitamin-D replacement therapy led to a significant decrease in pain symptoms based on pain visual analog scale. Benefits was greatest in severely vitamin D deficient patients	(9)
Low back pain	Insufficient; deficient	44	India	M37, F31	68	Cholecalciferol (D3)	8 weeks	60,000 IU/week For those patient with vitamin D <5 ng/ml received 60,000 IU/day for 5 days and then 60,000 IU/week for the next 8 weeks. Treatment was stopped for patients who achieved vitamin D level >60 ng/ml	Oral	Significant reduction in pain (VAS) and improvement in functional ability was observed at 2, 3, and 6 months. Interestingly, progressive improvement from 2, 3 to 6 months are both VAS and functional ability	(10)
Cancer	Insufficient; deficient	62.4 ± 13	Sweden	36M, 42F	78	Cholecalciferol (D3) dissolved in Miglyol	3 months	4,000 IE/day	Oral	Improvements in pain management (reduced Fentanyl dose) was seen as early as 1 month after treatment. After 3 months, significantly reduced antibiotic usage	(11)
Osteoarthritis	Insufficient; deficient	64.58 ± 0.55	Thailand	17M, 158F	175	Ergocalciferol (D2)	6 months	40,000 IU/week	Oral	Ergocalciferol supplementation decreased pain (VAS), improved LDL cholesterol levels, reduced oxidative protein damage and improved quality of life in osteoarthritis patients	(12)
Growing pains	Insufficient; deficient	10	Italy	18M, 15F	33	Cholecalciferol (D3)	3 months	40,000–100,000 IU/week	Oral	After the first 3 months of vitamin D treatment significant >60% reduction in pain intensity was reported	(13)
Locomotion and daily activities in elderly	Insufficient	58–89	Romania	17M, 28W	45	Cholecalciferol (D3)	12 months	125 μg	Oral (fortified in bread)	Improvement in reported pain symptoms	(14)
Sickle cell disease	Insufficient; deficient	13.2 ± 3.1	USA	19M, 27F	39	Cholecalciferol (D3)	6 weeks	40,000–100,000 IU/week	Oral	Significant reduction in the number of pain-days per week was reported at week 8 following treatment with cholecaciferol supplementation	(15)
Chronic pain	Insufficient; deficient	44.5	USA	M 28	28	Cholecalciferol (D3) [insufficient group]; Ergocalciferol (D2) [deficient group];	3 months	D3 1,200 IU/day; D2 50,000 IU/week	Oral	Reduced pain and reduced number of pains was reported following vitamin D supplementation in patients with multiple areas of chronic pain	(16)

ACUTE PAIN

The ability to feel acute "normal pain," protecting the injured tissue of the body to facilitate recovery and the amelioration of the resulting pain from an injury, is one of mother nature's gift to mankind and the rest of animal kingdom. We evolved to feel pain because the sensation and pain-induced responses serve as an alarm system that is necessary for self-preservation, and this evolutionary advantage is maintained in every animal with a sensory nervous system.

CHRONIC PAIN

However, the "normal pain" can change to "abnormal pain" and thereby the benefit as a necessary alarm to self-preservation becomes instead incapacitating to the individual and is then seen as nature's curse. This change is often due to damage to the nervous system, resulting in a lowering of the pain threshold to otherwise innocuous stimuli such as warmth and gentle touch and is now recognized as painful (allodynia). Or, when the degree of pain from a painful stimulus becomes intensified/exaggerated (hyperalgesia). These "abnormal pain" conditions often fail to resolve within 3 months (chronic pain), long after the acute injury, and are generally intractable to conventional analgesia and become debilitating. Chronic pain is broadly defined as "pain caused by a lesion or disease of the somatosensory nervous system" (www.iaps.org). The lesion could be due to damage to the nervous system (as observed in neuropathic pain) or inflammation (as found in arthritis). There are several types of chronic pain- [chronic primary pain, chronic postsurgical and posttraumatic pain, chronic neuropathic pain, chronic headache and orofacial pain, chronic visceral pain, and finally chronic musculoskeletal pain (32)] of which lower back pain probably being one of the most frequently experienced chronic pain conditions, and has a clear association between deficiency in vitamin D levels and pain as a result of softening of the bone (6).

VISCERAL PAIN, VITAMIN D AND GUT MICROBIOTA

Similar to nociception where the stimuli are sensed by the skin (see section below on Pain Signaling), the lining of the gut is also an "inside out" interface to our external environment and is essential in visceral nociception. Visceral pain is defined as that which originates from the tissues of the internal organs in the body, and pathologically affects more than 20% of the world's population. There is some homology between visceral nociceptors and those in the skin. For example, similar to the skin, the DRG nerve fibers that innervate the serosa and the muscle layer in the intestine also synapse in the spinal cord (**Figure 1**). Most visceral nociceptors are highly sensitive to pro-inflammatory signals, ischemia, and to chemicals released from the ischemic tissues.

Thus, visceral nociceptors are exquisite sensors of local changes in blood flow (ischemia) and inflammation within an organ, both of which can induce chronic visceral pain. Visceral pain can also emanate from factors in the lumen of the gut, including the microbiota and products derived from them.

The luminal microbiota aids digestion and generates short-chain fatty acids (SCFAs) that are an important energy source for the host. In addition, some of these SCFAs also serve as signaling molecules in host immunity and pain signaling. For example, we have shown, in mice, that SCFA can induce the release of glucagon-like peptide-1 (GLP-1) from intestinal L-cells (33). GLP-1 is a pleiotropic hormone mainly known for stimulating insulin release and appetite suppression. However, recent studies show that the GLP-1 receptor is also expressed in the sensory nerve fibers (34) and GLP-1 agonists have an anti-inflammatory action and can ameliorate pain (35, 36).

Several gastrointestinal neurotransmitters, in addition to their physiological role, also impact on pain sensing mechanisms. For example, the neurotransmitter serotonin is involved in motility of the gut, to propel the content in the lumen. However, it also modulates pain-sensing through potentiating the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel (37, 38). Interestingly, the synthesis of serotonin in the enterochromaffin cell in the intestine is regulated in response to metabolites generated from gut microbiota (39). The importance of the gut microbiota in regulating gut motility was recently highlighted by Obata et al. (40). In this animal study it was shown that microbial colonization of the gut led to upregulation of several genes in the enteric neurons of the colon, including aryl hydrocarbon receptor, a transcription factor that regulates intestinal motility through neuronal programming (40). Furthermore, neuronal GPCRs that are involved in nociception are activated by SCFAs and other metabolites secreted or degraded from the gut microbiome (41, 42).

Under pathological conditions such as in irritable bowel syndrome, or in chemotherapy drug-induced mechanical hyperalgesia, a heightened pro-inflammatory state of the intestines is observed and results in exaggerated visceral pain. Thus, it is likely that the gut microbiota is an important regulator for the expression of pain-genes not only locally but also in distal organs, such as expression of Toll-like receptors (TLRs) and cytokines in the spinal cord that mediate visceral pain response, and are vital to the development of mechanical hyperalgesia (43, 44).

It is, therefore, perhaps unsurprising that dysbiosis of the gut microbiome results in chronic intestinal inflammation and other diseases. For example, the pathogenic bacteria- *Salmonella enterica*- commonly known to cause food poisoning, can disable the host's defenses against inflammation through activation of the TLR4. It also degrades intestinal alkaline phosphatase, an enzyme that is important in detoxifying endotoxin (lipopolysaccharide-phosphate) and thereby inducing gut microbiota-mediated autoimmunity and chronic inflammation (45).

Several studies indicate that vitamin D supplementation and/or deficiency changes the gut microbiota profile (46, 47); as such, this can potentially modulate visceral pain. Emerging data suggest vitamin D/VDR play a role in pain-sensing through modulating key pain-genes. Some of these pain-genes are common to both nociception

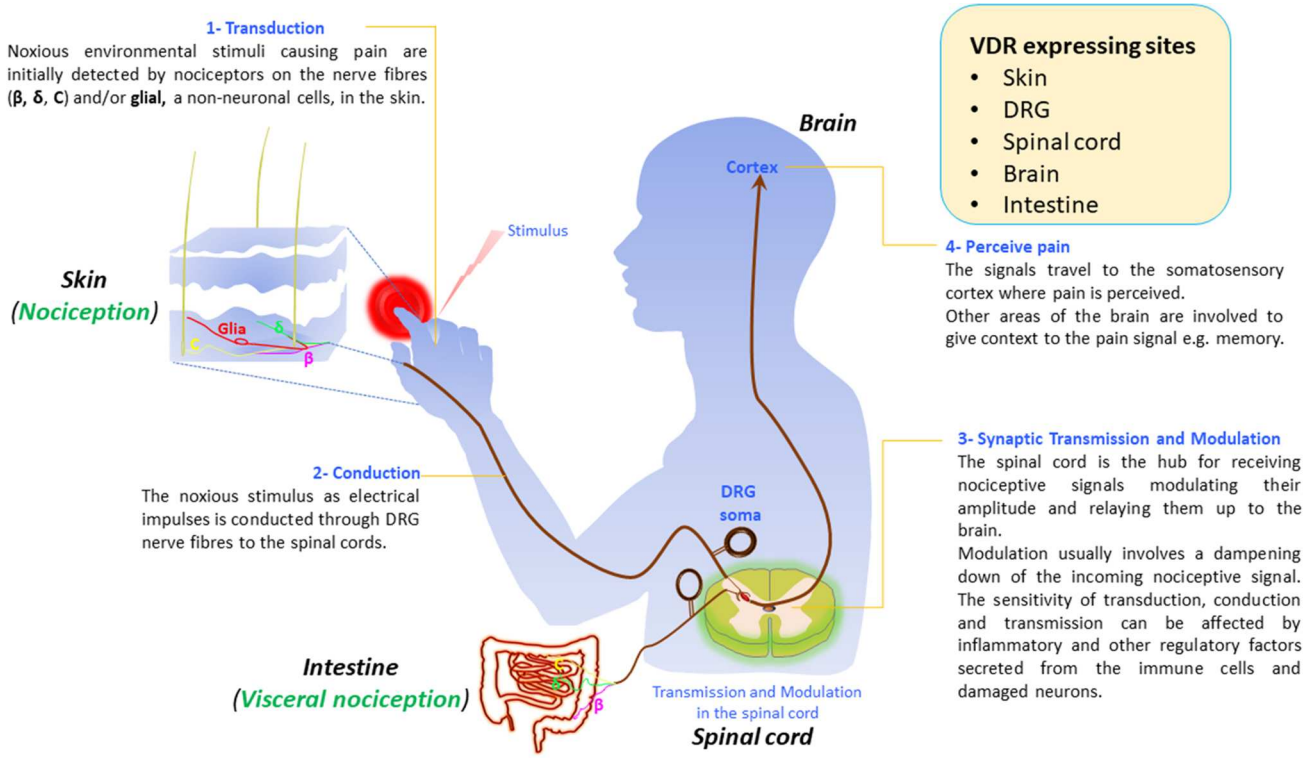

FIGURE 1 | Vitamin D and its receptor in pain signaling pathways. Vitamin D is synthesized in the skin. Vitamin D receptor (VDR) is expressed in neurons in the skin, dorsal root ganglia (DRG), spinal cord, brain, and the intestine. Nociceptors, from the specific area of the periphery and viscera respond to environmental nociceptive stimuli such as thermal, mechanical, or chemicals by converting the stimuli into a chemoelectrical signal (1). The signal travels as a nerve impulse through dorsal root ganglia (DRG) nerve fibers (c, β, and δ) from the skin to the spinal cord (2) where it gets transmitted to secondary neurons in the dorsal horns of the spinal cord (3). After modulating the signal, the secondary neurons relay it to the cortex where the message gets decoded and pain is perceived (4).

and visceral nociception, for example, TRPV1, Toll-like receptor, trophic factors, such as NGF, BDNF and GPCR. The interplay between vitamin D and these pain-genes are further discussed below.

SENSITIZATION- INTERPLAY BETWEEN IMMUNE AND NEURONAL CELLS

Spinal sensitization involves interactions between several neuronal and glial cells. The glial cells in the spinal cord play a significant role by responding to various cytokines and neurochemicals released from infiltrating macrophages, neutrophils and from the damaged peripheral nerve fibers. This response can be considered as part of protective measures, akin to acute pain, and vitamin D is thought to have a role in regulating the synthesis of cytokines (6).

There is growing evidence indicating that a family of TLR play a role in activating glial cells in the spinal cord and DRG neurons and thereby influence the sensitization process following nerve injury (48, 49). In a mouse neuropathic pain model of TLR2 knockout mice, the activity of the immune cells following the peripheral nerve injury is attenuated. Furthermore, reduced sensitivity to pain was observed in a behavior assay, suggesting that TLR2 could be a factor in neuropathic pain and in the activation of the glia in the spinal cord (50, 51). A similar result was also observed in TLR4 knockout mice (52). Vitamin D is known to be anti-inflammatory and inhibits the release of several cytokines and TLRs (53, 54). Expression of both receptors, TLR2 and TLR4, was shown to be modulated by vitamin D in human studies (55, 56). Identifying the molecular and neurochemical basis that cause/modulate the sensitization process may help enable us to identify therapeutic targets for the treatment of pain. In the next sections, we will focus on the potential ways by which vitamin D might play a role in the pain system.

PAIN SIGNALING

Skin is unique in that, apart from it being the most substantial organ system that protects the body from damage, it is also the largest damage-sensing organ and the only site for vitamin D synthesis in the body. Nociceptors, the specialized mammalian sensory neurons that react to tissue injury are

critical components of the somatosensory nervous system, pain-sensing machinery, proposed initially by Charles Sherrington a 100 years ago (57). These neurons with their nuclei in the dorsal root ganglia (DRG) and their fibers stretching into the skin, the gut, and other organs in the body convey the information about potential harm to the central nervous system. However, a more recent study indicates that the ability to initiate the signal for sensing damage to the tissue is no longer exclusive to nociceptive free nerve endings but can also occur through glial cell signaling (58). Nonetheless, the role of nociceptors in pain signaling has been validated by several studies over the years, and their healthy development is a prerequisite for sensing pain.

Glial and Schwann cells, together with nociceptors, constitute the peripheral nervous system. These neuron and non-neuronal cell complexes require a healthy vascular system that supplies oxygenated blood and nutrients through small blood vessels. Vitamin D supplementation is known to significantly improve vascular functions in type 2 diabetic patients with vitamin D deficiency (59). Vitamin D supplementation was also shown to enhance myelination of DRG neurons and to regulate expression of genes involved in axon growth in an animal model of nerve damage (60). More recently, the existence of a previously unknown sensory organ: the skin glial cell that is intimately associated with unmyelinated small thin fibers which sense pain, has been reported. These nociceptive glio-neural complexes have been shown to evoke pain signaling, as evidenced by whole-cell current recordings in response to mechanical pressure (58) and therefore are essential to our ability to sense damage to the skin. However, further validation of this ground-breaking finding in humans is required. Several studies have reported an association between vitamin D insufficiency with pain in general and or following injury (61–63); however, knowledge of the underpinning molecular mechanisms is limited.

VITAMIN D AND VDR IN PAIN SIGNALING PATHWAYS

There now exists several clinical studies (see **Table 1**) and some animal studies, mostly in rodents (see **Table 2**), that show that vitamin D deficiency leads to a worsening of pain whereas appropriate vitamin D supplementation leads to better outcomes relating to pain. However, there are no definitive studies with knockout mice lacking VDR or one of the key vitamin D metabolizing enzymes that have been performed which directly links vitamin D/VDR to pain pathways. This is most likely as a result of these knockout mice being infertile (68).

Nonetheless, the hypothesis that vitamin D perhaps influences pain-signaling pathways is biologically plausible because vitamin D and/or vitamin D receptor gene expression has been shown in relevant tissues such as skin (pain signal transduction), DRG neurons (conduction), spinal cord (transmission/modulation), and brain (pain perception) (see also **Figure 1**).

TABLE 2 | Selective examples of *in vivo* studies with Vitamin D receptor (VDR) KO mice and those examining the effect of Vitamin D and/or VDR on pain using animal models.

Vitamin D status	Pain model	Species	Strain	Age	Body weight (g)	Pain behavior assay	Vitamin D treatment if any			Main findings/conclusion	References
							Duration	Dose	Route		
Normal	Neuropathic pain	Rat	Sprague Dawley	NA	250–350	Mechanical nociceptive threshold method, Thermal cold allodynia	4 weeks	1,000 IU/kg	Gavage/diet	Vitamin D supplementation significantly reduced pain symptoms in monoarthiritic animals and accelerated recover from nerve injury compared with those on normal diet	(64)
Normal	Neuropathic pain (sciatic nerve injury)	Rat	Sprague Dawley	NA	200–250	Heat hyperalgesia (radian heat plantar), Cold (acetone), mechanical allodynia (von Frey)	Daily for 3 weeks post surgery	Up to 1,000 IU/kg	ip	Chronic vitamin D administration attenuates the behavioral scores of neuropathic pain	(65)
Deficient	Musculoskeletal and deep muscle Pain	Rat	Sprague Dawley	48 days	NA	Von Frey, Randall selitto test, paw pressure	4 weeks	2.2 IU/gm	Oral (diet)	Rats on cholecalciferol fortified diet showed less muscle pain than those on vitamin D deficient diet	(66)
Deficient (VDR KO)	NA	Mice	C57BL6	NA*	NA*	NA*	NA*	NA*	NA*	A viable transgenic animals using CRISPR Cas-9 were generated but infertile	(67)
Deficient (VDR KO)	NA	Mice	C57BL6	NA*	NA*	NA*	NA*	NA*	NA*	Vitamin D important in calcium homeostatic, bone formation as well as fertility	(68)

NA is information not available; NA is not applicable.*

VDR AND VITAMIN D REGULATING ENZYMES EXPRESSION IN TISSUES INVOLVED IN PAIN SIGNALING PATHWAYS

Vitamin D receptor expression has been reported in the peripheral and central neurons involved in pain-sensing and processing. Indeed, there appears to be a local cell or tissue-specific processing of vitamin D in the neuronal system. Expressions of the transcript for nuclear vitamin D receptor and/or the enzymes regulating the active form of vitamin D levels have been shown in DRG neuron bare nerve fiber endings in the skin and the soma (69–71), in the spinal cord neurons (71, 72), and the brain (71, 73–75). The level of VDR transcript in DRG neurons is higher when compared to the different regions in the brain in humans, and interestingly, this expression pattern is inversed in the mouse (71). The reason for this is unclear, but it is noteworthy as mouse models of pain are widely used in studying pain signaling. Vitamin D activity is determined by two enzymes, CYP27B1 (activates vitamin D in the kidney) and CYP24A1 (inactivates the active vitamin D) (70). These two enzymes, along with VDR, are also expressed in nociceptor neurons as well as in the brain.

VITAMIN D AND VDR IN PAIN SIGNAL TRANSDUCTION

Transduction of the pain signal involves the activation of ion channels that are expressed in nociceptive neurons in the skin in response to external stimuli, such as thermal, mechanical, or chemical inputs (see also **Figure 1**). Channels that are represented in the dermal and epidermal layers of the skin include the TRPV1, TRPV2, TRPV3, TRPV4 (for detecting heat); TRPM8, TRPA1 (for detecting cold), and TRPV4, TRPC (for detecting mechanical pressure sensing). The activation of these channels in response to stimuli leads to a transient change in cellular Ca^{2+} and Na^{+} concentration in the nociceptive neurons and thereby affecting their intrinsic electrical properties. The sensitivity of the skin to pain, touch or temperature differs depending on the sensory nerve ending whose sensitivity determines the kind of sensation evoked from the spot. The external stimulus detected by these receptor channels is then converted into an electrical signal and is transmitted along the axon by voltage-gated sodium channels. Therefore, for example, we and others have shown that a mutation in sodium channel Nav1.7 results in a complete loss of pain sensation (29, 76) or in extreme pain phenotypes (77, 78). Vitamin D, which is synthesized in the skin is thought to interact with nociceptive neuron nerve endings in the skin to directly sense noxious proinflammatory stimuli (79), and also to control TRPV1 channel activity in T cells (38, 80).

Taken together, the above findings suggest that VDR may play a role in modulating the expression of pain-genes, for example, those involved in the development of neurons and Schwann cells (60, 81) as well as ion channels expressed in nociceptor neurons that innervate the skin as discussed above. The presence of a tissue-specific "vitamin D enzyme system" that regulates vitamin D activity locally in specific tissues or cells including neuronal cells implies that the active vitamin D levels might be different from those in the general circulation. Therefore, alterations in the expression or function of vitamin D regulating enzymes, VDR expression, VDR targets in the skin and/or in sensory neurons or the associated glial cells could likely impact on chronic pain disorders listed above including neuropathic pain (82) and painful diabetic neuropathy (83).

THE INTERPLAY BETWEEN NGF AND VITAMIN D IN PAIN SIGNALING

Studies *in vivo* and *in vitro* have shown that vitamin D increases nerve growth factor (NGF) expression from the DRG neurons innervating the skin in rats (84), as well as in hippocampal neurons (85). NGF is a necessary neurotrophic factor for the development and maturity of the nociceptors. It is composed of three subunits, 2-α, 2-γ and 1-β, and primarily exists in its precursor proNGF form. Cells that produce NGF include macrophages, mast cells, and bone marrow-derived macrophages and keratinocytes. The levels of NGF in disease conditions seems to increase in response to inflammation (86, 87).

NGF's role in pain signaling has been clearly demonstrated in families with a hereditary mutation in NGF or its receptor, tropomyosin kinase receptor type A (TrkA) (which is a typical tyrosine kinase receptor), resulting in a pain-free phenotype and impairment in the sensing of temperature (88, 89). NGF also stimulates the release of calcitonin gene-related peptide (CGRP) from the DRG peripheral endings (90). CGRP is thought to promote and maintain sensitized nociceptive neurons which implicates its role in chronic pain. The sensitization is also enhanced by increasing the insertion of TRPV1, the heated gated ion channel into the cell surface membrane, which is facilitated by NGF (91).

Furthermore, the transcription level of various isoforms of sodium channels (e.g., Nav1.6, Nav1.7, Nav1.8, and Nav1.9) is modulated by NGF and ultimately results in an increase in sodium current density and the floodgates to nociception, primarily through Nav1.8. The development of hyperalgesia during inflammation is also thought to arise from an increase in Nav1.7 expression promoted by NGF (92, 93). Since Nav1.7 expression is restricted to DRG neurons only, selective drugs modulating its channel activity have the potential to be a useful therapy for chronic pain (29). In a more recent study, lack of NGF is reported to cause retraction of nociceptors from the skin (94), hence severely affecting pain signaling pathways.

These studies suggest that NGF is critical for nociceptor neuron development and in pain processing; however, it is not clear whether this is a direct effect of vitamin D on NGF or if the outcome is indirect via extranuclear or nuclear signaling pathways.

VITAMIN D IN REGULATING GDNF SIGNALING

The glial cell line-derived neurotrophic factor (GDNF) is yet another neurotrophic factor that is expressed in a small population of DRG neurons (95, 96) and is implicated in

promoting the survival and activity of large cutaneous sensory, proprioceptive neurons (97). In an animal model of neuropathic pain (spinal nerve ligation), GDNF has been shown to reverse the sensory abnormality induced by nerve damage and thereby ameliorate the pain, possibly thorough a tetrodotoxin (TTX) sensitive channel inhibition (98, 99). The neuropathic pain, following nerve damage is thought to be due to ectopic activity in the damaged myelinated TTX-sensitive neurons, i.e., those nociceptor neurons expressing, for example, fast-acting and fast-inactivating Nav1.7 channels. The utility of this channel has been exemplified in conditional deletion of Nav1.7 in nociceptive neurons, animals becoming pain-free in response to painful mechanical and inflammatory stimuli (100). Subsequent work by Minett et al. (101) showed a loss of pain-sensation to a noxious thermal stimulus. A similar pain phenotype has been documented in humans with inherited loss of function mutations in Nav1.7 (76). These findings demonstrate a central role for GDNF in pain signaling. Interestingly, a recent study shows that GDNF and its receptor, C-Ret are directly regulated by vitamin D (102). It is tempting to speculate that perhaps both vitamin D and its receptor could play a role in sodium channel-mediated neuropathic pain through modulating GDNF expression; however, experimental verification is needed.

EGFR SIGNALING IN PAIN PROCESSING: INTERPLAY WITH VITAMIN D?

The epidermal growth factor receptor (EGFR) and its downstream effectors have been recently identified as novel signaling pathways involved in pain processing (103) and their expression is also known to be regulated by the vitamin D pathway (104).

EGFR is a 175 KDa glycoprotein that is a member of the larger EGFR (also known as ErbB or HER) family of receptor tyrosine kinases which are important regulators of vital cellular processes such as cell growth, proliferation, differentiation, and apoptosis (105). The three other members of this family are known as EGFR2 (ErbB2, HER2), EGFR3 (ErbB3 or HER3), and EGFR 4 or (HER4) (106). EGFR signaling can be activated by either ligand-dependent or ligand-independent pathways such as via G-protein coupled receptor (GPCR) mediated transactivation that includes Angiotensin II (Ang II)-type 1 (AT1) receptors which are known to be involved in mediating several types of pain (107). In addition, EGFR transactivation has also been reported to occur via Beta-adrenergic receptors (108) as well as opioid and cannabinoid receptors (109–112) that also have important functions in regulating pain (see **Figure 2**).

For ligand-dependent EGFR activation, at least seven ligands have been described that bind to the mammalian receptor: Epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), heparin-binding EGF-like growth factor (HBEGF), betacellulin (BTC), amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN). All EGFR ligands are synthesized as precursors that are tethered to the plasma membrane and require extracellular domain matrix metalloproteinases (MMPs) or members of the disintegrin and metalloprotease (ADAM) family (115). The soluble ligands released can then bind to and activate the EGFR (see also **Figure 2**). Autophosphorylation at specific tyrosine sites within the intracellular tyrosine kinase domain induces EGFR homo- or hetero-dimerization with other EGFR family members. This results in subsequent activation of downstream signaling pathways including MAP kinases and PI3K/AKT/mTOR (106), both of which are also known to be involved in pain regulation (116, 117).

EGFR is expressed widely in the cells of the body including epithelial cells, pain-transmitting neurons and keratinocytes of the skin—a primary source of vitamin D for the body (118, 119).

Dysregulated EGFR signaling is well-known in leading to several cancers but there is now strong evidence for its importance in other pathologies that result in pain as well as in the underlying mechanisms of pain sensing and processing. For example, in diabetes, microvascular dysfunction/microangiopathy is critically involved in the etiology of disease-associated neuropathy and subsequent neuropathic pain (120, 121). In this regard, we were the first to show that increased EGFR signaling was a critical mediator of diabetes-induced microvascular dysfunction (122–124). Mechanistic studies from our group identified that EGFR signaling via multiple pathways including those already known to be involved in pain signaling, such as MAP kinases and PI3K/AKT, were critical mediators of diabetes-induced microvascular dysfunction (125–128). Further we showed that EGFR inhibition could prevent vascular dysfunction by normalizing up to 90% of the 1,100+ gene changes observed during the development of diabetes-induced microvascular dysfunction and that upregulation of EGFR appears to be a key early change leading to vascular pathology associated with diabetes (125, 129, 130). Thus, our work implied that treatment of diabetes-induced vascular dysfunction- a precursor to the development of disease associated neuropathy- with EGFR inhibitors might also be potentially useful in preventing or at least delaying diabetic neuropathy and attenuating neuropathic pain. Vitamin D has been shown to prevent vascular dysfunction in diabetes patients and is known to attenuate the development of neuropathic pain (5, 59) suggesting that common pathways might be responsible in mediating vascular dysfunction and pain. Indeed, there appears to be a significant overlap of EGFR signaling pathways involved in microvascular dysfunction and the more recent studies on the role of EGFR and its ligands in pain processing including chronic neuropathic pain (118, 131, 132).

Martin et al. (103) reported that increased EGFR phosphorylation and upregulation of its ligand epiregulin (EREG) occurred in mouse models of chronic pain. In an elegant study conducted in human subjects, mice, and Drosophila, it was shown that activation of EGFR, specifically by EREG (and not other ligands such as EGF), activates DRG neurons and pain behaviors via mechanisms involving the TRPV1-a non-selective cation channel involved in nociception (discussed above), the PI3K/AKT/mTOR pathway as well as the matrix metalloproteinase, MMP-9 (103)-a molecule previously known to have a role in mediating inflammation and the early stages of neuropathic pain (133). In several mouse models of pain,

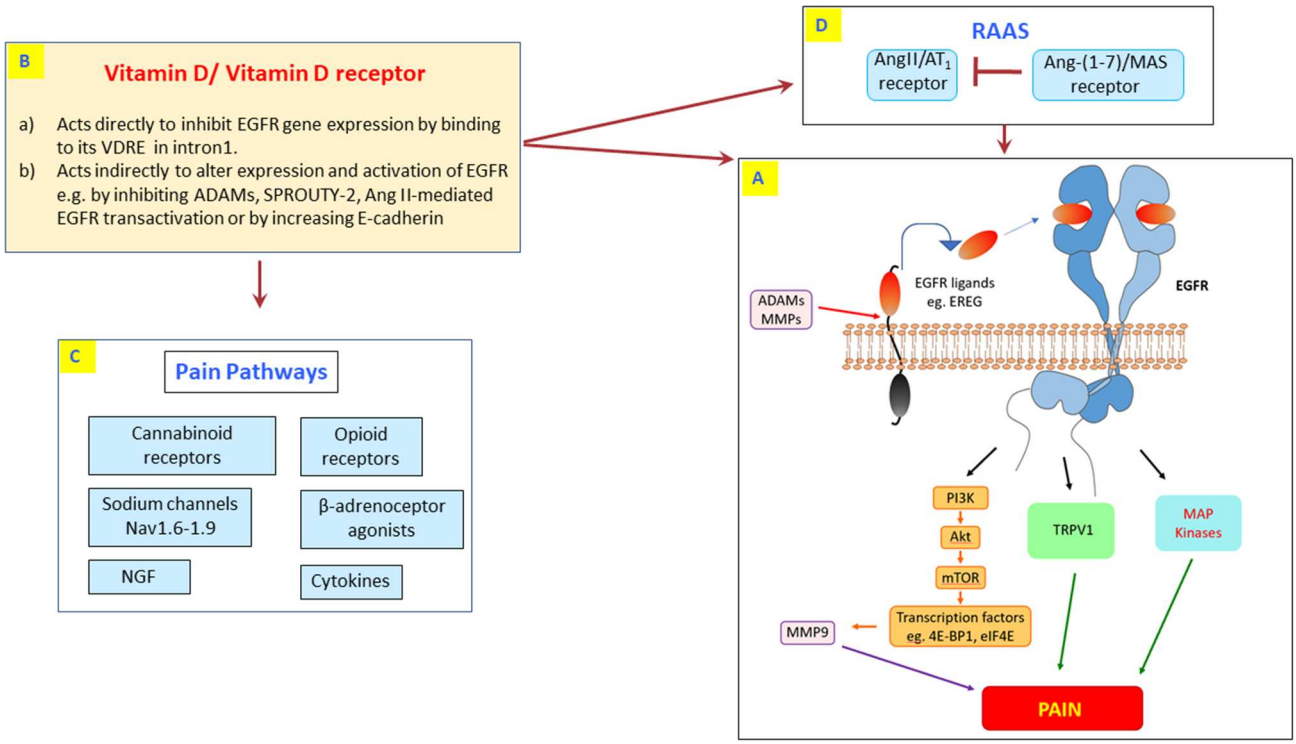

FIGURE 2 | The potential cross-talk between vitamin D and EGFR pain signaling. **(A)** Highlights the known EREG/EGFR signaling pathways involved in pain sensing and processing including downstream PI3K/AKT, TRPV1, MAP kinases, and MMP-9 effector molecules. Vitamin D might exert its analgesic effect through direct inhibition of EGFR mRNA expression via binding to its VDRE in intron 1 **(B)**. Alternatively, vitamin D can act indirectly through modulation of other intermediary signaling cascades that lead to inhibition/modulation of EGFR **(B–D)**. This includes vitamin D-mediated regulation of known pain signals via opioid receptors, cannabinoid receptors, beta-adrenoceptors, sodium channels, cytokines, and NGF **(C)** as well as inhibiting the Ang II/AT1 receptor-mediated transactivation by the renin-angiotensin-aldosterone system (RAAS; **D**). In the RAAS, activation of the Ang-(1-7)/Mas receptor can counter-regulate the actions of Ang II/AT1 receptor and also inhibit EGFR signaling (113, 114) **(D)**. Additionally, vitamin D can also inhibit EGFR by inhibiting ADAMs, SPROUTY-2, or by increasing E-cadherin (**B**; see main text for details).

intrathecal administration of EREG promoted nociception and led to pain hypersensitivity whereas treatment with preclinical (AG1478) or clinically used EGFR tyrosine kinase inhibitors (Gefitinib and Lapatinib) resulted in analgesia with efficacies and potencies comparable to that of morphine- an opioid analgesic. All EGFR inhibitors were effective in blocking inflammatory pain in two different animal models. They inhibited tonic inflammatory pain induced by formalin and completely attenuated thermal hypersensitivity induced by carrageenan- an inflammatory mediator. EGFR inhibitors were also completely and dose-dependently effective in reversing allodynia in three models of chronic pain including neuropathic pain. Knockdown of the EGFR ortholog specifically in peripheral neurons expressing pickpocket (ppk)—a nociceptive marker- in Drosophila resulted in prolonged withdrawal latencies in response to a heated probe at 46°C. This effect was reversed when EGFR gene was expressed in the neurons implying that the role of EGFR in pain processing is conserved across species. This study also showed that a single nucleotide polymorphism (rs1140475) in EGFR gene and one of its ligands (rs1563826 in epiregulin) was associated with development of chronic pain in humans suffering from temporomandibular disorder (103). Thus, this study demonstrated for the first time the direct association of EGFR and EREG with chronic pain in a clinical cohort of patients and further advocates the use of EREG/EGFR inhibitors as potential analgesics in the management of non-cancer pain in humans. These findings are supported by a recent study of lumbar radicular pain after disc herniation in rats, in which EREG was also thought to be released from herniated disc and mediated pain via PI3K/AKT pathway (134).

The analgesic effects of EGFR inhibition in several other human clinical studies have also been reported where diminished reporting of pain and an overall improvement in quality of life of patients was observed (131, 132, 135–137). In contrast to opioid analgesics, EGFR inhibitors showed no drug tolerance over the treatment period and the main side effect appeared to be skin rashes (131).

Taken together these studies suggest that EGFR is a key player in pain processing and sensation. Since it is already known that EGFR acts as an important hub and relay of signals from a variety of stimuli, its new role in processing pain signals would only provide added value to its proposed appellation as a "master hub or relay" of cell signaling (138) (see also **Figure 2**). Thus, inhibition of this important EGFR signaling hub by vitamin

D might explain the latter's analgesic effects. Indeed, there are several studies suggesting that vitamin D inhibits EGFR gene expression either directly or indirectly via other intermediary signaling pathways (104, 107, 139–141).

Vitamin D through its nuclear receptor can directly regulate expression of EGFR mRNA by binding to VDRE identified in intron 1 of the EGFR gene (104). Interestingly, this study identified EGFR as the primary vitamin D/VDR target gene in these cells (104). Indirect actions of vitamin D in regulating EGFR signaling have also been reported. $1\alpha,25(OH)_2D_3$ increases the level of E-cadherin protein at the plasma membrane, which downregulates EGFR (142, 143), or by decreasing levels of SPROUTY-2, a cytosolic protein that normally inhibits EGFR ubiquitination, internalization and degradation (144, 145). A vitamin D3 derivative increased nerve growth factor levels in streptozotocin-diabetic rats (84) that is known to inhibit EGFR gene expression (146). Vitamin D supplements such as paricalcitol (a vitamin D analog) have also been reported to decrease EGFR signaling via inhibition of ADAM17 (141)- a key cleaving enzyme that mediates the release of EGFR ligands from the plasma membrane. In renal tubular cells, paricalcitol inhibited aldosterone-induced EGFR transactivation, and its resulting proinflammatory effects via inhibition of TGF-α/ADAM-17 gene expression (141). The fact that anti-inflammatory actions of paricalcitol in tubular cells were dependent on the inhibition of TGF-α/ADAM17/EGFR pathway may explain why both EGFR inhibitors and vitamin D are effective in models of inflammatory pain (64, 103). Vitamin D can also inhibit GPCR-mediated EGFR transactivation by blocking the actions of members of the renin-angiotensin system such as Ang II (107). The presence of these direct and indirect regulatory mechanisms of vitamin D in reducing EGFR signaling in pain neurons is not known but if reproduced in neuronal tissue, they might account for how vitamin D exerts its pain relief.

As a recent genetic study has implicated polymorphisms in EGFR and its ligand, epiregulin, to the development of chronic pain (103), it might be noteworthy that low 25(OH)D3 levels are associated with EGFR mutations in pulmonary adenocarcinoma (147). Whether hypovitaminosis D is responsible for EGFR polymorphisms in pain models is not known and is worthy of further study.

As EGFR is differentially expressed (129), it should also be noted that vitamin D regulation of EGFR signaling is also likely to be cell or tissue specific. In contrast to the above studies showing vitamin D-induced inhibition of EGFR in several cell types, in BT-20 breast cancer cells $1,25(OH)_2D_3$ increases EGFR expression (148). Also, vitamin D supplementation with calcitriol led to the beneficial increase in neuregulin (a ligand for EGFR3 and 4 receptors) levels in the heart (149). Incidentally, Neuregulin is also required for remyelination and regeneration after nerve injury (150) and this may represent another mechanism by which vitamin D exerts its beneficial effects in models of neuropathic pain.

Like vitamin D, existing and newly discovered analgesic agents might also exert their pharmacological effects, at least in part, via EGFR inhibition. For example, opioids and cannabinoids are also known to regulate EGFR activity (109–112). Indeed, a novel role of cannabinoid receptor 2 in inhibiting EGF/EGFR pathway in breast cancer has recently been reported (111). Thus, the addition of vitamin D to the list of reagents modulating pain pathways via regulating EGFR signaling (see **Figure 2**) further implies that EGFR acts as a key convergent pathway in pain and as such might represent a novel pharmacological target for the development of new drugs and/or repurposing of existing anti-cancer EGFR inhibitors for pain therapy.

VITAMIN D AND REGULATION OF OPIOID SIGNALING

Despite the recent harrowing crisis of opioid dependency and its adverse sequalae in patients, opioids remain among the most widely used and indispensable analgesics for the treatment of moderate to severe pain. At present, no other oral therapeutic drug provides instant and effective relief of severe pain (151, 152). In rats, a vitamin D-deficiency diet was able to modulate the analgesic effect of the opioid, morphine- an effect that was blunted following administration of cholecalciferol (153). Cholecalciferol supplementation also induced dysregulation of opioid peptide genes, specifically POMC, PDYN, and PENK that code for endogenous opioids in the cerebrum (64, 154). In addition to modulating the expression of opioid agonists that are involved in pain perception, cholecalciferol supplementation did not affect the expression levels of opioid receptors. However, this supplementation was shown to dysregulate transcription levels of G protein subunits (Gαo) and effectors like subunits of the voltage-gated calcium channels (Cav2) and the adenylyl cyclase type 5 (AC5) enzymes as well as many other genes involved in opioid receptor signaling (64).

Calcium Channels

Voltage-gated Cav2 channels are effectors of opioid receptors that initiate their activity through direct binding of G protein subunits. Following receptor activation, the Gβγ dimer released from Gαi/o protein binds to the intracellular loop between the transmembrane (TM) TM I and TM II of Cav2α1 channel subunits (155, 156). This binding shifts channel activation to more depolarized potentials (157) and decreases the activation kinetics of the calcium currents (158, 159), resulting in reduced synaptic vesicle fusion with the plasma membrane for neurotransmitter release (160). This opioid modulation of channel activity is thus involved in the analgesic effect of opioids.

Functional studies showed that expression of delta opioid receptors (DORs) in sensory neurons from rat trigeminal (161) or dorsal root ganglia (162) inhibited voltage-gated calcium channels following stimulation with DOR agonists. In addition, characterizing the different subtypes of calcium channels subject to DOR inhibition was elucidated using specific toxins and nominated Cav2.1 (P/Q-type) and Cav2.2 (N-type) channels as notable effectors of DORs (163–165) in DRG sensory neurons. Both Cav2.1 and Cav2.2 are localized in axon presynaptic terminals and play an important role in synaptic transmission between somatosensory neurons and dorsal horn neurons (166). However, while Cav2.1 is found in large-diameter DRG neurons,

Cav2.2 is more expressed in small-diameter neurons (167–169). Surprisingly, similar to opioid receptors, vitamin D receptors have a widespread expression within the rat central nervous system and are found in high levels within distinct portions of the sensory, motor and limbic systems (72). Vitamin D receptors, like Cav2 channels, are also expressed in DRG neurons (69, 70, 170) and whether those receptors could affect opioid signaling by dimerizing with opioid receptors or by directly modulating the expression levels of Cav2 channels or other effectors, like the G protein-coupled inwardly rectifying potassium (GIRK or Kir3) channels involved in the analgesic effects of opioids (171–174), is still unknown.

Adenylate Cyclase

The cAMP signaling pathway regulated via the stimulation of AC enzymes is one of the key mediators of opioid actions (172, 175, 176). Membrane-bound AC isoforms are highly expressed in the central nervous system and have overlapping expression patterns with opioid receptors. In the striatum, stimulation of DORs or mu opioid receptors (MORs) which are found to be co-expressed with AC5 (177–179), inhibits cAMP production and causes locomotor activation (176, 180). Those responses were attenuated in mice lacking AC5 ($AC5^{-/-}$), making this enzyme an important component of the signal transduction mechanisms induced by opioid receptors.

cAMP signaling mediated by DORs may also contribute to the analgesic effects of opioids. In $AC5^{-/-}$ mice, the analgesic effect of acute opioid stimulation was reduced compared to wild-type mice (180). Moreover, those results were specific for the AC5 enzymes since acute pain responses did not differ in mice lacking either AC1, AC8 or both isoforms compared to wild-type animals (181). However, like DOR and AC5, the vitamin D receptor is also expressed in the striatum and the prefrontal cortex (72). In addition, cholecalciferol supplementation was shown to induce down-regulation of AC5 gene in the cerebrum (64) and might than define a potential effect of vitamin D on the analgesic effects of opioids via the cAMP pathway.

VITAMIN D LEVELS AND OPIOID USE

Low serum vitamin D levels have been linked to the development of opioid side effects and dependency. Among opioid-dependent patients recruited from the methadone maintenance treatment (MMT), more than half recorded a low vitamin D status (182). This common association deserves attention since high prevalence of chronic pain has also been observed in drug-dependent populations (183, 184). Chronic muscle and joint pain are common features of opioid withdrawal and are usually attributed to the effect of methadone (185). But, whether these conditions or other forms of chronic pain are affected by altered nutritional or metabolic factors related to vitamin D deficiency in drug-dependent populations is still unknown. Also, it should be noted that long-term therapy with some medications commonly used to treat chronic pain, such as steroids, antiepileptic drugs, and anticonvulsants can decrease levels of vitamin D (186–188). Therefore, it could be appropriate to recommend vitamin D supplements when levels are either deficient or insufficient with regards to the patient's medical conditions, current medications and skin exposure to sunlight. Nonetheless, vitamin D supplementation was recently shown to improve cognitive function and mental health in patients undergoing the MMT program (189).

Moreover, it has been suggested, using animal models, that ultraviolet B (UVB) light exposure induces strong analgesic effects due to elevation of endogenous opioids in the skin (190). This elevation in the POMC-derived peptide, β-endorphin, generated in response to UV radiation (191–193) was suggested to produce an opioid receptor-mediated addiction that leads to an increase in the nociceptive thresholds with pain relieving actions. However, since UVB light is responsible for the formation of vitamin D (194), it would be of interest to know if vitamin D plays a role in releasing β-endorphins to develop such addiction and if signaling pathways involved in this addiction are similar to those involved in exogenous opioid addiction.

It is known that sustained activation of opioid receptors results in compensatory mechanisms that increase cAMP production (195), referred to as AC superactivation- one of the key mechanisms leading to the development of physical dependence (196–198) and tolerance to opioids (172, 199–202). However, since cholecalciferol supplementation was shown to dysregulate AC5 gene expression (64), it might also be believed to play a critical role in modulating the development of opioid dependence and tolerance by affecting the AC superactivation mechanism. This correlation was partially proved by demonstrating the involvement of vitamin D deficiency in quickening the development of tolerance to opioid analgesics (153). In this study, rats raised for 3–4 weeks on a vitamin D-deficient rachitogenic diet developed tolerance to morphine faster than control rats, an effect that was prevented by cholecalciferol treatment.

Association between vitamin D levels and opioid use has also been investigated in human subjects. In an observational study, palliative cancer patients with 25-OHD levels <50 nmol/L required higher opioid (fentanyl) dose for pain relief compared to those with 25-OHD levels above 50 nmol/L (203). In a follow-up study, palliative cancer patients with 25-OHD levels <75 nmol/L were given vitamin D with a dose of 4,000 IU daily. After 1 month of supplementation, a decrease in fentanyl doses was observed compared to untreated controls (11). Together, these observations describe a significant correlation between low vitamin D levels and increased opioid consumption.

VITAMIN D TOXICITY

Both hypovitaminosis D (low levels of vitamin D) and hypervitaminosis D (high Levels; also known as vitamin D toxicity) are linked with unwanted effects and symptoms. Low levels of serum 25(OH)D3 concentration typically below 12 ng ml^{-1} (30 nmol l^{-1}) are associated with an increased risk of rickets/osteomalacia (204), however the precise serum levels of 25(OH)D3 that cause toxicity are not known. Those patients with serum levels >100 ng/ml are defined as being "at risk of toxicity"

whereas serum values >150 ng/ml are considered "toxic" (205–207). However, another report suggests that serum levels above 50 ng/ml may be "at risk of toxicity" (208).

Hypervitaminosis D is usually diagnosed from presenting symptoms (see below), high serum 25(OH)D3 levels as well as elevated serum and urine level of calcium and reduced serum level of intact parathyroid hormone. Vitamin D toxicity is more likely in the modern age where excessive screening and media attention have led to widespread (mis)use of vitamin D supplements including as a result of over-prescribing by physicians (209, 210). It is thought to arise from chronic use of high dose vitamin D supplements and not from abnormally high exposure of skin to the sun or from eating a regular diet (211, 212). Data from National Health and Nutrition Examination Survey (NHANES) of US residents gathered between 1999 and 2014 found a 3% increase in the number of subjects taking potentially unsafe amounts of vitamin D—more than 4,000 international units (IU) per day- and a nearly 18% increase in the number of people taking more than 1,000 IU of vitamin D daily, well exceeding the Institute of Medicine recommended dose of 600 to 800 IU (213). These findings are consistent with another report showing concentrations of 25(OH)D3 have also modestly increased over this time frame from a similar population data-set (214). As a result of increasing use of vitamin D usage, a substantial increase in the number of reports of vitamin D intoxication, with the majority (75%) of reports published since 2010 (207). Hypervitaminosis D may also result from medication/prescription errors as well as formulation errors such as under reporting (wrong strength) on labels by unlicensed manufacturers of supplements and fortified foods (207, 215, 216).

Hypervitaminosis D, by definition, arises from elevated plasma concentrations of 25(OH)D3 and its metabolites such as 24,25(OH), 25,26(OH)2D3, and 25(OH)D3-26,23-lactone (217). Although the exact role of the individual metabolites is not well-understood, vitamin D toxicity is thought to arise from a saturation of its catabolizing enzymes such as CYP24A1 and hyperactivation of the vitamin D/VDR signaling pathways especially those regulating calcium levels (210, 218, 219).

It appears that short excursions into very high vitamin D serum levels such as after a single megadose of 300,000 IU might be safe (220) whereas chronic high doses of vitamin D in human subjects results in the following clinical manifestations: GI abnormalities >90%, Azotaemia ∼80%, Polydipsia/Polyurea ∼50% Encephalopathy 40%, Nephrocalcinosis 40%, Pancreatitis ∼10% (207, 221, 222). Persistent vomiting as a result of vitamin D toxicity has also been recently reported (223). These clinical effects are largely thought to result from the vitamin-D-induced hypercalcemia that also leads to fatigue, generalized weakness and anorexia. The main mechanism of increased calcium in serum is due to vitamin D-induced bone resorption that can be blocked by bisphosphonates- known specific inhibitors of bone resorption- that are used clinically to treat hypercalcemia (224). In addition, hypervitaminosis D can also lead to increased absorption of dietary calcium from the GI tract, though this likely represents a minor contribution to the raised calcium levels in serum (224).

Hypervitaminosis D has been associated with increased risk of falls and bone fractures in post-menopausal women receiving high dose supplements (209). In extreme cases, vitamin D toxicity results in acute kidney injury (AKI). Chronic administration of mega doses (600,000 IU) has been shown to cause AKI and symptoms associated with hypercalcemia (210, 221, 225–227). In one study, 51 out of 62 patients receiving unusually high doses of vitamin D supplements developed AKI (226). The exact mechanisms for vitamin D associated renal complications are poorly understood but one rational explanation for the renal injury is hypercalcemia-induced severe dehydration. Renal toxicity may also be precipitated by and/or exacerbated by nephrocalcinosis (kidney stones) due to increased calcium deposition in renal tubules (228).

Vitamin D toxicity may also result from increased vitamin D/VDR signaling and/or decreased levels of catabolizing enzymes that could conceivably lead to hypervitaminosis D and subsequent toxic pharmacological effects (211, 212). However, as to what role is played by the "local vitamin D system" in cell/tissue specific toxicity remains poorly understood. For example, could overactivity of the "local vitamin D system" lead to a state of "local hypervitaminosis D" that then results in local cell/tissue toxicity? This would be important not only for its effects on the kidney but in other tissues such as in neurons involved in pain sensing and processing. Although, the contribution of the local vitamin D enzyme system to specific local tissue injury or damage is not well-understood, recent data suggests that the kidneys may be particularly prone to vitamin D toxicity. In addition to the normal uptake of vitamin D, the kidney proximal tubules also have the capability for re-uptake of DPB bound-vitamin D (DBP-D) through a Megalin and cubilin complex (229, 230). Thus, renal uptake of vitamin D by these multiple mechanisms could potentially result in higher accumulation and toxicity of vitamin D in the kidneys during hypervitaminosis D.

It should be noted, however, that vitamin D toxicity is rare and not all individuals with high serum 25(OH)D3 levels (even those with >150 ng/mL) manifest with clinical symptoms of vitamin D toxicity (24, 217, 218, 231, 232). Indeed, a recent study suggests that despite 167 patients having serum 25(OH)D3 levels >150 ng/mL, only 19 patients went on to develop vitamin D toxicity with AKI (210).

Vitamin D toxicity is more likely in extremes of age (children and elderly), in patients with impaired renal function, and those on certain prescription drugs such as thiazide diuretics (that decrease urinary calcium excretion) as their co-administration with vitamin D (that enhances intestinal calcium absorption) may theoretically result in hypercalcemia (233, 234).

Hypervitaminosis D and associated toxicity is largely preventable as it arises mainly from over-administration of high doses of vitamin D either by the patient, given by the physician/health care professional or because of manufacturer/formulation errors (207). Nonetheless, it is clear that further studies are needed to achieve a better understanding and definition of the precise cut-off levels for vitamin D toxicity in serum as well as in understanding the role of the local vitamin D system in mediating cell-specific toxicities ("local

hypervitaminosis D") that might arise even when circulating serum 25(OH)D3 levels are not abnormally high.

CONCLUDING REMARKS AND PERSPECTIVES

One in 5 of the adult population in the US is reported to suffer from chronic pain and 20% of these patients do not gain significant pain relief from currently available analgesic therapies. Vitamin D is speculated to provide clinical benefit in patients with chronic pain and several observational studies exist that have shown pain relief with vitamin D supplements (see **Table 1**). However, systematic reviews do not conclusively show patients benefit from vitamin D use in chronic pain (3, 235). There are multiple reasons for this discrepancy between the different clinical trials on vitamin D in pain which are discussed in more detail elsewhere [e.g., (3, 4)].

One of the concerns is the precise definition for serum 25(OH)D3 levels for its deficiency, for normal range and cut-off for toxicity. The difficulty in establishing pathophysiologically deficient 25(OH)D3 levels has been attributed to variations in methodological (statistical tools), the difference in experimental assays used (technical), and in the geographical latitude or other variations in the individuals being studied (236). Thus, it is argued that the so called "normal" range for serum 25(OH)D3 levels should be defined on an individual basis and in the clinical context (236). Serum variations can also arise from genetic polymorphisms in vitamin D processing enzymes and changes in vitamin D pharmacokinetics and pharmacodynamics. Another layer of complexity can arise from the specific variations in the individuals' disease state and this is particularly important in chronic pain that exhibits extreme heterogeneity amongst individuals and its perception can be very personalized. This leads to great challenges in the accurate assessment of pain especially when relying on self-reporting of pain by sufferers. Thus, there is an urgent need for the development of reliable biological markers of pain that can be accurately used in assessing pain in clinical trials. There is therefore a need for well-controlled large randomized clinical trials that account for the many variations, including having reliable biomarkers of pain, to conclusively determine the analgesic benefit of vitamin D in chronic pain.

The underlying molecular mechanism by which vitamin D/VDR modulates pain are not fully understood. In this review we have highlighted the potential interaction of pain signaling genes/pathways that are regulated by vitamin D/VDR. The reviewed literature suggests that vitamin D and/or its receptor can play a key role in modulating pain. Known pain genes such as NGF, GDNF, and EGFR, and opioid signaling are important components in pain signaling. We present here literature evidence for how vitamin D/VDR signaling might cross-talk with and regulate these genes that are critical in pain signaling. Further experimental studies *in vitro* and *vivo* are necessary to study these potential interactions specifically in pain models. Such studies could highlight the potential usefulness of vitamin D either alone or in combination with existing analgesics to better treat chronic pain.

AUTHOR CONTRIBUTIONS

AH and SA conceived the idea for the review. AH, SA, KN, NT, and VS wrote and edited the final draft of the manuscript.

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REFERENCES

1. Dahlhamer J, Lucas J, Zelaya C, Nahin R, Mackey S, DeBar L, et al. Prevalence of chronic pain and high-impact chronic pain among adults - United States, 2016. *MMWR Morb Mortal Wkly Rep.* (2018) 67:1001–6. doi: 10.15585/mmwr.mm6736a2
2. Vos T, Allen C, Arora M. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet.* (2017) 390:1211–59. doi: 10.1016/S0140-6736(17)32154-2
3. Straube S, Derry S, Straube C, Moore RA. Vitamin D for the treatment of chronic painful conditions in adults. *Cochrane Database Syst Rev.* (2015) 2015:CD007771. doi: 10.1002/14651858.CD007771.pub3
4. Wu Z, Malihi Z, Stewart AW, Lawes CM, Scragg R. Effect of vitamin D supplementation on pain: a systematic review and meta-analysis. *Pain Phys.* (2016) 19:415–27.
5. Shillo P, Selvarajah D, Greig M, Gandhi R, Rao G, Wilkinson ID, et al. Reduced vitamin D levels in painful diabetic peripheral neuropathy. *Diabet Med.* (2019) 36:44–51. doi: 10.1111/dme.13798
6. Holick MF. Vitamin D deficiency. *N Engl J Med.* (2007) 357:266–81. doi: 10.1056/NEJMra070553
7. Brady SRE, Naderpoor N, de Courten MPJ, Scragg R, Cicuttini F, Mousa A, et al. Vitamin D supplementation may improve back pain disability in vitamin D deficient and overweight or obese adults. *Steroid Biochem Mol Biol.* (2019) 185:212–7. doi: 10.1016/j.jsbmb.2018.09.005
8. Sari A, Akdogan Altun Z, Arifoglu Karaman C, Bilir Kaya B, Durmus B. Does vitamin D affect diabetic neuropathic pain and balance? *J Pain Res.* (2020) 13:171–9. doi: 10.2147/JPR.S203176
9. Kucukceran H, Ozdemir O, Kiral S, Berker DS, Kahveci R, Ozkara A, et al. The impact of circulating 25-hydroxyvitamin and oral cholecalciferol treatment on menstrual pain in dysmenorrheic patients. *Gynecol Endocrinol.* (2019) 35:53–7. doi: 10.1080/09513590.2018.1490407
10. Ghai B, Bansal D, Kanukula R, Gudala K, Sachdeva N, Dhatt SS, et al. Vitamin D Supplementation in patients with chronic low back pain: an open label, single arm clinical trial. *Pain Phys.* (2017) 20:E99–105. doi: 10.36076/ppj.2017.1.E99
11. Helde-Frankling M, Höijer J, Bergqvist J, Björkhem-Bergman L. Vitamin D supplementation to palliative cancer patients shows positive effects on pain and infections-results from a matched case-control study. *PLoS ONE.* (2017) 12:e0184208. doi: 10.1371/journal.pone.0184208
12. Manoy P, Yuktanandana P, Tanavalee A, Anomasiri W, Ngarmukos S, Tanpowpong T, et al. Vitamin D supplementation improves quality of life and physical performance in osteoarthritis patients. *Nutrients.* (2017) 9:E799. doi: 10.3390/nu9080799

13. Morandi G, Maines E, Piona C, Monti E, Sandri M, Gaudino R, et al. Significant association among growing pains, vitamin D supplementation, and bone mineral status: results from a pilot cohort study. *J Bone Miner Metab*. (2015) 33:201–6. doi: 10.1007/s00774-014-0579-5
14. Costan AR, Vulpoi C, Mocanu V. Vitamin D fortified bread improves pain and physical function domains of quality of life in nursing home residents. *J Med Food.* (2014) 17:625–31. doi: 10.1089/jmf.2012.0210
15. Osunkwo I, Ziegler TR, Alvarez J, McCracken C, Cherry K, Osunkwo CE, et al. High dose vitamin D therapy for chronic pain in children and adolescents with sickle cell disease: results of a randomized double blind pilot study. *Br J Haematol*. (2012) 159:211–5. doi: 10.1111/bjh.12019
16. Huang W, Shah S, Long Q, Crankshaw AK, Tangpricha V. Improvement of pain, sleep, and quality of life in chronic pain patients with vitamin D supplementation. *Clin J Pain*. (2013) 29:341–7. doi: 10.1097/AJP.0b013e318255655d
17. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev*. (2016) 96:365–408. doi: 10.1152/physrev.00014.2015
18. Heaney RP, Horst RL, Cullen DM, Armas LA. Vitamin D3 distribution and status in the body. *J Am Coll Nutr.* (2009) 28:252–6. doi: 10.1080/07315724.2009.10719779
19. Oliai Araghi S, van Dijk SC, Ham AC, Brouwer-Brolsma EM, Enneman AW, Sohl E, et al. BMI and body fat mass is inversely associated with vitamin D levels in older individuals. *J Nutr Health Aging*. (2015) 19:980–5. doi: 10.1007/s12603-015-0657-y
20. Arunabh S, Pollack S, Yeh J, Aloia JF. Body fat content and 25-hydroxyvitamin D levels in healthy women. *J Clin Endocrinol Metab*. (2003) 88:157–61. doi: 10.1210/jc.2002-020978
21. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. *J Biol Chem.* (2003) 278:38084–93. doi: 10.1074/jbc.M307028200
22. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. *J Steroid Biochem Mol Biol.* (2014) 144:132–7. doi: 10.1016/j.jsbmb.2013.09.012
23. Nykjaer A, Fyfe JC, Kozyraki R, Leheste JR, Jacobsen C, Nielsen MS, et al. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D3. *Proc Natl Acad Sci USA*. (2001) 98:13895–900. doi: 10.1073/pnas.241516998
24. Jones G, Prosser DE, Kaufmann M. Cytochrome P450-mediated metabolism of vitamin. *D J Lipid Res.* (2014) 55:13–31. doi: 10.1194/jlr.R031534
25. Jones G, Prosser DG. The activating enzymes of vitamin D. *Vitamin D.* (2011) 45:23–42. doi: 10.1016/B978-0-12-381978-9.10003-4
26. Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, et al. Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D3 in rat kidney but not in intestine. *J Biol Chem.* (1992) 267:13757–62.
27. Jones G, Prosser DE, Kaufmann M. 25-Hydroxyvitamin D-24-hydroxylase (CYP24A1): its important role in the degradation of vitamin D. *Arch Biochem Biophys.* (2012) 523:9–18. doi: 10.1016/j.abb.2011.11.003
28. Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). *Endocrinol Metab Clin North Am.* (2010) 39:255-69. doi: 10.1016/j.ecl.2010.02.007
29. Habib AM, Wood JN, Cox JJ. Sodium channels and pain. *Handb Exp Pharmacol.* (2015) 227:39–56. doi: 10.1007/978-3-662-46450-2_3
30. Habib AM, Okorokov AL, Hill MN, Bras JT, Lee MC, Li S, et al. Microdeletion in a FAAH pseudogene identified in a patient with high anandamide concentrations and pain insensitivity. *Br J Anaesth.* (2019) 123:e249–53. doi: 10.1016/j.bja.2019.02.019
31. Habib AM, Matsuyama A, Okorokov AL, Santana-Varela S, Bras JT, Aloisi AM, et al. A novel human pain insensitivity disorder caused by a point mutation in ZFHX2. *Brain.* (2018) 141:365–76. doi: 10.1093/brain/awx326
32. Treede RD, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, et al. A classification of chronic pain for ICD-11. *Pain*. (2015) 156:1003–7. doi: 10.1097/j.pain.0000000000000160
33. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, et al. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. *Diabetes.* (2012) 61:364–71. doi: 10.2337/db11-1019
34. Anand U, Yiangou Y, Akbar A, Quick T, MacQuillan A, Fox M, et al. Glucagon-like peptide 1 receptor (GLP-1R) expression by nerve fibres in inflammatory bowel disease and functional effects in cultured neurons. *PLoS ONE*. (2018) 13:e0198024. doi: 10.1371/journal.pone.0198024
35. Que Q, Guo X, Zhan L, Chen S, Zhang Z, Ni X, et al. The GLP-1 agonist, liraglutide, ameliorates inflammation through the activation of the PKA/CREB pathway in a rat model of knee osteoarthritis. *J Inflamm*. (2019) 16:13. doi: 10.1186/s12950-019-0218-y
36. Lee YS, Jun HS. Anti-inflammatory effects of GLP-1-based therapies beyond glucose control. *Mediat Inflamm*. (2016) 2016:3094642. doi: 10.1155/2016/3094642
37. Loyd DR, Weiss G, Henry MA, Hargreaves KM. Serotonin increases the functional activity of capsaicin-sensitive rat trigeminal nociceptors via peripheral serotonin receptors. *Pain*. (2011) 152:2267–76. doi: 10.1016/j.pain.2011.06.002
38. Bertin S, Aoki-Nonaka Y, de Jong PR, Nohara LL, Xu H, Stanwood SR, et al. (2014). The ion channel TRPV1 regulates the activation and proinflammatory properties of $CD4^+$ T cells. *Nat Immunol.* (2014) 15:1055–63. doi: 10.1038/ni.3009.
39. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*. (2015) 161:264–76. doi: 10.1016/j.cell.2015.02.047
40. Obata Y, Castaño Á, Boeing S, Bon-Frauches AC, Fung C, Fallesen T, et al. Neuronal programming by microbiota regulates intestinal physiology. *Nature*. (2020) 578:284–9. doi: 10.1038/s41586-020-1975-8
41. Torres-Fuentes C, Golubeva AV, Zhdanov AV, Wallace S, Arboleya S, Papkovsky DB, et al. Short-chain fatty acids and microbiota metabolites attenuate ghrelin receptor signaling. *FASEB J.* (2019) 33:13546–59. doi: 10.1096/fj.201901433R
42. Suez J, Elinav E. The path towards microbiome-based metabolite treatment. *Nat Microbiol.* (2017) 2:17075. doi: 10.1038/nmicrobiol.2017.75
43. O'Mahony SM, Dinan TG, Cryan JF. The gut microbiota as a key regulator of visceral pain. *Pain*. (2017) 158(Suppl 1):S19–S28. doi: 10.1097/j.pain.0000000000000779
44. Shen S, Lim G, You Z, Ding W, Huang P, Ran C, et al. Gut microbiota is critical for the induction of chemotherapy-induced pain. *Nat Neurosci.* (2017) 20:1213–6. doi: 10.1038/nn.4606
45. Yang WH, Heithoff DM, Aziz PV, Sperandio M, Nizet V, Mahan MJ, et al. Recurrent infection progressively disables host protection against intestinal inflammation. *Science.* (2017) 358:eaao5610. doi: 10.1126/science.aao5610
46. Waterhouse M, Hope B, Krause L, Morrison M, Protani MM, Zakrzewski M, et al. Vitamin D and the gut microbiome: a systematic review of in vivo studies. *Eur J Nutr.* (2019) 58:2895–910. doi: 10.1007/s00394-018-1842-7
47. Zhu W, Yan J, Zhi C, Zhou Q, Yuan X. 1,25(OH)2D3 deficiency-induced gut microbial dysbiosis degrades the colonic mucus barrier in Cyp27b1 knockout mouse model. *Gut Pathog.* (2019). 11:8. doi: 10.1186/s13099-019-0291-z
48. Lacagnina MJ, Watkins LR, Grace PM. Toll-like receptors and their role in persistent pain. *Pharmacol Ther*. (2018) 184:145–58. doi: 10.1016/j.pharmthera.2017.10.006
49. Bruno K, Woller SA, Miller YI, Yaksh TL, Wallace M, Beaton G, et al. Targeting Toll-Like receptor-4 (TLR4)-an emerging therapeutic target for persistent pain states. *Pain.* (2018) 159:1908–15. doi: 10.1097/j.pain.0000000000001306
50. Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, et al. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem.* (2007) 282:14975–83. doi: 10.1074/jbc.M607277200
51. Shi XQ, Zekki H, Zhang J. The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. *Glia*. (2011) 59:231–41. doi: 10.1002/glia.21093
52. Stokes JA, Cheung J, Eddinger K, Corr M, Yaksh TL. Toll-like receptor signaling adapter proteins govern spread of neuropathic pain and recovery following nerve injury in male mice. *J Neuroinflamm.* (2013) 10:148. doi: 10.1186/1742-2094-10-148

53. Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J.* (2001) 15:2579–85. Review. doi: 10.1096/fj.01-0433rev
54. Saul L, Mair I, Ivens A, Brown P, Samuel K, Campbell JDM, et al. 1,25-Dihydroxyvitamin D 3 restrains CD4 + T cell priming ability of CD11c + dendritic cells by upregulating expression of CD31. *Front Immunol.* (2019) 10:600. doi: 10.3389/fimmu.2019.00600
55. Ojaimi S, Skinner NA, Strauss BJ, Sundararajan V, Woolley I, Visvanathan K. Vitamin D deficiency impacts on expression of Toll-like receptor-2 and cytokine profile: a Pilot Study. *J Transl Med.* (2013) 11:176. doi: 10.1186/1479-5876-11-176
56. Do JE, Kwon SY, Park S, Lee E-S. Effects of vitamin D on expression of Toll-like receptors of monocytes from patients with Behcet's disease. *Rheumatology.* (2008) 47:840–8. doi: 10.1093/rheumatology/ken109
57. Foulkes T, Wood JN. Pain genes. *PLoS Genet.* (2008) 4:e1000086. doi: 10.1371/journal.pgen.1000086
58. Abdo H, Calvo-Enrique L, Lopez JM, Song J, Zhang M-D, Usoskin D, et al. Specialized cutaneous schwann cells initiate pain sensation. *Science.* (2019) 365:695–9. doi: 10.1126/science.aax6452
59. Al-Mheid I, Patel R, Murrow J, Morris A, Rahman A, Fike L, et al. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. *J Am Coll Cardiol.* (2011) 58:186–92. doi: 10.1016/j.jacc.2011.02.051
60. Chabas JF, Stephan D, Marqueste T, Garcia S, Lavaut M-N, Nguyen C, et al. Cholecalciferol (Vitamin D3) improves myelination and recovery after nerve injury. *PLoS ONE.* (2013) 8:e65034. doi: 10.1371/journal.pone.0065034
61. Hirani V. Vitamin D status and pain: analysis from the health survey for England among English adults aged 65 years and over. *Br J Nutr.* (2012) 107:1080–4. doi: 10.1017/S0007114511003965
62. Mauck MC, Hu J, Sefton C, Swor RA, Peak DA, Jones JS, et al. Obesity increases the risk of chronic pain development after motor vehicle collision. *Pain.* (2019) 160:670–5. doi: 10.1097/j.pain.0000000000001446
63. Dzik KP, Skrobot W, Kaczor KB, Flis DJ, Karnia MJ, Libionka W, et al. Vitamin D deficiency is associated with muscle atrophy and reduced mitochondrial function in patients with chronic low back pain. *Oxid Med Cell Longev.* (2019) 2019:6835341. doi: 10.1155/2019/6835341
64. Poisbeau P, Aouad M, Gazzo G, Lacaud A, Kemmel V, Landel V, et al. Cholecalciferol (Vitamin D3) reduces rat neuropathic pain by modulating opioid signaling. *Mol Neurobiol.* (2019) 56:7208–21. doi: 10.1007/s12035-019-1582-6
65. Banafshe HR, Khoshnoud MJ, Abed A, Saghazadeh M, Mesdaghinia A. Vitamin D supplementation attenuates the behavioral scores of neuropathic pain in rats. *Nutr Neurosci.* (2019) 22:700–5. doi: 10.1080/1028415X.2018.1435485
66. Tague SE, Clarke GL, Winter MK, McCarson KE, Wright DE, Smith PG. Vitamin D deficiency promotes skeletal muscle hypersensitivity and sensory hyperinnervation. *J Neurosci.* (2011) 31:13728–38. doi: 10.1523/JNEUROSCI.3637-11.2011
67. Zhang T, Yin Y, Liu H, Du W, Ren C, Wang L, et al. Generation of VDR knock-out mice via Zygote injection of CRISPR/Cas9 system. *PLoS ONE.* (2016) 11:e0163551. doi: 10.1371/journal.pone.0163551
68. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, et al. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet.* (1997) 16:391–6. doi: 10.1038/ng0897-391
69. Filipović N, Ferhatović L, Marelja I, Puljak L, Grković I. Increased vitamin D receptor expression in dorsal root Ganglia neurons of diabetic rats. *Neurosci Lett.* (2013) 549:140–5. doi: 10.1016/j.neulet.2013.05.023
70. Tague SE, Smith PG. Vitamin D receptor and enzyme expression in dorsal root ganglia of adult female rats: modulation by ovarian hormones. *J Chem Neuroanat.* (2011) 41:1–12. doi: 10.1016/j.jchemneu.2010.10.001
71. Ray P, Torck A, Quigley L, Wangzhou A, Neiman M, Rao C, et al. Comparative transcriptome profiling of the human and mouse dorsal root Ganglia: an RNA-seq-based resource for pain and sensory neuroscience research. *Pain.* (2018) 159:1325–45. doi: 10.1097/j.pain.0000000000001217
72. Prufer K, Veenstra TD, Jirikowski GF, Kumar R. Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. *J Chem Neuroanat.* (1999) 16:135–45. doi: 10.1016/S0891-0618(99)00002-2
73. Eyles DW, Smith S, Kinobe R, Hewison M, McGrath JJ. Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. *J Chem Neuroanat.* (2005) 29:21–30. doi: 10.1016/j.jchemneu.2004.08.006
74. Garcion E, Wion-Barbot N, Montero-Menei CN, Berger F, Wion D. New clues about vitamin D functions in the nervous system. *Trends Endocrinol Metab.* (2002) 13:100–5. doi: 10.1016/S1043-2760(01)00547-1
75. Eyles DW, Liu PY, Josh P, Cui X. Intracellular distribution of the vitamin D receptor in the brain: comparison with classic target tissues and redistribution with development. *Neuroscience.* (2014) 268:1–9. doi: 10.1016/j.neuroscience.2014.02.042
76. Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, et al. An SCN9A channelopathy causes congenital inability to experience pain. *Nature.* (2006) 444:894–8. doi: 10.1038/nature05413
77. Emery EC, Habib AM, Cox JJ, Nicholas AK, Gribble FM, Woods CG, et al. Novel SCN9A mutations underlying extreme pain phenotypes: unexpected electrophysiological and clinical phenotype correlations. *J Neurosci.* (2015) 35:7674–81. doi: 10.1523/JNEUROSCI.3935-14.2015
78. McDermott LA, Weir GA, Themistocleous AC, Segerdahl AR, Blesneac I, Baskozos G. Defining the functional role of Na V 1.7 in human nociception. *Neuron.* (2019) 101:905–19 e8. doi: 10.1016/j.neuron.2019.01.047
79. Cohen JA, Edwards TN, Liu AW, Hirai T, Ritter Jones M, Wu J, et al. Cutaneous TRPV1 + neurons trigger protective innate type 17 anticipatory immunity. *Cell.* (2019) 178:919–32.e14. doi: 10.1016/j.cell.2019.06.022
80. Long W, Fatehi M, Soni S, Panigrahi R, Rees Kelly G, Philippaert K, et al. LIGHT. 258-LB: vitamin D directly regulates TRPV1 channel activity may play an important role in the autoimmune disease of type 1 diabetes. *Diabetes.* (2019) 68(Supplement 1). doi: 10.2337/db19-258-LB
81. Chabas JF, Alluin O, Rao G, Garcia S, Lavaut M-N, Jacques Risso J, et al. Vitamin D2 potentiates axon regeneration. *J Neurotrauma.* (2008) 25:1247–56. doi: 10.1089/neu.2008.0593
82. Harty BL, Monk KR. Unwrapping the unappreciated: recent progress in remak Schwann cell biology. *Curr Opin Neurobiol.* (2017) 47:131–7. doi: 10.1016/j.conb.2017.10.003
83. Basit A, Basit KA, Fawwad A, Shaheen F, Fatima N, Petropoulos IN, et al. Vitamin D for the treatment of painful diabetic neuropathy. *BMJ Open Diabetes Res Care.* (2016) 4:e000148. doi: 10.1136/bmjdrc-2015-000148
84. Riaz S, Malcangio M, Miller M, Tomlinson DR. A vitamin D(3) derivative (CB1093) induces nerve growth factor and prevents neurotrophic deficits in streptozotocin-diabetic rats. *Diabetologia.* (1999) 42:1308–13. doi: 10.1007/s001250051443
85. Gezen-Ak D, Dursun E, Yilmazer S. The effect of vitamin D treatment on nerve growth factor (NGF) release from hippocampal neurons. *Noro Psikiyatr Ars.* (2014) 51:157–62. doi: 10.4274/npa.y7076
86. Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *Br J Pharmacol.* (1997) 121:417–24. doi: 10.1038/sj.bjp.0701148
87. Mizumura K, Murase S. Role of nerve growth factor in pain. *Handb Exp Pharmacol.* (2015) 227:57–77. doi: 10.1007/978-3-662-46450-2_4
88. Einarsdottir E, Carlsson A, Minde J, Toolanen G, Svensson O, Solders G, et al. A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum Mol Genet.* (2004) 13:799–805. doi: 10.1093/hmg/ddh096
89. Carvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, et al. A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSAN5 neuropathy. *J Med Genet.* (2011) 48:131–5. doi: 10.1136/jmg.2010.081455
90. Lyengar S, Ossipov MH, Johnsona KW. The role of calcitonin gene–related peptide in peripheral and central pain mechanisms including migraine. *Pain.* (2017) 158:543–59. doi: 10.1097/j.pain.0000000000000831
91. Zhan X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J.* (2005) 24:4211–23. doi: 10.1038/sj.emboj.7600893
92. Gould HJ, Gould TN, England JD, Paul D, Liu ZP, Levinson SR. A possible role for nerve growth factor in the augmentation of sodium channels in models of chronic pain. *Brain Res.* (2000) 854:19–29. doi: 10.1016/S0006-8993(99)02216-7

93. Schaefer I, Prato V, Arcourt A, Taberner FJ, Lechner SG. Differential modulation of voltage-gated sodium channels by nerve growth factor in three major subsets of TrkA-expressing nociceptors. *Mol Pain*. (2018) 14:1744806918814640. doi: 10.1177/1744806918814640
94. Nocchi L, Portulano C, Franciosa F, Doleschall B, Panea M, Roy N, et al. Nerve growth factor-mediated photoablation of nociceptors reduces pain behavior in mice. *Pain*. (2019) 160:2305–15. doi: 10.1097/j.pain.0000000000001620
95. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci*. (2002) 3:383–94. doi: 10.1038/nrn812
96. Brumovsky P, Villar MJ, Hökfelt T. Tyrosine hydroxylase is expressed in a subpopulation of small dorsal root Ganglion neurons in the adult mouse. *Exp Neurol*. (2006) 200:153–65. doi: 10.1016/j.expneurol.2006.01.023
97. Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM. GDNF is an age-specific survival factor for sensory and autonomic *Neurons Neuron*. (1995) 15:821–8. doi: 10.1016/0896-6273(95)90173-6
98. Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB. Potent analgesic effects of GDNF in neuropathic pain states. *Science*. (2000) 290:124–7. doi: 10.1126/science.290.5489.124
99. Boucher TJ, McMahon SB. Neurotrophic factors and neuropathic pain. *Curr Opin Pharmacol*. (2001) 1:66–72. doi: 10.1016/S1471-4892(01)00010-8
100. Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, et al. Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proc Natl Acad Sci USA*. (2004) 101:12706–11. doi: 10.1073/pnas.0404915101
101. Minett MS, Nassar MA, Clark AK, Passmore G, Dickenson AH, Wang F, et al. Distinct Nav1.7-dependent pain sensations require different sets of sensory and sympathetic neurons. *Nat Commun*. (2012) 3:791. doi: 10.1038/ncomms1795
102. Pertile RAN, Cui X, Hammond L, Eyles DW. Vitamin D regulation of GDNF/Ret signaling in dopaminergic neurons. *FASEB J*. (2018) 32:819–28. doi: 10.1096/fj.201700713R
103. Martin LJ, Smith SB, Khoutorsky A, Magnussen CA, Samoshkin A, Sorge RE, et al. Epiregulin and EGFR interactions are involved in pain processing. *J Clin Invest*. (2017) 127:3353–66. doi: 10.1172/JCI87406
104. Shen Z, Zhang X, Tang J, Kasiappan R, Jinwal U, Li P, et al. The coupling of epidermal growth factor receptor down regulation by 1alpha,25-dihydroxyvitamin D3 to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells. *Mol Cell Endocrinol*. (2011) 338:58–67. doi: 10.1016/j.mce.2011.02.023
105. Roskoski R Jr. The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacol Res*. (2014) 79:34–74. doi: 10.1016/j.phrs.2013.11.002
106. Wee P, Wang Z. Epidermal growth factor receptor cell proliferation signaling pathways. *Cancers*. (2017) 9:52. doi: 10.3390/cancers 9050052
107. Dougherty U, Mustafi R, Sadiq F, Almoghrabi A, Mustafi D, Kreisheh M, et al. The renin-angiotensin system mediates EGF receptor-vitamin d receptor cross-talk in colitis-associated colon cancer. *Clin Cancer Res*. (2014) 20:5848–59. doi: 10.1158/1078-0432.CCR-14-0209
108. Nackley-Neely AG, Tan KS, Karamarie F, Patrick F, Luda D, William M. Catechol-O-methyltransferase inhibition increases pain sensitivity through activation of Both β2 and β3 adrenergic receptors. *Pain*. (2007) 128:199–208. doi: 10.1016/j.pain.2006.09.022
109. Araldi D, Ferrari LF, Levine JD. Role of GPCR (mu-opioid)-receptor tyrosine kinase (epidermal growth factor) crosstalk in opioid-induced hyperalgesic priming (type II). *Pain*. (2018) 159:864–75. doi: 10.1097/j.pain.0000000000001155
110. Weingaertner IR, Koutnik S, Ammer H. Chronic morphine treatment attenuates cell growth of human BT474 breast cancer cells by rearrangement of the ErbB signalling network. *PLoS ONE*. (2013) 8:e53510. doi: 10.1371/journal.pone.0053510
111. Elbaz M, Ahirwar D, Ravi J, Nasser MW, Ganju RK. Novel role of cannabinoid receptor 2 in inhibiting EGF/EGFR and IGF-I/IGF-IR pathways in breast cancer. *Oncotarget*. (2017) 8:29668–78. doi: 10.18632/oncotarget.9408
112. Ravi J, Elbaz M, Wani NA, Nasser MW, Ganju RK. Cannabinoid receptor-2 agonist inhibits macrophage induced EMT in non-small cell lung cancer by downregulation of EGFR pathway. *Mol Carcinog*. (2016) 55:2063–76. doi: 10.1002/mc.22451
113. Akhtar S, Yousif MH, Dhaunsi GS, Chandrasekhar B, Al-Farsi O, Benter IF. Angiotensin-(1-7) inhibits epidermal growth factor receptor transactivation via a Mas receptor-dependent pathway. *Br J Pharmacol*. (2012) 165:1390–400. doi: 10.1111/j.1476-5381.2011.01613.x
114. Akhtar S, Chandrasekhar B, Attur S, Dhaunsi GS,Yousif MHM, Benter IF. Transactivation of ErbB family of receptor tyrosine kinases is inhibited by angiotensin-(1-7) via its Mas receptor. *PLoS ONE*. (2015) 10:e0141657. doi: 10.1371/journal.pone.0141657
115. Singh B, Carpenter G, Coffey RJ. EGF receptor ligands: recent advances version 1. *F1000Res*. (2016) 5:F1000 Faculty Rev-2270. doi: 10.12688/f1000research.9025.1
116. Popiolek-Barczyk K, Mika J. Targeting the microglial signaling pathways: new insights in the modulation of neuropathic pain. *Curr Med Chem*. (2016) 23:2908–28. doi: 10.2174/0929867323666160607120124
117. Cheng JK, Ru-Rong J. Intracellular signaling in primary sensory neurons and persistent pain. *Neurochem Res*. (2008) 33:1970–8. doi: 10.1007/s11064-008-9711-z
118. Bikle DD. Vitamin D and the skin: Physiology and pathophysiology. *Rev Endocr Metab Disord*. (2012) 13:3–19. doi: 10.1007/s11154-011-9194-0
119. Ahrens JM, Jones JD, Nieves NJ, Mitzey AM, DeLuca HF, Clagett-Dame M. Differential activity of 2-methylene-19-nor vitamin D analogs on growth factor gene expression in rhino mouse skin and comparison to all-trans retinoic acid. *PLoS ONE*. (2017) 12:e0188887. doi: 10.1371/journal.pone.0188887
120. Tesfaye S, Chaturvedi N, Eaton SE, Ward JD, Manes C, Ionescu-Tirgoviste C, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med*. (2005) 352:341–50. doi: 10.1056/NEJMoa032782
121. Malik RA, Tesfaye S, Thompson SD, Veves A, Sharma AK, Boulton AJ, et al. Endoneurial localisation of microvascular damage in human diabetic neuropathy. *Diabetologia*. (1993) 36:454–9. doi: 10.1007/BF00402283
122. Benter IF, Yousif MH, Griffiths SM, Benboubetra M, Akhtar S. Epidermal growth factor receptor tyrosine kinase-mediated signalling contributes to diabetes-induced vascular dysfunction in the mesenteric bed. *Br J Pharmacol*. (2005) 145:829–36. doi: 10.1038/sj.bjp.0706238
123. Benter IF, Yousif MH, Hollins AJ, Griffiths SM, Akhtar S. Diabetes-induced renal vascular dysfunction is normalized by inhibition of epidermal growth factor receptor tyrosine kinase. *J Vasc Res*. (2005) 42:284–91. doi: 10.1159/000085904
124. Yousif MH, Benter IF, Akhtar S. The role of tyrosine kinase-mediated pathways in diabetes-induced alterations in responsiveness of rat carotid artery. *Autonomic Autacoid Pharmacol*. (2005) 25:69–78. doi: 10.1111/j.1474-8673.2004.00333.x
125. Benter IF, Benboubetra M, Yousif M, Hollins AJ, Canatan H, Akhtar S. Early inhibition of EGFR signaling prevents diabetes-induced up-regulation of multiple gene pathways in the mesenteric vasculature. *Vasc Pharmacol*. (2009) 51:236–45. doi: 10.1016/j.vph.2009.06.008
126. Benter IF, Sarkhou F, Al-Khaldi AT, Chandrasekhar B, Attur S, Dhaunsi GS, et al. The dual targeting of EGFR and ErbB2 with the inhibitor Lapatinib corrects high glucose-induced apoptosis and vascular dysfunction by opposing multiple diabetes-induced signaling changes. *J Drug Target*. (2015) 23:506–18. doi: 10.3109/1061186X.2015.1057150
127. Akhtar S, Yousif MHM, Dhaunsi GS, Sarkhouh F, Chandrasekhar B, Attur S, et al. Activation of ErbB2 and downstream signalling via rho kinases and ERK1/2 contributes to diabetes-induced vascular dysfunction. *PLoS ONE*. (2013) 8:e67813. doi: 10.1371/journal.pone.0067813
128. Yousif MH, Benter IF, Hares N, Canatan H, Akhtar S. Phosphoinositide 3-kinase mediated signalling contributes to development of diabetes-induced abnormal vascular reactivity of rat carotid artery. *Cell Biochem Funct*. (2006) 24:13–22. doi: 10.1002/cbf.1278
129. Akhtar S, Benter IF. The role of epidermal growth factor receptor in diabetes-induced cardiac dysfunction. *BioImpacts*. (2013) 3:5–9. doi: 10.5681/bi.2013.008
130. Akhtar S, Chandrasekhar B, Yousif MHM, Renno W, Benter IF, El-Hashim AZ. Chronic administration of nano-sized PAMAM dendrimers *in vivo*

inhibits EGFR-ERK1/2-ROCK signaling pathway and attenuate diabetes-induced vascular remodeling and dysfunction. *Nanomedicine*. (2019) 18:78–89. doi: 10.1016/j.nano.2019.02.012
131. Kersten C, Cameron MG, Laird B, Mjåland S. Epidermal growth factor receptor-inhibition (EGFR-I) in the treatment of neuropathic pain. *Br J Anaesth*. (2015) 115:761–7. doi: 10.1093/bja/aev326
132. Kersten C, Cameron MG, Bailey AG, Fallon MT, Laird BJ, Paterson V, et al. Relief of neuropathic pain through epidermal growth factor receptor inhibition: a randomized proof-of-concept trial. *Pain Med*. (2019) 20:2495–505. doi: 10.1093/pm/pnz101
133. Kawasaki Y, Xu ZZ, Wang X, Park JY, Zhuang ZY, Tan PH, et al. Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. *Nat Med*. (2008) 14:331–6. doi: 10.1038/nm1723
134. Kongstorp M, Schjølberg T, Jacobsen DP, Fred H, Gjerstad J. Epiregulin is released from intervertebral disks and induces spontaneous activity in pain pathways. *Pain Rep*. (2019) 4:718. doi: 10.1097/PR9.0000000000000718
135. Kersten C, Cameron MG, Mjåland S. Epithelial growth factor receptor (EGFR)-inhibition for relief of neuropathic pain-a case series. *Scand J Pain*. (2013) 4:3–7. doi: 10.1016/j.sjpain.2012.11.011
136. Erschbamer M, Pernold K, Olson L. Inhibiting epidermal growth factor receptor improves structural, locomotor, sensory, and bladder recovery from experimental spinal cord injury. *J Neurosci*. (2007) 27:6428–35. doi: 10.1523/JNEUROSCI.1037-07.2007
137. Kersten C, Cameron MG. Cetuximab alleviates neuropathic pain despite tumour progression. *BMJ Case Rep*. (2012) 2012:bcr1220115374. doi: 10.1136/bcr.12.2011.5374
138. Klapper LN, Kirschbaum MH, Sela M, Yarden Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv Cancer Res*. (2000) 77:25–79. doi: 10.1016/S0065-230X(08)60784-8
139. Tong WM, Kállay E, Hofer H, Hulla W, Manhardt T, Peterlik M, et al. Growth regulation of human colon cancer cells by epidermal growth factor and 1,25-dihydroxyvitamin D3 is mediated by mutual modulation of receptor expression. *Eur J Cancer*. (1998) 34:2119–25. doi: 10.1016/S0959-8049(98)00267-6
140. Tong WM, Hofer H, Ellinger A, Peterlik M, Cross HS. Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells: relevance for suppression of epidermal growth factor-stimulated cell growth. *Oncol Res*. (1999) 11:77–84.
141. Arcidiacono MV, Yang J, Fernandez E, Dusso A. The induction of C/EBPβ contributes to vitamin D inhibition of ADAM17 expression and parathyroid hyperplasia in kidney disease. *Nephrol Dial Transplant*. (2015) 30:423–33. doi: 10.1093/ndt/gfu311
142. Pálmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol*. (2001) 154:369–87. doi: 10.1083/jcb.200102028
143. Andl CD, Rustgi AK. No one-way street: cross-talk between e-cadherin and receptor tyrosine kinase (RTK) signaling: a mechanism to regulate RTK activity. *Cancer Biol Ther*. (2005) 4:28–31. doi: 10.4161/cbt.4.1.1431
144. Cabrita MA, Christofori G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis*. (2008) 11:53–62. doi: 10.1007/s10456-008-9089-1
145. Barbáchano A, Ordóñez-Morán P, García JM, Sánchez A, Pereira F, Larriba MJ, et al. SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumourigenicity. *Oncogene*. (2010) 29:4800–13. doi: 10.1038/onc.2010.225
146. Cohen G, Ettinger K, Lecht S, Lelkes PI, Lazarovici P. Transcriptional down-regulation of epidermal growth factor (EGF) receptors by nerve growth factor (NGF) in PC 12 cells. *J Mol Neurosci*. (2014) 54:574–85. doi: 10.1007/s12031-014-0388-2
147. Shin DY, Kim S, Park S, Koh JS, Kim CH, Baek H, et al. Serum 25-hydroxyvitamin D levels correlate with EGFR mutational status in pulmonary adenocarcinoma. *Endocr Relat Cancer*. (2014) 21:715–21. doi: 10.1530/ERC-14-0259
148. Desprez PY, Poujol D, Falette N, Lefebvre MF, Saez S. 1,25-Dihydroxyvitamin D3 increases epidermal growth factor receptor gene expression in BT-20 breast carcinoma cells. *Biochem Biophys Res Commun*. (1991) 176:1–6. doi: 10.1016/0006-291X(91)90880-G
149. Dang R, Guo Y, Zhu Y, Yang R, Cai H, Jiang P. Chronic administration of calcitriol enhanced neuregulin-1/ErbB signaling in rat myocardium. *Pharmazie*. (2016) 71:192–5.
150. Fricker FR, Lago N, Balarajah S, Tsantoulas C, Tanna S, Zhu N, et al. Axonally derived neuregulin-1 is required for remyelination and regeneration after nerve injury in adulthood. *J Neurosci*. (2011) 31:3225–33. doi: 10.1523/JNEUROSCI.2568-10.2011
151. Szigethy E, Knisely M, Drossman D. Opioid misuse in gastroenterology and non-opioid management of abdominal pain. *Nat Rev Gastroenterol Hepatol*. (2018) 15:168–80. doi: 10.1038/nrgastro.2017.141
152. Chen Q, Larochelle MR, Weaver DT, Lietz AP, Mueller PP, Mercaldo S, et al. Prevention of prescription opioid misuse and projected overdose deaths in the United States. *JAMA Netw Open*. (2019) 2:e187621. doi: 10.1001/jamanetworkopen.2018.7621
153. Bazzani C, Arletti R, Bertolini A. Pain threshold and morphine activity in vitamin D-deficient rats. *Life Sci*. (1984) 34:461–6. doi: 10.1016/0024-3205(84)90501-0
154. Rossier J. Opioid peptides have found their roots. *Nature*. (1982) 298:221–2. doi: 10.1038/298221a0
155. De Waard M, Liu H, Walker D, Scott VE, Gurnett CA, Campbell KP. Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature*. (1997) 385:446–50. doi: 10.1038/385446a0
156. Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP. Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. *Nature*. (1997) 385:442–6. doi: 10.1038/385442a0
157. Bean BP. Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature*. (1989) 340:153–6. doi: 10.1038/340153a0
158. Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca^{2+} channels by G-protein beta gamma subunits. *Nature*. (1996) 380:258–62. doi: 10.1038/380258a0
159. Ikeda SR. Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature*. (1996) 380:255–8. doi: 10.1038/380255a0
160. Sudhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci*. (2004) 27:509–47. doi: 10.1146/annurev.neuro.26.041002.131412
161. Pettinger L, Gigout S, Linley JE, Gamper N. Bradykinin controls pool size of sensory neurons expressing functional delta-opioid receptors. *J Neurosci*. (2013) 33:10762–71. doi: 10.1523/JNEUROSCI.0123-13.2013
162. Bardoni R, Tawfik VL, Wang D, François A, Solorzano C, Shuster SA, et al. Delta opioid receptors presynaptically regulate cutaneous mechanosensory neuron input to the spinal cord dorsal horn. *Neuron*. (2014) 81:1443. doi: 10.1016/j.neuron.2014.03.006
163. Acosta CG, Lopez HS. Delta opioid receptor modulation of several voltage dependent Ca(2+) currents in rat sensory neurons. *J Neurosci*. (1999) 19:8337–48. doi: 10.1523/JNEUROSCI.19-19-08337.1999
164. Khasabova IA, Harding-Rose C, Simone DA, Seybold VS. Differential effects of CB1 and opioid agonists on two populations of adult rat dorsal root ganglion neurons. *J Neurosci*. (2004) 24:1744–53. doi: 10.1523/JNEUROSCI.4298-03.2004
165. Wu ZZ, Chen SR, Pan HL. Distinct inhibition of voltage-activated Ca^{2+} channels by delta-opioid agonists in dorsal root ganglion neurons devoid of functional T-type Ca^{2+} currents. *Neuroscience*. (2008) 153:1256–67. doi: 10.1016/j.neuroscience.2008.03.031
166. Simms BA, Zamponi GW. Neuronal voltage-gated calcium channels: structure, function, and dysfunction. *Neuron*. (2014) 82:24–45. doi: 10.1016/j.neuron.2014.03.016
167. Bell TJ, Thaler C, Castiglioni AJ, Helton TD, Lipscombe D. Cell-specific alternative splicing increases calcium channel current density in the pain pathway. *Neuron*. (2004) 41:127–38. doi: 10.1016/S0896-6273(03)00801-8
168. Murali SS, Napier IA, Mohammadi SA, Alewood PF, Lewis RJ, Christie MJ. High-voltage-activated calcium current subtypes in mouse DRG neurons adapt in a subpopulation-specific manner after nerve injury. *J Neurophysiol*. (2015) 113:1511–9. doi: 10.1152/jn.00608.2014
169. Yusaf SP, Goodman J, Pinnock RD, Dixon AK, Lee K. Expression of voltage-gated calcium channel subunits in rat dorsal root ganglion neurons. *Neurosci Lett*. (2001) 311:137–41. doi: 10.1016/S0304-3940(01)02038-9

170. Johnson JA, Grande JP, Windebank AJ, Kumar R. 1,25-Dihydroxyvitamin D(3) receptors in developing dorsal root ganglia of fetal rats. *Brain Res Dev Brain Res.* (1996) 92:120–4. doi: 10.1016/0165-3806(95)00204-9
171. Richard-Lalonde M, Nagi K, Audet N, Sleno R, Amraei M, Hogue M, et al. Conformational dynamics of Kir3.1/Kir3.2 channel activation via δ-opioid receptors. *Mol Pharmacol.* (2013) 83:416–28. doi: 10.1124/mol.112.081950
172. Nagi K, Pineyro G. Kir3 channel signaling complexes: focus on opioid receptor signaling. *Front Cell Neurosci.* (2014) 8:186. doi: 10.3389/fncel.2014.00186
173. Nagi K, Charfi I, Pineyro, G. Kir3 channels undergo arrestin-dependant internalization following delta opioid receptor activation. *Cellular and molecular life sciences: CMLS.* (2015) 72:3543–57. doi: 10.1007/s00018-015-1899-x
174. Gendron L, Nagi K, Zeghal M, Giguère PM, Pineyro G. Molecular aspects of delta opioid receptors. *Vitam Horm.* (2019) 111:49–90. doi: 10.1016/bs.vh.2019.06.001
175. Charfi I, Nagi K, Mnie-Filali O, Thibault D, Balboni G, Schiller PW, et al. Ligand- and cell-dependent determinants of internalization and cAMP modulation by delta opioid receptor (DOR) agonists. *Cell Mol Life Sci.* (2014) 71:1529–46. doi: 10.1007/s00018-013-1461-7
176. Gendron L, Cahill CM, von Zastrow M, Schiller PW, Pineyro G. Molecular pharmacology of delta-opioid receptors. *Pharmacol Rev.* (2016) 68:631–700. doi: 10.1124/pr.114.008979
177. Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, et al. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. *J Compar Neurol.* (1994) 350:412–38. doi: 10.1002/cne.903500307
178. Mons N, Harry A, Dubourg P, Premont RT, Iyengar R, Cooper DM. Immunohistochemical localization of adenylyl cyclase in rat brain indicates a highly selective concentration at synapses. *Proc Natl Acad Sci USA.* (1995) 92:8473–7. doi: 10.1073/pnas.92.18.8473
179. Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ, et al. Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics *in vivo*. *Proc Natl Acad Sci USA.* (2006) 103:9691–6. doi: 10.1073/pnas.0603359103
180. Kim KS, Lee KW, Lee KW, Im JY, Yoo JY, Kim SW, et al. Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proc Natl Acad Sci USA.* (2006) 103:3908–13. doi: 10.1073/pnas.0508812103
181. Li S, Lee ML, Bruchas MR, Chan GC, Storm DR, Chavkin C. Calmodulin-stimulated adenylyl cyclase gene deletion affects morphine responses. *Mol Pharmacol.* (2006) 70:1742–9. doi: 10.1124/mol.106.025783
182. Kim TW, Alford DP, Holick MF, Malabanan AO, Samet JH. Low vitamin d status of patients in methadone maintenance treatment. *J Addict Med.* (2009) 3:134–8. doi: 10.1097/ADM.0b013e31819b736d
183. Larson MJ, Paasche-Orlow M, Cheng DM, Lloyd-Travaglini C, Saitz R, Samet JH. Persistent pain is associated with substance use after detoxification: a prospective cohort analysis. *Addiction.* (2007) 102:752–60. doi: 10.1111/j.1360-0443.2007.01759.x
184. Rosenblum A, Joseph H, Fong C, Kipnis S, Cleland C, Portenoy RK. Prevalence and characteristics of chronic pain among chemically dependent patients in methadone maintenance and residential treatment facilities. *JAMA.* (2003) 289:2370–8. doi: 10.1001/jama.289.18.2370
185. Karasz A, Zallman L, Berg K, Gourevitch M, Selwyn P, Arnsten JH. The experience of chronic severe pain in patients undergoing methadone maintenance treatment. *J Pain Symptom Manage.* (2004) 28:517–25. doi: 10.1016/j.jpainsymman.2004.02.025
186. Misra A, Aggarwal A, Singh O, Sharma S. Effect of carbamazepine therapy on vitamin D and parathormone in epileptic children. *Pediatric neurology.* (2010) 43:320–4. doi: 10.1016/j.pediatrneurol.2010.05.013
187. Pascussi JM, Robert A, Nguyen M, Walrant-Debray O, Garabedian M, Martin P, et al. Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia. *J Clin Invest.* (2005) 115:177–86. doi: 10.1172/JCI21867
188. Zhou C, Assem M, Tay JC, Watkins PB, Blumberg B, Schuetz EG, et al. Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia. *J Clin Invest.* (2006) 116:1703–12. doi: 10.1172/JCI27793
189. Ghaderi A, Rasouli-Azad M, Farhadi MH, Mirhosseini N, Motmaen M, Pishyareh E, et al. Exploring the effects of vitamin D supplementation on cognitive functions and mental health status in subjects under methadone maintenance treatment. *J Addict Med.* (2020) 14:18–25. doi: 10.1097/ADM.0000000000000550
190. Fell GL, Robinson KC, Mao J, Woolf CJ, Fisher DE. Skin beta-endorphin mediates addiction to UV light. *Cell.* (2014) 157:1527–34. doi: 10.1016/j.cell.2014.04.032
191. Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, et al. Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell.* (2007) 128:853–64. doi: 10.1016/j.cell.2006.12.045
192. Skobowiat C, Dowdy JC, Sayre RM, Tuckey RC, Slominski A. Cutaneous hypothalamic-pituitary-adrenal axis homolog: regulation by ultraviolet radiation. *Am J Physiol Endocrinol Metab.* (2011) 301:E484–93. doi: 10.1152/ajpendo.00217.2011
193. Slominski A, Wortsman J. Neuroendocrinology of the skin. *Endocrine Rev.* (2000) 21:457–87. doi: 10.1210/er.21.5.457
194. Wacker M, Holick MF. Sunlight and vitamin D: a global perspective for health. *Dermato Endocrinol.* (2013) 5:51–108. doi: 10.4161/derm.24494
195. Christie MJ. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *Br J Pharmacol.* (2008) 154:384–96. doi: 10.1038/bjp.2008.100
196. Cao JL, Vialou VF, Lobo MK, Robison AJ, Neve RL, Cooper DC, et al. Essential role of the cAMP-cAMP response-element binding protein pathway in opiate-induced homeostatic adaptations of locus coeruleus neurons. *Proc Natl Acad Sci USA.* (2010) 107:17011–6. doi: 10.1073/pnas.1010077107
197. Han MH, Bolaños CA, Green TA, Olson VG, Neve RL, Liu RJ, et al. Role of cAMP response element-binding protein in the rat locus ceruleus: regulation of neuronal activity and opiate withdrawal behaviors. *J Neurosci.* (2006) 26:4624–9. doi: 10.1523/JNEUROSCI.4701-05.2006
198. Yang HY, Wu ZY, Wood M, Whiteman M, Bian JS. Hydrogen sulfide attenuates opioid dependence by suppression of adenylate cyclase/cAMP pathway. *Antioxidants Redox Signal.* (2013) 20:31–41. doi: 10.1089/ars.2012.5119
199. He L, Whistler JL. The biochemical analysis of methadone modulation on morphine-induced tolerance and dependence in the rat brain. *Pharmacology.* (2007) 79:193–202. doi: 10.1159/000100893
200. Nagi K, Pineyro G. Regulation of opioid receptor signalling: implications for the development of analgesic tolerance. *Mol Brain.* (2011) 4:25. doi: 10.1186/1756-6606-4-25
201. Shy M, Chakrabarti S, Gintzler AR. Plasticity of adenylyl cyclase-related signaling sequelae after long-term morphine treatment. *Mol Pharmacol.* (2008) 73:868–79. doi: 10.1124/mol.107.042184
202. Wang L, Gintzler AR. Morphine tolerance and physical dependence: reversal of opioid inhibition to enhancement of cyclic AMP formation. *J Neurochem.* (1995) 64:1102–6. doi: 10.1046/j.1471-4159.1995.64031102.x
203. Bergman P, Sperneder S, Höijer J, Bergqvist J, Björkhem-Bergman L. Low vitamin D levels are associated with higher opioid dose in palliative cancer patients–results from an observational study in Sweden. *PLoS ONE.* (2015) 10:e0128223. doi: 10.1371/journal.pone.0128223
204. Sempos CT, Heijboer AC, Bikle DD, Bollerslev J, Bouillon R, Brannon PM, et al. Vitamin D assays and the definition of hypovitaminosis D: results from the First International Conference on Controversies in Vitamin D. *Br J Clin Pharmacol Rev.* (2018) 84:2194–207. doi: 10.1111/bcp.13652
205. Scientific Committee on Food. *Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Vitamin D*. Brussels: European Commission (2002).
206. Vieth R. Why the optimal requirement for Vitamin D3 is probably much higher than what is officially recommended for adults. *J Steroid Biochem Mol Biol.* (2004) 89–90:575–9. doi: 10.1016/j.jsbmb.2004.03.038
207. Taylor PN, Davies JS. A review of the growing risk of vitamin D toxicity from inappropriate practice. *Br J Clin Pharmacol.* (2018) 84:1121–7. doi: 10.1111/bcp.13573
208. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab.* (2011) 96:53–8. doi: 10.1016/j.jada.2011.01.004

209. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA*. (2010) 303:1815–22. doi: 10.1001/jama.2010.594
210. Chowdry AM, Azad H, Najar MS, Mir I. Acute kidney injury due to overcorrection of hypovitaminosis D: a tertiary center experience in the Kashmir Valley of India. *Saudi J Kidney Dis Transpl*. (2017) 28:1321–9. doi: 10.4103/1319-2442.220873
211. Marcinowska-Suchowierska E, Kupisz-Urbanska M, Łukaszkiewicz J, Płudowski P, Jones G. Vitamin D toxicity-a clinical perspective. *Front Endocrinol*. (2018) 9:550. doi: 10.3389/fendo.2018.00550
212. Marcinowska-Suchowierska E, Płudowski P, Witamina Z, Tałałaj M. Vitamin D toxicity. *Post N Med*. (2016) 29:756–9.
213. Rooney MR, Harnack L, Michos ED, Ogilvie RP, Sempos CT, Lutsey PL. Trends in use of high-dose vitamin D supplements exceeding 1000 or 4000 international units daily, 1999-2014. *JAMA*. (2017) 317:2448–50. doi: 10.1001/jama.2017.4392
214. Schleicher RL, Sternberg MR, Lacher DA, Sempos CT, Looker AC, Durazo-Arvizu RA, et al. The vitamin D status of the US population from 1988 to 2010 using standardized serum concentrations of 25-hydroxyvitamin D shows recent modest increases. *Am J Clin Nutr*. (2016) 104:454–61. doi: 10.3945/ajcn.115.127985
215. Klontz KC, Acheson DW. Dietary supplement-induced vitamin D intoxication. *N Engl J Med*. (2007) 357:308–9. doi: 10.1056/NEJMc063341
216. Koutkia P, Chen TC, Holick MF. Vitamin D intoxication associated with an over-the-counter supplement. *N Engl J Med*. (2001) 345:66–7. doi: 10.1056/NEJM200107053450115
217. Shephard RM, Deluca HF. Plasma concentrations of vitamin D3 and its metabolites in the rat as influenced by vitamin D3 or 25-hydroxyvitamin D3 intakes. *Arch Biochem Biophys*. (1980) 202:43–53. doi: 10.1016/0003-9861(80)90404-X
218. Gupta AK, Jamwal V, Sakul, Malhotra P. Hypervitaminosis D and systemic manifestations: a comprehensive review. *JIMSA*. (2014) 27:236–7.
219. Jones G. Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr*. (2008) 88:582S–6S. doi: 10.1093/ajcn/88.2.582S
220. Apaydin M, Can AG, Kizilgul M, Beysel S, Kan S, Caliskan M, et al. The effects of single high-dose or daily low-dosage oral colecalciferol treatment on vitamin D levels and muscle strength in postmenopausal women. *BMC Endocr Disord*. (2018) 18:48. doi: 10.1186/s12902-018-0277-8
221. Misgar RA, Sahu D, Bhat MH, Wani AI, Bashir MI. Vitamin D toxicity: a prospective study from a tertiary care centre in Kashmir Valley. *Indian J Endocrinol Metab*. (2019) 23:363–6. doi: 10.4103/ijem.IJEM_116_19
222. Alshahrani F, Aljohani N. Vitamin D: deficiency, sufficiency and toxicity. *Forum Nutr*. (2013) 5:3605–16. doi: 10.3390/nu5093605
223. Ghauri MI, Bareeqa SB, Riaz A, Kumar A. Redundancy is of no good; iatrogenic hypervitaminosis d: a rare case of persistent vomiting due to hypercalcemia. *Clin Med Insights Case Rep*. (2019) 12:1179547619828688. doi: 10.1177/1179547619828688
224. Selby PL, Davies M, Marks JS, Mawer EB. Vitamin D intoxication causes hypercalcaemia by increased bone resorption which responds to pamidronate. *Clin Endocrinol*. (1995) 43:531–6. doi: 10.1111/j.1365-2265.1995.tb02916.x
225. De Francesco Daher E, Mesquita Martiniano LV, Lopes Lima LL, Viana Leite Filho NC, de Oliveira Souza LE, Duarte Fernandes PH, et al. Acute kidney injury due to excessive and prolonged intramuscular injection of veterinary supplements containing vitamins A, D and E: a series of 16 cases. *Nefrologia*. (2017) 37:61–7. doi: 10.1016/j.nefro.2016.05.017
226. Wani M, Wani I, Banday K, Ashraf M. The other side of vitamin D therapy: a case series of acute kidney injury due to malpractice-related vitamin D intoxication. *Clin Nephrol*. (2016) 86:236–41. doi: 10.5414/CN108904
227. Kaur P, Mishra SK, Mithal A. Vitamin D toxicity resulting from overzealous correction of vitamin D deficiency. *Clin Endocrinol*. (2015) 83:327–31. doi: 10.1111/cen.12836
228. Ammenti A, Pelizzoni A, Cecconi M, Molinari PP, Montini G. Nephrocalcinosis in children: a retrospective multi-centre study. *Acta Paediatr*. (2009) 98:1628–31. doi: 10.1111/j.1651-2227.2009.01401.x
229. Bikle DD, Schwartz J. Vitamin D binding protein, total and free vitamin D levels in different physiological and pathophysiological conditions. *Front Endocrinol*. (2019) 10:317. doi: 10.3389/fendo.2019.00317
230. Nielsen R, Christensen EI, Birn H. Megalin and cubilin in proximal tubule protein reabsorption: from experimental models to human disease. *Kidney Int*. (2016) 89:58–67. doi: 10.1016/j.kint.2015.11.007
231. Vogiatzi MG, Jacobson-Dickman E, DeBoer MD. Drugs, and therapeutics committee of the pediatric endocrine society. Vitamin D supplementation and risk of toxicity in pediatrics: a review of current literature. *J Clin Endocrinol Metab*. (2014) 99:1132–41. doi: 10.1210/jc.2013-3655
232. Maalouf J, Nabulsi M, Vieth R, Kimball S, El-Rassi R, Mahfoud Z. Short- and long-term safety of weekly high-dose vitamin D3 supplementation in school children. *J Clin Endocrinol Metab*. (2008) 93:2693–701. doi: 10.1210/jc.2007-2530
233. Robien K, Oppeneer SJ, Kelly JA, Hamilton-Reeves JM. Drug-vitamin D interactions: a systematic review of the literature. *Nutr Clin Pract*. (2013) 28:194–208. doi: 10.1177/0884533612467824
234. Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. *Am J Clin Nutr*. (2007) 85:6–18. doi: 10.1093/ajcn/85.1.6
235. Manson JE, Bassuk SS. Vitamin D research and clinical practice: at a crossroads. *JAMA*. (2015) 313:1311–2. doi: 10.1001/jama.2015.1353
236. Hollis BW, Wagner CL. Normal serum vitamin D levels. *N Engl J Med*. (2005) 352:515–6. doi: 10.1056/NEJM200502033520521

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Allogenic Adipose Tissue-Derived Stromal/Stem Cells and Vitamin D Supplementation in Patients With Recent-Onset Type 1 Diabetes Mellitus: A 3-Month Follow-Up Pilot Study

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Objective: To evaluate the short term safety and potential therapeutic effect of allogenic adipose tissue-derived stromal/stem cells (ASCs) + cholecalciferol in patients with recent-onset T1D.

Methods: Prospective, phase II, open trial, pilot study in which patients with recent onset T1D received ASCs (1×10^6 cells/kg) and cholecalciferol 2000 UI/day for 3 months (group 1) and were compared to controls with standard insulin therapy (group 2). Adverse events, C-peptide (CP), insulin dose, HbA1c, time in range (TIR), glucose variability (continuous glucose monitoring) and frequency of $CD4^+FoxP3+$ T-cells (flow cytometry) were evaluated at baseline (T0) and after 3 months (T3).

Results: 13 patients were included (8: group 1; 5: group 2). Their mean age and disease duration were 26.7 ± 6.1 years and 2.9 ± 1.05 months. Adverse events were transient headache ($n = 8$), mild local reactions ($n = 7$), tachycardia ($n = 4$), abdominal cramps ($n = 1$), thrombophlebitis ($n = 4$), mild floaters ($n = 2$), central retinal vein occlusion ($n = 1$, complete resolution). At T3, group 1 had lower insulin requirement (0.22 ± 0.17 vs. 0.61±0.26IU/Kg; $p = 0.01$) and HbA1c (6.47 ± 0.86 vs. 7.48 ± 0.52%; $p = 0.03$) than group 2. In group 1, 2 patients became insulin free (for 4 and 8 weeks) and all were in

honeymoon at T3 (vs. none in group 2; $p = 0.01$). CP variations did not differ between groups ($-4.6 \pm 29.1\%$ vs. $+2.3 \pm 59.65\%$; $p = 0.83$).

Conclusions: Allogenic ASCs + cholecalciferol without immunosuppression was associated with stability of CP and unanticipated mild transient adverse events in patients with recent onset T1D.

ClinicalTrials.gov registration: NCT03920397.

Keywords: type 1 diabetes, pancreatic function, transplant, adipose tissue-derived stromal/stem cells, vitamin D

INTRODUCTION

Type 1 Diabetes (TID) is a chronic disease caused by T-cell mediated autoimmune destruction of pancreatic β-cells. Patients with T1D require lifelong insulin treatment, which may lead to hypoglycemic episodes and interfere in quality of life. Interventions to cure T1D have been pursued. Even preservation or recovery of some residual mass of β cells, albeit not enough to cure the disease, could have potential benefits such as reduction in glycemic variability, severe hypoglycemia, insulin requirement and risk of chronic complications (1–5).

Clinical interventions aiming to cure T1D target the prevention of autoimmune destruction of pancreatic β cells, regression of insulitis, preservation or recovery of β cells residual mass (3, 6, 7). Various therapeutic methods have been evaluated for patients with T1D, including cytostatic drugs, monoclonal antibodies, and pancreas/islets transplantation. They have some limitations in clinical practice, such as adverse events of immunosuppressants, risk of immune rejection and surgical complications (bleeding, portal vein thrombosis and bile leakage) (8–12). Although some of these treatments have initially shown satisfactory outcomes, their effects have not been sustained for long periods and/or their potential side effects limit their repetitive use. In previous studies, most favorable metabolic and pancreatic function outcomes were linked to a better T regulatory cell function (13–15).

Adult stem cells transplantation has emerged as a potential treatment for T1D due to its intrinsic regenerative capacity and immunomodulatory properties. The rationale for its use is to arrest β cells autoimmune destruction and generate functional cells. Mesenchymal stromal/stem cells seem attractive as they have been tested for other autoimmune diseases with promising results (16–24) and do not require immunosuppression, even when allogenic sources are used. *In vivo* and *in vitro* studies showed that MSCs are capable of suppressing immune response by inhibiting the maturation of dendritic cells, suppressing T cells function and inducing expansion of regulatory T cells (16–19). A recent meta-analysis of the clinical efficacy and safety of stem cell therapy for T1D indicated that the treatment seems relatively safe and effective, but most studies are small, use hematopoietic stem cells with immunosuppression and autologous origin (20). In that meta-analysis, patients with recent-onset T1D that received MSCs (from bone marrow or umbilical cord tissue) did not have significant reduction in HbA1c or improvement in C-peptide levels, but 20% of treated T1D patients achieved exogenous insulin independence at some point (20). Adipose tissue-derived stromal/stem cells (ASCs) have not been evaluated for this purpose.

ASCs are an abundant source of adult stromal/stem cells, easily accessible by liposuction. These cells seem to display more potential immunosuppressive properties than other mesenchymal stem cells, with more pronounced cytokines secretion, suggesting a promising therapeutic application in autoimmune diseases, such as T1D. As ASCs do not express co-stimulatory molecules on their surface, they are unable to activate alloreactive T cells and could therefore be used for allogenic transplantation without the need for immunosuppression (18, 19). Studies that tested ASC for musculoskeletal disorders, perianal fistula in Crohn's disease and psoriasis showed potential therapeutic effects (21–23). Their use is currently been tested for autoimmune diseases, especially multiple sclerosis (24, 25).

Vitamin D (VitD) seems to have immunomodulatory effects. *In vitro* and *in vivo* studies showed that sufficient levels of VitD could preserve residual β cells and insulin secretion. VitD appears to inhibit lymphocyte proliferation, inhibit cellular autoimmune pathways and stimulate T regulatory response (26–28). However, results with the use of vitamin D for patients with T1D are still inconsistent (29–31).

Since T1D pathogenesis is multifactorial, interventions to approach islet autoimmunity should probably include a combination of agents with different mechanisms of action. Some authors have already suggested that acting at different points of the autoimmune process is more effective than treatment with a single therapy (32–34). The agents used for intervention in patients with T1D should have the lowest possible toxicity potential, especially if periodic repetition of the proposed treatment is considered. Our aim was to evaluate the short-term safety and efficacy of ASCs infusion from healthy donors and daily cholecalciferol (VitD) supplementation in patients with recent-onset T1D, a combined therapy that offers the opportunity of immunomodulation without the need of immunosuppression.

RESEARCH DESIGN AND METHODS

Patients and Study Design

This is a prospective, single-center, open trial, phase II, in which patients (Group 1) with recent onset T1D received a single dose of allogenic adipose tissue derived stem/stromal cells (ASCs) and cholecalciferol (Vit D) 2,000 IU/day for 3 months or were included in a control group that received standard insulin therapy with multiple injections (Group 2). This trial was registered at ClinicalTrial.gov (NCT03920397) and

approved by Ethics Research Board of the University Hospital Clementino Fraga Filho (HUCFF)/ Federal University of Rio de Janeiro (UFRJ). Participants or their legal representatives (in minors) signed an informed consent before inclusion (protocol 17488313.1.0000.5257).

Inclusion criteria were: diagnosis of T1D according to American Diabetes Association (ADA) criteria for < 4 months; age between 16 and 35 years; pancreatic autoimmunity (anti glutamic acid decarboxylase or GADA+). Exclusion criteria were: current or prior malignant diseases; pregnancy or desire to become pregnant within 12 months of the study; breastfeeding; HIV(+), Hepatitis B or C (+); diabetic ketoacidosis at diagnosis; glomerular filtration rate < 60 ml/min and use of immunosuppressors or glucocorticoids. Similarly to Voltarelli JC and Couri CE, we decided to exclude patients with previous diabetic ketoacidosis as the first patient included in their trial with diabetic ketoacidosis failed to benefit from AHST (35).

Stem/Stromal Cells Differentiation Procedures

ASCs differentiation potential into adipocytes, osteoblasts, and chondrocytes, were evaluated in third passage with a commercial medium (Lonza, Walkersville, EUA). The medium inducer was changed every 3 days during 3 weeks. To adipogenic and osteogenic differentiation, cells were seeded on glass coverslips (Sarstedt, Newton, NC, USA) in 24-well plates (Sarsted). Briefly, cells were treated with Bouin's fixative (Biotec, Labmaster, Paraná, Brazil) for 10 min, washed twice with 70% ethanol and once with Milliq water. Oil Red O (Sigma-Aldrich) was used to visualize lipid-rich vacuoles and hematoxylin-eosin (HE) (Biotec) was used for nuclear staining. Osteogenic differentiation was evaluated by Alizarin Red S (Fluka Chemie, Buchs, UK) and light green (Sigma-Aldrich) was used to counterstain. For chondrogenic differentiation, cells were grown in micromass culture. Briefly, 5 × 105 cells in 0.5 ml of medium were centrifuged at 300 g for 10 min in a 15 mL polypropylene tube to form a pellet. Without disturbing the pellet, cells were cultured for 21 days with medium inducer. On day 21, cell aggregates were fixed in 10% formaldehyde for 1 h dehydrated in serial ethanol dilutions and embedded in paraffin blocks. Toluidine Blue staining (Sigma-Aldrich) demonstrate the presence of intracellular matrix proteoglycans. Control cells were kept in DMEM-F12 medium with 15% FCS.

Stem/Stromal Cells Lipoaspirate and Culture

Adipose tissue samples were obtained through liposuction procedures of three healthy female individuals undergoing aesthetic surgery at *Hospital Universitário da Universidade Federal do Rio de Janeiro*, RJ, Brazil. Donors'sorology was negative for syphilis, Chagas disease, Hepatitis B virus (HBV), Hepatitis C, HIV 1 and 2, HTLV I/II. All donors were Cytomegalovirus (CMV) IgG positive with PCR negative in blood samples and in ASCs.

ASCs were isolated, cultured and characterized as previously described (18). Samples were processed at Core Cell Technology of Pontifícia Universidade Católica do Paraná. Briefly, 100 ml of adipose tissue were washed in sterile phosphate-buffered saline (PBS) (Gibco Invitrogen). One-step digestion by 1 mg/ml collagenase type I (Invitrogen) was performed for 30 min at 37°C during permanent shaking, followed by filtration through 100 μm mesh filter (BD FALCON, BD Biosciences Discovery Labware, Bedford, MA, USA). Cell suspension was centrifuged at 800 g for 10 min, and erythrocytes were removed by lysis buffer, pH 7.3. The remaining cells were washed at 400 *g* for 10 min and cultured at a density of 1×10^5 cells/cm^2 in T75 culture flasks and DMEM-F12 (Gibco Invitrogen) supplemented with 10% of fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Culture medium was replaced 3 days after seeding, and then twice a week. ASCs were sub-cultured after reaching 80% confluence, with 0.5% trypsin/EDTA (Invitrogen) solution. Cells were replated at a density of 4×10^3 cells/cm^2 for expansion.

Quality control of cell suspension sterility was evaluated by tests to detect bacteria and fungi (Bact/Alert 3D, Biomerieux), endotoxins (Endosafe™ PTS, Charles River) and Mycoplasma (KIT MycoAlert™ PLUS Mycoplasma Detection, Lonza). Cell viability was performed by flow cytometry using vital dye 7-AAD (7-Aminoactinomycin D—BD#559925) to determine percentage of viable cells and Annexin V protein (BD#51-65875X) to determine percentage of cells in apoptosis. Cytogenetic analysis was performed by GTG-banding method.

Cells were characterized by flow cytometry before the clinical application, using the following monoclonal antibodies: FITC-labeled CDç/14 (BD#555397), CD45 (BD#555482), CD19 (BD#555412), CD44 (BD#555478); PE-labeled CD73 (BD#550257), CD90 (BD#555596), CD166 (BD#559263), PerCP-labeled HLA-DR (BD#551375); APC-labeled CD34 (BD#555824), CD105 (BD#562408), CD29 (BD#559883) (BD Pharmingen). At least 100.000 events were acquired on BD FACSCalibur™ flow cytometer (BD Biosciences), and data were analyzed using FlowJo 10 (TreeStar) software[11A] (**Supplementary Material 1**).

ASCs Infusion

At the day of infusion, ASCs monolayer were dissociated as described above and 1×10^6 cells/kg of the donor patient were suspended in 5 ml of saline solution with 50% albumin and 5% ACD (Anticoagulant Citrate Dextrose Solution). Cell suspension was sent to the hospital in cooler with recycled ice.

An aliquot of cells was evaluated after transportation for monitoring viability and phenotype of ASCs by flow cytometry. Cells were washed with PBS-BSA 3% (bovine serum albumin) and incubated at 4°C for 30 min with the following monoclonal antibodies conjugated to fluorescent dyes: CD105-FITC (fluorescein isothiocyanate), CD73-PE (phycoerythrin) and CD90-APC (allophycocyanin), all from BD Biosciences, Franklin Lakes, NJ, USA. Unstained cells were used as controls. Then, cells were washed with PBS-BSA 3% and incubated with 7AAD (7 Amino Actinomycin D). Twenty thousand events were acquired in FACSAria III cytometer (BD Biosciences) and data were analyzed using FACSDiva 8.0 software (BD Biosciences). The percentage of viable cells was estimated by 7AAD exclusion.

Patients that received ASCs were admitted into hospital in the day of the infusion. A single dose of ASCs was infused in a peripheral upper arm vein during 15–20 min. Patients were discharged from hospital 24 h after infusion. Patients started taking oral cholecalciferol 2,000 IU in the same day.

Clinical and Biochemical Evaluation

All participants were followed for 3 months. At baseline, they were interviewed and had a physical exam. Weight, height, body mass index (BMI), blood pressure, heart frequency, frequency of hypoglycemia and insulin dose/kg of body weight were evaluated in the first visit (T0) and after 1 (T1) and 3 (T3) months. Insulin dose adjustments were done at each visit as necessary, according to glycemic control. Cases and controls received the same diabetes education, nutritional recommendations and help with management from health care providers. Honeymoon phase was defined as insulin dose $\leq$ 0.5 IU/Kg and HbA1C $<$ 7.5% (36). Blood samples were collected prior to ASCs infusion and at T1 and T3 for the following measurements: HbA1c—Glycated hemoglobin method- HPLC-High Performance Liquid Chromatography by boronate affinity (Trinity Biotech, USA), blood count and biochemistry analysis. Pancreatic function was evaluated through C-Peptide (CP) measurement (Microparticle Chemiluminescent Immunoassay method, Architect Abbott, Spain, following the manufacturer's protocol) 0 (basal; 8 h fasting), 30, 60, 90, and 120 min after a liquid mixed meal (Glucerna®). The area under the curve (AUC) was calculated. Insulin usual dose was administered after each test was completed. All tests were performed with fasting glucose between 70 and 250 mg/dL. Glutamic acid decarboxylase antibodies (GADA) and Islet Tyrosine Phosphatase 2 (IA2) were analyzed (ELISA-human antibodies tested by quantitative ELISA method—Enzyme-Linked Immunosorbent Assay, Euroimmun brand and Molecular Devices Spectra max reader, Germany).

Glucose Variability Assessment

Retrospective analysis of 72 h continuous glucose monitoring system (CGM—*Ipro Medtronic*) data was performed in 7 T1D patients and 4 controls at T0 and T3.

Mean glucose, glucose standard deviation (SD), J-Index, M-value, glycemic risk assessment in diabetes equation (GRADE), high blood glucose index (HBGI), low blood glucose index (LBGI), mean amplitude of glucose excursions (MAGE), mean of daily differences (MODD) and continuous overall net glycemic action at 1 h (CONGA 1) were calculated as described in original publications (37–39). The percentage of glucose reading in time in range (TIR) established as 70–180 mg/dL was recorded (40).

All volunteers received individual face-to-face consultation sessions, which included individualized diet prescriptions based on *American Diabetes Association* current recommendations (dietary energy content of 45–55% carbohydrates, 15–20% protein, and 25–35% total fat, $\leq$7% saturated fatty acids (SFA), 5–15% monounsaturated fatty acids (MUFA); $\leq$10% polyunsaturated fatty acids (PUFA), and 30–50% total fiber intake) (41) and advice on food selection and carbohydrate counting method. During CGM period, all foods and drinks consumed were documented.

Flow Cytometry

Mononuclear cells were isolated from peripheral blood samples by density centrifugation on Ficoll (Ficoll-Paque, GE Healthcare). Blood samples diluted in an equal volume of phosphate buffered saline (PBS) 0.01 M were overlaid on Ficoll-Paque and centrifuged at 500 g for 15 min, with centrifugation stop break turned off. The ring of mononuclear cells formed in tubes was harvested, washed twice with PBS containing 3% of bovine serum albumin (3% PBS-BSA) and incubated for 30 min at 4°C, shielded from light, with the following fluorochrome-conjugated anti-human monoclonal antibodies: CD45RA-PE (Clone HI100), CD3 PE-CF594 (Clone UCHT1), CD4-PERCP CY5.5 (Clone RPA-T4) and CD8-APCH7 (Clone SK1), with anti-human FoxP3—Alexa 647 (Clone 259 D/C7) (BD Biosciences, Franklin Lakes, NJ, USA). Cells were washed with PBS and followed the intracellular FoxP3 staining protocol, according to manufacturer's recommendations (BD Biosciences). Briefly, surface-stained cells were fixed and permeabilized using the buffers from Human FoxP3 Buffer Set (BD Biosciences). Cells were washed in PBS and incubated with anti-human FoxP3-Alexa 647 antibody (BD Biosciences) for 30 min at room temperature in the dark. After the incubation period, cells were washed with PBS and treated with 0.5 mL of BD FACS™ Lysing Solution (BD, Biosciences) during 10 min at room temperature, to remove residual erythrocytes. A hundred thousand events were acquired on FACSAria III (BD Biosciences), which was calibrated using Cytometer Settings and Tracking (CST) beads (BD Biosciences) according to the cytometer manufacturer's recommendations. Background staining was determined using unstained cells. An FMO (Fluorescence Minus One) control was used to set the boundary between negative and positive fluorescence for FoxP3. Cells incubated with a single antibody coupled with a fluorescent dye were used for compensation purposes. Data were analyzed using FACSDiva 6.0 software. A gate on lymphocytes was defined in a forward scatter (FSC) vs. side scatter (SSC) dot plot, followed by gating on $CD45RA^+CD3^+$ lymphocytes, followed by gating on $CD4^+$ or $CD8^+$ cells. Finally, a gate was set to determine the percentage of $FoxP3^+$ cells among $CD45^+CD3^+CD4^+$ cells or $CD45^+CD3^+CD8^+$ cells.

T-cells were evaluated on blood samples before (T0), one (T1) and three (T3) months after ASCs infusion.

Safety Tests

Adverse events were recorded during the procedure and during the hospitalization. At each outpatient visit (T0, T1, and T3), patients underwent an interview, clinical exam and biochemical evaluation to monitor for adverse events. Laboratorial evaluation included: blood count, lipids, renal, and hepatic function, TSH, free tyroxine, calcium, phosphorus and 25 OH vitamin D (performed using the automated biochemical equipment CMD 800 IX1).

Statistical Analysis

The primary outcome was the presence of severe adverse events. The secondary outcomes were other adverse events, changes in insulin uses, A1c and CP AUC. The sample size was established

by convenience sampling according to the number of cells that were available for the intervention procedure. Data are expressed as mean +/– standard deviation. Descriptive statistics have been used to summarize patient's characteristics. Comparisons of categorical variables have been performed by means of Chi Square test. Continuous variables have been compared by Mann-Whitney test. Wilcoxon test were used to compare results at baseline and after follow-up. Statistical tests are based on a 2-sided significance level of 0.05. SPSS software, version 21.0 was used for statistical analyses.

RESULTS

Characteristics of the Study Group

Sixteen newly diagnosed T1D volunteers fulfilled inclusion criteria and agreed to participate. Three were excluded due to glucocorticoid use, impaired renal function and pulmonary tuberculosis. Thirteen cases were evaluated: eight patients received ASCs infusion + vitamin D (Group 1) and five were included as controls (Group 2) (**Figure 1**). The study was disclosed in brazilian endocrinologists social media. The first five candidates were included in group 1. The next eight were included by randomization. All completed 3 months of follow-up.

Clinical characteristics of the study group are described in **Tables 1**, **2**. The mean age was 26.75 ± 6.11 years for group 1 and 20.6 ± 3.84 years for group 2 ($p = 0.04$). Their disease duration were 2.9 +/−1.05 and 3.0 ± 0.70 months, respectively ($p = 0.84$). All received insulin through subcutaneous injections. Biochemical, metabolic and immunological characteristics before and after intervention are reported in **Table 2**.

ASCs Infusion

Differentiation to adipocytes, osteoblasts, and chondrocytes was qualitatively assessed based on cell morphology and cytochemistry (**Figure 2**).

After 21 days, large, rounded cells with cytoplasmic lipid-rich vacuoles, for adipogenic differentiation were observed. Osteogenic differentiation was assessed by mineralization of extracellular matrix. In chondrogenic differentiation assays, the

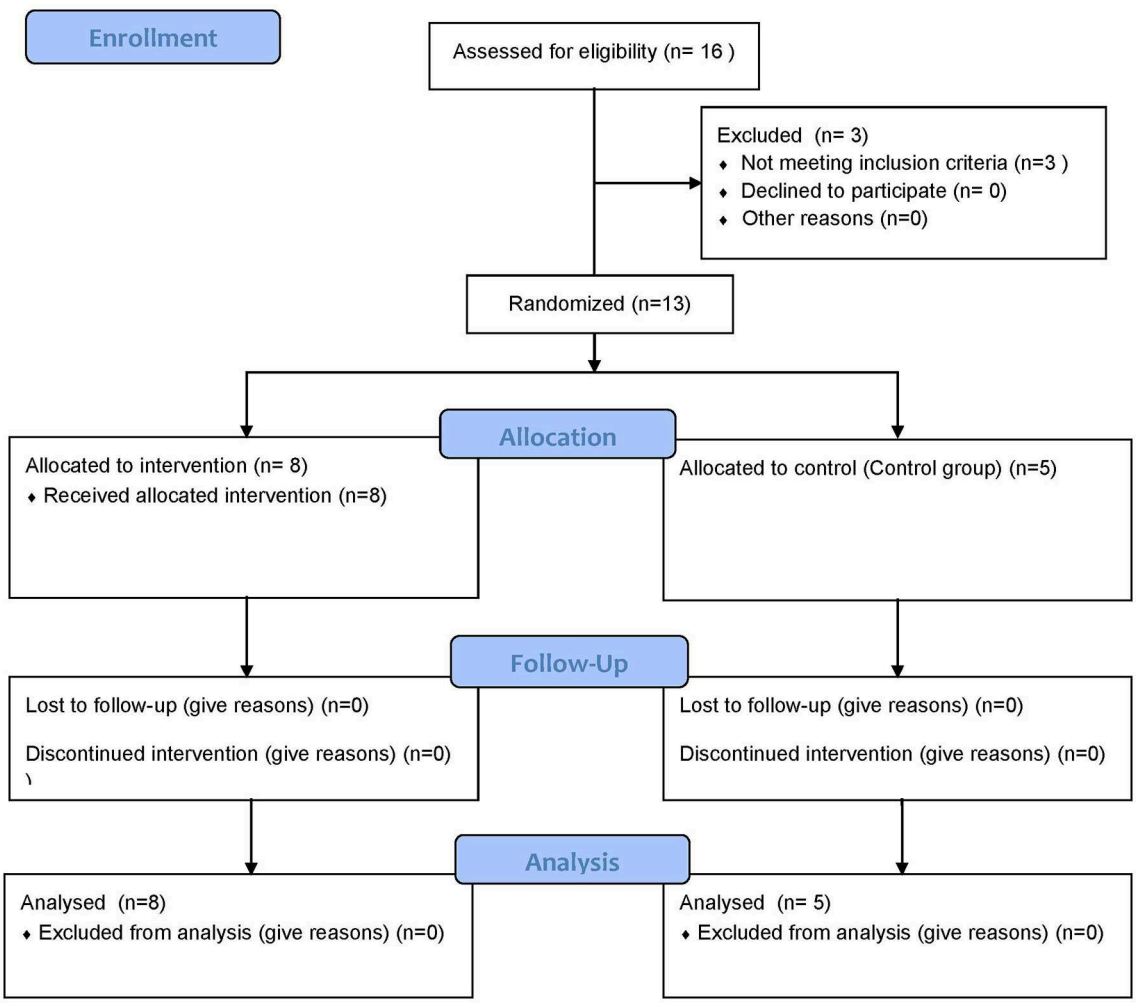

FIGURE 1 | Flow Diagram of the clinical trial.

TABLE 1 | Characteristics of the study group.

Group	Age (Gender)	Ethnicity	BMI	T1D	ASCs	Ins T0	Ins T1	Ins T3	HbA1C T0	HbA1C T1	HbA1C T3	CP T0	CP T1	CP T3
#1 (G1)	26 (M)	W	26.06	4	78	0.84	0.64	0.47	9.90	7.60	6.20	104.85	63.60	157.05
#2 (G1)	35 (M)	NW	25.91	4	74	0.17	0.15	0.18	8.0	7.50	6.60	148.95	162.60	149.70
#3 (G1)	28 (M)	W	23.38	2	65	0.21	0.18	0.17	6.30	5.60	5.50	340.50	470.7	388.95
#4 (G1)	34 (F)	W	23.56	2	73	0.07	0.07	0.11	7.50	6.90	6.20	328.50	317.7	291.90
#5 (G1)	23 (F)	NW	20.76	1.7	60	0.15	0.15	0.12	7.90	6.30	6.40	178.95	158.85	99.30
#6 (G1)	16 (F)	W	20.96	3.5	55	0.47	0.47	0.49	6.90	5.80	5.70	318.75	354.30	251.10
#7 (G1)	28 (F)	W	23.71	2	69	0.25	0.20	0.25	7.60	6.60	6.90	233.70	270.00	243.45
#8 (G1)	24 (M)	W	26.06	4	66	0.30	0.18	0	7.40	7.20	8.30	153.00	204.00	108.15
#9 (G2)	16 (M)	W	18.25	2	–	0.92	0.82	0.92	10.6	6.90	7.80	86.25	140.70	173.70
#10 (G2)	20 (F)	NW	23.71	3	–	0.92	0.69	0.64	6.80	6.80	6.90	90.30	127.95	78.75
#11 (G2)	18 (M)	NW	18.20	3	–	0.60	0.60	0.70	6.90	7.40	7.20	102.75	118.65	106.20
#12 (G2)	25 (F)	W	20.60	3	–	0.20	0.20	0.20	7.70	7.10	8.20	160.2	147.90	123.15
#13 (G2)	24 (M)	W	19.30	3	–	0.50	0.50	0.60	10.10	7.40	7.30	113.25	101.55	48.45

Body mass index (BMI) expressed as kilogram of body weight/centimeter^2 height was measured at the time of the transplant. ASCs number of cells was calculated according to patient weight × 10^6. G1, patient that received ASCs + VitD or Group 1; G2, control or Group 2; W, white; NW, non-white; T1D, Type 1 Diabetes duration in months; T0, basal period, T1, 1 month after intervention, T3, 3 months after intervention; Ins, insulin dose (IU/Kg); A1C, glycated hemoglobin (mg/dl,%); CP, the area under the curve of C-Peptide (ng/ml).

TABLE 2 | Comparison between group 1 and 2 before and after ASCs infusion + VitD.

Indexes	T0			T1			T3		
	Group 1	Group 2	*P*-value	Group 1	Group 2	*P*-value	Group 1	Group 2	*P*-value
HbA1C (mg/dl,%)	7.68 ± 1.04	8.42 ± 1.80	0.36	6.68 ± 0.75	7.12 ± 0.27	0.24	6.47 ± 0.86	7.48 ± 0.52	**0.03**
Insulin dose (IU/Kg)	0.31 ± 0.24	0.62 ± 0.30	0.08	0.25 ± 0.19	0.56 ± 0.23	**0.02**	0.22 ± 0.17	0.61 ± 0.26	**0.01**
GADA (mIU/L)	261.69 ± 138.77	182.13 ± 130.06	0.32	674.33 ± 1348.57	144.87 ± 151.81	0.41	697.75 ± 1338.72	245.08 ± 219.77	0.47
IA2 (mIU/L)	158.28 ± 270.59	818.74 ± 1718.42	0.31	156.68 ± 267.33	817.66 ± 1779.01	0.31	163.19 ± 280.34	815.77 ± 1780.05	0.32
TSH (mIU/L)	3.51 ± 3.06	3.76 ± 2.47	0.88	3.34 ± 3.30	3.48 ± 2.32	0.94	3.27 ± 3.08	3.45 ± 2.57	0.92
Vitamin D (ng/ml)	35.31 ± 14.07	21.74 ± 7.03	0.07	39.42 ± 6.28	25.35 ± 7.22	**0.01**	46.96 ± 8.13	24.73 ± 4.80	**0.01**
CD4+ FOXP3+ (%)	10.22 ± 7.14	15.28 ± 8.34	0.27	17.55 ± 6.29	11.72 ± 3.18	0.12	14.62 ± 8.97	10.76 ± 6.27	0.42
CD8+ FOXP3+ (%)	13.85 ± 9.74	5.52 ± 2.78	0.09	15.85 ± 7.33	5.87 ± 1.85	**0.03**	10.72 ± 9.04	11.25 ± 9.02	0.09
CP AUC (ng/ml min)	225.90 ± 92.91	110.55 ± 29.72	**0.02**	250.22 ± 129.49	127.35 ± 18.31	**0.03**	211.20 ± 100.42	106.05 ± 47.25	0.06
Media	6.94 ± 1.44	7.37 ± 1.13	0.66	––	––	–	6.79 ± 1.87	6.94 ± 0	0.94
SD	1.66 ± 0.69	1.84 ± 1.05	0.75	––	––	–	1.67 ± 0.93	2.36 ± 0	0.51
M-Value	0.95 ± 5.02	3.61 ± 2.96	0.42	––	––	–	1.02 ± 5.48	9.19 ± 0	0.20
MAGE	1.31 ± 0.34	0.59 ± 0.27	0.56	––	––	–	1.14 ± 0.80	0.67 ± 0.28	0.95
J Index	25.58 ± 13.15	29.39 ± 11.42	0.67	––	––	–	25.53 ± 16.88	29.00 ± 0	0.85
MODD	––	––	–	––	– – –	–	2.20 ± 0.68	3.27 ± 0	0.50
GRADE	0.61 ± 0.82	0.61 ± 0.32	0.99	– – –	––	–	256.69 ± 722.05	0.99 ± 0	0.75
LBGI	2.75 ± 1.97	2.77 ± 2.20	0.99	––	––	–	2.57 ± 1.87	6.17 ± 0	0.11
HBGI	4.55 ± 4.38	3.87 ± 2.75	0.81	––	––	–	3.92 ± 4.23	5.82 ± 0	0.68
CONGA-1	1.36 ± 1.02	1.91 ± 0.47	0.41	––	––	–	1.37 ± 0.79	1.83 ± 0	0.60
Time in range (%)	92.71 ± 4.27	81.00 ± 18.16	0.12	––	––	–	89.43 ± 13.50	57.50 ± 9.19	0.11

Glycemic variability indexes are expressed in mmol/L. Data are expressed as mean ± standard deviation. T0, basal period, T1, 1 month after intervention, T3, 3 months after intervention, HbA1C, glycated hemoglobin; GADA, Glutamic acid decarboxylase antibodies; IA2, Islet Tyrosine Phosphatase 2; CP AUC, the area under the curve of C-Peptide; SD, standard deviation; MAGE, mean amplitude of glucose excursions; MODD, mean of daily differences; GRADE, glycemic risk assessment in diabetes equation; LBGI, low blood glucose index; HGBI, high blood glucose index; CONGA-1, continuous overall net glycemic action at 1 h. GADA and IA2A titers were considered positive > 10I U/ml. Bold values means p value ≤ 0.05.

presence of intracellular matrix proteoglycans and chondrocyte-like lacunae were observed. Untreated control cultures did not exhibit spontaneous differentiation after 21 days of cultivation.

Group 1 ($n = 8$) received intravenous transfusion of ASCs (mean dose: 67.37± 7.65 × 10^6 cells). ASCs were characterized by immunophenotyping, and mean percentage labeling of cells

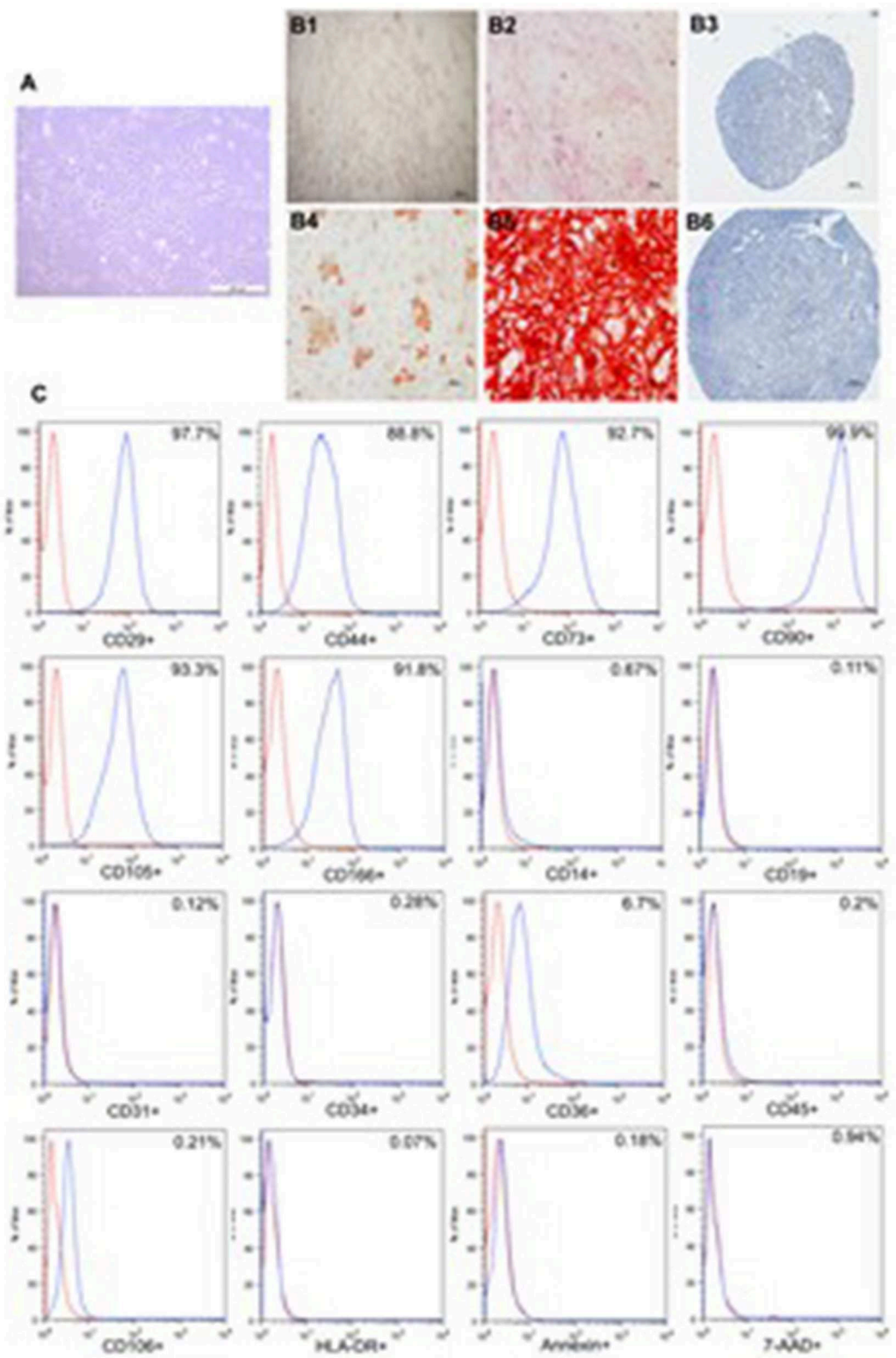

FIGURE 2 | Characterization of adipose tissue-derived stromal/stem cells. **(A)** Adipose tissue-derived stromal/stem cells morphology. **(B)** BM-MSC differentiation. Cells were incubated for 21 days in the presence of specific differentiation agents for adipocytes, osteoblasts and chondrocytes. Differentiation into adipocyte lineage was demonstrated by staining with Oil Red O **(B4)**, Alizarin Red S staining shows mineralization of the extracellular matrix in osteogenic differentiation **(B5)** and toluidine blue shows the deposition of proteoglycans and lacunaes in chondrogenic differentiation **(B6)**. Untreated control cultures without adipogenic, osteogenic or chondrogenic differentiation stimuli are shown **(B1–B3)**. **(C)** Representative figure of the flow cytometric analysis. The red line indicates isotype control and the blue line represent.

was as follows: CD105 93.47%; CD73 96.20%; CD90 99.77%; CD29 98.83%; CD166 94.75%; CD44 89.76%; CD14 1.57%; CD34 0.52%; CD45 0.84%; CD19 0.64% and HLA-DR 0.68%. All tests for microorganism's growth control were negative. All samples were approved by cytogenetic quality control for therapeutic use and no clonal chromosomal rearrangements were detected. After isolation and culture, 95.1% of viability was obtained.

Adverse Events

No serious adverse events were observed. Patients in group 1 presented the following immediate transient adverse events: transient headache ($n = 8$), mild local infusion reactions ($n = 7$), tachycardia ($n = 4$), and abdominal cramps ($n = 1$). Four patients developed local superficial thrombophlebitis within the first week without systemic manifestations and two reported transient mild eye floaters during infusion, with no subsequent visual abnormalities. One patient developed central retinal vein occlusion at T3, with complete resolution.

Pancreatic Function

At baseline, group 1 presented higher CP levels than group 2 (225.90 ± 92.91 vs. 110.55 ± 29.72 ng/ml; $p = 0.02$). At T1, the difference between groups was significant (250.22 ± 129.49 vs. 127.35 ± 18.31 ng/ml; $p = 0.03$), which was not observed at T3 (211.20 ± 100.42 vs. 106.05 ± 47.25 ng/ml; $p = 0.06$) (**Table 2** and **Figure 3**). There was a correlation between CP at T0 and at T3 ($p = 0.03$; $R = 0.66$). Groups 1 and 2 had similar percentage of CP AUC decrease during follow-up (−4.6 ± 29.1% vs. +2.3 ± 59.65%; $p = 0.83$). Neither cases or controls had significant changes in CP over time ($p = 0.57$ e $p = 0.68$).

Glycemic Control

At baseline, there were no significant differences in HbA1c (7.68 ± 1.04 vs. 8.42 ± 1.8%; $p = 0.60$), insulin dose (0.31 ± 0.24 vs. 0.62 ± 0.30 IU/Kg; $p = 0.06$) or TIR (92.71 ± 4.27% vs. 89.42 ± 13.5%; $p = 0.35$) between groups. One month after infusion, 2 patients in group 1 became insulin free, for 4 and 8 weeks each. At this time, lower insulin requirement were observed in group 1 when compared to group 2 (T1: 0.25 ± 0.19 vs. 0.56 ± 0.23 IU/Kg, $p = 0.02$) and this difference persisted after 3 months (T3: 0.22 ± 0.17 vs. 0.61 ± 0.26IU/Kg, $p = 0.01$). At T3, group 1 also had lower HbA1c than group 2 (6.47 ± 0.86 vs. 7.48 ± 0.52%; $p = 0.03$). Mean glucose readings in TIR were 89.43 ± 13.5 in group 1 vs. 57.50 ± 9.19% in group 2 ($p = 0.11$). The decline in HbA1c over time was significant in cases ($p = 0.04$) but not in controls ($P = 0.68$) and changes in insulin dose were not significant in either of the groups ($p = 0.27$ and $p = 1.00$). Moreover, at T3 all patients ($n = 8$) of group 1 were in honeymoon phase vs. none in group 2 ($p = 0.01$). Both groups had similar percentage of HbA1C reduction (−14.79 ± 13.68% vs. −8.37 ± 17.1%; $p = 0.72$) without statistically significant differences in insulin requirement (−14.48 ± 13.68% vs. +1.2 ± 19.97%; $p = 0.52$). **Table 2** and **Figure 3** summarizes these results. Neither CP at T0 or age were correlated to the rate of decline in HbA1c ($p = 0.57$; $R = 0.17$ and $p = 0.87$; $R = -0.04$). There was an inverse correlation between insulin dose requirement and Vit D levels at T1 ($p = 0.05$; $r = 0.58$) and T3 ($p = 0.01$; $r = 0.77$). Although there was no association between vitamin D and HbA1c at T1 ($p = 0.99$; $r = 0.01$), a negative correlation was found at T3 ($p = 0.03$; $r = 0.67$).

Retrospective analysis of blinded 72 h CGM did not show significant differences in SD, M-Value, MAGE, J-Index, MODD, GRADE, LBGI, HBGI, CONGA-1 between groups, as shown in **Table 2**. Glucose profiles are shown in **Supplementary Material 2**.

Frequency of FOXP3-Expressing Lymphocytes

There was no significant difference between groups for % CD4+FoxP3^{+}cells, but group 1 showed higher frequency of CD8+FoxP3^{+} cells at T1 when compared to group 2 ($p = 0.03$), as shown in **Table 2** and **Figure 4**.

There was a negative correlation between %CD4+FoxP3+ cells and: (1) HbA1C ($p = 0.01$; $r = 0.71$), (2) mean glucose ($p = 0.02$, $r = 0.77$); (3) SD ($p = 0.01$, $r = 0.82$); (4) M-Value ($p = 0.02$, $r = 0.03$); (5) J-Index ($p = 0.01$, $r = 0.02$); (6) HBGI ($p = 0.01$, $r = 0.87$) and (7) MODD ($p = 0.04$, $r = 0.77$) at T3. No significant correlation was found between % CD4+FoxP3+ cells and MAGE ($p = 0.77$; $r = 0.12$). Frequency of CD4+FoxP3+ cells was positively correlated to TIR ($p = 0.04$; $r = -0.76$).

At T1, %CD8+FoxP3+ cells were positively correlated with CP ($p = 0.04$; $r = 0.72$). At T3, a negative correlation was found between % CD8+FoxP3+ vs. HbA1C ($p = 0.04$; $r = 0.73$) and HBGI ($p = 0.02$, $r = 0.78$).

Additional data are shown in **Supplementary Materials 1, 2**.

DISCUSSION

We evaluated the short-term safety of ASCs infusion from healthy donors and daily oral cholecalciferol in patients with recent-onset T1D, as well as their potential therapeutic effect on glycemic control and pancreatic function. In most cases, mild and transient adverse events were observed (local reactions, tachycardia), except for one patient that presented central vein occlusion 3 months after infusion, with complete resolution. This reaction was probably not associated with the therapy since it occurred months after the intervention. A higher risk for this condition has been previously reported in individuals with diabetes (42) but the association between recent-onset T1D and this condition has not been shown. Two cases of central retinal vein occlusion have been previously reported in patients with hematologic malignancies receiving hematopoietic stem cell transplantation but not with adipose tissue-derived stromal/stem cells (43, 44). Tachycardia and thrombophlebitis may be associated with high cellularity concentration, high viscosity or other cell stabilizing products.

This was the first trial to test allogenic ASCs without immunosuppression plus VitD supplementation in newly diagnosed T1D patients. Allogenic source of cells was chosen due to the possibility of impairment of mesenchymal stromal/stem cells immune properties in individuals with T1D (44–46). The use of allogenic cells in this short-term evaluation was associated

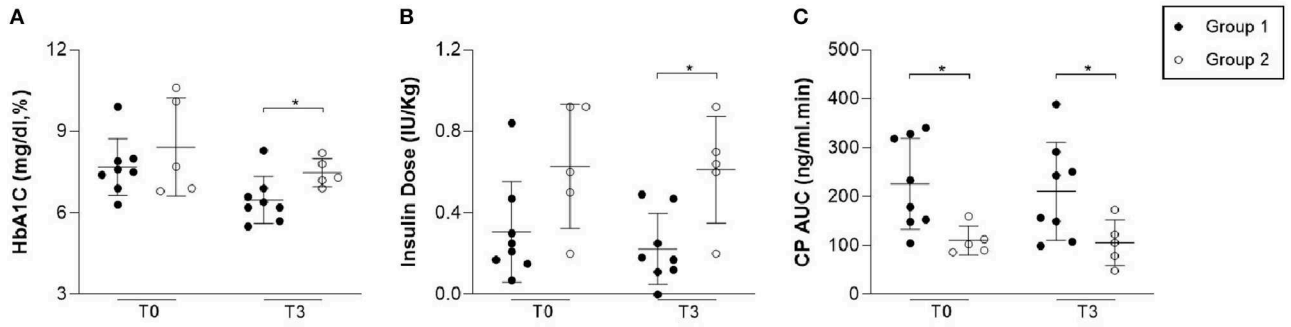

FIGURE 3 | Levels of HbA1C, insulin dose and C-Peptide during follow-up. The levels were evaluated on blood samples before (T0) and three (T3) months after ASCs infusion. Group 1 presented lower HbA1C ($p = 0.03$) and insulin dose ($p = 0.01$) in comparison to group 2. Group 1 presented similar CP AUC ($p = 0.06$) in comparison to group 2.

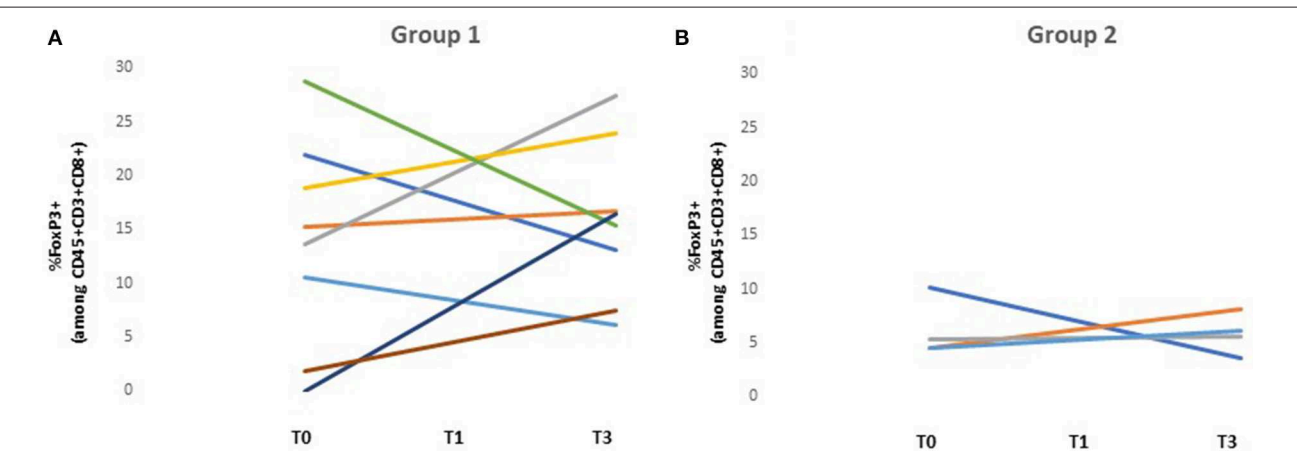

FIGURE 4 | Variation of FoxP3+ cells among CD45+CD3+CD8+ cells. The levels of T-cells were evaluated on blood samples before (T0), one (T1) and three (T3) months after ASCs infusion. **(A)** Individual variation of FoxP3+ cells among CD45+CD3+CD8+ cells of group 1. **(B)** Individual variation of FoxP3+ cells among CD45+CD3+CD8+ cells of group 2. At T1, group 1 presented a higher frequency of CD45+CD3+CD8+FoxP3+ cells in comparison to group 2 ($p = 0.03$). At T0 and T3 no significant difference was found ($p = 0.17$ and $p = 1.00$, respectively).

with mild and transient unanticipated adverse events, although a longer follow-up is still required.

After a 3 month follow-up, those that underwent ASCs infusion + VitD had a better glycemic control and lower insulin requirement than the group in standard treatment. We cannot exclude that the better glycemic control in the treated individuals are due to the higher baseline CP or slightly older age, which have been associated with more sustained residual β-function in previous studies (5, 47). However, neither basal CP or age were correlated to A1c decrease in this study and the sample comprised mostly young adults. Moreover, separate longitudinal analyses were performed for each group and indicated an improvement in HbA1c in the intervention group but not in controls. This suggests that the intervention itself might have a potential therapeutic effect (2, 4, 5, 48).

A therapeutic effect of the intervention in glycemic control could be related to the ASCs, VitD or more probably to the combination of both acting in multiple pathways to arrest β cell destruction. The inverse correlation between VitD levels and both insulin requirements and HbA1C seen in group 1 suggests a role of VitD in the process (26–28). However, most results with vitamin D supplementation from previous clinical trials are inconsistent and do not indicate a significant effect on glycemic indices in patients with recent-onset T1D (29, 31). Interestingly, Gabbay et al. have shown lower CP decline in patients with recent-onset T1D that received vitamin D supplementation (30).

Although it is not possible to determine if a potential beneficial effect of the combined intervention was caused by immune modulation or by the differentiation of ASCs in β cells, the higher frequency of CD8+FoxP3+ T cells in those that underwent the intervention suggests an increase in T cell regulatory response. Immunoregulatory CD8+ T cells have been reported before (49) and were also associated with clinical response in a T1D trial with humanized Fc-mutated anti-CD3 monoclonal antibody hOKT3 (50). However, as CD127 staining or other markers for cell characterization were not performed, we cannot exclude

that the higher FOXP3 expression represents solely T cell activation (51, 52).

Both CD4+FoxP3$^+$ and CD8+FoxP3$^+$ cells were inversely correlated not only to HbA1C but also to various metrics of glucose variability, which suggests that a T cell regulatory response in early onset T1D might reduce glucose excursions, but might also be related to T cell activation. (2, 6–8, 50–52).

This study had some limitations. Firstly, the small sample size could explain the lack of statistical significance observed in some parameters. However, this was a pilot study with the priority to establish the safety of ASCs and the feasibility of the trial. Other limitations were the lower baseline CP and age in the control group, when compared to the intervention group. Nevertheless, we compared the percentage of changes in CP, insulin requirements and A1c variation as well as longitudinal changes in these parameters within each group. An additional limitation was the absence of a group treated solely with vitamin D and insulin, to determine if the positive results were related to ASCs, vitamin D or both. Moreover, a longer follow-up is still necessary to determine the long-term safety and efficacy of this intervention. The use of Glucerna® instead of higher glycemic index carbohydrate preparations for CP evaluation might also be a limitation as this might have slightly underestimated pancreatic residual secretion but the same test was performed for all subjects with the carbohydrate content recommended for mixed meal test.

In summary, this was the first study to test allogenic ASCs infusion + VitD without immunosuppression in patients with recent onset T1D. After a 3 month follow-up, patients that underwent intervention showed stability of CP, better glucose control and lower insulin requirement than patients that received only standard treatment, but the favorable outcome may be related to random baseline differences between groups. A larger sample and a longer follow-up period are necessary to further investigate the safety of the treatment and to determine the efficacy of ASCs infusion combined to VitD supplementation for recent-onset T1D. However, this pilot study is an important early assessment of ASCs and vitamin D supplementation as a potential combined therapy for T1D.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This clinical trial has been registered at ClinicalTrial.gov (NCT03920397) and approved by the Ethics Research Board of the University Hospital Clementino Fraga Filho (HUCFF) from the Federal University of Rio de Janeiro (UFRJ). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DA, MP, CC-D-S, and DS researched data. JD, KS, and LB wrote the manuscript and researched data. MR, CR, and LZ reviewed/edited the manuscript and contributed with the discussion. RL and JM contributed with statistical analysis supervision. CC, MG, SD, JO, and AM contributed to the discussion and reviewed the manuscript. PB, AS, and DD processed the ASC cells.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00993/full#supplementary-material

REFERENCES

1. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet.* (2001) 358:221–9. doi: 10.1016/S0140-6736(01)05415-0
2. Achenbach P, Bonifacio E, Koczwara K, Ziegler AG. Natural history of type 1 diabetes. *Diabetes.* (2005) 54(Suppl. 2):S25–31. doi: 10.2337/diabetes.54.suppl_2.S25
3. Couri CEB, de Oliveira MC, Simões BP. Risks, benefits, and therapeutic potential of hematopoietic stem cell transplantation for autoimmune diabetes. *Curr Diabetes Rep.* (2012) 12:604–11. doi: 10.1007/s11892-012-0309-0
4. Dantas JR, Almeida MH, Barone B, Serfaty F, Raggio LR, Kupfer R, et al. Continuous C-peptide loss in patients with type 1 diabetes and multiethnic background. *Diabetes Res Clin Pract.* (2013) 99:e33–6. doi: 10.1016/j.diabres.2012.12.019
5. Rodacki M, Milech A, de Oliveira JEP. A secreção residual do peptídeo C faz diferença no tratamento do diabetes melito tipo 1? *Arq Bras Endocrinol Metab.* (2008) 52:322–33. doi: 10.1590/S0004-27302008000200020
6. Fiorina P, Voltarelli J, Zavazava N. Immunological applications of stem cells in type 1 diabetes. *Endocr Rev.* (2011) 32:725–54. doi: 10.1210/er.2011-0008
7. Lernmark Å, Larsson HE. Immune therapy in type 1 diabetes mellitus. *Nat Rev Endocrinol.* (2013) 9:92–103. doi: 10.1038/nrendo.2012.237
8. Rydén AK, Wesley JD, Coppieters KT, Von Herrath MG. Non-antigenic and antigenic interventions in type 1 diabetes. *Hum Vaccines Immunother.* (2014) 10:838–46. doi: 10.4161/hv.26890
9. Davis IC, Randell J, Davis SN. Immunotherapies currently in development for the treatment of type 1 diabetes. *Expert Opin Invest Drugs.* (2015) 24:1331–41. doi: 10.1517/13543784.2015.1075973
10. Vudattu NK, Herold KC. Treatment of new onset type 1 diabetes with teplizumab: successes and pitfalls in development. *Expert Opin Biol Ther.* (2014) 14:377–85. doi: 10.1517/14712598.2014.881797
11. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med.* (2009) 361:2143–52. doi: 10.1056/NEJMoa0904452

12. Marmont AM. Coincidental autoimmune disease in patients transplanted for conventional indications. *Best Pract Res Clin Haematol.* (2004) 17:223–32. doi: 10.1016/j.beha.2004.04.004
13. Kuhn C, Besançon A, Lemoine S, You S, Marquet C, Candon S, et al. Regulatory mechanisms of immune tolerance in type 1 diabetes and their failures. *J Autoimmun.* (2016) 71:69–77. doi: 10.1016/j.jaut.2016.05.002
14. Okubo Y, Torrey H, Butterworth J, Zheng H, Faustman DL. Treg activation defect in type 1 diabetes: correction with TNFR2 agonism. *Clin Trans Immunol.* (2016) 5:e56. doi: 10.1038/cti.2015.43
15. Hamari S, Kirveskoski T, Glumoff V, Kulmala P, Simell O, Knip M, et al. Analyses of regulatory CD4 + CD25 + FOXP3 + T cells and observations from peripheral T cell subpopulation markers during the development of type 1 diabetes in children. *Scand J Immunol.* (2016) 83:279–87. doi: 10.1111/sji.12418
16. Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. *Diabetes.* (2008) 57:1759–67. doi: 10.2337/db08-0180
17. Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci USA.* (2006) 103:17438–43. doi: 10.1073/pnas.0608249103
18. Baptista LS, do Amaral RJFC, Carias RBV, Aniceto M, Claudio-da-Silva C, Borojevic R. An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples. *Cytotherapy.* (2009) 11:706–15. doi: 10.3109/14653240902981144
19. García-Olmo D, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA. A phase I clinical trial of the treatment of crohn's fistula by adipose mesenchymal stem cell transplantation: diseases of the colon & rectum. *Dis Colon Rectum.* (2005). 48:1416–23. doi: 10.1007/s10350-005-0052-6
20. Gan J, Wang Y, Zhou X. Stem cell transplantation for the treatment of patients with type1 diabetes mellitus: a meta-analysis. *Exp Ther Med.* (2018) 16:4479–92. doi: 10.3892/etm.2018.6769
21. Rivera-Izquierdo M, Cabeza L, Láinez-Ramos-Bossini A, Quesada R, Perazzoli G, Alvarez P, et al. An updated review of adipose derived-mesenchymal stem cells and their applications in musculoskeletal disorders. *Expert Opin Biol Ther.* (2019) 19:233–48. doi: 10.1080/14712598.2019.1563069
22. Georgiev-Hristov T, Guadalajara H, Herreros MD, Lightner AL, Dozois EJ, García-Arranz M, et al. A step-by-step surgical protocol for the treatment of perianal fistula with adipose-derived mesenchymal stem cells. *J Gastrointest Surg.* (2018) 22:2003–12. doi: 10.1007/s11605-018-3895-6
23. De Jesus MM, Santiago JS, Trinidad CV, See ME, Semon KR, Fernandez MO Jr., et al. Autologous adipose-derived mesenchymal stromal cells for the treatment of psoriasis vulgaris and psoriatic arthritis: a case report. *Cell Transplant.* (2016) 25:2063–9. doi: 10.3727/096368916X691998
24. Maria AT, Maumus M, Le Quellec A, Jorgensen C, Noël D, Guilpain P. Adipose-derived mesenchymal stem cells in autoimmune disorders: state of the art and perspectives for systemic sclerosis. *Clin Rev Allergy Immunol.* (2017) 52:234–59. doi: 10.1007/s12016-016-8552-9
25. Daumas A, Magalon J, Jouve E, Truillet R, Casanova D, Giraudo L, et al. Long-term follow-up after autologous adipose-derived stromal vascular fraction injection into fingers in systemic sclerosis patients. *Curr Res Transl Med.* (2017). 65:40–3. doi: 10.1016/j.retram.2016.10.006
26. Riachy R, Vandewalle B, Kerr Conte J, Moerman E, Sacchetti P, Lukowiak B, et al. 1,25-dihydroxy vitamin D 3 protects RINm5F and human islet cells against cytokine-induced apoptosis: implication of the antiapoptotic protein a20. *Endocrinology.* (2002) 143:4809–19. doi: 10.1210/en.2002-220449
27. Al-Zubeidi H, Leon-Chi L, Newfield RS. Low vitamin D level in pediatric patients with new onset type 1 diabetes is common, especially if in ketoacidosis: Vitamin D in new onset Type 1 Diabetes. *Pediatr Diabetes.* (2016) 17:592–8. doi: 10.1111/pedi.12342
28. Treiber G, Prietl B, Fröhlich-Reiterer E, Lechner E, Ribitsch A, Fritsch M, et al. Cholecalciferol supplementation improves suppressive capacity of regulatory T-cells in young patients with new-onset type 1 diabetes mellitus - a randomized clinical trial. *Clin Immunol.* (2015) 161:217–24. doi: 10.1016/j.clim.2015.08.002
29. Gregoriou E, Mamais I, Tzanetakou I, Lavranos G, Chrysostomou S. The effects of vitamin D supplementation in newly diagnosed type 1 diabetes patients: systematic review of randomized controlled trials. *Rev Diabet Stud.* (2017) 14:260–8. doi: 10.1900/RDS.2017.14.260
30. Gabbay MAL, Sato MN, Finazzo C, Duarte AJ, Dib SA. Effect of cholecalciferol as adjunctive therapy with insulin on protective immunologic profile and decline of residual β-cell function in new-onset type 1 diabetes mellitus. *Arch Pediatr Adol Med.* (2012) 166:601–7. doi: 10.1001/archpediatrics.2012.164
31. Thorsen SU, Mortensen HB, Carstensen B, Fenger M, Thuesen BH, Husemoen L, et al. No association between type 1 diabetes and genetic variation in vitamin D metabolism genes: a Danish study: T1D and SNPs in vitamin D metabolism genes. *Pediatr Diabetes.* (2014) 15:416–21. doi: 10.1111/pedi.12105
32. Vija L, Farge D, Gautier J-F, Vexiau P, Dumitrache C, Bourgarit A, et al. Mesenchymal stem cells: stem cell therapy perspectives for type 1 diabetes. *Diabetes Metab.* (2009) 35:85–93. doi: 10.1016/j.diabet.2008.10.003
33. Cai J, Wu Z, Xu X, Liao L, Chen J, Huang L, et al. Umbilical cord mesenchymal stromal cell with autologous bone marrow cell transplantation in established type 1 diabetes: a pilot randomized controlled open-label clinical study to assess safety and impact on insulin secretion. *Diabetes Care.* (2016) 39:149–57. doi: 10.2337/dc15-0171
34. Trivedi HL, Vanikar AV, Thakker U, Firoze A, Dave SD, Patel CN, et al. Human adipose tissue-derived mesenchymal stem cells combined with hematopoietic stem cell transplantation synthesize insulin. *Transpl Proc.* (2008) 40:1135–9. doi: 10.1016/j.transproceed.2008.03.113
35. Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA.* (2007) 297:1568–76. doi: 10.1001/jama.297.14.1568
36. Sokołowska M, Chobot A, Jarosz-Chobot P. Department of pediatrics, the upper silesian child health center, the honeymoon phase - what we know today about the factors that can modulate the remission period in type 1 diabetes. *Pediatr Endocr Diabetes Metab.* (2016) 22:66–70. doi: 10.18544/PEDM-22.02.0053
37. Langendam M, Luijf YM, Hooft L, DeVries JH, Mudde AH, Scholten RJ. Continuous glucose monitoring systems for type 1 diabetes mellitus. *Cochrane Datab Syst Rev.* (2012) 1:CD008101. doi: 10.1002/14651858.CD008101.pub2
38. Kohnert K-D, Heinke P, Fritzsche G, Vogt L, Augstein P, Salzsieder E. Evaluation of the mean absolute glucose change as a measure of glycemic variability using continuous glucose monitoring data. *Diabetes Technol Ther.* (2013) 15:448–54. doi: 10.1089/dia.2012.0303
39. Hill NR, Oliver NS, Choudhary P, Levy JC, Hindmarsh P, Matthews DR. Normal reference range for mean tissue glucose and glycemic variability derived from continuous glucose monitoring for subjects without diabetes in different ethnic groups. *Diabetes Technol Ther.* (2011) 13:921–8. doi: 10.1089/dia.2010.0247
40. Battelino T, Danne T, Bergenstal RM, Amiel SA, Beck R, Biester T, et al. Clinical targets for continuous glucose monitoring data interpretation: recommendations from the international consensus on time in range. *Diabetes Care.* (2019) 42:1593–603. doi: 10.2337/dci19-0028
41. Evert AB, Boucher JL, Cypress M, Dunbar SA, Franz MJ, Mayer-Davis EJ, et al. Nutrition therapy recommendations for the management of adults with diabetes. *Diabetes Care.* (2013) 36:3821–42. doi: 10.2337/dc13-2042
42. Wang Y, Wu S, Wen F, Cao, Q. Diabetes mellitus as a risk factor for retinal vein occlusion: a meta-analysis. *Medicine.* (2020) 99:e19319. doi: 10.1097/MD.0000000000019319
43. Yoo, YS; Na, KS; Shin, JA; Park, YH; Lee, JW. Posterior eye segment complications related to allogeneic hematopoietic stem cell transplantation. *Retina.* (2017) 37:135–43. doi: 10.1097/IAE.0000000000001122
44. Zhang Y, Chen W, Feng B, Cao H. The clinical efficacy and safety of stem cell therapy for diabetes mellitus: a systematic review and meta-analysis. *Aging Dis.* (2020) 11:141–53. doi: 10.14336/AD.2019.0421
45. Bertuzzi F, De Carlis L, Marazzi M, Rampoldi AG, Bonomo M, Antonioli B, et al. Long-term effect of islet transplantation on glycemic variability. *Cell Transplant.* (2018) 27:840–6. doi: 10.1177/0963689718763751
46. Hugle T, Daikeler T. Stem cell transplantation for autoimmune diseases. *Haematologica.* (2010) 95:185–8. doi: 10.3324/haematol.2009.017038
47. Rickels MR, Evans-Molina C, Bahnson HT, Ylescupidez A, Nadeau KJ, Hao W, et al. High residual C-peptide likely contributes to glycemic control in type 1 diabetes. *J Clin Invest.* (2020) 130:1850–62. doi: 10.1172/JCI134057

48. Redondo MJ, Connor CG, Ruedy KJ, Beck RW, Kollman C, Wood JR, et al. Pediatric diabetes consortium type 1 diabetes new onset (NeOn) study: factors associated with HbA1c levels one year after diagnosis: characteristics associated with HbA1c. *Pediatr Diabetes*. (2014) 15:294–302. doi: 10.1111/pedi.12061
49. Christoffersson G, von Herrath M. Regulatory immune mechanisms beyond regulatory T cells. *Trends Immunol*. (2019) 40:482–91. doi: 10.1016/j.it.2019.04.005
50. Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. *J Clin Invest*. (2005) 115:2904–13. doi: 10.1172/JCI 23961
51. Hagopian W, Ferry RJ Jr., Sherry N, Carlin D, Bonvini E, Johnson S, et al. Teplizumab preserves C-peptide in recent-onset type 1 diabetes: two-year results from the randomized, placebo-controlled Protégé trial. *Diabetes*. (2013) 62:3901–8. doi: 10.2337/db13-0236
52. Aronson R, Gottlieb PA, Christiansen JS, Donner TW, Bosi E, Bode BW, et al. Low-dose otelixizumab anti-CD3 monoclonal antibody DEFEND-1 study: results of the randomized phase III study in recent-onset human type 1 diabetes. *Diabetes Care*. (2014) 37:2746–54. doi: 10.2337/dc13-0327

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SYSTEMATIC REVIEW
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Immunomodulatory Effects of Diet and Nutrients in Systemic Lupus Erythematosus (SLE): A Systematic Review

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multiple organ involvement, including the skin, joints, kidneys, lungs, central nervous system and the haematopoietic system, with a large number of complications. Despite years of study, the etiology of SLE remains unclear; thus, safe and specifically targeted therapies are lacking. In the last 20 years, researchers have explored the potential of nutritional factors on SLE and have suggested complementary treatment options through diet. This study systematically reviews and evaluates the clinical and preclinical scientific evidence of diet and dietary supplementation that either alleviate or exacerbate the symptoms of SLE. For this review, a systematic literature search was conducted using PubMed, Scopus and Google Scholar databases only for articles written in the English language. Based on the currently published literature, it was observed that a low-calorie and low-protein diet with high contents of fiber, polyunsaturated fatty acids, vitamins, minerals and polyphenols contain sufficient potential macronutrients and micronutrients to regulate the activity of the overall disease by modulating the inflammation and immune functions of SLE.

Keywords: diet, nutrients, autoimmune diseases, systemic lupus erythematosus (SLE), polyunsaturated fatty acids, vitamins, minerals, polyphenols

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INTRODUCTION

Systemic lupus erythematosus (SLE), an autoimmune disease, is characterized by abnormal inflammatory responses due to complex, aberrant humoral and cellular immune responses. The pathogenesis of SLE is largely unknown; however, data from the literature suggest that manifestation of this disease is the result of several environmental, hormonal, and nutritional factors that, in predisposed subjects, contribute to impaired cellular, and humoral immune responses (1–3). Accordingly, the presence of autoantibodies is nearly universal among patients with SLE. Indeed, having antinuclear antibodies specifically against double-stranded DNA (dsDNA) is a hallmark classification criterion for SLE; this and other clinical and immunological criteria must be satisfied for SLE classification as proposed by the European League Against Rheumatism and American College of Rheumatology (4). Autoantibodies also contribute to the synthesis of multiple immune complexes and exert direct cytotoxic effects. As a result, SLE affects the whole body with no system spared; finally, damage occurs in multiple organs including the kidneys, central nervous system (CNS), skin, joints and haematopoietic system (5, 6). Although SLE

represents a prototypical autoimmune disorder, its prevalence is relatively low, estimated between 6.5 and 178.0 per 100,000 people, with an incidence ranging from 0.3 to 23.7 per 100,000 people per year (7).

The contribution of lifestyle-associated factors is still a matter of controversy in SLE; however, dietary habits and dietary-related microbiome composition are receiving more attention from researchers (8, 9). Indeed, some SLE-related clinical features are associated with nutrition; perhaps not as an aetiological factor but as a clinical repercussion (10). Thus, SLE represents a mosaic of metabolic changes and mineral and vitamin deficiencies superimposed by the systemic presentation of arthritis, nephritis, vascular events and organ damage to the heart, CNS, kidneys, and skin, which contribute to increases in the morbidity and mortality of these patients (6, 11, 12). In the last two decades, many clinical, and preclinical studies have investigated the impact of diet and nutrients on SLE inflammatory response and disease activity. This has become an important highlighted topic and remains under investigation by many researchers. Nutritional therapy including restrictions on carbohydrate and protein and the use of nutritional supplements (*i.e.*, vitamins, minerals and polyphenols) is a promising way to control inflammatory responses in SLE (8, 13). Nutritional supplements may exert potentially prophylactic effects with fewer or no side effects than those of the classic pharmacological therapies besides reducing co-morbidities and improving the quality of life of patients with SLE.

As a broad range of evidence has demonstrated that some diets and nutrients have antioxidant, anti-inflammatory and immunomodulatory effects on immunoinflammatory diseases, the present study evaluates the impact of diet and nutrients on SLE based on the existing *in vivo* studies in animal models and human subjects.

METHODS: SEARCH STRATEGY

A systematic search strategy was developed by combining the terms SLE, "Systemic lupus erythematosus," lupus, food, nutrient*, diet, intake, antioxidant*, nutrition*, benefit*, nutrition*, physicochemical, dietary, bioactive, composition, supplement*, vitamin*, mineral*, phenol*, "olive oil," and curcumin, where quotations represented an exact term and an asterisk (*) denoted a root word or wildcard term. PubMed, Scopus, and Google Scholar electronic databases were searched combining the appropriate keywords with Boolean logical operators "AND" and "OR" using "Advanced" and "Expert" search options (**Appendix**). Only English-language articles were searched. There was no year restriction, and the final systematic search was conducted on 22 December 2019. Review articles, non-English articles, errata, letters, comments, editorials, and duplicate articles among different databases were excluded. Duplicate studies that resulted from different electronic databases were removed and managed by EndNote software (version X8). Study selection methodology is illustrated in **Figure 1**.

MACRONUTRIENTS

Macronutrients represent the group of environmental substances widely used by organisms for vital processes such as growth, body development, and bodily functions. Several theories have described the effects of macronutrient- or macronutrient-derived molecules including glucose, amino acids, and fatty acids on body weight regulation, maintenance of homeostasis, and the immune response.

Carbohydrates

Carbohydrates are among the macronutrients that provide energy and, when consumed in excess, contribute to increased energy storage and subsequent weight gain. Although there is no clear evidence that altering the proportion of total carbohydrate in the diet is an important determinant of energy intake, nutritional imbalance, and excess carbohydrate dietary intake have been suggested as risk factors that exacerbate clinical manifestations of several autoimmune diseases such as rheumatoid arthritis and SLE (14). Obesity is a well-known risk factor for low-grade inflammation characterized by activation of several pathways involved in the expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6. Activation of these proinflammatory pathways significantly contributes to the perpetuation of the inflammatory response, which are at least partly responsible for the severe co-morbidities seen in SLE patients (15). Indeed, patients with SLE are characterized by a high-risk of developing metabolic syndrome, insulin resistance and type 2 diabetes mellitus (T2DM) (16), which can contribute to increased risk of developing cardiovascular co-morbidities, a leading cause of premature death in SLE patients (17).

Indeed, several studies have shown that up to 35% of SLE patients are overweight and 39% are obese, and these patients are characterized by a higher concentration of inflammatory markers including C-reactive protein (CRP) (18, 19). Recent studies have suggested that obesity is associated independently with SLE disease activity (20, 21). Corticosteroids remain the first choice of treatment for SLE, but their administration is linked to excess weight gain and the development of corticosteroid-induced diabetes. Obesity was detected as an independent risk factor in worsening the functional capacity, fatigue, and inflammation status of patients with SLE (20, 22–24).

Mouse Models

Mouse models provide excellent insight into the pathogenesis of SLE and the observation of dietary-induced changes. Notably, the restriction of calorie intake leads to substantial changes in the immune response. For example, in a study with a lupus-prone mouse model (NZB/NZW F1), calorie intake restriction effectively delayed the onset of proteinuria and significantly decreased serum levels of anti-dsDNA antibodies. Calorie restriction also had a significant impact on the B-cell population, resulting in a reduction of their frequency and activity. Parallel to this, a decline in $CD8^+$ T cells and a higher proportion of naïve $CD4^+$ and $CD8^+$ T cells have been observed (25). This finding is of special importance as it

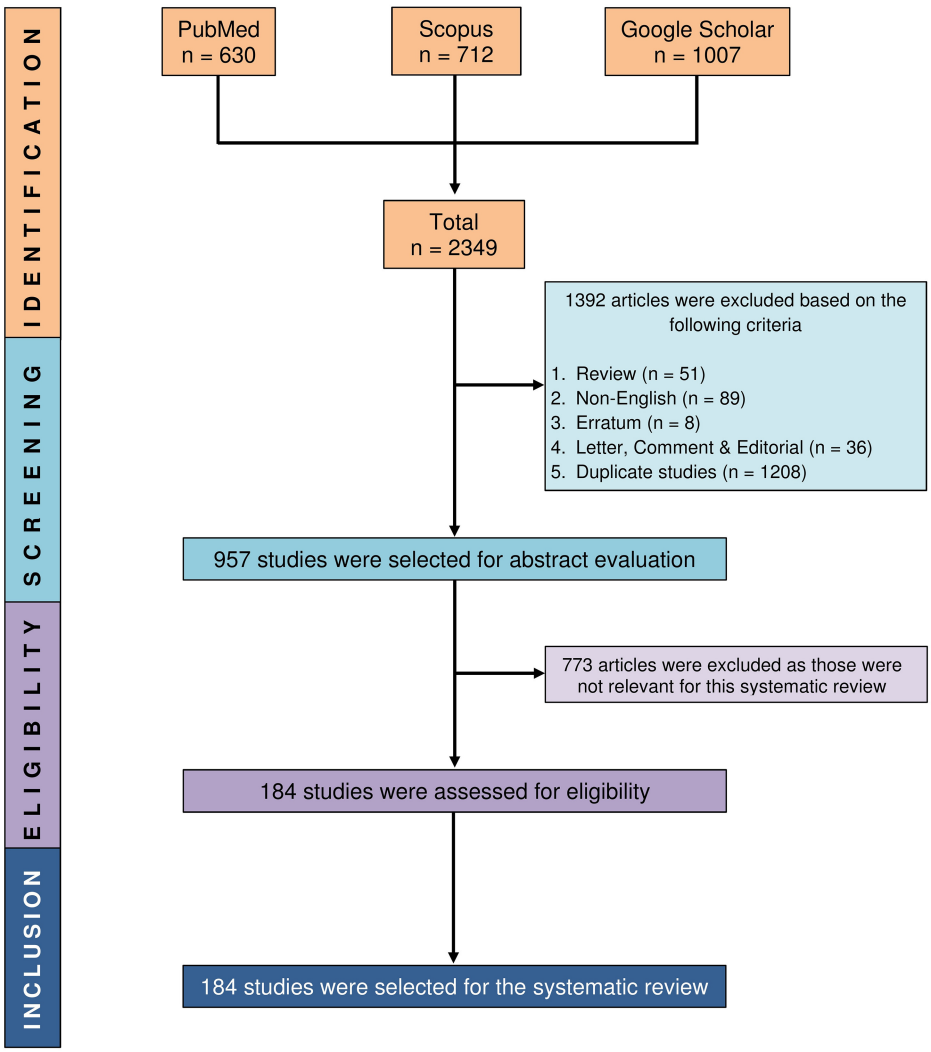

FIGURE 1 | PRISMA flow diagram showing the process of selecting relevant studies.

shows the direct impact of calorie restriction on B-cell and T-cell compartments, key immunocompetent cell compartments involved directly in SLE pathogenesis (26, 27). In another SLE-prone mouse model, when a 40% calorie-restricted diet was provided, B:T cell and $CD4^+$:$CD8^+$ T-cell ratios were significantly lowered compared with the control group (28). This suggests a decline in the predominance of abnormally activated T cells and regulatory T (Treg) cells that potentially reduces disease activity.

The impact of calorie intake on autoimmune system functioning is not restricted to immune executive cells. At the end of the last century, Troyer et al. (29) showed that a calorie-restricted diet was successful in modulating the key inflammatory ligand platelet-derived growth factor (PDGF) subunit A and the thrombin receptor, resulting in the suppression of murine lupus nephritis. Quite recently, the direct mechanism of this finding has been explained, elucidating the role of PDGF as a growth factor that regulates cell proliferation and is responsible for mesangial proliferation, periostin formation and progressive glomerulosclerosis (30).

Less is known about the humoral response in this regard. The influence of calorie restriction on the expression of main cytokines and synthesis of immunoglobulin (Ig)G has been tested in NZB/NZW F1 mice. In the study, reduced calorie intake contributed to the reduction of *interferon (IFN)-γ*, *IL-10*, and *IL-12* mRNA expression besides the reduction of IgG secretion in the submandibular glands (31).

Human Study

During the last several decades, the prevalence of excess body weight has increased rapidly worldwide and is now recognized as a main public health crisis (32). Obesity has a strong impact on organism functioning and is linked to the development of all diseases of civilization, including metabolic syndrome, atherosclerosis and T2DM. Strong evidence also links obesity to many autoimmune disorders including SLE (8, 33, 34). Obesity

in SLE patients is associated with a poorer outcome, higher disease activity and higher cumulative organ damage (34, 35). Therefore, the importance of prevention and treatment of obesity is widely acknowledged. Unfortunately, these recommendations are chiefly driven from cross-population studies; thus, data on the direct impact of body weight reduction on disease activity in SLE are limited.

The influence of body weight reduction on SLE activity has been addressed by Davies et al. (36). They enrolled 23 overweight female subjects (BMI: >25 kg/m^2) with SLE who were on corticosteroid therapy. A 6-week controlled trial where a low glycaemic index diet ($n = 11$) or a calorie-restricted diet ($n = 12$) was implemented resulted in significant weight loss, reduction in waist and hip measurements and fatigue in both treatment groups. Of note, caloric restriction did not cause any flares of disease. However, these results must be interpreted with caution because they from only one study that addresses this issue, and the population studied was small. Nevertheless, it is surprising that such an important problem has not yet attracted greater attention, and more studies in this field are required. Presently, evidence supports the idea that a hypocaloric diet reduces the disease activity of SLE. By contrast, however, not all obese patients share inflammatory profiles, and not all individuals in a healthy weight range are metabolically healthy (37, 38). This underscores the need for proper nutrition or weight loss as only two factors of many for lupus patients (18). Lifestyle modifications such as meditation and exercise can also ameliorate lupus symptoms (39–43). Therefore, patients with SLE should maintain a balanced diet with caution to avoid excess daily calorie intake besides avoiding a sedentary lifestyle, especially in the case of obese patients or patients with a tendency toward obesity.

Proteins

The restriction of dietary protein has been addressed in several studies in patients with SLE and animal models. These data should be interpreted in a wider context as diet composition rather than protein restriction alone may show beneficial effects on SLE course. As an example, a traditional Mediterranean diet provides protection from certain chronic diseases including autoimmune disorders. This diet consists of vegetables, fruits, nuts, grains, olive oils and fish with limited meat consumption. Reduction of protein intake may be a reasonable approach in cases of lupus nephropathy as high protein intake contributes to reduced renal filtration, directly leading to the progression of kidney damage (44). Indeed, Milovanov et al. (45) observed that early restriction of dietary protein (0.6 g/kg/day) slowed the decline in glomerular filtration rate in patients with SLE-induced chronic kidney disease. In a cross-sectional study on Brazilian juvenile subjects with SLE ($n = 22$), consistent bone mineral loss was detected when subjects were treated with excessive proteins (46).

It has also been postulated that not only proteins but also selected amino acids may influence the course of SLE. In a case-controlled study, levels of serum L-canavanine (a non-proteinogenic amino acid) was significantly high ($p < 0.01$) in a group of Mexican patients with SLE ($n = 100$) compared with those of healthy controls ($n = 100$). This was therefore postulated to be a risk factor in developing SLE (47).

Among the many mechanisms by which amino acids modulate the immune response, regulation of mechanistic target of rapamycin (mTOR) attracts special attention. The signaling of mTOR is recognized as the most important intracellular pathway that coordinates local nutrients and systemic energy status at the organismal and cellular levels (48). Moreover, it is deeply involved in the immune response; thus, any dysfunction in this pathway may result in an aberrant immune response and predisposition to the development of autoimmunity (49). The role of the mTOR pathway is usually discussed in the context of T-cell function (50). It has been shown recently that T-cell dysfunction observed in SLE is at least partially due to the activation of mTOR caused by reduced glutathione (GSH) depletion via mitochondrial hyperpolarisation (51, 52). It was established that supplementation with N-acetylcysteine (NAC), a precursor of GSH, significantly improved the activity of SLE disease by profoundly blocking mTOR activity ($p < 0.0009$) in T cells and reversing GSH depletion (53). Regarding the other amino acids, in case-controlled studies (54, 55), lower levels of tryptophan and higher levels of kynurenine (a metabolite of tryptophan) with activation of the kynurenine pathway were detected in patients with SLE. Indeed, an interventional study showed that NAC treatment significantly reduced the levels of kynurenine ($p = 2.8 \times 10^{-7}$) in patients with SLE (56). Therefore, supplementation with NAC or a GSH-rich diet and nutrients may exert a therapeutic role in the management of SLE.

Contrary to this, a diet low in phenylalanine and tyrosine showed a protective role against nephropathy (57) and reduced autoantibody production in a mouse model (NZB/W) of SLE (58). In another study, high L-arginine intake was associated with renal fibrosis and shortened the life span of MRL/*lpr* SLE mice (59).

Considering the role of proteins and amino acids, it should be emphasized that a diet of moderate protein intake is recommended, and a high-protein diet should be avoided especially by patients with lupus-related kidney diseases (*e.g.,* overt lupus nephropathy). Major sources of dietary proteins are shown in **Table 1** based on the *United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference* (60).

Essential Fatty Acids

Fatty acids (FAs), especially polyunsaturated FAs (PUFAs), are an effective and essential dietary factor for patients with SLE (61). Among PUFAs, omega-3 (ω-3) fatty acids [*i.e.,* docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)] can reduce the level of inflammatory mediators (**Figure 2**) as well as CRP. EPA and DHA can further reduce lymphocyte proliferation, macrophage-mediated and cytotoxic T-cell-mediated cytotoxicity, synthesis of proinflammatory cytokines, and chemotaxis from monocytes and neutrophils.

In a lupus-prone mouse model, when animals were fed with DHA, IFN-related genes were suppressed besides reduced autoantibody levels and glomerulonephritis (62). In another lupus-prone mouse model (female NZBWF1), the

TABLE 1 | Major nutrient sources related to systemic lupus erythematosus.

Nutrients		Sources*
MACRONUTRIENTS		
Amino acids		Eggs, meat, dairy products, pulses/legumes, whole cereals, royal jelly, and seafoods.
Polyunsaturated fatty acids	Omega-3	Fish oil, krill oil, flaxseed oil, canola oil, soybean oil, olive oil, nuts, margarine, and fishes (*i.e.*, salmon, tuna, sardine, herring, mackerel, sablefish, whitefish).
	Omega-6	Safflower oil, sunflower oil, soybean oil, maize oil, sesame oil, canola oil, corn oil, poppyseed oil, nuts, walnut oil, primrose oil, margarine, ruminant, meat, eggs, and milk.
Fiber		Beans, cereals, pulses/legumes, whole grains, vegetables, fruits, curry powder, cinnamon, dried rosemary, dried oregano, coriander seed, dried basil, chili powder, and cloves.
MICRONUTRIENTS		
Vitamins	A	Carrots, sweet potatoes, pumpkins, spinach, shallots, kale, pepper, liver, fish oil, various meats, and tropical fruits.
	B complex	Fortified cereals, peanut butter, potatoes, dried peppers, nuts, banana, avocado, eggs, chicken, various red meats, liver, mollusks, salmon, and sardine.
	C	Tangerine, orange juice, apple, papaya, guava, litchis, kiwi, broccoli, tomato, carrot, pepper, and whole cereals, green tea, coriander leaf.
	D	Sunlight exposure, eggs, liver, fatty fishes (*i.e.*, salmon eel, mackerel, trout sturgeon, swordfish, and sardine), fish oil, cod liver, mushrooms, and supplemented dairy products.
	E	Wheat germ, sunflower oil and seeds, canola oil, soybean, whole cereals, nuts, almonds, peanut butter, milk, fish, spinach, pepper, and margarine.
Minerals	Calcium	Dairy products, dried basils, dried tofu, kale, soybean, spinach, sardine, and fortified whole cereals.
	Zinc	Mollusks, whole cereals, peanut butter, seeds, white beans, soybean, spinach, milk, beef, turkey, and lamb.
	Sodium	Table salt, soy sauce, salted fishes (*i.e.*, mackerel and salmon), wasabi, salted tofu, chili powder, canned foods, and cheeses with salt.
	Selenium	Pike, carp, rainbow trout, mollusks, wheat germ, whole cereals, sunflower seeds, nuts, fish (tuna, cod, haddock, salmon, crayfish, herring), egg, chicken liver, turkey, lamb, beef, mustard seed, fortified flours and products, and ricotta.
	Iron	Fortified whole cereals, dried basil, dried spearmint, seaweed, cumin seed, fenugreek seed, turmeric, bay leaf, soybeans, kale, pulses/legumes, mollusks, various meats, spinach, and broccoli.
	Copper	Beans, sesame seeds, sunflower seeds, dried basil, lentils, mushrooms, seaweed, nuts, mollusks, various meats, and liver.
Polyphenols		Various fruits and vegetables (*i.e.*, grapes, oranges, watermelon, kiwi, apple, tomato, lettuce, broccoli, asparagus, spinach, lentils, celery, parsley, thyme, and peppermint), green tea, coffee, walnut, green, and white beans, olive oil, chamomile olives, and legumes.

**Based on the USDA, National Nutrient Database for Standard Reference 2018.*

autoimmune response (*i.e.*, increased levels of proinflammatory cytokines) was triggered by airway silica exposure, resulting in glomerulonephritis, lung damage and autoantibody formation. It was established that an isocaloric diet containing DHA reduced cytokine and chemokine synthesis, lymphocyte infiltration and autoantibody synthesis by plasma cells in this model (63).

In the mid-1980s, Kelley et al. in his pioneer study (64) demonstrated the direct impact of a fish oil diet (rich in EPA) on prostanoid metabolism and function that ultimately led to reduction of the inflammatory response in MRL/*lpr* mice. Many years later, the mechanism of this phenomenon was explained. The direct anti-inflammatory effect of EPA was due to the inhibition of cyclooxygenase (COX)-1, a key enzyme in the prostanoid synthesis pathway (65). Parallel to the inhibition of COX, fish oil ω-3 PUFA suppressed autoantibody production and reduced the gene expression of inflammatory response-related products, especially in the spleen and kidney of a murine SLE model. These mechanisms ultimately lead to the inhibition of glomerulonephritis and inflammation and prolong the lifespan of lupus-prone mice (66, 67). Fish oil maintained the enzymatic ratios of reduced GSH to oxidized GSH (GSH:GSSG) and the antioxidant profile in lupus-prone aged B/W mice (68). Furthermore, DHA-enriched fish oil reduced IgG deposition and caspase 1 in the kidney and anti-dsDNA antibody production and lipopolysaccharide-induced IL-18 production in the serum. It downregulated genes related to $CD4^+$ T cells and decreased the expression of *TNF-α*, *CD80*, *CXCR3*, *CTLA-4,* and various ILs (*i.e., IL-6*, *IL-10,* and *IL-18*) in the kidneys and spleens of SLE-prone mice (66, 67).

Translationally, these immunomodulatory mechanisms assist to minimize inflammatory responses in patients with SLE (69–71). Omega-3 PUFA from fish oil improved the endothelial function of patients with SLE in one study (72) but not in another (73). Besides their anti-inflammatory activity, dietary fish oil supplements also have played important roles in decreasing symptoms related to neuromotor and cardiovascular involvement and have improved conditions of fatigue and depression in patients with SLE (72, 74). Recently, in a double-blind randomized controlled trial, Seluang fish (*Rasbora* Spp.) oil (500 μL/day) reduced the inflammatory response in

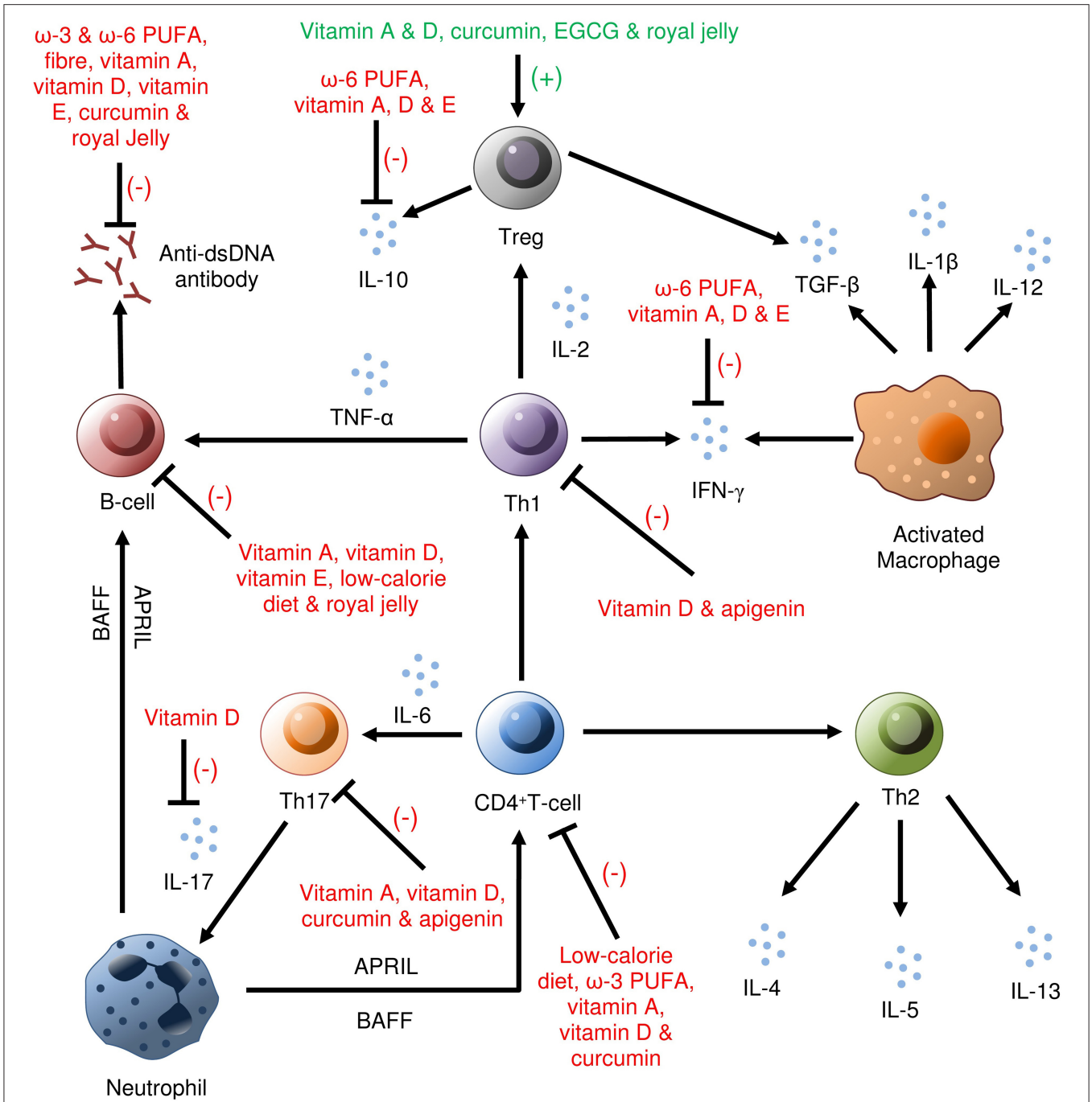

FIGURE 2 | Immunomodulatory benefits of nutrients in SLE (based on the studies on human and animal models). In the immune-mediated pathogenesis of SLE, vitamin A inhibits anti-dsDNA antibodies, activation of B-cells, IFN-γ, $CD4^+$ T-cell, Th17 cells, IL-10; vitamin D inhibits IL-10, IL-17, IFN-γ, B-cells, Th1, Th17, $CD4^+$ T cells; ω-3 PUFAs inhibit anti-dsDNA antibodies, $CD4^+$ T-cells and ω-6 PUFAs inhibit anti-dsDNA antibodies, IL-10, and IFN-γ. Vitamin A, vitamin D, curcumin, EGCG, and royal jelly were found to activate Treg cells. Anti-dsDNA, Anti-double-stranded DNA; PUFA, Polyunsaturated fatty acids; EGCG, Epigallocatechin-3-gallate.

patients with SLE by increasing serum levels of vitamin D and reducing IL-1, IL-6, and IL-17 (75). Altogether, concentrated fish oil increased the activity of renal antioxidant enzymes (*i.e.*, GSH peroxidase and catalase) and serum vitamin D levels and reduced IL-1, IL-6, IL-1β, IL-17, and TNF-α besides anti-dsDNA antibody production in the kidney, spleen and liver.

A similar conclusion comes from studies on linoleic acid (ω-6 PUFA). Conjugated linoleic acid (CLA, an isomer of linoleic acid) demonstrated significant efficacy in SLE-induced rats by

hindering cytokine and autoantibody production by controlling splenomegaly. It also obstructed oxidative stress as well as the nuclear factor kappa B (NF-κB) pathway and thus improved SLE activity and minimized mortality rates (76–78). Moreover, CLA demonstrated beneficial effects in SLE by improving the lipid profile in animal models by exhibiting antioxidant and anti-sclerotic activities (76–78).

Extra virgin olive oil (EVOO), as a main source of unsaturated fatty acids, reduced disease activity in a pristane-induced SLE mouse model by influencing proinflammatory cytokine expression in the spleen and kidney besides regulating prostaglandin E_2 levels in the kidney and matrix metalloproteinase-3 in the serum. The potential mechanisms by which EVOO may exert beneficial effects on SLE activity may involve such pathways as mitogen-activated protein kinase, Janus kinase/signal transducer and activator of transcription, NF-κB, NF-E2-related factor-2, and heme oxygenase-1 pathways (79).

Only two studies addressed the role of fatty acids in patients with SLE. In a cross-sectional study on women with SLE (n = 105), a diet with inflammatory potential (measured here as high dietary inflammatory index) was associated with a less-favorable lipid profile in patients with SLE (80). A population-based study (n = 456) suggested that a higher dietary intake of ω-3 fatty acids and lower ω-6:ω-3 ratios were favorably associated with self-reported lupus activity and sleep quality (81). This is an interesting finding that suggests a direct influence of a ω-3-rich diet on the disease activity and quality of life in patients with SLE. Among many mechanisms that may potentially explain this finding, the impact of EPA and DPA on the synthesis of serotonin has been recently proposed (82). Higher serotonin levels may simply alleviate dysfunction in the serotoninergic system, thereby contributing to the reduction of neuropsychiatric symptoms, depression and sleep disturbance (82).

Despite certain fatty acids significantly improving SLE conditions, some research has indicated ambiguity concerning their effectiveness in SLE (especially of ω-3 and ω-6 PUFAs), which are related to overconsumption, dose-dependent complexity, adverse immune responses and accelerated autoimmune symptoms; these should be carefully considered (83–85). Major sources of dietary PUFAs (ω-3 and ω-6), based on the *United States Department of Agriculture (USDA), National Nutrient Database for Standard Reference* (60), are shown in **Table 1**.

Fiber

Dietary fiber consists of edible carbohydrate polymers with three or more monomeric units that are resistant to endogenous digestive enzymes and are thus neither hydrolysed nor absorbed in the small intestine (86). Although fiber is present in a wide range of plant-based food sources, consumption is low in Western countries, contributing to changes in gut microbiota that may influence the development of serious gastrointestinal, cardiovascular and autoimmune disorders (87). Indeed, Statovci et al. (88) observed that a westernized diet with low fiber stimulates pathogenic bacterial growth inside the gut. Other studies also found a connection between fiber intake and diversity of intestinal microbiota (89, 90). Low levels of short-chain fatty acids (SCFAs), an end product of fiber fermentation, due to lower availability of dietary fiber can result in inflammation and imbalance in innate and adaptive immunity (91, 92). Parallel to this dietary residue, complex carbohydrates (fiber) are substrates for fermentation that produces SCFAs, and these serve as an energy source for gut microbiota. Thus, any changes in fiber intake may lead to a reduction of the population of gut microbiota (dysbiosis). Dysbiosis is not an uncommon phenomenon in patients with SLE and can be partially explained by the insufficient intake of dietary fiber (93–97). In a prospective study on Japanese female patients with SLE (n = 43) (98), an inverse correlation was observed between high intake of dietary fiber and risk of active, possibly through improving overall immune functions and suppressing active inflammation.

Therefore, an adequate intake of dietary fiber is recommended in patients with SLE because of the beneficial effects of fiber in reducing the disease activity by decreasing serum levels of autoantibodies and inflammatory cytokines. The major sources of dietary fiber are shown in **Table 1** based on the *United States Department of Agriculture (USDA), National Nutrient Database for Standard Reference* (60).

MICRONUTRIENTS

Micronutrients comprise chemical compounds and essential elements that are required by organisms in varying quantities (usually very low) throughout life to orchestrate a range of metabolic activities to maintain health. This term usually refers to the vitamins and minerals that cannot be synthesized by the body and must be derived from the diet (99). Micronutrients typically can be divided into four categories: water-soluble vitamins, fat-soluble vitamins, microminerals and trace minerals.

Vitamin A

Vitamin A, a member of the fat-soluble vitamins, is an essential factor with multiple functions, including maintaining immune system, integrity and proper function besides acting upon nuclear retinoic acid receptors regulating transcription of several genes. In the MRL/*lpr* mouse model of lupus, vitamin A exerts paradoxical effects on the development of autoimmune lupus, resulting in decreasing inflammation in some organs or exacerbation of disease course in others (100). In another study with the (MRL/MPJ-*lpr/lpr*) SLE mouse model, vitamin A deficiency exerted a suppressive effect on the activities of abnormal T and B cells and suppressed serum anti-dsDNA antibody production (**Figure 2**) (101). Ikeda et al. (102) observed that 5–10 mg/kg oral administration of etretinate (a synthetic vitamin A derivative) significantly reduced dermal thickness ($p < 0.05$) in this SLE mouse model (MRL/*lpr*) compared with that of the controls by inducting apoptosis in dermal infiltrating cells and regulating cytokine production.

Considering human studies, Kinoshitak et al. (103) observed that after treatment with retinoids, levels of anti-dsDNA antibody and proteinuria were improved without side effects in patients with lupus nephritis. Daily intake of 100,000 U of vitamin A produced enhanced antibody-dependent cellular cytotoxicity, natural killer cell and IL-2 activities in patients with SLE (104).

In a case-controlled study with Indonesian patients with SLE ($n = 62$), vitamin A modulated T-helper type (Th17), and Treg cell balance (105).

Vitamin B

The role of the vitamin B group in autoimmune patients is usually discussed in the context of its insufficiency. Indeed, several studies have indicated a low level of vitamin B in SLE patients in comparison with those of healthy controls. Vitamin B_2 (riboflavin) deficiency was detected in 88% of a cohort of Chinese patients with SLE ($n = 258$) in an observational study (106). Similarly, significantly low levels of vitamin B_{12} (cobalamin) were observed (<180 pg/mL) in another cohort of patients with SLE ($n = 43$) when compared with those of healthy individuals ($p < 0.0005$); however, this was not significantly associated with disease activity (107). Later, in a prospective study on female Japanese patients with SLE ($n = 43$) (98), an inverse correlation was observed between a high intake of vitamin B_6 (pyridoxine) (1.7 mg/day) and the risk of active SLE, possibly through improving overall immune functions and suppressing active inflammation. Furthermore, immunotherapy with vitamin B_9 (folate) alleviated SLE-related symptoms in two lupus-prone mouse models (NXBW/F1 and MRL/MpJ*Tnfrsf6*^{lpr}), leading to a significant prolongation of survival ($p = 0.005$) in both cases (25–53 vs. 16–31 weeks for untreated mice) (108).

Vitamin C

In a 4 year prospective study on Japanese patients with SLE ($n = 279$), it was observed that vitamin C intake (109.99 mg/day) was significantly inversely associated ($p = 0.005$) with the risk of developing active SLE (109). These researchers postulated that the antioxidant properties of vitamin C modulated immune functions, regulated the release of inflammatory mediators, decreased oxidative stress and suppressed autoantibody production in the SLE subjects. In a double-blind randomized placebo-controlled study of patients with SLE ($n = 39$), after a 12 week combined therapy with vitamin C (500 mg/day) and vitamin E (800 IU/day), reduction in lipid peroxidation was observed without affecting other oxidative stress markers or endothelial function (110).

Vitamin D

Recently, the role of vitamin D in the development of various autoimmune diseases has attracted attention. Vitamin D is commonly recognized as a pleiotropic compound, and its role goes beyond managing calcium metabolism. Not long ago, it was confirmed that vitamin D exerted a strong effect on cellular proliferation, differentiation and immune modulation. Congruently, vitamin D exerted protective effects in several diseases like hypertension, diabetes, cardiovascular diseases (CVDs), autoimmune diseases and cancer development (111). Studies on the role of vitamin D are especially important because vitamin D deficiency is commonly observed in people from developed countries and may potentially contribute to the increasing burden of autoimmune diseases in the last few years (112, 113).

A significantly high prevalence of vitamin D deficiency has been observed in patients with SLE in Saudi Arabia (114), Bahrain (115), the USA (116, 117), Canada (118), Jamaica (119), Brazil (120), France (121), Hungary (122), Denmark (123), and Spain (124, 125). In clinical studies on patients with SLE, significant inverse correlations were observed between serum levels of vitamin D and SLE disease activity index (SLEDAI) score in Malaysian (126), Taiwanese (127), Thai (128), Indian (129), Egyptian (130, 131), Saudi Arabian (132), Brazilian (133, 134), Australian (135), American (136), Hungarian (122), and Chinese (137) populations with an exception of Serbian SLE subjects ($n = 46$) (138). Interestingly, two prolonged follow-up studies (up to 329 months) observed no associations between reported dietary intake of vitamin D during adolescence and risk of developing SLE in adulthood (139) and in adult women (140), suggesting that vitamin D metabolism may be at least partially related to inflammatory status in autoimmune patients. Several reasons for vitamin D inadequacy are independent of vitamin D dietary intake. Specifically, in lupus patients, vitamin D deficiency may be due to the avoidance of sunshine, photoprotection, impaired vitamin D synthesis (renal insufficiency) or the use of medications such as glucocorticoids, anticonvulsants, antimalarials, and calcineurin inhibitors, which exert a negative impact on the metabolism of vitamin D or downregulate the functions of the vitamin D receptor (113).

Vitamin D and SLE Activity

Considering the role of vitamin D deficiency on the development of SLE, Yu et al. (141) recently demonstrated that vitamin D plays a protective role in the autoantibody-induced injury of podocytes (kidney cells that wrap around capillaries of the glomerulus) in patients with lupus nephritis, which could be a potential novel therapeutic target to treat lupus nephritis. From a 24 week double-blind randomized placebo-controlled trial by Lima et al. (142), it was established that supplementation of vitamin D in patients with juvenile-onset SLE effectively improved parameters of bone microarchitecture ($p = 0.024$). Bone marrow mesenchymal stem cells from patients with SLE show impaired proliferative capacity compared with that of normal controls (143). Administration of an analog of vitamin D (EB1089) promoted proliferation and osteogenic differentiation of stem cells by the Smad 1/5/8 signaling pathway (144). Although vitamin D deficiency was insignificantly correlated with higher SLEDAI scores in Egyptian patients with juvenile-onset SLE (145), it was later suggested that vitamin D supplementation was effective for a significant reduction of SLEDAI ($p = 0.01$) and improving fatigue ($p = 0.008$) in Brazilian patients with juvenile-onset SLE (146). It was observed from two recent meta-analyses that the serum levels of vitamin D were significantly low ($p < 0.00001$) in patients with SLE compared with those of healthy controls (147), and vitamin D supplementation was significantly effective ($p < 0.001$) in increasing these low serum vitamin D levels in patients with SLE with improved fatigue (148). In a group of patients with SLE, levels of *mTOR* mRNA were higher in a group with severe vitamin D deficiency compared with a group with vitamin D insufficiency ($p = 0.036$) indicating that

severe vitamin D deficiency contributes to SLE pathogenesis via increased expression of mTOR (149).

Cytokines and Innate Immunity

Increased levels of antinuclear antibodies, IL-23, and IL-17 (important for Th17 development and function) were also significantly associated ($p < 0.05$) with a vitamin D deficiency (120). Abnormal activation of toll-like receptors (TLRs) contributes to the pathogenesis of SLE, and, interestingly, vitamin D exerts some of its immunomodulatory effects in patients with SLE by significantly downregulating the expression levels of TLR3 ($p = 0.03$), TLR7 ($p = 0.0001$), and TLR9 ($p = 0.007$) (150). Autoimmune diseases like SLE may result from a conversion of Treg cells into Th17 cells, and in the serum of patients with SLE, IL-17 levels were abnormally high. After 6 months of vitamin D supplementation in a group of Portuguese patients with SLE ($n = 24$), the $FoxP3^+$:IL-17A ratio was higher than that in the baseline ($p < 0.001$) (151). Similar conclusions arose from studies on IFNs. Low levels of vitamin D were significantly associated with high expression of IFN-α ($p < 0.05$) (130) and high levels of IFN-α ($p = 0.02$) (152) and IFN-γ ($p = 0.03$) (153) in the SLE group compared with the control group. Vitamin D deficiency was associated with impaired endothelial repair mechanisms and induction of CVDs with the potential to activate the type I IFN signaling pathway in SLE (154). Reynolds et al. (155) demonstrated that vitamin D positively modifies endothelial repair mechanisms by reducing *CXCL-10* expression and thus reduces risks of CVDs in SLE. Handono et al. (156) showed that vitamin D significantly prevented endothelial damage ($p < 0.05$) induced by increased neutrophil extracellular trap formation in patients with SLE.

Adaptive Immunity

Vitamin D also induced the expansion of SLE Treg cells ($CD25^{high}$ $Foxp3^+$). However, Schneider et al. (157) did not find any significant association between levels of vitamin D and cytokine profiles in SLE patients. After long-term (12 months) monthly treatment with vitamin D in patients with SLE, a significant enhancement ($p = 0.03$) of Treg cells ($CD4^+CD45RA^+CCR7^-$) was observed (158). Similarly, lower levels of vitamin D caused a significant reduction ($p = 0.015$) in the percentage of migrated Treg cells ($CD4^+CD25^+CCR4^+$) compared with that of healthy controls (159). The $CD4^+/CD8^+$ ratio was significantly higher ($p < 0.001$) in patients with severe vitamin D deficiency compared with a vitamin D-insufficient group (149), and vitamin D supplementation elevated $CD4^+CD8^+$ double-positive T cells (160) or inhibited the activation of $CD4^+$ T cells (**Figure 2**) through inhibition of the PKCδ/ERK pathway and reduced expression of inflammatory factors (161). Through vitamin D supplementation for 6 months in patients with SLE ($n = 20$), hypovitaminosis D, Treg-cell balance (increased Treg cells and decreased effector Th1 and Th17 cells) and B-cell homeostasis were restored with reduced anti-dsDNA antibodies (162). Low levels of vitamin D (<20 ng/mL) were associated with reduced micro (mi)-RNA expression in T cells (*i.e.*, miRNA-377, miRNA-342, miRNA-10a, miRNA-374b, miRNA-125a, and miRNA-410) of patients with SLE compared with those of healthy controls, indicating that unfavorable immunological alterations take place because of a lack of vitamin D in patients with SLE (163).

Vitamin E

Considering the pivotal role of vitamin D in the development of SLE, data on the fat-soluble vitamin E are still scanty. Some animal studies have addressed this problem with contradictory results. In the one study using SLE-prone NZB/W F1 mice as a model, high-dose vitamin E supplementation (550 mg/kg all-rac-α-tocopheryl acetate) decreased anti-dsDNA IgG antibody (**Figure 2**), oxidative stress, regulated cytokines (IFN-γ and IL-6 secretion) and production of lymphocytes (B-cell differentiation), subsequently alleviating the severity of SLE with a prolonged lifespan (164). By contrast, Hsieh et al. (165) observed that low-dose vitamin E supplementation (250 mg/kg all-rac-α-tocopheryl acetate) was more beneficial for the survival of SLE-prone MRL/*lpr* mice, lowering the levels of anti-dsDNA, and anticardiolipin antibodies when compared with those of the high-dose group (500 mg/kg all-rac-α-tocopheryl acetate).

Our current understanding of the role of vitamin E in the development of SLE in humans is also limited. From a prospective case-control study, lower levels of serum vitamin E were detected in a group of SLE patients (0.64 ± 0.09 mg/dL) compared with those of normal controls (0.80 ± 0.21 mg/dL) (166). According to the study of Maeshima et al. (167), oral administration of vitamin E (150–300 mg/day) in patients with SLE suppressed synthesis of antibodies via a mechanism independent of antioxidant activity. In a study with Egyptian patients with SLE ($n = 25$) (168), when *Nigella sativa* and vitamin E were supplemented together for 3 months, levels of antioxidant enzymes such as reduced GSH and superoxide dismutase were increased significantly when compared with the levels before supplementation ($p < 0.001$). Moreover, levels of IL-10, malondialdehyde, nitric oxide and inducible nitric oxide synthase decreased significantly compared with pre-supplementation levels ($p < 0.001$). Additionally, there was a significant decrease in antinuclear antibodies ($p < 0.001$), anti-dsDNA levels ($p < 0.001$), and SLEDAI score ($p < 0.01$).

Minerals

Like other micronutrients, minerals such as calcium, zinc, sodium, selenium, iron and copper generally attenuate SLE activity via different immunomodulatory mechanisms (**Table 2**). However, special attention must be given to mineral intake because it is best to restrict the consumption of some minerals like sodium. **Table 2** shows the major dietary mineral sources based on the *United States Department of Agriculture (USDA), National Nutrient Database for Standard Reference* (60).

NUTRACEUTICS

Evidence accumulated over the past decade has demonstrated that various nutrients possess additional effects on body function

TABLE 2 | Effects of dietary minerals on disease activities of SLE.

Minerals	Study model	Country	Findings	Year, References
Calcium	Cross-sectional	Spain	Vitamin D-calcium supplementation increased the serum levels of vitamin D, however, do not modify the serum calcium levels rather increased arterial stiffness significantly (IMT; $p = 0.041$).	2019, (169)
	Prospective interventional	Saudi Arabia	Vitamin D-calcium supplementation significantly improved the bone mineral density in vitamin D-deficient SLE patients, however, not significantly attenuated immune markers or disease activity.	2018, (170)
	Case-control	Egypt	There was no significant correlation between SLEDAI score and calcium supplementation ($p = 0.861$).	2016, (171)
	Animal model	USA	In presence of high-calcium diet, vitamin D supplement markedly suppress inflammatory T-cell activity in experimental MRL/*lpr* SLE mice.	2001, (172)
Zinc	Animal model	USA	Zinc restriction reduced autoantibodies (*i.e.*, anti-dsDNA antibody) and lymphoproliferation in MRL/*lpr* SLE mice.	2001, (173)
	Animal model	USA	Zinc-deficient diet retarded autoantibody (*i.e.*, anti-dsDNA antibody) production in NZB/NZW SLE mice.	1982, (174)
	Animal model	USA	Depot-zinc therapy significantly reduced kidney damage in the B/W SLE mice ($p < 0.01$).	1981, (175)
Sodium	Cross-sectional	Mexico	A positive correlation was detected between sodium intake and levels of $CD4^+CD25^+Foxp3^+$ Treg cells in SLE.	2018, (176)
	Prospective	Italy	Due to dietary sodium intake, Th17 and Treg cells significantly decreased ($p = 0.01$) and increased ($p = 0.04$), respectively. Additionally, serum IL-9 levels were significantly reduced in SLE patients ($p = 0.03$).	2017, (177)
	Animal model	China	Excessive intake of sodium in diet aggravated lupus nephritis through SGK1 pathway by significantly increasing the Th1/Th2 and Th17/Treg ratios in MRL/*lpr* SLE mice.	2015, (178)
Selenium	Animal model	USA	Selenium supplementation leads to impaired differentiation and maturation of macrophages.	(179)
			Selenium in the drinking water significantly improved the survival rate ($p < 0.04$) and increased NK cell activity ($p < 0.001$) of the NZB/NZW SLE mice though there was no effect on autoantibody (*i.e.*, anti-ssDNA antibody) production.	1988, (180)
Iron	Animal model	USA	Anemia and incidence of skin lesions were high in severely iron deficient MRL/MPJ-*lpr*/*lpr* SLE mice.	1995, (181)
Copper	Double blind, double placebo-controlled trial	Ireland	No significant effect on SLAM-R was observed.	2004, (182)

SLEDAI, Systemic lupus erythematosus disease activity index; dsDNA, Double stranded DNA; ssDNA, Single stranded DNA; IMT, Intima-media thickness; SGK1, Serine/threonine protein kinase 1; Th, T helper; Treg, Regulatory T; IL, Interleukin; NK, Natural killer; SLAM-R, Revised Systemic Lupus Activity Measure.

and metabolism. Concordantly, several studies have shown that nutrients modify the immune response as well as the integrity of the organs and tissues. In connective tissue diseases where a pharmacological approach is limited and introduces the risk of several severe side effects, there is a demand for compounds that potentially exert beneficial effects without side effects. Such compounds are commonly referred to as nutraceuticals, and this approach, although a bit controversial, has been tested in several SLE studies.

Polyphenols

Curcumin

Curcumin, a major natural polyphenol of turmeric, exerted a protective effect against lupus nephritis in SLE-prone MRL/*lpr* mice by reducing serum anti-dsDNA antibody levels and inhibiting the expression of the NLRP3 inflammasome, which is believed to be a key player in the development of lupus nephritis (183). An experimental study with SLE-prone MRL/*lpr* mice demonstrated that curcumin aggravated CNS pathology (184). Moreover, curcumin decreased anti-dsDNA IgG antibodies and attenuated lupus nephritis upon interaction with Treg cells in SLE-prone NZB/W F1 female mice (185).

Direct translation of these promising data into clinical application is a difficult task; data in this field are scarce and frequently controversial. In a small double-blind randomized controlled trial ($n = 39$), when curcumin was administered along with vitamin D in a group of patients with SLE who displayed hypovitamin D, no significant differences arose in SLEDAI reduction or decreased serum IL-6 and increased TGF-β1 (186). Contrary to this, another study revealed that administration of low doses of curcumin (0.1 and 1.0 μg/mL) to cultured $CD4^+$ cells from patients with SLE significantly ($p < 0.001$) modulated the Th17/Treg balance without affecting healthy subjects (187). Similar to this *in vitro* experiment, low-dose curcumin (0.1 μg/mL) inhibited the expression and activation of *PYK2* in peripheral blood mononuclear cells (PBMCs) and reduced the proliferation of PBMCs in patients with lupus nephritis (188).

Although the results of completed studies are somewhat promising, they are inconclusive, and more studies on the role of curcumin in SLE are warranted.

Virgin Olive Oil

In a pristane-induced BALB/c mouse model of SLE, administration of virgin olive oil (VOO) and its phenol fraction (with major components of hydroxytyrosol, tyrosol, oleuropein aglycone, and ligstroside aglycone) counteracted inflammatory pathways in cells of the monocyte–macrophage lineage (189). Therefore, both VOO and its phenol fraction are promising immunomodulators of SLE activity. Epigallocatechin-3-gallate (EGCG), the major bioactive polyphenol present in green tea, enhanced the Nrf2 antioxidant signaling pathway, decreased renal NLRP3 inflammasome activation and increased systemic Treg cell activity in NZB/W F1 SLE-prone mice (190). EGCG attenuated inflammation in mesangial cells of SLE-prone MRL/*lpr* mice via the PI3K/Akt/mTOR pathway by decreasing Akt phosphorylation (191).

Limited data exist on the role of VOO in modulating the immune response in SLE patients. An *in vitro* study on PBMCs derived from SLE patients (192) showed that the phenol fraction of VOO was successful in modulating cytokine production and attenuated induced T-cell activation, most likely through the NF-κB signaling pathway.

Polyphenols and Microbiota

Cuervo et al. (193) determined an association between polyphenol intake and fecal microbiota in Spanish patients with SLE compared with healthy controls. Major dietary sources of polyphenols include various fruits and vegetables (oranges, lettuce, watermelon, kiwi, tomato, apple, lentils and celery) as assessed through a semi-quantitative food-frequency questionnaire. Interestingly, a well-balanced diet with a high intake of apples and oranges besides other fruits and vegetables rich in flavonoids has been associated with the presence of beneficial microorganisms (*i.e.*, *Lactobacillus*, *Blautia,* and *Bifidobacterium*) in the fecal matter of these patients with SLE.

Flavonoids

The results of a 6 year survey collected in 2016 showed that higher serum levels of lycopene (enriched in tomatoes, red carrots and watermelons) have a significant protective effect on mortality when compared with lower levels (5.3 vs. 33.3%; $p = 0.04$) in subjects with SLE (194). Apigenin (a mutagenic flavonoid rich in parsley, thyme, peppermint, olives and chamomile) inhibited autoantigen presentation and stimulatory functions of antigen-presenting cells necessary for activation and expansion of autoreactive Th1 and Th17 cells (**Figure 2**) and B cells in SLE by downregulation of COX-2 expression (195). Nevertheless, Pocovi-Gerardino et al. (196) documented no significant correlation between the dietary intake of macronutrients, micronutrients or antioxidants, and serum levels of CRP in patients with SLE.

Royal Jelly

Royal jelly, a creamy product secreted by young nurse worker bees (*Apis mellifera*), is composed of 10–12% carbohydrates, 12–15% proteins, and 3–7% lipids, vitamins, minerals and polyphenols. In an open-label study, children with SLE ($n = 20$) received 2 g of freshly prepared royal jelly daily for 12 weeks. The SLEDAI score significantly improved ($p = 0.01$) after 3 months of royal jelly therapy. Complement C3 ($p = 0.009$) and C4 ($p = 0.014$) levels also significantly increased at the end of the 12-week treatment. Additionally, percentages of $CD4^+CD25^{+high}$ and $CD4^+CD25^{+high}FOXP3^+$ cells ($CD4^+$ Treg cells) were significantly increased after royal jelly treatment ($p < 0.01$); however, the frequency of Treg cells remained significantly lower than that in the control group ($p = 0.01$). Moreover, $CD8^+CD25^{+high}$ and $CD8^+CD25^{+high}FOXP3^+$ cells ($CD8^+$ Treg cells) significantly increased after royal jelly treatment ($p < 0.01$). Percentages of $CD8^+CD25^{+high}$ and $CD8^+$ Treg cells in patients with SLE at baseline (prior to royal jelly treatment) were significantly lower than in the control group of children without SLE ($p = 0.003$) (197).

In another study, when royal jelly was administered in a group of SLE-prone NZB/NZW F1 female mice, a significant delay in the onset of the disease and prolonged lifespan were observed. Additionally, serum levels of anti-ssDNA, anti-dsDNA antibodies and autoreactive B cells were significantly decreased (198).

CONCLUSIONS

Because dietary supplementation of various macronutrients and micronutrients has exhibited immunomodulatory effects including maintenance of homeostasis and improvement of physical and mental well-being of patients with SLE, it is recommended that these patients consume a balanced diet that is low in calories and protein but contains plenty of fiber, PUFAs (ω-3 and ω-6), vitamins (A, B, C, D, and E), minerals (calcium, zinc, selenium, iron and copper) and polyphenol-containing foods.

AUTHOR CONTRIBUTIONS

MI contributed to the conception of the work, developed the search strategy, conducted the literature search and drafted the manuscript. SK assisted in writing and drawing **Figure 2**. PK and RH substantively revised the manuscript. All the authors approved the final submitted version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01477/full#supplementary-material

REFERENCES

1. Ali M, Firoz CK, Jabir NR, Rehan M, Khan MS, Tabrez S. An insight on the pathogenesis and treatment of systemic lupus erythematosus. *Endocr Metab Immune Disord Drug Targets.* (2018) 18:110–23. doi: 10.2174/1871530318666171207145003
2. Jeong DY, Lee SW, Park YH, Choi JH, Kwon YW, Moon G, et al. Genetic variation and systemic lupus erythematosus: a field synopsis and systematic meta-analysis. *Autoimmun Rev.* (2018) 17:553–66. doi: 10.1016/j.autrev.2017.12.011
3. Farivar S, Aghamaleki FS. Effects of major epigenetic factors on systemic lupus erythematosus. *Iran Biomed J.* (2018) 22:294–302. doi: 10.29252/ibj.22.5.294
4. Aringer M, Costenbader K, Daikh D, Brinks R, Mosca M, Ramsey-Goldman R, et al. European league against rheumatism/american college of rheumatology classification criteria for systemic lupus erythematosus. *Arthritis Rheumatol.* (2019) 71:1400–12. doi: 10.1002/art.40930
5. Maidhof W, Hilas O. Lupus: an overview of the disease and management options. *Pharm Ther.* (2012) 37:240–6.
6. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med.* (2011) 365:2110–21. doi: 10.1056/NEJMra1100359
7. Stojan G, Petri M. Epidemiology of systemic lupus erythematosus: an update. *Curr Opin Rheumatol.* (2018) 30:144–50. doi: 10.1097/BOR.0000000000000480
8. Aparicio-Soto M, Sánchez-Hidalgo M, Alarcón-de-la-Lastra C. An update on diet and nutritional factors in systemic lupus erythematosus management. *Nutr Res Rev.* (2017) 30:118–37. doi: 10.1017/S0954422417000026
9. Katz-Agranov N, Zandman-Goddard G. The microbiome and systemic lupus erythematosus. *Immunol Res.* (2017) 65:432–7. doi: 10.1007/s12026-017-8906-2
10. de Medeiros MCS, Medeiros JCA, de Medeiros HJ, Leitão JCGdC, Knackfuss MI. Dietary intervention and health in patients with systemic lupus erythematosus: a systematic review of the evidence. *Crit Rev Food Sci Nutr.* (2018) 59:2666–73. doi: 10.1080/10408398.2018.1463966
11. Perricone C, Agmon-Levin N, Colafrancesco S, Shoenfeld Y. Vitamins and systemic lupus erythematosus: to D or not to D. *Expert Rev Clin Immunol.* (2013) 9:397–9. doi: 10.1586/eci.13.23
12. Teng X, Brown J, Choi SC, Li W, Morel L. Metabolic determinants of lupus pathogenesis. *Immunol Rev.* (2020) 295:167–86. doi: 10.1111/imr.12847
13. Constantin MM, Nita IE, Olteanu R, Constantin T, Bucur S, Matei C, et al. Significance and impact of dietary factors on systemic lupus erythematosus pathogenesis. *Exp Ther Med.* (2019) 17:1085–90. doi: 10.3892/etm.2018.6986
14. dos Santos FdMM, Borges MC, Telles RW, Correia MIT, Lanna CCD. Excess weight and associated risk factors in patients with systemic lupus erythematosus. *Rheumatol Int.* (2013) 33:681–8. doi: 10.1007/s00296-012-2402-8
15. Elkan A, Anania C, Gustafsson T, Jogestrand T, Hafström I, Frostegård J. Diet and fatty acid pattern among patients with SLE: associations with disease activity, blood lipids and atherosclerosis. *Lupus.* (2012) 21:1405–11. doi: 10.1177/0961203312458471
16. Kotyla P. Insulin resistance varies across connective tissue diseases patients: comparison between rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis. *Clin Exp Rheumatol.* (2019) 37:14.
17. Liu Y, Kaplan MJ. Cardiovascular disease in systemic lupus erythematosus: an update. *Curr Opin Rheumatol.* (2018) 30:441–8. doi: 10.1097/BOR.0000000000000528
18. Borges MC, dos Santos FdMM, Telles RW, Lanna CCD, Correia MIT. Nutritional status and food intake in patients with systemic lupus erythematosus. *Nutrition.* (2012) 28:1098–103. doi: 10.1016/j.nut.2012.01.015
19. Oeser A, Chung CP, Asanuma Y, Avalos I, Stein CM. Obesity is an independent contributor to functional capacity and inflammation in systemic lupus erythematosus. *Arthritis Rheum.* (2005) 52:3651–9. doi: 10.1002/art.21400
20. Cozier YC, Barbhaiya M, Castro-Webb N, Conte C, Tedeschi S, Leatherwood C, et al. A prospective study of obesity and risk of systemic lupus erythematosus (SLE) among black women. *Semin Arthritis Rheum.* (2019) 48:1030–4. doi: 10.1016/j.semarthrit.2018.10.004
21. Teh P, Zakhary B, Sandhu VK. The impact of obesity on SLE disease activity: findings from the Southern California Lupus Registry (SCOLR). *Clin Rheumatol.* (2019) 38:597–600. doi: 10.1007/s10067-018-4336-3
22. Castrejon I, Shakoor N, Block J, Pincus T. Higher rates of obesity and associations with poorer clinical status in patients with RA, OA and SLE: a cross-sectional study from routine care. *Ann Rheum Dis.* (2017) 76:1451–2. doi: 10.1136/annrheumdis-2017-eular.3812
23. Sinicato NA, Postal M, Peres FA, Peliçari KdO, Marini R, dos Santos AdO, et al. Obesity and cytokines in childhood-onset systemic lupus erythematosus. *J Immunol Res.* (2014) 2014:1–10. doi: 10.1155/2014/162047
24. Patterson SL, Schmajuk G, Jafri K, Yazdany J, Katz P. Obesity is independently associated with worse patient-reported outcomes in women with systemic lupus erythematosus. *Arthritis Care Res.* (2019) 71:126–33. doi: 10.1002/acr.23576
25. Sun D, Krishnan A, Su J, Lawrence R, Zaman K, Fernandes G. Regulation of immune function by calorie restriction and cyclophosphamide treatment in lupus-prone NZB/NZW F1 mice. *Cell Immunol.* (2004) 228:54–65. doi: 10.1016/j.cellimm.2004.04.001
26. Feng Y, Yang M, Wu H, Lu Q. The pathological role of B cells in systemic lupus erythematosus: From basic research to clinical. *Autoimmunity.* (2020) 53:56–64. doi: 10.1080/08916934.2019.1700232
27. Yap DY, Chan TM. B cell abnormalities in systemic lupus erythematosus and lupus nephritis—role in pathogenesis and effect of immunosuppressive treatments. *Int J Mol Sci.* (2019) 20:6231. doi: 10.3390/ijms20246231
28. Urao M, Ueda G, Abe M, Kanno K, Hirose S, Shirai T. Food restriction inhibits an autoimmune disease resembling systemic lupus erythematosus in (NZB× NZW) F1 mice. *J Nutr.* (1995) 125:2316–24. doi: 10.1093/jn/125.9.2316
29. Troyer D, Chandrasekar B, Barnes J, Fernandes G. Calorie restriction decreases platelet-derived growth factor (PDGF)-A and thrombin receptor mRNA expression in autoimmune murine lupus nephritis. *Clin Exp Immunol.* (1997) 108:58–62. doi: 10.1046/j.1365-2249.1997.d01-970.x
30. Zhao X, Hao J, Duan H, Rong Z, Li F. Phosphoinositide 3-kinase/protein kinase B/periostin mediated platelet-derived growth factor-induced cell proliferation and extracellular matrix production in lupus nephritis. *Exp Biol Med.* (2017) 242:160–8. doi: 10.1177/1535370216668050
31. Muthukumar AR, Jolly CA, Zaman K, Fernandes G. Calorie restriction decreases proinflammatory cytokines and polymeric Ig receptor expression in the submandibular glands of autoimmune prone (NZB× NZW) F1 mice. *J Clin Immunol.* (2000) 20:354–61. doi: 10.1023/a:1006620130114
32. Bristow C, Meurer C, Simmonds J, Snell T. Anti-obesity public health messages and risk factors for disordered eating: a systematic review. *Health Promot Int.* (2020). doi: 10.1093/heapro/daaa018. [Epub ahead of print].
33. Thomas PE, Jensen BW, Sørensen KK, Jacobsen S, Aarestrup J, Baker JL. Early life body size, growth and risks of systemic lupus erythematosus–A large Danish observational cohort study. *Semin Arthritis Rheum.* [Epub ahead of print]. (2020). doi: 10.1016/j.semarthrit.2020.01.011
34. Meza-Meza MR, Vizmanos-Lamotte B, Muñoz-Valle JF, Parra-Rojas I, Garaulet M, Campos-López B, et al. Relationship of excess weight with clinical activity and dietary intake deficiencies in systemic lupus erythematosus patients. *Nutrients.* (2019) 11:2683. doi: 10.3390/nu11112683
35. Kang JH, Xu H, Choi SE, Park DJ, Lee JK, Kwok SK, et al. Obesity increases the incidence of new-onset lupus nephritis and organ damage during follow-up in patients with systemic lupus erythematosus. *Lupus.* (2020) 6:578–86. doi: 10.1177/0961203320913616
36. Davies R, Lomer M, Yeo S, Avloniti K, Sangle S, D'Cruz D. Weight loss and improvements in fatigue in systemic lupus erythematosus: a controlled trial of a low glycaemic index diet versus a calorie restricted diet in patients treated with corticosteroids. *Lupus.* (2012) 21:649–55. doi: 10.1177/0961203312436854
37. Ding C, Chan Z, Magkos F. Lean, but not healthy: the 'metabolically obese, normal-weight'phenotype. *Curr Opin Clin Nutr Metab Care.* (2016) 19:408–17. doi: 10.1097/MCO.0000000000000317
38. Lavie CJ, De Schutter A, Milani RV. Healthy obese versus unhealthy lean: the obesity paradox. *Nat Rev Endocrinol.* (2015) 11:55–62. doi: 10.1038/nrendo.2014.165
39. Ayán C, Martin V. Systemic lupus erythematosus and exercise. *Lupus.* (2007) 16:5–9. doi: 10.1177/0961203306074795

40. Bantornwan S, Watanapa WB, Hussarin P, Chatsiricharoenkul S, Larpparisuth N, Teerapornlertratt T, et al. Role of meditation in reducing sympathetic hyperactivity and improving quality of life in lupus nephritis patients with chronic kidney disease. *J Med Assoc Thai.* (2014) 97:101–7.
41. Clarke-Jenssen AC, Fredriksen PM, Lilleby V, Mengshoel AM. Effects of supervised aerobic exercise in patients with systemic lupus erythematosus: a pilot study. *Arthritis Care Res.* (2005) 53:308–12. doi: 10.1002/art.21082
42. Tench C, McCarthy J, McCurdie I, White P, D'Cruz D. Fatigue in systemic lupus erythematosus: a randomized controlled trial of exercise. *Rheumatology.* (2003) 42:1050–4. doi: 10.1093/rheumatology/keg289
43. Benatti FB, Miyake CN, Dantas WS, Zambelli VO, Shinjo SK, Pereira RM, et al. Exercise increases insulin sensitivity and skeletal muscle AMPK expression in systemic lupus erythematosus: a randomized controlled trial. *Front Immunol.* (2018) 9:906. doi: 10.3389/fimmu.2018.00906
44. Milovanov I, Lysenko L, Milovanova L, Dobrosmyslov I. The role of balanced low-protein diet in inhibition of predialysis chronic kidney disease progression in patients with systemic diseases. *Ter Arkh.* (2009) 81:52–7.
45. Milovanov YS, Milovanova LY, Mikhailov A, Aleksandrova I. Influence of diet balanced with essential amino acids/keto acid analogs and high-nutrient blend on the progression of renal failure in patients in the pre-dialysis stage of chronic kidney disease caused by systemic autoimmune diseases. *Int J Biomed.* (2013) 3:184–7.
46. Caetano MC, Ortiz TT, Terreri MTS, Sarni RO, Silva SG, Souza FI, et al. Inadequate dietary intake of children and adolescents with juvenile idiopathic arthritis and systemic lupus erythematosus. *J Pediatr.* (2009) 85:509–15. doi: 10.2223/JPED.1941
47. Ruíz-Bedolla E, López-Martínez B, Parra-Ortega I. Evaluation of the amino acid L-canavanine in the serum of patients with systemic lupus erythematosus. *Rev Latinoamer Patol Clin.* (2016) 63:87–90. doi: 10.1016/B978-0-12-801917-7.00009-7
48. Mao Z, Zhang W. Role of mTOR in glucose and lipid metabolism. *Int J Mol Sci.* (2018) 19:2043. doi: 10.3390/ijms19072043
49. Liu Y, Zhang DT, Liu XG. mTOR signaling in T cell immunity and autoimmunity. *Int Rev Immunol.* (2015) 34:50–66. doi: 10.3109/08830185.2014.933957
50. Bi Y, Liu G, Yang R. mTOR regulates T-cell differentiation and activation in immunity and autoimmunity. *Crit Rev Eukaryot Gene Expr.* (2011) 21:313–22. doi: 10.1615/CritRevEukarGeneExpr.v21.i4.20
51. Gergely P Jr, Grossman C, Niland B, Puskas F, Neupane H, Allam F, et al. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. *Arthritis Rheum.* (2002) 46:175–90. doi: 10.1002/1529-0131(200201)46:1<175::AID-ART10015>3.0.CO;2-H
52. Shah D, Aggarwal A, Bhatnagar A, Kiran R, Wanchu A. Association between T lymphocyte sub-sets apoptosis and peripheral blood mononuclear cells oxidative stress in systemic lupus erythematosus. *Free Radic Res.* (2011) 45:559–67. doi: 10.3109/10715762.2011.555765
53. Lai ZW, Hanczko R, Bonilla E, Caza TN, Clair B, Bartos A, et al. N-acetylcysteine reduces disease activity by blocking mammalian target of rapamycin in T cells from systemic lupus erythematosus patients: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* (2012) 64:2937–46. doi: 10.1002/art.34502
54. Bengtsson AA, Trygg J, Wuttge DM, Sturfelt G, Theander E, Donten M, et al. Metabolic profiling of systemic lupus erythematosus and comparison with primary Sjögren's syndrome and systemic sclerosis. *PLoS ONE.* (2016) 11:e0159384. doi: 10.1371/journal.pone.0159384
55. Zhang Q, Yin X, Wang H, Wu X, Li X, Li Y, et al. Fecal metabolomics and potential biomarkers for systemic lupus erythematosus. *Front Immunol.* (2019) 10:1–10. doi: 10.3389/fimmu.2019.00976
56. Perl A, Hanczko R, Lai Z-W, Oaks Z, Kelly R, Borsuk R, et al. Comprehensive metabolome analyses reveal N-acetylcysteine-responsive accumulation of kynurenine in systemic lupus erythematosus: implications for activation of the mechanistic target of rapamycin. *Metabolomics.* (2015) 11:1157–74. doi: 10.1007/s11306-015-0772-0
57. Dubois EL, Strain L. Effect of diet on survival and nephropathy of NZB-NZW hybrid mice. *Biochem Med.* (1973) 7:336–40. doi: 10.1016/0006-2944(73)90091-4
58. Corman LC. The role of diet in animal models of systemic lupus erythematosus: possible implications for human lupus. *Semin Arthritis Rheum.* (1985) 15:61–9. doi: 10.1016/0049-0172(85)90010-1
59. Peters H, Border WA, Rückert M, Krämer S, Neumayer HH, Noble NA. L-arginine supplementation accelerates renal fibrosis and shortens life span in experimental lupus nephritis. *Kidney Int.* (2003) 63:1382–92. doi: 10.1046/j.1523-1755.2003.00881.x
60. US Department of Agriculture. *Agricultural Research Service, Nutrient Data Laboratory. USDA, National Nutrient Database for Standard Reference. Legacy Release*, Beltsville (2018).
61. Huerta MDR, Trujillo-Martin MM, Rua-Figueroa I, Cuellar-Pompa L, Quiros-Lopez R, Serrano-Aguilar P, et al. Healthy lifestyle habits for patients with systemic lupus erythematosus: a systemic review. *Semin Arthritis Rheum.* (2016) 45:463–70. doi: 10.1016/j.semarthrit.2015.09.003
62. Wierenga KA, Harkema JR, Pestka JJ. Lupus, silica, and dietary omega-3 fatty acid interventions. *Toxicol Pathol.* (2019) 47:1004–11. doi: 10.1177/0192623319878398
63. Bates MA, Brandenberger C, Langohr II, Kumagai K, Lock AL, Harkema JR, et al. Silica-triggered autoimmunity in lupus-prone mice blocked by docosahexaenoic acid consumption. *PLoS ONE.* (2016) 11:1–31. doi: 10.1371/journal.pone.0160622
64. Kelley V, Ferretti A, Izui S, Strom T. A fish oil diet rich in eicosapentaenoic acid reduces cyclooxygenase metabolites, and suppresses lupus in MRL-lpr mice. *J Immunol.* (1985) 134:1914–9.
65. Smith WL. Cyclooxygenases, peroxide tone and the allure of fish oil. *Curr Opin Cell Biol.* (2005) 17:174–82. doi: 10.1016/j.ceb.2005.02.005
66. Pestka JJ, Vines LL, Bates MA, He K, Langohr I. Comparative effects of n-3, n-6 and n-9 unsaturated fatty acid-rich diet consumption on lupus nephritis, autoantibody production and CD4+ T cell-related gene responses in the autoimmune NZBWF1 mouse. *PLoS ONE.* (2014) 9:1–18. doi: 10.1371/journal.pone.0100255
67. Halade GV, Rahman MM, Bhattacharya A, Barnes JL, Chandrasekar B, Fernandes G. Docosahexaenoic acid-enriched fish oil attenuates kidney disease and prolongs median and maximal life span of autoimmune lupus-prone mice. *J Immunol.* (2010) 184:5280–6. doi: 10.4049/jimmunol.0903282
68. Kim YJ, Yokozawa T, Chung HY. Effects of energy restriction and fish oil supplementation on renal guanidino levels and antioxidant defences in aged lupus-prone B/W mice. *Br J Nutr.* (2005) 93:835–44. doi: 10.1079/BJN20051440
69. Everett ST, Wolf R, Contento I, Haiduc V, Richey M, Erkan D. Short-term patient-centered nutrition counseling impacts weight and nutrient intake in patients with systemic lupus erythematosus. *Lupus.* (2015) 24:1321–6. doi: 10.1177/0961203315582284
70. Borges MC, Santos FdMM, Telles RW, Correia MITD, Lanna CCD. Polyunsaturated omega-3 fatty acids and systemic lupus erythematosus: what do we know? *Rev Bras Reumatol.* (2014) 54:459–66. doi: 10.1016/j.rbre.2013.12.002
71. Calder PC. The 2008 ESPEN sir david cuthbertson lecture: fatty acids and inflammation–from the membrane to the nucleus and from the laboratory bench to the clinic. *Clin Nutr.* (2010) 29:5–12. doi: 10.1016/j.clnu.2009.11.003
72. Wright SA, O'Prey FM, McHenry MT, Leahey WJ, Devine AB, Duffy EM, et al. A randomised interventional trial of ω-3-polyunsaturated fatty acids on endothelial function and disease activity in systemic lupus erythematosus. *Ann Rheum Dis.* (2008) 67:841–8. doi: 10.1136/ard.2007.077156
73. Bello KJ, Fang H, Fazeli P, Bolad W, Corretti M, Magder LS, et al. Omega-3 in SLE: a double-blind, placebo-controlled randomized clinical trial of endothelial dysfunction and disease activity in systemic lupus erythematosus. *Rheumatol Int.* (2013) 33:2789–96. doi: 10.1007/s00296-013-2811-3
74. Arriens C, Hynan LS, Lerman RH, Karp DR, Mohan C. Placebo-controlled randomized clinical trial of fish oil's impact on fatigue, quality of life, and disease activity in systemic lupus erythematosus. *Nutr J.* (2015) 14:82–92. doi: 10.1186/s12937-015-0068-2
75. Partan RU, Hidayat R, Saputra N, Rahmayani F, Prapto H, Yudha TW. Seluang fish (rasbora spp.) oil decreases inflammatory cytokines via increasing vitamin D level in systemic lupus erythematosus. *Open Access Maced J Med Sci.* (2019) 7:1418–21. doi: 10.3889/oamjms.2019.308

76. Bergamo P, Maurano F, Rossi M. Phase 2 enzyme induction by conjugated linoleic acid improves lupus-associated oxidative stress. *Free Radic Biol Med.* (2007) 43:71–9. doi: 10.1016/j.freeradbiomed.2007.03.023
77. Bergamo P, Luongo D, Maurano F, Mazzarella G, Stefanile R, Rossi M. Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Faslpr mice. *J Lipid Res.* (2006) 47:2382–91. doi: 10.1194/jlr.M600187-JLR200
78. Yang M, Cook ME. Dietary CLA decreased weight loss and extended survival following the onset of kidney failure in NZB/W F1 mice. *Lipids.* (2003) 38:21–4. doi: 10.1007/s11745-003-1026-8
79. Aparicio-Soto M, Sanchez-Hidalgo M, Cárdeno A, Rosillo MÁ, Sanchez-Fidalgo S, Utrilla J, et al. Dietary extra virgin olive oil attenuates kidney injury in pristane-induced SLE model via activation of HO-1/Nrf-2 antioxidant pathway and suppression of JAK/STAT, NF-κB and MAPK activation. *J Nutr Biochem.* (2016) 27:278–88. doi: 10.1016/j.jnutbio.2015.09.017
80. Pocovi-Gerardino G, Correa-Rodríguez M, Callejas-Rubio JL, Ríos-Fernández R, Martín-Amada M, Cruz-Caparros MG, et al. Dietary inflammatory index score and cardiovascular disease risk markers in women with systemic lupus erythematosus. *J Acad Nutr Diet.* (2019) 120:280–7. doi: 10.1016/j.jand.2019.06.007
81. Charoenwoodhipong P, Harlow SD, Marder W, Hassett AL, McCune WJ, Gordon C, et al. Dietary omega polyunsaturated fatty acid intake and patient-reported outcomes in systemic lupus erythematosus: the michigan lupus epidemiology & surveillance (MILES) program. *Arthritis Care Res.* (2019) 72:874–81. doi: 10.1002/acr.23925
82. Patrick RP, Ames BN. Vitamin D and the omega-3 fatty acids control serotonin synthesis and action, part 2: relevance for ADHD, bipolar disorder, schizophrenia, and impulsive behavior. *FASEB J.* (2015) 29:2207–22. doi: 10.1096/fj.14-268342
83. Fenton JI, Hord NG, Ghosh S, Gurzell EA. Immunomodulation by dietary long chain omega-3 fatty acids and the potential for adverse health outcomes. *Prostaglandins Leukot Essent Fatty Acids.* (2013) 89:379–90. doi: 10.1016/j.plefa.2013.09.011
84. Yang M, Pariza MW, Cook ME. Dietary conjugated linoleic acid protects against end stage disease of systemic lupus erythematosus in the NZB/W F1 mouse. *Immunopharmacol Immunotoxicol.* (2000) 22:433–49. doi: 10.3109/08923970009026004
85. Benjamin S, Prakasan P, Sreedharan S, Wright ADG, Spener F. Pros and cons of CLA consumption: an insight from clinical evidences. *Nutr Metab.* (2015) 12:4–23. doi: 10.1186/1743-7075-12-4
86. Jones JM. CODEX-aligned dietary fiber definitions help to bridge the 'fiber gap'. *Nutr J.* (2014) 13:34. doi: 10.1186/1475-2891-13-34
87. Makki K, Deehan EC, Walter J, Bäckhed F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe.* (2018) 23:705–15. doi: 10.1016/j.chom.2018.05.012
88. Statovci D, Aguilera M, MacSharry J, Melgar S. The impact of western diet and nutrients on the microbiota and immune response at mucosal interfaces. *Front Immunol.* (2017) 8:838. doi: 10.3389/fimmu.2017.00838
89. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science.* (2011) 334:105–8. doi: 10.1126/science.1208344
90. Kong LC, Holmes BA, Cotillard A, Habi-Rachedi F, Brazeilles R, Gougis S, et al. Dietary patterns differently associate with inflammation and gut microbiota in overweight and obese subjects. *PLoS ONE.* (2014) 9:e109434. doi: 10.1371/journal.pone.0109434
91. Kim M, Qie Y, Park J, Kim CH. Gut microbial metabolites fuel host antibody responses. *Cell Host Microbe.* (2016) 20:202–14. doi: 10.1016/j.chom.2016.07.001
92. Bishehsari F, Engen P, Preite N, Tuncil Y, Naqib A, Shaikh M, et al. Dietary fiber treatment corrects the composition of gut microbiota, promotes SCFA production, and suppresses colon carcinogenesis. *Genes.* (2018) 9:1–17. doi: 10.3390/genes9020102
93. Silverman GJ, Azzouz DF, Alekseyenko AV. Systemic lupus erythematosus and dysbiosis in the microbiome: cause or effect or both? *Curr Opin Immunol.* (2019) 61:80–5. doi: 10.1016/j.coi.2019.08.007
94. López P, Sánchez B, Margolles A, Suárez A. Intestinal dysbiosis in systemic lupus erythematosus: cause or consequence? *Curr Opin Rheumatol.* (2016) 28:515–22. doi: 10.1097/BOR.0000000000000309
95. Hevia A, Milani C, López P, Cuervo A, Arboleya S, Duranti S, et al. Intestinal dysbiosis associated with systemic lupus erythematosus. *MBio.* (2014) 5:1–14. doi: 10.1128/mBio.01548-14
96. He Z, Shao T, Li H, Xie Z, Wen C. Alterations of the gut microbiome in Chinese patients with systemic lupus erythematosus. *Gut Pathog.* (2016) 8:1–7. doi: 10.1186/s13099-016-0146-9
97. Rodríguez-Carrio J, López P, Sánchez B, González S, Gueimonde M, Margolles A, et al. Intestinal dysbiosis is associated with altered short-chain fatty acids and serum-free fatty acids in systemic lupus erythematosus. *Front Immunol.* (2017) 8:23. doi: 10.3389/fimmu.2017.00023
98. Minami Y, Hirabayashi Y, Nagata C, Ishii T, Harigae H, Sasaki T. Intakes of vitamin B6 and dietary fiber and clinical course of systemic lupus erythematosus: a prospective study of Japanese female patients. *J Epidemiol.* (2011) 21:246–54. doi: 10.2188/jea.JE20100157
99. Gernand AD, Schulze KJ, Stewart CP, West KP Jr, Christian P. Micronutrient deficiencies in pregnancy worldwide: health effects and prevention. *Nat Rev Endocrinol.* (2016) 12:274–89. doi: 10.1038/nrendo.2016.37
100. Liao X, Ren J, Wei CH, Ross AC, Cecere TE, Jortner BS, et al. Paradoxical effects of all-trans-retinoic acid on lupus-like disease in the MRL/lpr mouse model. *PLoS ONE.* (2015) 10:e0118176. doi: 10.1371/journal.pone.0118176
101. Liao CH, Erdman J Jr, Voss E Jr, Johnston P. Dietary vitamin A deficiency and the immune system in a murine model of systemic lupus erythematosus. *Nutr Res.* (1996) 16:279–92. doi: 10.1016/0271-5317(96)00012-7
102. Ikeda T, Nishide T, Ohtani T, Furukawa F. The effects of vitamin A derivative etretinate on the skin of MRL mice. *Lupus.* (2005) 14:510–6. doi: 10.1191/0961203305lu2144oa
103. Kinoshita K, Kishimoto K, Shimazu H, Nozaki Y, Sugiyama M, Ikoma S, et al. Successful treatment with retinoids in patients with lupus nephritis. *Am J Kidney Dis.* (2010) 55:344–7. doi: 10.1053/j.ajkd.2009.06.012
104. Vien C, González-Cabello R, Bodó I, Gergely P. Effect of vitamin A treatment on the immune reactivity of patients with systemic lupus erythematosus. *J Clin Lab Immunol.* (1988) 26:33–5.
105. Handono K, Firdausi SN, Pratama MZ, Endharti AT, Kalim H. Vitamin A improve Th17 and Treg regulation in systemic lupus erythematosus. *Clin Rheumatol.* (2016) 35:631–8. doi: 10.1007/s10067-016-3197-x
106. Sun LD, Zeng K, Wang Q. Study of riboflavin nutritional status in patients with systemic lupus erythematosus. *Mod Prev Med.* (2007) 1:53–5.
107. Molad Y, Rachmilewitz B, Sidi Y, Pinkhas J, Weinberger A. Serum cobalamin and transcobalamin levels in systemic lupus erythematosus. *Am J Med.* (1990) 88:141–4. doi: 10.1016/0002-9343(90)90463-N
108. Varghese B, Haase N, Low PS. Depletion of folate-receptor-positive macrophages leads to alleviation of symptoms and prolonged survival in two murine models of systemic lupus erythematosus. *Mol Pharm.* (2007) 4:679–85. doi: 10.1021/mp0700615
109. Minami Y, Sasaki T, Arai Y, Kurisu Y, Hisamichi S. Diet and systemic lupus erythematosus: a 4 year prospective study of Japanese patients. *J Rheumatol.* (2003) 30:747–54.
110. Tam LS, Li EK, Leung VY, Griffith JF, Benzie IF, Lim PL, et al. Effects of vitamins C and E on oxidative stress markers and endothelial function in patients with systemic lupus erythematosus: a double blind, placebo controlled pilot study. *J Rheumatol.* (2005) 32:275–82.
111. Lai YH, Fang TC. The pleiotropic effect of vitamin D. *ISRN Nephrol.* (2013) 2013:898125. doi: 10.5402/2013/898125
112. Dankers W, Colin EM, van Hamburg JP, Lubberts E. Vitamin D in autoimmunity: molecular mechanisms and therapeutic potential. *Front Immunol.* (2017) 7:697. doi: 10.3389/fimmu.2016.00697
113. Mok CC. Vitamin D and systemic lupus erythematosus: an update. *Expert Rev Clin Immunol.* (2013) 9:453–63. doi: 10.1586/eci.13.19
114. Damanhouri LH. Vitamin D deficiency in Saudi patients with systemic lupus erythematosus. *Saudi Med J.* (2009) 30:1291–5.
115. Farid E, Jaradat A, Al-Segai O, Hassan A. Prevalence of vitamin D deficiency in adult patients with systemic lupus erythematosus in Kingdom of Bahrain. *Egypt J Immunol.* (2017) 24:1–8.
116. Willis R, Smikle M, DeCeulaer K, Romay-Penabad Z, Papalardo E, Jajoria P, et al. Clinical associations of proinflammatory cytokines, oxidative biomarkers and vitamin D levels in systemic lupus erythematosus. *Lupus.* (2017) 26:1517–27. doi: 10.1177/0961203317706557

117. Kamen DL, Cooper GS, Bouali H, Shaftman SR, Hollis BW, Gilkeson GS. Vitamin D deficiency in systemic lupus erythematosus. *Autoimmun Rev.* (2006) 5:114–7. doi: 10.1016/j.autrev.2005.05.009
118. Toloza SM, Cole DE, Gladman DD, Ibanez D, Urowitz MB. Vitamin D insufficiency in a large female SLE cohort. *Lupus.* (2010) 19:13–9. doi: 10.1177/0961203309345775
119. McGhie TK, DeCeulaer K, Walters CA, Soyibo A, Lee MG. Vitamin D levels in Jamaican patients with systemic lupus erythematosus. *Lupus.* (2014) 23:1092–6. doi: 10.1177/0961203314528556
120. Souto M, Coelho A, Guo C, Mendonca L, Argolo S, Papi J, et al. Vitamin D insufficiency in Brazilian patients with SLE: prevalence, associated factors, and relationship with activity. *Lupus.* (2011) 20:1019–26. doi: 10.1177/0961203311401457
121. Schoindre Y, Jallouli M, Tanguy ML, Ghillani P, Galicier L, Aumaitre O, et al. Lower vitamin D levels are associated with higher systemic lupus erythematosus activity, but not predictive of disease flare-up. *Lupus Sci Med.* (2014) 1:1–7. doi: 10.1136/lupus-2014-000027
122. Szodoray P, Tarr T, Bazso A, Poor G, Szegedi G, Kiss E. The immunopathological role of vitamin D in patients with SLE: data from a single centre registry in Hungary. *Scand J Rheumatol.* (2011) 40:122–6. doi: 10.3109/03009742.2010.507220
123. Muller K, Kriegbaum NJ, Baslund B, Sorensen OH, Thymann M, Bentzen K. Vitamin D3 metabolism in patients with rheumatic diseases: low serum levels of 25-hydroxyvitamin D3 in patients with systemic lupus erythematosus. *Clin Rheumatol.* (1995) 14:397–400. doi: 10.1007/BF02207671
124. Salman-Monte TC, Torrente-Segarra V, Almirall M, Corzo P, Mojal S, Carbonell-Abelló J. Prevalence and predictors of vitamin D insufficiency in supplemented and non-supplemented women with systemic lupus erythematosus in the Mediterranean region. *Rheumatol Int.* (2016) 36:975–85. doi: 10.1007/s00296-016-3497-0
125. Munoz-Ortego J, Torrente-Segarra V, Prieto-Alhambra D, Salman-Monte TC, Carbonell-Abello J. Prevalence and predictors of vitamin D deficiency in non-supplemented women with systemic lupus erythematosus in the Mediterranean region: a cohort study. *Scand J Rheumatol.* (2012) 41:472–5. doi: 10.3109/03009742.2012.697189
126. Yeap SS, Othman AZ, Zain AA, Chan SP. Vitamin D levels: its relationship to bone mineral density response and disease activity in premenopausal Malaysian systemic lupus erythematosus patients on corticosteroids. *Int J Rheum Dis.* (2012) 15:17–24. doi: 10.1111/j.1756-185X.2011.01653.x
127. Lin TC, Wu JY, Kuo ML, Ou LS, Yeh KW, Huang JL. Correlation between disease activity of pediatric-onset systemic lupus erythematosus and level of vitamin D in Taiwan: a case-cohort study. *J Microbiol Immunol Infect.* (2018) 51:110–4. doi: 10.1016/j.jmii.2015.12.005
128. Sumethkul K, Boonyaratavej S, Kitumnuaypong T, Angthararuk S, Cheewasat P, Manadee N, et al. The predictive factors of low serum 25-hydroxyvitamin D and vitamin D deficiency in patients with systemic lupus erythematosus. *Rheumatol Int.* (2013) 33:1461–7. doi: 10.1007/s00296-012-2537-7
129. Mandal M, Tripathy R, Panda AK, Pattanaik SS, Dakua S, Pradhan AK, et al. Vitamin D levels in Indian systemic lupus erythematosus patients: association with disease activity index and interferon alpha. *Arthritis Res Ther.* (2014) 16:1–8. doi: 10.1186/ar4479
130. Abdel Galil SM, El-Shafey AM, Abdul-Maksoud RS, El-Boshy M. Interferon alpha gene expression and serum level association with low vitamin D levels in Egyptian female patients with systemic lupus erythematosus. *Lupus.* (2018) 27:199–209. doi: 10.1177/0961203317716321
131. Hamza RT, Awwad KS, Ali MK, Hamed AI. Reduced serum concentrations of 25-hydroxy vitamin D in Egyptian patients with systemic lupus erythematosus: relation to disease activity. *Med Sci Monit.* (2011) 17:711–8. doi: 10.12659/MSM.882131
132. AlSaleem A, AlSaghier A, Al-Mayouf SM. Vitamin D status in children with systemic lupus erythematosus and its association with clinical and laboratory parameters. *Clin Rheumatol.* (2015) 34:81–4. doi: 10.1007/s10067-014-2811-z
133. Eloi M, Horvath DV, Ortega JC, Prado MS, Andrade LEC, Szejnfeld VL, et al. 25-Hydroxivitamin D serum concentration, not free and bioavailable vitamin D, is associated with disease activity in systemic lupus erythematosus patients. *PLoS ONE.* (2017) 12:e0170323. doi: 10.1371/journal.pone.0170323
134. Borba VZ, Vieira JG, Kasamatsu T, Radominski SC, Sato EI, Lazaretti-Castro M. Vitamin D deficiency in patients with active systemic lupus erythematosus. *Osteoporos Int.* (2009) 20:427–33. doi: 10.1007/s00198-008-0676-1
135. Yap K, Northcott M, Hoi AB, Morand E, Nikpour M. Association of low vitamin D with high disease activity in an Australian systemic lupus erythematosus cohort. *Lupus Sci Med.* (2015) 2:1–6. doi: 10.1136/lupus-2014-000064
136. Robinson AB, Tangpricha V, Yow E, Gurion R, McComsey GA, Schanberg LE. Vitamin D deficiency is common and associated with increased C-reactive protein in children and young adults with lupus: an atherosclerosis prevention in pediatric lupus erythematosus substudy. *Lupus Sci Med.* (2014) 1:1–7. doi: 10.1136/lupus-2014-000011
137. Gao C, Liu S, Wu Z, Li T, Gao G, Liu Z, et al. Severe vitamin D deficiency increases the risk for moderate to severe disease activity in Chinese patients with SLE. *Lupus.* (2016) 25:1224–9. doi: 10.1177/0961203316635289
138. Miskovic R, Plavsic A, Raskovic S, Jovicic Z, Bolpacic J. Vitamin D status in patients with systemic lupus erythematosus in Serbia: correlation with disease activity and clinical manifestations. *Open Access Maced J Med Sci.* (2015) 3:256–61. doi: 10.3889/oamjms.2015.052
139. Hiraki LT, Munger KL, Costenbader KH, Karlson EW. Dietary intake of vitamin D during adolescence and risk of adult-onset systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Care Res.* (2012) 64:1829–36. doi: 10.1002/acr.21776
140. Costenbader KH, Feskanich D, Holmes M, Karlson EW, Benito-Garcia E. Vitamin D intake and risks of systemic lupus erythematosus and rheumatoid arthritis in women. *Ann Rheum Dis.* (2008) 67:530–5. doi: 10.1136/ard.2007.072736
141. Yu Q, Qiao Y, Liu D, Liu F, Gao C, Duan J, et al. Vitamin D protects podocytes from autoantibodies induced injury in lupus nephritis by reducing aberrant autophagy. *Arthritis Res Ther.* (2019) 21:19–30. doi: 10.1186/s13075-018-1803-9
142. Lima G, Paupitz J, Aikawa N, Alvarenga J, Pereira RR. A randomized double-blind placebo-controlled trial of vitamin D supplementation in juvenile-onset systemic lupus erythematosus: positive effect on trabecular microarchitecture using HR-pQCT. *Osteoporos Int.* (2018) 29:587–94. doi: 10.1007/s00198-017-4316-5
143. Sun LY, Zhang HY, Feng XB, Hou YY, Lu LW, Fan LM. Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. *Lupus.* (2007) 16:121–8. doi: 10.1177/0961203306075793
144. Xu JJ, Sun YB, Zhang XL, Wang XF. Vitamin D analog EB1089 could repair the defective bone marrow-derived mesenchymal stromal cells in patients with systemic lupus erythematosus. *Int J Clin Exp Med.* (2015) 8:916–21.
145. Garf KE, Marzouk H, Farag Y, Rasheed L, El Garf A. Vitamin D status in Egyptian patients with juvenile-onset systemic lupus erythematosus. *Rheumatol Int.* (2015) 35:1535–40. doi: 10.1007/s00296-015-3245-x
146. Lima G, Paupitz J, Aikawa NE, Takayama L, Bonfa E, Pereira RM. Vitamin D supplementation in adolescents and young adults with juvenile systemic lupus erythematosus for improvement in disease activity and fatigue scores: a randomized, double-blind, placebo-controlled trial. *Arthritis Care Res.* (2016) 68:91–8. doi: 10.1002/acr.22621
147. Islam MA, Khandker SS, Alam SS, Kotyla P, Hassan R. Vitamin D status in patients with systemic lupus erythematosus (SLE): a systematic review and meta-analysis. *Autoimmun Rev.* (2019) 18:1–19. doi: 10.1016/j.autrev.2019.102392
148. Zheng R, Gonzalez A, Yue J, Wu X, Qiu M, Gui L, et al. Efficacy and safety of vitamin D supplementation in patients with systemic lupus erythematosus: a meta-analysis of randomized controlled trials. *Am J Med Sci.* (2019) 358:104–14. doi: 10.1016/j.amjms.2019.04.020
149. Zhao M, Duan XH, Wu ZZ, Gao CC, Wang N, Zheng ZH. Severe vitamin D deficiency affects the expression of autophagy related genes in PBMCs and T-cell subsets in active systemic lupus erythematosus. *Am J Clin Exp Immunol.* (2017) 6:43–51.
150. Yazdanpanah E, Mahmoudi M, Sahebari M, Rezaieyazdi Z, Esmaeili SA, Tabasi N, et al. Vitamin D3 alters the expression of toll-like receptors in peripheral blood mononuclear cells of patients with systemic lupus erythematosus. *J Cell Biochem.* (2017) 118:4831–5. doi: 10.1002/jcb.26155

151. Marinho A, Carvalho C, Boleixa D, Bettencourt A, Leal B, Guimarães J, et al. Vitamin D supplementation effects on FoxP3 expression in T cells and FoxP3+/IL-17A ratio and clinical course in systemic lupus erythematosus patients: a study in a Portuguese cohort. *Immunol Res.* (2017) 65:197–206. doi: 10.1007/s12026-016-8829-3
152. Ritterhouse LL, Crowe SR, Niewold TB, Kamen DL, Macwana SR, Roberts VC, et al. Vitamin D deficiency is associated with an increased autoimmune response in healthy individuals and in patients with systemic lupus erythematosus. *Ann Rheum Dis.* (2011) 70:1569–74. doi: 10.1136/ard.2010.148494
153. Kokic V, Martinovic Kaliterna D, Radic M, Perkovic D, Cvek M, Capkun V. Relationship between vitamin D, IFN-γ, and E2 levels in systemic lupus erythematosus. *Lupus.* (2016) 25:282–8. doi: 10.1177/0961203315605367
154. Reynolds JA, Rosenberg AZ, Smith CK, Sergeant JC, Rice GI, Briggs TA, et al. Brief report: vitamin D deficiency is associated with endothelial dysfunction and increases type I interferon gene expression in a murine model of systemic lupus erythematosus. *Arthritis rheumatol.* (2016) 68:2929–35. doi: 10.1002/art.39803
155. Reynolds JA, Haque S, Williamson K, Ray DW, Alexander MY, Bruce IN. Vitamin D improves endothelial dysfunction and restores myeloid angiogenic cell function via reduced CXCL-10 expression in systemic lupus erythematosus. *Sci Rep.* (2016) 6:1–11. doi: 10.1038/srep22341
156. Handono K, Sidarta YO, Pradana BA, Nugroho RA, Hartono IA, Kalim H, et al. Vitamin D prevents endothelial damage induced by increased neutrophil extracellular traps formation in patients with systemic lupus erythematosus. *Acta Med Indones.* (2014) 46:189–98.
157. Schneider L, Colar da Silva A, Werres Junior L, Alegretti A, dos Santos AP, Santos M, et al. Vitamin D levels and cytokine profiles in patients with systemic lupus erythematosus. *Lupus.* (2015) 24:1191–7. doi: 10.1177/0961203315584811
158. Piantoni S, Andreoli L, Scarsi M, Zanola A, Dall'Ara F, Pizzorni C, et al. Phenotype modifications of T-cells and their shift toward a Th2 response in patients with systemic lupus erythematosus supplemented with different monthly regimens of vitamin D. *Lupus.* (2015) 24:490–8. doi: 10.1177/0961203314559090
159. Handono K, Marisa D, Kalim H. Association between the low levels of vitamin D and Treg function in systemic lupus erythematosus patients. *Acta Med Indones.* (2013) 45:26–31.
160. Ding Y, Liao W, He XJ, Xiang W. Effects of 1, 25 (OH)2D3 and vitamin D receptor on peripheral CD4+/CD8+ double-positive T lymphocytes in a mouse model of systemic lupus erythematosus. *J Cell Mol Med.* (2017) 21:975–85. doi: 10.1111/jcmm.13037
161. He XJ, Ding Y, Xiang W, Dang XQ. Roles of 1, 25 (OH) 2D3 and vitamin D receptor in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus by regulating the activation of CD4+ T cells and the PKCδ/ERK signaling pathway. *Cell Physiol Biochem.* (2016) 40:743–56. doi: 10.1159/000453135
162. Terrier B, Derian N, Schoindre Y, Chaara W, Geri G, Zahr N, et al. Restoration of regulatory and effector T cell balance and B cell homeostasis in systemic lupus erythematosus patients through vitamin D supplementation. *Arthritis Res Ther.* (2012) 14:1–10. doi: 10.1186/ar4060
163. Chen DJ, Li LJ, Yang XK, Yu T, Leng RX, Pan HF, et al. Altered microRNAs expression in T cells of patients with SLE involved in the lack of vitamin D. *Oncotarget.* (2017) 8:62099–110. doi: 10.18632/oncotarget.19062
164. Hsieh CC, Lin BF. The effects of vitamin E supplementation on autoimmune-prone New Zealand black × New Zealand white F1 mice fed an oxidised oil diet. *Br J Nutr.* (2005) 93:655–62. doi: 10.1079/BJN20051413
165. Hsieh CC, Lin BF. Opposite effects of low and high dose supplementation of vitamin E on survival of MRL/lpr mice. *Nutrition.* (2005) 21:940–8. doi: 10.1016/j.nut.2004.11.021
166. Comstock G, Burke A, Hoffman S, Helzlsouer K, Bendich A, Masi A, et al. Serum concentrations of α tocopherol, β carotene, and retinol preceding the diagnosis of rheumatoid arthritis and systemic lupus erythematosus. *Ann Rheum Dis.* (1997) 56:323–5. doi: 10.1136/ard.56.5.323
167. Maeshima E, Liang XM, Goda M, Otani H, Mune M. The efficacy of vitamin E against oxidative damage and autoantibody production in systemic lupus erythematosus: a preliminary study. *Clin Rheumatol.* (2007) 26:401–4. doi: 10.1007/s10067-006-0477-x
168. Shahba A, Esheba NE, Fooda AA, El-Dardiry S, Wagih A, el-Deeb O. Effect of nigella sativa and vitamin E on some oxidative/nitrosative biomarkers in systemic lupus erythematosus patients. *Life Sci J.* (2015) 12:157–62.
169. Mellor-Pita S, Tutor-Ureta P, Rosado S, Alkadi K, Granado F, Jimenez-Ortiz C, et al. Calcium and vitamin D supplement intake may increase arterial stiffness in systemic lupus erythematosus patients. *Clin Rheumatol.* (2019) 38:1–10. doi: 10.1007/s10067-018-04416-x
170. Al-Kushi AG, Azzeh FS, Header EA, ElSawy NA, Hijazi HH, Jazar AS, et al. Effect of vitamin D and calcium supplementation in patients with systemic lupus erythematosus. *Saudi J Med Med Sci.* (2018) 6:137–42. doi: 10.4103/sjmms.sjmms_134_17
171. Tayel MY, El-Zawawy AI, Said MI, Soliman EA, Mohamed MK. A study on the role of calcium homeostasis and vitamin D deficiency in premenopausal systemic lupus erythematosus patients and its relation with disease activity. *Egypt J Obes Diabetes Endocrinol.* (2016) 2:95–107. doi: 10.4103/2356-8062.197571
172. Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J.* (2001) 15:2579–85. doi: 10.1096/fj.01-0433rev
173. Leiba A, Amital H, Gershwin M, Shoenfeld Y. Diet and lupus. *Lupus.* (2001) 10:246–8. doi: 10.1191/096120301674681790
174. Beach R, Gershwin ME, Hurley L. Nutritional factors and autoimmunity. II. prolongation of survival in zinc-deprived NZB/W mice. *J Immunol.* (1982) 128:308–13.
175. Kretzschmar P, Brewer G, Walker S. Depot-zinc therapy of systemic lupus erythematosus in B/W mice. *Proc Soc Exp Biol Med.* (1981) 168:301–5. doi: 10.3181/00379727-168-41276
176. Vitales-Noyola M, Layseca-Espinosa E, Baranda L, Abud-Mendoza C, Niño-Moreno P, Monsiváis-Urenda A, et al. Analysis of sodium chloride intake and Treg/Th17 lymphocytes in healthy individuals and patients with rheumatoid arthritis or systemic lupus erythematosus. *J Immunol Res.* (2018) 2018:1–12. doi: 10.1155/2018/9627806
177. Scrivo R, Massaro L, Barbati C, Vomero M, Ceccarelli F, Spinelli FR, et al. The role of dietary sodium intake on the modulation of T helper 17 cells and regulatory T cells in patients with rheumatoid arthritis and systemic lupus erythematosus. *PLoS ONE.* (2017) 12:1–14. doi: 10.1371/journal.pone.0184449
178. Yang X, Yao G, Chen W, Tang X, Feng X, Sun L. Exacerbation of lupus nephritis by high sodium chloride related to activation of SGK1 pathway. *Int Immunopharmacol.* (2015) 29:568–73. doi: 10.1016/j.intimp.2015.09.027
179. Soni C, Sinha I, Fasnacht MJ, Olsen NJ, Rahman ZS, Sinha R. Selenium supplementation suppresses immunological and serological features of lupus in B6. Sle1b mice. *Autoimmunity.* (2019) 52:57–68. doi: 10.1080/08916934.2019.1603297
180. O'Dell J, McGivern JP, Kay H, Klassen L. Improved survival in murine lupus as the result of selenium supplementation. *Clin Exp Immunol.* (1988) 73:322–7.
181. Leiter LM, Reuhl KR, Racis Jr SP, Sherman AR. Iron status alters murine systemic lupus erythematosus. *J Nutr.* (1995) 125:474–84.
182. Duffy EM, Meenagh GK, McMillan SA, Strain JJ, Hannigan BM, Bell AL. The clinical effect of dietary supplementation with omega-3 fish oils and/or copper in systemic lupus erythematosus. *J Rheumatol.* (2004) 31:1551–6.
183. Zhao J, Wang J, Zhou M, Li M, Li M, Tan H. Curcumin attenuates murine lupus via inhibiting NLRP3 inflammasome. *Int Immunopharmacol.* (2019) 69:213–6. doi: 10.1016/j.intimp.2019.01.046
184. Foxley S, Zamora M, Hack B, Alexander RR, Roman B, Quigg RJ, et al. Curcumin aggravates CNS pathology in experimental systemic lupus erythematosus. *Brain Res.* (2013) 1504:85–96. doi: 10.1016/j.brainres.2013.01.040
185. Lee H, Kim H, Lee G, Chung HS, Bae H. Curcumin attenuates lupus nephritis upon interaction with regulatory T cells in New Zealand Black/White mice. *Br J Nutr.* (2013) 110:69–76. doi: 10.1017/S0007114512004734
186. Singgih Wahono C, Diah Setyorini C, Kalim H, Nurdiana N, Handono K. Effect of curcuma xanthorrhiza supplementation on systemic lupus erythematosus patients with hypovitamin d which were given Vitamin D3 towards Disease Activity (SLEDAI), IL-6, and TGF-β1 serum. *Int J Rheumatol.* (2017) 2017:1–9. doi: 10.1155/2017/7687053
187. Handono K, Pratama MZ, Endharti AT, Kalim H. Treatment of low doses curcumin could modulate Th17/Treg balance specifically on CD4+ T cell

cultures of systemic lupus erythematosus patients. *Cent Eur J Immunol.* (2015) 40:461–9. doi: 10.5114/ceji.2015.56970

188. Wang M, Zhou G, Lv J, Zeng P, Guo C, Wang Q. Curcumin modulation of the activation of PYK2 in peripheral blood mononuclear cells from patients with lupus nephritis. *Reumatologia.* (2017) 55:269–75. doi: 10.5114/reum.2017.72623
189. Aparicio-Soto M, Montserrat-de la Paz S, Sanchez-Hidalgo M, Cardeno A, Bermudez B, Muriana FJ, et al. Virgin olive oil and its phenol fraction modulate monocyte/macrophage functionality: a potential therapeutic strategy in the treatment of systemic lupus erythematosus. *Br J Nutr.* (2018) 120:681–92. doi: 10.1017/S0007114518001976
190. Tsai PY, Ka SM, Chang JM, Chen HC, Shui HA, Li CY, et al. Epigallocatechin-3-gallate prevents lupus nephritis development in mice via enhancing the Nrf2 antioxidant pathway and inhibiting NLRP3 inflammasome activation. *Free Radic Biol Med.* (2011) 51:744–54. doi: 10.1016/j.freeradbiomed.2011.05.016
191. Peairs A, Dai R, Gan L, Shimp S, Rylander MN, Li L, et al. Epigallocatechin-3-gallate (EGCG) attenuates inflammation in MRL/lpr mouse mesangial cells. *Cell Mol Immunol.* (2010) 7:123–32. doi: 10.1038/cmi.2010.1
192. Aparicio-Soto M, Sánchéz-Hidalgo M, Cárdeno A, Lucena JM, Gonzáléz-Escribano F, Castillo MJ, et al. The phenolic fraction of extra virgin olive oil modulates the activation and the inflammatory response of T cells from patients with systemic lupus erythematous and healthy donors. *Mol Nutr Food Res.* (2017) 61:1–9. doi: 10.1002/mnfr.201601080
193. Cuervo A, Hevia A, López P, Suárez A, Sánchez B, Margolles A, et al. Association of polyphenols from oranges and apples with specific intestinal microorganisms in systemic lupus erythematosus patients. *Nutrients.* (2015) 7:1301–17. doi: 10.3390/nu7021301
194. Han GM, Han XF. Lycopene reduces mortality in people with systemic lupus erythematosus: a pilot study based on the third national health and nutrition examination survey. *J Dermatolog Treat.* (2016) 27:430–5. doi: 10.3109/09546634.2015.1133879
195. Kang HK, Ecklund D, Liu M, Datta SK. Apigenin, a non-mutagenic dietary flavonoid, suppresses lupus by inhibiting autoantigen presentation for expansion of autoreactive Th1 and Th17 cells. *Arthritis Res Ther.* (2009) 11:1–13. doi: 10.1186/ar2682
196. Pocovi-Gerardino G, Correa-Rodríguez M, Rubio JLC, Fernández RR, Ortego-Centeno N, Rueda-Medina B. Diet quality and high-sensitivity C-reactive protein in patients with systemic lupus erythematosus. *Biol Res Nurs.* (2019) 21:107–13. doi: 10.1177/1099800418803176
197. Zahran AM, Elsayh KI, Saad K, Eloseily EM, Osman NS, Alblihed MA, et al. Effects of royal jelly supplementation on regulatory T cells in children with SLE. *Food Nutr Res.* (2016) 60:1–10. doi: 10.3402/fnr.v60.32963
198. Mannoor M, Shimabukuro I, Tsukamotoa M, Watanabe H, Yamaguchi K, Sato Y. Honeybee royal jelly inhibits autoimmunity in SLE-prone NZB× NZW F1 mice. *Lupus.* (2009) 18:44–52. doi: 10.1177/0961203308094765

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