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The role of vitamin D as an immunomodulator

Edited by
Mourad Aribi, Franck J. D. Mennechet and Chafia Touil-Boukoffa

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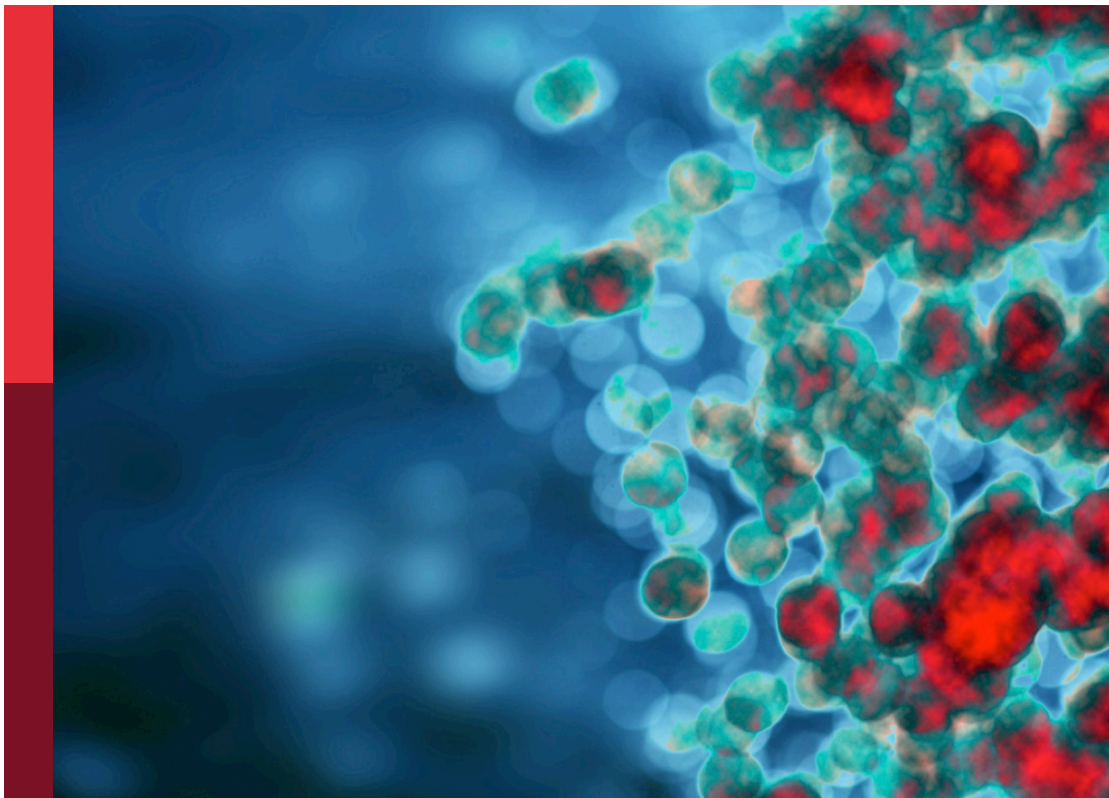

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The role of vitamin D as an immunomodulator

Topic editors

Mourad Aribi — University of Abou Bekr Belkaïd, Algeria

Franck J. D. Mennechet — University of Montpellier 1, France

Chafia Touil-Boukoffa — University of Science and Technology Houari Boumediene, Algeria

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Table of contents

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Landos Biopharma, Inc., United States

*CORRESPONDENCE
Mourad Aribi
mourad.aribi@univ-tlemcen.dz

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Editorial: The role of vitamin D as an immunomodulator

Mourad Aribi[1]*, Franck J. D. Mennechet[2] and Chafia Touil-Boukoffa[3]

[1]Laboratory of Applied Molecular Biology and Immunology, W0414100. University of Tlemcen, Tlemcen, Algeria, [2]Pathogenesis and Control of Chronic and Emerging Infections, The Institut National de la Santé et de la Recherche Médicale (INSERM) U1058, University of Montpellier, Etablissement Français du Sang, Antilles University, Montpellier, France, [3]Cytokines and Nitric Oxide (NO) Synthases Team, Laboratory of Cellular and Molecular Biology (LBCM), Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), Algiers, Algeria

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Editorial on the Research Topic
The role of vitamin D as an immunomodulator

The metabolism and immunomodulatory role of vitamin D: An abbreviated overview

Considered as a vitamin and a pre-hormone, vitamin D, in its biologically active form (1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]) is able to immunomodulate the functional activities of both innate and adaptive immune cells (1), including B-cells (2), T-cells (3), monocytes (4), macrophages (5), dendritic cells (6), neutrophils (7), and platelets (8), as these cells express the vitamin D receptor (VDR), a nuclear receptor and ligand-activated transcription factor/a ligand-dependent transcription regulator molecule belonging to the superfamily of nuclear receptors (9). Mechanistically, the action of the $1,25(OH)_2D_3$ on the target cells is performed by its binding to the VDR in the cytoplasm, followed by its heterodimerization with the retinoid X receptor-α (RXR-α) in the nucleus, resulting in the formation of the $1,25(OH)_2D_3$-RXR-VDR complex that binds to vitamin D response elements (VDRE) located on DNA (10) (Figure 1).

Humans and some animals have the ability to synthesize vitamin D in the form of previtamin D_3 in the skin as a result of localized conversion of 7-dehydrocholesterol (7-DHC) when exposed to solar ultraviolet B rays (UVB, 290-320 nm) (11–13). Previtamin D_3 isomerizes to vitamin D_3 (cholecalciferol) in the skin, in a thermo-sensitive but noncatalytic process (14), and both previtamin D_3 and vitamin D_3 are converted into a variety of photoproducts thanks to UVB radiation (15). Vitamin D_3 is considered to be best metabolized by the body. Its metabolism takes place consecutively in the liver, where it undergoes a first hydroxylation at the carbon 25-position into calcifediol/calcidiol ([25 $(OH)D_3$]) by the enzyme microsomal vitamin D 25-hydroxylase, also known as a cytochrome p450 2R1, which is encoded in humans by the CYP2R1 gene (16), and then, according to the cell needs and in response to cellular signals, it is hydrolyzed mainly in the kidneys at the 1α-position into $1,25(OH)_2D_3$, *i.e.*, calcitriol (17) by the enzyme 25-

hydroxyvitamin D 1-alpha-hydroxylase (VD 1A hydroxylase), referred to as cytochrome p450 27B1 (CYP27B1)/1-alpha-hydroxylase, which is encoded by the CYP27B1 gene in humans (18). As VDRs, the most vitamin D metabolizing enzymes are present in various immune cells, including T-cells, B-cells and antigen-presenting cells (APCs) (19).

Vitamin D deficiency and potential immunomodulatory interventions

Although the bioactive form of vitamin D is calcitriol, its status is assessed by determining the concentration of the so-called "circulating/blood" vitamin D form which corresponds to the result of the first vitamin D conversion, *i.e.*, calcifediol. In addition, it should be accepted that there are no single criteria and methods defining normal values for vitamin D. Nevertheless, it has been suggested that the normal levels of circulating blood vitamin D in human measured in late winter or early spring is greater than or equal to 75 nmol/L (30 ng/mL) as a cutoff value (20). While, greater than 20 ng/mL as optimal level, 11-20 ng/mL as insufficient, and vitamin D deficiency has been defined by a level less than or equal to 10 ng/mL (to convert to nmol/L, multiply by 2.496) (21).

Vitamin D deficiency may expose to increased risk of developing various immune-related diseases, including systemic and organ-specific autoimmune diseases, tuberculosis, sepsis, respiratory infection, and COVID-19, etc. (22, 23). This appears to be generalized in many geographical localities, and can be considered as a global public health problem in all age groups (24). This involves (i) insufficient sun exposure levels during the winter period (25), (ii) air pollution and low ground level of UVB rays as a result of overcast clouds (iii) a diet poor in vitamin D_2 (ergocalciferol) and vitamin D_3, (iv) aging, as it affects the formation of 1,25 $(OH)_2D_3$ (26), and (v) pigmented skin, as melanin absorbs the UVB that initiates vitamin D synthesis (27), etc.

Two bioequivalent forms of vitamin D can be recommended as vitamin D supplements, in order to correct such a deficiency (28), and include vitamin D_2 (obtained from dietary vegetable sources and oral supplements) and vitamin D_3 (present in plants and animals and obtained from oily fish and variably fortified foods and oral supplements) (29). In this context, they may also be the subject of nutritional immunomodulation intervention, since vitamin D has been shown to play a key role in stimulating immune responses, and protecting against infections, including respiratory tract infection (30), allergies (31), auto-immune diseases (32), and the development of malignant tumors (33). Vitamin D can also strengthen the barrier function of intestinal cells and different epithelial junctions, thus preventing the translocation of the intestinal microbiota to the bloodstream. Finally, vitamin D can suppresses the activity of β-catenin and then inhibits cell proliferation, modulates the expression level and functions of tight junction (TJ) proteins, such as claudin2 and 12, and other cell junction proteins, including E-cadherin, Occludin and ZO-1 (34).

The role of vitamin D as an immunomodulator: A review of the key findings from the research topic

This Editorial looks at the key findings related to *The Role of Vitamin D as an Immunomodulator* that have been announced in our Research Topic. It covers a compilation of six papers that were given precedence for publication within the topic by diverse research teams.

The Carlberg team has been able to gain knowledge on how vitamin D_3 supplementation affects immunity by examining the transcriptome of peripheral blood mononuclear cells (PBMCs) when stimulated with either lipopolysaccharide (LPS) and β-glucan (BG), both in the presence or absence of 1,25$(OH)_2D_3$. Their findings point out that while 1,25$(OH)_2D_3$ does affect immune responses, its effect is largely dependent on when it is administered regarding a real-life situation where vitamin D_3 has been supplemented after, before or during an infection. This evidence should be beneficial to clinicians as this indicates that timing plays a huge role in considering vitamin D_3 supplementation. Additionally, the findings of Carlberg and colleagues provide further proof for the role of vitamin D_3 in supporting a healthy immune system and could give insight into when and how it can be used to treat or prevent certain illnesses through altering functional consequences of immune challenges. These results should have substantial implications for medical practice, thus warranting more studies to support them.

The human blood transcriptome is an important factor in the understanding of immune system health and function. Despite this, there has been a lack of research into how vitamin D_2 and D_3 supplementation affects it. Durrant et al. have addressed this gap by conducting an extensive study on the effects of physiological doses of these vitamins on the human blood transcriptome using microarrays. They sought to gain greater insight into this question by extending an earlier study exploring changes in the blood transcriptome of healthy European and South Asian women who were given daily doses of either vitamin D_2 or D_3 over 12 weeks during wintertime in the UK. Their research provides valuable insight into how the two forms of vitamin D can affect gene expression within individuals over time, which may have implications for the treatment of a variety of conditions, including influence of ethnic background. Their study provides compelling evidence for the differential effects of vitamin D_2 and D_3 on gene expression, including gene expression associated with type I and type II interferon activity, and highlights potential differences between ethnicities. The results suggest that further research is needed to determine the physiological roles of each form of vitamin D, as well as their effects on gene expression among different populations. This ground-breaking research offers important insights into the molecular mechanisms underpinning the physiological roles of each form of vitamin D, highlighting potential differences between them. This study is particularly timely given increasing rates of both deficiency and supplementation with either or both forms of vitamin D across many populations worldwide. The findings have important implications for understanding how these two forms may interact differently at a cellular level to modulate immune responses, potentially leading to

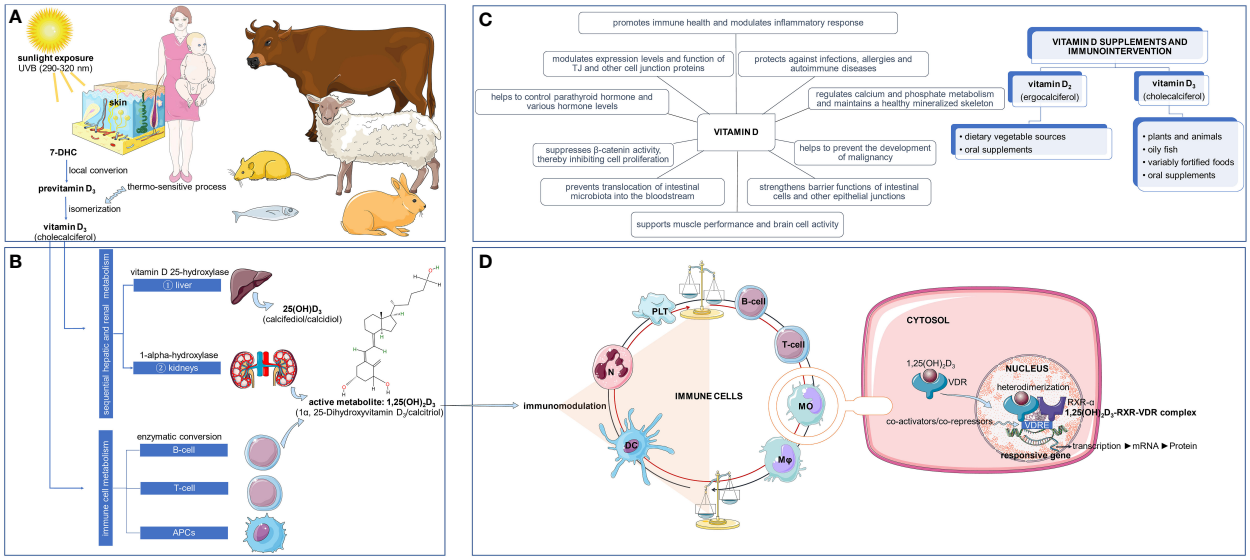

FIGURE 1
The complexity of vitamin D3: synthesis, metabolism, mechanism of action, and immunointervention. The bioactive form of vitamin D_3 plays an important role in the modulation of immune system. The skin of humans and some animals synthesize vitamin D_3 when exposed to ultraviolet B (UVB) radiation (290–320 nm) by acting on 7-dehydrocholesterol (7DHC) **(A)**. After undergoing enzymatic processes in the liver and kidneys, vitamin D_3 is then metabolized into its active form, $1,25(OH)_2D_3$ (1α, 25-Dihydroxyvitamin D_3/calcitriol) by vitamin D 25-hydroxylase and 1-alpha-hydroxylase, respectively. Some immune cells, including B-cells, T-cells and antigen presenting cells (APCs), are also able to transform vitamin D_3 into its active metabolite through a process of enzymatic conversion. **(B)**. Not only does vitamin D_3 perform many vital roles, it has been shown to possess powerful effects that can control immune function, allowing it to be utilized in immunologic interventions to keep the immune system functioning optimally and to correct any existing vitamin D deficiencies (C). The immune effects of vitamin D_3 are ensured by its active metabolite, i.e., $1,25(OH)_2D_3$. Acting upon specific immune cells, $1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) in the cytoplasm and then heterodimerizes with the retinoid X receptor-a (RXR-a) in the nucleus, forming the $1,25(OH)_2D_3$-RXR-VDR complex which attaches to vitamin D response elements (VDRE) on the DNA **(D)**. DC, dendritic cell; MO, monocyte; Mφ, macrophage; N, neutrophil; PLT, platelet; TJ, tight junction. Some illustrations were sourced from the Servier Medical Art platform.

different outcomes when considering disease susceptibility, severity or treatment efficacy. In conclusion, this work serves as an essential foundation upon which to build further research into the cellular and molecular mechanisms by which vitamin D_2 and D_3 differentially influence gene expression, ultimately impacting on immune responses. It is clear that a greater understanding of this area is needed to inform clinical management strategies for individuals at risk of deficiency or requiring supplementation. An improved comprehension of how these two forms may interact differently could lead to more effective treatments and interventions in many diseases associated with dysregulation of the immune system.

The intricate relationship between exercise and immunity is one that has been explored by scientists for many years. It is now becoming increasingly clear that physical activity, intensity of effort, as well as dietary factors like vitamin D can all play a role in modulating our immune system. Thanks to a narrative review, Crescioli highlights how vitamin D shapes human immunity through its effects on both the immune system and skeletal muscle in athletes, who are particularly exposed to oxidative stress and inflammation due to intense training. She highlights the pros and cons associated with its immunomodulatory properties and outlines future research directions for further investigation into this important topic. Therefore, several aspects have been reported, including more specifically effort intensity effect on immunity, vitamin D-induced regulation of skeletal muscle immunity, and vitamin D's effect on muscular immunity, etc. Overall, physical activity should be monitored closely when training at high intensities; additionally, athletes should strive for adequate levels of vitamin D intake as well as regular sun exposure in order to maintain a healthy immune system. Further research is needed to better understand the relationship between exercise, vitamin D, and immunity; however, some important findings should be considered for developing new and more adequate training regimens for athletes.

It has been established that deep vein thrombosis (DVT) is a major health issue around the world. Risk factors for DVT include age, extended periods of immobility or being confined to a wheelchair due to medical issues such as cancer and heart disease, hormone replacement therapy, post-surgery complications, obesity, smoking and taking medications containing estrogen. People who fly often may also be at an increased risk since they spend long stretches without moving. Treatment usually involves anticoagulant drugs which prevent clotting but can have serious side effects. There is now evidence that vitamin D plays an important role in preventing DVT; people with lower levels of vitamin D are more likely to experience it. This inspired Tao et al. to investigate whether the gender gap in DVT and vitamin D levels might explain why female stroke patients have a greater chance of developing DVT due to their lower vitamin D levels. Their research has found that women are more likely than men to develop DVT, and this could be related to differences in their vitamin D levels. The implications of this study suggest further investigation into whether providing extra

vitamin D through supplementation can reduce the risk of DVT, especially for female stroke patients.

The relationship between vitamin D, the immune system, and the gut microbiota has not been extensively researched yet; therefore, there is not much knowledge on how taking vitamin D supplements impacts intestinal traits in individuals prone to autoimmune diseases. Martens et al. conducted a study to explore the long-term effects of varying levels of dietary vitamin D supplementation (400 and 800 IU/day) on type 1 diabetes (T1D) development in nonobese diabetic (NOD) mice, as well as its link with $25(OH)D_3$ concentration levels and their impact on peripheral/gut immunity, exploring specifically $FoxP3^+$ Treg cells, $CD39^+CD73^+$ T-cells, IL-10-secreting T-cells, and Tr1 cells, intestinal barrier function, and gut microbiota composition. Results showed that the 800 IU/day dose was associated with delayed disease onset; significantly decreased incidence of T1D; increased rate of Treg cells in various lymphoid organs; and a modified intestinal microbiota signature, with an increase in *Ruminiclostridium_9* and decrease of *Marvinbryantia* at the genus level. Despite this research, more studies are needed to gain further knowledge on how taking vitamin D supplements impacts intestinal traits in people prone to T1D and other autoimmune diseases.

Although vitamin D supplementation has been shown to have potential anti-inflammatory effects by reducing platelet-mediated inflammation, its effects on platelet-mediated inflammation in patients with type 2 diabetes (T2D) have yet to be sufficiently studied. Thus, Johny et al. conducted a single-center, randomized, double-blind, placebo-controlled study to evaluate the potential impact of vitamin D supplementation on platelet activation and systemic inflammation in patients with T2D who were deficient in vitamin D. The trial was supported by an extensive analysis of clinical and biochemical variables, vitamin D binding protein (VDBP), immunome profiling, platelet–immune cell aggregate, oxidative and nitrosative stress biomarkers as well as pro- and anti-inflammatory cytokines and chemokines. The results suggest that supplementing vitamin D may be beneficial for T2D patients by alleviating oxidative stress and reducing platelet-mediated inflammation - thereby potentially slowing down the progression of the disease and decreasing cardiovascular risk.

Conclusions

In conclusion, the current Research Topic yielded important knowledge regarding the complexity of the connection between vitamin D, human health and immunity. It has highlighted that vitamin D can have a wide range of impacts, including influencing gene expression, modulating immune response, inflammation, oxidative stress, and the gut microbiota signature. These findings suggest that vitamin D supplementation may be beneficial in the treatment or prevention of a variety of illnesses and conditions. Nevertheless, further research into the molecular and cellular mechanisms of vitamin D is warranted to substantiate these claims and to further explore the intricate relationship between vitamin D and human immunity in health and disease.

Author contributions

MA made a significant impact on the progression and presentation of the Research Topic, including actively aiding in the writing of the text, preparing the manuscript, designing the Editorials' accompanying Figure, and providing analyses and conclusions featured in the articles included in this Research Topic. FM was instrumental in the formation and organization of the Research Topic, aiding in the writing of the text and the preparation of the manuscript. CT-B conducted thorough research on the topic and supplied key concepts for the manuscript, while also supplying helpful data to the Research Topic proposal. All the authors were actively involved in the editing of the articles submitted for publication.

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Conflict of interest

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

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The Role of Vitamin D in the Relationship Between Gender and Deep Vein Thrombosis Among Stroke Patients

Jiejie Tao[1†], *Feiling Lou*[1†] *and Yuntao Liu*[2*]

[1] Department of Radiology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [2] Department of Neurology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

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Edited by:
Chafia Touil-Boukoffa,
University of Science and Technology Houari Boumediene, Algeria

Reviewed by:
Rachida Raache,
University of Science and Technology Houari Boumediene, Algeria
Houda Belguendouz,
University of Science and Technology Houari Boumediene, Algeria
Hanane Touil,
Columbia University Irving Medical Center, United States

***Correspondence:**
Yuntao Liu
18267850815@163.com

[†] These authors have contributed equally to this work and share first authorship

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Introduction: Accumulating evidence had demonstrated that females had a higher risk of deep vein thrombosis (DVT) than males, but the mechanism was still unknown. Vitamin D was found to play an essential role in DVT, and gender may influence the serum vitamin D levels. This study aimed to explore whether vitamin D played a role in the gender difference in DVT.

Materials and Methods: A total of 444 patients with acute stroke were recruited, which were divided into the DVT group ($n = 222$) and the non-DVT group ($n = 222$). Serum vitamin D levels were measured after admission and were split into three categories, including deficiency (<50 nmol/L), insufficiency (52.5–72.5 nmol/L), and sufficiency (more than 75 nmol/L). Hierarchical regression analysis was adopted to analyze the relationship between gender and DVT, controlling the confounding factors.

Results: Females showed a higher proportion of DVT than males (60.7 vs. 42.5%, $p < 0.001$), and lower serum vitamin D levels than males (53.44 ± 16.45 vs. 69.43 ± 23.14, $p < 0.001$). Moreover, serum vitamin D levels were lower in the DVT group than in the non-DVT group (59.44 ± 19.61 vs. 66.24 ± 23.86, $p < 0.001$). Besides, the DVT group showed a lower proportion of vitamin D sufficiency than the non-DVT group (21.2 vs. 32.9%, $p < 0.05$). Hierarchical regression analysis showed that females had 2.083-fold ($p < 0.001$, unadjusted model) and 1.413-fold ($p = 0.155$, adjusted model) risk to develop DVT. In addition, the sufficiency status of vitamin D showed an independent protective effect on DVT (unadjusted model OR, 0.504, $p = 0.004$; adjusted model OR, 0.686, $p = 0.011$).

Conclusion: Females had a higher risk of DVT than males, and vitamin D may play an essential role in this relationship. Further studies are needed to explore whether vitamin D supplementation could reduce DVT risk in stroke patients, especially females.

Keywords: stroke, deep venous thrombosis, gender, vitamin D, seniors

INTRODUCTION

Venous thromboembolic disease (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE) (1, 2), affects nearly 1 per 1,000 individuals annually (3, 4) and has become a significant issue that threatens public health in China (5). DVT was prevalent in stroke patients, occurring in nearly 80% of those who did not receive preventive treatments (6). Without any prevention, DVT would happen in 2 to 7 days after stroke onset (7, 8).

The risk of DVT has been found to vary by gender and age. Females under 50 years old had a decreased incidence, whereas over 65 years old had a higher incidence of DVT (4). Moreover, previous studies in stroke patients also found that females had a higher risk of DVT than males (9, 10). The mechanisms underlying the increased risk of DVT in females

TABLE 1 | Characteristics of all stroke patients.

Characteristic	Total (*N* = 444)	Non-DVT group (*N* = 222)	DVT group (*N* = 222)	Statistic	*P*-value
Demographic characteristics					
Age (years), mean ± SD	70.80 ± 9.54	70.46 ± 9.67	71.13 ± 9.42	−0.736	0.462
Females, *n* (%)	183 (41.22)	72 (32.43)	111 (50.00)	13.423	<0.001***
BMI (kg/m2), mean ± SD	23.56 ± 2.53	23.49 ± 2.89	23.63 ± 2.12	−0.578	0.564
Vital signs					
Temperature (°C)	37.05 ± 1.04	36.90 ± 1.36	37.20 ± 0.52	−3.020	0.003*
Respiratory rate (breaths per min)	20.00 (19.00, 20.00)	20.00 (19.00, 20.00)	20.00 (19.00, 20.00)	2,4677.500	0.976
Heart rate (beats per min)	77.18 ± 14.73	76.26 ± 14.97	78.11 ± 14.46	−1.326	0.186
SBP (mmHg)	156.10 ± 24.04	156.92 ± 23.01	155.28 ± 25.05	0.718	0.473
DBP (mmHg)	84.84 ± 14.00	85.68 ± 14.17	83.99 ± 13.81	1.275	0.203
Risk factors for stroke, *n* (%)					
History of hypertension	307 (69.14)	152 (68.47)	155 (69.82)	0.042	0.837
History of diabetes mellitus	100 (22.52)	47 (21.17)	53 (23.87)	0.323	0.570
CAD	44 (9.91)	27 (12.16)	17 (7.66)	2.043	0.153
Current smoking	138 (31.08)	74 (33.33)	64 (28.83)	0.852	0.356
Current drinking	121 (27.25)	65 (29.28)	56 (25.23)	0.727	0.394
Atrial fibrillation	71 (15.99)	37 (16.67)	34 (15.32)	0.067	0.796
Laboratory results					
WBC ($\times 10^9$/L), mean ± SD	7.88 ± 2.71	8.02 ± 2.86	7.74 ± 2.56	1.100	0.230
RBC ($\times 10^{12}$/L), mean ± SD	4.37 ± 0.48	4.42 ± 0.49	4.33 ± 0.47	2.033	0.027*
HB (g/L), mean ± SD	133.70 ± 15.14	135.94 ± 13.78	131.46 ± 16.11	3.150	0.002**
PLT ($\times 10^9$/L), mean ± SD	218.04 ± 69.54	217.43 ± 69.17	218.65 ± 70.06	−0.185	0.851
PT(s), mean ± SD	13.93 ± 1.43	14.06 ± 1.59	13.79 ± 1.23	2.006	0.046*
APTT(s), mean ± SD	37.42 ± 4.73	37.67 ± 4.63	37.18 ± 4.83	1.099	0.273
FIB(g/L), mean ± SD	3.51 ± 1.17	3.33 ± 1.09	3.70 ± 1.22	−3.335	0.001**
D-dimmer, median (IQR)	1.72 (0.19–3.25)	1.28 (0, 2.98)	2.24 (0.54–3.94)	19075	0.001**
EGFR (ml/ min/l.73m2), mean ± SD	83.76 ± 16.90	83.76 ± 15.78	83.77 ± 17.98	−0.099	0.921
Vitamin D (nmol/L), mean ± SD	62.84 ± 22.08	66.24 ± 23.86	59.44 ± 19.61	3.276	0.001**
Clinical characteristics					
Intracerebral hemorrhage (%)	153 (34.46)	80 (36.04)	73 (32.88)	0.359	0.485
NIHSS score, median (IQR)	5.00 (2.00, 10.00)	5.00 (2.00, 10.00)	6.00 (2.00, 10.00)	23852.500	0.558
Lower limb NIHSS score ≥ 2, median (%)	211 (47.55)	94 (42.30)	117 (52.70)	4.371	0.029*
NIHSS score at admission ≥ 11	92 (20.7)	45 (20.30)	47 (21.2)	0.055	0.815
Padua score, median (IQR)	4.00 (4.00, 5.00)	4.00 (3.25, 5.00)	4.00 (4.00, 5.00)	2,1498.000	0.015*
Infection (%)	71 (16.00)	45 (20.30)	26 (11.70)	6.052	0.014*

DVT, deep vein thrombosis; BMI, body mass index; SBP, systolic pressure; DBP, diastolic pressure; CAD, Coronary Heart Disease; WBC, white blood cell count; RBC, red blood cell count; HB, hemoglobin value; PLT, platelet count; PT, Prothrombin Time; APTT, activated partial thromboplastin time; FIB, fibrinogen; EGFR, estimated glomerular filtration rate; NIHSS, National Institute of Health stroke scale; SD, standard deviation; IQR, interquartile.

****$p < 0.001$, **$p < 0.01$, *$p < 0.05$.*

Values were presented as the mean ± standard deviation (SD) or median (median± interquartile range, IQR) for continuous variables, and categorical variables were indicated as numbers (percentages). Student t-tests and Mann-Whitney U-tests were used according to data distribution. Chi-squared tests were used for categorical variables.

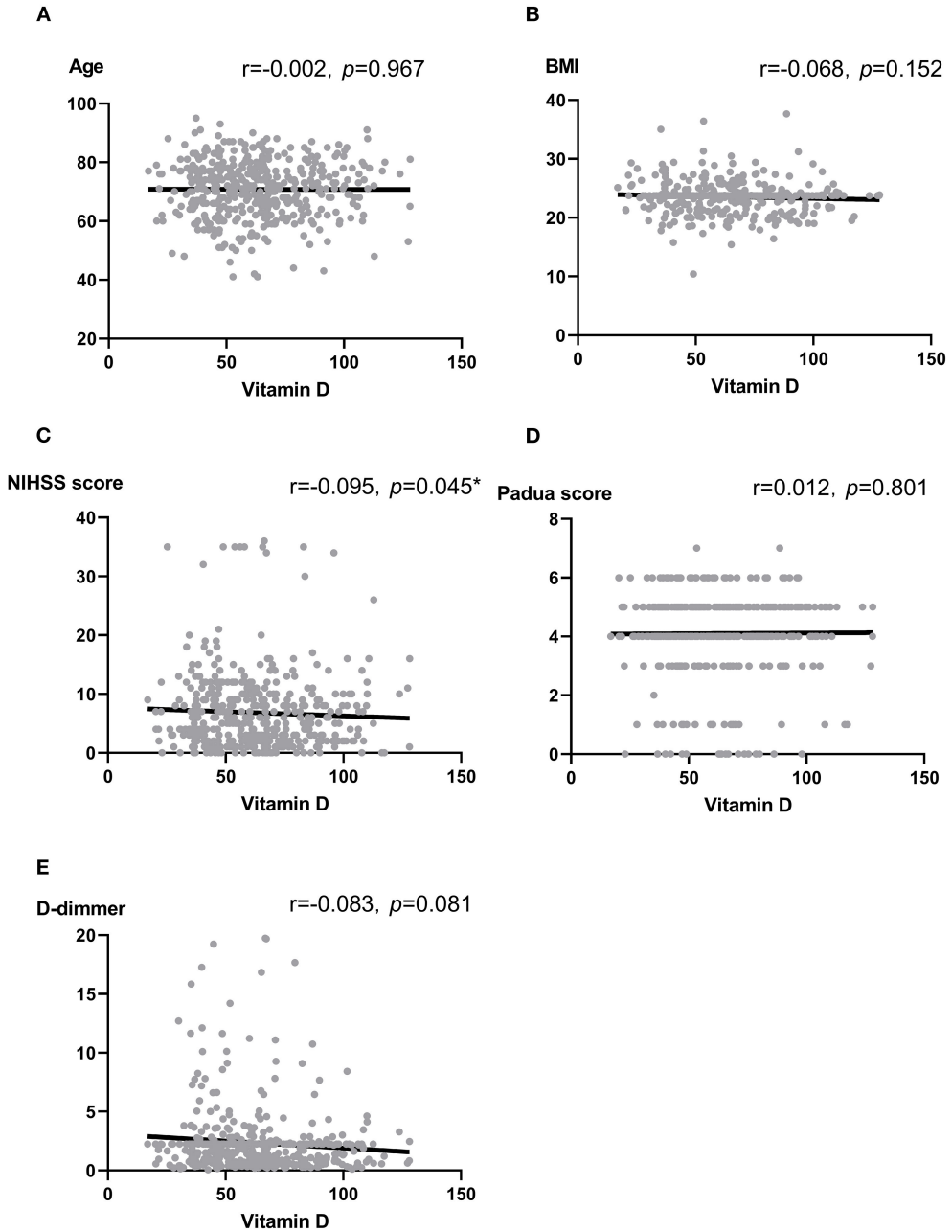

FIGURE 1 | (A–E) Scatter plots: Association between vitamin D and Age **(A)**, BMI **(B)**, NIHSS score **(C)**, Padua score **(D)** as well as D-dimmer **(E)**. Note: r, correlation coefficient; BMI, body mass index; NIHSS, National Institute of Health stroke scale. Pearson correlation and Spearman analysis were used to evaluate the correlation according to the type of data. *$p < 0.05$.

may be as follows: pregnancy (11), hormone replacement therapy (12), estrogen antagonist therapy (13) and the antiphospholipid antibody syndrome (14), hormone loss during menopause (15), oral contraceptives (OCPs) (16). However, most of these factors were not common among stroke patients in China due to the differences such as age, culture, race, and disease (17). Therefore, further studies were needed to identify why female stroke patients had a higher risk of DVT.

Vitamin D is a fat-soluble vitamin involved in calcium and phosphorus metabolism to maintain bone health (18). The deficiency of vitamin D is a worldwide health issue (19, 20). Low vitamin D levels may increase the risk of stroke (21) and lead to a worse prognosis of stroke patients (22). It was worth noting that vitamin D was also crucial in developing DVT in stroke patients, and a small sample study showed that vitamin D deficiency showed a 4.683-fold risk for DVT (23). While the

TABLE 2 | Characteristics of the patients categorized by gender.

Characteristic	Total ($N = 444$)	Female group ($N = 183$)	Male group ($N = 261$)	Statistic	*P*-value
Demographic characteristics					
Age (years), mean ± SD	70.80 ± 9.54	70.97 ± 8.93	70.67 ± 9.96	0.33	0.741
BMI (kg/m2), mean ± SD	23.56 ± 2.53	23.60 ± 2.48	23.53 ± 2.58	0.258	0.796
Vital signs					
Temperature (°C)	37.05 ± 1.04	37.20 ± 0.45	36.94 ± 1.29	2.956	0.003**
Respiratory rate (breaths per min)	20.00 (19.00, 20.00)	20.00 (19.00, 20.00)	20.00 (19.00, 20.00)	2,4715	0.471
Heart rate (beats per min)	77.18 ± 14.73	80.52 ± 15.43	74.84 ± 13.77	3.994	<0.001***
SBP (mmHg)	156.10 ± 24.04	155.66 ± 24.99	156.41 ± 23.40	−0.321	0.749
DBP (mmHg)	84.84 ± 14.00	83.97 ± 14.19	85.45 ± 13.86	−1.093	0.275
Risk factors for stroke, *n* (%)					
History of hypertension	307 (69.14)	129 (70.49)	178 (68.20)	0.168	0.681
History of diabetes mellitus	100 (22.52)	43 (23.50)	57 (21.84)	0.088	0.767
CAD	44 (9.91)	25 (13.66)	19 (7.28)	4.218	0.04
Atrial fibrillation	71 (15.99)	36 (19.67)	35 (13.41)	2.691	0.101
Laboratory results					
WBC ($\times 10^9$/L), mean ± SD	7.88 ± 2.71	7.65 ± 2.61	8.04 ± 2.78	−1.505	0.133
PLT ($\times 10^9$/L), mean ± SD	218.04 ± 69.54	232.11 ± 82.93	208.18 ± 56.47	3.391	<0.001***
PT(s), mean ± SD	13.93 ± 1.43	13.86 ± 1.76	13.97 ± 1.13	−0.746	0.456
APTT(s), mean ± SD	37.42 ± 4.73	36.51 ± 4.84	38.06 ± 4.55	−3.404	<0.001***
FIB (g/L), mean ± SD	3.51 ± 1.17	3.56 ± 1.04	3.48 ± 1.25	0.685	0.494
D-dimmer (mg/L), median (IQR)	1.72 (0.19,3.25)	2.24 (0.46, 4.02)	1.44 (0,3.06)	20,809	0.02*
EGFR (ml/min/l.73m2), mean ± SD	83.76 ± 16.90	84.95 ± 15.01	82.93 ± 18.08	1.279	0.202
Vitamin D (nmol/L), mean ± SD	62.84 ± 22.08	53.44 ± 16.45	69.43 ± 23.14	−8.511	<0.001***
Vitamin D status				54.049	<0.001***
Sufficiency, *n* (%)	120 (27.00)	17 (9.30)	103 (39.50)		<0.05*
Insufficiency, *n* (%)	180 (40.50)	83 (45.40)	97 (37.20)		>0.05
Deficiency, *n* (%)	144 (32.40)	83 (45.40)	61 (23.40)		<0.05*
Clinical characteristics					
Intracerebral hemorrhage (%)	153 (34.46)	56 (30.60)	97 (37.16)	1.772	0.183
NIHSS score, median (IQR)	5.00 (2.00, 10.00)	6.00 (2.00, 11.00)	5.00 (2.00, 9.00)	25,526	0.215
Lower limb NIHSS score ≥ 2, median (%)	211 (47.50)	106 (40.60)	105 (57.4)	12.123	<0.001***
NIHSS score at admission ≥ 11	92 (20.7)	49 (28.60)	43 (16.50)	6.949	0.008**
Padua score, median (IQR)	4.00 (4.00, 5.00)	4.00 (4.00, 5.00)	4.00 (4.00, 5.00)	25,804	0.129
Infection (%)	71 (16.00)	32 (17.50)	39 (14.90)	0.518	0.472
The proportion of IDDVT (%)	222 (50.00)	111 (60.70)	111 (42.50)	14.139	<0.001***

DVT, deep vein thrombosis; BMI, body mass index; CAD, Coronary Heart Disease; WBC, white blood cell count; RBC, red blood cell count; HB, hemoglobin value; PLT, platelet count; PT, Prothrombin Time; APTT, activated partial thromboplastin time; FIB, fibrinogen; EGFR, estimated glomerular filtration rate; NIHSS, National Institute of Health stroke scale; SD, standard deviation; IQR, interquartile.

****$p < 0.001$, **$p < 0.01$, *$p < 0.05$.*

Values were presented as the mean ± standard deviation (SD) or median (median± interquartile range, IQR) for continuous variables, and categorical variables were indicated as numbers (percentages). Student t-tests and Mann-Whitney U-tests were used according to data distribution. Chi-squared tests were used for categorical variables (Bonferroni correction was used if multiple comparisons).

sample size of this study was small ($n = 180$), larger sample size was needed further to identify the relationship between vitamin D and DVT. Besides, previous research had also shown that vitamin D deficiency was more common in elderly and middle-aged females (24, 25), which may be owing to the factors such as gender difference in dietary intakes (26), less sunlight exposure (27), and estrogen loss (28).

Considering the gender difference in DVT and vitamin D levels and the role of vitamin D in DVT, this study assumed that lower vitamin D levels might be why female stroke patients had a higher risk of DVT.

MATERIALS AND METHODS

Study Design

This research was a cross-sectional design. Patients were screened from March 2015–May 2020 in the First Affiliated Hospital of Wenzhou Medical University. The following were the criteria for

inclusion: (1) ages over 40; (2) within seven days of the stroke onset; (3) stroke (ischemic or intracranial hemorrhage) validated by computed tomography (CT) and magnetic resonance imaging (MRI). The following were the criteria for exclusion: (1) Patients with a history of DVT; (2) Patients taking vitamin D, calcium supplements; (3) Patients with active cancer or other serious medical diseases. (4) Patients taking estrogen, OCPs, estrogen antagonists, or anticoagulant therapy before admission. (5) pregnancy. (6) antiphospholipid antibody syndrome.

Ultimately, 222 patients with DVT were admitted, and 222 age-matched patients without DVT were also admitted. This study was agreed by the Medical Ethics Committee of First Affiliated Hospital of Wenzhou Medical University and abide by the Declaration of Helsinki's principles.

Data Collection

Data including demographic and clinical characteristics were collected. All blood samples were obtained between 6:00 and 8:00 a.m., after a night of fasting within 24 h of admission.

We selected serum 25-hydroxyvitamin D [25 (OH) D] as the indicator for vitamin D levels because it is the best indicator of its status (29). The fasting venous blood samples were obtained from patients within 24 h after admission and sent to the clinical laboratory within 4 h. The blood samples were centrifuged at 3,000 rpm for 10 min, and the supernatant was taken for further evaluation. The serum 25 (OH) D levels were measured by a competitive electrochemiluminescence protein binding assay (Cobas e602.Roche Diagnostics, Germany) in the lab of our hospital. The intraassay variation was between 7 and 10%, and the level over 75 nmol/l is considered vitamin D sufficiency in our hospital.

In the present study, vitamin D deficiency was specified as a 25 (OH) D of <20 ng/ml (50 nmol/L), insufficiency as 21–29 ng/ml (52.5–72.5 nmol/L), and sufficiency as more than 30 ng/ml (75 nmol/L) referred to the Endocrine Society's Practice Guidelines on vitamin D (30, 31).

Diagnostic Criteria for DVT

Patients were examined using color Doppler ultrasonography (HDI 5,000 system with a 3–7 MHz linear array transducer, Philips ATL, Bothell, WA, USA) within seven days after admission.

Statistical Analysis

Baseline demographic and clinical variables were compared between the groups. For a normal distribution test, the Kolmogorov-Smirnov test was used. The Mann-Whitney test was used for non-normally distributed variables, shown as the median (quartile). The normally distributed variables were represented by the mean and standard deviation (SD), analyzed by the student's *t*-test. Percentage and numbers were expressed for categorical variables and analyzed with chi-squared tests. The correlation analysis was conducted by the Pearson or Spearman rank analysis. Hierarchical regression analysis was further adopted to examine the relationship between gender and DVT, controlling the confounding factors. The confounding factors were categorized by accepted and validated classification criteria (such as WBC count, FIB, D-dimmer). Other variables such as PT, APTT were categorized according to the mean or median of the present values. SPSS 20.0 was used to conduct all analyses (IBM, SPSS, and Chicago, IL). Based on a two-sided test, $p < 0.05$ was regarded as statistically significant in all studies.

RESULTS

Characteristics Between DVT and Non-DVT Groups

There was no statistical difference between the two groups regarding age, BMI, vital signs (except for body temperature), and stroke risk factors (all $p > 0.05$). In the DVT group, laboratory indicators including vitamin D levels, red blood cell (RBC), hemoglobin (HB), and Prothrombin Time (PT) were lower than the Non-DVT group (shown in **Table 1**, all $p < 0.05$). Padua score, the proportion of infection, fibrinogen (FIB), and D-dimmer levels were higher in the DVT group (shown in **Table 1**, all $p < 0.05$). Besides, RBC ($r = 0.171$, $p < 0.001$) and HB ($r = 0.221$, $p < 0.001$) were positively associated with vitamin D levels, other biochemistry markers such as WBC, PLT, PT, APTT, FIB, D-dimmer and EGFR were not related to vitamin D (all $p > 0.05$).

The DVT group showed a higher percentage of females than the non-DVT group [(50.00%) vs. (32.43%), $p < 0.001$] (shown in **Table 1**). Patients with vitamin D sufficiency in the DVT group were fewer than the non-DVT [(21.2%) vs. (32.9%), $p < 0.05$]. In contrast, the proportion of insufficiency [(45.5%) vs. (35.6%), $p < 0.05$] and deficiency [(33.3%) vs. (31.5%), $p > 0.05$] were higher in the DVT group (though deficiency group did not reach statistically significance) (shown in **Table 4**).

Correlation Between Vitamin D and Other Risk Factors

We analyzed the correlation between vitamin D and some thrombotic risk factors reported in stroke patients (17, 32). Vitamin D was not associated with age, BMI, Padua score, and D-dimmer (all $p > 0.05$). In contrast, there was a marginally significant association between vitamin D and National Institute of Health stroke scale (NIHSS) score at the admission ($r = -0.095$, $p = 0.045$) (**Figure 1**).

Characteristics Between Male and Female

As was shown in **Table 2**, variables such as temperature and heart rate were higher in females (all $p < 0.01$). Moreover, blood biomarkers of the females, such as APTT levels, were lower while PLT and D-dimmer levels were higher than the males (all $p < 0.05$). The proportions of NIHSS score ≥11 and lower limb NIHSS score ≥2 were also higher among the females ($p < 0.01$) (**Table 2**). It was worth noting that females showed lower levels

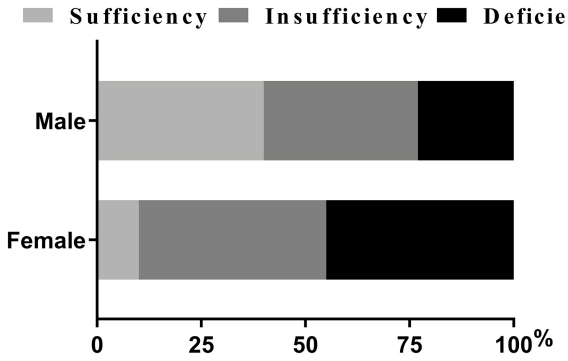

FIGURE 2 | Percentage of patients with vitamin D status (%). Chi-squared tests and *post hoc* Bonferroni corrections for multiple comparisons were used for comparison between genders. Our data showed there were statistical differences in insufficiency and deficiency groups when categorized by genders.

of vitamin D and a higher proportion of DVT than the males (all $p < 0.001$) (**Table 2**). Besides, females had a higher proportion of deficiency [(45.40%) vs. (23.40%), $p < 0.05$] and insufficiency of Vitamin D status [(45.40%) vs. (37.20%), $p > 0.05$] than males, and had a lower proportion of sufficiency [(9.30%) vs. (39.50), $p < 0.05$] (**Figure 2**; **Table 2**).

Gender, Vitamin D and DVT

We used hierarchical regression analysis to examine the role of vitamin D in gender and DVT (**Tables 3**, **4**). Firstly, univariate binary regression suggested that female gender, infection, lower limb NIHSS score, Padua score, HB, PT, FIB, D-dimmer, and vitamin D status was found to be correlated with the presence of DVT (all $p < 0.05$) (**Table 4**). Then, multivariate-adjusted regression was conducted. In Model 1–3 (**Table 3**), the association between gender and DVT remained significant after adjusting the confounding factors (all $p < 0.05$) (**Table 3**). However, when categorized vitamin D status was added in model 4, the association between gender and DVT became insignificant ($p = 0.155$). The established model was shown in **Table 5**. Moreover, we also examined the interaction effect of DVT and gender on vitamin D levels using Two-way ANOVA analysis. However, the interaction effect was insignificant ($F = 1.430$, $p = 0.232$).

TABLE 3 | Binary logistic models to examine the effect of gender on DVT.

	OR (95%CI)	*P*-value
Female		
Unadjusted model	2.083 (1.418–3.062)	<0.001***
Model 1	2.069 (1.401–3.055)	<0.001***
Model 2	2.055 (1.377–3.068)	<0.001**
Model 3	1.582 (1.010–2.479)	0.045*
Model 4	1.413 (0.878–2.273)	0.155

Take insufficiency of vitamin D group as the reference.
Model 1 included age ≥ 65 years, temperature ≥ 38°C, intracerebral hemorrhage, and infection.
Model 2 included model 1 and added NIHSS score at admission ≥ 11, Lower limb NIHSS score ≥ 2, BMI ≥ 25, and Padua score ≥ 4.
Model 3 included model 2 and added RBC (categorized), HB (categorized), PLT (categorized), PT (categorized), APTT (categorized), FIB (categorized) and D-dimmer (categorized).
Model 4 included model 3 and added vitamin D (categorized).
DVT, deep vein thrombosis; BMI, body mass index; CAD, Coronary Heart Disease; WBC, white blood cell count; RBC, red blood cell count; HB, hemoglobin value; PLT, platelet count; PT, Prothrombin Time; APTT, activated partial thromboplastin time; FIB, fibrinogen; EGFR, estimated glomerular filtration rate; NIHSS, National Institute of Health stroke scale; OR, odds ratios; CI, confidence intervals.
****p < 0.001, **p < 0.01, *p < 0.05.*

DISCUSSION

As far as we know, this was the first research to explore the three-way association among gender, vitamin D, and DVT. There were three main findings in this study. Firstly, females had lower vitamin D levels and a higher risk of DVT than males. Secondly, higher vitamin D levels were associated with a lower risk of DVT. Thirdly, vitamin D may act as a mediating role between gender and DVT.

Accumulating studies had found that elderly females had a higher risk of DVT than males (4), which was in line with our research. In general populations, risk factors such as pregnancy or estrogen antagonist therapy may account for the gender difference in DVT (14), but these factors were not common among elder stroke patients (17). The present study observed that vitamin D might play an essential role between gender and DVT.

Previous research has shown that vitamin D deficiency was more common in elderly, middle-aged, and perimenopausal females (24, 25, 33). According to a retrospective cohort study, as high as 43.9% of elderly females had vitamin D insufficiency (34). Decreased serum levels of vitamin D led to reduced calcium absorption and osteoporosis, which is prevalent among perimenopausal women (33). The previous study has reported the gender difference in dietary intakes of vitamin D. Women tended to intake less amount of vitamin D in wintertime and autumn in a nationwide nutritional survey (26). In an animal experiment, adult ovariectomized female rats had lower recovery levels of 1.25 $(OH)_2$ D after 25 (OH) D administration than intact ovary rats, and beta-estradiol administration could restore this decline (28), indicating the critical role estrogen plays in vitamin D transformation. In China, females were more reluctant to be exposed to sunshine and participate in outdoor activities (35), which may be one of the primary causes of hypovitaminosis D. In addition, the gender difference in the amount of body fat may be another possible cause. A previous study demonstrated that vitamin D deficiency was associated with a higher percentage of fat mass in females (36). Given above, the following reasons may partially explain the high prevalence of vitamin D deficiency in elderly females: difference in dietary intake, estrogen deficiency, lower sunlight exposure, and the higher amount of fat in females.

This study also found that vitamin D played a protective role in DVT among stroke patients. The potential role of vitamin D in thrombolytic diseases has been reported formerly. The possible mechanism was that Vitamin D might exert an anti-inflammatory effect through IL-10 receptor expression induction

TABLE 4 | Stratified variables of stroke patients and binary logistic regression model testing predicting the value of variables with IDDVT.

	DVT patients	Non-DVT patients	Unadjusted model		Adjusted model 4	
	(*N* = 222)	(*N* = 222)	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (years)						
<65, *n* (%)	54 (24.30)	53 (23.90)	1 (ref)		1 (ref)	
≥65, *n* (%)	168 (75.70)	169 (76.10)	0.976 (0.632–1.507)	0.912	0.765(0.461–1.270)	0.3
Gender						
Male, *n* (%)	111 (50.00)	150 (67.60)[a]	1 (ref)		1 (ref)	
Female, *n* (%)	111 (50.00)	72 (32.43)[a]	2.083 (1.418–3.062)	<0.001***	1.413 (0.878–2.273)	0.155
Body temperature (°C)						
<38°C, *n* (%)	209 (94.1)	217 (97.7)	1 (ref)		1 (ref)	
≥38°C, *n* (%)	13 (5.9)	5 (2.3)	2.700 (0.946–7.705)	0.063	2.289 (0.723–7.252)	0.159
Stroke type						
Ischemic stroke, *n* (%)	149 (67.10)	142 (64.00)	1 (ref)		1 (ref)	
Intracerebral hemorrhage, *n* (%)	73 (32.90)	80 (36.00)	0.870 (0.588–1.287)	0.485	1.493 (0.805–2.768)	0.203
Infection						
Yes, *n* (%)	45 (20.30)	26 (11.70)[a]	1.917 (1.135–3.236)	0.015*	1.485 (0.807–2.736)	0.204
No, *n* (%)	177 (79.70)	196 (88.30)[a]	1 (ref)		1 (ref)	
NIHSS score at admission						
<11, *n* (%)	175 (78.8)	177 (79.70)	1 (ref)		1 (ref)	
≥11, *n* (%)	47 (21.20)	45 (20.30)	1.056 (0.667–1.672)	0.815	0.762 (0.433–1.343)	0.347
Lower limb NIHSS score						
<2, *n* (%)	105 (47.30)	128 (57.70)[a]	1 (ref)		1 (ref)	
≥2, *n* (%)	117 (57.70)	94 (42.30)[a]	1.517 (1.043–2.207)	0.029*	1.246 (0.782–1.984)	0.355
Padua score						
<4, *n* (%)	31 (14.00)	56 (25.20)[a]	1 (ref)		1 (ref)	
≥4, *n* (%)	191 (86.00)	166 (74.80)[a]	2.079 (1.279–3.378)	0.003**	2.704 (1.261–5.796)	0.011*
RBC ($\times 10^{12}$/L)						
<4.375, *n* (%)	124 (55.90)	107 (48.20)	1 (ref)		1 (ref)	
≥4.375, *n* (%)	98 (44.10)	115 (51.80)	0.735 (0.506–1.068)	0.107	1.229 (0.686–2.202)	0.488
WBC ($\times 10^{9}$/L)						
<10, *n* (%)	190 (85.60)	185 (83.30)	1 (ref)		1 (ref)	
≥10, *n* (%)	32 (14.40)	37 (16.70)	0.842 (0.503–1.409)	0.513	0.702 (0.385–1.281)	0.249
H.B. (g/L)						
<135, *n* (%)	103 (63.50)	141 (46.50)[a]	1 (ref)		1 (ref)	
≥135, *n* (%)	119 (53.60)	81 (36.50)[a]	0.497 (0.340–0.727)	<0.001***	0.598 (0.324–1.103)	0.100
PLT ($\times 10^{9}$/L)						
<213, *n* (%)	119 (53.60)	124 (55.90)	1 (ref)		1 (ref)	
≥213, *n* (%)	103 (46.40)	98 (44.10)	1.095 (0.75401.592)	0.634	0.957 (0.628–1.458)	0.837
PT (s)						
<13.750, *n* (%)	124 (55.9)	98 (44.1)[a]	1 (ref)		1 (ref)	
≥13.750, *n* (%)	98 (44.1)	124 (55.9)[a]	0.657 (0.448–0.966)	0.014*	0.608 (0.389–0.951)	0.029*
APTT(s)						
<36.600, *n* (%)	110 (49.5)	106 (47.7)	1 (ref)		1 (ref)	
≥36.600, *n* (%)	112 (50.5)	116 (52.3)	0.912 (0.625–1.329)	0.631	0.926 (0.600–1.429)	0.728
FIB (g/L)						
<4, *n* (%)	143 (64.40)	174 (78.40)[a]	1 (ref)		1 (ref)	
≥4, *n* (%)	79 (35.6)	48 (21.60)[a]		0.001**	1.670 (1.027–2.713)	0.039*
D–dimmer (mg/L)						
<0.5, *n* (%)	24 (10.80)	51 (23.00)[a]	1 (ref)		1 (ref)	
≥ 0.5, *n* (%)	198 (89.20)	171 (77.00)[a]	2.461 (1.453–4.166)	0.001**	2.241 (1.242–4.045)	0.007**
Vitamin D status				0.016*		0.033*
Sufficiency, *n* (%)	47 (21.2)	73 (32.9)[a]	0.504 (0.315–0.806)	0.004**	0.507 (0.300–0.858)	0.011*
Insufficiency, *n* (%)	101 (45.5)	79 (35.6)[a]	1 (ref)		1 (ref)	
Deficiency, *n* (%)	74 (33.3)	70 (31.5)	0.827 (0.533–1.284)	0.397	0.686 (0.424–1.111)	0.126

[a]*indicates statistical difference between DVT and Non-DVT patients (Bonferroni correction was used if multiple comparisons).*

DVT, deep vein thrombosis; WBC, white blood cell count; RBC, red blood cell count; HB, hemoglobin value; PLT, platelet count; PT, Prothrombin Time; APTT, activated partial thromboplastin time; FIB, fibrinogen; NIHSS, National Institute of Health stroke scale; OR, odds ratios; CI, confidence intervals.

****p < 0.001, **p < 0.01, *p < 0.05.*

TABLE 5 | Multivariate logistic model of DVT among stroke patients.

Variables	β	OR (95% CI)	*P*-value
Padua score (≥4)	1.001	2.704 (1.261–5.796)	0.011*
PT (≥13.75 s)	−0.489	0.608 (0.389–0.951)	0.029*
FIB (≥4 g/L)	0.524	1.670 (1.027–2.713)	0.039*
D-dimmer (≥0.5 mg/L)	0.804	2.241 (1.242–4.045)	0.007**
Vitamin D status			0.033*
Sufficiency	−0.655	0.507 (0.300–0.858)	0.011*
Insufficiency	Reference	1	Reference
Deficiency	−0.372	0.686 (0.424–1.111)	0.126

DVT, deep vein thrombosis; WBC, white blood cell count; RBC, red blood cell count; HB, hemoglobin value; PLT, platelet count; PT, Prothrombin Time; APTT, activated partial thromboplastin time; FIB, fibrinogen; NIHSS, National Institute of Health stroke scale; β, regression coefficient; OR, odds ratios; CI, confidence intervals.
***p < 0.01, *p < 0.05.*

and suppression of NF-κB activation (37, 38) and alleviate oxidative stress in endothelial cells (39) to reduce the chance of blood clots. Besides, it was also worth noting that vitamin D and its analogs may have antithrombotic properties (40, 41) and was reported to modulate coagulation pathways directly or indirectly (42). A pilot randomized clinical trial on DVT and PE patients with vitamin D deficiency found that warfarin's anticoagulation efficacy was enhanced after vitamin D supplement (43). Evidence from a cell study suggested that in monocytic cells, vitamin D inhibited coagulation by downregulating tissue factor (TF), upgrading thrombomodulin (TM), reducing the effects of tumor necrosis factor, and oxidized low-density lipoprotein to exert the antithrombotic activity (44). Other mechanisms of anticoagulation were as follows: modulation of plasminogen activator inhibitor-1 (PAI-1) and thrombospondin-1 expression in smooth cells (45), downregulation, and modulation of coagulation indicators (highly sensitivity c-reactive protein, tissue factor pathway inhibitor, and TNF-α) (46–48).

Hierarchical regression analysis to examine the role of vitamin D in gender and DVT indicated that females had a higher risk of DVT in the univariate model but tended to be more insignificant after multiple adjustments, especially when vitamin D was added. These results showed that vitamin D had a higher statistical power, which could also be interpreted that vitamin D might act as a mediating factor between DVT and the female gender. We speculated that elderly females had lower levels of vitamin D and thus were more likely to develop DVT after stroke. The underlying mechanism might be the decreased properties in anti-inflammation and anti-thromboembolism due to vitamin D insufficiency/deficiency.

Interestingly, our data did not show that vitamin D deficiency could promote DVT after stroke. Current research on the link between vitamin D and VTE has generated inconsistent results. Several studies found that there was no relationship between VTE and Vitamin D deficiency. One study examined the effect of categorized variables of vitamin D (<44 nmol/l or <30 nmol/l) and failed to show a significantly higher risk of VTE (49). Another prospective study over two decades of follow-up also concluded similar results (50). Whereas, other studies reported the lower levels of vitamin D were correlated with DVT or VTE (23, 51, 52), and a pooled HR estimate of 1.25 (95% CI 1.07–1.45) in a Meta-analysis was calculated (50). The inconsistency could be attributed to the difference in study design, race, sample size, latitude, season, criteria of vitamin D deficiency, and dietary habits. In this study, the undefined correlation between vitamin D deficiency and DVT might be due to the limited sample size and cross-sectional design. Further studies including a larger sample, prospective design, and more covariates (such as season) were needed to explore the relationship between vitamin D deficiency and DVT.

There were some limitations in this study. Firstly, as mentioned above, this cross-sectional study could not draw a causal relationship among gender, vitamin D, and DVT. Secondly, we did not record the fat mass and measured the estrogen levels, which may help us justify the relationship between vitamin D levels and elderly females. Thirdly, we only measured the vitamin D concentration once at admission, and we could not observe its fluctuations in stroke patients during hospitalization. Finally, we have not collected the dietary habits and duration of sunlight exposure of the patients daily, which may influence the vitamin D levels.

In conclusion, females had a higher risk of DVT than males among stroke patients, and vitamin D may play an essential role in this relationship. Further studies are needed to explore whether vitamin D supplementation could reduce DVT risk in stroke patients, especially females.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of First Affiliated Hospital of Wenzhou Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JT: conceptualization, methodology, and formal analysis. FL: data curation and writing—original draft preparation. YL: writing—reviewing and editing and supervision. All authors contributed to the article and approved the submitted version.

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Vitamin D Treatment Sequence Is Critical for Transcriptome Modulation of Immune Challenged Primary Human Cells

*Henna-Riikka Malmberg[1], Andrea Hanel[1], Mari Taipale[1], Sami Heikkinen[1,2] and Carsten Carlberg[1]**

[1] Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland, [2] Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland

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Plateforme d'Expertise Maladies Rares- Hôpitaux Universitaires de Marseille, France

***Correspondence:**
Carsten Carlberg
carsten.carlberg@uef.fi

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Microbe-associated molecular patterns, such as lipopolysaccharide (LPS) and β-glucan (BG), are surrogates of immune challenges like bacterial and fungal infections, respectively. The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), supports the immune system in its fight against infections. This study investigated significant and prominent changes of the transcriptome of human peripheral blood mononuclear cells that immediately after isolation are exposed to 1,25$(OH)_2D_3$-modulated immune challenges over a time frame of 24-48 h. In this *in vitro* study design, most LPS and BG responsive genes are downregulated and their counts are drastically reduced when cells are treated 24 h after, 24 h before or in parallel with 1,25$(OH)_2D_3$. Interestingly, only a 1,25$(OH)_2D_3$ pre-treatment of the LPS challenge results in a majority of upregulated genes. Based on transcriptome-wide data both immune challenges display characteristic differences in responsive genes and their associated pathways, to which the actions of 1,25$(OH)_2D_3$ often oppose. The joined BG/1,25$(OH)_2D_3$ response is less sensitive to treatment sequence than that of LPS/1,25$(OH)_2D_3$. In conclusion, the functional consequences of immune challenges are significantly modulated by 1,25$(OH)_2D_3$ but largely depend on treatment sequence. This may suggest that a sufficient vitamin D status before an infection is more important than vitamin D supplementation afterwards.

Keywords: immune challenge, infection, lipopolysaccharide, β-glucan, PBMCs, vitamin D, transcriptome, responsive genes

INTRODUCTION

After infection or vaccination, cells of the innate immune system, such as monocytes in circulation and macrophages in tissues, show long-term changes in their epigenome, transcriptome and cell physiology (1). This reprogramming of immune cells can be induced by microbe-associated molecular patterns (2), i.e., by molecules that are preferentially or even exclusively found on the surface of microbes, such as the glycolipid LPS on the outer membrane of Gram-negative bacteria

(3) or the polysaccharide BG in the cell wall of the fungus *Candida albicans* (4). Both LPS and BG induce in monocytes and macrophages signal transduction cascades that start at the pattern-recognition receptors TLR4 (Toll like receptor 4) (5) and CLEC7A (C-type lectin domain containing 7A) (6), respectively, use either kinases of the MAPK (mitogen-activated protein kinase) family or the RAF1 (Raf-1 proto-oncogene, serine/threonine kinase)/AKT1 (AKT serine/threonine kinase 1) pathways and end with well-known transcription factors, such as CREB1 (cAMP responsive element binding protein 1), AP1 (activating protein 1) and NF-κB (nuclear factor κB). Thus, LPS and BG serve as surrogates of bacterial and fungal infections and induce significant changes in the transcriptome of innate immune cells (7, 8). The functional consequences of this so-called trained immunity are an enhanced response to a re-stimulation with microbial molecules, an extended production of pro-inflammatory cytokines and the increased ability to eliminate infectious microbes (9, 10). Trained immunity is mostly beneficial to the host, but it may also become maladaptive in the context of sepsis or autoinflammatory disorders (11).

Vitamin D is a secosteroid that activates *via* its metabolite $1,25(OH)_2D_3$ a transcription factor, the nuclear receptor VDR (vitamin D receptor) (12), i.e., in contrast to LPS and BG, $1,25(OH)_2D_3$ has a direct effect on gene regulation (13). The main endocrine site of $1,25(OH)_2D_3$ production are proximal tubule cells of the kidneys, but also a number of immune cells are able to produce the nuclear hormone for para- and autocrine purposes (14). The general role of vitamin D is to maintain energetic and survival homeostasis of VDR-expressing cells (15), while its main specific functions are calcium homeostasis for supporting bone mineralization (16) and a modulation of the immune system (17). *Via* the latter vitamin D efficiently reacts on infectious diseases (18) and at the same time it helps to avoid overreactions, such as in autoimmune diseases (19). The modulatory role of vitamin D on the function of the immune system as a whole, i.e., on innate and adaptive immunity, is beneficial to the host (20). In contrast, vitamin D deficiency often associates with increased rates of complications of infectious diseases, such as tuberculosis (21) or COVID-19 (22), chronic inflammation, such as in inflammatory bowel disease (23), and autoimmune diseases, such as the onset and progression of multiple sclerosis (24, 25).

Vitamin D and its metabolites as well as their synthetic analogs have not only a disease preventive potential (26) but are also used for the therapy of diseases, such as the autoimmune disorder psoriasis (27). In this study, we ask the question, whether on the level of the transcriptome of primary immune cells there is a difference between $1,25(OH)_2D_3$ treatment before, during or after immune challenge by LPS or BG. An answer should enable to judge, whether it is critical to have a sufficient vitamin D status before, during or after experiencing an infection. We investigate the transcriptome of peripheral blood mononuclear cells (PBMCs) that were immediately after isolation stimulated with either LPS or BG in the presence or absence of $1,25(OH)_2D_3$. PBMCs represent a natural mixture of monocytes, undifferentiated macrophages, natural killer (NK) cells, T and B cells, i.e., of cells of the innate and adaptive immune system, of which monocytes and macrophages are the most vitamin D-responsive cell types (28). The modulation of the immune challenge with $1,25(OH)_2D_3$ was 24 h after, 24 h before or in parallel corresponding to an *in vivo* situation of vitamin D_3 supplementation after, before or during an infection. The results indicate that the functional consequences of immune challenges are significantly modulated by $1,25(OH)_2D_3$ but largely depend on treatment sequence.

MATERIALS AND METHODS

PBMC Isolation

Blood samples were collected from a single healthy individual (male, age 56 years, body mass index 25.1, vitamin D status 87.6 nM 25-hydroxyvitamin D_3 in serum), who gave written informed consent to participate in the study. All experiments were performed in accordance with relevant guidelines and regulations related to the VitDbol trial (NCT02063334, ClinicalTrials.gov). The research ethics committee of the Northern Savo Hospital district had approved the study protocol (#9/2014). PBMCs were isolated from freshly collected peripheral blood using Vacutainer CPT Cell Preparation Tubes with sodium citrate (Becton Dickinson) according to manufacturer's instructions. Deconvolution of RNA-seq data from triplicate solvent-treated samples of each of the three models determined the relative amount of B cells (5.5%), T cells (49.1%), NK cells (19.4%), monocytes/macrophages (23.8%) and other cells (2.2%) within the pool of PBMCs.

PBMC Culture

PBMCs were washed with phosphate-buffered saline and immediately cultured at a concentration of 0.5 million cells/ml in 5 ml RPMI 1640 medium supplemented with 10% charcoal-depleted fetal calf serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Cells were kept at 37 °C in a humidified 95% air/5% CO2 incubator. PBMCs were treated within one hour after taking them into culture with 100 ng/ml LPS (Sigma-Aldrich), 5 μg/ml β-1,3(D)-glucan (BG) (Sigma-Aldrich) or their solvent dimethyl sulfoxide (DMSO) (final concentration 0.1%) and 10 nM $1,25(OH)_2D_3$ (Sigma-Aldrich) or its solvent ethanol (EtOH) (final concentration 0.1%) using three different models (**Figure 1A**). In model 1, cells were first exposed for 24 h to LPS, BG or DMSO and then either $1,25(OH)_2D_3$ or EtOH were added for another 24 h without a wash-out step. In model 2, cells were first stimulated for 24 h with $1,25(OH)_2D_3$ or EtOH and then for additional 24 h with LPS, BG or DMSO. In model 3, cells were incubated for 24 h simultaneously with LPS, BG or DMSO and $1,25(OH)_2D_3$ or EtOH. Each *in vitro* experiment had been performed in three biological repeats within one week with cells from the same donor.

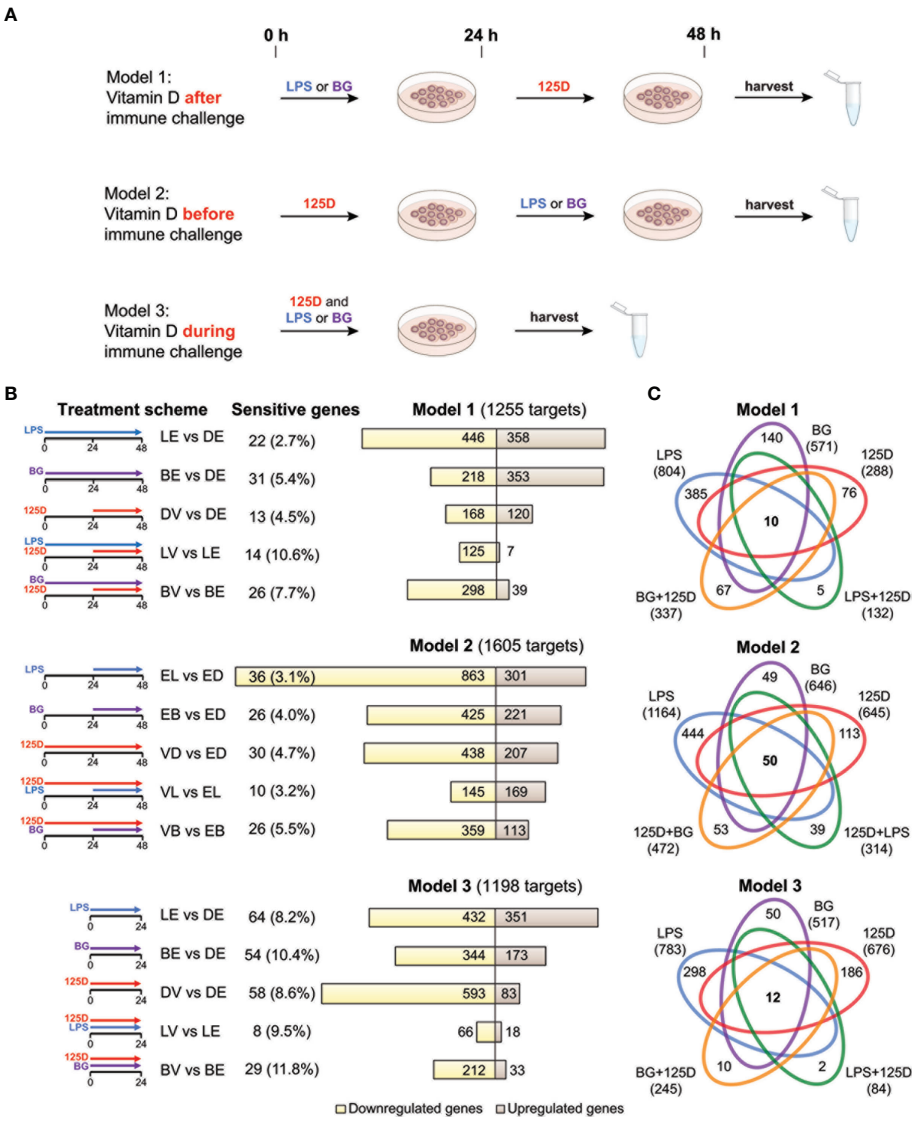

FIGURE 1 | Transcriptomic changes of immune challenged PBMCs. PBMCs of one individual were isolated and treated in three repeats with 100 ng/ml LPS (L), 5 µg/ml BG **(B)** or solvent (0.1% DMSO (D)) in combination with 10 nM 1,25$(OH)_2D_3$ (V) or solvent (0.1% EtOH (E)) using three different models **(A)**. Freshly isolated PBMCs are stimulated with 1,25$(OH)_2D_3$ (125D) after (model 1), before (model 2) or during (model 3) immune challenge with LPS or BG. RNA is extracted and RNA-seq analysis indicates differentially expressed genes for the 15 different treatment conditions indicated by pictograms **(B)**. The number of cell culture sensitive genes is calculated in reference to the 165 differently regulated genes found between models 1 and 2 (for models 1 and 2) and the 152 differently regulated genes found between models 1 and 3 (for model 3) (**Figure S3B**). Bar charts monitor counts of up- (brown) and downregulated (yellow) genes for the indicated gene set comparisons. Venn diagrams display the overlap of different treatments within each model **(C)**. Gene numbers in brackets represent the total number of genes found responsive to the indicated treatment, while gene numbers in bold highlight common genes of all treatment conditions. Blue: LPS, purple: BG, red:1,25D, green: LPS/1,25D, orange: BG/1,25D.

RNA-seq Analysis

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) according to manufacturer's instructions. RNA quality was assessed on an Agilent 2100 Bioanalyzer system (RNA integrity number ≥ 8). rRNA depletion and cDNA library preparation were performed using New England Biolabs kits NEBNext rRNA Depletion Kit, NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (Index Primers Sets 1 and 2) according to manufacturer's protocols. RNA-seq libraries went through quality control with an Agilent 2100 Bioanalyzer and were sequenced on a NextSeq 500 system (Illumina) at 75 bp read length using standard protocols at the Gene Core facility of the EMBL (Heidelberg, Germany).

The single-end, reverse-stranded cDNA sequence reads were aligned (without any trimming) to the reference genome (version

GRCh38) and Ensembl annotation (version 93) using STAR (version 2.6.0c) with default parameters. Read quantification was performed within the STAR alignment step (–quantMode GeneCounts). Mapped and unmapped read counts are listed in **Table S1**. Ensembl gene identifiers were annotated with gene symbol, description, genomic location and biotype by accessing the Ensembl database (version 101) *via* the R package BiomaRt (version 2.44.1) (29). Gene identifiers missing external gene name annotation, genomic location or being mitochondrially encoded were removed from the datasets. When a gene name appeared more than once, the entry with the highest average number of counts was kept.

Differential gene expression analysis was computed in R (version 3.6.3) using the tool EdgeR (version 3.28.1) (30) that uses negative binomial distribution to model gene counts. The gene-wise statistical test for differential expression was computed using the generalized linear model quasi-likelihood pipeline (31). In order to mitigate the multiple testing problem, only expressed genes were tested for differential expression. The filtering threshold was adjusted to the expression of the low expressed but highly specific vitamin D responsive gene *CYP24A1* (cytochrome P450 family 24 subfamily A member 1). For this purpose, read counts were normalized for differences in sequencing depth to counts per million (CPM). Each gene needed to have an expression of > 0.5 CPM in at least 36 out of 54 samples, in order to be considered. This requirement was fulfilled by 16,861 genes. After filtering, library sizes were recomputed and trimmed mean of M-value normalization applied, in order to eliminate composition bias between libraries. The underlying data structure was explored by visualizing the samples *via* multidimensional scaling (MDS) (**Figure S1**). MDS was computed *via* EdgeR's function plotMDS() in which distances approximate the typical log2 fold change (FC) between the samples. This distance was calculated as the root mean square deviation (Euclidean distance) of the largest 500 log2FCs between a given pair of samples, i.e., for each pair a different set of top genes was selected. The two principal factors distinguishing the samples' expression profiles were the type of immune challenge and whether they were treated with $1,25(OH)_2D_3$. Thus, the meaningful clustering of samples confirmed the similarity of the triplicates and demonstrates the effects of the treatments. In this line, a design matrix was constructed for the following pairwise comparisons: i) LPS/EtOH (LE) with DMSO/EtOH (DE) reference, ii) BG/EtOH (BE) with DE, iii) DMSO/$1,25(OH)_2D_3$ (DV) with DE, iv) LPS/$1,25(OH)_2D_3$ (LV) with LE and v) BG/$1,25(OH)_2D_3$ (BV) with BE. Trended negative binomial dispersion estimate was calculated using CoxReid profile-adjusted likelihood method and together with empirical Bayes-moderated quasi-likelihood gene-wise dispersion estimates used for generalized linear model fitting. The empirical Bayes shrinkage was robustified against outlier dispersions as recommended (31). Finally, quasi-likelihood F-test was applied to inspect, whether the observed gene counts fit the respective negative binomial model. Only genes with a false discovery rate (FDR) < 0.001 and an absolute FC > 2 were considered. Mean-Difference (MA) plots were generated with vizzy (version 1.0.0), (https://github.com/ATpoint/vizzy) to display the expression profile of each of the 15 comparisons (**Figure S2**).

Data Analysis and Presentation

Relative cell type composition within the PBMC pool was estimated by deconvolution *via* the algorithm CIBERSORTx (32) using the default LM22 validated gene-signature matrix and gene expression data of solvent-treated samples of all three models. Estimations are based on 1000 permutations. Venn diagrams were created applying the webtool jvenn (33) (http://jvenn.toulouse.inra.fr) and Manhattan plots were produced in R by using packages ggbio (version 1.36.0) (34) and GenomicRanges (version 1.40.0) (35). Based on transcriptome-wide data pathway analysis was performed *via* the webtool Enrichr (36, 37) (https://maayanlab.cloud/Enrichr/) utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) 2019 Human pathways (38). Adjusted P-values were employed for pathway ranking and the threshold < 0.001 was applied. Integrative database Genecards (https://www.genecards.org) was used for gene product locations and functions.

RESULTS

Transcriptome Changes Due to Immune-Challenges or Vitamin D Stimulation

PBMCs of a single healthy individual were stimulated immediately after isolation with LPS, BG or solvent control (DMSO) in the presence of $1,25(OH)_2D_3$ or its solvent (EtOH) (**Figure 1A**). Three different models were applied: in model 1 the cells were first exposed to LPS or BG for 24 h and then for another 24 h to $1,25(OH)_2D_3$, in model 2 the sequence was changed, i.e., first $1,25(OH)_2D_3$ stimulation for 24 h and then treatment with LPS or BG, and in model 3 immune challenges and $1,25(OH)_2D_3$ were applied simultaneously for 24 h. The experiments of each model were performed in three repeats followed by RNA-seq and differential gene expression analysis. When the thresholds FDR < 0.001 and absolute FC > 2 were applied, 1255, 1605 and 1198 differentially expressed genes were detected in models 1, 2 and 3, respectively (**Table S2** and **Figure S3A**). For comparison, the influence of cell culture conditions like different treatment times (48 h in models 1 and 2 versus 24 h in model 3) were estimated by differential gene expression analysis of solvent-treated samples of each model (**Figure S3B**). These differences were largely model specific (75.1% of all) and only the five genes *ACP5* (acid phosphatase 5, tartrate resistant), *ALDH1A1* (aldehyde dehydrogenase 1 family member A1), *CCL24* (C-C motif chemokine ligand 24), *CD302* (cluster of differentiation 302) and *SPARC* (secreted protein acidic and cysteine rich) were identified as common genes that are sensitive to cell culture conditions.

In 13 of the 15 single and combined treatments the majority of the responsive genes were downregulated (**Figure 1B**). Within a given model, 23.6 to 33.4% of the responsive genes were

downregulated in all treatments, while only 7.4 to 11.1% were exclusively upregulated. Thus, the majority (59.2 to 68.5%) of the responsive genes showed a mixed regulation profile (**Figure S3C**). In total of the three models, 1580 genes responded to LPS, 966 to BG and 1006 to $1,25(OH)_2D_3$, from which 503, 388 and 201, respectively, have been previously reported (7, 39) (**Figure S3D**).

In all models, a treatment with LPS alone resulted in the highest count of responsive genes, while lowest numbers were obtained by a combined LPS/$1,25(OH)_2D_3$ treatment (**Figure 1C**). The number of responsive genes was also reduced by BG/$1,25(OH)_2D_3$ co-treatment but the effect was less prominent. LPS and BG showed 336, 505 and 375 overlapping genes in models 1, 2 and 3, respectively (**Figure S3E**). For comparison, in the presence of $1,25(OH)_2D_3$ there were only 107, 177 and 57 common genes (**Figure S3F**). The count of 1,25 $(OH)_2D_3$-responsive genes was only 288 in model 1, but 645 and 676 in models 2 and 3, respectively. Interestingly, the co-treatment with BG in model 1 increased the number of 1,25 $(OH)_2D_3$-responsive genes, while in models 2 and 3 as well as in combination with LPS the numbers declined, i.e., the count and identity of vitamin D responsive genes was dependent on the co-treatment. The LPS treatment in model 2 is an exception, since in this case the ratio between up- and downregulated genes increased from 0.35 to 1.17 due to pre-treatment with 1,25 $(OH)_2D_3$. The number of genes that are responsive to all three treatments, single and in combination, is rather low: 10 in model 1, 50 in model 2 and 12 in model 3 (**Figure 1C**). In contrast, there are 385, 444 and 298 genes that are in models 1, 2 and 3, respectively, exclusively responsive to the single treatment with LPS. These numbers are significantly higher than the counts for single treatments with BG (140, 49 and 50) or $1,25(OH)_2D_3$ (76, 113 and 186).

In summary, the transcriptome of freshly isolated PBMCs shows in a time frame of 1-2 days significant (FDR < 0.001) and prominent (absolute FC > 2) changes in 1580 and 966 genes after immune challenges with LPS and BG, respectively, and in 1006 genes following $1,25(OH)_2D_3$ treatment. The counts of the primarily downregulated LPS and BG responsive genes are clearly reduced to a total of 407 and 595, respectively, when the cells are treated 24 h after, 24 h before or in parallel with 1,25 $(OH)_2D_3$. Interestingly, only a pre-treatment of the LPS challenge with $1,25(OH)_2D_3$ leads to a majority of upregulated genes, while in the five remaining treatment protocols the proportion of downregulated genes even further increases.

Key Genes and Pathways Representing Immune Challenge and Modulation by Vitamin D

In order to identify key genes responding to either immune challenges by LPS or BG or $1,25(OH)_2D_3$ modulation, we focused first on single treatments in all models. From the in total 1580 LPS responsive genes only 24.3% responded in all three models (**Figure 2A**). Similarly, only 27.3% of the 966 BG responsive genes (**Figure 2B**) and 15.5% of 1006 $1,25(OH)_2D_3$ responsive genes (**Figure 2C**) were common to all models. Thus, most responsive genes have a specificity for one or two models suggesting that the sequence of treatment has a major impact on the responsiveness of the cells.

For understanding the common aspects of the three models, we concentrated on joined responsive genes of the single treatments. Manhattan plots displayed the regular genome-wide distribution of the common responsive genes of LPS (**Figure 2D**), BG (**Figure 2E**) and $1,25(OH)_2D_3$ (**Figure 2F**). The number of downregulated responsive genes was at all three treatment conditions higher than the count of upregulated genes. Despite the dominance of downregulation, the most prominent gene expression changes were observed for upregulated genes. Applying an absolute FC > 32 (= 25) threshold highlighted 19 LPS responsive genes (13 up and 6 down), 18 BG responsive genes (16 up and 2 down) and 12 $1,25(OH)_2D_3$ responsive genes (6 up and 6 down) (named in **Figures 2D–F**). The vast majority of these responsive genes are protein coding, but *HMGN2P46* is a pseudogene and *FAM198B-AS1*, *AC022509.1* and *AC037198.1* are non-coding RNA genes. Interestingly, the top responding genes indicated a number of common responsive genes for LPS and BG treatment [*CXCL5* (C-X-C motif chemokine ligand 5), *CCL1*, *CD163*, *ITGB8* (integrin subunit beta 8), *INHBA* (inhibin subunit beta A), *MMP7* (matrix metallopeptidase 7)] but no overlap with $1,25(OH)_2D_3$ stimulation.

We used the transcriptome-wide data for pathway analysis using the webtool Enrichr with the 384, 264 and 156 common responsive genes of LPS, BG and $1,25(OH)_2D_3$, respectively, pointed to their top five functions based on KEGG pathways. LPS treatment associated with "Cytokine-cytokine receptor interaction", "Rheumatoid arthritis", "NOD-like receptor signaling pathway", "Salmonella infection" and "Osteoclast differentiation" (**Figure 2G**). The first two functions were also found with BG treatment, in addition to "Toll-like receptor signaling pathway", "Legionellosis" and "Proteoglycans in cancer" (**Figure 2H**). The latter pathway was also associated with $1,25(OH)_2D_3$ treatment alongside "Phagosome", "Hematopoietic cell lineage", "ECM-receptor interaction" and "Staphylococcus aureus infection" (**Figure 2I**). When the top five pathways were analyzed for each model separately (**Figure S4**), LPS treatment resulted for all models in "Rheumatoid arthritis" and "Osteoclast differentiation", the functions "Cytokine-cytokine receptor interaction" and "NOD-like receptor signaling pathway" were found for models 1 and 3 and "Hematopoietic cell lineage" for models 1 and 2, while "Phagosome", "Leishmaniasis" and "Influenza A" were model-specific (**Figures S2A–C**). BG treatment highlighted the pathways "Rheumatoid arthritis" and "Cytokine-cytokine receptor interaction" in all models, "Leishmaniasis" in models 2 and 3, while "Proteoglycans in cancer", "Complement and coagulation cascades", "ECM-receptor interaction", "Hematopoietic cell lineage", "Inflammatory bowel disease", "Legionellosis" and "Salmonella infection" showed a model-specific fashion (**Figures S2D–F**). In contrast, $1,25(OH)_2D_3$ triggered pathways in a more diverse way: "Phagosome", "Staphylococcus aureus infection", "Tuberculosis", "Rheumatoid arthritis" and "Leishmaniasis" associated with two models, while "Hematopoietic cell lineage",

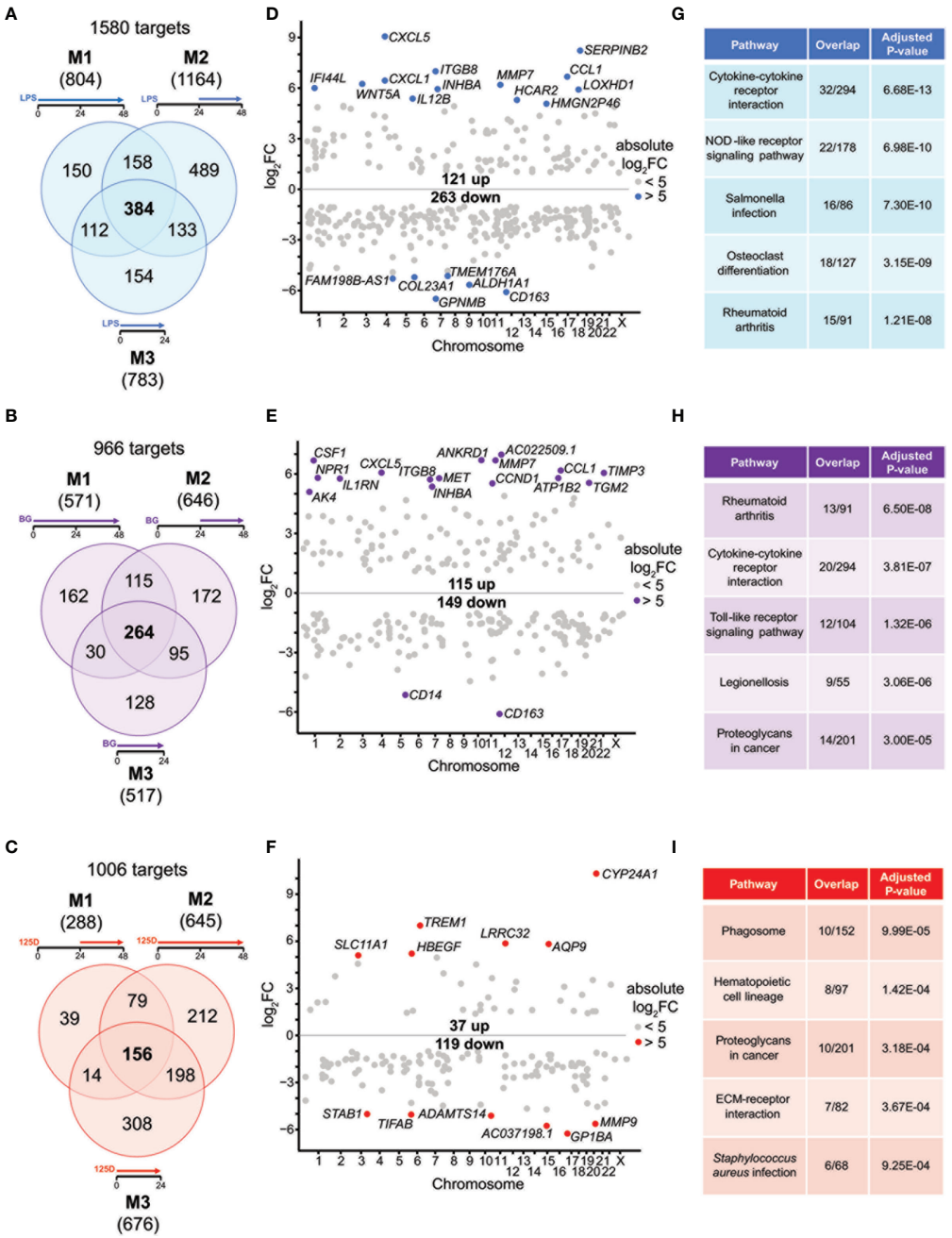

FIGURE 2 | Genes and pathways affected by single stimulations. Venn diagrams display responsive genes obtained after single treatment with LPS **(A)**, BG **(B)** or 1,25 $(OH)_2D_3$ (125D) **(C)** in all models. Gene numbers in brackets represent the total number of genes found responsive to the indicated treatment, while gene numbers in bold highlight common genes of all treatment conditions. Genome-wide distribution of overlapping genes is monitored by Manhattan plots of log2FC values from 48 h treatments, which are obtained from model 1 for LPS **(D)** and BG **(E)** and model 2 for 1,25$(OH)_2D_3$ **(F)**. Highly prominent (absolute log2FC > 5) responsive genes are named and marked by colored dots, whereas the other genes are indicated by grey dots. Top five KEGG pathways representing the most significantly enriched functions of the overlapping genes sorted by adjusted P-value **(G–I)**. Blue: LPS, purple: BG, red:1,25D. M1, model 1; M2, model 2; M3, model 3.

"Toxoplasmosis", "Cytokine-cytokine receptor interaction", "Osteoclast differentiation" and "Fluid shear stress and atherosclerosis" were found to be model-specific (**Figures S2G–I**).

Representative responsive genes were selected on the criteria i) being responsive to all treatments in at least one model ii) displaying prominent changes in expression and iii) being involved in the top KEGG pathways. The genes *TMEM176A* (transmembrane protein 176A), *WNT5A* (WNT family member 5A), *CXCL1*, *S100A8* (S100 calcium binding protein A8), *TNFSF15* (TNF superfamily member 15), *CSF1* (colony

stimulating factor 1), *CD163*, *INHBA*, *CCL1*, *MMP9*, *CDKN1A* (cyclin dependent kinase inhibitor 1A) and *TREM1* (triggering receptor expressed on myeloid cells 1) all represent previously reported LPS, BG or 1,25$(OH)_2D_3$ responsive genes (7, 40, 41) (**Figure S5**). They represent a 4x3 matrix indicating that the whole group of responsive genes can be classified into 12 categories, such as being primarily responsive only to LPS or BG, both LPS and BG, or only 1,25$(OH)_2D_3$, as well being all down- or upregulated or showing a mixed response. This highlighted interesting specificities, such as that *CCL1* is clearly responsive both immune challenges but it barely responded to treatment with 1,25$(OH)_2D_3$, whereas *TREM1* showed distinct preference for 1,25$(OH)_2D_3$. The examples of the mixed regulation category indicated that immune challenges led to increased gene expression while 1,25$(OH)_2D_3$ showed opposite regulation. Furthermore, model-specific differences were observed, where, e.g., *TNFSF15* showed distinct responsiveness while *CSF1* responded almost the same in all models.

Taken together, the immune challenges LPS and BG display characteristic differences in responsive genes and the respective functions mediated by them, but also reasonable overlap in responding genes and regulated pathways. In contrast, 1,25 $(OH)_2D_3$ primarily regulates a distinct set of genes and in case of joined responsive genes often show opposite direction of gene regulation. Despite these differences, all observed top functions relate to innate and adaptive immunity.

Genes and Pathways Representing Vitamin D-Modulated Immune Challenges

For all models, the effects of either single treatments with LPS or BG and 1,25$(OH)_2D_3$ were compared with their respective combinations (**Figure 3**). In model 1, LPS and 1,25$(OH)_2D_3$ treatments overlapped in 112 genes, only 16 of which responded to the combined treatment of LPS and 1,25$(OH)_2D_3$ (**Figure 3A**). Individual LPS and 1,25$(OH)_2D_3$ treatments had in model 2 406 identical genes, 97 of which responded also to the combination of both treatments (**Figure 3B**). In model 3, LPS and 1,25$(OH)_2D_3$ treatments shared 343 genes, only 23 of which were found with their combination (**Figure 3C**). Similar results were obtained for immune challenge with BG, but compared to LPS the overlaps were larger: in model 1 127 BG and 1,25$(OH)_2D_3$ responsive genes overlapped, 47 of which in the context of dual stimulation (**Figure 3D**), in model 2 there were 321 identical genes, 123 of which responded to both stimuli (**Figure 3E**), and 320 shared genes in model 3, 89 of which occurred with both treatments (**Figure 3F**).

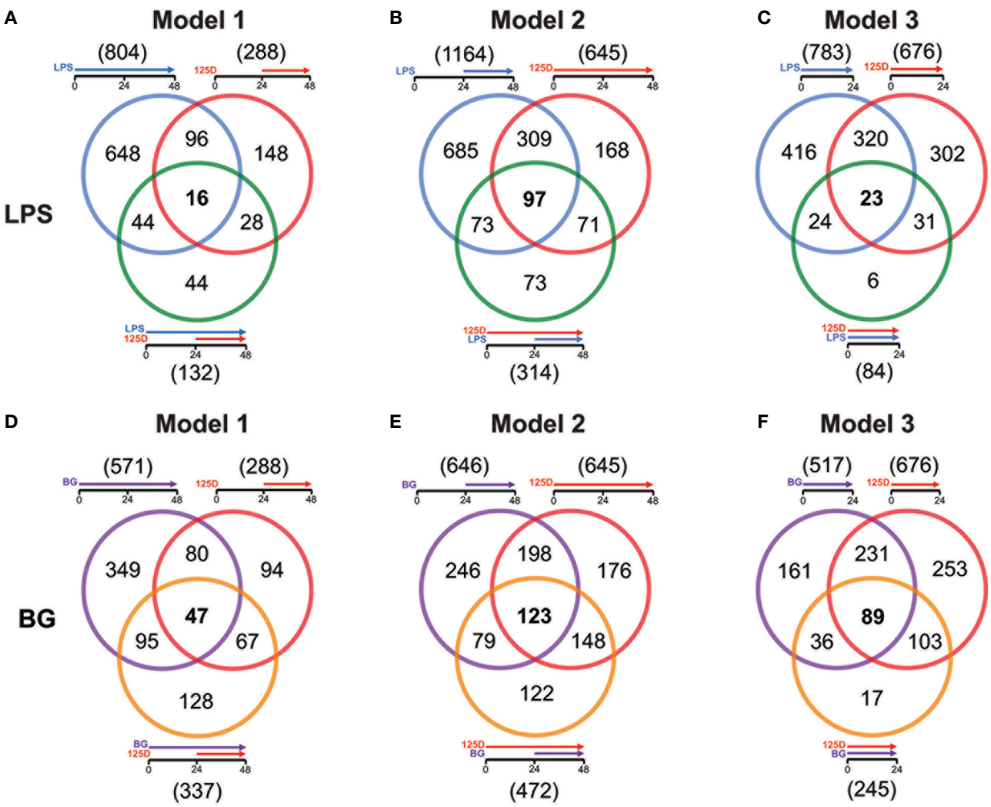

FIGURE 3 | Genes responding to single treatment in relation to combined treatment. Venn diagrams display for the three models the overlap of genes responding to single treatment with LPS **(A–C)** or BG **(D–F)**, 1,25$(OH)_2D_3$ (125D) and the combination of both. Gene numbers in brackets represent the total number of genes found responsive to the indicated treatment, while gene numbers in bold highlight common genes of all treatment conditions. Blue: LPS, purple: BG, red: 1,25D, green: LPS/1,25D, orange: BG/1,25D.

The combined treatments had reduced the total number of vitamin D responding genes to 407 in presence of LPS (**Figure S6A**) and 595 together with BG (**Figure S6B**). Interestingly, only 23 genes were commonly responding in all models to LPS/1,25$(OH)_2D_3$, while for BG/1,25$(OH)_2D_3$ the number was with 166 far higher. Furthermore, the model-specific combined responsive genes were in model 2 with 226 and 191 genes for LPS and BG co-treatment, respectively, clearly higher than in model 1 (66 and 94 genes) and model 3 (15 and 17 genes). Although model 2 had for combined LPS/1,25$(OH)_2D_3$ treatment a larger responsive gene count than models 1 and 3, only the pathways "ECM-receptor interaction" and "Cytokine-cytokine receptor" passed the threshold (**Figure S6C**). The latter function was also found in model 3, while all five top pathways of model 1 ("Phagosome", "Proteoglycans in cancer", "Legionellosis", "Tuberculosis", "Amoebiasis") as well as the remaining four of model 3 ("Allograft rejection", "Malaria", "Rheumatoid arthritis" and "Pertussis") were model-specific. In contrast, for the BG/1,25$(OH)_2D_3$ combination models 2 and 3 shared the top five pathways "Hematopoietic cell lineage", "Phagosome", "Tuberculosis", "Cytokine-cytokine receptor interaction" and "Osteoclast differentiation" and model 1 at least the first three of them (**Figure S6D**). The two specific pathways of model 1 were "*Staphylococcus aureus* infection" and "Asthma". Compared with the pathways highlighted by single treatments, the combined treatments relate more to infectious diseases and their specific pathogens.

Responsive genes serving as representative examples for the effects of combined treatments in comparison with single treatments (**Figure S7**) were selected by the same criteria as in case of the latter (**Figure S5**). The combined treatments showed either a boosting, inhibitory or mixed effect on gene expression. Moreover, genes were sorted by being under all conditions downregulated, upregulated or showing a mixed response providing each a 3x3 matrix for LPS and BG. Representative genes for LPS response were *FPR3* (formyl peptide receptor 3), *TGFBI* (transforming growth factor beta induced), *ITGB2* (integrin subunit beta 2), *CD14*, *FBP1* (fructose-bisphosphatase 1), *SEMA6B* (semaphoring 6B), *SLC22A23* (solute carrier family 22 member 23), *CXCL5* and *STAG3* (stromal antigen 3) (**Figure S7A**). The genes *TLR4*, *HLA-DRB5* (major histocompatibility complex, class II, DR beta 5), *CCL2*, *CLMN* (calmin), *IL1RN* (interleukin 1 receptor antagonist), *IL1R1* (interleukin 1 receptor type 1), *GAL3ST4* (galactose-3-O-sulfotransferase 4), *HBEGF* (heparin binding EGF like growth factor) and *G0S2* (G0/G1 switch 2) represent the BG response (**Figure S7B**). With exception of the genes HLA-*DRB5*, *SLC22A23*, *STAG3* and *GAL3ST4* the example genes are already known as LPS, BG and/or 1,25$(OH)_2D_3$ responsive genes (7, 39, 42).

In summary, the number of genes responding both to immune challenge and vitamin D, alone and in combination, indicate a descending ranking of models 2, 3 and 1. The joined response to BG and vitamin D shows a far better consensus between the models than that of LPS and vitamin D, both in gene count as well as by pathways. Responsive genes are either boosted or inhibited by dual treatments and often show mixed responses depending on the chosen model.

Common and Specific Responses to Treatment Models

Integrating the functional consequences of the treatment sequence based on pathway analysis of single (**Figures 2G–I** and **S2**) and combined (**Figures S6C, D**) stimulation highlighted the differences of the three models. In model 1, immune challenge with LPS caused chemotaxis and induced cytokine signaling, whereas BG treatment affected proliferation, cell growth and cell migration but also increased cytokine signaling (**Figure 4A**). In contrast, stimulation with 1,25$(OH)_2D_3$-modulated genes and pathways involved in antigen recognition and phagocytosis. Interestingly, the combined treatment changed the effects of the immune challenges. The modulation of the LPS challenge with 1,25$(OH)_2D_3$ caused a shift towards phagocytosis, proliferation and cell migration, while the response to BG converted by modulation with 1,25$(OH)_2D_3$ into differentiation and phagocytosis. In model 2, the effects of all single treatments associated with inflammation, which in case of the immune challenges related to cytokines but with 1,25$(OH)_2D_3$ linked to pathogen inhibition (**Figure 4B**). Vitamin D modulated both immune challenges so that cytokine signaling was inhibited and in case of BG also phagocytosis was affected. In model 3, single treatment with LPS caused chemoattraction and affected pathogen recognition, while that of BG related to cytokine signaling and inflammation induced by pathogens (**Figure 4C**). In contrast, stimulation with 1,25$(OH)_2D_3$ alone affected differentiation and caused downregulation of phagocytosis, while in combination with LPS it inhibited cytokine signaling and together BG it initiated differentiation.

Only the genes *STAB1* (stabilin 1) and *HCAR3* (hydroxycarboxylic acid receptor 3) were in all models responsive to all types of treatments and serve as master examples for monitoring the differences between the models (**Figure 4D**). The *STAB1* gene encodes for a highly expressed membrane protein involved in endocytosis, which in every model was downregulated by all types of treatments (**Figure 4E**). The LPS/1,25$(OH)_2D_3$ co-treatment clearly reduced the change of downregulation being caused by respective single treatments. In contrast, the BG/1,25$(OH)_2D_3$ treatment resulted in model 1 in an enhanced change in downregulation, in model 2 in no significant effect and in model 3 in a slightly reduced change in downregulation. The *HCAR3* gene encodes for a G protein-coupled receptor with low affinity for nicotinic acid. In PBMCs the gene shows a low basal expression, was upregulated by both immune challenges but downregulated by 1,25$(OH)_2D_3$ and combined treatment. However, the combined treatments led to less change in downregulation than 1,25$(OH)_2D_3$ alone. Changes in *HCAR3* gene expression did not vary much between the three models, although in model 2 LPS had the lowest and BG the highest effect.

Taken together, a co-stimulation with 1,25$(OH)_2D_3$ is able to change the functional consequences of immune challenges but there are large differences as consequence of treatment sequence, i.e., of the chosen model. The genes *STAB1* and *HCAR3* are master examples monitoring the complex model-specific response to the modulation of immune challenges by vitamin D.

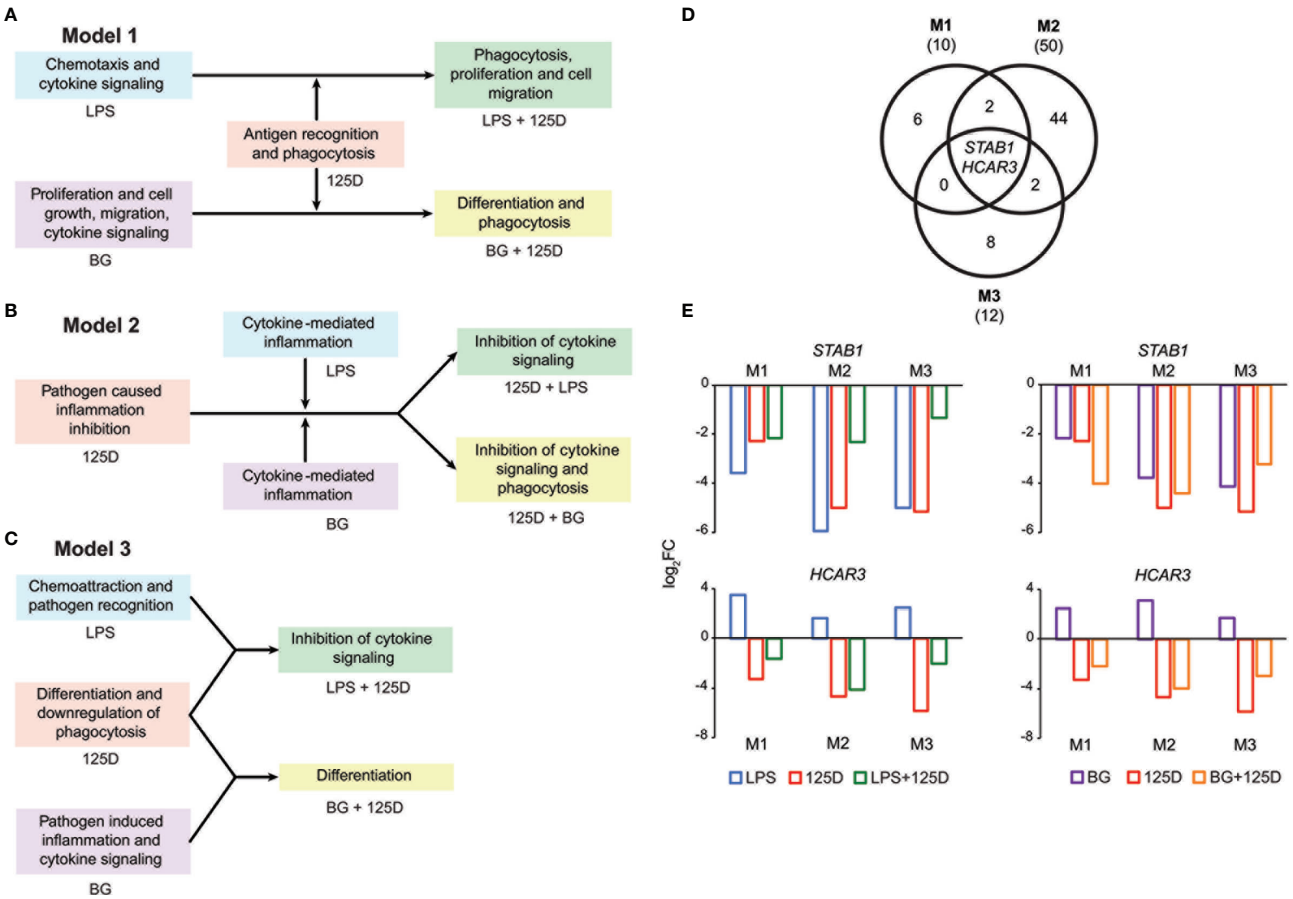

FIGURE 4 | Consequences of single and combined treatments for common pathways and master genes. Key functions affected by single and combined treatments in model 1 **(A)**, model 2 **(B)** and model 3 **(C)**. A Venn diagram indicates the number of genes responding to the treatment combinations **(D)**. Gene numbers in brackets represent the total number of genes found responsive to the indicated treatment, while gene numbers in bold highlight common genes of all treatment conditions. Bar charts monitor the expression profiles of *STAB1* and *HCAR3* **(E)**. Blue: LPS, purple: BG, red: 1,25$(OH)_2D_3$ (125D), green: LPS/1,25D, orange: BG/1,25D. M1, model 1; M2, model 2; M3, model 3.

DISCUSSION

This study investigated on the level of significant (FDR < 0.001) and prominent (absolute FC > 2) changes of the transcriptome, whether 1,25$(OH)_2D_3$ stimulation affected transcriptional programing of primary human immune cells by immune challenges, such as the surrogates of bacterial and fungal infections, LPS or BG. Since there are ethical concerns against voluntary infections or *in vivo* treatments with LPS or BG, this study was designed *in vitro*, where PBMCs were treated immediately after isolation, in order to minimize transcriptional changes due to *in vitro* culture. Moreover, we retained from isolation of the most active and vitamin D responsive cell compartment (43), monocytes and undifferentiated macrophages, which together represent nearly a quarter of the PBMC population. Furthermore, we focused on the first 24-48 h after onset of stimulation, since transcriptional programming of the immune cells takes place within this time frame (7). Another important aspect in the design of this study was the sequence of treatment, where i) immune challenge before 1,25 $(OH)_2D_3$ stimulation (model 1) mimicked the situation where an individual got infected at a low vitamin D status and vitamin D is used for treatment, ii) 1,25$(OH)_2D_3$ stimulation before LPS or BG treatment (model 2) represents infections at a high vitamin D status and iii) a simultaneous application of 1,25$(OH)_2D_3$ and LPS or BG (model 3) served as a reference. Nevertheless, this study has the limitation that not an isolated cell type was studied but a mixture of monocytes, macrophages, NK cells, and different subtypes of B and T cells. Moreover, the longer total treatment time of models 1 and 2 (48 h) limited the compatibility with model 3, in which the treatment was only for 24 h. However, models 1 and 2 were well comparable to each other. The focus of the study was on changes of the transcriptome, but its major findings need to be confirmed by proteome-wide approaches and functional assays, such as testing changes phagocytosis potential. Finally, humans have a personal vitamin D response index, i.e., they show inter-individual variations and respond with different strength to vitamin D_3 supplementation (44). Therefore, the results of this study may not be generalized for the whole population.

The *in vitro* stimulated PBMCs showed to be most responsive to LPS (783 to 1164 responsive genes) and less affected by single treatments with BG (517 to 646 genes) and $1,25(OH)_2D_3$ (288 to 676 genes). However, there are marked differences between the treatment models, so that in all three models only 384, 264 and 156 genes are responding to LPS, BG and $1,25(OH)_2D_3$, respectively, while there are reasonable counts of model-specific responsive genes. For example, 489 LPS and 172 BG responsive genes are specific to model 2, while 308 $1,25(OH)_2D_3$ responsive genes are exclusively found in model 3. This is one important indication that the sequence of treatment has a large impact on the response of the transcriptome.

With the exception of BG treatment in model 1, the single treatments with LPS, BG and $1,25(OH)_2D_3$ resulted in a majority of downregulated genes, i.e., all three stimuli rather diminish gene expression than enhance it. Moreover, the co-stimulations of the immune challenges with $1,25(OH)_2D_3$ derived in a clearly reduced number of responsive genes, i.e., vitamin D appears to neutralize the responsiveness of a large number of LPS and BG responsive genes. Furthermore, with the exception of joint LPS/$1,25(OH)_2D_3$ stimulation in model 2, the co-treatments by vitamin D and the two immune challenges still mostly produced downregulated genes. It should be noted that the downregulation of a gene by one signal transduction pathway requires that first other signals upregulated of the gene. Thus, $1,25(OH)_2D_3$-activated VDR seems to interfere with the responsiveness of many LPS and BG responsive genes, i.e., VDR counteracts to their mechanism of regulation. For example, $1,25(OH)_2D_3$ and its receptor antagonize the pro-inflammatory actions of the transcription factors NF-κB (45). Interestingly, the interference of $1,25(OH)_2D_3$ signaling with that of immune challenges does not require that the respective genes are primary vitamin D responsive genes, i.e., they do not have to contain VDR binding sites in their regulatory regions (46).

Although a rather large number of genes (112 to 406) respond in the different models to two different individual treatments, only 16 to 123 of these genes are responsive to the respective joint treatment. This is another indication that a co-treatment neutralizes the effects of the individual treatments. Nevertheless, model 2 displayed for both types of immune challenges a clearly higher number of genes with joint responsiveness than the two other models, i.e., the assumed beneficial effects of a pre-treatment with vitamin D are only in part neutralized by immune challenges. Interestingly, there are also cases where vitamin D and immune challenges boost each other. Since LPS, BG and $1,25(OH)_2D_3$ mediate their signaling *via* different signal transduction pathways, it is not surprising that only two genes, *STAB1* and *HCAR3*, are in all models responsive to the three types of stimuli. The two genes serve as master genes demonstrating that the downregulation by $1,25(OH)_2D_3$ affects their response to immune challenges. In case of the *STAB1* gene, $1,25(OH)_2D_3$ reduces the amount of downregulation by LPS in all three models and it even further promotes the downregulation by BG in models 1 and 2. In contrast, the upregulation of *HCAR3* by LPS and BG is reversed by $1,25(OH)_2D_3$ co-stimulation to a downregulation of the gene.

In total, we selected 32 genes as representative examples for the different types of responses of PBMCs (**Figure 5**). The proteins encoded by these genes are located either within the plasma membrane (20/32) or are secreted (8/32). The majority of these proteins are either membrane receptors or cytokines and chemokines. Only two of the proteins, which are encoded by the representative genes, are found in the nucleus (*CDKN1A* and *STAG3*), whereas *FBP1* is located in the cytosol and *G0S2* in mitochondria. Most of the example genes are responsive to all treatments but not in all models. In contrast, some genes were only regulated by one stimulus, most of which are LPS responsive, while only *S100A8* is a specific responsive gene of BG. Interestingly, the example genes that are responsive to all treatments at least in one model show preference towards $1,25(OH)_2D_3$ and BG or were equal between both. Out of the three applied treatments LPS signaling seems to be most independent. This is related to the fact that infection with bacteria carrying LPS on their surface are detrimental (47), while intake of vitamin D or BG are primarily beneficial (48, 49).

The stimulation of PBMCs with either LPS or BG affects the expression of genes that are involved in biochemical pathways of first line immune responses, such as enhancing cytokine signaling and inflammation. Furthermore, both immune challenges support pathogen recognition, but LPS has a focus on the extracellular and BG on the intracellular. In contrast, the stimulation of the cells with $1,25(OH)_2D_3$ downregulates phagocytosis, induces differentiation and inhibits inflammation, i.e., pathways are activated that are rather contrary to those induced by immune challenges. While LPS and BG induce stress to cells and direct them to early responses like inflammation, vitamin D increases the potency of the immune system and boosts later steps in innate immune responses like destroying pathogens or initiating differentiation. Thus, the observed responses of PBMCs are most likely caused by their monocyte and macrophage compartment than by lymphocytes. When vitamin D is applied after immune challenge (model 1) both LPS- and BG-treated PBMCs initiate phagocytosis, but LPS-challenged cells activate proliferation and cell migration, while BG-treated cells differentiate. In contrast, a pre-treatment with vitamin D (model 2) reduces the activating effects of both LPS and BG on cytokine signaling as well as on inflammation and together with BG it activates phagocytosis. Interestingly, the simultaneous treatment with immune challenges and vitamin D (model 3) causes in case of LPS the inhibition of cytokine signaling and with BG the induction of differentiation. In all three models the co-treatment significantly changes the functional outcomes of immune challenges, which are directed towards disease- and pathogen-specific responses. Thus, the most disease-preventive reactions are caused by a pre-treatment with vitamin D (model 2).

In conclusion, this study provides a transcriptome-wide view how vitamin D modulates responses of the innate immune system to immune challenges like bacterial and fungal infections. A pre-treatment with vitamin D (model 2) appears

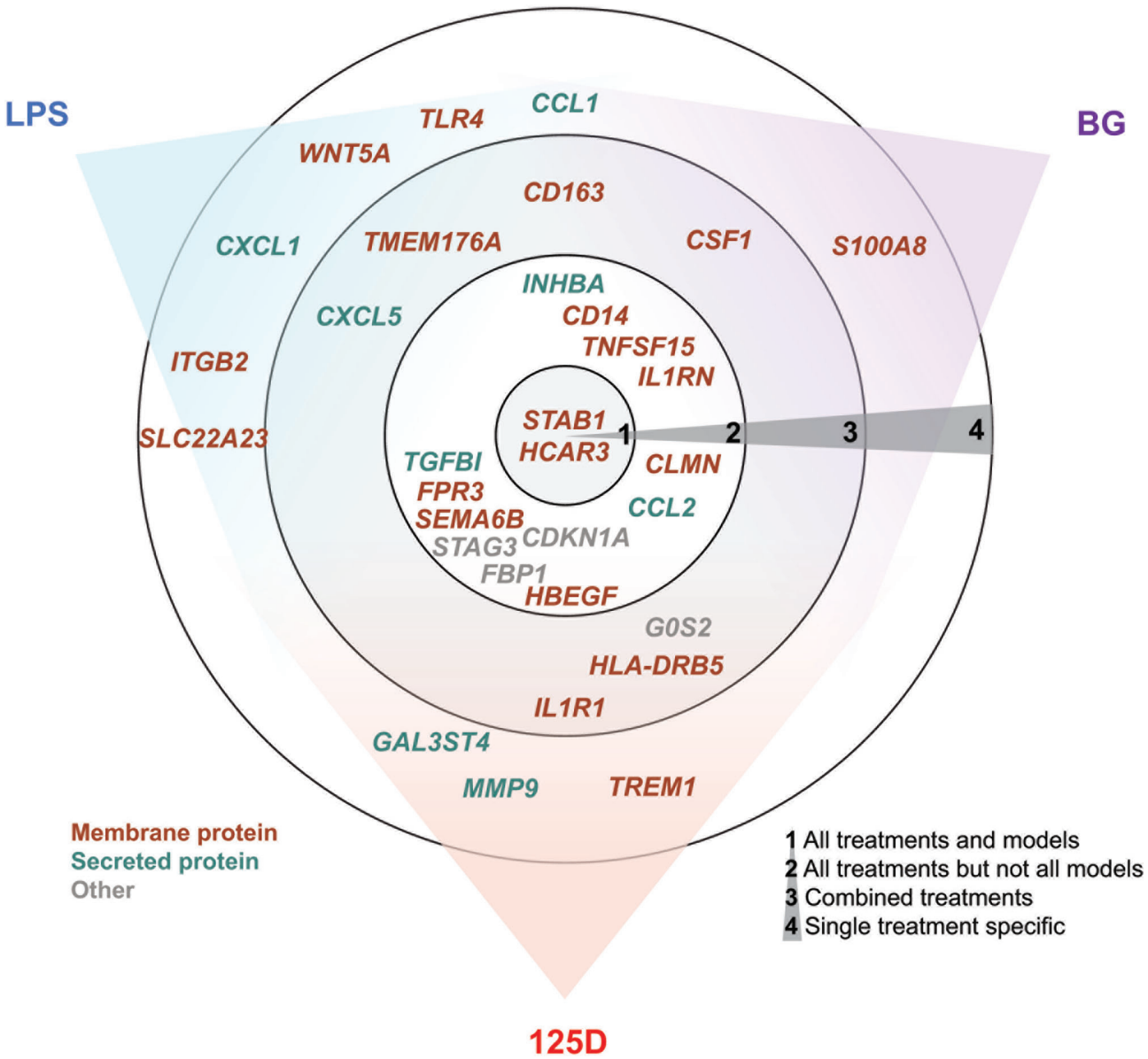

FIGURE 5 | Summary of key responsive genes. LPS, BG and 1,25$(OH)_2D_3$ (125D) responsive genes are categorized based on their response to different treatments in different models. Circle 1: genes that are responsive to all treatments and models, circle 2: genes that are responsive to all treatments but not in every model, circle 3: genes that are responsive to at least two stimuli and their combination and circle 4: genes that are specifically responsive to stimuli. The gene positions in relation to colored sectors represent their preferences to the indicated stimuli. Genes are color-coded based on the main cellular location of their protein products. Blue: LPS, purple: BG, red: 1,25D.

to be more effective than its application after microbial challenges, such as infections with pathogens. Vitamin D_3 supplementation will improve the vitamin D status of an individual and make vitamin D signaling *via* VDR and its target genes more effective. Since a large number of these responsive genes are involved in improving the functionality of immunity, their vitamin D-triggered activity can be considered as training of in particular the innate immune system (43). This suggests that vitamin D_3 supplementation may have an important role in preventing infectious diseases or reducing their severe consequences.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the research ethics committee of the Northern Savo Hospital District had approved the study protocol (#9/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

H-RM and CC performed all experiments. AH performed differential gene expression analysis. H-RM performed data analysis. H-RM and CC wrote the manuscript, which was reviewed by AH, MT, and SH. All authors contributed to the article and approved the submitted version.

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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.2021.754056/full#supplementary-material

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Vitamins D_2 and D_3 Have Overlapping But Different Effects on the Human Immune System Revealed Through Analysis of the Blood Transcriptome

Louise R. Durrant[1†‡], Giselda Bucca[1,2†‡], Andrew Hesketh[2‡], Carla Möller-Levet[1], Laura Tripkovic[1], Huihai Wu[1], Kathryn H. Hart[1], John C. Mathers[3], Ruan M. Elliott[1], Susan A. Lanham-New[1] and Colin P. Smith[1,2†]*

[1] Department of Nutritional Sciences, School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, United Kingdom, [2] School of Applied Sciences, University of Brighton, Brighton, United Kingdom, [3] Human Nutrition Research Centre, Population Health Sciences Institute, Newcastle University, Newcastle, United Kingdom

Vitamin D is best known for its role in maintaining bone health and calcium homeostasis. However, it also exerts a broad range of extra-skeletal effects on cellular physiology and on the immune system. Vitamins D_2 and D_3 share a high degree of structural similarity. Functional equivalence in their vitamin D-dependent effects on human physiology is usually assumed but has in fact not been well defined experimentally. In this study we seek to redress the gap in knowledge by undertaking an in-depth examination of changes in the human blood transcriptome following supplementation with physiological doses of vitamin D_2 and D_3. Our work extends a previously published randomized placebo-controlled trial that recruited healthy white European and South Asian women who were given 15 µg of vitamin D_2 or D_3 daily over 12 weeks in wintertime in the UK (Nov-Mar) by additionally determining changes in the blood transcriptome over the intervention period using microarrays. An integrated comparison of the results defines both the effect of vitamin D_3 or D_2 on gene expression, and any influence of ethnic background. An important aspect of this analysis was the focus on the changes in expression from baseline to the 12-week endpoint of treatment *within* each individual, harnessing the longitudinal design of the study. Whilst overlap in the repertoire of differentially expressed genes was present in the D_2 or D_3-dependent effects identified, most changes were specific to either one vitamin or the other. The data also pointed to the possibility of ethnic differences in the responses. Notably, following vitamin D_3 supplementation, the majority of changes in gene expression reflected a down-regulation in the activity of genes, many encoding pathways of the innate and adaptive immune systems, potentially shifting the immune system to a more tolerogenic status. Surprisingly, gene expression associated with type I and type II interferon activity, critical to the innate response to bacterial and viral infections, differed following supplementation with either vitamin D_2 or vitamin D_3, with only vitamin D_3 having a stimulatory effect. This study suggests that further investigation of the respective physiological roles of vitamin D_2 and vitamin D_3 is warranted.

Keywords: adaptive immunity, ethnicity, immunomodulation, innate immunity, human transcriptome, vitamin D supplementation, vitamin D_3, vitamin D_2

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Mourad Aribi, University of Abou Bekr Belkaïd, Algeria

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Ouliana Ziouzenkova, The Ohio State University, United States

****Correspondence:***
Colin P. Smith
c.p.smith@surrey.ac.uk; genomics@cpsmith.net

†Present address:
Louise R. Durrant, British Nutrition Foundation, London, United Kingdom, formerly known as Louise Wilson
Colin P. Smith, Department of Nutritional Sciences, University of Surrey, Guildford, United Kingdom
Giselda Bucca, School of Applied Sciences, University of Brighton, Brighton, United Kingdom

‡The authors have contributed equally to this work and share first authorship

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INTRODUCTION

Vitamin D is a pro-hormone that is essential for human health. While vitamin D is best known for its role in maintaining bone health and calcium homeostasis, it also exerts a broad range of extra-skeletal effects on cellular physiology and on the immune system (1–6) and multiple studies have linked poor vitamin D status with increased risk of osteoporotic and stress fractures, increased risk of developing cardiovascular diseases and some cancers, poor modulation of the immune system, higher mortality including death from cancer, and the pathogenesis of immune mediated inflammatory diseases (7–10). It is recommended that individuals maintain serum 25-hydroxyvitamin D (25(OH)D) concentrations (the accepted biomarker of systemic vitamin D status) of at least 25 nmol/L throughout the year and throughout the life course (11). However, vitamin D deficiency and inadequacy (defined as serum 25(OH)D concentrations below 25 nmol/L and 50 nmol/L, respectively) is considered a global pandemic and a public health issue of great importance in the human population, especially in older people, individuals not exposed to sufficient sunlight and, importantly, ethnic groups with darker skin tone (12–14).

Vitamin D is the overarching term used to describe both vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Both the plant/fungus-derived vitamin D_2 and the animal-derived vitamin D_3 forms can be found in some (albeit limited) human foods or are available as food supplements. However, vitamin D_3 is also produced in the skin by the action of ultraviolet B radiation from the sun. Following two successive hydroxylation steps, in the liver and kidney, respectively, the active form of the vitamin, 1,25-dihydroxyvitamin D_3 (1,25(OH)D_3) binds to the intracellular vitamin D receptor (VDR) and, in complex with the retinoid X receptor (RXR), the heterodimer regulates expression of hundreds of genes through the vitamin D response element (VDRE) (15); other tissues also express the 25(OH)D 1-α-hydroxylase although kidney is considered the most important for production of circulating 1,25(OH)D (16). Both forms of vitamin D can be double hydroxylated into their active metabolites and they bind to the VDR with similar affinity (17), but there remains some controversy around whether or not they elicit identical biological responses (17–19). Moreover, there are differences in their catabolism and in their binding affinity to vitamin D binding protein (DBP), the major vitamin D transport protein in blood. Vitamin D_2 binds DBP with lower affinity and is catabolised faster (20, 21).

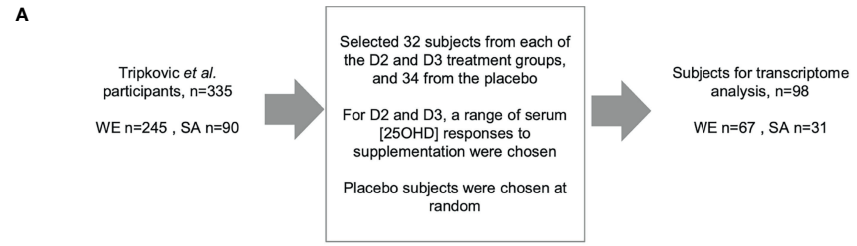
The functional equivalence, or otherwise, of vitamins D_2 and D_3 for human health has been a subject of much debate in recent years, with some authors suggesting that the two compounds have equal efficacy while others provide evidence that vitamin D_3 increases circulating serum 25(OH)D concentration more efficiently than vitamin D_2 (22–26). In acute studies comparing the efficacy of vitamins D_2 and D_3 in raising serum 25(OH)D concentration, vitamin D_2 is less effective than D_3 when given as single bolus (22). However, the findings from studies following the daily administration of D_2 or D_3 over longer time-periods are more equivocal, with clinical trials showing higher efficacy of D_3 (27, 28) or equal efficacy (29, 30). Furthermore, a recent meta-analysis of vitamin D supplementation trials found that reduced cancer mortality was seen only with vitamin D_3 supplementation, not with vitamin D_2 supplementation, and indicated that all-cause mortality was significantly lower in trials with vitamin D_3 supplementation than in trials with vitamin D_2 (9). Moreover, a recent study of US women makes the intriguing observation that the two independent metabolites, 25(OH)D_3 and 25(OH)D_2 appear to have totally different associations with depression (31). Supplementation with one form of vitamin D can reduce circulating concentrations of the other form. For example, our recent comparative trial of vitamin D_2 versus D_3 revealed that serum 25(OH)D_3 concentration was reduced over the 12-week intervention in participants given vitamin D_2, compared with the average 12-week concentration in participants given placebo [(28), see **Figure 1C**, for example]. The implications of such reciprocal depletion remain to be explored.

There is limited understanding of the effects of vitamin D supplementation on gene expression *in vivo* in humans because of the diversity of experimental designs used. This includes: (i) diverse sampling intervals, ranging from hours to years following vitamin D supplementation (32, 33); (ii) use of substantially different doses ranging from physiological (moderate) to supra-physiological doses; and (iii) relatively small participant numbers so most studies were considerably underpowered (33, 34). Currently, there is no robust evidence, from *in vivo* human genome-wide expression analysis, about which specific cellular pathways are influenced by vitamin D supplementation. Moreover, the influence of vitamin D_2, as distinct from vitamin D_3, on gene expression in humans has not yet been evaluated, even though vitamin D_2 is also used widely as a supplement and food fortificant.

We have addressed this gap in knowledge by investigating gene expression in a relatively large cohort of healthy white European and South Asian women who participated in a randomised double-blind placebo-controlled trial – the D2-D3 Study - that compared the relative efficacy of vitamins D_2 and D_3 in raising serum 25(OH)D concentration. The study concluded that vitamin D_3 was superior to vitamin D_2 in raising serum 25(OH)D concentration (28). As an integral part of the original study design, we also investigated gene expression in the participants over the 12-week trial period. This allowed us to examine the effects of vitamin D supplementation on the transcriptome and to determine whether these effects might differ following supplementation with vitamin D_2 compared with vitamin D_3. We provide evidence in support of the hypothesis that the biological effects of vitamin D_2 and D_3 differ in humans. Consequently, a more comprehensive analysis of the biological effects of the two forms of vitamin D on human physiology is warranted.

MATERIALS AND METHODS

The recruitment of individuals as part of the 'D2-D3 Study' was described previously (28) and the clinical trial has been registered



B

Group	White Euro (n)	South Asian (n)	Total (n)
D2	21	11	32
D3	21	11	32
Placebo	25	8	33

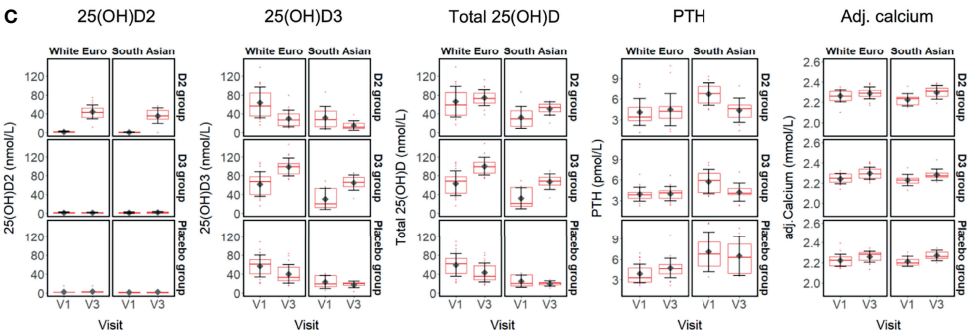


FIGURE 1 | **(A)** Selection of 98 subject participants from the Tripkovic et al. (2017) study (28) for transcriptomic analysis of their V1 (baseline) and V3 (12-week) samples in the present study; **(B)** Ethnicity and treatment group membership for the 97 subjects for which transcriptomic data was obtained (data for one South Asian subject from the placebo group did not pass quality control); **(C)** Metadata on serum concentrations of 25(OH)D_2, 25(OH)D_3, total 25(OH)D, PTH and calcium (albumin-adjusted) for the 97 subjects (see **Supplementary Data File 1** for details). Participants were selected to provide comparable numbers between the placebo and the two vitamin D treatment groups, covering the full range of serum responses to supplementation within the D_2 and D_3 treatment groups, as judged from the measured changes in serum 25(OH)D_2 or 25(OH)D_3 concentrations between V1 and V3.

(ISRCTN23421591). This study received ethical approval from the South-East Coast (Surrey) National Health Service Research Ethics Committee (11/LO/0708) and the University of Surrey Ethics Committee (EC/2011/97/FHMS). All participants gave written informed consent in agreement with the Helsinki Declaration before commencing study activities. Briefly, 335 women of both South Asian (SA) and white European (WE) descent were randomised to one of three intervention groups for 12-weeks and provided with daily doses of vitamin D within fortified foods (both orange juice and biscuits) (28). Participants were randomised to placebo, 15 µg/d vitamin D_2 or 15 µg/d vitamin D_3. Of the 335 participants reported previously by Tripkovic et al (28), 97 were selected for transcriptome analysis, representing 67 WE and 30 SA participants; allocations to each treatment group are reported in **Figure 1** and **Supplementary Data File 1**. Participants were selected at random from both ethnic groups to form a balanced placebo group. We undertook transcriptome analysis at baseline (V1) and at 12 weeks after treatment commenced (V3).

Blood Transcriptome Analysis

Whole peripheral blood (2.5 ml) was collected using PAXgene Blood RNA tubes (BD Biosciences and Diagnostics). The tubes were inverted ten times immediately after drawing blood, stored upright at 15-25°C for 24 hours, followed by a -20°C freezer for 24 hours and then into a -80°C freezer for long-term storage. Transcriptomic analysis was conducted essentially as described in previous studies (35, 36). Total RNA was isolated using the PAXgene Blood RNA Kit (Qiagen) following the manufacturer's recommendations. cRNA was synthesised and fluorescently labelled with Cy3-CTP from 200 ng of total RNA using the Low Input QuickAmp Labelling Kit, One Color (Agilent Technology). Labelled cRNA was hybridised on a Sure Print G3 Human Gene Expression 8 x 60K v2 microarray slide (Agilent Technologies). Standard manufacturer's instructions for one colour gene-expression analysis were followed for labelling, hybridisation and washing steps. Extracted RNA was quantified using NanoDrop ND2000 spectrophotometer (Thermo Scientific). RNA quality and integrity was evaluated

using either the Bioanalyzer 2100 or the TapeStation 4200 (Agilent Technologies). Only RNA samples with an RNA Integrity Number (RIN) of >7.0 were subjected to DNA microarray analysis. Microarrays were hybridised at 65°C for 17 hours in an Agilent Hybridization Oven on a rotisserie at 10 rpm. The washed microarrays were scanned using an Agilent Microarray Scanner with a resolution of 2 μm.

Transcriptome Data Processing and Differential Expression Analysis

Raw scanned microarray images were processed using Agilent Feature Extraction software (v11.5.1.1) with the Agilent 039494_D_F_20140326_human_8x60K_v2 grid, and then imported into R for normalization and analysis using the LIMMA package (37). Normalised data for all participants (V3 – V1) was assessed by principal component analysis to screen for any batch effects (**Supplementary Figure 3**). Microarray data were background-corrected using the 'normexp' method (with an offset of 50) and quantile normalised, producing expression values in the log base 2 scale. The processed data were then filtered to remove probes exhibiting low signals across the arrays, retaining non-control probes that are at least 10% brighter than negative control probe signals on at least 41 arrays (~20% of the arrays in the analysis). Data from identical replicate probes were then averaged to produce expression values at the unique probe level. Initial data exploration identified one sample (participant 0017, V3 time point) with array data that was a notable outlier from the group and therefore both the V1 and V3 microarrays for this subject were excluded from all subsequent analysis, reprocessing the data as above in their absence before proceeding.

Tests for differential expression were performed using LIMMA, applying appropriate linear model designs to identify: (i) significant differences in the transcriptional responses occurring across the 12-week V1 to V3 period of the study between the treatment and placebo groups for each ethnic cohort (example contrasts tested, in the format ethnicity_treatment_time: (WE_D2_V3 - WE_D2_V1) - (WE_P_V3-WE_P_V1) = 0, and (WE_D3_V3 - WE_D3_V1) - (WE_P_V3 - WE_P_V1) = 0); and ii) to determine significant changes for each ethnic cohort within each treatment group between the V3 and V1 sampling points, blocking on subject identity (example contrasts tested: WE_D2_V3 - WE_D2_V1 = 0, WE_D3_V3 - WE_D3_V1 = 0). Blocking on subject identity was used to control for inter-subject variability. Significance p-values were corrected for multiplicity using the Benjamini and Hochberg method, obtaining adjusted p-values (adj.P.Val).

Functional Enrichment and Network Analysis

Functional enrichment analysis of lists of genes of interest possessing valid ENTREZ gene identifiers was performed using the R package clusterProfiler (38). The software produces adjusted p-values (p.adjust) using the Benjamini and Hochberg correction method. Construction and analysis of protein-protein interaction networks from sets of genes was undertaken in Cytoscape (39) using the STRING plugin (40). Cytoscape was also used to construct and visualise commonality in the functional categories identified as being significantly enriched in the genes responding to the experimental treatments. To visualise categories identified from the D_2 or D_3 treatment groups but not from the placebo (e.g. **Figure 4**) significantly enriched categories (p.adjust < 0.01) from all groups were imported such that treatment group nodes (D_2, D_3 and placebo (P)) are linked to functional category nodes by edges assigned the corresponding p.adjust values. All nodes with an edge connection to the placebo treatment node were then removed and the resulting networks further filtered to retain only those nodes with at least one edge connection with p.adjust<=0.001 (p.adjust<=0.01 for Reactome pathways). The results were finally summarised as heatmap plots (**Figure 4**).

Weighted gene co-expression network analysis was performed in R using the WGCNA (41) and CoExpNets (42) packages. A normalised expression data matrix generated by filtering to retain probes with signals more markedly above background (30% brighter than negative control probe signals on at least 41 arrays) was used as input, consisting of 12,169 unique probes. Signed scale-free networks were constructed separately for the data for the SA and WE ethnic groups using 50 iterations of the k-means clustering option in the CoExpNets 'getDownstreamNetwork' function to refine the clustering process (**Supplementary Data File 6**). Pearson correlations between cluster module eigengenes and metadata for serum 25$(OH)D_2$, 25$(OH)D_3$, total 25(OH)D and PTH concentrations were calculated using pairwise complete observations.

Gene Set Enrichment Analysis (GSEA)

GSEA was performed using the R package clusterProfiler (38), applying the default parameters which implement the fgsea algorithm and correct for multiple testing using the Benjamini and Hochberg method. Gene sets were obtained from MSigDB *via* the msigdbr package (43). Ranked gene lists for interrogation were derived from LIMMA analysis of the unfiltered microarray data, pre-ranking genes according to their t-statistic.

RESULTS

The study volunteers were South Asian (SA) and white European (WE) women based in the United Kingdom, aged between 20 and 64 years [n=335; (28)] and participation was for 12 weeks over the winter months (October to March, in Surrey, UK; latitude, 51°14' N). Serum measurements, including concentrations of total 25(OH)D, 25$(OH)D_2$ and 25$(OH)D_3$, were determined from fasting blood samples taken at the start [baseline defined as Visit 1 (V1)] and at weeks 6 (V2) and 12 (V3). To determine whether changes in serum 25(OH)D concentration as a result of vitamin D supplementation are associated with physiological changes at the level of gene expression, we investigated the whole blood transcriptome from a representative subset of the study participants (n=98) using total RNA isolated from V1 and V3 blood samples and Agilent Human Whole Genome 8 × 60K v2 DNA microarrays

(**Figure 1**). Participants for transcriptome analysis were selected to provide comparable numbers between the placebo and the two vitamin D treatment groups, covering the full range of serum responses to supplementation within the D_2 and D_3 treatment groups, as judged from the measured changes in serum 25(OH)D_2 or 25(OH)D_3 concentration between V1 and V3 (**Supplementary Data File 1**).

Metadata for the Transcriptome Analysis Cohort

Following microarray data quality control, transcriptome data were available for both the V1 and V3 samples of 97 study participants (**Figure 1B**); the RNA from one participant failed quality control and was excluded. Similar to data reported previously for the entire study cohort (28), the sub-set of participants in the present study showed increased concentrations of 25(OH)D_2 and 25(OH)D_3 only after supplementation with vitamin D_2 and vitamin D_3, respectively, and higher total 25(OH)D in both treatment groups (**Figure 1C**). In the vitamin D_3 treatment groups the mean 25(OH)D_3 serum levels rose by 59% (WE) and 116% (SA) over the 12-week intervention. Conversely, mean serum 25(OH)D_3 concentrations fell across the 12 weeks of the study in the placebo groups who did not receive additional vitamin D; 25(OH)D_3 baseline vitamin D concentration in the SA ethnic group tended to be lower than for the WE group and dropped, respectively, by 23% and 29% relative to baseline V1 (**Figure 1C** and **Supplementary Data File 1**). It is notable that, in the vitamin D_2 treatment group, serum 25(OH)D_3 concentration decreased to a greater extent over the 12-week period, by 52% (SA) and 53% (WE). Serum 25(OH)D_2 was low in the absence of specific supplementation, typically less than 5 nmol/L (152/162 samples analysed). Serum calcium concentration, appropriately adjusted for serum albumin concentration, was maintained within a normal clinical range across all sample groups, while the concentration of parathyroid hormone (PTH) was stable between V1 and V3 only within the SA placebo group, the WE vitamin D_2 and WE vitamin D_3 intervention groups. PTH concentrations increased at V3 compared with V1 in the WE placebo group, but decreased in both the vitamin D_2 and D_3 intervention groups in the SA ethnic cohort.

Effects of Supplementation With Either Vitamin D_2 or D_3 on Global Gene Expression

Filtering of the normalised microarray data to select for probes showing signals at least 10% above background in at least 41 (~20%) of the arrays yielded transcript abundance data from 20,662 probes corresponding to 17,588 different genomic features (12,436 of which were annotated with an ENTREZ gene identifier). Using the filtered data, we identified significant differences in the transcriptional responses occurring across the 12-week V1 to V3 period of the study *between* the vitamin D_2, vitamin D_3 and placebo treatment groups for each ethnic cohort. In a separate analysis, we determined significant changes *within* each group between the V3 and V1 sampling points (**Figure 2** and **Supplementary Data File 2**). The former was tested in a 'difference-in-difference' analysis according to the generalised null hypothesis [treatment1.V3 – treatment1.V1] - [treatment2.V3 – treatment2.V1] = 0 (**Figure 2A**, rows 7-12), and the latter using treatment.V3 – treatment.V1 = 0 (**Figure 2A**, rows 1-6).

No significant changes in probe signals (adj.P.Val<=0.05) were identified in the 'difference-in-difference' analysis for the data for the WE ethnic group, but five were found in the corresponding analysis of the SA cohort (**Figure 2A**). Furthermore, difference-in-difference analysis of the entire study data, ignoring ethnicity, did not yield statistically significant changes between the D_2 or D_3 treatment groups and the placebo control (data not shown). We note that another 'difference-in-difference' study on the influence of vitamin D on gene expression (the BEST-D trial) failed to identify any significant changes in gene expression following long-term vitamin D treatment in a Caucasian cohort (32). The observed $\log_2$ fold-change ($\log_2$FC) in signal abundances between V3 and V1 in the study typically fell well within +/- 1, and, in such a context, it is likely that the sample sizes used within the study restrict our ability to reliably identify individual gene expression responses using this highly conservative approach.

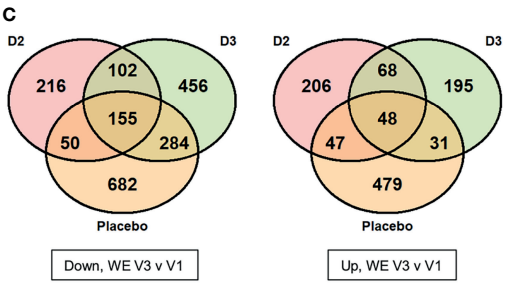
We consider that the strong interpersonal differences in gene expression across individuals in the study population are likely to hinder detection of small statistically significant gene expression changes *across* treatment groups from microarray-derived data. It is known that expression of any particular gene and its magnitude of change over time can vary widely across individuals in a human population, and the differences in response across time *within* an individual is considered more physiologically meaningful. This issue is considered further in the **Discussion**. In this study we observe that many statistically significant differences are detected when evaluating gene expression changes within an individual across time (as described below where we examine paired observations with two time points per subject, V1 and V3).

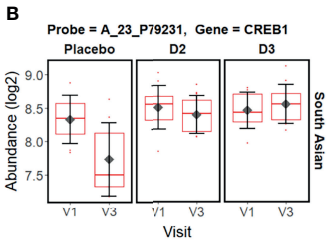
The five significant changes that were identified as occurring in response to treatment of the SA ethnic group with vitamin D_3, relative to the placebo, are summarised in **Supplementary Figure 1**. These are driven primarily by the marked changes in the placebo group between the V1 and V3 sampling points, and may be associated with the sustained low serum 25(OH)D_3, or high PTH, concentrations observed in this group of subjects. One of these differentially expressed genes encodes the cAMP response element binding protein CREB1 (**Figure 2B**) which is part of the cAMP-PKA-CREB signaling pathway in bone cells which contributes to the regulation of skeletal metabolism in response to PTH concentrations (44).

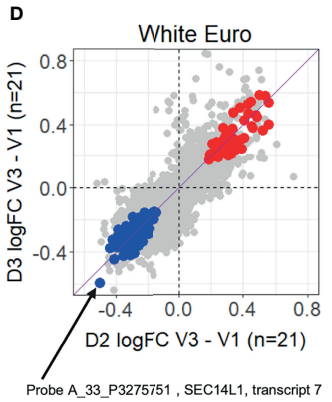
In contrast to the difference-in-difference analysis, the direct *within*-group comparisons of transcript abundance measurements at V3 versus V1 per subject identified large numbers of significant changes in gene expression, most notably in the WE D_2, WE D_3 and WE placebo groups (**Figures 2A, C** and **Supplementary Data File 2**). These statistically significant gene expression changes in the WE ethnic cohort arising in the vitamin D treatment groups are considered in more detail below. While there is some overlap in the groups of differentially expressed genes, only 13% of down-regulated differentially expressed genes (102 of 774) were common between the two vitamin D treatment groups. Conversely, 28% (216 of 774) and 59% (456 of 774) were uniquely down-regulated

A

Comparison	Sig. down probes (genes)	Sig. up probes (genes)
WE D2 V3 v V1	523 (498)	369 (350)
WE D3 V3 v V1	997 (943)	342 (329)
WE P V3 v V1	1171 (1096)	605 (565)
SA D2 V3 v V1	1 (1)	0 (0)
SA D3 V3 v V1	1 (1)	0 (0)
SA P V3 v V1	191 (189)	436 (408)
[WE D2 V3 v V1] v [WE P V3 v V1]	0 (0)	0 (0)
[WE D3 V3 v V1] v [WE P V3 v V1]	0 (0)	0 (0)
[WE D3 V3 v V1] v [WE D2 V3 v V1]	0 (0)	0 (0)
[SA D2 V3 v V1] v [SA P V3 v V1]	0 (0)	0 (0)
[SA D3 V3 v V1] v [SA P V3 v V1]	2 (2)	3 (3)
[SA D3 V3 v V1] v [SA D2 V3 v V1]	0 (0)	0 (0)







Comparison	logFC	adj.P.Val
WE D2 V3 v V1	-0.503	2.29E-03
WE D3 V3 v V1	-0.593	5.25E-05
WE P V3 v V1	-0.182	0.209

FIGURE 2 | **(A)** Summary of limma differential expression test results identifying significant changes (adj.P.Val ≤ 0.05) in transcript probe abundance within and between the experimental groups (see **Supplementary Data File 2** for full details). Both the number of unique microarray probes, and the number of unique genome loci they represent, are indicated. WE = "white European"; SA = "South Asian"; D2 = vitamin D_2 treatment group; D3 = vitamin D_3 treatment group; P = placebo group. **(B)** The CREB1 gene probe signal is significantly different (adj.P.Val=0.021) between the vitamin D_3-treated group and the placebo group over the course of the V1 to V3 study period in the South Asian cohort (i.e. [SA D3 V3 v V1] v [SA P V3 v V1] comparison from **Figure 2A**). **(C)** Venn diagrams showing the numbers of probes in the white European (WE) cohort that are specifically significantly (adj.P.Val ≤ 0.05) down- or up-regulated in V3 compared to V1 in each treatment group. **(D)** Comparison of the $\log_2$ fold-changes (FC) in abundance occurring from V1 to V3 in the WE cohort in the D_2 and D_3 treatment groups. Probes significantly up-regulated in both D_2 and D_3 groups but not the placebo are coloured red, while those similarly down-regulated are shown in blue (see **Supplementary Data File 3**). A probe detecting *SEC14L1* shows the largest decreases in abundance in those down-regulated by D_2 and D_3 but not placebo.

by D_2 and D_3, respectively (excluding those additionally down-regulated in the placebo group over the equivalent 12-week intervention)(**Figure 2C**). As noted above, serum 25(OH)D_3 concentration fell over the 12-week intervention period in the vitamin D_2-treated group; thus, changes in gene expression observed in this group could *a priori* either be due to the influence of vitamin D_2 itself, or could be attributable to the depletion of the endogenous 25(OH)D_3 reserves. It was therefore important to have included comparative transcriptomic analysis of the placebo group in this study to distinguish gene expression changes resulting from vitamin D_2 *per se* from those changes arising due to the depletion of 25(OH)D_3 in the vitamin D_2 treatment group.

It is notable that expression of a large number of genes was altered between the first and last visits (V1 to V3) in the non-treated placebo group. While some of these changes will be attributable to the reduced synthesis of vitamin D that is seen in northern latitudes over the winter months, there is also significant over-representation of genes known to exhibit seasonal differences in gene expression (45) (**Supplementary Figure 2**). There is however no unique association of seasonally expressed genes with the data for the WE placebo group; a similar significant enrichment in 'seasonal genes' is also observed for the significant changes in gene expression identified in the WE D_2 and WE D_3 treatment groups (**Supplementary Figure 2**) and therefore it is unlikely that this apparent seasonal effect exclusively reflects vitamin D-specific effects. There was no overlap between genes significantly down-regulated exclusively in the placebo group and those significantly up-regulated exclusively in the vitamin D treatment groups (or vice versa). Consistent with the 12-week period of the study, seasonal changes in gene expression are therefore a background feature of all the data collected, and is therefore a factor that needs to be considered when undertaking such vitamin D supplementation studies.

Probe A_33_P3275751, detecting *SEC14L1* gene transcription, showed the largest decrease in response to both forms of vitamin D, but not placebo participants, in the group of probe signals with significantly reduced abundance in the V3 samples compared with

V1 (**Figure 2D**). This probe was originally designed to detect 'transcript variant 7' and hybridizes to a location at the 3' end of the *SEC14L1* locus, detecting several splice variants. High expression of *SEC14L1* is significantly associated with lymphovascular invasion in breast cancer patients, where transcript abundance correlates positively with higher grade lymph node metastasis, and poor prognostic outcome (46). Its overexpression is also frequent in prostate cancer where *SEC14L1* has been identified as a potential biomarker of aggressive progression of the disease (47, 48).

Genes Significantly Down-Regulated by Both Vitamins D_2 and D_3, But Not Placebo, Are Enriched for Functions Associated With Immune Responses

Given the relatively small sample sizes used in this study, it is considered more appropriate to examine enrichment of cellular pathways among differentially expressed genes rather than focusing on changes in individual genes. Functional enrichment analysis of the genes represented by the probes specifically repressed by vitamins D_2 and D_3 (blue data points in **Figure 2D**) suggests that both supplements have suppressive effects on the immune response in the WE group (**Figure 3** and **Supplementary Data File 3**). Indeed, a significant ($p = 1.98 \times 10^{-6}$) protein-protein interaction network derived from the *Homo sapiens* medium confidence interactions curated in the STRING database (49) is centred on a highly connected group of proteins associated with the innate immune response, neutrophil degranulation and leukocyte activation (**Figure 3A**; all protein groups are detailed in **Supplementary Data File 3**). This includes the histone acetyltransferase, EP300, known to function as a transcriptional coactivator with VDR, the vitamin D receptor protein (50). Collectively, these findings are consistent with the emerging view that vitamin D exposure leads to a shift from a pro-inflammatory to a more tolerogenic immune status (1, 2).

Genes Significantly Up-Regulated by Both Vitamins D_2 and D_3, But Not Placebo, Include Components of Histone H4 and the Spliceosome

A similar analysis of the probes specifically induced by D_2 and D_3 (red points in **Figure 2D**) produced a significant ($p = 4.99 \times 10^{-9}$) protein-protein interaction network enriched primarily for mitochondrial and ribosomal proteins, but also involving two subunits of histone H4 and SNRPD2, a core component of the SMN-Sm complex that mediates spliceosomal snRNP assembly (**Figure 3B**).

Comparative Functional Enrichment Analysis Supports Roles for Vitamins D_2 and D_3 in the Suppression of Immunity, and in Chromatin Modification and Spliceosome Function

As a complementary approach to the functional enrichment analysis of specific subsets of genes whose expression is altered by supplementation with vitamin D_2 and D_3 but not by placebo treatment, a comparative functional enrichment analysis of all significant changes in each treatment group was performed (see Methods). This aimed to identify functional categories that are more extensively affected by vitamin D_2, or by vitamin D_3, or by both vitamins D_2 and D_3, than by the placebo, and took into consideration the changes occurring in the placebo treatment group across the 12 weeks of the study (**Figure 4** and **Supplementary Data File 4**). Consistent with the earlier analysis, functional categories associated with immunity and immune response pathways are prominent among those genes repressed by vitamin D supplementation (**Figure 4A**), while mitochondrial, ribosomal and spliceosomal functions are prominent in the induced genes (**Figure 4B**). Although the two vitamin treatments share many common categories identified from this analysis, the results also highlight some differences between the respective effects of D_2 or D_3 supplementation. For example, 'histone exchange' is significant only in the vitamin D_2 up-regulated genes, and 'chromatin modifying enzymes' are significant only in the D_3 down-regulated genes.

Overall, the observed differences in gene expression from the blood transcriptome presented in this study suggest that the physiological effects of vitamin D_3 and D_2 may be dissimilar.

Weighted Gene Correlation Network Analysis (WGCNA) Identifies Modules of Co-Expressed Genes That Significantly Correlate With Serum Markers of Vitamin D Supplementation in the WE and SA Ethnic Groups

WGCNA quantifies both the correlations between individual pairs of genes or probes across a data set, and also the extent to which these probes share the same neighbours (41). The WGCNA process creates a dendrogram that clusters similarly abundant probes into discrete branches, and subsequent cutting of the dendrogram yields separate co-expression modules, representing putatively co-regulated sets of genes. The first principal component of the expression matrix of each module defines the expression profile of the eigengene for the module, and this can then be correlated with experimental metadata. By allowing phenotypic traits to be associated with relatively small numbers (tens) of module eigengenes, instead of thousands of individual variables (*i.e.* gene probes), WGCNA both alleviates the multiple testing problem associated with standard differential expression analysis and also directly relates experimental traits to gene expression data in an unsupervised way that is agnostic of the experimental design.

WGCNA was used to construct separate signed co-expression networks for the WE and SA ethnic groups as described in the Methods, and Pearson correlations between expression of the module eigengenes and serum 25(OH)D_2, 25(OH)D_3, total 25 (OH)D and parathyroid hormone (PTH) concentrations were calculated (**Figure 5** and **Supplementary data files 6–8**). These results therefore ignore whether the data originates from the vitamins D_2, D_3 or placebo treatment groups and focus solely on the relationship between the serum metabolite concentrations and gene expression. A significant negative correlation was observed between 25(OH)D_2 concentration and expression

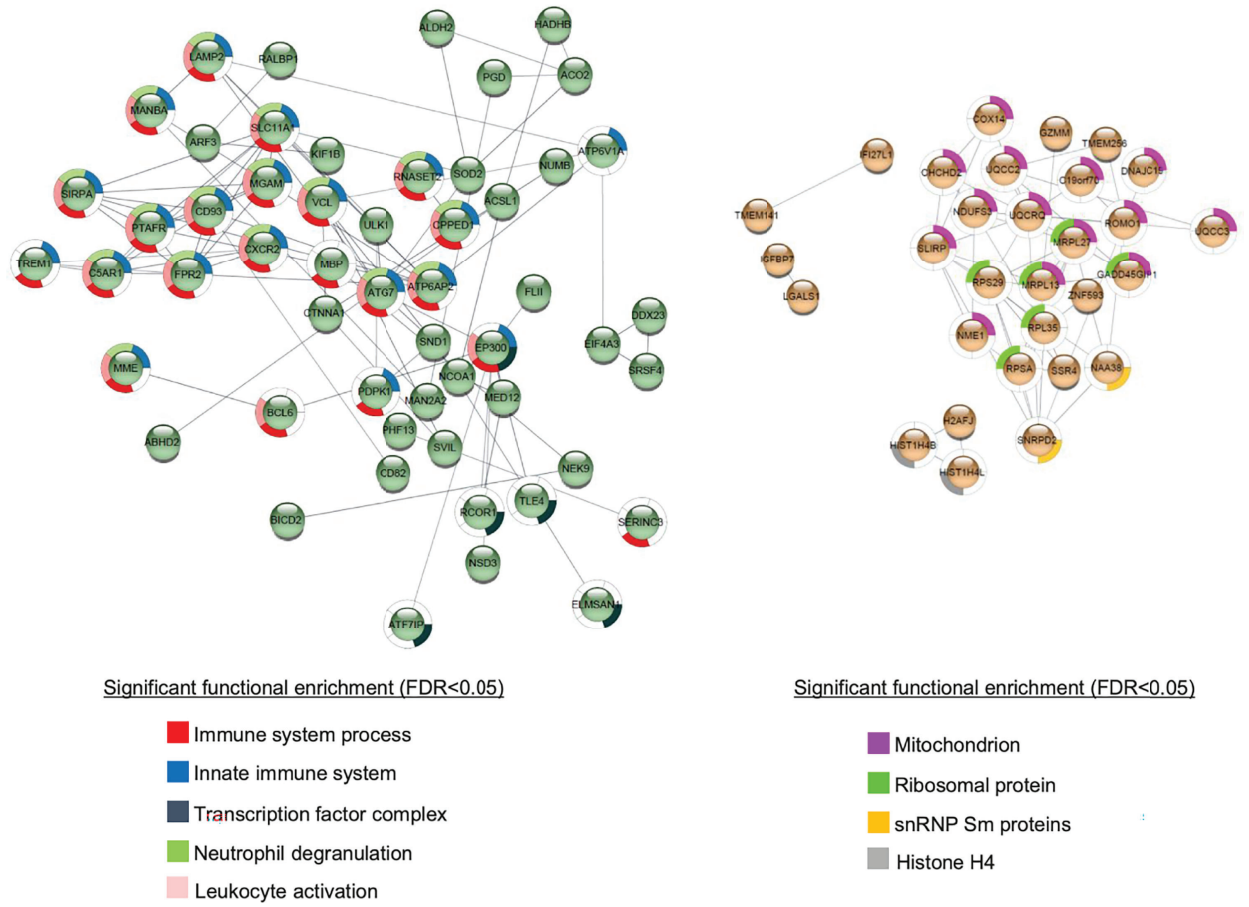


FIGURE 3 | Protein-protein interaction networks for gene products corresponding to the probes **(A)** significantly down-regulated or **(B)** significantly up-regulated in both the D_2 and D_3 treatment groups of the WE cohort, but not the placebo group. Details given in **Supplementary Data File 3**. The networks were generated using the STRING database of *Homo sapiens* medium confidence (0.4) interactions, and only connected nodes are shown. Networks for both **(A, B)** are significantly enriched for interactions compared to randomised sets, yielding p-values of 1.98×10^{-6} and 4.99×10^{-9}, respectively.

modules in the WE co-expression network that are enriched for immune-associated functions (midnight blue and pink modules in **Figure 5A**). This is consistent with the results from the different analytical approaches (presented in **Figures 3**, **4**). Similarly, the only module exhibiting a significant positive correlation with 25(OH)D_2 (and total 25(OH)D concentration), the red expression module, is enriched for GO categories associated with the ribosome and mRNA processing (**Supplementary Data File 6**). No significant correlations with serum 25(OH)D_3 concentrations were detected in the WE cohort.

Interestingly, stronger and more numerous correlations were observed in the co-expression network for the SA cohort (**Figure 5B**). In agreement with the WE network, an expression module significantly associated with the ribosome (black module in **Figure 5B**) showed a general positive correlation with total 25(OH)D concentration whereas a module enriched for histone binding (turquoise) had a significant negative correlation with 25(OH)D concentration. Strikingly however, modules enriched for immune response functions (midnight blue, orange, purple, yellow in **Figure 5B**) were consistently positively correlated with total 25(OH)D and usually 25(OH)D_3, but not 25(OH)D_2 concentrations, in the SA network, and negatively correlated with PTH concentrations. This raises the possibility that vitamin D supplementation may exert different effects on the immune system depending on ethnicity of the individual, may indicate that PTH status has an influence on the outcome and may reflect physiological differences resulting from the low baseline vitamin D status in SA women. In this context we observed that PTH concentration at the V1 sampling point in the SA cohort was elevated compared with that in the WE cohort (**Figure 1C**).

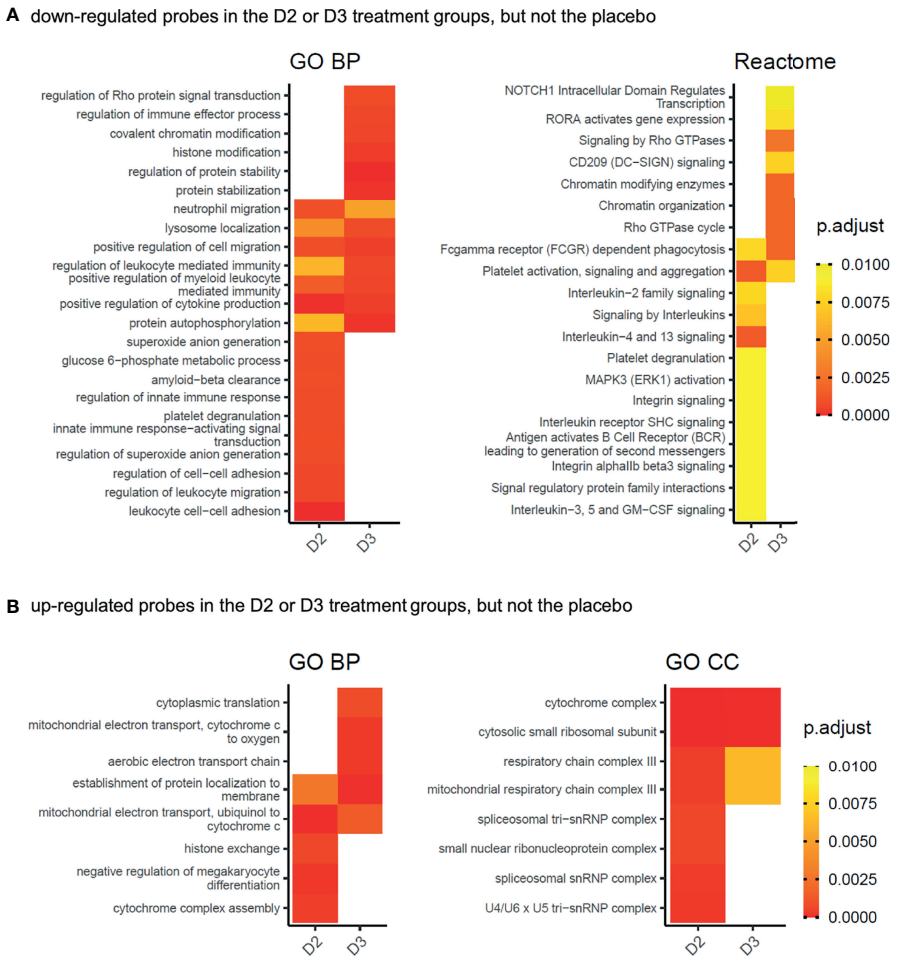


FIGURE 4 | Gene Ontology biological process (GO BP), cellular compartment (GO CC), or Reactome pathway functional categories significantly enriched in the gene products represented by the probes **(A)** significantly down-regulated (adj.P.Val ≤ 0.05) in the D2 or D3 treatment groups of the WE cohort, but not in the placebo group, and **(B)** significantly up-regulated (adj.P.Val ≤ 0.05) in the D2 or D3 treatment groups of the WE cohort, but not in the placebo group. Gene products represented by the significantly down-regulated probes in the comparisons WE D2 V3 v V1, WE D3 V3 v V1 and WE P V3 v V1 from **Figure 2A**, and possessing ENTREZ identifiers, were subjected separately to functional enrichment analysis using compareCluster (38). The details for each group are given in **Supplementary Data File 4**. Significantly enriched categories (p.adjust ≤ 0.01) from all groups were processed as described in the Methods section to visualise categories identified from the D_2 or D_3 treatment groups but not by the placebo. Heatmap tiles that are blank correspond to categories that did not meet the significance criteria applied during the processing. The complete networks for each differentially expressed group of genes are shown in **Supplementary Data File 5**.

Gene Sets That Respond to Interferon Alpha and Gamma Show Divergent Behaviour in the Vitamin D_2 and D_3 Treatment Groups of the WE Cohort

The molecular signatures database (MSigDB) hallmark gene sets represent coherent gene expression signatures related to well-defined biological states or processes (https://www.gsea-msigdb.org/gsea/msigdb/). Gene set enrichment analysis (GSEA) was performed to identify statistically significant (padjust<=0.05), concordant changes in expression of these hallmark sets between the V3 and V1 sampling times for the placebo, vitamin D_2, and vitamin D_3 treatment groups (**Figure 6** and **Supplementary Data File 9**). Interestingly, in the WE cohort, the gene sets defining the signature responses to interferon alpha and gamma exhibited divergent behaviour in the vitamin D_2 and D_3 treatment groups. This is evident as significant up-regulation over the course of the intervention study in the D_3 group but significant down-regulation in the D_2 group (see also **Supplementary Figures 4**, **5** illustrating the core genes accounting for the enrichment signals). This differed from the SA cohort where significant down-regulation was observed in the D_3 group (see also **Supplementary Figure 6**). It is acknowledged that some of the observed differences between the WE and SA groups could reflect the differences in the respective sample sizes. However, in cases where statistically significant differences are found, such as the reciprocal trend in the respective interferon responses these are not likely to be attributable to sample size differences.

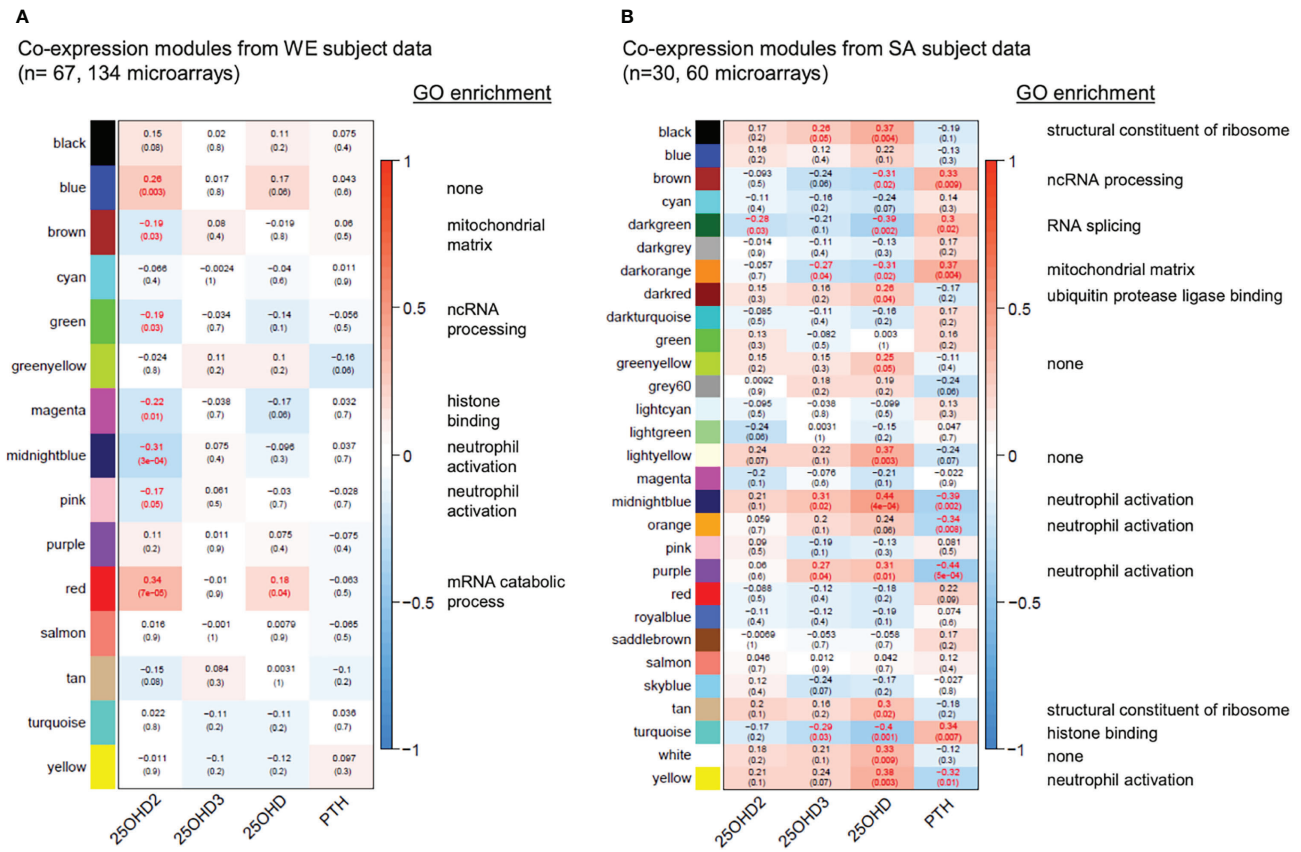


FIGURE 5 | Gene co-expression networks for the WE **(A)** and SA **(B)** ethnic groups. For each group of subjects, signed scale-free networks were constructed from the expression data using WGCNA to cluster probes with similar expression characteristics across all samples into discrete modules (colours on the y-axis: colours in **(A, B)** are independent). Module membership for the modules of genes identified in the WGCNA analysis of the data from the South Asian and white European cohorts are detailed in **Supplementary Data File 6**. The colour scale to the right of each panel represents the Pearson correlation (from -1 to +1) between the expression profile for each module's eigengene and the respective serum 25(OH)D_2, 25(OH)D_3, total 25(OH)D and PTH concentrations (x-axis). The Pearson correlation coefficients are also provided in each box, followed in brackets by an adjusted p-value, testing for the significance of each correlation. Statistically significant correlations (adjusted p-value ≤ 0.05) are indicated in red. A headline significant GO functional enrichment category (p.adjust ≤ 0.01) for each significant module is shown to the right; GO test results for each module are summarised in **Supplementary Data Files 7**, **8**).

DISCUSSION

In the present study, we conducted a longitudinal blood transcriptome analysis in 97 of the original cohort of 335 women from two ethnic backgrounds, South Asian (SA) and white European (WE), reported by Tripkovic at al (28). Extensive changes in gene expression in all three treatment groups were observed, some of which were unique to the vitamin D_2-treated or D_3-treated groups, with the majority exhibiting downregulation of transcription over the 12-week intervention period. Gene expression changes in the placebo group are likely to be attributable, at least in part, to seasonal drops in vitamin D status (**Figure 2**) (45, 51). The effects of vitamin D supplementation on gene expression take place superimposed on the natural seasonal changes that would have taken place in the absence of intervention. **Figure 7** presents a schematic diagram to help visualize the possible interplay between genetic factors, seasonal changes, vitamin D status and whole blood transcriptome expression.

Statistically significant gene expression changes were mainly detected as differences *within* each treatment group (i.e. differences at baseline (V1) versus 12 weeks after the intervention (V3) *within* each individual participant). In this respect, our longitudinal study design partly circumvents the problems that are often encountered with heterogeneity in inter-individual responses in omic studies of human populations, which make it difficult to detect robust changes between different groups of individuals. Recent longitudinal multi-omics studies are revealing strong interpersonal differences among individuals that can hinder statistical comparisons across different treatment populations or across disease cases and controls [e.g (52, 53).; M.P. Snyder, personal communication]. Our strategy to examine the *changes* in gene expression within each individual, from baseline to the treatment endpoint has enabled us to detect differences in the trajectory and magnitude of gene expression and, in combination with pathway analysis, has allowed us to extract physiologically meaningful information from the transcriptome data; this could

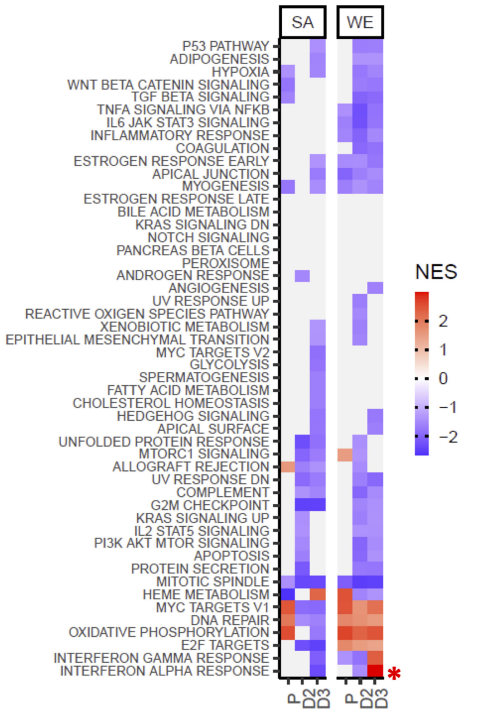


FIGURE 6 | Gene Set Enrichment Analysis using the MSigDB hallmark gene sets indicates divergent behaviour for the interferon alpha and gamma response gene sets following supplementation with vitamin D_2 and D_3 in the WE cohort. Coloured tiles in the heatmap correspond to gene sets exhibiting a statistically significant (padjust≤ 0.05), concordant change between the V3 and V1 sampling times for the placebo (P), vitamin D_2 (D2) or vitamin D_3 (D3) treatment groups in the SA or WE cohorts. Grey tiles indicate non-significance. A positive normalised enrichment score (NES) indicates up-regulation of a gene set in V3 relative to V1, and conversely down-regulation is indicated by a negative NES score. Full results are provided in **Supplementary Data File 9**, and the behavior of the leading edge, core genes accounting for the significant enrichment signals in the interferon alpha and gamma response sets are illustrated in **Supplementary Figures 4–6**.

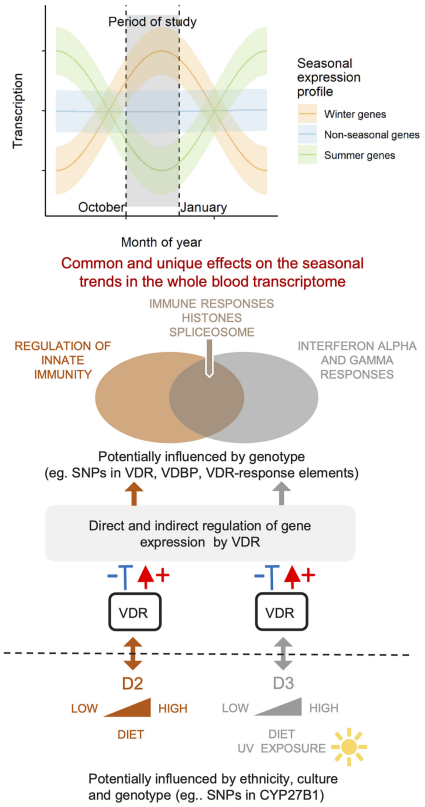


FIGURE 7 | A schematic diagram of this study in the context of genetic and seasonal factors that can influence physiological vitamin D status and the whole blood transcriptome. Dietary supplementation with vitamin D_2 or D_3 boosts the native levels of the active forms of these vitamins in the blood, and generates overlapping but different effects on the seasonal trends in gene expression. Non-supplemented placebo subjects remain on their natural trajectories for levels of active D_2 and D_3, and for their seasonal expression profile. Selected differentially expressed pathways from this study are indicated on the Venn diagram.

not have been achieved by examining differences across the different treatment groups. This *in vivo* human transcriptome study illustrates long-term effects on gene expression. The majority of differentially expressed genes identified in this study were down-regulated by vitamin D supplementation, with many of these encoded pathways involved in immunity. Observed gene expression changes are consistent with vitamin D exerting a modulating effect on the immune system, leading towards a more tolerogenic state, a concept reviewed by (1).

Vitamins D_2 and D_3 Do Not Influence Expression of the Same Genes in Whole Blood

Excluding genes that were also differentially expressed over the 12-week intervention in the placebo group, only 13% of down-regulated differentially expressed genes were common between the two treatment groups while 28% and 59% were uniquely down-regulated by vitamins D_2 and D_3, respectively (**Figure 2C**). For example, some biological processes such as histone modification and covalent chromatin modification are downregulated following vitamin D_3 supplementation only, while spliceosomal function are upregulated by vitamin D_2 only. Functional categories of genes enriched among the upregulated genes, following supplementation with either vitamin D_2 or D_3, include translation, mitochondrial and spliceosome function (**Figure 3B** and **Supplementary Data File 4**); statistically enriched biological cellular component terms in these functional categories are ribosomal proteins, components of the mitochondrial respiratory chain, two subunits of the histone H4 and snRNP Sm protein components of the spliceosome assembly. It is known that, in addition to influencing transcription, vitamin D can also influence post-transcriptional events by recruiting co-regulators (54). In this context it is relevant that components of the spliceosome, such as snRNP Sm proteins that mediate both transcriptional control and splicing decisions, leading to alternatively spliced transcripts (55), were upregulated by vitamin D supplementation in this study.

In light of the finding that vitamins D_2 and D_3 influence expression of different genes in the human blood transcriptome, recently we undertook a parallel *in vitro* study with a model rat cell line (56). We examined the respective influences of the two physiologically active forms of vitamin D, 1,25(OH)D_3 and 1,25 (OH)D_2 on differentiation and global gene expression in differentiating rat CG4 oligodendrocyte precursor cells and revealed considerable differences in the influence of the two types of vitamin D on gene expression at 24 h and after 72 h following onset of differentiation. We demonstrated that 1,25 (OH)D_3 and 1,25(OH)D_2 respectively influenced expression of 1,272 and 574 genes at 24 h following addition of the vitamin to the culture, where many of the changes in expression were specific to one or the other form of the vitamin (56). This study provides evidence of the different direct effects of the two active vitamin D metabolites on gene expression *in vitro* in cultured cells and provides some evidence that the changes we observed in the *in vivo* study may reflect the influence of the physiologically active forms of the vitamin.

Influence of Vitamin D on Expression of Genes Encoding Immune Pathways

In common to both the D_2 and D_3 treatment groups, but not the placebo group, we found that many different pathways of the immune system are differentially expressed (largely down-regulated) by vitamin D, consistent with the notion that one physiological role of vitamin D is to restrain, or balance, the activity of the immune system (1, 2) (**Figures 3A**, **4** and **Supplementary Data Files 3–5**). The immunomodulatory effects of vitamin D on both innate and adaptive immunity are well documented (57–59). In this regard, our observation that vitamin D_2 and D_3 supplementation in the WE cohort was associated with divergent patterns of expression for interferon alpha (type I) and interferon gamma (type II) signature gene sets stands out as particularly interesting (**Figure 6**). Vitamin D appears to modulate type I interferon activity. For example, it enhances the effects of type I interferon treatment on mononuclear cells from patients with multiple sclerosis (60), which parallels evidence for modest benefits of vitamin D as an adjunct treatment with type II interferon in multiple sclerosis patients (61, 62). Vitamin D may also help suppress symptoms in autoimmune diseases such as systemic lupus erythematosus (63), which are associated with chronic over-activity of interferon signalling and tentatively designated interferonopathies. Moreover, type I interferons play a critical role in defence against viral infections. Basal expression of type I interferon-stimulated genes, in the absence of infection, is key to priming a rapid and effective response to viral infection (64). There is currently intense interest in vitamin D as both a potential prophylactic and a therapeutic agent for treatment of SARS-CoV-2 infection. One of the proposed modes of action of vitamin D is modulation of interferon activity (65); in this context our observation that vitamin D_3 (but not vitamin D_2) enhances the expression of genes involved in the interferon alpha response, is highly relevant to susceptibility to viral infection. Indeed, a recent genome-wide association study (GWAS) in 2,244 critically ill Covid-19 patients identified genetic variants leading to reduced interferon type I signalling that are associated with severe Covid-19 disease (66).

Gene Expression Changes in Response to Vitamin D Are Partially Attributable to Ethnicity

We have also found differences in gene expression response to vitamin D according to ethnicity (with the caveat that the sample size of the SA group was smaller than the WE group and the average baseline vitamin D status was significantly lower relative to the WE group). Unlike the white European group, differences in gene expression *across* treatment groups were found in the South Asian group (**Figures 2A, B**) who received supplementation with vitamin D_3, where three genes were significantly upregulated and two down-regulated after the 12-week intervention relative to the placebo group (**Figure 2** and **Supplementary Figure 1**). cAMP-responsive element binding protein 1 (*CREB1*), one of the three upregulated genes, encodes a transcription factor that binds as a homodimer to the cAMP-responsive element. CREB1 protein is phosphorylated by several protein kinases and induces transcription of genes in response to hormonal stimulation of the cAMP pathway. The protein kinase A (PKA) pathways are activated by the parathyroid hormone PTH in response to low serum calcium levels to maintain serum calcium homeostasis, primarily by promoting vitamin D 1α-hydroxylation in the kidney. Both PTH and 1,25 $(OH)_2$ vitamin D have similar effects in promoting the maturation of osteocytes and opposing the differentiation of osteoblasts into osteocytes (67, 68). It is relevant to note that those in the SA cohort had elevated serum PTH at baseline relative to the WE cohort.

Ethnic differences in response are also suggested from the Weighted Gene Correlation Network Analysis (WGCNA) and the Gene Set Enrichment Analysis (GSEA), where the response to vitamin D_3 intake appeared to have the opposite effect on the type I and II interferon pathways in the SA group compared with the WE group (**Figure 6** and **Supplementary Figures 4**, **6**). However, direct correlation between the stimulation of immune responses, other than the interferon pathways, with an increase in serum 25(OH)D_3 concentration was evident in the SA group only. This is the opposite of that observed in the WE group where the effect of vitamin D supplementation was to suppress several immune pathways.

The transcriptome results differ considerably between the two different ethnic groups. While some of the differences may be attributable to differences in sample sizes studied between the two groups it is also possible that some of the differences represent genuine differences in the respective physiological responses of the two ethnic groups. Alternatively, the differences may reflect the starting physiological status of the SA participant group, who had considerably lower baseline vitamin D concentrations (and higher PTH concentrations) than the WE group. The importance of clarifying these different responses is given particular impetus by the emerging evidence for the interplay between ethnicity, skin tone, vitamin D status and susceptibility to viral infection, Covid-19 being of particular current relevance here (12, 13, 69, 70). The

transcriptomic data presented in this study might provide a useful context for further studies aimed at understanding the role of vitamin D in influencing the immune response to SARS-CoV-2 infection, particularly in relation to severe Covid-19. It is notable that the recent GenOMICC study of severe Covid-19 disease highlighted a link between severe Covid-19 and reduced type I interferon signalling (66). Our finding that vitamin D_3 appears to stimulate type I interferon signalling could be relevant in the context of its use as a prophylactic treatment. It should be noted here that in order to have a beneficial effect, a vitamin D replete status would be required prior to exposure to the virus, contributing innate immunity; administration of vitamin D following admission to hospital, even at high bolus doses, would not provide any clinical benefit because the virus would have already established itself. Recent clinical trials have borne out this latter point.

Concluding Remarks

Our ability to detect differences in the effects of vitamin D_2 and vitamin D_3 may have been negatively impacted by the relatively low statistical power and by the inclusion of two different ethnic groups among the 97 participants, since the transcriptome results from the two ethnic groups are clearly different; the number of participants represents a weakness of the present study. It will be important to replicate this study using a larger cohort in order to verify, or otherwise, the key findings from this study. From power calculations it is considered that for a whole human microarray-based gene expression study of this nature, and with gene expression changes of the magnitude we observe, at least 400 participants of each ethnic group should be recruited for each treatment, giving a total cohort size of 2,400. In this context, the biological interpretation of our findings should be considered as preliminary, requiring independent verification. A second limitation of this study was our failure to take account of the contribution of potential changes in blood cell composition across the seasons and across ethnicity; it is known that blood cell composition can vary significantly throughout the year (51, 71).

It is difficult to compare the findings of the present study with other reported *in vivo* studies because of the considerable differences in experimental design, including the use of supra-physiological vitamin D doses (up to 2,000 μg single bolus doses), different human population types and small sample sizes, which make statistical analysis not feasible [e.g. (33, 34)]. Furthermore, our study is unique in that it compared gene expression in participants given either of the two commonly used forms of vitamin D, vitamin D_2 and vitamin D_3. The present study evaluated overall expression changes in a complex blood cell population, which is also known to change in cellular composition across seasons (51). Furthermore, this study has focussed on identification of statistically significant pathway, or gene set, enrichment following sustained vitamin D supplementation, rather than a gene-focussed analysis; the latter approach is limited given the effect sizes and sample size of this study.

This study suggests that a more detailed consideration of the system-wide effects of these two forms vitamin D is warranted. This is perhaps of particular importance to at-risk ethnic groups, including black and South Asian populations who reside in northern latitudes. The studies would need to be time course-based (temporal) to track gene expression changes *within* individuals from baseline to defined sampling times and would need in-built control to account for seasonal gene expression changes. They would need to be designed specifically to answer whether different ethnicity or different vitamin D baseline concentrations give rise to different responses to vitamin D supplementation.

Since some pathways appear to be regulated specifically by vitamin D_3, or in some cases, in opposing directions by vitamin D_3 and D_2, future studies should investigate whether vitamin D_2 supplementation might counteract some of the benefits of vitamin D_3 on human health. This possibility is prompted by the findings from this cohort that the circulating concentration of 25(OH)D_3 within vitamin D_2-treated participants was significantly lower after the 12-week intervention than in the placebo group, who received no vitamin D supplements – suggesting that the former might be depleted by the latter. The results from this study suggest that guidelines on food fortification and supplementation with specific forms of vitamin D may need revisiting.

DATA AVAILABILITY STATEMENT

All microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-8600.

ETHICS STATEMENT

This study received ethical approval from the South-East Coast (Surrey) National Health Service Research Ethics Committee (11/LO/0708) and the University of Surrey Ethics Committee (EC/2011/97/FHMS). All participants gave written informed consent in agreement with the Helsinki Declaration before commencing study activities. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CS and SL-N conceived of the study. CS, GB, and SL-N supervised the project. LD, GB, and LT carried out the experiments, AH, GB, CS, LD, CM-L, HW, and RE analyzed and interpreted the transcriptome data. CS, AH, and GB wrote the first draft of the manuscript. AH generated the figures and all authors contributed to the final 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.2022.790444/full#supplementary-material

Supplementary Data File 1 | Metadata on serum concentrations of 25(OH)D_2, 25(OH)D_3, total 25(OH)D, PTH and calcium (albumin-adjusted) for the 97 subjects subjected to transcriptomic analysis [selected from the cohort described by Tripkovic et al. (28)].

Supplementary Data File 2 | [Note: data file is 9.1 MB.] Significant differences in the transcriptional responses occurring across the 12-week V1 to V3 period of the study *between* the vitamin D_2, vitamin D_3 and placebo treatment groups for each ethnic cohort, and significant changes *within* each group between the V3 and V1 sampling points (see **Figure 2**).

Supplementary Data File 3 | Functional analysis of the genes represented by the probes specifically repressed by D_2 and D_3 in the white European cohort (blue data points in) or induced by D_2 and D_3 in the white European cohort (red data points in **Figure 2D**).

Supplementary Data File 4 | Comparative functional enrichment analysis of all significant changes in each treatment group in the white European cohort (**Figure 2C**; and see **Figure 4**).

Supplementary Data File 5 | Networks illustrating all the functional categories significantly enriched (p.adjust < 0.01) in analysis of the gene products represented by the probes significantly up- or down-regulated (adj.P.Val < 0.05) in the white European cohort treatment groups (from data presented in **Supplementary Data File 4**).

Supplementary Data File 6 | Module membership for the modules of genes identified in the WGCNA analysis of the data from the South Asian and white European cohorts, as presented in **Figure 5**.

Supplementary Data File 7 | Gene ontology functional enrichment analysis results for the modules of genes identified in the WGCNA analysis of the data from the white European cohort.

Supplementary Data File 8 | Gene ontology functional enrichment analysis results for the modules of genes identified in the WGCNA analysis of the data from the South Asian cohort.

Supplementary Data File 9 | Gene Set Enrichment Analysis results for the analysis reported in **Figure 6**.

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Vitamin D Supplementation Modulates Platelet-Mediated Inflammation in Subjects With Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Trial

*Ebin Johny[1], Aishwarya Jala[2], Bishamber Nath[1], Md Jahangir Alam[3], Indra Kuladhipati[4], Rupam Das[4], Roshan M. Borkar[2] and Ramu Adela[1]**

[1] Department of Pharmacy Practice, National Institute of Pharmaceutical Education and Research, Guwahati, India, [2] Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education and Research, Guwahati, India, [3] Department of Biotechnology, National Institute of Pharmaceutical Education and Research, Guwahati, India, [4] Downtown Hospital, Guwahati, India

Background: Recently, our group identified increased platelet-mediated inflammation in type 2 diabetes (T2DM) patients, and it is a well-established risk factor for diabetes complications, particularly for the development of cardiovascular diseases (CVD). Furthermore, vitamin D is reported to play an important role in the modulation of platelet hyperactivity and immune function, although the effect of vitamin D on platelet-mediated inflammation is not well studied. Hence, we aimed to investigate the effect of vitamin D supplementation on platelet-mediated inflammation in T2DM patients.

Methods: After screening a total of 201 subjects, our randomized, double-blind, placebo-controlled trial included 59 vitamin-D-deficient T2DM subjects, and the participants were randomly assigned to placebo (n = 29) or vitamin D3 (n = 30) for 6 months. Serum vitamin D metabolite levels, immunome profiling, platelet activation, and platelet–immune cell aggregate formation were measured at baseline and at the end of the study. Similarly, the serum levels of inflammatory cytokines/chemokines were assessed by a multiplex assay.

Results: Six months of vitamin D supplementation increases the serum vitamin D3 and total 25(OH)D levels from the baseline ($p < 0.05$). Vitamin D supplementation does not improve glycemic control, and no significant difference was observed in immune cells. However, platelet activation and platelet immune cell aggregates were altered after the vitamin D intervention ($p < 0.05$). Moreover, vitamin D reduces the serum levels of IL-18, TNF-α, IFN-γ, CXCL-10, CXCL-12, CCL-2, CCL-5, CCL-11, and PF-4 levels compared to the baseline levels ($p < 0.05$). Our *ex vivo* experiment confirms that a sufficient circulating level of vitamin D reduces platelet activation and platelet intracellular reactive oxygen species.

Conclusion: Our study results provide evidence that vitamin D supportive therapy may help to reduce or prevent the disease progression and cardiovascular risk in T2DM patients by suppressing oxidative stress and platelet-mediated inflammation.

Clinical Trial Registration: Clinical Trial Registry of India: CTRI/2019/01/016921.

Keywords: type 2 diabetes, vitamin D, platelet, inflammation, oxidative stress

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****Correspondence:***
Ramu Adela
ramu@niperguwahati.in

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INTRODUCTION

Type 2 diabetes (T2DM) is a chronic metabolic disorder characterized by elevated blood glucose levels and insulin resistance. Diabetes remains a critical public health concern worldwide, with an estimated 537 million adults suffering from the disease and with T2DM accounting for 90% of all diabetes cases (1). Diabetes affects multiple organs, including the heart, muscle, skin, eyes, brain, and kidneys, and causes microvascular and macrovascular complications. Diabetic subjects exhibit a two- to four-fold more significant risk of developing cardiovascular disorders when compared to non-diabetic individuals (2). Insulin resistance and hyperglycemia together trigger inflammation, oxidative stress, and endothelial dysfunction, contributing to the development of cardiovascular diseases in type 2 diabetes patients. Platelet activation and platelet-mediated inflammation are other critical factors implicated in the pathogenesis of coronary artery disease in type 2 diabetes patients. Recently, our group has shown that hyperglycemia in type 2 diabetes causes platelet activation; aggregation further contributes to systemic inflammation and the complexity of both type 2 diabetes and the development of cardiovascular diseases (3).

Vitamin D is classified as a secosteroid and exists in two forms, *i.e.*, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Our previous article reported that lower vitamin D levels are associated with T2DM with coronary artery disease (2). Furthermore, vitamin D has recently gained prominence as a diabetes risk modifier as vitamin D supplementation has reduced the incidence of T2DM and improved glycemic control, owing to increased insulin production (4), decreased insulin resistance (5), and reduced inflammation (6). Several observational studies have also reported an inverse relationship between platelet activation and vitamin D levels in different disease conditions (7, 8). Recently, Sultan et al. reported that lower vitamin D levels significantly contribute to increased platelet aggregation in type 2 diabetes patients (9). However, there is a lack of evidence on the effect of vitamin D supplementation on platelet-mediated inflammation in type 2 diabetes. This study aimed to determine the effect of vitamin D supplementation on vitamin-D-deficient type 2 diabetes patients, focusing on platelet activation and platelet-mediated systemic inflammation.

METHODS

Study Design

The study was a single-center, randomized, double-blind, placebo-controlled study, and the participants were recruited from the outpatient unit of the departments of diabetes and cardiology at Downtown Hospital, Guwahati, Assam, India. The study was approved by Downtown Hospital Ethical Committee with approval number EC/DTH/CT/2018/10 and registered in the Clinical Trial Registry of India (CTRI) with registration number CTRI/2019/01/016921. The study was conducted from July 2019 to January 2021. The clinical trial was conducted following the principles outlined in the Declaration of Helsinki and the institutional and ethical standards. The individuals supplied written informed consent at the screening visit, and data on clinical history, physical examinations, and blood samples were obtained to confirm the eligibility criteria. The study design is shown in **Supplementary Figure S1A**.

Study Population

The study includes uncontrolled type 2 diabetes subjects with HbA1c ≥7% as defined by the American Diabetes Association (10, 11). Another inclusion criterion was subjects aged between 35 to 65 years with vitamin D deficiency [total 25(OH) D ≤20 ng/ml] at the baseline visit. The exclusion criteria were subjects who had a prior treatment with vitamin D, clinical or laboratory evidence of chronic disease of liver failure, renal failure, type 1 diabetes, cancer, and thyroid disease, and pregnancy. The overall flow chart of the study is shown in **Figure 1**.

Randomization and Intervention

With computer-generated randomization codes, the volunteers were randomly assigned to either the placebo block or the vitamin D3 block. The vitamin D3 group received 60,000 IU cholecalciferol/week for the initial 3 months as a management dose, followed by 60,000 IU/month for 3 months as a maintenance dose. The placebo group received a matching placebo (composed of starch powder) similar to vitamin D. After completing 6 months of intervention, data on clinical history, physical examinations, and blood samples were obtained. The vitamin D dosage pattern is shown in **Supplementary Figure S1B**.

Measurement of Clinical and Biochemical Parameters

Standardized methodologies were used to assess the height, weight, and hip and waist circumference. The body mass index (BMI) was determined by dividing the weight in kilograms (kg) by the square of the height in meters (m^2). The waist-to-hip ratio was obtained by dividing the waist circumference by the hip circumference. Blood pressure (BP) was measured in triplicate with Omron HEM automated BP monitor. The Xpress A1C analyzer (Accurex Biomedical Pvt. Ltd) was used to determine the glycated hemoglobin (HbA1c) level. The serum levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), creatinine, and uric acid were analyzed using Erba Chem 7 Biochemistry Analyser (Transasia Bio-Medicals, India). The urinary creatinine levels were measured by a colorimetric detection method (catalog number 40620, Accurex Biomedical). Human insulin levels

Abbreviations: ACD, acid citrate dextrose; BMI, body mass index; CVD, cardiovascular diseases; DBP, diastolic blood pressure; ELISA, enzyme-linked immunosorbent assay; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; IS, internal standard; IU, international unit; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; ROS, reactive oxygen species; SBP, systolic blood pressure; SOD, superoxide dismutase; T2DM, type 2 diabetes; VDBP, vitamin D-binding protein.

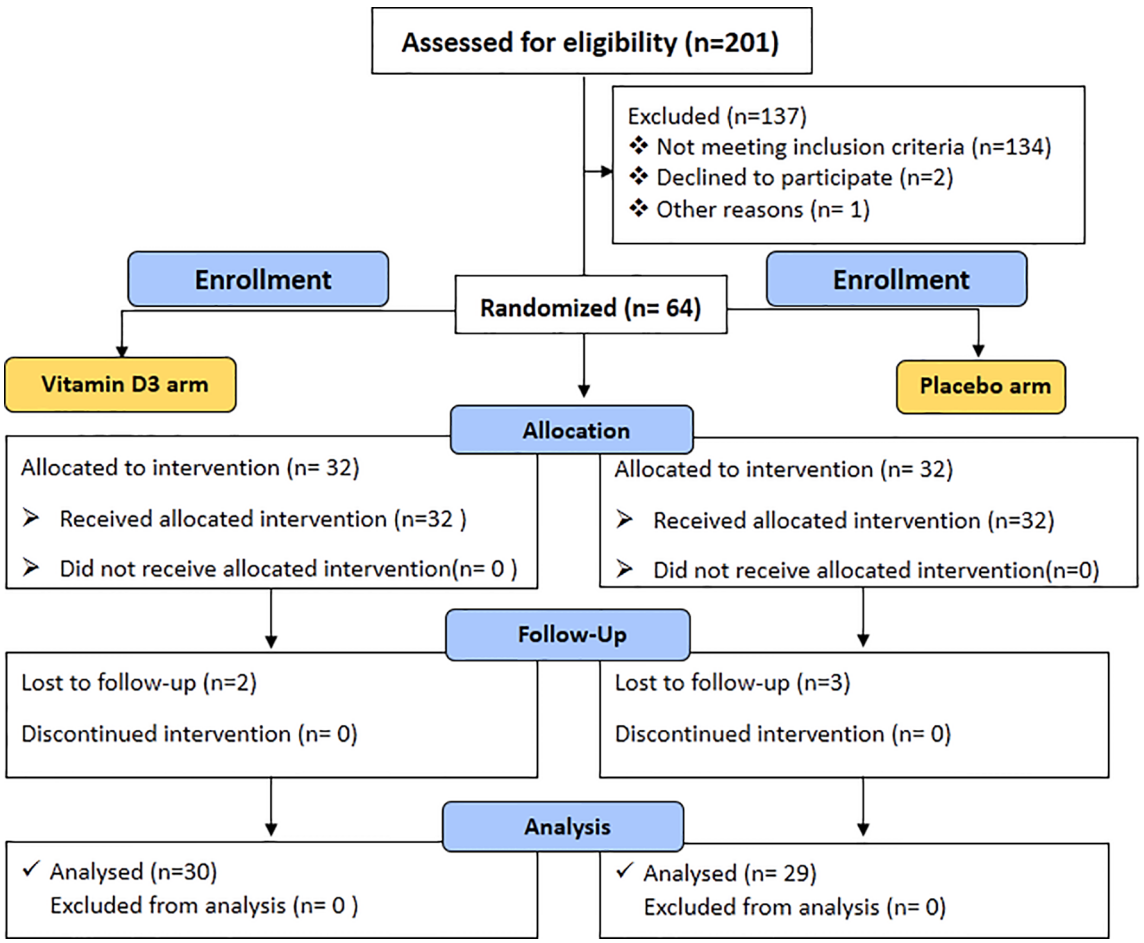

FIGURE 1 | Consort 2010 flow diagram.

were measured by using the ELISA kit (catalog number KBH0001, Krishgen Biosystems). HOMA-IR values were calculated by using the following formula: fasting blood sugar (FBS, mmol/L) × fasting insulin (mIU)/22.5.

Sample Preparation and Quantification of Vitamin D Metabolites by LC–MS/MS

The stock solutions of vitamin D3, vitamin D2, and their metabolites [25(OH)D3, 1,25(OH)2D3, 25(OH)D2, and 1,25 (OH)2D2] were prepared in ethanol. Furthermore, the working solution for the calibration curve (5–200 ng/ml) was prepared in methanol from the primary stock solutions. Similarly, an internal standard (IS) was prepared, *i.e.*, dihydrotachysterol of 50 ng/ml.

The extraction of vitamin D metabolites from serum samples was performed following the protocol described in our previously reported studies (2, 12). Briefly, liquid–liquid extraction technique was used to extract vitamin D metabolites from serum samples. A mixture of hexane/heptane/acetone—in the ratio of 45:40:15—was used as an extraction solvent. To 100 µl of serum sample, 10 µl of IS was added, followed by the addition of 1 ml extraction solvent. The mixture was vortexed thoroughly and placed in a shaker for 10 min. The samples were centrifuged at 6,000 rpm for 10 min at 4°C. The resulting supernatant was collected and dried under vacuum using an Eppendorf concentrator plus a centrifuge concentrator. The concentrated sample is reconstituted in 100 µl of methanol and subjected to liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) analysis. The details of the method, validation data (**Supplementary Table S1**), and LC–MS/MS chromatograms for vitamin D (**Supplementary Figure S2**) and its metabolite are provided in the supplementary file.

Measurement of Vitamin-D-Binding Protein

The human vitamin-D-binding protein (VDBP) was measured in the serum samples using a standard enzyme-linked immunosorbent assay protocol according to the manufacturer's instructions (catalog number DVDBP0B, R&D systems).

Flow Cytometry Analysis

The flow cytometry analysis was performed to measure platelet activation, detect immunome profiling, and perform platelet–

immune cell aggregate analysis following the protocol described in our previously reported study (3). Briefly, blood was collected by venepuncture in acid citrate dextrose (BD Vacutainer®) tubes for all the flow cytometry experiments and processed for analysis within 2 h of blood collection.

Measurement of Platelet Activation

Platelet-rich plasma (PRP) was isolated, and 25 µl of PRP was incubated with platelet activation markers PAC-1-FITC (BD, catalog number 340507) and CD62P)-APC (BD, catalog number 550888) for 20 min at room temperature. The platelets were washed with phosphate-buffered saline (PBS), resuspended in PBS, and analyzed in Attune NxT flow cytometer (Thermo Fisher Scientific, Singapore). The gating strategy for measuring the percent of platelet activation is described in **Supplementary Figure S3**.

Measurement of Platelet Factor 4

Platelet activation marker platelet factor 4 (PF-4) was measured in the serum samples using the ELISA method (catalog number K12-4574), according to the manufacturer's instructions.

Immunome Profiling and Measurement of Platelet–Immune Cell Aggregate Formation

Immunome profiling and platelet–immune cell aggregates were measured, as mentioned in our previous article (3). Briefly, whole blood was lysed, and cells were suspended in PBS with 1% w/v bovine serum albumin. Furthermore, the cells were incubated with respective antibodies for 20 min at room temperature. The cells were washed with PBS and analyzed by a flow cytometer. For compensation purposes, unstained and single antibody-stained cells were used. Work was divided into three panels. Each panel consists of one stained and unstained sample. Leucocytes were identified based on the forward scatter (FSC) and side scatter characteristics, and the same was confirmed using specific cell surface markers. Monocytes were identified based on FSC *vs*. SSC, were confirmed by CD14 APC Cy 7, and were further subdivided based on their differential expression of CD14 and CD16 into classical (CD14++ CD16-), intermediate (CD14++ CD16+), and non-classical (CD14+ CD16++) monocytes. Similarly, T cells were confirmed by their surface expression of CD3 from lymphocyte populations and further gated to obtain CD4 T cells (T-helper cells, CD3+ CD4+), CD8 T cells (cytotoxic T cells, CD3+ CD8+), and natural killer T cells (CD3+ CD56+). From the CD3-ve cells, natural killer cells (CD3- CD56+) were also identified. Dendritic cells (lineage–HLA DR+) were identified from WBC populations and were further subdivided into myeloid (HLA DR+ CD11C+) and plasmacytoid (HLA DR+ CD123+) dendritic cells. Platelet surface marker CD41a–PECY5 (BD, catalog number 559768) was included in each panel to detect platelet–immune cell interaction (3). The gating strategy for immunome profiling and detection of platelet–immune cell aggregation is described in **Supplementary Figures S4**, **S5**. The fluorescence minus one method for identifying platelet–immune cell aggregates is demonstrated in **Supplementary Figure S6**. AttuneTM NxT software v3.1.2 was used to perform the flow cytometry data analysis.

Measurement of Urinary 11-Dehydrothromboxane B2 by LC–MS/MS

Urinary 11-dehydrothromboxane B2 was measured by LC–MS/MS method. The extraction of 11-dehydroythromboxane B2 from urine samples was performed following solid-phase extraction (13). The separation of 11-dehydroythromboxane B2 and IS from endogenous substances was achieved using ZORBAX Eclipse Plus C18 Rapid Resolution HD (2.1 × 50 mm, 1.8 µm) and mobile phase consisting of a mixture of 2 mM ammonium formate with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) in a gradient program mode. The details of the method, method validation data (**Supplementary Table S2**), and LC–MS/MS chromatograms for 11-dehydroythromboxane B2 (**Supplementary Figure S7**) are provided in the supplementary file.

Measurement of Circulatory Inflammatory Markers

Circulating levels of cytokines and chemokines, *i.e*., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, TNFα, IFN-γ, GM-CSF, CXCL-1, CXCL-10, CXCL-12, CCL-2, CCL-3, CCL-4, CCL-5, and CCL-11, were measured using Invitrogen ProcartaPlex Hu Th1/Th2/multiplex immunoassay kit (catalog number EPX200-12173-901). Stored serum samples were thawed and centrifuged (10,000 rpm) to remove the debris. All experiments were performed according to manufacturer's protocol using an automated microplate washer (Bio-Rad) and analyzed using Bio-Plex 200 systems (Bio-Rad). Four of the 20 cytokines analyzed (IL-2, IL-5, IL-6, and GM-CSF) were omitted from further analysis because either more than 95% of the analyte concentration was below the lowest standard or its maximal fluorescence intensity value was near the background. The manufacturer provided standards for all cytokines and chemokines. A serum sample of 25 µl was used, and 100 bead events/bead region was acquired. The mean florescent intensity was measured using Bio-Plex manager software, version 6.2. All samples were measured in singlet, whereas blank and standards were measured in duplicate.

Measurement of Oxidative Stress Markers and Nitric Oxide Levels

To find the effect of vitamin D supplementation on oxidative stress, we have measured the circulatory superoxide dismutase (SOD), glutathione (GSH), and total nitric oxide (TNO) levels. Total nitric oxide (catalog number K023-H1, Arbor Assays), SOD activity (catalog number 19160-1KT-F, Sigma Aldrich), and glutathione (catalog number CS0260, Sigma Aldrich) were measured in stored serum samples using the colorimetric detection protocol according to manufacturer's instructions.

Ex Vivo Measurement of Platelet Activation and Intracellular ROS

To further examine the influence of vitamin D on platelet activation, platelets from healthy volunteers were incubated at 37°C in 5% CO_2 atmosphere in the plasma of healthy control

subjects, plasma of T2DM subjects with vitamin D deficiency (baseline), and plasma of T2DM subjects with sufficient vitamin D (6 months). After 1, 3, and 6 h of incubation, the cells were washed and stained with CD62P-APC for 20 min at room temperature. The percentage of P-selectin-positive cells was determined by flow cytometry.

Similarly, to measure the influence of vitamin D on platelet intracellular reactive oxygen species (ROS), platelets from healthy subjects were incubated with different plasma samples from subjects of healthy control, T2DM with vitamin D deficiency, and T2DM with sufficient vitamin D as mentioned above. Furthermore, the cells were washed and stained with DCFDA (catalog number D6883, Sigma Aldrich). The mean fluorescent intensity of DCFDA was assessed using a flow cytometer to measure platelet intracellular ROS.

Statistical Analysis

Parametric data were presented as mean ± standard deviation, whereas non-parametric data were summarized as median with interquartile range (25th to 75th quartiles). We used Shapiro–Wilk tests to examine the normality of the data. Comparisons between placebo and vitamin D3 groups at baseline were tested using unpaired *t*-test or Mann–Whitney *U*-test, whereas paired Student's *t*-test or Wilcoxon matched-pair test was used to compare the difference between the baseline and 6-month data. Different heat map visualizations of circulating inflammatory markers were made using the "pheatmap" v1.0.12 package of R programming interface. Box plots were produced using the packages ggplot2 (v0.4.0) and readr (v2.0.2) of R 4.0.2 running under RStudio. The statistical analysis was performed using GraphPad Prism, version 8.0.2 (263) (GraphPad Software, San Diego, CA, USA).

RESULTS

Characteristics of the Study Group

A total of 201 patients were screened for the study. A total of 137 participants were excluded from the trial, of whom 134 did not meet the inclusion criteria. Two declined to participate in the study, while one subject was excluded from the study due to access concerns for the subsequent visit. Finally, 64 subjects were enrolled, randomized, and allocated an intervention. Two patients in the vitamin D3 group and three in the placebo group did not attend the follow-up visit. A total of 59 patients [placebo (n = 29) and vitamin D3 (n = 30)] completed the 6-month follow-up. The baseline characteristics of the participants in each arm are shown in **Table 1**. There was no significant difference observed between the study groups regarding demographic details and clinical and biochemical characteristics. No statistically significant difference was reported in baseline total 25-OH vitamin D levels in the two treatment groups.

Serum Vitamin D Metabolite Levels Before and After Intervention

We employed LC–MS/MS to assess vitamin D and its metabolites in the serum at baseline and 6 months after the intervention to confirm that vitamin D supplementation increased the serum levels of its metabolites. The results are shown in **Supplementary Table S3**. Vitamin D3 supplementation showed a significant increase in the serum levels of vitamin D3 (baseline, 1.79 ± 0.70; 6 months, 14.17 ± 15.74), 25-OH vitamin D3 (baseline, 14.02 ± 5.76; 6 months, 53.12 ± 16.44), and total 25-OH vitamin D (baseline, 14.15 ± 5.8; 6 months, 51.99 ± 16.46) at 6 months as compared to the baseline levels ($p < 0.0001$). However, no significant changes were observed in the placebo group (**Figures 2A–C**).

Similarly, VDBP, the major regulator of the delivery of vitamin D metabolites to target cells, was measured in serum using the ELISA method. We observed a significant ($p < 0.05$) increase in VDBP level at 6 months (170.6 ± 60.24) of vitamin D supplementation compared to the baseline level (205.4 ± 88.30) (**Figure 2D**).

Effect of Vitamin D on Glycemic Parameters

Glycemic parameters (FBS and HbA1c) were measured, and HOMA-IR values were calculated to know the effect of vitamin D supplementation on managing type 2 diabetes. However, no significant differences were observed between the values at baseline and at 6 months in the placebo or vitamin D3 group (**Figure 3**).

Effect of Vitamin D on Immune Cells

Immunome profiling of monocyte cells, natural killer cells, and dendritic cells was done using flow cytometry at baseline and at 6 months of intervention in both the placebo and vitamin D treatment groups. The results are shown in **Table 2**. However, no significant difference was observed in the percentage of different immune cells after the intervention.

Effect of Vitamin D Supplementation on Platelet Activation Markers

The vitamin D supplementation effects on the expression levels of platelet activation markers (P-selectin and PAC-1) at baseline and at 6 months of placebo and vitamin D are presented in **Figures 4A, B**. Our results showed that vitamin D supplementation significantly reduced both PAC-1 and p-selectin expression in the platelets ($p < 0.05$).

Effect of Vitamin D Supplementation on the Levels of Circulating Platelet Factor 4

The serum levels of platelet factor 4 (serum marker for platelet activation) were also measured in both placebo and vitamin D treatment groups by the ELISA method. Our result showed that the PF-4 (pg/ml) levels decreased after vitamin D supplementation compared to the baseline levels (**Figure 4C**).

Effect of Vitamin D Supplementation on the Levels of 11-Dehydrothromboxane B2

We also measured the urinary levels of 11-dehydrothromboxane B2, a urine marker for platelet activation, in both placebo (n = 12) and vitamin D treatment (n = 12) groups at baseline and at 6 months

TABLE 1 | Baseline clinical and biochemical variables in the study groups.

Variables	Study groups	
	Placebo (*n* = 29)	Vitamin D3 (*n* = 30)
Age (years)	55.06 ± 9.57	53.6 ± 9.6
Sex (M/F)	20/9	22/8
Body mass index (kg/m^2)	26.03 ± 3.28	25.72 ± 4.09
Waist-to-hip ratio	0.97 ± 0.06	0.97 ± 0.07
Systolic BP (mmHg)	137.76 ± 16.47	133.3 ± 15.34
Diastolic BP (mmHg)	82.03 ± 7.63	79.2 ± 7.33
FBS (mg/dl)	191.31 ± 55.49	212.47 ± 63.36
HbA1c (%)	7.93 ± 1.43	8.22 ± 1.30
Total 25-OH vitamin D (ng/ml)	11.95 ± 5.05	14.15 ± 5.8
Duration of T2DM (years)[a]	8 (2–12)	10 (4.75–15)
Total cholesterol (mg/dl)	178.14 ± 32.64	166.7 ± 23.47
Triglycerides (mg/dl)	150.17 ± 51.05	163.43 ± 50.31
HDL (mg/dl)	44.76 ± 8.45	44.07 ± 8.80
LDL (mg/dl)	99.45 ± 19.27	91.4 ± 13.03
Uric acid (mg/dl)	5.94 ± 1.70	5.88 ± 1.38
Creatinine (mg/dl)	1.17 ± 0.26	1.29 ± 0.24
Alcoholic history (yes/no)	5/24	4/26
Smoking history (yes/no)	5/24	6/24
Diabetic medications (%)		
Metformin	3 (10.3)	2 (6.7)
Metformin + sulfonylureas	18 (62.06)	19 (63.3)
Metformin + α-glucosidase inhibitors	2 (6.9)	2 (6.7)
Metformin + DPP-4 inhibitors	1 (3.4)	2 (6.7)
Metformin + sulfonylureas + α-glucosidase inhibitors	5 (17.2)	4 (13.3)
Metformin + sulfonylureas + DPP-4 inhibitors	0 (0)	1 (3.3)

Data are presented as mean ± SD, and unpaired test is used for comparisons between outcome groups.

DPP-4, dipeptidyl peptidase 4.

[a]*Presented as median (Q1–Q3) and compared using Mann–Whitney U-test.*

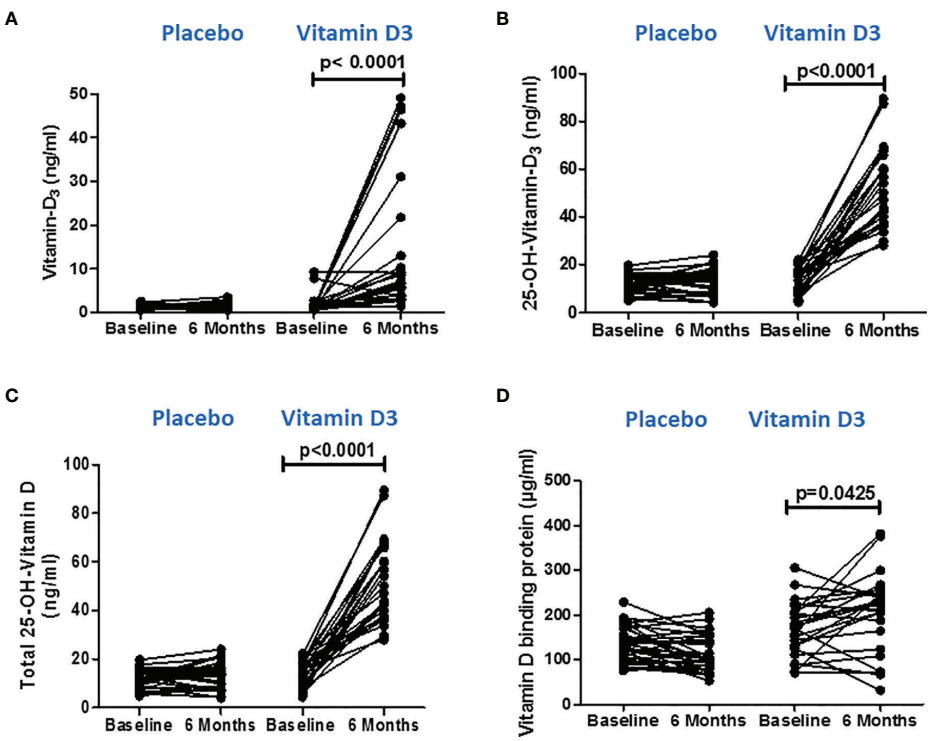

FIGURE 2 | Serum vitamin D metabolite levels after vitamin D supplementation. **(A)** Vitamin D3, **(B)** 25(OH) D3, and **(C)** total 25(OH) D levels at baseline and 6 months in the placebo and vitamin-D3-treated groups. **(D)** Serum vitamin-D-binding protein values at baseline and at 6 months in the placebo and vitamin-D3-treated groups. Wilcoxon matched-pair test is used for the comparison between baseline and 6 months in the placebo and vitamin-D3-treated groups.

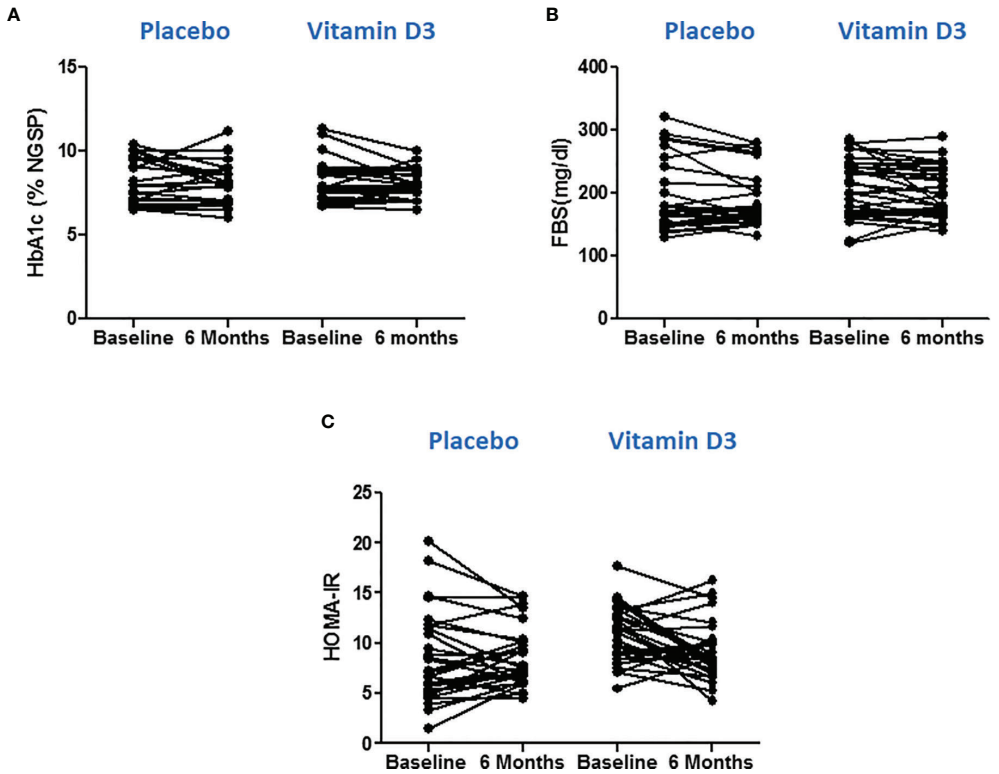

FIGURE 3 | Effect of vitamin D on glycemic parameters. **(A)** Glycated hemoglobin, **(B)** fasting blood sugar, and **(C)** HOMA-IR values at baseline and 6 months in placebo and vitamin D3 treated group. Wilcoxon matched pair test is used for comparison between baseline and 6 months in the placebo and vitamin-D3-teated groups.

using the LC–MS/MS technique. We observed a significant decrease in 11-dehydrothromboxane B2 (ng/mg creatinine) levels after 6 months of vitamin D supplementation (**Figure 4D**).

Ex Vivo Assessment of Vitamin D on Platelet Activation

To further confirm the effect of the circulatory levels of vitamin D on platelet activation, we performed an *ex vivo* assay. Platelets that were incubated with the plasma of vitamin-D-sufficient T2DM subjects showed a decreased platelet activation than the platelets incubated in the plasma of vitamin-D-deficient T2DM subjects (**Figure 4E**).

Effect of Vitamin D on Platelet Aggregate Formation With Immune Cells

The vitamin D supplementation effects on platelet–immune cell aggregate formation at baseline and at 6 months of placebo and vitamin D treatment are presented in **Table 2** and **Figure 5**. Platelet aggregate formation with monocytes, classical (CD14++ CD16-) monocytes, intermediate (CD14++ CD16+) monocytes, non-classical (CD14+ CD16++) monocytes, neutrophils, T cells, natural killer (CD3- CD56+) cells, and dendritic (lineage– HLA DR+) cells were significantly reduced after 6 months of vitamin D supplementation. Platelet aggregate with CD4 (CD3+ CD4+) T cells, CD8 (CD3+ CD8+) T cells, natural killer T (CD3+ CD56+) cells, plasmacytoid dendritic (HLA DR+ CD123+) cells, and myeloid dendritic (HLA DR+ CD11C+) cells were also found to be decreased after vitamin D supplementation. However, the changes were not statistically significant. There were no significant changes observed in the placebo group.

Effect of Vitamin D Supplementation on Circulating Inflammatory Markers

A heat map showing the serum levels of the 16 cytokines and chemokines is presented in **Figure 6A**. The median values of each cytokine/chemokine at baseline and at 6 months of the placebo and vitamin D treatment groups are further presented in **Figure 6B**. Our data showed that serum IL-18, TNF-α, IFN-γ, CXCL-10, CXCL-12, CCL-2, CCL-5, and CCL-11 levels significantly decreased after vitamin D supplementation (**Figure 7**). However, no statistically significant changes were observed in the placebo treatment group.

Effect of Vitamin D on Superoxide Dismutase Activity, Glutathione, and Nitric Oxide Levels

We assessed the SOD activity, glutathione, and total nitric oxide levels in the serum samples to find the mechanism of decreased platelet activation after vitamin D supplementation. We observed increased SOD activity (% inhibition rate) in the

TABLE 2 | Percentage of immune cells, platelet activation, and platelet–immune cell aggregation in the study groups.

	Placebo (*n* = 29)			Vitamin D3 (*n* = 30)		
	Baseline	**6 Months**	***P*-value**	**Baseline**	**6 months**	***P*-value**
Monocyte subsets						
Classical monocytes (%)	82.44 (76.62–88.36)	78.94 (73.99–83.36)	0.12	84.89 (80.86–87.50)	83.47 (77.58–88.23)	0.76
Intermediate monocytes (%)	9.75 (4.16–12.35)	10.09 (7.62–14.99)	0.07	8.43 (5.80–10.93)	8.49 (6.20–13.55)	0.76
Non-classical monocytes (%)	7.87 (5.13–12.73)	11.79 (9.16–16.30)	0.06	6.93 (4.39–9.11)	8.37 (5.46–11.21)	0.26
T cell subsets						
CD4 cells (%)	58.26 (53.29–67.50)	60.12 (49.70–66.31)	0.49	53.14 (49.62–65.37)	58.65 (49.23–67.02)	0.24
CD8 cells (%)	28.69 (20.24–31.06)	26.45 (21.70–32.42)	0.74	26.07 (21.12–33.30)	28.37 (22.40–32.17)	0.94
NKT cells (%)	4.76 (3.59–7.25)	3.75 (2.5–5.30)	0.19	5.17 (3.25–8.35)	3.93 (3.16–5.89)	0.06
NK cells (%)	1.96 (1.30–3.0)	1.32 (0.81–2.37)	0.06	1.98 (1.58–3.08)	1.58 (0.81–3.21)	0.31
Dendritic cells and subset						
Dendritic cells (%)	0.33 (0.19–0.43)	0.34 (0.18–0.39)	0.07	0.35 (0.21–0.51)	0.33 (0.20–0.56)	0.74
Myeloid dendritic cells (%)	0.19 (0.12–0.27)	0.13 (0.08–0.24)	0.06	0.24 (0.13–0.37)	0.19 (0.11–0.35)	0.65
Plasmacytoid dendritic cells (%)	0.098 (0.05–0.14)	0.06 (0.03–0.12)	0.26	0.08 (0.05–0.15)	0.08 (0.04–0.16)	1.00
Platelet activation markers						
Pac-1 expression (%)	0.29 (0.10–0.51)	0.22 (0.12–0.49)	0.53	0.20 (0.07–0.57)	0.10 (0.09–0.18)	**0.03**
P-selectin expression (%)	56.0 (40.72–63.08)	53.17 (41.45–60.)	0.45	53.83 (42.51–59.76)	34.10 (25.76–47.96)	**<0.001**
Platelets aggregate formation with innate immune cells						
Platelet–monocyte aggregates (%)	85.25 (74.33–90.83)	85.35 (76.25–87.33)	0.43	80.0 (71.75–87.337)	49.80 (36.80–67.88)	**<0.001**
Platelet–classical monocyte aggregates (%)	87.55 (73.23–92.96)	84.80 (72.85–86.81)	0.27	84.33 (74.67–89.75)	45.14 (33.11–65.03)	**<0.001**
Platelet–intermediate monocyte aggregates (%)	93.65 (87.11–91.64)	89.92 (83.66–96.95)	0.18	94.80 (88.09–98.12)	72.41 (42.69–87.38)	**<0.001**
Platelet–non-classical monocyte aggregates (%)	92.85 (87.45–95.91)	91.84 (84.73–95.61)	0.96	91.39 (85.44–100)	64.40 (52.73–81.04)	**<0.001**
Platelet–neutrophil aggregates (%)	70.36 (58.60–78.14)	76.01 (59.05–86.83)	0.07	62.96 (54.53–69.92)	54.10 (39.78–65.40)	**0.004**
Platelet–T cell aggregates (%)	25.52 (19.82–33.90)	24.92 (19.69–35.63)	0.39	28.55 (20.91–34.83)	23.25 (19.96–28.66)	**0.001**
Platelet–CD4 cell aggregates (%)	17.90 (12.99–27.16)	19.38 (12.97–29.36)	0.25	20.44 (15.23–30.19)	19.19 (13.85–22.87)	0.07
Platelet–CD8 cell aggregates (%)	28.76 (20.24–34.15)	26.81 (22.23–36.93)	0.82	29.12 (21.12–35.83)	24.36 (21.67–30.22)	0.08
Platelet–NKT cell aggregates (%)	27.52 (18.45–33.77)	30.76 (21.25–37.09)	0.39	29.73 (25.87–36.77)	28.12 (22.04–35.64)	0.48
Platelet–NK cell aggregates (%)	24.55 (18.57–35.50)	19.20 (15.70–29.85)	0.09	26.80 (13.54–33.21)	17.58 (13.24–25.56)	**0.03**
Platelet–dendritic cell aggregates (%)	44.63 (32.33–63.31)	44.85 (35.35–64.25)	0.24	44.11 (34.0–55.89)	31.49 (17.33–48.69)	**0.004**
Platelet–myeloid dendritic aggregates (%)	48.54 (34.91–78.45)	76.66 (50.30–89.20)	0.06	46.86 (26.88–58.70)	39.10 (21.24–70.13)	0.91
Platelet–plasmacytoid dendritic aggregates (%)	43.94 (31.14–51.25)	50.57 (34.13–66.60)	0.23	46.66 (17.39–62.50)	39.53 (27.84–52.72)	0.85

Data are presented as median (25th–75th percentile). Baseline and 6-month intervention data were compared using paired t-test or Wilcoxon rank sum test according to the data distribution. Bold values denote statistical significance p<0.05.

serum samples after vitamin D supplementation ($p < 0.05$) (**Figure 8A**). Similarly, the serum levels of glutathione increased after 6 months of vitamin D supplementation ($p < 0.05$) (**Figure 8B**). Total nitric oxide, an inhibitor of platelet activation, was significantly increased in serum samples after vitamin D supplementation ($p < 0.05$) (**Figure 8C**).

Ex Vivo Assessment of Vitamin D on Platelet Intracellular ROS

To confirm the effect of vitamin D on oxidative stress, we have performed an *ex vivo* experiment; platelets incubated in the plasma samples of vitamin-D-sufficient T2DM subjects showed lower intracellular ROS levels than the platelets incubated in the plasma samples of vitamin-D-deficient T2DM subjects (**Figure 8D**).

DISCUSSION

In the present randomized, placebo-controlled trial, vitamin D3 supplementation improved the circulatory vitamin D levels in type 2 diabetes patients. Our study provides the first and strongest evidence that vitamin D supplementation reduced platelet activation, platelet–immune cell aggregates, inflammation, and oxidative stress in vitamin-D-deficient type 2 diabetes patients. According to previously published literature, vitamin D deficiency is a global health concern, and the prevalence of vitamin D deficiency in India is 80–90% (14). Vitamin D deficiency and the significance of vitamin D in the pathogenesis of type 2 diabetes and associated complications have piqued the researchers' curiosity. In agreement with previous studies, we observed that vitamin D3 supplementation improved the circulatory levels of vitamin D metabolites. Different doses of vitamin D have been used in clinical trials to treat vitamin D deficiency (15–17). However, our study demonstrates that cholecalciferol (vitamin D3) supplementation with 60,000 IU/week for 3 months, followed by 60,000 IU/month up to 6 months, improved and helped maintain sufficient vitamin D levels in vitamin-D-deficient type 2 diabetes patients. After vitamin D supplementation, the serum levels of total 25-OH vitamin D in all patients were below the toxic levels (<100 ng/ml). Further to this, no clinically significant adverse events were detected in either of the two groups during

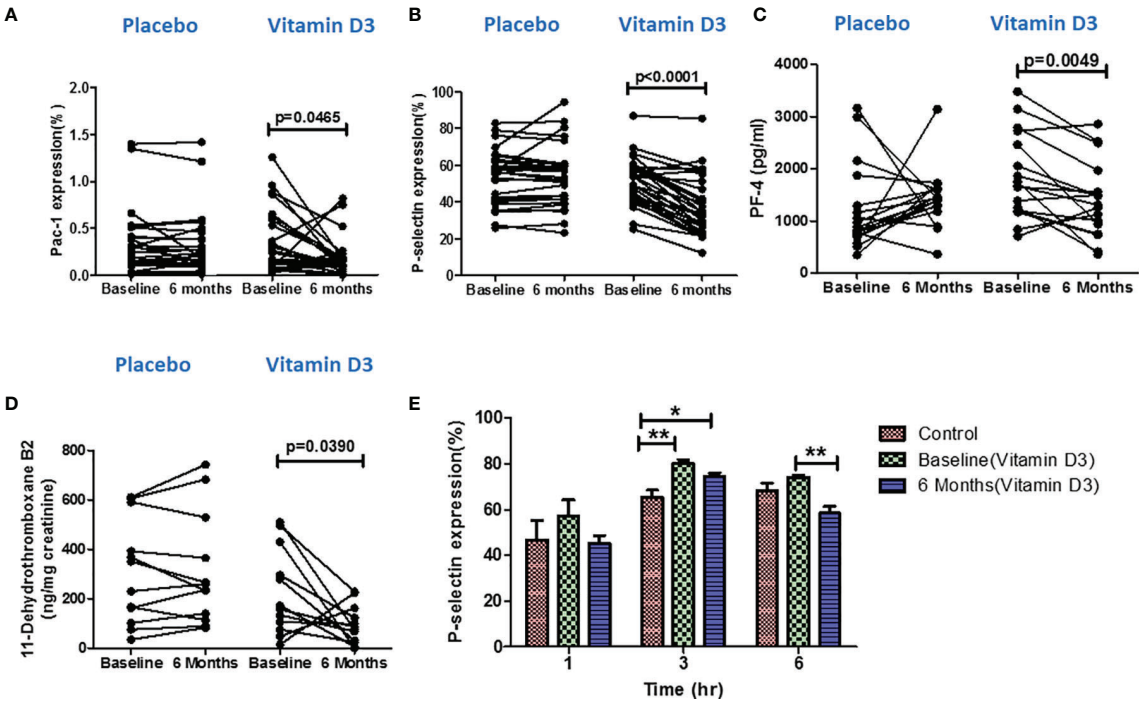

FIGURE 4 | Effect of vitamin D on platelet activation. Percentage of **(A)** PAC-1 expression, **(B)** P-selectin expression, **(C)** platelet factor-4 levels in serum samples, and **(D)** 11-dehydrothromboxane B2 (ng/mg creatinine) level in urine samples at baseline and 6 months in the placebo and vitamin-D3-treated groups. Wilcoxon matched-pair test is used for the comparison between baseline and 6 months in the placebo and vitamin-D3-treated group. **(E)** *Ex vivo* analysis of platelet activation following the incubation of platelets in the plasma samples of healthy control at baseline and 6 months of the vitamin D3 treatment group at different time points (1, 3, and 6 h). One-way ANOVA with Bonferroni test was used for the comparison between outcome groups. * denotes $P < 0.05$ and ** denotes $P < 0.01$.

the research period.

Apart from the vitamin D metabolites, the serum levels of VDBP were also shown to be higher after vitamin D treatment. The vitamin-D-binding protein is a key factor for regulating 25-OH-vitamin D concentrations in the circulation and controls 25-OH vitamin D and 1, 25(OH) $_2$ vitamin D bioavailability to target tissues. Similar to our findings, Berg et al. reported increased serum levels of VDBP after vitamin D2 supplementation (18).

The results of the current clinical trial demonstrated that 6 months of vitamin D supplementation did not improve the FBS, HbA1c, or HOMA-IR value. A recent meta-analysis that included 20 randomized controlled trials found that oral vitamin D supplementation did not influence the FBS, HbA1c, and fasting insulin levels in type 2 diabetes (19). Furthermore, another meta-analysis comprised of 22 randomized clinical trials in type 2 diabetes showed a modest 0.32% reduction of HbA1c after vitamin D supplementation (20). However, the effect of vitamin D supplementation on glycemic control in type 2 diabetes is not clear; the difference in ethnicity and genetic background could be the reason behind the variability of observations among the different clinical trials (21).

Our clinical study identified a decreased percentage of PAC-1-positive and P-selectin-positive platelets after the vitamin D intervention. Two individuals in the vitamin D arm had higher PAC-1 expression at six months compared to baseline, which could be attributed to the complexity of the disease state prevalent in T2DM patients. It is reported that insulin resistance, hyperglycemia, inflammation, oxidative stress, and endothelial damage in type 2 diabetes contribute to platelet activation by altering calcium hemostasis, ROS generation, impairing NO release and glycation of platelet proteins (22, 23). Our finding of decreased platelet activation is further confirmed by decreased urine levels of 11-dehydrothromboxane B2 and serum levels of platelet factor 4 after vitamin D supplementation. The 11-dehydrothromboxane B2 is a metabolite of thromboxane A2 and an important urinary marker for platelet activation (24). PF-4 is an inflammatory marker and a circulating platelet activation marker, according to experts (25). Our observation was in agreement with the previous study that vitamin D deficiency contributes to the increased platelet reactivity and platelet aggregation in type 2 diabetes (9, 26).

Our previous research has proven that platelet activation and platelet-immune cells aggregate formation may contribute to inflammation and complexity of both type 2 diabetes and coronary artery disease among type 2 diabetes subjects (3). Hui Min et al. reported that increased platelet-monocyte aggregation in vitamin D deficient healthy subjects (27). It is reported that activated platelets adhere to immune cells and form platelet immune cell aggregation by tethering of platelet surface ligands with their counter receptors on the immune cells (28). In our study, we studied vitamin D effect on platelet-aggregate

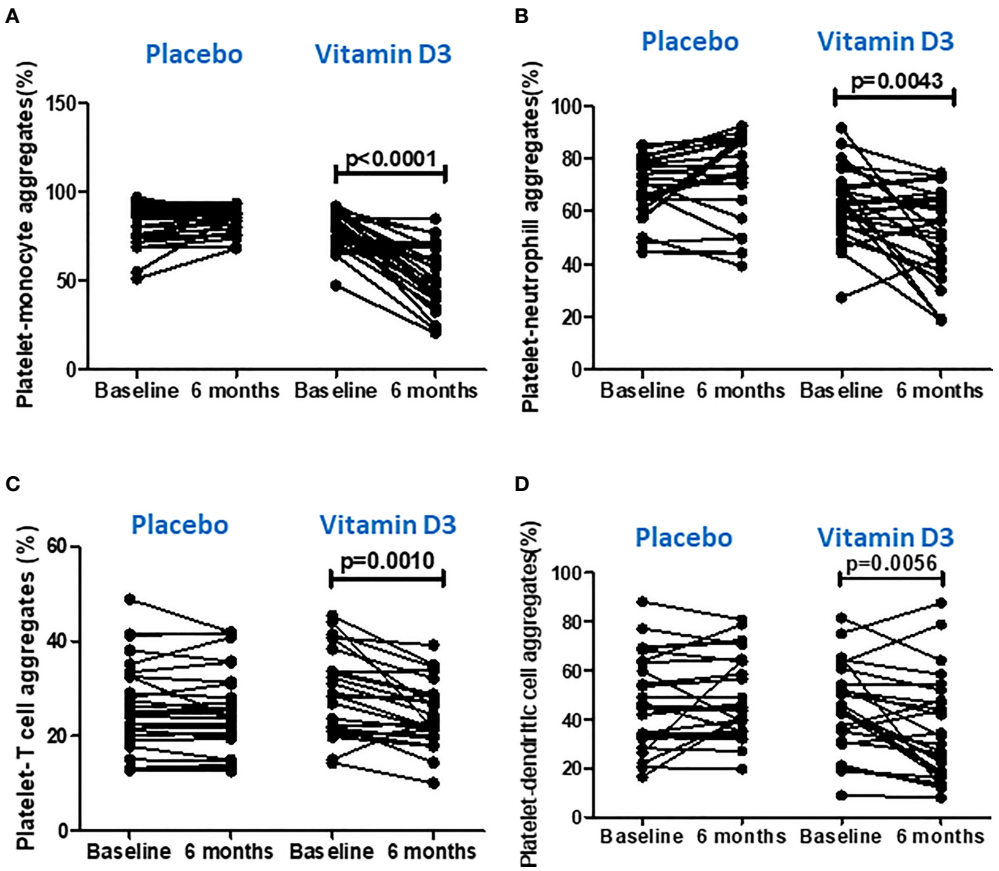

FIGURE 5 | Effect of vitamin D on platelet aggregation with immune cells. **(A)** Platelet–monocyte aggregates, **(B)** platelet–neutrophil aggregates, **(C)** platelet–T cell aggregates, and **(D)** platelet–dendritic cell aggregates at baseline and 6 months in the placebo and vitamin-D3-treated groups. Wilcoxon matched-pair test was used for the comparison between baseline and 6 months in the placebo and vitamin-D3-treated groups.

formation with different immune cells. We observed vitamin D supplementation decreased platelet aggregation with monocytes (and monocyte subsets), neutrophils, T cells, natural killer cells and dendritic cells.

As per available research evidence, the activated platelets release reactive oxygen and nitrogen species, which are critical in developing inflammation and thrombosis (29). Further increased levels of ROS, such as superoxide anion, hydrogen peroxide can directly contribute to platelet activation (30). In the present study we have measured superoxide dismutase (SOD) activity, glutathione (GSH), and total nitric oxide (TNO) to understand the effect of vitamin D on oxidative stress and the mechanism by which vitamin D reduces platelet activation. Following vitamin D administration, we noticed increased superoxide dismutase (anti-oxidant) activity and glutathione levels. Similar to our observation, researchers reported decreased oxidative DNA damage after vitamin D supplementation in patients with metabolic disorders (31). Recently, Imanparast et al. reported that vitamin D3 improves endothelial dysfunction by reducing oxidative stress (32). Nitric oxide is a key marker for endothelial function and also acts as a platelet activation inhibitor (33, 34). Increased nitric oxide after vitamin D intervention may also contribute to reducing the platelet-mediated inflammation in the study subjects. Further, we performed *ex-vivo* experiment to confirm the effect of circulating vitamin D metabolites on platelet activation. We observed decreased activation of healthy platelets when incubated in plasma samples from vitamin D sufficient subjects. Similarly, we observed decreased intracellular ROS in platelets when incubated in vitamin D sufficient plasma as compared to vitamin D deficient plasma of the same subject.

Furthermore, we examined serum cytokines and chemokines to understand the effect of vitamin D supplementation on systemic inflammation in type 2 diabetes. We observed significant decrease in circulating levels of IL-18, TNF-α, IFN-γ, CXCL-10, CXCL-12, CCL-2, CCL-5, and CCL-11. However, we observed a difference in the serum IL-18, IL-8, and IFN-gamma levels between the study groups at baseline. This could be due to the complexity of disease conditions in the study groups. We were more interested in investigating changes in inflammatory markers after vitamin D supplementation. Compared to baseline levels, we observed a significant decrease in IL-18 and IFN-gamma after the vitamin D treatment. No statistically significant difference was observed in most of the clinical and biochemical parameters between the study groups. Apart from this, we have also measured platelet factor 4, and

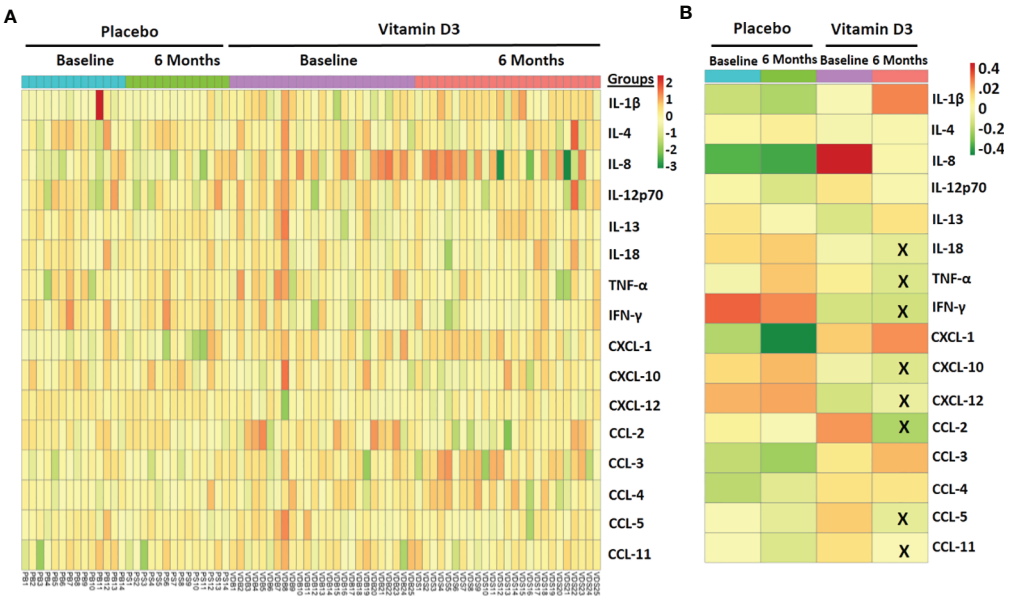

FIGURE 6 | Effect of vitamin D supplementation on circulating inflammatory markers. **(A)** Heat map showing the circulatory inflammatory marker samples at baseline and 6 months in the placebo and vitamin-D3-treated group. **(B)** The median level of circulating markers across different study groups is also shown. The X mark indicates statistically significant change as compared to the baseline level ($p < 0.05$).

we observed decreased PF-4 levels after vitamin D supplementation in T2DM subjects. It was interesting to know from previous publications that the release of inflammatory mediators like IL-18 (35), TNF-α (36, 37), CXCL-12 (38), PF-4 (39), and CCL-5 (40) can be induced by platelet activation and platelet–immune cell aggregation formation. Moreover, researchers also reported that inflammatory mediators such as IL-18, TNF-α, and PF-4 can significantly contribute to platelet activation in various disease conditions (35, 39, 41). Our findings suggest that decreased platelet activation and platelet immune cell aggregation following vitamin D treatment may reduce systemic inflammation and *vice versa*.

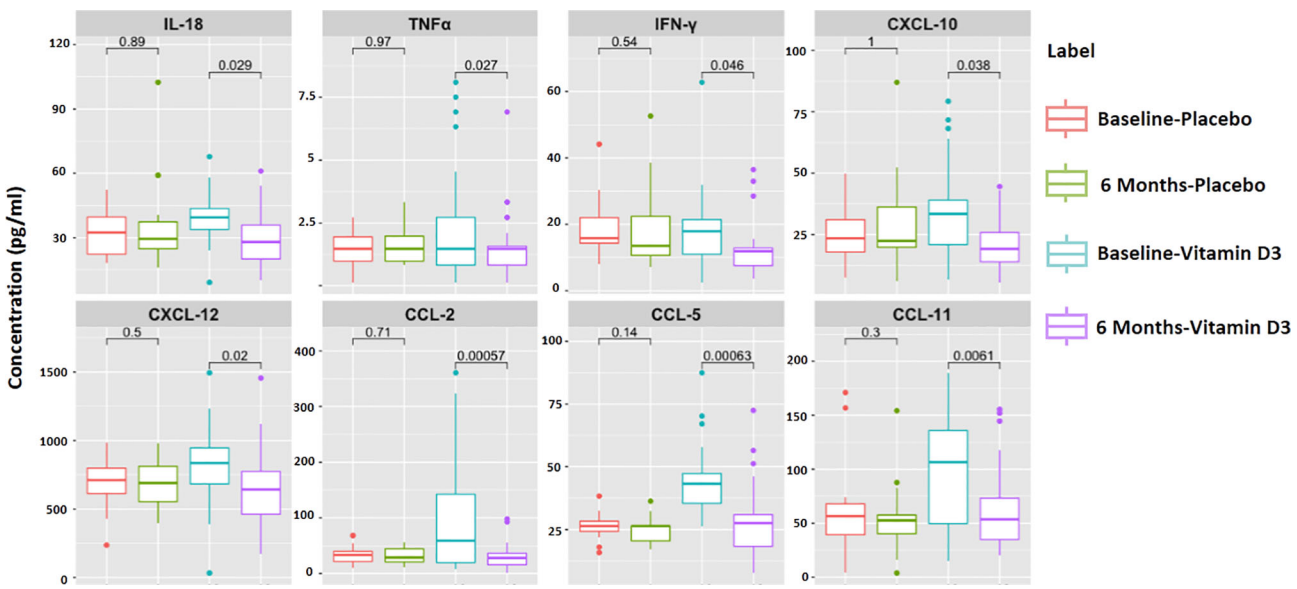

FIGURE 7 | Significantly altered cytokines and chemokines in 6 months of treatment in the placebo and vitamin-D3-treated groups as compared to the baseline level. Wilcoxon matched-pair test is used for the comparison between baseline and 6 months in the placebo and vitamin-D3-treated groups.

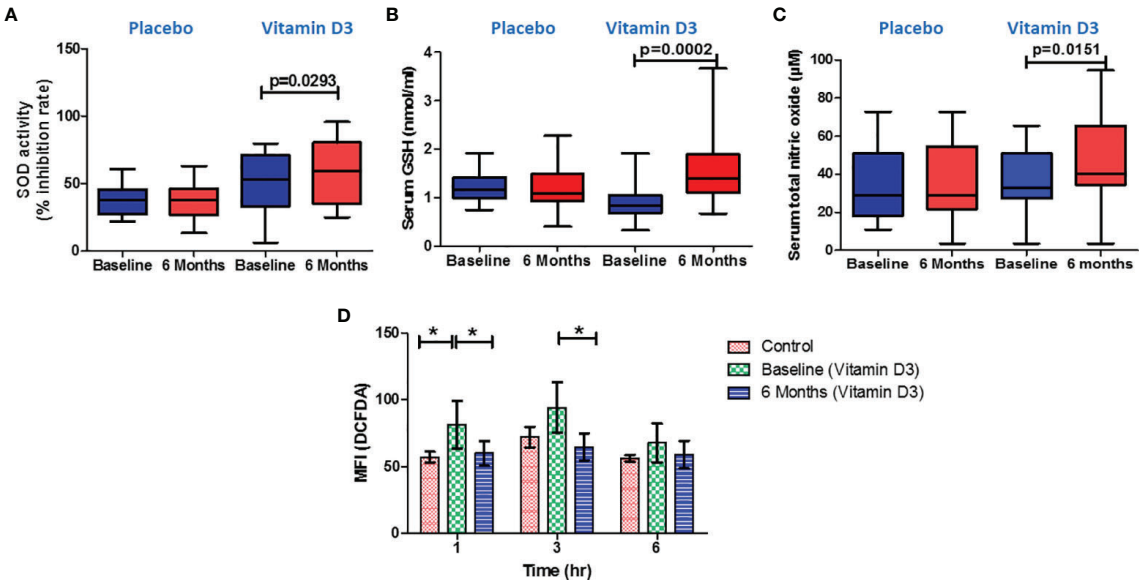

FIGURE 8 | Effect of vitamin D on oxidative stress markers and serum nitric oxide levels. **(A)** Serum superoxide dismutase activity (% inhibition), **(B)** reduced glutathione (nmol/ml), and **(C)** total nitric oxide (μM) at baseline and 6 months in the placebo and vitamin-D3-treated groups. Paired *t*-test or Wilcoxon matched-pair test was used for the comparison between baseline and 6 months in the placebo and vitamin-D3-treated groups. **(D)** *Ex vivo* measurement of intracellular reactive oxygen species in incubated platelets from the plasma samples of healthy control, baseline, and 6 months of the vitamin D3 treatment group at different time points (1, 3, and 6 h). One-way ANOVA with Bonferroni test was used for the comparison between outcome groups. * denotes $P < 0.05$.

Limitations

In the present study, we attempted to find the effect of vitamin D supplementation on platelet-mediated inflammation in type 2 diabetes patients. However, our study has a few limitations. The study's main limitation is being a single-center study design with a small sample size and a short duration. Another limitation of our study is that it does not focus on the molecular mechanism responsible for reducing platelet-mediated inflammation at the cellular level. Further *in vitro* experiments, to evaluate the direct impact of vitamin D on platelets, are required to firmly conclude that vitamin D reduces platelet-mediated inflammation. However, by assessing markers in cells, serum, and urine, our study provides strong evidence that vitamin D reduces platelet-mediated inflammation in type 2 diabetes subjects.

CONCLUSION

In conclusion, the correction of vitamin D deficiency in type 2 diabetes patients by vitamin D3 supplementation does not improve the glycemic parameters. Our study highlights that vitamin D supplementation reduces platelet activation, platelet–immune cell aggregates, and platelet-mediated inflammation in type 2 diabetes patients. Our study results provide evidence that cholecalciferol supportive therapy may help to reduce or prevent the disease progression and cardiovascular risk in type 2 diabetes patients. A better understanding of the molecular mechanism behind platelet-mediated inflammation reduction by vitamin D3 needs to be elucidated in future *in vitro* experiments and clinical studies with a large number of type 2 diabetes patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Downtown Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EJ and RA conceived and designed the study. EJ, AJ, and BN performed the experiments. Analysis of the data was done by EJ, RB, and RA. Heat map visualizations were made by MJA and EJ. Subject recruitment and clinical characteristic measurement done by RD, IK, and EJ. EJ, RB, and RA wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.869591/full#supplementary-material

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High Serum Vitamin D Concentrations, Induced *via* Diet, Trigger Immune and Intestinal Microbiota Alterations Leading to Type 1 Diabetes Protection in NOD Mice

Pieter-Jan Martens[1†], Javier Centelles-Lodeiro[2†], Darcy Ellis[1], Dana Paulina Cook[1], Gabriele Sassi[1], Lieve Verlinden[1], Annemieke Verstuyf[1], Jeroen Raes[2], Chantal Mathieu[1‡] and Conny Gysemans[1‡]*

[1] Clinical and Experimental Endocrinology (CEE), Katholieke Universiteit Leuven, Leuven, Belgium, [2] Laboratory of Molecular Bacteriology, Rega-Institute, Katholieke Universiteit (KU) Leuven, Leuven, Belgium

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Edited by:
Chafia Touil-Boukoffa,
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Nassima Behairi,
University of Sciences and Technology Houari Boumediene (USTHB), Algeria

***Correspondence:**
Conny Gysemans
conny.gysemans@kuleuven.be

[†]These authors share first authorship

[‡]These authors share senior authorship

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The hormonally-active form of vitamin D, 1,25-dihydroxyvitamin D_3, can modulate both innate and adaptive immunity, through binding to the nuclear vitamin D receptor expressed in most immune cells. A high dose of regular vitamin D protected non-obese diabetic (NOD) mice against type 1 diabetes (T1D), when initiated at birth and given lifelong. However, considerable controversy exists on the level of circulating vitamin D (25-hydroxyvitamin D_3, 25(OH)D_3) needed to modulate the immune system in autoimmune-prone subjects and protect against T1D onset. Here, we evaluated the impact of two doses of dietary vitamin D supplementation (400 and 800 IU/day), given to female NOD mice from 3 until 25 weeks of age, on disease development, peripheral and gut immune system, gut epithelial barrier function, and gut bacterial taxonomy. Whereas serum 25(OH)D_3 concentrations were 2.6- (400 IU/day) and 3.9-fold (800 IU/day) higher with dietary vitamin D supplementation compared to normal chow (NC), only the 800 IU/day vitamin D-supplemented diet delayed and reduced T1D incidence compared to NC. Flow cytometry analyses revealed an increased frequency of FoxP3$^+$ Treg cells in the spleen of mice receiving the 800 IU/day vitamin D-supplemented diet. This vitamin D-induced increase in FoxP3$^+$ Treg cells, also expressing the ecto-5'-nucleotidase CD73, only persisted in the spleen of mice at 25 weeks of age. At this time point, the frequency of IL-10-secreting CD4$^+$ T cells was also increased in all studied immune organs. High-dose vitamin D supplementation was unable to correct gut leakiness nor did it significantly modify the increased gut microbial diversity and richness over time observed in NOD mice receiving NC. Intriguingly, the rise in alpha-diversity during maturation occurred especially in mice not progressing to hyperglycaemia. Principal coordinates analysis identified that both diet and disease status significantly influenced the inter-individual microbiota variation at the genus level. The abundance of the genera *Ruminoclostridium_9* and *Marvinbryantia*

gradually increased or decreased, respectively in faecal samples of mice on the 800 IU/day vitamin D-supplemented diet compared to mice on the 400 IU/day vitamin D-supplemented diet or NC, irrespective of disease outcome. In summary, dietary vitamin D reduced T1D incidence in female NOD mice at a dose of 800, but not of 400, IU/day, and was accompanied by an expansion of Treg cells in various lymphoid organs and an altered intestinal microbiota signature.

Keywords: type 1 diabetes, vitamin D, immunomodulation, gut microbiota, gut permeability, microbiome

INTRODUCTION

The increase in the incidence of type 1 diabetes (T1D), the most common chronic immune-mediated disease in young children and adolescents, over the last 40 years cannot be explained by genetic drift but is probably related to one or more environmental exposures. The 'hygiene hypothesis' proposes that fewer early childhood infections (1), and poorer gut microbial diversity may deviate the immune system towards islet auto-reactivity (2, 3). Interestingly, enteroviruses (e.g., coxsackievirus B4 and rotavirus) have tropism for self-pancreatic tissues (4), and can directly instigate failure and complete destruction of the insulin-producing β cells (5). Moreover, T1D patients have enterovirus persistence associated with strong inflammation in their gut mucosa, which may promote islet autoimmunity by bystander activation mechanisms (6). The intestinal microflora in people with T1D seems less diverse, at both the abundance and functional level, compared to healthy individuals but whether this is cause or consequence is still under debate [reviewed in (7)]. Exactly how commensal bacteria and the immune system interact to provoke islet autoimmunity and ensuing T1D remains largely unknown. Intestinal epithelial barrier dysfunction may allow dietary antigens but also foreign microbial components to translocate into the periphery where they could subsequently trigger islet autoimmunity due to epitope mimicry (8–10).

Researchers also postulate that a lack of sun exposure and consequential vitamin D deficiency could lead to aberrant immune responses and more autoimmunity. Vitamin D deficiency is common among children with T1D and children with multiple islet autoantibodies compared to autoantibody-negative children, implying that it proceeds disease onset (11). In addition, we demonstrated that severe vitamin D deficiency in diabetes-prone non-obese diabetic (NOD) mice aggravated T1D development and was associated with immune system defects (12). Vitamin D is not only vital for calcium homeostasis and bone growth, but strong evidence points to its essential role in the functionality of both innate and adaptive immunity (13, 14). Its hormonally-active form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has powerful anti-inflammatory and immunomodulatory properties and functions by binding to the nuclear vitamin D receptor (VDR), present in most immune cell types [reviewed in (14, 15)]. Within the immune system, vitamin D has the ability to tolerise dendritic cells, inhibit IFN-γ- and IL-17-producing effector T (Teff) cells, induce regulatory $CD4^+$ T (Treg) cells, and stimulate production of antimicrobial peptides [reviewed in (13, 14)]. Early-life treatment with $1,25(OH)_2D_3$ protected against T1D development in NOD mice, although this regimen was associated with severe hypercalcemia and bone fractures (16). On the other hand, high-dose regular (dietary) vitamin D supplementation safely prevented T1D progression in NOD mice, but only when administered early and lifelong (17). Protection was associated with skewing of the Teff-to-Treg balance in favour of Treg cells in the pancreatic draining lymph nodes. Moreover, in humans, early life supplementation with regular vitamin D at a dose of 2,000 IU/day (50 μg/day) reduced the risk of developing T1D, up to an 80% decrease projected over the following 30 years (18). Based on these preclinical and observational data, a clinical trial was designed to study whether new-borns with an increased genetic risk for T1D will be protected from disease development when given 2,000 IU/day of regular vitamin D instead of the recommended 400 IU/day dose (ClinicalTrials.gov identifier: NCT00141986) (19). There remains however, a lot of discussion about the correct vitamin D substitution regimen and the (circulating) vitamin D concentrations needed to maintain immune homeostasis, as current practical guidelines are primarily focussing on bone health (20).

In regards to intestinal features, both vitamin D and its receptor have been reported to influence the gut commensal composition, maintain gut epithelial barrier function, and avoid pathogenic immune responses in the gut by inhibiting Teff responses and inducing $FoxP3^+$ Treg cells [reviewed in (21)]. Vitamin D deficiency has been associated with a dysbiosed gut microbiota (22), altered gut mucosal defence (23), impaired mucus, and increased gut permeability (23, 24). Moreover, vitamin D supplementation in vitamin D-deficient women significantly increased gut microbial diversity and richness with an increase in the *Bacteroidetes* to *Firmicutes* ratio, along with an increase in the abundance of particular health-promoting taxa (22). Interestingly, repeated UVB light exposure increased alpha- and beta-diversity in the gut microbiota of subjects that had not taken vitamin D supplements

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D3; 25,25-dihydroxyvitamin D_3; $25(OH)D_3$, 25-hydroxyvitamin D_3; DSS, dextran sulfate sodium; FoxP3, forkhead box P3; GARP, glycoprotein A repetitions predominant; IL, interleukin; LAG3, lymphocyte-activation gene 3; LAP, latency-associated peptide; LC-MS/MS, liquid chromatography tandem mass spectrometry; MLN, mesenteric lymph nodes; NC, normal chow; NOD, non-obese diabetic; NP, non-progressor; OTU, operational taxonomic unit; P, progressor; PLN, pancreatic lymph nodes; SPF, specific pathogen free; T1D, type 1 diabetes; Teff, effector T cell; Th, T helper cell; Treg, regulatory T cell; VDR, vitamin D receptor.

prior to study enrolment (25). At the genus level, vitamin D seems to connect to some genera of the *Lachnospiraceae* family (25, 26). Circulating 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations showed a strong correlation with the relative abundance of *Lachnospiraceae* genera (25). A recent genome-wide association study (GWAS) study established significant associations between gut microbial features and the VDR gene (27). Interestingly, the expression level of the VDR gene is not only regulated by vitamin D but also by other hormonal components, like secondary bile acids and metabolites produced by the gut microbiota (28, 29).

The complex interplay between vitamin D, the immune system, and the gut microbiota is clearly understudied and not much is known about how vitamin D supplementation affects the intestinal characteristics in autoimmune-prone subjects. In the current study, we evaluated the impact of two different doses of vitamin D supplementation (400 and 800 IU/day), administered lifelong *via* the diet, on T1D development and studied whether vitamin D intake and circulating concentrations of $25(OH)D_3$ were associated with peripheral and gut immunity (i.e., $FoxP3^+$ Treg cells, $CD39^+CD73^+$ T cells, IL-10-secreting T cells, and T regulatory type 1 (Tr1) cells), intestinal barrier function, and gut microbiota composition in diabetes-prone NOD mice. We found that only the 800 IU/day vitamin D-supplemented diet, which increased $25(OH)D_3$ serum concentration up to a mean value of 193.9 nmol/L, delayed disease onset, significantly reduced T1D incidence, and was accompanied by an increased frequency of Treg cells in the studied immune organs, and an altered intestinal microbiota composition favouring *Ruminiclostridium_9* and diminishing *Marvinbryantia* at the genus level.

MATERIALS AND METHODS

Animals and Experimental Design

The NOD Leuven strain is bred in specific pathogen free (SPF) environment and maintained under semi-barrier conditions in the animal facility of the KU Leuven since 1989. For this study, 93 female NOD mice were randomly assigned at the time of weaning to a grid cage and group housed (5/cage) per dietary condition to either a vitamin D-sufficient diet (normal chow, NC) or two different doses (400 or 800 IU/day) of a vitamin D-supplemented diet until 25 weeks of age. In NC the natural-ingredient was ssniff® R/M-H maintenance diet containing 1% calcium, 0.7% phosphorus, and 1,000 IU vitamin D/kg diet (BioServices BV, Uden, the Netherlands), meeting the recommended concentrations for rodents according to the criteria described in the 'Nutrient Requirements of Laboratory Animals' (30). Based on a daily consumption of 4 g food per 20 g body weight, this corresponds to an intake of 4 IU/day of vitamin D. The 400 and 800 IU/day vitamin D-supplemented diet was the ssniff® R/M-H maintenance diet containing 1% calcium, 0.7% phosphorus but supplemented with 100,000 or 200,000 IU vitamin D/kg diet, respectively. The animals had *ad libitum* access to both food and water. Mice were screened three times weekly for diabetes onset by evaluating glucose concentrations in urine (Diastix; Ascensia Diabetes Care, Machelen, Belgium) and venous blood (Accu-Chek; Roche Diagnostics, Vilvoorde, Belgium). Mice were diagnosed as diabetic (progressor mice; P) when having positive glycosuria and two consecutive blood glucose measurements above 200 mg/dL. The remaining mice that maintained normal blood glucose concentrations were labelled as non-progressor (NP) mice (**Supplementary Figure 1**).

Serum Vitamin D, Calcium, and Phosphate Measurements

At 8 weeks of age, blood was collected by submandibular vein puncture. Serum or plasma was stored at −80°C until biochemical determinations were performed. Serum $25(OH)D_3$, $1,25(OH)_2D_3$ and both serum and plasma $24,25(OH)_2D_3$ levels were analysed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Sciex, Framingham, MA) (31). Serum calcium (product code: OSR60117) and phosphate (product code: OSR6122) were analysed on a Beckman Coulter DxC 700 AU chemistry analyser (Analis, Vilvoorde, Belgium).

Flow Cytometric Analysis

Single-cell suspensions of spleen, pancreatic lymph nodes (PLN), and mesenteric lymph nodes (MLN) were prepared by mechanical disruption from mice at 8 and 25 weeks of age. The following antibodies were used: CD4, CD25, CD39, CD49b, CD73, lymphocyte-activation gene 3 (LAG3), and IL-10 (eBiosciences, Fisher Scientific SPRL, Merelbeke, Belgium). Intracellular staining was performed with FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Cells were acquired on a Canto II AIG flow cytometer (BD Biosciences, Erembodegem, Belgium) and analysed with FlowJo software (FlowJo, LLC, Ashland, OR). All analyses were performed on fixable viability dye negative singlet population as outlined in the gating strategy (**Supplementary Figure 2**).

Intestinal Barrier Function Using FITC-Dextran Assay

Dextran sulfate sodium (molecular mass of 70,000 Da, DSS, Fisher Scientific SPRL) was added at 5% (w/v) in drinking water *ad libitum* for 8 days to 8-week-old C57BL/6 mice, which has been described to destroy the intestinal barrier function and result in intestinal permeability and colitis-like symptoms. All mice including the DSS-treated C57BL/6 mice (as positive control) were food and water-starved overnight and kept in a cage without bedding to limit the coprophagic behaviour. FITC-dextran (Sigma-Aldrich, Overijse, Belgium) dissolved in PBS (100 mg/mL) was administered to each mouse (44 mg/100 g body weight) by oral gavage. After 4 hours, mice were sacrificed, serum was collected and FITC-dextran measurements were performed in duplicate by fluorimeter (excitation, 485 nm; emission, 535 nm; VICTOR^{3TM}, PerkinElmer, Zaventem, Belgium). Serial dilutions of FITC-dextran in PBS were used to calculate a standard curve.

Sequencing and Processing of Bacterial 16S rRNA

Faecal pellets were collected longitudinally from mice at both 3 and 8 weeks of age and were stored within 2 hours after sampling

at −80°C until processing. The data set comprised 38-paired samples, 18 originated from P mice and 20 from NP mice. Nineteen samples (9 P mice and 10 NP mice) belonged to mice receiving NC, while 10 (5 P mice and 5 NP mice) and 9 (4 P and 5 NP) samples belonged to mice receiving the 400 and 800 IU/day vitamin D-supplemented diet, respectively.

Bacterial genomic DNA was extracted from faecal pellets using the Qiagen QIAamp DNA Stool Mini Kit following manufacturer's instructions (Qiagen Benelux BV, Antwerp, Belgium). Samples for paired-end Illumina MiSeq (the VIB Nucleomics core laboratory, Leuven, Belgium) were constructed using a two-step PCR amplicon approach targeting the V4 region of the 16S rRNA gene as described (32). The 16S rRNA read de-multiplexing was performed using LotuS pipeline v1.62.1 (33). Data pre-processing was performed using DADA2 v.1.14.1 (34), including trimming, quality control, merging of sequencing pairs and taxonomy assignment with the Silva classifier v1.32 (35), using the default parameters. Genera appearing in less than 50% of each dietary or outcome group were excluded for the differential microbiota abundance analyses. Samples were variance stabilization (VS) transformed using the *DESeq2* package (36) in R.

Statistical Analysis

Data were plotted as mean ± SEM and statistics calculated with GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). Diabetes incidence was evaluated by Kaplan–Meier survival analysis with Mantel-Cox log-rank test. For all other data derived from mice receiving NC or vitamin D-supplemented diets throughout life, differences were estimated by a Student's two–tailed t test or Mann–Whitney U test if the data did not assume Gaussian distribution. However, when two or more groups were compared, normally distributed data sets were analysed by one–way ANOVA with Bonferroni's multiple-comparison test, whereas the Kruskal–Wallis test with subsequent Dunn's multiple-comparison test was used for non-normally distributed data.

Gut microbiota data were analysed by R statistical software using the packages vegan v.2.5-7 (37), phyloseq v1.30.0 (38), FSA v.0.9.1 (39), and stats v.3.6.3 (40). Genera alpha-diversity indices (Shannon diversity and Chao1 richness) were calculated using phyloseq. Due to small sample size, Mann–Whitney U or Kruskal–Wallis test were used to test the differences in microbial taxa and alpha-diversity indices. Only after the Kruskal-Wallis test, we performed a *post-hoc* Dunn's test, to determine which diets were driving the differences in the corresponding features. The contribution of metadata variables on genus-level microbiota community variation was determined using multivariate distance-based redundancy analysis (dbRDA) using the Bray-Curtis dissimilarity index as implemented in the R package vegan. Microbiome inter-individual variation was visualized by canonical correspondence analysis (CCA) on the genus-level VS transformed abundance matrix using the ggplot2 v.3.3.5 R package (41). P values ≤ 0.05 were considered significant (* ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001).

RESULTS

Daily Vitamin D Supplementation With 800 IU, But Not 400 IU, Delayed Disease Onset and Reduced T1D Incidence in NOD Mice

Few data exist on circulating vitamin D concentrations needed to restore immune tolerance in autoimmune-prone subjects. Here, we studied the effect of two doses (400 and 800 IU) of daily lifelong dietary vitamin D supplementation on T1D development in the NOD mouse model and evaluated whether serum vitamin D concentrations correlated with disease outcome.

We first determined the effects of oral vitamin D supplementation on circulating vitamin D metabolites 5 weeks upon dietary intervention (at 8 weeks of age) (**Figure 1A**). A 2.6- and 3.9-fold increase in serum concentrations of 25(OH)D_3, the major circulating form of vitamin D, was recorded in mice receiving the 400 (129.0 ± 9.6 nmol/L, P ≤ 0.0001) or 800 (193.9 ± 2.9 nmol/L; P ≤ 0.001) IU/day vitamin D-supplemented diet respectively, compared to mice receiving NC (49.5 ± 2.8 nmol/L). Serum values of 25(OH)D_3 further increased 1.5-fold with the 800 IU/day vitamin D-supplemented diet compared to values obtained under the 400 IU/d vitamin D-supplemented diet. The amount of circulating 1,25$(OH)_2D_3$, the active form of vitamin D, is strictly regulated in a renal negative feedback loop and is independent of circulating 25(OH)D_3. We observed that serum concentrations of 1,25$(OH)_2D_3$ increased 6.9- and 6.8-fold in mice receiving a 400 (1,644.3 ± 113.6 pmol/L; P ≤ 0.0001) or 800 (1,624.0 ± 109.5 pmol/L; P ≤ 0.0001) IU/day vitamin D-supplemented diet respectively, compared to mice receiving NC (237.1 ± 26.4 pmol/L). Serum 24,25$(OH)_2D_3$ concentrations serve as an indicator of vitamin D catabolic status and depend on the amount of 25(OH)D_3. We found that serum 24,25$(OH)_2D_3$ concentrations increased 11.5- and 11.4-fold in mice receiving a 400 (529.6 ± 13.2 nmol/L; P ≤ 0.0001) or 800 (522.2 ± 16.4 nmol/L; P ≤ 0.0001) IU/day vitamin D-supplemented diet respectively, compared to mice receiving NC (45.9 ± 1.7 nmol/L).

High vitamin D concentrations may cause hypercalcemia and hyperphosphatemia, which can negatively affect bone and kidney health. Serum calcium values were 1.08- and 1.24-fold higher in mice receiving the 400 (2.65 ± 0.04 mmol/L; P ≤ 0.01) or 800 (3.06 ± 0.21 mmol/L; P ≤ 0.05) IU/day vitamin D-supplemented diet compared to mice receiving NC (2.53 ± 0.02 mmol/L) (**Figure 1A**). Serum phosphate levels were also higher in mice receiving the 800 (3.06 ± 0.19 mmol/L; P ≤ 0.05), but not in those receiving the 400 (2.64 ± 0.10 mmol/L; P = NS) IU/day vitamin D-supplemented diet compared to mice receiving NC (2.69 ± 0.05 mmol/L), indicating signs of moderate hypercalcemia and -phosphatemia.

Interestingly, only the 800 IU/day vitamin D-supplemented diet was able to significantly reduce T1D development in female NOD mice compared to mice receiving NC (13 vs. 50% at 25 weeks of age; P ≤ 0.01), with a delay in disease onset of 5 weeks compared to mice receiving NC (**Figure 1B**). T1D onset and incidence in mice receiving the 400 IU/day vitamin D-

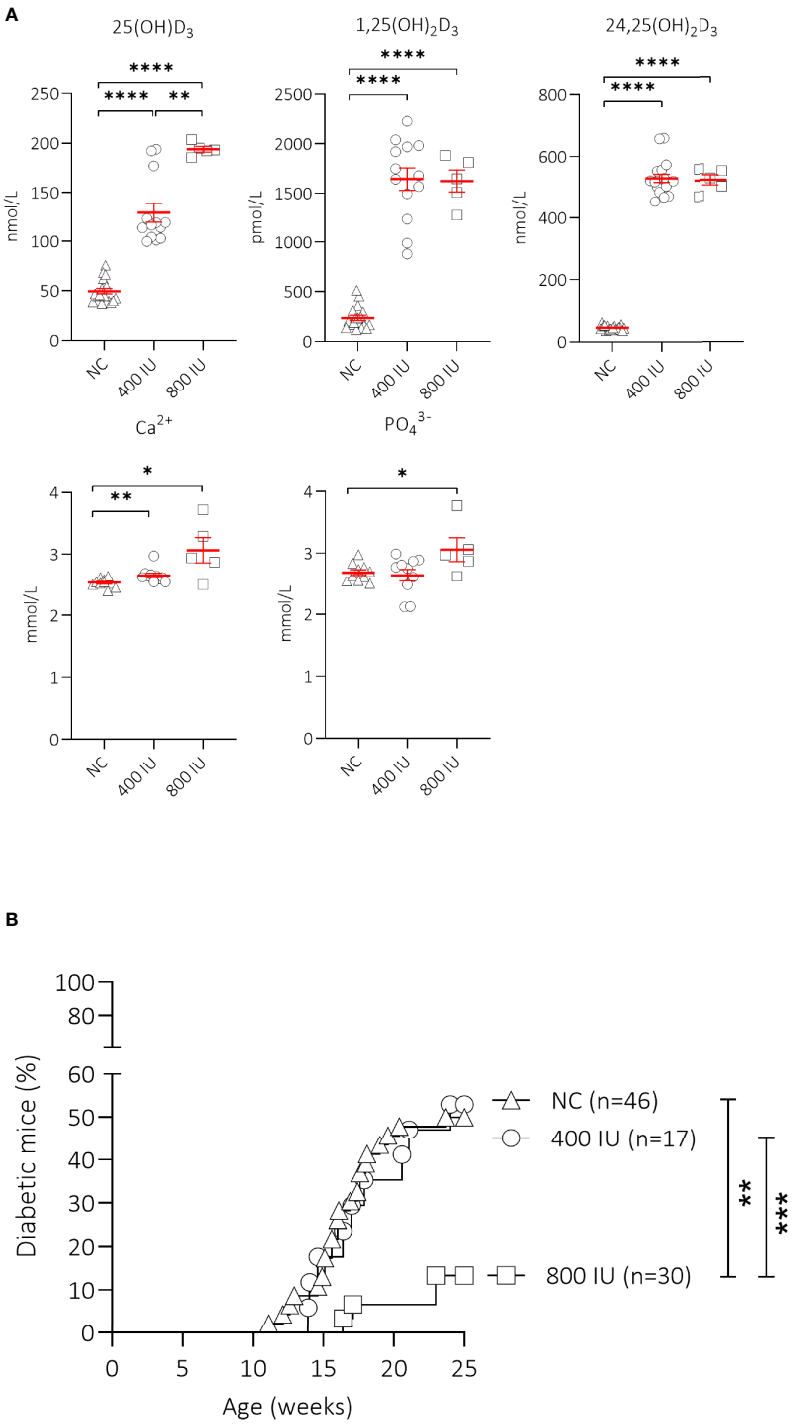

FIGURE 1 | Effect of vitamin D substitution regimen on serum metabolites and type 1 diabetes (T1D) incidence. NOD mice were fed normal chow (NC) or two different doses of vitamin D-supplemented (400 or 800 IU/day) diet from 3 until 25 weeks of age. Serum levels of 25(OH)D_3, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_3$, calcium, and phosphate are shown for NOD mice of 8 weeks of age (5 weeks on the respective diets). Symbols represent individual mice (N = 5-18), and the line reflects the group mean with SEM. **(A)** Kaplan-Mayer survival curves depict T1D incidence over time. Mice (N = 17-46) with two consecutive measurements of blood glucose values >200 mg/dL were considered diabetic. **(B)**. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

supplemented diet was comparable to values obtained in mice receiving NC (disease onset at 14 vs. 12 weeks of age, T1D incidence 53 vs. 50% respectively, P = NS).

Only High-Dose Vitamin D Supplementation Maintained Increased Frequencies of Splenic FoxP3$^+$ Treg Cells

Treg cells are the central component for maintaining peripheral tolerance. Here, we studied FoxP3$^+$ Treg cells in spleen, and draining lymph nodes (of the pancreas and gut) at both 8 and 25 weeks of age using multi-colour flow cytometry.

The frequencies of FoxP3$^+$ Treg cells in all studied immune organs were similar between mice receiving the 400 IU/day vitamin D-supplemented diet compared to those on NC at 8 and 25 weeks of age. However, the percentages of FoxP3$^+$ Treg cells in mice receiving the 800 IU/day vitamin D-supplemented diet were increased in the spleen, PLN, and MLN, compared to mice receiving the 400 IU/day vitamin D-supplemented diet at 8 weeks of age (**Figure 2A**). These values further augmented however, only in the spleen of mice receiving the 800 IU/day vitamin D-supplemented diet compared to mice receiving either the 400 IU/day vitamin D-supplemented diet or NC at 25 weeks of age (**Figure 2A**).

Although expression of the IL-2Rα chain (CD25) by CD4$^+$ T cells follows activation, its expression by CD4$^+$ T cells is utilised extensively as a marker to classify Treg cells. The percentages of CD25$^+$FoxP3$^+$ Treg cells were significantly increased in the spleen of mice receiving the 800 IU/day vitamin D-supplemented diet compared to mice receiving a 400 IU/d vitamin D-supplemented diet at 8 and 25 weeks of age (**Supplementary Figure 3A**). Moreover, the frequencies of the CD25$^-$FoxP3$^+$ Treg cell subset were also expanded however, only by the 800 IU/d vitamin D diet in the spleen, PLN and MLN at 25 weeks of age (**Supplementary Figure 3B**).

FoxP3$^+$ Treg Cell Expansion Upon High-Dose Vitamin D Supplementation Was Associated With Co-Expression of the Ecto-5'-Nucleotidase CD73 in the Spleen

Several mechanisms have been proposed as to how Treg cells exert their suppressive function including cell-to-cell contact and the release of soluble mediators. Recent data point to CD39 and CD73 as novel markers of FoxP3$^+$ Treg cells that subsequently degrade the extracellular ATP pool and catalyse the formation of adenosine, which has the ability to dampen aberrant immune reactions (42). Surprisingly, the frequency of CD39$^+$ and of CD39$^+$CD73$^+$ T cells within the CD4$^+$Foxp3$^+$ T-cell gate was reduced in the spleen, MLN, and PLN of mice receiving the 800 IU/day vitamin D-supplemented diet compared to mice receiving the 400 IU/day vitamin D-supplemented diet or NC. This observation was present at 8 weeks of age, but more pronounced at 25 weeks of age (**Figures 2B, D**). On the other hand, the CD73$^+$ T-cell population within the CD4$^+$FoxP3$^+$ T-cell gate was significantly enlarged in the spleen of mice receiving the 800 IU/day vitamin D-supplemented diet compared to mice receiving the 400 IU/d vitamin D-supplemented diet or NC at 25 weeks (**Figure 2C**), indicating that under a high-dose vitamin D substitution regimen FoxP3$^+$ T cells in the periphery may function in a CD73-dependent manner.

High-Dose Vitamin D Supplementation Increased Frequencies of IL-10-Secreting CD4$^+$ T Cells

Apart from FoxP3$^+$ Treg cells, IL-10-secreting T cells may constitute a supplementary mechanism responsible for peripheral tolerance. Different IL-10-secreting T cells have been described such as Tr1 cells (3), Treg cells expressing the latency-associated peptide (LAP), but also IL-10-secreting CD4$^+$CD25^{hi}CD127$^-$FoxP3$^-$ T cells induced by vitamin D in healthy and T1D individuals (17). Supplementing NOD mice with the 800 IU/day vitamin D diet increased the percentages of CD4$^+$IL-10$^+$ T cells within the spleen, PLN, and MLN compared to values obtained in mice on the 400 IU/day vitamin D diet or NC at 25 weeks of age (**Figure 3A**). We further explored whether Tr1 cells, which are characterized by co-expression of CD49b and LAG3, high secretion of IL-10, and lack of FoxP3 expression, were responsible for the vitamin D-induced rise in IL-10$^+$CD4$^+$ T cells (3). However, we did not find an expansion of Tr1 cells in any of the studied immune organs by vitamin D at 8 nor at 25 weeks of age (**Figure 3B**). Recent data based on the use of an IL-10-GFP/FoxP3-RFP dual reporter transgenic model demonstrated that co-expression of CD49b and LAG3 was not restricted to the FoxP3$^-$ Tr1 cells, but was also observed in Foxp3$^+$ Treg cells (43). Percentages of Foxp3$^+$ Tr1 cells were not increased but on the contrary diminished in the spleen of NOD mice upon the 800 IU/day vitamin D-supplemented diet compared to the other groups at 25 weeks of age (**Figure 3C**).

High-Dose Vitamin D Supplementation Did Not Correct Gut Leakiness in NOD Mice

Loss of gut epithelial barrier function has been linked to the activation of autoreactive T cells and T1D onset (44). Moreover, vitamin D was demonstrated to maintain gut barrier integrity (45). Here, we evaluated the effect of the highest dose (800 IU/day) of the vitamin D diet on gut epithelial barrier function in non-diabetic female NOD mice. As a negative and positive control, we included female healthy C57BL/6 as well as DSS-treated C57BL/6 mice of 8 weeks of age, respectively. We observed that NOD mice at 8 weeks of age already had a leaky gut compared to age-matched healthy C57BL/6 mice as exemplified by increased serum levels of FITC-labelled dextran ($P \leq 0.01$; **Figure 4**). We also demonstrated that gut leakiness further increased with age in the NOD mice, while supplementation with 800 IU/day of regular vitamin D did not correct the breakage of the gut barrier integrity (P = NS; **Figure 4**).

Microbiota Diversity and Richness Increases With Aging in NOD Mice, Especially in Mice Not Progressing to Diabetes

Previous publications have reported that the gut microbiome from NOD mice undergoes significant modifications between 3 and 8 weeks of age (46). Here, we assembled a *phyloseq* object

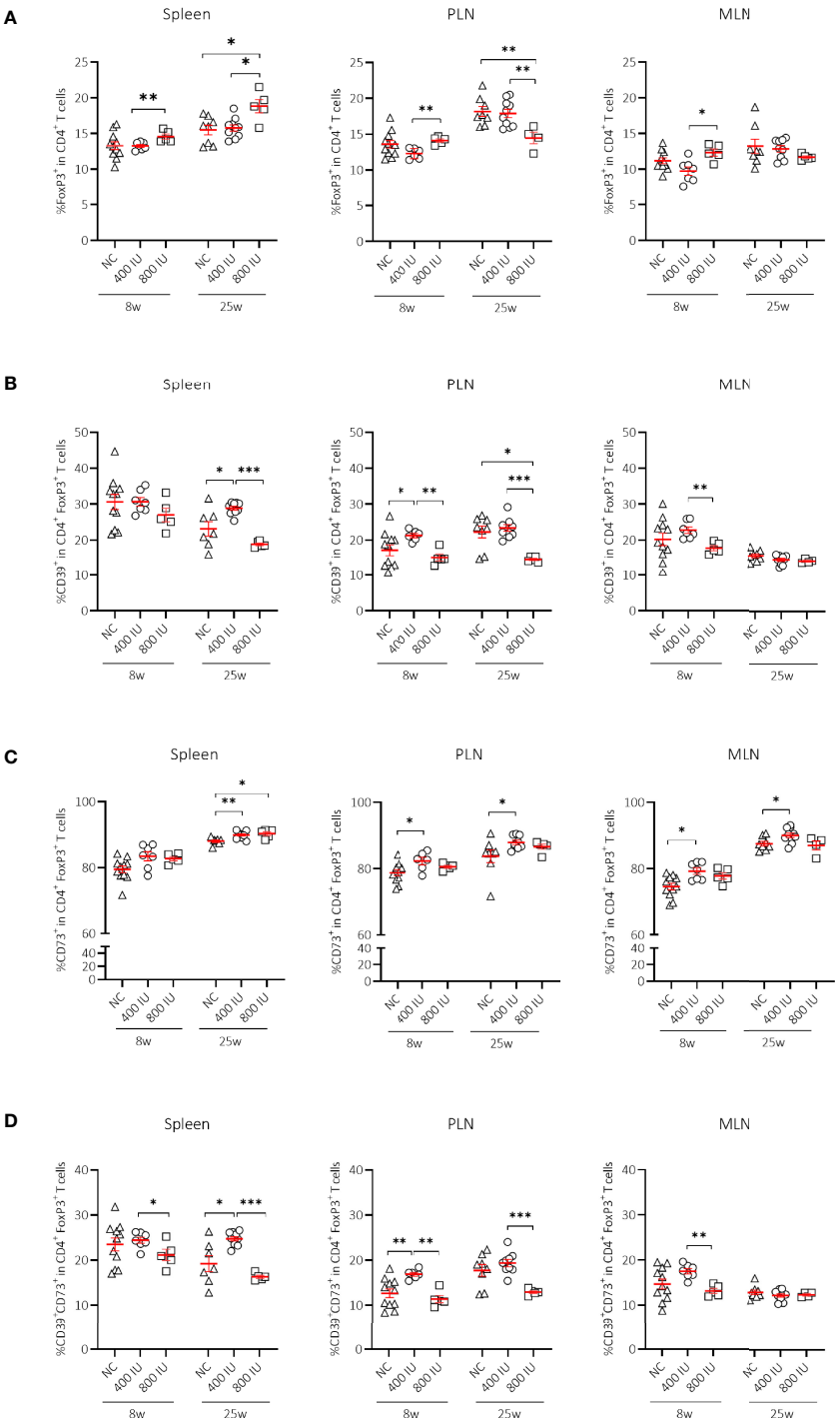

FIGURE 2 | Effect of vitamin D substitution regimen on frequency and functionality of Treg cells. Frequency of FoxP3$^+$ T cells within CD4$^+$ T-cell gate **(A)** and their functionality as expressed by the frequency of CD39$^+$ **(B)**, CD73$^+$ **(C)** or CD39$^+$CD73$^+$ T cells **(D)** within FoxP3$^+$CD4$^+$ T-cell gate are shown at both 8 and 25 weeks of age in spleen, pancreatic draining lymph nodes (PLN), and mesenteric draining lymph nodes (MLN). Female NOD mice were fed normal chow (NC) or two different doses of vitamin D-supplemented (400 or 800 IU/day) diet from 3 until 25 weeks of age (lifelong). Symbols (N = 4-11) represent individual mice, and line reflects group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

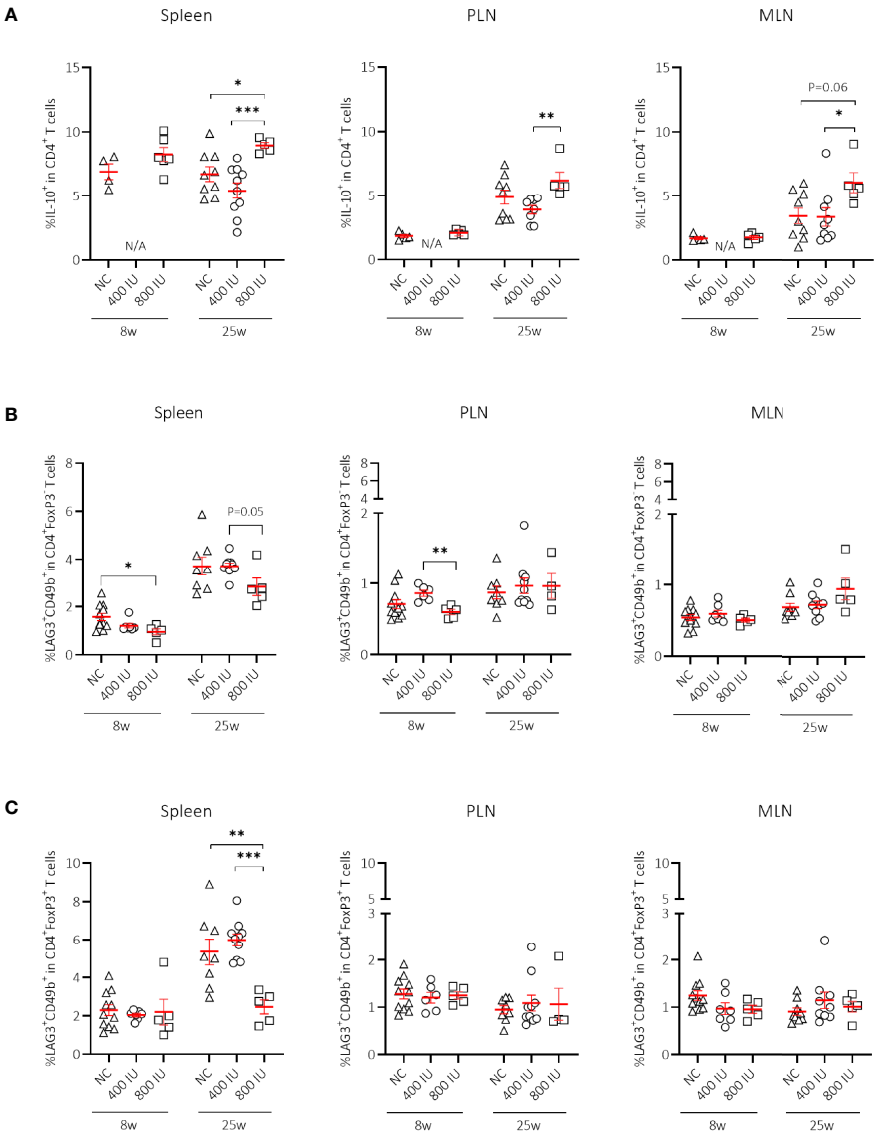

FIGURE 3 | Effect of vitamin D substitution regimen on frequency of IL-10-secreting CD4$^+$ T cells. Frequency of IL-10-secreting CD4$^+$ T cells within CD4$^+$ T-cell gate **(A)** and frequency of Tr1 cells as defined as LAG3$^+$CD49b$^+$ within the CD4$^+$FoxP3$^-$ **(B)**, and CD4$^+$FoxP3$^+$ T-cell gate **(C)** are shown at both 8 and 25 weeks of age in spleen, pancreatic draining lymph nodes (PLN), and mesenteric draining lymph nodes (MLN). Female NOD mice were fed normal chow (NC) or two different doses of vitamin D-supplemented (400 or 800 IU/day) diet from 3 until 25 weeks of age (lifelong). Symbols (N = 4-11) represent individual mice, and the line reflects the group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

consisting of 105 genera and 10 phyla on 38 paired samples. Phyla or genera not present in at least 50% of each experimental group were filtered-out, leaving 7 phyla and 35 genera.

We first demonstrated that the composition of the gut microbiome at 3 weeks of age (weaning) in mice receiving NC was indeed significantly different from that at 8 weeks of age, a time when all mice were still normoglycaemic. We observed that the Shannon's alpha-diversity index (genera diversity in terms of genera richness and relative abundance) and the Chao1 index (genera richness) significantly increased between 3 and 8 weeks of age in NOD mice receiving NC (**Figure 5A**; **Supplementary Table S1**), indicating increased taxonomic complexity of bacterial communities. At the phylum level, NOD mice receiving NC showed a significant difference over time in 4 out of 7 tested phyla with an increase in *Tenericutes*, *Proteobacteria*, and *Actinobacteria*, in addition to a decrease in *Epsilonbacteraeota* (**Figure 5B**; **Supplementary Table S2**), but overall we did not find a significant change in the *Bacteroidetes*: *Firmicutes* ratio between 3 and 8 weeks of age in NOD mice receiving NC (data not shown). At the genus level, NOD mice receiving NC showed an increase in the abundance of *Lachnospiraceae UCG-001* and a decrease in *Helicobacter* between 3 and 8 weeks of age (**Figure 5C**; **Supplementary Table S3**).

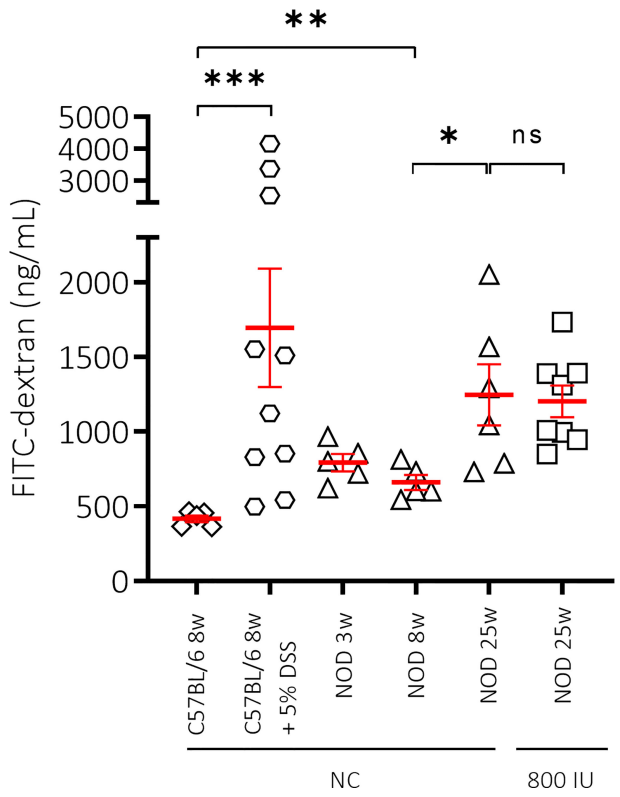

FIGURE 4 | Effect of mouse strain, age and vitamin D substitution regimen on intestinal permeability. Concentration of FITC-dextran as measurement for intestinal permeability is shown for female C57BL/6 mice at 8 weeks of age in addition to female NOD mice at increasing ages (3, 8, and 25 weeks of age). Mice received either normal chow (NC) or the 800 IU/day vitamin D-supplemented diet from 3 until 25 weeks of age. To validate our technique, dextran sulfate sodium (DSS) was added at 5% in drinking water *ad libitum* for 8 days to 8-week-old C57BL/6 mice. Symbols (N = 3-10) represent individual mice, and the line reflects the group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. NS means non significant.

We next examined whether there were characteristics of the microbial community that could discriminate the T1D status. We assessed the Shannon and Chao1 indices across time in P and NP mice receiving NC. Interestingly, the greater diversity and richness of the different taxa over time was only significant in NP mice receiving NC (**Figure 6A**; **Supplementary Table S1**). The increased alpha-diversity in NP went along with a significant increase in *Tenericutes* and a decrease in *Epsilonbacteraeota* phyla. *Proteobacteria* and *Actinobacteria* were also increased, although this was not statistically significant (**Figure 6B**; **Supplementary Table S2**). Moreover, the *Bacteroidetes*: *Firmicutes* ratio was not related to disease outcome (data not shown). At the genus level, we demonstrated that the differential abundance observed over time in *Lachnospiraceae UCG-001* and *Helicobacter* was only significant in the NP mice receiving NC (**Figure 6C**; **Supplementary Table S3**). We also identified a drop in alpha-diversity in faecal samples collected at 8 weeks of age of P compared to NP mice receiving NC (**Figure 6A**; **Supplementary Table S2**). Intriguingly, this divergence in alpha-diversity occurred at a time before the P mice presented with clinical disease.

Both Diet and Disease Outcome Influence the Microbial Community Composition in NOD Mice

Although the 400 and 800 IU/day vitamin D-supplemented diets did not significantly modify the increased alpha-diversity nor the different abundance in specific phyla observed over time in NOD mice receiving NC, a multivariate dbRDA analysis revealed that diet in addition to disease outcome induced significant changes in the composition of the gut microbiome at 8 weeks of age at the genus level (multivariate dbRDA, total adjusted $R^2 = 9.4\%$; diet P = 0.001; individual adjusted $R^2 = 7.57\%$. disease outcome P = 0.046. individual adjusted $R^2 = 1.58\%$; N = 38) (**Figures 7A, B**). Furthermore, a *post hoc* comparison of the diets revealed that the 800 IU/day vitamin D diet significantly deviated in microbial community composition from mice receiving the 400 IU/day vitamin D-supplemented diet or NC (univariate dbRDA, adjusted R2 = 2.34%; P = 0.012; N = 28 and R2 = 7.57%; P = 0.001; N = 19; respectively (**Figure 7B**).

High-Dose Vitamin D Supplementation Altered the Abundance of *Ruminiclostridium_9* and *Marvinbryantia* in Faecal Samples of 8-Week-Old NOD Mice, Irrespective of Disease Outcome

We demonstrated that vitamin D supplementation correlated with non-redundant changes in the NOD microbial community composition at 8 weeks of age (**Figure 7**). First, we noticed that the vitamin D-supplemented diet had no influence on the increased abundance of *Lachnospiraceae UCG-001* and *Helicobacter* identified in faecal samples of NOD mice at 8 weeks of age receiving NC (data not shown). On the other hand, we identified two bacterial genera that discriminated the 800 IU/day vitamin D group including *Ruminiclostridium_9* and *Marvinbryantia* from both the 400 IU/day vitamin D and NC groups at 8 weeks of age (**Figure 8A**; **Supplementary Table S3**). The abundance of the *Ruminiclostrium_9* and *Marvinbryantia* significantly increased or decreased, respectively with accumulating doses of vitamin D (**Figure 8A**; **Supplementary Table S3**). The abundance of the genus *Lachnospiraceae_FCS020_group* increased in faecal samples of mice receiving the 400 IU/day vitamin D-supplemented diet compared to both NC and 800 IU/day vitamin D-supplemented diet (**Figure 8A**). The diet-induced changes in the gut microbial community in NOD mice at 8 weeks of age did not correspond to disease outcome, although faecal samples of NP mice on the 800 IU/day vitamin D diet had a tendency for an increased abundance in the genus *Ruminiclostridium_9* (**Figure 8B**; **Supplementary Table S3**).

DISCUSSION

In the past, our team demonstrated that an 800 IU/day vitamin D-supplemented diet safely reduced T1D incidence in

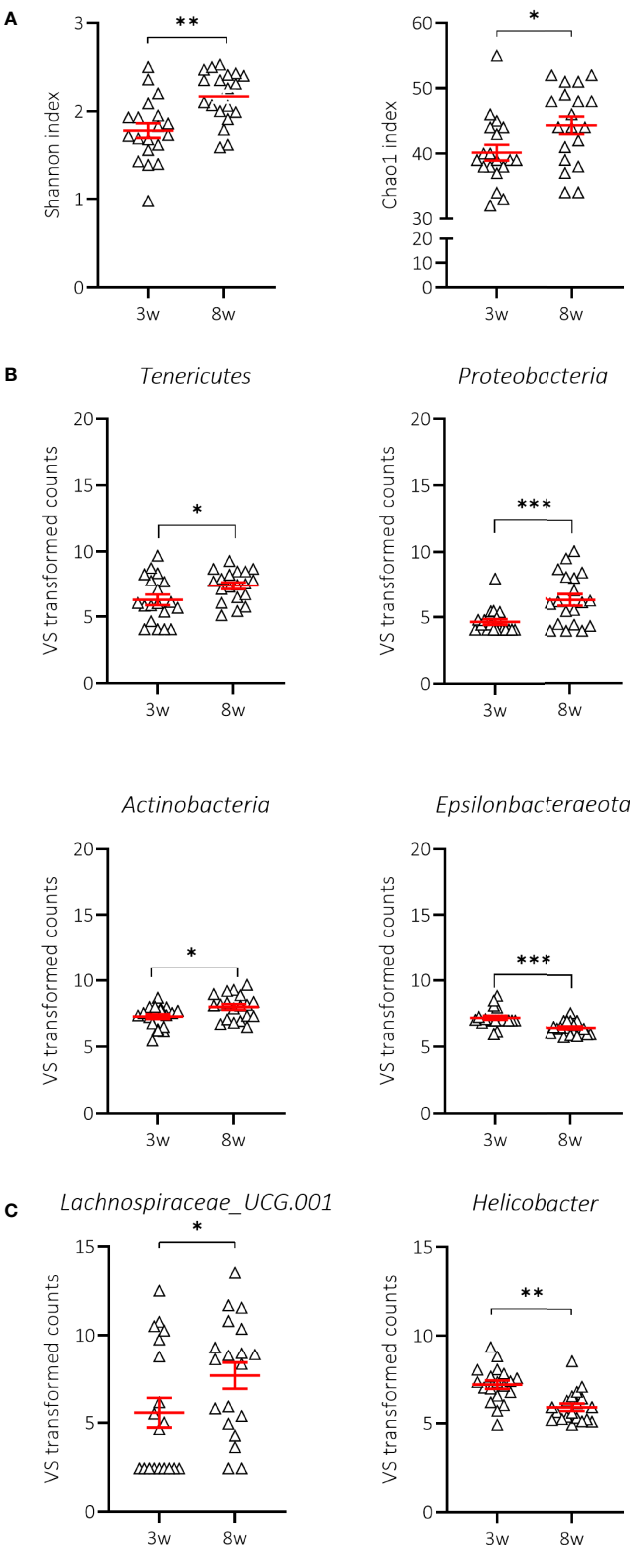

FIGURE 5 | Change in alpha-diversity and taxa abundances of NOD mice receiving normal chow (NC). Changes in Shannon and Chao1 indices **(A)**, phyla **(B)** and genera **(C)** are shown for female NOD mice receiving normal chow (NC) (N = 38 paired samples). Symbols (N = 9-19) represent individual mice, and the line reflects the group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

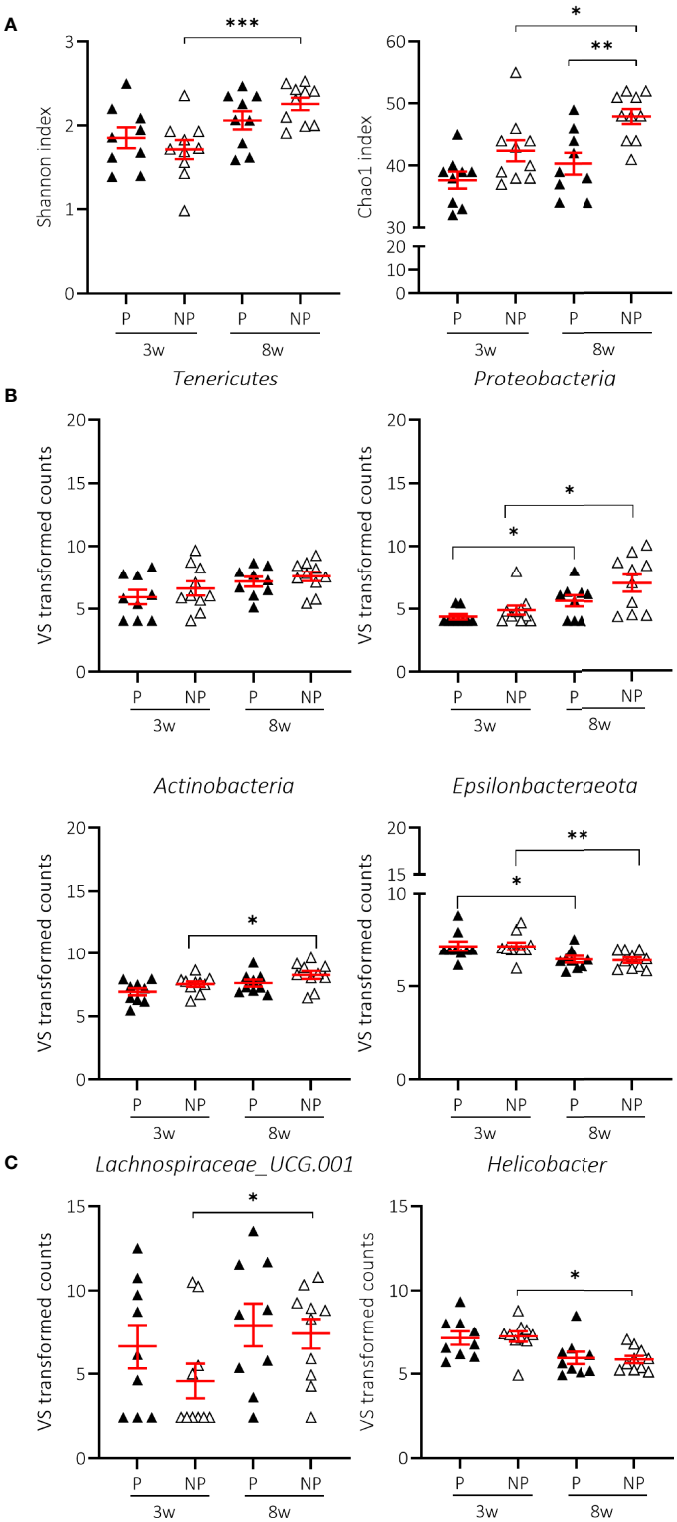

FIGURE 6 | Change in alpha-diversity and taxa abundances of T1D progressor (P) and non-progressor (NP) NOD mice receiving normal chow (NC). Changes in Shannon and Chao1 indices **(A)**, phyla **(B)**, and genera **(C)** are shown for female NOD mice receiving NC and progressing (P) (N = 18 paired samples) or not progressing towards T1D (NP) (N = 20 paired samples). Symbols (N = 4-10) represent individual mice, and the line reflects the group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

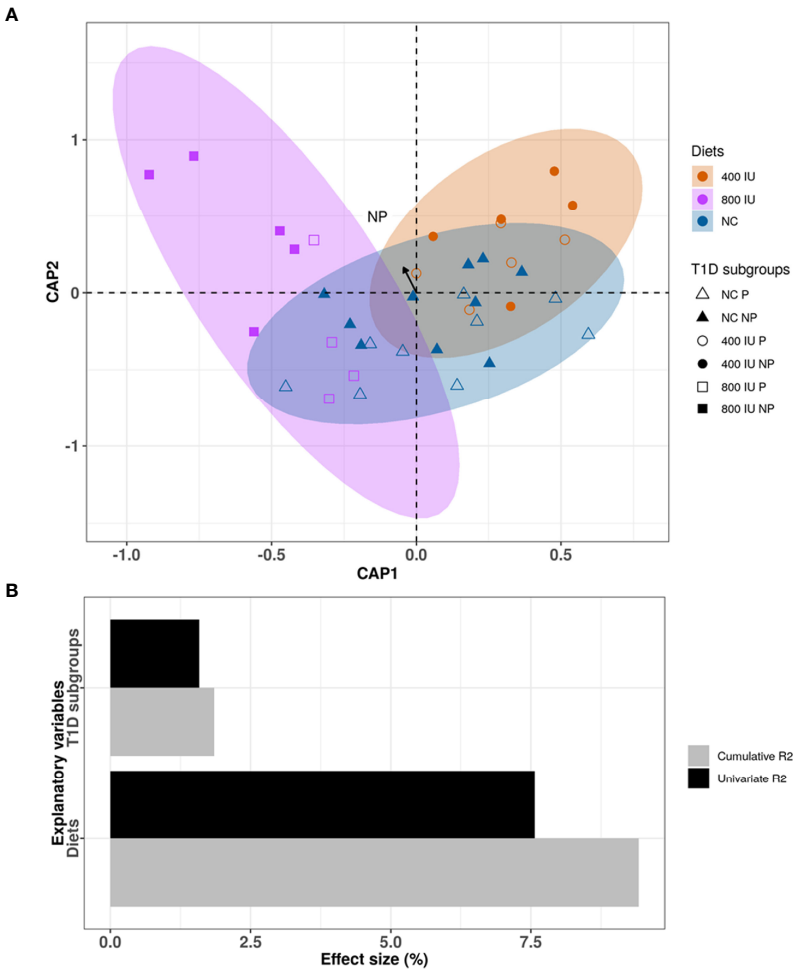

FIGURE 7 | Differences in microbial community composition between NOD mice receiving normal chow (NC), 400 IU/day or 800 IU/day vitamin D-supplemented diet and T1D subgroups at 8 weeks of age. Canonical analysis of principal coordinates (CAP) visualization of inter-mice differences in the microbiome profiles of the NOD mice cohort at 8 weeks of age (N = 38 biologically independent samples, data points coloured by diet and disease outcome subgroups denoted by shape). The figure is stratified by dietary subgroups depicting the differences in microbial composition of the mice receiving NC (N = 38 paired samples, blue), 400 (N = 20 paired samples, orange) or 800 IU/day (N = 18 paired samples, purple) vitamin D-supplemented diets at 8 weeks of age. The arrow points to the centroid of the T1D NP subgroup **(A)**. Bar plot representation of the explanatory power of the diet and disease outcome on the NOD microbiota compositional variation in a single variable model (univariate effect size [R2]) or combined in a multivariate model (cumulative R2) **(B)**. Statistical significance was calculated by distance-based redundancy analysis (Bray-Curtis dissimilarity index).

autoimmune diabetes-prone NOD mice, when given lifelong (17). While the scientific community is convinced that vitamin D deficiency (<25 nmol/L 25(OH)D_3 concentrations) should be avoided to prevent skeletal defects (47), there is less consensus on which prescriptions of oral vitamin D supplements or circulating 25(OH)D_3 concentrations are needed to interfere with the initiation and progression of inflammatory and autoimmune pathologies including T1D. Our previous study indicated that serum 25(OH)D_3 concentrations reaching a mean value of 290 nmol/L at the end of the study, in 35-week-old NOD mice, was linked to T1D protection. These concentrations are way beyond the 50 and 75 nmol/L that are respectively recommended by the Institute of Medicine (IOM) (48) and the Endocrine Society Task Force (20) for maximum bone health for children and adults. On the other hand, many authorities proclaim that optimal serum 25 (OH)D_3 concentrations for all health issues should be above 100 nmol/L (49). Behind this background, the Endocrine Society advocated an ideal range for circulating 25(OH)D_3 between 100 and 150 nmol/L, and judged a level up to 250 nmol/L as risk-free (20, 50, 51).

Hence, we studied whether a lower dose, 400 IU/day, of dietary vitamin D supplements would be equally effective in delaying and preventing T1D onset in NOD mice. Moreover, by studying not only the peripheral and local (PLN) immune systems, also the effects of vitamin D on different characteristics of the gut (i.e., MLN, intestinal barrier function, and intestinal microbiota composition), we hoped to acquire insights into the mechanisms by which vitamin D elicits disease

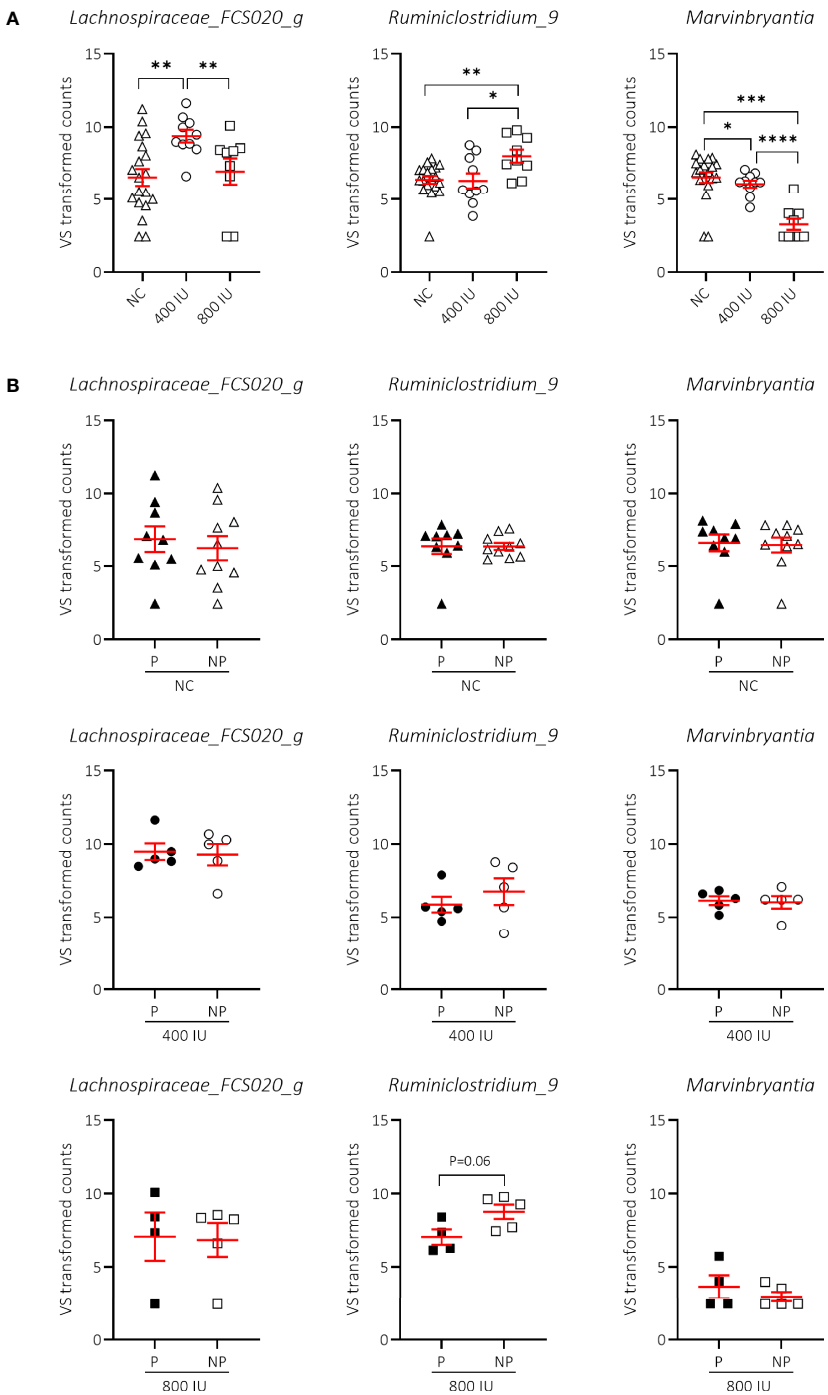

FIGURE 8 | Difference in genera abundances between diets and T1D subgroups at 8 weeks of age. Differences in genera abundances between diets at 8 weeks of age are shown for female NOD mice receiving normal chow (NC), 400 or 800 IU/day vitamin D-supplemented diets **(A)**. Differences in genera abundance for the corresponding diets shown in **(A)** are separated based on disease outcome **(B)**. P = progressor mice, NP = non-progressor mice. Symbols (N = 4-19) represent individual mice, and the line reflects the group mean with SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.

protection in NOD mice. We demonstrated that only the 800, and not the 400, IU/day vitamin D-supplemented diet could significantly delay and prevent T1D development in NOD mice by 25 weeks of age. We also found that at 5 weeks on the diet (at 8 weeks of age), serum $25(OH)D_3$, but not $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, concentrations positively correlated with T1D outcome later in life. Although $1,25(OH)_2D_3$ is the active (hormonal) form of vitamin D, its circulating values are 1,000-

fold less compared to $25(OH)D_3$ values and it has a short half-life (4-6 hours), limiting its utility as biomarker for vitamin D status. Both $25(OH)D_3$ and $1,25(OH)_2D_3$ undergo further metabolism, primarily by renal 24-hydroxylase, to generate $24,25(OH)_2D_3$ (52). The production of $24,25(OH)_2D_3$, the major circulating catabolite of vitamin D, is only modestly affected by vitamin D supplementation (53), and its physiological role remains elusive. While we observed that a particular threshold value for circulating $25(OH)D_3$, above 150 nmol/L, was linked to T1D protection and accompanied by moderate hypercalcemia and -phosphatemia in mice, hard evidence from other disease models is currently lacking to make strong recommendations on the optimal vitamin D concentrations needed to avoid development of cancer, infections, metabolic and autoimmune diseases in humans.

Amongst the many reported extra-skeletal effects of vitamin D, its capability to modulate both the innate and adaptive systems has obtained significant attention. It is traditionally believed that bioactive $1,25(OH)_2D_3$ functions directly *via* its receptor, being present on almost all cells of the immune system (14). Many immune cells also possess vitamin D metabolizing enzymes which can affect the autocrine/paracrine vitamin D system and subsequently promote or abrogate response of 25 $(OH)D_3$. In this context, vitamin D can promote a shift from an inflammatory T helper (Th)1 towards a tolerogenic response *via* the induction or expansion of Th2 cells and Treg cells (13, 54). While some researchers observed only increases in the frequency of Treg cells (55), others reported improved suppressive capacity of Treg cells without alterations in their abundance (56). Although not completely straightforward in this study, we did detect a persistent increase in the percentages of $FoxP3^+$ Treg cells in the spleen of the 800 IU/day vitamin D-supplemented mice at 25 weeks of age compared to the other dietary groups. Treg cells can exert their suppressive function by several mechanisms, including the release of immunosuppressive cytokines such as IL-10 and TGF-β, or *via* cellular communication (57). Active vitamin D has been shown to instruct $CD4^+$ T cells to convert into $CD4^+CD25^{hi}IL\text{-}10^+$ Treg cells. However, there is currently no consensus on whether these cells also express the lineage-specifying transcription factor *FoxP3* (58, 59). In this study, we also observed an increase in the frequencies of IL-10-producing $CD4^+$ T cells in all studied immune organs of mice at 25 weeks of age receiving the 800 IU/day vitamin D-supplemented diet but this increase was not due to an expansion of $FoxP3^-$, nor of $FoxP3^+$, Tr1 cells, co-expressing LAG3 and CD49b. A recent transcriptomic analysis substantiated our results that vitamin D does not enrich for genes in the Tr1 cell signature. On the other hand, they found that vitamin D, through autocrine/paracrine production, can generate IL-10-producing $CD4^+$ T cells *via* IL-6 and STAT3 signalling (60).

A few remarkable reports prompted us to study whether vitamin D could influence the frequency of $FoxP3^+$ Treg cells endowed with the expression of the cell surface ecto-enzymes CD39 and CD73 that subsequently regulate pericellular adenosine accumulation from extracellular nucleotide catabolism (61, 62). Adenosine has been shown to suppress Teff functions by binding to a number of adenosine receptors (i.e., A1R, A2AR, A2BR, and A3R) that are expressed in some immune cell subsets and endothelial cells (42, 63). While vitamin D has been shown to upregulate the expression of CD39 and CD73 on $CD4^+FoxP3^+$ and/or $CD4^+Foxp3^-$ T cells, expressing the late-stage Treg activation markers glycoprotein A repetitions predominant (GARP) and LAP, in addition to neuropilin-1 (61, 62), we did not study the expression level (i.e., mean fluorescent intensity) of CD39 and CD73 on $FoxP3^+$ Treg cells. However, we did identify an 800 IU/day vitamin D-induced increase in the abundance of $CD73^+FoxP3^+$ Treg cells in mice at 25 weeks of age, but only in the systemic circulation. CD73 has been shown to be critical in mediating many immunosuppressive features of T cells. Whether CD73-expressing $FoxP3^+$ T cells exert their function in T1D development by patrolling the systemic circulation and subsequently suppressing Teff activation and/or limiting Teff migration to the PLN and pancreas warrants further investigation. Intriguing observations in the T1D field indicate that $CD73^+CD4^+$ T cells can inhibit islet-reactive T-cell proliferation by the production of the anti-inflammatory cytokine TGF-β, yet not of IL-10 (64).

As mentioned before, intestinal dysbiosis, low-grade intestinal inflammation, and intestinal barrier dysfunction at an early age, allowing increased antigen (i.e., dietary or microbial) trafficking, could trigger T1D initiation *via* the activation of diabetogenic T cells in the intestinal mucosa (44, 65). Alternatively, loss of intestinal barrier function could allow the release of bacterial pathogens with molecular mimicry to islet antigens in the periphery, which could directly damage insulin-producing β cells or activate islet-reactive T cells within the PLN and pancreas (8). We confirmed by a FITC-dextran assay that NOD mice of 3 weeks of age (at weaning) already have augmented gut permeability, a time point before the development of insulitis and overt hyperglycaemia, which further increased with age. Although the regimen with 800 IU/d of dietary vitamin D did not correct the intestinal barrier dysfunction in NOD mice by 25 weeks of age, we tested *via* 16S rRNA sequencing whether intestinal dysbiosis could be another gut-related trait leading to T1D initiation and could be modified by vitamin D. An aberrant microbial composition of the gut may result in a distorted maturation of the immune system and increase the vulnerability to immune-mediated diseases. We found that bacterial Chao1 richness, Shannon diversity, and the relative abundance of particular phyla (i.e., *Proteobacteria*, *Tenericutes*, and *Actinobacteria*) increased in NOD mice during aging (from 3 until 8 weeks of age), specifically in NOD mice not further progressing to overt hyperglycaemia. A greater intestinal microbiota diversity is conducive to enrichment of bacterial taxa that can produce bile acids and short chain fatty acids (SCFA) like acetate, butyrate, and propionate, which are beneficial for not only gut barrier function, and intestinal immunomodulation, but also help maintain homeostasis and health during the whole lifespan (66–68). The *Bacteroidetes* phylum is associated with the production of acetate and propionate, while the *Firmicutes* phylum mainly produces butyrate. Here, the ratio *Bacteroidetes*

to Firmicutes was not modified in NOD mice over time nor was it related to disease outcome. Conflicting reports have been published with some authors demonstrating a successive decline in *Firmicutes* and increase in *Bacteroidetes* species in the intestinal microbiome of children during the first 6 months of life before T1D development (69), while others did not found any correlation between *Bacteroidetes* abundance and T1D onset (70). Still, our data propose that the phyla *Proteobacteria*, *Tenericutes*, and *Actinobacteria* were associated with T1D protection in NOD mice later in life.

Diet is an important factor in intestinal microbiota shaping (68). Although vitamin D did not alter the bacterial richness, diversity, and phyla abundance in NOD mice over time, a multivariate dbRDA analysis revealed a significant association between community composition and vitamin D supplementation at the genus level. The 800 IU/day vitamin D-supplemented group had a higher relative abundance of the genus *Ruminiclostridium_9* and a lower relative abundance of the genus *Marvinbryantia* compared to the 400 IU/day vitamin D-supplemented and NC groups at 8 weeks of age. We also observed a trend of a higher relative abundance of *Ruminiclostridium_9* in mice not further progressing to T1D. Little is known about the physiological and pathogenic roles of *Ruminiclostridium_9* and *Marvinbryantia* in the gastrointestinal tract. A recent report found a decrease in *Ruminiclostridium_9* to be associated with exacerbated insulitis in NOD mice (71). Both *Ruminiclostridium_9* and *Marvinbyrantia* belong to the phylum *Firmicutes* and are proposed to be involved in the conversion of primary to secondary bile acids and butyrate production. The beneficial effects of butyrate on local and systemic immunity are well defined. Butyrate can recruit Treg cells in the colon as well as the pancreas (72) and modulate their function, linking them to crosstalk between the intestinal microbiota and immune system. Moreover, butyrate has been observed to increase intestinal VDR expression (73), which could further potentiate the effect of vitamin D. Vitamin D seems to have opposing effects on the abundance of these two genera, necessitating more in depth studies with larger sample size.

In summary, our investigation of the influence of dietary vitamin D supplementation on T1D development revealed that only the higher dose (800 IU/day) could delay disease onset and significantly reduce T1D incidence in NOD mice. In an attempt to characterise the mechanisms of T1D protection elicited by this dosing regimen, we identified a peripheral expansion of $FoxP3^+$ Treg cells that may modulate autoimmune inflammation in a CD73-dependent manner. Increased frequencies of IL-10-producing $CD4^+$ T cells in various immune organs of mice given the 800 IU/d vitamin D-supplemented diet may constitute a supplementary mechanism in restoring peripheral tolerance. In addition, high-dose vitamin D supplementation and T1D protection were associated with alterations in microbial community composition favouring *Ruminiclostridium_9* and diminishing *Marvinbryantia* at the genus level. The results of this preclinical study are promising, but also warrant more research into the interplay between vitamin D, the gut microbiota, and T1D progression. Indeed, further insights are needed into the specific molecular interactions with the host immune responses and how the gut microbiota may alter T1D development. Additionally, human studies will have to be designed to ensure translatability of our findings. This study sheds further light on the potential of vitamin D as an environmental exposure in delaying and preventing T1D in genetically predisposed individuals. Integration of these findings into our understanding of T1D may help to advance therapeutic efficacy in the new age of preventative treatments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/ena/browser/view/PRJEB51877.

ETHICS STATEMENT

All animal experiments were approved by the Ethics Committee of the KU Leuven Animal Care and Use Committee and compiled with Belgian animal protection law under animal experiment license 114/2015.

AUTHOR CONTRIBUTIONS

P-JM, JC-L, DC, and DE designed and performed research as well as analysed and discussed the data and wrote the manuscript. LV did experiments and analysed data. LV, AV, and JR offered resources. JR discussed the data. CM and CG conceptualized the research goals, acquired major funding, designed research, analysed and discussed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.902678/full#supplementary-material

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EDITED BY
Nabiha Yusuf,
University of Alabama at Birmingham, United States

REVIEWED BY
Chander Raman,
University of Alabama at Birmingham, United States
Mohammad Asif Sherwani,
University of Alabama at Birmingham, United States

*CORRESPONDENCE
Zhiying Luo
lzhy199089@csu.edu.cn
Wenhui Liu
504474@csu.edu.cn

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Vitamin D metabolism pathway polymorphisms are associated with efficacy and safety in patients under anti-PD-1 inhibitor therapy

Jianquan Luo[1,2], Huiqing Chen[1,2], Fang Ma[3], Chenlin Xiao[1,2], Bao Sun[1,2], Yiping Liu[1,2], Haoneng Tang[1,4], Yue Yang[5], Wenhui Liu[1,2]* and Zhiying Luo[1,2]*

[1]Department of Pharmacy, The Second Xiangya Hospital, Central South University, Changsha, China, [2]Institute of Clinical Pharmacy, Central South University, Changsha, China, [3]Department of Oncology, The Second Xiangya Hospital, Central South University, Changsha, China, [4]Department of Laboratory Medicine, The Second Xiangya Hospital, Central South University, Changsha, China, [5]Department of Spine Surgery, The Second Xiangya Hospital of Central South University, Changsha, China

Aim: Vitamin D (VitD) signaling has been increasingly investigated for its role in stimulating the innate and adaptive immune systems and suppressing inflammatory responses. Therefore, we examined the associations between VitD-related genetic polymorphisms, plasma 25-hydroxyvitamin D (25(OH)D), and the efficacy and safety of immune checkpoint inhibitors (ICIs).

Patients and methods: A total of 13 single-nucleotide polymorphisms (SNPs) in VitD metabolic pathway genes were genotyped in 343 cancer patients receiving ICI treatment using the MassARRAY platform. In 65 patients, the associations between plasma 25(OH)D levels and ICI treatment outcomes were investigated further.

Results: We found that the *CYP24A1* rs6068816TT and rs2296241AA genotypes were significantly higher in patients who responded to ICIs. Furthermore, patients with higher plasma 25(OH)D levels had a better treatment response. The distribution of allele and genotype frequencies showed that three SNPs (rs10877012, rs2762934, and rs8018720) differed significantly between patients who had immune-related adverse events (irAEs) and those who did not. There was no statistically significant relationship between plasma 25(OH)D levels and the risk of irAEs.

Conclusion: In summary, our findings showed that genetic variations in the VitD metabolism pathway were associated with ICI treatment outcomes, and VitD supplementation may be useful in improving ICI treatment efficacy.

KEYWORDS

immune checkpoint inhibitors, anti-PD-1 inhibitors, immune-related adverse effects, polymorphism, vitamin D

Introduction

Immune checkpoint inhibitors (ICIs) are a novel class of drugs that target the programmed death ligand-1 (PD-L1)/programmed cell death protein-1 (PD-1) pathway and have been approved as first-line therapy for serious cancers (1). ICIs are essentially humanized monoclonal antibodies that can activate T cells and relieve the immune system from recognizing and assaulting cancer cells. Successful immunotherapy-induced anti-tumor immune responses require $CD8^+$ and $CD4^+$ T cells (2, 3). Because of the unsatisfactory efficacy of ICI monotherapy, ICIs combined with chemotherapy, radiotherapy, or anti-angiogenesis therapy have been approved as successful first-line therapy for several malignant tumors regardless of the PD-L1 expression level in tumor tissues (4). Investigating the mechanisms of insensitivity to immunotherapy has emerged as one of the most important challenges in cancer immunotherapy.

Paradoxically, combination therapy is often associated with a high incidence of immune-related adverse events (irAEs) (5, 6). The unleashed immune response could promote T-cell activation and autoimmunity, resulting in many systemic autoinflammatory reactions (7). IrAEs manifest differently in different patients, with some developing irAEs in a single organ and others developing irAEs in multiple organs (8). Several of these irAEs are self-limiting and easily manageable. Others may limit treatment, causing interruption that will require treatment with methylprednisolone or tumor necrosis factor-α antibody or even directly threaten life (9). Recent studies indicate that irAEs likely result from abnormal T- and B-cell activation and an overall increased inflammatory response, resembling the hyperimmune responses observed in autoimmune patients (10). Undoubtedly, the mechanisms of irAEs are complex and not fully understood. Because irAEs limit the therapeutic benefits of ICIs, identifying and investigating potential biomarkers that can predict the efficacy and safety of ICIs have received much attention in recent years.

Vitamin D (VitD) signaling has been increasingly investigated for its non-classical actions in stimulating the innate and adaptive immune systems and suppressing inflammatory responses (11, 12). It is known that VitD deficiency decreases the numbers of CD4+ and CD8+ T lymphocytes, while VitD supplementation increases CD4+ lymphocytes. In the direct and indirect pathways, VitD can induce and stimulate T-regulatory cells (Tregs), which can suppress proinflammatory responses by other immune cells and prevent exaggerated or autoimmune reactions (13). Similarly, VitD suppresses the tumor microenvironment by increasing the Treg/T-helper 17 (Th-17) cell ratio (14, 15). VitD has been shown to induce the expression of PD-L1 on human gut epithelial cells and PD-1 on immune cells in patients with inflammatory bowel diseases (16, 17). Numerous studies in the last decade have linked VitD deficiency and genetic polymorphisms in genes involved in the VitD metabolism pathway to an increased risk of several autoimmune diseases and cancers (18–20).

Recent studies suggest that the genetic background of patients receiving ICIs could play a role in susceptibility to irAEs (21). Several single-nucleotide polymorphisms (SNPs) located in genes related to VitD metabolism have been linked to plasma 25(OH)D levels and immune disease (22). Given the immunoregulatory activity of VitD, we hypothesize a possible link between polymorphisms associated with VitD physiological disposition and ICI treatment outcomes. We aimed to analyze the relationship between genetic variants underlying VitD metabolism (*VDR*, *CYP24A1*, *CYP27B1*, *CYP2R1*, *GC*, *DHCR7*, *RXRA*, and *SEC23A*) and the efficacy and safety in patients treated with anti-PD-1 inhibitors.

Materials and methods

Study population

A unicentric and retrospective study was conducted to elucidate the effect of genetic polymorphisms on the efficacy and safety of inter-individual differences. We collected the blood samples from patients receiving anti-PD-1 inhibitor (nivolumab or pembrolizumab) therapies regardless of treatment lines between October 2018 and January 2022 in the Department of Oncology, Second Xiangya Hospital, Central South University (Changsha, China). The Ethics Committee of Second Xiangya Hospital at Central South University (Changsha, China) approved this study, and all procedures followed the Declaration of Helsinki. The study was registered with the Chinese Clinical Trial Registry (ChiCTR2100045873). All patients provided written informed consent for blood banking and clinical information follow-up.

The inclusion criteria for patients are as follows: 1) age >18 years; 2) clinical symptoms, physical signs, imaging examination, and histologically or cytologically consistent with the diagnostic criteria for tumors; 3) treatment with anti-PD-1 inhibitor monotherapy or combination therapy at the recommended dose; and 4) no prior history of inflammatory or serious autoimmune diseases in the same affected organs.

Collection of clinical variables and follow-up method

Electronic medical records were reviewed for demographics (such as age and sex), smoking and drinking status, primary tumor sites, histological types, Eastern Cooperative Oncology Group (ECOG) performance status (PS), PD-1 expression level, treatment strategies, and past medical history. All elements were

abstracted and entered into a clinical data sheet. Patients in this study were prescribed five types of anti-PD-1 inhibitors (nivolumab, pembrolizumab, terelizumab, sindilizumab, and carrelizumab) and administered intravenously at a dose of 200 mg every 3 weeks as recommended. Other drugs, particularly broad-spectrum antibiotics, were also used. According to our previously published study (23), the follow-up lasted 6 months with regular clinic visits by an oncologist (Dr. Fang Ma) and two pharmacists (Pharm. Wenhui Liu and Pharm. Jianquan Luo).

The primary objective of this study was to determine the efficacy of an anti-PD-L1-based treatment strategy. According to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria, the objective remission rate (ORR) and disease control rate (DCR) were used to assess the treatment efficacy. ORR was defined as the percentage of patients who achieved a complete response (CR) or partial response (PR) to treatment. The proportion of patients with CR, PR, or stable disease (SD) was defined as DCR. The second objective was irAEs, which were assessed and graded using the Common Terminology Criteria for Adverse Events version 4.0 (CTCAE4.0).

Single-nucleotide polymorphism selection

The inclusion criterion for the candidate polymorphisms in our research is SNPs that may result in a functional alteration of the vitamin D metabolism pathway. The following were the selection criteria for the candidate SNPs: minor allele frequency (MAF) >1% in the Chinese population, with potential functions or associated with VitD concentration. Finally, we selected 13 SNPs from the VitD metabolism pathway, including *VDR* (rs1544410, rs731236, rs7975232, and rs2228570), *CYP24A1* (rs2296241, rs6068816, and rs2762934), *CYP27B1* (rs10877012), *CYP2R1* (rs2060793), *GC* (rs7041), *DHCR7* (rs12785878), *RXRA* (rs9409929), and *SEC23A* (rs8018720), which have been related to VitD circulating concentrations and immune disease. HaploReg showed that these polymorphisms were regulated by Enhancer histone marks, DNAse, proteins bound, motifs changed, and so on. However, the effects of the selected polymorphisms on gene expression remain unclear in previous studies.

DNA extraction and genotyping

The Wizard Genomic DNA Purification kits (Promega, Madison, WI, USA) were used to extract genomic DNA from 2 ml of peripheral blood samples, according to the manufacturer's protocol. Genotyping was performed using the SNP Sequenom MassARRAY platform (Bioyong Technologies Inc., Beijing, China). AssayDesigner (Ver. 3.1) designed the primers. The primer and probe sequences are shown in Supplementary Table 1. Subsequently, SNPs were genotyped using iPLEX Gold technology (Sequenom, San Diego, CA, USA), and automated data analysis was performed. SNPs with >1% MAF and >95% call rate have been sorted out for analysis. Additionally, 10% of randomly selected samples were retested using Sanger Sequencing, yielding a >99.9% concordance. The positive rates for these SNPs exceeded 90%, with some data missing due to competition in the genotyping reaction system in such a high-throughput technology.

Measurement of plasma 25(OH)D

The plasma 25(OH)D level was measured at the Second Xiangya Hospital's Department of Laboratory Medicine (Changsha, China). In this study, an additional 2 ml of peripheral blood was collected from 65 patients under ICI therapy using an EDTA anticoagulant tube. The plasma 25 (OH)D level was determined using a chemiluminescent immunoassay (CLIA) by Roche, Elecsys 2010 (Basel, Switzerland). CLIA is the most commonly used clinical test for the VitD state. The Elecsys VitD total assay uses a VitD binding protein as a capture protein to bind vitamin D3 (25-OH) and vitamin D2 (25-OH). The measuring range was 3.00–70.0 ng/ml or 7.50–175 nmol/L (defined by the limit of detection and the maximum of the master curve). VitD deficiency is defined as a VitD (25-OH) concentration of ≤20.0 ng/ml (≤50.0 nmol/L). VitD insufficiency is recognized as 21.0–29.0 ng/ml.

Statistical analyses

Both SPSS 19.0 (IBM Corp., Chicago, IL, USA) and Plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) were used to carry out the statistical analysis. All tests were two-tailed, and p-values <0.05 were considered a significant difference. Quantitative data were presented as mean values and standard deviation (SD). The chi-squared test or Fisher's exact test was used to compare categorical data and evaluate the Hardy–Weinberg equilibrium (HWE). Direct counting was used to compare genotype and allele frequencies, and a t-test was used for between-group comparisons. The associations between polymorphisms and the risk of treatment outcomes were expressed as the odds ratios (ORs) and 95% confidence intervals (CIs).

Results

Clinical parameters and their impact on efficacy and safety of immune checkpoint inhibitors

This study included 343 qualified patients who received anti-PD-1 inhibitors regardless of treatment line and had an available

blood sample. Table 1 shows the demographic and baseline characteristics of the patients involved. The overall median age of the patients was 58.41 ± 10.63 years, with 283 (82.51%) being male. More than half of the patients (51.89%) had a history of smoking, and 30.03% had a history of drinking alcohol. Most of the enrolled patients had advanced malignant tumors (stage III–IV), particularly non-small cell lung cancer (NSCLC). Most patients had good health conditions (ECOG PS score ≤1). Most patients had PD-L1 expression levels tested before being administered with anti-PD-1 inhibitors, and approximately two-thirds (≥1%) of patients had positive PD-L1 expression levels. In 301 (87.75%) patients, anti-PD-1 inhibitors combined with chemotherapy or radiotherapy were the most frequently used first-line treatment strategy.

The median follow-up duration for this cohort was 11.7 months [interquartile range, 7.2–16.5 months]. We considered therapy effective when patients were classified as CR, PR, or SD. Patients were evaluated as PD when their treatment was infective. Only 296 (86.30%) patients had available data to assess efficacy. Sixteen (4.66%) patients achieved CR, 129 (37.61%) patients achieved PR, 96 (27.99%) patients experienced SD, and 55 (16.03%) patients suffered PD. ORR was 70.26%, and DCR was 42.27%. Table 2 illustrates the treatment efficacy of anti-PD-1 inhibitors in this study. During the follow-up period, a total of 215 patients developed irAEs, and most patients (159, 73.95%) presented with mild irAEs. The most frequently reported irAEs in our patient population were pruritus and/or rash, thyroid dysfunction, and peripheral neuritis, as shown in Figure 1A. Figure 1B shows that 120 patients had at least two types of irAEs. Most patients (159, 46.35%) experienced mild irAEs, and only 56 (16.33%) suffered from severe irAEs (grade 3–5).

Clinical parameters and their impact on efficiency

Five patients died due to disease progression, and we were unable to evaluate efficacy in 47 patients during follow-up. Because most patients were still alive at the end of the follow-up period, obtaining mean overall survival for patients was not feasible. In univariate analysis, positive factors in patients with good efficacy (DCR) included NSCLC diagnosis ($p = 0.012$), higher PD-L1 expression level ($p = 0.006$), and use of anti-PD-L1 inhibitors ($p = 0.001$) as first-line treatment. Negative factors, such as patients with a higher PS score (≥2) and ICI monotherapy, were associated with lower treatment efficacy. Our findings confirmed that the incidence of irAEs was associated with a more favorable prognosis (66.39% *vs.* 50%, $p = 0.029$). Age, sex, body mass index, smoking and drinking habits, or disease stage ($p > 0.05$) did not affect treatment efficacy. These findings are presented in Supplementary Table 2.

Pharmacogenomic association of vitamin D pathway polymorphisms with the efficacy of immune checkpoint inhibitors

First, we genotyped 13 SNPs in 343 patients, and the complete list of candidate SNPs for our sample is shown in Table 3. The potential function of these polymorphisms was analyzed based on HaploReg. The allele frequencies in the studied sample were similar to the MAF value of CHS: Southern Han Chinese from the 1000 Genomes Project. The genotyping results revealed that each SNP had a higher than 95% call rate. One SNP (*VDR* rs2228570) ($p < 0.05$) was excluded from the subsequent analyses due to its deviation from HWE.

Second, we established a link between genotypes and treatment response (DCR). These findings are shown in Supplementary Table 3. Two SNPs (rs6068816 and rs2296241) were significantly associated with DCR (Table 4). The frequency of rs6068816T allele was significantly lower in the patients with ineffective responses than in those with effective responses (47.11% *vs.* 66.38%, OR (95% CI): 0.45 (0.29–0.69); p = 3.14E−4). The frequency of the rs2296241A allele in patients with ineffective treatment was significantly lower than in those with effective treatment (33.96% *vs.* 46.86%, OR (95% CI): 0.58 (0.37–0.91); $p = 0.017$). These two SNPs were also associated with DCR ($p = 0.00058$ and $p = 0.048$) after adjusting for baseline PS score, cancer type, treatment line, PD-L1 expression level, anti-PD-1 monotherapy, and treatment line. The linkage disequilibrium (LD) information presented here for the SNP pair (rs6068816 and rs2296241) is based on haplotype frequencies estimated using the expectation–maximization (EM) algorithm, $R^2 = 0.25$, $D' = 0.73$. This result showed that these two SNPs had a weak LD association.

Pharmacogenomic association of vitamin D pathway polymorphisms with the safety of immune checkpoint inhibitors

Except for the disease stage, the demographic and baseline characteristics were nearly identical between patients with or without irAEs (as shown in Supplementary Table 4). As shown in Supplementary Table 5, the association analysis results in this cohort revealed that two SNPs (rs10877012 and rs8018720) were significantly associated with the development of irAEs. The allele and genotype frequency distribution showed that three SNPs

TABLE 1 Demographic and baseline characteristics of enrolled samples.

Characteristics	Patient count (N = 343)
Age, years	
Mean ± SD	58.41 ± 10.63
Sex	
Male	283 (82.51%)
Female	60 (17.49%)
BMI, mean ± SD	22.20 ± 3.70
BMI < 18.5	36 (10.50%)
18.5 ≤ BMI ≤ 24.9	244 (71.14%)
25 ≤ BMI ≤ 29.9	59 (17.20%)
BMI > 30	4 (1.17%)
Smoke habit	178 (51.89%)
Drink habit	103 (30.03%)
Disease stage	
I–II	41 (11.95%)
III–IV	302 (88.05%)
Cancer type	
Non-small cell lung cancer	213 (62.10%)
Esophagus cancer	31 (9.04%)
nasopharyngeal carcinoma	14 (4.08%)
Malignant melanoma	5 (1.45%)
Other types	80 (23.32%)
ECOG PS score before treatment	
0	6 (1.70%)
1	320 (93.30%)
≥2	17 (5.0%)
Patients with PD-L1 expression level	245 (71.43%)
<1%	84 (34.28%)
1%–49%	79 (32.24%)
≥50%	82 (33.48%)
Anti-PD-1 plus chemotherapy or radiotherapy	301 (87.75%)
Anti-PD-1 monotherapy	42 (12.25%)
Treatment line	
First line therapy	242 (70.55%)
Second- or third-line therapy	101 (29.45%)

BMI, body mass index; ECOG PS score, Eastern Cooperative Oncology Group performance status.

differed significantly between groups. In *CYP27B1* gene, TT genotype carriers had a significantly lower risk of irAEs than rs10877012 GG+GT genotype carriers (50.41% *vs.* 33.49%, OR (95% CI): 0.51 (0.32–0.80); p = 0.0037). The G allele frequency was significantly lower in patients who did not experience irAEs than in those who did (29.34% *vs.* 40.09%, OR (95% CI): 0.61 (0.44–0.86); p = 0.0057). In *CYP24A1* gene, the rs2762934G allele was significantly lower in patients with irAEs compared to patients without irAEs (92.58% *vs.* 87.80%, p = 0.043). For *SEC23A* rs8018720, GC genotype carriers were more likely to develop irAEs (53.05% *vs.* 39.37%, p = 0.049). These results are presented in Table 5.

The relationship between plasma 25(OH)D concentration and immune checkpoint inhibitor clinical outcomes

Finally, plasma 25(OH)D levels were measured in 65 patients in this study, with a mean age of 57.56 ± 9.43 years. Eight patients responded to ICIs, and 41 patients developed irAEs. The plasma level of 25(OH)D was significantly higher in patients with effective responses (44.56 ± 13.15 ng/ml) than in patients with ineffective outcomes (33.67 ± 11.36 ng/ml) (p = 0.001). In contrast, no difference in 25(OH)D levels was found between patients with and without irAEs (43.81 ± 12.15 *vs.* 38.11 ± 12.43 ng/ml, p = 0.38).

Discussion

Recently, VitD signaling has been increasingly investigated for its non-classical actions in stimulating innate immunity and suppressing inflammatory responses. In this retrospective study, we examined the association between polymorphisms in the VitD metabolism pathway and the efficacy and safety of anti-PD-L1 treatment. We identified two SNPs in *CYP24A1* gene linked to a higher likelihood of treatment response. Furthermore, three SNPs were statistically related to the risk of irAEs.

Previous studies have shown that clinical parameters, such as PD-L1 expression level, tumor histology, and anti-PD-1 monotherapy, are significantly associated with ICI treatment efficacy (24, 25). Our findings are consistent with those studies, implying that our findings are reliable. For instance, our study found that patients with NSCLC who had lower PS scores before ICI treatment, high PD-L1 expression levels, combination therapy, and ICIs as first-line therapy were more likely to benefit from ICI treatment. Furthermore, we confirmed that patients who respond to ICIs are more likely to develop irAEs. This finding raises the possibility of shared genetic relationships between treatment-related toxicity and efficacy. One previous study, for example, found that two human leukocyte antigen (HLA) alleles (HLA-DRB1*11:01 and HLA-DQB1*03:01) are predisposed to autoimmune diseases and are associated with an increased risk of developing pruritus or colitis during immunotherapy (26). Similarly, HLA genotyping was previously performed in patients with melanoma and NSCLC treated with ICIs; patients with HLA-B44 had longer survival, whereas those with HLA-B62 had worse disease outcomes (27).

VitD comprises a group of structurally related fat-soluble compounds that regulate over 200 genes and are essential for a wide range of physiological processes (28). VitD is first hydroxylated at the 25 position to 25(OH)D by *CYP27A1* and *CYP2R1*, 25(OH)D is considered active VitD, and its plasma concentrations can be used to determine the VitD status of

TABLE 2 The evaluated treatment efficacy by RECIST1.1.

Primary endpoints (efficacy)	
Complete response	16 (4.66%)
Partial response	129 (37.61%)
Stable disease	96 (27.99%)
Progressive disease	55 (16.03%)
Not evaluable	47 (13.70%)
DCR	241 (70.26%)
ORR	145 (42.27%)
Second endpoints (safety)	343 (100%)
irAEs	215 (62.68%)
Severe irAEs (grades 3–5)	56 (16.33%)
Mild irAEs (grades 1–2)	159 (46.35%)
No immune-related adverse events	128 (37.32%)

RECIST, Response Evaluation Criteria in Solid Tumors; DCR, disease control rate; ORR, objective remission rate; irAEs, immune-related adverse events.

patients. In the kidney, 1-hydroxylase (encoded by *CYP27B1*) hydroxylates to 1,25-dihydroxy VitD [1, 25(OH)2D]. Finally, 24-hydroxylase (*CYP24A1*) converts the active forms of 25(OH)D and 1,25(OH)2D into inactive forms (13). Previously, studies found that variants near genes involved in VitD transport could regulate VitD levels, and the presence of SNPs might influence autoimmune disease susceptibility by causing VitD deficiency (29, 30). Previous research has rarely reported on the role of genes involved in the VitD metabolic pathway in ICI treatment. According to one recently published randomized controlled trial, supplementing with VitD at a dose of 2,000 IU/day for approximately 5 years resulted in a lower incidence of autoimmune disease (22%) than placebo (31). Osama et al. first identified VitD as a protective factor against the development of ICI-induced colitis (32). Furthermore, 1,25(OH)2D has been shown to trigger tumor resistance by maintaining elevated PD-L1 and PD-L2 signaling in the tumor

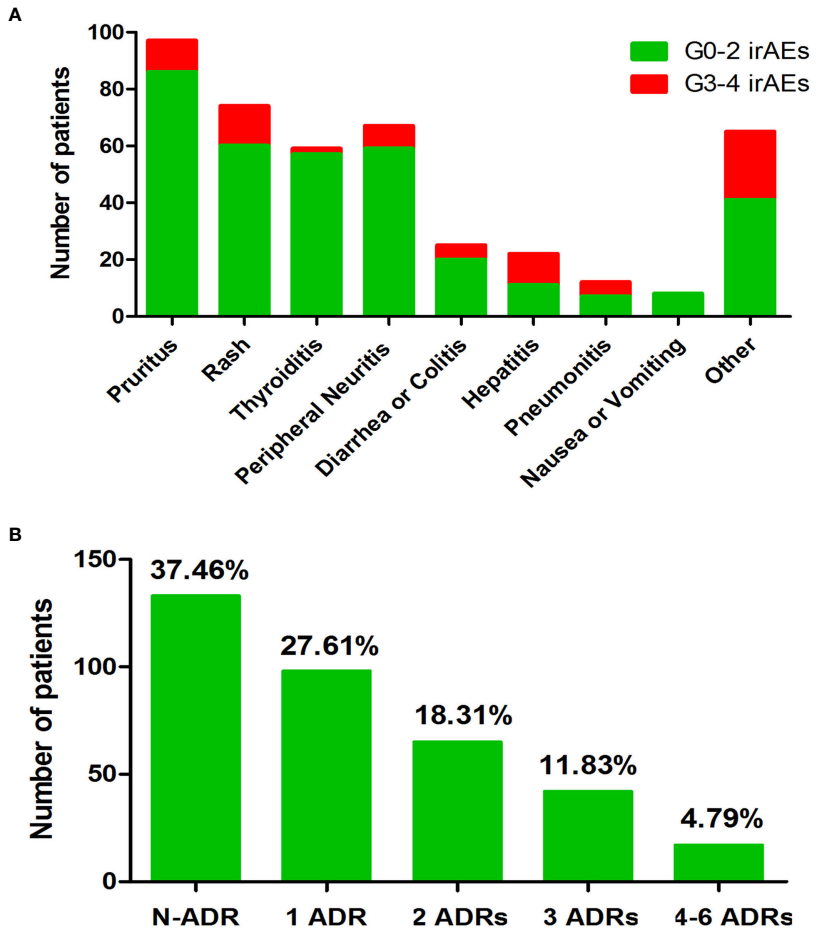

FIGURE 1
Overview of the irAEs that occurred during follow-up. **(A)** Number of patients of each type of irAE. **(B)** Number of irAEs that occurred during follow-up time. Note. N-ADR, number of patients without adverse drug response (ADR); 1 ADR, number of patients with one kind of ADR; 2 ADR, number of patients with two kinds of ADR; 3 ADR, number of patients with three kinds of ADR; 4–6 ADR, number of patients with four to six kinds of ADR; irAEs, immune-related adverse events.

TABLE 3 Characteristics of studied SNPs from vitamin D metabolism pathway.

Gene	Chr	SNP	MAF (ref[a])	Alleles	Location	Call rate (%)	p[b]
VDR	12	rs1544410	0.041 (0.087)	T:C	Intron variant	99.71	0.557
	12	rs731236	0.05 (0.078)	G:A	Synonymous variant	98.25	0.329
	12	rs7975232	0.31 (0.28)	A:C	Intron variant	97.38	0.98
	12	rs2228570	0.47 (0.42)	A:G	Missense variant	97.96	3E−04
CYP24A1	20	rs2296241	0.45 (0.41)	A:G	Synonymous variant	98.83	0.82
	20	rs6068816	0.37 (0.37)	T:C	Synonymous variant	96.79	0.18
	20	rs2762934	0.10 (0.092)	A:G	3′ prime UTR variant	98.25	0.34
CYP27B1	12	rs10877012	0.36 (0.36)	G:T	2KB Upstream Variant	97.08	0.54
CYP2R1	11	rs2060793	0.36 (0.34)	A:G	5′ prime UTR variant	98.54	0.04
GC	4	rs7041	0.29 (0.27)	C:A	Missense variant	99.13	0.26
DHCR7	11	rs12785878	0.42 (0.47)	T:G	5′ flanking	98.54	0.38
RXRA	9	rs9409929	0.20 (0.18)	A:G	NA	95.04	0.33
SEC23A	14	rs8018720	0.38 (0.40)	G:C	Missense variant	99.13	0.80

SNP, single-nucleotide polymorphism; MAF, minor allele frequency.
[a]ref indicates the MAF value of CHS from 1000 Genomes Project.
[b]p-Value for Hardy–Weinberg equilibrium analysis.

TABLE 4 SNPs significantly associated with treatment efficacy.

SNP	Model	Genotype/allele	Ineffective (N = 54)	Effective (N = 215)	OR (95 CI)	p
rs6068816	Genotypic model	TT	10 (19.23%)	97 (41.28%)	Reference	5.06E−04
		CT	29 (55.77%)	118 (50.21%)	0.42 (0.19–0.90)	0.033
		CC	13 (25.00%)	20 (8.51%)	0.16 (0.061–0.41)	1.77E−4
	Dominant model	TT *vs.* CT+CC	10 (19.23%)/42 (80.77%)	97 (41.28%)/138 (58.72%)	0.34 (0.016–0.71)	0.003
	Recessive model	CC *vs.* TC+TT	13 (25.00%)/39 (75.00%)	20 (8.51%)/138 (91.49%)	0.28 (0.13–0.61)	0.003
	Allelic model	T *vs.* C	49 (47.12%)/55 (52.88%)	312 (66.38%)/158 (33.62%)	0.45 (0.29–0.69)	3.14E−4
rs2296241	Genotypic model	CC	21 (39.62%)	67 (28.03%)	Reference	0.029
		CA	28 (52.83%)	120 (50.21%)	1.34 (0.71–2.54)	0.41
		AA	4 (7.55%)	52 (21.76%)	4.07 (1.31–12.60)	0.012
	Dominant model	CC *vs.* CA+AA	21 (39.62%)/32 (60.38%)	67 (28.03%)/172 (71.97%)	1.68 (0.91–3.13)	0.10
	Recessive model	AA *vs.* CC+CA	4 (7.55%)/49 (92.45%)	52 (21.76%)/187 (78.24%)	0.29 (0.10–0.85)	0.019
	Allelic model	A *vs.* C	36 (33.96%)/70 (66.04%)	224 (46.86%)/254 (53.14%)	0.58 (0.37–0.91)	0.017

SNP, single-nucleotide polymorphism.

TABLE 5 SNPs significantly associated with the development of irAEs.

SNP	Model	Genotype/allele	N-irAEs group (N = 128)	irAE group (N = 215)	OR (95 CI)	p
rs10877012	Genotypic model	GG	11 (9.09%)	30 (13.95%)	Reference	0.012
		GT	49 (40.50%)	110 (51.16%)	0.82 (0.38–1.77)	0.70
		TT	61 (50.41%)	72 (33.49%)	0.43 (0.20–0.93)	0.045
	Dominant model	GG+GT *vs.* TT	60 (49.59%)/61 (50.41%)	140 (66.51%)/72 (33.49%)	0.51 (0.32–0.80)	0.0037
	Recessive model	GG *vs.* GT+TT	11 (9.09%)/110 (90.91%)	30 (14.15%)/182 (85.85%)	0.61 (0.29–1.26)	0.22
	Allelic model	G *vs.* T	71 (29.34%)/171 (70.66%)	170 (40.09%)/254 (59.91%)	0.62 (0.44–0.86)	0.0055
rs2762934	Allelic model	A *vs.* G	19 (7.42%)/237 (92.58%)	51 (12.20%)/367 (87.80%)	0.58 (0.33–1.00)	0.043
rs8018720	Genotypic model	CC	54 (42.52%)	74 (34.74%)	Reference	0.041
		CG	50 (39.37%)	113 (53.05%)	1.65 (1.02–2.67)	0.049
		GG	23 (18.11%)	26 (12.21%)	0.82 (0.43–1.60)	0.61

microenvironment, suppressing T cell-mediated anti-tumor immunity (33).

In this current study, we found that *CYP24A1* rs6068816 and rs2296241 polymorphisms were significantly associated with ICI efficacy. After adjustment for clinical factors, patients with the rs6068816T and rs2296241A alleles were more likely to benefit from immunotherapy. Moreover, we found that patients with higher plasma levels of 25(OH)D responded better to ICIs. One recent study showed that the rs6068816T allele is associated with higher levels of 25(OH)D concentration and a lower risk of NSCLC, whereas no such association was found for the rs2296241 allele (34). However, inconsistent results were found in other studies, which found that both the rs6068816 and rs2296241 polymorphisms were not associated with 25(OH)D concentration levels (35, 36). Because of a synonymous polymorphism, rs6068816 and rs2296241 cannot alter the amino acid sequence of *CYP24A1*. Therefore, we hypothesized that rs6068816 and rs2296241 might be involved in the treatment efficacy of ICIs by affecting VitD status, and the mechanism needed to be validated by more rigorous studies with a larger sample size and a different ethnic population.

Despite the lack of association between 25(OH)D level and the risk of irAEs, our study found that several mutations, including *CYP27B1* rs10877012, *CYP24A1* rs2762934, and *SEC23A* rs8018720, significantly reduced the risk of irAEs. *CYP27B1* rs10877012 is located in the promoter of *CYP27B1*, and the rs10877012GG genotype is associated with significantly higher serum 25(OH)D levels compared to GT/TT genotypes (37). Rs2762934 is found in the 3′ untranslated region of *CYP24A1* gene, and AA/AG genotypes are associated with an increased risk of VitD deficiency (38). A previous genome-wide association study identified that rs8018720 in *SEC23A* is significantly associated with serum 25(OH)D concentration (39). However, further studies failed to validate this association (40, 41). These inconsistencies might be explained by the genetic backgrounds of different disease types, sample sizes, and races.

In conclusion, our findings demonstrated that NSCLC patients with lower PS scores had high PD-L1 expression levels, combination therapy, and ICIs as first-line therapy were more likely to benefit from ICI treatment. The pharmacogenomics results revealed that *CYP24A1* rs6068816 and rs2296241 polymorphisms act as independent beneficial factors in the treatment response of ICIs. The allele and genotype frequency distribution showed three SNPs (rs10877012, rs2762934, and rs8018720) associated with the risk of irAEs. Furthermore, we found that plasma VitD levels were significantly higher in patients who responded to ICIs. The abo-e findings implied that investigating the role of VitD in the treatment outcomes of ICIs was of great clinical importance. However, our study also has limitations. Despite enrolling a large number of patients, the population studied for the role of plasma VitD concentration on ICI treatment was relatively small. Second, only DCR and ORR were used to assess ICI treatment efficacy. The role of genetic factors and plasma VitD concentration in other efficacy-related indicators (progression-free survival, overall survival, and so on) deserves further study. Finally, because the genotyping and chemiluminescent immunoassay tests were not performed on the same samples, we could not further investigate the relationship between gene polymorphism and 25(OH)D level. The precise role of VitD metabolic pathway genes and plasma VitD levels in ICI treatment outcomes needs to be explored further in future repetitive and functional studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This study was reviewed and approved by the Ethic Committee of Second Xiangya Hospital at Central South University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.937476/full#supplementary-material

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EDITED BY
Mourad Aribi,
University of Abou Bekr Belkaïd, Algeria

REVIEWED BY
Alessandro de Sire,
University of Magna Graecia, Italy
Malik Hamaidia,
University of Liège, Belgium

*CORRESPONDENCE
Clara Crescioli,
clara.crescioli@uniroma4.it

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Vitamin D, exercise, and immune health in athletes: A narrative review

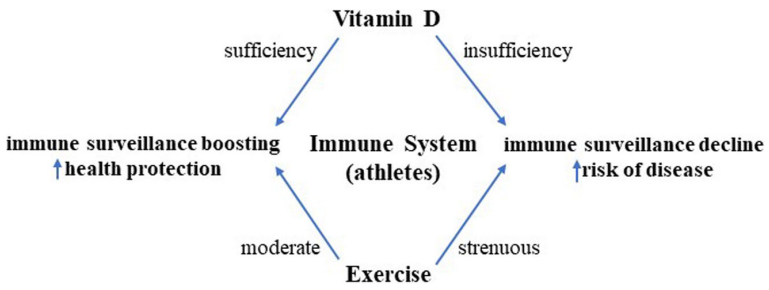
Clara Crescioli*

Department of Movement, Human and Health Sciences, University of Rome "Foro Italico", Rome, Italy

Vitamin D exerts important extra-skeletal effects, exhibiting an exquisite immune regulatory ability, affecting both innate and adaptive immune responses through the modulation of immunocyte function and signaling. Remarkably, the immune function of working skeletal muscle, which is fully recognized to behave as a secretory organ with immune capacity, is under the tight control of vitamin D as well. Vitamin D status, meaning hormone sufficiency or insufficiency, can push toward strengthening/stabilization or decline of immune surveillance, with important consequences for health. This aspect is particularly relevant when considering the athletic population: while exercising is, nowadays, the recommended approach to maintain health and counteract inflammatory processes, "too much" exercise, often experienced by athletes, can increase inflammation, decrease immune surveillance, and expose them to a higher risk of diseases. When overexercise intersects with hypovitaminosis D, the overall effects on the immune system might converge into immune depression and higher vulnerability to diseases. This paper aims to provide an overview of how vitamin D shapes human immune responses, acting on the immune system and skeletal muscle cells; some aspects of exercise-related immune modifications are addressed, focusing on athletes. The crossroad where vitamin D and exercise meet can profile whole-body immune response and health.

KEYWORDS

Vitamin D, immune system, skeletal muscle, exercise, myokines, health, athletes



GRAPHICAL ABSTRACT

Introduction

There is robust evidence of causative links between exercise, improved immunity, and disease prevention. Indeed, an optimal functioning immune system plays a central role in health maintenance, promoting a well-balanced defense against microorganisms or aberrant cells. In this light, exercise training is recommended as a multifaceted intervention for health (1, 2). Nevertheless, intense and prolonged exercise bouts seem to produce a temporary immunodepression, associated with a decreased host protection and, in turn, an increased risk of diseases, particularly infections, as documented by studies on athletes (3, 4). The human immune system is intensely shaped by exercise and by a variety of stimuli, such as stress, lack of sleep, general health status, environmental extremes (altitude), competition, and nutrients. Among others, vitamin D is a well-known regulator of the immune response, acting on several immune cell types, including macrophages, antigen-presenting cells (APC), dendritic cells (DCs), T cells, and B cells, which express vitamin D receptor (VDR), either constitutively or upon activation (5). Adequate levels of vitamin D are recommended to maintain immunity and prevent illness. Currently, overwhelming evidence suggests that D hypovitaminosis is similarly widespread in the general population and in athletes (6–9). In fact, albeit athletes are generally healthy subjects, many of them are vitamin D deficient, likely as a consequence of combined factors like poor/inadequate diet and sun underexposure (10, 11). A vitamin D-deficient athlete may be at an increased risk of potential problems like stress, fractures, respiratory infections, muscle injuries, and immune system depression. Remarkably, vitamin D remodels and strengthens immunity, not only acting directly on immune cells but also modulating the so-called immune ability of nonproperly working immune tissues, such as the skeletal muscle (12–14). Indeed, besides resident immune compartments, which exert inflammatory, protective, and reparative functions, the skeletal muscle can behave as a proper immune secretory organ, functioning as a checkpoint within a complex integrated network of immune-endocrine signals, malleable by exercise and vitamin D. Thus, the interplay between exercise and vitamin D status seems to play a pivotal role in immune health homeostasis.

This review aims to provide an overview of how vitamin D shapes human immunity, acting on both the immune system and skeletal muscle, and how it interplays with exercise to profile whole-body immune response, focusing on athletes. In the first part of the paper, some aspects of exercise-related modifications in the immune system are summarized with pros and cons.

Exercise and the immune system: Is there a limit separating health and disease?

The importance of exercise on human health has been clarified since 400 BCE by Hippocrates, who stated, "…if there is any deficiency in food or exercise the body will fall sick." It is recognized that the immune system is intensely modified by physical activity and exercise (15, 16).

A sedentary lifestyle is associated with an increased risk of comorbidities, including cardiovascular and metabolic diseases, cancer, neurodegeneration, and depression. These clustering diseases, reported as "diseasome of physical inactivity" (17), are essentially ascribable to immunity polarization toward T helper (Th)1/Th17-dominance and chronic inflammation, mediated by a plethora of immune/inflammatory active biomolecules, arising from immunocytes and adipocytes in consequence of inactivity-derived visceral fat accumulation (often accompanied by muscle mass decline) (18–20). The expanded adipose tissue, along with infiltrated resident macrophages, is recognized to be the main source of prototypic inflammatory Th1 cytokines, such as tumor necrosis factor (TNF)-α (21). Exercise-induced anti-inflammatory effects keep under control immune/inflammatory signaling with acknowledged benefits for health maintenance (22, 23). However, "too much" exercise, as much as experienced by athletes, likely does not support immunity, as addressed hereafter.

Pros: An example from the elderly

In addition to restoring an optimal muscle/fat ratio, exercising is currently recognized to significantly decrease inflammation, protect against several immune/inflammatory diseases (24, 25), and the reduce morbidity or mortality rate in adulthood and older age by counteracting frailty and cognitive decline (26–29). Those positive effects are promoted by improvements in immune function and opposition to immune senescence, a biological age-related decline of immune surveillance, leading to higher susceptibility to infections, lower efficacy of vaccination, and higher risk of cancer (30–32). In the elderly, physical activity is associated with better immune response and better protection of the influenza vaccine (33, 34). In addition to the benefits of some age-related alterations, excellently summarized in a recent review (35), studies on young subjects document that exercise boosts the immune system by acting on circulating inflammatory cytokines and decreasing the secretion of several inflammatory cytokines, including TNF-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-6, and IL-8 (36, 37).

In early investigations, the anti-inflammatory effect was associated with an increased risk of infections due to exercise-induced immunodepression, as addressed hereafter.

Nowadays, regularly exercising is a well-recognized adjuvant of immune surveillance by balancing the Th1/Th2 ratio and opposing the interplay between inflammatory and oxidant processes, recently referred to as "oxinflammation" (38).

The decrease of reactive oxygen and nitrogen species (ROS and RNS, respectively) and the simultaneous increase of antioxidant defense—by potentiating enzymatic activity of catalase, superoxide dismutase, glutathione peroxidase—are examples of the multiple mechanisms involved in exercise-induced support to the immune system (38, 39). A recent meta-analysis on oxidative stress parameters concludes that training improves health-related outcomes, reducing the pro-oxidants/antioxidants ratio, regardless of the studied population, and independently of intensity/volume/type of exercise (40). Exercise significantly supports immune response by promoting immune cell recirculation from lymphoid tissues and their interchange with blood: intensity-dependent leukocytosis is followed by an increase in the number and redistribution of effector cells to peripheral tissues. Leukocyte recirculation following exercise likely depends on cell mobilization and demargination of previously circulating cells, driven by surface modifications of adhesion molecules, rather than de novo bone marrow release (41).

Even a single bout of exercise can promote the redistribution of natural killer (NK) and viral-specific T cells—thus limiting latent viral reactivation and reducing the antigenic load on T cells—and can prevent exhausted/senescent T-cell accumulation *via* apoptosis (35).

It has been suggested that the modifications to natural killer (NK) and T-cell trafficking promoted by exercise might potentially have important implications for health, i.e., by isolating mobilized lymphocytes for immune cell therapeutics (35, 41).

Acute exercising for less than 1 h transiently promotes the recirculation of B cells, NK cells, and CD8+ T cytotoxic lymphocytes exhibiting effector-memory phenotype, highly active in immune host defense (16, 42–44). Furthermore, during moderate exercise (lasting less than 1 h), stress hormones do not reach the high concentration needed to act as a suppressor of immunocyte activity (45). This transient effect results in immune surveillance boosting.

Conversely, intense/prolonged exercise is known for quite a long to increase circulating stress hormones, such as cortisol or catecholamines, which alter leukocyte trafficking and redistribution; in particular, catecholamines exert a greater impact on NK than T or B cells, in keeping with the density gradient of cell β receptors (41, 46, 47). Stress hormone-induced modifications to cell number, surface molecule expression, and cell deformation, found in different cell subsets, are greater with prolonged intense exercise, as exhaustively reported elsewhere (41).

Exercise-induced positive regulation of the immune system involves several mechanisms, including the qualitative shift from a Th1 to a Th2 response, the enhancement of mitochondrial function in peripheral blood mononuclear cells, and the regulation of immunometabolism toward more oxidative phenotypes (48–54). Thus far, albeit research in exercise immunology is still emergent and gaps in the knowledge exist, the summation of the effects induced by each bout of moderate exercise repeated over time significantly strengthens immune surveillance against pathogens, inflammatory disorders, and cancer cells by several mechanisms, collectively supporting the therapeutic potential of exercising (55, 56), as summarized in Table 1.

Nevertheless, it is undeniable that heavy exertion as practiced by athletes may be associated with increased inflammation, oxidative stress, and increased risk of illness.

Cons: An example from the athletes

The attention on immune response in athletes is currently high since heavy training workloads might turn to an immune dysfunctional response and increased risk of illness.

This phenomenon gives rise to questions on a possible edge separating the immune-depressive from the immune-boosting effect of exercise, particularly in athletes (57, 58).

The pioneering studies on changes in basic immune cell counts and function evidenced profound perturbations of leukocyte subsets linked to endeavor-related stress (57, 59–62).

TABLE 1 The effect of effort intensity on immunity.

Effort intensity	Immune-related modification	Summation of effects
Moderate-to-vigorous regular exercise (less than 1) Stress hormones do not reach the concentration to suppress immune activity	+ macrophage antipathogen activity + immunosurveillance against cancer cells + immunoglobulins + anti-inflammatory cytokines + neutrophils + NK cells + T cells (particularly cytotoxic CD4+ T cells)	Immune defense activity enhancement, systemic inflammation decrease, diminished risk of illness
Prolonged and intensive endurance exercise Stress hormones reach the concentration to suppress immune activity	– macrophage function (altered MHC-II) – immunosurveillance against pathogens and cancer cells – neutrophil function – NK cell activity – salivary IgA output	Prolonged immune system alteration, systemic inflammation increase, increased risk of illness

The effects induced by moderate/vigorous exercise and prolonged/intensive endurance exercise on different immune components and cell types are compared, and the summation of the effect on immunity is depicted. The signs "+" and "–" indicate up- and downregulation, respectively.

Indeed, after prolonged/intensive endurance exercise, critical alterations in immunity biomarkers—salivary immunoglobulin (Ig) A output (suppressed), the function of NK cells, neutrophils, T cells, and B cells (reduced), expression of major histocompatibility complex II (MHC-II) in macrophages (downregulated), just to mention some—persist for hours to days, expose the athletes to illness higher risk (16, 63–68), in primis to an increased risk of upper respiratory tract infections (URTI) (61, 62, 69–76). Table 1 summarizes the effects induced by prolonged/strenuous exercise. Indeed, overexercise suppresses MHC-II expression and negatively impacts macrophages' ability to present the antigen to T lymphocytes, further impairing immune surveillance (77).

The relationship between the risk of URTI and exercise intensity in humans mostly emerged from self-reported sickness logs and was substantially confirmed in animals, albeit mechanistic experimental studies are often not immediately translated to humans, considering the difficult comparison across species due to the high variability of exercise protocols or adaptation (78–80).

The drastic reduction in lymphocyte number and function is observed within 1-2 h after exercise, a timeframe known as an "open window" similar to a break in immune surveillance as represented by a J-curve model (81). To date, this hypothesis has been argued and it is still under debate (68).

Over time, exercise immunology has received growing attention, and investigators have clarified that exercise-dependent immunity modulation specifically mirrors intensity, duration, and type of the effort, with different responses to acute/chronic, and moderate/vigorous regimens (differentiation criteria: 60% intensity threshold of oxygen and heart rate reserve, 60 min duration threshold).

In fact, exercise in a moderate regimen on a regular basis can decrease illness incidence by dampening inflammation and infections, as previously addressed (57, 82). Accordingly, consistent results from several randomized clinical trials show exercise-reduced URTI incidence and duration: summarizing, at least 5 days/week of aerobic exercise (from 20 min) can decrease by 43% the number of days with URTI vs. sedentary habits (exercising less than 1 day/week), as recently reviewed in an exhaustive paper on this topic (56, 83, 84). This result persists after adjustment of confounders, such as age, gender, education level, marital status, and mental stress (56, 57). The comparison between heavy and moderate exertion, such as marathon races and 30/45 min walking, respectively, supports the hypothesis that the perturbation of immune function specifically reflects the extent of stress experienced by the exerciser (56).

Athletes undergoing repeated heavy exertion cycles, i.e., in proximity to competitions, often experience concomitant stressors such as traveling, nutrition changes, sleep deprivation, and mental stress, all together merging in reduced immune surveillance, which, in turn, associated with the higher illness of respiratory tract, skin, digestive and genitourinary tract (58, 85). Albeit pure cause–effect relationships between heavy exertion and risk of diseases (either infective or not) have not yet been clarified, some chief organizations, such as the International Olympic Committee and the International Association of Athletic Federation, have introduced surveillance programs to prevent and manage this important problem (58, 86–88).

Thus, consensus statements with the ultimate goals of achieving performance and maintaining athlete's health provided some key guidelines (4, 58, 89).

Remarkably, exercise stress represents such a challenge for the immune system, requiring biosynthetic and oxygen

bioavailability to promptly reprogram and support effector cell metabolism and production of specific mediators, like cytokines, involved in the inflammatory response.

Indeed, intensely trained athletes show important alterations in the bioactive lipidome and proteome, like metabolites from lipid super pathways (oxylipins) or immune-related proteins, largely involved in immune cell chemotaxis and migration, mediating organ cross-talk during inflammatory responses (90–92).

With the development of high-resolution omics technologies, the recent hypothesis is that the transient immune dysfunction in the "open window" is due to a significant decrease in cell metabolic capacity during recovery immediately after intense exercise bouts, rather than being a general immune depressing response (93–95).

In this scenario, the multiomics approach highlights the importance of nutritional interaction on immune modifications in response to exercise. Of several factors, vitamin D status is highly critical, considering that this molecule can control whole-body immunity, affecting the immune system and the immune activity of skeletal muscle.

Vitamin D and athletes: Dialoguing with the immune system

The diet of athletes should provide sufficient nutrients and micronutrients—proteins, carbohydrates, minerals, and vitamins—to meet their energy needs and maintain at best their immune health (4).

Even short-term deficiencies from dietary restrictions, often aimed to rapidly reduce athlete's weight while continuing hard training, immediately turn into impaired immune surveillance (96–99). Furthermore, larger increases in circulating stress hormones and greater immune perturbation have been reported in athletes exercising in a carbohydrate-depleted state (96).

Vitamin D is a well-recognized upregulator of immunity, and matters arise from the observation that D insufficiency/deficiency is a common feature in athletes from different sports disciplines, including dancing, taekwondo, running, jockeying, and weightlifting (100–106).

The explanation for this widespread D inadequacy is likely due to different factors. First, ultraviolet (UV)B sunrays insufficient exposure, which is the main source of vitamin D, in addition to the diet (few foods naturally contain it) or vitamin D-fortified foods, as previously reported (107). The effectiveness of vitamin D endogenous synthesis seems to be affected by several factors, including latitude, season, atmospheric pollution, type of sport, indoor/outdoor training, lifestyle, sunscreen use, skin pigmentation (dark-skinned people need about 10-time longer sun exposure due to melanin concentration), albeit contradictory data are reported on this topic (108). Regardless of the cause, vitamin D hypovitaminosis is acknowledged in the global athletic population and attracts growing attention.

Studies on vitamin D inadequacy among athletes often are focused only on performance, as this molecule seems to act as a "performance enhancer, although conclusive data on this topic are still missing (109).

Instead, concerns should be addressed about general health rather than limited to performance, considering the tight control exerted by vitamin D on some important functions, broadly affecting health in all individuals, including athletes.

Vitamin D, behaving as a typical steroid hormone or as a micronutrient with rapid mechanisms, exerts pleiotropic effects *via* interaction with vitamin D receptor (VDR), virtually expressed by every human tissue (110, 111).

In addition to homeostasis regulation in bone, which is the classical tissue target of this molecule, it is well recognized that vitamin D significantly impacts the inflammatory status, which, in turn, is acknowledged as the common link in several noxious conditions, including infections, joint degenerative diseases, and disturbance of metabolism, to mention some (112–115).

Unfortunately, the ability of vitamin D to modulate the immune response can be listed among the main mechanisms underlying its anti-inflammatory effects.

Albeit immunocytes are considered nonclassical target cells of vitamin D, almost all types of immune cells, including CD4+ and CD8+ T cells, B cells, neutrophils, APCs, like macrophages, and dendritic cells (DCs), express vitamin VDR, which upon ligand binding modulates cell number and function (116). The multifaceted effects on the different immune cell types are extensively reported in the literature; essentially, they converge in promoting a shift from the Th1/Th17 inflammatory subset to protolerogenic dominance, in association with enhancement of T regulatory (Treg) cells and impairment of APC. Vitamin D signaling, indeed, ensures the suppression of proinflammatory status, downregulating T cells and cytokines like IL-2, IL-6, IL-8, IL-12, tumor growth factor (TGF)-β, IFN-γ, IL-17, and IL-21, simultaneously enabling Treg subset expansion with increased production of protolerogenic mediators, such as IL-4, IL-5, IL-13, IL-10, and CCL2 (117–121).

Vitamin D-dependent inhibition of DC differentiation from monocytes, antigen processing, and antigen presentation decrease—due to the downregulation of costimulatory molecules/major histocompatibility complex (MHC)-II-complexed antigen—and IL-10 upregulation, further supporting protolerogenic signals (122).

In particular, vitamin D can modify DC morphology to a more adherent spindle shape, and surface markers drive the cells to a less mature/more tolerogenic phenotype, in association with a decrease in cluster of differentiation (CD) 80, CD86 (costimulatory molecules), and CD54 (adhesion molecule), in addition to MHC-II downregulation, whereas the expression of CCR5 (chemokine receptor), DEC205 (antigen-uptake

receptor), F4/80 (macrophage marker), and CD40 increases, resulting in a general downregulation of antigen presentation function (123, 124).

The overall effect likely acts as a "balance" of the inflammatory response evoked by long/high-intensity exercise (above 80% VO2max, 120 min) characterized by proinflammatory cytokine rise, i.e., IL-6, IL-1, IL-8, and TNF-α, as reported, whereas it seems to merge with the effect of short-term/moderate exercise (50%–75% VO2max, 45–60 min), associated with an expansion in T-cell-derived Th2 mediators, as IL-4 and IL-10.

Interestingly, IL-10 promotes long-lasting antigen-specific T-cell anergy and plays a driving role for type 1 T regulatory (Tr1) cells, the cell subset known to be critical for maintaining tolerance to self and nonself antigens in humans and animals, in the presence of APC, as emerged from *in vitro* experiments (125–128).

In line with previous *in vitro* investigations reporting IL-10 increase and IFN-γ reduction in peripheral blood mononuclear cells after vitamin D, recent *in vivo* experimental studies on atheroprotection document that vitamin D added to dexamethasone significantly promotes IL-10 by DC as well as other APC, thus establishing IL-10 network of lymphoid and myeloid immune cells, and simultaneously reduces Th1 response by inhibiting IFNγ-producing CD4+ and CD8+ T cells (129).

Noticeably, vitamin D-induced mechanisms underlying the transition from proinflammatory IFN-γ+ Th1 cells to suppressive IL-10+ cells seem primed by wide-epigenetic T-cell remodeling, which promotes VDR expression and enzyme cytochrome P450 family 27 subfamily B member 1 (CYP27B1) activation by autocrine/paracrine mode, leading to Th1/Th17 program repression (*via* STAT3, c-JUN, and BACH2) and IL-10 enhancement (*via* IL-6–STAT3 signaling), as recently shown by elegant research in coronavirus disease 2019 (COVID-19) patients (130).

Subsequent studies are encouraged to verify whether similar/different vitamin D-dependent mechanisms occur in immune adaptation to exercise.

As already addressed, regular short-term/moderate-intensity exercise strengthens the immune system by increasing macrophage activity, which is further potentiated by the vitamin D effect on monocytes. Of note, vitamin D induces macrophage and epithelial cells to produce cathelicidin, a protein with marked antimicrobial activity, able to improve macrophage bacterial capacity involved in host-first-line defense (131–134). Furthermore, vitamin D impairs macrophage inflammatory cascade by targeting cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) and, therefore, reducing nitric oxide (NO) and prostaglandin (PG)E2 (135). Figure 1 summarizes the main effects induced by vitamin D in different types of immunocytes.

The higher production of cathelicidin and defensin (another host-defense peptide) induced by vitamin D along with its anti-inflammatory action would promptly reduce the cytokine storm during infection by COVID-19 (136). Indeed,

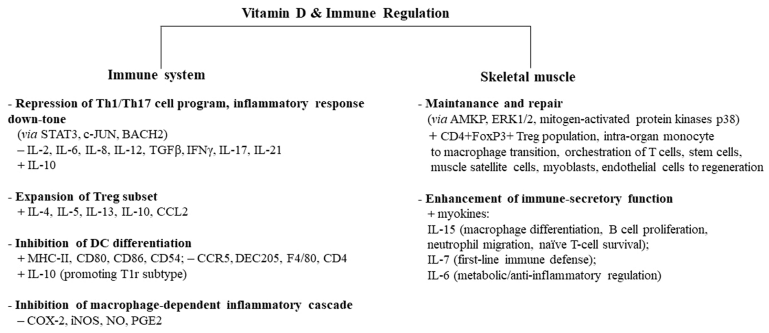


FIGURE 1
Vitamin D-induced immune regulation of the immune system and skeletal muscle. Vitamin D controls inflammation and promotes tolerogenic status, acting on several types of circulating immune cells, skeletal muscle cells, and intraorgan immune cells. Some cell types, biomediators, and signaling paths/molecules mainly involved in this process are indicated. Similar immune regulatory mechanisms are promoted by moderate exercise, while intense/prolonged exercise leads to a decline in immune surveillance, recalling the immunodepression allowed by sedentary status. Adequate vitamin D levels can downtone exercise-induced inflammatory-like response and converge to exercise-induced immunosurveillance boosting, with protective effects on whole-body health. Overtraining athletes in hypovitaminosis D can be at higher risk of infectious and noninfectious diseases. The signs "+" and "–" indicate up- and downregulation, respectively. AMPK, 5′ adenosine monophosphate-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; Tr1, type 1 T regulatory cells; MHC-II, major histocompatibility complex II; STAT3, signal transducer and activator of transcription 3; c-JUN, transcription factor Jun; BACH2, transcription regulator protein broad complex-tramtrack-bric a brac and Cap'n'collar homology 2; COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PGE2, prostaglandin E2.

recent investigations in COVID-19 patients document the usefulness of vitamin D administration due to the protective effects against mortality and intensive care unit admission (137, 138).

Adequate vitamin D status combined with the practice of exercise seems to promote positive outcomes in COVID-19, albeit the research on this specific topic is still in its infancy (139).

It has been reported that sex-dependent dimorphism in vitamin D metabolism likely explains the greater immune vulnerability to perinatal infections observed in male vs. female fetuses/neonates due to the testosterone-induced decrease of cathelicidin gene, through the inhibition of alpha hydroxylase CYP27B1, the enzyme necessary for D bioactive form (140). It would be interesting to extend this kind of investigation into adulthood to verify whether similar mechanism(s) may underlie some sex-dependent differences in response to infections (including COVID-19), which are also seen in athletes and are frequently mistreated (141, 142).

In B cells, vitamin D inhibits proliferation and immunoglobulin production similarly to heavy exertion, whereas repeated bouts of moderate-intensity exercise enhance B-cell proliferation.

Since B and T cells, macrophages, and DCs can self-synthesize vitamin D, a kind of "local" anti-inflammatory effect occurs within infiltrated target tissues (5, 143). Exercise upregulates VDR expression in T cells regardless of exercise-induced T-cell mobilization (144), therefore enhancing the anti-inflammatory loop. Thus far, vitamin D can impact both innate and adaptive immunity with a decisive anti-inflammatory profile. While this effect was initially simplistically considered immunosuppressive, the current concept focuses on the exquisite modulating role of vitamin D toward tolerogenic homeostasis (145).

Conversely, low vitamin D levels are associated with deficits in immune surveillance, including lower salivary IgA and increased risk of long-lasting respiratory infection, as observed in elite athletes (11). Indeed, hypovitaminosis D-shaped modifications in the immune system often converge on and amplify heavy exertion-induced effects.

Furthermore, vitamin D deficiency is described in the pathophysiology of Th2-driven allergic diseases such as asthma, in which the lower hormone levels are associated with IL-4, IL-5, IL-9, and IL-13 deregulation, increase in asthma markers (IgE and eosinophil), and more severe clinical disease manifestation, as reported (146–149).

To date, vitamin D deficit-dependent damage is not limited to the immune system but highly impacts the function of skeletal muscles; since this tissue exhibits important immunocompetent capacity, whole-body immune surveillance is further compromised.

Vitamin D and the immunity of skeletal muscle

Skeletal muscle is a nontraditional target tissue of vitamin D and is finely regulated by this molecule at several levels. According to experimental and human studies, insufficient vitamin D levels and VDR deletion cause critical muscular dysfunctions (150–153).

In fact, skeletal muscle development, myocyte differentiation, muscular volume, tissue functional maintenance, and physical performance are processes tightly dependent on the intact vitamin D/VDR system, as confirmed by studies in humans with VDR mutations or in VDR knockout (VDRKO) mice (151–153).

Lower levels of vitamin D are associated with a significant reduction in muscle fiber size and atrophy (mainly of type II fiber), and overall, determine muscular defects in energy handling (as insulin resistance), plasticity, and contraction, in the general population and in athletes as well (154, 155).

Conversely, higher vitamin D levels are reported to be linked with lower injury rates and improved sports performance (156).

The beneficial effects of vitamin D on skeletal muscle function are related to the fine-tuned regulation exerted at the cell level through VDR interaction, albeit, in the past, the presence of this receptor in human muscle was questioned (157). To date, VDR is mainly detected in fast-twitch muscle fibers (committed to rapid actions) and expressed at different levels in human isolated cells, depending on the cell fusion stage (upregulation upon myotube formation) (150, 158–161).

Intramyonuclear VDR concentration is directly associated with vitamin D serum level, suggesting that the circulating vitamin D/muscular VDR system, plays a pivotal role in the integrity of skeletal muscle, rather than hormone deficiency alone (158). In line with this hypothesis, VDR/D deficit promotes a series of biomolecular alterations, including increased oxidative stress and decreased antioxidant activity, converging in muscle deterioration and ending in atrophy (107, 162).

Type II fiber atrophy significantly ameliorates with vitamin D, as documented in biopsies from vitamin D-deficient patients before and after the treatment with the hormone (163). Vitamin D helps faster recovery from muscle injury and inflammation after high-intensity exercise (164, 165), whereas vitamin D-deficient athletes show a delayed recovery. Upon VDR expression increase, the intracellular signaling cascade involved in repair processes—such as 5′ adenosine monophosphate-activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK)1/2, mitogen-activated protein kinases p38—is activated and interferes with proinflammatory molecule genes (166). Generally, it can be stated that vitamin D affects almost all stages of the myogenic program toward regeneration, also acting on satellite cells. Due to myocyte's ability to uptake vitamin D

from the bloodstream, skeletal muscle tissue accumulates this hormone and acts as a functional reservoir, ready to release it upon blood-level decline. Interestingly, regularly exercising maintains and enhances this functional feature (167).

Exercise is a well-known strategy against muscle wasting and atrophy, not only because it counteracts mass loss but because it exquisitely regulates the mitochondrial function and the internal immune component, both critical for muscle integrity maintenance during stress, as shown by multiomics analysis in astronauts during spaceflights (168).

Of note, exercise- and vitamin D-induced signals converge in the dynamic remodeling of mitochondria, promoting correct genomic reprogramming and skeletal muscle cell remodeling (169).

Beyond those beneficial effects, it is mandatory to highlight the function of vitamin D in maintaining the immune-secretory function of skeletal muscle, which is closely in line with the topic of this review.

Nowadays, the renewed and proven concept is that skeletal muscle is a proper secreting organ with immunoregulatory function. Indeed, upon contraction the muscle releases many trophic/immunoreactive small peptides, the myokines, which can control the function of nearby or distant organs, acting in an autocrine/paracrine/endocrine fashion, as recently reviewed in an exhaustive paper (170). Those factors, before their full identification, were referred to as the "work factors" or "exercise factors", to clearly state that their release occurs exclusively upon muscular contraction and work (170). Currently, more than 650 myokines are identified by the proteomic analysis of the muscular secretory profile, which is constantly updated (171). Among this plethora of biomolecules, some myokines drew attention due to their ability to modulate the immune response, introducing a novel view of immunity-muscle crosstalk, which was previously considered to be a unidirectional route, with muscle being under immune system control (and not vice versa).

Indeed, like other tissues, skeletal muscle has its resident immune cell population to warrant the regenerative potential and tissue homeostasis. The CD4+FoxP3+ Treg population is the main subset infiltrating damaged muscle upon to micro- or macroinjuries, as well as during exercise, and drives muscle regeneration and satellite niche fate; intraorgan monocyte to macrophage conversion plays a pivotal role in orchestrating T cells, mesenchymal stem cells, muscle satellite cells, myoblasts, and endothelial cells towards muscle regeneration or pathogen clearance (94, 162, 172).

Fiber damage due to different injuries, including contusions, strains, hyperextensions, avulsions, or ruptures, promptly activates neutrophils resident in skeletal muscle to release within the microenvironment high concentrations of inflammatory factors necessary for repair (173, 174).

Albeit several types of leukocytes, such as mast cells, neutrophils, eosinophils, and lymphocytes, participate in the repair/regeneration, the monocyte/macrophage population controls all stages of this process (175). Indeed, after neutrophils, macrophages represent the second subpopulation reaching the injured areas (peak at 3 to 6 days and persisting 2 weeks after extensive damage), gradually shifting from a phagocytic to pro-myogenic phenotype, from M1 to M2 macrophages, respectively (172). The shift in macrophage phenotype orchestrates the time of myogenic sequence, supporting first cell proliferation and migration, while delaying differentiation, and then facilitating alignment and fusion (176, 177). During regenerative processes, soluble molecules as growth factors, cytokines, and prostaglandins regulate immune and muscle cell communications, but interestingly, close cell-to-cell contacts between myogenic cells and macrophages occur *via* adhesion molecules, macrophage pseudopodial extensions, and myogenic cell cytoplasmic protrusions (174, 178, 179). T cells show a delayed response, roughly 4 days after the initial damage (172, 180).

Adequate vitamin D levels support the function of the immune intraorgan component: its role, generally considered pro-tolerogenic, is, indeed, to dampen the damaging effects of cell stress and immune response during excessive or chronic reactions, and, in this view, this molecule is defined "pro-survival" (181). Furthermore, exercise-induced production of some myokines, in particular, IL-6, IL-7, and IL-15, by skeletal muscle cells, gives the muscle an "immune-like feature" and the capacity to impact leukocyte subset trafficking, immune cell function, and inflammation (35, 182).

Interleukin-6 is the prototypic myokine, the first one and most extensively studied. Differently from systemic proinflammatory "bad IL-6", deriving from immune cells and adipocytes, muscular "good IL-6" is transiently released in the blood during exercise (up to a 100-fold increase, depending on intensity) and exhibits an unquestionable anti-inflammatory and metabolic profile (183).

Exercise-related pulsatile release of IL-6 promotes the anti-inflammatory macrophage subset (M2-like), involving suppressor of cytokine signaling 3 (SOCS3) ablation, and IL-1 receptor antagonist (IL-1ra) and IL-10, resulting in overall downregulation of inflammatory responses (52, 184). Interleukin-6 likely plays a central role in exercise-induced leukocytosis and late lymphopenia mediated by cortisol, as shown by IL-6 infusion in athletes (52).

In humans, IL-6 is known to counteract TNFα production and signaling from monocytes (185, 186).

Furthermore, IL-6 behaves as an energy biosensor in conditions of energy shortage/demand, such as during physical exercise, enhancing hepatic glucose production, and promoting fat oxidation (187).

Thus far, the myokine IL-6 likely characterizes exercise adaptation, as it is involved in long-term beneficial effects, related to an exercise-training reduction in abdominal fat and anti-inflammatory actions (188). Vitamin D can enhance the

biological effects of IL-6, as shown by the improved metabolic function observed in vitamin D-deficient trained men after a single intramuscular injection of vitamin D, which was associated with a significant rise of IL-6 1 h after resistance exercise (189). The lack of modification in inflammatory parameters is likely due to the short duration of the treatment and the use of a single dose.

Interleukin-6 output from human skeletal muscle cells maintained in nutrient restriction, to mimic energy-demanding conditions such as postexercise, was significantly increased after the treatment with a VDR agonist (162). Conversely, the addition of a VDR agonist to human muscle cells challenged by a strong proinflammatory environment significantly counteracted inflammation-induced intracellular cascade underlying Th1-type chemokine release (190). Thus far, vitamin D modulation seems to be beneficial with prometabolic or anti-inflammatory effects, depending on the microenvironmental needs of skeletal myocytes.

Muscle-derived IL-15 regulates macrophage differentiation, B-cell proliferation, neutrophil migration, and naïve T-cell survival (191). This myokine tightly cooperates with vitamin D, promoting the conversion into the active hormone, the upregulation of VDR, and the induction of cathelicidin (192, 193).

IL-7 also plays a pivotal role in first-line immune defense; the age-dependent decline of this myokine can be counteracted by exercise and by vitamin D, which can help to restore aberrant IL-7-dependent signal, i.e., occurring in immunosenescence or autoimmune processes (194–196).

Thus far, vitamin D is a good enhancer of some exercise-induced "immune" adaptations of the skeletal muscle. The main immune regulatory effects of vitamin D on intraorgan immunocytes and myocytes are depicted in Figure 1.

Conclusions

Vitamin D insufficiency/deficiency is so extensive across the world to ideally meet the criteria for the statement "pandemic", and, in addition to the general population, it seems to affect the athletic population as well.

Athletes are thought to be in good health almost by definition, considering that many human diseases are tightly related to sedentary behavior and inflammation; importantly, the latter is a well-recognized bridge linking different (and clustering) illnesses.

Nevertheless, the condition of overexercising, too often experienced in several sports disciplines, exposes athletes to a higher risk of inflammation and, consequently, a higher risk of diseases.

This phenomenon is essentially related to the modulation of the immune system by exercise, which can enhance or decrease human immune surveillance, essentially depending on the athlete's experienced effort. In this scenario, vitamin D status plays a critical role in immune health, as possible exercise-induced detrimental effects might merge with the poor immune health status determined by hypovitaminosis D. Conversely, vitamin D adequacy counteracts inflammation, enhancing the immune defense and shaping the immune response of skeletal muscle, which is recognized to be a proper secreting organ with immune-like features.

Thus far, screening for vitamin D status would be mandatory in the athletic population as well. This topic still represents a hot topic in literature, as important issues regarding vitamin D determination and supplementation, representing a possible strategy to limit the "pandemic" hypovitaminosis, are still far from being translated into practice, as thoroughly reported by the Consensus statement from the 2nd International Conference on Controversies in Vitamin D (197). The lack of discussion on these aspects is among the limits of this review, which does not include sex-dependent differences in immune response or in vitamin D levels, or cardiovascular features. Nevertheless, recalling the attention to the crossroad where exercise and vitamin D are likely to meet to shape immune health hopefully will help to bring further attention to an issue that is highly significant for athletes and the general population's wellbeing.

Author contributions

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

Conflict of interest

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

Publisher's note

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Matthew Cook,
University of Cambridge,
United Kingdom

REVIEWED BY
Claudia Raja Gabaglia,
Biomedical Research Institute of
Southern California, United States

*CORRESPONDENCE
Endrit Shahini
endrit.shahini@irccsdebellis.it

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Can vitamin D status influence seroconversion to SARS-COV2 vaccines?

Endrit Shahini[1]*, Francesco Pesce[2], Antonella Argentiero[3] and Antonio Giovanni Solimando[4]

[1]Gastroenterology Unit, National Institute of Gastroenterology S. De Bellis Research Hospital (IRCCS), Castellana Grotte, Italy, [2]Nephrology, Dialysis and Transplantation Unit, Department of Precision and Regenerative Medicine and Ionian Area - (DiMePRe-J), University of Bari "A. Moro", Bari, Italy, [3]Medical Oncology Unit, IRCCS Istituto Tumori "Giovanni Paolo II" of Bari, Bari, Italy, [4]Guido Baccelli Unit of Internal Medicine, Department of Precision and Regenerative Medicine and Ionian Area - (DiMePRe-J), University of Bari "A. Moro", Bari, Italy

Existing data indicate an association between vitamin D deficiency and increased severity of respiratory distress due to COVID-19 infection, especially in high-risk populations. To date, the effect of vitamin D on immunogenicity to SARS-CoV-2 vaccines has been investigated solely in young healthcare workers in a few studies, yielding conflicting findings, yet highlighting that the response to immunization is inversely related to age. Vitamin D status can potentially influence the antibody titers in people with a previous (or naïve) SARS-CoV-2 infection and vaccination, given its role in immune regulatory functions. From this standpoint, vitamin D supplementation can help reduce the risk of SARS-CoV-2 infection, COVID-19 severity/mortality and rebalance immunological function, particularly in subjects with vigorous T lymphocyte responses to COVID-19. However, more research is needed to establish a correlation between vitamin D status and the generation of protective serological responses to SARS-CoV-2 vaccination.

KEYWORDS

COVID-19, coronavirus, vitamin D, antibodies, serology, autoimmune disorders, pneumonia

1 Introduction

Altmann D.M. et al., in their comment, have elaborated elegantly on the plethora of unresolved issues with the reliability of serological tests used for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) identification, after coronavirus disease 2019 (COVID-19) resolution (1). U.S. has been widely tested for SARS-CoV-2 infection from March to April 27, 2020 resulting in 5.44 million people infected, followed by Italy (1.76 million). The identification of infected individuals relies on polymerase chain reaction (PCR) based tests for SARS-CoV-2. In contrast, antibody blood tests determine whether a

past infection has occurred by detecting serum antibodies against the virus (1). Despite the higher sensitivity of IgA enzyme-linked immunosorbent assay (ELISA) in detecting antibody titers, their false-negative rates due to lower specificity suggest a particular caution when interpreting the results (2).

It has been observed that COVID-19 outbreaks, and mortality, in particular, have a higher prevalence in the Northern hemisphere, exhibiting a gradient according to the geographical distribution of hypovitaminosis D (3). This is mainly mediated by distinct Earth's seasonal ultraviolet (UV) exposure, although another reasonable explanation could rely on certain atmospheric phenomena (3, 4).

UV radiation strength, exposure time, and skin colour are all known to influence vitamin D biosynthesis (5). Optimal conditions for vitamin D biosynthesis may differ in each individual, and seasonal changes in sunlight exposure may also have an impact (6–9). Notably, previous research has also shown that vitamin D levels can drastically decrease from summer to winter in adult and young (apparently) healthy subjects of different ethnicities (10–15).

Additionally, several studies on the relationship between sunlight exposure and the global COVID-19 pandemic have been conducted (6–8), with solar UV showing a direct effect on SARS-CoV-2 inactivation (16, 17). Furthermore, COVID-19 transmission in South America was aided by cold, dry, and windless conditions (18). Even though no conclusive studies are available, seasonal variations in SARS-CoV-2 illness may be influenced in several cases by a lack of sun exposure, which may impair vitamin D status, especially in at-risk patients (19, 20).

2 Vitamin D immunomodulatory properties in the COVID-19 setting

Vitamin D has pleiotropic effects on the immune system (3, 21). Active vitamin D (1,25-dihydroxyvitamin D3 [1,25-(OH)2D3] is synthesized in renal tubules and acts as a steroid hormone, influencing the expression of hundreds of genes (22). It induces cathelicidins and defensins, which concurrently down-regulate viral replication and pro-inflammatory cytokines that lead to potential interstitial pneumonia and acute respiratory distress syndrome (ARDS) after the onset of a cytokine storm (3, 21). Besides, vitamin D modulates adaptive immunity by suppressing T Helper cell type-1 (TH1) responses and promotes the induction of T regulatory cells that counterbalance inflammatory responses (21). Vitamin D inhibits the expression of renin and, therefore also affects the renin-angiotensin system (RAS)/angiotensin-converting enzyme 2 (ACE2) signaling axis (3). Interestingly, vitamin D exerts propitious anti-thrombotic actions in tissues directly or indirectly involved in thrombosis pathophysiology (21).

Chauss and Colleagues have presented new evidence that severe COVID-19 may be caused, in some people, by the lack of the ability to resolve an exuberant type I immune response. They specifically implicated vitamin D receptor (VDR) signaling in this process (23), showing that when vitamin D levels are low, human CD4+ T cells express more type 1 (IFNG) and type 17 genes. In contrast, IL-6R and IL-10 levels are lower. Vitamin D induces STAT3, BACH2, and JUN to increase IL-6R (reinforcing STAT3 activation) and IL-10, which may be necessary for converting the pro-inflammatory TH1 cell-type to that crucial in resolving type 1 inflammation in the setting of severe COVID-19 (23). *Minton K* also proposed that the impaired transcriptional response to vitamin D in patients with severe COVID-19 could be due to vitamin D deficiency or dysregulation of complement-induced autoregulatory VDR signaling (24). Thus, vitamin D supplementation may modulate IL-6 towards a favorable profile in COVID-19.

3 Evidences linking vitamin D and COVID-19 outcomes

A 2021 meta-analysis of fifty-four observational studies including a total of 1,403,715 individuals, found that all patients with severe deficiency, and/or insufficiency of vitamin D present an increased risk of ARDS requiring admission to intensive care unit (ICU) or mortality due to COVID-19 and a higher susceptibility to SARS-CoV-2 infection and related hospitalization (25).

Current evidence supports a solid relationship between vitamin D deficiency and increased severity of ARDS associated with COVID-19, especially in high-risk populations for hypovitaminosis D (*e.g.*, elderly, Northern people, hospitalized, cardiovascular disease, gastrointestinal diseases, chronic kidney disease, diabetics, obese, impaired immune function, dark-skinned ethnicities, vegetarians or vegans) (3, 21–44). The studies listed in the Supplementary Material support the association between vitamin D levels and COVID-19 outcomes.

Several studies have investigated vitamin D deficiency in the context of COVID-19 severity/mortality based on specific evidence of a protective effect of vitamin D supplementation (400-1000 IU daily) against respiratory tract infections resulting from two rigorous and large meta-analyses of randomized controlled trials (RCTs), particularly in individuals with baseline 25-hydroxyvitamin D3 [25(OH)D3] levels < 25 nmol/L (44, 45).

4 Vitamin D status and COVID-19 immunization

Antibody titers, after recovery from COVID-19, indicate healing, but still, there is uncertainty about their neutralizing

nature. The levels of such antibodies can be theoretically influenced also by vitamin D status in patients with previous SARS-CoV-2 infection given the immunomodulatory properties of vitamin D, which also include the inhibition of lymphocyte proliferation and immunoglobulin production in healthy conditions (46).

4.1 Seroconversion in immune-mediated diseases

A meta-analysis published in 2022 found that seroconversion rates after SARS-CoV-2 vaccination are lower in patients with immune-mediated inflammatory diseases. Specific therapies (anti-TNF, anti-integrin, anti-IL17, anti-IL6, anti-IL12/23) do not affect seroconversion rates, whereas others (anti-CD20, anti-CTLA-4) have a negative impact (47). Notably, there is an inverse relationship between vitamin D status and the development of several autoimmune diseases (48). It is known that vitamin D counteracts the suppressive effect of inflammatory cytokines on CTLA-4 expression and regulatory function (49). Furthermore, 1,25-(OH)2D3 has a critical positive regulation of CTLA4, an essential negative regulator in immune responses (50).

4.2 Vitamin D supplementation

The conundrum has been raised of whether vitamin D would be beneficial in reducing the severity of COVID-19 illness, its requirement for hospitalization, and the length of symptoms.

Vitamin D supplementation may reduce the risk of SARS-CoV-2 infection, COVID-19 severity, and mortality risk (3). It may also help rebalance immunological function in high-risk individuals, particularly those with COVID-19 who demonstrated strong T lymphocyte responses (1, 51).

To decrease the risk of infection and enhance the immunological reactivity against COVID-19, oral supplementation in patients with vitamin D deficiency has been initially suggested at a dosage of 10,000 IU/day of vitamin D3 for a few weeks, followed by a daily dosage of 5,000 IU (3, 52). The ideal target is a 25(OH)D3 over 40-60 ng/mL.

A meta-analysis of 6 RCTs involving 551 COVID-19 patients published in 2022 concluded that despite the heterogeneity of the included studies, vitamin D supplementation was beneficial in COVID-19 and was associated with a lower rate of ICU admission, mortality events, and RT-PCR positivity (53).

Based on a pilot study and several observational intervention studies in which the use of high doses of calcifediol dramatically reduced the need for ICU admission and the mortality rate, it was proposed a rapid correction of 25(OH)D3 deficiency in all patients in the early stages of COVID-19 (54). In particular, early administration of high-dose versus standard-dose vitamin D3 to at-risk older patients with COVID-19 improved the two-week mortality (55). Similarly, other *Authors* in a recent meta-analysis of thirteen observational and RCTs involving a total of 2,933 COVID-19 patients (56), concluded that high-dose cholecalciferol supplementation may be associated with better clinical outcomes, particularly when administered after the diagnosis of COVID-19.

On the contrary, another recent RCT found that a single high oral dose of vitamin D3 (200,000 IU) did not significantly reduce hospital length of stay in 237 COVID-19 patients hospitalized (57). Other previous RCTs produced mixed and inconsistent results, with no clear positive results, and two 2021 meta-analyses found no significant difference between vitamin D supplementation and major health outcomes in COVID-19 (58–61).

Bergman P discussed vitamin D's role in protecting against COVID-19 infection in a recent Editorial (62). He hypothesized that the association could be due to reverse causality or confounding. However, because COVID-19 vaccination was being rolled out during the previous null studies, he did not rule out the possibility that the highly effective vaccination could have masked any vitamin D effect.

Therefore, questions about the correct dosage, period, and methods of administration of vitamin D still need to be answered. A group from Harvard Medical School is currently investigating this question in a new pragmatic, cluster-randomized, double-blinded trial officially named VIVID (Vitamin D for COVID Trial), which is experimenting with the effects of a higher dose of vitamin D above 3,000 IU per day on disease progression and post-exposure prophylaxis for COVID-19 infection (63).

5 Vitamin D and gastrointestinal diseases during the COVID-19 scenario

Notably, even though SARS-CoV-2 is a lung-tropic virus damaging the respiratory tract by binding to the ACE2 cell-surface compounds found on alveolar pulmonary epithelial cells, gastrointestinal symptoms are common in COVID-19 patients and often precede respiratory tract disease. Recently, it was discovered that SARS-CoV-2 actively replicates in the gut, particularly in mature enterocytes expressing the ACE2 viral receptor and the TMPRSS4 protease (64).

According to current research, the host's gut microbiota is key in influencing their health, particularly as a mediator of

chronic systemic low-grade inflammation (65). During SARS-CoV-2 infection, the viral balance in the gastrointestinal tract may be disrupted, influencing the equilibrium of the intestinal microbiota (66).

There is mounting evidence that a cross-talk between the gut microbiome, vitamin D, and the RAS/ACE2 system is key for the elderly immune system's balanced functioning. It has been proposed that the state of the gut microbiome prior to infection determines the outcome of COVID-19 sepsis and may also be a critical factor in vaccination success. Evidence suggests a complex relationship between COVID-19 severity and gut microbiota, ACE-2 expression, and vitamin D. Vitamin D promotes the growth of commensal strains of Bifida and Firmicutes species in the gut (67).

Anyway, for those with low vitamin D levels below 50 nmol/L, the 1000-2000 IU/day supplementation may be appropriate. Importantly, high-risk individuals with gastrointestinal malabsorption diseases such as celiac disease, Crohn's disease, intestinal bypass surgery, hepatobiliary/pancreatic diseases, and sarcopenia, should continue to receive vitamin D and calcium.

Taking vitamin D supplements could have multifaced advantages, including the fact that COVID-19 may act as a potential trigger factor for several autoimmune diseases, such as celiac disease, in predisposed patients (68, 69).

6 Vitamin D levels and immune response to SARS-CoV-2 vaccines

Grifoni A and *Sette A* found early that CD4+ T cell responses to spike protein, the main target of most vaccine efforts, were robust and correlated with the anti-SARS-CoV-2 IgG and IgA magnitude titers in COVID-19 convalescent patients. Notably, they also found a significant prevalence of SARS-CoV-2-reactive CD4+ T cells in uninfected people, implying cross-reactive T cells between circulating commonly coronaviruses and SARS-CoV-2 (70).

On the other hand, a 2022 Chinese multicenter study found that, when compared to seronegative healthy controls, patients with solid malignancies who failed the standard 2-dose inactivated COVID-19 vaccines had a relatively poor humoral response to the third dose of vaccines, which was associated with low vitamin D levels and intake (71).

Several studies have confirmed that vitamin D and its pathway polymorphism can improve vaccine efficacy for infectious diseases like influenza, measles, rubella, pneumococcal/meningococcal/human papillomavirus disease, tuberculosis, or hepatitis B in a variety of ways (72, 73). Only a few studies excluded that serum 25(OH)D3 concentrations affected the immunogenicity of influenza vaccination in the elderly (74, 75).

To date, the impact of vitamin D on immunogenicity to SARS-CoV-2 vaccines has been studied mainly in healthy healthcare workers (HCWs) in a few studies with contrasting results.

In a 2021 German observational trial, SARS-CoV-2 IgG and neutralization potency and 25(OH)D3 concentrations were measured in a cohort of 126 mostly female and young healthy HCWs 24 weeks after BNT162b2 vaccination. The antibody response was inversely related to age. A similar trend in neutralizing antibodies was observed, though significance was lost at the final sampling time point. Still, the dynamic change of SARS-CoV-2 IgG as a function of 25(OH)D3 status showed no significant differences (76). Nevertheless, the results could have been influenced by the fact that almost 50% of participants were supplementing vitamin D during winter, and the final blood sample was taken in summer when vitamin D levels usually increase even in those not supplementing. Also, the number of patients whose antibody level values were available at the end of the study was drastically reduced compared to the beginning to make reliable results (n = 56 vs. 126). Finally, the authors did not specify the proportion of vitamin D deficiency/insufficiency among participants.

Similarly, three sub-studies were conducted within the CORONAVIT RCT UK adults (n = 2,808) with low vitamin D levels to see if vitamin D supplements could improve the immunogenicity and efficacy of SARS-CoV-2 vaccination. Vitamin D supplementation did not affect the risk of SARS-CoV-2 infection in vaccinated participants when 800 IU/day or 3200 IU/day supplementation was used versus no supplementation, nor on combined IgG-IgA-IgM anti-Spike or neutralizing antibody titers. However, the study had some biases because 69.3% of the mostly white participants (with various comorbidities) received two doses of ChAdOx1nCoV-19, while the remaining participants received two doses of BNT162b2; nearly 5% of subjects had SARS-CoV-2 infection before vaccination and only 11% of patients had vitamin D deficiency, whereas vitamin D levels were not determined in the non-supplemented group (n = 908) (77).

Another Romanian study looked at antibody responses after BNT162b2 vaccination concerning previous SARS-CoV-2 infection status and age and looked for potential biomarkers associated with changes in immune responses. They discovered that prior infection yielded an 8-fold growth in antibody titers, an effect that was weaker in people over 60 years old and unaffected by vitamin D serum levels (78).

Conversely, in an Italian study, the *Authors* explored the relationship between serum 25(OH)D3 levels and the immune response evoked by the BNT162b2 vaccine in a group of 101 HCWs, the majority of whom were young females naïve for SARS-CoV-2 infection (79). There were significant correlations between baseline 25(OH)D3 concentration and anti-spike IgG response and neutralizing antibody titer 24 weeks after the second dose when

serum 25(OH)D3 levels increased significantly. These findings may be justified by the coincidence of the summer season after the six months when sun exposure usually increases vitamin D levels. As a result, 25(OH)D3 levels at the time of vaccination may influence the persistence of the antibody response, even if further studies are required to corroborate these findings, particularly in vulnerable populations.

Interestingly, a recent Indonesian study followed 194 volunteers for eight months after receiving two inactivated SARS-CoV2 vaccination injections. The subjects with low vitamin D levels had lower IFN-γ and IL-12 levels 6 to 7 weeks after the second vaccine injection. Also, those with low IFN-γ levels had a higher risk of COVID-19 infections during follow-up. Therefore, inadequate vitamin D levels were associated with a lower Th1 immune response, whereas adequate IFN-γ levels were necessary to improve vaccine efficacy (80).

Similarly, a 2022 single-center study examined the effect of vitamin D on the response to SARS-CoV-2 immunization after the first BNT162b2 vaccine dose as measured by anti-SARS-CoV-2 Spike IgG concentration in 97 UK HCWs, primarily white and female, over 8 weeks. After two months, the response to immunization was inversely related to age and significantly affected by vitamin D status. Younger people with 25(OH)D3 levels greater than 50 nmol/L had a 29.3% higher IgG peak value. Antibody concentrations decreased in an age-dependent manner, with younger HCWs with higher peaks decreasing

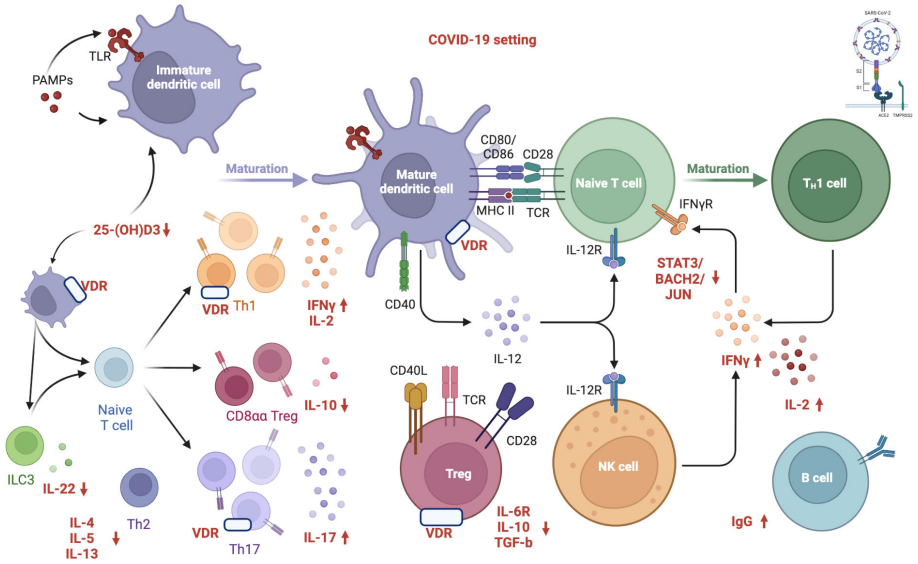

FIGURE 1
Proposed mechanism linking vitamin D deficiency to COVID-19 antibody response. "Created with BioRender.com" (Agreement number: MW24DBIWVD, Academic License). Vitamin D inhibits both the acquired immune system and stimulates the innate immune response as well. VDR modulates immunity processes, such as anti-microbial/anti-viral defense and the induction of T-cell tolerance. Several immune cells express the VDR and CYP27B1 (enzyme 1α-hydroxylase that converts vitamin D to the active form, 1,25-dihydroxyvitamin D3). The activation of innate immunity in SARS-CoV-2 infection seems to result in an increased local 1,25(OH)2D3 production and viral clearance while altering the pro-inflammatory response. The active form of vitamin D, 1,25(OH)2D3, is an inhibitor of dendritic cell maturation and functions as an immune modulator, reducing the activation of the acquired immune system. Furthermore, vitamin D induces transcription factors, including STAT3, BACH2, and JUN, which repress TH1 and TH17 responses and increase IL-10 via IL-6-STAT3 signaling, which is crucial in resolving type 1 inflammation in severe COVID-19 patients. Low vitamin D levels impair innate immune function, inactivating T reg cells and expanding the activated T and B cells, along with the immunoglobulin production. Specifically, human CD4+ T cell activation is associated with high levels of IFNγ, IL-2, IL-17, and low levels of IL-6R, TGF-β, and IL-10, resulting in the pro-inflammatory TH1 skewing and potentially severe tissue damage. COVID-19: Coronavirus Disease 2019; SARS CoV 2: Severe Acute Respiratory Syndrome Coronavirus 2; S1: S1 domain of the coronavirus spike (S) protein; TMPRSS2: Transmembrane protease serine 2; S2: S2 domain of the coronavirus spike (S) protein; ACE2: Angiotensin-Converting Enzyme 2; PAMP: Pathogen Associated Molecular Patterns; TLR: Toll-like receptor; 25(OH)D3: 25-hydroxycholecalciferol; ILC3: Type 3 Innate Lymphoid Cells; IL-22: Interleukin 22; IL-4: Interleukin 4; IL-5: Interleukin 5; IL-13: Interleukin 13; Th2: Type 2 T Helper Cell; Th1: Type 1 T Helper Cell; Th17: Type 17 T Helper Cell; VDR: Vitamin D Receptor; CD8αα: Cluster of Differentiation 8αα; Treg: Regulatory T cells; IFNγ: Gamma Interferon; IL-2: Interleukin 2; IL-10: Interleukin 10; IL-17: Interleukin 17; CD40: Cluster of Differentiation 40; CD80: Cluster of Differentiation 80; CD86: Cluster of Differentiation 86; CD28: Cluster of Differentiation 28; MHCII: Major Histocompatibility Complex; TCR: Toll-like receptor; IL-12: Interleukin 12; IL-12R: Interleukin-12 Receptor; CD40L: Cluster of Differentiation 40 Ligand; CD28: Cluster of Differentiation 28; IL-6R: Interleukin-6 Receptor; TGF-β: Transforming Growth Factor Beta; NK: Natural Killer; IFNγR: Gamma Interferon Receptor; STAT3: Signal Transducer And Activator Of Transcription 3; BACH2: BTB Domain And CNC Homolog 2; JUN: Jun proto-oncogene; IgG: Immunoglobulin G. APC: Antigen-presenting cells; VDR: vitamin D receptor.

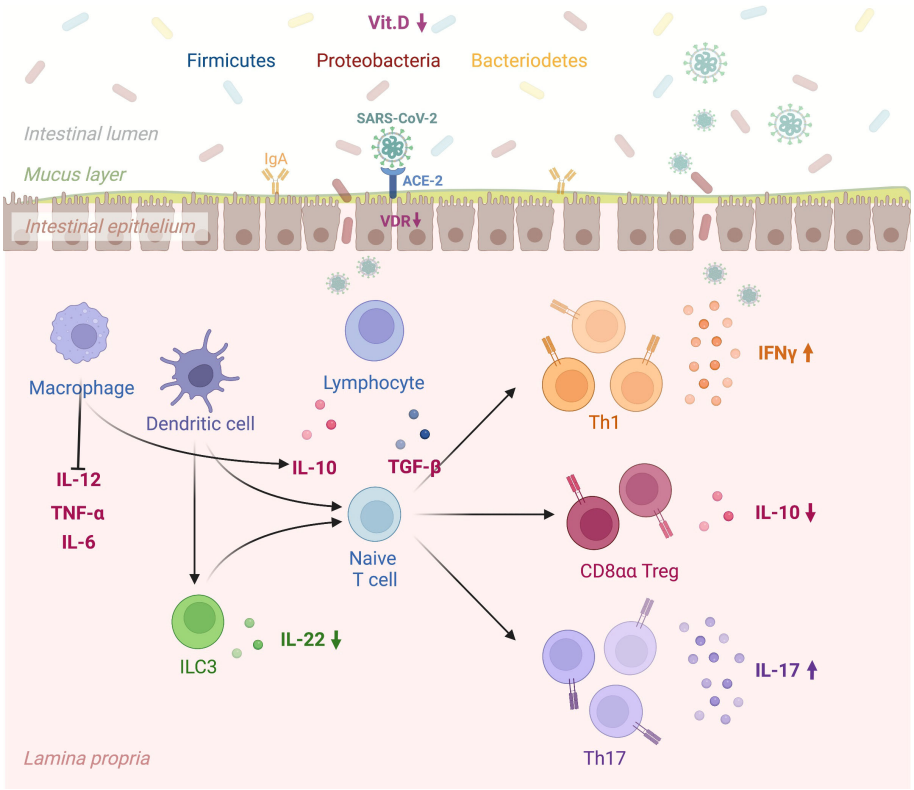

FIGURE 2
A schematic view linking vitamin D deficiency, the gut microbiome, and the intestinal immune response to COVID-19. "Created with BioRender.com" (Agreement number: ON24KBPV47, Academic License). Vitamin D promotes the expression of gap junction proteins within the gastrointestinal tract, which maintain barrier integrity and thus prevent tissue entry by bacteria from the gut microbiome. The VDR-vitamin D endocrine system can modulate acquired and innate immune system functions in viral infections. Specifically, SARS-COV-2 infection of the intestinal epithelium results in rapid viral replication. In the presence of vitamin D deficiency intestinal dendritic cells activated by viruses migrate to lymph nodes, where they earn an enhanced capacity to present antigen or T cell activation (e.g., CD4, CD8, TH, T reg, and ILC). Lower Vitamin D levels alter immune balance promoting pro-inflammatory cytokines and T Helper cell type-1 (TH1), 17 (TH17) and suppressing T regulatory response. Vit.D: Vitamin D; SARS CoV 2: Severe Acute Respiratory Syndrome Coronavirus 2; ACE-2: Angiotensin-Converting Enzyme 2; VDR: Vitamin D Receptor; IgA: Immunoglobulin A; IL-12: Interleukin 12; TNF-α: Transforming Growth Factor; IL-6: Interleukin-6; ILC3: Type 3 Innate Lymphoid Cells; IL-22: Interleukin 22; IL-10: Interleukin 10; TGF-β: Transforming Growth Factor Beta; Th1: Type 1 T Helper Cell; CD8αα T reg: Cluster of Differentiation 8αα T Regulatory Cell; Th17: Type 17 T Helper Cell; IFNγ: Gamma Interferon; IL-17: Interleukin 17. VDR: vitamin D receptor.

faster to comparable concentrations across ages at week 8 (81). These findings support the authors' conclusion that a booster immunization program should be planned after sun exposure (end of summer or early autumn) or following vitamin D supplementation.

However, conclusive data associating antibody titers against the virus with vitamin D serum levels in COVID-19 patients still need to be included. Figure 1 depicts in detail a proposed mechanism linking vitamin D deficiency to COVID-19 antibody response while Figure 2 illustrates the role of vitamin D deficiency in the context of gut microbiome and the intestinal immune response concerning COVID-19.

Furthermore, as the next Gordian knot, we need to establish whether the host residual immunological activation after SARS-CoV-2 eradication will latently progress into full-blown B-cell polyclonal or monoclonal expansion. At the state of our knowledge, this enigma is currently unsolvable as it requires long-term surveillance.

7 Conclusion

Further research is needed to determine a link between vitamin D status and the generation of protective serological responses to SARS-CoV-2 vaccination.

Author contributions

Study conceptualization, ES. Drafting the original manuscript, ES. Supervision, FP, AA, and AS. Data collecting and curation, ES. Data gathering, manipulation, analysis, ES, FP, AA, and AS. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1038316/full#supplementary-material

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EDITED BY
Harumichi Ishigame,
RIKEN Yokohama, Japan

REVIEWED BY
Alessandra Pontillo,
University of São Paulo, Brazil
Ioanna Aggeletopoulou,
University of Patras, Greece

*CORRESPONDENCE
Xinxia Li
Lxx-patho@xjmu.edu.cn

†These authors have contributed equally to this work

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Vitamin D3 alleviates inflammation in ulcerative colitis by activating the VDR-NLRP6 signaling pathway

Hongliang Gao[1,2†], He Zhou[3†], Zhiqiang Zhang[2], Jianshu Gao[2], Jian Li[2] and Xinxia Li[1*]

[1]Pathology Center, Xinjiang Medical University Affiliated Tumor Hospital, Urumqi, Xinjiang, China, [2]The Second Department of Gastroenterology, the First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China, [3]State Key Laboratory of Cancer Biology, National Clinical Research Center for Digestive Diseases and Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi'an, Shaanxi, China

Inflammation is a key factor in the development of ulcerative colitis (UC). 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$, VD_3), as the major active ingredient of vitamin D and an anti-inflammatory activator, is closely related to the initiation and development of UC, but its regulatory mechanism remains unclear. In this study, we carried out histological and physiological analyses in UC patients and UC mice. RNA sequencing (RNA-seq), assays for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), chromatin immunoprecipitation (ChIP) assays and protein and mRNA expression were performed to analyze and identify the potential molecular mechanism in UC mice and lipopolysaccharide (LPS)-induced mouse intestinal epithelial cells (MIECs). Moreover, we established nucleotide-binding oligomerization domain (NOD)-like receptor protein *nlrp6*$^{-/-}$ mice and siRNA-NLRP6 MIECs to further characterize the role of NLRP6 in anti-inflammation of VD_3. Our study revealed that VD_3 abolished NOD-like receptor protein 6 (NLRP6) inflammasome activation, suppressing NLRP6, apoptosis-associated speck-like protein (ASC) and Caspase-1 levels *via* the vitamin D receptor (VDR). ChIP and ATAC-seq showed that VDR transcriptionally repressed NLRP6 by binding to vitamin D response elements (VDREs) in the promoter of NLRP6, impairing UC development. Importantly, VD_3 had both preventive and therapeutic effects on the UC mouse model *via* inhibition of NLRP6 inflammasome activation. Our results demonstrated that VD_3 substantially represses inflammation and the development of UC *in vivo*. These findings reveal a new mechanism by which VD_3 affects inflammation in UC by regulating the expression of NLRP6 and show the potential clinical use of VD_3 in autoimmune syndromes or other NLRP6 inflammasome-driven inflammatory diseases.

KEYWORDS

VDR, NLRP6 inflammasome, ulcerative colitis, VD_3, ATAC-seq

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Introduction

UC is a kind of local recurrence of intestinal inflammatory disease because of the complexity and long course of the disease and its wide range of lesions and is recognized as a cancer-plus lesion of colon cancer (1). At present, hormones and amino salicylic acid drugs are still used as the first-choice drugs in treatment, but the long-term use of such drugs has large adverse reactions (2). Therefore, it has become a research topic to seek ideal drugs with weak adverse reactions that are effective, especially for the remission period.

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, VD_3), as the active metabolite of vitamin D, is a modulator in immunology and can stimulate the production of transforming growth factor beta1 (TGF-β1) and interleukin (IL)-4, thereby reducing inflammation (3). Studies in IL-10 knockout mice have found that when vitamin D is deficient, the animals spontaneously develop symptoms of colitis, such as blood in the stool and wasting, accompanied by a high mortality rate (4). Administration of enough VD_3 relieves symptoms, showing that VD_3 is associated with the pathogenesis of UC. In fact, there is increasing evidence that deficiency of active vitamin D plays an important role in the development and severity of inflammatory bowel diseases (IBDs) (5, 6). Animal experiments have shown that lack of cytochrome P450 family 27 subfamily B1 (CYP27b1) and decreased vitamin D secretion increase both the prevalence and severity of IBD (7). VD_3 has been shown to have important regulatory functions in many autoimmune diseases (8, 9). Studies have shown that vitamin D directly regulates the T-cell antigen receptor (TCR) (10). In naive T cells, low expression of phospholipase C-y 1 (PLC) was associated with low cellular responses. The induction of PLC-yl is dependent on vitamin D and its receptor (VDR) (10). VDR is a nucleophilic protein that is an intranuclear biological macromolecule that mediates the biological effects of VD_3 (11). In essence, it is a ligand-dependent nuclear transcription factor (12, 13). Active vitamin D binds to VDR and transcribes its downstream genes to exert diverse biological functions (14). VDR plays an important role in maintaining the body's calcium-phosphorus metabolism and regulating cell proliferation and differentiation (15). The VDR level has a significant effect on IBD (5, 16). Experiments have shown that VDR gene polymorphisms and serum 25(OH) vitamin D levels are closely related to UC (17). Mutated VDR genotypes increase susceptibility to UC (18). All these studies show strong evidence for the application of vitamin D as an anti-inflammatory immunomodulator in IBD. However, the molecular mechanism underlying VD_3-VDR-mediated alleviation of UC is still unclear.

Nucleotide-binding oligomerization domain (NOD)-like receptor protein 6 (NLRP6) is a novel member of the NOD-like receptor (NLR) protein family discovered to inhibit the innate immune response-related signaling pathway, and its encoding gene is located on human chromosome 11 (19). NLRP6 can interact with cysteine-containing aspartate specific proteinase-1 (Caspase-1) and apoptosis associated speck-like protein (ASC) containing caspase recruitment domain (CARD), forms an intracellular polyprotein complex (NLRP6 inflammasome) through the protein-protein linkage of the N-terminal pyrin domain (PYD), and finally produces interleukin (IL)-1β. IL cytokines such as IL-18 are involved in the inflammatory and immune responses (20, 21). NLRP6 is highly expressed in intestinal tissue (22). The NLRP6 inflammasome is considered an important player in maintaining intestinal homeostasis, and any perturbations of this pathway, especially NLRP6, ASC, caspase-1, or IL-18, may promote human IBD initiation or progression in some cases (23, 24).

In this study, we carried out a series of histological, physiological and molecular analyses in UC patients and UC mice and lipopolysaccharide (LPS)-induced MIECs to determine the role and regulatory mechanism of VD_3 in UC progression. We demonstrated that VD_3 was beneficial in the treatment of acute UC through VD_3-VDR inhibiting NLRP6 transcription, which led to decreased NLRP6 inflammasome activity. These data provide an alternative for the diagnosis and treatment of UC. Considering this evidence, well-conducted clinical trials of vitamin D or its analogues in human UC patients are strongly indicated to further assess the potential therapeutic immunomodulatory properties of this underestimated nutrient.

Materials and methods

Patients

Inflammatory colorectal tissues were collected from January 2022 to June 2022 from patients first visiting the hospital for acute UC (n = 30), and the control tissues were from persons whose physical examinations were normal (n = 30). The diagnosis of UC complied with the relevant standards in the 'Consensus Opinions on the Diagnosis and Treatment of IBD in China'. The study was approved by the Research and Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Ethical approval number: 20211015-33). Patient consent was also obtained from all subjects before research.

Construction of the dextran sulfate sodium induced acute colitis model

C57BL/6 mice (male, 8 weeks old) were purchased from Vital River Laboratories (Beijing, China). The mice were maintained in a 12 h light/dark cycle and allowed free access to food and water in the animal facility with a temperature-controlled environment. All animal experiments were performed according to the guidelines of the National Institutes of Health for Animal Care and Use and were approved by the Committee for Animal Research of Henan University. Acute UC was induced in mice by administering 3% DSS (MP Biomedical, Santa Ana, California, USA) into their drinking water for 7 d (Days 0 to 7). The mice were randomly assigned to the control, DSS, and DSS+VD_3 groups, with 6 mice in each group, as per the Animal Research Reporting of *in vivo* Experiments (ARRIVE) guidelines. The control mice were given clean drinking water. Each mouse received a single injection of VD_3 (100 μg/kg) in the abdomen every 2 days (Days 0, 2, 4 and 6), and the drug treatments carried out on DSS-treated mice were blinded.

Construction of the *nlrp6*$^{-/-}$ deficient mouse model

The *nlrp6*$^{loxp/loxp}$ mice and Villin-Cre mice were purchased from a commercial company (Cyagen, China) and were mated to produce offspring. The resulting offspring were mated with *nlrp6*$^{loxp/loxp}$ mice to obtain *nlrp6*$^{loxp/loxp}$Villin-Cre (*nlrp6*$^{-/-}$) mice.

Data source and differential expression analysis

The GSE155301 dataset consists of 6 microarray expression profiles and was downloaded from the Gene Expression Omnibus Comprehensive Website (GEO, http://www.ncbi.nlm.nih.gov/geo). Sample data were obtained from the colons of 3 dextran sulfate sodium-treated mice with acute UC and 3 healthy controls. The platform of the dataset was GPL21103 (Illumina HiSeq 4000 (Mus musculus)). The differentially expressed genes in the TLE and control groups were analysed using the GEO2R tool and were selected according to an at least 1.5-fold difference.

ATAC-seq

In total, 6×10^5 tissue-adherent MIECs were processed according to a previously published protocol (25), and 150 bp paired-end sequencing was performed on an Illumina Xten to yield an average of 97 M reads/sample.

ChIP assay

The SimpleChip Plus Enzymatic Chromatin IP Kit (Cat# 9004, Cell Signalling, China) was used in the ChIP assay with mouse clone tissues. Immunoprecipitation was carried out with a specific VDR antibody (ab109234, Abcam) negatively controlled by nonspecific IgG (Cat# 3420, Cell Signalling, China). Twenty percent of the samples were reserved as 'Input'. The primer sequences specific for the three VDREs are shown in Supplemental Table 1. The enriched DNA amounts were quantified with the results of IgG and the input DNA.

Immunofluorescence staining

Immunofluorescence staining of mouse colon sections was performed as previously described (26). The sections were incubated with primary antibodies against VDR (ab109234, Abcam), NLRP6 (ab58705, Abcam), Caspase-1 (ab138483, Abcam) and ASC (ab175449, Abcam) at 4°C overnight, followed by an incubation with fluorescently labelled secondary antibodies. The sections were examined using a Leica confocal microscope (LEICA TCS SP5).

Quantitative real-time PCR

The total RNA of each mouse colon tissues was extracted with TRIzol reagent (T9429, Sigma, US) according to the manufacturer's instructions and reverse-transcribed with the PrimeScript™ RT Reagent Kit (TaKaRa, Japan). qRT–PCR was performed with SYBR Green Detection Mix (TaKaRa, Japan). The relative expression levels of genes in this study were normalized to actin expression and analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The collected MIECs and mouse colon tissues were extracted using protein lysis buffer (Sigma-Aldrich, USA) and quantified *via* a bicinchoninic acid assay (Pierce, USA). Protein samples were then electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, EMD Millipore, MA, USA), which was probed with antibodies against VDR (ab109234, Abcam), NLRP6 (ab58705, Abcam), Caspase-1 (ab138483, Abcam) and ASC (ab175449, Abcam) at a dilution of 1:1000. Blots were subsequently detected and visualized using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) according to protocols provided by the manufacturer. A Bio-Rad scanning system was used to detect immunoreactive protein bands, and GAPDH (ab204276, Abcam) was used as a control.

Intestinal permeability test

Fluorescein isothiocyanate (FITC)-labelled dextran (FD) was used to detect the intestinal permeability. After overnight fasting, the mice in each group were orally administered the permeability tracer FD (400 mg/kg) for 4 h. Blood was collected from the medial canthus, and the fluorescence intensity in serum was measured by spectrophotometer (excitation wavelength 490 nm, emission wavelength 530 nm).

Transmission electron microscopy

The mouse colon tissues were collected and analysed as previously described (27). The ultrathin colon sections were detected with an electron microscope (HITACHI, Tokyo, Japan).

Evaluation of disease activity index

Weigh the mice every day and observed their behaviors, stool characteristics, diarrhea degree and whether death occurred. Calculate as follows: (a) Stool consistency: 0, Normal; 2, Loose stool; 4, Watery diarrhea; (b) Blood stool: 0, Normal; 2, Slight bleeding; 4, Massive hemorrhage; (c) Weight loss: 0, None; 1, Decrease by 1%~5%; 2, Decrease by 5~10%; 3, Decrease by 11%~15%; 4, Decrease>15%. Evaluate each item of the mice, and the sum of a, b and c is the DAI score.

Gene ontology and Kyoto encyclopedia of genes and genomes analysis

The functional enrichment of the differentially expressed genes (DEGs) was evaluated to obtain the genes associated with UC through GO and KEGG analyses. The functional terms for the GO enrichment analysis, including cellular component (CC), biological process (BP) and molecular function (MF) categories, were performed *via* the online tool Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Version 6.8; https://david.ncifcrf.gov/home.jsp). KEGG analysis of DEGs was performed using KEGG Orthology-Based on KOBAS 2.0. The results of the enrichment were analysed by Fisher's exact test, using $P \leq 0.05$ as the significance threshold.

Protein-protein association network analysis

The PPI network centred on VDR, NLRP6, ASC and Caspase-1 was constructed using the Search tool for the retrieval of interacting

genes/proteins (STRING) database (Version 11.0; www.string-db.org), and a PPI score (medium confidence) ≥ 0.4 was defined as the cut-off value.

Statistical analysis

SPSS 22.0 (IBM Corporation, USA) and GraphPad Prism 5.0 (GraphPad Inc., USA) were employed for statistical analyses. All data are presented as the means ± SD (standard deviation). Student's *t test* or one-way ANOVA with Bonferroni's *post-hoc* test were utilized for analysis of mRNA and protein levels between distinctive groups. A value of $P<0.05$ was considered statistically significant.

Results

VDR expression is negatively correlated with inflammation in UC patients

To investigate the histopathological damage of colitis in UC patients, pathological features were analyzed through HE staining and TEM observation. The results of HE staining showed that the colonic tissue of normal individuals was integral, the mucosa had no obvious defect, the glands were neatly arranged without atrophy, and no obvious inflammatory cells infiltrated the mucosal lamina propria. However, in UC patients, the mucosal layer of the gland had atrophied or had even disappeared, replaced by a large number of inflammatory cells infiltrating the submucosa (Figure 1A). Furthermore, the mitochondria, endoplasmic reticulum, lysosome and nucleus in normal colon epithelial cells with regular shapes were clearly visible by TEM, and the mitochondria clearly revealed double membranes (Figure 1A). However, these intracellular organelles were reduced in number and blurred in UC patients (Figure 1A). Thus, the occurrence of UC could induce obvious pathological changes in colon tissue.

In addition, the IL-1β and IL-18 contents in UC patients were significantly increased compared to those in normal patients (**$P < 0.01$, ***$P < 0.001$; Figure 1B). The LPS (10 μg/ml)-treated MIECs confirmed the results from UC patients, as both the IL-1β and IL-18 contents were increased significantly compared to the controls (***$P < 0.001$; Figure 1C).

VD_3 was shown to present anti-inflammatory actions and generated the active metabolite $1\alpha,25\text{-}(OH)_2D_3$ mainly by the metabolizing enzymes CYP27A1, CYP27B1 and CYP24A1, which were recognized by the nuclear transcription factor VDR regulating a series of gene expressions (28, 29) (Figure 1D). According to the qPCR results, the mRNA expression levels of CYP24A1, CYP27A1, and CYP27B1 changed unconspicuously, but the VDR mRNA levels were decreased in UC patients and LPS-treated MIECs (Figure 1E).

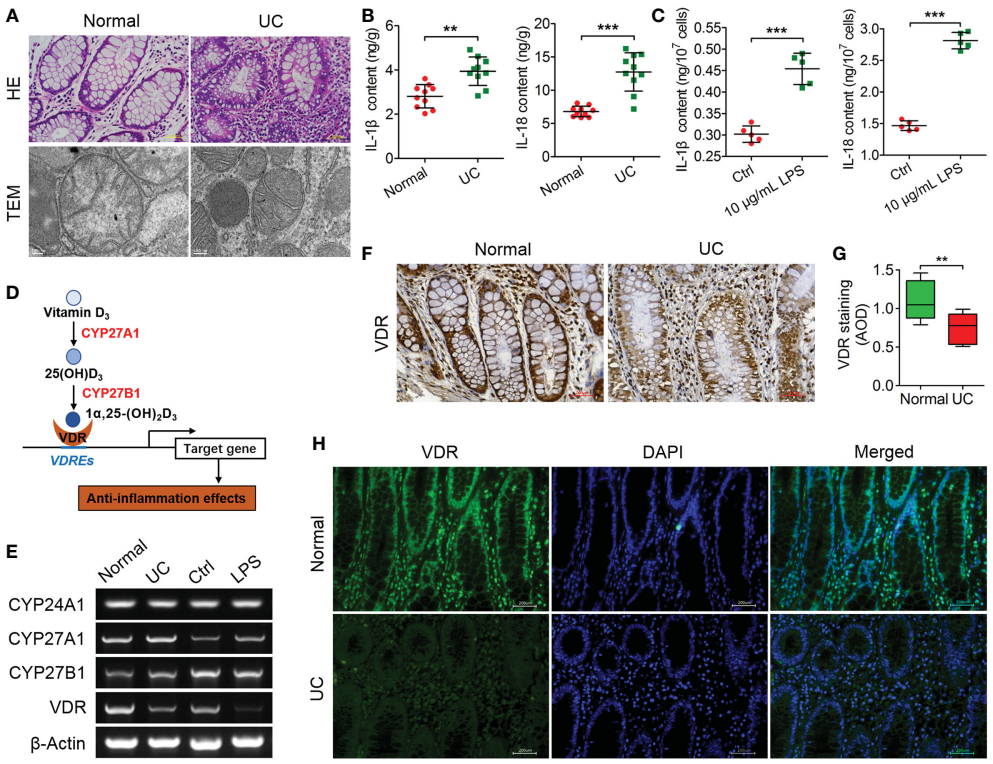

FIGURE 1
VDR is negatively correlated with inflammation in UC patients. **(A)** The pathological features of colitis are depicted by HE staining and TEM observation. **(B, C)** The contents of IL-1 and IL-18 were analysed through ELISA in UC tissues **(B)** and LPS-treated cells **(C)**. **(D)** Schematic diagram illustrating the key factors (CYP27A1, CYP27B1, VDR and CYP24A1) involved in the anabolism and catabolism of $1\alpha,25(OH)_2D_3$ and the regulation of target genes. **(E)** qPCR was used to analyse the mRNA expression of CYP24A1, CYP27A1, CYP27B1 and VDR in colon tissue from UC patients and LPS-treated MIECs. **(F, G)** Representative Immunohistochemical images **(F)** and quantified data **(G)** for VDR in colons from normal and representative UC patients. **(H)** The expression of VDR was measured by immunofluorescence assay in colon sections from normal and representative UC patients. The data are shown as the means ± SD; n ≥ 3, **$P < 0.01$, ***P <0.001.

Furthermore, the immunohistochemical results showed that VDR expression was downregulated significantly in the colon tissues of UC patients (***$P < 0.001$; Figures 1F, G). The VDR immunofluorescence signals were weak in UC patients, which confirmed the results from immunohistochemistry (Figure 1H).

VD_3 treatment attenuates pathological damage to DSS-induced UC in mice

To investigate the effect of VD_3 on UC development, mice were given 3% DSS in drinking water to induce acute colitis and administered VD_3. Seven days later, the UC colons were shorter and smaller than the normal colons (Figure 2A), and the body weights of the mice were significantly reduced (**$P < 0.01$; Figure 2B). However, VD_3 treatment significantly mitigated the effect of UC on mouse colon tissues, and the body weight of the UC + VD_3 group was significantly increased compared to that of the UC group (**$P < 0.01$; Figure 2B). Moreover, the value of DAI was significantly higher in UC mice than in normal mice, but it decreased significantly after VD_3 treatment (**$P < 0.01$; Figure 2C). The UC mice showed a significantly high intestinal permeability rate of FD, but the UC + VD_3 group revealed a significantly lower FD intestinal permeability rate than the UC mice (**$P < 0.01$; Figure 2D). The UC mice exhibited damaged structure and disordered glands, massive destruction of mucosal epithelial cells, and infiltration of a large number of inflammatory cells in the submucosa, as shown by HE staining of colons, and the number of intracellular organelles in UC mice was reduced and difficult to distinguish by TEM, similar to the findings in UC patients (Figure 2E). However, the pathological damage after VD_3 treatment was significantly reduced compared with that in UC mice (Figure 2E).

The VDR-NLRP6 signaling pathways are involved in DSS-induced UC

To determine the molecular mechanism underlying the effects of VD3 on UC progression, we carried out gene expression profile analysis in DSS-induced UC mouse colon tissues. In total, 1461

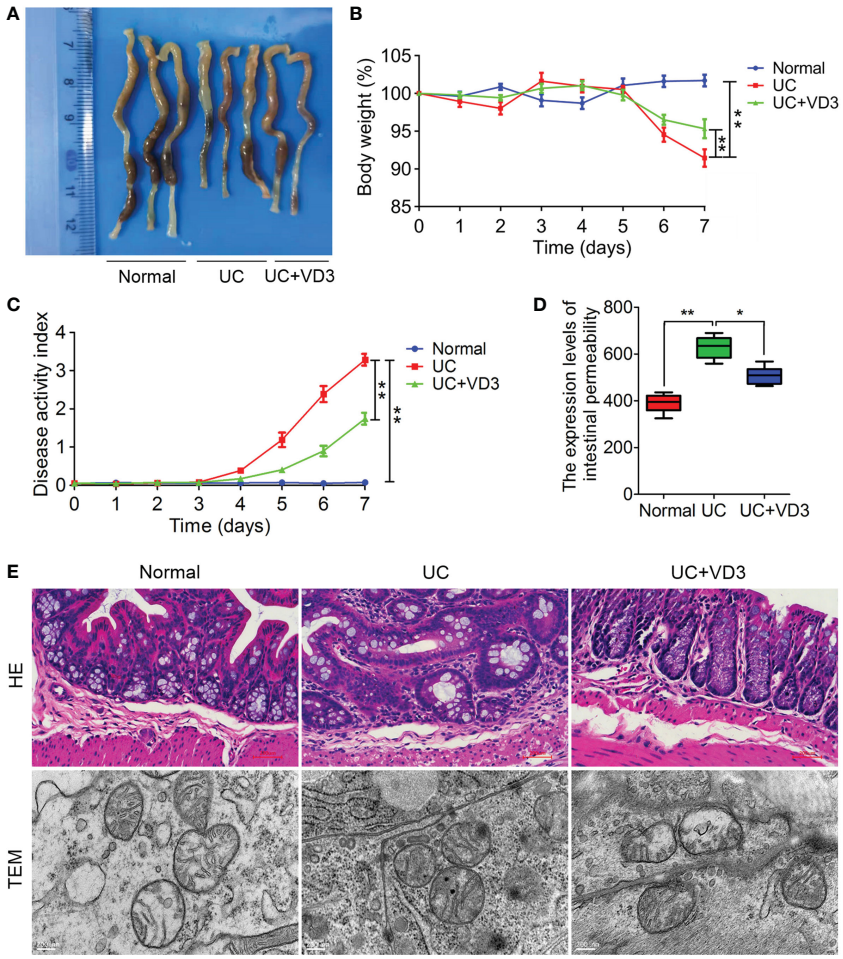

FIGURE 2
$1,25(OH)_2D_3$ alleviates DSS-induced UC in mice. **(A)** Macroscopic appearances and colon lengths of the mice were measured. **(B)** Mice were given 3% DSS in drinking water for 7 d to induce acute colitis. Body weight loss. **(C)** The DAI of these mice during the experimental period is depicted. **(D)** The intestinal permeability rate of FITC-dextran was measured. **(E)** Representative HE-stained and TEM colon sections. The data are shown as the means ± SD; n ≥ 3, *$P < 0.05$, **$P < 0.01$.

significant DEGs were identified, among which 798 were upregulated and 663 were downregulated (Figure 3A). Furthermore, we performed GO term and KEGG pathway analyses on the identified DEGs (Figures 3B–D). The gene expression levels of 16 enriched DEGs were depicted in a heatmap that were classified into vitamin B6 metabolism and pertussis signalling pathways, as determined by KEGG pathway analysis (Figure 3C). Among these 16 genes, VDR showed lower expression, but NLRP6, Caspase-1, PYD and CARD domain-containing (PYCARD) showed relatively higher expression in UC mouse colons (Figure 3C).

VD_3 suppressed NLRP6 inflammasome activation

NLRP6 acts as an innate immune sensor that recruits ASC and Caspase-1 and forms an inflammasome mediating the release of the inflammatory cytokines IL-1β and IL-18 (30). Thus, we detected the expression of VDR and the main mediators of the NLRP6 inflammasome, NLRP6, ASC and Caspase-1, in UC mice and LPS-treated MIECs. The western blotting results showed that VDR protein expression decreased in both UC mice and LPS-treated MIECs compared to the controls (**$P < 0.01$; Figures 4A–D). In contrast, the protein expression levels of NLRP6, ASC and Caspase-1 increased significantly in UC mice and LPS-treated MIECs (**$P < 0.01$; Figures 4A–D). Moreover, the mRNA expression levels of these genes in UC mice and LPS-treated MIECs were consistent with the results of western blotting analysis (**$P < 0.01$; Supplemental Figure 1). However, the expression of VDR was enhanced, but NLRP6 inflammasome gene expression was reduced by VD_3 administration (*$P < 0.05$, **$P < 0.01$; Figure 4, Supplemental Figure 1). The immunohistochemistry results further confirmed these findings that VD_3 significantly increased VDR expression but decreased NLRP6, ASC and Caspase-1 expression in UC mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Figures 4E, F).

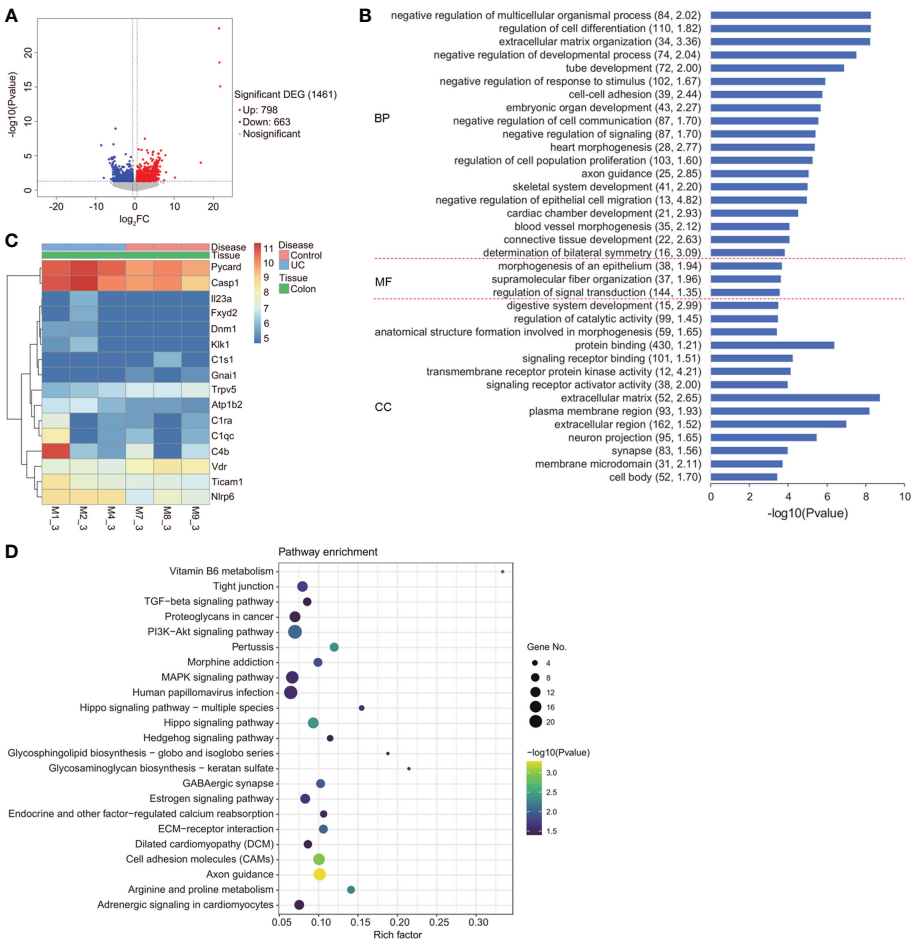

FIGURE 3
Gene expression profile of DSS-induced UC in mice. **(A)** Volcano plot of differentially expressed genes. X axis: log 2 FC; Y axis: -log10 (FDR). Red represents upregulated genes, and blue represents downregulated genes. **(B)** GO term analysis of BP, MF, and CC for the genes. The minus logarithm of the *P value* (x-axis) indicates the significance of the gene set belonging to predefined categories under the coexpression network gene background. The y-axis represents each GO category. **(C)** A heatmap depicting the gene expression profiles of vitamin B6 metabolism and the pertussis signalling pathway in the colon of the DSS mouse model and healthy controls. X axis: sample name; Y axis: gene name. **(D)** Enriched upregulated and downregulated genes, as determined by KEGG pathway analysis.

VD_3 transcriptionally regulates NLRP6 expression in mouse colons, and NLRP6 is a key player in the regulation of NLRP6 inflammasome activity in UC

We performed ATAC-seq to map genome-wide chromatin accessibility to the colon tissues of UC mice. The results showed a peak in the NLRP6 promoter regions in the normal controls but not in the UC group (Figure 5A), which implied that NLRP6 was transcriptionally regulated during UC development. The potential binding sites in the NLRP6 promoter regions are shown in Figure 5B. In addition, the ChIP assay revealed that the DNA fragments containing -852/-846 and -1179/-1173 VDREs were enriched significantly compared to the IgG controls, while the fragment containing the -1939/-1933 VDRE was not enriched (Figures 5B, C). Meanwhile, the enriched DNA fragments were normalized to the input. Thus, the ATAC-seq and ChIP assays suggested that VDR transcriptionally bound to VDREs in mouse clone tissues.

To identify the effect of NLRP6 on UC progression, we established *nlrp6*$^{-/-}$ mice and detected the expression levels of VDR, NLRP6, ASC and Caspase-1 in WT and *nlrp6*$^{-/-}$ mice treated with DSS and VD_3. The western blotting results from the UC and UC + VD_3 mice controlled by the WT mice were consistent with the above findings that VDR expression was reduced, but NLRP6, ASC and Caspase-1 expression levels were enhanced in UC mice (*$P < 0.05$, **$P < 0.01$; Figure 6A, C–F). However, VD_3 significantly upregulated VDR but downregulated the expression of NLRP6 inflammasome genes (*$P < 0.05$, **$P < 0.01$; Figures 6A, C–F). The results from the LPS- and VD_3-treated MIECs controlled by the siRNA-NC groups further confirmed these findings. However, in *nlrp6*$^{-/-}$ mice, the NLRP6 level was almost undetectable (**$P < 0.01$; Figures 6A, D). DSS-induced UC increased NLRP6 levels in *nlrp6*$^{-/-}$ mice, but VD_3 treatment decreased these levels (*$P < 0.05$; Figures 6A, D). The levels of Caspase-1 and ASC were similar to those of NLRP6 in *nlrp6*$^{-/-}$ mice treated with UC and UC + VD_3 (*$P < 0.05$, **$P < 0.01$; Figures 6A, E, F). Additionally, the results from siRNA-

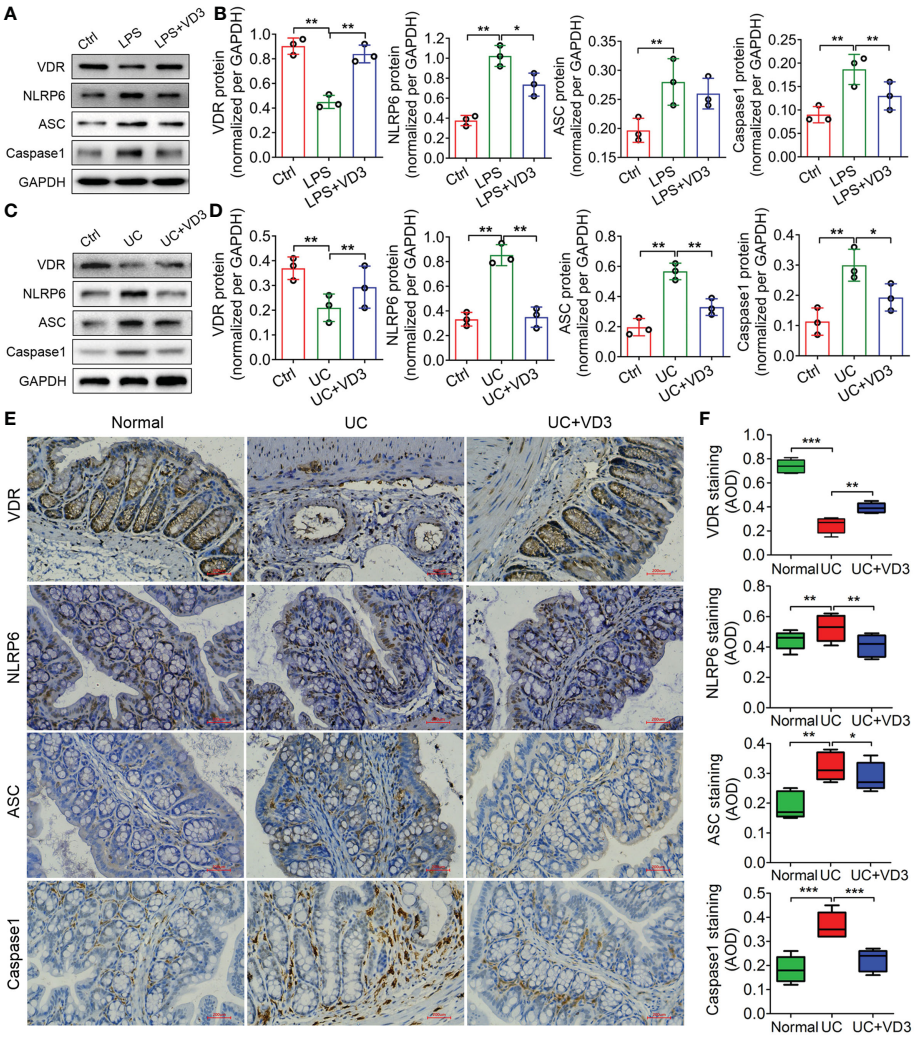

FIGURE 4
1,25 $(OH)_2D_3$ inhibits expression of the NLRP6, ASC and Caspase-1 inflammasomes in LPS-treated primary intestinal epithelial cells and DSS-induced UC mice. **(A)** Western blot analyses were performed to evaluate the expression of the VDR, NLRP6, ASC and Caspase-1 proteins in LPS-primed MIECs treated with VD_3 for 3 h. **(C)** Western blot analyses were performed to evaluate the expression of the VDR, NLRP6, ASC and Caspase-1 proteins in UC mice treated with VD_3. **(B)** and **(D)** The quantification of protein expressions in A and C. **(E)** Immunohistochemical analysis of VDR, NLRP6, ASC and Caspase-1 expression in colon sections from UC mice treated with VD_3. **(F)** Quantitative analysis of the average optical density by immunohistochemistry. Scale bar represents 500 μm. The data are shown as the means ± SD; n ≥ 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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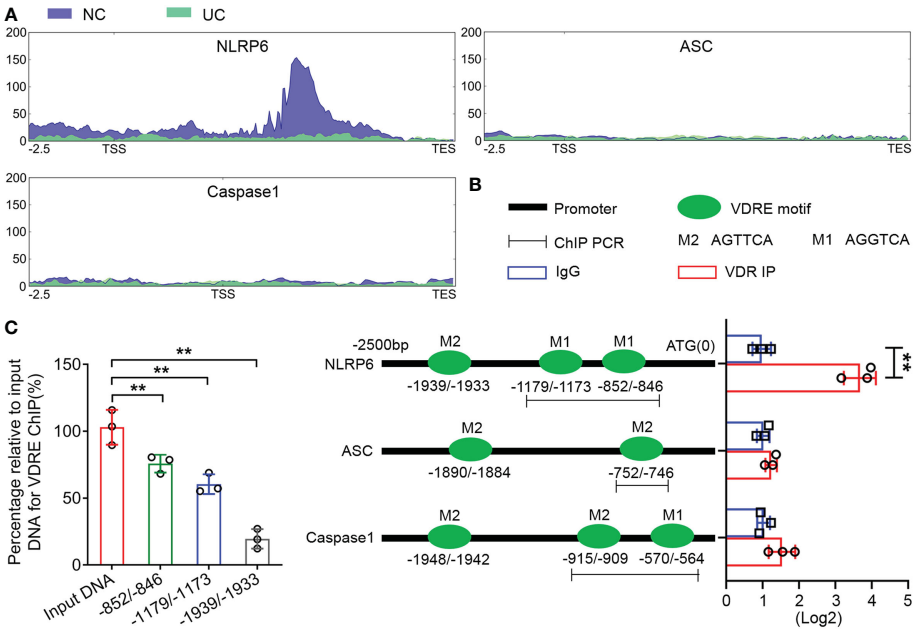

FIGURE 5
VDR downregulates NLRP6 expression by inhibiting the transcriptional activity of the NLRP6 promoter. **(A)** ATAC-seq enrichment from 2500 bp upstream of the TSSs throughout the whole ranges of the NLRP6, ASC and Caspase-1 genes in normal and UC tissues. **(B)** Presence of the AGTTCA and AGGTCA motifs in the promoters of NLRP6, ASC and Caspase-1 (left) and qChIP-PCR results (right) showing the binding of VDR to the promoter fragments containing the AGTTCA and AGGTCA motifs in the promoters of NLRP6, Caspase-1 and the AGTTCA motifs in the promoters of ASC, respectively. **(C)** The percentage relative to the input for the enriched fragments of the VDRE ChIP. The data are shown as the mean ± SD, n ≥ 3, $^{**}P < 0.01$.

NLRP6 cells treated with LPS and LPS + VD_3 further confirmed the results in *nlrp6*$^{-/-}$ mice (Figures 6G–J). Interestingly, VDR expression was increased in both *nlrp6*$^{-/-}$ mice compared to WT mice and in siRNA-NLRP6 cells compared to siRNA-NC controls ($^{*}P < 0.05$; Figures 6A–C, G). All these findings were confirmed by qPCR results ($^{*}P < 0.05$, $^{**}P < 0.01$; Supplemental Figure 2).

VDR is negatively correlated with NLRP6 expression in the initiation and development of UC

The results from protein-protein association network analysis of the NLRP6-(Caspase-1)/IL-1β signalling pathway in STRING showed that VDR, NLRP6 and IL-1β were closely associated at the protein level (Figure 7A). Moreover, VDR expression was negatively correlated with NLRP6 and Caspase-1 expression (Figure 7B). Thus, we identified the expression of these NLRP6 inflammasome genes in UC patients, which showed that the protein expression levels of NLRP6, ASC and Caspase-1 were all increased compared to those in normal individuals in immunohistochemical and immunofluorescence analysis ($^{***}p < 0.001$; Figure 7C, Supplemental Figure 3).

Discussion

UC is a chronic inflammatory disease of the colon resulting in digestive disorders (2, 31). Although the incidence and prevalence have continued to increase worldwide in recent years, the current treatment methods for UC have not shown good clinical efficacy in most patients, who ultimately require colectomy, seriously affecting their quality of life (2). Factors associated with the onset and pathogenesis of UC have been uncovered recently, including genetic susceptibility, environmental factors, intestinal epithelial barrier dysfunction, and immune response dysregulation (32). However, many efforts are needed to enhance the understanding of UC development.

Vitamin D exerts various anti-inflammatory, antioxidant, immunomodulatory, and antifibrotic effects (33). Typically, patients with UC have low serum vitamin D levels, which are associated with complications such as low bone mineral density (34–37). The inverse association of vitamin D with IBD or UC disease has been confirmed recently, and vitamin D supplements have been shown to help relieve disease symptoms (33, 36, 38, 39). In this study, DSS-induced UC mice treated with VD_3 showed a significant increase in body weight compared to UC mice, and VD_3 administration successfully reduced the DAI and intestinal permeability rate in UC mice. Moreover, the results from colon sections revealed that VD_3 supplements improved the integrity of the intestinal mucosal barrier. Thus, these results suggested that VD_3 treatment attenuates pathological damage in UC mice, which confirmed previous findings. As the nuclear receptor of VD_3, low expression of VDR and dysfunction of VD_3-VDR signaling in IBD patients have been reported (40). In this study, the UC patients exhibited typical symptoms with obvious mucosal tissue defects and inflammatory cell infiltration. Meanwhile, the contents of the proinflammatory factors IL-1β and IL-18 increased significantly. However, the VDR protein level was reduced in UC patients. These results confirmed that VDR expression was inhibited in the intestinal tissue of patients with colitis. It is worth mentioning that the expression of the key enzymes in vitamin D metabolism releasing

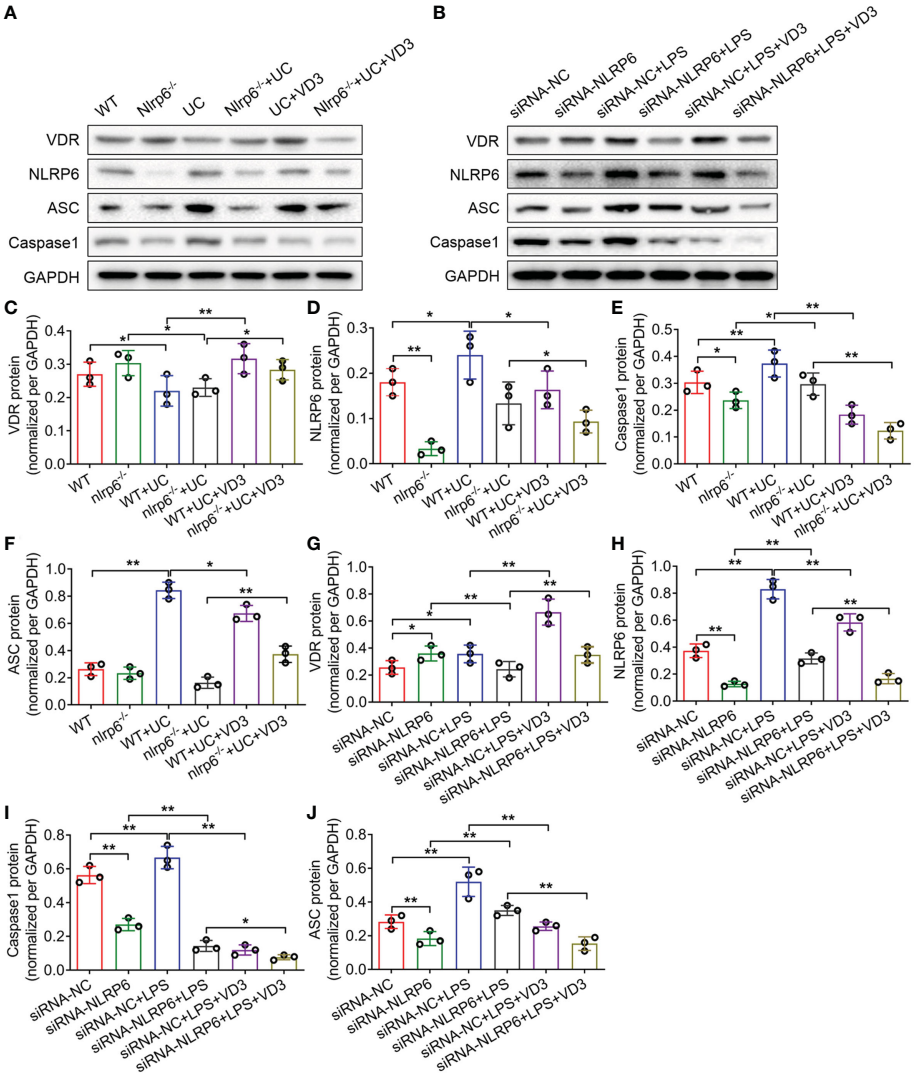

FIGURE 6
1,25(OH)$_2$D$_3$ promotes NLRP6 expression *in vivo* and *in vitro*. **(A)** Western blot analyses were performed to evaluate the expression of the VDR, NLRP6, ASC and Caspase-1 proteins in *Nlrp6*$^{-/-}$ mice and UC mice subsequently treated with VD$_3$. **(B)** Western blot analyses were performed to evaluate the expression of the VDR, NLRP6, ASC and Caspase-1 proteins in NLRP6 siRNA MIECs that were then treated with LPS and incubated with VD$_3$. **(C–J)** Quantitative analysis of the protein levels. The data are shown as the mean ± SD, n ≥ 3, *$P < 0.05$, **$P < 0.01$.

the active ingredient VD$_3$ did not change in UC patients in this study, which indicated that UC development might not rely on the dysregulated metabolism of vitamin D, although its aberrances normally confer resistance to the protective effect of vitamin D in many diseases. Reduced VDR levels and the subsequent inactivation of VD$_3$-VDR signaling contributed to UC progression.

According to the results from RNA-seq in DSS-induced UC mice, the expression levels of NLRP6 and Caspase-1 increased. NLRP6 is a recently defined inflammasome that plays key roles in regulating inflammation and intestinal homeostasis (41, 42). Although the association between NLRP6 expression and intestinal integrity has been confirmed, in some cases, NLRP6 expression was observed to be upregulated, while in others, it was downregulated (20). In this study, the expression levels of the NLRP6 inflammasome genes NLRP6, ASC and Caspase-1 were enhanced in UC patients and mice and in LPS-treated MIECs. Moreover, in UC mice and LPS-stimulated MIECs, the expression levels of these NLRP6 inflammasome genes were decreased, but VDR expression increased with VD$_3$ treatment, which indicated that VD$_3$-VDR signaling might be closely associated with NLRP6 inflammasome activation. The ATAC-seq and ChIP assays showed that VDR transcriptionally bound to VDRE in the NLRP6 promoter region, and the protein-protein association network indicated that VDR expression was negatively correlated with NLRP6 expression. Taken together, these results suggest that VDR transcriptionally represses NLRP6 expression and subsequently inhibits NLRP6 inflammasome activation. VDR-NRPL6 signaling might be responsible for the anti-inflammatory activity of VD$_3$ in UC.

Interestingly, VDR expression increased in *nlrp6*$^{-/-}$ mice and siRNA-NLRP6 MIECs, which suggested that NLRP6 suppressed VDR expression in mouse colons. The feedback loop regulation between VDR and NLRP6 expression may benefit NLRP6 upregulation under UC conditions. In addition, the expression levels of ASC and Caspase-1 decreased with NLRP6 deficiency. Thus, NLRP6 might exert key roles in inflammasome assembly and

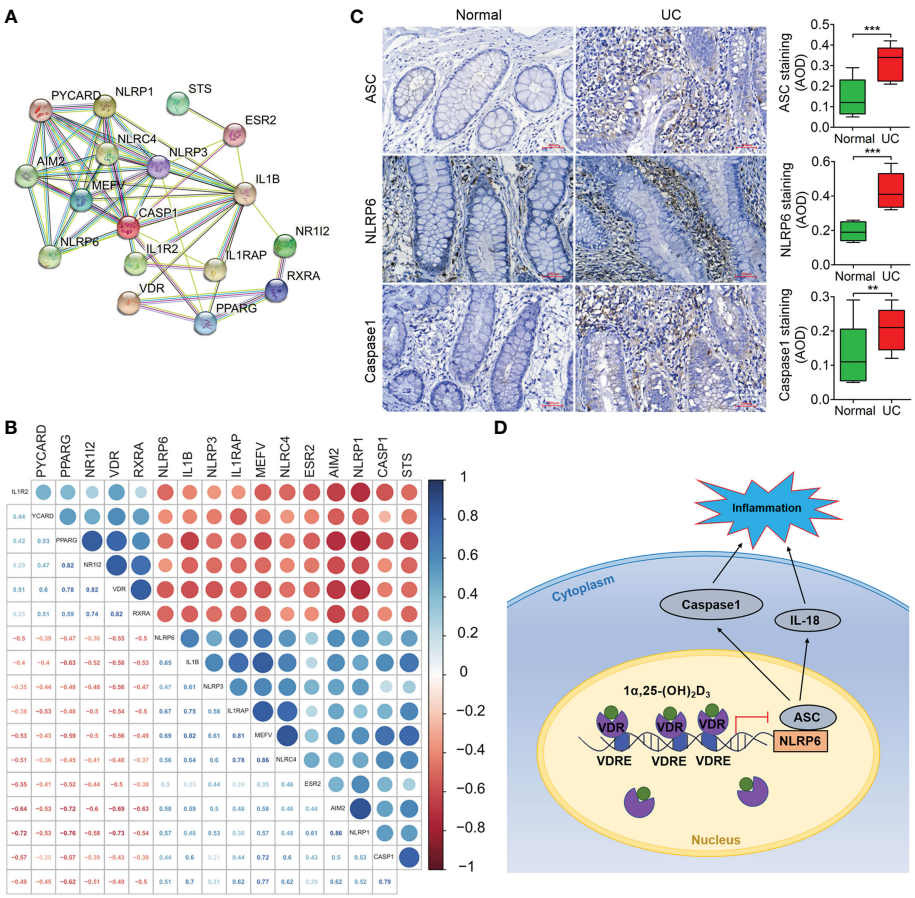

FIGURE 7
VDR expression is negatively correlated with NLRP6 expression and has the potential to be used for clinical prognosis prediction. **(A)** Protein–protein association network of the NLRP6-(Caspase-1)/IL-1β signalling pathway in STRING. VDR, NLRP6, ASC and Caspase-1 were selected as input. **(B)** Correlation of gene expression was analysed in GSE128682 ChiP data. The correlation coefficient ranges from –1 (red colour) to +1 (blue colour). The red region represents absolute negative correlations, afnd the blue region represents absolute positive correlations. Hclust, hierarchical clustering order. A value of 0.05 was chosen as the significance level. **(C)** Immunohistochemical analysis of ASC, NLRP6 and Caspase-1 expression in normal and UC tissue samples from representative patients. **(D)** Schematic illustration of VDR-NLRP6 signalling in MIECs. There are three VDREs in the promoter region of NLRP6. The transcription factor VDR transcriptionally represses NLRP6 by binding to the VDRE, reducing NLRP6 expression. The data are shown as the mean ± SD, n ≥ 3, *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.

activation. Recent research indicated that the disturbed interaction of NLRP6 with ASC prevented excessive inflammation (42).

In conclusion, we suggest that VDR might inhibit NLRP6 inflammasome activation by transcriptionally repressing NLRP6 expression in the animal intestine and that vitamin D exerts anti-inflammatory actions *via* VDR-NLRP6 signaling in UC development to maintain the stability of the intestinal mucosa (Figure 7D). Thus, this study clarified the molecular mechanism relating vitamin D protective roles in UC and accumulated evidence for the use of vitamin D in the clinical treatment of UC patients. Nonetheless, more investigations are still needed in the future, as the aetiological relationship between vitamin D and the onset of UC is far from clear.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA929335 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA929335).

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Xinjiang Medical University Affiliated Tumor Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of Xinjiang Medical University Affiliated Tumor Hospital.

Author contributions

HG performed the experiments, acquired the data and drafted the manuscript. HZ and JG revised the manuscript and analyzed and interpreted the data. ZZ and JL provided material and technological support. XL and HG conceived and designed the study, obtained funding and supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1135930/full#supplementary-material

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