

AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF VITAMIN D ON THE DIFFERENTIATION
OF TH17 CELLS

by

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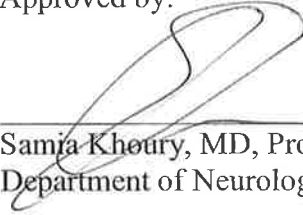
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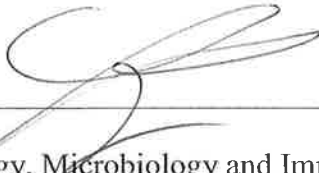
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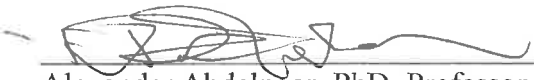
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AN ABSTRACT OF THE THESIS OF

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Title: The Effect of Vitamin D on the Differentiation of Th17 Cells

Background: Th17 cells play an important role in the pathogenesis of Multiple Sclerosis, a demyelinating neurodegenerative disease of the central nervous system, by the secretion of several pro-inflammatory cytokines. The active form of Vitamin D, 1 α ,25-dihydroxyvitamin D₃, has several anti-inflammatory properties. Studies have shown its negative effect on Th1 and Th17 responses and its positive effect on regulatory T-cells and Th2 cellular responses. The aim of this study was to investigate the effect of 1 α ,25-dihydroxyvitamin D₃ on the differentiation of naïve CD4⁺ T cells into Th17 cells *ex-vivo* in healthy controls, and to characterize the phenotype of Th17 cells and its modulation by vitamin D.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Isopaque density gradient centrifugation from collected blood samples or Leukopacks obtained from healthy donors. Naïve CD4⁺ T helper cells were isolated by microbead negative selection. The cells were then cultured in Th17 polarizing conditions in the presence of the cytokines: rIL-6, rTGF- β , rIL-1 and rIL-23, in the presence or absence of 1 α ,25-Dihydroxyvitamin D₃ at a 10 nM concentration. On the 6th day of culture, cells were stained with antibodies conjugated to different fluorochromes against IL-17, IFN γ , TNF α , IL-10, CCR6, CD161, CCR4, CD25, ROR γ t and Foxp3. The acquisition of stained cells was done on a fluorescence-activated cell sorter (FACS Aria III SORP) analyzer. The secretion of IL17, IFN γ , TNF α , IL-6, IL-2, IL-4, and IL-10 by the cells was quantitatively measured by ELISA and cytometric bead array (CBA).

Results: Results obtained from healthy controls showed an effect for 1 α ,25-dihydroxyvitamin D₃ on Th17 polarization as measured by the frequency of IL-17⁺ CD4⁺ T cells by flow cytometry. Vitamin D also decreased the frequency of ROR γ t⁺, IFN γ ⁺, TNF α ⁺, CCR6⁺, CD161⁺ and CCR4⁺ cells, all associated with the pro-inflammatory phenotype of Th17 cells. Conversely, vitamin D increased the frequency of Foxp3⁺, CD25⁺ and IL-10⁺ cells. There was a shift in the frequency of IL-17⁺ROR γ t⁺ towards IL-17⁺Foxp3⁺ double positive cells as well. ELISA and CBA results were consistent with flow cytometry findings; a decrease in IL-17, IFN γ and TNF α in culture supernatants was observed in the presence of vitamin D in contrast to an increase in levels of IL-4. No statistical changes were reported in the levels of IL-6, IL-2 and IL-10.

Conclusion: $1\alpha,25$ -dihydroxyvitamin D₃ has a direct negative effect on the differentiation of Th17 cells and its addition to CD4⁺ naïve T cells in culture, in Th17-polarizing conditions, decreases their pro-inflammatory phenotype and shifts it to a more regulatory one. Vitamin D supplementation trials would help us evaluate whether vitamin D should be implemented within treatment strategies for MS.

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CHAPTER I

INTRODUCTION

T helper cells play an important role in the adaptive immune response. Among the different T helper cell subsets already characterized, a new subset has recently emerged: T helper 17 cells (Th17 cells). Th17 cells normally play a protective role in the immune system by mediating an immune response against fungi and extracellular bacteria mainly through the recruitment of neutrophils to sites of inflammation. However, they are also considered to be pathogenic in a number of autoimmune diseases, such as Multiple Sclerosis (MS), through the secretion of several pro-inflammatory cytokines.

Multiple Sclerosis is a demyelinating neurodegenerative disease of the central nervous system (CNS). Risk factors for MS include genetic pre-disposition accounting for 30% of the risk and environmental factors. One of the environmental risk factors is vitamin D deficiency.

Vitamin D is a secosteroid hormone that is known to maintain mineral homeostasis and to regulate bone metabolism, but it also has immune-modulating activity and anti-inflammatory properties. Several reports have demonstrated a suppressive effect on Th1 immune responses and enhancing effects on regulatory T cells and Th2 responses. However, the role of Vitamin D on Th17 cells *ex-vivo* hasn't been fully investigated, although some reports suggest a suppressive effect on Th17 cell differentiation.

The aim of this study is to investigate the effects of the active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃, on the *ex-vivo* differentiation of CD4⁺ naïve human T cells

into Th17 cells and to characterize the phenotype of the differentiated T cells exposed to vitamin D.

CHAPTER II

LITERATURE REVIEW

A. Th17 cells

1. Different subsets of CD4⁺ T cells

The adaptive immune response is a fascinating phenomenon. It constitutes the body's third line of defense against pathogens and microorganisms and all its components work in a synchronized manner to fight infection and disease (1). In addition to B cells and cytotoxic T cells, another distinct subset exists: CD4⁺ T helper cells. Upon activation, naïve CD4⁺ T cells differentiate into different subsets depending on the local cytokine environment, each subset having its own particular phenotype, effector function and mechanism of action (2, 3).

Initially, two main different CD4⁺ T cell subsets were described: the T helper 1 cells (Th1) and T helper 2 cells (Th2) (4). However, several other T helper cell subsets have since been described as well: T helper 9 cells (Th9), T helper 22 cells (Th22) and T helper 17 cells (Th17) (5).

Th1 cells play a role in the cell-mediated immune response. They are known to produce significant amounts of pro-inflammatory cytokines such as Interferon-gamma (IFN- γ), interleukin 2 (IL-2), interleukin 3 (IL-3) and are responsible of activating macrophages in order to kill intracellular pathogens (2, 6). They have been associated for

many years with autoimmune diseases. Th2 cells, on the other hand, produce anti-inflammatory cytokines including interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 13 (IL-13) thus protecting against extracellular pathogens. This subset plays a role in the humoral immune response and is a mediator of antibody class switching in B cells as well (2, 7).

The less described T helper cell subsets, Th9 cells are known as interleukin-9 producing cells. Their physiological role is yet to be clearly determined despite reports suggesting a role in generating an immune response against helminthes and associating them with allergic reactions (8). Th22 cells, which mainly secrete interleukin-22, are thought to be implicated in both skin homeostasis, more specifically tissue repair and wound healing, and pathology (9). Furthermore, recent studies have demonstrated an inflammatory role of Th22 cells in autoimmune diseases (6).

Regulatory T cells (Tregs) are also a distinct $CD4^+$ T cells subset that is unique in terms of regulating the body's immune response in addition to suppressing immune responses against self antigens thus preventing autoimmunity and inducing self tolerance (10). Regulatory T cells are characterized by expression of the transcription factor forkhead box p3 (Foxp3) and the high expression of the extracellular marker CD25 (11).

2. Th17 cells overview

In 2005, a new door was opened in immunological research when a new $CD4^+$ T helper cell subset was identified as Interleukin-17 (IL-17) producing T cells, later named Th17 cells (12).

Th17 cells play an important role in the adaptive immune response by inducing the production of several pro-inflammatory cytokines, chemokines and metalloproteinases that coordinately mediate tissue inflammation (13, 14). They function in recruiting neutrophils to sites of infection as well as mediating immune responses against fungi and extracellular bacteria (15). Moreover, the interesting aspect of Th17 cells lies in the fact that this subset is able to fight pathogenic infections that Th1 or Th2 cells are usually unable to handle (14).

a. Role in the immune system

Th17 cells produce several effector molecules including IL-17A, IL-17F, Interleukin 21 (IL-21), Interleukin 22 (IL-22), Tumor necrosis factor- α (TNF- α), Interleukin 6 (IL-6), Interleukin 8 (IL-8) and CCL20 (14, 16).

No surface marker has been specifically identified for Th17 cells however, a combination of surface markers and chemokine receptors have been associated with Th17 cell phenotype. In fact, Th17 cells are characterized by the expression of the cluster of differentiation marker CD161 whose biological function is still not very clear (17). Th17 cells also co-express the chemokine receptor CCR6 (also named CD196) that binds the chemokine CCL20 and the chemokine receptor CCR4 (CD194) that binds several chemokines such as CCL4 and CCL22. Both receptors play a role in recruiting Th17 cells to target tissues (18). Furthermore, there has been several conflicting reports about the expression of the chemokine receptor CXCR3 (CD183) that binds CXCL9 and CXCL10 and plays an essential role in the migration of Th17 cells to sites of inflammation (16, 19).

IL-17A and IL-17F are the most studied cytokines of the IL-17 family that includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (14, 20). Both cytokines are genetically linked and have similar functions. IL-17 is a pro-inflammatory cytokine that coordinates and mediates tissue inflammation through several mechanisms (13). First of all, it acts on several non-immune cells including fibroblasts, epithelial cells and endothelial cells by inducing the secretion of pro-inflammatory mediators: Interleukin 6 (IL-6), IL-8, Granulocyte-macrophage colony-stimulating factor (GM-CSF) (16). It also increases the production of a number of chemokines (CXCL1, CXCL8, CCL20) and certain matrix metallo-proteinases, namely MMP3 and MMP13 (21).

Physiologically, IL-17 induces tissue infiltration and destruction by the action of the previously stated molecules (13). Th17 cells are involved as well in the activation, expansion and recruitment of neutrophils by regulating the GM-CSF and secreting IL-8, a potent chemoattractant of neutrophils (22). In fact, several studies have shown that mice over-expressing the IL-17 cytokine exhibited a strong neutrophil expression and tissue inflammation, in contrast to mice that were deficient in the IL-17 receptor and were found to be susceptible to bacterial infection due to a reduced neutrophil recruitment (13,22).

Another important effector molecule of Th17 cells, IL-22, acts on non-immune cells and promotes antimicrobial defenses by inducing the secretion of antibacterial proteins (23).

These different mechanisms of action as well as the different molecules secreted by Th17 cells make these cells important in generating an immune response against mostly Gram negative extracellular bacteria such as *Klebsiella pneumonia* and *Borrelia* spp. and more importantly, against systemic fungal infections such as *Candida albicans*, by

neutrophil recruitment to sites of infections and by inducing the secretion of antibacterial peptides (20, 24).

However, despite the protective role of Th17 cells in the immune system, Th17 cells are pathogenic in a number of autoimmune diseases such as multiple sclerosis, and this will be further expanded in the following chapters (15). This shift between the protective and pathogenic function of Th17 cells depends on successive differentiation stages (25).

b. Th17 cell differentiation

The differentiation of Th17 cells is regulated by a unique transcription factor named ROR γ T, a retinoid orphan nuclear receptor (26). The induction of this transcription factor is dependent on the signal transducer and activator of transcription 3 (STAT-3) that transduces signals from IL-6 and IL-21 activating cytokines. (25)

Another transcription factor that contributes to a lesser extent to the differentiation of Th17 cells is also a member of the retinoid nuclear receptor family, ROR α . In fact, ROR γ T and ROR α , in addition to other transcription factors still unidentified, all act in synergy to transduce extracellular signals and induce the differentiation of Th17 cells. (21)

The differentiation of T cells in general involves an interaction between antigen presenting cells (APCs), more specifically dendritic cells, and T cells: In fact, T cell differentiation is driven by immune signals provided by APCs. These signals are grouped into antigenic, co-stimulatory and cytokine signals. (25)

The first antigenic signal is the engagement of the T cell receptor (TCR) on T cells by the antigen/MHC complex on the APC. The differentiation of Th17 cells is affected by the concentration of the antigen in question. If the concentration of the antigen is high, IL-17 production is favored (25).

The second signal in the differentiation process involves the expression of several co-stimulatory molecules. A high antigen concentration will lead to a strong up-regulation of the co-stimulatory molecule CD40L by T cells and thus to a strong CD40-CD40L interaction between T cells and dendritic cells that enhances the production of IL-6. In turn, IL-6 further drives the differentiation process. (25, 27) Other co-stimulatory signals include the expression of CD80 (B7.1) and CD86 (B7.2) molecules by dendritic cells and their interaction with their receptor on T cells, CD28. This interaction transmits signals for T cell expansion and survival. In contrast, CTLA-4 also expressed by T cells can interact with the B7 molecules as well causing inhibition of Th17 cell differentiation (1, 2).

The third signal of differentiation is the most important step in defining the phenotype of the differentiated cell, depending on the specific cytokine milieu that is present (28) The differentiation of naïve CD4⁺ T cells into Th17 cells is initiated in the presence of immunoregulatory cytokine transforming growth factor beta (TGF- β) and pro-inflammatory cytokine IL-6 (29).

IL-1 β , a cytokine produced by dendritic cells, also plays a role in the early differentiation process (25). While IL-21 is produced by differentiating Th17 cells in an autocrine manner and propagates the differentiation in a positive feedback loop (26).

As for IL-23, it plays a role at a later stage when its receptor becomes expressed after stimulation with IL-6 and IL-21. It maintains and stabilizes the Th17 phenotype rather

than playing a direct role in its differentiation by driving the proliferation, expansion and survival of Th17 cells. As a result, IL-23 is indispensable for the pathogenicity of Th17 cells (21). In fact, several studies have shown that IL-23p19-deficient mice were completely resistant to the development of experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Conversely, T cells expanded by IL-23 were able to induce EAE when adoptively transferred into mice (14). These data underline the key role of IL-23 in controlling autoimmune diseases (30).

In contrast to positive polarizing cytokines, Th17 cell differentiation is negatively modulated by a number of cytokines as well. IL-27 is the most potent cytokine that limits the cells' differentiation and decreases their pathogenic auto-inflammatory roles. Its mechanism of action is defined by the induction of Interleukin-10 (IL-10) (25). IL-10 is an anti-inflammatory cytokine that plays an essential role in suppressing Th-17 induced auto-inflammation as well. Another mechanism of action of IL-27 is inhibiting ROR γ T and ROR α expression (21).

Other cytokines such as IL-4, IL-2 and IFN- γ that are secreted by T cells also contribute to the inhibition of Th17 cell differentiation (26).

c. Pathogenic and non-pathogenic Th17 cells

As previously discussed, Th17 cells are regarded as key player in the adaptive immune response. However, not all Th17 cells are pathogenic. Some Th17 cells are pro-inflammatory and significantly contribute to autoimmune inflammation, while other Th17 cells co-express IL-10 with IL-17 and are non-pathogenic. These two different Th17 cell

phenotypes are particularly distinguished from one another by the different secretion of a number of cytokines (28, 31).

The shift between pathogenic and protective Th17 cells depends on the cytokine milieu during naïve T cell differentiation. The major influencing factor is the amount of IL-23 versus TGF- β present: High doses of TGF- β decrease the expression of the IL-23 receptor thus resulting in a “classical” non-pathogenic Th17 phenotype. Alternatively, Th17 cells could be generated in-vitro in low amounts of TGF- β -signaling and presence of IL-23, but show more severe pathogenicity and were dubbed “alternative” Th17 cells (14). This has been confirmed by the fact that Th17 cells polarized in the presence of IL-6 and TGF- β without IL-23 were unable to induce disease when transferred into mice. However, cells that were polarized in the presence of IL-23 were able to induce EAE in mice (30).

The alternative Th17 cells have an interesting cytokine profile: In addition to IL-17A and IL-17F, they have been described as producers of IFN- γ as well. The IL-17⁺ IFN- γ ⁺ double positive cells have been found in the target tissues of various autoimmune diseases specifically in the central nervous system (CNS) of mice during the development of EAE (25, 32). The cytokine profile of pathogenic Th17 cells includes the pro-inflammatory cytokine TNF- α and IL-22 as well (22). Furthermore, GM-CSF production (induced by IL-23) has been associated with alternative Th17 cells and is also thought to be crucial to the pathogenicity of Th17 cells during EAE, especially that it stimulates antigen presenting cells to produce IL-23 in a positive feedback loop (33).

The classical more regulated Th17 cells produce IL-17A and IL-17F as well but are specifically characterized by their high production of IL-10, IL9 and IL-21. In fact, since IL-10 is an anti-inflammatory cytokine, the increased pathogenicity of alternative

Th17 cells has been associated with the decreased production of IL-10 by these subtypes. IL-10 is believed to reduce Th17-induced inflammation in the absence of IL-23 (28, 30 and 34).

All of these findings underline the importance of TGF- β and IL-23 in understanding the diversity of Th17 responses and their effect on shifting Th17 phenotypes towards classical and alternative phenotypes respectively (28).

d. The special relationship between Th17 cells and Tregs

As previously mentioned, Th17 cells differentiation is initiated by TGF- β and IL-6.

Interestingly, TGF- β alone induces the transcription factor Foxp3 in naïve T cells in the periphery, thus generating the immune suppressing regulatory T cells (Treg). However, when IL-6 is present along with TGF- β , it inhibits the induction of Foxp3⁺ cells and generates instead Th17 cells. IL-6 is therefore considered crucial in determining whether naïve T cells differentiate into Tregs or Th17 cells. That is why several studies have elaborated on a very tight and reciprocal relationship existing between inhibitory Treg cells and pro-inflammatory Th17 cells, in addition to a certain plasticity present between these two cell types (14, 21 and 25).

This relationship was further supported by results observed in IL-6 knockout mice which showed to have a defect in the generation of Th17 cells in contrast to an increased proportion of Foxp3⁺ T regs (14). Additional evidence of a balance between Th17 cells and regulatory T cells came from the findings that ROR γ t and Foxp3, the transcription factors

for Th17 cells and Tregs respectively, are able to physically bind to each other and neutralize each other's functions (21, 24).

In fact, it is believed that during the “steady state” in the absence of infection or inflammation, TGF- β is produced and induces the differentiation of Tregs by Foxp3 in order to maintain self-tolerance. However in case of infection, IL-6 (an acute phase reactant) is produced, and together with TGF- β , it inhibits the generation of Tregs by overcoming the suppressive effect of Foxp3 and shifts the balance towards Th17 cells in order to mount an immune response against the infecting organism. Conversely, cytokines produced by Tregs have been shown to have a regulatory effect on Th17 mediated inflammation (14, 34).

The plasticity of Th17 and Treg subsets has raised a major question regarding the dedifferentiation or reprogramming of committed Tregs or Th17 cells. Were this to be possible, it would present a major therapeutic avenue for autoimmune diseases, such as multiple sclerosis, by manipulating pathogenic Th17 cells and switch them to a more regulatory Foxp3⁺ phenotype (14).

B. Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating neurodegenerative autoimmune disease of the central nervous system (CNS) (35). It affects mainly young adults from the ages of 20 to 40 years of age and is considered to be T cell mediated since myelin-specific autoreactive CD4⁺ T cells are thought to play an essential role in orchestrating CNS inflammation (36). Multiple sclerosis is characterized by significant neurological disability mediated by

damage to myelin, the protective coating around nerve fibers in the CNS, oligodendrocyte destruction, and axonal loss (37).

1. Clinical forms and symptoms of MS

Multiple sclerosis is a multi-faced heterogeneous disease, both clinically and pathologically. In fact, it can be divided into four different clinical forms: relapsing-remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS) and progressive relapsing (PRMS) (38).

RRMS is the most common subtype of multiple sclerosis. Most patients (80 to 85%) start with RRMS that is characterized by defined clinical attacks followed by complete or incomplete remission phases where symptoms improve or even disappear. Most patients usually progress from RRMS to SPMS which can be described as a clinical worsening of neurological disability without remission periods and is refractory to treatment. On the other hand, PPMS accounts for 10% of patients and is characterized by a progressive disease course from onset without any remissions. It is considered to be resistant to current therapeutic strategies as well. As for PRMS, it is the rarest subtype of the disease, occurring in less than 5% of patients, and is characterized by clinical neurological worsening from the onset of the disease with some occasional relapses (38, 39).

Symptoms of multiple sclerosis could include any neurological disability. MS patients could suffer from mental changes such as decreased concentration, attention deficit or even memory loss, in addition to loss of sensation and swallowing, speech and

coordination difficulties. Other symptoms comprise of visual disturbances including blurred or double vision and color distortions, muscle spasms, fatigue, numbness sensations, limb weaknesses and loss of balance along with bladder and bowel dysfunction. Patients could suffer from uncontrolled mood swings as well such as severe depression and paranoia (36, 40).

2. Risk factors

Multiple sclerosis is thought to be caused by a complex interaction between genetic predisposition and a number of environmental factors (35).

a. Genetic risk factors

Although multiple sclerosis is not a hereditary disease, it occurs in people who are considered as genetically susceptible. In fact, family studies based on assessing the risk of MS amid relatives have shown an increased risk of developing MS among first-degree relatives (41).

Genetic epidemiological studies have revealed a number of risk alleles that are associated with MS. The HLA class II haplotype HLA-DRB1*1501 has shown a strong association with multiple sclerosis especially in northern Europe, in addition to HLA-DRB5*0101, HLA-DQA1*0102 and HLA-DQB1*0602 (39, 42). However, their association with MS could differ from one region to the other (35). Other genes in the non-HLA regions have also been implicated with MS risks and these include the genes encoding the interleukin-7 receptor α and interleukin-2 receptor α (42).

Ethnic origins have been thought to play a role in acquiring MS as well. As a matter of fact, people of African-American descent in addition to Chinese and Filipinos are considered to be less susceptible to disease development than other ethnicities (41).

Moreover, in addition to the previously stated risk factors, the fact that women are more susceptible to multiple sclerosis than men has also supported the association of genetic factors with MS, although studies have failed to find an actual risk allele on the X chromosome (41).

b. Environmental risk factors

It is true that genetic pre-disposition is needed to develop multiple sclerosis; however environmental triggers have been shown to contribute to the disease as well. The most common environmental factors known to play a role include viral and microbial infections, smoking and vitamin D deficiency (40, 42).

Potential infectious agents associated with MS comprise of bacterial agents such as *Chlamydia pneumoniae* (43) and viral agents such as Human herpesvirus 6 (HHV-6) and Epstein-Barr virus (EBV) (44, 45). Research is currently focusing on EBV as an MS risk factor due to the fact that approximately all MS patients (more than 99%) have been found to be seropositive for this particular virus. Additionally, people that present increased titers of anti-EBV antibodies such as antibodies against the EBV nuclear antigen 1 (EBNA1) have been shown to be more prone to disease development (41, 47).

Another non-infectious environmental MS risk factor is smoking. Several studies have demonstrated its dose-dependent relationship with MS risk. In fact, substances present in smoked tobacco have been shown to increase the risk of multiple sclerosis (48).

Even so, for the last 30 years the main focus has been on vitamin D deficiency as an important risk factor for multiple sclerosis (49). This was first supported by observations made about an increased MS prevalence in certain geographical areas of the world, notably in areas far from the equator (41). Researchers subsequently attributed this association of MS incidence with increasing latitudes to a decreased sunlight exposure and thus a decreased vitamin D status (50).

In fact, studies have shown that serum levels of 25-hydroxyvitamin D3 (25(OH)D) and 1,25-dihydroxyvitaminD3 (1,25(OH)₂D) were lower in MS patients compared to controls (51). Conversely, a study done on active-duty US military personnel demonstrated that increased levels of 25(OH)D lowered the risk of developing multiple sclerosis among white subjects alone, but not black or hispanic populations (52). Furthermore, a large epidemiological study done on women taking vitamin D supplementation revealed a 40% decrease in the risk of multiple sclerosis (53). Correlations have also been made between increased outdoor activities and sunlight exposure during adolescence, associated with high levels of 25(OH)D and 1,25(OH)₂D, and a decreased risk of disease development (50).

In addition to its role in preventing the occurrence of the disease, Vitamin D has an interesting effect on disease progression as well. In fact, vitamin D plays a prominent role in decreasing the rate of relapses in patients suffering from relapsing-remitting multiple sclerosis. This observation was supported by vitamin D supplementation trials which showed a decrease in the relapse rates in RRMS patients compared to the expected disease

course (54). Additionally, 25(OH)D concentrations were found to be lower during MS relapses than in remission phases which further confirmed the potential role of vitamin D in regulating disease progression and MS severity (55).

Since vitamin D appears to have such positive effects on several aspects of the disease simultaneously, researchers are currently attempting to use it as a potential therapeutic agent (56, 57).

3. Th17 cells and MS

Multiple sclerosis' pathogenesis has been extensively studied by its analogy with the animal model of the disease, experimental autoimmune encephalomyelitis (EAE) (58). In fact, the autoimmune hypothesis of MS states that the disease is the side effect of immune responses against non-self antigens such as viral and microbial agents. This side effect can be the result of either molecular mimicry or bystander activation (59).

Molecular mimicry involves antigens derived from infectious agents that share a structural homology with self antigens such as myelin and more famously myelin basic protein (MBP). This will cause initiation of an immune response against myelin proteins in the CNS and thus, CNS-mediated autoimmunity. On the other hand, bystander activation happens when an immune response against infectious agents leads to the activation of self reactive T cells that will in turn mount an immune response against myelin proteins. This will also contribute to the initiation of the disease (35, 59).

Another hypothesis implies that defects in immunoregulatory mechanisms and self tolerance, rather than the activation of self reactive T cells themselves, are behind disease development (39, 50).

a. Evidence of Th17 cell implication in MS

Autoreactive T cells specific for myelin antigen have been found in the peripheral blood and central nervous system (CNS) of MS patients (60). As a result, MS has been long thought to be a Th1-mediated autoimmune disease (61). However, after the discovery of Th17 cells in 2005, this theory was put into perspective (31, 37).

In fact, studies done on IL-12 knockout mice (the cytokine involved in the differentiation of Th1 cells) initially showed that these mice were protected from the induction of EAE. Nevertheless when the two subunits of IL-12, IL-12p35 and IL-12p40, were targeted separately only IL-12p40 knockout mice were protected from disease development while mice deficient in IL-12p35 were unprotected from the disease and even suffered from exacerbated symptoms. Later on it was shown that the IL-12p40 subunit can also bind the IL-23p19 subunit and together, they form the IL-23 cytokine which is involved in the differentiation of Th17 cells. When blocking separately IL-23, IL-12p40 and IL-23p19, all of the knockout mice were resistant to EAE which further confirmed that mice deficient in IL-23 were not susceptible to EAE whereas mice deficient in IL-12 were either susceptible or had an increased susceptibility to EAE (31, 62).

These findings refuted the initial theory of a Th-1 mediated disease and shifted the focus instead to the crucial role of IL-23 and Th17 cells' pathogenicity in autoimmune diseases (31, 37 and 62).

The current emphasis on Th17 cells in MS pathogenesis has emerged from the following observations: first of all, high levels of Interleukin-17 have been found in the peripheral blood, cerebrospinal fluid (CSF) and brain lesions of multiple sclerosis patients (63). High expression of IL-17 correlates with disease severity and with the number of active plaques on magnetic resonance imaging (MRI) (64). Pathogenic Th17 cells that co-produce IL-17A and IFN- γ together have also been reported in brain lesions of MS patients (65). Moreover, the percentage of Th17 cells in the CSF of RRMS patients was found to be higher during relapses compared to remission phases and to patients that have non-inflammatory neurological diseases (66).

Other cytokines related to Th17 cells, including IL-6 and IL-23, are also more abundant in the blood and brain lesions of MS patients compared to healthy controls (35).

In addition to evidence from patients, studies done on animal models have described a link between Th17 cells and the disease. In fact, IL-17-producing T cells generated in-vitro in the presence of IL-23 are able to induce severe EAE when adoptively transferred into mice (67). Conversely, mice deficient in IL-17 develop EAE with decreased severity and incidence (68).

b. Th17 cells' pathogenicity in MS

The main characteristic of multiple sclerosis is auto-inflammation and neurodegeneration of the central nervous system. Consistently, Th17 cells present a high inflammatory potency due to the fact that they express higher activation markers and adhesion molecules than any other CD4⁺ T cell subtype including Th1 cells. That is why the IL-23/IL-17/Th17 axis has been regarded as the most crucial aspect in the pathogenesis of MS (36, 39).

First of all, Th17 cells and Th17-associated cytokines such as IL-17A, IL-17F and IL-22 can target several cell types including myeloid cells, fibroblasts, epithelial cells and endothelial cells (14, 16). Because the IL-17 receptor (IL-17R) is ubiquitously expressed on various tissues, IL-17 is considered a pleiotropic cytokine that exerts potent pro-inflammatory activities (14, 21). Its main function is to breach the blood-brain-barrier (BBB) and transmigrate across it by simultaneously attracting other effector T cells and immune cells. In fact, the breakdown of the BBB is a successive process that requires the involvement of adhesion molecules, chemokines and matrix metallo-proteinases (36, 39).

The first step involves an interaction between adhesion molecules on endothelial cells such as the vascular-cell adhesion molecule 1 (VCAM-1) and adhesion molecules on lymphocytes such as the very late activation antigen-4 (VLA-4) (36). Next, IL-17 production induces the production of a number of matrix metalloproteinases (MMPs), most importantly MMP3, MMP13 and MMP9. The MMPs are proteolytic enzymes that are involved in attracting neutrophils to sites of inflammation by degrading the extracellular matrix and basement membrane. Consequently, they are implicated in the axonal and sustained myelin damage as well (13, 36). IL-17 also contributes to the pathogenesis of MS

by inducing the secretion of a wide range of chemokines. It induces the production of IL-8, CCL3 (major inflammatory protein (MIP)-1 α), CCL2 (monocyte chemoattractant protein (MCP)-1) and CCL20 (macrophage inflammatory protein (MIP)-3 α) (31, 65). All of these chemokines will bind chemokine receptors expressed on monocytes, macrophages and circulating lymphocytes and will recruit them from peripheral blood into the CNS (37). Furthermore, IL-17 itself is considered as a chemokine that can bind the IL-17R expressed on monocytes to enhance its migration (69).

IL-22, also produced by Th17 cells, has the ability to disrupt the BBB in combination with IL-17 by breaking tight junctions between endothelial cells (14, 16).

This process will ultimately lead to a neutrophil-mediated activation of MMPs, proteases and gelatinases that coordinately participate in the disruption of the BBB. Following the large infiltration of various inflammatory cells into the CNS, sustained myelin damage and axonal loss become inevitable under these circumstances (35, 36).

The cytokine profile of Th17 cells found in the brain lesions of MS patients includes the production of several pro-inflammatory cytokines along with IL-17A such as TNF- α , IL-6 and more importantly IFN- γ (14). This specific Th17 cell subtype of IL-17⁺ IFN- γ ⁺ double positive has been the main focus of researchers since it is thought to be a transient phenotype between Th17 and Th1 cells. No clear explanation has been elaborated so far for its existence. However, this phenotype of Th17 cells found in MS patients is in accordance with the “pathogenic” Th17 cells described in earlier paragraphs (14, 35).

c. Targeting Th17 cells in MS

After confirming the pathogenic role of Th17 cells in MS by several studies, multiple trials attempted to target this cell type for MS treatment.

A study done on mice to evaluate the therapeutic efficacy of IL-17 neutralization showed that the addition of an IL-17 neutralizing antibody to mice immunized with myelin antigen resulted in a decrease of the severity of EAE (70). Another study attempted to use microRNAs in order to block Th17 cells' differentiation. This also led to promising results where the knockdown of miR-326 (a microRNA involved in the differentiation of Th17 cells) alleviated EAE (71). Furthermore, a number of drugs currently used as therapeutic agents in MS were found to target Th17 cells (35, 39). That is why modulating the differentiation of Th17 cells by immune regulation has become a priority in managing multiple sclerosis.

C. Vitamin D

1,25-dihydroxyvitamin D₃, the biologically active form of vitamin D₃, is a secosteroid hormone that plays a role in regulating calcium homeostasis and bone metabolism.

However, it is extremely associated with other biological functions as well including the modulation of immune responses (50, 56).

1. Sources and mechanism of action of vitamin D

Vitamin D₃ is derived from two different sources: photosynthesis in the skin and, to a more limited extent, dietary intake. In fact, when the skin is exposed to ultraviolet (UV) light, it converts 7-dehydrocholesterol in the skin into pre-vitamin D₃ which in turn spontaneously isomerizes into vitamin D₃. Dietary sources are dairy products and fatty fish that are rich in vitamin D₃ (57, 72).

Vitamin D₃ from both sources is then hydroxylated in the liver by the enzyme 25-hydroxylase to become 25-hydroxyvitamin D₃ (25(OH)D). 25(OH)D is the major circulating form of vitamin D and is the metabolite measured in order to assess the vitamin D status of patients. Next, 25(OH)D is further hydroxylated in the kidney by the enzyme 1 α -hydroxylase to become the active form of vitamin D 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)D). This is the metabolite responsible of the biological properties of vitamin D₃ (56, 73).

1,25(OH)D mediates its functions through the vitamin D receptor (VDR) which is a member of the superfamily of nuclear hormone receptors (74). Upon cytoplasmic binding of 1,25(OH)D to VDR, the receptor undergoes conformational changes and heterodimerizes with the retinoid X receptor (RXR). The 1,25-dihydroxyvitamin D-VDR-RXR complex binds then to vitamin D response elements (VDRE) found in the promoter region of vitamin D responsive genes and recruits nuclear receptor co-regulators. Co-activators and co-repressors will then either enhance or inhibit the transcription of the target gene, respectively (50, 75). This process was found to affect the function and phenotype of numerous immune cells, and this will be further elaborated in the following pages (76).

2. Vitamin D and the immune system

It wasn't until the 1980s that vitamin D was discovered to have immune modulating abilities. In fact, this observation was made when VDR expression was first discovered in monocytes and then later on in activated T lymphocytes and antigen presenting cells such as dendritic cells. The fact that VDR expression was also found to be present in the central nervous system in certain cell types along with the enzyme 1α -hydroxylase further supported this hypothesis. Contrary to the liver and kidney, this enzyme is not regulated by a negative feedback mechanism and is thus activated extensively in the metabolism of vitamin D (57, 77).

In fact, vitamin D can be catabolized in the central nervous system as well as throughout the body in order to mediate its immune potential (57).

a. Vitamin D and autoimmune diseases

As reported in the previous chapters, vitamin D deficiency is strongly associated with an increased risk of autoimmune diseases, notably multiple sclerosis (50, 76).

The normal plasma level of 25-hydroxyvitamin D can reach the value of 90-100 nmol/L for optimal bone metabolism; however it is thought that this metabolite is needed at much higher levels in order to exert its other functions. In all cases, the reported plasma level of 25(OH)D in patients suffering from autoimmune diseases is lower than in healthy controls (50, 76).

The active form of vitamin D₃, $1\alpha,25$ -dihydroxyvitamin D, is found in the plasma at a physiological level of 100-150 pmol/L in healthy individuals. This metabolite has not

been extensively measured in MS patients since the vitamin D status is normally assessed using the 25(OH)D form and not 1,25(OH)D (54).

Nevertheless, 1,25(OH)D is needed at higher levels locally for it to affect the phenotype and effector functions of immune cells. This supraphysiological level can be obtained by the conversion of vitamin D₃ precursors into its active form at a cellular level, due to the presence of the 1 α -hydroxylase enzyme locally and in the CNS (56, 57).

Several studies have been done on animal models in order to support the hypothesis of 1,25(OH)D's implication in MS. In fact, mice that were exposed to ultraviolet light, and thus have high levels of 1,25(OH)D, were found to be protected from EAE when immunized with myelin antigen. Other studies have shown that giving vitamin D supplementation to mice during immunization with myelin components alleviated the symptoms of EAE. Conversely, in VDR knockout mice, none of the previously stated effects took place and vitamin D was unable to have protective effects against EAE. This was also the case in mice that were given a vitamin D-free diet during induction of EAE and were shown to have more severe symptoms (76, 78).

b. Vitamin D's effect on immune cells

Vitamin D's direct effect on several specific immune cells has been described in previous studies. In fact, the targets of vitamin D include antigen presenting cells, like dendritic cells and macrophages, and T lymphocytes (40, 56).

Vitamin D was found to inhibit the proliferation of dendritic cells and decrease their expression of major histocompatibility complex II (MHC II) and of co-stimulatory

molecules, thus decreasing their ability to provide secondary signals for T cells activation and differentiation. Moreover, it decreases the expression of both pro-inflammatory cytokines IL-12 and IL-23 by APCs which are involved in the differentiation of Th1 cells and Th17 cells respectively whereas the expression of the anti-inflammatory cytokines IL-4 and IL-10 are increased. This highlights the indirect effect of vitamin D in shifting the immune phenotype from pro-inflammatory Th1 and Th17 cells to anti-inflammatory Th2 cells and regulatory T cells (56, 57 and 79).

Resting T lymphocytes express low levels of vitamin D, but when activated their expression of the receptor is significantly increased which allows vitamin D to exert strong effects on this particular subtype. This hypothesis has been extensively studied in in-vitro models where naïve or memory CD4⁺ T cells were put in culture in Th1, Th2 or regulatory T cell polarizing conditions. These different studies have emphasized the fact that vitamin D inhibits the effector function of Th1 cells by decreasing their expression of several cytokines such as IL-2 and IFN γ . Conversely, vitamin D was found to enhance regulatory T-cells' activity by increasing the secretion of the anti-inflammatory cytokine IL-10 and to improve Th2 cell responses by increasing the secretion of IL-4, IL-5 and IL-10 through the VDR signaling pathway (56, 76 and 80).

The effect of vitamin D on Th1 cells, Th2 cells and Tregs has been the focus of many studies. However, the effect of vitamin D on Th17 cells hasn't been fully described yet. There has been a limited number of studies that showed a decrease of the expression of IL-17 in naïve CD4⁺ T cells put in Th17 polarizing conditions together with a decrease in other pro-inflammatory cytokines related to Th17 cells such as IL-22 and in matrix-metalloproteinases including MMP-1 and MMP-3. A decrease in surface markers such as

CCR6 has also been described. But the effect of vitamin D on Th17 cells still lacks a broader and much more detailed description (81- 83 and 92).

CHAPTER III

MATERIALS AND METHODS

A. Control subjects and blood collection

Three healthy participants, 2 males and 1 female, were recruited for blood sample collection after obtaining the approval of the Institutional Review Board (IRB). The participants' ages ranged from 19 to 28 years old. All participants signed a consent form prior to their enrollment to the study. The consent form clearly stated that blood up to 8 tablespoons will be drawn from the volunteer and will be used as a normal control. The subjects were notified that the participation is voluntary and could be discontinued freely at any time they decide to end it.

Three different leukopack male donors were obtained from the American University of Beirut Medical Center's Blood Bank as well. Blood was tested prior to collection to confirm that the participants are healthy controls. The participants' ages ranged from 22 to 34 years old.

Blood from both healthy donors and leukopacks were collected in Vacutainer lithium heparin tubes (BD Biosciences, Franklin Lakes, NJ, USA). The steps following blood collection, including blood processing and cell culture, were all performed in sterile conditions under a biosafety cabinet (BSC).

B. Th17 cell polarization *ex-vivo*

1. Isolation of Peripheral blood mononuclear cells (PBMCs)

The isolation of PBMCs was done by the Ficoll-Isopaque density gradient centrifugation technique. Collected Blood was transferred to a 50 mL falcon tube (Corning, NY, USA) and was diluted with Dulbecco's Phosphate Buffer Saline (PBS) modified, without calcium chloride and magnesium chloride, suitable for cell culture (Sigma-Aldrich, St. Louis, MO, USA). Ficoll-Paque Plus (GE Healthcare, SE-751 84 Uppsala, Sweden) was added to a 50 mL falcon tube at a Ficoll to blood 1:2 ratio and blood was layered gently on the ficoll. The tube was then centrifuged at 800g for 30 minutes with the brake off (Acceleration=1; Deceleration=1). The buffy coat was collected and transferred to a 50 mL falcon tube and washed with PBS. The tube was centrifuged at 300g for 15 minutes. The supernatant was discarded and the pellet was resuspended in PBS.

Alive peripheral blood mononuclear cells (PBMCs) were counted by the trypan blue 0.4% (Sigma-Aldrich, St. Louis, MO, USA) exclusion method using the Neubauer Chamber. The centrifugation step was then repeated at 300g for 15 minutes and depending on the cell count, the pellet was resuspended in Fetal Bovine Serum (FBS) heat inactivated, sterile-filtered, suitable for cell culture (Sigma-Aldrich, St. Louis, MO, USA) and flash freezing media constituted of 80% FBS and 20% dimethyl sulfoxide (DMSO) sterile-filtered (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10×10^6 cells/ mL. The cell suspension was then distributed on 2 mL cryogenic vials (Corning, NY, USA) in order to have 10 million cells per cryovial. The vials were put in a -80 °C freezer overnight and then transferred the next day to a liquid nitrogen tank at -196 °C to be stored for later usage.

2. Naïve CD4⁺ T cells isolation

Naïve CD4⁺ T cells isolation was performed by microbead negative selection on a magnetic column using the Naïve CD4⁺ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The principle of this separation consists of magnetically depleting non-T helper cells and memory CD4⁺ T cells by indirectly labeling them with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and Anti-biotin Microbeads, as secondary labeling reagent. The magnetically labeled non-T helper cells and memory CD4⁺ T cells are depleted by retaining them on a column in the magnetic field, while the unlabeled naïve CD4⁺ T cells pass through the column.

In order to isolate the naïve CD4⁺ T cells from the PBMCs, depending on the number of cells needed to put in culture, a number of cryovials was taken from the liquid nitrogen tank. The frozen PBMCs were semi-melted by shaking the cryovials in a water bath set at 37°C. The cells were washed with PBS+5% FBS and centrifuged at 1200 rpm, 22°C, for 15 minutes. The supernatant was discarded without disturbing the pellet and the pellet was resuspended in PBS+5% FBS. This step was repeated 3 times in order to remove all traces of freezing media from the cells. Cells were then resuspended in EDTA 2 mM (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes to remove any cell clumps and obtain a single cell suspension prior to the magnetic separation. The cells were then counted by the trypan blue exclusion method using the Neubauer chamber.

According to the manufacturer's instructions, cells were centrifuged at 300g for 10 mins and resuspended in a buffer especially prepared for the magnetic separation containing PBS+0.5% FBS+2 mM EDTA. Depending on the cell count, 40 µL of buffer

was added for each 10^7 cells with 10 μ L of Biotin-Antibody Cocktail (Cocktail of biotinylated CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCR γ/δ , anti-HLA-DR, and CD235a (glycophorin A) antibodies) per 10^7 cells as well, directed against memory CD4⁺ T helper cells and non-T helper cells. The suspension was mixed thoroughly and incubated for 10 minutes at 4°C. Cells were washed with 5 mL buffer and centrifuged at 300g for 10 minutes. The pellet was resuspended in 80 μ L of buffer for each 10^7 cells with 20 μ L of Anti-Biotin microbeads (microbeads conjugated to a monoclonal anti-biotin antibody) per 10^7 cells as well. The suspension was mixed thoroughly and incubated for 15 minutes at 4°C. The cells were then washed with 5 mL buffer and centrifuged at 300 g for 10 minutes and resuspended in 0.5 mL buffer, regardless of the cell count.

In order to prepare the column prior to the separation, an LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) was placed in the magnetic field of a MidiMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and was rinsed with 3 mL buffer. The cells suspension was then applied onto the column and the effluent was collected as fraction with unlabeled cells. This constituted the enriched naïve CD4⁺ T cells fraction. The column was further washed with 9 mL buffer and the effluent was collected in the same tube. This represents the unlabeled naïve CD4⁺ T cells as well.

Cells were then centrifuged at 300 g for 10 minutes and counted. Depending on the cell count, the pellet was resuspended with RPMI (Gibco Life technologies, Carlsbad, CA, USA) in the presence of 10% FBS+1% Penicillin Streptomycin in order to have a 2×10^6 cells/mL concentration. The cells were left to rest in the incubator for one hour.

3. Evaluation of naïve CD4+ T cells purity

The evaluation of the purity of naïve CD4+ T cells obtained by the magnetic microbead selection was evaluated by flow cytometry. Prior to incubation, 50×10^3 cells were taken from the fraction of naïve CD4+ T cells obtained by negative selection. The cells were centrifuged at 1400 rpm, 4°C for 8 minutes and resuspended in 100 µL staining buffer (PBS+1% FBS) in the presence of fluorochrome conjugated-antibody against CD4, PerCP-antiCD4 (BD Biosciences, Franklin Lakes, NJ, USA). The suspension was incubated for 30 minutes at 4°C and then washed twice with staining buffer in the same centrifugation conditions. After the second wash, the pellet was resuspended in 200 µL staining buffer prior to flow cytometry acquisition.

The cells were then acquired on a fluorescence activated cells sorter, BD FACSAria™ III (BD Biosciences, Franklin Lakes, NJ, USA) to determine the percentage and purity of CD4+ T cells present.

4. Culture and activation of naïve CD4+ T cells

After the incubation time was over, the naïve CD4+ T cells were centrifuged at 1200 rpm for 15 minutes and resuspended in serum free media X-Vivo 15 (Lonza, Basel, Switzerland). The cells were cultured in a 96-well plate (Corning, NY, USA) by adding 5×10^4 cells per well and activated using the T cell activation/expansion kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany) which consists of beads coated with anti-CD2, anti-CD3 and anti-CD28 at a 1:4 beads to cell ratio in each well.

The following recombinant human cytokines were added to each well under Th17 polarizing conditions: IL-6 (50ng/mL), TGF- β (2ng/mL), IL-1 β (10 ng/mL) and IL-23 (20ng/mL) (R&D Systems, Minneapolis, MN).

The active form of Vitamin D, 1,25-dihydroxvitamin D3 (Enzo Life Sciences Inc., Farmingdale, New York), was added at a 10 nM concentration in the indicated wells. The optimal concentration of vitamin D used was chosen after several optimization experiments using the same protocol of Th17 polarization *ex-vivo* in the presence of 1 nM, 10 nM and 100 nM of vitamin D to determine the best effect of vitamin D with the lowest concentration possible.

On day 3 of culture, recombinant human IL-2 (8ng/mL) (R&D Systems, Minneapolis, MN) was added to each well.

C. Flow cytometry staining

On day 6 of culture, cells to be stained intracellularly were restimulated with phorbol 12-myristate 13-acetate PMA (Sigma-Aldrich, St. Louis, MO, USA) at a 50 ng/mL concentration in the presence of calcium ionophore ionomycin (Invitrogen Life technologies, Carlsbad, CA, USA) at a 1 μ g/mL concentration and protein transport inhibitor, brefeldin A, Golgi plug (BD Biosciences, Franklin Lakes, NJ, USA) at a ratio of 1 μ L of brefeldin A to 1 mL of cell suspension.

The cells were left in the incubator at 37 °C in restimulating conditions for 5 hours prior to the intracytoplasmic staining.

1. Staining of live cells

After incubation time was over, cells were harvested and transferred to a V-bottom 96-well plate (Corning, NY, USA) and were centrifuged at 1400 rpm, 4°C for 8 minutes. All the following centrifugation steps were done in the same conditions. The supernatant was discarded and the pellet was resuspended in EDTA (2mM) to remove all cells clumps and incubated for 10 minutes. Cells were then pelleted and stained with LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen Life technologies, Carlsbad, CA, USA) in the presence of PBS at 1 µL dye per 1 mL cell suspension concentration and incubated on ice for 30 minutes, according to the manufacturer's instructions. This stain is used to evaluate the viability of cells by flow cytometry by differentiating live cells from dead ones. It is based on the reaction of a fluorescent reactive dye with cellular amines. The reactive dye can permeate the compromised membranes of dead cells and react with free amines both in the interior and on the cell surface, resulting in intense fluorescent staining. In contrast, only the cell-surface amines of viable cells are available to react with the dye, resulting in relatively dim staining. The cells were pelleted and washed twice with staining buffer (PBS+1% FBS).

2. Extracellular staining of surface markers

The cells were then resuspended in 100 µL staining buffer per well and stained extracellularly in the presence of the following fluorochrome-conjugated antibodies, depending on the staining conditions: FITC-anti-CD161, PE-anti-CCR6, Alexa Fluor-700-anti-CD25, Brilliant Violet 450-anti-CCR4 extracellular markers. Cells were incubated for

30 minutes at 4 °C and then pelleted and washed twice with staining buffer in order to remove any residual unbound fluorochrome-conjugated antibody.

3. *Intracytoplasmic staining of cytokines and transcription factors*

After extracellular staining was completed, the protocol was proceeded in order to stain the cells intracellularly. For that, the cells were fixed by resuspending thoroughly the pellet with 100 µL per well of the BD Cytotfix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ, USA) for 20 mins at 4°C. This solution is used for the simultaneous fixation and permeabilization of cells prior to the intracytoplasmic staining. They were then pelleted and washed twice with the 1× BD Perm/Wash™ buffer (BD Biosciences, Franklin Lakes, NJ, USA). The pellet was resuspended in 50 µL of the 1× BD Perm/Wash™ buffer in the presence of the following fluorochrome-conjugated antibodies, depending on the staining conditions: Alexa Fluor 647-anti-IL-17A, Brilliant Violet 450-anti-IFN γ or FITC-anti-IFN γ , PE-anti-TNF α , cytokines and PE-anti-ROR γ t transcription factor for subtyping the Th17 pro-inflammatory phenotype and PE-anti-IL-10 cytokine and PE-anti-Foxp3 transcription factor for checking for a more anti-inflammatory phenotype. The cells were incubated at 4 °C for 40 minutes, and then pelleted and washed twice with the 1× BD Perm/Wash™ buffer in order to remove any residual unbound fluorochrome-conjugated antibody. After the last centrifugation step, the cells were resuspended in 200 µL staining buffer per well for their acquisition by flow cytometry.

The volume of all antibodies used in the intracellular staining was done according to the manufacturer's instructions, except for Alexa Fluor 647-anti-IL-17A. The optimal

volume of Alexa Fluor 647-anti-IL-17A used was chosen carefully after several optimization experiments using the same protocol of Th17 polarization *ex-vivo* in the presence of a serial 3 fold-dilution of the antibody in order to get the best possible positive signal, considering that the concentration of the IL-17 cytokine found in culture is low.

The cells were then acquired on a fluorescence activated cells sorter, BD FACSAria™ III Special Order Research Product (SORP).

4. Negative controls

Cells that were not activated with beads on the first day of culture, or that were activated with beads without Th17 polarizing conditions were used as negative controls for the staining of the different fluorochrome conjugated-antibodies. An unstained negative control for cells that were activated with beads in Th17 polarizing condition was used as a negative control as well.

Moreover, in order to discriminate the specific staining of Alexa Fluor 647-anti-IL-17A from an artifactual staining, a ligand blocking control was performed. This was done to confirm the specificity of the low staining signal that was found after acquisition of the cells on the flow cytometer. Alexa Fluor 647-anti-IL-17A fluorochrome conjugated antibody was pre-incubated with human IL-17A recombinant cytokine (eBioscience Inc, San Diego CA 92121, USA) at a 0.1 mg/mL concentration and incubated for 30 minutes at 4 °C. After incubation, the fixed/permeabilized cells, prepared as stated previously, were resuspended in 50 µl of the pre-blocked labeled anti-cytokine antibody and incubated for 30

min at 4°C. The cells were then washed and steps were preceded as mentioned in the intracellular staining protocol.

5. Compensation beads

Because the staining protocol involves several multicolor staining panels, correction of spectral overlap between different channels was accomplished by software compensation using compensation beads or single stained controls. The compensation beads were prepared using the AbC™ Anti-Mouse Bead Kit and the ArC™ Amine Reactive Compensation Bead Kit (Invitrogen Life technologies, Carlsbad, CA, USA). The kits provided capture beads as a positive stained control and negative beads as a negative unstained control.

According to the manufacturer's instructions, sample tubes were labeled for each fluorochrome-conjugated antibody and one drop of the reactive capture beads was added to each labeled tube. A pre-titrated amount of each fluorochrome conjugated antibody was then added to the appropriate tube and mixed. The beads were incubated at room temperature for 30 minutes. After incubation time was over, 3 mL of PBS was added to each tube and the tubes were centrifuged at 300g for 5 minutes to collect the beads. The supernatant was discarded and the bead pellet was resuspended in 0.5 mL PBS. One drop of negative beads was then added to each tube containing the capture beads. The tubes were vortexed thoroughly prior to flow cytometry acquisition.

The beads were then acquired as compensation controls prior to the stained cells on a fluorescence activated cells sorter, BD FACSAria™ III (BD Biosciences, Franklin Lakes, NJ, USA).

D. Enzyme-Linked Immunosorbant Assay (ELISA)

The Enzyme-linked immunosorbant assay (ELISA) was used in order to quantitatively measure Interleukin 17-A (IL-17A) production in culture supernatants of the naïve CD4⁺ T cells put in Th17 polarizing conditions in the presence or absence of 1,25-dihydroxyvitamin D₃. This procedure was done on the cultures supernatants of 6 different donors.

All culture supernatants were obtained after 6 days of culture prior to flow cytometry staining of cells, in order to investigate the cumulative secretion of IL-17A by the cells throughout the 6 days. They were initially frozen at -20°C and then thawed on the day the ELISA was performed. The kit used is the Human IL-17A (homodimer) ELISA Ready-SET-Go!® (eBioscience Inc, San Diego CA 92121, USA). All steps in the procedure were performed according to the manufacturer's instructions.

The kit provided the following components: capture antibody, standards, biotin-conjugated detection antibody, ELISA coating buffer, assay diluent, pre-titrated Avidin-Horseradish peroxidase (Avidin-HRP) detection enzyme, Tetramethylbenzidine (TMP) substrate solution and a 96 well plate. The wash buffer was prepared using 1X PBS and 0.05% Tween-20. The stop solution was prepared by diluting phosphoric acid (H₃PO₄) 14.8 M to a 1 M concentration.

- The ELISA 96 well plate was coated with 100 μL /well coating buffer. The plate was sealed and incubated overnight at 4°C. The coating buffer was prepared by diluting 1.25 mL 10X coating buffer to a final volume of 12.5 mL double distilled water.
- On the next day, the plate was washed 3 times with the wash buffer and blotted each time on absorbent paper to remove any residual buffer.
- The wells were blocked with 1X assay diluent and incubated at room temperature for 1 hour. 1X assay diluent was prepared by diluting 10 mL of 5X assay diluent to a final volume of 50 mL double distilled water.
- Standard dilutions were prepared in 8 different 12 \times 75-mm polypropylene tubes (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.625 pg/mL, 7.8125 pg/mL, 3.90625 pg/mL) to make the standard curve for a total of 8 points. The top concentration of the standard dilution was prepared by adding 10 μL of standard to a final volume of 20 mL 1X assay diluent (500 pg/mL). 250 μL of assay diluent was then added to the each of the other 7 tubes. A 2-fold serial dilution of the top standard was performed by transferring 250 μL of the top standard to the second tube and so on until the eighth tube. A ninth tube served as a blank zero standard with a concentration of 0 pg/mL.
- 100 μL of each standard concentration and 100 μL of each sample were added to the appropriate wells. Each sample and standard dilution was run in

duplicates. The plate was then sealed and incubated at room temperature for 2 hours.

- After incubation, the plate was washed 5 times with the wash buffer and blotted each time on absorbent paper to remove any residual antigen.
- 100 μL of diluted detection antibody was added to each well and the plate was incubated for 1 hour at room temperature. The detection antibody was diluted by adding 48 μL of the antibody to a final volume of 12 mL 1X assay diluent
- After incubation, the plate was washed 5 times with the wash buffer and blotted each time on absorbent paper to remove any residual detection antibody.
- 100 μL of diluted Avidin-HRP was added to each well and the plate was sealed and incubated for 30 minutes at room temperature. The Avidin-HRP was diluted by adding 48 μL of enzyme to a final volume of 12 mL 1X assay diluent.
- After incubation, the plate was washed 7 times with the wash buffer and blotted each time on absorbent paper to remove any residual enzyme.
- 100 μL of substrate solution was added to each well and the plate was incubated for 15 minutes at room temperature in the dark,
- After incubation, 50 μL of stop solution was added to each well.
- The absorbance was read at 450 nm using the Bio-Tek/ELx800 micro-plate reader.

E. Cytometric Bead Array (CBA)

The Cytometric bead array (CBA) was used in order to quantitatively and simultaneously measure interleukin 17-A (IL-17A), tumor necrosis factor (TNF), interferon- γ (IFN- γ), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-2 (IL-2) levels in culture supernatants of the naïve CD4⁺ T cells put in Th17 polarizing conditions in the presence or absence of 1,25-dihydroxyvitamin D₃. This procedure was done on the cultures supernatants of 3 different donors.

All culture supernatants were obtained after 6 days of culture prior to flow cytometry staining of cells, in order to investigate the cumulative secretion of these cytokines by the cells throughout the 6 days. Culture supernatants were initially frozen at -20°C and then thawed on the day the CBA was performed. The kit used is the Cytometric Bead array Human Th1/Th2/Th17 cytokine kit (BD Biosciences, Franklin Lakes, NJ, USA). All steps in the procedure were performed according to the manufacturer's instructions.

The kit provides a method to capture a set of cytokines with beads of known sizes and fluorescence and thus makes it possible to detect the cytokines using flow cytometry. Each capture bead and detection antibody will form a sandwich complex with the cytokine and give a fluorescent signal in proportion to the amount of cytokines present in the culture supernatants.

The kit provided the following components: human IL-2 capture beads, human IL-4 capture beads, human IL-6 capture beads, human IL-10 capture beads, human TNF capture beads, human IFN- γ capture beads, human IL-17A capture beads, Human

Th1/Th2/Th17 PE detection reagents, Human Th1/Th2/Th17 cytokine standards, cytometer setup beads, wash buffer and assay diluent.

- Standard dilutions were prepared in 9 different 12 × 75-mm polypropylene tubes (5000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 312.5 pg/mL, 156.25 pg/mL, 78.13 pg/mL, 39.06 pg/mL, 19.53 pg/mL) to make the standard curve for a total of 9 points. The top concentration of the standard dilution was prepared by reconstituting standard spheres from lyophilized Human Th1/Th2/Th17 standards with 2 mL of assay diluents (500 pg/mL). The reconstituted standard was left 15 minutes at room temperature to let it equilibrate.
- 300 µL of assay diluent was then added to the each of the other 8 tubes. A 2-fold serial dilution of the top standard was performed by transferring 300 µL of the top standard to the second tube and so on until the ninth tube. A tenth tube served as a blank zero standard with a concentration of 0 pg/mL.
- The different capture beads corresponding to each cytokine (IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , IL-17A) were then mixed together. Each capture bead suspension was vigorously vortexed and a 10 µL aliquot of each capture bead, for each assay tube, was taken and added into a single tube.
- The mixed capture beads tube was vortexed and 50 µL is added to all assay labeled tubes.
- 50 µL of the Human Th1/Th2/Th17 cytokine standard dilutions was added to the corresponding control tube.
- 50 µL of each unknown sample was added to the appropriately labeled sample tube.

- 50 μ L of the Human Th1/Th2/Th17 PE detection reagent was added to all assay tubes.
- The assay tubes were then incubated for 3 hours at room temperature protected from light.
- After incubation time was over, 1 mL of wash buffer was added to all assay tubes and the tubes were centrifuged for 5 minutes at 200g.
- The supernatants were aspirated and discarded from each tube.
- Each bead pellet was resuspended with 300 μ L wash buffer.
- The sample tubes were then acquired on the fluorescence activated cells sorter, BD FACSAria™ III.

Prior to flow cytometry acquisition, instrument setup was performed using the Cytometer setup beads. This was done by adding 100 μ L of cytometer setup beads to a 12 \times 75-mm sample acquisition tube with 400 μ L of wash buffer. The tube was then vortexed to gently mix the contents. The tube was acquired on the fluorescence activated cells sorter, BD FACSAria™ III in order to adjust photomultiplier tube (PMT) settings in preparation for running the BD CBA assay using BD FACSDiva software.

F. Flow cytometric analysis

All samples were acquired on a fluorescence activated cells sorter, BD FACSAria™ III after setting the PMT voltages of the Forward and Side Scatter in addition to the voltages of

each conjugated fluorochrome. The parameters were collected as logarithmic signals. In total 10 000 events were measured from each sample.

The obtained data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

G. Cytomeric bead array analysis

The obtained CBA data was analyzed using the **FCAP Array™** Software.

H. Statistical analysis

Whenever applicable, data were presented as means \pm standard deviations. Data was normalized and statistical differences in the results were determined by a two-tailed paired Student's t-test using Graphpad Prism 5.0 statistical software (Graphpad Software Inc, La Jolla, CA, USA). P values <0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Flow cytometry

1. Gating strategy

Sequential gating was done in order to analyze the stained cells. In all flow cytometric analyses, dead cells were first excluded from the gate on the basis of the Live/Dead stain kit by gating on the live cells which are more dimly stained than the dead cells (Figure 1A). Then, T cells were gated based on forward scatter (FSC-A) related to size, and side scatter (SSC-A) related to granularity in order to exclude any cellular debris from the gating (Figure 1B). Finally, cells were analyzed depending on the fluorochromes in question (Figure 1C).

2. Effect of Vitamin D on the viability of cells

Naïve CD4⁺ T helper cells (96% purity after isolation from PBMCs) were cultured in Th17 polarizing conditions in the absence or presence of 1 α ,25-Dihydroxyvitamin D3 (10nM) for 6 days. On the 6th day of culture, the percentages of live cells were assessed by the Live/Dead stain kit. Vitamin D increased the viability of the cells by 69%, going from 46.7% live cells in the absence of vitamin D to 78.9% in the presence of vitamin D (Figure 2).

3. Effect of different doses of Vitamin D on the frequency of IL-17-producing cells

Naïve CD4⁺ T helper cells were cultured in Th17 polarizing conditions in the absence or presence of different concentrations of 1 α ,25-Dihydroxyvitamin D3 (1 nM, 10 nM, 100 nM). On the 6th day of culture, cells were restimulated with PMA/ionomycin in the presence of Brefeldin A and stained to detect intracellular Interleukin-17.

The frequency of IL-17-producing CD4⁺ T cells decreased from 8.36% in the absence of vitamin D to 6.76%, 2.32% and 2.42% in the presence of 1 nM, 10 nM, 100 nM vitamin D respectively. The maximal decrease of 72.3% in IL-17 producing cells was obtained with 10 nM vitamin D. Therefore, for the subsequent experiments vitamin D was used at a 10 nM concentration to ensure the best effect of vitamin D with the lowest concentration (Figure 4). The mean fluorescence intensity (MFI) of IL-17 was also reduced by 65%, decreasing from 409 to 143 (Figure 5).

The specificity of the stain was confirmed by the use of several negative controls (Figure 3).

4. Effect of Vitamin D on the phenotype of Th17 cells

Naïve CD4⁺ T helper cells were cultured in Th17 polarizing conditions in the absence or presence of 1 α ,25-Dihydroxyvitamin D3 (10nM). On the 6th day of culture, cells were restimulated with PMA/ionomycin in the presence of Brefeldin A and stained with different fluorochromes.

a. Pro-inflammatory phenotype

The frequency of total IFN γ -producing CD4⁺ T cells was decreased by 47% in the presence of vitamin D (Figure 6). Similarly, the frequency of total TNF α -producing CD4⁺ T cells was reduced by 64% when vitamin D was added to culture (Figure 8). When IL-17⁺IFN γ ⁺ T cells and IL-17⁺TNF α ⁺ T cells were analyzed, the frequency of both subsets were found to be decreased by 85% and 92%, respectively, in the presence of vitamin D (Figure 7 and Figure 9). Moreover, when looking into high expression of the surface marker CD25 (CD4⁺CD25^{hi} associated with regulatory functions) within the gated IL-17⁺TNF α ⁺ Th17 cells, vitamin D showed a 4-fold increase in the percentages of CD25^{hi} Th17 (Figure 10).

Vitamin D also reduced the expression of extracellular markers usually found on the surface of Th17 cells. It decreased the frequency of total CCR6⁺ CD4⁺ T cells by 59% in addition to decreasing the percentage of CCR6⁺ cells within the Th17 cell subsets which was reduced by 46% in the presence of vitamin D (Figure 11 and Figure 12). The frequency of total CD161⁺ CD4⁺ T cells was also decreased by 42% in the presence of vitamin D (Figure 13). As for the frequency of total CCR4⁺ CD4⁺ T cells, it remained unchanged when cells were exposed to vitamin D. However, its mean fluorescence intensity was reduced by 24% (Figure 14).

The mean fluorescence intensity of the transcription factor ROR γ t was decreased by 41% in the presence of vitamin D (Figure 15). Accordingly, the frequency of ROR γ t⁺ IL-17⁺ double positive cells was inhibited by 79% (Figure 16).

b. Non-pathogenic phenotype

Vitamin D increased both the percentage of total Foxp3⁺ cells by 2.2 folds and the mean fluorescence intensity of Foxp3 by 1.7 folds (Figure 17 and Figure 18). The frequency of cells expressing high levels of CD25 (CD25^{hi}) was increased by 2 folds in the presence of vitamin D (Figure 19). Consistently, vitamin D increased the frequency of total Foxp3⁺CD25^{hi} cells by 1.8 folds (Figure 20).

Foxp3⁺CD25^{hi} cells were also analyzed after gating on Th17 cells. A 4.4-fold increase was found in the frequency of Foxp3⁺CD25^{hi} Th17 cells in the presence of vitamin D (Figure 21).

Finally, vitamin D slightly increased the total percentage of IL-10 producing CD4⁺ T cells by 1.3 folds (Figure 22).

B. Enzyme-Linked Immunosorbant Assay (ELISA)

Naïve CD4⁺ T helper cells were cultured in Th17 polarizing conditions in the absence or presence of 1 α ,25-Dihydroxyvitamin D3 (10nM). On the 6th day, culture supernatants were obtained from 5 donors to measure the cumulative production of IL-17A in both conditions.

The data was normalized in order to calculate statistical significance because the initial production of IL-17A by cultured cells differed from one donor to the other (Table 2, Figure 23A). Levels of IL-17A secreted in culture supernatants significantly decreased (p<0.0001) in the presence of vitamin D compared to cells put in Th17 polarizing conditions alone (Table 3, Figure 23B).

C. Cytometric Bead Array (CBA)

Naïve CD4⁺ T helper cells were cultured in Th17 polarizing conditions in the absence or presence of 1 α ,25-Dihydroxyvitamin D3 (10nM). On the 6th day, culture supernatants were obtained from 3 donors to measure the cumulative production of IL-17, IFN- γ , TNF α , IL-6, IL-2, IL-4 and IL-10 in both conditions.

The data was normalized in order to calculate statistical significance because the initial production of all cytokines by cultured cells differed from one donor to the other (Tables 4 and 5). Levels of IL-17A significantly decreased ($p < 0.05$) in the presence of vitamin D compared to cells put in Th17 polarizing conditions alone (Figure 25). Similarly, Vitamin D significantly decreased the levels of IFN- γ and TNF α ($p < 0.01$) (Figures 24 and 25). The levels of IL-6 remained unchanged in the presence of vitamin D (Figure 27). However, levels of IL-2 slightly increased even though this was not statistically significant (Figure 28). Levels of IL-4 significantly increased ($p < 0.05$) in the presence of vitamin D (Figure 29) whereas levels of IL-10 slightly decreased with a very high standard deviation and thus, no statistical relevance (Figure 30).

Table 1: Protocol followed to study the effect of Vitamin D on the differentiation of Th17 cells.

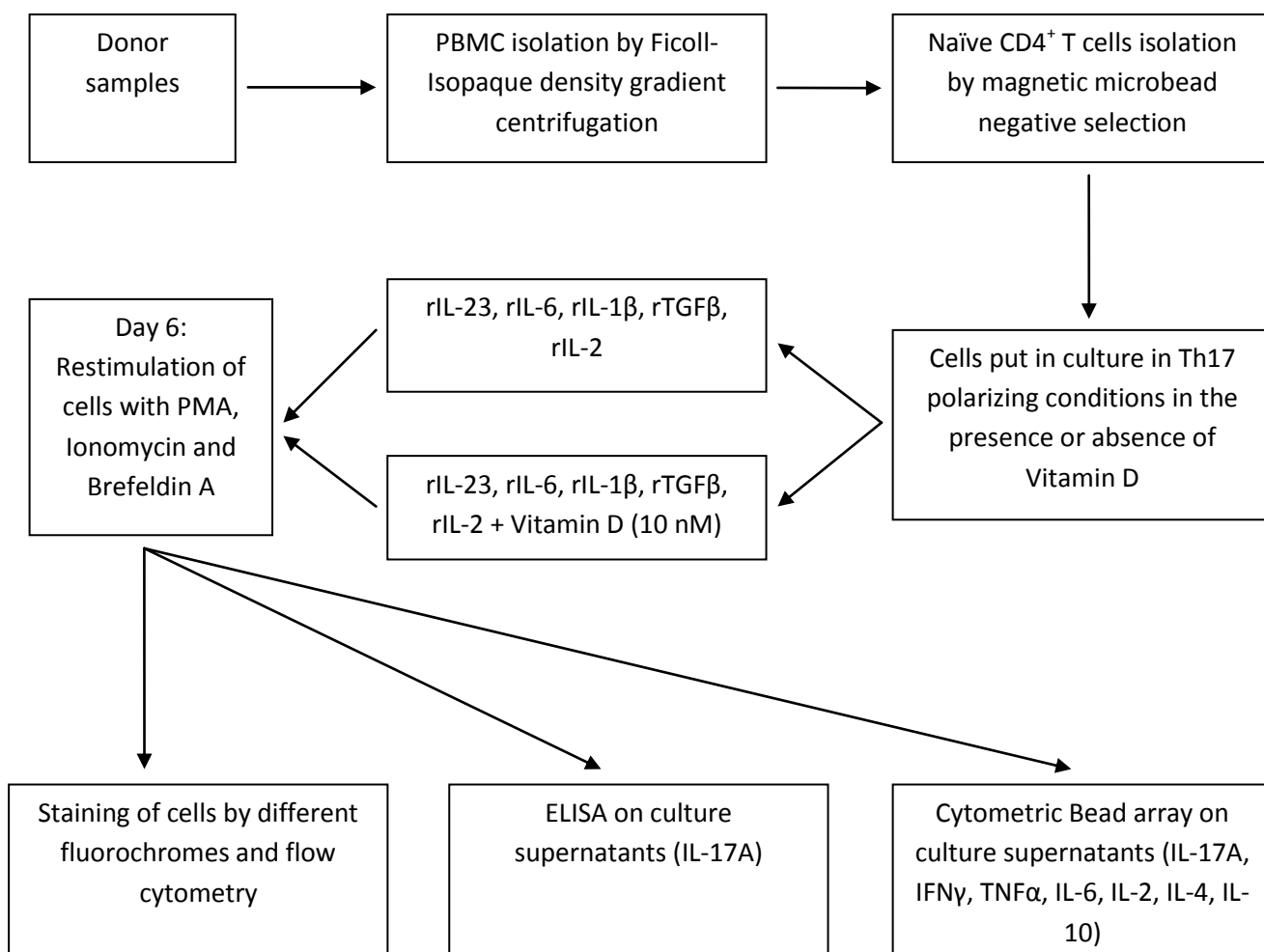


Table 2: Concentrations of IL-17A (pg/mL) in culture supernatants obtained by ELISA, in the absence or presence of Vitamin D.

	Th17 polarizing conditions	Th17 polarizing conditions +VitD (10 nM)
Donor 1	279.05 ± 19.19	35.86 ± 6.82
Donor 2	261.02 ± 15.90	16.33 ± 3.45
Donor 3	60.75 ± 6.69	16.63 ± 3.54
Donor 4	59.05 ± 1.26	7.40 ± 0.084
Donor 5	18.25 ± 0.13	6.15 ± 0.17

Table 3: Normalized data of IL-17A concentrations in culture supernatants obtained by ELISA, in the absence or presence of Vitamin D. Data is representative of 5 different donors and is presented as means ± SD.

	Th17 polarizing conditions	Th17 polarizing conditions +VitD (10 nM)
IL-17A	100	18.54 ± 11.48

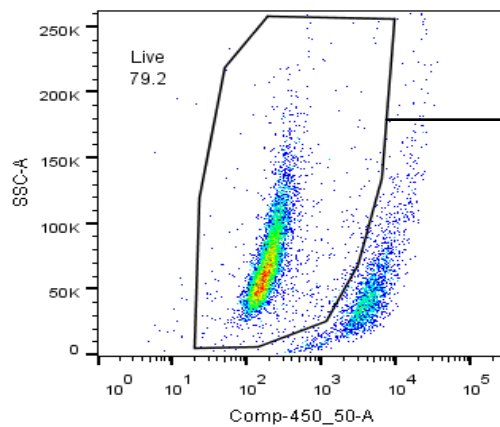
Table 4: Concentrations of IL-17A, IFN γ , TNF α , IL-6, IL-2, IL-4, IL-10 (pg/mL) in culture supernatants obtained by CBA, in the absence or presence of Vitamin D.

		Th17 polarizing conditions	Th17 polarizing conditions +VitD (10 nM)
IL-17A	Donor 1	16.63	4.27
	Donor 2	60.15	19.39
	Donor 3	445.87	10.11
IFN γ	Donor 1	28.13	8.79
	Donor 2	431.49	68.86
	Donor 3	59.5	4.19
TNF α	Donor 1	410.91	113.26
	Donor 2	1219.65	119.34
	Donor 3	46.49	13.48
IL-6	Donor 1	17508.04	17120.89
	Donor 2	12968.54	13258.41
	Donor 3	13258	12685.25
IL-2	Donor 1	1654.18	1588.92
	Donor 2	3036.44	4300.25
	Donor 3	1063.64	2194.23
IL-4	Donor 1	0.28	0.6
	Donor 2	0.64	1.82
	Donor 3	0.31	0.83
IL-10	Donor 1	92.47	79.79
	Donor 2	107.03	121.22
	Donor 3	182.69	21.77

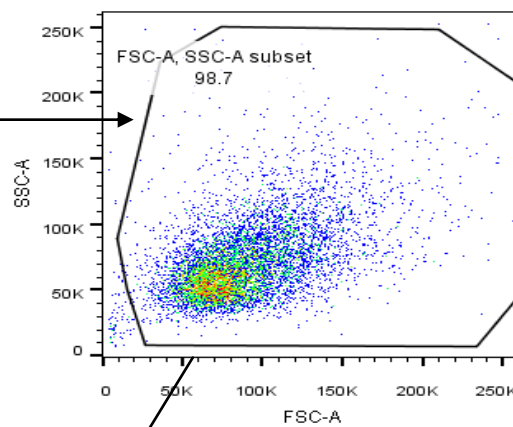
Table 5: Normalized data of IL-17A, IFN γ , TNF α , IL-6, IL-2, IL-4, IL-10 concentrations in culture supernatants obtained by CBA, in the absence or presence of Vitamin D. Data is representative of 3 different donors and is presented as means \pm SD.

	Th17 polarizing conditions	Th17 polarizing conditions +VitD (10 nM)
IL-17A	100	20.06 \pm 15.75
IFN γ	100	18.08 \pm 12.24
TNF α	100	22.11 \pm 10.70
IL-6	100	98.57 \pm 3.35
IL-2	100	147.99 \pm 55.40
IL-4	100	255.47 \pm 36.62
IL-10	100	70.49 \pm 52.49

(A) Live cells



(B) T Cells



(C) IL-17⁺ Cells

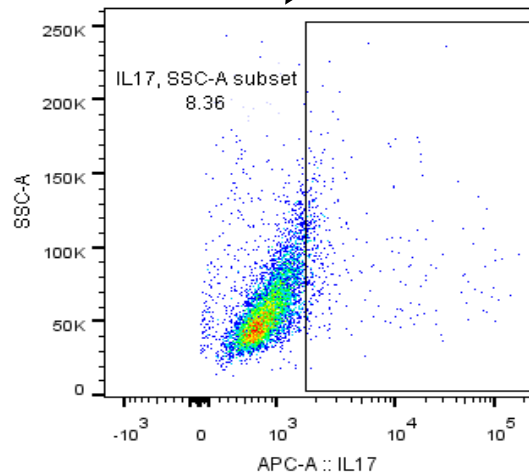
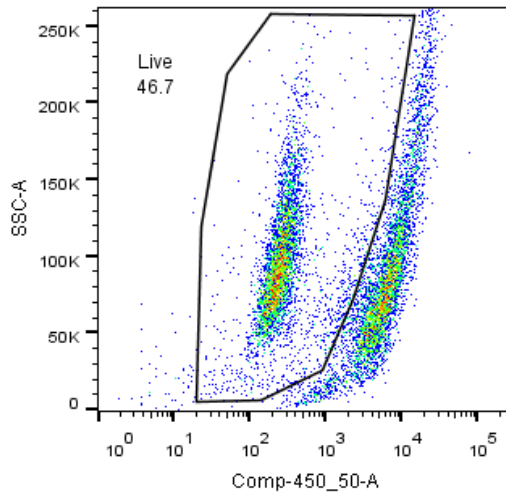


Figure 1: Gating strategy for flow cytometry analysis of Interleukin-17⁺ (IL-17⁺) cells. Sequential gating is shown from A to C. (A) Dead cells were excluded from the analysis on the basis of the Live/Dead stain kit. Gating was done on the live cells which are dimly stained. (B) Gating of cells based on forward scatter (FSC-A) related to size, and side scatter (SSC-A) related to granularity. (C) Gating on IL-17⁺ cells.

(A) Th17 cells



(B) Th17 cells + VitD (10 nM)

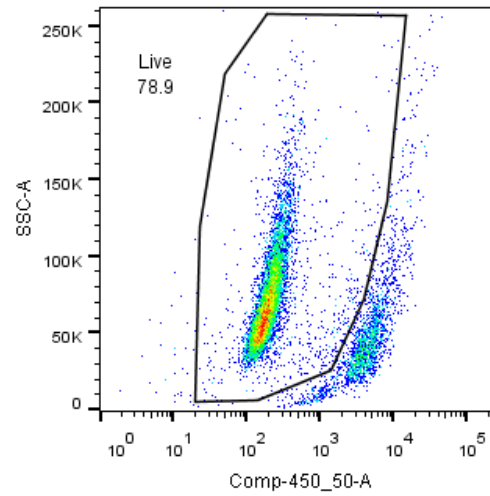
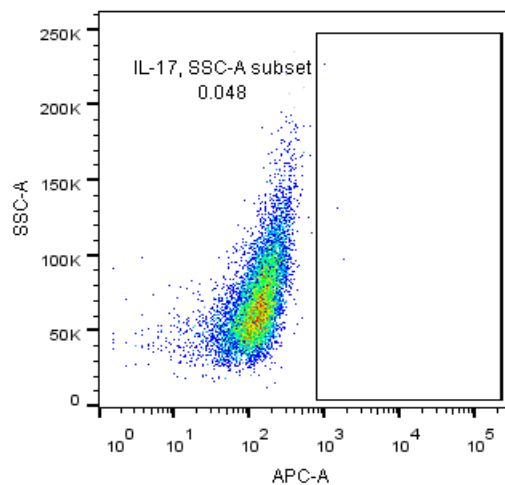


Figure 2: Percentages of live CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10nM) (B) for 6 days. The percentages of live cells were assessed by gating on the dimly stained cells. The dot plots of live cells are shown above. Data is representative of 10 independent experiments.

(A) Unstained negative control



(B) Ligand blocking control

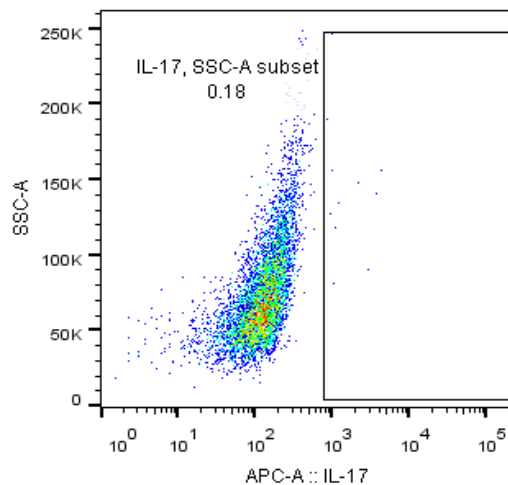
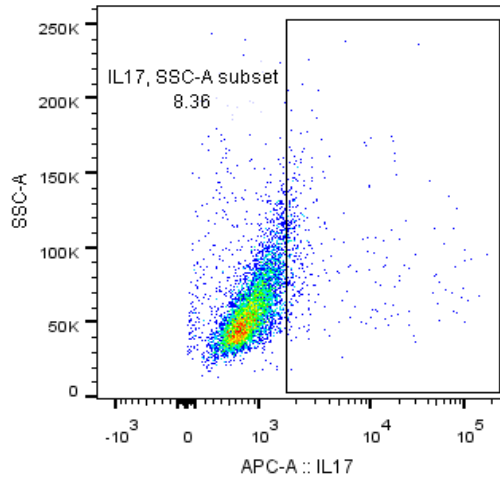
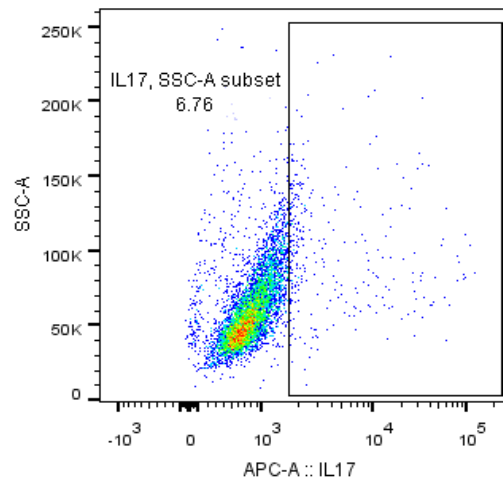


Figure 3: Negative controls to confirm the specificity of the fluorochromes used. The controls include an unstained negative control for cells cultured in Th17 polarizing conditions (A) or a ligand blocking control performed on polarized cells (B). Representative data for the specificity of the anti-IL17A-Alexa Fluor 647 stain is shown.

(A) Th17 cells

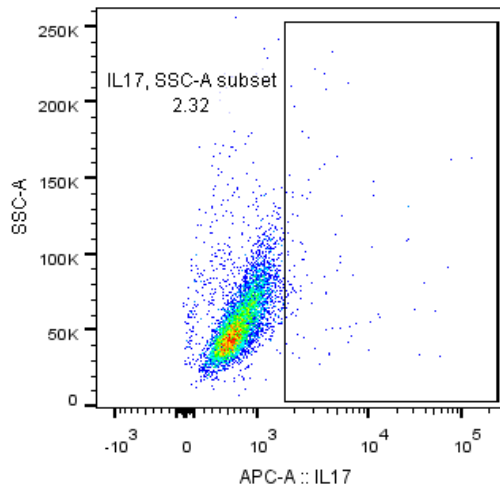


(B) Th17 cells + VitD3 (1 nM)

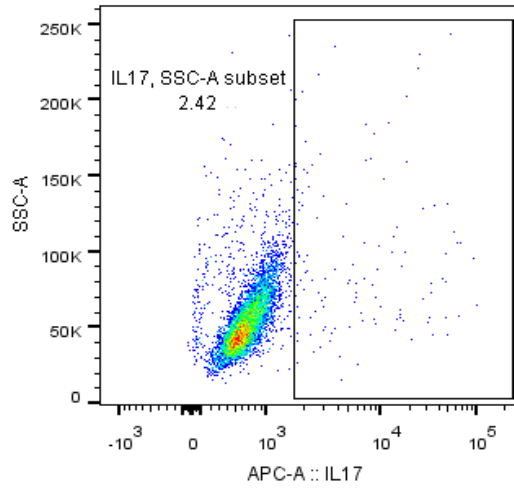


→ IL-17A

(C) Th17 cells + VitD3 (10 nM)



(D) Th17 cells + VitD3 (100 nM)



→ IL-17A

Figure 4: Percentages of IL-17A⁺ CD4⁺ T cells in the absence or presence of different Vitamin D concentrations. Naïve CD4⁺ T helper cells were cultured in Th17 polarizing conditions in the absence (A) or presence of different 1 α ,25-Dihydroxyvitamin D3 concentrations of 1 nM (B), 10 nM (C), 100 nM (D). The percentages of IL-17-producing CD4⁺ T were assessed by flow cytometry. The dot plots of IL-17⁺ cells are shown.

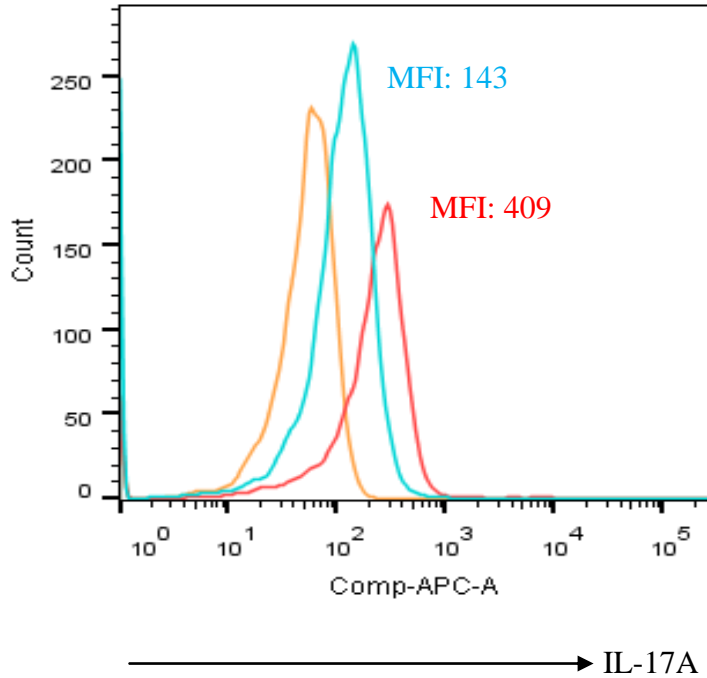


Figure 5: Mean fluorescence intensity (MFI) of IL-17A in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (red) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (blue) for 6 days. IL-17 MFI is shown for both conditions. The unstained negative control is shown as well (orange). Data is representative of 15 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

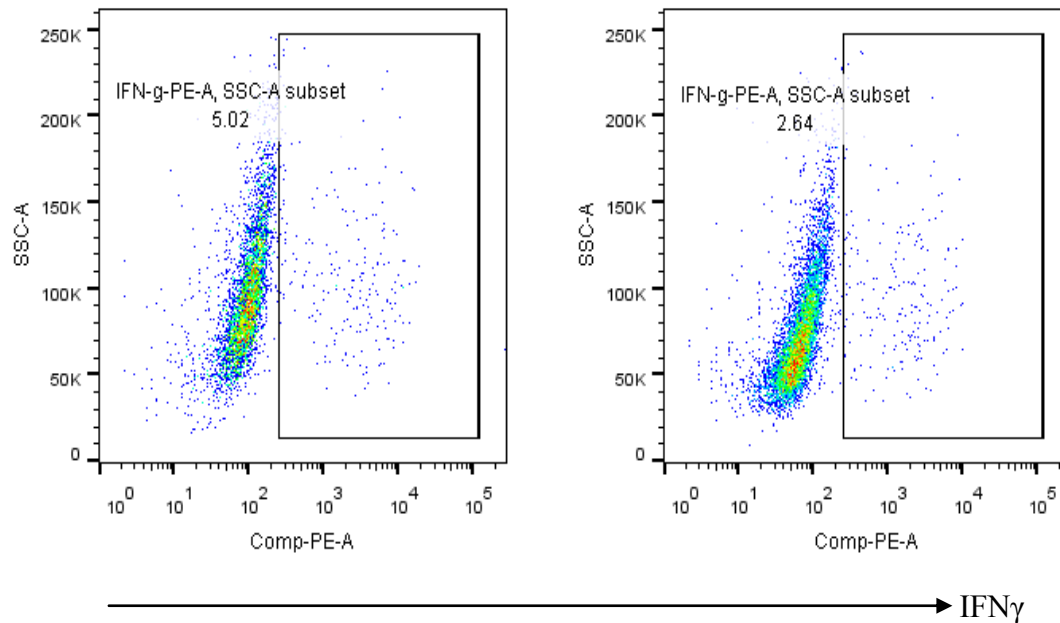


Figure 6: Percentages of IFN γ ⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of IFN γ -producing CD4⁺ T cells were assessed by flow cytometry. The dot plots of IFN γ ⁺ cells are shown. Data is representative of 4 independent experiments.

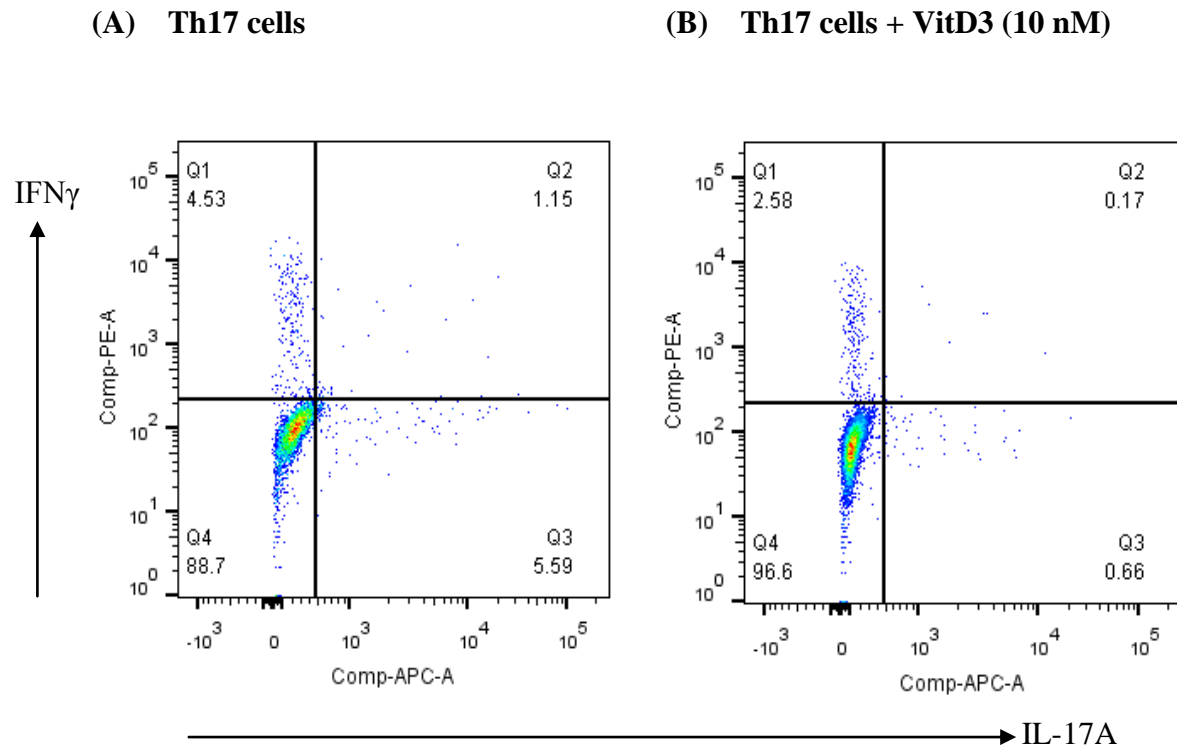


Figure 7: Percentages of IL-17A⁺IFN γ ⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of IFN γ and IL-17A double-producing CD4⁺ T cells were assessed by flow cytometry. The dot plots of IL-17A⁺IFN γ ⁺ cells are shown. Data is representative of 4 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

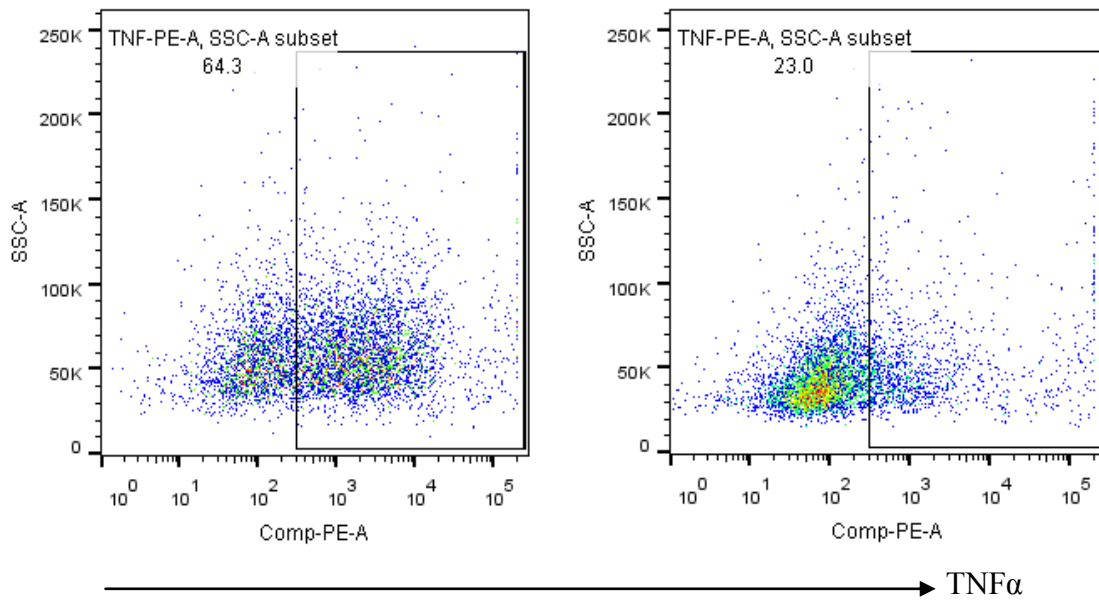


Figure 8: Percentages of TNF α ⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of TNF α -producing CD4⁺ T cells were assessed by flow cytometry. The dot plots of TNF α ⁺ cells are shown. Data is representative of 3 independent experiments.

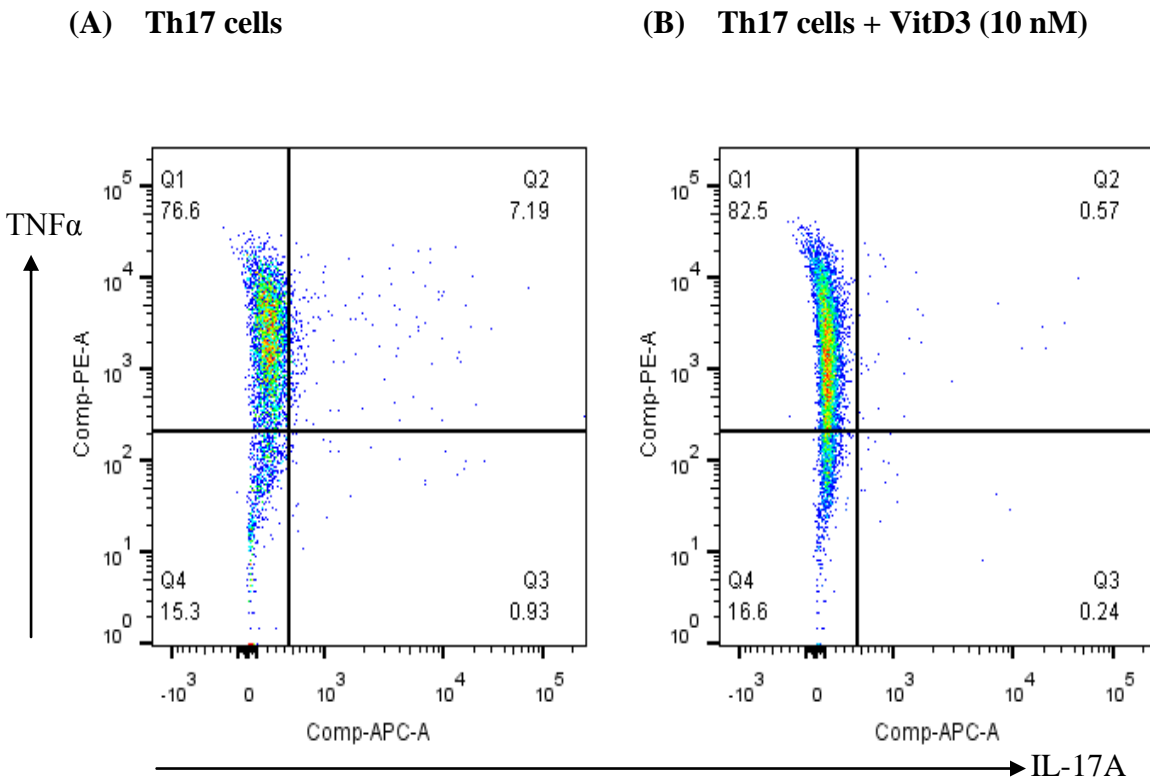


Figure 9: Percentages of IL-17A⁺TNFα⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1α,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of TNFα and IL-17A double-producing CD4⁺ T cells were assessed by flow cytometry. The dot plots of IL-17A⁺ TNFα⁺ cells are shown. Data is representative of 3 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

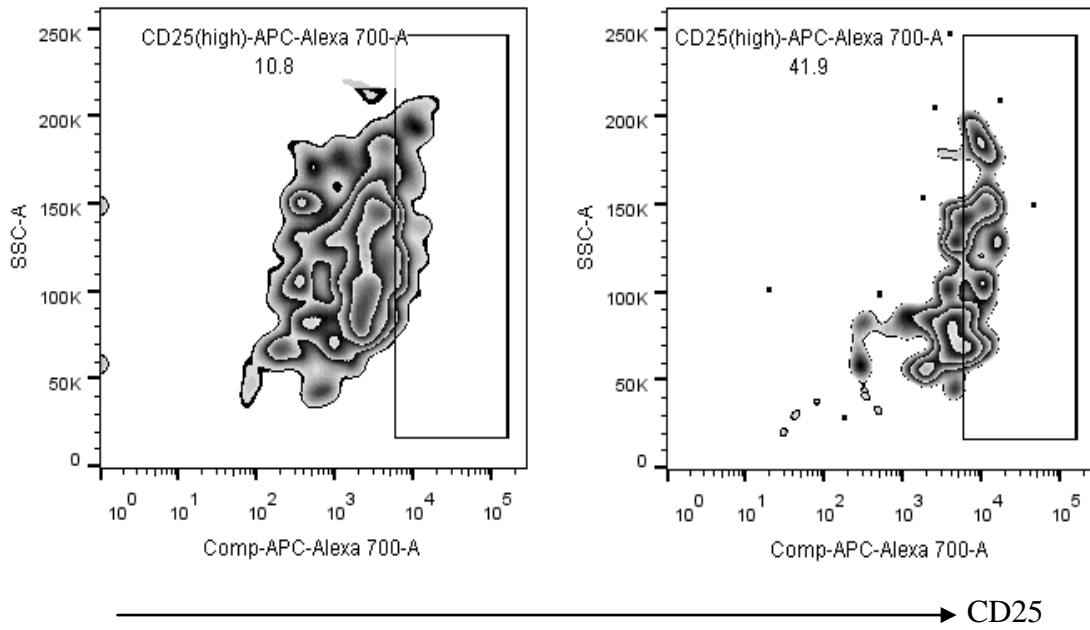


Figure 10: Percentages of CD25^{hi} Th17 cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of highly positive CD25 cells (CD25^{hi}) within IL-17A⁺ TNF α ⁺ gated cells were assessed by flow cytometry. The zebra plots of CD25^{hi} cells in the IL-17A⁺ TNF α ⁺ Th17 cells are shown. Data is representative of 2 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

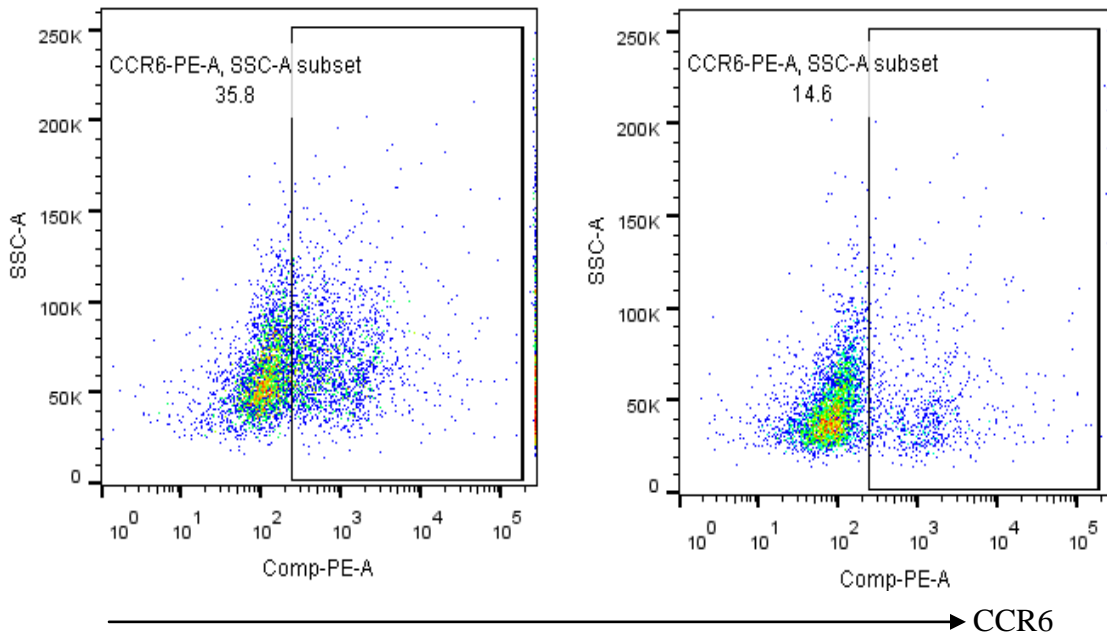


Figure 11: Percentages of CCR6⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of the chemokine receptor CCR6⁺ T cells were assessed by flow cytometry. The dot plots of CCR6⁺ cells are shown.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

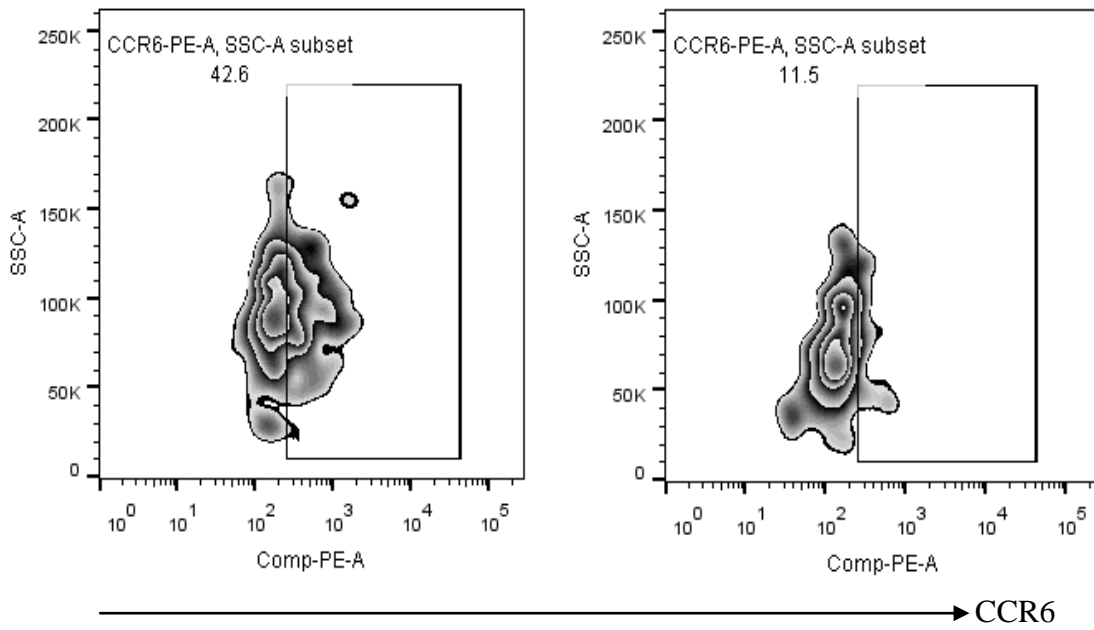


Figure 12: Percentages of CCR6⁺ Th17 cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of CCR6⁺ cells within IL-17A⁺ gated cells were assessed by flow cytometry. The zebra plots of CCR6⁺ Th17 cells are shown.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

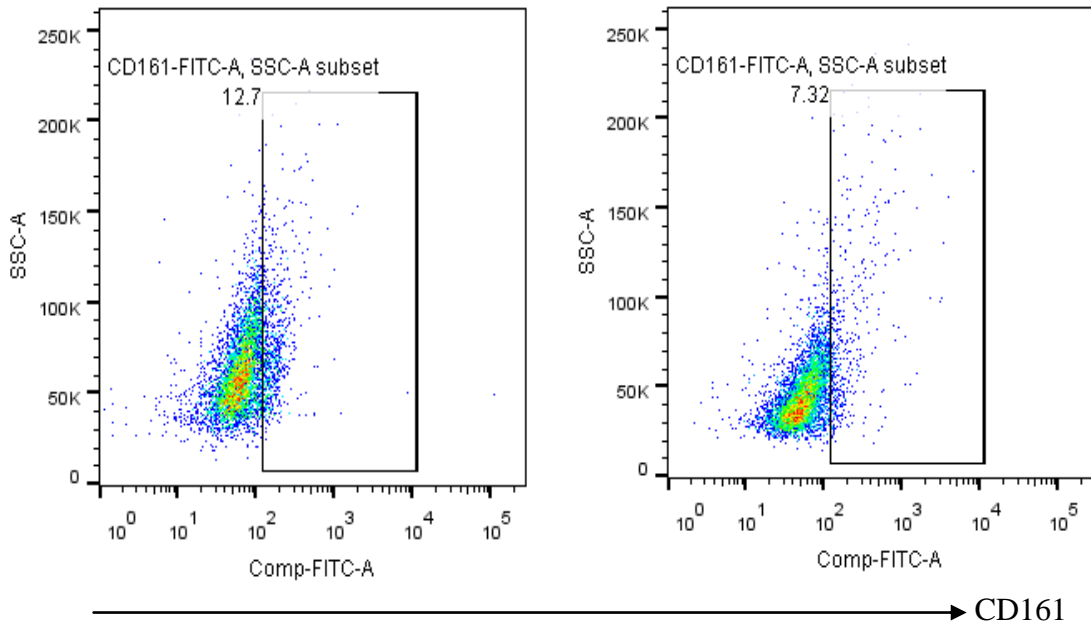


Figure 13: Percentages of CD161⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of the surface marker CD161⁺ T cells were assessed by flow cytometry. The dot plots of CD161⁺ cells are shown.

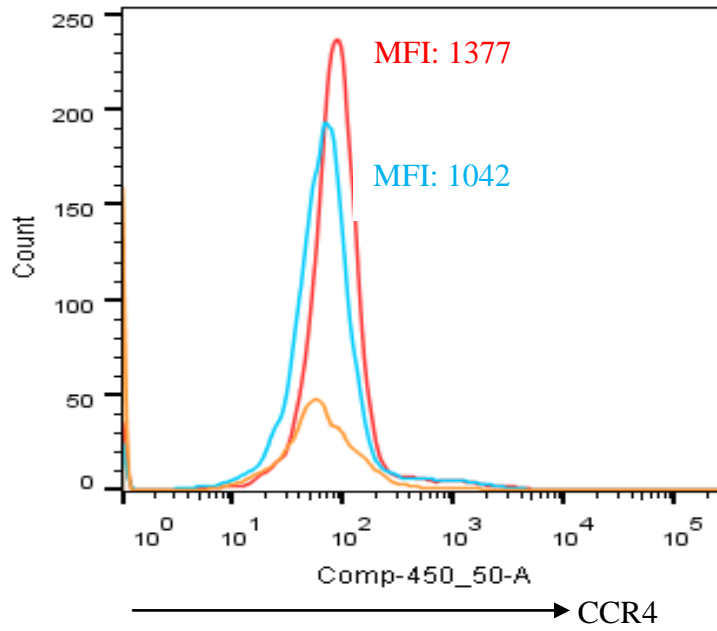


Figure 14: Mean fluorescence intensity (MFI) of CCR4 in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (red) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (blue) for 6 days. The chemokine receptor CCR4 MFI is shown for both conditions. The unstained negative control is shown as well (orange).

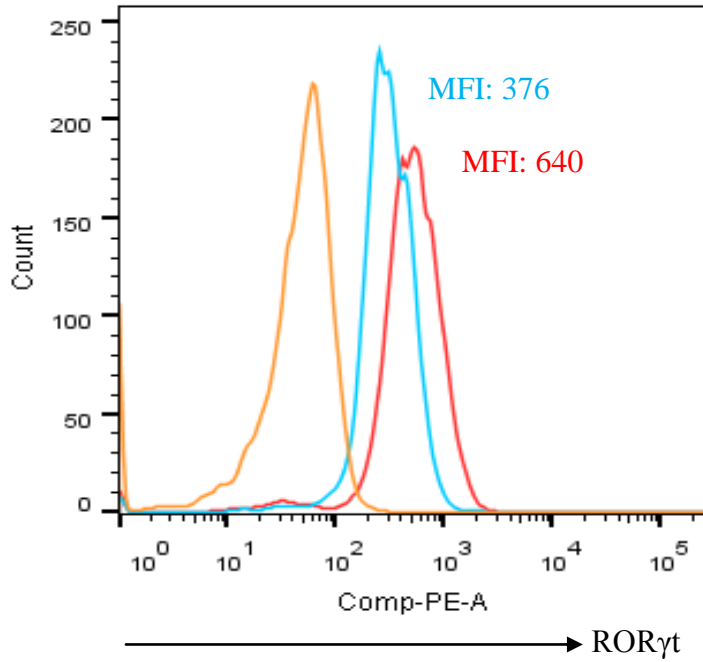


Figure 15: Mean fluorescence intensity (MFI) of ROR γ t in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (red) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (blue) for 6 days. The transcription factor ROR γ t MFI is shown for both conditions. The unstained negative control is shown as well (orange).

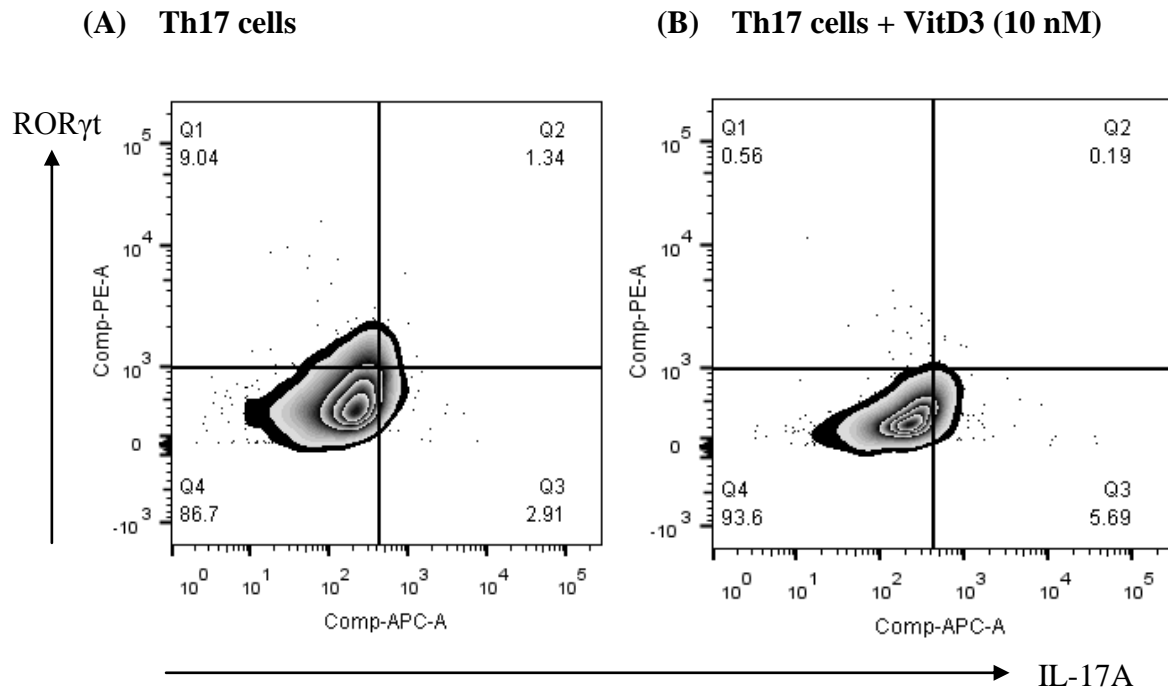


Figure 16: Percentages of IL-17A⁺ RORγt⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of RORγt⁺ and IL-17-producing cells were assessed by flow cytometry. The zebra plots of IL-17A⁺ RORγt⁺ double positive cells are shown.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

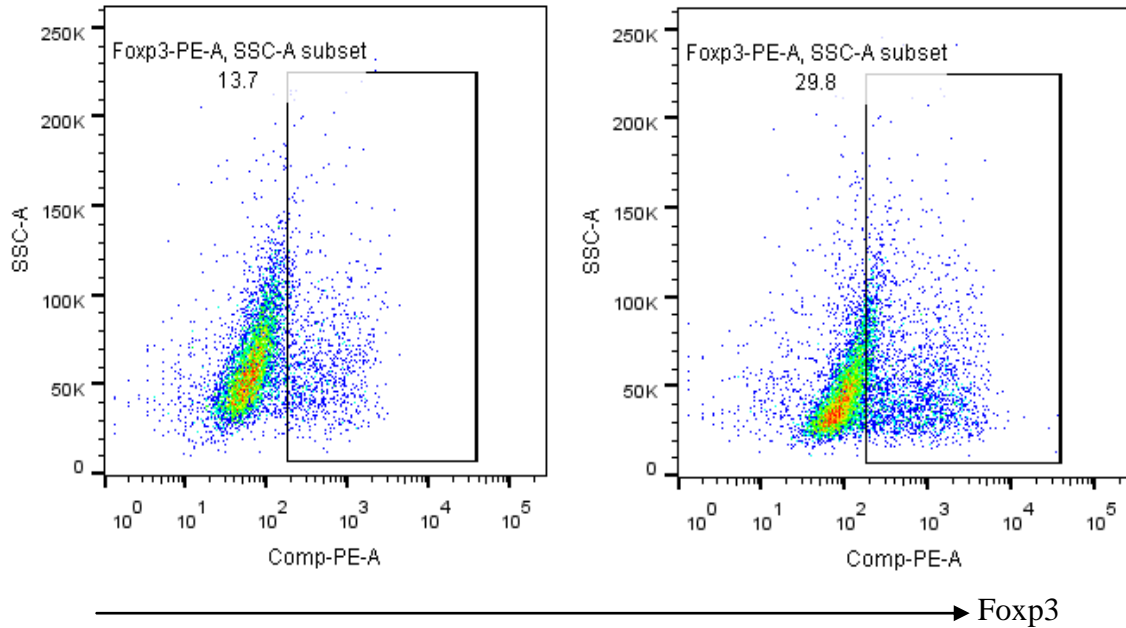


Figure 17: Percentages of Fxp3⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of the transcription factor Fxp3⁺ T cells were assessed by flow cytometry. The dot plots of Fxp3⁺ cells are shown. Data is representative of 3 independent experiments.

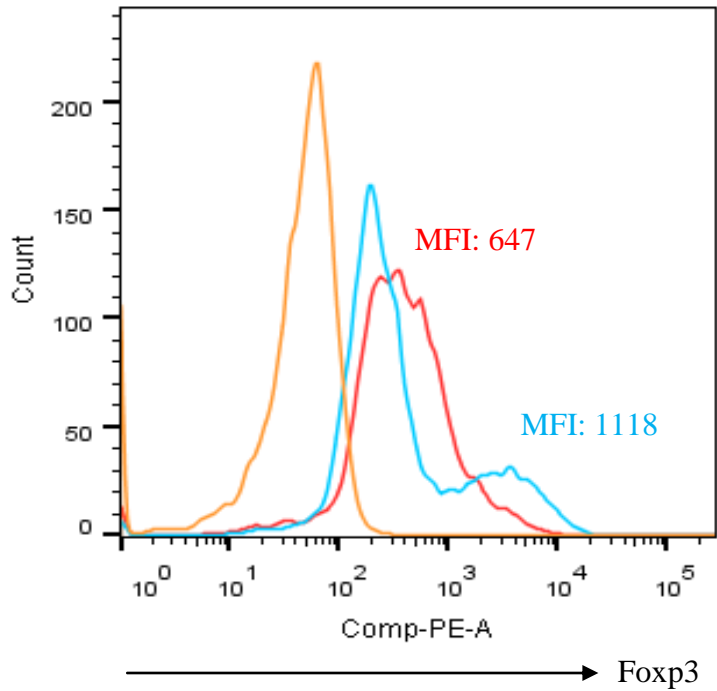


Figure 18: Mean fluorescence intensity (MFI) of Foxp3 in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (red) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (blue) for 6 days. The transcription factor Foxp3 MFI is shown for both conditions. The unstained negative control is shown as well (orange). Data is representative of 3 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

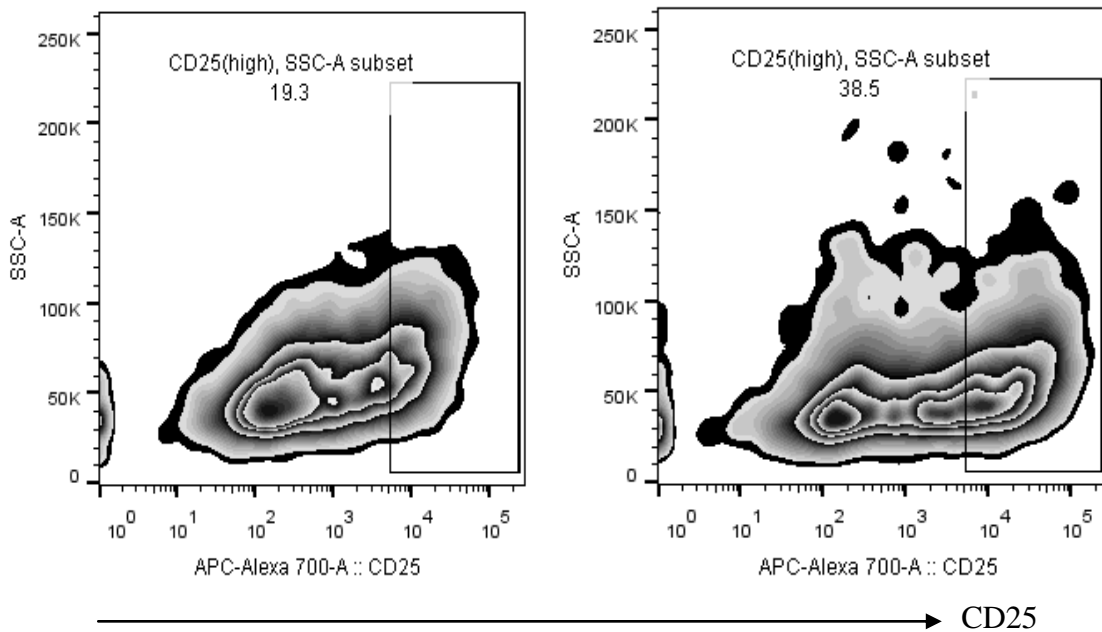


Figure 19: Percentages of CD25^{hi} CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of highly positive CD25 cells (CD25^{hi}) were assessed by flow cytometry. The zebra plots of CD25^{hi} cells are shown. Data is representative of 2 independent experiments.

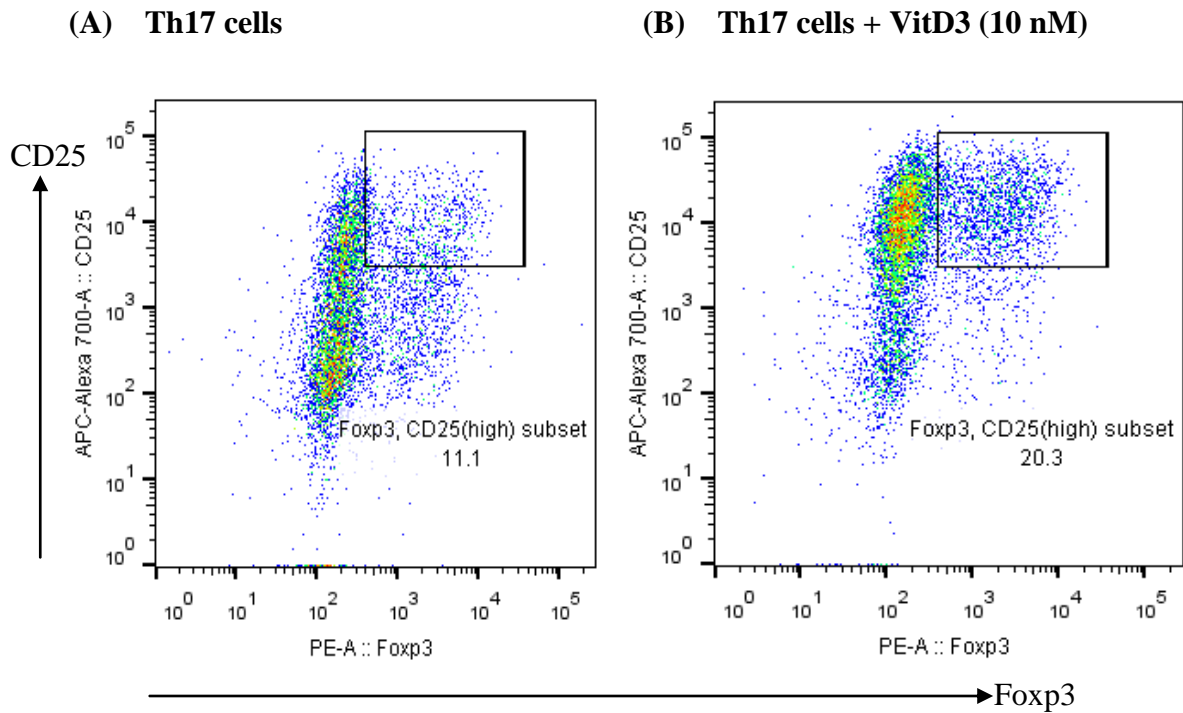


Figure 20: Percentages of Foxp3⁺CD25^{hi} CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of Foxp3⁺CD25^{hi} were assessed by flow cytometry. The dot plots of Foxp3⁺CD25^{hi} cells are shown. Data is representative of 2 independent experiments.

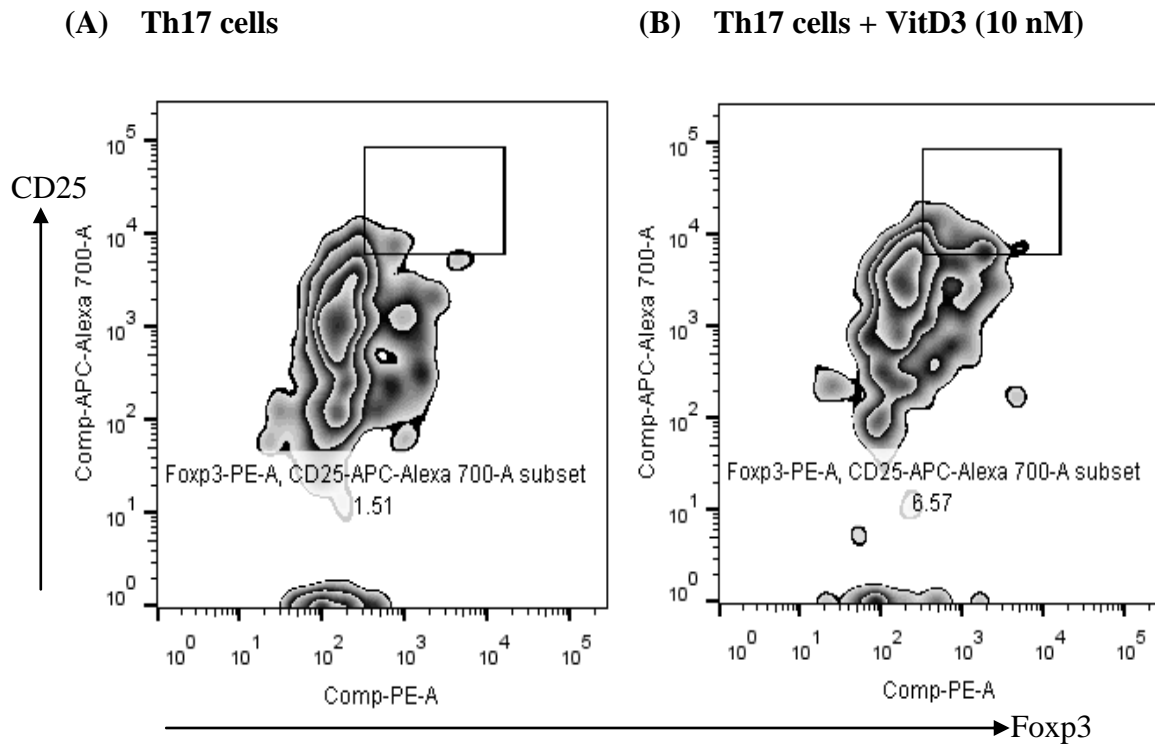


Figure 21: Percentages of Foxp3⁺CD25^{hi} Th17 cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of Foxp3⁺CD25^{hi} within IL-17A⁺ gated cells were assessed by flow cytometry. The dot plots of Foxp3⁺CD25^{hi} Th17 cells are shown. Data is representative of 2 independent experiments.

(A) Th17 cells

(B) Th17 cells + VitD3 (10 nM)

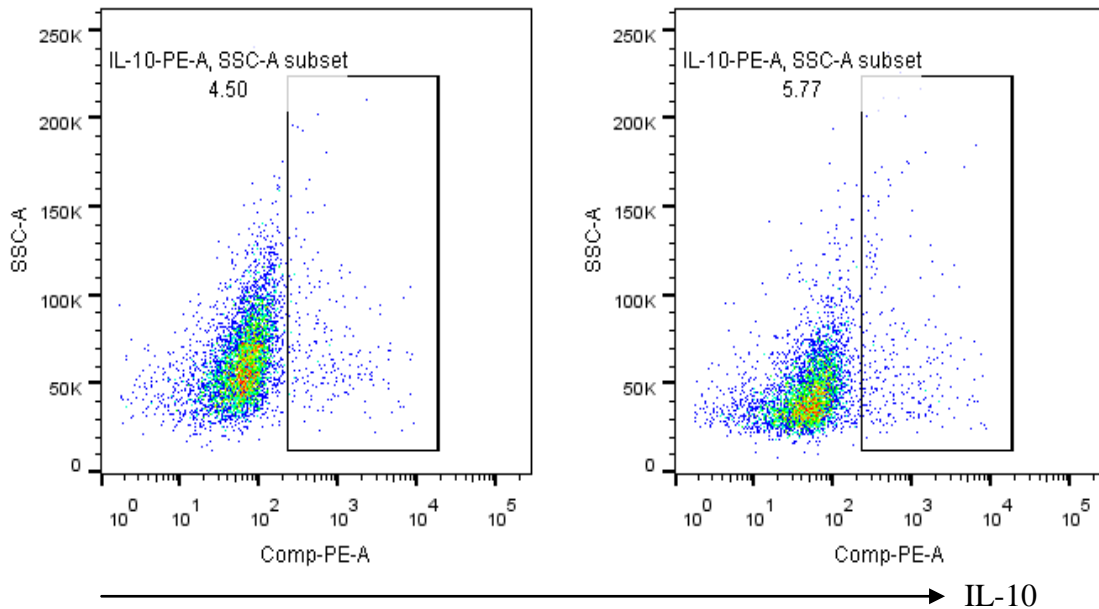


Figure 22: Percentages of IL-10⁺ CD4⁺ T cells in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence (A) or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) (B) for 6 days. The percentages of IL-10-producing CD4⁺ T cells on were assessed by flow cytometry. The dot plots of IL-10⁺ cells are shown. Data is representative of 2 independent experiments.

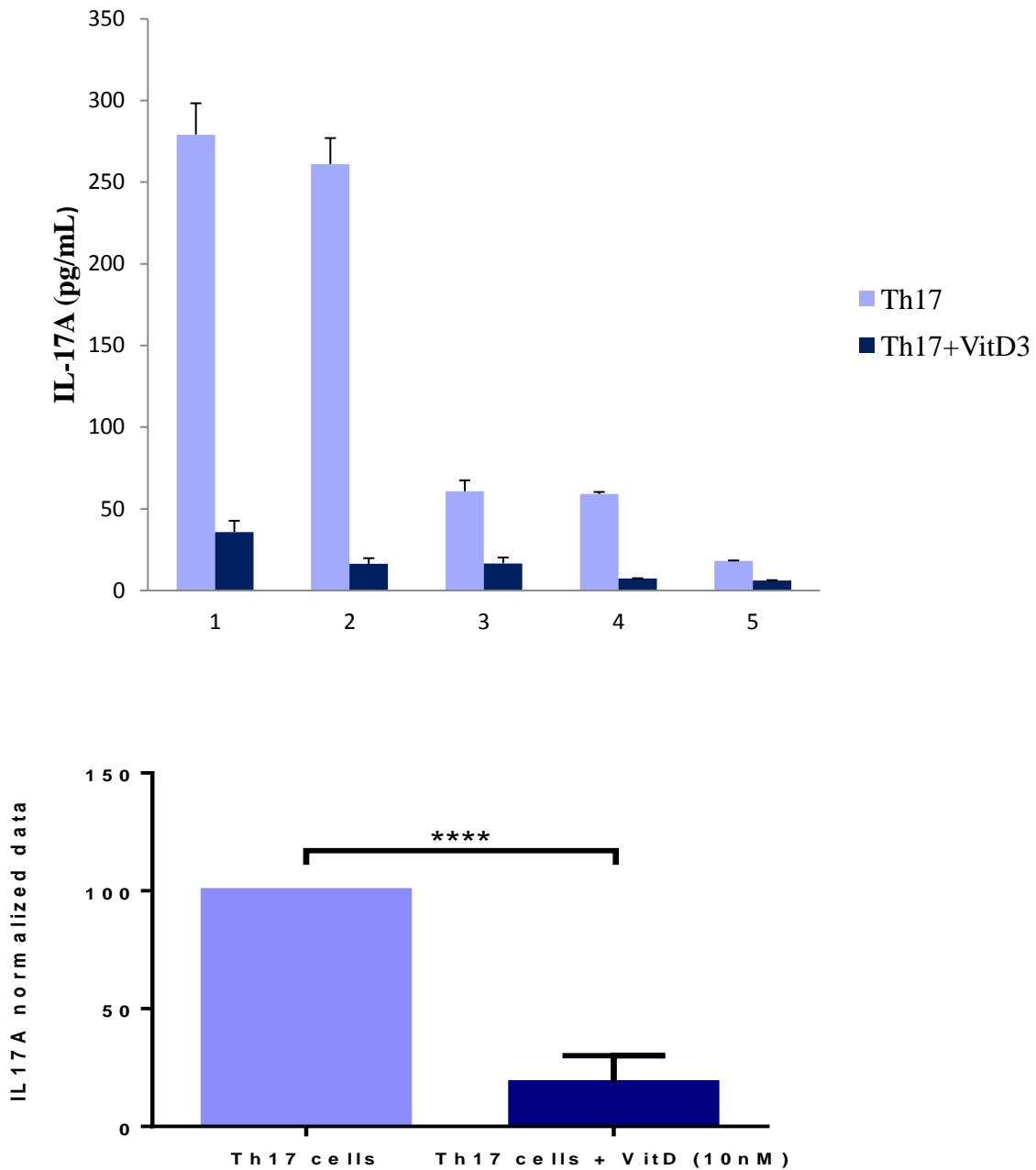


Figure 23: Concentrations of IL-17A (pg/mL) in culture supernatants in the absence or presence of Vitamin D. (A) ELISA of IL-17A production in culture supernatants of 5 different donors. (B) Normalized data of IL-17A production in culture supernatants. Data is representative of 5 independent donors and is expressed as means \pm SD. ****, $P < 0.0001$ by paired Student's t test.

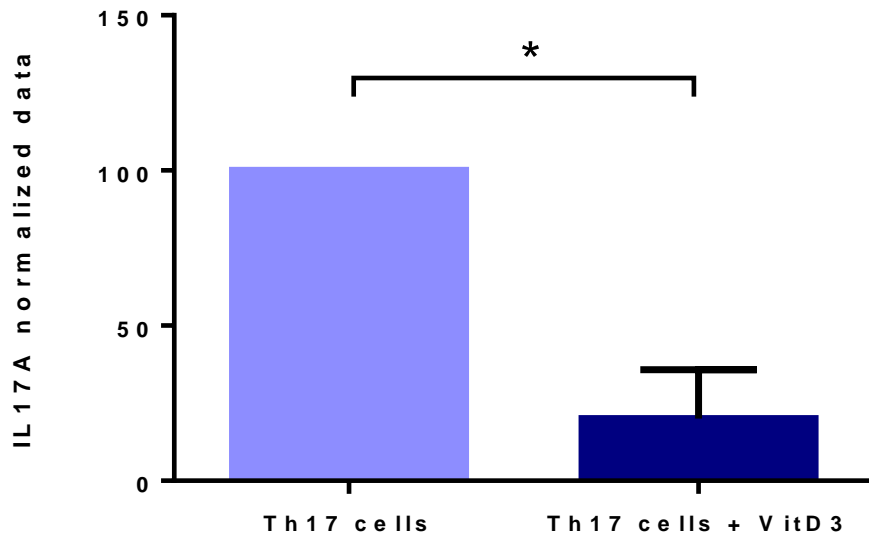


Figure 24: Normalized data of cumulative IL-17A production in culture supernatants in the absence or presence of Vitamin D. Naïve CD4⁺ T helper cells were put in culture in Th17 polarizing conditions in the absence or presence of 1 α ,25-Dihydroxyvitamin D3 (10 nM) for 6 days. The concentrations of IL-17A in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD. *, P < 0.05 by paired Student's t test.

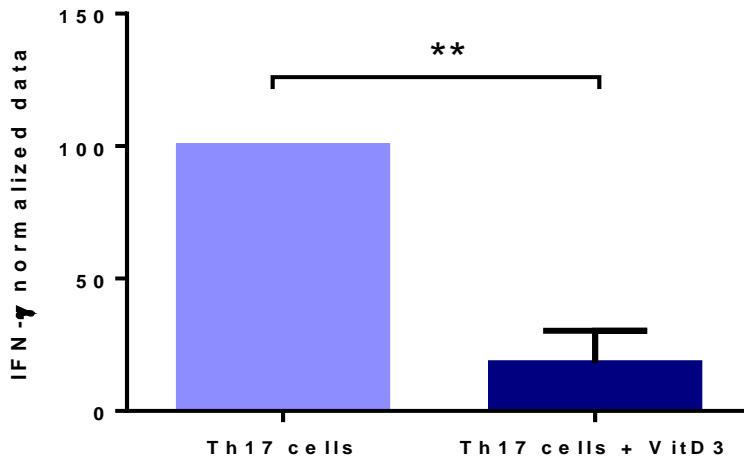


Figure 25: Normalized data of cumulative IFN γ production in culture supernatants in the absence or presence of Vitamin D. The concentrations of IFN γ in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD. **, P <0.01 by paired Student's t test.

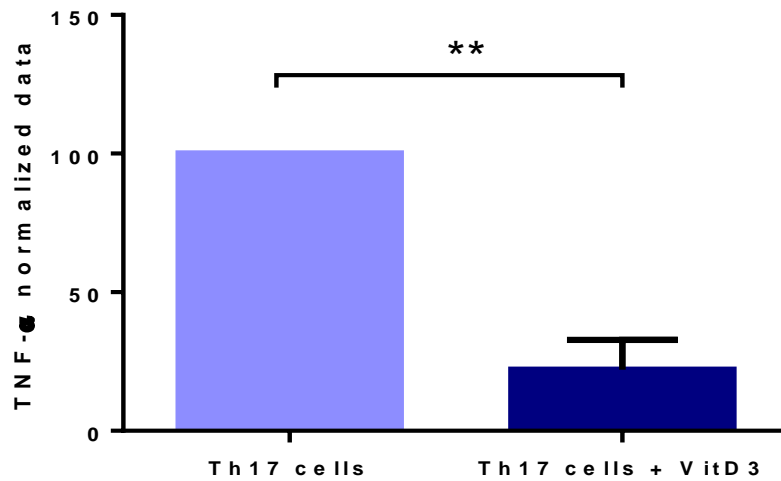


Figure 26: Normalized data of cumulative TNF α production in culture supernatants in the absence or presence of Vitamin D. The concentrations of TNF α in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD. **, P <0.01 by paired Student's t test.

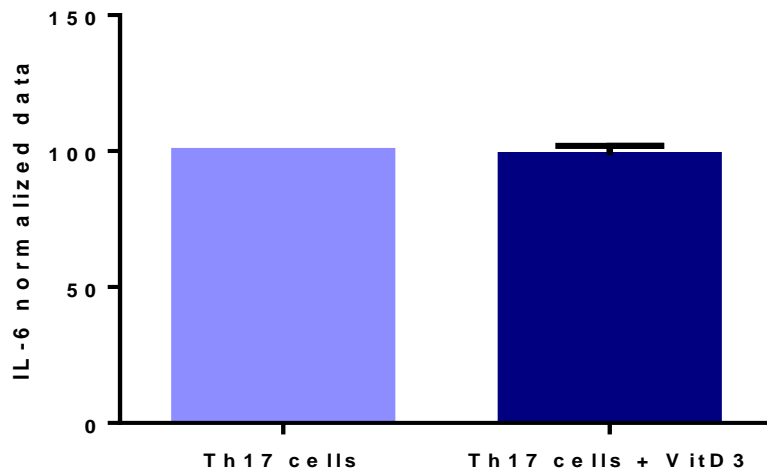


Figure 27: Normalized data of cumulative IL-6 production in culture supernatants in the absence or presence of Vitamin D. The concentrations of IL-6 in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD.

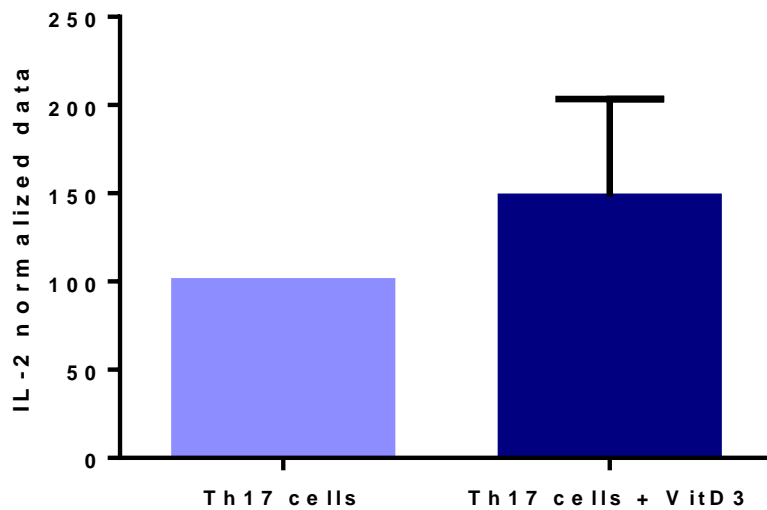


Figure 28: Normalized data of cumulative IL-2 production in culture supernatants in the absence or presence of Vitamin D. The concentrations of IL-2 in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD.

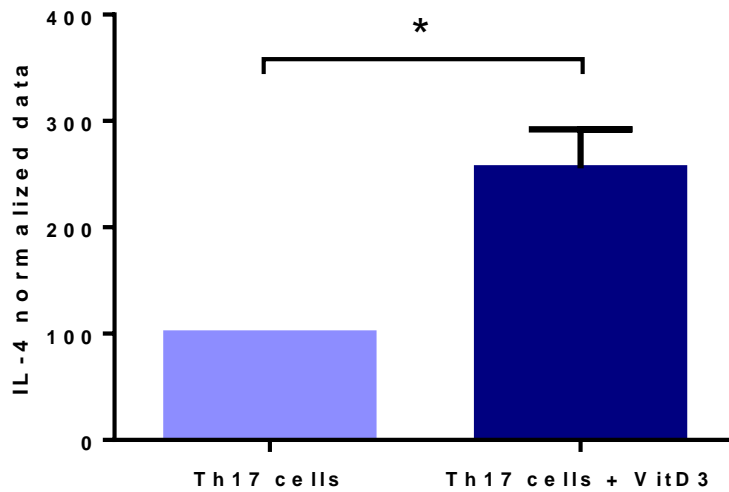


Figure 29: Normalized data of cumulative IL-4 production in culture supernatants in the absence or presence of Vitamin D. The concentrations of IL-4 in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD. *, $P < 0.05$ by paired Student's t test.

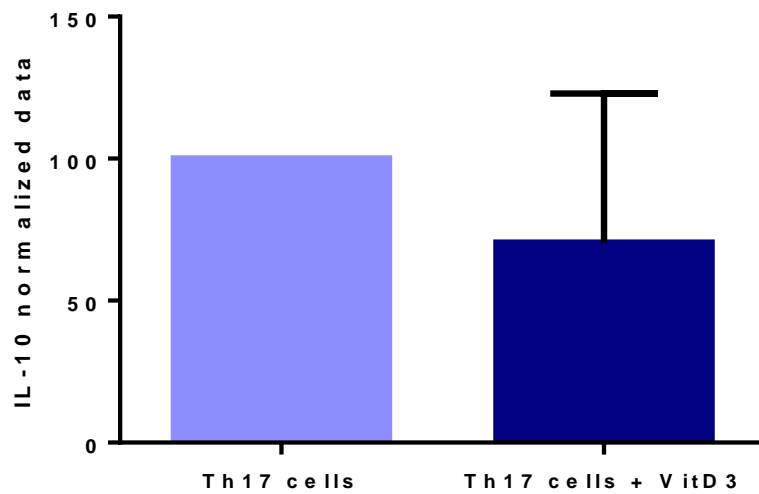


Figure 30: Normalized data of cumulative IL-10 production in culture supernatants in the absence or presence of Vitamin D. The concentrations of IL-4 in culture supernatants were measured by CBA. Data is representative of 3 independent donors and is expressed as means \pm SD.

CHAPTER V

DISCUSSION

Pathogenic Th17 cells play a central role in the development of multiple sclerosis. High levels of Th17 cells are found in the peripheral blood and central nervous system of multiple sclerosis patients, hence the importance of targeting this particular subtype in treatment strategies for MS (63, 65).

So far, studies conducted by Boonstra et al. (84), Reichel et al. (85) and Gorman et al. (86) have shown the negative effect of vitamin D on Th1 responses and its positive effect on Th2 cell responses and regulatory T-cells respectively. Furthermore, vitamin D has already demonstrated immune-modulating abilities *in-vivo* on Th17 responses in animal models of MS such as EAE (70) in addition to its indirect negative effect on Th17 cells *ex-vivo* through antigen presenting cells (APCs) (81). However, its direct effect on human Th17 cells and on the differentiation of naïve CD4⁺ T cells into Th17 cells *ex-vivo* has yet to be fully addressed. The aim of this study was to investigate the effect of 1,25-dihydroxyvitamin D3 on the differentiation of Th17 cells and to further characterize the phenotype of this cell type after its exposure to vitamin D.

When naïve CD4⁺ T cells were put in Th17 polarizing conditions for 6 days, vitamin D increased the overall viability of cells that were restimulated with PMA/ionomycin prior to flow cytometric staining. This could be explained by the fact that vitamin D inhibits activation-induced cell death (AICD) by negatively regulating the expression of Fas ligand (FasL) on T cells as reported in a study conducted by Cippitelli et

al. (87). FasL is part of the Fas/FasL system that usually mediates AICD that could be caused by the PMA restimulation. However, the effect of vitamin D on the viability of naïve CD4⁺ T cells needs to be investigated further by studying possible cell death pathways, but this was beyond the scope of this thesis.

When naïve CD4⁺ T cells were cultured in Th17 polarizing conditions for 6 days, the combination of cytokines used (IL-6, TGFβ, IL-1β and IL-23) induced their differentiation into pathogenic Th17 cells. However, when vitamin D was present in the culture media, it decreased the frequency of IL-17 producing CD4⁺ T cells in a dose dependent manner with maximal inhibition obtained at 10 nM. This is consistent with studies reported by Tian et al. (81) and Ikeda et al. (82).

After confirming the inhibitory effect of vitamin D on Th17 cells' differentiation, we investigated its effect on the phenotype of Th17 cells. In fact, vitamin D significantly reduced both total IFNγ⁺ and IL-17⁺ IFNγ⁺ double positive cells, in addition to significantly reducing total TNFα⁺ cells and IL-17⁺ TNFα⁺ double positive cells. IFNγ and TNFα are pro-inflammatory cytokines associated with “alternative” Th17 cells and are both essential in the pathogenicity of Th17 cells in multiple sclerosis (22, 25). The decrease in the frequency of these subsets suggests that vitamin D could modulate the phenotype of Th17 cells rendering it less pro-inflammatory in autoimmune diseases. This could control inflammation in MS. The reduction of IFNγ⁺ cells by vitamin D has been described by Tian et al. (81), but the decrease of TNFα⁺ cells put in Th17 polarizing conditions by vitamin D was not reported in previous studies making it an interesting characteristic that should be further explored.

To further explore the effect of vitamin D on Th17 phenotype, surface markers associated with Th17 cells were also studied. The chemokine receptor CCR6 and surface marker CD161 were both significantly reduced in the presence of vitamin D. As for the chemokine receptor CCR4, its frequency remained the same however vitamin D decreased its overall expression and fluorescence intensity on Th17 cells. CCR6 and CCR4 are responsible of Th17 cell trafficking to the central nervous system (18). Their decrease by vitamin D could make the cells less prone to migrate to the CNS and breach the blood brain barrier. This finding was supported by a study done by Tian et al. (81) where cells cultured in the presence of vitamin D showed a decrease in the relative expression of CCR6 mRNA. CD161's biological function is still not very clear but it has been reported as a reliable marker for Th17 cells and its decrease further confirms vitamin D's suppressive effect on Th17 cells (17). No studies examining the effect of vitamin D on CCR4 and CD161's expression by naïve T cells put in Th17 conditions have been reported. These data suggest that vitamin D may inhibit Th17 cell chemotaxis, but would need to be confirmed by a chemotaxis assay.

ROR γ t, the master transcription factor for Th17 cells, was significantly reduced by vitamin D. In fact, its fluorescence intensity was decreased, and the frequency of ROR γ t⁺ IL-17⁺ double positive cells was decreased as well. Tian et al. (81) reported a decrease in the mRNA expression of ROR γ t by vitamin D suggesting that vitamin D may have a direct effect on Th17 cells' transcription and differentiation.

It is important to note that all of these described markers are reportedly highly expressed on memory Th17 cells rather than naïve CD4⁺ T cells polarized *ex-vivo* (88). This could explain why their percentages were relatively low by flow cytometric analysis

even though vitamin D still exerted a clear negative effect on their frequencies and expression.

Th17 cells have a very tight relationship with regulatory T cells. It was interesting to see if vitamin D was able to shift the balance from pro-inflammatory Th17 cells to a more regulatory phenotype. In fact, vitamin D not only increased the frequency of total Foxp3⁺ cells but it also increased its mean fluorescence intensity. Similarly, vitamin D increased the frequency of total CD25^{high} cells. CD25 expression alone is not an indicator of regulatory function; it is rather an activation marker since it is expressed on all activated CD4⁺ T cells. However, when expressed at high levels it is associated with regulatory phenotype (89). This was the case of cells put in Th17 conditions in the presence of vitamin D. The total frequency of Foxp3⁺CD25^{hi} T cells was also enhanced by vitamin D. A study conducted by Joshi et al. (83) showed that vitamin D supplementation in mice immunized with myelin antigen increased the frequency of CD25⁺Foxp3⁺ cells. Another study done by Baeke et al. (89) reported that vitamin D analogs were able to promote the expression of CD4⁺CD25^{high} in regulatory T cells, but this should be further investigated for cells put in Th17 polarizing conditions.

Foxp3 and ROR γ t have been reported to physically bind to each other and neutralize each other's functions. This could explain the decrease in ROR γ t that was encountered with an increase of Foxp3 expression (14).

As previously mentioned, vitamin D reduced the differentiation of Th17 cells but it did not inhibit it completely. When looking into the phenotype of the remaining Th17 cells present, vitamin D showed an increase in the frequency of Foxp3⁺CD25^{hi} Th17 cells compared to Foxp3⁺CD25^{hi} Th17 cells without vitamin D exposure. These results, along

with the fact that vitamin D enhanced the frequency of IL10-producing CD4⁺ T cells (consistent with a study done by Palmer et al. (80)), suggest that vitamin D reduces the differentiation of naïve CD4⁺ T cells into Th17 cells, but also shifts the balance towards a more regulatory phenotype.

The results described here are suggestive for increase on regulatory function but will need to be supplemented with functional assays (e.g. suppression assay) to demonstrate increased regulatory activity.

In addition to analyzing the frequency and phenotype of Th17 cells, we measured the secretion of different cytokines during the 6 days in culture. Flow cytometry is used to measure intracytoplasmic cytokines at a certain time point after restimulation with PMA/ionomycin but it does not show the cumulative effect of vitamin D on the production of cytokines. Therefore, it is considered a more qualitative method than a quantitative one. That is why ELISA and CBA were done on culture supernatants.

Consistent with flow cytometry results, the pro-inflammatory cytokines secreted by Th17 cells IL-17, IFN γ and TNF α were all significantly decreased by vitamin D. Accordingly, vitamin D has been shown to decrease IL-17 and IFN γ in culture supernatants of cells put in Th17 polarizing conditions by Tian et al. (81) without any reports on its effect on TNF α .

IL-6 production remained the same. Vitamin D has been shown to reduce the production of IL-6 by APCs but this effect has not been studied in naïve cells put in Th17 polarizing conditions (90). One explanation could be that recombinant IL-6 is added to the media at high concentrations on the first day of culture in order to induce the differentiation of naïve CD4⁺ T cells into Th17 cells. That is why a change in its concentration could not

be observed. One way to overcome this issue is to analyze the mRNA IL-6 expression in the absence or presence of vitamin D rather than looking at culture supernatants.

The secretion of the anti-inflammatory cytokine IL-4 was increased by vitamin D. The result seems promising since the cells in culture might be shifted by vitamin D towards a Th2 phenotype. Moreover, this increase has not been previously described for cells put in Th17 conditions. However, the concentrations found in the culture supernatants were still extremely low, even after exposure to vitamin D.

Vitamin D had mixed effects on both IL-2 and IL-10 depending on the donor. Some donors showed an increase in their production whereas others showed a decrease. IL-2 is a growth factor and is related to cell survival rates (91). Moreover, just like IL-6, IL-2 was added to culture media on the 3rd day of culture. This could explain its slight increase, though not statistically significant. Studies done on cells put in Th1 polarizing conditions showed a decrease in IL-2 secretion but this has not been previously investigated in cells put in Th17 polarizing conditions (40).

Vitamin D has shown positive effects on the production of IL-10 by Th17 cells in a study done by Joshi et al. (83). In this study, there was no statistical difference in the culture supernatants measured. This could be explained by the kinetics of the production of IL-10 by the cells. IL-10 reportedly peaks after 3 days of culture and then gradually declines (83). Our culture supernatants were taken after 6 days not 3, therefore the peak of IL-10 secretion could have been missed. However, it is important to note that the frequency of IL-10⁺ cells was shown to be increased by flow cytometry in the presence of vitamin D, indicating that cellular production of IL-10 is increased.

In order further investigate vitamin D's effect on IL-2 and IL-10, these results should be repeated in a larger pool of donors.

In conclusion, vitamin D has repeatedly shown a consistent immunomodulatory effect by targeting both T cells and other immune cells including APCs (76, 80). Its negative effect on Th17 cells' differentiation along with shifting their balance to a regulatory phenotype, suggests that vitamin D could be implemented in preventive or therapeutic strategies for multiple sclerosis.

BIBLIOGRAPHY

- (1) Chaplin DD. Overview of the human immune response. *Journal of Allergy and Clinical Immunology* 2006 2;117(2, Supplement 2):S430-S435.
- (2) Bonilla FA, Oettgen HC. Adaptive immunity. *Journal of Allergy and Clinical Immunology* 2010;125(2):33-40.
- (3) Parkin J, Cohen B. An overview of the immune system. *The Lancet* 2001 6/2;357(9270):1777-1789.
- (4) Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology* 1986;136(7):2348-2357.
- (5) Annunziato F, Romagnani S. Heterogeneity of human effector CD4⁺ T cells. *Arthritis Research & Therapy* 2009;11(6):257-264.
- (6) Veldhoen M. The role of T helper subsets in autoimmunity and allergy. *Curr Opin j Immunol* 2009 12;21(6):606-611.
- (7) Romagnani S. The Th1/Th2 paradigm. *Immunol Today* 1997 6;18(6):263-266.
- (8) Jabeen R, Kaplan MH. The symphony of the ninth: the development and function of Th9 cells. *Current Opinion in Immunology* 2012;24:303-307.
- (9) Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nature immunology* 2009;10:864-871.
- (10) Sakaguchi S, Wing K, Miyara M. Regulatory T cells – a brief history and perspective. *European Journal of Immunology* 2007;37(1):116-123.
- (11) Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *International Immunology* 2009;21(10):1105-1111.
- (12) Marwaha AK, Leung NJ, McMurchy AN, Levings MK. Th17 cells in Autoimmunity and Immunodeficiency: Protective or Pathogenic? *Frontiers in Immunology* 2012;3:1-8.
- (13) Bettelli E, Oukka M, Kuchroo VK. Th-17 cells in the circle of immunity and autoimmunity. *Nature immunology* 2007;8(4):345-350.

- (14) Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annual Review of Immunology* 2009;27:485-517.
- (15) O'Connor Jr W, Zenewicz LA, Flavell RA. The dual nature of Th17 cells: shifting the focus to function. *Nature immunology* 2010;11:471-476.
- (16) Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *The American Journal of Pathology* 2012;181(1):8-18.
- (17) Maggi L, Santarlasci V, Capone M, Peired A, Frosali F, Crome SQ, et al. CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol* 2010;40(8):2174-2181.
- (18) Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, et al. CCR6 regulates the migration of inflammatory and regulatory T cells. *The Journal of Immunology* 2008;181(12):8391-8401.
- (19) Oo YH, Banz V, Kavanagh D, Liaskou E, Withers DR, Humphreys E, et al. CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *Journal of Hepatology* ;57(5):1044-1051.
- (20) Oukka M. Th17 cells in immunity and autoimmunity. *Annals of the Rheumatic Diseases* 2008;67:26-29.
- (21) Dong C. Differentiation and function of pro-inflammatory Th17 cells. *Microbes and Infection* 2009;11:584-588.
- (22) Gaffen SL. An overview of IL-17 function and signaling. *Cytokine* 2008 9;43(3):402-407.
- (23) Laurence A, O'Shea JJ, Watford WT. Interleukin-22: a sheep in wolf's clothing. *Nature Medicine* ;14:247-249.
- (24) Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of Th17 cells. *Nature* 2008;453:1051-1055.
- (25) Huang G, Wang Y, Chi H. Regulation of Th17 cell differentiation by innate immune signals. *Cellular & Molecular Immunology* 2012;9:287-295.
- (26) Zhou L, Littman DR. Transcriptional regulatory networks in Th17 cell differentiation. *Current Opinion in Immunology* 2009;21(2):146-152.

- (27) Lezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4⁺ T cells. *Proc Natl Acad Sci USA* 2009;106:876-881.
- (28) Peters A, Lee Y, Kuchroo VK. The many faces of Th17 cells. *Current Opinion in Immunology* 2011;23:702-706.
- (29) McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity* 2008;4:445-453.
- (30) Croxford AL, Mair F, Becher B. IL-23: One cytokine in control of autoimmunity. *Eur J Immunol* 2012;42(9):2263-2273.
- (31) Koenders MI, van den Berg, W. B. Translational Mini-Review Series on Th17 cells: Are T helper 17 cells really pathogenic in autoimmunity? *Clinical & Experimental Immunology* 2010;159(2):131-136.
- (32) Abromson-Leeman S, Bronson RT, Dorf ME. Encephalitogenic T cells that stably express T-bet and ROR gamma t consistently produce IFN γ but have a spectrum of IL-17 profiles. *Journal of Neuroimmunology* 2009;215(1):10-24.
- (33) El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of T(H)17 cells is dependent of IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nature immunology* 2011;12(6):568-575.
- (34) Akdis M, Palomares O, van de Veen W, van Splunter M, Akdis CA. TH17 and TH22 cells: A confusion of antimicrobial response with tissue inflammation versus protection. *Journal of Allergy and Clinical Immunology* 2012;129(6):1438-1449.
- (35) McFarland H, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. *Nature immunology* 2007;8(9):913-919.
- (36) Jadidi-Niaragh F, Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. *Scandinavian Journal of Immunology* 2011;74:1-13.
- (37) Rostami A, Ciric B. Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. *Journal of the Neurological Sciences* 2013;333(1):76-87.
- (38) Compston A, Coles A. Multiple Sclerosis. *The Lancet* 2008;372(9648):1502-1517.
- (39) Comabella M, Khoury SJ. Immunopathogenesis of multiple sclerosis. *Clinical Immunology* 2012;142:2-8.

- (40) Ho S, Alappat L, Awad AB. Vitamin D and Multiple Sclerosis. *Critical Reviews in Food Science and Nutrition* 2012;52(11):980-987.
- (41) Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *The Lancet* 2010;9:727-739.
- (42) Korn T. Pathophysiology of multiple sclerosis. *Journal of neurology* 2008;255:2-6.
- (43) Sriram S, Mitchell W, Stratton C. Multiple sclerosis associated with Chlamydia pneumoniae infection of the CNS. *Neurology* 1998;50(2):571-572.
- (44) Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *The Journal of Experimental Medicine* ;204(12):2899-2912.
- (46) Soldan SS, Berti R, Salem N, Secchiero P, Flamand L, Calabresi PA, et al. Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nature Medicine* 1997;3:1394-1397.
- (47) Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal Relationship Between Elevation of Epstein-Barr Virus Antibody Titers and Initial Onset of Neurological Symptoms in Multiple Sclerosis. *JAMA* 2005;293(20):2496-2500.
- (48) Hernán MA, Olek MJ, Ascherio A. Cigarette smoking and incidence of multiple sclerosis. *Am J Epidemiol* 2001;154(1):69-74.
- (49) Simon KC, Munger KL, Ascherio A. Vitamin D and multiple sclerosis: epidemiology, immunology, and genetics. *Current opinion in neurology* 2012;25(3):246-251.
- (50) Yang CY, Leung PS, Adamopoulos IE, Gershwin ME. The Implication of Vitamin D and Autoimmunity: a Comprehensive Review. *Clinical Reviews in Allergy and Immunology* 2013;45(2):217-226.
- (51) Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* 2009 May;132(Pt 5):1146-1160.
- (52) Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* 2006 Dec 20;296(23):2832-2838.
- (53) Munger KL, Zhang SM, O'Reilly E, Hernan MA, Olek MJ, Willett WC, et al. Vitamin D intake and incidence of multiple sclerosis. *Neurology* 2004 Jan 13;62(1):60-65.

- (54) Arnson Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. *Ann Rheum Dis* 2007 Sep;66(9):1137-1142.
- (55) Soilu-Hanninen M, Airas L, Mononen I, Heikkila A, Viljanen M, Hanninen A. 25-Hydroxyvitamin D levels in serum at the onset of multiple sclerosis. *Mult Scler* 2005 Jun;11(3):266-271.
- (56) Van Etten E, Mathieu C. Immunoregulation of 1,25-dihydroxyvitamin D₃: Basic concepts. *Journal of steroid biochemistry & molecular biology* 2005;97:93-101.
- (57) Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in multiple sclerosis, a review. *Journal of Neuroimmunology* 2008;194:7-17.
- (58) Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann Neurol* 2006 Jul;60(1):12-21.
- (59) Weissert R. The immune pathogenesis of multiple sclerosis. *J Neuroimmune Pharmacol* 2013 Sep;8(4):857-866.
- (60) Frohman EM, Racke MK, Raine CS. Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* 2006 Mar 2;354(9):942-955.
- (61) Voskuhl RR, Martin R, Bergman C, Dalal M, Ruddle NH, McFarland HF. T helper 1 (Th1) functional phenotype of human myelin basic protein-specific T lymphocytes. *Autoimmunity* 1993;15(2):137-143.
- (62) Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003 Feb 13;421(6924):744-748.
- (63) Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 2008 Jan;172(1):146-155.
- (64) Matusevicius D, Kivisakk P, He B, Kostulas N, Ozenci V, Fredrikson S, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* 1999 Apr;5(2):101-104.
- (65) Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. *Blood* 2013 Mar 28;121(13):2402-2414.

- (66) Brucklacher-Waldert V, Stuermer K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* 2009 Dec;132(Pt 12):3329-3341.
- (67) Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005 Jan 17;201(2):233-240.
- (68) Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006 Jul 1;177(1):566-573.
- (69) Shahrara S, Pickens SR, Dorfleutner A, Pope RM. IL-17 induces monocyte migration in rheumatoid arthritis. *J Immunol* 2009 Mar 15;182(6):3884-3891
- (70) Hofsetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, Toyka KV, et al. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cellular immunology* 2005;237:123-130.
- (71) Martin AJ, Zhou L, Miller SD. MicroRNA--managing the TH-17 inflammatory response. *Nat Immunol* 2009 Dec;10(12):1229-1231.
- (72) Holick MF, MacLaughlin JA, Clark MB, Holick SA, Potts JT, Jr, Anderson RR, et al. Photosynthesis of previtamin D3 in human skin and the physiologic consequences. *Science* 1980 Oct 10;210(4466):203-205.
- (73) Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. *Physiol Rev* 1998 Oct;78(4):1193-1231.
- (74) Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, et al. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* 1998 Mar;13(3):325-349.
- (75) Carlberg C, Polly P. Gene regulation by vitamin D3. *Crit Rev Eukaryot Gene Expr* 1998;8(1):19-42.
- (76) Cantorna MT. Vitamin D and its role in immunology: multiple sclerosis, and inflammatory bowel disease. *Prog Biophys Mol Biol* 2006 Sep;92(1):60-64.
- (77) Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab* 1983 Dec;57(6):1308-1310.

- (78) Hauser SL, Weiner HL, Che M, Shapiro ME, Gilles F, Letvin NL. Prevention of experimental allergic encephalomyelitis (EAE) in the SJL/J mouse by whole body ultraviolet irradiation. *J Immunol* 1984 Mar;132(3):1276-1281.
- (79) Kongsbak M, Levring TB, Geisler C, Rode von Essen M. The vitamin D receptor and T cell function. *Frontiers in Immunology* 2013;4(148):1-10.
- (80) Palmer MT, Lee YK, Maynard CL, Oliver JR, Bikle DD, Jetten AM, et al. Lineage-specific effects of 1,25-dihydroxyvitamin D(3) on the development of effector CD4 T cells. *The Journal of Biological Chemistry* 2011;286(2):997-1004.
- (81) Tian Y, Wang Chaokui, Ye Z, Xiao X, Kijlstra A, Yang P. Effect of 1,25-Dihydroxyvitamin D3 on Th17 and Th1 Response in Patients with Behcet's Disease. *Immunology and Microbiology* 2012;53(10):6434-6441.
- (82) Ikeda U, Wakita D, Ohkuri T, Chamoto K, Kitamura H, Iwakura Y, et al. 1 α ,25-Dihydroxyvitamin D3 and all-trans retinoic acid synergistically inhibit the differentiation and expansion of Th17 cells. *Immunology Letters* 2010;134:7-16.
- (83) Joshi S, Pantalena LC, Liu XK, Gaffen SL, Liu H, Rohowsky-Kochan C, et al. 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol Cell Biol* 2011 Sep;31(17):3653-3669.
- (84) Reichel H, Koeffler HP, Tobler A, Norman AW. 1 alpha,25-Dihydroxyvitamin D3 inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes. *Proc Natl Acad Sci U S A* 1987 May;84(10):3385-3389.
- (85) Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001 Nov 1;167(9):4974-4980.
- (86) Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, et al. Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. *J Immunol* 2007 Nov 1;179(9):6273-6283.
- (87) Cippitelli M, Fionda C, Di Bona D, Di Rosa F, Lupo A, Piccoli M, et al. Negative regulation of CD95 ligand gene expression by vitamin D3 in T lymphocytes. *J Immunol* 2002 Feb 1;168(3):1154-1166.
- (88) Ramesh R, Kozhaya L, McKeivitt K, Djuretic IM, Carlson TJ, Quintero MA, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* 2014 Jan 13;211(1):89-104.

(89) Baeke F, Korf H, Overbergh L, Verstuyf A, Thorrez L, Van Lommel L, et al. The vitamin D analog, TX527, promotes a human CD4⁺CD25^{high}CD127^{low} regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation. *J Immunol* 2011 Jan 1;186(1):132-142.

(90) Lefebvre d'Hellencourt C, Montero-Menei CN, Bernard R, Couez D. Vitamin D3 inhibits proinflammatory cytokines and nitric oxide production by the EOC13 microglial cell line. *J Neurosci Res* 2003 Feb 15;71(4):575-582.

(91) Kelly E, Won A, Refaeli Y, Van Parijs L. IL-2 and related cytokines can promote T cell survival by activating AKT. *J Immunol* 2002 Jan 15;168(2):597-603.

(92) van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Cornelissen F, van Leeuwen JP, et al. TNF blockade requires 1,25(OH)₂D₃ to control human Th17-mediated synovial inflammation. *Ann Rheum Dis* 2012 Apr;71(4):606-612.

