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Vitamin D responsiveness of multiple sclerosis associated genes in CD4+ T cells

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- Ina Skaara Brorson

Abstract

Multiple Sclerosis (MS) is an inflammatory autoimmune disease. Several lines of evidence indicate that vitamin D sufficiency acts to suppress T-cell driven autoimmune diseases. Active vitamin D ($1,25(OH)_2D_3$) acts through the vitamin D receptor (VDR), which is a nuclear receptor that forms dimers with the retinoic X receptor to bind to vitamin D responsive element (VDRE), and alter gene expression. Recently, 110 MS associated loci have been identified, where the majority contains VDRE in their gene promoter or regulatory region. CD4+ T cells are thought to be the major players in MS, hypothesized from the animal model with a MS-like disease, experimental encephalomyelitis (EAE). Active vitamin D acts directly on the T lymphocyte VDR to inhibit EAE. Today, there are few genes known to be direct targets of VDR in T cells. Naive T cells have low expression of VDR, which can be induced upon T cell activation. The upregulation of VDR mediates a shift of active T cells to a more anti-inflammatory profile.

Our lab has studied the vitamin D responsiveness of a selection of MS associated gens. In an initial screening, 13 genes were analyzed for vitamin D responsiveness in CD4+ T cells, and *IL2RA* and *TAGAP* was vitamin D responsive when co-cultured with aCD3/CD28 beads. These genes are known to have a role in T cell immunity. s. The aims of this project were to study if vitamin D alone is sufficient to trigger gene regulation and to investigate whether genotype of MS associated loci correlates with gene expression of *TAGAP* and *IL2RA* in response to vitamin D. In addition, gene expression of *IL2RA* and *TAGAP* in MS patients and healthy controls were compared. Samples from MS patients (n = 32) and healthy controls (n = 30) were genotyped for rs1738074 and rs7090512 in *TAGAP* and *IL2RA*, respectively. CD4+ T cells were cultivated and treated with vitamin D as described. Cells were harvested after 6, 24 and 48 hours and gene expression was measured by qPCR,

We concluded that (1) vitamin D (without co-culture with αCD3/CD28) is sufficient for regulation of *IL2RA* and *TAGAP* expression, they were revealed to be upregulated and repressed, respectively, (2) the presence of MS risk variant in *IL2RA* correlated with higher expression in CD4+ T cells treated with vitamin D and co-cultured with αCD3/CD28 beads after 24 hours in culture and (3) vitamin D treatment results in lower expression of *TAGAP* in

CD4+ T cells from MS patients than in healthy controls, however, no expression differences of *IL2RA* were observed in these groups.

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1 Introduction

The immune system exists to protect our body from infections and keep us healthy. If the immune system of an organism mistakenly recognizes its host as foreign, or becomes overactive – autoimmunity occurs. Multiple sclerosis **(MS)** is one out of many diseases that are possibly caused by our own immune system. Although it is unclear what the exact disease etiology is, numerous environmental and genetic factors have been shown to be associated with the disease.

This section will briefly address the background of MS and MS genetics, before the functions of T lymphocytes and how T cell regulation maintains immune homeostasis are described. Bearing in mind the various functions of T cells, vitamin D and its receptor are subsequently discussed with respect to their proposed function in T cell regulation before the rationale and aims of this study are finally addressed.

1.1 Multiple Sclerosis

1.1.1 Background

MS is a demyelinating and inflammatory disorder affecting the central nervous system **(CNS)**. The prevalence of MS cases in Norway is one of the highest prevalence worldwide, with approximately 200 cases per 100 000 inhabitants [1]. The cause of MS is unknown, however, a general consensus is that MS is a multifactorial disease that develops in individuals with a genetic susceptibility, triggered by common environmental factors such as smoking, low level of vitamin D in serum and viral infections [2, 3]. In addition, the current understanding of the genetic factors involved in MS pathogenesis also implies that both epigenetic factors as well as post-genomic regulatory events are involved in disease etiology [4].

1.1.2 Vitamin D and MS

At higher degrees of latitude, and with less exposure of sunlight, an increased risk of MS is observed [5, 6]. Low serum levels of $25(OH)D_3$, the major circulating form of vitamin D, are also associated with MS prevalence and disease activity [7, 8]. Recently, a large-scale genetic screen also implicated the relevance of vitamin D processing molecules in MS [9]. $1,25(OH)_2D_3$ signaling modulates both innate and adaptive immune responses [10], even

though it was originally described as an essential hormone for mineral bone homeostasis. Naive T cells have a low expression of vitamin D receptor **(VDR)**, which is induced upon T cell activation [11, 12], whereby $1,25(OH)_2D_3$ mediates a shift towards a more anti-inflammatory cytokine profile [13].

1.1.3 MS genetics

As early as in the 1970s, the first genetic factors in MS were recognized with the identification of DNA variations in the human leukocyte antigen **(HLA) (**termed MHC when not specified to humans) gene complex that conferred MS risk. However, the complexity of the genetic component became clearer in recent years when increasingly large genome-wide association studies (GWAS) revealed numerous associated risk variants outside the HLA-complex [9, 14]. Of the genes found outside the HLA-complex, there is a strong overrepresentation of immunologically relevant genes, particularly genes known to regulate T cells [9].

1.1.4 MS susceptibility loci

The HLA gene complex allele *DRB1**15:01 has the strongest association with MS in the Western populations [9]. The HLA genes are located on the short arm of chromosome 6 at p21.3. The gene stretches for almost 4000 kilo bases **(kb)** of DNA. The HLA molecules are encoded on multiple genes that form the complexes. There are now more than 110 established multiple sclerosis risk variants outside the MHC complex [9] [4] [15]. The majority of the non-HLA MS susceptibility loci are located in or close to immune regulatory genes. The majority of single nucleotide polymorphisms **(SNPs)** identified as MS risk loci are found in non-coding regions of the genome [9]. When one SNP allele occurs more or less frequently in a group of patients than in healthy groups, it is considered disease associated and protective associated, respectively. The functional consequence of most of these SNPs is not known, however, MS associated SNPs are enriched at deoxyribonuclease I **(DNase I)** hypersensitive sites **(DHS)**. DNase I hypersensitive sites are precise and reliable indicators of open and active chromatin, indicating a functional role of MS associated SNPs in the regulation of gene transcription [16].

1.1.5 MS – a T cell mediated autoimmune disease

CD4+ T cells have for a long time been thought to be the major player in MS pathogenesis, hypothesized from animal models with a MS-like disease, experimental autoimmune

encephalomyelitis **(EAE)**, induced by myelin reactive T cells [17-20]. Interestingly, $1,25(OH)_2D_3$ acts directly on the T lymphocyte VDR to inhibit EAE [21]. Both CD4+ T cells and CD8+ T cells have been found in MS lesions, where CD4+ T cells predominate in acute lesions and CD8+ T cells are more frequently present in chronic lesions [22]. In addition, a higher proportion of activated T cells are observed in the peripheral blood of MS patients [22], further strengthening the hypothesis that T cell activation is part of the disease etiology.

Autoimmune diseases are caused by the adaptive immune system that initiates an immune response directed against healthy cells and tissues, as opposed to tolerance for self-components. During the development of B- and T-cells, various mechanisms contribute to this immunological self-tolerance. Already in thymus and bone marrow, there is a negative selection where cells binding too strongly to self-peptides are deleted or arrested. For T-cells in the thymus, potential auto-reactive cells are terminated when binding too strongly to the major histocompatibility complex **(MHC)** presenting self-peptides. Despite an effective negative selection in thymus, some T cells reactive to self-peptides escape deletion and enter circulation. For a T-cell mediated autoimmune disease, genes that alter the capacity of lymphocytes to become autoreactive may influence development of such diseases. Immature B-cells in the bone marrow are exposed to self-antigens expressed by different cells circulating in the blood plasma. Immature B-cells with receptors binding self-antigens are retained in the bone marrow and given a chance to lose their self-reactivity by altering their B-cell receptor. In humans, it is thought that autoimmunity is induced by a combination of genetic and environmental factors [23].

1.2 The immunology of T lymphocytes

The focus of this section is T cell function in the immune response to underline why T cells, in particular CD4+ T cells, are interesting to investigate in autoimmune diseases like MS. Central to this section will be the activation of T cells, and the different subsets they differentiate into. T cells are a type of leukocytes, and comprise the main component of the adaptive immune system, together with B cells. Both cell subtypes possess specific antigen receptors, which are critical for pathogen recognition and to start an immune response upon infection. B cells secrete immunoglobulin **(Ig)** molecules that target specific antigens, whereas T cells are characterized by expression of the T cell receptor **(TCR)** on the cell

surface. The effector function of T cells is carried out through antigen specific interactions with other cells in the body.

1.2.1 CD4+ T cell subsets

T cells can be grouped depending on the expression of CD4 or CD8 surface molecules, *i.e*. CD4+ or CD8+ T cells. In short, CD4+ T cells – T helper cells - provide help for B cell differentiation, and CD8+ T cells are involved in class I restricted lysis of antigen-specific targets, and are termed cytotoxic T cells. The CD4 molecule binds to the MHC class II β-chain, expressed on APCs. In contrast, the CD8 molecule binds the α-domain of the MHC class I, which is expressed in most cell types. Here, a focus will be placed on CD4+ T cells, as these are the cells used in this study.

Naive CD4+ T cells can differentiate into several T cell subsets, including T helper 1 **(Th1),** T helper 2 **(Th2),** T helper 17 **(Th17)** and T regulatory cells **(Treg).** Each of these subsets possesses unique properties for pathogen elimination. Th1 cells produce mainly IL-2, tumor necrosis factor α **(TNF-α)** and interferon-γ **(IFN-γ),** which lead to macrophage activation and inflammation. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, mainly leading to B cell differentiation and the production of neutralizing antibodies. Th17 cells produce mainly IL-17. Tregs, which are CD4+CD25+Foxp3+ T cells, suppress T-cell proliferation by producing immunosuppressive cytokines, thereby maintaining immunological tolerance [24]. In contrast to other effector T cells, Tregs are not able to produce IL-2, but are highly dependent on IL-2 for survival [25].

1.2.2 T cell activation and co-stimulation

Upon infection, the challenge for the immune system is to bring naive T cells specific for the pathogen into contact with pathogenic antigens, presented by an antigen-presenting cell **(APC)**. T cells only recognize peptide antigens when these peptides are presented in a complex with MHC on the surface of APCs [26].

Dendritic cells **(DCs)** are examples of APCs, whose main function is to trigger T cell responses. Naive T cells first encounter antigens presented by DCs in secondary lymphoid tissues, such as lymph nodes. When a T cell encounters an MHC:peptide complex to which the TCR binds, the T cell is retained in the lymph node and activated. Though the MHC:peptide complex is necessary, this alone is not sufficient to trigger the T cell activation and proliferation; a co-stimulatory signal is required. The cell surface protein on

naive T cells that receives the co-stimulatory signal is called cluster of differentiation 28 **(CD28)** [26]. The CD28 receptor is structurally related to its ligands B7.1 and B7.2 proteins, also known as CD80 and CD86, presented by APC. In the absence of co-stimulatory signals, T cells can die or become anergenic, thus arresting proliferation or end in apoptosis terminating the initiation of the immune response [27].

In addition, expression of co-receptors in T cells is also required for adequate activation. These co-receptors are either CD4 or CD8, and assist TCR in APC communication. Via their intracellular domains they amplify the signals through the TCR [28]. After T cell activation, an additional receptor for B7 becomes expressed on T cells [29], called cytotoxic T lymphocyte associated protein 4 **(CTLA-4).** CTLA-4 is structurally similar to CD28, but binds more strongly to B7, and functions as antagonist to CD28 [26]. In other words, CTLA-4 reduces the immune response [30], resulting in inhibition of proliferation. Hence, the balance between the positive and negative regulatory signals will determine the immune response by the T cells.

1.2.3 Intracellular signaling

The signal provided upon binding of TCR to the MHC:peptide complex is transmitted to the cytoplasmic tails of CD3 proteins, which are associated to the TCR (figure 1.1). The cytoplasmic tail of CD3 contains immune-receptor tyrosine-based activation motifs **(ITAMs)**, which are associated with cytoplasmic protein tyrosine kinases. CD4 (and CD8) have cytoplasmic tails associated with the protein tyrosine kinase **Lck.** Upon binding of MHC ligand, ITAMs are phosphorylated by this kinase, and the cytoplasmic protein tyrosine kinase zeta-chain associated protein **(ZAP-70)** binds the phosphorylated ITAMs, initiating intracellular signaling pathways leading to altered gene expression produced by the transcription factor nuclear factor of activated T cells **(NFAT),** nuclear factor kappa-light-chain-enhancer of activated B cells **(NFκB)** and activator-protein 1 **(AP-1).** The combined actions of these transcription factors activate genes involved in T cell proliferation and differentiation into different T cell subsets, thereby initiating continuation of the immune response.

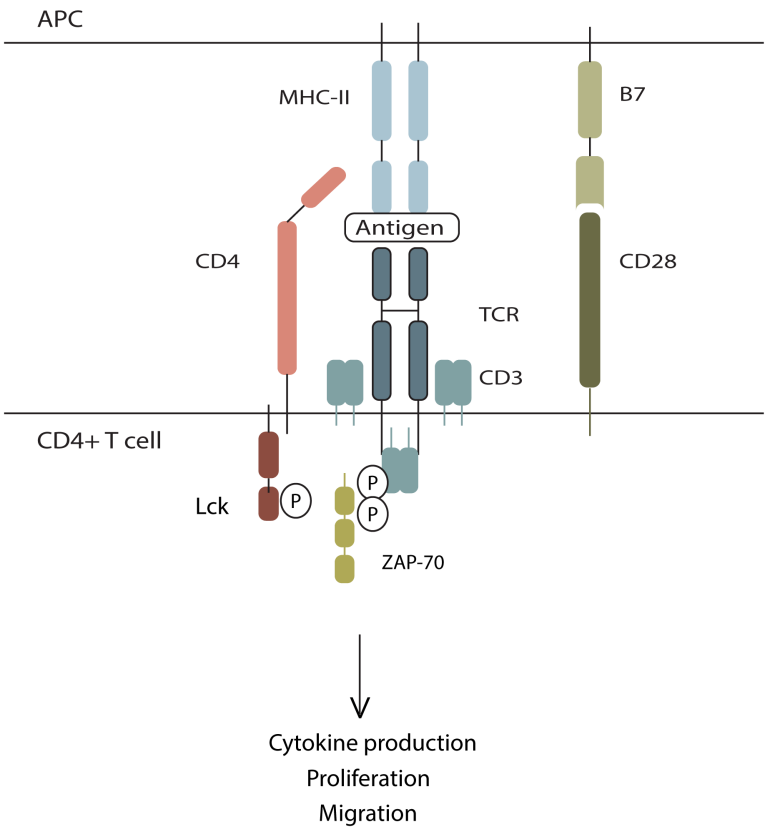

Figure 1.1: TCR and MHC:antigen complex in activation of CD4+ T cells in presence of co-stimulatory molecules. Upon T-cell activation, the MHC complex presents the antigen to a T cell through its TCR. In the presence of costimulatory signals, which are delivered by the interaction between B7 on the APC with CD28 in T cells, the co-receptor CD4 activates Lck through its intracellular ITAM-domain, which in turn phosphorylates ZAP-70, leading to a chain of events leading to gene regulation and finally production of cytokines and proliferation and migration of T cells.

1.3 Vitamin D and vitamin D receptor

1.3.1 Transcription

Before vitamin D and its receptor, which is a nuclear receptor and a transcription factor, are described in more detail, transcription of genes is briefly discussed. Transcription is the process where DNA is copied into a complementary, single stranded RNA strand. This process is catalyzed by RNA polymerases and regulated by transcription factors, co-factors and chromatin remodeling complexes and histone modifiers. RNA polymerase II transcribes genes into mRNA resulting in proteins. Transcription factors are sequence specific DNA-bindings proteins that can directly associate with the promoter regions in proximity of genes or with enhancers further away, thereby regulating expression of their target gene. Depending on the transcription factor as well as their interaction partner and

post-translational modifications, the gene can be either enhanced or repressed. Various mechanisms are in play to perform these regulations. Transcription factors might block or enhance the ability of the RNA polymerase to interact with the coding DNA, and might recruit co-activators or co-repressors to the transcription complex [31], or influence chromatin state and thus DNA accessibility.

1.3.2 Vitamin D metabolism

Vitamins *(*from: *vital amines)* are organic compounds, which organisms are not able to synthesize themselves in sufficient amounts [32]. Nowadays, it is known that vitamins have an important role in the immune system, and vitamin A and D in particular act on the immune system in specific ways [33].

The major source of vitamin D is sun exposure and diet. Vitamin D exists in a biologically inert form and requires hydroxylation by a member of the cytochrome P **(CYP)** family to become the major circulating form of vitamin D, 25-hydroxy vitamin D_3 ($25(OH)D_3$), also called cholecalciferol. In the liver, several of such CYP $25(OH)D_3$ enzymes (CYP2R1, CYP27A1 and CYP3A4) are found [34]. A second hydroxylation of the circulating form of vitamin D, catalyzed by 1α-hydroxylase **(**encoded by ***CYP27B1*)** is required to become its active metabolite 1,25-dihydroxy vitamin D_3 ($1,25(OH)D_3$), also called calcitriol [34]. The vitamin D 24-hydroxylase, encoded by *CYP24A1* is able to induce an inactivating pathway for vitamin D metabolites. This enzyme is transcriptionally regulated by the levels of $1,25(OH)_2D_3$, as well as by the fibroblast growth factor 23 **(FGF23)** [35]. In a negative feedback loop, $1,25(OH)_2D_3$ induces expression of *CYP24A1*, which in turn limits the production by repressing the *CYP27B1* gene and also initiates degradation of $1,25(OH)_2D_3$ by catalyzing hydroxylation of $1,25(OH)_2$ or 25(OH) to produce inactivating metabolites [36, 37].

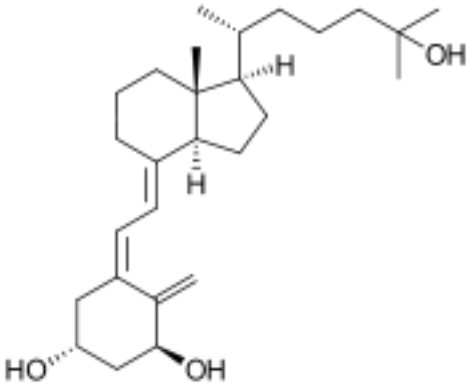

Figure 1: Chemical structure of $1,25(OH)_2D_3$. The precursor of the active form of vitamin D requires hydroxylation by members of the CYP-family, (CYP2R1, CYP27A1 and CYP3A4) to become the major circulating form of vitamin D, $25(OH)D_3$. A second hydroxylation is required to become the active metabolite $1,25(OH)_2D_3$, illustrated in the figure, catalyzed by 1α-hydroxylase (CYP27B1).

1.3.3 VDR – a nuclear transcription factor

Signaling through the Vitamin D receptor **(VDR)** appears to have a dampening immune effect as VDR knock out mice **(VDR-KO)** have a vigorous immune response and increased risk of development of autoimmune diseases [38]. Naive T cells have a low expression of VDR, which is induced upon activation or exposure to $1,25(OH)_2D_3$ [11, 12]. $1,25(OH)_2D_3$ has a direct effect on T lymphocyte proliferation and cytokine production [39], and it has also been shown to inhibit transcription of the IL-2 gene [40], suggesting that up-regulation of VDR serves as a control mechanism to prevent possible overreaction of the immune system, and thereby shifting the T cell to a more anti-inflammatory profile. Moreover, $1,25(OH)_2D_3$ signaling represses the transcription of genes encoding Th1 pro-inflammatory cytokines, such as IFN-y, IL17 and IL21 [10, 41].

Vitamin D receptors (VDRs) are discovered in monocytes and later in APCs, also suggesting a role for $1,25(OH)_2D_3$ [11]-[42]-[43]. The Vitamin D receptor belongs to a family of ligand induced transcription factors. The protein is 427 amino acids long. The two major and functional units are the N-terminal zinc finger DNA binding domain **(DBD)** and the C-terminal ligand-binding domain **(LBD)**[44], illustrated in figure 1.3. The LBD contributes to binding of ligand, dimerization and transcriptional activity.

Vitamin D, together with its many metabolites are transported in the blood by the vitamin D-binding protein **(DBP)** [45]. The vitamin dissociates from DBP when entering a target

cell with VDR in the cytoplasm and binds VDR before the complex enters nucleus and forms a heterodimer with the nuclear receptor retinoid X receptor **(RXR)** [45].

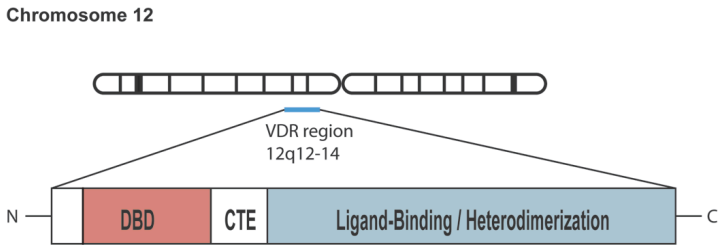

Figure 1.3: Schematic drawing of the VDR gene and protein. The different domains of the 427-amino acid human VDR, located at chromosome 12, are highlighted in the figure. The two major functional units are the N-terminal zinc-finger DNA binding domain (DBD) and the C-terminal ligand binding (LBD) / heterodimerization domain. (CTE = C-terminal extension)

1.3.4 VDR mediated regulation of gene expression

$1,25(OH)_2D_3$ binds VDR with high affinity, and triggers association of the VDR to its heterodimeric partner, RXR. The heterodimer then binds to a specific sequence in the promoter region of a target gene, called vitamin D responsive elements **(VDREs)** [46]. VDREs consists of a direct repeat of two hexanucleotide half-elements consisting of three nucleotides (direct repeat 3, DR3) spacing, or an everted repeat of the two half-elements with a six nucleotide (everted repeat 6, ER6) spacing [35], where DR3s are being more common [35].

Ligand induced heterodimerization of VDR and RXR leads to conformational changes, which in turn will recruit co-modulators for transcriptional regulation. It is suggested that DNA looping can facilitate contact between co-modulators and enhancer elements to the transcriptional start site [46]. Target genes are repressed or activated dependent on whether the VDR-RXR heterodimer attracts co-repressor or co-activator proteins. Several genes regulated by vitamin D contain only one copy of VDRE in the proximal promoter. However, later studies have revealed multiple target genes possessing multiple VDREs [47]. These genes, containing multiple VDREs, require all VDR-RXR docking sites for maximal induction by $1,25(OH)_2D_3$ [35]. Nontheless, there are VDR regulated genes lacking DR3 or ER6, for which the mechanism of regulation is not understood [40].

1.3.5 VDR can induce and repress gene expression

Numerous gene targets are identified for VDR, and these include genes involved in the regulation of calcium metabolism and bone formation. More recently it has also been established that VDR may have a role in all phases of T cell differentiation [48]. The transcriptional activation of genes regulated by $1,25(OH)_2D_3$ through VDR is currently hypothesized to be a multistep process initiated at the promoter region of expressed genes. Transactivation requires an open chromatin state at the targets' gene regulatory region [49]. Proteins conferring such activity are recruited by nuclear receptors in response to ligand binding. On the other hand, ligand-dependent repression of gene transcription by VDR-RXR happens by recruitment of nuclear co-repressors to alter the chromatin in proximity to the gene, to heterochromatin. Enzymes that modulate chromatin accessibility are those with chromatin remodeling activities and histone modifying abilities, like ATP dependent chromatin remodelers and histone methyl- or acetyl- transferases.

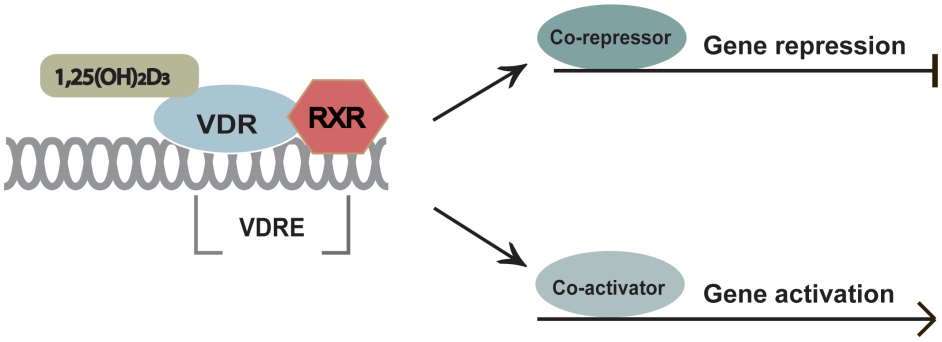

Figure 1.2: VDR can act as a transcriptional activator or repressor. The active form of vitamin D, $1,25(OH)_2D_3$ mediates its biological effect through binding to the vitamin D receptor (VDR), which is a nuclear receptor dimerizing with the retinoic X receptor (RXR). This heterodimer binds to vitamin D responsive elements (VDRE) in regulatory regions of genes, and alter gene expression according to the co-factor. Recruitment of co-repressors in complex with VDR-RXR leads to suppression of gene transcription, whereas recruitment of co-activators enhances gene expression.

1.4 The rationale of this study

By now, approximately 110 MS susceptibility genes are identified [9, 15] The majority of these are thought to play a role in T cell biology. To date, only few genes are proven to be direct targets of vitamin D in T cells [42]. Since T cells are important in MS pathogenesis, and because low levels of vitamin D in serum are associated with an increased risk of MS, it is of interest to see if MS susceptibility genes are regulated by vitamin D in T cells. Using

data from previous *in silico* analyses [47] 80 % of the MS associated gene have one or more VDREs in their gene promoter or regulatory regions (-10kb-(+)5kb). Of these genes, 23 genes contained two or more VDREs. Our lab has studied the vitamin D responsiveness of a selection of these genes in T cells. Genes that had either (i) more than one VDRE in their regulatory region or (ii) that contained an MS risk SNP and one VDRE in the regulatory region of the gene and that were potentially expressed in CD4+ T cells (analyzed *in silico*; [50, 51]) were chosen for the study. 13 genes were analyzed for vitamin D responsiveness in CD4+ T cells isolated from six healthy donors. *IL2RA* was consistently upregulated by vitamin D, whereas *TAGAP* was down regulated in these cells (Berge, unpublished).

When gene expression was measured in these initial studies, the cells were co-cultured with αCD3/28 beads at the time of vitamin D addition and during the whole $1,25(OH)_2D_3$ treatment period (Berge, unpublished). Whether $1,25(OH)_2D_3$ can modulate expression of *TAGAP* and *IL2RA* also in the absence of αCD3/28 beads is not known. Both genes did contain an MS associated SNP in their promoter and they have at least one VDRE in their regulatory gene regions, see table 1.

Table 1: The genes of interest selected for this study.

	IL2-Rα	***TAGAP***
Approved name	Interleukin 2 receptor, alpha	T-cell activation Rho GTPase activating protein
Gene family	Interleukin and interleukin receptors	Rho GTPase activating proteins
Chromosome nr	10	6
Number of VDREs	1	2
MS associated SNPs	rs2104286 (Intronic) rs3118470 (Intronic) rs7090512 (Promoter)	rs212405 (Promoter) rs1738084 (5`UTR)

1.4.1 **Interleukin 2 and its receptor**

One of the genes activated by the intracellular pathway initiated by ZAP-70, encodes the cytokine interleukin 2 **(IL-2)** through the transcription factor NFAT. IL-2 is essential for expansion and differentiation of T-cells. In naive T cells, the IL-2 receptor is a heterodimer consisting of two chains, a β- and γ-chain, which bind IL-2 with low affinity. However, upon T cell activation, the naive T cell expresses a third component, the IL-2 receptor α

chain **(**encoded by the ***IL2RA*** gene**)**, also called CD25. The β:γ heterodimer with the α chain constitutes a high affinity IL-2 receptor, subsequently making the cell more responsive to IL-2. Binding of IL-2 to its high affinity receptor triggers cell proliferation, and initiates a series of phosphorylation events. Phosphorylation results in transcriptional activation of cytokine targeted genes, including the Treg transcription factor Foxp3 and the IL2RA itself [52].The expression of CD25 on effector T cells is reduced through a negative feedback loop that will direct the cells towards becoming inactivated [52, 53]. CD25 can also be cleaved by enzymes from the T cell surface upon activation, resulting in soluble IL2RA **(sIL2RA)** [54].

It has been demonstrated in knock-out mice that a deficiency of *IL2, IL2RA* or *IL2RB* lead to early death due to severe autoimmunity [55, 56], and in humans it is known that that a deficiency in the *IL2RA* gene can result in autoimmunity [57, 58]. In contrast, it is well documented that vitamin D results in decreased IL2 secretion *in vivo* [40, 59, 60], hence less ligand for the IL2RA. Evidence supporting an important role of the IL2R-pathway in immunological tolerance is revealed by GWAS, which have identified several genetic variants in the IL-2/IL2R pathway to be associated with MS [9].

1.4.2 Current knowledge about *TAGAP*

TAGAP encodes a protein that is a member of the Rho GTPase-activating **(GAP)** family [61]. T-cell activation RhoGTPase-activating protein ***(TAGAP)*** has a role in activation of T cells, as suggested by its name. The Rho family of GTPases functions as molecular switches that are turned on or off in response to a variety of extracellular stimuli. Rho proteins in the guanine-triphosphate protein **(GTP)-**bound active state can interact with effectors leading to diverse biological responses including gene transcription, cell cycle regulation and control of apoptosis. *TAGAP,* being a GAP, induces the inactive form of the Rho molecule by dephosphorylation of Rho and thereby releasing GTP, which in turn allows guanine-diphospohate protein **(GDP)** to bind. Guanine nucleotide exchange factors **(GEFs)** on the other hand, stimulate the release of GDP, allowing GTP to bind, returning Rho to an active state in a cyclical fashion. *TAGAP* propagates the inactive form of the Rho molecule, illustrated in figure 1.4, which in turn will have a negative effect downstream of T cell activation, thereby being an interesting factor to study regarding T-cell driven autoimmune disease. The expression of *TAGAP* increases transiently in activated T cells and is more highly regulated than other GAP- proteins [61].

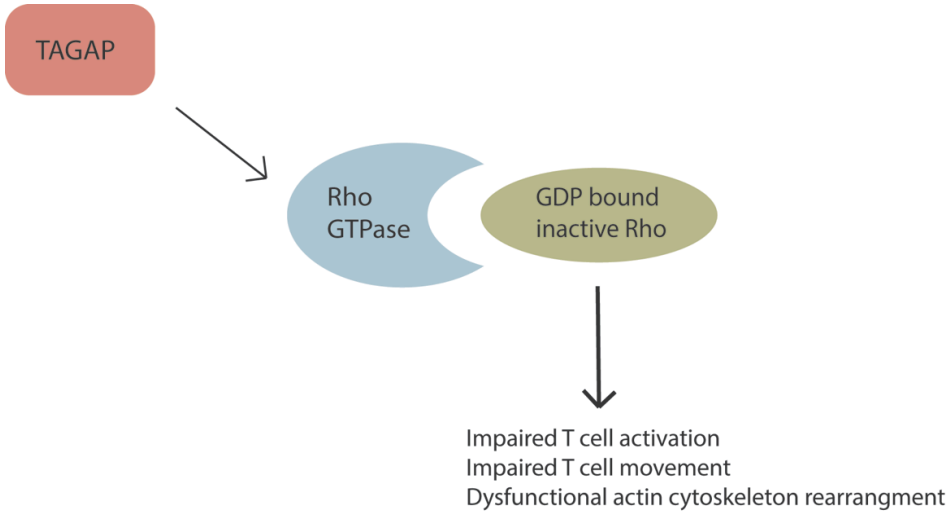

Figure 1.4: A simplified biochemical model of the function of T cell activation Rho GTPase-activating protein ***(TAGAP)***. *TAGAP* encodes a member of the Rho GTPase activator superfamily[1]. TAGAP increases the rate of Rho GTPases, which hydrolyzes Rho and mediates the inactive form of the Rho molecule with negative downstream effects. The figure is inspired from [62].

High expression levels of *TAGAP* are shown to be associated with other immune mediated diseases like rheumatoid arthritis, coeliac disease and diabetes mellitus [63]. Increased *TAGAP* expression has also been established in severe diseased Chrons` patients [62].

1.5 Aims of the study

Naive T cells have low expression of VDR, which can be induced upon T cell activation [11, 12]. The up-regulation of VDR mediates a shift of active T cells into a more anti-inflammatory profile [13]. Recent data suggests that vitamin D plays an important role for T cell homeostasis in MS as it acts directly on CD4+ T cells to induce cytokine secretion and reduce proliferation [12].

Two of the recently identified MS susceptibility genes (Berge, unpublished); *TAGAP* and *IL2RA,* known to regulate T cell mediated immunity, contain VDREs in their regulatory region, and are thereby possible candidates for vitamin D regulation.

The main aim of this study is addressed through the following questions:

1. Is vitamin D alone a sufficient signal to regulate expression of *TAGAP* and *IL2RA* at both the mRNA- and protein level?

[1] http://www.ncbi.nlm.nih.gov/gene/117289

2. Is there a correlation between the presence of an MS risk variant in both *TAGAP* and *IL2RA* and their vitamin D response in CD4+ T cells?

3. Is there a difference in expression of *TAGAP* and *IL2RA* in healthy controls and MS patients?

To answer these questions, gene expression was measured in CD4+ T cells by quantitative real-time PCR in conditions with and without vitamin D treatment, in the presence and absence of T cell activation.

2 Methods

This section describes the laboratory techniques used in this study. An introduction to each method is briefly described prior to the following procedures. Where commercial kits were used, the supplied protocols from manufacturer are referred to.

2.1 Isolation of human CD4+ T cells from whole blood

Human primary CD4+ T cells were isolated from whole blood of MS patients and healthy volunteers. Authorized personnel collected blood in ethylenediaminetettraacetic acid **(EDTA)** coated tubes to prevent blood from coagulating.

Human blood needs to be handled with care to avoid possible infections, as blood can be potential carrier of infections. Gloves were always used, and gloves and other plastics contaminated with blood were disposed in special waste. Cell work requires a sterile environment to avoid any kind of contamination, such as bacteria, fungi, virus or other cultured cells. To preserve a sterile environment, sterile techniques are at all times practiced when working with cells. Procedures are performed in low air flow **(LAF)** - benches, and frequently change of gloves in addition to use of sterile equipment and filtered tips, all contributes to a sterile environment.

2.1.1 Procedure for isolation of PBMC from whole blood

Peripheral blood mononuclear cells **(PBMC)** can be isolated from whole blood using density gradient centrifugation [64] (Lymphoprep). In this method, erythrocytes and granulocytes will sediment through the medium while PBMC is held at the sample/medium interphase, illustrated in figure 2.1. Following this procedure, isolation of desired lymphocytes, such as CD4+ T cells can be performed.

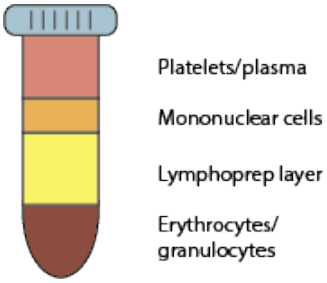

Figure 2.1: Schematic illustration of PBMC separation with lymphoprep. Lymphoprep (Stem cell Technologies) is a density gradient medium that separates PBMC from blood by a density gradient generated through centrifugation. Erythrocytes and granulocytes have a higher density than lymphoprep and mononuclear cells, which makes the separation of PBMC possible.

Procedure:

1. Cover the LAF-bench with paper towels

2. Add 1 ml of 100 mM EDTA to a cell culture flask, 75 ml. Pipette the freshly collected blood to the cell culture flask. Use 10 ml cold Roswell Park Memorial Institute **(RPMI)** 1640 medium (Life Technologies) to wash out the remaining blood in the tubes, and transfer this to the cell culture-flask with EDTA and blood.

3. Dilute the blood by adding RPMI 1640, to a minimum of 20 ml RPMI1640/5ml of blood, and mix the content by pipetting.

4. Prepare 10 ml of room temperature **(RT)** lymphoprep per 25 ml solution of blood and RPMI1640.

5. Carefully pipette 25 ml of blood/RPMI1640 to a 50 ml tube, use several tubes if necessary. Insert a long needle (40 x 1,25 mm, BD Medical) with 10 ml lymphoprep through the blood and to the very bottom of the tube. Gently apply pressure to the syringe so that the lymphoprep is placed in the bottom, distinct from the blood.

6. Centrifuge by 800 x g for 30 minutes at RT. Be sure to adjust brake to 0 and acceleration to 1!

7. The mononuclear cells have formed a band on the mediums interphase, see figure 2.1. This can be ”vacuumed” up by using a Pasteur pipette, or a 10 ml pipette.

Make sure to avoid the upper layer when transferring the mononuclear cells. Transfer the PBMCs to two 50 ml tubes.

8. Adjust the volume to 50 ml by adding phosphate buffered saline **(PBS)** to the tubes, to reduce the density of the sample. Centrifuge for 10 min, 650 x g at RT to form a pellet.

9. Remove supernatant **(SN)** from both tubes. Resuspend cells in one of the tubes in 10 ml PBS before transferring it into the other tube. If more than one tube were used, add 10 ml PBS into the first tube to wash out the PBMCs, total volume is now 20 ml. Centrifuge for 10 min at 200 x g at RT to wash away blood plates and to form a cell pellet.

10. Carefully remove the supernatant without disturbing the pellet. Add 10 ml PBS and resuspend. *Take out 50 µl cell suspension for cell count (section 2.2.4).* Centrifuge at 200 x g at RT to remove all contaminating blood plates.

11. Carefully remove supernatant, use a 1000 µl pipette for the last ml without disturbing the cell pellet. If the isolated cells are to be further processed by Dynabeads, add 500 µl Isolation Buffer (PBS with 0,1% BSA and 2 mM EDTA) per 50 x 10^6 cells. See section 2.2.1 for activation with dynabeads

2.1.2 Isolation of CD4+ T cells from PBMC

Human primary CD4+ T cells were isolated from PBMC by Dynabeads Untouched™ Human CD4 T cells Kit (Invitrogen™ by Life Technologies). Dynabeads are magnetic, monosized and spherical polymer beads used for cell separation. The Dynabeads Untouched™ Human CD4 T cells Kit isolates CD4+ T cells by negative selection, leaving the CD4+ T cells in the cell suspension. The isolation procedure is based upon the Dynabeads attribution to deplete B cells, NK cells, monocytes, platelets, dendritic cells, CD8+ T cells, granulocytes and erythrocytes. The resulting isolated CD4+ T cells are left without antibody and beads, thereby being suitable for further analysis. The isolation procedure was done according to the protocol supplied by the manufacturer.

2.2 Cultivation, activation and vitamin D treatment of primary human CD4+ T cells

Purified CD4+ T cells were cultivated in X-vivo 15^{TM} medium (BioWhittaker ®, Lonza) already containing the antibiotic gentamycin and phenol red as pH indicator. X-vivo media is optimized for proliferation of purified CD3+ T cells isolated from peripheral blood and human tumors[2]. The cell density was kept at 0.8 x 10^6 – 1.5 x 10^6 cells per milliliter (ml) X-vivo. To maintain the desired density, 1,0 µl 10 ng/µl of the T-cell growth factor interleukin 2 (IL-2; R&D systems) was added per ml of X-vivo. Cultivated cells were carefully monitored by cell counting, see section 2.2.4, as well as by microscopy. Cells were incubated in 37°C 5% CO_2.

2.2.1 Activation of CD4+ T cells

In order for the isolated CD4+ T cells to proliferate and get activated, the cells need to be stimulated. Dynabeads Human T- Activator αCD3/CD28^{TM} (Life Technologies) was used. The human T- activator beads are coated with anti-CD3 and anti-CD28 monoclonal antibodies, which interact with their corresponding molecules on the T cell surface, resulting in an intracellular signaling cascade that leads to activation of the T cells. The procedure for T cell activation was performed according to the manufacturers' instructions, where table 1 below shows the requirements.

Table 1. Reagents for cultivation of human peripheral CD4+ T cells

Reagent	Volume added
Volume of Dynabeads	25 ml
Volume of cell suspension	1 ml
Volume of IL-2 (10ng/µl)	1 ml

2.2.2 Vitamin D treatment of CD4+ T cells

Human CD4+T cells in culture were treated with the active form of vitamin D, 1,25-dihydroxy D_3 (calcitriol, Sigma Aldrich). Prior to the procedure, the 1,25-dihydroxy D_3 was solubilized in ethanol **(EtOH)** to 1 nM, and further diluted 1:100 in preheated X-VIVO, where 1 µl was added per ml cell suspension in a total volume of 1x10^6 cells. As negative control, the vehicle, EtOH, was also diluted 1:100 in preheated X-VIVO and added to the

[2] http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_BenchGuides_X-VIVO_Media_Information_Sheet.pdf

same number of cells in a separate well. As vitamin D is extremely toxic in high concentrations, dilutions were done in a fume hood to prevent inhalation.

Prior to addition of vitamin D/EtOH, the αCD3/CD28 activator Dynabeads were removed from the cells. The removal of αCD3/CD28 activator Dynabeads were done by transferring the cell suspension to a 15 ml tube, and then put the tube on magnet (Dyna Mag™ -2, Invitrogen), allowing the cells to rest for 24 hours in the CO_2 incubator. Vitamin D and EtOH, with and without αCD3/CD28 activator Dynabeads were then added to cell cultures. The time point when vitamin D or vehicle was added was designated time 0. Cells were harvested as described in the next subsection.

2.2.3 Collection of cell lysates

Cells were collected both for RNA isolation and for generating whole cell lysates. Harvesting of the CD4+ T cells was done after treatment as described in the previous section, and the specific time points are given in the result section.

It is critical to stabilize RNA immediately after cell harvesting to ensure high integrity and quality of the RNA, both important criteria for subsequent gene expression analysis. RNA protect Cell Reagent (Qiagen) was used for stabilization of RNA in cultured cells.

Procedure for harvesting cells on RNA protect:

1. Spin down cells for 5 min, 300 x g at RT.
2. Discard SN, and resuspend cells in 1 ml PBS.
3. Spin down as in step 1.
4. Discard SN.
5. Roughly mix cell pellet in 350 µl RNA protect.
6. Store at -70 ^{o}C.

Cell harvesting for protein lysate: The αCD3/CD28 activator Dynabeads were removed prior to cell collection to prevent unwanted bands on Western blot, which happen when the antibodies are dissolved from the Dynabeads upon heating of the samples with loading buffer prior to gel electrophoresis (see section 2.6.3)

Procedure for harvesting cells to generate protein lysate:

1. Spin down cells for 5 min, 300 g at RT.
2. Discard SN, and resuspend cells in 1 ml PBS.
3. Spin down as in step 1.
4. Remove ALL SN.
5. Store cell pellet in -20°C prior to generating protein lysis, described in section 2.6.1.

2.2.4 Cell count

Quantification of human CD4+ T cells was done by the use of TC20^{TM} automated cell counter (Bio-Rad). The cell counter can measure the viability when tryphan blue solution (Sigma Aldrich) is mixed in 1:1 ratio to the cell suspension. In addition, the settings on the cell counter can be adjusted in order to gate according to size, enabling definition of the population of interest.

Procedure for cell count:

1. Mix 10 µl cell suspension with 10 µl tryphan blue.
2. Pipet 10 µl into Counting Slides dual chamber (Bio-Rad).
3. Insert the slide into the TC20 automatic cell counter and wait for the cells to be counted.

2.3 Sample storage – MS Biobank

The MS research group has been collecting material from MS patients and healthy controls over the last years. CD4+ T cells have been isolated from from MS patients and healthy controls by members of the MS group. Live CD4+ T cells are stored in liquid nitrogen **(LN)**, which in this thesis will be referred to as the MS biobank. Samples collected were from untreated, female Norwegian MS patients with relapsing-remitting MS **(RRMS)** aged between 18 and 65. These were recruited from the MS clinic at the Department of Neurology, Oslo University Hospital. Controls are aged-matched to MS cases, and all female.

In this study, CD4+ T cells from healthy donors (n = 10) and MS patients (n = 8) from the MS biobank were thawed and expanded with αCD3/CD28 coated beads for 7-8 days, as described in section 2.3.1, before removal of beads for 24 hours to rest the cells prior to addition of vitamin D or vehicle control with or without αCD3/CD28 beads. Prior to addition of vitamin D or vehicle with or without αCD3/CD28 beads, an appropriate number of cells were frozen down again, described in section 2.3.2. Below is a flow chart indicating the major steps during expansion and cultivation of CD4+ T cells, which are described in detail in the next subsection.

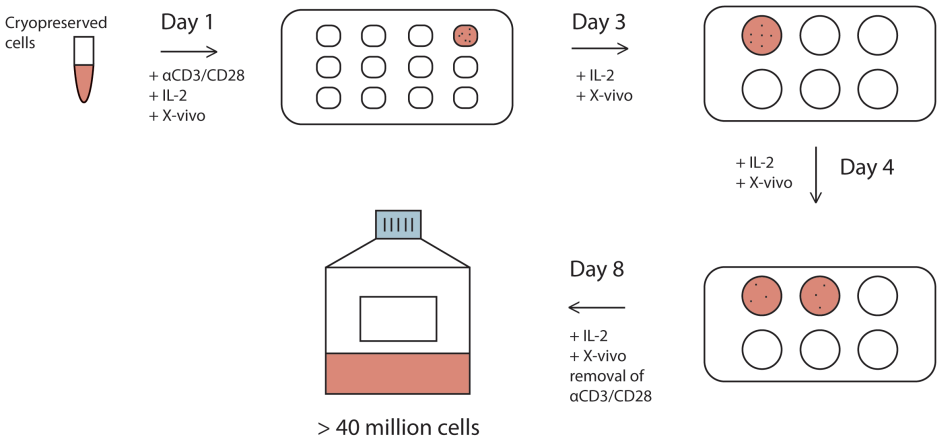

Figure 2.2: Flow chart showing CD4+ T cell expansion after thawing. Cell density was kept at 1-1,5 x 10^6 cells per ml. For each ml of X-vivo added, 1-1,4 µ1 IL-2 was also added.

All cultures were closely monitored by frequent cell count and by microscopy to observe morphology/cell size. On average, the cells needed 7-9 days in culture to achieve a sufficient number of cells to complete the experiments. To obtain optimal growth- and expanding conditions, cell density was kept at 1-1,5 x 10^6 cells per ml.

2.3.1 Thawing protocol of cryopreserved cells

When samples from the biobank are taken into use, it needs to be thawed and cultivated for a number of days in order to achieve a sufficient number of cells. The following procedure has been developed and optimized for proliferation of CD4+ T cells stored on liquid nitrogen. However, some minor modifications must still be taken into account when performing this procedure, as cell cultures from different donors do not have the same viability or growth rate.

Procedure:

Day 1:

- Thaw the cells (1 ml) in 37°C water bath, rocking gently until the ice is melted. Wipe the outside of the tube with alcohol.
- Transfer cells to a fresh 15 ml tube with 2 ml room tempered **(RT)** RPMI1640 medium. Add 7 ml of RT RPMI (wash cryotube with some of it).
- Spin cells down immediately (8 min. 1200 rpm at RT) and remove media as soon as possible. Use a 1000 µl pipette to remove the last drops of the RPMI.
- Resuspend cell pellet gently (5 ml pipette) in 1 ml warm (37 ^{o}C) X-VIVO medium and transfer to a 12 well plate. Wash out the remaining cells (1000 µl pipette) in 1 ml (37 ^{o}C) X-VIVO and add to the 12 well plate.
- Add 1µl IL-2 (10 ng/µl) per ml X-VIVO, and spread evenly in the well.
- Wash and add αCD3/28 Dynabeads, see section 2.2.1. 25 µl beads per well.

Day 3:

- Transfer the cells from the 12-well plate to a 6-well plate.
- Add 4 ml pre-heated (37 ^{o}C) X-VIVO (with 1,4 µl IL-2/ml medium).

Day 4:

- Split cells into 2 wells by transferring half the volume (~3 ml) into a new well, and add 3 ml pre-heated X-VIVO (with 1,4 µl IL-2/ml medium) to both wells.
- Leave for 4 nights before harvesting.

Day 7:

- If the media is turning yellow, add 1 ml new X-VIVO medium.

Day 8:

- At this point, the cell amount has increased to >40x10^6 cells. Dynabeads were removed allowing the cells to rest for 24 hours prior to stimulation with vitamin D or vehicle control, +/- αCD3/CD28 beads.

2.3.2 Cryopreservation of CD4+ T cells

CD4+ T cells can be stored for longer periods in liquid nitrogen in the presence of the detergent sulfoxide **(DMSO),** and can be thawed up for cultivation. To achieve the optimal conditions for thawing, cells must be frozen by addition of a mixture of fetal calf serum **(FCS)** and DMSO. The DMSO will protect the cells from puncture upon freezing by preserving the membrane integrity. For each tube of cells to be thawed up for cultivation in the study, described in the previous section, an appropriate number of cells were again frozen down after expansion.

Procedure:

1. Make freezing solution (make 0,5 ml solution per tube of CD4+ T cells to be frozen, 2 x 10^6 CD4+ T cells/tube) If several tubes of the same cell type should be frozen, make a "master mix" of 1 ml freezing solution per 4 million cells containing 20 % DMSO and 80 % FCS.

2. Prepare by adding DMSO to serum on ice. Keep on ice until use.

3. Place cryotubes on ice.

4. Add slowly (approximately 1 minute), one drop at the time, freezing solution (0,5 ml per tube to be frozen) to the cells. Keep on ice at all times. Swirl the tube with the cells during the procedure.

5. Mix the whole solution before transferring 1 ml of the solution to chilled cryotubes on ice and transfer them to an isopropanol box (4 ºC).

6. Place the box in -80ºC for minimum 24 hours before transferring them (use the box to carry the tubes) to liquid nitrogen.

2.4 RNA techniques

RNA was isolated from cells resuspended in RNA protect, see section 2.2.3. When working with RNA, gloves are used at all times and changed frequently as RNases will contaminate the samples. RNase catalyzes degradation of RNA. Pipettes, bench, and boxes were frequently washed with RNase Away (Qiagen). Filter tips and RNase-free reagents were used. RNA-isolations were done in a hood as β-mercaptoethanol (Sigma) is a serious irritant and is added to the lysis buffer provided by the RNA isolation kit.

2.4.1 RNA isolation

RNA is isolated with the RNeasy Plus Mini Kit using QIAshredder columns for lysate homogenization (both from Qiagen). RNA isolation is performed as described in the protocol provided by the manufacturer. Briefly, after cell lysis and homogenization, genomic DNA is removed by binding to the gDNA eliminator columns, provided by the RNeasy Plus Mini Kit. Ethanol is added to the solution enabling proper binding conditions for RNA to bind to the RNeasy membrane. RNA is then transferred to RNeasy mini spin column (Qiagen), where contaminations are washed away, while the RNA will be bound to the membrane in the spin column. The RNA is then eluted in RNase-free H_2O.

After each RNA isolation, 1,5 µl is saved for concentration measurements, described in the next subsection. In addition, from each round of RNA isolation (4-8 samples was isolated per round), 1,5 µl RNA is taken out from random samples to analyze the RNA integrity on the Bioanalyzer, see section 2.4.3

2.4.2 Assessment of nucleic acid concentration

Nanodrop 2000 is a micro-volume UV-Vis spectrophotometer for nucleic acid and protein quantification. Absorbance is measured at 230 nm, 260 nm and 280 nm. The NanoDrop ND-2000 software evaluates the purity of the samples by calculations of the absorbance ratios at 230/260 nm and 260/280 nm. The expected ratios of 260/230 nm and 260/280 nm are 2,0-2,2 and 1,8-2,0, respectively. Measurements of RNA were done according to the manufacturer's instructions.

2.4.3 RNA integrity

RNA quality is a concern when measuring gene expression. A number of RNA isolations have been performed in this project, and a selection of those were picked out for RNA integrity measurements.

The Agilent RNA 6000 Nano Kit (Agilent Technologies) is used to estimate the integrity of isolated RNA samples. The samples are loaded onto a chip that contain a special gel matrix with an RNA-intercalating dye in a miniature capillary system. The quality of RNA is a calculation of the RNA integrity number **(RIN)** generated by the software 2100 Bioanalyzer Expert, which also measures the ratio of 28S:18S ribosomal RNA.

RIN values are numerical assessments of the RNA integrity. RIN is given between 1 and 10, where 10 represent intact RNA and 1 represent total RNA degradation. A RIN value between 6 and 10 is considered sufficient RNA quality to be used for expression profiling. The procedure was followed in accordance to the manufacturer's instructions.

2.4.4 Complementary DNA synthesis

The enzyme reverse transcriptase (RT) generates complementary DNA **(cDNA)** from RNA template. The cDNA synthesis is done routinely prior to quantitative real time PCR **(qPCR)**. Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) was utilized in accordance to the protocol applied by the kit. The cDNA was stored at -20°C for short time storage, or at -80°C for long time storage.

Reverse transcriptase minus **(RT-)** negative control is important in the real-time PCR reactions to assess for genomic DNA **(gDNA)** contaminations of the RNA samples. The RT- has all components in the reaction mixture, except for the enzyme. In addition, a non-template control **(NTC)** is also included to assess for reagent contaminations. NTC contains all components except RNA template.

2.5 Real-time PCR

2.5.1 Quantitative real-time PCR

qPCR is a technique for amplification and quantification of targeted DNA, or cDNA in the case of gene expression. Expression of specific genes can be analyzed by qPCR where the amount of DNA is measured after each cycle via fluorescent dyes that gives increasing signal in direct proportion to the number of PCR products generated. If a particular

sequence is abundant in the sample, amplification is observed in early cycles. If a particular sequence is at low concentrations, amplification can be observed at later cycles.

Before initiation of PCR, the Taqman® probe is intact, and the reporter and quencher have a natural affinity for each other, see figure 2.3, thereby allowing fluorescent resonance energy transfer **(FRET)** to occur. During PCR, the primers and probe anneal to the target. DNA polymerase extends the primer upstream of the probe. If the probe is attached to the correct target sequence, the polymerase 5´nuclease activity cleaves the probe, releasing a fragment containing the reporter dye. Once cleavage takes place, the reporter and quencher are no longer attached to each other, and the released reporter molecule will not be quenched.

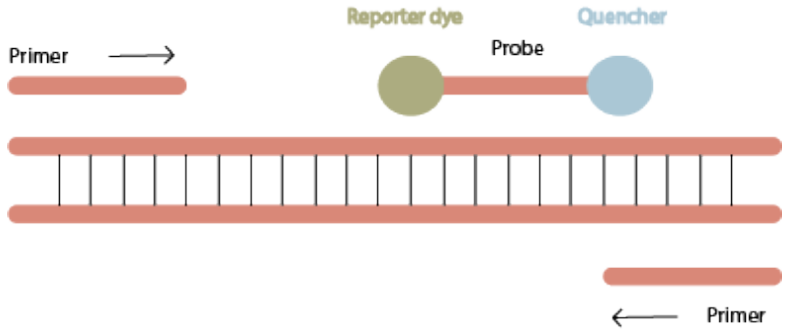

Figure 2.3: Schematic illustration of the TaqMan® assay. Before initiation of PCR, the Taqman® probe is intact, and the reporter and quencher have affinity for each other.

In this study, the relative standard curve method was utilized to analyze gene expression. A stock of eight-point standard curve with 1:2 dilution-series was made. VIC-labeled TBP and 18S rRNA (Life Technlogies) detection probes and gene specific FAM-labeled detection probe for *IL2RA* (taqman assay ID: HS00907777_m1), *TAGAP* (taqman assay ID:HS00299284_m1), *VDR* (taqman assay ID: HS00172113_m1) and *CYP24A1* (taqman assay ID: HS00167999_m1) was used in the qPCR runs in this study. Before the main qPCR runs, quality controls of the various assays were performed, as well as optimization of standard curves. These include (1) check of their specificity by running the PCR product on agarose gel (see section 2.5.3), (2) make sure the cDNA synthesis is not saturated by making standard curves of RNA, (3) ensure that the standard curves with cDNA are approximately 100% efficient reactions, which means a 10-fold increase in PCR amplicon

every 3,32 cycle, resulting in a standard curve slope of -3,3, and (4) dilution of samples to ensure they are amplified within the standard curve range.

All qPCR experiment preparations were done according to the instructions delivered by Applied Biosystems, Life Technologies, and run on Applied Biosystems Viia™ 7 Real-Time PCR system (Life Technologies) and the data was analyzed by Viia™ 7 Software (Life Technologies).

2.5.2 Single nucleotide polymorphism (SNP) genotyping

Genetic variation analysis is another application of real-time PCR. The technique also uses the 5´-nuclease activity of the polymerase. Each predesigned SNP Genotyping Assay contains one VIC–labeled probe and one FAM-labeled probe enabling to distinguish between the two alleles. The Taqman predesigned SNP assays IDs used were rs7090512 (taqman assay ID: C_1841420_20) and rs1738074 (taqman assay ID: C_2966098-10). DNA from 63 donors (MS patients n = 33, HC n = 30), had previously been extracted by members in the MS research group in connection with other research projects. The DNA extraction was performed by using a Qiagen Gentra Autopure LS Automated DNA Purifier machine, or manually by QIAamp DNA Blood Mini Kit extraction (Qiagen). 12 ng DNA from each donor was used for each genotyping assay, and the procedure was done according to the manufacturer's instructions and run on Viia™ 7 Real-Time PCR system. The data was analyzed by Viia™ 7 Software (Life Technologies)

2.5.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a standard method used to separate DNA molecules according to size. As DNA is negatively charged, DNA molecules migrate through the gel towards the anode, which is the positively charged electrode. DNA is visualized under UV light in a gel-dock station (GeneGenius Gel Light Imaging System, Syngene). The visualization is possible as the DNA is run in an agarose gel containing ethidium bromide **(EtBR)** (MP biomedical). EtBR intercalates into the structure of DNA making it visible in UV light. The percentage of agarose influences the migration and separation of the DNA molecules. A higher agarose percentage leads to slower migration rate. The percentage used in this thesis was 2%.

Procedure:

1. Mix agarose powder and 1 x Tris-acetate-EDTA **(TAE)** buffer in accordance to your desired percentage of agarose. For 2 % gel, mix as indicated in the table.

Reagent	Amount
Agarose	1 g
1xTAE buffer	50 ml
Total volume	50 ml

2. Boil solution until the agarose powder is completely dissolved.
3. Cool the solution to approximately 50ºC and add 2 µl (final concentration of 0,5 µg/ml) of EtBr.
4. Pour the solution into the gel tray and insert the well comb.
5. Let the gel harden.
6. Remove well comb and place the gel into the electrophoresis chamber.
7. Add 1 x TAE buffer until the gel is covered.
8. Add loading buffer to your samples and apply on the gel.

2.6 Protein techniques

Proteins are rapidly degraded, it is therefore important to work on ice, centrifuge at 4°C and include protease inhibitors in lysis buffer.

2.6.1 Protein extraction

This method describes how to generate radioimmunoprecipitation assay **(RIPA)** buffer protein lysate. RIPA (RIPA buffer, BioRad) is a lysis buffer, which can be used to extract proteins from cultured mammalian cells, including pelleted suspension cells.

Procedure:

1. Add 20 µl lysis buffer containing 1x protease inhibitor cocktail **(PIC)** (Roche) in 25 x stock concentration, to 1 million cells. Adjust the volume dependent on the cell number you lyse.

2. Vortex tubes to loosen the clumps and put them on the rotator machine at 4°C for 30 min to 1 hour.

3. Centrifuge at 12000 x g or maximum speed for 15 minutes at 4°C.

4. Transfer supernatant to a new chilled eppendorf tube on ice and mark the tubes. Store the lysate at -20°C. For long time storage it is recommended to store lysate at -70°C.

2.6.2 Protein concentration assessment

The Bradford method is a spectrophotometric method for determining concentration of protein in solution. The procedure is based on the proportional binding of Coomassie blue to proteins. The higher concentration of protein, the more Coomassie will bind and the darker the protein solution becomes. The Bradford method was done prior to SDS-PAGE, described in the next subsection, for estimation of protein concentration in the lysate, to be able to apply equal amounts of proteins to each well.

Procedure:

1. Prepare Bradford tubes according to your number of samples (two tubes per sample). Also prepare 10 tubes for generating a standard curve.

2. Add 1 ml of Coomassie Blue dyes to each tube.

3. For standards, add BSA (2 mg/ml) according to the table (two tubes of each):

Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
0,5 ml	1,0 µl	2,0 ml	4,0 ml	8,0 ml

4. Add 2 µl of your sample to the dye and mix. Check the color of the dye, it should be within the range of the standard, otherwise the concentration is too high or low and you need to adjust the amount added.

5. Vortex the tubes gently.

6. Incubate for 10-15 min and transfer samples to cuvettes before measuring absorbance at 595 nm on Ultraspec 2100 Pro UV/Vis Spectrophotometer

(Amershan Bioscience). Use 1 ml dye + 2 µl RIPA buffer as a reference for your samples lysed in RIPA buffer, and 1 ml dye as reference for the standard curve samples.

7. Make a standard curve of the standard samples, with protein concentration on the x-axis and absorbance at the y-axis. Calculate amount of protein from this standard curve.

2.6.3 SDS-PAGE

Sodium dodecylsulphate polyacrylamide gel electrophoresis **(SDS-PAGE)** is a method used routinely prior to Western blotting. Proteins are separated according to mass, after treatment with SDS. SDS is an anionic detergent that denatures the proteins and creates complexes between the protein and SDS. Due to the negative charge of SDS, the original charge of the proteins is irrelevant, and the proteins are given an overall negative charge.

As the molecular mass is the determining factor in the electrophoresis, small proteins will move faster in the gel than larger proteins. High percentage of acrylamide will make proteins migrate slower in a gel than low percentage of acrylamide. By the use of a molecular standard with known molecular weight in kilo Dalton **(kDa),** the sizes of the separated proteins can be estimated. Protein separation was performed using Criterion^{TM} TGX^{TM} 10% (Bio-Rad) precast gels and 10 % mini protean TGX Precast gels (Bio-Rad).

Preparing samples for SDS-PAGE:

1. Dilute all samples to the same volume in RIPA-buffer,
2. Add 6 x SDS loading buffer to each sample, recipe in appendix 3.
3. Heat for 5 minutes at 96°C.

Procedure:

1. Carefully pull out the comb of the premade gel. Wash the wells with dH_2O to remove any excess gel or air bubbles.
2. Place the gel into the Electrode Assembly.

3. Fill the inner chamber with 1 x running buffer, recipe in appendix 3, so that the wells in the gel are completely covered with buffer. Fill also the lower chamber with running buffer to cover the bottom of the gel.

4. Carefully apply the samples by letting it sink down in the well. The Sample Loading Guide makes it easier to apply. Avoid overflow of the samples to neighboring wells.

5. Apply the molecular standard; 6 µl Precision Plus Protein^{TM} dual color standard (Bio-Rad).

6. Run the gel at 150 volts (V) for 15 min, followed by 200 V for approximately 45 min.

7. Dissemble the gel electrophoresis equipment.

8. Wash the gel in dH_2O

Following SDS-PAGE, the gel can be further processed for Western blotting. Western blot is described in the next section, and recipes for the different buffers used in both SDS-PAGE analysis and in Western blotting are listed in appendix 2.

2.6.4 Western blotting

Western blotting is a technique used for detection of proteins by the use of antibodies after protein transfer from gel to membrane. After separation of proteins with SDS-PAGE, the proteins in the gel are transferred to a membrane in an electrical field. In this study, polyvinylidine fluorine **(PVDF)** (Immune-blot® PVDF, BioRad) membrane was used. Once the proteins are bound, blocking is done to prevent unspecific binding to the membrane. Proteins in the blocking solution will bind to all regions of the membrane, except for parts already occupied by proteins transferred from the gel. This will prevent the antibodies from binding non-specifically to the membrane. The next step is to incubate the membrane with the desired primary antibody, specific for a protein. The secondary antibody, which will bind the primary antibody, is linked to the enzyme Horseradish Peroxidase **(HRP)**. This enzyme will produce light upon developing, when a suitable substrate is added, in proportion with the amount of protein on the membrane.

Procedure:

Protein transfer:

1. Put the gel, outlined in the previous section, in transfer buffer (recipe in appendix 3) for 1-2 min.

2. Cut the PVDF membrane to appropriate size and activate it in methanol (Sigma Aldrich) for 15 sec.

3. Wash the membrane in dH_2O and put it in transfer buffer.

4. Soak two 3 mm filters (BioRad) in transfer buffer. Thereafter, place one of the filters in the blotting apparatus (Amersham ECL Semi-Dry Blotters TE 77 PWR, Amersham Bioscience).

5. Place the membrane on the top of the filter.

6. Place the gel on the membrane and the last filter on top of the gel. Press out air bubbles. Place the lid on the apparatus.

7. Run the blot at $1mAmp/cm^3$, equivalent to 130 mA for Criterion™ TGX™ gels, at 50 V for one hour.

Detection with antibody:

8. Block the membrane with 3% skimmed milk in tris-buffered saline with tween 20 (TBS/T) (see recipe in appendix 3) at room temperature (RT) for 1 hour.

9. Add the primary antibody in 3% skimmed milk/TBS/T, the dilution of antibody is different for different antibodies, dilute as recommended for your antibody.

10. Incubate for 1 hour at RT or at 4°C overnight.

11. Wash the membrane 3 x 20 min in TBS/T.

12. Add the secondary antibody in 3% skimmed milk in TBS-T, dilute as recommended. Incubate for 1 hour at RT.

13. Wash the membrane 3 x 20 min in 3% skimmed milk/TBS/T.

Developing blots:

14. Place the membrane in a plastic beaker and add the Super-Signal solution in 1:1 ratio (SuperSignal® West Pica Chemiluminescent substrate, Thermo Scientific).

15. Incubate for 5 min

16. Place the membrane in the developer cassette. Close the cassette and proceed in the developer room.

17. Turn off the light (only red light should be on). Remove the film (ECL hyperfilm, GE healthcare) from the box. Make a cut in the upper right corner of the film. Open the cassette and place the film on top of the membrane (a plastic film should be between the film and the membrane). Close the lid and expose the film before developing it (Curix 60, AGFA HealthCare).

Stripping of membranes:

If more than one protein is to be investigated on the same blot, it needs to be stripped prior to reprobing with another antibody. Restore^{TM} PLUS Western Blot Stripping Buffer (Thermo Scientific) was used in this project for stripping.

Procedure:

1. Reactivate the membrane in methanol for 15 sec.

2. Wash the membrane x 1 in dH_2O

3. Wash the membrane x 1 in TBS/T

4. If the signals are strong, cover the membrane in 10 x SDS running buffer, and heat it in the microwave until the point of boiling. If no strong signals are present, continue at point 7.

5. Discard the buffer.

6. Wash x 2 with TBS/T for 15 min, leave on tilting table at RT.

7. Add the stripping buffer, leave on a tilting table for 15 min at RT.

8. Block the membrane with 3% skimmed milk for 1 hour on tilting table at RT.

After stripping, the membrane is ready for incubation with a new primary antibody.

2.6.5 Cell preparation for flow cytometry

In this project, flow cytometry was used to analyze the purity of the CD4+ T cells isolated from PBMC (section 2.1.2). Flow cytometry uses light scatter to measure intrinsic size and granularity of the cell. Prior to the flow cytometry analysis, the cells were labeled with a fluorochrome–conjugated antibody, fluorescein isothiocyanate **(FITC)** or allophycocyanin **(APC**) conjugated antibodies were used. The procedure for preparation of cells for flow cytometry analysis is described below. 500 000 cells were taken out for immunostaining prior to flow cytometry.

Procedure:

1. Transfer cells to a 96-well tip-bounded plate (Nunc). Pipet 250 000 cells into each well.
2. Spin down at 340 x g for 2 minutes at 4^{o}C. Discard SN.
3. Wash x1 by adding 150 µl PBS w/1% FCS and 0,05% Sodium Azid.
4. Spin down as in step 2.
5. Add antibody solution. Dilute the desired antibody according to the manufacturer's instructions in PBS w/1% FCS and 0,05% NaAzid. 100 µl Ab-solution is used per 1 x 10^6 cells. Adjust the volume accordingly.
6. Leave on ice for 20 minutes. Cover with aluminum foil.
7. Spin down as in step 2 and discard SN.
8. Wash x 2 by adding 150 µl PBS w/1% FCS and 0,05% NaAzid, spin down as in step 2 in between. Discard SN.
9. Resuspend in 100 µl PBS with PFA for fixation of the cells. Incubate for 10 min at RT.

10. Transfer suspension to a flow tube with 200 μl PBS w/1% FCS and 0,05% NaAzid. Cover tube with aluminum foil, and leave at 4 °C. The cells can now be stored up to 7 days before the flow analysis begins.

Flow cytometry for purity assessment of isolated CD4+ T cells

250 000 prepared cells, see section above, were analyzed by flow cytometry (Facs Calibur, BD Biosciences) and data was processed and analyzed by the computer software CellQuestPro (BD Biosciences).

2.7 Statistical analyses

The data produced by gene expression analysis and protein expression was analyzed by statistical methods. Statistical analysis utilized in this project is Student`s paired and un-paired T-Test. Standard deviation **(SD),** standard error of the mean **(SEM)** were calculated by GraphPad Prism 6, in addition to the statistical analysis.

3 Results

The main objectives of this thesis are: (1) to investigate whether vitamin D alone is sufficient for regulation of target genes in CD4+T cells at the mRNA and the protein level, (2) correlate genotype of MS associated loci in *TAGAP* and *IL2RA* to their expression upon vitamin D treatment and (3) investigate if expression of target genes differ in healthy controls and MS patients.

3.1 VDR expression in CD4+ T cells

In the initial screening performed by Tone Berge, freshly isolated CD4+ T cells were stimulated to investigate vitamin D responsiveness of selected MS associated genes. In the current project, we wanted to use CD4+ T cells stored on liquid nitrogen. These cells have been isolated from MS patients and controls, and we have DNA available for genotyping. Each vial contains 2 million frozen cells, and to achieve sufficient numbers for our study, these cells have to be expanded prior to vitamin D treatment. To investigate vitamin D response in cells after expansion in the presence of αCD3/CD28 beads, cells were allowed to rest for 24 hours before addition of vitamin D or vehicle control in the presence or absence of αCD3/CD28 beads. In order for cells to be vitamin D responsive, the presence of the VDR is required and therefore we first wanted to evaluate whether cells treated in this manner did express VDR at the time of vitamin D addition *i.e.* after 24 hours resting. Freshly isolated CD4+ T cells from three healthy donors were activated with αCD3/CD28 coated beads for 40 hours prior to bead removal and resting of the cells for 24 hours. Cells were harvested before stimulation (time 0), after 40 hours of stimulation (time 40) and after an additional 24 hours of resting (time 40+24). Protein expression of VDR was analyzed by western blotting, presented in figure 3.1, showing one out of the three repeated experiments.

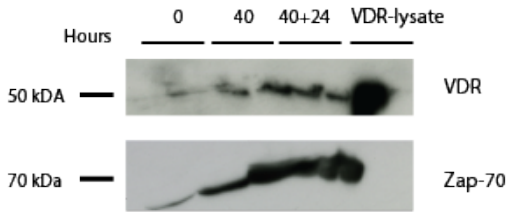

Figure 3.1: Western blot showing expression of VDR in CD4+ T cells. Freshly isolated CD4+ T cells were cultivated with αCD3/CD28 for 40 hours prior to removal of beads and a subsequent

resting of the cells for 24 hours. Cells were harvested at the time of activation (time 0); after 40 hours (time 40) and after an additional 24 hours of rest (time 40+24). Lysates from 1 x 10^6 cells were applied per well. Lysate from 293T cells, overexpressing VDR (Santa Cruz) was used as positive control. The blot was reprobed with anti-ZAP70 that functions as loading control. The figure shows one out of three experiments.

From the western blot (figure 3.1), we can see that VDR expression persisted 24 hours after removal of the beads. In conclusion, this means that the same stimulation conditions can be used for biobank samples.

3.2 Vitamin D responsiveness of MS biobank samples

Tone Berge has shown that 10 nM (as compared to 1 and 100 nM) of the active form of vitamin D, $1,25(OH)_2D_3$, induces maximum vitamin D response in CD4+ T cells as evaluated by measuring expression of *CYP24A1* (Berge, unpublished data), a well-known VDR target gene [42]. Stimulation by addition 10 nm of $1,25(OH)_2D_3$ will be referred to as "addition of vitamin D" throughout the results section. Vitamin D is solubilized in ethanol, which is used as vehicle control throughout the study.

3.2.1 Vitamin D responsiveness in CD4+ T cells

Upon drawing fresh blood from MS patients, peripheral blood mononuclear cells (PBMC) were isolated and CD4+ T cells were purified from PBMCs by negative selection (AutoMACS Pro Separator, Miltenyi Biotech, performed by members of the MS research group). Live cells were stored in liquid nitrogen as part of the 'Oslo MS Biobank'. To establish a protocol for optimal cultivation- and thawing- conditions of cells, we performed a pilot study. The aim of this pilot study was to verify that the stressful environment inflicted on the cells when performing cryopreservation did not affect the vitamin D responsiveness of selected MS associated genes, as well as to establish a protocol to analyze vitamin D responsiveness of MS susceptibility genes in CD4+ T cells that were previously stored in our local biobank. CD4+ T cells from healthy controls (n = 2) were therefore thawed and expanded with αCD3/CD28 for 8 days. Removal of beads after 8 days allowed cells to rest for 24 hours before addition of αCD3/CD28 beads together with vitamin D or vehicle control. Expression of *VDR* and *CYP24A1*, both established VDR target genes [42] were measured relative to the reference genes *TBP* and *18S rRNA*, which gave comparable results. Expression relative to the *TBP* is presented in Figure 3.2.

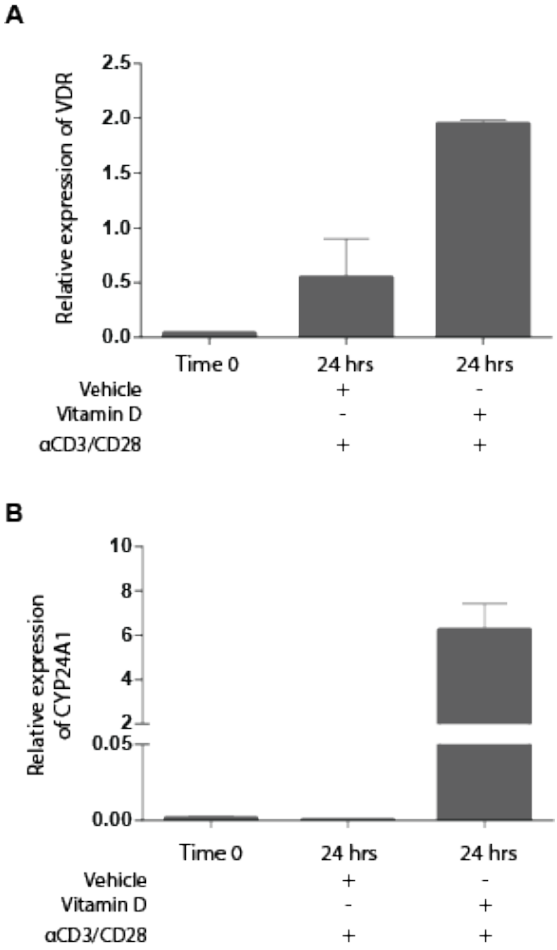

Figure 3.2: CD4+ T cells from the MS biobank are responsive to vitamin D. CD4+ T cells from healthy controls (n = 2) were cultured with αCD3/CD28 beads for 8 days, before removal of beads for 24 hours to rest the cells prior to activation with αCD3/CD28 and addition of vitamin D or vehicle control. Cells were harvested after resting (time 0) and after 24 hours with αCD3/CD28 and vitamin D or vehicle control as indicated in the figure. Expression of *VDR* and *CYP24A1* was measured by qPCR. Bars display mean expression of *VDR* **(A)** and *CYP24A1* **(B)** relative to *TBP* with standard error of the mean (**SEM**).

The relative expression of *VDR* in rested cells shows low expression, however the expression was induced in αCD3/CD28 activated CD4+ T cells after 24 hours, and even further induced in activated CD4+ T cells cultivated with vitamin D. *CYP24A1* was induced in activated cells with vitamin D. These results indicated that cryopreserved CD4+ T cells are vitamin D responsive upon thawing.

3.2.2 Is vitamin D alone sufficient to regulate gene expression of its target genes in CD4+ T cells?

Two vials of CD4+ T cells from the MS biobank were thawed for cultivation and expanded with αCD3/CD28 and IL-2 as described in the method chapter, section 2.3.1, in two separate cultivations. After 8 days in culture, αCD3/CD28 beads were removed. The cells

were rested for 24 hours (time point 0) prior to treatment with vitamin D or vehicle control with or without αCD3/CD28 beads. Cells were harvested after 24 and 48 hours of treatment and the relative expression of *VDR, CYP24A1, TAGAP* and *IL2RA* (figure 3.3 and 3.4) was measured. We know from the previous section that *VDR* and *CYP24A1* are vitamin D responsive genes using this stimulatory regime (in the presence of αCD3/CD28) and that *TAGAP* and *IL2RA* are vitamin D responsive in the presence of αCD3/CD28 beads in freshly isolated cells (Berge, unpublished).

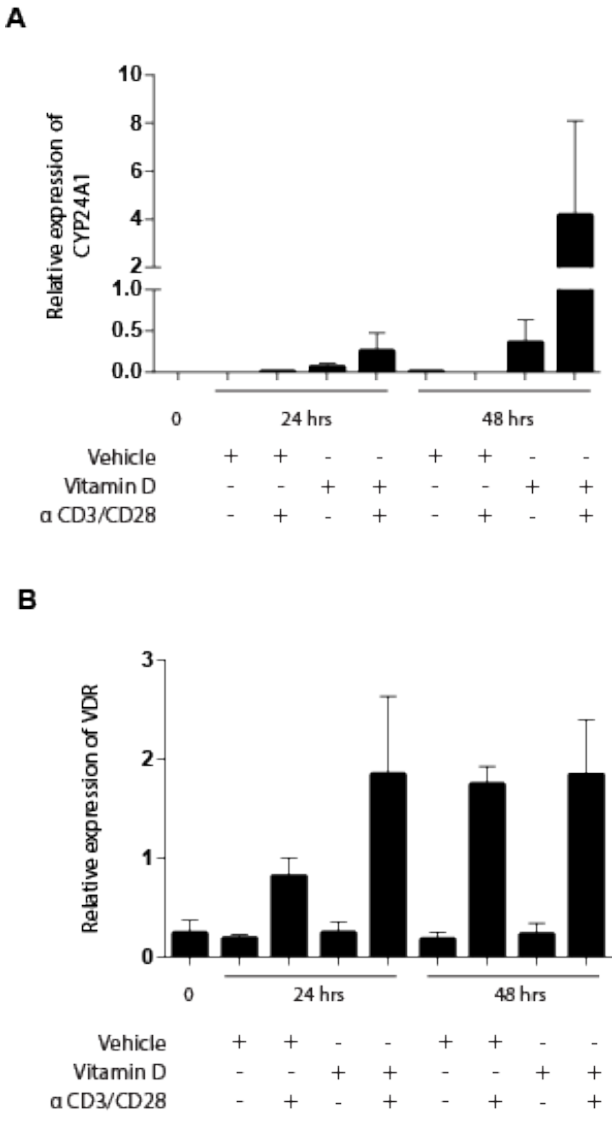

Figure 3.3: Cryopreserved CD4+ T cells are responsive to vitamin D. CD4+ T cells from the MS biobank were co-cultured with αCD3/CD28 for 8 days before 24 hours rest by removal of beads. After 24 hours, αCD3/CD28 beads were added and cells stimulated with vitamin D or vehicle control in the absence or presence of αCD3/CD28, and harvested after 3, 24 and 48 hours. Expression of genes is presented relative to *TBP*. Bars display mean +/- SEM. **A)** Relative expression of *VDR*. **B)** Relative expression of *CYP24A1*.

As expected from the pilot experiment presented in figure 3.2, VDR expression is induced after 24 hours by αCD3/CD28 beads alone (figure 3.3 A), however, addition of vitamin D further enhances VDR expression. No further increase in *VDR* expression was observed after 48 hours in culture. Vitamin D alone did not give rise to increased *VDR* expression even after 48 hours. *CYP24A1* expression was not induced upon activation of the cells with αCD3/CD28 beads, although, addition of vitamin D to the αCD3/CD28 treated cells resulted in a rise of *CYP24A1* expression, which reached a maximum after 48 hours. Addition of vitamin D alone gave rise to expression of *CYP24A1,* although to a lower level than in combination with αCD3/CD28 beads. This indicates that vitamin D alone is sufficient to trigger a VDR response in CD4+ T cells *in vitro*.

The relative expression of *TAGAP* (figure 3.4.A) seemed to be reduced in activated cells after 48 hours, and the expression was even further reduced with the addition of vitamin D. Vitamin D alone does not seem to have the same effect as in the presence of αCD3/CD28 beads. The results in figure 3.4 B indicates an induction of *IL2RA* expression in αCD3/CD28 stimulated cells, which appears to be induced further with addition of vitamin D after 48 hours. Vitamin D alone is not sufficient to trigger a rise of *IL2RA* expression.

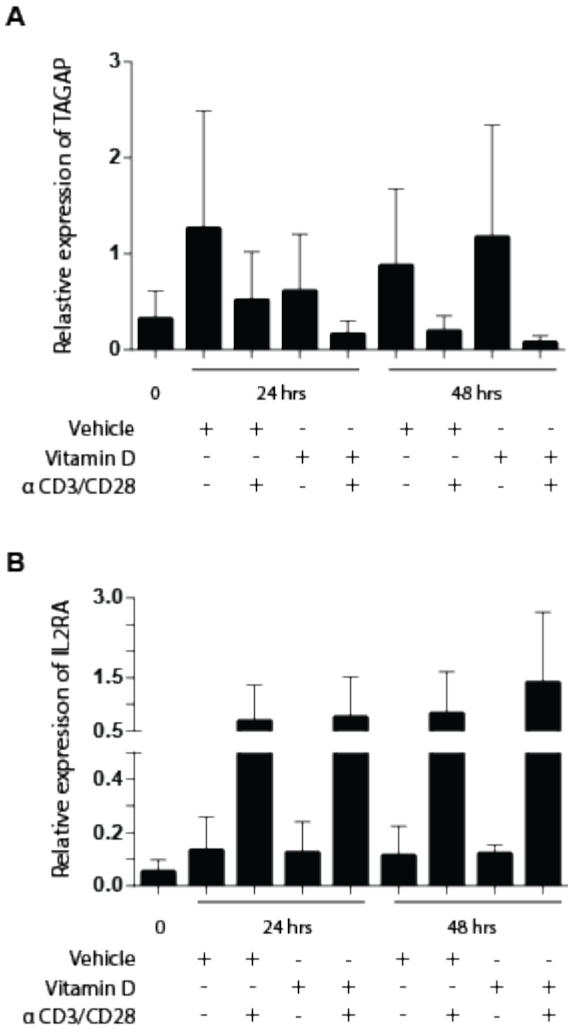

Figure 3.4. Cryopreserved cells are responsive to vitamin D. CD4+ T cells from the MS biobank were co-cultured with αCD3/CD28 for 8 days before 24 hours rest by removal of beads. After 24 hours, cells were activated and stimulated with vitamin D or vehicle control in the absence or presence of αCD3/CD28, and harvested after 3, 24 and 48 hours. Expression of genes is presented relative to *TBP*. Bars display mean +/- SEM. **A)** Relative expression of *TAGAP*. **B)** Relative expression of *IL2RA*.

From these initial experiments we concluded that CD4+ T cells are vitamin D responsive after cryopreservation, however, a higher vitamin D dependent response is observed in the presence of αCD3/CD28 beads.

3.3 Genotyping

To investigate whether the *in vitro* vitamin D responsiveness of the MS associated genes identified, *i.e. TAGAP* and *IL2RA*, is dependent on the genotype of their respective MS associated loci, the next step was to genotype the SNPs of interest in samples from MS patients and controls where live CD4+ T cells are stored in our local biobank. Samples were genotyped for each specific SNP by allele specific PCR, using TaqMan probes with

one VIC-labeled probe and one FAM-labeled probe for the different alleles as described in section 2.5.2.

3.3.1 Genotyping of MS biobank samples

For *TAGAP*, rs1738074 was genotyped as this SNP has been reported in the immunochip study to display the highest MS association signal in the *TAGAP* gene region [9]. For *IL2RA*, the rs7090512 was genotyped. This SNP is in the promoter region of *IL2RA*, and was identified as a secondary signal to the rs3118470 SNP identified in the GWAS study published in 2011 [14]. Figure 3.5 shows the genotyping results of rs173804 and rs7090512, presented in an allelic discrimination plot for each SNP.

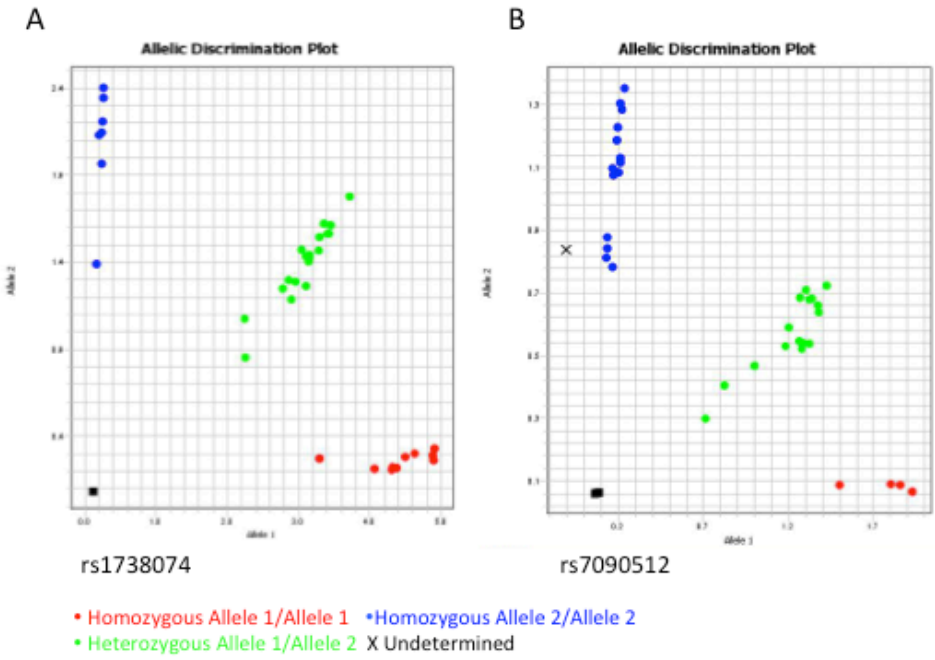

Figure 3.5: Genotyping of rs1738074 and rs7090512. Each dot represents one sample, either from an MS patient or a healthy control for rs1738074 (**A**) and rs7090512 (**B**). Allele 1 is represented in red where the VIC-labeled probe is attached, and represent the nucleotide G for both SNPs. Allele 2 is showed in blue where the FAM-labeled probe is attached, which represent the nucleotide A in both SNPs. The green dots represent heterozygous samples, where signals from both the VIC-labeled and the FAM-labeled probe are detected.

The 1000 genomes project [65] suggests a minor allele frequency of 0,47 for the nucleotide A in rs1738074 and 0,31 for the nucleotide G in rs7090512 in Utah residents with ancestry from northern and western Europe (CEU). Table 3.1 presents an overview over the genotype frequency expected calculated from the allele frequencies and the observed genotype frequency for the SNPs in this study.

Table 3.1: Expected and observed genotype frequency of rs1738074 (*TAGAP)* and rs7090512 *(IL2RA)*

SNP	Combinations	Theoretical genotype freq.	Observed genotype freq.
rs1738074	AA	25,0 %	19,0 %
	AG	50,0 %	57,0 %
	GG	25,0 %	24,0 %
rs7090512	AA	52,0 %	47,6 %
	AG	40,0 %	42,9 %
	GG	8,0 %	9,5 %

3.4 Preparing qPCR set-up

3.4.1 Primer limited – optimizing qPCR conditions

We decided to explore duplexing of qPCR for the genes of interest, *TAGAP, IL2RA* or *CYP24A1* with the *TBP* reference gene. If successful, this would be both time- and reagent-saving, and more accurate since cDNA are pipetted simultaneously into two assays thereby eliminating pipetting variation. Standard curves and amplification plots were closely studied for each assay run alone or in combination with *TBP*, and results indicated that both *CYP24A1* and *IL2RA* allowed duplexing based on the optimal PCR efficiency calculated from the data. However, that was not the case for *IL2RA* (data not shown). When *IL2RA* was tested in duplexing with *TBP,* the amplification of *TBP* failed as the *IL2RA* assay used up all the reagents in the PCR-reaction. In order to solve this problem, an experiment with different dilutions of *IL2RA* probe was executed. Optimizing primer-limited conditions is about testing different dilutions of the primer utilizing most of the reagents. The experiment was executed with eight different dilutions of the *IL2RA* probe, in eight different samples indicated by numbers in the figure below presenting the results from the primer-limited experiment.

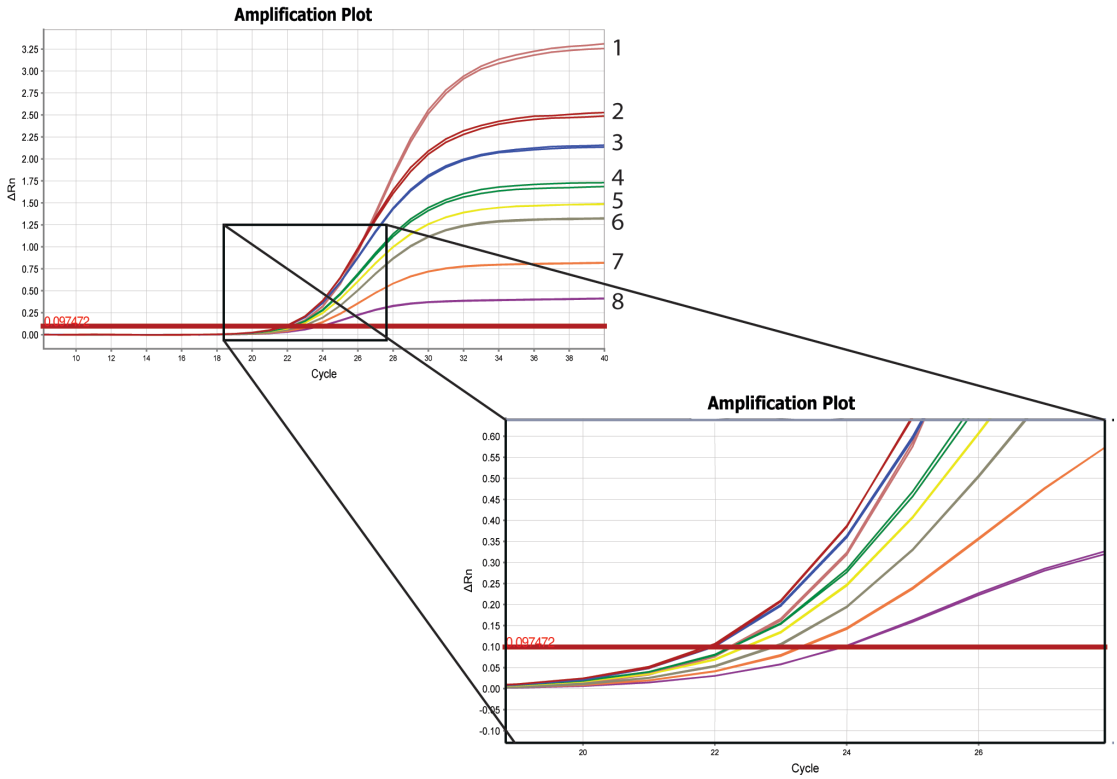

Figure 3.6: Amplification plot of the primer-limited experiment, with eight samples containing different amounts of the FAM-labeled *IL2RA* probe. The amplification plot is numbered according to primer dilution, representing amplification of the different samples. The plot in the lower right provides a detailed overview at the point of amplification to the threshold.

By making a primer-limited experiment, the delta Rn is decreased, which means less usage of the common PCR reagents when amplifying the most highly expressed gene. However, a condition where the cycle number (**CT)** was unchanged was chosen. Using the optimal condition gave rise to optimal amplification of the *TBP* reference gene when run in duplex.

3.4.2 Quality control of reference genes

Two reference genes were included in the qPCR analyses, *TBP* (run in duplex with the test genes) and *18S rRNA*. The comparison of *TBP* and *18S rRNA* (figure 3.7) shows a lower standard deviation for *TBP* than 1*8S rRNA*. We concluded that *TBP* was the most stable reference gene in our samples. All data was analyzed for both reference genes, but only one of the sets with relative to *TBP* will be presented here.

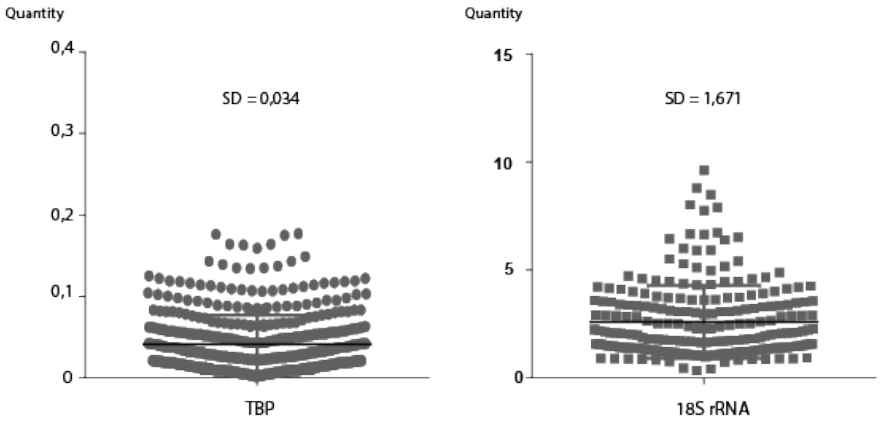

Figure 3.7: *TBP* and *18S rRNA* quantified in qPCR using the standard curve method. The graph displays the quantity of *TBP* and *18S rRNA* for all samples. Standard deviation **(SD)** was measured within each group.

Despite extensive attempts to optimize the duplexing conditions, it was revealed that the quantification of *TBP* in duplex with *IL2RA* deviated from the quantification of *TBP* in duplex with *TAGAP* and *CYP24A1*. However, *TBP* in duplex with the other two genes were perfectly aligned throughout all samples, see appendix 4. In conclusion to this, the expression of *IL2RA* is presented as relative to the mean of the other two *TBP* measurements throughout the rest of the result section.

3.5 qPCR of VDR target genes in vitamin D treated CD4+ T cells from the MS biobank

Samples from the MS biobank were selected according to the genotype of rs7090512 and rs1738074. Samples from 18 donors, both healthy controls (n = 10) and MS cases (n = 8), were thawed for cultivation for the remaining analysis in this study. Figure 3.8 displays an aligned scatter plot of all 18 cell cultures cultivated and expanded in X-vivo medium with IL-2 and αCD3/CD28 beads, to show the variation in growth rates. Each culture was monitored independently to maintain the optimal growing conditions.

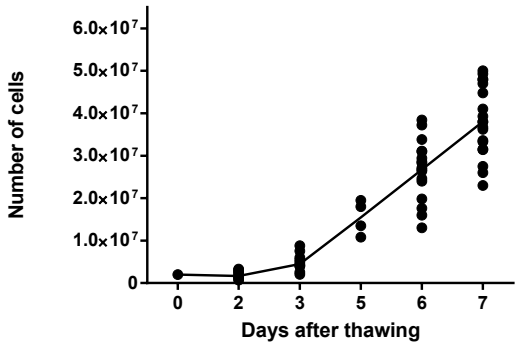

Figure 3.8. Aligned scatter plot of CD4+ T cell growth-rate. CD4+ T cells from 18 donors were thawed and expanded for seven to eight days in culture with αCD3/CD28 beads, X-vivo medium and IL-2 before removal of beads,

The aim was to investigate if vitamin D regulation of *TAGAP* and *IL2RA* expression in in CD4+ T cells correlate with the genotype of MS associated loci in *TAGAP* and *IL2RA* to their expression upon vitamin D treatment. Finally, expression of these target genes was compared in healthy controls and MS patients. Cells were treated with vitamin D alone or in combination with αCD3/CD28. Cells were allowed to rest for 24 hours by removal of beads, prior to addition of vitamin D or vehicle control alone or in combination with αCD3/CD28 beads. Cells were harvested at the time of bead removal (designated time 0) and after 6, 24 and 48 hours of stimulation.

3.5.1 **CYP24A1 as a positive control for vitamin D response**

As for the initiating experiments, relative expression of *CYP24A1* was measured as a control for the ability of vitamin D to induce transcription in activated CD4+ T cells (figure 3.9)

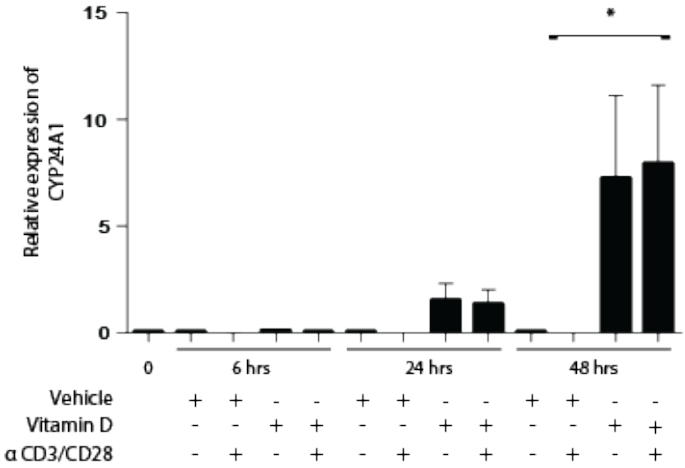

Figure 3.9: Relative expression of *CYP24A1* CD4+ T cells. CD4+ T cells were left unstimulated or stimulated and exposed to vitamin D or vehicle control, with and without αCD3/CD28 beads for indicated time points, relative to *TBP*. The graph represents mean +/- SEM. Paired Student`s test was performed, where $P < 0,05$ was considered significant. Symbol is defined as * = $p \leq 0,05$

CD4+ T cell stimulated with αCD3/CD28 beads in the presence of vitamin D gave rise to significantly higher expression levels as opposed to αCD3/CD28 beads in the absence of vitamin D after 48 hours. Although vitamin D alone did not give rise to significant increase in expression levels as opposed to vehicle control, the result indicates that vitamin D alone is enough to trigger *CYP24A1* expression.

3.5.2 *TAGAP* is responsive to vitamin D

When relative *TAGAP* expression was measured, the relative expression at time point 0 was used to normalize against. The results presented indicate that vitamin D alone (figure 3.10 A) is sufficient to regulate *TAGAP* expression through all time points. Expression of *TAGAP* is significantly reduced in cells treated with vitamin D. However, CD4 + T cells stimulated with αCD3/CD28 beads showed a transient increase of expression after six hours, before the expression of *TAGAP* was significantly reduced after 24 and 48 hours. Addition of vitamin D resulted in significant lower *TAGAP* expression after 24 and 48 hours compared to vehicle control. We concluded that the expression level of *TAGAP* is reduced upon vitamin D treatment.

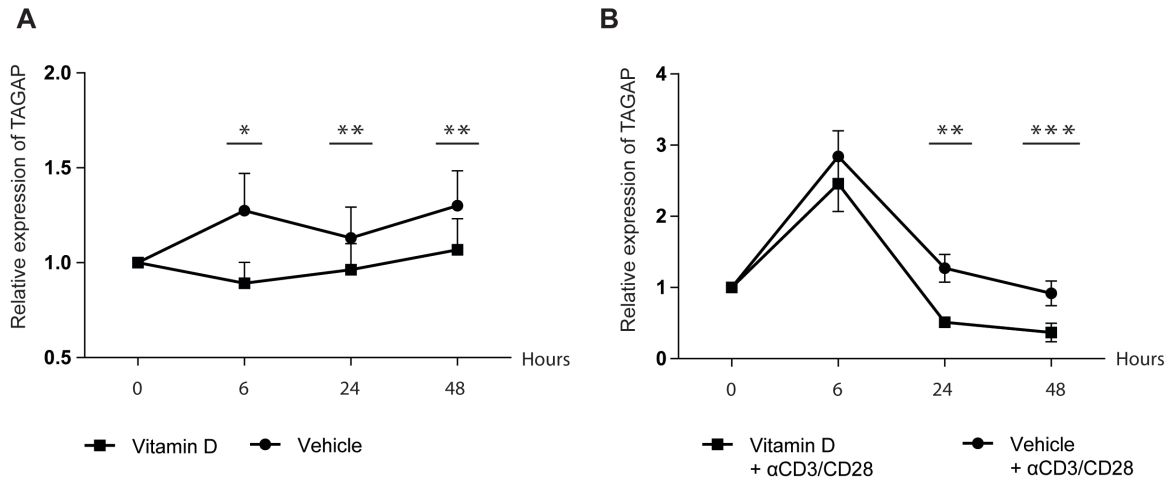

Figure 3.10: **Relative expression of *TAGAP* is repressed upon vitamin D treatment**. Human CD4+ T cells (n = 18) were cultivated for 8 days, and rested for 24 hours prior to addition of vitamin D or vehicle without (**A**) or without (**B**) addition of αCD3/CD28 as indicated in the figure legend. Graphs display mean relative expression of *TAGAP* divided by the relative expression in time point 0 +/- SEM. Paired Student`s T-test was performed, where $P < 0,05$ was considered significant. Symbols are defined as * = $p \leq 0,05$, ** = $p \leq 0,001$ and *** = $p \leq 0,0001$

3.5.3 *IL2RA* is responsive to vitamin D

When measuring *IL2RA* in the same samples, we observed a significantly higher expression in vitamin D treated samples compared to corresponding control after 48 hours (Figure 3.11 A). Vitamin D treatment co-cultured with αCD3/CD28 beads gave rise to even higher level of *IL2RA* expression (approximately three-fold increase at 48 hours). When comparing vitamin D treatment of αCD3/CD28 stimulated cells with vehicle control, a significantly higher level of expression was observed after 24 and 48 hours. With this, we concluded that vitamin D alone is enough to trigger an induction of *IL2RA* expression after 48 hours, and vitamin D in the presence of αCD3/CD28 beads trigger the expression even further.

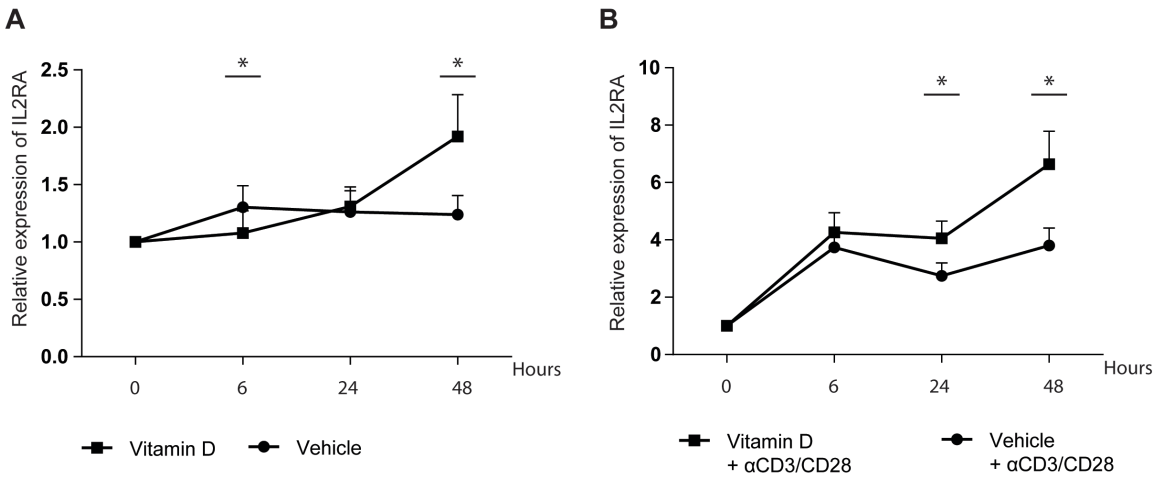

Figure 3.11: Relative expression of *IL2RA* is induced upon vitamin D treatment. Human CD4+ T cells (n = 18) were cultivated for 8 days, and rested for 24 hours prior to addition of vitamin D or vehicle without (**A**) or with (**B**) αCD3/CD28 beads as indicated in the figure legend. Both graphs display mean relative expression of *IL2RA* divided by the relative expression in time point 0 +/- SEM. Paired Student`s T-test was performed, where $P < 0,05$ was considered significant. Symbols are defined as * = $p \leq 0,05$.

3.6 Expression of *IL2RA* and *TAGAP* in individuals sorted on genotype

As one of the main objectives in this study, the next step was to analyze whether the vitamin D responsiveness of MS associated genes was dependent on the genotype of their MS associated loci.

3.6.1 Does *TAGAP* expression after vitamin D addition associate with rs1738074 genotypes?

The samples were sorted according to the genotype of the MS-associated rs1738074, to analyze whether there were any differences in *TAGAP* expression. The SNP analyzed is located in the 5` UTR region of *TAGAP*. The risk allele of rs1738074 is 'G'. Vitamin D responsiveness of *TAGAP* in CD4+ T cells from donors homozygous for 'G' (n = 7) or homozygous for the non-risk allele, 'A' (n = 6), were compared. The results are presented in figure 3.12.

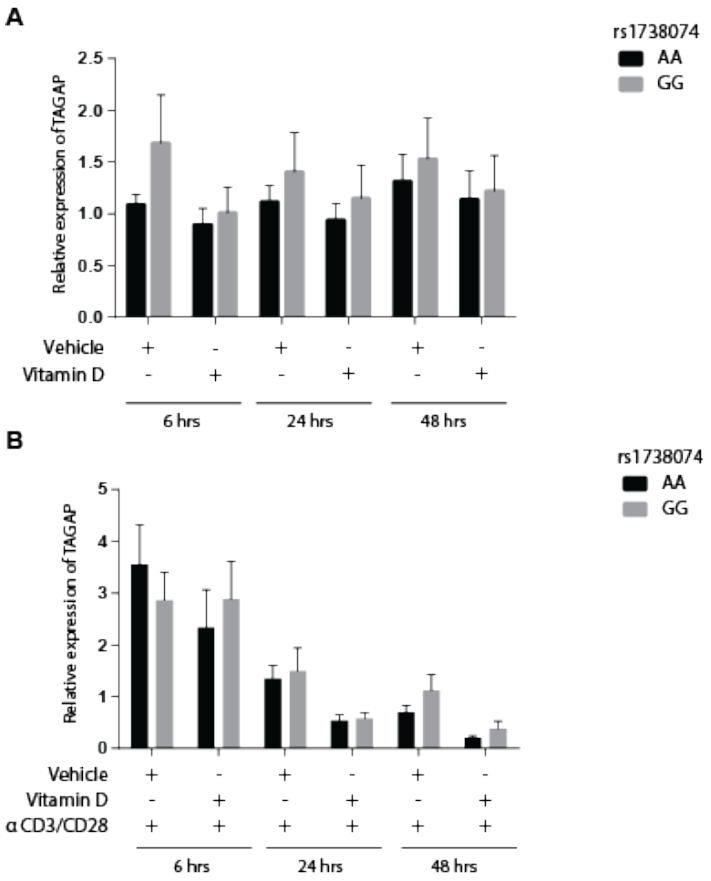

Figure 3.12: *TAGAP* expression in samples from individuals sorted on rs1738074 genotype. The graphs display the mean relative *TAGAP* expression relative to *TBP*+/- SEM,(n (AA) = 6, n (GG) = 7) in (**A**) vitamin D treated cells and in vehicle control and (**B**) in vitamin D treated cells in the presence of αCD3/CD28 beads and the corresponding vehicle control. Unpaired Student`s T test was performed, P = <0,05 was considered significant. No significant changes were detected

For all time points, both in untreated and vitamin D treated samples (except 6 hours with αCD3/CD28), an increase in *TAGAP* expression was observed in individuals homozygous for the G-allele. This was however not significant. Although, in vitamin D treated cells with αCD3/CD28 beads as well as the corresponding control, a reduction of *TAGAP* expression was seen after 24 and 48 hours in both homozygous groups (figure 3.12 B).

3.6.2 Does *IL2RA* expression after vitamin D addition associate with rs7090512 genotype?

The samples were sorted according to the genotype of the MS-associated rs7090512 in the *IL2RA* promoter region, where G is the risk allele. The gene expression data obtained from the qPCR experiments was used to compare the 13 individuals genotyped of which eight donors were of genotype 'AA' and five were of genotype 'GG'.

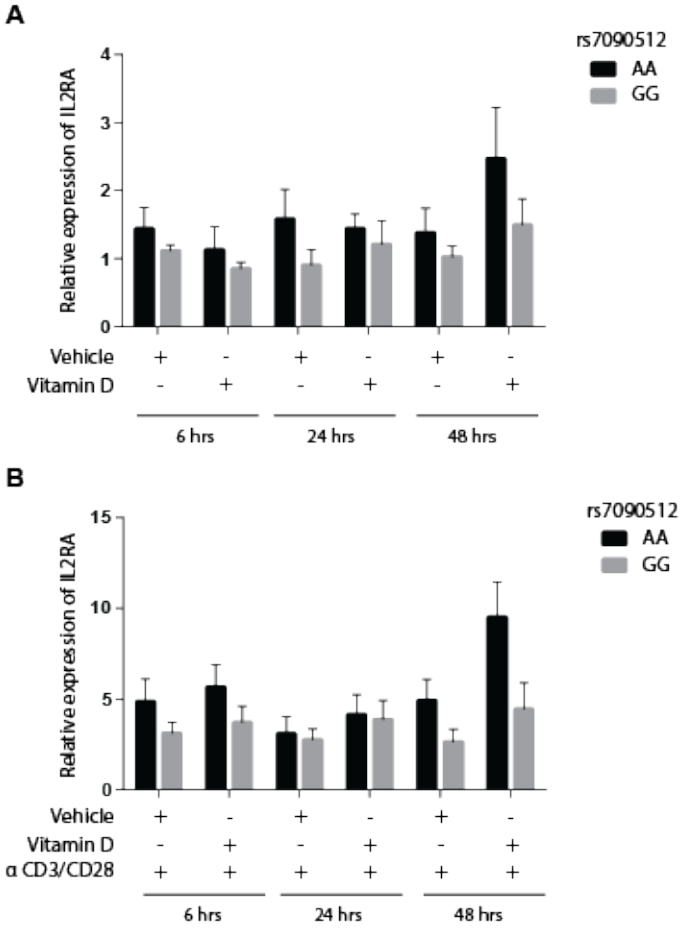

Figure 3.13. *IL2RA* expression in samples from individuals sorted on rs7090512 genotype. The graphs display the mean *IL2RA* expression relative to *TBP* +/- SEM, (n(AA) =8, n(GG) = 6), in **(A)** vitamin D treated cells and vehicle control and (**B**) in vitamin D treated cells in the presence of αCD3/CD28 beads and the corresponding vehicle control. Unpaired Student`s T test was performed, P = <0,05 was considered significant. No significant changes were detected.

Although no significant changes were detected in the analysis, also here some consistent trends were observed. Samples homozygous for G showed lower expression as opposed to A in all indicated groups. Increased *IL2RA* expression compared to corresponding controls is observed at time point 48 in samples treated with vitamin D alone or together with αCD3/CD28. However, no significant difference is observed between the two groups.

3.7 Comparison of *CYP24A1*, *TAGAP* and *IL2RA* expression in MS cases and healthy controls

As mentioned earlier, the cells used in this study are from both healthy controls **(HC)** (n = 10) and MS patients (n = 8). We wanted to see if the expression pattern of the vitamin D responsive genes, *i.e*. *CYP24A1*, *TAGAP* and *IL2RA* differs between MS patients and healthy controls upon vitamin D treatment. The data presented in this section is therefore

divided into “MS” and “HC” groups. Although no significant changes were detected in the relative expression of *CYP24A1* (figure 3.14), a non-significant trend can be observed where MS patients appear to express higher levels than healthy controls.

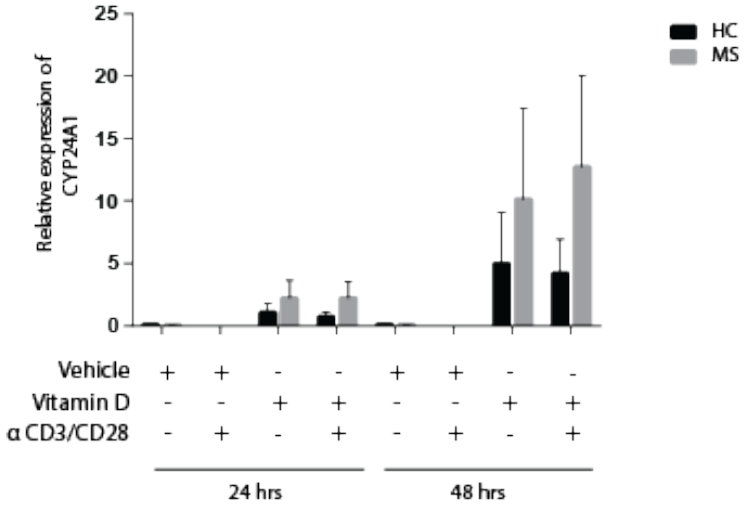

Figure 3.14 Relative *CYP24A1* expression in CD4+ T cells from MS patients and healthy controls. The graph displays the mean *CYP24A1* expression relative to *TBP* +/- SEM, (n(HC) =10, n(MS) = 8) in samples treated as indicated in the figure. Unpaired Student`s T-test was performed on all time points, $P < 0,05$ was considered significant. No significant changes were detected

3.7.1 Relative expression of *TAGAP* differ between healthy controls and MS patients upon vitamin D treatment

When comparing *TAGAP* expression in MS cases and healthy controls, as seen in figure 3.15, one can see that CD4+ T cells from healthy controls display higher expression in all groups compared with CD4+ T cells from MS patients. Significant groups are labelled with a star in the figure. Interestingly, upon vitamin D treatment alone there is a significantly reduction of *TAGAP* expression in MS patients compared to controls after 24 and 48 hours. CD4+ T cells stimulated with αCD3/CD28 beads in addition to vitamin D treatment also indicated lower expression levels of *TAGAP* as opposed to the respective vehicle, but this was only significant after 24 hours. In conclusion, the relative expression of *TAGAP* is lower in MS patients than in HC, in particular upon vitamin D treatment.

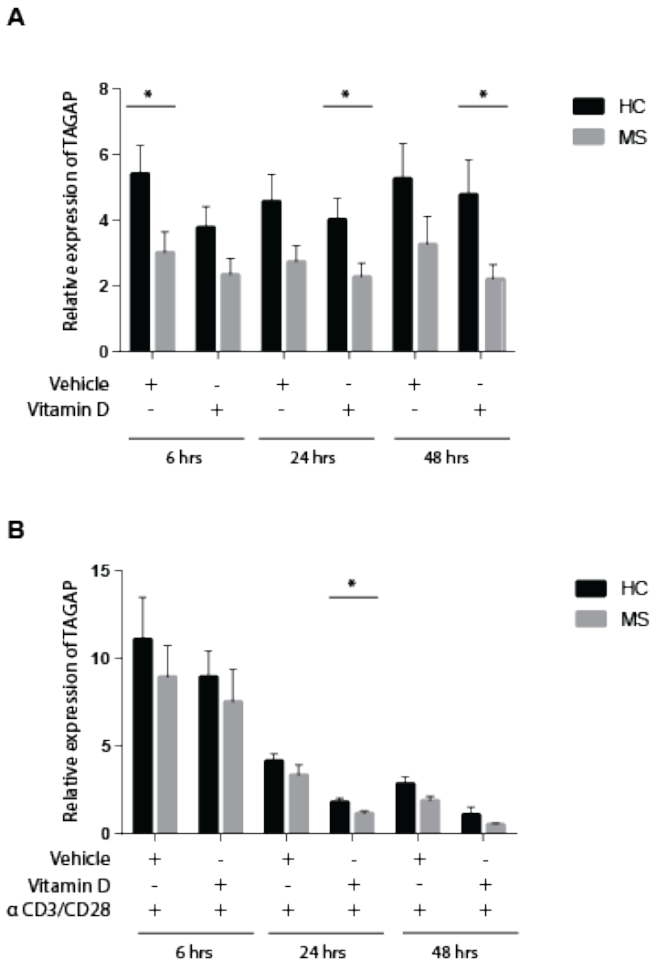

Figure 3.15: Relative *TAGAP* expression is lower in MS than in HC. The graphs display the mean *TAGAP* expression relative to *TBP* +/- SEM, (n(HC) =10, n(MS) = 8) in samples treated as indicated in the figure. Unpaired Student`s T-test was performed on all time points, P < 0,05 was considered significant. Symbols are defined as: * = p ≤ 0,05 **A)** Expression of *TAGAP* relative to *TBP* in CD4+ T cells treated with vitamin D, and vehicle control. ***B)*** Expression of *TAGAP* relative to *TBP* in CD4+ T cells treated with vitamin D + αCD3/CD28 and the corresponding vehicle control.

3.7.2 Does the vitamin D responsiveness of *IL2RA* differ in CD4+ T cells from healthy controls or MS cases?

The relative *IL2RA* expression in CD4+ T cells from MS cases and healthy controls was evaluated and the data is presented in figure 3.16. The figure is divided into MS and healthy controls groups. In all groups, *IL2RA* expression was lower in CD4+ T cells from MS patients compared to healthy controls, however, this was not significant. To conclude, the expression level of *IL2RA* does not differ significantly between MS cases and healthy controls.

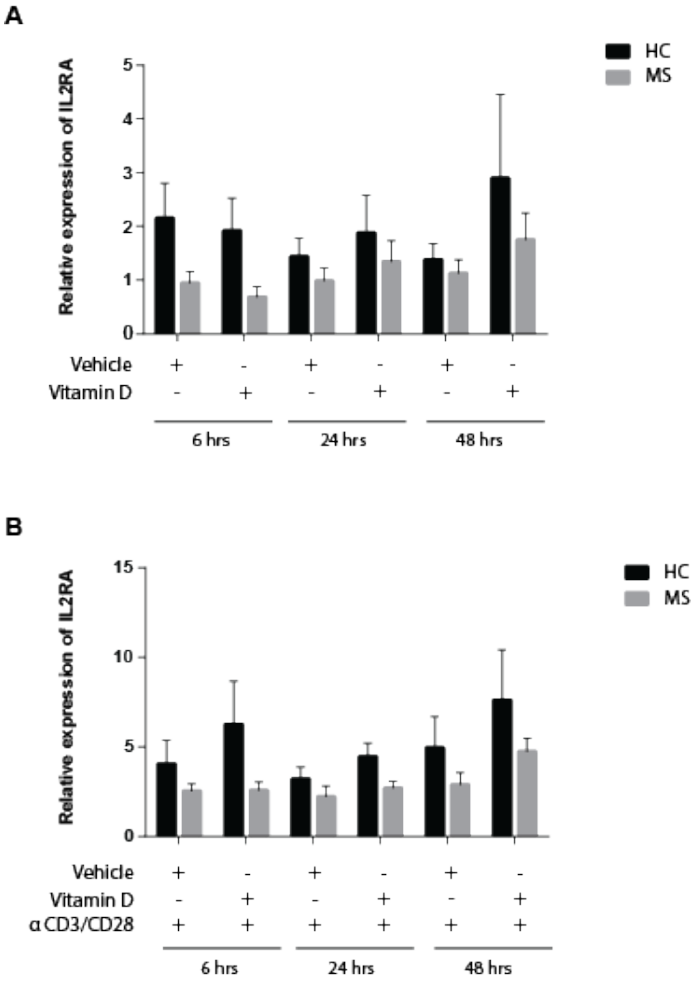

Figure 3.16: Relative *IL2RA* expression in CD4+ T cells from MS cases and healthy controls. The graphs display the mean *IL2RA* expression relative to *TBP* +/- SEM, (n(HC) =10, n(MS) = 8) in samples treated as indicated in the figure. Unpaired Student`s T-test was performed on all time points, $P < 0,05$ was considered significant. No significant changes were detected. **A)** Expression of *IL2RA* relative to *TBP* in samples treated with vitamin D and the corresponding vehicle. **B)** Expression of *IL2RA* relative to *TBP* in samples treated with vitamin D in the presence of αCD3/CD28 beads and the corresponding vehicle.

3.8 Protein expression analysis

3.8.1 Does vitamin D affect protein expression of TAGAP and IL2RA?

We have shown that gene expression of *TAGAP* and *IL2RA* is regulated by vitamin D in CD4+ T cells (figure 3.10 and 3.11 in this thesis and Berge, unpublished). To analyze whether this effect at the mRNA level is also detected at the protein level, CD4+ T cells from the MS biobank (n = 3) were thawed up and treated as described in section 2.3.1. Whole cell lysates were made from cells harvested prior to (time 0) and 24 and 48 hours after vitamin D and αCD3/CD28 addition. TAGAP and IL2RA expression was evaluated

by immunoblotting, figure 3.17 shows expression in one of the three donors. The remaining immunoblots are provided in appendix 4

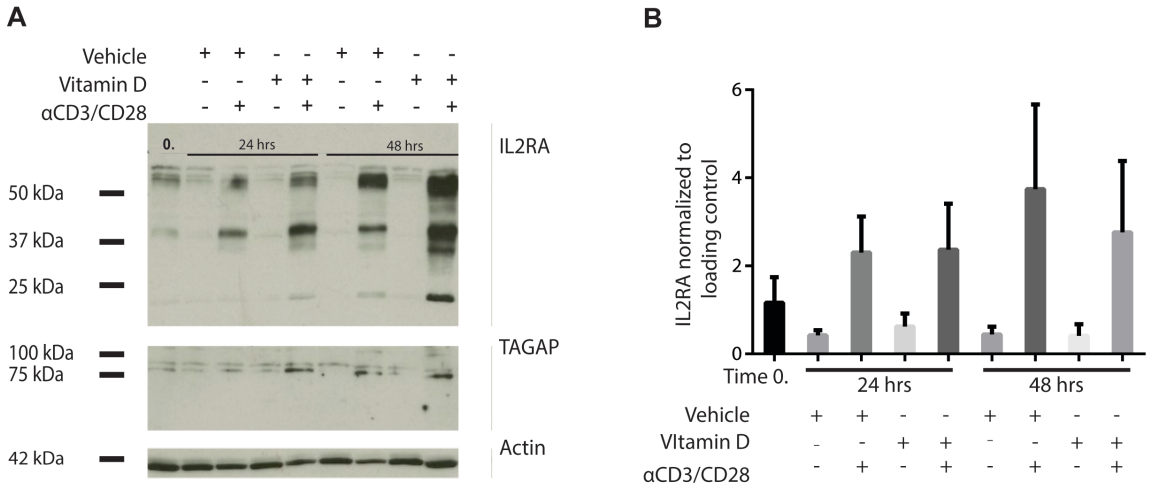

Figure 3.17: **Western blot analysis of IL2RA and TAGAP in vitamin D treated CD4+ T cells.** CD4+ T cells were treated as indicated and whole cell lysates were applied on an SDS polyacrylamide el prior to immunoblotting. **A)** The blot was probed with anti-IL2RA antibody. The blot was stripped and reprobed with anti-TAGAP antibody and anti-aectin antibody, which served as loading control. IL2RA was detected by using primary anti-IL2RA polyclonal antibody, TAGAP was detected by using primary anti-TAGAP polyclonal antibody and loading control (beta-actin) was detected by anti-actin polyclonal primary antibody. All secondary antibodies were HRP-conjugated. 17 µg of total protein was applied in each well in this experiment. **B)** Quantification of the IL2RA protein level in CD4+ T cell lysates from three donors normalized to loading control signals. The graph display mean and bars represent the SEM**.** Multiple paired Student`s T test was performed to check for significant changes in vitamin D treated CD4+ T in the absence and presence of αCD3/CD28 beads compared to the corresponding control and to time 0. $P < 0,05$ was considered significant. No significant changes were detected.

When using the IL2RA antibody, several bands, *i.e*. at approximately 60, 55, 40, 35 and 20 kDa were observed. Ensembl[3] lists four different isoforms at 30, 29, 22 and 12 kDa, and these do not exactly match the size of the bands we observed. We chose to quantify the band slightly above 50kDa, as this is the size of the IL2RA isoform recognized by the IL2RA antibody according to the distributer of the antibody. An up-regulation of IL2RA protein expression after stimulation with αCD3/CD28 beads is observed, which is expected as IL2RA increases upon T cell activation [53].Vitamin D in addition to αCD3/CD28 beads also implies further increase of IL2RA expression in the donor presented in figure 3.17 A. However, this varies from donor to donor, as this trend was not

3
http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000134460;r=10:6010689-6062325

observed when the signal from all three donors were quantified by ImageJ [66] and normalized to loading control (figure 3.17 B). When vitamin D was added alone, this only gives rise to weak bands that are comparable to the corresponding vehicle. Regardless, the intensity of the signals detected at approximately 40 and 20 kDa increased additionally in lysates with vitamin D and αCD3/CD28 beads, which are further discussed in chapter 4.

Only two faint bands between 75 and 100 kDa were observed when the blot was reprobed with TAGAP antibody. Ensembl[4] lists three isoforms at 80, 61 and 29 kDa. However, the distributor of the antibody lists four different isoforms, with one at 57 kDa in addition to the three already mentioned. When inspecting the band at approximately 80 kDa, αCD3/28 seems to induce its expression. Signals detected in the blot incubated with anti-TAGAP were not quantified since these were weak and in too close proximity to other bands, in addition to uncertainties regarding what bands should be considered.

To conclude, expression at the mRNA level is not fully in correspondence with the expression at the protein level. For the IL2RA protein, increased expression in activated cells correlates to the expression pattern at the mRNA level, although vitamin D alone had little effect at the protein level. For the TAGAP protein, the opposite effect is observed at the protein level in contrast to the mRNA level.

4 http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000164691;r=6:159034468-159045152

4 Discussion

In this study we have explored the vitamin D responsiveness of two MS associated genes in CD4+ T cells, and analyzed whether the expression is regulated by the genotype of the MS associated loci. At the end, data were sorted on healthy control and MS patients to study whether expression differs in CD4+ T cells from diseased and non-diseased individuals, and finally expression at the protein level was investigated. Subsequently, the discussion chapter is divided in three main subchapters, before some methodological considerations are discussed.

4.1 Successful system to monitor gene regulation in CD4+ T cells upon vitamin D treatment *in vitro*

The starting point for this project was the identification of several MS associated genes, containing VDREs in their regulatory region. Screening done in our lab revealed that two of these genes are regulated by vitamin D *in vitro.* Several groups have reported that naive T cells have low expression of VDR, which is induced upon T cell activation [11, 12, 41, 42] (Berge, manuscript in preparation). It has previously been shown that VDR is efficiently triggered only if $1,25(OH)_2D_3$ is added to T lymphocytes with already high levels of VDR expression [42]. We could confirm that under the cultivation conditions used during this thesis, providing the two signals necessary for full T cell activation, that the cells are vitamin D responsive as evidenced by induction of *CYP24A1* at the mRNA level. By Western blotting, we were able to show that VDR expression sustained 24 hours after αCD3/CD28 removal, which is the time when vitamin D were added in these studies. In the VDR Western blot, ZAP-70 was used as a loading control. Since it did not give similar band intensities in all lanes, we cannot conclude whether the expression of VDR increased after αCD3/CD28 stimulation, we know however, that it was expressed.

4.1.1 Vitamin D alone vs. vitamin D and αCD3/CD28 beads

Transcription of *CYP24A1* was confirmed at the mRNA level in αCD3/CD28 activated CD4+ T cells treated with vitamin D, and in cells treated with vitamin D alone after 24 hours. The expression of *CYP24A1* was further enhanced after 48 hours in the presence of vitamin D alone or in combination with αCD3/CD28, however, this increase of expression was only significant for cells that were treated with vitamin D in combination with αCD3/CD28. The

results imply that that vitamin D alone give rise to induction of *CYP24A1* expression, which indicates that vitamin D alone is sufficient to trigger regulation of VDR target genes in CD4+ T cells. Whether this is the case for other vitamin D responsive genes in CD4+ T cells, in addition to *TAGAP* and *IL2RA,* remains to be studied. However, after 24 and 48 hours, a non-significant trend for higher expression of *CYP24A1* in MS cases was observed, which has also been reported in a Dutch cohort by Smolders and colleagues [67]. The observed result is consistent with MS patients being associated with low levels of vitamin D, as the function of *CYP24A1* is to degrade the active form of vitamin D. Although, impact of other factors` involvement in decreased vitamin D response cannot be excluded, and in that case, one would expect reduced *CYP24A1* expression as MS patients have lower levels of vitamin D. For instance, there are no recordings regarding serum level of vitamin D, outside activity level or dietary uptake of vitamin D of the patients included in this study.

4.1.2 Addition of vitamin D diminishes *TAGAP* expression *in vitro*

In the main analysis, it was revealed that the expression of *TAGAP,* by the addition of vitamin D, was repressed. A decrease in *TAGAP* expression was significantly through all time points in cells treated with vitamin D alone, as well as in T cells activated with αCD3/CD28 and treated with vitamin D. Since *TAGAP* is reduced already after 6 hours, it could indicate that the *TAGAP* gene is a direct target of VDR.

In addition, it has previously been reported by Mao *et al*. [61] that the expression levels of *TAGAP* in peripheral blood T cells rose transiently and reached maximum after approximately 4-6 hours stimulation with a combination of αCD3 and αCD28 monoclonal antibodies. This supports the findings in this study, where the same transiently increase was observed after 6 hours before a decrease in expression after 24 and 48 hours (figure 3.10) in both activated CD4+ T cells in the addition to vitamin D, as well as in the corresponding control. The fact that the expression levels of *TAGAP* rise transiently upon T cell activation may also be indicative of its role in T cell activation.

If the expected expression pattern of *TAGAP* in this study is similar to the findings done by Connelly *et.al* [62], where higher expression levels of *TAGAP* were observed in Chron`s patients than in healthy controls, a possible outcome could be higher levels of *TAGAP* expression in MS patients in comparison to samples from healthy controls. Surprisingly, a significant effect contradictory to this outcome was observed in vehicle control after 6 hours. There was significantly decreased *TAGAP* expression in CD4+ T cells from MS

patients compared to healthy controls after 24 and 48 hours with vitamin D, and also in vitamin D in combination with the co-stimulatory activation signals after 24 hours. The expression of *TAGAP* is apparently lower in MS patients regardless of vitamin D or vehicle control. Treatment of CD4+ T cells with vitamin D *in vitro* lead to reduced expression of *TAGAP* in both healthy controls and MS patients, although not significant.

Since the outcome differs from findings in other autoimmune diseases, new questions arise. Has *TAGAP* a different role in MS compared to *i.e.* Chron`s disease? Given the cohort more power, would samples from MS patients still be more responsive to vitamin D?

4.1.3 Addition of vitamin D induces *IL2RA* expression *in vitro*

The study revealed that *IL2RA* is responsive to vitamin D alone. The expression of *IL2RA* at the mRNA level is induced in CD4+ T cells upon treatment with vitamin D alone or in combination with αCD3/CD28 beads. However, a significant increase of expression was only observed after 48 hours in vitamin D treated cells, and after 24 and 48 hours after vitamin D in addition to T cell activation. In contrast to *TAGAP*, the prolonged time to achieve a change in *IL2RA* regulation may imply that it is an indirect target gene of VDR. It could also be advantageous to measure the expression levels of *IL2RA* at a later time point in order to investigate at what time point it reaches maximum levels.

As high levels of *IL2RA* expression is associated to autoimmune diseases [57, 58], one can hypothesize that MS patients show a decrease in expression of this gene in comparison to healthy controls. Although no significance changes were found, the observed trend in the vehicle controls supports this suggestion. Higher expression of *IL2RA* in vitamin D treated cells, with and without αCD3/CD28, was observed after 48 hours in both groups compared to the corresponding controls.

Furthermore, it should be kept in mind that the patient cohort in this study has a mild course of disease, and all are untreated. Disease severity and treatment can alter the gene expression, in which case would not be discovered in this study, for neither of the studied genes.

4.1.4 Does the mRNA expression level correlate with the protein level?

The results from the protein expression analysis (section 3.8) showed increased IL2RA protein expression in activated cells together with vitamin D. This is consistent with the expression shown at the mRNA level. However, the same was not observed for cell lysates from cells treated with vitamin D alone. Interestingly, two other bands at approximately 40 and 20 kDa showed the same expression pattern, also seen in the two other donors (Appendix 4C). Still, whether this is of scientific relevance, is to be determined. As described in the result section, there are four different isoforms of the IL2RA protein, and the sizes of the bands detected in the immunoblotting don't comply with the suggested isoforms. Shift in migration pattern could be caused by different posttranslational modifications **(PTMs)** *i.e.* acetylation, methylation, phosphorylation or glycosylation.

Two faint bands were observed for the TAGAP protein at approximately 80 and 90 kDa, for the blot showed in the result section. Multiple bands were also observed in the other two experiments, although these were not at the same sizes. At the mRNA level, the expression of *TAGAP* is transiently induced after 6 hours in the presence of αCD3/CD28 beads. It cannot be excluded that this response is more slow at the protein level, hence the stronger bands after 24 and 48 hours.

Due to time constraint, optimizing the conditions for the two antibodies was not performed. Among others, positive controls should be included to estimate the size of the expected protein bands.

4.2 How is the expression of *TAGAP* and *IL2RA* regulated by the presence of vitamin D?

Neither of the MS associated SNPs studied in this project are located within the VDRE, but they are located within the regulatory regions of the genes. Next, I will discuss the possible roles of location of the respective VDREs and SNPs in gene regulation.

4.2.1 How does vitamin D diminish *TAGAP* expression?

As previously described in the introduction, there are two VDREs, -10 kb to +5kb of the *TAGAP* gene. These are located in the non-coding regulatory region and in the first intron of the coding gene, and the MS associated rs1730874 SNP is located in the 5' untranslated region **(UTR)**, illustrated in figure 4.1.

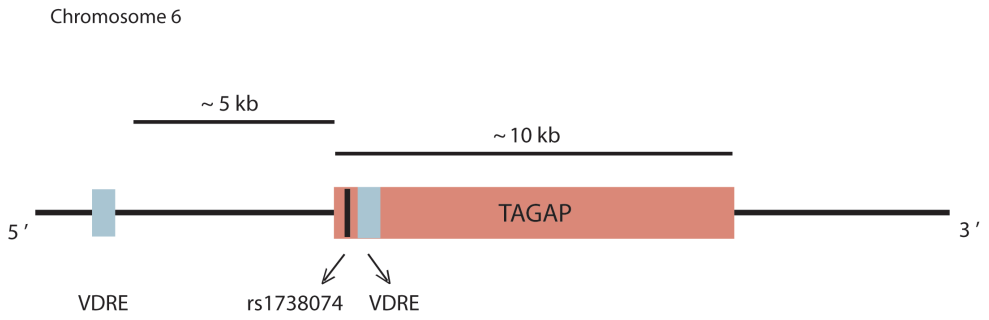

Figure 4.1: Schematic illustration of VDRE and SNP localization relative to the *TAGAP* gene. There are two VDREs in the *TAGAP* gene. One of the VDREs is located at chr6: 159,471,316-159.471,330, which is about 5000 bases upstream from the transcription site, and the other one is located in intron 1, chr6: 159,465,841-159,465,855. Our SNP of interest, rs1738074, is located at chr6: 159,465,977, which is in the promoter region of the gene. The chromatin assembly used is Human February 2009 (GRh37/hg19).

The SNP is associated with a number of different binding sites for transcription factors revealed from ChIP-seq directed by ENCODE factorbook [51]. Handel *et al.* have also recently located rs1738074 within the DNA that is immunoprecipitated from CD4+ T cells in VDR chromatin immunoprecipitation combined with DNA sequencing **(ChIP-seq)** experiments [68]. These findings show that VDR bind DNA in a region where this MS associated SNP is located, indicating that this SNP may impact the binding of VDR, or binding of possible co-factors. Due to the location, one could speculate whether this SNP can alter the binding affinity of transcription factors in this genetic region, resulting in diminished expression of the gene. Of the two VDREs in the *TAGAP* gene, the one located in the non-coding regulatory region is not associated with DHS or binding sites for transcription factors. However, the intronic VDRE is located on DHS clusters [51], and in close proximity to rs1738074 and is also within suggested binding region for some general transcription factors.

4.2.2 How does vitamin D enhance *IL2RA* expression?

There is one VDRE, -10kb to +5 kb of the *IL2RA* gene. This is located in the non-coding region upstream from the transcriptional start site. The SNP rs7090512 is also located in this region, as illustrated in the figure 4.2.

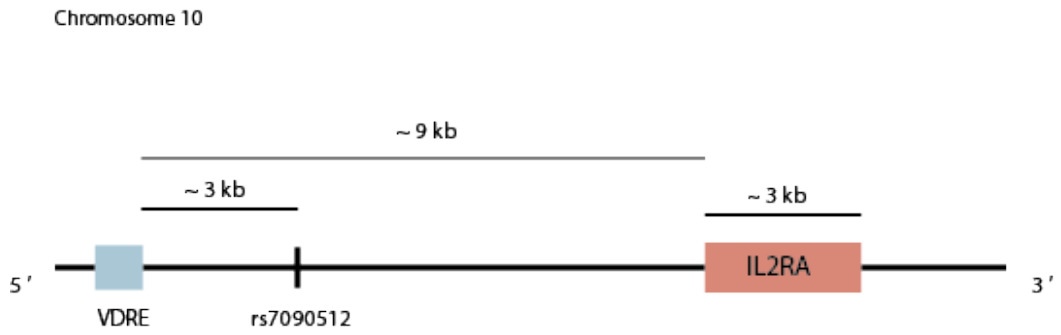

Figure 4.2: Schematic illustration of VDRE and SNP localization relative to the *IL2RA* gene. There is one VDRE in the regulatory region of the *IL2RA* gene, located at chr10: 6,113,820 – 6,113,839. The SNP of interest, rs7090512, is located at chr10: 6,110,829, upstream from the transcription site. The chromatin assembly used is Human February 2009 (GRh37/hg19).

The rs7090512 SNP is not located within the VDRE, or within the actual coding region. It is located between two DHS cluster sites, indicated by the ENCODE project [51]. There are no transcription factors known to bind in the area around rs7090512 or the region around the VDRE. However, the VDRE is associated with DHS clusters indicating a potential role in gene regulation. Although there are no suggested transcription factors binding sites located within the region of the VDR or rs7090512, there are also several proposals where VDR bound to VDRE can interact with transcription factors by DNA looping, or by the recruitment of co-regulators [35, 46, 69].

4.3 Vitamin D responsiveness may correlate to genotype of MS associated loci

No significant changes in expression of *IL2RA* and *TAGAP* were observed when sorting on genotype of rs7090512 and rs1738074, respectively. In addition to compare expression in samples from homozygous individuals for each SNP, we gave the cohort more power by increasing the size of the groups. This was done by including samples from heterozygous individuals in the analyses and thus group samples from individuals homozygous for the risk or non-risk allele, with samples from individuals that are carriers of risk or non-risk allele (data not shown). However, that did not result in any significant changes for the expression of *TAGAP,* even when correcting for the disease-induced differences, by excluding these from the analysis. One cannot exclude that this SNP is an imperfect proxy marker for a true MS risk SNP, meaning that there is a possibility for SNP-driven gene regulation to exist outside the local linkage disequilibrium **(LD)** structure, but this would not be detected it in this study. Although, for the *IL2RA* associated SNP, there was a

significant higher expression of *IL2RA* in in all carriers of the risk allele compared to homozygous individuals for the non-risk allele after 48 hours in activated and vitamin D treated cells after 24 hours (Appendix 4C). This suggests that rs7090512 is functional, or is in high LD with a functional SNP.

4.4 Methodological considerations

4.4.1 Vitamin D treatment – locally high concentrations

In initial dose-response analyses, 1, 10 or 100 nM of vitamin D was added to CD4+ T cells in addition to αCD3/CD28. *CYP24A1* reached maximum expression levels with 10 nM. Therefore, 10 nM of vitamin D has been utilized in the experiments performed in this project. Normal serum levels of the vitamin D precursor, $25(OH)D_3$, is about 25-170 nM, and serum levels of the active $1,25(OH)_2D_3$ is a 1000 fold lower, 60-110 pM. The conditions in which these experiments were executed in were therefore all in apparently high concentrations. However, the enzyme CYP27B1 is widely expressed [12, 42, 70, 71], and has earlier this year also been identified in activated T cells by Kongsbak with colleagues [72], meaning that T cells possess the ability to convert the vitamin D precursor, $25(OH)D_3$, to active vitamin D, thereby being able to make locally high concentrations of vitamin D *in vivo*. Further, it has also been shown that DBP in serum sequesters $25(OH)D_3$ and thereby inhibits the production of $1,25(OH)_2D_3$ from $25(OH)D_3$ in T cells *in vivo* [72]. However, the medium utilized in this project is serum free.

4.4.2 RNA and qPCR

RNA concentration, as well as the 260/230 and 280/230 ratios were measured for every sample on Nanodrop, although, these values do not say anything about degradation of RNA. However, Bioanalyzer measures the RNA integrity, and gives a RIN value that says something about the RNA quality, and whether it has been degraded. In this project, only a selection of samples was processed on the Bioanalyzer to obtain their respective RIN value. There were no indications of possible RNA degradation, but the possibility for degraded samples should still be considered.

Due to the big number of samples, cDNA synthesis was performed in two rounds and each qPCR assay was run in two separate 384-well plates, and considering comprehensive pipetting, this was performed on two subsequent days. Run-to-run variations were limited to

the best of our abilities by making a common mastermix for each assay. The remaining mastermix was stored at 4°C until the next day.

5 Summary of findings

In chapter 1 the aims of this study were outlined, and in chapter 3 the results were presented, and subsequently discussed in chapter 4. In this final chapter, I will summarize and conclude the findings of this project, as well as suggest studies for future work.

5.1 Conclusion – part 1

The first aim of this study was to investigate if vitamin D alone is sufficient to regulate expression of *TAGAP* and *IL2RA* at the mRNA- and the protein level in CD4+ T cells. We concluded that;

- *TAGAP* and *IL2RA* are vitamin D responsive in CD4+ T cells.
- In CD4+ T cells, vitamin D alone seems to be sufficient to trigger regulation of *TAGAP*, hence repression of *TAGAP* expression *in vitro* after 6, 24 and 48 hours.
- Vitamin D alone is sufficient to trigger an induction of *IL2RA* expression in CD4+ T cells after 48 hours, although, by addition of activation beads, the expression is further induced *in vitro.*
- No conclusions can be drawn regarding expression at the protein level, although there is a trend for higher expression of IL2RA protein in vitamin D treated cells co-cultured with αCD3/CD28.

5.2 Conclusion – part 2

Further, the study investigated if genotype of MS associated loci correlates with mRNA expression of *IL2RA* and *TAGAP*.

- We concluded that there is no correlation between *TAGAP* expression and the presence of risk allele at rs1738074 in *TAGAP* in CD4+ T cells treated with vitamin D with or without αCD3/CD28 beads.
- There is a correlation between the presence of risk allele at rs7090512 and increased expression levels of *IL2RA* in CD4+ T cells treated with vitamin and co-

cultured with αCD3/CD28 beads after 24 hours, but there is no correlation in CD4+ T cells treated with vitamin D alone.

5.3 Conclusion – part 3

The third aim was to compare gene expression of vitamin D responsive genes in CD4+ T cells from healthy controls and MS patients.

- We concluded that vitamin D treatment results in lower expression of *TAGAP* in CD4+ T cells from MS patients as opposed to healthy controls *in vitro.*
- We concluded that vitamin D treatment does not result in expression differences of *IL2RA* in CD4+ T cells from healthy controls and MS patients *in vitro.*

5.4 Future work

In this study, the expression of the VDR target genes *IL2RA* and *TAGAP* have been investigated upon vitamin D treatment by qPCR. For the short-term future perspective, I would optimize the immunoblotting conditions for both the IL2RA antibody and the TAGAP antibody. To confirm what bands to investigate for both proteins, additional optimization of the method should be performed. Furthermore, flow cytometry could be utilized to investigate the surface expression of *IL2RA*, as it will be transported to the cell surface to dimerize with the other two IL2 receptor chains. It would also be of great interest to study the expression of *TAGAP* and *IL2RA* in naive T cells. The MS research group is currently about to finish RNA sequencing of naive T cells isolated from patients and controls, enabling us to analyze such expression data for both *IL2RA* and *TAGAP*. In addition, monitoring of expression of both genes in T cells from patients before and after vitamin D supplement would also be of great interest to study the gene regulation.

Further studies of *TAGAP* would also be of great interest to assess its particular role in MS, as very little is known about this gene in contrast to *IL2RA.* Excessive functional studies in T cells, *i.e.* by making TAGAP-plasmids for over expression, use siRNA for knock down or by the use of a mutated version can be performed and subsequently analyze T cell activation *in vitro.* In addition, it would be interesting to study how the *TAGAP* gene is regulated by VDR-RXR using ChiP assays, electro mobility shift assay **(EMSA)** or DNA foot printing, as it has been suggested that mechanisms involved in gene repression by

VDR may exclude binding to VDREs. Also, reporter gene assay could be performed to investigate the cellular mechanism of gene regulation.

6 References

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Appendix 1: List of abbreviations

Abbreviation	Description
18S rRNA	18S ribosomal RNA subunit
Ab	Antibody
AP-1	Activator protein 1
APC	Antigen presenting cell
APC	Allophycocyanin
bp	Base pairs
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CT	Cycle threshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CYP	Cytochrome P
Da	Dalton
DBD	DNA binding domain
DC	Dendritic cell
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetettraacetic acid
EMSA	Electro mobility shift assay
ER	Everted repeat
et al.	And others (Latin: et alii)
EtOH	Ethanol
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FRET	Fluorescent resonance energy transfer
g	Gram
gDNA	Genomic DNA
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GWAS	Genome wide association study

Abbreviation	Description
HC	Healthy control
HCL	Hydrogen chloride
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
i.e	That is (latin: id est)
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-2	Interleukin 2
IL-2Rα	Interleukin 2 receptor alpha
IMSGC	International MS Genetics Consortium
ITAM	Immunoreceptor tyrosine-based activation motif
k	Kilo
kb	kilo bases
kDA	kilo Dalton
l	Liter
LAF bench	Low air flow bench
LBD	Ligand binding domain
LD	Linkage Disequilibrium
LN	Liquid nitrogen
m	Milli
M	Molar
MHC	Major histocompability complex
ml	Milliliter
mRNA	Messenger RNA
MS	Multiple sclerosis
n	Nano
NaAzid	Sodium Azid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor-κB
ng	Nano gram
nm	Nanometer
NTC	Non-template control
PAGE	Polyacrylamide gelelctrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Poly chain reaction

Abbreviation	Description
PTM	Post translational modifications
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
rcf	Relative centrifugation force
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RRMS	Relapsing Remitting MS
RT	Room temperature
RT -	Reverse transcriptase negative control
RXR	Retinoid X receptor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard deviation of mean
siRNA	Short interfering RNA
SN	Supernatant
SNP	Single nucleotide polymorphism
TAE buffer	Tris-acetate EDTA buffer
TAGAP	T cell activation Rho GTPase protein
TBP	TATA-box binding protein
TBS	Tris buffered saline
TBS/T	Tris Buffered Saline with Tween 20
TCR	T cell receptor
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory
UTR	Untranslated region
UV	Ultra violet
VDR	Vitamin D receptor
VDR-KO	Vitamin D receptor knock out
VDRE	Vitamin D responsive elements
ZAP-70	Zeta-chain associated protein
µ	Micro

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Aspargine	Asn	N
Aspartic acid	Asp	D
Cystein	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucin	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Nucleotide	One letter code
Adenine	A
Cytosine	C
Guanine	G
Thymine	T
Purine (A/G)	R
Pyrimidine (C/T)	Y
Any nucleotide	N

Appendix 2: Materials, equipment & computer software

Material	Catalog number	Producer
Cell culture		
1α,25 dihydroxy vitamin D3	D1530-.1MG	Sigma
Counting slides, dual chamber for cell counter	145-0015	BioRad
Cryotubes™ 1 ml	375418	Nunc™
Dimethyl Sulfoxide	D8418-100ml	Sigma Aldrich
Dyna Mag™-2	123.21D	Invitrogen
Dynabeads Human T activator CD3/CD28	11131D	Invitrogen
Dynabeads Untouched Human CD4	11346D	Invitrogen
Fetal Bovine serum EU approved	1147-9289	Fischer scientific
Lymphoprep	1115754	Axis Shield
Recombinant Human IL-2	202-IL	R&D Systems
RPMI 1640	21875-091	GIBCO® Invitrogen
Tryphan Blue Solution (0,4 %)	T8154	Sigma Aldrich
Hypodermic Needles, Microlance™ 3, 18G, 40x1,25mmm	613-3945	BD MEDICAL
X-vivo m/Gentamycin and phenolred	04-418F	Lonza
Gene expression		
DNase, RNase and protein free water	2500010	5 PRIME
Eukaryotic 18S rRNA Endogenous Control (VIC/MGB probe, primer limited)	4319413E	Life Technologies
Human TBP Endogenous Control (VIC/MGB probe, primer limited)	4326322E	Life Technologies
Maxima First Strand cDNA synthesis Kit for RT-qPCR	K1642	Thermo Scientific
TagMan gene expression assay CYP24A1 Hs00167999_m1, 750 rxn	4351370	Life Technologies
TagMan gene expression assay VDR Hs00172113_m1, 750 rxn	4331182	Life Technologies
Tagman Gene expression master mix	4369512	Life Technologies
TaqMan gene expression assay IL2RA HS00907777_m1, 750 rxn	4351370	Life Technologies
TaqMan gene expression assay TAGAP HS00299284, 750 rxn	4351370	Life Technologies

Genotyping

DNase, RNase and protein free water	2500010	5 PRIME
Genotyping assay, Assay ID: C_16095542-10, SNP ID: rs2104286 (IL2RA)	4351379	Life Technologies
Genotyping assay, Assay ID: C_1841420-20, SNP ID: rs7090512	4351379	Life Technologies
Genotyping assay, Assay ID: C_2966098-10, SNP ID: rs1738074 (TAGAP)	4351379	Life Technologies
TaqMan Genotyping Mastermix	4371355	Life Technologies

SDS-PAGE and Western blot

10 % mini protean TGX Precast gels, 10 well 30 ul	456-1033	BioRad
Actin (1-19) antibody	sc-1616	Santa Cruz
ECL developer, Super Signal west pico	PIER34080	Pierce
ECL Hyperfilm Amerham	28-9068-37	GE Healthcare
Gel loading tips	613-1046	VWR
IL2RA antibody (N-19)	sc-665	Santa Cruz Biotech
Immun-blot PVDF membrane for protein blotting	162-0177	BioRad
Methanol	32213-5L	Sigma
Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG	115-035-146	Jackson ImmunoResearch
Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG	111-001-003	Jackson ImmunoResearch
Peroxidase-conjugated AffiniPure Mouse Anti-Goat IgG	205-035-108	Jackson ImmunoResearch
Precision Plus Protein™ Standard	161-0374	Bio Rad
Restore Plus Western blot stripping buffer	PIER46430	Thermo Scientific
Skim Milk powder	70166-5006	Sigma Aldrich
TAGAP antibody (N-16)	sc-324393	Santa Cruz Biotech
ZAP-70 antibody	sc-32760	Santa Cruz Biotech

Gel electrophoresis

1 Kb Plus DNA ladder, 200 ug	10787-018	Invitrogen
6x DNA Loading Dye	R0611-PR143	Thermo Scientific
Ethidium Bromide	190202	MP Biomedicals
Saekem® LE Agarose	50004	Lonza
TAE Buffer (50 x)	R023	G-Bioscience

Bradford assay

Quick Start™ Bradford 1x Dye Reagent	500-0205	BioRad
Quick Start™ Bradford protein assay kit 1	500-0201	BioRad
RIPA buffer	11542461	Termo Scientific

RNA isolation and quality assessment		
Agilent RNA 6000 Nano Kit	5067-1511	Agilent Technologies
Qiashredder	79656	Qiagen
RNase AWAY®	7002	VWR
Beta mercaptoethanol	M6250	Sigma Aldrich
RNeasy Plus mini kit	74134	Qiagen

Computer Software	Version	Company
Bioanalyzer Expert Software	2100	Agilent technologies, Inc.
Excel®	14.2.5 121010	Microsoft
Graph Pad Prism 6		GraphPad Software, Inc.
Illustrator CC 214	18.01.00	Adobe
NanoDrop 2000	1.6	Thermo Scitentific
Viia™ 7 Software	1.0	Applied Biosystems

Equipment	Producer
2100 Bioanalyzer	Agilent
Centrifuge	Eppendorf
ECL Semi-Dry Blotter TE 77 PWR	Amersham Biosciences
Electrophoresis Chamber	Amersham bioscienses
FACS Calibur	BD sciences
GeneGenius Gel Light Imaging System	Syngene
Hypercasette™	Amersham Biosciences
Nanodrop UV/Vis spectrophotometer 2000	Thermo Scientific
TC20™Automated Cell Counter	Bio-Rad
Ultra 2100 Pro UV/Vis Spectrophotometer	Amersham Biosciences
Viia™ 7 Applied Biosystems	Life Technologies

Appendix 3: Recipes

Buffers for SDS-PAGE and Western blot analysis:

10 x Tris/Glycine/SDS running buffer	
(25 mM Tris, 192 mM 0,1% SDS)	**2,5 liters**
Tris base (Mm = 121,14 g/ml)	75 g
Glycine (Mm = 75,0666 g/mol)	360 g
20% SDS (v/w)	125 ml

1 x Tris/Glycine/SDS running buffer	
(25 mM Tris, 192 mM 0,1% SDS)	**1 litre**
10 x Tris/Glycine/SDS running buffer	100 ml
dH_2O	990 ml

Transfer buffer for 1 hour semi-dry	
(25 mM Tris, 192 mM glycine, 10 % methanol)	**2,5 liters**
Tris base (Mm = 121,14 g/ml)	7,5 g
Glycine (Mm = 75,0666 g/mol)	36 g
Methanol	250 ml
dH_2O	2,25 liters

10 x TBS, pH 7.6	
(100 mM Tris, 1,5 M NaCl)	**3 liters**
Tris base (Mm = 121,14 g/ml)	30,275 g
NaCl (mM = 58,44)	216,4 g
dH_2O	2,5 liters
Add HCL	to pH of 7,6

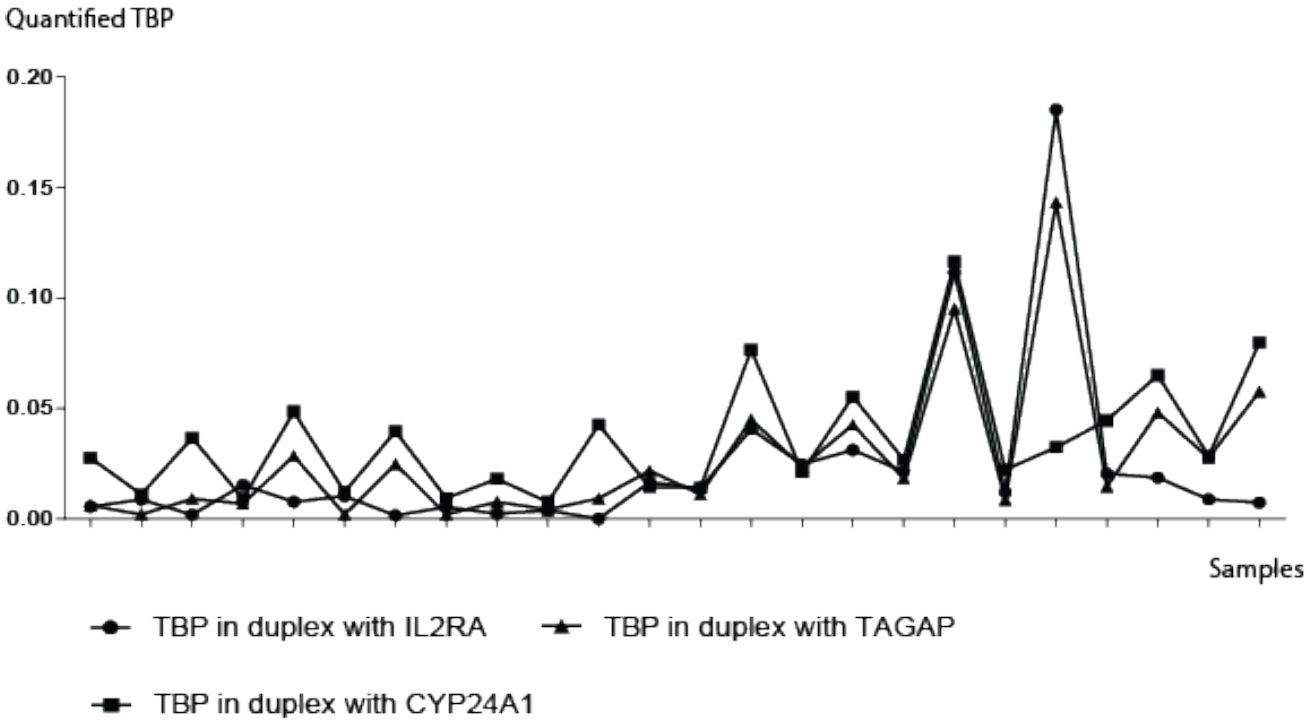
1 x TBS-T	
(10 mM Tris, 150 mM NaCl, Tween 20)	**3 liters**
10 x TBS, pH 7,6	300 ml
Tween 20	3 ml
dH_2O	2,7 liters

3% skimmed milk in 1 x TBS-T:	
(10 mM Tris, 150 mM NaCl, Skimmed milk powder)	**300 ml**
Skimmed milk powder	9,0 g
1 x TBS-T	up to 300 ml

6 x Sample loading buffer:	
(350 nM Tris-HCL pH 6,8, 10% SDS, 30% Glycerol, 0,175mM Bromophenolblue)	
1,5 M Tris-HCL, pH 6,8	5,8 ml
SDS (Mw = 288,4 g/mol)	2,5 g
Glycerol	7,5 ml
Bromophenolblue (Mm = 691,9 g/mol)	3 mg
β-mercaptoethanol	830 µl

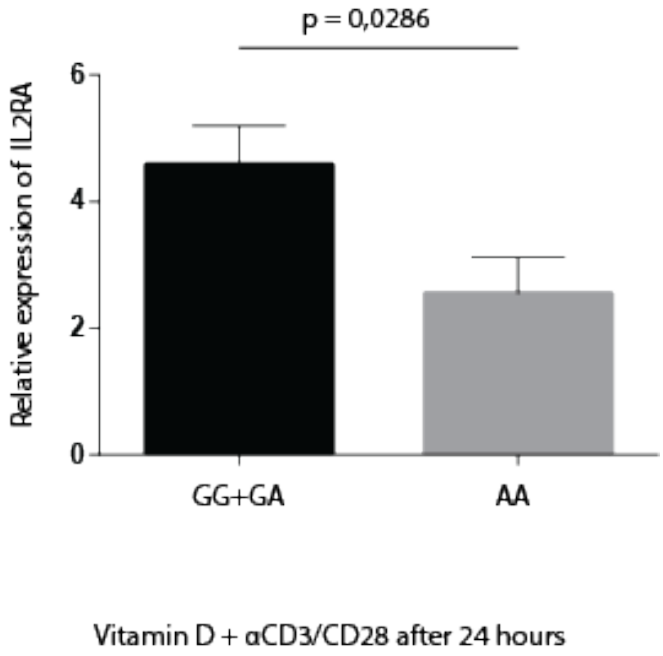
Appendix 4: Results

Quality control of reference genes



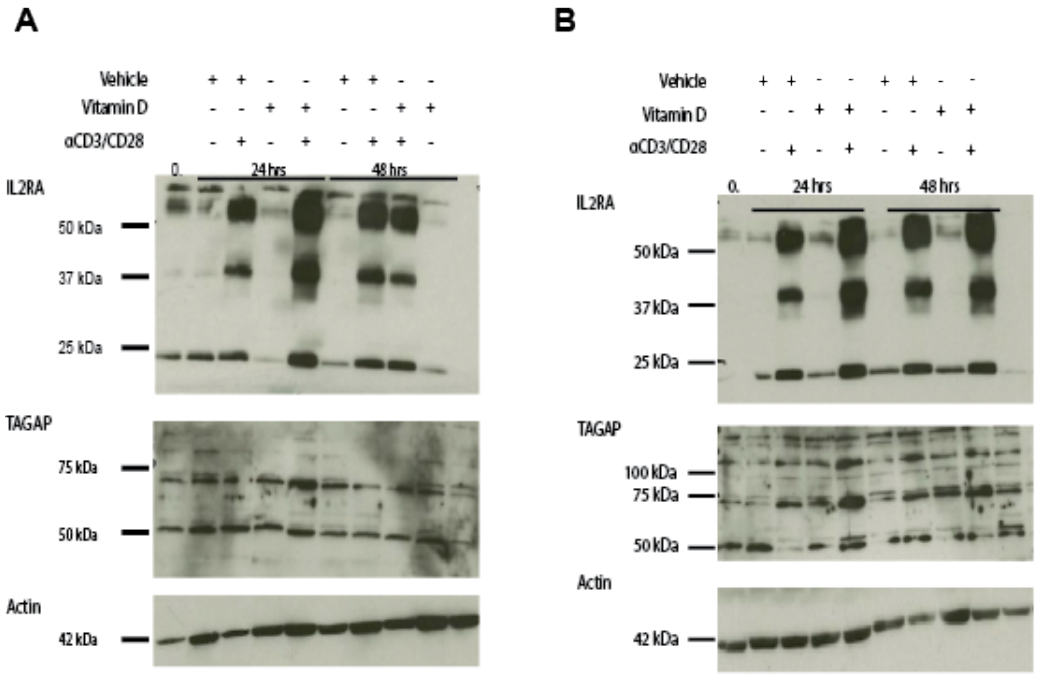
Supplementary figure 1: Quantified TBP from all duplex runs. TBP from all three runs aligned. TBP in duplex with *IL2RA* is not aligned with the other two.

Rs7090512 – GG+GA versus AA after 24 hours



Supplementary figure 2: Expression of *IL2RA* in individuals sorted on rs7090512 genotype. All carriers of the risk allele give rise to higher expression levels than non-risk allele in vitamin D treated cells co-cultured with αCD3/CD28 after 24 hours.

Western blots of IL2RA and TAGAP protein



Supplementary figure 3: Western blotting analysis (n = 2) IL2RA was detected by using primary anti-IL2RA polyclonal antibody produced in rabbit, and secondary anti-rabbit-HRP antibody, TAGAP

was detected by using primary anti-TAGAP polyclonal antibody produced in goat and secondary anti-goat-HRP antibody and loading control Actin was detected by anti-Actin polyclonal antibody produced in rabbit and secondary antibody anti-rabbit-HRP antibody. **A)** 13,125 µg of total protein was used in the experiment, but in sample with vitamin D+αCD3/CD28 24 and 48 hours, only 7,125 µg and 10,5 µg was applied in due to lack of material. **B)** 18, 75 µg was used, except for the well with vitamin D+αCD3/CD28 beads 24 hours, where only 2 µg material was available.