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The multiple sclerosis susceptibility genes *TAGAP* and *IL2RA* are regulated by vitamin D in CD4+ T cells

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Multiple sclerosis (MS) is an inflammatory, demyelinating disorder of the central nervous system that develops in genetically susceptible individuals. The majority of the MS-associated gene variants are located in genetic regions with importance for T-cell differentiation. Vitamin D is a potent immunomodulator, and vitamin D deficiency has been suggested to be associated with increased MS disease susceptibility and activity. In CD4+ T cells, we have analyzed *in vitro* vitamin D responsiveness of genes that contain an MS-associated single-nucleotide polymorphism (SNP) and with one or more vitamin D response elements in their regulatory regions. We identify *IL2RA* and *TAGAP* as novel vitamin D target genes. The vitamin D response is observed in samples from both MS patients and controls, and is not dependent on the genotype of MS-associated SNPs in the respective genes.

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory, demyelinating disorder of the central nervous system leading to various degrees of physical and cognitive disability. The prevalence is 0.5–2.0 cases per 1000 inhabitants, with the highest prevalence reported in Scandinavia.[1,2] MS typically appears in young adults and affects females more than twice as often as males.[3] The leading hypothesis is that MS is caused by a complex interaction between multiple genes and environmental factors, which leads to central nervous system inflammation causing demyelination and axonal degeneration. To date, the best supported environmental risk factors in MS are low vitamin D status, Epstein–Barr virus infection and smoking.[4]

It is now well established that the HLA class II-DRB1 locus causes the primary genetic association in MS, with the HLA-DRB1*15:01 allele as the major genetic risk factor in most populations (odds ratio = 3.1).[5,6] In 2011, the International Multiple Sclerosis Genetics Consortium and the Wellcome Trust Case Control Consortium 2 completed a genome-wide association study including more than 9000 MS cases and found evidence for 52 non-HLA genetic loci that are associated with MS. A strong presence of immunologically relevant genes, especially genes known to regulate T cell-mediated immunity, is evident within these loci. Apart from the HLA-DRB1*15:01 allele, all other genetic MS-risk loci each exert a relatively small effect (odds ratio = 1.1–1.3).[6] Interestingly, these genome-wide association studies implicated the relevance also for vitamin D-processing enzymes (that is, *CYP27B1* and *CYP24A1*) in MS[6] and one study shows that HLA-DRB1*15:01 is regulated by vitamin D in lymphoblastoid cell lines.[7]

These studies were followed by genome-wide analyses of immune-related loci using the ImmunoChip, which enhanced the catalog of MS-risk variants to 110.[8] The vast majority of single-nucleotide polymorphisms (SNPs) identified as MS-risk loci are located in non-coding regions of the genome.[6,8] Interestingly, the MS-associated SNPs are enriched in DNase I hypersensitive sites, indicating a functional role in regulation of gene transcription.[9]

Sun exposure and diet are the major sources of vitamin D. It exists as a biologically inert compound, vitamin D, which requires hydroxylation in the liver to 25-hydroxy vitamin D (25(OH)D). This is the major circulating form of vitamin D that has to be further hydroxylated to become the active metabolite 1,25-dihydroxy vitamin D ($1,25(OH)_2D$). This bioactive form of vitamin D was originally described as an essential hormone for mineral and bone homeostasis, and its biological effects are mediated by the nuclear receptor vitamin D receptor (VDR).[10,11] The discovery of VDR expression in monocytes and later in antigen-presenting cells suggested a potential role for $1,25(OH)_2D$ also in immune cells.[12–15] More recently it has become clear that vitamin D is indeed an important regulator of innate as well as adaptive immunity.[16–20] Naive T cells have a low expression of VDR, which is enhanced upon T-cell activation,[13,21,22] whereby $1,25(OH)_2D$ mediates a shift towards a more anti-inflammatory cytokine profile.[23–25] T cells express 1α-hydroxylase (encoded by *CYP27B1*), the enzyme responsible for the hydroxylation of 25(OH)D to $1,25(OH)_2D$ and can themselves metabolize circulating 25(OH)D to active $1,25(OH)_2D$.[22,26] Interestingly, $1,25(OH)_2D$ acts directly on the CD4+ T-lymphocyte VDR to inhibit experimental autoimmune encephalomyelitis (EAE), an animal model for MS,[27] whereas CD8+ T cells are dispensable for vitamin D-mediated experimental autoimmune encephalomyelitis protection.[24] Furthermore, data from Correale and colleagues suggest that $1,25(OH)_2D$ has an important role for T-cell homeostasis in MS, as $1,25(OH)_2D$ acts directly on CD4+ T cells to modulate cytokine secretion and reduce proliferation, and induce regulatory T cells (T_{reg}).[28,22] Taken together, these studies highlight the importance

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for $1,25(OH)_2D$ on T cells, especially CD4+ T cells, in relation with MS.

VDR is a nuclear receptor and a DNA-binding transcription factor that heterodimerizes with the retinoid X receptor (RXR). Upon $1,25(OH)_2D$ ligation, the VDR–RXR heterodimer recognizes vitamin D response elements (VDREs) within regulatory regions of its target genes, thereby affecting gene transcription.[11] VDREs are composed of tandem motifs with the consensus PuG(G/T)TCA, which are often arranged as direct repeats separated by 3 bp (DR3). VDR–RXR can also recognize everted repeats of hexameric motifs spaced by 6 bp (ER6).[29–31] A gene that is directly activated by a transcription factor such as VDR generally harbors a binding site for the transcription factor. Moreover, gene expression is normally rapidly induced upon activation of that particular transcription factor.

In the current study, we measured vitamin D response in human CD4+ T cells on expression of MS-associated genes,[6,8] with more than one VDREs in their regulatory region or with one VDRE and an MS-associated SNP in the same regulatory region. Exogenous addition of $1,25(OH)_2D_3$ to CD4+ T cells *in vitro* induced the expression of *IL2RA*, whereas it repressed *TAGAP* expression. This change in gene expression correlated with increased cell-surface expression of the *IL2RA* encoded CD25 and reduced TAGAP protein levels in activated CD4+ T cells. The vitamin D response was independent of MS disease and MS-risk genotype in the two genes. However, in MS patients there was a significant correlation between *IL2RA* expression in CD4+ T cells and serum levels of 25 (OH)D.

RESULTS

VDR and *CYP24A1* expression in CD4+ T cells

It has previously been reported that VDR expression is low in naive T cells, and that it is upregulated upon T-cell activation.[12,19,22,32,33] In agreement with this, we observed low VDR expression both at the mRNA and protein levels in freshly purified CD4+ T cells. Upon stimulation with αCD3/CD28-coated beads, VDR expression increased (Figures 1a and b) and reached its maximum protein expression after 24–40 h stimulation (Figures 1b and c). To verify the ability of $1,25(OH)_2D_3$ to induce activation of VDR signaling pathways in αCD3/CD28-stimulated human CD4+ T cells, we measured the expression levels of the well-documented VDR-target gene *CYP24A1* encoding 24-hydroxylase.[34] It has previously been shown that VDR is efficiently triggered only if $1,25(OH)_2D_3$ is added to T lymphocytes, with high levels of VDR expression at the time of $1,25(OH)_2D_3$ addition.[19] Therefore, CD4+ T cells were stimulated with αCD3/CD28-coated beads for 40 h before the addition of 1, 10 or 100 nM $1,25(OH)_2D_3$. After 24 h, *CYP24A1* was efficiently induced, reaching maximum induction with 10 nM $1,25(OH)_2D_3$ (Figure 1d).

Selection of MS-risk genes

In a recent genome-wide association study including samples from 9772 MS cases from 14 different countries, 23 previously suggested associations were replicated and 29 novel susceptibility loci were identified.[6] Combining these data with analysis of 14 498 MS cases on an ImmunoChip custom genotyping array, additional MS susceptibility variants were identified. The 110 established MS-risk variants represent 103 discrete loci outside the major histocompatibility complex.[8] Using data from previous *in silico* analyses,[35] we found that 80% of the 103 non-HLA MS-associated genes have one or more DR3-type VDREs in their gene promoter or regulatory regions (−10 to +5 kb). Of these genes, 23 genes contained two or more VDREs (19 genes with two VDREs and 2 genes each with three or four VDREs; see Supplementary Table 1). We chose to study genes that had either (i) more than one VDRE in their regulatory region (20 genes, see Supplementary Table 1) or

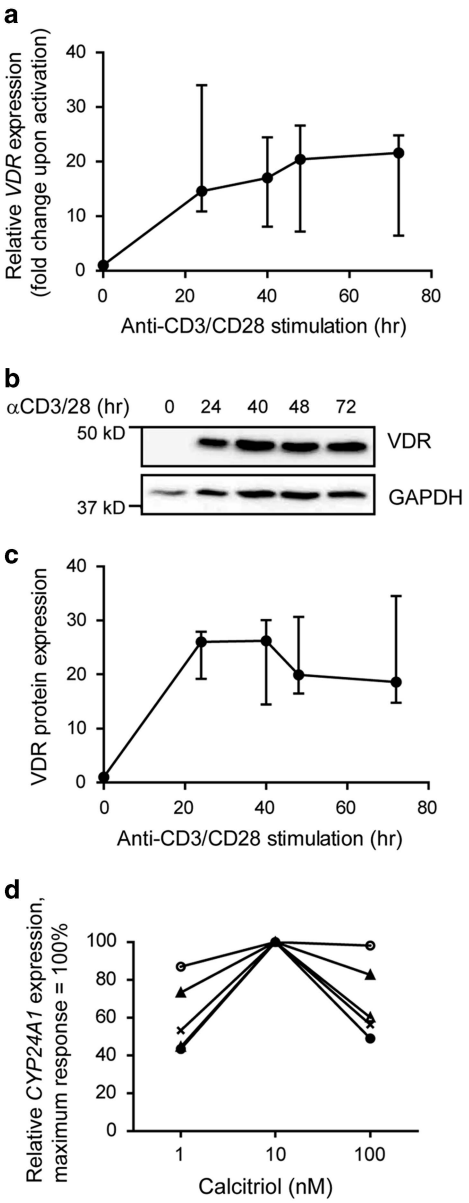

Figure 1. *VDR* and *CYP24A1* expression in CD4+ T cells. (**a**–**c**) Human CD4+ T cells were left unstimulated (0) or stimulated for indicated hours with αCD3/CD28 beads before cell harvesting. (**a**) mRNA expression levels of *VDR* were measured relative to *RNaseP*, and expression in the unstimulated cells was set to 1. The graph represents the median with error range from four independent experiments. (**b**) Whole-cell lysates from untreated or stimulated CD4+ T cells were immunoblotted with indicated antibodies. The immunoblot shows one representative experiment out of four. (**c**) Bands (as shown for one representative in **b**) were quantified and normalized as described. The graph shows the median level with error range of VDR from four independent experiments. (**d**) Human CD4+ T cells were stimulated with αCD3/CD28 beads for 40 h before the addition of indicated amounts of $1,25(OH)_2D_3$ or vehicle control for 24 h. *CYP24A1* mRNA expression was measured by quantitative real-time PCR using *RNaseP* as reference gene. The graph represents *CYP24A1* expression in $1,25(OH)_2D_3$-treated cells relative to its expression in vehicle-treated cells, with a maximum of 100% assigned for the group with maximum $1,25(OH)_2D_3$-induced *CYP24A1* expression, that is, 10 nM $1,25(OH)_2D_3$. Each line represents measurements from CD4+ T cells from one healthy donor.

(ii) that contained one VDRE and an MS-risk SNP in the regulatory region of the gene (3 genes; *IL12B*, *SCO2* and *IL2RA*). These MS-risk genes were analyzed *in silico* for expression in CD4+ T cells. We further limited the number of genes by selecting those that showed high expression in the https://genome-euro.ucsc.edu/ genome browser (labeled 'red' in Supplementary Table 1) and that displayed gene expression higher than the average at http://biogps.org/ (that is, *CD5*, *IL2RA*, *MALT1*, *RGS14*, *SP140*, *STAT3*, *STAT4*, *TAGAP*, *TCF7*, *TYK2*, *BATF* and *MYC*); see Table 1. In addition, *CD6* was analyzed, as the MS-associated SNPs in this region are located between the two VDRE-containing *CD5* and *CD6* genes (Table 1).

Calcitriol affects the expression of *IL2RA*, *TAGAP*, *BATF* and *MYC* in CD4+ T cells

A total of 13 genes (Table 1) were analyzed for 1,25$(OH)_2D_3$ responsiveness in CD4+ T cells isolated from six healthy donors. The cells were activated for 40 h with αCD3/CD28 to induce VDR expression before the addition of 10 nM 1,25$(OH)_2D_3$ or vehicle control (ethanol). Cells were collected 3, 6 and 24 h after addition of 1,25$(OH)_2D_3$ or vehicle, and gene expression was measured by quantitative real-time PCR relative to the three reference genes *18S rRNA*, *TBP* and *GAPDH* as described in the Materials and Methods section. Genes that displayed pair-wise significant differences in expression in 1,25$(OH)_2D_3$-treated samples compared with vehicle-treated samples (paired Student's *t*-test for each time point) for the three different reference genes were considered as 1,25$(OH)_2D_3$-responsive. *CYP24A1* has previously been shown to be regulated by VDR.[34] As expected, its expression was induced upon addition of 1,25$(OH)_2D_3$ compared with vehicle control. Of the tested MS-associated VDRE-containing genes (Table 1), *IL2RA* expression was induced by 1,25$(OH)_2D_3$ treatment, whereas *TAGAP, MYC* and *BATF* expression was downregulated (Figure 2). No significant 1,25$(OH)_2D_3$ response was observed for the other genes tested (data not shown).

Using a paired Student's *t*-test, we were able to compare 1,25$(OH)_2D_3$-induced versus vehicle-induced gene expression at each individual time point. To determine whether there is a change in differential expression over time for all the genes listed in Table 1, we performed a functional analysis of variance test. After Benjamini–Hochberg correction, only three of the genes from Figure 2 showed a significant *P*-value for all three reference genes, that is, *CYP24A1*, *IL2RA* and *TAGAP* (corrected *P*-values for gene expression relative to *TBP*; 1.9 e-13, 2.6 e-6 and 4.7 e-7, respectively). For further analyses, we chose to focus on genes that displayed significantly altered expression when normalized to each of the three reference genes in both statistical tests, that is, *IL2RA* and *TAGAP*. As before, *CYP24A1* was used as a positive control.

Table 1. Overview of the genes tested for vitamin D responsiveness in human CD4+ T cells

Chr	*Gene*	*VDRE*			*SNP*		*Gene expression*
		#	*Location*	*Sequence*	*ID*	*Location*	
2	*SP140*	3	−6384	AGGTCAacaGGT**C**CA	rs9989735[a]	Intronic	72.3 (14.7)
			−1842	AGG**C**CAagtAGGTCA	rs10201872[b]	Downstream/intronic	
			166	A**C**TTCAggtGGGTCA			
2	*STAT4*	2	−7814	AGTTCAttg**T**GGTCA	rs9967792[a]	Intronic	327 (25.7)
			−361	AGGTCAggaAGG**G**CA			
5	*RGS14*	2	−2840	GGG**G**CAgcaGGGTCA	rs4976646[a]	Intronic	39.5 (17.6)
			1546	AGTTC**T**gggGGTTCA			
5	*TCF7*	2	−5908	AGTTCAcctAGGTC**C**	rs756699[a]	Intergenic	166 (13)
			2954	GGG**C**CAcatGGGTCA			
6	*TAGAP*	2	−5146	GGTTCAagtG**A**TTCA	rs212405[a]	Promoter	752 (70.5)
			329	AGGTCActcAG**A**TCA	rs1738074[b]	5′-UTR	
8	*MYC*	2	−8026	G**A**TTCAgagAGGTCA	rs4410871[b]	Intergenic	847 (231)
			−7277	GGTTCAggcAGTTC**C**			
10	*IL2RA*	1	−9537	AGG**A**CAagaAGTTCA	rs2104286[a]	Intronic	8.6 (7.2)
					rs3118470[b]	Intronic	
					rs7090512[c]	Promoter	
11	*CD5/ CD6*[d]	3	−6734	AGGTC**T**ctgAGGTCA	rs34383631[a]	Intergenic	10.0 (9.1)/36 (7.6)
			2129	GGTTCAaggAGGTC**C**	rs650258[b]	Intergenic	
			3553	GGTTCAagcGGTTC**T**			
			1453	AG**C**TCAgggAGGTCA			
14	*BATF*	4	−8775	A**A**GTCAtttGGTTCA	rs2300603[b]	Intronic	51.3 (8.7)
			−8329	GGTT**G**AagtAGGTCA			
			−1426	AGGTCAgtgAGGT**A**A			
			−803	AGGTCAcatAG**C**TCA			
17	*STAT3*	2	−8560	GGTTCAaagAGGT**T**A	rs4796791[a]	Intronic	271 (135)
			−1155	**C**GTTCAgaaAGGTCA	rs9891119[b]	Intronic	
18	*MALT1*	2	−2840	G**A**GTCAgagAGGTCA	rs7238078[b]	Intronic	82.7 (15.1)
			1546	GGGTC**C**acaAGGTCA			
19	*TYK2*	2	−9110	GGTTCAagcAGTTC**T**	rs34536443[a]	Exonic	214 (61.1)
			−3574	GGTTCAagtG**A**TTCA	rs8112449[b]	Promoter (CDC27)	

Abbreviations: GWAS, genome-wide association study; ID, identity; SNP, single-nucleotide polymorphism; UTR, untranslated region; VDRE, vitamin D response element. The table indicates from the left, the chromosome number (Chr), the gene name, the number (#) of VDREs in the −10 to +5 kb region of the indicated genes,[35] the location of the VDREs relative to ATG (Build 35 version 1; August 2004),[35] the sequence of the VDRE consensus sequences; A/G G G/T T C A n n n A/G G G/T T C A (bold labels the nucleotides that deviates from the consensus sequence), the identity of the MS-associated SNPs,[6,8] the location of the MS-associated SNPs http://snpper.chip.org/,[6,8] and gene expression data from http://biogps.org of the indicated genes compared with mean value of gene expression in all tissues/cells tested in brackets. [a]SNPs identified/verified by the ImmunoChip.[8] [b]SNPs identified/verified by the GWAS.[6] [c]Secondary signal ($P < 10–14$) in the GWAS.[6] [d]The MS-associated SNPs in this region were located in the intergenic regions between *CD5* and *CD6*, however, it was denoted to *CD6*.[6,8]

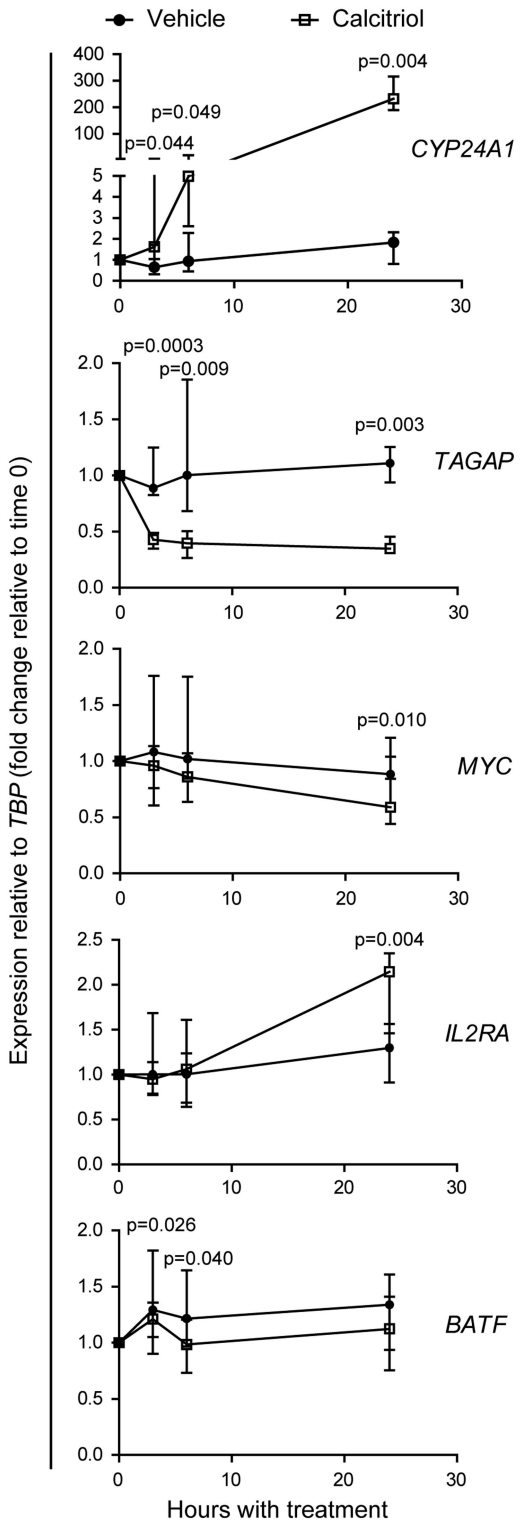

Figure 2. Calcitriol response of MS susceptibility genes in CD4+ T cells. Human CD4+ T cells from six donors were treated as described in Figure 1d, with 10 nM $1,25(OH)_2D_3$ before quantitative real-time PCR analysis of the MS-associated genes listed in Table 1. The graphs show median expression relative to *TBP* of indicated genes with error ranges in $1,25(OH)_2D_3$- and vehicle-treated cells. Expression at the time of $1,25(OH)_2D_3$ or vehicle addition is set to 1. Significant *P*-values, $P < 0.05$, are given (Student's paired *t*-test for each single time point). Only data from genes with significantly increased expression after $1,25(OH)_2D_3$ addition are shown.

Calcitriol inhibits TAGAP protein expression and induces cell-surface expression of CD25 in activated CD4+ T cells

To assess whether vitamin D has an impact on the protein level of TAGAP and CD25, encoded by *TAGAP* and *IL2RA*, respectively, CD4+ T cells were purified from healthy donors, activated for 40 h with αCD3/CD28 before addition of 10 nM $1,25(OH)_2D_3$ or vehicle control (ethanol). Cells were collected 24 and 48 h after addition of $1,25(OH)_2D_3$ or vehicle, and protein expression was measured by flow cytometry. We observed that TAGAP protein expression was induced upon T-cell activation as measured by flow cytometry and western blotting (Figures 3a and b). Addition of calcitriol resulted in a small but significant reduction of total TAGAP expression after 48 h of incubation (Figure 3b), whereas cell-surface expression of CD25 was slightly induced (Figure 3c). No significant changes in protein levels of either of the proteins were observed after 24 h with calcitriol (data not shown).

Expression of *TAGAP* and *IL2RA* is not influenced by MS or MS-risk genotype

To evaluate whether there is a correlation between the presence of an MS-risk variant in *TAGAP* or *IL2RA*, and their vitamin D response in CD4+ T cells *in vitro*, we analyzed gene expression in CD4+ T cells from 18 individuals (8 relapsing-remitting MS (RRMS) patients and 10 age- and sex-matched healthy controls) that were genotyped for rs1738074 (ImmunoChip top-hit[8] in *TAGAP* 5′ untranslated region) and rs7090512 (*IL2RA*, a promoter SNP that appeared as secondary signal of association in the genome-wide association study[6]). Cells were treated with 10 nM $1,25(OH)_2D_3$ or vehicle in the presence of αCD3/CD28 beads, collected after 6, 24 and 48 h, and relative gene expression of *CYP24A1*, *TAGAP* and *IL2RA* was measured by quantitative real-time PCR. *CYP24A1* expression was induced in the cultivated cells upon $1,25(OH)_2D_3$ treatment (data not shown). As in the initial screening in CD4+ T cells from healthy donors (Figure 2), we observed an increase in *IL2RA* and a decrease in *TAGAP* expression upon $1,25(OH)_2D_3$ addition. No difference was observed between MS patients and healthy controls (Figure 4a). As the presence or absence of MS did not give rise to any difference in vitamin D response, all samples were combined and grouped according to genotype of MS-associated SNPs in *IL2RA* and *TAGAP*. No significant genotype-dependent difference in vitamin D response was observed for either *TAGAP* or *IL2RA* (Figure 4b).

IL2RA expression in CD4+ T cells correlates with serum levels of 25(OH)D

Our *in vitro* experiments indicate that *TAGAP* and *IL2RA* gene expression is regulated by $1,25(OH)_2D_3$. To investigate whether there is a correlation between the expression of these vitamin D-responsive genes in immune cells and serum 25(OH)D levels, we measured the expression of *IL2RA* and *TAGAP* in CD4+ T cells purified from RRMS patients with known vitamin D status.[36] Within this cohort, 14 patients had used vitamin D supplements within 6 months before sampling.[37] Accordingly, the range of serum 25(OH)D levels was relatively large (52–260 nM).[36] In these samples, we observed a positive correlation between relative *IL2RA* expression and 25(OH)D serum levels in MS patients (Figure 5). This was most likely not due to differential *VDR* expression levels, as *VDR* expression did not correlate with the serum 25(OH)D levels in the MS patients,[36] neither did *VDR* expression correlate with *IL2RA* expression (data not shown). We did not observe any correlation between serum 25(OH)D levels and *TAGAP* expression (Figure 5).

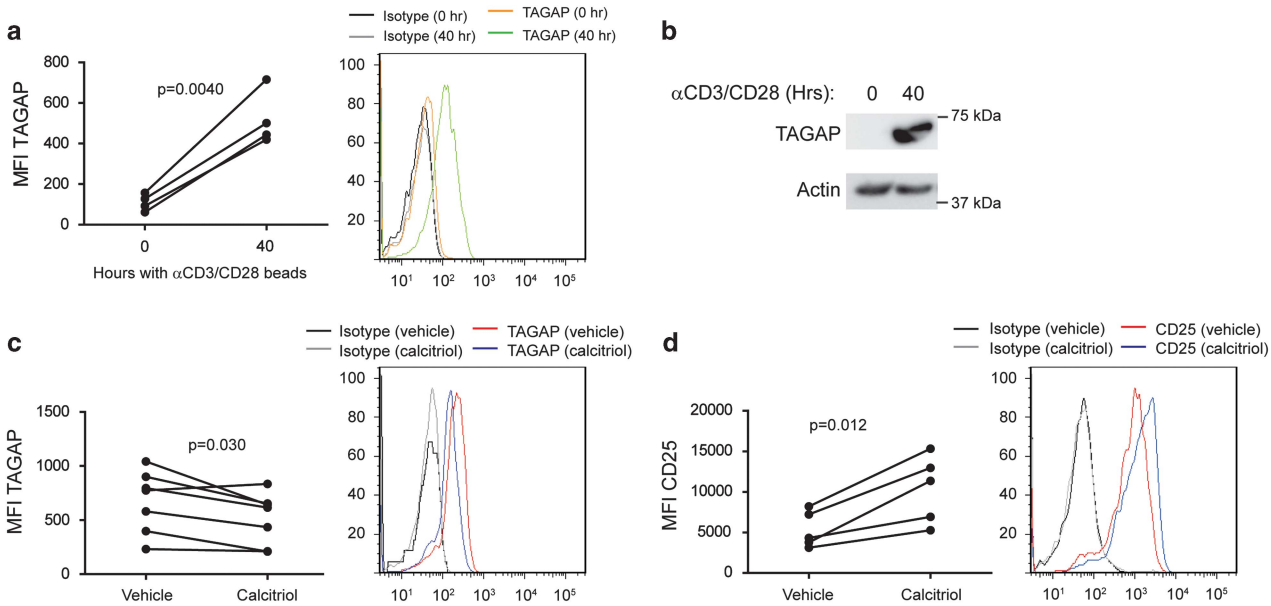

Figure 3. Calcitriol inhibits TAGAP protein expression and induces cell-surface expression of CD25 in CD4+ T cells. (**a**) Human CD4+ T cells were left unstimulated (0) or stimulated for 40 h with αCD3/CD28 beads before intracellular staining of TAGAP and flow cytometry. Median fluorescent intensity (MFI) before (0) and after (40) stimulation is indicated for each donor ($N=4$). Paired Student's *t*-test, $P=0.0040$. The histogram plot shows one representative experiment (black line, isotype control unstimulated cells (0 h); gray line, isotype control stimulated cells (40 h); orange line, TAGAP-stained unstimulated cells (0 h); green line, TAGAP-stained stimulated cells (40 h). (**b**) Whole-cell lysates from unstimulated or stimulated CD4+ T cells were immunoblotted with indicated antibodies. Human CD4+ T cells were treated as described in Figure 1d, with 10 nM $1,25(OH)_2D_3$ for 48 h before flow cytometry to measure (**c**) TAGAP ($N=7$) and (**d**) CD25 ($N=5$) expression. The graphs show the MFI of TAGAP and CD25 in vehicle- and calcitriol-treated cells. Paired Student's *t*-test $P=0.030$ (TAGAP) and $P=0.012$ (CD25). Histogram plots show one representative experiment (black line, isotype control vehicle-treated cells; gray line, isotype control calcitriol-treated cells; red line, TAGAP (**c**) or CD25 (**d**) vehicle-treated cells, blue line, TAGAP- (**c**) or CD25 (**d**)-stained calcitriol-treated cells).

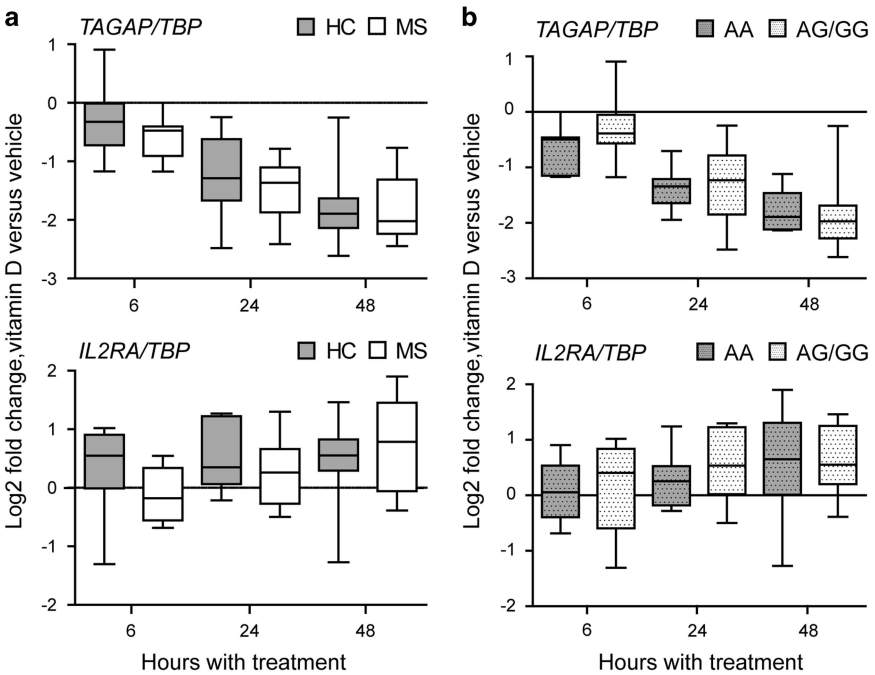

Figure 4. Calcitriol response of *TAGAP* and *IL2RA* in CD4+ T cells is not influenced by MS or MS-risk genotype. CD4+ T cells from 8 RRMS patients (MS) and 10 healthy controls (HC) genotyped for MS-risk SNPs in *TAGAP* (rs1738074) and *IL2RA* (rs7090512) were cultivated for 7–8 days with αCD3/CD28 and IL-2, treated as described in Materials and Methods before harvesting after 6, 24 and 48 h, with $1,25(OH)_2D_3$ or vehicle control. The box plots with whiskers defining minimum and maximum show the log2 values of relative expression of indicted genes (relative to *TBP*) in $1,25(OH)_2D_3$-treated cells divided by relative expression in vehicle-treated cells, that is, log2 of fold induction by $1,25(OH)_2D_3$ at each time point for (**a**) MS patients compared with HCs and (**b**) the same samples sorted on *IL2RA* and *TAGAP* genotype, that is, samples carrying the minor allele (*IL2RA*: $N=10$, MAF (G) = 0.3480; *TAGAP*: $N=11$, MAF (G) = 0.46563) compared with samples homozygous for major allele ($N=8$ (*IL2RA*); $N=7$ (*TAGAP*)). The horizontal lines within the boxes represent the median of the groups, and Mann–Whitney *U*-test was performed to compare the groups (MS vs HC, AA vs AG/GG). The horizontal line at 0 indicates no induction by $1,25(OH)_2D_3$.

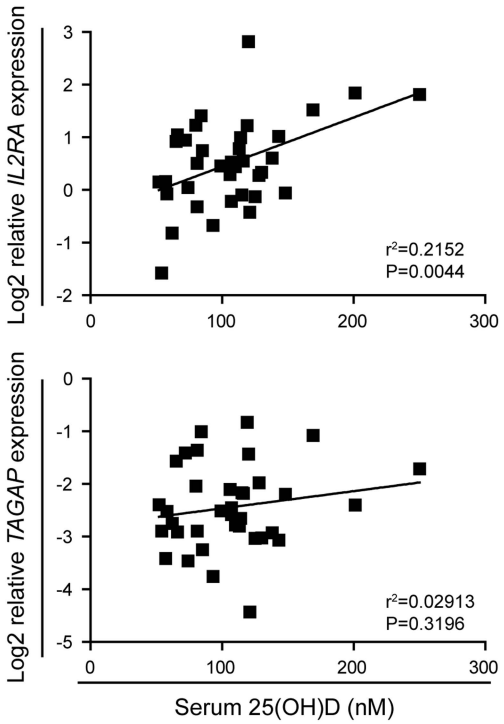

Figure 5. *IL2RA* expression correlates with serum levels of 25(OH)D in CD4+ T cells from MS patients. CD4+ T cells were purified from a Dutch cohort of RRMS patients with known serum 25(OH)D levels as described in Materials and Methods. The graphs display linear regression analyses of serum 25(OH)D levels with the log2 of the expression of *IL2RA* and *TAGAP* relative to *TBP* in CD4+ T cells. r^2 represents the coefficient of determination and *P* the uncorrected *P*-values.

DISCUSSION

Low levels of vitamin D is a risk factor in MS, and active vitamin D can act directly on immune cells, as these cells express VDR. In the present study, we demonstrate that two MS-associated genes, *IL2RA* and *TAGAP*, that both contain VDREs, are regulated by vitamin D in CD4+ T cells *in vitro*. The change in gene expression correlated with change also at the protein level for CD25 and TAGAP. The vitamin D response of these genes was comparable in T cells from MS cases and age- and sex-matched healthy controls, and was independent of the genotype of their corresponding MS-associated SNPs within the *IL2RA* and *TAGAP* genes, respectively. Finally, there was a positive correlation between *IL2RA* expression in CD4+ T cells and the serum level of 25(OH)D in MS cases, indicating that vitamin D might have an impact on *IL2RA* expression *in vivo*.

For *TAGAP* and *IL2RA*, a twofold change in gene expression was observed after $1,25(OH)_2D_3$ treatment. This rather modest response to vitamin D is comparable to what has been detected in other studies when analyzing *IL-2* expression in the Jurkat T cell line[38] or *IFN-γ* and *IL-10* expression in human primary T cells.[19] Furthermore, in a transcriptome-wide study performed in THP-1 cells, only 67 (16%) of 408 vitamin D-upregulated genes were induced more than 1.5-fold after 4 h of $1,25(OH)_2D_3$ treatment.[39] As $1,25(OH)_2D_3$ triggers VDR signaling in T lymphocytes efficiently only in the presence of high VDR levels,[19] T cells were activated with αCD3/CD28-coated beads to induce VDR expression before $1,25(OH)_2D_3$ addition, and these beads were kept in the media during the $1,25(OH)_2D_3$ treatment period. In the current work, we used 10 nM $1,25(OH)_2D_3$ throughout the study. This is a concentration that is frequently used in equivalent studies[19,22] and is considered to be a pharmacological dose.[22] This level of $1,25(OH)_2D_3$ is ~100-fold higher than that of the circulating levels of $1,25(OH)_2D_3$ (⩽100 pM) in healthy individuals.[40] However, it is likely that the local concentration of $1,25(OH)_2D_3$ in tissues and within cells expressing the 1-α-hydroxylase (from the *CYP27B1* gene) may be considerably higher than the levels in the circulation.[19,22,41] The initial screening (Figure 2) was performed in CD4+ T cells from six healthy donors that were not genotyped. The minor allele frequency of the MS-associated genes tested for vitamin D responsiveness differs between 0.010 (rs34536443 in *TYK2*) and 0.47 (rs1738074 in *TAGAP*). The genotype distribution will vary from gene to gene, and we cannot rule out the possibility that for some genes, especially those with low minor allele frequency, only one genotype is present, which could influence the vitamin D responsiveness of the gene. However, for *IL2RA* and *TAGAP*, the genotype of the MS-associated SNPs did not affect vitamin D responsiveness in the corresponding genes (Figure 4). The primary selection criterion for the current analyses of vitamin D responsiveness of MS-associated genes was that the genes to study should contain at least one VDRE. There are examples of genes that are regulated by VDR in a VDRE-independent manner where VDR inhibits gene expression by antagonizing certain transcription factors.[42–44] Thus, our analyses are not comprehensive. However, when analyzing genome-wide chromatin immunoprecipitation (ChIP) data of VDR binding focusing on the top VDR sites (based on fold enrichment scoring), the majority of these genes contain a DR3–VDRE sequence.[39,45–47] Genome-wide VDR ChIP-sequencing studies have been performed in five different cell lines (GM10855 and GM10861,[45] THP-1 cells,[39,48] LS180 colorectal cancer cells[49] and LX3 hepatic stellate cells[50]) and one study was performed in primary human CD4+ T cells.[51] Taken together, these data indicate that there are far more genomic VDR-binding sites (1000–10 000) per cell than target genes (100–500 per tissue). With a total of 21 776 non-overlapping VDR-binding sites, only 54 are common within the six data sets and 67% are unique for one of the analyzed data sets[47] (summarized for the MS susceptibility genes in Supplementary Table 2). This indicates that VDR displays cell-specific binding patterns, and functional follow-up of each potential target gene in relevant tissue is therefore of great importance.

Among the 13 VDRE-containing genes tested in CD4+ T cells, all genes were occupied by VDR in T cells, although in some genes more than 10 000 bp upstream of the coding sequence (that is, *IL2RA* and *TYK2*;[51] summarized in Supplementary Table 2), but only two of them were regulated by vitamin D *in vitro*. Whereas VDR-mediated gene repression occurs through a variety of mechanisms (reviewed in ref. 11), transcriptional activation by VDR is mediated through binding to its cognate VDREs. Surprisingly, the VDRE-containing *TAGAP* gene was downregulated upon $1,25(OH)_2D_3$ addition, and a response was observed after only 3 h. Owing to this rapid response it is likely that *TAGAP* is regulated by VDR itself and not indirectly by another factor that is transcriptionally induced by VDR. Interestingly, in ChIP experiments from CD4+ T cells, one of the VDREs in *TAGAP* (+329 VDRE in Table 1) lies within the VDR ChIP-sequence-binding interval.[51] Combined with our expression data, this suggests that VDR can bind *TAGAP* and thereby can regulate gene expression in human CD4+ T cells. Our data further indicated that the genotype of the MS-associated rs1738074, only 121 nucleotides away from the VDRE (+329), does not have any impact on vitamin D-mediated repression of *TAGAP* in CD4+ T cells. As *TAGAP* contains a VDRE in its 5′ region, it is likely that the vitamin D-activated VDR binds to the *TAGAP* promoter, as shown in CD4+ T cells,[51] thus reducing the transcriptional activity of the gene. This might occur by recruiting corepressors to the site, which is the case for instance of the VDR-regulated *MYC* gene in human prostate cells.[52] Alternatively, data from the encode database indicate that the VDRE in the 5′ region of *TAGAP* overlaps with a RELA ChIP-sequencing signal.[53] This suggests that VDR might compete with RELA inhibiting

TAGAP transcription in a mechanism similar to VDR-mediated *IL-2* repression, where VDR blocks the binding of the transcription factor nuclear factor of activated T cells-1 to the *IL-2* promoter.[42]

On the other hand, *IL2RA* was consistently induced after 1,25 $(OH)_2D_3$ treatment. This was observed at a much later time point, that is, 24 h and could indicate that *IL2RA* induction was not directly mediated by VDR. A significant correlation between *IL2RA* expression in CD4+ T cells and serum levels of vitamin D in MS patients further indicates that vitamin D, although not necessarily directly, might modulate *IL2RA* expression. *VDR* expression can be induced by calcitriol,[54] however, as there was no significant correlation between *VDR* expression and 25(OH)D levels[36] or *IL2RA* expression (data not shown), the positive correlation between *IL2RA* expression and vitamin D status is not due to different VDR expression levels. In the current analyses we could not find any correlation between *IL2RA* response and the genotype of rs7090512. Furthermore, the MS-associated SNPs in *IL2RA* were not pulled down in the VDR ChIP-sequencing experiment in CD4+ T cells.[51] The MS-associated SNPs in *IL2RA* are not within a predicted VDRE. The VDRE in *IL2RA* is situated almost 10 kb upstream of the *IL2RA* transcriptional start site (TSS), but VDR loci in a distance even more than 1 Mb from the gene's transcriptional start site are accepted as regulatory sites.[47] It remains to be shown whether this VDRE in *IL2RA* is a functional site for VDR in these cells.

TAGAP encodes the T-cell activation Rho-GTPase-activating protein (TAGAP). It has previously been shown that *TAGAP* mRNA expression is induced during T-cell activation,[55] and we here show that TAGAP is induced also at the protein level upon T-cell activation (Figures 3a and b). The function of TAGAP as such is currently unknown. However, as GAPs are involved in several key immunological processes in T cells,[56,57] TAGAP might have a potential regulatory role in T-cell activation. In the current study, we show that 1,25 $(OH)_2D_3$ down-modulated *TAGAP* expression in activated CD4+ T cells. Polymorphisms in *TAGAP* have shown association to other autoimmune diseases such as type-1 diabetes, rheumatoid arthritis and Crohn's disease,[58–61] and studies of colonic tissues in Crohn's disease patients show elevated *TAGAP* expression in samples from patients with a more severe phenotype.[62] It is not known whether *TAGAP* expression correlates with MS severity. Thus, whether vitamin D-mediated reduction in TAGAP expression in activated CD4+ T cells is beneficial for MS remains to be studied.

IL2RA expresses CD25, the alpha chain of the high-affinity IL-2 receptor, and increased cell-surface expression of CD25 observed in 1,25$(OH)_2D_3$-treated CD4+ T cells might indicate an increase in interleukin (IL)-2 responsiveness. Interestingly, there is a correlation between 25(OH)D levels and T_{reg} cell function.[63] Moreover, 1,25$(OH)_2D_3$ induces the development of T_{reg} cells, with an enhanced suppressive activity *in vitro*.[22] Even though IL-2 acts as trophic factor for anti-inflammatory T_{reg} cells, it is also aiding the expansion of pro-inflammatory effector T cells.[64] In the samples we have analyzed, both effector CD4+ T cells as well as T_{reg} cells are likely to be present. An increase in *IL-2* response of T_{reg} cells would probably be beneficial for autoimmunity, whereas the opposite would be the case for effector CD4+ T cells.

Low vitamin D status has been suggested to be a risk factor in several diseases, and VDR ChIP sequencing of human primary CD4+ T cells indicates enrichment of VDR binding within regions associated with autoimmunity.[51] In the current analyses we find that 80% of the MS-associated genes have one or more VDRE in their regulatory region; however, only 2 out of 13 selected VDRE-containing genes display vitamin D responsiveness in CD4+ T cells *in vitro*. This indicates that the presence of VDRE and binding of VDR is not sufficient to induce gene expression *in vitro*, and it remains to be analyzed whether this is different in an *in vivo* setting. Altogether our data show that the MS susceptibility genes *TAGAP* and *IL2RA* are regulated by vitamin D in CD4+ T cells. The mechanism behind this regulation remains to be elucidated; however, neither presence nor absence of MS nor the genotype of the MS-associated risk variants do seem to affect the vitamin D responsiveness of these genes.

MATERIALS AND METHODS

Reagents and antibodies

Calcitriol (1,25$(OH)_2D_3$; Sigma-Aldrich Corp., St Louis, MO, USA) was solubilized in absolute ethanol to 1 mM and further diluted in X-VIVO 15 medium (04–418, Lonza, Basel, Switzerland) before addition to the cell culture. The antibodies used were fluorescein isothiocyanate-conjugated mouse anti-human CD4 (clone RTF-4 g, Southern Biotech, Birmingham, AL, USA), fluorescein isothiocyanate-conjugated mouse IgG1 isotype control (15H6, Southern Biotech), allophycocyanin-conjugated anti-human CD25 (IgG1, Immunotools, Friesoythe, Germany), allophycocyanin-conjugated mouse isotype IgG1 (Immunotools), mouse anti-VDR (D-6, sc-13133, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-GAPDH (6C5, sc-32233, Santa Cruz Biotechnology), rabbit anti-TAGAP (EPR15593, Abcam, Cambridge, UK), rabbit anti-Actin (A2066, Sigma-Aldrich Corp.), rabbit monoclonal IgG (EPR25A, Abcam), horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Europe Ltd., Suffolk, UK) and Alexa Fluor 647-conjugated goat anti-rabbit IgG (A-21245, Molecular Probes, Life Technologies, Carlsbad, CA, USA).

Isolation of human CD4+ T cells and cell culture

Peripheral blood mononuclear cells were isolated from whole blood by Lymphoprep (Axis Shield, Dundee, Scotland), before negative selection of CD4+ T cells using autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) or Dynabeads Untouched Human CD4 T Cell Isolation kit (Life Technologies) to more than 95% purity as measured by flow cytometry (FacsCalibur, BD Biosciences, San Jose, CA, USA or Attune Acoustic Focusing Flow Cytometer, Life Technologies). Freshly purified CD4+ T cells were either frozen on liquid nitrogen for later use or resuspended in X-VIVO medium (Lonza) with human IL-2 (R&D Systems, Minneapolis, MS, USA) and αCD3/CD28 beads (1:1) (Human T-activator CD3/CD28 Dynabeads for cell expansion and activation, Life Technologies) for activation and 1,25$(OH)_2D_3$ treatment. If the cell density reached 2.5 million cells per ml, the wells were split. Cells from genotyped individuals were stored on liquid nitrogen (two million cells per tube). After thawing, the cells were expanded to achieve sufficient cell numbers for the analyses by growing them as the freshly isolated CD4+ T cells for 7-8 days before removal of aCD3/CD28 beads 24 h prior to addition of 1,25$(OH)_2D_3$ or vehicle control together with aCD3/CD28 beads. At the time intervals indicated in the text, cells were counted and collected by centrifugation. Before cell lysis for western blot analyses, magnetic beads were removed before cell harvesting.

Cell lysis, western blotting and flow cytometry

The cell pellet was resuspended in reducing SDS-loading buffer, sonicated and heated to 95 °C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis using pre-made Criterion gels (BioRad, Hercules, CA, USA) and transferred to polyvinylidene fluoride membrane (BioRad) using a Hoefer Semi-Phor Seri-Dry transfer unit (Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked in 3% skimmed milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 (Sigma-Aldrich) (TBS-T) before incubation with antibodies in TBS-T with 3% skimmed milk. Bound antibodies were visualized by incubation with secondary horseradish peroxidase-conjugated antibodies and Super Signal West Pico stable peroxide solution (Pierce Biotechnology, Rockford, IL, USA). Densitometry of the western blots was analyzed by the ImageJ software.[65] Cell-surface protein expression, that is, CD4 and CD25, was analyzed with flow cytometry (FacsCalibur, BD Biosciences; or Attune Acoustic Focusing Cytometer, Life Technologies) and FlowJo software (Tree Star, Ashland, OR, USA) or Attune Cytometric Software (Life Technologies). For intracellular staining of TAGAP, cells were fixed in 3% paraformaldehyde before permeabilization with 0.05% saponin (S7900, Sigma). In all steps proceeding permeabilization, 0.05% saponin was present in all staining and washing solutions. Samples were analyzed with Attune Acoustic Focusing Flow Cytometer (Life Technologies) and FlowJo software (Tree Star).

Genotyping

TAGAP and *IL2RA* were genotyped for rs1738074 and rs7090512 using pre-made TaqMan genotyping assays (assay ID 2966098 and 1841420, respectively, Applied Biosystems, Life Technologies) or genotypes were extracted from genome-wide SNP data assessed by the Human Omni Express Bead Chip (Illumina, San Diego, CA, USA).

MS patients and controls

The Norwegian sample collection consisted of CD4+ T cells that were isolated from whole blood[66] from untreated Norwegian females of Nordic origin with RRMS ($n = 8$, mean age 42 years, s.d. = 11) and female controls of Nordic origin ($n = 10$, mean age 36 years, s.d. = 6.4). The RRMS patients had not experienced a relapse or received steroids in the 3 months before enrollment. The Dutch collection consisted of cDNA from CD4+ T cells from RRMS patients ($n = 36$, mean age 40, s.d. = 10, 70% females) collected in the Netherlands.[36] The patients used either interferon-β or no immunomodulation drugs, and they had not had any relapse for the last 6 weeks. Serum levels of 25(OH)D in the Dutch cohort were measured as described previously.[36] All MS patients fulfilled the updated McDonald MS criteria.[67] The Regional Committee for Medical and Health Research Ethics South East, Norway, and the 'Atrium-Orbis-Zuyd' (Heerlen, The Netherlands) approved the study. Written informed consent was obtained from all study participants.

RNA isolation, cDNA conversion and real-time quantitative PCR

RNA and cDNA conversion from the Dutch cohort has previously been described.[36] For all other experiments, cells were resuspended in 350 µl RNA Protect Cell Reagent (Qiagen, Limburg, The Netherlands) and kept at − 80 °C before RNA extraction using QIAshredder (Qiagen) for homogenization of the cells, and RNAeasy Plus Mini Kit (Qiagen) for isolation of RNA. RNA concentration, quality and integrity were verified by Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (560 ng) was reverse transcribed in a 20-µl reaction using Maxima First Strand cDNA Synthesis Kit for RT-quantitative real-time PCR (Thermo Scientific, Waltham, MA, USA) and the GeneAmp PCR system 9700 thermo cycler (Applied Biosystems) for a one-step PCR reaction (25 °C for 10 min, 50 °C for 30 min and 85 °C or 5 min). The cDNA was stored at − 80 °C. Real-time quantitative PCR was performed on 7900HT Fast Real-Time PCR system or Viia7 Real-Time PCR system (both from Applied Biosystems, Life Technologies) with the standard curve method using TaqMan pre-made gene expression assays. A standard curve was generated for each RNA collection using a mixture of RNA for the specific collection. The samples were run in triplicates or duplicates, with 1–4 ng cDNA as input, and primers and probes (indicated below, all from Applied Biosystems, Life Technologies) were added to each reaction, including in addition 5 µl TaqMan Gene Expression Mastermix (Applied Biosystems, Life Technologies), 0,5 µl of 20× Primer Probe mix (Applied Biosystems, Life Technologies) and 4 µl RNase-free water (Qiagen). The samples were run on a MicroAmp Optical 384-well reaction plate (Applied Biosystems, Life Technologies), with the following PCR conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed by the sequence detection systems v. 2.3 (Applied Biosystems, Life Technologies). The following TaqMan gene expression assays were used (all from Applied Biosystems, Life Technologies): *BATF*, Hs00232390; *CD5*, Hs00204397; *CD6*, Hs00198752; *CYP24A1*, Hs00167999; *IQCB1*, Hs00274384; *KIF21B*, Hs01118430; *MALT1*, Hs01120052; *MYC*, Hs00153408; *RGS14*, Hs00374626; *SP140*, Hs00610654; *STAT3*, Hs01047580; *STAT4*, HS01028017; *TAGAP*, Hs00299284; *TCF7*, Hs00175273; *TYK2*, Hs00177464; and *VDR*, Hs00172113. All data were normalized to *TBP* and/or *GAPDH* and/or *18S rRNA*, also TaqMan probes from Applied Biosystems, Life Technologies: *18S rRNA*, 4319413E; *GAPDH*, Hs03929097; and *TBP*, 4326322E. Outliers from the triplets were excluded. In samples where only doublets were analyzed, samples showing a C_T s.d. more than 0.5 were re-run. A negative control without cDNA and a no-reverse transcriptase control were included in each experiment. Relative expression was calculated as the ratio between the target gene and *RNaseP* or *TBP*, *GAPDH* and *18S rRNA* reference genes. PCR specificity was verified by a single band after gel electrophoresis.

Statistics

Statistically significant differences between gene or protein expressions at single time points were assessed by a two-sided paired Student's *t*-test and gene expression between two groups (MS versus controls, groups with different genotypes) were assessed by two-sided Mann–Whitney *U*-test. Linear regression was used to asses correlation between gene expression and serum levels of 25(OH)D (all Graph Pad Prism Software, Inc., San Diego, CA, USA). *P*-values below 0.05 were considered significant. To assess the statistical significance for a change in gene expression over time, a functional analysis of variance was used. For each gene, a natural cubic spline with three degrees of freedom was fitted to the difference between the control samples and the vitamin D-treated samples and tested with analysis of variance against the null model. To account for the correlation between the series, we used a linear mixed model[68] with random effect for each series. As several independent genes were tested, Benjamini–Hochberg correction[69] was performed on the *P*-values using R.[70] Corrected *P*-values below 0.05 were considered significant.

CONFLICT OF INTEREST

TB has received an unrestricted grant for running expenses in this project from Biogen Idec Norway AS, ISL has received unrestricted grant for salary expenses from Novartis Norway AS and TH has received research grants from Merck Serono.

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