Journal of Cellular Biochemistry 89:922–932 (2003)

The Targets of Vitamin D Depend on the Differentiation and Activation Status of CD4 Positive T Cells

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Abstract Vitamin D is a potent immune system regulator. The active form of vitamin D ($1,25(OH)_2D_3$) suppresses the development of animal models of human autoimmune diseases. $1,25(OH)_2D_3$ decreased the proliferation of all T helper (h) cells and decreased the production of IFN-γ, IL-2, and IL-5. In Th2 cells $1,25(OH)_2D_3$ increased the production of IL-4. Quiescent CD4+ T cells express vitamin D receptors but only at a low level, which increased five-fold following activation. $1,25(OH)_2D_3$ treatment of Th0 cells, but not Th1 or Th2 cells, induced the expression of the transcription factor GATA-3. Microarray technology identified over 102 targets of $1,25(OH)_2D_3$ in CD4+ T cells. Of the 102 genes, 57 genes were down-regulated and 45 were up-regulated by $1,25(OH)_2D_3$ treatment of the CD4+ T cells. Two of the identified genes are regulators of NFkB. Other genes of interest included the IL-2Rβ gene and IgE binding factor. Th2 and Th0 cells produced more IgE binding factor after treatment with $1,25(OH)_2D_3$ while Th1 cell IgE binding factor expression was unaffected by $1,25(OH)_2D_3$ addition. It is unclear why some of the genes identified are expressed in CD4+ T cells and furthermore why $1,25(OH)_2D_3$ regulates the expression of these genes. Clearly CD4+ T cells can be direct targets of vitamin D. The targets of vitamin D in CD4+ T cells depend on the state of activation and differentiation status of the cells. J. Cell. Biochem. 89: 922–932, 2003.

Key words: T lymphocytes; vitamin D; cytokines; gene regulation

The active form of vitamin D (1,25 dihydroxyvitamin D3, $1,25(OH)_2D_3$) is a potent regulator of the immune system. Supplementation of mice with $1,25(OH)_2D_3$ halted the progression of experimental autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), and inflammatory bowel disease (IBD) [Cantorna et al., 1996; Cantorna et al., 2000]. The transfer of CD4+ T helper (h) cells from symptomatic mice with EAE or IBD induces disease in naïve mice [Raine, 1984; Morrissey et al., 1993]. The pathogenic T cells, which can induce EAE or IBD, have been identified as CD4+ T cells, which secrete cytokines like IFN-γ, and IL-2. The inhibition of EAE and IBD by $1,25(OH)_2D_3$ is likely due to both direct and indirect regulation of CD4+ T cells.

Activation of Th cells with mitogens and $1,25(OH)_2D_3$ has been shown to increase and stabilize vitamin D receptor (VDR) protein expression [Veldman et al., 2000]. Furthermore, $1,25(OH)_2D_3$ has been shown to inhibit Th cell proliferation, IL-2, TNF-α, and IFN-γ production in mixed cell cultures stimulated with T cell mitogens [Rigby et al., 1984, 1985, 1987]. The inhibition of proliferation and cytokine secretion by $1,25(OH)_2D_3$ was hypothesized to be a result of indirect regulation of T cell function by macrophage cells in the cultures [Lemire, 1992].

Recently, Boonstra et al. [2001] showed that $1,25(OH)_2D_3$ addition to purified CD4+ T cells, inhibited Th1 cell development and cytokine production and resulted in Th2 cell expansion and increased IL-4 production. Conversely, Staeva-Vieira and Freedman [2002] showed that $1,25(OH)_2D_3$ addition to purified CD4+ T cells inhibited Th1 and Th2 cell responses including the production of IL-4. The experimental design of Boonstra and Staeva-Vieira

Abbreviations used: $1,25(OH)_2D_3$, 1,25 dihydroxyvitamin D3; Th, T helper, OVA, ovalbumin; EAE, encephalomyelitis; IBD, inflammatory bowel disease; VDR, vitamin D receptor; TCR, T cell receptor.

Grant sponsor: NIH-NINDS; Grant number: 1R01 NS38888-01A2.

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Received 25 March 2003; Accepted 25 April 2003
DOI 10.1002/jcb.10580

were extremely similar and therefore it is unclear, why the results conflicted. Th cells have VDR and are direct targets of $1,25(OH)_2D_3$ in vitro. The cytokines and functions of CD4+ T cells, which are regulated by $1,25(OH)_2D_3$, have not been defined.

VDRs are part of the steroid hormone nuclear receptor family. When $1,25(OH)_2D_3$ is present the VDR binds with its partner nuclear receptor (the retinoid X receptor) to DNA sequences found in the promoters of targeted genes. All the members of the steroid hormone super family have been shown to regulate gene transcription. The targets of $1,25(OH)_2D_3$ in CD4+ T cells are unknown but are likely to include genes involved in cytokine production, cell proliferation, and a number of other novel genes. Here we present data, which shows that the targets of $1,25(OH)_2D_3$ in CD4+ T cells depend on the activation status and differentiation status of the Th cells. In addition a number of new genetic targets of $1,25(OH)_2D_3$ in CD4+ T cells have been identified.

MATERIALS AND METHODS

Mice

T cell receptor (TCR) transgenic mice (D011.10 specific for ovalbumin, OVA) were obtained from Jackson Laboratories (Bar Harbor, ME). Ninety percent of the CD4+ T cells, from the D011.10 transgenic mice, express the TCR from the D011.10 clone, which is specific for OVA. Normally only about 1 in 100,000 T cells expresses any given TCR. TCR transgenic mice were picked because they provide a good source of CD4+ T cells, which are 90% of the transgenic variety, and of a known antigenic specificity.

Th Cell Cultures

Cell culture medium was RPMI-1640 (Sigma-Aldrich; St. Louis, MO) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine (Sigma), 0.05 mM β2-mercaptoethanol (Sigma), 10 μg/ml gentimycin (Sigma), 4 mg/ml sodium bicarbonate (EA Science, Gibbstown, NJ) and adjusted to pH 7.2.

D011.10/Balb/c TCR transgenic mice were sacrificed at the age of 5–8 weeks. Splenocytes were collected and cultured at 4×10^6 cells/ml along with OVA (Sigma) at 1 mg/ml. The cultures were done under neutral conditions (Th0 priming) or with 500 U rIL-4 and 1 ng/ml anti-IFN-γ antibodies (Th2 priming), or with 100 U rIL-12 (Thl priming). Antibodies and cytokines were from Pharmingen (San Diego, CA). At 72 h rIL-2 was added at 20 ng/ml. On day 7 cells were collected and the dead cells removed by layering on Histopaque-1077 (Sigma). Flow cytometry showed that 95-98% of the cells were transgenic T cells [CD4+ (Ab from Pharmingen), and KJ1-26+ (Ab from Caltag, Burlingame, CA) for D011.10 transgenic T cells]. Cells were recultured at 4×10^6 cells/ml with phorbal myristiyl acetate (PMA, 10 ng/ml, Sigma) and ionomycin (2.5 μg/ml, Sigma). In some experiments Th0, Th1, and Th2 (Fig. 2 and Tables IV–VI) cells were treated with 50 nM $1,25(OH)_2D_3$ (Tetrionics, Inc., Madison, WI) or control treated (ethanol) 24 h following PMA and ionomycin addition. After an additional 24 h, cells were collected, and RNA extracted.

Measurement of Lymphocyte Proliferation and Cytokine Production

Th cells used for proliferation and ELISA analysis were cultured in 96 well plates for proliferation assays and 24 well plates for ELISA supernatant assays on day 7 (Corning Costar, Corning, NY). In all cases the Th0, Th1, and Th2 cells were stimulated with PMA and ionomycin in the presence or absence (controls) of optimal levels of $1,25(OH)_2D_3$ (10–50 nM). After 72 h (predetermined as optimal), supernatants were collected for ELISAs. For proliferation assays, 0.4 μCi of [3H]thymidine (ICN, Costa Mesa, CA) was added to each well and the cells were incubated for an additional 18 h. Radioactive thymidine incorporation was determined by liquid scintillation using a beta plate counter. Mouse IL-2, IL-4, IL-5, and IFN-γ production were detected using ELISA kits from Pharmingen, and the instructions provided. The ELISA detection limits were 25 pg/ml IL-2, 62 pg/ml IL-4, 312 pg/ml IL-5, and 1,000 pg/ml IFN-γ.

Quantitative and Semi-Quantitative mRNA Analysis

RNA isolation was done using the RNeasy kits and instructions exactly as described by the manufacturers (Qiagen, Valencia, CA). Semi-quantitative RT-PCR was done to confirm the microarray results. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), IgE binding factor, max binding protein, NFkB inducing kinase, p65 subunit of NFkB, IL-2 receptor β, and chemokine (C motif) XC receptor 1 were the genes analyzed. Total RNA was isolated and reverse

transcribed using oligo(dT) and the manufacturer's protocol (Promega, Madison, WI). Primers specific for each gene were designed to cross exon/intron borders and optimized prior to running the experiment. The products were resolved by 1.5% agarose gel electrophoresis and ethidium bromide stained. The gene products were identified by size with respect to molecular weight standards. Densitometry scanning software was used to quantitate the intensity of the bands (Scion Image For Windows, Frederick, MD). Values are reported as the intensity (arbitrary units) expressed in the gene of interest per 100 G3PDH units.

The primers used for semi-quantitative RT-PCR analysis were as follows: G3PDH, TGAAGGTCGGTGTGAACGGATTTGGC (forward), CATGTAGGCCATGAGG-TCCACCAC (reverse); IgEBF, TGCACGGGATAGAGTGAGTG (forward), ACAGGCTTTTACCCAGAGCA (reverse); max binding protein, GGAAGACAAAGGCACAGCAT (forward), AAAGGCCATTGGTGAGTGTC (reverse); NFkB inducing kinase, AAGAGGGGCTGAGGAAAGAG (forward), TGTTCGCCATTGAGAGACTG (reverse); p65 subunit of NFkB, GGTGTATTTCACGGGACCAG (forward), TTTGGAACAGGTGCAGACAG (reverse); IL-2 receptor β, GGCTCTTCTTGGAGATGCTG (forward), GCCAGAAAAACAACCAAGGA (reverse); and chemokine (C motif) XC receptor 1, TCTCAGCACAATGGAGTTGG (forward), CGGTGTCTCTCTGTCTGGACCT (reverse).

VDR, IgE binding factor and GATA-3 were analyzed using real-time quantitative PCR using methods as described by Heid et al. [1996]. PCR product accumulation using a dual-labeled fluorgenic probe (TaqMan Probe) was measured with a Perkin-Elmer ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Branchburg, NJ) as described by the manufacturer, at the Nucleic Acid Facility, Pennsylvania State University, University Park, PA. The primers were generated in the Nucleic Acid Facility, probes were generated by Biosearch Technologies, Inc. (Novato, CA). 18S ribosomal RNA served as the control gene for the quantitative VDR PCR. The IgE binding protein and GATA-3 real time PCR used G3PDH as the control gene. Values were normalized for the amount of 18S or G3PDH RNA in the samples. The primers and probes used to quantitate genes of interest were as follows: GATA-3, GAACCGGCCCCTTAACAAG (forward), CAGGATGTCCCTGCTCTCCTT (reverse), CCAAGCGAAGGCTGTCG-GCAG (probe); VDR, ACCTGCCCC TTCAATGGAG (forward), GGCCTGGCAGTGTCGC (reverse), TTGCCGCATCACCAAGGACAACC (probe); IgEBF, TGCTAATTTTACCTTGGTGCAGTT (forward), GTTTGCCAGTCAGCAGGAGTT (reverse), AGCCTCGCCGGCATGGCAC (probe).

Microarray

Microarrays were done using the methods reviewed by Schulze and Downward [2001] and slides printed at the Pennsylvania State University Microarray Facility. The source of oligonucleotides used to print microarray slides was the Array-Ready Mouse Genome Oligo Set (Qiagen Operon, Alameda, CA). This array allows for the assessment of over 7,000 different murine genes. Total RNA was reverse transcribed using superscript II Rnase H transcriptase (Gibco BRL/Life Technologies, Rockville, MD) as described by the manufacturer. During reverse transcription the cDNA was labeled with the reactive nucleotide aminoallyl dUTP (aa-dUTP, Sigma). The aa-dUTP labeled cDNA was purified with Nanosep 30 K concentrators (Pall Corporation, East Hills, NY) and dried. The aa-dUTP nucleotides in the cDNA were then coupled to succinimidyl ester derivatives of Cy3 (green; controls) or Cy5 (red; $1,25(OH)_2D_3$ treated) fluorescent dyes (Amersham/Pharmacia, Piscataway, NJ). The labeled cDNA was then purified using QiaQuick (Qiagen) columns and dried. The probes were combined and hybridized to preprinted and pretreated microarray slides for 20 h at 42°C in hybridization buffer (formamide-50%, Sigma), 5× standard saline citrate (SSC, Sigma), 0.1% SDS (Fisher Biotech, Fair Lawn, NJ), and 0.1 mg/ml Human Cot1 DNA (Gibco). The slides were then washed with increasing stringencies of SSC buffers and analyzed using the GenePix 4000 scanner and GenePix Pro 3.0 software (Axon Instruments, Foster City, CA).

The GenePix 4000 scanner uses a dual-laser scanning system that scans at 532 and 635 nm to excite and induce fluorescence in the Cy3 and Cy5 incorporated dyes respectively. Once scanned, GenePix Pro 3.0 automatically aligned each feature spot to a GenePix Array List file containing gene names, accession numbers, and two-dimensional location on the slide. Genes associated with feature spots that were not found by GenePix Pro 3.0 during alignment were flagged and eliminated from analysis.

Results are presented as the ratio of the median intensities of Cy5 fluorescence ($1,25(OH)_2D_3$-treated cells) to Cy3 fluorescence (control cells) for each respective gene. Ratios greater than one are associated with transcriptional upregulation of genes in $1,25(OH)_2D_3$-treated cells relative to control cells, and ratios less than one are associated with transcriptional downregulation.

Statistics

Data were analyzed using StatView (SAS, Cary, NC). Experiments were repeated as necessary and where possible, values were reported as means from multiple experiments. Differences between control treatment and in vitro addition of $1,25(OH)_2D_3$ were compared by paired t-test. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

$1,25(OH)_2D_3$ addition to purified CD4+ T cells decreased proliferation ($P = 0.07$ to 0.09) in response to PMA and ionomycin in Th0, Th1, and Th2 cells (Table I). Overall, the decrease was large and ranged from a 40–77% inhibition. Because of the variability from day to day in the proliferation assay the inhibition of proliferation by $1,25(OH)_2D_3$ did not reach statistical significance. The cells in the control and $1,25(OH)_2D_3$ cultures were still 90–93% viable after 72 h in culture as judged by trypan blue exclusion. Th0, Th1, and Th2 cells are direct targets of $1,25(OH)_2D_3$ in vitro.

As expected, Th1 cells secreted more IFN-γ and less IL-4 then Th2 cells (Table II). Surprisingly, Th2 cells secreted more IL-2 than Th1 cells and the IL-2 produced was repressed by $1,25(OH)_2D_3$. In Th0 cells IFN-γ, IL-5, and IL-2 production were significantly decreased by $1,25(OH)_2D_3$ addition (Table II). $1,25(OH)_2D_3$ treatment of Th cells repressed IFN-γ production in Th0, Th1, and Th2 cells. IL-4 production was enhanced when $1,25(OH)_2D_3$ was added to Th2 cells in vitro (Table III). All four experiments in Th2 cells showed an increase in IL-4 production with $1,25(OH)_2D_3$ addition and in three of the four experiments the increase in IL-4 secretion was significant (Table III).

TABLE I. Proliferation of CD4+ T Cells Stimulated With PMA and Ionomycin

	Control[a]	$1,25(OH)_2D_3$ (10 nM)
Th0	6.8 ± 2.7	2.4 ± 0.9
Th1	23.2 ± 4.7	5.3 ± 3.2
Th2	19.7 ± 4.7	11.8 ± 4.3

[a]Stimulation index = (cpm with PMA and ionomycin)/(cpm with no stimulation).
Values are the mean ± SE of three experiments.

OVA specific CD4+ Th0 cells were grown for one week in vitro. VDR mRNA expression, following activation with PMA and ionomycin, was measured as a function of time in Th0 cells (Fig. 1). VDR mRNA expression was low in quiescent Th0 cells and increased five- to six-fold within 24–48 h of stimulation (Fig. 1). The addition of $1,25(OH)_2D_3$ did not further increase the level of VDR mRNA transcription. Based on the low levels of VDR expression in unactivated CD4+ T cells, the remainder of the experiments used 24 h PMA and ionomycin preactivated Th cells.

As expected Th1 cells expressed less GATA-3 than Th2 cells (Fig. 2A). Interestingly $1,25(OH)_2D_3$ treatment of Th0 cells increased GATA-3 expression 2.5-fold (Fig. 2A). GATA-3 expression is a target of $1,25(OH)_2D_3$ in Th0 cells.

OVA specific CD4+ Th0 cells were grown for one week in vitro. The cells were collected and stimulated with PMA and ionomycin and 24 h later when VDR expression was maximal a 50 nM dose of $1,25(OH)_2D_3$ was added to the experimental cells for an additional 24 h period, while control cells received an equal volume of ethanol. The microarray experiment was repeated once. The inclusion criteria for genes reported in Tables IVand V were, (1) the gene was up or down-regulated by at least two-fold or more in one of the experiments, (2) the gene was up- or down-regulated by at least 1.3-fold in the second experiment in the same direction as in the first and, (3) genes that had combined median fluorescent intensities (median green fluorescence – median background + median red fluorescence – median background) of less than 100 were eliminated. One hundred two targets of $1,25(OH)_2D_3$were identified in CD4+ T cells using these criteria.

Among the genes repressed by $1,25(OH)_2D_3$ addition was 18S RNA, which is a commonly used house keeping gene (Table IV). 18S RNA was repressed by −2.06 fold in the presence of $1,25(OH)_2D_3$ (Table IV). Conversely, the expression of other house keeping genes, including G3PDH, were unaffected by $1,25(OH)_2D_3$ addition to CD4+ T cells (data not shown). Though

TABLE II. Cytokine Secretion of CD4+ T cells Stimulated With PMA and Ionomycin

	IFN-γ (ng/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
Th0				
Control	264 ± 123[a]	562 ± 200[a]	315 ± 136	1545 ± 669[a]
plus D 10 nM	13 ± 12	93 ± 72	268 ± 146	517 ± 160
Th1				
Control	3831 ± 1042[a]	90 ± 65	29 ± 29	187 ± 124
plus D 10 nM	1300 ± 1026	90 ± 42	31 ± 28	188 ± 116
Th2				
Control	419 ± 237[a]	456 ± 430[a]	2499 ± 1526	703 ± 484
plus D 10 nM	43 ± 22	34 ± 23	3115 ± 1895	703 ± 484

[a]Control is significantly higher than the $1,25(OH)_2D_3$ (plus D) value ($P < 0.05$).

18S RNA was used as the control for our quantitative PCR analysis of the VDR gene, all other quantitative and semi-quantitative PCR analyses used G3PDH.

Six genes were selected from Tables IVand V for further analysis by RT-PCR in RNA from Th0 cells treated as before with $1,25(OH)_2D_3$ or control treated (Table VI). With the exception of Chemokine (C motif) XC receptor 1, the RT-PCR confirmed the microarray analyses in Th0 cells. However, there were some major discrepancies in the magnitude of the differences reported using microarray and RT-PCR (Table VI). RT-PCR analysis was also done with Th1 and Th2 cells treated with and without $1,25(OH)_2D_3$ (Table VI). There seem to be differences in $1,25(OH)_2D_3$ regulation of many of these genes (IgE binding factor, max binding factor etc.) depending on the Th cell type examined.

IgE binding factor was one of the genes that seemed to be differentially regulated in different Th subsets (Table VI). IgE binding factor was selected for further quantitative analysis based on its differential expression in Th cell subsets and based on the high expression of this gene in Th0 cells (Table V, combined median intensity of 34,765). The results from quantitative RT-PCR (Fig. 2B) confirmed the semi-quantitative RT-PCR results. Th0 cells treated with $1,25(OH)_2D_3$ had eight-fold higher levels of IgE binding factor than control treated Th0 cells. Th2 cells had 2.3-fold higher levels of IgE binding factor than control treated Th2 cells. Th1 cell IgE binding factor expression was unaffected by $1,25(OH)_2D_3$ addition in vitro.

DISCUSSION

$1,25(OH)_2D_3$ is a direct regulator of differentiated Th cells. Overall $1,25(OH)_2D_3$ repressed proliferation and secretion of IFN-γ, IL-2, and IL-5 in Th cells. IL-4 was the only cytokine, which increased following $1,25(OH)_2D_3$ addition, and only in Th2 cells. Our data confirms a role for $1,25(OH)_2D_3$ as an inducer of Th2 cell differentiation [Boonstra et al., 2001]. Conversely, our data is in conflict with data suggesting that $1,25(OH)_2D_3$ inhibits IL-4 and Th2 cell development [Staeva-Vieira and Freedman, 2002]. Vitamin D is a direct regulator of T cell effector cell function.

Quiescent Th cells express very little of the VDR gene. Activation of Th cells for 24 h increased VDR mRNA five-fold. Our quantification of VDR mRNA expression used 18S RNA expression as a control for the amount of RNA in the samples. Unfortunately, $1,25(OH)_2D_3$ addition to Th cells resulted in a two-fold inhibition of 18S RNA expression (Table IV). In our assay $1,25(OH)_2D_3$ addition to activated Th0 cells did not further induce VDR mRNA levels over the controls. It is likely that the VDR mRNA values in $1,25(OH)_2D_3$ treated Th0 cells was underestimated, and if G3PDH was used as the

TABLE III. IL-4 Secretion in Th2 cells

Th2 cells	EXP 1	EXP 2	EXP 3	EXP 4
Control	8500 ± 10[a]	1487 ± 155 [a]	722 ± 30[a]	1487 ± 250
plus D 10 nM	10,600 ± 500	1639 ± 233	1247 ± 130	1639 ± 150

[a]Control is significantly lower than the $1,25(OH)_2D_3$ (plus D) value ($P < 0.05$).
In three of four experiments IL-4 secretion was significantly increased following $1,25(OH)_2D_3$addition in vitro. Values are mean ± SD of triplicate wells.

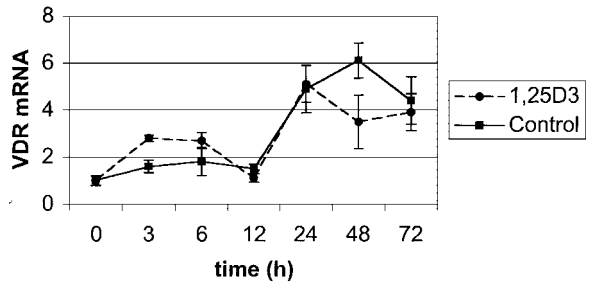

Fig. 1. VDR expression in CD4+ T cells as a function of time post-activation. VDR analyses were done using 18S RNA as the control. Data is representative of one of five independent experiments. Values are the ratios of VDR expression (corrected for 18S RNA) post stimulation over VDR expression (corrected for 18S RNA) at time 0 ± SD of triplicate samples.

control, $1,25(OH)_2D_3$ addition would have increased VDR mRNA levels an additional two-fold compared to controls. This would be consistent with previous measures of VDR protein levels in immune cells [Veldman et al., 2000]. The requirement of T cell activation for VDR expression suggests that vitamin D has little effect in the first 24 h of Th cell activation.

Likely targets of $1,25(OH)_2D_3$ in Th cells include transcription factors important in Th cell subset development. $1,25(OH)_2D_3$ treatment of Th0 cells induced GATA-3 expression. GATA-3 is a transcription factor whose expression has

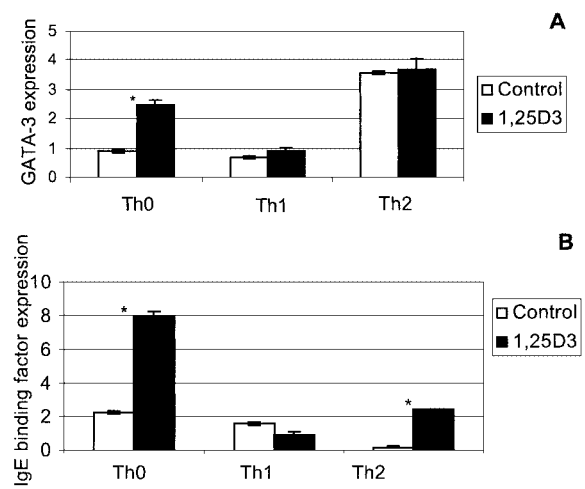

Fig. 2. GATA-3 and IgE binding factor expression in Th1 and Th2 cells. Twenty four hour PMA preactivated Th0, Th1, and Th2 cells were treated for an additional 24 h with $1,25(OH)_2D_3$ or control treated. GATA-3 (2A) and (2B) IgE binding factor analyses were done on RNA samples from the Th cells using G3PDH as the control. Values are the G3PDH corrected GATA-3 or IgE binding factor levels ± SD of triplicate samples. *Expression of GATA-3 or IgE binding factor was significantly higher in $1,25(OH)_2D_3$ treated cells compared to controls.

been closely linked to the development of the Th2 cell response [Rengarajan et al., 2000; Hofer et al., 2002]. The effect of $1,25(OH)_2D_3$ on GATA-3 mRNA levels in Th cells is controversial. Boonstra et al., showed an increase in GATA-3 mRNA (RT-PCR) and Staeva-Vieira and Freedman showed little to no change in GATA-3 mRNA (RT-PCR) [Boonstra et al., 2001; Staeva-Vieira and Freedman, 2002]. We have used quantitative RT-PCR and have clearly shown that GATA-3 is up-regulated by $1,25(OH)_2D_3$ in Th0 cells. The result of increased GATA-3 expression in Th0 cells would be to alter Th cell development by suppressing Th1 cells and increasing Th2 cells [Rengarajan et al., 2000; Hofer et al., 2002].

Clearly addition of 1,25(OH)2D3 to quiescent Th cells must be ineffective since VDR levels are so low. Based on this information, microarrays, and quantitative and semi-quantitative RT-PCR assays were done following a protocol where 24 h preactivated Th cells were treated or not with 1,25(OH)2D3. The microarray contained probes for cytokine genes (IL-4, IFN-γ, IL-2, etc.), and transcription factors (GATA-3, etc), which we expected were regulated by 1,25(OH)2D3. Under our experimental conditions (24 h activation, followed by 1,25(OH)2D3 or control treatment for 24 h) the microarrays failed to detect differences in GATA-3 or other relevant cytokine genes. If the culture conditions were changed we would certainly change the outcome. Since it is not practical to try every conceivable condition, the conditions picked were hypothesized to best reflect optimal vitamin D regulation. Furthermore, based on our quantitative RT-PCR analysis of IgE binding factor (Fig. 2) the microarray results underestimated the $1,25(OH)_2D_3$ mediated effects. An additional reason why GATA-3 and cytokines like IL-4 and IL-2 were not identified by the microarray as $1,25(OH)_2D_3$ targets in Th0 cells may be a result of the sensitivity of the microarray.

Microarray analysis of Th0 cells identified a number of genes that had clear ties to the regulation of immune function. Two chemokine genes (small inducible cytokine A21b or CCL21 and chemokine XC receptor 1 or lymphotactin) were repressed by $1,25(OH)_2D_3$ addition to Th0 cells. Both of these genes have been shown to be important in inflammation, and important for T cell migration [Bonecchi et al., 1998; Christopherson et al., 2001]. Another gene linked to inflammation and repressed by

TABLE IV. Genes Negatively Regulated by 1,25(OH)2D3 in CD4+ T Cells

Accession no.	Name	Ratio[a]	Combined median intensities
AB000490	Mouse mRNA for ELP3 complete	−1.66	195
AF229644	DNA segment Chr X Immunex 48 expressed	−1.67	432
Y15910	Diaphanous homolog 2 (Drosophila)	−1.76	246
U65586	Telomeric repeat binding factor 1	−1.77	225
D13473	Rad51 homolog (S. cerevisiae)	−1.81	492
AF001980	Small inducible cytokine A21b (serine)	−1.84	247
NM_011901	TATA box binding protein (TBP) associated	−1.84	285
AF057526	Rhesus blood group-associated A	−1.91	506
AF011422	Vomeronasal organ family 2 receptor 12	−1.92	320
AF200973	Exotoses (multiple)-like 2	−1.93	197
D84391	L1 repeat Tf subfamily member 14	−1.94	1222
AF177664	Rho GTPase activating protein 6	−1.96	264
M74555	House-keeping protein 1	−1.96	14094
M61215	Ferrochelatase	−1.99	243
U56650	Neurexophilin 2	−1.99	362
AF034610	Nuclear autoantigenic sperm protein	−2.00	4001
AJ231239	Mus musculus lgVk by9 pseudogene	−2.04	398
AB028459	Chemokine (C motif) XC receptor 1	−2.06	257
X56974	18S RNA	−2.06	111943
M38129	Mouse embryonic skeletal myosin heavy	−2.06	230
M92649	Nitric oxide synthase 2 inducible	−2.10	273
U12142	Thyroid hormone receptor alpha	−2.24	217
AF147785	Zinc finger protein regulator of apoptosis	−2.29	254
U40189	Pancreatic polypeptide receptor 1	−2.30	360
AF081797	Keratin associated protein 12-1	−2.38	327
Z22532	Syndecan 1	−2.46	442
AF025653	RNA guanylyltransferase and 5′-phosphatase	−2.61	175
AF126798	Delta-6 fatty acid desaturase	−2.97	725
M35732	Seminal vesicle protein 2	−3.06	398
X78543	Mast cell protease pseudogene 1	−3.13	214
AL078630	Mus musculus genomic DNA sequence	−3.25	316
AJ249198	Solute carrier family 7	−3.25	281
NM_013774	T-cell leukemia/lymphoma 1B 4	−3.44	382
M86751	Mouse lg L-chain gene variable region	−3.50	430
AB002693	Solute carrier family 10 member 2	−3.64	403
AF047726	Cytochrome P450 2c39	−3.68	498
U95783	Mus musculus endogenous provirus	−3.68	250
AF011418	Vomeronasal organ family 2 receptor 8	−3.71	634
D28599	Chondroitin sulfate proteoglycan 2	−3.79	357
AF077742	Transcription factor EC	−4.00	319
D84376	Phosphatidic acid phosphatase 2a	−4.14	561
M36780	Casein alpha	−4.21	241
X99915	High mobility group protein I isoform C	−4.30	376
NM_013616	Olfactory receptor 64	−4.41	230
AF104410	Vascular endothelial zinc finger 1	−4.46	332
AF227148	Candidate taste receptor T2R8 gene	−4.51	410
AF227147	Candidate taste receptor T2R5 gene	−4.64	559
AF091047	Quaking	−5.16	1143
M75721	Serine protease inhibitor 1-1	−5.39	1800
D31952	Melanocortin 2 receptor	−5.42	687
AF078905	Mus musculus zeta globin gene partial	−5.46	323
AB010152	Transformation related protein 63	−5.67	363
AB016275	Ornithine decarboxylase antizyme 3	−7.06	1007
AF082348	Bone morphogenetic protein 15	−7.41	253
NM_017396	Cytochrome P450 steroid inducible 3a41	−7.61	526
AF004927	Opioid receptor sigma 1	−12.62	2042

[a]The ratio is the inverse of the median intensities of red fluorescence over green fluorescence. Negative ratios represent genes, which are repressed in the presence of $1,25(OH)_2D_3$. All values are the means of two independent experiments.

$1,25(OH)_2D_3$ was nitric oxide synthase 2 inducible (iNOS). iNOS is the enzyme that makes the molecular messenger nitric oxide. Four helix bundle cytokine 10 (IL-20) is a gene that is linked to psoriasis [Rich and Kupper, 2001]. IL-20 is structurally related to IL-10 (20–40% homology) and plays a role in inflammation of the skin [Rich and Kupper, 2001]. $1,25(OH)_2D_3$ addition to Th0 cells strongly induced IL-20 mRNA 8.21 fold. Interestingly $1,25(OH)_2D_3$ is currently being used as a treatment for psoriasis [Kowalzick, 2001]. Perhaps, IL-20 induction by $1,25(OH)_2D_3$ in CD4+ T cells, is one mechanism that explains the efficacy of vitamin D in psoriasis. Max binding protein (Mnt) belongs to the *myc* family of proto-oncogenes that regulates cell cycle differentiation and progression [DePinho et al., 1991; Hurlin et al., 1997]. The

TABLE V. Genes Up-Regulated by $1,25(OH)_2D_3$ in CD4+ T Cells

Accession no.	Name	Ratio[a]	Combined median intensities
NM_011962	Procollagen-lysine	1.68	449
NM_008368	Interleukin 2 receptor beta chain	1.70	4368
AF022992	Period homolog (Drosophila)	1.70	411
M75135	Solute carrier family 2	1.74	851
AF052506	Adenosine deaminase RNA-specific	1.74	669
J03928	Phosphofructokinase liver B-type	1.77	1652
U49393	ATPase Ca^{++} transporting ubiquitous	1.80	569
D26077	Kinesin family member 3b	1.81	326
U34883	3′-phosphoadenosine 5′-phosphosulfate	1.82	202
M63114	Surfeit gene 4	1.82	1945
U46155	Patched homolog	1.83	297
U17343	Signal recognition particle receptor B	1.87	2483
Y12634	ATPase H^+ transporting lysosomal	1.88	306
M10062	IgE binding factor	1.89	34765
U90333	Aquarius	1.91	173
D87326	Germ cell-specific gene 2	1.93	442
U18658	Transcription factor 15	1.93	684
D11374	Signal-induced proliferation associated	1.94	417
AB032902	SEC61 alpha subunit (S. cerevisiae)	1.95	1410
NM_011395	Solute carrier family 22 member 3	1.98	220
AF126967	E4F transcription factor 1	1.99	219
M61909	p65 subunit of NFkB	2.01	3173
AF024637	TYRO protein tyrosine kinase binding	2.06	1269
M86750	Mouse lg H-chain gene variable region co	2.10	465
NM_012000	Ceroid-lipofuscinosis neuronal 8	2.10	2454
AF155913	ATPas class II type 9B	2.14	337
X65588	Per-pentamer repeat gene	2.21	470
U26176	Somatostatin receptor 4	2.26	123
AJ003128	Huntingtin-associated protein 1	2.27	274
U37543	CD6 antigen	2.31	3201
AF020184	Neuronal calcium sensor-1	2.33	232
U77356	Max binding protein	2.38	5557
AF297083	Guanylate cyclase 1 soluble beta 3	2.40	383
NM_013746	PH domain containing protein in retina 1	2.42	447
AJ007511	Mus musculus *hic-1* gene partial	2.53	323
AF030430	Sema domain transmembrane domain (TM)	2.58	163
X03491	Keratin complex 2 basic gene 4	2.58	804
U97572	Excision repair 2	2.68	230
U97149	Pale ear	2.82	193
D37874	Fc receptor IgG alpha chain transporter	2.93	808
L15325	Zinc finger protein 161	3.12	176
AF132744	Macrophage galactose N-acetyl-galactosamine	3.24	207
AF143094	NFkB inducing kinase	3.51	156
AF182033	Polycystic kidney disease 2-like	4.07	170
AF224267	Four helix bundle cytokine 10	8.21	149

[a]The ratio is the median intensities of red fluorescence over green fluorescence. Positive values represent genes, which are expressed in increased amounts in the presence of $1,25(OH)_2D_3$. All values are the means of two independent experiments.

reduced proliferation of CD4+ T cells treated with $1,25(OH)_2D_3$, may come as a result of the up-regulation of.

Two genes, which regulate the transcription factor NFkB were up-regulated in Th0, Th1, and Th2 cells by $1,25(OH)_2D_3$ treatment. NFkB is a crucial regulator of gene expression during immune and inflammatory responses [Baeuerle and Henkel, 1994]. These immunoregulatory genes may be the key to understanding $1,25(OH)_2D_3$ regulation of Th cell function.

The upregulation of the IL-2Rβ gene by $1,25(OH)_2D_3$ in Th0 cells was of particular interest. The IL-2 R signaling complex is made up of an α and β chain [Schimpl et al., 2002]. The expression of IL-2 R α (CD25+) on CD4+ T cells identifies these cells as regulatory T cells. CD25+ regulatory T cells have been shown to transfer tolerance to autoantigens [McHugh and Shevach, 2002; Szanya et al., 2002]. The IL-2 signaling pathway has been identified as a target of $1,25(OH)_2D_3$ in T cells in vitro and in vivo [Bemiss et al., 2002]. Furthermore, regulation of IBD symptoms by $1,25(OH)_2D_3$ was ineffective in the IL-2 KO mouse [Bemiss et al., 2002]. Interestingly, IL-2KO mice do not make CD4+ CD25+ regulatory T cells [Schimpl et al., 2002]. Barrat et al. [2002] has shown that a combination of vitamin D and dexamethasone induced IL-10-producing regulatory T cells in human and mouse CD4+ T cells. In vivo and in the absence of dexamethasone, $1,25(OH)_2D_3$

TABLE VI. RT-PCR Results for Selected Genes

Gene[a]	TH0 microarray	TH0 RT-PCR	TH1 RT-PCR	TH2 RT-PCR
IgE binding factor	1.89	1.33	−1.06	1.41
Max binding protein	2.38	1.05	3.44	1.39
NFkB inducing kinase	3.51	1.58	1.36	1.29
p65 subunit of NFkB	2.01	1.29	1.29	2.04
IL-2 receptor β	1.70	1.10	1.45	−1.05
Chemokine (C motif) XC receptor 1	−2.06	1.56	−1.91	1.51

[a]Th0, Th1, and Th2 cells were grown as described in the material and methods. After 1 week of antigen stimulation the cells were collected and restimulated with PMA and ionomycin for 24 h followed by an additional culture period of 24 h with $1,25(OH)_2D_3$ or ethanol controls. Values reported are corrected for G3PDH expression. Ratios of the expression of the gene of interest in the presence of $1,25(OH)_2D_3$ over the expression of the gene in the controls. Negative ratios represent gene values, which are repressed in the presence of $1,25(OH)_2D_3$.

treatment of experimental autoimmune diabetes induced a population of CD4+ CD25+ regulatory T cells that correlated with the protection of the mice from diabetes [Gregori et al., 2002]. The function of these CD25+ T cells is thought to involve IL-2 signaling, which requires both the α and β chain of the IL-2 receptor [Schimpl et al., 2002]. The $1,25(OH)_2D_3$ mediated induction of IL-2Rβ expression in Th cells might lead to the increased induction of CD4+ CD25+ regulatory T cells. It is likely that $1,25(OH)_2D_3$ regulation of IL-2R β is linked to CD25 expression supporting a role of vitamin D as an inducer of regulatory T cells.

Microarray, RT-PCR, and quantitative real-time PCR were used to investigate IgE binding factor regulation by $1,25(OH)_2D_3$ in Th cells. Microarray identified IgE binding factor as a gene, which was up-regulated by $1,25(OH)_2D_3$ in Th0 cells (1.89 fold induction). IgE binding factor was among the handful of genes, which were highly expressed in Th0 cells (Table V). RT-PCR analysis suggested that IgE binding factor was differentially regulated in Th1, Th2, and Th0 cells. The real-time PCR was the only quantitative approach of the three and the results confirmed the microarray and semi-quantitative PCR data for IgE binding factor expression in Th0 cells. The magnitudes of the effects due to $1,25(OH)_2D_3$ were dramatically different using the various approaches. It seems that RT-PCR (1.33) and the microarray (1.89) grossly underestimated the effects of $1,25(OH)_2D_3$ on IgE binding factor in Th0 cells (8.00). Microarray analyses are great tools to screen many genes at once but cannot be used as a quantitative means of measuring gene expression.

$1,25(OH)_2D_3$ increased the expression of IgE binding factor gene in Th0 and Th2 cells but not Th1 cells. Little is known about IgE binding factor. IgE binding factor was cloned in the year 1985 from a T cell hybridoma, which secreted an IgE-suppressive factor [Martens et al., 1985]. Other IgE binding factors (secreted by B cells) have since been identified (sCD23) [Delespesse and Sarfati, 1988]. sCD23/IgEBF has been shown to affect T cell proliferation [Armitage et al., 1989; Bertho et al., 1991], and to alter T cell and myeloid precursor differentiation through synergistic interactions with IL-1 [Mossalayi et al., 1990a,b]. The DNA sequence of CD23 and this IgE binding factor only share 43.8% homology. However, all of the sCD23 (the secreted form) is contained in the IgE binding factor we show is vitamin D regulated. Research on IgE binding factor apparently stopped in the late 1980s. It is unclear why vitamin D regulates IgE binding factor and many of the other 102 genes identified as $1,25(OH)_2D_3$ targets in CD4+ T cells. Further experimentation is required to determine the role of these genes in vitamin D mediated regulation of CD4+ T cells.

We have begun to identify the targets of vitamin D in CD4+ T cells. Certainly the targets of vitamin D depend on the stage of Th cell development, and activation status of the CD4+ T cells. The up-regulation of GATA-3 by $1,25(OH)_2D_3$ in Th0 cells shows that vitamin D potentiates T cell differentiation, driving the cells towards the Th2 phenotype and inhibiting Th1 cell development. Low VDR expression in quiescent Th cells suggests that Th cells are relatively unresponsive to $1,25(OH)_2D_3$ in the first 24 h of activation. Effector Th cells are direct targets of vitamin D as is evidenced by decreased proliferation, IL-2, IFN-γ, and IL-5 and increased IL-4 responses. One hundred two

novel targets of $1,25(OH)_2D_3$ were identified in CD4+ T cells. Because of their known functions, $1,25(OH)_2D_3$-regulated genes like NFkB kinase, p65 sub-unit of NFkB, and IL-2R β are of interest. For other $1,25(OH)_2D_3$ regulated genes (i.e., IgE binding factor) it is less clear how or why they are $1,25(OH)_2D_3$ regulated. Further experimentation is required to determine the role of the identified genes in Vitamin D-mediated regulation of CD4+ T cells, and more specifically, how these genes may affect T cell development and differentiation. CD4+ T cells are direct targets of vitamin D. The activation and differentiation status of the CD4+ T cells dictates, which genes are vitamin D regulated and when they are expressed.

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