Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 221–227

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D_3 in the immune system☆

Femke Baeke, Hannelie Korf, Lut Overbergh, Evelyne van Etten, Annemieke Verstuyf, Conny Gysemans, Chantal Mathieu*

Laboratory of Experimental Medicine and Endocrinology (LEGENDO), Katholieke Universiteit Leuven (KUL), Herestraat 49, O&N1, Bus 902, 3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 2 November 2009
Accepted 10 March 2010

Keywords:
Vitamin D
T lymphocytes
VDR signaling
Immune modulation

ABSTRACT

Besides its actions on minerals and bone, the bioactive vitamin D metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has important immunomodulatory properties. Within the immune system, dendritic cells represent key targets for this hormone and $1,25(OH)_2D_3$-induced changes in their phenotype and function ultimately affects T lymphocytes. However, the presence of vitamin D receptors (VDR) in activated T cells proposes additional mechanisms for $1,25(OH)_2D_3$ to directly regulate T cell responses. Here, we investigated the expression and kinetics of vitamin D-related genes in human activated T lymphocytes. Different activation stimuli elicited increased VDR- and 1-α-hydroxylase expression, with a highly similar kinetic pattern. Addition of $1,25(OH)_2D_3$ effectively triggered VDR signaling, as evidenced by 24-hydroxylase induction, but only when introduced to T lymphocytes expressing high levels of VDR. This enhanced degree of VDR signaling correlated with a stronger inhibition of cytokines (IFN-γ, IL-10) and modulation of homing receptor expression (CCR10, CLA) in long-term T cell cultures. Importantly, chronic $1,25(OH)_2D_3$-exposure further amplified VDR signaling and the concomitant T cell modulating effects. In conclusion, we validate T cells as direct targets for $1,25(OH)_2D_3$ and provide this optimized *in vitro* model to improve our understanding of the role of vitamin D as a direct regulator of T cell responses.

1. Introduction

The bioactive form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), was originally described as an essential hormone for mineral and bone homeostasis, but more recently, this hormone has emerged as an important regulator of innate and adaptive immune responses [1–3]. This notion is supported by the fact that many cell types within the immune system express the vitamin D receptor (VDR). The VDR constitutes a member of the nuclear receptor superfamily, which – upon interaction with its ligand – heterodimerizes with the retinoic X receptor (RXR) [4,5]. This $1,25(OH)_2D_3$–VDR–RXR-complex subsequently binds to vitamin D responsive elements (VDRE) in the promoter region of target genes to regulate gene transcription in a wide variety of cells [6].

Among the different immune cells, dendritic cells (DCs) are important targets of $1,25(OH)_2D_3$-mediated actions. $1,25(OH)_2D_3$ interferes with the differentiation and maturation of DCs and alters the production of DC-derived cytokines [7–10]. Consequently, $1,25(OH)_2D_3$-modulated DCs exhibit a markedly decreased T cell stimulatory capacity, while skewing T cell cytokine responses from an inflammatory T helper (Th)1 – and Th17 phenotype towards a Th2 – and a regulatory T cell phenotype [7,9,11–14]. These immunomodulatory actions of $1,25(OH)_2D_3$ have been confirmed *in vivo* by its ability to prevent and/or cure various autoimmune diseases and to inhibit allograft rejection in experimental models [15,16].

Thus, combined, these observations have implicated DC-dependent actions of $1,25(OH)_2D_3$ in the regulation of T cell responses. However, the presence of the VDR in activated T cells suggests also direct actions on these cells as a factor contributing to the immunoregulatory properties of $1,25(OH)_2D_3$. Indeed, several groups have demonstrated the ability of $1,25(OH)_2D_3$ to directly modulate human and murine T cell proliferation and cytokine responses [17–23]. However, varying results have been reported, depending on the activating stimuli, the source of T cells (primary cells versus cell lines, human versus mouse) and the concentration of $1,25(OH)_2D_3$ being used. Some groups have demonstrated anti-proliferative actions of $1,25(OH)_2D_3$ on T cells [17,22,24], whereas others reported no or even stimulatory effects of the hormone [20,25,26]. Conflicting data were also obtained regarding the modulation of T cell cytokine expression, varying from an overall suppression of both Th1- and Th2 cytokines [20], while others

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author at: Campus Gasthuisberg O&N1, Laboratory of Experimental Medicine and Endocrinology (LEGENDO), Herestraat 49, Bus 902, 3000 Leuven, Belgium. Tel.: +32 16 34 60 23; fax: +32 16 34 59 34.
E-mail address: Chantal.mathieu@med.kuleuven.be (C. Mathieu).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.03.037

observed a $1,25(OH)_2D_3$-mediated shift from a Th1- to a Th2 profile [19,27].

In the present study, we investigated the expression and kinetics of vitamin D-related genes in human activated T cells and we evaluated whether activation of these pathways was dependent on the type of T cell activating stimulus. Based on the observed kinetics, we developed an optimized *in vitro* model to study the direct immunomodulatory effects of $1,25(OH)_2D_3$ and analogs on human primary T lymphocytes.

2. Materials and methods

2.1. Materials

RPMI 1640 medium with Glutamax™-I, fetal calf serum and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Merelbeke, Belgium). Purified anti-CD3 mAb (clone UCHT1) and anti-CD28 mAb (clone 37407) were obtained from R&D Systems (Minneapolis, USA). Phytohaemagglutinin (PHA) was acquired from Remel (Lenexa, USA). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma (Saint Louis, USA). Human recombinant IL-2 and IL-12 were purchased from Peprotech (London, UK). $1,25(OH)_2D_3$ was a kind gift of J.P. Vandevelde (Solvay, Weesp, The Netherlands).

2.2. Isolation of human $CD3^+$ T cells and cell cultures

Peripheral blood mononuclear cells were isolated by Ficoll-gradient centrifugation (Axis-Shield Poc AS, Oslo, Norway) from buffycoats, obtained from healthy donors. $CD3^+$ T cells were subsequently purified by negative selection using the Pan T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol (purity routinely >96%). $CD3^+$ T cells, dissolved to a final concentration of 0.7×10^6 cells/ml in RPMI 1640 medium with Glutamax™-I, supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 UI/ml) were cultured in 24-well plates and activated by either plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml), PHA (5 μg/ml) or PMA (20 ng/ml) and ionomycin (1 μg/ml). If indicated, human recombinant IL-12 (2.5 ng/ml) was added at the beginning of the cell cultures. For culture periods longer than 48 h, cells were split every 2 days and supplemented with fresh medium containing human recombinant IL-2 (12.5 ng/ml). Depending on the treatment protocol, $1,25(OH)_2D_3$ (10^{-8} M) or vehicle (ethanol), was added on day 0, 2 or every other day starting at day 2. At indicated time points, samples were taken for real-time RT-PCR and FACS analysis.

2.3. RNA isolation and real-time RT-PCR

RNA was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Total RNA (0.5–1 μg) was reverse transcribed using 100U Superscript II Reverse Transcriptase (Invitogen, Merelbeke, Belgium) and 5 μM oligo$(dT)_{16}$ at 42 °C for 80 min. Real-time RT-PCR was performed using a MyIQ-Cycler (Bio-Rad) and the TaqMan system, as described previously [28,29]. Primer and probe sets for β-actin, 1-α-hydroxylase, IFN-γ and IL-10 were described previously [28,30]. Following primers and probe sets were used for VDR: TGGCTTTCACTTCAATGCTATGA (FW), CGTCGGTTGTCCTTGGTGAT (RV), AAGGCTGCAAAGGCTTCTTCAGGCG (TQ) and 24-hydroxylase: TATCGCGACTACCGCAAAGA (FW), CGGCCAAGACCTCATTGATT (RV), TCCGGACCCGCTGCCAGTCTT (TQ). All data were normalized to β-actin mRNA levels.

2.4. Flow cytometric analysis of skin-homing receptor expression

T cells were stained with directly conjugated monoclonal antibodies against CD3 (eBiosciences, San Diego, USA), CLA (Biolegend, San Diego, USA), CCR10 (R&D Systems, Minneapolis, USA) or matching isotype controls. Dead cells were excluded from analysis using 7-amino-actinomycin D (7-AAD) (eBiosciences). Stained cells were analyzed on a FACS Calibur or a FACS Canto, using the CellQuest or FACSDiva software, respectively (BD Biosciences).

2.5. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Significance was defined as $p < 0.05$.

3. Results

3.1. Kinetics of VDR- and vitamin D metabolizing gene expression in human activated $CD3^+$ T cells

To gain more insight into the role of vitamin D as a regulator of T cell responses and to verify whether human T cells would be responsive to the effects of active vitamin D, we analyzed the expression levels of VDR and vitamin D metabolizing genes, such as 1-α-hydroxylase and 24-hydroxylase, at different time points following T cell activation. For this purpose human $CD3^+$ T cells were isolated by negative magnetic cell separation of normal blood donors. In agreement with previous reports, resting $CD3^+$ T cells expressed only low basal VDR mRNA levels. However, full activation of T cells with immobilized anti-CD3/anti-CD28, providing the T cell receptor (TCR)- as well as co-stimulatory stimulus, resulted in a marked upregulation of VDR mRNA levels (Fig. 1A) [5,24,31]. Elevated VDR levels were detected as early as 8 h post-activation and further increased over time.

To evaluate whether the induction of VDR was dependent on the stimulus used, we mimicked the TCR- and co-stimulatory signals by using the mitogen PHA. This lectin induces T cell activation by binding to cell membrane glycoproteins, including the TCR–CD3 complex, resulting in sufficient cross-linking of the receptor to activate intracellular signaling events. In contrast to the results with anti-CD3/anti-CD28, PHA-induced activation of the cells only triggered a minor increase in VDR mRNA levels, peaking at 24 h (Fig. 1A). Also activation of T cells with PMA/ionomycin, which activates protein kinases downstream of TCR-engagement, was sufficient to induce VDR expression (Fig. 1A). Thus, although the kinetics and degree of VDR induction differed to some extent, all T cells exhibited the ability to upregulate VDR expression upon stimulation. Importantly, T cell activation by these stimuli also gradually increased the expression of 1-α-hydroxylase, which could already be detected 8 h post-activation (Fig. 1B). Whereas PHA and PMA/ionomycin-activated T cells featured maximum 1-α-hydroxylase levels after 24 h, a lower but progressive increment was observed in cells exposed to anti-CD3/anti-CD28.

Furthermore, we extended our analysis of the expression of vitamin D-associated genes in T cells on a more long-term basis by implementing anti-CD3/anti-CD28 as the most physiologically relevant activation stimulus. Our results demonstrated that VDR- and 1-α-hydroxylase levels ultimately peaked at 48 h post-activation, followed by a steady decline when T cell growth was maintained up to 10 days (Fig. 1C and D). Interestingly, 24-hydroxylase mRNA was not detectable in freshly isolated T cells and none of the activation stimuli induced its expression (data not shown).

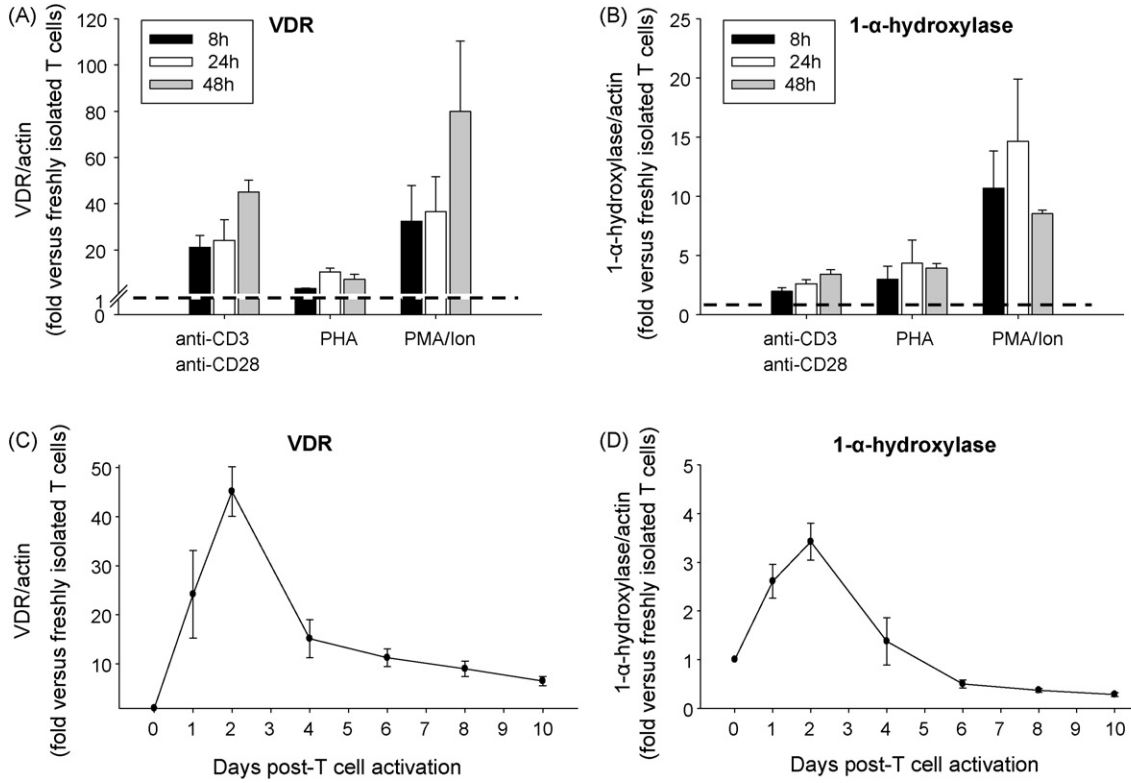

Fig. 1. Kinetics of vitamin D-related gene expression in human activated T cells. (A and B) Highly purified human T cells were activated with anti-CD3/anti-CD28, PHA or PMA/Ion, as described in Section 2. Samples for mRNA analysis were harvested after 8 (black bars), 24 (white bars) and 48 h (grey bars). mRNA expression levels of VDR (A) and 1-α-hydroxylase (B) were evaluated by quantitative real-time RT-PCR. (C and D) T cells, activated by anti-CD3/anti-CD28, were cultured up to 10 days and mRNA expression levels of VDR (C) and 1-α-hydroxylase (D) were analyzed by quantitative real-time RT-PCR at indicated time points. The results are depicted as relative expression levels compared to freshly isolated T cells, the latter which is indicated by the dashed line (A and B), and represent the mean ± SEM of 2–6 independent experiments.

3.2. 1,25(OH)$_2$D$_3$ activates VDR-dependent signaling pathways in human T cells

Next, we verified the ability of the natural ligand, 1,25(OH)$_2$D$_3$, to induce activation of VDR signaling pathways in human T cells by evaluating the expression levels of the well-documented VDR-target gene, 24-hydroxylase. Considering the kinetics of VDR expression, we compared the responsiveness of activated T cells to 1,25(OH)$_2$D$_3$ when treatment was started simultaneously with-, or 2 days after initial T cell activation. A once-off treatment of T cells with 1,25(OH)$_2$D$_3$ at the time of activation failed to effectively trigger VDR-dependent signaling pathways, since no significant increase in 24-hydroxylase mRNA levels could be detected (Fig. 2A). In contrast, treatment of the cells at a moment when expression levels of VDR peaked (48 h), resulted in a more pronounced, but still modest induction of 24-hydroxylase levels (Fig. 2B). Continuous exposure of the T cell cultures to 1,25(OH)$_2$D$_3$, by addition of the vitamin D$_3$ metabolite every second day starting 48 h after activation, further amplified 24-hydroxylase expression in long-term T cell cultures (10 days), whereas single addition of 1,25(OH)$_2$D$_3$ on day 2 did not suffice to maintain high levels of 24-hydroxylase up to this time point (Fig. 2C).

3.3. Long-term exposure to 1,25(OH)$_2$D$_3$ results in increased inhibitory effects on cytokine secretion by activated T cells and modulation of skin-homing receptors

We investigated whether the degree of VDR-dependent signaling would correlate with modulated cytokine expression of the cells. To study the effects on early T cell responses, T cells were treated with 1,25(OH)$_2$D$_3$ simultaneously with-, or 2 days after initial T cell activation. On day 4, mRNA expression levels of IFN-γ and IL-10 were measured by real-time RT-PCR. Interestingly, IFN-γ and IL-10 were equally suppressed in short term T cell cultures when a single dose of 1,25(OH)$_2$D$_3$ was administered simultaneously with-, or 2 days after T cell activation (Fig. 3A and B). Nevertheless, when cells were cultured up to 10 days, 1,25(OH)$_2$D$_3$ failed to suppress IL-10 when given at the time of T cell activation. In contrast, introduction of 1,25(OH)$_2$D$_3$ to activated T cells, expressing maximal VDR levels, resulted in a substantial inhibition of IL-10 and chronic addition of 1,25(OH)$_2$D$_3$ further enhanced this effect (Fig. 3C). Moreover, whereas a single dose of 1,25(OH)$_2$D$_3$, either given at the time of-, or 2 days after T cell activation, failed to modulate IFN-γ expression in long-term cell cultures, continuous exposure of T cells to the hormone significantly downregulated this cytokine (Fig. 3D).

Analysis of homing molecules, which have previously been reported to be regulated by 1,25(OH)$_2$D$_3$ [32,33], further confirmed the ability of the ligand to trigger immune-related VDR targets in the absence of antigen-presenting cells (APC) or APC-derived signals. Flow cytometric analysis revealed a higher induction of the skin-homing receptor CCR10 in cells that were activated prior to 1,25(OH)$_2$D$_3$-addition and repeated treatment further amplified this effect (Fig. 3E). Similarly, greater inhibition of cutaneous lymphocyte-associated antigen (CLA) expression, another surface receptor involved in T cell trafficking to the skin, was observed, in particular when cells were activated prior to 1,25(OH)$_2$D$_3$-exposure (42.3% CLA$^+$ T cells in control cells versus 35.5% in T cells treated at the time of activation and 33.3% in T cells activated prior to treatment). Also here, this effect was further stimulated by applying the hormone repeatedly (30.4% CLA$^+$ T cells).

In contrast to previous reports on the requirement of IL-12 in cell culture for efficient 1,25(OH)$_2$D$_3$-mediated induction of CCR10 on naïve T lymphocytes [32], in our experiments, IL-12 did not restore

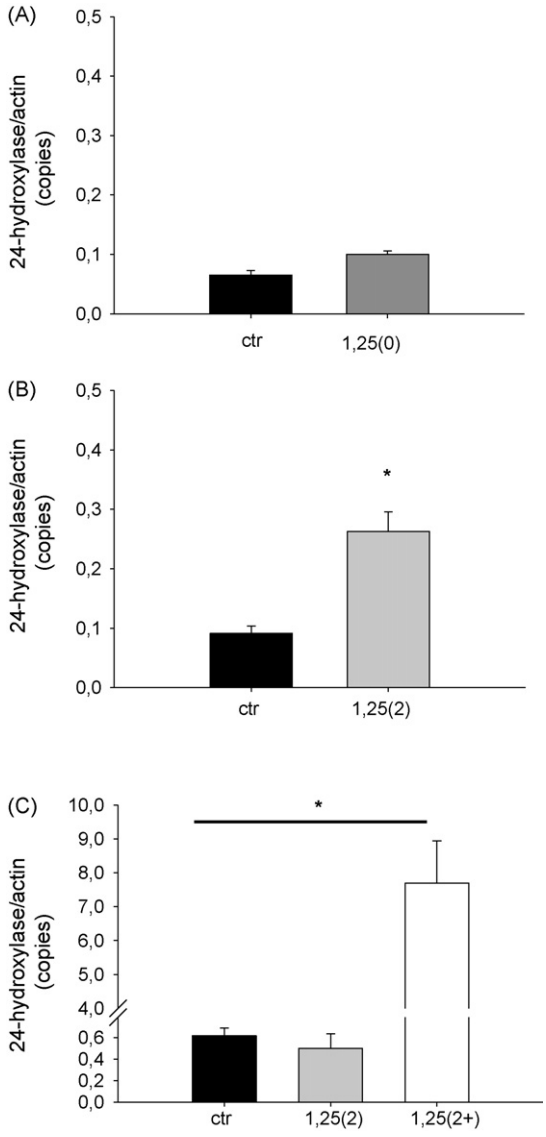

Fig. 2. $1,25(OH)_2D_3$ triggers VDR signaling pathways in human T cells. Purified human T cells were activated with anti-CD3/anti-CD28. (A and B) T cells received a single dose of vehicle (ctr, black bars) or $1,25(OH)_2D_3$ (10^{-8} M) at the time of activation (1,25(0), dark grey bar) (A) or on day 2 (1,25(2), light grey bar) (B). 48 h after $1,25(OH)_2D_3$-addition, 24-hydroxylase mRNA levels were determined by real-time RT-PCR. (C) Activated T cells received a single $1,25(OH)_2D_3$-dose on day 2 or were repeatedly treated with $1,25(OH)_2D_3$ on day 2, 4, 6 and 8 (1,25(2+), white bar). On day 10, 24-hydroxylase mRNA levels were determined by real-time RT-PCR. All results shown are representative for 3–6 independent experiments and are depicted as mean ± SEM. $^*p < 0.05$ vs. ctr.

the inability of resting T cells to respond to the hormone, since levels of CCR10 expression were not increased in T cells that were activated and simultaneously treated with $1,25(OH)_2D_3$ and IL-12 (0.41% $CCR10^+$ T cells) as compared to $1,25(OH)_2D_3$ alone (0.63% $CCR10^+$ T cells). Accordingly, the presence of IL-12 did not improve upregulation 24-hydroxylase when $1,25(OH)_2D_3$ was administered at the time of activation (data not shown).

We further tested whether $1,25(OH)_2D_3$ would affect the expression of its own receptor, a phenomenon that has been reported in several cell types, and possibly explaining the enhanced $1,25(OH)_2D_3$-mediated effects obtained in T cells upon repeated administration of the hormone. A single dose of $1,25(OH)_2D_3$ could indeed augment activation-induced VDR expression in T cells, whereas repeated treatment further amplified this effect (Fig. 3F).

4. Discussion

The discovery that receptors for active vitamin D are abundantly present throughout the immune system has stimulated the search for immunomodulatory actions of $1,25(OH)_2D_3$, revealing a plethora of effects targeting different immune cells [1,3]. Up to now, the well-documented actions of $1,25(OH)_2D_3$ on DCs are believed to be the central mechanism by which adaptive immune responses are regulated by this hormone. Nevertheless, the presence of the VDR and vitamin D metabolizing enzymes, such as 1-α-hydroxylase, in activated T cells suggests an important role for vitamin D in the direct regulation of T cell responses. Here, we investigated the expression and kinetics of vitamin D-related genes in human activated T cells to gain more insight into the role of vitamin D as a regulator of T cell responses. In addition, we evaluated the ability of the natural ligand to activate VDR signaling pathways and whether this translates into modulation of T cell responses.

Different groups have reported a strong induction of VDR expression upon T cell activation [5,24,31]. Our results confirm these findings and demonstrate that increased VDR expression can be elicited by various T cell activation stimuli, including anti-CD3/anti-CD28, providing the two signals necessary for full T cell activation. Elevated VDR levels were also observed by mimicking these signals with the lectin mitogen, PHA, or by triggering more downstream T cell signaling pathways with PMA/ionomycin. Nevertheless, the levels and kinetics of VDR expression seem to vary between the different activation stimuli. These findings might – at least in part – explain the conflicting results that have been reported regarding the effects of $1,25(OH)_2D_3$ on T cell proliferation and cytokine production [18,22,24,25,27], since the type of activation stimulus was not consistent throughout all these investigations.

Besides the induction of the receptor for $1,25(OH)_2D_3$, T cell activation was also accompanied by a strong increase in 1-α-hydroxylase expression, the enzyme responsible for conversion of circulating $25(OH)D_3$ into bioactive $1,25(OH)_2D_3$. Interestingly, the expression pattern of this enzyme in long-term T cell cultures closely mimicked the kinetics of VDR expression upon T cell activation, supporting the hypothesis that not only APCs, but also activated T cells may convert $25(OH)D_3$ into $1,25(OH)_2D_3$ for autocrine or paracrine use. In this context, other groups have indeed demonstrated the ability of activated T cells to convert the inactive vitamin D metabolite $25(OH)D_3$ into bioactive $1,25(OH)_2D_3$, allowing them not only to exhibit functional responses to the active ligand, but also to its precursor [24,32]. Thus, like in other cells of the immune system, 1-α-hydroxylase expression in T cells is controlled by immune signals and local processing of $25(OH)D_3$ into the active ligand is therefore likely to represent an important mechanism to prevent excessive inflammation during episodes of active immune responses.

Importantly, previous *in vitro* studies on the effects of $1,25(OH)_2D_3$ on T cells have mostly been performed by exposing T cells to $1,25(OH)_2D_3$ prior to or simultaneous with activation of the cells. Considering the kinetics of VDR expression upon T cell activation and the fact that $1,25(OH)_2D_3$-mediated effects largely depend on binding to its receptor, we investigated whether the extent of VDR-pathway activation was dependent on the timing of $1,25(OH)_2D_3$-introduction to the cultures. By using 24-hydroxylase, a well-documented target gene, as readout for functional activation of VDR-dependent pathways, we demonstrated that $1,25(OH)_2D_3$ effectively triggered VDR signaling in activated T cells, but introduction of the hormone at a time when VDR is present, was a prerequisite for efficient induction of 24-hydroxylase. Furthermore, to maintain high levels of the VDR-target gene, a periodic re-exposure to the ligand was required. Paradoxically, the need for continuous $1,25(OH)_2D_3$-exposure for long-term responsiveness of the T cells might be due to break-

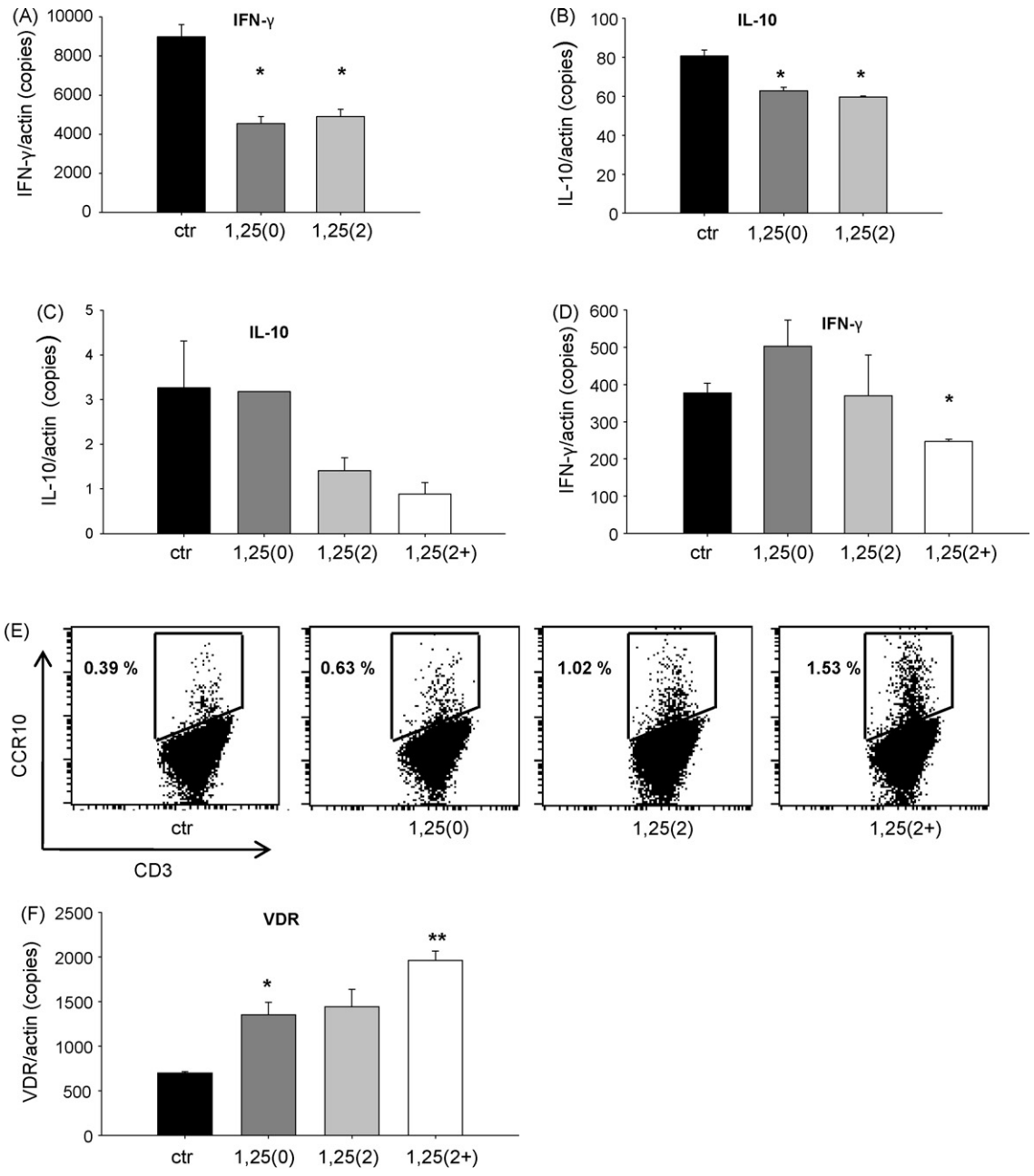

Fig. 3. 1,25$(OH)_2D_3$-mediated modulation of T cell responses. T cells were activated with anti-CD3/anti-CD28 and received a single dose of vehicle (ctr, black bars) or 1,25$(OH)_2D_3$ (10^{-8} M) at the time of activation (1,25(0), dark grey bars) or on day 2 (1,25(2), light grey bars) or cells were repeatedly treated with 1,25$(OH)_2D_3$ on day 2, 4, 6 and 8 (1,25(2+), white bars). (A–D) The impact of the various 1,25$(OH)_2D_3$-treatment protocols on T cells was analyzed by measurement of IFN-γ and IL-10 by real-time RT-PCR, both at early (day 4) (A and B) and long-term (day 10) (C and D) time points. (E) Additionally, flow cytometric analysis of CCR10 expression was verified. # (F) Effects of 1,25$(OH)_2D_3$ on VDR expression in long-term cultured T cells. The results shown are mean ± SEM of one representative experiment out of 3–6 donors tested. $^{*}p < 0{,}05$; $^{**}p < 0.01$ vs. ctr. # Flow cytometric data shown here are representative for three independent experiments.

down of the ligand by the degrading 24-hydroxylase enzyme. On the other hand, the multiple treatment-schedule with 1,25$(OH)_2D_3$ also enhanced the expression of its own receptor, which would explain the enhanced VDR associated gene expression in the long-term cultures. Recently, similar findings were published by Correale et al. who found that 1,25$(OH)_2D_3$ could increase VDR expression both in resting and activated T cells [24]. Our results confirm these findings, but additionally suggest the opportunity to further enhance this autoregulatory system by exposing T cells repeatedly to 1,25$(OH)_2D_3$.

However, the degree of VDR signaling triggered by 1,25$(OH)_2D_3$ did not fully reflect the ability of the ligand to interfere with early T cell cytokine responses, since 1,25$(OH)_2D_3$-addition at the time of T cell activation led to a similar inhibition of cytokine expression as compared to treatment of cells exhibiting maximal VDR levels. We cannot provide a clear explanation for this discrepancy, but propose that the inability of 1,25$(OH)_2D_3$ to induce the degrading enzyme, 24-hydroxylase, when introduced at basal VDR levels, prevents breakdown of the hormone and allows 1,25$(OH)_2D_3$ to remain intact in the cell cultures until activation-induced VDR appears. In this way, 1,25$(OH)_2D_3$ can affect early T cell function, irrespective of the timing of treatment. Alternatively, VDR-independent actions of the ligand could possibly account for this inconsistency [34]. Nevertheless, in long-term T cell cultures, we observed a substantial increment in the cytokine-inhibitory function of 1,25$(OH)_2D_3$ if administered when VDR is abundantly present. In addition, not

only cytokine responses of the cells were modulated by the active vitamin D metabolite, but also surface expression of skin T cell-associated homing molecules, as illustrated by the induction of CCR10 and inhibition of CLA. In this context, our data are in part supported by the findings of Sigmundsdottir et al., who demonstrated enhanced induction of CCR10 when naive T cells were activated prior to $1,25(OH)_2D_3$-exposure [32]. In addition, the investigators of this study could restore the inability of $1,25(OH)_2D_3$, introduced at the time of activation, to efficiently induce CCR10 expression on naive T cells, by adding IL-12 at the beginning of the cultures. However, in our experiments, a once-off treatment with $1,25(OH)_2D_3$ at the time of T cell activation in the presence of IL-12 failed to overcome the inability of the hormone to trigger VDR signaling, neither did IL-12 restore the capacity to effectively induce CCR10 expression on T cells. Thus, in our hands, the modulation of immune-related function by $1,25(OH)_2D_3$ on T cells could be elicited in the absence of APC or APC-derived factors such as IL-12.

In summary, to gain more insight into the role of vitamin D as a direct modulator of T cell responses, we have investigated the expression of vitamin D-related genes in human T cells, with special focus on the kinetics and expression levels during T cell activation. Different stimuli, each triggering activation of the cells at a specific level in the T cell signaling cascade, were capable of increasing the levels of VDR as well as 1-α-hydroxylase in human T cells and importantly, expression levels of 1-α-hydroxylase closely mimicked the kinetics of the receptor. Based on these findings, we demonstrated that the timing of $1,25(OH)_2D_3$-treatment is a critical factor for efficient activation of VDR signaling pathways. Moreover, we found that multiple addition of $1,25(OH)_2D_3$ dramatically enhanced signaling by the receptor and concomitantly amplified the T cell modulating effects of the ligand in long-term T cells cultures. Application of this optimized *in vitro* model could be used as screening system to identify new immunomodulatory actions of $1,25(OH)_2D_3$ or synthetic agonists on T cells, thereby improving our understanding of the role of vitamin D as a direct regulator of T cell responses.

Acknowledgements

The technical experience of Wim Cockx, Nursen Gol and Dirk Valckx is greatly appreciated. This work was supported by the Flemish Research Foundation (FWO) (Grants G.0552.06 and G.0649.08, a postdoctoral fellowship for Conny Gysemans and Hannelie Korf and a clinical fellowship for Chantal Mathieu), the Juvenile Diabetes Research Foundation (a postdoctoral fellowship for Evelyne van Etten (JDRF3-2006-33)), the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgian State (IAP P5/17 and P6/40), the Katholieke Universiteit Leuven (GOA 2004/10 and GOA 2009/10) and the 7th Framework Program of the European Union with acronym NAIMIT and of the JDRF Center for Prevention of Beta Cell Therapy in Diabetes [grant number 4-2005-1327].

References

[1] F. Baeke, E. van Etten, C. Gysemans, L. Overbergh, C. Mathieu, Vitamin D signaling in immune-mediated disorders: evolving insights and therapeutic opportunities, Mol. Aspects Med. 29 (6) (2008) 376–387.
[2] D. Bikle, Nonclassic actions of vitamin D, J. Clin. Endocrinol. Metab. 94 (1) (2009) 26–34.
[3] J.S. Adams, M. Hewison, Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity, Nat. Clin. Pract. Endocrinol. Metab. 4 (2) (2008) 80–90.
[4] C.M. Veldman, M.T. Cantorna, H.F. DeLuca, Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system, Arch. Biochem. Biophys. 374 (2) (2000) 334–338.
[5] D.M. Provvedini, C.D. Tsoukas, L.J. Deftos, S.C. Manolagas, 1,25-Dihydroxyvitamin D3 receptors in human leukocytes, Science 221 (4616) (1983) 1181–1183.
[6] A.S. Dusso, A.J. Brown, E. Slatopolsky, Vitamin D, Am. J. Physiol. Renal Physiol. 289 (1) (2005) F8–28.
[7] G. Penna, L. Adorini, 1Alpha, 25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation, J. Immunol. 164 (5) (2000) 2405–2411.
[8] M.D. Griffin, W. Lutz, V.A. Phan, L.A. Bachman, D.J. McKean, R. Kumar, Dendritic cell modulation by 1alpha, 25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo, Proc. Natl. Acad. Sci. U.S.A. 98 (12) (2001) 6800–6805.
[9] A.G. van Halteren, E. van Etten, E.C. de Jong, R. Bouillon, B.O. Roep, C. Mathieu, Redirection of human autoreactive T-cells Upon interaction with dendritic cells modulated by TX527, an analog of 1,25 dihydroxyvitamin D(3), Diabetes 51 (7) (2002) 2119–2125.
[10] A.W Pedersen, K. Holmstrom, S.S. Jensen, D. Fuchs, S. Rasmussen, P. Kvistborg, M.H. Claesson, M.B. Zocca, Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells, Clin. Exp. Immunol. 157 (1) (2009) 48–59.
[11] A.G. van Halteren, O.M. Tysma, E. van Etten, C. Mathieu, B.O. Roep, 1Alpha, 25-dihydroxyvitamin D3 or analogue treated dendritic cells modulate human autoreactive T cells via the selective induction of apoptosis, J. Autoimmun. 23 (3) (2004) 233–239.
[12] J. Tang, R. Zhou, D. Luger, W. Zhu, P.B. Silver, R.S. Grajewski, S.B. Su, C.C. Chan, L. Adorini, R.R. Caspi, Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response, J. Immunol. 182 (8) (2009) 4624–4632.
[13] G. Penna, S. Amuchastegui, N. Giarratana, K.C. Daniel, M. Vulcano, S. Sozzani, L. Adorini, 1,25-Dihydroxyvitamin D3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells, J. Immunol. 178 (1) (2007) 145–153.
[14] G. Penna, A. Roncari, S. Amuchastegui, K.C. Daniel, E. Berti, M. Colonna, L. Adorini, Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1, 25-dihydroxyvitamin D3, Blood 106 (10) (2005) 3490–3497.
[15] L. Adorini, G. Penna, Control of autoimmune diseases by the vitamin D endocrine system, Nat. Clin. Pract. Rheumatol. 4 (8) (2008) 404–412.
[16] L. Adorini, S. Amuchastegui, K.C. Daniel, Prevention of chronic allograft rejection by Vitamin D receptor agonists, Immunol. Lett. 100 (1) (2005) 34–41.
[17] W.F. Rigby, T. Stacy, M.W. Fanger, Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol), J. Clin. Invest. 74 (4) (1984) 1451–1455.
[18] W.F. Rigby, S. Denome, M.W. Fanger, Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA, J. Clin. Invest. 79 (6) (1987) 1659–1664.
[19] A. Boonstra, F.J. Barrat, C. Crain, V.L. Heath, H.F. Savelkoul, A. O'Garra, 1Alpha, 25-dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells, J. Immunol. 167 (9) (2001) 4974–4980.
[20] T.P. Staeva-Vieira, L.P. Freedman, 1,25-Dihydroxyvitamin D3 inhibits IFN-gamma and IL-4 levels during in vitro polarization of primary murine CD4+ T cells, J. Immunol. 168 (3) (2002) 1181–1189.
[21] A.K. Bhalla, E.P. Amento, B. Serog, L.H. Glimcher, 1,25-Dihydroxyvitamin D3 inhibits antigen-induced T cell activation, J. Immunol. 133 (4) (1984) 1748–1754.
[22] J.M. Lemire, J.S. Adams, V. Kermani-Arab, A.C. Bakke, R. Sakai, S.C. Jordan, 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro, J. Immunol. 134 (5) (1985) 3032–3035.
[23] B.D. Mahon, A. Wittke, V. Weaver, M.T. Cantorna, The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells, J. Cell. Biochem. 89 (5) (2003) 922–932.
[24] J. Correale, M.C. Ysrraelit, M.I. Gaitan, Immunomodulatory effects of Vitamin D in multiple sclerosis, Brain 132 (Pt 5) (2009) 1146–1160.
[25] L.E. Jeffery, F. Burke, M. Mura, Y. Zheng, O.S. Qureshi, M. Hewison, L.S. Walker, D.A. Lammas, K. Raza, D.M. Sansom, 1,25-Dihydroxyvitamin D(3) and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3, J. Immunol. 183 (9) (2009) 5458–5467.
[26] D.L. Lacey, J. Axelrod, J.C. Chappel, A.J. Kahn, S.L. Teitelbaum, Vitamin D affects proliferation of a murine T helper cell clone, J. Immunol. 138 (6) (1987) 1680–1686.
[27] E. Borgogni, E. Sarchielli, M. Sottili, V. Santarlasci, L. Cosmi, S. Gelmini, A. Lombardi, G. Cantini, G. Perigli, M. Luconi, G.B. Vannelli, F. Annunziato, L. Adorini, M. Serio, C. Crescioli, Elocalcitol inhibits inflammatory responses in human thyroid cells and T cells, Endocrinology 149 (7) (2008) 3626–3634.
[28] L. Overbergh, A. Giulietti, D. Valckx, R. Decallonne, R. Bouillon, C. Mathieu, The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression, J. Biomol. Technol. 14 (1) (2003) 33–43.
[29] A. Giulietti, L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon, C. Mathieu, An overview of real-time quantitative PCR: applications to quantify cytokine gene expression, Methods 25 (4) (2001) 386–401.
[30] K. Stoffels, L. Overbergh, A. Giulietti, L. Verlinden, R. Bouillon, C. Mathieu, Immune regulation of 25-hydroxyvitamin-D3-1alpha-hydroxylase in human monocytes, J. Bone Miner. Res. 21 (1) (2006) 37–47.
[31] A.K. Bhalla, E.P. Amento, T.L. Clemens, M.F. Holick, S.M. Krane, Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation, J. Clin. Endocrinol. Metab. 57 (6) (1983) 1308–1310.
[32] H. Sigmundsdottir, J. Pan, G.F. Debes, C. Alt, A. Habtezion, D. Soler, E.C. Butcher, DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attrac-

tion to the epidermal chemokine CCL27, Nat. Immunol. 8 (3) (2007) 285–293.
[33] K. Yamanaka, C.J. Dimitroff, R.C. Fuhlbrigge, M. Kakeda, I. Kurokawa, H. Mizutani, T.S. Kupper, Vitamins A and D are potent inhibitors of cutaneous lymphocyte-associated antigen expression, J. Allergy Clin. Immunol. 121 (1) (2008) 148–157.
[34] A.W. Norman, Minireview: vitamin D receptor: new assignments for an already busy receptor, Endocrinology 147 (12) (2006) 5542–5548.