

Impaired T cell activation and cytokine production by calcitriol-primed human B cells

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Summary

The biologically active form of vitamin D₃, 1, 25-dihydroxyvitamin D₃ (calcitriol), is a potent modulator of the immune response. We have shown previously that calcitriol modulates the immunoglobulin response *in vitro* and *in vivo* in mice and humans. To analyse the underlying molecular mechanisms we studied whether calcitriol-primed B cells modulate T cell activation and function. Human B cells were stimulated with anti-CD40 and interleukin (IL)-4 in the presence of increasing concentrations of calcitriol. After removal of calcitriol, primed B cells were co-cultured with autologous CD4⁺ T cells; the B cell phenotype T cell activation and their consecutive cytokine production were also assessed. Naive T cells co-cultured with calcitriol-primed naive B cells showed a reduced expansion, nuclear factor of activated T cells, cytoplasmic 2 (NFATc2) expression and cytokine production upon restimulation. CD86 expression on B cells after calcitriol priming was identified as an underlying mechanism, as T cell activation and expansion was rescued by activating anti-CD28 antibodies. Our data indicate that calcitriol-primed B cells display an impaired capacity to activate T cells. Taken together, we identified a novel B cell-dependent vitamin D immune regulatory mechanism, namely by decreased co-stimulation of calcitriol-primed B cells.

Keywords: B cells, co-stimulatory molecules, T cells, vitamin D

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Introduction

The initiation of T cell responses requires T cell receptor triggering and co-stimulatory signals from antigen-presenting cells (APCs). Isolated T cell receptor stimulation leads to T cell anergy [1]. B cells have been recognized not only as antibody-producing but also as potent APCs, which play an important role in the initiation of T cell responses [2]. They encounter B cell receptor (BCR)-specific antigens in secondary lymphoid organs and internalize, process and present them to T cells [3]. Upon CD40 activation, B cells express T cell co-stimulatory molecules such as CD80 and CD86 and thus provide naive T cell activation [4,5]. Numerous studies suggest that vitamin D deficiency is associated with immunological diseases, e.g. atopic dermatitis (AD) [6] and allergic asthma [7], but also autoimmune diseases [8–11]. Calcitriol (1, 25-dihydroxyvitamin D₃) is the bioactive form of vitamin D, and modulates lymphocyte function following binding to its receptor (VDR). In re-

sponse to calcitriol, T cells show a reduced proliferation and production of proinflammatory cytokines, but an increased expression of interleukin (IL)-10 [12,13]. Calcitriol-treated B cells display impaired immunoglobulin (Ig)E class-switch recombination and secretion [14], but produce enhanced IL-10 [15]. In dendritic cells (DCs), calcitriol results in an inhibition of differentiation and maturation and leads to a modulation of the T cell co-stimulatory capacity promoting the generation of suppressor T cell phenotypes [16,17]. Because B cells also act as APCs to T cells we hypothesized similarly that VDR activation of B cells may result in impaired T cell activation. First, therefore, we investigated the activation profile of calcitriol-primed B cells, and secondly their impact on T cell activation.

Our data show that calcitriol-primed B cells display a diminished co-stimulatory activation profile which results in reduced expansion and cytokine production of autologous T cells, delineating a novel immunomodulatory mechanism of vitamin D in the context of B cells.

Material and methods

B and T cell isolation and cultivation *in vitro*

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of four to five independent donors per experiment, and B cells were purified to >98% purity using anti-CD19-coupled multi-sort magnetic beads as described previously [18]. The pre-enriched CD19⁺ B cells were separated after release of magnetic beads in naive/memory B cells, additionally using anti-CD27-coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) [19]. Naive CD4⁺ T cells were positively isolated from CD19^{neg} fraction following depletion of CD45RO⁺, CD14⁺, CD25⁺ cells and memory CD4⁺ T cells upon removal of CD45RA⁺, CD14⁺ cells by using respective magnetically coupled monoclonal antibodies by magnetic cell sorting (all Miltenyi Biotec; purity > 98%). Purified B cells (1×10^6 /ml), activated with anti-CD40 monoclonal antibody (mAb) (1 µg/ml, clone 626-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and recombinant human IL-4 (10 ng/ml; Miltenyi Biotec), were cultured in RPMI-1640 culture medium (Life Technologies, Carlsbad, CA, USA) for 2 days in the presence of 1 µmol/l calcitriol (Sigma-Aldrich, St Louis, MO, USA) [19]. After 2 days' priming phase B cells were washed three times with 50 ml phosphate-buffered saline (PBS) (PAA, Pasching, Austria) and resuspended at concentration of 5×10^7 /ml in RPMI-1640 culture medium. Naive and memory T cells were labelled with 5(6)-carboxyfluorescein diacetate N-succinimidylester (CFSE; Sigma-Aldrich), as described elsewhere [20], and resuspended at a concentration of 2×10^6 /ml. Based on previous titration experiments, T cells were co-cultured with B cells at ratio of 4:1 in the presence of superantigen toxic shock syndrome toxin 1 (TSST-1, 50 pg/ml; Sigma-Aldrich) or platebound anti-CD3 (1 µg/ml, clone HIT3; BD Pharmingen, San Jose, CA, USA) for 7 days in RPMI-1640 culture medium (Life Technologies). In some experiments anti-CD28 (1 µg/ml, clone 28-2; BD Pharmingen) antibodies were added to T cell–B cell co-culture.

Functional assay to determine remaining amounts of calcitriol in the co-cultures

The human promyelocytic leukaemia HL-60 cell line was used to determine the remaining amount of calcitriol upon B cell priming and to rule out a possible spillover to the T cell co-culture. The HL-60 cell line expresses CD38 upon contact with calcitriol. B cells were treated as described and rested at 37°C in 5% CO₂ for 1 and 3 days in the absence of T cells and the supernatant was added to 2.5×10^5 /ml HL-60 cells. Calcitriol was added in concentrations from 1 µmol/l–10 pmol/l to 2.5×10^5 /ml HL-60 cells. In addition, RPMI-1640 medium supplemented with 1 µmol/l calcitriol

and kept in a similar manner to B cells was used as a control for the cell-free material spillover effect. The samples were incubated for 3 days at 37°C in 5% CO₂. On day 3, CD38 surface expression on HL-60 cells was determined.

Flow cytometric analysis

CD19⁺ B from whole blood were phenotyped using fluorochrome-conjugated monoclonal antibodies and characterized according to the surface expression of CD19, CD27, CD80, CD86 (all BD Pharmingen) and CD38 (Beckman Coulter, Krefeld, Germany). HL-60 cells were stained accordingly with antibodies against CD38 (Beckman Coulter) and CD14 (BD Pharmingen). On day 7, co-cultured cells were restimulated by 10 mg/ml phorbol 12-myristate 13-acetate (PMA), 1 mg/ml ionomycin and 2 mg/ml brefeldin A (all from Sigma-Aldrich) for 5 h. After permeabilization (Perm 2 solution; BD Pharmingen), T cells were stained intracellularly for CD4, CD45RA, IL-2, interferon (IFN)-γ, IL-4 (all BD Pharmingen) and nuclear factor of activated T cells, cytoplasmic 2 (NFATc2) (DRFZ). Flow cytometric analyses were performed using fluorescence activated cell sorter (FACS) Calibur and FACSaria II cytometer (BD Pharmingen). Data were analysed using FlowJo7 software (TreeStar, Ashland, OR, USA).

Quantitative real-time polymerase chain reaction (PCR)

B cells were harvested after 48 h activation and calcitriol-priming and RNA was isolated using the RNA isolation kit (NucleoSpin® RNA II; Macherey-Nagel GmbH & Co, Dueren, Germany), according to the manufacturer's instructions. The cDNA synthesis was performed by a reverse transcriptase-PCR with TaqMan® reverse transcription reagents (Applied Biosystems, Darmstadt, Germany). Gene expression analysis was performed using quantitative real-time PCR with LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany). Primers were synthesized by TIB Molbiol (Berlin, Germany) and the sequences are listed in the Supporting information, Table S1. The expression of CD86 was normalized to the expression of *hprt* as a house-keeping gene.

Statistical methods

Statistical evaluations were performed with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Data displayed the percentage of observations and columns in graphs using mean ± standard deviation (s.d.). Normal distribution was judged by the Kolmogorov–Smirnov test and these parameters were tested by Student's *t*-test or analysis of variance. *P*-values ≤ 0.05 were considered statistically significant.

Results

Reduced expansion of naive T cells in the presence of calcitriol-primed naive B cells

Activated B cells can serve as potent APCs and are able to generate robust proliferative CD4⁺ T cell responses [21]. We first addressed whether priming of B cells with calcitriol alters T cell activation and expansion. Purified naive and memory B cells were activated by anti-CD40/IL-4 and primed with or without 1 μM calcitriol, as identified by dose–response analysis (Supporting information, Fig. S1a). Thereafter primed B cells were co-cultured with naive or memory T cells in the presence of TSST-1, which binds to major histocompatibility complex (MHC)-II of B cells and Vβ2⁺ T cell receptor (TCR)-bearing T cells. A

concentration of 50 pg/ml of TSST-1 was added to the co-culture to ensure sufficient T cell activation and proliferation (Supporting information, Fig. S1b). After 7 days of co-culture we observed reduced percentages of expanding naive T cells upon co-culture with calcitriol-primed naive B cells (Fig. 1a,b). Accordingly, the expansion of naive T cells activated with anti-CD3 mAb in the presence of calcitriol-primed naive B cells was also reduced (Fig. 1c). However, a direct comparison revealed that the reduction of T cell expansion was more pronounced upon stimulation with TSST-1 (mean value of five experiments, 48% ± 18, *P* < 0.05) than with anti-CD3 mAb (mean value of five experiments, 29% ± 8, *P* = 0.07) (Fig. 1d). The T cell survival was comparable in the presence of activated and activated/calcitriol-primed B cells (data not shown). Upon co-culture with memory B cells, CFSE-labelled naive

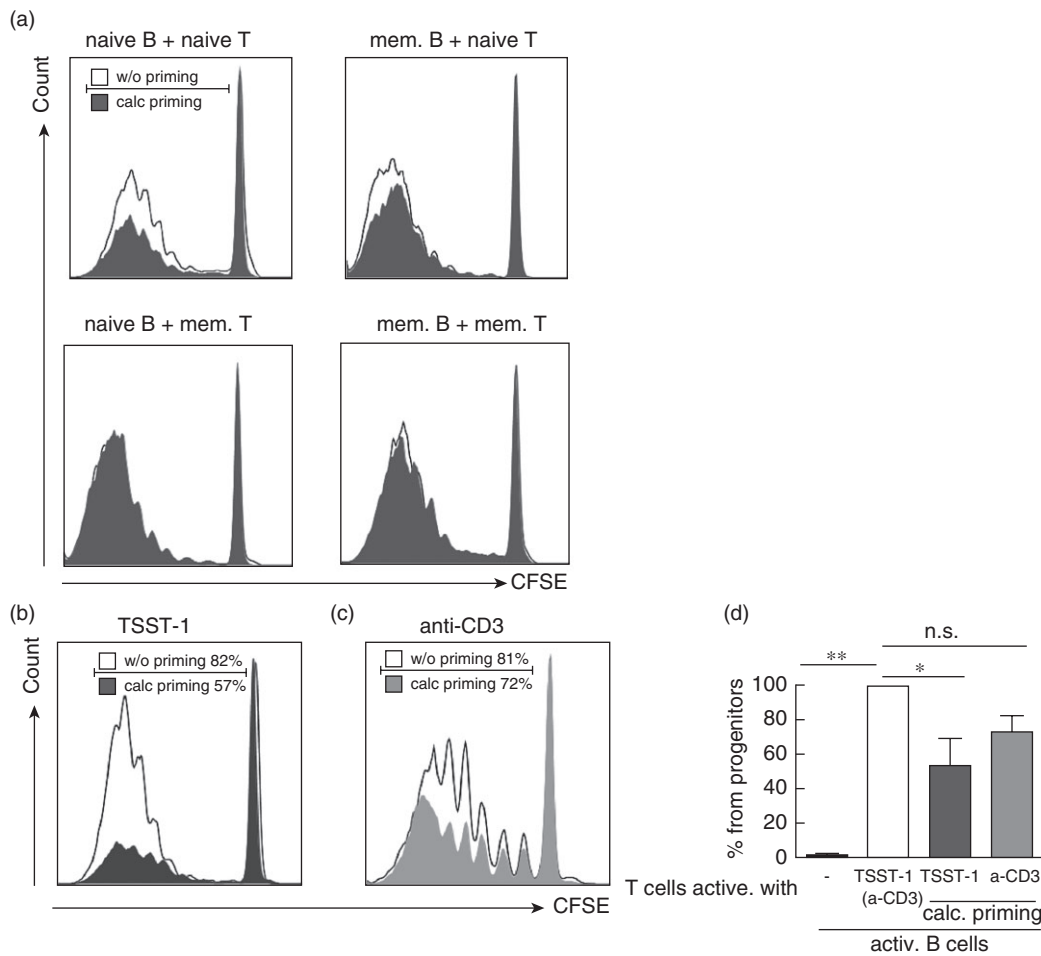


Fig. 1. Reduced proliferation of naive but not memory CD4⁺ T cells in the presence of calcitriol-primed naive B cells. Carboxyfluorescein diacetate N-succinimidylester (CFSE)-labelled and proliferated T cells after 7 days co-culture with anti-CD40 (1 μg/ml) and interleukin (IL)-4 (10 ng/ml) preactivated (solid line, open bar) or preactivated and calcitriol-primed B cells (filled area, open bars). (a) Toxic shock syndrome toxin 1 (TSST-1)-induced proliferation of naive and memory T cells in the presence of naive or memory B cells. (b) T cells activated with TSST-1. (c) T cells activated with anti-CD3. Dot-blots are gated on living T lymphocytes of a representative donor. (d) Graph bars summarized results of five independent experiments and represent difference in % of progenitor T cells in the presence of activated B cells, set as 100% (open bar). Data are shown as mean ± standard deviation. **P* ≤ 0.05; ***P* ≤ 0.01 considered significant.

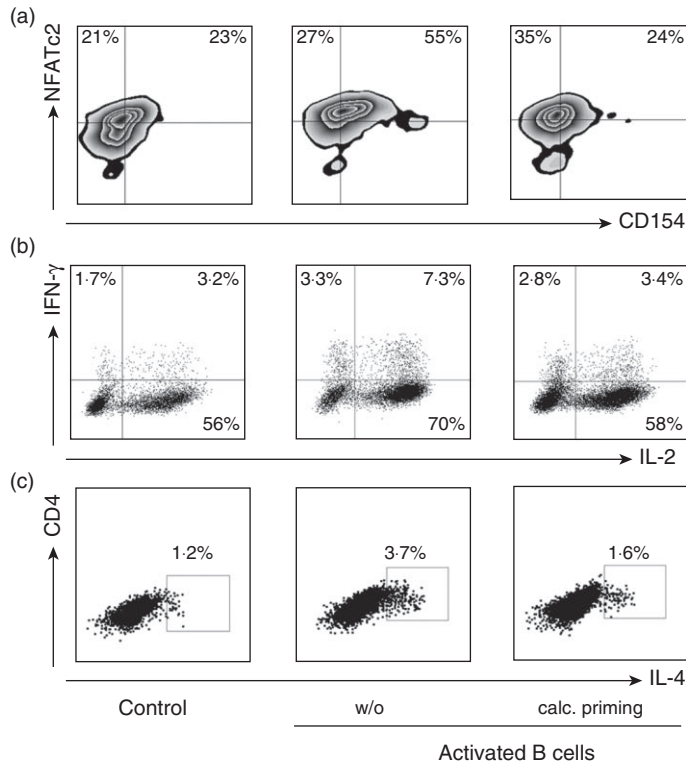
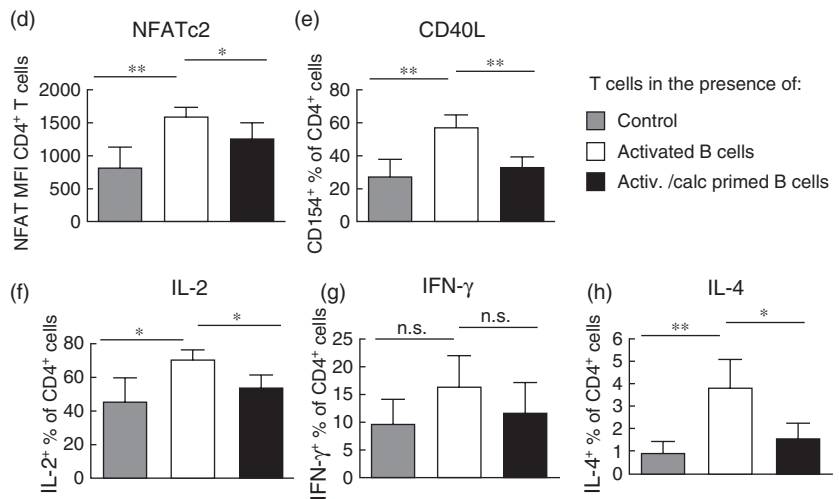


Fig. 2. Reduced expression of nuclear factor of activated T cells, cytoplasmic 2 (NFATc2) protein, CD40L and frequencies of interleukin (IL)-2, interferon (IFN)- γ , IL-4-producing T cells upon co-culture with calcitriol-primed B cells and restimulation. Naive T cells co-cultured with anti-CD40 (1 μ g/ml), IL-4 (10 ng/ml) activated naive B cells (middle column, open bars) or activated and calcitriol-primed B cells (right column, black bars). T cells alone served as control (left column, grey bars). On day 7 T cells were restimulated for 5 h with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (1 μ g/ml); for the last 4-5 h brefeldin A was added. Expression of NFATc2 protein and CD40L (a), IL-2 and IFN- γ (b) and IL-4 (c) was determined by flow cytometry. Plots are gated on newly generated CD45RA^{neg} cells. (d,f,g,h) Graph bars summarize the results of four independent experiments. Data are shown as mean \pm standard deviation. * P \leq 0.05; ** P \leq 0.01 considered significant.



and/or memory T cells show no significant reduction in expansion ($17\% \pm 3$, $P = 0.2$ and $4\% \pm 14$, $P = 0.9$). The following experiments were focused upon naive B and naive T cells.

Impact of calcitriol-primed B cells on T cell cytokine expression

Upon antigen-driven TCR activation, naive T cells differentiate into memory cells with characteristic patterns of cytokine expression. After 7 days of co-culture T cells were restimulated with PMA/ionomycin. NFATc2, CD40L and cytokine expression were measured by multi-colour flow

cytometry in newly generated CD45RO⁺ memory T lymphocytes (Fig. 2a-c). Our data show a significantly decreased NFATc2 protein expression in T cells co-cultured with calcitriol-primed B cells in comparison to the controls (mean fluorescence intensity from 1598 to 1259, $P < 0.05$; Fig. 2d). Similar observations were obtained when analysing the frequencies of T cells expressing CD40L (from 57 to 33%, $P < 0.01$; Fig. 2e), IL-4 (from mean 3.8 to 1.5%, $P < 0.01$; Fig. 2h), IL-2 (from mean 70 to 55%, $P < 0.05$; Fig. 2f) and less pronounced IFN- γ (from 16.1 to 11.7%, $P = 0.22$; Fig. 2g) upon restimulation and previous culture with calcitriol-primed B cells. However, no significant changes in expression of investigated markers could be

observed upon co-culture of naive T cells with calcitriol-primed memory B cells (data not shown).

To exclude any spillover effects, we tested the B cell supernatants for a possible remaining amount of calcitriol using the HL-60 cell line, which expressed CD38 upon contact with calcitriol. We found no signs of a biological relevant calcitriol carryover (<1 nM; Supporting information, Fig. S2). Moreover, upon addition of the same calcitriol amount (10 pM–1 nM) to the T–B cell co-culture, no alteration in proliferation and frequencies of cytokine-producing T cells was observed (data not shown).

In conclusion, T cells co-cultured with calcitriol-primed B cells display reduced activation, resulting in a diminished T cell effector development.

Calcitriol-priming promotes a reduced co-stimulatory molecule expression profile in B cells

We have shown previously that activated B cells express VDR and that VDR ligation results in diminished isotype switching and increased IL-10 production [14,15,18]. We now wished to address whether or not the APC function of B cells is also altered by calcitriol, as suggested recently from data with DCs [16,22,23]. Therefore, we first determined the expression profile of the co-stimulatory molecule CD86 (B7-2) on the B cell surface but also at the mRNA level upon CD40 + IL-4 activation and calcitriol priming. Activated naive B cells showed a higher CD86 cell surface expression ($8.7 \pm 2.3\%$ to $35.2 \pm 12.4\%$, $P < 0.01$, Fig. 3a,b) and CD86 mRNA expression ($0.29 \pm 0.12\%$ to $0.58 \pm 0.26\%$, $P < 0.05$, Fig. 3c). In summary, calcitriol-priming of B cells results in a significant decrease of surface CD86 (58%, $P < 0.05$, Fig. 3a) and moderate mRNA (57%, $P = 0.059$, Fig. 3c) expression compared to activated B cells (set as 100%).

Restoring of T cell activation by anti-CD28

To examine whether the reduction of the B cell co-stimulatory profile by calcitriol is biologically functional, naive T cells were co-cultured with calcitriol-primed B cells in the presence of activating anti-CD28 antibodies. As shown in Fig. 4a,b, agonistic anti-CD28 antibodies indeed enhanced activation and restored IL-2 (Fig. 4c) and IFN- γ (Fig. 4d) cytokine production in T cells when co-cultured with calcitriol-primed B cells and in comparison to the T cell–B cell co-culture without anti-CD28.

Discussion

In this study we show impaired naive but not memory T cell activation, cytokine production and transcription factor expression in the presence of calcitriol-primed naive B cells. Moreover, calcitriol priming resulted in a diminished co-stimulatory surface molecule expression of anti-CD40-

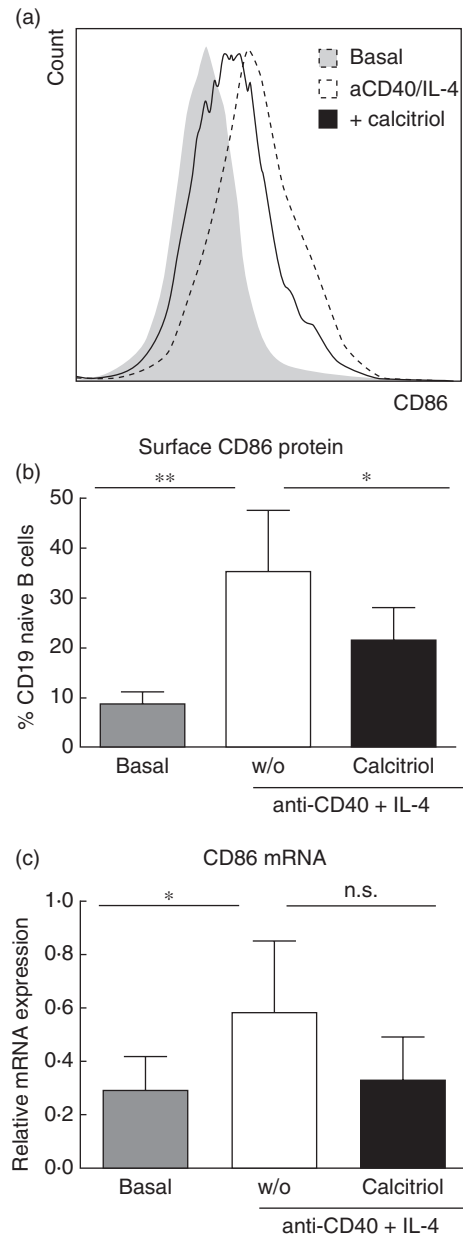


Fig. 3. Vitamin D receptor (VDR) activation diminishes B cell co-stimulatory molecule expression. Naive B cells stimulated with anti-CD40 (1 $\mu\text{g}/\text{ml}$), interferon (IL)-4 (10 ng/ml) (dashed line), without calcitriol (solid line). Reduced expression of surface co-stimulatory CD86 molecules (a), (b) and CD86 mRNA (c) determined 48 h after incubation. (a) Overlays show living B lymphocytes of a representative donor and (b,c) summarize results from four independent experiments. Data are shown as mean \pm standard deviation. * $P \leq 0.05$; ** $P \leq 0.01$ considered significant.

and IL-4-activated B cells. Finally, we could prove that the reduction of T cell activation was dependent upon a calcitriol-dependent down-regulation of B cell co-stimulation signalling, as the addition of anti-CD28

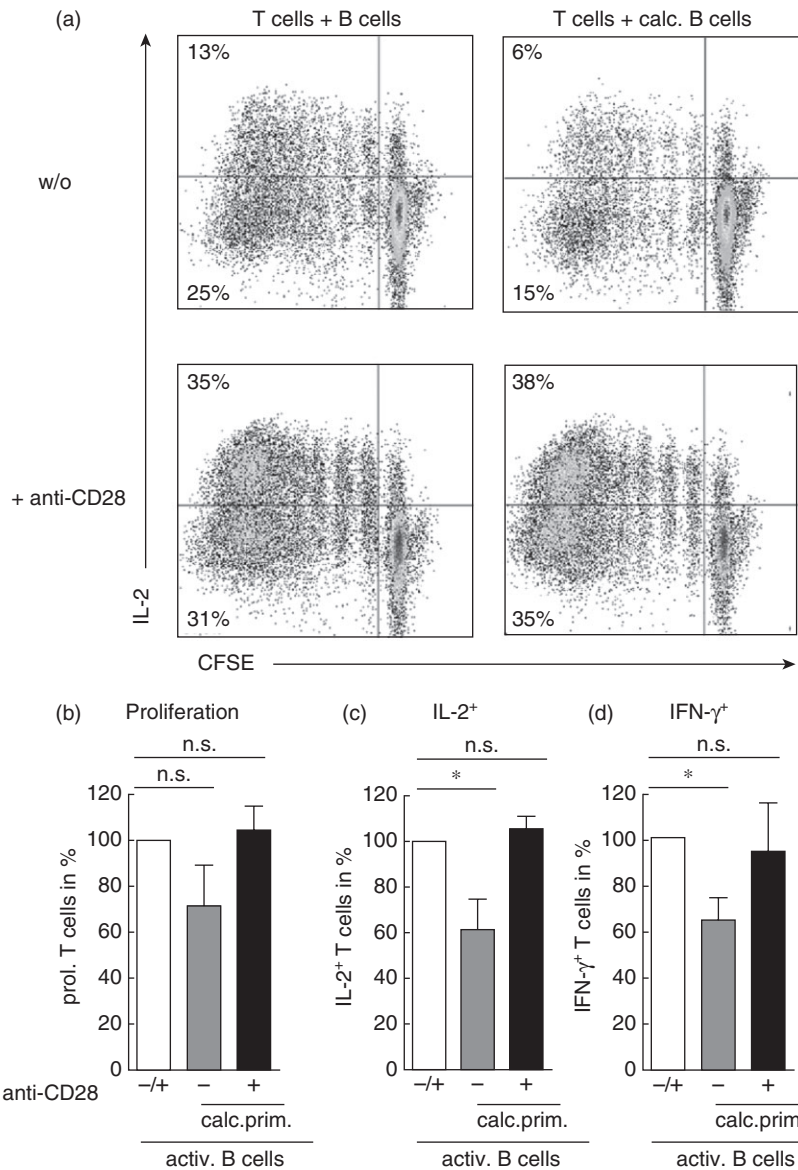


Fig. 4. Cytokine production in T cells is restored upon addition of anti-CD28 antibodies. Carboxyfluorescein diacetate N-succinimidylester (CFSE)-labelled T cells were co-cultured with naive, anti-CD40 [(1 µg/ml), interleukin (IL)-4 (10 ng/ml)] preactivated B cells on anti-CD3 coated plates. (a) Addition of anti-CD28 (1 µg/ml) to co-culture with calcitriol-primed naive B cells enhances proliferation and restores the production of IL-2 in restimulated T cells. (b) T cell proliferation in co-culture with calcitriol-treated B cells with or without addition of anti-CD28 antibodies. T cell proliferation in the co-culture with preactivated B cells ± anti-CD28 antibodies set as 100% (open bar). (c) Similar to (b) but percentages of IL-2⁺ T cells. (d) Similar to (b) but percentages of interferon (IFN)-γ⁺ T cells. Dot-blots were gated on T lymphocytes of a representative donor and (b–d) summarize results from four independent experiments. Data are shown as mean ± standard deviation. **P* ≤ 0.05 considered significant.

restored both the T cell cytokine production and also expansion in the presence of calcitriol-primed B cells.

The induction of T cell activation and cytokine production requires simultaneous signalling of the TCR and activating co-stimulatory molecules such as CD28 [24]. In contrast, TCR signalling alone induces anergy [1]. In this study, we addressed the consequences of B cell priming with calcitriol, first regarding the expression of the co-stimulatory molecules and their impact upon T cell activation. Recently, it has been shown that VDR-mediated signals in DCs can control naive T cell activation [16]. This was associated with a reduced T cell proliferation and decreased proinflammatory cytokine expression, including IL-2 and IFN-γ, but an induction of IL-10 [12,25]. Results obtained in *in-vivo* models for transplantation and autoimmunity have shown that VDR ligands can act as potent inducers of regulatory APCs [26,27]. Additionally, differen-

tiation, maturation and co-stimulatory capacity of human DCs were reduced in the presence of calcitriol or its low-calcaemic analogue in a dose-dependent manner [28]. In the present study we used anti-CD40 + IL-4-activated B cells, which can present antigens with the simultaneous expression of co-stimulatory molecules to T cells with comparable efficiency to mature DCs [5]. We demonstrated that human B cells express VDR and respond to calcitriol upon anti-CD40 + IL-4 stimulation [14,15,18,19,29]. In line with this, VDR expression is higher in naive than in memory B cells [19]. In this study, the expression of the co-stimulatory molecule CD86 was reduced by calcitriol in the naive B cell subpopulation and to a lesser extent in memory B cells. Indeed, calcitriol-mediated inhibition of T cell expansion via primed B cells was preferentially restricted to the naive, but not the memory, subset. These data are in line with findings from human monocyte-derived DCs which also

down-regulate CD86 upon calcitriol [16]. As the T cell co-stimulatory molecule CD86 is a nuclear factor kappa B (NF- κ B)-responsive target [30–32], and as VDR inhibits NF- κ B activation in B cells and other cell types, e.g. via inhibition of nuclear translocation of p65 and p50 nuclear expression or a direct I κ B α stabilization [14,19,33], this is most probably the underlying mechanism by which calcitriol-primed B cells modulate the T cell response. CD86 can activate CD28 and thereby provide critical co-stimulatory signals during antigen-specific T cell activation and cytokine responses [34]. In a murine model of rheumatoid arthritis, autoreactive T cell activation and the induction of arthritis were inhibited upon specific depletion of CD86 in B cells [35]. Blocking of the B7/CD28 ligation pathway results in changes of the immunological synapse and in reduced T cell activation proliferation and cytokine production consecutively [36,37]. Additionally, T cells co-stimulated by an activating antibody against CD28 express enhanced cytokine expression [37]. This pathway is currently targeted in the therapy of rheumatoid arthritis by using a dimeric recombinant cytotoxic T lymphocyte antigen 4 (CTLA4)/IgG Fc fusion protein, abatacept, which blocks B7/CD28 interaction and DC-dependent T cell activation [38]. In consequence, the circulating frequencies of Th1, Th2 and Th17 cells in the blood are reduced [39]. These findings support our observation that the addition of anti-CD28 to the B–T cell co-culture could mimic antigen-presenting help and was sufficient to restore T cell activation and cytokine production.

T cells co-cultured in the presence of calcitriol-primed B cells showed reduced proinflammatory cytokine production and expression of activation marker CD154 (CD40L) upon restimulation.

T cell activation, as a prerequisite for proliferation, differentiation into effector cells and cytokine expression, is dependent upon several transcription factors, including calcineurin-dependent NFAT [40]. The engagement of co-stimulatory molecule signalling increases NFAT activation mainly via enhanced nuclear import of NFAT [41] and shortening the mean time for the first T cell division [42]. We observed lower amounts of NFATc2 protein in T cells co-cultured with calcitriol-primed B cells. Additionally, the level of NFATc2 mirrored the CD40L expression and cytokine production upon restimulation. Thus, T cell activation was most probably reduced following contact with the calcitriol-primed B cells.

As published previously, VDR activation induces IL-10 production in B cells [15]. However, in this setting IL-10 was not induced in B cells, due to the type of stimulation and time-frame [43]. Accordingly, the concentrations of secreted IL-10 were at the detection limits and neutralizing IL-10 antibodies did not affect the co-culture (data not shown).

VDR activation in T cells has a direct impact upon proliferation [44] and transcriptional modulation of cytokine-

producing T cells, as also observed in this study [45]. However, in our setting calcitriol was not added to the T–B cell co-culture. We tested the co-cultures for possible calcitriol spillover effects to exclude any direct effects of calcitriol on T cells. Indeed, we did not find any biologically relevant remaining calcitriol concentrations (<1 nM). In addition, small amounts of calcitriol were titrated into the co-cultures without any measurable effects using up to 1 nM. Thus, we conclude that the direct modulation of T cell activation is most probably dependent upon VDR activation in B cells.

Taken together, in this study we demonstrate that T cell activation, cytokine and transcription factor expression were impaired in the presence of calcitriol-primed naive B cells. We describe a novel mechanism by which the bioactive form of vitamin D controls T cell activation, namely via reduced co-stimulation primed B cells.

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Disclosure

The authors declare that they have no conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. T cell proliferation following 7-day co-culture with activated B cells. Carboxyfluorescein diacetate N-succinimidylester (CFSE) dilution was determined in CD4⁺ T cells after 7-day co-culture activated [anti-CD40, 1 µg/ml + interleukin (IL)-4, 10 ng/ml, for 48 h] B cells. (a) T cell proliferation detected upon 7-day co-culture with activated and calcitriol primed, as indicated in the figure, B cells. (b) T cell proliferation upon 7-day co-culture with activated B cells in the presence of increasing concentrations of toxic shock syndrome toxin 1 (TSST-1). Data represent four to five independent experiments and analysed using the analy-

sis of variance (ANOVA) test. Data are shown as mean ± standard deviation. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ are considered significant.

Fig. S2. No relevant calcitriol spillover from B cell culture into T–B cell co-culture. The human promyelocytic leukaemia HL-60 cell line was incubated with calcitriol 1 µmol/l–100 pmol/l (upper plots) or supernatants from anti-CD40 (1 µg/ml), interleukin (IL)-4 (10 ng/ml) preactivated and calcitriol (as indicated in the Figure)-treated B cells; 48 h preactivation following washing and additional incubation with RPMI-1640 medium for 1 and 3 days. Supernatants were tested for calcitriol spillover with calcitriol-dependent expression of CD38 by the HL-60 cell line.

Table S1. Primer sequences for quantitative polymerase chain reaction (qPCR).