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ORIGINAL ARTICLE

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Physiological levels of 25-hydroxyvitamin D_3 induce a suppressive $CD4^+$ T cell phenotype not reflected in the epigenetic landscape

Carina Matos[1] | **Kathrin Renner**[1] | **Alice Peuker**[1] | **Gabriele Schoenhammer**[1] | **Laura Schreiber**[1] | **Christina Bruss**[1] | **Ruediger Eder**[1] | **Heiko Bruns**[3] | **Cindy Flamann**[3] | **Petra Hoffmann**[1,2] | **Claudia Gebhard**[2] | **Wolfgang Herr**[1] | **Michael Rehli**[1,2] | **Katrin Peter**[1] | **Marina Kreutz**[1,2]

[1]Department of Internal Medicine III, Hematology and Medical Oncology, University Medical Center of Regensburg, Regensburg, Germany

[2]Leibniz Institute for Immunotherapy, University Hospital Regensburg, Regensburg, Germany

[3]Department of Internal Medicine 5 – Hematology/Oncology, University Hospital of Erlangen, Erlangen, Germany

Correspondence
Marina Kreutz, Department of Internal Medicine III, Hematology and Medical Oncology, University Medical Center of Regensburg, Regensburg, Germany.
Email: marina.kreutz@ukr.de

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Abstract

1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D3 has a strong impact on the differentiation and function of immune cells. Here we analysed the influence of its precursor 25-hydroxyvitamin D3 ($25(OH)D_3$) on the differentiation of human $CD4^+$ T cells applying physiological concentrations in vitro. Our data show that $25(OH)D_3$ is converted to its active form $1,25(OH)_2D_3$ by T cells, which in turn supports FOXP3, CD25 and CTLA-4 expression and inhibits IFN-γ production. These changes were not reflected in the demethylation of the respective promoters. Furthermore, we investigated the impact of vitamin D3 metabolites under induced Treg (iTreg) polarization conditions using TGF-β. Surprisingly, no additive effect but a decreased percentage of FOXP3 expressing cells was observed. However, the combination of $25(OH)D_3$ or $1,25(OH)_2D_3$ together with TGF-β further upregulated CD25 and CTLA-4 and significantly increased soluble CTLA-4 and IL-10 secretion whereas IFN-γ expression of iTreg was decreased. Our data suggest that physiological levels of $25(OH)D_3$ act as potent modulator of human $CD4^+$ T cells and autocrine or paracrine production of $1,25(OH)_2D_3$ by T cells might be crucial for the local regulation of an adaptive immune response. However, since no epigenetic changes are detected by $25(OH)D_3$ a rather transient phenotype is induced.

1 | INTRODUCTION

Beside its well-known role in the regulation of bone and calcium homeostasis there is increasing evidence that 1alpha,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) plays a crucial role in the regulation of immune responses and the prevention of autoimmunity. The vitamin D3 precursor is present in high amounts in the skin, where it is photochemically converted from 7-dehydrocholesterol, or in the gut, where it is directly absorbed from the diet. Vitamin D3 is converted into its active metabolite by two consecutive hydroxylation steps. The first one occurs in

the liver, which is thought to be the major site of conversion of vitamin D3 to 25-hydroxyvitamin D3 ($25(OH)D_3$) by cytochrome P450 mixed-function oxidases (CYPs) such as CYP27A1 (localized in the mitochondria) or CYP2R1 (localized in the endoplasmic reticulum). The next step is the conversion of $25(OH)D_3$ to the active metabolite $1,25(OH)_2D_3$ by CYP27B1 in the kidney [1,2]. Normally $25(OH)D_3$ is determined to access a person's vitamin D status. The use of the precursor rather than the active metabolite is due to its higher half-life and increased concentration in serum. While $25(OH)D_3$ levels can be found in the range of 25-250 nM, concentrations of $1,25(OH)_2D_3$ are approximately 1000-fold lower in the pM range [3,4]. Nevertheless, a consensus in the adequate $25(OH)D_3$ level has not been reached worldwide but it is widely accepted that levels below 30 nM constitute vitamin D deficiency [5,6].

Several reports describe the extrarenal production of $1,25(OH)_2D_3$ from $25(OH)D_3$ and suggest a local paracrine immune regulatory function of $1,25(OH)_2D_3$ [7-9]. We and others have shown that CYP27B1 is expressed in monocyte-derived dendritic cells (DCs) and its expression is increased after stimulation with lipopolysaccharide (LPS). Accordingly, DCs show low constitutive production of $1,25(OH)_2D_3$, but activation with LPS increased $1,25(OH)_2D_3$ synthesis and inhibits their allo-stimulatory potential [10,11]. DCs are present in the skin as well as in the gut, the only organs where high levels of the $1,25(OH)_2D_3$ precursor vitamin D3 are found. In both organs the direct local conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ by DCs might be of importance for immune homeostasis at these immunological "barriers" and $25(OH)D_3$ may contribute to the development of tolerance, eg via the induction of regulatory T cells (Tregs).

Even though DCs and macrophages are thought to be the major immune cells capable of converting $25(OH)D_3$ to its active form, there is some evidence that activated human T cells also express the hydroxylase CYP27B1 on mRNA level [12,13]. Nevertheless, whether activated T cells have the ability to produce sufficient amounts of $1,25(OH)_2D_3$ and are capable to induce a vitamin D3-related response is controversially discussed. Kongsbak et al demonstrated that human $CD4^+$ T cells are able to convert $25(OH)D_3$ into $1,25(OH)_2D_3$ but only under serum-free conditions [3] whereas Jeffery et al stated that $25(OH)D_3$ cannot be metabolized by pure T cell cultures and DCs need to be added to produce the active metabolite $1,25(OH)_2D_3$ [14]. In contrast, Fawaz et al described that $25(OH)D_3$ is capable of decreasing IL-17 secretion by T cells independent of the presence of DCs [13].

In humans it has been shown that variations in $25(OH)D_3$ serum levels are paralleled by changes in the percentage of Tregs and the expression of FOXP3 [15,16]. Accordingly, high dose vitamin D3 supplementation (140 000 IU monthly) resulted in a significantly increased frequency of Tregs in a randomized controlled trial [17]. In a pilot study, we irradiated patients during the course of stem cell transplantation with low dose UVB to improve graft-versus-host disease (GvHD) outcome and observed an increase in $25(OH)D_3$ and in the number of circulating $CD4^+FOXP3^+$ Tregs [18]. Similar results were obtained by Milliken et al in patients with inflammatory and immune-mediated dermatoses [19].

Several studies investigated the impact of $1,25(OH)_2D_3$ on Treg differentiation in vitro. The majority of these studies were performed with an 'indirect system'. Here, $1,25(OH)_2D_3$ induces a tolerogenic dendritic cell phenotype that in turn leads to the upregulation of FOXP3 expression in T cells [20-22]. Tregs are specialized T cells that suppress immune responses, thereby maintaining homeostasis and self-tolerance. Sakaguchi's group and others [23-25] identified the transcription factor FOXP3 as a specific marker for regulatory T cells, and its stable expression is necessary for their suppressive capacity. Epigenetic mechanisms, such as DNA methylation/demethylation, play a crucial role for stable maintenance of Treg-specific gene expression [26]. Natural Tregs are characterized by hypomethylated regions, eg the Treg-specific demethylated region (TSDR) [27], whereas induced Tregs (iTregs) present an intermediate level of demethylation resulting in reduced FOXP3 stability [28]. Natural Treg are limited in number and therefore protocols were developed to generate iTregs from conventional T cells in vitro. The best characterized protocol is the culture of $CD4^+CD25^-$ in the presence of IL-2 and TGF-β which leads to the expression of FOXP3 [29,30]. However, there is controversy about the suppressive function of these cells [31] as iTregs generated by the addition of TGF-β still produce significant amounts of effector cytokines, a feature not observed in natural Tregs [32]. Beside indirect effects mediated by DCs, T cells can also be direct targets of $1,25(OH)_2D_3$ and high concentrations of $1,25(OH)_2D_3$ can lead to upregulation of FOXP3, CTLA-4 and regulate cytokine secretion in vitro independent of TGF-β [33-35].

Based on the known effects of $1,25(OH)_2D_3$ on FOXP3 and cytokine expression, we sought to combine the effect of vitamin D3 metabolites and TGF-β in this study. As the majority of studies make use of high, non-physiological $1,25(OH)_2D_3$ levels, we aimed to investigate physiological concentrations of $25(OH)D_3$. Our results demonstrate that these amounts are sufficient to induce a vitamin D3-related response, such as enhanced IL-10 and reduced IFN-γ secretion in human T cells independent of FOXP3 expression.

2 | MATERIALS AND METHODS

2.1 | Isolation of monocytes and T lymphocytes

With approval from the local ethics committee, monocytes and T lymphocytes were isolated from healthy donors as described previously [36]. All human participants gave written informed consent.

2.2 | Culture of monocyte-derived DC

For DC differentiation, 0.5 to 1.0 × 10^6 monocytes/mL were cultured for 5 days in RPMI medium supplemented with 10% foetal calf serum (PAN Biotech), IL-4 (144 U/mL), and granulocyte macrophage colony-stimulating factor (GM-CSF, 225 U/mL; both from PeproTech, Hamburg, Germany). iDCs were stimulated with 100 ng/mL LPS (from Salmonella abortus equi S-form, Enzo Life Sciences, Lörrach, Germany) for 48 h.

2.3 | Mixed-lymphocyte reactions

For the mixed-lymphocyte reaction (MLR), monocyte-derived DCs were harvested on day 7. DCs were washed and co-cultured with allogeneic human T lymphocytes in RPMI containing 5% AB serum, L-glutamine (2 mmol/L), penicillin (50 U/mL) and streptomycin (50 mg/mL). On day 5 of co-culture, 0.5 μCi/0.2 mL [3H]-thymidine (Hartmann Analytic, Braunschweig, Germany) was added and incorporated radioactivity was quantified after 24 h by means of a beta counter (Perkin Elmer, Gaithersburg, Waltham, MA, USA). All samples were analysed in triplicate.

2.4 | $CD4^+25^-$ T cell cultures

Peripheral blood mononuclear cells (PBMCs) were separated by leukapheresis of healthy volunteer donors, followed by density gradient centrifugation over Ficoll/Hypaque. Afterwards, $CD4^+$ cells were enriched using a $CD4^+$ T cell isolation kit from Miltenyi Biotec. Purified $CD4^+CD25^-$ T cells were obtained by FACS (fluorescence-activated cell sorting) using a BD FACSAria™ II high-speed cell sorter (Becton Dickinson, Heidelberg, Germany). Sorted cells were co-cultured with Dynabeads Human T-Activator CD3/CD28 (TCE) (1 bead/cell), in RPMI medium containing 2% AB serum, 100 U/ml IL-2, L-glutamine (2 mmol/L), penicillin (50 U/mL) and streptomycin (50 mg/mL) for 4 days in the presence or absence of TGF-β (10 ng/ml), $25(OH)D_3$ (50 nM), $1,25(OH)_2D_3$ (5nM) or combination. For CYP27B1 inhibition, T cells were pre-treated with either 1 or 10 μM ketoconazole.

2.5 | DNA methylation analysis

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA was bisulphite converted using the EZ DNA Methylation Kit (Zymo Research) with an alternative conversion protocol (Agena). The bisulphite converted DNA was amplified by PCR using region-specific primers described in [37]. PCR was followed by treatment with shrimp alkaline phosphatase, in vitro transcription, RNA-specific cleavage (MassCLEAVE, Sequenom) and MALDI-TOF MS (MassARRAY Compact MALDI-TOF, Sequenom) as previously described [37].

2.6 | Determination of cytokines and $1,25(OH)_2D_3$ in the culture supernatants

Determination of cytokines in culture supernatants was performed using ELISA kits from R&D Systems (Minneapolis, MN, USA). For $1,25(OH)_2D_3$ determination, supernatants were analysed by the MVZ Laborzentrum, Ettlingen, Germany.

2.7 | Preparation of RNA, reverse transcription and quantitative real-time PCR

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). Reverse transcription was performed with 500 ng RNA in a total volume of 20 μl using an M-MLV Reverse Transcriptase from Promega (Mannheim, Germany). For reverse transcription-quantitative real-time PCR, 1 μl cDNA, 0.5 μl of primers (10 μM) and 5 μl QuantiFast SYBR Green PCR Kit (Qiagen) in a total of 10 μl were applied. Primer sequences (all from Eurofins MWG Operon, Ebersberg, Germany) were as follows (−5′-3′); (F- Forward; R- Reverse): FOXP3_F: GAAACAGCACATTCCCAGAGTTC; FOXP3_R: ATGGCCCAGCGGATGAG; VDR_F: GTTGCGCTCCAATGAGTCCTTCAC; VDR_R: CAGTCCCACCTGGAACTTGATGAG; CYP27B1_F: TGGCAGAGCTTGAATTGCAAATGG; CYP27B1_R: ACTGTAGGTTGATGCTCCTTTCAGGT; IFN_F: CTAATTATTCGGTAACTGACTTGA; IFN_R: ACAGTTCAGCCATCACTTGGA; CTLA-4_F:

GACACGGGACTCTACATCTGCAAGG; CTLA-4_R: GAGGAAGTCAGAATCTGGGCACGG; IL-10_F: GCAA CCTGCCTAACATGCTTCGAG; IL-10_R: CTGGGTCTT GGTTCTCAGCTTGGG; 18S_F: ACCGATTGGATGGT TTAGTGAG; 18S_R: CCTACGGAAACCTTGTTACGAC.

2.8 | Preparation of whole cell lysates and western blotting

Whole cell lysates were prepared using RIPA buffer (Sigma-Aldrich) and quantified with the Qubit Protein Assay Kit (Thermo Fisher Scientific). After blotting, membranes were stained with anti-VDR (Cell Signaling Technology, Danvers, MA, USA), anti- CYP27B1 (Abcam, Cambridge, UK) or anti-actin (Sigma Aldrich) antibodies and analysed using the chemiluminescence system Fusion Pulse 6 (Vilber Lourmat).

2.9 | Flow cytometry analysis

Cells were washed twice with cold PBS (Gibco, Karlsruhe, Germany) containing 0.1% sodium azide and 0.6 mg/mL human immunoglobulin, and were stained with the following monoclonal antibodies: FOXP3 (eBioscience); CD25 (BD); CD28 (Biolegend); CTLA-4 (BD); ICOS (Biolegend); PD-1 (BD); OX40 (Biolegend); CCR4 (BD). For intracellular staining, the eBioscience FOXP3/Transcription Factor Fixation/Permeabilization Kit was used (Thermo Fisher). FACS data were acquired on a FACS LSR II™ or FACSCelesta™ cytometer and analysed using FACS Diva (all BD, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, OR, USA).

2.10 | Statistical analysis

Statistics were calculated using Graphpad Prism, Version 8 (La Jolla, CA, USA). Comparison between groups was performed using the appropriate statistical methods depending on Gaussian distributions and number of groups and variables. A value of $P < .05$ was considered statistically significant.

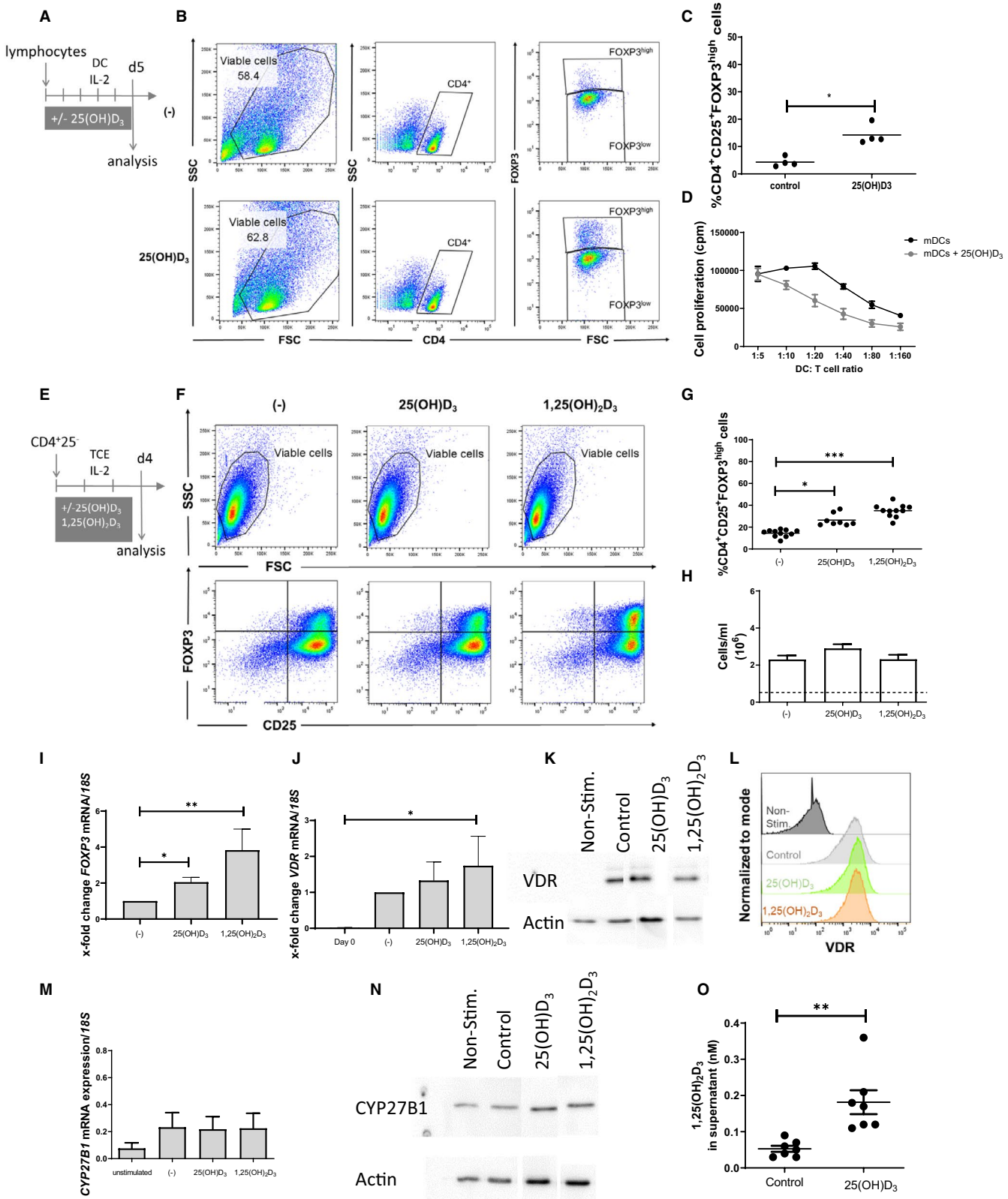
3 | RESULTS

3.1 | Physiological amounts of 25(OH)D_3 induce FOXP3 expression in human conventional $CD4^+$ T cells via conversion of 25(OH)D_3 into 1,25(OH)$_2D_3$

The induction of $FOXP3^+$ regulatory T cells by 1,25-dihydroxyvitamin D_3 is considered to be crucial for the local immune homeostasis. Since mature dendritic cells (mDCs) have been shown to produce 1,25(OH)$_2D_3$ from 25-hydroxyvitamin D_3 [10,11] and thereby induce $FOXP3^+$ regulatory T cells [14] we aimed to further analyse this local immune regulatory effect of 25(OH)D_3.

We first analysed T lymphocytes stimulated in a mixed lymphocyte reaction (MLR) with allogeneic mDCs for 5 days in the presence or absence of 50 nM 25(OH)D_3 (Figure 1A–D). This concentration is generally considered to represent an adequate 25(OH)D_3 serum level in humans [4,38]. In accordance with published data by Jeffery et al [14] we found a significant increase in the percentage of $CD4^+FOXP3^{high}$ cells in the presence of 25(OH)D_3 together with a decrease in cell proliferation (Figure 1C,D) when T cells were stimulated with DCs in the presence of 25(OH)D_3. Although mDCs have been described to be indispensable for the induction of $FOXP3^+$ T cells by 25(OH)D_3 [14], we also performed our experiments in the absence of DCs, stimulating the cells with Human T-Activator CD3/CD28 (TCE) beads instead (Figure 1E–H). To our surprise, the percentage of $FOXP3^{high}$ T cells in these mDC-free cultures was significantly increased after 4 days in the presence of 25(OH)D_3 which indicates either a direct induction of $FOXP3^{high}$ T cells by 25(OH)D_3 or the hydroxylation of 25(OH)D_3 to 1,25(OH)$_2D_3$ by T cells. As a control we used the active metabolite 1,25(OH)$_2D_3$ which was more potent to induce $FOXP3^{high}$ T cells than 25(OH)D_3 indicating that the conversion from 25(OH)D_3 to 1,25(OH)$_2D_3$ is necessary (Figure 1F–H). Increased FOXP3

FIGURE 1 Vitamin D induces FOXP3 expression in human T cells. Cells were stimulated with DCs for 5 days in the presence or absence of 25(OH)D3. Schematic representation of the experimental set-up (A). Representative FACS plot (B), percentage of **$FOXP3^{high}$** among $CD4^+$ cells (C), cell proliferation (D). $CD4^+25^-$ sorted T cells were stimulated with TCE beads (1:1 bead/cell ratio) for 4 days in the presence or absence of 25(OH)D3 or 1,25(OH)2D3 (E-O), representative plot (F), percentage of $FOXP3^{high}$ cells (G), cell proliferation (H). Each dot represents an individual donor. The relative *FOXP3* (I), *VDR* (J) gene expression was determined by means of quantitative real-time PCR (qPCR). Protein expression of VDR was analysed by Western blot (K) and by flow cytometry (L). The relative expression of *CYP27B1* was determined by qPCR (M) and protein expression by Western blot (N). The amount of 1,25(OH)2D3 in the supernatant was determined using a radioimmunoassay according to the manufacturer's instructions (O). Data shown as means ±SEM ($n \geq 3$). Statistical analysis was performed using Mann– Whitney (C) and (O) or Kruskal–Wallis test (G- N)



expression was also detectable on mRNA level (Figure 1I), upon treatment with both $25(OH)D_3$ and $1,25(OH)_2D_3$. These results indicate that not only $1,25(OH)_2D_3$ but also its precursor $25(OH)D_3$ seem to be potent regulators of FOXP3 in human $CD4^+$ T cells.

As the vitamin D receptor (VDR) mediates most effects of $1,25(OH)_2D_3$, we next analysed its expression in $CD4^+$ T cells. According to the literature non-stimulated T cells do not express the VDR, but upregulate its expression upon stimulation [12,39,40]. In line with these findings, we

detected no VDR expression in non-stimulated $CD4^+25^-$ T cells but VDR mRNA expression increased significantly after 4 days of TCE stimulation (Figure 1J). Western blot and flow cytometry analyses confirmed this result on protein level (Figure 1K,L). The addition of $25(OH)D_3$ or $1,25(OH)_2D_3$ did not significantly affect VDR expression (Figure 1J–L).

Conflicting reports have been published regarding T cells ability to produce $1,25(OH)_2D_3$ from its precursor. To determine the capacity of the T cells to hydroxylate $25(OH)D_3$ in the absence of [3,14,41] mDCs, we first analysed the CYP27B1 expression on mRNA level as CYP27B is the responsible enzyme catalysing the hydroxylation step from $25(OH)D_3$ to $1,25(OH)_2D_3$. Freshly isolated $CD4^+$ T cells exhibited a low expression level of CYP27B1 mRNA, which was slightly increased after stimulation. The presence of $25(OH)D_3$ or $1,25(OH)_2D_3$ did not affect this expression (Figure 1M). Since the mRNA expression of CYP27B1 does not automatically reflect protein expression and $1,25(OH)_2D_3$ production, we investigated CYP27B1 expression by western blot analyses and determined $1,25(OH)_2D_3$ in culture supernatants of T cells 4 days after TCE stimulation. CYP27B1 was expressed on protein level and correlated with a significant production and secretion of $1,25(OH)_2D_3$ in the presence of 50 nM $25(OH)D_3$ (Figure 1O). These data indicate that conventional $CD4^+$ T cells have the capability to produce $1,25(OH)_2D_3$ and provide a mechanism for the DC-independent induction of $FOXP3^{high}$ T cells in the presence of $25(OH)D_3$.

3.2 | Ketoconazole treatment dampens the 25(OH)D3-induced FOXP3 expression

Although we determined that $CD4^+$T cells have the availability to convert $25(OH)D_3$ to $1,25(OH)_2D_3$ and express CYP27B1, a direct effect of 25(OH)D3 on FOXP3 expression cannot be excluded. Therefore, we determined the effect of ketoconazole on FOXP3 upregulation. Ketoconazole is an imidazole anti-fungal agent with broad spectrum activity [42] and a known cytochrome P-450 enzymes inhibitor [43].

Therefore, we pre-incubated $CD4^+$ T cells with different concentrations of ketoconazole (1 and 10 μM) for 2 h and afterwards stimulated the cells with TCE beads in the presence or absence of 100 nM of $25(OH)D_3$. As observed in Figure 2A and B, the addition of 10 μM of ketoconazole to the cultures dampened the $25(OH)D_3$-induced FOXP3 upregulation.

Analysing the culture supernatants, we observed that the addition of $25(OH)D_3$ suppressed IFN-γ secretion (see also Figure 7) but 10 μM of ketoconazole counteracted this effect (Figure 2C). Ketoconazole abolished the $1,25(OH)_2D_3$ production at a concentration of 10 μM whereas $1,25(OH)_2D_3$ production was still observed applying 1 μM ketoconazole (Figure 2D).

3.3 | The $25(OH)D_3$-induced $CD4^+$ T cell phenotype is independent of promoter demethylation

Several groups have demonstrated an association of the differentiation and function of Treg with epigenetic modifications [26,37,44-46]. In particular, natural Treg but not TGF-β-induced Treg are characterized by the demethylation of a CpG-rich element within the FOXP3 locus, the Treg-specific demethylated region (TSDR) [26,44]. Therefore, we sought to investigate epigenetic modifications in our T cell cultures. For the epigenetic analysis we stimulated the $CD4^+CD25^-$ T cells for 4 days with either mDC or TCE in the presence or absence of $25(OH)D_3$, restimulated them another 7 days with TCE in the absence of $25(OH)D_3$, and 'rested' the cultures (Figure 3A). Next, we FACS-purified the resulting $FOXP3^{high}$ T cells, isolated their DNA and performed quantitative DNA methylation analysis on the EpiTYPER mass spectrometry platform [47]. As control, we included FACS-purified $CD4^+CD25^+$ natural Treg (nTreg) as well as $CD4^+CD25^-$ conventional T cells into our DNA methylation analysis. The examined genomic regions have been previously described to exhibit different methylation patterns in conventional T cells and nTreg [26,37]. As expected, the EpiTYPER DNA methylation analysis revealed an almost completely unmethylated state of the analysed regions in nTreg (Figure 3B). In contrast, conventional T cells exhibited a high degree of methylation. We detected no difference in the DNA methylation of the $FOXP3^{high}$ T cells generated in the presence of DC or in the presence of TCE. In addition, in most analysed regions the methylation pattern of T cells cultured in the presence of $25(OH)D_3$ resembled the methylation of conventional T cells. The presence of $25(OH)D_3$ did not affect the DNA methylation levels of the examined regions. Figure 3C–H depict the mean methylation level of selected analysed regions. As shown in Figure 3C, demethylation of the FOXP3 promoter occurred independently of the presence of $25(OH)D_3$. A region near a recently described vitamin D-response element (VDRE) in the intronic conserved noncoding region of the human FOXP3 gene [48] as well as the TSDR were demethylated in the nTreg but not in the $25(OH)D_3$-induced $FOXP3^{high}$ T cells (Figure 3D and E). Unmethylated CpG dinucleotides were also detected in the IL2RA gene as well as in the CTLA-4 gene of nTreg but not of $25(OH)D_3$-induced $FOXP3^{high}$ T cells (Figure 3F and G). In contrast, the IFN-γ gene which was previously

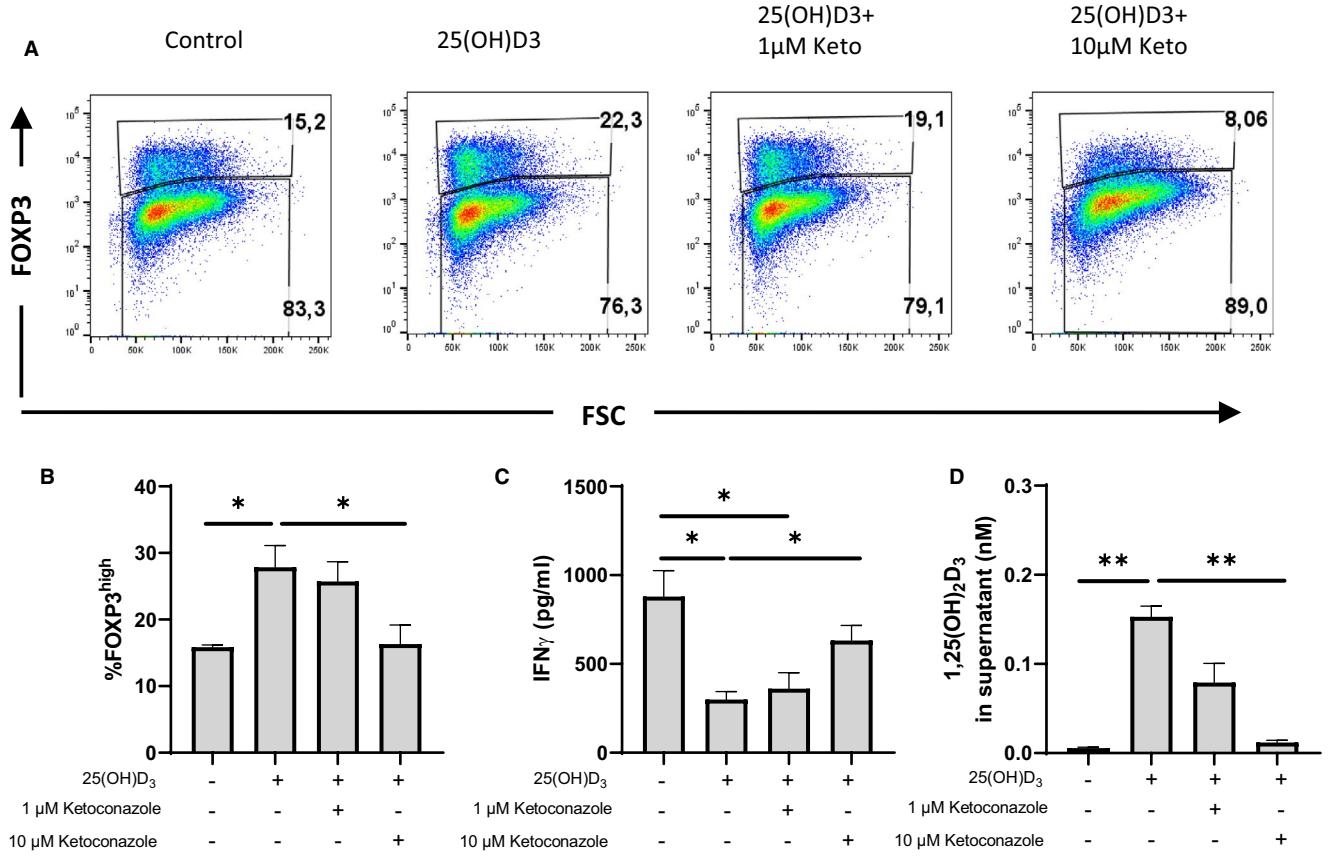


FIGURE 2 Ketoconazole treatment dampens 25(OH)D3-induced FOXP3 expression. $CD4^+$ T cells were pre-incubated for 2 hours with ketoconazole (1 μM and 10 μM) and activated with anti-CD3/28 beads in the presence of 100 nM 25(OH)D3. After 4 days, cells were harvested, counted and stained by means of flow cytometry. A representative FACS plot is presented in (A) and the percentage of $FOXP3^{high}$ among $CD4^+$ cells in (B). IFN-γ (C) and 1,25(OH)2D3 (D) were measured in the supernatants and normalized in relation with cell number. Data shown as means ±SEM ($n \geq 4$). Statistical analysis was performed using one-way ANOVA and Tukey multiple comparisons test

found to be mainly expressed in expanded conventional T cells [37] exhibited an almost 100% methylated CpG island in all studied cell types (Figure 3H). These results suggest that the gene regulation by $25(OH)D_3$ in conventional $CD4^+$ T cells is independent of epigenetic changes in the promoter of these genes and indicate that vitamin D3 metabolites more likely dampen a classical Th1 response rather than inducing a regulatory phenotype.

3.4 | Vitamin D3 increases the expression of CD25 and CTLA-4 independent of FOXP3 expression

In order to characterize our T cell populations, we stained different T cell markers such as CD25, CD28, CTLA-4, inducible T cell costimulator (ICOS), programmed cell death 1 (PD-1) and CCR4 on $FOXP3^{high}$ and in comparison on $FOXP3^{low}$ cells (gating strategy depicted in Figure 1F and Figure 4A,B). Although CD25 (IL-2R), an activation marker, is upregulated upon T cell activation, it is also known to be highly expressed on regulatory T cells. Nevertheless, we found that the vitamin D3 effect on CD25 seems to be independent of FOXP3 expression, since the upregulation of CD25 by both $25(OH)D_3$ and $1,25(OH)_2D_3$ was observed in both $FOXP3^{high}$ and $FOXP3^{low}$ cells (Figure 4C,D).

CD28 is the first member of a subfamily of costimulatory molecules, constitutively expressed by virtually all T cells and plays an important role in the activation of both conventional and regulatory T cells [49]. CD28 staining revealed an upregulation of this marker upon $1,25(OH)_2D_3$ treatment independent of FOXP3 expression (Figure 4E,F). Other members of the CD28 family include ICOS, CTLA-4 and PD-1. Both vitamin D3 metabolites significantly increased the expression of CTLA-4 on both $FOXP3^{high}$ T cells and $FOXP3^{low}$ T cells (Figure 4G,H). However, we did not observe a difference in PD-1 or ICOS expression upon vitamin D3 treatment in both $FOXP3^{high}$ and $FOXP3^{low}$ T cells (Figure 4K,L). CCR4 is highly expressed on most immunosuppressive Treg cells [50,51] and plays a dominant mediating Treg migration to the skin. In our hands, there was no difference on CCR4 expression upon vitamin D3 supplementation independent of FOXP3 expression (Figure 4M,N). Our

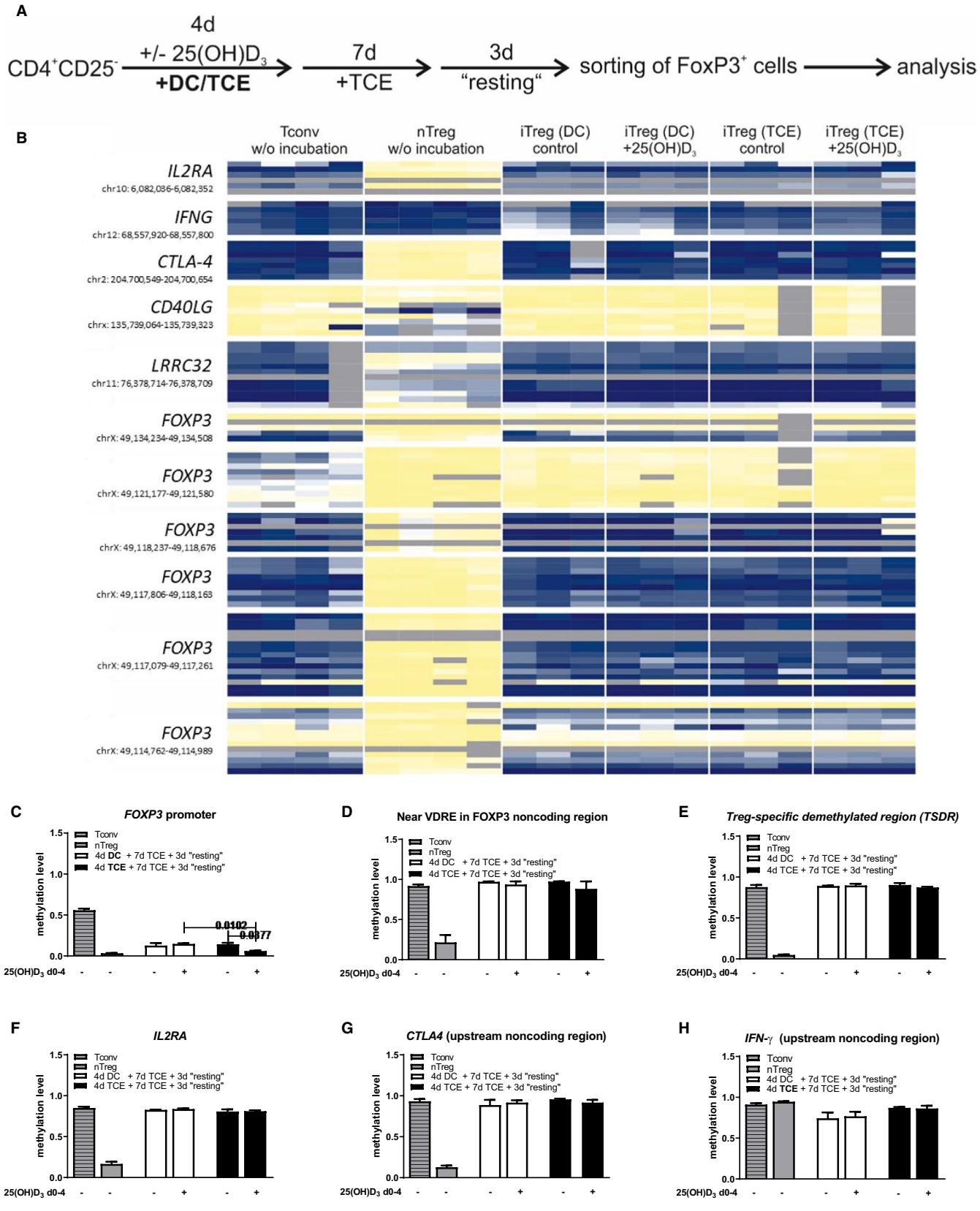


FIGURE 3 The phenotype of iTregs includes the demethylation of the FOXP3 promoter but not associated enhancers like the TSDR. $CD4^+CD25^-$ T cells were stimulated for 4 days with either mDC or TCE in the presence or absence of 25(OH)D3. Afterwards, the cells were restimulated for additional 7 days with TCE in the absence of 25(OH)D3 and 'rested' (A). Next, the DNA of FACS-purified $FoxP3^{high}$ T cells was isolated and quantitative DNA methylation analysis on the EpiTYPER mass spectrometry platform was performed. Data (representing means of $n > 3$) are presented as a heatmap with yellow and blue, representing low and high methylation, respectively (B). Rows correspond to individual CpGs and columns represent independent experiments. Data are summarized in (C-H)

data show that vitamin D3 metabolites can profoundly alter the surface expression of activation markers in conventional $CD4^+$ T cells but the regulation is not restricted to highly FOXP3 expressing cells.

3.5 | Vitamin D3 reduces the frequency of TGF-β–induced human $FOXP3^+$ Tregs

The importance of TGF-β for the generation of iTregs is well established. Based on the strong impact of vitamin D3 on typical Treg marker such as FOXP3 or CTLA-4, we hypothesized that vitamin D3 could support the generation of iTreg by TGF-β. Contrary to what we expected, the percentage of $FOXP3^{high}$ cells was even reduced when cells were treated with TGF-β and $1,25(OH)_2D_3$ (Figure 5A–C), although we did not find a significant difference on mRNA level or T cell proliferation (Figures 5D–4E).

Moreover, we analysed the VDR expression on mRNA (Figure 5F) and protein (Figure 5G,H) level. Both Western blot (Figure 5G) and flow cytometry (Figure 5H) analysis revealed a strong upregulation of VDR upon T cell stimulation but no significant differences were found between T cell cultures with TGF-β alone or in combination with $25(OH)D_3$ or $1,25(OH)_2D_3$.

Next we evaluated CYP27B1 expression in iTregs and detected no differences upon addition of vitamin D3 metabolites to TGF-β-treated T cells (Figure 5I). Neither on mRNA nor protein level CYP27B1 expression was altered by vitamin D3, but T cells were capable to convert $25(OH)D_3$ in the vitamin D3 active metabolite in the presence of TGF-β shown by measuring $1,25(OH)_2D_3$ in the culture supernatants (Figure 5K). These data clearly demonstrate that conventional CD4+ T cells and TGF-β-induced iTregs are capable to convert $25(OH)D_3$ into the active vitamin D3 metabolite $1,25(OH)_2D_3$ which in turn regulates FOXP3 expression. However, vitamin D3 metabolites seem not to support the 'regulatory phenotype' of iTregs as they even decreased the percentage of $FOXP3^{high}$ cells.

3.6 | Vitamin D3 leads to enhanced CD25 and CTLA-4 expression in TGF-β-induced human Tregs but reduces CCR4 expression

To address whether vitamin D3 is capable of modulating T cell markers in iTregs comparable to conventional $CD4^+$ T cells, we analysed CD25, CD28, CTLA-4, ICOS, PD-1 and CCR4 expression in the presence of TGF-β cells (gating strategy depicted in Figure 5B and Figure 6A,B). Comparable to our data with conventional $CD4^+$ T cells (Figure 4), we observed that both vitamin D3 metabolites upregulate CD25 but the effect was again not restricted to $FOXP3^{high}$ cells but also detected in $FOXP3^{low}$ T cells (Figure 6A–D). Similar results were obtained for CD28 and CTLA-4 expression. In contrast, ICOS and PD-1 were significantly downregulated in cultures containing $1,25(OH)_2D_3$ in combination with TGF-β (Figure 6I–L). Surprisingly and contrary to what we observed for conventional T cells, $1,25(OH)_2D_3$ significantly decreased CCR4 expression in iTregs cells (Figure 6M,N).

3.7 | Vitamin D3 modulates the cytokine profile of conventional T cells and iTregs

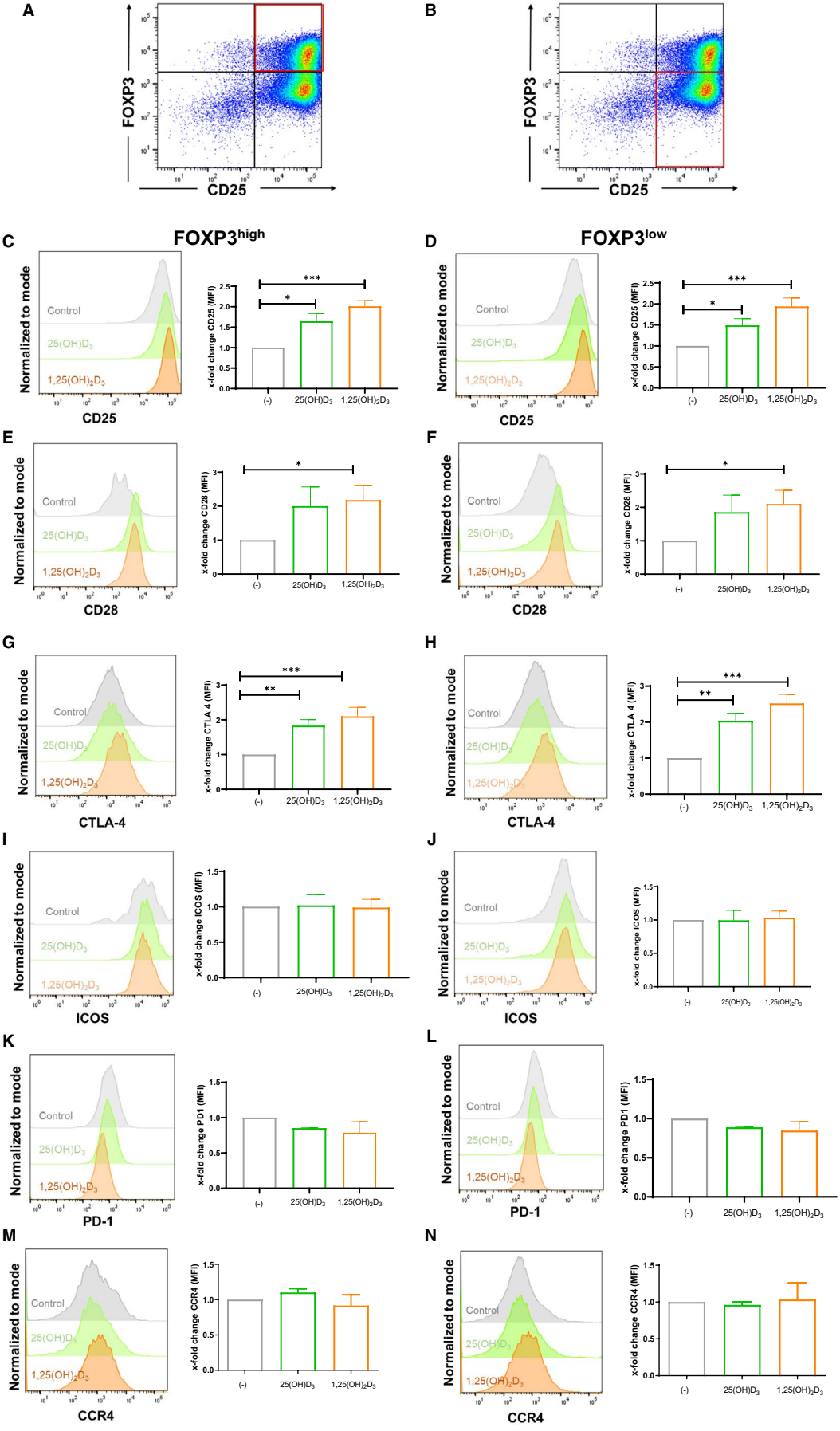
Although FOXP3 expression is strongly induced by TGF-β treatment, these iTregs still produce significant amounts of cytokines which is an atypical feature for natural Tregs [32]. Since $1,25(OH)_2D_3$ is known to downregulate IFN-γ [34,52,53], we investigated the impact of vitamin D3 metabolites on cytokine expression in iTreg in comparison to conventional $CD4^+$ T cells. Not only $1,25(OH)_2D_3$ but also $25(OH)D_3$ reduced IFN-γ mRNA expression both in conventional T cells and iTreg (Figure 7A).

IFN-γ secretion was also decreased; however, statistical significance was only reached in conventional T cells (Figure 7B). The opposite regulation was found regarding IL-10 expression, a cytokine implicated in the function of iTregs. Here, $1,25(OH)_2D_3$ significantly upregulated the mRNA expression only in iTregs which was reflected by high IL-10 secretion of $1,25(OH)_2D_3$-treated iTregs (Figure 7D-F).

Since vitamin D3 metabolites upregulated CTLA-4 protein expression in conventional and iTregs (Figures 4E,F and 6E,F), we also analysed CTLA-4 mRNA expression and measured sCTLA-4 in culture supernatants. In line with the results obtained by flow cytometry, CTLA-4 was strongly upregulated by the addition of $1,25(OH)_2D_3$ in conventional T cells and iTreg (Figure 7G). In line, the soluble form of CTLA-4 was significantly enhanced in the presence of vitamin D metabolites (Figure 7H,I).

4 | DISCUSSION

Besides its well-known role in calcium homeostasis, the active form of vitamin D3, $1,25(OH)_2D_3$, is an emerging immune regulatory drug. Effects of $1,25(OH)_2D_3$ on immune cells have been investigated in a variety of in vitro studies; however, the normal serum level of $1,25(OH)_2D_3$ is relatively low (up to 250 pM), and does not reflect the doses predominantly used for in vitro experiments (around 100 nM). Its precursor, $25(OH)D_3$ represents the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FOXP3
CD25
B
FOXP3
CD25
C
FOXP3high
Normalized to mode
Control
25(OH)D3
1,25(OH)2D3
CD25
x-fold change CD25 (MFI)
(-)
25(OH)D3
1,25(OH)2D3
D
FOXP3low
Normalized to mode
Control
25(OH)D3
1,25(OH)2D3
CD25
x-fold change CD25 (MFI)
E
Normalized to mode
CD28
x-fold change CD28 (MFI)
F
Normalized to mode
CD28
x-fold change CD28 (MFI)
G
Normalized to mode
CTLA-4
x-fold change CTLA 4 (MFI)
H
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CTLA-4
x-fold change CTLA 4 (MFI)
I
Normalized to mode
ICOS
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J
Normalized to mode
ICOS
x-fold change ICOS (MFI)
K
Normalized to mode
PD-1
x-fold change PD1 (MFI)
L
Normalized to mode
PD-1
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M
Normalized to mode
CCR4
x-fold change CCR4 (MFI)
N
Normalized to mode
CCR4
x-fold change CCR4 (MFI)

FIGURE 4 Vitamin D3 increases the expression of CD25 and CTLA-4 independent of FOXP3 expression. $CD4^{+}25^{-}$ sorted T cells were stimulated with TCE beads (1:1 bead/cell ratio) for 4 days in the presence or absence of 25(OH)D3 or 1,25(OH)2D3. Afterwards, the cells were harvested and stained for FOXP3 (A, B) and in addition CD25 (A–D), CD28 (E, F) and CTLA-4 (G, H), ICOS (I, J), PD-1 (K, L), CCR4 (M,N) were stained. One representative plot is presented. Data are means ±SEM ($n \geq 3$). Statistical analysis was performed using the Kruskal–Wallis test

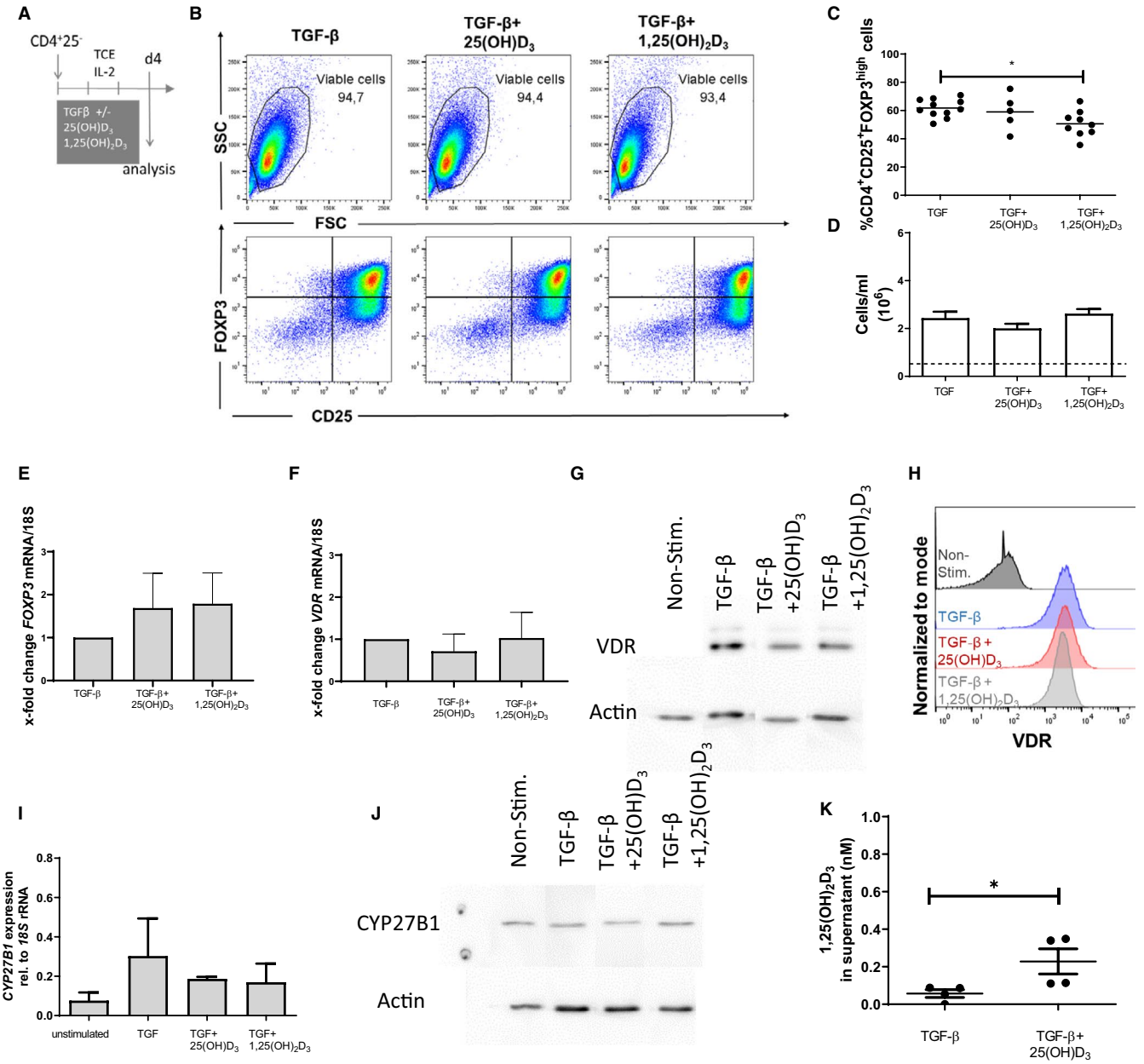

FIGURE 5 Vitamin D3 does not increase the frequency of TGF-β–induced human $FOXP3^{high}$ Tregs. Sorted $CD4^{+}25^{-}$ cells were stimulated with TCE beads (1:1 bead/cell ratio) for 4 days in the presence of 10 ng/ml TGF-β alone or in combination with 50 nM 25(OH) D3 or 5 nM 1,25(OH)2D3 (A). Representative flow cytometry plots (B), percentage of $FOXP3^{high}$ cells (C), cell proliferation (D). The relative *FOXP3* (E) and *VDR* (F) gene expression was determined by means of qPCR. Protein expression of VDR was analysed by Western blot (G) and by flow cytometry (H). The relative expression of *CYP27B1* was determined by qPCR (I) and protein expression by Western blot (J). The amount of 1,25(OH)2D3 in the supernatant was determined using a radioimmunoassay according to the manufacturer's instructions (K). Data are means ±SEM ($n \geq 3$). Statistical analysis was performed using Kruskal–Wallis test(C-I) or Mann–Whitney T-test (K)

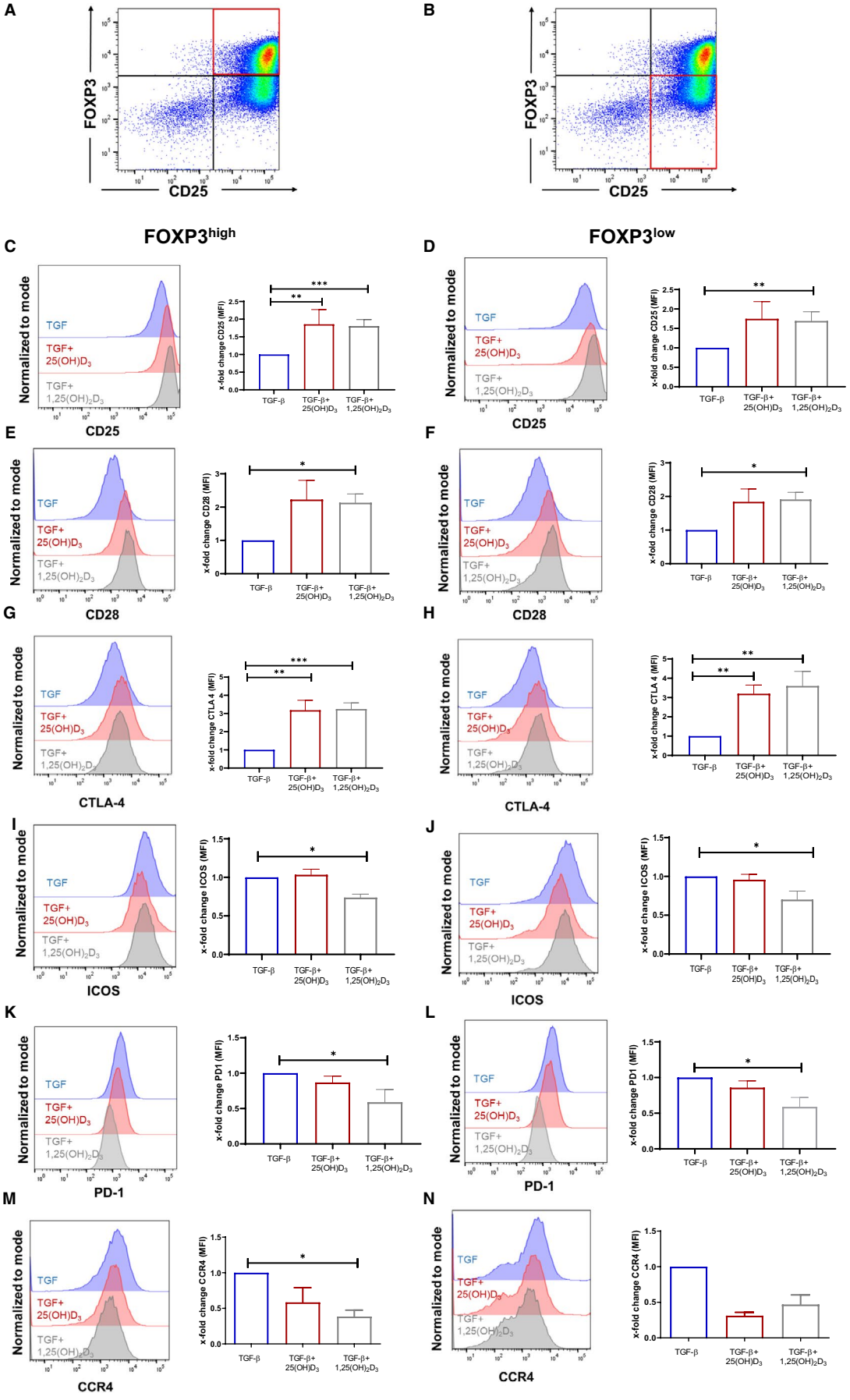
A
FOXP3
CD25
B
FOXP3
CD25
FOXP3high
FOXP3low
C
D
E
F
G
H
I
J
K
L
M
N
Normalized to mode
TGF
TGF+ 25(OH)D3
TGF+ 1,25(OH)2D3
CD25
CD28
CTLA-4
ICOS
PD-1
CCR4
x-fold change CD25 (MFI)
x-fold change CD28 (MFI)
x-fold change CTLA 4 (MFI)
x-fold change ICOS (MFI)
x-fold change PD1 (MFI)
x-fold change CCR4 (MFI)
TGF-β
TGF-β+ 25(OH)D3
TGF-β+ 1,25(OH)2D3

FIGURE 6 Vitamin D3 increases the expression of CD25 and CTLA4 in TGF-β- induced Tregs, independent of FOXP3 expression. Sorted CD4+25- cells were stimulated with TCE beads (1:1 bead/cell ratio) for 4 days in the presence of 10 ng/ml TGF-β alone or in combination with 50 nM 25(OH)D3 or 5 nM 1,25(OH)2D3. Afterwards, the cells were harvested and stained for FOXP3 (A, B) and in addition CD25 (A–D), CD28 (E, F) and CTLA4 (G, H), ICOS (I, J), PD1 (K, L), CCR4 (M,N). One representative plot is presented. Data are means ±SEM (n ≥ 3). Statistical analysis was performed using the Kruskal–Wallis test

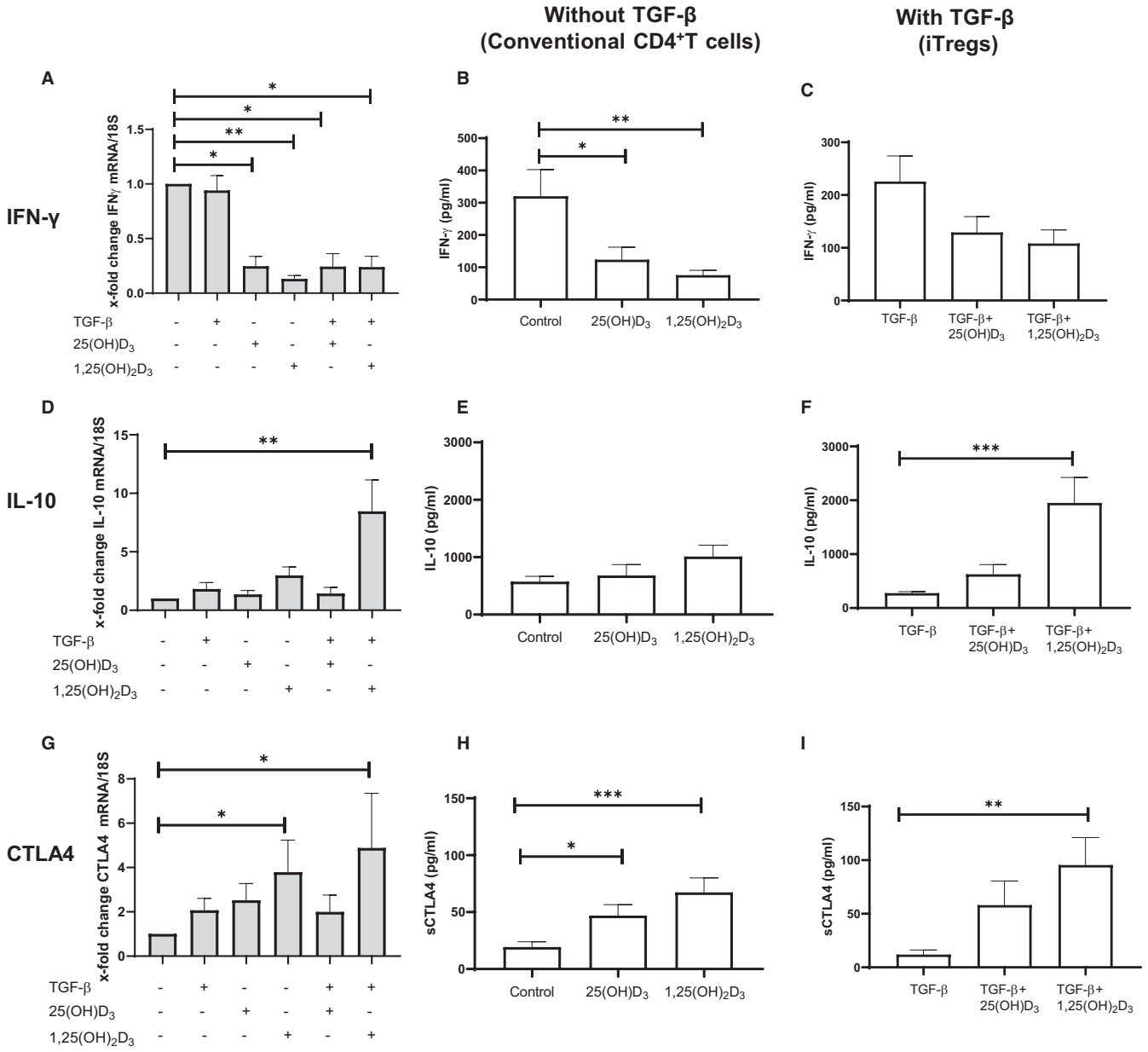

FIGURE 7 Vitamin D3 modulates the cytokine profile of T cells. Sorted CD4+25- cells were stimulated with TCE beads (1:1 bead/cell ratio) for 4 days in the presence of 10 ng/ml TGF-β alone or in combination with 50 nM 25(OH)D3 or 5 nM 1,25(OH)2D3. The amount of IFN-γ (A), IL-10 (D) and CTLA4 (G) was determined at mRNA level by means of qPCR. In addition, cell culture supernatants were analysed by ELISA: INF-γ (B-C); IL-10 (E-F) and sCTLA4 (H-I). Data are means ±SEM ($n \geq 4$). Statistical analysis was performed using the Kruskal–Wallis test

major circulating vitamin D3 metabolite and serum levels are about 1000-fold higher. In summer elevated serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels are associated with a higher number of peripheral $CD4^+$ and $CD8^+$ T cells and increased levels of FOXP3 expression in the Treg population [15]. Accordingly, high dose vitamin D3 supplementation (140 000 IU monthly) resulted in a significantly increased frequency of Tregs in a randomized controlled

trial [17]. The therapeutic potential of Tregs makes this cell population attractive in the context of GvHD, solid organ transplantation, multiple sclerosis or inflammatory bowel disease. As natural Tregs constitute only a small proportion of circulating $CD4^+$ T cells, several approaches have been developed to isolate and expand this cell type in vitro [54]. Alternatively, iTregs can be induced from conventional $CD4^+$ cells in the presence of TGF-β in vitro [30]. For natural Treg and induced Treg, the transcription factor FOXP3 is essential for differentiation and function [55] and $1,25(OH)_2D_3$ is capable of inducing FOXP3 expression in conventional human $CD4^+$ T cells [34,56]. IL-2 seems to play an important role in $1,25(OH)_2D_3$-induced FOXP3 upregulation, especially in monocyte-stimulated T cell cultures due to the relative lack of IL-2. We added IL-2 in all T cell experiments and in line with published data FOXP3 was induced in bead-stimulated cultures in the presence of $1,25(OH)_2D_3$ [34]. Since our cultures were also supplemented with serum, the observed effects may also depend on endogenous TGF-β which is an important factor regulating FOXP3 induction. Therefore, we combined vitamin D3 metabolites with a defined amount of TGF-β in a next step. In contrast to Chambers et al, who observed a synergistic effect of TGF-β and 1,25(OH)2D3 with respect to FOXP3 induction [33], we did not see such an effect in our culture system. Of note, the authors did not use $CD4^+CD25^-$ T cells as starting population, but bulk $CD4^+$ T cells, which may already contain natural $FOXP3^+$ Treg. Their findings rather support a selective expansion of $FOXP3^+$ cells within the bulk T cell culture than de novo induction of FOXP3 in T cells. What is more, the vitamin D3 concentration was 20-fold higher than the one used in the present study.

In our experiments $1,25(OH)_2D_3$ served as a positive control, and we focused on the question whether physiological amounts of the main vitamin D3 serum metabolite $25(OH)D_3$ would have similar effects on T cells. Regarding FOXP3 induction, $25(OH)D_3$ significantly induced FOXP3 in $CD4^+CD25^-$ T cells. $1,25(OH)_2D_3$ was more potent than $25(OH)D_3$ in inducing FOXP3 indicating that T cells may have to convert $25(OH)D_3$ into its active metabolite $1,25(OH)_2D_3$ which is the main ligand for VDR mediating most of the effects of vitamin D3. Therefore, we investigated the expression of the responsible enzyme CYP27B1 and found that both conventional T cells and iTregs expressed this enzyme. Two conflicting manuscripts were published on this issue up to now. In line with our findings, Fawaz et al [13] described induction of FOXP3 expression by $25(OH)D_3$ but they used a CD4 bulk population, which makes it difficult to discriminate FOXP3 induction from expansion of pre-existing $FOXP3^+$ T cells in the culture. In contrast, Jeffery and colleagues reported that T cells are not able to convert $25(OH)D_3$ into the active metabolite [14]. However, our data clearly show that $CD4^+$ T cells express CYP27B1 independent of the culture conditions on mRNA and protein level which is a strong indicator that T cells in principle should be able to convert $25(OH)D_3$. What is more, analyses of T cell culture supernatants demonstrated the capacity of the T cells to convert $25(OH)D_3$ into its active metabolite.

Although the paracrine secretion of $1,25(OH)_2D_3$ by our T cells was relatively low in vitro, autocrine production seems to be sufficient to regulate T cell function whenever a T cells is in an environment with high vitamin D3 precursor level. Essen et al demonstrated that T cell activation strongly depends on their capacity to produce $1,25(OH)_2D_3$ and the presence of the VDR [57].

Nevertheless, we cannot exclude a direct effect of $25(OH)D_3$ on T cells. Human VDR binds $1,25(OH)_2D_3$ with high affinity, whereas $25(OH)D_3$ binds roughly 50 times less efficiently [58]. In a study by Lou et al the authors demonstrated that a deletion of CYP27B1 did not abolish effects of $25(OH)D_3$ in mouse and human cells [58] which also suggests that $25(OH)D_3$ is a possible agonist of the VDR. However, effects on cell proliferation were not observed with 50 nM but only with concentrations higher than 500 nM $25(OH)D_3$ which is 10-fold higher than the physiological concentration used in our study. Therefore, it seems unlikely that direct effects of $25(OH)D_3$ are responsible for the gene regulation in human T cells in our study. In line, pre-treatment of our T cell cultures with 10 μM ketoconazole abolished the $25(OH)D_3$-induced FOXP3 upregulation, indicating that the T cell needs to convert $25(OH)D_3$ to $1,25(OH)_2D_3$ in order to upregulate FOXP3.

The importance of cell culture conditions for the effect of $25(OH)D_3$ was discussed by Kongsbak and colleagues [3]. The authors demonstrated that activated T cells can convert $25(OH)D_3$ to $1,25(OH)_2D_3$ but only in serum-free conditions as vitamin D binding protein (DBP) in serum can sequester $25(OH)D_3$ and limit its availability for T cells. But as the authors show, inflammation-induced oxidative stress leads to DBP carbonylation and thereby could result in higher free concentrations of 25(OH)D3. In our study, we used a rather low percentage of serum (2%) which may allow the uptake of free $25(OH)D_3$ and its conversion by T cells. In accordance, we were able to detect a significant increase in $1,25(OH)_2D_3$ production in T cells stimulated in the presence of $25(OH)D_3$.

Besides increased FOXP3 induction, $25(OH)D_3$ and $1,25(OH)_2D_3$ also significantly upregulated CD25 and CTLA-4 expression, both established markers of Treg which are normally co-expressed with FOXP3. However, when we analysed $FOXP3^{high}$ and $FOXP3^{low}$ T cells separately, the effect of vitamin D3 metabolites was

not restricted to FOXP3^{high} T cells but also detected in FOXP3^{low} T cells. These results suggest that vitamin D3 metabolites do not primarily act on Treg differentiation but target all CD4$^+$ T cells. In line, we did not find any evidence that 25(OH)D_3 changes the epigenetic landscape of conventional CD4$^+$ T cells, eg at the CTLA-4 promoter or TSDR region which is a typical feature of natural Treg. These results suggest that the gene regulation by 25(OH)D_3 is not related to epigenetic changes and indicate that vitamin D3 metabolites do not induce a regulatory T cell phenotype but rather suppress a classical Th1 differentiation. However, we cannot exclude that vitamin D3 has an impact on other genes or other epigenetic marks such as histone modifications which is a limitation of our study. Interestingly, a recent publication by Mijnheer and colleagues [59] identified a specific effector Treg (eTreg) signature that includes VDR as a predictor for eTreg differentiation. This specific Treg profile was also accompanied by epigenetic changes. Therefore a more global analysis including transcriptional and epigenetic profiling could shed some more light on the role of 25(OH)D_3 and 1,25(OH)$_2D_3$ for the modulation of the T cell phenotype.

Interestingly, CTLA-4 was not only upregulated on mRNA and protein level but we also detected increased amounts of soluble CTLA-4 in T cell supernatants. CTLA-4 is an immune suppressive protein and a CD28 homolog with higher binding affinity for B7 molecules present at the surface of APCs. CTLA-4 binding to B7 does not result in a stimulatory signal but counteracts the stimulatory signals from CD28 and limits T cell activation [60]. Upregulation of membrane bound CTLA-4 and soluble CTLA4- by vitamin D3 metabolites may thereby lead to an immunoregulatory phenotype of CD4+ T cells. Other co-inhibitory receptors such as ICOS and PD-1 were not altered by vitamin D3 treatment in our study, although other reports showed that vitamin D3 upregulates PD-1 expression [61,62].

Furthermore, the cytokine pattern was profoundly altered in the presence of vitamin D3 metabolites. Vitamin D3 metabolites reduced IFN-γ secretion but promoted IL-10 secretion. This is of importance especially for TGF-β-induced iTregs as several studies report that iTregs produce high amounts of Th1 effector cytokines, which is not the case for natural Treg [32]. Moreover, it is known that Tregs can exert their suppressive activity via the secretion of anti-inflammatory cytokines, such as IL-10 [63,64]. In this regard, the combination of vitamin D3 metabolites with TGF-β may promote iTreg function even though the percentage of FOXP3^{high} cells was decreased. Although the combination of the active vitamin D3 metabolite was already studied in combination with TGF-β [33,65], to the best of our knowledge the present study is the first which demonstrated an effect of 25(OH)D_3 in combination with TGF-β.

In vitro studies have limitations, and the complexity of an organism cannot be reflected by in vitro systems, but regulatory responses by vitamin D3 metabolites have also been studied in vivo using mouse models and clearly demonstrated the impact of vitamin D3 on immune cells [53,66,67]. In addition, patient data indicate that 25(OH)D_3 serum levels play a role in several disease settings [68]. However, 25(OH)D_3 serum levels not always reflect the levels found in tissues. In a study analysing the impact of vitamin D in rheumatoid arthritis (RA), Danyang Li and colleagues [69] observed no differences in 25(OH)D_3 levels in the serum of RA patients compared with healthy controls. However, a key observation of their study was a lower vitamin D metabolite concentration in synovial fluid versus paired serum samples. These observations demonstrate that the local 25(OH)D_3 level may play an important role for the resolution of inflammation that is not always reflected by the serum levels.

We believe that our study is of importance for several reasons. Even though 1,25(OH)$_2D_3$ is a well-known regulator of immune cells in vitro, the concentration necessary to obtain effects is far from what can be reached in vivo. Therefore it is very important that physiological concentrations of 25(OH)D_3 which correspond to human serum levels can indeed have biological effects comparable to high 1,25(OH)$_2D_3$ concentrations. Only a very limited number of studies addressed the relevance of 25(OH)D_3 for T cell activation and function even though local metabolism of 25(OH)D_3 is most likely important for regulation of an adaptive immune response especially at immunological barrier regions such as skin and gut where 25(OH)D_3 level are high. In addition, our study analysed the interplay between 25(OH)D_3 and TGF-β, two factors that are present under inflammatory conditions side by side in skin and gut. Our results further support the notion that adequate levels of vitamin D3 are important for the regulation of an adaptive immune response and may contribute to the maintenance of peripheral tolerance.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

Conceptualization, KP and MK; methodology, CM; AP; GS; LS; CG; RE; KR; PH investigation, CM; AP; GS; LS; MR; CB; CF writing – original draft preparation, CM; writing – review and editing, MK; KR; PH; WH supervision, MK funding acquisition, MK; K.P; and HB All authors have read and agreed to the published version of the manuscript.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

INSTITUTIONAL REVIEW BOARD STATEMENT

Collection of blood cells from healthy donors was performed in compliance with the Helsinki Declaration. All donors signed an informed consent. Blood sampling, the leukapheresis procedure and subsequent purification of peripheral blood monocytes were approved by the ethical committee of the University of Regensburg (reference number 15-101-0021**)**.

DATA AVAILABILITY STATEMENT

All data is available on request.

ORCID

Carina Matos https://orcid.org/0000-0002-1980-1380

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