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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[1]

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The active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), has potent immunomodulatory properties that have promoted its potential use in the prevention and treatment of infectious disease and autoimmune conditions. A variety of immune cells, including macrophages, dendritic cells, and activated T cells express the intracellular vitamin D receptor and are responsive to 1,25(OH)$_2$D$_3$. Despite this, how 1,25(OH)$_2$D$_3$ regulates adaptive immunity remains unclear and may involve both direct and indirect effects on the proliferation and function of T cells. To further clarify this issue, we have assessed the effects of 1,25(OH)$_2$D$_3$ on human CD4$^+$CD25$^-$ T cells. We observed that stimulation of CD4$^+$CD25$^-$ T cells in the presence of 1,25(OH)$_2$D$_3$ inhibited production of proinflammatory cytokines including IFN-γ, IL-17, and IL-21 but did not substantially affect T cell division. In contrast to its inhibitory effects on inflammatory cytokines, 1,25(OH)$_2$D$_3$ stimulated expression of high levels of CTLA-4 as well as FoxP3, the latter requiring the presence of IL-2. T cells treated with 1,25(OH)$_2$D$_3$ could suppress proliferation of normally responsive T cells, indicating that they possessed characteristics of adaptive regulatory T cells. Our results suggest that 1,25(OH)$_2$D$_3$ and IL-2 have direct synergistic effects on activated T cells, acting as potent anti-inflammatory agents and physiologic inducers of adaptive regulatory T cells. *The Journal of Immunology*, 2009, 183: 5458–5467.

I n response to stimulation, CD4$^+$ T cells can adopt a variety of different phenotypes which are geared toward specific immune functions. It is well established that Th1 cytokines such as IFN-γ provide help for macrophages and cellular immune responses while Th2 cytokines, such as IL-4, help B cell class switching and Ab production (1). The involvement of these T cell subsets in autoimmunity and allergy highlights the importance of correct and controlled T cell differentiation in immune function. Recently, additional CD4$^+$ T cell lineages including IL-17-secreting T cells (Th17), regulatory T cells (Tregs),[3] and T follicular helper cells (T$_{FH}$) have been identified, which arise in response to T cell activation under appropriate conditions (2, 3). Like Th1 and Th2 responses, Th17 cells carry out specific immune functions and are thought to be important in the response to extracellular bacteria such as *Klebsiella* (4). However, IL-17 is also widely implicated in the development of autoimmune diseases (5), including inflammatory bowel disease, arthritis, and experimental autoimmune encephalomyelitis (6), making understanding of its regulation important. IL-21 is a new member of the IL-2 family and plays a crucial role in the development of T$_{FH}$ cells which provide help for B cells in germinal centers (3) as well as providing an alternative pathway for Th17 differentiation. Indeed, overproduction of cells with a T$_{FH}$ phenotype also leads to autoimmunity (7).

Given the importance of T cell differentiation to both immunity and autoimmunity, there is much interest in understanding the factors that influence T cell differentiation. For Treg and Th17 differentiation, these factors are now emerging and studies indicate that TGF-β is central to both Treg and Th17 lineages (8). In the presence of inflammatory cytokines such as IL-1β and IL-6, TGF-β appears to promote IL-17 development (6, 9), whereas TGF-β alone stimulates the generation of adaptive Tregs or at least the induction of FoxP3 (10–13). Thus, inflammatory cytokines are important switch factors in deciding whether inducible Tregs or Th17 are produced. In addition, it is now clear that IL-2 plays a critical role in the induction and maintenance of Tregs being important for TGF-β effects and in the maintenance of FoxP3 expression (13–15).

In addition to cytokines produced by the immune system, environmental or dietary factors can also influence T cell differentiation. Strikingly, retinoic acid (vitamin A) and its derivatives produced by mucosal dendritic cells (DCs) have recently been shown to profoundly affect T cell differentiation (16–18), resulting in a bias toward Tregs and the inhibition of Th17 cells. In contrast, other compounds such as environmental dioxins can promote Th17 differentiation and suppress Treg development via the aryl hydrocarbon receptor (19, 20). Another dietary/environmental factor that

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[3] Abbreviations used in this paper: Treg, regulatory T cell; T$_{FH}$, T follicular helper cell; DC, dendritic cell; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; VDR, vitamin D receptor; MFI, mean fluorescence intensity.

FIGURE 1. 1,25(OH)$_2$D$_3$ inhibits T cell cytokine production without affecting cell division. *A*, Purified CD4$^+$CD25$^-$ T cells–were CFSE labeled and stimulated with anti-CD3/CD28-coated beads in the presence or absence of 100 nM 1,25(OH)$_2$D$_3$ for 5 days. Following stimulation, cells were stained for IL-2, IFN-γ, and IL-17 production and analyzed for cell division by FACS. *B*, Purified CD4$^+$CD25$^-$ were treated as in *A* except that cells were stimulated with autologous monocytes plus anti-CD3. Data are from a single experiment representative of three performed.
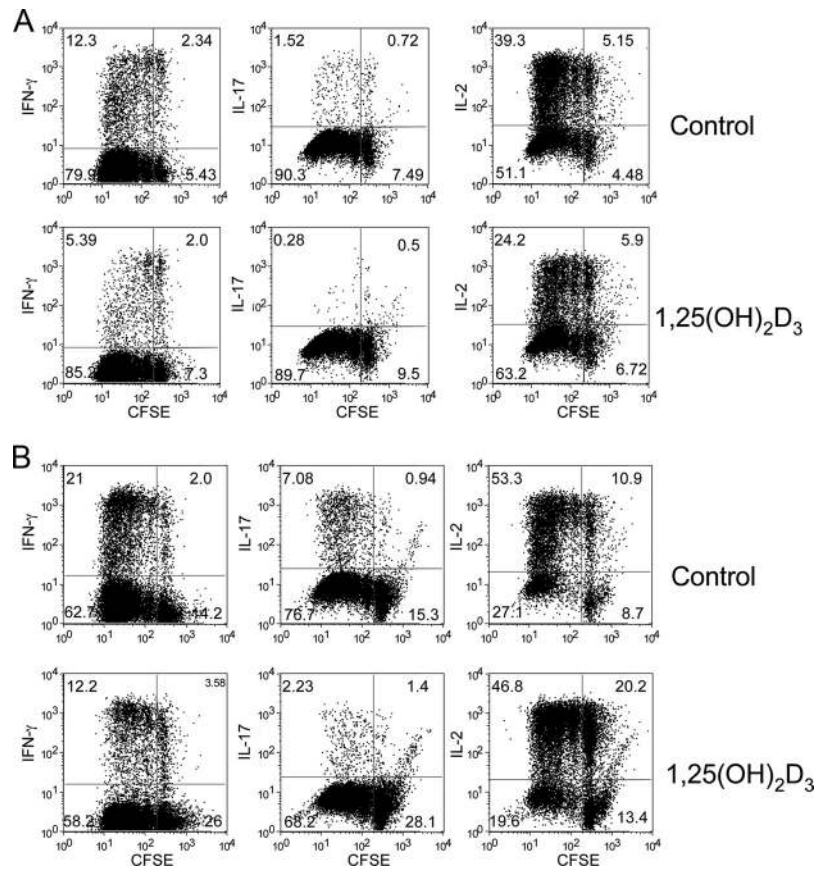
may play a significant role in immune regulation is the active form of vitamin D (1,25(OH)$_2$D$_3$) (21) (22). Recent studies have suggested that APCs that express the nuclear vitamin D receptor (VDR) are the major target for the immune suppressive actions of 1,25(OH)$_2$D$_3$ (23–25), although it is known that activated T cells also express the VDR (26). DCs treated with 1,25(OH)$_2$D$_3$ remain in an immature state with reduced expression of costimulatory molecules and IL-12 (23, 25, 27, 28). Although these observations have underlined a potential role for 1,25(OH)$_2$D$_3$ in the control of autoimmunity (21), its effect on newly described T cell subsets remains unclear.

Previous studies have demonstrated effects of 1,25(OH)$_2$D$_3$ on CD4$^+$ T cells, including the transcriptional regulation of cytokines such as IL-2 (29), IFN-γ (30, 31) and IL-4 (32), suggesting that it may suppress Th1 and promote Th2 differentiation. However, in some cases, these early studies did not adequately dissect between effects of 1,25(OH)$_2$D$_3$ on APCs or direct effects on T cells. The aim of the current study was to investigate the potential effects of 1,25(OH)$_2$D$_3$ on expression of inflammatory cytokines and regulatory markers in T cells. We observed that 1,25(OH)$_2$D$_3$ can act directly on CD4$^+$ T cells to influence their phenotype, down-regulating production of the effector cytokines IFN-γ, IL-17, and IL-21. Strikingly, we also observed that following 1,25(OH)$_2$D$_3$ treatment, T cells adopted the phenotypic and functional properties of adaptive Tregs, expressing high levels of CTLA-4 and FoxP3. T cells cultured in the presence of both 1,25(OH)$_2$D$_3$ and IL-2 expressed the highest levels of CTLA-4 and FoxP3 and possessed the ability to suppress proliferation of resting CD4$^+$ T cells. These data suggest that 1,25(OH)$_2$D$_3$ and IL-2 can directly suppress the production of proinflammatory cytokines and may therefore be important factors which influence susceptibility to autoimmune diseases.

Materials and Methods

Reagents

1,25(OH)$_2$D$_3$ was obtained as a gift from Dr. L. Binderup (Leo Pharma, Ballerup, Denmark). In each experiment, 1,25(OH)$_2$D$_3$ was used at a concentration of 100 nM or at the concentrations stated. Dilutions to 100 μM were made in ethanol from a 4 mM stock in isopropanol and then to the concentration desired in medium. Ethanol (control) was used as a vehicle control in all cases. Cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin and streptomycin, and 200 μM glutamine (Life Technologies/Invitrogen).

Antibodies

The following mAbs were purchased from BD Biosciences: anti-human CD4 conjugated to FITC (RPA-T4), anti-human CTLA-4, CD152 (BN13) conjugated to PE, anti-IFN-γ (B27) conjugated to allophycocyanin and anti-IL-2 (MQ1-17H12) conjugated to PE, and anti-human IL-10 (JES3-9D7) conjugated to PE. The following mAbs were purchased from eBioscience: anti-human IL-17 (ebio64CAP17) conjugated to PE, anti-human IL-21 (ebio3A3-N2) conjugated to PE, and anti-human FoxP3 (PCH101) conjugated to allophycocyanin, which was purchased as part of a FoxP3 staining kit. Isotype-matched controls conjugated to the appropriate fluorochrome were purchased from BD Biosciences and eBioscience and used to control for nonspecific binding.

Isolation of PBMC

PBMC were isolated from fresh buffy coats (provided by the National Blood Service, Birmingham, U.K.), using Ficoll-Paque density gradient centrifugation. Cells were washed by centrifugation twice in PBS and then resuspended at 1 × 10^8/ml in MACS buffer (0.5% BSA and 2 mM EDTA in PBS) for magnetic cell separation (see below). Blood was obtained from anonymous donors following informed consent and in line with institutional and ethical committee approval.

Purification of CD4$^+$CD25$^-$ T cells

CD4$^+$ T cells were separated by negative selection using human CD4 enrichment Ab mixture and magnetic colloid (StemSep) according to
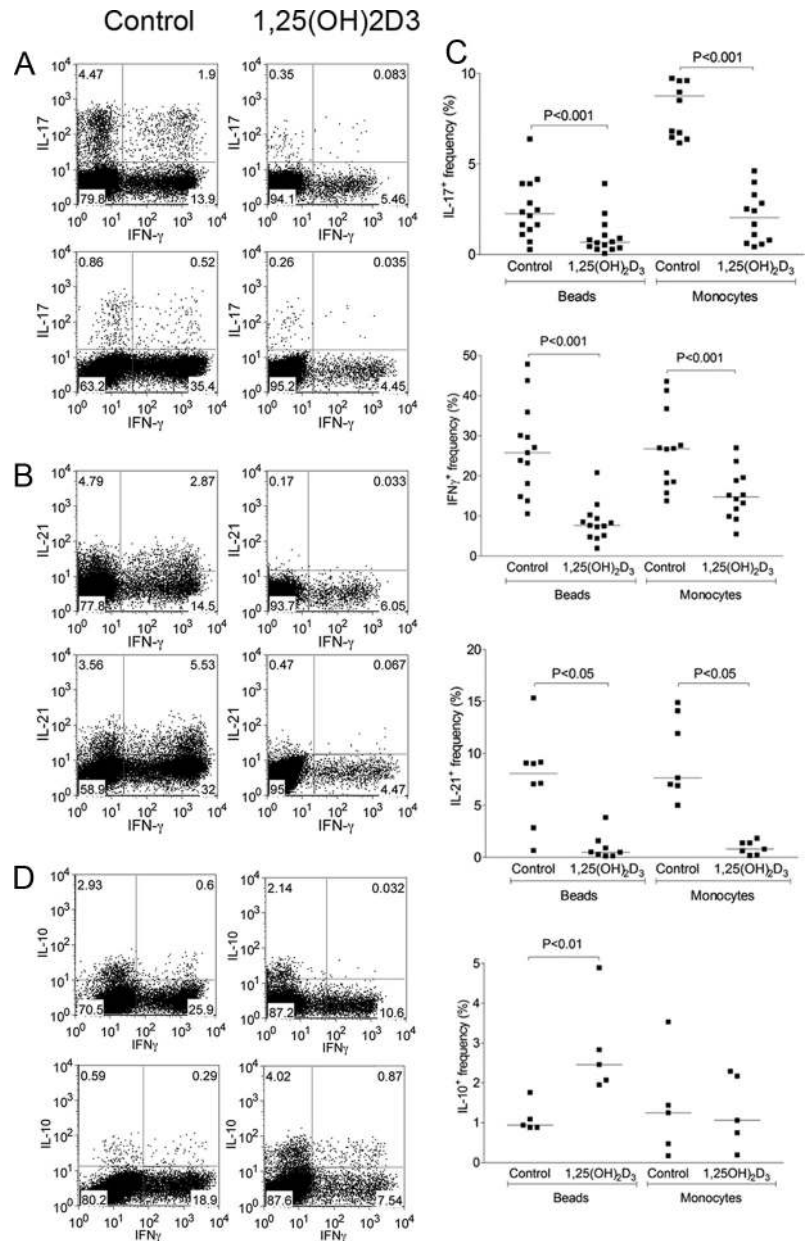
FIGURE 2. $1,25(OH)_2D_3$ inhibits IL-17, IFN-γ, and IL-21 but promotes IL-10. Purified $CD4^+CD25^-$ T cells were stimulated with anti-CD3/CD28-coated beads or autologous monocytes plus anti-CD3 (0.1 μg/ml) in the presence of 100 nM $1,25(OH)_2D_3$ or vehicle control for 5 days. Following stimulation, cells were stained for IFN-γ and costained for IL-17 (*A*) IL-21 (*B*), and IL-10 (*D*). *Upper panels* were stimulated with autologous monocytes plus anti-CD3 and *lower panels* with beads. Data from multiple experiments are represented in *C*. Horizontal bars indicate the median frequency. Significance was tested by a two-tailed Wilcoxon-matched pairs test.

the manufacturer's instructions (StemCell Technologies). To separate $CD4^+CD25^+$ T cells from $CD4^+CD25^-$ T cells, the $CD4^+$ T cells were incubated with anti-CD25-conjugated microbeads (Miltenyi Biotec) for 30 min at 4°C and then passed over a column. The negative fraction was collected from the column and used as described.

$CD4^+CD25^-$ T cell stimulation

One $\times$ 10^5 anti-CD3- and anti-CD28 Ab-coated beads (Dynal/Invitrogen) were used to activate $CD4^+$ T cells at a ratio of 1: 1 in the presence or absence of $1,25(OH)_2D_3$ at a concentration of 100 nM unless otherwise indicated. Cells were cultured in RPMI 1640 plus 10% FBS at 37°C. For experiments using monocytes, monocytes were separated from PBMC by negative selection using human monocyte enrichment Ab mixture and magnetic colloid (StemSep) according to the manufacturer's instructions (StemCell Technologies) and used to stimulate T cells at a ratio of 1:4 in the presence of 0.1 μg/ml anti-CD3 (clone OKT3).

Flow cytometry

For analysis of surface proteins, $CD4^+$ T cells were washed by centrifugation in PBS and labeled with the indicated mAb for 30 min on ice. For intracellular staining, cells were washed by centrifugation in PBS and fixed in 2% paraformaldehyde for 10 min before permeabilization in buffer containing 0.1%

saponin. Nonspecific Ab binding to Fc receptors was blocked by incubation in 0.1% saponin containing 2% goat serum for 30 min at room temperature. Labeled mAbs were then added and cells were incubated a further 30 min at room temperature. Finally, cells were washed by centrifugation once in permeabilization buffer and twice in PBS before acquisition on the flow cytometer. For intracellular cytokine staining, before labeling as described above, $CD4^+$ T cells were stimulated with 10 ng/ml PMA and 1 μM ionomycin for 7 h in the presence of 10 μg/ml brefeldin A for the last 5 h (all purchased from Sigma-Aldrich). FoxP3 staining was performed according to the manufacturer's instructions using a FoxP3 staining kit purchased from eBioscience. For staining of recycling CTLA-4, T cells were incubated with anti-CTLA-4-PE at 37°C as previously described (33). For cytokine staining, quadrants were set according to the position of the density of the nonstaining cells and for CTLA-4 and FoxP3 were set according to unstimulated cells. Cells were acquired on the FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

$CD4^+$ T cell proliferation assays

Proliferation of activated $CD4^+$ T cells was measured by dilution of CFSE. For these experiments, T cells were washed two times in PBS and then incubated with 2.5 μM CFSE for 10 min at room temperature with gentle agitation. The reaction was quenched by addition of medium and the cells
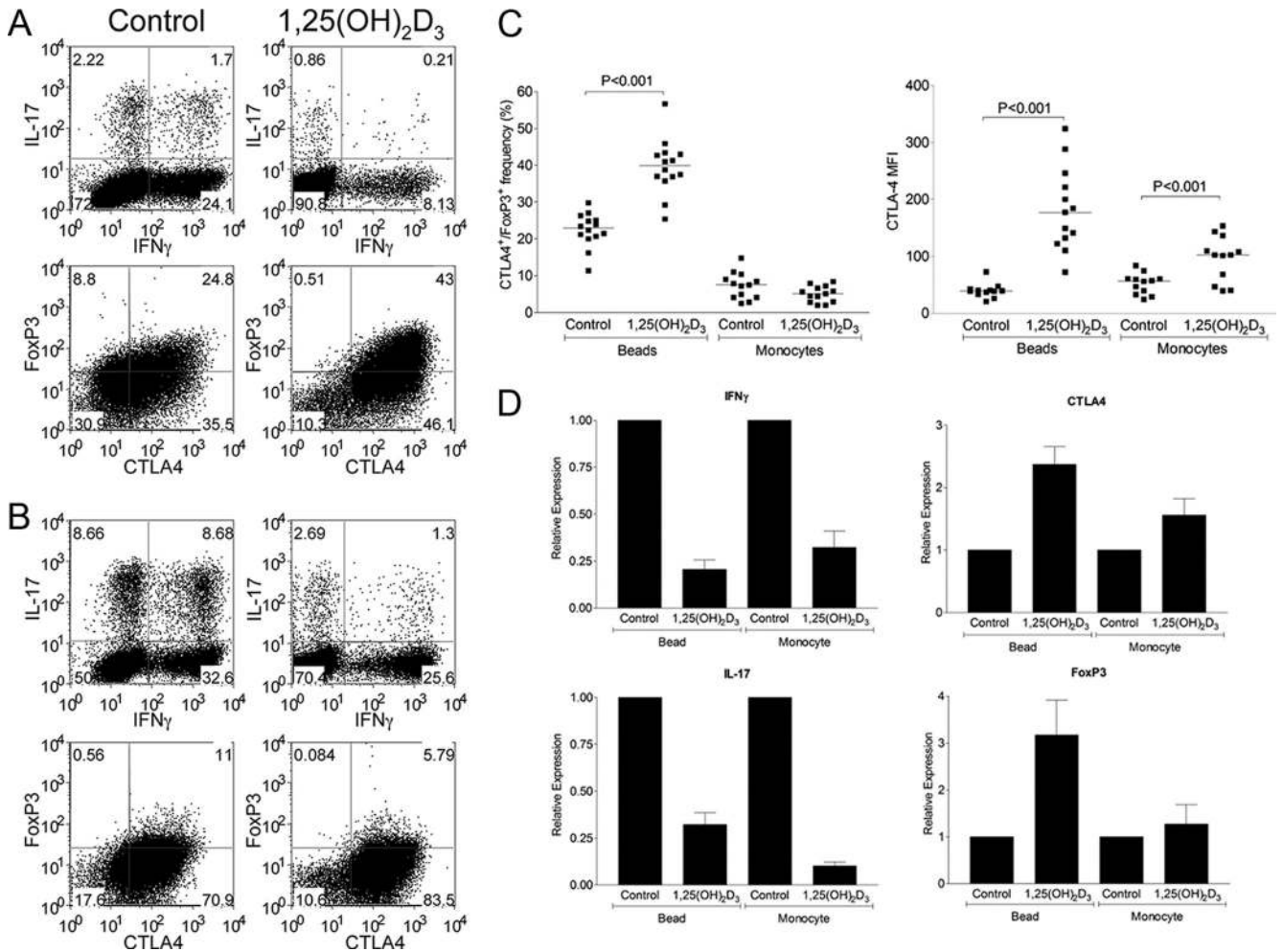
FIGURE 3. $1,25(OH)_2D_3$ promotes expression of CTLA-4 and FoxP3. Purified $CD4^+CD25^-$ T cells were stimulated with anti-CD3/CD28-coated beads (*A*) or autologous monocytes plus anti-CD3 (0.1 μg/ml; *B*) in the presence of 100 nM $1,25(OH)_2D_3$ or vehicle control for 5 days. Following stimulation, cells were stained for total expression of CTLA-4 and FoxP3 or IFN-γ and IL-17. Numbers in quadrants refer to percentage of cells. Data from multiple experiments for CTLA-4 and FoxP3 expression are represented in *C*. Horizontal bars indicate the median value and significance was tested by a two-tailed Wilcoxon-matched pairs test. Quantitative PCR analysis of mRNA expression for CTLA-4, FoxP3, IL-17, and IFN-γ is shown in *D*. Bars indicate the mean relative expression with respect to the control for $n = 3$ donors. Error bars show the SE.

were washed two times by centrifugation in medium. Cells were then re-suspended at 2×10^6/ml in medium and plated at a concentration of 10^5 cells/well in 96-well plates and activated as described above. At day 5, CFSE dilution was measured by flow cytometry.

Restimulation and suppression assays

For restimulation assays, $CD4^+$ T cells were primed with monocytes plus anti-CD3 (0.1 μg/ml) in the presence or absence of $1,25(OH)_2D_3$ (100 nM) and IL-2 (200 IU/ml) for 7 days. T cells were restimulated as shown for a further 4 days and stained by flow cytometry. For suppression assays, $CD4^+CD25^-$ T cells were primed with monocytes and anti-CD3 in the presence or absence of $1,25(OH)_2D_3$ and IL-2 for 8 days. Cells were then added at the ratio of 1:10 with fresh CFSE-labeled responder $CD4^+CD25^-$ T cells in a proliferation assay stimulated by autologous monocyte-derived DCs grown as previously described plus anti-CD3 (0.5 μg/ml). Proliferation was measured by CFSE dilution analysis.

Real-time PCR analysis

Total RNA was extracted using the TRIzol method (Invitrogen). One microgram per reaction was reverse transcribed with random hexamers (Fermentas Life Sciences). Quantitative real-time PCR was then performed on an Applied Biosystems 7500 using assay-on-demand from Applied Biosystems for IL-17A (Hs99999082), IFN-γ (Hs00174143), CTLA-4 (Hs00175480), and FoxP3 (Hs00203958). The reactions were multiplexed with VIC-labeled 18S (Applied Biosystems) as an endogenous control and analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Values of *p* were calculated by a two-tailed Wilcoxon-matched pairs test or two-tailed Mann-Whitney *U* test, as indicated in the figure legends, using GraphPad Prism3 software.

Results

$1,25(OH)_2D_3$ treatment inhibits T cell inflammatory cytokine production, but not proliferation

To assess the effect of $1,25(OH)_2D_3$ on $CD4^+CD25^-$ T cell responses, we CFSE labeled and activated cells with anti-CD3- and anti-CD28-coated beads (beads) in the presence or absence of 100 nM $1,25(OH)_2D_3$. This dose was found to be optimal, although similar effects were readily observed at doses of between 10 and 100 nM (data not shown). The effect of $1,25(OH)_2D_3$ on the production of IL-2, IFN-γ, and IL-17 was then determined in the context of T cell division. As shown in Fig. 1*A*, $1,25(OH)_2D_3$ treatment caused a marked decrease in the total number of cells producing IFN-γ. Interestingly, although the number of IL-17-producing cells was small, we observed a decrease in response to $1,25(OH)_2D_3$. IL-2 production was also reduced in the presence of $1,25(OH)_2D_3$. Thus, $1,25(OH)_2D_3$ appeared to have a general inhibitory effect on effector cytokine production. Despite these

decreases in cytokine production, $1,25(OH)_2D_3$ had no obvious effect on CD4$^+$ T cell proliferation with ~90% of cells entering cell division.

Although these experiments established that $1,25(OH)_2D_3$ could exert direct effects on T cells in the absence of APCs, we also determined whether the inhibition of inflammatory cytokines, in particular IL-17, could be modulated by $1,25(OH)_2D_3$ in the presence of a more potent IL-17 stimulus. The above experiment was therefore repeated with CD4$^+$CD25$^-$ T cells in the presence of autologous monocytes plus anti-CD3 to promote the Th17 population as previously described (35). Consistent with our observations using beads, we observed that $1,25(OH)_2D_3$ potently inhibited IFN-γ and IL-17 production (Fig. 1B). There was also a slight reduction in the number of cells entering division, suggesting that there may be an effect of $1,25(OH)_2D_3$ on the ability of monocytes to stimulate in these cultures.

Additional experiments were then performed to look in more detail at the effect of $1,25(OH)_2D_3$ on the ability of T cells to coproduce IL-17 plus IFN-γ as these populations are increased during autoimmune disease in vivo (36, 37). This revealed that $1,25(OH)_2D_3$ potently inhibited the appearance of cells producing IL-17 or IFN-γ alone, as well as cells that produced both cytokines (Fig. 2A). Likewise, the production of IL-21 by T cells in both IFN-γ- positive and -negative subsets was inhibited (Fig. 2B). Again, the effect of $1,25(OH)_2D_3$ on coproduction of cytokines was observed using either bead or monocyte stimulations, indicating that $1,25(OH)_2D_3$ was capable of inhibiting production of IL-17, IL-21, and IFN-γ through a direct effect on T cells. Collectively these data showed that irrespective of whether the T cells were stimulated in the presence or absence of APCs, the addition of $1,25(OH)_2D_3$ resulted in a robust and highly significant inhibition of proinflammatory cytokines, which was reproducibly observed across multiple donors examined (Fig. 2C). To determine whether $1,25(OH)_2D_3$ resulted in inhibition of all cytokines, we also examined its effect on IL-10, since $1,25(OH)_2D_3$ has previously been reported to enhance IL-10 production (38). Consistent with previous findings, we observed that $1,25(OH)_2D_3$ increased IL-10 (Fig. 2D) and was therefore clearly selective in its inhibitory action.

$1,25(OH)_2D_3$ up-regulates CTLA-4 and FoxP3

We next wished to establish whether treatment with $1,25(OH)_2D_3$ could promote alternative T cell lineages since Th17 and Tregs appear to be related fates in T cell differentiation (6, 8, 9). We therefore examined FoxP3 and CTLA-4 expression during CD4$^+$ T cell activation along with IL-17 and IFN-γ production (Fig. 3). Stimulation with beads generated a population of cells expressing both CTLA-4 and FoxP3 and the addition of $1,25(OH)_2D_3$ significantly increased this population (Fig. 3, A and C). Furthermore, in addition to the increase in the number of cells expressing FoxP3 and CTLA-4, the level of CTLA-4 expression also increased. These changes were accompanied by the characteristic inhibition of IFN-γ and IL-17 expression upon $1,25(OH)_2D_3$ treatment, indicating that although $1,25(OH)_2D_3$ suppressed inflammatory outcomes it also promoted regulatory ones.

Surprisingly, when T cells were stimulated with monocytes plus anti-CD3, we did not observe a significant population of cells expressing FoxP3 either in the presence or absence of $1,25(OH)_2D_3$ (Fig. 3B). These above effects were observed in multiple experiments (Fig. 3C) using different donors. Interestingly, we did observe increased CTLA-4 expression in the absence of any changes in FoxP3 using monocyte stimulations, suggesting that the effect on CTLA-4 expression was independent of its induction of FoxP3. Finally, we also examined whether these changes in the expression pattern were reflected at the transcriptional level using quantitative
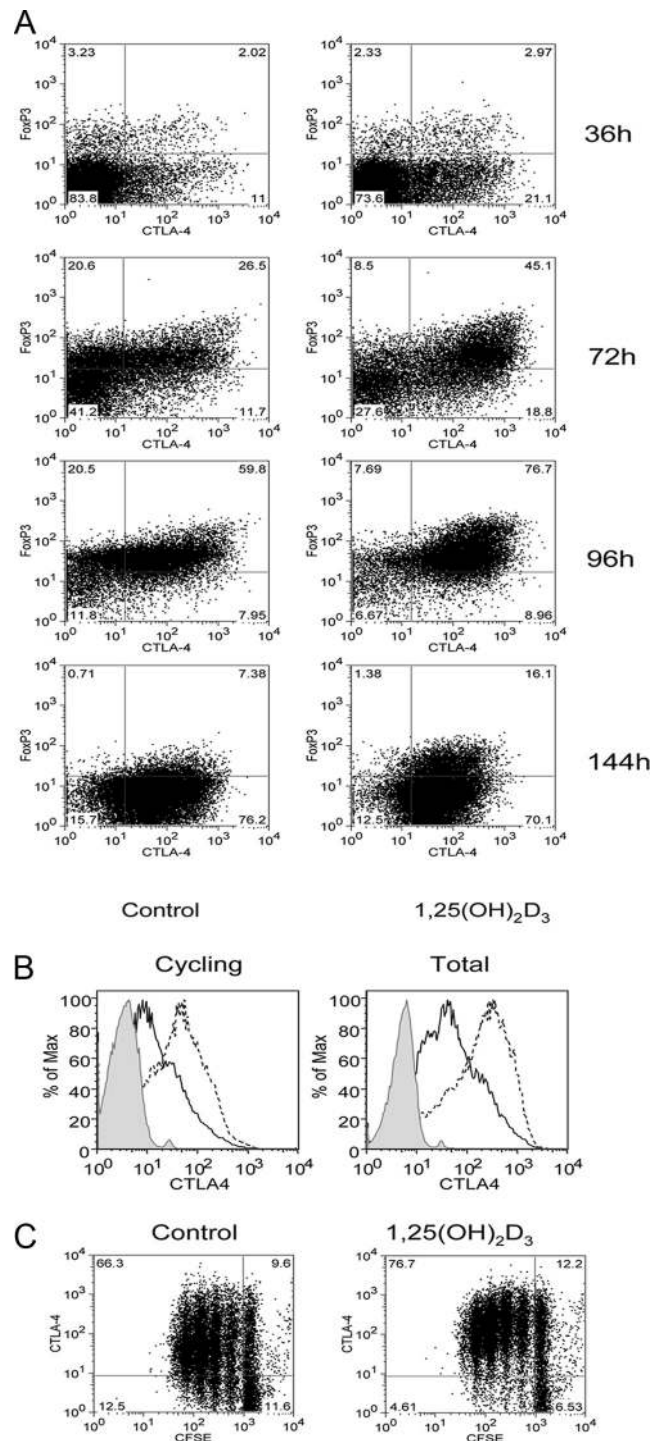
FIGURE 4. Expression of FoxP3 is not stably maintained by $1,25(OH)_2D_3$. A, Purified CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28-coated beads in the presence of 100 nM $1,25(OH)_2D_3$ or vehicle control for the times shown. Cells were analyzed for expression of CTLA-4 and FoxP3. B, Expression was determined for both the total and recycling pool of CTLA-4. Dotted lines indicate expression in the presence of $1,25(OH)_2D_3$ whereas solid lines indicate vehicle control. The shaded histogram represents isotype control staining. C, CFSE-labeled CD4$^+$ CD25$^-$ T cells were stimulated as above and analyzed for CTLA-4 expression in combination with cell division. Numbers in quadrants refer to percentage of cells. Data shown are from a single experiment representative of three conducted.

PCR (Fig. 3D). This revealed that for CTLA-4, FoxP3, IL-17, and IFN-γ changes in mRNA paralleled those of the protein, suggesting that $1,25(OH)_2D_3$ acts to affect transcription of these genes.
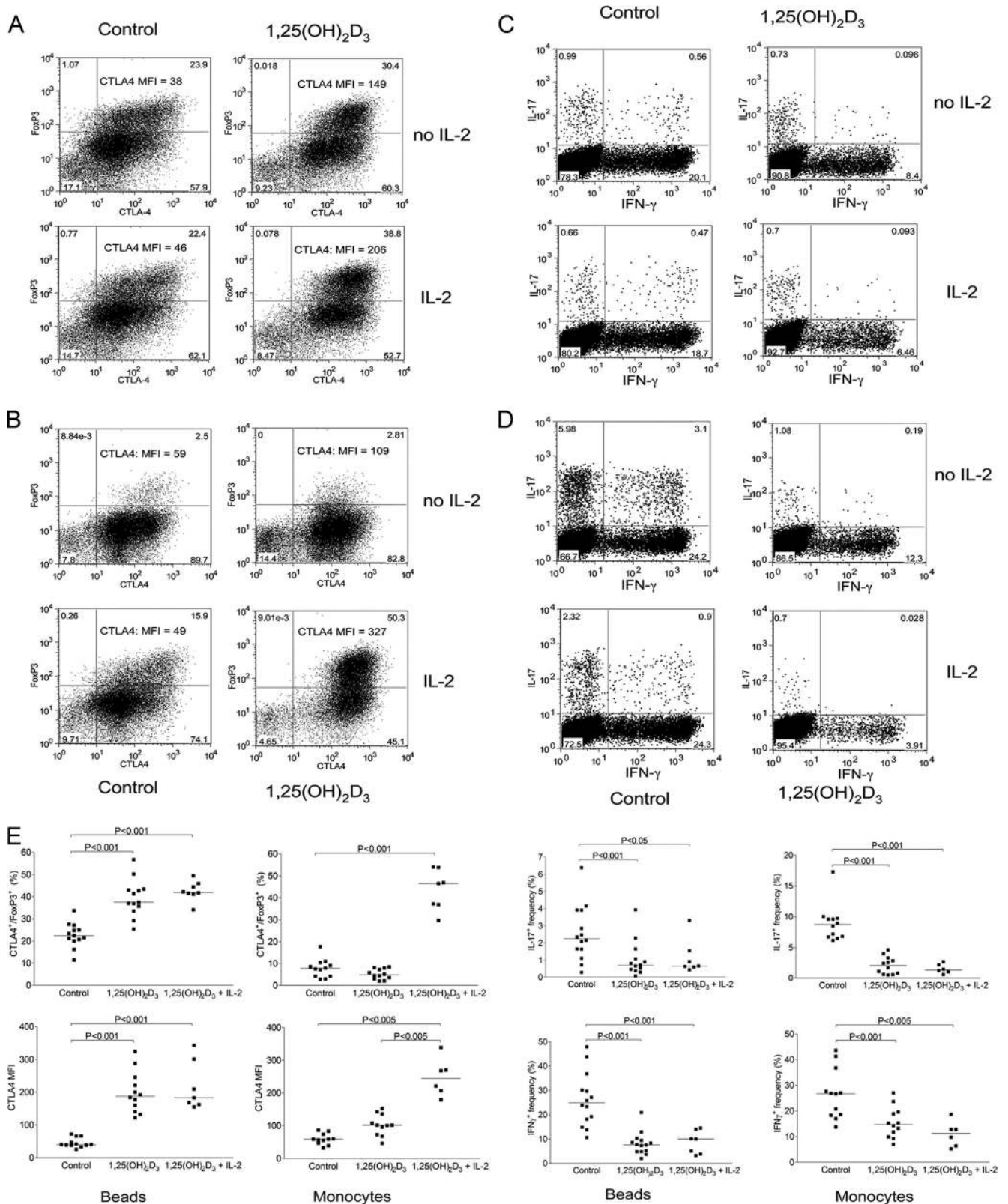
FIGURE 5. 1,25(OH)$_2$D$_3$ and IL-2 synergize to promote Treg development and inhibit inflammatory cytokine production. *A* and *C*, CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28-coated beads in the presence of 100 nM 1,25(OH)2D$_3$ or vehicle control with or without the addition of IL-2 (200 IU/ml). *B* and *D*, Purified CD4$^+$CD25$^-$ were stimulated with autologous monocytes plus anti-CD3 in the presence of 100 nM 1,25(OH)$_2$D$_3$ or vehicle control with or without the addition of IL-2 (200 IU/ml). Cells were analyzed for CTLA-4 and FoxP3 expression (*A* and *B*) or for cytokine expression (*C* and *D*). Numbers in quadrants refer to percentage of cells. CTLA-4 MFI is also shown. Summary data from multiple independent experiments are shown in *E*. Horizontal bars indicate median values. Significance was tested using a two-tailed Wilcoxon matched pairs test.

Because the expression of FoxP3 in induced Tregs is thought to be transient (39, 40), we analyzed the kinetics of the response to determine whether $1,25(OH)_2D_3$ treatment was able to sustain the expression of CTLA-4 and FoxP3. As shown in Fig. 4, treatment with $1,25(OH)_2D_3$ resulted in cells that expressed higher levels of CTLA-4 at all time points as well as an increased population of cells expressing FoxP3. However, by the end of the stimulation period, the expression of CTLA-4 and FoxP3 had reduced considerably both in the presence and absence of $1,25(OH)_2D_3$. Thus, whereas $1,25(OH)_2D_3$ promoted expression of characteristic Treg markers, it did not result in stable expression of these markers after a single round of stimulation. Furthermore, readdition of $1,25(OH)_2D_3$ at day 4 did not prevent this down-regulation (data not shown). We also investigated whether the increase in total CTLA-4 staining reflected the amount of CTLA-4 that was actually turned over at the plasma membrane and therefore functionally relevant. As shown in Fig. 4B, treatment with $1,25(OH)_2D_3$ increased both the total and recycling pools of CTLA-4 in a similar manner. We also determined whether cells with increased CTLA-4 expression were the result of outgrowth of a selected CTLA-4^{high} population (Fig. 4C). By staining for CTLA-4 expression in CFSE-labeled cells, we observed that $1,25(OH)_2D_3$ increased CTLA-4 expression even in undivided cells (control mean fluorescence intensity (MFI) = 95 vs treated MFI = 174). Since we did not observe increased cell death in the presence of $1,25(OH)_2D_3$, this supported the possibility that $1,25(OH)_2D_3$ could be influencing the differentiation T cells expressing high levels of CTLA-4.

Induction of FoxP3 and CTLA-4 involves synergy between $1,25(OH)_2D_3$ and IL-2

A feature of T cell stimulation by anti-CD3 and anti-CD28 Abs is the production of large amounts of IL-2. Because IL-2 is known to play a key role in regulating FoxP3 expression, we reasoned that the lack of FoxP3 expression in monocyte-stimulated cultures could be due to a relative lack of this cytokine. We therefore examined the effect of IL-2 supplementation to both bead- and monocyte-stimulated cultures to determine whether or not this affected responses to $1,25(OH)_2D_3$. As shown in Fig. 5, the addition of IL-2 alone to bead stimulations had no obvious effect on FoxP3 and CTLA-4 expression (Fig. 5A) or the cytokine profile (Fig. 5C). However, IL-2 in combination with $1,25(OH)_2D_3$ resulted in a robust population of CTLA-4 and FoxP3 double-positive cells and was associated with a concomitant decrease in IL-17- or IFN-γ-expressing cells (Fig. 5C).

In contrast, T cells cultured with monocytes and anti-CD3 alone did not generate FoxP3-expressing cells; however, the addition of IL-2 clearly allowed the induction of FoxP3 (Fig. 5B). The addition of $1,25(OH)_2D_3$ alone increased expression of CTLA-4 but not FoxP3. However, the addition of both IL-2 and $1,25(OH)_2D_3$ resulted in a strong synergistic effect with approximately one-half of the cells expressing high levels of CTLA-4 and FoxP3, characteristic of Tregs. Furthermore, the development of CTLA-4$^+$FoxP3$^+$ cells coincided with the strong inhibition of cells expressing IL-17 or IFN-γ (Fig. 5D). These findings were robustly observed in several experiments using different donors (Fig. 5E) and overall indicate that IL-2 and $1,25(OH)_2D_3$ have a synergistic anti-inflammatory and pro-regulatory effect on human CD4$^+$CD25$^-$ T cells.

To determine whether T cell cultures treated with $1,25(OH)_2D_3$ and IL-2 exhibited suppressive activity, we conducted suppression assays where CFSE-labeled responder T cells were stimulated by DCs plus anti-CD3 in the presence of potential suppressor T cells. Potential suppressors were generated by stimulating T cells with
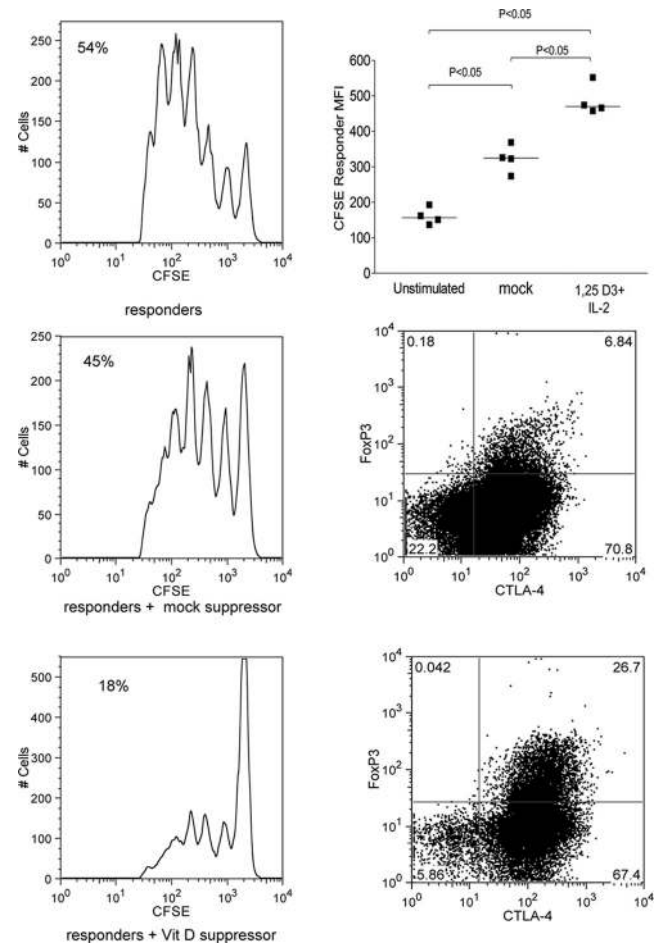

FIGURE 6. T cell culture in the presence of $1,25(OH)_2D_3$ and IL-2 results in suppressive function. Suppressor CD4$^+$ T cells were generated by stimulating purified CD4$^+$CD25$^-$ with autologous monocytes plus anti-CD3 in the presence of 100 nM $1,25(OH)_2D_3$ and the addition of IL-2 (200 IU/ml) for 7 days. Cells cultured without $1,25(OH)_2D_3$ and IL-2 were used as mock suppressor populations. Suppressor and mock suppressor populations were added at a ratio of 1:10 CFSE-labeled responder T cells and stimulated by DCs plus anti-CD3 at a ratio of 1:5 DCs:T cells. Responses were also compared with unstimulated (resting CD4$^+$ cells) as suppressor controls. Responder CFSE profiles are shown with the percentage of cells entering cell division (*left panels*) along with the phenotype of suppressor and mock suppressor cells (*right panels*).CFSE profiles are from a single experiment of four performed. Data showing individual experiments are summarized as MFI (CFSE responder MFI) in the *top right panel*. Data were analyzed using the Mann-Whitney *U* test. Dot plots show typical expression of CTLA-4 and FoxP3 in cells with and without treatment with 1,25(OH)2D3 plus IL-2.

monocytes plus $1,25(OH)_2D_3$ and IL-2 and compared with T cells stimulated in their absence. The result of this experiment (Fig. 6) clearly showed that T cells stimulated with monocytes plus anti-CD3 in the presence of IL-2 and $1,25(OH)_2D_3$ comprised an increased population of cells expressing CTLA-4 and FoxP3. Furthermore, when compared with T cells from untreated cultures, those treated with $1,25(OH)_2D_3$ and IL-2 demonstrated greater suppression of responder T cell proliferation as measured by CFSE dilution.

The effect of $1,25(OH)_2D_3$ and IL-2 on FoxP3 and CTLA-4 expression is sustained on restimulation

Since potential immunotherapies will most likely be directed at conditions where T cell activation and differentiation are already
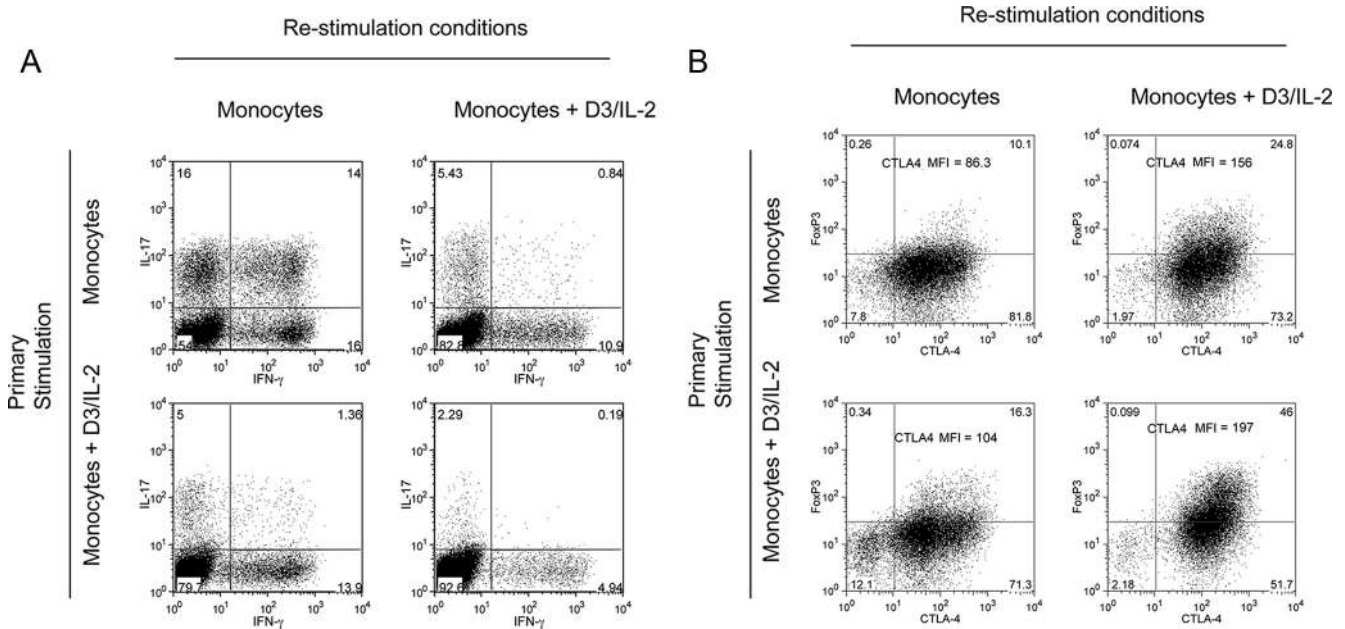
FIGURE 7. Effect of $1,25(OH)_2D_3$ and IL-2 is maintained on activated T cells. $CD4^+CD25^-$ T cells were stimulated in primary culture with monocytes and anti-CD3 for 7 days with or without $1,25(OH)_2D_3$ plus IL-2 (D3/IL-2; y-axis) and then restimulated with monocytes and anti-CD3 for a further 4 days under conditions shown (x-axis). The phenotype of the restimulated cells was then assessed for cytokine production (A) or FoxP3 and CTLA-4 expression (B). Numbers in quadrants refer to percentage of cells and CTLA-4 MFI intensity is also shown. Data are from a single experiment representative of three performed.

established, we conducted restimulation experiments to determine the effect of $1,25(OH)_2D_3$ and IL-2 on previously activated cells. Cultures were initially stimulated with monocytes plus anti-CD3 in the presence or absence of $1,25(OH)_2D_3$ and IL-2 for 8 days to allow initial responses to develop. The cells were then restimulated under a variety of conditions and their expression of cytokines CTLA-4 and FoxP3 was determined. Data in Fig. 7 revealed that cells initially stimulated and then restimulated with monocytes underwent further polarization toward IL-17 and IFN-γ production, with some 30% of cells expressing IL-17 (Fig. 7A). These cells expressed little FoxP3 and low levels of CTLA-4 (Fig. 7B). In contrast, cells that were both primed and restimulated in the presence of $1,25(OH)_2D_3$ and IL-2 contained <3% of cells expressing IL-17 and strongly reduced IFN-γ expression. As expected, these cells expressed high CTLA-4 and FoxP3. Importantly, cells that had been primed in the presence of monocytes to express IL-17 and IFN-γ were still inhibited by the addition of $1,25(OH)_2D_3$ during restimulation. Thus, in keeping with our previous observations, cells with the greatest inhibition of cytokine production also demonstrated the greatest increase in CTLA-4 and FoxP3 expression (Fig. 7B). Taken together, these data indicate that $1,25(OH)_2D_3$ and IL-2 act to inhibit inflammatory cytokine production and promote a Treg phenotype, an effect which can be maintained by T cell restimulation.

Discussion

Vitamin D status is emerging as a global health issue with an increasing number of human diseases being linked to vitamin D deficiency (40). These observations have been supported by recent studies highlighting a diverse array of nonclassical responses to vitamin D. Prominent among these are studies demonstrating potent effects of $1,25(OH)_2D_3$ on immune cells (22), in particular cells of the innate immune system (41, 42). Indeed, the effects of active $1,25(OH)_2D_3$ have focused primarily on the effects on DCs (23, 43–46). Consequently, the effects on $CD4^+$ T cells have been seen as indirect via DC modulation. Immature DCs express VDR

and respond directly to $1,25(OH)_2D_3$ which inhibits their differentiation, maturation, and activation (25). This results in decreased expression of costimulatory molecules, inhibition of IL-12 production (25, 23, 45), and impaired T cell responses. Interestingly, we did not observe any effect of $1,25(OH)_2D_3$ on the number of T cell divisions when stimulated in the absence of APCs. Therefore, it seems likely that the decreased proliferation of T cells ascribed to $1,25(OH)_2D_3$ results from impaired APC function.

Importantly, DCs and macrophages may be an important immune source of active $1,25(OH)_2D_3$ for direct effects on T cells since it is known that they are able to generate $1,25(OH)_2D_3$ due to their expression of the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (25). This complicates the issues of physiologically relevant doses of $1,25(OH)_2D_3$, which may be present at higher concentrations locally within immune tissue than measured systemically. Although we have used a relatively high dose of $1,25(OH)_2D_3$ in the present studies, we were able to observe responses at lower concentrations (1–10 nM) which are comparable to levels generated by DCs in vitro (47) and with therapeutically achievable doses in clinical studies.

In addition to the effect of $1,25(OH)_2D_3$ on T cell proliferation, we have examined its role in $CD4^+ CD25^-$ T cell differentiation. Recent observations have underlined the increasing plasticity found within the T cell lineage. This has established that resting $CD4^+CD25^-$ T cells can be differentiated toward a $FoxP3^+$ regulatory lineage under the influence of cytokines such as TGF-β (39, 48). Interestingly, this effect is further enhanced by vitamin A derivatives (16). However, inflammatory cytokines such as IL-1 and IL-6 have the ability to redirect this differentiation toward the IL-17 lineage (8). In the present experiments, we observed unequivocally that purified human T cells activated in the presence of $1,25(OH)_2D_3$ expressed markedly reduced levels of IL-17, IFN-γ, and IL-21, hallmark cytokines of Th17, Th1 and T_{FH} effector responses, respectively. Thus, $1,25(OH)_2D_3$ is able to inhibit T cell differentiation toward effector functions independently of effects on APCs. However, rather than simply inhibiting effector cytokine

production, our data show that $1,25(OH)_2D_3$ promotes a regulatory outcome as evidenced by high levels of CTLA-4 expression and FoxP3 and the induction of IL-10. Taken together with the fact that we see no preferential expansion of T cells or increased death in our cultures, these observations are consistent with an effect of $1,25(OH)_2D_3$ on T cell differentiation possibly similar to that of vitamin A (16, 18). The effect on FoxP3 and CTLA-4 was initially more noticeable using T cells stimulated by Ab-coated beads. However, the lack of FoxP3 in monocyte-stimulated cultures appears to be due to the relative lack of IL-2 since addition of IL-2 resulted in strong up-regulation of FoxP3. These data are therefore in line with several reports indicating that IL-2 is a key regulatory cytokine controlling FoxP3 expression (13, 15), further emphasizing the regulatory role of IL-2.

Although the study of in vitro effects on T cell responses has limitations, the induction of regulatory responses by $1,25(OH)_2D_3$ has also been studied in vivo using mouse models. In particular, administration of $1,25(OH)_2D_3$ to nonobese diabetic mice results in decreased Th1 cell infiltration in the pancreas, prevention of insulitis, and the onset of diabetes (49). Adorini (44) also showed that these effects were paralleled by an increase in the frequency of regulatory-type cells in draining pancreatic lymph nodes, although the FoxP3 status of these cells was not ascertained. $1,25(OH)_2D_3$ has also been shown to have effects on regulatory outcomes involving IL-10 in experimental autoimmune encephalomyelitis models (50) and in an induced colitis mouse model $1,25(OH)_2D_3$ demonstrated a change toward a Treg phenotype (51). Furthermore, the in vivo observation that $1,25(OH)_2D_3$ can inhibit IL-17 responses provides considerable support for our in vitro observations using human T cells (52). The concept that vitamin D may enhance CTLA-4 expression and therefore Treg function is also consistent with a recent study where the application of topical vitamin D resulted in enhanced Treg activity (53). More intriguingly, in human sarcoidosis where there is dramatically increased production of $1,25(OH)_2D_3$, an unusually high number of Tregs with high expression of CTLA-4 are observed (54, 55). This observation is entirely in keeping with the results presented here and supports the contention that $1,25(OH)_2D_3$ can significantly affect Treg differentiation in vivo. Although it is not clear from in vivo studies whether the induction of Tregs by $1,25(OH)_2D_3$ is due to indirect actions via DCs or whether this occurs as a result of generation of de novo adaptive Tregs, data presented here would argue that such an effect could result from the direct induction of CTLA-4 and FoxP3 during differentiation of T cells in response to $1,25(OH)_2D_3$.

The generation of de novo FoxP3-expressing T cells is now an accepted facet of Treg biology. A number of reports have now convincingly demonstrated the differentiation of adaptive FoxP3$^+$ Tregs from FoxP3$^-$ CD4 T cells. The conditions for such conversion vary widely and include activation in the presence of immunomodulatory agents, such as TGF-β (12, 56), the use of low doses of peptides (57) and simple adoptive transfer of T cells (58). In humans, the requirements for FoxP3 up-regulation appear less stringent with studies showing that essentially normal activation is sufficient to induce a cohort of cells that express FoxP3 (59), although it seems likely TGF-β is present in serum. While it is recognized that TGF-β can be a significant factor involved in Treg differentiation, the effects of $1,25(OH)_2D_3$ seem distinct from this pathway. Since our cultures were conducted in serum-containing medium, the effects we observed are in addition to the endogenous TGF-β present. Furthermore, we have not observed up-regulation of CTLA-4 or suppression of cytokines by adding TGF-β to our cultures in contrast to $1,25(OH)_2D_3$. Although we do not rule out an interaction between the $1,25(OH)_2D_3$ and TGF-β pathways, the

effects of $1,25(OH)_2D_3$ appear to be distinct and in addition to those of TGF-β.

The importance of CTLA-4 expression in Treg function is now beginning to clearly emerge. Several reports have indicated that CTLA-4 can play an important role in the suppressive activity of Tregs (60–62). Recently, we have observed that CTLA-4-deficient Tregs have compromised function in a model of diabetes (63) and conditional deletion of CTLA-4 in Tregs has been shown to cause fatal autoimmunity (64). We have also shown that expression of CTLA-4 confers regulatory activity to resting human T cells, whereas FoxP3 does not (65). Thus, expression of CTLA-4 appears to represent an important effector mechanism for Treg suppression. It is therefore most significant that stimulation of T cells in the presence of $1,25(OH)_2D_3$ results in the strong up-regulation of CTLA-4 and associated suppressive function.

It is now established that FoxP3$^+$ Tregs are critical to the prevention of catastrophic autoimmunity. The maintenance of a significant population of such cells is therefore required for lifelong health. Our data show that $1,25(OH)_2D_3$ has a significant and direct effect on the generation of FoxP3$^+$CTLA-4$^+$ Tregs which are capable of potent immune suppression. Coupled with increasing data linking autoimmune disease with low vitamin D status and polymorphisms in the gene for VDR (34, 66, 67), our data offer potential insight into the importance of vitamin D in the prevention and/or treatment of autoimmune disease.

Disclosures

The authors have no financial conflict of interest.

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