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The role of 1α,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3$^+$ and IL-10$^+$ CD4$^+$ T cells

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Abstract

1α,25-Dihydroxyvitamin D3 (1α25VitD3) has potent immunomodulatory properties. We have previously demonstrated that 1α25VitD3 promotes human and murine IL-10-secreting CD4$^+$ T cells. Because of the clinical relevance of this observation, we characterized these cells further and investigated their relationship with Foxp3$^+$ regulatory T (Treg) cells. 1α25VitD3 increased the frequency of both Foxp3$^+$ and IL-10$^+$ CD4$^+$T cells in vitro. However, Foxp3 was increased at high concentrations of 1α25VitD3 and IL-10 at more moderate levels, with little coexpression of these molecules. The Foxp3$^+$ and IL-10$^+$ T-cell populations showed comparable suppressive activity. We demonstrate that the enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10. 1α25VitD3 enables the selective expansion of Foxp3$^+$ Treg cells over their Foxp3$^-$ T-cell counterparts. Equally, 1α25VitD3 maintains Foxp3$^+$ expression by sorted populations of human and murine Treg cells upon in vitro culture. A positive in vivo correlation between vitamin D status and CD4$^+$Foxp3$^+$ T cells in the airways was observed in a severe pediatric asthma cohort, supporting the in vitro observations. In summary, we provide evidence that 1α25VitD3 enhances the frequency of both IL-10$^+$ and Foxp3$^+$ Treg cells. In a translational setting, these data suggest that 1α25VitD3, over a broad concentration range, will be effective in enhancing the frequency of Treg cells.

Keywords

1α,25-Dihydroxyvitamin D3; Asthma; Immune regulation; Regulatory T cells

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Introduction

Considerable interest exists in the therapeutic potential of regulatory T (Treg) cells to treat a range of immune-mediated pathologies in humans. This is partly based on evidence obtained from animal models of human disease demonstrating the capacity of Treg cells to control transplant rejection, and to successfully treat autoimmune and allergic disease [1]. Two broad therapeutic strategies are being considered in research initiatives worldwide: (i) adoptively transferring Treg cells that have previously been expanded in vitro into patients and (ii) inducing or boosting endogenous Treg cells directly in patients. The latter approach may be more applicable in highly prevalent conditions such as allergy and asthma, which cause considerable morbidity, but are generally not life threatening. The rationale for such a strategy is further strengthened by evidence that existing therapies for allergic diseases, such as allergen immunotherapy and glucocorticoids, are associated with the induction of Treg cells in patients [2]. Nevertheless, considerable scope for improving the safety and efficacy of these treatments exists.

Recent studies have focused on the capacity of vitamin D to modulate Treg-cell subsets. For example, culturing dendritic cells (DCs) with the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α25VitD3) leads to impaired DC maturation, development of tolerogenic properties [3], and the capacity to induce $CD4^+Foxp3^+$ cells with suppressive activity [4], or IL-10 expressing Treg cells [5]. In animal models of human disease, administration of 1α25VitD3 successfully treats transplant rejection [6] and a range of autoimmune conditions, including antiretinal autoimmunity [7], acute colitis [8], diabetes [6], arthritis [9], and EAE [10], as well as allergic airway disease [11]. These studies demonstrate a correlation between therapeutic efficacy and increased frequency or quantities of $CD4^+CD25^+$ T cells, IL-10, TGF-β, and CTLA-4.

Our earlier studies have highlighted the capacity of 1α25VitD3 to promote human $CD4^+$ IL-10 secreting Treg cells (IL-10-Treg) in culture both alone [12] and in concert with glucocorticoids such as dexamethasone [13,14]. Furthermore, treatment of severe steroid refractory asthma patients with 1α25VitD3 in vivo directly increased IL-10 gene expression in $CD3^+CD4^+$ T cells [12], and restored the impaired steroid-induced IL-10 response in $CD4^+$ cells in vitro [14,15].

The present study was designed to further investigate the mechanisms underlying the therapeutic potential of 1α25VitD3 in the context of asthmatic disease, and to determine effects on the induction of both $IL\text{-}10^+$ and $Foxp3^+$ T cells. Specifically, we have examined the effects of 1α25VitD3 on total, unfractionated $CD4^+$ T-cell populations, representative of those likely to be encountered in vivo. The data demonstrate that 1α25VitD3 increases the frequency not only of IL-10-Treg cells, but also of $Foxp3^+$ Treg cells, that these cells express increased levels of the inhibitory receptors CTLA-4 and PD-1, and exhibit inhibitory function. The data further suggest that 1α25VitD3 functions to maintain Foxp3 expression in the existing $Foxp3^+$ Treg-cell pool.

Results

1α25VitD3 increases the frequency of both $IL\text{-}10^+$ and $Foxp3^+$ human $CD4^+$ T cells in culture

We have previously described the induction of IL-10 secreting cells following culture of human $CD4^+$ T cells with 1α25VitD3 in vitro and directly ex vivo following administration of calcitriol to asthma patients [12,14]. An unusual dose response was observed in vitro with 1α25VitD3 at the very highest concentration tested (10^{-6} M 1α25VitD3) resulting in considerably lower IL-10 secretion than the optimal concentrations of 10^{-7} M and 10^{-8} M

1α25VitD3 [12]. Here, we analyzed this dose response further and investigated whether IL-10 was being synthesized by Foxp3 positive or negative T cells in response to 1α25VitD3 in culture.

Human peripheral blood $CD4^+$ T cells were stimulated with anti-CD3, IL-2, and IL-4 under conditions previously determined to optimally induce IL-10-Treg cells [12]. The expression of Foxp3 and IL-10 in the presence or absence of 1α25VitD3 was determined by flow cytometry. 1α25VitD3 at 10^{-6} M led to an increase in Foxp3 expression as compared with control cultures (No VitD3), whereas lower doses of 1α25VitD3 minimally affected Foxp3 expression. In contrast, maximal IL-10 induction was observed, as expected, at 10^{-7} M and 10^{-8} M 1α25VitD3 [12]. This response was confirmed using a panel of donors. A statistically significant increase in the frequency of $Foxp3^+$ T cells was observed at 10^{-6} M, but not at 10^{-7} M 1α25VitD3, while significant induction of $IL\text{-}10^+$ T cells was seen at 10^{-7} M, but not at 10^{-6} M (Fig. 1A). In summary, 1α25VitD3 enhances both the percentage of $Foxp3^+$ cells and the expression of Foxp3 transcripts (data not shown), but at a distinct concentration of 1α25VitD3 than required for optimal IL-10 induction.

In our early studies, cells were analyzed for expression of Foxp3 and IL-10 independently by flow cytometry. To confirm and extend the finding of differential effects of 1α25VitD3 on these molecules, a protocol for costaining was developed. $CD4^+$ T cells were cultured with 10^{-6} M or 10^{-7} M 1α25VitD3 and then restimulated with anti-CD3 and IL-2 for 16 h and analyzed for expression of IL-10 and Foxp3 by secretion assay and then intranuclear staining.

In two representative donors, shown in Figure 1B, virtually no (≤0.2%) cells stained positive for both Foxp3 and IL-10 in the presence of 10^{-7} M 1α25VitD3. When cells from a panel of healthy donors were screened, we observed that cell cultures preferentially expressed a high frequency of $Foxp3^+$ cells and low levels of IL-10, or conversely low Foxp3 and a high frequency of $IL\text{-}10^+$ cells in response to culture with 1α25VitD3 (Fig. 1B and C).

Since Foxp3 expression may not always reflect inhibitory function, the functional consequences of 1α25VitD3 modulation of Foxp3 versus IL-10 expression by human $CD4^+$ T cells was next investigated.

1α25VitD3-enhanced IL-10 and $Foxp3^+$ T cells show regulatory function and phenotype

$CD4^+$ T-cell lines generated from the same donor in the presence of either high (10^{-6} M; Foxp3-promoting Treg conditions) or lower (10^{-7} M; IL-10-Treg favoring conditions) concentrations of 1α25VitD3 were tested for their capacity to inhibit the proliferation of autologous, naïve CFSE-labeled responder T cells. Both populations showed comparable inhibitory activity (Fig. 2A). The suppression by cells generated with 10^{-7} M 1α25VitD3 could be diminished by the addition of anti-IL-10 receptor antibody to the co-culture, while in T-cell cultures generated with 10^{-6} M 1α25VitD3, the antibody had little effect (Fig. 2B), suggesting both IL-10-dependent and IL-10-independent mechanisms of suppression existed in the two different populations.

To further investigate the phenotype of 1α25VitD3-treated cells, T cells were cultured with a range of concentrations (10^{-9}–10^{-6} M) of 1α25VitD3 and analyzed for expression of surface markers by flow cytometry. A number of Treg-associated molecules, including the inhibitory molecules PD-1 and CTLA-4, as well as CD38 and CD25 were shown to be increased following exposure to 1α25VitD3, although the expression of the Treg-associated marker, GITR, and also CD62L, were inhibited by 1α25VitD3 (Fig. 3).

The enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10

We have previously shown that IL-10 expression is reduced when IL-10 signaling is neutralized in culture [12,13]. Cells were stimulated in the absence or presence of 10^{-8} – 10^{-6} M 1α25VitD3 together with either an anti-IL-10R antibody or the appropriate isotype control reagent. In a representative donor shown in Fig. 4A, a high frequency of $Foxp3^+$ cells was observed following culture with 10^{-6} M 1α25VitD3 and the presence of anti-IL-10R antibody in culture did not alter this. In contrast, considerably less $Foxp3^+$ cells were detected in cell cultures containing 10^{-7} M or 10^{-8} M 1α25VitD3, and the addition of anti-IL-10R to these cultures resulted in a marked increase in the frequency of $Foxp3^+$ cells (Fig. 4A; mean data from four healthy donors depicted in Fig. 4C). These data were also replicated at the mRNA level using real time RT-PCR where addition of anti-IL-10R antibody resulted in a significant increase in Foxp3 transcripts, with a reciprocal decrease in IL-10 transcripts (Fig. 4B).

To confirm these findings of the effects of IL-10 on 1α25VitD3-enhanced Foxp3 expression, a complimentary approach was used. $CD4^+$ T-cell stimulation cultures were established with high 10^{-6} M 1α25VitD3 in the presence or absence of recombinant IL-10. As predicted, the presence of IL-10 significantly inhibited the frequency of $Foxp3^+$ T cells compared with 10^{-6} M 1α25VitD3 alone (Fig. 4D).

TGF-β is required for the peripheral induction of Foxp3, both alone and in conjunction with retinoic acid (RA) [16–20]. Note in this study, no significant increase in Foxp3 expression was observed when exogenous TGF-β alone was added to cultures containing 10^{-6} M or 10^{-7} M 1α25VitD3 (data not shown). However, neutralization of endogenous TGF-β (by the addition of an antibody specific for TGF-β to the culture) decreased 1α25VitD3-enhanced Foxp3 expression (Supporting Information Fig. 1), suggesting a possible role for TGF-β.

1α25VitD3 maintains the expression of $Foxp3^+$ T cells in culture

Human $CD4^+CD25^{high}$ cells, which are largely $Foxp3^+$, are known to lose expression of Foxp3 over time upon culture in vitro. To determine if 1α25VitD3 acted to maintain the expression of Foxp3 in this population, $CD4^+CD25^{high}$ (>99% $CD25^+$; 86% $Foxp3^+$; Fig. 5A) T cells were isolated by cell sorting and cultured for 7 days with or without 1α25VitD3. The frequency of $Foxp3^+$ cells diminished from 86 to 11.7% upon culture with anti-CD3 and low dose IL-2 alone, shown in a representative plot in Figure 5B. In contrast, stimulation of $CD4^+CD25^{high}$ cells in the presence of 10^{-7} M and 10^{-6} M 1α25VitD3 resulted in 32% and 63.7% of the cells remaining $Foxp3^+$, respectively, in the representative data shown in Fig. 5B. These data suggest 1α25VitD3 contributes to the retention of $Foxp3^+$ expression by human $CD4^+CD25^{high}$ T cells.

To confirm and extend these data, these experiments were repeated with mouse T cells. When total unfractionated $CD4^+$ cells (>99% pure) were cultured in the absence or presence of 1α25VitD3, Foxp3 expression was increased from 3% to 7.3% with 10^{-7} M 1α25VitD3 in the example shown (Supporting Information Fig. 2A). When purified $CD4^+Foxp3GFP^+$ cells (>97% $Foxp3^+$) were stimulated with anti-CD3 and IL-2, in the absence of 1α25VitD3, Foxp3 expression was greatly reduced following 7 days of culture. In contrast, in cultures containing 10^{-7} M and 10^{-6} M 1α25VitD3, more than 50% of the cells remained $Foxp3^+$ (Supporting Information Fig. 2B?). The addition of RA plus TGF-β to all cell cultures enhanced Foxp3 expression as predicted from independent published data. Collectively, these data support the evidence from experiments with human T cells that 1α25VitD3 enhances the frequency of $Foxp3^+$ cells by maintaining Foxp3 expression in culture.

1α25VitD3 favors the expansion of Foxp3$^+$ over Foxp3$^-$ T cells

An enrichment in the percentage of Foxp3$^+$ cells was observed in the presence of 10^{-6} M 1α25VitD3, or in the presence of lower concentrations of 1α25VitD3 plus anti IL-10R antibody. As 1α25VitD3 has well-documented inhibitory effects on T-cell cycle and proliferation, we investigated the capacity of 1α25VitD3 to directly modify the proliferation of Foxp3$^+$ versus Foxp3$^-$ T cells using CellTrace Violet. This highly stable dye enabled monitoring of cell division of Foxp3$^+$ and Foxp3$^-$ cells for up to 14 days of culture by flow cytometry.

In the absence of 1α25VitD3, comparable proportions of the major Foxp3$^-$ and the minor Foxp3$^+$ T-cell populations had proliferated by day 7 and day 14 of culture. The addition of 1α25VitD3 10^{-6} M to the culture, impaired both FoxP3$^-$ and Foxp3$^+$ T-cell proliferation at days 7 and 14 (Fig. 6A). However, whereas the Foxp3$^-$ T-cell proliferative response was almost completely abrogated, a clear Foxp3$^+$ T-cell response, albeit reduced, could still be observed. The difference in the proliferative response between these two populations was significant (Fig. 6B). The addition of anti-IL-10R into cultures containing 10^{-7} M 1α25VitD3 resulted in a significant increase in cell division in the Foxp3$^+$, but not the Foxp3$^-$ T cells at day 7 (Supporting Information Fig. 3) and to a lesser extent at day 14 (data not shown). Together these data suggest that a contributory mechanism by which 1α25VitD3 increases the frequency of Foxp3$^+$ cells is via the preferential inhibition of the proliferation of Foxp3$^-$ cells.

In order to distinguish between effects of 1α25VitD3 on existing Treg cells compared with newly generated adaptive and/or activation-dependent Foxp3 expression arising from the effector population, Treg (CD4$^+$CD25^{high}CD127^{low}) and T-effector (CD4$^+$CD25$^-$CD127^{high}) cells were isolated from the same donor to high purity (Supporting Information Fig. 4). The two populations were individually labeled with CellTrace and then co-cultured at the original ratio (one Treg to nine effector cells), combining either labeled Treg with unlabeled T-effector cells, or conversely labeled T-effector cells with unlabeled Treg cells. These experiments demonstrate that a very low frequency of Foxp3$^+$ T cells arise from the labeled effector T-cell population, cultured alone or with labeled Treg cells, in the absence or presence of 1α25VitD3 (<2% at day 14; data not shown). These data suggest that 1α25VitD3 is not acting to enhance adaptive/activation-dependent Foxp3 expression. Furthermore, across a dose titration of 1α25VitD3, Treg cell proliferation was only reduced at 10^{-6} M 1α25VitD3, whereas at all other concentrations proliferation was unaffected or even enhanced (Fig. 6C and D). In contrast, proliferation of labeled effector T cells in co-culture was reduced at all concentrations of 1α25VitD3 tested (10^{-9}–10^{-6} M 1α25VitD3; Fig. 6C and D). These data imply that culture of T cells with 1α25VitD3 preferentially expands Treg over T-effector cells.

Evidence for a role of 1α25VitD3 in the maintenance of Foxp3$^+$ T-cell frequencies in vivo

Our earlier studies demonstrated that 1α25VitD3 enhances IL-10 expression by CD4$^+$ T cells not only in culture, but also following ingestion of standard formulary doses of 1α25VitD3 by both steroid refractory asthma patients and healthy subjects [12,14]. Subsequent work has demonstrated that no parallel increase in Foxp3 gene expression occurred in the same peripheral blood CD3$^+$CD4$^+$ T cells, analyzed directly ex vivo pre- and post-1α25VitD3 ingestion (data not shown). To investigate whether vitamin D might influence Foxp3 expression in the tissues, we analyzed the frequency of CD4$^+$Foxp3$^+$ cells in bronchoalveolar lavage (BAL) samples available from a pediatric severe asthma cohort under study, where serum 25-hydroxyvitamin D3 status was also being assessed (Supporting Information Table 1) [21]. Strikingly the majority of these patients showed a vitamin D status reflecting insufficiency (<75nmol/L) or deficiency (<50 nmol/L) [22]. A statistically

significant correlation between serum vitamin D status, and the frequency of $CD4^+Foxp3^+$ T cells in the BAL was observed ($r = 0.71$, $p = 0.02$), suggesting an in vivo correlate of our in vitro observations on the capacity of 1α25VitD3 to influence $Foxp3^+$ Treg cell prevalence (Fig. 7 and Supporting Information Fig. 5).

Discussion

Interest in enhancing Treg cells in patients is clearly driven by the therapeutic potential of these cells. An attractive approach would be the use of pharmacological agents such as 1α25VitD3, or vitamin D supplementation, to induce the expansion and/or maintenance of Treg cells. This approach is especially suited to ongoing chronic diseases such as asthma that occur at high prevalence, where a simple treatment such as vitamin D supplementation would be relatively safe, acceptable to patients, and cost effective. The present study aimed to investigate the capacity of 1α25VitD3 to promote Treg cells and whether dose-dependent limitations exist.

Early indications from clinical studies suggest vitamin D treatment of patients enhances T-cell expression of IL-10 in vivo, although data on the impact on $Foxp3^+$ Treg cell frequencies in human peripheral blood are less clear [12,23–26]. Here, we demonstrate that the active form of vitamin D3 increases the frequency of both $IL\text{-}10^+$ and $Foxp3^+$ cells in cultures of human peripheral blood derived $CD4^+$ T cells. The two Treg cell subsets promoted by 1α25VitD3 are distinct cell populations that are optimally induced by different concentrations of 1α25VitD3 in culture.

Both $Foxp3^+$ and $IL\text{-}10^+$ 1α25VitD3-promoted T cells exhibited comparable regulatory activity in a conventional in vitro suppression assay. However, more than one inhibitory mechanism appears to exist. Inhibition by T cells generated under conditions that optimally promoted IL-10 was reversed upon addition of an antibody that blocked IL-10 signaling to the co-culture suppression assay. In contrast, the suppressive activity of $Foxp3^+$ cells, generated in the presence of high-dose 1α25VitD3, was not reversed by neutralization of IL-10. A number of additional mechanisms of suppression by $Foxp3^+$ Treg cells have been reported [27].

To investigate how vitamin D modulates the frequency of $Foxp3^+$ cells in culture, initial studies focused on the capacity of 1α25VitD3 to maintain expression of Foxp3 by existing Treg cells. 1α25VitD3 maintained the levels of Foxp3 expression in human $CD4^+CD25^{high}$ Treg cells, which otherwise were lost upon in vitro culture. This observation was reproduced using Foxp3GFP $CD4^+$ cells from reporter mice. Using the CellTrace together with Foxp3 staining, we further demonstrated that 1α25VitD3 allowed the preferential expansion of $Foxp3^+$ T cells over $Foxp3^-$ (effector) T cells and this could provide a contributory or additional mechanism by which 1α25VitD3 promotes $Foxp3^+$ Treg cells. These data, together with earlier studies suggesting that vitamin D increases Foxp3 expression in human naïve T-cell cultures [10,28], indicate that vitamin D acts through several different mechanisms to enhance Foxp3 expression. IL-2 plays a central role in the maintenance of a functional Treg cell compartment [29,30]. Interestingly, our data suggest that one mechanism by which 1α25VitD3 may act to maintain Treg cells is via the observed increased expression of the alpha chain of the IL-2 receptor, CD25, and this could be relevant to all of the pathways proposed above.

An unprecedented finding of the present study is the reciprocal regulation of Foxp3 and IL-10 by 1α25VitD3. The phenotype of the Treg cell population generated is likely to depend not only upon the level of vitamin D available, but also the local cytokine milieu. In addition to the well-documented requirement for IL-2 to maintain Treg homeostasis, we

envisage that in the presence of higher concentrations of IL-10, IL-10-Treg induction will be favored. Conversely, elevated TGF-β and reduced IL-10 in 1α25VitD3-driven cultures will result in $Foxp3^+$ Treg cell generation. Our observations that exogenous IL-10 in 1α25VitD3-driven cultures reduces the frequency of Foxp3+ T cells, while blocking IL-10 signaling in these cultures increases $Foxp3^+$ T-cell frequency, further indicate reciprocity in control of IL-10 and Foxp3 expression. We show that Foxp3 expression was significantly enhanced by 1α25VitD3 following 14 days of culture (as previously reported for IL-10 [12]), while enhancement at day 7 was variable and did not achieve statistical significance (data not shown). This may indicate that longer-term exposure to vitamin D, arguably reflecting the situation in a vitamin D replete individual, will favor Treg cells in patients.

A high prevalence of vitamin D insufficiency has been documented in asthma cohorts worldwide. A strong association between low vitamin D status with severity and poor control of asthma has been shown by several independent groups of investigators [31–36]. Our own studies have addressed this in a severe therapy-resistant pediatric asthma cohort. We observe highly significant associations between serum 25-hydroxyvitamin D3 levels with lung function, asthma severity, and control [21]. Using this unique patient cohort, we recorded a positive correlation between serum 25-hydroxyvitamin D3 levels with the frequency of $CD25^+Foxp3^+$ T cells in the airways, complimenting our in vitro observations. Additionally, we have very recently observed that the frequency of $CD4^+CD127^{low}Foxp3^+$ T cells in the periphery of steroid sensitive is higher than in steroid refractory adult moderate to severe asthmatics, and go on to demonstrate a significant correlation between serum vitamin D status and the number of these cells in the periphery [37]. Together, these association data support the concept that vitamin D status may control $Foxp3^+$Treg frequencies in vivo, which could represent a mechanism whereby vitamin D treatment dampens asthma symptoms. However, two recently published studies using either a hypocalcaemic vitamin D analogue [24] or high-dose vitamin D supplementation in patients with multiple sclerosis [23] showed no increase in the frequency of peripheral blood $CD4^+Foxp3^+$ T cells following vitamin D treatment. Clearly further translational studies in patients are required to fully understand the impact of vitamin D on Treg cells in humans.

Although these studies were designed to investigate a role for vitamin D in a therapeutic context, they also have implications regarding a physiological role for vitamin D in immune modulation, including Treg frequency as highlighted by the data from pediatric BAL. Extrarenal synthesis of active vitamin D is increasingly being recognized as important for modulation of both innate and adaptive immunity [38]. Vitamin D3 can be metabolized into 1α25VitD3 by a variety of immune and structural cells including DCs [38,39], macrophages [40], and epithelial cells [41], which is proposed to result in relatively high 1α25VitD3 concentrated locally in the lymphoid microenvironment for presentation to interacting T-cells [42]. Thus, local synthesis of 1α25VitD3 in tissues may influence Treg frequency, although what constitutes “physiological” levels of 1α25VitD3 generated locally in tissues, and how these reflect observations from in vitro studies is as yet difficult to ascertain. Production of 1×10^{-9}–6×10^{-8} M 1α25VitD3 by antigen presenting cells has been reported [39,42], which is not that dissimilar to what is used in the present study.

In summary, vitamin D deficiency and insufficiency is increasing being associated with a wide range of immune-mediated pathologies [22,43]. In a translational setting, these data suggest that 1α25VitD3, over a broad concentration range, is likely to be safe and effective in enhancing the frequency of both $Foxp3^+$ and $IL\text{-}10^+$ Treg cell populations in patients. We believe, supported by our data and others, that vitamin D delivered either through supplementation or pharmacologically, including novel derivatives that lack the side effect of hypercalcaemia, could prove candidates for increasing the frequency of Treg cell

populations in patients. This type of approach may be particularly amenable in patients where individually tailored therapies are impractical.

Materials and methods

Mice

Wild-type C57BL/6 and genetically modified Foxp3GFP C57BL/6 [44] and TCR transgenic (TCR7) mice on a $Rag1^{-/-}$ background specific for hen egg lysozyme [45] crossed to Foxp3GFP C57BL/6 (Foxp3GFP TCR7 $Rag1^{-/-}$) mice [46] were bred and maintained under specific pathogen-free conditions at NIMR according to the Home Office UK Animals (Scientific Procedures) Act 1986 and used at 8–12 weeks of age.

Human studies

PBMCs were obtained from normal healthy individuals in the majority of experiments. The Ethics Committee at Guy's Hospital approved the study and all donors provided informed consent.

Twelve pediatric patients with severe therapy-resistant asthma were also studied (Supporting Information Table 1). Severe therapy-resistant asthma was defined as persistent chronic symptoms of airway obstruction, despite treatment with high-dose inhaled corticosteroids and trials of add on drugs, and/or recurrent severe asthma exacerbations. All children had been through a detailed protocol to optimize adherence and other aspects of basic management, as far as possible [21,47]. Bronchoscopies in the pediatric subjects were performed as previously described [48]. The Royal Brompton Hospital Ethics Committee approved the study; written age-appropriate informed consent was obtained from parents and children. Serum 25-hydroxyvitamin D was measured using a two-dimensional high performance liquid chromatography system–tandem mass spectrometry.

Cell purification and culture

Human PBMCs were isolated as previously described [12]. $CD4^+$ T cells were purified by positive selection using Dynabeads (Invitrogen; typical purity 98.5%) or cell sorting (typical purity 99.5%) using a FACSARIA flow cytometer (Becton Dickinson). $CD4^+CD25^{high}$ (purity >99%) cells were isolated by cell sorting from Buffy coats from the National Blood Service. Treg ($CD4^+CD25^{high}CD127^{low}$; typical purity >98%) and effector T cells ($CD4^+CD25\text{-}CD127^+$; purity >99%) were cell sorted from cones obtained from the National Blood Service.

Human $CD4^+$ T cells (1×10^6 cells/mL) were stimulated with plate-bound anti-CD3 (1 μg/mL; OKT-3) in RPMI containing 50 U/mL recombinant hIL-2 (Eurocetus), 10 ng/mL hIL-4 (NBS), and calcitriol (1α25VitD3; BIOMOL Research Labs) as indicated, for 7 day cycles. In some experiments, 5 ng/mL IL-10 (R&D), 5 μg/mL anti-TGF-β (clone 1D11; R&D), 5 μg/mL anti-IL-10R (clone 3F9-2; BD-Pharmingen), or the appropriate isotype control antibody were added, as indicated. Note cells used for proliferation analysis were stained at day 0 with 5 mM CellTrace™ Violet (Invitrogen), according to manufacturers' instructions.

Murine $CD4^+$ T cells were FACS sorted on a MoFlo cytometer (Beckman Coulter) for $CD4^+$ (purity >99%), $CD4^+CD44^{low}CD25^-$ ($Foxp3GFP^-$; purity >99%), or $CD4^+Foxp3GFP^+$ (purity >97%) from CD4-enriched spleen cells. Cells were stimulated in flat-bottom 96-well plates (0.25×10^6 cells/mL) with plate-bound anti-CD3 (145-2C11) at 2.5 mg/mL in cRPMI medium [45] containing 5 ng/mL recombinant mIL-2 (Insight Biotechnology) for 7 days. Cells were fed with IL-2 on day 3. Where indicated, 1α25VitD3,

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5 ng/mL recombinant hTGF-b1 (Insight Biotechnology), and 10 nM all trans RA (Sigma-Aldrich) were added to T-cell cultures.

Functional assays of regulatory function

$CD4^+$ T-cell lines were generated as described above. $CD4^+CD45RA^+$ naïve T cells were labeled with 2 μM CFSE (Molecular Probes, Eugene) and co-cultured with the autologous line at the ratios indicated, with 0.1 μg/mL plate-bound anti-CD3 and 1 μg/mL anti-CD28 (clone 15E8; Sanquin). In some experiments, anti-IL-10R or IgG control was added to the co-culture. On day 5, cells were stained with propidium iodide (PI; Sigma-Aldrich) for dead cell exclusion and 30,000 CFSE positive viable responder cells were acquired on a FACSCaliber flow cytometer (Becton Dickinson).

Analysis of IL-10 and Foxp3 expression by flow cytometry

Human $IL\text{-}10^+$ cells were identified using a commercially available IL-10 Secretion Assay Detection Kit (Miltenyi Biotec). Foxp3 (clone PCH101) expression was determined by cell staining using the Foxp3 staining buffer set from Ebiosciences. Quadrant markers were set according to the matched isotype control antibody staining. Antibodies used for cell surface phenotyping (BD Biosciences) were PD-1 (clone MIH4), CTLA-4 (clone BN13), CD62L (clone DREG-56), CD25 (clone M-A251), GITR (clone 110416), and CD38 (clone HIT2).

Expression of Foxp3 in murine $CD4^+$ T cells was determined by excluding dead cells with LIVE/DEAD Fixable Red Dead Cell Staining Kit (Invitrogen) and intracellular staining for Foxp3 with staining buffer set from eBiosciences. Samples were acquired on LSR II (BD) flow cytometer.

Real time PCR

RNA was extracted from cell pellets using RNeasy Mini kit (Qiagen). RNA was reverse transcribed using random hexamer primers (Fermantas Life Sciences). Real time (RT) PCR was performed in triplicate using FAM-labeled Assay-on-Demand reagent sets for IL-10 (Hs00174086 m1) and Foxp3 (Hs00203958 m1). RT-PCR reactions were multiplexed using VIC-labeled 18S primers and probes (Hs99999901 s1) as an endogenous control and analyzed using SDS software version 2.1 (Applied Biosystems), according to the $2^{-(\Delta\Delta Ct)}$ method.

Statistics

Results are presented as mean ± SEM, unless indicated. Data were assessed for normality and equal variation after which the appropriate parametric or nonparametric test was performed (see individual figure legends). Differences were considered significant at the 95% confidence level. Correlations were verified with the Pearson's correlation test or the Spearman's rank correlation coefficient, as indicated in the figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IL-10-Treg	IL-10-secreting Treg cell
RA	retinoic acid
1α25VitD3	1α,25-dihydroxyvitamin D3

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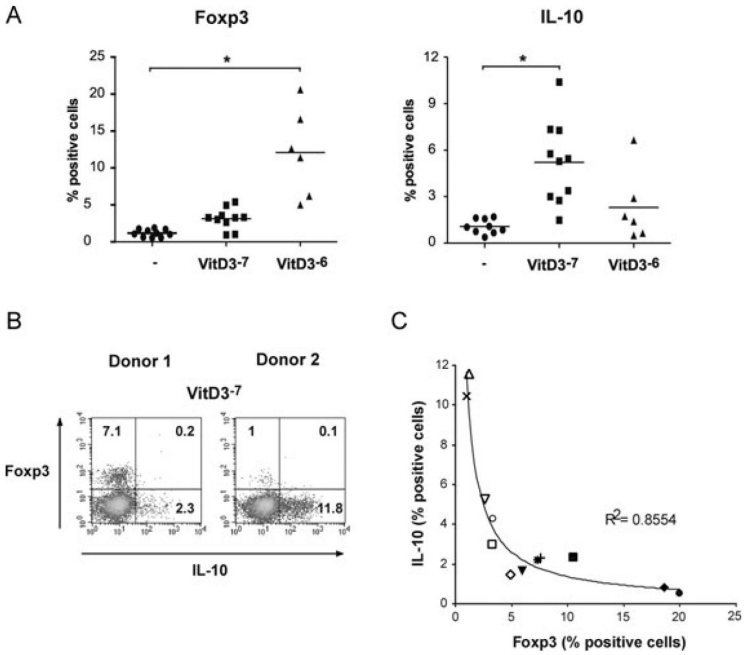

Figure 1.

1α25VitD3 increases the frequency of IL-10$^+$ and Foxp3$^+$ human CD4$^+$ T cells. Human CD4$^+$ T cells were stimulated for two 7-day cycles with anti-CD3, IL-2, and IL-4 (No VitD3) or additionally with the indicated concentration of 1α25VitD3 (VitD3; 10× M). (A) At day 14, cells were restimulated for 16 h with anti-CD3 and IL-2. IL-10$^+$ cells were identified using an IL-10 secretion assay kit. FoxP3$^+$ cells were assessed by intranuclear staining. Values represent the percentage of gated live CD4$^+$ cells. Each symbol represents an individual donor and lines represent the mean. *$p < 0.05$ as determined by the Mann–Whitney rank sum test. (B) Cells were costained for expression of both IL-10 and FoxP3 in the presence of 10^{-7} M 1α25VitD3. Two representative flow cytometry plots from different donors are shown. Note the absence of FoxP3$^+$IL-10$^+$ cells. Data are representative of seven independent experiments. (C) Data from the costaining experiments are depicted in a correlation analysis. R^2 value was determined by Spearman's rank correlation coefficient. Each symbol represents a different donor ($n = 8$); closed symbols = 10^{-6} M 1α25VitD3, open symbols = 10^{-7} M 1α25VitD3.

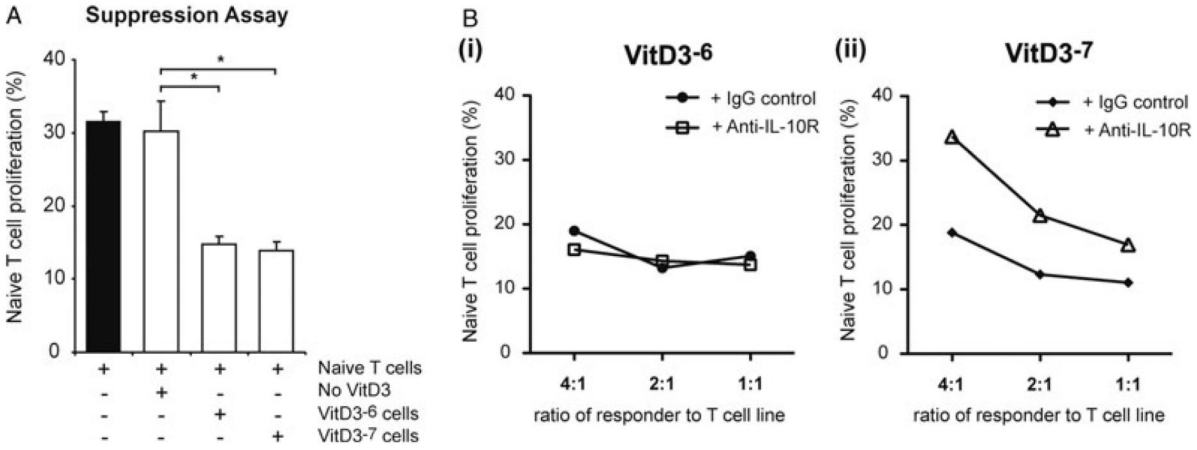

Figure 2.

1α25VitD3-treated $CD4^+$ T-cell populations acquire suppressive properties. (A) Autologous $CD45RA^+$ T cells were isolated, CFSE-labeled, and co-cultured with the cell lines (No VitD3 or VitD3, as indicated) at a ratio of 2:1 responder to cell line, for 5 days with anti-CD3 and CD28. The percentage of proliferating, viable CFSE-labeled responders is shown, as assessed by flow cytometry. Data are shown as mean ± SEM from four independent experiments from different healthy donors. $*p < 0.05$ as determined by the Mann–Whitney rank sum test. (B) CFSE-labeled ± responder cells were co-cultured with cell lines — (i) VitD3 10^{-6}M T cells, (ii) VitD3 10^{-7}M T cells–at the ratios indicated in the graph, in the presence of control IgG (closed symbols) or anti-IL-10R (hollow symbols); both at 5 μg/mL. Data are representative of four independent experiments.

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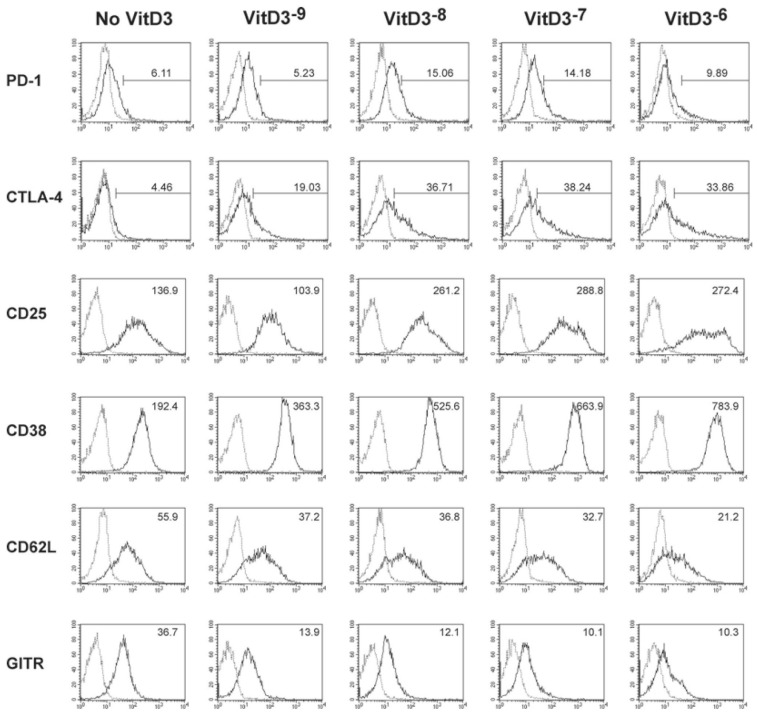

Figure 3.

1α25VitD3 promotes the expression of Treg-cell-associated surface markers. Human $CD4^+$ T cells were stimulated alone (No VitD3) or additionally in the presence of the indicated molar concentrations of 1α25VitD3 (VitD3). At day 14, cells were stained and analyzed for the surface expression of CD25, CD38, PD-1, CTLA-4, CD62L, and GITR (black lines; gray lines = matched isotype control) by flow cytometry. For PD-1 and CTLA-4, values represent percentage of positive cells; all other antigens values shown are indicative of the geometric mean fluorescence intensity. Data are representative of a minimum of six independent experiments.

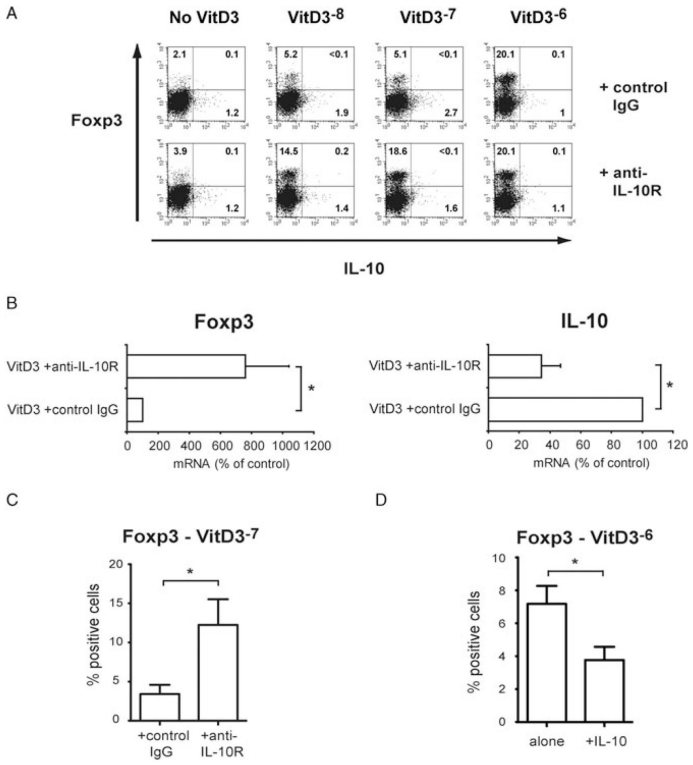

Figure 4.

The enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10.Human $CD4^+$ T cells were cultured for two 7 day cycles with anti-CD3, IL-2, and IL-4 (No VitD3) or additionally with the indicated concentration of 1α25VitD3 (VitD3; 10× M) in the presence of IL-10, anti-IL-10R, or control IgG, as indicated. (A) At day 14, cells were re-stimulated for 16 h with anti-CD3 and IL-2. $IL\text{-}10^+$ cells were determined using a commercially available IL-10 secretion assay and subsequently stained for intranuclear expression of Foxp3. Values represent the percentage of gated live cells. (B) Foxp3 and IL-10 gene expression, as determined by real time RT-PCR. Data are shown normalized to an endogenous control (18s rRNA) and expressed relative to IgG control-treated cells. Data are shown as mean +SEM from four independent experiments from different healthy donors. (C) $CD4^+$ T cells were cultured with a concentration of 10^{-7} M 1α25VitD3 (VitD3) in the presence of control IgG or anti-IL-10R. Foxp3 expression was determined by intranuclear staining for Foxp3. (D) $CD4^+$ T cells were cultured with a concentration of 10^{-6} M 1α25VitD3 (VitD3) in the presence or absence of IL-10. Foxp3 expression was determined by intranuclear staining for Foxp3. (C and D) Data are shown as mean +SEM of four experiments each performed with an individual donor. $*p < 0.05$ as determined by the Mann–Whitney rank sum test.

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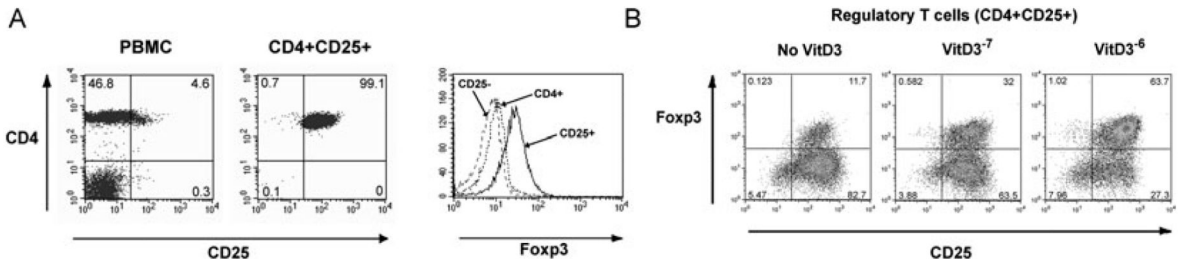

Figure 5.

Human $CD4^+CD25^+$ T cells retain Foxp3 expression in the presence of 1α25VitD3. (A) Human $CD4^+CD25^{high}$ Treg cells were sorted from $CD4^+$ T cells by flow cytometry. Foxp3 expression is depicted in the overlay histogram on the right.(B) Sorted $CD4^+CD25^+$ cells were cultured with anti-CD3 and IL-2 (50 U/mL) in the absence (No VitD3) or the presence of 10^{-7} M or 10^{-6} M 1α25VitD3 (VitD3) as indicated for 7 days and then assessed for expression of CD25 and Foxp3 by flow cytometry. Data are representative of three independent experiments each performed with a different healthy donor.

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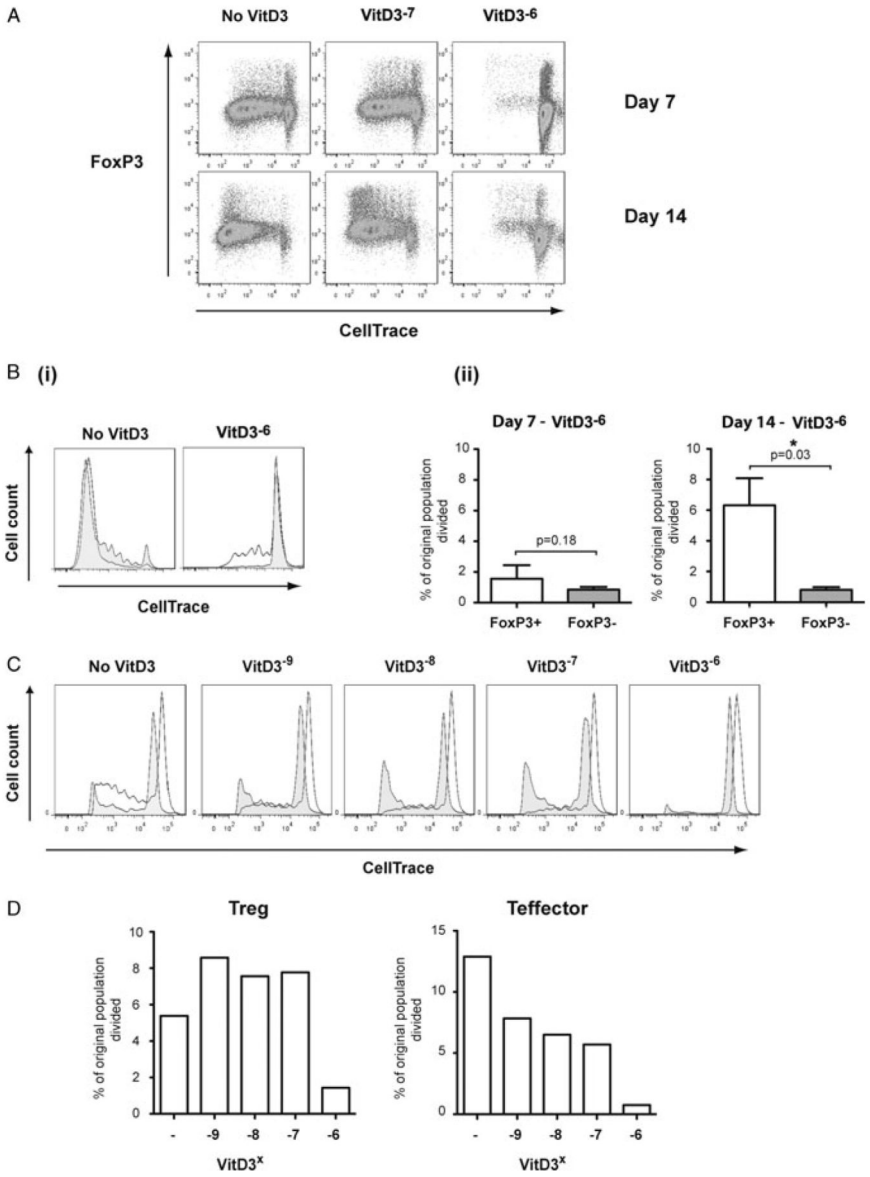

Figure 6.

1α25VitD3 favors the proliferation of Foxp3$^+$ over Foxp3- T cells.(A) Human CD4$^+$ T cells were labeled with CellTrace^{TM} Violet and then cultured for one or two 7-day cycles with anti-CD3, IL-2 (No VitD3), or additionally with the indicated concentration of 1α25VitD3 (VitD3; 10× M). At day 7 and 14, cells were stained for Foxp3 and analyzed by flow cytometry. Proliferation was assessed by loss of expression of CellTrace with each cell division. (B) (i) Representative histograms showing CellTrace expression in Foxp3$^-$ (filled histogram) and Foxp3$^+$ (open histogram) T cells at day 14. Note the peaks represent successive generations of cells. (ii) Data from day 7 (n = 7) and day 14 (n = 4) of the percentage of original population divided are shown as mean ± SEM of the indicated number of experiments. *$p < 0.05$ as determined by the Mann–Whitney rank sum test. (C) Overlay histograms from a representative donor showing CellTrace expression in individually labeled Treg or T-effector populations that were then co-cultured with the nonlabeled population for 14 days at the original ratio of 1:9 in the absence or presence of

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1α,25VitD3 as indicated. Filled histograms show proliferation of labeled Treg cells that were co-cultured with unlabeled T-effector cells. Open histograms assess proliferation of labeled T-effector cells, co-cultured with unlabelled Treg cells. (D) Data from (C) presented as percentage of original population divided. Representative data from three independent experiments each performed with different donors are shown.

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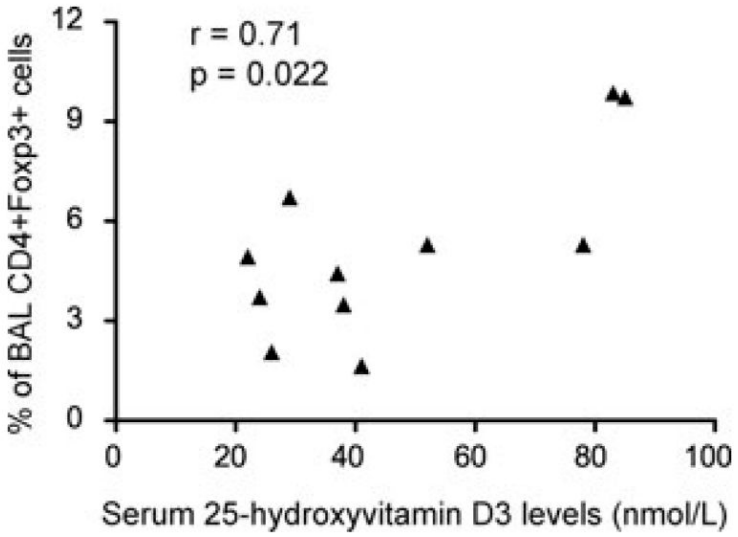

Figure 7.

Serum 25-hydroxyvitamin D3 correlates with the frequency of $CD4^+$ $FoxP3^+$ cells in BAL of pediatric asthma patients. Pediatric patients with severe therapy resistant asthma (STRA) were analyzed for the presence of Treg cells (percentage of $CD4^+$ cells expressing Foxp3) in bronchoaveolar lavage fluid. Serum was collected from the same patients and the concentration of the circulating form of vitamin D3, 25-hydroxyvitamin D3 was assessed by two-dimensional high performance liquid chromatography system–tandem mass spectrometry. Each point represents an individual patient from 11 experiments performed.The *r* and *p* values were assessed using Pearson's correlation test.

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