

1 α ,25-dihydroxyvitamin D₃ acts via transforming growth factor- β to up-regulate expression of immunosuppressive CD73 on human CD4⁺ Foxp3⁻ T cells

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Introduction

Vitamin D insufficiency has been negatively associated with multiple immune-mediated diseases which is believed to be attributed to its numerous immunomodulatory properties (recently reviewed in refs 1,2). We and others have shown that the frequency of Foxp3⁺ regulatory T cells can be increased by either high concentrations (10⁻⁶ M) of the active form of vitamin D (1 α ,25-dihydroxyvitamin D₃; 1,25(OH)₂D₃), or lower doses (10⁻⁷ to 10⁻⁸ M)

Summary

Vitamin D deficiency is associated with increased incidence and severity of various immune-mediated diseases. Active vitamin D (1 α ,25-dihydroxyvitamin D₃; 1,25(OH)₂D₃) up-regulates CD4⁺ T-cell expression of the purine ectonucleotidase CD39, a molecule that is associated with the generation of anti-inflammatory adenosine. Here we aimed to investigate the direct impact of 1,25(OH)₂D₃ on expression of the downstream ecto-5'-nucleotidase CD73 by human CD4 T cells, and components of the transforming growth factor- β (TGF- β) pathway, which have been implicated in the modulation of CD73 by murine T cells. At 10⁻⁸ to 10⁻⁷ M, 1,25(OH)₂D₃ significantly increased expression of CD73 on peripheral human CD4⁺ T cells. Although 1,25(OH)₂D₃ did not affect the mRNA expression of latent TGF- β ₁, 1,25(OH)₂D₃ did up-regulate expression of TGF- β -associated molecules [latency-associated peptide (LAP), glycoporphin A repetitions predominant (GARP), GP96, neuropilin-1, thrombospondin-1 and α_v integrin] which is likely to have contributed to the observed enhancement in TGF- β bioactivity. CD73 was highly co-expressed with LAP and GARP following 1,25(OH)₂D₃ treatment, but unexpectedly, each of these cell surface molecules was expressed primarily on CD4⁺ Foxp3⁻ T cells, rather than CD4⁺ Foxp3⁺ T cells. Notably, neutralization of TGF- β significantly impaired 1,25(OH)₂D₃-mediated induction of CD73. Collectively, we show that 1,25(OH)₂D₃ enhances expression of CD73 on CD4⁺ Foxp3⁻ T cells in a process that is at least partially TGF- β -dependent. These data reveal an additional contributing mechanism by which vitamin D may be protective in immune-mediated disease.

Keywords: 1 α ,25-dihydroxyvitamin D₃; CD73; Foxp3; transforming growth factor- β .

in the presence of high T-cell stimulation.^{3,4} Between 10⁻⁷ and 10⁻⁸ M 1,25(OH)₂D₃ can also increase the frequency of a population of regulatory CD4⁺ IL-10⁺ Foxp3⁻ T cells.³ Importantly these experimental data are supported by *in vivo* evidence whereby serum vitamin D status is positively associated with the frequency of Foxp3⁺ T cells in the periphery and airways, as well as levels of IL-10 in the airways.^{3,5-7} The downstream effects of 1,25(OH)₂D₃ are significantly influenced by the cytokine milieu, such that the combination of 1,25(OH)₂D₃ and

Abbreviation: 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃

transforming growth factor- β_1 (TGF- β_1) induces Foxp3 expression to a greater extent than either compound alone.⁸

Regulatory T cells employ various immunoregulatory mechanisms including the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) and TGF- β , as well as modulating dendritic cell behaviour and granzyme deployment.⁹ Of particular relevance here, cells can take up extracellular adenosine by two main mechanisms, either through specific adenosine transporters or through binding to one of four G-protein-coupled P1 purinergic receptors (A1, A2A, A2B and A3). Binding to the purinergic receptors can result in suppression of T-cell receptor signalling and induction of various immunomodulatory effects as reviewed elsewhere.^{10–12} In contrast, ATP is sensed by P2X and P2Y purinergic receptors and generally promotes a pro-inflammatory environment.¹³ The cell surface ecto-enzymes CD39 (ectonucleoside triphosphate dephosphorylase) and CD73 (ecto-5'-nucleotidase) regulate levels of ATP and adenosine, and therefore inflammation, by dephosphorylating ATP into ADP and AMP (CD39), and then into adenosine (CD73).^{10,11} We have previously shown that 1,25(OH)₂D3 can modulate this pathway by increasing expression of CD39 on peripheral blood human CD4⁺ T cells, which contributes to dampening IL-17A secretion.¹⁴

A multifaceted cytokine, TGF- β is involved in processes such as differentiation of T helper type 17 cells and Foxp3⁺ regulatory T cells, proliferation of CD4⁺ T cells and innate immune cell chemotaxis.¹⁵ Humans express three TGF- β isoforms (TGF β_{1-3}), all of which are synthesized in a latent form bound to latency-associated peptide (LAP) either alone or with latent TGF- β binding protein. Latent TGF- β can be expressed on the cell surface by the transmembrane orphan toll-like receptor glycoprotein A repetitions predominant (GARP), which itself requires the chaperone heat-shock protein GP96 for correct conformational folding.¹⁶ The active form of TGF- β is only released following cleavage of LAP or conformational remodelling by a range of molecules including neuropilin-1, thrombospondin-1 and various α_v integrins.^{17,18} The active moiety can then bind to a heterodimer of type I and II TGF- β receptors to mediate downstream effects.^{15,19}

Cross-talk between the TGF- β and adenosine pathways is indicated by the fact that treating mouse CD4⁺ T cells with TGF- β increases expression of CD73, and in CD73 knockout mice, experimental induction of TGF- β mRNA was blocked.^{20,21} Furthermore, the ability of CD4⁺ CD73⁺ T cells to delay the development of diabetes in a mouse model was impaired by anti-TGF- β , indicating a key protective role for this cytokine.²²

1,25(OH)₂D3 is known to enhance the frequency of human CD4⁺ CD39⁺ T cells; however, whether it controls the expression of the related ecto-5'-nucleotidase CD73 is

unknown. We further investigated the role of TGF- β in this process given existing evidence for cross-talk between the purine and TGF- β pathways in mice.

Materials and methods

Cell isolation and culture

Ethical approval was granted by Guy's Hospital Ethics Committee (09/H0804/77) and full written informed consent was obtained from all donors. CD4⁺ cells were isolated from peripheral venous blood as previously described using Dynabeads (Invitrogen, Paisley, UK).³ Then, 1×10^6 cells/ml were cultured in RPMI-1640 containing 10% fetal calf serum, 2 mM L-glutamine and 50 μ g/ml gentamycin, and stimulated with plate-bound anti-CD3 (1 μ g/ml; OKT-3) and 50 IU/ml recombinant human IL-2 (Eurocetus, Harefield, UK) in the presence or absence of 1,25(OH)₂D3 (BIOMOL Research Labs, Exeter, UK). For cultures going beyond 7 days, cells were counted and re-stimulated at 1×10^6 cells/ml on day 7 with fresh medium containing drugs. Where relevant, anti-TGF- β^{1-3} or an isotype control (5 μ g/ml; R&D Systems, Abingdon, UK) was added to the cultures on days 0 and 7. Viability did not significantly differ between culture conditions (data not shown).

Quantitative RT-PCR

RNA was extracted from cell pellets using the RNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and quantified using a Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, NC). RNA (250 ng) was reverse transcribed into cDNA then quantitative RT-PCR was performed in triplicate using an Applied Biosystems 7900 HT system and FAM labelled assay-on-Demand reagent sets (*cd73* – Hs00159686_m1; *tgfb1* – Hs00998133_m1; *lrcc32* – Hs00194136_m1; *gp96* – Hs00427665_g1; *nrp-1* – Hs00826128_m; *tsp-1* – Hs00962908_m1; α_v *integrin* – Hs00233808_m1). Quantitative RT-PCR were multiplexed using VIC-labelled *18s* probe (Hs99999901_s1) as an endogenous control and analysed using SDS software version 2.1 (Applied Biosystems, Foster City, CA) in accordance with the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

The following antibodies were used for cell surface phenotyping – CD73 (AD2; eBiosciences, Hatfield, UK), GARP and LAP (7B11 and TW4-2F8, respectively; Biolegend, London, UK). Where relevant, cells were further stained for intranuclear Foxp3 (PCH101; eBiosciences) using the eBiosciences Foxp3 staining kit as per the man-

manufacturer's instructions. Unstained cells, isotype controls, single cell stains and fluorescence minus one controls were employed. Dead cells (7-aminoactinomycin D positive; Sigma-Aldrich, Gillingham, UK) were gated out.

ATP assay

After 7 days of culture alone or in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$, 25,000 $\text{CD}4^+$ T cells were seeded in 100 μl culture medium with 500 μM ATP. Consumption of ATP was assessed 90 minutes later using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Southampton, UK) in accordance with the manufacturer's instructions. A standard curve was generated immediately before running the assay by dissolving ATP disodium salt hydrate (Sigma-Aldrich) in cell culture medium (100 μM to 100 mM).

Suppression assay

$\text{CD}4^+$ T cells were cultured with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 7 days as described, then live cells (7-aminoactinomycin-D-negative) were sorted based on CD73 expression using a BD FACSAria (BD Biosciences, Oxford, UK). $\text{CD}73^-$ or $\text{CD}73^+$ cells were subsequently cultured at a ratio of 0.25 : 1 with freshly isolated autologous CellTrace violet-labelled $\text{CD}4^+$ T cells for a further 7 days. Cell proliferation was assessed by the loss in fluorescence intensity of CellTrace Violet on an NxT attune (Life Technologies, Paisley, UK).

TGF- β bioassay

Bioactivity of TGF- β was assessed using mink lung epithelial cells transfected with a construct containing the 5' end of the human plasminogen activator inhibitor-1 gene fused to the firefly luciferase reporter gene (kindly donated by Prof. Daniel B Rifkin; New York University).²³ Then, 3×10^5 mink lung epithelial cells/ml were incubated for 14 hr at 37°C in 5% CO_2 with cell culture supernatants. Culture media were aspirated, cells were lysed and then luciferase activity (which corresponds to TGF- β activity) was determined using a Biotium Firefly Luciferase Assay Kit (Promega, Southampton, UK) in accordance with the manufacturer's instructions. Serum-free RPMI-1640 was used for these experiments due to high levels of TGF- β found in fetal calf serum.

Data analysis

Flow cytometry data were analysed using FLOWJO (Trestar Inc., Ashland, OR; version 10) and cumulative data analysis was performed in GRAPHPAD PRISM version 6.00 for Windows (Graphpad Software Inc., San Diego, USA). Data were assessed for Gaussian distribution and the

appropriate statistical test was then performed as described in the figure legends. Data are shown as mean \pm standard error of mean.

Results

1,25(OH) $_2$ D3 up-regulates $\text{CD}4^+$ T-cell expression of CD73

It has previously been shown that human $\text{CD}4^+$ T cells cultured in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ express elevated levels of CD39.¹⁴ Here this observation is extended to reveal that expression of downstream ecto-5'-nucleotidase CD73 is also significantly up-regulated at the gene (Fig. 1a) and protein level (% $\text{CD}73^+$ cells and mean fluorescence intensity; Fig. 1b and c) following 7 or 14 days culture with $1,25(\text{OH})_2\text{D}_3$. Furthermore, a trend towards lower ATP levels 90 min after spiking cells with 500 μM ATP was observed in $1,25(\text{OH})_2\text{D}_3$ -treated $\text{CD}4^+$ T cells ($P = 0.06$; see Supplementary material, Fig. S1A). $\text{CD}73^+$ cells were also shown to be regulatory because they suppressed the proliferation of autologous CellTrace Violet-labelled $\text{CD}4^+$ T cells (ratio of 0.25 : 1) to a greater extent than $\text{CD}73^-$ cells (lower percentage of cells divided ($P < 0.05$) and division index ($P = 0.09$); see Supplementary material, Figure S1B,C).

1,25(OH) $_2$ D3 up-regulates expression of TGF β -associated molecules

To investigate whether enhancement of CD73 expression was TGF- β dependent, we first assessed the impact of $1,25(\text{OH})_2\text{D}_3$ on components of the TGF- β pathway. After 7 and 14 days in culture alone or in the presence of the indicated concentration of $1,25(\text{OH})_2\text{D}_3$, $\text{CD}4^+$ T-cell expression of latent *tgfb2* and *tgfb3* was typically undetectable by quantitative RT-PCR (data not shown). Although latent *tgfb1* was highly expressed at the mRNA level, its expression was not modulated by $1,25(\text{OH})_2\text{D}_3$ treatment (Fig. 2a). In contrast, *lrrc32* (gene for GARP) mRNA was significantly up-regulated by $1,25(\text{OH})_2\text{D}_3$ with a trend ($P = 0.13$) towards increased expression of *gp96*, a chaperone for GARP, by day 14. At the protein level, $1,25(\text{OH})_2\text{D}_3$ treatment significantly increased overall expression of GARP and LAP as well as the percentage of cells co-expressing both of these molecules (Fig. 2b–d).

In order to generate active TGF- β , LAP must be cleaved. Within the immune cell compartment, this is reported to be primarily carried out by the cell surface expressed molecules thrombospondin-1, neuropilin-1 and α_v integrin,^{17,18} each of which were also up-regulated by $1,25(\text{OH})_2\text{D}_3$ treatment at the mRNA level, most notably after 14 days in culture ($P = 0.09$, $P < 0.05$ and $P < 0.05$, respectively; Fig. 2e, and see Supplementary material, Fig. S2).

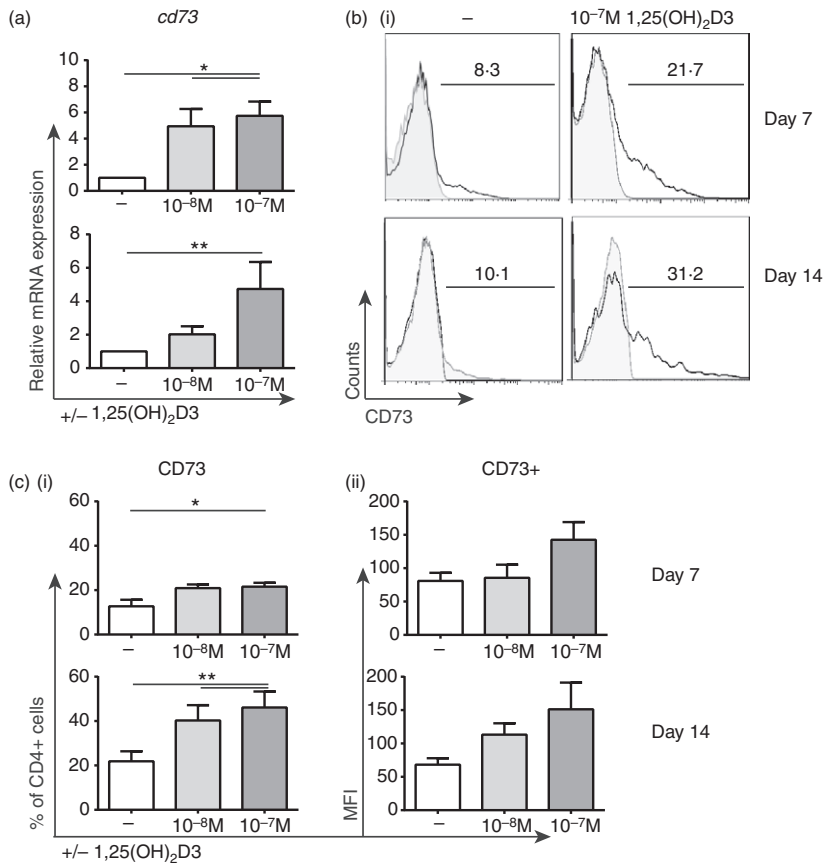


Figure 1. $1,25$ -dihydroxyvitamin D₃ ($1,25$ (OH)₂D₃) up-regulates CD73 expression on CD4⁺ T cells. CD4⁺ T cells stimulated with anti-CD3 and interleukin-2 (IL-2) for 7 or 14 days alone (−) or in the presence of the indicated concentration of $1,25$ (OH)₂D₃ (10^{-x} M). (a) Relative gene expression of *cd73* as determined by quantitative RT-PCR at days 7 ($n = 8$) and 14 ($n = 6$). (b) Representative histograms of CD73 expression (bold line) compared with isotype control (grey shaded). (c) Cumulative data of the percentage of CD4⁺ CD73⁺ T cells (c i) and CD73 mean fluorescence intensity (c ii) at days 7 (top; $n = 8$) and 14 (bottom, $n = 6$). Data assessed by repeated measures one-way analysis of variance with Tukey's multiple comparison test. * $P \leq 0.05$; ** $P \leq 0.01$.

$1,25$ (OH)₂D₃-mediated up-regulation of CD73, LAP and GARP occurs primarily on CD4⁺ Foxp3[−] T cells

Foxp3 is conventionally seen as the lineage defining transcription factor of natural and induced regulatory T cells, but Foxp3[−] suppressor cells do exist.²⁴ As the purinergic and TGF- β pathways have been linked to the function of regulatory T cells,⁹ the interaction between intranuclear Foxp3 expression and surface expression of CD73, LAP and GARP was assessed. In agreement with previous findings, no significant increase in Foxp3 expression was observed in the presence of 10^{-7} M $1,25$ (OH)₂D₃ (Fig. 3).³ Furthermore, $1,25$ (OH)₂D₃-mediated up-regulation of CD73 (Fig. 3a, b), GARP (Fig. 3c, d) and LAP (Fig. 3e, f) was principally seen on CD4⁺ Foxp3[−] T cells as opposed to CD4⁺ Foxp3⁺ cells.

Cross-talk between CD73 and TGF- β in $1,25$ (OH)₂D₃-treated CD4⁺ T cells

Mouse studies have shown that cross-talk can occur between the adenosine and TGF- β pathways,^{20,22} but whether this is true in humans and any impact of $1,25$ (OH)₂D₃ remains to be ascertained. Indeed, cell surface staining showed a significant increase in the percentage of cells co-expressing CD73 with TGF- β -associated molecules

LAP, GARP and neuropilin -1 following $1,25$ (OH)₂D₃ treatment (Fig. 4).

Furthermore, TGF- β bioactivity was elevated in supernatants harvested after 7 days of culture with $1,25$ (OH)₂D₃ ($P = 0.09$), as determined using mink lung epithelial cells and a luciferase assay (Fig. 5a). These observations were extended by culturing CD4⁺ T cells in the presence of a neutralizing TGF- β antibody or the relevant isotype control. Anti-TGF- β impaired the $1,25$ (OH)₂D₃-mediated induction of CD73 at the mRNA level ($P = 0.08$; Fig. 5a) and in both the percentage of CD4⁺ CD73⁺ T cells and the mean fluorescence intensity of CD73 ($P < 0.05$; Fig. 5b).

Discussion

The frequency of regulatory T-cell populations is known to be enhanced by vitamin D both *in vitro* and *in vivo*. However, how these cells suppress in different environments and what markers identify them is not clear.^{3,4} The focus of this work was to assess the immunomodulatory effects of $1,25$ (OH)₂D₃ on the induction of two complex immunoregulatory molecules, CD73 and TGF- β .

In the present study a significant up-regulation of CD73 expression in CD4⁺ T cells by $1,25$ (OH)₂D₃ was observed, complementing the previously published finding

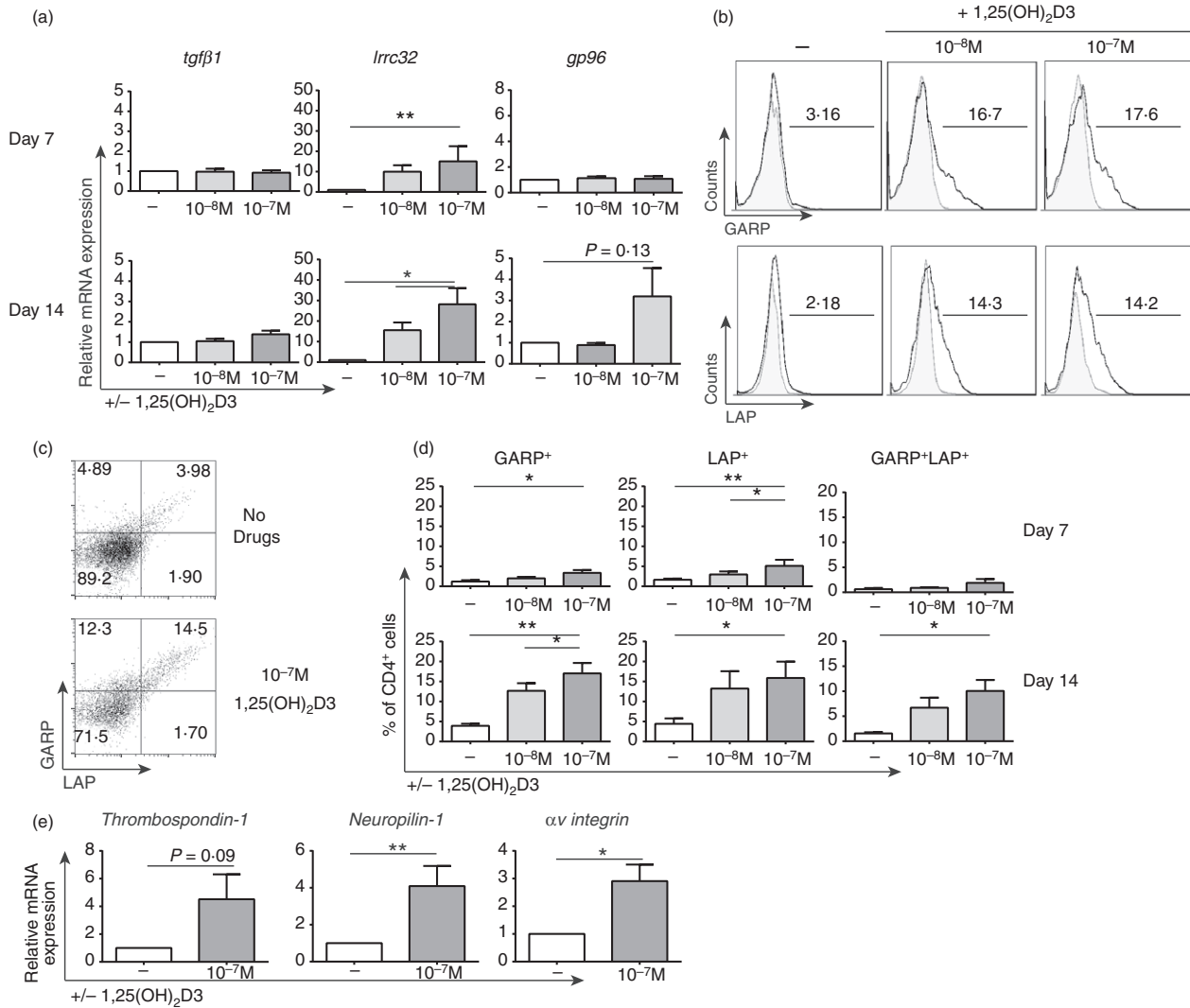


Figure 2. 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) up-regulates expression of transforming growth factor- β (TGF- β)-associated molecules. CD4⁺ T cells stimulated (anti-CD3/IL-2) for 7 or 14 days alone (-) or in the presence of the indicated concentration of 1,25(OH)₂D₃ (10^{-x} M). (a) Relative gene expression of *tgfb1*, *lrcc32* and *gp96* at days 7 (top) and 14 (bottom) as determined by quantitative RT-PCR [*n* = 7; repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple comparison test]. Representative histograms (b) and dot plots (c) of glycoprotein A repetitions predominant (GARP) and latency-associated peptide (LAP) expression at day 14. (d) Cumulative data showing the frequency of GARP⁺ (left), LAP⁺ (middle) and LAP⁺GARP⁺ (right) CD4⁺ T cells at day 7 (top) and day 14 (bottom) (*n* = 7; repeated measures one-way ANOVA with Tukey's multiple comparison test). (e) Relative gene expression of *thrombospondin-1*, *neuropilin-1* and α_v integrin at day 14 as determined by quantitative RT-PCR (*n* = 8; paired *t*-test). **P* ≤ 0.05; ***P* ≤ 0.01.

that 1,25(OH)₂D₃ enhances surface expression of CD39.¹⁴ Heightened expression of these two ecto-nucleotidases most likely contributes to the observed faster consumption of ATP by 1,25(OH)₂D₃-treated cells (Fig. S1A) and to the enhanced generation of downstream adenosine.²⁵ Studies have shown that CD73 is critical in mediating many immunosuppressive properties of cells in mice and man^{26,27} and that adenosine can impair T-cell proliferation in humans.^{28,29} The latter data correlate with the anti-proliferative properties of CD73⁺ cells that are reported both here and elsewhere.³⁰ As well as the

traditional CD39 and CD73 pathway for ATP catabolism, a CD39-independent pathway involving CD38 and CD73 has been documented in human T cells.³¹ As CD38 is known to be highly up-regulated on T cells in response to 1,25(OH)₂D₃,^{3,32} we hypothesize that 1,25(OH)₂D₃ is further enhancing ATP breakdown and adenosine generation by acting via the CD39-dependent and -independent pathways.

Since 10⁻⁷ M 1,25(OH)₂D₃ has previously been shown to increase the frequency of CD4⁺ Foxp3⁺ cells in the presence of TGF- β ,⁸ the relationship between 1,25

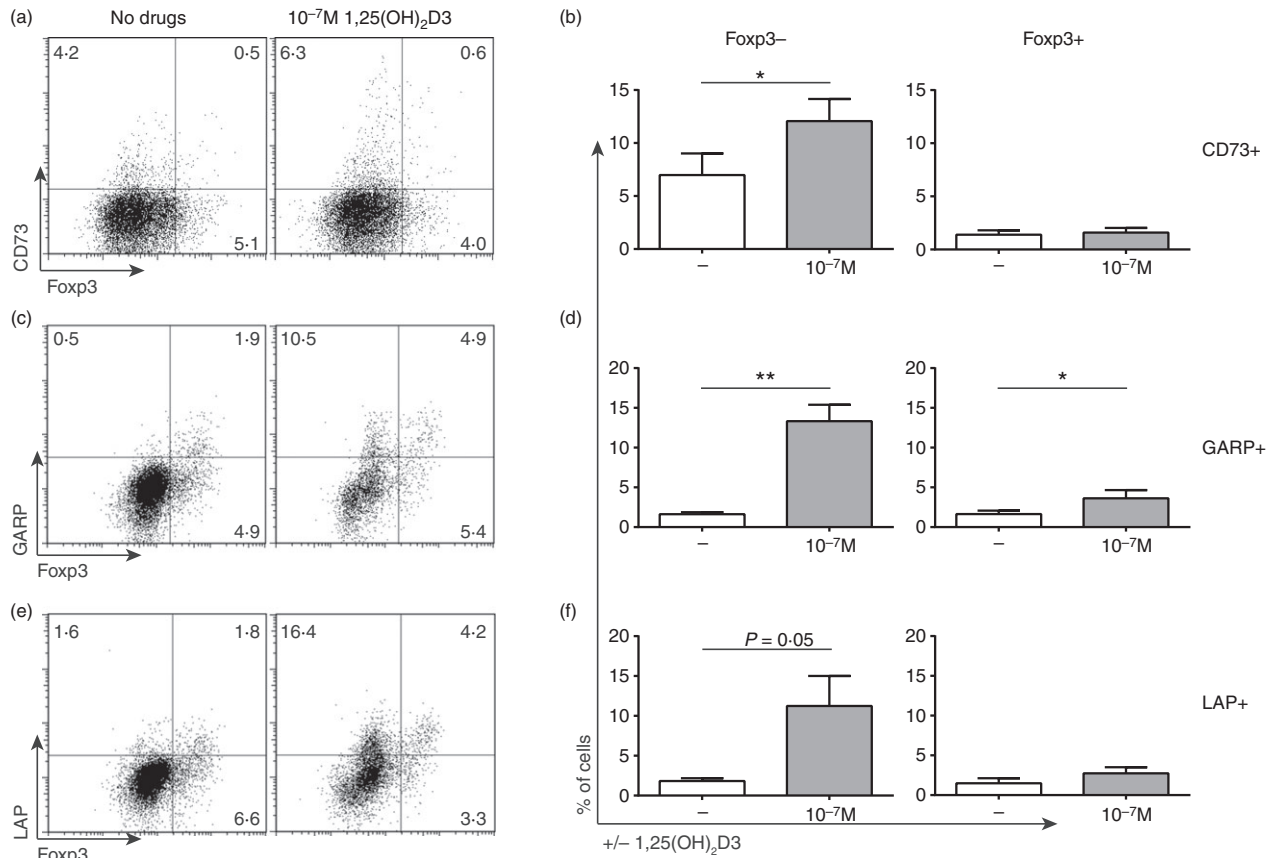


Figure 3. $1\alpha,25$ -dihydroxyvitamin D3 ($1,25(\text{OH})_2\text{D}_3$)-mediated up-regulation of CD73, latency-associated peptide (LAP) and glycoprotein A repetitions predominant (GARP) occurs primarily on Fcpx3⁻ CD4⁺ T cells. CD4⁺ T cells stimulated [anti-CD3/interleukin-2 (IL-2)] alone (-) or in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 14 days. Surface staining for CD73, GARP and LAP was performed before intranuclear Fcpx3 staining. Representative dot plots showing expression of Fcpx3 and CD73 (a), GARP (c) or LAP (e). Cumulative data of the percentage of cells expressing CD73 (b; $n = 6$), GARP (d; $n = 6$) or LAP (f; $n = 6$) on either Fcpx3⁻ or Fcpx3⁺ CD4⁺ T cells. Data assessed by paired *t*-test. * $P \leq 0.05$; ** $P \leq 0.01$.

(OH)₂D3 and TGF- β was investigated further. $1,25(\text{OH})_2\text{D}_3$ increased expression of LAP (component of latent TGF- β), GARP (latent TGF- β receptor) and GP96 (latent TGF- β chaperone), which together result in cells that have an enhanced capacity to both generate and present latent TGF- β . These data are in line with a study in patients with multiple sclerosis in whom vitamin D supplementation resulted in increased levels of LAP in the serum relative to those in the placebo group.³³ Although a whole host of molecules and environmental conditions such as heat and extreme pH have been implicated in activating TGF- β by cleaving LAP, α_v integrins, neuropilin-1 and thrombospondin-1 are the primary mediators within the immune cell compartment;¹⁸ here expression of each of these molecules, at least at the mRNA level, was shown to be higher in $1,25(\text{OH})_2\text{D}_3$ -treated cells, which most likely contributed to the enhanced TGF- β bioactivity observed.

In addition to its anti-inflammatory role, TGF- β is involved in tissue remodelling and therefore can have

potentially negative consequences in diseases such as asthma.³⁴ However, $1,25(\text{OH})_2\text{D}_3$ has also been shown to inhibit the proliferation of airway smooth muscle cells.³⁵ Moreover, *in utero* vitamin D deficiency in mice leads to increased airway smooth muscle mass and airway resistance³⁶ and in children with severe asthma, lower levels of vitamin D were associated with increased airway smooth muscle mass.^{37,38} As with many *in vitro* studies, the data presented here are observational. Actual levels of molecules as well as their expression relative to other mediators *in vivo* are critical; for example, lung remodelling in mice has been shown to result from an imbalance between TGF- β_1 and bone morphogenic protein 7 (BMP-7).³⁹ Whether TGF- β acts in a regulatory or tissue remodelling manner in various disease contexts is most likely dependent on the cytokine milieu as a whole and so warrants studying *in vivo*.

A major limitation in the study of regulatory T cells is a lack of specific cell surface markers, particularly in

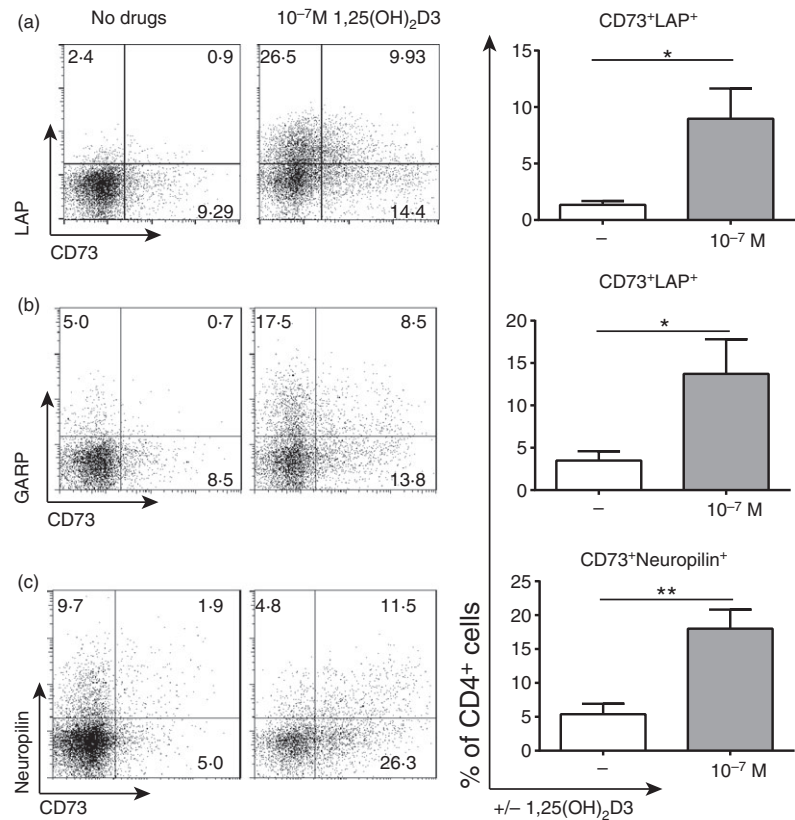


Figure 4. CD73 is co-expressed with latency-associated peptide (LAP) and glycoprotein A repetitions predominant (GARP) following $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25(\text{OH})_2\text{D}_3$) treatment. CD4⁺ T cells stimulated [anti-CD3/interleukin-2 (IL-2)] alone (-) or in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 14 days. Shown are representative dot plots and cumulative data of the percentage of cells co-expressing CD73 with LAP (a; $n = 7$), GARP (b; $n = 6$) or neuropilin-1 (c; $n = 5$). Data assessed by paired t -test. * $P \leq 0.05$; ** $P \leq 0.01$.

humans.^{40,41} Foxp3 is a widely accepted marker of many regulatory T-cell populations, but is intracellular so cells must be permeabilized in order to perform staining, which limits the isolation of pure and live cells. Since CD73, GARP and LAP are putative markers of regulatory T cells,^{42–44} their relationship with Foxp3 was examined in our culture conditions. CD73 was expressed primarily on CD4⁺ Foxp3⁻ T cells and $1,25(\text{OH})_2\text{D}_3$ -mediated induction of CD73, GARP and LAP was only clearly evident on CD4⁺ Foxp3⁻ T cells. In support of this, Foxp3 expression has been found to be independent of GARP expression in cells from HIV-positive patients.⁴⁵ Although not absolute markers, these data support the idea that CD73, GARP and LAP help to identify Foxp3⁻ regulatory cells.

Previous studies have shown that cross-talk between the TGF- β and adenosine pathways exists in mice,^{20–22} and here we confirm this in humans too. A significant proportion of CD4⁺ T cells co-express CD73 with GARP, LAP and neuropilin-1 following $1,25(\text{OH})_2\text{D}_3$ treatment and by impairing TGF- β activity, the induction of CD73 by $1,25(\text{OH})_2\text{D}_3$ was reduced.

A high concentration of $1,25(\text{OH})_2\text{D}_3$ ($10^{-8/7}$ M/ 10^{-100} nM) was used throughout this work for optimal *in vitro* responses, although trends were seen at 10^{-9} M (data not shown). Previous *in vitro* studies have shown that monocyte-derived DCs, alveolar macrophages and bronchial epithelial cells express the enzyme 25-hydrox-

vitamin D3- 1α -hydroxylase and can consequently produce up to 5×10^{-9} M functional $1,25(\text{OH})_2\text{D}_3$ from 25(OH)D.^{46–49} Although the levels of $1,25(\text{OH})_2\text{D}_3$ produced depend upon the concentration of 25(OH)D,⁵⁰ there is evidence that systemic levels of $1,25(\text{OH})_2\text{D}_3$ may not reflect an individual's vitamin D status;⁵¹ these findings highlight the importance of local conversion of 25(OH)D within immune tissues producing currently undefined levels of $1,25(\text{OH})_2\text{D}_3$.

In summary, we show that $1,25(\text{OH})_2\text{D}_3$ can enhance expression of CD73 on CD4⁺ Foxp3⁻ T cells in part by up-regulating autocrine TGF- β bioactivity. These data help to further elucidate the mechanisms by which vitamin D may skew the immune system towards a more regulatory phenotype and consequently protect against various immune-mediated diseases. Further work to probe the functional consequences of these observations is warranted both *in vitro* and *in vivo*.

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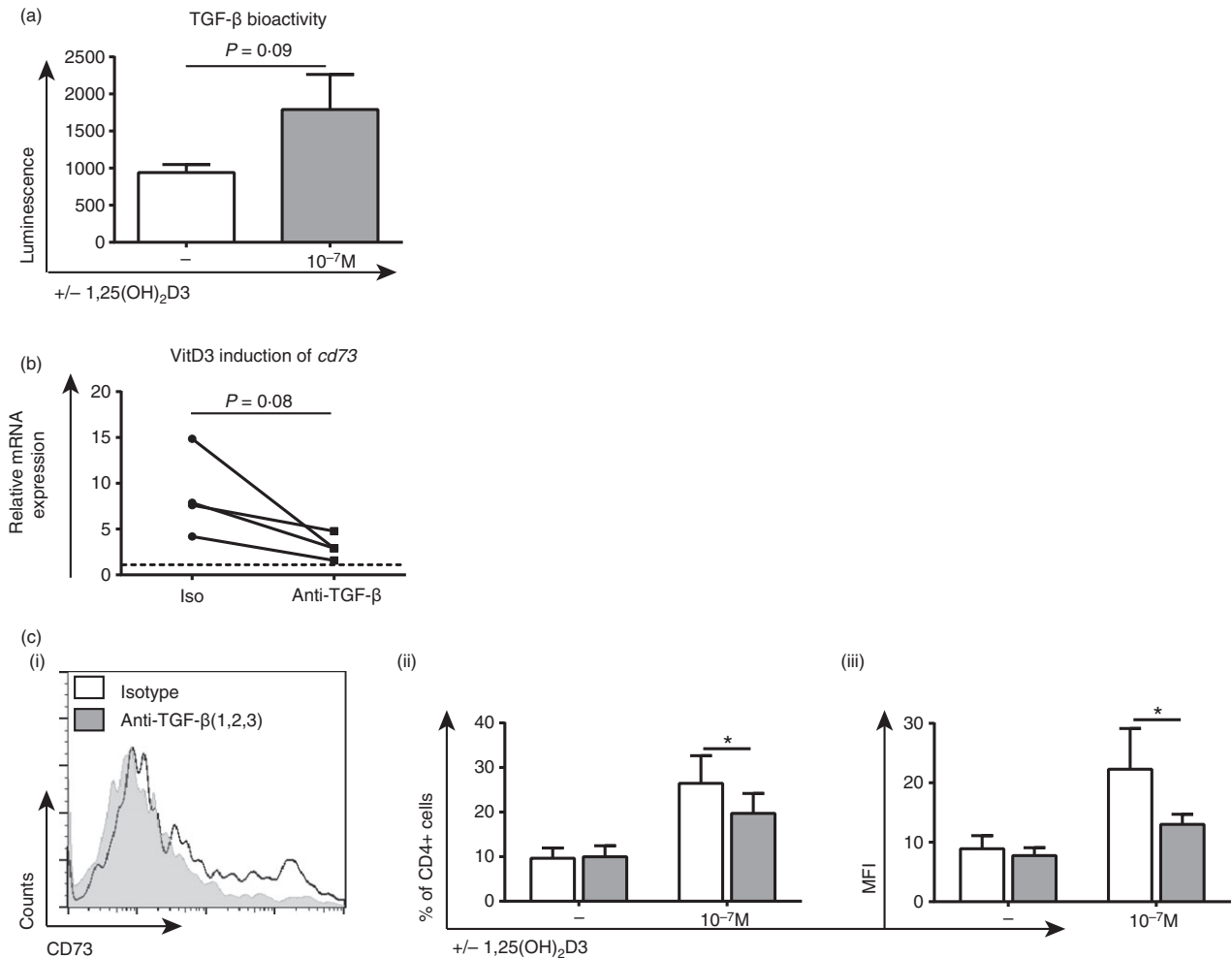


Figure 5. 1,25-dihydroxyvitamin D3 (1,25(OH)₂D3)-mediated up-regulation of CD73 is partially inhibited by anti-transforming growth factor-β (TGF-β). CD4⁺ T cells stimulated [anti-CD3/interleukin-2 (IL-2)] alone (–) or in the presence of 10^{–7} M 1,25(OH)₂D3. (a) Day 7 supernatants were harvested and TGF-β bioactivity was assessed using transfected mink lung epithelial cells and a luminescence assay; levels of luminescence were proportional to TGF-β bioactivity ($n = 7$; paired t -test). (b, c) Cells were cultured with anti-TGF-β (grey) or an isotype control for 14 days. (b) 1,25(OH)₂D3-mediated induction of *cd73* gene expression as determined by quantitative RT-PCR ($n = 4$; two-tailed paired Wilcoxon test). (c i) representative histograms of CD73 expression and cumulative data of the percentage of CD4⁺ CD73⁺ T cells (c ii) and CD73 mean fluorescence intensity (c iii) ($n = 5$; data assessed by two-way analysis of variance with Sidak's multiple comparison test). * $P \leq 0.05$.

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Disclosures

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CD4⁺ CD73⁺ T cells suppress the proliferation of autologous CD4⁺ T cells to a greater extent than CD4⁺ CD73⁻ T cells.

Figure S2. The effect of $1\alpha,25$ -dihydroxyvitamin D3 ($1,25(\text{OH})_2\text{D}_3$) treatment on the mRNA expression of transforming growth factor- β -associated molecules.