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1α,25- dihydroxyvitamin D3 (vitamin D3) catalyzes suppressive activity on human natural regulatory T cells, uniquely modulates cell cycle progression, and augments FOXP3

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Abstract

Human natural regulatory T cells (nTregs) show great promise for therapeutically modulating immune-mediated disease, but remain poorly understood. One explanation under intense scrutiny is how to induce suppressive function in non-nTregs and increase the size of the regulatory population. A second possibility would be to make existing nTregs more effective, like a catalyst raises the specific activity of an enzyme. The latter has been difficult to investigate due to the lack of a robust short-term suppression assay. Using a microassay described herein we demonstrate that nTregs in distinct phases of cell cycle progression exhibit graded degrees of potency. Moreover, we show that physiological concentrations of 1α,25-dihydroxyvitamin D3 (vitamin D3) boosts nTregs function. The enhanced suppressive capacity is likely due to vitamin D3's ability to uniquely modulate cell cycle progression and elevate FOXP3 expression. These data suggest a role for vitamin D3 as a mechanism for catalyzing potency of nTregs.

Keywords

regulatory T cells; FOXP3; vitamin D3; immune regulation; immunotherapy; tolerance

1. Introduction

Immune-mediated disease causes substantial morbidity and mortality. Therapeutic modalities to reign in auto-aggressive immune reactions are limited and compromise a patient's ability to respond to life-threatening infections. Studies to understand the immune system's own rheostat mechanism for preventing autoimmunity, i.e. natural regulatory T

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cells (nTregs), have shown promising efficacy in numerous animal models of disease. Many investigations have focused on the process of “infectious tolerance” or how non-regulatory T cells acquire suppressive function. Fewer investigations regarding mechanisms to augment or “catalyze” regulatory activity from existing nTregs have been reported and parallel studies using human nTregs have been particularly limited [1].Given the challenges associated with obtaining therapeutic quantities of nTregs, elucidation of mechanisms which enhance suppressive potency may provide insight to develop immunotherapies which achieve clinical efficacy with fewer cells.

To investigate the mechanisms controlling the potency of human nTregs, we first considered how to best demonstrate superior function when comparing samples from a heterogeneous group of individuals. Since most experiments do not yield sufficient primary nTregs for elegant *in vivo* models using immunocompromised mice [2;3;4], most studies have been relegated to *in vitro* proliferation assays. However, proliferation assays can be cumbersome and are not always suitable for smaller amounts of peripheral blood. Another caveat is that standard proliferation assays most commonly used to show suppressive function (generally inhibition of CFSE dilution or ^{3}H-thymidine incorporation) take days to complete, thereby obscuring early molecular events. Thus, we developed a short-term suppression assay (6 hours) requiring yet fewer nTregs. Importantly this assay is amenable to a wide titration of nTregs, allowing an objective measure of nTregs quality. Using this assay, we demonstrate graded degrees of activity for primary human nTregs in specific phases of cell cycle progression, directly linking this process with functional capacity. The ability to modulate cell cycle progression and known immunomodulatory properties prompted us to evaluate vitamin D3 as a physiologically important candidate for modulating nTregs function [5;6;7;8]. Utilizing the criteria described above, enhanced suppression across titrated doses of nTregs, we show that vitamin D3 significantly augments suppressive activity. Moreover, vitamin D3 likely mediates its effects through modulation of cell cycle progression and increased FOXP3 expression. These data support the notion that increasing the quality of regulatory function may be a promising strategy in settings where large quantities of therapeutic nTregs are not available.

2. Materials and Methods

2.1 Peripheral Blood Samples

Peripheral blood was obtained from either healthy platelet donors at St. Jude Children's Research Hospital Blood Donor Center with permission from the Institutional Review Board (IRB) or purchased from Lifeblood Biological Services (Memphis, TN). Peripheral blood was obtained from a severely ill child diagnosed with IPEX at St. Jude Children's Research Hospital with permission from the Institutional Review Board (IRB) and parental consent.

2.2 Purification of $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+}$ T lymphocytes

$CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+}$ populations were isolated using an AutoMACS® cell sorter following manufacturer's instructions ($CD4^{+}CD25^{+}$ Regulatory T Cell Isolation Kit, Miltenyi Biotec Inc., Auburn, CA). Purities (>95%) were assessed by flow cytometry.

2.3 Cell culture

$CD4^{+}CD25^{+}$ Tregs in culture medium (X-Vivo supplemented with 2mM L-glutamine, 15% Human Serum, and 10U/ml recombinant human IL2) were activated with anti-CD2/CD3/CD28 antibody-coated beads (MACS® T Cell Activation/Expansion Kit – Miltenyi Biotec Inc.) following manufacturer's conditions for the indicated times. Vitamin D3 was added at 20nM where indicated.

2.4 Suppression microassay

10,000 $CD4^+CD25^-$ T cells were activated as described above in the presence or absence of nTregs for 6 hours, unless otherwise indicated. Control conditions included nTregs cell cultures and $CD4^+CD25^-$ T cell cultures alone.

2.5 nTregs identification by labeling with fluorescent probes

Isolated nTregs were labeled with 1μM CMRA Cell Tracker™ (Molecular Probes, Invitrogen, CA) using serum-free media. Cells were incubated for 45 minutes at 37°C, followed by two washes using serum-free media prior to resuspension in culture media.

2.6 Propidium Iodide staining

Cell samples were washed once with PBS followed by labeling with Propidium Iodide solution (Propidium Iodide, sodium citrate and Triton X-100, Sigma, St. Louis, MO). Samples were treated with RNAse (Calbiochem, USA) for 30 minutes at room temperature prior to analysis.

2.7 Hoescht 33342

Cells were labeled with 10μM Hoescht 33342 in media containing human serum (Sigma, St. Louis, MO) for 40 minutes at 37°C, followed by two washes using PBS/2% human serum. Hoescht 33342 was added at a final concentration of 1μM to avoid efflux of the dye.

2.8 mRNA analysis

Total RNA was extracted using the QIAGEN RNeasy® Micro Kit (QIAGEN, Valencia, CA) and reverse transcribed using the TaqMan® Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Human *IL2* mRNA was tested using *ABL1* as the endogenous control. nTregs mRNA was used as a calibrator sample and mRNA from $CD4^+CD25^-$ T cells as a positive control. Human *VDR* and *FOXP3* mRNA were tested using the TaqMan® Gene Expression Assays. Gene Assays: *IL2*: Hs00174114_m1, *ABL1*: Hs00245445_m1, *FOXP3*: Hs00203958_m1, *VDR*: Hs00172113_m1.

2.9 Confocal Microscopy Analysis

nTregs were activated as described above in the presence or absence of 20nM vitamin D3 in 35mm Poly-d-lysine dishes with 10mm coverslips for five days. Cells were fixed with 1.5% paraformaldehyde (PFA) and permeabilized with 0.3% Triton X-100 followed by anti-FOXP3 Alexa 488 (clone 259D – BioLegend, San Diego, CA) staining. ProLong® Gold anti fade reagent with DAPI was used as mounting solution prior to study. Analysis was performed using Nikon C1Si confocal/ TE2000 microscope and NIS Elements software (Nikon Inc., Melville, NY).

2.10 Flow cytometry

Cellular surface markers were assessed by flow cytometry using the following antibodies: anti-CD4 APC (clone SK3, Becton Dickinson, CA) anti-CD25 PE (clone 4E3, Miltenyi Biotec); anti-CD127 FITC (clone hIL-7R-M21, Becton Dickinson). Samples were run on a FACS Calibur using Cell Quest software (BD Biosciences) and analyzed using FlowJo 8.8.2 software (Ashland, OR).

2.11 Intracellular Staining

Cells were fixed in 1.5% PFA and permeabilized with cold 100% methanol prior to staining (clone 259D BioLegend, San Diego, CA; clone PCH101 eBioscience, San Diego, CA; and the isotype controls recommended by the respective manufacturers). FOXP3 protein in

nTregs from the patient with IPEX was detected using the anti-human FOXP3 Flow kit (BioLegend); Alexa-Fluor 488 anti-FOXP3 (clone 259D), Alexa-Fluor 488 mouse IgG1 k isotype control and anti-human CD4-PE-Cy5/CD25-PE cocktail (clones RPA-T4/BC96) as per manufacturer's instructions. Plots show the comparison between the anti-FOXP3 staining or respective IgG antibody isotype control using FlowJo 8.8.2 software.

2.12 Protein analysis by Western Blot

Cell extracts were lysed and fractionated (cytoplasmic and nuclear extracts) using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL) as per manufacturer's conditions. Quantitation was done using the BCA™ Protein Assay Kit (Thermo Scientific). 10μg of protein was loaded per lane, separated by SDS-PAGE and transferred to an Immobilon™ PVDF Transfer Membrane (Millipore, Bedford, MA), followed by overnight blocking. Primary antibodies were incubated for 2 hours at RT, followed by several washes and incubation with secondary antibodies for 1 hour at RT. Membranes were developed using the SuperSignal® West Pico Chemiluminescent Substrate as per manufacturer's instructions (Thermo Scientific). FOXP3 detection: clone H190 rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.13 Analysis and statistics

Where possible, samples were set up in triplicate and each respective cDNA was analyzed for *IL2* in triplicate. Standard deviations (SD) were determined by paired t test using GraphPad Prism software (San Diego, CA). If culture samples were set up in duplicate followed by analyzing the cDNA for *IL2* content in triplicate, standard errors (SE) were determined by Excel software (Henderson, NV).

3. Results

Initial attempts from our laboratory to identify physiological parameters, which catalyze human nTregs activity, suggested that control of cell cycle progression may be linked to suppressive potency (Riberdy *et al.*, manuscript in preparation). However, to more carefully explore the relationship between regulatory potency and cell cycle progression, an assay meeting several criteria was needed. First, to interpret events that occur during a given round of division it was critical to reduce the assay duration from days (as required for standard proliferation assays) to one completed within hours. Secondly, a functional assay sufficiently robust to measure suppression across a heterogeneous range of responses, yet adequate to detect those approaching the lower end of the spectrum was important. Thirdly, since human nTregs investigations are hampered by the limited availability of cells, particularly in clinical studies of various immune-mediated diseases, suppression assays necessitating fewer cells would be highly desirable. The crucial role of IL2 production and features such as rapid mRNA up-regulation [9;10], combined with sensitive methods for detection, suggested that such a readout would be superior to the standard proliferation assay [9;11;12;13;14]. Figure 1A illustrates the dynamic spectrum of *IL2* mRNA signal from isolated $CD4^+CD25^-$ T cells at various times after activation, with 7 0f 9 samples peaking between 6 and 12 hours after activation before declining. Since the priority was to develop a short-term assay, subsequent experiments were done using a 6 hour activation period. Of note, we have empirically observed that despite the 6-12 hour range for peak *IL2* mRNA production among individuals, the majority of $CD4^+CD25^-$ T cells tested at 6 hours post-activation yielded sufficient signal to reliably assess suppression (Fig. 1B and data not shown). Similarly prioritizing the need to reduce the number of cells in a short-term suppression assay, titrated doses of $CD4^+CD25^-$ T cells from different individuals identified 10,000 cells as the minimum number of cells most reliably yielding signal above the negative controls (negative control CT values ranged 35-40; Fig. 1B and data not shown). It

is interesting to note that a minority of samples remain below the limits of detection at the highest dose of cells, perhaps reflecting the lower end of the *IL2* response range or the donors' health status (Fig. 1B).

Having reduced the time for a $CD4^+CD25^-$ T cell functional readout assay from days to 6 hours, we were poised to investigate whether cell cycle progression was linked to nTregs potency. However, three technical issues needed to be addressed. First, since an exclusive marker for human nTregs is lacking, we purified anergic cells expressing a $CD4^+CD25^{bright}CD127^{lo}FOXP3^+$ phenotype (Supplemental Figure 1 and data not shown). These characteristics comprise a broad consensus phenotype for human nTregs, at least until a definitive lineage specification marker is elucidated [15; 16] Secondly, conventional cell cycle analysis evaluates DNA content by propidium iodide staining of fixed cells [17], precluding functional analysis of specific subsets. Thirdly, although nTregs show evidence of rapid proliferation *in vivo* [18; 19], freshly isolated cells are largely anergic unless provided additional signals [20; 21; 22; 23]. To circumvent the latter two hurdles, primary human nTregs were activated and labeled for DNA content with Hoechst 33342. Five days of activation was the shortest activation period where sufficient numbers of cells were found in the S and G2/M phases (Supplemental Figure 2). Next, viable nTregs in indicated phases of cell cycle progression were sorted by flow cytometry and immediately assayed for regulatory function. As an additional control, DNA content of the sorted cells was evaluated at time 0 and 6 hours to verify that the nTregs remained within the desired phases for the duration of the assay (Fig. 1C). Surprisingly, nTregs in either S/G2/M combined, or S and G2/M fractionated, exhibited significantly more potent suppressor activity than those in the G0/G1 pool, further substantiating the notion that cell cycle progression is directly linked to nTregs potency (Fig. 1D and 1E).

If modulating cell cycle progression is a physiologically relevant mechanism for controlling nTregs function, then one would predict that important biological molecules participate in this process. Since vitamin D3 has been shown to alter cell cycle progression [24] and is emerging as a key immunomodulator [8;25;26], we investigated whether this molecule could potentiate nTregs function. Figure 2A shows that nTregs activated in the presence of physiological concentrations of vitamin D3 are significantly more effective at suppression than mock treated nTregs. Importantly, this enhancement is maintained when a fixed number of nTregs interacts with an increasing number of $CD4^+CD25^-$ T cells, providing an objective demonstration of amplified potency. We were curious whether vitamin D-mediated enhancement of suppressor function could also be observed at the level of IL2 protein production. However, the differing turnover time for *IL2* mRNA versus IL2 protein makes directly adapting the above mRNA suppression assay to intracellular cytokine staining challenging and additional kinetic studies will be required for optimization. Albeit modest, an increase in suppression of IL2 protein production was observed when vitamin D3-treated nTreg were assayed, suggesting that regulatory activity can be detected at both the protein and mRNA levels (Fig. 2B). Heightened function is not due to carry over of vitamin D3 acting on the responding cells themselves, as addition of vitamin D3 directly to $CD4^+CD25^-$ T cells does not diminish the level of detectable *IL2* mRNA or protein (Supplemental Figure 2). All nTregss populations we have tested thus far have exhibited increased regulatory activity when exposed to vitamin D3, but the kinetics of this boost varies among individuals (data not shown). This heterogeneity likely reflects genetic modifiers, placing the delineation of a precise time course beyond the scope of this study. Thus, to investigate whether the timing of vitamin D3 exposure was important in a more generalized manner, the potency of nTregs receiving a single dose either at the beginning or end of T cell receptor (TCR) stimulation was compared to those receiving a continually replenished supply. Figure 2C shows graded degrees of suppression among the groups, with those getting vitamin D3 throughout the activation program being most effective.

To better understand the molecular mechanism by which vitamin D3 acts on nTregs, we examined the effects of TCR stimulation on vitamin D receptor (*VDR*) expression. Purified nTregs were activated *ex vivo*, either in the presence or absence of vitamin D3 and assayed at the indicated times for *VDR* mRNA. Expression of *VDR* peaks around 12 hours after activation, remains partially elevated for at least 14 days, and is not differentially regulated in the presence of ligand (Fig. 3A and data not shown). Thus, the profile of *VDR* regulation in nTregs is similar to that described for conventional T cells (i.e. *VDR* increases upon TCR stimulation in a ligand-independent fashion) [27;28].

It has been reported for multiple cell types that vitamin D3 blocks the transition from G0/G1 to S [29;30]. Consistent with such a block, vitamin D3 treated $CD4^+CD25^-$ T cells exhibited fewer cells in the S and G2/M phases at both 48 and 120 hours, compared to mock treated cells (Fig. 3B). At 48 hours, nTregs similarly showed decreased numbers of cells in S and G2/M phases. However, the percentage of cells in S or G2/M phases unexpectedly increased in the vitamin D3 treated nTregs after 120 hours (approximately 3- and 10-fold, respectively), suggesting a release or less stringent block in cell cycle progression at this time (Fig. 3C). Next, we determined whether this pattern was unique to a specific individual or reflective of a more generalized trend, by performing a similar time course of cell cycle analysis on nTregs from 6 additional donors. To normalize for variations among different individuals, we combined the percentage of cells in S with those in G2/M as an overall measurement of “proliferating” cells within the total population (referred to as S/G2/M). Table 1 illustrates that after 120 hours of activation in the presence of vitamin D3, 5 of 6 individuals increase the number of cells in S/G2/M, indicating that nTregs exhibit a late release from the G0/G1 block.

FOXP3 is critical for nTregs function [31;32;33;34;35;36] and *in silico* studies suggested that the promoter for this gene has two potential *VDR* binding elements (Riberdy, unpublished). Hypothesizing that vitamin D3 may be mediating its activity through FOXP3, mRNA levels of *FOXP3* were examined at various time points after TCR stimulation where vitamin D3 was added at the onset of activation. As expected, *FOXP3* mRNA levels increased after activation, but addition of vitamin D3 did not show differential regulation (Fig. 4A). To examine FOXP3 levels more carefully, we looked at protein levels by intracellular flow cytometry using multiple antibodies to avoid epitope-specific variations. The number of $FOXP3^+$ cells increased when vitamin D3 was supplied during the 5-day activation, irrespective of the antibody used (Fig. 4B, Table 2, and data not shown). Table 2 illustrates that the number of FOXP3 expressing cells increases (n=5) and the mean fluorescence intensity per cell is typically elevated as well (4 of 5 individual samples). Using a second method, confocal microscopy, we demonstrated that exposure to vitamin D3 resulted in increased FOXP3 expression both at the single cell and population levels (Fig. 4C, D, and E). Employing a third method, western blot, and probing with a polyclonal rabbit antisera against FOXP3, we confirmed that protein levels were increased in vitamin D3 treated nTregs (Fig. 4F). A parallel study following the immune reconstitution of a patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) after hematopoietic stem cell transplantation provided a unique opportunity to examine the effect of vitamin D3 on the regulation of FOXP3 containing a point mutation in the forkhead domain. Prior to transplantation, nTregs from this patient poorly expressed FOXP3 (Kasow *et al.*, manuscript in preparation). Vitamin D3 treatment during *ex vivo* activation showed a three-fold increase in $FOXP3^+$ cells compared to control stimulation (Fig. 4G). Taken together, these data demonstrate that vitamin D3 modulates FOXP3 protein expression in human nTregs.

4. Discussion

It is clear from many studies that any single readout does not reflect the breadth of T lymphocyte function (i.e. all tetramer binding cells do not necessarily produce identical cytokine profiles or exhibit cytotoxicity) [37]. Similarly, solely assessing the ability of human nTregs to inhibit proliferation is not likely to reflect the full arsenal of capabilities ascribed to these cells. Broadening our ability to monitor nTregs by developing assays which survey additional functions will be critical to illuminate how this population functions at the molecular level. Moreover, the heterogeneity of human lymphocyte responses increases the likelihood that even among healthy donors, there will occasionally be weak responses below the limits of detection, as we show in Figure 1B. This caveat is particularly relevant in clinical samples where cell number is limited and responses are likely to be muted due to disease or therapy. These considerations highlight the urgent need to expand methodology, and improve sensitivity. The development of additional assays is also important for elucidating early vs. late molecular events during the orchestration of the suppressive program. For example, the short-term nature of the *IL2* mRNA suppression assay described in this work was critical for the cell cycle progression studies, which could not have been done using a proliferation-based method. Additionally, the vitamin D3-mediated enhancement of nTregs potency would have been missed without a 6-hour assay. It is tempting to speculate that because an immune response grows exponentially, a small enhancement of *IL2* suppression during the first 6 hours will likely result in a large impact on the overall magnitude.

Initial attempts to examine the effect of vitamin D3 using proliferation assays were inconclusive and prompted us to examine whether augmented potency may be transient. Indeed altering the dosing of vitamin D3 during nTregs activation suggests that this molecule make act temporally (Figure 2C). It is intriguing to consider that the transient effect of vitamin D3 may provide an additional fine-tune adjustment such that autoimmunity is ablated in one anatomical locale, yet at another site the body remains poised to respond to pathogens. It has been suggested that vitamin D3 can facilitate the recruitment of induced-Tregs into the modulation process [8; 38], while our data demonstrates a novel role for this molecule, augmenting function from existing nTregs. Such a scenario would support the concept that vitamin D3 rapidly acts on nearby nTregs to blunt immune genesis. However, in physical environments requiring a larger number of regulatory T cells such as the gut, induced-Tregs could be enrolled to assist.

Given the lack of a single cell suppression assay, it remains unknown whether all the nTregs subjected to *in vitro* analysis actually participate in the modulatory process or whether only a subpopulation mediates the bulk of activity. This is perhaps most relevant in the cell cycle studies. Given the observation that nTregs in S or G2/M are more potent, it is reasonable to speculate that the increase in S/G2/M cells after vitamin D3 exposure results in the enhanced suppressive function. To test this directly and compare treated vs. non-treated nTregs in S/G2/M, additional methods requiring even fewer cells need to be developed because sufficient cells cannot readily be obtained from peripheral blood. Nonetheless, it is important to point out the hypothetical scenario described above does not necessarily imply that the nTregs in S/G2/M are the subset most efficient at mediating other suppressive functions such as inhibiting proliferation, modulating dendritic cell function, or arresting cytotoxicity. Rather, it is likely that population of cells characterized by a $CD4^{+}CD25^{bright}CD127^{lo}FOXP3^{+}$ phenotype will be further partitioned into functional subsets, perhaps by markers such as CD45RA or HLA-DR, which are already known to correlate with specific functional capabilities [39;40].

The ability of vitamin D3 to enhance nTregs function, possibly via increasing FOXP3, emphasizes the need to consider both quality and quantity when considering the dynamics of immunomodulation. This issue is highlighted by the demonstration that vitamin D3 increases the number of nTregs expressing FOXP3, as well as the amount of protein per cell (Figure 4 and Table 2). However, it is unclear whether the nTregs expressing the most FOXP3 are actually more potent. Additional studies are necessary to elucidate how quantity (amount of FOXP3) and quality (perhaps as a post-translational modification) relate to suppressive function. The unique regulation of FOXP3 in human T cells poses additional constraints on interpretation of quality and quantity. For example, FOXP3 is restricted to murine T cells with regulatory function while all human T lymphocytes transiently express FOXP3 upon TCR stimulation, without necessarily acquiring suppressive function [41]. Thus, simple enumeration studies using FOXP3 alone may not reflect bona fide nTregs. An additional qualitative point is demonstrated by ectopic expression of FOXP3 in $CD4^+CD25^-$ T cells to induce regulatory function. In these studies ectopic expression of FOXP3 in murine $CD4^+CD25^-$ T cells is sufficient to induce potent regulatory function [4;31;42], while similar experiments with human T lymphocytes have resulted in only modest levels of activity [32;43;44;45]. However, potent suppressive activity can be obtained if high levels of ectopic FOXP3 are sustained for several days, perhaps reflecting a requirement for epigenetic changes [46]. The need for epigenetic alterations is further supported by the observation that bona fide regulatory cells fully demethylate a CpG island in the promoter of FOXP3. In contrast, those exhibiting a partial demethylation status show only transient expression and lack functional activity [47;48;49].

The ability to overcome the cell cycle block mediated by vitamin D3 is reminiscent of the observation that nTregs selectively expand in the presence of rapamycin, an anti-proliferative agent known to inhibit the activity of the mammalian target of rapamycin (mTOR) [50;51]. Histone deacetylases (HDAC) are key molecules in regulating cell cycle progression not only by virtue of their activity on histones and therefore control of transcription, but also on transcription factors themselves. Interestingly, HDAC inhibitors increase acetylation of FOXP3 itself and this modification is associated with enhanced nTregs function [52]. The combined observations that nTregs potency is enhanced by vitamin D3, rapamycin, and HDAC inhibitors, strongly suggest that cell cycle progression is uniquely regulated in nTregs. Understanding the molecular events controlling cell cycle progression in human nTregs may reveal novel targets for enhancing their function and will be critical for translating these observations into strategies which harness the full therapeutic potential of this elusive population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

nTregs	natural regulatory T cells
vitamin D3	1α,25-dihydroxyvitamin D3
PFA	1.5% paraformaldehyde
TCR	T cell receptor

VDR	vitamin D receptor
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
mTOR	mammalian target of rapamycin
HDAC	Histone deacetylases

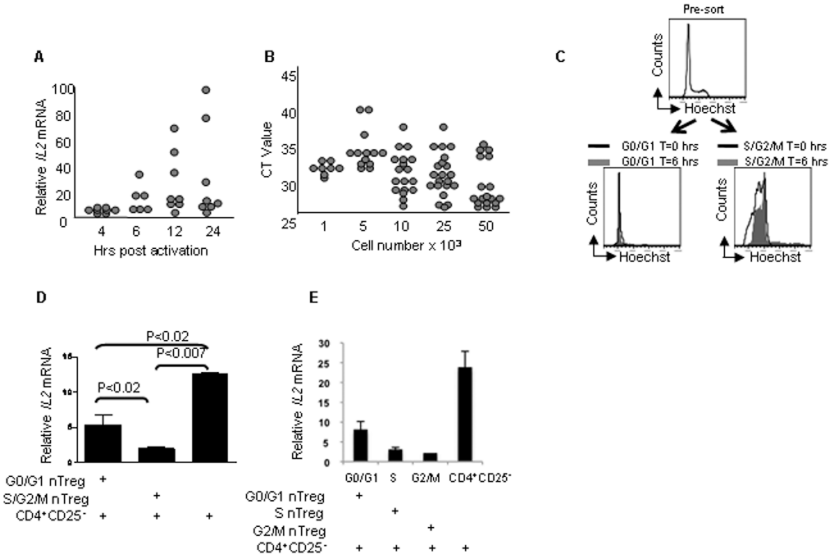

Figure 1. Cell cycle progression modulates nTregs function

(A) Optimal time for *IL2* mRNA detection was determined by activating 10,000 CD4+CD25- T cells *in vitro* for the indicated times, followed by qRT-PCR analysis for cytokine [n=9]. (B) The minimum number of cells for reliable *IL2* mRNA detection was determined by assaying titrated doses of *in vitro* activated CD4+CD25- T cells for cytokine via qRT-PCR [1k n=8, 5k n=12, 10k n=18, 25k n=21, 50k n=18]. (C) nTregs were activated *in vitro* for 5 days, labeled with Hoescht 33342, sorted into the indicated fractions and assayed for DNA content at time 0 and 6 hrs under suppression assay conditions. This is representative of 2 experiments. (D) 10,000 nTregs in the indicated cell cycle phases were sorted and incubated with 10,000 CD4+CD25- T cells under the described suppression microassay conditions for 6hrs prior to *IL2* mRNA analysis. Error bars represent SD and this is representative of 3 experiments. (E) Purified nTregs were activated and fractionated as in 1d, prior to assaying for suppressive activity. Error bars represent SE and this is representative of 4 experiments.

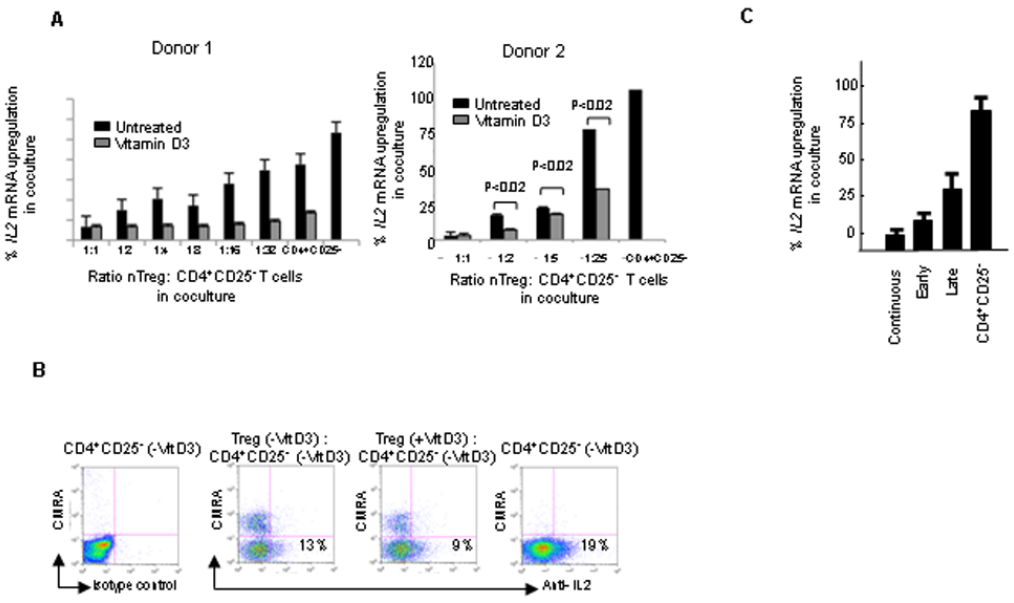

Figure 2. Vitamin D3 modulates human nTregs potency at both the mRNA and protein levels

(A) nTregs were either mock treated or treated with vitamin D3 for 5 days as described in Methods, assayed for suppressive potency with 10,000 CD4+CD25- T cells at the indicated ratios, and analyzed for *IL2* mRNA. Data represents 2 of 7 experiments. Sufficient nTregs were available from Donor 2 to set up each condition in triplicate, while the yield of nTregs from Donor 1 only permitted a single well for each condition. Error bars in the panel from Donor 2 represent SD. (B) Mock treated or vitamin D3 treated nTregs were assayed for the ability to inhibit IL2 protein production by CD4+CD25- T cells. nTregs were labeled with CMRA Cell Tracker™ to distinguish them from the CD4+CD25- T cells, mock treated or vitamin D3 treated, and assayed for suppressive function with the following changes to the microassay. 10,000 CD4+CD25- T cells were incubated with an equivalent number of nTregs under the described microassay conditions for 10 hrs. Brefeldin A was added during the last 5 hrs to inhibit cytokine secretion prior to intracellular IL2 staining. (C) Exposure to vitamin D3 at different stages of nTregs activation differentially alters potency. 20nM vitamin D3 was added to the nTregs activation cultures at 0hrs (Early); 96 hrs (Late); or 0, 48, and 96 hrs (Continuous). nTregs were assayed for suppressive function during the described 6hr suppression microassay (n=5 for the different nTregs groups).

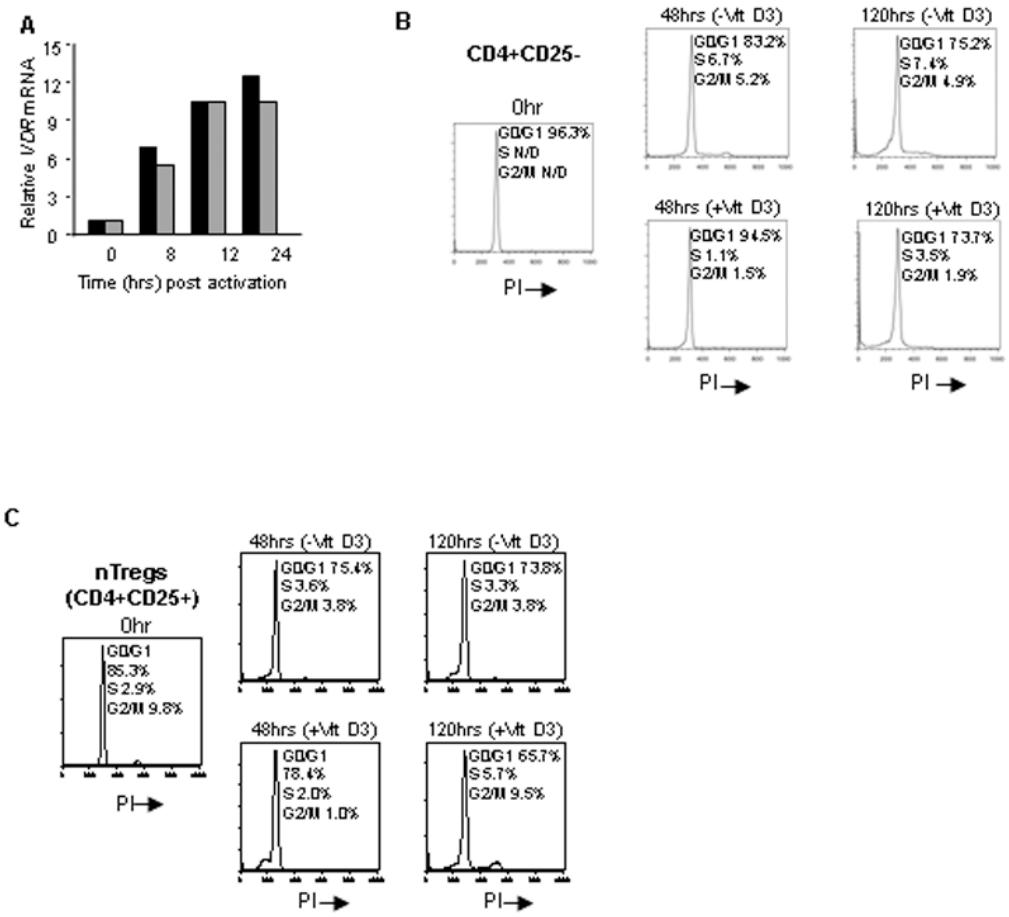

Figure 3. Vitamin D3 treatment modulates cell cycle progression

(A) Freshly isolated nTregs were activated for the indicated times in the presence or absence of vitamin D3 and assayed for *VDR* mRNA by qRT-PCR analysis (n=5). (B) CD4+CD25- T cells were activated in the presence or absence of vitamin D3 and labeled with propidium iodide for DNA content for the indicated time points. (C) nTregs were activated for 5 days and labeled for DNA content on days 0, 2, 5 (n=6).

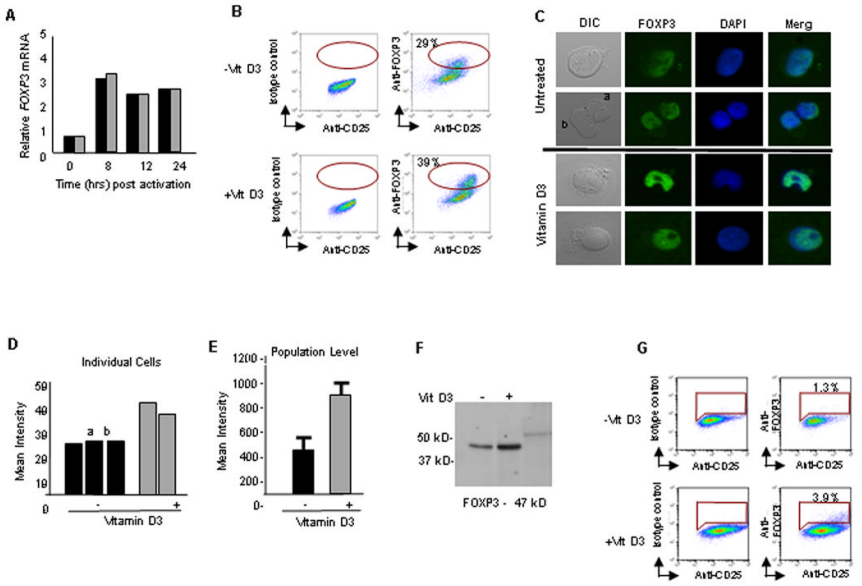

Figure 4. FOXP3 protein expression is augmented upon Vitamin D3 treatment

(A) Freshly isolated nTregs were activated and assayed for *FOXP3* mRNA by qRT-PCR analysis (n=5). (B) Freshly isolated nTregs were activated for 5 days as previously described in text. Gating strategy: CD4+ T cells (99%) were selected and analyzed for CD25 vs. FOXP3. IgG isotype controls and unlabelled cells were used to define gates. The percentage of CD4+ T cells expressing both CD25 and FOXP3 as depicted by the oval gate, are indicated. (C-E) Confocal microscopy analysis of nTregs. (C) Activated cells were fixed and permeabilized, followed by anti-FOXP3 staining. Controls included unlabeled cells and isotype controls (data not shown). Anti-fade reagent with DAPI was used as mounting solution and nuclear marker. Mean intensity of anti-FOXP3 was analyzed both at the single cell (D) and population (E) levels (representing 1 of 3 experiments) using NIS Elements Software. (F) Western blot analysis for FOXP3 expression using 10 mg of protein per lane as described in Methods. (G) nTregs purified from a patient with IPEX were either mock treated or vitamin D3 treated during *ex vivo* activation and stained for FOXP3 expression. Gating strategy: CD4+ T cells (99%) were selected and analyzed for CD25 vs. FOXP3. IgG isotype controls and unlabeled cells were used to define gates. The percentage of CD4+ T cells expressing both CD25 and FOXP3, as depicted by the gate are indicated.

Table 1

Percentage of mock and vitamin D3 treated nTregs in S/G2/M 120 hours after activation.[a]

	Mock S/G2/M	Vitamin D3 S/G2/M
Donor 1	3.7%	5.6%
Donor 2	1.9%	7.2%
Donor 3	2.7%	1.8%
Donor 4	7.8%	9.5%
Donor 5	7.0%	5.9%
Donor 6	2.2%	5.2%

[a]Freshly isolated nTregs were mock treated or vitamin D3 treated during 5-day activation as previously described. The percentage of cells in the indicated stages of cell cycle progression was determined after propidium iodide labeling.

Table 2

FOXP3 expression in mock and vitamin D3 treated nTregs during activation.[b]

	Vitamin D3	%CD4+CD25+FOXP3+	MFI FOXP3
Donor 1	-	29	195
	+	38.6	304
Donor 2	-	29.5	28
	+	48	35
Donor 3	-	75.9	7.5
	+	83.7	8
Donor 4	-	19.7	19
	+	35.9	12.3
Donor 5	-	25.8	170
	+	38.6	295

[b] Freshly isolated nTregs were activated for 5 days in the presence or absence of vitamin D3 followed by flow cytometry analysis of FOXP3 expression. The same gating strategy described in Figure 4 was used.