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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.

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.

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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 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 [2–4], 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 [5–8]. 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 2 mM 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 20 nM 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 1 μM 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 10 μM 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 1 μM 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,