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Requirements for growth and IL-10 expression of highly purified human T regulatory cells

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Abstract

Human regulatory T cells (T_R) cells have potential for the treatment of a variety of immune mediated diseases but the anergic phenotype of these cells makes them difficult to expand *in vitro*. We have examined the requirements for growth and cytokine expression from highly purified human T_R cells, and correlated these findings with the signal transduction events of these cells. We demonstrate that these cells do not proliferate or secrete IL-10 even in the presence of high doses of IL-2. Stimulation with a superagonistic anti-CD28 antibody (clone 9D4) and IL-2 partially reversed the proliferative defect, and this correlated with reversal of the defective calcium mobilization in these cells. Dendritic cells were effective at promoting T_R cell proliferation, and under these conditions the proliferative capacity of T_R cells was comparable to conventional CD4 lymphocytes. Blocking TGF- β activity abrogated IL-10 expression from these cells, while addition of TGF- β resulted in IL-10 production. These data demonstrate that highly purified populations of T_R cells are anergic even in the presence of high doses of IL-2. Furthermore, antigen presenting cells provide proper co-stimulation to overcome the anergic phenotype of T_R cells, and under these conditions they are highly sensitive to IL-2. In addition, these data demonstrate for the first time that TGF- β is critical to enable human T_R cells to express IL-10.

Introduction

T regulatory cells (T_R) are essential regulators of immune responses, as demonstrated by patients lacking T_R cells due to mutations in the transcription factor Foxp3. These patients

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Authorship Contributions

Benedetta Bonacci M.S. performed the majority of the cell culture experiments and aided in manuscript preparation. Brandon Edwards B.S. and Shuang Jia performed research. Calvin Williams M.D. aided in data analysis and manuscript preparation.

Martin J. Hessner Ph.D. analyzed the gene expression experiments and aided in manuscript preparation. Stephen Gauld Ph.D. performed research and aided in manuscript preparation. James Verbsky M.D., Ph.D. oversaw and planned all experiments, analyzed data, and wrote the manuscript

Conflict of interest statement

No authors have any competing financial interests.

develop the fatal autoimmune disease X-linked syndrome of immune dysfunction, polyendocrinopathy, and enteropathy (IPEX)^{1–5}. These patients exhibit a variety of autoimmune features from an early age, including diabetes mellitus and other endocrinopathies, inflammatory bowel disease, severe allergies and eczema. Defects in T_R cells have been implicated in numerous immune mediated illnesses, including autoimmune diseases and graft versus host disease⁶. T_R cells constitutively express the IL-2 receptor subunit CD25 implicating IL-2 in the function of T_R cells. In support of this, we have described a patient lacking CD25 due to compound heterozygous gene mutations who expressed a disease very similar to patients with IPEX because of Foxp3 mutations⁷. T_R cells suppress immune responses by a number of mechanisms, including CTLA-4 engagement of B7 molecules on target cells, surface expression of TGF- β ^{8–10}, cytotoxicity of target cells through the perforin/granzyme pathway¹¹, and by the expression of immunosuppressive cytokines, including TGF- β , and IL-10^{12;13}.

Given the central role T_R cells play in immune regulation, there is considerable interest in developing these cells for immunotherapy^{14–18}. One difficulty in utilizing these cells as a therapeutic agent is the relative small number of Foxp3⁺ T_R cells in human peripheral blood, accounting for only 5–10% of peripheral blood CD4 cells. It is important to note that CD25 is not specific for T_R cells as activated lymphocytes also express this marker. Thus interpreting studies where T_R cells have been isolated based on CD25 expression can be problematic. Finding ways to expand human T_R cells would be beneficial, however, T_R cells fail to proliferate *in vitro* in response to TCR activation¹⁹. One possible explanation of why these cells fail to grow under these conditions is that T_R cells fail to express IL-2 upon TCR activation, and it has been shown that Foxp3 binds to the IL-2 promoter and blocks transcription of the IL-2 gene^{20;21}. In support of this notion, treatment of human CD25⁺ cells with high doses of IL-2 has been reported to restore their growth¹⁹. We have previously shown that highly purified T_R cells from human blood do not proliferate in response to activation with antibodies to CD3 and CD28 even in the presence of IL-2¹¹. These studies utilized only the highest 1–2% of CD25 expressing CD4⁺ lymphocytes from human peripheral blood, a population that is highly enriched in T_R cells. To better characterize the requirements for growth and IL-10 production from human T_R cells, we utilized a strategy to isolate highly purified T_R without other contaminating cell populations. Using this strategy, we performed a comprehensive analysis of the requirements for T_R cells growth, cytokine expression, and the signal transduction events of these cells.

Materials and Methods

Reagents

Antibodies to CD25 (clone M-A251), CD4 (clone OKT-4), CD28 (clone 28.2), CD45RA (clone UCHL1), CD45RO (MEM-S6) were purchased from BDBiosciences. CD14 antibody (clone TuK4) was purchased from Caltag. Foxp3 antibody was purchased from eBioscience (clone PCH101). Antibodies to CD3 (clone OKT3), CD14 (clone HB247), and CD28 (clone 9.3) were isolated from tissue culture supernatants by protein G isolation (ATCC). CD46 antibody (clone Tra-2–10) was a gift of Dr. John Atkinson (St Louis, MO). Goat anti-mouse Ig magnetic beads were produced as follows: 10 mg of 50nm magnetic beads (fluidMAG-

ARA) (Chemicell, Germany) were incubated with 40mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (Sigma) and 40mg NHS (N-hydroxysuccinimidyl (NHS) ester) (Sigma) in 25mM MES (2-(N-morpholino)ethanesulfonic acid) (pH 6.0) for 30 minutes at room temperature. Beads were run on a magnetic column (Miltenyi) and washed with MES. Beads were then eluted with MES and incubated with 1 mg of goat anti-mouse antibody (Sigma) at 4°C rotating overnight. Goat-anti mouse conjugated beads were column purified, washed in storage buffer (0.1%BSA, 0.02% sodium azide, and 10mM Tris), and stored at 4°C until used.

Cell isolations

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of peripheral blood from random blood donors using Ficoll-Paque (Amersham). PBMCs were stained with CD25-PE antibodies in buffer consisting of PBS, 2% newborn calf serum, 2mM EDTA, and 50µg/ml of human immunoglobulin (Gammaguard, Baxter). Cells were washed with staining buffer and incubated with Goat anti-Mouse Ig coated magnetic beads for 30 minutes at room temperature. Cells were washed and purified on a magnetic column (Miltenyi). Eluted CD25⁺ cells were subsequently stained with a CD4 antibody, and sorted based on CD25 expression using a FACS Aria cell sorter (BDBiosciences).

Monocytes were isolated from PBMCs using CD14 antibodies and goat anti- mouse IgG magnetic beads, followed by magnetic column isolation. The isolated population was then stained with CD14 Alexa-700 (clone TuK4, Invitrogen), then purified by cell sorting based on CD14 expression.

Generation of Immature and Matured Myeloid Dendritic Cells

Dendritic cells were generated from CD14⁺ monocytes isolated as described above by culturing for 4 days in 1000U/ml IL-4 and 50ng/ml GM-CSF. These cells expressed high levels of HLA Class-II and CD86 (data not shown). Maturation of dendritic cells was induced by adding 300ng/ml LPS for an additional 24–36 hours as previously described.

T cell activation assay

Isolated cells were activated by plating on 96-well flat-bottom plates coated with 2.5 µg/ml of purified antibody in PBS. Cells were cultured in RPMI-1640 (Hi-Clone) supplemented with 1 mM glutamine, 1 mM penicillin/streptomycin, 1 mM MEM and 10mM sodium pyruvate along with 10% FBS and 0.5% β-mercaptoethanol (R10 media). Supernatants were removed 4 days later and IL-10 expression quantified by ELISA. ³H-thymidine was added for an additional 16 hours, and uptake of radioactivity was measured using a beta-plate scintillation counter.

For studies with TGF-β and TGF neutralizing antibodies, cells were activated with CD3/CD28 antibody in R10 with IL-2 at 10 U/ml with or without added human TGF-β1 (5 ng/ml). A pan-TGF blocking antibody (clone 1D11) was used at 100µg/ml. After 3–4 days proliferation and IL-10 production was measured.

Elisa

High protein binding plates (Polysorb, Nunc) were coated with an IL-10 capture antibody (clone JES5–2A5 BDBiosciences) in PBS at 5µg/ml overnight at 4°C. Plates were washed with PBS plus 0.05% Tween-20 (PBST) and blocked with 1% BSA solution in PBST for 30 minutes at room temperature. Culture supernatants were added and incubated overnight at 4°C. Plates were then washed and a biotinylated anti-IL-10 antibody (clone JES5–16E3 EBiosciences) diluted 1:1000 in blocking buffer was incubated for 1–2 hours at room temperature. Plates were washed, and Streptavidin-HRP (Pierce) diluted 1:10,000 in blocking buffer was added and incubated for one hour at room temperature. Plates were then washed and developed using ABTS (Pierce) according to the manufacturer's instructions.

Flow Cytometric Intracellular Calcium Analysis

Changes in intracellular calcium were measured by flow cytometry using Fluo-4 AM (Invitrogen). Cells were washed and resuspended in R10 medium at a density of 5×10^6 /ml and warmed to 37°C for 15 minutes. Fluo-4 AM was added at a final concentration of 2.5µM. Cells were incubated at 37°C for 45 minutes (protected from light), then centrifuged and resuspended in R10. Cells were then incubated with antibodies to CD3 and CD28, and stained with fluorochrome labeled antibodies against CD4 and CD8 for 15 minutes at room temperature. Cells were washed twice with R10 media and rested for 15 minutes. Cells were then analyzed on a LSRII flow cytometer for 30 seconds to establish the baseline Fluo-4 fluorescence. Goat anti-mouse IgG crosslinking antibody(Sigma) was then added and cell analyzed by flow cytometry for 5 minutes.

Western Blot Protocol

Cells were resuspended in R10 media with antibodies to CD3 and CD28 were added to cells on ice for 20 minutes, washed, then pre-warmed to 37°C. Goat anti-mouse cross-linking antibodies was then added and incubated at 37°C for 15 minutes. Cells were incubated in lysis buffer(150mM NaCL, 10mM Tris-base, 10mM tetrasodium pyrophosphate, 1% NP-40, 1mM PMSF, 1mM NaF, 0.5 EDTA, 2mM sodium orthovanadate, and 1µg/ml each of Aprotinin, Pepstatin, and Leupeptin), vortexed thoroughly and incubated on ice for 10 minutes, then centrifuged at 4°C for 10–15 minutes at 10,000 rpm and supernatants were collected and frozen until analyzed. Lysates were run on a 4–12% gradient polyacrylamide gel (Invitrogen), blotted onto PVDF Immobilon-FL (Millipore), blocked with Li-Cor Blocking Solution, then incubated with phospho-tyrosine specific antibody 4G10. Blots were washed then incubated with AlexaFluor-A680 goat anti-mouse IgG antibody, washed, then analyzed with a Li-Cor Odyssey imaging system.

Microarray analysis

Microarray analysis was performed in accordance to the manufacturer's protocol. Briefly, purified RNA (~100 ng) was amplified using a two-cycle cDNA synthesis kit, and cRNA was synthesized, labeled, fragmented, and hybridized to the GeneChip human genome U133 plus 2.0 array (Affymetrix). After hybridization arrays were washed and stained with the GeneChip Hybridization Wash and Stain Kit (Affymetrix) and scanned. Image data were quantified with Affymetrix Expression Console Software and normalized with Robust

Multichip Analysis (RMA; www.bioconductor.org/) to determine signal log ratios. All raw data files have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus (Accession Number **GSE22045**).

Results

Isolation of highly purified T_R cells

We utilized a sorting strategy to isolate pure populations of human T_R cells. PBMCs isolated from random healthy blood donors were first labeled with a CD25-PE antibody, and then isolated with goat anti-mouse labeled magnetic beads by magnetic column separation. Staining of the isolated populations with Foxp3 specific antibody revealed that roughly 42% of the positively selected cells expressed Foxp3, and these cells correlated with the CD25^{hi} population (Figure 1A). The percent purity of Foxp3⁺ cells in the CD25 isolated cells varied from 50% to 95% depending on the experiment (data not shown). The CD25 selected cells could be sorted based on CD25^{hi} expression. Staining of pre- and post-sorted populations with Foxp3 antibodies showed an increase in purity of T_R cells from approximately 42% to 95% following cell sorting in this experiment, with purities over 90% on average (Figure 1B). Similar purities were obtained when cells were sorted based on CD25 and CD127 (data not shown). CD25^{hi} sorted cells were able to inhibit the proliferation of autologous CD4 cells in co-culture assays (data not shown).

Microarray analysis of highly purified human T_R cells

In order to confirm that our isolation strategy resulted in cells with characteristics of T_R cells, we performed gene expression profiling of highly purified T_R cells from two different individuals, which were compared to the gene expression profile of naïve CD4⁺CD45RA⁺ cells. We first performed hierarchical clustering of the gene expression profiles of unactivated naïve and T_R cells from the two individuals. We show that the gene expression patterns of T_R cells were highly similar between the two samples (correlation coefficient of 0.82), as were the gene expression profiles of naïve CD4 cells between the two samples (correlation coefficient of 0.91). After averaging the gene expression signal from these two individuals, we compared the gene expression profile of T_R cells with naïve CD4 cells and show that T_R cells over-express a variety of genes known to be associated with a T_R phenotype (e.g. CTLA-4, IKZF2, IL-2RA). In addition, we also detected very high levels of expression of genes not typically associated with a T_R phenotype, with the highest level of expression of IL-1 receptors in T_R cells (Figure 2). These data confirm that the sorting strategy was effective at isolating T cells expressing a T_R phenotype.

Monocytes allow for activation of human T_R cells

Using these human T_R cells, we first tested if IL-2 was able to overcome the anergic phenotype of these cells. We chose to utilize antibodies to CD3 and the complement regulatory protein CD46 to activate these cells since prior studies have shown that antibodies to CD3 and CD46 are better able to activate human CD4 T cells compared with CD3 and CD28 antibodies²². Furthermore, we wanted to be able to control for the amount of IL-2 in this system, and CD46 activating antibodies are not able to provide the necessary co-stimulation required for IL-2 production²². Purified T_R cells from healthy random donors

were stimulated with plate bound antibodies to CD3 and CD46 in the presence or absence of IL-2. As shown in Figure 3A, T_R cells were unable to efficiently proliferate when activated in this manner and increasing the dose of IL-2 was unable to restore proliferation even at doses up to 1000U/ml (data not shown).

Preliminary experiments of CD25 selected, unsorted cells demonstrated efficient proliferation of T_R cells in the presence of IL-2, implying that other cell populations in these cultures allowed for T_R proliferation (data not shown). These unsorted populations of CD25⁺ cells did contain monocytes, therefore we tested whether the presence of monocytes was able to overcome the anergic phenotype of pure T_R cells. When monocytes were added to the cultures of purified T_R cells and activated with antibodies to CD3 and CD46 without added IL-2, little proliferation or IL-10 production was detected (Figure 3A). However, when IL-2 was added to these cultures, a significant increase in proliferation and IL-10 production was detected. Since reports have suggested that high dose IL-2 allows for T_R cell activation, we tested the dose of IL-2 required to activate T_R cells when activated in the presence of monocytes. As shown in Figure 3B, T_R cells were able to proliferate and produce IL-10 in the presence of monocytes even at doses of IL-2 as low as 0.1U/ml. Thus, in the presence of proper activation conditions, T_R cells efficiently utilize IL-2 to proliferate and produce IL-10.

Dendritic cells efficiently activate human T_R cells, and this response is independent of CD80/CD86 interactions

Studies in mice have shown that T_R cells exist predominantly in close proximity to dendritic cells *in vivo*²³, and other studies have demonstrated expansion of human T_R cells when activated in the presence of dendritic cells²⁴. Knowing that monocytes were able to stimulate pure human T_R cells, we next tested whether dendritic cells (DCs) were able to efficiently activate human T_R cells. Human CD14⁺ monocytes were cultured in the presence of GM-CSF and IL-4 for 3 days to generate immature DCs. In some experiments, LPS was added for 16 hours prior to use to generate mature DCs. In addition, to test the contribution of CD80 and CD86 in the activation of T_R cells, neutralizing antibodies to CD80 and CD86 were added to some of the cultures. Finally, all data from these experiments were normalized to the proliferation and IL-10 expression of CD4⁺ cells in order to compare the proliferative capacity of T_R cells to conventional T cells.

As shown in figure 4A, when T_R cells were activated alone, little proliferation was detected even in the presence of exogenous IL-2. When immature DCs were added to the cultures of T_R cells, we saw strong proliferation and IL-10 production of T_R cells even in the absence of IL-2 (Figure 4A). CD80/CD86 neutralizing antibodies had no effect on the proliferation of T_R cells in the presence of immature DCs (Figure 4, black bars). These antibodies were able to block the proliferation of naïve CD4 cells activated on LPS stimulated monocytes, demonstrating that they were effective at neutralizing CD80/CD86 mediated signals (data not shown). When LPS matured DCs were utilized, little increase in IL-10 production or proliferation were detected in the absence of IL-2 above that observed with immature DCs. When IL-2 was added to these cultures, proliferation and IL-10 production was enhanced with both immature and mature dendritic cells (Figure 4B). Under these conditions, CD80/

CD86 blocking antibodies did inhibit the increase in proliferation and IL-10 production. Importantly, the proliferative capacity of pure T_R cells was comparable to conventional CD4 cells when LPS matured DCs were added to the cultures in the presence of IL-2, indicating that under the proper activating conditions these cells proliferate and produce anti-inflammatory cytokines efficiently.

Superagonist CD28 antibodies allow for proliferation and IL-10 production of human T_R cells

Since CD80/CD86 blocking antibodies did show some effect on T_R activation, and since CD28 appears important in T_R development in mice, we next tested if signals transmitted by CD28 are able to reverse the anergic phenotype of human T_R cells. We tested two different CD28 antibodies (clones 28.2 and 9.3) and compared that with the response to CD46 antibodies. When T_R cells were activated alone with antibodies to CD3 and CD46 or CD28(clone 28.2), no proliferation or IL-10 production was detected (Figure 5). However, when T_R cells were activated alone with antibodies to CD28(clone 9.3), a significant increase in proliferation and IL-10 production was detected, although at lower levels than when DCs are present in the cultures. When T_R cells were activated in the presence of monocytes, all three antibodies were able to induce the proliferation of human T_R cells. Exogenous IL-2 was required for the proliferation and IL-10 production of pure T_R cells, indicating that although the CD28 clone 9.3 was able to allow for the proliferation and IL-10 production of human T_R cells, it did not allow for IL-2 production from T_R cells.

Signal transduction of human T_R cells

To further understand the anergic phenotype of pure T_R cells, we examined the signal transduction events following TCR activation of T_R cells. We examined the phosphotyrosine profile of T_R cells, naïve CD4 lymphocytes, and memory CD4 lymphocytes activated with antibodies to CD3 and CD46, or CD3 and CD28 (clone 9.3). As shown in Figure 6A, human T_R cells induced the phosphorylation of numerous proteins in response to CD3/CD46 and CD3/CD28 (clone 9.3) activating antibodies. Immunoprecipitation with specific antibodies to Zap-70, CD3 ζ , and LAT were used to establish the identity of the phosphorylated proteins (data not shown). There was no difference in phosphorylation of Zap-70, CD3 ζ , or LAT in T_R cells in response to CD3/CD46 activation compared with CD3/CD28 antibodies despite clear differences in proliferation of T_R cells in response to CD3/CD28 (Figure 5).

Since human T_R cells do not show defects in the phosphorylation of proteins involved in the TCR activation, we next tested whether these cells demonstrated an increase in intracellular calcium following activation with CD3/CD46 or CD3/CD28 (clone 9.3) antibodies and compared this to the response of naïve and memory CD4 cells. When naïve or memory CD4 lymphocytes are activated with antibodies to CD3 and CD46 or CD3 and CD28, a strong increase in intracellular calcium is detected (Figure 6B). Naïve lymphocytes stimulated with CD3/CD46 or CD3/CD28 demonstrated a higher prolonged intracellular calcium flux compared with memory cells. When human T_R cells were stimulated with antibodies to CD3/CD46, a detectable but weak increase in intracellular calcium was detected. When stimulated with antibodies to CD3 and CD28, a strong increase in intracellular calcium was detected, but still remained below the maximum calcium level in naïve or memory cells. The

level of intracellular calcium appeared to correlate with the proliferative capacity of T_R cells.

TGF- β is required for IL-10 production but not proliferation of human T_R cells

Studies have suggested a close relationship between TGF- β and T_R cell function. TGF- β induces Foxp3 expression in human and murine CD4 cells, and the resulting Foxp3 positive cells exhibit suppressive function in murine models^{25;26}, although studies with human cells remain controversial²⁷. Other studies suggest that TGF- β is important to maintaining peripheral T_R cell numbers and suppressive function²⁸. Therefore, we tested the effect of exogenous TGF- β 1 as well as the effect of a pan-TGF family neutralizing antibody(clone 1D11) on T_R cell proliferation and IL-10 production. When human T_R cells are activated with CD3 and CD28 antibodies in the presence of IL-2, proliferation and IL-10 production was detected(Figure 7). When TGF- β 1 was added to these cultures, proliferation of purified T_R cells was inhibited, but IL-10 levels remained elevated. When all TGF- β family members were neutralized, we detected significant proliferation of T_R cells, but the expression of the IL-10 was severely decreased. These data indicated that TGF- β is critical to maintaining IL-10 expression in T_R cells independent of its effect on proliferation.

Discussion

To better understand the requirements for growth and immunosuppressive cytokine expression of highly pure human T_R cells, we developed a novel sorting strategy to isolate human T_R to greater than 90% purity that express functional characteristics of T_R cells and a gene expression profile consistent with a T_R phenotype. We demonstrate that flow cytometry purified T_R cells fail to proliferate or express the immunosuppressive cytokine IL-10, even at high doses of IL-2. Dendritic cells are able provide the necessary signals to induce T_R cell proliferation and IL10 production, and under these conditions T_R cells are highly sensitive to IL-2, likely due to the constitutive expression of the high affinity IL-2 receptor CD25. Monocytes are less effective than DCs in this regard, although this could be partly explained by the use of a CD14 positive selection strategy that has been shown to affect the costimulatory function of these cells²⁹. In addition, IL-2 is critical to IL-10 production and proliferation and under the correct activating conditions, IL-2 drives IL-10 production in a dose dependent manner. Studies have shown that STAT5 is able to induce IL-10 expression due to STAT5 binding to an enhancer element of the IL-10 locus³⁰. In addition, studies examining the expression of IL-10 *in vivo* have shown that T_R cells are the predominant IL-10 expressing cell, and one explanation of this observation would be that the high expression of CD25 in T_R cells allows for effective utilization of IL-2 to drive IL-10 production³¹.

Dendritic cells are the most effective antigen presenting cell that are able to induce T_R cell proliferation and IL-10 production, and in the presence of DCs, T_R cells are able to proliferate to levels comparable to conventional CD4 cells. Prior studies have shown that signals mediated through CD28 are critical for T_R cell development. CD28^{-/-} animals have significantly reduced numbers of T_R cells compared with IL-2 or IL-2 receptor deficient animals³². However, neutralizing antibodies to CD80/CD86 were unable to abrogate T_R

activation in the presence of immature DCs, but did attenuate the proliferation of T_R cells stimulated in the presence of LPS treated DCs. Although this finding could be explained by technical reasons, such as failure of access of blocking antibodies in an immune synapse, it is also possible that DCs provide other co- stimulatory signals that drive the activation of T_R cells. In support of this, CD28 co- stimulation of human T_R cells was unable to fully activate these cells compared to the affect of DCs. In addition, unlike monocytes, DCs are able to induce T_R cell proliferation and IL-10 production in the absence of exogenous IL-2. Previous reports have shown that DCs express IL-2^{33–36}, although production of this cytokine is dispensable for effector T cell responses. Whether IL-2 production by dendritic cells is important for T_R expansion *in vivo* remains to be determined.

We also examined the proximal TCR phosphorylation events of pure human T_R cells. Our studies did not detect any detectable difference in phosphorylation of proteins in response to TCR activation even though these cells did not proliferate. There was no change in phosphorylation of Zap-70, CD3 ζ , or LAT in response to CD3 and superagonistic CD28 antibodies, also arguing that defects in proximal signaling proteins does not account for the rescue of proliferation by CD28 antibody (clone 9.3). When intracellular calcium levels were analyzed, we demonstrate that the failure of human T_R cells to proliferate to CD3/CD46 antibodies correlated with a failure to increase intracellular calcium levels compared to conventional CD4 memory and naïve CD4 cells. When CD3 and superagonistic CD28 antibodies(clone 9.3) were utilized, human T_R cells did display an increase in intracellular calcium, which correlated with the extent of proliferation of T_R cells. These data argue that a defect in CD28 signaling pathway at least partially accounts for the anergic phenotype of human T_R cells, and that strong stimulation with CD28 agonists is able to partly overcome this defect, which is supported by other studies^{37;38}.

These studies also examined the role of TGF- β on T_R cell proliferation and IL-10 production. We demonstrate that TGF- β actually inhibited the proliferation of pure human T_R cells. Surprisingly, the addition of TGF- β resulted in high levels of IL-10 to be produced, even though the cells proliferated less, implying that proliferation and IL-10 production in these cells occur due to divergent pathways. When an antibody that neutralizes all TGF- β family members was added to the cultures, proliferation was restored, but IL-10 production was severely inhibited. This is the first data that suggests that one of the main functions of TGF- β in T_R cells is to induce the expression of IL-10 separate from the role of TGF- β in inducing the expression of Foxp3. We have shown that blocking TGF- β also blocked the upregulation of Foxp3 in T_R cells, although these cells did express baseline levels of Foxp3(data not shown). TGF- β stimulation of Th1 cells was able to induce the expression of IL-10 that was dependent on Smad4 activation of the IL-10 locus³⁹, providing further evidence that one function of TGF- β signaling is to induce IL-10 expression. Collectively, these data indicate that in addition to the role of TGF- β in inducing Foxp3 in CD4 cells and a regulatory phenotype, TGF- β is critical to the expression of IL-10 from human T_R cells.

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Figure 1. T_R isolation protocol

A. PBMCs from healthy random donors were labeled with anti-CD25PE antibody and goat anti-mouse magnetic beads, and column purified. Flow plot shows representative CD4 and CD25 profile before and following column selection. **B.** The CD25^{hi} cells were sorted, then stained with Fxp3 antibodies. Representative flow diagrams showing the purity of column purified cells prior to cell sorting (42% Fxp3⁺ cells, left), and the purity cells post sorting (95% Fxp3⁺ cells, right).

Figure 2. Gene expression profiling in highly pure human T_R cells

Pure T_R cells and naïve CD4 cells were isolated by cell sorting, then RNA isolated and microarray analysis performed. Gene set of immunologically relevant genes are presented that are preferentially overexpressed or underexpressed in T_R cells compared to naïve CD4 cells.

Figure 3. CD14⁺ cells reverse the anergic phenotype of T_R cells

A. Pure T_R cells were cultured alone or together with irradiated CD14⁺ monocytes, and activated with plate bound antibodies to CD3 and CD46 with or without IL-2 as indicated. Three days later proliferation and IL-10 production was measured. **B.** Co-cultures of human T_R cells and monocytes were activated as in **A** with increasing doses of IL-2. Results are representative data of three separate experiments. *** p<0.001, ** p<0.01, *p<0.05

Figure 4. Dendritic cells are able to break the anergic phenotype of T_R cells

Pure T_R cells were cultured with immature(DC) or LPS treated dendritic cells (DCIps) or CD14⁺ monocytes without (A) and with IL-2 (B), and activated with CD3/CD46 antibodies. Neutralizing antibodies (Ab) to CD80 and CD86 were added (black bars) in some experiments. Three days later proliferation and IL-10 production were measured. All results were normalized to the proliferation and IL-10 production of conventional CD4⁺ cells activated similarly from each individual experiment. Data presented is the average from 4 separate experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ when compared to CD25^{hi} cells activated without other cell populations unless otherwise indicated.

Figure 5. A CD28 superagonist antibody is capable of inducing proliferation and IL-10 production in human T_R cells

Pure T_R cells were cultured alone or together with irradiated CD14 monocytes and activated with plate bound antibodies to CD3 and CD46, or CD3 and CD28 with or without IL2 as indicated. Proliferation(A) and IL-10 production(B) were measured 3 days later. Results are normalized to the maximal proliferation and IL-10 production of conventional CD4 cells.

Figure 6. Signal transduction of pure human T_R cells

A. Pure T_R cells, naïve CD4 cells(RA), or memory CD4 cells(RO) were labeled with CD3/CD28(clone 9.3) or CD3/CD46 antibodies and cross-linked with goat anti-mouse antibody for 5 minutes, western blots prepared and probed with phospho-tyrosine specific antibody (4G10). Blots were stripped then stained with an anti-Erk antibody to control for protein loading. UA represents unactivated CD4 cells and is representative of unactivated memory, naïve, and T_R cells(data not shown) **B.** T_R cells, naïve CD4 cells(RA), or memory CD4

cells(RO) were labeled with CD3/CD28(clone 9.3) or CD3/CD46 antibodies, loaded with fluo-4, then cross-linked with goat anti-mouse antibody and analyzed by flow cytometry.

Figure 7. Role of TGF- β in human T_R cells proliferation and IL-10 production

Pure T_R cells were cultured in media alone or activated with antibodies to CD3 and CD28, together with 10U/ml of IL-2 with TGF- β 1(TGF) or the pan-TGF family neutralizing antibody 1D11(block). Three days later proliferation and IL-10 production was measured.