Published in final edited form as:

Hum Immunol. 2007 June ; 68(6): 478–490.

Expansion of CD4⁺CD25⁺ Suppressive Regulatory T Cells From Rhesus Macaque Peripheral Blood By FN18/anti-human CD28 Coated Dynal Beads

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Abstract

CD4⁺CD25⁺ regulatory T cells (Tregs) play an important role in allograft and self-tolerance and thus have potential therapeutic application in transplantation, autoimmunity and allergy. Although non human primate (NHP) provide the most accepted preclinical models for translational studies in allograft tolerance and infectious diseases, CD4⁺CD25⁺ Tregs have been rarely studied in NHP. The low frequencies of Tregs in peripheral blood will likely necessitate *ex vivo* expansion to enable Tregs adaptive immune therapy in NHP and humans. Tregs were isolated by magnetic and flow sorting then stimulated weekly with anti-rhesus CD3 clone FN18 and anti-human CD28 coated Dynal beads plus 100U/ml rhIL-2. Under these conditions, the Tregs were expanded 300–2000-fold in 4 weeks. Expanded CD4⁺CD25⁺ Tregs expressed high to moderate levels of FOXP3 as well as CD95, CD62L, CD69 and CCR7 surface antigens. Expanded rhesus Tregs were anergic and suppressed the proliferation of autologous PBMC in a dose-dependent fashion, and the suppression was partially reversed by anti-TGF-β1 neutralizing Ab. These results demonstrate that rhesus macaque suppressive regulatory CD4⁺CD25⁺FOXP3⁺ Tregs can be efficiently expanded *in vitro* under rhesus specific stimulation, which would enable preclinical testing of Treg therapy in NHP model.

Keywords

Non-human primate; rhesus macaques; CD4⁺CD25⁺ Tregs expansion; FOXP3; memory

Introduction

A small subset of CD4 T lymphocytes, expressing the IL-2Rα chain CD25, has been identified as naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs) [1]. Many reports have demonstrated a role for Tregs in regulation of alloimmune responses and autoimmune diseases, suggesting that CD4⁺CD25⁺ Tregs play a critical role in immune regulation [2,3].

CD4⁺CD25⁺ Tregs are a heterogeneous population with regard to the brightness of CD25s as well as CD62L, CD69, CD38 and CD45RB expression [4,5]. Human CD4⁺CD25⁺ Tregs highly

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express memory markers CD45RO and CD95 [1,6,7]. The majorities of CD4⁺CD25⁺ Tregs preferentially migrate into lymphoid organs and express the lymphoid-homing chemokine receptor CCR7 and the adhesion molecule CD62L [8]. Thus, CD4⁺CD25⁺ Tregs represent a mixed population of central memory and effector memory cells [1,8].

CD4⁺CD25⁺ Tregs consistently express FOXP3, a transcription factor of the fork-head/winged helix family, and the glucocorticoid-induced TNF-receptor GITR. These molecules are important markers of CD4⁺CD25⁺ Tregs and also have regulatory functions [9,10]. The negative immunoregulatory molecules, CTLA-4 and TGF- β , are also expressed by CD4⁺CD25⁺ Tregs [11,12]. Although, CD4⁺CD25⁺ Tregs express IL-2R α , they do not produce IL-2 [13], and are anergic [14] following TCR-induced activation. However, CD4⁺CD25⁺ Tregs require IL-2 for growth, both *in vivo* and *in vitro* [15,16]. Activated CD4⁺CD25⁺ Tregs inhibit proliferation and cytokine production by responder CD4⁺CD25⁻ T cells [5]. The mechanisms mediating suppression by CD4⁺CD25⁺ Tregs are controversial. The *in vitro* suppressive effect of CD4⁺CD25⁺ Tregs appears to be cell-to-cell contact-dependent, but partial involvement of TGF- β 1 and IL-10 has also been reported [13,14,17,18]. Some *in vivo* studies have suggested a cytokine-dependent mechanism of suppression [19,20].

Recognizing the low Treg frequency and their high potential for adoptive immunotherapy, there is interest in developing Treg expansion protocols for translational application in humans with transplants and/or autoimmune diseases. It was reported that when culture medium was supplemented with anti-CD3 mAb and IL-2, efficient expansion of murine CD4⁺CD25⁺ Tregs ensued. Expanded murine Tregs retained their anergic and suppressive properties [5]. Of note, this stimulation protocol enhanced the suppressive effect of CD4⁺CD25⁺ Tregs 4–6-fold, compared with freshly isolated CD4⁺CD25⁺ Tregs. Similarly, anti-human CD3/CD28 coated Dynal beads combined with high concentration of rhIL-2 expanded human CD4⁺CD25⁺ Tregs, which retained suppressive effect on autologous responder cell proliferation [21,22]. Thus, functional Tregs can be expanded *ex vivo* in two diverse species.

Although NHP serve as surrogate preclinical models for human allograft transplantation and infectious diseases, studies of NHP CD4⁺CD25⁺ Tregs lag behind those in humans and rodents. Understanding the safety and effectiveness of Tregs awaits preclinical studies in large outbred species such as non-human primates. Thus, developing methods for expanding NHP Tregs have potential to advance this field. Like human and rodent CD4⁺CD25⁺ Tregs, most rhesus macaque CD4⁺CD25⁺ Tregs display a central memory phenotype, are anergic and suppress proliferation of autologous effector cells *in vitro* (Asiedu CK et al. manuscript submitted). As an extension of those studies we examined conditions for efficient expansion of rhesus macaque CD4⁺CD25⁺ Tregs *ex vivo*. Since, human specific CD3/CD28 T cell expander (anti-CD3 mAb, clone spvT3b) does not cross-react with a rhesus macaque, we generated rhesus specific reagent for the expansion of CD4⁺CD25⁺ Tregs. We found that rhesus CD4⁺CD25⁺ Tregs can be expanded 300–2000-fold within 4 weeks following stimulation with anti-rhesus CD3 (clone FN18) and anti-human CD28 coated Dynal beads plus 100 U/ml of rhIL-2. The expanded rhesus CD4⁺CD25⁺ Tregs expressed high levels of FOXP3 and exhibited a heterogeneous activated, central memory phenotype. Functionally, expanded rhesus Tregs was anergic and significantly suppressed proliferation of autologous PBMC. The mechanism of suppression was partially TGF- β 1 dependent. These studies provide a platform for forthcoming preclinical examination of NHP Tregs as cellular immunomodulators.

Materials and Methods

Animals

Male rhesus macaques (*Macaca mulatta*) from several NIH supported colonies and free of known primate pathogens were used in these experiments. All procedures were performed in

accordance with the National Institute of Health Guide for the Care and Use of Primates under supervision of the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Antibody coating of Dynal beads

Anti-mouse IgG beads (Dynal Biotech, Oslo, Norway) were washed twice with 0.1M Na borate pH 8.24 and incubated with purified anti-rhesus CD3 clone FN18 (2.5 $\mu\text{g}/10^7$ beads) and anti-human CD28 mAb (2.5 $\mu\text{g}/10^7$ beads) (total 5 μg mAbs per 10^7 beads) overnight at 4°C with rotation. 20 min after initial incubation with mAbs, bovine serum albumin (BSA) was added (0.5% final concentration). FN18/anti-human CD28 coated Dynal beads were washed 3 times with phosphate-buffered saline (PBS) at 4°C with rotation. Finally, antibody coated Dynal beads were suspended in PBS (0.02% NaN_3) at the concentration of 4×10^8 beads/ml according to the manufacturer's instructions.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained, heparinized peripheral blood samples by Ficoll-Hypaque density gradient (1.077 g/dl) centrifugation at 1500 rpm for 30 min. After washing twice, PBMC were resuspended in magnetic-activated cell sorting (MACS) buffer (0.5% FBS, 2mM EDTA in PBS, pH 7.2) and stained with anti-CD4 FITC (BD PharMingen, San Diego, CA) and anti-CD25 PE (Miltenyi Biotec, Auburn, CA). PBMC were incubated with mAbs for 15 min at room temperature (RT) and washed twice with cold MACS buffer. Then CD4⁺ cells were positively selected with MACS multi-sort anti-FITC microbeads according to the manufacturer's instructions. After releasing the anti-FITC microbeads, anti-PE microbeads were used to positively select CD4⁺CD25⁺ Tregs according to the manufacturer's protocol. In some cases, CD4⁺CD25⁺ expression were directly sorted by flow cytometry.

Expansion of CD4⁺CD25⁺ Tregs in vitro

Freshly isolated CD4⁺CD25⁺ Tregs were resuspended in X-VIVO 20 (Cambrex Bio Science, Walkersville, MD) medium supplemented with 100U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10% heat inactivated normal rhesus monkey serum (NMS), pooled from adult male rhesus monkeys (Labs of Virginia, Yemassee, SC) and stimulated with supermagnetic, polystyrene Dynal beads coated with rhesus specific FN18/anti-human CD28 mAbs at 1:1 ratio and 20U/ml of rhIL-2 (R&D Systems, Minneapolis, MN). 10^5 cells were cultured per well in 96 well U bottom plates. On day 3, the cells were transferred into 96 well flat bottom plates and 50% of the spent medium was replaced with fresh medium containing 100U/ml of rhIL-2. The cultured CD4⁺CD25⁺ Tregs were stimulated weekly with FN18/anti-human CD28 coated Dynal beads and cultured in 12 well plates at a density of 10^6 cells/ml. Culture medium was replenished with 100U/ml of rhIL-2 every 3–4 days. Viable cells were counted after trypan blue exclusion (Sigma-Aldrich, St Louis, MO). Phenotypic and functional analyses were performed 5–7 days following the 3rd stimulation.

Co-culture experiments

Freshly isolated PBMC were used as responder cells. 96 well round bottom plates were coated with anti-rhesus CD3 clone FN18 (10 $\mu\text{g}/\text{ml}$) overnight at 4°C. PBMC were resuspended in X-VIVO 20 medium supplemented with 100U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10% NMS at $2.5 \times 10^5/\text{ml}$. Anti-human CD28 mAb was added to a final concentration of 1 $\mu\text{g}/\text{ml}$ to provide costimulation. After washing FN18 coated plates with PBS, the responder cells (2×10^4) were added in a volume of 100 μl per well. Then expanded CD4⁺CD25⁺ Tregs were added to achieve responder: Treg (R: Treg) ratios of 1:1, 1:0.5, and 1:0.25. Where indicated, 100 μl of culture supernatant, harvested from expanded and rested CD4⁺CD25⁺ Tregs were

added to responder cells. Neutralizing anti-TGF- β 1 mAb was added to some co-culture wells as indicated. Co-cultures were incubated at 37°C for 5 days and labeled with ^3H thymidine (1 $\mu\text{Ci}/\text{well}$) for an additional 16 hours. Then the cells were harvested and ^3H thymidine uptake was measured as described previously [23].

Antibodies

Anti-human mAbs known to cross-react with rhesus macaques were selected for this study. These included mAbs CD4 FITC (M-T477, PharMingen); CD25 PE (4E3, Miltenyi); CD62L PE (SK11, PharMingen); and CD95 FITC (DX2, PharMingen), CD69 PC5 (TP1.55.3, Immunotech, Marceille Cedex, France), and purified anti-human CCR7 (150503, R&D Systems), which was biotinylated in our laboratory using the EZ-Link NHS-PEO solid phase biotinylation kit (Pierce, Rockford, IL) according to the manufacturer's instructions. SA-ECD (Immunotech) was used as a secondary reagent for biotinylated CCR7. For intracellular staining PE conjugated anti-human CTLA-4 (BN13, Pharmingen) mAb was used. Anti-mouse IgG1 FITC, IgG1 PE, IgG1 PC5 and SA-ECD (all from Immunotech) reagents were used as controls. Anti-mouse IgG2a biotin, IgG2a PE (Immunotech) and IgG2b RD1 (Beckman Coulter) were used as controls for biotinylated CCR7, CTLA-4 and CD25, respectively. For intracellular staining of FOXP3, FITC anti-human FOXP3 staining set (clone PCH101, eBioscience, San Diego, CA, USA) and rat IgG2a relevant isotype control (eBioscience) were used. Purified rabbit polyclonal anti-human FOXP3 antibody (ab 10563, abcam, Cambridge, MA, USA) and goat anti-rabbit IgG (HRP, ab 6721, abcam) were used for western blot analysis. Neutralizing anti-TGF- β 1 (AF-101-NA, R&D Systems) antibody was used in blocking assays.

Cell surface phenotyping by flow cytometry

5×10^5 expanded CD4⁺CD25⁺ Tregs were washed twice and resuspended in PBS supplemented with 1% BSA and 0.01% NaN_3 . The cells were initially blocked with normal mouse serum for 10 min. For multi-color staining, biotinylated anti-human CCR7 mAb was added first. Subsequently, SA-ECD and other directly conjugated mAbs were applied. All incubations were performed at RT for 20 min followed by 2 washes. Finally, the stained cells were fixed with cytofix (BD PharMingen) and subjected to flow cytometry using Cytomics FC500 cytometer (Beckman Coulter). Data were analyzed using RXP software (Beckman Coulter).

Intracellular staining by flow cytometry

1×10^6 expanded CD4⁺CD25⁺ Tregs were harvested 5–7 days after the 3rd stimulation with FN18/anti-human CD28-coated Dynal beads plus rhIL-2. Cells were washed twice and incubated for 4 h at a concentration of $1 \times 10^7/\text{ml}$ in X-VIVO 20 supplemented with 10% NMS in the presence or absence of 25 ng/ml of phorbol myristate acetate (PMA), 1 $\mu\text{g}/\text{ml}$ of ionomycin and 10 $\mu\text{g}/\text{ml}$ of brefeldin A (BFA) (all from Sigma-Aldrich). Then the cells were harvested, blocked with mouse serum for 10 min at RT and stained with cell surface markers or relevant isotype controls as mentioned above. Next, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) at 4°C overnight prior to staining with PE-conjugated anti-human CTLA-4 mAb or relevant isotype control. For FOXP3 intracellular staining, the cells were incubated with Fixation/Permeabilization buffer for 1 h at 4°C. After washing twice with 1X permeabilization buffer 1×10^6 cells were incubated with normal rat serum overnight at 4°C in permeabilization buffer. Then, the cells were stained with FITC conjugated anti-human FOXP3 clone PCH101 or relevant isotype control for 1 hour at 4°C. After washing twice with permeabilization buffer, the cells were resuspended in PBS and analyzed by flow cytometry. Data were analyzed using WinMDI and RXP software.

RT-PCR

First strand cDNA was generated from mRNA using random hexamers in the Superscript 1st strand synthesis system as per the manufacturer's protocol (Invitrogen, Carlsbad, CA). The sequences of the primers used were as follows: rhesus FOXP3 forward primer, 5'-CATTTTCATGCACCAGCTCTC-3', rhesus FOXP3 reverse primer, 5'-GTTTCGTCCATCCTCCTTTCCTTGATCTTGAGGTCA-3' (996 bp product) and rhesus β -actin forward primer, 5'-GTGGGGCGCCCCAGGCACCA-3', rhesus β -actin reverse primer, 5'-CTCCTTAATGTCACGCACGATTTC-3' (550 bp product). Amplification of rhesus FOXP3 was carried out in a total volume of 50 μ l, containing cDNA, 20 pmol of each primer, 5U of Taq Polymerase (Promega, Madison, WI) and 10 nmol of dNTP and 1.5 nM of $MgCl_2$. Amplification conditions were: initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were analyzed on 2% agarose gel containing ethidium bromide (Invitrogen). Amplification of rhesus β -actin was performed as described previously [24].

Quantitative Real Time RT-PCR

RNA was extracted from expanded $CD4^+CD25^+$ Tregs, freshly isolated $CD4^+CD25^+$ Tregs, and $CD4^+CD25^-$ cell subsets using μ MACS mRNA isolation kits (Miltenyi Biotec), according to conditions stipulated by the manufacturer. One-step real-time TaqMan PCR was used to analyze FOXP3 and CTLA-4 mRNA expression in expanded Tregs and isolated T cell subsets using the ABI/PRISM 7900HT sequence detector system (Applied Biosystems, Foster City, CA). Target mRNA expression was normalized to mRNA levels of the rhesus pyruvate dehydrogenase (PDH) gene using comparative Ct analysis. The amplification efficiencies for PDH and target genes were similar (Applied Biosystems bulletin #2). The primers and FAM probe mix for human CTLA-4, catalog number 4329510F was purchased from Applied Biosystems. The sequences for other primers and probes used are as follows: rhesus PDH forward primer, 5'-GCTGCTGAAGAGGCGCTT-3', rhesus PDH reverse primer, 5'-GCTCCTCATCCATACCCTGATTTT-3', rhesus PDH probe, FAM-5'-CACGAAGTGTACCTGCAGCGCAG-3'-TAMRA [25]; Rhesus FOXP3 forward primer, 5'-CACCTGGCTGGGAAAATGG-3', rhesus FOXP3 reverse primer, 5'-CCTTGTCAGATGATGCCACAGAT-3', rhesus FOXP3 probe, FAM-5'-CACTGACCAAGGCTTC-3'-NFQ (primers custom made by Applied Biosystems).

Western Blot

1×10^6 cells were washed 3 times with ice-cold PBS and the pellets were lysed directly in 1X sample loading buffer containing β -ME. Proteins in the whole cell lysates were denatured and separated by 10% SDS-PAGE. After electrophoresis the proteins were transferred to a PVDF membrane (Millipore, MA). The membrane was blocked with 5% non-fat milk (Bio-Rad Laboratories, Hercules, CA), stained with polyclonal rabbit anti-human FOXP3 antibody (1:500 in TBST, Abcam, ab10563) and incubated at 4°C overnight with gentle agitation. The membrane was washed 3 times with TBST for 5 min and incubated with HRP-conjugated goat polyclonal to rabbit IgG (1:5000, Abcam, ab 6721) for 1 h at room temperature followed by development using ECL detection kit (Amersham Biosciences, Uppsala, Sweden). The membranes were stripped and reprobed with anti-human β -actin (Sigma-Aldrich) to evaluate loading equivalency. The relative size of the specific band was estimated from a standard curve of molecular weight markers.

Luminex bead-based protein assay

Expanded Tregs and expanded $CD25^-$ T cells were washed 3 times with complete medium to wash out residual IL-2. Then the expanded cells were cultured (5×10^5 /ml) with or without FN18/CD28 coated dynal beads in the absence of additional rhIL-2. Supernatants from

expanded Tregs and expanded CD25⁻ T cells were collected after 72 hours culture. Culture supernatants of co-culture experiments were collected at day 5 before labeling with ³H thymidine and cytokine production was analyzed by Luminex bead-based assay according to manufacturer's instruction (BioSource-Invitrogen Corp., Carlsbad, CA).

Statistics

Differences between groups were analyzed by 2-tailed Student t test. P value <0.05 was considered significant (GraphPad Instat, San Diego, CA).

Results

FN18/anti-human CD28 coated Dynal beads effectively expand rhesus CD4⁺CD25⁺ Tregs *in vitro*

CD4⁺CD25⁺ Tregs were isolated from PBMC by sorting. The purity of isolated CD4⁺CD25⁺ Tregs was >90% and >98% by magnetic and flow sorting, respectively (Figure 1A and data not shown). On day 0, the CD4⁺CD25⁺ Tregs were stimulated with FN18/anti-human CD28 coated Dynal beads at 1:1 ratio and 20U/ml rhIL-2. Half of the medium was replenished with fresh medium containing increased rhIL-2 concentration of 100U/ml on day 3. Then, CD4⁺CD25⁺ Tregs were stimulated with FN18/anti-human CD28 coated Dynal beads plus 100U/ml rhIL-2 weekly. As shown in Figure 1B, a 300–2000-fold expansion of cultured CD4⁺CD25⁺ Tregs was achieved in a month. These findings demonstrate that rhesus CD4⁺CD25⁺ Tregs could be greatly expanded *in vitro* by repeated stimulation with FN18/anti-human CD28 coated Dynal beads plus rhIL-2.

FOXP3 expression of expanded rhesus CD4⁺CD25⁺ Tregs

As FOXP3 expression is a signature for CD4⁺CD25⁺ Tregs, we examined FOXP3 expression in expanded rhesus Tregs at mRNA and protein levels. First, cDNA from expanded CD4⁺CD25⁺ Tregs yielded a 996 bp product by RT-PCR using FOXP3 primers that were designed based on previously documented rhesus FOXP3 sequence analysis (Genbank accession #[NM_001032918](#), data not shown). Next, FOXP3 mRNA expression was quantitatively analyzed by real-time RT-PCR. As shown in Figure 2A, expanded CD4⁺CD25⁺ Tregs express comparable to higher levels of FOXP3 mRNA than freshly isolated CD4⁺CD25⁺ T cells. Notably, *in vitro* expanded CD4⁺CD25⁻ T cells showed slightly increased FOXP3 mRNA expression (Figure 2B). To follow up on the FOXP3 RNA expression, expanded Tregs and CD4⁺CD25⁻ T cells were stained for intracellular FOXP3 protein expression. As shown in Figure 2C, 90% of expanded CD4⁺CD25⁺ Tregs expressed intracellular FOXP3. FOXP3 was also detected in expanded CD4⁺CD25⁻ T cells as others have described [26]. Finally, FOXP3 protein expression in expanded CD4⁺CD25⁺ Tregs was confirmed by western blot analysis. Total cell lysates prepared from expanded rhesus Tregs were subjected to western blotting using anti-human FOXP3 polyclonal Ab (Abcam). A doublet band, ~ 47 kd representing FOXP3 protein was found only in expanded CD4⁺CD25⁺ Treg sample but not in cell lysates prepared from freshly isolated CD4⁺CD25⁻ T cells (Figure 2C). These findings indicate that *in vitro* expanded rhesus macaque CD4⁺CD25⁺ Tregs differentially express high levels of FOXP3 message and protein.

Phenotypic profiles of expanded CD4⁺CD25⁺ Tregs

After 3 consecutive stimulations with FN18/anti-human CD28 coated Dynal beads the *in vitro* expanded Tregs were almost homogeneously double positive for CD4 and CD25 expression (Figure 3A). This finding is consistent with a recent report by Hoffman et al., [27]. As shown in Figure 3B, 80% of the expanded CD4⁺CD25⁺ Tregs expressed CD95 indicating a memory phenotype [28]. In repeated experiments (N=3) an overall mean 66.8%

± 13.1 SD were CD95 positive. More than one third of the CD95⁺CD4⁺CD25⁺ Tregs shown in Figure 3B co-expressed CD69 (mean 29.5% ± 1.8 SD in 3 experiments), demonstrating an activated memory phenotype. The expression of CD62L, a secondary lymphoid organ homing receptor was retained on most of the expanded CD4⁺CD25⁺ Tregs (88% in Figure 3B, mean 74.7% ± 13.3 SD in 3 experiments); and nearly 80% of CD62L⁺ Tregs co-expressed CD95 (72% ± 15 SD), suggesting a dominant central memory phenotype. CCR7, another secondary lymphoid organ homing chemokine receptor was detected on a minority of expanded Tregs (15% ± 1.4 SD). Most of the CCR7⁺ subset co-expressed CD62L but only 20% co-expressed CD95. We did not find significant differences in memory markers between expanded CD4⁺CD25⁺ Tregs and expanded CD25⁻ T cells, but found some variations in CD25 and CTLA-4 expression. *In vitro* expanded Tregs were uniquely bright for the expression of CD25, while the majority of expanded CD25⁻ T cells were CD25^{dim} (Figure 3C). In addition, the expanded Tregs showed higher levels of intracellular CTLA-4 protein and message than those in expanded CD25⁻ T cells (Figure 3C, D). Although, the expanded Tregs display the same memory phenotype with expanded CD25⁻ T cells, the former exhibits homogeneously high expression of CD25 and to some extent CTLA-4, which might represent phenotypic distinctions between expanded macaque Tregs and expanded CD25⁻ T cells, that have been reported in humans [22, 27].

We also compared expanded and freshly isolated CD4⁺CD25⁺ Tregs for the expression of memory, homing and activation antigens. Like expanded CD4⁺CD25⁺ Tregs, the freshly isolated CD4⁺CD25⁺ Tregs highly expressed CD95 in combination with CD28 and CCR7 (Asiedu CK et al, manuscript submitted), suggesting a central memory phenotype. In contrast to expanded CD4⁺CD25⁺ Tregs, freshly isolated CD4⁺CD25⁺ Tregs showed negligible staining for CD69 expression, implying that freshly isolated rhesus macaque CD4⁺CD25⁺ Tregs represent a resting memory population (Figure 3E).

Cytokine production by expanded CD4⁺CD25⁺ Tregs

Next, we examined cytokine secretion of expanded T cells by a Luminex bead-based assay. In cultures without FN18/CD28 coated dynal beads, the expanded CD25⁻ T cells exhibited a 10–20 times higher secretion of IL-2, whereas expanded Tregs showed baseline level of IL-2 (data not shown). After stimulation with FN18/CD28 coated dynal beads for 72 hours, the expanded Tregs still secreted minimum amount of IL-2, almost equal with baseline level, while it was up-regulated >200-fold in expanded CD25⁻ T cells, suggesting that the expanded Tregs are anergic (Figure 4A). Interestingly, GM-CSF was also significantly elevated in expanded CD25⁻ T cells, but not in expanded Tregs. The level of IL-5, MIG, and MIP-1 β was never increased more than baseline level in all expanded Tregs, whereas it was variably increased in expanded CD25⁻ T cells (data not shown). In addition, mRNA expression of TGF- β was analyzed by real-time RT-PCR. The message of TGF- β was 2–3-fold higher in expanded Tregs than those in expanded CD25⁻ T cells (Figure 4B). These findings suggest that expanded Tregs are functionally quiescent.

In vitro expanded CD4⁺CD25⁺ Tregs are suppressive

The *in vitro* expanded CD4⁺CD25⁺ Tregs were functionally characterized in co-culture experiments, in which autologous PBMC were stimulated by plate-bound FN18 and soluble anti-human CD28. In contrast to normal responder PBMC, *in vitro* expanded CD4⁺CD25⁺ Tregs proliferated poorly (Figure 5A). Furthermore, expanded CD4⁺CD25⁺ Tregs significantly suppressed responder cell proliferation when co-cultured with responders in 1:1 ratio ($p < .05$). The suppression was dose-dependent (Figure 5A and Figure 5B). Culture supernatant from expanded CD4⁺CD25⁺ Tregs had a slight suppressive effect that was not statistically significant relative to the responder only. However, the suppression of responder cells by

expanded CD4⁺CD25⁺ Tregs was partially reversed when anti-TGF- β 1 neutralizing antibody was added to co-cultures (R:Treg 1:1 vs R:Tregs 1:1+anti-TGF- β 1) (Fig 5A).

In contrast to expanded CD4⁺CD25⁺ Tregs, expanded CD25⁻ T cells did not suppress responder cell proliferation, suggesting that expanded CD4⁺CD25⁺ Tregs and expanded CD25⁻ T cells are functionally distinct populations (Figure 5A). Although, the percent inhibition was variable in individual samples, the suppressive activity of expanded CD4⁺CD25⁺ Tregs on responder cell proliferation at 1:1 ratio was relatively stronger than those of freshly implanted CD4⁺CD25⁺ Tregs (Figure 5C).

CD4⁺CD25⁺ Tregs are known to compete with responder cells for IL-2, produced by activated responder cells during co-culture [29–32]. The consumption of IL-2 by Tregs is thought to be crucial to their up-regulation of Foxp3 [33]. CD4⁺CD25⁺ Tregs also inhibit IL-2 production [5]. It is difficult to resolve the degree to which early IL-2 consumption by Tregs or their inhibition of IL-2 production might have contributed to the suppressive activity of the expanded Tregs. When IL-2 levels were examined in the co-culture supernatants, we found that IL-2 production was decreased in co-cultures of responder and Tregs, while it was exuberantly increased when responder cells were co-cultured with expanded CD25⁻ T cells at 1:1 ratio (Figure 5D). We suggest that inhibition of responder cell IL-2 production by expanded FOXP3⁺CD4⁺CD25⁺ Tregs could be a major mechanism of suppression rather than IL-2 consumption, since expanded CD25⁻ T cells, which also express IL-2 receptor CD25 upon stimulation, did not inhibit, but rather enhanced IL-2 secretion in co-cultures with responder cells. Additionally, the findings that IFN- γ levels in culture supernatants of responder cells alone were similar to levels in co-cultures of responder cells with expanded Tregs, and were 10 fold higher in co-cultures of responder cells with expanded CD25⁻ T cells, support our contention that expanded CD4⁺CD25⁺ Tregs are functionally distinct from expanded CD25⁻ T cells.

Discussion

In vitro expansion of rare cell populations is widely used to facilitate definitive characterization and therapeutic applications using adoptive transfer. In murine models, wherein the supply of CD4⁺CD25⁺ Treg is essentially unlimited, adoptive transfer of these cells has successfully prevented allograft rejection and graft versus host disease [34,35]. Of note, a recent study showed that adoptive transfer of an unfractionated population of anergic T cells prolonged allograft acceptance in 50% of rhesus recipients for 1–2.4 years [36]. Here we have shown that isolated rhesus macaque CD4⁺CD25⁺ Tregs are expanded efficiently (up to 2000-fold) *in vitro* by repeated stimulation with rhesus specific FN18/anti-human CD28 coated Dynal beads plus 100U/ml of rhIL-2 over a period of one month. These cells significantly suppressed responder cell proliferation at 1:1 ratio. To our knowledge, this is the first report of *ex vivo* expansion of rhesus macaque CD4⁺CD25⁺ Tregs using a rhesus specific reagent.

CD4⁺CD25⁺ Tregs are anergic, but their survival and growth is exquisitely dependent on IL-2. Consequently, CD4⁺CD25⁺ Tregs are lacking in the thymus and periphery of both IL-2 KO and IL-2R KO mice [15,37]. Although, exogenous IL-2 is critical for TCR activated CD4⁺CD25⁺ Tregs to express suppressive activity [16,38], the high concentration of IL-2 or anti-CD28 abrogates both anergy and suppressive activity of Tregs in mice [39]. Conversely, human CD4⁺CD25⁺ Tregs expanded with anti-CD3/CD28 coated Dynal beads in the presence of high dose IL-2 in the range of 100U/ml–2000U/ml retained their phenotype as well as suppressive effects [21,22,26]. In our initial studies, rhesus CD4⁺CD25⁺ Tregs, expanded with FN18/CD28 coated Dynal beads in the presence of higher concentration of rhIL-2 (500U/ml) were anergic but had no suppressive activity on responder cell proliferation (data not shown). However, rhesus Tregs expanded with 100U/ml of rhIL-2 showed dose-dependent suppression

of autologous responder cell proliferation. Thus, there is an effect of recombinant IL-2 on CD4⁺CD25⁺ Tregs suppressive activity, which will likely require optimization for different species.

Scurfin/FOXP3 is a regulatory gene which encodes a transcription factor Scurfin. Deficiency of FOXP3 and Scurfin causes many autoimmune and inflammatory diseases as a consequence of uncontrolled activation and expansion of CD4⁺ T cells [40]. FOXP3 mRNA and protein expressions are highly correlated with CD4⁺CD25⁺ cells in both mice and human and thus considered as a key molecule for CD4⁺CD25⁺ Tregs [9,41]. We have determined previously that rhesus macaque FOXP3 cDNA (Genbank accession #**NM_001032918**) and amino acid sequences are >95% and >98% identical with human counterparts, respectively. Like human FOXP3 [42], two isoforms of FOXP3 were easily detected in lysates from rhesus CD4⁺CD25⁺ expanded Tregs by western blot analyses, however, the role of the individual isoforms of rhesus FOXP3 remains to be determined.

CD4⁺CD25⁺ Tregs are heterogeneous with regard to brightness of CD25, expression of integrins as well as activation/memory markers [13,18]. In addition to FOXP3 and CTLA-4, naturally occurring CD4⁺CD25⁺ Tregs express CD62L, CCR7, CD45RO and CD95. Similar to human and murine Tregs, freshly isolated rhesus macaques CD4⁺CD25⁺ exhibited a heterogeneous phenotype, with a dominant central memory subset (Asiedu CK et al. manuscript submitted). *In vitro* expanded rhesus CD4⁺CD25⁺ Tregs were also largely heterogeneous and the majority of them displayed CD95⁺CD62L⁺ central memory phenotype. In this context, the expanded CD4⁺CD25⁺ Tregs bearing CD62L should bind to peripheral node addressin on high endothelial venules to enable entry into lymph nodes, where interactions with APC, B cells and other T cells takes place [43,44]. Of interest is the minor subset of expanded rhesus CD4⁺CD25⁺ Tregs expressing the chemokine receptor CCR7 that directs cells to the organized T and B cell areas of the lymph node. Curiously, most of these cells appeared to be deficient in expression of Fas, i.e. CD95. Presumably, the absence of Fas would provide survival advantage in the midst of activated T cells that express FasL [45,46]. We speculate that these CD62L⁺CD95⁻ and CCR7⁺CD95⁻CD4⁺CD25⁺ Treg cells represent a single population of long lived, lymph node seeking memory cells. Alike, *in vitro* expanded CD4⁺CD25⁺ Tregs, the expanded CD25⁻ T cells also exhibited activated, memory profiles. However, the expression of CD25 was heterogeneous in expanded CD25⁻ T cells, in which the majority was CD25^{dim}. Although, it is known that CTLA-4 is not specific marker for Tregs, the CTLA-4 expression was consistently higher in both message (3–5-fold) and in single cell levels in expanded Tregs than expanded CD25⁻ T cells, suggesting that CD25 and CTLA-4 expression might be helpful in discriminating between *in vitro* expanded CD4⁺CD25⁺ Tregs and CD25⁻ T cells as reported previously [22,27]. Of note, the recent study of Liu et al., suggest that CD127 (IL-7R) is an excellent marker for human Tregs might be better than CD25, since the some CD4⁺CD25⁻CD127⁻FOXP3⁺ subset was anergic and as functionally suppressive as CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg subset [47]. Further studies are needed to determine whether CD127 expression will also coincide with the absence of FOXP3 expression in CD4⁺CD25⁺ macaque cells.

CD4⁺CD25⁺ Tregs are potent inhibitors of TCR mediated proliferation of autologous CD4⁺ and CD8⁺ T cells *in vitro* [38]. The suppressive phenotype of Tregs appears to differ between *in vitro* and *in vivo* models. Although both CD62L⁺ and CD62L⁻ subpopulations of CD4⁺CD25⁺ T cells can suppress *in vitro*, only the CD62L⁺CD4⁺CD25⁺ subset delayed adoptive transfer of diabetes and prevented acute graft versus host disease *in vivo* [8]. In addition, Levings et al. (2005) demonstrated that cloned human CD4⁺CD25⁺ T cell populations are heterogeneous with only the CD25^{hi} subset exhibiting suppressive activity. The intensity of CD25, CTLA-4 and GITR expression correlated with the suppressive activity of the clones [13]. It is now well known that the *in vitro* suppressive activity of naturally occurring

CD4⁺CD25⁺ T cells is mainly by cell-to-cell contact dependent mechanism, although some degree of involvement of TGF- β has also been reported [13,17].

To reveal whether the expanded CD4⁺CD25⁺ Tregs are potent regulators, we have compared the functional suppressive activity of expanded CD4⁺CD25⁺ Tregs versus expanded CD25⁻ T cells. As a result, expanded CD4⁺CD25⁺ Tregs showed a dose-dependent suppression on autologous responder cell proliferation, while expanded activated CD25⁻ T cells did not. Of note, anti-TGF- β 1 neutralizing antibody reversed the suppression of responder cell proliferation in co-cultures with expanded CD4⁺CD25⁺ Tregs, in contrast it suppressed the responder cell proliferation in co-cultures with expanded CD25⁻ T cells, i.e. in the absence of CD4⁺CD25⁺ Tregs. These results are consistent with expanded CD4⁺CD25⁺ Tregs and expanded CD25⁻ T cells being functionally distinct populations; with the *in vitro* suppressive effect of the former is at least partially TGF- β 1-dependent. The biological significance of TGF- β 1 dependence in rhesus Tregs is uncertain, since others have demonstrated TGF- β 1 producing CD4 T cells in long-term rhesus renal allograft recipients with metastable tolerance and evidence of ongoing chronic rejection [48]. Taken as a whole, these findings suggest that *ex vivo* expansion of anergic primate CD4⁺CD25⁺ Tregs is feasible for preclinical testing of Treg therapy in NHP model.

It has been demonstrated that CD4⁺CD25⁺ Tregs compete for IL-2 uptake with responder T cells both *in vitro* and *in vivo* [29–32]. CD4⁺CD25⁺ Tregs do not produce IL-2 but require continuous uptake of IL-2 to exert their suppressive function, even if the Tregs have been previously activated [16,29]. Murine studies have shown that T helper cell-derived IL-2 controls the number of CD25⁺Foxp3⁺ T cells in the periphery, and IL-2 signaling is required for the maintenance and function of Foxp3⁺ T cells *in vivo* [33]. Conceptually, high affinity IL-2 receptor CD25 expression on expanded Tregs could promote selective and constitutive capture of local IL-2, produced by activated responder cells during co-cultures, which has been reported to increase FOXP3 expression by CD4⁺CD25⁺ Tregs [33]. It is probable that IL-2 is necessary for continuous signaling of CD4⁺CD25⁺ Tregs to enhance their ability to inhibit IL-2 production by responder cells, rather than simply being an IL-2 sink. If IL-2 consumption was the primary mechanism of suppression in this study, the responder cell proliferation should also be suppressed in the co-cultures with expanded CD25⁻ T cells, which also express IL-2 receptor CD25. However, expanded CD25⁻ T cells up-regulated responder cell proliferation as well as IL-2 production in co-cultures, suggesting that an IL-2 sink mechanism is unlikely to be the primary mechanism of suppression by expanded rhesus CD4⁺CD25⁺ Tregs.

The successful *ex vivo* expansion of rhesus macaques CD4⁺CD25⁺ Tregs provides new experimental opportunities for in depth study of regulatory cytokine dependence, antigen requirements, and immunoregulatory mechanisms, e.g. ILT3 role, in the activation of expanded CD4⁺CD25⁺ Tregs to mediate suppression *in vivo* in an important surrogate human model.

Acknowledgements

The authors thank to Drs. Sakaguchi S and Nomura T for their help to design primers and experiments for rhesus specific FOXP3. We are also grateful to Ms. Lina Rodriguez and Jennifer McCairn for expert assistance and care of the rhesus macaques. This work was supported by NIH grants 1U19DK57958 and U19A10566542 to JMT.

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Abbreviations

BFA	brefeldin A
BSA	bovine serum albumin
MACS	magnetic-activated cell sorting
NHP	non human primate
NMS	normal monkey serum
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDH	pyruvate dehydrogenase
PMA	

phorbol myristate acetate

Treg

regulatory T cells

R:Treg

responder:Treg

RT

room temperature

Figure 1. Expansion of CD4⁺CD25⁺ Tregs with anti-rhesus CD3 clone FN18 and anti-human CD28 coated Dynal beads plus rhIL-2 *in vitro*

(A) CD4⁺CD25⁺ Tregs were isolated from PBMC by labeling with anti-human CD4 FITC and anti-human CD25 PE mAbs followed by positive selection using anti-FITC and anti-PE MACS microbeads. The purity of positively selected CD4⁺CD25⁺ Tregs was >90% by flow cytometry. The data are representative of >3 experiments performed. (B) 10⁵ purified CD4⁺CD25⁺ Tregs were stimulated weekly with Dynal beads coated with anti-rhesus CD3 clone FN18 and anti-human CD28 mAbs at 1:1 ratio in the presence of rhIL-2 (20U/ml for 3 days and 100U/ml thereafter). Viable cells were counted every week by trypan blue exclusion. 300–2000 fold expansion of CD4⁺CD25⁺ Tregs was obtained from 7 different donors (D1–D7) that was calculated as following: The number of viable cells at the indicated time points was divided by the number of viable cells at initiation of the culture.

A

B

C

D

Figure 2. Expanded rhesus CD4⁺CD25⁺ Tregs highly express FOXP3

(A) One-step real-time RT-PCR analysis of FOXP3 mRNA expression. mRNA extracted from different expanded (expTreg) and freshly isolated (fr) CD4⁺CD25⁺ Tregs as well as freshly isolated CD4⁺CD25⁻ T cells were analyzed in every set of experiments. FOXP3 mRNA expression was normalized to mRNA levels of the rhesus house keeping PDH gene using comparative C_t analysis. Freshly isolated CD4⁺CD25⁻ T cells were chosen as the calibrator. Representative data from 3 independent experiments are shown.

(B) Comparison of FOXP3 message in expanded Tregs and in expanded CD25⁻ T cells. mRNA extracted from expanded Tregs and expanded CD25⁻ T cells from the same donors were analyzed for the expression of FOXP3 mRNA by one-step real-time RT-PCR. FOXP3 mRNA expression was normalized to mRNA levels of the rhesus house keeping PDH gene using comparative C_t analysis. Freshly isolated CD4⁺CD25⁻ T cells were chosen as the calibrator. Representative data from 2 independent experiments are shown.

(C) Intracellular FOXP3 expression of expanded Tregs and expanded CD25⁻ T cells in single cell level by flow cytometry. 1×10⁶ cells from each population were permeabilized and incubated with normal rat serum overnight in permeabilization buffer. Then, the cells were stained with FITC conjugated anti-human FOXP3 or relevant isotype control for 1 hour and analyzed by flow cytometry. Data were analyzed using RXP software. Representative data of repeated experiments from 6 different samples are shown.

(D) Western blot analysis for FOXP3 protein expression. Whole cell lysates from 1×10⁶ cells were applied for this experiment. Representative data from 5 different donors including expanded CD4⁺CD25⁺ Tregs and freshly isolated CD4⁺CD25⁻ T cells are shown to compare relative levels of FOXP3.

A **B**

Figure 3. Phenotypic characterization of expanded CD4⁺CD25⁺ Tregs

(A) 5×10^5 expanded CD4⁺CD25⁺ Tregs were stained with CD4 FITC and CD25 PE for 20 min at RT and analyzed by flow cytometry. (B) Since essentially all the expanded Tregs were CD4⁺CD25⁺, additional 4-color staining was done with anti-CD95 FITC, anti-CD69 PC5, anti-CD62L PE, and biotinylated anti-CCR7 primary mAbs and relevant isotype controls. SA-ECD was used as a secondary reagent for detection of biotinylated CCR7. Since, the expanded cells were homogenously double positive for CD4/CD25, the total lymphocyte gate was used for this analysis. Quadrants were set based on isotype controls. These data are representative of 3 independent experiments performed. (C) 10^6 expanded CD4⁺CD25⁺ Tregs and expanded CD25⁻ T cells were analyzed for cell surface expression of CD25 and intracellular expression

of CTLA-4 by flow cytometry. Histogram profiles gated on lymphocyte population from both expanded Tregs and expanded CD25⁻ T cells are shown. The markers are based on isotype controls. Representative data from 3 donors are shown. **(D)** mRNA was extracted from expanded Tregs and expanded CD25⁻ T cells from the same donors and analyzed for CTLA-4 expression by one-step real-time RT-PCR. CTLA-4 mRNA expression was normalized to mRNA levels of the rhesus house keeping PDH gene using comparative C_t analysis. Freshly isolated CD14⁺ cells were chosen as the calibrator. Representative data from 3 independent experiments are shown. **(E)** Histograms show cell surface expression of CD69 on expanded and freshly isolated CD4⁺CD25⁺ Tregs. Markers are based on relevant isotype controls. Representative data from 4 donors are shown.

A **B** **Figure 4. Cytokine production by expanded Tregs**

(A) Analysis of cytokine secretion by Luminex bead-based assay. 72 hours culture supernatants were collected from expanded Tregs and expanded CD25⁻ T cells. The expanded cells were washed 3 times with complete medium to wash out residual IL-2. Then the expanded cells were cultured with or without FN18/CD28 coated dynal beads in the absence of additional rhIL-2. The culture supernatants were applied for Luminex bead-based assay. The concentration of cytokines is shown in "log" scale. Representative data from 2 independent experiments of 3 donors are shown. (B) Comparison of TGF- β message in expanded Tregs and in expanded CD25⁻ T cells. mRNA extracted from expanded Tregs and expanded CD25⁻ T cells from the same donors were analyzed for the expression of TGF- β mRNA by one-step real-time RT-PCR. TGF- β mRNA expression was normalized to mRNA levels of the rhesus house keeping PDH gene using comparative C_t analysis. Freshly isolated CD4⁺CD25⁻ T cells were chosen as the calibrator. Representative data from 2 independent experiments are shown.

A **B** **C**

D

Figure 5. Expanded rhesus CD4⁺CD25⁺ Tregs suppress the proliferation of autologous PBMC (A) For co-culture assays, freshly isolated PBMC (2.5×10^4 /well) were stimulated with plate-bound FN18 (10 μ g/ml) and soluble anti-human CD28 (1 μ g/ml) and cultured with expanded CD4⁺CD25⁺ Tregs and expanded CD25⁻ T cells from the same donors. The co-cultures were set in various (1:1, 1:0.5 and 1:0.25) responder to expanded Treg and responder to expanded CD25⁻ T cell ratios. 100 μ l of supernatants, harvested from expanded Tregs and expanded CD25⁻ T cells, were added in indicated wells. 25 μ g/well of anti-TGF- β 1 neutralizing mAb was applied for blocking experiments. All experiments were performed in triplicate wells. Cultures were incubated at 37°C for 5 days and labeled with ³H thymidine (1 μ Ci/well) for an additional 16 h. The data are representative of 4 independent experiments. (B) Line graph shows the individual dose-dependent inhibition of autologous PBMC proliferation by CD4⁺CD25⁺ Tregs expanded from 4 different donors. (C) Mean inhibition percentage of responder cells in co-cultures with expanded CD4⁺CD25⁺ Tregs (filled black bar) and freshly isolated CD4⁺CD25⁺ Tregs (hatched bars) at 1:1 ratio is shown. The data are representative of 4 independent experiments for each group. (D) Bar graph shows IL-2 and IFN- γ production in co-culture supernatants of responder:expanded CD4⁺CD25⁺ Tregs and responder:expanded CD25⁻ T cells at 1:1 ratio, analyzed by Luminex bead-based assay. Y axis represents concentration of cytokines in pg/ml. X axis represents IL-2 and IFN- γ production by responder cells only (open white bars), responder:expanded CD4⁺CD25⁺ Tregs (filled black bars) and responder:expanded CD25⁻ T cells (hatched bars). The data is representative of 2 donors.