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# Generation, cryopreservation, function and in vivo persistence of ex vivo expanded cynomolgus monkey regulatory T cells

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ABSTRACT

We expanded flow-sorted Foxp3$^+$ cynomolgus monkey regulatory T cells (Treg) >1000-fold after three rounds of stimulation with anti-CD3 mAb-loaded artificial antigen-presenting cells, rapamycin (first round only) and IL-2. The expanded Treg maintained their expression of Treg signature markers, CD25, CD27, CD39, Foxp3, Helios, and CTLA-4, as well as CXCR3, which plays an important role in T cell migration to sites of inflammation. In contrast to expanded effector T cells (Teff), expanded Treg produced minimal IFN-γ and IL-17 and no IL-2 and potently suppressed Teff proliferation. Following cryopreservation, thawed Treg were less viable than their freshly-expanded counterparts, although no significant changes in phenotype or suppressive ability were observed. Additional rounds of stimulation/expansion restored maximal viability. Furthermore, adoptively-transferred autologous Treg expanded from cryopreserved second round stocks and labeled with CFSE or VPD450 were detected in blood and secondary lymphoid tissues of normal or immunosuppressed recipients at least two months after their systemic infusion.



## 1. Introduction

Regulatory T cells (Treg) characterized by expression of the transcription factor forkhead box P3 (Foxp3) function to maintain immune tolerance and prevent inflammatory diseases [1]. Thus, considerable effort has been expended to exploit their immunomodulatory properties. Adoptive transfer of Treg has been shown to prevent rejection of organ allografts in mice and humanized mouse models [2–4], and to prevent experimental graft-versus-host disease (GVHD) [5]. These preclinical studies support Treg as a highly promising potential therapeutic agent to suppress adverse immune-mediated inflammatory reactions and promote immune tolerance in transplantation and autoimmune disease [6–8].

A major challenge in the clinical application of Treg is their paucity in peripheral blood. Expansion of isolated, naturally-occurring Treg ex vivo may provide a solution via generation of massive numbers of these cells for therapeutic application [5,9]. It has been reported that combination of high dose IL-2, rapamycin, anti-CD3 and (or) anti-CD28 beads or monoclonal antibodies and artificial APCs (aAPC) can expand Treg massively in mice and humans [10–12]. In contrast to these studies in mice and humans, few reports have addressed expansion of Treg from non-human primates (NHP), -important pre-clinical models in organ transplantation [13–18]. Given the challenge of manufacturing Treg on a large scale and the potential advantage of banking these cells, cryopreservation may facilitate their clinical application [19]. However, there is evidence that cryopreservation can affect Treg recovery, viability [11], expression of essential surface markers, cytokine secretion and function [20,21]. Here, we optimized a protocol for expansion of NHP polyclonal Treg that retained their phenotype and suppressive capacity; after two rounds of expansion we cryopreserved the Treg, then resuscitated and restimulated them for an additional round of expansion to overcome the negative effect of cryopreservation.

An additional important issue concerns the stability, trafficking and fate of Treg after their infusion in vivo. Like other immune cells,

*Abbreviations:* Treg, regulatory T cells; Teff, effector T cells; PBMC, peripheral blood mononuclear cells; Foxp3, forkhead box P3; APC(s), antigen-presenting cell(s); NHP, non-human primate; r, round(s) of expansion; d, days in a round of expansion; CFSE, carboxyfluorescein succinimidyl ester; VPD450, violet proliferation dye 450; MFI, mean fluorescence intensity; mAb, monoclonal antibody; IS, immunosuppressed; PI, propidium iodide; LN, lymph node(s); ing., inguinal; mes., mesenteric.

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Treg vary in their homing and chemokine receptor expression, that controls their in vivo tissue distribution [22]. In the setting of transplantation or autoimmune disease, immunosuppressive (IS) drug administration may affect the properties of infused Treg. For optimal function, adoptively-transferred Treg must be able to survive and migrate to host lymphoid tissues [23,24] and/or sites of inflammation [25], even in the presence of IS therapy. We therefore also investigated the persistence of expanded autologous Treg in blood and lymphoid tissues following their infusion in cynomolgus monkey recipients, with or without IS treatment. The IS regimen used comprised a lymphocyte-depleting agent (anti-thymocyte globulin; ATG [26,27]), tacrolimus and rapamycin. The latter agents inhibit T cell proliferative responses. Both ATG [28] and rapamycin [29] have been considered "Treg-sparing" IS agents.

## 2. Materials and methods

### 2.1. Animals and cell lines

Healthy male cynomolgus macaques (Macaca fascicularis) of Indonesian origin, weighing 3–5 kg, were obtained from specific pathogen-free colonies at Alpha Genesis, Inc, or the NIAID NHP colony (both Yemassee, SC). All animal procedures were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and conducted under a University of Pittsburgh Institutional Animal Care and Use Committee-approved protocol. Specific environment enrichment was provided.

Artificial antigen-presenting cells (aAPC) (L-32) that stably express CD32, CD80 and CD58, were kindly provided by Dr. M. K. Levings, University of British Columbia, Vancouver, Canada.

### 2.2. PBMC isolation

Fresh peripheral blood mononuclear cells (PBMC) were isolated from blood by one of the three methods (Fig. 1Ai–iii). The first method was to dilute the blood with PBS at 1:1 ratio, overlay 8 ml diluted blood on 4 ml Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB), spin for 20 min at 1500 rpm, then collect the buffy coat after centrifugation. The second method was to overlay 4 ml blood, diluted as in the first method, on 3 ml Lympholyte-mammal (Cedarlane, Burlington, NC) and collect the buffy coat post centrifugation. The third method was to mix 10 ml blood with 1.25 ml OptiPrep (Axis-Shield PoC AS, Oslo, Norway) thoroughly, overlay with 1 ml PBS, spin down, and then collect the PBMC layer between plasma and PBS. PBMC layers obtained using these three approaches were treated with red blood cell (RBC) lysis buffer to remove red blood cells. RBC lysis buffer-treated whole blood was used as a control. The yield and purity of the mononuclear cells was affected to a considerable extent by the efficiency of red blood cell lysis. PBMC composition was determined by flow cytometry. Absolute counts of each cell type were determined by CountBright™ absolute counting beads (Invitrogen), according to the manufacturer's protocol. Percent recovery was calculated as: absolute number of cells of interest in PBMC isolated from 1 ml blood/absolute number of cells of interest in 1 ml whole blood $\times$ 100%.

### 2.3. Treg isolation and ex vivo expansion

PBMCs isolated by Ficoll-Paque Plus or OptiPrep were labeled with fluorochrome-labeled anti-CD4, anti-CD25 and anti-CD127 (BD PharMingen, Franklin Lakes, NJ, or BioLegend, San Diego, CA) antibodies, and then flow-sorted into populations of $CD4^+CD25^+$ $CD127^-$ Treg and $CD4^+CD25^-CD127^+$ effector T cells (Teff) (Fig. 2) on a BD FACS Aria II high-speed cell sorter (BD Biosciences, San Jose, CA). Irradiated (80 Gy) and anti-CD3 mAb-preloaded artificial antigen-presenting cells (aAPCs) L-32 were cultured with sorted Treg or Teff control at 1:1 T to aAPC ratio in complete RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Mediatech, Inc., Herndon, VA), 100 U/ml penicillin–streptomycin (BioWhittaker), 10 mM HEPES (Mediatech) and 55 μM β-2 mercaptoethanol (Invitrogen) in the presence of 300 U/ml recombinant human IL-2 (R&D Systems, Minneapolis, MN) and 100 ng/ml rapamycin (LC Laboratories, Woburn, MA) for 3–4 days. Then the cells were split and transferred to larger vessels without L-32 cells or anti-CD3 mAb for an additional 4 days. At the end of the initial round of culture (7–8 days), non-adherent T cells were harvested and re-stimulated with L-32 cells as in the first round for an additional 2 rounds, except that no rapamycin was added. During each round, cultures were supplemented with fresh medium and 300 U/ml IL-2 at intervals. The protocol used for Treg expansion and investigation is shown in Fig. 3. To cryopreserve cells at the end of each round, they were first suspended in 80% medium, 20% FCS at $<2 \times 10^7$ cells per ml, and then diluted 1:1 with 60% medium, 20% FCS, 20% dimethyl sulfoxide (DMSO, Fisher Scientific, Fairlawn, New Jersey) and stored in liquid nitrogen. In some experiments, cryopreserved expanded Treg were re-stimulated and expanded for an additional round, as described above.

### 2.4. Flow cytometry

Single cell suspensions of T cells were stained as described [17] at 4 °C with fluorochrome-labeled anti-CD3, anti-CD4, anti-CD25, anti-CD27-PE, anti-CD39, anti-CD44, anti-CD45RA, anti-CD62L, anti-CD127, anti-CXCR3, anti-CCR7 (BD PharMingen, Franklin Lakes, NJ, or BioLegend, San Diego, CA). Intracellular Foxp3, cytotoxic T lymphocyte Ag-4 (CTLA-4), Helios, T-bet, IL-2, IL-17 and IFN-γ were stained using an eBioscience Foxp3 Staining kit, according to the manufacturer's instructions. Data were acquired on a LSR II or LSR Fortessa (BD Bioscience) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

### 2.5. Cytokine detection

Ex vivo-expanded Treg or for comparison, Teff, were stimulated with anti-CD3-preloaded L-32 cells at a Treg to L-32 ratio of 1:1 for 3 days. Supernatants were collected on day 1, 2 and 3, and IL-2, IL-17 and IFN-γ measured using a Cytometric Bead Assay (CBA) Flex set system following the manufacturer's instructions. Data were acquired on a LSR Fortessa and analyzed using FCAP Array Software (BD Bioscience). For intracellular cytokine staining, Treg or Teff were further activated on day 1 for 4 h with phorbol 12-myristate 13-acetate (PMA) (1 μg/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (BD Bioscience), followed by staining with LIVE/DEAD fixable dye (Molecular Probes, Invitrogen) and fluorescent-labeled anti-CD4 mAb. Intracellular expression of IL-2, IFN-γ or IL-17 was detected according to the eBioscience Intracellular Foxp3 Staining Protocol.

### 2.6. Apoptosis assay

Cells were stained using Annexin V Apoptosis Detection Kits (eBioscience) following the manufacturer's instructions. Briefly, expanded Treg were resuspended in 50 μl Annexin V binding buffer and incubated with 2.5 μl FITC or eFluor 405-labeled Annexin V at room temperature for 15 min. Two microliters propidium iodide (PI) were added to each sample before flow cytometry was performed. Annexin-$V^+$ $PI^-$ cells and Annexin $V^+$ $PI^+$ cells were considered early-apoptotic and late-apoptotic/necrotic, respectively.