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Rhesus Monkey Immature Monocyte-Derived Dendritic Cells Generate Alloantigen-Specific Regulatory T Cells From Circulating CD4⁺CD127^{-/lo} T cells

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Abstract

Background—Generation of non-human primate regulatory T cells (Treg) with alloantigen (alloAg) specificity would allow their testing in pre-clinical transplant models. Low recovery of Treg from peripheral blood limits their potential utility. In small animals and humans, conventional myeloid dendritic cells (DC) have been shown to select or induce alloAg-specific Treg.

Methods—We combined enrichment of rhesus macaque blood CD4⁺ Treg based on IL-7R α (CD127) expression with their stimulation in mixed leukocyte cultures with immature, allogeneic, control or vitamin (Vit) D3/IL-10-conditioned monocyte-derived DC. Following co-culture in IL-2 and IL-15 for up to 14 days, the ability of the resulting T cells to suppress alloreactive effector T cell proliferation was assessed.

Results—CD4⁺CD127^{-/lo} T cells represented approximately 7% of normal rhesus circulating CD4⁺ T cells, and were enriched for forkhead box P3 (Foxp3)⁺ cells. When stimulated with control allogeneic DC, they exhibited much inferior proliferative responses compared with bulk CD4⁺ or CD4⁺CD127⁺ cells. This anergic state was reversed by exogenous IL-2 and IL-15. Following 10–14 days culture of CD4⁺CD127^{-/lo} T cells with immature allogeneic DC, particularly maturation-resistant VitD3/IL-10 DC, the frequency of Foxp3⁺ T cells was increased. The cultured cells markedly inhibited CD4⁺ effector T cell proliferation in a dose-related and donor alloAg-specific manner.

Conclusions—Stimulation of rhesus CD4⁺CD127^{-/lo} T cells with immature and especially maturation-resistant allogeneic DC, generated highly-suppressive, alloAg-specific Treg. Without resorting to a more highly-purified starting population, this approach may have therapeutic utility in clinically-relevant transplant models.

Keywords

rhesus monkey; dendritic cells; vitamin D3; interleukin-10; regulatory T cells

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Introduction

Studies in non-human primates (NHP) have revealed both the potential and the limitations of experimental therapies for the induction of clinical, donor-specific transplant tolerance (1–3). Based on small animal work, there is currently considerable interest in the potential of regulatory T cells (Treg) for therapy of allograft rejection (4–8) and graft-versus-host disease (9,10). The most extensively studied Treg are murine, naturally-occurring, thymus-derived CD4⁺CD25⁺ Treg, that constitutively express the transcription factor forkhead box P3 (Foxp3) (11,12). In human peripheral blood, Treg constitute only a small fraction of all CD25⁺ cells, mostly CD4⁺CD25^{hi} cells. In both mice and humans, naturally-occurring Treg express high intracellular levels of cytotoxic T lymphocyte antigen (Ag) 4 (CTLA4) and Foxp3, and suppress CD4⁺CD25⁺ T cell proliferation and cytokine production (6) through multiple mechanisms (11,13).

One of the major limitations to the characterization/purification of naturally-occurring Treg is the lack of Treg-specific markers. Unlike the mouse, where Foxp3 is a Treg-restricted (albeit intranuclear) marker (14), Foxp3 (and CTLA4) is transiently expressed by activated human T cells that lack regulatory function (15,16). In vivo, murine Treg inhibit T cell responses to alloAg and suppress allograft rejection (17,18). Treg can be expanded polyclonally in vitro, with retention of suppressor function, by stimulation with anti-CD3/CD28 monoclonal antibodies (mAbs) (19,20) in the presence of IL-2. In mice or humans, alloAg-specific Treg can be selected/enriched/expanded in vitro/ex vivo using either allogeneic blood- or spleen-derived mononuclear cells (21), autologous/syngeneic dendritic cells (DC) pulsed with allopeptide (7,22), or allogeneic DC (23,24). These oligoclonal Treg exert a stronger and more selective activity than polyclonal Treg (4).

Phenotypic and functional characterization of NHP naturally-occurring Treg (CD4⁺CD25⁺) in blood or lymphoid tissues has been reported previously for the rhesus macaque (25–27), cynomolgus macaque (28) and baboon (29), and their expansion documented in response to polyclonal stimuli (25,30), xenogeneic peripheral blood mononuclear cells (PBMC) (29) or allogeneic DC (28). As in humans, however, more precise characterization of NHP Treg is needed, and to date, the generation of alloAg-specific Treg in NHP has not been described.

Recently, it has been reported that human Treg can be distinguished from conventional T cells by the absence of cell surface CD127 (IL-7R α), and that expression of CD127 correlates inversely with Foxp3 and the suppressive function of human Treg (31,32). Moreover, CD127 depletion enriches for human functional Treg, that retain Foxp3 expression and potent suppressive activity after CD25⁺ cell isolation and alloAg- or Ab-induced expansion (21). In cynomolgus macaques, CD4⁺CD25⁺CD127⁺ cells that express Foxp3 and exhibit suppressive activity, have been isolated and expanded using ‘semi-mature’ allogeneic DC and IL-2 (28), although alloAg specificity of the Treg was not ascertained. In rhesus macaques, circulating Treg have been expanded ex vivo using anti-CD3/CD28 mAbs and IL-2, and shown to potently inhibit effector T cell proliferative responses in a non-specific manner (30).

Herein, we report that, by co-culturing rhesus CD4⁺CD127^{+/lo} T cells with donor-derived immature DC, in particular vitamin/D3 (VitD3) IL-10-conditioned DC, a highly suppressive and alloAg-specific Treg population is rendered. This approach bypasses the need for a high level of Treg purification (a costly step that also reduces the number of available cells) and renders cells with therapeutic potential.

MATERIALS AND METHODS

Animals

Captive-bred, simian immunodeficiency virus-negative, herpes B virus-negative male indian rhesus macaque monkeys (*Macacca mulatta*), aged between 5 and 7 years, were maintained within the Primate Infectious Disease Research Facility of the University of Pittsburgh School of Medicine. All experiments were conducted according to the guidelines set forth in the National Institutes for Health *Guide for the Care and Use of Laboratory Animals* and approved by the appropriate Institutional Animal Care and Use Committee. Specific environment enrichment was provided.

Flow cytometry

The following fluorochrome-conjugated mAbs were used for T cell surface phenotypic analysis: CD127-PE and Biotin (hIL-7R-M21) from BD Biosciences, San Jose, CA and CD4-Pacific Blue (OKT4) and CD25-PE-Cy7 (BC96) from eBioscience, San Diego, CA. Briefly, cells were suspended in cell staining buffer (phosphate-buffered saline [PBS] with 1% v/v fetal bovine serum [FBS; Atlanta Biologicals, Atlanta, GA] and 0.1% sodium azide) and Fc receptor binding inhibited by incubation on ice for 15 min with 10% v/v goat serum (Sigma-Aldrich, St. Louis, MO). Cells were then incubated on ice for 30 min with mAbs, washed, centrifuged and suspended in cell staining buffer. Fluorochrome-conjugated mAbs FoxP3-APC (3G3; Miltenyi Biotec, Auburn, CA) and CD152-APC (CTLA4, BNI3; BD Biosciences) were used for intracellular staining. Intracellular staining for both markers was performed using the FoxP3 intracellular staining reagent set from eBioscience, according to the manufacturer's specifications. Data were collected and analyzed using a BD Biosciences LSR II flow cytometer.

DC generation

PBMC were isolated from peripheral blood via Ficoll density gradient (GE Healthcare, Piscataway, NJ) centrifugation, according to the manufacturer's instructions. Blood monocytes were positively selected using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA). Control DC were generated as described (33), by culturing CD14⁺ cells in the presence of recombinant (r) human (hu) granulocyte-macrophage colony-stimulating factor and r hu IL-4 (1,000 U/ml each; R&D Systems, Minneapolis, MN) for 7 days at 0.7×10^6 cells/ml in RPMI-1640 media (Invitrogen, Carlsbad), supplemented with 10% v/v fetal bovine serum, 2 mM L-glutamine (Mediatech, Inc., Herndon, VA), 100 U/ml penicillin-streptomycin (BioWhittaker), 25 mM HEPES (Mediatech) and 55 μ M β -2 mercaptoethanol (Invitrogen) (complete medium; CM). Non-adherent cells were harvested on day 5 and plated at 10^6 cells/ml in fresh, cytokine-supplemented CM. Vitamin D3/IL-10 (VitD3/IL-10) DC were generated by adding VitD3 (20nM; Sigma-Aldrich) to DC cultures on day 1 and 5, and r hu IL-10 (20 U/ml; Peprotech, Rocky Hill, NJ) on day 5 of culture.

CD4⁺CD127^{-/lo} T cell isolation

'Untouched' CD4⁺ T cells were recovered from PBMC using a NHP CD4⁺ T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. CD3⁺CD4⁺ T cell purity was confirmed by flow cytometry and was routinely > 90%. An enriched CD4⁺CD127^{-/lo} T cell population was isolated from the bulk CD4⁺ T cell population by depleting T cells expressing high levels of cell surface CD127. CD4⁺ T cells were incubated with 660 μ l of biotin-conjugated anti-hu CD127 mAb and 340 μ l of microbead buffer (10^6 cells/ml) for 20 min at 4°C. After centrifugation and washing, anti-biotin microbeads (Miltenyi Biotec) were added, according to the manufacturer's instructions and the cells incubated at 4°C for 20 min.

CD127⁺ cells were depleted by positive selection using LD columns (Miltenyi Biotec), resulting in an enriched CD127^{-/-} T cell population.

CD4⁺CD127^{-/-} T cell co-culture with allogeneic DC

Freshly-isolated CD127^{-/-} T cells (0.2×10^6) were cultured with irradiated (2.5 Gy) allogeneic control or VitD3/IL-10 DC (1×10^5) in 96-well, round-bottom plates for 10–14 days in 200 μ l of CM supplemented with r hu IL-2 (12.5 U/ml; Peprotech) and r hu IL-15 (10 ng/ml; Peprotech). On days 4 and 7, 100 μ l of media were removed and replaced with fresh IL-2/IL-15-supplemented CM. The cells were harvested and washed on day 10, then rested for 2 days in CM, before phenotypic and functional analyses.

T cell anergy analysis

Freshly-isolated CD127^{-/-} T cells (0.2×10^6) were cultured with irradiated (2.5 Gy) allogeneic control or VitD3/IL-10 DC (1×10^5) in 96-well, round-bottom plates for 5 days in 200 μ l of CM supplemented with or without r hu IL-2 (12.5 U/ml; Peprotech) and r hu IL-15 (10 ng/ml; Peprotech). One μ Ci of [³H] TdR (Perkin Elmer, Downers Grove, IL) was added on day 4, and isotope incorporation measured using a TopCount NXT Scintillation counter (Perkin Elmer) on day 5. Tests were set up in quadruplicate; results were expressed as mean counts per minute (cpm) $\pm$ 1 standard deviation (SD).

T cell suppression assay

Decreasing numbers of freshly-isolated CD127^{-/-} T cells, or allogeneic control or VitD3/IL-10 DC-expanded CD127^{-/-} T cells, were co-cultured for 5 days with irradiated (2.5 Gy), allogeneic PBMC (0.1×10^6) depleted of T cells using CD2⁺ microbeads (Miltenyi Biotec), and syngeneic CD4⁺CD127^{pos} T cells or third party, MHC mis-matched CD4⁺CD127^{pos} T cells (0.1×10^6), to determine whether suppression was alloAg-specific. One μ Ci of [³H] TdR was added on day 4, and proliferation measured on day 5 by isotope incorporation. Tests were set-up in quadruplicate; results were expressed as the mean cpm $\pm$ 1SD.

Statistics

Two-tailed paired Student's 't'-test was performed using Microsoft Excel computer software (Microsoft Corp., Richmond, WA). 'P' values < 0.05 were considered significant.

RESULTS

Isolation of CD4⁺ CD127^{-/-} T cells from fresh normal rhesus blood and cryopreserved PBMC

Peripheral venous blood was drawn on multiple occasions from 5 monkeys. The total numbers of PBMC, CD4⁺ T cells and CD4⁺CD127^{-/-} cells isolated from each sample are shown in Table 1. The mean incidence of CD4⁺CD127^{-/-} T cells was $7 \pm 1.2\%$ of bulk CD4⁺ T cells and $1.3 \pm 0.3\%$ of total PBMC. Based on these determinations, the average number of CD4⁺CD127^{-/-} cells that could be obtained from 100 ml fresh normal whole blood was $3.7 \pm 0.8 \times 10^6$. In addition, we examined the recovery of CD4⁺CD127^{-/-} cells from cryopreserved samples of rhesus PBMC. The mean incidence of CD127^{-/-} cells that was recovered ($6 \pm 1.2\%$ of bulk CD4⁺ T cells) was similar to that achieved for fresh blood. Based on this determination, 5.1×10^6 CD4⁺CD127^{-/-} T cells could theoretically be obtained from 10^9 cryopreserved PBMC.

Phenotypic characterization of rhesus CD4⁺CD127^{-/-} and CD127⁺ T cell populations

We next stained freshly-isolated blood CD4⁺CD127^{-/-} and CD4⁺CD127⁺ T cells for molecules that have been associated with and used to isolate Treg in humans and NHP. As shown in Fig. 1, the CD4⁺CD127^{-/-} population comprised a ten-fold greater proportion of

Foxp3⁺ cells than was present in bulk CD4⁺ or CD4⁺CD127⁺ populations, and expressed much higher levels of CD25 and intracellular CTLA4 than CD127⁺ cells, only a small/minor percentage of which were positive for these markers.

Rhesus CD4⁺CD127^{-/-} T cells are anergic to stimulation with allogeneic, monocyte-derived DC

We then examined the capacity of freshly-isolated CD4⁺CD127^{-/-} T cells to respond to stimulation with in vitro-generated, allogeneic monocyte-derived DC in primary MLR. As shown in Fig 2, CD4⁺CD127^{-/-} cells exhibited much weaker proliferative responses than bulk CD4⁺ T cells when stimulated with control DC or VitD3/IL-10-conditioned DC. However, proliferative responses to either DC population were partially restored when IL-2 and IL-15 were incorporated at the start of the MLR cultures, confirming that the CD4⁺CD127^{-/-} T cells were anergic. Notably, the proliferative responses induced by VitD3/IL-10-conditioned rhesus DC, either in the absence or presence of exogenous IL-2 and IL-15, were significantly lower than those induced by unmodified DC, consistent with the ability of VitD3 and IL-10 to inhibit DC maturation (34,35). This suggested that, like maturation-resistant murine DC (24), these rhesus monkey VitD3/IL-10-conditioned DC could further enrich for Foxp3⁺ Treg.

CD4⁺CD127^{-/-} T cells co-cultured with control or VitD3/IL-10-conditioned allogeneic DC markedly inhibit proliferative responses of CD4⁺CD127⁺ T cells to alloAg

We next examined the ability of CD4⁺CD127^{-/-} T cells that had been cultured with control allogeneic DC in the presence of IL-2 and IL-15, to suppress proliferative responses of freshly-isolated CD4⁺CD127⁺ (conventional) T cells to the same alloAg. Whereas freshly-isolated CD4⁺CD127^{-/-} cells failed to affect CD4⁺CD127⁺ T cell proliferation, even at a 1:2 ratio (Fig 3A), CD4⁺CD127^{-/-} cells that had been co-cultured with either control or VitD3/IL-10-conditioned DC inhibited conventional CD4⁺ T cell proliferation in a dose-related manner (Fig. 3B&C). Significant inhibition of proliferation was observed at CD4⁺CD127^{-/-}:CD4⁺CD127⁺ ratios as low as 1:80, indicating a potent suppressive effect of the cultured CD4⁺CD127^{-/-} cells, that we refer to subsequently as Treg.

CD4⁺CD127^{-/-} T cells co-cultured with VitD3/IL-10 DC are more efficient inhibitors of T cell proliferation than those expanded with control DC

Human or mouse DC that have been propagated in the presence of maturation inhibitors such as IL-10, VitD3 or various immunosuppressive drugs, not only have impaired T cell stimulatory ability, but can spare or promote the expansion of Treg (24,36). As shown in Fig. 4A, when the suppressive capacity of rhesus CD4⁺CD127^{-/-} Treg that had been stimulated in culture with control or VitD3/IL-10-conditioned DC was compared, a significantly greater inhibitory effect of the VitD3/IL-10-conditioned DC was observed. Furthermore, expression of Foxp3 in the regulatory cells stimulated with VitD3/IL-10-conditioned DC was higher than in those stimulated with control DC (Fig. 4B), consistent with an ability of the former DC to enrich for Treg that correlates with more potent suppressive capacity.

CD4⁺CD127^{-/-} Treg suppress T cell proliferation in an alloAg-specific manner

We next ascertained the functional specificity of the Treg that had been stimulated either with control or VitD3/IL-10-conditioned DC. As shown in Fig. 5A & B, CD4⁺CD127^{-/-} cells stimulated with either DC population, inhibited proliferative responses of CD4⁺CD127⁺ T cells to the initial, but not third party stimulators. In these experiments, the Treg stimulated with VitD3/IL-10 DC again proved more effective inhibitors of conventional CD4⁺ T cell proliferation (Fig. 5C).

DISCUSSION

In the present studies, we show that immature, blood monocyte-derived rhesus DC, and especially maturation-resistant DC propagated in VitD3 and IL-10, can enrich for alloAg-specific Treg in 10–14-day cultures from anergic CD4⁺CD127^{-/lo} T cells present in the peripheral blood of normal rhesus monkeys. These cells retained expression of the transcription factor Foxp3, a master regulator of the Treg signature (37), the expression of which correlates with Treg function in mice. These rhesus Treg were highly effective inhibitors of CD4⁺ effector T cell proliferation in response to alloAg stimulation compared with naïve CD4⁺CD127^{-/lo} T cells. Their efficiency as downregulators of purified CD4⁺ T cell alloproliferation was evident at a 1:80 regulator/effector ratio, which compares to similar potency of polyclonally-expanded rhesus Treg (30), generated from flow-sorted CD4⁺CD25⁺CD127^{lo} cells.

Recent studies have shown that isolation of human CD4⁺ Treg results in significant loss of Foxp3⁺ cells (31), and that sorting of CD4⁺ T cells based on low or negative expression of CD127, combined with a broader CD25 gate, enhances recovery of (Foxp3⁺) Treg from peripheral blood, but provides a very low number of cells. In rhesus macaques, sorting of CD4⁺CD25⁺CD127^{lo} cells, as opposed to CD4⁺CD25^{bright} cells, has been reported to enhance the purity of Treg that can then be expanded several-fold in response to polyclonal stimulation over a 3-week period (30).

It is generally considered that, if Treg are to be used clinically in adoptive cell therapy protocols to control undesired immune responses and to promote allograft tolerance, efficient methods for their production and enhanced insight into their functional biology and specificity of activity in NHP is required. In the present study, generation of alloAg-specific rhesus Treg was not dependent on the acquisition of a starting population of highly-purified, flow-sorted, CD4⁺CD127⁻CD25⁺ Treg, as described recently by others (28,30), and thus represents a comparatively convenient and cost-effective approach to rendering Treg in culture. Previous reports have shown that naturally-occurring NHP (rhesus) Treg expand polyclonally in response to anti-CD3 and anti-CD28 mAb stimulation in the presence of IL-2 (25,30). Indeed, potent polyclonal rhesus Treg can expand several hundred-fold over 3–4 weeks of culture (30). However, these cells lack Ag specificity, a consideration that may limit their potential clinical utility.

Although immature DC are comparatively weak stimulators of effector T cell proliferation, they can differentiate/enrich/expand mouse or human Ag-specific Treg in vitro and in vivo (7,22,24,38,39). Various diverse anti-inflammatory and immunosuppressive agents, that include IL-10 (35), VitD3 (40), dexamethasone (41), and rapamycin (42), can inhibit DC maturation and induce a persistent state of immaturity, both in vitro and in vivo, that appears to promote their tolerogenicity. In previous studies, we have shown that exposure of rhesus monocyte-derived DC to a combination of VitD3 and IL-10 renders a maturation-resistant DC population, that inhibits alloreactive T cell responses, in vitro and in vivo (33). The present study extends these findings to provide a mechanism (enrichment of alloAg-specific Treg) for the ability of these pharmacologically-modified rhesus DC to subvert effector T cell responses. Currently, we are investigating whether these maturation-resistant rhesus DC may induce the conversion of Foxp3⁻ CD4⁺CD127^{-/lo} to T regulatory type-1 cells (43).

In recent rodent studies, we have shown that alloAg-specific Treg selected by co-incubation of freshly-isolated CD4⁺ Treg with donor bone marrow-derived immature DC, and that potently inhibit effector T cell proliferation in vitro, achieve superior in vivo therapeutic effects to polyclonal Treg in organ allograft recipients (44). An important question for future studies in NHP and humans is the number and frequency of Treg administration that will be required to promote lasting inhibition of donor-specific responses. Clearly, immature DC do not expand

the large numbers of Treg that can be achieved using powerful polyclonal stimulants, such as anti-CD3 + anti-CD28 mAbs. However, use of maturation-resistant DC to generate alloAg-specific rhesus Treg from fresh or cryopreserved CD4⁺ CD127^{-lo} cells may allow adequate numbers of these potent inhibitors of effector T cell proliferation to be administered locally or systemically to (transiently) T cell-depleted graft recipients, thus conferring a graft-specific protective effect. Such an effect has been described recently in T cell-depleted murine organ transplant recipients for Treg expanded initially with anti-CD3/CD28 mAbs, then with donor bone marrow-derived DC and IL-2 (8). In these circumstances, the ratio of adoptively-transferred Treg to host conventional T cells is shifted markedly in favor of Treg function. Significantly, VitD3 (in combination with dexamethasone) has been shown recently to generate human tolerogenic monocyte-derived DC that induce hyporesponsiveness in memory CD4⁺ T cells, as determined by both their proliferative capacity and cytokine production (45). This additional property of pharmacologically-conditioned tolerogenic DC may be of added benefit in relation to the potential therapeutic application of these cells in cell and organ transplantation.

In summary, these observations reveal that, without resorting to a more purified starting population, highly-suppressive, alloAg-specific CD4⁺ rhesus monkey Treg can be enriched from circulating CD4⁺CD127^{-lo} cells using immature and especially maturation-resistant, monocyte-derived DC. This approach may have potential utility for evaluation of Treg therapy in clinically-relevant transplant models.

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Abbreviations

DC, dendritic cells; Foxp3, forkhead box P3; NHP, non-human primate; Treg, regulatory T cells; VitD3, vitamin D3.

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FIGURE 1.

Differential expression of Foxp3, CD25 and CTLA4 by CD4⁺CD127^{-/-} and CD4⁺CD127⁺ rhesus normal peripheral blood T cells. T cell populations were purified and labeled as described in the Materials and Methods, then examined by flow cytometry. Foxp3 expression in normal bulk CD4⁺ T cells is shown for comparison. Data are from one experiment representative of 4 performed.

(†) p < 0.05 (*) p < 0.001

FIGURE 2.

Normal rhesus (R364) CD4⁺CD127^{-/-} T cells were co-cultured for 5 days with allogeneic (R362) control DC or VitD3/IL-10-conditioned DC, in the absence or presence of exogenous IL-2 (12.5 U/ml) and IL-15 (10 ng/ml) ('cytokines'). Proliferative responses, including those of bulk CD4⁺ T cells and CD4⁺CD127^{-/-} cells in the absence of stimulation, are shown as mean cpm ±1SD. Results are from 1 experiment representative of 3 performed.

A [Control DC]-T reg

(†) $p < 0.05$ (*) $p < 0.001$

B [VitD3/IL-10 DC]-T reg

(†) $p < 0.05$

(*) $p < 0.001$

C Freshly-isolated $CD4^+CD127^{-/lo}$ cells

FIGURE 3.

Normal rhesus (R194) $CD4^+CD127^{-/lo}$ T cells (TR) were expanded for 10 days with (A) allogeneic (R360) control DC, or (B) VitD3/IL-10-conditioned DC (B), in the presence of IL-2 (12.5 U/ml) and IL-15 (10 ng/ml). After harvesting, decreasing numbers of responder T cells were co-cultured for 5 days with irradiated (R360) PBMC (10^5) stimulators (S) (1:1) and R194 $CD4^+CD127^{-/lo}$ T cell responders (R) (10^5). (C) Freshly-isolated $CD4^+CD127^{-/lo}$ T cells did not suppress. 10^5 irradiated (R194) PBMC stimulators (S), 10^5 allogeneic (R360) $CD4^+CD127^+$ responder T cells (R), and 5×10^4 freshly-isolated $CD4^+CD127^{-/lo}$ R360 T cells were co-cultured for 5 days. Results are means $\pm$ 1 SD, and are from one experiment representative of 3 performed.

(†) $p < 0.05$

(*) $p < 0.001$

FIGURE 4.

(A) Normal rhesus (R364) CD4⁺CD127^{int} T cells were expanded for 10 days with allogeneic (R360) control DC, or VitD3/IL-10-conditioned DC, in the presence of IL-2 (12.5 U/ml) and IL-15 (10 ng/ml). After harvesting, decreasing numbers of R364 T reg were co-cultured for 5 days with irradiated, R360 PBMC (10⁵) stimulators (S) and R364 CD4⁺CD127⁺ T cell responders (R) (10⁵). (B) Expression of Foxp3 by CD4⁺- and CD4⁺CD25⁺-gated T cells in the control DC- and VitD3/IL-10DC-expanded populations were tested for suppressor function. Results are means $\pm$ 1SD from 1 experiment, representative of 3 (A) or 2 (B) performed.

A [Control DC]-T reg

(†) p < 0.05

(*) p < 0.001

B [VitD3/IL-10 DC]- T reg

(†) $p < 0.05$

(*) $p < 0.001$

(†) $p < 0.05$

FIGURE 5.

Rhesus (R363) $CD4^+CD127^{-/lo}$ cells (TR) were expanded with either R194 control DC (A) or VitD3/IL-10-conditioned DC (B) for 14 days in the presence of IL-2 (12.5 U/ml) and IL-15 (10 ng/ml). After harvesting, decreasing numbers of R363 Treg were co-cultured with irradiated, allogeneic R194 PBMC or third party R360 PBMC stimulators (S) (1:1) and R194 $CD4^+CD127^+$ responder T cells (R) for 5 days. Results are means $\pm$ 1SD from 1 experiment representative of 2 performed.

Table 1

Isolation of CD4⁺ CD127^{-lo} T cells from normal rhesus peripheral blood

Monkey	Volume Blood (ml)	PBMC ($\times 10^6$)	CD4 ⁺ T cells ($\times 10^6$)	CD4 ⁺ T cells (% PBMC)	CD127 ^{-lo} ($\times 10^6$)	CD127 ^{-lo} (% CD4 ⁺ T cells)	CD127 ^{-lo} (% PBMC)
R364	81	430	65	15	5.7	9	1.3
	81	360	54	15	3.0	6	0.8
	84	280	45	16	3.4	8	1.3
	84	150	39	26	2.5	7	1.6
	45	175	27	15	1.9	7	1.1
	84	280	45	16	3.4	8	1.3
R194	84	150	39	26	2.5	7	1.6
	85	300	64	21	3.9	6	1.3
R363	64	204	23	13	1.9	8	0.9
	85	224	71	32	3.6	5	1.6
R362	90	250	68	27	4.7	7	1.9
	80	380	52	14	3.7	7	1.0
R655	48	132	33	25	1.5	5	1.1
Mean $\pm$ 1SD				20 $\pm$ 6.1		7 $\pm$ 1.2	1.3 $\pm$ 0.3