Published in final edited form as:

Transplantation. 2010 October 27; 90(8): 811–816. doi:10.1097/TP.0b013e3181ebf782.

Non-Human Primate Regulatory T Cells: Current Biology and Implications for Transplantation

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Abstract

Regulatory T cells (Treg) offer potential for improving long-term outcomes in cell and organ transplantation. The non-human primate (NHP) model is a valuable resource for addressing issues concerning the transfer of Treg therapy to the clinic. Herein we discuss the properties of NHP Treg and prospects for their evaluation in allo- and xenotransplantation.

Keywords

Regulatory T cells; Non-human primates; Transplantation; Xenotransplantation

Treg and regulation of immune reactivity

The importance of regulatory T cells (Treg) in the control of immunity (1) and their involvement in tolerance to auto- and alloantigens (Ags) is well-documented in rodents and humans (2,3). Treg offer potential for therapy (3–5), and the promise of avoiding many toxicities and morbidities associated with current immunosuppressive drug regimens that, except in rare circumstances, fail to promote clinical transplant tolerance. Treg offer the possibility of being highly-specific and effective, with potential to provide long-term tolerance. Naturally-occurring, conventional CD4⁺CD25⁺Treg (nTreg) that constitute approximately 10% of peripheral CD4⁺ T cells are the most extensively investigated. They are identified by intra-nuclear expression of the transcription factor forkhead/winged-helix box protein 3 (Foxp3) (6), which, at least in mice, is expressed selectively by Treg. Recent reviews (7,8) describe their development and function. nTreg can suppress the activation, proliferation, differentiation and effector function of various immune cells, including CD4⁺ and CD8⁺ T cells, B cells, dendritic cells (DC) and natural killer cells (7) via different mechanisms, depending on the target and location of their action.

Human and NHP versus rodent Treg

Multiple ways of improving Treg activity to control alloimmunity are being pursued. Adoptive transfer of purified Treg is the most widely-studied approach. However, many practical concerns need to be addressed, while ethical issues limit the testing that can be

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Authors Contributions: EMD, GR, DKCC and AWT each participated in the writing of the mini-review

done in humans. Moreover, rodent models of tolerance induction are rather poor predictors of tolerance in NHP and humans (9). This singles out the non-human primate (NHP) as a valuable pre-clinical resource, reinforced by the reported similarity in phenotype and function between human and NHP (rhesus macaque) Treg (10,11) and the differences between the latter and rodent Treg, on which much experimental work has been done. Thus, unlike mouse Treg, human Treg constitute only a minor proportion of all CD25⁺ cells, with only CD4⁺CD25^{hi} cells exhibiting regulatory properties. Furthermore, whereas Treg of conventional, specific pathogen-free mice are immunologically naïve cells, the majority of human Treg express a memory phenotype. Furthermore, while Foxp3 is clearly correlated with Treg suppressive function in mice, its expression in humans is also observed in activated T cells, that lack regulatory function.

Development of Treg therapeutic protocols

Adoptive cell therapies are at a very early stage of evaluation in NHP, and there are as yet no reports of conventional Treg therapy in NHP transplantation. However, in the rhesus macaque, Bashuda et al (12) administered autologous 'suppressive T cells' (approx 10⁷/kg) rendered anergic by co-culture with donor alloAg in the presence of anti-CD80 and -CD86 monoclonal antibodies (mAbs). The anergic T cells were infused 13 days after renal transplantation to splenectomized recipients given brief cyclosporine A and cyclophosphamide therapy. Significantly, long-term graft survival and donor-specific tolerance were achieved in 50% of the recipients, demonstrating the potential of a regulatory T cell approach in NHP organ transplantation.

Realization of a therapeutic protocol based on adoptive transfer of Treg requires that multiple issues be addressed (Fig 1A): 1) the efficacy of Treg and the number of cells necessary to obtain a therapeutic effect; 2) the Ag specificity necessary for safe and effective control of rejection; 3) the stability of the suppressive phenotype of adoptively-transferred Treg; 4) the Treg migratory pattern that guarantees the strongest regulatory function; 5) the conditions permissive to regulation of the memory response; 6) the ability of Treg to control the xeno-reactive response; 7) the impact of lymphocyte depletion/concomitant immunosuppressive therapy on Treg function. In light of the strong similarity between human and NHP Treg, NHP transplant models are well-positioned to resolve these issues.

Purification, induction, and expansion of NHP Treg

Although rare cells, new methods have emerged for the purification and expansion of polyclonal and Ag-specific Treg (13). Phenotypic and functional characterization of NHP nTreg (CD4⁺CD25⁺) in blood or lymphoid tissues has been reported for cynomolgus (14,15) and rhesus macaques (10,16,17) and baboons (18). Their ex vivo expansion following immunomagnetic bead or/and flow sorting has been documented in response to either polyclonal stimuli (11,16), peripheral blood mononuclear cells (PBMC) (18) or allogeneic DC (14) (Table 1). As in humans, more precise characterization of NHP Treg is needed. Recently, human Treg have been better distinguished from conventional T cells by their lack of cell surface CD127 (IL-7R α); CD127 expression correlates inversely with Foxp3 and suppressive function (13). In cynomolgus macaques, circulating CD4⁺CD25⁺CD127⁻ cells that express Foxp3 and exhibit suppressive activity have been isolated and expanded using 'semi-mature' allogeneic DC and IL-2 (14), although the alloAg specificity of the Treg was not ascertained. In rhesus macaques, circulating CD4⁺CD25⁺CD127^{lo/-} T cells have been expanded ex vivo using anti-CD3/CD28 mAbs and IL-2, and shown to potently inhibit effector T cell proliferation in a non-specific manner (11). Our recent findings (19) are the first to show that a highly-suppressive population of alloAg-specific rhesus Treg can be

generated in vitro from circulating CD4⁺CD127^{-lo} T cells (6±1% of bulk CD4⁺ T cells) in response to immature allogeneic DC and stimulation with IL-2 and IL-15.

Since the frequency of nTreg is comparatively low, de novo generation of Treg from conventional T cells is an appealing strategy to obtain large numbers of cells. In vitro activation of human CD4⁺CD25⁻ T cells via T cell receptor stimulation in the presence of transforming growth factor (TGF)β upregulates Foxp3, conferring suppressive capacity (20). As with genetic manipulation of conventional T cells to express Foxp3, the reproducibility of Treg induction (iTreg) using this approach has been questioned, as has the stability of their suppressive capacity (21). Despite these concerns, considerable promise has now been shown by the ability of all-trans-retinoic acid (RA) to synergize with TGFβ during T cell stimulation and induce highly suppressive murine or human Foxp3⁺ T cells (22,23). These iTreg suppress immune-mediated disorders in mice (22), and potentially represent a valid alternative to expanded nTreg. However, clinical testing is not foreseeable due to safety concerns, and NHP models represent the best tool to establish the feasibility of this approach.

Function of NHP nTreg and iTreg

We have characterized and sorted nTreg from cynomolgus blood and also ascertained whether iTreg can be generated from conventional T cells. We tested in vitro conditions for expansion of nTreg and iTreg, and at the same time, determined their suppressive capacity. Flow-sorted CD4⁺CD25⁺CD127^{lo} T cells were stimulated with anti-CD3/CD28-coated beads and IL-2. Additionally, TGF-β was tested for its ability to preserve the expression of Foxp3 and nTreg suppressive ability. As shown in Fig 1B, both conditions caused significant proliferation of the cells, with up to 700-fold expansion over a 15-day period. In our amplification system, when nTreg were stimulated with IL-2 only, high Foxp3 expression was lost (Fig 1C). Addition of TGFβ preserved the level of this transcription factor necessary for Treg suppressive activity. Expanded nTreg were tested for their ability to suppress proliferation of autologous responder T cells stimulated by allogeneic PBMC. As expected from the absence of Foxp3 expression, nTreg expanded without TGFβ did not exhibit significant suppressive capacity. By contrast, nTreg expanded with TGFβ were highly suppressive (Fig 1D).

Cynomolgus CD4⁺CD25⁻CD127^{hi} T cells were also flow-sorted and stimulated in vitro with anti-CD3/CD28-coated beads in the presence of IL-2 and TGFβ1, with or without RA. Both conditions induced Foxp3 expression and favored significant expansion of the stimulated population (Fig 1B). Interestingly, as reported by Wang et al (23) for human T cells, only cynomolgus T cells stimulated with TGFβ and RA demonstrated profound inhibition of autologous alloreactive PBMC (Fig 1E). These data indicate the feasibility of testing the therapeutic potential of NHP iTreg and comparing their suppressive activity to that of expanded nTreg.

Several NHP studies have shown strong in vitro suppressive effects with Treg:responder cell ratios from 1:1 to 1:100, depending on type of expansion. Thus, Anderson et al (11) not only demonstrated impressive expansion of rhesus Treg, but also enhanced suppressive activity of polyclonally-expanded Treg with ratios up to 1:100. Studies in rhesus or cynomolgus macaques using alloAg-specific stimulation indicate however, that cell numbers cannot be expanded significantly (from no increase, up to 25-fold) (14,19). On the other hand, pig PBMC and IL-2 were used to expand baboon Treg up to 2000-fold after 4 weeks of culture (18). These Treg suppressed effector T cell responses even at 1:256 ratio. Most likely, large numbers of Treg will be required to achieve the desired suppressive effect in vivo. An alternative solution could be incorporation of a T-cell depleting strategy (e.g. alemtuzumab

[humanized anti-CD52; (24)] or anti-thymocyte globulin [ATG]) in NHP prior to Treg infusion, as fewer Treg would be needed for a favorable Treg:T cell ratio. Under these circumstances, delayed infusion of Treg would be indicated in order to avoid Treg depletion by the Ab (the terminal half-life of alemtuzumab in humans is 15–21 days [(25,26)]). In our experience in cynomolgus monkeys, initial recovery of lymphocytes was evident 7 days after infusion of alemtuzumab (27), suggesting that Treg infused at this or a later time-point would remain intact.

Migratory properties of NHP Treg

Recently, Treg migratory ability has gained increased attention as a correlate of their in vivo suppressive function (28). While regulatory T cell markers are expressed both in accepted and rejected NHP renal allografts (29), recruitment of CD4⁺ Treg expressing TGFβ to the graft interstitium has been implicated in metastable renal transplant tolerance in rhesus monkeys (30). Multiple studies, including those in transplant models, have indicated that expression of specific chemokine receptors is essential for Treg activity in vivo (31,32). There is also evidence that maximal Treg protective activity occurs when the cells migrate to both the transplant site and draining lymphoid tissue. Based on this paradigm, optimization of a procedure for rendering highly-suppressive NHP Treg should include testing for expression of a pattern of chemokine receptors (CCR2, CCR4, CCR5, CCR7) compatible with their migration to both the graft and lymphoid tissues. Recently, associations between Treg numbers and the expression of ligands for CXCR3, CCR4 and CCR7 in lymphoid tissue of simian immunodeficiency virus-infected cynomolgus monkeys have been reported (33).

Control of memory T cells

Donor-reactive memory T cells (Tmem) resistant to immunosuppressive agents undermine strategies for tolerance induction. Moreover, recent studies have shown that lymphoablative strategies (e.g. T cell depletion) may lead to preferential expansion and accumulation of Tmem, that derive from homeostatic proliferation of naïve T cells (34). In mice, CD4⁺ and CD8⁺ allospecific Tmem are more resistant to Treg-mediated suppression than naïve T cells (35). However, these Treg still suppress Tmem proliferation when present in equivalent number or at 1:3 ratio (Treg:Tmem). Human nTreg can suppress both naïve and memory T cell proliferation (36). While these reports suggest the potential of Treg to control rejection in sensitized patients, they also indicate the likely need for complementary treatments to control Tmem without affecting Treg activity. Thus it is noteworthy that proliferation of sirolimus-resistant rhesus monkey Tmem is inhibited by combined use of bortezomib (a proteasomal inhibitor that blocks nuclear factor κβ nuclear translocation) and sirolimus, a regimen that preserves pre-existing Treg survival (37).

Xenotransplantation

Although most NHP Treg studies have concerned the allogeneic response, a recent report by Porter et al (38) focused on baboon Treg in a xenogeneic (pig to baboon) setting. Treg were expanded in a donor-specific manner using irradiated pig PBMC. The expanded Treg not only were strongly suppressive at a ratio of 1:1, but donor-specific suppression was achieved. Further development of NHP Treg in the context of xenotransplantation, which offers the consistent advantage of donor identification before transplantation, could justify preclinical studies in this field [reviewed in (39)].

Transitioning to the clinic: the importance of NHP research

Safe and reliable protocols for the selection/induction and expansion of Treg that could be tested clinically are at a very early stage of development. A recent report (40) describes the first two cases of (donor-derived) CD4⁺CD25⁺CD127⁻ Treg therapy in humans with acute or chronic graft-versus-host disease following hematopoietic cell transplantation. The findings indicate that the required Treg number may depend on clinical symptoms and underlying disease, as well as CD4⁺ T cell numbers, rather than on host body weight. NHP models have the inherent advantage of closely resembling the human condition and allow for preclinical testing, although such studies provoke ethical considerations, demand relevant experience, are expensive and time-consuming. They also require specialized facilities and resources that may only be available in large academic centers. Whether pre-clinical studies of Treg in NHP are essential before proceeding to a clinical trial is certainly debatable. Of importance is whether the therapeutic agents to be employed are effective in NHP, which is not always the case. However, if they are known to be effective, studies in NHP do provide a greater indication of both efficacy and safety than can be achieved from investigations in rodents. A clear example is the possible suppression of the immune response to infectious agents that could be induced by Treg infusion. Most would agree that experience of potential detrimental effects in a NHP model is much preferred, as it provides a greater assurance that the approach will be safe and efficacious when applied clinically. An innovative and more cost-effective approach to assess NHP Treg in vivo (and particularly relevant to rapid testing of primate-specific reagents) could be the use of 'primatized' mice, similar to 'humanized' mice (41), engrafted with NHP lymphohematopoietic stem cells giving rise to a complete repertoire of NHP immune cells. Such a model could be beneficial for initial testing of NHP Treg, without the need for, and the aforementioned limitations of, the large animal model. Taken together, and with due consideration to questions regarding efficacy and safety, NHP Treg hold considerable potential for preclinical testing of tolerance-promoting strategies.

Acknowledgments

This work was supported by National Institutes of Health grant U01 AI 51698. Eefje M. Dons is the recipient of fellowships from the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences and the Stichting Professor Michael van Vloten Fund, The Netherlands. Giorgio Raimondi is in receipt of an American Heart Association Beginning Grant-in-Aid, an American Diabetes Association Junior Faculty Grant and the Thomas E. Starzl Transplantation Institute Joseph A. Patrick Fellowship.

Abbreviations

Ag	antigen
DC	dendritic cells
NHP	non-human primate
PBMC	peripheral blood mononuclear cells
RA	retinoic acid
Teff	T effector cells
TGFβ	transforming growth factor β
Tmem	memory T cells
Treg	regulatory T cells

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FIGURE 1.

(A), Issues that govern the successful application of Treg therapy in transplantation.

(B–D), Highly-suppressive Treg can be expanded/induced from cynomolgus monkey blood.

(B), CD4⁺CD127^{lo}CD25⁺ (nTreg) were flow-sorted from PBMC. They were stimulated in vitro with anti-CD3/CD28-coated beads, in the presence of human IL-2 (500U/ml), with or without TGFβ1 (5ng/ml). In parallel, CD4⁺CD127^{hi}CD25⁺ T cells were sorted and induced to convert into regulatory cells (iTreg) by stimulation with anti-CD3/CD28-coated beads in the presence of IL-2 and TGFβ1, with or without retinoic acid (RA; 5ng/ml). The fold-expansion obtained under each condition at multiple time points is indicated. **(C),** Preservation of Foxp3 expression in expanded nTreg was tested by intracellular staining after 16 days of culture. **(D),** The suppressive capacity of expanded nTreg was tested in MLR. PBMC were obtained from the same source as nTreg and stimulated in vitro with irradiated allogeneic PBMC (1:2 responder to stimulator ratio). Titrated numbers of nTreg or nTreg (TGFβ1) were added to the cultures at the indicated Treg to responder ratios. Responder cell proliferation was quantified by thymidine incorporation on day 4. nTreg expanded with IL-2 alone did not inhibit proliferation significantly (data not shown). **(E),** as in D, T cells induced to convert into iTreg were tested for their suppressive capacity in MLR.

p<0.02; **, p<0.05 for all comparisons indicated.

TABLE 1
Reports of NHP Treg isolation, expansion and ability to inhibit allo- or xenoreactive T cell proliferation

Species	Treg selection (purity)	Cell yield	Expansion method	Expansion rate	Suppressive activity	Reference
Rhesus (macaque)	T cells from spleen	200×10 ⁶ cells per recipient (splenectomized)	Donor splenocytes + anti-CD80/CD86; 13 d	2–4-fold	In vivo infusion 13d post allo kidney transplant; donor-specific suppression of rejection	(12)
Rhesus	MACS (90%) or FACS (98%) CD4 ⁺ CD25 ⁺	Not mentioned	Anti-CD3/CD28 beads + IL-2; 4wk	300–2000-fold	Up to 1:8 ^a ratio, inhibition of autologous PBMC proliferation	(16)
Rhesus	Anti-CD8 and anti-CD20 Dynabeads, or CD4 ⁺ MACS, followed by anti-CD25 MACS (82%)	10% of CD3 ⁺ CD4 ⁺ T cells	Fresh cells used; no expansion	n/a	Proliferation of T _{eff} to anti-CD3 or irradiated PBMC decreased at 1:1 ^a ratio, but variation between animals	(10)
Rhesus	FACS CD4 ⁺ CD25 ^{hi} or CD4 ⁺ CD25 ⁺ CD127 [−]	10×10 ⁴ cells/ml blood	Anti-CD3/CD28 beads + IL-2; 4 wk	Up to 450-fold	CFSE-MLR, up to 1:100 ^d ratio; suppression of allo response by responder-specific or third-party T _{reg}	(11)
Rhesus	MACS CD4 ⁺ CD127 [−] lo	7% of CD4 ⁺ /1.3% of total PBMC; 3.7 × 10 ⁴ cells/ml blood	Immature Mo-DC + IL-2 + IL-15; 14d, or 10d followed by 2d without DC	No expansion	Suppression up to 1:40 ^d ratio; donor-specific	(19)
Cynomolgus (macaque)	FACS CD4 ⁺ CD25 ⁺ CD127 [−] (>98%)	0.4% of PBMC	Allo DC (BMDC/Mo-DC) + IL-2; 7days	12–25-fold	CFSE-MLR, PBMC+ anti-CD3/CD28+ T _{reg} ; 30% inhibition at 1:3 ^a ratio. T _{reg} suppressive capacity stimulated by BMDC > Mo-DC	(14)
Cynomolgus (MS model)	MACS CD4 ⁺ CD25 ⁺ (>90%)	6.4% of T cells	Fresh cells used; no expansion	n/a	Proliferation to anti-CD3/CD28 or CD3/CD46 stimulation is impaired during active MS	(15)
Baboon	MACS→FACS sorting (>95%) CD4 ⁺ CD25 ^{hi}	1.7% spleen, 3.1% LN, 1.9% blood T cells; 10×10 ⁴ cells/ml blood	Pig PBMC + IL-2; 3–4 wk + 7–10d without PBMC	≤ 2000-fold, depending on IL-2 concentration	1:1 ^a , strong xenogeneic suppression, donor-specific; 4–10× less efficient to third-party	(18)

^aT_{reg}:effector cell ratio;

n/a, not applicable

BMDC, bone-marrow derived dendritic cells; d, day; FACS, fluorescence-activated cell sorting; LN, lymph node; MACS, magnetic-activated cell sorting; Mo-DC, monocyte-derived dendritic cells; MS, multiple sclerosis; wk: week