Mammalian Target of Rapamycin Inhibition and Alloantigen-Specific Regulatory T Cells Synergize To Promote Long-Term Graft Survival in Immunocompetent Recipients

Giorgio Raimondi, Tina L. Sumpter, Benjamin M. Matta, Mahesh Pillai, Natasha Corbitt, Yoram Vodovotz, Zhiliang Wang and Angus W. Thomson

*J Immunol* 2010; 184:624-636; Prepublished online 9 December 2009;
doi: 10.4049/jimmunol.0900936
http://www.jimmunol.org/content/184/2/624

**References**

This article cites 79 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/184/2/624.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Mammalian Target of Rapamycin Inhibition and Alloantigen-Specific Regulatory T Cells Synergize To Promote Long-Term Graft Survival in Immunocompetent Recipients

Giorgio Raimondi,*† Tina L. Sumpter,*† Benjamin M. Matta,*† Mahesh Pillai,*† Natasha Corbitt,*† Yoram Vodovotz,†‡§ Zhiliang Wang,*† and Angus W. Thomson*†‡

Minimization of immunosuppression and donor-specific tolerance to MHC-mismatched organ grafts are important clinical goals. The therapeutic potential of regulatory T cells (Tregs) has been demonstrated, but conditions for optimizing their in vivo function posttransplant in nonlymphocyte-depleted hosts remain undefined. In this study, we address mechanisms through which inhibition of the mammalian target of rapamycin (Rapa) synergizes with alloantigen-specific Treg (AAsTreg) to permit long-term, donor-specific heart graft survival in immunocompetent hosts. Crucially, immature allogeneic dendritic cells allowed AAsTreg selection in vitro, with minimal expansion of unwanted (Th17) cells. The rendered Treg potently inhibited T cell proliferation in an Ag-specific manner. However, these AAsTreg remained unable to control T cells stimulated by allogeneic mature dendritic cells, a phenomenon dependent on the release of proinflammatory cytokines. In vivo, Rapa administration reduced danger-associated IL-6 production, T cell proliferation, and graft infiltration. Based on these observations, AAsTreg were administered posttransplant (day 7) in combination with a short course of Rapa and rendered >80% long-term (>150 d) graft survival, a result superior to that achieved with polyclonal Treg. Moreover, graft protection was alloantigen-specific. Significantly, long-term graft survival was associated with alloreactive T cell anergy. These findings delineate combination of transient mammalian target of Rapa inhibition with appropriate AAsTreg selection as an effective approach to promote long-term organ graft survival. The Journal of Immunology, 2010, 184: 624–636.

The ability to induce donor-specific tolerance in organ transplantation would obviate allograft rejection and patients' dependency on life-long immunosuppressive drug therapy (1). Currently, there is considerable interest in developing cell-based therapies that promote tolerance induction in experimental models (2, 3) and that provide a feasible approach to achieving this goal in humans. Thus, the suppressive activity of naturally occurring regulatory T cells (Treg), necessary to control immune reactivity and prevent autoimmunity, has captured considerable attention (4–7) since before the availability of markers by which to specifically purify Treg (8). Extensive studies in animal models and screening of patients have established the importance of Tregs in the control of allograft rejection (9, 10) and other inflammatory or immune-mediated disorders (11–13). Moreover, early clinical reports suggest a correlation between organ graft survival and circulating Treg function (14). Polyclonal Tregs have been used successfully to prevent rejection responses in rodents. However, Ag-specific Tregs (commonly obtained from TCR transgenic mice) have proved more powerful, with the added benefit of minimizing suppression of unrelated responses (4, 5, 15). Additionally, several groups have shown that, following their isolation from normal, unmanipulated hosts, Treg can be expanded in culture with appropriate specificity, then adoptively transferred to suppress organ or bone marrow allograft rejection (16–18).

To date, the capacity of adoptively transferred Tregs to promote MHC-incompatible organ graft survival in rodents has been demonstrated only in immunologically impaired hosts, such as T cell- and B cell-deficient (RAG−/−) (19), T cell-depleted (20), or irradiated animals (17). Additionally, the approach employed in most of these models has involved the injection of Treg before transplantation, precluding application to deceased donor transplantation. These limitations can be rationalized by the need to harness multiple mechanisms encompassing deletion, anergy, and regulation (21) to establish a robust state of tolerance, an outcome difficult to obtain with a single infusion of Treg. Moreover, recent findings suggest that the suppressive activity of Treg is diminished by danger signal-mediated maturation of APCs, an effect mediated by a still poorly defined mixture of cytokines (22, 23). Although the influence of these cytokines on Ag-specific Tregs is unknown, it is reasonable to speculate that in unmanipulated graft
recipients, the combination of proinflammatory cytokines released following surgery (24) and the activation of large numbers of effector T cells in response to transplantation may override the protective ability of Tregs (endogenous and exogenous). An additional factor that limits Treg function is the capacity of calcineurin inhibitors (cyclosporine A or tacrolimus), used to prevent acute rejection, to block the protolerogenic activity of Treg. By contrast, inhibition of the mammalian target of rapamycin (Rapa), a downstream target of rapamycin (Rapa), downstream target of PI3K/Akt signaling, spares the induction of transplant tolerance (25–27). Moreover, CD4+CD25+ Tregs are relatively resistant to the Rapa (28, 29) and can expand in its presence (6, 29–31).

Isolation of comparatively pure human Treg is difficult due to the lack of Treg-specific markers. We have recently developed a purification strategy based on differential expression of the regulatory molecule programmed cell death-1 (PD-1) that allows distinction between Treg and activated T cells (30). Additionally, an alternative strategy based on reduced expression of CD127 (IL-7R) on Treg has been proposed (32, 33). Availability of these strategies renders the therapeutic use of Treg a potential clinical reality and urges the identification of a protocol that would exploit and support their therapeutic capacity.

We report in this study that coculture of Treg with immature, donor-derived dendritic cells (DCs) in a conditioned medium is preferable over the use of mature DCs for the selection of potent allograft-specific Treg (AAsTreg). We show also that the highly suppressive function of AAsTreg is susceptible to modulation by danger signal-induced DC maturation. However, posttransplant suppression of AAsTreg is susceptible to modulation by calci-}

Materials and Methods

Animals

Eight- to 12-wk-old C57BL/10 (B10; H-2Kb), C3H/HeJ (C3H; H-2Kk) and BALB/c (H-2Kd) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of University of Pittsburgh School of Medicine (Pittsburgh, PA). B6.SJL-PepRpr+/BoyAiTac (B6.SJL; H-2Kb) were purchased from Taconic Farms (Hudson, NY). IL-6−/− C57BL/6 mice were a gift from Dr. A.J. Demetris (Department of Pathology, University of Pittsburgh). Experiments were conducted under an institutional animal care and use committee-approved protocol and in accordance with National Institutes of Health-approved guidelines.

Reagents

Complete medium (CM)-comprised RPMI 1640 (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with 10% volume to volume ratio FCS (Atlanta Biologicals, Lawrenceville, GA), nonessential amino acids, l-glutamine, sodium pyruvate, penicillin-streptomycin, and 2-ME (all from Invitrogen). Anti–mouse IL-2, IFN-γ, IL-17, CD4, CD25, glucocorticoid-induced TNFR-related protein, CD62L, CTLA-4, CCR5, CD103, and Lag-3 mAbs were from BD Pharmingen (San Diego, CA). Purified mouse CCL19-Fc (for detection of mouse CCR7) and anti-forkhead box P3 (Foxp3; clone FJK-16s) were purchased from eBioscience (San Diego, CA). Anti–CCR4 mAb was purchased from Capradoses (Hardwick, MA). Vybrant CFDA SE cell tracer kit was purchased from Molecular Probes (Invitrogen). Magnetic microbeads and separation columns were from Miltenyi Biotec (Auburn, CA) or Invitrogen, as indicated.

T cells and Treg purification

T cells were purified from mouse spleens and lymph nodes using the following procedure. Single-cell suspensions were incubated with anti–CD11b, anti–CD8, anti–B220, anti–Gr-1, anti–CD16/32, anti–Ter119 (BD Biosciences, San Jose, CA), and anti–I–A/E (eBioscience) mAbs. Cells were washed and then incubated with anti-rat dynabeads (Dynal, Invitrogen) according to the manufacturer’s instructions. CD4+ T cells were purified by magnetic negative selection. These represented “bulk” CD4+ T cells. For Treg isolation, bulk CD4+ T cells were incubated with anti–CD25-PE mAb and CD4+CD25+ T cells isolated by positive selection using anti-PE microbeads and MS separation columns (Miltenyi Biotec). Purity was assessed by cytofluorometric analysis and was consistently 90–95%. The remaining cells were used as CD4+CD25− T cells for assays of suppressor function. For experiments on Foxp3 upregulation, the remaining CD4+CD25− T cells were depleted of residual CD25+ cells by depletion in an AutoMACS automatic separator (Miltenyi Biotec) using the depletionS program.

Generation of bone marrow-derived DC

Myeloid DC were propagated from bone marrow cells, as described (34). Briefly, femoral bone marrow was flushed with a PBS solution containing 2 mM EDTA. RBCs were lysed and remaining cells plated in Petri dishes at 2 × 10⁷/plate with recombinant murine GM-CSF (300 U/ml) and recombinant murine IL-4 (200 U/ml) (both from R&D Systems, Minneapolis, MN). Media was changed every 3 d. On day 7, nonadherent cells were washed thoroughly and CD11c+ DC purified using anti-CD11c immumomagnetic beads and isolation columns (Miltenyi Biotec; manufacturer’s protocol). When indicated, mature DCs were generated by adding LPS (200 ng/ml; Escherichia coli K12; Invivogen, San Diego, CA) on day 6 of culture and harvesting the cells 24 h later.

Generation of supernatant of MLR

Bulk CD4+ T cells (B10) were cocultured with mature allogeneic DC (BALB/c) at 20 T cells:1 DC ratio. Cells were cultured in 96-well, round-bottom plates in 200 μl total volume/well of complete media at 37°C. After 5 d coculture, supernatant was collected, centrifuged at 500 × g for 5 min, filtered through 0.22-μm filters, and stored at −80°C.

Quantification of cytokines

Supernatant of MLR (MLRsup) cytokine levels were assessed with the luminex multiplexing platform (MiRiBio, Alameda, CA) using the BioSource 20-plex mouse cytokine bead set (BioSource-Invitrogen, San Diego, CA) and additional beadsets for the detection of IL-7, IL-9, IL-15, and IL-21 (Millipore, Billerica, MA), as per the manufacturer’s specifications. The concentration of active TGF-β1 in the MLRsup was determined using the Quantikine ELISA (R&D Systems), which detects the binding of active TGF-β1 to immobilized TGF-β1 type II receptors. The concentration of active TGF-β1 present in the supernatant was assessed after PBS treatment for 3 h in vitro according to the manufacturer’s recommendation.

Enrichment of AAsTreg and phenotypic analysis

Freshly isolated B10 Tregs (CD4+CD25+) were incubated with allogeneic bone marrow-derived DC (BALB/c or C3H, depending on the experiment) at 3 Treg:1 DC ratio in 96-well plates. Total volume per well was 150 μl composed of 75 μl Cm + 75 μl of MLRsup. Human rIL-2 (a gift from Dr. M.T. Lotze, Department of Surgery, University of Pittsburgh; 500 U/ml) was used instead of MLRsup where indicated. Cells were incubated at 37°C. After 5 d coculture, 50 μl/well of media was removed and replaced with 100 μl of MLRsup (or Cm + IL-2). On days 9 and 10, cells were collected and remaining DCs removed by incubation with anti–CD11c-conjugated microbeads (Miltenyi Biotec) and AutoMACS separation. The remaining cells (AAsTreg) were used for experimentation. To investigate the proliferation and cytokine release profile of Treg during coculture, freshly isolated Tregs were CFSE stained for marker expression or restimulated with PMA + ionomycin for 5 h and analyzed for intracellular expression of Foxp3 and the indicated cytokines (eBioscience; manufacturer’s staining protocol).
Suppressor assays
Graded numbers of γ-irradiated (2000 rad) BALB/c DC were used as stimulators of purified allogeneic (B10) bulk CD4 or CD4*CD25− T cells (2 × 10^5/well) in 72-h MLR using 96-well, round-bottom plates, as described (35). Titrated numbers of AAsTreg or freshly isolated Tregs were added where indicated. For the final 16–18 h of culture, individual wells were pulse-labeled with 1 μCi [3H]thymidine. Radioisotope incorporation was determined using a β scintillation counter. Results are expressed as mean cpm ± 1 SEM calculated from triplicate wells. To evaluate T cell proliferation in more detail, bulk B10 CD4 or CD4*CD25− T cells were CFSE labeled. These CFSE+ T cells (2 × 10^5) were cocultured with 2 × 10^5 allogeneic (C3H) DC in 96-well, round-bottom plates. The indicated numbers of AAsTreg or freshly isolated Treg were added at the beginning of the culture. After 4–5 d, cells were collected and stained for surface CD4 and intracellular Foxp3. The CFSE dilution profile of CD4*Foxp3− T cells was then determined by flow cytometry.

In vivo Rapa treatment and IL-6 production by primary DCs
B10 mice were treated for 10 d with daily i.p. injections of Flt3 ligand (Flt3L) to increase the number of DCs in secondary lymphoid tissue (2, 36, 37). Animals were separated into two groups based on the administration of Rapa (1 mg/kg daily) during the last 7 d of FL treatment. On day 10, animals were euthanized and splenic DC purified by density gradient centrifugation followed by CD11c+ cells isolation. DCs were then cultured overnight with or without LPS (1 μg/ml) and the level of IL-6 expression quantified by ELISA (ELISA Max, Biolegend, San Diego, CA).

Vascularized heart transplantation and AAsTreg administration
Heterotopic (intra-abdominal) heart transplantation was performed from BALB/c to B10 mice, as described (38). Groups of mice received a sub-therapeutic regimen of 1 mg/kg/day i.p. Rapa (LC Laboratories, Woburn, MA) in a vehicle containing 0.02% Tween 80 and 0.26% polyethylene glycol (both from Sigma-Aldrich, St. Louis, MO) for 10 consecutive days (days 0–9). Additional groups received autologous, freshly isolated Tregs. AAsTreg (B10) enriched using BALB/c-derived DC, or AAsTreg (B10) enriched using C3H-derived DC. Transplant survival was assessed by daily transabdominal palpation. Rejection was defined by the complete cessation of cardiac contraction and confirmed histologically. Bulk CD4* or CD4*CD25− T cells were purified from mice with long-surviving grafts (>150 d) or normal, age-matched mice. A fraction was analyzed for Foxp3 expression. The remaining cells were cocultured with irradiated BALB/c-derived DC. Their proliferative response, in the presence or absence of recombinant human IL-2 (100 U/ml), was assessed after 3 d by thymidine incorporation, as described above.

Statistics
Results are expressed as means ± 1 SEM. The significances of differences between means were determined using Student t test. p < 0.05 was considered significant. Prism 5 Software package (GraphPad Software, GraphPad, San Diego, CA) was used to generate survival curves, and the significance of differences in graft survival between groups was determined by Kaplan-Meier analysis and the log-rank test.

Results
Alloreactive Tregs are present in the pool of naturally occurring Tregs
The Ag specificity of naturally occurring Tregs remains a topic of intense investigation (39–44). Although preliminary reports indicate that Tregs can regulate alloreactive T cells, it remains unclear whether cells with specificity, or cross-reactivity (45), for alloantigens are present within the pool of naturally occurring Tregs. To confirm the presence of alloreactive Tregs, bulk CD4* or CD4*CD25− T cells were purified from normal B10 mice and cultured in 3-d MLR with allogeneic (BALB/c) bone marrow-derived DC (alloDC). As shown in Fig. 1A, T cell proliferation increased significantly in the absence of Treg, suggesting the presence, in the CD4*CD25− population, of cells able to suppress alloreactive T cell expansion/activation. To further characterize the behavior of Treg, we stained bulk CD4* T cells with CFSE and cocultured them with alloDC. Flow cytometric analysis of the CFSE dilution profile, in combination with Foxp3 staining (Fig. 1B), revealed that the buffering (suppressive) activity of Treg was associated with proliferation of a significant fraction of Foxp3+ cells (~5% of the proliferating cells). To confirm that these Foxp3+ cells were derived from the pre-existing population of Treg, CFSE-stained CD4*CD25− T cells (Foxp3+ confirmed by intracellular staining; data not shown) were stimulated with alloDC and the expression of Foxp3 quantified in proliferating cells after 4 d. Under these conditions, <1% of the proliferating cells showed upregulated Foxp3 (Fig. 1C). Interestingly, when congenic (CD45.1+) Tregs were added back to the culture at physiological concentration (10% of CD4* cells), but excluded from the analysis through appropriate gating strategy (Fig. 1D), ~2% of the proliferating CD4* cells upregulated Foxp3. These results support the hypothesis that, although a fraction of effector T cells upregulate Foxp3 (and are possibly converted to induced Treg) in response to allostimulation, alloreactive (or cross-reactive) cells are present in the Treg pool and are induced to proliferate extensively in MLR.

Selection of alloreactive Treg: mature DCs induce IL-17+ allogeneic T cell expansion
A different outcome was obtained when purified CD4*CD25− T cells (Treg) were cocultured with allogeneic DC (Fig. 2A). By staining the purified population with CFSE before culture, then analyzing the dilution profile in combination with Foxp3 expression at day 5, a reduced rate of proliferation in comparison with that seen with bulk CD4* T cells was evident. The proliferating cells displayed an activated phenotype, as shown by upregulation of surface PD-1 (35), indicating that exposure to
allogeneic DC induced their activation, but that factors able to fully sustain their expansion were absent. In an attempt to sustain their in vitro proliferation, CFSE-stained Treg were cocultured with allogeneic DC matured by overnight exposure to LPS (46). Analysis of the CFSE dilution profile indicated increased proliferation of Foxp3+ cells (Fig. 2B); however, ∼20% of the proliferating cells were Foxp3−. To investigate the nature of these Foxp3− cells, CFSE-stained Treg were cocultured with mature DC for 5 d, restimulated with PMA/ionomycin for 5 h, then analyzed for expression of IL-2, IFN-γ, and IL-17 by intracellular staining (Fig. 2C). The results show that the Foxp3− cells were a heterogeneous population, including a major proportion of IL-17-producing (Th17) cells. As Yamazaki et al. (46) did not report this phenomenon, we tested whether the addition of exogenous IL-2, as used by these authors, could sustain Treg expansion and prevent Foxp3− T cell proliferation. We then repeated the same experiment in the presence of 500 U/ml IL-2 (Fig. 2D). Unexpectedly, the exogenous IL-2 promoted Foxp3− Th17 cell proliferation even further. As Th17 cells have been characterized extensively as proinflammatory cells (47, 48), their expansion and the inability to separate them from (Foxp3+) Treg undermine the use of mature DC for selection of AAs Treg.
Our observation that Foxp3− cells were not obtained when Treg were incubated with immature DC (Fig. 2A) prompted us to explore an alternative approach to sustain alloreactive Treg expansion. Fig. 1B indicates that during a MLR, Treg proliferate extensively. This suggests that a mixture of cytokines, probably produced by activated T cells, is released and sustains Treg expansion. We tested this hypothesis by adding a fraction of the supernatant obtained from MLR induced using bulk CD4+ T cells (MLRsup) to CFSE-stained Treg cocultured with allogeneic immature DC. For comparative purposes, we added IL-2 (500 U/ml) to some of the cultures. The resulting CFSE dilution profile (Fig. 3) indicated that both approaches promoted strong expansion of Foxp3+ cells. Additionally, intracellular staining for IL-17 production in response to restimulation with PMA/ionomycin revealed the presence of a minimal proportion of Th17 cells, under both conditions. However, exogenous IL-2 yielded a significant proportion of proliferating Foxp3− T cells. As the suppressive ability of Treg is strictly linked to maintenance of Foxp3 expression (49, 50), we opted to use the combination of immature DC and MLRsup in subsequent alloreactive Treg selection procedures.

The difference between IL-2 and MLRsup underscored the presence in the latter of important factors that sustained the expression of Foxp3 during Treg proliferation. In an attempt to identify possible candidates, we quantified the cytokine content of the MLRsup using the Luminex multiplex platform and a 20-plex mouse cytokine bead set (BioSource-Invitrogen), with the addition of common γ-chain signaling cytokines (IL-7, -9, -15, and -21), and ELISA-based measurement of active TGF-β1 (Fig. 3B). Surprisingly, the most obvious candidates (TGF-β1, IL-4, IL-7, IL-9, and IL-15) were present at extremely low or even undetectable levels, compromising our ability to identify the key mediators for sustained Foxp3 expression.

Selected Treg retain a regulatory phenotype and are alloantigen-specific

Purified host-derived CD4+CD25+ T cells were cultured for 8–10 d with donor-derived immature DC in the presence of MLRsup (Fig. 4A). We then evaluated the characteristics of the resulting T cell population following immunomagnetic bead-based depletion of the remaining CD11c+ cells. The cells exhibited a conventional regulatory phenotype (Fig. 4A, 4B); >80% of the cells expressed a high level of Foxp3, confirming that the enrichment protocol favored Treg expansion and prevented downregulation of Foxp3 (20, 51, 52). The cells were uniformly glucocorticoid-induced TNFR-related+ (Fig. 4B), with minimal surface CTLA-4, but a high level of intracellular CTLA-4, another characteristic of conventional Treg (11). CD62L expression analysis revealed a mixed population with, on average, a majority (∼60% of Foxp3+ cells) having downregulated CD62L. This profile, associated with homogenous expression of CCR4 and minimal expression of CCR5, indicates that the majority of these T cells have the potential to migrate directly to peripheral tissues, a possible advantage for exhibition of their function (53). Significant fractions of the selected Foxp3+ cells expressed CD103 and Lag3, two molecules associated with powerful regulatory activity (54, 55). Moreover, almost all the cells were positive for CCR7, a molecule crucial for Treg migration to secondary lymphoid tissue and the control of the initiation of immune reactivity (56).

We next determined the suppressive capacity of the enriched Treg on CFSE-stained CD4 T cell proliferation induced by immature alloDC at a Treg:T cell ratio of 1:20, known to be ineffective when freshly isolated Treg are employed (11, 57). As indicated in
are gated on CD4+Foxp3+ cells. Data are representative of six independent experiments.

**FIGURE 4.** Phenotypic characterization of Treg obtained using the selection protocol. A, Following coculture of purified CD4+CD25+ T cells with immature allogeneic DC in the presence of MLRsup, CD11c+ cells were removed by magnetic bead-mediated depletion and the remaining cells stained for surface CD4 and intracellular Foxp3 then analyzed by flow cytometry. B, Same as A, after DC depletion, remaining cells were stained for CD4, Foxp3, and the indicated surface or intracellular markers. Graphs are gated on CD4+Foxp3+ cells. Data are representative of six independent experiments.

Fig. 5A, although freshly isolated natural Tregs (nTreg) did not suppress the proliferation of alloreactive T cells at this ratio, the enriched Treg exerted a profound inhibitory effect. Because it has been clearly shown that, once activated, Treg exert profound nonspecific function (58), we tested the suppressive activity of enriched Treg that were rested without DC for 2 d before their addition to cultures of CFSE-stained CD4 T cells and alloDC. As expected, significant reduction (10-fold), especially in CD8+ T cells by Rapa, was observed (G. Raimondi, unpublished data). We then investigated the influence of Rapa administration on host peripheral T cells. To this end, it was important to distinguish the direct effects of systemic Rapa administration from those related to the presence of Rapa during Ag exposure (the transplanted organ). Naive B10 mice were treated for 8 d with the 1 mg/kg i.p. daily to prevent heart graft infiltration by CD4+ and CD8+ T cells on day 7 posttransplant.

As expected, Rapa administration to otherwise unmanipulated mice significantly reduced (almost 2-fold) the total number of circulating lymphocytes (G. Raimondi, unpublished data). This decrease was associated with thymic involution that correlated with a compromised transition of thymocytes from single-positive to double-positive and consequent reduced thymic output. Surprisingly, the frequency of Foxp3+ cells in the blood dropped from 6.3 ± 0.9% to 2.7 ± 0.3% of CD4+ T cells. A similar decrease was observed in the spleen, from 16.3 ± 0.6% to 10.2 ± 1.4% (G. Raimondi, unpublished data).

Rapa treatment of heart graft recipients for 8 d posttransplant markedly affected the cellularity of secondary lymphoid tissue (Table I). As expected, Rapa prevented the increase in absolute numbers of both lymph node and spleen cells observed in untreated graft recipients. In addition, a generalized reduction (2.3-fold in lymph node and 1.3-fold in spleen) in absolute lymphocyte numbers was evident in comparison with normal animals, and the proportion of CD4+ T cells was decreased. This latter effect was particularly evident in the spleen, the principal lymphoid tissue draining the transplant. Moreover, although the unexpected decrease in frequency of Foxp3+ cells was still evident in the blood, there was a marked increase in the proportion of CD4+Foxp3+ T cells in the spleens of Rapa-treated graft recipients compared with untreated hosts (Table I). These results confirmed that, in addition to preventing alloreactive T cell expansion, Rapa reduced the absolute number of lymphocytes in lymphoid tissues, thus creating an environment that would favor Treg activity as the T: Treg balance was significantly altered. This balance was also altered by the capacity of Rapa to favor the accumulation (or induction) of Treg in the lymphoid tissue draining the graft.

**APC maturation reduces T cell sensitivity to AAsTreg function**

As we aimed to use AAsTreg postoperatively without irradiation or (selective) lymphodepletion, three important points needed to be addressed: 1) suppression of acute graft rejection during AAsTreg enrichment; 2) induction of an environment favorable to AAsTreg function; and 3) minimal manipulation of the recipient. To address these requirements, we first tested the capacity of a short, postoperative course of low-dose Rapa (1 mg/kg i.p. daily) to prevent heart graft infiltration by CD4+ and CD8+ T cells on day 7 posttransplant. Collectively, these results support the validity of the devised protocol for the enrichment of AAsTreg.

**Rapa administration creates an environment conducive to AAsTreg function**

APC maturation reduces T cell sensitivity to AAsTreg, and dependence on IL-6

Maturation of APC, and DC in particular, in response to danger signals is associated with the release of multiple soluble factors (IL-6 and other, still unidentified molecules) that, when all present at the same time, can render conventional T cells refractory to Treg suppression. Organ transplantation is associated with a potent
inflammatory response caused by the release of danger signals that induce APC maturation (24). We speculated that modulation of alloreactive T cell susceptibility to suppression, due to exposure to this mixture of proinflammatory cytokines, could affect the therapeutic efficacy of AAsTreg. Additionally, all reports investigating inflammation-induced T cell refractoriness to Treg suppression (22, 59) have used anti-CD3 mAb as the stimulatory agent, and no information exists relating to the Ag specificity of this effect. We investigated whether AAsTreg were as susceptible as freshly isolated Treg in their ability to control alloreactive T cells stimulated by maturing APC. CD4⁺CD25⁻ T cells were stimulated with allogeneic, immature, or LPS-exposed DC and T cell proliferation measured after 3 d by [³H]thymidine incorporation. The suppressive capacity of AAsTreg added at two different AAsTreg:T cell ratios (1:20 and 1:40) was then measured and compared with that of freshly isolated Treg (at 1:4 ratio). T cells stimulated by immature DC were strongly suppressed by either AAsTreg in a dose-related manner or fresh Treg (Fig. 6A, and G. Raimondi, unpublished data). As expected, fresh Treg did not affect T cell proliferation induced by mature DC (Fig. 6B). Surprisingly, AAsTreg exerted a reduced but significant suppressive effect at a 1:20 ratio. However, the extent of suppression was not equivalent to that exerted on immature DC-stimulated T cells (Fig. 6A), and suppression at the 1:40 ratio was completely abolished. This confirmed that APC maturation favors T cell activation by reducing their sensitivity to Treg regulation, even in the context of alloantigen-specific reactivity.

As Treg proliferative capacity is highly influenced by cytokines (57), we questioned whether the regulation of suppression observed by measuring [³H]thymidine incorporation was truly associated with T cell proliferation. Thus, the above experiment was repeated using CFSE-stained CD4 T cells and the indicated ratios of AAsTreg. The CFSE dilution profile measured after 4 d of coculture
Table I. Influence of Rapa administration on T lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Untreated</th>
<th>Rapa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood % of nucleated cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>3.3 ± 0.2</td>
<td>8 ± 2.6</td>
<td>10 ± 0.1*</td>
</tr>
<tr>
<td>CD4</td>
<td>4.5 ± 0.5</td>
<td>13.3 ± 1.6**</td>
<td>11.9 ± 0.5*</td>
</tr>
<tr>
<td>% of CD4+ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4<em>Foxp3</em></td>
<td>6.3 ± 0.8</td>
<td>5.8 ± 2.2</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>Lymph nodes % of total cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cellularity (× 10^6)</td>
<td>42.8 ± 10.2</td>
<td>53.5 ± 6.4</td>
<td>18.3 ± 6.8***</td>
</tr>
<tr>
<td>CD8</td>
<td>19.5 ± 2.0</td>
<td>15.6 ± 1.3</td>
<td>24.9 ± 1.6***</td>
</tr>
<tr>
<td>CD4</td>
<td>26 ± 0.7</td>
<td>23.2 ± 3.5</td>
<td>21.4 ± 0.5*</td>
</tr>
<tr>
<td>% of CD4+ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4<em>Foxp3</em></td>
<td>20.5 ± 1.4</td>
<td>27.4 ± 0.2*</td>
<td>25.7 ± 1.7</td>
</tr>
<tr>
<td>Spleen % of total cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cellularity (× 10^6)</td>
<td>114 ± 9.8</td>
<td>217 ± 19.4**</td>
<td>87.8 ± 7.6***</td>
</tr>
<tr>
<td>CD8</td>
<td>7.9 ± 1.4</td>
<td>8.8 ± 0.2</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td>CD4</td>
<td>13.6 ± 1.70</td>
<td>13.2 ± 0.5</td>
<td>8.4 ± 1.2***</td>
</tr>
<tr>
<td>% of CD4+ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4<em>Foxp3</em></td>
<td>16.3 ± 0.6</td>
<td>17.3 ± 1.2</td>
<td>21.1 ± 2.1*</td>
</tr>
</tbody>
</table>

B10 animals received BALB/c heart transplants and were divided into two groups (n = 3/group). One group was left untreated and the other was given daily i.p. injections of Rapa (1 mg/kg) starting on the day of transplant. On day 7 posttransplant, animals were euthanized, and the absolute numbers of total cells (total cellularity) determined in parallel to flow cytometric analysis of the frequency of the indicated T cell populations.

*p < 0.05 between Rapa and normal animals; **p < 0.05 between untreated and normal animals; ***p < 0.05 between untreated and Rapa animals.

indicated that AAsTreg significantly suppressed T cell proliferation when immature DCs were used as the stimulators (Fig. 6C). However, when mature DCs were used, alloreactive T cell proliferation was restored, confirming the results obtained with thymidine incorporation. Additionally, as indicated previously, this effect was dependent on the release of multiple proinflammatory cytokines, the precise identity of which remains unclear, but certainly includes IL-6. Indeed, as all the components of the mixture must be present at the same time to exert an effect (22), when mature IL-6^-/- DC were used as stimulators (Fig. 6D), alloreactive T cells remained susceptible to AasTreg (and freshly isolated Treg)-mediated suppression and to the same extent as T cells stimulated by immature DC.

DCs from Rapa-treated mice show marked reduction in IL-6 production

The above results clearly indicated that even though AAsTreg showed stronger regulatory capacity than nTreg, their function could still be affected significantly by the release of proinflammatory cytokines by APC. We next tested whether Rapa could also exert antinflammatory activity that could (additionally) support the protolerogenic activity of AAsTreg. B10 mice were treated with the DC poietin Flt3L for 10 d to markedly increase DC (2). During the last 7 d, Rapa was administered using the same regimen as for transplant recipients. On day 10, the animals were euthanized, splenic DC isolated, and stimulated ex vivo with the TLR4 ligand LPS. After overnight incubation, the amount of IL-6 released in the supernatant was quantified. As indicated in Fig. 6E, DC isolated from Rapa-treated animals released 3-fold less IL-6. This result indicated that Rapa would promote graft survival by inducing an environment that supports Treg activity.

AAsTregs promote indefinite graft survival, associated with anergy of donor-reactive T cells

Using the above insights, we devised the therapeutic protocol depicted in Fig. 7A and tested its efficacy. Heart graft survival data shown in Fig. 7B, and summarized in Fig. 7C, indicate that, in comparison with the untreated control group (median survival time [MST] = 11 d), Rapa alone (days 0–9) significantly inhibited rejection (MST = 30 d), but with no long-term survivors (LTS). By contrast, infusion of AAsTreg to Rapa-treated mice on day 7 posttransplant resulted in long-term survival in >80% of graft recipients (MST > 150 d). The addition of freshly isolated Treg (polyTreg) prolonged graft survival (MST = 45), but only 40% of the grafts survived long-term. These results confirmed that AAsTreg possess an inherent advantage in vivo over nonselected nTreg. Heart grafts in recipients treated with Rapa and Treg selected against third party (C3H) Ags (Fig. 7B) survived a similar length of time to those in the Rapa-only group (and with an inferior MST to those in the polyTreg-treated group).

We also investigated the cellular mechanisms responsible for the long-term maintenance of the allografts. LTS (>150 d) were euthanized and the frequency of Foxp3^+ CD4 T cells analyzed (Fig. 8A). In comparison with age-matched controls (normal B10), the two LTS analyzed showed a 2-fold increase in the frequency of Treg in the spleen. This suggested that long-term graft acceptance was associated with sustained Treg activity. We then tested the reactivity of bulk CD4 T cells isolated from the spleens of the two LTS against donor-derived DC (Fig. 8B). LTS showed a significant reduction (1.8-fold on average) in T cell proliferation. However, removal of CD25^+ T cells from the tested cells did not compensate for the difference in responsiveness between LTS and control T cells, as a 2-fold difference was maintained. This result called into question the role of Treg in long-term maintenance of graft survival, even though these findings do not rule out a protective effect of the Treg mediated within the transplanted tissue (60, 61). Interestingly, addition of exogenous IL-2 to the assay with CD4^+CD25^- T cell responders caused an almost complete restoration of responsiveness, with no significant difference between the groups. This suggests that AAsTreg + Rapa created conditions that favored induction of an anergic state in alloreactive T cells that permitted long-term allograft survival (without relying on the suppressive function of endogenous or exogenous Treg).
Discussion

Our data and others’ (46, 57) indicate that coinubation of freshly isolated Treg with donor-derived DC can expand potent AAsTreg. This was demonstrated in the current study by comparison of the transplant survival curves for donor-selected AAsTreg-infused recipients and those given third-party–selected AAsTreg (Fig. 7B). Graft MST in the latter group was equivalent to that of animals treated with Rapa only, indicating that the injected Treg were

FIGURE 6. AAsTreg are potent suppressors of alloreactive T cells but susceptible to IL-6–dependent modulation of their suppressive activity by maturing DC. A, AAsTreg were tested for their ability to suppress alloreactive CD4 T cell proliferation in response to stimulation with allogeneic immature DC (1:10 DC to T cell ratio). Ratios shown in parentheses (x-axis) indicate AAsTreg to CD4 T cell proportions. T cell proliferation was quantified on day 4 of coculture by thymidine incorporation during the last 16 h of culture. Freshly isolated CD4⁺CD25⁺ Treg (Fresh Treg) were used for comparison. Asterisks indicate statistically significant differences between experimental groups (n = 3). B, Same as A, but DCs were matured by overnight exposure to LPS before use as stimulators in the cultures. C, To confirm that the reduction in AAsTreg suppressive activity observed in B was not an artifact caused by induced proliferation of AAsTreg by mature DC, purified CD4 T cells were stained with CFSE and stimulated by immature (imm) or mature (mat) DC in the presence of the indicated ratios of AAsTreg:T cells. Following 4 d of coculture, cells were collected and stained for surface CD4 and intracellular Foxp3. Graphs are gated on Foxp3⁺ cells to exclude any contribution of the added AAsTreg to the number of total CD4⁺Foxp3⁺ T cells. Data are representative of three independent experiments. D, Same as C, but IL-6⁻/⁻ animals were used as the source of allogeneic DC. Data (gated on Foxp3⁺ cells) are representative of three independent experiments. E, B10 mice were treated for 10 d with Flt3L to increase the number of DC. During the last 7 d, one group of animals received daily i.p. injection of Rapa (1 mg/kg). On day 10, DC were purified from the spleens and stimulated (where indicated) with LPS. Following overnight incubation, IL-6 in the supernatants was quantified by ELISA. Data are from two independent experiments conducted in triplicate. *p < 0.05.
undertaken to prevent DC maturation.

Our observations made during development of the enrichment protocol underscore DC maturation as a key variable relevant to the successful therapeutic application of Treg. Recent reports (46) have advocated the use of specific isolating factors (the composition of which remains undefined, besides identification of the necessary, but insufficient, role of IL-6) that render Treg expansion (57). As the use of a single cytokine (MLRsup) contains specific factors that support the activation and expansion of AAsTreg. Additionally, it has been reported that IL-2 expansion of AAsTreg significantly while minimizing Th17 cell

These findings prompted us to develop an AAsTreg selection protocol based on immature DC. In conventional MLR, Treg proliferate extensively (Fig. 1B), suggesting that the supernatant (MLRsup) contains specific factors that support the activation and expansion of AAsTreg. Additionally, it has been reported that IL-2 supports Treg expansion (57). As the use of a single cytokine would facilitate translation of the protocol to the clinical setting, we compared the influence of MLRsup or IL-2 addition to co-cultures of Treg and immature donor DC. Both methods improved the expansion of Treg significantly while minimizing Th17 cell expansion. Co-culture with added IL-2, however, resulted in significant Foxp3+ cell proliferation. These cells were not IL-17+.

Two hypotheses can be formulated regarding the identity of these cells. It is possible that they are derived from a small fraction of contaminating cells released from Treg control by the addition of large amounts of exogenous IL-2. Adding back a small number of Foxp3+ cells are Th17 cells (62). This result is not unexpected (63), although not reported by Yamazaki et al. (46). The main reason for these discordant findings is probably the different Treg purification methods used in each study. Magnetic bead-mediated isolation does not guarantee the same level of purity as flow sorting, but it is fast becoming the strategy of choice for clinical-grade Treg purification. Maturing DC release a mixture of factors (the composition of which remains undefined, besides identification of the necessary, but insufficient, role of IL-6) that renders T cells less susceptible to Treg suppression (22). This could result in the release of small numbers of contaminating cells from Treg-mediated suppression and permit their faster proliferation and differentiation toward a Th17 phenotype. This hypothesis was tested in a preliminary experiment in which we added CFSE-stained CD4+CD25+ to the isolated CD4+CD25+ (Treg), cultured them with mature DC, and analyzed their CFSE dilution profile after 5 d (G. Raimondi, unpublished data). Differently from the pool of CD4+CD25+ cells, CD4+CD25+ cells did not proliferate significantly. This indicated that, under the conditions tested, Treg could still control the expansion of naive T cells and suggested that Th17 cells were expanding from the original pool of CD4+CD25+ T cells. This result is in accordance with a recent study (52) that used Foxp3-restricted GFP expression to show that, following activation in the presence of IL-6, a significant proportion of Treg converted to Foxp3+ IL-17+ Th17 cells. This phenomenon requires further in-depth analysis that is beyond the scope of the present investigation. Despite the fact that such data could reduce the positive impact of the proposed methodology, they underscore the need for caution in translation of Treg-based therapies to the clinic. Compared with rodents, human Treg isolation is more difficult, even though useful surface markers have been identified (32, 33, 35). This could render the process of human Ag-specific Treg selection susceptible to the expansion of potentially harmful effector T cells (47) if appropriate protocols are not implemented to prevent DC maturation.

These findings prompted us to develop an AAsTreg selection protocol based on immature DC. In conventional MLR, Treg proliferate extensively (Fig. 1B), suggesting that the supernatant (MLRsup) contains specific factors that support the activation and expansion of AAsTreg. Additionally, it has been reported that IL-2 supports Treg expansion (57). As the use of a single cytokine would facilitate translation of the protocol to the clinical setting, we compared the influence of MLRsup or IL-2 addition to co-cultures of Treg and immature donor DC. Both methods improved the expansion of Treg significantly while minimizing Th17 cell expansion. Co-culture with added IL-2, however, resulted in significant Foxp3+ cell proliferation. These cells were not IL-17+. Two hypotheses can be formulated regarding the identity of these cells. It is possible that they are derived from a small fraction of contaminating cells released from Treg control by the addition of large amounts of exogenous IL-2. Adding back a small number of Foxp3+ cells are Th17 cells (62). This result is not unexpected (63), although not reported by Yamazaki et al. (46). The main reason for these discordant findings is probably the different Treg purification methods used in each study. Magnetic bead-mediated isolation does not guarantee the same level of purity as flow sorting, but it is fast becoming the strategy of choice for clinical-grade Treg purification. Maturing DC release a mixture of factors (the composition of which remains undefined, besides identification of the necessary, but insufficient, role of IL-6) that renders T cells less susceptible to Treg suppression (22). This could result in the release of small numbers of contaminating cells from Treg-mediated suppression and permit their faster proliferation and differentiation toward a Th17 phenotype. This hypothesis was tested in a preliminary experiment in which we added CFSE-stained CD4+CD25+ to the isolated CD4+CD25+ (Treg), cultured them with mature DC, and analyzed their CFSE dilution profile after 5 d (G. Raimondi, unpublished data). Differently from the pool of CD4+CD25+ cells, CD4+CD25+ cells did not proliferate significantly. This indicated that, under the conditions tested, Treg could still control the expansion of naive T cells and suggested that Th17 cells were expanding from the original pool of CD4+CD25+ T cells. This result is in accordance with a recent study (52) that used Foxp3-restricted GFP expression to show that, following activation in the presence of IL-6, a significant proportion of Treg converted to Foxp3+ IL-17+ Th17 cells. This phenomenon requires further in-depth analysis that is beyond the scope of the present investigation. Despite the fact that such data could reduce the positive impact of the proposed methodology, they underscore the need for caution in translation of Treg-based therapies to the clinic. Compared with rodents, human Treg isolation is more difficult, even though useful surface markers have been identified (32, 33, 35). This could render the process of human Ag-specific Treg selection susceptible to the expansion of potentially harmful effector T cells (47) if appropriate protocols are not implemented to prevent DC maturation.

These findings prompted us to develop an AAsTreg selection protocol based on immature DC. In conventional MLR, Treg proliferate extensively (Fig. 1B), suggesting that the supernatant (MLRsup) contains specific factors that support the activation and expansion of AAsTreg. Additionally, it has been reported that IL-2 supports Treg expansion (57). As the use of a single cytokine would facilitate translation of the protocol to the clinical setting, we compared the influence of MLRsup or IL-2 addition to co-cultures of Treg and immature donor DC. Both methods improved the expansion of Treg significantly while minimizing Th17 cell expansion. Co-culture with added IL-2, however, resulted in significant Foxp3+ cell proliferation. These cells were not IL-17+. Two hypotheses can be formulated regarding the identity of these cells. It is possible that they are derived from a small fraction of contaminating cells released from Treg control by the addition of large amounts of exogenous IL-2. Adding back a small number

FIGURE 7. Combined posttransplant AAsTreg and Rapa administration promotes long-term (>150 d) alloantigen-specific heart graft survival. A, Schematic representation of the protocol used for the posttransplant treatment of recipients of vascularized hearts by administration of AAsTreg under the cover of short-term Rapa. B, Survival curves of heart allografts in recipients treated as indicated: Rapa, mice received daily Rapa injections; Rapa+AAsTreg, animals received the full treatment as depicted in A; Rapa+polyTreg, animals received poly Treg instead of AAsTreg; Rapa+3rd AAsTreg, mice received the full treatment, but AAsTreg were selected against third party C3H-derived DC. C, Individual graft survival times in the different treatment groups. &p;0.05 compared with group 1; b&p;0.05 compared with group 2; c&p;0.05 compared with group 3.
factors, including cytokines such as TGF-β, expression is highly regulated and influenced by many external factors. This is not to say that Foxp3 is not important, as some reports have documented the loss of suppressive capacity in Treg with ablation of Foxp3 expression (69–71). This raises doubt over the use of a selection protocol that does not support Foxp3 expression. Interestingly, high levels of IL-13 were revealed in the MLRsup, but conflicting and only indirect evidence exists regarding its role in Treg and Foxp3 expression (64–68). Based on these observations, further investigations are necessary to identify specific factor(s) that could be used as an alternative to MLRsup.

Recent reports have documented the loss of suppressive capacity in Treg when transferring a patient could elicit no effect if the recipient T cells are no longer sensitive to Treg control. Although there is evidence that exogenous danger signals can break an established Treg-dependent tolerant state (24), no information exists on the role of this phenomenon in regulation of Ag-specific responses and alloreactivity in particular. Our data clearly indicate that, although AAsTreg are more effective than polyclonal Treg in suppressing alloreactive T cells, they remain susceptible to the capacity of maturing DC to promote a Treg-independent T cell response. As indicated by Pasare et al. (22), this effect remains strictly dependent on the secretion of multiple cytokines. We observed that the absence of IL-6 (so far, the only cytokine shown to be a necessary, albeit insufficient, component of the mixture) from the system allowed recovery of T cell susceptibility to AAsTreg suppression. Our observation that DC isolated from Rapa-treated animals released significantly lower levels of IL-6 following maturation indicates that Rapa can also support the function of Treg by reducing inflammation. Such activity is underscored by the effects on graft survival. All these properties point to Rapa as a unique drug that favors the balance of the immune system toward regulation.

Long-term graft survival (>150 d) was achieved by combining Rapa and AAsTreg. This raised the question of the extent and duration of the activity associated with AAsTreg. Analysis of T cell reactivity in LTS indicated that, at 200 d posttransplant, Treg (both endogenous and exogenous) did not play a dominant role in controlling T cell activation in the spleen and lymph nodes. Although we cannot exclude a dominant, sustained role of Treg within the graft (60, 61), our data can be interpreted using the following model: combination of Rapa and AAsTreg permits transitory control of the alloimmune response that is probably associated with partial elimination of the reactive T cells and conversion of the remaining cells to an anergic state. Persistence of Ag sustains a tolerant state (74, 75) that is dependent on the maintenance of circulating alloreactive T cells in a state of anergy. We must emphasize, however, that long-term survival was unexpected. The Treg selection process was directed toward those cells activated via the direct pathway of Ag recognition/presentation. These cells were then able to control directly alloreactive T cells, but not indirectly activated cells. Our protocol targeted the indirect pathway of T cell activation only for the initial 10 d posttransplant (due to Rapa administration). In fact, histological analysis of LTS revealed cellular infiltration and graft tissue damage, suggesting a slowly progressive immune response (G. Raimondi, unpublished data). Such a response does not result in cessation of graft function, as indicated recently by Joffre et al. (17). This underscores the need to develop a selection protocol for AAsTreg that are activated through the indirect pathway. Although it demonstrates the relevance of the spectrum of specificity of Treg to generate a therapeutic effect, the method proposed by Joffre et al. is unfortunately not applicable to clinical settings, as it requires F1-derived APCs. However, other approaches have been delineated in the literature to select Treg with indirect specificity (76, 77).

![Long-term graft survival](http://www.jimmunol.org/)

**FIGURE 8.** Long-term surviving graft recipients exhibit a high frequency of Foxp3+ cells, but Treg-independent alloreactive T cell hyporesponsiveness. A, LTS (n = 2) were euthanized, CD4 T cells purified from the spleens, and the frequency of Foxp3+ T cells determined by flow cytometry. Age-matched normal control mice were analyzed for comparison. B, The reactivity of LTS-derived splenic CD4+ T cells (bulk or depleted of CD25+ cells) was determined by measuring their proliferation in response to donor DC. The influence of exogenous IL-2 (100 U/ml final concentration) to cultures of CD4+CD25− T cells was also tested (as indicated). *p < 0.05.

of CFSE-stained CD4+CD25− cells to Treg before the coculture excluded this possibility, as no significant proliferation was evident (G. Raimondi, unpublished data). Alternatively, these Foxp3− cells could derive from Treg that have lost Foxp3 expression. Foxp3 expression is highly regulated and influenced by many external factors, including cytokines such as TGF-β1, IL-2, IL-4, IL-7, and IL-15. Our Luminex-based quantification of the cytokines present in the MLRsup did not single out specific factor(s) responsible for sustaining Foxp3 expression. Interestingly, high levels of IL-13 were revealed in the MLRsup, but conflicting and only indirect evidence exists regarding its role in Treg and Foxp3 expression (64–68). Based on these observations, further investigations are necessary to identify specific factor(s) that could be used as an alternative to MLRsup.

Recent reports have documented the loss of suppressive capacity in Treg with ablation of Foxp3 expression (69–71). This raises doubt over the use of a selection protocol that does not support Foxp3 expression. Conversely, MLRsup sustained consistently high levels of Foxp3 expression, favoring its use for AAsTreg enrichment.

The short course of Rapa given to graft recipients shifted the effector T cell:Treg ratio in favor of the latter. It is recognized that immunosuppressive agents affect intrathymic selection of new T cells. At the dose tested, Rapa caused a 2-fold reduction in the absolute number of peripheral T cells. This probably resulted from a combination of highly reduced thymic output and minimized homeostatic proliferation of circulating T cells. The influence of Rapa on the ratio of Treg among T cells is noteworthy. In the absence of antigenic stimulation, Rapa reduced the percentage of circulating Foxp3+ cells. When Ag stimulation was present (in the form of a heart allograft), the proportion of Foxp3+ cells increased significantly. Three nonexclusive properties of Rapa could account for this observation. First, Rapa promotes activation-induced cell death of effector T cells while sparing Treg (31). Second, although Rapa prevents expansion of effector T cells, it allows proliferation of Treg (6, 29). Third, Rapa has been reported to promote the expression of Foxp3 (31, 72) and that could contribute to the alterations in proportions of Foxp3+ and Foxp3− cells we observed. On the whole, these changes are conducent to an effector T cell:Treg ratio that promotes the activity of the latter, even though it does not ultimately prevent graft rejection (Rapa-only group; Fig. 7).

The innate immune response converts T cells to a state refractory to Treg suppression (59, 73). This indicates that Treg transfer into a patient could elicit no effect if the recipient T cells are no longer sensitive to Treg control. Although there is evidence that exogenous danger signals can break an established Treg-dependent tolerant state (24), no information exists on the role of this phenomenon in regulation of Ag-specific responses and alloreactivity in particular. Our data clearly indicate that, although AAsTreg are more effective than polyclonal Treg in suppressing alloreactive T cells, they remain susceptible to the capacity of maturing DC to promote a Treg-independent T cell response. As indicated by Pasare et al. (22), this effect remains strictly dependent on the secretion of multiple cytokines. We observed that the absence of IL-6 (so far, the only cytokine shown to be a necessary, albeit insufficient, component of the mixture) from the system allowed recovery of T cell susceptibility to AAsTreg suppression. Our observation that DC isolated from Rapa-treated animals released significantly lower levels of IL-6 following maturation indicates that Rapa can also support the function of Treg by reducing inflammation. Such activity is underscored by the effects on graft survival. All these properties point to Rapa as a unique drug that favors the balance of the immune system toward regulation.

Long-term graft survival (>150 d) was achieved by combining Rapa and AAsTreg. This raised the question of the extent and duration of the activity associated with AAsTreg. Analysis of T cell reactivity in LTS indicated that, at 200 d posttransplant, Treg (both endogenous and exogenous) did not play a dominant role in controlling T cell activation in the spleen and lymph nodes. Although we cannot exclude a dominant, sustained role of Treg within the graft (60, 61), our data can be interpreted using the following model: combination of Rapa and AAsTreg permits transitory control of the alloimmune response that is probably associated with partial elimination of the reactive T cells and conversion of the remaining cells to an anergic state. Persistence of Ag sustains a tolerant state (74, 75) that is dependent on the maintenance of circulating alloreactive T cells in a state of anergy. We must emphasize, however, that long-term survival was unexpected. The Treg selection process was directed toward those cells activated via the direct pathway of Ag recognition/presentation. These cells were then able to control directly alloreactive T cells, but not indirectly activated cells. Our protocol targeted the indirect pathway of T cell activation only for the initial 10 d posttransplant (due to Rapa administration). In fact, histological analysis of LTS revealed cellular infiltration and graft tissue damage, suggesting a slowly progressive immune response (G. Raimondi, unpublished data). Such a response does not result in cessation of graft function, as indicated recently by Joffre et al. (17). This underscores the need to develop a selection protocol for AAsTreg that are activated through the indirect pathway. Although it demonstrates the relevance of the spectrum of specificity of Treg to generate a therapeutic effect, the method proposed by Joffre et al. is unfortunately not applicable to clinical settings, as it requires F1-derived APCs. However, other approaches have been delineated in the literature to select Treg with indirect specificity (76, 77).
In summary, we provide strong evidence of the existence of key molecular and cellular variables that significantly impact the successful application of experimental Treg therapy. Although further investigations are necessary to better characterize these variables, the results obtained underscore the potential advantage of the devised approach. As the number of Tregs required for therapeutic efficacy remains a major consideration for their clinical application, having identified parameters that can be targeted to strengthen (or preserve) their function permits the use of a much lower number in comparison with what is required by other approaches (17, 20). The present approach overcomes concern over the use of Treg generated by genetic modification (forced expression of Foxp3) (78, 79), as the approach overcomes concern over the use of Treg generated by genetic modification (forced expression of Foxp3) (78, 79), as the long-term safety of such an approach has not yet been demonstrated. Additionally, our protocol is compatible with conventional immunosuppression. More importantly, this approach results in long-term alloantigen-specific graft survival, a fundamental goal in the design of improved therapies.

Acknowledgments

We thank Derek Barclay for conducting Luminex-based quantifications on our samples. We thank our colleagues for constructive critiques of the manuscript and Miriam Freeman for excellent administrative support. We are grateful to Dr. Hongmei Chen and the Starzl Transplantation Institute flow cytometry core facility for expert advice and assistance.

Disclosures

The authors have no financial conflicts of interest.

References


