This information is current as of April 17, 2017.

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,*‡ Tina L. Sumpter,*† Benjamin M. Matta,*† Mahesh Pillai,*† 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 MHCI-compatible 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 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 coregulatory 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 alloantigen-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 administration of Rapa creates conditions that favor the tolerance-promoting function of Treg. In particular, Rapa alters the relative proportions of effector and endogenous Tregs in favor of the latter and suppresses proinflammatory cytokines produced by activated DC. Small numbers of adoptively transferred AAsTreg induced long-term (>150 d), donor-specific heart allograft survival in unmanipulated recipients when given 1 wk posttransplantation under cover of a short, postoperative course of low-dose Rapa. Additionally, graft survival was associated with anergy induction in directly alloreactive host T cells. By combining a reliable method of AAsTreg enrichment with a treatment (mammalian target of Rapa inhibition) conducive to their protective function, we have identified a promising approach for the induction of long-term, donor-specific organ transplant survival with minimal host manipulation/drug-based immunosuppression.

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). Eight- to 12-wk-old C57BL/6J-Ptprc<sup>b<sup>+/+<sup>BoyAItac</sup> (B6.SJL-H-2K<sup>d</sup>) were purchased from Taconic Farms (Hudson, NY). IL-6<sup>−/−</sup> C57BL/6 mice were a gift from Dr. A.J. Demetrius (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 Caprablex (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-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<sup>+</sup> cells were purified by magnetic negative selection. These represented “bulk” CD4<sup>+</sup> T cells. For Treg isolation, bulk CD4<sup>+</sup> T cells were incubated with anti-CD25-PE mAb and CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated by positive selection using anti-PE microbeads and MS separation columns (Miltenyi Biotec). Purity was assessed by cytfluorometric analysis and was consistently 90–95%. The remaining cells were used as CD4<sup>+</sup>CD25<sup>+</sup> T cells for assays of suppressor function. For experiments on Foxp3 upregulation, the remaining CD4<sup>+</sup>CD25<sup>+</sup> T cells were depleted of residual CD25<sup>+</sup> 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 × 10<sup>6</sup>/plate with recombinant murine GM-CSF (300 U/ml) and recombinant murine IL-4 (200 U/ml) (both from R&amp;D Systems, Minneapolis, MN). Media was changed every 3 d. On day 7, nonadherent cells were washed thoroughly and CD11c<sup>+</sup> DC purified using anti-CD11c immunomagnetic 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<sup>+</sup> 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 (MiraBio, 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&amp;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<sup>+</sup>CD25<sup>+</sup>) were incubated with allogeneic bone marrow-derived DC (BALB/c or C3H, depending on the experiment) using the Luminex multiplexing platform (MiraBio, Alameda, 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&amp;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.
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 T cell proliferation quantified by thymidine incorporation. Results are expressed as means ± 1 SEM. The significances of differences between groups was determined by Student t test. p < 0.05 was considered significant.

Statistics

Data are expressed as means ± 1 SEM. The significances of differences between means were determined using 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 activation/expansion. 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+T cells. 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.

FIGURE 2. Coculture of nTreg and mature DC favors the expansion of Th17 cells. A, Purified CD4+CD25+ T cells were stained with CFSE and cultured with immature allogeneic DC. After 4 d, the cells were collected and stained for surface PD-1 and intracellular Foxp3. Histograms are gated on the proliferating population. B, Same as in A, but Treg were cocultured with LPS-matured allogeneic DC. Data are representative of five independent experiments. C, To further characterize the Foxp3− cells induced to proliferate in the presence of mature allogeneic DC, CD4+CD25+ T cells were stained with CFSE and cocultured with mature DC. After 4 d, cells were isolated, stimulated with PMA/ionomycin, then stained for surface CD4 and the intracellular cytokines indicated. Dot plots are gated on the Foxp3− population obtained. Percentages indicate frequency of cytokine positive cells relative to the total of proliferating CD4+ cells. Representative of two independent experiments. D, Same strategy as B, but cells were cocultured with the addition of 500 IU/ml IL-2. Analysis of Foxp3 expression in proliferating T cells (left) is gated on CD4+ cells. Analysis of IL-17 expression (right) is gated on CD4+ Foxp3− cells. Numbers indicate percentage of positive cells in the proliferating cell gate. Data are representative of three independent experiments.
MLRsup enhances Foxp3+ T cell expansion induced by immature DC

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

FIGURE 3. MLRsup contains factors that stimulate the expansion of Treg, preserve Foxp3 expression, and minimize Th17 cell proliferation. A, CFSE-stained CD4+CD25+ T cells were cultured with allogeneic DC for 4 d in the presence of exogenous IL-2 (500 IU/ml) or supernatant obtained from MLR reactions (MLRsup; see Materials and Methods). Cells were then isolated and stained with PMA/ionomycin as described in the Materials and Methods before staining for surface CD4 (used to gate the events depicted) and intracellular Foxp3 or IL-17, as indicated. Data are representative of three independent experiments. B, Bulk CD4 T cells were purified by negative selection and then cocultured for 4 d with irradiated allogeneic DC. The supernatant (MLRsup) was then collected and the cytokine content measured by Luminex assay. Active TGF-β (+) was measured by conventional ELISA. Data are representative of two experiments.
A

B

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. Strong suppressive activity was still evident, even at a lower Treg:T cell ratio of 1:40 (Fig. 5B). Moreover, we tested whether our enrichment protocol generated AAsTreg. Treg (B10) were enriched against C3H DC and their ability to inhibit the proliferation of CFSE-stained (B10) CD4 T cells against C3H-derived DC. The data, expressed as percentage of proliferating CD4 over the total CD4 T cells (Fig. 5D) confirm the absence of nonspecific suppression. Collectively, these results support the validity of the devised protocol for the enrichment of AAsTreg.

Rapa administration creates an environment conducive 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. 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 Rapa, euthanized, and the absolute numbers and incidence of CD8+ T cells, and Foxp3+ T cells quantified in different tissues. 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 in the blood 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, 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 mature APC. CD4^+CD25^− T cells were stimulated with allogeneic, immature, or LPS-exposed DC and T cell proliferation measured after 3 d by [H]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 [H]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
Using the above insights, we devised the therapeutic protocol depicted in Fig. 7 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).

Table I. Influence of Rapa administration on T lymphocytes

<table>
<thead>
<tr>
<th>Blood</th>
<th>Normal</th>
<th>Untreated</th>
<th>Rapa</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 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+Foxp3+</td>
<td>6.3 ± 0.8</td>
<td>5.8 ± 2.2</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cellularity (× 10⁶)</td>
<td>42.8 ± 10.2</td>
<td>53.5 ± 6.4</td>
<td>18.3 ± 6.8***</td>
</tr>
<tr>
<td>% of total cells</td>
<td></td>
<td></td>
<td></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+Foxp3+</td>
<td>20.5 ± 1.4</td>
<td>27.4 ± 0.2**</td>
<td>25.7 ± 1.7</td>
</tr>
</tbody>
</table>

| Spleen | | | |
| Total cellularity (× 10⁶) | 114 ± 9.8 | 217 ± 19.4** | 87.8 ± 7.6*** |
| % of total cells | | | |
| CD8 | 7.9 ± 1.4 | 8.8 ± 0.2 | 6.3 ± 1.4 |
| CD4 | 13.6 ± 1.7 | 13.2 ± 0.5 | 8.4 ± 1.2*** |
| % of CD4+ cells | | | |
| CD4+Foxp3+ | 16.3 ± 0.6 | 17.3 ± 1.2 | 21.1 ± 2.1* |

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 antiinflammatory activity that could (additionally) support the protogenic 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 coincubation 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
unable to regulate the response to third party. This observation draws attention to graft survival in recipients given nonselected (poly) Treg, with an MST of 45 d. Multiple data, including ours (Fig. 1), suggest that, as the T cell pool comprises a high frequency of alloreactive cells, the repertoire of Treg is similarly represented. This could explain the development of 40% LTS in the polyTreg-treated group, together with the level of success achieved in early preclinical studies using polyclonally expanded Treg to promote bone marrow transplant acceptance and prevention of graft-versus-host disease (5, 6). The significant increase in frequency of LTS following injection of AAsTreg underscores how delicate the balance between tolerance and reactivity may be and also that the Ag-specific enrichment protocol may be crucial for the successful clinical application of such therapy.

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 indicated that mature DCs (CD86hi) have a stronger Treg stimulatory effect than immature DCs. 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 expansion of AAsTreg. Additionally, it has been reported that IL-2 expands 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 proliferation 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 cocultures of Treg and immature donor DC. Both methods improved the expansion of Treg significantly while minimizing Th17 cell expansion. Coculture 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. †Animal death in cage; *p < 0.05 compared with group 1; **p < 0.05 compared with group 2; ***p = 0.05 compared with group 3.
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 (31, 72) and that could contribute to the alterations in proportions of Foxp3+ and Foxp3− cells we observed. On the whole, these changes are conducive 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. 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 process 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 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. We thank Jaromir Hutka for excellent administrative support. We are grateful to Dr. Hongmei Chen and the Stariž Transplantation Institute flow cytometry core facility for expert advice and assistance.

Disclosures
The authors have no financial conflicts of interest.

References


