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Contrasting Effects of Cyclosporine and Rapamycin in *De Novo* Generation of Alloantigen-Specific Regulatory T Cells

W. Gao[a,*], **Y. Lu**[a], **B. El Essawy**[a], **M. Oukka**[b], **V.K. Kuchroo**[c], and **T.B. Strom**[a,*]

[a]Department of Medicine, Division of Transplant Immunology and Transplant Research Center, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

[b]Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA

[c]Center for Neurologic Diseases, Brigham and Woman's Hospital, Harvard Medical School, Boston, MA

Abstract

The outcome of T-cell-mediated responses, immunity or tolerance, critically depends on the balance of cytopathic versus regulatory T (T_{reg}) cells. In the creation of stable tolerance to MHC incompatible allografts, reducing the unusually large mass of donorreactive cytopathic T effector (T_{eff}) cells via apoptosis is often required. Cyclosporine (CsA) blocks activation-induced cell death (AICD) of T_{eff} cells, and is detrimental to tolerance induction by costimulation blockade, whereas Rapamycin (RPM) preserves AICD, and augments the potential of costimulation blockade to create tolerance. While differences between CsA and RPM in influencing apoptosis of activated graft-destructive T_{eff} cells are apparent, their effects on graft-protective T_{reg} cells remain enigmatic. Moreover, it is unclear whether tolerizing regimens foster conversion of naïve peripheral T cells into alloantigen-specific T_{reg} cells for graft protection. Here we show, using reporter mice for T_{reg} marker Foxp3, that RPM promotes *de novo* conversion of alloantigen-specific T_{reg} cells, whereas CsA completely inhibits this process. Upon transfer, *in vivo* converted T_{reg} cells potently suppress the rejection of donor but not third party skin grafts. Thus, the differential effects of RPM and CsA on T_{eff} and T_{reg} cells favor the use of RPM in shifting the balance of aggressive to protective type alloimmunity.

Keywords

Antigen specific; cyclosporine; *de novo* generation; Foxp3; Rapamycin; regulatory T cells; tolerance

Introduction

In the past two decades, a large array of immunosuppressive agents has expanded the armamentarium used by transplant physicians and surgeons to prevent acute allograft rejection, evidenced by the greatly improved rates of short-term graft survival. The focus of transplantation medicine is now more shifted towards tackling issues associated with side effects of long-term immunosuppression and chronic rejection. The goal is to achieve transplantation tolerance that is specific and indefinite acceptance of transplanted graft without ongoing immunosuppression. Animal studies indicate that the hallmark of

*Corresponding author: W. Gao or T. B. Strom, wgao@bidmc.harvard.edu or tstrom@bidmc.harvard.edu.

transplantation tolerance is the generation of donor-specific regulatory T (T_{reg}) cells that are capable of suppressing cytopathic T effector (T_{eff}) cells. Among different subsets of T cells with regulatory properties, naturally occurring $CD4^+CD25^+$ T_{reg} cells are the most often and thoroughly studied.

It has recently been reported that the forkhead/winged-helix transcription factor Foxp3 is the 'master switch' for the development and function of $CD4^+CD25^+$ T_{reg} cells, and serves as their lineage-specific marker (1–3). These thymus-derived cells are essential in maintaining self-tolerance (4), and are also indispensable for induction of peripheral tolerance in animal models of transplantation (5). Nonetheless, it is a matter of debate whether upon antigen stimulation, formerly naïve T cells in the extrathymic compartment can adopt a T_{reg} phenotype and exert antigen-specific regulatory function (6–8). In transplantation setting, it has not been addressed at molecular details whether peripheral generation of T_{reg} cells occurs and whether such adaptive or induced T_{reg} (iT_{reg}) cells contribute to donor-specific tolerance. Hence we aim to examine the existence and role of *de novo*-generated T_{reg} cells by potential tolerance-inducing protocols. However, the intracellular localization of Foxp3 limits its usefulness in isolating and transferring live cells for functional studies. To overcome this difficulty, we created a knock-in mouse (Foxp3GFP) with a bicistronic EGFP reporter introduced into the endogenous *Foxp3* locus (9). Compared with the $CD4^+GFP^-$ T cells, $CD4^+GFP^+$ T cells are anergic and immunosuppressive *in vitro*, similar to the wild type $CD4^+CD25^+$ counterparts. The GFP^+ cells also robustly express T_{reg}-associated markers, including Foxp3, CTLA-4, CD25 and GITR (Supplementary Figure 1). The coordinated but independent expression of wild type Foxp3 and GFP proteins allows us to faithfully track Foxp3-expressing cells with the green fluorescence marker and study the factors that modulate Foxp3 expression (9). With this system, we compared the effects of the two commonly used immunosuppressive drugs, CsA and RPM, on *de novo* generation of alloantigen-specific T_{reg} cells.

Materials and Methods

Mice

The Foxp3GFP knock-in mice in C57BL/6 background ($CD45.2^+$) were generated as reported (9). They were further crossed once with the $CD45.1^+$ homozygous mice in C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) to obtain both the CD45 congenic alleles. The other mouse strains used in this study were all purchased from the Jackson Laboratories. All animal experiments were performed in compliance with the approval of the Harvard Medical Area Standing Committee on Animals (Boston, MA).

In vitro Foxp3 induction

FACS-sorted $CD4^+GFP^-$ cells (2×10^5) from naïve Foxp3GFP mice were stimulated in 48-well culture plates coated with anti-CD3 (3 μg/mL) and anti-CD28 (5 μg/mL) (Pharmingen). In some wells, TGF-β1 (eBioscience, 1 ng/mL), anti-TGF-β1 (R & D Systems, 20 μg/mL), and different doses of RPM or CsA (both from LC Laboratories) were added. After 3 days, cells were analyzed on FACS for GFP expression or processed for realtime PCR quantification of Foxp3 message, using RNeasy Mini Kit (Qiagen), cDNA reverse transcription kit and Taqman primer-probe set (both from Applied Biosystems). A comparative threshold cycle (C_T) method was used to determine Foxp3 gene relative expression analyzed against the endogenous gene of murine GAPDH. For each sample, the Foxp3 C_T value was normalized with the formula $\Delta C_T = C_T$ Foxp3 − C_T GAPDH. The mean ΔC_T was determined, and relative Foxp3 expression was calculated with the formula $2^{-\Delta CT}$.

In vivo T_{reg} conversion

FACS-sorted $CD4^+GFP^-$ cells (1×10^7, $CD4^+GFP^+$ <0.1%) from naïve Foxp3GFP mice ($CD45.1^+CD45.2^+$) were i.v. injected into nonirradiated BDF1 hosts. After cell transfer, BDF1 mice were treated on days 0, 1, 2 with HBSS or RPM (3 mg/kg, i.p.) or CsA (20 mg/kg, s.c.), alone or together with anti-CD154 (MR1, BioExpress, 0.25 mg, i.p.). On day 4, lymph node and spleen cells from BDF1 hosts were analyzed on FACS by gating on $CD45.1^+CD4^+$ cells. In some experiments, *de novo*-generated $CD4^+GFP^+$ T_{reg} cells were FACS-sorted for *in vitro* and *in vivo* function assays.

CFSE labeling, Foxp3 and Annexin V staining

Lymph node and spleen cells from naïve C57BL/6 mice were depleted of $CD4^+CD25^+$ cells by anti-CD25 beads (Miltenyi) and labeled with CFSE (5 μM, Invitrogen) at room temperature for 6 min. Cells ($4–6 \times 10^7$) were then i.v. injected into BDF1 mice. The doses and times for treating recipients with RPM, CsA and/or anti-CD154 were the same as above. After 4 days, spleen and lymph node cells from the treated animals were intracellularly stained with anti-Foxp3 (eBioscience), and gated on the $H\text{-}2D(d)^-CD4^+$ fraction. Annexin V staining was carried out with a kit from BD Pharmingen.

In vitro suppression assay

Spleen cells from DBA/1 or DBA/2 mice were depleted of T cells by anti-CD4/CD8 beads (Miltenyi), treated with Mitomycin C (Sigma) at 50 μg/mL for 30 min, and used as stimulators (8×10^4) in round-bottomed 96-well plates. Naïve $CD4^+GFP^-$ cells from Foxp3GFP mice were FACS-sorted and used as responders (8×10^4) in MLR. Varying ratios of FACS-sorted, *de novo*-induced $CD4^+GFP^+$ T_{reg} (iT_{reg}) cells from BDF1 hosts treated with RPM plus anti-CD154 were added to the MLR culture for 4 days. Cells were pulsed with [^{3}H]methylthymidine (0.5μCi/well; NEN) for the last 8 h before harvesting, and incorporated radioactivity of triplicate wells was counted.

Skin transplantation

FACS-sorted $CD4^+GFP^-$ T cells (2×10^5, from naïve knock-in mice) were transferred by tail vein injection into C57BL/6 RAG-1-deficient mice. One day later, mice were transplanted with allogeneic tail skin grafts from DBA/2 donor. One group of animals was treated with RPM (3 mg/kg, i.p.) for three consecutive days, then every other day for total 14 days. The second group was treated with CsA (20 mg/kg, s.c.) every day for 14 days. The third group was left untreated. In some recipients of the three groups, spleen and lymph node cells were examined for GFP expression by FACS on day 18 and day 30.

To test the alloantigen-specific suppressive activity of iT_{reg} cells, allogeneic tail skin grafts from DBA/1 and DBA/2 mice were simultaneously transplanted onto C57BL/6 RAG-1-deficient mice on the opposite sides of the flank. $CD4^+GFP^-$ T_{eff} cells (2×10^5, from naïve knock-in mice) with or without *de novo*-induced iT_{reg} cells (3×10^4, from BDF1 hosts treated with RPM plus anti-CD154) were transferred by tail vein injection. Each graft was examined daily beginning at day 7 post-adoptive transfer and was considered rejected when the graft is completely necrotic. Difference of graft survival times was assessed by Kaplan-Meier survival analysis with StatView software. $P < 0.01$ is considered statistically significant.

Results

RPM, but not CsA, induces Foxp3 expression in activated $CD4^+$ $Foxp3^-$ T cells in vitro

First, we examined *in vitro* whether RPM or CsA can affect *de novo* expression of Foxp3 by peripheral T cells. Naïve $CD4^+GFP^-$ T cells were sorted by FACS (99% purity), and stimulated with plate-bound anti-CD3 and anti-CD28. Addition of TGF-β1 resulted in the conversion of 30–40% of naïve $CD4^+GFP^-$ T cells into the $Foxp3^+$ T_{reg} phenotype, as previously reported (9). RPM also promotes conversion, albeit less vigorously, with about 10% of $CD4^+Foxp3^-$ cells stimulated with plate-bound anti-CD3 and anti-CD28 converting into the $Foxp3^+$ phenotype after 3 days of culture (Figure 1A). Induction of Foxp3 by RPM is dose-dependant, though transient peaking at day 3 (Figure 1B).

Previous work has demonstrated that RPM induces TGF-β1 expression (10). We found that anti-TGF-β1 antibody completely blocked RPM-induced Foxp3 expression (Figure 1C), suggesting a potential link between RPM effect and TGF-β1 signaling. Although CsA is also known to induce TGF-β1 (11), it not only failed to induce Foxp3 (not shown), but also blocked Foxp3 induction by TGF-β1 (Figure 1D) or RPM (not shown). Interestingly, TGF-β-converted $GFP^+(Foxp3^+)$ T_{reg} cells are mainly Annexin V negative, indicating that they are more resistant to apoptosis than $GFP^-(Foxp3^-)$ T_{eff} cells. Contrary to RPM being permissive to TGF-β, CsA blocked TGF-β-induced Foxp3 expression in cells that were still Annexin V negative (Figure 1D,E), suggesting its inhibitory effect is not simply due to compromising cell viability. Another calcineurin inhibitor FK506 (Tacrolimus) exerted the same effects as CsA (not shown). Thus, both TGF-β1 and calcineurin-dependent signals are required for *de novo* induction of Foxp3.

RPM, but not CsA, induces *in vivo* conversion of naïve T cells into T_{reg} cells

Next, we compared the effects of RPM and CsA upon *in vivo* conversion of naïve $CD4^+GFP^-$ T cells into T_{reg} cells. To optimally stimulate naïve T cells with alloantigen, FACS-sorted $CD4^+GFP^-$ cells (GFP^+ cells <0.1%) from the knock-in mice (C57BL/6 background, H-2^b, CD45.1/2^+) were adoptively transferred into naïve nonirradiated BDF1 mice (F1 of C57BL/6 and DBA/2, H-$2^{b,d}$, CD45.2^+) (Figure 2A). In this GVHD-like model, transferred alloreactive $CD4^+$ T cells respond to host H-2^d alloantigen, proliferate and complete 7–8 cell divisions within 3 days as demonstrated by CFSE dye-dilution assay (not shown). After transfer of $CD4^+GFP^-$ C57BL/6 T cells, BDF1 hosts were treated on days 0, 1, 2 with HBSS or RPM (3 mg/kg, i.p.) or CsA (20 mg/kg, s.c.), alone or with MR1 anti-CD154 mAb (0.25 mg, i.p.).

On day 4 post cell transfer, lymph node and spleen cells from BDF1 hosts were analyzed by flow cytometry via gating onto the $CD45.1^+CD4^+$ cells. Six percent of C57BL/6 cells residing in the spleens of the hosts treated with RPM became $GFP^+(Foxp3^+)$, while 2% were positive in hosts receiving buffered saline. Provision of anti-CD154 mAb alone elicited a similar outcome. Combined RPM and anti-CD154 treatment was synergistic as nearly 20% of transferred C57BL/6 $CD4^+$ T cells residing in the host spleens were $Foxp3^+$ (Figure 2B), and the ratio reached 25% for C57BL/6 cells harvested from the host lymph nodes (not shown). In sharp contrast, CsA not only abrogated the positive effect of anti-CD154 upon conversion of $Foxp3^-$ into the $Foxp3^+$ phenotype, but also blocked the low basal level conversion (about 2%) in control nontreated (HBSS) hosts. Clearly, RPM induces, whereas CsA blocks, the *in vivo* conversion of naïve $CD4^+Foxp3^-$ T cells into $Foxp3^+$ cells that arises with alloantigen stimulation.

The kinetics of *in vivo* conversion is fairly fast. In a separate experiment, we determined the percentages and absolute numbers of GFP^+ cells among transferred $CD4^+$ cells at different time points. The peak of conversion induced with RPM+anti-CD154 occurs by day 4 in

spleen and by day 7 in lymph nodes (Supplementary Figure 2). Conversion declines afterwards, and on day 15, much fewer GFP^+ cells can be recovered from spleen and lymph nodes (Supplementary Table 2). Two months after cell transfer, no GFP^+ cells can be detected in $CD45.1^+CD4^+$ population (not shown). As suggested by the *in vitro* observation (Figure 1B), RPM-induced conversion may also be transient *in vivo*.

Differences between T_{eff} cells and *de novo*-generated T_{reg} cells in cell cycle progression and susceptibility to apoptosis

Although RPM and anti-CD154 synergize in inducing higher percentage of converted T_{reg} cells, RPM itself paradoxically induces more T_{reg} cells by absolute number in both spleen and lymph nodes (Figure 2B, Supplementary Table 1a and 1b). It might be that anti-CD154 differentially inhibits the proliferation of GFP^- T_{eff} cells, thus increasing the percentage of GFP^+ T_{reg} cells. To resolve this issue, we studied *in vivo* T_{reg} conversion using a method that combines staining of Foxp3 intracellular proteins and CFSE labeling for cell division analysis. We adopted this strategy because both GFP (Foxp3) and CFSE dye emit green fluorescence, and cannot be simultaneously analyzed on the FL1 channel of FACScan. Lymph node and spleen cells from naïve C57BL/6 mice were depleted of $CD4^+CD25^+$ T_{reg} cells by magnetic beads, labeled with CFSE and transferred into BDF1 hosts. RPM alone or RPM+anti-CD154 vigorously induced conversion of alloactivated $CD4^+$ T cells into the T_{reg} phenotype in the early cell divisions (e.g., 30–40% cells became $Foxp3^+$ within divisions 1 and 2), whereas T cells undergoing more extensive proliferation (divisions 5–7) negligibly expressed Foxp3 (Figure 3A,B). Again, CsA completely blocked Foxp3 induction (Figure 3B). Interestingly, the percentages of $Foxp3^+$ cells at divisions 1–4 were virtually the same between RPM and RPM^+anti-CD154 treatments, suggesting anti-CD154 has little effect on the conversion process. Nonetheless, as compared to RPM treatment alone, treatment with RPM+anti-CD154 more significantly inhibited the proliferation of alloactivated $Foxp3^-$ T_{eff} cells, but only slightly inhibited the apparent proliferation of $Foxp3^+$ T_{reg} cells (Figure 3C). In this experimental setting, however, the division peaks of $Foxp3^+$ T_{reg} cells do not necessarily represent their actual division rate, due to the fact that the initial CFSE label was on the $Foxp3^-$ T_{eff} cells. $Foxp3^+$ cells within a particular division could be the descendants of proliferating induced T_{reg} cells and/or those converted “on spot” from proliferated $Foxp3^-$ T_{eff} cells into $Foxp3^+$ phenotype. Regardless of the interpretation, costimulation blockade with anti-CD154 shifts the balance of T_{reg} vs. T_{eff} by inhibiting more on the proliferation of T_{eff} cells, albeit the absolute number of converted T_{reg} cells is slightly reduced.

Since activation-induced cell death (AICD) accompanies vigorous T-cell proliferation (12,13), we investigated whether T_{eff} and newly converted T_{reg} cells are differentially susceptible to apoptosis. Indeed, when recipients of allogeneic T cell transfers were treated with RPM+anti-CD154, $CD4^+$ T cells with Annexin V positive staining were primarily noted within the GFP^- compartment (Figure 3D). Therefore, RPM-based treatment fosters *de novo* conversion of naïve T cells into $Foxp3^+$ T_{reg} cells. Moreover, unlike T_{eff} cells, the converted T_{reg} cells are more resistant to apoptosis, a conclusion also supported by the *in vitro* finding (Figure 1D).

De novo-generated T_{reg} cells are alloantigen-specific, and can protect donor-strain skin graft upon transfer

We then tested the antigen specificity of *de novo*-generated T_{reg} cells in the mixed lymphocyte reaction (MLR). $CD4^+GFP^-$ T_{eff} cells from naïve knock-in mice were stimulated with Mitomycin C-treated DBA/1 ($H\text{-}2^q$) or DBA/2 ($H\text{-}2^d$) splenocytes. The *de novo*-generated T_{reg} cells, sorted by FACS from RPM+anti-CD154 treated BDF1 mice, potently suppressed the proliferation of T_{eff} cells against donor DBA/2 but not third-party

DBA/1 allogeneic cells (Figure 4A), indicating they are alloantigen specific. To test the specificity of their graft protective function *in vivo*, we simultaneously grafted DBA/1 and DBA/2 skins onto recipient RAG-1-deficient mice on the opposite sides of the flank, and then transferred FACS-sorted naïve $CD4^+GFP^-$ T_{eff} cells, with or without GFP^+ induced T_{reg} (iT_{reg}) cells sorted from RPM+anti-CD154 treated BDF1 hosts. Transfer of 3×10^4 iT_{reg} cells powerfully suppressed the ability of 2×10^5 T_{eff} cells to reject DBA/2, but not DBA/1, skin grafts (Figure 4B). Thus, such *de novo*-generated $Foxp3^+$ cells are *bona fide* antigen-specific T_{reg} cells that, like *in vitro* activated naturally occurring T_{reg} cells (14, 15), can mediate donor graft protection, even at a low ratio of transferred 6 effectors to 1 regulatory T cell.

RPM, but not CsA, induces long-term skin allograft survival with concomitant *de novo* generation of $Foxp3^+$ cells

To correlate RPM-induced *in vivo* T_{reg} cell generation with allograft tolerance in a transplant setting more physiological than the alloantigen-driven GVHD-like model (Figures 2 and 3), we adoptively transferred 2×10^5 $CD4^+GFP^-$ naïve T cells into C57BL/6 RAG-1-deficient mice bearing transplanted DBA/2 skin grafts, and then treated the reconstituted recipients with RPM or CsA for 14 days. Untreated animals rejected DBA/2 skins promptly (MST = 25.7 days, n = 6). CsA treatment prolonged graft survival (MST = 40.7 days, n = 6) with statistical difference ($p = 0.003$), but all the grafts were eventually rejected. In contrast, RPM treatment induced indefinite graft survival (MST > 100 days, n = 7) (Figure 5A). FACS analysis on day 18 and day 30 post-treatment showed that RPM promoted a significant portion of transferred naïve T cells to become $Foxp3^+$ T_{reg} cells. These cells first appeared in the spleen, and later were enriched in the graft-draining lymph nodes of RPM-treated hosts. CsA, on the other hand, had a detrimental effect and even inhibited the basal level (1–3%) induction of $Foxp3^+$ T_{reg} cells by homeostatic proliferation (Figure 5B and W.G. unpublished observation). In a separate cell dosing experiment, 10-fold more (2×10^6) $CD4^+GFP^-$ naïve T cells were adoptively transferred into RAG-1-deficient mice bearing DBA/2 skin grafts, and the recipient mice were treated with RPM or CsA by the same protocol. FACS analysis on day 18 post-treatment revealed a similar pattern of T_{reg} cell induction as above (not shown). Moreover, the MST of DBA/2 skin grafts were 10.0 days (untreated, n = 3), 17.5 days (CsA-treated, n = 2) and 26.0 days (RPM-treated, n = 2) respectively. We terminated the experiment because the mice started to show signs of wasting disease. Nevertheless, these results collectively suggest that RPM-induced *de novo* generation of $Foxp3^+$ T_{reg} cells may contribute to its graft protecting activities.

Discussion

Cyclosporine (CsA) and Rapamycin (RPM) are widely used to effectively prevent transplant rejection. Both drugs are potent and reasonably well tolerated immunosuppressive agents, but their effects on graft-destructive T_{eff} and graft-protective T_{reg} cells are drastically different. In collaboration with the Turka laboratory, we have previously reported that RPM promotes, whereas CsA blocks, AICD of allore-active T cells (13,16). In several other models, RPM enhances while CsA abrogates the efficacy of costimulation blockade-based therapy to induce graft tolerance (17–20). These and work on $CD8^+$ T cells (21) collectively indicate that AICD of alloreactive T cells in general is prerequisite for tolerance induction by costimulation blockade. Nonetheless, the effects of RPM and CsA on subsets of alloreactive T cells, namely T_{eff} versus T_{reg} cells, especially those *de novo*-generated T_{reg} cells, were not tested in all the abovementioned studies. Here we demonstrated that RPM promotes and synergizes with anti-CD154, to convert peripheral alloreactive $CD4^+Foxp3^-$ T cells into apoptosis-resistant $Foxp3^+$ T_{reg} cells that can mediate donor-specific skin graft protection upon transfer, whereas CsA completely inhibits this process. In a companion

study using a pre-transplant conditioning regimen of donor-specific transfusion plus anti-CD154 mAb, the enhanced donor-directed T_{reg} activity in the $CD4^+CD25^+$ pool could be further strengthened by addition of RPM but abolished by CsA cotreatment (Kang et al., submitted for publication). It has been reported that CsA treatment reduces Foxp3 expression in natural T_{reg} cells (22), and fails to support the differentiation of the highly suppressive $CD4^+CD25^+CD27^+$ subset upon alloantigen stimulation (23). On the contrary, RPM does not show adverse effects but sustains a high ratio of natural $CD4^+CD25^+$ T_{reg} cells during IL-2-mediated expansion (24). In addition, naïve human T cells exhibit regulatory activities upon TCR stimulation in the presence of RPM (25), although direct evidence for T_{eff} to T_{reg} conversion was not fully established due to promiscuous expression of Foxp3 in human T cells after activation (26). The effects of CsA versus RPM on T_{reg} conversion was not assessed in that study (25). In renal transplant recipients, calcineurin inhibitors, but not RPM, were found to reduce the frequencies of $CD4^+CD25^+Foxp3^+$ T_{reg} cells (27). The detrimental effect of calcineurin inhibitors on T_{reg} cells could partly lie in their activity to block IL-2 production, which is required for T_{reg} function and homeostasis (28), as replenishing CsA-treated hosts with exogenous IL-2/Fc (or IL-2) restores T_{reg} activity in transplantation (Kang et al.) and GVHD models (29). Additionally, calcineurin inhibitors may more profoundly affect T_{reg} cell programming by directly interfering with NFAT:Foxp3 interaction (30,31). Such an effect could be fatal for the newly converted T_{reg} cells when Foxp3 levels are delicately low. In support of this notion, excessive amount of IL-2/Fc failed to revert CsA blockade on T_{reg} conversion *in vivo* (not shown).

The extrathymic *de novo* generation of T_{reg} cells is of considerable interest, as this process may underline producing allograft-specific suppressors important for transplant tolerance, as well as new T_{reg} recruits in the vicinity of tumor or infection that deter specific immunity. By using a Foxp3-GFP fusion protein knock-in mouse, Fontenot et al. reported that induction of Foxp3 expression in $CD4^+GFP^-$ T cells does not occur *in vivo* during pathogen-driven immune responses (7). Our study demonstrated for the first time that *de novo* generation of graft-protective T_{reg} cells indeed occurs *in vivo* under tolerizing conditions. Such conversion of T_{eff} into T_{reg} may very likely depend on cytokine milieu, as proinflammatory cytokines highly secreted during alloactivation, pathogen infection or immunization with complete adjuvant would inhibit Foxp3 induction while favoring the development of pathogenic Th17 cells (9). RPM is a potent antiinflammatory agent (32), and as well an inducer of TGF-β directly and/or indirectly through causing apoptosis (10,33). Although RPM effect is TGF-β dependent (Figure 1C), TGF-β might not be the sole factor responsible for RPM-induced conversion. During *in vitro* MLR with allogeneic splenocytes, RPM and TGF-β showed a synergistic effect in inducing T_{reg} cells. The effect by RPM cannot be supplemented by increasing TGF-β dose (Supplementary Figure 4). Suppression of cell cycle progression by RPM may also favor T_{reg} conversion, as T_{eff} cells undergoing extensive proliferation failed to induce Foxp3 ((8); Figure 3). This inverse correlation between proliferation and conversion is also supported by our findings that antigen presenting cells (APCs) possessing weak costimulatory activity promote better conversion of naïve T cells into the T_{reg} phenotype than APCs with potent costimulatory properties, and co-stimulation blockade has an added beneficial effect (Zhong et al., submitted for publication).

In summary, RPM and CsA differentially affect both T-cell death and T-cell regulation. RPM induces *de novo* generation of biologically active T_{reg} cells that mediate graft protection selectively for stimulating cells/tissues. Therefore, RPM but not CsA should be included in tolerance-inducing protocols, in order to recruit not only natural but also induced $Foxp3^+$ cells into the overall T_{reg} pool. How much natural and induced T_{reg} cells contribute relatively to transplant tolerance warrants further study, especially with the use of

genetically modified mouse lines that enable specific depletion of one or the other population at ease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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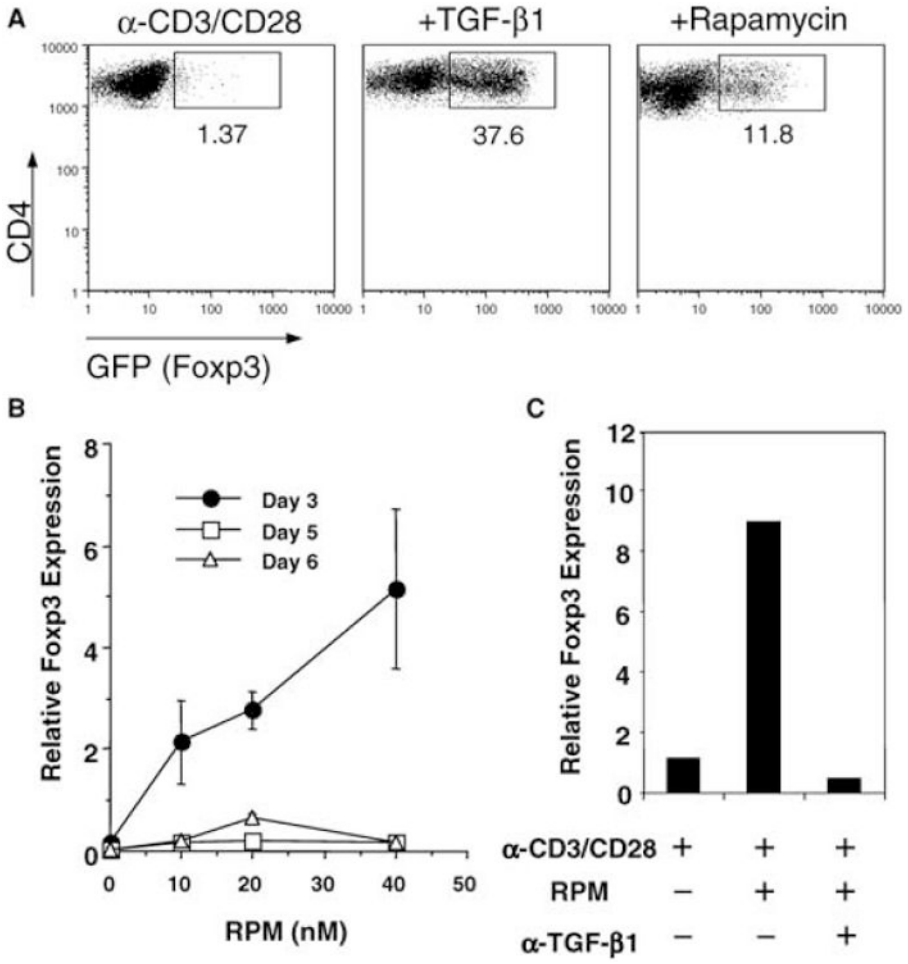

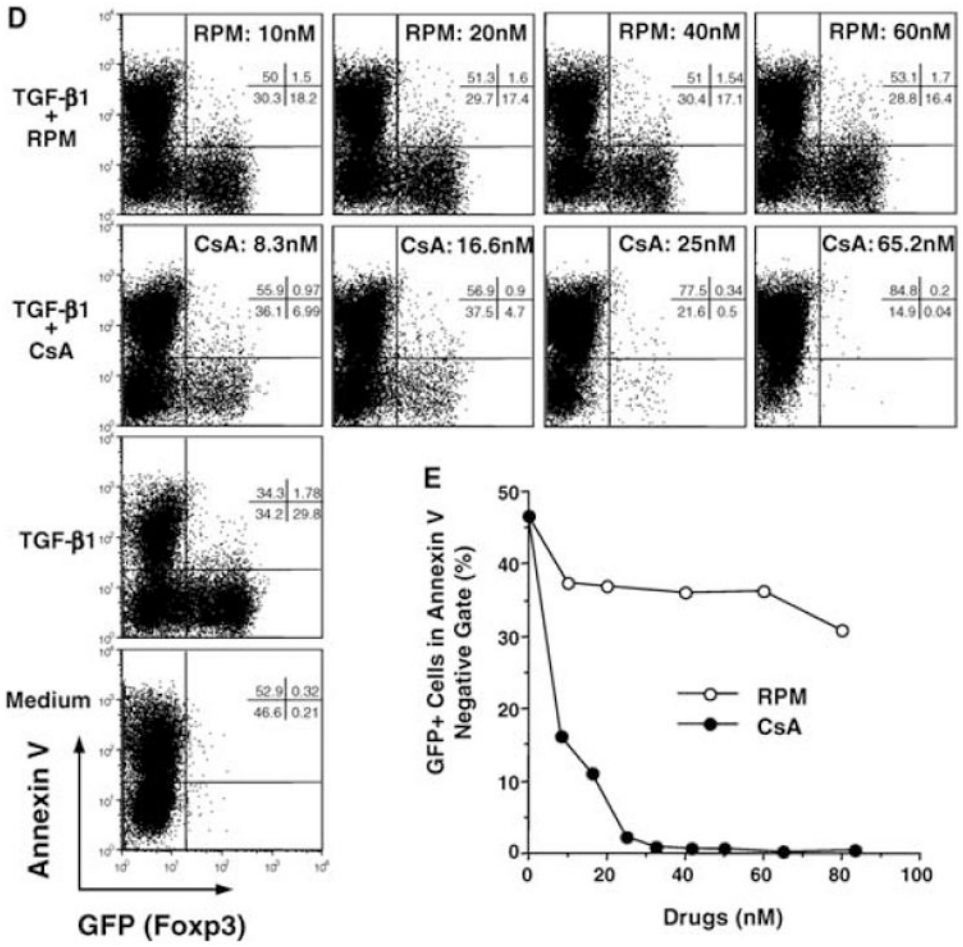

Figure 1. RPM but not CsA induces Foxp3 expression in CD4$^+$Foxp3$^-$ T cells*in vitro*

(**A**) FACS-sorted CD4$^+$Foxp3$^-$(GFP$^-$) cells from naïve Foxp3GFP knock-in mice were stimulated with plate-bound anti-CD3 and anti-CD28 alone or in the presence of TGF-β1 (1 ng/mL) or RPM (20 nM) for 3 days. Foxp3 induction was monitored by the GFP signal by FACS. The percentages of the Foxp3$^+$(GFP$^+$) cells within the total CD4$^+$ T-cell population are indicated. (**B**) Kinetics of Foxp3 induction by different doses of RPM. Error bars represent two measurements of Foxp3 message (relative to that of GAPDH) by real-time PCR in cells stimulated *in vitro* as above. (**C**) Anti-TGF-β1 blocked the induction of Foxp3 message by RPM. The neutralizing antibody (20 μg/mL) was added at the beginning of the 3-day culture. (**D**) Differential effects of RPM and CsA on TGF-β induction of Foxp3$^+$(GFP$^+$) cells. FACS-sorted CD4$^+$Foxp3$^-$(GFP$^-$) cells were stimulated as in (A) in the presence of TGF-β1 (1 ng/mL) with increasing doses of RPM or CsA. After 3 days, total cells were gated for Annexin V staining. The percentages of GFP$^+$ cells in Annexin V negative population were plotted against the drug doses in (**E**). Data represent three independent experiments.

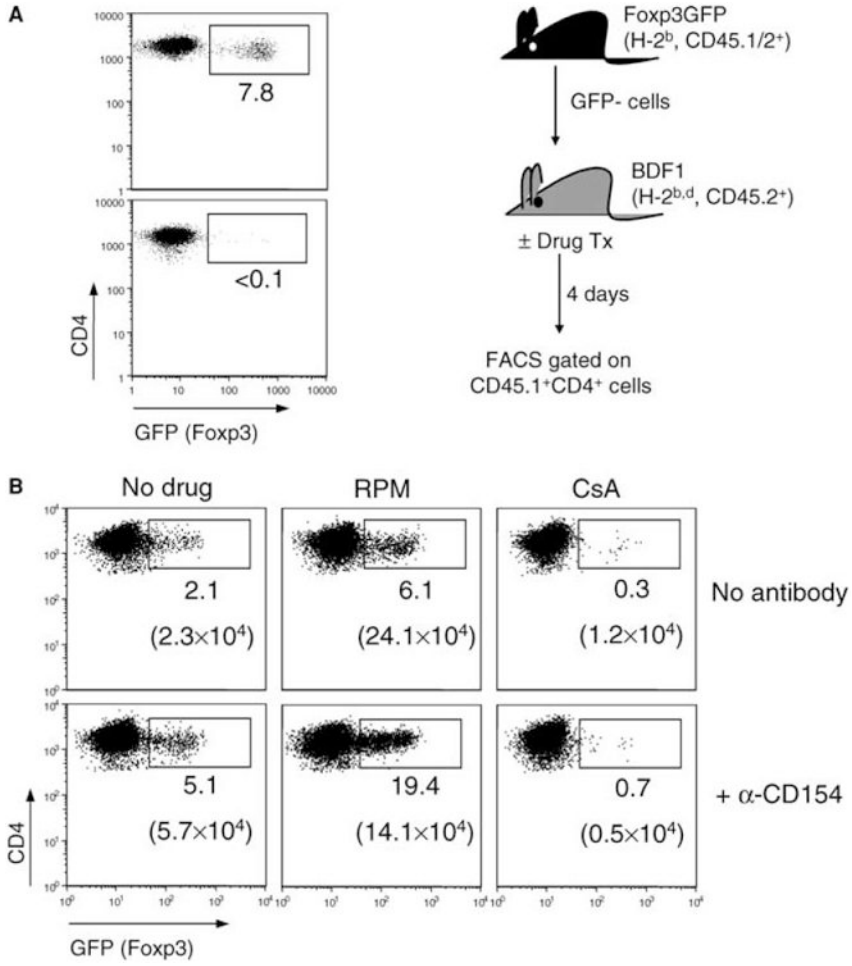

Figure 2. The influence of RPM and CsA upon alloantigen-driven extrathymic *de novo* generation of Foxp3$^+$ cells

(**A**) Naïve CD4$^+$GFP$^-$ cells from Foxp3GFP mice (H-2^b, CD45.1$^+$CD45.2$^+$) were enriched by FACS sorting. The pre- (*upper*) and post-sort (*lower*) FACS plots, and the scheme of adoptive cell transfer are shown. BDF1 hosts (H-2^{b,d}, CD45.2$^+$) were injected (i.v.) with 10 million of sorted C57BL/6 CD4$^+$GFP$^-$ cells, and treated on days 0, 1, 2 with HBSS, RPM (3 mg/kg, i.p.), CsA (20 mg/kg, s.c.), alone or together with anti-CD154 (MR1, 0.25 mg, i.p.). (**B**) C57BL/6 CD4$^+$ T cells residing in the spleens of BDF1 hosts were analyzed on day 4 via FACS gating on the CD45.1$^+$ population. The percentages of induced Foxp3$^+$(GFP$^+$) cells within the gated C57BL/6 CD4$^+$ T cells are indicated. Note that induced T_{reg} cells (RPM and/or anti-CD154 treatment in B) express lower levels of GFP than natural T_{reg} cells (upper panel in A). The absolute numbers of GFP$^+$ cells recovered from the spleens of animals under different treatments were presented in parenthesis. See Supplementary Tables 1a and b for detailed calculations. Data are representative of the results obtained in 10 different experiments.

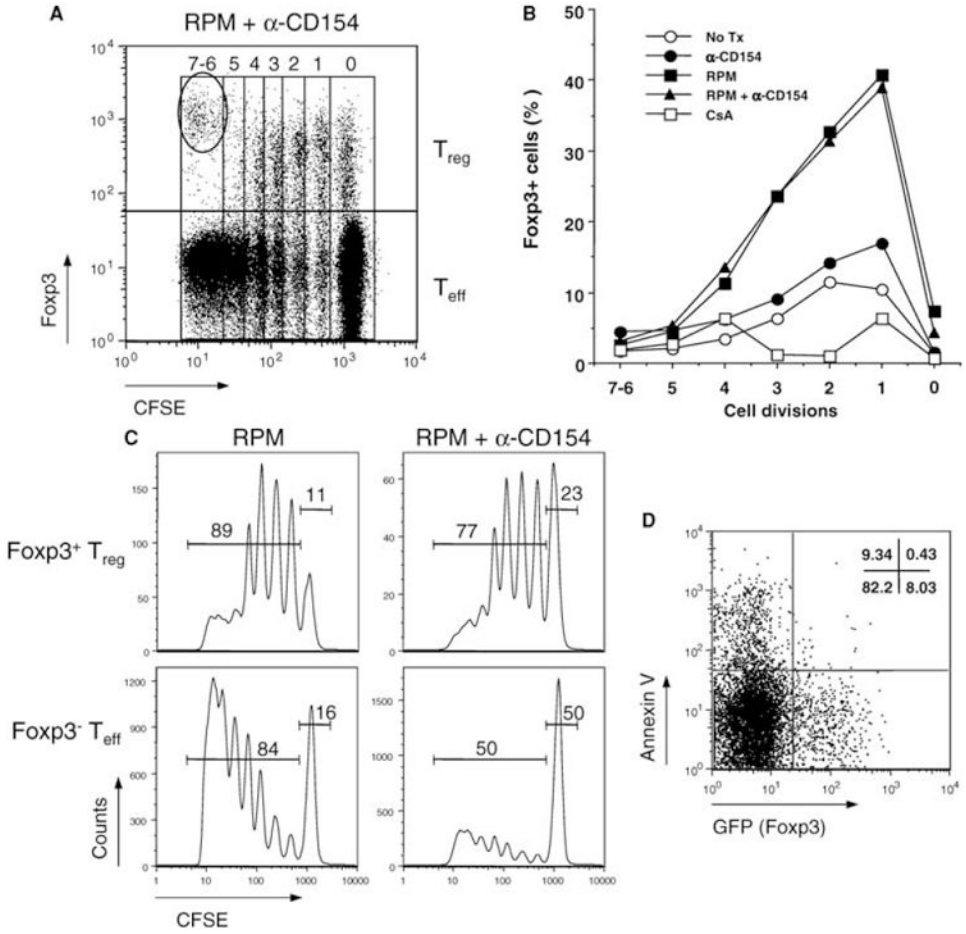

Figure 3. Differences between T_{eff} cells and *de novo* generated T_{reg} cells in cell cycle progression and susceptibility to apoptosis

(**A**) BDF1 hosts injected with CFSE-labeled $CD4^+CD25^-$ cells from naïve C57BL/6 mice were treated with RPM+anti-CD154 to induce *de novo* conversion of T_{reg} cells as described in Methods. Lymph node cells from BDF1 hosts were stained with anti-Foxp3 and the gated H-2D(d)$^-$CD4$^+$ (C57BL/6) fraction was analyzed by FACS on day 4. The oval gate indicates the small contaminating natural T_{reg} population within the starting $CD4^+CD25^-$ pool, which has higher Foxp3 expression than *de novo* generated T_{reg} cells. (**B**) $Foxp3^+$ cells were induced in the early cell divisions. The percentages of C57BL/6 $Foxp3^+$ cells induced 4 days after adoptive transfer into BDF1 hosts and subsequent drug treatment were plotted as a function of the number of cell divisions. (**C**) Anti-CD154, in conjunction with RPM, preferentially inhibits the proliferation of $Foxp3^-$ T_{eff} cells. Day 4 CFSE dilution profiles of H-2D(d)$^-$CD4$^+$ T_{eff} cells ($Foxp3^-$ gating in A) and T_{reg} cells ($Foxp3^+$ gating in A) with frequencies of divided and nondivided cells are shown. (**D**) The converted GFP^+ T_{reg} cells, but not GFP^- T_{eff} cells, are more resistant to apoptosis as demonstrated by Annexin V negative staining. T_{reg} cells were *de novo* generated from GFP^- naïve T cells of the knock-in mice upon adoptive transfer into BDF1 hosts and subsequent RPM+anti-CD154 treatment. Total H-2D(d)$^-$CD4$^+$ T cells were gated for Annexin V staining. Data are representative of three different experiments.

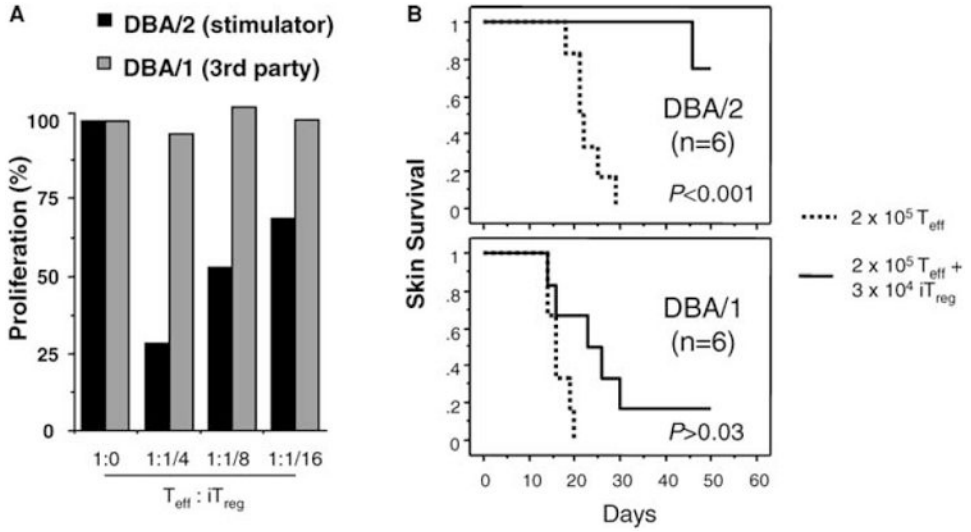

Figure 4. *De novo* generated T_{reg} cells exert alloantigen-specific suppression and donor-selective graft protection

(**A**) *In vitro* MLR. Naïve $CD4^+GFP^-$ T_{eff} cells from the knock-in mice (H-2^b) were stimulated with Mitomycin C-treated DBA/1 (H-2^q) or DBA/2 (H-2^d) splenocytes for 4 days. $CD4^+GFP^+$ induced T_{reg} (iT_{reg}) cells were FACS-sorted and added at varying ratios to T_{eff} cells in the MLR. T-cell proliferation in these cultures, as measured by the mean values of incorporated thymidine of triplicate wells, is compared to that of MLR cultures with T_{eff} alone (normalized as 100%). (**B**) Protection of donor but not third party skin grafts by iT_{reg} cells. C57BL/6 RAG-1-deficient mice were simultaneously transplanted on the opposite sides of the flank with allogeneic tail skin grafts from DBA/1 and DBA/2 mice. $CD4^+GFP^-$ T_{eff} cells (2×10^5) alone (dotted line) or together with $CD4^+GFP^+$ iT_{reg} cells (3×10^4) (solid line) were then transferred by tail vein injection. $P<0.01$ in Kaplan-Meier survival analysis is considered statistically significant.

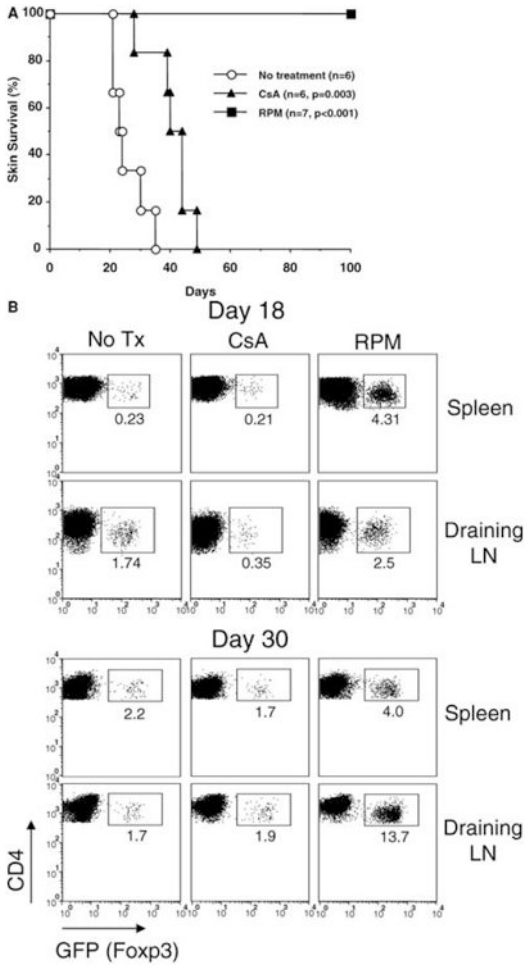

Figure 5. RPM induces long-term skin allograft survival with concomitant *de novo* generation of Foxp3$^+$ cells

(**A**) RPM, but not CsA, induces long-term skin graft survival in an adoptive transfer model. FACS-sorted CD4$^+$GFP$^-$ T cells (2×10^5, from naïve knock-in mice) were transferred into C57BL/6 RAG-1-deficient mice receiving allogeneic tail skin grafts from DBA/2 donor. Mice were either not treated, or treated with RPM (3 mg/kg, i.p., daily for the first 3 days and then every other day for 11 days) or CsA (20 mg/kg, s.c, daily for 14 days). (**B**) Cells from spleens and graft-draining lymph nodes were examined for GFP expression by FACS on day 18 and 30. The percentages of GFP$^+$ cells among transferred CD4$^+$ T cells were indicated. Data represent the mean values of two individual mice from each group.