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Cutting Edge: Allogeneic CD4⁺CD25⁺Foxp3⁺ T Regulatory Cells Suppress Autoimmunity while Establishing Transplantation Tolerance

Dennis Adeegbe,* Allison L. Bayer,*† Robert B. Levy,* and Thomas R. Malek2*†

An important unresolved question with regard to T regulatory (Treg) cell specificity and suppressive activity is whether allogeneic Treg cells inhibit self-reactive T cells. In the present study, this issue was addressed using IL-2Rα-deficient mice that develop rapid lethal autoimmunity due to impaired production of Treg cells. We show that adoptive transfer of completely MHC-mismatched Treg cells into IL-2Rα⁻/⁻ mice resulted in life-long engrafment of the donor cells, which exhibited skewed reactivity toward host alloantigens, and prevented autoimmunity. Thus, Treg cells that underwent thymic selection by peptide/MHC class II complexes distinct from those recognized by autoreactive T cells, still effectively suppress autoimmunity. Remarkably, when such animals were skin grafted, they exhibited dominant tolerance to those grafts bearing MHC molecules that were shared with donor Treg cells. Collectively, these data demonstrate that effective engrafment by allogeneic Treg cells controls autoimmunity and results in permissive conditions for long-term acceptance of allografts. The Journal of Immunology, 2006, 176: 7149–7153.

Natural CD4⁺CD25⁺Foxp3⁺ T regulatory (Treg) cells control autoreactive T cells that escape thymic deletion contributing to peripheral self-tolerance (1–3). These cells suppress graft-vs-host disease (4–7) and mediate tolerance to allografts (8–13). Adoptive transfer of Treg cells promises novel therapies to alleviate autoimmune diseases and other unwanted immune responses. One difficulty is that Treg cells are relatively difficult to grow, although there has been some progress (14–18). Another issue concerns the specificity of Treg cells. The TCRs of both Treg cells and autoaggressive T cells are selected based on reactivity toward self-peptides associated with the same MHC-restricting elements. Recent work indicates that Treg cells specific for a self-Ag are more effective than polyclonal-activated Treg cells in suppressing autoimmune diabetes in NOD mice (19). Nevertheless, there does not appear to be a strict requirement for Treg cells to recognize the same autoantigen targeted by autoreactive T cells (4, 20). Therefore, we hypothesized that allogeneic Treg cells are competent to suppress self-reactive T cells. The ability to use mismatched Treg cells is an important practical issue because the pool of donors for adoptive Treg cell immunotherapy becomes much larger for eventual clinical application. The present study was undertaken to examine whether MHC disparate CD4⁺CD25⁺ Treg cells could inhibit severe systemic autoimmunity. We show that small numbers of adoptively transferred allogeneic Treg cells controlled lethal autoimmunity while establishing transplantation tolerance to donor alloantigens.

Materials and Methods

Mouse studies

C57BL/6, BALB/cBy, and C3H/HeJ mice were obtained from The Jackson Laboratory. C57BL/6 mice congenic for CD45 and expressing the CD45.1 allele (B6.SJL-Ptprc/BoAiTac) were obtained from Taconic Farms. C57BL/6 IL-2Rα⁻/⁻ (B6.129P2-IL2rbtm1Mak/J) mice, backcrossed 12 generations to C57BL/6 mice, have been described previously (21, 22). These mice were backcrossed to BALB/cBy mice for five generations to generate BALB/c IL-2Rα⁻/⁻ mice. Homozygous IL-2Rα⁻/⁻ mice were maintained in our animal colony as described previously (21).

CD4⁺CD25⁺ T cells (2 × 10⁵ in 50 µl) were adoptively transferred iv. into a superficial facial vein of 1- to 2-day-old IL-2Rα⁻/⁻ mice. Skin grafting was performed as a modification of the technique used by Billingham and Medawar (23). Anesthetized mice received full thickness donor tail skin on separate graft beds on the tail of 8- to 10-wk-old sex-matched recipient mice. Protective glass tubing was placed over the length of the tail bearing the grafts for 7 days. Grafts were monitored every other day and scored as rejected when >75% or more of the original graft tissue had been lost or become necrotic as assessed by visual examination.

Abs and FACS analysis

mAbs to CD4, CD8, CD45.1, CD62L, CD44, and CD25 were prepared in our laboratory. Anti-Foxp3 (FJK16s) was obtained from eBioscience and was used according to the manufacturer’s instructions. All other Abs and reagents for flow cytometric analysis were purchased from BD Pharmingen. FACS analysis was performed as described previously (24) using a BD Biosciences LSR1 and CellQuest software. Typically, 30,000–50,000 events were collected per sample.

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3 Abbreviations used in this paper: Treg, T regulatory; WT, wild type; LN, lymph node.
Cell purification and Treg cell suppressor assay

CD4⁺CD25⁺ T cells were purified from normal or adoptive transferred mice as described previously (21). To obtain CD4⁺CD25⁺ T cells, the negative fraction from the CD25⁺ purification was incubated with anti-CD4 magnetic beads followed by positive selection on MS MACS separation columns (Miltenyi Biotec). APCs were T cell-depleted mitomycin C-treated spleen cells from normal mice (21). CD4⁺CD25⁺ responder cells (5 × 10⁵/well) and APC (5 × 10⁶/well) were cultured with the indicated number of Treg cells in 96-well, round-bottom microtiter wells in the presence or absence of anti-CD3 (1 μg/ml) for 72 or 96 h, respectively, the latter for the MLR. [3H]Thymidine (25 Ci/mmol; Amersham) was added during the last 6 h of culture.

Results and Discussion

Allogeneic Treg cells engraft IL-2Rβ⁻/⁻ mice

IL-2Rβ⁻/⁻ mice develop rapid systemic autoimmune disease accompanied by a lymphoproliferative disorder due to failed production of an effective population of Treg cells such that the mice die between 4 and 12 wk of age (21, 22). We previously reported that the adoptive transfer of 1–2 × 10⁶ wild-type (WT) syngeneic CD4⁺CD25⁺ Treg cells into neonatal IL-2Rβ⁻/⁻ led to life-long engraftment of the donor Treg cells and prevented this lethal disease (21). This system was used to test the efficacy of fully allogeneic Treg cells by transfer of BALB/c (H2b) or C57BL/6 (H2b) Treg cells into neonatal C57BL/6 or BALB/c IL-2Rβ⁻/⁻ mice, respectively. From 2.4–3.6% of the cells in the lymph nodes (LN) (Fig. 1A) and spleen (data not shown) were donor-derived T cells, the majority of which were CD4⁺ T cells (Fig. 1A). This level of donor CD4⁺ T cell engraftment was first noted at 1 wk after transfer within LNs but not until 3–4 wk posttransfer within the spleen, after which engraftment remained at this level for >1 year (Fig. 1B). Notably, the majority of the engrafted donor CD4⁺ T cells coexpressed CD25 and Foxp3 (Fig. 1, A and C) such that the donor Treg cells were at a level comparable to that present in WT mice. We calculated that this level of donor engraftment tains the number of allogeneic Treg cells at 2–4 × 10⁶ cells in peripheral lymphoid tissue in IL-2Rβ⁻/⁻ recipient mice. This result indicates that there was considerable expansion of the initial donor inoculum (2 × 10⁵ cells), which has also been reported when syngeneic Treg cells were used as the donor cells (24), such that essentially all CD4⁺ T cells that express CD25 or Foxp3 in these recipients were of donor origin (Fig. 1C). Thus, even though IL-2Rβ⁻/⁻ mice contain a few CD4⁺ Foxp3⁺ CD25⁻ T cells (25), these cells do not favorably compete with the transferred WT CD4⁺ CD25⁺ Foxp3⁺ Treg cells.

Key symptoms of autoimmunity in untreated IL-2Rβ⁻/⁻ mice include autoimmune hemolytic anemia and unchecked proliferation of peripheral lymphocytes that exhibit an activated phenotype (21, 22). Examination of these parameters in IL-2Rβ⁻/⁻ mice that received fully allogeneic Treg cells indicate that virtually all recipients remained autoimmune-free because cellularity of the LNs (Fig. 2A) and hematocrits (Fig. 2B) were in the normal range, and peripheral lymphocytes were not activated as judged by the low percentage of CD4⁺ and CD8⁺ (data not shown) LN T cells that expressed the activation marker CD69 (Fig. 2C) or were CD62Llow or CD44high (data not shown). Importantly, these mice lived a symptom-free and normal life span. In more limited experiments, similar results were seen when C3H (H2b) Treg cells were transferred into BALB/c IL-2Rβ⁻/⁻ recipient mice (data not shown). Collectively, these data indicate that allogeneic Treg cells efficiently engraft, normally repopulate, and persist in IL-2Rβ-deficient mice while effectively controlling lethal autoimmunity. Because the number of host Foxp3⁺ cells remained low (Fig. 1C), it is unlikely that these cells contributed to suppression of disease.

FIGURE 1. Donor cell engraftment in IL-2Rβ⁻/⁻ mice. Purified CD4⁺CD25⁺ Treg cells were adoptively transferred into neonatal IL-2Rβ⁻/⁻ mice and denoted as the donor Treg strain—recipient IL-2Rβ⁻/⁻ strain. Multicolor FACS analysis was performed to establish the phenotype of donor and host T cells. A, Representative FACS plots 8 wk posttransfer (left) and summary 8–60 wk posttransfer (right) of donor cells in LNs. B, Time course of donor CD4⁺ cell engraftment in the spleen and LNs based on FACS analysis. C, Representative FACS dot plots (left) and summary (right) of host vs donor CD4⁺ CD25⁺ T cells or CD4⁺ Foxp3⁺ T cells in the LNs of the indicated mice. Data represent the mean ± SE for 10–15 mice/group, except for Foxp3 staining and the time course (n = 2–4).
Although the input donor Treg cells were always $> 90\%$, and often $> 95\%$, CD4$^+$ CD25$^+$ T cells, we found donor-derived CD8$^+$ T cells in IL-2R$^{-/-}$ recipient mice (Fig. 1A). Their proportion ranged from 10 to 20% of all donor cells in young (<12 wk of age) recipients to somewhat as abundant as 40% in older (>16 wk of age) mice. Although others have claimed that CD8$^+$ T cells suppress autoimmunity in IL-2R$^{-/-}$ mice (26), it does not appear that these donor CD8$^+$ T cells are required for suppression because CD4$^+$ CD25$^+$ T cells isolated from C57BL/6 CD8-deficient mice effectively prevented autoimmunity after transfer into BALB/c IL-2R$^{-/-}$ recipients (Fig. 2). In addition, syngeneic or allogeneic WT CD8$^+$ T cells did not prevent autoimmunity after adoptive transfer into neonatal IL-2R$^{-/-}$ mice (data not shown). Thus, allogeneic CD4$^+$ CD25$^+$ Foxp3$^+$ T cells are sufficient to effectively control autoimmune disease in IL-2R$^{-/-}$ recipients without a contribution by CD8$^+$ T cells.

Allogeneic Treg cells are primed to host alloantigens

Prevention of autoimmunity by allogeneic Treg cells raised the possibility that the TCR repertoire of these cells was skewed toward host MHC Ags. This point was evaluated by testing the suppressive activity of the donor Treg cells in the presence of various populations of APCs in vitro. When compared with BALB/c Treg cells from normal mice, BALB/c Treg cells isolated from adoptively transferred C57BL/6 IL-2R$^{-/-}$ mice were approximately four times more effective on a per cell basis in suppressing proliferation by normal BALB/c CD4$^+$ CD25$^-$ T cells to C57BL/6 APCs in the MLR (Fig. 3A). This level of inhibition was comparable to that seen when both populations of Treg cells were used to suppress polyclonal anti-CD3-stimulated T cell proliferation using the same population of responding CD4$^+$ T cells (Fig. 3B). Furthermore, BALB/c Treg cells from C57BL/6 IL-2R$^{-/-}$ mice minimally suppressed the MLR by BALB/c CD4$^+$ CD25$^+$ T cells to C3H APC (Fig. 3C), while effectively inhibiting anti-CD3-induced proliferation (Fig. 3B). Taken together, these data indicate that the TCR repertoire of donor Treg cells are largely skewed to react against host MHC alloantigens, yet this has not compromised their capacity to suppress autoimmune disease.

Allogeneic Treg cells lead to tolerance to skin grafts

We also investigated whether the successful engraftment of allogeneic donor Treg cells in the now autoimmune-free IL-2R$^{-/-}$ mice led to tolerance of skin grafts bearing MHC molecules identical with the donor Treg cells. Three contiguous skin grafts from C57BL/6, BALB/c, and C3H mice were applied to the tail of normal or BALB/c IL-2R$^{-/-}$ mice that previously received either syngeneic or allogeneic Treg cells. Syngeneic skin survived indefinitely in all groups, whereas allogeneic grafts were rejected by normal control as well as BALB/c IL-2R$^{-/-}$ mice that received syngeneic Treg cells (Fig. 4, A and B). The somewhat slower rejection time by IL-2R$^{-/-}$ mice is likely the result of host T cells that cannot

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Interestingly, when C57BL/6 Treg cells rejecting third party C3H skin grafts (data not shown). Inter- indicated population of Treg cells was adoptively transferred into neonatal BALB/c IL-2R receptors served as a positive control for graft rejection. A, Tolerance to B6 skin. B, Tolerance to B6 and (B6 × C3H)F1 skin. C, B6→BALB/c IL-2Rβ−/− mice previously grafted with BALB/c, B6, and C3H skin and judged to be tolerant to B6 skin were challenged with (B6 × C3H)F1 skin 90 days after the first grafts. The number of mice per group and the mean survival time of allogeneic skin grafts are listed within each panel of the figure.

FIGURE 4. IL-2Rβ−/− mice adoptively transferred with allogeneic Treg cells are tolerant to skin grafts bearing the MHC molecules of the donor Treg cells. The indicated population of Treg cells was adoptively transferred into neonatal BALB/c IL-2Rβ−/− mice and denoted as the donor Treg strain→BALB/c−/−. WT IL-2Rβ−/− BALB/c (BALB/c−/−) recipients served as a positive control for graft rejection. A, Tolerance to B6 skin. B, Tolerance to B6 and (B6 × C3H)F1 skin. C, B6→BALB/c IL-2Rβ−/− mice previously grafted with BALB/c, B6, and C3H skin and judged to be tolerant to B6 skin were challenged with (B6 × C3H)F1 skin 90 days after the first grafts. The number of mice per group and the mean survival time of allogeneic skin grafts are listed within each panel of the figure.

respond to IL-2 and IL-15, which somewhat impairs the effector response. Importantly, >85% of C57BL/6 Treg cells→BALB/c IL-2Rβ−/− mice retained C57BL/6 skin grafts (Fig. 4A) for >90 days while rejecting “third party” grafts from C3H mice. Similar results were observed when allogeneic donor C57BL/6 Treg cells were derived from CD8-deficient mice (Fig. 4A). Similarly, 75% of BALB/c Treg cells→C57BL/6 IL-2Rβ−/− mice retained BALB/c skin grafts for >90 days while rejecting third party C3H skin grafts (data not shown). Interestingly, when C57BL/6 Treg cells→BALB/c IL-2Rβ−/− mice received grafts from (C57BL/6 × C3H)F1 (Fig. 4B) rather than C3H donors, or mice that were identified as tolerant to C57BL/6 skin were then grafted with (C57BL/6 × C3H)F1 skin (Fig. 4C), both groups of mice were tolerant toward F1 skin, which was more striking in the serial grafted animals. Thus, the presence of allogeneic Treg cells in IL-2Rβ−/− mice not only prevented autoimmune disease but established dominant transplantation tolerance toward MHC Ags in which the Treg cells were initially selected.

This study illustrates several important points concerning the potential application of allogeneic Treg cells in adoptive immunotherapy. First, Treg cells effectively suppress autoreactive T cells even when derived from a MHC-disparate donor. Thus, at least theoretically, the pool of donors for Treg cell immunotherapy is large. Second, the efficacy of allogeneic Treg cells further supports the notion that operationally Treg cell-suppressive activity is nonspecific with respect to autoreactive T cells. The allogeneic donor Treg cells were initially selected and maintained by recognition of peptides in the context of MHC class II molecules distinct from that of the recipient before the adoptive transfer into IL-2Rβ−/− recipients. Thus, this control of autoimmunity is most likely accounted for by cross-reactive recognition of self-peptides and/or MHC molecules of the host by the TCR of allogeneic Treg cells. Lastly, the successful long- term engraftment of allogeneic Treg cells led to transplantation tolerance toward grafts bearing the MHC of the donor Treg cells, even those grafts that coexpressed unrelated MHC molecules. This dominant suppression has been seen by others (9, 27, 28) and suggests that tolerance is, at least in part, directly mediated by suppressive activity of donor Treg cells. Several other features of this system, i.e., an attenuated effector cell response due to IL-2Rβ deficiency of the host responding T cells and preconditioning of the recipient toward potential graft Ags through chimerism of the allogeneic donor Treg cells, may also contribute to skin graft tolerance. In any case, these findings provide proof of principle that tolerance of organ transplants is possible by establishing stable engraftment of Treg cells that are highly reactive toward Ags expressed by cells of the transplanted tissue. Lastly, we speculate that two relevant features that promote the therapeutic effectiveness of donor Treg cells in IL-2Rβ-deficient mice are the lack of host Treg cells and adoptive transfer at a time when the host T cell compartment was just developing and likely contains relatively few autoreactive or host antidonor T cells. Thus, application of this approach in normal adults may require reduction of host Treg cells to facilitate engraftment of donor cells and using sufficient number of autoantigen- or alloantigen-reactive Treg cells.

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