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Generation of Adaptive Regulatory T Cells by Alloantigen Is Required for Some But Not All Transplant Tolerance Protocols

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Abstract

Background—Because CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are essential for the maintenance of self-tolerance, significant interest surrounds the developmental cues for thymic-derived natural Tregs (nTregs) and periphery-generated adaptive Tregs (aTregs). In the transplant setting, the allograft may play a role in the generation of alloantigen-specific Tregs, but this role remains undefined. We examined whether the immune response to a transplant allograft results in the peripheral generation of aTregs.

Methods—To identify generation of aTregs, purified graft-reactive CD4⁺CD25[−] T cells were adoptively transferred to mice-bearing skin allograft. To demonstrate that aTregs are necessary for tolerance, DBA/2 skin was transplanted onto C57BL/6-RAG-1-deficient recipients adoptively transferred with purified sorted CD4⁺CD25[−] T cells; half of the recipients undergo tolerance induction treatment.

Results—By tracking adoptively transferred cells, we show that purified graft-reactive CD4⁺CD25[−] T lymphocytes up-regulate Foxp3 in mice receiving skin allografts in the absence of any treatment. Interestingly, cotransfer of antigen-specific nTregs suppresses the up-regulation of Foxp3 by inhibiting the proliferation of allograft-responsive T cells. In vitro data are consistent with our in vivo data—Foxp3⁺ cells are generated on antigen activation, and this generation is suppressed on coculture with antigen-specific nTregs. Finally, blocking aTreg generation in grafted, rapamycin-treated mice disrupts alloantigen-specific tolerance induction. In contrast, blocking aTreg generation in grafted mice treated with nondepleting anti-CD4 plus anti-CD40L antibodies does not disrupt graft tolerance.

Conclusions—We conclude that graft alloantigen stimulates the de novo generation of aTregs, and this generation may represent a necessary step in some but not all protocols of tolerance induction.

Keywords

Treg; Adaptive Tregs; Tolerance

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Regulatory T cells (Tregs) are critical for the maintenance of self-tolerance (1). The forkhead/winged helix transcription factor Foxp3 is the “master switch” for the development and function of CD4⁺CD25⁺ Treg cells and is the best marker of this T-cell subset (2–4). The importance of Foxp3 and Treg lineage is illustrated in mice and humans with a genetic deficiency in Foxp3, which results in severe autoimmune disease, T-cell hyperproliferation, and premature death (5, 6). Tregs represent only 5% to 10% of CD4⁺ T cells (7) yet clearly exert a dominant suppression in vivo (8, 9).

Two subsets of Tregs have been classified based on their site of development. Natural Tregs emerge from the thymus (10, 11), whereas adaptive Tregs (aTregs) or induced Tregs are generated in the periphery under certain conditions (10, 12–16). The aTregs have also been generated in vitro; for example, stimulation of CD4⁺CD25[−]Foxp3[−] T cells through the T-cell receptor (TCR) in the presence of transforming growth factor (TGF)-β results in Foxp3 up-regulation (17–19). In vivo it remains difficult to distinguish peripheral generation of Tregs versus thymic generation of Tregs, because there is no established marker that differentiates them (20).

Evidence for CD4⁺ T-cell-mediated immunoregulation in transplantation has been noted for more than 20 years (21–25), yet the direct contribution of periphery-generated Tregs to transplant tolerance is unknown. In this study, we demonstrate that a small fraction of alloactivated CD4⁺ T cells converts into aTregs and that the presence of existing alloantigen-specific Tregs inhibits this conversion, suggesting a critical interplay between natural Tregs (nTregs) and aTregs in peripheral self-tolerance. Furthermore, we demonstrate that aTreg generation represents a necessary step for tolerance induction using the immunosuppressant rapamycin but not by treatment with nondepleting anti-CD4 antibody.

RESULTS

CD4⁺CD25[−] T Cells Up-Regulate Foxp3 in Response to Allograft

The TS1 transgenic mouse expresses the TS1 TCR receptor specific for peptides 107 to 119 from influenza hemagglutinin (HA). TS1 TCR⁺ T cells can be identified with the clonotypic 6.5 antibody. When this mouse is mated to the HA28 mouse that expresses HA ubiquitously, the TS1×HA28 mouse develops roughly a 1:1 mixture of regulatory Foxp3⁺ and nonregulatory Foxp3[−] HA-reactive CD4⁺ T cells (26). We have previously shown that CD4⁺CD25[−] conventional T (Tconv) cells from TS1 mice will reject HA⁺ skin grafts within 23 days in an adoptive transfer model (27, 28) (Fig. 1A). Cotransfer of HA-reactive CD4⁺CD25⁺ Tregs results in long-term graft survival in part because Tregs prevent the proliferation of responding CD4⁺ T cells.

On in vivo encounter with administered cognate antigen, CD4⁺CD25[−] T cells may convert into aTregs (29). We asked whether aTregs are generated in response to transplanted alloantigen in vivo. HA⁺ skin grafts are transplanted onto otherwise syngeneic immunocompetent BALB/c mice and do not generate an allograft response on this host. Purified, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4⁺CD25[−] T cells from TS1 mice were adoptively transferred to the grafted mouse 30 days after the skin graft procedure to allow the graft to heal. Although these transferred cells will eventually reject the HA⁺ skin graft, a small percentage of these cells become Foxp3⁺ in response to the graft alloantigen (16.4±5.2-fold increase; *P* < 0.03; *n* = 4; Fig. 1B, center). We also observe this conversion when HA⁺ islets or HA⁺ heart graft is used instead of skin grafts (data not shown). These data demonstrate that some Tconv up-regulate Foxp3 on activation in response to allograft.

Others have demonstrated that preexisting tolerogenic Tregs may provide the mechanism of “infectious” tolerance by facilitating the conversion of Tconv to Foxp3⁺ aTregs (24, 30). To determine whether the presence of antigen-specific Tregs would improve the efficiency of generation of aTregs, Tregs were purified from TS1×HA28 mice and cotransferred with naive Tconv cells. Surprisingly, cotransfer of Tregs did not facilitate generation of aTregs (Fig. 1B, right and graph). In fact, natural Tregs suppressed conversion of the responding 6.5+ CD4⁺ Tconv population by suppressing their proliferation that is necessary for conversion to take place (27, 30). In the presence of nTregs, nearly the same percentage of Tconv remains undivided compared with culture in the absence of nTregs—more than 80% of Tconv remain undivided. Although some Tconv divide and up-regulate in the presence of nTregs, conversion is significantly suppressed relative to culture with peptide alone (3.3±1.0-fold, in presence of nTregs). Similar results were obtained at days 6, 9, 11, and 14 (data not shown).

Antigen-Activated CD4⁺CD25[−] T Cells Up-Regulate Foxp3 in the Absence of Addition of Exogenous TGF-β In Vitro

To confirm the in vivo results, we set up an in vitro culture system in which purified CFSE-labeled CD4⁺CD25[−]Foxp3[−] T cells from TS1 mice were stimulated with HA peptide. Similar to the in vivo conversion of Tconv to aTregs, 6.5+ CD4⁺CD25[−] T cells underwent significant proliferation and conversion in vitro when cultured with the HA peptide at 10 μM (Fig. 2A, center and bar graph). We observed Treg conversion at peptide doses as low as 1.5 μM (data not shown). This conversion occurs in the absence of addition of exogenous TGF-β (17). The development of aTregs likely results from endogenous TGF-β production by CD4 T cells or antigen-presenting cells (APCs) (31).

Again, to determine whether the presence of antigen-specific Tregs could facilitate the conversion of CD4⁺CD25[−] T cells into Foxp3⁺ cells, we cocultured CD4⁺CD25⁺ T cells with the CFSE-labeled CD4⁺Foxp3[−] T cells. In the presence of purified antigen-specific Tregs, both proliferation and conversion were suppressed (Fig. 2A, right). These data support the in vivo observation that in the presence of antigen-specific Tregs, conversion of Foxp3[−] T cells to Foxp3⁺ T cells is inhibited.

Preactivation of Tregs has been demonstrated to be necessary to facilitate the conversion of aTregs on coculture (30). We activated our Tregs with anti-CD3 plus anti-CD28 plus interleukin (IL)-2 or with HA peptide plus IL-2 for 5 days in culture and used these activated Tregs in the in vitro conversion assay. Neither preactivation condition changed the outcome of the conversion (data not shown).

We repeated this coculture experiment in the presence of IL-2. IL-2 allows the Tconv to overcome the suppression and thereby proliferate, and we hypothesized that this proliferation would restore aTreg conversion. In the presence of exogenous IL-2 and coculture with Tregs, proliferation was, at least, partially restored, and aTreg conversion occurred at levels comparable with those observed in cultures of Tconv stimulated with HA peptide (Fig. 2B, right).

IL-2 also stabilized levels of Foxp3 in nondividing Tregs (Fig. 2B, left and right), and these cells complicated interpretation of the source of the dividing Foxp3⁺ population (7, 32). To eliminate the possibility that the conversion was the result of contaminating CD4⁺CD25[−]Foxp3[−] cells, we crossed the TS1 mice with Foxp3[−]green fluorescence protein (GFP) mice, which express GFP under the Foxp3 promoter (33). We repeated the in vitro conversion experiment by sorting 6.5+ CD4⁺CD25[−] GFP[−] T cells by flow cytometry and culturing them with or without HA peptide. In the presence of peptide, GFP expression is activated in a

subset of Tconv, which demonstrates that we are observing de novo expression of Foxp3 on activation (Fig. 2C).

aTreg Generation Is Necessary for Rapamycin-Mediated Allograft Tolerance But Not Anti-CD4/Anti-CD40L-Mediated Tolerance

We have demonstrated that in the transplantation setting, aTreg generation occurs in vivo. Next, we addressed whether aTregs are necessary for donor-specific tolerance in a nontransgenic setting. In the mouse, the immunosuppressant rapamycin promotes tolerance, in part, by de novo generation of alloantigen-specific Foxp3⁺ cells (34). To demonstrate that this in vivo generation of aTregs is necessary for allograft tolerance, we adoptively transferred sorted CD4⁺CD25⁻ wild-type (WT) or scurfy T lymphocytes (both on C57BL/6 background) into C57BL/6-RAG-1-deficient mice bearing acutely transplanted DBA/2 skin grafts and then treated half the recipients with rapamycin for 14 days. Scurfy mice are deficient in functional Foxp3 and thus are unable to generate aTregs (1, 9). If generation of aTregs is necessary for transplant tolerance, rapamycin should prolong graft survival with WT cells but not with scurfy cells because of their inability to become Foxp3⁺. WT and scurfy Tconv promptly and consistently rejected DBA/2 skin grafts although rejection by scurfy cells was slightly delayed (median survival time=19 days, n=3 WT cells; MST=30 days, n=8 scurfy cells; Fig. 3A). Rapamycin significantly prolonged skin graft survival in mice with WT transferred T cells, and more than half the grafts survived more than 100 days ($P<0.03^*$; n=5). In contrast, the inability of scurfy cells to become Foxp3⁺ blocked long-term graft survival and graft survival prolongation by rapamycin treatment (MST=34 days, n=5; Fig. 3A and B). These data provide a direct demonstration that aTreg generation is necessary for some protocols of graft tolerance.

To identify whether necessary aTreg conversion can be generalized to other protocols of transplant tolerance, we repeated our experiment using nondepleting anti-CD4 antibody in combination with anti-CD40L (35). The generation of aTregs has been implicated as a mechanism of their tolerance induction for both antibodies, and alone, neither antibody significantly prolongs full major histocompatibility complex (MHC)-mismatched skin graft survival (1, 21, 35). Naive CD4⁺CD25⁻CD44^{lo}CD62L^{hi} cells were sorted by fluorescence-activated cell sorter (FACS) from WT or scurfy mice and adoptively transferred into C57BL/6-RAG-deficient mice. Vehicle-treated WT and scurfy cells reject skin grafts with a similar tempo (median survival: day 20 WT vs. day 22 scurfy). Unexpectedly, grafts from treated recipients receiving WT or scurfy cells survived longer than 100 days (Fig. 3C). These data demonstrate that aTreg conversion is not necessary for anti-CD4/anti-CD40L-mediated graft tolerance, and thus aTreg conversion is necessary for some but not all protocols of tolerance induction.

DISCUSSION

Our data demonstrate that during an immune response, a fraction of graft-reactive cells up-regulates Foxp3. Rapamycin-mediated transplant tolerance correlates with the generation of aTregs, whereas in the absence of aTregs, rejection ensues. Moreover, transplant rejection on depletion of Tregs has also been inferred to demonstrate the necessity of aTregs; however, this is also indirect and cannot distinguish between aTregs and nTregs. By using responder T cells that cannot become Foxp3⁺, we demonstrate that tolerance can exist in the absence of aTregs. We continue to explore other transplant tolerance protocols and their dependence on aTreg generation.

We are careful not to overinterpret the data from an immunodeficient host when extending conclusions to an immunocompetent host. That is, therapy for anti-CD4 and anti-CD154 is a powerful combination of costimulatory blockade. After 1 week of treatment with these

antibodies, one may have eliminated the population of graft-reactive T cells by deletion or anergy, at which point the absence of aTreg conversion is secondary to the absence of adoptively transferred cells to reject the graft. In contrast, in an immunocompetent host, once costimulatory blockade treatment is over, the continual generation of graft-reactive T cells may only be halted by the contribution of aTreg conversion.

Adaptive Tregs are likely generated during many immune responses depending on the quantity and quality of antigenic stimulus, costimulatory molecules, and perhaps most critically the cytokine environment (36, 37). Suppression of Tconv by Tregs is overcome by addition of IL-2, and conversion of Tconv is inhibited by anti-TGF- β antibody. We speculate that both these cytokines are involved in the conversion of Tconv. nTregs suppress proliferation of Tconv by consumption of IL-2. Production of TGF- β by nTregs also suppresses proliferation. In the acute setting, Tconv may encounter proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor- α along with the TGF- β produced by nTregs; this combination would likely inhibit Foxp3 induction while favoring the development of pathogenic Th17 cells (33).

Rapamycin alters the local cytokine environment during an immune response. In addition to its antiinflammatory properties (38), rapamycin directly or indirectly induces TGF- β , which likely is critical for its ability to generate aTregs (39). In the absence of a tolerizing protocol, generation of graft-reactive Tregs in response to an allograft clearly is not sufficient to protect the graft from rejection. Rapamycin not only boosts the generation of Tregs but also maintains their suppressor function by dampening the counter-regulatory effects of inflammation and activation of the innate immune response (40–42).

Cotransfer or coculture of Tregs with naive responder T cells, followed by activation, has been demonstrated to result in induction of Foxp3 expression in responder T cells. This mechanism of “infectious tolerance” is believed to represent how Tregs maintain tolerance and expand their suppressive capacities. Unexpectedly, we find that antigen-specific Tregs prevent up-regulation of Foxp3 in responder T cells both in vitro and in vivo. Differences in experimental design likely account for the difference in results, such as mode of TCR stimulation, use of IL-2 in culture, and use of a different TCR transgenic system.

Parallel experiments were performed in the acute (data not shown) and established settings. When adoptively transferred within 24 hr of skin grafting, at day 14, we find that the 6.5+ Tconv proliferated poorly, and thus, we were unable to assess the effect of cotransfer of natural Tregs. We cannot distinguish whether this is because of the proinflammatory setting or because of the less-abundant accumulation of antigen in the draining lymph node. We have previously demonstrated that the acute setting inactivates the suppressor function of Tregs (27, 41). We hypothesize that in contrast to the established setting, natural Tregs in the inflammatory setting will not be able to suppress the proliferation and differentiation of Tconv.

MATERIALS AND METHODS

Animals

TS1 transgenic mice possess a high frequency of CD4⁺ T cells specific for the immunodominant (site 1) epitope (amino acid sequence, SFERFEIFPK) of the influenza HA protein in the context of MHC class II I-E^d (43). HA104 mice provide a source of HA-expressing grafts as they carry the HA transgene controlled by the SV40 early region promoter/enhancer that results in ubiquitous transgene expression (44). HA28 mice also have HA expression driven by the SV40 promoter and hence have ubiquitous tissue expression. TS1×HA28 mice develop a roughly 1:1 mixture of regulatory Foxp3⁺ and

nonregulatory Foxp3⁻ HA-reactive CD4⁺ T cells (26). TS1, HA28, and HA104 transgenic lines are maintained as hemizygotes backcrossed with BALB/cJ mice (Jackson Labs, Bar Harbor, ME). Foxp3-GFP mice were generously provided by Oukka and coworkers (33). For MHC-mismatched skin graft experiments, WT, scurfy, and RAG-1-deficient mice are on the C57BL/6 background (Jackson). All animals are maintained in a pathogen-free environment under Institutional Animal Care and Use Committee–approved protocols.

Adoptive Transfer Model of Graft Rejection

Thirty days after the skin graft procedure, 4×10^6 CFSE-labeled magnetic bead-sorted CD4⁺CD25⁻ TS1 TCR transgenic T cells were transferred with or without 2.5×10^6 CD4⁺CD25⁺ TS1 TCR transgenic T cells to BALB/c mice-bearing HA skin grafts. Cells were separated using Miltenyi MACS Beads (Miltenyi Biotec GmbH, Germany). Purity of the sorted populations ranged from 95% to 99%.

For rapamycin experiments, C57BL/6-RAG-1-deficient mice were transplanted with skin grafts from DBA/2 donor (Jackson Laboratories). One day later, magnetic bead sorted CD4⁺CD25⁻ T cells (2×10^5 from naive C57BL/6 or scurfy mice) were transferred by tail vein injection into grafted RAG-1-deficient recipients. One group of animals was treated with rapamycin (3 mg/kg intraperitoneally) for 3 consecutive days, then every other day for total 14 days. One group was injected with vehicle.

For anti-CD4/anti-CD40L experiments, cells were sorted by FACS Aria (BD Biosciences; San Jose, CA). Anti-CD4 Pacific Blue, anti-CD25 APC, anti-CD44 fluorescein isothiocyanate, and anti-CD62L (all ebioscience) were used to sort CD4⁺CD25⁻CD44^{low}CD62L^{hi} cells. Mice were treated with 1mg YTS-177 and with 250µg MR1 anti-CD40L (Bio X Cell, NH) on days 0, 2, 4, and 6. The 2×10^5 sorted cells were transferred into each recipient.

CFSE Labeling

Spleen and lymph nodes were harvested and purified, and cells were labeled and prepared as previously described (45). Briefly, single-cell suspensions were prepared by passage of lymph node tissue through a cell strainer (70 µm; Falcon, NJ). Cells were resuspended at a density of 10^7 cells/mL in minimum essential medium (MEM). An equal volume of 5 mM CFSE (Invitrogen, Carlsbad, CA) diluted 1:300 (for in vivo experiments) or 1:1200 (for in vitro experiments) in MEM was added, and cells were cultured at 37°C for 5 min. The reaction was quenched through the addition of an equal volume of fetal calf serum. Labeled cells were washed at least two times with cold MEM containing 5% fetal calf serum.

Flow Cytometric Analysis

Cells from spleen and lymph node were washed in biotin-free Roswell Park Memorial Institute (culture medium) (Irvine Scientific, Santa Ana, CA), and 2 to 4×10^6 cells were stained per sample. The following antibodies were used for analysis: anti-CD4 PE-Cy7 (ebioscience) and anti-Foxp3 APC (ebioscience Foxp3 Staining Kit). In addition, 6.5 biotin followed by Streptavidin-APC-A750 (Invitrogen) secondary were used to detect the transgenic TCR (26, 43). Flow cytometric analysis was performed on a BD Immunocytometry System (San Jose, CA) FACSCalibur or on an LSR II. FACSCalibur data acquisition and analysis were accomplished with Becton Dickinson CellQuest software, whereas LSR II used Diva and FlowJo Software (Tree Star, Stanford, CA).

Skin Grafting

Skin grafts were transplanted to mice according to the technique of Billingham and Medawar (46) as previously described. Grafts were scored as rejected when more than 75% of the grafted tissue area had been lost.

In Vitro Suppression Assays

A total of 4×10^5 CFSE-labeled, purified $CD4^+CD25^-$ T cells from TS1 mice were cultured with 10- μ M HA peptide, 2×10^5 irradiated BALB/c lymph node cells, and in the presence or absence of 2×10^5 Tregs for 4 to 5 days. Cells were grown in complete 10% Roswell Park Memorial Institute (culture medium) 1640 (Sigma, St Louis, MO). Recombinant mouse IL-2 was purchased from R&D Systems and used at 100 U/mL.

Statistical Analysis

Survival data were compared with the Kaplan–Meier method and analyzed by the log-rank test. For normally distributed data, Student's *t* test was applied. *P* values less than 0.05 were considered significant.

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FIGURE 1.

CD4⁺CD25⁻ T cells up-regulate Foxp3 in response to graft alloantigen. (A) Adoptive transfer model. BALB/c mice receive HA⁺ skin grafts 30 days before adoptive transfer. CD4⁺CD25⁻ T cells are magnetically sorted from TS1 mice, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled, then transferred with or without CD4⁺CD25⁺ T cells purified from TS1×HA28 mice. After transfer, lymphocytes from draining lymph node are analyzed by flow cytometry. (B) Cotransfer of antigen-specific regulatory T cells (Tregs) prevents conversion into adaptive Tregs. In response to the graft, there is a significant conversion of naive T cells to adaptive Tregs (aTregs; 16.4±5.2-fold increase; $P<0.03$; n=4). Cotransfer of nTregs with naive T cells significantly decreases the conversion to aTregs (3.3±1.0-fold; $P<0.03$; n=4). Analyses are of live forward scatter/side scatter, CD4-gated cells. Number represents the percentage of cells per total CFSE⁺ cells. Recipient endogenous cells are non-CFSE-labeled cells observed in fluorescence-activated cell sorter plots and not included in percentages. This experiment, in which cells were harvested at day 11 for all three panels, is representative of four experiments. HA, hemagglutinin.

FIGURE 2.

In vitro antigen-activated conventional T cells (Tconv) up-regulate Foxp3. (A, *left and center*) BALB/c splenocytes and carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4⁺CD25⁻ naive T cells are cultured with or without hemagglutinin (HA) peptide. (*Right*) BALB/c splenocytes, CFSE-labeled CD4⁺CD25⁻ naive T cells, HA peptide, and CD4⁺CD25⁺ regulatory T cells (Tregs) are cultured for 5 days. Tconv and natural regulatory T cells (nTregs) are cultured at a 1:1 ratio. No peptide versus peptide, 12.5±2.0-fold increase, ***P* less than 0.01; peptide versus peptide plus nTregs, ***P* less than 0.01. (B) Interleukin (IL)-2 restores conversion in the presence of nTregs. BALB/c splenocytes, CFSE-labeled CD4⁺CD25⁻ naive T cells with or without CD4⁺CD25⁺ Tregs are cultured with HA peptide and IL-2. IL-2 restores the rate of conversion when compared with cultures without nTregs. (A and B) Representative of four experiments. Number represents the percentage of CFSE⁺Foxp3⁺ cells per total CFSE⁺ cells. Peptide plus nTregs±IL-2, *P*=0.31, no significant difference. (C) Antigen-stimulated Tconv activate Foxp3-GFP expression. TS1 mice were mated with Foxp3-GFP mice. Spleen and lymph node cells from TS1×Foxp3-GFP mice were sorted based on 6.5, CD4, CD25, and GFP expressions. Tconv were cultured 4 to 5 days with HA peptide and BALB/c splenocytes. Data shown are representative of at least three separate experiments. APC, antigen-presenting cell.

FIGURE 3.

Adaptive regulatory T cell (aTreg) conversion is necessary for rapamycin (rapa)-mediated transplantation tolerance but not anti-CD4/anti-CD40L-mediated transplantation tolerance. (A) CD4⁺CD25⁻ wild-type (WT) or scurfy lymphocytes were adoptively transferred into C57BL/6-RAG-deficient mice bearing DBA/2 skin grafts. Sixty percent of rapa-treated mice receiving WT T cells survived more than 100 days, whereas rapa treatment did not prolong mice receiving scurfy cells. Control mice were injected with vehicle alone. (B) Rapa converts conventional T cells to Foxp3⁺ aTregs in vivo. Lymphocytes from grafted, rapa-treated, and untreated mice were analyzed 3 weeks after adoptive transfer. The percentages of adaptive Foxp3⁺ CD4⁺ T cells from RAG-deficient hosts are indicated as analyzed by flow cytometry. (C) FACSARIA-sorted CD4⁺CD25⁻CD44^{low} CD62L^{high} WT or scurfy lymphocytes were adoptively transferred into C57BL/6-RAG-deficient mice bearing DBA/2 skin grafts. Recipients receiving cells from WT or scurfy donors and no antibody treatment rejected grafts with similar tempo. In contrast, grafts on anti-CD4/anti-CD40L-treated mice survived long-term regardless of whether they received WT or scurfy cells (***P* < 0.01). tx, transplantation; YTS, YTS-177.