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Homeostatic Proliferation of Lymphocytes Results in Augmented Memory-Like Function and Accelerated Allograft Rejection

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Homeostatic proliferation is a normal physiological process triggered by lymphopenia to maintain a constant level of T cells. It becomes the predominant source of new T cells in adulthood after thymus regression. T cells that have undergone homeostatic proliferation acquire the memory phenotype, cause autoimmune disease, and are resistant to tolerance induction protocols. Transplantation is a rare example in which lymphopenia is deliberately induced for its immunosuppressive effect. However, it is not known whether the homeostatic proliferation that follows will have the opposite effect and accelerate rejection. We show that T cells that have undergone homeostatic proliferation acquire a memory phenotype, spontaneously skew toward the Th1 phenotype, even in the absence of antigenic stimulus. Interestingly, in contrast, the percentage of Foxp3+ regulatory T cells increased by 28-fold following homeostatic proliferation. Using a mouse life-sustaining kidney transplant model, we showed that T cells that have gone through homeostatic proliferation in lymphopenic hosts transformed chronic rejection to acute rejection of a single MHC class II-mismatched kidney allograft. T cells that have undergone homeostatic proliferation consistently cause reliable rejection even when bona fide memory T cells cannot. These functional changes are long-lasting and not restricted to the acute phase of homeostatic proliferation. Our findings have important implications for tolerance induction or graft-prolonging protocols involving leukocyte depletion such as irradiation bone marrow chimera, T cell-depleting Abs, and lymphopenia induced by infections such as CMV and HIV. The Journal of Immunology, 2008, 180: 3910–3918.

Each individual needs to maintain a diverse repertoire of naive and memory T cells to defend against new and previously encountered pathogens. The level of peripheral T cells is continuously being closely regulated. As a result, the absolute number of circulating lymphocytes falls within a narrow range throughout life (1). From birth to early adulthood, this is maintained by thymic emigration of newly formed naive T cells, but from early adulthood onward, with thymic involution, this is maintained by homeostatic proliferation of peripheral mature T cells (2).

Both naive and memory T cells can undergo homeostatic proliferation, probably as a result of availability of “niche” such as access to self-MHC-peptide complexes and IL-7 (3) for naive CD8+ and CD4+ T cells, with the addition of IL-15 for CD8+ memory T cells and the chemokine CCL21 for CD4+ T cells (4).

Homeostatic proliferation takes place within the T cell area of the spleen and lymph nodes (5). Physical space is required because hosts devoid of lymphoid tissues cannot sustain homeostatic proliferation (6). Transferred T cells do not go through exaggerated homeostatic proliferation in wild-type (WT)3 hosts but do so in TCR-transgenic mice that have normal numbers of T cells but a highly restricted repertoire, suggesting regulation by clonal competition (7).

Despite being an Ag-independent process, T cells that have undergone homeostatic proliferation show up-regulation of memory markers such as CD44 (8) but not activation markers such as CD25, CD67, and CD71 (9) and can masquerade as memory T cells by responding rapidly to antigenic challenge (10–12) with a lower requirement for costimulation through CD28 (8).

Although the surface phenotypic changes of T cells after homeostatic proliferation have been well described, whether they can function as bona fide memory T cells in vivo is uncertain. Lymphopenia in humans and rodents is associated with autoimmunity (reviewed in Ref. 13). CD4+CD45RBhigh T cells transferred into lymphopenic hosts have been shown to cause colitis (14, 15).

In experimental models of organ transplantation, homeostatic proliferation has been shown to be a barrier to tolerance induction using costimulatory blockade (16). In clinical transplantation, irradiation bone marrow chimera and depleting mAbs such as anti CD52 (Campath-1H, alemtuzumab) are increasingly being used either to prolong graft survival or as part of an experimental tolerance induction protocol (17). This renders the recipients lymphopenic and the residual T cells will undergo exaggerated homeostatic proliferation. Indeed, CD4+ T cells with the cell surface phenotype CD45RA–CD62L– consistent with effector memory

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cells are thought to be responsible for the rejection episodes following alemtuzumab treatment (18). This has been postulated as the reason for the disappointing results of clinical trials using this mAb without conventional immunosuppression (19, 20).

Much is known about the mechanisms of homeostatic proliferation, mainly from experiments using TCR-transgenic T cells. However, data on the functional effect of this transformation of WT polyclonal naive T cells in a clinically relevant model is still lacking. Since lymphopenia and the resultant homeostatic proliferation are a common and often deliberately induced phenomenon in recipients of organ transplantation, we investigated the effect of homeostatic proliferation of T cells on graft rejection and survival.
to highlight the potential danger of deliberate induction of lymphopenia in organ transplant recipients.

**Materials and Methods**

**Animal models**

All animals were between the age of 8 and 12 wk and used in accordance with the Animals (Scientific Procedures) Act 1986. C57BL/6 (BL/6) (H-2b) mice were purchased from Harlan. BL/6 RAG^−/−, BM1 (H-2bm1), and BM12 (H-2bm12) mice were bred in-house. All mice were kept under specific pathogen-free conditions, with the exception of the RAG^−/− mice that were kept in individually ventilated cages. Mouse renal transplantation was performed using a technique adapted from that described by Kalina et al. (21) and Han et al. (22). The left kidney was removed at the time of transplant, the right 7 days later, leaving the transplanted kidney life-sustaining. Skin grafts were performed as previously described (23).

**Recipient priming**

Donor splenocytes were prepared using RBC lysing solution (4.17 g of NH₄Cl/0.0185 g of EDTA disodium (BDH)/0.5 g of NaHCO₃ (Sigma-Aldrich) in 500 ml of water). Recipients were primed with a s.c. injection of 10 × 10⁶ donor splenocytes 14 days before renal transplant.

**Reconstitution of RAG^−/− mice**

Splenocytes were prepared by centrifugation through a Ficoll gradient (Sigma-Aldrich) and isolated by positive magnetic beads separation (Miltenyi Biotec) and used according to the manufacturer’s instruction. Preparations used were at least 97% pure and free from CD8 T cell contamination (data not shown). Fifteen million whole splenocytes or 5 × 10⁶ CD4 T cells were injected i.v. on the days indicated. To demonstrate homeostatic proliferation of injected lymphocytes, cells were labeled with CFSE before injection (Molecular Probes) according to the manufacturer’s instructions.

**Flow cytometry**

Cells were stained by using the appropriate mAb. Anti-CD4 (clone GK1.5), anti CD8 (clone 53-6.7), anti-CD44 (clone IM7; all from BD Pharmingen), anti-Foxp3 (clone FJK-16s (eBioscience), or HY-specific UTY Db tetramer (Beckman Coulter) (24). For tracking experiments, cells were labeled with

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**FIGURE 1.** (continued)
CFSE before injection according to the manufacturers’ instructions. Intracellular cytokine and Foxp3 staining was performed as previously described (25) using anti-IL-4 (clone 11B11), anti-IL-17 (clone TC11-18H10) and anti-IFN-γ (clone XMG1.2; all from BD Pharmingen), and anti-Foxp3 (clone FJK-16s; eBioscience). Briefly, cells were activated on plate-bound anti-CD3 (1 μg/ml, clone 145-2C11; BD Pharmingen) and anti-CD28 (5 μg/ml, CD28 clone 37.51; BD Pharmingen) for 5 h, the last 4 h in the presence of brefeldin A (5 μg/ml; Sigma-Aldrich). Cells were then harvested, fixed in paraformaldehyde (4%), permeabilized with 0.5% saponin (Sigma-Aldrich), and stained intracellularly. Cells were acquired using a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software version V3.3 (BD Biosciences).

Serum creatinine measurement

Serum creatinine was measured on a regular basis from tail vein bleeds using an electrospray mass spectrometer according to the manufacturer’s instructions.

Histological analysis

Grafts were processed and embedded in paraffin blocks, cut, and stained with H&E, periodic acid-Schiff, modified martius scarlet blue, and Masson trichrome (reagents from RALamb and Sigma-Aldrich). A system that we have specifically devised for assessing mouse kidney allografts (26) was used to grade histological changes in all of the kidney allografts. Immunohistology was performed as described elsewhere (27). Frozen tissue samples were cut into 5-μm sections and fixed in acetone. Foxp3 was identified using a biotinylated Ab (clone FJK-16s; eBioscience) incubated overnight, while CD4 was stained with a purified primary rat anti-mouse mAb (clone H129.19) followed by a biotinylated goat anti-rat (both from BD Pharmingen). A rat IgG2a, clone R35-95 (BD Pharmingen), was used as an isotype control. Positive cells were counted in 20 random high-power fields (HPF, ×400) of each sample by two independent observers blinded to the experimental conditions, and the means were calculated. Rejection of allografts was confirmed by examination of H&E and periodic acid-Schiff sections using the Banff criteria (28). Acute rejection grade IA was used as the minimum criteria for acute rejection to be diagnosed (significant interstitial infiltration; >25% of the parenchyma was affected and foci of moderate tubulitis, >4 mononuclear cells/tubular cross-section of a group of 10 tubular cells). Banff criteria was also used to score chronic allograft nephropathy.

Statistical analysis

Graft survival between different experimental groups was tested for statistical significance using the log-rank sum test. Median survival times (MST) are given, as the kidney allografts are life sustaining, graft failure will result in the death of the recipient, and was always confirmed by blood urea nitrogen reading and histology. Therefore, MST refers to both graft and recipient survival. The t test was used for data obtained from FACS analysis.

Results

Chronic rejection of BM12 kidney grafts by WT recipients

When a single MHC class II-mismatched kidney allograft from BM12 donors was transplanted into BL/6 recipients, there was a slow deterioration of graft function as measured by serum creatinine. Recipients died of graft failure between 49 and 77 days with a MST of 55 days (Fig. 1, A and B). Histological examination revealed glomerular sclerosis, tubular atrophy with interstitial fibrosis, and intimal hyperplasia but little mononuclear cell infiltration (grade III, chronic allograft nephropathy; Fig. 2A). Syngeneic graft recipients survived indefinitely with normal function and histology (Fig. 2B).

Lymphocytes undergo homeostatic proliferation in RAG−/− recipients

To investigate the effect of homeostatic proliferation, we reconstituted RAG−/− recipient mice with 15 × 10⁶ WT splenocytes. Fig. 3A shows that a small population of CFSE-labeled WT splenocytes could be seen 1 day postreconstitution. Halving of the CFSE concentration could be demonstrated by distinct peaks on flow cytometry (Fig. 3B). By day 6, the peak corresponding to CFSE had shifted to the far left, indicating extensive homeostatic proliferation (Fig. 3C). The level of reconstitution after 14 days was calculated from the percentage of CD4+ and CD8+ T cells on FACS analysis and absolute cell count in spleens of reconstituted RAG−/− animals. The number of CD4+ and CD8+ T cells was 3.82 ± 0.78 × 10⁹ and 1.51 ± 0.24 × 10⁹, respectively (n = 6), respectively.
significantly lower than WT mice, 8.08 ± 2.12 × 10⁶ (p = 0.0005) and 6.31 ± 1.34 × 10⁶ (p = 0.0002), respectively.

Expansion of CD4⁺ Foxp³⁺ T cells after homeostatic proliferation

It has been proposed that the outcome of an immune reaction depends on the balance of effector and regulatory T cells. To determine whether Foxp³⁺ regulatory T cells go through homeostatic proliferation to the same extent as nonregulatory T cells, reconstituted RAG⁻/⁻ mice were sacrificed 14 days after reconstitution. Although only 0.73 ± 0.16% of the CD4⁺ T cells from spleens of naive animals also stained positive for intracellular Foxp3 (n = 10), 20.2 ± 4.9% of the CD4⁺ T cells from spleens of reconstituted RAG⁻/⁻ mice were double positive, representing a 28-fold increase (n = 6, p = 0.0002; Fig. 4).

Expression of CD44 by T cells after homeostatic proliferation

It has been demonstrated previously that T cells that have undergone homeostatic proliferation up-regulate their expression of the memory marker CD44 (29). To confirm this in our model and to determine the extent to which this can happen, we reconstituted RAG⁻/⁻ mice with 15 × 10⁶ WT splenocytes and harvested the spleens 8 days later for flow cytometric analysis. Only 9.37 ± 2.57% of the CD4⁺ T cells expressed high levels of the memory marker CD44 in naive WT mice. However, 8 days after reconstitution of RAG⁻/⁻ recipients with naive WT splenocytes, this increased to 82.19 ± 1.50% (Fig. 5A). Likewise, the percentage of CD8⁺ CD44high T cells increased from 17.56 ± 3.29% to 86.02 ± 2.40% after reconstitution (Fig. 5B). Neither the CD4⁺ or CD8⁺ subsets demonstrated an up-regulation of the activation marker CD25 (data not shown).

Cytokine profile of T cells after homeostatic proliferation

To determine whether T cells are polarized toward Th1, Th2, or Th17 following homeostatic proliferation, reconstituted RAG⁻/⁻ mice were sacrificed 14 days after reconstitution and their intracellular cytokine profile was determined by FACS analysis. Fig. 6 shows that intracellular IFN-γ was not detected from CD4⁺ T cells of naive WT mice. However, after homeostatic proliferation in RAG⁻/⁻ hosts, T cells were skewed toward the Th1 phenotype with 10.71 ± 4.2% staining positive for IFN-γ (p < 0.0001), demonstrating that T cell polarization has taken place despite the lack of antigenic encounter. Intracellular cytokine staining for IL-4 and IL-17 did not show any cytokine production (data not shown).

Transformation of chronic to acute rejection of BM12 kidney grafts after homeostatic proliferation

BM12 kidney grafts were transplanted into RAG⁻/⁻ recipients. One day after transplantation, they were reconstituted with either 15 × 10⁶ WT splenocytes or 5 × 10⁶ purified CD4⁺ T cells. Unlike WT BL/6 recipients, reconstituted RAG⁻/⁻ recipients rejected the BM12 allografts acutely, MST = 11.5 and 8.0 days for WT splenocytes and purified CD4⁺ T cells, respectively (p = 0.0011 and p = 0.0022, respectively, compared with WT recipients; Fig. 1, C and D). Histological examination of the grafts

FIGURE 4. Intracellular Foxp3 staining following homeostatic proliferation. CD4⁺ T cells from spleen of control naive mice show a low percentage of Foxp3⁺ cells (A and C), but when allowed to homeostatically proliferate in lymphopenic hosts, the percentage of CD4⁺ T cells that are also positive for Foxp3 increased significantly to 20.2 ± 4.9% (B and C).

FIGURE 5. A, CD44high memory marker on WT CD4⁺ cells in RAG⁻/⁻ mice before reconstitution and 8 days after reconstitution with 15 × 10⁶ splenocytes following homeostatic proliferation in a RAG⁻/⁻ recipient demonstrating the development of the surface phenotype characteristic of memory T cells (p = 0.0001 WT compared with postreconstitution CD4⁺ T cells). B, CD44high memory marker on CD8⁺ cells (p = 0.0001 WT compared with postreconstitution CD8⁺ T cells).
showed mononuclear cell infiltrate and tubulitis, typical of severe acute rejection, grade IB according to the Banff criteria (Fig. 2B). Control RAG−/− recipients reconstituted in the same manner, but when transplanted with syngeneic BL/6 kidneys did not reject their grafts (Fig. 1, C and D).

Acquisition of “augmented” memory function following homeostatic proliferation

Having demonstrated that homeostatic proliferation can convert chronic rejection of BM12 kidney grafts to acute rejection, we investigated whether rejection of kidney grafts from other donor strains that normally have a different rejection tempo and characteristics would also be affected by homeostatic proliferation. Only 56% of single-class I MHC-mismatched BM1 kidney allografts transplanted into naive WT recipient BL/6 mice were rejected, as confirmed by histological analysis (MST = 104 days; Fig. 1, E and F). Even in recipients that have been primed with donor splenocytes 14 days before kidney transplantation, only 57% of the recipients rejected subsequent BM1 kidney allografts (MST = 98 days; Fig. 1, E and F). By sharp contrast, BM1 kidney grafts were rejected acutely by all naive whole-splenocyte-reconstituted RAG−/− recipients (MST = 9 days, p = 0.0005 compared with WT recipients, p = 0.0088 compared with primed WT recipients; Fig. 1, G and H), demonstrating that lymphocytes that have gone through homeostatic proliferation were able to respond with more vigor than bona fide memory T cells in this strain combination.

Acceleration of rejection kidneys from a single minor Ag-mismatched donor after homeostatic proliferation

To test the limit of homeostatic proliferation on accelerating rejection by T cells, we chose one of the least immunogenic donors to be transplanted into reconstituted RAG−/− recipients. Male donors only differ from female recipients of the same strain by a single minor Ag, HY. Even skin grafts, normally regarded as highly immunogenic, are not rejected in most HY-mismatch combinations, except in mice of the H-2k haplotype (30, 31), while heart grafts are not rejected at all (32). Whether male kidney grafts are rejected by female recipients is previously unknown. When we transplanted male BL/6 kidney grafts into syngeneic female recipients, all of the recipients and grafts survived indefinitely (Fig. 1, I and J). Graft function was within the range accepted as normal and did not differ significantly from the function of syngeneic grafts. However, all male kidney grafts transplanted into reconstituted female RAG−/− recipients were rejected acutely (MST = 10 days, p = 0.0003 compared with WT recipients; Fig. 1, K and L). In this model, the use of HY-specific tetramer allowed the detection of HY-specific CD8+ T cells following an immune response. A small population of HY-specific CD8+ T cells could be seen in a minority of WT female recipients of male kidney grafts (Fig. 7, A and B), but not in reconstituted female RAG−/− recipients, suggesting that the number of responding cells was too small to be detected and implies that this small population must have enhanced functional capability to cause rejection.

Homeostatic proliferation is required for accelerated rejection

To ensure that homeostatic proliferation is required for the observed acceleration in rejection, WT instead of RAG−/− recipients were injected with WT splenocytes 1 day after transplantation. Because WT recipients already have a full repertoire of T cells, injected splenocytes will not go through exaggerated homeostatic proliferation. As expected, there was no acceleration of rejection of BM12 kidney grafts (MST = 49 days (Fig. 1, M and N), p = 0.4351 compared with noninjected WT recipients of BM12 grafts, MST = 55 days).

Accelerated rejection is not limited to the acute phase of homeostatic proliferation and is long-lasting

To test whether accelerated rejection was limited to the acute phase of homeostatic proliferation, when splenocytes injected into hosts completely devoid of T cells and niche are available in abundance, we reconstituted RAG−/− recipients 50 days before transplantation instead of 1 day after transplantation. In this setting, T cells undergo homeostatic proliferation in the absence of alloantigen, thus allowing us to address the contribution of alloantigen recognition to the acquisition of enhanced effector function by T cells. BM12 kidney grafts were still rejected acutely and more rapidly than those reconstituted on day 1 after transplantation (MST = 9 days; Fig. 1, O and P; p = 0.0047) compared with day 1-reconstituted recipients, suggesting that accelerated rejection was not limited to the acute phase of homeostatic proliferation and that the functional change of the T cells is long-lasting, a hallmark of immunological memory.

Accelerated rejection in the absence of inflammation

To further test the functional capability of the T cells after homeostatic proliferation, we set out to determine whether these cells can reject an allograft in the absence of inflammation. Acutely transplanted organs up-regulated markers such as MHC class II as a

FIGURE 6. IFN-γ production by CD4+ T cells after homeostatic proliferation. A, Naive isotype Ab-stained control. B, Following homeostatic proliferation, a distinct population of cells staining positive for IFN-γ could be seen with 10.71 ± 4.2% staining positive for IFN-γ, while only background staining could be detected for CD4+ T cells from naive mice (p < 0.0001; C).

FIGURE 7. FACS plots demonstrating the absence of detectable HY tetramer-positive CD8+ T cells in reconstituted RAG−/− recipients of HY-mismatched grafts (A) while in the WT even without graft rejection a discrete population can be demonstrated (B). This further supports the notion that the small numbers of cells that have undergone homeostatic proliferation have enhanced functional capabilities.
result of tissue injury sustained during the harvesting, preservation, and transplantation processes. With time these proinflammatory features become quiescent, making the graft less prone to immunological identification and subsequent damage. To do this, RAG−/− recipients were reconstituted 50 days after transplantation, instead of 1 day, when most of the inflammatory signals had subsided. Furthermore, because MHC class II molecules are upregulated as a result of inflammation and the donor strain used here is only mismatched to the recipient by a single MHC class II molecule, the level of alloantigen being expressed will be much lower by day 50. Even under these circumstances, lymphocytes that have undergone homeostatic proliferation were able to reject BM12 allografts at the same tempo as mice reconstituted 1 day after transplant (MST = 14 days; Fig. 1, Q and R; p = 0.886.

Acceleration of rejection despite Foxp3+ T cell infiltration of allografts

We have previously demonstrated that the level of Foxp3+ T cells is higher in spleens and within grafts of WT mice tolerant to renal allografts (33). The demonstration that the proportion of Foxp3+ T cells increased dramatically following homeostatic proliferation in the spleens of reconstituted RAG−/− mice (Fig. 4) therefore suggest that the non-Foxp3+ T cells do indeed have enhanced effector function since they can resist regulation by a higher number of Foxp3+ T cells. To investigate whether the increased proportion of Foxp3+ T cells was also reflected in BM12 allografts transplanted into reconstituted RAG−/− recipients compared with those transplanted into WT recipients (104.8 ± 3.9 and 101.9 ± 16.9 cells per HPF, respectively, n = 4). Surprisingly, the level of Foxp3+ T cells infiltrating grafts transplanted into reconstituted RAG−/− recipients was more than two times higher than those transplanted into WT recipients (27.7 ± 6.7 and 13.5 ± 3.5 cells per HPF, respectively, n = 4; Fig. 8B), although it did not reach statistical significance. The ratio of Foxp3+ T cells to CD4+ T cells was also higher in BM12 kidney grafts transplanted into RAG−/− recipients compared with WT recipients (1:3.8 compared with 1:7.6, respectively). Despite a higher level of Foxp3+ cells within the grafts, rejection was accelerated, further supporting the notion that effector T cells that have undergone homeostatic proliferation have enhanced functional capabilities.

Retention of enhanced effector function acquired during homeostatic proliferation after adoptive transfer

To determine whether the enhanced effector function and cytotoxic capability seen in T cells that have undergone homeostatic proliferation is long-lived and can be maintained outside a lymphopenic environment, where they no longer continue to undergo homeostatic proliferation, RAG−/− recipients were reconstituted with 15 × 10⁶ WT splenocytes. They were then left for 14 days for the T cells to go through homeostatic proliferation. Splenocytes were harvested and 15 × 10⁶ cells that had undergone homeostatic proliferation were adoptively transferred into WT recipients. One day later, the recipients were transplanted with BL/6 male skin grafts. This again resulted in acceleration of the rejection tempo: WT recipients injected with reconstituted RAG−/− splenocytes rejected the grafts with an accelerated tempo, a MST of 19 days (n = 6), whereas WT recipients injected with splenocytes from saline-injected RAG−/− mice rejected the grafts with a MST of 45 days (n = 4, p = 0.0048).

Discussion

The life-sustaining mouse kidney allograft model is clinically relevant and allows accurate objective measurement of graft function using serum creatinine to demonstrate any augmentation in the rejection response. WT lymphocytes were used for reconstitution rather than TCR-transgenic T cells, such as ABM or Marylin mice that recognize H-2bm12 and the male HY Ag, respectively, because they are more physiological and contain a full repertoire of T cells with different affinity to self-MHC ligands. TCR affinity to the self-MHC is thought to play a role in the selection of high-affinity clones for preferential homeostatic proliferation.

Homeostatic proliferation invariably takes place as a result of lymphopenia, regardless of its cause, which can include genetic mutation such as SCID or athymic nude and RAG−/−, irradiation, mAb treatment (34–40), viral infection, and cessation of cyclosporin treatment (41). Using the RAG−/− host is convenient and the level of lymphopenia is absolute as well as consistent. However, the implications should be valid for all other situations of lymphopenia-induced homeostatic proliferation.

It has been shown that the extent of homeostatic proliferation following lymphopenia is related to the microbiological status of the mice (34). All of the RAG−/− recipients were housed in individually ventilated cages and, therefore, exposure to pathogens is limited, but not completely absent. Nonetheless, there was enough exposure to support rigorous homeostatic proliferation in our model. We have not observed any evidence of autoimmune diseases in our RAG−/− colony due to this. RAG−/− mice have been extensively used to study various immunological phenomena. They lack T cells which are known to interact with dendritic cells. Whether dendritic cells in RAG−/− mice function normally has not received much attention and is beyond the remit of this study. However, a recent study showed that dendritic cells form RAG−/− mice differentiate and function normally (42).

Since nephrotoxic immunosuppressive therapies such as cyclosporin were not used in our model, the damage sustained by the class II mismatched compared with syngeneic grafts could only be due to immunological differences between donor and recipient. In naïve recipients, BM12 kidney grafts were rejected slowly with an insidious loss-of-function and histological features characteristic of chronic allograft nephropathy. In reconstituted RAG−/− recipients, despite the lower number of total CD4+ and CD8+ T cells and higher percentages of Foxp3+ T cells, not only was the tempo of the rejection accelerated, the qualitative histological changes were also markedly different with transformation from features associated with chronic allograft nephropathy, such as tubular atrophy, interstitial fibrosis, and glomerular sclerosis to features consistent with acute rejection, namely, tubulitis and mononuclear cell infiltrate. It has been proposed that direct Ag presentation to T cells is mainly responsible for acute rejection while Ag presentation via
the indirect pathway leads to chronic rejection of allografts (43–48). Our data neither support nor disprove this notion because we have not studied the route of Ag presentation. Nevertheless, the acceleration of graft dysfunction is clear, demonstrating that T cells that have gone through homeostatic proliferation have augmented effector function.

This change in function is not limited to a single class II MHC mismatch as demonstrated by the single class I MHC mismatch and single minor Ag mismatch transplant models. In the case of the single class I MHC mismatch model, even primed recipients, such as those that received donor splenocytes i.p., rejected only 57% of kidney allografts acutely. By contrast, all reconstituted lymphopenic hosts rejected their grafts acutely, suggesting that T cells which have undergone homeostatic proliferation have augmented memory T cell function compared with bona fide memory T cells.

Reconstituted RAG−/− hosts were able to reject allografts even if reconstitution took place 50 days after transplantation, when the level of MHC class II, other inflammatory, and costimulatory molecules should have returned to baseline level. Reconstitution 50 days before transplantation also produced the same result. This enduring functional alteration and the relative independence from costimulation makes the T cells following homeostatic proliferation further resemble memory T cells in terms of function.

It has been suggested that during a rejection response, both effector lymphocytes and regulatory T cells are formed and it is the balance between these two populations that determine graft outcome (49). Indeed, higher urinary Foxp3 mRNA levels (presumed to be derived from graft-infiltrating leukocytes leaking into the urine) in patients undergoing rejection are associated with better outcome and long-term graft survival (50). Previous reports on the expansion of regulatory T cells during homeostatic proliferation have yielded contradictory results, with one study showing reduced proportion (51) and another showing full restoration (52) of regulatory T cells following homeostatic proliferation. In this study, we have shown that the proportion of CD4+ T cells that are also Foxp3+ is 28-fold higher following homeostatic proliferation in this model and that there was a higher number of Foxp3+ T cells infiltrating BM12 kidney grafts. The reason for reduced regulatory T cell proliferation in one report, normal in another, and enhanced proliferation reported here is not apparent, but most likely is due to the exact experimental conditions used and requires further investigation. Despite higher percentages of Foxp3+ cells in our model, reconstituted RAG−/− recipients still rejected their allografts in an accelerated fashion, further demonstrating the “memory” characteristics of the T cells that have undergone homeostatic proliferation, since memory T cells are typically thought to be resistant to regulation by regulatory T cells (53).

The mechanism through which T cells that have undergone homeostatic proliferation acquire the ability to respond like memory T cells is unclear. Selection of high-affinity self-reactive T cell clones has been put forward as a possible explanation (34, 35, 43–48, 54–58). If this is the case, then in all of our experiments (with the exception of day −50 reconstitution; Fig. 1, Q and R), T cell clones with high affinity for donor Ags presented via either the direct or indirect pathway would be selected and expand preferentially, because they would have been proliferating in the presence of the donor organ which has been recently transplanted and has a proinflammatory environment caused by surgical trauma and ischemic reperfusion damage during the transplant process. This may be the reason for the accelerated rejection we observed in these experiments. In the case of the day −50 reconstitution in which the RAG−/− hosts were reconstituted before transplantation, homeostatic proliferation would have taken place in the absence of donor Ags. Nonetheless, accelerated rejection was observed. T cell clones may have been selected for their high affinity for self-MHC molecules and it is possible that these clones cross-react with donor-mismatched Ags to accelerate rejection in a manner similar to heterologous immunity (59, 60). It remains unclear, however, why cells that have undergone homeostatic proliferation express the cell surface marker CD44+ traditionally associated with activated and memory T cells. Given that T cells acquire memory surface phenotype following homeostatic proliferation, it is not surprising that they also become polarized. In this study, we demonstrated that they do indeed become polarized toward Th1 (but not Th2 or Th17), which provides a logical and convincing mechanistic explanation for their enhanced effector function.

Results of studies in animal models are not directly translatable into the clinical setting and caution should be exercised when extrapolating these results. With that in mind, depleting mAb are now being used in the clinic in tolerance-inducing or graft survival-prolonging protocols. Even using an extremely effective depleting mAb such as alutenzumab, there are residual T cells that escape depletion. The resulting lymphopenia will trigger homeostatic proliferation and may account for the high rejection rate in organ transplantation clinical trials using anti-CD52 mAb Campath-1H, which required the addition of adjuvant immunosuppressive agents (18–20) such as calcineurin inhibitors.

Although our data highlight the potential adverse consequences of using T cell depletion in the transplantation setting, clinical trials must continue because the potential impact of successful outcomes on the management of patients posttransplantation is potentially dramatic, providing that investigators are aware of the effects of homeostatic proliferation. A recent report (61) suggests that regulatory T cells can control homeostatic proliferation. The notion of combining anti-T cell treatment to deplete the bulk of alloreactive T cells with regulatory T cells to control the remaining T cell population has already been suggested and experimentally demonstrated to be effective as an adjunct to tolerance induction by Turka and colleagues (51). If this strategy is used, then careful use of regulatory T cells after T cell depletion may serve the dual purpose of controlling the rejection process by the remaining T cells as well as preventing their excessive homeostatic proliferation and acquisition of memory phenotype and function, at the same time maintaining host defense against external pathogens.

Disclosures
The authors have no financial conflict of interest.

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