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Homeostatic proliferation is a barrier to transplantation tolerance

Zihao Wu^{1,6}, Steven J Bensinger^{1,6}, Jidong Zhang¹, Chuangqi Chen¹, Xueli Yuan², Xiaolun Huang³, James F Markmann³, Alireza Kassaei⁴, Bruce R Rosengard³, Wayne W Hancock⁵, Mohamed H Sayegh², and Laurence A Turka¹

¹ Department of Medicine, University of Pennsylvania, Philadelphia, Philadelphia 19104, USA

² Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02131, USA

³ Department of Surgery, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

⁴ Department of Radiation Oncology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

⁵ Pathology and Biesecker Pediatric Liver Center, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA

Abstract

Despite the ease of inhibiting immune responses by blockade of T-cell costimulation in naive rodent models, it is difficult to suppress those responses in animals with memory cells^{1,2}. Studies demonstrating the importance of alloreactive T-cell deletion during tolerance induction have promoted use of peritransplant T-cell-depleting therapies in clinical trials^{3–6}. But potentially complicating wide-scale, nonspecific T-cell depletion is the finding that extensive T-cell proliferation can occur under conditions of lymphopenia. This process, termed homeostatic proliferation^{7,8}, may induce acquisition of functional memory T cells^{9–13}. Here, using clinically relevant mouse models of peripheral T-cell depletion, we show that residual nondepleted T cells undergo substantial homeostatic expansion. In this setting, costimulatory blockade neither significantly suppresses homeostatic proliferation nor prevents allograft rejection. In addition, T cells that have completed homeostatic proliferation show dominant resistance to tolerance when adoptively transferred into wild-type recipients, consistent with known properties of memory cells *in vivo*. These findings establish the importance of homeostatic proliferation in clinically relevant settings, demonstrate the barrier that homeostatic proliferation can present to the induction of transplantation tolerance, and have important implications for transplantation protocols that use partial or complete peripheral T-cell depletion.

T cells undergo homeostatic proliferation when transferred into severe combined immunodeficiency (*scid*), recombination-activating gene-deficient or irradiated recipients^{7,8}. In many clinical transplantation protocols, T-cell depletion is less profound. We therefore asked whether residual nondepleted cells could undergo homeostatic proliferation. We treated mice with two doses (100 µg/dose) of depleting monoclonal antibodies directed at CD4 and CD8 (days 0 and 5). This induced 80–90% T-cell depletion, which was maintained for >10 d after the last dose (data not shown). Five days after the last

Correspondence should be addressed to L.A.T. (turka@mail.med.upenn.edu).

⁶These authors contributed equally to this work.

COMPETING INTERESTS STATEMENT

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antibody doses (day 10), some mice also received 700 rad of focal thymic irradiation. One day later (day 11), the mice were fed bromodeoxyuridine (BrdU) until sacrifice at day 17. We observed substantial BrdU labeling of peripheral T cells in thymic-irradiated, antibody-treated mice (34.6%; $n = 3$), compared with minimal BrdU labeling of peripheral T cells in unmanipulated control mice (7.75%; $n = 3$; Fig. 1a). The dose of thymic irradiation used kills virtually all double- and single-positive thymocytes¹⁴. In addition, mature thymocytes are not generated for at least 10 d after irradiation, and new thymic emigrants do not appear in the periphery for at least 14 d (M. Sykes, personal communication, and data not shown). Thus, BrdU staining of peripheral T lymphocytes reflects division occurring in mature residual T cells not removed by depleting monoclonal antibodies; that is, homeostatic proliferation. BrdU labeling is also increased (albeit to a lower extent) in T cells from mice that did not receive thymic irradiation, suggesting that homeostatic proliferation can occur even in the face of continued thymic export.

In the experiments above, it was formally possible that BrdU labeling reflected intrathymic division of maturing T cells, as opposed to homeostatic proliferation of peripheral lymphocytes. To exclude this, we adoptively transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled syngeneic T cells (as a tracer population) into mice treated with depleting monoclonal antibodies, with or without thymectomy, and killed the mice 10 d later. This revealed substantial division of the donor CFSE-labeled T cells in both populations of mice (Fig. 1b). No proliferation was observed when cells were transferred into control recipients not depleted of T cells (Fig. 1c and data not shown). Host origin of the CFSE-negative cells was confirmed by experiments using congenic Thy1.1 CFSE-labeled donor cells transferred into Thy1.2 recipients (Fig. 1c). Collectively, these data show that homeostatic proliferation can occur in euthymic mice that are partially T-cell depleted.

Having found that homeostatic proliferation occurs in the aftermath of T-cell depletion, we next asked whether it influences susceptibility to tolerance induction. First, we sought a model in which homeostatic proliferation and graft rejection both occurred. C57BL/6 *scid* (B6-*scid*) mice were injected with lymph node mononuclear cells from congenic B6 mice, and simultaneously transplanted with BALB/c vascularized cardiac allografts. As anticipated, unreconstituted B6-*scid* mice accept cardiac allografts indefinitely (Fig. 2a). Transfer of 0.5×10^6 cells at the time of transplantation is sufficient to induce graft rejection, and a dose of 2×10^6 cells reliably led to BALB/c cardiac allograft rejection in 18 d.

We determined the susceptibility of newly reconstituted mice to tolerance induction by costimulatory blockade, using either CTLA4Ig, to block CD28/CTLA-4:B7 interactions) or antibodies to CD154, plus donor antigen (5×10^6 donor lymph node cells, used here as donor-specific transfusion, or DST). Because irradiated (600 rad) and viable DSTs both synergize with costimulatory blockade to prevent graft rejection¹⁵, we used irradiated cells to prevent the development of graft-versus-host disease in the *scid* hosts. Whereas CTLA4Ig with DST induces long-term cardiac allograft survival in wild-type B6 hosts, it is virtually ineffective in B6-*scid* hosts simultaneously reconstituted with 2 million B6 lymph node cells (Fig. 2b). In models where single doses of CTLA4Ig do not induce long-term allograft survival, multiple doses and/or a combination of CTLA4Ig with antibodies to CD154 can be effective¹⁶. However, neither three doses of CTLA4Ig, nor a combination of CTLA4Ig with CD154-specific antibody, led to consistent long-term allograft survival (Fig. 2b). In addition, costimulatory blockade only minimally suppressed homeostatic proliferation (Fig. 2c).

Although costimulatory blockade did not induce tolerance in cells undergoing homeostatic expansion in lymphodeficient mice completely lacking T cells, the extent of the homeostatic

proliferation in that system clearly exceeds that observed after incomplete T-cell depletion of normal mice (compare Fig. 1b,c with Fig. 2c). Thus, it was important to test whether costimulatory blockade could induce long-term graft survival under the latter set of circumstances. To this end, wild-type, euthymic B6 mice were treated with 100 µg each of CD4- and CD8-specific monoclonal antibodies, on days -14 and -9. This protocol, which was used for the experiments in Figure 1, induces 80–90% T-cell depletion. On day 0, mice received BALB/c cardiac allografts and were treated with either CTLA4Ig and DST, or with control Ig and DST. Based on the experiments shown in Fig. 1, the timing of the transplantation relative to T-cell depletion was chosen to occur during the period of homeostatic proliferation of residual T cells.

All depleted mice treated with control Ig and DST rejected their grafts (Fig. 2d), with some delay that was probably caused by pretransplant T-cell depletion ($P = 0.0038$ compared with nondepleted mice treated with DST and CTLA4Ig). Notably, most depleted mice treated with CTLA4Ig and DST also rejected their grafts ($P = 0.0255$ compared with nondepleted mice treated with DST and CTLA4Ig). These data support the concept that T cells undergoing homeostatic proliferation after clinically relevant strategies of T-cell depletion resist the induction of transplant tolerance by T-cell costimulatory blockade.

Resistance to tolerance during homeostatic proliferation might be a reversible consequence of proliferation itself, or an irreversible fate of cells that have previously undergone division. To discriminate between these two possibilities, it was necessary to create conditions in which T cells would undergo homeostatic proliferation and then cease dividing. We reconstituted B6-*scid* mice with 50×10^6 B6 lymph node cells, which resulted in both homeostatic proliferation and associated tolerance resistance (data not shown). We then pulsed the mice with BrdU for 6 d at different time points. Ten days after reconstitution, 42.7% of T cells were BrdU⁺ (mean value of 37.9% for three experiments; Fig. 3a). This percentage decreased until 8–9 weeks after reconstitution, when the percentage of BrdU⁺ cells was identical to that observed in wild-type B6 mice (~10%) due to basal T-cell proliferation.

We next used B6-*scid* mice, 12 weeks after reconstitution with 50×10^6 cells, as allograft recipients (Fig. 3b). These mice continued to resist induction of tolerance with DST and CTLA4Ig, despite the fact that active homeostatic proliferation had ceased. Only a single graft survived beyond 50 d, and histologic examination of that graft at day 90 revealed severe parenchymal injury with extensive myocardial necrosis, mononuclear cell infiltration and fibrosis, and advanced arteriosclerosis (data not shown).

Failure to induce tolerance in mice after reconstitution by homeostatic proliferation might be caused by a defect in the expansion of regulatory cells. This seems unlikely, however, given studies showing that CD4⁺CD25⁺ T cells undergo a similar level of homeostatic proliferation as CD4⁺CD25⁻ T cells, and retain their suppressive function afterwards¹⁷. Furthermore, in our experiments, reconstituted *scid* mice killed after cessation of homeostatic proliferation (at 12 weeks) had the same percentages of CD4⁺CD25⁺ T cells in their lymph nodes (~9% of CD4⁺ T cells) and spleens (~3.5%) as did naive control B6 mice, and both groups of mice exhibited equivalent suppressive capabilities (Fig. 3c).

Intrinsic immunologic abnormalities of *scid* mice could have confounded the interpretation of our results. To address this, we reconstituted B6-*scid* mice with syngeneic T cells that were allowed to complete homeostatic proliferation over a 3-month period. These *scid* mice were killed, and 10 million 'post-homeostatic-proliferation' T cells were adoptively transferred into naive, syngeneic, wild-type B6 recipients that then received an allogeneic BALB/c cardiac allograft. This small number of post-homeostatic-proliferation T cells

dominantly blocked tolerance induction in otherwise susceptible recipients (Fig. 4a). In contrast, adoptive transfer of control T cells transferred from a wild-type syngeneic B6 mouse into a B6 recipient did not interfere with tolerance induction (Fig. 4a).

Although we considered it unlikely, it remained formally possible that the *scid* environment in which homeostatic proliferation had occurred might still bias the results of the adoptive transfer experiments in Fig. 4a, even though the transplants took place in normal mice. To test this, we thymectomized wild-type B6 mice (day 0) and then rigorously depleted them of T cells using large doses (200 μ g/dose) of antibodies to CD4 and CD8 (days 8 and 13) and 600 rad of total-body irradiation (day 7). The use of high-dose T-cell-specific antibodies, coupled with thymectomy and total body irradiation, was sufficient to eliminate all peripheral T cells. This was verified by fluorescence-activated cell sorting and, more importantly, by function, as the mice were unable to reject BALB/c cardiac allografts (data not shown). Next, the thymectomized, T-cell-depleted mice were reconstituted with syngeneic B6 T cells (day 27), which were allowed to undergo homeostatic proliferation. BrdU labeling verified that, as with reconstituted *scid* mice, homeostatic proliferation had ceased by roughly 8 weeks (data not shown). Five weeks later (13 weeks after reconstitution), the mice were given BALB/c cardiac allografts. All (five of five) of the grafts from recipients treated with control Ig and DST were readily rejected, as were the majority (five of seven) of those from mice treated with CTLA4Ig and DST (Fig. 4b). The two grafts in the latter group continued beating very weakly until the mice were killed at days 116 and 120. In our experience, this is associated with severe scarring and is indicative of a rejection response. As a control, thymectomy itself did not inhibit tolerance induction (BALB/c cardiac allografts survived >1 year in thymectomized B6 recipients treated with CTLA4Ig and DST; $n = 10$).

We hypothesized that resistance to tolerance is linked to the development of effector-like function during homeostatic proliferation, and memory-like function after proliferation. One known characteristic of memory cells is their relative lack of dependence on CD28 costimulatory signals for proliferation¹³. To examine this question using our model, naive T cells and post-homeostatic-proliferation B6 T cells (collected 12 weeks after reconstitution of *scid* mice) were stimulated with BALB/c T-cell-depleted spleen cells at a 1:3 ratio, and CTLA4Ig (10 μ g/ml) was added to the culture to block CD28 costimulation¹⁵. Proliferation was assessed by [³H]thymidine incorporation at 96 h. Naive T cells yielded $4,919 \pm 1,210$ c.p.m., whereas post-homeostatic-proliferation T cells yielded $10,327 \pm 301$ c.p.m. ($P < 0.05$; representative of two experiments). Thus, when stimulated by allogeneic antigen-presenting cells, post-homeostatic-proliferation T cells were less dependent on CD28 signaling than naive T cells. This is consistent with their resistance to tolerance by costimulatory blockade (Figs. 3b-4a) and with previous studies on antigen-induced memory cells^{1,2}.

Cells arising from homeostatic proliferation infiltrate cardiac allografts (and in most instances, effect rejection) in mice lacking secondary lymphoid organs; this is an ability of memory, but not naive, cells¹⁸. Induction of alloresponsive memory cells by immunization with specific antigen¹ or as a result of heterologous immunity to viral infections¹⁹ renders the host resistant to tolerance induction through costimulatory blockade. Taken together with the present report, these data further support the concept that resistance to tolerance induction after homeostatic proliferation is a consequence of the development of memory-like cells.

A recent report showed that T-cell regulation of disease could be a byproduct of homeostatic proliferation²⁰. In the inflammatory bowel disease model used in that study, however, disease is not spontaneous; it is only induced by naive cells undergoing homeostatic

proliferation in lymphopenic hosts. We concur with the authors' interpretation that their findings reflect competition during the expansion process between a specific pathogenic population of cells and a broad-based naive polyclonal population. In our studies, homeostatic expansion clearly did not result in inhibition of pathogenic T cells. We suspect this is due to a combination of factors, including the high frequency of alloreactive T cells in a normal repertoire²¹, and the fact that cells in normal unmanipulated mice are able to mount the pathologic response (that is, rejection) without a requirement for homeostatic expansion.

Our studies do not imply that lymphodepletion is necessarily inconsistent with tolerance induction. The ability of mixed hematopoietic chimerism, in which T-cell depletion is used to kill mature alloreactive T cells and facilitate bone marrow engraftment²²; to induce tolerance in humans^{23,24} or primates²⁵ clearly shows that this is not the case, and emphasizes some of the unique features of that approach. In the specific case of tolerance to vascularized allografts induced by DST and costimulatory blockade, a key issue may be whether or not T-cell depletion is complete. If incomplete, residual T cells undergoing homeostatic expansion may ultimately prevent the establishment of tolerance (Fig. 2d).

The importance of homeostatic proliferation in immune responses has recently been demonstrated in tumor models^{26,27}. Those data and the present study have important implications in clinical settings. Lymphodepletion is increasingly used as part of newer strategies in solid organ transplantation^{5,6}. The occurrence of homeostatic proliferation during lymphoid reconstitution, an event that may be especially relevant in the setting of age-related thymic involution²⁸ or protocols that employ thymic irradiation, may present a barrier to the establishment of transplantation tolerance, and may also be relevant to self-tolerance and autoimmunity.

METHODS

Reagents

Human CTLA4Ig was a gift of R. Peach (Bristol-Myers Squibb). Purified monoclonal antibody to CD154 (MR-1 clone) and the depleting CD4-specific (GK1.5) and CD8-specific (2.43) monoclonal antibodies were purchased from BioExpress. Control human Ig was purchased from Jackson ImmunoResearch. CTLA4Ig, control Ig, and/or monoclonal antibodies were given by intraperitoneal injection.

Mice and surgical procedures

BALB/c, C57BL/6.Thy1.1⁺, C57BL/6.Thy1.2⁺ and C57BL/6.Thy1.2⁺ *scid* (B6-*scid*) mice were purchased from The Jackson Laboratory and were housed under pathogen-free conditions. Heterotopic vascularized cardiac allografts were performed using BALB/c donors and B6 or B6-*scid* recipients. As indicated in the text, mice received 5×10^6 irradiated donor-specific cells (DST) by intravenous injection on the day of transplantation. Graft survival was monitored by palpation; rejection, defined as the absence of palpable contractions, was verified by visual inspection and pathologic examination. Loss of function within 48 h of transplantation was defined as a technical failure (<2% incidence). Where indicated, thymectomy was performed under direct visualization. All procedures were approved by institutional animal use committees of the University of Pennsylvania and the Brigham and Women's Hospital.

Adoptive transfers

Varying numbers of unfractionated mononuclear lymph node cells (prepared by tissue mincing, followed by hypotonic lysis of red blood cells) from B6.Thy1.1⁺ mice were delivered intravenously by retro-orbital injection into B6-*scid* Thy1.2⁺ recipients. Where

indicated, the cells were labeled with CFSE prior to transfer as described¹³. Purified T cells and CD8-depleted lymph node cells were prepared by negative selection using Miltenyi MACS separation columns (Miltenyi Biotec) as per the manufacturer's instructions. The purity of the population was >95%.

Flow cytometry

Fluorochrome-labeled monoclonal antibodies (CD4-allophycocyanin (APC), CD8-APC, CD25-biotin, Thy1.2-phycoerythrin (PE), Thy1.1-PE, Thy1.1-PerCP, BrdU-FITC, streptavidin-APC) and isotype controls were purchased from PharMingen. At the indicated time points, recipients were killed, spleen and lymph nodes (superficial inguinal, axillary, lateral axillary, cervical, submandibular and para-aortic) were collected and mononuclear cells were analyzed by multicolor flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems). Data was obtained using CellQuest acquisition software, and analyzed using FlowJo (Tree Star). A live lymphocyte gate was set using forward- and side-scatter properties. Between 0.5 and 2.0×10^6 events were collected. Where indicated, donor cells were identified by CFSE labeling and/or Thy1.1 staining. For suppressor cell assays (see below), CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated by sorting on a FACS Vantage (Becton Dickinson). The purity of these populations exceeded 95%.

Assays for suppression

Purified CD4⁺CD25⁻ T cells obtained from the spleens and lymph nodes of naive B6 mice were used as responders. After labeling with CFSE, they were stimulated by culture with 0.5 μ g/ml monoclonal antibody to CD3, in the presence of B6 T depleted splenocytes (as antigen-presenting cells), for 72 h. Purified CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were obtained from mice as indicated in Results and tested for suppressor function by adding them to the responders in a 1:1 ratio (final cell concentration was 1×10^6 cells/ml). Proliferation and responder frequency were assessed by CFSE dilution. Responder frequency was calculated as described²⁹.

T-cell depletion

Mice were injected intraperitoneally with 100 μ g each of depleting monoclonal antibodies to CD4 and CD8, on days 0 and 5. Where indicated, mice received 600 rad of total-body irradiation (cesium source) or 700 rad of localized thymic irradiation (X-ray source, Varian Clinac 2100CD) with narrow-beam geometry using a stereotactic cone.

BrdU labeling

Mice were given drinking water containing BrdU (0.8 mg/ml) for 6 d before they were killed. BrdU-labeled cells were detected after fixation and permeabilization using Fix and Perm media from Caltag.

Histology

BALB/c cardiac allografts ($n = 10$) were collected 23–90 d after transplantation from reconstituted B6-*scid* recipients treated with DST and CTLA4-Ig, and were evaluated by H&E, trichrome and elastin staining of paraffin sections³⁰.

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Figure 1.

Homeostatic proliferation after partial T-cell depletion. **(a)** BrdU labeling of live CD3⁺ cells from B6 mice treated with depleting antibodies to CD4 and CD8 (mAb) and/or thymic irradiation. Six days after the last antibody dose, mice were fed BrdU for six days until sacrifice. **(b)** B6 mice underwent thymectomy and were treated with depleting antibodies to CD4 and CD8. Five days after the last antibody dose, 20 million CFSE-labeled syngeneic T cells were adoptively transferred. **(c)** Same protocol as in **b**, except congenic B6.Thy1.1 CFSE-labeled T cells were used for transfer into B6.Thy1.2 mice. Data are representative of three experiments.

Figure 2.

Homeostatic proliferation induces resistance to tolerance induction by costimulatory blockade. **(a)** B6 (WT) or B6-*scid* (SCID) mice received BALB/c cardiac allografts. B6-*scid* mice were injected intravenously with the indicated numbers of B6 lymph node cells on the day of transplantation ($n = 5$ –10 mice per group). **(b)** B6-*scid* mice received BALB/c cardiac allografts plus 2 million B6 lymph node cells. Mice were also treated with DST, CTLA4Ig, control Ig or antibody to CD154 (200 μ g/dose). All mice received irradiated DST on the day of transplantation ($n = 5$ –10 mice per group). **(c)** B6-*scid* Thy1.2⁺ mice were injected with 2 million CFSE-labeled B6.Thy1.1⁺ lymph node cells and treated as indicated. Mice were killed on day 5 (left panels) or day 10 (right panels). Data are for live Thy1.1⁺ cells (representative of four experiments). **(d)** Euthymic B6 mice were given depleting antibodies to CD4 and CD8, received a BALB/c cardiac allograft 9 d later, and were treated with the indicated agents. Control mice (Δ) did not undergo prior T-cell depletion. $P = 0.0038$ for T-cell-depleted mice treated with control Ig and DST, compared with non-depleted mice treated with DST and CTLA4Ig ($n = 6$); $P = 0.0255$ for T-cell-depleted mice treated with CTLA4Ig and DST, compared with non-depleted mice treated with DST and CTLA4Ig ($n = 12$).

Figure 3.

Kinetics of homeostatic proliferation and long-term effect on function and tolerance induction. **(a)** B6-*scid* mice were injected intravenously with 50 million B6 lymph node cells and followed for the indicated time points. Six days before sacrifice, BrdU was added to the drinking water. Normal B6 mice were fed BrdU for six days prior to sacrifice as a control. Data for live T cells are displayed (representative of six experiments). **(b)** B6-*scid* mice were injected intravenously with 50 million B6 lymph node cells 12 weeks before receiving a BALB/c heterotopic vascularized cardiac allograft. Mice were also treated with DST and control Ig or CTLA4Ig ($n = 6$ mice per group). **(c)** B6-*scid* mice were injected with 50 million B6 lymph node cells and killed after 12 weeks. Purified CD25⁺ or CD25⁻ cells were tested for suppressor function. CD25⁺ cells from wild-type, unmanipulated B6 mice were used as controls. Histograms show proliferation, as CFSE dye dilution, of the responder population. R_f , calculated responder frequency (percentage of responder T cells that divided). Data shown are representative of two experiments.

Figure 4.

Tolerance resistance in wild-type mice. **(a)** T cells that underwent homeostatic proliferation in *scid* mice dominantly transferred tolerance resistance. B6-*scid* mice were adoptively transferred with 50 million B6 lymph node cells. Mice were killed after 12 weeks, and post-homeostatic-proliferation T cells were purified. Unmanipulated B6 mice, B6 mice given 10 million post-homeostatic-proliferation T cells, and B6 mice given 10 million T cells from naive syngeneic mice received BALB/c cardiac allografts and treatment with DST and CTLA4Ig ($n = 5-6$ mice per group). $P < 0.0001$ for mice receiving post-homeostatic-proliferation cells, compared with unmanipulated mice or mice that received control naive cells. **(b)** T cells that underwent homeostatic proliferation in thymectomized wild-type recipients acquired tolerance resistance. Wild-type B6 mice were thymectomized, treated with 600 rad of irradiation and depleting antibodies to CD4 and CD8, and adoptively transferred with 50 million B6 lymph node cells. Thirteen weeks later, the animals received BALB/c cardiac allografts and treatment with DST and either control Ig ($n = 5$) or CTLA4Ig ($n = 7$). $P = 0.7507$.