Long-term persistence of IL-2-unresponsive allogeneic T cells in sublethally irradiated SCID mice

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Abstract
Donor T cells that are activated by host alloantigens initiate graft versus host disease (GVHD) but their long-term fate is poorly understood. The behavior of alloreactive donor T cells was studied in sublethally irradiated SCID mice. Intravenous injection of 10^6 allogeneic lymphocytes caused a severe form of GVHD, characterized by host hematopoietic atrophy. Fifty-fold fewer donor cells did not induce disease and were not simply rejected by radioresistant host mechanisms. Instead, low numbers of allogeneic T cells expanded 20- to 50-fold and remained for >1 year without causing evidence of GVHD. Persistent non-cycling donor cells with an activated phenotype were mainly found in the spleen. Tolerance was inferred by the recovery of host hematopoiesis, despite the presence of donor allogeneic T cells, and the inability of long-term persisting donor T cells to mediate cellular cytotoxicity or proliferate in response to exogenous IL-2 or antigenic stimulation in vitro. The TCR density of long-term persisting donor T cells was down-regulated. These findings suggest that the development of GVHD depends on the magnitude of the initial anti-host response. Subsequently donor cells differentiate, over several months, into a senescent-like state. This behavior questions the rationale for current treatment approaches to GVHD and is of relevance to any clinical situation where chronic T cell activation takes place in the absence of thymic development.

Introduction
Mature donor T cells initiate clinical graft versus host disease (GVHD) after allogeneic stem cell transplantation (BMT) (1). T cell-containing donor leukocyte infusions are increasingly used to provoke GVHD and treat relapsed malignancies after BMT through a poorly characterized graft versus leukemia effect (2). In both these clinical situations, the long-term fate of donor T cells is poorly understood, possibly because it has been difficult to separate them from the T cells that develop from donor hematopoietic stem cells. An important question is: do chronically activated mature host-reactive donor T cells sustain GVHD or an anti-tumor effect, or is their effect only temporary?

Development of GVHD appears to require the injection of at least 10^5 donor T cells/kg (3). Are T cell numbers below this threshold simply rejected by host defense mechanisms that survive the conditioning regimen? If small numbers of mature donor T cells survive, it is not clear why GVHD is prevented. Donor cells expand to reconstitute the peripheral T cell compartment (4,5) and, a priori, alloreactive cells should be preferentially selected. In principle, a single alloreactive cell should be able to expand and cause GVHD.

To study the fate of mature alloreactive donor T cells and the effect of initial cell number on GVHD, we have used a previously characterized model in which enriched T cells from the spleens of C57BL/6J mice (H-2^b) are injected into sublethally irradiated C.B-17-SCID mice (H-2^d) (6,7). This...
model is especially useful for tracking the fate of donor T cells in vivo because the lack of host T cells precludes host versus donor lymphocyte interactions and donor marrow is not required for survival. In addition, the specific pathogen-free conditions required for the maintenance of SCID mice partially control for the effects of exogenous infections. In this paper, the behavior of low numbers (<3×10^5) of alloreactive B6 T cells in sublethally irradiated SCID hosts was compared with that of higher numbers (10^6) that were previously shown to cause acute GVHD (7). Peripheral lymph node cells (PLNC) that do not contain hematopoietic stem cells (8) were used to ensure that the peripheral T cell compartment was not reconstituted by thymic regeneration. Low numbers of alloreactive B6 T cells failed to cause GVHD but repopulated the peripheral T cell compartment to the same extent as an initial injection of 50-fold higher cell numbers. Moreover, these cells differentiated into a long-lived, IL-2-unresponsive state with a characteristic surface phenotype.

**Methods**

**Mice**

C57BL/6J (B6) (H-2^b^, Thy-1.2), B6.PL-Thy-1a/Cy (H-2^b^, Thy-1.1), BALB/c (H-2^d^) and C3H/HeJ (H-2^k^) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). BALB.K (H-2^k^), B6-SCID (H-2^b^, Thy-1.2), (B6×C.B-17) F1, SCID (H-2^k^) and C.B-17-SCID (H-2^k^) mice were bred and maintained in the defined flora animal colony at the Ontario Cancer Institute (Toronto, Ontario, Canada). C.B-17 is congenic to BALB/c, differing only in the region of the Ig heavy chain allele. BALB/c are IgHa and C.B-17 are IgHb (9). Breeding pairs of B6-SCID mice were initially provided by L. Shultz of the Jackson Laboratories.

**Antibodies, reagents, and cell lines**

Anti-CD3 (145-2C11) (10) was purified from hybridoma culture supernatants by Protein G column chromatography. Phycoerythrin (PE)- or FITC-labeled CD4 and CD8 antibodies, 7-AAD, propidium iodide, and streptavidin–PE were purchased from Sigma (St Louis, MO). The anti-FcyRIII-α antibody (2.4G2) (11) and the anti-heat stable antigen (HSA) antibody (J11d) were obtained from ATCC (Rockville, MD) and used as culture supernatants. PE- and FITC-labeled antibodies against Thy-1.1, Thy-1.2, Mac-1, H-2^b^, H-2^d^, HSA, IFN-γ, IL-2, CD3 and CD44, and biotinylated antibodies against Vγ3 and CD25 were purchased from PharMingen (San Francisco, CA). Anti-CD4– and anti-CD8–TriColor antibodies were purchased from Caltag (Burlingame, CA). The hamster antimurine CD28 hybridoma (37.51) was obtained from Dr James Allison (University of California, Berkeley, CA) (12) and antibodies were purified from culture supernatants by Protein G-affinity chromatography (Pharmacia, Uppsala, Sweden) in our laboratory.

Phorbol myristate acetate (PMA) and Brefeldin A were purchased from Sigma, and ionomycin was purchased from Calbiochem (La Jolla, CA). Murine IL-2 and IL-4 cDNA-transfected X63Ag8-653 cells were a generous gift of H. Karasuyama (13). CT.4S and P-815 lines were obtained from ATCC and maintained in exponential growth by serial passage in complete medium (CM) (α-MEM, 10% FCS, 5×10^{-5} 2-mercaptoethanol, 15 mM HEPES, 2 mM glutamine) at 37°C in an atmosphere of 5% CO₂.

**Cell preparations**

Where multiple sites were to be studied for the presence of donor T cells, mice were anesthetized in vaporess Enfluane USP (Abbott Laboratories, Montreal, Canada) and exsanguinated by cutting the right axillary artery. Blood was collected in CM with 100 U/ml of heparin. Cell suspensions of spleen, thymus and PLNC were made by passage through metal screens. Bone marrow cell suspensions were made from the femurs and tibiae of each mouse by injecting ~6–10 ml of CM via a 25-gauge needle and a 3-cm³ syringe. Peritoneal washings were collected by infusion of approximately 6 ml of cold PBS via a 10-cm³ syringe and an 18-gauge needle. A small cut was made in the peritoneal membrane and the infusate was collected using a sterile Pasteur pipette. The process was repeated once.

To purify donor T cells from chimeric spleens by negative selection, red cells were first depleted by ammonium chloride treatment. To remove J11d⁺ erythroid cells, spleen cells were washed and incubated for 30 min at 4°C in the presence of J11d hybridoma supernatant at a 1:4 dilution. Cells were then washed and incubated with rabbit complement (Cedarlane, Hornsby, Ontario, Canada) at a 1:10 dilution in cytotoxicity medium (Cedarlane) at 37°C for 45 min. Viable cells were then harvested over Lympholyte columns (Cedarlane).

**DNA analysis**

T cells (~1×10^6) were washed and fixed in 70% ethanol at ~20°C for several days at 10^6 cells/ml. The cells were then washed and resuspended in 1 ml of Ca^{2+}, Mg^{2+}-free PBS to which 0.1% Triton X-100, 0.1 mM EDTA and 50 µg/ml RNase were added, and incubated for 1 h at 37°C. This incubation period allows low mol. wt DNA to escape through the permeabilized membranes (14). Cells were then washed and resuspended in staining buffer (0.1 mM EDTA, 0.1% Triton X-100 and 50 µg/ml of propidium iodide) at room temperature in the dark for 4–12 h. Cells were then filtered through nylon mesh and analyzed on a Becton Dickinson (Mountain View, CA) FACScan flow cytometer using Lysys II software.

**Proliferation assays and mixed lymphocyte responses**

In some assays, responder T cells were diluted to 2×10^5 cells/ml in CM and cultured with syngeneic irradiated (2000 cGy) spleen cells as filler cells and 0.1 µg/ml of soluble anti-CD3 antibody for 72 h. Then 1 µCi of [³H]thymidine was added to each culture for 18 h and the amount of incorporated thymidine was measured in a β-scintillation counter. In other assays, donor T cells were stimulated with plate-bound anti-CD3 plus or minus anti-CD28 antibodies as previously described (15). For mixed lymphocyte reactions, responder spleen cells were diluted to a concentration of 2.5×10^6 T cells/ml in CM. Irradiated spleen cell stimulators (2000 cGy) were from BALB/c and BALB.K or C3H mice at 5×10^6 cells/ml. The mixed lymphocyte reactions were incubated for 72 h and then 1 µCi of [³H]thymidine was added to the cultures for a subsequent 18 h. The cells were then harvested and the
amount of thymidine incorporation measured in a 
\(\gamma\)-scintillation counter.

**Redirected lysis assays**
P815 tumor targets in exponential growth phase were col-
clected by centrifugation, resuspended in two drops of 100% 
FCS and radiolabeled with 50 \(\mu\)l of sodium chromate 
(7.14 mCi/ml) (Dupont, NEN, Boston, MA) for 1 h. Effector 
cells, purified from spleen cells using Lympholyte separation 
medium, were added at varying effector:target ratios in 100 
\(\mu\)l of CM to individual wells of a U-bottom plate. Anti-CD3 
antibody (0.2 \(\mu\)g) was then added at a final concentration of 
1 \(\mu\)g/ml (10). Chromium-labeled targets were washed 3 times 
with \(\alpha\)-MEM + 1% FCS and 100 \(\mu\)l of target cells (2\(\times\)10^4/ml 
in CM) were added to each well. The plates were centrifuged 
at 600 r.p.m. for 3 min and then incubated at 37°C for 4 h. Plates 
were then centrifuged at 800 r.p.m. for 5 min and 100 
\(\mu\)l of the supernatant transferred to Fisherbrand flint glass 
tubes (Fisher Scientific, Pittsburgh, PA) and counted in a 
\(\gamma\)-counter (CompuGamma Model 1282; LKB, Stockholm, 
Sweden). Total release (TR) was measured by lysis of tumor 
targets with 1% acetic acid and spontaneous release (SR) 
was measured in the absence of effector cells. Percent 
cytotoxicity was determined by the ratio (c.p.m. – SR)/ 
(TR – SR)\times 100%.

**Immunofluorescence**
Cell staining was performed after first blocking non-specific 
binding with a 10 min incubation at room temperature with 
10 \(\mu\)l of mouse serum (Cedarlane) and 40 \(\mu\)l of 2.4G2 culture 
supernatant. Cells (5\(\times\)10^5) were then allowed to react with 
pre-titrated doses of antibodies for 20 min, washed, incubated 
with 7-AAD to exclude dead cells and then analyzed on a 
FACScan flow cytometer (Becton Dickinson) using Lysys II 
software.

**Intracellular cytokine staining**
The method of Ferrick et al. (16) was mainly followed. Cells 
were suspended at a concentration of 5\(\times\)10^6/ml in CM and 
incubated at 37°C for 4 h in the presence of 5 \(\mu\)g/ml Brefeldin 
A (unstimulated) or Brefeldin A plus 10 ng/ml PMA and 500 
ng/ml ionomycin (stimulated). Cells were then washed and 
non-specific binding blocked with 2.4G2 and mouse serum 
in a total volume of 90 \(\mu\)l, and fixed in 75 \(\mu\)l of Solution A 
(Caltag) for 30 min at room temperature. After washing in 
Ca^{2+}-Mg^{2+}-free PBS, cells were stained at room temperature 
with 0.2 \(\mu\)g of CD4– and CD8–TriColor, and IL-4–PE (0.2 
\(\mu\)g) and IFN-\(\gamma\)–FITC (0.1 \(\mu\)g) in 75 \(\mu\)l of solution-B (Caltag) for 30 
min. Cells were then washed and analyzed as described 
above.

![Fig. 1](image1.png)

**Fig. 1.** The severity of GVHD is directly proportional to the number of injected donor cells. C.B-17-SCID mice were sublethally irradiated 
with 275 cGy and immediately injected with 10^6 (open circles) (n = 
19) or 3\(\times\)10^4 (closed circles) (n = 24) PLNC from B6 mice. Cages 
were inspected every 1–2 days and animals were sacrificed when 
they were moribund or had lost 30% of their starting body weight. Results were independent of the sex of donor or recipient mice.

![Fig. 2](image2.png)

**Fig. 2.** Absence of histological changes of GVHD in mice injected with low numbers of allogeneic PLNC. Liver sections were prepared and 
stained with hematoxylin & eosin. (A) The liver of a sublethally irradiated SCID mouse injected 3 months earlier with 10^6 B6 spleen cells 
showing lymphocytic periportal infiltrates and bile ducts demonstrating apoptosis and drop-out of duct cells. Piece-meal necrosis and fatty 
infiltration are found in the surrounding liver parenchyma. (B) From a mouse injected with 3\(\times\)10^5 PLNC after sublethal irradiation 120 days 
earlier, the portal areas are intact with scant cellular infiltrates.
Production of GVHD

C.B-17-SCID mice were irradiated with 275 cGy from a γ source (137Cs) (Gammacell 40 Exactor, Nordion International, Kanata, Ontario, Canada) on the same day as the injection. Inguinal lymph node cells were obtained from donor mice and varying numbers were injected into the tail veins of SCID hosts. Mice were examined daily and sacrificed if moribund.

Results

Small numbers of donor T cells do not cause acute GVHD in sublethally irradiated SCID mice

Injection of 10^6 B6 PLNC into sublethally irradiated SCID mice caused an aggressive form of GVHD with a mean survival time of ~15 days (6) (Fig. 1). Injection of 3×10^4 or 3×10^3 B6 LNC did not cause noticeable GVHD (Fig. 1). More than 100 mice have been injected with low numbers of donor cells and have remained healthy for up to 1 year post-injection.

Characteristic pathological liver changes of GVHD (17) could be found after 3 months in sublethally irradiated SCID mice injected with B6 spleen cells (Fig. 2a). These lesions were not found even at 4 months post-injection of low numbers of PLNC (Fig. 2b). Note that SCID mice injected with high numbers of PLNC could not survive for 3 months.

Low numbers of allogeneic T cells persist for long periods in sublethally irradiated SCID mice

A trivial explanation for the absence of GVHD after injection of low numbers of T cells might be clearance of donor cells by radioresistant host NK cells that recognize the complete MHC mismatch between B6 donor cells and C.B-17-SCID mice (18). Injection of graded numbers of B6 PLNC into C.B-17-SCID mice not given irradiation confirmed that 5×10^5 donor cells were rejected within the first few days (Fig. 3).

The number of donor cells in the spleens of sublethally irradiated hosts was determined in order to document their survival. As shown in Fig. 3, 3×10^4 T cells reached a plateau level of ~2×10^5 donor cells per spleen by the second week and remained at this level for up to 1 year.

Samples of 3×10^3 PLNC behaved either the same as 3×10^5 PLNC and reached the plateau level after a couple of weeks or else were not found from the first determination at around days 5–7. This behavior suggested that 3×10^3 PLNC was near the limit below which injected cells could be rejected by radioresistant mechanisms in SCID mice. Consequently, the rest of the experiments described in this paper were performed using 3×10^4 B6 PLNC as the initial inoculum.

When 10^6 PLNC were injected, cell numbers also increased during the first week but surprisingly reached approximately the same plateau level as an initial injection of 3×10^4 PLNC. They could not be followed any longer than 2–3 weeks due to the morbidity of the injected animals.

In summary, Fig. 3 shows that, by ~2 weeks after injection, the load of donor T cells was the same in animals initially injected with 3×10^4 or 10^5 PLNC. However, in the former case the animals survived without evidence of disease, while, in the latter, they became moribund.

Similar expansion of potentially alloreactive T cells in F1 SCID mice

To determine the effect of host H-2^D expression on donor T cell accumulation, B6 T cells were injected into sublethally irradiated (B6×C.B-17) F1 SCID mice. At least for the first 2 months after injection, B6 T cells behaved much the same in F1 SCID mice as they did in fully MHC disparate C.B-17-SCID mice (Fig. 3). In contrast, syngeneic BALB/c T cells expanded to reach a plateau level in sublethally irradiated C.B-17-SCID mice that was higher than for allogeneic cells (Fig. 3). Moreover the ratio of CD4^+ to CD8^+ cells was skewed towards CD4, whereas it was inverted for B6 cells (legend to Fig. 3).

Destruction of host hematopoietic cells by 10^6 but not by 3×10^5 B6 PLNC

The number of host spleen cells was also determined as a function of time after irradiation and injection of varying numbers of donor T cells (Fig. 4). The number of mononuclear spleen cells in an unirradiated SCID mouse is ~10^7. Shortly after a radiation dose of 275 cGy, this number fell to ~10^5 cells but recovered by the end of the second week to the level of an otherwise untreated mouse (Fig. 4).

After injection of 10^6 B6 PLNC, no recovery of host hematopoietic cells was observed. Instead, their numbers in spleen fell <10^5 by the second week, consistent with the interpretation that hematopoietic atrophy was the terminal event.

In contrast, the recovery of host cells from radiation was virtually unimpaired in animals injected with low numbers of allogeneic T cells. If anything, there was an initial overshoot of host cells, possibly in response to secretion of growth factors such as IL-3 or granulocyte macrophage colony stimulating factor from the proliferating donor cells. The majority of host cells were erythroblasts (by examination of Wright's-stained cytopins). Approximately 10% were Mac-1^+ and many were in cycle (not shown). After several months, the total number of host cells declined regardless of whether donor cells were injected. The explanation for this decline was not clear but may be related to exhaustion of SCID hematopoietic precursors. Cells homozygous for the SCID defect are known to be more radiosensitive than wild-type cells (19).

The results of Figs 3 and 4 showed that allogeneic T cells could persist in a sea of host antigen for many months after an initial injection of low numbers of cells. For the rest of this paper, such animals will be called low-dose chimeras (LDC).

Characterization of donor T cells in GVHD: persisting cells develop an intrinsic proliferative defect that cannot be reversed by IL-2

Figures 3 and 4 demonstrated the simultaneous presence of donor cells and normal numbers of host hematopoietic cells in LDC. Despite this potentially inflammatory situation, there was no overt GVHD suggesting that the donor cells were functionally tolerant of host antigens.

To study the in vitro proliferative responses of donor T cells from short-term LDC was technically difficult due to their low numbers. Two strategies that gave equivalent results were
Exhaustion of allogeneic cells in irradiated SCID mice

Fig. 3. Expansion and long-term persistence of allogeneic T cells in irradiated SCID mice. PLNC from B6 or BALB/c (congenic with C.B-17 mice and differing at the Ig heavy chain allotype locus) donors were injected into sublethally irradiated (four groups) or unirradiated (1 group) SCID mice. At serial times thereafter, host spleen cell suspensions were stained with antibodies against Thy-1.2 and H-2b, and the percentage of H2b\(^+\)Thy-1.2\(^+\) cells was determined using flow cytometry. The number of donor cells was then calculated by multiplying this number by the total number of splenocytes. The number of cells in mice initially injected with \(10^6\) B6 PLNC could not be determined beyond 15 days because of the state of the animals. Approximately 70% of PLNC are T cells and the number of cells in the spleens on day 0 was assumed to be 20% of the injected dose (58). The average ± SE of the results from at least 5 mice per data point are reported. Only two mice at 365 days were examined. The initial ratio of CD4\(^+\) to CD8\(^+\) T cells, measured by flow cytometry and the appropriate antibodies, was ~1.5:1 for B6 or BALB/c mice while the CD4/CD8 ratio at \(>100\) days was 0.56 ± 0.03 \((n = 4)\) for LDC mice and 3.8 ± 1.2 \((n = 2)\) for host mice injected with syngeneic PLNC. Key: ○, \(10^6\) B6 LNC into irradiated C.B-17-SCID hosts; ●, \(3 \times 10^4\) B6 LNC into irradiated C.B-17-SCID hosts; □, \(3 \times 10^4\) B6 LNC into irradiated F1 SCID hosts; ■, \(3 \times 10^4\) BALB/c LNC into irradiated C.B-17-SCID hosts; Δ, \(5 \times 10^5\) B6 LNC into unirradiated C.B-17-SCID hosts.

employed. The first was to sort H-2\(^b\)\(^+\)Thy-1.2\(^+\) cells or Thy-1.1\(^+\) cells, when Thy-1.1 was used as a marker for donor cells, on a cell sorter and to use similarly purified naive B6 PLNC as controls in proliferation assays. Purity of the sorted T cells was >95% (unpublished results). The second strategy used the HSA-specific mAb, J11d, to deplete HSA\(^+\) cells by complement-mediated cytotoxicity. Preliminary studies had revealed that the majority of host cells in the spleens of SCID mice and LDC were J11d\(^+\) while the donor cells were J11d\(^-\). This negative selection procedure could only enrich the percentage of donor cells several fold, to ~10% (unpublished results). The percentage of donor T cells in the spleens of long-term LDC was usually high enough (10–20%) that functional assays could be performed without need for further enrichment procedures.

The functional properties of B6 donor T cells from long-term LDC were compared to B6 T cells, from normal donors and after injection into C.B-17- and B6-SCID mice, and to BALB/c T cells, after injection into C.B-17-SCID mice. Naive B6 cells were used to compare the behavior of donor T cells after adoptive transfer to their initial behavior. The most important control is the study of B6 T cells in irradiated B6-SCID mice in the absence of alloantigenic stimuli. Unfortunately, B6-SCID mice are inherently 'leaky' (6) so that, without a genetic marker, it is difficult to distinguish adoptively transferred B6 T cells from cells that undergo thymic development. We have used B6-Thy-1.1 T cells in order to distinguish exogenous from endogenous T cells in B6-SCID (Thy-1.2\(^+\)) mice. However the thy-1 polymorphism provides an antigenic stimulus for transferred Thy-1.1 T cells (7). Indeed, at low doses, we found evidence for activation of Thy-1.1\(^+\) donor cells [i.e. inversion of the CD4/CD8 ratio and \(\text{in vitro}\) anergy (see below)]. Irradiated adult C.B-17-SCID mice are much less leaky. Therefore, the adoptive transfer of syngeneic BALB/c T cells into sublethally irradiated C.B-17-SCID mice was used to ensure that the behavior of long-term surviving T cells was not due to some non-specific, poorly understood mechanism.

Observations on the \(\text{in vitro}\) functions of T cells after adoptive transfer into allogeneic and syngeneic hosts as a function of initial cell number are summarized in Table 1. Cells from long-term LDC could not respond to host or third-party antigens (Table 1, rows 7 and 16; column 4) and this lack of response could not be rescued by IL-2 (Table 1, row 8, column 4). The response to a mitogenic stimulus (anti-CD3) was depressed compared with fresh B6 T cells and only minimally increased by provision of co-stimulation by simultaneous cross-linking of CD28 molecules (Fig. 5). T cells taken
In comparison, T cells actively mediating GVHD directly produced IFN-γ ex-vivo and the level of production was significantly enhanced by stimulation (Fig. 6). Note that if cells are not making a cytokine directly but do so after stimulation, this is thought to mean that they previously produced the cytokine (20).

IL-4 production by mitogen-activated long-term LDC spleen cultures could not be detected in a bioassay and was not measurable in cultures of mitogen activated naïve T cells (data not shown). IL-4 production was also not demonstrable using intracellular cytokine staining of T cells from long-term LDC or from mice actively undergoing GVHD (Fig. 6). Taken together with their potential ability to produce IFN-γ (Fig. 6), this finding suggests that donor cells have not differentiated into Tc2 or Tr2 cells (21).

Donor cells reside in multiple organs

Donor cells could be found in multiple organs of host mice as shown in Fig. 7. The majority of donor cells were found in the spleen as described in the figure legend. Low percentages of donor cells were also found in the thymus, bone marrow and blood. Note that animals were exsanguinated before collection of thymuses and bone marrow to avoid contamination with peripheral blood. A significant increase in the percentage of donor cells was found in the lymph nodes and the peritoneal cavity. Despite the increased proportion, the total number of donor cells was low at these sites because of their low total cellularity (legend to Fig. 7).

PLNC were used as the source of donor T cells to avoid potential reconstitution by donor hematopoietic stem cells (8). Evidence that the long-term persisting T cells derived from the initial inoculum of mature T cells is shown in Fig. 7. The only H-2b+ cells were also Thy-1.2+. If donor hematopoietic reconstitution had occurred, Thy-1.2+H-2b+ cells, such as B cells, should have developed. Note that a population of Thy-1.2+H-2b– cells is seen in Fig. 7. This population may represent NK or T cells that develop from ‘leaky’ SCID stem cells after recovery from sublethal irradiation (22,23) but was an inconsistent finding.

The cell cycle status of donor cells was determined by flow cytometry analysis of propidium iodine staining of DNA. Figure 7(g and i) shows that the number of donor T cells from long-term LDC in the G2/S phase of the cell cycle was little higher than the number for T cells from naïve B6 mice. In contrast, significantly higher numbers of donor cells were in cycle within the first few weeks after injection (Fig. 7h).

The surface phenotype of donor cells in LDC is consistent with prior activation

Cell size and surface expression of CD25, CD3 and CD44 on donor T cells from LDC were determined by flow cytometry (Fig. 8). As described for memory T cells (24), CD44 was up-regulated in LDC (MFI = 1485) and in SCID mice given initial injections of high numbers of donor T cells (MFI = 1993) when compared with donor B6 mice (MFI = 675) (Fig. 8). Despite this phenotypic change, donor T cells in LDC over 100 days after initial injection were only slightly larger on forward scatter (mean = 120) than naïve T cells (mean = 112) (Fig. 8) although, shortly after the injection of high numbers of donor T cells, the majority were significantly larger
Exhaustion of allogeneic cells in irradiated SCID mice

Table 1. Functional properties of donor T cells in LDC

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Stimulators</th>
<th>IL-2 (25 U/ml)</th>
<th>Stimulation indexa</th>
<th>Maximal response (%)b</th>
<th>Cytotoxic activity (%) (10:1 E:T ratio)c</th>
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<tbody>
<tr>
<td>B6</td>
<td>–</td>
<td>+</td>
<td>7.4 ± 2.3 (n = 11)</td>
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<tr>
<td>Long-term LDC</td>
<td>–</td>
<td>+</td>
<td>1.5 ± 0.2 (n = 6)</td>
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<tr>
<td>Short-term LDC</td>
<td>–</td>
<td>+</td>
<td>27.6 ± 19.4 (n = 4)</td>
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<tr>
<td>HD</td>
<td>–</td>
<td>+</td>
<td>30.1 ± 10.3 (n = 4)</td>
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<tr>
<td>B6</td>
<td>BALB/c</td>
<td>–</td>
<td>5.8 ± 2.1 (n = 4)</td>
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<tr>
<td>B6</td>
<td>BALB/c</td>
<td>+</td>
<td>9.6 ± 2.9 (n = 7)</td>
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<td>Long-term LDC</td>
<td>BALB/c</td>
<td>+</td>
<td>0.8 ± 0.1 (n = 3)</td>
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<tr>
<td>Short-term LDC</td>
<td>BALB/c</td>
<td>+</td>
<td>1.1 ± 0.1 (n = 5)</td>
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<tr>
<td>Short-term LDC</td>
<td>BALB/c</td>
<td>+</td>
<td>0.95 ± 0.3 (n = 3)</td>
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<tr>
<td>HD</td>
<td>BALB/c</td>
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<td>0.4 ± 0.1 (n = 4)</td>
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<tr>
<td>HD</td>
<td>BALB/c</td>
<td>+</td>
<td>1.7 ± 0.3 (n = 3)</td>
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<td>Syngeneic HDd</td>
<td>BALB/c</td>
<td>–</td>
<td>32.9 ± 3.0 (n = 2)</td>
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<tr>
<td>Syngeneic HD</td>
<td>C3H</td>
<td>–</td>
<td>11.4 ± 0.8 (n = 2)</td>
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<tr>
<td>B6</td>
<td>BALB.K</td>
<td>–</td>
<td>4.6 ± 1.8 (n = 4)</td>
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<tr>
<td>Long-term LDC</td>
<td>BALB.K</td>
<td>–</td>
<td>1.4 ± 0.6 (n = 5)</td>
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<tr>
<td>Short-term LDC</td>
<td>BALB.K</td>
<td>–</td>
<td>1.4 ± 0.1 (n = 2)</td>
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<tr>
<td>B6 + LDC</td>
<td>BALB/c</td>
<td>–</td>
<td>101 ± 3 (n = 2)</td>
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<tr>
<td>B6 + LDC</td>
<td>BALB.K</td>
<td>–</td>
<td>144 ± 29 (n = 3)</td>
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Donor T cells were obtained from the spleens of normal B6 donor mice, long-term LDC and short-term LDC or from sublethally irradiated SCID mice injected with high numbers of B6 PLNC (HD). T cells were purified from short-term LDC by either FACS sorting or depletion of J11D+ cells by complement cytotoxicity and the equivalent number of T cells was compared in mixed lymphocyte reactions. Proliferation was measured by thymidine uptake assays as described in Methods.

aStimulation index is the thymidine uptake in the mixed lymphocyte reaction divided by the sum of the uptake of the stimulator and effector cells cultured alone or of the effector cells alone for the first four rows. The average ± SE of the indicated number of individual experiments is reported.

bTo measure the suppressor activity of donor T cells in long-term LDC, the same number of B6 and long-term LDC T cells were mixed and used as effectors in mixed lymphocyte reactions. The thymidine uptake of the mixture, calculated as the difference between the mixed lymphocyte reaction and the individual stimulator and effector cells cultured alone, was divided by the thymidine uptake of the mixed lymphocyte reaction without the addition of LDC cells and multiplied by 100%. The average ± SE of the indicated number of individual experiments is reported.

cRedirected lysis assays were performed as described in Methods. The same number of donor T cells, determined by flow cytometry, from a LDC that had been injected 6 months earlier and a sublethally irradiated SCID mouse given 10^6 donor T cells, 6 days earlier, was compared. Percent cytotoxicity ± SEM of four different measurements is reported.

dAs a syngeneic control, 10^6 B6-Thy-1.1 T cells were injected into sublethally irradiated B6-SCID mice and studied 22 days later.

(mean = 147) (Fig. 8, right column). The TCR receptor density on persisting donor T cells in LDC was also significantly less (MFI = 45) than on both naive cells (MFI = 167) and cells actively mediating GVHD in vivo (MFI = 160) (Figs 8 and 10b). TCR down-regulation is also a marker for recent activation (25). CD25 expression on T cells from long-term LDC was about the same as naive donor T cells and not as high as on donor T cells 6 days after initiation of GVHD with high doses of cells (Fig. 8).

Rechallenge of LDC with naive, congenic B6 T cells

A possible explanation for the behavior of donor cells in LDC was that the host environment could no longer support the proliferation and differentiation of alloreactive cells. Down-regulation of immunogenic antigen and co-stimulatory signals required to support an alloresponse or the presence of a suppressor cell population, despite evidence that donor T cells in LDC were not suppressors in vitro (Table 1, rows 18-19), could possibly account for this altered host environment. To evaluate these possibilities, LDC were challenged with a second injection of PLNC from B6-Thy-1.1 mice. The allelic difference at the Thy-1 locus allowed the rechallenge cells to be distinguished from the pre-existing tolerant donor cells.

As shown in Fig. 9(a), B6-Thy-1.1 PLNC increased in number and percentage compared with pre-existing Thy-1.2+ cells in the spleens of LDC, arguing that the tolerant state of the latter was not caused by suppressor cells or insufficient immunogenic host antigen. Further evidence that Thy-1.1+ T cells were responding to host antigens was that some of the rechallenged mice became moribund within 1 week, likely due to GVHD. Moreover, the number of Thy-1.1+ T cells increased to the same level as a primary challenge of sublethally irradiated SCID mice with >10^9 B6 T cells (Fig. 3). Note that the Thy-1.2+ CD3+ cells in Fig. 9(b) represent the donor cells remaining from the initial injection which clearly have down-regulated TCR densities compared to the responding cells from the second injection (see also Fig. 8).

Discussion

In this paper, low numbers of allogeneic B6 T cells (low dose or LD) were shown to increase ~50-fold in number after adoptive transfer into sublethally irradiated C.B-17-SCID mice and persist for up to a year without causing GVHD. In contrast, an initial injection of 50-fold higher numbers of donor T cells...
Radiation was presumably required to prevent the immediate rejection of donor PLNC by NK cells (18,26) that recognize the full MHC mismatch between B6- and C.B-17-SCID mice (Fig. 3). Prolonged persistence of allogeneic cells suggests that the host NK cells that recover from irradiation are tolerant of donor cells (27) or that another cell type required for allogeneic resistance does not survive sublethal irradiation.

Despite the clear relationship to clinical outcome, the early behavior of the donor T cells after injection did not seem to depend on their initial number. In both cases, the total number of donor T cells reached a plateau level of ~3×10^5 cells/spleen (Fig. 3). Even with initially low numbers of donor cells, host superantigen-reactive T cells, represented by CD4^+ Vβ3^+ T cells stimulated by the open reading frame protein of the mtv-6 provirus in the SCID genome, expanded up to 10^4-fold within the first month (unpublished results). This result suggests that failure of low doses of T cells to cause GVHD was not because the number of transferred immunocompetent precursors was below the frequency required to respond to host antigens. Significant numbers of donor T cells could not be found in other sites (Fig. 7).

Superficially, the apparent expansion of initially high numbers of donor T cells (Fig. 3), followed by a phase of deletion, is similar to other in vivo experiments that lead to peripheral tolerance, such as the response of T cells following superantigen injection or transgenic T cells after their injection into mice expressing their specific antigen (28–30). However, the decrease in donor T cell numbers may simply reflect the morbidity of mice with GVHD at this time. In contrast to normal superantigen responses (31), CD4^+ Vβ3^+ T cells were not deleted at the time that total numbers of donor T cells were decreasing (unpublished results and manuscript in preparation).

Within the first month after injection, both LD and HD T cells secreted IFN-γ directly ex vivo (Fig. 6 and unpublished results), and HD T cells could mediate perforin-dependent cellular cytotoxicity (Table 1, row 4, column 6). Also within the first month, HD and LD T cells proliferated strongly in response to exogenous IL-2 (Table 1) but were growth inhibited after reactivation through the TCR in vitro (Table 1 and Fig. 5). We and others have previously shown that this growth arrest reflects activation-induced T cell death, and is mediated by multiple factors including perforin, tumor necrosis factor-α and IFN-γ (7,32,33).

Subsequently, LD T cells entered a long-lived non-functional state, characterized by small size (Fig. 8), unresponsiveness to exogenous IL-2 or allogeneic stimuli (Table 1), lack of direct ex-vivo secretion of IFN-γ (Fig. 6), or killing in a redirected lysis assay (Table 1). Failure of third-party killing could possibly be due to an alteration in frequency of antigen-reactive cells, following the expansion phase of the response to BALB/c antigens, rather than to a tolerance process. However, third-party responses were decreased early (Table 1), even before the expansion process was finished. Moreover, the persisting cells showed evidence of prior activation indicated by up-regulation of CD44 and down-regulation of the TCR cell surface density (Fig. 8), and the failure to respond to mitogens suggested a global defect in the persisting T cells. The fate of HD T cells could not be determined after several weeks because of the morbidity of host mice.

Further evidence for the proliferative arrest of T cells in LDC was provided by their behavior after transfer into a

Fig. 5. Proliferative unresponsiveness of T cells from long-term LDC cannot be restored by activation with anti-CD3 or anti-CD28 antibodies. (Top panel) The equivalent numbers of T cells (2×10^5) from the spleens of normal B6 donors (1), sublethally irradiated SCID mice injected with 10^6 allogeneic (B6) (2) or syngeneic (BALB/c) T cells (4) 6 days earlier, or LDC injected at least 100 days earlier with 3×10^4 PLNC (3) were plated on microtiter plates coated with FCS, anti-CD3 or anti-CD3 and anti-CD28 antibodies in the presence of 50 U/ml IL-2, as described in Methods. The proliferative response, determined by [3H]thymidine uptake, is reported as the average ± SE of four replicate cultures. The reported results are representative of at least six separate experiments, except for the syngeneic transfer which was repeated twice. (Lower panel) The equivalent T cell numbers (2×10^5) from the spleens of normal B6-Thy-1.1 donors and sublethally irradiated B6-SCID mice injected 21 days earlier with 10^6 B6-Thy-1.1 PLNC and also from BALB/c donors or sublethally irradiated C.B-17-SCID mice injected 20 days earlier with 3×10^4 BALB/c PLNC were stimulated with mitogens in vitro. The average ± SE of three replicate cultures is reported and is representative of two separately injected host mice.

(high dose or HD) caused a severe form of GVHD within 2 weeks.

Radiation was presumably required to prevent the immediate rejection of donor PLNC by NK cells (18,26) that recognize
Exhaustion of allogeneic cells in irradiated SCID mice

Fig. 6. Donor cells in LDC can produce limited amounts of IFN-γ after mitogenic stimulation. The capability of donor T cells from long-term LDC (b) compared with donor T cells from mice 8 days after initiation of acute GVHD (a) to produce IFN-γ and IL-4 was determined by intracellular cytokine staining and flow cytometric analysis. Cells were stained after a 4 h incubation in the presence of Brefeldin A with and without PMA and ionomycin.

Freshly irradiated secondary host (data not shown). Secondary transfer of $3 \times 10^4$ T cells from LDC caused no apparent morbidity. Several weeks after passage, H-2${}^b$ T cells could only be found in the peritoneal cavity. The total number was $5 \times 10^3$, suggesting that donor T cells from the LDC did not proliferate although they could persist in vivo. In contrast, the same number of donor T cells expanded 50- to 100-fold after primary transfer, although clinical GVHD was not observed (Fig. 3).

The finding that polyclonal, alloreactive T cells eventually became unresponsive after chronic stimulation in vivo is consistent with, and extends, recent observations with TCR transgenic T cells that encounter persistent antigen. Lanoue et al. (34) showed that transgenic CD4$^+$ T cells, specific for an influenza hemagglutinin epitope, initially proliferated after transfer into hemagglutinin transgenic hosts. However, by day 30 after transfer, the majority of transgenic CD4$^+$ T cells had been deleted and the remainder were anergic. Tanchot et al. (35) showed that transgenic CD8$^+$ T cells specific for the HY male antigen became anergic after adoptive transfer under conditions of male antigen excess. This state of proliferative anergy was not restored by ionomycin and PMA, which restored IL-2 and IFN-γ production and IL-2 R expression, and was associated with increased IL-10 production. In contrast, TCR down regulation (Fig. 8), rather than production of Th2 cytokines, was associated with the tolerant state of chronically in vivo allo-activated CD4$^+$ and CD8$^+$ T cells.

Previous attempts to follow the fate of mature donor T cells in GVHD were performed in irradiated hosts given donor stem cells and peripheral T cells that could be distinguished by congenic markers (36). The results of such studies suggested that the mature T cells disappeared within several months after the initiation of GVHD and that chronic GVHD was maintained by T cells of donor origin that developed in the host thymus. Although these findings may apply to pediatric bone marrow transplantation, thymic reconstitution is limited in adult recipients of stem cell transplants (37) and may be non-existent for the first 6 months (38). The model of GVHD used in the studies presented here, involving sublethally irradiated SCID hosts and donor T cells from peripheral lymph nodes, may be more representative of BMT in adults where T cell reconstitution is dominated by adoptively transferred peripheral T cells (39).

The long-term survival of tolerant donor T cells described in this paper may depend on the absence of thymic development in SCID mice. Anergic autoreactive transgenic B cells
Fig. 7. Non-cycling donor T cells are found at multiple sites in LDC. Mice were exsanguinated and cells from the (a) spleen, (b) thymus, (c) pooled inguinal and mesenteric lymph nodes, (d) peritoneal washings, and (e) bone marrow were harvested, stained with anti-Thy-1.2–PE and H-2b–FITC antibodies, and analyzed by flow cytometry. Spleen cells from a mouse that was only sublethally irradiated were analyzed in the same way (f) as a negative control. Shown are the results from an LDC 77 days post-initial injection but are representative of animals up to 160 days. The total cellularity at each site for this example was: spleen \((7 \times 10^6)\), bone marrow \((1.8 \times 10^7)\), thymus \((5.3 \times 10^6)\), peritoneum \((2.5 \times 10^5)\) and pooled lymph nodes \((4 \times 10^4)\). DNA content histograms from B6 control animals (g) and a day 162 LDC (i), gated on H-2b+ spleen cells that were CD4+ or CD8+, show that donor T cells in the LDC were mainly in the G1/G0 phase of the cell cycle. A significantly higher number were in cycle 6 days after induction of GVHD with high doses of donor T cells (h).

Persist in the absence of B cells with a normal Ig repertoire but, in their presence, do not compete successfully for access to survival niches in the germinal centers of secondary lymphoid organs. A similar competition for T cell survival niches between functional and tolerant donor T cells, probably in the parafollicular areas of secondary lymphoid organs, may ‘dilute out’ the latter and account for their disappearance in other models of GVHD. Indeed, Tanchot and Rocha (41,42) have recently shown that virgin and tolerant CD8+ TCR transgenic T cells persist for prolonged times in the absence of thymic output and that thymic emigrants cause the deletion of tolerant, but not memory, cells.

Note that syngeneic T cells appeared to expand more than allogeneic cells (Fig. 2) after adoptive transfer into sublethally irradiated SCID mice. This somewhat paradoxical finding may be related to different TCR affinities for host antigens. Contact...
Exhaustion of allogeneic cells in irradiated SCID mice

Fig. 8. Activation markers on persisting donor T cells in LDC. Spleen cells from a B6 control (left column) and LDC (center column) mouse were harvested and, after gating on H-2^{b+} cells that were CD4^{+} or CD8^{+}, subjected to flow cytometric analysis of the following activation markers: (a) forward scatter (cell size), (b) CD44, (c) IL-2Rα (CD25), (d) TCR density and (e) isotype controls. The results shown are from a day 360 mouse but are representative of mice at least 6 weeks post-injection of allogeneic cells. For comparison, the same activation markers on HD T cells 6 days after initiation of GVHD is shown (right column).

with host antigens may be required for the prolonged survival of donor cells as has been recently reported for endogenous peripheral T cells (43,44). All BALB/c T cells that were positively selected in the thymus would have weak binding affinities for host H-2^{d} molecules (45). The antigen reactivity of the initially injected allogeneic cells may be considered in three groups: (i) superantigen reactive cells [V_{p}3 and V_{p}5 cells reactive against mtv-6 (~5% of the TCR repertoire)] (46), (ii) alloreactive cells (1–2% of the TCR repertoire) (47) and (iii) the remaining cells which, although not host reactive, must have been positively selected on H-2^{b} molecules. The latter cells should not persist in host mice if antigen is required as a survival signal (48). This population might have been expected to survive in F_{1} SCID mice where the selecting molecules are present on host hematopoietic cells. However, the expansion of donor T cells was no higher than in fully allogeneic SCID mice (Fig. 3, open squares), possibly suggesting their suppression by the alloreactive cells. Based on their increased CD44 and down-regulated TCR expression, possibly representing recent contact with antigen (24,25), the remaining T cells may represent alloreactive cells. The lower plateau level reached by proliferating allogeneic T cells may be related to their exhaustive differentiation (29) in response to alloantigens. In contrast, the affinity of survival signals for
syngeneic cells may be low enough that they can expand without being driven to exhaustion.

We speculate that the behavior of donor T cells is independent of their initial number and that chronic activation causes them to enter a globally unresponsive state. These results are consistent with the 'geographical' view of immune responses espoused by Zinkernagel and co-workers (49). Because host antigens are 'everywhere' in lymphoid organs in GVHD, the fate of host reactive cells is to become 'exhausted'. However, before exhaustion they may cause significant immunopathology. In this case, immunopathology is GVHD caused by the resulting 'cytokine storm' (1). Low doses of T cells also become exhausted but do not cause clinically significant immunopathology simply because the 'cytokine storm' is not large enough. Our ability to identify them in sublethally irradiated SCID hosts suggests that 'exhausted' cells are small, their surface phenotype is CD44hi, TCRlo, and they are irradiated SCID hosts suggests that 'exhausted' cells are large enough. Our ability to identify them in sublethally irradiated SCID hosts simply because the 'cytokine storm' is not large enough. Low doses of T cells also become exhausted but do not cause clinically significant immunopathology simply because the 'cytokine storm' is not large enough. Our ability to identify them in sublethally irradiated SCID hosts suggests that 'exhausted' cells are small, their surface phenotype is CD44hi, TCRlo, and they are

Acknowledgements

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Abbreviations

BMT bone marrow transplantation
C57BL/6J B6
CM complete medium
GVHD graft versus host disease
HD high dose
HSA heat-stable antigen
LD low dose
LDC low-dose chimera
mtv mammary tumor virus
PE phycoerythrin
PLNC peripheral lymph node cells
PMA phorbol myristate acetate

References


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Exhaustion of allogeneic cells in irradiated SCID mice