Correlation between Lymphocyte-induced Donor-specific Tolerance and Donor Cell Recirculation
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Summary
Intravenous infusion of mice with major histocompatibility complex (MHC) incompatible lymphocytes can inhibit the response of recipient T cells capable of recognizing the injected cells, and can enhance survival of grafts sharing MHC with the injected cells. However, neither T cell inactivation nor graft survival enhancement is always achieved. This is particularly true for donor cells that are fully allogeneic (as compared to semiallogeneic) to the recipient. We show here that both donor-specific induced response reduction and graft survival enhancement are directly correlated with the ability of the injected lymphoid cells to persist in the recirculating lymphocyte pool of the host. Whether donor cells persist correlates inversely with the level of natural killer cell (NK) activity in the host. Fully allogeneic cells can only persist in hosts with low NK activity and can then induce response reduction. Both persistence and response reduction are abrogated by injection of the host with polyI:C, a treatment that boosts host NK activity. The same treatment also destroys the ability of semiallogeneic injected cells to persist, to induce response reduction, and to enhance skin graft survival.

Materials and Methods
Mice. (C57BL/6 × DBA/2)F1 (B6D2F1, H-2b/d), C57BL/6 (B6, H-2b), DBA/2 (D2, H-2b), SJL (H-2s), SWR (H-2s), C57BL/6-beige (B6-beige, H-2b), 6-12 F1, C57BL/6-mu (B6-nu), and BALB/c-nude were from The Jackson Laboratory (Bar Harbor, ME). All mice were used at least 1 wk after delivery at an age of 7–12 wk and were maintained under specific pathogen-free (SPF) conditions.

Preparation and Infusion of Donor Cells. Lymphoid cells were prepared by gently pressing spleen and pooled cervical, inguinal, and mesenteric lymph nodes through a wire mesh into our complete medium (CM). This consists of MEM supplemented with 10% (vol/vol) FCS (Gibco, Grand Island, NY), 5 × 10⁻⁵ M 2-ME (Eastman Kodak Co., Rochester, NY) and 10 mM Heps buffer (Sigma Chemical Co., St. Louis, MO). The cells were then washed

Abbreviations used in this paper: ALC, allogeneic lymphocyte cytotoxicity; CM, complete medium; LNC, lymph node cell; polyI:C, polymerized inosine, cytidine; and SPF, specific pathogen-free.
through 6% BSA in PBS, resuspended in 5 ml CM, underlaid with
5 ml lymphocyte-M (Cedarlane Laboratories, Hornby, Ontario,
Canada), and centrifuged at 500 g for 30 min to remove red blood
and dead cells. After washes in CM, a FITC solution (30 µg/ml
PBS final) was added to 4-6 × 10^7 cells in 1 ml PBS, and
incubated at 37°C in an 11% CO₂ incubator. Excess FITC was re-
moved by centrifuging the cells through 3 ml 6% BSA/PBS. After
more two washes in 1% BSA/PBS, 3 × 10^7 cells in 0.3 ml 1% BSA/PBS were injected into the lateral tail vein of recipient mice.
Entry of FITC-labeled donor cells into the lymphocyte recirculating
pool was monitored by killing recipients after injection, and
preparing suspensions of blood, lymph nodes, or spleen cells, and
analyzing them on a Becton Dickinson & Co. FACScan® flow
cytometer.

Treatment of Host with Polymerized InosineCytidine (polyI:C) or
AGMI. PolyI:C (100 µg, Sigma Chemical Co.) in 0.3 ml PBS
was administered intraperitoneally into each mouse 30-60 min after
donor cell infusion, and again 1 d later. 50 µg antiasialo GM1 (rabbit)
(AGMI, Wako Chemicals USA, Dallas, TX) in 0.3 ml PBS was
injected intraperitoneally into each mouse 1 d before donor cell
infusion.

MLR and Cytotoxicity Assay. Standard MLR conditions were
used as previously described (5, 7). Briefly three titrations of re-
sponder lymph node cells (LNC) (10^5, 3 × 10^5, 10^6) in five repi-
cates were cultured with 3 × 10^5 irradiated (2,000 rad) stimulator
spleen cells in 96-well V-bottomed plates in 0.2 ml CM for 5 d
at 37°C in an 11% CO₂ incubator. To overcome possible
deficiency in help, mouse rIL-2 culture supernatant (SN) (2 U/ml
final) was added (16). The cultures were tested for cytotoxic ac-
tivity in a standard 4-h ¹¹Cr-release assay using appropriate Con
A splenoblasts as targets. Con A blasts were prepared by culturing
10^6 spleen cells in 10-ml CM in flasks with 4 µg/ml Con A for
2-3 d, centrifuging over lympholyte-M and washing twice in CM
containing 20 mM 2-methyl-D-mannoside. Individual cultures re-
ceived 2,000 target cells labeled with Na¹¹CrO₄ (New England
Nuclear, Boston, MA) as described (5, 7). The fractional specific
lysis was measured as [(Experimental release - spontaneous re-
lease)/Total release - spontaneous release].

Tail Skin Grafting. This was done as described by Kast et al.
(8). Briefly, pieces of donor tail skin ∼4 × 8 mm and of a thickness
including the epidermis and most of the dermis were removed
with a sharp scalpel and transferred to sites on the recipient tail
from which an equivalent amount of skin had been removed. Grafts
were covered with a clear spray bandage (New-Skin, Medtech Labs,
Jackson, WY) and further protected with a light, loosely fitting
masking tape. Tubes were removed 7-10 d after grafting. Each re-
cipient received two skin grafts, experimental and control, placed
lengthwise along the taft, and separated by about 5 mm. Grafts
were visually monitored daily and scored as rejected when >90%
dead.

Results

Induced Donor-specific Response Reduction Is Correlated with
Donor Cell Recirculation. D2 mice were injected intravenously
with 3 × 10^7 FITC-labeled viable B6D2F1 lymphoid cells and
killed 3 d later. Lymph node cells were tested in an in vitro MLR for their ability to generate CTL, and were also checked by flow
cytometry for the presence of FITC-labeled donor cells. Fig. 1 A (left) shows that FITC-labeled F1 cells can be detected in the LN cells where they comprise 2.5% of all cells. As argued previously (5), these cells appear to have entered the recirculating lymphocyte pool of the D2 host. The antidonor (anti-F1) CTL responses of these LN cells was reduced compared to uninjected controls (Fig. 1 A, middle) whereas the CTL response against a third party (SWR) was unaffected (Fig. 1 A, right). Similar results were seen on injecting FITC-labeled B6D2F1 lymphocytes into B6 mice (not shown), in agreement with previous work, i.e., the, injected
cells entered the recirculating pool (5) and produced a donor-specific response reduction (2-7).

Injection of B6 mice with (fully allogeneic) D2 or BALB/c
Table 1. Fate of Donor Lymphoid Cells after Injection into Syngeneic or Allogeneic Hosts

| Donor-Host | LN | spleen | Blood |
|------------|----|--------|-------|
|            | Day 1 | Day 2 | Day 1 | Day 2 |
| D2-D2      | 4.4 ± 0.2 | 4.9 ± 0.4 | 4.8 ± 1.0 | 4.4 ± 0.2 |
| D2-B6      | 1.9 ± 0.1 | 0.05 ± 0.01 | 2.6 ± 0.2 | 0.04 ± 0.01 |

3 × 10⁷ FITC-labeled D2 lymphoid cells were infused into D2 or B6 mice. 1 or 2 d later, host LN, spleen, and blood cells were analyzed by flow cytometry. The table lists mean ± SD percent FITC-labeled cells obtained from two different experiments.

lymphoid cells does not induce response reduction (7) even though the differences potentially detectable by the host on the injected cells are identical to those seen when B6D2F₁ lymphoid cells are injected. We injected 3 × 10⁷ FITC-labeled D2 lymphoid cells into B6 mice. 3 d later, mice were killed and their LN cells tested as above. In agreement with previous results, no donor-specific response reduction was seen (Fig. 1 B). However, we also found that the injected cells could not be detected in LN (Fig. 1 B), i.e., they were not in the recirculating lymphocyte pool.

We reasoned that the failure to detect FITC-labeled D2 cells in B6 LN after injection into B6 mice was either because the injected cells could not enter the lymphocyte recirculating pool and would thus stay in blood for some time, or that they were being rapidly removed. The latter appears to be the case. FITC-labeled allogeneic donor cells were detected in LN and spleen 1 d after injection, i.e., they could enter the recirculating pool, but had disappeared from these tissues as well as from blood by day 2 (Table 1).

It is possible that fully allogenic donor cells are being removed by host NK cells. B6 mice homozygous for the beige mutation have very low levels of NK activity (17), and NK activity of SJL mice is known to be less than half that of B6 mice (18). We injected 3 × 10⁷ FITC-labeled BALB/c lymphocytes into B6-bg, SJL, and B6 hosts. 3 d later, host LN cells were tested for the presence of donor cells by flow cytometry. One sees (Table 2) that the injected cells were detected in B6-bg and SJL LN, but not in B6 LN, which is consistent with NK cells being responsible for removing the cells. As a direct test that host T cells were not responsible, we injected D2 cells into B6-nu mice. They were rapidly removed (Table 2, Expt. 2).

In vivo administration of antiasialo GM1 antibody is known to decrease NK activity (19). When B6 mice were treated with this antibody, BALB/c donor cells could be found in LN 3 d later (Table 2). In vivo administration of polyI:C is known to increase endogenous NK activity (20). We therefore tested the effect of poly I:C treatment on SJL mice injected with BALB/c lymphoid cells (Table 2), and on D2 or B6 mice injected with B6D2F₁ lymphoid cells (Table 3). In both cases, polyI:C treatment led to disappearance of donor cells from LN when tested 3 d after injection of the lymphoid cells (Tables 2 and 3). Note that polyI:C treatment had no influence on syngeneic donor cell recirculation (Table 3).

We next asked whether fully allogeneic cells that enter the recirculating lymphocyte pool will also induce donor-specific response reduction. B6-bg mice were infused with 3 × 10⁷ FITC-labeled D2 lymphocytes. 3 d later, both donor cell recirc-

Table 2. Persistence of Fully Allogeneic Donor Cells Correlates Inversely with Host NK Activity

| Host | polyI:C | AGM1 | Percent donor cells in LN |
|------|---------|------|--------------------------|
|      |         |      |                          |
| Expt. 1 | B6-bg | -    | -                        | 4.2 ± 0.3 |
|        | SJL    | -    | -                        | 1.7 ± 0.2 |
|        | B6     | -    | -                        | 0.02 ± 0.01 |
|        | B6     | -    | +                        | 4.3 ± 0.03 |
|        | SJL    | +    | -                        | 0.03 ± 0.01 |
| Expt. 2 | B6-mu | -    | -                        | 0.24 ± 0.08 |
|        | BALB/c-mu | -    | -                        | 5.4* |
|        | D2     | -    | -                        | 7.5* |

Groups of three recipients were infused with 3 × 10⁷ FITC-labeled BALB/c (Expt. 1) or 4 × 10⁷ FITC-labeled D2 (Expt. 2) lymphoid cells with or without polyI:C or AGM1 treatment as indicated. 3 d later, recipient LN were analyzed by flow cytometry for percent donor cells. * Single recipient.

Table 3. polyI:C Treatment of Host Influences Persistence of Semiallogeneic but Not Syngeneic Donor Cells

| Host | No treatment | polyI:C-treated |
|------|--------------|-----------------|
|      | %            |                 |
| D2   | 2.5 ± 0.2    | 0.01 ± 0.01     |
| B6   | 1.6 ± 0.5    | 0.01 ± 0.02     |
| B6D2F₁ | 4.9 ± 0.1  | 4.9 ± 0.2 |

In each group, three mice were infused with 3 × 10⁷ FITC-labeled B6D2F₁ lymphocytes with or without polyI:C treatment as indicated. 3 d later, LN cells were analyzed by flow cytometry for percent donor cells.
Intravenous injection of mice with viable allogeneic lymphoid cells often, but not always, renders the recipient tolerant to the transplantation antigens carried by the donor lymphocytes. Although MHC differences are clearly implicated, detailed analysis of such differences between donor and host does not enable one to predict the outcome with complete accuracy. Often tolerance is not induced for situations in which the MHC differences predict it might have been. Thus, other factors must be involved. We show here that one of these factors, perhaps the decisive one, is whether the injected allogeneic cells can persist in the recirculating lymphocyte pool of the host. We further show that the extent of persistence is modulated by both genetic and environmental factors.

Recipient mice were injected intravenously with allogeneic or semiallogeneic donor lymphoid cells differing from the recipient at both class I and class II MHC. For the combinations tested, we found that 3 d later there was a donor-specific reduction in the ability of the host to generate CTL in an in vitro MLR if and only if a significant fraction (at least one third compared with the same cells injected into a syngeneic host) of the injected donor cells were still recirculating in the host (Fig. 1). Although tested less critically, the same criterion may also predict the long term survival of a donor skin graft (Fig. 2).

Irrespective of their ultimate fate, injected cells appear capable of traversing from blood to LN. 1 d after injection, injected cells can be found in comparable frequencies in LN, spleen, and blood (Table 1). Disappearance from the recirculating pool appears to be due to an active process mediated by the host. Mediators of this process could be either host NK cells, host T cells, or host antibody. We feel the first to be most likely:

(a) Injected cells can be removed very rapidly (Table 1) in a time shorter than that required for a specific host antidonor immune response to develop (21).

(b) Cells injected into allogeneic athymic nude mice (few or no T cells but at least normal levels of NK cells [18]) are removed as effectively as in allogeneic normal mice (Fig. 2), only consistent with removal by NK cells.

(c) Cells from strain B mice injected into strain A mice are removed much more rapidly than are F1 (A x B) cells. Host T cells or antibody should recognize B and F1 (A x B) equivalently, or nearly so. See below for a discussion of how NK cells might make this distinction.

(d) Persistence of allogeneic cells is more likely in hosts with low endogenous NK activity. In Fig. 1, compare the rapid removal of D2 cells in B6 (medium NK activity, reference 18) with their persistence in B6-bg (very low NK activity, reference 17) or persistence of B6 cells in SJL hosts (low NK activity, reference 18).

(e) Persistence of allogeneic cells can be increased by removal of NK cells. In Table 2, injection of B6 mice with antiasialo GM1 antibody greatly increased the persistence of injected D2 cells. These mice also showed a CTL response reduction against donor cells as in Fig. 1, but also had a reduced response against unrelated stimulators (data not shown), suggesting that antiasialo GM1 antibody reacts with CTL precursors as well as NK cells. We were unable to find a dose of antiasialo GM1 that permitted recirculation to continue without also having some effect on the response to unrelated stimulators (unpublished).

(f) Persistence of allogeneic cells can be decreased by boosting NK cell activity. In Fig. 1, injection of SJL mice with polyI:C, known to boost NK activity (20), led to the removal of injected B6 cells which otherwise would have persisted. In Table 3, injection of DBA/2 or B6 mice with polyI:C also led to the removal of B6D2F1 cells which otherwise would have persisted, but had no effect on the persistence
of the same F1 cells injected into syngeneic F1 recipients.

We conclude that, although circumstantial, the evidence is quite strong that host NK cells are removing the injected cells. There is also evidence that the mechanism responsible for removing the cells is more effective at removing fully allogeneic than semiallogeneic cells.

NK cells can recognize and kill intravenously injected lymphoid cells that lack class I MHC molecules carried by the NK cells (22, 23, 24). These observations have been used to support the hypothesis that NK cells recognize and respond to absence of self class I MHC molecules (25, 26). This would explain why we found that fully allogeneic cells (e.g., B6 into D2) were rapidly removed, but predicts that semiallogeneic cells (e.g., B6D2F1 into D2) should not be removed at all, whereas we found they were removed after polyI:C priming.

Extensive studies have been made in the rat of what has been called allogeneic lymphocyte cytotoxicity (ALC) (27, 28). Unsensitized rats injected with allogeneic viable lymphocytes will start killing these lymphocytes within hours of injection. The effector cells are NK cells. At least some target structures are linked (identical ?) to MHC molecules. In order of decreasing strength, destruction of injected cells is seen with inbred strain A recognizing strain B, F1 (A × B) recognizing A, and A recognizing F1 (A × B), with the extent of removal observed also being influenced by the genetic backgrounds of both the host and donor strains. The first two patterns can be explained by the missing self class I MHC model (25, 26). The third cannot and is analogous to our situation of injecting F1 into parent. In this third pattern, killing is much weaker and is not observed in many strain combinations (27). One can speculate that all three patterns of killing have a similar biological basis, perhaps resulting in some way from impaired self recognition (27, 29).

It has been long established that performing multiple blood transfusions before kidney transplantation improves kidney graft survival in the human situation (30, 31). Recently, it has been shown that the donor blood must share at least one HLA-DR antigen with the recipient (32). Further, in in vitro studies, T cell unresponsiveness to donor cells was induced only if donor and recipient shared one HLA haplotype or at least one HLA-B and HLA-DR antigen (33). We hypothesize that this matching is required to enable the donor cells to persist in the recipient long enough to mediate response reduction.

Rammensee and Hügin (34) have reported that in vivo administration of anti-CD4 mAb before injection of allogeneic cells can enhance the ability of injected allogeneic cells to produce donor-specific response reduction in an MLR. More recently, Kitagama et al. (35) have reported that treatment of the recipient with either anti-CD8 or anti-CD4 enhances donor-specific response reduction either as measured in an MLR or as assessed by skin graft survival. Anti-CD4 was effective only if administered before allogeneic cell injection. Anti-CD8 was also effective when given later. It is hypothesized that these mAb treatments are affecting either the interactions between the infused allogeneic cells and host cells that can recognize them, or are directly removing host alloreactive cells. Our results suggest a third possibility i.e., that the mAbs (particularly anti-CD4) may be enhancing survival of the injected allogeneic cells in the recirculating lymphocyte pool of the host by eliminating cells whose lymphokine production (particularly IFN-γ [20]) would up-regulate endogenous NK activity.

Our studies have been done with strictly pathogen-free animals that should minimize environmental activation of both NK cells and CD4+ cells. The widely variable results obtained by different groups studying donor-specific transfusion effects in very similar systems could reflect in part differences in environmental activation of host NK cells as well as strain-dependent differences in NK activity that occur independent of environmental factors. This reasoning leads to the prediction that appropriate selection of the MHC of the infused cells combined with a drug that blocked NK cell activation or, better still, blocked NK cell action, would produce a profound enhancement in donor-specific response reduction and graft survival.

The main conclusion of this study is that intravenously injected allogeneic lymphoid cells must persist in the recirculating lymphocyte pool of the host to induce response reduction. This conclusion is fully consistent with the veto hypothesis (11-15) but does not rule out other mechanisms for explaining response reduction. Knowing that the persistence of the injected cells is greatly enhanced if they share MHC with the host, and that persistence can be reduced by host NK activity, should not only facilitate future studies of the phenomenon but enhance the prospects for its practical application.

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