

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.

Intravenous injection of mice with viable allogeneic lymphoid cells can produce a donor-specific reduction in the ability of the mice to proliferate (1), generate CTL (2–6), and generate Th (7) in a subsequent MLR, and can also enhance survival of a graft syngeneic to the lymphoid cell donor (6, 8–10). One possible explanation is that the injected donor lymphoid cells act as deletional APC or veto cells (11–15) and produce functional deletion rather than activation of host lymphocytes that recognize them (3–8). However, if this hypothesis is correct, an explanation for the following must be found. As reviewed elsewhere (7), injection of viable semiallogeneic F₁ (A × B) lymphoid cells into either parent A or parent B usually (but not always) induces response reduction and/or enhanced graft survival. However, injection of fully allogeneic B lymphoid cells into A seldom induces either response reduction or enhanced graft survival, although the veto hypothesis predicts it should occur.

Previous work from this laboratory has shown that when B6D2F₁ lymphoid cells are injected into B6 recipients, they induce a donor-specific inactivation of both CTLp (5) and Thp (7). The lymphoid cells to be injected were first FITC labeled, and could thus be easily detected and enumerated in the host by flow cytometry. It was thus found that the injected cells persisted in the host for at least 10 d and appeared to have entered the recirculating lymphocyte pool. Using the same methodology, we show here that those lymphoid cells which, on injection, fail to induce response reduc-

tion, also disappear in <3 d from the recirculating pool, whereas those that do induce response reduction are still readily detectable on day 3. Cells that fail to persist in the recirculating pool appear to be removed by an active process, possibly NK-cell mediated. A similar correlation appears to govern whether injected F₁ lymphoid cells can enhance F₁ skin graft survival on a parent.

Materials and Methods

Mice. (C57BL/6 × DBA/2)F₁ (B6D2F₁, H-2^{b/d}), C57BL/6 (B6, H-2^b), DBA/2 (D2, H-2^d), SJL (H-2^s), SWR (H-2^g), C57BL/6-beige (B6-bg, H-2^b), C57BL/6-nude (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)¹ 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 α 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 Hepes buffer (Sigma Chemical Co., St. Louis, MO). The cells were then washed

¹ 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 lympholyte-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 μ g/ml PBS final) was added to 4–6 $\times 10^7$ cells in 1 ml PBS, and incubated at 37°C in an 11% CO₂ incubator. Excess FITC was removed by centrifuging the cells through 3 ml 6% BSA/PBS. After two more washes in 1% BSA/PBS, 3 $\times 10^7$ 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 Inosine:Cytidine (polyI:C) or AGMI. PolyI:C (100 μ g, 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 μ g antisialo GM1 (rabbit) (AGM1, 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 responder lymph node cells (LNC) (10^5 , 3×10^4 , 10^4) in five replicates 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 activity in a standard 4-h ⁵¹Cr-release assay using appropriate Con A splenoblasts as targets. Con A blasts were prepared by culturing 10^7 spleen cells in 10-ml CM in flasks with 4 μ g/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 received 2,000 target cells labeled with Na ⁵¹CrO₄ (New England Nuclear, Boston, MA) as described (5, 7). The fractional specific lysis was measured as [(Experimental release – spontaneous release)/(Total release – spontaneous release)].

Tail Skin Grafting. This was done as described by Kast et al. (8). Briefly, pieces of donor tail skin $\sim 4 \times 8$ 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 transparent glass tube slipped over the tail and held in place with masking tape. Tubes were removed 7–10 d after grafting. Each recipient received two skin grafts, experimental and control, placed lengthwise along the tail, 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%

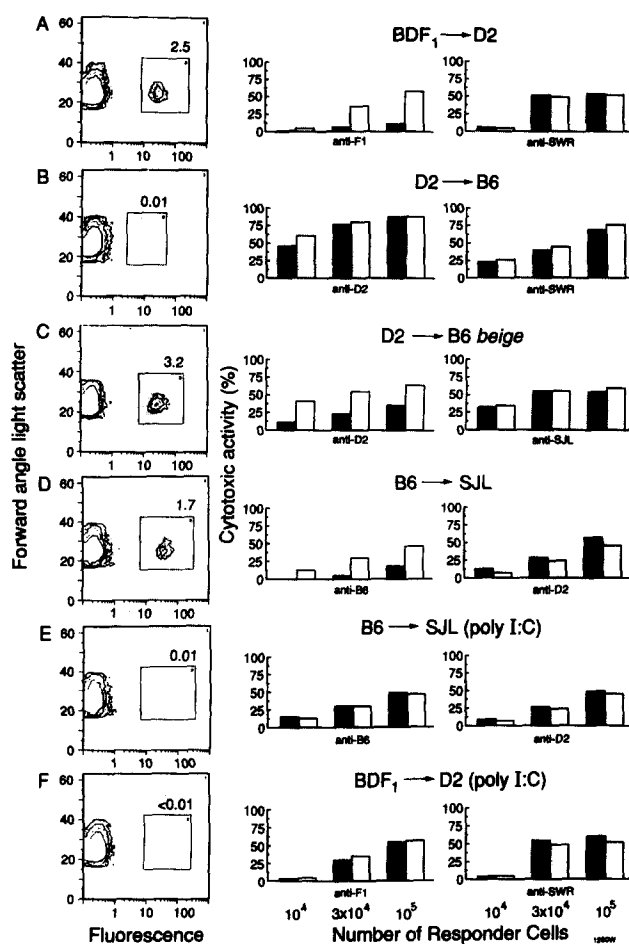


Figure 1. Continued presence of injected foreign lymphoid cells is correlated with their ability to induce donor-specific response reduction. Recipient mice were injected intravenously with 3×10^7 FITC-labeled foreign lymphoid cells. 3 d later, mice were killed and LN cells analyzed for the presence of FITC-labeled cells by flow cytometry (left, forward angle light scatter (FSC) vs fluorescence) and for their ability to generate CTL against donor stimulator cells (middle) and unrelated third party stimulator cells (right) in a 5-d MLR. The MLR data are shown as cytotoxic activity developed by 10^4 , 3×10^4 , or 10^5 responder cells from injected (solid bars) or control (open bars) mice. (A) B6D2F₁ lymphoid cells injected into D2 recipients; (B) D2 lymphoid cells injected into B6 recipients; (C) D2 lymphoid cells (from same pool as in B) injected into B6-bg/bg recipients; (D) B6 lymphoid cells injected into SJL recipients; (E) B6 lymphoid cells injected into polyI:C-treated SJL recipients. D and E were done at the same time using the same donor pool and matched recipients; and (F) B6D2F₁ lymphoid cells injected into polyI:C-treated D2 recipients. A and F were done at the same time using the same donor pool and matched recipients.

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 response 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	Day 1	Day 2
D2-D2	4.4 ± 0.2	4.9 ± 0.4	4.8 ± 1.0	4.4 ± 0.2	2.0 ± 0.3	0.9 ± 0.1
D2-B6	1.9 ± 0.1	0.05 ± 0.01	2.6 ± 0.2	0.04 ± 0.01	1.3 ± 0.2	0.04 ± 0.03

3×10^7 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 3×10^7 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 allogeneic 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 3×10^7 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 3×10^7 FITC-labeled D2 lymphocytes. 3 d later, both donor cell cir-

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- <i>bg</i>	-	-	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- <i>nu</i>	-	-	0.24 ± 0.08
	BALB/c- <i>nu</i>	-	-	5.4*
	D2	-	-	7.5*

Groups of three recipients were infused with 3×10^7 FITC-labeled BALB/c (Expt. 1) or 4×10^7 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	Donor cells in LN	
	No treatment	PolyI:C-treated
	%	
D2	2.5 ± 0.2	0.01 ± 0.01
B6	1.6 ± 0.5	0.01 ± 0.02
B6D2F ₁	4.9 ± 0.1	4.9 ± 0.2

In each group, three mice were infused with 3×10^7 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.

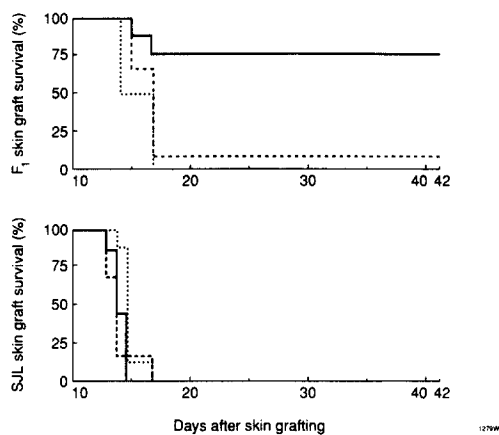


Figure 2. Skin graft survival after intravenous injection of foreign lymphoid cells. Groups of D2 mice were infused with 3×10^7 B6D2F₁ lymphoid cells with (dashed lines) or without (solid lines) also being given polyI:C or were left untreated (dotted line). The groups contained nine, eight, and six mice, respectively. All mice were then given two tail skin grafts, B6D2F₁ (top) and SJL (bottom) and graft survival monitored daily. Similar results were obtained in an earlier experiment using fewer graft recipients.

ulation and donor-specific response reduction were seen (Fig. 1 C). Similarly, when SJL mice (low NK level) were infused with B6 lymphoid cells, both donor cell recirculation and donor-specific response reduction were seen (Fig. 1 D). However, when the recipient SJL mice were also treated with polyI:C, the injected cells failed to recirculate, and also failed to induce response reduction (Fig. 1 E). B6D2F₁ cells injected into D2 mice both recirculate and induce donor-specific response reduction (Fig. 1 A). However, when the recipient mice were also injected with poly I:C, neither recirculation nor donor-specific response reduction was seen (Fig. 1 F).

Enhanced Donor-specific Graft Survival Is Correlated with Donor Cell Recirculation. D2 mice were infused with 3×10^7 B6D2F₁ lymphocytes and divided into two groups, one of which was treated with polyI:C as described. 4 d after donor cell infusion, tail skin grafting (B6D2F₁ and SJL) was done on all these mice plus a third group of normal D2 mice age and sex matched to the first two groups. Fig. 2 shows significant enhancement of F₁ (the infused antigen) but not SJL skin graft survival (third party) in the mice infused with B6D2F₁ cell and not given polyI:C, but not in the other two groups.

Discussion

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 (7). 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 (Table 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 × B) cells. Host T cells or antibody should recognize B and F₁ (A × 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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