failed to reject H2s marrow grafts (Fig. 4B). In addition, pretreatment of mice with F(ab')2 antibody fragments that block the Ly49C receptor prior to transplant partially restored the ability of SHIP$^{-/-}$ hosts to reject BALB/C BM grafts (Fig. 4C). These results demonstrate that overrepresentation of an inhibitory receptor contributes directly to the compromised ability of SHIP$^{-/-}$ hosts to reject allogeneic BM grafts. To exclude the possibility that the NK compartment was simply impaired, we examined the ability of SHIP$^{-/-}$ mice to reject MHC class I–deficient BM (*7*) as compared to their wild-type littermates (Fig. 4D). SHIP$^{-/-}$ mice showed complete rejection of β2m$^{-/-}$ BM grafts comparable to that seen in SHIP$^{+/+}$ littermates. Thus, the NK compartment in SHIP$^{-/-}$ hosts was not broadly disabled.

To determine whether engraftment of MHC mismatched BM could lead to severe graft-versus-host disease (GVHD), we transplanted a cohort of SHIP$^{-/-}$ mice and their SHIP$^{+/+}$ littermates with BM from BALB/C mice (*7*). The large majority of SHIP$^{-/-}$ mice survived transplant without developing GVHD, whereas less than half of the SHIP$^{+/+}$ mice survived (Fig. 4E) (*7*). These findings suggest a previously unappreciated role for host NK cells in the initiation of GVHD. Potentially, SHIP$^{-/-}$ NK cells fail to produce inflammatory cytokines (γ-IFN, TNF-α) in response to allogeneic BM cells, thereby reducing the likelihood of a significant GVH reaction by donor T cells. Alternatively, other host cell types that contribute to GVHD, such as antigen-presenting cells (*21*), could also be altered by SHIP deficiency.

Although Ly49 inhibitory receptors prevent inappropriate killing by NK cells, the interaction of these receptors with self MHC ligands may also elicit signals that promote the survival or proliferation of these cells in vivo (*22*). SHIP may counteract these signals and thus prevent the expansion of NK subsets expressing more than one self-restricted inhibitory receptor. We propose that inhibiting SHIP activity prior to BM transplant could restrict the NK inhibitory repertoire, such that selecting a donor with an appropriate MHC ligand, or ligands, might enable engraftment in the absence of GVHD. Thus, inhibition of SHIP signaling should be explored as a means to increase both the efficacy and utility of allogeneic BM transplantation.

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Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants

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T cells that accompany allogeneic hematopoietic grafts for treating leukemia enhance engraftment and mediate the graft-versus-leukemia effect. Unfortunately, alloreactive T cells also cause graft-versus-host disease (GVHD). T cell depletion prevents GVHD but increases the risk of graft rejection and leukemic relapse. In human transplants, we show that donor-versus-recipient natural killer (NK)–cell alloreactivity could eliminate leukemia relapse and graft rejection and protect patients against GVHD. In mice, the pretransplant infusion of alloreactive NK cells obviated the need for high-intensity conditioning and reduced GVHD. NK cell alloreactivity may thus provide a powerful tool for enhancing the efficacy and safety of allogeneic hematopoietic transplantation.

Human leukocyte antigen (HLA)–matched allogeneic hematopoietic transplantation has revolutionized the treatment of leukemia, lymphoma, and inherited hematopoietic stem cell diseases (*1*). Donor T cells in the allograft are vital for promoting engraftment, eradicating malignant cells [the graft-versus-leukemia (GVL) effect], and reconstituting immunity. Unfortunately, they mediate GVHD, which is an attack on recipient tissues. GVHD and the global immunosuppression needed to prevent or treat it underlie the major reasons for transplant failures: infection and neoplastic relapse. Furthermore, only 60% of patients have matched sibling or unrelated donors, and even fewer make it to transplant because of the delays due to the donor search and bone marrow harvesting (*2*). However, virtually every patient has a family member who is identical for one HLA haplotype and fully mismatched for the other, and thus could immediately serve as a donor.

Transplantation across the histocompatibility barrier has been made possible by extensive T cell depletion of the graft to help prevent GVHD and transplantation of large numbers of hematopoietic stem cells to help overcome rejection (*2–6*). These grafts result in the rapid generation of natural killer (NK) cells. NK cells are negatively regulated by major histocompatibility complex (MHC) class I–specific inhibitory receptors (*7*, *8*). In humans, receptors termed killer Ig-like receptors (KIRs) recognize groups of HLA class I alleles. Although KIRs and other class-I inhibitory receptors (*9–11*) may be coexpressed by NK cells, in any given individual's NK repertoire there are cells that express a single KIR and are blocked only by a specific class I allele group. Missing expression of the KIR ligand on mismatched allogeneic cells can therefore trigger NK cell alloreactivity (*12–17*). In hematopoietic

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transplants, when the recipient's class I alleles do not block all donor NK cells, donor alloreactive NK clones are generated, which kill host targets, including acute myeloid leukemia (AML) cells (*18*).

In clinical transplantation and in mouse transplant models, we determined the impact of donor-versus-recipient NK cell alloreactivity on relapse, rejection, GVHD, and survival. One hundred and twelve high-risk acute leukemia patients received hematopoietic transplants from HLA haplotype–mismatched family donors (*2*, *3*, *5*). Typing of the HLA-C locus was available in 92 of these individuals [of whom 57 had AML and 35 had acute lymphoid leukemia (ALL)], and so only these transplants were analyzed in this study (*19*). Primary engraftment was achieved in 90.2%, GVHD occurred in 8.6%, and event-free survival was seen in 26% of AML patients and 8% of ALL patients (*20*).

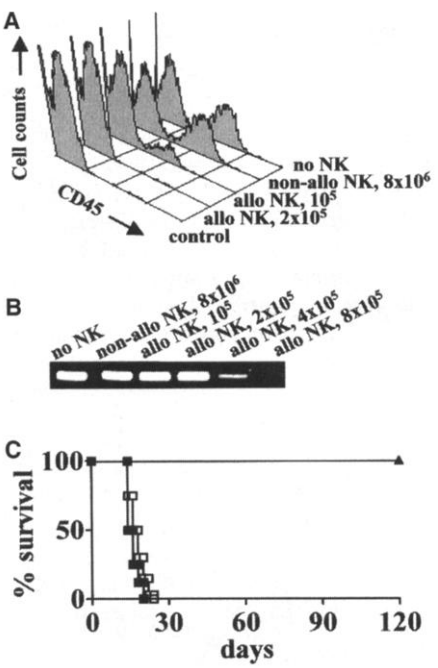

Fig. 1. A single infusion of alloreactive NK cells eradicates advanced human leukemia in NOD/SCID mice (*21*). Bone marrow infiltration by chronic myeloid leukemia (CML) myeloid blastic crisis, as evaluated by flow cytometric analysis of human CD45 (**A**), and by polymerase chain reaction analysis of the BCR/ABL gene (**B**), before (no NK) and 1 week after the infusion of human nonalloreactive NK clones (non-allo-NK) or alloreactive NK clones (allo NK) at the indicated cell doses (data are representative of five mice in each group). In (A), "control" denotes mice not given human leukemia. (**C**) Survival of leukemic mice that received no alloreactive NK cells (solid squares), 8×10^6 nonalloreactive NK cells (open squares), or 8×10^5 alloreactive NK cells (solid triangle) (data are from 10 mice in each group). Identical results were obtained from four additional experiments with cells from three AML patients and one CML patient in myeloid blastic crisis.

To evaluate the role of donor-versus-recipient NK cell alloreactivity in transplantation outcomes, donor-recipient pairs were divided into two groups: the first without and the second with KIR ligand incompatibility in the graft-versus-host (GVH) direction (Table 1). Donors were evaluated for NK cell alloreactivity by screening ≥200 NK clones (≥100 on each of two separate occasions) and were scored positive when the frequency of lytic clones was ≥1 in 50 [as a rule, frequencies of positive clones were either high (1 in 2 to 1 in 20) or nondetectable (<1 in 200)] (*21*). KIR ligand incompatibility correlated closely with the detection of donor NK clones killing recipient targets. Transplantation from NK cell alloreactive donors totally protected patients against rejection, GVHD, and AML relapse (Table 1). In AML, the probability of event-free survival at 5 years was 5% in the first group versus 60% in the second ($P < 0.0005$) (*22*). Multivariate analysis (*22*) that considered crucial variables affecting transplantation outcome, such as conditioning regimens, number of stem cells and T cells in the graft, and status of disease at transplant (*21*), showed that KIR ligand incompatibility in the GVH direction was the only

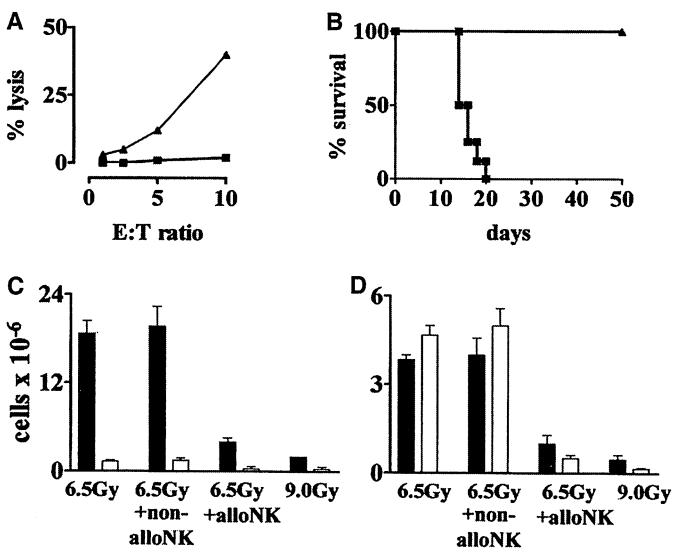

Fig. 2. Alloreactive NK cells cause immune- and myeloablation but not GVHD. (**A**) Donor *H-2*$^{d/b}$ mouse NK cell alloreactivity against *H-2*^{b} recipient targets (curves illustrate representative results of five cytotoxicity assays). Donor Ly49A$^+$/G2$^+$ (Ly49C/I$^-$) NK cells, alloreactive against recipient targets (solid triangles), were used for conditioning. Nonalloreactive, Ly49C/I$^+$ (Ly49A$^-$/G2$^-$) cells (solid squares) served as controls (*21*). (**B**) In vivo infusion of alloreactive NK cells does not cause GVHD. The solid triangle represents survival in lethally (9 Gy) irradiated *H-2*^{b} mice given *H-2*$^{d/b}$ bone marrow plus 16×10^6 alloreactive *H-2*$^{d/b}$ NK cells versus survival of mice (solid squares) given *H-2*^{d} bone marrow plus 10^6 *H-2*^{d} T cells (number of mice in each group = 10). (**C** and **D**) Mice were given nonlethal TBI (6.5 Gy), nonlethal TBI plus 4×10^6 nonalloreactive NK cells (nonallo NK), nonlethal TBI plus 4×10^6 alloreactive NK cells (allo NK), or lethal (9 Gy) TBI alone. Alloreactive NK cells ablated bone marrow (C) and spleen (D) granulocytes (black bars) and T cells (white bars) in nonlethally irradiated mice (mean ± SD of data from a total of nine mice, three each in three independent experiments).

Table 1. Clinical data and transplantation outcomes in HLA haplotype-mismatched transplants with and without KIR ligand incompatibility in the GVH direction. KIR ligand incompatibility in the GVH direction was defined as absence in recipients of donor class I allele group(s) recognized by KIRs (*9*–*11*). Such groups are HLA-C alleles with Asn^{77}-Lys^{80}, Cw2, 4, 5, 6, and related alleles; HLA-C alleles with Ser^{77}-Asn^{80}, Cw1, 3, 7, 8, and related alleles; HLA-Bw4 alleles; and HLA-A3/A11. Twenty-six pairs (11 in ALL and 15 in AML) were mismatched for HLA-C groups, 8 (3 in ALL, and 5 in AML) were mismatched for HLA-Bw4 group; the HLA-A3/A11 mismatch was never found alone but only in conjunction with HLA-C group mismatches (2 pairs).

KIR ligand incompatibility in GVH direction	No	Yes
Number of transplants	58	34
Donors displaying antirecipient NK clones	1/58	34/34*
Disease		
ALL	21	14
AML	37	20
Transplantation outcomes		
Rejection	15.5%	0%*
Acute GVHD, ≥ grade II	13.7%	0%*
Probability of relapse at 5 years		
ALL	90%	85%
AML	75%	0%**

*$P \leq 0.01$; **$P < 0.0008$ (*22*).

independent predictor of survival in AML. Conversely, the absence of KIR ligand incompatibility in the GVH direction was the only independent factor predicting poor outcome (hazard ratio = 0.33, 95% confidence interval 0.11 to 0.94, $P < 0.04$). KIR ligand incompatibility in the GVH direction had no effect on ALL.

Our data on the human system suggested that alloreactive NK cells are responsible for GVL effects. In light of these observations, we explored these effects in a model system, using transfer of alloreactive NK cells. In these experiments, human alloreactive NK clones were infused into human AML-engrafted nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (*21*). Mice infused with human AML developed advanced disease in 5 to 6 weeks (Fig. 1, A and B). If left untreated, or given nonalloreactive human NK clones, mice died over the following 3 weeks (Fig. 1C). In contrast, many fewer human alloreactive NK cells cleared leukemia (Fig. 1, A and B), and mice survived until they were killed (120 days) (Fig. 1C).

We next tested whether alloreactive NK cells could obviate the need for lethal conditioning in an MHC mismatched $F_1 \rightarrow$ parent mouse bone marrow transplant (BMT) model. In F_1 *H-2*$^{d/b} \rightarrow$ parent *H-2*^{b} transplants, donor T cells were tolerant of the recipient MHC, but donor NK cells not expressing *H-2*^{b}–specific Ly49C/I inhibitory receptor and bearing instead *H-2*^{d}–specific Ly49A/G2 receptors were activated to kill the recipient's targets (Fig. 2A) (*21*, *23*). Alloreactive NK cells did not cause GVHD, even when infused in large numbers into lethally irradiated recipients (Fig. 2B). However, in nonlethally irradiated recipients, alloreactive NK cells but not control nonalloreactive NK cells reduced recipient-type T cell and granulocyte counts in marrow and spleen to levels observed after lethal irradiation (Fig. 2, C and D).

Mice conditioned with nonlethal [≤7 grays (Gy)] total-body irradiation (TBI) alone rejected donor marrow grafts (Fig. 3A). In contrast, all recipients conditioned with nonlethal irradiation and alloreactive NK cells engrafted with durable, donor-type hematopoietic chimerism (Fig. 3A) (*21*). As few as 2×10^5 alloreactive NK cells resulted in major donor hematopoiesis, and 4×10^6 nonalloreactive NK cells had no effect (Fig. 3B). Similar results were obtained in F_1 *H-2*$^{d/b} \rightarrow$ parent *H-2*^{d} transplants. In this case, donor NK cells used for conditioning did not express *H-2*^{d}–specific Ly49A/G2 receptors but expressed instead the *H-2*^{b}–specific Ly49C/I receptor (*24*). Alloreactive NK cells allowed mismatched BMT, even when combined with the reduced-intensity conditioning regimens adopted from matched human transplants (*25*). Thus, mice given these regimens plus low doses of alloreactive NK cells achieved >80% donor chimerism (Fig. 3C), unlike controls that received nonalloreactive NK cells. Even mice conditioned with fludarabine alone plus alloreactive NK cells displayed 30% donor chimerism (Fig. 3 E). In addition, infusion of alloreactive NK cells 6 weeks after transplant was able to convert mixed chimeras to stable full-donor chimerism.

Because our clinical data suggested that NK cell alloreactivity does not cause GVHD but actually protects against it, we next tested whether NK cell conditioning could replace the need for T cell depletion. Lethally irradiated *H-2*^{b} mice transplanted with *H-2*^{d} bone marrow containing 10^6 T cells died from GVHD in 2 to 4 weeks (Fig. 4A). After conditioning with TBI plus alloreactive NK cells, cohorts of transplanted mice were given escalating doses of *H-2*^{d} T cells. Even with as many as 2×10^7 T cells, 100% of these mice survived until they were killed (120 days) with no signs of GVHD (*21*). In contrast, administration of nonalloreactive NK cells, even in very large numbers, provided no protection. We hypothesized that this protection might be mediated by alloreactive NK cells attacking recipient antigen-presenting cells (APCs), shown to be responsible for initiating GVHD (*26*, *27*), and that consequently, mice with APCs that are resistant to alloreactive NK cell killing might not be protected against GVHD. We therefore made B6 × BALB/c → B6 bone marrow chimeras (*21*) to replace the alloreactive NK cell–sensitive *H-2*^{b} mouse hematopoietic cells,

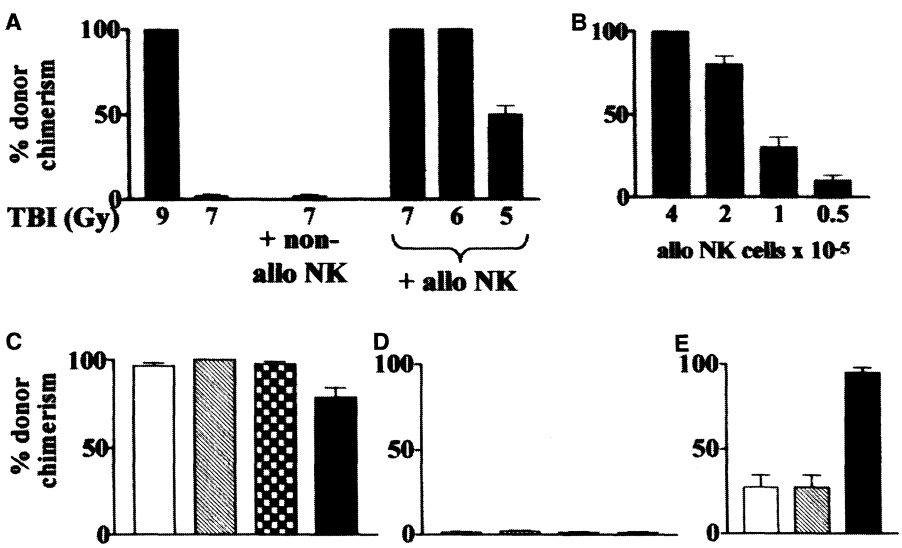

Fig. 3. Successful MHC haplotype-mismatched transplantation after alloreactive NK cell-based conditioning. (**A**) Donor *H-2*$^{d/b}$ chimerism (*21*) after conditioning *H-2*^{b} recipients with lethal TBI (9 Gy), nonlethal TBI (7 Gy), nonlethal TBI plus 4×10^6 nonalloreactive NK cells (nonallo NK), or decreasing nonlethal TBI doses (7, 6, and 5 Gy) plus 4×10^6 alloreactive NK cells. (**B**) Alloreactive NK cell doses yielding major donor chimerism in transplantation of nonlethally (6.5 Gy) irradiated mice are low, i.e., $\geq 2 \times 10^5$. (**C** and **D**) Mismatched transplantation after conditioning with reduced-intensity fludarabine-based regimens (*25*). Conditioning regimens were fludarabine (180 mg/m^2) plus busulfan (8 mg/Kg) (white bars), fludarabine (120 mg/m^2) plus melphalan (120 mg/m^2) (hatched bars), fludarabine (120 mg/m^2) plus 2 Gy TBI (checked bars), or fludarabine (120 mg/m^2) plus cyclophosphamide (120 mg/Kg) (black bars). Survival without transplant after any of these schemes was 100% (*24*). (C) Donor chimerism of mice receiving the drugs plus 8×10^5 alloreactive NK cells. (D) Donor chimerism of mice receiving either the drugs alone or the drugs plus 8×10^6 nonalloreactive NK cells [in panels (A) through (D), data are from three independent experiments, each using six mice]. (**E**) Post-engraftment infusion of alloreactive NK cells converted mixed chimeras to full-donor chimeras. Donor chimerism in transplanted mice conditioned with fludarabine (120 mg/m^2) plus 2×10^5 alloreactive NK cells (white bar), as determined 6 months after transplant. The black bar shows donor chimerism, determined 6 months after transplant, in another group of mice that received the same conditioning [fludarabine (120 mg/m^2) plus 2×10^5 alloreactive NK cells], transplant, and a post-engraftment infusion of 8×10^5 alloreactive NK cells given 6 weeks after transplant. The hatched bar shows a control group of mice that received the same conditioning, transplant, and an infusion of 8×10^6 nonalloreactive NK cells. In all panels in this figure, the bars represent mean percentages ± SD of donor chimerism as evaluated by two-color flow cytometric analysis. Identical degrees of donor chimerism were found in granulocytes and lymphocytes in bone marrows and spleens (*24*). Granulocyte and lymphocyte values were pooled and are illustrated as a single bar (all experiments were repeated at least three times).

including APCs, with $H\text{-}2^{d/b}$ cells that would be resistant to NK cell killing ($H\text{-}2^{d/b} \rightarrow H\text{-}2^{b}$ chimeras). Although the $H\text{-}2^{d}$ allele protects against alloreactive NK cells, the $H\text{-}2^{b}$ molecules can still present antigen to donor $H\text{-}2^{d}$ T cells, thus priming GVH reactions. When analyzed 4 months after transplant, >90% of bone marrow, spleen, and gut dendritic cells in these chimeras were of $H\text{-}2^{d/b}$ origin (*21*). When these chimeras were reconditioned with TBI plus alloreactive NK cells and reconstituted with $H\text{-}2^{d}$ BMT containing 10^6 T cells, 100% died from GVHD (Fig. 4A). Control $H\text{-}2^{b} \rightarrow H\text{-}2^{b}$ chimeras given 2×10^7 T cells survived with no signs of GVHD. We also found that alloreactive NK cells accelerated the loss of bone marrow, spleen, and gut APCs, as compared to mice conditioned with either TBI or TBI plus nonalloreactive NK cells (Fig. 4, B, through D). Taken together, these data indicate that alloreactive NK cells prevent GVHD by elimination of recipient APCs.

Our clinical data show that spontaneously generated NK cell alloreactions from stem cell grafts are associated with a remarkable GVL effect and total control of rejection and GVHD. This dramatically affects survival of AML patients (5% in the absence versus 60% in the presence of NK cell alloreactivity). This is far better than survival after matched unrelated-donor transplant, which is 34% in first complete remission, 27% in second complete remission, and 7% in third or more complete remission or in relapse (*1*). This survival rate is striking, as most of our AML patients were in their third or more complete remission or in relapse (*21*).

Direct involvement of NK cell alloreactivity is provided by our transplant models, which demonstrate that infusion of alloreactive NK cells eradicates human leukemia in vivo, prepares mice for MHC-mismatched BMT by killing host lymphohematopoietic cells, and reduces GVHD by eliminating recipient-type APCs. In humans as in mice, NK cells had no effect unless the target was susceptible to alloreactive NK cell killing; for instance, they failed to control ALL, a leukemia histotype that is resistant to alloreactive NK lysis in vitro (*18*).

Alloreactive NK cell infusions have the potential to improve outcomes of KIR ligand-mismatched transplants even further and are therefore of extraordinary therapeutic interest. In mice, they were successfully combined with reduced-intensity conditioning to achieve durable full-donor engraftment. Even alone, alloreactive NK cells converted mixed to full-donor chimerism and eradicated leukemia. NK cell conditioning even protected against GVHD efficiently enough to allow the safe infusion of otherwise lethal doses of allogeneic T cells for immune reconstitution.

Alloreactive NK cells emerge as a form of cell therapy that might be used in conditioning regimens for host immune suppression and leukemia ablation. Their ability to prevent GVHD could allow a greater T cell content in the graft and consequently reduce the infection-related morbidity and mortality that are associated with extensive T cell depletion (*3*, *5*). With this approach, mismatched transplants can be envisaged for the elderly and for heavily pretreated patients.

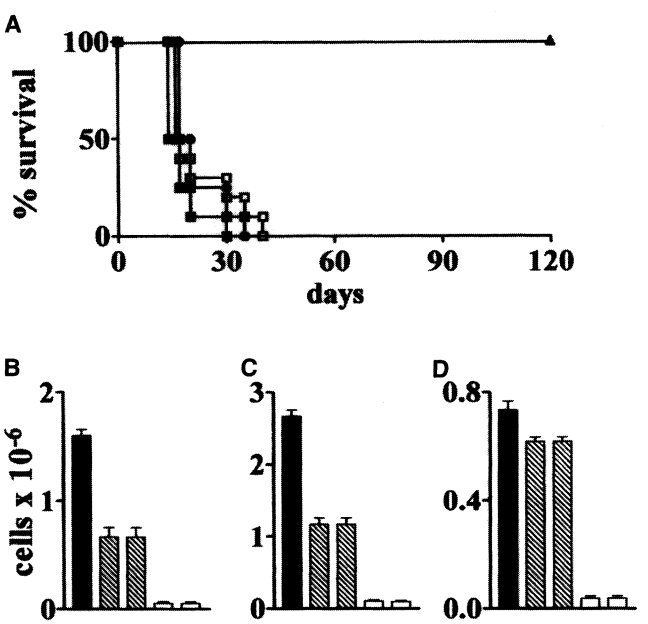

Fig. 4. Conditioning by alloreactive NK cells protects against GVHD by ablating host APCs. (**A**) Survival of $H\text{-}2^{b}$ mice conditioned with TBI (9 Gy) (solid circles) or TBI plus nonalloreactive NK cells (4×10^6) (solid squares), and transplanted with $H\text{-}2^{d}$ bone marrow containing 10^6 T cells, versus that of $H\text{-}2^{b}$ mice conditioned with TBI (9 or 6.5 Gy) plus alloreactive NK cells (4×10^5) and given $H\text{-}2^{d}$ BMT containing 2×10^7 T cells (solid triangle). Survival of $H\text{-}2^{b}$ mice bearing $H\text{-}2^{d/b}$ APCs and therefore resistant to alloreactive NK cell killing ($H\text{-}2^{d/b} \rightarrow H\text{-}2^{b}$ chimeras), conditioned with TBI plus alloreactive NK cells and transplanted with $H\text{-}2^{d}$ bone marrow containing 10^6 T cells (open squares) versus survival of control $H\text{-}2^{b} \rightarrow H\text{-}2^{b}$ chimeras (with susceptible APCs) conditioned with TBI (9 or 6.5 Gy) plus alloreactive NK cells (4×10^5) and given $H\text{-}2^{d}$ BMT containing 2×10^7 T cells (same as solid triangle). (**B**) Bone marrow, (**C**) spleen, and (**D**) gut APC ($CD11c^{+}$ dendritic cell) counts in untreated mice (black bar) versus mice conditioned with 9 Gy TBI with or without 4×10^6 nonalloreactive NK cells (the two hatched bars, respectively) versus mice conditioned with either 9 or 6.5 Gy TBI plus 4×10^5 alloreactive NK cells (the two white bars, respectively).

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19. HLA was assessed by serologic and molecular typing. All transplant pairs were mismatched at the HLA-A, -B, -C, and -DR loci of one haplotype and matched at the other.
20. "Event-free" denotes survival without rejection, chronic GVHD, leukemia relapse, or infection. All patients had a minimum follow-up of 1 year and were evenly distributed across the 1- to 8-year follow-up range.
21. A supplementary table and experimental procedures are available on *Science* Online at www.sciencemag.org/cgi/content/full/295/5562/2097/DC1.
22. Differences among frequencies were analyzed by the Fisher exact test. Probability rates were evaluated by Kaplan-Meier analysis. The log-rank test was used for comparisons. The Cox model was applied for multivariate analyses. The Shoenfeld test was used to test the assumption of proportional hazard.
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