The variability-generating system is theoretically capable of generating over 7.0×10^{13} different nucleotide sequences and 9.2×10^{12} amino acid sequences in VR1 and the encoded product, respectively (*18*). The close proximity of required genetic elements suggests that it operates as a variability-generating cassette with three major components: a reverse transcriptase, a template repeat, and a second repeated sequence capable of variation. A more detailed understanding of the variability mechanism should allow us to engineer constructs designed to promote in vivo targeted mutagenesis of specific DNA sequences. Such capability could be useful in applications where massive parallel screening of diverse protein sequences is desirable.

Reverse transcriptases are ubiquitous in nature. They are frequently found in both prokaryotic and eukaryotic genomes and are often associated with mobile genetic elements (*19*). Indeed, over 40% of the human genome appears to have resulted from the process of reverse transcription (*20*). Variations of the RT-dependent diversity-generating mechanism described here could confer powerful selective advantages in a variety of biological contexts. It will be of interest to determine if this adaptive mechanism has found utility in nature in addition to its role in facilitating tropism switching by *Bordetella* bacteriophages.

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10. To identify the *mtd*-encoded polypeptide, rabbit antibody was raised against a recombinant glutathione S-transferase–Mtd (GST-Mtd) fusion protein. Western blotting of concentrated phage lysates resulted in recognition of a 40-kD protein corresponding to the predicted size of Mtd. No significant similarity to *mtd* was found in GenBank database. Annotation of the phage genome did not reveal any other predicted open reading frames (ORFs) with similarity to known bacteriophage tail fiber proteins.
11. In-frame deletions were carried out using allelic exchange essentially as described by R. A. Edwards, L. H. Keller, and D. M. Schifferli [*Gene* **207**, 149 (1998)].
12. His_6-tagged derivatives of the wild-type Brt protein and the YMDD to SMAA mutant (Brt_{SMAA}) were constructed, overexpressed in *E. coli*, and purified from insoluble inclusion bodies. [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.] Proteins were solubilized with the use of 6 M guanidine hydrochloride and renatured in the presence of 1 M nondetergent sulfobetaines (Novagen, Madison, WI) and dialyzed overnight in 40 mM Tris pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 50% glycerol. RT assays were performed essentially as described by M. Matsuura *et al.* [*Genes Dev.* **11**, 2910 (1997)] with the use of poly(rA) as the template primed with oligo$(dT)_{18}$ primers.
13. BPP-1 or BMP-1 lysogens were induced with mitomycin C to eliminate selection for infectious phages. The lysates were used to generate polymerase chain reaction (PCR) products containing VR1, which were purified and digested with Afl III or Alu I for BMP-1 or with Mbo II for BPP-1. The amplification-restriction cycle was repeated until no further cutting was observed, and the products were cloned into pBluescript KS+ for sequencing. See supplementary information (*8*) for the primers used.
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18. At the nucleotide level, variations were consistently seen at 23 positions in VR1 and, in most cases, all four nucleotides were observed [Web fig. 2 (*8*)]. Therefore, the maximum theoretical diversity is $4^{23} = 7 \times 10^{13}$. Of the 23 variable bases, 20 are distributed in 10 separate dinucleotides capable of encoding one of 15 possible amino acids, and 2 are in the first base of a codon. The theoretical amino acid diversity is then $15^{10} \times 4^2 = 9.2 \times 10^{12}$.
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21. We would like to thank the members of the Miller lab for helpful comments throughout the course of this work. M.L. was supported by a research fellowship from the American Lung Association and a training grant GM-08042 to the UCLA-CalTech Medical Scientist Training Program from the NIH. This work was supported by NIH grants AI38417 (J.F.M.), AI43986 (P.A.C.), and T32A107323 (R.D.) and by USDA grant 1999-02298 (J.F.M and P.A.C). The sequencing of BPP-1, BMP-1, and BIP-1 genomes were supported by the Wellcome Trust.

26 October 2001; accepted 24 January 2002

Influence of SHIP on the NK Repertoire and Allogeneic Bone Marrow Transplantation

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Natural killer cell (NK) receptors for major histocompatibility complex (MHC) class I influence engraftment and graft-versus-tumor effects after allogeneic bone marrow transplantation. We find that SH2-containing inositol phosphatase (SHIP) influences the repertoire of NK receptors. In adult $SHIP^{-/-}$ mice, the NK compartment is dominated by cells that express two inhibitory receptors capable of binding either self or allogeneic MHC ligands. This promiscuous repertoire has significant functional consequences, because $SHIP^{-/-}$ mice fail to reject fully mismatched allogeneic marrow grafts and show enhanced survival after such transplants. Thus, SHIP plays an important role in two processes that limit the success of allogeneic marrow transplantation: graft rejection and graft-versus-host disease.

Certain intracellular phosphatases contain SH2 domains that enable their recruitment to phosphorylated tyrosine residues in the cytoplasmic tails of inhibitory receptors (*1*). Following recruitment to the plasma membrane, these enzymes remove phosphate groups on other proteins or inositol phospholipids and thus counteract signaling cascades necessary for cell survival, proliferation or differentiation. SHIP is one of these signaling phosphatases (*2–4*). SHIP has been shown to limit the number of myeloid cells produced in vivo demonstrating it as a crucial mediator of survival signals in a hematopoietic lineage (*5, 6*).

In order to examine the role SHIP plays in NK development and function, we generated mice with a targeted mutation in the SHIP gene resulting in SHIP-deficient mice (*7*). Analysis of the peripheral NK compartment at different stages of ontogeny (*7*) indicated NK cells develop normally in juvenile $SHIP^{-/-}$ mice (Fig. 1A). However, in adult mice an abnormal population of NK cells appears that expresses approximately 10-fold higher surface levels of the NK receptor, NK1.1 ($NK1.1^{hi}$) (Fig. 1A). The $NK1.1^{hi}$ population lacks CD3 and thus is not an NK-T cell population. The appearance of the

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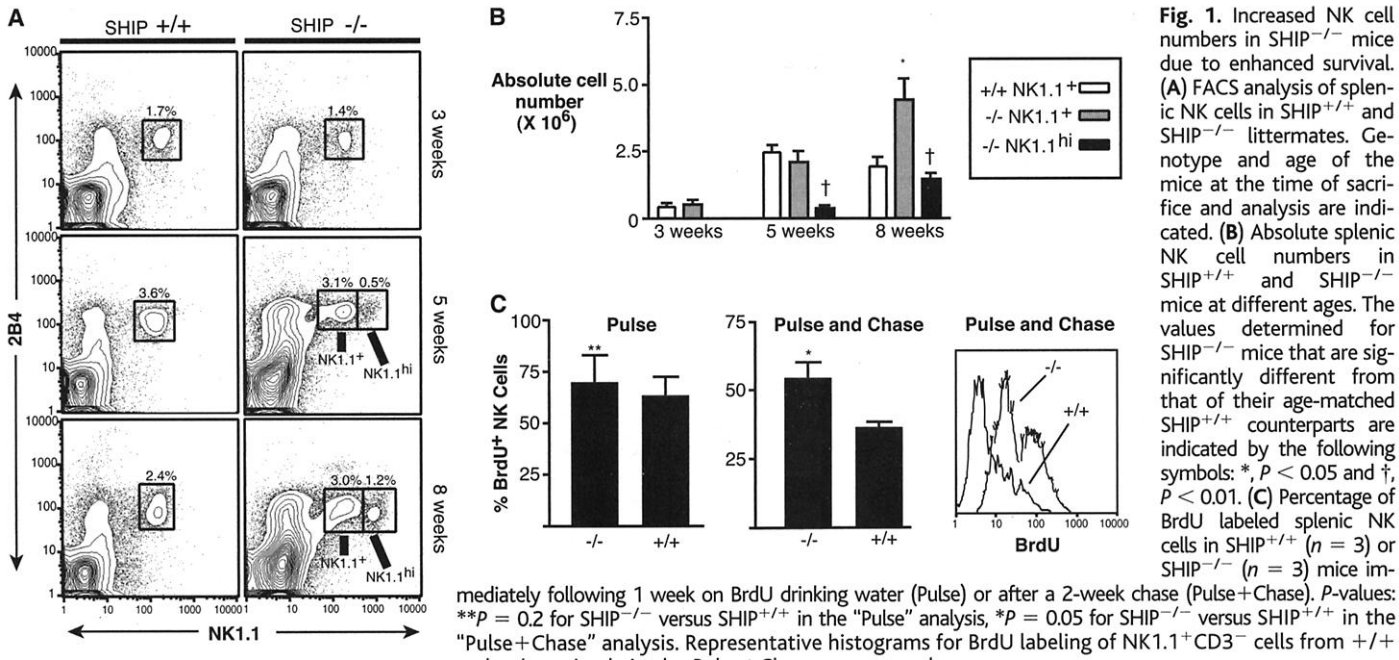

Fig. 1. Increased NK cell numbers in SHIP$^{-/-}$ mice due to enhanced survival. (**A**) FACS analysis of splenic NK cells in SHIP$^{+/+}$ and SHIP$^{-/-}$ littermates. Genotype and age of the mice at the time of sacrifice and analysis are indicated. (**B**) Absolute splenic NK cell numbers in SHIP$^{+/+}$ and SHIP$^{-/-}$ mice at different ages. The values determined for SHIP$^{-/-}$ mice that are significantly different from that of their age-matched SHIP$^{+/+}$ counterparts are indicated by the following symbols: *, $P < 0.05$ and †, $P < 0.01$. (**C**) Percentage of BrdU labeled splenic NK cells in SHIP$^{+/+}$ ($n = 3$) or SHIP$^{-/-}$ ($n = 3$) mice immediately following 1 week on BrdU drinking water (Pulse) or after a 2-week chase (Pulse+Chase). *P*-values: **$P = 0.2$ for SHIP$^{-/-}$ versus SHIP$^{+/+}$ in the "Pulse" analysis, *$P = 0.05$ for SHIP$^{-/-}$ versus SHIP$^{+/+}$ in the "Pulse+Chase" analysis. Representative histograms for BrdU labeling of NK1.1$^+$CD3$^-$ cells from +/+ and −/− animals in the Pulse+Chase group are shown.

NK1.1^{hi} population coupled with an increase in NK cells with a normal 2B4$^+$NK1.1$^+$ staining profile (NK1.1$^+$ cells) leads to a two- to threefold increase in peripheral NK cells in SHIP$^{-/-}$ adult mice (≥8 weeks), relative to wild-type littermates (Fig. 1B).

We then examined whether the increased number of NK cells present in SHIP$^{-/-}$ mice is due to increased proliferation or due to enhanced survival (*7*). When SHIP$^{-/-}$ and SHIP$^{+/+}$ mice were labeled for 1 week with BrdU and their NK compartments were analyzed, we found no significant difference in BrdU uptake (Fig. 1C) indicating NK cells in both genotypes have similar rates of production and proliferation. However, when mice were pulsed with BrdU for 1 week and the mice analyzed 2 weeks later, a significantly larger proportion of the SHIP$^{-/-}$ NK compartment retained the BrdU label (Fig. 1C), demonstrating that SHIP$^{-/-}$ NK cells survive longer in vivo than do their SHIP$^{+/+}$ counterparts.

Murine NK cells detect MHC class I molecules using receptors encoded by the Ly49 or CD94/NKG2 genes (*8*). Expression of these MHC receptors is distributed among different NK subsets during the transition from neonate to adult. Because the number of peripheral NK cells increases in SHIP$^{-/-}$ mice during this period, we asked whether the relative representation of NK subsets expressing certain Ly49 and CD94 receptors might account for this increase (*7*). Indeed, the relative representation of several Ly49 receptors and CD94 was significantly altered in the SHIP$^{-/-}$ NK compartment of older mice when compared to SHIP$^{+/+}$ littermates (Fig. 2) (*7*). However, SHIP$^{-/-}$ weanlings showed no skewing of their NK repertoire, relative to wild-type littermates (Fig. 2)

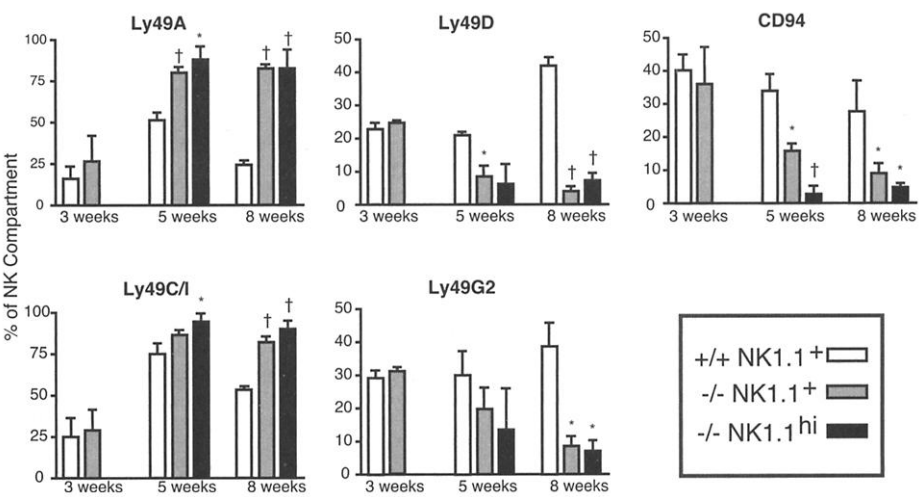

Fig. 2. MHC class I receptors on peripheral NK cells in SHIP$^{-/-}$ mice. The mean percentage of peripheral NK cells expressing the indicated Ly49 or CD94 molecule after gating on 2B4$^+$NK1.1$^+$ cells. The age and genotype of the mice are indicated. Values determined for SHIP$^{-/-}$ mice that are significantly different from their age-matched SHIP$^{+/+}$ littermates are indicated: *, $P < 0.05$; †, $P < 0.01$.

(*7*). This repertoire distortion was most pronounced in mice ≥8 weeks of age and was found in both the NK1.1$^+$ and the NK1.1^{hi} populations. We found that expression of Ly49A$^+$ and C/I$^+$ was overrepresented in adult SHIP$^{-/-}$ mice, while Ly49D, G2 and CD94 were underrepresented (*7*). Because the overwhelming majority of the NK1.1$^+$ and NK1.1^{hi} cell populations lacked Ly49I in adult SHIP$^{-/-}$ mice (*7*), then the majority of the Ly49C/I$^+$ NK cells express only Ly49C. Thus, the repertoire distortion in adult SHIP$^{-/-}$ mice leads to an NK compartment dominated by a subset of cells with the following repertoire: Ly49A$^+$C$^+$D$^-$G2$^-$I$^-$CD94$^-$. In vitro and in vivo studies indicated that Ly49C and Ly49A can bind ligands in the H2b haplotype of SHIP$^{-/-}$ mice; however, these two receptors also bind and transmit inhibitory signals from ligands in most or all H2 haplotypes (*9–12*). Therefore, SHIP deficiency leads to an NK inhibitory repertoire that is both self-specific and promiscuous for other ligands.

A potential explanation for the repertoire disruption seen in SHIP$^{-/-}$ NK cells is that SHIP is recruited to certain inhibitory receptors expressed by NK cells to oppose intracellular signals that mediate survival of specific NK subsets expressing these receptors. Indeed, SHIP binds the phosphorylated ITIM motif of Ly49A in vitro (*13*). These findings prompted us to examine whether SHIP associates in vivo

with Ly49 receptors expressed by NK cells (*7*). This analysis revealed that SHIP is associated with Ly49A and Ly49C under physiological conditions (Fig. 3A), but not Ly49G2, Ly49F, or Ly49I (Fig. 3B). As further confirmation that the protein coprecipitating with Ly49A and Ly49C is SHIP, we analyzed NK lysates from $SHIP^{+/+}$ and $SHIP^{-/-}$ mice (Fig. 3C). This analysis detected coprecipitation of SHIP with Ly49A and Ly49C only in the $SHIP^{+/+}$ NK lysates, confirming the coprecipitating protein is SHIP.

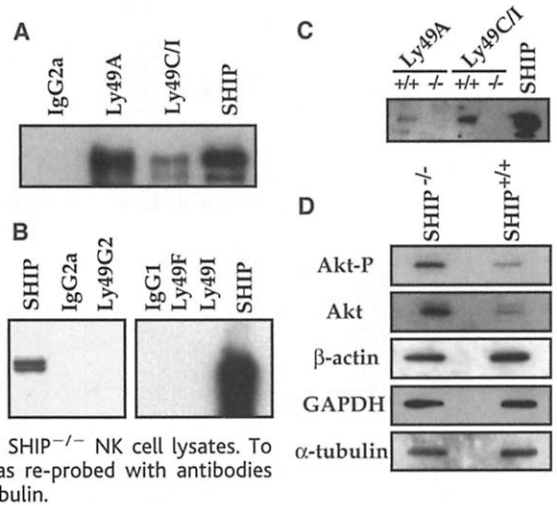

Fig. 3. SHIP is recruited to NK inhibitory receptors in vivo to oppose activation of Akt. (**A**) Western blot detection of SHIP in Ly49A and Ly49C immunoprecipitates. Immunoprecipitation with a murine IgG2a antibody (IgG2a) was analyzed as a negative control and SHIP was immunoprecipitated as a positive control (SHIP). (**B**) Western blotting for SHIP in other Ly49 immunoprecipitates (Ly49G2, Ly49F, and Ly49I). (**C**) Western blot analysis of SHIP in Ly49A and Ly49C immunoprecipitates prepared from lysates of $SHIP^{+/+}$ (+/+) and $SHIP^{-/-}$ (−/−) NK cells. (**D**) Western blot analysis of Akt phosphorylation at Thr^{308} and total Akt protein in $SHIP^{+/+}$ and $SHIP^{-/-}$ NK cell lysates. To control for equal loading the blot was re-probed with antibodies specific for β-actin, GAPDH, and α-tubulin.

Because SHIP limits the in vivo survival of myeloid cells by opposing the PI3K/Akt pathway (*3, 4*), we examined whether Akt is activated in $SHIP^{-/-}$ NK cells in vivo based on its phosphorylation at Thr^{308} (*14*). We found that both Akt phosphorylation and total Akt protein levels were significantly increased in $SHIP^{-/-}$ NK cells relative to those in $SHIP^{+/+}$ NK cells (Fig. 3D) (*7*). The increase in total Akt levels is surprising; however, primary B cell activation leads to increased Btk levels in a PI3K-dependent manner (*15*). This additional level of regulation may further amplify signals from PH domain-containing kinases (Akt, Btk) that are recruited to $PI(3,4,5)P_3$. Consistent with the activation of the PI3K/Akt pathway in $SHIP^{-/-}$ NK cells, we find that *Bcl-2* levels were increased in $SHIP^{-/-}$ NK cells and the p85 subunit of PI3K is recruited to Ly49A and Ly49C/I (*16*). Taken together, these findings suggest the interplay of SHIP and PI3K may influence the relative survival of NK subsets expressing MHC class I receptors capable of recruiting these enzymes. Interestingly, PI3K is recruited to human KIR and can activate Akt in human NK cells (*17*). Thus, despite their evolutionary divergence in how they bind MHC class I, murine Ly49 receptors and human KIR may recruit SHIP to limit the in vivo survival of NK subsets, just as both receptors recruit SHP-1 to limit NK effector functions (*18, 19*).

We speculated that the distortion of the NK repertoire toward inhibitory receptors with ligands in most MHC haplotypes might hamper responses to allogeneic targets in $SHIP^{-/-}$ mice. Indeed, transgenic mice with enforced expression of Ly49A are unable to reject allogeneic bone marrow (BM) grafts from H2d donors (*20*). Thus, we examined whether $SHIP^{-/-}$ mice on an H2b background could acutely reject fully H2 mismatched BM grafts (*7*). We first analyzed whether $SHIP^{-/-}$ mice reject fully allogeneic BM grafts from BALB/C mice whose H2d haplotype forms strong interactions with both Ly49A and Ly49C. Consistent with our hypothesis, we observed that BALB/C BM was not rejected by $SHIP^{-/-}$ mice, whereas their wild-type littermates could reject equivalent grafts (Fig. 4A). However, the failure of $SHIP^{-/-}$ mice to reject H2d grafts might also have been due to underrepresentation of the activating receptor, Ly49D, which also binds H2d. To rule out this possibility, we tested the ability of $SHIP^{-/-}$ hosts to reject BM grafts from A/SW(H2s)/Sn mice whose H2s haplotype possesses inhibitory ligands for both Ly49A and Ly49C (*9*), but not activating ligands for Ly49D. Consistent with our proposed mechanism, $SHIP^{-/-}$, but not $SHIP^{+/+}$ hosts,

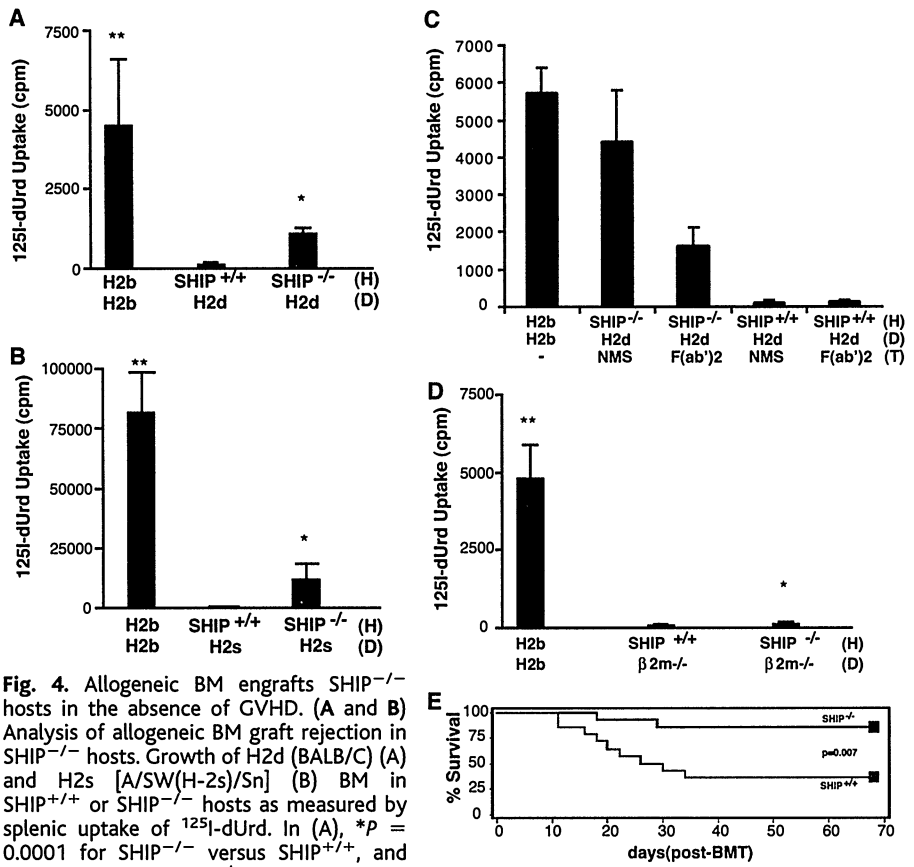

Fig. 4. Allogeneic BM engrafts $SHIP^{-/-}$ hosts in the absence of GVHD. (**A** and **B**) Analysis of allogeneic BM graft rejection in $SHIP^{-/-}$ hosts. Growth of H2d (BALB/C) (A) and H2s [A/SW(H-2s)/Sn] (B) BM in $SHIP^{+/+}$ or $SHIP^{-/-}$ hosts as measured by splenic uptake of ^{125}I-dUrd. In (A), $*P$ = 0.0001 for $SHIP^{-/-}$ versus $SHIP^{+/+}$, and $**P$ = 0.0633 for $SHIP^{-/-}$ versus syngeneic control. In (B), $*P$ = 0.0006 for $SHIP^{-/-}$ versus $SHIP^{+/+}$ and $**P$ = 0.002 for $SHIP^{-/-}$ versus syngeneic control. (**C**) Growth of BALB/C donor BM in $SHIP^{+/+}$ or $SHIP^{-/-}$ (H2b) hosts after pretreatment with blocking antibodies against Ly49C receptors [F(ab')2] or with 0.2 ml of normal mouse serum (NMS) as a source of irrelevant mouse IgG. *P*-values: F(ab')2-treated $SHIP^{-/-}$ versus NMS-treated $SHIP^{-/-}$, P = 0.0476; F(ab')2-treated $SHIP^{+/+}$ versus NMS-treated $SHIP^{+/+}$, P = 0.4654; Syngeneic control versus NMS-treated $SHIP^{-/-}$, P = 0.4000; NMS-treated $SHIP^{-/-}$ versus NMS-treated $SHIP^{+/+}$, P = 0.0179; F(ab')2-treated $SHIP^{-/-}$ versus F(ab')2-treated $SHIP^{+/+}$, P = 0.0022. (**D**) Growth of $\beta2m^{-/-}$ BM in $SHIP^{+/+}$ (C57BL6/J) or $SHIP^{-/-}$ (C57BL6/J) hosts, $*P$ = 0.2894 for $SHIP^{-/-}$ versus $SHIP^{+/+}$, $**P$ = 0.0001 for $SHIP^{-/-}$ versus syngeneic control (D- donor; H- host; T- treatment). (**E**) Survival plots for $SHIP^{-/-}$ and $SHIP^{+/+}$ recipients receiving fully H2 mismatched BM grafts.

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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23. We thank S. Highfill for genotyping of SHIP mice, A. Cantor for Kaplan-Meier analysis of survival, and M. Bennett for providing the 5E6 F(ab')2 fragments and helpful comments on the manuscript. This work is supported in part by grants from the NIH (RO1 DK54767, PO1 NS27405).

28 November 2001; accepted 24 January 2002

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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