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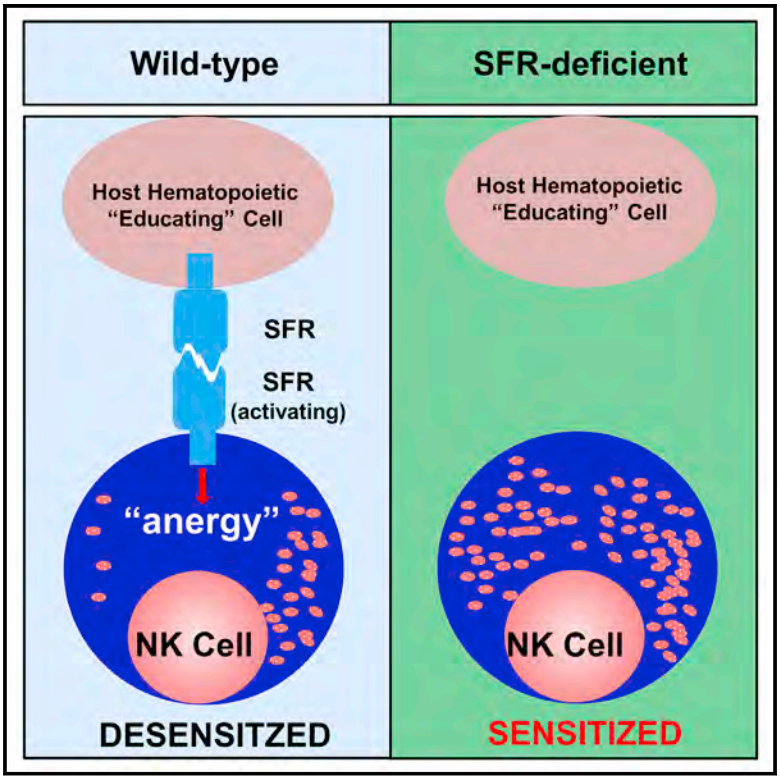
The Self-Specific Activation Receptor SLAM Family Is Critical for NK Cell Education

Graphical Abstract



Highlights

- Mice lacking seven SFR members were generated using the sequential CRISPR technique
- SFR-deficient NK cells displayed enhanced reactivity against hematopoietic cells
- SFRs acted as activation receptors in NK cell rejection of hematopoietic cells
- Chronic engagement of activating SFRs desensitized NK cell responsiveness

Authors

Shasha Chen, Meixiang Yang, Juan Du, ..., Lianfeng Zhang, Zhigang Tian, Zhongjun Dong

Correspondence

tzg@ustc.edu.cn (Z.T.), dongzj@biomed.tsinghua.edu.cn (Z.D.)

In Brief

NK cell education describes a process for NK cell acquisition of functional competence, which is primarily achieved by self-MHC-I-specific inhibitory receptors. Dong and colleagues demonstrate that the self-specific activation receptor SLAM family is critical for NK cell education and activation.

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The Self-Specific Activation Receptor SLAM Family Is Critical for NK Cell Education

Shasha Chen,[1,5] Meixiang Yang,[2,5] Juan Du,[1] Dan Li,[1] Zehua Li,[1] Chenxu Cai,[1] Yuanwu Ma,[3] Lianfeng Zhang,[3] Zhigang Tian,[4,*] and Zhongjun Dong[1,*]

[1]Institute for Immunology, School of Medicine and Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing 100084, China
[2]Biomedical Translational Research Institute, Jinan University, Guangzhou 510632, China
[3]Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Comparative Medical Center, Peking Union Medical College, Beijing 100021, China
[4]School of Life Sciences, University of Sciences and Technology of China, Hefei 230026, China
[5]Co-first author
*Correspondence: tzg@ustc.edu.cn (Z.T.), dongzj@biomed.tsinghua.edu.cn (Z.D.)
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SUMMARY

NK cell education, a term describing a process for NK cell acquisition of functional competence, is primarily achieved by self-MHC-I-specific inhibitory receptors. In this study, we have demonstrated that SLAM family receptors (SFRs) redundantly expressed on hematopoietic cells function as self-specific activation receptors critical for NK cell education. To overcome gene redundancy, we generated mice simultaneously lacking seven SFRs, revealing that NK-cell-mediated rejection of semi-allogeneic hematopoietic cells largely depended on the presence of SFRs on target cells. This stimulatory effect was determined by the presence of SFR-coupled adaptors; however, SFR-deficient mice displayed enhanced reactivity to hematopoietic cells. These findings demonstrate that SFRs endow NK cells with an ability to kill hematopoietic cells during the effector phase; however, the sustained engagement of SFRs can desensitize NK cell responses during an education process. Therefore, self-specific activating ligands may be "tolerogens" for NK cells, akin to self-antigens that induce T cell tolerance.

INTRODUCTION

The activation of natural killer (NK) cells plays an important role in immunosurveillance against abnormal cells, such as virally infected cells, malignant cells, and allogenic bone marrow. In contrast to T and B cells, NK cells are innate lymphocytes without antigen-specific receptors. These cells possess germline-encoded activation receptors responsible for killing these "unwanted" cells. NK cells must maintain tolerance to self-tissues, which is primarily achieved by self-major histocompatibility complex (MHC) class I molecules (Shifrin et al., 2014; Raulet and Vance, 2006; Elliott and Yokoyama, 2011; Orr and Lanier, 2010). These ubiquitous MHC molecules, which independently act as molecular signatures of "self," are sensed by NK cell inhibitory receptors, including inhibitory Ly49 family receptors and NKG2A in mice. However, NK cells exhibit self-tolerance to the target cells despite a lack of self-MHC-I (Bix et al., 1991; Liao et al., 1991; Kim et al., 2005). Similarly, the absence of these inhibitory receptors also leads to NK cell hyporesponsiveness (Bélanger et al., 2012; Fernandez et al., 2005). Therefore, Ly49-mediated recognition of self-MHC-I molecules on host cells is vital to the education (also called "licensing") of NK cells, a process that describes NK cell functional competence.

In contrast, the knowledge concerning the roles of activation receptors in NK cell education is still incomplete. The potential of activation receptors to influence NK cell education has been investigated by overexpressing the ligands on normal cells in transgenic mice. For examples, transgenic expression of m157, a mouse CMV (MCMV)-encoded protein specific for activation receptor Ly49H, causes a reduced NK cell responsiveness (Sun and Lanier, 2008; Tripathy et al., 2008). Similarly, ectopic expression of Rae1, a ligand for NKG2D, results in reduced capacity of NK cells to reject mismatched cells (Coudert et al., 2008). Consistently, the inactivation of NK cell activation receptors, such as NKp46 and NKG2D, results in enhanced NK cell effector function (Narni-Mancinelli et al., 2012; Deng et al., 2015; Zafirova et al., 2009). These studies imply that ligands for NKp46 or NKG2D must be expressed on normal cells, but the prediction has not been tested because the relevant ligands have not yet been discovered. Therefore, convincing evidence is still lacking to support the fine-tuning of NK cell reactivity by self-specific activation receptors that recognize endogenous self-ligands.

"Hybrid resistance," which refers to the failure of first-generation hybrid mice to accept parental semi-allogeneic hematopoietic grafts, is thought to be executed by NK cells (Bennett et al., 1995; Daley and Nakamura, 1984). The mechanism involving the preference of NK cells to kill mismatched hematopoietic cells is poorly understood. Unless "missing-self" status is sufficient for the activation of NK cells, hematopoietic targets must display active ligands that are recognized by self-specific activation receptors on NK cells so that they are subject to being killed by wild-type (WT) NK cells when hematopoietic cells lack self-MHC-I molecules. Unfortunately, none of the hematopoietic-cell-specific ligands are involved in NK-cell-mediated hybrid resistance (Beilke et al., 2010).

We have previously shown that signaling lymphocyte activating molecule (SLAM) family receptors (SFRs) are solely detectable on hematopoietic cells (Dong et al., 2009). This family consists of seven members: 2B4, Ly9, CRACC, CD48, SLAM, CD84, and Ly108. These receptors recognize each other, so they mediate homotypic interactions between hematopoietic cells (Cannons et al., 2011; Schwartzberg et al., 2009; Veillette et al., 2007). SFRs signal through their cytoplasmic immunoreceptor tyrosine switch motifs (ITSMs), which recruit a family of SH2-domain-only-containing adaptors, including SLAM-associated protein (SAP), which has ''loss-of-function'' mutations in human X-linked lymphoproliferative (XLP) patients, and its homologs EAT-2 and ERT. Mice lacking these three adaptors have a severe defect in killing hematopoietic cells (Dong et al., 2009). This may be the indication that SFRs most likely are self-specific activation receptors. Because the activity of SFRs can be activating or inhibitory, which largely depends on the presence of SAP family adaptors or not (Dong et al., 2012; Kageyama et al., 2012; Zhao et al., 2012), the severe defect of NK cell function observed in SAP-family-deficient mice is most likely attributed to a failure of SFRs to transmit activating signaling and/or the functional switch of SFRs to be inhibitory. Most likely, both of these mechanisms are involved. Genetic removal of SFRs, either on NK cells or on hematopoietic targets, will be able to disclose the underlying mechanism.

All of the SFRs, except SLAM, are ubiquitously distributed on mature NK cells (Dong et al., 2009). Unfortunately, genetic deletion of any individual member of the SFRs cannot recapitulate the phenotype in SAP-family-deficient mice (Lee et al., 2004; Johnson et al., 2003; Cruz-Munoz et al., 2009). Moreover, the genes encoding these SFRs are closely linked and positioned within the same locus on chromosome 1. For these reasons, redundancy must exist in the family and prevents us from further investigating the important roles of SFRs for NK cell activation and education.

Herein, mice simultaneously lacking seven SFR members were generated using sequential multiplex genome engineering. We reveal that SFRs act as endogenous self-specific NK cell activation receptors in mediating rejection of semi-allogeneic hematopoietic cells. These activation receptors desensitize NK cell reactivity when NK cells persistently interact with hematopoietic cells. Hence, we provide the evidence to support that self-tissue-specific ligand may be ''tolerogens'' for NK cells in a manner akin to self-antigens, which induce T cell tolerance.

RESULTS

Mice Lacking Seven SFR Members Can Be Generated Using a Sequential CRISPR Technique

To explore whether SFRs are self-specific activation receptors involved in NK cell activation and education, we aimed to generate mice that completely lacked seven SFRs. However, the genes encoding SFRs are closely linked on chromosome 1, as shown in Figure 1A, which prevented us from obtaining such mice using conventional approaches. Instead, we used a CRISPR-based technique to sequentially delete individual genomic sequences of SFRs (Wang et al., 2013). A mixture of synthetic guide RNAs was injected into the blastocysts isolated from pure C57BL/6 (B6) mice together with the enzyme Cas9 (Figure 1B and Table S1). We used a flow-cytometry-aided approach to simplify the screening procedure. After first-round targeting, founder mice lacking SLAM, Ly108, CRACC, and CD84 were established (Figures 1B and 1C). The male founder was further bred with female WT (B6 background) mice for the second-round genome editing. A series of mutants, including three founders that were completely deficient in seven SFRs, was then obtained (Figure 1C). The expression of SFRs on immune cells isolated from the spleens of SFR-deficient mice was completely abolished (Figure S1A). Importantly, these SFR gene deletions were further confirmed by genome sequencing (Figure S1B). Total-SFR-deficient mice bred normally and produced offspring according to the expected Mendelian ratio. Histological examination of the lungs, liver, and intestine indicated no obvious autoimmunity phenotype (Figure S1C).

NK Cell Differentiation Is Moderately Altered in SFR-Deficient Mice

We initially examined the expression of SFRs on developing NK cells. SFR-deficient NK cells were used as a negative control to distinguish non-specific binding. A high proportion of NK cell progenitors expressed both SLAM and Ly108 when NK cells were committed. During NK cell differentiation, the presence of these two SFR members gradually decreased. SLAM disappeared at the terminal stage. The other SFR members, including 2B4, CRACC, Ly9, and CD84, maintained a high expression throughout all stages (Figure 1D and Table S2).

This distinct expression profile of SFRs on developing NK cells led us to investigate whether global SFR deficiency interfered with NK cell development. In fact, the percentages and absolute numbers of NK cells in both the spleen and the bone marrow (BM) were not obviously altered (Figure 1E), but a slight but significant increase in immature $CD27^{+}CD11b^{-}$ NK cells was observed in the SFR-deficient mice (Figures 1F, 1G, and S2A). In contrast, SFR-deficient NK cells consistently showed lower expression of the markers that represent NK cell maturation, such as KLRG1 (Figure S2B). Other molecules that begin to be expressed in the immature stage, such as CD27, NKG2A, and most of the Ly49 family receptors (I, D, G2, and H), were expressed on NK cells in the BM and spleen in amounts comparable to the control (Figure S2B). Furthermore, a higher percentage of SFR-deficient $CD27^{+}CD11b^{-}$ cells was still observed in chimera mice that were reconstituted with a mixture of BM cells from WT (CD45.1) and SFR-deficient mice (CD45.2), suggesting a cell-intrinsic requirement of SFRs for NK cell differentiation (Figure S2C).

SFR-Deficient NK Cells Exhibit Higher Responsiveness to Hematopoietic Cells

Next, we began to evaluate the effect of SFR deficiency on NK cell activation in a steady state. Freshly isolated resting NK cells were stimulated with RMA-S and YAC-1 cells. Contrary to the phenotype observed in SAP-family-deficient NK cells (Dong et al., 2009), SFR-deficient NK cells unexpectedly displayed an increased production of interferon-γ (IFN-γ) and an upregulated release of CD107a, a marker for degranulation, when incubated with hematopoietic RMA-S and, to a lesser extent, with YAC-1 (Figure 2A). We further sought to study the activation of polyI:C-activated NK cells. SFR-def NK cells also showed higher

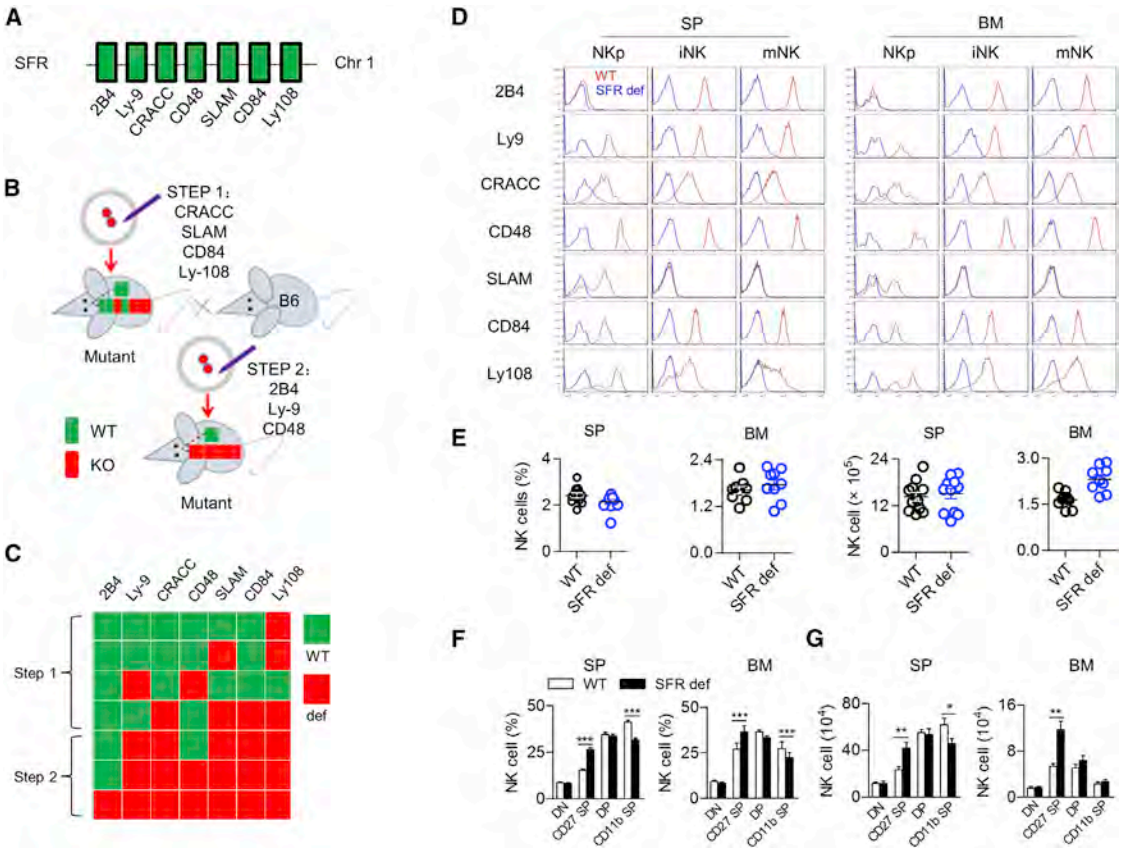

Figure 1. NK Cell Differentiation Is Slightly Altered in SFR-Deficient Mice

(A) Gene location of SLAM family receptors (SFRs).

(B) A strategy to generate multiple SFR-deficient mice using CRISPR-based multiple genome engineering.

(C) A collection of SFR mutants was generated after two-step sequential genome editing.

(D) Flow cytometry analysis of SFR expression on progenitor NK cells (NKp, $CD3^-CD122^+$ $NK1.1^-CD11b^-$), immature NK cells (iNK, $CD3^-CD122^+NK1.1^+$ $CD11b^-$), and mature NK cells (mNK, $CD3^-CD122^+NK1.1^+CD11b^+$) of WT (red line) mice. SFR-deficient (blue line) NK cells served as a negative control.

(E–G) Analysis of NK cell development. Percentages and total numbers of splenic and BM $CD3^-$ $CD19^-NK1.1^+$ cells are shown. Each symbol represents an individual mouse (E). Percentages (F) and absolute NK cell numbers (G) of four-stage development, including $CD27^-CD11b^-$ (DN), $CD27^+CD11b^-$ (CD27 SP), $CD27^+CD11b^+$ (DP), and $CD27^-CD11b^+$ (CD11b SP) of the gated $CD3^-CD19^-NK1.1^+$ NK cells in the spleen and BM of WT and SFR-deficient mice. (E–G) The data represent the mean ± SEM of 9–12 mice pooled from three independent experiments.

Please see also Figures S1 and S2.

responsiveness to RMA-S stimulation (Figure 2B). The difference was less dramatic when YAC-1 was used (Figure 2B). Due to the complexity of tumor cell lines in ligands for NK cell activation receptors, we examined the activation of SFR-deficient NK cells in response to primary MHC-I-lacking cells. Similarly, SFR-deficient NK cells activated by polyI:C exhibited increased production of IFN-γ and degranulation when triggered by hematopoietic immature DC (iDC) and T cells that were pre-activated by Con A, but not mouse embryonic fibroblasts (MEFs) (Figure 2C). Therefore, SFR-deficient NK cells have an increased ability to kill MHC-I-lacking hematopoietic cells in vitro.

Next, this finding was further validated by two NK-cell-mediated in vivo assays. Notably, SFR-deficient mice could eliminate MHC-I-lacking hematopoietic cells slightly but reproducibly faster than WT mice (Figure 2D). To ascertain whether the increased ability of SFR-deficient mice to reject hematopoietic cells in vivo could be attributed to the action of NK cells, the recipient mice were administered with anti-NK1.1 antibody to deplete NK cells. This treatment completely abolished the hyperresponsiveness of SFR-deficient mice in response to hematopoietic cells (Figure 2D). We then observed that SFR-deficient mice also rejected RMA-S tumor cells better than WT mice (Figure 2E). Taken together, these results show that SFR-deficient mice display an unexpected phenotype of NK cell hyperresponsiveness.

Furthermore, other possibilities that could cause the altered responsiveness of NK cells in SFR-deficient mice were also excluded. First, NK cells from two other SFR-deficient founders, 2# and 3#, consistently had an increased ability to secret IFN-γ following in vitro stimulation with RMA-S (Figure 2F) and to eliminate β_2m-deficient splenocytes in vivo (Figure 2G). Thus, the hyperresponsiveness phenotype in SFR-deficient mice was not due to an off-target effect, possibly caused by CRISPR-based targeting. Therefore, we chose founder 7# as the representative in the subsequent experiments. Second, based on developmentally matched comparison, the

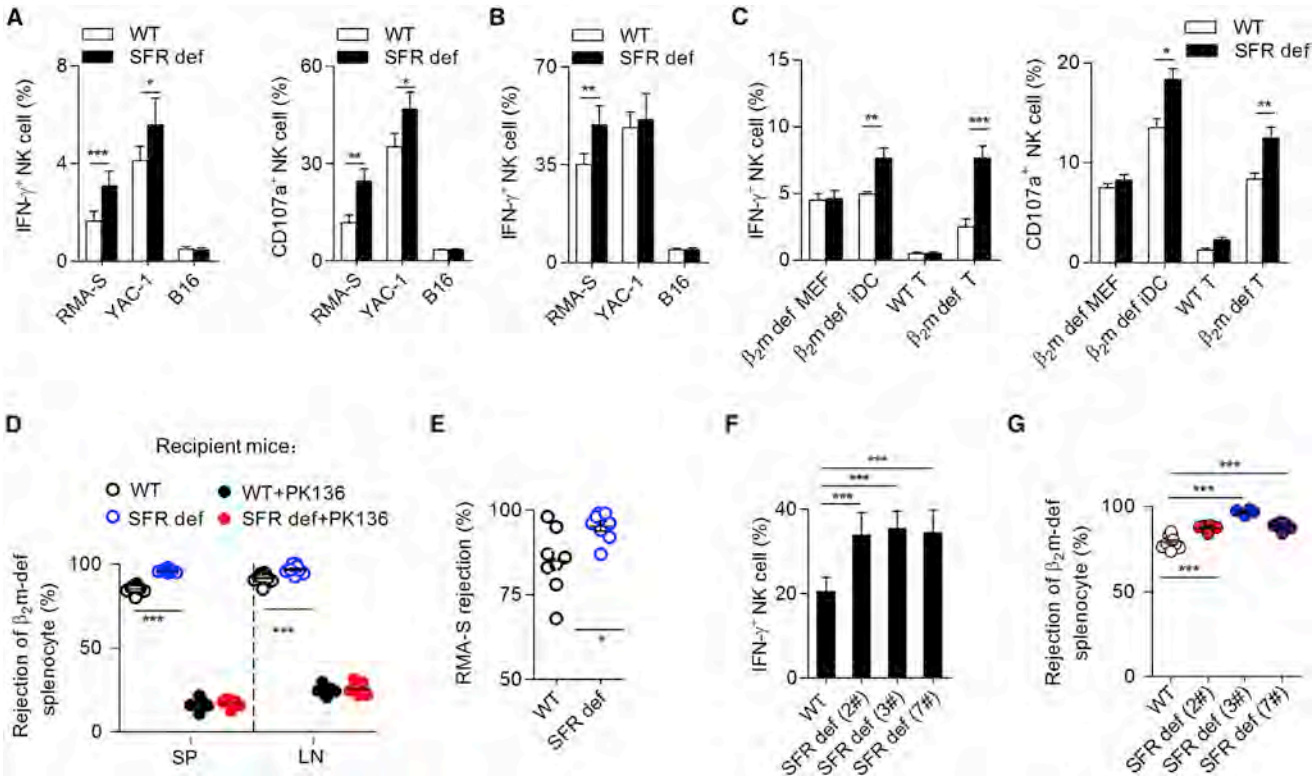

Figure 2. SFR-Deficient NK Cells Exhibit Higher Responsiveness to Hematopoietic Cells

(A and B) Naive splenocytes from WT and SFR-deficient mice were incubated with the indicated tumor targets. (A) $CD3^-$ $NKp46^+$ NK cells were analyzed for intracellular IFN-γ (left) or CD107a (right). (B) Similar to (A), while polyI:C-activated splenocytes were used. The data represent the mean ± SEM of 5–10 mice per group analyzed in two separate experiments.

(C) PolyI:C-activated splenocytes from WT and SFR-deficient mice were incubated with the indicated primary cells, including β_2m-deficient MEFs and β_2m-deficient iDCs, as well as Con-A-activated T cells with (WT T) or without β_2m (β_2m def T). NK cells were analyzed for intracellular IFN-γ (left) and CD107a (right). The data represent the mean ± SEM of 3–6 mice per group analyzed in three separate experiments.

(D) In vivo rejection of β_2m-deficient splenocytes. Percent rejection of CFSE-labeled cells obtained from the spleen (SP) and LNs of WT and SFR-deficient mice. The β_2m-deficient splenocytes were used as donor cells, and the WT (B6) splenocytes were used as an internal control. WT and SFR-deficient mice were injected with anti-NK1.1 antibody (+PK136) to deplete NK cells. Each symbol represents an individual mouse; small horizontal lines indicate the average. The data are pooled from two experiments. n = 7–8 mice per group.

(E) In vivo RMA-S peritoneal clearance. Each symbol represents an individual mouse. The data are pooled from two representative experiments. n = 7–8 mice per group.

(F) Frequency of IFN-γ-producing cells among polyI:C-activated NK cells from WT or three SFR-deficient founders (2#, 3#, and 7#) stimulated with RMA-S. The data represent the mean ± SEM of 3 mice per group analyzed in three independent experiments.

(G) In vivo rejection of β2m-deficient splenocytes. Similar to (D). The data are pooled from two representative experiments (n = 4–9 mice per group).

Please see also Figure S3.

four SFR-deficient NK cell subpopulations, $CD27^-CD11b^-$, $CD27^+CD11b^-$, $CD27^+CD11b^+$, and $CD27^-CD11b^+$, consistently displayed higher responsiveness to RMA-S stimulation than WT NK cells (Figure S3A). Thus, the hyperresponsiveness was not due to the slight modification to NK cell differentiation in SFR-deficient mice. In addition, this higher reactivity of SFR-deficient NK cells was not associated with an increase in the expression of major NK-cell-activation receptors on the cell surface (Figure S2B), and SFR deficiency did not increase the stability and duration of NK cell contact with RMA-S cells, although a critical role of SAP is the formation of NK-target conjugates (Qi et al., 2008) (Figure S3B). Lastly, but importantly, we confirmed that the hyperresponsiveness resulted from a cell-autonomous mechanism in a competitive chimera assay (Figure S3C).

SFRs Are General Activation Receptors on NK Cells

Given that the activity of SFRs on NK cells is highly complex due to their dual (activating or inhibitory) activity (Dong et al., 2009, 2012), the most plausible interpretation of the higher NK activity in SFR-deficient mice may have two possibilities. One possibility is that SFRs on NK cells may be globally inhibitory during the effector phase. The other possibility is that SFRs probably function as activation receptors that desensitize NK cell responsiveness during an "education" process because the sustained engagement of activation receptors induces cross-tolerance of multiple distinct NK cell activation pathways (Coudert et al., 2008).

To understand the total activity of SFRs on NK cells, we first determined that ectopic expression of ligands for SFRs on B16 melanoma could trigger NK cell activation, as evidenced by the

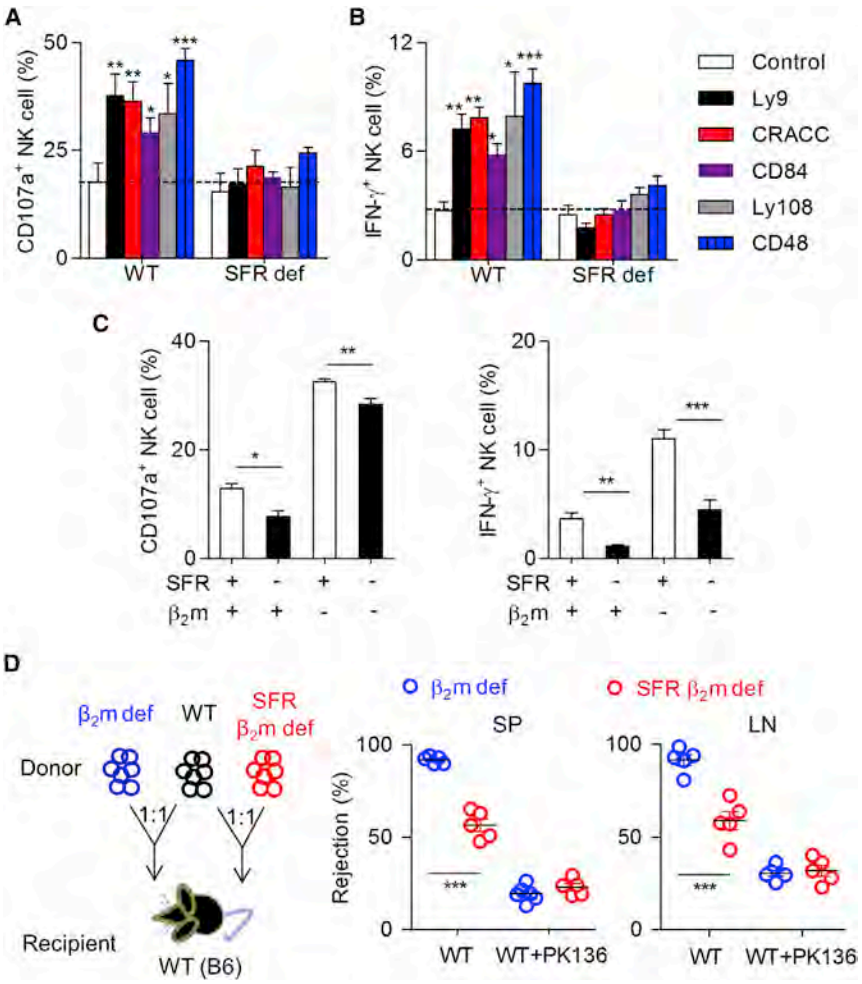

Figure 3. SFRs Are Overall Activation Receptors on NK Cells

(A and B) Frequency of CD107a$^+$ (A) and IFN-γ-producing (B) cells among polyI:C-activated NK cells from WT and SFR-deficient NK cells following stimulation by B16 cells with or without (control) the expression of indicated ligands for various SFRs. The data represent the mean ± SEM of 3–5 mice per group analyzed in two independent experiments.

(C) Frequency of CD107a$^+$ (left) and IFN-γ-producing (right) polyI:C-activated WT (B6) NK cells following stimulation by immature DCs. Targets differentially expressed with (+) or without (–) SFRs and/or β_2m were used. The data represent the mean ± SEM of 7–9 mice per group pooled from two separate experiments.

(D) In vivo rejection of β_2m-deficient splenocytes. Percent rejection of CFSE-labeled cells obtained from the spleen and LNs of recipient WT (B6) mice. Splenocytes from β_2m-deficient or SFR β_2m-deficient mice were used as donor cells, and WT (B6) splenocytes were used as an internal control. WT (B6) mice were injected with anti-NK1.1 antibody (+PK136) to deplete NK cells. Each symbol represents an individual mouse. The data are representative of three individual experiments, n = 5–6 mice per group.

Please see also Figure S4.

increased production of IFN-γ and expression of CD107a; however, the stimulatory effect was almost completely abolished in SFR-deficient NK cells (Figures 3A and 3B). These results confirmed that SFRs play a stimulatory role in the activation of WT NK cells, as previously reported (Dong et al., 2009).

Given that SFRs are self-recognizing, the deletion of SFRs on target cells will also lead to the failure of SFRs on NK cells to transmit signaling. To further ascertain the activity of SFRs, WT NK cells were chosen as effectors, while MHC-I-deficient hematopoietic cells with or without SFRs were chosen as targets. In this situation, the potential effect of SFR deficiency on NK cell education was avoided. As predicted, the loss of SFRs on WT or MHC-I-deficient iDCs led to the failure of iDC to maximize NK cell effector functions, such as the production of IFN-γ and CD107a in vitro (Figure 3C). These findings were further proven using an in vivo model in which SFR-sufficient β_2m-deficient hematopoietic cells as targets were easily eliminated in WT (B6) mice, whereas SFR β_2m-deficient cells remained relatively resistant to be rejected (Figure 3D). As expected, SFR-deficient mice were able to equally reject β_2m-deficient and SFR β_2m-deficient hematopoietic cells but better than WT mice did (Figure S4). These experiments demonstrate that SFRs function overall as activation receptors on NK cells.

To address whether the residual rejection of SFR-deficient hematopoietic cells is mediated by NK cells, recipient mice were depleted of NK cells by anti-NK1.1 antibody. This treatment caused more severe defects in the rejection of adoptively transferred lymphocytes, with or without SFRs, suggesting that the residual rejection of SFR-deficient lymphocytes was also mediated by NK cells, perhaps through additional NK cell activation receptors (Figure 3D).

SFRs Are Required for Acute Rejection of Semi-Allogeneic Hematopoietic Cells, a Model for Hybrid Resistance

To test whether SFRs are NK cell activation receptors and involved in NK-mediated hybrid resistance, first, an in vitro assay was performed to reveal that SFR-sufficient parental hematopoietic cells that lack MHC-I could efficiently trigger the activation of WT hybrid NK cells, as evidenced by the elevated production of IFN-γ and release of CD107a, but the absence of SFRs on these targets greatly diminished the stimulatory effect (Figure S5). Notably, an in vivo model mimicking hybrid resistance also revealed that SFR-deficient parental BM cells were considerably resistant to in vivo rejection in the irradiated WT (F1) mice (Figure 4A). A similar difference was also noted when non-irradiated hybrid mice were shown to reject splenocytes (Figure 4B). To determine whether the difference was due to the differential activation of NK cells caused by SFR expression, WT (F1) recipient mice were injected with anti-NK1.1 antibody to deplete NK cells in vivo. As expected, NK-depleted recipients accepted SFR-sufficient targets as comparable to SFR-deficient ones (Figure 4B).

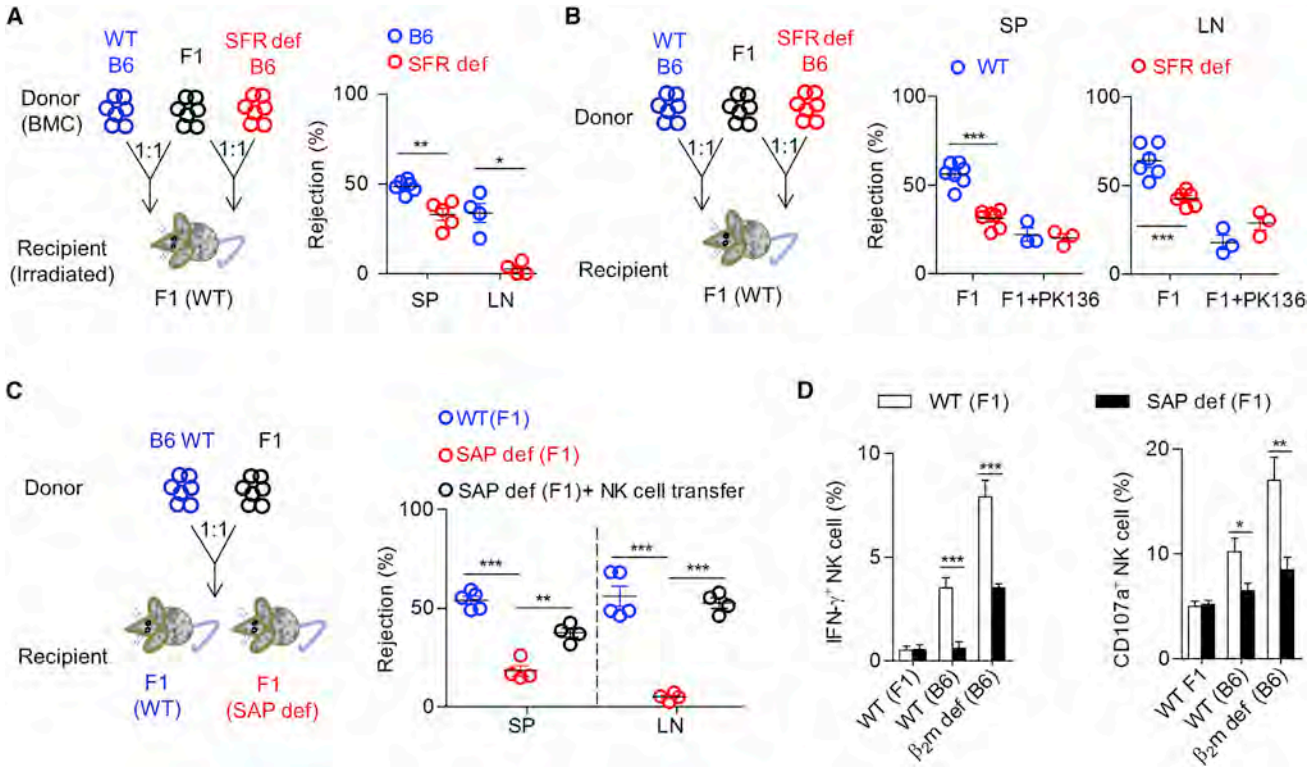

Figure 4. SFRs and Adaptor Protein SAPs Are Required for NK-Cell-Mediated Acute Rejection of Semi-Allogeneic Hematopoietic Cells

(A) In vivo model for hybrid resistance. Left, experimental flow, irradiated WT (F1) mice were injected with a mixture of parental WT or SFR-deficient BMCs (B6) mixed with an equal number of WT (F1) BMCs as an internal control. Right, percent rejection of CFSE-labeled cells obtained from the spleen and LNs of WT (F1) mice was quantified. Each symbol represents an individual mouse. The data represent the mean ± SEM of 4–6 mice per group. The data are representative of two experiments.

(B) Left, experimental design. Right, percent rejection of CFSE-labeled cells obtained from the spleen (SP) and LNs of WT (F1) mice 6 hr after injection of WT or SFR-deficient splenocytes, together with WT (F1) splenocytes as an internal control. To confirm NK dependence, WT (F1) mice were injected with anti-NK1.1 antibody (+PK136) to deplete NK cells. Each symbol represents an individual mouse. The data are representative of three experiments are expressed as the mean ± SEM of 3–7 mice per group.

(C) In vivo model for hybrid resistance. Left, experimental flow, WT or SAP-deficient mice (F1) were injected with a mixture of WT (B6) splenocytes together with WT (F1) splenocytes as an internal control. Right, the percentage of rejection was quantified. Purified NK cells (2×10^6) from WT (F1) mice were adoptively transferred to SAP-deficient mice (F1) to confirm NK cell-dependence. The data are representative of three experiments and are expressed as the mean ± SEM of 4–5 mice per group.

(D) Frequency of IFN-γ-producing (left) and $CD107a^+$ (right) polyI:C-activated NK cells from WT or SAP-deficient mice (F1) stimulated with BM-derived iDCs derived from WT or β_2m-deficient WT (B6) mice, and F1 as the control. The data representing the mean ± SEM of 3–4 mice per group are representative of two experiments.

Please see also Figure S5.

Thus, the ligands for SFRs on BM cells could trigger the NK-cell-mediated rejection of semi-allogenic hematopoietic cells. NK-cell-depleted mice exhibited more severe defect in the clearance of hematopoietic targets either with or without SFRs (Figure 4B), suggesting that other self-specific activation receptors probably exist. Therefore, our findings demonstrate that SFRs act as at least one type of self-specific activation receptor that is involved in the model for hybrid resistance.

SAP, an SFR-Coupled Adaptor, Is Required for the Model for Hybrid Resistance

To further validate SFR-mediated downstream activating signaling in the model for hybrid resistance, we investigated whether SFR-coupled adaptors are involved. SAP was chosen as a representative of the family because the *Sh2d1a* gene encoding SAP lies on the X chromosome and male SAP-deficient mice (F1) could be obtained by crossing WT (BALB/c) mice with *Sh2d1a* heterozygous mice ($Sh2d1a^{+/-}$, H-2^b). Notably, SAP-deficient mice (F1) displayed more severe defects in the rejection of the transferred lymphocytes in the detected spleens and LNs (Figure 4C). To validate whether the action of SAP occurs through NK cells, NK cells (F1) were purified and adoptively transferred to SAP-deficient mice (F1). This treatment could largely rescue the severe defect of recipient mice in the rejection of semi-allogeneic hematopoietic cells (Figure 4C). A further in vitro assay was then performed. When SAP-deficient NK cells (F1) were co-cultured with parental iDC cells, they failed to be fully activated (Figure 4D). In parallel, similar results were achieved when NK cells were exposed on MHC-I-deficient DC cells as stimuli (Figure 4D). These experiments demonstrate

that SFRs mediate NK-cell-mediated rejection of parental hematopoietic cells through coupling SAP family adaptors or at least through SAP.

Chronic Engagement of SFRs Desensitizes NK-Cell Responsiveness during Their Education

The data discussed above clearly exclude the possibility that the increased activity of SFR-deficient NK cells was due to a loss of SFR inhibitory activity during the effector phase. We hypothesized that SFRs might function as self-specific activation receptors that weaken NK cell responsiveness when NK cells persistently make contact with SFR-expressing hematopoietic cells during the "education" process. However, the question is difficult to test because the repertoire of activation receptors on NK cells that recognize primary hematopoietic cells is not completely defined. As an alternative, we analyzed the activation of individual polyI:C-activated NK cells by stimulation through NK1.1 and Ly49D, as the antibodies against them have widely been used for the analysis of NK cell education. These antibodies stimulate NK cell activation in a dose-dependent manner (Kim et al., 2005). We found that crosslinking NK1.1 using a high concentration of plate-coated antibody led to IFN-γ production by WT NK cells, while SFR-deficient NK cells displayed a stronger ability to secrete this cytokine (Figure 5A). Similar results were also observed upon stimulation through Ly49D (Figure 5A), as well as Rae1-expressing B16 cells (Figure S6A). The difference between the two genotypes seemed to be less dramatic at low antibody concentrations (Figure S6B). We also noted that the naive NK cells were more sensitive to crosslinking through NK1.1 or Ly49D (Figure 5B). Therefore, the increased activity of these activation receptors can probably be attributed to the hyperresponsiveness phenotype in SFR-deficient mice. Taken together, SFR-mediated interactions between NK cells and hematopoietic cells most likely weaken NK cell responsiveness, probably by affecting the activity of other activation receptors.

SFR-Involved NK Cell Education Is Affected by Inhibitory Receptors that Recognize Self-MHC-I Molecules

Furthermore, we sought to understand whether SFR-involved NK cell education is influenced by self-MHC-I dependent inhibitory signaling, which is primarily necessary for NK cell functional competence. As we have known that B6 (H-2^b) mice express four well-characterized inhibitory Ly49 receptors, Ly49A, Ly49C, Ly49G2, and Ly49I, on overlapping NK cell subsets. NK cells that express Ly49C and/or Ly49I bind to H-$2K^b$ and are licensed in H-2^b mice. Based on the overlapping expression of Ly49C and Ly49I, which could be specifically recognized by two monoclonal antibodies, 4LO33 and YLI-90, respectively, NK cells were divided into four subsets (Figure 5C).

To investigate whether the expression of self-MHC-I-specific Ly49C and Ly49I affects the activity of SFR-deficient NK cell subsets in response to hematopoietic, MHC-I-deficient RMA-S cells were used as stimuli to stimulate NK cells. We observed that SFR-deficient NK cells bearing Ly49C and/or Ly49I produced more cytokine than the respective WT subsets (Figure 5C). SFR-deficient Ly49C$^-$Ly49I$^-$ NK cells were also more activated by the stimulation. These findings indicate that the sustained engagement of SFRs can desensitize the responsiveness of both Ly49C$^-$Ly49I$^-$ and Ly49C$^+$Ly49I$^+$ NK subsets.

In addition to inhibitory Ly49s, NKG2A is inhibitory and most likely required for NK cell licensing by self-MHC-I. Presumably, fully educated NK cells in B6 mice are positive for Ly49C, Ly49I, and NKG2A. To further understand whether Ly49C$^-$Ly49I$^-$ NK cells contain NKG2A$^+$ NK cells, we examined these three molecules on NK cells (Figure 5D), revealing that NKG2A, Ly49C, and Ly49I overlapped on some NK cells. Indeed, half of Ly49C$^-$Ly49I$^-$ NK cells were NKG2A$^+$. Thus, we wondered whether the presence of NKG2A$^+$ NK cells among the Ly49C$^-$Ly49I$^-$ population was attributed to the increased activity of this population. To exclude this possibility, NK cells were further divided into four subsets: Ly49C$^-$Ly49I$^-$NKG2A$^-$, Ly49C$^-$Ly49I$^-$NKG2A$^+$, Ly49C$^+$Ly49I$^+$NKG2A$^-$, and Ly49C$^+$Ly49I$^+$NKG2A$^+$ (Figure 5D). We then analyzed the activity of these subpopulations of NK cells in response to RMA-S stimulation. We noted that Ly49C$^-$Ly49I$^-$NKG2A$^-$ NK cells that are "non-licensed" showed much stronger responsiveness when SFRs were absent on NK cells. Two SFR-deficient NK cell subsets expressing Ly49C$^-$Ly49I$^-$NKG2A$^+$ and Ly49C$^+$Ly49I$^+$NKG2A$^-$ showed moderately higher activity than WT NK cells (Figure 5D). However, SFR-deficient Ly49C$^+$Ly49I$^+$NKG2A$^+$ NK cells displayed comparable secretion of IFN-γ to SFR-sufficient NK cells. Therefore, SFRs differentially fine-tune NK cell subsets during the education process, probably more efficiently in the NK cells with less or no self-MHC-I dependent receptors.

In MHC-I-deficient mice, all of NK cells, either Ly49C$^-$Ly49I$^-$ or Ly49C$^+$Ly49I$^+$, are presumably not licensed by self-MHC-I molecules. To finally confirm the conclusion that SFR deficiency affects the activity of non-licensed NK cells, SFR β_2m-deficient mice were used. The in vitro stimulation assay revealed that deleting MHC-I could dampen NK cell production of IFN-γ in response to the stimulation with NK1.1 antibody, as seen previously (Kim et al., 2005). Despite this, SFR β_2m-deficient NK cells, either Ly49C$^-$Ly49I$^-$ or Ly49C$^+$Ly49I$^+$, reproducibly showed higher hyperresponsiveness to the stimulation by NK1.1 antibody compared to β_2m-deficient NK cells (Figure 5E). Thus, we conclude that the SFR deficiency indeed affects the functional acquisition of non-licensed NK cells.

The Presence of SAP Family Adaptors Determines the Activation of SFRs on NK Cells

The above data clearly elucidated the dual role of SFRs in NK cell education and activation; that is, SFRs act as self-specific activation receptors that desensitize NK cell responses during functional competence and also act as hematopoietic-specific activation receptors that trigger NK-mediated rejection of semi-allogeneic hematopoietic cells. The remaining question is how the activation of SFRs is regulated. SFRs primarily recruit SAP family members. We previously proposed two mechanisms involving the actions of SAP family members in the SFR-triggered activation of NK cells (Dong and Veillette, 2010). One is the "active signaling molecule" model, which suggests that SFRs deliver an activating signal through the recruitment of SAPs. The "natural blockers" model suggests that the SAP family prevents SFRs from coupling with SH2 domain-containing inhibitory molecules (Dong et al., 2009, 2012). As expected, normal parental B6 hematopoietic cells could not be effectively destroyed by SAP-deficient F1 NK cells. However, when the SFRs were genetically removed from these target cells, the

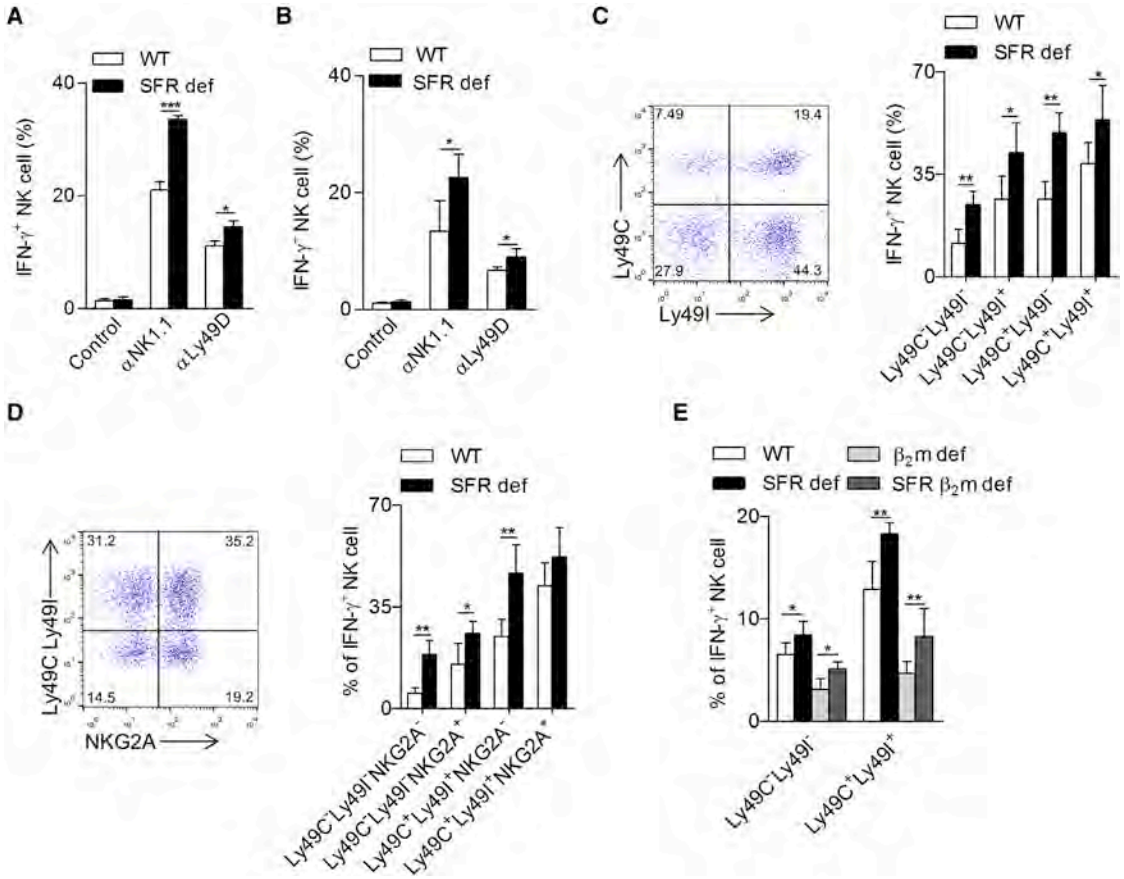

Figure 5. Chronic Engagement of Activating SFRs Desensitizes NK Cell Responsiveness during Education Process

(A and B) Poly I:C-activated (A) or resting (B) splenocytes from WT and SFR-deficient mice were stimulated by plate-coated antibody against NK1.1 or anti-Ly49D (10 μg/mL), isotype antibody as the control. $CD3^-NKp46^+$ NK cells were analyzed for intracellular IFN-γ. The percentages of IFN-γ^+ NK cells were quantified. The data represent the mean ± SEM of 3–6 mice per group analyzed in three independent experiments.

(C) Gating strategy (left), the expression of Ly49C versus Ly49I on splenic $CD3^-NKp46^+$ NK cells was distinguished by staining with antibodies, clone 4LO33 and YLI-90, respectively (right). Poly I:C-activated splenocytes from WT and SFR-deficient mice were stimulated by RMA-S. Four NK ($CD3^-NKp46^+$) subpopulations were analyzed for intracellular IFN-γ. The percentages of IFN-γ^+ NK cells were quantified. The data represent the mean ± SEM of 4–5 mice per group in two independent experiments.

(D) Gating strategy (left). Ly49C and Ly49I were first co-stained by biotin-conjugated antibodies, clone 4LO33 and YLI-90. The differential expression of NKG2A versus Ly49C and Ly49I on splenic $CD3^-NKp46^+$ NK cells was examined by flow cytometry and showed as dot plots (right). Poly I:C-activated splenocytes from WT and SFR-deficient mice were stimulated by RMA-S. Four NK ($CD3^-NKp46^+$) subpopulations were analyzed for intracellular IFN-γ. The percentages of IFN-γ^+ NK cells were quantified. The data represent the mean ± SEM of 3–5 mice per group in three independent experiments.

(E) Poly I:C-activated splenocytes from the indicated mice were stimulated by high concentration of NK1.1 antibody (10 μg/mL). Percentages of IFN-γ-producing cells among $Ly49C^-Ly49I^-$ and $Ly49C^+Ly49I^+$ (including $Ly49C^+$, $Ly49I^+$, and $Ly49C^+Ly49I^+$) NK cell populations were analyzed. Ly49C and Ly49I were distinguished by the antibodies, clone 4LO33 and YLI-90, respectively, as seen in (C). The percentages of IFN-γ^+ NK cells were quantified. The data represent the mean ± SEM of 4–5 mice per group in two independent experiments.

Please see also Figure S6.

compromised NK cell activity was rescued (Figure 6A). Similar results were also obtained when using SFR β_2m-deficient cells as donor cells (Figure 6B). These data suggest that the presence of SAP partly determines the activity of SFRs on NK cells.

To explore whether the deletion of SFRs on donor cells could reverse the severe defect in NK cells that we previously observed in SAP-family-deficient mice in the 129sv background (Dong et al., 2009), we re-generated SAP-family-deficient mice in a B6 background to ascertain that the genetic backgrounds were comparable (Jordan et al., 2007). Consistent with the data previously observed, parental B6 hematopoietic cells that express SFRs could not be effectively rejected in our newly generated SAP-family-deficient mice. However, when SFR-deficient hematopoietic targets were injected into SAP-family-deficient mice, the compromised NK cell activity was completely rescued (Figure 6C). This experiment suggests that the dominant signal from SFRs with SAP family deficiency is inhibition.

As a final test of whether the removal of SFRs on SAP-family-deficient NK cells could also diminish the inhibitory role of SFRs, we generated mice simultaneously lacking seven SFRs and three SAP family adaptors (SFRDs). However, because EAT-2 and ERT, two members of the SAP family, are also linked with the SFR locus on chromosome 1, we failed to obtain such

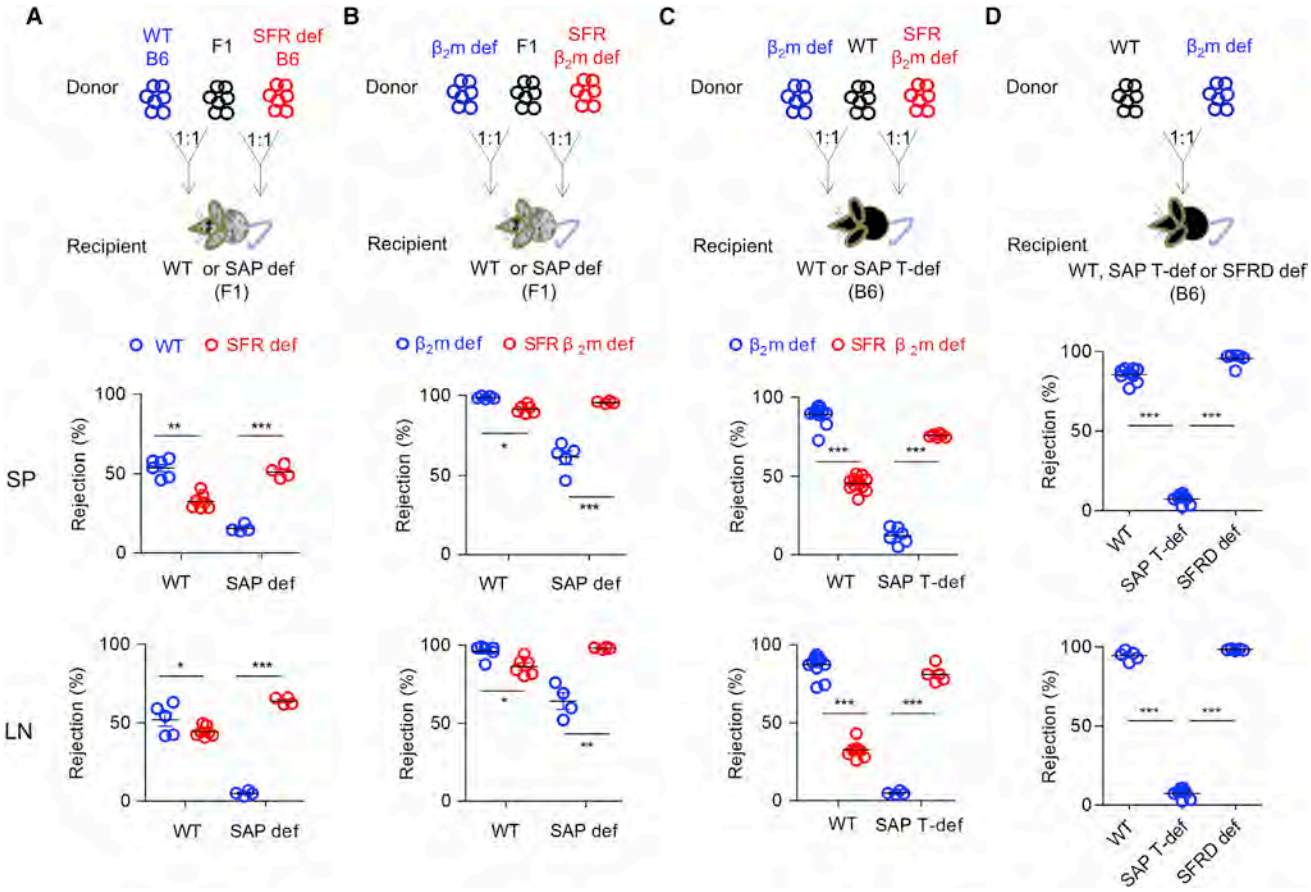

Figure 6. Presence of SAP Family Adaptors Determines the Activation of SFRs on NK Cells

(A–D) Top, experimental design.

(A) Rejection of CFSE-labeled cells obtained from the spleens (middle) and LNs (bottom) of the indicated mice after injection of parental WT or SFR-deficient B6 splenocytes mixed with an equal number of WT (F1) splenocytes as internal control. Each symbol represents an individual mouse. The data represent the mean ± SEM of 4–7 mice per group analyzed in two independent experiments.

(B) Rejection of CFSE-labeled cells obtained from the spleens (middle) and LNs (bottom) of indicated mice after the injection of parental β_2m-deficient or SFR-β_2m-deficient WT (B6) splenocytes together with an equal number of WT (F1) splenocytes as internal control. Each symbol represents an individual mouse. The data represent the mean ± SEM of 4–6 mice per group analyzed in two independent experiments.

(C) Rejection of CFSE-labeled cells obtained from the spleens (middle) and LNs (bottom) of WT and SAP-family-triply-deficient (SAP T-def) mice after injection of β_2m-deficient or SFR β_2m-deficient splenocytes, together with an equal number of WT (B6) splenocytes as internal control. Each symbol represents an individual mouse. The data represent the mean ± SEM of 6–12 mice pooled from two experiments.

(D) Rejection of CFSE-labeled cells obtained from the spleens (middle) and LNs (bottom) of WT, SAP T-def, and SLAM-SAP-family-deficient (SFRD-def) mice after an injection of equal proportions of WT (B6) and β_2m-deficient splenocytes. Each symbol represents an individual mouse. The data are pooled from two representative experiments, n = 6–9 mice per group.

mice by the routine method. We then developed mice deficient in SFRs, EAT2, and ERT using sequential CRISPR-based genome engineering. The loss of EAT-2 and ERT was validated by detecting genomic sequences (Figures S7A and S7B). After the resulting mice were further bred onto the SAP-deficient mice, SFRD mice were finally obtained. We found that the combined deletion of SFRs on NK cells could completely reverse the severe defect of SAP-family-deficient mice in the clearance of hematopoietic cells (Figure 6D). Furthermore, we performed in vitro assays at a single-cell level to directly address whether the in vivo phenotypic reversal was due to the improvement of NK cell effector functions. Notably, the deletion of SFRs on NK cells could largely correct the severe defect of IFN-γ secretion in NK cells without the SAP family (Figures S7C and S7D). Thus, SFRs on the surface of SAP-family-deficient NK cells are indeed inhibitory.

Taken together, SFRs act as self-specific activating receptors, which are strictly dependent on the presentence of SAP family adaptors. When the SAP family is absent in NK cells, SFRs are converted and become inhibitory, which may aggravate the NK-cell defect in rejecting self-MHC class I mismatched hematopoietic cells.

The Altered NK Cell Phenotypes Observed in SFR-Deficient Mice Are Not due to Alternative Signaling by SAP Family

Although SAP family adaptors are mainly recruited by SFRs, they also have the potential to bind to other immunoreceptor

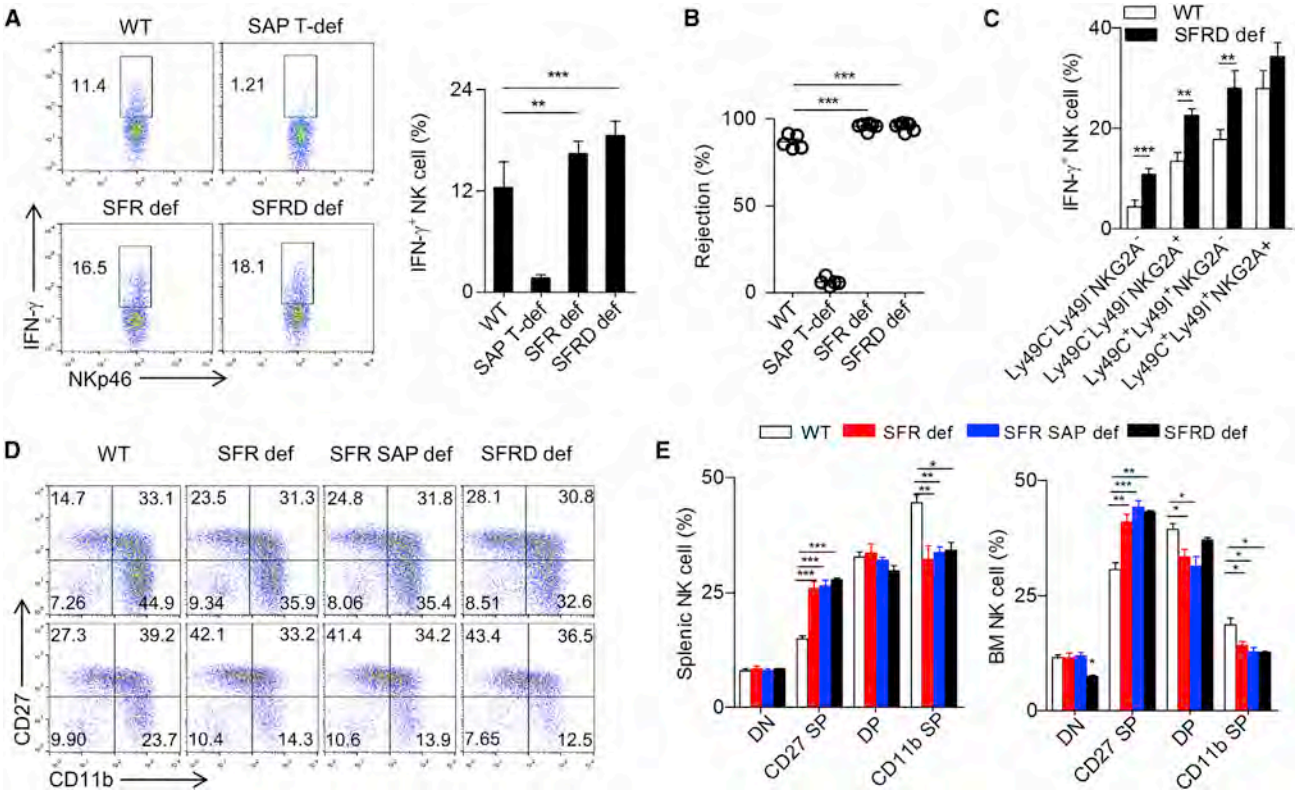

Figure 7. The Altered Phenotypes in SFR-Deficient Mice Are Not due to the Alternative Signaling by SAP Family Members

(A) Splenocytes isolated from polyI:C-activated mice as indicated were incubated with RMA-S. $CD3^-NKp46^+$ NK cells were analyzed for intracellular IFN-γ. The representative flow cytometry plots were presented (left) and the percentages of IFN-γ^+ NK cells were quantified (right). The data are representative of three experiments and represent the mean ± SEM of 3-5 mice per group.

(B) Rejection of CFSE-labeled splenocytes obtained from the spleen of the indicated mice 6 hr after injection of β_2m-deficient splenocytes. Each symbol represents an individual mouse. The data represent the mean ± SEM of 5–7 mice per group analyzed in three separate experiments.

(C) PolyI:C-activated NK cells from indicated mice that were stimulated with RMA-S. Frequency of IFN-γ-producing cells among four subpopulations were quantified. The data are representative of three experiments and expressed as the mean ± SEM of 4–5 mice.

(D and E) Representative flow cytometry plots (D) and quantifications (E) of the four-stage development (similar to Figure 1G) of the gated $CD3^-NK1.1^+$ NK cells in the spleens and BM of the indicated mice. The data are representative of three experiments and expressed as the mean ± SEM of 4–6 mice per group (E). Please see Figure S7.

tyrosine-based activation motif (ITAM) (Proust et al., 2012). To exclude this possibility that other ITAM-containing activation receptors recruit SAP family adaptors, most likely leading to the abnormal phenotypes observed in SFR-deficient mice, we performed further experiments using SFRD-deficient mice. Compared with SFR-deficient NK cells, polyI:C-activated NK cells from SFRD-deficient mice exhibited an equivalent ability to produce IFN-γ (Figure 7A). When primary splenocytes lacking self-MHC-I were transferred, the capacity of SFRD-deficient NK cells to clear these abnormal cells was also greater than in control WT mice but comparable to SFR-deficient mice (Figure 7B). SFR deficiency also caused a higher activity of NK cell subsets, including $Ly49C^-Ly49I^-NKG2A^-$, $Ly49C^-Ly49I^-NKG2A^+$, and $Ly49C^+Ly49I^+NKG2A^-$ NK cells, even in the absence of SAP family adaptors (Figure 7C), as observed in SFR-deficient mice. Moreover, the altered NK cell differentiation was also recapitulated in SFRD-deficient mice (Figures 7D and 7E). Therefore, we conclude that the altered NK cell phenotypes observed in SFR-deficient mice are not due to alternative signaling, possibly caused by SAP family.

DISCUSSION

Studies of gene function are often hampered by gene redundancy, which widely exists in the immune system. In this study, we developed sequential multiplex genome engineering based on the established CRISPR technique. We successfully obtained mice that lack SFRs, SAP family members, or both. With these multiple (up to 10)-gene-deficient mice, we revealed three critical roles for SFRs in the physiology of NK cells: an essential role for the activation of NK cells in rejection of MHC-I-mismatched hematopoietic cells, an unexpected role important for the induction of NK cell tolerance to self-hematopoietic cells, and another regulatory role implicated in NK cell differentiation. These novel findings have not been disclosed by individual deletion of any member of SFRs. Thus, we believe that the technical quality of this study is undoubtedly strong and is a good example for successfully resolving immune redundancy in mice.

The discrimination of self from non-self is a central topic in immunology. The prevention of self-tissues from immune attack is tightly controlled by the induction of immune tolerance, which

is originally defined as T or B cell unresponsiveness to a self-antigen that is induced by previous exposure to that antigen (Goldrath and Bevan, 1999; Hogquist et al., 2005). To date, the dissection of NK cell tolerance has mainly focused on the role of inhibitory Ly49 receptors recognizing self-MHC class I molecules (Kim et al., 2005; Fernandez et al., 2005). Although activation receptors were also involved in the induction of NK cell tolerance in transgenic models (Coudert et al., 2008; Tripathy et al., 2008), the physiological relevance of those studies remains unclear. We have demonstrated here that SFRs are one of the self-specific activation receptors that can sense endogenous ligands on hematopoietic cells and thus induce NK cell tolerance. The mechanism may be more naturally occurring and universally applicable within the vertebrate immune system, as SFRs are widely expressed on other immune cells (Cannons et al., 2011). Ubiquitous SFR expression functions as an endogenous checkpoint in maintaining NK cell peripheral tolerance to self-hematopoietic cells, akin to self-antigen induced T cell anergy (Kyewski and Klein, 2006). Overall, NK cell tolerance would be achieved by multiple mechanisms, somewhat analogous to central versus peripheral tolerance for T cells (Kyewski and Klein, 2006). NK cells ensure tissue-specific peripheral tolerance most likely through self-specific activation receptors, including SFRs. It will be interesting to further understand how NK cells maintain tissue-specific tolerance to other organs or tissues.

Our finding demonstrates that self-specific activation receptors can definitely educate NK cells physiologically. Thus, NK cell functional acquisition can be achieved by at least two distinct signals derived from self-specific activation receptors and/or from Ly49-mediated inhibitory receptors. Moreover, we have revealed here that SFR-induced NK cell tolerance is affected by self-MHC-I. Our interpretation is that SFR-mediated interactions among hematopoietic cells work together with Ly49-dependent inhibitory receptors not only during NK cell effector functions but also NK cell education. The balance between signaling emanated from these two types of interactions will determine the outcome of NK cell education, which is highly similar to the scenario when NK cells attack their targets. Due to the ubiquitous expression of SFRs on hematopoietic cells, SFR-mediated interactions persistently stimulate all the NK cells, inducing hyporesponsiveness of NK cells, but they induce less hyporesponsiveness of Ly49C$^+$ Ly49I$^+$ NKG2A$^+$ NK cells, probably because MHC-I dependent inhibitory signaling decreases the SFR-mediated activation signaling. Therefore, we probably identified one of the key stimulatory signaling that works in conjunction with inhibitory signals, particularly self-MHC-I-dependent signaling, to determine the outcome of NK cell education.

It is notable that the loss of SFRs on hematopoietic target cells could significantly inhibit NK cell-mediated degranulation. However, the effect is not completely abolished, inferring that other hematopoietic cell-specific receptors play a role in rejection of semi-allogeneic hematopoietic cells, a model for hybrid resistance. Moreover, SFRD-deficient NK cells that lack all SFRs and SAP family members presumably not only fail to generate SFR-mediated inhibitory signaling but also lose SFR-triggered activating signaling. Indeed, the ability of NK cells in SFRD-deficient mice to kill hematopoietic cells was slightly increased. The remaining question is which receptors are responsible for NK cell recognition of hematopoietic cells in the absence of SFRs. Although NKG2D has been highlighted to be involved in hybrid resistance, their relevant ligands are not hematopoietic-cell specific and have not been tested on normal WT (B6) mice (Beilke et al., 2010), suggesting that NKG2D is not the receptor that facilitates NK-mediated hybrid resistance when SFRs are absent. We assume that NK cells most likely possess multiple hematopoietic-cell-specific receptors, including SFRs. Given that SFRs, such as 2B4, could synergize with other activation receptors, such as NKG2D and CD16, for NK cell activation (Dong et al., 2009), SFRs may alternatively serve as co-stimulatory receptors for full activation of NK cells in mediating hybrid resistance.

Inhibitory Ly49 receptors license NK cells through a cytoplasmic tail composed of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Kim et al., 2005), and SHP-1 is thought to be involved. Mice deficient in SHP-1 display compromised NK cell education (Viant et al., 2014), although it could not be recapitulated in a second study (Kim et al., 2005). Thus, it remains controversial how NK cells are licensed by inhibitory signaling. However, licensing by activating signaling also remains controversial. Currently, no evidence supports a role for DAP10 or DAP12, two adaptors essential for NKG2D and Ly49H signaling, in NKG2D- or Ly49H-mediated NK cell education (Coudert et al., 2008). Although SAP family adaptors are well-known to be downstream of SFR signaling, SAP-family-deficient mice show a phenotype completely opposite to that in SFR-deficient mice (Dong et al., 2009). This discrepancy between two mutant mice is reasonably explained by the fact that SFRs are converted to be inhibitory in the absence of SAP family members. Thus, it is impossible for us to obtain direct evidence supporting that the SAP family is involved in NK cell tolerance. Generating mice bearing SAP family mutants that lack the ability to transduce active signaling at no expense to the activity of natural blocker will help to determine whether SFR-induced tolerance of NK cells relies on the SAP family.

Overall, our study has shown that SFRs are hematopoietic-specific activation receptors involved in a model for hybrid resistance. These receptors most likely account for the preference of NK cells to kill hematopoietic cells. Therefore, this finding paves the way for the potential use of a cocktail of SFR antibodies for the short-term blockade of NK cell rejection of allogeneic hematopoietic cell transplants. Our study also suggests that long-term blockage of SFR-mediated desensitization of NK cells, which mimics the scenario in SFR-deficient mice, may help awaken NK cells, which may represent a potential strategy for boosting NK cell function for the clinical treatment of hematopoietic malignancies.

EXPERIMENTAL PROCEDURES

Mice

Mice differentially lacking SLAM-SAP family members were generated by a sequentially CRISPR-Cas9-based genome editing (see Supplemental Experimental Procedures). C57BL/6 (B6, H-2^b), BALB/c (H-2D^d), and β_2m-deficient mice lacking MHC-I were obtained from The Jackson Laboratory. RAG1 γc-deficient mice were described previously (Yang et al., 2015). SAP-deficient F1-hybrid mice were generated by crossing male BALB/c with female SAP-deficient mice (B6, H-2^b). All of the mice were bred and maintained in specific pathogen-free animal facilities at Tsinghua University. All of the procedures

involving animals were approved by the Animal Ethics Committee of Tsinghua University.

Cells

RMA-S, YAC-1, and B16 have been described previously (Dong et al., 2009). B16 expressing Rae1 or SLAM-family ligands were generated using the retroviral vector pMSCV-Puro. For the generation of T cell blasts, the splenocytes from WT, SFR-deficient, β_2m-deficient, or SFR β_2m-deficient mice were activated with 3 μg/mL of concanavalin (Con A, Sigma) for 48 hr. Then, the cells were expanded by the low concentration of mouse interleukin (IL)-2 (50 U). For the derivation of iDCs, BM cells were grown for 8 days with the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). MEFs were isolated from the embryos of β_2m-deficient mice at gestation days 12.5–13.5.

In Vitro NK Cell Assays

To detect NK cell degranulation and IFN-γ production, splenocytes (2×10^6) from polyI:C-treated or naive mice were cultured together with an equal number of the indicated target cells or stimulated by the plate-coated antibodies against NK1.1 or Ly49D at a serial of concentrations (low, 1 μg/mL or high, 10 μg/mL) for 5 hr in the presence of GolgiStop (BD Biosciences) and CD107a (eBioH4A3). Cells were stained with the antibodies against NKp46 and CD3 and then were fixed and made permeable with Cytofix/Cytoperm Buffer (BD Biosciences). Cells were then stained with IFN-γ (XMG1.2). For control experiments, the splenocytes were stimulated with PMA (50 ng/mL) plus ionomycin (1 mM) or IL-12/IL-18 (10 ng/mL each).

In Vivo Detection of NK-Mediated Rejection of Hematopoietic Cells

Splenocytes (1×10^6) from B6, SFR-deficient, β_2m-deficient, or SFR-β_2m-deficient mice labeled with 5 μM CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) were equally mixed with splenocytes of F1-hybrid mice that were labeled with 0.5 mM CFSE and then injected intravenously into F1-hybird mice with or without SAP, which were primed with 100 μg of PolyI:C. When WT, SFR-deficient, SAP-family-deficient, and SFRD-deficient mice were recipients, β_2m-deficient or SFR-β_2m-deficient splenocytes were labeled with 5 μM CFSE and then equally mixed with the splenocytes of B6 mice that were labeled with 0.5 μM CFSE. $CFSE^+$ cells were identified in spleens and lymph nodes (LNs) by flow cytometry 6 hr after intravenous injection. The results are expressed as the ratio of rejected target cells. For hybrid resistance assay, hybrid mice as recipients are lethally irradiated and transferred with 1×10^6 of WT, SFR-deficient, β_2m-deficient, or SFR-β_2m-deficient parental B6 BM cells that are labeled with 5 μM CFSE together with an equal number of BM cells of F1 (WT) mice that were labeled with 0.5 μM CFSE.

Statistical Analyses

Prism 5 software was used for unpaired Student's t tests (two-tailed). A p value of less than 0.05 was considered significant. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.07.013.

AUTHOR CONTRIBUTIONS

S.C. and M.Y. conceived the project and designed and performed most experiments. J. D., D.L., Z. L., and C.C. performed experiments and analysis. Y.M. and L.Z. helped to generate SFR-deficient KO mice. Z.T. and Z.D. co-led the investigation. Z.D. wrote the paper.

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REFERENCES

Beilke, J.N., Benjamin, J., and Lanier, L.L. (2010). The requirement for NKG2D in NK cell-mediated rejection of parental bone marrow grafts is determined by MHC class I expressed by the graft recipient. Blood *116*, 5208–5216.

Bélanger, S., Tu, M.M., Rahim, M.M., Mahmoud, A.B., Patel, R., Tai, L.H., Troke, A.D., Wilhelm, B.T., Landry, J.R., Zhu, Q., et al. (2012). Impaired natural killer cell self-education and "missing-self" responses in Ly49-deficient mice. Blood *120*, 592–602.

Bennett, M., Yu, Y.Y., Stoneman, E., Rembecki, R.M., Mathew, P.A., Lindahl, K.F., and Kumar, V. (1995). Hybrid resistance: 'negative' and 'positive' signaling of murine natural killer cells. Semin. Immunol. *7*, 121–127.

Bix, M., Liao, N.S., Zijlstra, M., Loring, J., Jaenisch, R., and Raulet, D. (1991). Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. Nature *349*, 329–331.

Cannons, J.L., Tangye, S.G., and Schwartzberg, P.L. (2011). SLAM family receptors and SAP adaptors in immunity. Annu. Rev. Immunol. *29*, 665–705.

Coudert, J.D., Scarpellino, L., Gros, F., Vivier, E., and Held, W. (2008). Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. Blood *111*, 3571–3578.

Cruz-Munoz, M.E., Dong, Z., Shi, X., Zhang, S., and Veillette, A. (2009). Influence of CRACC, a SLAM family receptor coupled to the adaptor EAT-2, on natural killer cell function. Nat. Immunol. *10*, 297–305.

Daley, J.P., and Nakamura, I. (1984). Natural resistance of lethally irradiated F1 hybrid mice to parental marrow grafts is a function of H-2/Hh-restricted effectors. J. Exp. Med. *159*, 1132–1148.

Deng, W., Gowen, B.G., Zhang, L., Wang, L., Lau, S., Iannello, A., Xu, J., Rovis, T.L., Xiong, N., and Raulet, D.H. (2015). Antitumor immunity. A shed NKG2D ligand that promotes natural killer cell activation and tumor rejection. Science *348*, 136–139.

Dong, Z., and Veillette, A. (2010). How do SAP family deficiencies compromise immunity? Trends Immunol. *31*, 295–302.

Dong, Z., Cruz-Munoz, M.E., Zhong, M.C., Chen, R., Latour, S., and Veillette, A. (2009). Essential function for SAP family adaptors in the surveillance of hematopoietic cells by natural killer cells. Nat. Immunol. *10*, 973–980.

Dong, Z., Davidson, D., Pérez-Quintero, L.A., Kurosaki, T., Swat, W., and Veillette, A. (2012). The adaptor SAP controls NK cell activation by regulating the enzymes Vav-1 and SHIP-1 and by enhancing conjugates with target cells. Immunity *36*, 974–985.

Elliott, J.M., and Yokoyama, W.M. (2011). Unifying concepts of MHC-dependent natural killer cell education. Trends Immunol. *32*, 364–372.

Fernandez, N.C., Treiner, E., Vance, R.E., Jamieson, A.M., Lemieux, S., and Raulet, D.H. (2005). A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood *105*, 4416–4423.

Goldrath, A.W., and Bevan, M.J. (1999). Selecting and maintaining a diverse T-cell repertoire. Nature *402*, 255–262.

Hogquist, K.A., Baldwin, T.A., and Jameson, S.C. (2005). Central tolerance: learning self-control in the thymus. Nat. Rev. Immunol. *5*, 772–782.

Johnson, L.A., Vaidya, S.V., Goldfarb, R.H., and Mathew, P.A. (2003). 2B4(CD244)-mediated activation of NK cells reduces metastases of B16F10 melanoma in mice. Anticancer Res. *23* (5A), 3651–3655.

Jordan, M.A., Fletcher, J.M., Pellicci, D., and Baxter, A.G. (2007). Slamf1, the NKT cell control gene Nkt1. J. Immunol. *178*, 1618–1627.

Kageyama, R., Cannons, J.L., Zhao, F., Yusuf, I., Lao, C., Locci, M., Schwartzberg, P.L., and Crotty, S. (2012). The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. Immunity *36*, 986–1002.

Kim, S., Poursine-Laurent, J., Truscott, S.M., Lybarger, L., Song, Y.J., Yang, L., French, A.R., Sunwoo, J.B., Lemieux, S., Hansen, T.H., and Yokoyama, W.M. (2005). Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature *436*, 709–713.

Kyewski, B., and Klein, L. (2006). A central role for central tolerance. Annu. Rev. Immunol. *24*, 571–606.

Lee, K.M., McNerney, M.E., Stepp, S.E., Mathew, P.A., Schatzle, J.D., Bennett, M., and Kumar, V. (2004). 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. J. Exp. Med. *199*, 1245–1254.

Liao, N.S., Bix, M., Zijlstra, M., Jaenisch, R., and Raulet, D. (1991). MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. Science *253*, 199–202.

Narni-Mancinelli, E., Jaeger, B.N., Bernat, C., Fenis, A., Kung, S., De Gassart, A., Mahmood, S., Gut, M., Heath, S.C., Estellé, J., et al. (2012). Tuning of natural killer cell reactivity by NKp46 and Helios calibrates T cell responses. Science *335*, 344–348.

Orr, M.T., and Lanier, L.L. (2010). Natural killer cell education and tolerance. Cell *142*, 847–856.

Proust, R., Bertoglio, J., and Gesbert, F. (2012). The adaptor protein SAP directly associates with CD3ζ chain and regulates T cell receptor signaling. PLoS ONE *7*, e43200.

Qi, H., Cannons, J.L., Klauschen, F., Schwartzberg, P.L., and Germain, R.N. (2008). SAP-controlled T-B cell interactions underlie germinal centre formation. Nature *455*, 764–769.

Raulet, D.H., and Vance, R.E. (2006). Self-tolerance of natural killer cells. Nat. Rev. Immunol. *6*, 520–531.

Schwartzberg, P.L., Mueller, K.L., Qi, H., and Cannons, J.L. (2009). SLAM receptors and SAP influence lymphocyte interactions, development and function. Nat. Rev. Immunol. *9*, 39–46.

Shifrin, N., Raulet, D.H., and Ardolino, M. (2014). NK cell self tolerance, responsiveness and missing self recognition. Semin. Immunol. *26*, 138–144.

Sun, J.C., and Lanier, L.L. (2008). Tolerance of NK cells encountering their viral ligand during development. J. Exp. Med. *205*, 1819–1828.

Tripathy, S.K., Keyel, P.A., Yang, L., Pingel, J.T., Cheng, T.P., Schneeberger, A., and Yokoyama, W.M. (2008). Continuous engagement of a self-specific activation receptor induces NK cell tolerance. J. Exp. Med. *205*, 1829–1841.

Veillette, A., Dong, Z., and Latour, S. (2007). Consequence of the SLAM-SAP signaling pathway in innate-like and conventional lymphocytes. Immunity *27*, 698–710.

Viant, C., Fenis, A., Chicanne, G., Payrastre, B., Ugolini, S., and Vivier, E. (2014). SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. Nat. Commun. *5*, 5108.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell *153*, 910–918.

Yang, M., Li, D., Chang, Z., Yang, Z., Tian, Z., and Dong, Z. (2015). PDK1 orchestrates early NK cell development through induction of E4BP4 expression and maintenance of IL-15 responsiveness. J. Exp. Med. *212*, 253–265.

Zafirova, B., Mandarić, S., Antulov, R., Krmpotić, A., Jonsson, H., Yokoyama, W.M., Jonjić, S., and Polić, B. (2009). Altered NK cell development and enhanced NK cell-mediated resistance to mouse cytomegalovirus in NKG2D-deficient mice. Immunity *31*, 270–282.

Zhao, F., Cannons, J.L., Dutta, M., Griffiths, G.M., and Schwartzberg, P.L. (2012). Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis. Immunity *36*, 1003–1016.