Immunoreceptor tyrosine-based inhibitory motif–dependent functions of an MHC class I-specific NK cell receptor

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Natural killer (NK) cells express MHC class I (MHC-I)-specific receptors, such as Ly49A, that inhibit killing of cells expressing self–MHC-I. Self–MHC-I also "licenses" NK cells to become responsive to activating stimuli and regulates the surface level of NK-cell inhibitory receptors. However, the mechanisms of action resulting from these interactions of the Ly49s with their MHC-I ligands, particularly in vivo, have been controversial. Definitive studies could be derived from mice with targeted mutations in inhibitory Ly49s, but there are inherent challenges in specifically altering a single gene within a multigene family. Herein, we generated a knock-in mouse with a targeted mutation in the immunoreceptor tyrosine-based inhibitory motif (ITIM) of Ly49A that abolished the inhibitory function of Ly49A in cytotoxicity assays. This mutant Ly49A caused a licensing defect in NK cells, but the surface expression of Ly49A was unaltered. Moreover, NK cells that expressed this mutant Ly49A exhibited an altered inhibitory receptor repertoire. These results demonstrate that Ly49A ITIM signaling is critical for NK-cell effector inhibition, licensing, and receptor repertoire development.

tolerance | immunity | NK cells | lymphocytes | MHC class I

Natural killer (NK) cells are innate lymphocytes that provide crucial immunity to intracellular pathogens and tumors. NK cells carry out these functions through direct cytotoxicity of stressed cells and through the production of cytokines, such as IFN-γ, both of which are regulated through balanced signaling through germline-encoded activation and inhibitory receptors (1). Mouse NK cells express inhibitory receptors belonging to the Ly49 family of lectin-like receptors that can bind to target cell MHC class I (MHC-I) molecules and inhibit killing of healthy cells that express normal levels of MHC-I (2). During infection or tumorigenesis, cells may down-regulate MHC-I, which leads to evasion of MHC-I–restricted T cells but also NK-cell attack by "missing-self" recognition (3), presumably due to lack of Ly49 engagement. However, the in vivo function of inhibitory Ly49s remains incompletely understood. Novel tumor immunotherapies have been developed that block the inhibitory killer immunoglobin-like receptors (KIRs) on human NK cells that are functionally homologous to murine Ly49s (4). However, clinical trials of anti-KIR checkpoint blockade failed to show efficacy in treating patients with smoldering multiple myeloma (5). A better understanding of the in vivo functions of inhibitory NK-cell receptors is important for improving checkpoint blockade of NK cells.

MHC-I–deficient mice, such as those lacking β_2-microglobulin ($\beta 2m^{-/-}$) or H-2K^b and H-2D^b [$K^{b-/-}D^{b-/-}$, double-knockout (KODO)], do not possess autoreactive NK cells as predicted by the missing-self hypothesis (6–8). Instead, NK cells from MHC-I–deficient mice are hyporesponsive to stimulation through their activation receptors (8, 9). Interestingly, some NK cells in wild-type (WT) mice lack inhibitory receptors that recognize self MHC-I, and these cells are also hyporesponsive (9). To explain these results, we previously proposed that a second function of inhibitory Ly49s is to "license" or educate NK cells by self–MHC-I so that NK cells become competent to be triggered by their activation receptors (8).

In addition to inhibiting and licensing NK cells, binding between Ly49s and MHC-I influences the surface levels of Ly49 receptors on NK cells. Binding between Ly49A and H-2D^d down-regulates the mean fluorescence intensity (MFI) of Ly49A by flow cytometry (10). In vitro acid stripping of NK cells from H-2^d mice destabilizes surface MHC-I and enhances MHC-I tetramer staining of NK cells; this result has been used to argue that H-2D^d down-regulates the Ly49A MFI by binding to Ly49A in *cis* on the NK cell surface to mask antibody-binding sites (11). Although acid stripping elevates the MFI of Ly49A when stained with the YE1/48 monoclonal antibody, surprisingly, acid stripping does not increase the MFI of Ly49A when stained with the JR9 antibody. These results have been used to support an alternative hypothesis that the Ly49A MFI shift may be due to other mechanisms, such as signaling-dependent Ly49 receptor internalization (12).

Binding between Ly49s and MHC-I also influences the percentage of NK cells that express other Ly49s. Evidence for MHC-I–dependent receptor repertoire skewing comes from mice with different MHC-I haplotypes that have been shown to contain NK cells with distinct Ly49 repertoires (13). This repertoire

Significance

Natural killer (NK) cells are cytotoxic immune cells that are regulated by inhibitory receptors, such as murine Ly49s, that bind to MHC class I (MHC-I). Cancer immunotherapies are currently in clinical trial that target inhibitory NK-cell receptors analogous to checkpoint inhibitors that block inhibitory receptors on T cells. To improve checkpoint blockade of NK cells, it is critical to better understand the in vivo functions of inhibitory NK-cell receptors. Here, we developed a knock-in mouse with a targeted mutation predicted to abolish the signaling motif of the inhibitory receptor Ly49A. This mutant mouse revealed multiple mechanisms by which inhibitory receptor signaling controls NK-cell self-tolerance that could impact the efficacy of checkpoint blockade of NK cells.

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skewing has been suggested to be dependent on Ly49s binding to MHC-I because Ly49A transgenic mice show skewing of the Ly49 repertoire in the presence of H-2^{d} with reduced coexpression of Ly49A and Ly49G2, which both bind H-2D^{d} (14, 15). However, the mechanism of receptor repertoire development has been challenging to investigate without Ly49 mutant mice.

Inhibitory Ly49s are generally thought to function by signaling through a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) with the consensus sequence (I/L/V/S)xYxx(L/V), the only known signaling motif in the Ly49s (16). Upon binding to MHC-I, Ly49s become tyrosine-phosphorylated in the ITIM, which leads to recruitment and activation of the phosphatases SHP-1, SHP-2, and SHIP that counteract kinases acting downstream of activation receptors (17–19). Evidence that ITIM signaling is required for Ly49-mediated inhibition comes from transfection of a rat NK-cell line and primary NK cells from transgenic mice expressing Ly49A^{BALB} with an ITIM mutation (Ly49A-Y8F^{BALB}) (17, 20). However, these experiments, particularly with Ly49 transgenic mice, carry the caveats that the site of transgene insertion is unknown and that transgenic Ly49 is expressed at nonphysiological levels and times during NK-cell development, and on cells other than NK cells. As a result of these caveats, one Ly49A transgenic line has been shown to exhibit a complete block in NK-cell development (21, 22), which appears to be inconsistent with studies of WT mice. Thus, the role of ITIM signaling in inhibiting cytotoxicity by primary murine NK cells remains incompletely understood.

Transgenic mouse (Ly49A-Y8F^{BALB}-tg) and retroviral bone marrow chimeric approaches have been used to suggest that ITIM signaling is required for NK-cell licensing (8, 23). However, both of these approaches are potentially limited by caveats similar to those associated with Ly49 transgenic mice mentioned previously. Furthermore, conflicting evidence exists regarding the role of downstream SHP-1 signaling in licensing. Initial studies using mixed bone marrow chimeras with SHP-1–deficient, motheaten-viable (*me-v*) mice suggested that licensing is SHP-1–independent (8). In contrast, *Ptpn6*$^{fl/fl}$ × NKp46-Cre mice that lack SHP-1 only in NK cells have subsequently been used to argue that SHP-1 is required for licensing (24). This conflict highlights the importance of further evaluation of the role of ITIM signaling in NK-cell licensing.

Specific targeted mutations in *Ly49*s have been challenging to generate because the *Ly49* genes are highly related and clustered in the NK gene complex (NKC), the *Ly49* cluster has a high concentration of repetitive elements (25), and the *Ly49* cluster in B6 mice encodes Ly49s distinct from those in the 129-strain initially favored for embryonic stem (ES)-cell targeting. Ly49 knockout mice have been successfully generated for only Ly49Q^{129}, which is expressed exclusively on myeloid cells (26), and Ly49E, which is expressed exclusively on liver tissue-resident NK cells but not on conventional splenic NK cells (27, 28). Another attempt with an *Ly49* targeting construct led to generation of the NKC knockdown (NKC^{KD}) mouse that contains a concatemerized targeting construct inserted in the NKC (29). Although the NKC^{KD} mouse was shown to express reduced levels of Ly49s, results from this mouse are confounded because the NKC is derived from the 129-strain, Ly49 expression is not completely lost, and expression of other NKC receptors encoded near the *Ly49* gene cluster are also affected by the concatemer insertion.

In this study, we generated mice with a targeted mutation in *Klra1* (*Ly49a*) within the C57BL/6 NKC. This "AYF" allele encoded Ly49A with a single amino acid mutation predicted to abolish ITIM signaling that was validated. The AYF mouse allowed us to study the effect of losing endogenous Ly49 ITIM signaling on the in vivo development and function of primary NK cells.

Results

Generation of Ly49A ITIM Mutant Mice. To investigate the functions of Ly49A ITIM signaling in primary NK cells, we introduced a single-nucleotide mutation in exon 4 of the *Klra1* gene encoding Ly49A directly in C57BL/6 ES cells that conferred a tyrosine-to-phenylalanine mutation in the ITIM of Ly49A (Fig. 1*A*). Southern blot analysis verified correct targeting of the *Klra1* locus in seven (1.9%) of 370 clones (Fig. 1*B*), and two of these clones were found to have a normal karyotype. Correctly targeted ES-cell clones were microinjected into Albino B6 blastocysts. Mice containing the germline-transmitted targeted allele were bred to mice of a CMV-Cre (Cre transgene under the control of the CMV promoter) transgenic line to remove the neomycin resistance cassette. The resulting allele is referred to as "AYF" (Fig. 1*A*). AYF homozygous mice were found to contain a normal percentage and number of Ly49A^{+} NK cells (Fig. S1 *A–C*).

ITIM Signaling Is Required for Ly49A to Inhibit NK-Cell Killing. To test if the ITIM is required for Ly49A to inhibit killing by primary murine NK cells, chromium release assays were performed with purified Ly49A^{+} lymphokine-activated killer cells (LAKs) from

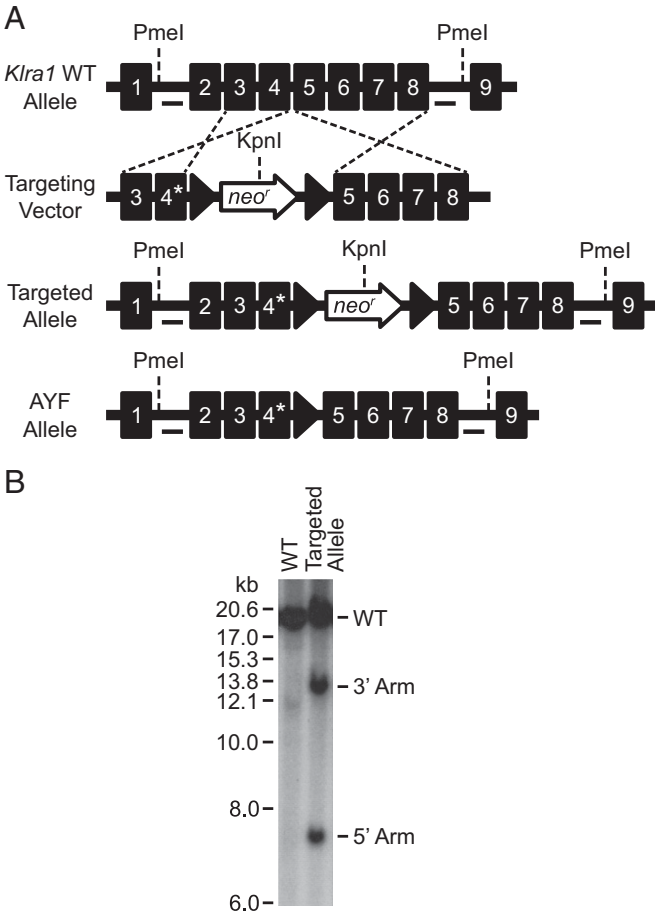

Fig. 1. Generation of the AYF mouse. (*A*) Targeted *Klra1* locus encoding Ly49A is depicted before and after removal of the neomycin resistance gene by Cre recombinase. LoxP sites are indicated as black triangles. The AYF allele contains a point mutation in exon 4 indicated as 4* that codes for Ly49A^{Y8F} with a tyrosine-to-phenylalanine mutation in the ITIM of Ly49A. (*B*) Parallel bars indicate the binding sites of probes that were used to generate the Southern blot on genomic DNA digested with PmeI and KpnI from ES cells that were homozygous for WT *Klra1* or heterozygous for the targeted allele. Bands representing the 3′ and 5′ arms are indicated. Removal of the neomycin resistance gene was confirmed by PCR.

WT B6 or homozygous AYF mice, in a manner similar to our original assays (2). Ly49A$^+$ LAKs from WT and AYF mice exhibited similar levels of cytotoxicity toward C1498 (H-2^b) target cells at high effector-to-target (E:T) ratios (Fig. 2*A*). C1498-D12 target cells that express transfected H-2D^d were not killed by Ly49A$^+$ LAKs from WT mice due to the inhibitory interaction between Ly49A and H-2D^d as previously described (2). In contrast, Ly49A$^+$ LAKs from AYF mice killed C1498-D12 targets at higher levels than WT LAKs at all high E:T ratios tested (Fig. 2*B*). These data indicate that ITIM signaling is required for Ly49A to inhibit cytotoxicity by primary NK cells and that the AYF allele functionally inactivates the Ly49A ITIM.

Mutation of the Ly49A ITIM Does Not Affect NK-Cell Development. To study the function of Ly49A ITIM signaling in vivo, we bred the AYF mouse to KODO mice that lack expression of H-2K^b and H-2D^b (AYF KODO), and we introduced the ligand for Ly49A by breeding these mice to the D8 mouse that expresses an H-2D^d transgene to generate the AYF D8 KODO mouse. Although bone marrow competition experiments with Ly49A transgenic mice suggested that the Ly49A–H-2D^d interaction promotes NK-cell development (30), we found that total NK-cell number and Ly49A$^+$ NK-cell number were both unchanged between D8 KODO and AYF D8 KODO mice (Fig. 3 *A*–*C*). Furthermore, mutation of the ITIM of Ly49A did not alter the maturation profile of Ly49A$^+$ NK cells as indicated by CD27 and CD11b expression (Fig. 3 *D* and *E*). Thus, these data indicate that Ly49A ITIM signaling is not required for NK-cell development.

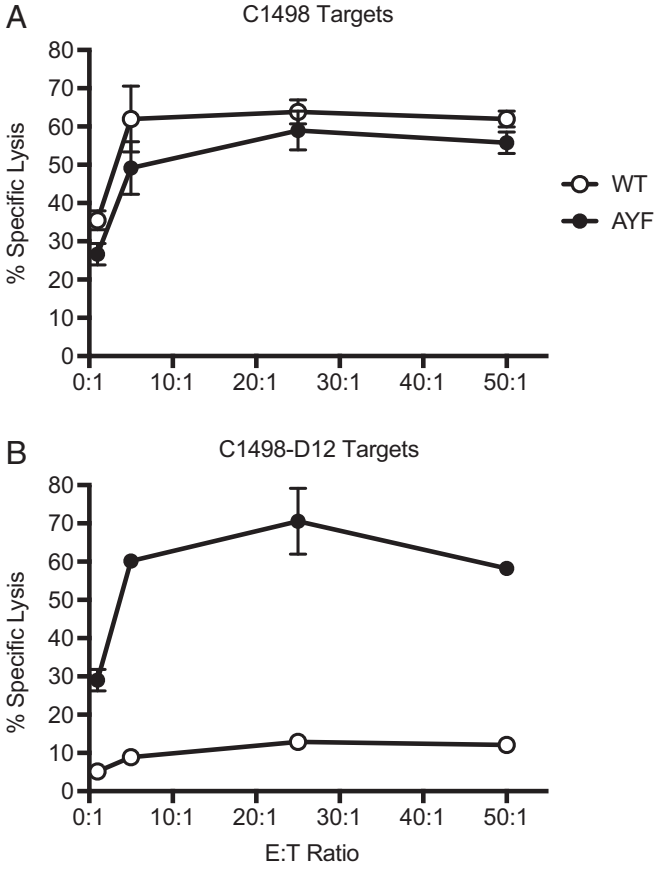

Fig. 2. The ITIM is required for Ly49A to inhibit NK-cell cytotoxicity. Chromium release assays were performed at the indicated E:T ratios using Ly49A$^+$ LAKs from WT or AYF mice. C1498 cells (*A*; H-2^b) or C1498 cells transfected with H-2D^d (*B*; C1498-D12) were used as targets. LAKs were generated from splenocytes pooled from three mice per group. Error bars indicate SD of technical triplicates. Data are representative of two independent experiments.

NK Cell Licensing by Ly49A Is ITIM-Dependent. To investigate the role of ITIM signaling in NK-cell licensing, we stimulated NK cells from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice with plate-bound anti-NK1.1 antibody and analyzed IFN-γ production, as described previously (8, 9). To quantify the degree of NK-cell licensing by a given Ly49, a licensing ratio was calculated as the ratio of the percentage of IFN-γ$^+$ cells within the Ly49$^+$ subset over the Ly49$^-$ subset as previously described (31). Ly49G2$^+$ and NKG2A$^+$ NK cells were gated out before calculating the licensing ratio for Ly49A to eliminate the potential confounding effects of licensing through these other receptors by H-2D^d. Ly49A$^+$ NK cells from the MHC-I–deficient KODO and AYF KODO mice produced little IFN-γ after stimulation with anti-NK1.1, which corresponded to a licensing ratio of less than 1 (Fig. 4 *A* and *B*). In contrast, a large fraction of Ly49A$^+$ NK cells from D8 KODO mice produced IFN-γ due to licensing by Ly49A on H-2D^d, corresponding to a Ly49A licensing ratio of greater than 1 in D8 KODO mice, as previously reported (8, 32). Remarkably, Ly49A$^+$ NK cells from AYF D8 KODO mice produced dramatically lower levels of IFN-γ compared with D8 KODO mice, with a reduction in the Ly49A licensing ratio to below 1 in AYF D8 KODO mice (Fig. 4 *A* and *B*). Importantly, when activation receptor signaling was bypassed with phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation, Ly49A$^+$ and Ly49A$^-$ NK cells from all strains produced equivalent levels of IFN-γ (Fig. 4*C*), verifying that Ly49A$^+$ NK cells from AYF D8 KODO mice remained capable of producing IFN-γ. Thus, licensing through the interaction between Ly49A and H-2D^d is completely abolished in AYF D8 KODO mice.

To test whether licensing through other Ly49 receptors is affected by the AYF mutation, we assessed NK-cell licensing by Ly49G2 in Ly49A$^-$ NKG2A$^-$ NK cells after stimulation with plate-bound anti-NK1.1 antibody. The Ly49G2 licensing ratio was elevated in D8 KODO mice compared with KODO mice (Fig. 4 *D* and *E*), indicating that Ly49G2$^+$ NK cells were licensed by H-2D^d as has been previously suggested (33). In contrast to the Ly49A licensing ratio (Fig. 4 *A* and *B*), the Ly49G2 licensing ratio was unchanged between D8 KODO and AYF D8 KODO mice (Fig. 4 *D* and *E*). Ly49G2$^+$ and Ly49G2$^-$ NK cells from all strains responded equivalently to stimulation with PMA and ionomycin (Fig. 4*F*). Collectively, these data indicate that mutation of the ITIM of Ly49A impairs NK-cell licensing in a manner that is cell-intrinsic to Ly49A-expressing cells.

Down-Regulation of the Ly49A MFI by H-2D^d Does Not Require ITIM Signaling. To determine whether ITIM signaling is required for the H-2D^d–dependent MFI shift in Ly49A, we compared the MFI of Ly49A in KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice (Fig. 5). The MFI of Ly49A was reduced in D8 KODO mice compared with KODO mice as previously reported (32, 34). However, the MFI of Ly49A was also significantly down-regulated in AYF D8 KODO mice compared with AYF KODO mice, and we did not observe a difference in the MFI of Ly49A in D8 KODO and AYF D8 KODO mice (Fig. 5). We observed a very small but reproducible reduction in the Ly49A MFI in AYF KODO mice compared with KODO mice. We also observed a similar small reduction in the Ly49A MFI on NK cells from AYF mice compared with WT mice (Fig. S1 *D* and *E*), suggesting that ITIM signaling may modulate the expression level of Ly49A in the absence of classical MHC-I molecules expressed as self or that the Ly49A targeting strategy slightly altered Ly49A expression. Overall, these data show that H-2D^d down-regulates the MFI of Ly49A in an ITIM-independent manner, which argues against a role for

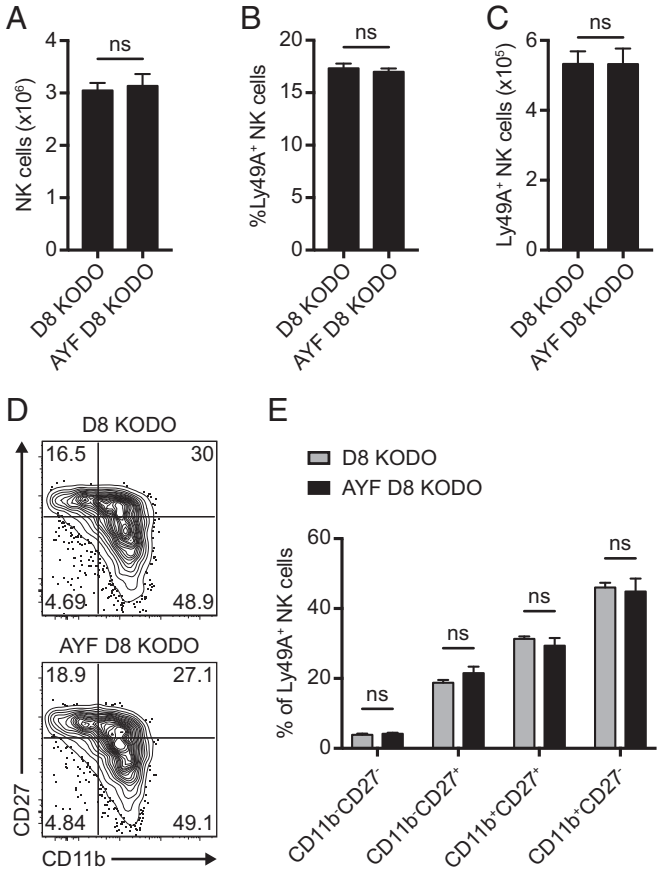

Fig. 3. NK-cell development is normal in AYF D8 KODO mice. Total splenic NK cell ($CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$) number (*A*), percentage of NK cells that express Ly49A (*B*), and $Ly49A^+$ NK-cell number (*C*) from D8 KODO and AYF D8 KODO mice are shown for 16–20 mice per group. Data are pooled from at least five independent experiments. There were no significant differences as indicated (Student's *t* test). (*D*) Representative dot plots of $Ly49A^+$ NK-cell maturation stages. (*E*) Maturation stages of $Ly49A^+$ NK cells separated by CD27 and CD11b expression. Data in *E* are pooled from two independent experiments with a total of six mice per group (two-way ANOVA with Bonferroni correction). Error bars indicate SEM. ns, not significant.

signaling-dependent internalization of Ly49A by classical MHC-I molecules expressed as self.

Ly49A ITIM Signaling Regulates the NK-Cell Receptor Repertoire. To determine the role of ITIM signaling in the development of the NK-cell receptor repertoire, we assessed the expression of Ly49s and NKG2A/CD94 in splenic NK cells freshly isolated from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice (Fig. 6). As previously reported in $H\text{-}2^d$ mice (13), there was a substantial reduction in the percentage of $Ly49A^+$ cells expressing Ly49G2 in D8 KODO mice compared with KODO mice. Moreover, we observed a significant reduction in the percentage of $Ly49A^+$ cells expressing Ly49F and Ly49I in D8 KODO mice compared with KODO mice. Remarkably, there were substantially elevated percentages of $Ly49A^+$ NK cells expressing Ly49G2, Ly49F, and Ly49I in AYF D8 KODO mice compared with D8 KODO mice (Fig. 6). In contrast, we did not detect any significant differences in the percentages of NK cells expressing these Ly49s on $Ly49A^-$ NK cells from D8 KODO and AYF D8 KODO mice (Fig. S2*B*), which suggests that these repertoire changes are not due to off-target mutations in other *Ly49* genes. Thus, these data suggest that the effect of Ly49A ITIM signaling on the receptor repertoire is cell-intrinsic to Ly49A-expressing cells and that ITIM signaling is required for $H\text{-}2D^d$–dependent skewing of the receptor repertoire on $Ly49A^+$ NK cells.

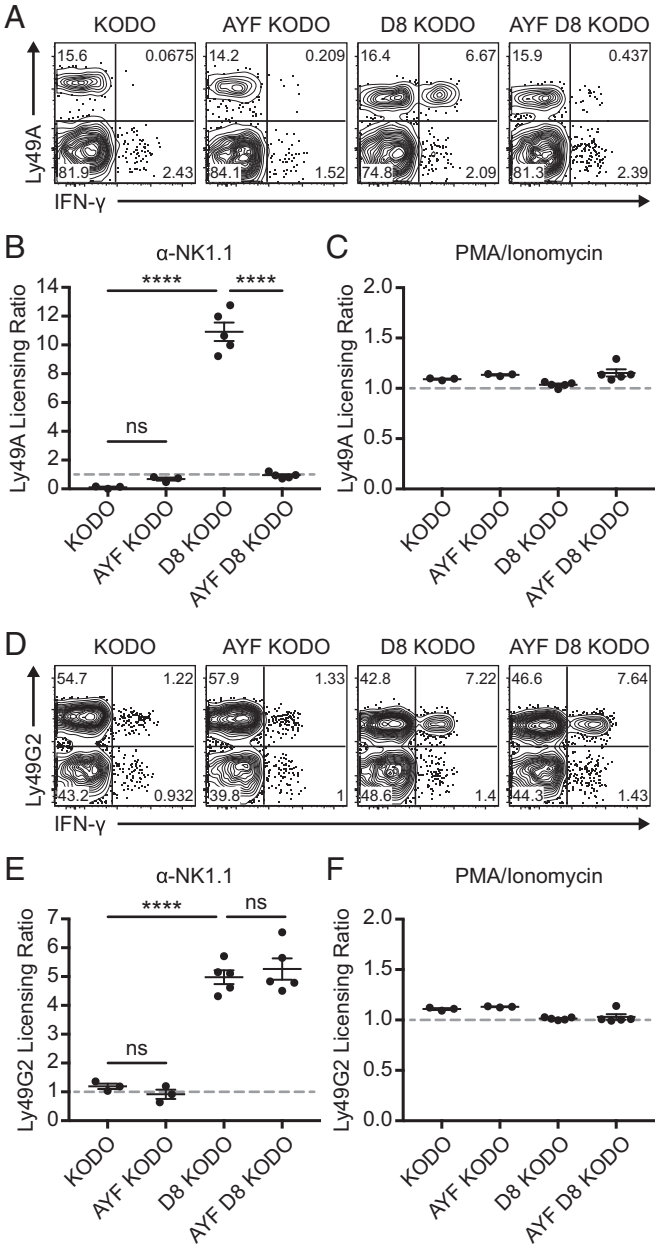

Fig. 4. NK-cell licensing by Ly49A is impaired in AYF D8 KODO mice. Splenocytes from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice were stimulated with plate-bound anti-NK1.1 antibody or with PMA and ionomycin and analyzed for intracellular IFN-γ production by NK cells ($CD3^-$ $CD19^-$ $NKp46^+$). (*A*) Representative dot plots showing IFN-γ production by $Ly49G2^-$ $NKG2A^-$ NK cells after stimulation with plate-bound anti-NK1.1 antibody. Relative production of IFN-γ by $Ly49A^+$ and $Ly49A^-$ cells within the $Ly49G2^-$ $NKG2A^-$ NK cell population is quantified by a licensing ratio (*Materials and Methods*) for stimulation with plate-bound anti-NK1.1 (*B*) or PMA and ionomycin (*C*). (*D*) Representative dot plots showing IFN-γ production by $Ly49A^-$ $NKG2A^-$ NK cells after stimulation with plate-bound anti-NK1.1 antibody. Relative production of IFN-γ by $Ly49G2^+$ and $Ly49G2^-$ cells within the $Ly49A^-$ $NKG2A^-$ NK-cell population is quantified by a licensing ratio for stimulation with plate-bound anti-NK1.1 (*E*) or PMA and ionomycin (*F*). Data are representative of four independent experiments with a total of 12–15 mice per group. Error bars indicate SEM. $^{****}P < 0.0001$ (one-way ANOVA with Bonferroni correction). ns, not significant.

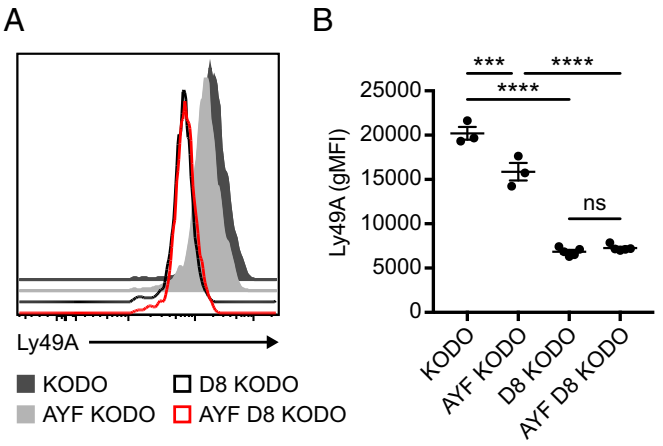

Fig. 5. H-2D^d–dependent down-regulation of the Ly49A MFI is ITIM-independent. (*A*) Representative histogram of Ly49A expression on Ly49A$^+$ splenic NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ Ly49A$^+$) from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice. (*B*) Summary of the geometric MFI (gMFI) of Ly49A on Ly49A$^+$ NK cells from the indicated strains. Data are representative of four independent experiments with a total of 10–13 mice per group. Error bars indicate SEM. ****$P < 0.0001$; ***$P < 0.001$ (one-way ANOVA with Bonferroni correction). ns, not significant.

Discussion

Here, we describe a knock-in mouse with a targeted mutation in a self-MHC–specific Ly49 expressed by mature NK cells. The AYF mouse expresses Ly49A with a point mutation known to inactivate ITIM signaling in vitro, and we show here that Ly49A ITIM signaling inhibits cytotoxicity by primary NK cells. This mouse allowed us to definitively study the role of the ITIM in the functions and attributes of a self-MHC–specific inhibitory NK-cell receptor. To study the role of Ly49A in NK-cell development and education, we bred AYF D8 KODO mice that express the ligand for Ly49A, H-2D^d, and lack all other classical MHC-I molecules. We used AYF D8 KODO mice to show that ITIM signaling is not required for NK-cell development but is required for NK-cell licensing. Moreover, we show that ITIM signaling is not required for H-2D^d to down-regulate the Ly49A MFI. In contrast, our data provide evidence that Ly49A ITIM signaling significantly shapes the NK-cell receptor repertoire.

Studies with Ly49A transgenic mice have led to conflicting reports of the impact of Ly49 signaling on NK-cell development. Ly49A transgenic mice contain a normal number of NK cells (14, 35); however, in competitive mixed bone marrow chimeras, Ly49A transgene expression promotes NK-cell development (30). Moreover, a separate Ly49A transgenic line was found to exhibit a complete block in NK-cell development (21). Our results clearly show that endogenous Ly49A ITIM signaling alone does not substantially affect NK-cell development, even in the presence of its MHC ligand, expressed as self, which distinguishes NK-cell development from licensing.

Ly49s have been suggested paradoxically to both inhibit NK cells and license them to become responsive to stimulation (2, 8). The "disarming" hypothesis resolves these seemingly opposing functions by proposing that Ly49s promote NK-cell responsiveness through inhibiting overstimulation by endogenous activating ligands. In contrast, the "arming" hypothesis predicts that binding between Ly49s and MHC-I triggers a signaling pathway distinct from inhibitory signaling to educate NK cells (36). Our results here show that both inhibition and licensing of NK cells require signaling through the same ITIM, which rules out the possibility that Ly49s might educate NK cells through a previously unidentified signaling motif. Although this result appears to support the disarming hypothesis, it remains possible that distinct signaling pathways downstream of the ITIM mediate inhibition and licensing.

The disarming hypothesis predicts that SHP-1 is required for NK-cell education; however, conflicting results have been found with SHP-1 mutant *me-v* mice and *Ptpn6*$^{fl/fl}$ × NKp46-Cre mice (8, 24). Importantly, *Ptpn6*$^{fl/fl}$ × NKp46-Cre mice exhibit a moderate defect in NK-cell maturation, as indicated by CD27 and CD11b expression, which we did not observe in Ly49A$^+$ NK cells from AYF D8 KODO mice. Because SHP-1 is known to signal downstream of various receptors other than Ly49s, our results bring up the possibility that the defects in NK-cell maturation and responsiveness seen in *Ptpn6*$^{fl/fl}$ × NKp46-Cre mice may not be due

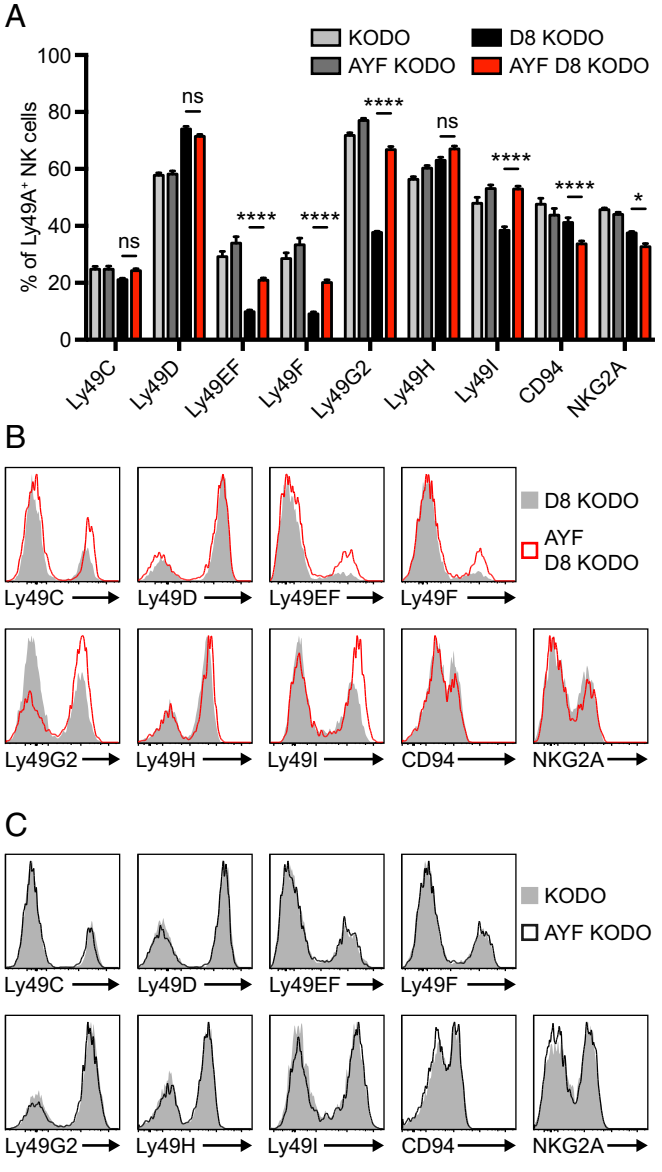

Fig. 6. Mutation of the Ly49A ITIM causes skewing of the NK-cell receptor repertoire. (*A*) Summary of receptor expression on splenic Ly49A$^+$ NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ Ly49A$^+$). (*B*) Representative histograms showing receptor expression on Ly49A$^+$ NK cells from D8 KODO and AYF D8 KODO mice. (*C*) Representative histograms showing receptor expression on Ly49A$^+$ NK cells from KODO and AYF KODO mice. Data in *A* are pooled from three independent experiments with eight to 11 mice per group total. ****$P < 0.0001$; *$P < 0.05$ (two-way ANOVA with Bonferroni correction). ns, not significant.

to a loss of Ly49 signaling and may be due to interactions between SHP-1 and other receptors. Consequently, the pleiotropic functions of SHP-1 may make it challenging to use SHP-1–deficient mice to study NK-cell education by Ly49s. In contrast, our results clearly identify that Ly49 ITIM signaling is required for NK-cell licensing, which highlights the need to better characterize the signaling pathways downstream of the Ly49 ITIM to differentiate between the arming and disarming hypotheses. Moreover, it will be important to investigate whether context can influence ITIM signaling similar to how the immunoreceptor tyrosine-based switch motif of SLAM family receptors can signal through distinct pathways to activate or inhibit NK cells depending on the setting (37).

A previous study showed that NK cells from NKC^{KD} mice express reduced levels of Ly49s due to concatemer insertion in the NKC, which leads to impaired killing of MHC-I–deficient splenocytes and tumor cell lines. However, NK cells from NKC^{KD} mice produce normal levels of IFN-γ after in vitro stimulation with plate-bound anti-NKp46 antibody or tumor cell lines (29). These data were used to conclude that NK-cell responsiveness is not dependent on Ly49 expression. In contrast, our results here show that in vitro NK-cell responsiveness is strongly impacted by ITIM-dependent Ly49 signals when its cognate MHC-I ligand is expressed as self. It is likely that previous studies with the NKC^{KD} mouse provided different results because NK cells from these mice exhibit abnormal expression of other NKC receptors encoded adjacent to the *Ly49* genes and because Ly49 expression is not completely abolished. These conflicting results underscore the importance of developing mice with targeted mutations in specific *Ly49* genes, such as the AYF mouse.

Binding between Ly49A and H-$2D^d$ has been shown to down-regulate the MFI of Ly49A, but the role of signaling-dependent receptor internalization had not been previously studied. Stimulation of G-protein–coupled receptors and various other receptors leads to receptor internalization mediated by β-arrestins (38). Intriguingly, β-arrestin 2 has been shown to bind the ITIM of the human inhibitory NK receptor KIR2DL1 to reduce its surface expression, which suggests that ITIM signaling can trigger receptor endocytosis (39). In contrast, our data show that the MFI of Ly49A stained with the JR9 antibody was not significantly different between D8 KODO and AYF D8 KODO mice. This result clearly shows that the H-$2D^d$–dependent MFI shift in Ly49A is not caused by ITIM signaling-induced internalization of Ly49A. Our data are consistent with the idea that the MFI shift is due to binding between the receptor and ligand in *cis* to block the antibody-binding epitope. However, the *cis* interaction may not fully explain the Ly49A MFI shift because staining with the JR9 antibody has been reported to not be affected by acid stripping that impairs MHC-I expression (12). Alternatively, our results may indicate that Ly49A harbors additional signaling motifs outside of the ITIM that modulate surface expression.

A prior study using Ly49A-$Y8F^{BALB}$-tg mice suggested that ITIM signaling might modulate the NK-cell receptor repertoire (23). However, these conclusions were based on comparing Ly49A-$Y8F^{BALB}$ with a separate $Ly49A^{BALB}$ transgenic line that likely expresses $Ly49A^{BALB}$ at different levels. Moreover, that study focused on $Ly49A^{BALB}$, for which the ligands have been less studied compared with $Ly49A^{B6}$, and also reported ITIM-dependent skewing of Ly49D and Ly49H that is inconsistent with our current findings. Moreover, the WT $Ly49A^{BALB}$ transgene caused an ITIM-dependent increase in the percentage of $Ly49F^+$ NK cells that is opposite of our data reported here and is inconsistent with studies of WT $Ly49A^{B6}$ transgenic mice (40). Because the AYF D8 KODO mouse reported here expressed mutated $Ly49A^{B6}$ at similar levels to WT Ly49A in D8 KODO mice, our results provide strong evidence that Ly49A ITIM signaling regulates the repertoire of inhibitory receptors.

Two models have been proposed to explain the impact of MHC-I on the repertoire of inhibitory Ly49s (41). The sequential model proposed that NK cells sequentially and stochastically acquire expression of Ly49s until a signaling threshold is reached, at which point the acquisition of additional Ly49s is terminated. In contrast, the two-step selection model proposed that a diverse pool of NK cells stochastically expressing Ly49 repertoires develops initially and is subsequently subjected to positive and negative selection, similar to T-cell development. Surprisingly, neither model fully explains the repertoire changes that we observed here in AYF D8 KODO mice.

The sequential model predicts that $Ly49A^+$ NK cells in AYF D8 KODO mice should up-regulate expression of all Ly49s compared with D8 KODO mice regardless of binding specificity. In contrast, the two-step selection model predicts that $Ly49A^+$ NK cells in AYF D8 KODO mice should only up-regulate the expression of inhibitory Ly49s that bind to H-$2D^d$. Here, we show that $Ly49A^+$ NK cells from AYF D8 KODO mice up-regulate Ly49G2, Ly49F, and Ly49I. In contrast, we did not detect an increase in Ly49C, Ly49D, or Ly49H expression on $Ly49A^+$ NK cells. Because Ly49G2, Ly49F, and Ly49I have all been reported to bind to MHC molecules of the H-2^d haplotype (15), the results here are consistent with the two-step selection model. In contrast, both models predict that mutation of the Ly49A ITIM should result in a higher fraction of $Ly49C^+$ NK cells because Ly49C has been reported to bind to H-$2D^d$ (15, 42), but we did not observe a significant difference in Ly49C expression on $Ly49A^+$ NK cells between D8 KODO and AYF D8 KODO mice. We did, however, observe a subtle trend toward more $Ly49C^+$ NK cells in AYF D8 KODO mice compared with D8 KODO mice. Regardless, neither model explains the variable degree of receptor skewing that we observed between the different Ly49s that bind H-2^d. While these data indicate that neither the sequential nor two-step selection model fully explains the receptor repertoire, it remains possible that the NK-cell repertoire is determined by a combination of these two models. Alternatively, there may need to be a modification of the basic premise that the Ly49s are stochastically expressed. Finally, a precise reevaluation of the MHC-I specificities of the various Ly49s, in addition to Ly49A–H-$2D^d$, particularly in the context of self-MHC expression in vivo, may be required to fully understand Ly49 repertoire development.

Our results, nonetheless, clearly indicate that ITIM signaling via self–MHC-I–specific receptors within NK cells shapes the inhibitory receptor repertoire. Interestingly, however, expression of the Ly49D activation receptor on $Ly49A^+$ NK cells was unchanged between D8 KODO and AYF D8 KODO mice even though Ly49D has been shown to bind H-$2D^d$ and activate NK cells (43, 44). This result suggests that the ITIM may modulate the receptor repertoire through signaling pathways that are independent of inhibiting activation signals. Alternatively, these results may indicate that the expression of activation receptors like Ly49D is established at a different time than inhibitory receptors during NK-cell development. Consistent with this model, Ly49D expression has been reported to lag behind Ly49A and Ly49G2 expression in NK cells from neonatal mice (45).

Although the AYF allele was shown to abolish the inhibitory interaction between Ly49A and H-$2D^d$, we did not observe any overt autoimmunity in AYF D8 KODO mice. This finding is consistent with $\beta 2m^{-/-}$ mice that lack MHC-I surface expression but do not contain autoreactive NK cells. Herein, we show that Ly49A ITIM signaling is required not only to inhibit NK cells but also to license NK cells and to regulate receptor repertoire development. All three of these processes likely contribute to self-tolerance and are relevant to the development of more potent tumor immunotherapies that target NK-cell tolerance that could affect all three of these processes (46). Our results support the possibility that checkpoint blockade with anti-KIR2D antibodies

has been ineffective in clinical trials due to unintended effects on NK-cell education and receptor expression, as has been previously hypothesized (46, 47).

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from the National Cancer Institute and from Charles River Laboratories. Albino B6 mice [B6(Cg)-*Tyr*$^{c-2J}$/J] were purchased from The Jackson Laboratory. H-2K$^{b-/-}$ H-2D$^{b-/-}$ (KODO) mice were purchased from Taconic Farms. D8 transgenic mice expressing an H-2D^{d} transgene were provided by D. Marguiles, National Institute of Allergy and Infectious Diseases, Bethesda, MD. D8 KODO mice were generated by crossing D8-transgenic mice to KODO mice. CMV-Cre mice (48) backcrossed to the C57BL/6 background were provided by Marco Colonna, Washington University, St. Louis, MO.

Development of AYF Mice. BAC recombineering (Red/ET; GeneBridges) was used to subclone an 11.9-kb region of *Klra1* from BAC RP23-446O7 (AC087336; Children's Hospital Oakland Research Institute) into the pACYC177 vector (New England Biolabs). The pACYC177 capture vector contained a 146-bp 5′ capture arm and a 301-bp 3′ capture arm. The loxP-pGK-gb2-neo-loxP selection cassette (GeneBridges) was cloned into the NsiI site between exons 4 and 5 of the *Klra1* gene. The tyrosine at position 8 of Ly49A was replaced with phenylalanine by cloning in a 122-bp PCR product with XhoI and Bsu36I. Primers used to generate the Ly49A^{Y8F} mutation were the following: 5′-CACTCGAGGCACCATTTGAACTGAGAACATACTTTATATATCAATCCCAAGATGAGTGAGCAGGAGGTCACTTTTTC-3′ and 5′-TTTAGTCTCCTCAGGTCTCACTTGTTTCTGCAATCCTGCAGATTTATGAAATCTCACCATTGAAAAAG-3′. The targeting construct was electroporated into C57BL/6NTac-derived ES cells generated in-house (49), and positive clones were screened by Southern blot analysis. Seven of 370 ES cell clones were positive by Southern blot analysis. Karyotyping revealed that two of seven positive clones contained a normal karyotype. Positive clones with a normal karyotype were microinjected into Albino B6 blastocysts by the Transgenic, Knockout, and Micro-Injection Core at Washington University. Chimeric mice were bred to Albino B6 mice, and the targeted allele from one of the two ES-cell lines was germline-transmitted. Mice containing the germline-transmitted targeted *Klra1* allele were bred to C57BL/6 mice to remove the Albino allele, and subsequently to C57BL/6 CMV-Cre mice (48) to delete the selection cassette. The CMV-Cre transgene was removed by breeding to C57BL/6 mice. The AYF allele was then bred to homozygosity for all experiments. AYF mice were subsequently crossed to KODO or D8 KODO mice to generate the AYF KODO and AYF D8 KODO strains, respectively. D8 KODO and AYF D8 KODO mice were homozygous for the D8 transgene in all experiments.

Antibodies and Flow Cytometry. The following antibodies and reagents were purchased from eBioscience: anti-CD3e (145-2C11), anti-CD19 (eBio1D3), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-CD27 (LG.7F9), anti-CD11b (M1/70), anti-Ly49D (eBio4E5), anti-Ly49E/F (CM4), anti-Ly49G2 (eBio4D11), anti-Ly49H (3D10), anti-Ly49I (YLI-90), anti-CD94 (18d3), anti-NKG2A^{B6} (16a11), anti–IFN-γ (XMG1.2), and Fixable Viability Dye eFluor 506. The following antibodies and reagents were purchased from BD Biosciences: anti-Ly49F (HBF-719), anti-Ly49G2 (4D11), and streptavidin (SA)-phycoerythrin. The following antibodies and reagents were purchased from BioLegend: anti-NK1.1 (PK136) and SA-allophycocyanin. Anti-Ly49I (YLI-90) was purchased from Abcam. Anti-Ly49A (JR9) was purified in our laboratory from hybridoma supernatants and subsequently conjugated to biotin or FITC. The JR9 hybridoma was generously provided by Jacques Roland, Pasteur Institute, Paris, France. Anti-Ly49C (4LO33) was purified in our laboratory from hybridoma supernatants and subsequently conjugated to biotin. The 4LO hybridoma was generously provided by Suzanne Lemieux, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada. Anti-NK1.1 (PK136) was purified in our laboratory from hybridoma supernatants. The PK136 hybridoma was purchased from American Type Culture Collection. Fc receptor blocking was performed with 2.4G2 (anti-FcγRII/III) hybridoma (American Type Culture Collection) culture supernatants. Surface staining was performed on ice in staining buffer (1% BSA and 0.01% NaN_3 in PBS). Samples were collected using a FACSCanto (BD Biosciences), and data were analyzed using FlowJo (TreeStar).

Preparation of Ly49A^{+} LAKs. Nylon-wool nonadherent splenocytes were cultured in R10 media supplemented with 800 IU/mL IL-2. On day 6, Ly49A^{+} LAKs were purified by panning with the JR9 (anti-Ly49A) antibody as previously described (2, 32). LAKs were harvested for chromium release assays on day 9 or 10. LAKs were 94–97% Ly49A^{+} by flow cytometry.

Chromium Release Assay. Four-hour ^{51}Cr release assays were performed as previously described (2). Day 9 or 10 Ly49A^{+} LAKs were used as effectors. C1498 target cells were purchased from American Type Culture Collection. The C1498-D12 target cell line was generated previously by transfecting C1498 cells with H-2D^{d} (2).

In Vitro Stimulation and Intracellular Cytokine Staining. Splenocytes were stimulated with anti-NK1.1 (PK136) as previously described (8, 31). Briefly, 24-well culture plates were coated with 500 μL of purified PK136 (1 μg/mL). Plates were washed with PBS, and 5×10^6 splenocytes were then added to each well in 500 μL of R10 media. Splenocytes were stimulated in parallel with 0.5 μg/mL PMA (Sigma–Aldrich) and 4 μg/mL ionomycin (Sigma–Aldrich). Splenocytes were incubated at 37 °C and in 5% CO_2 for a total of 7 h. Brefeldin A (GolgiPlug; BD Biosciences) was added to the cells after 1 h. After staining surface antigens, cells were fixed and permeabilized (Cytofix/Cytoperm; BD Biosciences) followed by staining for IFN-γ. NK cells were gated as viable CD3^{-} CD19^{-} NKp46^{+} lymphocytes.

The licensing ratio was calculated as [(%Ly49^{+}IFN-γ^{+})/(%Ly49^{+})]/[(%Ly49^{-}IFN-γ^{+})/(%Ly49^{-})] as previously described (31). The Ly49A licensing ratio was calculated on Ly49G2^{-} NKG2A^{-} NK cells, and the Ly49G2 licensing ratio was calculated on Ly49A^{-} NKG2A^{-} NK cells.

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