Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo

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Natural killer (NK) cells are lymphocytes of the innate immune system able to recognize and kill tumors lacking self-MHC class I molecules. This “missing-self” recognition is mediated by the lack of engagement of MHC class I-specific inhibitory NK cell receptors that include the killer cell Ig-like receptors (KIR) in humans and Ly49 molecules in mice. A promising immunotherapeutic strategy against MHC class I+ cancer is to block NK cell inhibitory receptors using monoclonal antibodies (mAb). However, interactions between MHC class I molecules and their inhibitory receptors are also required for the acquisition of NK cell functional competence, a process referred to as “education.” In addition, inhibitory receptors are involved in self-tolerance on educated NK cells. Here, we developed a preclinical mouse model in which all NK cells are educated by a single transgenic inhibitory receptor, human KIR2DL3, through the engagement with its HLA-Cw3 ligand. This approach revealed that NK cells could be reprogrammed to control the development of mouse syngeneic tumors in vivo. Moreover, in vivo anti-KIR mAb treatment induced the killing of HLA+ target cells without breaking self-tolerance. Finally, the long-term infusion of anti-KIR mAb neither abolished NK cell education nor tumor cell recognition. Therefore, these results strongly support the use of inhibitory receptor blockade in cancer patients.

anti-tumor therapy  innate immunity  pre-clinical model  tolerance

Natural killer (NK) cells are lymphocytes of the innate immune system, initially identified by their capacity to kill tumors. They are also involved in antimicrobial responses and act as regulatory cells during inflammation (1). NK cell effector functions include direct cytotoxicity, as well as cytokine and chemokine productions (e.g., IFN-γ). NK cell activation is regulated by an array of stimulatory and inhibitory cell surface receptors that sense potential target cells. Inhibitory receptors include several killer cell Ig-like receptors (KIR) in humans, Ly49 molecules in mice, and CD94/NKG2A heterodimers in both species, which recognize respectively classical and nonclassical major histocompatibility complex (MHC) class I molecules, constitutively expressed by most nucleated cells (2–5). MHC class I-specific inhibitory receptors and their ligands (H-2 in mice and HLA in humans) are highly polymorphic molecules encoded by multigenic, multiallelic families of genes that are inherited independently. NK cells have thus to discriminate self in a context where self-molecules differ from individuals to individuals. Like T lymphocytes, NK cells are educated to self versus altered-self discrimination, but the molecular strategies involved in this education are different. T cell education involves the stimulatory T cell receptor whereas NK cell education is mediated through the engagement of the MHC class I-specific inhibitory receptors (4, 6–10). This education, also termed “licensing,” leads to the maturation of NK cell functional repertoire (i.e., the ensemble of stimulation toward which NK cells are reactive), which is adapted to self-MHC class I environment (4, 9–11). Consequently, NK cells in MHC class I-deficient hosts are hypersensitive to stimulatory receptor stimulation and thereby tolerant to self. In physiological conditions, 2 types of self-tolerant NK cells coexist: functionally competent NK cells, whose effector responses are inhibited by the recognition of self MHC class I molecules, and hyporesponsive NK cells that cannot detect self-MHC class I (9, 10). NK cell education does not result in an on/off switch, but rather in a quantitative tuning of NK cell responsiveness: The more inhibitory receptors recognizing self-MHC class I are expressed, the more NK cells are responsive to cells lacking self-MHC class I (11–15).

The molecular mechanisms underlying the MHC-dependent NK cell education are still unknown, but have been shown in mice to require a functional immunoreceptor tyrosine-based inhibitory motif in the intracytoplasmic tail of Ly49 inhibitory receptors (6).

Several studies have suggested that the manipulation of NK cell missing-self recognition may have important clinical benefit in leukemic patients (16–19). In particular, retrospective studies of KIR-HLA mismatched stem cell transplantation in acute myeloid leukemia patients showed that the lack of KIR engagement on donor NK cells by patient MHC class I molecules was associated with a significant reduced risk for leukemia relapse (20, 21). The manipulation of NK cell alloreactivity in these settings implies haploidentical hematopoietic transplantations, which are associated with considerable adverse effects, including graft versus host disease mediated by allogenic T cells. A safer strategy would be to block NK cell inhibitory receptors in an autologous setting (17). Such a strategy is currently tested in phase I clinical trials with a fully human mAb (1–7F9). This mAb recognizes KIR2D inhibitory receptors and blocks their interaction with the human MHC class I molecules HLA-C, leading to NK cell-mediated lysis of leukemic cells (38). However, one of the main concerns for using this therapeutic approach in humans is the risk of generating a strong reactivity against normal self-tissues. Here, we report the generation of transgenic mice expressing a single inhibitory KIR in the context of its HLA ligand and in absence of endogenous mouse MHC class I genes. These mice were used to manipulate NK cell education and to serve as a preclinical model for testing the effects of anti-KIR mAb treatment in vivo.

Results and Discussion

In Vivo Coexpression of KIR2DL3 and HLA-Cw3 Leads to Mouse NK Cell Education. Mouse inhibitory receptors are not uniformly expressed on NK cells leading to a heterogeneous population of


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Our previous studies on TgKIR mice indicated that KIR2DL3 acts as an inhibitory receptor in mouse cells (22, 24). To confirm these results in different settings, we performed in vivo transfer experiments of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells. Splenocytes from KdKO, WT, and KdKO-TgHLA mice were adoptively transferred to WT or TgKIR recipients. The ability of NK cells to reject mouse cells lacking mouse or human MHC class I molecules was assessed in spleen and blood of recipient mice 40 h after injection (Fig. 1 and supporting information (SI) Fig. S1). These experiments showed that the rejection of KdKO cells by TgKIR mice is reversed by the transgenic expression of HLA-Cw3 on injected cells. In contrast, both KdKO and KdKO-TgHLA splenocytes were rejected in WT animals showing that HLA molecules are not recognized by mouse inhibitory receptors. Therefore, the engagement of transgenic KIR is sufficient to inhibit mouse NK cell activating pathways similarly to MHC class I specific mouse inhibitory receptors, validating this genetic model for further investigations on NK cell education.

A result of education resides in the increase of NK cell ex vivo responsiveness (IFN-γ production or granule-dependent cytotoxicity) toward tumor targets or after activating receptor stimulation (6–8). To assess the ability of a human KIR to promote NK cell education in vivo in the presence of its ligand, we compared NK cell responses in WT, KdKO, and KdKO-TgKIR/HLA mice. KdKO-TgKIR and KdKO-TgHLA mice were used as controls. Splenic NK cell functions were tested ex vivo using cross-linking of the NK1.1 activating receptor, the tumor cells YAC-1, interleukin (IL)-12 and IL-18 or phorbol 12-myristate 13-acetate (PMA), and ionomycin (Fig. 2). As expected, KdKO NK cells were hyporesponsive to stimulation with the tumor YAC-1 (Fig. 2 A and B) as judged by both IFN-γ production and degranulation (i.e., CD107a cell surface exposure, a surrogate marker of NK cell cytotoxicity). The single transgenic expression of human KIR or HLA did not modify the responsiveness of mouse KdKO NK cells (Fig. 2 A and B). In contrast, when both molecules were coexpressed in the same animals, NK cell reactivity was comparable with that of WT NK cells. Mouse NK cells could thus be genetically reprogrammed for activation by tumor cells using a human inhibitory receptor and its HLA ligand.

NK1.1 (Nkrl) is an activating receptor expressed by all mature NK cells in B6 mice (3). Signaling through this receptor leads to INF-γ production and CD107a cell surface expression in WT NK cells, but is impaired in uneducated NK cells (6, 7, 11). This cell-free system allows the assessment of the intrinsic responsiveness of the NK cells without possible interference with unknown inhibitory ligand potentially expressed on target cells. The surface density of NK1.1, detected by flow cytometry, was the same in KdKO-TgKIR/HLA, KdKO-TgKIR, KdKO-TgHLA, WT, and KdKO mice. As expected, NK cells from KdKO and KdKO-TgKIR-TgHLA mice were hyporesponsive to NK1.1 triggering (Fig. 2C). In contrast, NK cell IFN-γ production after NK1.1 stimulation was restored in KdKO-TgKIR/HLA animals (Fig. 2C). As KIR2DL3 directly interacts with HLA-Cw3 (25), these results showed that KIR-HLA interaction leads to NK cell education in vivo. KIR-HLA education modulated some, but not all, activating pathways. Indeed, NK cells were equally responsive to cytokine (IL-12 and IL-18) or PMA/ionomycin stimulation in all of the strains of mice tested (Fig. 2 A and B). These last results are consistent with our current knowledge of mouse NK cell education (6, 7), as KdKO and WT NK cells are also equally responsive to these stimulations (Fig. 2B). Of note, the frequency of reactive NK cells was not significantly increased in KdKO-TgKIR/HLA compared with WT mice despite the fact that they uniformly express an inhibitory receptor for self and should theoretically be all educated by HLA-Cw3. These results suggest that other signals regulate NK cell responsiveness. Along this line, cytokines like IL-15 or IL-18 involved in NK cell proliferation (26–28) may also act as limiting factors for NK cell reactivity in KdKO-TgKIR/HLA mice, as they do in WT animals.

**Reprogramming of NK Missing-Self Recognition.** NK cell reprogramming was then tested in vivo. Upon transfer, healthy hematopoietic cells lacking self-MHC class I ligands are known to be rejected by NK cells (29, 30). We asked whether the education of mouse NK cells by KIR-HLA interaction was sufficient to confer them the ability to recognize and reject HLA− healthy cells. To address this point, we measured the in vivo rejection of HLA− splenocytes in KdKO-TgKIR/HLA mice. A mix of CFSE-labeled WT, KdKO, and KdKO-TgHLA splenocytes (Fig. S2) was adoptively transferred into KdKO and KdKO-TgKIR/HLA recipient mice. WT and KdKO-TgKIR recipients were used as positive and negative control, respectively. In vivo cytotoxicity was assessed 20–40 h after transfer in spleen (Fig. 3A). As expected, WT mice rejected both KdKO and KdKO-TgHLA cells,
confirming that human HLA-Cw3 was not able to engage mouse inhibitory receptors. Uneducated KbDbKO NK cells as well as NK cells from KbDbKO-TgKIR spared the 3 subsets equally, indicating that the mere expression of an inhibitory receptor in absence of ligand interaction does not induce NK cell education. However, not only KbDbKO cells but also WT splenocytes were eliminated in the KbDbKO-TgKIR/HLA model, whereas HLA\(^{+}\) cells were recognized as self (Fig. 3A). The expression of KIR and HLA was thus sufficient to increase the NK cell repertoire to missing-HLA...
interpretation of these data is that NK cells in KbDbKO-TgKIR/HLA cells was injected in KbDbKO-TgKIR, KbDbKO-TgKIR, KbDbKO, or WT mice. Monitoring the survival of the transferred animals, we observed an increased lifespan of ~50% in KbDbKO-TgKIR/HLA mice when compared with KbDbKO-TgKIR controls (Fig. 3B). The survival curve of WT mice after RMA transfer was almost parallel to that of KbDbKO or KbDbKO-TgKIR mice, showing that even if NK cells of WT mice are fully responsive (Fig. 2), MHC class I molecules (H-2b) expressed by RMA impaired their killing by NK cells. Despite the expression of H-2-specific inhibitory receptors on a fraction of NK cells from KbDbKO-TgKIR/HLA, these NK cells were efficient in the antitumor response delaying the onset of the disease. This delay was abolished by pretreatment of KbDbKO-TgKIR/HLA with anti-NK1.1 antibodies, which deplete NK cells (Fig. 3B). The simplest interpretation of these data is that NK cells in KbDbKO-TgKIR/HLA mice were educated to missing-HLA recognition. Particularly, the KIR “Ly49C/I”-NKGA2” NK cell subset should be responsible for this effect. We cannot formally exclude that these NK cells have acquired an additional stimulatory receptor specific for RMA. However, we show that educating signals mediated by KIR-HLA interactions conferred to NK cells the capacity to control the growth of a syngenic tumor expressing mouse MHC class I but lacking HLA.

Altogether, the results presented in Figs. 2 and 3 show that mouse MHC class I is not necessary for NK cell education, granting that other inhibitory receptor–ligand interactions are at work. Previous works have shown that human NK cell responsiveness was correlated with the expression of inhibitory receptor for self-MHC class I (8, 12, 13). We show here in a mouse model that in vivo engagement of an inhibitory KIR by its HLA ligand is sufficient to reprogram uneducated mouse NK cells into competent effector cells. Moreover, the expression of this inhibitory receptor confers to NK cells a new repertoire of recognition resulting in missing-HLA recognition. Two main models of NK cell education have been proposed (9, 10). According to the first one, called the “stimulatory” or “arming” model, NK cells are initially hyporesponsive and become educated when their Ly49 receptors engage self-specific stimulatory ligands. Nevertheless, our results unambiguously show that a human inhibitory KIR can branch to the mouse signaling pathways involved in NK cell education.

**In Vivo Elimination of HLA+ Cells Upon Anti-KIR mAb Infusion.** A fully human IgG4 anti-KIR2DL3 mAb, 1–7F9, was generated with the aim of being used in anticancer therapy (38). Because the affinity of the well-characterized mouse IgG1 anti-KIR2DL3 mAb, GL183 (32), is comparable with that of 1–7F9 and the 2 mAb compete with each other on the KIR2DL3 molecule, we tested both mAbs in parallel. To assess the capacity of this antibody to promote HLA-Cw3+ target cell elimination in vivo, we performed adoptive transfers of a mix (1:1 ratio) of KdpKO-TgHLA and WT splenocytes in TgKIR mice. To avoid the recognition of HLA-Cw3 by T and B cells, the experiments were performed using TgKIR mice bred onto a Rag-deficient background (RagKO-TgKIR). In this model, all NK cells expressed KIR2DL3 but were educated via mouse MHC class I specific receptors. The ratio between KdpKO-TgHLA and WT donor splenocytes was detected by flow cytometry in recipients’ spleen and blood 20 h after transfer (Fig. 4 and Fig. S3). Rag-TgKIR mice rejected KdpKO cells, but were tolerant to KdpKO-TgHLA and WT cells. In contrast, when mice were pretreated for 24 h with increasing doses of GL183 mAb, KdpKO-TgHLA cells were eliminated in vivo (Fig. 4). NK cells mediated this effect, as it was abolished when NK cells were depleted with an anti-NK1.1 mAb (Fig. 4). In this assay, the blocking effect of 1–7F9 was equivalent to that of GL183 (Fig. 4). Thus, anti-KIR mAb infusion triggers missing-HLA recognition by educated NK cells in vivo.

In vivo blocking of KIR with mAb does not break self-tolerance. Previous studies in the mouse have shown that infusion of anti-Ly49C/I blocking mAbs could be efficient against MHC class I+ syngenic tumors without major deleterious effect for the recipient mice (33–35). However, in these experiments, a F(ab’)2 fragment with short half-life and low affinity was used, and in WT B6 mice only half of Ly49C/I+ NK cells also express the inhibitory receptor CD94/NKG2A that could be engaged by Qa-1, reducing the risk of self-reactivity. In humans, the size of KIR2D+ NK cell subsets is highly variable among individuals (4). In patients expressing several KIR2D and their ligands, the use of high-affinity, long half-life, anti-KIR2D mAbs could target the activation of a high number of NK cells and thus significantly increase the risk of autoimmunity. To evaluate the toxicity of anti-KIR mAb infusion, we used the KdpKO-TgKIR/HLA model in which NK cells are monoclonal regarding their MHC class I-induced education and in which the blocking anti-KIR mAb bind to self-specific inhibitory recognition, resulting in the killing of WT cells. Of note, we observed a slight trend toward a weaker elimination of WT cells than that of KdpKO cells, suggesting that mouse Ly49 and CD94/NKG2A inhibitory receptors that can recognize mouse MHC class I molecules on WT CFSE-labeled cells might still be functional for the negative regulation of NK cell effector function, despite their lack of role in NK cell education in KdpKO-TgKIR/HLA mice.

NK cells are known to recognize and kill tumors in vivo. Rauscher murine leukemia virus antigen (RMA) cells are T cell lymphomas from a B6 origin. A MHC class I-deficient variant of this cell line, RMA-S, is a well-characterized NK cell target (31). RMA cells thus express stimulatory ligands, but their NK-mediated lysis is inhibited by the engagement of inhibitory receptors by MHC class I molecules. To test whether KIR-HLA-educated NK cells would be able to control mouse tumor growth, a lethal dose of RMA cells was injected in KdpKO-TgKIR/HLA, KdpKO-TgKIR, KdpKO, or WT mice. Monitoring the survival of the transferred animals, we observed an increased lifespan of ~50% in KdpKO-TgKIR/HLA mice when compared with KdpKO-TgKIR controls (Fig. 3B). The survival curve of WT mice after RMA transfer was almost parallel that of KdpKO or KdpKO-TgKIR mice, showing that even if NK cells of WT mice are fully responsive (Fig. 2), MHC class I molecules (H-2b) expressed by RMA impaired their killing by NK cells. Despite the expression of H-2-specific inhibitory receptors on a fraction of NK cells from KdpKO-TgKIR/HLA, these NK cells were efficient in the antitumor response delaying the onset of the disease. This delay was abolished by pretreatment of KdpKO-TgKIR/HLA with anti-NK1.1 antibodies, which deplete NK cells (Fig. 3B). The simplest interpretation of these data is that NK cells in KdpKO-TgKIR/HLA mice were educated to missing-HLA recognition. Particularly, the KIR “Ly49C/I”-NKGA2” NK cell subset should be responsible for this effect. We cannot formally exclude that these NK cells have acquired an additional stimulatory receptor specific for RMA. However, we show that educating signals mediated by KIR-HLA interactions conferred to NK cells the capacity to control the growth of a syngenic tumor expressing mouse MHC class I but lacking HLA.

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receptors expressed on 100% of NK cells. We only used the mouse GL183 mAb and not the human 1–7F9 mAb in these long-term settings because the mouse immune system reacted against the human mAb, avoiding a complete saturation of KIR2DL3. KdKO-TgKIR/HLA mice were treated every 4 days with 400 μg of anti-KIR mAb GL183 during 3 weeks. GL183 was tolerated by the mouse immune system and allowed a complete KIR2DL3 saturation during the course of the treatment (Fig. 5A). The blood hematopoietic compartment was analyzed at 4 time points (days 0, 7, 14, and 21) after the first injection. Mice treated with the vehicle only were analyzed in parallel. NK cell frequency in spleen and blood was not significantly modified, and, phenotypically, NK1.1, NKp46, and KLRG1 expression levels at NK cell surface were not significantly affected by mAb treatment. Multiple parameters were measured, including leukocyte, erythrocyte, and platelet counts, hemoglobinemia, hematocrit, mean corpuscular volume, red cell distribution width, and mean platelet volume (Table S1). Among leucocytes, the frequency of lymphocytes, monocytes, neutrophils, eosinophils, and basophils was also assessed (Table S2). Using these readouts, no adverse hematological effect was observed even after 21 days of KIR2DL3 saturation. In addition, mice did not present any sign of disease, and their weight remained stable during antibody infusion (Table S1). Finally, spleen cell counts did not vary significantly at any time during the treatment. Similar results were obtained when KdKO-TgKIR mice were treated with GL183 mAb. Thus, the blocking of inhibitory receptor in vivo using saturating concentration of mAbs did not induce detectable signs of autoimmune disorder.

**Long-Term Treatment with Anti-KIR mAb Does Not Abolish NK Cell Education.** Because KIR blocking in vivo did not induce detectable hematopoietic toxicity in KdKO-TgKIR/HLA model, NK cell tolerance was ensured. This result suggested either a modulation in NK cell education or that NK cells were not toxic enough to lead to clinical autoimmune symptoms in vivo, even when blocked through anti-inhibitory receptor. Answering these questions is critical for the use of blocking inhibitory receptors in clinical settings, because a potential negative effect of anti-KIR mAb on NK cell education could lead to a complete inhibition of NK cell functions, abolishing their antitumor effects. To address these issues, we tested whether the continuous KIR blocking over a long period (21 days) resulted in a reduction of NK cell reactivity against tumors. In KdKO-TgKIR/HLA mice the responsiveness (IFN-γ production and CD107a surface exposure) toward the tumor YAC-1 remained stable over the course of mAb treatment (Fig. 5A). Similar results were observed when NK cells were stimulated by NK1.1 cross-linking using mAb-coated plates (Fig. 5B). These data thus showed that despite the long-term blocking of the interaction between KIR and HLA, NK cell responsiveness was not drastically impaired. Anti-KIR mAb treatment in vivo thus did not abolish NK cell education.

It is somewhat surprising that after a 3-week-long blockade of KIR, NK cells retained responsiveness, because the KIR-HLA interaction is the only one that can theoretically educate NK cells in these mice. A recent paper has shown an involvement of cis-interactions (i.e., engagement of inhibitory Ly49A and H-2Dd on the same cell) in NK cell education (36). This process might also apply to KIR-HLA interactions. In this case, the mAb may not be as efficient in inhibiting cis-interactions than trans-interactions, explaining why education is maintained upon antibody infusion in our model. Another possible explanation would be that education takes place in a compartment that is poorly accessible to mAb. Further studies will be needed to address this question.

In addition, the stimulatory signals provided to NK cells by activating ligands expressed on normal cells are likely to be qualitatively and/or quantitatively different as compared with those provided upon tumor encountering (or antibody-mediated receptor stimulation). These differences may account for the different outcomes regarding self-tolerance and reactivity to YAC-1 tumor cells observed in our experiments when inhibitory receptors were blocked. It is also possible that the isolation of normal splenocytes in vitro and their subsequent transfer in recipient mice (Fig. 1 and Fig. 3A) induced at their surface the up-regulation of stimulatory ligands that were not present at the steady state. Rejection is often partial in these models, and a fraction of cells may be altered during the in vitro processing, rendering them more sensitive to NK cells. Irrespective of these possibilities, it remains that anti-KIR treatment did not break self-tolerance and preserved NK cell responsiveness to tumors emphasizing the interest of this immunotherapy strategy. Along this line, the KIR repertoire in human is polyclonal.
and includes also stimulatory receptors (KIR-S). 1–7F9 mAb recognizes inhibitory KIR2DL1/L2/L3 but also KIR2DS1/2 stimulatory molecules (38). We could not address the effect of the antibody binding to KIR-S receptors by using the K^D^–TgKIR/HLA model, but we have shown that KIR-2DS triggering on NK cells from human PBMC did not induce any significant activation (38). However, the effect of the antibody on KIR-2DS-expressing patients in vivo remains to be addressed.

In conclusion, using a genetic model of NK cell education, we showed that mouse NK cells could be educated with a human inhibitory receptor only in the context of engagement with its HLA ligand. This education “across species” was sufficient to reprogram NK cell cytotoxicity toward mouse tumors in vitro and in vivo. Moreover, the infusion of blocking anti-KIR mAbs did not break tolerance to self-hematopoietic cells and did not induce a detectable autoimmune. Finally, the long-term blocking of inhibitory receptors by anti-KIR mAbs in vivo did not reverse their responsiveness toward tumors, at least in vitro. Further studies will be needed to test whether the in vivo elimination of tumors upon treatment with blocking antibodies is conserved to maintain clinical benefits and to evaluate the best protocols of mAb infusion (doses, kinetics of administration). However, these results strongly support the blockade of inhibitory NK cell receptors in cancer patients.

Materials and Methods

CFSE Assay for in Vivo Rejection of Target Cells. This method for quantitative measurements of in vivo killing has been adapted from a previous study (37).


Briefly, populations of spleen cells expressing or not a missing-self phenotype were labeled with 0.5 or 3 μM CFSE (Invitrogen) and mixed at 1:3 or at 1:1 ratios. In some experiments, an additional staining was performed using an anti-H2K^d^ mAb (28.8.6, mlgG2a, BD PharMingen) to discriminate 2 populations stained with 0.5 μM CFSE (Fig. S2). The ratio between the different populations before conjection was determined by FACS analysis and compared with the ratio in the blood or spleen at various time points after inoculation, allowing a quantitative measurement of the rejection of donor cells in the recipients. Donors and recipients were sex-matched.

Antibody Treatments in Vivo. Purified anti-KIR mAbs 1–7F9 (human IgG4) and GL183 (mouse IgG1) were injected i.v. at the indicated doses and time points. GL183 antibody recognizes KIR2DS2L/3L2 molecules (32), and 1–7F9 is a fully human monoclonal antibody that recognizes KIR2DS1/2L/L2/L3. Receptor saturation was tested 2 h after mAb injection in the peripheral blood and then when mice were killed for analysis at days 7, 14, or 21. For NK1.1 depletions, 100 μg of the PK136 (mlgG2a) antibody was given i.v. 24 h before the experiment.

SI. See SI Materials and Methods for more details.

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Supporting Information

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SI Materials and Methods

Mice and Cell Lines. All mice were bred and maintained at the CIML or at Innate Pharma animal facilities in specific pathogen-free conditions. C57BL/6J (B6) mice were purchased from Charles River. KbDbKO mice were kindly provided by F. Lemonier (Pasteur Institute, Paris). C57BL/6-HLA-Cw3 transgenic (TgHLA) mice were previously described (1). TgKIR mice were previously generated in the laboratory and were backcrossed on B6 background (2). These 3 strains were crossed to obtained KbDbKO-TgKIR, KbDbKO-TgHLA and KbDbKO-TgKIR/HLA. YAC-1 is a Moloney virus-induced lymphoma of the A/Sn strain that down-regulates its MHC class I expression upon prolonged in vitro culture. RMA (H-2b) is a sub-line of RBL-5, originating from the EL-4 T cell lymphoma, induced in a B6 mouse.

Receptor Saturation Assay. Blood or spleen lymphocytes from mice treated or not with anti-KIR mAb were purified, washed 2 times, and stained with PE-labeled GL183 mAb for detection of free KIR2DL3 receptors. For each mouse, the mean fluorescence intensity (MFI) of PE-GL183 before dosing was measured by FACS and compared with the MFI of GL183-PE staining obtained at each time point. Acquisition of the samples was standardized to allow the comparison of MFI values across several time points. Percentages of KIR2DL3 receptor occupancy were calculated for each mouse using the following formula: %KIR2DL3 receptor occupancy = 100 – [100×(MFI time X/MFI PD)], where MFI PD is the mean fluorescence intensity of PE-GL183 at predose and MFI time X is the MFI of PE-GL183 obtained at each time point.

Antibodies and FACS Analysis. MAbs used for cytometry were: anti-NK1.1 (PK136)-APC, anti-CD3 (145–2C11)-PerCP-Cy5.5, anti-IFN-γ (XMG1.2)-PE, anti-CD107a (1B4B)-FITC (BD Biosciences), anti-NKp46 (29A1.4)-Alexa 647 (3). Fc receptors were blocked by incubation with anti-FcyRII/III (2.4G2) during staining. Results were acquired with a FACSCanto or a FACSDiva (BD Biosciences) and analyzed using the flowJo software (Three Star).

In Vitro Stimulation Assay. Splenocytes (5 × 10^5) from naïve mice were either co-cultured with YAC-1 cells (2 × 10^5) or added in plates precoated (overnight at 4 °C) with 25 µg/mL purified anti-NK1.1 mAb (PK136) or isotype control. For negative and positive controls, cells were incubated in culture medium alone or were stimulated with a mix of IL-12 (25 ng/mL, Cliniscience) and IL-18 (20 ng/mL, MDL) or with a mix of PMA (200 ng/mL) and ionomycin (5 µg/mL). Plates were incubated at 37 °C for 4 h followed by cell surface staining as described above. For intracellular IFN-γ detection, cells were fixed and permeabilized using the Cytofix/cytoperm kit (BD Pharmingen) followed by intracellular staining using Perm/wash (BD Pharmingen).

Monitoring of Blood Parameters. A fully automated hematology analyzer, the ABX Pentra 60 C (HORIBA MEDICAL Corporate) was used for in vitro diagnostic of whole blood specimen after calibration to mouse cells.

Statistical Analysis. Statistical analyses were performed using the Prism software. Data obtained in different groups of mice were compared using a nonparametric, one-tailed Mann–Whitney (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Fig. S1. Engagement of KIR2DL3 by its ligand HLA-Cw3 protects target cells from lysis in vivo. WT, K<sup>Db</sup>D<sup>Kb</sup>KO, and K<sup>Db</sup>D<sup>Kb</sup>KO-TgHLA were stained with the fluorescent dye CFSE, mixed at an equal ratio, and transferred into WT or TgKIR recipient. Mice were killed 40 h after injection, and the percentage of CFSE-labeled cells in spleen cells was assessed by flow cytometry, 3 mice per group (n = 4).
Fig. S2. Identification of 3 populations of CFSE labeled splenocytes after transfer in recipient mice. WT and KβDβKO-TgHLA splenocytes were labeled with CFSE (0.5 µM) and subsequently differentiated by staining with an anti-H-2Kb/Dβ mAb by FACS analysis. KβDβKO cells were labeled with CFSE (3.0 µM). The 3 subsets were mixed at a ratio 1:1:1 before transfer to recipient mice.
Fig. S3. 1–7F9 and GL183 anti-KIR mAbs induce the in vivo elimination of HLA⁺ cells in TgKIR mice. WT and KbDbKO-TgHLA splenocytes were stained with CFSE, mixed at an equal ratio, and transferred into RagKO-TgKIR recipients which were pretreated or not with increasing doses of the anti-KIR mAbs 1–7F9 or GL183 (from 3 to 300 μg per mouse). For a group of mice, treated with the highest dose of mAbs (300 μg per mouse), NK cells were depleted with anti-NK1.1 mAbs a day before cell transfer. Recipient mice were killed 20 h after injection, and the percentage of each cell type among CFSE-labeled cells was assessed in peripheral blood lymphocytes of the recipients by flow cytometry. The mean (± SD) of the ratio between KbDbKO-TgHLA and WT CFSE⁺ cells is shown (3 mice per group).
Fig. S4. KIR2DL3 receptor saturation during the course of long-term treatment with GL183 mAb. GL183 mAb (400 μg) was injected in K<sup>o</sup>D<sup>DKO</sup>-TgKIR/HLA mice every 4 days starting at day 0. KIR2DL3 receptor saturation was assessed on peripheral NK cells by staining peripheral blood mononuclear cell with PE-labeled-GL183 mAb. Percentages of KIR2DL3 receptor occupancy were calculated for each mouse at each time point: 2 h (n = 12), 7 days (n = 4), 14 days (n = 4), or 21 days (n = 4) after the first injection.
Table S1. Blood parameters before and after treatment with anti-KIR mAb

<table>
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<tr>
<th></th>
<th>WBC, 10^3/ml</th>
<th>RBC, 10^6/ml</th>
<th>Hgb, g/dL</th>
<th>Hct, %</th>
<th>MCV, μm^3</th>
<th>RDW, %</th>
<th>Pit, 10^3/ml</th>
<th>MPV, μm^3</th>
<th>Mouse weight, g</th>
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<td>Anti-KIR mAb</td>
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<td>Day 0 (n = 12)</td>
<td>13.9 (3.9)</td>
<td>9.1 (0.7)</td>
<td>13.3 (1.7)</td>
<td>41.0</td>
<td>44.7 (4.4)</td>
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<td>13.3 (2.4)</td>
<td>41.7</td>
<td>45.5 (4.7)</td>
<td>14.1</td>
<td>1198.8 (346.7)</td>
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<td>13.3 (2.7)</td>
<td>41.8</td>
<td>44.5 (4.8)</td>
<td>13.8</td>
<td>1134.1 (440.6)</td>
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<td>12.9 (2.9)</td>
<td>40.6</td>
<td>44.2 (5.0)</td>
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<td>1026.0 (507.5)</td>
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<td>Mock-injected</td>
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<td>13.2 (2.8)</td>
<td>40.0</td>
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<td>811.5</td>
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K^dD^bKO-TgKIR/HLA mice were treated every 4 days by 400 μg of anti-KIR mAb GL183 starting at day 0 or with PBS in mock-injected mice. Blood parameters were measured at day 0, 7, 14, and 21 using the hematology analyzer ABX Pentra 60 C (HORIBA ABX Diagnostic), which has been calibrated for mouse cells. Multiple parameters were measured, including white blood cells (WBC), red blood cells (RBC) or platelet (Pit) counts, hemoglobinemia (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), red cell distribution width (RDW), mean platelet volume (MPV), and mouse weight. Numbers represent means (SD). The number of mice tested at each time point is indicated.
### Table S2. Leukocyte subsets before and after treatment with anti-KIR mAb

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>%</th>
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<th>%</th>
<th>%</th>
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<td></td>
<td>Lymphocytes</td>
<td>Monocytes</td>
<td>Neutrophils</td>
<td>Eosinophils</td>
<td>Basophils</td>
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<td>Anti-KIR mAb</td>
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<td>72.7 (5.7)</td>
<td>10.0 (3.5)</td>
<td>14.7 (2.3)</td>
<td>2.3 (1.8)</td>
<td>0.3 (0.1)</td>
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<tr>
<td>Day 7 (n = 12)</td>
<td>72.3 (4.5)</td>
<td>10.2 (2.8)</td>
<td>14.9 (2.6)</td>
<td>2.3 (1.0)</td>
<td>0.2 (0.1)</td>
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<tr>
<td>Day 14 (n = 8)</td>
<td>74.2 (3.4)</td>
<td>8.9 (2.0)</td>
<td>14.7 (3.2)</td>
<td>2.0 (1.1)</td>
<td>0.2 (0.2)</td>
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<tr>
<td>Day 21 (n = 4)</td>
<td>74.3 (3.8)</td>
<td>15.0 (7.0)</td>
<td>9.6 (9.1)</td>
<td>0.9 (1.2)</td>
<td>0.3 (0.2)</td>
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<tr>
<td>Mock-injected</td>
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<td>64.1 (11.3)</td>
<td>11.2 (2.2)</td>
<td>20.1 (8.3)</td>
<td>2.0 (0.7)</td>
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<tr>
<td>Day 7 (n = 6)</td>
<td>63.7 (6.9)</td>
<td>12.3 (1.7)</td>
<td>20.3 (6.4)</td>
<td>2.3 (1.2)</td>
<td>1.4 (1.7)</td>
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<td>Day 14 (n = 4)</td>
<td>62.8 (15.3)</td>
<td>10.2 (1.3)</td>
<td>16.2 (3.2)</td>
<td>2.5 (1.0)</td>
<td>0.3 (0.1)</td>
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<tr>
<td>Day 21 (n = 2)</td>
<td>74.0</td>
<td>17.0</td>
<td>7.4</td>
<td>1.4</td>
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</table>

K<sup>d</sup>D<sup>Db</sup>KO-TgKIR/HLA mice were treated every 4 days by 400 μg of anti-KIR mAb GL183 starting at day 0 or with PBS in mock-injected mice. Blood parameters were measured at day 0, 7, 14, and 21. Among leukocytes the percentage of lymphocytes, monocytes, neutrophils, eosinophils, and basophils was assessed by using the ABX Pentra 60 C+R. Numbers represent means (SD). The number of mice tested at each time point is indicated.