THE JOURNAL OF IMMUNOLOGY

HLA Reduces Killer Cell Ig-like Receptor Expression Level and Frequency in a Humanized Mouse Model

Jeroen van Bergen, Allan Thompson, Melissa van Pel, Christelle Retière, Daniela Salvatori, David H. Raulet, John Trowsdale and Frits Koning

J Immunol 2013; 190:2880-2885; Prepublished online 6 February 2013;
doi: 10.4049/jimmunol.1200650
http://www.jimmunol.org/content/190/6/2880

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

HLA Reduces Killer Cell Ig-like Receptor Expression Level and Frequency in a Humanized Mouse Model

Jeroen van Bergen,* Allan Thompson,* Melissa van Pel,* Christelle Retière,† Daniela Salvatori,‡ David H. Raulet,§ John Trowsdale,¶ and Frits Koning*

NK cells use NK cell receptors to be able to recognize and eliminate infected, transformed, and allogeneic cells. Human NK cells are prevented from killing autologous healthy cells by virtue of inhibitory NKRs, primarily killer cell Ig-like receptors (KIR) that bind "self" HLA class I molecules. Individual NK cells stably express a selected set of KIR, but it is currently disputed whether the fraction of NK cells expressing a particular inhibitory KIR is influenced by the presence of the corresponding HLA ligand. The extreme polymorphism of the KIR and HLA loci, with wide-ranging affinities for individual KIR and HLA allele combinations, has made this issue particularly hard to tackle. In this study, we used a transgenic mouse model to investigate the effect of HLA on KIR repertoire and function in the absence of genetic variation inside and outside the KIR locus. These H-2K$^{b-/-}$ and H-2D$^{b-/-}$ mice lacked ligands for inhibitory Ly49 receptors and were transgenic for HLA-Cw3 and a KIR B haplotype. In this reductionist system, the presence of HLA-Cw3 reduced the frequency of KIR2DL2$^+$ cells, as well as the surface expression levels of KIR2DL2. In addition, in the presence of HLA-Cw3, the frequency of NKG2A$^+$ cells and the surface expression levels of NKG2A were reduced. In line with these findings, both transgene-encoded KIR and endogenous NKG2A contributed to the rejection of cells lacking HLA-Cw3. These findings support the idea that HLA influences the human KIR repertoire. ***The Journal of Immunology*****, 2013, 190: 2880–2885.**

Under steady-state conditions, NK cells are prevented from killing autologous cells by inhibitory NKRs binding "self" MHC class I molecules. In humans, this role is fulfilled by multiple inhibitory killer cell Ig-like receptors (KIRs), binding classical HLA class I molecules (HLA-A, -B, -C), and CD94/NKG2A, binding the nonclassical HLA-E molecule. Whereas the CD94/NKG2A–HLA-E system is highly conserved, the KIR locus on chromosome 19 and the classical HLA class I locus on chromosome 6 display extensive polymorphism, with only particular KIR and HLA allele products binding each other (1). KIR3DL1, for example, binds HLA-A and -B molecules expressing the Bw4 motif, but the affinity and functionality of this interaction depend on the specific KIR3DL1 and HLA-A and -B alleles involved (2). Furthermore, individual NKR are expressed on only a fraction of NK cells, ranging from 0 to 100% of NK cells depending on the receptor and the receptor allele. As a result, the NK cell repertoire consists of many NK cell subsets expressing distinct combinations of receptors, and these repertoires differ greatly among individuals.

The mechanisms underlying NK cell tolerance in humans are the subject of intense investigation and have been informed by experiments in inbred mouse strains (3). Two non–mutually exclusive mechanisms have been proposed: 1) HLA dictates NKR expression patterns, and 2) HLA modulates NK cell responsiveness, depending on the combination of NKR expressed. Initially, on the basis of NK cells randomly cloned from two individuals, it was postulated that every NK cell clone expresses at least one inhibitory NKR specific for self HLA class I (4). This postulate would ensure NK cell self-tolerance and implied a major influence of HLA allotype on KIR repertoire, making responsiveness modulation redundant. Yet, subsequent analyses of the NK cell repertoires of a larger panel of HLA- and/or KIR-identical siblings showed that HLA had only a limited impact on KIR repertoire (5, 6). Consistent with this finding, potentially autoreactive NK cells lacking inhibitory NKR binding autologous HLA class I were found to constitute a significant fraction (>10%) of the mature NK cell repertoire (7, 8). These cells were hyporesponsive to stimulation (7), which may explain why they do not appear to kill autologous cells.

Recent developments in multiparameter flow cytometry provided more refined analyses, allowing the simultaneous detection of up to five NKR on NK cells at the single-cell level (8–11). The results of these experiments are conflicting. One study in Japanese individuals (n = 132) showed an HLA ligand–induced increase in the frequency of NK cells expressing cognate KIR, but this effect was detectable only in the case of high-affinity KIR–HLA combinations and neutralized by the presence of additional KIR–HLA interactions (9). This finding was corroborated in Germans (n = 150) (8). In contrast, such an effect was undetectable in Swedes (n = 44) (11). Rather, the latter study suggested a model in which KIR expression frequencies are genetically hardwired and repertoires low in KIR are buffered by CD94/NKG2A. The overall

*Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; †Etablissement Français du Sang, Université de Nantes, Immunovirologie et Polymorphisme Génétique, Nantes Cedex 01, France; ‡Experimental Animal Core Facility, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; §Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720; and ¶Immunology Division, Department of Pathology, Cambridge University, Cambridge CB2 1QP, United Kingdom

Received for publication February 23, 2012. Accepted for publication January 8, 2013.

This work was supported by Landsteiner Foundation for Blood Transfusion Research Grant 0515.

Address correspondence and reprint requests to Dr. Jeroen van Bergen, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. E-mail address: J.van_Bergen@lumc.nl

The online version of this article contains supplemental material.

Abbreviations used in this article: KIR, killer-cell Ig-like receptor; MFI, mean fluorescence intensity.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200650

conclusion from these studies is that much larger studies would be necessary to examine the effect of cognate HLA on the inhibitory KIR repertoire (11). Furthermore, such studies should be based on allele-level KIR genotyping because KIR polymorphism affects the level and frequency of KIR expression (9, 10).

To circumvent these issues, we used a KIR and HLA-Cw3 transgenic mouse model on a H-2K^b– and H-2D^b–deficient C57BL/6 background. This mouse is transgenic for an almost intact and fully sequenced KIR B haplotype (12, 13), which includes the HLA-Cw3–specific KIR2DL2. Similar to KIR genes in humans, these genes are expressed stochastically in this mouse, so that individual KIR genes are expressed only on a subset of NK cells. The absence of H-2K^b and H-2D^b eliminated any impact of the endogenous inhibitory interactions between Ly49 and MHC class I. We show that in these mice, a functional interaction between HLA-Cw3 and KIR2DL2 reduces the expression intensity and frequency of KIR2DL2.

Materials and Methods

Mice

Mice transgenic for a KIR B haplotype and on a mixed (C57BL/6 and CBA) genetic background (13) were back-crossed 8 times onto C57BL/6 (Jackson) mice (14). This KIR B haplotype has been sequenced in full and contains the following intact genes: KIR3DL3*003, KIR2DS2*001, KIR2DL2*003, KIR2DL4*005, KIR3DS1*013, KIR2DL5A*001, KIR2DS5*002, and KIR2DS1*002 (12). The presence and integrity of the KIR locus was checked after every backcross by KIR genotyping. C57BL/6 (B6) mice transgenic for genomic HLA-Cw*0304 construct (15) were a kind gift from Eric Vivier (Centre d'Immunologie de Marseille-Luminy, Marseille, France). To obtain KIR or HLA transgenic mice on a H-2K$^{b-/-}$ and H-2D$^{b-/-}$ background, KIR$^{+/+}$, HLA-Cw3$^{+/+}$, or control B6 mice were crossed with H-2K$^{b-/-}$H-2D$^{b-/-}\beta_2m^{-/-}$ mice, also on a B6 background (16). Mice with the desired phenotype (Kb$^-$Db$^-\beta_2m^+$KIR$^+$, Kb$^-$Db$^-\beta_2m^+$HLA-Cw3$^+$, or Kb$^-$Db$^-\beta_2m^+$) were selected from the F2 and used for further breeding to obtain K$^{b-/-}$D$^{b-/-}$KIR$^{+/+}$, K$^{b-/-}$D$^{b-/-}$HLA-Cw3$^{+/+}$, and K$^{b-/-}$D$^{b-/-}$ mice. To avoid integration artifacts, mice heterozygous for the KIR and HLA transgenes were used for experiments: KIR$^{+/-}$, HLA-Cw3$^{+/-}$, and KIR$^{+/-}$HLA-Cw3$^{+/-}$ mice, all on a K$^{b-/-}$D$^{b-/-}$background. The experiments were approved by the Leiden University Medical Center (Leiden, The Netherlands) animal experimental committee, and performed according to local guidelines.

NK cell expression of KIR and Ly49

Mononuclear cells were isolated from spleen using a Ficoll-Hypaque gradient and incubated with fluorescently labeled Abs. Samples were acquired on an LSRII (BD Biosciences) and analyzed using FACSDiva software (BD). NK cells were identified as CD3$^-$NK1.1$^+$ cells, using a combination of CD3-Pacific Blue (clone 500A2; BD) and NK1.1-PE-Cy7 (clone PK136; BD). For staining of mouse NKRs, the following FITC-conjugated Abs were purchased from BD: Ly49A (clone A1), Ly49C/I (clone 5E6), Ly49D (clone 4E5), Ly49G2 (clone 4D11), and NKG2A/C/E (clone 20d5). As B6 NK cells do not express appreciable levels of NKG2C and NKG2E (17), the specificity of the latter Ab was designated as NKG2A. The NKG2A-specific 16a11 Ab conjugated in-house to Alexa Fluor 647 was also used (18). KIR2DL4 was also detected using an in-house Alexa Fluor 647–conjugated Ab (clone 181703; R&D Systems). A PE-conjugated Ab to KIR2DL2/KIR2DL3/KIR2DS2 (clone GL183, Coulter Immunotech) was used to detect KIR2DL2/KIR2DS2, because the mouse does not carry the KIR2DL3 gene. APC-labeled HLA-Cw3/GAVDPLLAL tetramers (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) were used to selectively stain KIR2DL2, and Ab 1F12 to selectively stain KIR2DS2 (19). Stained samples were acquired on a Becton Dickinson LSRII and analyzed using BD FACSDiva software. For each NKR, the results from HLA-Cw3$^{+/-}$ and HLA-Cw3$^{-/-}$ mice were compared using a two-sided Student *t* test, without correcting for multiple comparisons.

NK responsiveness to crosslinking of Nkrp1c (NK1.1)

Splenic NK cells were stimulated with plate-bound PK136 Ab (specific for NK1.1/Nkrp1c/Klrb1c) for 5 h, with addition of brefeldin A after 1 h, and analyzed for intracellular accumulation of IFN-γ (using Ab clone XMG1.2; BD), as described (20, 21). To test whether NK cell subsets expressed different levels of IFN-γ after stimulation, the results were analyzed using the Friedman test, and if the resulting p value was < 0.05, post hoc analysis using the Dunn multiple comparison test determined whether IFN-γ production differed significantly between NK cells staining with specific NKR Abs (e.g., KIR2DL2/S2$^+$NKG2A$^-$ or KIR2DL2/S2$^-$NKG2A$^+$) and NK cells that did not stain with these Abs (e.g., KIR2DL2/S2$^-$NKG2A$^-$).

In vivo rejection of target cells

In vivo rejection of CFSE-labeled spleen cells was performed as described (22). Briefly, two different populations of spleen cells, one internal syngeneic control expressing HLA-Cw3 and one lacking HLA-Cw3, were labeled with 0.5 μM and 5 μM CFSE (Invitrogen), respectively, and mixed in a 1:1 ratio. On days 1, 3, and 6 (or days 2, 4, and 6) after i.v. injection (day 0) of this mixture (10^7 of each type), peripheral blood of recipient mice was collected and analyzed by FACS. The relative rejection of HLA-Cw3$^{-/-}$ target cells was calculated as follows: [1 − (acquired number of CFSE^{high} cells in sample/acquired number of CFSE^{low} cells in sample)/(acquired number of CFSE^{high} cells in injection mix/acquired number of CFSE^{low} cells in injection mix)] × 100%. In some experiments, NK cells or NKG2A$^+$ cells were depleted by i.p. injection of 200 μg protein A–purified Ab PK136 or 16a11, respectively, in 200 μl PBS on day −4 and day −1. As both Abs are mouse IgGs, plain PBS was used instead of an isotype-matched control Ab. To compare the resulting survival curves, the areas under the curve (AUC) were calculated for each individual mouse, and they were compared between groups of mice, using the two-tailed Mann–Whitney *U* test. However, when testing the effect of Ab-mediated depletion, one-tailed p values were calculated, because depletion of NK cells or NK cell subset should reduce rejection rates.

Results

HLA-Cw3 tetramers stain KIR2DL2

To assess the influence of HLA on KIR expression in a system with minimal genetic variation and minimal influence of endogenous mouse NKR systems, we compared KIR expression between MHC class I–deficient (H-2K$^{b-/-}$ H-2D$^{b-/-}$) mice expressing a human KIR locus in the presence or absence of HLA-Cw3. To detect the corresponding inhibitory receptor KIR2DL2, we used HLA-Cw3 tetramers. These should bind KIR2DL2, but not the very similar activating receptor KIR2DS2, because only KIR2DL2 shows measurable binding to HLA-Cw3 (23).

To confirm the specific detection of KIR2DL2 by these tetramers, spleen NK cells were costained (Fig. 1A) with HLA-Cw3 tetramer, a KIR2DL2/KIR2DS2-specific Ab (GL183), and a KIR2DS2-specific Ab (1F12). These staining reagents did not interfere with each other's binding (data not shown). The tetramer stained GL183$^+$, but not GL183$^-$, NK cells, demonstrating that it did not bind an endogenous mouse receptor or another KIR encoded by the transgene (Fig. 1A). Furthermore, the tetramer bound a subset of GL183$^+$ cells, consistent with the idea that GL183$^+$ cells include both KIR2DL2$^+$ (tetramer$^+$) and KIR2DL2$^-$ (tetramer$^-$) cells. The latter population (GL183$^+$tetramer$^-$) of cells stained homogeneously with KIR2DS2-Ab 1F12, confirming that the tetramer did not stain KIR2DS2. Finally, the specificity of these reagents was confirmed using human T cell clones expressing either KIR2DL2 or KIR2DS2 (ref. 24; Supplemental Fig. 1). In conclusion, the HLA-Cw3 tetramer provided a sensitive and specific tool to detect KIR2DL2 expression in the KIR transgenic mice.

HLA-Cw3 reduces the frequency and intensity of NK cell KIR2DL2 expression

The percentage of NK cells expressing KIR2DL2 and/or KIR2DS2 varied greatly between mice (Fig. 1B). We compared KIR2DL2 expression in the presence or absence of its ligand HLA-Cw3. Consistent with reports in humans, the presence of the HLA-Cw3 transgene reduced the KIR2DL2 mean fluorescence intensity (MFI) nearly 2-fold (Fig. 1B), while leaving KIR2DS2 staining unaffected. On average, the frequency of KIR2DL2$^+$ cells within NK cells was also ~ 2-fold reduced (Fig. 1B), and again no

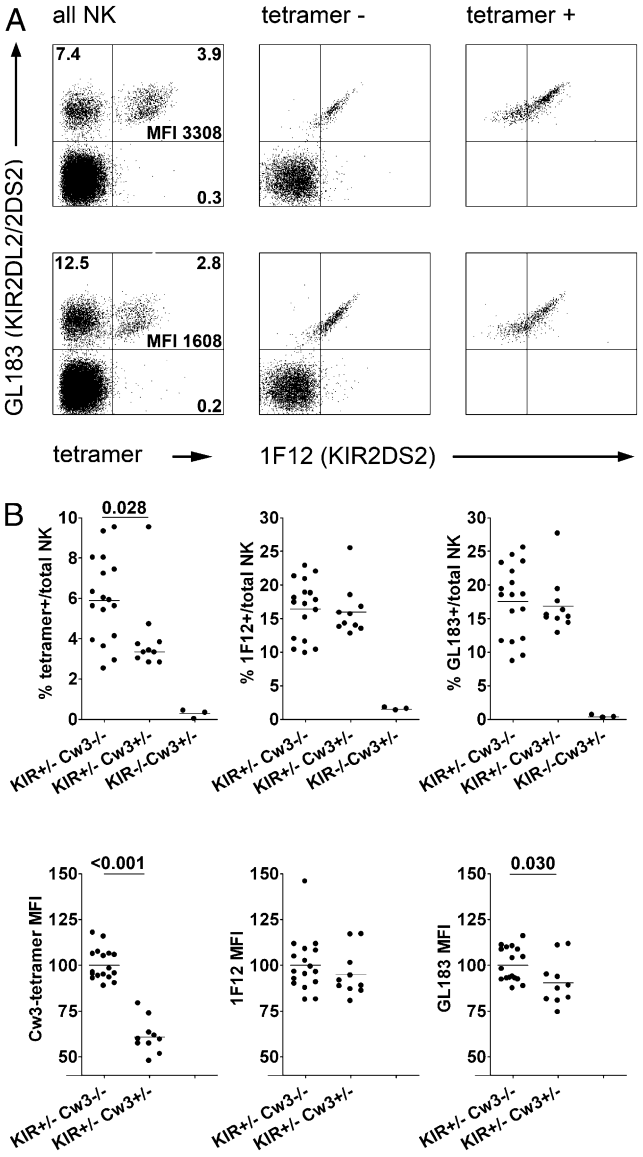

FIGURE 1. The presence of HLA-Cw3 reduces KIR2DL2 expression frequency and intensity on NK cells from $K^{b-/-}D^{b-/-}KIR^{+/-}$ mice. Spleen cells from $Kb^{-/-}Db^{-/-}$ mice transgenic for a KIR B haplotype and/or HLA-Cw3 were first stained with HLA-Cw3/GAVDPLLAL tetramers and then with Abs to CD3, NK1.1, KIR2DS2 (1F12), and KIR2DL2/KIR2DS2 (GL183). (**A**) Representative dot plots for $K^{b-/-}D^{b-/-}KIR^{+/-}$ (*top panel*) and $K^{b-/-}D^{b-/-}KIR^{+/-}HLA\text{-}Cw3^{+/-}$ (*bottom panel*) splenocytes, gated–from left to right–on all NK cells, HLA–Cw3 tetramer-negative cells, or HLA–Cw3 tetramer-positive NK cells. The numbers represent the percentages of cells within each quadrant, as well as the MFI of the tetramer staining (tetramer$^+$ cells only). (**B**) Summary of four experiments comparing the proportions of NK cells staining with HLA-Cw3 tetramer, KIR2DS2 Ab 1F12, and KIR2DL2/KIR2DS2 Ab GL183 between $K^{b-/-}D^{b-/-}KIR^{+/-}HLA\text{-}Cw3^{-/-}$ ($n = 17$), $K^{b-/-}D^{b-/-}KIR^{+/-}HLA\text{-}Cw3^{+/-}$ ($n = 10$), and $K^{b-/-}D^{b-/-}KIR^{-/-}HLA\text{-}Cw3^{+/-}$ ($n = 3$) mice (*top panel*). In each experiment, the MFI values for NK cells staining with these reagents were normalized for the average MFI values obtained with $K^{b-/-}D^{b-/-}KIR^{+/-}HLA\text{-}Cw3^{-/-}$ mice before the experiments were pooled (*bottom panel*). The p values < 0.05 are shown.

such effect was seen for KIR2DS2. In addition, the expression of KIR2DL4, another (non–HLA-Cw3–specific) receptor encoded by the KIR transgene, was also unaffected by HLA-Cw3 (Fig. 2A). As previously reported for these same KIR transgenic mice on a wild-type C57BL/6 background (14), GL183 (KIR2DL2/KIR2DS2) staining was not significantly different between HLA-Cw3$^-$ and HLA-Cw3$^+$ mice (Figs. 1B, 2A). This lack of difference resulted because the majority of GL183$^+$ NK cells expressed KIR2DS2, whereas only a minority expressed KIR2DL2 (Fig. 1B). Thus, HLA-Cw3 reduced the frequency as well as the intensity of NK cell KIR2DL2 expression, but not of other, non–HLA-Cw3–binding KIR present in the transgene.

The introduction of HLA-Cw3 did not alter the expression frequencies of mouse Ly49 receptors (Fig. 2A), but was associated with a slight, but significant, reduction in the expression frequency and intensity of NKG2A (Fig. 2A), an inhibitory mouse receptor for the MHC class Ib molecule Qa-1. With another NKG2A-specific Ab (18) in an additional set of mice, this effect of HLA-Cw3 on NKG2A expression frequency and intensity was confirmed (Supplemental Fig. 2). Even though these mice do not express a ligand for it, Ly49G2 surface expression intensity, but not frequency, was elevated in the presence of HLA-Cw3. In general, individual KIR and Ly49 genes are expressed largely independently of other KIR and Ly49 genes. Yet, in our mice KIR2DL4 was preferentially coexpressed on the same cell with KIR2DL2/KIR2DS2, and the opposite was true for NKG2A and KIR2DL2/KIR2DS2 (Fig. 2B). These coexpression biases were unaffected by the presence of HLA-Cw3. In summary, HLA-Cw3 significantly reduced the expression frequency and intensity of the endogenous CD94/NKG2A receptor.

HLA-Cw3 increases responsiveness of NKG2A$^+$ NK cells

NK cells expressing inhibitory receptors that bind endogenous MHC class I are more responsive to activating stimuli than are NK cells that do not express such "useful" inhibitory receptors (7, 20, 21). Hence, the presence of HLA-Cw3 might also influence the potency of NK cells expressing an HLA-Cw3–specific inhibitory receptor. In mice lacking both mouse and human MHC class Ia molecules (K^b, D^b, and HLA-Cw3), NK cells were poorly responsive to NK1.1 crosslinking, irrespective of the receptors they carried (Fig. 3). In the presence of HLA-Cw3, KIR2DL2/KIR2DS2$^+$ NK cells produced slightly more IFN-γ than did KIR2DL2/KIR2DS2$^-$ NK cells, but this increase was not statistically significant. In contrast, the responsiveness of NKG2A$^+$ NK cells was greatly increased in the presence of HLA-Cw3, suggesting that NKG2A$^+$ NK cells are also educated by HLA-Cw3 in this model system.

KIR- and NKG2A-dependent rejection of "missing self" HLA-Cw3 in vivo

To test whether KIR and HLA mediated "missing self"-recognition in these mice, we analyzed the rejection of $K^{b-/-}D^{b-/-}$ spleen cells by KIR and HLA transgenic $K^{b-/-}D^{b-/-}$ mice (Fig. 4). "Missing self"-rejection was tested by an in vivo assay based on differential labeling of donor cells using CFSE dye (22). Mixed $CFSE^{high}$ $K^{b-/-}D^{b-/-}$ and control $CFSE^{low}$ $K^{b-/-}D^{b-/-}HLA\text{-}Cw3^{+/-}$ spleen cells were injected i.v. into $Kb^{-/-}Db^{-/-}KIR^{+/-}HLA\text{-}Cw3^{+/-}$ or control $K^{b-/-}D^{b-/-}KIR^{-/-}HLA\text{-}Cw3^{+/-}$ mice. Both types of recipient mice swiftly rejected ~ 80% of HLA-Cw3–negative $K^{b-/-}D^{b-/-}$ target cells (Fig. 4A). The presence of the KIR transgene did not significantly increase the rejection rate, showing that KIR were not necessary for rejection.

Because the presence of HLA-Cw3 affected NK cell NKG2A expression levels, as well as the frequency and functionality of NKG2A$^+$ NK cells, we next tested whether these cells contributed to rejection (Fig. 4B). In $K^{b-/-}D^{b-/-}HLA\text{-}Cw3^{+/-}$ mice, depletion of NKG2A$^+$ cells before and during the experiment indeed greatly reduced the rejection of $Kb^{-/-}Db^{-/-}$ cells. To isolate the effect of KIR on rejection, the fate of injected $K^{b-/-}D^{b-/-}$ cells

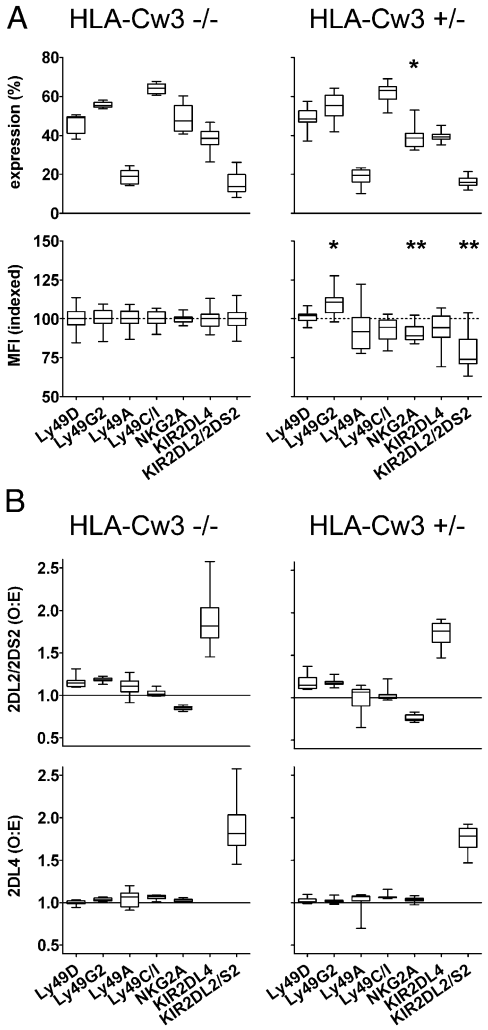

FIGURE 2. The presence of HLA-Cw3 reduces mouse NKG2A expression frequency and intensity on NK cells from $K^{b-/-}D^{b-/-}KIR^{+/-}$ mice. Spleen cells from $K^{b-/-}D^{b-/-}KIR^{+/-}$ mice transgenic (HLA-$Cw3^{+/-}$) or not (HLA-$Cw3^{-/-}$) for HLA-Cw3 were stained with Abs to CD3, NK1.1, KIR2DL2/2DS2, and KIR2DL4 in combination with Abs directed against mouse Ly49 receptors or NKG2A. (**A**) Frequency of surface expression of individual mouse (Ly49s, NKG2A) or human (KIR2DL4, KIR2DL2/KIR2DS2) NKRs by spleen NK ($CD3^-NK1.1^+$) cells from $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{-/-}$ and $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ mice. The MFI values for NK cells positive for individual receptors were normalized for the MFI values obtained using $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{-/-}$ mice, as in Fig. 1B. (**B**) Coexpression of transgenic KIR2DL2/KIR2DS2 (*top panel*) or KIR2DL4 (*bottom panel*) with endogenous mouse NKRs was quantified in terms of deviation from the "product rule" (4). O represents the observed frequency of cells coexpressing KIR2DL2/KIR2DS2 or KIR2DL4 and a particular mouse NKR among NK cells, and E represents the product of the individual expression frequencies of these human and mouse receptors on NK cells, that is, the expected frequency of cells expressing these receptors. Coexpression values for KIR2DL2/KIR2DS2 and KIR2DL4 were also calculated. Data are from eight $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{-/-}$ and eight $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ mice. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the total range of measurements. $*p < 0.05$, $**p < 0.005$.

was compared between NKG2A-depleted $K^{b-/-}D^{b-/-}$HLA-$Cw3^{+/-}$ mice having or lacking the KIR transgene (Fig. 4C). When $NKG2A^+$ cells were depleted, rejection of $Kb^{-/-}Db^{-/-}$ cells was dependent on the presence of the KIR transgene (Fig. 4C). Additional depletion of NK cells in these KIR transgenic mice reduced rejection to the level of control KIR-less mice,

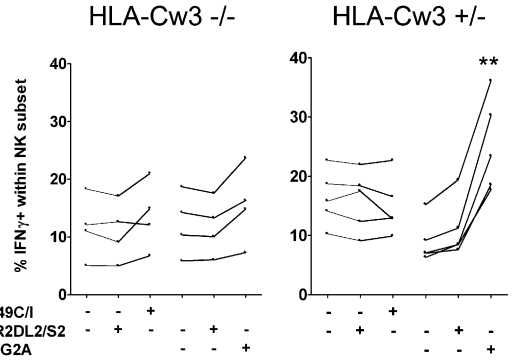

FIGURE 3. The presence of HLA-Cw3 increases the responsiveness of $NKG2A^+$ NK cells. Spleen cells from $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{-/-}$ (HLA-$Cw3^{-/-}$) and $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ (HLA-$Cw3^{+/-}$) mice were stimulated with plate-bound anti-NK1.1 for 5 h, and accumulation of intracellular IFN-γ in NK cells ($CD3^-DX5^+$) was subsequently analyzed by flow cytometry. NK cells were subdivided according to their expression of KIR2DL2/KIR2DS2, of which the inhibitory KIR2DL2 binds HLA-Cw3, and their expression of inhibitory mouse receptors binding mouse MHC class I. Ly49C and Ly49I both bind H-$2K^b$ (absent from the mice), and NKG2A binds Qa-1. Data are from two experiments using a total of four $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{-/-}$ and five $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ mice. Lines connect data from individual mice. For each receptor combination, the results were compared between cells expressing a particular receptor (e.g., $KIR2DL2/S2^+NKG2A^-$) and those lacking that receptor (e.g., $KIR2DL2/S2^-NKG2A^-$). $**p < 0.01$.

supporting the idea that all KIR-dependent rejection in NKG2A-depleted mice was mediated by NK cells. In conclusion, the mouse CD94/NKG2A receptor dominated the "missing HLA" response in KIR and HLA transgenic mice, and only upon depletion of $NKG2A^+$ NK cells did KIR-mediated rejection become apparent.

Discussion

We used humanized inbred mice to investigate the effect of HLA on KIR repertoire and function in a controlled reductionist system. In these MHC class Ia–deficient ($K^{b-/-}D^{b-/-}$) and KIR transgenic mice, the presence of HLA-Cw3 reduced the proportion of $KIR2DL2^+$ cells as well as the surface expression levels of KIR2DL2. In addition, HLA-Cw3 reduced the expression frequency and intensity of NKG2A. In line with these observations, both KIR and NKG2A contributed to the rejection of "missing self" target cells lacking HLA-Cw3.

Studies on human NK cell repertoires in most cases showed no HLA effect on KIR expression frequencies (5, 6, 9, 11), except in very specific circumstances. For example, in individuals homozygous for specific inhibitory KIR binding their ligand with high affinity (KIR2DL1 or KIR3DL1*001/KIR3DL1*015/KIR3DL1*020), the presence of ligand was associated with increased frequencies of NK cells expressing these receptors, but only in the absence of too many additional inhibitory KIR–ligand interactions (8, 9). A similar, albeit less pronounced, effect was observed for KIR2DL3 and C1 (8). These effects were detected in individuals homozygous for KIR A haplotypes, characterized by the absence of KIR2DL2, KIR2DL5, and most activating receptors (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1). In whites, such A-homozygous individuals make up less than half of the population.

KIR repertoires in individuals carrying KIR B haplotypes have been more difficult to study, mainly because Abs specific for inhibitory KIR cross react with activating KIR present in B, but not A, haplotypes. Group 1 HLA-C (HLA-C^{Asn-80}) effects on KIR2DL2

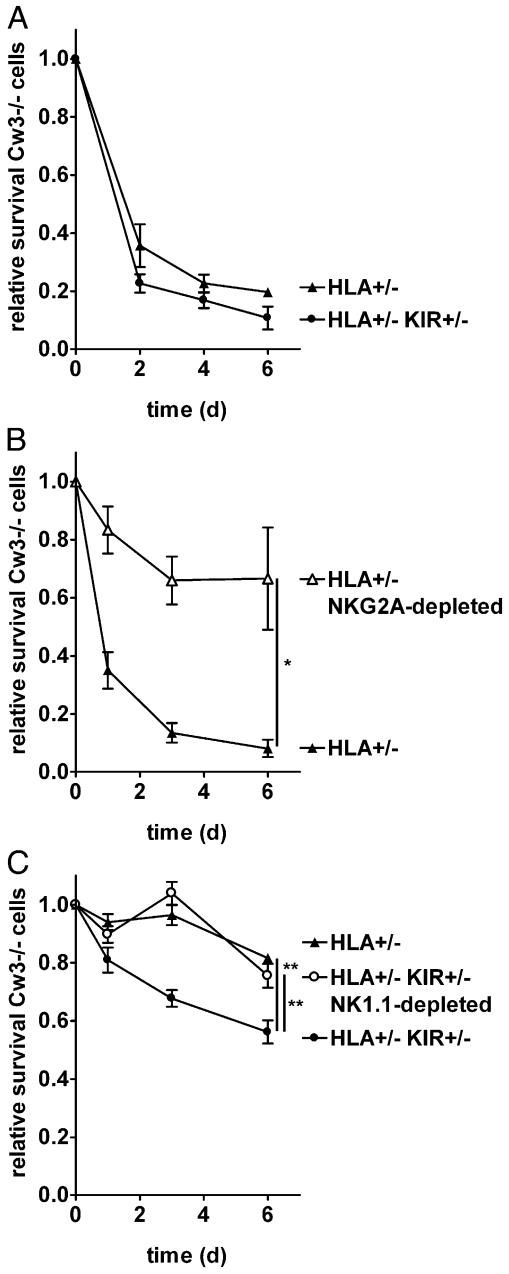

FIGURE 4. In KIR and HLA-Cw3 transgenic $K^{b-/-}D^{b-/-}$ mice, KIR and NKG2A contribute to the rejection of $K^{b-/-}D^{b-/-}$ grafts. $K^{b-/-}D^{b-/-}KIR^{+/-}$ HLA-$Cw3^{+/-}$ ($HLA^{+/-}$ $KIR^{+/-}$) or control $K^{b-/-}D^{b-/-}KIR^{-/-}$HLA-$Cw3^{+/-}$ ($HLA^{+/-}$) mice were injected i.v. with mixed CFSE-labeled $K^{b-/-}D^{b-/-}$ ($CFSE^{high}$) and control $K^{b-/-}D^{b-/-}$HLA-$Cw3^{+/-}$ ($CFSE^{low}$) spleen cells. The relative survival of $CFSE^{high}$ ($Cw3^{-/-}$) cells in peripheral blood, normalized for the $CFSE^{high}/CFSE^{low}$ ratio in the injected cells, was tracked in (**A**) nondepleted $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ ($n = 4$) or control $K^{b-/-}D^{b-/-}KIR^{-/-}$HLA-$Cw3^{+/-}$ ($n = 3$) mice, in (**B**) nondepleted ($n = 4$) versus 16a11(NKG2A)-depleted ($n = 3$) $K^{b-/-}D^{b-/-}KIR^{-/-}$HLA-$Cw3^{+/-}$ mice, and in (**C**) 16a11-depleted $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ ($n = 5$), 16a11-depleted $K^{b-/-}D^{b-/-}KIR^{-/-}$HLA-$Cw3^{+/-}$ ($n = 5$), and 16a11- and PK136 (NK1.1)-depleted $K^{b-/-}D^{b-/-}KIR^{+/-}$HLA-$Cw3^{+/-}$ ($n = 5$) mice. $*p < 0.05$, $**p < 0.01$.

expression frequencies have been particularly difficult to detect, not only because the available Abs cross react with the activating KIR2DS2, nearly always present on the same haplotype, but also because KIR2DL2 also binds some group 2 HLA-C alleles (HLA-C^{Lys-80}; ref 25). These problems were circumvented in our humanized mice, because we were able to use HLA-Cw3 (group 1 HLA-C) tetramers to detect specifically KIR2DL2, and because we compared mice lacking or having an HLA-C group 1 allele. In these mice, the presence of HLA-Cw3 decreased the frequency of $KIR2DL2^+$ NK cells. Thus, in this model system with a genetically homogeneous background, the presence of an HLA ligand clearly did influence the expression frequency of the corresponding inhibitory KIR.

KIR expression frequencies varied greatly between mice, and the degree of variation was similar to that of the endogenous Ly49 and CD94/NKG2A receptors. It is unlikely that this was due to genetic variation between mice, as they had been backcrossed extensively to a C57BL/6 background. Furthermore, interindividual variation in Ly49 and CD94/NKG2A expression frequencies was also observed in the nontransgenic inbred C57BL/6 strain (26). The probability of Ly49 expression is regulated by overlapping and bidirectional promoter elements and differs between Ly49 genes, depending on the relative efficiencies of the forward and reverse promoters (27). Bidirectional promoters have also been found for KIR genes, suggesting that such probabilistic transcriptional switches also control stochastic expression of the KIR genes (28). Taken together, these findings indicate that, as with mice carrying a genomic Ly49A transgene (29), regulatory elements necessary for stochastic KIR expression were contained within the transgene, which contained an almost intact KIR locus, including intronic and intergenic sequences. In all cases, it is unclear what causes the variation in receptor expression frequencies among genetically identical mice.

In addition to the effect of HLA-Cw3 on the proportion (i.e., the percentage) of NK cells expressing KIR2DL2 and CD94/NKG2A, the presence of HLA-Cw3 also reduced the expression levels (i.e., the MFI) of these receptors. This effect of ligand on receptor MFI was previously reported both in humans (9) and in mice (30). It most likely results from ligand-induced receptor downmodulation, and may help to correct for differences in receptor-ligand affinity (30). For example, strong rejection induced by high-affinity interactions would be dampened by lowering the expression level of the NKR involved.

In the $K^{b-/-}D^{b-/-}$ mice transgenic for KIR and HLA-Cw3, $NKG2A^+$ cells contributed to the rejection of "missing HLA-Cw3." In line with this finding, the introduction of HLA-Cw3 in KIR transgenic $K^{b-/-}D^{b-/-}$ mice affected the expression of NKG2A, as well as the responsiveness of $NKG2A^+$ NK cells. Importantly, $K^{b-/-}D^{b-/-}$ mice transgenic for HLA-Cw3 only already rejected $K^{b-/-}D^{b-/-}$ target cells, and this rejection was inhibited by Ab-mediated depletion of $NKG2A^+$ cells. NKG2A is an inhibitory receptor binding Qa-1, whose surface expression in C57BL/6 mice largely depends on its loading with the $H\text{-}2D^b$ leader peptide AMAPRTLLL. The very similar HLA-Cw3 leader peptide VMAPRTLIL also binds $Qa\text{-}1^b$ and thereby induces a functional ligand for CD94/NKG2A in $K^{b-/-}D^{b-/-}$ mice (31, 32). Thus, the recognition by CD94/NKG2A of HLA-Cw3 leader peptides bound to $Qa\text{-}1^b$ likely contributes to the education, selection, and function of the NK cell repertoire in HLA-Cw3 transgenic $K^{b-/-}D^{b-/-}$ mice, irrespective of the presence of KIR. Therefore, NKG2A may also have contributed to the rejection of "missing HLA-Cw3" targets in the KIR2DL3 and HLA-Cw3 transgenic $K^{b-/-}D^{b-/-}$ mice described by Sola and colleagues (33).

Upon the deletion of $NKG2A^+$ cells, KIR-dependent rejection of "missing" HLA-Cw3 by KIR and HLA-Cw3 transgenic $K^{b-/-}D^{b-/-}$ mice was considerably greater than what we observed previously in KIR and HLA-Cw3 transgenic mice on a wild-type C57BL/6 background (14). This finding is reminiscent of the experiments of Johansson et al. (30), who found that the education

of NK cells by weak Ly49 ligands was attenuated by the presence of strong Ly49 ligands. Hence, in transgenic mice on a wild-type C57BL/6 background, the presence of the strong interaction between Ly49C and H-K^b may have obscured the detection of "missing" HLA-Cw3. In agreement with the differences in KIR-mediated rejection, the presence of KIR2DL2/KIR2DS2 on NK cells did not detectably increase their responsiveness in mice on a wild-type C57BL/6 background (14), but on a $K^{b-/-}D^{b-/-}$ background a small but nonsignificant increase in responsiveness was detected. Because only a third of KIR2DL2/KIR2DS2$^+$ NK cells expressed KIR2DL2, this weak response may have been due to dilution rather than poor responsiveness of KIR2DL2$^+$ NK cells. As in our hands HLA-Cw3 tetramer stains did not work in combination with intracellular IFN-γ staining, we were unable to discriminate between these possibilities.

Our data show that in a setting with little or no variation in genetic background, a functional interaction between the products of a single inhibitory KIR allele and a single HLA allele reduced both the surface expression levels of that KIR and the fraction of NK cells expressing it. This finding supports the idea that HLA influences the human KIR repertoire.

Acknowledgments

We thank the National Institutes of Health Tetramer Facility for providing HLA-Cw3 tetramers, Michel Mulders, Suzanne van Duikeren, and Arie Boon for assistance with animal experiments, and Geert Haasnoot for advice on statistical analyses. We are also greatly indebted to Eric Vivier (Centre d'Immunologie de Marseille-Luminy, Marseille, France) and François Lemonnier (Institut Pasteur, Paris, France) for providing HLA-Cw3$^{+/+}$ and H-$2K^{b-/-}$H-$2D^{b-/-}\beta_2m^{-/-}$ mice, respectively.

Disclosures

The authors have no financial conflicts of interest.

References

1. Parham, P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat. Rev. Immunol.* 5: 201–214.
2. Carr, W. H., M. J. Pando, and P. Parham. 2005. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J. Immunol.* 175: 5222–5229.
3. Höglund, P., and P. Brodin. 2010. Current perspectives of natural killer cell education by MHC class I molecules. *Nat. Rev. Immunol.* 10: 724–734.
4. Valiante, N. M., M. Uhrberg, H. G. Shilling, K. Lienert-Weidenbach, K. L. Arnett, A. D'Andrea, J. H. Phillips, L. L. Lanier, and P. Parham. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity* 7: 739–751.
5. Gumperz, J. E., N. M. Valiante, P. Parham, L. L. Lanier, and D. Tyan. 1996. Heterogeneous phenotypes of expression of the NKB1 natural killer cell class I receptor among individuals of different human histocompatibility leukocyte antigens types appear genetically regulated, but not linked to major histocompatibility complex haplotype. *J. Exp. Med.* 183: 1817–1827.
6. Shilling, H. G., N. Young, L. A. Guethlein, N. W. Cheng, C. M. Gardiner, D. Tyan, and P. Parham. 2002. Genetic control of human NK cell repertoire. *J. Immunol.* 169: 239–247.
7. Anfossi, N., P. André, S. Guia, C. S. Falk, S. Roetynck, C. A. Stewart, V. Breso, C. Frassati, D. Reviron, D. Middleton, et al. 2006. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 25: 331–342.
8. Schönberg, K., M. Sribar, J. Enczmann, J. C. Fischer, and M. Uhrberg. 2011. Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. *Blood* 117: 98–107.
9. Yawata, M., N. Yawata, M. Draghi, A. M. Little, F. Partheniou, and P. Parham. 2006. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J. Exp. Med.* 203: 633–645.
10. Yawata, M., N. Yawata, M. Draghi, F. Partheniou, A. M. Little, and P. Parham. 2008. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood* 112: 2369–2380.
11. Andersson, S., C. Fauriat, J. A. Malmberg, H. G. Ljunggren, and K. J. Malmberg. 2009. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. *Blood* 114: 95–104.
12. Wilson, M. J., M. Torkar, A. Haude, S. Milne, T. Jones, D. Sheer, S. Beck, and J. Trowsdale. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc. Natl. Acad. Sci. USA* 97: 4778–4783.
13. Belkin, D., M. Torkar, C. Chang, R. Barten, M. Tolaini, A. Haude, R. Allen, M. J. Wilson, D. Kioussis, and J. Trowsdale. 2003. Killer cell Ig-like receptor and leukocyte Ig-like receptor transgenic mice exhibit tissue- and cell-specific transgene expression. *J. Immunol.* 171: 3056–3063.
14. van Bergen, J., A. Thompson, C. Retière, J. Trowsdale, and F. Koning. 2009. Cutting edge: killer Ig-like receptors mediate "missing self" recognition in vivo. *J. Immunol.* 182: 2569–2572.
15. Dill, O., F. Kievits, S. Koch, P. Ivanyi, and G. J. Hämmerling. 1988. Immunological function of HLA-C antigens in HLA-Cw3 transgenic mice. *Proc. Natl. Acad. Sci. USA* 85: 5664–5668.
16. Grigoriadou, K., C. Ménard, B. Pérarnau, and F. A. Lemonnier. 1999. MHC class Ia molecules alone control NK-mediated bone marrow graft rejection. *Eur. J. Immunol.* 29: 3683–3690.
17. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* 190: 1801–1812.
18. Vance, R. E., A. M. Jamieson, D. Cado, and D. H. Raulet. 2002. Implications of CD94 deficiency and monoallelic NKG2A expression for natural killer cell development and repertoire formation. *Proc. Natl. Acad. Sci. USA* 99: 868–873.
19. David, G., M. Morvan, K. Gagne, N. Kerdudou, C. Willem, A. Devys, M. Bonneville, G. Folléa, J. D. Bignon, and C. Retière. 2009. Discrimination between the main activating and inhibitory killer cell immunoglobulin-like receptor positive natural killer cell subsets using newly characterized monoclonal antibodies. *Immunology* 128: 172–184.
20. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416–4423.
21. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709–713.
22. Oberg, L., S. Johansson, J. Michaëlsson, E. Tomasello, E. Vivier, K. Kärre, and P. Höglund. 2004. Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo—role of KARAP/DAP12-dependent and -independent pathways. *Eur. J. Immunol.* 34: 1646–1653.
23. Saulquin, X., L. N. Gastinel, and E. Vivier. 2003. Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *J. Exp. Med.* 197: 933–938.
24. van Bergen, J., A. Thompson, A. van der Slik, T. H. Ottenhoff, J. Gussekloo, and F. Koning. 2004. Phenotypic and functional characterization of CD4 T cells expressing killer Ig-like receptors. *J. Immunol.* 173: 6719–6726.
25. Moesta, A. K., P. J. Norman, M. Yawata, N. Yawata, M. Gleimer, and P. Parham. 2008. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J. Immunol.* 180: 3969–3979.
26. Fahlén, L., U. Lendahl, and C. L. Sentman. 2001. MHC class I-Ly49 interactions shape the Ly49 repertoire on murine NK cells. *J. Immunol.* 166: 6585–6592.
27. Saleh, A., G. E. Davies, V. Pascal, P. W. Wright, D. L. Hodge, E. H. Cho, S. J. Lockett, M. Abshari, and S. K. Anderson. 2004. Identification of probabilistic transcriptional switches in the *Ly49* gene cluster: a eukaryotic mechanism for selective gene activation. *Immunity* 21: 55–66.
28. Davies, G. E., S. M. Locke, P. W. Wright, H. Li, R. J. Hanson, J. S. Miller, and S. K. Anderson. 2007. Identification of bidirectional promoters in the human KIR genes. *Genes Immun.* 8: 245–253.
29. Tanamachi, D. M., D. C. Moniot, D. Cado, S. D. Liu, J. K. Hsia, and D. H. Raulet. 2004. Genomic Ly49A transgenes: basis of variegated Ly49A gene expression and identification of a critical regulatory element. *J. Immunol.* 172: 1074–1082.
30. Johansson, S., M. Johansson, E. Rosmaraki, G. Vahlne, R. Mehr, M. Salmon-Divon, F. Lemonnier, K. Kärre, and P. Höglund. 2005. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J. Exp. Med.* 201: 1145–1155.
31. Kurepa, Z., C. A. Hasemann, and J. Forman. 1998. Qa-1b binds conserved class I leader peptides derived from several mammalian species. *J. Exp. Med.* 188: 973–978.
32. Sivakumar, P. V., A. Gunturi, M. Salcedo, J. D. Schatzle, W. C. Lai, Z. Kurepa, L. Pitcher, M. S. Seaman, F. A. Lemonnier, M. Bennett, et al. 1999. Cutting edge: expression of functional CD94/NKG2A inhibitory receptors on fetal NK1.1+Ly-49- cells: a possible mechanism of tolerance during NK cell development. *J. Immunol.* 162: 6976–6980.
33. Sola, C., P. André, C. Lemmers, N. Fuseri, C. Bonnafous, M. Bléry, N. R. Wagtmann, F. Romagné, E. Vivier, and S. Ugolini. 2009. Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo. *Proc. Natl. Acad. Sci. USA* 106: 12879–12884.