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

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Under steady-state conditions, NK cells are prevented from killing autologous cells by inhibitory NKR 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 allelotype 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 hypo-responsive 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
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-2Kb– and H-2Db–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-2Kb and H-2Db 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*001, 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-Cw3*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-2Kb– and H-2Db–deficient background, KIR+/+, HLA-Cw3+/+, and control B6 mice were crossed with H-2Kb+/–/H-2Db+/–/β2m–/– mice, also on a B6 background (16). Mice with the desired phenotype (Kb Db 3a 3b KIR+/, H-2Kb–/– and H-2Db–/–) were obtained from the National Institute of Health Tetrramer Core Facility, Emory University, Atlanta, GA) were used to selectively stain B6 NK cells do not express appreciable NKG2A/C/E (clone 20d5). As B6 NK cells do not express appreciable FITC-conjugated Abs were purchased from BD: Ly49A (clone A1), KIR2DL4 was also detected using a combination of CD3-Pacific Blue (clone 500A2; BD) and NK1.1-PE (clone 100.1), 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 CFSEhigh cells in sample/acquired number of CFSElow cells in injection mix)/acquired number of CFSE+ cells in injection mix) × 100%. In some experiments, NK cells or NK2Gα 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 NK systems, we compared KIR expression between MHC class I–deficient (H-2Kb–/–/– H-2Db−/−) 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 specificity for the KIR and HLA transgenes were used for experiments: KIR3DL3*003, KIR2DS2*001, HLA-Cw3+, and KIR2DL2, all on a K-β2m–/– 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 LSR II (BD Biosciences) and analyzed using FACSDiva software (BD). NK cells were identified as CD3− NK1.1+ cells, using a combination of CD1-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), Ly49C1 (clone 5E6), Ly49D1 (clone 4E5), Ly49G2D (clone 4D1), and NK2Gα/ε (clone 20d5). As B6 NK cells do not express appreciable levels of NK2GαC and NK2Gε, the specificity of the latter Ab was designated as NK2GαA. The NK2GαA-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). An 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/ G2A/TGpALL tetramers were used to selectively stain KIR2DL2, and Ab 1F12 to selectively stain KIR2DS2 (19). Stained samples were acquired on a Becton Dickinson LSR II and analyzed using BD FACSDiva software. For each NK, 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/Nkrk1c) for 5 h, with addition of brefeldin A after 1 h, and analyzed for intracellular accumulation of IFN-γ (using Ab clone XMGI1.2; 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 cell 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 synthetic 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 CFSEhigh cells in sample/acquired number of CFSElow cells in sample)/acquired number of CFSEcells 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.

HLA-Cw3 reduces KIR 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
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 Ia 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 did 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 (Kb, Db, 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 KIR−/− Db−/− spleen cells by KIR and HLA transgenic Kb−/− Db−/− 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 CFSEhigh Kb−/− Db−/− and control CFSElow Kb−/− Db−/− HLA-Cw3−/− spleen cells were injected i.v. into Kb−/− Db−/− KIR−/− HLA-Cw3−/− or control Kb−/− Db−/− KIR+/− HLA-Cw3−/− mice. Both types of recipient mice swiftly rejected ~80% of HLA-Cw3−/− negative Kb−/− Db−/− 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 Kb−/− Db−/− HLA-Cw3−/− mice, depletion of NKG2A+ cells before and during the experiment greatly reduced the rejection of Kb−/− Db−/− cells. To isolate the effect of KIR on rejection, the fate of injected Kb−/− Db−/− cells

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**FIGURE 1.** The presence of HLA-Cw3 reduces KIR2DL2 expression frequency and intensity on NK cells from KIR−/− Db−/− mice. Spleen cells from Kb−/− Db−/− mice transgenic for a KIR B haplotype and/or HLA-Cw3 were first stained with HLA-Cw3/GAVDPALL tetramers and then with Abs to CD3, NK1.1, KIR2DS2 (1F12), and KIR2DL2/KIR2DS2 (GL183). (A) Representative dot plots for Kb−/− Db−/− KIR+/+ (top panel) and Kb−/− Db−/− KIR−/− HLA-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 Kb−/− Db−/− KIR+/+ (n = 17), Kb−/− Db−/− KIR−/− HLA-Cw3+ (n = 10), and Kb−/− Db−/− KIR−/− HLA-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 Kb−/− Db−/− KIR+/+ HLA-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)
was compared between NKG2A-depleted KIR+/-/HLA-Cw3/- mice having or lacking the KIR transgene (Fig. 4C). When NKG2A+ cells were depleted, rejection of KIR+/-/D+/- 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, 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 (Kb+/-/D+/-) 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 HLA” and “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*0207) effects on KIR2DL2...
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-CW3+). For example, strong rejection induced by high-affinity interactions would be dampened by lowering the expression level of the NKR involved.

In the KIR+/D’/ mice transgenic for KIR and HLA-Cw3, NKGA2+ cells contributed to the rejection of “missing” HLA-Cw3. This line with this finding, the introduction of HLA-Cw3 in KIR transgenic KIR+/D’/ mice affected the expression of NKGA2+ cells, as well as the responsiveness of NKGA2+ NK cells. Importantly, KIR+/D’/ mice transgenic for HLA-Cw3 only already rejected KIR+/D’/ target cells, and this rejection was inhibited by Ab-mediated depletion of NKGA2+ cells. NKGA2 is an inhibitory receptor binding QA-1, whose surface expression in genetically identical mice. 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.
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^a^b^b^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

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Disclosures

The authors have no financial conflicts of interest.

References

Supplementary figure 1. The 1F12 antibody and HLA-Cw3 tetramer bind KIR2DS2 and KIR2DL2, respectively. Human GL183+ CD4+ T cell clones from a donor homozygous for a KIR B-haplotype (lacking KIR2DL3) and expressing either KIR2DS2 (ref. 24, Fig. 5A, clone 6) or KIR2DL2 (ref. 24, Fig. 5A, clone 1) transcripts, but no other KIR, were stained with GL183-PE (KIR2DL2/KIR2DS2-specific), 1F12-FITC (19) or HLA-Cw3-APC tetramer. A control KIR- CD4+ T cell clone (top) was also stained. Black areas indicate staining with the specific reagents, while grey areas represent staining with the appropriate control reagents (IgG1-PE, IgG1-FITC, streptavidin-APC). Note that the 1F12 antibody gives some non-specific background staining, which was also seen on KIR-negative cells.
Supplementary 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 (Cw3^+/+, n=10) or not (Cw3^--/, n=17) for HLA-Cw3 were stained with antibodies to CD3, NK1.1, together with NKG2A-specific antibody 16a11 (18). Both (A) the frequency of NKG2A+ cells among NK cells and (B) the NKG2A mean fluorescence intensity (MFI) values are shown. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the total range of the measurements. MFI values were normalized as in Fig. 1B, and the results were analyzed using a 2-sided Student’s t-test (numbers are P values).