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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 NKR, 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-2Kb−/− and H-2Dd−/− 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: 000–000.

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 imply a major influence of HLA allotype on KIR repertoire, making responsive 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
Materials and Methods

Mice

Mice transgenic for a KIR B haplotype and on a mixed (C57Bl/L6 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 (15). The presence and integrity of the KIR locus was checked after every backcross by karyotyping. C57BL/6 (B6) mice transgenic for genomic HLA-Cw*0304 construct (15) were a kind gift from Eric Vivier (Centre d’Immunoologie de Marseille-Luminy, Marseille, France). To obtain KIR or HLA transgenic mice on a H-2K<sup>b</sup>/H-2D<sup>b</sup> background, KIR<sup>+</sup> or HLA-Cw3<sup>+</sup> (clone CMG1L2), or control B6 mice were crossed with H-2K<sup>?</sup>/H-2D<sup>?</sup>/β<sup>m</sup>−/− mice, also on a B6 background (16). Mice with the desired phenotype (Kb Db β<sup>m</sup> KIR<sup>+</sup>, Kβ β<sup>m</sup> HLA-Cw3<sup>+</sup>, or Kβ Db β<sup>m</sup> were) selected from the F2 and used for further breeding to obtain K<sup>β−/−</sup>D<sup>−/−</sup> KIR<sup>+</sup>, K<sup>β−/−</sup>D<sup>−/−</sup> HLA-Cw3<sup>+</sup>, and K<sup>β−/−</sup>D<sup>−/−</sup> mice. To avoid integration artifacts, mice heterozygous for the KIR and HLA transgenes were used for experiments: KIR<sup>+</sup>, HLA-Cw3<sup>+</sup>, and KIR<sup>+</sup> HLA-Cw3<sup>+</sup> mice, all on a K<sup>β−/−</sup>D<sup>−/−</sup> 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), 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*NGK2A<sup>+</sup> or KIR2DL2/S2*NGK2A<sup>−</sup>) and NK cells that did not stain with these Abs (e.g., KIR2DL2/S2*NGK2A<sup>−</sup>).
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 (Kβ, Dβ, 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 Kβ−/−/Dβ−/− spleen cells by KIR and HLA transgenic Kβ+/−/Dβ+/− 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 Kβ−/−/Dβ−/− and control CFSElow Kβ+/−/Dβ+/−/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 Kβ−/−/Dβ−/−/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 Kβ+/−/Dβ+/− cells...
was compared between NKG2A-depleted Kb⁻/⁻Dᵇ⁻/⁻/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, 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⁻/⁻Dᵇ⁻/⁻) 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^*0401-05) 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). 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 NK ligand involved.

In the Kb+/−/Db+/− 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 Kb+/−/Db+/− mice affected the expression of NKG2A, as well as the responsiveness of NKG2A+ NK cells. Importantly, Kb+/−/Db−/− mice transgenic for HLA-Cw3 only already rejected Kb+/−/Db−/− 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-2Db leader peptide MAPRPTLLL. The very similar HLA-Cw3 leader peptide VMAPRTLIL also binds Qa-1L and thereby induces a functional ligand for CD94/NKG2A in Kb+/−/Db+/− mice (31, 32). Thus, the recognition by CD94/NKG2A of HLA-Cw3 leader peptides bound to Qa-1 likely contributes to the education, selection, and function of the NK cell repertoire in HLA-Cw3 transgenic Kb+/−/Db+/− 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 Kb+/−/Db−/− 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 Kb+/−/Db+/− 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-Kb 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 KIR-deficient 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.

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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).