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*J Immunol* 2003; 171:915-923; doi: 10.4049/jimmunol.171.2.915

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Enhanced Recognition of Human NK Receptors After Influenza Virus Infection


The NK cell cytotoxic activity is regulated by both inhibitory and activating NK receptors. Thus, changes in the expression levels and in the affinity or avidity of those receptors will have a major effect on the killing of target cells. In this study, we demonstrate that the binding of NK-inhibitory receptors is enhanced after influenza virus infection. Surprisingly, however, no change in the level of class I MHC protein expression was observed on the surface of the infected cells. The increased binding was general, because it was observed in both the killer cell Ig-like receptor 2 domain long tail 1 and leukocyte Ig-like receptor-1. The increased binding was functional, was not dependent on the interaction with viral hemagglutinin-neuraminidase, was not dependent on the glycosylation site, and was not abolished after mutating the transmembrane or cytosolic portions of the class I MHC proteins. Confocal microscopy experiments showed increased binding of NK receptor-coated beads to infected cells expressing the appropriate class I MHC proteins. In addition, specific cell-free bead aggregates covered with class I MHC proteins were observed only in infected cells. We therefore suggest that the influenza virus use a novel mechanism for the inhibition of NK cell activity. This mechanism probably involves the generation of class I MHC complexes in infected cells that cause increased recognition of NK receptors. The Journal of Immunology, 2003, 171: 915–923.

Natural killer cells are able to kill tumor and virus-infected cells (1). The NK cell-mediated killing is controlled by inhibitory, costimulatory, and lysis receptors (2). The main lysis receptors include the Nkp46, Nkp44, and Nkp30 proteins (3). We have recently demonstrated that the viral hemagglutinin (HA) protein of influenza virus and the HA-neuraminidase (HN) protein of Sendai virus (SV) can interact with both the Nkp44 and Nkp46 receptors and that this interaction leads to increased killing that can overcome the class I MHC-mediated inhibition (4, 5).

Three different types of inhibitory receptors inhibit NK cytotoxicity via interaction with class I MHC proteins (6), including the killer cell Ig-like receptor (KIR) family (6, 7), the C-type lectin family (7), and the Ig-like transcript/leukocyte Ig-like receptor (LIR) family (8). NK cell cytotoxicity can be also inhibited in a class I MHC-independent manner, e.g., via the homotypic interactions of CEACAM1 proteins (9, 10).

In this study, we investigated the involvement of the killer cell Ig-like receptor 2 domain long tail 1 (KIR2DL1) and the leukocyte Ig-like receptor-1 (LIR1) in the recognition of influenza virus-infected cells. The KIR2DL1 recognizes HLA-C proteins containing lysine at position 80 (11, 12), and the LIR1 receptor recognizes a broad spectrum of class I MHC proteins.

We show that the binding of KIR2DL1-Ig is specifically increased after infection with different strains of influenza viruses. This increased binding is not dependent on HN recognition nor on the cytoplasmatic and transmembrane portions of the HLA-Cw4 protein. The enhanced binding is general and was also observed with LIR1-Ig. The increased binding is functional and possibly results from the generation of complexes of class I MHC proteins after infection. Finally, we show that the killing of infected targets by NK cells is determined by the net result of the interactions between the various NK receptors present on NK cells and their ligands on the infected cells.

Materials and Methods

Cells and transfectants

The cell lines used in this work were the MHC class I-negative human EBV-transformed B cell line 721.221 (.221) and the murine thymoma BW cell line, which lacks the TCR expression. Human NK cells were isolated from PBL using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec, Auburn, CA). The generation of .221 transfectants expressing HLA-Cw3 (.221/Cw3), HLA-Cw3-GFP (green fluorescence protein) (.221/Cw3-GFP), HLA-Cw4 (.221/Cw4), HLA-Cw4-GFP (.221/Cw4-GFP), HLA-Cw6 (.221/Cw6), HLA-Cw7 (.221/Cw7), HLA-E (.221/E), HLA-G (.221/G), HLA-A201 (.221/A201), and HLA-B2705 (.221/B2705) proteins was previously described (11, 13). The generation of most of the mutated Cw4 proteins has also been previously described (14). The asparagine residue located in position 86 of the HLA-Cw4 protein (containing the glycosylation site) was mutated to glutamine by PCR using the following sense primer, 5’-CGCTCTGCTGTAGTACGCG-3’, and the antisense primer, 5’-CCCAAGCTTG-3’.

Generation of BW cells expressing the chimeric KIR2DL1ζ protein and the detection of IL-2 production

The generation of chimeric KIR2DL1ζ cDNA used for transfection into BW cells was performed by PCR amplification. The primers used for the PCR of the extracellular portion of KIR2DL1 were: 5’-CCCAAGCTTG-3’.
The KIR2DL1-Ig binding is specifically enhanced after SV infection

The results presented above were surprising, first because the enhanced KIR2DL1-Ig binding did not correlate with increased W6/32 binding and second because the increased binding did not result from interaction with viral HN. To further confirm our results, we used another assay, based on confocal microscopy, which enables direct visualization of the increased binding.

The .221 cells transfected either with CW3 or CW4 proteins attached to GFP (.221/CW4-GFP and .221/CW3-GFP, generated previously (13)) were incubated with beads coated with different proteins and imaged using a confocal microscope, as described in
Materials and Methods. Fig. 3A shows an increased number of KIR2DL1-Ig-coated beads bound to .221/Cw4-GFP cells infected with A/Sydney. This increased binding was specific, as no increase was observed when .221/Cw3-GFP cells were used (Fig. 3A). In addition, no change in the binding of the control CD7-Ig-coated beads to infected or noninfected .221/Cw4-GFP cells was detected. Similar results were obtained when other strains of influenza viruses were used (data not shown). Examples of the increased binding of KIR2DL1-Ig-coated beads to the infected cells are presented in Fig. 3, B and C. It can be clearly seen that the reduced binding of the KIR2DL1-Ig-coated beads to the noninfected cells was not due to the shortage of beads in the assays, as free beads can be seen in the picture (Fig. 3B). Interestingly, cell-free aggregates of KIR2DL1-Ig-coated beads were observed after the viral infection (Fig. 3, C and D) and not in uninfected cells (Fig. 3, B and D). It was previously reported that class I MHC proteins can be transferred from one cell to another (21). Strikingly, and in agreement with these observations, it can be seen that the clustered KIR2DL1-Ig-coated beads are covered with Cw4-GFP (red color in Fig. 3E). The aggregate formation was specific, as no KIR2DL1-Ig aggregates were observed in infected or noninfected Cw3-GFP cells and no aggregates were formed when the control CD7-Ig-coated beads were used (data not shown and Fig. 3A). It can, therefore, be strongly suggested that the class I MHC molecules derived from the infected cells are found in certain complexes, which induced the specific aggregate formation after viral infection.

The increased KIR2DL1 binding to the infected .221/Cw4 cells resulted in increased functional activity

To test the functional relevance of the above observations, mouse BW cells were stably transfected with a chimeric molecule composed of the extracellular portion of KIR2DL1 fused to mouse ζ-chain, as described in Materials and Methods. Coincubation of .221/Cw4, but not of .221/Cw3 cells, with BW/KIR2DL1ζ cells led to the secretion of mouse IL-2, as measured by ELISA, thus indicating that the system is specific (Fig. 4). In agreement with our binding results, incubation of BW/KIR2DL1ζ cells with .221/Cw4 cells infected with influenza A/Moscow, A/Beijing, or A/Sydney resulted in an increase in IL-2 secretion compared with the uninfected .221/Cw4 cells (OD at 650 nm 0.862, 0.836, 0.66, and 0.478, respectively) (Fig. 4). This increased secretion was the result of KIR2DL1ζ interaction with HLA-Cw4, as preincubation of the BW/KIR2DL1ζ cells with the anti-KIR2DL1 mAb HP-3E4 abrogated IL-2 secretion (Fig. 4). No significant secretion of IL-2 was observed when BW/KIR2DL1ζ cells were incubated with infected or uninfected .221/Cw4 cells.
noninfected .221/Cw3 cells or when cells were incubated with untransfected BW cells (Fig. 4 and data not shown). No significant change in the IL-2 secretion was observed when the control 12E7 mAb directed against CD99 protein was added (data not shown).

The tail and transmembrane portions of HLA-Cw4 are not specifically involved in the increased KIR2DL1-Ig binding to infected cells

We have previously shown that the tail of the HLA-C protein is involved in the inhibition of NK killing by an unknown NK-inhibitory receptor (22). One of the possible explanations for the increased KIR2DL1-Ig binding to the infected cells might be that other proteins are bound via the tail or the transmembrane portion of HLA-Cw4 molecule in the infected cells and that these proteins interact with the KIR2DL1 protein. Similar mechanisms of specific binding of the HIV nef protein to HLA-A and HLA-B, but not to HLA-C proteins, have previously been reported (14). The following HLA-Cw4 chimeric proteins were therefore generated. The extracellular portion of the HLA-Cw4 protein and the cytoplasmic tail of HLA-B27 were fused in frame. The extracellular portion of HLA-Cw4 protein and the cytoplasmic tail of HLA-A201 were fused in frame. The extracellular portion of HLA-Cw4 protein was fused in frame to the transmembrane portion and cytoplasmic tail of HLA-A201. Transfected cells expressing the various chimeras were infected with the various influenza virus strains and then tested for KIR2DL1-Ig and W6/32 binding. Importantly, increased KIR2DL1-Ig binding after infection was observed in all transfec-tants, whereas no significant change in the W6/32 staining was detected (data not shown). These results suggest no specific role for the transmembranal portion and cytoplasmic tail of HLA-C in the increased KIR2DL1-Ig binding to the infected cells. Similar results were observed when the cells were infected with A/Moscow, A/Beijing, and A/Sydney (data not shown).

It is also possible that the HA or HN molecules generate complexes of HLA-C proteins in the infected cells via the interaction between HA or HN and the sialic acid residues presented by HLA-C. To exclude this possibility, we mutated the sugar-containing Asn residue located in position 86 to Gln (N86Q) and abolished the only glycosylation site present in HLA-C. Increased KIR2DL1-Ig binding was observed in the SV-infected .221/Cw4-N86Q cells, indicating that the sugar-carrying residue 86 is not involved in the increased KIR2DL1 recognition of the infected cells (data not shown). Similar results were observed when the .221/Cw4-N86Q cells were infected with A/Moscow, A/Beijing, and A/Sydney (data not shown).

Complexes of HLA-C proteins efficiently trigger IL-2 secretion from BW/KIR2DL1Δ cells

The above results demonstrate that the increased KIR2DL1 binding to the infected cells is functional, not dependent on HN interaction, and not abolished after exchanging the transmembrane or the cytosolic portions of HLA-C proteins. The mechanism controlling the increased binding is unclear. It is possible that proteins other than HLA-C are involved in such interactions; however, immunoprecipitation experiments performed on the infected cells showed no significant existence of additional protein bands, other than HLA-Cw4 (data not shown). Alternatively, it is possible that complexes of HLA-C are formed in the infected cells, via unknown mechanisms, and that the KIR2DL1 receptor that binds HLA-Cw4 with very fast on and off rates (23) recognizes HLA-C with increased avidity after infection with the influenza viruses. This increased avidity cannot be observed when high affinity interactions are formed, for example, the interactions between the W6/32 mAb and HLA-Cw4. Indeed, aggregates of KIR2DL1-Ig-coated beads covered with Cw4-GFP were specifically observed in the infected cells only (Fig. 3). This suggests that the class I MHC proteins are found in certain complexes in the infected cells. To directly demonstrate that complexes of HLA-C can efficiently trigger the KIR2DL1 receptor, .221/Cw4 cells were preincubated with increasing concentrations of W6/32 mAb, which is known not to block the KIR2DL1/Cw4 interaction (24). Indeed, increased IL-2 secretion was observed when .221/Cw4 cells were incubated with increased concentrations of W6/32 mAb (Fig. 5A). This increased secretion was the result of the generation of HLA-Cw4 complexes, as incubation with W6/32 mAb in the form of Fab did not augment secretion (Fig. 5A). The controls, 12E7 mAb or 12E7 in the form of Fab, had no effect (Fig. 5A).

Next, we tested the effect of W6/32 mAb with infected .221/Cw4 cells. If our assumption is correct and the HLA-Cw4 proteins are found in complexes in the infected cells, then lower amounts of W6/32 mAb would be needed for the induction of IL-2 secretion from BW/KIR2DL1Δ cells. Indeed, increased IL-2 secretion was
observed when low concentrations of W6/32 (50–200 ng/ml) were incubated with .221/Cw4 cells infected with A/Moscow, whereas no significant change in the IL-2 secretion was observed when the same W6/32 concentrations were incubated with uninfected cells (Fig. 5B). No significant changes in IL-2 secretion were observed when the parental BW cells were used or when BW/KIR2DL1/H9256 cells were incubated with infected or uninfected .221/Cw3 cells (data not shown). Similar results were obtained when .221/Cw4 cells were infected with other strains of influenza virus (data not shown).

The killing of .221/Cw4-infected cells is influenced by the balance between inhibitory and activating receptors

The NK cells’ killing activity is controlled by a balance between inhibitory and stimulating signals. We have previously shown that the interaction between NKp46, NKp44, and viral HA or HN can overcome the class I MHC-mediated inhibition in some infected cells (5). In this study, we demonstrate that the binding of the inhibitory KIR2DL1 is increased after infection. We therefore tested what the net result of the interaction between influenza-infected cells and NK cells would be by using various NK clones and bulk NK cultures derived from different healthy donors. NK cells were assayed against infected or noninfected .221 and .221/Cw4 cells. In general, when no inhibition was observed, infection with the various influenza strains had no effect (see, for example, Fig. 6A). Three different NK phenotypes were observed when NK cells expressing KIR2DL1 were used (Fig. 6, B–D). 1) No significant change in the inhibition was observed in 51% of the clones (for example, see Fig. 6B); 2) complete or partial abolishment of the inhibition was observed in 40% of the clones (for example, see Fig. 6C); and 3) enhancement of the inhibition was observed in 9% of the clones (for example, see Fig. 6D). Similar results were obtained when other virus strains were used (data not shown). Thus, the killing of infected cells is determined by the NK receptor repertoire and can vary significantly among different NK clones.

The specific recognition of LI1-Ig is enhanced after infection with various strains of influenza viruses

The LIR-Ig was used to test whether the effect observed with KIR2DL1 is general and whether infecting cells with the various influenza strains would result in the increased recognition of other NK-inhibitory receptors. The LIR1 was used for two main reasons: its broad recognition of various class I MHC proteins (25) and the fact that the binding site of LIR1 on class I MHC is different from...
that of KIR2DL1 (20, 24, 26). The .221, .221/E, .221/G, .221/A201, .221/B2705, .221/Cw3, .221/Cw4, or .221/Cw6 cells were infected with various strains of influenza viruses, and the binding of KIR2DL1-Ig, LIR1-Ig, and CD7-Ig proteins was analyzed. In agreement with the above results, no significant changes were observed in the levels of class I MHC protein expression on infected vs noninfected cells detected by staining with W6/32 mAb directed against class I MHC or by MEM-E/06 mAb directed against HLA-E (data not shown). In agreement with the above observations (Fig. 1), specific increased binding of the KIR2DL1-Ig protein was observed to .221/Cw4 and .221/Cw6 cells infected with the different influenza viruses, while no KIR2DL1-Ig binding was observed to infected or noninfected .221 cells or .221 cells expressing HLA-E, -G, -A201, -B2705, and Cw3 proteins (Fig. 7A).

Similar results were obtained with LIR1-Ig fusion protein. Increased binding of LIR1-Ig was observed in all cells that were specifically recognized by the receptor, including .221/G (~2-fold), .221/A201 (4.5- to 22-fold), .221/B2705 (2- to 3.5-fold), .221/Cw3 (4- to 8-fold), and .221/Cw6 (2- to 5-fold) (Fig. 7B). In contrast, no binding of LIR1-Ig was observed to infected or noninfected .221, .221/E, or .221/Cw4 cells (Fig. 7B). No binding of the control CD7-Ig was observed in any of the cells tested (data not shown).

To further confirm our results, we used confocal microscopy experiments to image the recognition between the LIR1-Ig-coated beads and the various transfectants.

**Discussion**

We have previously shown that both the NKp46 and the NKp44 receptors, but not the NKp30 receptor, interact with the HN or HA proteins present on infected cells, leading to enhanced NK-mediated killing (4, 5). In this study, we demonstrate a specific increased binding of KIR2DL1 and LIR1 to infected cells expressing the appropriate ligands (Figs. 1, 7, and 8). The specific increased binding to the infected cells was detected by three different assays, including Ig fusion proteins (Figs. 1 and 7), BW cells transfectected with KIR2DL1 (Fig. 4), and confocal microscopy (Figs. 3 and 8).
Surprisingly, the increased binding to the infected cells was not accompanied with increased class I MHC expression (Fig. 1 and data not shown). It is unlikely that another protein expressed on the cell surface binds to class I MHC proteins and to KIR2DL1 or LIR1 as well, because the increased binding of KIR2DL1 and LIR1 after influenza infection is specific. In addition, swapping of the HLA-Cw4 transmembranal or cytosolic portions with other class I MHC proteins had no effect (data not shown). Finally, no significant additional protein bands, other than HLA-Cw4, were observed in immunoprecipitation experiments performed on infected cells (data not shown).

One possible explanation for the increased KIR2DL1 and LIR1 binding is the generation of class I MHC protein complexes in the infected cells. NK receptors bind class I MHC proteins in very fast on and off rates (23, 24). Therefore, it is possible that when complexes of class I MHC proteins are formed in the infected cells, increased binding avidity of the various NK receptors would be observed. This increased avidity cannot be observed in cases when high affinity interactions are formed, such as the interactions between W6/32 mAb and the various class I MHC proteins. Similar observations were reported by Fan et al. (27), showing higher binding avidity of KIR2DL1 for HLA-C when artificially made dimmers of KIR2DL1 were used. Indeed, artificially formed complexes of HLA-Cw4 generated with the intact W6/32 mAb enhanced the IL-2 secretion from BW/KIR2DL1α cells (Fig. 5). In addition, low concentrations of W6/32 mAb were needed to jump start the IL-2 secretion from BW/KIR2DL1α cells, suggesting that the complexes of HLA-Cw4 already existed in the infected cells (Fig. 5B). Furthermore, aggregates of KIR2DL1-Ig-coated beads covered with HLA-Cw4-GFP were observed only in the infected cells (Fig. 5B). As previously reported (21), such aggregates might result from the transfer of clustered HLA-Cw4 proteins from the infected cells to the coated beads.

The class I MHC complexes might also affect the binding avidity of the TCR and therefore might increase the lysis of viral-infected cells. However, TCR-MHC complexes have much slower off and on rates than that of the NK receptors (23). In addition, the formation of soluble class I MHC complexes in the infected cells might inhibit the killing of NK cells, as previously demonstrated (28), and block CTL killing.

The activity of NK cells is balanced by inhibitory and killing receptors (2), and killing will occur only if the killing signals are dominant. Indeed, we observed different NK-killing phenotypes of infected cells that probably represent the different NK receptor repertoire on the various clones (Fig. 6). It is possible that in the
beginning of the infection, when NK cells are found in low numbers and are not activated, the increased binding of the inhibitory receptors to the infected cells gives the virus a certain advantage. The virus, however, has to be cleared from the body. Therefore, later on, when NK cells are activated, the recognition of the infected cells by NK-activating receptors such as NKp44, NKp46, NKG2D, and other short tail NK receptors would counteract immunosurveillance (29, 30), including manipulation of MHC class I or MHC class I-like protein expression (25, 31–33). The immune system, in contrast, is trying to eliminate the virus. The work presented in this study demonstrates a similar situation with regard to influenza virus infection. NK cells try to eliminate the virus-infected cells via several mechanisms, among which are the interactions of NKp44 and NKp46 with viral HA and HN (4, 5). The influenza virus, in contrast, modulates NK activity via the increased binding of the NK receptors, probably due to the formation of class I MHC complexes.

Acknowledgments
We thank Dr. M. López-Botet (CEXS-Immunologia, Universitat Pompeu Fabra, Barcelona, Spain) for providing the mAb HP-3E4, Dr. A. Bernard (Hôpital de L’Archet, Nice, France) for providing the mAb 12E7, Dr. M. Tarshish (Biological Services, Hebrew University-Hadassah Medical School, Jerusalem, Israel) for his excellent assistance in the confocal microscopy experiments, Dr. A. Ben-Yehuda and Dr. Y. Barenholz for providing the anti-virus sera, and Dr. Václav Høřejší (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic) for providing the mAb MEM-E/06.

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FIGURE 8. The binding of LIR1-Ig-coated beads is specifically increased after viral infection. A/Sydney-infected or noninfected 221 cells transfected with HLA-E (A), HLA-G (B), HLA-A201 (C), HLA-Cw3 (D), HLA-Cw4 (E), HLA-Cw6 (F), and HLA-B2705 (G) were incubated with beads coated with LIR1-Ig protein and imaged using a confocal microscope. Figure shows representative of numerous cells in two independent experiments.


