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Recognition of HLA-Cw4 but Not HLA-Cw6 by the NK Cell Receptor Killer Cell Ig-Like Receptor Two-Domain Short Tail Number 4

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NK cells are cytotoxic to virus-infected and tumor cells that have lost surface expression of class I MHC proteins. Target cell expression of class I MHC proteins inhibits NK cytotoxicity through binding to inhibitory NK receptors. In contrast, a similar family of activating NK receptors, characterized by the presence of a charged residue in their transmembrane portion and a truncated cytoplasmic tail, augment lysis by NK cells when ligated by an appropriate class I MHC protein. However, the class I MHC specificity of many of these activating NK receptors is still unknown. Here, we show enhanced lysis of HLA-Cw4 but not HLA-Cw6-expressing cells, by a subset of NK clones. This subset may express killer cell Ig-like receptor two-domain short tail number 4 (KIR2DS4), as suggested by staining with various mAb. It is still possible, however, that these clones may express receptors other than KIR2DS4 that might recognize HLA-Cw4. Binding of KIR2DS4-Ig fusion protein to cells expressing HLA-Cw4 but not to those expressing HLA-Cw6 was also observed. The binding of KIR2DS4-Ig to HLA-Cw4 is weaker than that of killer cell Ig-like receptor two-domain long tail number 1 (KIR2DL1)-Ig fusion protein; however, such weak recognition is capable of inhibiting lysis by an NK transfectant expressing a chimeric molecule of KIR2DS4 fused to the transmembrane and cytoplasmic portion of KIR2DL1. Residue α14 is shown to be important in the KIR2DS4 binding to HLA-Cw4. Implications of the role of the activating NK receptors in immunosurveillance are discussed. The Journal of Immunology, 2001, 166: 7260–7267.

Natural killer cells that belong to the innate immunity system, as well as CTL that are part of the acquired immune response, are able to destroy foreign or infected tissue (1). In contrast to CTL, which are activated in the presence of class I MHC molecules and an appropriate specific peptide, one well-defined function of NK cells is the lysis of target cells deficient in expression of MHC class I proteins (2, 3).

Recognition of polymorphic determinants on HLA molecules by human NK cell-inhibitory receptors is mediated by three types of class I MHC-binding receptors (reviewed in Ref. 4). The Ig-like transcript 2 receptor, a member of the Ig-like transcript family of receptors (5), is able to bind to distinct class I MHC protein and to deliver an inhibitory signal (6). The C-type lectin complex CD94/NKG2A can deliver an inhibitory signal on binding to HLA-E (7, 8). Finally, members of the Ig superfamily of receptors, in particular the 58-kDa and 70-kDa NK-inhibitory receptors, containing two and three domains, respectively, can inhibit NK cell cytotoxicity when binding to the appropriate class I MHC molecules (9–11). In contrast, related 50-kDa receptors associated with DAP12 (12) augment NK cell cytotoxicity (13) and T cell proliferation (14). However, the class I MHC molecules recognized by many of these activating NK receptors and functions of activating NK receptors are currently poorly defined. Due to the possible expression of a multitude of NK receptors on any given NK cell, it is difficult to define the class I MHC-binding specificity of any given NK receptor using cellular assays with peripheral NK cells. One example of such difficulty is the analysis of the binding of killer cell Ig-like receptor two-domain short tail number 4 (KIR2DS4, also known as CL 39 or NKAT8) to class I MHC molecules. The expression of KIR2DS4, as well as other NK receptors, was reported on a CD4+ T cell clone (14). Enhanced proliferation was observed when this T cell clone (named TANK-1) was incubated with superantigen-coated 721.221 cells expressing Cw4 or Cw7 class I MHC proteins, but not with Cw3 or Cw6 (14). In contrast, KIR2DS4 has been postulated to interact with HLA-Cw3 (15), but again, this suggestion was based on cellular assays using NK cells expressing many NK receptors.

Here, we show binding of the KIR2DS4-Ig to HLA-Cw4, but not to HLA-Cw6, although both alleles belong to the same allo-type-specific group characterized by the presence of K30 (16). We demonstrate that the W residue at position 14 of HLA-Cw4 is important in the binding of KIR2DS4 to HLA-Cw4. The binding of KIR2DS4-Ig to HLA-Cw4 was weak compared with the killer cell Ig-like receptor two-domain long tail number 1 (KIR2DL1)-Ig binding to the same HLA-C molecules. However, it was still functionally significant in that it was sufficient to inhibit the lysis of Cw4-expressing target cells by YTS cells (an NK tumor line) expressing the chimeric protein of KIR2DS4 fused to the transmembrane and cytoplasmic tail portion of KIR2DL1 (YTS/KIR2DS4*) and to cause enhancement of lysis by NK cells expressing KIR2DS4.

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0022-1767/01/$02.00 3 Abbreviation used in this paper: KIR2DS4, killer cell Ig-like receptor two-domain short tail number 4; KIR2DL1, killer cell Ig-like receptor two-domain long tail number 1. Other moieties are similarly abbreviated.
Materials and Methods

Cells and mAb

The cell lines used in this work are the class I MHC-negative human cell line 721.221 (17) and the YTS NK tumor line (a kind gift from Dr. Eshhar, The Weizmann Institute, Rehovot, Israel). Point mutation in HLA-Cw4 and HLA-Cw6 cDNA was performed by PCR. Transfection of 721.221 cell lines with the cDNA of various class I MHC molecules (wild-type and mutants) was performed as previously described (16). NK cell lines and clones were isolated from PBL using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec, Auburn, CA). NK cell lines were grown in culture as previously described (16).

mAb used in this work were mAb W632, G46-2.6, and HP1F7 directed against class I MHC molecules. mAb HP3E4 (18) and EB6 (Immunotech, Westbrook, ME) are both directed against KIR2DL1. The HP3E4 and HP1F7 mAb were a kind gift from M. Lopez-Botet (Hospital de la Princesa, Madrid, Spain). The anti-CD99 mAb 12E7, used as a control, was a kind gift from A. Bernard (Hôpital de L’Archet, Nice, France).

Cytotoxic assays

The cytotoxic activity of YTS cells and NK cells against the various targets was assessed in 5-h ³¹⁵S release assays as described previously (16). In experiments where mAb were included, the final mAb concentration was 10 µg/ml, or 1/2 dilution in cases where the mAb were used as tissue culture supernatants. In all presented cytotoxic assays, the spontaneous release was <15% of maximal release.

Ig fusion proteins

The generation of KIR2DL1-Ig and CD99-Ig fusion proteins was previously described (19). The sequence encoding the extracellular portion of KIR2DS4 protein was amplified by PCR from cDNA isolated from human NK clones. The KIR2DS4 specific primers were: 5’-primer (including Hinc II site and Kozak sequence), 5’-CTTACGCTTGAGGCTCCCCACCTGTGGCGCTGCTCATGGTCATCATC-3’; 3’-primer (including BamHI site), 5’-CGGGATCCAGAACATGTAGGTGTCTGGG-3’. These PCR-generated fragments were cloned into the mammalian expression vector containing the Fc portion of human IgG1 as previously described (19). Sequencing of the constructs revealed that cDNA of all Ig fusion proteins were in frame with the human Fc genomic DNA and were identical with the reported sequences. The production of CD99-Ig, KIR2DL1-Ig, and KIR2DS4-Ig fusion proteins and the FACS staining procedure were previously described (19). All Ig fusion proteins used in this work run as a single protein band on nonreduced SDS-PAGE. The Ig fusion proteins were routinely tested on SDS-PAGE for protein degradation.

Generation of YTS cells transfected with the chimeric protein containing the extracellular portion of KIR2DS4 fused to the transmembrane and cytoplasmic tail portion of KIR2DL1

For the expression of KIR2DS4 with the transmembrane and cytoplasmic tail portion of KIR2DL1, plasmid constructs were prepared that replaced an EcoRI-AgeI fragment of the KIR2DL1 cDNA with that of the KIR2DS4 cDNA. Sequencing of the constructs revealed that KIR2DL1 cDNA was in frame with the KIR2DS4 cDNA and was identical with the reported sequences. YTS cells were transfected with the cDNA encoding for the chimeric KIR2DS4/KIR2DL1 (KIR2DS4*) protein, as previously described (20, 21).

Results

721.221/Cw4 cells are protected from lysis by YTS cells expressing KIR2DS4* chimeric protein

cDNA encoding for KIR2DS4 was amplified by PCR from peripheral NK clones and cloned into the mammalian expression vector containing the Fc portion of human IgG1 to produce the KIR2DS4-Ig fusion protein. KIR2DS4-Ig was then used to stain 721.221 cells transfected with various class I MHC molecules. Strikingly, specific KIR2DS4-Ig binding was observed only to cells expressing the Cw4 class I MHC protein (data not shown). No binding was observed to 721.221 transfected either with Cw3 or Cw7, while little or no binding was observed to 721.221 cells transfected either with HLA-Cw6 or with HLA-Cw6 in which the cysteine at position 309 was replaced by tryptophan (Cw6/C309W) (Ref. 22 and data not shown). The KIR2DL1 binds to both Cw4 and Cw6 class I MHC molecules (reviewed in Ref. 4). Indeed, efficient binding of KIR2DL1-Ig, which was about 20 times higher than that of KIR2DS4-Ig binding, was observed to 721.221/Cw4 cells (data not shown). The expression of all class I MHC molecules on the surface of 721.221 cells measured by W632 binding was similar (data not shown). KIR2DS4-Ig fusion protein did not bind to 721.221 expressing HLA-A alleles (HLA-A2) or HLA-B alleles (B7, B8, B44, B2702, B2705, B58, and B73) or to 721.221 cells expressing nonclassical class I MHC proteins (HLA-E, HLA-G, and CD1d; data not shown).

Two main conclusions can be drawn from the above experiments. 1) The KIR2DS4-Ig protein specifically binds to 721.221/Cw4 but not to 721.221/Cw6 cells and thus can discriminate between the two alleles that belong to the same allotype group characterized by the presence of K80 (16). 2) The binding of KIR2DS4-Ig to 721.221/Cw4 cells is much weaker than that of KIR2DL1-Ig. Thus, the question arises as to whether the low level of binding between KIR2DS4 and 721.221/Cw4 is sufficient to induce cellular response. An NK tumor line, YTS, was infected with the cDNA encoding for KIR2DS4, KIR2DS2, and CD16 using the retroviral vector pBABE as previously described (20). Although surface expression of all receptors was detected using the mAb HP3E4, GL183, and 3G8 for KIR2DS4, KIR2DS2, and CD16, respectively, none of the transfectants was able to induce lysis in redirected killing assays against P815 cells using appropriate mAb (data not shown). This may be because the signaling pathway, which these receptors use to enhance NK cytotoxicity, is impaired in YTS cells. In contrast, inhibition of lysis of 721.221/Cw4 and Cw6 cells was observed when YTS infected with cDNA encoding for KIR2DL1 (YTS/KIR2DL1) were used (Ref. 20 and Figs. 2 and 3), thus indicating that the inhibitory signal cascade is functioning in YTS cells.

The functional differences between the inhibitory and activating NK receptors are made possible due to the different transmembrane and cytoplasmic tail portions that exist between the two receptors (reviewed in Ref. 4). A chimeric cDNA construct was therefore made that included the extracellular portion of KIR2DS4 cDNA fused in frame to the transmembrane and cytoplasmic tail portion of KIR2DL1 (KIR2DS4*), cDNA were inserted into the retroviral vector pBABE and transfected into the YTS cell line as previously described (20, 24). Six different transfectants were obtained expressing the KIR2DS4* chimeric protein. All transfectants were positively stained with HP3E4 mAb, but not with EB6 mAb (two representative clones, 1 and 2, are seen in Fig. 1). In contrast, YTS/KIR2DL1 cells were positively stained with both mAb (Fig. 1). No staining was observed to YTS or to YTS/MOCK cells (Fig. 1). Thus, mAb HP3E4 can recognize the extracellular portion of KIR2DS4.

We next tested whether incubation of YTS/KIR2DS4* with 721.221/Cw4 cells will result in inhibition of lysis. YTS/MOCK, YTS/KIR2DL1, and YTS/KIR2DS4* were incubated with [³¹⁵S]methionine-labeled target cells in 5-h killing assays (Fig. 2). YTS/MOCK lysed all target cells tested to the same extent (Fig. 2, top). Efficient inhibition of YTS/KIR2DL1 lysis was observed when cells were incubated with either 721.221/Cw4 or 721.221/Cw6 cells in all E/T ratios tested (Fig. 2, middle). In contrast, inhibition of YTS/KIR2DS4* was observed only when target cells expressing HLA-Cw4 were used (Fig. 2, bottom). Correlating to the weak staining of 221/Cw4 by KIR2DS4-Ig compared to KIR2DL1-Ig (Fig. 6), the inhibition of YTS/KIR2DS4* was weaker than that observed with YTS/KIR2DL1 (Fig. 2). The results presented above suggest that the interaction between the KIR2DS4* and the HLACw4 molecules is sufficient to transmit the inhibitory signal. To test whether this inhibition is indeed the result of the binding of the extracellular portion of KIR2DS4 to the HLA-Cw4, mAb HP3E4, by guest on July 29, 2017 http://www.jimmunol.org/ Downloaded from
shown to bind KIR2DS4 in Fig. 1, was used to block the KIR2DS4 protein binding to HLA-Cw4. HP3E4 was indeed able to reverse the inhibition mediated by 721.221/Cw4 cells, whereas a control mAb 12E7 had no effect (Fig. 3).

Lysis of 721.221 cells expressing the Cw4 class I MHC protein is enhanced by NK clones expressing the KIR2DS4

The functional relevance of the above observation suggesting a specific interaction between KIR2DS4 and HLA-Cw4 was tested by using NK clones derived from various healthy donors. NK clones were generated as described in Materials and Methods. Two hundred fifty independent NK clones were tested in killing experiments against various class I MHC-transfected target cells. Three killing phenotypes were observed with regard to the killing of 721.221/Cw4 cells, of which three representatives are shown in Fig. 4. NK clone 3 was stained equally brightly with mAb EB6 and HP3E4. This clone probably expresses KIR2DS1, because activation of lysis was observed against 721.221/Cw4 and 721.221/Cw6 cells, and both EB6 and HP3E4 mAb blocked the enhancement of lysis (Fig. 4, top). Among the 250 NK clones tested, 5 (2%) were stained brightly with mAb HP3E4 and dimly with mAb EB6 (representative NK clone 6 is shown in Fig. 4, middle). This unique staining suggests the existence of KIR2DS4 (Fig. 1). Indeed, enhancement of lysis of 721.221/Cw4 cells only was observed when cells were incubated with all five clones mentioned. The addition of HP3E4 mAb blocked this enhancement, whereas EB6 or 12E7 mAb had no effect (representative NK clone 6 is seen in Fig. 4, middle). This is in agreement with our above observation suggesting that the ligand for KIR2DS4 is the HLA-Cw4 only. Finally, inhibition of lysis was observed when 721.221/Cw4 and 721.221/Cw6 cells were tested against NK clone 52. As expected, this clone was stained equally brightly with EB6 and HP3E4 mAb (Fig. 4, bottom), suggesting the existence of the KIR2DL1.

The W residue at position 14 of HLA-Cw4 is important in the binding to KIR2DS4

It has been demonstrated that peptides bound in the groove can influence recognition by NK receptors (23–25). Thus, the binding of KIR2DS4 to HLA-Cw4, but not -Cw6 could be the result of the
influence of the repertoire of peptides bound by -Cw4 vs -Cw6. All four HLA-C molecules (Cw3, Cw4, Cw6, and Cw7) analyzed here exhibit related peptide motifs, although each allelic product shows individual characteristics in their fine specificity of peptide binding (26). Alternatively, and perhaps more likely, given the limited effect of peptides on NK recognition (23, 24), it is possible that KIR2DS4 binds to the class I MHC proteins on a site that is different from the one identified for the inhibitory NK receptors KIR2DL1 and KIR2DL2 (16, 27, 28). Indeed, the KIR2DS4 inhibition could not be blocked with any of the anti-HLA mAb tested.
including HP1F7, G46-2.6, W632, and W632 F(ab')2 (data not shown). Moreover, comparison of the amino acid sequences of all known HLA-C proteins characterized by the presence of R80 revealed the existence of a pair of residues that can be found in HLA-Cw4 only (S11 and W14; Fig. 5A). The location of both residues is shown in Fig. 5C. Although S11 can also be found in the sequence of other HLA-C alleles that belong to the other allotype-specific group, characterized by the presence of N80 (e.g., Cw0101, Cw0102, Cw1402, and Cw1403), W14 is unique to HLA-Cw4 (Fig. 5A).

The involvement of S11 and W14 of HLA-Cw4 in the binding to KIR2DS4 was therefore tested using site-directed mutagenesis.
experiments in which residues S11 and W14 were replaced with the corresponding residues found in HLA-Cw6 (Cw4 S11A and Cw4 W14R). Mutations were performed by PCR, sequenced, and transfected into 721.221 cells. The expression of both Cw4 S11A and Cw4 W14R was assayed by staining with W632 mAb and was similar to that observed with the wild-type HLA-Cw4 (see legends to Fig. 6). Whereas binding of the KIR2DL1-Ig fusion was observed to all HLA-Cw4 proteins (wild-type and mutants), binding of KIR2DS4-Ig was observed to 721.221/Cw4 and 721.221/Cw4 S11A but not to 721.221/Cw4 W14R (Fig. 6), thus suggesting that residue W14 is important in the KIR2DS4 binding to HLA-Cw4.

To further demonstrate the importance of residue W14 in the binding to HLA-Cw4, we mutated by PCR residue R14 in HLA-Cw6 cDNA to W (Cw6 R14W). cDNA was sequenced and transfected into 721.221 cells. Whereas KIR2DL1-Ig fusion protein binds all HLA-Cw4 and HLA-Cw6 expressing cells, including wild types and mutants, binding of KIR2DS4-Ig was observed only to cells expressing HLA-C alleles containing the W14 residue, i.e., 721.221/Cw4 W14R, 721.221/Cw6 transfectant (these cells do not bind KIR2DS4-Ig (Fig. 6)). In contrast, activation of lysis was observed when 721.221/Cw4, 721.221/Cw6 R14W, or 721.221/Cw4 S11A cells were used (all cells bind KIR2DS4-Ig (Figs. 6 and 7A)). In all targets in which activation of lysis was observed, the activation could be blocked with HP3E4 mAb but not with EB6 mAb or 12E7 mAb.

**Discussion**

Three main conclusions can be drawn from our results. First, KIR2DS4 can bind to cells expressing HLA-Cw4 but not to cells expressing -Cw6 class I MHC molecules (Fig. 6) and that binding
Materials and Methods

of various targets by YTS/KIR2DS4* cells. The E:T ratio was 2.5:1. NK
KIR2DS4 to either Cw6 or Cw7 class I MHCproteins (29). This
with previous results demonstrating no binding of soluble

FIGURE 7. Mutation in residue 14 affects the lysis by NK cells ex-
ing NK receptors to class I MHC proteins. A soluble form of the
experiments have been reported analyzing the binding of activat-
ing NK receptors to class I MHC proteins. A soluble form of the
KIR2DS4-Ig but not by KIR2DL1-Ig (Fig. 6). The second
conclusion is that the binding of KIR2DS4-Ig to Cw4 is much
weaker than that observed with KIR2DL1-Ig (Figs. 2 and 6). Few
experiments have been reported analyzing the binding of activat-
ing NK receptors to class I MHC proteins. A soluble form of the
activating NK receptor molecule EB6ActI (KIR2DS1) bound very
weaker than that observed with KIR2DL1-Ig (Fig. 5B). Site-directed mutagenesis
the W14 residue in Cw4 to R abolished recognition by
KIR2DS4. This might account for the weak interaction observed
between KIR2DS4 and HLA-Cw4 (Figs. 2 and 6). In addition, the
the KIR2DS4 receptor based on the crystal structure of KIR2DL1
predicted that the surface potential of KIR2DS4 is dramatically
from that of KIR2DL2 (29). Moreover, the mAb EB6, which
recognizes an epitope important for the binding of
KIR2DL1 to HLA-Cw4 (33), does not recognize KIR2DS4 (Fig. 1).
This suggests that the site on these two NK receptors involved
in the binding to HLA-C is distinct. Residues at positions 67–70,
GPMM, found in KIR2DL2, which binds to HLA-Cw3, can also
be found in KIR2DS4 (Fig. 5B). Some of these residues were also
reported to be important in the HLA-C binding (28, 30, 32). Thus,
of seven amino acid residues that are found in the two regions of
KIR sequences reported to be important in HLA-Cw4 binding
(30–32), only three, namely, F45, N46, and M69, are found in
KIR2DS4. This might account for the weak interaction observed
between KIR2DS4 and HLA-Cw4 (Figs. 2 and 6). In addition, the
crystal structure of KIR2DL2 in complex with HLA-Cw3 was re-
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crystal structure of KIR2DL2 in complex with HLA-Cw3 was re-
cently solved (28). Sixteen contact residues were identified in the
KIR2DL2 protein interacting with HLA-Cw3 (Ref. 28 and Fig.
5B). Among these residues, three were unique for KIR2DS4 only,
namely, P71, V72, and A184. Site-directed mutagenesis analysis
of these residues would determine their involvement in the
KIR2DS4 binding to HLA-Cw4.

The third conclusion is that the low binding affinity between the
activating NK receptor KIR2DS4 and HLA-Cw4 is likely to be
sufficient for the function of this receptor (Figs. 2–4 and 7).
Accumulating evidence suggests that activating NK receptors can
function on binding to various class I molecules. A correlation
between the expression of p50 receptor stained with EB6 mAb and
enhanced cytotoxicity against Cw4-expressing target cells was ob-
served (13). Enhanced proliferation was observed when a T cell
clonel (TANK-1) positive for KIR2DS4 expression was incubated
with superantigen-coated 721.221 expressing Cw4 or Cw7 class I
MHC proteins, but not with Cw3 or Cw6 (14). This last observa-
tion (14) seems to be in contrast with the results presented here
demonstrating binding of KIR2DS4 to HLA-Cw4 only. However,
as the field developed and more sequence information became
available, a later analysis using additional PCR primers revealed
that the TANK-1 clone expresses mRNA for other NK receptors
(29). Using YTS cells transfected with KIR2DS4 and KIR2DS4-Ig
protein, it is now clear that KIR2DS4 binds to HLA-Cw4-express-
ing cells only.

Finally, a model for the interplay of NK-activating and inhibi-
tory receptors can be speculated. Inhibitory receptors bind with
higher affinity to MHC class I proteins but have a higher threshold
for the number of MHC molecules required for triggering signaling,
whereas the activating receptors bind with lower affinity and have a
higher threshold for triggering signaling. Thus, on recognition of
normal MHC expression, signaling through the inhibitory receptors
dominates, whereas on recognition of reduced class I MHC expres-
sion, e.g. in viral-infected cells, the activating NK receptors dominate
the NK cell response. Alternatively, it might be that the activating NK
receptors function only in cases where allele-specific down-regulation can be observed, e.g., after HIV infection (20) or tumor spread (reviewed in Ref. 34). In these situations, after down-regulation of HLA molecules for which specific inhibitory NK receptors are present, activating NK receptors may be triggered via other HLA molecules remaining on the cell surface.

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