MHC Class I-Independent Recognition of NK-Activating Receptor KIR2DS4

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Natural killer cells are capable of killing tumor and virus-infected cells. This killing is mediated primarily via the natural cytotoxicity receptors, including Nkp46, Nkp44, Nkp30, and by the NK2D receptor. Killer cell Ig-like receptors (KIRs) are mainly involved in inhibiting NK killing (inhibitory KIRs) via interaction with MHC class I molecules. Some KIRs, however, have been found to enhance NK killing when interacting with MHC class I molecules (activating KIRs). We have previously demonstrated that KIR2DS4, an activating KIR, recognizes the HLA-Cw4 protein. The interaction observed was weak and highly restricted to HLA-Cw4 only. These findings prompted us to check whether KIR2DS4 might have additional ligand(s). In this study, we show that KIR2DS4 is able to also interact with a non-class I MHC protein expressed on melanoma cell lines and on a primary melanoma. This interaction is shown to be both specific and functional. Importantly, site-directed mutagenesis analysis reveals that the amino acid residues involved in the recognition of this novel ligand are different from those interacting with HLA-Cw4. These results may shed new light on the function of activating KIRs and their relevance in NK biology. The Journal of Immunology, 2004, 173: 1819–1825.

In addition to the inhibitory KIR2Ds, activating KIR2Ds also exist, which activate NK killing after interacting with HLA-C-expressing cells (15, 20). These activating KIR2Ds resemble the inhibitory KIR2Ds in their extracellular domains, but contain a charged residue in their transmembrane domain (Lys) and also lack any known motifs in their short cytoplasmic tail (21). The activating receptors are associated with the signal transduction protein DAP12, which contains the ITAM motif in its cytoplasmic domain (22). The ligands that are recognized by the activating KIR2Ds are only poorly defined.

The KIR2DS4, an activating KIR2D, is a unique molecule. It contains a mixture of residues from both group 1 and 2 KIR2Ds. For example, KFN, instead of MFN or KFK in positions 44–46, and also expresses its own unique residues (23). We have recently shown that KIR2DS4 interacts with HLA-Cw4, but not with HLA-Cw6 (24). The observed interaction was of low affinity, compared with that of KIR2DL1, an inhibitory KIR2D (24). We suggested that the presence of group 2 residues in positions 67–70 (GPMM instead of SRMT) weakens the interaction between KIR2DS4 and HLA-Cw4. We further demonstrated that the W14 residue of HLA-Cw4 is critical for the recognition by KIR2DS4 (24).

These results were puzzling, first because KIR2DS4 could only interact in a very restricted specificity, and second because the interaction was of low affinity. We, therefore, investigated whether the KIR2DS4 might have an additional ligand(s). In this study, we demonstrate binding of KIR2DS4 to a non-class I MHC protein, expressed on melanoma cell lines and on a primary melanoma. The interaction observed was also functional, resulting in NK-mediated killing of the target cells expressing this, yet unidentified ligand.

Materials and Methods

Cells and mAb

The cell lines used in this work are the human B lymphoblastoid cell line 721.221 and other human melanoma lines, all of which are MHC class I negative. Transfection of 721.221 cells with the cDNA of various class I MHC molecules was performed by electroporation, as previously described (13). Primary NK cells (NK clones) were isolated from PBL using the
human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotech, Auburn, CA).

mAb used in this work are mAb W6/32, directed against MHC class I molecules; mAb HP5E4 (a kind gift from M. Lopez-Botet, DCEXS Universitat Pompeu Fabra, Barcelona, Spain), directed against KIR2DL1, KIR2DL1, and KIR2DS4 (24-26); and mAb EB6 (Immunootech, Westbrook, ME), directed against KIR2DL1 and KIR2DS1 (24, 25). The anti-CD99 mAb 12E7 (a kind gift from A. Bernard, Hôpital de L’Archet, Nice, France) was used as a control.

Cytotoxic assays
The cytotoxic activity of primary NK cells against the various targets was assessed in 5-h ³⁵S release assays, as previously described (13). In experiments in which mAb were included, the final mAb concentration was 5 µg/ml or 1/2 dilutions in cases in which the mAb were used as tissue culture supernatants.

Ig fusion proteins
The Ig fusion proteins used in this work are CD99-Ig, KIR2DL1-Ig, and KIR2DS4-Ig (wild type and mutants) (24). Briefly, the sequence encoding the extracellular portion of the receptor was amplified by PCR from cDNA isolated from human NK clones. These PCR-generated fragments were cloned into a mammalian expression vector, containing the Fc portion of human IgG1. The construct was transfected into COS-7 cells, and the protein produced was purified using protein G column. Point mutations in the cDNA coding for the KIR2DS4 were performed by PCR.

Flow cytometry
Cells were stained either with mAb or Ig fusion proteins. Second reagents were FITC-conjugated F(ab’2) goat anti-mouse IgG (ICN Biomedicals, Aurora, OH) and PE-conjugated F(ab’2) goat anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, PA), directed against mAb and Ig fusion proteins, respectively. mAbs were used at a final concentration of 3 µg/ml, and Ig fusion proteins at 100 µg/ml. The staining procedure was as follows: 30,000 cells were washed once in FACS medium (1× PBS, 0.5% BSA, 0.05% NaN₃) and then incubated in 100 µl of FACS medium containing either mAb or Ig fusion protein for 1 or 2 h on ice (4°C), respectively. Incubations were performed in 96 U-shaped plates (Nunc, Roskilde, Denmark). Cells were then washed twice in FACS medium and incubated on ice for 1 h with the appropriate second reagents. Following the incubation, cells were washed twice, resuspended in 200 µl of FACS medium, and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). For blocking experiments, Ig fusion proteins (1 µg/ml) were co-incubated with mAb (4 µg/l) for 4 h on ice (in a final volume of 100 µl of FACS medium), before the incubation with the cells. In cases in which proteolysis was performed, cells were washed once in RPMI 1640 (Sigma-Aldrich, Rehovot, Israel) and incubated for 30 min at 37°C in the presence of increasing concentrations of proteasome K (Sigma-Aldrich). Cells were then washed three times in RPMI 1640 supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) and stained, as described.

Results

KIR2DS4-Ig binds MHC class I-negative cells

We have previously shown that the binding of KIR2DS4-Ig to class I MHC molecules is of low affinity compared with that of KIR2DL1-Ig, and also further demonstrated that this interaction is highly restricted, i.e., KIR2DS4-Ig binds HLA-Cw4 only (24). We, therefore, speculated that KIR2DS4 might have an additional ligand(s). To test this hypothesis, various cell lines were stained with KIR2DS4-Ig (HeLa, HT-29, SW-480, Jeg3, SJS-A-1, 1106mel, HUVEC, COS-7, RPMI 8866, 721.221, Daudi, YTS, P815). Indeed, among the cell lines tested, the MHC class I-negative melanoma 1106mel (27, 28) (Fig. 1, left panel) was found to bind the KIR2DS4-Ig, whereas little or no binding of either KIR2DL1-Ig or CD99-Ig was observed (Fig. 1, right panel). The 1106mel cells were derived from a metastatic melanoma specimen obtained from a patient who had undergone tumor-infiltrating lymphocyte-based therapy (27). The immunotherapy protocol resulted in a more aggressive form of the disease that eventually caused patient death. When screened for cell surface expression, 1106mel cells were shown to be devoid of MHC class I molecules. This deficiency was found to be due to the apparent absence of β₂-microglobulin protein (27). As 1106mel cells were stained with KIR2DS4-Ig, we tested whether additional MHC class I-negative melanomas 1074mel, 1259mel (27, 28), and FO-1 (29) would also be recognized by KIR2DS4-Ig. In addition, we obtained 13 primary melanomas, directly derived from patients and screened for the expression of MHC class I. One of these melanomas, designated M-24, was found to be MHC class I negative. As can be seen in Fig. 1, left panel, none of the melanoma cells tested expressed MHC class I molecules. Importantly, binding of KIR2DS4-Ig to all melanomas was observed (Fig. 1, right panel). The levels, however, of KIR2DS4-Ig staining were variable. Relatively weak staining was observed to 1074mel and M-24 cells, moderate staining was observed to 1259mel and 1106mel cells, and the FO-1 cells were strongly recognized by KIR2DS4-Ig (Fig. 1, right panel). Thus, additional ligand(s) for KIR2DS4, other than MHC class I molecules (HLA-Cw4), exists on the various melanomas.

KIR2DS4 is involved in the killing of 1106mel cells

To test whether the specific KIR2DS4 binding observed to 1106mel cells is functional, NK killing assays were performed. We selected the 1106mel for further investigation due to several reasons: 1106mel cells are shown to be moderately killed by NK cells,
compared with 1259mel cells (28), and it is therefore possible that the blocking of one activating receptor will result in a significant effect. The staining of KIR2DS4 was higher on 1106mel cells compared with 1074mel or M-24 cells. Finally, there was a background staining of KIR2DL1 on FO-1 that might influence the activity of inhibitory receptors in an NK killing assay.

We have previously shown that the expression of KIR2DS4 on NK cells can be distinguished by the dim EB6 and the bright HP3E4 mAb staining (24). These results were confirmed by the Sixth International Leukocyte Typing Workshop (www.ncbi.nlm.nih.gov/prow/guide/68273991_g.htm), and by several publications (25, 26). Moreover, the KIR2DS4 cDNA was cloned from NK clones that were EB6 dim and HP3E4 bright (data not shown). We, therefore, stained >300 NK clones derived from different individuals with both mAb. Four clones that met with the above criteria were identified, and a representative clone is shown in Fig. 2, upper panel.

To verify that we indeed isolated KIR2DS4-positive clones, we then assayed the clones in killing against 721.221 and 221/Cw4 cells. As can be seen in Fig. 2, middle panel, the killing of 221/Cw4 cells was enhanced by all four clones compared with the killing of 721.221 cells (shown is one representative clone). This increased killing resulted from KIR2DS4 interaction with HLA-Cw4, as it was blocked by the HP3E4 mAb. We next assayed the same clones against 1106mel cells that were found to bind the KIR2DS4-Ig (Fig. 1, right panel). Importantly, the killing of 1106mel cells was markedly reduced when the HP3E4 mAb was included in the assays, whereas the control mAb 12E7 had no effect (shown is one representative clone in Fig. 2, lower panel). These results suggest that the binding of KIR2DS4 to 1106mel cells is functional and that 1106mel cells express a yet unidentified ligand for KIR2DS4.

As an additional control for the experiment, we also assayed the same target cells against KIR2DL1-positive NK clones. NK clones positive for KIR2DL1 were identified based on the similar staining of both EB6 and HP3E4 mAb (a representative clone is shown in Fig. 3, upper panel). As previously reported (24), such clones were inhibited by 221/Cw4 cells, and this inhibition could be reversed.
using the HP3E4 mAb (Fig. 3, middle panel). In contrast, however, to the enhanced killing of 1106mel cells observed with the KIR2DS4-positive clones, no difference in the percentages of killing of 1106mel cells was observed when the KIR2DL1-positive clones (preincubated with or without the various mAb) were used (Fig. 3, lower panel). The killing of 1106mel cells was consistently higher when KIR2DS4− NK clones were used. We do not as yet know what reasons account for this phenomenon. Comparison of NK-activating receptors such as Nkp46 and NKG2D revealed no apparent differences between KIR2DS4− and KIR2DS4+ NK clones (data not shown). It might be that additional, yet unidentified NK-activating receptors are primarily expressed on KIR2DS4− NK clones only.

To further confirm the functional results, we used the HP3E4 mAb to block the KIR2DS4-Ig binding to 1106mel cells. KIR2DS4-Ig was coincubated with the HP3E4 mAb and then incubated with 1106mel cells. As expected, a significant reduction in the KIR2DS4-Ig binding was observed after incubation with HP3E4 (Fig. 4). No effect was observed when other fusion proteins such as CD99-Ig and KIR2DL1-Ig were preincubated with HP3E4 (Fig. 4). Preincubation with the control 12E7 mAb had no effect on KIR2DS4-Ig binding to 1106mel cells (data not shown).

Different amino acid residues control KIR2DS4 binding to 1106mel and 221/Cw4 cells

The binding of KIR2DS4 to HLA-Cw4 is unique. It is of low affinity and of limited specificity (24). To characterize the amino acid residues involved in the binding of KIR2DS4 to HLA-Cw4 and to the unknown tumor ligand, we performed an extensive site-directed mutagenesis of potential contact residues. The amino acid residues that were selected for mutagenesis are those that were previously shown to participate either in KIR2DL1 or KIR2DL2 binding to their ligands (residues 44–45, 67–72, 184) (15–19) or those that are in proximity to such residues and differ between KIR2DS4 and KIR2DL1 (residues 50 and 190) or KIR2DL2 (residue 46) (24) (Fig. 5A). Residues 44–46, 50, and 67–72 are within the membrane-distant domain D1, and residues 184 and 190 are located in the membrane-proximal domain D2 (18, 19) (Fig. 5B). Site-directed mutagenesis was performed by PCR, and the various cDNA were sequenced, cloned in frame with the human IgG1, and produced in COS-7 cells, as previously described (24). As shown in Fig. 6, the binding of KIR2DS4-Ig to 221/Cw4 cells was abrogated when residues 44, 50, and 190, which are present in KIR2DL2, were replaced by the KIR2DL1 residues (K44M, H50R, N190K) or when the unique KIR2DS4 residues were replaced by residues present in both KIR2DL1 and KIR2DL2 (PV71,72QD, A184S). Mutating residues 67–70 that are present in KIR2DL2 to the corresponding residues in KIR2DL1 (GPMM67–70SRMT) resulted in an enhanced binding of KIR2DS4-Ig to 221/Cw4 cells, as well as to 221/Cw6 cells (which are not bound by the intact KIR2DS4-Ig) (Fig. 6). Thus, these particular residues determine the fine specificity of KIR2DS4 to HLA-Cw4 only. Mutating residue 46 present in KIR2DL1 to the corresponding residue in KIR2DL2 (N46K) slightly increased the binding of KIR2DS4-Ig to 221/Cw4 cells, while, remarkably, it had a dramatic effect in facilitating KIR2DS4-Ig binding to 221/Cw3 cells (which, like 221/Cw6 cells, are not bound by the intact KIR2DS4-Ig) (Fig. 6).

The binding of the various KIR2DS4-Ig mutants was then assayed on 1106mel cells (Fig. 7). Similar to the results obtained with 221/Cw4 cells, the binding of KIR2DS4-Ig to 1106mel cells was abolished when residues 44 (K44M) and 71–72 (PV71,72QD) were mutated. Also, a slight increase in the binding of KIR2DS4-Ig to 1106mel cells was observed when mutating residue 46 (N46K). In contrast, however, mutating residues 50, 67–70, 184, and 190 differently affected the binding of KIR2DS4-Ig to 1106mel cells compared with 221/Cw4 cells. Mutating residues 50 (H50R) and 190 (N190K) had little or no effect on the binding of KIR2DS4-Ig to 1106mel cells (Fig. 7), while mutating the same residues abrogated the binding of KIR2DS4-Ig to 221/Cw4 cells (Fig. 6). Mutating residues 67–70 (GPMM67–70SRMT) prevented KIR2DS4-Ig binding to 1106mel cells (Fig. 7), while the same mutation resulted in enhanced binding of KIR2DS4-Ig to 221/Cw4 and 221/Cw6 cells (Fig. 6). Inversely, mutating residue 184 (A184S) increased KIR2DS4-Ig binding to 1106mel cells (Fig. 7), while eliminating binding to 221/Cw4 cells (Fig. 6). Thus, we show in this study the molecular mechanisms controlling the unique and specific recognition of HLA-Cw4 by KIR2DS4. In addition, we show that KIR2DS4 uses different amino acid residues to recognize two different ligands: class I MHC ligand, HLA-Cw4, and an as yet unknown, non-class I MHC ligand.

![FIGURE 4. KIR2DS4-Ig binding to 1106mel cells is blocked by mAb HP3E4. Upper panel, 1106mel cells were incubated with or without various Ig fusions: no protein (thin line), CD99-Ig (dotted line), KIR2DL1-Ig (dashed line), KIR2DS4-Ig (bold line), followed by staining with PE-conjugated F(ab’)2 goat anti-human IgG. Lower panel, The various Ig fusions were preincubated with mAb HP3E4 and then used as above. Vertical line indicates border of background staining (no protein). The figure shows one representative experiment of three performed.](http://www.jimmunol.org/Downloadedfrom)
The 1106mel ligand is sensitive to proteolysis

The nature of the 1106mel ligand can be versatile. It can be a lipid, a carbohydrate, a protein, or a combination of all of these, i.e., a glycolipid, a proteoglycan, etc. Despite several attempts to immunoprecipitate the 1106mel ligand using the KIR2DS4-Ig reagent, no specific protein band was observed (data not shown). Therefore, to obtain more general information regarding the nature of the 1106mel ligand, we used the proteinase K enzyme, a highly reactive serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic, or hydrophobic amino acids, and therefore considered a nonspecific protease (30). Treatment of 1106mel cells with proteinase K eliminated the binding of KIR2DS4-Ig in a dose-dependent manner (Fig. 8). Thus, the novel ligand for KIR2DS4 that is expressed on the surface of 1106mel cells is most probably a protein.

Discussion

Several reasons may exist to explain the role of activating KIRs. 1) Allele-specific down-regulation: the particular class I MHC protein that interacts with a given inhibitory KIR is down-regulated,
while the same cells still express the particular class I MHC protein that interacts with the activating KIR. In such a situation, killing of these abnormal cells would be enhanced. 2) Peptide-specific interactions: it is possible that some, as of yet unidentified peptides will enhance the binding of activating KIRs in certain viral spread or tumor transformation, resulting in enhanced killing of these hazardous cells. However, no distinct observations were found to support these explanations. Additionally, it is still difficult to understand the very restricted specificity of KIR2DS4, an activating KIR, toward HLA-Cw4 only (24).

We, therefore, speculated that the activating KIRs might interact with non-class I MHC ligands. Indeed, we show in this study that KIR2DS4-Ig, a soluble form of the activating receptor KIR2DS4, binds to various MHC class I-negative melanoma cells. This binding was specific and functional (Figs. 1–4). Supporting our observation, a recent report (31) demonstrated that subjects with activating KIR2DS1 and/or KIR2DS2 genes are susceptible to developing psoriatic arthritis, but only when HLA ligands for their homologous inhibitory receptors, KIR2DL1 and KIR2DL2/3, are missing, thus suggesting that other, non-class I MHC ligands might interact with these activating KIRs.

Importantly, different residues control the binding of KIR2DS4 to both the MHC class I ligand, HLA-Cw4, and the ligand present on 1106mel cells (Figs. 5–7). Although few residues had the same effect when mutated, i.e., decreased binding of KIR2DS4-Ig to both 221/Cw4 and 1106mel cells (residues 44, 71–72) or slightly increased binding (residue 46), the majority of the critical residues (7 of 11) had a different effect on KIR2DS4-Ig binding to both ligands. Residues 50, 184, and 190 were critical in the KIR2DS4-Ig binding to 221/Cw4 cells, but either had no effect on the binding to 1106mel cells (residues 50 and 190) or even enhanced the binding (residue 184). The opposite effect is seen when mutating residues 67–70 (GPMM67–70SRMT). These residues enhanced KIR2DS4-Ig binding to 221/Cw4 cells, while decreasing the binding to 1106mel cells. We, therefore, suggest that residues 50, 184, and 190 are crucial to the specific interaction of KIR2DS4 with HLA-Cw4, and that residues 67–70 are important in the specific recognition of the non-class I MHC ligand. Furthermore, in agreement with other publications (15, 17), mutating residue 46 (within the context of residues 44–46) or residues 67–70 to the corresponding residues in KIR2DL2 (N46K) or KIR2DL1 (GPMM67–70SRMT) partially restored the capability of KIR2DS4 to bind HLA-Cw3 or HLA-Cw4/HLA-Cw6 molecules, respectively (Fig. 6). These results provide a molecular basis to explain the highly restricted manner in which KIR2DS4 binds its class I MHC ligand, HLA-Cw4. Finally, it seems as if residues 67–70 might play a dual role: first, they weaken the interaction with HLA-Cw4, while preventing the binding to HLA-Cw6; second, they control the specific binding to the non-class I MHC ligand present on 1106mel cells.

In an attempt to further characterize the 1106mel ligand for KIR2DS4, we treated the cells with proteinase K, a nonspecific protease. As was observed, such treatment reduced the KIR2DS4-Ig binding in a dose-dependent manner (Fig. 8). It, therefore, might be deduced that the newly detected ligand is a protein. Despite numerous trials, we were not able to immunoprecipitate a specific protein from 1106mel cells using the KIR2DS4-Ig as the precipitating agent (data not shown). This could be either because the interactions between KIR2DS4 and the unknown ligand are too weak to permit precipitations or because the ligand is expressed on 1106mel cells in low levels (Fig. 1, right panel).

The reasons that activating KIRs are expressed on NK cells are not completely understood. The results presented in this work...
bring a new perspective as to the function of the activating KIR. It would be interesting to test whether the activating KIRs might also recognize some viral ligands, and whether infection of a particular population would result in the acquisition of a particular activating KIR.

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**References**