Brief Definitive Report

A p70 Killer Cell Inhibitory Receptor Specific for Several HLA-B Allotypes Discriminates among Peptides Bound to HLA-B*2705

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Summary

Natural killer (NK) cells express a repertoire of killer cell inhibitory receptors (KIR) for major histocompatibility complex (MHC) class I molecules. KIR specificity for MHC class I can be broad, as in the case of a single p70 KIR that can recognize several HLA-B allotypes, including HLA-B*2705. On the other hand, recognition of MHC class I can also be highly specific, as in the case of NK clones that recognize HLA-B*2705 in a peptide-specific manner. Most NK cells express multiple KIR sequences. To determine whether the broad and specific types of HLA-B recognition by NK cells reflect the use of different receptors or a property of a single KIR we analyzed the recognition of HLA-B*2705 by the p70 KIR-11, known to recognize several HLA-B allotypes. Vaccinia virus–mediated expression of KIR-11 in NK clones resulted in inhibition by HLA-B*2705 molecules on wild type but not on target cells deficient in the transporter for antigen presentation (TAP). Two peptides (FRYNGLIHR and RRSKEITVR) loaded onto HLA-B*2705 molecules on TAP-deficient cells provided protection from lysis by NK cells expressing KIR-11 but three other B27-specific peptides did not. As the five peptides bound to HLA-B*2705 with similar stability, these data demonstrate that a single KIR specific for several HLA-B allotypes recognizes a subset of peptides bound to HLA-B*2705.

Human NK cells express inhibitory receptors specific for distinct HLA-A, -B, and -C molecules (1–3). As a result of interaction with HLA molecules on target cells, NK cells receive an inhibitory signal through these receptors that prevents lysis (reviewed in references 4–6). The killer cell inhibitory receptors (KIR) on NK cells comprise a family of molecules called p58 and p70 (2, 7) that belong to the Ig superfamily and are encoded by a family of genes on human chromosome 19 (8–10). Soluble recombinant forms of p58 KIR can directly bind to HLA-C molecules expressed on transfected cells (11). Expression of individual p58 KIR in NK clones using recombinant vaccinia viruses demonstrated that these receptors provide both specific recognition of HLA-C and the inhibitory signal that prevents target cell lysis (11). By use of the same vaccinia virus–mediated expression in NK clones, a p70 KIR was shown to recognize several HLA-B allotypes of the Bw4 serological group (11). Another p70 KIR was reported to bind to HLA-A3 (12). Most NK clones that express NKB1, a determinant shared by p70 KIR molecules reactive with mAb DX9 (6, 7), were inhibited by HLA-B*2705 (13). However, recognition of HLA-B*2705 by NK cells is complex in that several p70 molecules are reactive with DX9 and that recognition of HLA-B*2705 can also be mediated by receptors other than NKB1 (6, 7, 13).

Recognition of HLA-B*2705 by NK cells was reported to involve some degree of peptide specificity (14). A group of HLA-B*2705-specific NK clones recognized only one of four peptides bound to HLA-B*2705 molecules expressed by RMA-S or by T2 transporter for antigen presentation (TAP)-deficient cells whereas another group of HLA-B*2705-specific NK clones failed to recognize any of the four peptides (14). More recently, we have shown that NK clones can recognize additional unrelated peptides bound to HLA-B*2705 but not several other peptides (14a). All the peptides tested were known endogenous ligands of HLA-B*2705 molecules in human B cells.

One puzzle raised by these findings is that KIR appear to be both broadly reactive and very specific at the same time. On the one hand, a single p70 KIR (KIR-11 encoded by cDNA cl-11) recognized several HLA-B allotypes (HLA-B*2705 and HLA-B*5101) on target cells (11) and, on the other hand, NK clones specific for HLA-B*2705 displayed selectivity for peptides (14, 14a). Given that the p70 KIR are encoded by at least four different sequences (reviewed in reference 6) and that individual NK clones can express multiple KIR (3, 8, 11, 15), it was possible that different members of the p70 family accounted for these distinct specificities.

To test whether discrimination among peptides bound

to HLA-B*2705 could be achieved by a single KIR and whether peptide-specific recognition of HLA-B*2705 was a property carried by the same KIR that recognized other HLA-B allotypes, we combined two experimental approaches. First, HLA-B*2705 molecules on TAP-deficient cells were loaded with individual peptides and, second, NK clones that did not recognize HLA-B*2705 were infected with recombinant vaccinia viruses encoding KIR-11. We show here that KIR-11 can discriminate among peptides bound to HLA-B*2705 molecules on TAP-deficient cells.

Materials and Methods

NK Clones and Cell Lines. NK clones were generated from PBMC obtained by separation on Ficoll-Hypaque. PBMC were washed with ice-cold PBS containing 1% BSA and 5 mM EDTA, and incubated with anti-CD3 mAb conjugated to magnetic microbeads for 20 min at 4°C. After one wash PBMC were passed through a MACS® column (Miltenyi Biotec Inc., Auburn, CA). The flow-through fraction was collected and analyzed for purity by flow cytometry (<5% $CD3^+$ T cells). NK cells were cloned, expanded, and maintained as described (16). The human NK cell line NK-92 (gift of H.G. Klingemann, Terry Fox Laboratory, Vancouver, Canada) was cultured as described (17, 18). The TAP-deficient T2 cells transfected with HLA-B*2705 (T2-B27) and RMA-S transfected with the human β2-microglobulin gene and with the HLA-B*2705 gene (RMA-S-B27) have been described (19). These cell lines were maintained in Iscove's modified essential medium containing 10% FCS and 2 mM glutamine. RMA-S (expressing human β2-microglobulin) and T2-B27 were maintained in 0.5 mg/ml G418. RMA-S-B27 was maintained in 0.5 mg/ml G418 and 250 U/ml hygromycin B.

Antibodies and Immunostaining. The phenotype of NK cells was determined as described (16) with the anti-p58 mAbs EB6 and GL183 (20, 21) (gift from A. Moretta, University of Genova, Genova, Italy), and the anti-p70 DX9 (7) (gift from L. Lanier, DNAX Research Institute, Palo Alto, CA). Surface expression of HLA-B*2705 molecules and of influenza virus hemagglutinin H3 molecules was monitored with mAb ME.1 (American Type Culture Collection, Rockville, MD) and a pool of anti-H3 mAbs (gift from R. Webster, St. Jude's Children's Hospital, Memphis, TN), respectively. 2×10^5 cells were incubated for 30 min on ice with the primary mAb, washed in PBS containing 2% FCS, and stained with PE-conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed twice, and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, CA).

*HLA-B*2705-specific Peptides.* Peptides were synthesized and purified as described (22). Purity was confirmed by analytical reverse-phase HPLC and by mass spectrometry. To evaluate the ability of each peptide to bind HLA-B*2705 molecules expressed on RMA-S-B27 and on T2-B27 cells, the surface level of HLA-B*2705 was measured by flow cytometry, after a 24-h incubation at 25°C in the absence or presence of each B*2705-specific peptide. 5×10^5 cells were incubated with peptides added at two separate time points (onset of the culture and 12 h later) to reach a final concentration of 50 μM. To establish the stability of HLA-B*2705 surface expression induced by the specific peptides, RMA-S-B27 and T2-B27 cells were then harvested, washed at room temperature in PBS containing 2% FCS to remove unbound peptide, and divided into three aliquots. Two aliquots were incubated at 37°C, the remaining one placed on ice. Aliquots were removed after 1 and 3 h at 37°C and placed on ice. Surface levels of HLA-B*2705 were measured by flow cytometry using the mAb ME.1 as described above.

Vaccinia Virus Infections. NK clones or the cell line NK-92 were infected for 1.5 h with purified recombinant vaccinia viruses encoding KIR-11 (Vac-11) (11) or the influenza hemagglutinin H3 (Vac-H3) (construct D in reference 23) as reported (11). Under the conditions used >90% of the cells expressed surface KIR-11 (5–10 PFU/cell) and ≥80% of the cells expressed surface H3 molecules (10–30 PFU/cell). In all experiments two different doses of PFU/cell were used for each vaccinia infection.

Cytotoxicity Assay. Frozen NK clones were thawed and cultured for 3–5 d in NK medium (Iscove's containing 10% human serum, 2 mM glutamine, 50 U/ml rIL-2 [gift of Hoffmann-LaRoche, Nutley, NJ]) and 5% purified IL-2 (Schiapparelli Eni Diagnostic, Fairfield, NJ) before being tested in the ^{51}Cr-release assay. NK cells infected with vaccinia as described above, or uninfected were collected, counted, resuspended in Iscove's modified essential medium containing 10% FCS, 2 mM glutamine, 50 U/ml rIL-2, and plated in a final volume of 100 μl in V-bottomed 96-well plates. NK clones were tested at several effector to target ratios in duplicate wells. During the last 12 h of the incubation with peptide at 25°C, targets were labeled with sodium ^{51}chromate (100 μCi/well, Amersham Corp., Arlington Heights, IL). After two washes, cells were counted and resuspended at a final concentration of 2.5×10^4 cells/ml in RPMI 1640, 5% FCS, and 2 mM glutamine. 100 μl of the cell suspension were added to the NK clones and incubated in a 3-h ^{51}Cr-release killing assay (16).

Single Strand Conformation Polymorphism. First strand cDNA was synthesized from total RNA of human NK clones and of NK-92 as described (8). PCR primers and amplification of p58 cDNA have been reported (8). For amplification of p70 cDNA the forward and backward primers were GCCTCGAGGAGGACACACGT and GAGGGAGTTTTCTGTGGT, respectively. PCR amplification of p70 was as described (8) except that the annealing temperature was 65°C, instead of 62°C. Samples were electrophoresed on 6% nondenaturing polyacrylamide gels containing 10% glycerol, as described (8).

Results and Discussion

*HLA-B*2705 Molecules Loaded with Peptide FRYNGLIHR Inhibit NK Clones Expressing the p70 KIR-11.* HLA-B*2705 molecules on TAP-deficient cells loaded with peptide FRYNGLIHR inhibited lysis by some but not by other NK clones, even though all NK clones tested were inhibited by HLA-B*2705 on wild-type cells, such as C1R-B27 (14). To test whether KIR-11, known to recognize HLA-B*2705 and HLA-B*5101 (11), was able to recognize the peptide FRYNGLIHR bound to HLA-B*2705 molecules on TAP-deficient cells, two NK clones that did not recognize HLA-B*2705 were selected for vaccinia virus–mediated expression of KIR-11. These NK clones did not express p58 and p70 KIR reactive with mAbs EB6, GL183, and DX9, and they lysed C1R, C1R-B27, RMA-S, and RMA-S-B27 cells (data not shown). To control for nonspecific inhibition of lysis caused by vaccinia virus infection, target cell lysis by each NK clone was tested in three different conditions: with uninfected NK clones, with NK clones after infection by a control vaccinia virus encoding the influenza virus hemagglutinin H3 molecule (Vac-H3),

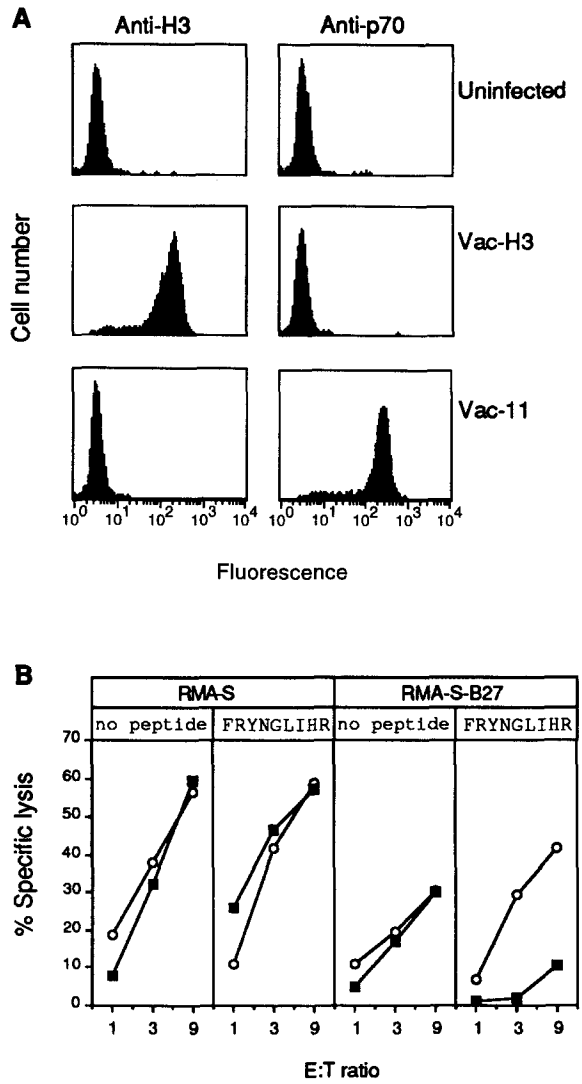

Figure 1. Expression of KIR-11 in NK clones confers recognition of HLA-B*2705 molecules loaded with peptide FRYNGLIHR. (*A*) Flow cytometry analysis of NK clone 5w-A06 either uninfected, infected with 30 PFU/cell of Vac-H3, or infected with 10 PFU/cell of Vac-11, as indicated. Cells were stained with anti-H3 mAb or anti-p70 mAb DX9. Identical results were obtained with NK clone 10w-A18. (*B*) NK clone 5w-A06 infected with Vac-H3 (*circles*) or Vac-11 (*squares*) was tested for its ability to lyse RMA-S (*left*) and RMA-S-B27 (*right*) cells. Target cells were preincubated at 25°C for 24 h in the absence or presence of peptide FRYNGLIHR, as indicated. Similar results were obtained in other experiments with this and another NK clone (10w-A18).

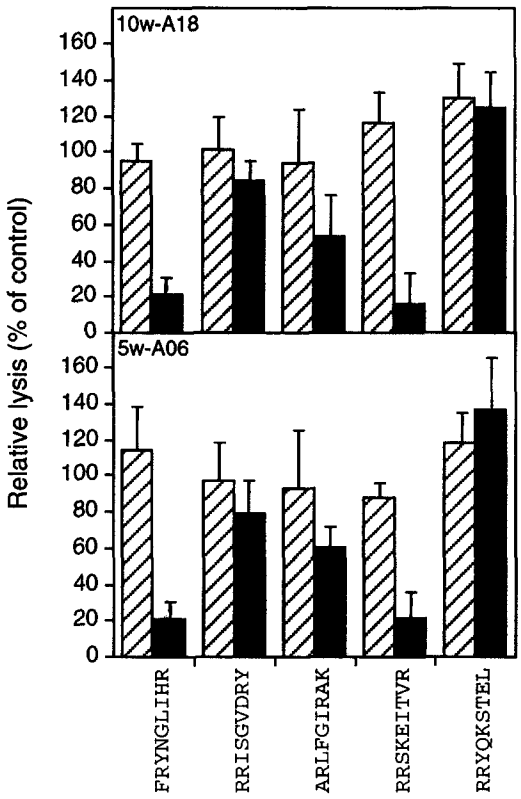

Figure 2. Discrimination among several peptides bound to HLA-B*2705 by NK clones expressing KIR-11. Lysis of RMA-S-B27 cells by the two indicated NK clones infected with Vac-H3 (*hatched bars*) or with Vac-11 (*filled bars*). Target cells were preincubated at 25°C for 24 h without or with the indicated peptides. The data are expressed as lysis of targets in the presence of peptide relative to the lysis of the same targets in the absence of peptide given as the average of two to five experiments for each peptide. The bars represent the SEM.

or by a vaccinia virus encoding KIR-11 (Vac-11). Infected NK clones expressed uniform surface levels of H3 or KIR-11 (Fig. 1 *A*). RMA-S cells were lysed by NK clones in all the conditions even after incubation with peptide FRYNGLIHR (Fig. 1 *B*). RMA-S-B27 cells were protected from lysis by NK clones only after incubation with peptide FRYNGLIHR and only after infection of NK clones by Vac-11 (Fig. 1 *B*). Therefore, expression of KIR-11 in NK clones resulted in specific recognition of peptide FRYNGLIHR in the context of HLA-B*2705 molecules expressed on target cells.

*Functional Transfer of KIR-11 into NK Clones Confers Recognition of a Subset of Peptides Bound to HLA-B*2705.* Five different B*2705-specific peptides, corresponding to endogenous peptides associated with HLA-B*2705 on normal cells (24), were tested for their ability to inhibit NK clones expressing KIR-11 (Fig. 2). In a separate study (14a), we showed that all five peptides caused a similar increase in surface HLA-B*2705 molecules on RMA-S-B27 cells and conferred to HLA-B*2705 molecules a similar stability at 37°C, but that only two of the five peptides (FRYNGLIHR and RRSKEITVR) provided protection from lysis by a panel of B27-specific NK clones. Vac-11 infection of NK clones resulted in the recognition of the same two peptides bound to HLA-B*2705 (Fig. 2). These two peptides did not provide protection from lysis by Vac-H3 infected NK clones and the other three peptides did not provide protection under any condition. These results demonstrate that KIR-11 can distinguish among HLA-B*2705 molecules loaded with different peptides and that expression of KIR-11 confers to NK clones specificity for at least two peptides that are unrelated by sequence. Therefore, recognition of different peptides in the context of

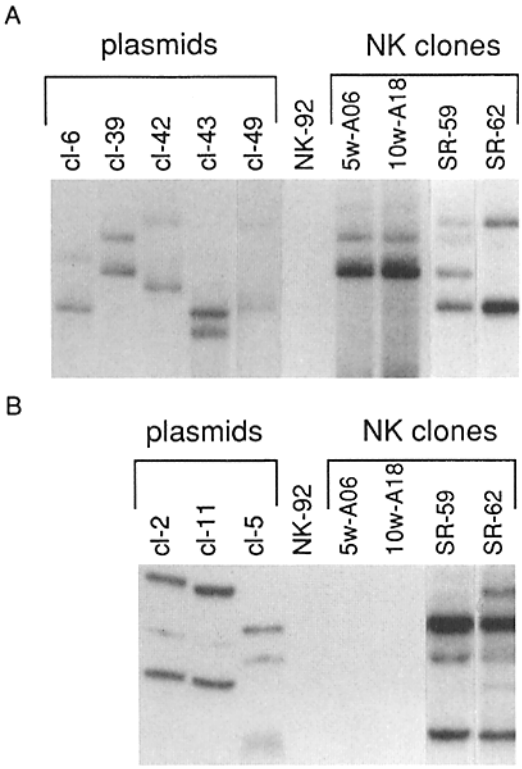

Figure 3. Expression of KIR mRNA in different NK cells. SSCP analysis of PCR amplification products derived from plasmids encoding p58 or p70 and from mRNA of the indicated NK cells. (*A*) SSCP pattern of p58 in the indicated NK cells. The picture was generated from different exposures of the same gel. (*B*) SSCP pattern of p70 in the indicated NK cells. The picture was generated from a single exposure of one gel.

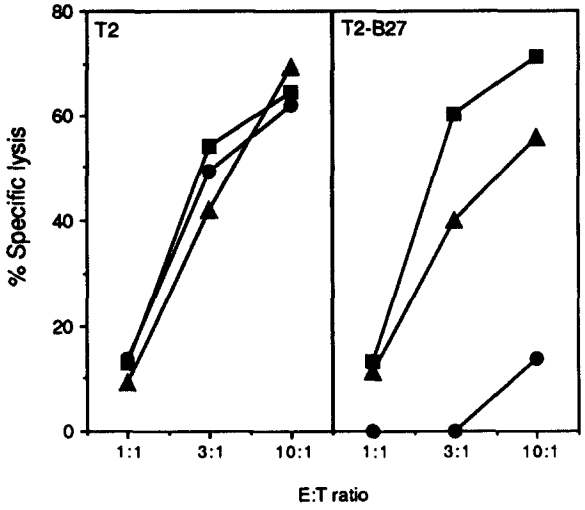

Figure 4. Expression of KIR-11 in the KIR-negative cell line NK-92 confers peptide-specific recognition of HLA-B*2705. NK-92 cells were infected with 10 PFU/cell of Vac-11. T2 and T2-B27 target cells were preincubated at 25°C for 24 h in the absence (*triangles*) or in the presence of peptide FRYNGLIHR (*circles*) or peptide RRYQKSTEL (*squares*). Similar results were obtained in two other experiments.

HLA-B*2705 is not mediated by a distinct KIR for each peptide but by a single KIR.

*Expression of KIR-11 in the Cell Line NK-92 Results in Peptide-specific Recognition of HLA-B*2705.* Human NK clones express at least one, and often several different KIR (3, 8, 15). The presence of multiple KIR in NK clones may complicate the assignment of specificity to a single KIR, even though the NK clones used in the vaccinia virus infections described above did not express molecules reactive with anti-KIR mAbs. The presence of mRNA encoding KIR was analyzed by single strand conformation polymorphism (SSCP) in the two NK clones (5w-A06 and 10w-A18) used in the infection experiments described above, in two control NK clones (SR-59 and SR-62) that express KIR, and in NK-92, an NK cell line (17) that does not express KIR reactive with available mAbs and that has been used to reconstitute KIR function (18).

NK clones 5w-A06 and 10w-A18 expressed only one p58 receptor (corresponding to cl-39) but no detectable p70 KIR (Fig. 3, *A* and *B*). NK clones SR-59 and SR-62 expressed several p58 and p70 receptors. In contrast, the NK-92 cell line did not detectably express any known p58 or p70 molecule. Therefore, NK-92 was infected with Vac-11 to determine whether KIR-11 could confer peptide-specific recognition of HLA-B27 in the absence of other known members of the KIR family. Lysis of the human TAP-deficient cells T2-B27 by NK-92 was more efficient than that of RMA-S-B27 (70 and 30% lysis, respectively, at an E/T ratio of 6, data not shown). Therefore, T2-B27 cells were used to test for peptide-specific recognition by NK-92 after vaccinia virus infections. As shown for NK clones, NK-92 cells expressed uniform surface levels of H3 and KIR-11 after infections with the relevant vaccinia viruses (not shown). T2-B27 cells were protected from lysis by NK-92 only after incubation with peptide FRYNGLIHR (Fig. 4). Peptide RRYQKSTEL did not provide protection, despite its ability to stabilize surface HLA-B*2705 molecules on T2-B27 cells (Table 1). The protective effect of peptide FRYNGLIHR was dependent on HLA-B*2705 molecules because it did not protect T2 cells. All cells were lysed indiscriminately by Vac-H3-infected

Table 1. *Stability of HLA-B*2705 Molecules on T2-B27 Cells*

	Hours at 37°C		
Peptide	0	1	3
None	84	50	42
FRYNGLIHR	119	103	100
RRYQKSTEL	145	101	101

T2-B27 cells were incubated at 25°C for 24 h in the absence or in the presence of peptide, washed, and incubated at 37°C for the indicated time. Surface levels of HLA-B*2705 were measured by flow cytometry using the B*2705-specific mAb ME.1. The data are expressed as median fluroescence intensity.

NK-92 cells (not shown). Thus, discrimination among peptides bound to HLA-B*2705 molecules was achieved by KIR-11 expressed in the cell line NK-92.

Conclusions. The combination of two experimental systems, TAP-deficient cells whose class I molecules can be loaded with defined peptides and reconstitution of KIR function by vaccinia virus-mediated expression in NK clones, made it possible to test the specificity of a single KIR for peptides bound to HLA-B*2705. The data clearly showed that a single p70 KIR discriminates among peptides bound to HLA-B*2705 molecules. Somewhat surprisingly, KIR-11 is the same receptor that inhibits NK cells upon interaction with different allotypes of the Bw4 subgroup (11). It will be interesting to test whether recognition of other HLA-Bw4 allotypes by KIR-11 is also influenced by peptide sequences. KIR-11 recognized HLA-B*2705 molecules loaded with two different peptides almost completely unrelated by sequence. This permissive peptide specificity of KIR-11 is quite distinct from the fine peptide specificity typically displayed by T cell receptors and from the lack of peptide specificity of the mouse NK inhibitory receptor Ly49A (25). These results suggest a different mode of interaction with MHC class I for KIR, Ly49, and TCR molecules.

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