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Killer Cell Inhibitory Receptors Specific for HLA-C and HLA-B Identified by Direct Binding and by Functional Transfer

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Summary

An inhibitory signal is delivered to natural killer (NK) cells and a subset of cytotoxic T cells upon recognition of HLA class I molecules on target cells. We demonstrate that soluble forms of killer cell inhibitory receptors (KIR) bind directly and specifically to HLA-C alleles on transfected cells. Furthermore, transfer of individual KIR into NK clones reconstituted recognition of HLA-C on target cells, leading to inhibition of lysis. Using such functional reconstitution, a related KIR that confers specificity for some HLA-B alleles was also identified. These KIR share conserved tyrosine phosphorylation motifs in their cytoplasmic tails. Thus, a single receptor in NK cells provides both specificity for HLA class I on target cells and the inhibitory signal that prevents lysis.

Introduction

Natural killer (NK) cells kill virus-infected cells (Biron et al., 1989; Malnati et al., 1993; Brutkiewicz and Welsh, 1995) and contribute to the rejection of bone marrow allo- or xenografts (Yu et al., 1992; Murphy et al., 1993). They also play an important regulatory role in immune responses to parasites and bacteria (Scott and Trinchieri, 1995). Despite their lack of immunoglobulin and T cell antigen receptors (TCR), NK cells display specificity in target cell recognition. In contrast with T cells, the specificity of target cell recognition by NK cells is not provided by triggering receptors but by inhibitory receptors associated with recognition of major histocompatibility complex (MHC) class I molecules (Yokoyama, 1995; Lanier and Phillips, 1995; Leibson, 1995; Raulet and Held, 1995). The importance of peptides bound to class I molecules for proper recognition by NK cells suggests that the same type of stably assembled class I complex of heavy chain, β2-microglobulin, and peptide is recognized by NK and T cells (Correa and Raulet, 1995; Malnati et al., 1995). Target cells that fail to express MHC class I molecules, or properly assembled MHC class I molecules, are generally lysed by NK cells (Storkus et al., 1989; Ljunggren et al., 1989; Ljunggren and Kärre, 1990; Liao et al., 1991). Thus, the outcome of MHC class I recognition is totally different for cytotoxic T cells and NK cells. Whereas class I–restricted $CD8^+$ T cells are triggered to kill target cells that express a given class I molecule, NK cells receive a negative signal upon recognition of class I molecules that overrides triggering signals for lysis (Karlhofer et al., 1992; Moretta et al., 1993; Correa et al., 1994; Vitale et al., 1995; Kaufman et al., 1995).

A family of molecules, called Ly49, expressed on mouse NK cells, provides specificity for MHC class I molecules on target cells (Karlhofer et al., 1992; Mason et al., 1995; Stoneman et al., 1995). These receptors are disulfide-linked homodimers belonging to the C-type lectin type II transmembrane protein superfamily (Yokoyama and Seaman, 1993). Ly49 molecules bind to class I molecules (Kane, 1994; Daniels et al., 1994a) and this binding requires the carbohydrate structure of class I molecules (Daniels et al., 1994b). In surprising contrast, a family of human NK receptors called p58 (Moretta et al., 1993) is composed of type I transmembrane proteins with two immunoglobulin-related extracellular domains (Wagtmann et al., 1995; Colonna and Samaridis, 1995). Antibody-blocking experiments have implicated p58 receptors in the recognition of HLA-C alleles (Moretta et al., 1993; Ciccone et al., 1994; Vitale et al., 1995). Cross-linking of these receptors delivers negative signals to killer cells that inhibits target cell lysis. Specificity of NK clones for HLA-Cw3 and related alleles (Colonna et al., 1993) correlated with surface expression of one member of the p58 NK receptor family, whereas other NK clones specific for HLA-Cw4 and related alleles express a different p58 receptor, as defined with monoclonal antibodies (MAbs) (Moretta et al., 1993; Ciccone et al., 1994; Vitale et al., 1995).

The family of p58 receptors displays sequence diversity in both the extracellular and intracellular domains (Wagtmann et al., 1995). Three different lengths of cytoplasmic tails were observed, even among p58 receptors that were closely related in their extracellular immunoglobulin domains. These data suggested that p58 receptors may differ both in their ligand specificities and in their signaling capabilities. The five different p58 members identified in a single individual exceed the minimal number of receptors needed to distinguish between the two groups of HLA-C alleles. Analysis of the expression of these receptors in NK cells by SSCP revealed a complex distribution, with individual NK clones expressing always at least one, and often more, receptors (Wagtmann et al., 1995).

Other inhibitory receptors have been described that correlate with specificity for HLA-B molecules (Litwin et al., 1994; Moretta et al., 1994). In particular, a molecule of 70 kDa called NKB1 is expressed on NK clones that are specific for HLA-B alleles belonging to the Bw4 subgroup (Gumperz et al., 1995). The recent isolation of a cDNA clone encoding NKB1 revealed that this molecule contains three immunoglobulin domains, two of which are related to p58 (D'Andrea et al., 1995). Additional receptors for HLA-B must exist, as some NK clones that do not express NKB1 can also recognize alleles of the Bw4 group (Litwin et al., 1994; Gumperz et al., 1995; Lanier et al., 1995). A regulatory role for p58 and NKB1 receptors in T cell responses has been suggested by their presence on a

subset of T cells (Ferrini et al., 1994; Mingari et al., 1995; Phillips et al., 1995). Cross-linking of these receptors on T cells, either with MAbs or by interaction with target cells, resulted in inhibition of activation signals delivered by the TCR.

The identification of the p58 family of receptors raises several interesting questions. Are p58 molecules specific receptors for MHC class I molecules or are they only signaling molecules? Which type of cytoplasmic tail among those found in p58 receptors is responsible for the inhibitory function? Does the p58 family include inhibitory receptors involved in the recognition of molecules other than HLA-C?

The existence of completely different receptors, such as Ly49 in mouse and p58 in humans, with apparently similar function, is surprising. To date, mouse homologs of p58 and human homologs of Ly49 receptors have not been isolated. Are p58 and Ly49 independent receptors with overlapping function, or do they function as coreceptors, with one providing specificity for MHC class I, the other providing signaling capability? Binding studies are required to determine whether the p58 receptors themselves provide specificity in NK recognition of HLA-C alleles.

Here, we provide answers to these questions. Specific recognition of HLA-C alleles by p58 receptors was demonstrated by direct binding with soluble recombinant p58 molecules. Second, a functional reconstitution system developed in human NK clones revealed that a single p58 molecule can confer specificity for an HLA-C allele and that receptors with the longer form of cytoplasmic tail containing tyrosine phosphorylation motifs can deliver the negative signal. Finally, a p58-related molecule with a similar cytoplasmic tail was identified that inhibited NK clones upon specific recognition of HLA-B molecules.

Results

Soluble p58 Receptors Bind Specifically to HLA-C Alleles

Studies on HLA-C allorecognition by NK cells indicated the existence of two main groups of NK clones (Moretta et al., 1993; Colonna et al., 1993; Vitale et al., 1995). One group of clones, which is inhibited by HLA-Cw3 and related alleles, including HLA-Cw1, HLA-Cw7, and HLA-Cw8, expresses p58 molecules reactive with the MAb GL183. Another group of clones that is inhibited by HLA-Cw4 and related alleles (HLA-Cw2, HLA-Cw5, and HLA-Cw6) expresses p58 molecules reactive with MAb EB6. To test whether these correlations reflect direct binding of p58 receptors to HLA-C alleles, soluble receptors were produced as p58–Ig fusion proteins. cDNA fragments encoding the entire extracellular part of p58–cl6 (reactive with MAb GL183) or p58–cl42 (reactive with MAb EB6) were cloned upstream of sequences encoding the hinge, CH2, and CH3 domains of human immunoglobulin G1 (IgG1) to produce soluble divalent receptors (Aruffo et al., 1990). Vectors encoding only the hinge CH2 and CH3 of IgG1 as controls or the p58–Ig fusion proteins cl6–Ig or cl42–Ig were transfected into COS7 cells. Supernatants of metabolically labeled transfectants were tested for the presence of the recombinant proteins by binding to protein A–sepharose followed by SDS–PAGE. In each case, proteins of the expected size (35 kDa for the control molecule and 75 kDa for p58–Ig) were secreted into the medium (Figure 1).

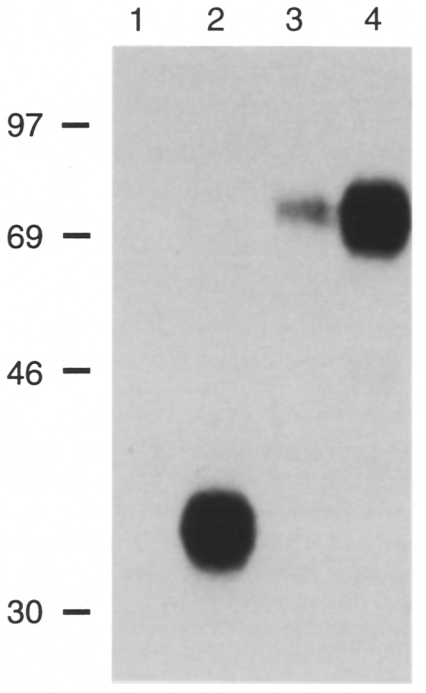

Figure 1. Production of Soluble p58–Ig Fusion Proteins

Reducing SDS–PAGE analysis of soluble p58–Ig molecules purified from supernatants of transfected COS7 cells. Cells were mock-transfected (lane 1), transfected with control vector encoding the Fc portion of human IgG1 (lane 2), or with vectors encoding the cl6–Ig (lane 3) or cl42–Ig (lane 4) p58–Ig fusion proteins. The position of size markers in kilodaltons is indicated on the left.

Purified soluble fusion proteins were tested for binding to the human B cell line 721.221, which lacks HLA-A, HLA-B, and HLA-C molecules, and to 721.221 cells transfected with the class I alleles HLA-Cw3, HLA-Cw4, or HLA-B27 (Figure 2). The soluble cl6–Ig molecule bound specifically to HLA-Cw3 transfectants, whereas the cl42–Ig molecule bound specifically to HLA-Cw4 transfectants. Neither molecule bound to 721.221 or to .221-B27 cells, and the control molecule did not bind to any of the four cell lines. These data indicate that p58–cl6 encodes a NK receptor for HLA-Cw3 and p58–cl42 encodes a receptor for HLA-Cw4. Titrations of the recombinant soluble molecules between 3 μg/ml and 100 μg/ml revealed dose-dependent binding of p58–Ig to cells expressing HLA-Cw3 or HLA-Cw4 (data not shown). These results suggest that the p58 NK receptors bind directly to HLA-C and demonstrate that discrimination between HLA-C alleles is achieved at the level of specific binding.

NK Clones Maintain their Target Cell Specificity after Infection with Vaccinia Virus

The existence of p58 molecules with very similar extracellular domains but different types of cytoplasmic tails sug-

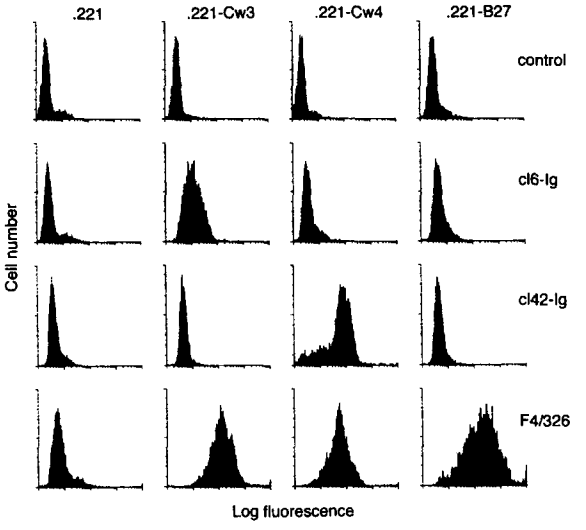

Figure 2. Soluble p58–Ig Fusion Proteins Bind Specifically to Distinct HLA-C Alleles

The human B cell line 721.221 (.221) and transfected 721.221 cells expressing HLA-Cw3 (.221-Cw3), HLA-Cw4 (.221-Cw4), or HLA-B27 (.221-B27) were incubated with 10 μg/ml of purified Fc portion of human IgG1 (control), the cl-6 (cl6–Ig) or the cl-42 (cl42–Ig) p58–Ig fusion proteins, or with MAb F4/326 (specific for HLA-C and some HLA-B alleles), as indicated. The cells were washed, incubated with FITC-conjugated goat anti-human immunoglobulin (top three panels) or anti-mouse immunoglobulin (bottom panel) antisera, and analyzed by flow cytometry.

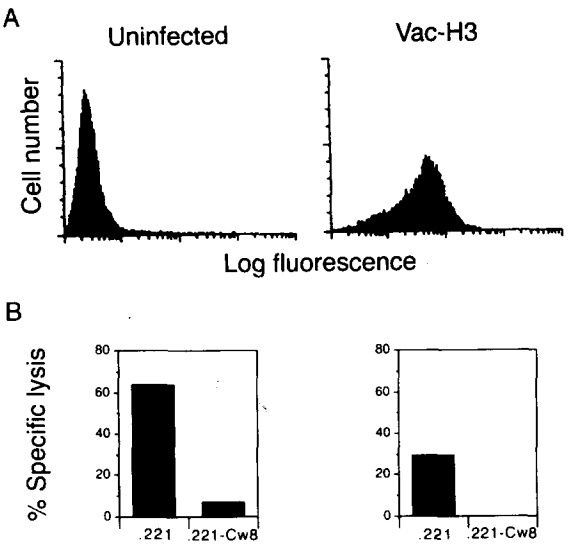

Figure 3. NK Clones Infected with Vaccinia Virus Retain their Lytic Capacity and MHC Class I Specificity

(A) NK clone SR70, either uninfected (left) or infected with 30 pfu/cell of a recombinant vaccinia virus encoding the influenza virus haemagglutinin H3 (Vac-H3), was tested for surface expression of H3 with anti-H3 MAbs followed by FITC goat anti-mouse IgG, and analyzed by flow cytometry. The histograms show relative log fluorescence of ungated cells.

(B) The uninfected (left) or Vac-H3-infected (right) NK cells shown in (A) were tested for their ability to lyse 721.221 (.221) or 721.221 stably transfected with HLA-Cw8 (.221-Cw8) in a 2 hr ^{51}Cr release assay. The data shown were obtained at an effector to target ratio of 5; similar results were obtained at a ratio of 1. Similar results were obtained in several other experiments with the same and with three other NK clones.

gested that receptors with similar ligand specificity may have different signaling capability. Therefore, it was important to test which members of the p58 family can mediate inhibition of target cell lysis. The most direct approach was to express functionally these receptors in NK cells. The large number of cells and the time required to obtain stable transfectants precluded the use of NK clones, which can only be established as short-term cultures. In the face of such limitations, and in view of the ability of a cytotoxic T cell line to retain lytic activity after infection with recombinant vaccinia viruses (Romeo and Seed, 1991), we explored the use of the recombinant vaccinia virus expression system to deliver individual receptor molecules into NK clones.

Three important requirements must be fulfilled to achieve functional reconstitution of inhibition of NK lysis after vaccinia virus infection. First, NK cells should retain cytotoxicity towards unprotected target cells. Second, infection should not interfere with the negative signaling machinery. Third, homogenous infection of NK cells is critical because the lytic activity of even a small fraction of uninfected cells could obscure inhibitory effects in the infected cells. Conditions were developed to obtain homogenous infections of small numbers of NK clones (less than 10^6). The effect of vaccinia virus infection on NK-mediated lysis was examined using NK clones specific for HLA-Cw8, an allele related to HLA-Cw3 (Colonna et al., 1993). These NK clones kill the human B cell line 721.221, but do not lyse the same line stably transfected with HLA-Cw8 (.221-Cw8). The NK clones were infected with a control recombinant vaccinia virus encoding the influenza virus haemagglutinin H3 molecule (Vac-H3) and simultaneously tested for surface expression of the H3 molecule and for cytolytic activity (Figure 3). The majority of the cells were productively infected and expressed high levels of H3 at their surface after 1.5 hr of infection (Figure 3A). Longer infections resulted in higher levels of surface H3 but diminished the lytic capacity of the infected cells (data not shown). Infection with Vac-H3 did not change the surface expression of endogenous p58 molecules (data not shown). Virus-infected NK cells were still able to kill unprotected 721.221 cells, although at a somewhat reduced level. Importantly, they could still receive inhibitory signals from protected targets, as they did not kill .221-Cw8 cells (Figure 3B). The killing by virus-infected cells was usually at least 50% of that by uninfected cells. To correct for this reduction in killing, lysis mediated by vaccinia virus–infected NK clones was always compared with that of cells infected with control viruses. In all the experiments described here, Vac-H3 was used as control virus.

These results indicated that although vaccinia virus infection causes a partial reduction in the lytic capacity of NK clones, it does not abrogate their ability to recognize class I or to receive inhibitory signals, suggesting that vaccinia-mediated gene transfer could be used to test the function of individual receptor molecules in NK cells.

Recombinant Vaccinia Viruses Encoding p58 and Related Molecules

To establish directly the role of p58 molecules in recognition of HLA-C by NK cells, recombinant vaccinia viruses encoding two of the p58 receptors (encoded by cl-6 and cl-42) were constructed. We also generated recombinant viruses encoding two molecules, cl-5 and cl-11, that are structurally related to p58 but contain an additional immunoglobulin-related domain (Figure 4A). Several cDNA clones encoding this type of molecule were isolated by hybridization during screening of a cDNA library with a p58 probe (Wagtmann et al., 1995), and sequenced completely. Two of these three-domain molecules, cl-2 and cl-11, encode molecules with only 4 aa differences in their extracellular domains. The third, cl-5, differs from cl-11 by 61 aa scattered throughout the entire length of the molecule; 45 of these differences occur in the extracellular domains. The second and third immunoglobulin domains of the three-domain molecules are about 74% and 85% identical to the corresponding domains in p58 receptors. During revision of this manuscript, a cDNA clone for NKB1 was reported (D'Andrea et al., 1995); its deduced amino acid sequence is identical to that of cl-2. Similar cDNA clones have been isolated by PCR amplification, including one, NKAT3 (Colonna and Samaridis, 1995), whose translation is identical to that of cl-11. The expression in NK clones of the mRNA encoding these three-domain molecules was evaluated with an SSCP assay similar to that previously described for p58 molecules (Wagtmann et al., 1995). Out of 35 NK clones that expressed at least one p58 molecule, 30 also expressed one or two three-domain molecules (data not shown).

The three-domain molecules have cytoplasmic tails of either 84 or 95 aa, which are very similar to the longer type of cytoplasmic tails found in the p58 receptors. The functional relationship of the three-domain molecules to p58 was not known, but conservation of known tyrosine-based signaling motifs in the cytoplasmic tails of each type of receptor (Wagtmann et al., 1995; Colonna and Samaridis, 1995; D'Andrea et al., 1995) suggested they may have similar signaling capacity.

To verify expression from the recombinant vaccinia viruses encoding p58 or three-domain molecules, human B cells were infected and metabolically labeled with [^{35}S]methionine. Immunoprecipitation with specific antibodies revealed proteins with the expected size for molecules bearing core N-linked glycosylation (Figure 4B). The expected molecular mass of the p58 molecule encoded by p58–cl6, including four N-linked glycosylations, is 47.5 kDa. It is 60 kDa for the one encoded by cl-5.

Transfer of a Single p58 Receptor into NK Clones Confers Specificity for HLA-C

To examine the role of individual p58 receptors in target cell recognition by NK clones and to identify the cytoplasmic tail involved in the delivery of a negative signal, two NK clones that did not recognize HLA-Cw3, HLA-Cw4, or HLA-Cw8 were selected. Such clones lyse the HLA-A-, HLA-B-, and HLA-C-negative 721.221 cells, 721.221 cells

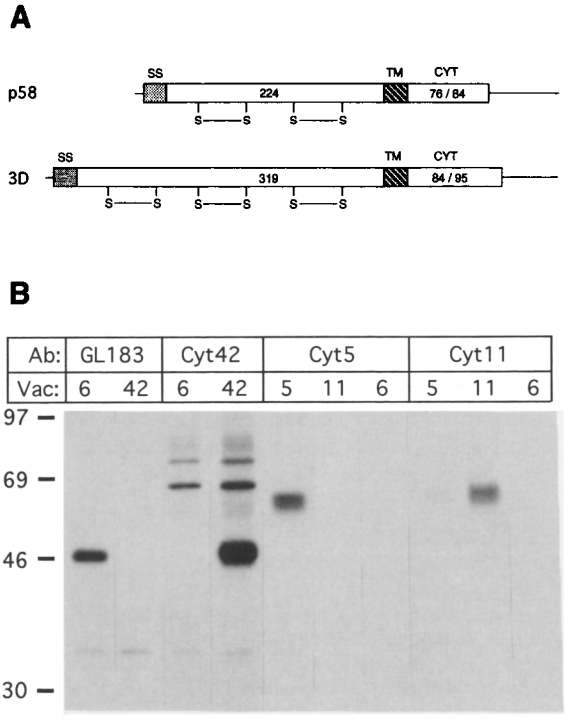

Figure 4. Expression of p58 and Related NK Receptors in Cells Infected with Recombinant Vaccinia Viruses

(A) Schematic diagram of the primary structure of p58 NK receptors and of related molecules with three immunoglobulin domains (3D). The signal sequence and transmembrane region are shaded and hatched, respectively. The cytoplasmic tails of the molecules encoded by p58–cl6 and p58–cl42 are 76 and 84 aa, respectively, and those of the three-domain molecules cl-5 and cl-11 are 84 and 95 aa. Noncoding sequences are depicted as a thin line.

(B) The B cell line 721.45 was infected with the indicated recombinant vaccinia viruses and labeled with [^{35}S]methionine. Cells (4×10^6) were infected with 30 pfu/cell and pulse-labeled for 30 min (four lanes on the left) or with 20 pfu/cell and pulsed for 15 min (six lanes to the right). The infected cells were lysed in detergent and subjected to immunoprecipitation with the MAb GL183, or with rabbit antisera to the cytoplasmic tails of p58–cl42, cl-5, or cl-11, as indicated. Immunoprecipitates were analyzed by SDS–PAGE. The position of markers (in kilodaltons) is indicated on the left.

transfected with HLA-Cw3, HLA-Cw4, and HLA-Cw8, as well as C1R cells that express HLA-Cw4 as the only serologically detectable class I molecule (Zemmour et al., 1992). The level of specific lysis of each of these targets by an individual NK clone may vary (data not shown). This small variability could complicate interpretation of the effects of vaccinia-mediated p58 expression. For this reason, and to control for the nonspecific reductions in overall NK lysis due to vaccinia infection (see Figure 3), we always compared the lysis of a target by an NK clone infected with vaccinia encoding p58 to the lysis of that target by the same clone infected with Vac-H3.

After infection of the two NK clones SR46 and SR50 with Vac-H3, Vac-6, or Vac-42, both clones expressed uniform surface levels of the relevant molecules (Figure 5A). Expression of surface p58 appeared to be higher in clones infected with Vac-6 than with Vac-42. NK clones infected

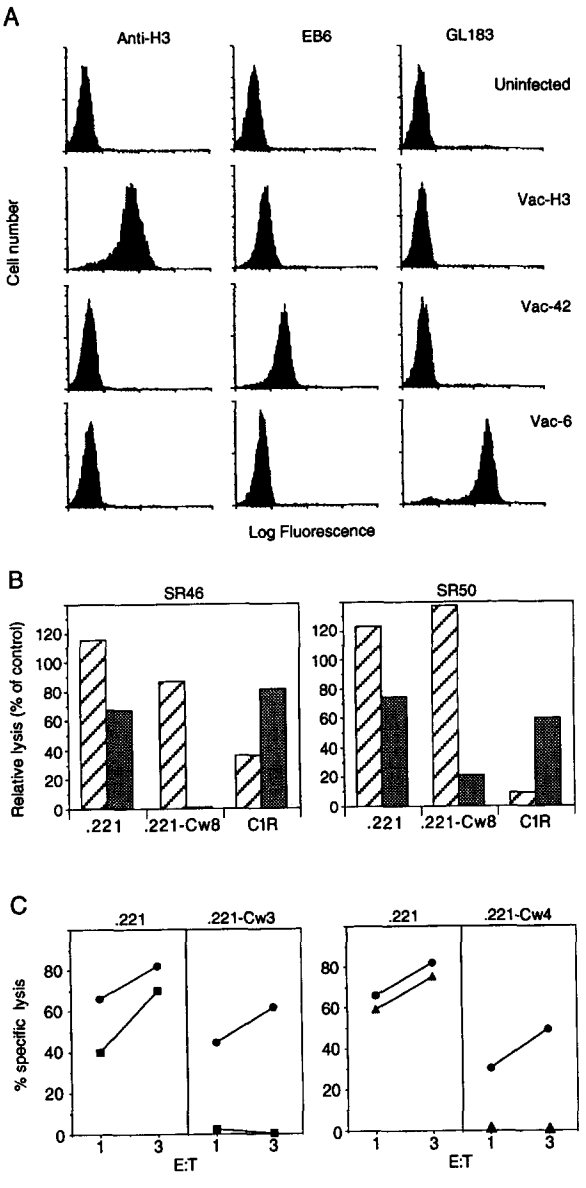

Figure 5. Expression of p58–cl42 in NK Clones Confers Specificity for HLA-Cw4 and p58–cl6 Confers Specificity for HLA-Cw8 and HLA-Cw3 on Target Cells

(A) Flow cytometry analysis of cell surface H3 and p58 molecules in NK clone SR46 either uninfected or infected with Vac-H3, Vac-42, or Vac-6, as indicated. The cells were stained with isotype-matched anti-H3 MAbs or the anti-p58 MAbs EB6 or GL183 (all IgG1), followed by FITC goat anti-mouse IgG. The histograms show log fluorescence of ungated cells. Essentially identical results were obtained with NK clone SR 50.

(B) Aliquots of the same infected SR46 (left) and SR50 (right) NK cells shown in (A) were tested for their ability to lyse 721.221, .221-Cw8, and C1R cells in a 3 hr ^{51}Cr release assay. Data are expressed as lysis by cells infected with Vac-6 (shaded) or Vac-42 (hatched) relative to lysis of the same targets by cells infected with Vac-H3. The specific lysis of 721.221, .221-Cw8, and C1R cells by Vac-H3-infected SR46 cells was 39%, 14%, and 47%, respectively, and for SR50 it was 62%, 19%, and 54%, respectively. Lysis is shown for an effector to target ratio of 9; similar data were obtained at a ratio of 3. Virtually identical results were obtained in a separate experiment with the same two NK clones.

(C) NK clone SR50 infected with Vac-H3 (circles) or Vac-6 (squares) was tested for its ability to lyse .221 or .221-Cw3 cells (left). NK clone SR50 infected with Vac-H3 (circles) or Vac-42 (triangles) was tested for its ability to lyse .221 or .221-Cw4 cells (right).

with any of the three viruses lysed the 721.221 target cells (Figure 5B). In contrast, the .221-Cw8 targets were lysed by Vac-H3- and Vac-42-infected NK clones but not by the Vac-6-infected NK clones (Figure 5B). Infection with Vac-6 also resulted in the inability to lyse .221-Cw3 targets (Figure 5C). Reciprocally, the Cw4-positive C1R cells were lysed by both Vac-H3- and Vac-6-infected NK clones, but to a much lesser extent by Vac-42-infected NK clones (Figure 5B). To confirm that Vac-42 conferred specificity for HLA-Cw4, rather than some other ligand on C1R, we tested the ability of Vac-42-infected cells to lyse .221 or 221-Cw4 cells. Infection with Vac-42 resulted in complete inhibition of lysis of .221-Cw4, whereas .221 cells were still lysed (Figure 5C).

Thus, in agreement with the binding data, expression of p58–cl42 in NK clones was sufficient to confer specificity for HLA-Cw4, while expression of p58–cl6 conferred specificity for HLA-Cw8 and HLA-Cw3. Furthermore, the negative signal delivered to the NK cells by these receptors identified them as killer cell inhibitory receptors (KIR; this name takes into account the existence of p58 inhibitory receptors on both T cells and NK cells). Both p58–cl42 and p58–cl6 have the longer form of cytoplasmic tail containing tyrosine-phosphorylation motifs, demonstrating that this type of cytoplasmic tail can deliver the negative signal leading to inhibition of target cell lysis.

Identification of an Inhibitory Receptor that Recognizes HLA-B27 and HLA-B51

To test whether the three domain molecules encoded by cl-5 and cl-11 were also inhibitory receptors that provide specificity for HLA molecules on target cells, NK clones infected with Vac-5 or Vac-11 were assayed on a panel of target cells, including C1R (Cw4) and 721.45 (HLA-A2, HLA-B5, HLA-Cw1). Lysis of 721.45 and C1R cells by an NK clone was maintained after infection with Vac-H3 or Vac-5 (Figure 6A). In contrast, infection with Vac-11 did not affect lysis of C1R but completely blocked lysis of 721.45 cells. Given that recognition of HLA-Cw1 is expected to involve one of the p58 receptors (Ciccone et al., 1992; Colonna et al., 1993), and that HLA-A2 is generally a poor protective allele (Storkus et al., 1991; Litwin et al., 1993), the HLA-B5 molecule on 721.45 was a good candidate for a protective element.

To examine this possibility, the same NK clone was tested after infection for its ability to lyse C1R cells or C1R transfected with HLA-B27 or HLA-B51 (a B5 allele). Both B27 and B51 belong to the Bw4 group of class I alleles. Whereas NK cells infected with Vac-5 lysed all three targets as efficiently as cells infected with Vac-H3, infection with Vac-11 strongly inhibited lysis of C1R-B27 and C1R-B51 targets, but not the lysis of C1R (Figure 6B). This inhibition of lysis of C1R-B27 mediated by cl-11 was further confirmed in two additional NK clones. Transfer of cl-11 into these NK clones inhibited their ability to lyse targets

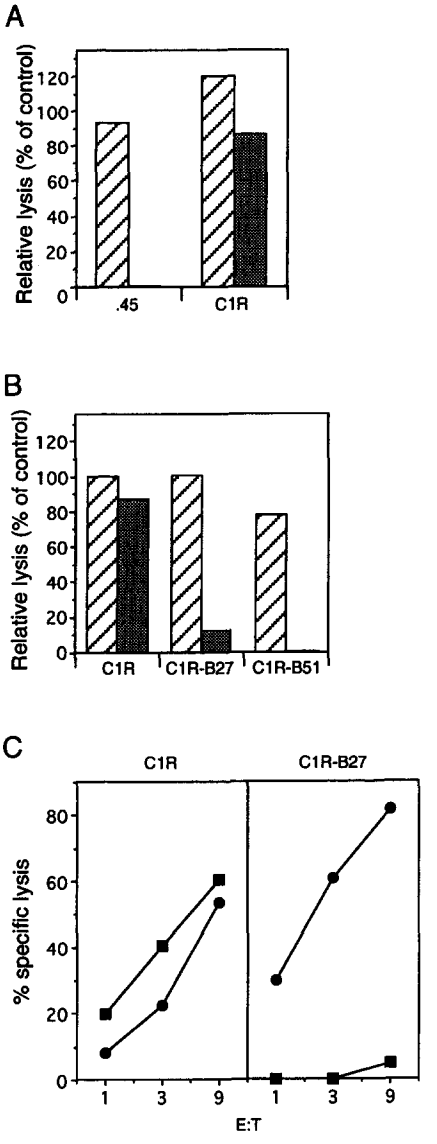

Figure 6. Expression of the Three-Domain Receptor Encoded by cl-11 in NK Clones Confers Specificity for HLA-B51 and HLA-B27 on Target Cells

(A) The NK clone SR50 was infected with Vac-H3, Vac-5, or Vac-11 and tested for its ability to kill 721.45 (HLA-A2, HLA-B5, HLA-Cw1) and C1R cells in a 3 hr ^{51}Cr release assay. Data represent lysis by cells infected with Vac-5 (hatched) or Vac-11 (shaded) relative to the lysis by cells infected with Vac-H3. The specific lysis by the Vac-H3-infected cells was 15% and 45% with 721.45 and C1R cells, respectively. Data shown were obtained at an effector to target ratio of 8; similar data were obtained at a ratio of 4. Virtually identical results were obtained in a separate experiment.

(B) NK clone SR50 was infected as above and tested for its ability to lyse C1R cells and C1R transfected with HLA-B27 or HLA-B51 in a 4 hr ^{51}Cr release assay. Data shown represent lysis by cells infected with Vac-5 (hatched) or Vac-11 (shaded) relative to the lysis by cells infected with Vac-H3 (43%, 16%, and 46% specific lysis against C1R, C1R-B27, and C1R-B51). Data shown were obtained at an effector to target ratio of 9; similar data were obtained at a ratio of 3. Similar results were obtained in a separate experiment.

(C) NK clone 5wA-06 was infected with Vac-H3 (circles) or Vac-11 (squares) as described above and tested for its ability to lyse C1R and C1R-B27 targets in a 4 hr ^{51}Cr-release assay. Data are presented as specific lysis. Similar results were obtained in another experiment with the same and with another NK clone.

bearing HLA-B27, while infection with control Vac-H3 had little effect on the efficiency of lysis. Data from one such clone is depicted in Figure 6C. Thus, the receptor with three immunoglobulin domains encoded by cl-11 is a KIR specific for HLA-B27 and HLA-B51.

Discussion

This study demonstrates direct binding of the KIR p58 to HLA-C molecules expressed on transfected cells. Specific binding was revealed in that two different recombinant soluble p58 receptors discriminated between two distinct HLA-C alleles. In addition, an expression system to evaluate p58 function was established in NK clones and was used to demonstrate that several receptors of the p58 family with a conserved cytoplasmic tail provided the inhibitory signal upon recognition of specific HLA class I molecules. This system was exploited to identify a receptor with specificity for HLA-B alleles, and provides a new tool for the molecular analysis of KIR.

A New Receptor for MHC Class I

Antibody-blocking experiments, as well as correlations between NK cell phenotype and specificity for target cells, previously showed that p58 receptors were associated with recognition of HLA-C on target cells (Ciccone et al., 1992; Moretta et al., 1993; Vitale et al., 1995). These experiments indicated that p58 was involved in target cell recognition in one of two ways. First, p58 itself could carry specificity for class I molecules. Alternatively, p58 could serve as a coreceptor or a signaling module associated with other receptors that provide class I specificity. The experiments described here demonstrate that p58 is a specific receptor for MHC class I on target cells. Studies with soluble class I molecules are required to establish whether HLA-C alone is sufficient for p58 binding.

The direct binding of p58 receptors to specific HLA-C molecules is incompatible with p58 serving as coreceptor for another class I–specific receptor such as Ly49. Therefore, coexpression of these two types of receptors in NK cells may represent a redundancy in NK inhibitory recognition systems, possibly serving to ensure that every NK cell will express at least one inhibitory receptor. Even though both types of receptors may exist in each species, or at least in their common ancestor, humans and mice may have adapted to the predominant use of only one type of receptor. There is precedence for such adaptation in MHC class II molecules. Whereas the evolutionary counterparts of the human MHC class II isotypes HLA-DR and HLA-DQ are I-E and I-A in mice, respectively, human HLA-DR and mouse I-A molecules, which are not structural counterparts, clearly predominate as restricting elements in cellular immune responses. Similarly, mice may use predominantly C-type lectin receptors to inhibit NK cells, whereas humans use immunoglobulin-related receptors for that function. The alternative scenario that they provide redundant systems for the inhibition of NK cells would be more effective if separate sets of NK cells expressed either p58 or a Ly49 counterpart.

Inhibition of NK Cells Mediated by KIR

An expression system in NK clones was successfully developed to demonstrate that a single receptor confers both specific recognition of MHC class I on target cells and the ability to inhibit NK-mediated lysis. The HLA-C allele specificities of p58 receptors transferred into NK clones matched those determined by the binding of soluble receptors. Thus, the HLA-Cw4 specificity was transferred to NK clones by expression of p58–cl42, while the HLA-Cw8 and HLA-Cw3 specificity was conferred by expression of p58–cl6. This system was further exploited to determine the function and the specificity of a p58-related molecule with three immunoglobulin domains. The receptor encoded by cDNA cl-11 conferred specificity for HLA-B27 and HLA-B51 to NK clones.

The functional reconstitution system is particularly useful because the multiplicity of p58 and related NK receptors, and their complex pattern of expression in NK clones, precludes the assignment of their specificities based simply on their endogenous expression in given NK clones. Specificities of NK clones did not correlate with expression of the molecule NKAT3 (Colonna and Samaridis, 1995), which is identical to that encoded by cl-11. In our own analysis of 16 NK clones that were inhibited by HLA-B27, using an SSCP assay that distinguishes expression of cl-5 from cl-2 and cl-11 (but not cl-2 and cl-11 from each other; data not shown), eight expressed cl-2/cl-11, two expressed cl-5, and six expressed one of each. Therefore, a receptor distinct from that encoded by cl-2 and cl-11 must also provide specificity for HLA-B27. Our data are consistent with the description of HLA-B27-specific NK clones that do not express NKB1 (Litwin et al., 1994; Gumperz et al., 1995), a receptor molecule identical to the one encoded by cl-2. The expression system described here can now be used to determine the specificities of different members of the p58 family and those of other inhibitory receptors.

The three KIR identified here share the same type of cytoplasmic tail with conserved YxxL motifs, which is distinct from a shorter type carried by other members of the p58 family (Wagtmann et al., 1995). The longer cytoplasmic tails include a motif related to the immune receptor tyrosine-based activation motif (ITAM) found in antigen receptors of B, T, and mast cells (Weiss and Littman, 1994). In contrast to TCR, FcR, and immunoglobulin, where ITAM motifs are located on specialized signaling polypeptides noncovalently associated with the antigen receptors, KIR have the potential to initiate the signal themselves through tyrosine phosphorylation of their YxxL motifs and recruitment of proteins with SH2 domains. The identification of a common cytoplasmic tail involved in delivery of the inhibitory signal for NK cells and the development of a functional expression system in NK cells opens the way to a molecular dissection of this signaling pathway.

Experimental Procedures

Cells and Antibodies

NK clones were generated and maintained as described (Malnati et al., 1993). The B-EBV cell line 721.45 has been described (Kavathas et al., 1980). Hmy2.C1R (C1R), a human B cell line expressing HLA-Cw4 as the only serologically detectable class I molecule (Zemmour et al., 1992), and C1R transfected with HLA-B*5101 were provided by W. E. Biddison (National Institute of Neurological Disorders and Stroke, National Institutes of Health). C1R transfected with HLA-B*2705 (Calvo et al., 1990) was a gift from J. Lopez de Castro (University of Madrid, Spain). The HLA-A-, HLA-B-, and HLA-C-negative mutant B-EBV cell 721.221 (Shimizu and DeMars, 1989) and 721.221 transfected with HLA-Cw3, HLA-Cw4, or HLA-B*2705 were a gift from J. Gumperz and P. Parham (Stanford University). 721.221 transfected with HLA-Cw8 was obtained from R. Biassoni (National Cancer Institute, Genova, Italy). Anti-H3 MAbs were a gift from R. Webster (St. Judes Children's Hospital, Memphis, Tennessee). The MAb F4/326 with specificity for HLA-C and some HLA-B alleles (Mizuno et al., 1989) was a gift from S. Y. Yang (Memorial Sloan-Kettering Cancer Center, New York, New York). The anti-p58 MAbs GL183 and EB6 (Moretta et al., 1990) were provided by A. Moretta (National Cancer Institute, Genova, Italy). Rabbit antisera to the carboxy-end of the cytoplasmic tails of p58–cl42 (Cyt42), cl-5 (Cyt5), or cl-11 (Cyt11) were raised by immunization with the synthetic peptides AESRSKVVSCP (Cyt42), PRAPQSGLEGVF (Cyt5), and AKPRSKVVSCP (Cyt11). Each antiserum was affinity purified on the peptide used for immunization (Research Genetics).

p58–Ig Fusion Proteins

The sequences encoding the extracellular portions of p58–cl6 and p58–cl42 (Wagtmann et al., 1995) were amplified by polymerase chain reaction (PCR) with the forward primer 5′- CAG GGG GCG CTA GCG CAT GAG GGA GTA CAC AGA AAA CC - 3′ corresponding to sequences immediately downstream of the signal sequence cleavage site and containing a NheI site, and the backward primer 5′- GAG GTC CCA GGA TCC GCA TGA TGC AGG TGT CTG GGG TTA CC - 3′ immediately upstream of the transmembrane region and containing a BamHI site. The amplification products were digested with NheI and BamHI and cloned into the NheI–BamHI cut Cd5Ineg1 expression vector (a gift of B. Seed; see Aruffo et al., 1990) in frame with the leader peptide of CD5 and the artificial splice site allowing fusion of the p58 cDNA to genomic DNA encoding the hinge, CH2, and CH3 regions of human IgG1 (Aruffo et al., 1990). The last 3 aa of p58 were HLH and the first 3 aa encoded by the vector were DPE, in the single letter code. COS7 cells were transfected using DEAE–Dextran. On day 4 after transfection, cells were labeled with *Trans*-label (ICN Biomedicals, Incorporated) (150 μCi/ml) for 4 hr and the supernatants were assayed for the presence of human immunoglobulin-containing proteins by precipitation with protein A–sepharose. For large-scale production of fusion proteins, serum-free COS cell supernatants were collected on days 4 and 8 after transfection and affinity purified on protein A–Sepharose as described (Linsley et al., 1991). The integrity of the purified proteins was verified by SDS–PAGE and Coomassie staining. Protein concentrations were determined with the Micro bicinchoninic acid assay (Pierce).

Direct Binding Assay

The human B cell line 721.221 and transfected 721.221 cells expressing HLA-Cw3, HLA-Cw4, or HLA-B27 were incubated with the MAb F4/326 or with 10 μg/ml of the purified p58–Ig fusion proteins cl6–Ig, cl42–Ig, or the Fc portion of human IgG1 (control) for 1 hr at 4°C. The cells were then washed and incubated with fluorescein isothiocyanate (FITC) conjugates of either goat anti-human or goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Fluorescence on a total of 5,000 cells was then analyzed by flow cytometry on a FACScan (Beckton Dickinson).

cDNA Cloning and Sequencing

The screening of a $CD3^-$ cDNA library with a probe corresponding to the coding region of the p58–cl6 cDNA has been described (Wagtmann et al., 1995). Several cDNA clones encoding molecules with three immunoglobulin domains were isolated that could be divided into three groups of identical sequences based on partial sequencing of their 5′ ends. Representative clones of each group, namely cl-2, cl-5, and cl-11, were sequenced completely in both directions using sequenase (United States Biochemical).

Recombinant Vaccinia Viruses

Recombinant vaccinia viruses encoding p58–cl6, p58–cl42, cl-5, and cl-11 were generated and purified as described (Earl and Moss, 1988). In brief, the full-length p58 cDNA were subcloned as SalI–NotI fragments into the vector pSC–65 (provided by B. Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health). They were then inserted into the WR strain of vaccinia virus by homologous recombination. Standard viral plaque assays on CV1 cells were used to determine the titer of each of the resulting recombinants. A recombinant vaccinia virus encoding the influenza virus hemagglutinin H3 molecule has been described (see Guéguen et al., 1994, construct D). The B cell line 721.45 was infected as previously described (Guéguen et al., 1994) with recombinant vaccinia for 1.5 hr at 37°C, starved in methionine-free medium for 1 hr at 37°C, and pulse-labeled with 0.1 mCi/ml of [^{35}S]methionine. After three washes at 4°C, the cells were lysed with 1% Triton X-100 and subjected to immunoprecipitation and SDS–PAGE on 10.5% gels as described (Wagtmann et al., 1995). Radiolabeled proteins were detected by autoradiography.

Vaccinia Virus Infection of NK Clones

NK clones were washed three times with infection medium (Iscove's, 0.5% bovine serum albumin, 1 × nonessential amino acids, 2 mM L-glutamine) at room temperature and resuspended at 0.5 × 10^6/ml in infection medium supplemented with 50 U/ml rIL-2 (a gift from Hoffmann-La Roche, Incorporated). Aliquots (0.5 ml) were given either no virus or different doses of recombinant vaccinia virus and incubated for 1.5 hr at 37°C on a rotator. After one wash with CTL medium (Iscove, 10% fetal calf serum, 2 mM glutamine, 50 U/ml rIL2), cells were resuspended in CTL medium and counted. The infected or uninfected control cells were simultaneously plated for standard ^{51}Cr-release killing assays (Malnati et al., 1993) and for antibody staining and flow cytometry as described (Wagtmann et al., 1995). For each NK clone used, a separate titration with each vaccinia virus was performed. Subsequent infections were with the minimal dose that resulted in homogeneous infection of the majority of the cells as monitored by surface staining.

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GenBank Accession Numbers

The sequences reported in this paper have been deposited in Genbank with accession number U30273 (KIR-cl2), U30272 (KIR-cl5), and U30274 (KIR-cl11).