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Degenerate Recognition of MHC Class I Molecules with Bw4 and Bw6 Motifs by a Killer Cell Ig-like Receptor 3DL Expressed by Macaque NK Cells

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The killer cell Ig-like receptors (KIRs) expressed on the surface of NK cells recognize specific MHC class I (MHC-I) molecules and regulate NK cell activities against pathogen-infected cells and neoplasia. In HIV infection, survival is linked to host KIR and MHC-I genotypes. In the SIV macaque model, however, the role of NK cells is unclear due to the lack of information on KIR–MHC interactions. In this study, we describe, to our knowledge, the first in-depth characterization of KIR–MHC interactions in pigtailed macaques (Macaca nemestrina). Initially, we identified three distinct subsets of macaque NK cells that stained ex vivo with macaque MHC-I tetramers loaded with SIV peptides. We then cloned cDNAs corresponding to 15 distinct KIR3D alleles. One of these, KIR049-4, was an inhibitory KIR3DL that bound MHC-I tetramers and prevented activation, degranulation, and cytokine production by macaque NK cells after engagement with specific MHC-I molecules on the surface of target cells. Furthermore, KIR049-4 recognized a broad range of MHC-I molecules carrying not only the Bw4 motif, but also Bw6 and non-Bw4/Bw6 motifs. This degenerate, yet peptide-dependent, MHC reactivity differs markedly from the fine specificity of human KIRs. The Journal of Immunology, 2012, 189: 4338–4348.

Natural killer cells are critical components of the innate immune system that contribute to protection against intracellular pathogens and neoplasia (1–4). The principal determinant of NK cell activation and function is the ability to discriminate between healthy cells and the altered signature of pathogen-infected or transformed cells. In particular, viral infection and neoplastic processes frequently downregulate the cell surface expression of MHC class I (MHC-I) molecules to escape immune pressure mediated by CD8+ T lymphocytes. These phenotypic changes can trigger NK cell activation.

NK cells recognize MHC-I molecules via a large array of germline-encoded cell surface receptors that provide both activating and inhibitory signals (5–7). In primates, these receptors comprise members of the C-type lectin-like family (NK2A-E) and a number of Ig superfamily molecules, such as leukocyte Ig-like receptors (LILRs) and killer cell Ig-like receptors (KIRs) (8). MHC-I molecules constitute a major ligand for several of these receptors, including KIRs (9).

KIR molecules are encoded by several highly polymorphic genes that produce cell surface proteins with either two (KIR2D) or three (KIR3D) extracellular Ig-like domains. In addition, the transmembrane domain and cytoplasmic tail occur in two distinct forms. KIR molecules with long tails (KIR2DL, KIR3DL) provide inhibitory signals due to the presence of ITIMs (10). In contrast, KIR molecules with short tails (KIR2DS, KIR3DS) provide activating signals mediated by the adaptor protein DAP12, which associates with a charged residue in the KIR transmembrane domain (5).

In humans, KIRs possess fine specificity for defined groups of MHC-I molecules characterized by specific motifs at the C-terminal end of the α1 helix. KIR2DL1 recognizes HLA-C molecules that possess asparagine and lysine at positions 77 and 80, respectively. KIR2DL2 and KIR2DL3 recognize all the other HLA-C molecules, which harbor serine and asparagine at positions 77 and 80, respectively. In contrast, KIR3DL1 specifically recognizes HLA-B molecules with a Bw4 motif (NLR[IT]ALR) between positions 77 and 83. Other HLA-B molecules, which lack the Bw4 motif, harbor the distinct Bw6 sequence (SLRNLRG); this is not recognized by any human KIRs. The vast majority of HLA-A molecules carry neither the Bw4 nor the Bw6 motif and are not recognized by KIRs, except for HLA-A*03 and HLA-A*11, which bind KIR3DL2 in a peptide-dependent manner (11). The ligands for most activating KIRs are currently unknown.

The polymorphic KIR genes have been shown to play a critical role during viral infection. For example, genetic studies have demonstrated that the presence of KIR2DL3 in hepatitis C virus-infected patients correlates with improved viral control (12).
Similarly, specific combinations of MHC-I (HLA alleles harboring the Bw4-80I motif) and KIR (KIR3DL1/KIR3DS1) genes are associated with slower progression to AIDS in HIV-1–infected patients (13, 14). Furthermore, KIR3DL1/S1+ NK cells expand in vivo during acute HIV-1 infection in individuals carrying HLA-Bw4 alleles (15). However, the precise mechanism of protection is still unclear, and ligands for KIR3DS1 have yet to be identified. Studies in animal models could help to elucidate the protective role of NK cells during HIV-1 infection.

Infections of Asian macaques with SIV or SIV/HIV chimeras (SHIV) are classical primate models of HIV disease. Rhesus macaques (Macaca mulatta), pig-tailed macaques (Macaca nemestrina), and cynomolgus macaques (Macaca fascicularis) are the most commonly used species for this purpose. In these animals, SIV or SHIV infection recapitulates the pathology and disease progression observed in HIV-1–infected humans (16). The organization of MHC genes in these three species is markedly different compared with the human MHC region. Specifically, macaques lack the MHC-C gene, but possess several copies of both the MHC-A and MHC-B genes. These are called Mamu, Mane, or Mafa genes in rhesus, pig-tailed, and cynomolgus macaques, respectively. Numerous macaque MHC-I alleles have been sequenced, and immunogenic viral epitopes presented by the expressed proteins have been identified in some cases (17–22). In addition, certain macaque MHC-I alleles are associated with lower viral loads and protection against SIV/SHIV disease development (23–25).

The role of NK cells during SIV/SHIV infection in macaques remains unclear. NK cell depletion experiments have failed to demonstrate a significant impact on SIV load and disease progression, potentially due to partial targeting of all NK cells (26). However, numerous KIR alleles have been identified in rhesus and cynomolgus macaques (27–30), and some studies have reported an effect of specific KIR alleles on viral load, independently of MHC background (31, 32).

Although macaque MHC and KIR sequence diversity has been studied extensively, little is known about macaque KIR specificity for MHC-I ligands. Only two studies have identified specific KIR–MHC pairs in rhesus macaques (33, 34). Colantoni et al. (34) demonstrated that KIR3DL05 recognizes Mamu-A1*002. Similarly, Rosner et al. (33) showed that KIR3DL05 and KIR3DLw03 interact with several Mamu-A, but not with Mamu-B molecules in vitro. These KIRs also interact strongly with human HLA-C molecules (35).

In this study, we describe, to our knowledge, the first in-depth characterization of KIR–MHC interactions in pig-tailed macaques. The identified receptor, named KIR049-4, is a member of the inhibitory KIR3DL family and exhibits broad peptide-dependent reactivity against both Bw4 and non-Bw4 MHC-I molecules.

### Materials and Methods

**Animals and viruses**

Pig-tailed macaques were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (36). Animal handling and experimental procedures were approved by the National Institute of Allergy and Infectious Diseases Institutional Animal Care and Use Committee. Macaques were housed in a biosafety level 2 facility. Biosafety level 3 practices were followed. Macaques were anesthetized with i.m. injections of ketamine hydrochloride (Ketaset; Phoenix Pharmaceutical, St. Joseph, MO) and acepromazine acetate (Fermenta Animal Health, Kansas City, MO) during phlebotomy and virus inoculations. Ten macaques had been inoculated i.v. several years earlier with CXCR4-tropic SHIV of low pathogenicity. At the time of study, plasma RNA viral loads were below the limit of detection in all infected macaques, and CD4+ T lymphocyte counts were within the normal range.

**KIR3D cloning and sequencing**

Macaque KIR3D cDNAs were cloned, as described previously (28). Briefly, 1 μg total RNA, extracted from macaque PBMCs using TRI Reagent (Molecular Research Center, Cincinnati, OH), was used to synthesize cDNA using random hexamers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. KIR cDNAs were amplified by PCR using KIR-S1 (5′-CGACCCAGCATGCAGCTCAATC-3′) sense and KIR-R1 (5′-GGGGTCAAGTGAAAGGGAGAGA-3′) reverse primers with high fidelity Phusion polymerase (New England Biolabs, Ipswich, MA). The PCR products were heated at 98°C for 30 s, and then amplifications were conducted over 28 cycles of 98°C for 5 s, 63°C for 1 s, and 72°C for 20 s. A final extension was conducted at 72°C for 5 min. The PCR product (~1.6 kb) was gel purified using the Qiagen gel extraction kit (Qiagen, Valencia, CA) and cloned into the pCR4-Blunt-TOPO vector (Invitrogen). Between 24 and 60 individual clones were sequenced per animal using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences of the pig-tailed macaque KIR3D alleles described in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/ genbank/) under accession number HG713453–HG713467.

**Phylogenetic analysis**

KIR3DL and KIR3DS sequences were aligned separately using the Cluster W program in the MacVector 11.1.2 software suite (MacVector, Cary, NC) with minor manual adjustments. Phylogenetic trees were constructed using the neighbor-joining method. Genetic distances were estimated using Kimura’s two-parameter method (37). Bootstrap analysis (1000 replicates) was performed to assign confidence values to tree nodes (38).

**Cell lines**

The MHC-I–deficient cell line 721.221 (39), provided by E. Long (Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD), was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 U/ml streptomycin (R10 medium).

To generate target cells expressing a single macaque MHC-I allele, full-length MHC-I cDNAs encoding the desired molecular information were amplified by PCR using a PE-conjugated MHC-I–specific mAb (clone W6/32; AbD Serotec, Raleigh, NC). Cells were cultured for 1 wk in R10 medium supplemented with 0.70 mg/ml geneticin (Invitrogen), and then positively selected for MHC-I expression on the cell surface using PE-conjugated anti-MHC-I mAb and MACS anti-PE microbeads (Miltenyi Biotech, Auburn, CA), as specified by the manufacturer. Stable cell lines were maintained thereafter in R10 medium supplemented with 0.70 mg/ml geneticin (Invitrogen).

Macaque KIR3D cDNAs were modified by PCR to insert a Flag epitope (DYKDDDDK) between the leader peptide and the D0 domain. Modified KIR3D cDNAs were cloned into the EcoRI restriction site of pcDNA3.1 (+) (Invitrogen). Plasmids encoding macaque Flag-KIR3D constructs were nucleaseoected into the 721.221 cell line, as described for MHC-I alleles. At 20 h postnucleofection, cell surface expression of Flag-KIR3D molecules was verified by flow cytometry using a PE-conjugated Flag–specific mAb (clone M2; Abcam, Cambridge, MA). Cells were cultured for 1 wk in R10 medium supplemented with 0.70 mg/ml geneticin (Invitrogen), and then positively selected for Flag expression on the cell surface using PE-conjugated anti-Flag mAb and MACS anti-PE microbeads (Miltenyi Biotech), as specified for the MHC-I cell lines. Stable cell lines were maintained afterward in R10 medium supplemented with 0.70 mg/ml geneticin (Invitrogen).

**MHC genotyping**

Macaque A1*082 genotyping was performed on genomic DNA extracted from macaque PBMCs using the Wizard Genomic DNA purification kit (Promega, Madison, WI). Briefly, a sequence–specific primer–PCR amplification was performed using Mane-A3S2 sense (5′-CAACACACAGAC- CATTCCAGAGAA-3′) and Mane-A3R reverse (5′-CTCTGATCTGCT- CCGGCTG-3′) primers with AmpliTaq DNA polymerase (Perkin Elmer, Wellesley, MA). An internal control for amplification was obtained using Mane-DRBS3 sense (5′-GAGTGTGATTTCTTCACACGGGA-3′) and Mane-DRBR2 reverse (5′-CCTCGGCGTGCAGTGT-3′) primers. The
PCRs were heated at 94°C for 5 min, and then amplifications were con-
ducted over 25 cycles of 94°C for 30 s, 65.5°C for 30 s, and 72°C for 20 s.
A final extension was conducted at 72°C for 7 min. PCRs were loaded on
to a 1.5% agarose gel and separated by electrophoresis. A 246-bp Mane-
DRB–specific product was amplified in all macaques, whereas a 493-bp prod-
cut was generated in Mane-A1*082 macaques. Mane-A1*082-
specific amplicons were gel purified using the Qiaquick gel extraction kit
(Qiagen) and directly sequenced using a 3130xl Genetic Analyzer (Ap-
plied Biosystems). Mane-A4*14 genotyping was performed similarly, as
described previously (18).

Tetramer production
Fluorochrome-conjugated tetrameric peptide–MHC class I (pMHC-I) com-
plexes were produced in-house, as described previously (40).

Tetramer staining of macaque PBMCs
One million freshly isolated macaque PBMCs were labeled with pMHC-I tetramers conjugated to either PE or allophycocyanin for 15 min at room temperature (RT) in the dark. Cells were labeled further with anti-CD3
(clone SP34-FTC; clone SP34-2-PE; or clone SP34-2-allophycocyanin; BD Biosciences), anti-CD8 (clone SK1-PerCP; BD Biosciences), anti-
CD14 (clone RM052-FTC or clone RM052-allophycocyanin; Beckman Coulter), and anti-CD20 (clone B9E9-FTC or clone B9E9-allophycocyan-
in; Beckman Coulter) mAbs for 30 min at 4°C, washed twice with PBS, and resuspended in PBS containing 1% formaldehyde. At least
200,000 events were acquired per test using a FACSComp flow cytometer (BD Biosciences). Data were analyzed with FlowJo software version 9.3.2 (Tree Star, Ashland, OR).

Tetramer staining of KIR-expressing cell lines
One million 721.221 parental cells and KIR-expressing cell lines were
stained with PE-conjugated pMHC-I tetramers for 30 min at RT in the dark. KIR surface expression was assessed independently for each cell line by staining with a PE-conjugated Flag-specific mAb (clone M2; Abcam) for 30 min at 4°C. Cell samples were washed twice with PBS, resuspended in PBS, and stained with 7-aminoactinomycin D (Via-Probe; BD Bio-
sciences) for 10 min at RT in the dark prior to acquisition. At least 60,000 events were acquired per test using a FACSComp flow cytometer (BD Biosciences). Data were analyzed with FlowJo software version 9.3.2 (Tree Star).

Immunophenotyping of macaque NK cells
Macaque peripheral blood samples were stained at RT with mAbs against CD3 (clone SP34-FTC; BD Biosciences), CD8 (clone SK1-PerCP; BD Biosciences), CD14 (clone RM052-allophycocyanin; Beckman Coulter), CD20 (clone B9E9-allophycocyanin; Beckman Coulter), and NK markers
coupled to PE. The following mAbs were used: 1) anti-CD16 clone 3G8,
anti-CD56 clone MY31, anti-CD2 clone S5.2, anti-NKG2D clone 1D11,
anti-CD57 clone HP-3E4, CD158b clone CH-L, and anti-CD158 clones
anti-CD2 clone BAB281, anti-NKG2A clone Z199, and anti-LILRB1 clone HP-F1, anti-LILRB2 clone 42D1, anti-LILRB3 clone MKTS.7H5.1, and anti-LILRB4 clone ZM41.2 (eBioscience); and 4) anti-
KIR2D clone NKVS1 (Miltenyi Biotec). After 15 min, RBCs were lysed
with FACS lysing solution (BD Biosciences) for 10 min, and then samples
were washed twice with PBS. Cells were then resuspended in PBS contain-
ing 1% formaldehyde. At least 200,000 events were acquired per test using a FACSComp flow cytometer (BD Biosciences). Data were analyzed using CellQuest Pro software version 6.0 (BD Biosciences).

Functional assays
To monitor NK cell activation or TNF-α production by NK cells, freshly
isolated macaque PBMCs were incubated for 5 h in the presence of brefeldin
A (GolgiPlug; BD Biosciences), either alone or at a NK:target ratio of
1:1 based on the percentage of NK cells in the PBMC sample, with pa-
rental 721.221 cells or 721.221 cell lines expressing Mane-A or Mane-B
alleles. Cells were subsequently washed once with PBS, labeled with either PE-conjugated pMHC-I tetramers for 15 min at RT or PE-conjugated anti-
KIR2D mAb (clone NKVS1; Miltenyi Biotec) for 30 min at 4°C, and then stained with anti-CD3 (clone SP34-FTC; BD Biosciences) and anti-
CD8 (clone SK1-PerCP; BD Biosciences) mAbs for 30 min at 4°C. After
two further washes with PBS, the cells were permeabilized for 10 min using Permeabilization Solution 2 (BD Biosciences), washed with PBS, and stained with anti-CD69 (clone FN50-allophycocyanin; BD Biosciences) or anti-TNF-α (clone mAb11-allophycocyanin; BD Biosciences) mAb for
30 min at 4°C. After two final washes with PBS, cells were resuspended in PBS containing 1% formaldehyde.

To monitor NK cell degranulation, macaque PBMCs were incubated for 5 h in the presence of anti-CD107a and anti-CD107b mAbs (clone H4A3-
FITC and clone H4B4-FITC; BD Biosciences) either alone or at a NK:target ratio of 1:1, with parental 721.221 cells or 721.221 cell lines expressing
Mane-A or Mane-B alleles. Monensin (GolgiStop; BD Biosciences) was
added for the last 4 h. Cells were then washed once with PBS, labeled with
PE-conjugated pMHC-I tetramers for 15 min at RT, and stained with anti-
CD3 (clone SP34-allophycocyanin; BD Biosciences) and anti-CD8 (clone SK1-PerCP; BD Biosciences) mAbs for 30 min at 4°C. After two further washes with PBS, cells were resuspended in PBS containing 1% formal-
dehyde.

For all assays, at least 300,000 events were acquired per test using a FACSComp flow cytometer (BD Biosciences). Data were analyzed with FlowJo software version 9.3.2 (Tree Star).

Results
Macaque CD3+ CD8+ lymphocyte subsets bind pMHC-I tetramers ex vivo
In the SIV/SHIV pig-tailed macaque model, CD8+ T cell responses
specific for the SIV Gag-derived epitope KP9 (KKFGAEVVVP) contrib-
ted to viral control and improved survival in monkeys carry-
ing the Mane-A1*084 allele (formerly known as Mane-A*10)
(20). We identified a different Gag-derived epitope (D19:
DHQAAMQII) presented by the Mane-A1*082 allele (formerly
known as Mane-A*03). Mane-A1*082 macaques infected with
SIV/SHIV were found to harbor CD8+ T lymphocytes specific for
this epitope, as measured by intracellular staining for IFN-γ and
TNF-α (data not shown). Although KP9-specific CD8+ T cell
responses were detected in all Mane-A1*084 SIV/SHIV-infected
macaques tested, only a fraction of infected Mane-A1*082 mae-
cques responded to the D19 epitope.

To investigate whether this poor response frequency was due to
the lack of peptide-specific CD8+ T cells, we stained PBMCs from
Mane-A1*082 SHIV-infected macaques directly ex vivo using Mane-A1*082 tetramers loaded with the D19 peptide or an N-
terminal truncated variant (H8: HQAAMQII). Each tetramer
detected a subset of CD8+ T cells in Mane-A1*082 macaques that
exhibited IFN-γ responses upon in vitro stimulation with the D19
peptide (Fig. 1A). We did not detect specific tetramer staining
of CD8+ T cells from Mane-A1*082 SHIV-infected macaques that
failed to produce IFN-γ upon D19 peptide stimulation, suggest-
ing that the lack of response was due to the absence of peptide-specific
CD8+ T cells. However, in the same samples, a significant fraction
(13.9–24.7%) of CD3+ CD8+ cells stained with the Mane-A1*082
tetramers (Fig. 1B). Both tetramers recognized the same CD3+ CD8+
lymphocyte subset, as demonstrated by co-staining experi-
ments using Mane-A1*082 tetramers conjugated with different
fluorochromes (Fig. 1C), suggesting that subsets of CD3+ CD8+
lymphocytes express a receptor capable of binding pMHC-I com-
plexes.

Tetramer-binding CD3+ CD8+ lymphocyte subsets are present
irrespective of MHC genetic background or infection status
Next, we analyzed freshly isolated PBMCs from a cohort of 20 pig-
tailed macaques that included 10 Mane-A1*082 animals (50%).
Ten macaques (50%) had been infected with a CXCR4-tropic
SHIV, and 10 macaques (50%) were uninfected. We identified
Mane-A1*082 tetramer-binding CD3+ CD8+ lymphocytes in seven
animals, but the presence of these cells was independent of infec-
tion status or Mane-A1*082 genotype (Table I). Five macaques
harbored CD3+ CD8+ lymphocytes that stained similarly with both
Mane-A1*082 tetramers. Two additional macaques possessed a
subset of CD3+ CD8+ cells that stained with the H8/Mane-A1*082
tetramer, but not with the D19/Mane-A1*082 tetramer.

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FIGURE 1. pMHC-I tetramers bind macaque CD3+CD8+ lymphocytes ex vivo. (A) Detection of virus-specific CD8+ T cells with Mane-A1*082 tetramers in a SHIV-infected, Mane-A1*082+ macaque (PT1246A). (B) Two Mane-A1*082 tetramers bind subsets of CD3+CD8+ lymphocytes in a SHIV-infected, Mane-A1*082+ macaque (PT93P049) lacking CD8+ T cell responses to the corresponding peptide epitopes. Lymphocytes are gated as CD3+ (Table I).

The observation of pMHC-I tetramer staining within the CD3+CD8+ cell subset was somewhat unexpected because these reagents are typically used to analyze epitope-specific CD8+ T cells, which express CD3 as part of the TCR complex. Thus, the absence of CD3 expression by tetramer-binding cells suggested the involvement of a different hematopoietic lineage. We hypothesized that the tetramer-positive cells were NK cells, which do express CD8, but lack CD3 molecules.

To evaluate the role of macaque NK cells in pMHC-I tetramer binding, we performed immunophenotyping assays on peripheral blood samples from 20 animals using a panel of 10 distinct markers (Supplemental Table I). First, CD16 and CD56, two markers used to distinguish human NK cell subsets, were evaluated (42). A large majority of CD3+CD8+ cells expressed high levels of CD16, but only a small fraction was positive for CD56, consistent with the expression of three different receptors. One receptor recognizes both Mane-A1*082 complexes, but not AF9/Mane-A4*14. A second receptor binds Mane-A1*082 in a peptide-dependent manner. A third receptor is specific for the Mane-A4*14 molecule.

To expand our observations, we used Mane-A4*14 (formerly known as Mane-A*17) tetramers loaded with another Gag-derived peptide (AF9: ALAPVPIPF) (41). Four macaques harbored a known as Mane-A*17 tetramers that bound the AF9/Mane-A4*14 tetramer. As with the Mane-A1*082 tetramers, the presence of a Mane-A4*14 tetramer-binding CD3+CD8+ lymphocyte subset was independent of infection status and MHC genotype (Table I).

The receptors responsible for binding the AF9/Mane-A4*14 and Mane-A1*082 tetramers are clearly distinct. Moreover, costaining experiments of CD3+CD8+ lymphocytes from one animal (PT98P033) with both AF9/Mane-A4*14 and HI8/Mane-A1*082 tetramers revealed distinct but overlapping subsets of cells reactive with each pMHC-I complex (Fig. 1D).

Thus, macaque CD3+CD8+ lymphocytes express receptors with diverse specificity for pMHC-I complexes. Indeed, three distinct patterns of tetramer binding were observed, most likely resulting from the expression of three different receptors. One receptor recognizes both Mane-A1*082 complexes, but not AF9/Mane-A4*14. A second receptor binds Mane-A1*082 in a peptide-dependent manner. A third receptor is specific for the Mane-A4*14 molecule.

**Peptide–MHC-I tetramers bind NK cell subsets directly ex vivo**

To evaluate the role of macaque NK cells in pMHC-I tetramer binding, we performed immunophenotyping assays on peripheral blood samples from 20 animals using a panel of 10 distinct markers (Supplemental Table I). First, CD16 and CD56, two markers used to distinguish human NK cell subsets, were evaluated (42). A large majority of CD3+CD8+ cells expressed high levels of CD16, but only a small fraction was positive for CD56, consistent with the expression of three different receptors. One receptor recognizes both Mane-A1*082 complexes, but not AF9/Mane-A4*14. A second receptor binds Mane-A1*082 in a peptide-dependent manner. A third receptor is specific for the Mane-A4*14 molecule.

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CD8+ cells was more variable between individual macaques. The expression of CD16, NKp30, and NKp46 was almost exclusively detected on CD3+ CD8+ cells and was virtually absent on CD3+ CD8- cells (Supplemental Table I); the other markers tested were less specific for NK cells.

Next, we examined expression of six distinct LILR receptors (LILRA2, LILRA4, LILRB1, LILRB2, LILRB3, LILRB4) on macaque cells. A mAb specific for LILRB3 (CD85a) reacted with monocytes only (data not shown). The five additional human LILR-specific mAbs did not cross-react with macaque molecules. Among six distinct mAbs specific for human KIR2D (HP-3E4, GL183, CHL, DX27, NKVFS1) and KIR3D (DX9) molecules, only one of them (NKVFS1) was reactive with macaque cells (Supplemental Table I and data not shown).

KIR2D was expressed on a subset of CD3+ CD8+ cells that displayed variable frequencies between macaques (mean 22.1 ± 15.6%; range 0.3–68.2%). Three distinct staining patterns were observed. Intense staining of KIR2D+ cells was observed in seven macaques further argues against a T cell origin. However, none of the markers tested displayed concordant expression profiles that could explain the observed patterns of tetramer reactivity.

Cloning of KIR3D alleles from pig-tailed macaques

To identify the receptors responsible for pMHC-I tetramer binding, we investigated the distribution of tetramer-binding cells among CD16+ and KIR2D+ NK cells. The Mane-A1*082 and Mane-A4*14 tetramer-binding cells were found mainly among CD16+ NK cells, which are known to preferentially express KIR molecules in humans (44) (Fig. 2A). However, tetramer staining did not cosegregate with KIR2D expression (Fig. 2B). These results led us to consider KIR3D molecules.

On this basis, we cloned KIR3D alleles from four pig-tailed macaques, including one animal (PT93P049) with NK cells that bound both Mane-A1*082 tetramers and one animal (PT98P033) with NK cells that bound both the H18/Mane-A1*082 and AF9/Mane-A4*14 tetramers. We identified nine KIR3DL and six KIR3DS alleles in this small group of macaques, with each animal expressing multiple activating and inhibitory receptors. All KIR3DL molecules possessed long cytoplasmic tails containing two ITIMs (Fig. 3). In contrast, the KIR3DS molecules had very short cytoplasmic tails, and their transmembrane domains harbored an arginine residue that was absent from the KIR3DL molecules (Fig. 3).

Next, we compared pig-tailed macaque KIR3DL and KIR3DS allele sequences by phylogenetic analysis with alleles previously identified from rhesus and cynomolgus macaques. Pig-tailed macaque KIR3DL sequences intermingled with alleles from the other two macaque species to form five distinct clusters containing representatives from each macaque species (Fig. 4). This observation suggests the presence of five conserved KIR3DL loci among pig-tailed, rhesus, and cynomolgus macaques. Similar phylogenetic comparisons performed with KIR3DS alleles demonstrated that the sequence diversity of pig-tailed macaque KIR3DS alleles was comparable to that of KIR3DS alleles found in rhesus and cynomolgus macaques. However, there was no evidence for the conservation of activating KIR loci among macaque species, as no clustering of KIR3DS alleles was observed (data not shown).

The KIR3DL molecule KIR049-4 recognizes Mane-A1*082

To identify KIR3D alleles encoding pMHC-I tetramer-binding receptors, we expressed four KIR3DS and four KIR3DL cDNAs, the latter from distinct allelic clusters, in 721.221 cells. Due to the lack of available mAbs specific for macaque KIR3D molecules, we inserted a Flag epitope between the leader peptide and the first extracellular Ig-like domain (D0), and established stable cell lines expressing high levels of each KIR3D molecule on the cell surface (Fig. 5A, top row). Whereas the KIR3DL alleles were expressed at high levels and remained stable, expression of the KIR3DS alleles was somewhat lower and generally less stable. Each cell line was tested for its ability to bind Mane-A1*082 and Mane-A4*14 tetramers. None of the KIR3D cell lines bound the AF9/Mane-A4*14 tetramer above background levels observed with parental 721.221 cells (Fig. 5B). Cells expressing KIR049-4, however, bound both Mane-A1*082 tetramers (Fig. 5A [bottom row], 5B). Consistent with these findings, the KIR049-4 allele was cloned from a macaque (PT93P049) with NK cells reactive...
against both Mane-A1*082 tetramers (Fig. 1B). In contrast, none of the other 7 KIR3D cell lines bound either of the Mane-A1*082 tetramers. The KIR049-4 cell line also bound weakly to the KP9/Mane-A1*084 tetramer (Fig. 5B).

**KIR049-4 interactions with Mane-A1*082 and Mane-A1*084 molecules inhibit NK cell responses**

The KIR049-4 allele encodes a KIR3DL molecule, which should generate inhibitory signals upon cognate ligand engagement. To formally address this, we monitored the responses of primary macaque NK cells after in vitro stimulation with the MHC-deficient cell line 721.221, or 721.221 variants expressing single macaque MHC-A or MHC-B alleles (Mane-A1*082, -A1*084, -A1*003, -A3*13, -B*109). After stimulation, NK cells were analyzed for induced expression of CD69, a marker of cellular activation. NK cells expressing KIR049-4 or other Mane-A1*082 tetramer-binding receptors were identified by staining with Mane-A1*082 tetramers, and activation status was assessed for both the tetramer-binding and nonbinding subsets (Fig. 6A, Supplemental Fig. 1A). In the absence of target cells, both NK subsets exhibited low activation levels. Stimulation with 721.221 cells or cells expressing Mane-A1*003, -A3*13, or -B*109 triggered high activation levels in both NK subsets. In contrast, activation of KIR049-4 + or other Mane-A1*082 tetramer-binding NK cells was almost completely inhibited after stimulation with 721.221 cells expressing Mane-A1*082 or Mane-A1*084. Moreover, tetramer-negative NK cells in the same samples maintained high levels of CD69 expression, similar to those observed after stimulation with other MHC alleles (Fig. 6A, Supplemental Fig. 1A). Additional analyses showed that KIR2D+ cells were not inhibited under the same conditions (Fig. 6B, Supplemental Fig. 1A). Thus, the inhibition of NK cell activation by Mane-A1*082 or Mane-A1*084 was restricted to the KIR049-4+ subset.

In further experiments, the specific inhibition of NK cell activation by Mane-A1*082 and Mane-A1*084 was associated with reduced degranulation, as monitored by surface mobilization of CD107, and decreased TNF-α production (Fig. 6, Supplemental Fig. 1B, 1C). Collectively, these results demonstrate that KIR049-4 or related Mane-A1*082 tetramer-binding molecules are inhibitory KIRs that recognize specific MHC-I molecules.

**KIR049-4 specificity is defined by the α1 helix of MHC-I molecules**

To characterize KIR049-4 specificity for MHC-I binding, the five MHC-I alleles tested were divided in two groups based on their capacity to interact with KIR049-4. Accordingly, Mane-A1*003, -A3*13, and -B*109 were classified as binders, whereas Mane-A1*003, -A3*13, -B*109 were classified as nonbinders. The protein sequences of all alleles were analyzed for polymorphic sites that cosegregated with the ability to bind KIR049-4. Fifty-six amino acid positions differed between the five alleles, but most of these were specific to one allele (32 positions; 57.1%). Furthermore, the amino acids present at 17 additional positions (30.3%) in the binder group were also found among the nonbinder MHC-I alleles. The seven remaining positions mapped to the MHC-I α1 and α2 helices at positions 67, 74, 76, 77, 114, 156, and 165 (Fig. 7). Five of these positions (67, 74, 77, 114, 156) contribute to peptide-binding pockets and may affect KIR–MHC-I interactions by altering peptide selection. The mutation at position 165 from leucine, which is found in binder MHC-I alleles, to the valine
residue present in the nonbinder MHC-I alleles is unlikely to affect KIR binding due to the similar physicochemical properties of these amino acids. In contrast, the glutamic acid present at position 76 in the binder MHC-I alleles is replaced by either a valine or an alanine in the nonbinder MHC-I alleles. The exchange between a charged residue and a small hydrophobic amino acid at a position exposed on the surface of the α1 helix could affect the KIR3D–MHC-I interaction (Fig. 7).

FIGURE 5. KIR049-4 binds Mane-A1*082 tetramers. (A) KIR expression and D9/Mane-A1*082 tetramer staining are shown for the indicated stable cell lines expressing macaque Flag-KIR3D molecules. Surface KIR expression was assessed by staining with PE-conjugated anti-Flag mAb (top row, white histograms). Staining with D9/Mane-A1*082 tetramer is shown for the same cell lines (bottom row, white histograms). Parental 721.221 cells stained under the same conditions are depicted in gray. (B) Eight stable cell lines expressing distinct KIR3Ds were stained with the following pMHC-I tetramers: H6b/Mane-A1*082, D9/Mane-A1*082, K9/Mane-A1*084, and AF9/Mane-A4*14. The relative binding index, defined as the mean fluorescence intensity of tetramer binding to the KIR cell line divided by the mean fluorescence intensity of tetramer binding to the parental 721.221 cell line stained in parallel under identical conditions, is shown in each case. Data shown represent the mean of up to nine experiments (**p < 0.0001 by one-way ANOVA).

FIGURE 6. KIR049-4 inhibits NK cell functions in a specific MHC-I–dependent manner. (A) Activation of NK cells (left) was monitored by induction of CD69 expression after stimulation with 721.221 cells or 721.221 variants expressing Mane-A or Mane-B alleles. NK cells stimulated under identical conditions were also monitored for degranulation by surface CD107a/b staining (middle) and for the production of intracellular TNF-α (right). Coexpression of KIR049-4 or related molecules was determined by HI8/Mane-A1*082 tetramer staining after stimulation. NK cells are gated as CD3−CD8+ lymphocytes. Data shown represent the mean of five independent experiments, each performed using a sample obtained from a distinct macaque (PT1670, PT93P049, PT97P027, PT98P021, PT98P056). ***p < 0.0001, **p < 0.001 by one-way ANOVA. (B) Activation (left), degranulation (middle), and TNF-α production (right) among KIR2D+ and KIR2D− NK cell subsets after stimulation with individual MHC-I molecules. These data include tetramer-positive cells present in both the KIR2D+ and KIR2D− NK cell subsets (Fig. 2). Data shown represent the mean of four independent experiments, each performed using a sample obtained from a distinct macaque (PT1670, PT93P049, PT97P027, PT98P021). PT98P056 was not included because < 2% of the NK cells were KIR2D+ in this animal. Error bars indicate SD. Representative raw data are shown in Supplemental Fig. 1.
KIR049-4 interacts with both the Bw4 and Bw6 motifs of MHC-I

Human KIR3DL1 recognizes MHC-I molecules bearing the Bw4 motif at the C-terminal end of the α1 helix, whereas KIR3DL2 specificity is limited to HLA-A*03 and HLA-A*11 in a peptide-dependent manner (45). Surprisingly, both macaque MHC-I molecules recognized by KIR049-4, Mane-A1*082 and -A1*084, possess the Bw6 motif, rather than the Bw4 motif.

To assess KIR049-4 specificity with regard to Bw4/Bw6 motifs, we stained the KIR049-4 cell line with multiple human tetramers comprising HLA molecules with Bw4, Bw6, and non-Bw4/Bw6 motifs (Table II). KIR049-4 bound three distinct Bw4+ HLA molecules (HLA-B*27:05, -B*51:01, and -B*57:01) with high avidity, but failed to bind another Bw4+ HLA molecule (HLA-B*44:02). These interactions were specific for KIR049-4, as four additional KIR3DL molecules failed to bind the same tetramers (Table II). Furthermore, the interaction was confirmed using primary macaque NK cells expressing KIR049-4 (data not shown).

Next, we tested three HLA-B molecules bearing Bw6 motifs and two HLA-A molecules without either the Bw4 or the Bw6 motif (Table II). KIR049-4 bound to HLA-B*07:02 and HLA-A*03:02 tetramers in a peptide-dependent manner, albeit with lower avidities compared with the Bw4+ tetramers. No binding was detected with HLA-A*02:01, -B*08:01, and -B*35:01 tetramers loaded with different peptides. Thus, KIR049-4 can recognize MHC-I molecules bearing both Bw4 and Bw6 motifs in peptide-dependent manner.

Discussion

In this study, we characterized the function of a pig-tailed macaque KIR3DL molecule that recognizes a broad range of MHC-I ligands in a peptide-dependent manner. Engagement of this receptor by its cognate ligands inhibits NK cell activation, degranulation, and cytokine production. Although the inhibitory properties of KIR049-4 are typical of a KIR3DL molecule, its degenerate MHC-I recognition profile contrasts with the focused recognition properties of human KIR molecules.

In humans, the three KIR3DL genes (KIR3DL1, KIR3DL2, and KIR3DL3) encode polymorphic receptors with distinct MHC-I specificities (45). KIR3DL1 binds to MHC-I molecules carrying the Bw4 motif at the C-terminal end of the α1 helix, particularly those possessing an isoleucine at position 80 (Bw4-80I) (46), whereas KIR3DL2 recognizes only HLA-A*03 and HLA-A*11 in a peptide-dependent manner (11). The ligands for KIR3DL3 are currently unknown. Similarly, KIR2D family members recognize HLA-C molecules based on the presence of motifs, called C1 and C2, in the same region of the MHC-I α1 helix as the Bw4 motif (45). There are no known human KIRs specific for MHC-I molecules carrying the Bw6 motif, which represent almost 50% of all MHC-I molecules.

**Table II.** KIR049-4 interacts with both the Bw4 and Bw6 motifs of MHC-I in a peptide-dependent manner

<table>
<thead>
<tr>
<th>HLA</th>
<th>Peptide Origin/Sequence</th>
<th>KIR033-2</th>
<th>KIR033-7</th>
<th>KIR049-4</th>
<th>KIR049-6</th>
<th>KIR049-7</th>
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<tr>
<td><strong>Bw4-80I</strong></td>
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<td>B*51:01</td>
<td>HIV Env YPPPIQGVI</td>
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<td>B*57:01</td>
<td>HIV Gag SPRTLNAW</td>
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<td>79.0</td>
<td>1.2</td>
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<td>HIV Gag KRWIILGLNK</td>
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<td>B*07:02</td>
<td>HIV Nef RPMTYKAAAL</td>
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<td>1.6</td>
<td>0.9</td>
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</table>

Values in bold represent positive tetramer-binding indexes.

1. The 721.221 cell line and stable derivatives thereof expressing five different KIR3DL alleles (KIR033-2, 033-7, 049-4, 049-6, and 049-7) from distinct clusters (Fig. 4) were stained with MHC-I tetramers and analyzed by flow cytometry.

2. Relative binding index, defined as the mean fluorescence intensity of tetramer binding to the KIR cell line divided by the mean fluorescence intensity of tetramer binding to the parental 721.221 cell line stained in parallel under identical conditions.
In contrast, the macaque KIR3DL molecule identified in this study recognizes Bw4⁺ MHC-I molecules with high avidity, some Bw6⁺ MHC-I molecules with lower avidity, and some non-Bw4/Bw6 MHC-I molecules. In each case, the nature of the peptide presented by MHC-I affects the strength of the interaction, with a more profound impact on the lower avidity interactions. Despite the relatively low binding avidity for macaque Bw6⁺ MHC-I molecules detected by pMHC-I tetramer staining, the interaction is sufficient to block NK cell activation in functional assays.

Although peptides have been shown to impact human KIR–HLA interactions (47–50), particularly in the case of KIR3DL2 molecules (11), this is often considered secondary to the role of the presenting MHC-I H chain because few KIR residues interact directly with the peptide (51–53). The degenerate MHC-I recognition characteristics of KIR049-4 suggest that macaque KIR3D molecules rely on somewhat different criteria than human KIR3DLs for MHC-I recognition, with the bound peptide perhaps playing a more influential role. A consequence of this peptide selectivity is that some permissive MHC-I molecules are unable to interact with KIR049-4 in the presence of antagonist peptides, potentially due to steric interference from peptide amino acid side chains. This is most likely exemplified in our dataset by the differential binding observed for HLA-A*07:02 tetramers bearing distinct peptides derived from HIV and CMV (Table II). Similarly, it is possible that some MHC-I molecules identified as nonbinders in our assays, such as HLA-A*02:01, -B*08:01, and -B*35:01, could be recognized by KIR049-4 if different, agonist peptides were loaded in the binding groove. Accordingly, the range of MHC-I molecules that can bind KIR049-4 is likely larger than currently described. However, not all MHC-I molecules are able to bind KIR049-4, as demonstrated in our functional assays with cell lines expressing Mane-A1*003, -A3*13, and -B*109 (Fig. 6). In these assays, the MHC-I molecules are loaded with numerous variable peptides derived from the intracellular compartment and most likely incorporate both agonist and antagonist peptides. The lack of recognition by KIR049-4 indicates that motifs within the MHC-I protein itself are necessary to stabilize the KIR–pMHC complex.

To gain a deeper understanding at the atomic level, we analyzed the degenerate MHC-I recognition properties of KIR049-4 based on the structure of human KIR3DL1 complexed with a Bw4⁺ MHC-I molecule (HLA-B*57:01) (53). The binding site between these two proteins is defined by 17 aa in the MHC-I molecule that contact 21 aa in the KIR3DL1 molecule. Most of the contact residues (11 of 17) in HLA-B*57:01 are shared with macaque MHC-I molecules that bind KIR049-4. The six residues that differ map to positions 19, 76, 80, 83, 142, and 151. The contact residues identified in human KIR3DL1 are equally divided between 11 aa (positions 9, 11, 13, 29, 34, 200, 201, 228, 230, 276, and 277) that are conserved in KIR049-4 and 10 aa (positions 138, 140, 165, 166, 167, 199, 227, 272, 279, and 282) that differ in KIR049-4. The pattern of conservation/polymorphism between the KIR3DL1 and KIR049-4 molecules suggests that the interactions mediated by the D0 and D2 domains of KIR3D with the side of the MHC-I molecule and the α2 helix, respectively, are conserved. Studies using KIR3DL1 mutants have shown that the D0 domain helps to stabilize the interaction mediated by D1 and D2 over the MHC-I groove (54). Additional studies have shown that KIR2DL1 recognition can be expanded beyond the confines of HLA-C molecules by fusion with the D0 domain of KIR3DL1 to include some HLA-B and HLA-G molecules (55). As the contact points present in the D0 domain of KIR3DL1 are conserved in KIR049-4, it is likely that the D0 domain of KIR049-4 is an important contributor to degenerate MHC-I recognition via stabilization of the KIR–MHC complex. The majority of the variable residues affect the D1 and D2 domain interactions with the α1 helix, particularly around position 76 and the Bw4/Bw6 motif region. In particular, the D1 region at positions 165–167, which contacts both the peptide and the MHC-I molecule around position 76 and 80, differs completely between KIR049-4 and human KIR3DL1. These clustered polymorphisms most likely contribute to the degenerate MHC-I recognition profile of KIR049-4 and its associated peptide dependence.

The ability of KIR049-4 to recognize Bw6⁺ MHC-I molecules is not unique. Rhesus macaque NK cells also express specific KIR3DLs encoded by Mamu-KIR3DL5 alleles that recognize Bw6⁺ MHC-I molecules in a peptide-dependent manner (33, 34). Two such KIR3DL molecules, KIR3DLw03*004 and KIR3DL05*007, display degenerate MHC-I recognition properties and are sensitive to amino acid changes at positions 77, 80, and 83 within the Bw4/ Bw6 motif (33). Furthermore, polymorphic residues located in the D1 domain encoded by these KIR3DL5 alleles contribute to the peptide-dependent recognition of Mamu-A1*002 tetramers, which carry the Bw6⁺ motif (34). Our analysis extends these findings beyond the rhesus macaque model.

The characteristics of KIR049-4 described in our study have implications for other macaque species. Indeed, phylogenetic analysis identified several KIR alleles from rhesus and cynomolgus macaques that are highly similar to KIR049-4 (Fig. 4). In particular, the cynomolgus macaque KIR3DL allele KIR55 differs from KIR049-4 by only one synonymous and six nonsynonymous mutations affecting the D0 domain, the D2 domain, and the cytoplasmic tail (28). As none of these mutations affect the predicted points of contact with MHC-I molecules, KIR55 most likely exhibits a degenerate MHC-I recognition profile similar to that of KIR049-4. Additional KIR molecules encoded by the same locus, such as KIR07 in cynomolgus (28) and KIR3DLw03*004 in rhesus (29) macaques, possess many additional polymorphic sites. However, these mutations map mainly outside of the predicted points of contact, and these KIR alleles most likely behave similarly to KIR049-4. Therefore, the degenerate MHC-I recognition profile observed with KIR049-4 is most likely common to the three macaque species for which we have KIR data, due to conservation of the encoding locus.

Two additional macaque NK cell receptors are apparent from the macaque pMHC-I tetramer reactivity patterns observed in our study. These are likely KIR molecules that remain to be identified. Based on the MHC-I–binding properties of KIR molecules, it would be expected that pMHC-I tetramer staining could be used more generally to characterize NK cells ex vivo. However, only one previous report has described such results in rhesus macaques (34). Multiple studies have reported staining of human NK cell clones or KIR transfectants with pMHC-I tetramers. Indeed, this approach has allowed the characterization of specific ligands for KIR3DL1 and KIR3DL2 (11, 48, 50, 56). In contrast, no other report has shown pMHC-I tetramer staining of human NK cells isolated directly from blood or tissue. The reasons for this lack of staining remain to be reconciled with our knowledge of KIR binding to MHC-I molecules.

Finally, the ability to characterize NK cells using pMHC-I tetramers provides us with a new approach that enables the study of these cells during SIV/SHIV infection. This is an important advance because relatively few reagents are currently available to monitor the diverse array of KIR molecules expressed in macaques.
Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


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