KIR3DL01 Recognition of Bw4 Ligands in the Rhesus Macaque: Maintenance of Bw4 Specificity since the Divergence of Apes and Old World Monkeys

Jamie L. Schafer, Arnaud D. Colantonio, William J. Neidermyer, Dawn M. Dudley, Michelle Conole, David H. O'Connor and David T. Evans

*J Immunol* 2014; 192:1907-1917; Prepublished online 22 January 2014; doi: 10.4049/jimmunol.1302883
http://www.jimmunol.org/content/192/4/1907

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/01/22/jimmunol.130288
3.DCSupplemental

**References**
This article cites 51 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/192/4/1907.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
KIR3DL01 Recognition of Bw4 Ligands in the Rhesus Macaque: Maintenance of Bw4 Specificity since the Divergence of Apes and Old World Monkeys

Jamie L. Schafer,*† Arnaud D. Colantonio,*† William J. Neidermyer,*† Dawn M. Dudley,‡ Michelle Connole,§ David H. O’Connor,‡ and David T. Evans*†

The identification of MHC class I ligands for rhesus macaque killer cell Ig-like receptors (KIRs) is fundamental to our basic understanding of KIR and MHC class I coevolution and to the study of NK cell responses in this nonhuman primate model for AIDS and other viral diseases. In this study, we show that Mamu-KIR3DL01, which is expressed by ∼90% of rhesus macaques, recognizes MHC class I molecules with a Bw4 motif. Primary NK cells expressing Mamu-KIR3DL01 were identified by staining with a mAb which, in this study, was shown to bind Mamu-KIR3DL01 alleles with an aspartic acid at position 233. The cytolytic activity of Mamu-KIR3DL01+ NK cells was suppressed by cell lines expressing the Bw4 molecules Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01. The Bw4 motif was necessary for Mamu-KIR3DL01 recognition because substitutions in this region abrogated Mamu-KIR3DL01+ NK cell inhibition. However, the presence of a Bw4 motif was not sufficient for recognition because another Bw4 molecule, Mamu-B*017:01, predicted to be KIR contacts based on the three-dimensional structure of the human KIR3DL1-HLA-Bw4 complex, with the corresponding residues at these positions for the other Mamu-Bw4 ligands restored Mamu-KIR3DL01+ NK cell inhibition. These results define the ligand specificity of one of the most polymorphic and commonly expressed KIRs in the rhesus macaque and reveal similarities in Bw4 recognition by Mamu-KIR3DL01 and human KIR3DL1, despite the absence of an orthologous relationship between these two KIRs or conservation of surface residues predicted to interact with MHC class I ligands. The Journal of Immunology, 2014, 192: 1907–1917.

Natural killer cells are able to recognize and kill virus-infected cells and tumor cells without prior antigenic stimulation and therefore constitute an important innate cellular defense against infectious diseases and cancers. NK cell responses in primates are regulated in part through interactions between two highly polymorphic molecules, the killer cell Ig-like receptors (KIRs) on NK cells and their MHC class I ligands on target cells. Depending on sequences in their transmembrane and cytoplasmic domains, KIRs can transduce either inhibitory or activating signals. In the case of inhibitory KIRs, NK cell activation is suppressed upon receptor engagement of MHC class I ligands on the surface of healthy cells. Thus, NK cells bearing inhibitory KIR may become activated upon disruption of ligand recognition, either as a consequence of MHC class I downregulation by viral infection (1–5), deletion of MHC class I genes during tumor progression (6), or MHC class I presentation of antagonistic peptides (7).

Genetic evidence suggests that KIR and MHC class I polymorphisms play a significant role in determining the course of infection for several human pathogens, including hepatitis C virus (8), hepatitis B virus (9, 10), human papilloma virus (11, 12), HSV (13), and HIV (14, 15). However, studies addressing the functional implications of these observations have been limited by the lack of a suitable animal model. Mice and other rodents do not express KIRs but instead use C type lectin–like molecules encoded by the Ly49 genes as polymorphic NK cell receptors for MHC class I ligands (16). Moreover, KIRs appear to be evolving at a particularly rapid pace in primates (17–20). As a consequence, there is little conservation among the KIR genes of different species, and it is not possible to predict the specificity of KIR–MHC class I interactions on the basis of sequence comparisons with human KIRs.

The rhesus macaque is an important animal model for AIDS research (21) and for other viral diseases caused by EBV (22), CMV (23), and Kaposi’s sarcoma–associated herpesvirus (24). Immunogenetic characterization of this species also has contributed to our basic understanding of the coevolution of KIR and MHC class I genes. Rhesus macaques have duplicated Mamu-A and -B genes, which correspond to HLA-A and -B in humans (25, 26). However, they do not have a C locus because HLA-C represents a duplication of an ancestral B gene that occurred after the divergence of apes and Old World monkeys (25, 26). There are as many as four Mamu-A genes and an undefined and variable number of Mamu-B genes on any given haplotype in the rhesus macaque (27, 28). In accordance with KIR and MHC class I coevolution, macaques lack

*Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115; †Division of Microbiology, New England Primate Research Center, Southborough, MA 01772; ‡Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53711; and §Division of Immunology, New England Primate Research Center, Southborough, MA 01772

Received for publication October 25, 2013. Accepted for publication December 10, 2013.

This work was supported by Public Health Service Grants AI095098 and AI098485 (to D.T.E.) and R01AI12745 (to D.H.O.). Additional support was provided by Public Health Service Grants OD011103 and OD010849. D.T.E. is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

Address correspondence and reprint requests to Dr. David T. Evans at the current address: Department of Pathology and Laboratory Medicine, University of Wisconsin, AIDS Vaccine Research Laboratory, 585 Science Drive, Madison, WI 53719.

E-mail address: dtevans2@wisc.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: CAM, calcein acetoxymethyl ester; HA, hemagglutinin; KIR, killer cell Ig-like receptor.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302883

lineage III KIR genes, which encode KIR2DL/S specific for HLA-C (29, 30), and instead have an expanded repertoire of KIR3DL/S genes characterized by extensive polymorphism (20, 29–32). Indeed, 19 distinct KIR3DL/S genes have been identified in rhesus macaques (31, 33).

The recognition of MHC class I molecules by human KIRs is primarily determined by sequences in the ligand α1 and α2 domains. All HLA-B molecules, and some HLA-A molecules, can be classified as either Bw4 or Bw6 allotypes on the basis of residues at positions 77–83 in the α1 domain (34). KIR3DL1 is the most polymorphic human KIR and recognizes diverse HLA class I ligands that share a Bw4 motif (35). The contribution of Bw4 residues to ligand recognition by KIR3DL1 was recently corroborated by a crystal structure of KIR3DL1*001 in complex with HLA-B^*5701, which revealed multiple contacts between the D1 domain of KIR3DL1*001 and Bw4 residues of HLA-B^*5701 (36). This structure also revealed additional contacts between the D1 and D2 domains of KIR3DL1*001 and the α1 and α2 domains of HLA-B^*5701, indicating that the Bw4 motif is not the sole determinant of ligand recognition (36).

Only a few MHC class I ligands have been defined for nonhuman primate KIRs. In the rhesus macaque, these include the identification of Mamu-A^*002 as a ligand for Mamu-KIR3DL05 by binding and cellular assays (37, 38), with additional binding partners for Mamu-KIR3DL05, -3DL03, -3DL11, and -3DS05 revealed by staining with soluble KIR-Fc fusion proteins (38). In the pig-tailed macaque, Mane-A^*082 and -A^*084 were also identified as ligands for KIR049-4 (39). However, the value of most of these interactions for studying NK cell responses is in question and MHC class I–defined animals remains limited because of the lack of reagents for staining specific KIR on primary macaque NK cells.

In this study, we show that a mAb to human KIR2D molecules binds allotypes of Mamu-KIR3DL01 bearing an aspartic acid at position 233. We use this Ab to define the ligand specificity of Mamu-KIR3DL01, a commonly expressed and highly polymorphic KIR in the rhesus macaque. Our results reveal similarities in the recognition of Bw4 molecules by Mamu-KIR3DL01 and human KIR3DL1, despite the absence of an orthologous relationship between these two KIRs. These observations provide an important foundation for studying NK cell responses in KIR- and MHC class I–defined animals and provides an ancient origin for Bw4 specificity prior to the divergence of apes and Old World monkeys.

Materials and Methods

Ethics statement

Indian origin rhesus macaques (Macaca mulatta) were used in this study. Housing and care of the animals at the New England Primate Research Center were in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Animal experiments were approved by the Harvard Medical Area Standing Committee on Animals and conducted according to the principles described in the Guide for the Care and Use of Laboratory Animals (40).

Phenotyping of NKVFS1+ lymphocytes

Unless otherwise noted, all Abs are from BD Biosciences. PBMC were isolated by Ficoll–Paque (GE Healthcare) and stained with anti–CD3-V500 (clone SP34-2), anti–NKG2A-Pacific Blue (clone Z199, in-house conjugation), anti–CD4-FITC (clone L200, anti–HLA-DR-Texas red (clone Immuno-357; Beckman Coulter), anti–CD20-PE-Cy5.5 (clone L27), anti–CD56-PE-Cy7 (clone B159), anti–KIR2D-APC (clone NKVFS1; Miltenyi Biotec), anti–CD8-PE-Alexa700 (clone RPA-T8), anti–CD16-APC-Cy7 (clone 3G8), and anti–CD14-PE (clone M4P9) or anti–NKG2D-PE (clone BAT221; Miltenyi Biotec) for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 2 × 10^6 lymphocyte events were collected using an LSRII flow cytometer (BD Biosciences), and the data were analyzed using FlowJo 8.8.7 (Tree Star).

Electroproportion of Jurkat cells with KIR expression constructs

Jurkat cells (10^7 cells) were electroporated (250 V, 950 μF) with 40 μg plasmid DNA encoding hemagglutinin (HA)-tagged KIR in the pCCG vector in 350 μl RPMI 1640 medium (Invitrogen) in a 0.4-cm cuvette using the GenePulser ShockPod system (Bio-Rad). Cells were rested for 10 min and then cultured overnight in RPMI 1640 medium supplemented with 10% FBS (Invitrogen), HEPEs, and glutamine (Invitrogen). Twenty-four hours later, cells were stained with anti–HA-PE (clone G68-IF3.3; Miltenyi Biotec) and anti–KIR2D-APC for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 3 × 10^6 events were collected using a FACSCalibur flow cytometer, and the data were analyzed, using FlowJo 8.8.7, after gating on GFP^+ cells as the transfected subset.

721.221 stable cell lines

Rhesus macaque MHC class I cDNAs were cloned into pQCXIP or pQCCXIN retroviral vectors (BD Clontech). These vectors were packaged into VSV-G pseudotyped murine leukemia virus–based particles by cotransfection with pVSV-G (BD Clontech) into GP2-293 cells (BD Clontech). Supernatant was collected from transfected GP2-293 cells 2 d posttransfection and concentrated by centrifugation in UltraTrex 50k filter centrifuge tubes (Millipore). 721.221 cells were transduced by incubation with concentrated virus for 3 h at 37°C. Three days later, cells were expanded under selection with 0.4 μg/ml purorycin (Invitrogen) or 500 μg/ml G418 (Calbiochem). Surface expression of transduced MHC class I molecules was verified by flow cytometry staining with a PE-conjugated pan-MHC class I–specific mAb (clone W6/32; DakoCytomation).

NK cell expansion and cell culture

PBMC (5 × 10^6 cells) were cultured with 1 × 10^7 gamma-irradiated K562 clone 9.mlbl21 cells (41) in a volume of 40 ml RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, glutamine, primocin (InvivoGen), and 50 U/ml IL-2 (AIDS Research and Reference Reagent Program). On days 3 and 5 after stimulation, cells were resuspended in fresh medium.

The expanded NK cells were restimulated on day 7 and weekly thereafter with additional gamma-irradiated K562 clone 9.mlbl21 cells at a 1:1 ratio. From day 7 onward, expanded cells were maintained at 4 × 10^5 cells/ml by resuspension in fresh medium twice to three times weekly.

NK cell sorting

T cells were depleted from expanded NK cell cultures by incubation with anti–CD3 Ab (clone 6G12), followed by immunomagnetic bead depletion with pan-mouse IgG Dynabeads (Dynal Biotech). Mamu-KIR3DL01- and KIR3DL01~ subsets were separated by FACS using a pan-KIR2D–specific mAb (clone NKVFS1) that cross-reacts with a subset of Mamu-KIR3DL01 allotypes. NK cells were stained with anti–CD3-Pacific Blue (clone SP34-2; BD Biosciences), anti–NKG2A-Pacific Blue, anti–CD3-FITC (clone SP34; BD Biosciences), and NKVFS1-APC. NKVFS1*CD3*NKG2A* and NKVFS1*CD3*NKG2A* subsets were sorted using a FACSAria (BD Biosciences). These sorted NK cell subsets were maintained as described above by restimulation with gamma-irradiated K562 clone 9.mlbl21 cells.

CD107a degranulation assay

PBMC (1 × 10^6 cells) were stimulated for 6 h with parental 721.221 cells, or with 721.221 cells that constitutively express rhesus macaque MHC class I molecules, at a 5:1 ratio in the presence of anti–CD107a-PE-Cy5 (clone HA3; BD Biosciences), GolgiStop, and GolgiPlug (BD Biosciences).

The cells were then stained with anti–CD3–FITC, anti–NKG2A-PE, and NKVFS1-APC for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 2 × 10^6 lymphocyte events were collected using a FACSCalibur flow cytometer, and the data were analyzed using FlowJo 8.8.7.

Calcine acetoxyxymethyl ester cytotoxicity assay

Parental 721.221 cells or 721.221 cells expressing rhesus macaque MHC class I molecules were stained with calcine acetoxyxymethyl ester (CAM) (Invitrogen) at a 1:100 dilution for 1 h at 37°C. Cells were washed and then incubated with KIR3DL01~ or KIR3DL01~ NK cells for 4 h at E:T ratios between 0.5:1 and 10:1. The release of CAM into the supernatant was measured.
measured using a fluorescent plate reader (excitation 485 nm, absorption 530 nm). Percent-specific lysis was calculated as (test release – spontaneous release)/maximum release – spontaneous release.

**KIR sequencing**

Total cDNA was isolated from PBMC using an AllPrep DNA/RNA mini kit (Qiagen). cDNA was synthesized using a SuperScript III cDNA first-strand synthesis system using Oligo(dT) primers (Invitrogen). cDNA was PCR amplified using Phusion high-fidelity DNA polymerase (New England Biolabs) with two primer sets for each animal. One primer set produced a 669-bp amplicon containing Roche/454 titanium adaptors and multiplex identifier tags attached to the following sequence-specific primer sequences: KIR-405-F, 5′-AGGTCCCCTGGTGAAATCAG-3′; and KIR-1004-R, 5′-GTCAGACCATGTCGCTCAT-3′. The full-length KIR alleles were amplified with Phusion high-fidelity DNA polymerase (New England Biolabs) using the following conditions: 98°C for 30 s, 28 cycles of 98°C for 5 s, 60°C for 10 s, and 72°C for 30 s, followed by a 5-min extension at 72°C. These amplicons were purified using AMPure XP size selection beads (Beckman Coulter), quantitated using picogreen dye (Qubit), and pooled at equimolar ratios. The pool was amplified using emulsion PCR, the emulsions broken, and beads containing DNA with both adaptors were enriched and sequenced on a Roche/454 GS Junior using Titanium technology and standard protocols provided with the sequencing kits.

The other primer set amplified the full-length KIR coding sequence with a variety of expected sizes up to 1500 bp, depending on the KIR alleles within the animal, with the following sequence-specific primer sequences: KIR5-UTR-3, 5′-AGGCTCACTAAGTGGAAGG-3′; and KIR3-UTR-5, 5′-CTCTGGTCAATGTTGGTTAAGG-3′. The PCR conditions for this amplicon were published previously (42). These amplicons were purified using Agencourt AMPure XP size selection beads (Beckman Coulter), quantitated using picogreen dye (Qubit), and pooled at equimolar ratios. The pool was amplified using emulsion PCR, the emulsions broken, and beads containing DNA with both adaptors were enriched and sequenced on a Roche/454 GS Junior using Titanium technology and standard protocols provided with the sequencing kits.

The other primer set amplified the full-length KIR coding sequence with a variety of expected sizes up to 1500 bp, depending on the KIR alleles within the animal, with the following sequence-specific primer sequences: KIR5-UTR-3, 5′-AGGCTCACTAAGTGGAAGG-3′; and KIR3-UTR-5, 5′-CTCTGGTCAATGTTGGTTAAGG-3′. The full-length KIR alleles were amplified with Phusion high-fidelity DNA polymerase (New England Biolabs) using the following conditions: 98°C for 30 s, 28 cycles of 98°C for 5 s, 60°C for 10 s, and 72°C for 30 s, followed by a 5-min extension at 72°C. These amplicons were purified using AMPure XP size selection beads (Beckman Coulter), quantitated using picogreen dye (Qubit), and pooled at equimolar ratios. The pool was amplified using emulsion PCR, the emulsions broken, and beads containing DNA with both adaptors were enriched and sequenced on a Roche/454 GS Junior using Titanium technology and standard protocols provided with the sequencing kits.

**KIR genotype analysis**

The Roche/454 sequences were trimmed for quality (error probability limit of 0.01), and then, reads >300 bp were de novo assembled with 100% minimum overlap identity and 0% mismatches per read using Geneious version 6.1 (43, 44). Consensus sequences generated from the assembly were trimmed with an error probability limit of 0.0001, 0 low-quality bases, and 0 maximum ambiguities and trimmed for primer sequences. These sequences were then de novo assembled at 99% minimum overlap identity and 1% maximum mismatches per read, and consensus sequences were trimmed with the parameters above. These consensus sequences representing each allele within an animal were used as a backbone against which the MiSeq 250-bp paired-end reads were mapped over multiple iterations using Geneious version 6.1 software. To begin, MiSeq-generated paired-end sequences were set to pair the ends and trimmed for a quality of 0.01. Sequences >150 bp were trimmed for primers, and sequences >100 bp were subjected to multiple iterations of assembly. The first iteration consisted of custom assembling the paired and trimmed MiSeq reads against a list of consensus sequences (backbones) representing the alleles found in each animal by the Roche/454 analysis with a minimum overlap identity of 100% over a minimum overlap of 95 bp, 0% mismatches, and mapping best matches to none to avoid sequences mapping to more than one backbone. These Roche/454-based consensus sequences were elongated by the paired ends during this assembly and were then trimmed with an error probability limit of 0.0001. The elongated and trimmed consensus sequences generated from both the Roche/454 and MiSeq sequences were used as the backbone in place of the Roche/454 sequences for the next iteration of mapping the paired MiSeq sequences against. This iterative mapping continued until the KIR alleles no longer extended. Many alleles were extended from the 669-bp Roche/454 read length to full-length including start and stop codons for each allele found within an animal. The final sequence assemblies were then blasted against a database of all KIRs published in the Genbank to determine the identity of the KIR alleles for each animal. Allele designations were assigned by the Immuno Polymorphism Database (45).

**Phylogenetic analysis of rhesus and human KIRs**

Full-length amino acid sequences were aligned using MacVector 12.0.6 (MacVector). Phylogenetic trees were generated by the Neighbor-Joining method using MEGA 5.2 (46). The bootstrap method with 500 replicates was used with pairwise deletion, and the Poisson correction was used in computing evolutionary distances.

**Results**

A mAb to human KIR2D stains rhesus macaque NK cells and CD8+ T cells

We previously observed that a mAb to human KIR2D (clone NKVFS1) stained a PBL population in some rhesus macaques (37). Further characterization of this population revealed that the majority of these cells express Ags typical of NK cells, including CD8, CD16, NK2G2D, and NK2G2A (Supplemental Fig. 1A). The NKVFS1 population consisted of NK cells (CD3− CD8− NK2G2A+) and, to a lesser extent, CD8+ T cells (CD3+ CD8− NK2G2A+) (Supplemental Fig. 1B). NKVFS1+ cells were observed in each of the four NK cell subsets defined by CD16 and CD56 expression (Supplemental Fig. 1C). These results, and the similar staining pattern observed for Mamu-KIR3DL05 on PBLs (37), suggest that NKVFS1 cross-reacts with a rhesus macaque KIR.

**NKVFS1 binds to Mamu-KIR3DL01 alleles with an aspartic acid residue at position 233 of the D2 domain**

To identify the rhesus macaque KIR bound by NKVFS1, each of the KIR alleles cloned from an NKVFS1+ animal were expressed from a construct with an HA tag at the N terminus of the D0 domain (37). Jurkat cells were transfected with these constructs and stained with NKVFS1 and an HA-specific mAb. For cells expressing Mamu-KIR3DL01*001, KIR expression correlated with NKVFS1 staining (Fig. 1). In contrast, cells expressing another Mamu-KIR3DL01 allele from this animal, Mamu-KIR3DL01*002, did not stain with NKVFS1. Because the Ig-like domains of these molecules differ by only 8 aa (Fig. 2A), reciprocal substitutions at each of these positions were tested to define the epitope bound by NKVFS1.
NKVFS1. An aspartic acid to histidine change at position 233 (D233H) of Mamu-KIR3DL01*001 abrogated staining by NKVFS1, whereas substitutions at positions 71, 93/96/98, 104, 173, or 181 did not affect NKVFS1 staining (Fig. 2B). Conversely, a histidine to aspartic acid substitution at position 233 (H233D) in Mamu-KIR3DL01*002 resulted in staining with NKVFS1. These results indicate that residue 233 of Mamu-KIR3DL01*001, predicted to be a surface residue in the D2 domain (36), differentiates allotypes of Mamu-KIR3DL01 that bind NKVFS1 from those that do not. This distinction was confirmed by positive NKVFS1 staining of cells expressing Mamu-KIR3DL01*014, *017, or *020 (Fig. 2C), all of which have an aspartic acid at position 233.

Identification of MHC class I ligands for Mamu-KIR3DL01

Having identified Mamu-KIR3DL01 D233 allotypes as the surface Ag bound by NKVFS1, we next sought to define the MHC class I ligands recognized by this KIR. The NKVFS1 Ab was used to identify additional rhesus macaques expressing Mamu-KIR3DL01 D233, and the presence of alleles coding for this KIR was confirmed by deep sequencing (Table I). PBMC from four Mamu-KIR3DL01+ macaques were stimulated with MHC class I–deficient 721.221 cells (47), or 721.221 cells that constitutively express rhesus macaque MHC class I molecules, and stained for CD107a as a degranulation marker. The frequency of NK cells with surface CD107a was compared between Mamu-KIR3DL01+ (NKVFS1+) and -KIR3DL01− subsets from the same animal. Whereas a similar percentage of Mamu-KIR3DL01+ and -KIR3DL01− NK cells degranulated in response to parental 721.221 cells and 721.221 cells expressing Mamu-A1*001:01, -B*017:01, -B*022:01, and -B*056:01, CD107a upregulation by the Mamu-KIR3DL01+ subset was selectively suppressed by incubation with 721.221 cells expressing Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 (Fig. 3A). This result was reproducible using NK cells from four unrelated animals (Fig. 3B).

To corroborate these results, NK cell recognition of 721.221 cells expressing rhesus macaque MHC class I molecules was evaluated in cytotoxicity assays. Primary NK cells were expanded by stimulation with gamma-irradiated K562 clone 9.mbIL21 cells, which express CD64 (FcyRI), CD86 (B7-2), CD137L (4-1BBL), truncated CD19, and membrane-bound IL-21 (48). Expanded NK cells were sorted into Mamu-KIR3DL01+ and -KIR3DL01− subsets by staining with the NKVFS1 Ab and were used as effector cells in cytotoxicity assays with 721.221 cells expressing rhesus MHC class I molecules as target cells. The 721.221 target cells were labeled with CAM, a cell-permeable dye that is converted to fluorescent calcein by cellular esterases (49, 50), and incubated with NK cells for 4 h. The release of CAM into the supernatant was measured using a fluorescent plate reader to calculate the percent-specific lysis. The Mamu-KIR3DL01− NK cells lysed all of the 721.221 cell lines regardless of MHC class I expression. However, the cytolytic activity of the Mamu-KIR3DL01+ NK cells was inhibited by certain MHC class I molecules. Whereas 721.221 cells expressing Mamu-A1*001:01, -B*017:01, or -B*056:01 were lysed as efficiently as parental 721.221 cells by Mamu-KIR3DL01− NK cells.

**FIGURE 2.** Residue 233D is essential for NKVFS1 binding to Mamu-KIR3DL01*001. (A) An amino acid alignment shows residues that differ between Mamu-KIR3DL01*001 and -KIR3DL01*002 in D0, D1, and D2. Jurkat cells were electroporated with constructs expressing HA-tagged KIR with reciprocal substitutions at positions that differ between Mamu-KIR3DL01*001 and -KIR3DL01*002 (B), or constructs expressing five different Mamu-KIR3DL01 alleles (C). Staining of GFP+ cells for HA and NKVFS1 is shown. Data shown is representative of results obtained in three independent experiments.
NK cells, target cells expressing Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 inhibited lysis by Mamu-KIR3DL01+ NK cells (Fig. 3C). The intermediate inhibition shown with target cells expressing Mamu-B*022:01 was only observed with NK cells from two of three animals. In contrast, the inhibition of Mamu-KIR3DL01+ NK cells by Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 was reproducible with NK cells from three animals (Fig. 3D). These data corroborate results obtained by CD107a staining and define these four molecules as ligands for Mamu-KIR3DL01.

**Residues in the Bw4 epitope contribute to recognition by Mamu-KIR3DL01**

Because the rhesus macaque MHC class I molecules identified as ligands for Mamu-KIR3DL01 all have a Bw4 motif at positions 77-83 in the α1 domain (34), substitutions were introduced into this sequence to determine if the Bw4 motif contributes to recognition by Mamu-KIR3DL01. Mamu-B*065:01 residues at positions that differ between Bw4 and Bw6 motifs (77, 80, 81, 82, and 83) were exchanged, separately and in combination, with the corresponding Bw6 residues at these positions. 721.221 cells expressing these Mamu-B*065:01 mutants were tested for recognition by sorted primary Mamu-KIR3DL01+ and -KIR3DL01− NK cells from the same animal in CAM cytotoxicity assays. Whereas none of the MHC class I mutants inhibited the cytolytic activity of the Mamu-KIR3DL01− NK cells, 721.221 cells expressing Mamu-B*065 with an alanine to lysine substitution at position 81 (A81L) or a lysine-to-arginine substitution at position 82 (L82R) inhibited the cytolytic activity of Mamu-KIR3DL01+ NK cells to a similar

---

**Table I. Mamu-KIR3DL01 alleles expressed by the rhesus macaques in this study**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Mamu-KIR3DL01</th>
<th>Residues 230–236</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*001</td>
<td>TSFDMYH</td>
</tr>
<tr>
<td>2</td>
<td>*016</td>
<td>TSFDMYH</td>
</tr>
<tr>
<td>3</td>
<td>*019</td>
<td>TSFDMYH</td>
</tr>
<tr>
<td>4</td>
<td>*019</td>
<td>TSFDMYH</td>
</tr>
<tr>
<td>5</td>
<td>*019</td>
<td>TSFDMYH</td>
</tr>
</tbody>
</table>

The residue indicated in bold is the aspartic acid at position 233 of Mamu-KIR3DL01 that is necessary for binding by the mAb NKVFS1.

---

**FIGURE 3.** Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 are ligands for Mamu-KIR3DL01. (A) PBMC were stimulated overnight with parental 721.221 cells or 721.221 cells expressing the indicated MHC class I molecules at a 5:1 ratio in the presence of a mAb to CD107a and stained with NKVFS1 and Abs to CD3 and CD8. After gating on CD3+CD8+ lymphocytes, the upregulation of CD107a on NKFSV1+ versus NKFSV1− NK cell subsets was assessed. (B) The frequency of CD107a upregulation for Mamu-KIR3DL01+ and -KIR3DL01− populations is summarized for four different animals where error bars indicate ±1 SD. Asterisks indicate a significant difference (*p < 0.05; **p < 0.01; ***p < 0.005 by two-way ANOVA with Dunnett’s test) between Mamu-KIR3DL01+ and -KIR3DL01− NK cells from the same animal that were coincubated with parental 721.221 cells or 721.221 cells expressing the indicated rhesus macaque MHC class I molecules at the indicated E:T ratios. Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. Percent-specific lysis is defined as (test release - spontaneous release)/(maximum release - spontaneous release). Results are representative of those obtained with expanded cells from three different animals and the compiled results for Mamu-KIR3DL01+ NK cells from these animals are presented in (D). Error bars, ±1 SD. Asterisks indicate a significant difference (*p < 0.05; ****p < 0.001 by two-way ANOVA with Dunnett’s test) between coincubation with 721.221 parental cells and coincubation with the indicated cell line at all E:T ratios shown.
extent as cells expressing wild-type Mamu-B*065 (Fig. 4A). However, 721.221 cells expressing Mamu-B*065 with an asparagine-to-serine substitution at position 77 (N77S), a threonine-to-asparagine substitution at position 80 (T80N), an arginine-to-glycine substitution at position 83 (R83G), or Bw6 residues at each of the five positions (Mamu-B*065-Bw6), were susceptible to lysis by Mamu-KIR3DL01+ NK cells (Fig. 4A). The susceptibility of cell lines expressing Mamu-B*065-N77S, -T80N, -R83G, or -Bw6 to lysis by Mamu-KIR3DL01+ NK cells was reproducible using primary NK cells isolated from three unrelated animals (Fig. 4B). Thus, residues 77, 80, and 83 within the Bw4 epitope contribute to ligand recognition by Mamu-KIR3DL01.

**Additional MHC class I residues necessary for recognition by Mamu-KIR3DL01**

Although Mamu-B*017:01 is identical to Mamu-B*007:01 and -B*065:01 at positions 77–83, Mamu-B*017:01 did not suppress the activation of Mamu-KIR3DL01+ NK cells, indicating that the presence of a Bw4 motif in the α1 domain is not sufficient for recognition by Mamu-KIR3DL01. To identify additional residues necessary for this interaction, reciprocal substitutions between Mamu-B*017:01 and -B*065:01 were introduced at polymorphic sites predicted to be contact residues based on the KIR3DL1-HLA-B*57 crystal structure (Fig. 5A) (36). 721.221 cells expressing these MHC class I mutants were tested for recognition in a CAM cytotoxicity assay with sorted Mamu-KIR3DL01+ and -KIR3DL01+ NK cells from the same animal. None of the MHC class I mutants inhibited the cytolytic activity of the Mamu-KIR3DL01+ NK cells. Whereas 721.221 cells expressing wild-type Mamu-B*065:01 suppressed killing by Mamu-KIR3DL01+ NK cells, substitutions in Mamu-B*065 at positions 76 (G76E), 142 (F142N), and 149 (A149G) abrogated this inhibition (Fig. 5B). Consistent with a role for these residues in recognition by Mamu-KIR3DL01, a combination of all three reciprocal substitutions was necessary to efficiently inhibit the cytolytic activity of Mamu-KIR3DL01+ NK cells in the context of Mamu-B*017:01 (Fig. 5B). These results were reproducible with Mamu-KIR3DL01+ NK cells from three different animals (Fig. 5C). Therefore, in accordance with the three-dimensional structure of human KIR3DL1 in complex with HLA-B*57, additional residues in the α1 and α2 domains, including G76, F142, and A149, contribute to ligand recognition by Mamu-KIR3DL01.

**Comparison of rhesus macaque and human Bw4 ligands**

Many of the residues required for Mamu-KIR3DL01 recognition of rhesus macaque Bw4 molecules are also contact residues in the human KIR3DL1-HLA-B*57 crystal structure (36). An amino acid alignment of HLA-B*57 and the seven Mamu-B molecules investigated in this study demonstrates that 13 of the 17 predicted KIR contact residues are conserved among these molecules (Fig. 6A). Notably, residues 76 and 142 differ between HLA-B*57 and the Mamu-KIR3DL01 ligands, despite their importance for KIR recognition in both species. These residues, along with residue 149, are also polymorphic among the Mamu-B molecules studied. Whereas the four molecules identified as ligands for Mamu-KIR3DL01 have residues G76, F142, and A149, the nonligands (Mamu-B*017:01, -B*022:01, and -B*065:01) have an asparagine at position 142 (N142), with Mamu-B*017:01 also differing at the two other positions. These three residues, and the Bw4 motif residues 77, 80, and 83, are located at surface exposed positions near the C terminus of the α1-domain and the N terminus of the α2-domain (Fig. 6B), partially overlapping the footprint of KIR3DL1 bound to HLA-B*57 (36).

**Mamu-KIR3DL01 is phylogenetically distinct from human KIR3DL1**

Despite their shared specificity for Bw4 ligands and a coincidental similarity in nomenclature, Mamu-KIR3DL01 and human KIR3DL1 are not orthologous gene products. This is illustrated by phylogenetic comparisons of the predicted amino acid sequences for human and rhesus macaque KIRs. In contrast to Mamu-KIR2DL04 and human KIR2DL4, which cluster together as the most conserved KIR between humans and rhesus macaques (20), Mamu-KIR3DL01 and human KIR3DL1 are separated by deep branch lengths and are more similar to other KIRs of their respective species than they are to each other (Fig. 7A). A nearly identical tree was obtained from an analysis that was restricted to the D1 and D2 domains, indicating that the shared specificity of Mamu-KIR3DL01 and human KIR3DL1 for Bw4 molecules is not the result of greater similarity in their ligand-binding domains (data not shown). Indeed, only 7 of the 16 residues in the D1 and D2 domains of KIR3DL1 that participate in interactions with HLA-B*57 match residues in Mamu-KIR3DL01 (Fig. 7B). Interestingly, all of these conserved residues are in the D2 domain. Whereas 7 of the 11 HLA contact residues of KIR3DL1 are conserved in the D2 domain of Mamu-KIR3DL01, none of five contacts matches D1 domain residues of Mamu-KIR3DL01 (Fig. 7B, 7C). Thus, the specificity of Mamu-KIR3DL01 for Bw4 ligands could not have been predicted based on sequence similarity with human KIR3DL1.
Discussion

The identification of MHC class I ligands for rhesus macaque KIRs is important for studying NK cell responses in this nonhuman primate model for AIDS and other infectious diseases. KIRs are of particular interest in the context of viruses because of genetic associations between KIR and MHC class I alleles and the ability to control certain viral infections, including HIV (14, 15). In this study, we identified a mAb that binds selectively to D233 allotypes of Mamu-KIR3DL01. Using this mAb to distinguish KIR3DL01+ NK cells, we identified Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 as ligands for Mamu-KIR3DL01. We also defined residues in the α1 and α2 domains of these molecules that are essential for recognition by Mamu-KIR3DL01, facilitating the prediction of additional ligands for this KIR. These results reveal similarities in the recognition of Bw4 ligands by rhesus macaque KIR3DL01 and human KIR3DL1, despite differences in the MHC class I contact residues of these receptors and the considerable divergence of KIR and MHC class I genes since these species last shared a common ancestor.

Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 are the first B molecules to be identified as KIR ligands in the rhesus macaque. As products of the extensively duplicated and highly polymorphic Mamu-B genes, the diversity of these molecules reflects the diversity of Mamu-KIR3DL01, which is the most polymorphic of the rhesus macaque KIRs (32). Mamu-KIR3DL01 is also one of the most commonly expressed rhesus macaque KIRs (expressed by ~85–95% of Indian origin animals (31–33, 42)) and is thus likely to play a significant role in regulating NK cell responses in a majority of animals. Therefore, the identification of MHC class I ligands for Mamu-KIR3DL01 is especially important for investigating the role of NK cells in vaccine and pathogenesis studies in rhesus macaques.

The Bw4 ligands of Mamu-KIR3DL01 and human KIR3DL1 share a number of features necessary for KIR recognition. Corresponding to the importance of arginine at position 83 for HLA-Bw4 recognition by human KIR3DL1 (36, 51), this residue is also critical for Bw4 recognition by Mamu-KIR3DL01. Likewise, just as threonine or isoleucine at position 80 contributes to interactions with human KIR3DL1 (36), a threonine at this position is important for Bw4 recognition by Mamu-KIR3DL01. Similar amino acid positions outside of the Bw4 motif are also important for ligand recognition by these two KIRs. Replacement of amino acids 76, 142, or 149 of Mamu-B*065:01 with the corresponding residues from Mamu-B*017:01 impaired recognition by Mamu-KIR3DL01. Conversely, replacement of these three residues in Mamu-B*017:01 with the corresponding residues of the Mamu-Bw4 ligands conferred an interaction with Mamu-KIR3DL01. These results illustrate the similarity in Bw4 recognition by KIR3DL1 and Mamu-KIR3DL01 because these positions are also HLA contact sites for KIR3DL1 (36).

There are also differences in the MHC class I residues required for recognition by Mamu-KIR3DL01 and human KIR3DL1. Whereas KIR3DL1 is dependent on leucine at position 82 (51), Mamu-KIR3DL01 recognizes ligands with leucine or arginine at this position equally well. Moreover, although position 77 is a not a contact residue for KIR3DL1 (36), substitutions at this position do not affect KIR3DL1 interactions with HLA-B*51:01 or -B*15:13 (51),
position 77 is essential for Bw4 recognition by Mamu-KIR3DL01. An asparagine-to-serine substitution at this position in Mamu-B*065 abrogates its ability to inhibit Mamu-KIR3DL01 + NK cells. Thus, Mamu-KIR3DL01 and human KIR3DL1 have overlapping, but distinct, specificities for their respective ligands. Although KIR recognition of Bw4 ligands has been maintained since humans and macaques last shared a common ancestor, the KIRs conferring this specificity have not. Fewer than half of the HLA-B*57 contact residues of KIR3DL1 are conserved with Mamu-KIR3DL01. Most of the differences between these receptors are in the D0 and D1 domains, which together retain only 2 of 10 MHC class I contacts. In contrast, 7 of 11 MHC class I contacts are retained in the D2 domain. Greater conservation of the D2 domain may reflect greater evolutionary constraint to preserve core MHC class I binding interactions, while allowing the D0 and D1 domains more freedom to fine-tune ligand specificity. This possibility is consistent with structural data revealing greater complementarity in shape and charge of residues at the D2 interface (36) and the observation that polymorphisms in the D0 and D1 domains can affect the avidity and peptide-specificity of MHC class I interactions (37).

The cross-reactivity of the anti-human KIR2D mAb NKVFS1 with D233 allotypes of Mamu-KIR3DL01, which make up 20 of the 27 defined Mamu-KIR3DL01 allotypes (45), effectively doubles the number of KIR-defined NK cell subsets that can be studied in the rhesus macaque. Although several mAbs to rhesus macaque KIRs were recently described, only one was specific for a single KIR (52). Coincidentally, this mAb bound to Mamu-KIR3DL05 (52), which also can be stained with Mamu-A1*002 tetramers (37). The availability of reagents for differentiating NK cells expressing specific KIRs is essential for dissecting the role of KIRs in primary NK cell activation in vitro and for monitoring longitudinal changes of specific NK cell subsets in vivo. NKVFS1 will therefore be especially useful for monitoring phenotypic and functional changes in Mamu-KIR3DL01 D233+ NK cells after experimental infection of rhesus macaques with SIV and other viral pathogens.

**FIGURE 6. KIR contact residues of rhesus macaque Bw4 molecules.** (A) An amino acid alignment of the MHC class I α1 and α2 domains of HLA-B*5701 and Mamu-B*065:01, -B*007:01, -B*017:01, -B*022:01, -B*041:01, -B*056:01, and -B*058:02 with the predicted KIR contact residues shaded based on the HLA-B*57:KIR3DL1*001 crystal structure (36). (B) Crystal structure of HLA-B*57 (yellow) and bound peptide (cyan). Highlighted in red are positions important for recognition by Mamu-KIR3DL01 identified in this study: residues 76, 77, 80, 83, 142, and 149.
In summary, the current study identifies Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 as ligands for Mamu-KIR3DL01. Further characterization of the determinants of ligand recognition revealed that this KIR has a similar specificity for Bw4 molecules as human KIR3DL1. As one of the most common and most polymorphic KIRs in the rhesus macaque, identification of the MHC ligands for Mamu-KIR3DL01 provides insights into the evolutionary conservation and functional diversity of KIRs in non-human primates.

FIGURE 7.  Phylogenetic analysis of human and rhesus macaque KIRs. (A) Midpoint-rooted phylogenetic tree generated from full-length amino acid sequences using the Neighbor-Joining (NJ) method. Bootstraps were calculated with 500 iterations. (B) An amino acid alignment of the D0, D1, and D2 domains of human KIR3DL1 and allotypes of Mamu-KIR3DL01 with the predicted MHC ligand contact residues shaded based on the HLA-B*57: KIR3DL1*001 crystal structure (36). (C) Crystal structure of human KIR3DL1 with HLA-B*57 contact residues highlighted. Residues highlighted in red are conserved between KIR3DL1 and Mamu-KIR3DL01*001, whereas residues highlighted in yellow differ between the two KIRs.
class I molecules recognized by Mamu-KIR3DL01 provides an important foundation for studying NK cell responses in this non-human primate model. These observations also suggest that, despite the divergence of KIR and MHC class I genes (53), KIR specificity for Bw4 ligands has been maintained since rhesus macaques and humans last shared a common ancestor ~25 million years ago (54).

Acknowledgments

We are grateful to Dean Lee from the University of Texas (Houston, TX) for providing the K562 Clone 9.mbl.21 cells, and to Jacqueline Gillis, Fay Eng, and Yi Yu at the New England Primate Research Center for flow cytometry services.

Disclosures

The authors have no financial conflicts of interest.

References


20. Ouwehand, A. M., A. J. Moreland, B. Annis, R. P. Johnson, and P. Parham. 2005. Characterization of killer Ig-like receptor (KIR) ligands in Mamu-KIR3DL01 provides an important foundation for studying NK cell responses in this non-human primate model. These observations also suggest that, despite the divergence of KIR and MHC class I genes (53), KIR specificity for Bw4 ligands has been maintained since rhesus macaques and humans last shared a common ancestor ~25 million years ago (54).


