A Viral Immunoevasin Controls Innate Immunity by Targeting the Prototypical Natural Killer Cell Receptor Family

Oscar A. Aguilar,1,2,11 Richard Berry,3,6,11 Mir Munir A. Rahim,4,9 Johanna J. Reichel,5 Branka Popović,5 Miho Tanaka,1,2 Zhihui Fu,3 Gautham R. Balaji,3,6 Timothy N.H. Lau,1,2 Megan M. Tu,4 Christina L. Kirkham,5,2 Ahmad Bakur Mahmoud,4,8 Aruz Mesci,1,2 Astrid Krmpotić,5 David S.J. Allan,1,2,10 Andrew P. Makrigiannis,4,9,* Stipan Jonjić,5,* Jamie Rossjohn,3,6,7,* and James R. Carlyle1,2,12,*

1Department of Immunology, University of Toronto, Toronto, ON M5S 1A8, Canada
2Sunnybrook Research Institute, Toronto, ON M4N 3M5, Canada
3Infection and Immunity Program, Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia
4Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada
5Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, 51000 Rijeka, Croatia
6ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, VIC 3800, Australia
7Institute of Infection and Immunity, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK
8College of Applied Medical Sciences, Taibah University, 30001 Madinah Munawwarah, Kingdom of Saudi Arabia
9Present address: Department of Microbiology and Immunology, Dalhousie University, Halifax, NS B3H 4R2, Canada
10Present address: National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA
11Co-first author
12Lead Contact
*Correspondence: andrew.makrigiannis@dal.ca (A.P.M.), stipan.jonjic@medri.uniri.hr (S.J.), jamie.rossjohn@monash.edu (J.R.), james.carlyle@utoronto.ca (J.R.C.)
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SUMMARY

Natural killer (NK) cells play a key role in innate immunity by detecting alterations in self and non-self ligands via paired NK cell receptors (NKR). Despite identification of numerous NKR-ligand interactions, physiological ligands for the prototypical NK1.1 orphan receptor remain elusive. Here, we identify a viral ligand for the inhibitory and activating NKR-P1 (NK1.1) receptors. This murine cytomegalovirus (MCMV)-encoded protein, m12, restrains NK cell effector function by directly engaging the inhibitory NKR-P1B receptor. However, m12 also interacts with the activating NKR-P1A/C receptors to counterbalance m12 decoy function. Structural analyses reveal that m12 sequesters a large NKR-P1 surface area via a “polar claw” mechanism. Polymorphisms in, and ablation of, the viral m12 protein and host NKR-P1B/C alleles impact NK cell responses in vivo. We identify the long-sought foreign ligand for this key immunoregulatory NKR family and reveal how it controls the evolutionary balance of immune recognition during host-pathogen interplay.

INTRODUCTION

Natural killer (NK) cells are a subset of innate lymphoid cells (ILC) capable of recognizing an array of pathological target cells. As efficient sentinels responsible for the clearance of cancerous, virus-infected, transplanted, antibody-opsonized, and stressed cells, they discriminate between healthy-self, altered-self, and non-self targets through the integration of balanced signals delivered via multiple families of paired germline-encoded NK cell receptors (NKR) (Raulet and Vance, 2006).

Several NKR families have been described, many of which are grouped together in large genomic regions, such as the natural killer gene complex (NKC). In rodents, most NKR are C-type lectin-like proteins encoded within the NKC. These include the Ly49 (Klra1) receptors, the CD94/NKG2 (Klrd1/Klrc1) receptors, the NKG2D (Klrk1) receptor, and the NKR-P1 (Klrb1) receptors, which interact with genetically-linked C-type lectin-related (Clr/Clec2) proteins (Kirkham and Carlyle, 2014; Raulet and Vance, 2006).

There exist at least five functional mouse NKR-P1 receptors, three stimulatory isoforms (NKR-P1A,C,F), and two inhibitory isoforms (NKR-P1B,G) (Carlyle et al., 2008; Kirkham and Carlyle, 2014). Originally described almost 40 years ago (Glimcher et al., 1977), the NKR-P1 proteins were the first receptors to be identified as selectively expressed by NK cells. Yet to date, NKR-P1C, the prototypical NK1.1 antigen in B6 mice (Glimcher et al., 1977; Koo and Peppard, 1984; Ryan et al., 1992), and NKR-P1A remain orphan-activating receptors with unknown physiological ligands. On the other hand, the paired NKR-P1F and NKR-P1G receptors recognize overlapping “self” Cln/Clec2 ligands to balance signals during NK cell education and effector responses, while the inhibitory NKR-P1B receptor recognizes the broadly expressed self Cln-b (Clec2) ligand (Carlyle et al., 2004; Chen et al., 2011; Iizuka et al., 2003; Kveberg et al., 2009). To date, Cln-b has
Figure 1. MCMV Encodes a Clr-b-Independent NKR-P1B Immunevasin, the m02 Family Member, m12
Mouse NIH 3T3 fibroblasts were infected with MCMV overnight and then used as stimulators for BWZ reporter cells expressing a CD3ζ-NKR-P1B129 fusion receptor (BWZ.P1B129).

(A) Cell-surface expression of Clr-b on mock, MCMV-infected, or UV-treated MCMV-infected cells.
(B and C) BWZ.P1B129 reporter cell assays using the above stimulators in the absence or presence of blocking Clr-b mAb (10 μg/ml).
(D and E) Clr-b-deficient NIH 3T3.ΔClr-b cells, targeted by CRISPR/Cas9 genome editing, analyzed by flow cytometry and BWZ.P1B129 reporter assays following MCMV infection.

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been shown to be involved in “missing-self” recognition under diverse pathological states (Kirkham and Carlyle, 2014), including cancer (Carlyle et al., 2004), genotoxic and cellular stress (Fine et al., 2010), hematopoietic transplantation (Chen et al., 2015), immune escape of primary lymphoma cells (Rahim et al., 2015), and cytomegalovirus and poxvirus infection (Aguiar et al., 2015; Voigt et al., 2007; Williams et al., 2012), while the remainder of the self Clr ligands remain less well characterized.

Among pathogens recognized by NK cells, cytomegaloviruses (CMV) demonstrate co-evolution with their natural hosts, likely due to cycles of natural selection for viral versus host fitness. Since early responses to CMV infection are largely NK cell mediated, these viruses have evolved a diverse array of immunoevasins that antagonize NK cell function. For example, the MCMV m157 decoy immunoevasin binds directly to inhibitory Ly49C/I receptors from certain mouse strains, while the stimulatory Ly49H receptor has been evolutionarily adapted to directly recognize m157 as a foreign antigen, in turn dominantly establishing MCMV resistance in B6 mice (Arase et al., 2002).

Here, we identify the MCMV m02 family member, m12, as a functional decoy ligand that directly engages the mouse NKR-P1B inhibitory receptor and inhibits NK cell cytotoxicity. Importantly, we also demonstrate that m12 directly interacts with two stimulatory orphan receptors, the prototypical NK1.1 antigen, NKR-P1C, and NKR-P1A. These findings demonstrate an important role for the NKR-P1 receptor family in NK cell-mediated immunity to infection and identify m12 as the first physiological NKR-P1C(B6) ligand, almost four decades since its discovery as the prototypical NK1.1 antigen (Glimcher et al., 1977).

RESULTS

MCMV Encodes an NKR-P1B Decoy Immunoevasin

The murine NKR-P1B ligand, Clr-b (Clec2d), is rapidly lost in response to cellular infection by diverse viruses (Aguiar et al., 2015; Voigt et al., 2007; Williams et al., 2012). However, MCMV-infected fibroblasts also induce a Clr-b-independent NKR-P1B ligand (Aguiar et al., 2015). To establish whether this was due to a viral immunoevasin, we infected cells with different MCMV strains and used them as stimulators for BWZ.CD3/NCrP1B reporter cells, a stimulus that was blocked using Clr-b mAb, while weaker stimulation was observed upon live MCMV infection, which could not be blocked using Clr-b mAb (Figures 1A–1C). Thus, live MCMV infection is required to both downregulate host Clr-b and induce an alternative Clr-b-independent ligand.

To confirm that this signal was Clr-b independent, we generated Clr-b-deficient NIH 3T3 cells (NIH 3T3.ΔClr-b) by CRISPR/Cas9-mediated genome editing. Only MCMV-infected, but not mock-treated, NIH 3T3.ΔClrb cells stimulated BWZ.P1B129 reporter cells (Figures 1D and 1E). Employing another approach, we compared MCMV infection of primary adult ear fibroblasts (AEF) from B6 WT and B6.Clr-b–/– mice (Chen et al., 2015); here, BWZ.P1B129 reporter cells responded only to infected B6.Clr-b–/– AEF, whereas WT AEF behaved similarly to NIH 3T3 cells (Figures S1A and S1B). Notably, similar results were observed upon MCMV infection of primary Clr-b+/β2m–/– AEF cells (Figure S1C) and rat fibroblasts (data not shown). Thus, a Clr-b/β2m-independent NKR-P1B ligand is induced upon MCMV infection of mouse and xenogeneic rat fibroblasts.

Since NKR-P1B is inhibitory, these results suggest that MCMV may encode a decoy immunoevasin. To test this, we analyzed distinct MCMV strains and large genomic deletion mutants to determine whether any isolates lacked the NKR-P1B reporter signal upon infection. The MCMVSmith and MCMVK181 strains both generated strong BWZ.P1B129 reporter signals; however, none of the mutants tested nor the parental BAC-generated MCMVMMW97 strain elicited significant BWZ.P1B129 reporter responses (Figures 1F and 1G). This suggested that MCMVMMW97 might possess a polymorphism or loss-of-function mutation that abrogated BWZ.P1B129 reporter cell recognition.

The MCMV-Encoded m12 Immunevasin Targets NKR-P1B-Mediated Inhibition

To take advantage of the discrepancy between the MCMV strains, we conducted unbiased whole-transcriptome RNA-sequencing (RNA-seq) using MCMV-infected NIH 3T3 cells to identify differences between MCMVSmith and MCMVMMW97. RNA-seq reads were then mapped to the MCMV genome sequence using TopHat analysis and visualized using the IGV browser. Since numerous MCMV immunoevasin genes are located at the left or right genomic termini, such as the m02 and m145 families, we analyzed differences within these regions (Figures 1H and 1I). Within the m145 family, few differences in transcript expression or genetic polymorphisms were noted between the two MCMV strains (Tables S1 and S2). In contrast, the m02 family displayed significantly more differences in transcript profiles and single-nucleotide polymorphisms (SNP). To screen these alleles functionally, we independently cloned all of the MCMVSmith m02 and m145 family members and some other known immunoevasins (in total, 32 gene products spanning ~15% of the MCMV genome) into a mammalian expression vector, and then we tested them individually using human 293T transfectants as stimulators for BWZ.P1B129 reporter cells. While none of the m145 family gene products yielded positive results, a single gene product in the m02 family, m12, was capable of directly stimulating BWZ.P1B129, but not parental BWZ reporter cells (Figures 1J and 1K). Thus, m12 is an NKR-P1B decoy ligand.
m12 Is a Glycosylated Type I Transmembrane Protein

We next characterized the topology, localization, and functional expression of m12. While a transmembrane (TM) domain was identified near the C terminus of m12 (Figure S2A), SignalP did not detect an N-terminal signal peptide. However, bioinformatic analyses identified a second in-frame ATG codon that yielded a canonical signal peptide (when analyzed by SignalP) and translational start site (according to the NetStart server). To validate the second ATG translational initiation site, we generated several m12 constructs, including native m12 (m12ATG1, m12ATG2) and N-terminal FLAG-tagged m12 with either a native or preprotrypsin signal peptide (m12NSP-FLAG, m12PSP-FLAG). These constructs were then analyzed using 293T transient transfectants, flow cytometry, and BWZ.P1B129 reporter cell assays (Figures S2B and S2C).

Notably, cell-surface m12FLAG was detected on transfectants using ATG2 and both signal peptides (m12NSP-FLAG, m12PSP-FLAG), confirming a type I TM topology (Figure S2B). In addition, all of the m12 constructs stimulated BWZ.P1B129 reporter cells, with m12ATG1 yielding the weakest signal (Figure S2C). Collectively, these results suggest that ATG2 is preferred, the native signal peptide is cleaved, and m12 is expressed as a functional type I TM cell-surface protein.

Recognition of m12 by the Prototypical NK1.1 Activating Receptor, NKR-P1CB6

We next tested whether m12 was capable of interacting with paralogous host receptor isoforms and alleles. To this end, BWZ reporter cells bearing CD3ζ-fusion NKR-P1A/B/C/F/G receptors were constructed from the FVB, B6, and 129/BALB-strain alleles. Notably, the NKR-P1B129/BALB alleles react with NK1.1 mAb, whereas the NKR-P1B129/BALB alleles do not (Carlyle et al., 1999, 2006; Ryan et al., 1992), and NKR-P1A/B/C forms a separate clade from NKR-P1F/G (Figure 2A).

When BWZ.NKR-P1 reporter cells were stimulated using 293T transfectants of the m12Smith allele, the stimulatory NKR-P1A, NKR-P1F, and inhibitory NKR-P1G receptors did not appreciably recognize m12Smith (data not shown), while all inhibitory NKR-P1B receptor alleles recognized m12 Smith (Figures 2B, 2D, and 2F). Remarkably, the m12Smith immunoevasin also significantly interacted with the stimulatory NKR-P1CB6 receptor, demonstrating that m12Smith is a much-anticipated natural ligand for the prototypical NK1.1 antigen (Glimcher et al., 1977; Koo and Peppard, 1984; Ryan et al., 1992) (Figure 2C).
Figure 3. Direct Binding of m12 Protein to NKR-P1 Receptors

(A and B) Surface plasmon resonance sensograms and equilibrium binding curves (where appropriate) are shown for the binding of (A) m12Smith (0.025–50 μM) and (B) m04Smith (100 μM) to various NKR-P1 or Ly49 receptors, as indicated. Equilibrium dissociation constants (Kd) were calculated from two independent measurements, with each experiment performed in duplicate. Error bars represent the SEM. NB, no binding; ND, not determined.

(C) Sensograms are shown for the binding of 50 μM m12Smith to NKR-P1BB6 and NKR-P1CB6 either before (solid lines) or after (dashed lines) exposure of immobilized receptor to the NK1.1 antibody.

(D) The relative binding of NK1.1 mAb to immobilized NKR-P1BB6 and NKR-P1CB6.

(E) m12Smith tetramer binding to HEK293T cells transfected with NKR-P1 alleles from B6, 129, and FVB strains. Numbers correspond to mean fluorescence intensities of the GFP+ gate.

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Interestingly, the NK1.1+ NKR-P1C\textsubscript{FVB} allele also recognized m12\textsubscript{Smith}, but the NK1.1+ NKR-P1C\textsubscript{129/BALB} allele did not (Figures 2E and 2G), demonstrating that paralogous stimulatory recognition of m12\textsubscript{Smith} is not unique to B6 mice.

To confirm these results reciprocally, we also generated BWZ.m12\textsubscript{Smith} reporter cells and used them to semiquantitatively assess interactions with all NKR-P1 alleles on 293T transfectants (Figure 2H). Using this approach, BWZ.m12\textsubscript{Smith} reporters recognized NKR-P1B\textsubscript{B6,129,FVB}, NKR-P1A\textsubscript{B6,129,FVB}, and NKR-P1C\textsubscript{B6,FVB}, but not NKR-P1C\textsubscript{129}; however, the stimulatory NKR-P1A/C paralogs interacted with m12\textsubscript{Smith} more weakly than the inhibitory NKR-P1B counterparts in this cellular context. This was not due to splice variants nor inefficient surface expression of the stimulatory isoforms, as reciprocal domain swaps of NKR-P1B and NKR-P1C stimulated m12\textsubscript{Smith} reporters similarly to native isoforms (Figures S2E and S2F). Collectively, dual recognition of m12 by paralogous NKR-P1 receptors with opposing signals suggests that NKR-P1:m12 interactions are evolving under selection pressures driven by host-pathogen interactions.

**Direct Interactions between the NKR-P1A/B/C and m12 Proteins**

Next, we expressed soluble m12 and NKR-P1 receptor ectodomains and assessed their interaction by surface plasmon resonance (SPR). Here, m12\textsubscript{Smith} bound with similar affinity to the inhibitory NKR-P1B\textsubscript{B6} (K\textsubscript{d} = 5.8 \textmu M) and stimulatory NKR-P1C\textsubscript{B6} (K\textsubscript{d} = 4.1 \textmu M) receptors (Figure 3A). As observed using reporter cell assays, m12 recognition was found to be highly allele sensitive, with m12\textsubscript{Smith} binding to NKR-P1B\textsubscript{129} and NKR-P1B\textsubscript{FVB} with affinities 8-fold (K\textsubscript{d} = 0.7 \textmu M) and 40-fold (K\textsubscript{d} = 0.15 \textmu M) higher than observed for NKR-P1B\textsubscript{B6} (Figure 3A). As controls, m12\textsubscript{Smith} did not bind to the structurally related Ly49A\textsubscript{B6} receptor and m04 did not bind appreciably to any NKR-P1 alleles tested (Figure 3B). Notably, binding of m12\textsubscript{Smith} to NKR-P1C\textsubscript{B6}, but not NKR-P1C\textsubscript{129}, could be blocked using NK1.1 mAb (Figure 3C), which only recognizes NKR-P1C\textsubscript{B6} (and NKR-P1C\textsubscript{FVB}) but not NKR-P1C\textsubscript{129} (Figure 3D) (Carlyle et al., 1999, 2006; Ryan et al., 1992).

We further generated m12\textsubscript{Smith} tetramers and used these to stain transient 293T.NKR-P1 transfectants using an IRES-GFP reporter vector. These m12 tetramers were found to bind directly to cells expressing NKR-P1A\textsubscript{B6,129,FVB}, NKR-P1B\textsubscript{B6,129,FVB}, and NKR-P1C\textsubscript{B6,FVB}, but not NKR-P1C\textsubscript{129}, NKR-P1F, or NKR-P1G (Figure 3E). Notably, m12\textsubscript{Smith} tetramer staining of the activating NKR-P1A/C receptors was significantly lower than for the inhibitory NKR-P1B receptors at similar IRES-GFP reporter levels, indicating a weaker avidity of m12\textsubscript{Smith} for the cellular NKR-P1A/C isoforms. In addition, the m12\textsubscript{Smith} tetramers bound to fresh ex vivo B6-strain lymphocyte subsets, which mainly consisted of the NKR-P1B\textsubscript{+} subset of NK cells, with significantly lower staining observed for the remaining NK cells, NKT cells, and T cells, likely due to the weaker m12:NKR-P1A/C interactions at the cell surface (Figure 3F). Interestingly, differential results were observed for ex vivo lymphocytes from a number of distinct mouse strains (B6.Cir-b\textsuperscript{-/-}, 129, BALB/c, FVB; Figure S3A), suggesting that differential susceptibility to MCMV m12 variants might be expected.

Reciprocally, we also generated NKR-P1B tetramers from the B6, 129, and FVB strains and used these to stain m12-expressing cells. Here, NKR-P1B\textsubscript{B6,129,FVB} tetramers variably stained transient 293T.m12 and 293T.Cir-b transfectants, stable BWZ.m12 transductants, and NIH 3T3 cells infected with MCMV variants, demonstrating their utility in the absence of m12 mAb (Figures S3B–S3C and data not shown). Importantly, differences in NKR-P1 tetramer staining levels were observed using distinct NKR-P1 receptors across several m12 variants from distinct MCMV strains; these differences likely reflect their respective avidities for m12 variants at the cell surface (as they follow a similar affinity hierarchy to SPR results, where FVB > 129 > B6 for m12\textsubscript{Smith}, see also below). Collectively, the above results demonstrate that there exists allelic and isoform specificity among the direct m12:NKR-P1A/B/C protein-protein interactions.

**Structure of m12\textsubscript{Smith} Complexed to NKR-P1B\textsubscript{B6}**

We next determined the structure of NKR-P1B\textsubscript{B6} bound to m12\textsubscript{Smith} (Figure 4; Figure S4; Table S3). NKR-P1B adopted a classical C-type lectin-like domain (CTLD) fold and possessed a compact j3-b4 loop rather than the extended conformation observed in murine NKR-P1A (Kolenko et al., 2011) (Figure S4A). Overall, m12 adopts an eight-stranded \( \beta \)-sandwich fold comprising one large (AIDEF) and one small (BGHC) \( \beta \) sheet, similar to that described for the m04 immunoevasin (Figures S4B–S4D) (Berry et al., 2014; Sgourakis et al., 2014). Like m04, the m12 j1 strand possesses two cysteine residues (Cys168 and Cys173) that form intra-molecular disulfide bonds with the CD loop (Cys88 and A strand (Cys58), respectively. However, m12 also contains an additional disulfide bridge between Cys40-Cys95 that serves to tether the relatively long N-terminal extension to the CD loop (Figure S4). Moreover, m12 possesses an additional \( \alpha \) helix between the B and C strands. Thus, m12 possesses unique structural features that are directly involved in NKR-P1B receptor binding.

Topologically, m12 lies across the top of the NKR-P1B CTLD, such that the m12 \( \beta \) sheets lie parallel to the interface (Figure 4A). Here, m12 straddles NKR-P1B in a claw-like manner, whereby a relatively flat “palm” engages the planar top surface of the NKR-P1B CTLD, while several “fingers” grasp around the periphery (Figures 4B–4C). This binding mode results in an extensive interface that buries a total solvent-accessible surface area of 2,180 A\(^2\), which is considerably larger than the 1,680 A\(^2\) observed upon binding of the KACL endogenous ligand to the human NKP65 receptor (Li et al., 2013) (Figures S4A and S4F).
The interface is characterized by high shape complementarity and is dominated by hydrophilic interactions, including 14 H bonds and 8 salt-bridges (Figure 4B and Table S4). The m12 palm encompasses residues from the A and B strands as well as the interconnecting loop (Ser60-Leu66) that form an extended surface that simultaneously engages the β2-β3 and β4-β5 loops as well as the β4 strand of NKR-P1B. Here, the interaction is stabilized by a number of H bonds that are primarily derived from the m12 and NKR-P1B main chains (Figures 4C–4D). In contrast to the flat, featureless palm, the m12 fingers are comprised of multiple large, primarily polar residues that form highly specific interactions around the edges of the docking site (Figures 4C and 4E–4G). For example, the m12 N terminus contains a highly charged ΔER50 motif that flanks the NKR-P1B CTLD (Figure 4E). In addition, the loop region connecting the NKR-P1B β1 sheet to the α1 helix is similarly flanked by a triad of residues (Lys69, Asn73, Val76) that emanate from the unique m12 α1 helix (Figure 4F). Further polar interactions are centered on the base of the m12 I strand, where Asp165 and Lys167 extend downward in order to engage Glu200, Asn206, Arg207, and Asn150 (Figure 4G). Overall, the breadth and the polar claw style of the interactions with NKR-P1B underlie the capacity of m12 to target this receptor family.

A Single Allelic SNP between m12Smith/MW97 Impacts NKR-P1B Immunoevasin Function

Despite differential stimulation of BWZ.P1B129,B6 reporter cells by NIH 3T3 fibroblasts infected using MCMVSmith versus MCMVMW97 (Figure 1G), quantitation of m12 transcripts by qRT-PCR revealed similar mRNA levels between the two viruses (Figure 5A). However, a single G271A SNP was observed in m12MW97 that resulted in a non-conservative E91K charge substitution. To determine whether this allelic m12 SNP affected immunoevasin function, we compared BWZ.P1B129,B6 reporter stimulation by m12MW97 versus m12Smith in 293T transfectants; indeed, m12MW97 was recognized more weakly than m12Smith and host Clr-b (Figure 5B). This suggests that m12Smith is a superior NKR-P1B immunoevasin versus m12MW97, at least in B6 mice. Importantly, this finding was confirmed and extended using NKR-P1B tetramers, whereby all three allelic NKR-P1BB6,129,FVB tetramers stained transient 293T.m12Smith transfectants better than 293T.m12MW97 transfectants (Figure S3B).

We further validated these results using cytotoxicity assays. Here, we generated stable retroviral transductant YAC-1 target cells expressing native m12Smith, m12MW97, or host Clr-b
As observed using 293T transfectants, BWZ.NKR-P1 reporter analyses using YAC-1 stimulators show that BWZ.P1B B6 cells recognize m12Smith more strongly than m12MW97 (Figure 5C). In contrast, BWZ.P1BFVB cells recognize m12Smith and m12MW97 similarly (Figure S5B), suggesting that the energetic profile of the interaction is allele specific. However, we could not detect significant stimulation of BWZ.P1CB6 reporter cells using YAC-1.m12Smith stimulators (Figure 5D), perhaps due to the lower m12 expression levels on these sorted stable transductants; notably, these data are consistent with reduced m12 tetramer staining of NKR-P1A/C relative to NKR-P1B (Figure 3E).

Next, YAC-1 transductants were incubated as targets with sorted NKp46+NKR-P1B+ or control NKp46+NKR-P1B– B6-strain lymphokine-activated killer (NK-LAK) effectors in 51Cr-release cytotoxicity assays. As shown in Figure 5, YAC-1.m12MW97 target cells were killed equivalently to control YAC-1(–) targets, while both YAC-1.m12Smith and YAC-1.Clr-b target cells significantly inhibited NK-LAK cell cytotoxicity (Figure 5E). Importantly, inhibition by m12Smith and host Clr-b was mediated via NKR-P1BB6, as it was not observed using NKR-P1B– NK-LAK effectors (Figure 5F).

Notably, similar results were observed using sorted FVB-strain NKR-P1B+ and NKR-P1B– NK-LAK effectors, partitioned using NK1.1 mAb (PK136), which recognizes NKR-P1BFVB (but not NKR-P1CFVB) (Carlyle et al., 1999) (Figures S5C and S5D). The similar recognition of m12Smith and m12MW97 using BWZ.P1BFVB reporters (Figure S5B) and NKR-P1BFVB tetramers (Figure S3B) but strong inhibition of cytotoxicity only by m12Smith suggests differential recognition of the m12 alleles by other stimulatory receptors, such as the NKR-P1A/C paralogs (Figure 2H; see also below).

The m12Smith:NKR-P1BB6 structure provided an opportunity to understand the molecular basis underpinning the highly allele-sensitive nature of this pivotal interaction. For example, NKR-P1B129 differs from NKR-P1BFVB6 by 16 residues within the CTLD, but only 4 contact m12Smith (S157R, Y158F, G201S, and S204T). Of these, the S157R and G201S polymorphisms would likely result in additional contacts with m12Smith and would thereby account for the marked increase in affinity of NKR-P1B129 compared to NKR-P1BB6 (Figure 5G). In addition, the polymorphic m12 residue, E91, did not directly contact NKR-P1BB6 but instead packed tightly against the m12 N terminus, thereby facilitating the interaction of the m12 48ERR50 motif with the m12Smith:NKR-P1BB6 structure (Figure 5G).

Figure 5. An Allelic MCMV m12 Polymorphism Controls NKR-P1B-Mediated Recognition and Inhibition of Cytotoxicity

(A) Quantitative real-time RT-PCR analysis of host Clec2d and MCMV m12 transcript expression during infection of NIH 3T3 cells with MCMVSmith or MCMVMW97.

(B) BWZ.P1BB6 reporter cell analysis of HEK293T stimulators transfected with host Clr-b, m12Smith, m12MW97, or empty vector.

(C and D) YAC-1 cells transduced with m12Smith, m12MW97, Clr-b, or empty vector were used as stimulators for BWZ.P1BB6 or BWZ.P1CB6 reporter cells.

(E and F) YAC-1 transductants as above were used as targets in 51Cr-release cytotoxicity assays with sorted NKp46+NKR-P1B+ or NKp46+NKR-P1B– NK-LAK effectors. Data are representative of at least two independent experiments. Mean ± SEM values are shown, with pairwise significance determined by two-way ANOVA.

(G) Mapping of polymorphisms on the m12:NKR-P1B structure. Residues that differ between NKR-P1BB6 and NKR-P1B129 are shown in pink, whereas those that differ between NKR-P1B129 and NKR-P1BFVB are shown in cyan. The E91K polymorphism identified in m12MW97 is highlighted in green, and the likely position of the A34V substitution found in m12CAV is indicated in black. The m12 48ERR50 motif is denoted by a dashed oval.
with NKR-P1BB6 (Figure 5G). Here, the m12MW97 E91K polymorphism would likely disrupt this packing and would thus indirectly abrogate the m12MW97:NKR-P1BB6 interaction.

Collectively, these data demonstrate that an allelic m12MW97 SNP results in less-efficient NKR-P1B immunoevasin function, while the m12Smith immunoevasin is capable of effective inhibition of both B6 and FVB NK-LAK effectors using YAC-1 targets. Moreover, the crystal structure provides a molecular basis for understanding the impact of host and viral allelic variation across the m12:NKR-P1B axis.

Wild MCMV Isolates Demonstrate Host-Driven Evolution of the m12 Gene Product

The above findings support the existence of both host- and pathogen-driven evolutionary selection pressures, including the loss-of-function m12MW97 SNP. Since NKR-P1B and NKR-P1C are polymorphic between mouse strains, we investigated whether additional m12 polymorphisms also exist in wild-derived MCMV isolates. Thus, we searched databases to compare m12 sequences from additional MCMV strains, including the Smith, K181, MW97.01, WP15B, CA4, CA4B, CA4C, CA4D, G4, AA18d, NO7, and N1 isolates (Smith et al., 2013); notably, with the exception of K181 and WP15B, each of the other strains differed from the m12Smith allele by at least one amino acid substitution (MW97.01, CA4/D/B), with extensive polymorphisms observed for some wild isolates (G4), including loss of signal peptide (AA18d), and premature truncations in the m04-like domain (CA4C,N1,NO7) (Figure 6A; Figure S6).

To assess these functionally, we cloned unique m12 gene products and then tested their recognition using BWZ.NKR-P1 reporters and 293T.m12 transfectant stimulators. We observed differences in recognition among all MCMV m12 alleles when tested against the different NKR-P1B and NKR-P1C alleles (Figures 6B–6G). The m12C4A variant has an A34V substitution yet engaged all NKR-P1B alleles similarly to m12Smith (Figures 6B, 6D, and 6F); this was also true using NKR-P1B tetramers (Figure S3B). This result is not surprising, since the most N-terminal m12 residue visible in the structure (S39) is distal to the NKR-P1B binding site (Figure 5G). The m12MW97 variant, as observed previously, has an E91K substitution and engaged NKR-P1BB6 much less efficiently than m12Smith in both reporter assays and using NKR-P1B tetramers, yet this difference was reduced using the NKR-P1B129 allele and was absent using the NKR-P1BFVB allele (Figures 6B, 6D, and 6F; Figure S3B). The highly diversified m12G4 variant was not recognized by NKR-P1BB6, interacted with NKR-P1B129 similarly to m12MW97, and interacted with NKR-P1BFVB less well than other m12 variants (Figures 6B, 6D, and 6F; Figure S3B).

When tested against the NKR-P1C paralogs, all m12 variants interacted with NKR-P1CBB6 to some extent, although m12G4 recognition was very weak (Figure 6C). None of the m12 variants were found to interact with NKR-P1C129 (Figure 6E), which may not be functionally expressed, due to the absence of a key cysteine residue (C122S) likely to be involved in intra-molecular stabilization. In contrast, NKR-P1CFVB was stimulated strongly...
Figure 7. In Vitro and In Vivo Analyses of MCMV m12 Allelic Variants in Distinct Inbred, Congenic, and Mutant Mouse Strains

(A–C) NIH 3T3 fibroblasts were infected with MCMVSmith or parental MW97-strain variants, including WT (m12MW97), Δm12 mutant (Δm12), and m12Smith revertant (m12Smith) then used as stimulators for (A) BWZ.P1BB6, (B) BWZ.P1B129, and (C) BWZ.P1BFVB reporter cells.

(D and E) NIH 3T3 fibroblasts infected with the above MCMV variants were used as targets in 51Cr-release cytotoxicity assays with sorted (D) NKR-P1B+ or (E) NKR-P1B– B6-strain splenic NK-LAK effectors.

(F) Viral PFU titers in spleens of WT B6 and B6.Nkrp1b−/− mice infected in vivo using the above MCMV variants, analyzed 3 days post-infection. Data are pooled from two independent infections using 1x10⁶ PFU/mouse.

(legend continued on next page)
by m12Smith and m12C4A4 but very weakly by m12MW97 and m12C4A4 (Figure 6G), in turn suggesting that m12Smith immunoevasin function may be counterbalanced by host NKR-P1CFVB activation relative to m12MW97 (see also below). Notably, NKR-P1B B6 and NKR-P1CFVB only differ in a single m12 contact residue (S204T), thereby explaining their similar responses to the m12 variants (Figure 5G). Interestingly, Clr-b also weakly stimulated NKR-P1CFVB reporters (Figures 6G and 6H), demonstrating that host Crr-b represents a novel activating self NKR-P1C ligand in the FVB strain. These data provide further evidence of the existence of host- and pathogen-driven evolution of the viral m12 decoy and reciprocal host adaptation of the NKR-P1B/C paralogs. These findings also validate the hypothesis that the NKR-P1B/C receptors have been co-evolving as a paired receptor clade to recognize similar host and viral ligands.

MCMV Mutants Reveal NKR-P1B-Dependent Inhibition In Vitro and Virulence In Vivo

To establish an immunoevasin role for m12, we generated m12-modified variants of the parental MCMVMW97 strain, including both m12-deficient (Δm12) and m12Smith-revertant (m12Smith) mutants. In vitro characterization revealed that all three MCMV variants similarly downregulated host Crr-b upon infection of NIH 3T3 cells (Figure S7A).

Using infected NIH 3T3 cells directly, the m12Smith-revertant virus stimulated BWZ.P1B B6 reporters similarly to the original MCMVMW97 virus, while the Δm12 mutant, parental MCMVMW97, and mock-infected NIH 3T3 cells lacked significant stimulation (notably, BWZ.P1B B6 reporters routinely fail to recognize endogenous host Crr-b levels on NIH 3T3 cells; Figure S7A). In contrast, using BWZ.P1B129 reporters, strong stimulation was observed with MCMVMW97 virus, m12Smith-revertant virus, and mock-infected NIH 3T3 cells, compared with weak stimulation using parental MCMVMW97, and no stimulation using Δm12 mutant MCMV (Figure 7B). Finally, using BWZ.P1B B6 reporter cells, stimulation was observed using mock-infected cells, m12Smith-revertant virus, MCMVMW97, and MCMVMMy, but not the Δm12 mutant virus (Figure 7C). The Crr-b-independent nature of these responses was confirmed using blocking Crr-b mAb (data not shown), as well as using mutant NIH 3T3ΔCrrb cells infected with each of the MCMV strains (Figures S7B–S7D). Together, these data demonstrate that m12 is a non-redundant NKR-P1B decoy immunoevasin and that the m12 G271A SNP (E91K polymorphism) identified between MCMVMW97 and MCMVMy is responsible for the differential stimulation of BWZ.P1B reporter cells.

Next, we conducted 51Cr-release cytotoxicity assays using sorted B6-strain NKR-P1B+ and NKR-P1B- NK-LAK effectors and MCMV-infected NIH 3T3 targets. Notably, mock, MCMVMW97, and Δm12-infected NIH 3T3 targets were efficiently lysed, whereas m12Smith-infected targets displayed significantly lower cytotoxicity in response to NKR-P1B+ effectors (Figure 7D). Importantly, inhibition by m12Smith was NKR-P1B dependent, as it was not observed using NKR-P1B− NK-LAK (Figure 7E). These data reveal that m12Smith, but not m12MW97, is directly capable of inhibiting B6-strain NK cytotoxicity in an NKR-P1B-dependent manner upon live MCMV infection.

To evaluate m12 immunoevasin function in vivo, the three variant MCMVMW97 strains were used to infect B6-strain WT, Clr-b−/−, and Nkrp1b−/− mice, then MCMV virulence was evaluated 3 days later using plaque assays and quantitative real-time PCR (qPCR) for viral genomic copy numbers in infected spleens. As expected, due to weak interaction between m12MW97 and NKR-P1B B6, no significant difference was observed between the parental MCMVMW97 and Δm12 mutant viruses in B6 WT mice in vivo (Figure 7F); remarkably, however, the m12Smith-revertant virus displayed significantly higher splenic viral titers (PFU) and viral genomic copy numbers in B6 WT mice (Figures 7F–7G). Importantly, the enhanced virulence of the m12Smith allele in vivo was NKR-P1B dependent and Crr-b independent, as it was not observed using B6-strain Nkrp1b−/− mice (Figures 7F–7G), yet Crr-b−/− mice displayed a similar trend to WT B6 mice (data not shown). Notably, increased virulence of MCMVMMy in vivo has been previously observed using WT B6 mice relative to B6.Nkrp1b−/− mice (Rahim et al., 2016). This finding is confirmed to be m12-dependent here using the three MCMVMW97-strain variants, where the m12Smith-revertant virus exhibits enhanced virulence versus parental MCMVMW97 and Δm12 mutant MCMV, in WT B6, but not B6.Nkrp1b−/− mice. These findings confirm a non-redundant NKR-P1B immunoevasin function for m12 in vivo.

To further elucidate the effects of the polymorphic host NKR-P1A/B/C-clade isoforms and alleles, we next performed in vivo experiments in the FVB, 129, and BALB inbred mouse strains using the three allelic MCMV variants. In the FVB strain, where strong interactions are observed between m12Smith and both the inhibitory NKR-P1CFVB and stimulatory NKR-P1BFVB receptors (Figures 6F–6G), m12Smith function may be counterbalanced by the paired receptors. However, the m12MW97 immunoevasin function should be intact, since a moderate interaction is observed for the inhibitory NKR-P1BFVB receptor yet a much weaker interaction for the stimulatory NKR-P1CFVB receptor (Figures 6F–6G). Interestingly, in FVB mice, no significant difference was observed between the m12Smith-revertant and Δm12 mutant viruses in vivo, while the parental MCMVMW97 virus maintained significantly elevated viral titers (Figure 7H). Thus, the m12MW97 allele possesses superior immunoevasin function in the FVB strain, where the m12Smith allele encounters opposing function from the activating NKR-P1CFVB (and perhaps NKR-P1A FVB) paralog.
On the other hand, in BALB/c and 129-strain mice, small but significant differences were observed only between the parental MCMV*AW97* and Δm12 mutant viruses in vivo, with the m12*Smih-* revertant virus showing an intermediate response (Figure 7I). Interestingly, the published BALB/c and 129-strain NKR-P1A/B/C alleles are identical. In keeping with this, we previously observed moderate interactions between the inhibitory NKR-P1B*BALB/129* receptor and most m12 alleles (Figure 6D), no interactions between the stimulatory NKR-P1C*BALB/129* receptor and any m12 alleles (Figure 6E), and a weak interaction for the stimulatory NKR-P1A*BALB/129* receptor and m12*Smih* (Figures 2H and 3E); thus, these in vivo results predict that the NKR-P1A*BALB/129* receptor may have a higher affinity for m12*AW97* versus m12*Smih*, in turn resulting in lower viral titers. Notably, while NKR-P1B*BALB/129* appears to be functional upon overexpression, m12*Smih* tetramers fail to stain ex vivo NK cells from the BALB/c and 129 strains (Figure S3A), suggesting that both the NKR-P1B/C*129/BALB* receptors may be non-functional in vivo, in turn resulting in reliance on NKR-P1A*129/BALB* alone.

To further validate the NKR-P1B-decoy function of the m12 immunoevasin, we also performed in vivo infections using co-horts of B6.Ly49H*−/−* mice, which lack the dominant Ly49H*B6* activating receptor that recognizes m157, and BALB.B*Gmp* congenic mice, which are NK1.1*−/−* and retain the B6-strain Nkrp1 genes. Here, the dominant decoy function of the m12*Smih*-revertant significantly augmented viral PFU titers in both the B6.Ly49H*−/−* (Figure 7J) and BALB.B*Gmp* (Figure 7K) congenic host strains, relative to the Δm12 mutant and parental m12*AW97* viruses. These data confirm that the superior decoy function of the m12*Smih* allele versus the m12*AW97* allele is enhanced in the absence of Ly49H, segregates with the B6-strain NKR-P1 (Klrb1) region, and is unaffected by epistatic effects from the BALB genomic background.

Taken together, the above findings demonstrate that the immunoevasin function of m12 in vivo is both host and virus strain dependent, and it can be functionally counterbalanced by both inhibitory and stimulatory NKR-P1A/B/C-clade polymorphisms and paralogs. These findings further confirm that complex interactions between host NKR and MCMV immunoevasins ultimately determine the outcome of host-pathogen interactions in vivo.

**DISCUSSION**

The NK1.1 (NKR-P1; Klrb1) receptor family comprises both activating and inhibitory members, with a bona fide ligand of the activating NK1.1 antigen remaining unknown, despite being described 40 years ago (Gilmich et al., 1977). We have identified m12 as a natural (viral) ligand for the prototypical NK1.1 antigen, the stimulatory NKR-P1C*m* orphan receptor (Gilmich et al., 1977; Koo and Peppard, 1984; Ryan et al., 1992). In addition, host Clr-b represents a second (self) NKR-P1C ligand in the FVB mouse strain. Moreover, we show that m12 functions as a decoy immunoevasin to subvert NK cell-mediated cytotoxicity by direct interaction with the inhibitory NKR-P1B receptor, which normally surveys infected cells for Clr-b downregulation during infection. The identification of the distantly Ig-related m02-family member, m12, as an NKR-P1A/B/C-clade ligand demonstrates sophisticated viral utilization of this protein fold to directly engage an NKR. Structurally, m12 clamped around the NKR-P1B receptor, in a polar claw mechanism, enveloping an extensive area of NKR-P1B that simultaneously provided insight into the specific targeting of the receptor by m12 and how allelic differences in this axis impacted upon recognition. Here, a number of the allelic polymorphisms in both the m12 immunoevasin and NKR-P1 receptors either mapped to the binding site or were in close proximity to it, thereby implying that both direct and indirect effects can impact the m12-NKR-P1 recognition axis.

The m12 footprint overlapped extensively with that observed for binding of the endogenous self ligand, KACL, to the closely related human NKp65 receptor (Li et al., 2013). Indeed, the utilization of existing binding sites on host receptors/ligands appears to be a common viral immune evasion strategy. Nonetheless, such structural mimicry does not necessarily underpin immunoevasin strategies, as structurally diverse immunoevasins, such as HCMV UL16 and MCMV m152, map to the same sites on ligands for the human and mouse activating NKG2D receptors, respectively (Müller et al., 2010; Wang et al., 2012). Further, some viral immunoevasins, including m157, can adopt unusual and unanticipated binding modes (Berry et al., 2013). Our work shows how the structurally divergent, viral Ig-like m12 immunoevasin utilizes binding mode mimicry to engage the inhibitory NKR-P1B receptor, which then underpins the ability of m12 to be reciprocally targeted by the structurally related activating NKR-P1A/C family members, in turn countering the m12 immunoregulatory properties.

The rodent Klrb1 genes display considerable polymorphism, being subdivided into two antigenic and phylogenetic clusters in mice and rats: the NKR-P1A/B/C and NKR-P1F/G clades (Carlyle et al., 2008; Kirkham and Carlyle, 2014; Kveberg et al., 2009). Strikingly, the NKR-P1B and NKR-P1C receptors appear to have undergone parallel divergence characteristic of paired recognition receptors, leading to the hypothesis that they are under selective pressure, within the constraints imposed by both endogenous self ligands and foreign viral ligands. Here, we provide compelling evidence of co-evolution at the host-pathogen interface, with host adaptation of the NKR-P1A and NKR-P1C (NK1.1) stimulatory receptor paralogs to directly recognize the m12 decoy, as well as the existence of wild-derived viral m12 immunoevasin alleles, including loss-of-function alleles, which may have evolved to avert direct NKR-P1A/C-mediated NK cell activation. We also provide strong evidence that m12 is a non-redundant NKR-P1B-targeting immunoevasin in vivo. However, the functional contribution of m12 to the outcome of host-pathogen interactions in vivo is ultimately determined by both the viral m12 and host NKR-P1A/B/C-clade polymorphisms.

For example, each m12 allele may contribute differentially to viral fitness in distinct mouse strains, and what may appear to be a superior immunoevasin in one mouse strain may be counterbalanced by opposing signals from both inhibitory NKR-P1B and activating NKR-P1A/C paralogs in another mouse strain. Our in-depth analyses employing eight inbred, mutant, or congenic mouse strains and three MCMV variants underscore how viral versus host fitness can be significantly impacted by polymorphisms in both the paired host receptors and viral immunoevasin.
The conserved use of decoy immunoevasins by MCMV (m12) and RCMV (RCTL) to subvert NKR-P1B-mediated NK cell immunosurveillance of pathological target cells highlights the role of the NKR-P1:Clr recognition axis in immunity to infectious disease and suggests that similar mechanisms may operate during HCMV infection of human cells (Aguilar et al., 2015; Voigt et al., 2007). Notably, the human NKR-P1A:LLT1 receptor-ligand pair are likely orthologous to some rodent NKR-P1B:LLT1 interactions. NKR-P1A (CD161) is inhibitory and is expressed on ~60% of human NK cells (Lanier et al., 1994), resembling NKR-P1B in function and expression. LLT1 has more restricted expression than Clr-b and is induced in response to PRR agonists and infection (Aguilar et al., 2015; Germain et al., 2011; Rosen et al., 2008). It remains to be determined whether HCMV similarly target the human NKR-P1A receptor.

The NK1.1 antigen was the prototypical receptor identified to be selectively expressed by NK cells. Thus, while being used as a tool to study and purify NK cells for almost four decades, the physiological ligand for the activating NK1.1 antigen has remained a mystery. Here, we showcase m12 as a natural virally encoded ligand for this important NK family. Moreover, NK1.1 is also found on some ILC and various T cell subsets, including invariant NKT cells, the ubiquitous mucosal-associated invariant T (MAIT) cells, CD4+ T helper cells, and activated CD8+ T cells. Thus, targeting of the NK1.1 antigen by a virally encoded ligand has important implications for both innate and adaptive immunity, virus-host co-evolution, and immunotherapy.

STAR★METHODS
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SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2017.03.002.

AUTHOR CONTRIBUTIONS
O.A.A. designed, performed, and analyzed experiments and contributed to the manuscript writing. R.B. and Z.F. generated the purified m12 and NKR-P1 receptor proteins and performed SPR analyses. R.B. solved, refined, and analyzed the structure and contributed to manuscript writing. M.M.A.R., M.T., B.P., M.M.T., and A.B.M. assisted with in vivo experiments. J.J.R. generated the m12 mutant and revertant viruses. T.N.H.L assisted with bioinformatic analysis. G.R.B. assisted with the structural analysis. C.L.K., A.M., B.P., and A.K. assisted with in vitro experiments. D.S.J.A., A.P.M., S.J., J.R., and J.R.C. contributed to the direction of the study. This work was done in the labs of A.P.M., S.J., J.R., and J.R.C. J.R.C. contributed to the design and interpretation of experiments, project management, and manuscript writing.

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