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Cmv4, a New Locus Linked to the NK Cell Gene Complex, Controls Innate Resistance to Cytomegalovirus in Wild-Derived Mice

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CMV can cause life-threatening disease in immunodeficient hosts. Experimental infection in mice has revealed that the genetically determined natural resistance to murine CMV (MCMV) may be mediated either by direct recognition between the NK receptor Ly49H and the pathogen-encoded glycoprotein m157 or by epistatic interaction between Ly49P and the host MHC H-2Dk. Using stocks of wild-derived inbred mice as a source of genetic diversity, we found that PWK/Pas (PWK) mice were naturally resistant to MCMV. Depletion of NK cells subverted the resistance. Analysis of backcrosses to susceptible BALB/c mice revealed that the phenotype was controlled by a major dominant locus effect linked to the NK cell gene complex. Haplotype analysis of 41 polymorphic markers in the Ly49h region suggested that PWK mice may share a common ancestral origin with C57BL/6 mice; in the latter, MCMV resistance is dependent on Ly49H-m157 interactions. Nevertheless, PWK mice retained viral resistance against m157-defective mutant MCMV. These results demonstrate the presence of yet another NK cell-dependent viral resistance mechanism, named Cmv4, which most likely encodes for a new NK activating receptor. Identification of Cmv4 will expand our understanding of the specificity of the innate recognition of infection by NK cells. The Journal of Immunology, 2006, 176: 5478–5485.

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5 Abbreviations used in this paper: MCMV, mouse cytomegalovirus; LOD, logarithm of odds; NKC, NK gene complex; ORF, open reading frame; SNP, single nucleotide polymorphism.
express m157 variants that do not trigger NK cell activation (25), and even resistant B6 mice become highly permissive to viral replication when infected with a mutant MCMV strain that lacks m157 (25, 26). Additionally, the function of other NK-encoded activating receptors such as NKG2D also appears to have been subverted by MCMV. NKG2D binds at least seven different MHC class I-like cell surface glycoproteins encoded by genes clustered on mouse chromosome 10 (27, 28). Infection of cells with MCMV induces the translocation of mouse NKG2D ligand genes; however, MCMV has evolved three viral genes (m152, m155, and m145) that prevent the expression of NKG2D ligands on the surface of infected cells from classical inbred mouse strains (29–32).

We reasoned that novel mechanisms of MCMV resistance might be found in strains of mice derived from wild specimens of the genus Mus that were recently trapped in different geographical locations. These mice are now kept as fully inbred strains and are amenable to genetic studies. Indeed, by crossing them with laboratory strains, offspring can be produced that carry polymorphisms not available in classical inbred strains (33, 34). We have quantified antiviral innate responses in six stocks of inbred mice derived from wild Mus musculus specimens and found one resistant to MCMV. Viral titers in spleen and liver were indistinguishable from classical inbred mouse strains (29–32).

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c strains were purchased from The Jackson Laboratory. PWK/Pas (henceforth referred to as PWK), MAI/Pas, MBT/Pas, CAST/Ei, WLA/Pas, WMP/Pas, SEG/Pas originate from specimens of wild M. musculus species derived from wild mice trapped near Prague, Czech Republic in 1974. MAI and MBT belong also to the M. m. musculus species, whereas CAST belongs to the Mus musculus castaneus species and WMP and WLA to the Mus musculus domesticus species. The mode of inheritance of the PWK resistance trait was studied in F1 and F2 offspring from crosses between MCMV-resistant PWK and MCMV-susceptible BALB/c mice. Intercrosses of F1 mice have failed to produce F2 mice, most probably due to the Haldane’s rule that predicts infertility in the heterogametic offspring of hybrid species (33). However, crossover F2 females with parental BALB/c males were fertile and, therefore, were used to produce 75 ([BALB/c × PWK]F1 × BALB/c] segregating backcross N2 mice. Five of the 75 N2 mice were excluded from the analysis because viral loads in the liver were unusually low (<2.5 log10 PFU), likely reflecting technical problems with the infection procedure. Animals were kept at the Central Animal Facilities of the Institut Pasteur and used for experiments at 6–12 weeks of age. All protocols for animal experiments conducted at the Institut Pasteur were reviewed by the Central Animal Facilities of the Institut Pasteur and were done in accordance with guidelines approved by the French Ministry of Agriculture. Some experiments were conducted at the Central Animal Facility of the Medical Faculty of Rijeka, Croatia. Animal work done at the Medical Faculty of Rijeka, Croatia was approved by the local ethical committee and done in agreement with the local regulations.

MCMV infection and plaque-forming assay

The wild-type Smith strain of MCMV was obtained from the American Type Culture Collection and propagated by salivary gland passages as previously described (35) or grown in tissue culture (26). The tissue culture-grown viruses A1057 MCMV, which lacks open reading frame (ORF) m157, and A6 MCMV, which contains a deletion spanning ORFs 144–158, have been previously described by us (26, 29). At 7 wk of age, mice were infected i.p. with 5 × 102 PFU of salivary gland passaged or 5 × 103 PFU of tissue culture-grown virus. The degree of infection was assessed by determining the number of MCMV PFU in the spleen and the liver 3–4 days postinfection by plaque assay in BALB/c mouse embryonic fibroblasts as described (35). Viral titers were expressed as log10 MCMV PFU per organ. In some experiments, depleting anti-asialo-GM1 or blocking anti-NKG2D (clone CX5) (36), provided by Dr. L. L. Lanier (University of California, San Francisco, CA) mAbs were injected i.p. 2 days before infection.

Flow cytometry analysis

Single cell suspensions of splenocytes were prepared as described (37). Cells were stained with mAbs specific for DX5, Ly49D, Ly49G1, 2B4, CD3, CD5, CD16, CD69, CD94, CD122, NKG2A/C/E (BD Pharmingen), NKG2D (see previous paragraph), and Ly49H/C1 (clone IF8 (21), provided by Dr. M. Bennett, University of Texas Southwestern Medical Center, Dallas, TX). In some experiments we used the polyclonal rabbit anti-Ly49H Ab (38), which was detected by a PE-conjugated donkey anti-rabbit IgG (The Jackson Laboratory). For Ly49H intracellular staining, IL-2 activated splenocytes were used in order to have a larger number of NK cells. Although IL-2 induces changes in the expression profile of certain NK cell receptors, it does not perturb significantly the Ly49 receptor repertoire. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). (BALB/c × PWK)F1 fibroblasts were mock treated or infected with either wild-type MCMV or Δ6 MCMV (1 PFU/ml). Twelve hours postinfection, fibroblasts were analyzed for expression of NKG2D ligands using NKG2D-PE tetramers (28) (provided by D. H. Busch, Technische Universität München, Munich, Germany) as previously described (28).

Cytotoxicity assay

A standard 4-h 51Cr release assay was used to measure NK activity in vitro. Target cells (YAC-1, Ba/F3, and m157-transfected Ba/F3 (22), the latter a gift from Dr. L. L. Lanier (University of California), were labeled with 100 μCi of 51Cr (ICN Pharmaceuticals). Red cell-depleted splenocytes were either used fresh or expanded or after culture in RPMI 1640 supplemented with 10% FCS, 5 × 10−3 M 2-ME, 100 μg/ml streptomycin, 100 U/ml penicillin, and 1000 U/ml human IL-2 (R&D Systems) for 5–8 days. Immediately before the assay, the cells were adjusted so as to have equivalent counts of CD122+DX5+CD3+ NK cells in all of the samples of the assay.

Haplotype analysis

We conducted haplotype mapping on genomic DNA using a set of 20 polymorphic markers, including 14 that were previously localized to the minimal genetic interval of Cow1 (20, 23, 38, 39). In addition, we used six new markers (SV175, Ly49b15R, SV20, SV151, SV168, and SV169) derived from the Ly49h genomic DNA sequence (40). Molecular characteristics of these markers are presented in Table I. PCRs were performed using 20 ng of genomic DNA in a 20-μl reaction containing 10 pmol of each primer, 0.2 U of Taq DNA polymerase (Boehringer Mannheim), and 100 nM dNTPs under previously described conditions (20, 23, 38, 39). Simple sequence and restriction fragment length polymorphisms were visualized by ethidium bromide staining following electrophoresis in 0.5% Tris-borate-EDTA buffer on either 1% regular agarose or 7% acrylamide gels. Products obtained with markers within the Ly49h gene, SV175, Ly49h15R, and SV20, were sequenced to confirm their identity in individual mouse strains.

Genotyping and statistical analysis

Genomic DNA was extracted from 70 [(BALB/c × PWK)F1 × BALB/c] mice tail tips as described (37). Genotypes in the NKC and H-2 region were

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primersa</th>
<th>PrimerSequence (5’−3’)</th>
<th>ProductSize (bp)</th>
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<td>SV175-R</td>
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<td>SV20-R</td>
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<tr>
<td>Ly49h</td>
<td>SV205-F</td>
<td>GGAGAAGTTCTTTCTACCTATTGGT</td>
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</tr>
<tr>
<td>Ly49h</td>
<td>SV205-R</td>
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<tr>
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<td>SV168-R</td>
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<td>SV169-F</td>
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<td>Ly49h</td>
<td>SV169-R</td>
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a F. forward primer; R. reverse primer.
determined by PCR-RFLP. The NKC was amplified using a Ly49e marker followed by PstI enzyme digest, as previously described (15). The H-2 locus was genotyped with the MHC class II-specific primers for IAa1. Digestions with both HindIII and PstI enzymes allowed the discrimination between the H-2a (BALB/c) or H-2e (PWK) alleles (41). The contribution of PWK alleles at NKC and H-2 to the segregation of the phenotype (i.e., the log10 of the number of PFU in the spleen) in [(BALB/c × PWK)F1 × BALB/c] backcross mice was estimated using the following linear model: phenotype = m + nkc + h-2 + e, where nkc and h-2 are used to represent the number of PWK alleles at each locus, m is the common mean value, and e represents the usual independent, normally distributed, random deviations. Logarithm of odds (LOD) scores for linkage were calculated by taking the log10 of the likelihood ratio of the model.

cDNA cloning

Total RNA from PWK NK cells was isolated with TRIzol reagent (Invitrogen Life Technologies) and reverse transcribed using SuperScript II polymerase (Invitrogen Life Technologies) with oligo(dT) primers. NK cell receptor CDNsAs were amplified with gene-specific oligonucleotide primers for Ly49s and Nkg2d. Oligonucleotide sequences are presented in Table II. Amplified products were analyzed by gel fractionation, purified with the QiAEX II gel extraction kit (Qiagen), and directly ligated into the pGEM-T Easy vector (Promega). A minimum of three identical clones from two independent PCRs were sequenced for each of the PWK novel genes. DNA and predicted amino acid sequence analysis of these clones was performed using standard nucleotide-nucleotide BLAST (blastn) and standard protein-protein BLAST (blastp) found on the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). The alignment program Clustal W was used for multiple sequence alignments (www.ebi.ac.uk/clustalw).

Results

NK cells confer resistance to MCMV in wild-derived PWK mice

We have measured early immune responses to MCMV in six wild-derived inbred strains of mice derived from the M. musculus species. Mice were infected with a sublethal dose of MCMV that readily distinguishes resistant B6 mice from susceptible BALB/c mice at the level of spleen viral titers, but less so in the liver (8, 9) (Fig. 1). Five of six M. musculus-derived strains presented high viral titers in the spleen and liver, comparable to those observed in the susceptible strain BALB/c (log10 PFU was 4.0 in the spleen 4.0 and 4.3 in the liver). In contrast, the PWK strain was resistant, showing viral titers of 1.9 log10 PFU in the spleen and 3.8 log10 PFU in the liver, both of which are comparable to titers found in the resistant B6 mouse strain. These results demonstrate that the viral replication pattern in PWK mice is most similar to B6, suggesting that NK cells may be involved.

Further supporting this hypothesis, injection of PWK mice with anti-asialo-GM1, which preferentially depletes NK cells (42), rendered PWK mice relatively susceptible to MCMV infection. Treated PWK mice presented viral loads that were more than two orders of magnitude greater than those of untreated mice (Fig. 2) in both spleen and liver, implicating NK cells in the control of these two organs.

Table II. Summary of PCR primers used for cDNA amplification

<table>
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<tr>
<th>Locus</th>
<th>Primersa</th>
<th>Primer Sequence (5′–3′)</th>
<th>Product Size (bp)</th>
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</thead>
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<td>Ly49h</td>
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<td></td>
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</tr>
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<td>Ly49k</td>
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<td>707</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>NKG2d</td>
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<td></td>
<td>NKG2d-R</td>
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</table>

a F, forward primer; R, reverse primer.

Mode of inheritance of the PWK resistance trait

To study the mode of inheritance of the PWK MCMV resistance trait, we determined the antiviral response in F1 and N2 backcross progeny issued from a cross between PWK and MCMV-susceptible BALB/c mice. (BALB/c × PWK)F1 progeny showed splenic viral loads comparable to or lower than those of resistant PWK and B6 mice (Fig. 3A). This finding indicated an autosomal dominant

FIGURE 1. Survey of wild-derived mouse strains for MCMV resistance. MCMV titers in the spleen (filled histograms) and in the liver (open histograms) were determined by plaque assay 3 days after i.p. injection of 5 × 103 PFU MCMV (Smith strain, salivary gland preparation). The dashed line indicates the level of detection of our assay (log10 PFU > 1.69). Statistically significant differences in comparison with observed viral titers in MCMV-susceptible BALB/c mice at p < 0.05 are indicated by an asterisk (*).

FIGURE 2. Depletion of NK cells abrogates MCMV resistance in PWK mice. Groups of four PWK mice were treated with anti-asialo-GM1 polyclonal Abs two days before MCMV infection (i.p. injection of 5 × 103 PFU MCMV Smith strain, salivary gland preparation). Viral titers from both treated (gray circles) and untreated (open circles) animals were determined in the spleen and liver by plaque assay 3 days postinfection.
mode of inheritance of the MCMV resistance phenotype. Phenotypes of the N2 progeny presented a bimodal distribution consistent with a major locus effect (Fig. 3). Means of each mode were log10 MCMV PFU 2.2 and 5.8, which are very similar to the values of the MCMV-resistant and MCMV-susceptible parental strains (Fig. 3A). We noted, however, that 10% of the N2 cohort had intermediate values, suggesting that additional genes may influence the phenotype.

Innate resistance to MCMV in PWK mice is genetically linked to the NKC complex

Because our data suggested that NK cells were key players in PWK natural resistance to MCMV (Fig. 2), we hypothesized that a gene in the NKC complex might control the resistance phenotype.

The MCMV-infected N2 progeny were individually genotyped using the polymorphic NKC marker Ly49e and the H-2 marker IAAT. Because the H-2 locus has been associated with MCMV resistance (15, 43), we decided to include it in our analysis to model the MCMV resistance trait. The statistics supported a two-locus additive model in which both H-2 and NKC genes play a significant role in the phenotype determination (Table III). The joint LOD score for the model was 25.1 (p < 2.2e-16). The proportion of the variation explained by the H-2 locus was estimated to be 4.4% with a LOD score of 2.7 (p < 1.9e-4), whereas that for the NKC was 77.7% with a LOD score of 21.9 (p < 2.2e-16). To visualize the effects of the parental alleles, N2 animals were also separated according to their combined H-2 and NKC genotypes (Fig. 3B). The results clearly demonstrated that PWK alleles at the NKC are associated with a 2–3 log10 PFU reduction of viral titers. In contrast, it was also clear that acquiring a PWK allele at H-2 results in an increase of the mean viral titer by more than one log10 unit, suggesting that the PWK allele at the H-2 locus is the susceptibility allele.

B6 and PWK may share a common ancestral origin in the Ly49h region

The results of the genetic linkage analysis demonstrated that a gene closely linked to Ly49e is responsible for resistance to MCMV infection in PWK mice. To explore the existence of other MCMV resistance alleles and to study the genotype/phenotype relationship we determined the allelic composition of a set of 41 linked loci in the vicinity of Cmv1 and studied their haplotypes in a panel of 11 mouse strains, including the six wild-derived inbred strains used in this study plus B6, BALB/c, and 129/J and two additional wild-derived strains belonging to more distantly related Mus spretus. We have previously shown that the three latter strains have distinct prototypical haplotypes at the NKC (24, 44). In addition to microsatellites or PCR-RFLP informative markers, we also used six novel markers and 24 single nucleotide polymorphisms (SNPs) overlapping the Ly49h gene (Fig. 4). The PWK haplotype presented a unique combination of alleles at the loci analyzed, clearly defining a new NKC haplotype. However, remarkable similarity between PWK and C57BL/6 was observed at the Ly49h region with the highest number of SNPs (18/24) conserved between these two strains, indicating a possible ancestral relationship at this region and suggesting a similar MCMV resistance mechanism.

Receptor repertoire in PWK NK cells

PWK NK cells were not stained with mAb specific for NK1.1, CD94, or 2B4 (data not shown). In contrast, they stained positive for NKG2D, NKG2A/C/E, CD69, Ly49C/I, Ly49D, and CD16 and, in line with haplotype results, were also labeled by the 1F8 mAb that detects Ly49H, Ly49C, and Ly49I (Fig. 5, and, in line with haplotype results, were also labeled by the 1F8 mAb that detects Ly49H, Ly49C, and Ly49I (Fig. 5, A and B, and data not shown). Without knowing the sequence of PWK receptors, the possibility that cross-reactivity accounts for positive results could not be excluded. To evaluate the significance of the 1F8 staining, we used an alternative strategy aimed at detecting a Ly49H-specific intracytoplasmic epitope. PWK cells showed some reactivity compared with Ly49H-negative BALB/c cells, however, they did not show the bimodal distribution and bright intensity typical of B6 cells (Fig. 5C).

Table III. Effects of quantitative trait loci controlling MCMV infection

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<th>Locus</th>
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<th>LOD Score</th>
<th>Variance (%)</th>
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<tr>
<td>LY49e</td>
<td>&lt;2.2e-16</td>
<td>21.9</td>
<td>77.1</td>
</tr>
<tr>
<td>IAAT (H-2)</td>
<td>1.90e-04</td>
<td>2.7</td>
<td>4.4</td>
</tr>
</tbody>
</table>
NKG2D-independent MCMV resistance

As expected from the Ab staining results, cDNA cloning and sequence analysis of the predicted amino acid sequence of the NKG2D receptor from PWK indicated the presence of a functional receptor with only two sequence variants in regard to the B6 sequence in the intracellular domain (H10Y) and the stalk region (I89V), but none in the ligand recognition domain. Thus, one possible explanation for the PWK resistance to MCMV is that PWK-infected cells are somewhat refractory to the immunomodulatory action of MCMV genes on NKG2D ligand expression (29 –32). This possibility was ruled out because of the evidence that NKG2D ligands were effectively down-modulated in infected (BALB/c × PWK)F1 fibroblasts; however, the virus containing a deletion spanning ORFs 144 –158 is not capable of interfering with the expression of NKG2D ligands (Fig. 6A). To validate the biological relevance of our findings, we specifically blocked NKG2D in vivo before infection. The blocking CX5 mAb (36) did not subvert MCMV resistance in PWK mice (Fig. 6B). These results ruled out NKG2D activating receptors as candidate for MCMV resistance in PWK mice.

Ly49H and m157-independent MCMV resistance in PWK mice

Although attempts to clone Ly49h from PWK NK cells were unsuccessful, we identified three closely related cDNAs coding an expressed pseudogene, Ly49k, and full-length Ly49n1 and Ly49n2 transcripts highly homologous to Ly49n from B6 (Table IV). Ly49n1 and Ly49n2 lack a highly conserved cysteine residue (position 154) involved in disulfide bond formation, suggesting that these receptors are not functional (Fig. 7). These data also support the idea that the Ly49 repertoires of PWK and B6 are related but not identical. Although PWK NK cells do not appear to express a bona fide Ly49H, it is possible that an activating receptor in PWK may recognize the viral m157 glycoprotein. A functional cytotoxicity assay was used to test this possibility. Tumor cells of the pre-B cell line Ba/F3 are relatively resistant to NK cell lysis; however, when transfected with m157 they become sensitive to lysis.
by Ly49H+ NK cells (22). As expected, Ly49H+ B6 cells could readily kill m157-transfected tumor cells. In contrast, PWK and BALB/c NK cells failed to do so, although they did show lytic activity against the prototypic YAC-1 lymphoma targets (Fig. 8A, and data not shown).

These results prompted us to test whether PWK mice would still respond to viruses that fail to activate NK cells through m157. Infection with m157 MCMV abrogates resistance in B6 mice, because dominant Ly49H-induced responses cannot take place (26). The results in Fig. 8B show that PWK mice, contrary to B6 mice, were highly resistant to m157 MCMV, providing additional evidence for a new resistance mechanism independent of Ly49H-m157 interactions.

Discussion
We have described in this study a new MCMV resistance locus found in the M. musculus wild-derived PWK strain. Compared with the reference resistant B6 strain, the PWK resistance pattern showed remarkable similarities and important new aspects. As in B6 mice, viral titers were low in the spleen and higher in the liver early after infection; the resistance was NK cell dependent and genetically linked to the Ly49 gene cluster at the NKC. Contrary to the B6 strain, however, Ab staining and cDNA cloning indicated the absence of a bona fide Ly49H receptor in PWK mice. PWK mice infected with mutated m157 MCMV were resistant to infection, indicating a mechanism of host resistance also independent of m157. This result is not entirely surprising, because a recent study by Voigt et al. (25) has shown that although m157 is crucial for the activation of Ly49H+ NK cells, most wild isolates of MCMV (~86%) present mutations in m157.

We have also shown that the NKC-encoded activating receptor NGK2D was properly expressed in PWK. NGK2D ligands, however, could not be detected in MCMV-infected (BALB/c × PWK)F1 fibroblasts, indicating that PWK cells were susceptible to evasion strategies adopted by MCMV to escape recognition by NGK2D (29–32). Finally, in vivo blocking experiments of NGK2D did not abolish MCMV resistance in PWK, formally excluding NGK2D as mediator of resistance. Altogether, these observations support the hypothesis that alternative mechanisms other than NGK2D or Ly49H-m157 interactions mediate NK cell dependent resistance to MCMV in the PWK mouse strain.

The NKC haplotypes of the wild strains had different combinations of alleles, but PWK and B6 were similar at the Ly49h region, indicating a possible ancestral relationship at this locus. However,
jection of 5/H9004 wild-type MCMV (open circles) or prototype YAC-1 target cells or m157-transfected Ba/F3 targets and the of free-living also demonstrated allelic heterogeneity at NKC loci in populations Ly49 receptor repertoire in this strain. A study by Scalzo et al. (45) related receptors in PWK, demonstrating the presence of a unique cDNA cloning indicated the presence of distinct albeit Ly49H- was assessed at the indicated E:T ratios in a 4-h 51Cr release assay against expanded in vitro in the presence of IL-2. Cytotoxic activity of NK cells to MCMV.

**FIGURE 8.** m157-independent mechanisms of NK cell immunity to MCMV. A. NK cells were purified from B6 or PWK mouse spleens and expanded in vitro in the presence of IL-2. Cytotoxic activity of NK cells was assessed at the indicated E:T ratios in a 4-h 51Cr release assay against prototype YAC-1 target cells or m157-transfected Ba/F3 targets and the control parental Ba/F3 targets. B. Groups of 3–4 mice were infected with wild-type MCMV (open circles) or Δm157 MCMV (gray circles). Viral titers were determined in the spleen by plaque assay 3 days after i.p. injection of 5 × 10^3 PFU of tissue-cultured MCMV viruses.

cDNA cloning indicated the presence of distinct albeit Ly49H-related receptors in PWK, demonstrating the presence of a unique Ly49 receptor repertoire in this strain. A study by Scalzo et al. (45) also demonstrated allelic heterogeneity at NKC loci in populations of free-living *M. m. domesticus* mice, of which only two of 18 specimens were relatively resistant to MCMV. Allelic variability at the NKC among wild-derived strains of mice is not surprising, because high level of variation is a common theme in chromosomal regions containing immune-related genes, deploying the possibility of a wide range of defense options against rapidly evolving pathogens (46–48). The rare occurrence of host resistance against MCMV in wild mice, which are constantly exposed to environmental pathogens, was somewhat unexpected, but the variation at the NKC may also reflect variation in MCMV immunoregulatory proteins (25) and the presence of specific NK cell receptor/ligand pairs occurring during natural infections with MCMV variants. It would be of interest to determine whether infection with wild MCMV isolates, originating from the same geographical location as the wild-derived mouse strains used here, reveals NKC-linked MCMV resistance mechanisms in mouse strains other than PWK.

The dominant NKC gene effect identified in PWK, together with our candidate gene and haplotype analysis, indicate the presence of yet another mechanism of MCMV resistance at a locus, which we named *Cmv4*. Remarkably, viral titers of mice carrying PWK alleles at the NKC and H-2 were significantly higher than those of mice homozygous at H-2, indicating that both H-2 and NKC loci are important for MCMV resistance. The H-2 effect may reflect a different affinity of PWK NK cell inhibitory receptors for BALB/c or PWK H-2 gene products, which determine an inhibitory effect on NK cell killing activity against infected cells. Alternatively, the H-2 effect may reflect an increased affinity of PWK activating receptors for BALB/c H-2 gene products expressed on MCMV-infected cells, resulting in enhanced NK cell killing activity as has been proposed for the *Cmv3*-mediated resistance in the MA/My model. Our genetic analysis, however, indicated that H-2 has only a minor contribution to host resistance in PWK in contrast to the NKC gene effect that explains 77% of the variance, suggesting that *Cmv4* operates in a manner similar to that of the *Cmv1/Ly49h* mechanism.

At this point, it is not possible to identify which of the NKC-linked genes, such as *Nkrp, Clr*, or other *Nkg2* or *Ly49* gene family members (17, 49), is identical with *Cmv4*. High-resolution linkage mapping and cDNA cloning experiments are warranted to tract down the PWK innate mechanism of host resistance. However, it is tempting to speculate that a novel Ly49 activating receptor that it is directly triggered by a viral product is likely to mediate MCMV resistance in PWK. Smith et al. (23) identified m157 and at least 11 other ORFs encoding molecules with putative MHC class I-like fold. As previously proposed for m157, which binds Ly49H in B6 mice, any other MHC class I-like molecule (for example m144; Ref. 50) could serve as a ligand for an unknown PWK activating receptor signaling target cell killing.

The study of activating receptors and their inheritance in PWK mice will be important for our understanding of the evolutionary role of activating NK receptors and may shed light on human immune resistance mechanisms to infectious diseases. Human killer Ig-like receptors, much like rodent Ly49, show allelic polymorphism (51) and control NK cell functions through conserved mechanisms of intracellular signal transduction (52) despite the structural divergence between Ly49 and killer Ig-like receptors. Human NK receptor genes have been implicated in viral infections (53–55), cell transplantation (56), and pre-eclampsia (57), making this a central topic in modern medicine (51). Mice offer a powerful tool to dissect the genetics of at least some of these associations and to understand the biology of NKK functions.

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**References**


