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

Sonia Girard Adam, Anouk Caraux, Nassima Fodil-Cornu, J. Concepcion Loredo-Osti, Sarah Lesjean-Pottier, Jean Jaubert, Ivan Bubic, Stipan Jonjic, Jean-Louis Guénet, Silvia M. Vidal and Francesco Colucci

J Immunol 2006; 176:5478-5485; doi: 10.4049/jimmunol.176.9.5478
http://www.jimmunol.org/content/176/9/5478

References
This article cites 57 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/176/9/5478.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
Cmv4, a New Locus Linked to the NK Cell Gene Complex, Controls Innate Resistance to Cytomegalovirus in Wild-Derived Mice

Sonia Girard Adam,*† Anouk Caraux,‡ Nassima Fodil-Cornu,*† J. Concepcion Loredo-Osti,*† Sarah Lesjean-Pottier,‡ Jean Jaubert,§ Ivan Bubic,¶ Jean-Louis Guénét,§ Silvia M. Vidal,2,3† and Francesco Colucci2,4‡

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 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 specificities of the innate recognition of infection by NK cells. The Journal of Immunology, 2006, 176: 5478–5485.

I

nfection with human CMV is a common cause of congenital disorders and can be life-threatening in organ transplant as well as AIDS patients (1–4). CMVs are strictly species specific; however, infection of mice with murine CMV (MCMV) provides an excellent experimental model with which to study the genetics and pathophysiology of the host immune response (5–7). Scoring the innate immune response in terms of viral titers in the organs of infected mice 2–5 days after infection gives an unambiguous outcome that depends on the genetic makeup of the host (8, 9). Whereas most mouse strains allow uncontrolled viral growth in multiple organs, some mouse strains such as C57BL/6 (B6) and MA/My can effectively control early viral replication (8–10). A complex network of cells, soluble factors, cellular receptors, and intracellular signaling pathways organize the innate response against the virus (7, 11). NK cells, however, play a central and nonredundant role in this process (12–14). Studies of natural variation in host resistance or susceptibility to MCMV infection have clarified several aspects of the function of NK cells in the innate immune response.

In B6 and MA/My mouse strains innate resistance to MCMV is controlled by alternative loci, namely Cmv1 and Cmv3, which reside on the NK cell gene complex (NKC) on distal mouse chromosome 6 (9, 10, 15). The NKC encodes many NK cell receptors, including activating and inhibitory members of the Ly49 family of MHC class I receptors (16–18). In B6, Cmv1 encodes the activating NK cell receptor Ly49H (19–21). Ly49H binds to m157, a viral MHC class I-like protein expressed at the surface of infected cells during the early phase of infection (22, 23). In MA/My, Cmv3-mediated innate resistance is expressed in epistatic interaction with H-2Dk (15). In this model, the activating receptor Ly49P was shown to interact with H-2-Dk only on the MCMV-infected cells (15). Thus, activating Ly49 receptors seem to recognize infection and trigger NK cells to kill infected cells, providing a mechanism of natural host resistance.

The use of classical laboratory mouse stocks sets serious limitations, as B6 and MA/My strains represent apparent exceptions. All of other strains tested either do not possess Ly49h or do not coexpress Ly49p and H-2Dk and are consequently susceptible to MCMV (15, 24). Moreover, other Ly49 receptors exist that can bind to m157, but the outcome of this interaction is thought to rather facilitate viral replication. One such receptor is the Ly49I inhibitory receptor on NK cells of the susceptible 129/J strain, where m157 may in fact switch off NK cells upon viral recognition (22). In contrast, most MCMV strains isolated from wild mice...
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 NKC-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 transcription 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 quantitatively analyzed strains, offspring can be produced that carry polymorphisms at specific 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 wild-type Smith strain of MCMV, which contains a deletion spanning ORFs 144–148, have been previously described by us (26, 27). At 7 wk of age, mice were infected i.p. with 5 × 10^6 MCMV PFU of salivary gland passage or 5 × 10^3 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 log_{10} 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, CD112, NKG2A/C/E (BD Pharmingen), and NKG2D (see previous paragraph), and Ly94HCl (clone 1F8 (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/cell). 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, Balb/C, and m157-transfected Balb/C (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 freshly explanted or after culture in RPMI 1640 supplemented with 10% FCS, 5 × 10^{-5} 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, cells were washed with anti-DX5, anti-CD122, and anti-CD3 mAb to quantify the number of effector cells that was adjusted so as to have equivalent counts of CD122 DX5 CD3 NK cells in all of the samples of the assay.

Haplotypic 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 Cmv1 (20, 23, 38, 39). In addition, we used six new markers (SV175, Ly49h(15R), SV50, 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 volume reaction containing 10 pmol of each primer, 0.2 μl of Taq 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 5% acrylamide gels. Products obtained with markers within the Ly49h gene, SV175, Ly49h(15R), and SV50, were sequenced to confirm their identity in individual mouse strains.

Genotyping and statistical analysis

Genomic DNA was extracted from 70 [(BALB/c × PWK)F1] segregating backcross N2 mice. Five of these N2 mice were excluded from the analysis because viral loads in the liver were unusually low (<2.5 log_{10} 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.

### Table I. Genetic markers

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers(^a)</th>
<th>Primer Sequence (5’→3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly49h</td>
<td>SV175-F</td>
<td>CAAGTCCTTCTAAAATAAGTTGA</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>SV175-R</td>
<td>GTGATAGGTGGTGAGTTAGAG</td>
<td>290</td>
</tr>
<tr>
<td>Ly49h</td>
<td>SV175-F</td>
<td>GTGATAGGTGGTGAGTTAGAG</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>SV175-R</td>
<td>CAAGTCCTTCTAAAATAAGTTGA</td>
<td>290</td>
</tr>
<tr>
<td>Ly49h</td>
<td>Ly49h-F</td>
<td>GGAGAGTTCTTTCTTAAAGT</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>Ly49h-R</td>
<td>TGTCAAGATATGAGTTAGAGG</td>
<td>348</td>
</tr>
<tr>
<td>Ly49h</td>
<td>SV50-F</td>
<td>GGAGAGTTCTTTCTTAAAGT</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>SV50-R</td>
<td>TGTCAAGATATGAGTTAGAGG</td>
<td>236</td>
</tr>
<tr>
<td>Ly49h</td>
<td>SV151-F</td>
<td>GTGCTCGATGACACATAATGG</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>SV151-R</td>
<td>GTGCTCGATGACACATAATGG</td>
<td>204</td>
</tr>
<tr>
<td>Ly49h</td>
<td>SV168-F</td>
<td>TTTTGTGTATAGGTGTTGTTG</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>SV168-R</td>
<td>TTTTGTGTATAGGTGTTGTTG</td>
<td>195</td>
</tr>
<tr>
<td>Ly49h</td>
<td>SV169-F</td>
<td>CAACATATATTCGAGTCAAGGAG</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>SV169-R</td>
<td>CAACATATATTCGAGTCAAGGAG</td>
<td>318</td>
</tr>
</tbody>
</table>

\(^{a}\) 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 I-A\(\alpha\). Digestions with both HindIII and PstI enzymes allowed the discrimination between the H-2\(^b\) (BALB/c) or H-2\(^a\) (PWK) alleles (41). The contribution of PWK alleles at NKC and H-2 to the segregation of the phenotype (i.e., 
\[\text{log}_{10} \text{PFU in the spleen} \right ) \text{in } [(\text{BALB/c} \times \text{PWK})_F_1 \times\text{BALB/c}] \text{backcross mice was estimated using the following linear model:} 
\text{phenotype} = m + nkc + h-2 + e,\] 
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 log_{10} 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 cDNAs 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 (log_{10} PFU = 4.0 in the spleen and 4.3 in the liver). In contrast, the PWK strain was resistant, showing viral titers of 1.9 log_{10} PFU in the spleen and 3.8 log_{10} PFU in the liver, both of which are comparable to titers found in MCMV-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 (log_{10} PFU = 4.0 in the spleen and 4.3 in the liver). In contrast, the PWK strain was resistant, showing viral titers of 1.9 log_{10} PFU in the spleen and 3.8 log_{10} 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>
<thead>
<tr>
<th>Locus</th>
<th>Primers(^a)</th>
<th>Primer Sequence (5‘–3‘)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly49(h)</td>
<td>Ly49h-F</td>
<td>AACCTCTTAAAGAGGATACAGAC</td>
<td>1042</td>
</tr>
<tr>
<td>Ly49h-R</td>
<td>TCTCAAGATAGTAGGAGGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49k</td>
<td>Ly49k-F</td>
<td>GCATGCTTGAGCAAGAATTCTG</td>
<td>707</td>
</tr>
<tr>
<td>Ly49-k-R</td>
<td>CCAAAATTACAGTTAGGAGGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49n</td>
<td>Ly49n-F</td>
<td>TCTCAAGATAGTAGGAGGAGG</td>
<td></td>
</tr>
<tr>
<td>Ly49-R</td>
<td>CCAAAATTACAGTTAGGAGGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKG2d</td>
<td>NKG2d-F</td>
<td>ACACCATCAGATCAGTTCAGAGG</td>
<td>779</td>
</tr>
<tr>
<td>NKG2d-R</td>
<td>TTGCTGTTGTTGTTGAGGTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</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 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 \times 10^3 PFU MCMV (Smith strain, salivary gland preparation). The dashed line indicates the level of detection of our assay (log_{10} 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 (*).

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 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 \times 10^3 PFU MCMV (Smith strain, salivary gland preparation). The dashed line indicates the level of detection of our assay (log_{10} 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 (*).
mode of inheritance of the MCMV resistance phenotype. Phenotypes of the N2 progeny presented a bimodal distribution consistent with a major locus effect (Fig. 3A). Means of each mode were log_{10} 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 IAAl. 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 log_{10} 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 log_{10} 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 Cmvl 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 species. 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, C). They did not show the bimodal distribution and bright intensity typical of B6 cells (Fig. 5C).

![FIGURE 3. Genetic analysis of MCMV resistance in PWK. A, Phenotypic distribution of parental PWK (n = 17) and BALB/c (n = 11) strains and (BALB/c × PWK)F1 (n = 6) and [(BALB/c × PWK) F1 × BALB/c] N2 progeny (n = 70). Data is viral loads at 3 days after an i.p. injection of 5 × 10^3 PFU MCMV (Smith strain, salivary gland preparation). Mean is indicated for each group by the horizontal bar across the individual symbols. Each symbol represents an individual mouse. B, Combined effects of NKC and H-2 loci on spleen viral titers. The vast majority of BALB/c homozygous NKC genotypes at Ly49e (NKC^{Hom}) were susceptible (40/41). Most heterozygous NKC genotypes at Ly49e (NKC^{Het}) were resistant (21/29), yet a bimodal distribution could be appreciated within this group, and 7/29 showed intermediate titers. The two NKC genotypes were plotted against BALB/c homozygous H-2 genotypes at IAAl (H-2^{Hom}) and heterozygous H-2 genotypes at IAAl (H-2^{Het}). The box-and-whisker plot illustrates that, among heterozygous NKC genotypes, those that inherited a BALB/c homozygous H-2 were more resistant, indicating that the BALB/c H-2 allele is the resistant one.](image)

Table III. Effects of quantitative trait loci controlling MCMV infection

<table>
<thead>
<tr>
<th>Locus</th>
<th>p</th>
<th>LOD Score</th>
<th>Variance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>&lt;2.2e-16</td>
<td>25.1</td>
<td>82.1</td>
</tr>
<tr>
<td>LY49e</td>
<td>&lt;2.2e-16</td>
<td>21.9</td>
<td>77.1</td>
</tr>
<tr>
<td>IAAl (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.

FIGURE 5. Flow cytometry analysis. Cells were obtained from inbred mice B6, BALB/c, and PWK (top, middle, and bottom panels, respectively). A, Fresh splenocytes were stained with Abs specific for CD3, CD122, and NKG2D. CD3-CD122+ NK cells were electronically gated. The histogram shows NKG2D staining against a negative control on gated NK cells. Numbers in histograms represent mean fluorescence intensity. B, Fresh splenocytes were stained with Abs specific for CD3, CD122, Ly49C/I, and Ly49H/C/I. CD3-CD122+ NK cells were electronically gated. The dot plot shows the populations of gated NK cells stained with 1F8 (anti-Ly49C/F/I/H/I) and 5E6 (Ly49C/I). Numbers in parentheses indicate the percentage of cells stained positive with 1F8 only or with both 1F8 and 5E6 for the indicated mice. C, IL-2-activated NK cells were stained with Abs specific for CD3 and CD122 and then permeabilized and stained intracellularly with polyclonal Ab specific for Ly49H. CD3-CD122+ NK cells were electronically gated. The histogram shows Ly49H intracellular staining against a negative control on gated NK cells. Numbers in histograms represent percentages of cells staining positive.
by Ly49H+ NK cells (22). As expected, Ly49H+/H11001 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 /H9004 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 /H9004 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 /H9004 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+/H11001 NK cells, most wild isolates of MCMV (~86%) present mutations in m157.

We have also shown that the NKC-encoded activating receptor NKGD2 was properly expressed in PWK. NKG2D 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 NKG2D (29–32). Finally, in vivo blocking experiments of NKG2D did not abolish MCMV resistance in PWK, formally excluding NKG2D as mediator of resistance. Altogether, these observations support the hypothesis that alternative mechanisms other than NKG2D 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.
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 out 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 Cmv-4. 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 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 Cmv-3-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 Cmv-4 operates in a manner similar to that of the Cmv-1/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 Cmv-4. High-resolution linkage mapping and cDNA cloning experiments are warranted to track 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 NKC functions.

Acknowledgments

We thank Isabelle Lancin for skillful help with the wild-derived mouse strains, Seung-Hwan Lee for viral stocks, Michel Bennett, Dirk H. Busch, Martin Messerle, and Lewis L. Lanier for generously sharing reagents, James P. Di Santo for support, and Ute Rogner for critically reading the manuscript.

Disclosures

The authors have no financial conflict of interest.

References
