Requisite $H_2^k$ Role in NK Cell-Mediated Resistance in Acute Murine Cytomegalovirus-Infected MA/My Mice

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Requisite H2\(^k\) Role in NK Cell-Mediated Resistance in Acute Murine Cytomegalovirus-Infected MA/My Mice

Abhijit Dighe,* Marisela Rodriguez,*† Pearl Sabastian,* Xuefang Xie,*† Michael McVoy,‡ and Michael G. Brown²*†

Human CMV infections are a major health risk in patients with dysfunctional or compromised immunity, especially in patients with NK cell deficiencies, as these are frequently associated with high morbidity and mortality. In experimental murine CMV (MCMV) infections, Ly49H activation receptors on C57BL/6 (B6) NK cells engage m157 viral ligands on MCMV-infected cells and initiate dominant virus control. In this study, we report that MCMV resistance in MA/My relies on Ly49H-independent NK cell-mediated control of MCMV infection as NK cells in these mice do not bind anti-Ly49H mAb or soluble m157 viral ligands. We genetically compared MA/My resistance with MCMV susceptibility in genealogically and NK gene complex-Ly49 haplotype-related C57L mice. We found that MCMV resistance strongly associated with polymorphic H2\(^k\)-linked genes, including MHC and non-MHC locations by analysis of backcross and intercross progeny. The H2\(^b\) haplotype most frequently, but not absolutely, correlated with MCMV susceptibility, thus confirming a role for non-MHC genes in MCMV control. We also demonstrate a definite role for NK cells in H2\(^a\)-type MCMV resistance because their removal from C57L.M-H2\(^a\) mice before MCMV infection diminished immunity. NK gene complex-linked polymorphisms, however, did not significantly influence MCMV control. Taken together, effective NK cell-mediated MCMV control in this genetic system required polymorphic H2\(^b\) genes without need of Ly49H-m157 interactions. The Journal of Immunology, 2005, 175: 6820–6828.

M urine CMV (MCMV)\(^1\) infection in inbred mice has proven useful as an experimental model of human CMV infection and as a key toward identification of host genes associated with resistance or susceptibility to infectious disease (1). NK cells are well known for their role in controlling virus infections and these innate effectors provide critical antiviral functions in mice infected with MCMV. From experimental MCMV studies in the C57BL/6 (B6) and BALB/c genetic system, Scalzo et al. (2) found that MCMV replication in infected B6 spleens is restricted by the Cmv1' locus (now known as Ly49H), but similar infection in Cmv1'-type BALB/c spleens leads to overwhelming virus growth. Ly49H activation receptors expressed on B6 NK cells bind MHC class I-related m157 ligands encoded by MCMV on infected cells (3, 4). Without Ly49H receptors, BALB/c NK cells fail to similarly recognize MCMV infection. Thus, NK cells endowed with pathogen-sensing Ly49H receptors in MCMV-resistant B6 are activated and efficiently target MCMV-infected cells for destruction through m157 ligand recognition (5–8).

Despite the seemingly singular and dominant role of Ly49H\(^+\) NK cells in MCMV resistance, several additional gene products are known to contribute at least in part to the outcome in MCMV-infected mice. Indeed, Chalmer et al. (9) first noted that MHC-linked genes could contribute virus resistance following MCMV infection because H2\(^a\) in C3H, CBA, and H2\(^b\) congenic strains was associated with increased survival following high-titer MCMV infection (9, 10), however H2-linked MCMV resistance has not been further investigated and consequently its role in immune protection is not understood. Although MHC class I proteins can serve as cellular receptors of MCMV infection in some cultured cell lines (11), macrophages or embryonic fibroblasts of various H2 types, including H2\(^b\), are apparently equally permissive for MCMV infection and replication in vitro (9). Moreover, Tay et al. (12) and Polic et al. (13) demonstrated that β2-microglobulin (β2m) is not required for in vivo MCMV infection, nor is it needed for infection of embryonic fibroblasts taken from β2m\(^−/−\) mice. A role for the NKG2D activation receptor in MCMV immunity has also been noted in studies of innate immune responses toward MCMV with targeted mutations, in particular, virus encoded NK cell immunoevasins (14–17). NKG2D binds “stress-induced” MHC class I-related ligands including retinoic acid early inducible transcript (Rae1) family members, the minor histocompatibility Ag H-60 and MULT-1 in mice and MIC-A, MIC-B, and ULBP in humans (18). From studies of mice with spontaneous or chemical-induced mutations or targeted gene disruptions, it is also apparent that other innate proteins including cytokines and chemokines, cytokine receptors and TLRs, intracellular signal transducers, and cytotoxic effector proteins also are required components of the fully functional “resistome” that may include as many as 300 proteins (reviewed in Ref. 1).

Two additional inbred strains noted for effective NK cell control after acute MCMV infection include NZW (19) and MA/My (20).
NZW resistance is interesting in that Ly49H-like receptors on NZW NK cells do not bind m157 ligands and are otherwise not involved in controlling MCMV infection. Ongoing genetic studies of the New Zealand strains indicate that MCMV control by a complex trait determined by multiple gene products (19). MA/My MCMV resistance, in contrast, was thought to involve Cmv f-type immunity because immunodepletion of NK cells before infection led to full virus susceptibility (20), but resistance in this strain was not pursued perhaps because additional genetic tools were not previously available. Similar studies have also been performed in outbred wild mice. Despite fairly ubiquitous MCMV infection among wild-trapped mice from different geographical locations in Australia, Cmv f-like resistance was observed only in ~11% of outbred wild mice (21), thus, this type of MCMV resistance is actually rare, similar to that observed among inbred strains. Although human NK cells also contribute important antiviral defenses, especially against herpes viruses (22), but fail to express Ly49 receptors, we reasoned that NK cells likely use additional Ly49-independent sensors in control of MCMV and potentially other infectious pathogens.

To address this hypothesis, host innate antiviral defenses were studied in MA/My, C57L and their hybrid offspring at early time points after MCMV infection. Despite having related NK gene complex (NKC) Ly49 haplotypes (23), C57L and MA/My display entirely disparate MCMV control phenotypes. We demonstrate here that MA/My NK cell-mediated immune control of MCMV does not require Ly49H or recognition of m157 viral ligands. We instead found that MCMV control in MA/My requires polymorphic MHC and non-MHC genes outside the NKC. Ly49H-independent sensing and control of MCMV infection by NK cells in MA/My implicates multiple antiviral defense mechanisms used by NK cells that may also be analogous to NK cell-mediated control of pathogenic viruses in humans.

Materials and Methods

Mice

MA/My, C57L, C57BL/6, and BALB/cJ breeder pairs were purchased from The Jackson Laboratory and were housed in the MR-5 SPF Vivarium at the University of Virginia, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. (C57L × MA/My)F1 and F2 hybrids, backcross offspring on the C57L and MA/My backgrounds, congenic C57L.H-2b and C57L.H2-Kb/NKc GM, and (BALB/c × MA/My)F1 mice were bred and housed in the same vivarium. Mouse inventory and breeding records were recorded in a modified MouSeek 1.0 (http://groups.yahoo.com/group/mouseek/) database. All animal studies were approved by and conducted in accordance with Animal Care and Use Committee oversight.

Cell lines and tissue culture

Anti-Ly49H (IgG1) hybridoma 3D10 (a gift from Dr. W. M. Yokoyama, Washington University, St. Louis, MO) was grown in DMEM supplemented with 10% normal calf serum, penicillin/streptomycin (100 U/ml: 100 μg/ml), and l-glutamine (2.0 mM). HEK293 cells (Tissue Culture Facility, University of Virginia) were grown in similarly supplemented DMEM-F12 plus sodium-pyruvate (1.0 mM) and sodium-bicarbonate (1.5 mg/ml). NIH3T3 (ATCC CRL1658; American Type Culture Collection), 3T12 (ATCC CCL-164), and L929 (ATCC CCL-1) cell lines were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin (100 U/ml:100 μg/ml), and glutamine (2.0 mM).

Abs and FACS analysis

mAbs were purified from spent cell-free hybridoma supernatants (Lymphocyte Culture Center, Department of Anatomy and Cell Biology, University of Virginia). Purified anti-Ly49H mAb 3D10 was FITC-conjugated using a kit (Calbiocem). Purchased mAbs included anti-NK1.1 PK136 (IgG2b) PE (BD Pharmingen), eBMA2 PE (control IgG2a; eBioscience), anti-human IgG FITC (Jackson ImmunoResearch Laboratories), anti-mouse CD3ε PerCP 145-2C11 (eBioscience), and rat anti-mouse pan NK cells DX5 PE (IgM, eBioscience). Chimeric m157-IgG1 fusion protein was obtained from the spent cell-free supernatants of transiently transfected HEK293 cells as described previously (3, 19). For flow cytometric analysis using a FACSScan (BD Biosciences) and FlowJo (version 4.3.1; Tree Star), IL-2-activated NK cells, or mouse spleen leukocytes were bound with unlabeled mAb 2.4G2 (5 μg/ml) and subsequently stained with primary labeled mAbs 3D10 FITC (~5 μg/ml), PK136 PE (2 μg/ml), 145-2C11 PerCP (2 μg/ml), DX5 PE (2 μg/ml), or with m157-IgG (50 μg of supernatant/106 splenocytes) followed by anti-human IgG FITC (14 μg/ml) as described (24).

Recombinant Δm157-MCMV construction

Recombinant virus construction was performed essentially as described (25). Briefly, a deletion mutation spanning the m157 open reading frame (ORF) was introduced into the WT K181 MCMV genome using homologous recombination. MCMV targeting sequences (right = 214,490–215,536 and left = 217,872–218,831) were PCR amplified using high-fidelity TripleMaster polymerase mix (Eppendorf) and subcloned into pBluescript II sk+ for sequence verification. Right (ClaI/NotI) and left (EcoRI/SphI) restriction fragments were subsequently subcloned into pEGFP-Puro (25). NIH3T3 cells were transfected with the resultant targeting plasmid pTC-Δm157 and later infected with WT K181 MCMV. MCMV collected and purified from thoroughly lysed cell cultures went through several rounds of bulk selection in porcymic (1 μg/ml), followed by rounds of limiting-dilution cloning in 96-well plates without porcymic selection but with screening for green fluorescence. The targeted m156-m157-m158 sequence deletion was confirmed using MCMV sequence-specific PCR to detect the correctly targeted locus (data not shown). WT and Δm157- MCMV replicate with similar kinetics in 3T12, NIH3T3, L929, and mouse embryonic fibroblast cells and enhanced GFP fluorescence is visible in cells surrounding virus plaques formed in 3T12 monolayers or in less confluent 3T3 cells within 12 h after infection with the recombinant Δm157-MCMV (data not shown).

Virus assays

For experimental MCMV (Smith Strain, ATCC no. VR194) infections, salivary gland stock virus (SGV) was prepared after serial passage in BALB/c mice as described previously (19). Average virus titers (PFU per milliliter) for SGV and 3T12-passaged stocks were determined in multiple (3–5) independent titering experiments on NIH3T3 monolayers. Experimental mice (6–10 wk) were i.p. infected with MCMV (5–10 × 103 PFU) or 3T12-passaged MCMV stocks (1–5 × 103 PFU) were used in experimental infections for comparison of WT K181 and Δm157-MCMV levels in infected spleens and livers. To study the role of NK cells during MCMV infection, mice were i.p. injected (200 μg of mAb/200 μl of PBS) with anti-NK cell receptor mAb 48 h before MCMV infection. Spleens and livers were collected from euthanized mice 3.5 days postinfection.

For virus quantitation, spleen and liver genomic DNA (50 ng/25 μl of PCR mix) samples purified using a kit (Genta Systems) were analyzed using quantitative real-time PCR (QPCR) as described previously (26). All sample measurements were performed in triplicate. MCMV levels (average for triplicate measurements) were reported as the log10 (number of MCMV genome copies/number of β-actin genomic copies).

Genotyping and quantitative genetics analysis

Genome wide scans were performed using a panel of fluorescent-labeled simple sequence length polymorphism (SSLP) markers to distinguish C57L and MA/My alleles (Refs. 19 and 23 and our unpublished data). SSLP amplified products were analyzed on a Genetic Analyzer 3100 using Genescan and Genotyper software (Applied Biosystems) as described previously (19). Marker regression analysis using free (backcross cohort) or recessive (intercross cohort) models for spleen MCMV levels and genotypes was performed with MapManager QTX software (27, 28) to determine probable locations for putative quantitative trait loci (QTL). Note that 6 of 133 intercross mice under study were excluded from the QTL analysis because more than two SSLP genotypes have not been resolved. Permutation analysis (1000 permutations; 1 cM steps), interval mapping (1 cM steps), and bootstrap analysis also were performed to assess significance levels of putative QTL implicated in marker regression.

Results

Induced MCMV control in MA/My uses Ly49H-m157 independent NK cell control

We were intrigued by the finding of Scalzo et al. (20) demonstrating NK cell-mediated control of MCMV infection in MA/My, but not in C57L, because these strains share a common ancestry (29).
and also the same NKC-Ly49 (Ly49H resides in the Ly49 gene cluster) haplotype (23). Thus, we compared MA/My and C57L NK cells for Ly49H expression. Unlike in B6 or NZW, CD3^+ NK1.1^+ splenocytes from MA/My and C57L do not express 3D10-binding receptors, suggesting either that this Ly49H epitope is missing or that bona fide *Cmv1r*-type Ly49H receptors are not expressed by NK cells in these strains (Fig. 1). In support of the latter possibility, Ly49-related sequences were obtained from MA/My NK cell cDNA samples under low stringency conditions, but Ly49H sequences have not been amplified using gene-specific PCR strategies (data not shown). Thus, MA/My-type MCMV resistance was further characterized as a first step toward uncovering host genes responsible for the underlying dramatic differences in MCMV control in these strains.

A role for NK cells in MA/My MCMV immunity was confirmed (Fig. 2A) because virus levels in the spleens and livers of NK cell-depleted mice are considerably higher than those observed for control mice. A substantial increase in MCMV replication was also found in (C57L × MA/My)F₂ hybrids that have undergone similar immunodepletion of NK cells (Fig. 2B). MA/My NK cells therefore contribute vital innate MCMV control. Anti-Ly49H mAb 3D10 that blocks *Cmv1r*-type MCMV resistance in B6 mice (5, 19) however, does not similarly diminish resistance in (C57L × MA/My)F₂ hybrids when injected before MCMV infection (Fig. 2B). In further support of this finding, we also found that (BALB/c × MA/My)F₂ spleens have considerably higher viral burdens in acute MCMV infection than similarly infected MA/My spleens, in striking contrast to dominant *Cmv1r*-type resistance observed for (BALB/c × B6)F₂ hybrids (Fig. 2C). Thus, NK cells in MA/My and (C57L × MA/My)F₂ hybrids control MCMV infection through a Ly49H-independent mechanism.

Without Ly49H control, we questioned whether MA/My NK cells limit MCMV infection through recognition of MHC class I-related m157 ligands on infected cells. In Fig. 1, ~45% of control B6 NK cells express receptors for m157 viral ligands, but only minimal staining of CD3^+ NK1.1^+ MA/My NK cells with the same reagent was observed, thereby suggesting that MA/My NK cells should control MCMV through m157-independent recognition of infected cells. To further address this possibility, a mutant MCMV virus was produced with its m157 ORF replaced by an enhanced GFP gene cassette. Replication of m157 deletion mutant virus in NIH3T3, 3T12, L929 and mouse embryonic fibroblasts is not significantly different from the parental-type K181 strain (data not shown). We therefore compared virus levels in MA/My mice following MCMV infections with similarly titered deletion mutant and WT viruses. As expected, comparable low MCMV levels were observed in the spleens and livers of MA/My or B6 mice infected with WT virus (Fig. 3). Virus levels in MA/My spleens and livers from mice infected with Δm157-MCMV were slightly higher than WT virus infections probably because a higher inoculum was used.

**FIGURE 1.** MA/My and C57L NK cells lack receptors for 3D10 mAb or virus-encoded m157. Histogram plots of B6, MA/My, and C57L CD3^+ NK1.1^+ splenocytes stained with anti-Ly49H mAb (3D10-FITC) or m157-Ig and anti-IgG-FITC analyzed in flow cytometry are shown. Data are representative of three to five independently studied mice of each strain.

**FIGURE 2.** Ly49H-independent role of NK cells in MA/My and (C57L × MA/My)F₂ MCMV resistance. A, MA/My and anti-NK1.1-treated MA/My mice were infected with 5 × 10⁶ PFU SGV. Spleen (○) and liver (■) MCMV levels (3.5 days postinfection) determined in QPCR for individual mice of each group are shown. B, (C57L × MA/My)F₂ mice (untreated or preinjected with PBS, anti-NK1.1, or anti-Ly49H mAb) were infected with 5 × 10⁶ PFU SGV and similarly studied. C, MA/My, BALB/c, and (BALB/c × MA/My)F₂ hybrids were infected with 1 × 10⁶ PFU SGV. Spleen MCMV levels (3.5 days postinfection) determined in QPCR for individual mice of each group are shown. Data in B and C are representative of two and three independent experiments, respectively.
Similar virus levels were also observed in the livers of Δm157-MCMV infected B6 mice. B6 spleens, however, displayed significantly higher virus levels following Δm157-MCMV infection because NK cells in these mice could no longer efficiently limit MCMV. Moreover, we consistently observed in three independent experiments significantly higher Δm157-MCMV in infected B6 spleens than in similarly infected MA/My spleens. Together, these results show that MA/My NK cells use a distinct virus sensing mechanism, one that can be distinguished from Ly49H, to detect and subsequently limit MCMV infection without requirement for m157 expression.

**NK cell-mediated MCMV resistance controlled through H2-linked polymorphic genes**

A classical genetics approach was undertaken to further characterize MA/My-type MCMV resistance with the ultimate goal of identifying genes associated with increased MCMV resistance during acute virus infection. We found that similarly infected (C57L × MA/My)F1 hybrids and also backcross offspring on the MA/My background displayed only low level MCMV in their spleens, thus MA/My resistance was dominant to C57L susceptibility (Fig. 4). In fact, both the F1 and the backcross progeny had lower MCMV than in MA/My, suggesting that hybrid vigor actually augmented viral resistance observed in these animals. In striking contrast, virus levels in the spleens of (C57L × MA/My)F1 × C57L backcross progeny displayed a bimodal distribution of either low (MA/My-like) or high (C57L-like) MCMV that easily distinguished individual offspring (Fig. 5). Note that here also in the backcross hybrids, especially in the N2 generation, observed phenotypes are more extreme than in the progenitor strains. Because NKC- and H2-linked genes are known to impart MCMV control and because polymorphism(s) in their protein products could potentially explain the observed trait variation, NKC and H2 haplotypes were assessed for each of the infected backcross hybrids on the C57L background. Genotypes determined for the experimental cross revealed that H2 haplotypes were unmistakably linked with spleen virus levels in the backcross offspring (Fig. 5), but no association with NKC haplotypes was found (data not shown). In particular, high spleen virus levels were associated with H2h homozygosity, whereas low virus levels were only associated with H2 heterozygosity in the C57L backcross offspring (Fig. 5). High MCMV in one H2 heterozygous backcross mouse may have been due to an independent mutation in a H2-linked gene required in virus control, or alternately additional non-H2 loci might influence virus levels regardless of H2h regulation (see below). Nevertheless, H2h was unequivocally associated with lower MCMV replication levels at early times after infection in the backcross offspring on the C57L background.

It was not surprising then that only low virus levels were observed in the spleens of H2h homozygous F2 intercross offspring and that spleens with the greatest overall MCMV burden were from H2h homozygous F2 offspring (Fig. 5). Similar to the backcross study, NKC haplotypes were not correlated with MCMV control in the intercross cohort. Unexpectedly however, 5 of 10 H2h homozygous F2 spleens contained MCMV levels that were as low as those observed in the H2h F2 spleens (Fig. 5). In support of this finding, similar MCMV resistance in several additional homozygous H2h-type offspring were found in a second (MA/My × C57L)F1 cohort of MCMV-infected mice and virus levels in the livers of these MCMV-resistant H2h-F2 mice were not atypical (data not shown). Additionally, MCMV levels in H2h/F2 spleens ranged from low to intermediate. Thus, MCMV susceptibility is most pronounced and also most frequently associated with H2h in this genetic system, but non-H2 MCMV control is also apparent.

We next sought probable chromosome locations for MCMV control by searching for putative QTL in marker regression analysis. Virus levels and genotypes determined using a genome-wide screening panel of SSLP genetic markers that distinguish MA/My and C57L alleles were assessed in 38 backcross and 127 intercross combinations. The reliable limit of MCMV detection in this QPCR assay is also indicated (dotted line). Data are representative of three independent experiments.
offspring. H2-linked MCMV control in both cohorts was substantiated because ~50% of the trait variance in the backcross offspring or ~35% of the trait variance in the F2 setting was mapped on chromosome 17 (Tables I and II and Fig. 6). Interestingly, the strongest genetic association with MCMV control in the backcross cohort (p < 0.00001) coincided with D17Uvaa9 (~18.3–19 mega bp (Mbp)), whereas D17Uvaa12 linkage yielded the greatest likelihood ratio statistic (LRS) in our intercross analysis. Thus, MHC and non-MHC genes may contribute in MCMV control and as much as 50–65% of the trait variance may be determined by polymorphic genes beyond chromosome 17.

### Table I. Marker regression analysis in (C57L × MA/My)F2 intercross cohort (n = 127)

<table>
<thead>
<tr>
<th>Chr</th>
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<th>LRS (LOD)</th>
<th>% Variance</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
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<td>D17Mli6</td>
<td>50 (10.9)</td>
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<td>17</td>
<td>D17Uvaa12</td>
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<td>&lt;0.00001</td>
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<tr>
<td>17</td>
<td>D17Mli10</td>
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<td>26</td>
<td>&lt;0.00001</td>
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<tr>
<td>17</td>
<td>D17Mli152</td>
<td>18.6 (4.0)</td>
<td>14</td>
<td>&lt;0.00001</td>
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</table>

* Putative chromosome QTL locations determined in MapManager QTX (LRS > 18.6; p < 0.01) are listed.

* Genotypes determined using MIT (38) or our novel Uva SSLP genetic markers (see Fig. 6c) for the designated chromosome locations.

A role for H2-linked MCMV control through MA/My NK cells confirmed in interval-specific C57L.M-H2k^NKC^congenic mice

To assess the role of NK cells in H2^k-dependent MCMV resistance, CD3^+ DX5^+ splenocytes collected from control or PK136-treated mice before or after MCMV infections were first studied. PK136-mediated immunodepletion of MA/My DX5^+ NK cells was maximal by 96 h and maintained throughout the experimental time course (Fig. 7A). DX5^+ splenocytes in C57L however were only marginally compromised by the same time point and ~60% of these cells persisted through the experimental time course, presumably because MA/My and C57L NK1.1 Ags are encoded by distinct NK-C-Nkpr1 (NK1.1) haplotypes (24, 26). We also noted a substantial loss of NK cells from C57L and MA/My spleens by 48 h after MCMV infection, but only MCMV-infected MA/My spleens were repopulated by NK cells by 84 h after virus infection with an overall increase 150–170% of control levels in this compartment by comparison with NK cells in control MA/My mice (Fig. 7, B and C). This was consistent with an initial loss of total NK1.1^+ splenocytes and an overall decrease in the percent NK1.1^+CD3^- in MCMV-infected C57BL/6 spleens by day 2 postinfection (7). NK cell repopulation was more limited in MCMV-infected MA/My mice that also received PK136 treatment (Fig. 7D). DX5^+ NK cells in MCMV-infected C57L spleens in contrast remained low through 84 h postinfection. Thus, although NK cells were not efficiently depleted from C57L mice using mAb PK136, a substantial loss in NK cells does occur in this susceptible strain following MCMV infection.

We also produced an interval-specific C57L.M-H2k^NKC^congenic strain that displays innate MCMV resistance using SSLP marker-assisted selection of backcross offspring (P. Clark, P. Sebastian and M. G. Brown, unpublished data). MCMV resistance in this strain is determined by H2^k, but its NKC haplotype does not otherwise affect MCMV levels following infection. Heterozygous (het) C57L-M-H2^Ab,NKC^congenic mice, was backcrossed to C57L and the genotyped progeny were sorted into the following groups: (A) H2^het, NKC^congenic (B) H2^het, NKC^congenic (C) H2^b, NKC^congenic and (D) H2^b, NKC^congenic based on their MHC and NKC haplotypes. Mice from each group were i.p. injected with mAb PK136 before MCMV infection. Spleen and liver MCMV levels were subsequently determined and compared with C57L and MA/My controls. Of note, C57L.M-H2k^NKC^congenic and C57L.M-H2^k^ controls without PK136 treatment display comparable MA/My-like MCMV resistance (data not shown). As expected, spleen and liver MCMV levels in group A mice with their NK compartment intact displayed MCMV resistance that was comparable with that observed in MA/My or C57L.M-H2^k^ mice (Fig. 8). Virus levels were substantially higher in H2^b^ litter mate control spleens and livers regardless of the presence or absence of NK cells (Fig. 8). In fact, the virus levels in H2^b^ splenomes were almost certainly an underestimate of the extent of pathogenesis that was underway because total splenocytes in groups C and D were dramatically lower (~3%) than total splenocytes collected from H2^het^ group A mice (data not shown). In group B mice however, MCMV levels were significantly higher in 5 of 12 spleens or 11 of 12 livers than those observed for group A mice, consistent with PK136-mediated immunodepletion of NK cells from these NKC^het^ animals. It is curious however that MCMV levels ranged from very low to intermediate in 7 of 12 group B spleens. This is a consistent finding as similar results were obtained in two independent experiments. It is also unlikely that differences in PK136-mediated NK cell depletions could account for this outcome because similar NK cell losses were observed in seven experimental group B mice examined for DX5^+ cells on 3.5 days after infection, including mice that showed either high or low MCMV. Taken together, it is apparent that polymorphic H2^k^ gene(s) are required in innate control of acute MCMV infection with direct contributions through NK cells.

### Table II. Marker regression analysis in (C57L × MA/My)F1 × C57L backcross cohort (n = 38)

<table>
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<th>LRS (LOD)</th>
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<td>D17Mli10</td>
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Discussion

In this study the dominant role of MCMV resistance in MA/My mice was investigated using molecular and classical genetics strategies. We found that MA/My NK cells can recognize and control MCMV infection without need of m157-binding Ly49H receptors on NK cells. Our conclusion is based on several important findings: 1) Ly49H-like receptors were not detected on MA/My or C57L NK cells and anti-Ly49H mAb injections into (C57L × MA/My)F1 hybrids did not diminish innate MCMV immunity, 2) MA/My-type MCMV resistance, unlike Ly49H-dependent control, is attenuated by a gene(s) in the BALB/c genome, and 3) MA/My NK cells do not control MCMV infections through recognition of MCMV encoded m157 ligands and immunity in this strain is not perturbed by interfering with m157 expression through deletion of the m157 ORF from the MCMV genome.

Our findings clearly distinguish acute MCMV resistance in MA/My, NZW, and C57BL/6. Dominant Cmv1r resistance through Ly49H recognition of m157 is limited so far to the C57BL genetic background and this control mechanism is not negatively affected by a gene(s) in the BALB/c genetic background. In contrast, the BALB/c genetic background can attenuate NZW or MA/My MCMV resistance because MCMV levels in (BALB/c × NZW)F1...
and (BALB/c × MA/My)F1 outcross progeny are considerably higher than that observed for either NZW or MA/My (Fig. 2, Ref. 19). H2k MCMV control further distinguishes MA/My and NZW MCMV resistance mechanisms because H2 polymorphisms were not associated with innate MCMV immunity in New Zealand crosses so far studied (19). Thus, NK cells have harnessed multiple independent antiviral mechanisms to efficiently control this important viral pathogen. Human NK cells without Ly49 receptors may use similar antiviral defense mechanisms in controlling HCMV and perhaps other viral pathogens that are frequently found in patients with isolated NK cell deficiency (30). That multiple genes play a role in NK cell recognition and control of acute MCMV infection is evident in both the MA/My and New Zealand genetic systems, but the effects of hybrid vigor were quite remarkable in (C57L × MA/My)F1 and backcross progeny on the MA/My background. Whether hybrid vigor in this setting can be explained by the loss of potentially inhibitory MA/My allele(s) or by the addition of C57L alleles that could potentially augment MCMV resistance is not known. It will therefore be important to fully characterize such innate mechanisms of host antiviral defenses as these may lead to useful targets to modify host immune responses and defenses against viral pathogens.

We found that innate MCMV resistance in MA/My requires polymorphic H2k gene expression. This finding is consistent with the observations of Grundy (Chalmer) et al. (9, 10) that clearly defined an association between low mortality after MCMV infection and H2k in C3H, CBA and several H2k congenic strains. Grundy et al. (10) also noted that both the MHC class I K and D gene regions likely contribute in MCMV immunity and that such immunity correlated with induction of innate natural cytotoxicity functions in most inbred strains that were examined (31). But without further investigation into H2k-linked MCMV protection, H2-dependent MCMV resistance is still not understood mechanistically. In the current study, two distinct H2-linked locations genetically mapped in the backcross or intercross cohorts implicate multiple MCMV control QTL in this region of chromosome 17, including D17Uva09 that resides well beyond the MHC. In fact, in a search for potential QTL interactions, D17Uva09 and D17Uva12 were among the very highest (data not shown) thereby underscoring the likelihood that multiple chromosome 17 QTL contribute in innate MCMV control.

In related work published during preparation of this report, Desrosiers et al. (32) noted that Ly49P expression correlated with MCMV resistance by genetic comparison of MA/My with BALB/c or BALB.K. Because Ly49P activation receptors were specifically triggered on reporter cells in vitro by exposure to MCMV infected cells and anti-Dk mAb could block this activation, a role for Ly49P recognition of Dk ligands on MCMV-infected cells in MA/My has been implicated as a potential mechanism in NK cell-mediated resistance in acute MCMV infection (32).
Here an independent approach was used to examine genetic mechanisms in control of MCMV resistance. Although both reports demonstrate the importance of H2k in innate MCMV resistance, several informative differences have been uncovered. First, in the MA/My × BALB/c genetic system, relatively high virus levels were observed in MA/My and C57L spleens in A. MCMV-infected (1 × 10⁶ PFU SGV) MA/My and C57L spleens in B and C, and PK136-treated and MCMV-infected MA/My and C57L spleens in D. In B–D, 48 and 84 h time points after MCMV infection were studied. PK136 i.p. injections were given 48 h before MCMV infections in D. Data points represent average of two individual animals under study.

Second, NKC polymorphisms contributed in the MA/My × BALB/c genetic system (32), but were not associated with genetic variation in this report. Third, additional genes contribute to genetic variance that reside outside the MHC and NKC regions because H2b intercross spleens were observed with only low MCMV levels and H2k intercross spleens had either intermediate or low MCMV. It is also intriguing that discrete maximal LOD values were obtained for the backcross (D17Uva09) or intercross (D17Uva12) cohorts, thereby implicating distinct QTL effects in the context of the different genetic backgrounds. Finally, we have also extended the previous findings by demonstrating that H2k innate MCMV resistance actually involves NK cells because their depletion from MA/My or C57L.M-H2k-NKCmamm mice before MCMV infection was associated with a significant loss of splenocytes and also high virus burdens in the spleens and livers of most of these mice during the 3.5 day time course under study. Likewise, we observed full abrogation of NK cell-mediated resistance in all PK136-treated MA/My or (C57L × MA/My)F1, mice under study, whereas only ~42% of similarly treated C57L.M-H2b mice also were converted to MCMV susceptibility. Thus, additional non-NK cell-mediated MCMV resistance mechanisms must influence MCMV control in MA/My or intercross mice. The C57L.M-H2b congenic mice should prove invaluable in genetic dissection and identification of H2-linked genes required in MA/My MCMV resistance.

Its not clear why anti-NK1.1 treatments could more effectively diminish NK control of MCMV in the livers of infected mice than in their spleens, but as this is a consistent finding its possible that the distinction of NK cell control in livers and spleens has been revealed in the particular experimental conditions. Moreover, while NKC differences were not otherwise associated with MCMV control variation through NK cells in this genetic system, MA/My and C57L NK cells may express similar Ly49P activation receptors that could be triggered by exposure to Dk ligands during MCMV infection. This model however does not explain how NK cell recognition of Dk ligands on infected cells could keep pace with MCMV infection because cell surface MHC class I proteins, including Dk proteins (X. Xie and M. G. Brown, unpublished data), are efficiently decreased by MCMV encoded proteins (33).
H2k protection in acute MCMV infection may therefore also involve receptor-ligand interactions other than Ly49P with D6 that could potentially influence NK cell dependent resistance mechanisms, perhaps through H2 class I protein down-regulation that might be expected to deliver a strong signal alerting MA/My NK cells to MCMV infection. Paradoxically however, because MCMV also efficiently down-regulates Kd and Db proteins, it is not evident why NK cells in C57L do not similarly control MCMV infection. We have considered that allele-specific MHC class I down-regulation by MCMV (33) could potentially lead to more pronounced or more rapid down-regulation of H2k alleles during the course of acute MCMV infection, but so far we have not found evidence of this in MCMV infected cells in vitro assays (X. Xie and M. G. Brown, unpublished data). In contrast, if C57L NK cells or particular subsets of NK cells in C57L fail to recognize an important class I ligand during development their function could be impaired by comparison with NK cells in MA/My or in C57L.H2k because normal MHC class I expression is required for fully mature NK cell functioning (34, 35). In support of the latter possibility, NK cells in C57L.H2k or C57L must differ functionally because only the H2k environment supported NK cell competency in acute MCMV infection. Thus, H2k class I proteins may favor selective education of NK cells in MA/My so that they become competent for recognition of MCMV infected cells, whereas NK cells developing in the H2b environment of C57L are less fit. Importantly, background genes in these mice were similar and NKc region linked polymorphisms did not contribute to phenotypic differences in the mice. Hence, NKc linked receptor expression and/or selective maturation of a NK subset that can effect control virus growth may be modulated by H2k gene expression. Interestingly, Johansson et al. recently found that NK cell maturation and effector function is directly impacted by the MHC background in which development occurred (36). Because the educational impact of MHC class I on NK cell receptor expression and effector functions is dependent on the particular class I protein, and the environmental context in which it is recognized, it seems plausible that particular H2k class I proteins may contribute toward selective development and maturation of functionally competent NK cells that could then deliver effective H2-dependent antiviral defenses. There is also an apparent role for additional non-MHC proteins that contribute in overall MCMV resistance in this genetic system because marker regression analysis determined that as much as 65% of the genetic variance is not accounted for by H2-linked genes. Still undefined antiviral mechanisms may also be used by NK cells or other innate immune cells in these mice to efficiently control MCMV infection. Further study of MCMV resistance in this genetic setting is warranted and may prove useful in modeling human NK cell virus control mechanisms because expression of some inhibitory NK receptors and particular HLA alleles correlate with the capacity to resolve virus infections (37).

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Disclosures

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References


