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Gain of Virulence Caused by Loss of a Gene in Murine Cytomegalovirus‡

Ivan Bubić,[1]† Markus Wagner,[2]†§ Astrid Krmpotić,[1] Tanja Saulig,[1] Sungjin Kim,[3] Wayne M. Yokoyama,[3] Stipan Jonjić,[1]* and Ulrich H. Koszinowski[2]

Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, 51000 Rijeka, Croatia[1]; Max von Pettenkofer Institute, LMU, D-80336 Munich, Germany[2]; and Howard Hughes Medical Institute, Rheumatology Division, Washington University School of Medicine, St. Louis, Missouri 63110[3]

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Mouse strains are either resistant or susceptible to murine cytomegalovirus (MCMV). Resistance is determined by the *Cmv1*^{r} (*Ly49h*) gene, which encodes the Ly49H NK cell activation receptor. The protein encoded by the *m157* gene of MCMV has been defined as a ligand for Ly49H. To find out whether the m157 protein is the only Ly49H ligand encoded by MCMV, we constructed the *m157* deletion mutant and a revertant virus. Viruses were tested for susceptibility to NK cell control in Ly49H$^+$ and Ly49H$^-$ mouse strains. Deletion of the *m157* gene abolished the viral activation of Ly49H$^+$ NK cells, resulting in higher virus virulence in vivo. Thus, in the absence of *m157*, Ly49H$^+$ mice react like susceptible strains. 129/SvJ mice lack the Ly49H activation NK cell receptor but express the inhibitory Ly49I NK cell receptor that binds to the m157 protein. The Δ*m157* inhibitory phenotype was weak because MCMV encodes a number of proteins that mediate NK inhibition, whose contribution could be shown by another mutant.

Infection of mice with murine cytomegalovirus (MCMV) is an established model for studying human cytomegalovirus infection. Immune control of MCMV infection is organized in a hierarchical and redundant manner by diverse components of the innate and adaptive immune response (19, 21, 32, 34). NK cells play an important role in the innate control of cytomegalovirus infection. This has been demonstrated in humans with a rare disorder characterized by complete absence of NK cells and in genetically deficient mice lacking NK cells or being depleted of these cells by treatment with anti-NK cell antibodies (4). On the basis of their susceptibility to MCMV infection, mouse strains are either susceptible (e.g., BALB/c mice) or resistant (e.g., C57BL/6 mice) (17, 37). MCMV titers in the spleens of mice inversely correlate with their ability to mount an effective NK cell response, which is controlled by the single dominant locus, named *Cmv1*, located in the natural killer gene complex on mouse chromosome 6 (10, 14, 37–39). The alleles of the *Cmv1* locus can confer either susceptibility (*Cmv1*^{s}, a recessive allele) or resistance (*Cmv1*^{r}, a dominant allele) to MCMV (36, 39).

The *Cmv1*^{r} (*Ly49h*) gene encodes the Ly49H receptor (5, 9, 23, 24), which belongs to the Ly49 family of NK cell receptors and is expressed on approximately 50% of NK cells in C57BL/6 mice (41, 43, 47). Unlike the inhibitory Ly49 receptors, Ly49H lacks the immunoreceptor tyrosine-based inhibition motif and is noncovalently coupled with DAP12 at the cell surface, allowing transduction of an activation signal into the cell via its immunoreceptor tyrosine-based activation motif (16, 43) that is required for resistance to MCMV (40). However, Ly49H does not define resistance to vaccinia virus and gammaherpesvirus 68 (2, 12).

Unlike other members of the Ly49 receptor family, which use major histocompatibility complex (MHC) class I molecules as their cellular ligands, Ly49H binds to at least one MCMV-encoded protein, the *m157* gene product (2, 42). The m157 protein has structural homology to MHC class I molecules, similar to several other proteins encoded by MCMV *m145* gene family members (42). An MCMV deletion mutant restricted Ly49H activation to 15 genes in the HindIII-E region (2). Isolated open reading frames (ORFs) from this region, with the exception of *m157*, failed to activate Ly49H. However, a similar contribution of other genes in this region cannot be excluded since certain cytomegalovirus proteins encoded by different genes can only be expressed as a complex (27). Therefore, it remained an open question whether *m157* is the only viral gene that contributes to MCMV resistance defined by Ly49H.

To investigate the biological relevance of the *m157* gene, we constructed an *m157* deletion mutant, as well as the corresponding revertant virus. We studied the susceptibility of these recombinant viruses to control by NK cells in vivo in Ly49H$^+$ and Ly49H$^-$ mouse strains. Loss of the *m157* gene is associated with gain of virulence in Ly49H$^+$ but not in Ly49H$^-$ mouse strains. Therefore, m157 is the only MCMV-encoded protein that activates Ly49H$^+$ NK cells. The absence of the gene that encodes this protein in the *m157* deletion mutant gave us the opportunity to reveal the function of viral genes that down-modulate NK cell activity in *Cmv1*^{r} mice. *Cmv1* has been defined as a locus of resistance to MCMV, influencing virus control mainly in the spleen (37). Furthermore, since we could define m157 as the only MCMV-encoded ligand for the

* Corresponding author. Mailing address: Department of Histology and Embryology, Faculty of Medicine, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia. Phone: 385 51 651 206 or 170. Fax: 385 51 651 176. E-mail: jstipan@medri.hr.

† Ivan Bubić and Markus Wagner contributed equally to this work.

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§ Present address: Department of Pathology, Harvard Medical School, Boston, MA 02115.

Ly49H receptor, we could also address the question of NK cell control of infection at a different site of infection.

MATERIALS AND METHODS

Viruses and cells. Bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 has previously been shown to be biologically equivalent to MCMV strain Smith (ATCC VR-194, recently reaccessed as VR-1399) and is here referred to as wild-type (w.t.) MCMV (50). The ΔMS94.5 virus, which possesses a deletion of 15 genes (*m151* to *m165*) is described elsewhere (46). All viruses were propagated on third-passage BALB/c mouse embryonic fibroblasts (MEFs) and purified by sucrose cushion centrifugation. Tissue culture-grown virus preparations were used for mouse inoculations.

Cells of the mouse macrophage cell line IC-21 were obtained from the American Type Culture Collection (ATCC catalog no. TIB-186) and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Plasmid construction. Plasmid pori6k-pA, which contains the Zeocin resistance gene, the poly(A) signal from BHG, the origin of replication (ori*6k*), and an additional 34-bp FRT site, was generated by ligation of a 353-bp KpnI/PvuII fragment from plasmid pCDNA4TO (Invitrogen) into the KpnI/EcoRV sites of plasmid pori6kZeo (A. Bubeck, M. Wagner, Z. Ruzsics, M. Iglesias, I. R. Singh, and U. H. Koszinowski, submitted for publication). With primers SpeI-nt215895-918 and SpeI-nt217226-250 and pSM3fr as the template DNA, the *m157* gene and its putative promoter (nucleotide [nt] positions 215895 to 217250, as described in reference 33) were amplified by PCR and inserted into the SpeI site of plasmid pori6k-pA, thereby generating plasmid pori6k-m157-pA. The correct amplification of the *m157* promoter and the *m157* gene was confirmed by sequencing with primers M157-1 (5′-TGTTGACCGCCATCTGTTCTTGA), M157-2rev (5′-GGTAAGATTAATATTCAAGGATCA), and M157-3 (5′-GGATTGAAAATTGTTACAGCACG) (data not shown).

Insertion of an FRT site into MCMV BAC pSM3fr between genes *m16* and *m17*. Mutagenesis of the MCMV BACs was performed as previously described (49). The insertion of a 48-bp FRT site (5′-GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3′) into the intergenic region between MCMV ORFs *m16* and *m17* (nt positions 15678 to 15748) was achieved as follows. A linear PCR fragment containing a kanamycin resistance gene flanked by two 48-bp FRT sites and viral homologies to the noncoding region between ORFs *m16* and *m17* was generated by PCR with primers 5-m16-FRT-Kan-pCP15 (5′-CCCTCTTAATCATGACAATTATAAGTGTCTTATACGCAATACTTTTATCATAATTCGGGGGTGTCCAGGGTTTTCCC) and 3-m17-FRT-Kan-pCP15 (5′-GAGGAATAGGAATAACTCACCACCGATTTCAGCGTCTGCCCCAAGTCTGACTTCCGGCTCGTATGTTGTGTGG) and plasmid pCP15 (8). This fragment was inserted into pSM3fr by homologous recombination in *Escherichia coli*, thereby deleting 70 bp of the noncoding region between these two genes (nt positions 15678 to 15748). The kanamycin resistance-encoding gene was subsequently excised by FLP-mediated site-directed recombination as described previously (49), leaving only one FRT site, which can be used for site-directed insertion of any gene of interest into this site. The correct mutagenesis of resulting MCMV BAC pSM3fr-FRT was confirmed by restriction pattern analysis and sequencing of the *m16-m17* genome region with primers MCMV-15461-down (5′-GAAGTCCATGTATCTCCTTCA) and MCMV-15939-up (5′-TCGGACAAATTCTAAACCTCG) (data not shown). The w.t.-FRT-MCMV strain generated from pSM3fr-FRT was shown to replicate to w.t. MCMV titers in NIH 3T3 fibroblasts and also in the lungs, spleens, and livers of BALB/c mice infected with 2×10^5 PFU at days 3 and 7 postinfection (data to be published elsewhere). This confirmed that the insertion of short sequences into this intergenic genome region does not significantly interfere with virus replication in vitro or in vivo.

Deletion of the *m157* ORF in MCMV BACs pSM3fr and pSM3fr-FRT. For deletion of the *m157* ORF in the respective MCMV BACs, a linear DNA fragment was generated by PCR with primers 5-m157-Kan (5′-CGTGGTCAAGCCGGTCGTGTTGTACCAGAACTCGACTTCGGTCGCGTTCGATTTATTCAACAAAGCCACG) and 3-m157-Kan and plasmid pACYC177 as the template DNA. This fragment was subsequently inserted into MCMV BACs pSM3fr and pSM3fr-FRT, respectively, by homologous recombination in *E. coli* as described previously (49), generating recombinant MCMV BACs pΔm157 and pΔm157-FRT. These genomes lack most parts of the *m157* gene, including the ATG start codon (nt positions 216291 to 216874).

Reinsertion of the *m157* gene, including its promoter, at an ectopic position into the *m157* deletion genome. For reinsertion of the *m157* ORF including its promoter and an additional poly(A) signal from BHG into MCMV BAC pΔm157-FRT at the ectopic position between genes *m16* and *m17*, a linear DNA fragment that contains these elements and an additional Zeocin resistance gene was generated by PCR with primers 5-m157-Zeo-m16/17 (5′-CCCTCTTAATCATGACAATTATAAGTGTCTTATACGCAATACTTTTATCATAATACATGTGGAATTGTGAGC) and 3-m157-Zeo-*m16/17* (5′-GAGGAATAGGAATAACTCACCACCGATTTCAGCGTCTGCCCCAAGTCTGATTAGCACGTGTCAGTCCT) and plasmid pori6k-m157-pA as the template DNA. After homologous recombination of this fragment with pΔm157-FRT in *E. coli*, revertant MCMV BAC p*m157*rev was generated. The correct insertion of the *m157* gene, including its promoter, at this ectopic position was confirmed by restriction pattern analysis and sequencing with primers M157-1 (5′-TGTTGACCGCCATCTGTTCTTGA), M157-2rev (5′-GGTAAGATTAATATTCAAGGATCA), and M157-3 (5′-GGATTGAAAATTGTTACAGCACG) (data not shown).

Reconstitution of virus mutants from recombinant BACs. By transfection of 2 μg of the corresponding MCMV BAC DNA into MEFs, mutants Δ*m157*-MCMV and Δ*m157*-FRT-MCMV and revertant virus *m157*Rev-MCMV were reconstituted as previously described (51).

Northern blot analysis. NIH 3T3 fibroblasts were infected at a multiplicity of infection (MOI) of 3 with Δ*m157*, w.t. MCMV, or *m157*Rev, and total RNA was isolated 6 and 24 h postinfection with the TriZol reagent (Invitrogen) in accordance with the manufacturer's instructions. An [α-^{32}P]dCTP-labeled DNA probe specific for 528 bp of the *m157* gene (nt positions 216350 to 216878, according to reference 33) was generated with the nick translation kit from Amersham (Amersham Biosciences Europe) in accordance with the manufacturer's instructions. Three micrograms of total RNA from each sample of virus-infected cells was separated on a denaturing formaldehyde gel, blotted to a nylon membrane, and hybridized with the [α-^{32}P]dCTP-labeled DNA probe for detection of *m157*-specific mRNA transcripts.

Animals. BXD-8/Ty (*H-2*^{b}), 129/SvJ (*H-2*^{b}), and C57BL/6 RAG1$^{-/-}$ (*H-2*^{b}) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All of the mice used in this study, including congenic inbred BALB.B6-*Cmv1*^{r} (*H-2*^{d}) and inbred C57BL/6 (*H-2*^{b}) and BALB/c (*H-2*^{d}) mice, were housed and bred under specific-pathogen-free conditions at the Central Animal Facility of the Medical Faculty, University of Rijeka, in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. The Ethical Committee at the University of Rijeka approved all of the animal experiments described here. C57BL/6 RAG1$^{-/-}$ (*H-2*^{b}) mice were maintained under specific-pathogen-free conditions in the animal facility of the Washington University School of Medicine, and the experiments conducted with these mice were in accordance with institutional guidelines for animal care and use. Six- to eight-week-old female mice were used in all of the experiments.

Infection conditions, detection of infectious MCMV in tissues, and statistical evaluation. Mice were injected intravenously with 5×10^5 (Ly49H$^+$ mice) or 2×10^5 (Ly49H$^-$ mice) PFU of tissue culture-grown recombinant virus or w.t. MCMV in a volume of 500 μl of diluents. Organs were collected 3 days after infection, and viral titers were determined with a standard assay of viral plaque formation on MEFs (34). Each experiment shown here is representative of at least three independent experiments. The statistical significance of differences between experimental groups was determined by the Mann-Whitney exact rank test. Viral titers (from groups x and y) were considered significantly different for P (x versus y) $< \alpha = 0.05$ (one sided), where P is the observed probability value and α is a selected significance level.

Depletion of NK cell subsets in vivo. Depletion of NK1.1$^+$ cells was done with monoclonal antibody (MAb) PK136 (20) at a concentration of 1 mg per mouse by intraperitoneal inoculation 24 to 2 h before infection. The efficacy of depletion was assessed by cytofluorometric analysis of spleen cells with phycoerythrin (PE)-conjugated antibodies to mouse NK1.1 (BD Bioscience Pharmingen, San Diego, Calif.). Depletion of Ly49C/I$^+$ NK cell subsets was performed with MAb 5E6 (31) at a concentration of 150 μg per mouse, and depletion of Ly49C/H/I$^+$ NK cell subsets was performed with MAb 1F8 (9) at a concentration of 150 μg per mouse by intraperitoneal inoculation 24 h before infection.

Staining of intracellular IFN-γ. An in vitro assay to detect gamma interferon (IFN-γ) production was performed as previously described (42). Briefly, C57BL/6 RAG1$^{-/-}$ splenocytes were cocultured for 12 h with IC-21 cells that were either uninfected or infected for 24 h at an MOI of 5 with Δ*m157* mutant, *m157*Rev, or w.t. MCMV. Brefeldin A (BD Bioscience Pharmingen) was added for the last 11 h of coincubation. Cells were first surface stained with biotinylated 3D10 (α-Ly49H) (41) and then stained with PE-streptavidin and allophycocyanin-PK136. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Bioscience Pharmingen) in accordance with the manufacturer's instructions. Intracellular IFN-γ was stained with fluorescein isothiocyanate-XMG1.2 (BD Bioscience Pharmingen) in the permeabilization buffer. Cells were analyzed with a FACScalibur cytometer (BD Biosciences, San Jose, Calif.) gating on the NK1.1$^+$ CD3$^-$ populations.

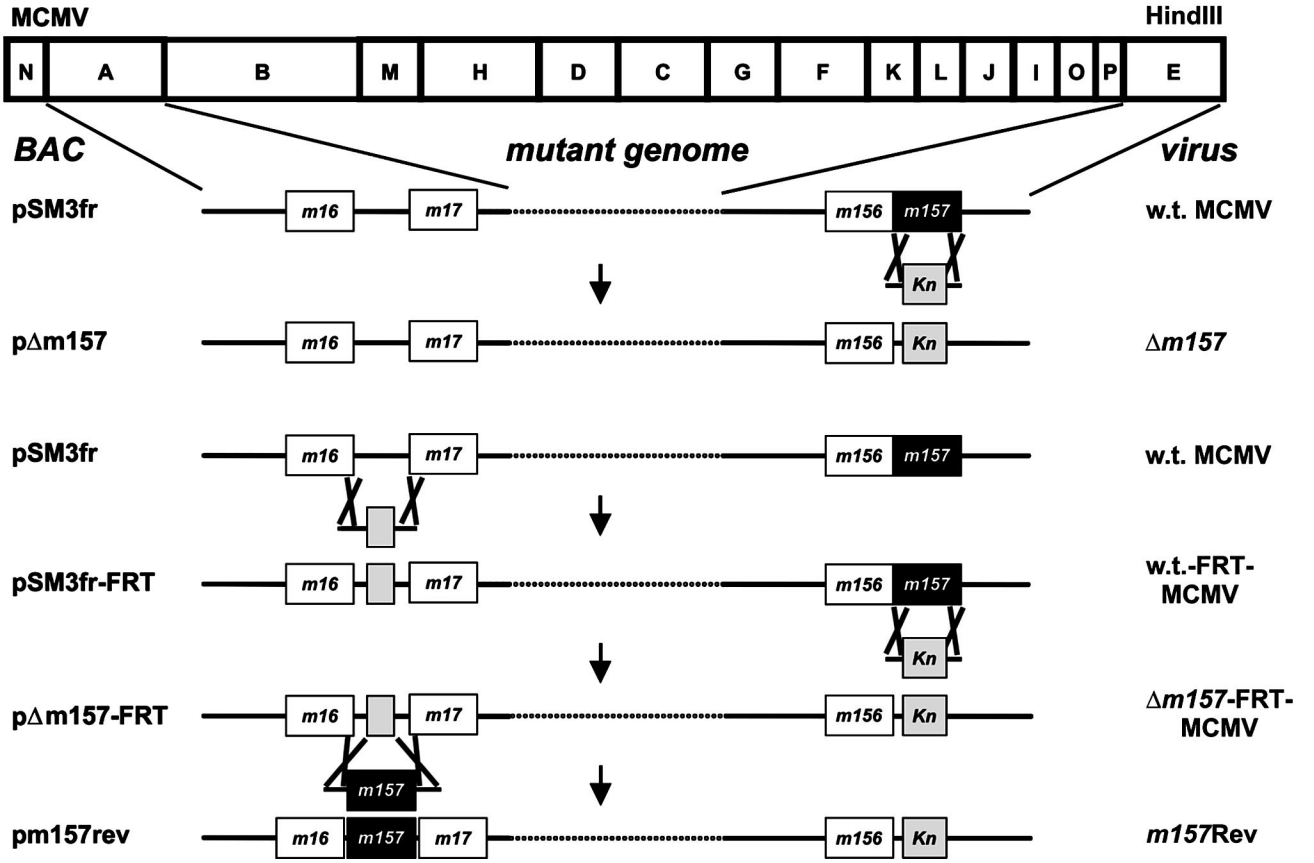

FIG. 1. Genome structure of recombinant MCMVs. The HindIII cleavage map of the MCMV (Smith strain) genome is shown at the top. Deletion and insertion of the *m157* gene, respectively, were achieved by homologous recombination between the parental MCMV BACs and a linear DNA fragment containing the desired sequence, a kanamycin resistance gene (*Kn*), and flanking homologies to the target site in the viral genome. The deletion genome pΔm157 was generated by homologous recombination of the linear DNA fragment and w.t. MCMV BAC pSM3fr. An independent Δ*m157* genome was constructed as follows. First, a 48-bp FRT site (grey box) was inserted into the intergenic region between genes *m16* and *m17*, generating pSM3fr-FRT, to prove that insertion of sequences at this positions does not interfere with virus replication. In a second mutagenesis step, the *m157* gene was deleted from pSM3fr-FRT, generating the second independent *m157* deletion genome, pΔm157-FRT. Finally, the *m157* gene, including its native promoter, was reinserted into pΔm157-FRT at the ectopic positions between genes *m16* and *m17*, thereby removing the FRT site. The names of the MCMV BACs are indicated on the left. Recombinant viruses were reconstituted by transfection of the recombinant MCMV BACs into permissive MEFs. The names of the corresponding reconstituted viruses are given on the right.

RESULTS

Generation of MCMV mutants. To investigate the significance of the *m157* gene product for virus control in vivo, two independent MCMV mutants were constructed. First, mutants with a targeted deletion of the *m157* gene (Δ*m157* and Δ*m157*-FRT) were prepared (Fig. 1). In vitro and in vivo testing in the lungs, spleens, and livers of BALB/c and C57BL/6 mice at day 3 postinfection revealed no difference between Δ*m157* and Δ*m157*-FRT (data not shown). In the experiments described here, only Δ*m157* was used. Thereafter, a revertant virus (*m157*Rev) was constructed. Rather than reconstructing the w.t. situation, in the revertant virus the *m157* gene was reintroduced at an ectopic position between ORFs *m16* and *m17* of the MCMV genome to selectively study the effect of the *m157* ORF. All mutants were derived from parental MCMV BAC pSM3fr, which has w.t. MCMV properties in vitro and in vivo (50). The correct mutagenesis of the resulting Δ*m157* and Δ*m157*-FRT strains, as well of the *m157*Rev MCMV strain, was confirmed by restriction pattern analysis and sequencing of the *m157* genome region.

No phenotype of Δ*m157* and *m157*Rev in cell culture. Multistep growth curves of recombinant and w.t. MCMVs served to assess whether deletion of the *m157* gene and its ectopic reinsertion affect virus growth in cell culture. After infection of primary BALB/c MEFs at 0.01 PFU per cell, the replication of Δ*m157* and the *m157*Rev was indistinguishable from that of w.t. MCMV (Fig. 2). These results indicated that the *m157* gene product is dispensable for virus growth in fibroblasts and that ectopic reinsertion of this gene between MCMV ORFs *m16* and *m17* does not compromise viral growth in vitro.

Δ*m157* gains virulence in Ly49H$^+$ mice through loss of NK cell-mediated control. Replication of MCMV in vivo during the early period after infection inversely correlates with the ability of mouse strains to mount an effective NK cell response, which is controlled by the *Ly49h* locus (15). The MCMV protein encoded by the *m157* gene is the only ligand for the Ly49H

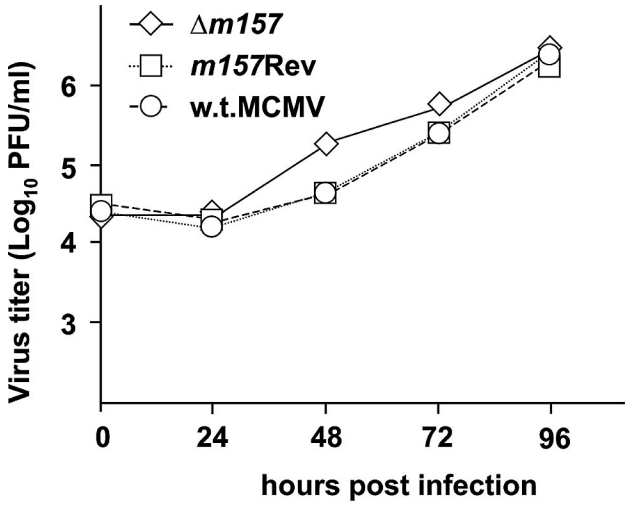

FIG. 2. In vitro growth of recombinant viruses. BALB/c MEFs were infected with Δ*m157*, *m157*Rev, or w.t. MCMV at 0.01 PFU per cell. Supernatants were harvested at the indicated time points after infection, and virus titers were determined.

receptor that has been identified so far (2, 42). To test whether the m157 protein is the only MCMV protein interacting with Ly49H, we used the Δ*m157* virus. C57BL/6 (Ly49H$^+$) mice (Fig. 3, top) and congenic BALB.B6-*Cmv1^r* mice (Fig. 3, bottom) were injected with Δ*m157* or w.t. MCMV, and virus titers in their organs were determined 3 days later. The spleens and lungs of mice infected with Δ*m157* showed significantly higher virus titers than did those of mice infected with w.t. MCMV. Consistent with published data (6, 52, 53), depletion of NK cells by anti-NK1.1 MAbs increased the virus titers in mice infected with w.t. MCMV, whereas it had no effect on the virus titers in the spleens and lungs of mice infected with Δ*m157*. We concluded that Δ*m157* virus is resistant to NK cell control in vivo and that there is no other viral ligand for Ly49H encoded by the MCMV genome.

Remarkably, NK cells were efficient in limiting virus replication in liver irrespective of the presence or absence of the *m157* gene. This finding is in line with previously published data on the protective effect of the *Cmv1* (*Ly49h*) locus in the spleen but not in the liver (5, 37, 44). Interestingly, contrary to the MCMV titer in the liver and similar to that in the spleen, the MCMV titer in the lungs was also controlled by the Ly49H NK cell activation receptor.

Reintroduction of the *m157* gene reverses the susceptibility of MCMV to NK cells. To confirm that resistance of mutant virus to NK cells in vivo is solely due to lack of the m157 protein and no other unwanted effect in Δ*m157*, we compared the abilities of Δ*m157*, *m157*Rev, and w.t. MCMVs to induce activation of Ly49H$^+$ NK cells. Splenocytes derived from C57BL/6 RAG1$^{-/-}$ mice were incubated for 12 h with

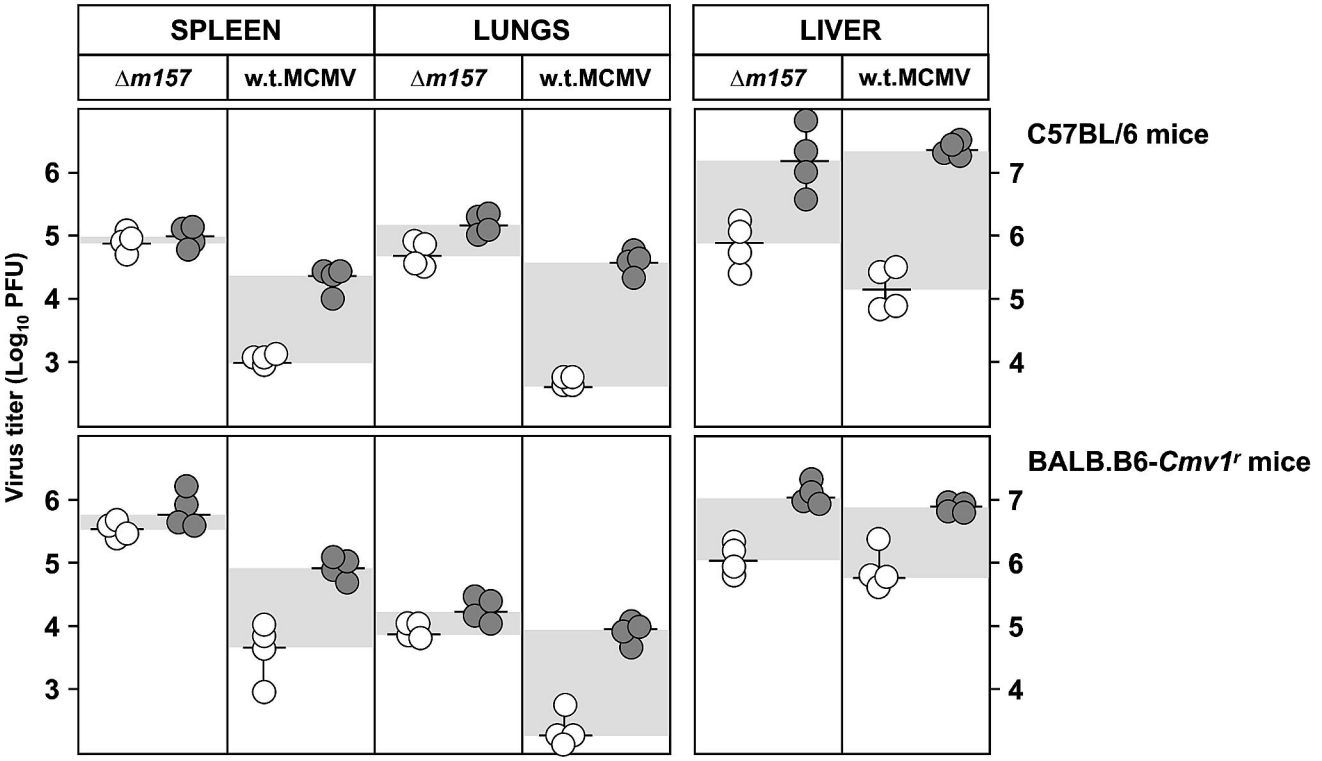

FIG. 3. Deletion of the *m157* gene abolishes MCMV susceptibility to NK cell-mediated control in Ly49H$^+$ mice. Undepleted (open circles) or NK1.1-depleted (shaded circles) C57BL/6 and congenic BALB.B6-*Cmv1^r* mice were injected intravenously with 5×10^5 PFU of Δ*m157* or w.t. MCMV. Three days after infection, the virus titers in the spleen, lungs, and liver were determined by standard plaque assay. Titers in individual mice (circles) and median values (horizontal bars) are shown. Virus titers were calculated per organ in the spleen and lungs and per gram of liver. The differences in viral titers between undepleted and NK cell-depleted groups of mice are shown by shaded areas. There were significant differences ($P < 0.025$) in virus titers between the groups of undepleted mice infected with Δ*m157* and w.t. MCMV in the spleen and lungs.

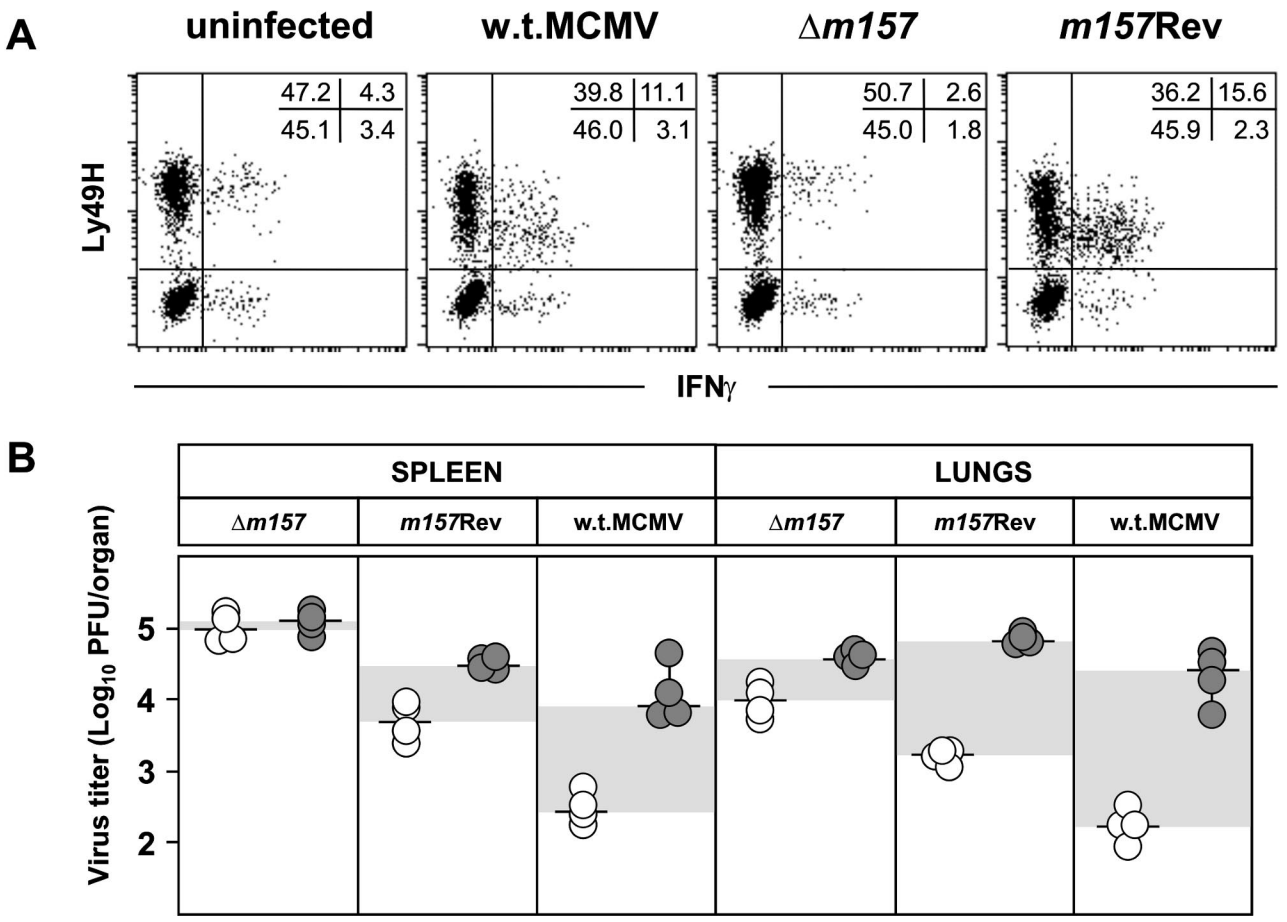

FIG. 4. NK cell activation and reversal of susceptibility to NK cell control by reintroduction of the *m157* gene. (A) C57BL/6 RAG1$^{-/-}$ splenocytes were incubated for 12 h (1 h without brefeldin A and 11 h with brefeldin A) with IC-21 cells that were either uninfected or infected (MOI = 5, 24 h) with Δ*m157*, *m157*Rev, or w.t. MCMV. Cells were stained with biotinylated anti-Ly49H, followed by PE-streptavidin and allophycocyanin–anti-NK1.1. The cells were fixed, permeabilized, and stained with fluorescein isothiocyanate–anti-IFN-γ antibody. Data represent NK1.1$^+$ cells. The values are the percentages of cells within the quadrants. (B) Undepleted (open circles) or NK1.1-depleted (shaded circles) C57BL/6 mice were injected intravenously with 5×10^5 PFU of Δ*m157*, *m157*Rev, or w.t. MCMV, and virus titers in the spleen and lungs were determined 3 days after infection. Titers in individual mice (circles) and median values (horizontal bars) are shown. The differences in viral titers between undepleted and NK cell-depleted groups of mice are shown by shaded areas. There were significant differences ($P < 0.025$) in virus titers between the groups of undepleted mice infected with Δ*m157* and *m157*Rev and between the groups of undepleted mice infected with Δ*m157* and w.t. MCMV.

MCMV-infected IC-21 cells, and the frequency of Ly49H$^+$ IFN-γ-secreting cells was measured (Fig. 4A). The incubation of splenocytes with w.t. MCMV- or *m157*Rev-infected IC-21 cells resulted in a comparable expansion of Ly49H$^+$ IFN-γ-secreting cells (11.1 and 15.6%, respectively). IC-21 cells infected with Δ*m157* failed to activate Ly49H$^+$ cells, and the percentage of activated Ly49H$^+$ NK cells was the same as in cultures incubated with uninfected IC-21 cells. These findings confirm previously published data, obtained by anti-Ly49H blockade and reporter cell assays, that showed that the isolated m157 protein is crucial for the activation of Ly49H$^+$ NK cells (2, 42). The results presented here extend the above studies to the situation found during virus infection. As shown in Fig. 4B, reintroduction of the *m157* gene into the Δ*m157* genome also reversed the sensitivity of the virus to NK cell control in vivo. This is illustrated by the fact that the titers of the *m157*Rev virus in C57BL/6 mice were significantly lower than those of Δ*m157*. Furthermore, the depletion of NK cells increased the titer of *m157*Rev whereas, consistent with the data presented in Fig. 3, Δ*m157* remained resistant to NK cell control. The reconstitution of viral sensitivity to NK cells essentially confirmed the role of the *m157* gene. Still, the in vivo function of the gene in *m157*Rev was not fully restored. This is probably due to the fact that the bona fide promoter of *m157* was transferred together with the ORF. Therefore, other sequences modulating gene expression may be lacking, and indeed, Northern blot analysis revealed that the *m157* transcripts appear earlier in *m157*Rev-infected cells and peak later in w.t.-infected cells (see supplemental material). This difference in the activity of ectopic *m157* might explain the variance in virus sensitivity to NK cells in vivo.

Δ*m157* lacks a phenotype in C57BL/6 mice depleted of Ly49H$^+$ NK cells and in mice lacking the Ly49H receptor. To address the role of the Ly49H$^+$ NK cell subset in the control of MCMV infection and the role of the m157 protein, C57BL/6 mice were injected with MAb 5E6 (αLy49C/I), 1F8 (αLy49C/

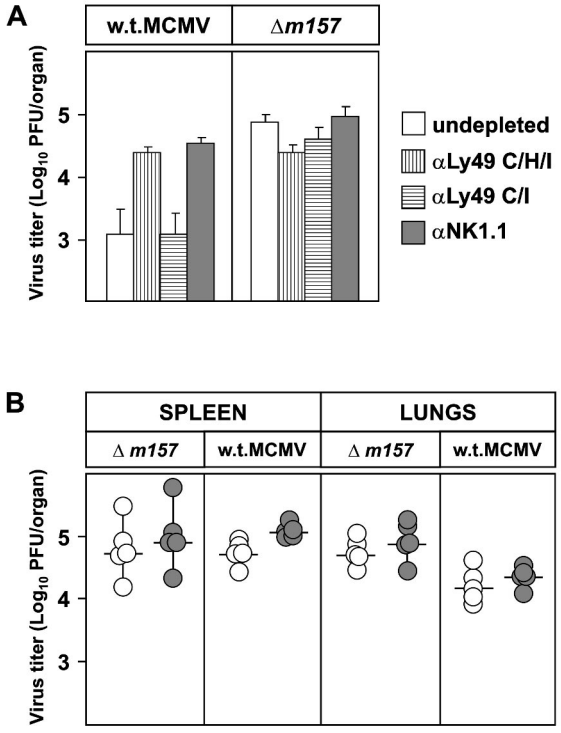

FIG. 5. Ly49H$^+$ NK cells play no role in control of Δ*m157* virus. (A) C57BL/6 mice were depleted with MAb 5E6 (αLy49 C/I), 1F8 (αLy49 C/H/I), or PK136 (αNK1.1) and injected intravenously with 5×10^5 PFU of Δ*m157* and w.t. MCMV. Virus titers in the lungs were determined 3 days after infection. Data represent the mean value ± the standard deviation of four or five mice. Depletion with MAb 1F8 or PK136 resulted in a significant increase ($P < 0.025$) in the w.t. MCMV titer. (B) Recombinant BXD-8 mice were injected with 2×10^5 PFU of Δ*m157* and w.t. MCMV, and virus titers in the spleen and lungs were determined 3 days after infection. Titers in individual mice (circles) and median values (horizontal bars) are shown.

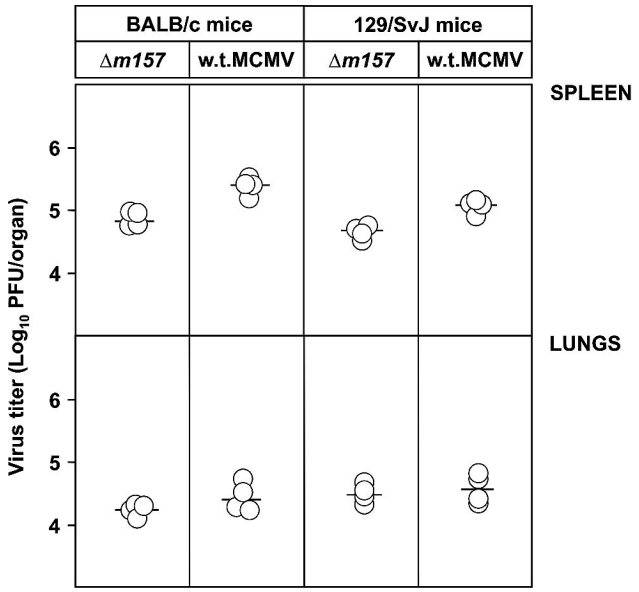

FIG. 6. Control of Δ*m157* virus in BALB/c and 129/SvJ mice. BALB/c and 129/SvJ mice were injected with 2×10^5 PFU of Δ*m157* and w.t. MCMV, and virus titers in the spleen and lungs were determined 3 days later. Titers in individual mice (circles) and median values (horizontal bars) are shown. There were significant differences ($P < 0.025$) in virus titers in spleen between groups of mice infected with Δ*m157* and w.t. MCMV.

H/I), or PK136 (αNK1.1) prior to infection with either w.t. or Δ*m157* MCMV. Depletion of 1F8$^+$ cells, but not 5E6$^+$ cells, in mice infected with w.t. MCMV led to an increase in virus titers comparable to the effect of NK1.1$^+$ cell depletion. This indicates that other NK cell subsets contribute very little, if at all, to virus control (Fig. 5A). In accordance with the data presented in Fig. 3, in mice infected with Δ*m157*, depletion of the Ly49H$^+$ subset did not increase the virus titers. Therefore, the *m157* effect on NK cell activation was mediated solely by the Ly49H$^+$ NK cell subset, thus confirming previous studies (5, 9).

The BXD-8 mouse strain is a recombinant inbred strain derived from C57BL/6 (Ly49H$^+$) and DBA/2 (Ly49H$^-$) progenitor strains (45). Although BXD-8 mice possess the entire natural killer gene complex from C57BL/6 mice, they are susceptible to MCMV infection because of a lesion in the *Ly49h* gene (5, 23). For that reason the m157 protein should play no role in NK cell-mediated virus control in these mice. Indeed, 3 days after infection, no differences in virus titers between the group of mice infected with Δ*m157* and that infected with w.t. MCMV were observed (Fig. 5B). Furthermore, NK depletion had no influence on virus titers, confirming that in the absence of Ly49H the m157 protein plays no role in virus control.

Weak phenotype of Δ*m157* in BALB/c and 129/SvJ mice. We tested the role of the m157 protein in mice lacking the Ly49H but expressing the Ly49I NK cell receptor. 129/J mice do not contain Ly49H or other NK cell activation receptors that recognize m157 (2). However, 129/J mice express the inhibitory NK cell receptor Ly49I (26). The m157 protein binds to 129/J NK cells but not to NK cells of BALB/c mice in vitro (2). If the in vitro conditions reflect the situation in vivo, Δ*m157* should be more attenuated in 129/J mice than in BALB/c mice. To test this, BALB/c and 129/SvJ mice were injected with Δ*m157* or w.t. MCMV and virus titers were tested. Remarkably, there was no difference in the reactivities of these two mouse strains. However, there was a small but statistically significant difference ($P < 0.025$) between Δ*m157* and w.t. MCMV control in the spleen, indicating a certain degree of attenuation (Fig. 6). Nevertheless, we concluded that the presence or absence of the *m157* gene has no strong phenotype in mice expressing the Ly49I NK receptor.

NK response to Δ*m157* in C57BL/6 mice is prevented by viral evasion genes. MCMV has several NK silencing genes, of which only *m152* and *m144* have been characterized (13, 22, 25). To show the impact of viral silencing genes in a Ly49H$^+$ strain, we compared Δ*m157* with w.t. MCMV and a virus in which in addition to *m157*, 14 other genes, including *m152*, were deleted (Fig. 7). W.t. MCMV is strictly controlled by NK cells, and the virus grows only after NK cell depletion. Δ*m157* lacks the NK activation via m157-Ly49H, and therefore NK depletion has no phenotype. The ΔMS94.5 virus lacks *m157* and, in addition, 14 other genes, including *m152*, that prevent NK cell activation by down-modulating NKG2D ligands (22, 25). We now have evidence that at least one more MCMV

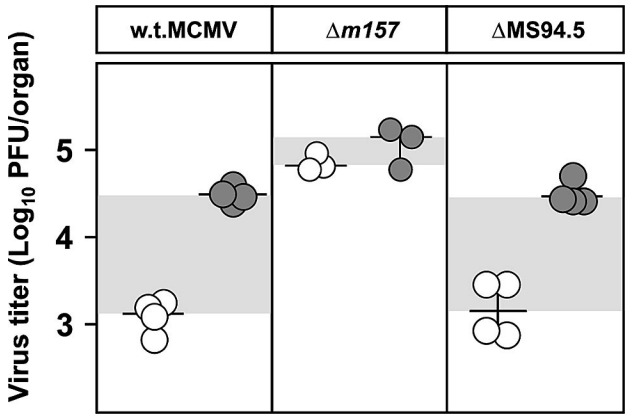

FIG. 7. NK cell response against Δ*m157* in C57BL/6 mice is prevented by viral evasion genes. Undepleted (open circles) or NK1.1-depleted (shaded circles) C57BL/6 mice were injected intravenously with 5×10^5 PFU of w.t., Δ*m157*, or ΔMS94.5 MCMV, and virus titers in the lungs were determined 3 days after infection. Titers in individual mice (circles) and median values (horizontal bars) are shown. The differences in viral titers between undepleted and NK cell-depleted groups of mice are shown by shaded areas. There were significant differences ($P < 0.025$) in virus titers between the groups of undepleted mice infected with Δ*m157* and ΔMS94.5 and between the groups of undepleted mice infected with Δ*m157* and w.t. MCMV.

gene, in addition to *m152*, can down-modulate ligands for the NKG2D receptor (S.J., unpublished data). This inhibitory effect is present in the Δ*m157* virus, and therefore the Δ*m157* virus grows to a higher titer than the ΔMS94.5 virus, which has lost this NK silencing gene(s). Deletion of NK cells abolishes this type of control, resulting in identical titers of the ΔMS94.5 and Δ*m157* viruses. This situation is comparable to the situation in *Cmv1*^{s} mice after infection in the absence or presence of the *m152* gene (22). Therefore, in the presence of a number of additional viral silencing genes the effect of the m157-Ly49I interaction is not expected to have a strong impact.

DISCUSSION

Viruses affect NK cell control by providing viral ligands that bind NK cell receptors. Depending on whether these receptors are activating or inhibitory, the resulting NK function is modulated (29). The second type of NK cell modulation affects cellular ligands for NK cell receptors that are regulated by infection or stress (3, 22, 25). Two MCMV genes are thought to encode ligands for NK cell receptors, *m144* and *m157*, but only the cognate NK cell receptor for the m157 protein, Ly49H, has been identified (2, 42). The interaction between Ly49H and the m157 protein is a unique situation and contributes to MCMV resistance in C57BL/6 mice, which express the Ly49H NK cell activation receptor. In previous studies the ligand-receptor interaction was demonstrated by expressing the isolated viral ligand (2, 42). By deleting *m157* from the MCMV genome, we show that m157 indeed is the only MCMV ligand for Ly49H. Accordingly, in congenic mice lacking the Ly49H receptor (BXD-8), as well as in mice depleted of Ly49H$^+$ NK cells, deletion of this gene from the virus has no phenotype. Ly49H$^+$ mice, after infection with Δ*m157*, lose their MCMV-encoded resistance phenotype.

m157 also serves as a ligand for the inhibitory NK receptor Ly49I in the MCMV-susceptible 129/J strain, and it also binds to NK cells of other MCMV-sensitive strains (1). Therefore, m157 is seen as an inhibitory NK cell ligand and Ly49H$^+$ mice are considered an exception to the rule. However, no experiments in which NK cell function is blocked through m157-Ly49I have been published. Loss of NK cell activation can be studied in the context of virus infection when the virus either expresses or lacks the gene of interest. However, when we studied the effect of *m157* deletion in the 129/SvJ strain no vigorous phenotype became apparent. A certain degree of virus attenuation was noticed in both BALB/c and 129/SvJ mice. Considering that the m157 protein binds to the Ly49I allelic form of 129/J but not of BALB/c mice (2), it is not clear whether this effect is related to the m157-Ly49I interaction. A strong signal was not to be expected, since in comparison to NK cells from the C57BL/6 strain only a minority of NK cells from 129/J mice binds m157 but does not lyse m157$^+$ targets in vitro (2).

The absence of NK activation in Ly49H$^+$ mice infected with Δ*m157* provided the opportunity to demonstrate the function of other viral genes inhibiting NK cell function. These genes act by down-modulating NKG2D ligands similarly to the function of *m152* (22, 25). There is another not yet identified gene(s) in the left end of the genome (28), and we have evidence of at least one additional gene elsewhere (U.H.K. and S.J., unpublished data). Most NK cells express NKG2D receptors (18). Therefore, the loss of one silencing signal from a chorus of several is difficult to detect. This situation is similar to that of the genes modulating MHC class I expression, in which identification of the three genes involved (*m04*, *m06*, and *m152*) had to precede the construction of virus mutants that lack or express class I modulating functions in all possible combinations (49). In addition, deletion of the viral gene that down-modulates a ligand for an activating NK cell receptor has an immediate effect on NK control, since virus infection upregulates stress-induced ligands (7, 11). On the other hand, deletion of a viral ligand for an inhibitory NK cell receptor should not have a strong phenotype, especially if the stress-induced activating ligands are down-modulated at the same time.

Our study also confirms and extends the known complexity of NK cell control in different organs (30, 44). The *Cmv1* locus has been described as a host resistance locus that regulates NK cell responses during acute MCMV infection in the spleen (37). Accordingly, Δ*m157* lacks this type of control. In addition, we show here for the first time that, akin to virus control in the spleen, Ly49H$^+$ NK cells also mediate MCMV control in the lungs. Additional evidence for this is provided by C57BL/6 mice depleted of Ly49H$^+$ NK cells, in which the titer of w.t. MCMV in the lungs reached a level comparable to that after depletion of NK1.1$^+$ NK cells. In contrast, MCMV control in the liver appears to be independent of m157-Ly49H interaction. This finding is in accordance with the minimal effects of anti-Ly49H MAbs on the virus titer in the liver (5), pointing at a different type of NK control of MCMV in that organ (30, 44). After MCMV infection, NK cells pass through two different stages of activation. The first, nonspecific phase, during the first 2 days after MCMV infection, is characterized by IFN-γ production and NK cell proliferation, irrespective of

Ly49H expression, while in the second, specific phase, there is a selective proliferation of Ly49H$^+$ NK cells (12). This early activation of NK cells may be sufficient to control virus infection in the liver but not in the spleen and lungs. The mode by which NK cells mediate their antiviral effector function also differs between the liver and spleen. While in the spleen NK cells act via a cytolytic mechanism, in the liver MCMV is controlled by cytokines including IFN-γ produced by NK and NKT cells (30, 44). In perforin-deficient C57BL/6 mice, no differences between Δ*m157* and w.t. MCMV titers in the spleen were observed, which suggests that Ly49H-positive cells mediate their effect almost exclusively via a perforin-dependent pathway (I.B., unpublished data).

The MCMV genome harbors genes, in addition to *m152* (22), whose products down-modulate NKG2D ligands, also in mice expressing *Cmv1^r* (*Ly49h*) (S.J., unpublished). However, this inhibition of NK cell activation is overridden by NK activation through m157, which makes these mice an exception to the rule. It has been proposed that natural isolates from wild mice, depending on the presence or absence of the Ly49H receptor, are expected to be variable in the *m157* gene (1, 35). Indeed, this has been recently demonstrated by Voigt et al., who reported that most of the MCMV strains they isolated from wild mice possessed a specific mutation of the *m157* gene (48). Furthermore, this study provides evidence that NK cells can exert sufficient immunological pressure on MCMV that it undergoes rapid and specific mutation in the *m157* gene region. Accordingly, we have been studying immunodeficient C57BL/6 mice that rely on NK functions to survive MCMV infection. Under these selective conditions, virus mutants arise that indeed do not respond to the Ly49H receptor (R. A. French, T. J. Pingel, M. Wagner, I. Bubic, L. Yang, S. Kim, U. H. Koszinowski, S. Jonjic, and W. M. Yokoyama, submitted for publication).

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