The Immunoevasive Function Encoded by the Mouse Cytomegalovirus Gene *m152* Protects the Virus Against T Cell Control In Vivo

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Summary

Cytomegaloviruses encode numerous functions that inhibit antigen presentation in the major histocompatibility complex (MHC) class I pathway in vitro. One example is the mouse cytomegalovirus (MCMV) glycoprotein gp40, encoded by the *m152* gene, which selectively retains murine but not human MHC class I complexes in the endoplasmic reticulum–Golgi intermediate compartment/cis-Golgi compartment (Ziegler, H., R. Thäle, P. Lucin, W. Muranyi, T. Flohr, H. Hengel, H. Farrell, W. Rawlinson, and U.H. Koszinowski. 1997. *Immunity*. 6:57–66). To investigate the in vivo significance of this gene function during MCMV infection of the natural host, we constructed recombinants of MCMV in which the *m152* gene was deleted, as were the corresponding virus revertants. We report on the following findings: Deletion of the *m152* gene has no effect on virus replication in cell culture, whereas after infection of mice, the *m152*-deficient virus replicates to significantly lower virus titers. This attenuating effect is lifted by reinsertion of the gene into the mutant. Mutants and revertants grow to the same titer in animals deprived of the function targeted by the viral gene function, namely in mice deficient in β2-microglobulin, mice deficient in the CD8 molecule, and mice depleted of T cells. Upon adoptive transfer of naive lymphocytes into infected mice, the absence of the *m152* gene function sensitizes the virus to primary lymphocyte control. These results prove that MHC-reactive functions protect CMVs against attack by $CD8^+$ T lymphocytes in vivo.

Key words: cytomegalovirus • immune evasion • virus mutants • MHC class I • CD8 T lymphocytes

The T cell–mediated immune response is decisive for control and clearance of most viral infections. $CD8^+$ T cells limit virus infections by secretion of cytokines with antiviral activity and by direct cytolysis of infected cells. To achieve effective surveillance and elimination of virus-infected cells, $CD8^+$ T cells need to recognize viral peptides in the context of MHC class I molecules at the surfaces of infected cells for maturation to CTLs or for reactivation from memory (1). Presentation of viral peptides via this pathway requires degradation of viral proteins by the proteasome and the translocation of the peptides into the endoplasmic reticulum (ER)[1] by transporters associated with antigen presentation for loading into the binding groove of nascent MHC class I molecules and subsequent egress of MHC complexes to the cell surface (for review see references 2 and 3).

Certain viruses make use of specific and unique genes to thwart this pathway of virus peptide presentation (for review see reference 4). For example, the adenovirus E3-19K protein binds and arrests MHC class I molecules in the ER, and the herpes simplex virus type I–infected cell protein (ICP)47 inhibits transport of peptides into the ER by the transporters associated with antigen presentation by competing for the peptide binding site (5–8). Remarkably, CMVs use multiple genes to interfere with the MHC class I pathway of antigen presentation. The human (H)CMV encodes at least four viral polypeptides, each of which can independently and by different molecular mechanisms interfere with MHC class I antigen presentation to inhibit efficient recognition of infected cells by CTLs (9–15). In mouse (M)CMV, at least three genes affect MHC class I molecules (16, 17, 18). The function of the viral proteins is usually of selective specificity for the target proteins of the natural host (16).

Detailed studies of the molecular mechanisms by which individual viral polypeptides act at various steps of the antigen presentation pathway in vitro are contrasted by the pau-

A. Krmpotic and M. Messerle contributed equally to this work.

[1]*Abbreviations used in this paper:* β2m, β2 microglobulin; E, early; ER, endoplasmic reticulum; IE, immediate-early; MEFs, mouse embryonic fibroblasts.

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city of data concerning the relevance of their function in vivo. Only the adenovirus E3-19K and herpesvirus ICP47 proteins have been investigated in mice so far (19, 20). However, mice do not represent the natural host of these viruses, and, given the species specificity of the functions, such studies might not completely reflect their physiological role for virus control.

Therefore, the question of the biological significance of the individual viral evasion mechanisms should be addressed in the natural host of the virus. MCMV offers the unique opportunity to study the biological impact of herpesviral immune evasion genes in vivo. Comparable to HCMV, MCMV already tightly controls the presentation of viral antigens at early (E) phases of infection (21). The effect is achieved by blocking transport of MHC class I molecules to the cell surface (22). We have identified the *m152* gene responsible for inhibition of MHC class I antigen presentation in the E phase of virus gene expression. The MCMV E glycoprotein gp40 encoded by the *m152* gene blocks the export of MHC class I complexes from the ER/cis-Golgi compartment and thereby prevents the presentation of viral peptides to CTLs (16). Similar to HCMV, additional MCMV functions exist that also control antigen presentation in the MHC class I pathway (23). Two additional proteins we have identified are the products of the *m04* and *m06* genes, which form complexes with MHC class I molecules (17, 18).

Here, we constructed mutants of MCMV that lacked the *m152* gene as well as the corresponding virus revertants. We investigated the susceptibility of these recombinant viruses to host immune control. We demonstrate that the deletion of the *m152* gene results in high susceptibility of the virus to CD8 T cell control. We conclude that even the deletion of a single viral gene from a group of genes that interfere with the MHC class I presentation pathway affects the fitness of CMV in vivo.

Materials and Methods

Cells and Viruses. Mouse NIH 3T3 cells (American Type Culture Collection [ATCC] CRL1658) were grown in DME supplemented with 10% newborn calf serum. Primary mouse embryonic fibroblasts (MEFs) prepared from BALB/cJ mice and B12 cells (24) were grown in MEM with 10% FCS. The Smith strain of MCMV (VR-194; ATCC) and the recombinant viruses were propagated on third-passage MEFs and purified by sucrose gradient centrifugation. Tissue culture–grown virus preparations were used throughout.

Construction of Recombination Plasmids and Recombinant Viruses. Plasmid constructions were performed by standard methods (25). Plasmid p152KO used for generating *m152*$^-$ recombinant viruses was constructed by ligation of a 5-kb NotI–BamHI fragment comprising a *loxP*-flanked *lacZ* cassette (26) into the XhoI/NheI-digested plasmid pEcoOΔMB (all sites were blunt-ended by treatment with Klenow DNA polymerase). Plasmid pEcoOΔMB contains a 5.0-kb EcoRI–MluI fragment of the MCMV genome (MCMV nucleotides 209,756–214,714) encompassing the *m152* gene (27). To generate recombination plasmid pm152gpt, the *Escherichia coli gpt* gene was flanked with *loxP* sites and inserted into an XhoI site of plasmid pEcoOΔMB at the 3′ end of the *m152* gene.

Recombinant viruses were generated by homologous recombination in NIH 3T3 as described previously (26). *LacZ*$^+$ recombinants were identified by 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) staining and isolated by at least five rounds of limiting dilution passage. Recombinant viruses carrying the *gpt* marker were first enriched by selection in medium that was supplemented with 12.5 μg/ml mycophenolic acid (GIBCO BRL) and 100 μg/ml xanthine (Sigma Chemical Co.) (28) and further purified by limiting dilution. *LacZ*$^-$ and *gpt*$^-$ mutants were generated by a single passage through the recombinase *Cre*$^+$ cell line, N2 (26). *LacZ*$^-$ recombinants were identified as white plaques after X-gal color screening and purified by limiting dilution. *Gpt*$^-$ mutants were selected on STO cells (ATCC CRL-1503) in medium containing 20 μg/ml 6-thioguanine (Sigma Chemical Co.) as described previously (29). To characterize the recombinant virus genomes, viral DNA was isolated from infected cells and analyzed by Southern blot analysis (26).

Characterization of Viral Proteins. B12 cells were infected with wild-type MCMV or *m152* recombinant viruses. Cells were pulse labeled at 37°C for 60 min with 500 μCi/ml [^{35}S]methionine (1,200 Ci/mmol; Amersham) in methionine-free MEM supplemented with 5% dialysed FCS and chased in the presence of 10 mM nonlabeled methionine for 2 h. Labeled cells were washed in ice cold PBS and disrupted in lysis buffer (140 mM NaCl, 20 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 1% NP-40, and 1 mM PMSF). Cytoplasmic extracts were precleared by incubation with normal mouse serum, antiactin mAb (Boehringer Mannheim), and protein A–coupled Sepharose (Pharmacia). Immunoprecipitations were performed with anti-K^d mAb MA-215 ascitic fluid, and immune complexes were retrieved using protein A–coupled Sepharose. Endoglycosidase H (Endo H; Boehringer Mannheim) digestion and SDS-PAGE were performed as described previously (23).

Cytolytic Assays. Target cells were labeled for 90 min with $Na_2{}^{51}CrO_4$, and a 4-h standard release assay was performed with 10^3 target cells and a graded number of effector cells in fivefold dilution steps as described (21, 30). In short, for selective and enhanced expression of immediate-early (IE) genes, MEFs were infected with 0.5 PFU of recombinant viruses or wild-type MCMV per cell by centrifugation (800 *g*, 30 min). Infection was performed in the presence of cycloheximide (50 μg/ml), which was removed 3 h later by washing with medium containing actinomycin D (5 μg/ml). Limited E gene expression after CH treatment was achieved by removal of cycloheximide using inhibitor-free medium and by adding actinomycin D to the final concentration of 5 μg/ml after 1.5 h. To generate pp89-specific polyclonal CTLs, MCMV-primed spleen cells were restimulated with pp89-derived antigenic peptide (21), and recombinant IL-2 (100 U/ml) was added 5 d later. Cultures were restimulated with gamma-irradiated syngeneic MEFs pulsed with antigenic peptide at a concentration of 10^{-8} M. Data represent the mean percentage of specific lysis from three replicate cultures (see Fig. 2 B).

Animals and Infection Conditions. BALB/c (H-2^d haplotype) and C57BL/6 mice (H-2^b haplotype) were bred at the Central Animal Facilities at the Medical Faculty, University of Rijeka. Mice homozygous for the μ chain mutation (C57BL/6 background; reference 31) were provided by Dr. Klaus Rajewsky (Institute for Genetics, Cologne, Germany) and were backcrossed on the BALB/c background for 10 generations. Mice heterozygous (μMT$^{-/+}$) and homozygous (μMT$^{-/-}$) for the μ chain mutation were distinguished by ELISA for the presence or absence of IgM in mouse sera, as described previously (32). Mice homozygous for the β2 microglobulin mutation ($\beta_2m^{-/-}$; supplied by Dr. Rudolf Jaehnisch, Whitehead Institute of Biomedical Research, Cambridge, MA) fail to express ternary MHC class I complexes and are devoid of

$CD8^+$ T lymphocytes (33). Mice homozygous for the deletion of the gene encoding the CD8 molecule ($CD8^{-/-}$) were obtained from the Centre de Developpement des Techniques Avancées pour l'Expérimentation Animale, Institut de Transgenose, Orleans, France. The absence of $CD8^+$ T lymphocytes in $\beta_2m^{-/-}$ and $CD8^{-/-}$ mice was verified by flow cytometry as described previously (34). Neonatal mice, 24 h and 4 d postpartum, were injected intraperitoneally with recombinant viruses or wild-type MCMV. 6–8-wk-old mice were injected either in the posterior footpad or i.p. with 2×10^5 PFU of virus in a volume of 50 and 500 μl of diluent, respectively, as described (35).

Detection of Infectious MCMV in Tissues and Statistical Evaluation. Plaque assays were performed in MEF as described previously (36, 37). Statistical significance of differences between the experimental groups was determined by the Mann-Whitney exact rank sum test. Virus titers (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 alpha is a selected significance level.

In Vivo Depletion of Lymphocyte Subsets. In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocyte subsets was performed by intraperitoneal injection of mAbs (rat anti–mouse) to CD4 (YTS 191.1) and/or CD8 (YTS 169.4) molecules (38). Adult and newborn mice received 1 mg and 250 μg of antilymphocyte antibodies, respectively, at the time of injection and every fifth day throughout the experiment. The efficacy of T lymphocyte depletion was >95%, as assessed by cytofluorometric analysis of spleen cells using FITC- or PE-conjugated antibodies directed against mouse CD4 and CD8 molecules (Becton Dickinson; nos. 1333 and 1447).

Adoptive T Lymphocyte Transfer. Donor T lymphocytes were harvested from spleens of uninfected (nonprimed) or latently infected (MCMV-primed) mice. Recipient mice were injected with 2×10^5 PFU of virus in a rear footpad 12 h after gamma irradiation (6.5 Gy). Immediately after infection, 2×10^5 nylon wool–purified cells were injected intravenously into recipient mice. Mice that did not receive cell transfer were used as negative controls. Mice were killed on day 14 after infection, and tissues were harvested for virus titer determinations.

Results

Generation of Recombinant MCMV. To investigate the significance of the *m152* gene product in the course of infection, a targeted deletion of the *m152* gene and subsequent reintroduction of this gene into the MCMV genome was performed (Fig. 1 A). The recombinant virus ΔMC95.21 was generated by homologous recombination between the wild-type MCMV genome and the recombination plasmid p152KO. In this plasmid, a 1.2-kb XhoI–NheI fragment containing the *m152* gene was replaced by a *lox*P-flanked *E. coli lacZ* gene. The *lacZ* marker was excised by passaging the ΔMC95.21 recombinant through the recombinase Cre^+ cell line N2 (26) to create the $m152^-lacZ^-$ deletion mutant ΔMC95.24. To generate a revertant virus, the *m152* gene, together with the *loxP*-flanked *gpt* gene, was reinserted by homologous recombination into the ΔMC95.21 genome. After positive selection (28) of the $m152^+gpt^+$ virus rMC95.26, the *gpt* marker gene was again removed by passaging the virus through the recombinase Cre^+ cell line N2 to generate the $m152^+gpt^-$ revertant virus (designated rMC96.27).

Southern blot analysis of the recombinant virus genomes confirmed the recombination events at the expected positions (Fig. 1 B). In the mutant virus genomes, the original 23.3-kb HindIII E fragment and the 5.9-kb EcoRI O fragment were

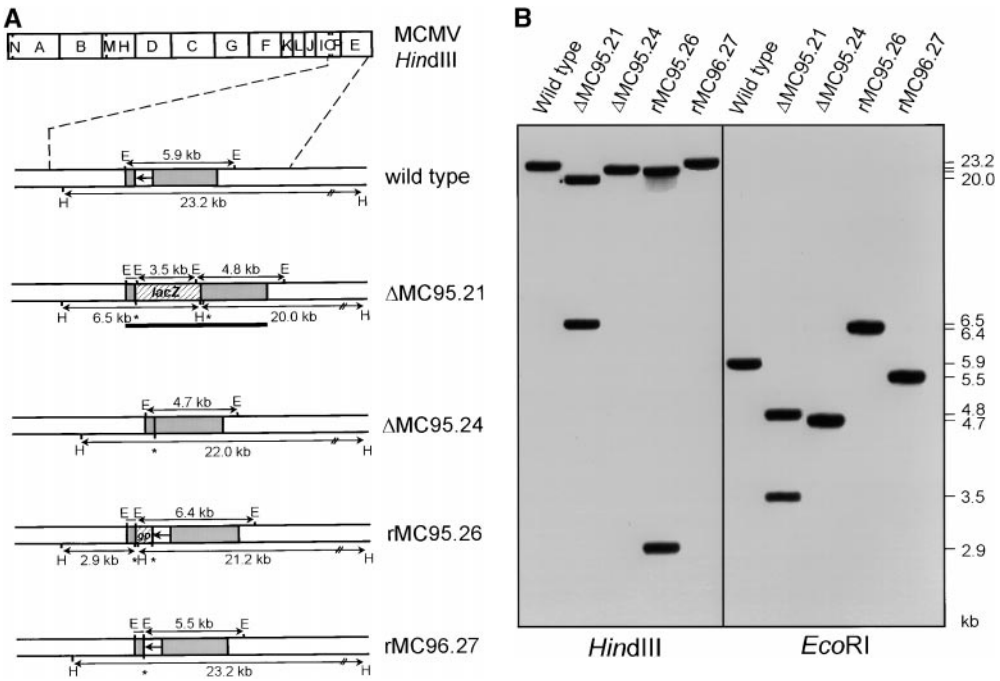

Figure 1. Characterization of *m152* recombinant viruses. (A) Genome structure of recombinant viruses. The HindIII cleavage map of the MCMV strain Smith genome is shown at the top, and the expanded HindIII E region of wild-type and recombinant viruses is shown below, with the HindIII (H) and EcoRI (E) cleavage sites indicated. The open box with the arrow depicts the position and orientation of the *m152* gene, and the shaded boxes represent viral sequences that were used for homologous recombination. The positions of the *loxP* sites are indicated by asterisks (*). The marker genes used for selection, *loxZ* and *gpt*, are indicated. The probe used for Southern blot analysis is represented by a bar. The expected sizes of the HindIII and EcoRI fragments are indicated by arrows. (B) Southern blot analysis of the recombinant virus genomes. DNA was isolated from infected NIH 3T3 cells and digested with restriction enzymes HindIII and EcoRI, respectively. Sizes of the DNA fragments are indicated in kb.

replaced by expected new HindIII and EcoRI fragments. HindIII fragments of 20.0 and 6.5 kb and EcoRI fragments of 4.8 and 3.5 kb are evident in ΔMC95.21, whereas in the *m152*$^{-}$*lacZ*$^{-}$ deletion mutant, ΔMC95.24, a 22-kb HindIII fragment and a 4.7-kb *Eco*RI fragment were found. In the genomes of the rMC95.26 and rMC96.27 revertant viruses, HindIII fragments of 21.2 and 2.9, and 23.2 kb, and EcoRI fragments of 6.4 and 0.4 kb, and 5.5 and 0.5 kb, were observed, respectively. Note that the size of the HindIII E fragment in the rMC96.27 genome is identical to that in the wild-type MCMV genome, whereas a new EcoRI site purposely introduced outside of the m152 open reading frame enabled us to discriminate between the constructed revertant and wild-type MCMV. Comparison of the HindIII, EcoRI, and XbaI digestion patterns of the recombinant genomes with those of the wild-type MCMV genome confirmed that the recombinant viruses were free of detectable deletions or insertions in any other region of the viral genome (data not shown).

Maturation of MHC Class I Molecules in Cells Infected with the m152 Deletion Mutant. The altered glycosylation pattern of newly synthesized molecules can be used to locate the export block of nascent MHC class I molecules in MCMV-infected cells (1, 22, 23). Correctly assembled MHC class I complexes retained by the *m152* gene product in the ERGIC/cis-Golgi compartment of MCMV-infected cells are not processed by medial-Golgi enzymes to complex glycans. Therefore, the majority of MHC class I molecules from cells infected with wild-type MCMV exhibit high mannose N-linked glycans typical for this compartment that are sensitive to Endo H and migrate faster in gels after digestion with Endo H (Fig. 2 A). In contrast, MHC class I complexes in cells infected with the *m152* deletion mutant ΔMC95.21 as well as in uninfected cells acquire Endo H–resistant glycans, indicating the normal egress from the ERGIC/cis-Golgi compartment. As expected, the MHC class I transport was affected again in cells infected with the revertant virus rMC96.27, demonstrated by the reappearance of molecules sensitive to Endo H digestion.

Deletion of the m152 Gene Restores Presentation of Viral Antigens to CTLs. The transport arrest of MHC class I molecules by the MCMV *m152* gene product at early (E) times of virus replication prevents surface expression of these molecules and thus the recognition and lysis of infected cells by specific CTLs (1). A deletion of this gene should restore the recognition of infected cells by CTLs under the experimental conditions. To test this, MEFs were infected with the *m152* deletion mutant ΔMC95.21, the revertant virus rMC96.27, or wild-type MCMV. Infected cells were arrested in the IE or E phase of the MCMV replication cycle and used in a CTL assay with MHC class I–restricted CTLs specific for the MCMV antigen pp89 (21, 30, 36). As expected, recognition and cytolysis were equivalent for cells infected with all three viruses during the IE phase of the viral replication cycle, a time at which the *m152* gene product is not yet expressed (Fig. 2 B). However, recognition was impaired during the E phase when cells infected with wild-type or revertant virus were used as targets. In contrast, efficient recognition of ΔMC95.21-infected cells was seen, confirming that retention of MHC class I molecules and the associated block in antigen presentation is mediated under these conditions exclusively by the *m152* gene.

Replication of m152 Deletion Mutant and Revertant Virus in Cell Culture. Multistep growth curves of recombinant

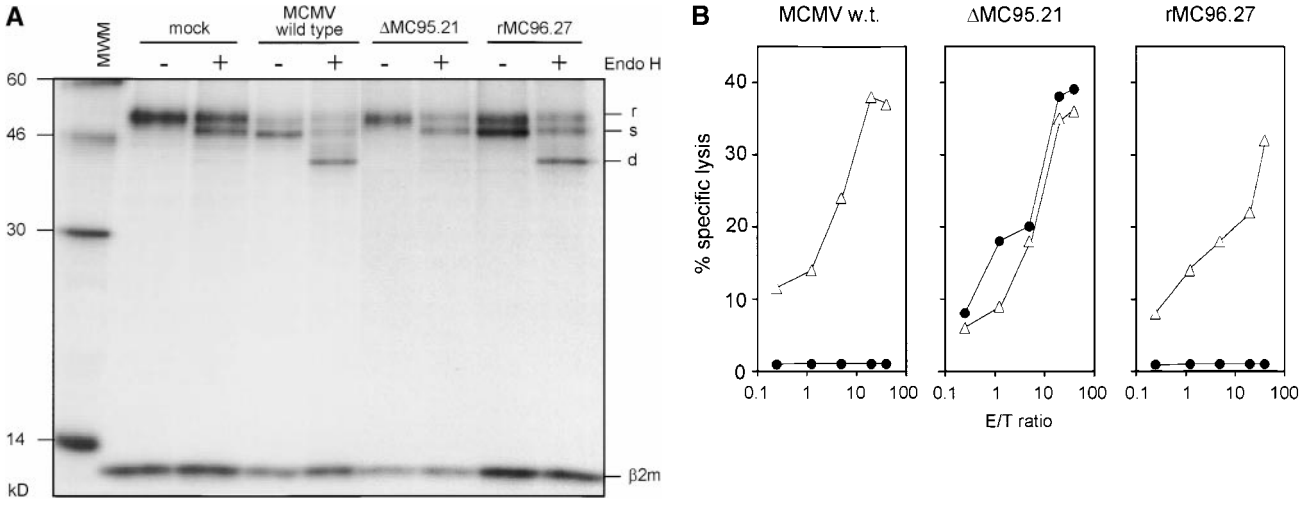

Figure 2. Functional characterization of the *m152* deletion mutants. (A) Normal maturation of newly synthesized MHC class I molecules in cells infected with the *m152* deletion mutant. B12 cells were either mock infected or infected with wild-type MCMV or ΔMC95.21 and rMC96.27 recombinants. 6 h after infection, cells were pulse labeled for 1 h with [^{35}S]methionine, and newly synthesized molecules were chased for 2 h. K^{d} MHC class I complexes were precipitated from cell lysates with anti-K^{d} mAb MA-215. Half of the precipitates were digested with Endo H or mock treated before separation by 12.5% SDS-PAGE. The different glycosylation forms of the MHC class I heavy chains with regard to Endo H sensitivity are denoted as r, Endo H resistant; s, Endo H sensitive; or d, Endo H digested. MWM, molecular weight marker. (B) Restoration of MHC class I antigen presentation in cells infected with the *m152* deletion mutant. BALB/c MEFs were infected with wild-type (w.t.) MCMV, the *m152* deletion mutant ΔMC95.21, and the revertant virus rMC96.27 under conditions that allowed expression of only IE (△) or IE and E viral proteins (●). Antigen presentation was tested with CTLs specific for the MCMV antigen pp89 at the indicated E/T ratios in a 4-h ^{51}Cr-release assay.

and wild-type viruses served to assess whether the deletion of the *m152* gene affects virus growth in cell culture. After infection of NIH 3T3 fibroblasts at a multiplicity of infection of 0.1 PFU per cell, replication of the *m152* deletion mutant and revertant were indistinguishable from that of MCMV wild-type virus (Fig. 3). Identical results were obtained by comparing the replication capacity of the *m152* deletion mutants, the revertant virus, and MCMV wild type on primary MEFs (not shown), indicating that the *m152* gene product is completely dispensable for virus growth in fibroblasts.

Replication of the m152 Deletion Mutant In Vivo. Considering the fact that three different MCMV genes affect nascent MHC molecules and that *m152* merely represents the gene that is expressed first, it was not clear whether or not the deletion of this gene would have any detectable impact on the susceptibility of the virus to immune control in vivo. Whereas adult mice control the infection with tissue culture–derived wild-type MCMV effectively, young mice allow virus replication to high titers (39, 40). To detect even minor differences in virulence due to deletion of the single *m152* gene, we assayed virus replication in neonatal mice. To avoid the potential influence of marker gene products on the biological properties of mutant viruses, the in vivo experiments were performed mainly with the *m152* deletion mutant ΔMC95.24 and the revertant virus rMC96.27, although the other mutants gave comparable results (data not shown). Neonatal mice were injected with 100 PFU of the *m152* deletion mutant, the revertant virus, or wild-type MCMV and monitored for 30 d. After infection with wild-type MCMV or revertant virus, 53 and 75%, respectively, of animals succumbed to infection (Fig. 4 A). In contrast, infection with the *m152* deletion mutant was survived by the majority of mice (25% mortality). With respect to clinical signs, all three groups of mice exhibited during the first week of infection significant runting and a general failure to thrive compared with mock-infected controls. By 14–20 d after infection, however, most animals that survived the infection with the *m152* deletion mutant had recovered. In contrast, clinical signs persisted throughout the course of observation for wild-type MCMV and revertant virus–infected mice. The different disease courses correlated with the body weights of infected mice. On day 26 after infection, the av-

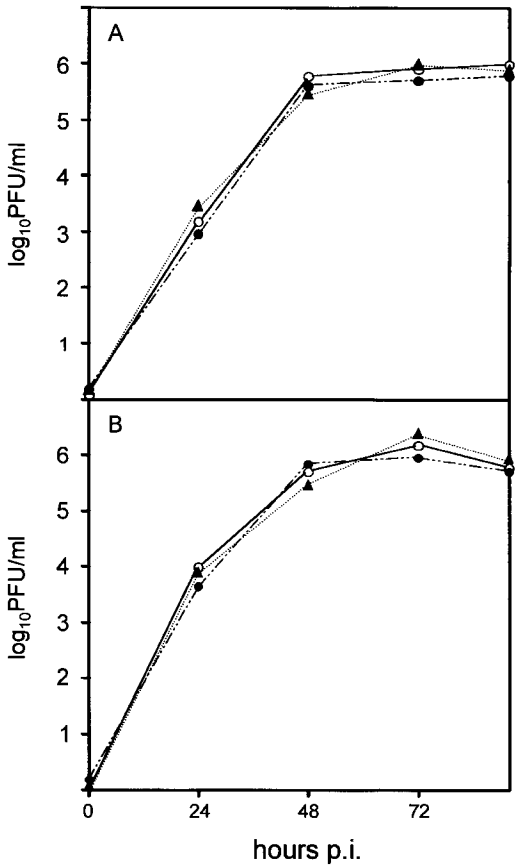

Figure 3. In vitro growth of recombinant viruses. NIH 3T3 cells were infected with wild-type MCMV (○), ΔMC95.24 (▲), or ΔMC95.21 (●) recombinants at a multiplicity of infection of 0.1 PFU per cell. Supernatants (A) and cells (B) were harvested at the indicated time points after infection (p.i.), and virus titers were determined.

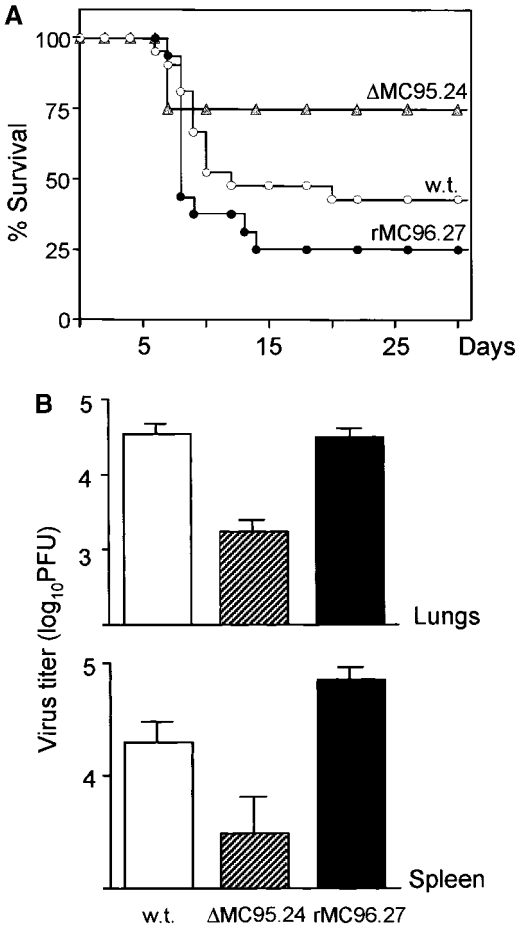

Figure 4. Reduced virulence and replication capacity of the *m152* deletion mutant in vivo. (A) Newborn BALB/c mice were inoculated with 100 PFU i.p. of wild-type (w.t.) MCMV (○), ΔMC95.24 (filled gray triangles), or rMC96.27 (●) virus 12 h post partum, and their survival was monitored daily. (B) Newborn BALB/c mice were infected as shown in A, and virus titers were determined 8 d after infection. Data represent the mean value of at least five mice.

erage body weight of mice that survived infection with ΔMC95.24 was comparable to that of the control group (9.79 ± 1.86 and 10.9 ± 1.16 g, respectively), whereas mice infected with the revertant virus still appeared runted (7.04 ± 1.70 g; data not shown).

To assess whether the differences were due to an altered tissue tropism associated with the *m152* deletion, virus titers were determined for lungs, spleen (Fig. 4 B), and salivary glands (data not shown). The mutant ΔMC95.24 yielded lower titers in the spleen and lungs as compared with wild-type MCMV and the revertant virus. Although the differences in virus titers in tissues of neonatal mice did not exceed 1–2 $\log_{10}$ steps, this finding was reproducible both in MCMV-sensitive (BALB/c) as well as MCMV-resistant (C57BL/6) mouse strains. In the salivary glands, this observation could not be made. In this organ, the virus titer yielded by the *m152* deletion mutant was indistinguishable from that of the wild-type and revertant virus. In this context, it is of interest to note that we have demonstrated earlier that the salivary gland represents the only organ in which MCMV replication is exempt from $CD8^+$ T cell control (41). Altogether, the lack of the *m152* gene results in an attenuated course of infection and in restricted virus growth.

The Attenuation of the m152 Deletion Mutant Is a Function of T Cells. Immunodeficient mice were used to assess whether the attenuated phenotype of the *m152* deletion mutant indeed reflected an enhanced sensitivity to T cell control. BALB/c mice were immunodepleted by gamma irradiation and by injection with cytolytic antibodies to T lymphocytes and NK cells. In immunodepleted animals, all three viruses replicated to high titers without significant titer differences (data not shown). This demonstrated already that the attenuated phenotype of the *m152* deletion mutant is caused by an increased sensitivity to immune control mechanisms. The *m152* deletion mutant replicates to lower virus titers than the revertant virus (Fig. 5 A, left panels; $P < 0.005$) in undepleted BALB/c as well as C57BL/6 mice (Fig. 5 A, top and bottom panels, respectively). This growth restriction was abrogated after depletion of T lymphocyte subsets (Fig. 5 A, right panels), indicating that the attenuated phenotype of the deletion mutant is caused by an enhanced sensitivity to T cell control.

B cell–deficient ($\mu MT^{-/-}$) mice were employed to identify the relative role of T cell subsets (Fig. 5 B). Due to the lack of specific antibodies, MCMV spreads rapidly in $\mu MT^{-/-}$ mice, and detection of infectious virus is facilitated (35). 8-wk-old $\mu MT^{-/-}$ mice were depleted of only $CD8^+$ T lymphocytes, depleted of both $CD8^+$ and $CD4^+$ T cell subsets, or left undepleted. Virus titers were determined 10 d after infection. The growth restriction of the *m152* deletion mutant was notable in particular in the lungs of nondepleted mice, resulting in titer differences ranging from 2 to 3 $\log_{10}$ (Fig. 5 B, left panels). After depletion of $CD8^+$ T lymphocytes or of both T cell subsets, mutant and revertant virus reached comparable virus titers (Fig. 5 B, center and right panels). These data demonstrate that $CD8^+$ T cells are the relevant cell subset responsible for the replication inhibition associated with the *m152* gene deficiency. Furthermore, the attenuating effect is also seen in adult mice. Although the differences are not significant, in BALB/c $\mu MT^{-/-}$ mice, the *m152* deletion mutant reached slightly

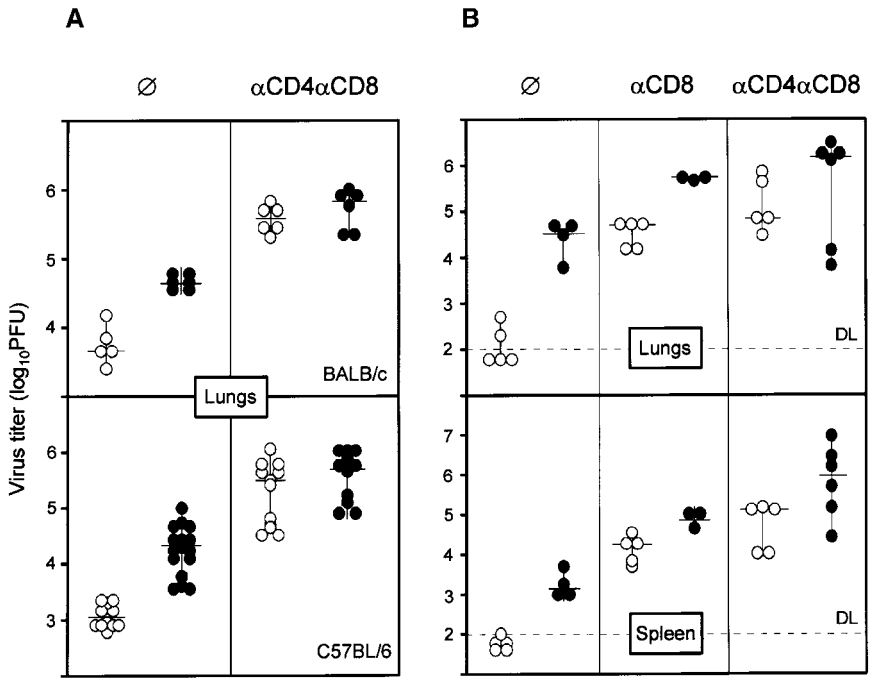

Figure 5. Attenuation of the *m152* deletion mutant is T cell dependent. (A) 4-d-old BALB/c and C57BL/6 mice were depleted of $CD4^+$ and $CD8^+$ T lymphocytes or were left untreated, and they were then infected with 1,000 PFU i.p. of the ΔMC95.24 (○) or rMC96.27 viruses (●). 10 d after infection, virus titers were determined. Titers of individual mice (circles) and median values (horizontal bars) are shown. There was a significant difference in virus titers between ΔMC95.24 and rMC96.27 in both mouse strains ($P < 0.005$; left panels). Depletion of T cells abrogated the difference (right panels). (B) 8-wk-old B cell–deficient mice ($\mu MT^{-/-}$, BALB/c background) were depleted of $CD8^+$ T cells or both $CD4^+$ and $CD8^+$ T lymphocytes, or they were left untreated. Mice were infected with 2×10^5 PFU i.p. of the ΔMC95.24 and rMC96.27 viruses, and virus titers were determined 10 d after infection. Titers in individual animals and median values (horizontal bars) are shown. The differences in virus titers between the groups of nondepleted mice infected with ΔMC95.24 and rMC96.27 were significant ($P < 0.005$) for titers in lungs and spleens (left panels). Depletion of both T cell subsets abrogated the differences between the two recombinants in both organs tested (right panels). Depletion of only $CD8^+$ T cells reduced but did not abolish the differences between the two viruses ($P < 0.05$; center panels). DL, detection limit.

lower titers than the revertant, even after depletion of T cell subsets.

No Phenotype of the m152 Deletion Mutant in MHC Class I–deficient and CD8$^+$ T Cell–deficient Mice. The *m152* gene function affects antigen presentation in the MHC class I pathway. Therefore, in mice in which this presentation pathway is defective, the specific defect of the virus should be phenotypically complemented. To test this, we used MHC class I–deficient C57BL/6 $\beta_2m^{-/-}$ mice (33) and mice deficient for CD8$^+$ T lymphocytes due to the deletion of the CD8 gene (C57BL/6 CD8$^{-/-}$ mice). 4-d-old mice were infected with 1,000 PFU of either the *m152* deletion mutant or the revertant virus. In contrast to the situation in immunocompetent mice, no difference in the titers between the two viruses was found in three replicate experiments performed in $\beta_2m^{-/-}$ and CD8$^{-/-}$ mice. One representative experiment is shown in Fig. 6. Essentially, the same message was obtained in adult CD8$^{-/-}$ mice infected with the *m152* deletion mutant or the revertant virus. However, adult mice of the C57BL/6 strain cleared both viruses so efficiently that the titers in tissues were below the threshold levels when assayed 10 d after infection. Therefore, to enhance the virus replication and to get a measurable virus load in tissues, we had to deplete NK cells in vivo (data not shown). Altogether, these experiments show that attenuation of the *m152* deletion mutant is directly linked to functions required for antigen presentation and recognition in the MHC class I pathway.

Increased Susceptibility of the m152 Deletion Mutant to Naive Lymphocytes. Adoptive cell transfer into immunodepleted recipients was used to determine the sensitivity of MCMV N2 virus-primed as well as to naive lymphocytes. Lethal MCMV infection in gamma-irradiated BALB/c mice is therapeutically prevented by adoptive transfer of as few as 10^5 MCMV-primed CD8$^+$ T cells, whereas the same number of naive lymphocytes or primed CD4$^+$ T cells is ineffective (37, 41, 42). As the product of the *m152* gene downregulates presentation of viral antigens in the MHC class I pathway, we expected to see an increased sensitivity of the mutant to primed T cells and perhaps also a more effective priming of T lymphocytes.

To test this, 2×10^5 lymphocytes derived from BALB/c mice, either MCMV primed or naive, were intravenously transferred into syngeneic gamma-irradiated recipients 12 h after infection with wild-type MCMV, the *m152* deletion mutant, or the revertant virus strain. Adoptive T cell control of MCMV is a selective function of CD8 T cells but not of CD4 T cells and is more effective in spleen and liver than in the lungs (37, 42). Accordingly, the replication of the *m152* deletion mutant is more efficiently controlled in these organs than the revertant virus.

Small numbers ($\sim 10^5$) of naive T lymphocytes fail to protect mice against MCMV infection (41). This was reproduced for mice infected with the revertant virus; however, the number of 2×10^5 naive lymphocytes already decreased the titers of the *m152* deletion mutant (Fig. 7). This is a function of T lymphocytes, as depletion of the CD8 T cell subset eliminated this activity (data not shown). Transfer of graded numbers of naive cells into gamma-irradiated mice showed that the number of naive T cells had to be increased by 100-fold to achieve an effect on wild-type MCMV comparable to the effect on revertant MCMV

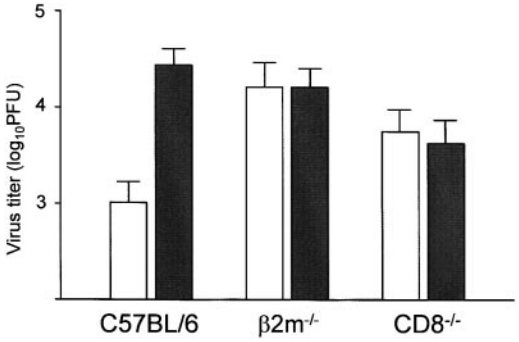

Figure 6. No growth difference of *m152* deletion and revertant viruses in $\beta_2m^{-/-}$ and CD8$^{-/-}$ mice. Normal C57BL/6, $\beta_2m^{-/-}$, and CD8$^{-/-}$ mice (all 4 d old) were inoculated with 1,000 PFU i.p. of the ΔMC95.24 (open bars) or rMC96.27 (shaded bars) recombinant viruses. Shown are virus titers in lungs 10 d after infection. Data represent the mean value of five mice. There was a significant difference in virus titers between ΔMC95.24 and rMC96.27 viruses ($P < 0.005$). The titer difference between the two viruses in $\beta_2m^{-/-}$ and CD8$^{-/-}$ mice is not significant.

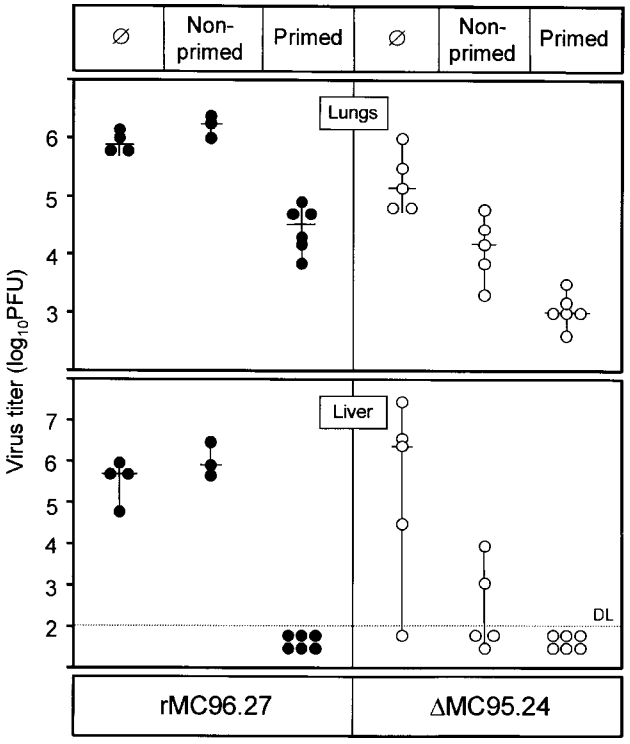

Figure 7. Susceptibility of the *m152* deletion mutant to MCMV-primed and naive T lymphocytes. 8-wk-old BALB/c gamma-irradiated mice were injected with 10^5 PFU of ΔMC95.24 or rMC96.27 virus. 2×10^5 T lymphocytes were obtained from latently infected or uninfected BALB/c mice, and cells were transferred intravenously into recipients immediately after infection. Mice that did not receive cell transfer were used as negative controls. Shown are titers in individual recipients, measured 13 d after transfer and infection. Horizontal bars indicate the median values. DL, detection limit.

(data not shown). We therefore concluded that deletion of the *m152* gene increases the antigenicity of the virus.

Discussion

Herpesvirus genomes contain several genes coding for potential immunomodulatory functions. Shared between viruses of the α- and the β-herpesvirus family is the expression of gene functions that interfere with peptide presentation in the MHC class I pathway in vitro. Herpesviruses are highly species specific, and so are the functions of the genes that affect this pathway. No cellular homologue has been detected for any of these genes so far (4). For a better understanding of the contribution of each individual gene to the biology of the virus infection, experiments in the natural host are required. Here, we report on the in vivo function of the immunomodulatory protein encoded by gene *m152* of MCMV during infection of its natural host.

To prove that a gene has a predicted immunoregulatory function in vivo, three aspects must be addressed. First, the deletion of the gene from the genome should not affect virus growth in cells in the absence of immune control. Second, a phenotype seen in vivo should be lifted by a targeted revertant of the virus. Third, the attenuation due to lack of the immunomodulatory function of the virus should be phenotypically complemented in a host that is genetically or functionally disabled to exert the control that is specifically affected by the deleted viral gene product.

Only the fulfillment of all three requirements confirms the prediction of the in vitro studies. Not all virus genes that have an effect on specific immune effector mechanisms in vitro show this effect in vivo as their main function. One such example is the Fc receptor function encoded by gene *m138* of MCMV. The Fc receptor is expressed at the cell surface and selectively binds mouse IgG in vitro. The deletion of *m138* results in strong attenuation of the mutant virus in vivo that is lifted by the specific revertant. However, in Ig-deficient mice, the attenuation is still present, proving that attenuation of the virus due to the deletion of the Fc receptor is not linked to Ig control (26, 43).

The *m152* gene encodes the glycoprotein gp40, which arrests the export of nascent mouse but not human MHC class I molecules (16). If this was the major function of the protein, then the deletion of the gene should be dispensable for virus growth in fibroblasts but should restrict replication in immunocompetent animals. This prediction was fulfilled by the *m152* deletion mutant viruses. Virus growth in vivo but not in fibroblasts was affected by the mutation. Furthermore, the MHC class I complex transport and the capacity to present viral peptides to CD8 T lymphocytes was restored.

The revertant virus regained wild-type properties in vivo and fulfilled the second requirement by proving the causal linkage between targeted deletion and biological phenotype. As with HCMV infection in humans, the primary infection of mice even with wild-type MCMV is usually asymptomatic. Newborn mice and mice that are a few days old are much more sensitive than adult mice to tissue culture–grown virus, due to the immaturity of the NK cell response (44). In neonates, the infection with 10^2 PFU causes a high percentage of mortality and runting in survivors. The attenuating effect of the *m152* gene deletion resulted in a higher number of survivors and an earlier cessation of runting.

The third requirement was also fulfilled: loss of the phenotypic difference between deletion mutant and revertant virus in the absence of the host immune function affected by the viral gene product. gp40 blocks the export of nascent MHC class I molecules already loaded with viral peptides. The predictable consequence is the inhibition of CD8 T cell priming and CD8 T cell effector function. Loss of the *m152* gene should lead to an increased sensitivity of the virus to lymphocytes. Indeed, the virus mutant grew to smaller titers in the various tissues tested. This attenuation did reflect a more stringent control of the deletion mutant by T cell functions, as elimination of T cells resulted in comparable tissue titers of mutant and revertant virus. Furthermore, the attenuating effect of the *m152* deletion mutant was absent in C57BL/6 mice that failed to form the functional MHC class I molecules due to the lack of β2-microglobulin expression and also in mice that have a defect in the maturation of MHC class I–restricted $CD8^+$ T cells due to the deletion of the CD8 gene. Altogether, this study proves for the first time that in their natural host, herpesviruses benefit from functions that inhibit antigen presentation in the MHC class I pathway in vivo.

It remains open whether the observed function is the only function of the *m152* gene product in vivo. MHC class I molecules activate $CD8^+$ T cells and, at the same time, inhibit NK cells (45, 46). Accordingly, a prediction of the transport block of MHC class I molecules due to *m152* gene expression is the susceptibility of MCMV-infected cells for NK cell–mediated destruction in vivo. A deletion of the *m152* gene and the restoration of MHC class I molecule transport should result in an enhanced resistance of infected cells to NK cell control in vivo. Our data do not support this assumption. Preliminary studies suggest that the lack of the *m152* gene certainly does not make the virus more resistant to control by NK cells (Krmpotic, A., B. Polic, and S. Jonjic, unpublished data). Both HCMV and MCMV genes code for glycoproteins that show homology to MHC class I molecules, *UL18* in HCMV (47) and *m144* in MCMV (27, 48). It has been hypothesized that these viral MHC class I homologues are capable of engaging NK cell inhibitory receptors to protect cells from lysis due to the downregulation of MHC class I expression. Attenuation of MCMV harboring a deletion in the *m144* gene has been explained by enhanced control by NK cells in vivo (48). However, a more recent study on *UL18* functions failed to confirm the inhibitory function of viral MHC class I homologues on NK cells (49). Therefore, the potential interaction of *m152* with *m144* needs to be addressed.

Another explanation is that the remaining functions of the genes *m04* and *m06* fully complement the expected NK cell effect of *m152*. The genes *m04* and *m06* have an effect on MHC class I molecules. Both genes are expressed later than *m152* during the MCMV replication cycle, and both

genes encode glycoproteins that bind tightly to MHC class I molecules. gp34, encoded by the *m04* gene, forms a complex with MHC class I molecules that can be detected on the surfaces of infected cells, but the functional consequence is not clear (17). The *m06* gene product gp48 binds to MHC class I molecules in the ER and reroutes them to lysosomes for rapid proteolytic degradation (18). This leads to the downregulation of MHC class I surface expression in the late phase of the replication cycle (18, 23). Here, we show that the *m04* and *m06* genes cannot fully compensate all aspects of the loss of the *m152* function. Thus, the interaction between the *m152* gene product and other viral gene functions is not yet clear and remains to be tested. We are in the process of constructing double and triple deletion mutants to determine the individual contribution of each of the MHC class I–reactive genes and MHC class I homologues in immune evasion. To this end, we have recently pioneered the cloning of infectious herpesvirus genomes and have developed targeted and random mutagenesis techniques (50, 51).

Our results show for the first time that genes that inhibit antigen presentation in the MHC class I pathway provide a significant growth advantage for CMV during primary infection. What is the potential benefit for the virus? The conditions of primary infection define the load of latent viral genomes and the risk of recurrence of the CMV infection (39). Accordingly, we predict that the *m152* gene allows a higher number of MCMV genomes to establish a latent infection, thereby enhancing the chance for reactivation and transmission to the next host and thus escaping extinction.

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References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601–624.
2. Heemels, M.T., and H.L. Ploegh. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu. Rev. Biochem.* 64:463–491.
3. Koopmann, J.O., G.J. Hämmerling, and F. Momburg. 1997. Generation, intracellular transport and loading of peptides associated with MHC class I molecules. *Curr. Opin. Immunol.* 9:80–88.
4. Hengel, H., and U.H. Koszinowski. 1998. Inhibition of MHC class I function by cytomegalovirus. *In* Herpesviruses and Immunity. P.G. Medvczky, H. Friedman, and M. Bendinelli, editors. Plenum Press, New York. 247–264.
5. Burgert, H.G., and S. Kvist. 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell.* 41:987–997.
6. York, I.A., C. Roop, D.W. Andrews, S.R. Riddell, F.L. Graham, and D.C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to $CD8^+$ T lymphocytes. *Cell.* 77:525–535.
7. Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature.* 375:411–415.
8. Früh, K., K. Ahn, H. Djaballah, P. Sempe, P.M. van Endert, R. Tampe, P.A. Peterson, and Y. Yang. 1995. A viral inhibitor of peptide transporters for antigen presentation. *Nature.* 375:415–418.
9. Wiertz, E.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from endoplasmic reticulum to the proteasome for destruction. *Nature.* 384: 432–438.
10. Wiertz, E.J., T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, and H.L. Ploegh. 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell.* 84:769–779.
11. Jones, T.R., E.J. Wiertz, L. Sun, K.N. Fish, J.A. Nelson, and H.L. Ploegh. 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. USA.* 93: 11327–11333.
12. Ahn, K., A. Angulo, P. Ghazal, P.A. Peterson, Y. Yang, and K. Früh. 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA.* 93:10990–10995.
13. Ahn, K., A. Gruhler, B. Galocha, T.R. Jones, E.J. Wiertz, H.L. Ploegh, P.A. Peterson, Y. Yang, and K. Früh. 1997. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity.* 6:613–621.
14. Hengel, H., J.O. Koopmann, T. Flohr, W. Muranyi, E. Goulmy, G.J. Hämmerling, U.H. Koszinowski, and F. Momburg. 1997. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. *Immunity.* 6:623–632.
15. Lehner, P.J., J.T. Karttunen, G.W. Wilkinson, and P. Cresswell. 1997. The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation. *Proc. Natl. Acad. Sci. USA.* 94: 6904–6909.
16. Ziegler, H., R. Thäle, P. Lucin, W. Muranyi, T. Flohr, H. Hengel, H. Farrell, W. Rawlinson, and U.H. Koszinowski. 1997. A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. *Immunity.* 6:57–66.

17. Kleijnen, M.F., J.B. Huppa, P. Lucin, S. Mukherjee, H. Farrell, A.E. Campbell, U.H. Koszinowski, A.B. Hill, and H.L. Ploegh. 1997. A mouse cytomegalovirus glycoprotein gp34 forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:685–694.
18. Reusch, U., W. Muranyi, P. Lucin, H.G. Burgert, H. Hengel, and U.H. Koszinowski. 1999. A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:1081–1091.
19. von Herrath, M.G., S. Efrat, M.B. Oldstone, and M.S. Horwitz. 1997. Expression of adenoviral E3 transgenes in beta cells prevents autoimmune diabetes. *Proc. Natl. Acad. Sci. USA*. 94:9808–9813.
20. Goldsmith, K., W. Chen, D.C. Johnson, and R.L. Hendricks. 1998. Infected cell protein (ICP) 47 enhances herpes simplex virus neurovirulence by blocking the $CD8^+$ T cell response. *J. Exp. Med.* 187:341–348.
21. del Val, M., K. Münch, M.J. Reddehase, and U.H. Koszinowski. 1989. Presentation of cytomegalovirus immediate-early antigens to cytolytic T lymphocytes is selectively blocked by viral genes expressed in the early phase. *Cell*. 58: 305–315.
22. del Val, M., H. Hengel, H. Häcker, U. Hartlaub, T. Ruppert, P. Lucin, and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J. Exp. Med.* 176: 729–738.
23. Thäle, R., U. Szepan, H. Hengel, G. Geginat, P. Lucin, and U.H. Koszinowski. 1995. Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility complex class I molecule transport. *J. Virol.* 69:6098–6105.
24. del Val, M., H.J. Schlicht, T. Ruppert, M.J. Reddehase, and U.H. Koszinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell*. 66:1145–1153.
25. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1659 pp.
26. Crnkovic-Mertens, I., M. Messerle, I. Milotic, U. Szepan, N. Kucic, A. Krmpotic, S. Jonjic, and U.H. Koszinowski. 1998. Virus attenuation after deletion of the cytomegalovirus Fc receptor is not due to antibody control. *J. Virol.* 72:1377–1382.
27. Rawlinson, W.D., H. Farrell, and B.G. Barrell. 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. *J. Virol.* 70:8833–8849.
28. Vieira, J., H.E. Farrell, W.D. Rawlinson, and E.S. Mocarski. 1994. Genes in the HindIII J fragment of the murine cytomegalovirus genome are dispensable for growth in cultured cells: insertion mutagenesis with a *lacZ/gpt* cassette. *J. Virol.* 68:4837–4846.
29. Greaves, R.F., J.M. Brown, J. Vieira, and E.S. Mocarski. 1995. Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the *Escherichia coli* guanosine phosphoribosyl transferase (*gpt*) gene. *J. Gen. Virol.* 76:2151–2160.
30. del Val, M., H. Volkmer, B. Rothbard, S. Jonjic, M. Messerle, J. Schickedanz, M.J. Reddehase, and U.H. Koszinowski. 1988. Molecular basis for cytolytic T-lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp89. *J. Virol.* 62:3965–3972.
31. Kitamura, D., J. Roes, R. Kühn, and K. Rajewsky. 1991. B cell-deficient mouse by targeting disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature*. 350: 423–426.
32. Klein-Schneegans, A.S., C. Gaveriaux, P. Fonteneau, and F. Loor. 1989. Indirect double sandwich ELISA for the specific and quantitative measurement of mouse IgM, IgA and IgG subclasses. *J. Immunol. Methods*. 119:117–125.
33. Zijlstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenish. 1990. β2-microglobulin deficient mice lack $CD4^-8^+$ cytolytic T cells. *Nature*. 344:742–746.
34. Polic, B., S. Jonjic, I. Pavic, I. Crnkovic, I. Zorica, H. Hengel, P. Lucin, and U.H. Koszinowski. 1996. Lack of MHC class I complex expression has no effect on spread and control of cytomegalovirus infection in vivo. *J. Gen. Virol.* 77:217–225.
35. Jonjic, S., I. Pavic, B. Polic, I. Crnkovic, P. Lucin, and U.H. Koszinowski. 1994. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J. Exp. Med.* 179:1713–1717.
36. Reddehase, M.J., G.M. Keil, and U.H. Koszinowski. 1984. The cytolytic T lymphocyte response to the murine cytomegalovirus. I. Distinct maturation stages of cytolytic T lymphocytes constitute the cellular immune response during acute infection of mice with the murine cytomegalovirus. *J. Immunol.* 132:482–489.
37. Reddehase, M.J., F. Weiland, K. Münch, S. Jonjic, A. Lüske, and U.H. Koszinowski. 1985. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. *J. Virol.* 55:264–273.
38. Cobbold, S.P., A. Jayasuriya, A. Nash, T.D. Prospero, and H. Waldman. 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature*. 312:548–550.
39. Reddehase, M.J., M. Balthesen, M. Rapp, S. Jonjic, I. Pavic, and U.H. Koszinowski. 1994. The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. *J. Exp. Med.* 179:185–193.
40. Trgovcich, J., E. Pernjak-Pugel, J. Tomac, U.H. Koszinowski, and S. Jonjic. 1998. Pathogenesis of murine cytomegalovirus infection in mice. *In* CMV-related Immunopathology. Monographs in Virology. Vol. 21. M. Scholz, H.F. Rabenau, H.W. Doerr, and J. Cinatl, Jr., editors. Karger Publishers, Basel, Switzerland. 42–53.
41. Jonjic, S., W. Mutter, F. Weiland, M.J. Reddehase, and U.H. Koszinowski. 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of $CD4^+$ T lymphocytes. *J. Exp. Med.* 169:1199–1212.
42. Reddehase, M.J., S. Jonjic, F. Weiland, W. Mutter, and U.H. Koszinowski. 1988. Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors. *J. Virol.* 62:1061–1065.
43. Thäle, R., P. Lucin, K. Schneider, M. Eggers, and U.H. Koszinowski. 1994. Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. *J. Virol.* 68: 7757–7765.
44. Bukowski, J.F., J.F. Warner, G. Dennert, and R.M. Welsh. 1985. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. *J. Exp. Med.* 161:40–52.
45. Lanier, L.L., B. Corliss, and J.H. Phillips. 1997. Arousal and inhibition of human NK cells. *Immunol. Rev.* 155:145–154.
46. Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni,

M.C. Mingari, and L. Moretta. 1996. Receptors for HLA class-I molecules in human natural killer cells. *Annu. Rev. Immunol.* 14:619–648.

47. Beck, S., and B.G. Barrell. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature*. 331:269–271.
48. Farrell, H.E., H. Vally, D.M. Lynch, P. Fleming, G.R. Shellam, A.A. Scalzo, and N.J. Davis-Poynter. 1997. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. *Nature.* 386:510–514.
49. Leong, C.C., T.L. Chapman, P.J. Bjorkman, D. Formankova, E.S. Mocarski, J.H. Phillips, and L.L. Lanier. 1998. Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog. *J. Exp. Med.* 187:1681–1687.
50. Messerle, M., I. Crnkovic, W. Hammerschmidt, H. Ziegler, and U.H. Koszinowski. 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc. Natl. Acad. Sci. USA*. 94:14759–14763.
51. Brune, W., C. Ménard, U. Hobom, S. Odenbreit, M. Messerle, and U.H. Koszinowski. 1999. Rapid identification of essential and nonessential herpesvirus genes by direct transposon mutagenesis. *Nat. Biotechnol.* 17:360–364.