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Research Article

Human CMV (HCMV) is a major cause of morbidity and mortality in both congenitally infected and immunocompromised individuals. Development of an effective HCMV vaccine would help protect these vulnerable groups. NK group 2, member D (NKG2D) is a potent activating receptor expressed by cells of the innate and adaptive immune systems. Its importance in HCMV immune surveillance is indicated by the elaborative evasion mechanisms evolved by the virus to avoid NKG2D. In order to study this signaling pathway, we engineered a recombinant mouse CMV expressing the high-affinity NKG2D ligand RAE-1 γ (*RAE-1 γ MCMV*). Expression of RAE-1 γ by MCMV resulted in profound virus attenuation in vivo and lower latent viral DNA loads. *RAE-1 γ MCMV* infection was efficiently controlled by immunodeficient hosts, including mice lacking type I interferon receptors or immunosuppressed by sublethal γ -irradiation. Features of MCMV infection in neonates were also diminished. Despite tight innate immune control, *RAE-1 γ MCMV* infection elicited strong and long-lasting protective immunity. Maternal *RAE-1 γ MCMV* immunization protected neonatal mice from MCMV disease via placental transfer of antiviral Abs. Despite strong selective pressure, the *RAE-1 γ* transgene did not exhibit sequence variation following infection. Together, our results indicate that use of a recombinant virus encoding the ligand for an activating NK cell receptor could be a powerful approach to developing a safe and immunogenic HCMV vaccine.

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Recombinant mouse cytomegalovirus expressing a ligand for the NKG2D receptor is attenuated and has improved vaccine properties

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Human CMV (HCMV) is a major cause of morbidity and mortality in both congenitally infected and immunocompromised individuals. Development of an effective HCMV vaccine would help protect these vulnerable groups. NK group 2, member D (NKG2D) is a potent activating receptor expressed by cells of the innate and adaptive immune systems. Its importance in HCMV immune surveillance is indicated by the elaborate evasion mechanisms evolved by the virus to avoid NKG2D. In order to study this signaling pathway, we engineered a recombinant mouse CMV expressing the high-affinity NKG2D ligand RAE-1 γ (RAE-1 γ MCMV). Expression of RAE-1 γ by MCMV resulted in profound virus attenuation in vivo and lower latent viral DNA loads. RAE-1 γ MCMV infection was efficiently controlled by immunodeficient hosts, including mice lacking type I interferon receptors or immunosuppressed by sublethal γ -irradiation. Features of MCMV infection in neonates were also diminished. Despite tight innate immune control, RAE-1 γ MCMV infection elicited strong and long-lasting protective immunity. Maternal RAE-1 γ MCMV immunization protected neonatal mice from MCMV disease via placental transfer of antiviral Abs. Despite strong selective pressure, the RAE-1 γ transgene did not exhibit sequence variation following infection. Together, our results indicate that use of a recombinant virus encoding the ligand for an activating NK cell receptor could be a powerful approach to developing a safe and immunogenic HCMV vaccine.

Introduction

Human CMV (HCMV) is an important human pathogen causing morbidity and mortality in congenitally infected and immunosuppressed individuals. CMVs are highly adapted to their mammalian hosts and are host species-specific in their replication, which precludes the study of HCMV in animal models. Research on murine CMV (MCMV) is the most advanced model with regard to the principles that govern the immune surveillance of CMVs. After primary infection, the host immune response effectively terminates virus replication; however, clearance of the viral genome is not achieved, and CMV establishes lifelong latency, with periodic reactivation and shedding of virus (1).

While HCMV infection is readily controlled by the immunocompetent host, the virus displays its pathogenic potential when host immunity is impaired. HCMV infection is the most common viral congenital infection and may result in lifelong neurological sequelae, including brain damage, sensorineural hearing loss, and mental retardation (2–5). Solid organ transplant recipients and hematopoietic stem cell transplant recipients are the second group of patients at risk for severe CMV infections (6–8). In HIV-infected

patients, CMV continues to be the most frequent viral opportunistic pathogen, although severe infections have become less common following the introduction of highly active antiretroviral therapy (9). Due to this immense public health challenge, the development of an HCMV vaccine has been ranked as a top priority for the 21st century by the U.S. Institute of Medicine (10).

Both innate and adaptive immune responses are important for the control of CMV infection (11–15). Innate immunity, in particular NK cells, plays a key role in limiting CMV infection at an early stage and in priming of the adaptive immune response (16, 17). CD8 $^{+}$ T cells are the principal effectors required for resolution of productive infection and establishment of latency (18). Although CD8 $^{+}$ T cells play a dominant role, CD4 $^{+}$ T cells and NK cells contribute to the maintenance of latent CMV infection (19). Antiviral Abs, although not essential for the control of primary CMV infection and the establishment of latency, play a critical role in limiting the dissemination of recurrent virus (20). Abs can modify the disease associated with HCMV infection in transplant recipients as well as congenital CMV infection in humans and experimental animal models (21–26). Consequently, a CMV vaccine should ideally aim to elicit an effective cellular and humoral immune response.

A number of subunit vaccine strategies and live, attenuated CMV vaccines have been developed (27–31). Recently, a phase II clinical trial was described that suggested a protective capacity against maternal infection by use of recombinant monovalent gB HCMV

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vaccine (32). While subunit vaccines induce an immune response to selected viral proteins, the advantage of live vaccines is that they elicit an immune response that mimics natural immunity and provides broader protection. Their use, however, carries the risk of CMV disease caused by the vaccine strain or reactivation in the immunocompromised state, unless the vaccine virus is efficiently controlled by residual immunity. One approach to generating such an immunogenic, yet safe live vaccine is deletion of viral genes that subvert the host immune response (33, 34) or essential genes resulting in spread-deficient virus (31). The other approach is the insertion of a ligand recognized by the activating receptor on immune cells into the CMV genome. In our study, we have used the latter approach and designed an experimental CMV vaccine encoding a ligand for NK group 2, member D (NKG2D), an activating receptor expressed on NK cells, activated CD8⁺ T cells, and γδ T cells (35, 36). The engagement of NKG2D can override inhibitory signals delivered by self-MHC class I proteins and trigger NK cell activation. NKG2D binds to a family of MHC class I-related proteins that are not constitutively expressed but are induced by cell transformation or stress, including infection. In mice, the RAE-1 family of proteins, H60, and MULT-1 are ligands for NKG2D. The importance of the NKG2D signaling pathway in CMV control is best illustrated by a sophisticated mechanism that HCMV and MCMV have developed to avoid NKG2D-mediated immune control. Four MCMV genes are dedicated to down-modulating all of the NKG2D ligands from the surface of infected cells: *m152* targets the RAE-1 family of molecules; *m145* targets MULT-1; and *m155* targets H60. MULT-1, H60, and RAE-1ε are additionally downregulated by *m138* (37, 38). The MCMV mutants lacking any of the NKG2D inhibitors are sensitive to the NK cell control in vivo, due to the preserved NKG2D ligand on the cell surface of infected cells (39). Similarly, HCMV encodes proteins that downregulate NKG2D ligands in humans (40).

To take the advantage of this powerful signaling pathway in the generation of a safe, yet immunogenic vaccine, we inserted RAE-1γ into the MCMV genome in place of the *m152* gene, which otherwise negatively regulates this NKG2D ligand. We hypothesized that (a) the deletion of *m152* from the MCMV genome should prevent downregulation of both endogenous RAE-1γ and RAE-1γ encoded by the transgene; and (b) the consistent expression of RAE-1γ on infected cells may override the effect of all other MCMV immunoevasins for NK cells and (c) may also augment the CD8⁺ T cell response through the costimulatory function of NKG2D on these cells. Furthermore, since *m152* additionally arrests the maturation of MHC class I molecules (41), the deletion of this gene may improve the presentation of viral proteins and enhance the T cell immune response. Here we demonstrate that RAE-1γ-expressing MCMV (*RAE-1γ*MCMV) was dramatically attenuated in vivo not only in the immunocompetent host but even in immunological immature neonatal and in immunodeficient mice. However, despite tight immune control, *RAE-1γ*MCMV infection elicited a potent, long-lasting cellular and Ab immune response able to protect animals against challenge infection. Moreover, maternal *RAE-1γ*MCMV infection resulted in the production and placental transfer of antiviral Abs that protected offspring from MCMV infection following neonatal infection.

Results

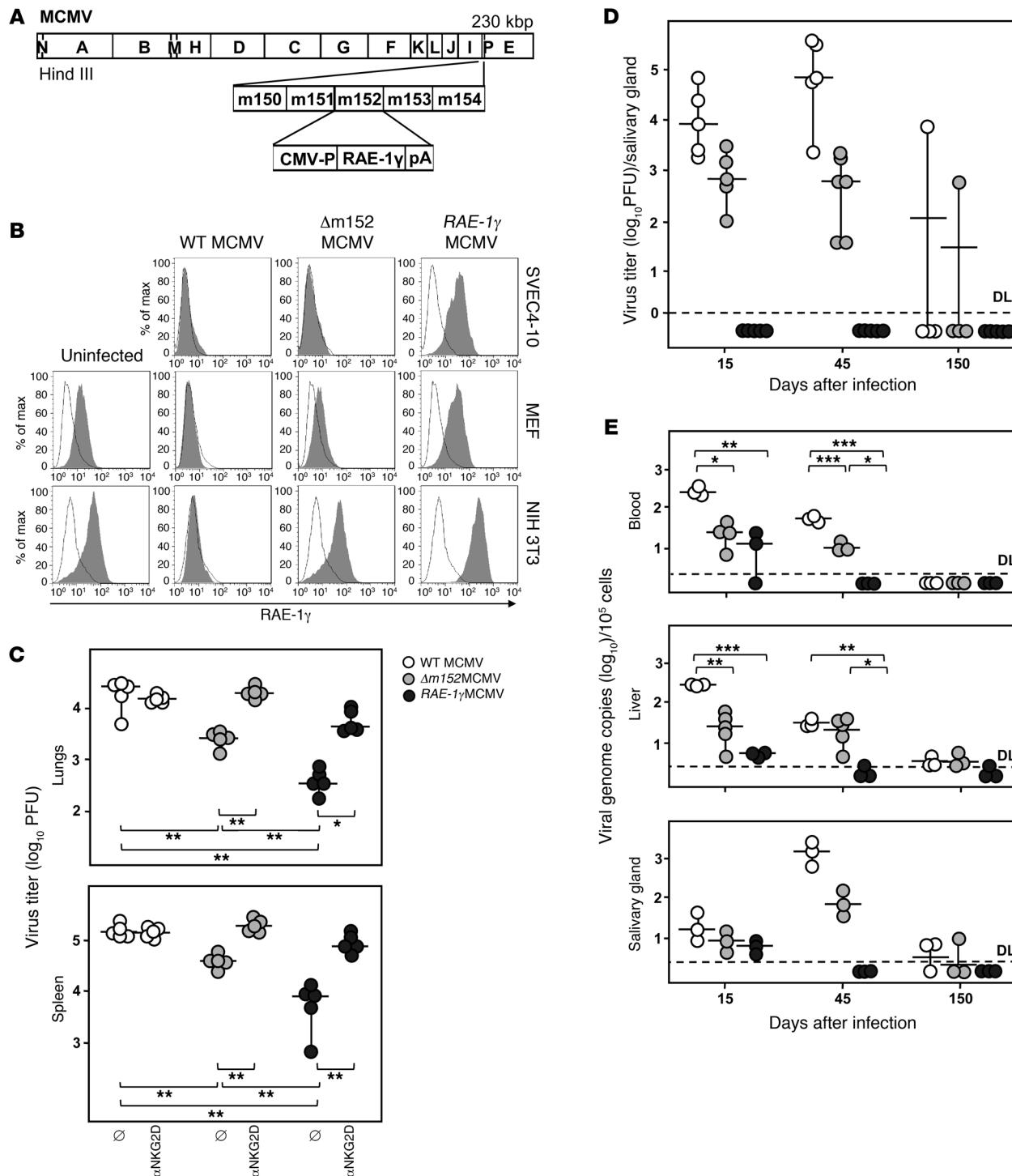
Generation and in vitro characterization of a recombinant MCMV expressing the NKG2D ligand RAE-1γ. To study how the expression of NKG2D

ligand by MCMV influences immunobiology of this virus infection, we designed a recombinant virus, referred to as *RAE-1γ*MCMV, that expresses RAE-1γ. *RAE-1γ*MCMV was constructed by replacing the *m152* ORF in the (BAC-cloned) MCMV genome with a cassette comprising the RAE-1γ ORF under control of the HCMV immediate-early promoter (Figure 1A). *RAE-1γ*MCMV replication assessed in a multistep growth kinetics assay was comparable to WT MCMV replication (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI43961DS1). Infection of SVEC4-10 cells, an endothelial cell line that does not express RAE-1γ, with the recombinant MCMV resulted in cell surface RAE-1γ expression (Figure 1B). As shown previously, WT MCMV infection downregulates endogenous RAE-1γ, which was prevented by the deletion of *m152* from the MCMV genome (*Δm152* MCMV). Introduction of *RAE-1γ* to the *Δm152* MCMV resulted in RAE-1γ overexpression on the surface of infected MEFs and NIH 3T3 cells (Figure 1B). Note that *RAE-1γ*MCMV infection did not change the pattern of cell surface expression of other NKG2D ligands compared with *Δm152* MCMV (Supplemental Figure 1B). Together, these data indicate that *RAE-1γ* insertion into the MCMV genome had no effect on virus replication in vitro and resulted in the expression of RAE-1γ on the surface of infected cells.

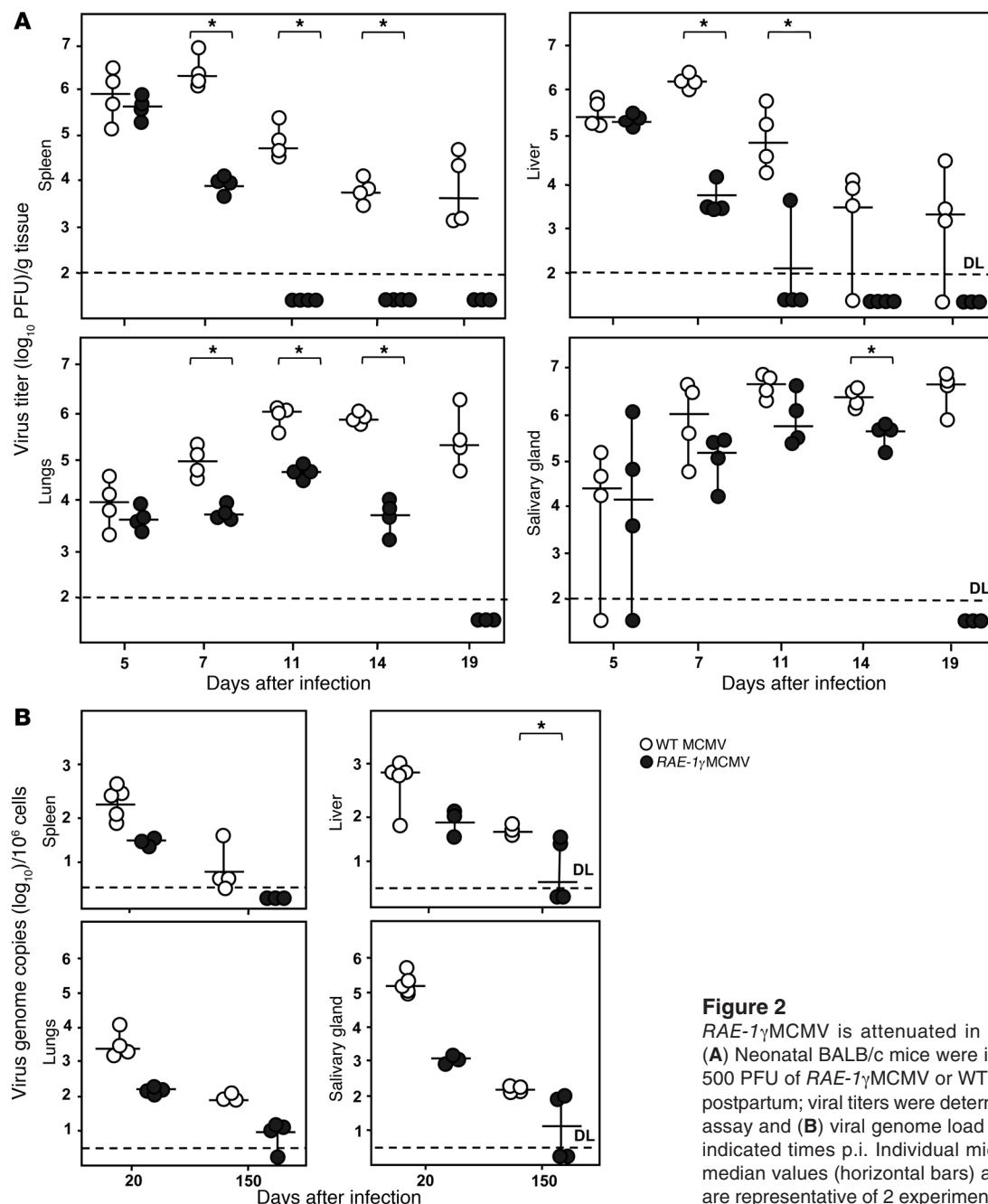
*RAE-1γ*MCMV is strongly attenuated in vivo and fails to establish persistent infection in salivary gland. Adult BALB/c mice were injected with *RAE-1γ*MCMV, WT MCMV, or *Δm152* MCMV to study whether expression of the NKG2D ligand by the MCMV influences virus control in vivo. In agreement with our previous results (42), at day 3 after infection (day 3 p.i.) replication of *Δm152* MCMV was attenuated in an NKG2D-dependent manner as compared with WT MCMV. Introduction of *RAE-1γ* into the *Δm152* MCMV genome further attenuated viral replication and resulted in significantly lower viral titers in all tested organs as compared with *Δm152* MCMV and WT MCMV (Figure 1C). The observed attenuation was NKG2D-dependent and was abolished by administration of anti-NKG2D blocking Abs that restored *RAE-1γ*MCMV titers almost to the WT MCMV level.

The salivary glands remain persistently infected with MCMV long after productive virus replication is terminated in other tissues (43, 44). NK cells and CD4⁺ T cells are essential for virus clearance in the salivary glands and prevention of horizontal virus spread (44, 45). We therefore compared the virus titers in salivary glands 15, 60, and 150 days after *RAE-1γ*MCMV, WT MCMV, and *Δm152* MCMV infection. In contrast to a high-titer persistent virus replication in WT MCMV-infected mice, no infectious virus was detected in salivary glands following *RAE-1γ*MCMV infection (Figure 1D). Although *Δm152* MCMV reached slightly lower virus titers compared with WT MCMV, replication kinetics of these two viruses in salivary glands were similar. We next determined whether marked differences between *RAE-1γ*MCMV and WT MCMV replication are reflected in the kinetics of viral clearance from blood and viral genome load in tissue during latency. Unlike in mice infected with WT MCMV, in which viral DNA was maintained in the blood for prolonged period of time (46), viral DNA was cleared from the blood of *RAE-1γ*MCMV-infected mice by day 45. At that time, *RAE-1γ*MCMV DNA remained in organs, but the viral load was reduced to a barely detectable level or, in some cases, to below the limit of detection. Viral DNA load in *Δm152* MCMV-infected mice corresponded to infectious virus titers (Figure 1E).

To study how the expression of NKG2D ligand by the MCMV affects virus control in mice with a constitutively more efficient

**Figure 1**

RAE-1 γ MCMV is attenuated in vivo in an NKG2D-dependent manner. (A) The HindIII cleavage map of the MCMV genome is shown at the top, with the genomic region encoding the m152 ORF below. The m152 ORF was replaced by an expression cassette (bottom) comprising the HCMV major immediate early promoter (CMV-P), the RAE-1 γ ORF, and the SV40 polyadenylation signal sequence. (B) SVEC4-10 cells, NIH 3T3 cells, and MEFs were infected with the indicated viruses and 12 hours later analyzed for the surface expression of RAE-1 γ by staining with anti-RAE-1 γ Ab, followed by PE-conjugated goat anti-rat IgG. Cells incubated with the secondary Ab in the absence of the primary Ab were used as negative control (thin line). Each histogram represents 10,000 gated propidium iodide-negative cells. (C) Untreated BALB/c mice or BALB/c mice treated with blocking anti-NKG2D mAb were i.v. injected with 10^5 PFU of the indicated viruses. Viral titers were determined in lungs and spleen 3 days p.i. by plaque assay. Ø, untreated BALB/c mice. (D) BALB/c mice were f.p. injected with 2×10^5 PFU of the indicated viruses. Viral titers were determined by plaque assay and (E) viral genome load by qPCR at different time p.i. Individual mice (circles) and median values (horizontal bars) are shown. DL, detection limit. Results from 1 of 2 similar experiments are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 2**

RAE-1 γ MCMV is attenuated in neonatal mice. (A) Neonatal BALB/c mice were i.p. injected with 500 PFU of RAE-1 γ MCMV or WT MCMV 6 hours postpartum; viral titers were determined by plaque assay and (B) viral genome load by qPCR at the indicated times p.i. Individual mice (circles) and median values (horizontal bars) are shown. Data are representative of 2 experiments. * P < 0.05.

NK cell response, we injected C57BL/6 with RAE-1 γ MCMV, WT MCMV, or $\Delta m152$ MCMV. MCMV resistance of C57BL/6 mice is due to the expression of Ly49H activating receptor on NK cells, which recognizes virally encoded protein m157 (47, 48). Similar to results in MCMV-sensitive BALB/c mice, RAE-1 γ MCMV reached significantly lower titers compared with WT MCMV and $\Delta m152$ MCMV. Thus, unlike in mice infected with MCMV mutant lacking m152 only, NKG2D-mediated control of RAE-1 γ MCMV was not overcome by NK cell activation via Ly49H (Supplemental Figure 2A). Taken together, the results indicate that expression of RAE-1 γ by MCMV resulted in a dramatic attenuation of virus replication in different organs and a lower latent viral DNA load.

RAE-1 γ MCMV is attenuated even in neonatal mice. Neonatal mice are highly sensitive to MCMV infection, and i.p. injection even with a low dose of cell culture-derived virus results in significant morbidity and mortality. Mice that survive MCMV infection establish a disseminated, high-titer virus replication and long-lasting persistent infection in salivary glands (43). To test RAE-1 γ MCMV replication in neonatal mice, we injected newborn animals i.p. with 500 PFU of RAE-1 γ MCMV or WT MCMV. During the first 5 days of infection, the two viruses replicated to comparable titers, but starting from day 7 RAE-1 γ MCMV replication was significantly reduced in all tested organs (Figure 2A). Productive RAE-1 γ MCMV infection was cleared by day 11 in spleen and liver and by day 19 in lungs and even in salivary glands. By contrast, around that time

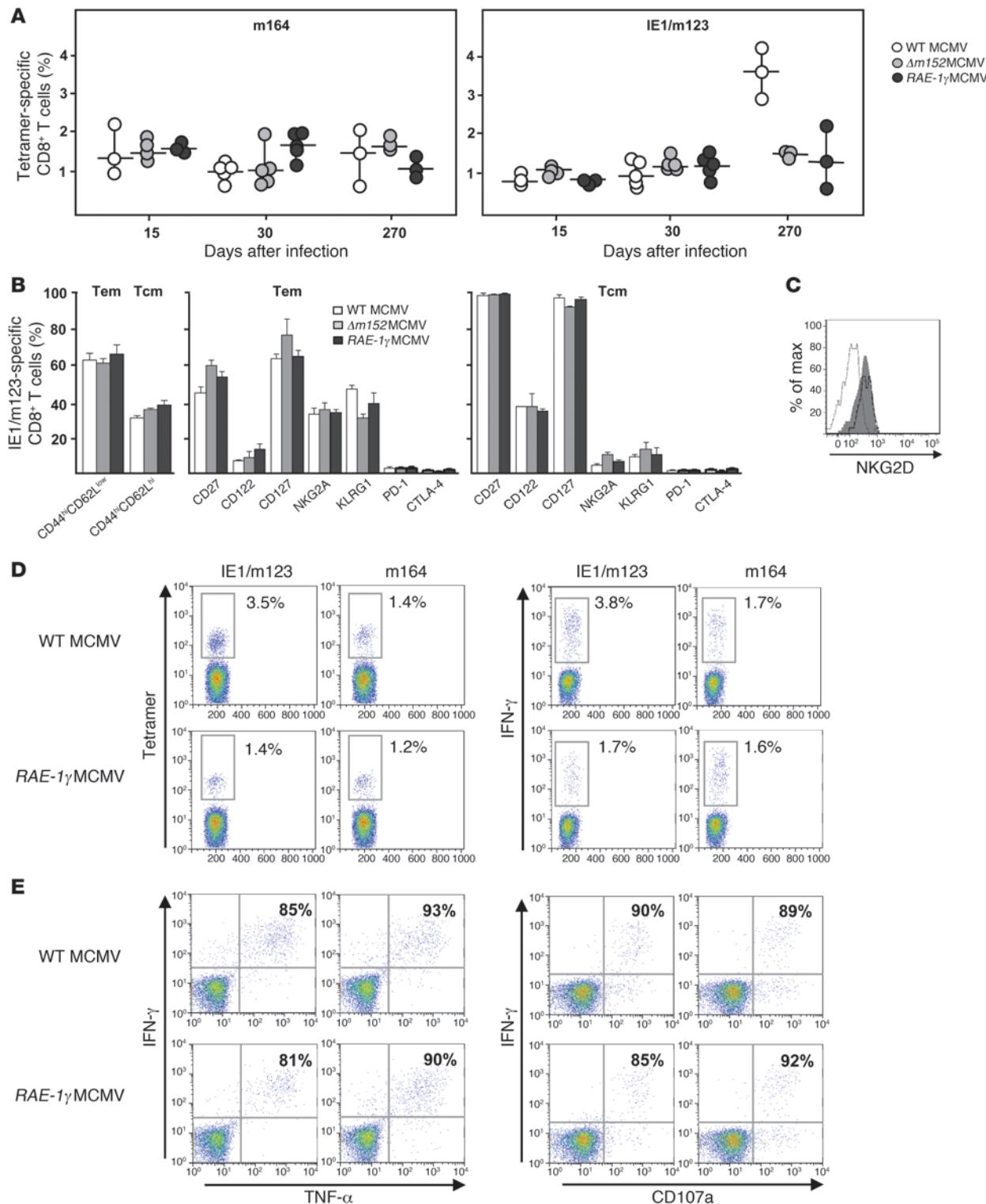


Figure 3

Comparable kinetics and phenotype of MCMV-specific memory CD8⁺ T cells in *RAE-1 γ MCMV*–, WT MCMV–, and $\Delta m152$ MCMV–infected mice. **(A)** BALB/c mice were f.p. injected with 2×10^5 PFU *RAE-1 γ MCMV*, WT MCMV, or $\Delta m152$ MCMV. Splenocytes were isolated at different times after infection and stained with IE1/m123 or m164 MHC class I tetramers and anti-CD8 Ab. The percentage of tetramer-specific CD8⁺ T cells for individual mice (circles) and median values (horizontal bars) are shown. **(B)** Splenocytes were isolated 9 months p.i. and stained with IE1/m123 MHC class I tetramer, anti-CD8 Ab, and Abs to indicated cell surface molecules. The percentage of IE1/m123-specific CD8⁺ T cells displaying effector memory (Tem) or central memory (Tcm) phenotype (left) and the percentage of Tem- and Tcm-expressing indicated cell surface molecules are shown (right). Error bars show mean \pm SEM. **(C)** Representative histogram showing NKG2D staining on IE1/m123-specific CD8⁺ T cells in spleen 9 months after WT MCMV (filled histogram) or *RAE-1 γ MCMV* (dotted line) infection. Tetramer-negative CD8⁺ T cells are indicated by the dashed line. **(D)** Splenocytes were isolated 9 months p.i. and stained with the indicated tetramers or stimulated with the indicated peptides and stained for IFN- γ production or **(E)** costained for IFN- γ and TNF- α production. Splenocytes were stimulated as above in the presence of the anti-CD107a Ab and costained for IFN- γ production. **(D and E)** Representative dot plots gated on CD8⁺ T cells of 3 mice per group are shown. Numbers indicate means. Results from 1 of 2 similar experiments are shown.

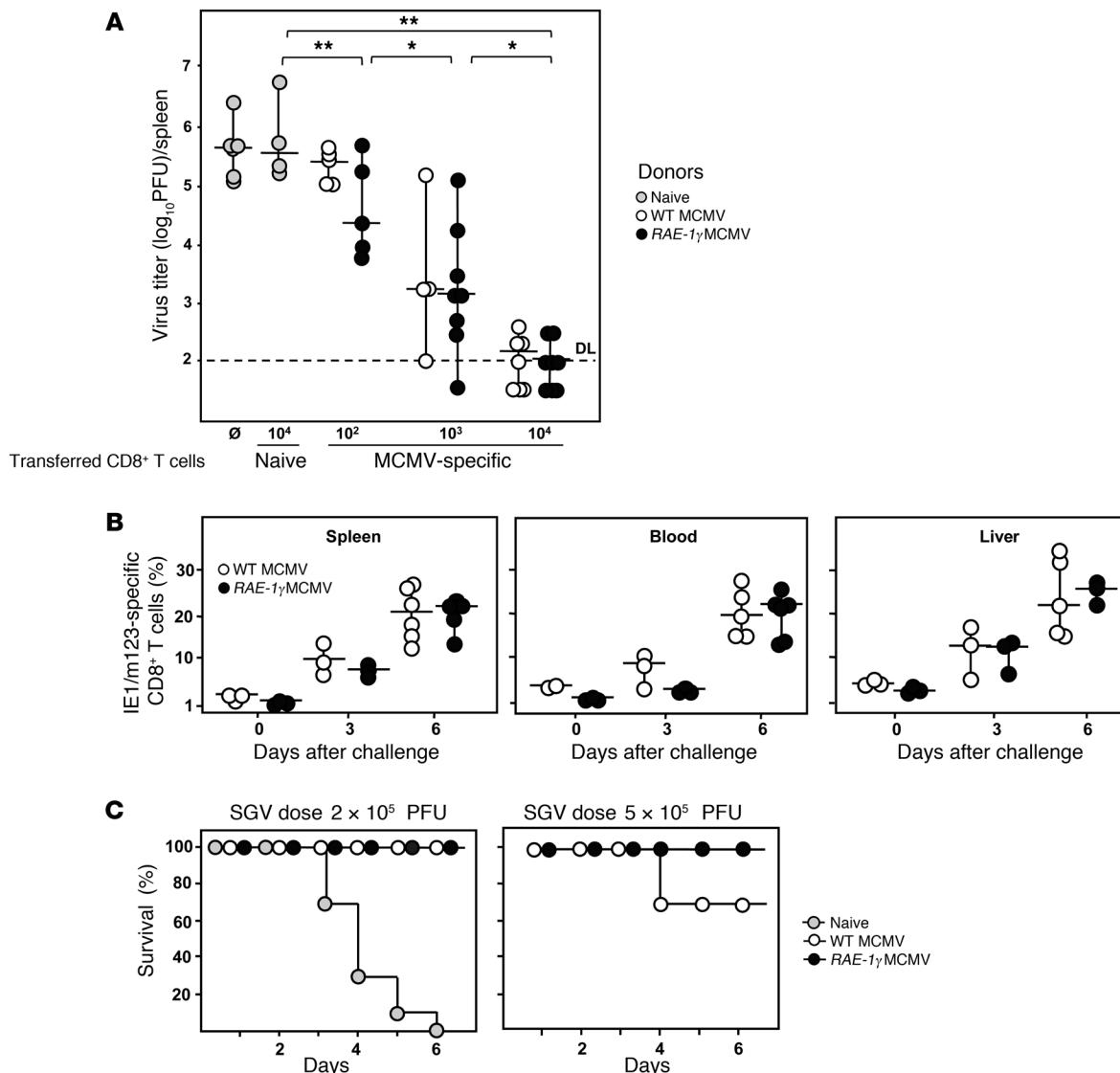
WT MCMV replication in salivary glands and lungs were at plateau levels (Figure 2A), and productive infection continued for several months (ref. 43 and data not shown). Similar to results obtained in adult mice, $\Delta m152$ MCMV replication was attenuated compared with WT MCMV but not to the level of *RAE-1 γ MCMV* attenuation. Furthermore about 3 weeks p.i., $\Delta m152$ MCMV still replicated to high titers in salivary glands (Supplemental Figure 3). Attenuated *RAE-1 γ MCMV* replication in neonates led to a lower load of viral DNA in various organs, while prolonged, high-level WT MCMV replication resulted in higher load of viral DNA in organs (Figure 2B). Collectively, the results indicate that *RAE-1 γ MCMV* infection in neonates is characterized by attenuated virus replication, shorter duration of the productive infection, and subsequent lower virus DNA load as compared with the WT MCMV.

Efficient priming and maintenance of adaptive immune response after *RAE-1 γ MCMV* infection. To test whether the *RAE-1 γ MCMV* attenuation impacts on the adaptive antiviral immune response, we injected adult BALB/c mice f.p. with 2×10^5 PFU of *RAE-1 γ MCMV*, WT MCMV, or $\Delta m152$ MCMV. The kinetics of the virus-specific T cell response was followed by use of MHC class I tetramers loaded with MCMV peptides (49). The CD8⁺ T cell response was dominated by IE1/m123-specific and m164-specific cells, while the response to the 4 other studied epitopes (m04, M83, M84, M45) was low or below the level of detection (Figure 3A and data not shown). Following infection with any one of the 3 viruses, the m164-specific CD8⁺ T cells displayed comparable stable memory kinetics. By contrast, immunoinflation of IE1/m123-specific T cells in spleen at 9 months p.i. was less prominent following *RAE-1 γ MCMV* and $\Delta m152$ MCMV than after WT MCMV infection (50). The kinetics of the antiviral CD8⁺ T cell response in the blood closely reflected that in spleen (data not shown). The phenotypic and functional properties of virus-specific CD8⁺ T cells were similar following *RAE-1 γ MCMV*, WT MCMV, and $\Delta m152$ MCMV infection (Figure 3B). Between 60% and 75% of IE1/m123-specific and m164-specific CD8⁺ T cells in spleen and blood retained effector memory pheno-

type up to 9 months after infection. It is important to note that the expression of NKG2D, a CD8⁺ T cell costimulatory receptor, was essentially identical following both *RAE-1 γ MCMV* and WT MCMV infection (Figure 3C). Also, the inhibitory receptors PD-1 and CTLA-4, described to be associated with T cell exhaustion during persistent infections (51), were not upregulated on memory CD8⁺ T cells, and the T cells remained fully functional throughout latent *RAE-1 γ MCMV* and WT MCMV infection (Figure 3, D and E). At each time point analyzed, the percentage of CD8⁺ T cells detected by tetramer staining was similar to the percentage of CD8⁺ T cells secreting IFN- γ upon stimulation with a viral antigenic peptide in vitro (Figure 3D), and most of the cells simultaneously secreted TNF- α , but not IL-2 (Figure 3E and data not shown), and extruded cytotoxic granules (externalized CD107a) (Figure 3E). Interestingly, in C57BL/6 mice the frequency of MCMV-specific CD8⁺ T cells at an early time point after *RAE-1 γ MCMV* infection was even greater compared with WT MCMV (Supplemental Figure 2B).

Similar priming capacity and the frequency of virus-specific CD8⁺ T cells after infection with *RAE-1 γ MCMV* or WT MCMV in spite of dramatic differences in the load of infectious virus in their tissues prompted us to test whether this can be explained by a differential effect of *RAE-1 γ MCMV* and WT MCMV on DCs in vivo. MCMV infection results in a reduction in conventional DCs (cDCs) in BALB/c mice that can be prevented by an efficient antiviral NK cell response in the C57BL/6 strain (16, 52). To test how the vaccine virus affects DCs in vivo, we compared DC subsets following *RAE-1 γ MCMV* and WT MCMV injection in BALB/c mice. While a marked reduction in cDCs occurred at early times after WT MCMV infection, both CD11b⁺ and CD8 α ⁺ subsets of cDCs were preserved following *RAE-1 γ MCMV* infection (Supplemental Figure 4). As reported by others (16), the frequency of cDCs in spleen of infected mice inversely correlated with type I IFN levels in sera of infected mice. At day 2 p.i., the average level of TNF- α in sera was significantly higher after WT MCMV ($5,212 \pm 1,266$ pg/ml) as compared with *RAE-1 γ MCMV* infection ($1,459 \pm 840$ pg/ml). Thus, an efficient early control of *RAE-1 γ MCMV* resulted in preservation of cDCs, possibly by preventing an overwhelming production of type I IFNs, providing optimal conditions for priming of MCMV-specific T cells.

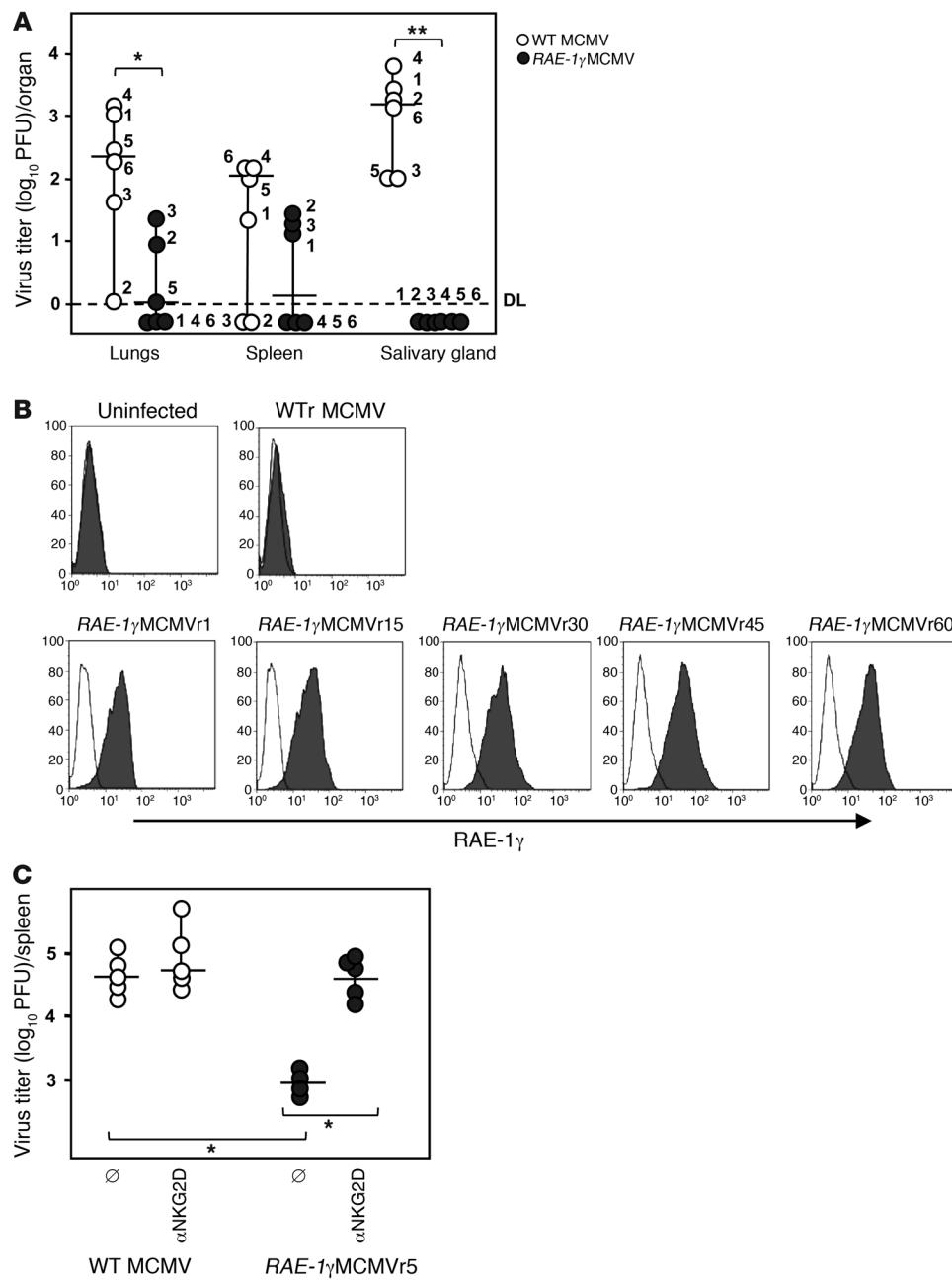
In vivo antiviral effector activity of MCMV-specific memory CD8⁺ T cells generated following *RAE-1 γ MCMV* and WT MCMV infection was compared by prophylactic adoptive transfer into immunodepleted MCMV-infected recipient mice. Adoptive transfer of only 10^3 MCMV-specific cells markedly limited virus multiplication, while 10^4 MCMV-specific cells nearly abolished virus replication in spleen. No differences in protective capacity of CD8⁺ T cells generated following *RAE-1 γ MCMV* and WT MCMV infection were observed (Figure 4A). Recall response of memory CD8⁺ T cells was tested 6 months after the primary infection (Figure 4B and data not shown). The IE1/m123-specific and m164-specific CD8⁺ T cells in spleen, blood, and tissue rapidly expanded upon challenge infection. Expansion peaked around day 6 after the challenge, resulting in T cell frequencies several orders of magnitude higher than before the challenge in both *RAE-1 γ MCMV*– and WT MCMV–infected mice. Thus, although the initial memory T cell pool was smaller in *RAE-1 γ MCMV*– than in WT MCMV–infected mice, the size of the resulting T cell pool after the challenge infection was similar in the two groups of mice. Collectively, these data indicate that despite tight innate immune control, *RAE-1 γ MCMV* infection elicited a strong, enduring antiviral immune response comparable to that following WT MCMV infection.

**Figure 4**

RAE-1 γ MCMV infection induces protective immunity. **(A)** Donors of memory CD8⁺ T cells were μ MT/ μ MT B cell-deficient mice either naive or latently infected with RAE-1 γ MCMV or WT MCMV (>6 months p.i.). Splenocytes from 3 donors per group were pooled, and the number of MCMV-specific CD8⁺ T cells was assessed by combined staining with IE1/m123, m164, M83, M84, and m04 MHC class I tetramers. 10^4 naive CD8⁺ T cells or graded numbers of MCMV-specific CD8⁺ T cells were i.v. transferred to recipient BALB/c mice immunocompromised by 6 Gy γ -irradiation. Recipients were f.p. injected with 10^5 PFU WT MCMV 6 hours after the cell transfer. Viral titers in spleen were determined 12 days p.i. by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) are shown. \emptyset , no transfer. **(B)** Mice infected as described in Figure 3 were i.p. challenged with 10^5 PFU of salivary gland-derived MCMV (SGV) 6 months p.i. Lymphocytes were isolated from blood, spleen, and liver at different time points after the challenge and stained with IE1/m123 MHC class I tetramer and anti-CD8 Ab. The percentage of IE1/m123-specific CD8⁺ T cells for individual mice (circles) and median values (horizontal bars) are shown. **(C)** Naive mice and mice infected as described in Figure 3 were i.p. challenged with 2×10^5 or 5×10^5 PFU of SGV 6 months p.i. Survival rates were monitored daily. Results from 1 of 2 similar experiments are shown. * P < 0.05, ** P < 0.01.

RAE-1 γ MCMV immunization protects mice from challenge infection. To test whether the immune response induced by the RAE-1 γ MCMV infection is sufficient to protect the host from challenge infection, we injected adult BALB/c mice f.p. with 2×10^5 PFU of RAE-1 γ MCMV or WT MCMV 6 months prior to lethal challenge with salivary gland-derived MCMV (SGV). SGV is more virulent than the cell culture-derived MCMV, and injection of only 10^5 PFU of SGV results in multiorgan damage and high mortality (53).

While naive mice failed to control the infection and succumbed to a dose of 2×10^5 SGV (2 LD₅₀), all of the mice immunized with RAE-1 γ MCMV, similar to the mice previously infected with WT MCMV, survived the challenge (Figure 4C). Notably, mice immunized with RAE-1 γ MCMV resisted challenge infection with 5 LD₅₀ of the SGV better than WT MCMV-infected mice, suggesting that expression of NKG2D ligand provides innate immune stimuli that enhance the effectiveness of the adaptive immune response.



Taken together, the results indicate that immunization with *RAE-1 γ MCMV* induced an immune response that conferred protection against lethal MCMV infection.

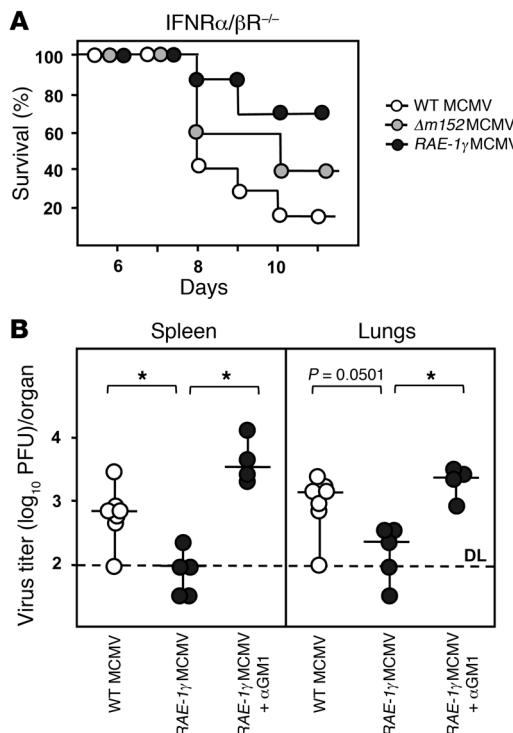
Strong attenuation in vivo does not prevent RAE-1 γ MCMV from establishing latent infection and reactivating upon immunosuppression. The burden of latent viral DNA in a tissue predetermines the risk of recurrent CMV infection (54). The barely detectable DNA load of *RAE-1 γ MCMV* during latent infection could limit viral reactivation and subsequent recurrent virus infection. However, kinetics and phenotype of MCMV-specific T cells observed during latent infection were indicative of repeated antigen exposure. Therefore, we investigated the potential of *RAE-1 γ MCMV* to reactivate from latency by combined depletion of NK cells and T cell subsets in latently infected B cell-deficient mice with a homozygous μ chain mutation

(μ MT/ μ MT mice). In this experimental system, the absence of Abs facilitates virus multiplication and dissemination after recurrence, which increases the sensitivity of virus detection (20). Following immunosuppression, recurrent infection occurred independently in different organs in 4 of 6 (66%) *RAE-1 γ MCMV*-infected mice and in all of the WT MCMV-infected mice (Figure 5A). In addition, while in WT MCMV-infected mice recurrent infection first occurred in salivary glands, which favors virus shedding, recurrence was not detected in salivary glands of any *RAE-1 γ MCMV*-infected mice. Thus, tight immune control of the *RAE-1 γ MCMV* during primary infection did not prevent viral recurrence after immunosuppressive treatment altogether but altered incidence and sites of recurrence.

RAE-1 γ remains intact during latent RAE-1 γ MCMV infection. Selective pressure from the immune system can result in the emergence of

Figure 5

RAE-1 γ remains intact during latent RAE-1 γ MCMV infection. (A) μ MT/ μ MT B cell-deficient mice latently infected with *RAE-1 γ MCMV* or WT MCMV were depleted of CD4 $+$, CD8 $+$, and NK cells by use of Abs. Viral titers were determined by plaque assay 13 days after immunodepletion. Titers of individual mice (circles) and median values (horizontal bars) are shown. Numbers indicate individual mice. (B) A total of 73 recurrent, plaque-purified viruses (termed *RAE-1 γ MCMVr1* to *RAE-1 γ MCMVr73*) and recurrent WT MCMV (WTr MCMV) were isolated from organ homogenates of B cell-deficient μ MT/ μ MT mice with recurrent *RAE-1 γ MCMV* infection. SVEC4-10 cells were infected with the indicated recurrent, plaque-purified *RAE-1 γ MCMV* viruses and analyzed for surface *RAE-1 γ* expression by FACS as described in Figure 1B. (C) Untreated BALB/c mice or BALB/c mice treated with blocking anti-NKG2D Ab were i.v. injected with 10⁵ PFU WT MCMV or recurrent, plaque-purified *RAE-1 γ MCMV* (clone *RAE-1 γ MCMVr5*). Viral titers were determined in spleen 3 days p.i. by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) from a representative of two independent experiments are shown. *P < 0.05, **P < 0.01.



virus mutants that escape from the immune control, even in herpes viruses with highly accurate mechanisms of genome replication (55, 56). To address whether a strong immune response can drive emergence of RAE-1 γ MCMV mutants that escape from NKG2D-mediated immunosurveillance, we prepared plaque-purified viruses from spleen and lung homogenates of B cell-deficient μ MT/ μ MT mice with recurrent RAE-1 γ MCMV infection (see above). A total of 73 plaque-purified isolates (termed RAE-1 γ MCMVr1 to RAE-1 γ MCMVr73) were tested for the expression of RAE-1 γ , and some of them were tested for sensitivity to the NKG2D-mediated immune control in vivo. Infection of SVEC4-10 cells with plaque-purified isolates resulted in cell surface expression of RAE-1 γ as detected by FACS analysis (Figure 5B), and infection of BALB/c mice with a RAE-1 γ MCMVr isolate (RAE-1 γ MCMVr5) resulted in NKG2D-dependent attenuation of virus replication similar to the attenuation of parental RAE-1 γ MCMV (Figure 5C). Finally, PCR amplification of RAE-1 γ was performed, and sequence analysis of PCR products did not reveal sequence variation in any of 30 RAE-1 γ MCMVr isolates tested (data not shown). These data indicate that despite strong selective pressure imposed by NKG2D-dependent immune control mechanisms, the RAE-1 γ transgene encoded by RAE-1 γ MCMV remained intact.

Control of RAE-1 γ MCMV in mice lacking the receptor for type I IFNs and after hematopoietic irradiation. Type I IFNs play an important role in limiting MCMV replication during the early stage of infection. Consequently, mice lacking the receptor for type I IFNs (IFN- α /βR $^{-/-}$ mice) are 1,000-fold more susceptible to MCMV infection than the parental mouse strain (57). To test whether RAE-1 γ MCMV is efficiently controlled even in the severely immunodeficient host, we injected IFN- α /βR $^{-/-}$ mice i.p. with RAE-1 γ MCMV, WT MCMV, or $\Delta m152$ MCMV. While most of the WT MCMV- and $\Delta m152$ MCMV-infected mice succumbed to the infection (85% and 60%, respectively), the mortality rate of the RAE-1 γ MCMV-infected animals was significantly lower (30%) (Figure 6A).

Figure 6

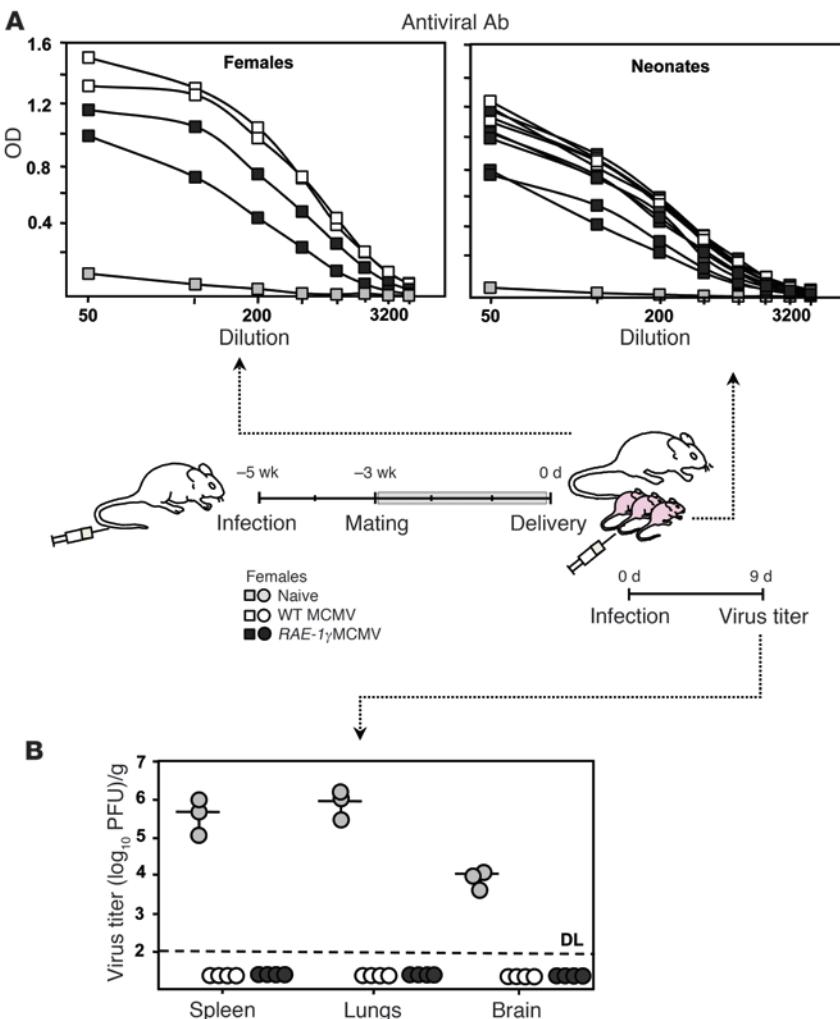
RAE-1 γ MCMV is attenuated in immunocompromised host. **(A)** IFN- α /βR $^{-/-}$ mice were i.p. injected with 2×10^5 PFU of RAE-1 γ MCMV, WT MCMV, or $\Delta m152$ MCMV, and survival rates were monitored daily. Combined results from 2 similar experiments are shown. **(B)** BALB/c mice were subjected to 6 Gy total-body γ -irradiation 6 hours prior to f.p. injection with 10^5 PFU of RAE-1 γ MCMV or WT MCMV. Some groups of mice were depleted of NK cells by anti-asialoGM1 (α GM1) Ab. Viral titers were determined 7 days p.i. by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) are shown. *P < 0.05.

NK cells are more resistant to irradiation than other lymphoid cells (58, 59), and RAE-1 γ MCMV is extremely sensitive to the NK cell control. We assessed whether residual NK cells, after hematopoietic treatment, are sufficient to control RAE-1 γ MCMV infection. BALB/c mice were hematopoietically ablated using a sublethal dose (6 Gy) of total body γ -irradiation 6 hours prior to f.p. injection with 10^5 PFU of RAE-1 γ MCMV or WT MCMV, and viral titers were compared on day 7 p.i. RAE-1 γ MCMV infection in hematopoietically ablated mice resulted in significantly lower viral titers as compared with WT MCMV infection, suggesting that residual NK cells are sufficient to restrain RAE-1 γ MCMV infection (Figure 6B). Together, these data indicate that infection with the RAE-1 γ MCMV presents a low risk for disease, even in severely immunodeficient hosts.

Maternal RAE-1 γ MCMV immunization protects neonatal mice from MCMV infection. Maternal preconception immunity to CMV provides substantial protection against congenital infection (60–62). The presence of maternal antiviral Abs is associated with a decreased incidence of intrauterine transmission and better neurological outcomes in the setting of congenital infection. The role of Abs in the prevention of congenital infection has also been emphasized in the guinea pig CMV model (63). Since the mouse hemoplacental barrier does not support MCMV transfer, we established a model of i.p. neonatal MCMV infection whose pathogenesis closely resembles congenital HCMV infection (64). To test whether the maternal Ab response induced by the RAE-1 γ MCMV immunization can protect neonatal mice from MCMV infection, we injected female BALB/c mice with RAE-1 γ MCMV or WT MCMV or mock infected them 2 weeks before mating. A number of neonates were sacrificed on the day of birth and tested for the presence of antiviral Abs in serum, while the others were i.p. injected with 500 PFU WT MCMV and tested for replicating virus in the tissue. No antiviral Abs were detected in the serum of neonates of naive females. By contrast, antiviral Abs were detected in serum of RAE-1 γ MCMV- and WT MCMV-immunized females and in serum of their neonates, confirming passive placental transfer of antiviral Abs (Figure 7A). Whereas MCMV infection in infected neonates of naive females resulted in disseminated virus replication, no replicating virus was detected in various tissues at day 9 after the infection in neonates of RAE-1 γ MCMV-immunized females or in neonates of WT MCMV-immunized females (Figure 7B). Thus, immunization with recombinant RAE-1 γ MCMV induced a maternal Ab response that, upon placental transfer, limited virus dissemination and protected neonatal mice from MCMV infection.

Discussion

Despite efforts, no effective HCMV vaccine is currently available. Several features of HCMV make vaccine development extremely difficult. First, a large number of viral immuno-evasion proteins subvert the host's immune responses at virtually every step. Sec-


Figure 7

Maternal immunization with *RAE-1 γ MCMV* protects offspring from MCMV disease. (A) Female BALB/c mice were i.v. injected with 2×10^5 PFU of *RAE-1 γ MCMV* or WT MCMV or mock injected 2 weeks before mating. Antiviral Ab titers in their serum and in serum of their neonates were determined by ELISA 6 hours postpartum. (B) A group of the neonates was i.p. injected with 500 PFU WT MCMV 6 hours postpartum, and viral titers were determined in various organs 9 days p.i. by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) are shown.

the design of rational mutants as CMV vaccine candidates (74, 75). The vaccination potential of CMV mutants lacking nonessential viral genes has already been proven (33, 34). Also, spread-deficient MCMV lacking the essential gene M94 induced a virus-specific immune response and proved to be safe in an immunodeficient host (31). The approach of the present study was to generate an experimental vaccine expressing NKG2D ligand, which is therefore attenuated due to strong immune control and, at the same time, resistant to viral immuno-evasion of this signaling pathway. The recombinant virus expressing the NKG2D ligand *RAE-1 γ* was severely attenuated in vivo in an NKG2D-dependent manner, not only in the immunocompetent host but also in immunologically immature neonates and mice immunodepleted by sublethal γ -irradiation or in mice lacking the receptor for type I IFNs.

The definition of herpes virus latency (76) implies that the viral genome is maintained and can reactivate to productive infection.

Second, immunity from naturally acquired infection is not completely protective against superinfection or CMV transmission from mother to fetus (60, 62, 65). Third, persistence of virus in the state of latency with the possibility of reactivation and disease in immunocompromised patients represents a safety concern when a replicating CMV is considered for use as vaccine candidate. Still, a live, attenuated vaccine approach has several characteristics that render it attractive. Unlike subunit vaccines, which induce cellular or humoral immune response to selected antigens only, live vaccines induce a much broader immunity that may mimic protection acquired following natural infection (31, 33, 66–69). Cellular immunity against CMV follows unique kinetics characterized by maintenance or even expansion of the virus-specific CD8 $^+$ T cell response over time (70, 71). In addition, recombinant CMVs that expressed heterologous simian immunodeficiency virus, lymphocytic choriomeningitis virus, and influenza virus peptides have been shown to induce protective immunity against the respective viruses (72, 73). Therefore, live, attenuated CMVs are attractive candidates for a CMV vaccine or a CMV-based vaccine vector provided that their pathogenicity is significantly attenuated but their immunogenicity is unaffected.

A better understanding of viral immunobiology and the introduction of BAC technology have made the CMV genome accessible to

The data from the MCMV model have shown that the conditions of the primary infection predetermine the risk of reactivation by modulating the latent viral load (43). Despite accelerated viral clearance during primary infection and barely detectable viral DNA load, *RAE-1 γ MCMV* was able to reactivate upon immunodepletion. Still, the incidence of recurrence and viral titers were lower in *RAE-1 γ MCMV*- than in WT MCMV-infected mice, most probably as a consequence of a lower load of latent viral genome. Under selective pressure imposed by the immune control mechanism, apathogenic vaccine strains may become pathogenic due to the emergence of escape mutants no longer sensitive to immunosurveillance (56, 77). However, sequencing of plaque-purified isolates from mice with recurrent *RAE-1 γ MCMV* infection did not reveal any *RAE-1 γ* mutations, and the susceptibility of the isolates to the NKG2D-mediated immune control was comparable to that of the parental *RAE-1 γ MCMV*. Efficient NK cell and T cell response may have suppressed virus replication to the extent that emergence of virus escape mutants was restricted. We speculate that such a low level of *RAE-1 γ MCMV* genome is sufficient to elicit nonproductive reactivations and boost cells of the immune system. Still, due to low-level recurrence and intact *RAE-1 γ* transgene, the virus would be efficiently controlled even in the severely immunocompromised host provided that some residual NK cell response remains.



The salivary glands are the privileged organ for CMV replication in that productive infection continues long after innate and adaptive immune responses have cleared MCMV from other organs (43, 44). *RAE-1 γ MCMV* infection of mice led to the emergence of new, extremely favorable biological characteristics, including the lack of detectable infectious virus in salivary glands during primary or recurrent infection in BALB/c mice. We propose that attenuated *RAE-1 γ MCMV* multiplication and dissemination during primary infection restricted salivary gland colonization, prevented productive infection, and prohibited *RAE-1 γ MCMV* reactivation in salivary gland upon immunosuppression. It is thus likely that salivary shedding of *RAE-1 γ MCMV* is absent and horizontal transmission via saliva could be expected to be eliminated or significantly reduced.

It is generally accepted that the innate immune system has a key role in determining the strength and quality of the adaptive immune response. However, whether a strong innate immune response is beneficial or, alternatively, detrimental for priming and maintenance of efficient adaptive immunity is a matter of debate. Robbins et al. reported that an efficient NK cell response promotes adaptive immunity, in part by preventing production of high, immunosuppressive levels of IFN- α / β and other innate cytokines (16). However, if viral replication is too attenuated, the low antigen supply may limit priming and maintenance of efficient immune response (78). Andrews et al. recently reported that Ly49H $^+$ NK cells negatively regulate T cell response following MCMV infection by limiting exposure of T cells to infected APCs (52). According to these authors, an efficient NK cell response negatively impacts the ability of specific immune responses to limit persistent viral replication. However, our results do not support the notion that strong NK cell response in general would compromise specific immune response against herpesviruses. We report that efficient early control of virus guided by NKG2D-dependent mechanisms does not affect generation, strength, and longevity of specific, protective immune response. Although it is currently difficult to explain the differences between the above-mentioned and our findings, it should be pointed out that unlike Ly49H, NKG2D receptor also serves as a costimulatory molecule on CD8 $^+$ T cells, which may additionally influence the quality of the CD8 $^+$ T cell response toward *RAE-1 γ MCMV*. Although NK cell activation via Ly49H or NKG2D can overcome the viral evasion of NK cells, it is worth mentioning that virus expressing *RAE-1 γ* is attenuated even in C57BL/6 mice, suggesting that effector functions mediated through these two receptors do not completely overlap. Furthermore, unlike the MCMV gene *m157*, the *RAE-1 γ* expressed in the context of virus genome was not subject to escape by mutation or deletion due to the strong selective pressure. Permanent and strong susceptibility of *RAE-1 γ MCMV* to the immune control may explain its inability to reach salivary glands of immunocompetent adult mice. Alternatively, the NKG2D-dependent immune control in salivary gland may be more efficient as compared with other tissues.

Virus-specific CD8 $^+$ T cells generated by *RAE-1 γ MCMV* infection have predominantly an effector memory phenotype and provide long-term protective immunity and thus were similar to those generated following WT MCMV infection. One reasonable explanation for the unique pattern of the CD8 $^+$ T cell phenotype and kinetics in MCMV-infected mice is repetitive exposure to antigen during low-level transcription of viral genes during latency leading to endogenous boosting. The frequency of abortive MCMV reactivation during latency depends on the amount of latent viral genome (54), which may account for the restricted immunoinfla-

tion of MCMV-specific CD8 $^+$ T cells after low-dose (2×10^5 PFU) intraplantar infection and the stable memory kinetics of IE1/m123- and m164-specific CD8 $^+$ T cells following *RAE-1 γ MCMV* infection. Despite of the predominance of the effector memory phenotype indicative of repeated antigen encounter, virus-specific CD8 $^+$ T cells did not show upregulation of inhibitory receptors such as PD-1 or CTLA-4, nor any signs of functional exhaustion (51). In addition, expression of *RAE-1 γ* by MCMV did not affect NKG2D expression by CD8 $^+$ T cells, which remained similar during *RAE-1 γ MCMV* and WT MCMV infection.

The *RAE-1 γ MCMV* was constructed by insertion of the *RAE-1 γ* ORF into the $\Delta m152$ MCMV genome. The product of the *m152* gene, in addition to downregulating *RAE-1 γ* , targets MHC class I presentation and impairs CD8 $^+$ T cells recognition of infected cells (41, 79, 80). However, previous work has shown that the presence or absence of MHC I immune evasion proteins has little impact on the size and specificity of the antiviral CD8 $^+$ T cell response over the course of infection (81). The latter finding is explained by the fact that the majority of MCMV-specific CD8 $^+$ T cells are primed not by infected professional APCs directly, but rather by antigen cross-presentation (69).

An important goal of CMV vaccine development is to develop a candidate vaccine that provides protection from disease associated with intrauterine CMV infection (10). Although mice have not been utilized as a model for congenital infection because of the restrictions of fetal infection secondary to the anatomy of the mouse placenta, newborn animals have been used to model the CNS disease and sequelae associated with congenital CMV infection (64). Importantly, the newborn mouse is developmentally similar to the late-second-trimester human fetus, and infection delivered by an i.p. inoculation can lead to CNS infection and disease whose pathogenesis closely resembles that which is thought to occur in the infected human fetus. Furthermore, passively acquired antiviral Abs are thought to modulate infection and disease in infected newborn mice in a fashion similar to transplacentally acquired maternal Abs in human fetuses infected in utero (24). Infected neonates born to *RAE-1 γ MCMV*-vaccinated mothers had diminished viral titers in all organs and no virus-induced organ damage, including CNS sequelae. Thus, our data provide evidence of protective immunity against neonatal infection and disease induced by maternal *RAE-1 γ MCMV* vaccination that is comparable to that induced by maternal WT MCMV infection. Together, these findings demonstrate the efficacy of an attenuated MCMV vaccine expressing the NKG2D ligand to generate a robust and persistent protective immune response integrating all components of the immune system.

Multiple approaches have been proposed for attenuation of CMVs; however, since the biological target of attenuation, i.e., prevention of infection, disease, or transmission, is experimentally ill-defined, optimal attenuation of this virus remains speculative. We hypothesize that optimal attenuation should result in a virus that can replicate sufficiently to induce adaptive immunity, thereby establishing immunological memory and an attenuated level of persistence at the same time. Here we demonstrated that CMV engineered to express ligand for NK cell-activating receptor NKG2D led to not only strong attenuation in immunocompetent host but also additional results beneficial for attenuated vaccine candidates, including (a) optimal priming and enduring protective T and B cell responses; (b) attenuated phenotype even in immunologically immature or immunodeficient hosts; (c) preservation of

transgene in spite of the strong selective pressure imposed by NK cells; (d) minimal risk of recurrence, and (e) altered virus distribution, as illustrated by the failure of such virus to colonize salivary gland. The latter property would provide a more favorable safety profile for a replicating vaccine by limiting the transmission within the population and possibly to offspring. Moreover, upon maternal vaccination with MCMV expressing NKG2D ligand, transplacental transfer of antiviral Abs protected neonatal mice from CMV disease. With respect to the use of live, attenuated viruses such as CMVs, one should also point out the large potential of these viruses to serve as live vaccine vectors (73). We believe that approaches such as the one described in this article will be feasible in the near future.

Methods

Cells and viruses. Mouse embryonic fibroblasts (MEFs) and SVEC4-10 (ATCC CRL-2181), NIH 3T3 (ATCC CRL-1658), and B12 fibroblasts (82) were grown as described previously (83). A BAC-derived MCMV, MW97.01, has previously been shown to be biologically equivalent to the MCMV Smith strain (VR-194 [reaccessioned as VR-1399]; ATCC) and is here referred as WT MCMV. The recombinant strain $\Delta m152$ MCMV was generated as described previously (84, 85). Viruses were propagated on MEFs and concentrated by sucrose gradient ultracentrifugation (83). The SGV MCMV was used as a third passage and prepared as described previously (83).

Construction of recombinant plasmids and recombinant viruses. To generate the RAE-1 γ MCMV mutant, an ORF encoding FLAG-tagged RAE-1 γ was first cloned into pGL3 (Invitrogen) together with a kanamycin resistance gene (kanR), which was inserted further downstream. Then, the RAE-1 γ expression cassette plus kanR were PCR amplified using the primers 5'-GCACCCGAC-GATCTGACATTGTCCAGTGTGCCGGTCGCAACATCCCTAGT-TATTAATAGTAATC-3' and 5'-TGTACCCGCTCCACGTTCACCGTCG-GTCTCCCGATCGCTAGCCTGTACACAGGAACACTTAACGGCTGA-3', which contained 50 nucleotides at their 5' ends homologous to the intended integration site in the BAC-cloned MCMV genome. The PCR fragment was integrated into the BAC by red α , - β , - γ -mediated recombination as described previously (86), thereby replacing the m152 ORF. The kanR cassette was subsequently excised with FLP recombinase (87, 88). The resulting MCMV BAC was characterized by restriction analysis, and the mutant was reconstituted by transfection of the BAC DNA into MEFs.

Animals, infection, and lymphocyte subset depletion. BALB/c (H-2 d), C57BL/6 (H-2 b), IFN type I receptor $^{-/-}$ mice on a 129 background (IFN- α /IFN- β R $^{-/-}$), and BALB/c (H-2 d) μ MT/ μ MT mice (89) were bred under specific pathogen-free conditions at the Central Animal Facility of the Faculty of Medicine, University of Rijeka. Animal handling, experimental procedures, and administration of anesthesia were performed in accordance with the guidelines contained in the Council for International Organizations of Medical Sciences *International Guiding Principles for Biomedical Research Involving Animals*. The Ethics Committee of the University of Rijeka approved all animal experiments described within this report. Unless otherwise indicated, mice were injected f.p. with 2×10^5 PFU of tissue culture-derived MCMV at the age of 6–8 weeks. Neonatal mice were i.p. injected with 500 PFU MCMV 6 hours postpartum. In vivo blocking of NKG2D and depletion of CD4 $^+$ T cells, CD8 $^+$ T cells, and NK cells were performed by i.p. injection of mAb (rat anti-mouse) to NKG2D (R&D Systems), CD4 (YTS 191.1), CD8 (YTS 169.4), and anti-asialoGM1 serum (Wako Chemicals), respectively.

Viral titers and real-time PCR. Viral titers were determined using a standard plaque assay (90). The detection limit of the assay was extended to 1 PFU per organ homogenate as described previously (19). Genomic DNA was extracted from mouse tissues (10 mg) or blood (300 μ l) using Wizard Genomic DNA Purification Kit (Promega), according to the instruc-

tion manual, and dissolved in 100 μ l of DNA Rehydration Solution. Viral genome was quantified by real-time PCR using the LightCycler system (Roche) and LightCycler Fast Start DNA MasterPlus SYBR Green I and analyzed by LightCycler data analysis software version 3.3.40. Primers *ie4fwd* (5'-TGACTTAACTCCCCAGGCAA-3') and *ie4rev* (5'-TAGGTGAG-GCCATAGTGGCAG-3'), nucleotide positions 6692–6672 and 6592–6612, respectively (GenBank L06816), were chosen to amplify a segment of exon 4 of the *ie1* gene. A cellular gene was detected with primers *gla1fwd* (5'-TGCCTGTTCTTGAGTCTGT-3') and *gla1rev* (5'-AGTCGAGT-GAAGGGTAACGAGC-3'), nucleotide positions 312–332 and 403–382, respectively (GenBank X75832). Specificity of PCR products was determined by melting curve analysis. Serial dilutions of pGEM-T Easy Vector (Promega) expressing partial MCMV *ie1* gene and of DNA extracted from MEFs were used as standards to determine the MCMV genome copy numbers and the number of cells, respectively. Tissue DNA samples from uninfected mice and multiple samples without template served as negative controls. The PCR amplification efficiencies (E) for the *ie1* and *gla1* standard curves as well as for both genes in a titration of sample DNA were calculated according to the formula $E = 10^{-1/\text{slope}}$ (technical note no. LC11/2000; Roche) and differed by $\Delta E < 0.05$ in the reported experiments. Likewise, for each of the two genes tested, amplification efficacies differed by $\Delta E < 0.05$ between a titration of sample DNA and the respective standard curve. To determine sensitivity of quantitative PCR (qPCR) detection of MCMV, genomic DNA samples were spiked with serial dilutions of target plasmid pGEM-T Easy Vector containing *ie1* genomic sequence as template in the PCR as previously described (91). The detection limit was found to be 6 copies of MCMV per 10^6 cells.

Adoptive transfer of MCMV-specific CD8 $^+$ T cells. Adoptive transfer experiments were performed as described previously (92). In short, donors of CD8 $^+$ T cells were naive or latently infected μ MT/ μ MT (B cell-deficient) mice, MCMV infected 6 months before the adoptive transfer. Splenocytes from 3 donors per group were pooled, and the number of MCMV-specific CD8 $^+$ T cells was assessed by combined staining with IE1/m123, m164, M83, M84, and m04 MHC class I tetramers. Unfractionated splenocytes containing 10^5 naive CD8 $^+$ T cells or graded numbers of MCMV-specific CD8 $^+$ T cells were i.v. transferred to recipient BALB/c mice immunocompromised with a single dose of 6 Gy γ -irradiation 12 hours prior to adoptive transfer. Recipients were f.p. injected with 10^5 PFU WT MCMV 6 hours after the adoptive transfer. Viral titers in the spleen were determined 12 days p.i. by plaque assay.

Flow cytometry and intracellular staining. Custom MCMV-specific H-2 d and H-2 b class I-restricted antigenic peptide synthesis to a purity of greater than 80% was performed by Jerni Peptide Technologies. Tetramers were synthesized by the NIH Tetramer Core Facility. Various fluorescently conjugated Abs were used: CD8 α (clone 53-6.7), CD27 (LG.7F9), CD62L (MEL-14), CD122 (5H4), CD127 (A7R34), KLRG-1 (2F1), NKG2A/C/E (20D5), NKG2D (M1-6), CTLA-4 (UC10-4B9), PD-1 (J43), IL-2 (JES6-SH4), IFN- γ (XMG1.2), TNF- α (MP6-XT22), CD3 β (H57-597), CD11c (N418), NKp46 (29A1.4), PDCA-1 (eBio927), RAE-1 γ (CX1), RAE-1 $\alpha\beta\delta\epsilon$ (clone 199205), MULT-1 (clone 237104), H60 (clone 205326). An in vitro assay to detect cytokine production and degranulation was performed as previously described (33). In short, splenocytes were resuspended in complete RPMI 1640 supplemented with 10% FCS and stimulated with 1 μ g of peptides IE1/m123 (168 YPH-FMPTNL 176), m164 (257 AGPPRYSRI 265), IE3 (416 RALEYKNL 423), m139 (419 TVYGFCLL 426), M45 (985 HGIRNASFI 993), or M38 (316 SSPPMFRV 323) for 6 hours at 37°C, with brefeldin A (eBioscience) added for the last 4 hours of stimulation. For degranulation assays, CD107a mAb (D4B) and monensin (eBioscience) were added to the cultured cells during peptide stimulation. For DC population analysis, splenocytes were digested by collagenase D (Roche) as described previously (16). All samples were acquired by FACSaria (BD) and analyzed with FlowJo software (Tree Star).



Sequence analysis of RAE-1 γ . Organ homogenates from μ MT/ μ MT B cell-deficient mice with recurrent infection were serially diluted 2-fold across 96-well trays and added to MEF cultures in 96-well trays. Wells showing a viral cytopathic effect derived from a single plaque were harvested for preparation of virus stocks. The RAE-1 γ ORF was PCR amplified by using purified viral DNA. MEFs were infected with the recovered viruses, and whole genomic DNA was extracted using a DNeasy blood and tissue kit (QIAGEN). The region of interest was amplified by PCR with primers m152fw GTGTATGTGGCCCGACGGCGG and m152rv CGCGGGCTACTCCGAAAGAGTAACATC. The amplicate was sequenced (3130 genetic analyzer, Applied Bioscience) using the primers m152fw, GTGTATGTGGCCCGACGGCGG, m152rv CGCGGGCTACTCCGAAAGAGTAACATC, RAEfw ATGGCCAAGGCAGCTGAC, and RAErv TGCTCGACCTGAGGTAAATTATAACCC. Sequences were aligned to the RAE-1 γ ORF of the input virus using Vector NTI 11 (Invitrogen).

Quantification of MCMV-specific Ab and serum IFN- α levels by ELISA. Serum MCMV-specific IgG titers were determined by ELISA as previously described (93). Serum levels of IFN- α were determined by ELISA Kit for IFN- α (PBL Biomedical Laboratories) according to the manufacturer's instructions.

Statistics. Statistical significance was calculated by unpaired 2-tailed Student's *t* test using Prism 4 software (GraphPad Software). Statistical

analyses of the virus titers were done by Mann-Whitney *U* test. *P* values less than 0.05 were considered significant.

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