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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-1γ (RAE-1γMCMV). Expression of RAE-1γ by MCMV resulted in profound virus attenuation in vivo and lower latent viral DNA loads. RAE-1γMCMV 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-1γMCMV infection elicited strong and long-lasting protective immunity. Maternal RAE-1γMCMV immunization protected neonatal mice from MCMV disease via placental transfer of antiviral Abs. Despite strong selective pressure, the RAE-1γ 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 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-1γ (RAE-1γMCMV). Expression of RAE-1γ by MCMV resulted in profound virus attenuation in vivo and lower latent viral DNA loads. RAE-1γMCMV 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-1γMCMV infection elicited strong and long-lasting protective immunity. Maternal RAE-1γMCMV immunization protected neonatal mice from MCMV disease via placental transfer of antiviral Abs. Despite strong selective pressure, the RAE-1γ 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 immunocompromised 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...
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-1 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-1γMCMV) 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-1γMCMV infection elicited a potent, long-lasting cellular and Ab immune response able to protect animals against challenge infection. Moreover, maternal RAE-1γMCMV 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-1γMCMV, that expresses RAE-1γ. RAE-1γMCMV 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-1γMCMV 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-1γMCMV 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-1γMCMV is strongly attenuated in vivo and fails to establish persistent infection in salivary gland. Adult BALB/c mice were injected with RAE-1γMCMV, 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-1γMCMV 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-1γMCMV, 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-1γMCMV 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-1γMCMV 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-1γMCMV–infected mice by day 45. At that time, RAE-1γMCMV 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-1γMCMV 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-1γ 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-1γ by staining with anti–RAE-1γ 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 i.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.
NK cell response, we injected C57BL/6 with RAE-1γMCMV, WT MCMV, or Δm152 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-1γMCMV reached significantly lower titers compared with WT MCMV and Δm152 MCMV. Thus, unlike in mice infected with MCMV mutant lacking m152 only, NKG2D-mediated control of RAE-1γMCMV was not overcome by NK cell activation via Ly49H (Supplemental Figure 2A). Taken together, the results indicate that expression of RAE-1γ by MCMV resulted in a dramatic attenuation of virus replication in different organs and a lower latent viral DNA load.

RAE-1γMCMV 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-1γMCMV replication in neonatal mice, we injected newborn animals i.p. with 500 PFU of RAE-1γMCMV or WT MCMV. During the first 5 days of infection, the two viruses replicated to comparable titers, but starting from day 7 RAE-1γMCMV replication was significantly reduced in all tested organs (Figure 2A). Productive RAE-1γMCMV 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 2

RAE-1γMCMV is attenuated in neonatal mice. (A) Neonatal BALB/c mice were i.p. injected with 500 PFU of RAE-1γMCMV 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.
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, Δm152 MCMV replication was attenuated compared with WT MCMV but not to the level of RAE-1γMCMV attenuation. Furthermore about 3 weeks p.i., Δm152 MCMV still replicated to high titers in salivary glands (Supplemental Figure 3). Attenuated RAE-1γMCMV 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-1γMCMV 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-1γMCMV infection. To test whether the RAE-1γMCMV attenuation impacts on the adaptive antiviral immune response, we injected adult BALB/c mice f.p. with 2 × 10⁶ PFU of RAE-1γMCMV, WT MCMV, or Δm152 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, immunoflation of IE1/m123-specific T cells in spleen at 9 months p.i. was less prominent following RAE-1γMCMV and Δm152 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-1γMCMV, WT MCMV, and Δm152 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 phenotype 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-1γMCMV 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-1γMCMV 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 CD107α) (Figure 3E). Interestingly, in C57BL/6 mice the frequency of MCMV-specific CD8⁺ T cells at an early time point after RAE-1γMCMV 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-1γMCMV 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-1γMCMV 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-1γMCMV and WT MCMV infection in BALB/c mice. While a marked reduction in cDCs occurred at early times after WT MCMV infection, both CD11b⁺ and CD80⁺ subsets of cDCs were preserved following RAE-1γMCMV 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 IFN-α in sera was significantly higher after WT MCMV (5,212 ± 1,266 pg/ml) as compared with RAE-1γMCMV infection (1,459 ± 840 pg/ml). Thus, an efficient early control of RAE-1γMCMV 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-1γMCMV and WT MCMV infection was compared by prophylactic adoptive transfer into immunodepleted MCMV-infected recipient mice. Adoptive transfer of only 10⁴ MCMV-specific cells markedly limited virus multiplication, while 10⁵ MCMV-specific cells nearly abolished virus replication in spleen. No differences in protective capacity of CD8⁺ T cells generated following RAE-1γMCMV 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-1γMCMV- and WT MCMV–infected mice. Thus, although the initial memory T cell pool was smaller in RAE-1γMCMV– 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-1γMCMV infection elicited a strong, enduring antiviral immune response comparable to that following WT MCMV infection.
RAE-1γMCMV immunization protects mice from challenge infection. To test whether the immune response induced by the RAE-1γMCMV infection is sufficient to protect the host from challenge infection, we injected adult BALB/c mice i.p. with 2 × 10^5 PFU of RAE-1γMCMV 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).

**Figure 4**
RAE-1γMCMV infection induces protective immunity. (A) Donors of memory CD8^+ T cells were μMT/μMT B cell–deficient mice either naive or latently infected with RAE-1γMCMV 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. Ø, 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 experiment are shown. *P < 0.05, **P < 0.01.
Taken together, the results indicate that immunization with RAE-1γMCMV induced an immune response that conferred protection against lethal MCMV infection. Strong attenuation in vivo does not prevent RAE-1γMCMV 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-1γMCMV 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-1γMCMV 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 (μMT/μMT 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-1γMCMV–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-1γMCMV–infected mice. Thus, tight immune control of the RAE-1γMCMV during primary infection did not prevent viral recurrence after immunosuppressive treatment altogether but altered incidence and sites of recurrence.

RAE-1γ remains intact during latent RAE-1γMCMV infection. (A) μMT/μMT B cell–deficient mice latently infected with RAE-1γMCMV 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-1γMCMVr1 to RAE-1γMCMVr73) and recurrent WT MCMV (WTr MCMV) were isolated from organ homogenates of B cell–deficient μMT/μMT mice with recurrent RAE-1γMCMV infection. SVEC4-10 cells were infected with the indicated recurrent, plaque-purified RAE-1γMCMV viruses and analyzed for surface RAE-1γ 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 10^5 PFU WT MCMV or recurrent, plaque-purified RAE-1γMCMV (clone RAE-1γMCMVr5). 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.
viruses 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-1γMCMV mutants that escape from NKG2D-mediated immunosurveillance, we prepared plaque-purified viruses from spleen and lung homogenates of B cell–deficient μMT/μMT mice with recurrent RAE-1γMCMV infection (see above). A total of 73 plaque-purified isolates (termed RAE-1γMCMVr1 to RAE-1γMCMVr73) were tested for the expression of RAE-1γ, 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-1γ as detected by FACS analysis (Figure 5B), and infection of BALB/c mice with a RAE-1γMCMVr isolate (RAE-1γMCMVr5) resulted in NKG2D-dependent attenuation of virus replication similar to the attenuation of parental RAE-1γMCMV (Figure 5C). Finally, PCR amplification of RAE-1γ was performed, and sequence analysis of PCR products did not reveal sequence variation in any of 30 RAE-1γMCMVr isolates tested (data not shown). These data indicate that despite strong selective pressure imposed by NKG2D-dependent immune control mechanisms, the RAE-1γ transgene encoded by RAE-1γMCMV remained intact.

Control of RAE-1γMCMV 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-1γMCMV is efficiently controlled even in the severely immunodeficient host, we injected IFN-α/βR−/− mice i.p. with RAE-1γMCMV, WT MCMV, or Δm152 MCMV. While most of the WT MCMV– and Δm152 MCMV–infected mice succumbed to the infection (85% and 60%, respectively), the mortality rate of the RAE-1γMCMV–infected animals was significantly lower (30%) (Figure 6A).

Figure 6
RAE-1γMCMV is attenuated in immunocompromised host. (A) IFN-α/βR−/− mice were i.p. injected with 2 × 106 PFU of RAE-1γMCMV, WT MCMV, or Δm152 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 irradiation 6 hours prior to f.p. injection with 106 PFU of RAE-1γMCMV or WT MCMV. Some groups of mice were depleted of NK cells by anti-asialoGM1 (αGM1)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-1γMCMV is extremely sensitive to the NK cell control. We assessed whether residual NK cells, after hematopoietic treatment, are sufficient to control RAE-1γMCMV infection. BALB/c mice were hematoablated using a sublethal dose (6 Gy) of total body γ-irradiation 6 hours prior to f.p. injection with 106 PFU of RAE-1γMCMV or WT MCMV, and viral titers were compared on day 7 p.i. RAE-1γMCMV infection in hematoablated mice resulted in significantly lower viral titers as compared with WT MCMV infection, suggesting that residual NK cells are sufficient to restrain RAE-1γMCMV infection (Figure 6B). Together, these data indicate that infection with the RAE-1γMCMV presents a low risk for disease, even in severely immunodeficient hosts.

Maternal RAE-1γMCMV 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 neurodevelopmental 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-1γMCMV immunization can protect neonatal mice from MCMV infection, we injected female BALB/c mice with RAE-1γMCMV 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-1γMCMV– 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-1γMCMV–immunized females or in neonates of WT MCMV–immunized females (Figure 7B). Thus, immunization with recombinant RAE-1γMCMV 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 immunoevasion proteins subvert the host’s immune responses at virtually every step. Sec-
ond, 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 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 immunoevasion of this signaling pathway. The recombinant virus expressing the NKG2D ligand RAE-1γ 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. 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-1γMCMV was able to reactivate upon immunodepletion. Still, the incidence of recurrence and viral titers were lower in RAE-1γMCMV– 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-1γMCMV infection did not reveal any RAE-1γ mutations, and the susceptibility of the isolates to the NKG2D-mediated immune control was comparable to that of the parental RAE-1γMCMV. 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-1γMCMV genome is sufficient to elicit nonproductive reactivations and boost cells of the immune system. Still, due to low-level recurrence and intact RAE-1γ 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). RAЕ-1γMCMV 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 RAЕ-1γMCMV multiplication and dissemination during primary infection restricted salivary gland colonization, prevented productive infection, and prohibited RAЕ-1γMCMV reactivation in salivary gland upon immunosuppression. It is thus likely that salivary shedding of RAЕ-1γMCMV 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-γ 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 RAЕ-1γMCMV. Although NK cell activation via Ly49H or NKG2D can overcome the viral evasion of NK cells, it is worth mentioning that virus expressing RAЕ-1γ 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 RAЕ-1γ 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 RAЕ-1γMCMV 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 RAЕ-1γMCMV 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-
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 to as WT MCMV. The recombinant strain Δm152 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-1YMCMV mutant, an ORF encoding FLAG-tagged RAE-1γ was first cloned into pGL3 (Invitrogen) together with a kanamycin resistance gene (kanR), which was inserted further downstream. Then, the RAE-1γ expression cassette plus kanR were PCR amplified using the primers 5′-GCACCGGAC-GATCTGAGATGTTGCAGGCGCAGGAACATCCCTAGTTATTAATGTTAATCT-3′ and 5′-TGTACCCGCCGTCACGTTCGTTGGAAGAACCTTACAGCCTGTAAC-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-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-2o), C57BL/6 (H-2b), IFN type I receptor−/− mice on a 129 background (IFN-α/β−/−), and BALB/c (H-2o) μMT/μMT 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 i.p. with 0.2 × 108 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 μl) using Wizard Genomic DNA Purification Kit (Promega), according to the instruc-
Sequence analysis of RAE-1. Organ homogenates from μMT/μMT 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 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 GTTGATGTGGCAGCCCCGGC and m152rv CCGGGGCGTACTCCCCAGGAAATGACATC. The amplified was sequenced (3130 genetic analyzer, Applied Bioscience) using the primers m152fw GTTGATGTGGCAGCCCCGGC, m152rv CCGGGGCGTACTCCCCAGGAAATGACATC, RAfw ATGGCCCAAGGCGCAGGTACG, and RAeq TGTCGCACCGTAGAATTATTACCC. Sequences were aligned to the RAE-1 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.

Statistical. Statistical significance was calculated by unpaired 2-tailed Student’s t test using Prism 4 software (GraphPad Software). Statistical analysis of the virus titers were done by Mann-Whitney U test. P values less than 0.05 were considered significant.

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