Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Virology. Author manuscript; available in PMC 2015 June 26.

Published in final edited form as:

Virology. 2009 September 30; 392(2): 153–161. doi:10.1016/j.virol.2009.05.035.

Investigation of the impact of the common animal facility contaminant murine norovirus on experimental murine cytomegalovirus infection

Carmen M. Doom[a], **Holly M. Turula**[a], and **Ann B. Hill**[a,*]

[a]Department of Molecular Microbiology and Immunology, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd, Portland OR 97214

Abstract

Murine norovirus (MNV) is a recently discovered pathogen that has become a common contaminant of specific pathogen-free mouse colonies. MNV-1 induces a robust interferon-β response and causes histopathology in some mouse strains, suggesting that it may impact other mouse models of infection. Despite many concerns about MNV-1 contamination, there is little information about its impact on immune responses to other infections. This study addresses whether MNV-1 infection has an effect on a model of murine cytomegalovirus (MCMV) infection. Exposure to MNV-1 resulted in a decreased CD8 T cell response to immunodominant MCMV epitopes in both BALB/c and C57BL/6 mice. However, MNV-1 did not impact MCMV titers in either mouse strain, nor did it stimulate reactivation of latent MCMV. These data suggest that while MNV-1 has a mild impact on the immune response to MCMV, it is not likely to affect most experimental outcomes in immunocompetent mice in the MCMV model.

Keywords

MNV; MCMV

Introduction

Animal models of infection and disease have been central to the study of pathogenesis, vaccine development, and drug treatment. While there are drawbacks to these models—particularly that they may not precisely model human disease—they are indispensable for making precise statements about the agent being studied, because they allow the investigator to control confounding variables. The timing and dose of infection can be strictly controlled, the impact of genetic polymorphisms is controlled by use of inbred mouse strains, mutant pathogens and animals can be generated, and exposure of animals to other pathogens can be controlled by housing them in specific pathogen-free (SPF) facilities.

*Corresponding Author: Ann B. Hill. OHSU, 3181 SW Sam Jackson Park Dr, L220, Portland, OR, 97239. Phone: (503) 494-0763. Fax: (503) 494-6662. hillan@ohsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

SPF facilities are particularly important for immunological and pathogenesis studies, as unintentional infections can alter immune system functioning and confound experiments using other infectious agents. Use of SPF mice for this sort of study has been virtually universal for decades. SPF mice are typically screened for pathogens such as mouse parvovirus (MPV) and mouse hepatitis virus (MHV) that are known to spread endemically and affect experimental results (Biggart and Ruebner, 1970; Bonnard et al., 1976; McKisic et al., 1993; Riley et al., 1960; Rowe et al., 1959). But how do we define a pathogen? Agents that cause overt disease in healthy animals are automatically classified as pathogens and are excluded from animal facilities. However, a range of other microorganisms can colonize animals, including normal gastrointestinal flora, some of which can cause disease in immunocompromised animals. Some agents, such as *Helicobacter (H.) hepaticus* and *H. bilis* are part of the regular screens performed to monitor SPF facilities, and yet they are not eradicated by many facilities, including our own. Eradication of agents from a facility can be extremely costly, frequently requiring elimination of most of the colony and rederivation of breeder stock. In practice, investigators and facility managers are generally motivated to eradicate new agents by evidence that the agents impact experimental outcomes.

Karst et al. discovered murine norovirus (MNV) in conventionally housed mice at Washington University by a screen for novel human pathogens capable of infecting mice (Karst et al., 2003). MNV-1 was subsequently isolated and identified as the first calicivirus to infect rodents. It has since been suggested that MNV infection of laboratory mice is endemic, presumably being spread by the oral-fecal route as in humans (Perdue et al., 2007). A recent study of mice in research facilities within North America found that 22% of serum samples were MNV seropositive, making MNV the most common mouse colony contaminant known (Hsu et al., 2005). Caliciviruses are single-stranded, positive-sense RNA viruses that are non-enveloped and non-segmented. There are ~7400 base pairs that code for three open reading frames (ORFs) and eight known proteins. The original strain was named MNV-1; 26 MNV-1 genomes have since been isolated from different institutions around the world and sequenced (Karst et al., 2003; Thackray et al., 2007). Of the 15 unique strains identified by Thackray et al., there is a maximum of 13% nucleotide divergence at the whole genome level (Thackray et al., 2007). Minor amino acid changes can quite significantly impact the virulence of strains, as mutation in only two amino acids can attenuate a virulent strain (Bailey et al., 2008). Currently, all known strains form a single genotype, genogroup, and serotype. It is important and convenient that there serological cross-reactivity exists, because infection with any of the isolated strains can be identified using a single antibody (Hsu et al., 2006; Lochridge and Hardy, 2007; Thackray et al., 2007).

There has been much concern, particularly in the immunology community, about the potential impact of MNV on infection and disease models in mice and thus, the need for its eradication. As a consequence, we embarked on the current study to investigate how MNV-1 impacts the outcome of experiments carried out in our own laboratory, which focuses on immunity to murine cytomegalovirus (MCMV). MNV seems most likely to pose a problem for experiments involving the immunological pathways that are important for its control. It is clear that the interferon (IFN) response is important in this regard, as MNV-1 infection is

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

lethal to mice that lack the receptors for both type I and type II interferons (IFN-α/β/γR$^{-/-}$). MNV-1 is also lethal to mice that lack signal transducer and activator of transcription (STAT)-1, a transcription factor activated by IFN signaling that ultimately induces expression of interferon stimulated genes (ISG) (Karst et al., 2003). MNV is not lethal, however, to mice that have *either* the IFN-α/β (type I) or the IFN-γ (type II) system intact (Karst et al., 2003; Muller et al., 2007). Mice that lack other aspects of the innate or adaptive immune systems, such as inducible nitric oxide synthase (NOS$^{-/-}$) or protein kinase RNA-activated (PKR)$^{-/-}$ and recombination-activating gene (RAG)-1$^{-/-}$ or RAG-2$^{-/-}$, respectively, do not succumb to infection (Karst et al., 2003).

MNV can be either acute or persistent; persistence is both virus strain- and mouse strain-specific. For example, wild type 129/SvJ mice clear MNV-1 CW after acute infection, but 129/SvJ RAG-2$^{-/-}$ mice are persistently infected with MNV-1 CW and have viral RNA in multiple tissues up to 90 days post-infection (p.i.) (Karst et al., 2003). C57BL/6 RAG-1$^{-/-}$ mice are also “chronic secreters” of MNV-1 CW, but wild type C57BL/6 mice clear the virus by day seven p.i. (Karst et al., 2003; Thackray et al., 2007). Strains that are acutely cleared in wild type animals include MNV-1 CW1, CW3, and WU11, while strains CR3, CR6, and CR7, and WU11 have been shown to be persistent (Thackray et al., 2007).

MNV infection is not as symptomatic in mice as its human counterpart, which causes gastrointestinal illness. There are a few examples where mice show overt signs of infection, however. Before STAT-1$^{-/-}$ mice succumb to MNV-1 CW3 infection, they display gastrointestinal symptoms, including diarrhea, decreased gastric emptying, and decreased fecal weight contents, in addition to splenitis and pneumonia at 72 hours p.i. (Karst et al., 2003; Mumphrey et al., 2007). While MNV-1 infection has a clear impact on the morbidity and mortality of some immunodeficient, knock-out mice, the nature of infection and immune response in immunocompetent animals is still under investigation. MNV-1 CW3-infected C57BL/6 and 129/SvJ are asymptomatic, but infected 129/SvJ mice have evidence of splenic red pulp hypertrophy and granulocyte infiltration in the intestine at three days p.i. (Mumphrey et al., 2007). Viral RNA is detectable in the liver, spleen, and intestine up to three days p.i. in 129/SvJ mice and up to seven days p.i. in C57BL/6 mice (Karst et al., 2003; Mumphrey et al., 2007). MNV-1 infection induces a strong serum type I IFN response in 129/SvJ mice, which peaks at 24 hours p.i. (Thackray et al., 2007). This cytokine response suggests that silent MNV infection could have a strong and polarizing effect on the immune system of immunocompetent mice.

MNV is present in most laboratory mouse colonies in North America, but now most major commercial suppliers are free of MNV. The virus is thought to be readily transmitted by fecal dust that is spread by normal handling of mouse cages. Thus, mice purchased for experiments have a significant chance of undergoing acute MNV infection at some stage during an experiment. Another laboratory at our institution experienced a dramatic change in the LD_{50} of their mice for West Nile virus (WNV) that appeared to coincide historically with the appearance of MNV in the colony (Janko Nikolich-Zugich, personal communication). This raised the suspicion that inadvertent MNV infection could seriously impact interpretation of other infectious models. Our laboratory routinely infects mice with the γ-herpesvirus MCMV, which establishes lifelong infection and is often used to study

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

immune evasion mechanisms and the immune response to a latent viral infection. We measure the CD8 T cell response by cytokine production and viral control by comparing titers in various organs. There is natural variability in this system, and we have the impression that responses and titers seem to be higher amongst an entire experimental cohort in some experiments than in others (Doom and Hill, 2008). The variability is greater than might be expected using inbred mice and standard, tissue culture-derived virus. When MNV was added to the panel of agents for which our sentinel mice are routinely tested, we became aware of the presence of MNV in our mouse colony. We therefore wondered whether MNV contamination of our experimental mice was responsible for some of the variation we see in our model.

The following study was performed in order to assess the effect on MNV on MCMV infection and the ensuing immune response. We tested two strains of mice (BALB/c and C57BL/6) and two strains of MNV (one persistent, one acute), varying the timing of both infections to model potential random MNV contamination. We did not find a dramatic impact of MNV on MCMV infection or the immune response to it under most conditions. However, MNV did impact the size of the CD8 T cell response to MCMV during acute infection in immunocompetent animals.

Materials and Methods

Cells

BALB/c 3T3 fibroblasts (ATCC) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% Fetalplex (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, and 100 U penicillin/mL, and 100 μg/mL streptomycin. The mouse macrophage cell line, RAW 264.7 (a kind gift from Fred Heffron, OHSU) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, and antibiotics (“DMEM-complete”).

Viruses

Wild-type (WT) BAC-derived MCMV strain MW97.01 was grown on C57BL/6 mouse embryonic fibroblasts (MEF) using a multiplicity of infection (MOI) of 0.01. Virus was purified by ultracentrifuging through a 15% sucrose cushion. Mice were infected by intraperitoneal (i.p.) injection with $5x10^5$ plaque-forming units (PFU) in a volume of 200 μL unless otherwise stated. MCMV strain K181 (a kind gift from Ed Mocarski, Stanford University, CA) was grown on C57BL/6 MEFs. Crude viral lysates were frozen and thawed three times and aliquoted. Mice were infected i.p. with $2x10^5$ PFU in a volume of 600 μL. Each virus stock was titered on BALB/c 3T3 cells. The final virus titers were calculated by taking the mean of 3 virus titrations.

MNV-1 CW3 and passage 2 of MNV-1 CR6 (both kind gifts from Skip Virgin, Washington University, MO) were grown on RAW 264.7 cells at an MOI of 0.05. Cell lysates were frozen and thawed three times and clarified by centrifugation at 3000 rpm for 20 min. For concentrated stocks, lysates were centrifuged at 27,000 rpm for 3 hr at 4°C. Each virus stock was titered on RAW 264.7 cells as described (Wobus et al., 2004). The final virus titers

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

were calculated by taking the mean of three virus titrations. Mice were infected orally (p.o.) with $3x10^7$ PFU in a volume of 10 μL, unless otherwise specified.

Mice

Female C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA), both certified MNV-free vendors. All mice were maintained according to institutional and federal guidelines in animal facilities at Oregon Health and Science University (Portland, OR). Mice were given access to food and water *ad libitum* and housed in a specific-pathogen-free facility that monitors sentinel animals for 13 different pathogens every three months. None of the sentinels for the animals used in these experiments were ever positive for any of the monitored pathogens. Mice were used in experiments between 7 and 12 weeks of age. At sacrifice, all organs were aseptically removed. The spleen and salivary glands were harvested into 1 mL of DMEM-complete and any other organs were snap frozen in liquid nitrogen. The spleen and salivary glands were homogenized the day of the experiment using tissue grinders (Wheaton, Millville, NJ). Snap frozen organs were thawed, suspended in 1 mL of DMEM-complete and homogenized. All homogenates were stored at −80°C and frozen and thawed 3 times before use.

Intracellular Cytokine Staining and Flow Cytometry

Splenocytes or peripheral blood mononuclear cells (PBMC) were isolated from MCMV-infected mice and incubated with 10^{-6} M MCMV peptides (Jerini, Berlin, Germany) or a no-peptide control for 6 hr at 37°C in the presence of brefeldin A (GolgiPlug; BD PharMingen, San Diego, CA), as described (Munks et al., 2006). Cells were washed and surface stained with PE-Cy5-conjugated anti-CD8-α (clone 53–6.7; eBioscience, San Diego, CA). Cells were then fixed and permeabilized using BD PharMingen's Cytofix/Cytoperm kit before staining with PE-conjugated anti-IFN-γ (clone XMG.1; eBiosciences). Cells were acquired by flow cytometry on an LSR-II (BD Bioscience, Franklin Lakes, NJ) using Cell Quest acquisition software (BD Bioscience). All further data analysis was done using FlowJo software (Treestar, San Carlos, CA).

MCMV Plaque Assay

BALB/c 3T3 cells were seeded at $1.5x10^5$ cells/well in six-well plates (Falcon) and grown overnight. Ten percent organ homogenates were clarified by centrifugation and added in 10-fold dilutions to 1 mL of freshly-replaced media/well. Ninety minutes later, the media was removed and the wells were overlaid with 4 mL of a 3:1 mixture of media and 15% w/v carboxymethylcellulose (CMC; Sigma). On day 6, the plates were rinsed with PBS and stained with 0.1% crystal violet in 1% formalin (Fisher) for 5 min. The plates were rinsed again in PBS and allowed to dry. Plaques were then visualized on a light source and counted. Plaques detected on 3T3 cells were MCMV and not MNV, because (a) MNV can only cause cytopathic effects (CPE) in 3T3 cells at extremely high MOI and (b) all plaques detected on 3T3 cells from dually infected mice were positive for the MCMV protein pp89 by immunofluorescence (not shown).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

MNV plaque assay

Plaque assays for MNV were performed as described (Wobus et al., 2004). Briefly, RAW 264.7 cells were seeded at $2x10^6$ cells/well in six-well plates and grown overnight. Either purified virus preparations or 10% organ homogenates that had been clarified by centrifugation were added in 10-fold dilutions to 500 μL of freshly-replaced media/well. After rocking for 1 hr at room temperature, each well was overlaid with 2 mL of a 1:1 mixture of 2X MEM and 3% Seaplaque (Lonza, Basel, Switzerland). Two days later, each well was overlaid with 2 mL of a 1:1 mixture of 2X MEM, 3% SeaKem (Lonza), including 0.01% neutral red dye. Twelve hours later, plaques were visualized by microscope and counted.

Enzyme-linked immunosorbent assay (ELISA)

All sera for ELISAs were collected by retro-orbital bleed. Blood samples were allowed to coagulate overnight at 4°C and then were spun at 2 rpm for 20 min. The sera were aliquoted into multiple tubes for further use. To test for MNV seropositivity, high-binding Immulon 4HBX microtiter plates (Thermo, Milford, MA) were coated with purified MNV. Purified MNV was prepared as described by Wobus et al. with some modification (Wobus et al., 2004). Briefly, RAW 264.7 cells were seeded at $4x10^7$ cells/well in T175 flasks and grown overnight. The cells were infected at an MOI of 0.05 with MNV-1 CW3 and incubated for 48 hrs. The cells and supernatant were harvested and frozen (−80°C) and thawed 3 times before being centrifuged at 3000 rpm for 20 min. The supernatant was then centrifuged through a 30% sucrose cushion at 27,000 rpm for 3 hr at 4°C. The supernatant was removed and virus was resuspended in a total of 2 mL of PBS, aliquoted, and stored at −20°C. ELISA plates were coated with purified MNV-1 particles and stored at −20°C until use. Sera was serially diluted at least four times and tested in triplicate. The primary antibody, horseradish peroxidase-conjugated, goat anti-mouse IgG (heavy and light chains, Biorad, Hercules, CA), was diluted 1:1000. The substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Calbiochem, Gibbstown, NJ) was added and chemiluminescence was measured at 405 nm by a Biorad Benchmark Plus spectrophotometer (Biorad). The data was acquired using the Microplate Manager 5.2 software program (Biorad). Some sera samples were also sent for commercial testing to University of Missouri Research Animal Diagnostic Laboratory (RADIL; Columbia, MO). All mice were tested for MNV seropositivity before use in an experiment and at the end of the experiment.

Statistics

Unpaired, two-tailed t-tests were used to analyze the statistical significance of MCMV-specific CD8 T cell responses. Responses of < 0.5% of all CD8-α^+ T cells were not reliably above background, and when the mean response to an epitope was below 0.5%, statistical analysis was not performed. The non-parametric Mann Whitney test was used to analyze organ viral titers when at least one sample per treatment contained virus above the limit of detection. All statistical analyses were performed using GraphPad Prism (La Jolla, CA).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Results

Handling of mice to avoid inadvertent MNV infection

Because these experiments were performed in an animal room in which some sentinel mice had tested positive for MNV, it was important to prevent accidental MNV infection of the study mice. MNV is thought to be transmitted by means of infectious fecal material spreading through the air as dust. To prevent inadvertent infection, we followed procedures established by the Virgin laboratory. Mice were housed in standard cages with filter tops and were only handled in a biosafety cabinet dedicated to these experiments and only by two individuals who did not handle other mice in the colony. All cage equipment was autoclaved as a unit and stored in the autoclave bag, which was opened in the biohazard cabinet to prevent exposure to airborne fecal dust. The water was autoclaved and supplied in individual bottles and all food was irradiated. Surfaces and supplies were disinfected before and after use for at least ten minutes in a 10% bleach solution. Whenever mice were handled, infected, or harvested, naïve mice were handled first, followed by MCMV-infected mice, followed by MNV (± MCMV) infected mice. Mice were tested for MNV seropositivity before use in an experiment and on the day of sacrifice. Seroconversion generally occurs by day 21 p.i, but does not peak until at least day 35 p.i. (Karst et al., 2003; Thackray et al., 2007); therefore, serological testing could not exclude inadvertent infection for many experiments. In total, 272 mice were used in this study. Over half of them (152) were in the facility for greater than 21 days; the average length of time in the facility before sacrifice was 63 days. For all of these experiments, the only mice that were seropositive at sacrifice were mice that had been intentionally infected with MNV. Furthermore, no sentinels from the rack of mice used in these experiments tested positive for MNV. Therefore, although we cannot conclusively exclude the possibility of inadvertent MNV infection shortly before sacrifice, it is likely that the vast majority of the control mice (MNV-non-infected) in this study remained MNV-free. We know that mice intentionally administered MNV were productively infected, however, as 17/20 mice infected with MNV for 23 days were MNV-seropositive (data not shown).

MNV infection does not impact the timing or magnitude of acute MCMV viral titers

We considered that mice purchased commercially arrive at our facility free of MNV, but may become infected at any time thereafter. We thought that MNV would be most likely to impact our experimental results if acute MNV infection occurred around the time of initial infection with MCMV. Hence, the first set of experiments (Figure 1) tested whether MNV infection at the time of or immediately before MCMV would impact the course of MCMV infection. We infected mice by mouth with MNV-1 CW3 strain of MNV, which causes acute, cleared infection in immunocompetent mice, and then infected these and control mice i.p. with MCMV, either the same day or 1 or 2 days later. We used the BAC-derived MCMV Smith strain MW97.01 (Wagner et al., 2002) (5×10^5 PFU) (Figure 1). We tested both BALB/c and C57BL/6 mice, which differ greatly in their ability to control acute MCMV infection due to a difference in efficacy of the NK cell response. MCMV titers were measured in multiple organs at day four post-infection, the peak of acute infection; the results are shown in Figure 1. As expected, MCMV titers were much higher in BALB/c than in C57BL/6 mice. These results also illustrate the variability between experiments that we

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

sometimes see in this model (e.g. compare the spleen titers in Figure 1 i. and iii.), which we had thought might be explained by concomitant MNV infection. Occasionally, comparisons revealed a small but significant difference between titers in individual organs within an experiment. For example, in Figure 1 C57BL/6 ii., 4/12 mice infected with MCMV only had MCMV detectable in the liver (mean titer = 125 PFU), while 10/12 mice infected with MCMV+MNV had MCMV detectable in the liver (mean titer = 220, p=0.0098). However, in the same experiment, no other organs showed a significant difference in titers. Furthermore, in a parallel experiment (Figure 1 C57BL/6 iii.), the titers did not differ in the liver (0.5476). Overall, there was no consistent impact of concomitant MNV infection on MCMV titers at day four post-infection. In addition, acute titers of the more virulent MCMV-K181 strain were not impacted by MNV-1 CW3 exposure two days prior to MCMV infection (2 x 10^5 PFU, i.p.) in C57BL/6 mice (data not shown).

After day four, virus titers decline in most organs, and MCMV MW97.01 appears in the salivary glands at about two weeks post-infection. In BALB/c mice, virus persists in the salivary glands until about four weeks post-infection. Even though uncloned MCMV-Smith can persist in the salivary glands of C57BL/6 mice, we have found salivary glands infection of C57BL/6 mice with the BAC-derived MW97.01 strain to be frequently undetectable. We investigated the impact of MNV on the kinetics of acute MCMV infection in C57BL/6 mice, on days three, seven and 14 post-infection (Figure 2A). The potential impact of MNV infection on the peak of MCMV salivary glands infection (21 days post-MCMV) in both BALB/c and C57BL/6 mice was also investigated (Figure 2B). In none of these experiments did we see an impact of MNV.

Finally, we also tested a persistent strain of MNV. MNV-1 CR6 (passage 2) persists in the distal ilea and mesenteric lymph nodes of C57BL/6 mice up to 35 days p.i. (Thackray et al., 2007). At 21 days post-infection with MCMV, despite ongoing MNV shedding in the feces (detected by PCR, data not shown), no MCMV was detected in any organ except for the kidney (Figure 2C). Together, the experiments in Figures 1 and 2 provide no consistent evidence that MNV impacts the early course of laboratory MCMV infection in immunocompetent mice.

Acute MNV affects the acute, immunodominant CD8 T cell response to MCMV

While MCMV virus titers were not impacted by concurrent MNV infection, it was possible that MNV infection could affect the CD8 T cell response to MCMV infection. The CD8 T cells responses can be affected by the cytokines, including IFN-α and IL-12, and MNV has been shown to induce a strong type I IFN response in 129/SvJ mice, which peaks at 24 hours p.i. (Mumphrey et al., 2007). Furthermore, it is possible that MNV would impact the CD8 T cell response to MCMV infection if there were any cross-reactive epitopes (Brehm et al., 2002; Selin et al., 1994). The MCMV-specific CD8 T cell response is robust and well-characterized. It peaks at day seven p.i. and the immunodominant epitopes to which CD8 T cells respond are known in both BALB/c and C57BL/6 mice (Holtappels et al., 2002a; Holtappels et al., 2002b; Munks et al., 2006; Reddehase and Koszinowski, 1984; Reddehase et al., 1986). Mice (C57BL/6 or BALB/c) infected with MNV-1 CW3 and control mice were infected two days later with MCMV. Seven days later the CD8 T cell response was

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

measured in splenocytes by intracellular cytokine staining (ICCS) for IFN-γ (Figure 3). In BALB/c mice, the percentage of IFN-γ^+ $CD8^+$ T cells specific for the two most immunodominant peptides, IE1 (pp89) and m164, was modestly but significantly lower in mice infected with MNV ($p=0.005$ and $p=0.0003$, respectively). The same result was seen in two separate experiments using T cells isolated from the peripheral blood (data not shown). Similarly, in C57BL/6 mice, the magnitude of the response to the most immunodominant peptide, M45, was lower in MNV-infected mice ($p=0.045$). The immunodominance hierarchy was not affected by MNV in either mouse strain. Thus, concomitant MNV infection did affect the CD8 T cell response to MCMV infection, although the impact was modest.

MCMV does not reactivate in response to MNV infection

By four weeks p.i., the BAC-derived MCMV MW97.01 is generally undetectable in any organ by plaque assay although latent infection, and perhaps undetected persistent replicative infection, continues. Reactivation of latent MCMV is an important, yet still poorly understood, part of the infectious cycle that can be experimentally induced by a number of stimuli (Cook et al., 2002; Cook et al., 2006; Koffron et al., 1999; Simon et al., 2005). Notably, inflammatory stimuli, such as bacterial infection, LPS, TNF-α, and IL-1, have been shown to induce CMV reactivation. We have sometimes found several mice in a single cage with MCMV in the spleen or other organs at a time post-infection at which the virus is usually undetectable, which has bedeviled experimental reproducibility and made us suspect a non-random adventitious event. Since MNV infects some of the cell types in which MCMV has been shown to be latent (Hanson et al., 1999; Koffron et al., 1998; Mercer et al., 1988; Pollock et al., 1997; Pomeroy et al., 1991; Wobus et al., 2004), and since it induces inflammatory cytokines, we thought it possible that MNV may induce reactivation of latent MCMV. In order to test this, mice were infected with MCMV MW97.01 for at least four weeks, after which half of the mice were also infected with acute MNV-1 CW3. MCMV reactivation models have different peaks of reactivation depending on the stimulus used, therefore, viral titers were measured at either one or two weeks post-*MNV* in the salivary glands, lung, and spleen, the organs in which MCMV reactivation is most readily detected (Balthesen et al., 1993). However, we detected no difference in viral titers in the presence or absence of MNV stimulation in either BALB/c (Figure 4A) or C57BL/6 mice (Figure 4B). MCMV MW97.01 is somewhat attenuated compared to either Smith or K181 strains of MCMV (unpublished data). We therefore repeated the reactivation experiments with the more virulent K181 strain (Figure 4C). K181 caused persistent infection of the salivary glands in C57BL/6 mice, as has been reported (M. Degli-Esposti, unpublished data). However, MNV infection did not cause any alteration of virus titers to indicate reactivation in either C57BL/6 or BALB/c mice.

Discussion

This study was undertaken in order to determine whether the recently discovered mouse pathogen, MNV, had an impact on a mouse model of MCMV infection. MNV is a common infection in mouse colonies around the world; there is much concern regarding this emerging pathogen but little published data on its impact on other infectious agents or

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

models. Recently, Hensley et al. reported that the persistent MNV-1 CR6 strain has no significant impact on the immune response to acute vaccinia or influenza viruses (Hensley et al., 2009). We wanted to investigate a possible impact of MNV on our *in vivo* model of MCMV infection, because there is a degree of variability that has always been puzzling. The variation segregates by experimental day and often by cage. Mice infected with the same dose on different days may have MCMV titers in their salivary glands that differ by up to two logs at 21 days p.i. This kind of variability could be explained by inadvertent infection with another agent. Upon learning that sentinel mice in our colony were MNV seropositive, we were keen to see whether MNV was responsible. It is important to note that the experiments performed for this study were solely concerned with the outcomes of MCMV infection, our own experimental system, and we did not address the impact of MCMV on MNV pathogenesis. In order to assess the effect of MNV-1 on MCMV infection, two well-characterized outcomes of infection were measured: the MCMV-specific CD8 T cell response and MCMV viral titers.

We focused primarily on the impact of MNV infection occurring around the time of MCMV infection, which we thought would be most likely to affect our experimental outcomes. However, we could not detect a consistent impact of MNV on acute MCMV infection, or on persistent salivary glands infection. Furthermore, acute MNV infection did not drive detectable reactivation of latent MCMV. We can cautiously conclude that MNV does not have a large impact on the course of MCMV infection in immunocompetent mice and is unlikely to be a major contributor to experimental variation within our model system. The cause of our experimental variation remains to be determined; possible explanations that have not yet been addressed include adventitious infection with a currently unknown agent (it is unlikely that MNV will be the last such agent to be identified), differences in commensal flora between commercial mice and those bred in our facility, and the impact of the estrus cycle (as we predominantly use female mice).

There are possible caveats to this conclusion, which is based on experimental infection with two laboratory-passaged strains of MNV. A growing number of MNV strains have been identified that possess different characteristics regarding persistence, etc., and it has also been reported that it does not take many generations for the phenotype of a strain to change quite drastically (Bailey et al., 2008; Mumphrey et al., 2007; Thackray et al., 2007). The strains that have spontaneously contaminated our mouse colony on the West coast may have very different characteristics than those propagated for use in this study. Nevertheless, we used high dose infection of the originally isolated MNV-1 strain (CW3) without detecting any impact on the course of MCMV infection. Furthermore, infection with a very different MNV strain (CR6), which causes chronic infection in C57BL/6 mice also failed to impact acute MCMV infection.

We did, however, reproducibly detect a small but significant impact of MNV infection two days prior to MCMV on the size of the CD8 T cell response to the most immunodominant epitopes in both BALB/c and C57BL/6 mice. Curiously, there was no impact on the magnitude of the CD8 T cell response to subdominant peptides, nor were there any changes in the hierarchy itself. As there was no increase in the size of the response to a particular MCMV epitope or the immunodominance hierarchy, in general, there was no evidence for

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

cross-reactive epitopes. As yet, CD8 T cell responses to MNV have not been described. The characterization of innate immune control of MNV is incomplete, but it is known that MNV-1 infection induces a strong serum type I IFN response in 129/SvJ mice, which peaks at 24 hours p.i. (Mumphrey et al., 2007). The CD8 T cell response is known to be regulated in part by inflammatory cytokines, including type I IFN, and we suspect that the impact of MNV on the cytokine milieu present at the time of CD8 T cell priming may be responsible for its impact on the CD8 T cell response to MCMV. This impact was rather small, and would not alter the interpretation of most experiments. Nevertheless, it should be borne in mind, particularly for studies on immunodominance and the size of the CD8 T cell response.

Our study with MCMV cannot predict the impact that MNV might have on other infectious models. Furthermore, we used only immunocompetent mice. MNV is much more likely to impact studies using immunologically impaired mice, since it is lethal for mice severely impaired in interferon responses. Further investigations are needed before the overall impact of this agent is clear. In the meantime, MNV remains a non-random variable that is superimposed on our experiments. Mice purchased from commercial vendors are free from MNV, whereas strains bred in-house at many facilities may be contaminated. In particular, immunodeficient mutant strains are more likely to be chronic carriers. Given these considerations, even without direct evidence of the impact of MNV on experimental models, it is likely that many facilities will decide to eradicate MNV, as some already have. Eradication of MNV from most facilities will require embryonic rederivation of mouse strains that cannot be purchased commercially, and is an expensive undertaking. It is of course highly unlikely that MNV is the last “adventitious” murine infection that will be discovered. A side benefit of rederivation of a colony is that it will likely remove other as yet undiscovered agents in addition to MNV. While the immune systems of inbred SPF mice may imperfectly model those of animals in the wild, rigorous control of variables using these mice has enabled remarkable progress in the fine dissection of immune responses. At present, the significance of MNV as a variable for different experimental outcomes remains to be determined.

Acknowledgments

This work was supported by NIH/NIAID AI47206.

References

Bailey D, Thackray L, Goodfellow I. A single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation in vivo. J Virol. 2008; 82:7725–7728. [PubMed: 18495770]

Balthesen M, Messerle M, Reddehase M. Lungs are a major organ site of cytomegalovirus latency and recurrence. J Virol. 1993; 67:5360–5366. [PubMed: 8394453]

Biggart J, Ruebner B. Lymphoid necrosis in the mouse spleen produced by mouse hepatitis virus (MHV3): an electron-microscopic study. J Med Microbiol. 1970; 3:627–632. [PubMed: 4323489]

Bonnard G, Manders E, Campbell DJ, Herberman R, Collins MJ. Immunosuppressive activity of a subline of the mouse EL-4 lymphoma. Evidence for minute virus of mice causing the inhibition. J Exp Med. 1976; 143:187–205. [PubMed: 1244418]

Brehm M, Pinto A, Daniels K, Schneck J, Welsh R, Selin L. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nat Immunol. 2002; 3:627–634. [PubMed: 12055626]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Cook CH, Trgovcich J, Zimmerman P, Zhang Y, Sedmak D. Lipopolysaccharide, tumor necrosis factor alpha, or interleukin-1 beta triggers reactivation of latent cytomegalovirus in immunocompetent mice. J Virol. 2006; 80:9151–9158. [PubMed: 16940526]

Cook C, Zhang Y, McGuinness B, Lahm M, Sedmak D, Ferguson R. Intra-abdominal bacterial infection reactivates latent pulmonary cytomegalovirus in immunocompetent mice. J Infect Dis. 2002; 185:1395–1400. [PubMed: 11992273]

Doom C, Hill A. MHC class I immune evasion in MCMV infection. Med Microbiol Immunol. 2008; 197:191–204. [PubMed: 18330598]

Hanson L, Slater J, Karabekian Z, Virgin HT, Biron C, Ruzek MC, Van Rooijen N, Ciavarra R, Stenberg R, Campbell A. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. J Virol. 1999; 73:5970–5980. [PubMed: 10364349]

Hensley S, Pinto A, Hickman H, Kastenmayer R, Bennink J, Virgin H, Yewdell J. Murine norovirus infection has no significant effect on adaptive immunity to vaccinia virus or influenza A virus. J Virol. 2009

Holtappels R, Grzimek N, Simon C, Thomas D, Dreis D, Reddehase M. Processing and presentation of murine cytomegalovirus pORFm164-derived peptide in fibroblasts in the face of all viral immunosubversive early gene functions. J Virol. 2002a; 76:6044–6053. [PubMed: 12021337]

Holtappels R, Thomas D, Podlech J, Reddehase M. Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2^{d} haplotype. J Virol. 2002b; 76:151–164. [PubMed: 11739681]

Hsu CC, Riley L, Wills H, Livingston R. Persistent infection with and serologic cross-reactivity of three novel murine noroviruses. Comp Med. 2006; 56:247–251. [PubMed: 16941951]

Hsu CC, Wobus C, Steffen E, Riley L, Livingston R. Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. Clin Diagn Lab Immunol. 2005; 12:1145–1151. [PubMed: 16210475]

Humphreys I, De Trez C, Kinkade A, Benedict C, Croft M, Ware C. Cytomegalovirus exploits IL-10-mediated immune regulation in the salivary glands. J Exp Med. 2007; 204:1217–1225. [PubMed: 17485516]

Karst S, Wobus C, Lay M, Davidson J, Virgin HT. STAT1-dependent innate immunity to a Norwalk-like virus. Science. 2003; 299:1575–1578. [PubMed: 12624267]

Koffron A, Varghese T, Hummel M, Yan S, Kaufman D, Fryer J, Leventhal J, Stuart F, Abecassis M. Immunosuppression is not required for reactivation of latent murine cytomegalovirus. Transplant Proc. 1999; 31:1395–1396. [PubMed: 10083617]

Koffron A, Hummel M, Patterson B, Yan S, Kaufman D, Fryer J, Stuart F, Abecassis M. Cellular localization of latent murine cytomegalovirus. J Virol. 1998; 72:95–103. [PubMed: 9420204]

Lochridge V, Hardy M. A single-amino-acid substitution in the P2 domain of VP1 of murine norovirus is sufficient for escape from antibody neutralization. J Virol. 2007; 81:12316–12322. [PubMed: 17804495]

McKisic M, Lancki D, Otto G, Padrid P, Snook S, Cronin DN, Lohmar P, Wong T, Fitch F. Identification and propagation of a putative immunosuppressive orphan parvovirus in cloned T cells. J Immunol. 1993; 150:419–428. [PubMed: 8419475]

Mercer J, Wiley C, Spector D. Pathogenesis of murine cytomegalovirus infection: identification of infected cells in the spleen during acute and latent infections. J Virol. 1988; 62:987–997. [PubMed: 2828694]

Muller B, Klemm U, Mas Marques A, Schreier E. Genetic diversity and recombination of murine noroviruses in immunocompromised mice. Arch Virol. 2007; 152:1709–1719. [PubMed: 17533553]

Mumphrey S, Changotra H, Moore T, Heimann-Nichols ER, Wobus C, Reilly M, Moghadamfalahi M, Shukla D, Karst S. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. J Virol. 2007; 81:3251–3263. [PubMed: 17229692]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Munks M, Gold M, Zajac A, Doom C, Morello C, Spector D, Hill A. Genome-wide analysis reveals a highly diverse CD8 T cell response to murine cytomegalovirus. J Immunol. 2006; 176:3760–3766. [PubMed: 16517745]

Perdue K, Green K, Copeland M, Barron E, Mandel M, Faucette L, Williams E, Sosnovtsev S, Elkins W, Ward J. Naturally occurring murine norovirus infection in a large research institution. J Am Assoc Lab Anim Sci. 2007; 46:39–45. [PubMed: 17645294]

Pollock J, Presti RM, Paetzold S, Virgin HT. Latent murine cytomegalovirus infection in macrophages. Virology. 1997; 227:168–179. [PubMed: 9007070]

Pomeroy C, Hilleren P, Jordan M. Latent murine cytomegalovirus DNA in splenic stromal cells of mice. J Virol. 1991; 65:3330–3334. [PubMed: 1851880]

Reddehase M, Fibi MR, Keil G, Koszinowski U. Late-phase expression of a murine cytomegalovirus immediate-early antigen recognized by cytolytic T lymphocytes. J Virol. 1986; 60:1125–1129. [PubMed: 2431160]

Reddehase M, Koszinowski U. Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature. 1984; 312:369–371. [PubMed: 6095095]

Riley V, Lilly F, Huerto E, Bardell D. Transmissible Agent Associated with 26 Types of Experimental Mouse Neoplasms. Science. 1960; 132:545–547. [PubMed: 17798830]

Rowe W, Hartley J, Estes J, Huebner R. Studies of mouse polyoma virus infection. 1. Procedures for quantitation and detection of virus. J Exp Med. 1959; 109:379–391. [PubMed: 13641563]

Selin L, Nahill S, Welsh R. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. J Exp Med. 1994; 179:1933–1943. [PubMed: 8195718]

Simon C, Seckert C, Dreis D, Reddehase M, Grzimek N. Role for tumor necrosis factor alpha in murine cytomegalovirus transcriptional reactivation in latently infected lungs. J Virol. 2005; 79:326–340. [PubMed: 15596827]

Thackray L, Wobus C, Chachu K, Liu B, Alegre E, Henderson K, Kelley S, Virgin HT. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. J Virol. 2007; 81:10460–10473. [PubMed: 17652401]

Wagner M, Gutermann A, Podlech J, Reddehase M, Koszinowski U. Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J Exp Med. 2002; 196:805–816. [PubMed: 12235213]

Wobus C, Karst S, Thackray L, Chang K, Sosnovtsev S, Belliot G, Krug A, Mackenzie J, Green K, Virgin H. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol. 2004; 2:e432. [PubMed: 15562321]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

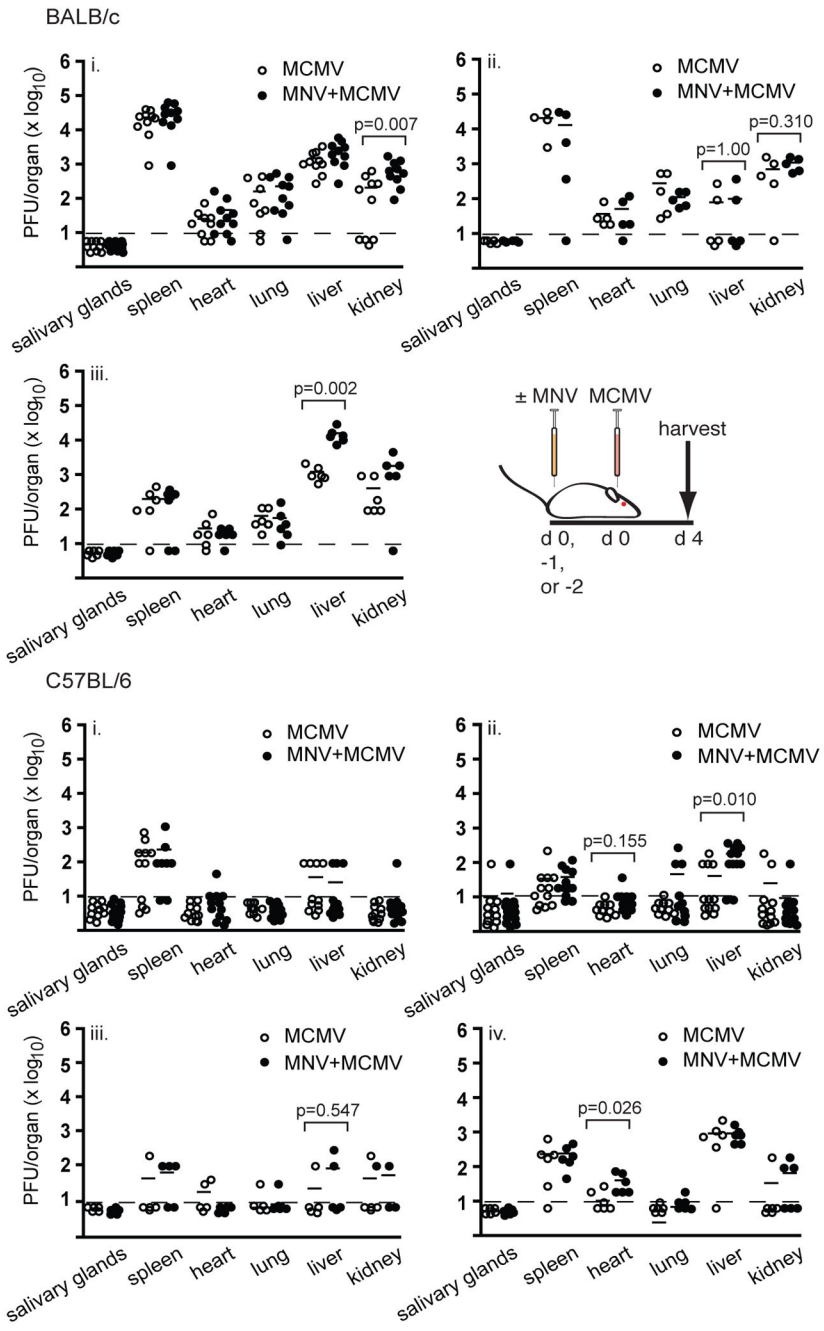

Figure 1.

MNV infection does not alter the magnitude of MCMV infection. A) BALB/c (i.–iii.) or C57BL/6 (i.–iv.) mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2, day -1, or day 0. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV MW97.01. Four days later (d 4) mice were sacrificed. The salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid nitrogen. MCMV titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 or 12 for each group. Roman

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

numerals (i.–iv.) represent similar experiments performed on different days. MNV was given on day 0 in BALB/c i. and C57BL/6 i. MNV was given on day -1 in BALB/c ii. and C57BL/6 ii. and iii. MNV was given on day -2 in BALB/c iii. and C57BL/6 iv. All mice were MNV seronegative. All p-values comparing organ virus titers with and without MNV infection were > 0.100, unless otherwise noted. In each case where there was a significant difference between organ virus titers, there is an example provided from a similar experiment where no significant difference was found.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

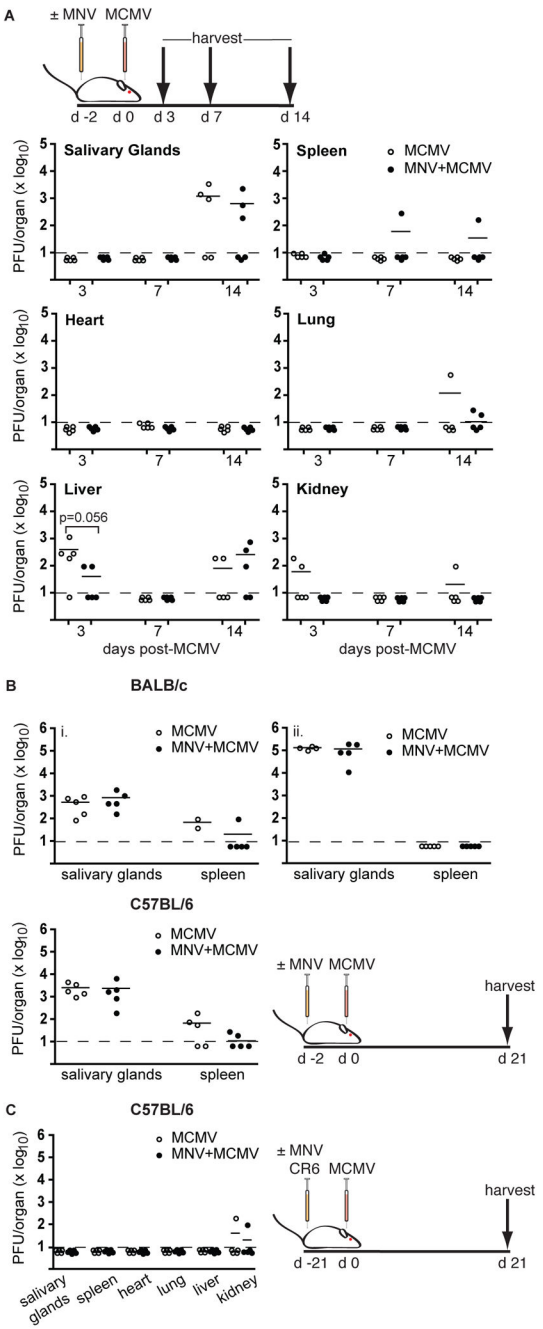

Figure 2.

MNV infection does not alter the kinetics of MCMV infection. C57BL/6 or BALB/c mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV MW97.01. A) 3, 7, and 14 days later or B) 21 days later, mice were sacrificed. C) C57BL/6 mice were infected p.o. with 10^8 PFU MNV-1 CR6 (passage 2) on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV MW97.01. Twenty-one days later, mice were sacrificed. The salivary glands and spleen were harvested into 1 mL DMEM-complete and the other organs were snap frozen in liquid nitrogen. MCMV

titers in different organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. B) i–ii. represent identical experiments performed on different days. All mice were MNV seronegative, except in B) 12/15 MNV-infected mice were MNV-seropositive and C) 5/5 MNV-infected mice were MNV-seropositive. All p-values comparing organ virus titers with and without MNV infection were > 0.200, unless otherwise noted.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

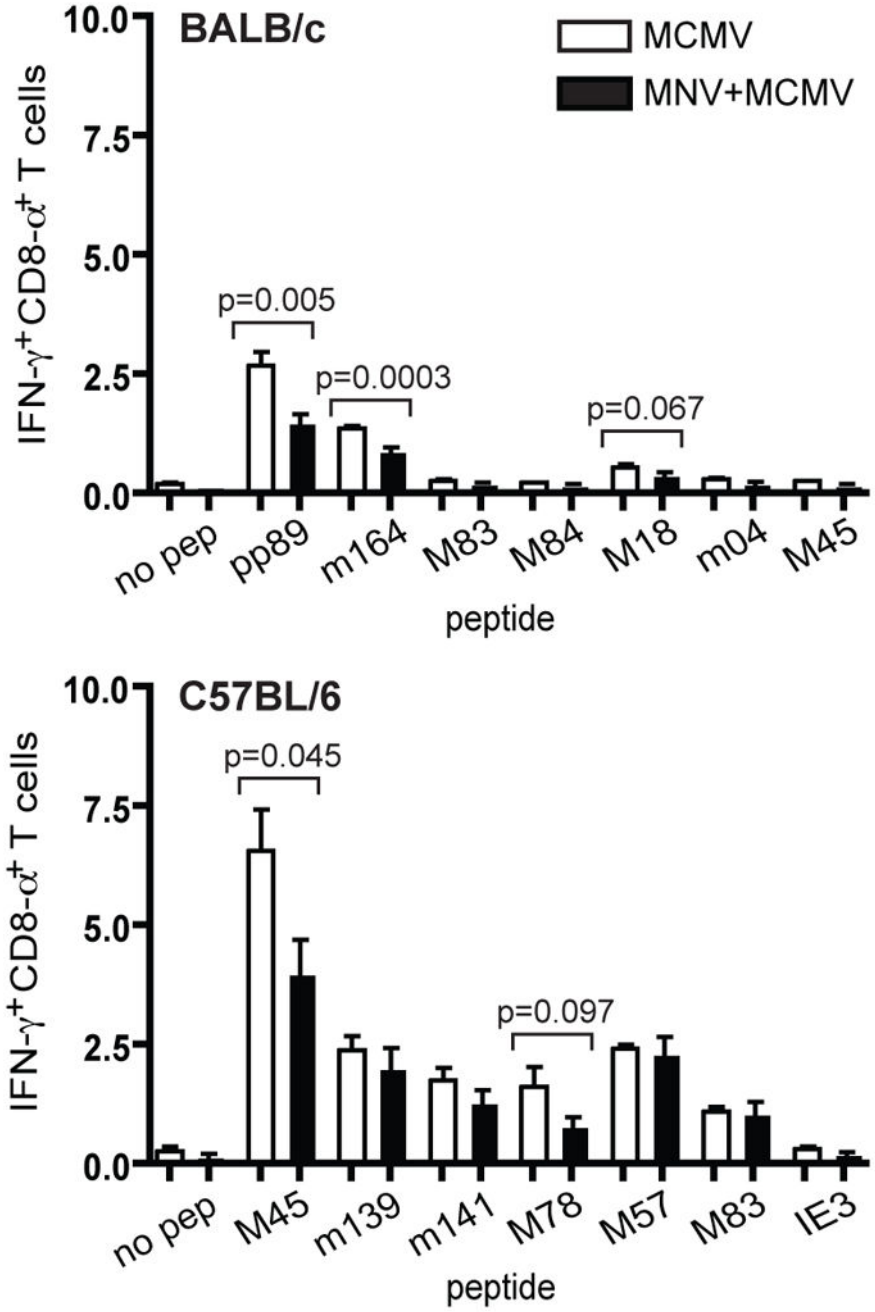

Figure 3.

MNV infection impacts the immunodominant CD8 T cell response to MCMV. BALB/c mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3 on day -2. On day 0, mice were infected i.p. with $5x10^5$ PFU MCMV MW97.01. A week later (d 7), the spleens were harvested and splenocytes were incubated with MCMV peptides (10^{-6} M) for 7 hours in the presence of brefeldin A. They were then stained for surface CD8-α, fixed, permeabilized, and stained for intracellular IFN-γ. The percentage of IFN-γ^+ CD8-α^+ T cells were measured by flow cytometry on an LSR II and analyzed by FloJo software. Error bars indicate SEM. n=6 for each group. All mice were MNV-1 seronegative. All p-values

comparing an epitope-specific response with and without MNV infection were > 0.100, unless otherwise noted.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

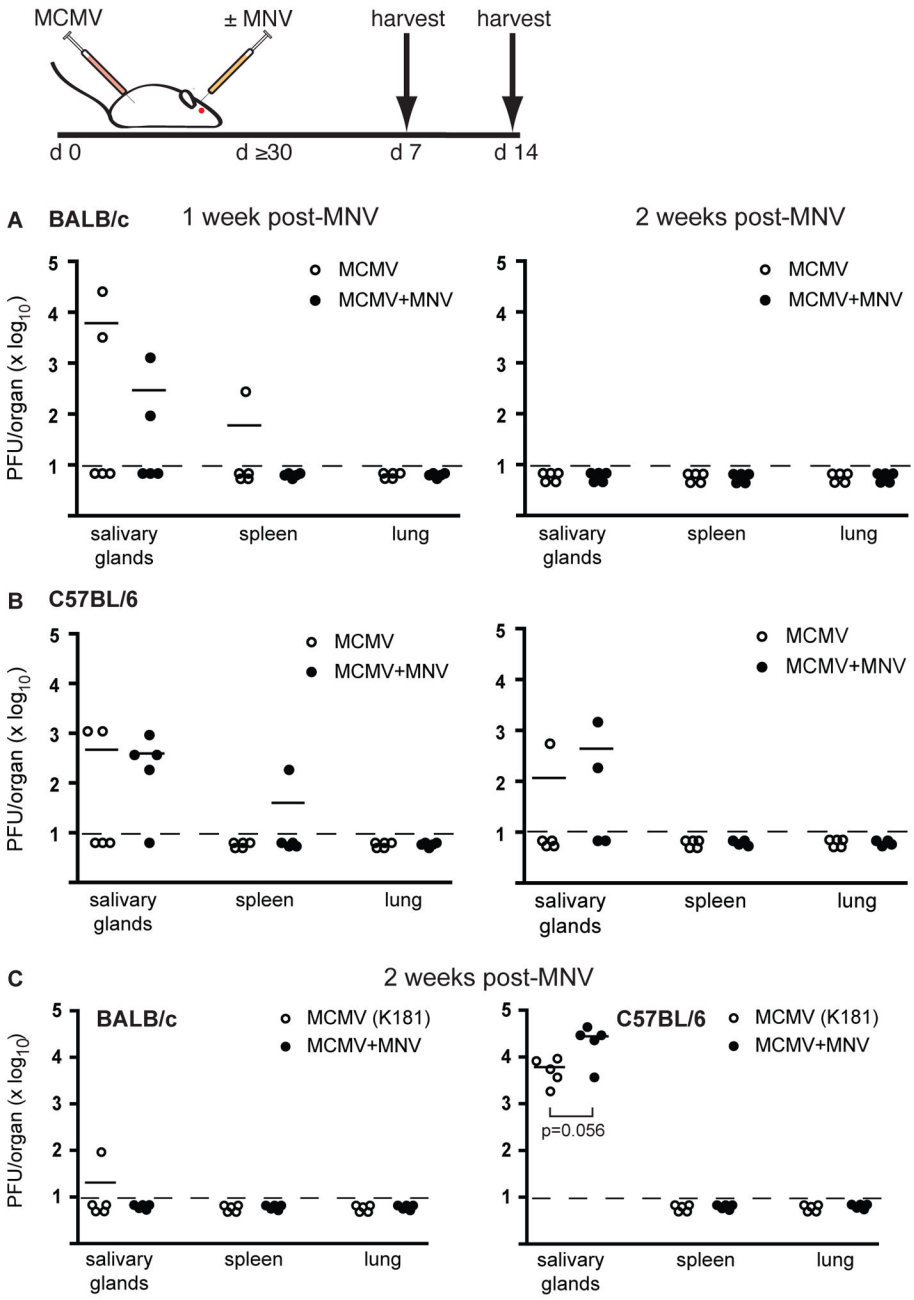

Figure 4.

MCMV MW97.01 does not reactivate in response to MNV infection. A) BALB/c or B) C57BL/6 mice were infected i.p. with $5x10^5$ PFU MCMV MW97.01 on day ≥ 30. On day 0, mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3. Either 1 (left panels) or 2 (right panels) weeks later, mice were sacrificed. C) BALB/c (left) or C57BL/6 mice (right) were infected i.p. with $5x10^5$ PFU MCMV-K181 on day ≥ 30. On day 0, mice were infected p.o. with $3x10^7$ PFU MNV-1 CW3. Two weeks later, mice were sacrificed. The salivary glands, spleen, and lung were harvested into 1 mL DMEM-complete. MCMV titers in these organs were tested by standard plaque assay using 10% tissue homogenates. Each dot represents an

individual mouse; the short, solid line represents the mean. The limit of detection is 10^1 PFU and is indicated by the dotted line. n=5 for each group. All mice were MNV-1 seronegative. All p-values comparing organ virus titers with and without MNV infection were > 0.400, unless otherwise noted.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript