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RESEARCH ARTICLE

Protective vaccination with hepatitis C virus NS3 but not core antigen in a novel mouse challenge model

Susanne El-Gogo,[1] Caroline Staib,[1] Juan José Lasarte,[2] Gerd Sutter,[3*†] Heiko Adler,[4*†]

[1]*Institute of Virology, Technical University München, Germany;* [2]*University of Navarra, Pamplona, Spain;* [3]*Paul-Ehrlich-Institut, Department of Virology, Langen, Germany;* [4]*GSF-National Research Centre for Environment and Health, Institute of Molecular Immunology, CCG HCT, München, Germany*

*Correspondence to: Gerd Sutter, Department of Virology, Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 51-59, D-63225 Langen, Germany.
E-mail: sutge@pei.de

**Correspondence to: Heiko Adler, GSF-Hämatologikum, Marchioninistrasse 25, D-81377 Munich, Germany.
E-mail: h.adler@gsf.de

†Both investigators contributed equally and should be considered as senior authors.

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Abstract

Background Efficient vaccines against hepatitis C virus (HCV) infection are urgently needed. Vaccine development has been hampered by the lack of suitable small animal models to reliably test the protective capacity of immmunization.

Methods We used recombinant murine gammaherpesvirus 68 (MHV-68) as a novel challenge virus in mice and tested the efficacy of heterologous candidate human vaccines based on modified vaccinia virus Ankara or adenovirus, both delivering HCV non-structural NS3 or core proteins.

Results Recombinant MHV-68 expressing NS3 (MHV-68-NS3) or core (MHV-68-core) were constructed and characterized *in vitro* and *in vivo*. Mice immunized with NS3-specific vector vaccines and challenged with MHV-68-NS3 were infected but showed significantly reduced viral loads in the acute and latent phase of infection. NS3-specific CD8+ T cells were amplified in immunized mice after challenge with MHV-68-NS3. By contrast, we did neither detect a reduction of viral load nor an induction of core-specific CD8+ T cells after core-specific immunization.

Conclusions Our data suggest that the challenge system using recombinant MHV-68 is a highly suitable model to test the immunogenicity and protective capacity of HCV candidate vaccine antigens. Using this system, we demonstrated the usefulness of NS3-specific immunization. By contrast, our analysis rather discarded core as a vaccine antigen. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords hepatitis C virus; immunization; recombinant adenovirus; recombinant MVA; recombinant MHV-68; vaccine

Introduction

Hepatitis C virus (HCV) infection causes severe human disease of high prevalence world wide. There is no effective vaccine available and, at present, the only relevant preclinical model is the infection of chimpanzees. Thus, for the development of promising candidate vaccines, a suitable small animal model system would be highly desirable. Recently, we proposed infection with recombinant murine gammaherpesvirus 68 (MHV-68) as a novel mouse challenge model to test the protective efficacy of heterologous vaccines based on recombinant modified vaccinia virus Ankara (MVA) [1]. Thus, infection of mice with recombinant MHV-68 expressing antigens of HCV could be a robust

test system for HCV vaccines. HCV-core protein is relatively conserved among HCV genotypes, and immunizations induced HCV-core specific antibody and cytotoxic T lymphocyte (CTL) responses [2,3]. However, there is some controvery regarding its usefulness as vaccine antigen because HCV-core appears to have many regulatory functions, being involved in the modulation of host cell apoptosis, transcription, transformation and immune presentation [4,5]. Non-structural protein 3 (NS3) is a highly conserved, bi-functional protein with a serine protease in the N-terminal one-third and a RNA helicase in the C-terminal two-thirds. Immune responses to HCV-NS3 have been implicated in viral clearance. Numerous CTL epitopes have been identified in NS3 [6–8], and strong T helper responses to NS3 have been correlated with recovery from hepatitis [8] and were demonstrated to be long lived [9].

In the present study, we established the recombinant MHV-68 challenge system to assess the efficacy of HCV-specific candidate vaccines upon delivery of HCV-core or NS3 antigens. Importantly, the MHV-68 challenge system first demonstrated the *in vivo* benefit of NS3- but not core-specific vaccination with recombinant MVA vaccines and revealed a significant enhancement of protective capacity when applying NS3 in a heterologous prime-boost regimen with recombinant adenovirus and MVA.

Materials and methods

Plasmid construction

To construct the recombination plasmid pST76K-SR-M1/M2-NS3, the 1.9-kb NS3 fragment (amino acids 1028–1658) [10] was cloned blunt end into the *Sma*I site of pMCMV4 (kindly provided by Martin Messerle) between the MCMV promoter and the polyA signal. To obtain the recombination plasmid pST76K-SR-M1/M2-core, the 573-bp core (amino acids 1–191) [10] fragment was cloned into the *Bam*HI/*Pst*I site of pMCMV4. The NS3- or core-expression cassettes (excised by *Hind*III) were cloned blunt end into the *Bgl*II site (position 3846 of the MHV-68 genome) of the plasmid pST76K-SR M1/M2. Thus, the NS3- or core-expression cassettes are flanked on both sides by a homologous sequence of the MHV-68 genome (position 2406–3846 as 5′ flank and position 3847–6261 as 3′ flank). The following two-step mutagenesis procedure was performed as previously described [11,12].

Reconstitution and characterization of recombinant MHV-68

BHK-21 cells, Ref-Cre cells, NIH3T3 cells and MC3T3 fibroblasts were used and recombinant MHV-68 were reconstituted and characterized as previously described [1]. Expression of NS3 and core was tested by western blot analysis using rabbit polyclonal anti-HCV protein antibodies (Antigenix America Inc./Bio-Trade, Vienna, Austria; 1 : 10 000, 1 : 5000, respectively).

Generation of viral vector vaccines MVA-NS3, MVA-core, Adeno-NS3 and Adeno-core

HCV-1b core (amino acids 1–191) and NS3 (amino acids 1028–1658) sequences were originally prepared from plasmids pCMV-C980 and pCMV-N-729–3010 [10] and expressed in recombinant MVA under transcriptional control of vaccinia virus promoter P7.5 [13–15]. Vaccine preparations were purified by ultracentrifugation through sucrose, and stored in 1 mM Tris-HCl, pH 9.0 at −80 °C [15]. Synthesis of recombinant HCV antigens was quality controlled by western blot analysis (data not shown). Non-recombinant MVA was used to immunize control animals. Generation and characterization of vaccine preparations based on recombinant adenovirus expressing HCV core (Adeno-core) and NS3 (Adeno-NS3) proteins have been previously described [16,17].

In vivo experiments

All animal experiments were in compliance with protocols approved by the local Animal Care and Use Committee and were performed as previously described [1]. Briefly, C57BL/6 (B6) mice (Charles River, Sulzfeld, Germany) were infected intranasally (i.n.) with 5×10^4 plaque forming units (PFU) or intraperitoneally (i.p.) with 1×10^6 PFU. To determine virus titers, lungs were harvested on day 6 after i.n. infection and spleens on day 6 after i.p. infection. For determination of spleen weight and frequency of virus reactivation, spleens were collected on day 17 or 21 after i.n. or i.p. infection. To assess protective capacity of vaccines, mice were immunized i.p. with 10^8 infectious units (IU) MVA-WT, MVA-NS3 or MVA-core or 10^9 IU Ad-NS3 or Ad-core in 250 µl phosphate-buffered saline (PBS). Mice were challenged i.n. with 5×10^4 PFU MHV-68-WT*, MHV-68-NS3 or MHV-68-core in 30 µl PBS or i.p. with 10^6 PFU in 250 µl PBS. Lungs and spleens were harvested as described above, and virus titers were determined as previously described [1].

Limiting dilution reactivation assay

The frequency of cells carrying virus reactivating from latency was determined as described previously [18].

Intracellular cytokine staining assay

Splenocytes were re-stimulated with MC3T3 fibroblasts infected with MHV-68-WT*, MHV-68-NS3 or MHV-68-core. In addition, in the case of NS3, splenocytes were re-stimulated by incubation with NS3 peptide

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LGAVQNEVTLTHPIT containing the H2-K^b restricted CTL epitope GAVQNEITL (position 1629–1637) [19]. MC3T3 cells were infected for 1 h with a different multiplicity of infection (MOI) of MHV-68-WT*, MHV-68-NS3 or MHV-68-core and grown over night at 37 °C. After harvest with 10 mM EDTA in PBS or medium, cells were incubated with splenocytes of vaccinated mice for 2 h at 37 °C. After adding Brefeldin A (Golgi Plug, PharMingen/Becton Dickinson) at a final concentration of 1 μg/ml, cells were incubated for 8 h at 37 °C and then kept at 4 °C over night. To measure NS3-specific T-cell response, spleen cells of vaccinated and challenged mice were incubated with peptide at a final concentration of 1 μg/ml for 2 h, with Brefeldin A at a final concentration of 1 μg/ml for 5 h, and then kept at 4 °C over night. Following steps were performed as previously described [20].

Chromium release assay

Splenocytes of naïve B6 mice were infected with MHV-68-NS3 or MHV-68-core (MOI = 0.1) for 1.5 h, washed, irradiated (3000 rad) and incubated with splenocytes from MVA-WT-, MVA-NS3-, MVA-core-, Ad-NS3/MVA-NS3- or Ad-core/MVA-core-vaccinated mice at 37 °C and 5% CO_2. After 5 days of culture, lytic activities of CTL were determined in a 6-h ^{51}Cr-release assay as previously described [1].

Measurement of latent viral load by quantitative real time polymerase chain reaction (PCR)

Latent viral load in spleens of infected mice was quantified by real-time PCR using the ABI 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described [1].

Statistical analysis

If not otherwise indicated, data were analysed by Student's *t*-test.

Results

Construction of MHV-68-NS3 and MHV-68-core and *in vitro* and *in vivo* characterization. After generation by BAC-technology, we analysed the genomes of the recombinant viruses MHV-68-NS3 and MHV-68-core by restriction enzyme analysis and Southern blot (Figures 1A, 1B and 1C). As designed, insertion of the 2.5-kb core- or 3.8-kb NS3-coding expression cassette, respectively, into the *Bgl*II site at nucleotide position 3846 of the MHV-68 genome [21] resulted in a shift of the *Hind*III-E fragment from 6.1 kb in the parental BAC-derived virus (= WT*) to 8.6 kb in MHV-68-core or 9.9 kb in MHV-68-NS3. *In vitro*, both MHV-68-core and MHV-68-NS3 showed comparable growth kinetics to MHV-68-WT* (Figure 1D). Western blot analysis demonstrated the ability of MHV-68-NS3 and MHV-68-core to produce the respective proteins upon infection of NIH3T3 cells (Figures 1E and 1F). Intranasal infection of mice with MHV-68 results in an acute, productive infection in the lung with viral titers reaching the peak around day 6 post infection [22,23]. Hence, to test for lytic replication *in vivo*, C57BL/6 mice were infected i.n. with 5×10^4 PFU of MHV-68-WT*, MHV-68-NS3 or MHV-68-core. At day 6 after infection, titration of infectious virus from lung homogenates demonstrated that all viruses produced very similar amounts of lytic virus (Figure 2A). Both after i.n. and i.p. MHV-68 infection, latency is established in the spleen and other lymphoid organs [22]. The latent infection in the spleen is associated with a marked splenomegaly, an increase in the number of splenocytes and a rise in the number of latently infected B cells which peak around 2–3 weeks post infection [24]. B lymphocytes are the major reservoir harboring latent MHV-68 [25], but macrophages [26], dendritic cells [27] and lung epithelial cells [28] have also been shown to harbor latent virus. Memory B cells are the major reservoir for long-term latency [29–31]. Following the amplification of latency, there is both a decrease of the splenomegaly and of the number of latently infected splenocytes back to a basal level [22]. Multiple immune mechanisms including CD8+ T cells, CD4+ T cells and antibodies contribute to the control of latency and preventing recrudescence of lytic virus [22,32]. Thus, to analyse virus latency, C57BL/6 mice were infected i.p. with 1×10^6 PFU of MHV-68-WT*, MHV-68-NS3 or MHV-68-core. At day 17 after infection, the weight of spleens from mice infected with MHV-68-NS3 was significantly higher than the spleen weight of naive mice but significantly lower than the spleen weight of mice infected with MHV-68-WT* (Figure 2B). By contrast, spleen weights of mice infected with MHV-68-core or MHV-68-WT* were close to equal (Figure 2B). The frequency of reactivating virus in cells in the *ex vivo* reactivation assay was 1 in 14 000 splenocytes from mice infected with MHV-68-WT*, 1 in 49 000 splenocytes from mice infected with MHV-68-NS3 and 1 in 2000 splenocytes from mice infected with MHV-68-core (Figure 2C). Moreover, the viral genomic load was significantly higher in mice infected with MHV-68-core, compared to MHV-68-WT* (Figure 2D). Similar results were obtained after i.n. infection (data not shown).

Ex vivo expansion of NS3-specific, but not of core-specific T cells, by stimulation with MHV-68-NS3 or MHV-68-core. The use of MHV-68-NS3 infected target cells allowed for efficient *ex vivo* amplification of NS3-specific, MHC class I-restricted CD8-positive T cells derived from MVA-NS3-vaccinated C57BL/6 mice. Only splenocytes from MVA-NS3 immunized mice contained CD8+ T cells (approximately 7%) being specifically activated by MHV-68-NS3 infected targets (Figure 3A). Notably, splenocytes from MVA-NS3 immunized mice

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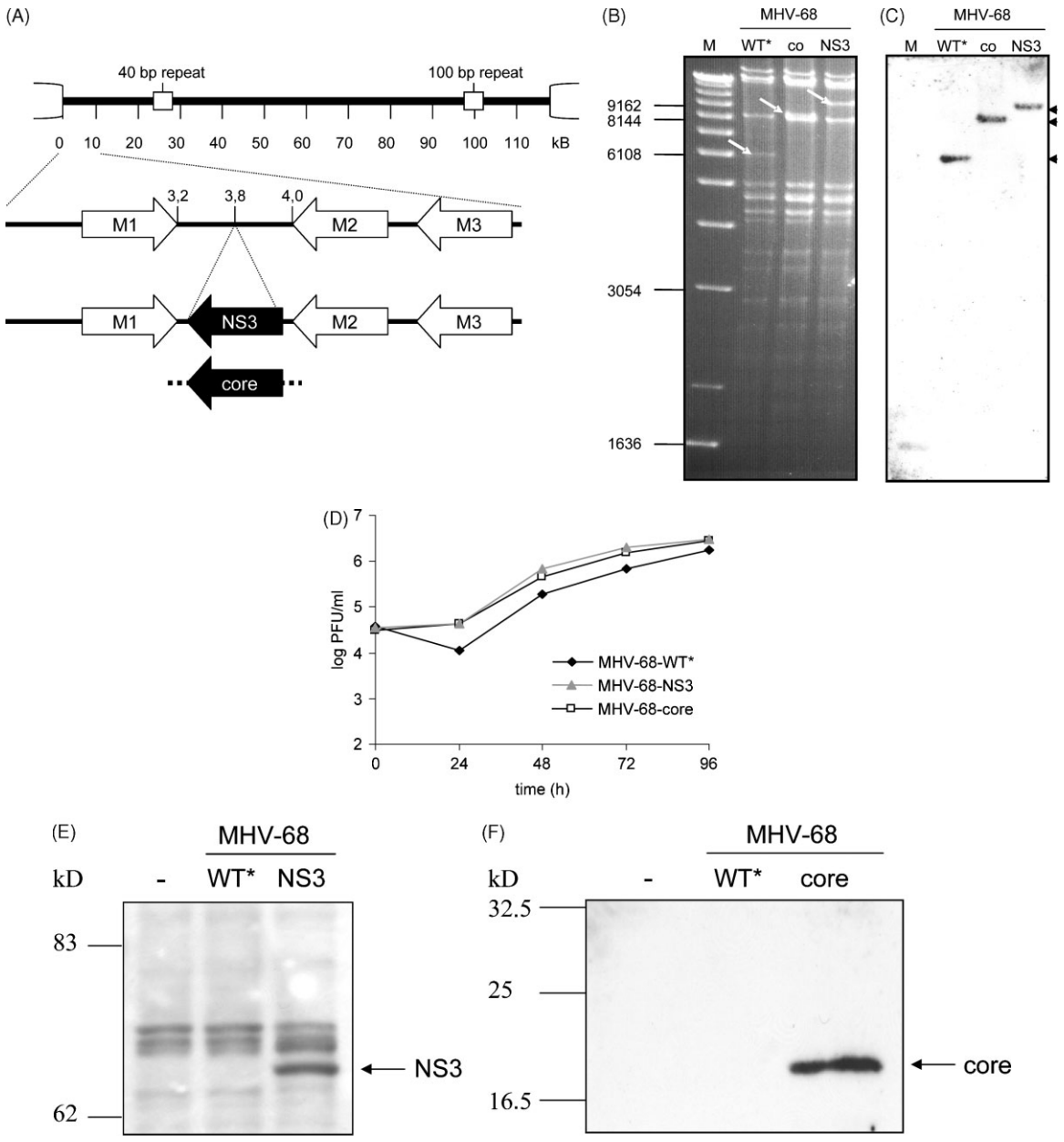

Figure 1. Construction and *in vitro* characterization of MHV-68-NS3 and MHV-68-core. (A) Schematic map of the MHV-68 genome with the inserted 3.8-kb NS3 and 2.5-kb core expression cassettes, respectively, indicated. (B) Ethidium bromide stained agarose gel of *Hind*III-digested viral DNA. (C) Southern blot analysis of the gel shown in (B) using a DIG-labelled probe (920 bp, nucleotide positions 2520–3440), recognizing a 6.1-kb-fragment in the parental BAC virus (= WT*), a 9.9-kb-fragment in the MHV-68-NS3 and a 8.6-kb-fragment in the MHV-68-core, indicated by arrows in (B) and arrowheads in (C). Marker sizes (in bp) are indicated on the left. (D) *In vitro* growth curves. NIH3T3 cells were infected with a MOI of 0.1 with MHV-68-WT*, MHV-68-NS3 or MHV-68-core. Cells were harvested at different timepoints and titers determined on BHK-21 cells. (E) and (F) Expression of NS3 and core, respectively, in infected fibroblasts. NIH3T3 cells were infected with MHV-68-WT*, MHV-68-NS3 or MHV-68-core with a MOI of 1. Forty-eight hours after infection, cells were harvested and expression of NS3 and core, respectively, was detected by western blot

were only activated with MHV-68-NS3 infected stimulator cells but not with MHV-68-WT* infected stimulator cells clearly demonstrating antigen specificity. By ^{51}Cr release assay, we confirmed that these activated T cells were also functional. Splenocytes from vaccinated mice were incubated with fibroblasts infected with MHV-68-NS3 or MHV-68-WT*, and only those derived from MVA-NS3 immunized mice were able to specifically lyse MHV-68-NS3 infected fibroblasts (Figure 3B). By contrast to NS3-specific vaccination, we were not able to demonstrate *ex vivo* amplification of core-specific CD8+ T cells from MVA-core-vaccinated C57BL/6 mice (Figures 3C and 3D).

Reduced viral loads of MHV-68-NS3 challenge virus after vaccination with NS-3 specific vaccines. To test the effect of vaccination with NS3 on the lytic replication of the challenge virus MHV-68-NS3, mice were immunized two times within 14 days with MVA-WT, MVA-NS3 or Ad-NS3/MVA-NS3. Eight weeks after the second immunization, mice were challenged i.n. with MHV-68-NS3, and lungs were harvested at day 6 after

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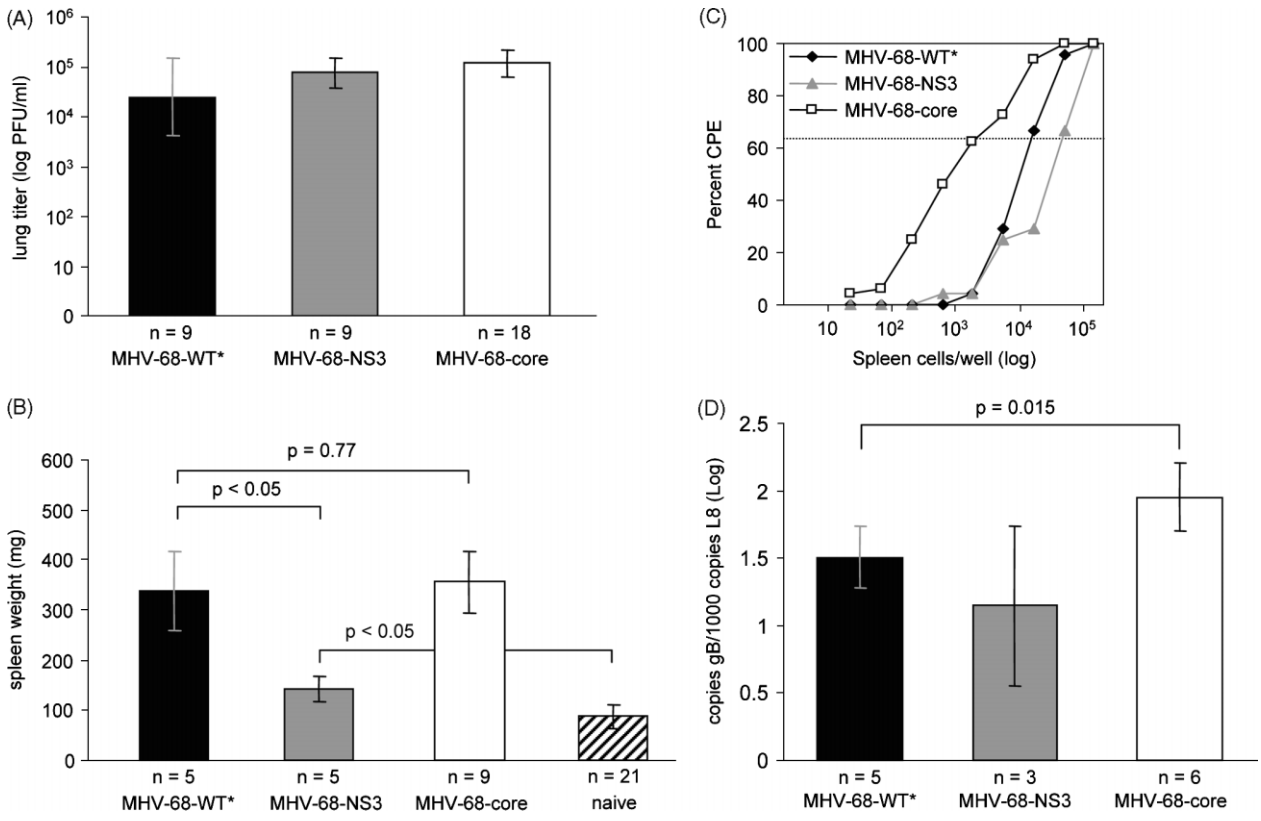

Figure 2. ***In vivo*** **characterization of MHV-68-NS3 and MHV-68-core. (A) Lytic virus titers in the lungs. C57BL/6 mice were infected i.n. with 5 x 10^4 PFU of MHV-68-WT*, MHV-68-NS3 or MHV-68-core. At day 6 after infection, lungs were harvested, and virus titers were determined from organ homogenates by plaque assay on BHK-21 cells. (B) Spleen weights, (C)** ***ex vivo*** **reactivation of splenocytes and (D) viral genomic load in the spleen. C57BL/6 mice were infected i.p. with 1 x 10^6 PFU of MHV-68-WT*, MHV-68-NS3 or MHV-68-core. At day 17 after infection, spleens were harvested and the spleen weights were taken. Single splenocyte suspensions were prepared and analysed in the** ***ex vivo*** **reactivation assay or used for DNA isolation for real time PCR analysis. Data shown in (A), (B) and (D) are the means ± SD of the number of individual mice indicated with** ***n*****. Data shown in (C) are from a representative experiment. For the** ***ex vivo*** **reactivation assay (C), splenocytes from three mice per group were pooled. The dashed line in (C) indicates the point of 63.2% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells reactivating lytic replication**

challenge. Mice immunized with MVA-NS3 showed a significant reduction of the lung titer compared to mice immunized with MVA-WT. This effect was even more pronounced when the mice were immunized with Ad-NS3/MVA-NS3 (Figure 4A). Importantly, immunization with Ad-NS3/MVA-NS3 did not reduce the lung titer of the challenge virus MHV-68-core, indicating that the observed effects are antigen-specific (compare Figure 4A with Figure 5A, left). To determine the influence of vaccination on the latent infection with MHV-68-NS3 as well as on T cell immunity, we immunized C57BL/6 mice twice within 14 days with MVA-WT, MVA-NS3 or Ad-NS3/MVA-NS3. Four weeks after the second immunization, mice were challenged i.p. with MHV-68-NS3 and spleens were harvested at day 21 after challenge. The frequency of reactivation was 1 in 120 000 for both the 2xMVA-WT/MHV-68-NS3 control group and the 2xMVA-NS3/MHV-68-NS3 group. By contrast, the mice immunized with Ad-NS3/MVA-NS3 showed a drastic reduction in the number of *ex vivo* reactivating splenocytes to an undetectable level in this assay (Figure 4B). Consistent with these results, mice immunized with Ad-NS3/MVA-NS3 and challenged with MHV-68-NS3 showed a substantial increase in the number of NS3-specific, interferon (IFN)-γ-secreting CD8+ T cells (Figure 4C). In addition, only mice immunized with Ad-NS3/MVA-NS3 showed a significant reduction of the viral genomic load in the spleen (Figure 4D) compared to the other groups. Similar results were obtained using different time points and routes of challenge infection (data not shown). By sharp contrast to the situation with NS3 but consistent with the failure to demonstrate *ex vivo* amplification of core-specific CD8+ T cells from MVA-core-vaccinated C57BL/6 mice (see above), vaccination with MVA-core or Ad-core/MVA-core did not reduce viral loads in the acute (Figure 5A) or latent phase (Figure 5B) of MHV-68-core challenge infection. This result was independent of the time point and route of challenge infection as tested in additional experiments (data not shown).

Discussion

The present study aimed to test the usefulness of recombinant MHV-68 expressing HCV candidate antigens for evaluation of the protective capacity of HCV-specific

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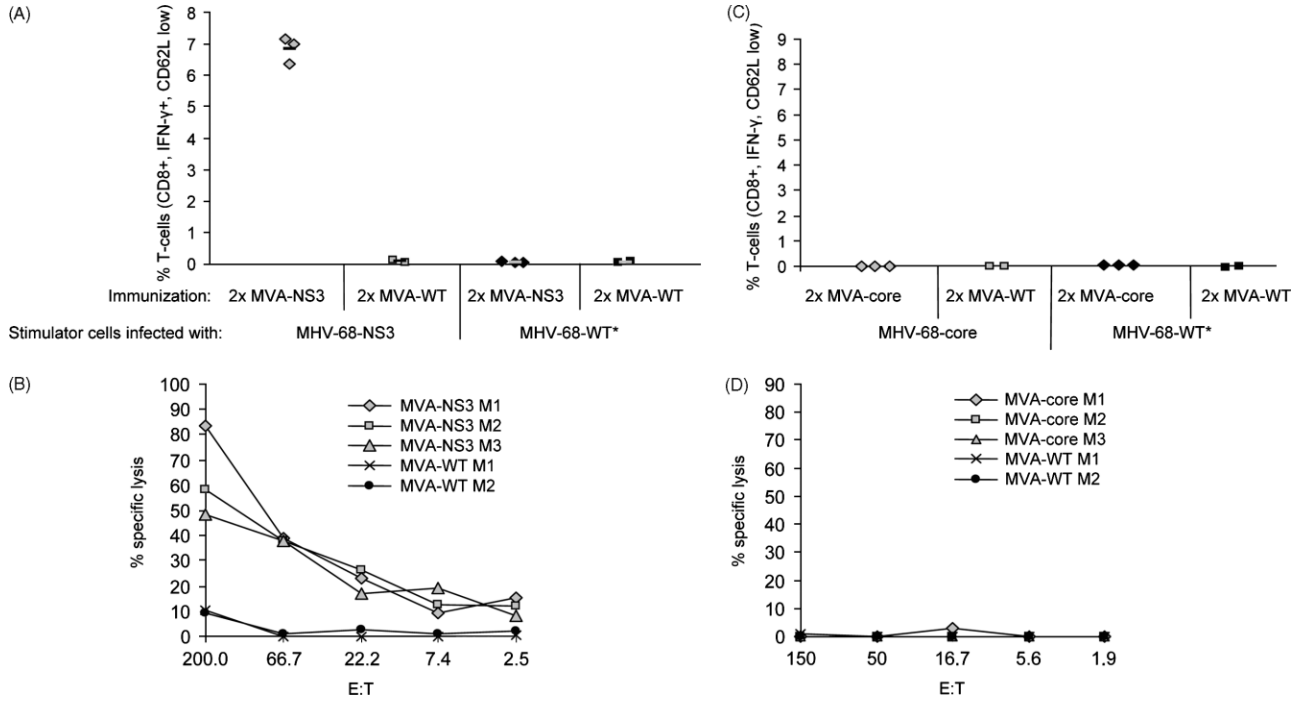

Figure 3. ***Ex vivo*** **expansion of NS3-specific, but not of core-specific T cells, by stimulation with MHV-68-NS3 or MHV-68-core, respectively. (A, C) Splenocytes isolated from MVA-WT, MVA-NS3 or MVA-core immunized C57BL/6 mice were re-stimulated** ***in vitro*** **with MC3T3 fibroblasts infected with MHV-68-WT*, MHV-68-NS3 or MHV-68-core (MOI = 1) as indicated. Subsequently, the intracellular cytokine staining assay was performed as described in the Materials and Methods. Only splenocytes from MVA-NS3 immunized mice contained CD8+ T cells being specifically activated by re-stimulation with the corresponding targets. Each symbol represents an individual mouse. (B, D) Splenocytes isolated from naive C57BL/6 mice were infected with MHV-68-WT*, MHV-68-NS3 or MHV-68-core (MOI = 0.1), washed, irradiated, and then used for re-stimulation of splenocytes isolated from mice vaccinated twice with MVA-WT, MVA-NS3 or MVA-core. After 5 days of culture, the lytic activity of the resultant CTL effector cells was determined at the indicated various effector to target cell (E/T) ratios in a 6-h ^{51}Cr-release assay as described in the Materials and Methods. Shown are the results from three individual mice immunized with MVA-NS3 or MVA-core, respectively (grey symbols), and two individual mice immunized with MVA-WT (black symbols). Data are from one representative experiment**

experimental vaccines. Spontaneous recovery from HCV infection in humans is typically associated with vigorous HCV antigen-specific T cell immune responses [33,34]. For assessment in the MHV-68 model system, we wished to test two different HCV-specific antigens. The NS3 protein of HCV represents a promising target for immunization because NS3-specific CD8+ and CD4+ T cell responses have been described as positive markers for viral clearance both in humans and chimpanzees [9,35–42]. Second, we chose the HCV core antigen, which represents a viral protein that is rather well conserved in different HCV genotypes and a potential target of cross-reactive immune responses [2,3]. Yet, this polypeptide has also been proposed as a potential viral modulator of host immune defense [4]. The core protein has been shown to bind the receptor of the complement protein C1q, to down-regulate interleukin (IL)-12 production by macrophages and consequently to inhibit proliferation and IL-2/IFN-γ synthesis of T cells [43–45].

Lytic replication *in vitro* of recombinant MHV-68-NS3 and MHV-68-core was indistinguishable from MHV-68-WT*. By contrast, *in vivo*, we found distinct differences. Although lytic replication of both MHV-68-NS3 and MHV-68-core was similar to MHV-68-WT*, we observed significant differences with regard to the parameters associated with latent infection. Whereas the spleen weight of mice infected with MHV-68-NS3 was significantly lower than the spleen weight of mice infected with MHV-68-WT*, the spleen weight of mice infected with MHV-68-core was very similar to the spleen weight of mice infected with MHV-68-WT*. The latent viral load was significantly lower in mice infected with MHV-68-NS3, but significantly higher in mice infected with MHV-68-core, compared to MHV-68-WT*. These data are suggestive for the immunogenicity and potential immunomodulatory functions of HCV-NS3 and HCV-core and further studies are clearly required on the underlying mechanisms.

Consistent with the initial observations, we were able to demonstrate that mice vaccinated with recombinant vector vaccines expressing HCV-NS3, but not with recombinant vector vaccines expressing HCV-core, showed significantly reduced viral loads in the acute and latent phase of challenge infection. Furthermore, the induction of CD8+ T cells specific for HCV-NS3 but not HCV-core could be demonstrated. These findings suggest a clear correlation of NS3-specific vaccine immunogenicity and protective capacity against challenge infection and are in agreement with the results obtained in a recent T cell-inducing viral vector vaccination study in chimpanzees [46]. That study suggested the principal

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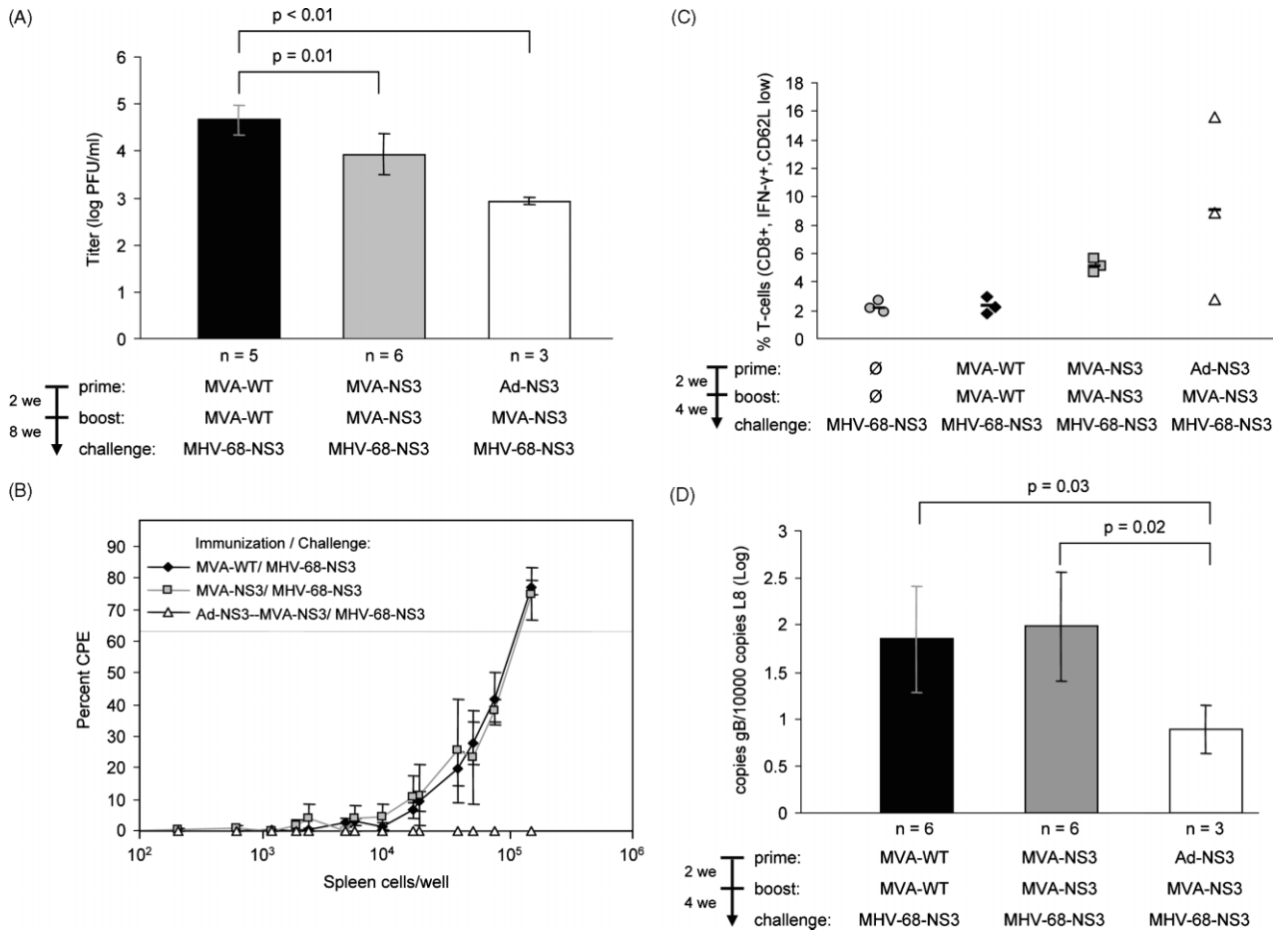

Figure 4. Reduced viral loads in the acute and latent phase of MHV-68-NS3 challenge infection after vaccination with MVA-NS3 or Ad-NS3/MVA-NS3 (A) Lung titers after i.n. challenge with MHV-68-NS3 8 weeks after prime-boost immunization as indicated. C57BL/6 mice were immunized twice within 14 days with MVA-WT, MVA-NS3 or Ad-NS3/MVA-NS3. Eight weeks after the second immunization, mice were challenged i.n. with MHV-68-NS3 and lungs were harvested at day 6 after challenge. Data shown are the mean ± SD of the number of individual mice indicated with *n*. (B) *Ex vivo* reactivation of splenocytes from mice challenged with MHV-68-NS3 4 weeks after prime-boost immunization. C57BL/6 mice were immunized twice within 14 days with MVA-WT, MVA-NS3 or Ad-NS3/MVA-NS3. Four weeks after the second immunization, mice were challenged i.p. with MHV-68-NS3 and spleens were harvested at day 21 after challenge. Single splenocyte suspensions were prepared and analysed in the *ex vivo* reactivation assay or used for DNA isolation for real-time PCR analysis (D). Data shown are the mean ± SEM pooled from two independent experiments for the groups immunized with MVA-WT and MVA-NS3, and from one experiment for the group immunized with Ad-NS3/MVA-NS3. In each experiment, splenocytes from three mice per group were pooled. The dashed line indicates the point of 63.2% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells reactivating lytic replication. To calculate significance, frequencies of reactivation events were statistically analysed by paired *t*-test over all cell dilutions. The statistical significance of the difference between the group immunized with Ad-NS3/MVA-NS3 and the other groups is $p = 0.03$. (C) Detection of NS3-specific, IFN-γ-secreting CD8-positive T cells by intracellular cytokine staining. C57BL/6 mice were immunized twice within 14 days with with MVA-WT, MVA-NS3 or Ad-NS3/MVA-NS3. Four weeks after the second immunization, mice were challenged i.p. with MHV-68-NS3 and spleens were harvested at day 21 after challenge. Single splenocyte suspensions were prepared, stimulated with LGAVQNEVTLTHPIT peptide and analysed by intracellular cytokine staining as described in the Materials and Methods. Each symbol represents an individual mouse. The horizontal bars indicate the mean. (D) Viral genomic load in the spleen of mice challenged with MHV-68-NS3 4 weeks after prime-boost immunization as indicated. Data shown are the mean ± SD of the number of individual mice indicated with *n*

feasibility of protective vaccination against HCV infection using non-structural antigens and demonstrated the prominent induction of NS3-epitope specific CD8+ T cells. In the present study, we observed a substantially enhanced immunogenicity of the Ad-NS3/MVA-NS3 prime boost regimen compared to a homologous MVA-NS3/MVA-NS3 prime boost. This result was anticipated given that other heterologous prime boost immunizations (e.g. the combination of influenza and MVA vector vaccines) has been shown to result in amplification of antigen-specific CD8+ T cell responses [47]. The first inoculation of recombinant MVA vaccine induces immune responses to MVA as well as HCV antigens. Upon homologous MVA booster immunization, it can be assumed that these responses compete with each other for preferential re-amplification [48]. By contrast, the heterologous Ad-NS3/MVA-NS3 prime boost regimen allows to exclusively intensify the NS3-specific response.

By contrast to the NS3-specific responses, we could not demonstrate the induction of CD8+ T cells specific

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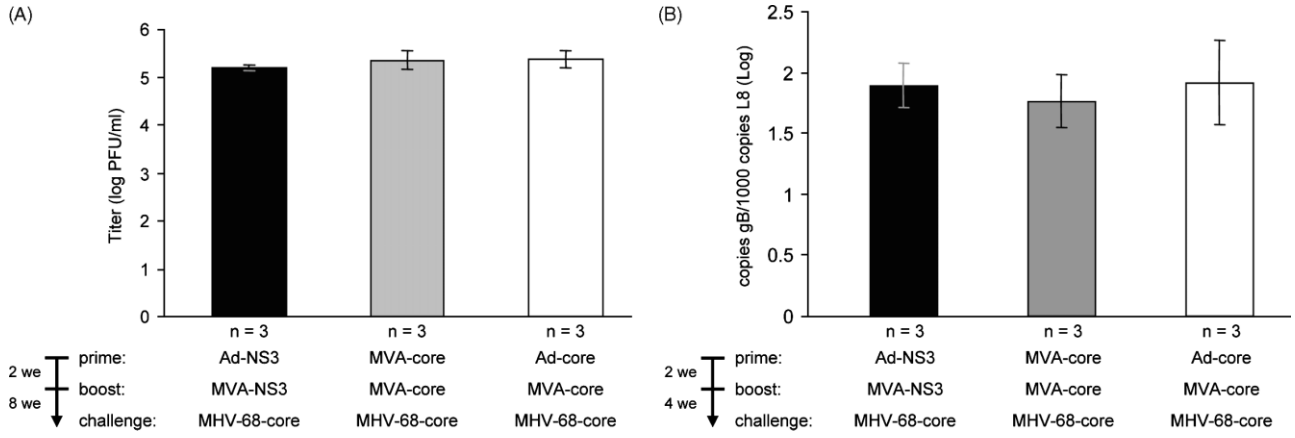

Figure 5. Vaccination with MVA-core or Ad-core/MVA-core does not reduce viral loads in the acute and latent phase of MHV-68-core challenge infection. (A) Lung titers after intranasal challenge with MHV-68-core 8 weeks after prime-boost immunization as indicated. C57BL/6 mice were immunized two times within 14 days with Ad-NS3/MVA-NS3 (as the control), MVA-core or Ad-core/MVA-core. Eight weeks after the second immunization, mice were challenged i.n. with MHV-68-core and lungs were harvested at day 6 after challenge. Data shown are the mean ± SD of the number of individual mice indicated with *n*. (B) Viral genomic load in the spleen of mice challenged with MHV-68-core 4 weeks after prime-boost immunization as indicated. C57BL/6 mice were immunized twice within 14 days with Ad-NS3/MVA-NS3 (as the control), MVA-core or Ad-core/MVA-core. Four weeks after the second immunization, mice were challenged i.p. with MHV-68-core and spleens were harvested at day 17 after challenge. Single splenocyte suspensions were prepared and used for DNA isolation for real-time PCR analysis. Data shown are the mean ± SD of the number of individual mice indicated with *n*

for HCV-core by using MC3T3 cells infected with MHV-68-core for re-stimulation. One possibility we considered was that the MVA-core vector used is non-immunogenic. However, we could demonstrate core-specific CD8+ T cells in MVA-core or MVA-1-830 (core, E1, E2, p7) immunized BALB/c or transgenic HHD mice, with the latter expressing human HLA-A*0201, by using a HCV-core specific peptide (HLA-A*0201-restricted epitope DLMGYIPLV; amino acids 132–140) [49] for stimulation, thus ruling out a possible malfunction of the vector (data not shown). Another possibility was that the sequence of core in MVA-core is not exactly the same as the one in the MHV-68-core. However, sequencing analysis confirmed that both sequences are identical (data not shown). It is possible that the mode of re-stimulation may have an impact on the detection of core-specific CD8+ T cells. However, *in vivo*, core-specific immunization did also not provide protection against MHV-68-core challenge infection. Thus, from our analysis of vector vaccine-induced immune responses, the HCV-core antigen must be considered as an extremely poor immunogen at least in mice. This is consistent with the suppression of vaccinia virus-specific T cell responses after infection of mice with recombinant vaccinia virus expressing HCV-core [50]. Thus, our data further corroborate the possible role of core to down-regulate host T cell responses [43–45] and to contribute to HCV-specific immune evasion. The underlying mechanisms of this potential viral immunomodulation clearly require more detailed study. By contrast to the findings by Folgori *et al*. [46], the vaccination of chimpanzees with an ISCOM adjuvanted NS-3-4-5-core polyprotein T cell vaccine or a DNA/MVA immunization delivering core-E1-E3-NS3 could not prevent chronic persistent HCV infection [51–52], and one might speculate about a negative impact due to core protein activity.

In summary, the present study suggests that recombinant MHV-68 is a highly suitable novel model to test immunogenicity and protective capacity of HCV candidate antigens, including specific T cell antigens. We clearly corroborate the usefulness of NS3-specific immunization. By contrast, our analysis discarded core as a vaccine antigen. Thus, the MHV-68 challenge model should prove useful for the evaluation of candidate vaccines not only against HCV, but also against other persisting virus infections.

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