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Corrigendum

Corrigendum to "MHV-68 producing mIFNα1 is severely attenuated *in vivo* and effectively protects mice against challenge with wt MHV-68" [Vaccine 29 (2011) 3935–3944]

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The authors would like to rectify an error that occurred in their article.
James P. Stewart was mistakenly omitted from the published list of authors. His details have now been added.
The authors apologize for any inconvenience caused.

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MHV-68 producing mIFNα1 is severely attenuated *in vivo* and effectively protects mice against challenge with wt MHV-68

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ABSTRACT

Human gammaherpesviruses such as Epstein–Barr virus (EBV) cause lifelong infections and associated diseases, by virtue of their ability to establish latent infection. Many studies performed in the past years in murine herpesvirus 68 (MHV-68) model of infection suggested that the limited immunity generated against isolated viral components by subunit vaccines cannot counteract the multiple immune evasion strategies operated by gammaherpesviruses. Indeed, a significant inhibition of long-term latency establishment could be observed in mice vaccinated with strains of genetically modified MHV-68 defective in reactivation or establishment of latency.

In this study, we focused on the effects of interferon-α (IFN-α) on both the lytic and latent phase of MHV-68 infection, as exerted by the constitutive release of IFN-α1 by a clone of MHV-68 genetically modified to produce this cytokine (MHV-68mIFNα1). Although the MHV-68mIFNα1 recombinant virus exhibited *in vitro* replication features indistinguishable from those of the wild type MHV-68, its pathological properties were severely attenuated *in vivo* in immunocompetent mice and not in mice rendered genetically unresponsive to type I IFN, suggesting that a stronger immune response was primed in the presence of the cytokine. Notably, MHV-68mIFNα1 attenuation did not result in a reduced level of long-term spleen latency establishment. These results prompted us to evaluate the efficacy of MHV-68mIFNα1 in a prophylactic vaccination regimen aimed at inhibiting the symptoms of acute virus infection and the establishment of long-term latency after MHV-68 challenge. Our results show that mice vaccinated with MHV-68mIFNα1, administered as a live-attenuated or partially inactivated (by Psoralen and UV treatment) vaccine, were protected against the challenge with wt MHV-68 from all phases of infection. The ability of MHV-68mIFNα1 to produce IFN-α at the site of the infection, thus efficiently stimulating the immune system in case of virus reactivation from latency, makes this recombinant virus a safer live-attenuated vaccine as compared to the previously reported latency-deficient clones.

1. Introduction

The search for an effective vaccine aimed at reducing the transmission of gammaherpesviruses, such as EBV or HHV8, causing severe diseases in humans, recently drove an increasing interest in studying the murine herpesvirus 68 virus (MHV-68) model of infection. In the last decades, this model has been exploited to understand the physiology of gammaherpesvirus infection and to test the efficacy of vaccination strategies against this class of viruses. The failure of most of the vaccination approaches based on structural proteins, lytic or putative latency-associated antigens in protecting against all phases of MHV-68 infection [1–11], suggested that the limited immunity generated against isolated viral components cannot counteract the multiple immune evasion strategies operated by the virus. It is a general opinion that vaccination against gammaherpesviruses should mainly minimize virus-induced lymphocyte proliferation and reduce the long-term latent viral load, since these are the features more likely responsible for the development of gammaherpesvirus-associated lymphoproliferative diseases [12].

A number of studies utilized DNA recombinant techniques to generate genetically modified MHV-68 viruses, not only to identify the function of viral genes, by assessing the changes in virus replication caused by genome targeted disruptions [13], but also to generate latency defective recombinant MHV-68 clones, and evaluate their efficacy as vaccines in protecting mice against

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the challenge with the "wild type" (wt) virus [5–8,11]. With one exception [14], the vaccination of mice with live attenuated latency-deficient recombinant MHV-68 gave the best results in terms of protection against the establishment of long-term latency [5–8,11,15]. In general, the advantage of live-attenuated *vs* replication-deficient virus vaccines relies on their high immunogenicity, based on their ability to stimulate both local and systemic response against a broader spectrum of epitopes for cell-mediated and humoral immunity. However, viruses replicating and persisting in a latent state are not considered completely safe, since their long-term persistence might have unpredictable consequences.

In the study presented herein, we generated a recombinant MHV-68 expressing the mouse IFN-α1 coding sequence to evaluate the effects of the constitutive production of this cytokine on MHV-68 replication *in vitro* and *in vivo*. Originally discovered for their antiviral activity, type I IFN (IFN-I) are now recognized to exert a variety of immunomodulatory activities in both human and mouse models [16,17]. Notably, by virtue of its well documented role in dendritic cell (DC) differentiation/maturation and activity, IFN-I are now considered as important cytokines linking the innate and adaptive immune response to infections [18].

As expected on the basis on their antiviral activity, endogenous IFN-I play a significant role in limiting MHV-68 replication at early stages of infection and before the onset of the adaptive immune response [19]. In addition, IFN-I counteract viral latency, both by contributing to the development and maintenance of an anti-MHV-68 innate immune response [20], and by controlling virus reactivation from latency [21]. Notably, like many herpesviruses, MHV-68 evolved several immune evasion strategies to neutralize IFN-I-mediated control of acute and latent infection [22–25]. In the light of all this, we hypothesized that a recombinant MHV-68 producing IFN-α could represent a useful tool for further understanding the role of this cytokine in the different phases of virus infection *in vivo*. Furthermore, we assumed that the secretion of IFN-α by the cells infected with the recombinant virus could neutralize the inhibitory strategies evolved by MHV-68 against IFN-I antiviral activities, thus allowing the immune stimulatory properties of the cytokine to be more effective.

In the study presented herein, we report the effects of the continuous production of IFN-α1 on the course of infection of a clone of MHV-68 genetically modified by the insertion of the mouse IFN-α1 coding gene (MHV-68mIFNα1). Moreover, we describe the efficacy of MHV-68mIFNα1 administered as a live-attenuated or partially inactivated virus (following Psoralen and UV treatment) in protecting mice from the challenge with wt MHV-68.

2. Methods

2.1. Mice

Four to five week-old 129Sv and C57BL/6 mice (H-2^b) were purchased from Charles River Italia (Italy). IFNARI$^{-/-}$ mice have a targeted disruption in an essential chain of the IFN-I receptor gene (IFNARI), and do not respond to IFN-I. A colony of IFNARI$^{-/-}$ 129Sv was established at the Department of Cell Biology and Neurosciences of the Istituto Superiore di Sanità, Rome. IFNARI$^{-/-}$ C57BL/6 mice have been obtained from EMBL, Monterotondo, Italy. Mice were housed in plastic cages and maintained under specific pathogen-free conditions in the animal house of the Istituto Superiore di Sanità.

2.2. Cell lines

IFNARI$^{-/-}$ cells were grown in Dulbecco's medium complemented with 10% FCS, 10% TPB, penicillin/streptomycin (70 μg/ml and 10 μg/ml) and L-glutamine (2 mM). IFNARI$^{-/-}$ cells were derived from embryonic fibroblasts isolated from an IFNARI$^{-/-}$ 129Sv mouse, immortalized *in vitro* by transfecting a SV40 T antigen expressing plasmid. αRec14 cells have been obtained by stably transfecting into a fibroblast cell line originated from IFNARI$^{-/-}$ 129Sv mouse a plasmid encoding the IFNARI coding sequence, to recover a functional IFN-I receptor.

2.3. Virus stocks

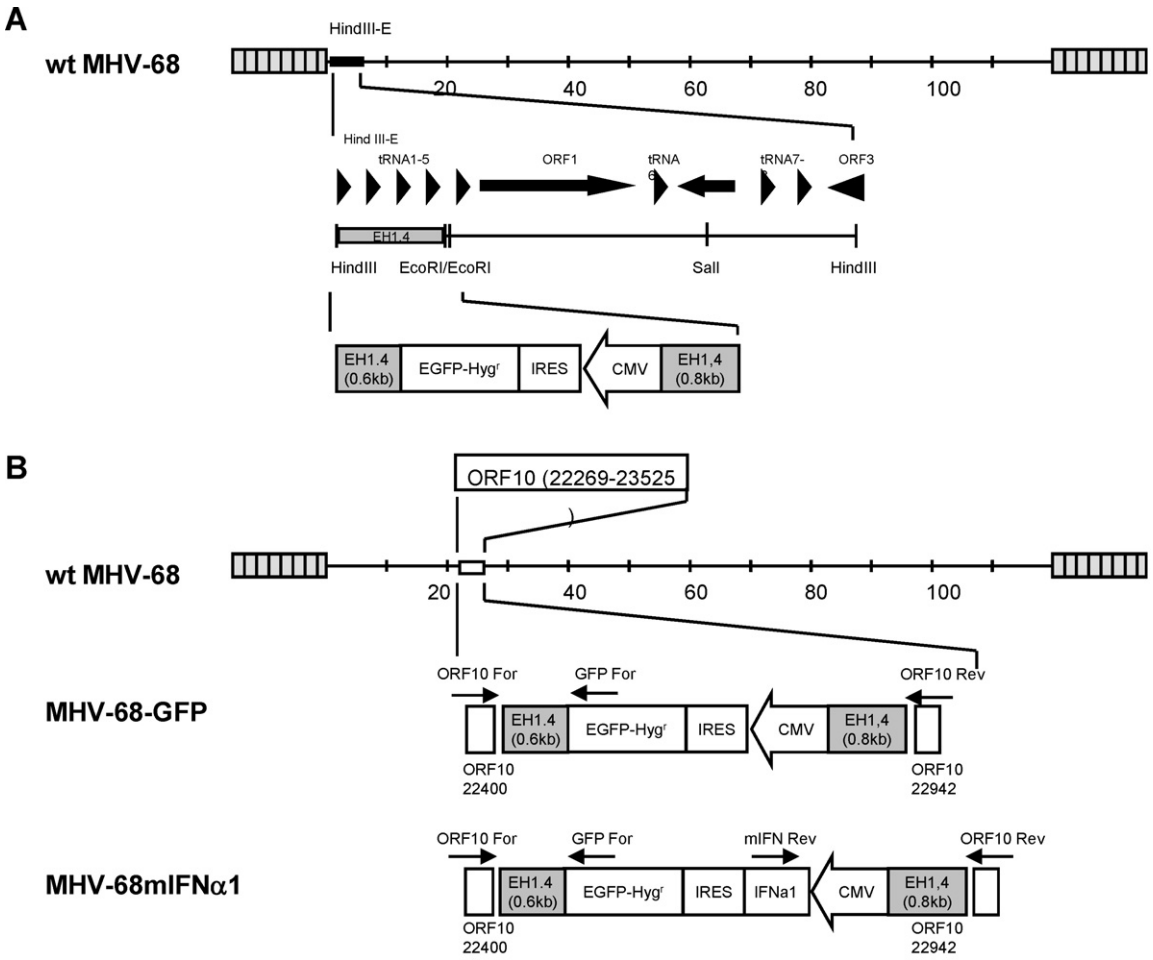
MHV-68 was originally obtained from Professor D. Blaskovic [26]. To avoid the possible interference of the antiviral activity of IFN-I produced by recombinant MHV-68, IFNARI$^{-/-}$ cells have been used for the production and titration of the wt and recombinant virus stocks. Working stocks of MHV-68 clone g2.4 [27], MHV-68mIFNα1 and MHV-68-GFP were prepared by infection of IFNARI$^{-/-}$ cells at 0.01 PFU/cell as previously described [28]. Virus stocks were titered by the previously described plaque assay [4] performed on IFNARI$^{-/-}$ cells.

2.4. Construction of recombinant viruses

Two clones of recombinant MHV-68 were produced by homologous recombination in IFNARI$^{-/-}$ cells. To obtain the insertion of the mIFN-α1 gene into the 1400 bp left end region of MHV-68 genome (named EH1,4), comprised between the HindIII and EcoRI restriction site, the IFN-α1 coding sequence was cloned into a plasmid specifically designed to drive homologous recombination in that site. The expression cassette consisted of the CMV-IE promoter and the EGFP-Hygr gene from EGFPHyg (Clontech), followed by EMCV IRES element. The gene encoding mIFN-α1 was cloned downstream from the IRES, followed by the SV40 poly(A) signal. The whole cassette was flanked by two portions of the EH1,4 MHV-68 region, of 0.6 and 0.8 kb, respectively. MHV-68 DNA (5 μg) and 10 μg of linearized plasmid were transfected by electroporation into 2×10^6 IFNARI$^{-/-}$cells by double-pulse setting (high-voltage setting = 600 V, 25 μF, 99 Ω; low-voltage setting = 260 V, 1500 μF, 329 Ω; 0.1-s interpulse delay) on an EasyJect electroporator (EquiBio). Electroporated cells were cultured in a six-well plate. Green fluorescent plaques were picked after 5 days and subjected to four rounds of plaque purification by limiting dilution (in 96-well plates) with hygromycin selection (100 μg/ml) on IFNARI$^{-/-}$ cells. Each round of purification was verified by a PCR to amplify mIFN-α1 or GFP, and by IFN-I titration on cell supernatant. Once a pure population of green fluorescent plaques was obtained, the genomic structure was analyzed. MHV-68 genomic DNA was prepared from purified virions as described previously [27]. PCR was performed on DNA samples to obtain the molecular characterization of the recombinant viruses. The following primers were used: ORF10-For: AAGTCTGCCCCCTCGATTAT (nucleotide position in MHV-68 genome: 22275), ORF10-Rev: TAGAGGGTCTGCCACTCCAT (nucleotide position in MHV-68 genome: 23505), mIFN-α1-Rev: TGAGTCTGAGGCAGGTCACA (nucleotide position in mIFN-α1 gene: 97), GFP-For: CGACCACTACCAGCAGAACA (nucleotide position in GFP gene: 31).

2.5. IFN-I titration

IFN-I was titrated on L929 cells as described previously [29]. IFN-I titers are expressed in International Units (IU). To avoid the possible infection of L929 cells by the virus present in test supernatants, making advantage of IFN-I pH stability, test samples underwent overnight pH 2 acidification by HCl addition, followed by NaOH neutralization before the inoculation on L929 monolayers.



C

	MHV68mIFNα1				MHV68GFP			
Primer	ORF10-For	ORF10-Rev	GFP-For	mIFNa1-Rev	ORF10-For	ORF10-Rev	GFP-For	mIFNa1-Rev
ORF10-For		-	+	-		-	+	-
ORF10-Rev	-		-	+	-		-	-
GFP-For	+	-		-	+	-		-
mIFNa1-Rev	-	+	-		-	-	-	

Fig. 1. Construction and molecular characterization of MHV-68mIFNα1 and MHV-68-GFP recombinant viruses. (A) The enlargement shows the structure of the unique portion of the left end of MHV-68 genome and the plasmid cassette designed to drive the homologous recombination in EH1,4 region. (B) The diagram represents the structures of the obtained recombinant viruses, in comparison with wtMHV-68 genome. The recombination caused the replacement of 542 bp of viral ORF10 by the plasmid expression cassettes. The name and positions of the PCR primers used to confirm the insertion and the orientation of the cassette into viral genome are also indicated. (C) The results of the PCR performed with different combinations of primers, complementary to specific sequences of the inserted genes (mIFN-α1 and GFP), or to ORF10 were confirmed by sequencing analysis.

2.6. Virus PSUV inactivation

Psoralen (49-aminomethyl-Trioxsalen; Calbiochem, La Jolla, CA) (stock solution = 100 μg/ml in H_2O) was added to wt MHV-68 and MHV-68mIFNα1 suspended at 10^6 PFU/ml in Hanks balanced salt solution (0.1% bovine serum albumin). Psoralen final concentration was 1.5 or 3 μg/ml. The suspension (1 ml in 35-mm-diameter wells) was incubated 10′ at room temperature and irradiated in a Stratalinker 1800 UV cross-linking unit equipped with 365-nm UV bulbs. Two different distances (2 cm and 4 cm) between the bulbs and the plates were tested. The remaining plaque-forming activities of the inactivated virus were determined by plaque assay (See Section 2.3).

2.7. Mice infection and analysis of tissues

Four to five week-old female mice were infected under halothane anesthesia by intranasal (i.n.) injection of 1×10^5 PFU of virus in 40 μl of sterile phosphate-buffer saline (PBS). At various time points p.i. mice were sacrificed by anterior neck dissection and tissues were harvested for analysis. For BAL collection, the trachea was cannulated with a 22-gauge angiocatheter and lungs lavaged with 1 ml of PBS. Whole blood was collected by cardiac puncture using a 25-gauge needle and syringe without heparin. Serum was isolated from whole blood using a serum separator tube (BD Biosciences). PBMC were isolated by Ficoll gradient separation and resuspended in PBS. Infectious virus titers in the lungs and latent

virus in the spleens were determined by the plaque assay and the infectious centers assay previously described by us [4], performed on IFNARI$^{-/-}$ cells.

2.8. Mice vaccination

Groups of 6 4–5 weeks old female C57BL/6 mice were vaccinated by i.n. injection (under halothane anesthesia) of 1×10^5 PFU of live-attenuated MHV-68mIFNα1, or the same dose of erstwhile PFU of wt MHV-68 or MHV-68mIFNα1 exposed to the PSUV treatment tested to cause partial virus inactivation (1.5 μg/ml, 4 cm distance from UV lamp). All mice were boosted two weeks later by a second administration of the same vaccine. Two weeks later, half of the vaccinated mice were anaesthetized and challenged i.n. with 4×10^5 PFU of wt MHV-68 (or lower doses, when indicated). At various time points p.i., mice were sacrificed by anterior neck dissection and tissues were harvested for analysis (see Section 2.7).

2.9. Immunophenotypic analysis

Cells isolated from spleens or PBMC were washed, resuspended in PBS containing 1% FBS, and incubated with fluorochrome-conjugated, anti-mouse CD19, CD69 CD8, CD62 or Vb4 mAbs (BD PharMingen) for 30 min at 4 °C. After two washings with PBS, cells were analyzed by flow cytometry. Data were collected and analyzed by using a FACSort (BD Biosciences, Bedford, MA) flow cytometer, and data analysis was performed by CellQuest software (BD Biosciences).

2.10. Real time PCR

Genomic DNA was extracted from mice splenocytes by the DNeasy Tissue Kit (QIAGEN, Valencia, CA). To specifically quantify wt MHV-68 *vs* MHV-68mIFNα1 genomes, primers specific for MHV-68 ORF50 gene (ORF50-For 5′-ATGGCACATTTGCTGCAGAAC-3′, ORF50-Rev 5′-ACGGCGCCTGTGTACTCAA-3′) were used to measure the total number of MHV-68 genomes (wt and recombinant); primers specific for the IRES sequence (IRES-For 5′-CTAACGTTACTGGCCGAAGC-3′, IRES-Rev 5′-GGAACTGCTTCCTTCACGAC-3′) quantified MHV-68mIFNα1 genomes. Quantitative measurements of virus DNA were acquired by real-time PCR using ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Wellesley, MA). For each sample, 200 ng of DNA was amplified in duplicate with QuantiTect SYBR Green PCR reagents (Qiagen) and 300 nM of each primer. To verify that a single product was amplified, a melting curve was generated at the end of every run. For each gene, standard curves were obtained by measuring 1–10^8 copies of plasmids containing ORF50 or IRES on a background of 200 ng of uninfected splenocyte DNA. The results obtained for ORF50 were confirmed by using DNA isolated from the S11 cell line [30] as standard. The detection limit was 1 copy/200 ng of genomic DNA.

2.11. Statistical analysis

Data were analyzed by Mann–Whitney test.

3. Results

3.1. Generation of recombinant MHV-68 expressing mIFN-α1

To avoid the disruption of critical virus functions, the insertion of the murine IFN-alpha1 (mIFN-α1) coding sequence was designed to occur in the left end of the unique portion of MHV-68 genome, containing four tRNAs-like sequences (Fig. 1A) and reported to be unessential for virus replication [31]. Southern blotting analysis of the DNA isolated from the clones of recombinant MHV-68 expressing mIFNα1 or GFP only (as control) showed that the recombination between the plasmid cassette and MHV-68 DNA had not occurred into the EH1,4 region of the virus genome (*data not shown*). Further investigations carried out by PCR specifically amplifying the inserted genes (mIFN-α1 and GFP), or the wt MHV-68 genome (Fig. 1B and C) revealed that the recombination event had replaced 542 bp of MHV-68 ORF10 with the plasmid cassette (Fig. 1B). Since ORF10 belongs to a group of MHV-68-coded genes proven to be nonessential for virus replication *in vitro* and *in vivo* [32], any modification in the *in vitro* and *in vivo* replication of the recombinant virus producing IFN-α could not be ascribed to the deletion of this viral gene. This possibility was further ruled out by performing the subsequent *in vitro* and *in vivo* experiments in the presence or absence of a functional IFN-I system and including the MHV-68-GFP as a control for the effects of the recombination *per se*.

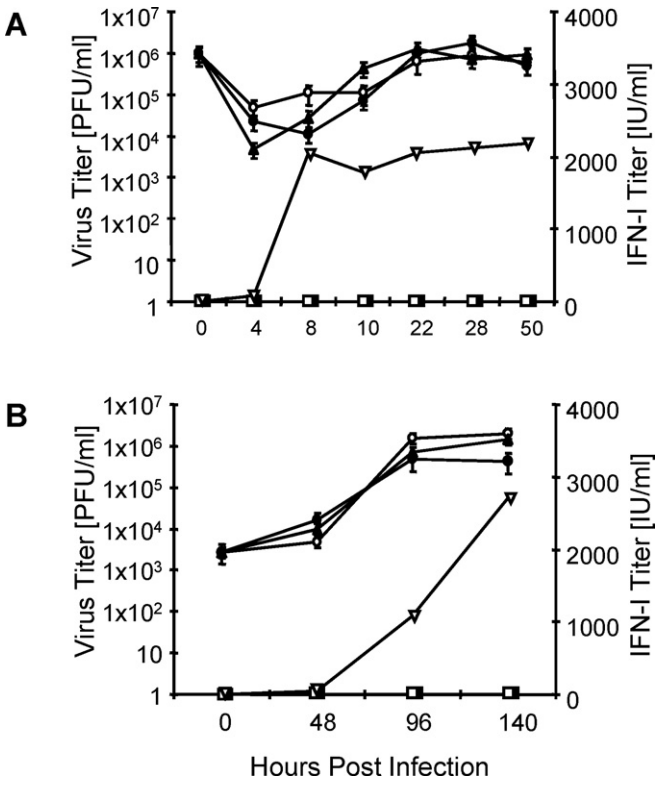


Fig. 2. *In vitro* replication of wtMHV-68 (●), MHV-68-GFP (○) and MHV-68mIFNα1 (▲) on aRec14 cells in a one-step (A, m.o.i. = 5) and multi-step experiment (B, m.o.i. = 0.05). The amount of IFN-α released in the culture supernatants by wtMHV-68 (■), MHV-68-GFP (□) and MHV-68mIFNα1 (▼) is also shown. Data are represented as mean virus (left scale) or IFN-I (right scale) titers ± SD and are representative of two separate experiments, each performed in triplicate.

3.2. In vitro replication of recombinant MHV-68mIFNα1

The replication kinetics of MHV-68mIFNα1 and wt MHV-68 were compared in a one-step (Fig. 2A) and a multi-step replication experiment (Fig. 2B), in which the amount of IFN-I released in the supernatant by the infected cells was also measured. Despite the high amount of the cytokine released in the supernatant of cells infected with the recombinant virus, the kinetics of viral replication *in vitro* was not affected by the genetic modification, and was similar to the one exhibited by wt and control virus, consistently with the observation that MHV-68 replication *in vitro* is not significantly impaired in IFN-α-treated cells (Supplementary Fig. 1).

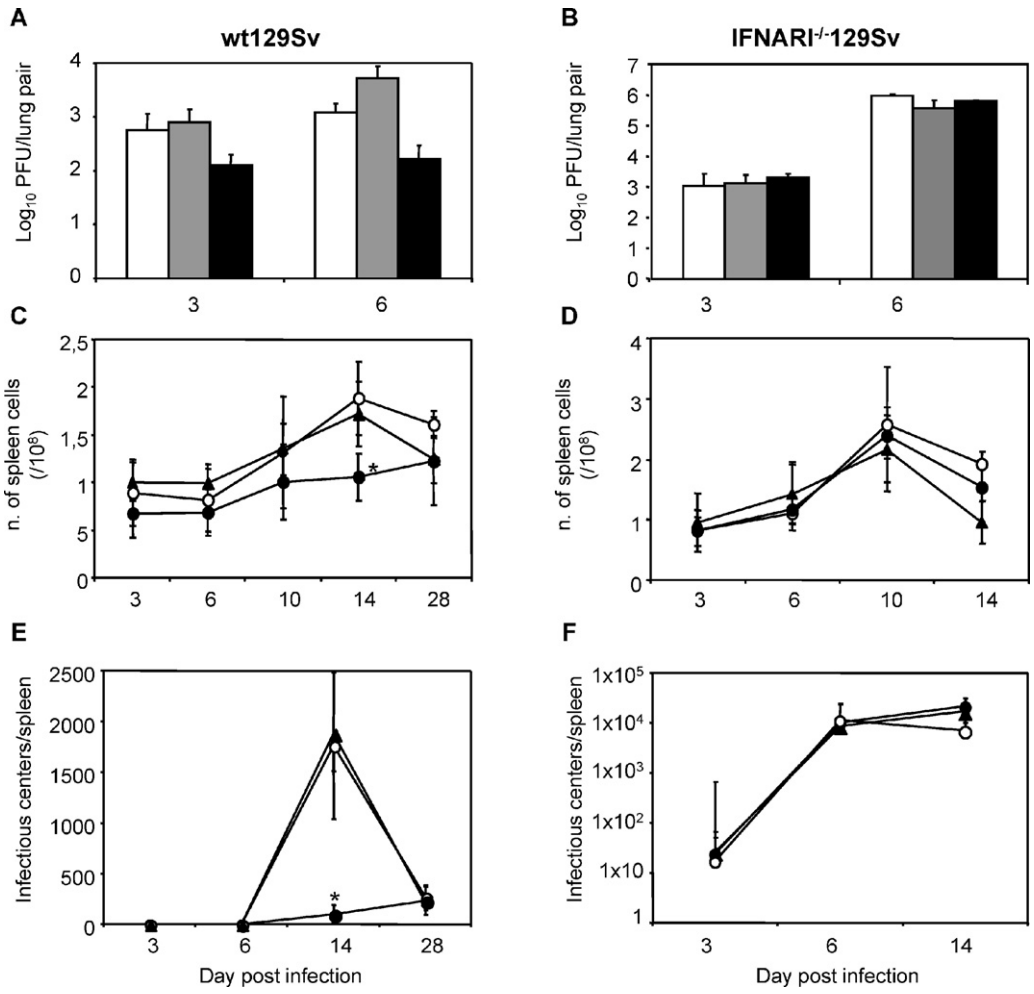


Fig. 3. Biological characterization of recombinant MHV-68mIFNα1 in wt (A, C, E) or IFNARI$^{-/-}$ 129Sv mice (B, D, F). (A and B) Viral replication in the lungs of mice infected i.n. with 1×10^5 PFU of wtMHV-68 (white bars), MHV-68-GFP (grey bars) or MHV-68mIFNα1 (black bars). The mean $\log_{10}$ virus titer ± SD for six mice per group is shown for each time point. (C and D) Number of spleen cells following i.n. infection of mice with 1×10^5 PFU of wtMHV-68 (○), MHV-68-GFP (▲) or MHVmIFNα1 (●). The mean total number of splenocytes ± SD for six mice per group is shown for each time point. (E and F) Latent virus in the spleens of mice infected with 1×10^5 PFU of wtMHV-68 (○), MHV-68-GFP (▲) or MHVmIFNα1 (●) as determined by IC assay. The mean number of IC per spleen ± SD for six mice per group is shown for each time point. $^*p < 0.005$ *vs* GFP and wt MHV-68.

3.3. *In vivo replication of recombinant MHV-68mIFNα1*

The *in vivo* effects of the continuous production of IFN-α by cells infected with MHV-68mIFNα1 were evaluated by infecting wt and IFNARI$^{-/-}$ 129Sv mice i.n. with 10^5 PFU of wt MHV-68 or each of the recombinant viruses. Notably, MHV-68mIFNα1 capability to induce the production of IFN-α *in vivo* was demonstrated by the detection of IFN-I in mice serum and bronchoalveolar lavages (BAL) six days after the infection (Supplementary Table 1). The viral load in the lungs of mice infected with MHV-68mIFNα1 was significantly reduced as compared to mice infected with wt or control virus on day 3 and 6 post infection (p.i.) (Fig. 3A). This reduction was specifically mediated by the IFN produced following infection with the recombinant MHV-68, since it was not observed in mice infected with the control virus encoding GFP (Fig. 3A) and in IFNARI$^{-/-}$ mice (Fig. 3B). MHV-68mIFNα1 also failed to induce the increase in splenocyte numbers observed in wt mice infected with wt MHV-68 and MHV-68-GFP and peaking 14 days after infection (Fig. 3C). Notably, no difference in the extent of virus-driven splenomegaly, typically caused by MHV-68-induced B cells activation, was observed in mice lacking a functional IFN-I system (Fig. 3D). Upon infection with MHV-68mIFNα1, the peak of splenocytes reactivating latency observed two weeks after infection with the wt or control virus was virtually absent in normal mice, whereas it occurred in IFNARI$^{-/-}$ mice indistinguishably when compared to mice infected with the wt or control virus (Fig. 3E and F). Notably, at the latest time point (28 days p.i.) the extent of splenic latency reactivation was similar and very close to the limit of detection of the IC assay for all mice (Fig. 3E).

3.4. *MHV-68-driven activation of B cells and infectious mononucleosis-like syndrome*

The IFN-mediated attenuation of MHV-68mIFNα1 replication *in vivo* was confirmed following infection of C57BL/6 mice (Supplementary Fig. 2). In these mice, we also assessed whether MHV-68mIFNα1-related reduced amplification of latently infected cells was associated with the impairment in the usual virus driven B-cells activation [33]. At 14 days p.i., mice infected with MHV-68mIFNα1 had a significantly lower level of activated B cells (CD19$^+$ CD69$^+$) with respect to the levels induced by wt MHV-68 (Fig. 4A). This reduction was mediated by the IFN produced following infection with the recombinant virus, since it was absent in IFNARI$^{-/-}$ (Fig. 4A).

To assess whether the attenuation of MHV-68mIFNα1 *in vivo* replication affected the virus-induced mononucleosis, also resulting from B-cells amplification, the presence of activated CD62L^{low} CD8$^+$ lymphocytes and the level of CD8$^+$ T cells expressing Vβ4

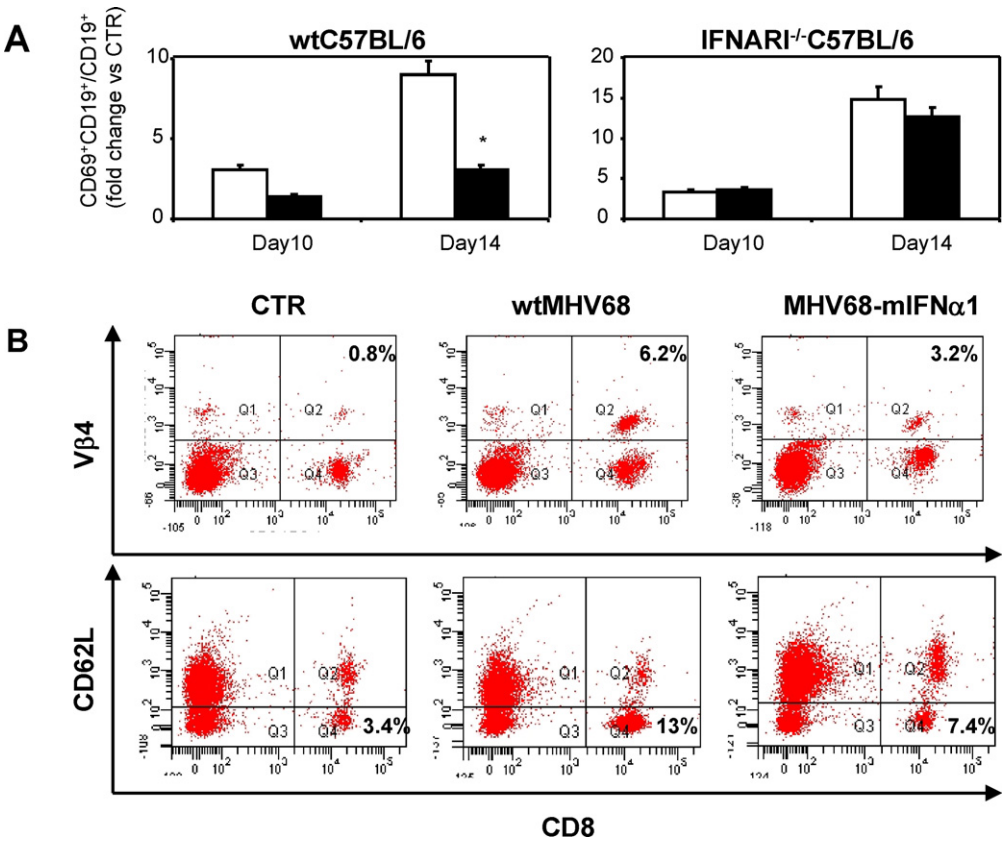


Fig. 4. Virus-driven B cell activation and Infectious Mononucleosis-like syndrome. (A) Spleen cells from wt or IFNARI$^{-/-}$ C57BL/6 mice infected with wt MHV-68 (white bars), or MHV-68mIFNα1 (black bars) were sampled at 14 days p.i. and analyzed by flow cytometry to determine the proportion of CD19$^+$ and activated CD69$^+$ CD19$^+$ B cells in the spleen. Mean percentage of CD19$^+$ CD69$^+$ ± SD for six mice per group is shown. *$p < 0.05$. (B) FACS analysis of PBMC recovered at day 45 from mice infected with the indicated viruses and stained with monoclonal antibodies specific for CD8, Vβ4 and CD62L. Data shown as plots are representative of at least three separate experiments.

were measured at 45 days p.i. As shown in Fig. 4B (*upper panels*), the percentage of CD8$^+$ Vβ4$^+$ T cells in the PBMC of mice infected with MHV-68mIFN-α1 was significantly increased with respect to control uninfected mice ($p < 0.05$). However, this increase was significantly lower than the one observed in mice infected with the wt virus ($p < 0.005$). The analysis of activated (CD62L^{low}) CD8$^+$ showed the same trend, with mice infected with MHV-68mIFNα1 having significantly lower levels of activated CD8$^+$ than animals receiving wt virus (Fig. 4B, *lower panels*).

3.5. *Effects of vaccination with live-attenuated or partially inactivated MHV-68mIFNα1 on the acute infection of wt MHV-68*

The ability of MHV-68mIFNα1 to elicit an immune response effective in protecting mice against wt MHV-68 challenge was tested in a vaccination experiment, in which MHV-68mIFNα1 was administered as a live-attenuated vaccine or in a partially inactivated formulation, obtained by Psoralen-mediated UV crosslinking (PSUV). As our objective was to prime an effective immune response against not only structural, but also lytic and latency-associated virus antigens, we selected a suboptimal dose of PSUV inactivation condition (1.5 μg/ml, 4 cm, see Section 2 for details), proven to cause a dramatic reduction but not the complete suppression of virus infectivity, while preserving MHV68mIFNα1 ability to drive the production of IFNα1 by the infected cells *in vitro* (Supplementary Fig. 3). Of note, the establishment of spleen latency by the residual infective virions was abolished in mice infected with partially inactivated MHV68mIFNα1 but not with the wt counterpart (Supplementary Table 2).

Mice vaccinated with live-attenuated or PSUV-inactivated MHV-68mIFNα1 were analyzed to check parameters of virus infection after the challenge with wt MHV-68. Virus replication in the lung was significantly reduced in mice vaccinated with partially inactivated MHV-68mIFNα1, and virtually null in mice receiving live-attenuated MHV-68mIFNα1 (Fig. 5A). The group of mice receiving inactivated wt MHV-68 as a vaccine exhibited on average a considerable viral load that, however, was significantly reduced as compared to unvaccinated control mice (Fig. 5A).

Mice vaccinated with partially inactivated MHV-68mIFNα1 also experienced a significant reduction of splenocytes reactivating latency on day 14 p.i. as compared to unvaccinated control mice (Fig. 5B). Of note, no virus reactivation was found in splenocytes of mice receiving the same vaccination but no virus challenge, suggesting that the vaccine virus *per se* was not able, at least at the time point considered, to cause long-term latency detectable with this *ex vivo* reactivation assay (Fig. 5B). A significant reduction of spleen latency amplification was observed also in mice vaccinated with live-attenuated MHV-68mIFNα1. However, mice receiving the same vaccine but no challenge showed detectable levels of latency reactivating splenocytes, proving that the infective virus present in the vaccine could establish latency and persist in spleen cells (Fig. 5B). Notably, vaccination with inactivated wt MHV-68 also reduced spleen latency amplification in infected mice (Fig. 5B), although to a lesser extent as compared to mice receiving IFNα-based vaccines. Finally, MHV-68 induced splenomegaly and non-specific B-cell activation (CD19$^+$ CD69$^+$) was completely absent at day 14 p.i. in all vaccinated mice (Fig. 5C and D).

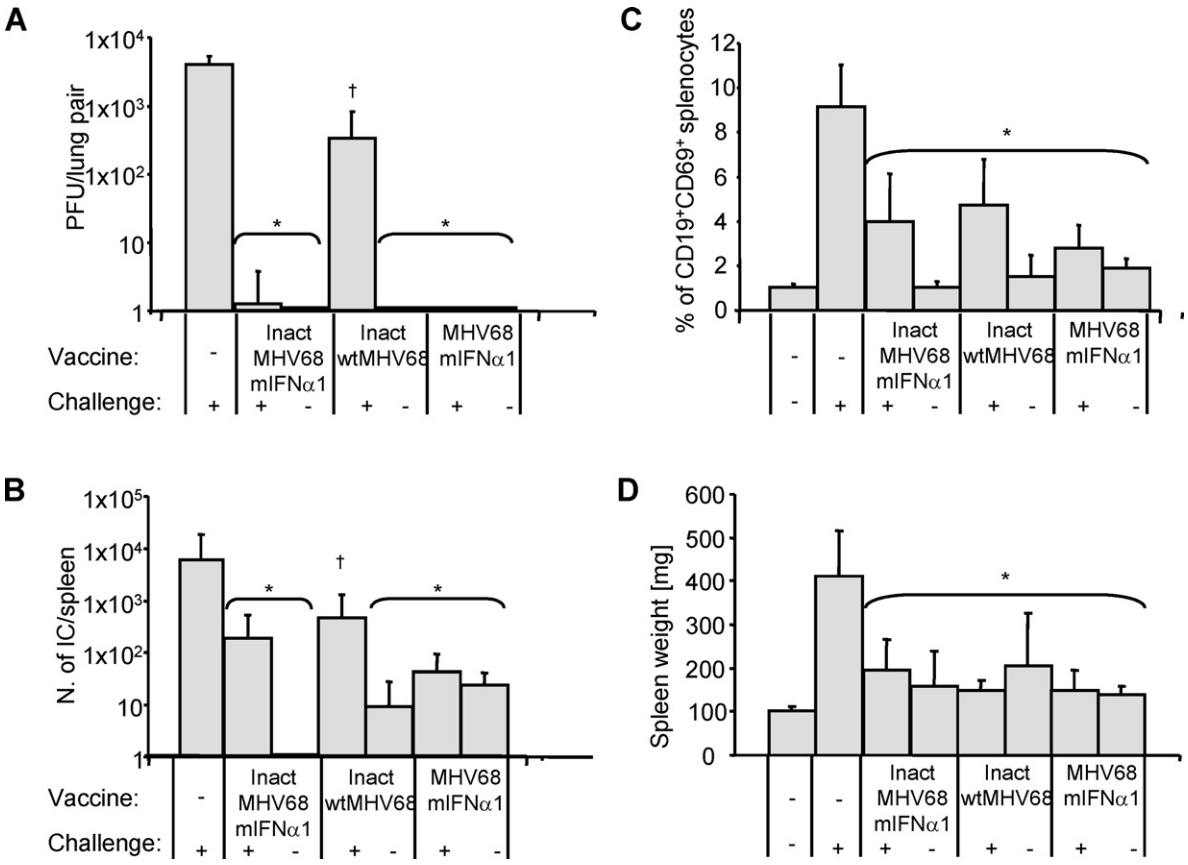


Fig. 5. Effect of vaccination with partially inactivated wt or recombinant MHV-68 or with live-attenuated MHV-68mIFNα1 on MHV-68 infection. C57BL/6 were vaccinated as indicated and infected with wt MHV-68 (see Section 2). At selected times p.i., samples were collected from infected and control mice to check the parameters of virus replication. (A) Virus titers in mice lungs (day 4 p.i.). The mean $\log_{10}$ virus titer ± SD for six mice per group is shown. (B) The peak mean number of latently infected splenocytes (as determined by IC assay) ± SD for six mice per group is shown for day 14 p.i. (C) As a marker of non-specific B-cells activation, the mean value of $CD19^+/CD69^+$ cells detected in mouse spleens on day 14 p.i., ± SD per group is reported. (D) MHV-68-induced splenomegaly is indicated by the mean spleen weight ± SD per group (measured on day 14 p.i.). $^*p < 0.01$, $^\dagger p < 0.05$ with respect to control unvaccinated mice.

3.6. Effects of vaccination with live-attenuated or partially inactivated MHV-68mIFNα1 on long-term latency

The spleens of mice vaccinated with live-attenuated or partially inactivated MHV-68mIFNα1 and challenged with escalating doses of wt MHV-68 virus underwent a biological and molecular analysis of MHV-68 long-term latency, conducted on day 80 p.i. Overall, all vaccinated mice receiving the highest dose of challenging MHV-68 (10^5 PFU) had levels of splenocytes reactivating latency similar to those detected in unvaccinated mice infected with the same dose of challenging virus, as assessed by infectious center assay (Fig. 6A). In contrast, after the challenge with the intermediate (10^4 PFU) or low (10^2 PFU) dose of wt MHV-68, a considerable fraction of vaccinated animals did not show any latent virus reactivating from splenocytes *ex vivo*, with the best protection achieved in mice vaccinated with live-attenuated MHV-68mIFNα1 and infected with 10^4 PFU of wt MHV-68 (Fig. 6A). Interestingly, mice receiving live-attenuated MHV-68mIFNα1 as a vaccine but not undergoing virus challenge exhibited levels of virus latency in the spleen similar to the ones detected in mice injected with the same vaccine that were also challenged with wt MHV-68, proving that the vaccine virus itself was able to establish long-term latency.

A real time PCR assay specifically designed to distinguish wt from recombinant MHV-68 genomes in mice splenocytes was performed at the same time point (day 80 p.i.) on mice infected with the low doses of viruses (Fig. 6B), experiencing the greatest inhibition of long-term latent infection. As expected on the basis of our previous results (Supplementary Table 2), the splenocytes of mice receiving inactivated MHV-68mIFNα1 as a vaccine were virtually free of recombinant MHV-68 genomes. Moreover, these mice had almost undetectable levels of wt MHV-68 genomes, proving that this vaccine was effective in protecting mice against the establishment of long-term latency by the challenging virus. On the contrary, the splenocytes of mice vaccinated with live-attenuated MHV-68mIFNα1 harbored genomes, although at very low level, belonging to the recombinant virus used as a vaccine, thus confirming that this virus vaccine was able to persist in mice spleens. Of note, among mice receiving live-attenuated MHV-68mIFNα1, the ones undergoing wt MHV-68 challenge had levels of recombinant virus genomes lower than those observed in uninfected mice.

4. Discussion

Genetic modification of virus genomes has been extensively applied for the development of live attenuated prophylactic vaccines [34]. In the last years, the co-expression of immunomodulating molecules by live recombinant viruses has also been evaluated as a strategy to enhance the immune response elicited by virus replication *per se*, thus boosting the response against the subsequent virus challenge [35–38]. Our study reports the characterization of the *in vitro* and *in vivo* replication of a recombinant clone of MHV-68 expressing the mIFN-α1 coding sequence, and the protection conferred to naïve mice by vaccines based on this recombinant virus.

Despite the high amounts of IFNα released in the supernatant of cells infected with the recombinant virus, the kinetics of viral

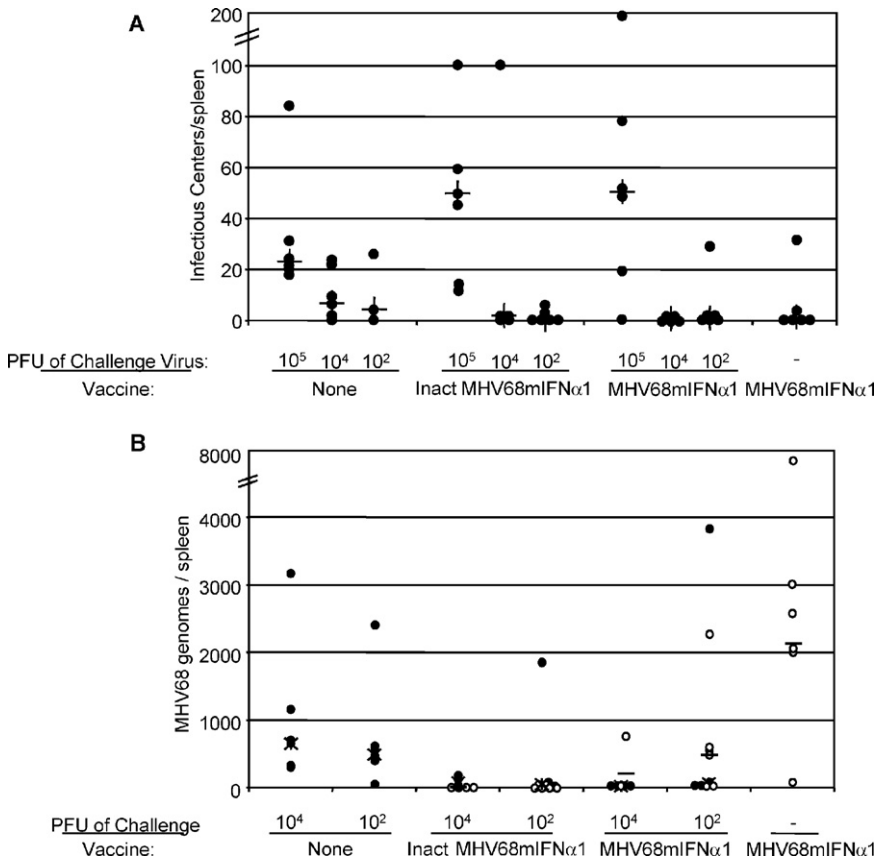


Fig. 6. Effect of vaccination with partially inactivated or live-attenuated MHV-68mIFNα1 on long-term MHV-68 latent infection in splenocytes. C57BL/6 were vaccinated as indicated and challenged (i.n.) with different doses of MHV-68. On day 80 p.i., virus latency was measured by IC (A) and a molecular assays designed to distinguish total MHV-68 from MHV-68mIFNα1 DNA (B). (A) The graph displays the number of splenocytes reactivating latency detected in each individual mouse (*black circles*) and the median value for each group (+). (B) The plot displays the amount of wt (*filled circles*) or MHV-68mIFNα1 (empty circles) genomes in the spleens of mice. The median number of wt MHV-68 (✳) and MHV-68mIFNα1 (–) genomes detected is also shown for each group.

replication *in vitro* was not affected by the genetic modification. Nevertheless, the MHV-68mIFNα1 proved to be markedly attenuated *in vivo*, since mice carrying a functional IFN-I system experienced a significant reduction of all the parameters of acute virus infection. Taken together, these results strongly suggested that the *in vivo* attenuation of MHV-68mIFNα1 was not caused by a direct antiviral effect elicited by the cytokine produced during virus replication *in vivo*, but rather mediated by the immunomodulatory effects exerted by IFN-α on the host immune system.

It has been reported that MHV-68 counteracts IFN-I activity in several ways [22–25], including the inhibition of the cytokine production *in vivo* [24]. Our results suggest that forcing the production of IFN-α at the site of virus replication, concomitantly with the viral proteins synthesis and release, can subvert this evasion mechanism and result in decreased acute virus replication as a consequence of the enhancement of the immune response against virus antigens. By reason of the proven role of IFN-α in the activation and maturation of DC [18,39], the production of IFN-α by the replicating virus may in fact compensate for the virus-mediated inhibition of DC differentiation [24], thus triggering a more efficient DC maturation and an antiviral immune response (Figs. 3, 5 and 6 and Supplementary Fig. 4).

It has been proven that the extent of latency is independent of the amount of virus seeding from the infected epithelium [40–42]. In this light, the reduced splenic lymphocytosis and expansion of latently infected cells observed in mice infected with MHV-68mIFNα1 (Fig. 4A) is unlikely to be an effect of the fewer virus particles spreading from the initial pulmonary *foci*, while it may reflect an impaired B cell infection by the virus in the presence of the cytokine. Virus-driven Vβ4$^+$ TCR CD8$^+$ T-cell expansion, also dependent upon the presence of MHV-68-infected B cells [43,44], is also reduced in mice infected with recombinant MHV-68 producing IFN-α1 (Fig. 4B).

No difference between wt MHV-68 and the recombinant MHV-68mIFNα1 could be observed in long-term latency reactivation (Fig. 3C) or establishment (*data not shown*). Although we cannot rule out the possibility that IFNα1 gene undergoes transcription and release by the recombinant virus during latency, we speculate that the cytokine produced in bigger amounts by recombinant MHV-68 during the productive replication enhances the immune response directed against the lytic more than latent antigens, in keeping with our results.

The attenuation exhibited by MHV-68mIFNα1 *in vivo*, together with the possibility of priming a significant immune response against a variety of MHV-68 antigens in the presence of a potent immune adjuvant (i.e., IFN-α) supports its use as a candidate live vaccine. Nevertheless, the capability of MHV-68mIFNα1 to establish long-term spleen latency represents a drawback for its utilization as a live-attenuated virus in prophylactic vaccination regimens, by which a sterilizing immunity should be pursued. With the dual aim of overcoming this limitation and of coupling the advantage of IFN-α1 production with an impaired infectivity of the vaccine virus *in vivo*, we exploited PSUV-induced virus inactivation, proven to preserve surface antigenicity [43] as well as the gene transcription under early but not late promoters control [44].

Interestingly, partially inactivated MHV-68mIFNα1, but not the wt counterpart, could not establish spleen latency *in vivo* (Supplementary Table 2), in apparent contrast with the reported evidence that the establishment and maintenance of MHV-68 latency are independent of the infection dose [42]. It has been suggested that the constant level of MHV-68 latently infected cells is maintained through the stimulation of sporadic reactivation phenomena and depends on host factors, such as the activation of the immune system and the availability of susceptible target cells for a *de novo* infection. We assume that the association of the release of viral antigens with IFN-α1 production by the residual replicating MHV-68mIFNα1 virions in the PSUV-partially inactivated preparations effectively stimulates the immune system against the virus, thus interfering with the establishment of the steady state long-term MHV-68 latency. In keeping with this assumption, PSUV-inactivated MHV-68mIFNα1 turned out to be the safest vaccine formulation tested in this study.

Although all vaccinated mice showed a significant inhibition of virus acute replication in the lungs with respect to control untreated animals, the extent of the reduction was much higher in mice receiving MHV-68mIFNα1-based vaccines (Fig. 5A), that were also the only animals showing a dramatic reduction of the peak of spleen latency reactivation (Fig. 5B). Of note, inactivated MHV-68mIFNα1 vaccine was more effective in counteracting the establishment of long-term latency by lower than higher doses of challenging virus (Fig. 6). This dose-dependent phenomenon, together with the dramatic reduction of all the parameters of acute infection in MHV-68mIFNα1 vaccinated mice, suggests that these vaccines may at least in part act through the block of the primary infection, the amount of infecting virus being a critical parameter for the starting and progression of infection. This hypothesis is in line with the proven capability of IFN-α to act as a vaccine adjuvant when administered in combination with a soluble antigen or other viral vaccines [45]. In fact, the cytokine released by cells infected with inactivated or replicating virions, together with the viral antigens released during vaccination, can prime the immune system to effectively recognize free infecting virions as well as infected cells expressing lytic viral antigens. Interestingly, the adjuvant effect of IFN-α was much more evident when associated to viruses inactivated with a procedure preserving the viral proteins antigenicity, such as PSUV, rather than when co-administered with heat-inactivated vaccines [4].

It is worth mentioning that vaccination strategies effective in inhibiting long-term MHV-68 latency were reported to be related to antibody production [5,46]. Of note, in mice vaccinated with live-attenuated or partially inactivated MHV-68mIFNα1 the typical MHV-68-driven non-specific B cells activation was abolished (Fig. 4A), while the production of virus-specific antibodies reached significant levels (Supplementary Fig. 4). Thus, the IFN-α released during the course of the infection or at the moment of the immunization against viral antigens may play a role in protecting plasma cells from the virus-mediated suppression that usually ensues in virus dissemination and persistence [47].

Overall, our data showed that MHV-68mIFNα1 administered as a partially inactivated or a live-attenuated vaccine is highly effective in reducing the extent of wt MHV-68 infection in terms of acute and long-term spleen latency, which was abolished in a significant percentage of mice (Figs. 5B and 6). These phenomena were likely mediated by IFN-α1, produced at the site of the infection by the vaccinating virus and acting as a vaccine adjuvant in stimulating a protective anti-MHV-68 immune response. We believe that the ability of MHV-68mIFNα1 to drive the production of IFN-α1 whenever it reactivates from latency makes this recombinant virus a valuable live-attenuated vaccine.

Gammaherpesvirus persistence is mediated by sporadic reactivation and replication events usually controlled by the immune systems, and it is intrinsically linked to the viral ability of promoting neoplastic transformation of the latently infected cells that, under conditions of immune suppression, can give rise to the insurgence of gammaherpesviruses-associated malignancies. In this scenario, a persisting virus specifically designed to induce the synthesis of a powerful immune modulator such as IFN-α during virus reactivation from latency may be particularly beneficial for immunocompromised hosts, where the cytokine can prompt the host immune system to mount an effective response against the latently infected cells, including those undergoing virus reactivation from latency, ultimately reducing the rate of *de novo* infection and, as a consequence, the risks of virus-mediated cell transformation. The possibility to combine the advantages offered by the release of an immune adjuvant such as IFNα1 with the higher safety of a MHV-68-latent-deficient mutant unable to colonize the vaccinated host will be considered in further studies, with the aim of finding the optimal conditions for a vaccine capable of conferring an effective and sterilizing anti-herpesvirus immunity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.03.092.

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