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Microspheres-prime/rMVA-boost vaccination enhances humoral and cellular immune response in IFNAR(-/-) mice conferring protection against serotypes 1 and 4 of bluetongue virus.

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Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT), which affects domestic and wild ruminants. At the present, 27 different serotypes have been documented. Vaccination has been demonstrated as one of the most effective methods to avoid viral dissemination. To overcome the drawbacks associated with the use of inactivated and attenuated vaccines we engineered a new recombinant BTV vaccine candidate based on proteins VP2, VP7, and NS1 of BTV-4 that were incorporated into avian reovirus muNS-Mi microspheres (MS-VP2/VP7/NS1) and recombinant modified vaccinia virus Ankara (rMVA). The combination of these two antigen delivery systems in a heterologous prime-boost vaccination strategy generated significant levels of neutralizing antibodies in IFNAR(-/-) mice. Furthermore, this immunization strategy increased the ratio of IgG2a/IgG1 in sera, indicating an induction of a Th1 response, and elicited a CD8 T cell response. Immunized mice were protected against lethal challenges with the homologous serotype 4 and the heterologous serotype 1 of BTV. All these results support the strategy based on microspheres in combination with rMVAs as a promising multiserotype vaccine candidate against BTV.

Keywords: bluetongue, microspheres, MVA, multiserotype, vaccine

1. Introduction

Bluetongue (BT) is a vector-borne viral disease of domestic and wild ruminants caused by Bluetongue virus (BTV), one of the most important livestock pathogens. (Saegerman et al., 2008). The disease is characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema in ruminants (Maclachlan et al., 2009) and IFNAR(-/-) mice (Marin-Lopez et al., 2016). BTV belongs to the genus *Orbivirus* within the family *Reoviridae* with a non-enveloped virion and an icosahedral capsid. The genome is composed of double-stranded RNA (dsRNA) distributed in ten segments, encoding for seven structural proteins (VP1-VP7) and five/six nonstructural proteins (NS1, NS2, NS3/3A, NS4 and NS5) (Ratinier et al., 2011; Roy, 1992; Stewart et al., 2015). The virus is mainly transmitted between ruminant hosts through certain species of hematophagous *Culicoides* (*Diptera, Ceratopogonidae*) midges (Mellor et al., 2000). To date, 27 serotypes of BTV have been identified (Zientara et al., 2014) with two further putative/novel BTV serotypes identified so far (Maan et al., 2015). Since 1998 at least 8 serotypes have been detected within the European Union (Zientara and Sanchez-Vizcaino, 2013) and the introduction of new BTV serotypes is a permanent threat to the region. The constant arrival of new BTV serotypes re-emphasizes the importance of making multiserotype and more effective vaccines than those that are currently available. Although conventional vaccines have controlled or limited BTV spreading in the past, they cannot address the need for cross-protection among serotypes (Marín-López et al., 2016). Furthermore, modified live virus (MLVs) and inactivated vaccines currently used in Europe do not allow the differentiation of infected from vaccinated animals (DIVA).

In previous work in our laboratory, we demonstrated that the immunization of IFNAR(-/-) mice with an experimental subunit vaccine based on VP2, VP7, and NS1 proteins of BTV-4 incorporated into avian reovirus (ARV) muNS-Mi microspheres

(MS-BTV) and without adjuvant protected against a homologous challenge with a lethal dose of BTV-4. Furthermore, the vaccine partially cross-protected against a heterologous challenge with a lethal dose of BTV-1 (Marin-Lopez et al., 2014). Immunized mice generated significant levels of neutralizing antibodies specific to BTV-4 and T cell responses, predominantly CD4+. Cellular immune responses, specially cytotoxic T lymphocyte responses (CTL) have been demonstrated to be important in clearance of homologous and heterologous serotypes of BTV (Jeggo et al., 1985) and in vitro studies showed that BTV-specific ovine and murine CTL were cross-reactive among serotypes (Jones et al., 1996; Takamatsu and Jeggo, 1989). Furthermore, CD8 T cell epitopes have been identified in sheep and the murine model of infection from VP7 and NS1 proteins of BTV-8 (Rojas et al., 2014; Rojas et al., 2011) and CTL epitopes have been described in sheep from VP2 and NS1 proteins of BTV-1 (Andrew et al., 1995; Janardhana et al., 1999).

In the present work, we developed a new vaccination strategy based on a heterologous prime-boost strategy with the particulate subunit vaccine MS-BTV and with the viral vaccine vector MVA expressing BTV antigens. Particulate immunogens are best for stimulating both humoral and cellular immune responses (Roy, 1996). Moreover, they are cheap, very stable, do not require the use adjuvants due to their intrinsic adjuvant effect, and are biologically safe. On the other hand, it has been described the use of rMVAs as a potent inductor of CD8 T cellular immune responses when used as a heterologous boost vaccination following a strong priming agent expressing the same antigen (Cottingham and Carroll, 2013; Whelan et al., 2009). In order to improve the serotype cross-protection of the experimental vaccine, we focused the vaccine composition on the VP2, VP7 and NS1 proteins of BTV-4. These proteins

have been generally described to induce cross-serotype helper T-cell or cytotoxic T-cell responses.

2. Materials and methods

2.1. Virus and cells.

Chicken embryo fibroblasts (DF-1) (ATCC, Cat. No. CRL-12203) and Vero (ATCC, Cat. No. CCL-81) cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Insect cells High Five (Invitrogen) were grown in TC-100 medium supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. BTV serotype 4 (Spain2005/05) (BTV-4) and serotype 1 (ALG2006/01) (BTV-1) were used in the experiments. BTV and MVA virus stocks and titrations were performed as previously described (Calvo-Pinilla et al., 2009).

2.2. Mice.

IFN $\alpha/\beta R^{o/o}$ IFNAR(-/-) 129/Sv mice and wild type 129/Sv mice were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout. Upon reception, the mice were held for 7 days for acclimatization under pathogen-free conditions in the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal (INIA-CISA), Madrid. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the INIA-CISA and Comunidad de Madrid (Permit number: PROEX 037/15).

2.3. Generation of muNS-Mi microspheres (MS-VP2/VP7/NS1), and recombinant MVAs expressing VP2, VP7 and NS1 BTV-4 proteins (rMVA-VP2/VP7/NS1).

The production and purification of muNS-Mi-VP2/VP7/NS1 microspheres using the baculovirus expression system and the generation of rMVA-VP2/VP7/NS1 have been previously described (Brandariz-Nunez et al., 2010; Marin-Lopez and Ortego, 2016; Marin-Lopez et al., 2014).

2.4. In silico T CD8 epitope prediction

Amino acid sequences for the non-structural NS1 protein (NCBI accession number: AM778441.1) and structural VP2 protein of BTV-4 (Spain2005/05) were analized using three prediction algorithms available on the web: Immuno Epitope DataBase (IEDB Analysis Resource) (www.iedb.org), SYFPEITHI (www.syfpeithi.de), and BIMAS (www.bimas.cit.nih.gov/) for the H-2-Db MHC class I for 129/Sv mice to identify T CD8 epitopes that could be good binders to H-2-Db MHC. Theoretical T-cell epitopes were chosen by a combination of the best score in these databases (table 1).

2.5. Peptides

The selected peptides (table 1) were purchased from Proteogenix (Schiltigheim, France) and Sigma-Aldrich (The Woodlands, USA). Peptides were >95 % pure (HPLC%). They were re-constituted in DMSO or Dimetilformamide according to the manufacturer instructions and kept frozen at −20 °C until use. All peptides were diluted in culture media: RPMI-1640 (Gibco, GreenIsland, NY) containing 10 % (v/v) heat-

inactivated fetal bovine serum (FBS), 1 % (v/v) antibiotic-antimycotic solution (Gibco), 1% (v/v) non-essential aminoacids and 2 mM glutamine (Gibco).

2.6. Studies of cellular immune response in IFNAR(-/-) mice.

Three groups of IFNAR(-/-) mice (n=4) were immunized following a homologous prime-boost regimen with rMVA-VP2, rMVA-NS1 or MVA-wild type (non-immunized group) three weeks apart. All animals were sacrificed at 14 days post-booster and their spleens were harvested for analysis by ELISPOT and intra-cellular cytokine staining (ICCS) as previously described (de la Poza et al., 2015; Marin-Lopez et al., 2014).

2.7. Prime-boost immunization and challenge with BTV in IFNAR$^{(-/-)}$ mice.

Groups of five IFNAR(-/-) mice were immunized by homologous prime-boost vaccination with MS-VP2/VP7/NS1 (50 μg of each per mice) or by heterologous prime-boost vaccination with MS-VP2/VP7/NS1 (50 μg/mice) and rMVA-VP2/VP7/NS1 (10^7 PFU/mice) , or prime-boosted with MS (50 μg/mice) and $3x10^7$ PFU per mice of MVA-wt (non-immunized) administered by intraperitoneal injection three weeks apart. Two weeks after the second immunization all mice were subcutaneously inoculated with $5x10^2$ PFUs of BTV-4 or 10^2 PFUs of BTV-1. Clinical data were recorded as described previously (de la Poza et al., 2013).

2.8. Detection of BTV-4 and BTV-1 in blood.

Whole blood was collected in EDTA from all animals at regular intervals after viral challenge. The viruses were released from whole blood by three freeze/thaw cycles. The amount of infectious virus was measured by plaque assay on Vero cells.

2.9. BTV-4 and BTV-1 neutralizing antibody detection in immunized mice by viral neutralization test (VNT) and isotyping ELISA assay for antibodies specific of VP2 protein.

The VNT was used to determine neutralizing antibody titers against BTV-4 prior infection. For plaque reduction assays, 2 fold dilutions of sera were mixed with 100 PFU of BTV-4, incubated for 1 hour at 37 °C and then plated into monolayers of Vero cells. After 1 hour, agar overlays were added and the plates were incubated for 5 days. The titer was determined as the highest dilution that reduced the number of plaques by 50%.

Isotype determination was performed by ELISA using a mouse immunoglobin isotyping kit (Biorad), using a fixed dilution of sera (1/50). As antigen in this assay, 96 well plate was coated with 150 ng/well of recombinant VP2 protein from BTV-4 expressed in Bac-To-Bac Baculovirus expression System, (Invitrogen) (Calvo-Pinilla et al., 2012).

3. Results

3.1. Heterologous prime boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 protects IFNAR(-/-) mice against homologous BTV-4.

With the aim to analyze the efficacy of the combination of MS-VP2/VP7/NS1-rMVA-VP2/VP7/NS1 as immunogen to protect against BTV-4 infection, two groups of mice were immunized with MS-VP2/VP7/NS1. Three weeks apart, one group of mice was boosted with rMVA-VP2/VP7/NS1 and the other group received a second immunization with MS-VP2/VP7/NS1. A third group of mice was primed with MS and boosted with MVA-wt (non-immunized group). Two weeks after the second immunization, mice were challenged subcutaneously with $5x10^2$ PFUs of BTV-4. All non-immunized animals showed clinical signs (the most severe signs, eye swelling, lethargy and hypothermia appeared at day 4) and died between days 4 to 5 post-infection. In contrast, 100% of the animals immunized with MS-VP2/VP7/NS1 or the combination of MS-VP2/VP7/NS1 with rMVA-VP2/VP7/NS1 did not show clinical signs throughout the experiment and they were completely protected against the lethal challenge with BTV-4 (Fig 1A). The titers of infectious virus recovered in the blood after challenge with BTV-4 were determined in all animals by plaque assay and titers up to $3.3\pm1.0 \times 10^2$ PFU/ml were observed at day 3 post-challenge in non-immunized animals (Fig 1B). Viremia was not detected in the immunized mice.

3.2. Prime boost immunization with MS-VP2/VP7/NS1 and MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 elicits neutralizing antibodies against BTV-4 in IFNAR(-/-) mice increasing the ratio IgG2a/IgG1.

In order to analyze the humoral immune response elicited in mice immunized with the homologous and the heterologous strategies, virus neutralization test (VNT) and isotyping ELISA assay were performed using the sera of immunized and non-immunized animals collected 2 weeks after the booster treatment. Similar titers of BTV-4 neutralizing antibodies were observed in the sera with a VNT_{50} of 1.825 ± 0.14 and 1.9

in MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 and MS-VP2/VP7/NS1 immunized mice, respectively (Fig 2A). Neutralization activity against BTV-4 was not detected in serum of non-immunized mice ($VNT_{50} \leq 0.6$) at the analyzed time. This observation revealed that combining microspheres and recombinant MVAs expressing BTV antigens achieved similar high levels of neutralizing antibodies to those induced by microspheres alone.

Sera analyzed by ELISA showed an increase in the induction of IgG subtypes 1, 2a and 2b in the animals boosted with rMVA-VP2/VP7/NS1 compared with those immunized with two doses of MS-VP2/VP7/NS1 (Figure 2B). Furthermore, the IgG2a/IgG1 ratio was clearly increased in the group boosted with MVA compared to the group immunized with two doses of microspheres (Figure 2C). These data indicate that the introduction of recombinant MVAs in the vaccine composition improve the humoral response induced just by microspheres, enhancing the ratio of IgG2a/IgG1, suggesting an activation of a Th1-biased immune response.

3.3. MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 immunization elicits strong cellular immune response.

To further analyze the cellular immune response elicited by the MS-VP2/VP7/NS1+ rMVA-VP2/VP7/NS1 vaccine, the amount of IFN-γ-producing spleen cells after the immunizations was determined by ELISPOT. IFNAR(-/-) mice were immunized by prime-boost vaccination with MS-VP2/VP7/NS1, MS-rMVA-VP2/VP7/NS1 or MS (control), administered three weeks apart. Two weeks after the second immunization spleens were harvested and the splenocytes were stimulated with MS, MS-VP2, MS-VP7, or MS-NS1 in ELISPOT plates. Animals immunized with the combined strategy significantly increased the level of IFN-γ producing cells after

stimulation when compared to the group immunized with MS or the non-immunized group (Figure 3).

3.4. Prediction and assessment of peptide binding from VP2 and NS1 protein to H-2-Db haplotype as T CD8 epitopes.

MVA vaccines are expected to induce a strong CD8+ T cell immune response. To identify epitopes triggering virus specific CD8+ T cell responses we used a combination of three epitope prediction algorithms and 6 peptides from VP2 and 4 peptides from NS1 proteins from BTV-4 were selected and synthetized (Table 1). The ability of these peptides to stimulate the proliferation of IFNγ positive CD8+ T cells and to induce the expression of CD107a on the surface of this population/ to bind H-2 Db and Kb molecules was assessed by ICCS assay in splenocytes from immunized animals with VP2 or NS1 proteins vectorized through recombinant MVAs. None of the six VP2 selected peptides induced significant levels of IFN-γ in $CD8^+$ T cells in the immunized animals after *in vitro* stimulation. (Figure 4A). In contrast, one of the four NS1 selected peptides, NS1-152 induced a significant recall INFγ response in $CD8^+$ T cells from the immunized animals (Figure 4B). These data confirm that NS1-152 peptide is a CD8 epitope in the 129 mouse strain. We next examined the ability of NS1-152 peptide to induce the expression of CD107a on the surface of IFN-γ positive $CD8^+$ T cells, as a surface marker of CTL degranulation. The NS1-152 peptide significantly induced the presence of CD107a on the surface of restimulated $CD8^+$ T cells indicating that NS1-152-specific CD8+ T cells display CTL activity (Figure 4C). These results suggest that NS1-152 is an immunodominant epitope for CD8+ T cell responses in IFNAR(-/-) mice.

3.5. MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 immunization induces strong CD8+ T cell immune response and promotes cytotoxic activity.

To assess the phenotype of the BTV-specific IFNγ producing cells after *in vitro* restimulation observed by ELISPOT assay, we immunized mice with MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1. T cell immune responses were measured by ICCS after the stimulation of splenocytes with the NS1-152 peptide. Re-stimulation with the peptide induced high expression of IFNγ by CD8+ T cells in the group of mice boosted with rMVA-VP2/VP7/NS1 (Figure 5A). Furthermore, the level of CD8+ T cells secreting CD107 augmented upon re-stimulation with NS1-152 peptide in this group of animals (Figure 5B). These data suggest that the booster with rMVA-VP2/VP7/NS1 of animals primed with MS-VP2/VP7/NS1 elicits an specific CD8+ T cell response, exhibiting marked cytotoxic activity

3.6. Heterologous prime boost immunization with MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 protects IFNAR(-/-) mice against heterologous BTV-1 infection

In order to analyze the efficacy of the combination of MS-VP2/VP7/NS1-rMVA-VP2/VP7/NS1 as a vaccine candidate against multiple serotypes of BTV, one group of animals was immunized following a prime-boost strategy, while a second group was primed with MS and boosted with MVA-wt (non-immunized mice). Both groups were challenged with a lethal dose of BTV-1. Non-immunized animals died between day 5 and 6 post-infection, showing the most severe clinical signs at days 4 and 5 and a peak of viraemia at day 5. In contrast, all immunized animals survived after challenge and they did not show clinical signs or viraemia after infection (Figure 6). These results indicate that the heterologous prime-boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 reaches the levels of protection conferred by

the homologous immunization against BTV-4 achieving a 100% of survival and also provides a total protection in absence of remarkable clinical signs and viraemia against the heterologous challenge with BTV-1.

4. Discussion

Current BT vaccines commercially available include both live attenuated and inactivated vaccines. They are effective but serotype specific and incompatible with serological assays to differentiate infected from vaccinated animals. In the last years, new experimental DIVA vaccines are being developed trying to generate broad cross-protection among BTV serotypes.

In previous work, we developed a subunit BTV vaccine candidate based on the proteins VP2, VP7, and NS1 of BTV-4 incorporated into avian reovirus muNS-Mi microspheres. IFNAR(-/-) mice immunized with MS-VP2/VP7/NS1 without adjuvant generated significant levels of neutralizing antibodies specific of BTV-4, a specific CD4 T cell response, and immunized mice were fully protected against an homologous challenge with a lethal dose of BTV-4 and partially protected against the heterologous BTV-1 (Marin-Lopez et al., 2014). In the present work, we combined this subunit vaccine with rMVAs expressing the same BTV antigens in a prime-boost regimen to improve the protection against heterologous serotypes of BTV. The results show that the prime boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 confers total protection against a homologous challenge with BTV-4 and heterologous with BTV-1 in IFNAR(-/-) mice. This new strategy elicits similar levels of neutralizing antibodies specific of BTV-4 that the strategy based only in microspheres. Interestingly, the boost with rMVA-VP2/VP7/NS1 increases IgG2a and IgG2b antibody levels in sera

and enhances the ratio of IgG2a/IgG1, suggesting that the rMVA-VP2/VP7/NS1 booster stimulate a Th1-type immune response.

Antibody isotypes play different roles in antiviral immunity. Influenza vaccines that stimulate IgG1 and IgG2a antibody subclasses induce better protection that those that induce only IgG1 with neutralizing activity (Huber et al., 2006). IgG2A antibodies are better suited to induce antibody-mediated cell cytotoxic responses and complement mediated responses (Liu et al., 2016; Tjiam et al., 2015; Yendo et al., 2016) which have been shown to contribute to the clearance of viruses from infected hosts (Huber et al., 2001). Although there are not studies about the role of the different isotypes in the BTV protection, these should be addressed in order to rationally improve BTV vaccine strategies.

Cellular immune responses, specially CTL have been demonstrated to be important in the clearance of homologous and heterologous serotypes of BTV (Jeggo et al., 1985) and MVA has been described as a potent inductor of CD8 T cell responses when used as heterologous boost vaccination following a strong priming agent expressing the same antigen (Cottingham and Carroll, 2013; Whelan et al., 2009). Previous work in our laboratory using rMVA as a boost in the vaccination strategy showed that the heterologous DNA/rMVA prime-boost immunization expressing VP2, VP7, and NS1 BTV-4 proteins protected IFNAR(-/-) mice against heterologous challenges with BTV-1 and BTV-8, and reduced viraemia significantly in sheep infected with the heterologous BTV-8 (Calvo-Pinilla et al., 2014; Calvo-Pinilla et al., 2012). In this work, ELISPOT assay showed that splenocytes of mice boosted with rMVA-VP2/VP7/NS1 elicited stronger cellular immune response than mice immunized twice with MS-VP2/VP7/NS1. To better analyze the cellular response phenotype induced by the BTV antigens included in the experimental vaccine and find

immunodominant epitope(s) to CD8+ T cell responses we studied *in silico* and *in vivo* the presence of CD8 epitopes in proteins VP2 and NS1 of BTV-4. Although CD4 and CD8 epitopes have been described for the VP7 protein of BTV-8 in mice and sheep (Rojas et al., 2011), we focused the study in proteins VP2 and NS1 where CTL epitopes have been described in BTV immunized sheep (Andrew et al., 1995; Janardhana et al., 1999). None of the six predicted VP2 peptides were capable of eliciting IFN-γ production in splenocytes from rMVA-VP2 immunized mice. In contrast, one peptide NS1-152 of NS1 protein was identified as a CD8+ T cell epitope that induced the expression of IFNγ and CD107a in CD8+ T cells, a marker of cytotoxic activity. Importantly, NS1-152 epitope is conserved among all BTV serotypes and it has been also previously characterized as a T cell epitope in sheep and C57BL/6 mice (Rojas et al., 2014).

Intracellular cytokine staining studies showed that peptide NS1-152 induced the activation of CD8+ T cells and CTLs *in vivo* in animals boosted with rMVA-VP2/VP7/NS1, in contrast with previous observations where the immunization with MS-VP2/VP7/NS1 predominantly induces a CD4+ T cell response (Marin-Lopez et al., 2014). In addition, 100% of the MS- rMVA-VP2/VP7/NS1 vaccinated IFNAR(-/-) mice survived to the challenge with the heterologous virus BTV-1 and viraemia was not observed in immunized animals after BTV-1 challenge.

In summary, the combination of microspheres and rMVA in a vaccination strategy improves the cellular immune response and cross-protection capacity of the experimental BTV subunit vaccine based on microspheres. We show that vaccination of IFNAR(-/-) mice with MS/rMVA expressing VP2, VP7, and NS1 proteins of BTV-4 achieves protective homotypic and heterotypic immunity and protection against homologous and heterologous infection with BTV-4 and BTV-1. This new experimental

strategy is an attractive approach to generate new effective, safe and cross-protective marker vaccines again the multiple BTV serotypes.

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FIGURE LEGENDS

Figure 1. Protection of MS-rMVA-VP2/VP7/NS1 vaccinated IFNAR(-/-) mice against a lethal challenge with BTV-4. Mice (8 weeks old, 5 per group) were immunized by prime-boost vaccination with MS-VP2/VP7/NS1 or heterologous MS-rMVA-VP2/VP7/NS1. Two weeks after immunization all mice were subcutaneously inoculated with $5x10^2$ PFUs of BTV-4 (lethal dose). (A) Survival rates of immunized and non-immunized IFNAR(-/-) mice after inoculation with BTV-4. The mice were observed every 24 h for 15 days. (B) Titers of BTV-4 recovered in blood of immunized

and non-immunized IFNAR(-/-) mice after challenge. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars.

Figure 2. Humoral response observed in IFNAR(-/-) mice vaccinated with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1. (A) Neutralizing antibodies specific of BTV-4 were analyzed in sera of immunized mice by VNT. Neutralization titers at day 15 post-boost treatment in sera of animals immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 are shown. Means are presented as bars and standard deviations are shown as error bars. Asterisks (*) indicate statistically significant differences ($P<0.05$) between immunized and non-immunized mice, calculated by signed rank test. (B) IgG1 and IgG2a, IgG2b, IgG3, and IgM antibody levels were measured in 15 post-booster treatment pools of sera of animals immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 by ELISA. (C) IgG2a/IgG1 O.D. ratio.

Figure 3. ELISPOT assays measuring IFN-γ-secreting T cells in the spleen of immunized IFNAR(-/-) mice. Mice were immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 as described in Materials and Methods. Splenocytes were harvested at day 14 post-boost. Mice inoculated with MS were used as non-immunized controls. Bars represent the IFN-γ spot-forming cells (SFC) mean number ± standard deviation within each group. 10μg of each protein (MS, MS-VP2, MS-VP7, and MS-NS1) per well were used as stimulus in each experiment.

Figure 4. Membrane labelling of CD107a and intracellular staining of IFN-γ in T CD8+ cells of MVA-VP2 or MVA-NS1 immunized IFNAR(-/-) mice. Two weeks after the second immunization, spleens were harvested and the splenocytes were

stimulated with 10 µg/ml of each peptide. Membrane CD107a and intracellular IFN-γ production was analyzed in CD8-positive cells by flow cytometry. Black bars: mice immunized with MVA-VP2 or MVA-NS1; grey bars: mice immunized with MVA-wt. The results represent the average of 4 mice ± SEM. Asterisks represent significant difference between samples, calculated by Man-Whitney non parametric test ($p \leq 0.05$).

Figure 5. Membrane labelling of CD107a and intracellular staining of IFN-γ in T CD8+ cells of MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 immunized IFNAR(-/-) mice. Two weeks after second immunization, spleens were harvested and the splenocytes were stimulated with 10 µg/ml of peptides 152 and 14 from NS1. At 5 h post-stimulation, intracellular IFN-γ production and labeling of CD107a were analyzed in CD8-positive cells by flow cytometry. Grey bars: peptide NS1-14; black bars: peptide NS-152. The results represent the average of 4 mice ± SD. Asterisks represent significant difference between immunized and non-immunized mice, calculated by Man-Whitney non parametric test ($p \leq 0.01$).

Figure 6. Protection of MS-rMVA-VP2/VP7/NS1 vaccinated IFNAR(-/-) mice against a BTV-1 challenge. Mice (8 weeks old, 5 per group) were immunized twice by homologous prime-boost vaccination with MS-rMVA-VP2/VP7/NS1 administered 3 weeks apart. Non-immunized group was immunized with MS-rMVA and used as a control. Two weeks after the second immunization all mice were subcutaneously inoculated with 10^2 PFUs of BTV-1. (A) Survival rates of immunized and non-immunized IFNAR(-/-) mice after inoculation with BTV-1. The mice were observed every 24 h for 15 days. (B) Titers of BTV-1 recovered in blood of immunized and non-

immunized IFNAR(-/-) mice after challenge. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars.

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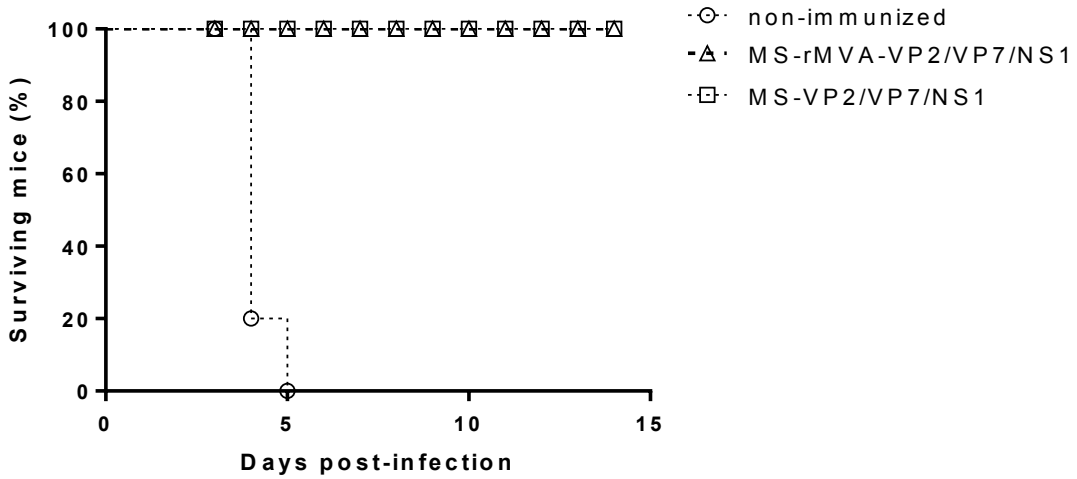
Table 1. Peptides selected from the epitope prediction in H-2 Db haplotype.

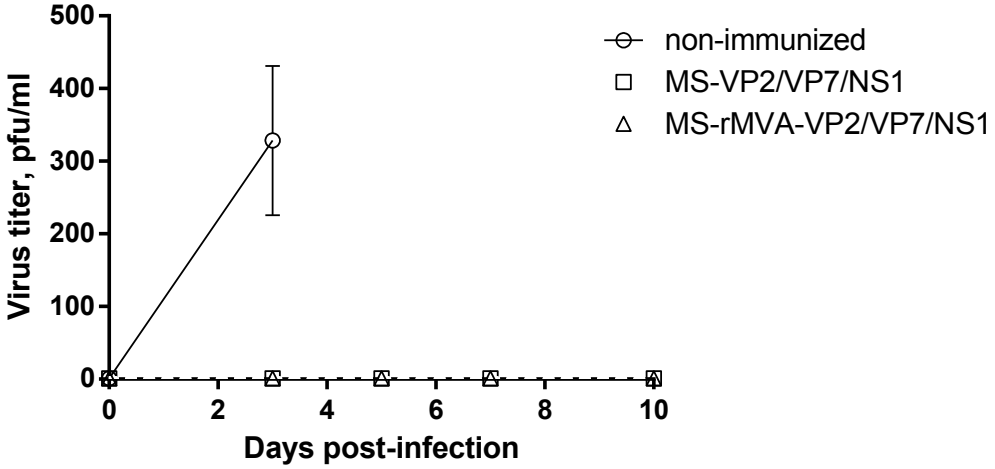
Protein	Position	Sequence	IEDB	SYFPEITHI	BYMAS
VP2	147	IYYDYFPL	0.3	-	-
	694	YLIQNSTGL	0.8	25	720.000
	731	LNVINFLPL	0.5	23	720.000
	743	VQDNISYW	0.8	-	-
	782	KSFYNFIRF	1.4	23	3120
	139	AKTANADTI	0.4	26	220.000
NS1	125	SALVNSERV	0.2	28	51.480
	152	GQIVNPTFI	0.2	28	720.000
	14	YANATRTFL	0.7	16	2.600
	222	IQLINFLRM	0.2	25	792.000

Using a combination of three epitope T prediction algorithms (IEDB, SYFPEITHI and BYMAS), peptides from VP2 and NS1 proteins of BTV-4 were selected and synthesized.

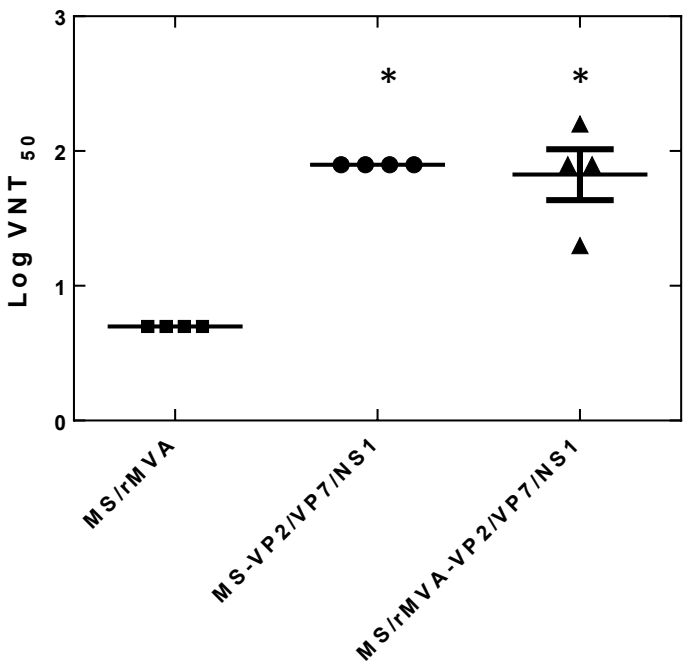
A


Surviving mice (%)
100
80
60
40
20
0
0
5
10
15
Days post-infection
non-immunized
MS-rMVA-VP2/VP7/NS1
MS-VP2/VP7/NS1

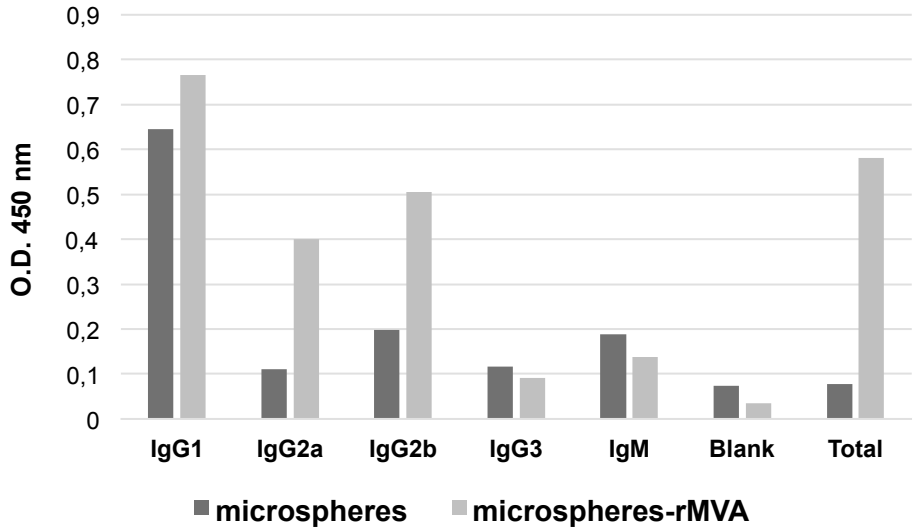
B


Virus titer, pfu/ml
500
400
300
200
100
0
0
2
4
6
8
10
Days post-infection
non-immunized
MS-VP2/VP7/NS1
MS-rMVA-VP2/VP7/NS1

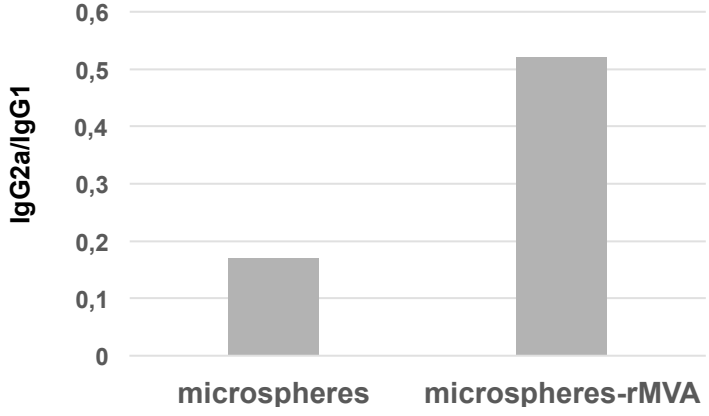
A

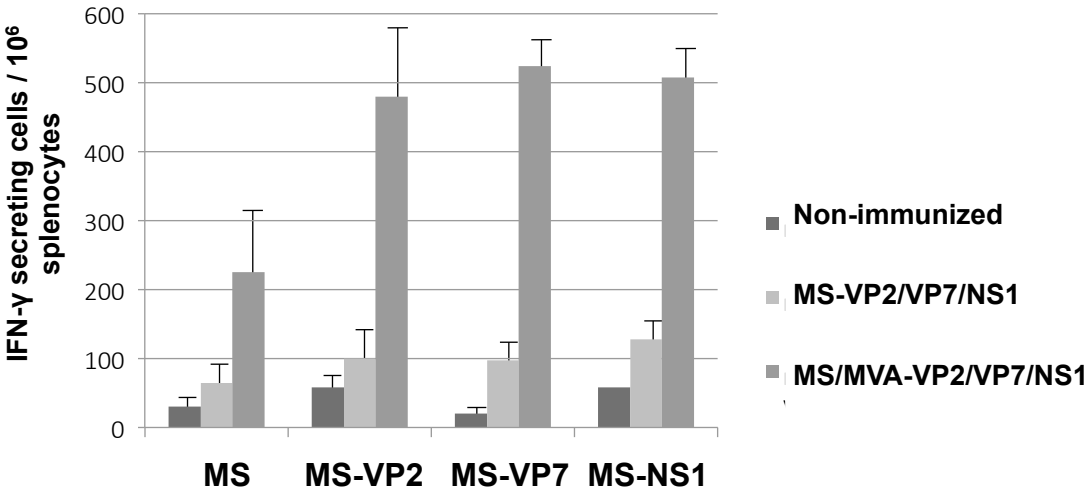

Log VNT 50
3
2
1
0
*
*
MS/rMVA
MS-VP2/VP7/NS1
MS/rMVA-VP2/VP7/NS1

B

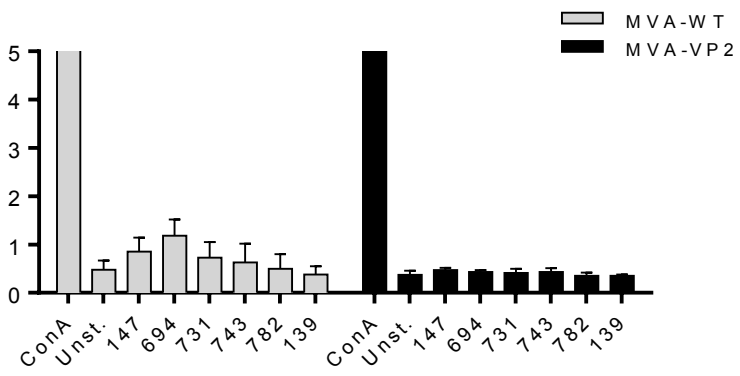

O.D. 450 nm
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
IgG1
IgG2a
IgG2b
IgG3
IgM
Blank
Total
microspheres
microspheres-rMVA

C

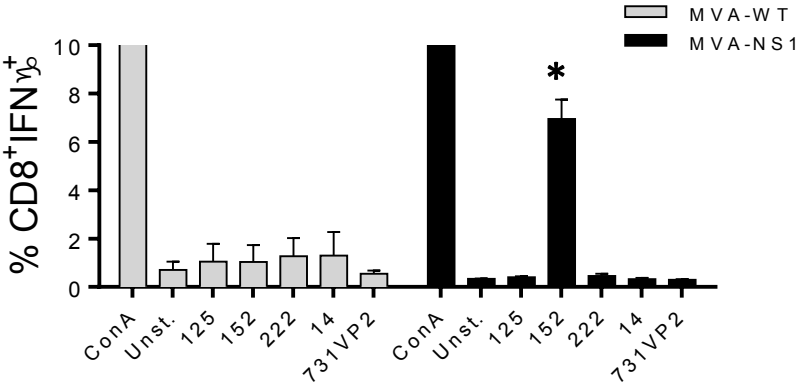

IgG2a/IgG1
0,6
0,5
0,4
0,3
0,2
0,1
0
microspheres
microspheres-rMVA


IFN-γ secreting cells / 10^6 splenocytes
600
500
400
300
200
100
0
MS
MS-VP2
MS-VP7
MS-NS1
Non-immunized
MS-VP2/VP7/NS1
MS/MVA-VP2/VP7/NS1

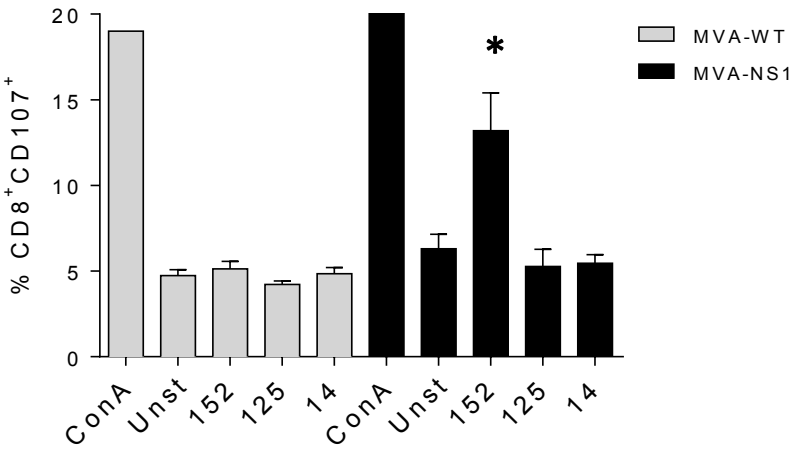
A


MVA-WT
MVA-VP2
5
4
3
2
1
0
ConA
Unst.
147
694
731
743
782
139
ConA
Unst.
147
694
731
743
782
139

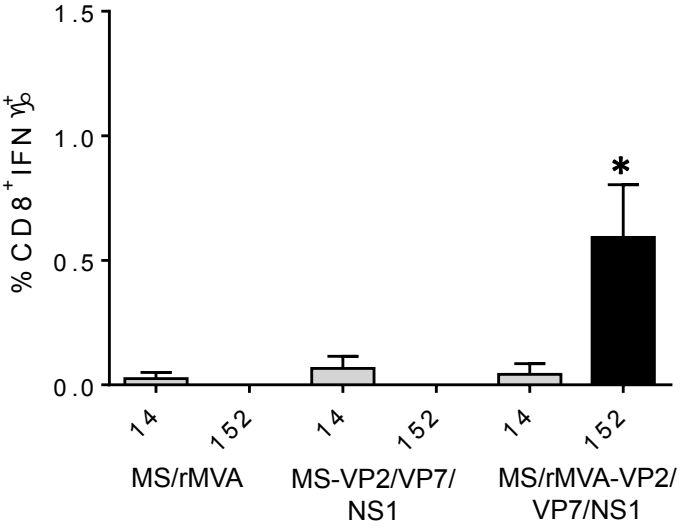
B


MVA-WT
MVA-NS1
% CD8+IFNγ+
10
8
6
4
2
0
*
ConA
Unst.
125
152
222
14
731VP2
ConA
Unst.
125
152
222
14
731VP2

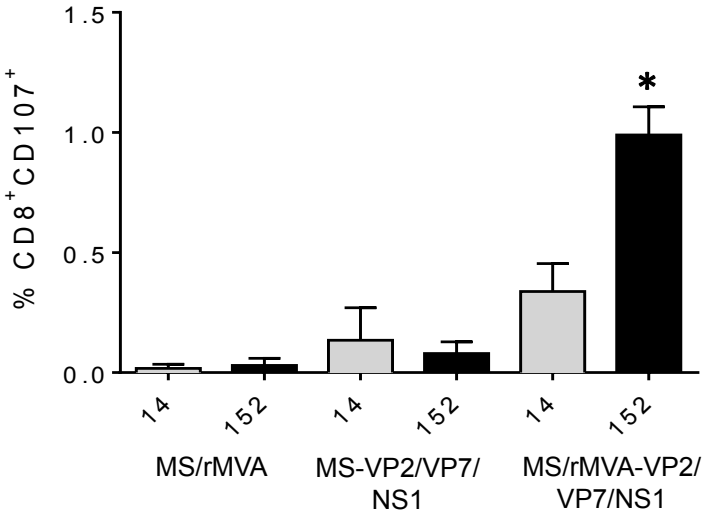
C


MVA-WT
MVA-NS1
% CD8+CD107+
20
15
10
5
0
*
ConA
Unst
152
125
14
ConA
Unst
152
125
14

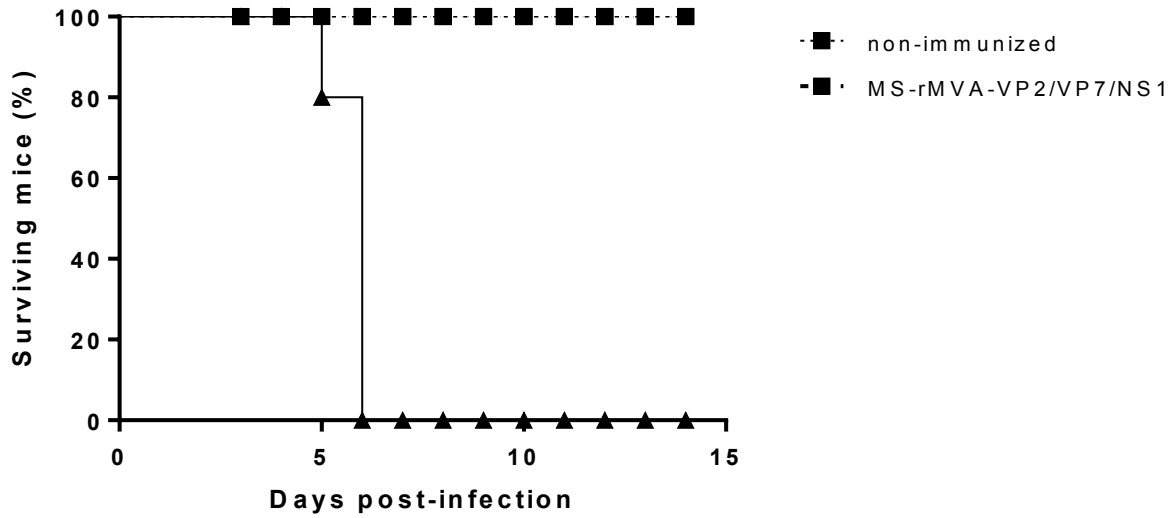
A


1.5
1.0
0.5
0.0
% CD8+IFN γ+
*
14
152
14
152
14
152
MS/rMVA
MS-VP2/VP7/
NS1
MS/rMVA-VP2/
VP7/NS1

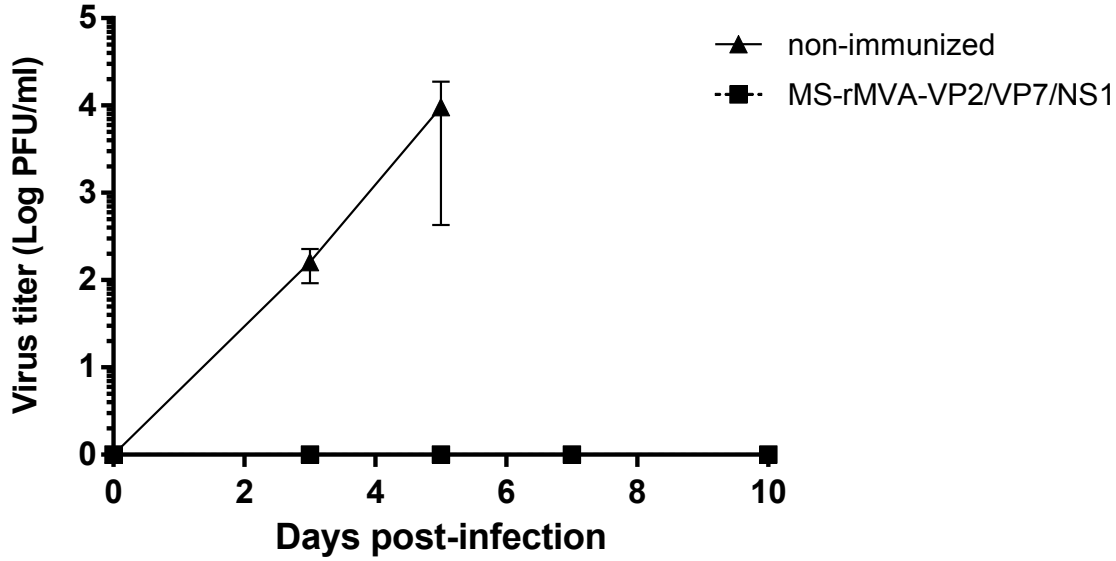
B


1.5
1.0
0.5
0.0
% CD8+CD107+
*
14
152
14
152
14
152
MS/rMVA
MS-VP2/VP7/
NS1
MS/rMVA-VP2/
VP7/NS1

A


100
80
60
40
20
0
Surviving mice (%)
0
5
10
15
Days post-infection
non-immunized
MS-rMVA-VP2/VP7/NS1

B


5
4
3
2
1
0
Virus titer (Log PFU/ml)
0
2
4
6
8
10
Days post-infection
non-immunized
MS-rMVA-VP2/VP7/NS1