Ns1 Is a Key Protein in the Vaccine Composition to Protect Ifnar(−/−) Mice against Infection with Multiple Serotypes of African Horse Sickness Virus

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Abstract

African horse sickness virus (AHSV) belongs to the genus Orbivirus. We have now engineered naked DNAs and recombinant modified vaccinia virus Ankara (mVMA) expressing VP2 and NS1 proteins from AHSV-4. IFNAR(−/−) mice inoculated with DNA/rMVA-VP2, NS1 from AHSV-4 in an heterologous prime-boost vaccination strategy generated significant levels of neutralizing antibodies specific of AHSV-4. In addition, vaccination stimulated specific T cell responses against the virus. The vaccine elicited partial protection against an homologous AHSV-4 infection and induced cross-protection against the heterologous AHSV-9. Similarly, IFNAR(−/−) mice vaccinated with an homologous prime-boost strategy with rMVA-VP2-NS1 from AHSV-4 developed neutralizing antibodies and protective immunity against AHSV-4. Furthermore, the levels of immunity were very high since none of vaccinated animals presented viraemia when they were challenged against the homologous AHSV-4 and very low levels when they were challenged against the heterologous virus AHSV-9. These data suggest that the immunization with rMVA/mVMA was more efficient in protection against a virulent challenge with AHSV-4 and both strategies, DNA/rMVA and rMVA/mVMA, protected against the infection with AHSV-9. The inclusion of the protein NS1 in the vaccine formulations targeting AHSV generates promising multiseroype vaccines.

Introduction

African horse sickness virus (AHSV) is an Orbivirus of the family Reoviridae that causes a severe disease in equids. In susceptible horses the mortality could reach 90%. Although African horse sickness (AHS) is mostly confined to sub-Saharan Africa, there are sporadic outbreaks in North Africa, Pakistan, India, Portugal and Spain [1]. Nine serotypes of the virus, AHSV-1 to AHSV-9, have been described [2,3]. The genus Orbivirus also includes bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV), which have similar morphological and biochemical properties but affect different hosts. Moreover, AHSV is transmitted by Culicoides midges [1,4], the same insect vectors as those that transmit BTV. Since 2008 there has been a dramatic northward spread of BTV in Europe related with the extension of the insect’s habitat due to climate change. Therefore the presence of insect vectors in Europe increases the probability that outbreaks of AHS may follow [5].

AHSV is a non-enveloped, icosahedral symmetric virus with ten linear segments of double-stranded RNA. AHSV virions are composed of seven structural proteins (VP1-VP7) arranged as three concentric layers surrounding the genome [6]. VP2 and VP5 are the outer capsid proteins, while the core surface layer is composed of VP7 and VP3 forms the inner capsid of the virion. Proteins VP1, VP4 and VP6 constitute core associated transcriptase complexes. There are four nonstructural proteins (NS1, NS2, NS3/SA, and NS4), involved in virus replication, morphogenesis and release from the infected cell [7,8,9].

Vaccination with a live-attenuated polyvalent AHSV vaccine is used to control the disease in Africa. However, this type of vaccine causes viraemia in the host and therefore has the potential to be acquired by the vector and transmitted in the field. In addition, a recent study showed that horses immunized against AHSV can be infected both clinically and subclinically with AHSV following natural infection in field conditions. Indeed, the level of viraemia observed in subclinically infected hores might be sufficient to infect midges with AHSV [10]. These attenuated vaccines have other disadvantages, such as the possible exchange of genome segments with field strains and the impossibility to distinguish (naturally) infected and vaccinated animals (‘DIVA’).

Recently, a recombinant vaccine based on MVA expressing VP2 protein (MVA-VP2) showed its efficacy eliciting neutralizing antibodies in ponies [11] and protection in mice against homologous challenge [12]. VP2 contains the major neutralizing epitopes; however, they are serotype-specific [13,14]. Other recombinant vaccines, expressing VP2 [15] or VP2/VP5 [16] protected against homologous challenge, however no previous heterotypic vaccination studies have been described.
Sequences of the NS1 gene are highly conserved between the different serotypes of AHSV [7]. Although little is known about the role of AHSV NS1 in host immune response, multiple CTL epitopes are present on non-structural NS1 protein of BTV [17,18]. Moreover, previous studies from our group demonstrated that the inclusion of NS1 in a vaccination strategy based on DNA/MVA expressing VP2 and VP7 proteins enhanced cross-protection against heterologous serotypes of BTV [19]. Therefore, we considered of interest to determine whether AHSV NS1 might be similarly able to enhance the level of cross-protection in a vaccination strategy against heterologous challenge.

Interferon alpha/beta receptor knockout (IFNAR−/−) mice have been characterized as a suitable animal model for AHSV, BTV and EHDV, since these mice are able to support the in vivo growth of these orbiviruses and they show viraemia and clinical signs. In addition, our previous results [12,19,20,21] and those of others [15,16] have shown that the inclusion of NS1 in a vaccination strategy is useful for the definition of effective recombinant vaccine candidates against several viruses.

In the present study, we have determined the protection of IFNAR−/− mice vaccinated with DNA/rMVA or rMVA/rMVA expressing VP2 and NS1 proteins from AHSV-4 against homologous or heterologous challenge (AHSV-9). As well the immune response elicited by these vaccination regimes was analyzed in the mouse model.

Materials and Methods

Virus and Cells

Baby hamster kidney (BHK-21) (ATCC, Cat. No. CCL-10), chicken embryo fibroblast (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 2 mM glutamine and 10% fetal calf serum (FCS). AHSV serotype 4 (Madrid-87) (AHSV-4) and AHSV serotype 9 (PAKrrah/09) (AHSV-9) were used in the experiments. Standard virus titrations were performed in Vero cells. Virus stocks were generated by multiplication of infection (MOI) of 1. At 48 hours post-infection (h.p.i.), or when cytopathic effect (CPE) was visible, the cells and supernatants were harvested and centrifuged. The virus were released from the cells after plaque assay by the addition of X-Gal to the agar overlay. 

Whole blood was collected in EDTA from all animals at regular intervals after inoculation. 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.

**AHSV-4 and AHSV-9 Neutralizing Antibody Detection in Immunized Mice by VNT**

The VNT was used to determine neutralizing antibody titers against AHSV-4 or AHSV-9. For plaque reduction assays, 2 fold dilutions of sera were mixed with 100 PFU of AHSV-4 or AHSV-9, 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%.

**UV Inactivation of AHSV**

Extracts of Vero cells infected with AHSV-4 or AHSV-9 were exposed to UV light (300 μJ/cm²) for 30 min [27]. The effectiveness of this treatment at inactivating the virus was confirmed by plaque assay. Confluent cultures of Vero cells were infected with the UV-inactivated virus as previously described by using the intact virus.

**IFN-γ ELISPOT Assays**

ELISPOT assays were performed with Mouse IFN gamma ELISPOT Ready-SET-Go (eBioscience), according to the method recommended by the manufacturer. A total of 4×10^5 splenocytes were added to the well and stimulated with 10^4 PFUs of UV inactivated virus. Plates were incubated at 37°C and 5% CO₂ for 18–20 hours. As a positive control, PHA was used. Plates were scanned on an ImmunoSpot reader (Cellular Technology Ltd.). Specific spots were counted using the Immuno-Spot software. The threshold values to consider a positive response by ELISPOT was that the number of specific spots/well had to be at least 2 times the average values found in positive response by ELISPOT was that the number of specific spots/well had to be at least 2 times the average values found in positive control wells of each group, and that after subtraction of background values, responses had to be higher than 20 SFC/well.

**Detection of Epitope-specific CD4^+ and CD8^+ T-cell Responses by Intra-cellular Cytokine Staining (ICC5)**

Mice immunized with rMVA or DNA were sacrificed at 14 days post-booster and their spleens were harvested for analysis by ICCS assay. A total of 10^6 splenocytes were stimulated with 10^4 PFUs of UV inactivated virus per well or left untreated during 18 hours in RPMI 1640 supplemented with 10% FCS and containing brefeldin A (5 μg/ml) to increase the accumulation of gamma interferon (IFN-γ) in the responding cells. After stimulation, cells were washed, stained for the surface markers, fixed, permeabilized and stained intracellularly using the appropriate fluorochromes. To analyze the adaptive immune responses, the following fluorochrome-conjugated antibodies were used: CD4-FITC, CD8-PerCP and IFNγ-PE. All antibodies were from BD Biosciences. Data were acquired by FACS analysis on a FACScalibur (Becton Dickinson) and were analyzed with CellQuest Pro software.

**Results**

**Evaluation of Protein Expression in Cells Tranfected with cDNAs or Infected with Recombinant MVAs Expressing VP2 and NS1 Proteins from AHSV-4**

In order to evaluate the expression of the AHSV-4 recombinant VP2 and NS1 proteins from pcDNA3 and rMVAs vectors in transfected BHK-21 and infected DF-1 cells, respectively, transient expression studies using immunofluorescence microscopy (IFA) were performed. Labeling was observed on BHK-21 cells transfected with pcDNA3-VP2 and pcDNA3-NS1 by using a serum of mice infected with serotype 4 of AHSV, but not on cells transfected with the control plasmid pcDNA3 (Fig. 1). Expression of VP2 and NS1 proteins was also observed in DF-1 cells infected with rMVA-VP2, and rMVA-NS1, respectively, but not in control MVA infected cells (Fig. 1). These data confirmed the efficient expression of the proteins from AHSV-4 cloned in the DNA and MVA vaccine vectors used for immunization of IFNAR(−/−) mice.

**Heterologous Prime-boost Immunization with pcDNA3-VP2/pcDNA3-NS1 and rMVA-VP2/rMVA-NS1 Partially Protects IFNAR(−/−) Mice against Homologous AHSV-4 and Heterologous AHSV-9 Infection**

Adult IFNAR(−/−) mice were immunized first with pcDNA3-VP2 and/or pcDNA3-NS1 by intramuscular route. After three weeks, mice were inoculated intraperitoneally with a booster of rMVA-VP2 and/or rMVA-NS1. Two weeks after the second immunization, immunized and control IFNAR(−/−) mice were challenged subcutaneously with 10^6 PFUs of AHSV-4 or 10^6 PFUs of AHSV-9. These doses of AHSV-4 have been previously described to induce clinical signs, viraemia and significant levels of lethality [12]. All mice infected with AHSV-4 presented clinical signs, which were more evident in non-immunized mice and animals immunized with NS1 (Table 1). These included presentation of rough hair coat, a hunched posture and reduction of mobility, which in some cases led to lethargy. Laccimation and swelling of the eyelids was also observed in non-immunized or NS1 immunized animals. Non-immunized mice infected with AHSV-9 presented a milder clinical syndrome characterised by reduction of mobility and eye swelling. In contrast, the groups of animals immunized with NS1, VP2 or both antigens remained healthy to the end of the study (Table 1).

While 20% of non-immunized and 40% of NS1 immunized animals died, 100% of the animals immunized with VP2 or VP2-NS1 survived to the challenge with AHSV-4. All the animals, immunized or not immunized, infected with AHSV-9 survived to the challenge (Fig. 2A).

The titers of infectious virus recovered in the blood after challenge with AHSV-4 or AHSV-9 were determined in immunized and non-immunized IFNAR(−/−) mice by plaque assay. In the animals infected with AHSV-4, titers up to 5×10^2 PFU/ml were observed at 5 days post-challenge in non-immunized and NS1 immunized animals. Lower level of viraemia was detected in the animals immunized with VP2 and a delay in the appearance of infective virus in blood in the animals immunized with VP2/NS1. In the groups of animals challenged with AHSV-9, titers up to 4×10^2 PFU/ml were detected at 3 days post-challenge in non-immunized mice. Titers up to 10^4 and 0.7×10^2 PFU/ml were detected in the animals immunized with VP2 and NS1, respectively, at the same day post-challenge. Mice immunized with VP2/NS1 showed lower viral titers than the other groups challenged with AHSV-9 (Fig. 2B).

These data suggested that the immunization of mice with VP2 partially protected the animals against an homologous challenge with AHSV-4. Furthermore, VP2/NS1 of AHSV-4 induced protection against serotypes 4 and 9 of AHSV upon heterologous prime-boost immunization using naked DNA and rMVA as vaccine vectors.
Homologous Prime-boost Immunization with rMVA-VP2/ rMVA-NS1 Protects IFNAR\(^{-/-}\) Mice against Homologous AHSV-4 and Heterologous AHSV-9 Infection

In order to improve the protection conferred by the antigens VP2 and NS1 against homologous and heterologous challenge with AHSV, adult IFNAR\(^{-/-}\) mice were immunized twice with rMVA-VP2 and/or rMVA-NS1 by intraperitoneal injection three weeks apart. Previous studies demonstrated that two immunizations with rMVA expressing VP2 protected IFNAR\(^{-/-}\) mice against AHSV-4 [12]. Two weeks after the vaccination boost, immunized and control IFNAR\(^{-/-}\) mice were challenged subcutaneously with 10\(^6\) PFUs of AHSV-4 or 10\(^6\) PFUs of AHSV-9. The percentage survival showed that all the mice vaccinated with VP2 or VP2/NS1 survived the challenge with AHSV-4 (Fig. 3A). In contrast, the non-immunized and the NS1 immunized groups presented a survival rate of 60% and 80%, respectively. All of the rMVA-VP2 and rMVA-VP2/NS1 vaccinated animals were also protected from clinical signs and were completely healthy until the end of the study. In contrast, all the non-vaccinated control mice and the animals immunized with rMVA-NS1 developed clinical signs similar to those described earlier during the previous challenge experiment. Non-immunized mice infected with AHSV-9 presented a milder clinical syndrome characterized by reduction of mobility and eye swelling. In contrast, the groups of animals immunized with NS1, VP2 or both antigens did not show clinical signs after challenge.

Viraemia after immunization and challenge was determined by virus isolation on cell culture from whole blood. Non-immunized and NS1 immunized animals infected with AHSV-4 showed titers up to 4.4\(^6\) 10\(^2\) PFU/ml and 3.5\(^6\) 10\(^2\) PFU/ml, respectively, at 5 days post-challenge. No viraemia was detected in the animals immunized with VP2 or VP2/NS1. After an heterologous challenge with AHSV-9, titers up to 3.4\(^6\) 10\(^2\) PFU/ml, 4.4\(^6\) 10\(^2\) PFU/ml, and 2.8\(^6\) 10\(^2\) PFU/ml were detected at 5 days post-challenge in non-immunized, VP2, and NS1 immunized mice, respectively. Lower titers up to 80 PFU/ml were detected in the animals immunized with VP2/NS1 but only at day 5 post-challenge with AHSV-9 (Fig. 3B).
14 days post-immunization (data not shown).

immunized mice and DNA/rMVA or rMVA immunized mice at
against AHSV-4 and AHSV-9 were not detected in sera from non-
immunized mice. In contrast, neutralizing antibodies against
DNA/rMVA-NS1 were similar to those measured in non-
immunized mice. In addition, neutralizing antibodies detected in mice immunized with
levels of neutralizing antibodies detected in mice immunized with
dna/rMVA or rMVA immunized mice was analyzed by virus neutralization tests (VNT)
before challenge. Neutralizing antibodies against AHSV-4 were
observed in mice immunized with DNA/rMVA-VP2 or DNA/
rMVA-VP2/NS1 two weeks after booster treatment with rMVAs
with a Log VNT50 of 1.51 and 1.45 respectively (Fig. 4A). The
levels of neutralizing antibodies detected in mice immunized with
dNA/rMVA-NS1 were similar to those measured in non-
immunized mice. In contrast, neutralizing antibodies against
heterologous AHSV-9 were not observed in the serum of
immunized or non-immunized mice (VNT50 ≤0.3) at the analyzed
time. Mice vaccinated with rMVA-VP2 or rMVA-VP2/NS1 developed neutralizing antibodies against AHSV-4 after the
second dose of vaccine was given with a Log VNT50 of 1.81
and 1.93, respectively [Fig. 4B]. In contrast, comparison of VNT50
specific of AHSV-9 between serum from vaccinated and non-
vaccinated mice did not show statistical significance by the
Student’s t-test (p≤0.05). In addition, neutralizing antibodies
against AHSV-4 and AHSV-9 were not detected in sera from non-
immunized mice and DNA/rMVA or rMVA immunized mice at
14 days post-immunization (data not shown).

These data indicate that the immunization of mice with rMVA-
VP2 or rMVA-VP2/NS1 induces total protection against an
homologous challenge with AHSV-4. Furthermore, VP2/NS1 of
AHSV-4 induced protection against an heterologous challenge
with AHSV-9 and reduced the viraemia almost completely.

Homologous and Heterologous Prime-boost Vaccination with DNA or rMVA Expressing VP2 from
AHSV-4 Elicit Neutralizing Antibodies against AHSV-4 but not against AHSV-9 in IFNAR(−/−) Mice

The presence of serotype-specific neutralizing antibodies against
AHSV-4 and AHSV-9 in the sera of DNA/rMVA or rMVA immunized mice was analyzed by virus neutralization tests (VNT)
before challenge. Neutralizing antibodies against AHSV-4 were
observed in mice immunized with DNA/rMVA-VP2 or DNA/
rMVA-VP2/NS1 two weeks after booster treatment with rMVAs
with a Log VNT50 of 1.51 and 1.45 respectively (Fig. 4A). The
levels of neutralizing antibodies detected in mice immunized with
DNA/rMVA-NS1 were similar to those measured in non-
immunized mice. In contrast, neutralizing antibodies against
heterologous AHSV-9 were not observed in the serum of
immunized or non-immunized mice (VNT50 ≤0.3) at the analyzed
time. Mice vaccinated with rMVA-VP2 or rMVA-VP2/NS1 developed neutralizing antibodies against AHSV-4 after the
second dose of vaccine was given with a Log VNT50 of 1.81
and 1.93, respectively [Fig. 4B]. In contrast, comparison of VNT50
specific of AHSV-9 between serum from vaccinated and non-
vaccinated mice did not show statistical significance by the
Student’s t-test (p≤0.05). In addition, neutralizing antibodies
against AHSV-4 and AHSV-9 were not detected in sera from non-
immunized mice and DNA/rMVA or rMVA immunized mice at
14 days post-immunization (data not shown).

The lack of or poor neutralizing antibody response to serotype 9
in vaccinated mice indicates that VP2 and NS1 from AHSV-4 do
not induce detectable cross-reactive neutralizing antibodies against
serotype 9.

Heterologous Prime-boost Vaccination with pcDNA3-
VP2/pcDNA3-NS1 and rMVA-VP2/rMVA-NS1 Induces the Generation of Specific T cell Responses

To further analyze the protective immune response elicited by
the DNA/rMVA and rMVA/rMVA vaccines, the amount of IFN-
γ-producing spleen cells after the immunizations was determined
by ELISPOT. IFNAR(−/−) mice were immunized by homologous
or heterologous prime-boost vaccination with DNAs and rMVAs
expressing VP2 and NS1 proteins from AHSV-4 or DNA and
MVA (controls), administered 3 weeks apart. Two weeks after
second immunization spleens were harvested and the splenocytes
were stimulated with UV inactivated AHSV-4 or AHSV-9 in
ELISPOT plates. As shown in Figure 5A, mice immunized with
both strategies, DNA/rMVA and rMVA/rMVA, developed
detectable specific IFN-γ producing cells after stimulation with UV
inactivated AHSV-4 and AHSV-9 when compared to the
non-immunized group. The mean value of IFN-γ producing cells
after stimulation with AHSV-4 was higher than after stimulation
with AHSV-9, although the difference was not significant when
the results were analyzed by Students t-test. In addition, higher
mean value of IFN-γ producing cells was observed in mice
immunized with rMVA/rMVA than in the DNA/rMVA immu-
nized mice, but the difference was not significant either.

To analyze the phenotype of the AHSV-specific IFN-γ
producing cells induced in vivo, intracellular cytokine staining
was performed. Whole splenocytes of DNA/rMVA-VP2/NS1 or
rMVA/rMVA-VP2/NS1 immunized mice were re-stimulated
with UV inactivated AHSV-4 or AHSV-9 for 24 h and

These data indicate that the immunization of mice with rMVA-
VP2 or rMVA-VP2/NS1 induces total protection against an
homologous challenge with AHSV-4. Furthermore, VP2/NS1 of
AHSV-4 induced protection against an heterologous challenge
with AHSV-9 and reduced the viraemia almost completely.

| Immunization | Challenge | Group | 3     | 5     | 7     | 10    |
|--------------|-----------|-------|-------|-------|-------|-------|
| DNA/rMVA     | AHSV-4    | Non immun. | 2.4 ± 0.54 | 4.2 ± 0.83 | 4.2 ± 0.83 | 2.75 ± 0.95 |
|              |           | VP2   | 0     | 2.8 ± 0.44 | 0     | 0     |
|              |           | NS1   | 1.4 ± 0.54 | 3.8 ± 0.83 | 3.6 ± 0.89 | 3 ± 1.22 |
|              |           | VP2+NS1 | 2 ± 0.70 | 2.6 ± 0.54 | 3.8 ± 1.3 | 46 ± 0.57 |
| AHSV-9       | Non immun. | 0     | 3.4 ± 0.61 | 1.4 ± 0.7 | 0     | 0     |
|              |           | VP2   | 0     | 0     | 0     | 0     |
|              |           | NS1   | 0     | 0     | 0     | 0     |
|              |           | VP2+NS1 | 0     | 0     | 0     | 0     |
| rMVA/rMVA    | AHSV-4    | Non immun. | 1.6 ± 0.54 | 4.8 ± 0.44 | 4.3 ± 0.57 | 0     |
|              |           | VP2   | 0     | 0     | 0     | 0     |
|              |           | NS1   | 2.4 ± 0.54 | 3.4 ± 0.54 | 4.2 ± 0.99 | 0     |
|              |           | VP2+NS1 | 0     | 0     | 0     | 0     |
| AHSV-9       | Non immun. | 0     | 3.2 ± 0.44 | 1.25 ± 0.5 | 0     | 0     |
|              |           | VP2   | 0     | 0     | 0     | 0     |
|              |           | NS1   | 0     | 0     | 0     | 0     |
|              |           | VP2+NS1 | 0     | 0     | 0     | 0     |

Mice were evaluated and scored for individual symptoms. Rough hair (absent = 0, present = 1), activity (normal = 0, reduced = 1, severely reduced = 2), hunched
(absent = 0, present = 1), eye swelling (absent = 0, present = 1). The final score was the addition of each individual score. The minimum score was 0 for healthy and 1–5
depending upon the severity. Each score represents the mean values of six animals and the standard deviation.

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Table 1. Post-challenge sickness score in mice vaccinated with indicated vaccine regimens.
intracellular IFNγ production by CD8+ T and CD4+ T cells was then determined by flow cytometry upon treatment of the cells with the golgi inhibitor, brefeldin A. UV inactivated AHSV-4 and AHSV-9 did not induce the expression of IFNγ by CD4+ T cells upon re-stimulation in rMVA/rMVA-VP2/NS1 or DNA/rMVA-VP2/NS1 immunized mice (data not shown). In contrast, CD8+ T cells were only stimulated in splenocytes of the animals immunized with rMVA/rMVA-VP2/NS1 after stimulation with both UV inactivated AHSV (Fig. 5B), although the level of stimulation was higher with AHSV-4 than with AHSV-9. These data suggest that although the two strategies of immunization assayed induced an immune cellular response in the mice, only the animals immunized with rMVA/rMVA-VP2/NS1 elicited an evident CD8+ T response that could be essential for a complete protection against multiple serotypes of AHSV.

Discussion

Recent outbreaks of bluetongue in Europe (particularly the northern European outbreak caused by BTV-8) have clearly demonstrated an increasing threat to animals posed by bluetongue virus (BTV), and consequently by other related orbiviruses that are transmitted by the same biting midge vectors such as: African horse sickness virus (AHSV), Equine Encephalosis virus (EEV), and Epizootic Haemorrhagic disease virus (EHDV). Consequently, the risk of African horse sickness (AHS) outbreaks may increase in areas traditionally free of the disease, including the Middle East,
North Africa and Europe and perhaps other parts of the world [16].

A variety of vaccines have been developed to prevent AHSV infection of equids. The major antigenic capsid protein ‘VP2’, either on its own, or co-expressed with VP5, VP3, and VP7 to form virus-like-particles (VLPs), induced virus neutralizing antibodies and protective immunity against AHSV [28,29]. Other AHSV vaccination strategies have been based on recombinant live viral vectors, specially the poxviruses. Thus, a recombinant wild-type vaccinia virus expressing VP2 of AHSV-4 was shown to protect horses against virulent challenge [13]. Similarly, MVA was also an effective vaccine-vector for AHSV. Good protein expression was demonstrated in equine skin fibroblast or avian cells infected with recombinant MVA viruses encoding AHSV VP2, VP7, or NS3. Furthermore, vaccination of ponies with recombinant MVA induced an AHSV-specific immune response that (in the case of rMVA-VP2) included development of neutralizing antibodies [11]. In addition, rMVA-VP2 was tested in IFNAR(−/−) mice and the immunization induced sterile protection in the vaccinated animals [12]. Similarly, recombinant canarypox virus, expressing both major outer-capsid antigens, VP2 and VP5, induced protective immunity [16,30].

In this work we have developed recombinant AHSV-vaccines that are inherently safer and compatible with a DIVA approach. First, the efficacy of a homotypic vaccination with MVA vectors, and a heterotypic vaccination with DNA and MVA vectors expressing VP2 and NS1 proteins from AHSV-4 has been assayed. IFNAR(−/−) mice, a small animal model of BTV, AHSV, and
EHDV infection [12,20,31], have been used in the present work in order to study the protection and immune response conferred by the homologous and heterologous prime-boost vaccination with DNA and rMVA expressing VP2 and NS1 from AHSV-4. Protection indicators used in our studies included primarily the observation of clinical signs and viraemia of challenged mice. Non-immunized mice infected with AHSV-4 or AHSV-9 showed similar clinical signs (rough hair coat, a hunched posture, reduction of mobility, and lacrimation and swelling of the eyelids), but in the case of the animals infected with serotype 9, these clinical signs were milder and the mortality rate was very low. In contrast, the level of viraemia was similar in the animals infected with the serotypes 4 or 9, although the period of viraemia was shorter in mice infected with AHSV-9. For this reason, in this study, viraemia has been the main protection indicator to analyze the potency of the vaccination strategies and the best vaccine composition to induce cross-protection.

Immunization with DNA/rMVA or rMVA/rMVA expressing VP2 alone or in combination with NS1 from AHSV-4 induced neutralizing antibodies specific of serotype 4. The serological cross-reactivity between certain AHSV serotypes has been described. Cross-reactivity between serotypes 1 and 2, serotypes 3 and 7, serotypes 5 and 8, and serotypes 6 and 9, has been described, while serotype 4 does not cross react with any other serotype [32]. Interestingly, we observed that heterologous neutralizing antibodies to serotype 9 were not detected in mice immunized with DNA/rMVA or rMVA/rMVA expressing AHSV-4 VP2 alone or in combination with AHSV-4 NS1. However, these mice were protected against AHSV-9. Previous studies reported that horses vaccinated with purified VP2 and VP7 of AHSV-4 or with the recombinant canarypox ALVAC-AHSV-4 expressing VP2 and VP5 did not seroconvert but were protected against virulent challenge with AHSV-4 [16,29]. These studies suggested that cell-mediated immune mechanisms were playing a role in the protection of these vaccinated animals. Studies performed with BTV, a related orbivirus, demonstrated that VP2 and NS1 are major CTL immunogens in sheep [17,18]. In addition, we showed that vaccination of IFNAR(-/-) mice with DNA/rMVA expressing VP2, VP7 and NS1 of BTV-4 achieved protective heterotypic immunity and protection against heterologous infection with BTV-8 and BTV-1 by inducing a strong T cell immune response [19]. The ELISPOT results showed that mice immunized with DNA/rMVA or rMVA/rMVA expressing VP2 alone or in combination with NS1 from AHSV-4 elicited specific IFN-α responses. UV inactivated AHSV-4 or AHSV-9 used as stimuli resulted in significant IFN-α responses by splenocytes of immunized mice, especially the rMVA/rMVA immunized mice, strategy that conferred more efficient protection against the virus. This data and the fact that not significant differences in IFN-γ responses

Figure 4. Humoral immune response observed in IFNAR(-/-) mice vaccinated with DNA/rMVA or rMVA/rMVA expressing VP2/NS1. Neutralizing antibodies specific of AHSV-4 or AHSV-9 were analyzed in sera of immunized mice by VNT. Neutralization titers at day 15 post-booster treatment in sera of animals immunized with DNA/rMVA (A) or rMVA/rMVA (B) expressing VP2, NS1, or VP2/NS1 are shown. (▲) Non-Immunized, (●) VP2 immunized, (■) NS1 immunized, and (●) VP2/NS1 immunized. Means are presented as bars (—). Asterisks (*) indicate statistically significant differences (P<0.05) between immunized and non-immunized mice, calculated by signed rank test. doi:10.1371/journal.pone.0070197.g004
were observed between stimulation with AHSV-4 and AHSV-9 confirming the importance of cell mediated immunity in the induction of homotypic and heterotypic protection against AHSV infection.

Protective immunization of horses with an attenuated AHSV-4 or a recombinant canarypox virus vectored vaccine co-expressing VP2 and VP5 induced an increase of CD8+ cells able to recognize multiple T-epitopes in the AHSV proteins [16,30,33]. The proliferation of virus-specific CD8+ T cells suggests that these T-cells may play a role in protective immunity. The ELISPOT results detected the stimulation of a number of activated cell populations that secrete IFN-γ in IFNAR(−/−) mice vaccinated with DNA/rMVA or rMVA/rMVA expressing VP2/NS1 as described in Material and Methods. Splenocytes were harvested at day 14 post-vaccination. Non-immunized mice were used as controls. Black (immunized) and white (non-immunized) bars represent the SFC mean number ± standard deviation for the ELISPOT within each group. 10⁴ PFUs of UV inactivated virus per well were used as stimulus in each experiment. Two weeks after second immunization, spleens were harvested and the splenocytes were stimulated with 10⁴ PFUs of UV inactivated virus per well. At 24 h post-stimulation, intracellular IFN-γ production was analysed in CD8-positive cells by flow cytometry. Grey bars: mice stimulated with AHSV-9; black bars: mice stimulated with AHSV-4. M3-MVA/MVA and M4-MVA/MVA are the values of two representative rMVA/rMVA immunized mice.

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In summary, two strategies of vaccination based on homologous or heterologous prime-boost of naked DNA and rMVA expressing VP2 and NS1 proteins of AHSV-4 have been assayed against the infection with serotypes 4 or 9 of AHSV. The immunization with rMVA/rMVA was more efficient in protection against a virulent challenge with AHSV-4 and both strategies, DNA/rMVA and rMVA/rMVA, protected against the infection with AHSV-9, serotype with a lower virulent behavior in the infections of IFNAR(−/−) mice. The inclusion of NS1 in the vaccine composition, as previously was described for BTV vaccines [19], elicits cross-protection against heterologous infections of AHSV. NS1 protein of orbiviruses has been reported to be conserved among serotypes and its inclusion in the composition of recombinant marker vaccines could be important to generate effective protective orbivirus multiserotype vaccines.

Figure 5. Cellular immune response observed in IFNAR(−/−) mice vaccinated with DNA/rMVA or rMVA/rMVA expressing VP2/NS1. (A) ELISPOT assays measuring IFN-γ secreting T cells in the spleen of immunized mice. Mice were immunized with DNA/rMVA or rMVA/rMVA expressing VP2 and NS1 as described in Material and Methods. Splenocytes were harvested at day 14 post-vaccination. Non-immunized mice were used as controls. Black (immunized) and white (non-immunized) bars represent the SFC mean number ± standard deviation for the ELISPOT within each group. 10⁴ PFUs of UV inactivated virus per well were used as stimulus in each experiment. (B) Intracellular staining of IFN-γ, in T CD8+ cells of rMVA/rMVA-VP2/NS1 immunized IFNAR(−/−) mice. Two weeks after second immunization, spleens were harvested and the splenocytes were stimulated with 10⁴ PFUs of UV inactivated virus per well. At 24 h post-stimulation, intracellular IFN-γ production was analysed in CD8-positive cells by flow cytometry. Grey bars: mice stimulated with AHSV-9; black bars: mice stimulated with AHSV-4. M3-MVA/MVA and M4-MVA/MVA are the values of two representative rMVA/rMVA immunized mice.

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Author Contributions

Conceived and designed the experiments: JO GL. Performed the experiments: FP EC EL AM FM GL JO. Analyzed the data: JO GL EC FP. Contributed reagents/materials/analysis tools: JC. Wrote the paper: FP EC JO.