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Intranasal IgG4/7 antibody responses protect horses against equid herpesvirus-1 (EHV-1) infection including nasal virus shedding and cell-associated viremia

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ABSTRACT

Equid herpesvirus-1 (EHV-1) outbreaks continue despite widely used vaccination. We demonstrated previously that an ORF1/ORF71 gene deletion mutant of the EHV-1 strain Ab4 (Ab4ΔORF1/71) is less virulent than its parent Ab4 virus. Here, we describe the Ab4 challenge infection evaluating protection induced by the Ab4ΔORF1/71 vaccine candidate. Susceptible control horses developed respiratory disease, fever, nasal shedding, and viremia. Full protection after challenge infection was observed in 5/5 previously Ab4 infected horses and 3/5 Ab4ΔORF1/71 horses. Two Ab4ΔORF1/71 horses developed short-lasting viremia and/or virus shedding. Protective immunity in the respiratory tract was characterized by pre-existing EHV-1-specific IgG4/7 antibodies, the absence of IFN-α secretion and rapidly increasing IgG4/7 upon challenge infection. Pre-existing systemic EHV-1-specific IgG4/7 highly correlated with protection. T-cell immunity was overall low. In conclusion, protective immunity against EHV-1 infection including prevention of viremia was associated with robust systemic and intranasal IgG4/7 antibodies suggesting immediate virus neutralization at the local site.

1. Introduction

Equid Herpesvirus-1 (EHV-1) is an alphaherpesvirus that infects equids worldwide. The clinical disease ranges from mild rhinopneumonitis, to abortion or equine herpesvirus myeloencephalopathy (EHM) (Gilkerson et al., 1999; Patel and Heldens, 2005; Goehring et al., 2006; Lunn et al., 2009; Perkins et al., 2009). The latter two severe disease outcomes adversely affect the equine industry with loss of life, closure of equine venues, quarantine, and the inability to move horses or participate in equine races, events or competitions (Henninger et al., 2007; Kohn et al., 2006). Commercially available killed and modified-live vaccines are commonly administered and routine vaccinations are required by various horse breeding and competition organizations (AAEP Equine Herpesvirus Guidelines). Vaccines have been shown to reduce severity of disease and nasal shedding, however, abortion and neurological outbreaks still occur despite widely used vaccination (Kydd et al., 1994a; Goodman et al., 2006; Goehring et al., 2010; Lunn et al., 2009; Perkins et al., 2009; Soboll Hussey et al., 2011; Wagner et al., 2017).

It is still not fully understood what constitutes protective immunity against EHV-1 and, consequently, which immune parameters a vaccine should enhance to improve protection and reduce the prevalence of outbreaks. EHV-1 is transmitted by nose-to-nose contact of horses or by fomites (Lunn et al., 2009; Perkins et al., 2009). It initially infects the respiratory epithelium, is taken up by antigen presenting cells which transport the virus from the apical side of the epithelium, through the basement membrane to the laminia propria and the regional lymph nodes (Baghi and Nauwynck, 2014; Kydd et al., 1994b). The virus then spreads from the local infection site throughout the body *via* cell-associated viremia (Kydd et al., 1994b; Slater et al., 1994). During severe disease outcomes, such as abortions or EHM, the peripheral white blood cells carrying infectious virus closely interact with vascular endothelial cells. The consequences are vasculitis, local inflammation, and thrombosis (Edington et al., 1986). Predilection sites are the blood vessels of the pregnant uterus and spinal cord resulting in late-term abortion and EHM, respectively (Edington et al., 1986, 1991). Cell-associated viremia is thus accepted to be a prerequisite for abortions and EHM (Vandekerckhove et al., 2011; Kydd et al., 1994b; Lunn et al., 2009),

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and preventing cell-associated viremia will likely protect against the severe clinical outcomes of EHV-1. In agreement with this prediction are data that a reduction in the magnitude and duration of cell-associated viremia decreases the likelihood of EHM (Edington et al., 1986; Allen, 2008).

EHV-1 is an immune-modulatory virus and has been shown to interfere with cellular immune responses *in vitro* (Ma et al., 2012; Hussey et al., 2014; Wimer et al., 2011). EHV-1-specific T-cell responses after EHV-1 infection of horses are typically of slow onset and low magnitude (Wagner et al., 2017; Wimer et al., 2018; Schnabel et al., 2018b). EHV-1 uses major histocompatibility complex (MHC) class I as an entry receptor (Kurtz et al., 2010; Sasaki et al., 2011). A transmembrane protein, pLU56 encoded by the ORF1 gene is involved in down-regulation of MHC class I on the cell surface of EHV-1 infected peripheral blood mononuclear cells (PBMC) *in vitro* (Ma et al., 2012). It was assumed that the decrease in MHC class I expression could be responsible for insufficient antigen presentation and consequently weakens EHV-1-specific T-cell responses *in vivo* (Ma et al., 2012). To test this hypothesis, experimental infection of ponies using ORF1/ORF2 (Ab4ΔORF1/2) or ORF1/71 (Ab4ΔORF1/71) double deletion mutants of the neuropathogenic EHV-1 strain Ab4 were performed. This resulted in reduced virulence indicated by reductions in fever and viral nasal shedding in comparison to the parent Ab4 virus (Soboll Hussey et al., 2011; Wimer et al., 2018). However, neither Ab4ΔORF1/2 nor Ab4ΔORF1/71 reduced viremia (Soboll Hussey et al., 2011; Wimer et al., 2018) and increased T-cell responses were not observed (Wimer et al., 2018).

The deletion of the ORF1 gene also suppressed IFN-α and IL-10 expression and chemokine secretion by PBMC *in vitro* (Ma et al., 2012; Hussey et al., 2014). Innate intranasal immunity after infection of EHV-1 naïve horses with the EHV-1 strain Ab4 was characterized by early IFN-α production on day 2 post infection (d2pi), followed by IL-10 (d2–5pi) and sCD14 (d3–6pi) (Wimer et al., 2018). In agreement with the *in vitro* findings, experimental infection with the double deletion mutant Ab4ΔORF1/71 decreased the secretion of all three cytokines at the respiratory viral entry site (Wimer et al., 2018). Nevertheless, infection of EHV-1 naïve horses with Ab4ΔORF1/71 and Ab4 induced strikingly similar immunological responses indicating that the deletion of the ORF1 and ORF71 genes fully maintained the immunogenicity of the virus. Both parent and deletion mutant virus, provoked high local and systemic antibody responses of rapid onset (d8–10pi) which were dominated by EHV-1-specific IgG4/7 antibodies while peripheral T-cell responses were overall low (Wimer et al., 2018).

Here we performed an EHV-1 Ab4 challenge infection of horses that were infected with either the Ab4ΔORF1/71 deletion mutant or the parental Ab4 virus (Wimer et al., 2018) six months prior to this challenge infection. The goal of this approach was to further test Ab4-ΔORF1/71 as a vaccine candidate and especially, to analyze if previous infection with Ab4ΔORF1/71 provided protection against EHV-1 challenge infection and to identity local and systemic immune parameters correlating with protection against EHV-1.

2. Materials and methods

2.1. Horses, groups and initial EHV-1 infection

Fifteen horses from the EHV-1-controlled herd of Icelandic horses at Cornell University (Wagner et al., 2015, 2017) were enrolled in this study. Six months prior to the EHV-1 challenge infection described here, all 15 horses were 2.5 years of age and EHV-1 naïve. They were randomly assigned to three groups (n = 5), and participated in an initial experimental EHV-1 infection previously described by Wimer et al. (2018). Briefly, one group of horses was not infected with EHV-1 (control). A second group was infected with the neurogenic EHV-1 strain Ab4 (Nugent et al., 2006). The third group was infected with Ab4ΔORF1/71 (Table 1). Overall, Ab4ΔORF1/71 was less virulent than the parent Ab4 virus. However, immune induction was markedly similar between the Ab4ΔORF1/71 and Ab4 infected groups. The outcomes of the EHV-1 infection performed by Wimer et al. (2018) are summarized in Suppl. Table 1. After release from initial EHV-1 infection and prior to the EHV-1 challenge infection described here, the horses were kept on pasture separated by group at an isolated facility at Cornell University without contact to other horses in the US prior to and for the duration of this study. The facility had restricted access for people to avoid infection with common US pathogens and to maintain the EHV-1-controlled status of the Icelandic herd. Grass hay was fed *ad libitum*. Horses were vaccinated against rabies, West Nile virus, Eastern and Western Encephalitis virus, and tetanus. They were dewormed on a regular basis but were not vaccinated or treated otherwise.

2.2. EHV-1 challenge infection

This EHV-1 challenge infection was performed six months after the initial EHV-1 infection when horses were 3 years of age. All 15 horses were infected with 1×10^7 plaque-forming units (PFU) of the neurogenic strain Ab4 resulting in three experimental groups determined by the initial infection: control/Ab4, Ab4ΔORF1/71/Ab4, and Ab4/Ab4 (Table 1). The Ab4 challenge infection was performed using a mucosal atomizer device (Wolfe Tory Medical, Salt Lake City, UT). The horses were moved into the isolation barn with individual box stalls one day prior to infection to acclimate. The barn had an entry area for donning personal protective equipment (PPE) including disposable coveralls, boots and gloves and biosecurity precautions were taken by all people entering the isolation barn. The box stalls did not allow direct nose-to-nose contact, however, the barn had a center hallway with shared airspace and all horses were handled as one group. No specific care was taken to prevent spread of virus from horse-to-horse within the barn by animal handlers with the exception of changing gloves after taking nasal swab samples from each horse.

The experimental EHV-1 infection and all sample collections for this study were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National

Table 1
Three groups of EHV-1 naïve horses were infected intranasally with different EHV-1 viruses (1×10^7 PFU) and then challenged with the neuropathogenic EHV-1 strain Ab4 six months later.

Group	n	Age (yrs)[a]	Gender[b]	Initial infection[c]	Challenge 6-months pii[d]
Non-infected control/Ab4	5	2.5	2 m; 3 g	saline	Ab4
Ab4ΔORF1/71/Ab4	5	2.5	1 m; 4 g	Ab4ΔORF1/71	Ab4
Ab4/Ab4	5	2.5	2 m; 3 g	Ab4	Ab4

[a] age in years at initial infection.
[b] m = mare; g = gelding.
[c] details on the initial infection are described in Wimer et al. (2018).
[d] pii = post initial infection.

Institute of Health and Animals in Agricultural Research and Teaching. The animal protocol was approved by the Institutional Animal Care and Use Committee at Cornell University (protocol #2011-0011). All efforts were made to minimize discomfort of the animals during sampling, for example by short sedation. After the end of this experimental study, all horses were kept at the facility at Cornell University as research horses.

2.3. Samples

Blood and nasal secretion (swab) samples were obtained and processed as previously described (Wimer et al., 2018). Blood samples included sodium heparinized samples for PBMC isolation and those without anti-coagulant for serum collection. Nasal swabs were obtained by using two sterile, polyester tipped swabs (Puritan Medical Products Company, LLC, Guilford, ME) and placing them in the nostril contacting the nasal mucosa for about 2–3 s. The swabs containing nasal secretions were subsequently transferred into tubes containing 1 ml of PBS and maintained at 4 °C until processing within a few hours after collection. Small amounts of the nasal swab samples were used for virus isolation the day of collection and the rest was frozen at −80 °C for measuring cytokines and antibodies at the end of the study. Baseline blood and nasal secretion samples were taken two days before EHV-1 challenge infection (d-2). Serum samples were also taken on the day of challenge infection (d0) prior to infection. Afterwards, blood and nasal secretion samples were taken daily (d1–10pi), and on d15 and d22pi. Additional blood samples were taken on d57 and d92pi. Horses were released from the isolation barn on d10pi after sampling and kept in their experimental groups on separate pastures without contact between the different groups.

2.4. Clinical evaluation

Baseline physical examination measurements were taken on d-1, immediately before EHV-1 infection (d0), and in the mornings of d1–10pi. Body temperatures were taken in the morning and evening until d7pi and then once daily in the mornings before samples were obtained. A fever was defined as a rectal temperature of > 38.5 °C. Clinical scoring, including gait evaluation for ataxia, was done as previously described (Wimer et al., 2018) according to the system described by Furr and coworkers (Furr and Reed, 2008). In brief, the numerical clinical score ranged from 0 to 22 and was calculated as the sum of scores for nasal discharge (0−6), ocular discharge (0−3), lymph node enlargement (0−3), ataxia and neurological signs (0−5), depression (0−3) and reduced appetite (0−2). The clinicians taking samples and performing the scoring were not aware of the group assignments of the horses during the initial EHV-1 infection (Table 1). A fever was defined as a rectal temperature of > 38.5 °C.

2.5. Quantification of EHV-1 (PCR or Virus Isolation) in nasal secretions and PBMC

Virus isolation from nasal secretions was performed in a plaque assay to assess viral shedding as previously described (Wimer et al., 2018). Briefly, for virus isolation from nasal secretions RK13 cells were cultured in MEM medium supplemented with 0.292 g/l L-glutamine, 1 mM sodium pyruvate, 50 µg/ml gentamycin, 0.75 ng/l Amphotericin B (all Thermo Fisher Scientific Waltham, MA, USA), and 10% fetal calf serum (Atlanta biological, Flowery Branch, GA, USA). Serial dilutions of the nasal swab fluid were added to the RK13 cells. Plates were incubated for 4 h at 37 °C in a humidified CO_2 incubator to allow the virus to attach to the cells. Afterwards, the medium was replaced by medium containing 0.5% w/v Methylcellulose (Sigma Aldrich, St. Louis, MO) and plates were incubated for 5 days. Then, plates were washed twice with PBS, followed by fixation and staining with a crystal violet solution (PBS containing 0.05% w/v crystal violet, 4% v/v paraformaldehyde, 1% v/v methanol (all Sigma Aldrich, St. Louis, MO)) for 20 min at room temperature. Afterwards plates were washed with tap water and allowed to dry. They were evaluated for viral plaques by eye and by microscopy if necessary. Results were expressed as PFU per 1 ml PBS solution

PCR was performed to determine cell-associated viremia in PBMC as previously described (Wimer et al., 2018). In brief, PBMC were isolated from heparinized blood by density gradient centrifugation (Ficoll-Paque™ Plus, GE Health- care, Piscataway, NJ). DNA was isolated from aliquots of 5×10^6 snap frozen PBMC to determine viral genome copy numbers by quantitative PCR targeting the gB gene (Elia et al., 2006). The quantitative PCR was performed at the Animal Health Diagnostic Center (AHDC) at Cornell University.

2.6. Quantification of EHV-1-specific antibodies in nasal secretion and serum

Antibodies specific for EHV-1 glycoproteins gB, gC and gD were measured by a fluorescent bead-based EHV-1 Multiplex assay as previously described (Wimer et al., 2018). Briefly, a monoclonal anti-equine IL-4 antibody (clone 25; Wagner et al., 2012) was initially coupled to all three beads numbered 33, 35 and 36 (Luminex Corp.). Afterwards, bead 33 was incubated with IL-4-tagged EHV-1 gB, bead 35 with IL-4 tagged EHV-1 gC, and bead 36 with IL-4-tagged EHV-1 gD. All three IL-4-tagged EHV-1 glycoproteins were expressed as described previously (Wagner et al., 2015). After EHV-1 gB, gC and gD incubation, the beads were washed. Then, the beads were incubated with serum or nasal secretion, respectively. Serum was measured at a dilution of 1:400 and nasal secretions were run undiluted. After washing the beads again, a polyclonal biotinylated anti-IgG(H+L) detection antibody (Jackson Immunoresearch Laboratories, West Grove, PA) followed by streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA) was added to the assay to analyze total EHV-1 gB, gC or gD-specific antibodies. The assay was performed at the AHDC at Cornell University. In addition to EHV-1-specific total Ig detection, isotype-specific assays were performed by replacing the polyclonal detection antibody with equine isotypes-specific monoclonal antibodies against IgG1, IgG1/3, IgG4/7, IgG3/5, IgG6 or IgM (Wagner et al., 2015, 2017; Wimer et al., 2018).

The immunogenic EHV-1 and EHV-4 gB, gC, and gD antigens share a high amino acid and structural homology resulting in serological cross-reactions between the two viruses (Sinclair et al., 1993a, 1993b; Love et al., 1993). We have expressed the EHV-4 gC and gD genes and confirmed that this also applies to the antibody responses of immunologically mature Icelandic horses (data not shown). However, the pre-infection anti-EHV-1 gB, gC, and gD antibody values in all horses in the control group infected with Ab4 during this study were low. This indicates that the control/Ab4 group had also no or only very low amounts of anti-EHV-4 antibodies prior to infection. In the protected Ab4ΔORF1/71/Ab4, and Ab4/Ab4 groups, similarly low anti-EHV-1 gC and gD antibody values were detected prior to the initial Ab4 or Ab4-ΔORF1/71 infection (Wimer et al., 2018). Then, these horses were infected, their anti-EHV-1 antibody values reached maximum values by d32–72pi, and continuously decreased afterwards until this Ab4 challenge studies was performed (Wimer et al., 2018). This indicates that anti-EHV-4 antibodies did not exist in any substantial amounts in the two protected groups prior to their initial infection and prior to the Ab4 challenge infection described here. Thus, the antibody responses measured here are in all likelihood EHV-1-specific.

2.7. Cellular in vitro re-stimulation and cytokine detection by multiplex assay

PBMC were cultured in cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Grand Island, NY) containing 10% (v/v) FCS (Thermo Scientific, Logan, UT), 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 mM 2-mercaptoethanol,

50 mg/ml gentamicin). EHV-1 re-stimulation of PBMC was performed with the EHV-1 strain Ab4 at a MOI of 1 as previously described in detail (Goodman et al., 2012; Wagner et al., 2015, 2017; Wimer et al., 2018). In addition, aliquots of PBMC from each horse were kept in medium as negative control or were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) and ionomycin (1 μM; both Sigma, St. Louis, MO). The latter stimulation was used as a viability control of the PBMC. Supernatants from EHV-1 re-stimulated PBMC and control supernatants were harvested after 48 h of incubation for cytokine analysis.

Nasal secretion samples and cell culture supernatants from EHV-1 re-stimulation assays of PBMC were evaluated using equine Cytokine Multiplex assays detecting IFN-α, IL-4, IL-10, IL-17A, IFN-γ, CCL2, or soluble CD14 (sCD14) as previously described (Wagner and Freer, 2009; Wagner et al., 2013; Schnabel et al., 2018a) and were performed at the AHDC at Cornell University.

2.8. Flow cytometric analysis of EHV-1-specific T-cells

For detection of EHV-1-specific T-cells, PBMC were cultured as described above in the presence of Brefeldin A (10 μg/ml; Sigma, St. Louis, MO) for the last 24 h of incubation to block cellular secretion. PBMC were then harvested, washed in PBS, fixed in 2% formaldehyde, and washed again. Tri-color staining and flow cytometric evaluation of EHV-1-specific T-cells was performed as previously described (Wagner et al., 2010, 2015; Goodman et al., 2012; Perkins et al., 2014; Wimer et al., 2018). Briefly, cell aliquots were triple stained for either cell surface CD4, CD8 and intracellular IFN-γ production, or intracellular IL-10, IL-4, and IL-17A and analyzed in a FACS Canto II flow cytometer (BD Biosciences, San Diego, CA), using FlowJo software version 10.2 (FlowJO LLC, Ashland, OR, USA). An analysis gate was set on the small lymphocytes and these cells were analyzed for cytokine production. Isotype controls at equivalent Ig concentrations were included for each reagent. The percentages of EHV-1-specific IFN-γ positive lymphocytes after re-stimulation were reduced by those of respective medium controls and evaluated as previously described (Goodman et al., 2012; Wagner et al., 2015, 2017).

2.9. Statistical analysis

D'Agostino & Pearson normality tests indicated that values on most days were not normally distributed. Correlations between virus isolation (PFUu/ml nasal secretion sample) and inflammatory cytokine concentrations in nasal secretions on the same day, or those between clinical parameters, viral shedding, viremia, and intranasal or serum antibody values or cellular immune parameters were analyzed using Spearman rank correlations. Pre-existing antibody values in nasal secretion or serum samples or pre-existing cellular immune parameters were compared between the three experimental groups by Kruskal-Wallis Tests with Dunn's tests for multiple comparisons. All clinical, viral, humoral and cellular immune parameters were also compared over time post challenge infection by repeated-measures ANOVAs with Tukey's post tests for multiple comparisons between the three groups. To increase power of the statistical analysis all time points from pre-challenge to d10pi were compared between the three groups, *i.e.* until most systemic immune parameters were similar between the three groups by inspection of the longitudinal data graphs. This applied to the intranasal and systemic protective immune parameters, including all antibody data and cytokine secretion after PBMC re-stimulation. Because of the fewer analysis time points, all longitudinal data points were compared for cellular flow cytomeric data such as IFN-γ producing T-cells and T-cell subpopulations. P-values of < 0.05 were considered significant. The statistical analyses were performed and the graphs were created using GraphPad Prism 6 for MacOSX, version 6f.

3. Results and discussion

3.1. Previous infection with the neuropathogenic EHV-1 strain Ab4 or its deletion mutant Ab4ΔORF1/71 provides protection from Ab4 challenge infection

Fifteen horses were intranasally challenged with EHV-1 Ab4. Six months prior to the challenge infection, horses (n = 5 per group) were intranasally infected with either Ab4 (Ab4/Ab4 group), Ab4ΔORF1/71 (Ab4ΔORF1/71/Ab4 group), or not-infected (control/Ab4 group). At the time of the Ab4 challenge infection, horses in the control/Ab4 group were fully susceptible to EHV-1 infection. They showed a high fever at d2–3pi and a secondary mild fever between d3–6pi along with an increase in clinical scores between d2–4pi (all $p < 0.05$–0.0001). In contrast, horses previously infected with Ab4 or Ab4ΔORF1/71 did not develop clinical signs of disease in response to Ab4 challenge infection. This was confirmed by a lack of fever and a consistently low clinical score following challenge infection with Ab4 in all horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups (Fig. 1A and B). None of the horses developed neurological signs.

Compared to the horses previously infected with Ab4 or Ab4ΔORF1/71, the control/Ab4 group shed EHV-1 in nasal secretions from d1–3pi with a significant increase on d3pi ($p > 0.01$) (Fig. 1C). The control/Ab4 group also developed viremia with significantly higher EHV-1 DNA amounts detectable in PBMC between d4–10pi (all $p > 0.05$) (Fig. 1D). Peak virus shedding and viremia values are also shown for all three groups in Suppl. Table 2. Comparison of the clinical course of disease, nasal shedding and viremia of the control/Ab4 group with the primary Ab4 infections described previously (Wimer et al., 2018; Schnabel et al., 2018b), confirmed that horses in the control/Ab4 group were fully susceptible to the Ab4 challenge.

In contrast, EHV-1 was neither isolated from nasal secretions nor detected in PBMC from all horses in the Ab4/Ab4 group (5/5) and most horses in the Ab4ΔORF1/71/Ab4 group (3/5) after challenge infection with Ab4. We concluded that 100% of horses previously infected with Ab4 and 60% of those infected with Ab4ΔORF1/71 (3/5) were fully protected from Ab4 challenge infection as assessed by the absence of fever, clinical signs of disease, nasal virus shedding and cell-associated viremia. This suggests the absence of virus replication in the upper respiratory tract of horses that are fully protected against EHV-1. The other two horses in the Ab4ΔORF1/71/Ab4 group showed no fever or clinical signs. However, low amounts of virus (10–40 PFU/ml) were isolated from their nasal secretions on d1pi or d1–3pi respectively, and one of them also was viremic on d6pi. These two horses were considered partially protected from Ab4 challenge infection. We concluded that the initial infection with Ab4ΔORF1/71 induced full or partial protection against EHV-1 infection for at least 6 months post initial infection.

Although viral shedding or viremia outcomes between the Ab4/Ab4 or Ab4ΔORF1/71/Ab4 groups were not significantly different, the two partially protected horses in the latter group indicated that the ORF1/71 deletion mutant virus induced less robust protection than the parent Ab4 virus. However, initial infection with Ab4ΔORF1/71 which preceded this study resulted in significantly reduced fever and nasal shedding than infection with Ab4 (Wimer et al., 2018). Based on these characteristics, the Ab4ΔORF1/71 virus is of low virulence which provides protection from EHV-1 infection for up to six months. However, to be considered as a vaccine candidate that improves protection against EHM, the Ab4ΔORF1/71 virus would need additional modifications to fully reduce its virulence and especially cell-associated viremia.

3.2. Absence of virus replication in protected horses correlates with the lack of IFN-α and inflammatory markers in the upper respiratory tract secretion

On d-2 prior to EHV-1 infection, all 15 horses had undetectable IFN-

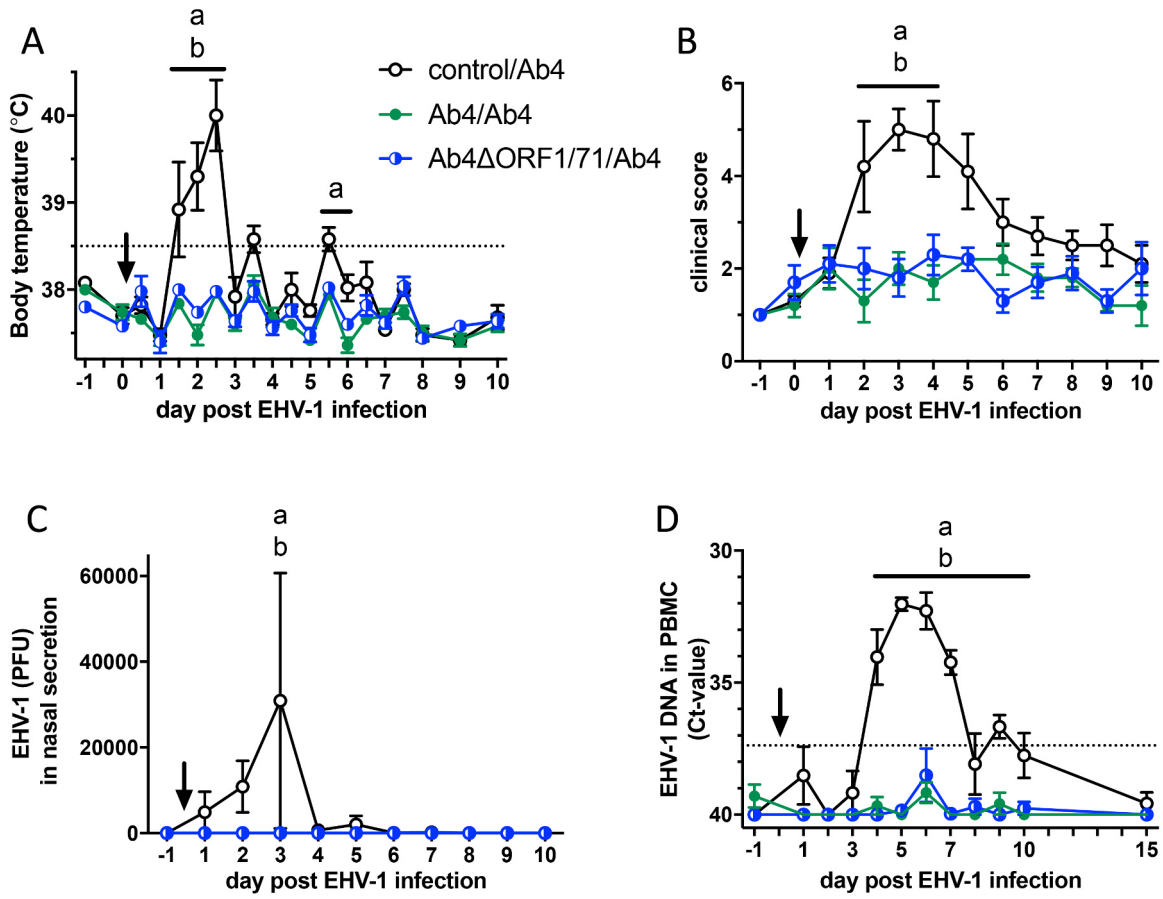

Fig. 1. Clinical findings and virus detection after intranasal challenge infection with EHV-1 strain Ab4. All horses were experimentally infected with either Ab4 (n = 5), a deletion mutant strain Ab4ΔORF1/71 (n = 5), or received saline (non-infected control; n = 5) six months prior to this challenge infection. Then, all 15 horses were challenged intranasally with 1×10^7 PFU Ab4. The arrow points to the time of Ab4 challenge infection (d0). (A) Body temperature; the dotted line shows the cut-off value for a fever at 38.5 °C; (B) total clinical score determined by several clinical variables evaluated on a numerical scale; (C) infectious EHV-1 in nasal secretions measured by virus isolation; (D) viremia measured by quantitative PCR from PBMC; the dotted horizontal line shows the cut-off value for a positive PCR result. PFU = plaque forming units, Ct value = cycle threshold value. Means and standard errors are displayed. Significant differences between groups are marked: a = Ab4/Ab4 and control/Ab4; b = Ab4ΔORF1/71/Ab4 and control/Ab4.

α and CCL2, and low concentrations of sCD14 (median 930 pg/ml; range 370–2480 pg/ml) in their nasal secretions without differences between the three groups. After Ab4 challenge, horses in the Ab4/Ab4 or Ab4ΔORF1/71/Ab4 groups were lacking IFN-α induction in their nasal secretions (Fig. 2A) and only slightly enhanced their intranasal CCL2 and sCD14 concentrations (Fig. 2B/C). In contrast, IFN-α, CCL2 and sCD14 increased in the nasal secretions of the susceptible horses in the control/Ab4 group shortly after EHV-1 infection (Fig. 2A-C). Nasal secretions of control/Ab4 horses had significantly elevated IFN-α on d2pi ($p < 0.0001$), CCL2 on d2pi ($p < 0.0001$) and d3pi ($p < 0.01$), and sCD14 on d3–5pi ($p < 0.05$ to < 0.0001) compared to the Ab4/Ab4 or Ab4ΔORF1/71/Ab4 groups. Afterwards, all three inflammatory markers were quickly downregulated in the control/Ab4 group within the first week pi. Similar rapid increases and decreases in intranasal IFN-α and sCD14 secretion were reported after previous experimental Ab4 infection studies of EHV-1 susceptible horses (Soboll Hussey et al., 2011; Wimer et al., 2018; Schnabel et al., 2018b), while CCL2 induction after *in vivo* EHV-1 infection of horses is reported here for the first time. Other intranasal cytokines such as IFN-γ, IL-17A and IL-10 were of overall low concentrations despite few significant increases in their expression during the first week pi in the control/Ab4 group (Suppl. Fig. 1).

Type I IFNs including IFN-α are induced in the early stages of innate immune responses, especially after viral infection. They have long been known for their anti-viral activity characterized by inhibition of viral infection and replication (Samuel, 2001). Moreover, type I IFNs affect many downstream effects in both innate and adaptive immune responses, such as the induction of dendritic cell maturation, activation and maintenance of Th1-cells, and regulation of various immune mediators (Theofilopoulos et al., 2005; Murira and Lamarre, 2016). Because of its beneficial anti-viral effects, IFN-α has been used for treatment of patients with viral diseases such as Hepatitis B or HIV (Tan et al., 2018; Noël et al., 2018). As in this current EHV-1 challenge infection study (Fig. 2A), EHV-1 infection induced pronounced IFN-α production by local respiratory epithelial cells in EHV-1 naïve or susceptible horses (Schnabel et al., 2018b; Wimer et al., 2018). This suggests that the innate intranasal IFN-α response to EHV-1 infection supports the anti-viral stage and adaptive immune induction.

Chemokines such as the pro-inflammatory, chemotactic CCL2 are primarily expressed by immune cells, like monocytes, macrophages or dendritic cells (Schnabel et al., 2018a). CCL2 is also upregulated after EHV-1 infection of equine nasal mucosal explants suggesting that infected epithelial cells at the local infection site contribute to CCL2 secretion (Zhao et al., 2017). CCL2 recruits monocytes and T-cells into tissues in mice and people (Carr et al., 1994; Lu et al., 1998; Mantovani et al., 2004) and supports the migration of monocytic cells in EHV-1 infected nasal explants in horses (Zhao et al., 2017). In agreement with the findings in the equine nasal explant model *in vitro*, EHV-1 infection induced intranasal CCL2 secretion in the control/Ab4 group (Fig. 2B) suggesting that this chemokine plays important roles in attracting immune cells to the site of EHV-1 entry.

As shown in Fig. 2C and previous studies, we have repeatedly

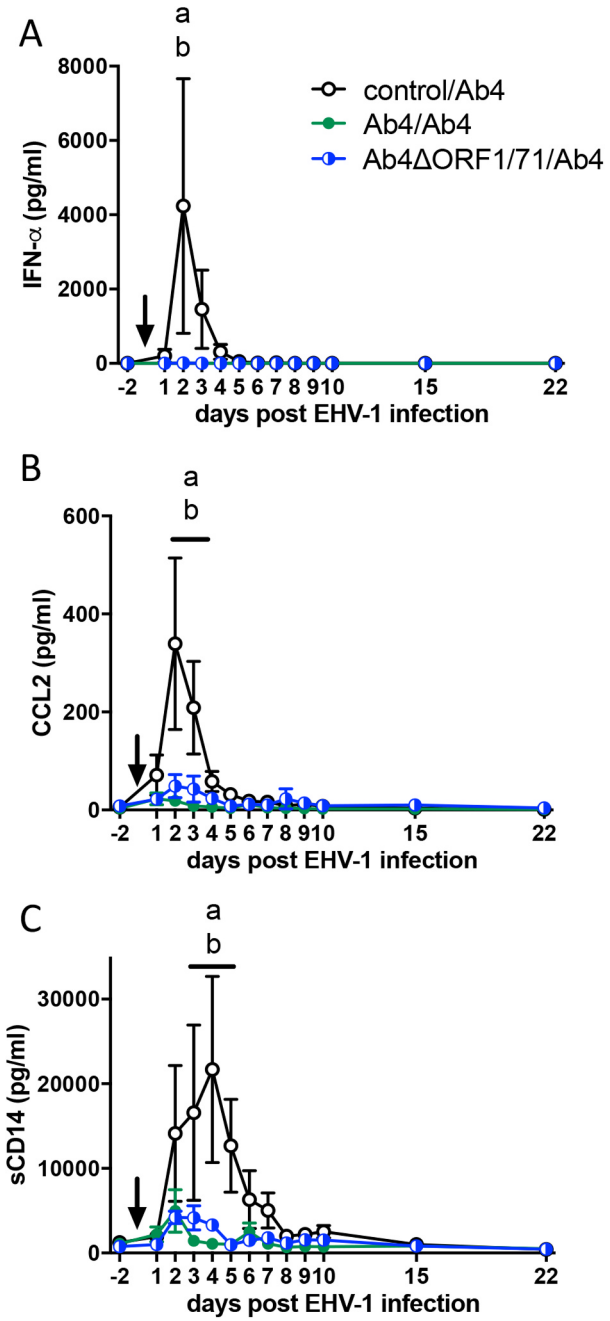

Fig. 2. Cytokines in nasal secretion after Ab4 challenge infection of EHV-1 susceptible and protected horses. Horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 were infected six months prior to this challenge infection with Ab4 or Ab4ΔORF1/71, respectively. Susceptible control horses were not infected previously. Intranasal Ab4 challenge infection with 1×10^7 PFU was performed on d0 (arrow). Cytokines were measured in nasal secretion samples using different fluorescent bead-based multiplex assays. Rapid increases in A) IFN-alpha, B) CCL2, and C) sCD14 were detected in the control/Ab4 group. Significant differences between groups are shown: a = Ab4/Ab4 and control/Ab4; b = Ab4ΔORF1/71/Ab4 and control/Ab4.

observed robust intranasal sCD14 secretion after EHV-1 infection of susceptible horses (Schnabel et al., 2018b; Wimer et al., 2018). CD14 is expressed on the surface of equine monocytes and macrophages (Kabithe et al., 2010) where it functions as the receptor for bacterial LPS and LPS-binding protein by associating with Toll-like receptor 4 (Landmann et al., 2000). Shedding of CD14 from cell surfaces results in sCD14 (Bazil et al., 1986; Landmann et al., 1992) which acts as an LPS-inhibitor *in vivo* by protecting cells against LPS-induced death (Schütt et al., 1992; Haziot et al., 1995). As a sink mechanism from the circulation, sCD14 progressively removes LPS from membrane CD14 and transfers it to plasma lipoproteins (Kitchens and Thompson, 2005). Consequently, humans and horses have high baseline concentrations of sCD14 in their serum (Marcos et al., 2010; Wagner et al., 2013). In humans, systemic sCD14 is used as a soluble biomarker to evaluate disease susceptibility and progression in neonatal sepsis (Mussap et al., 2011), pneumonia in children (Marcos et al., 2010), and during chronic HIV infection (Leeansyah et al., 2013). In horses, increased systemic sCD14 concentrations were associated with neonatal sepsis, recurrent airway obstruction (Wagner et al., 2013), and endotoxaemia (Fogle et al., 2017). As discussed previously, healthy horses routinely have existing sCD14 concentrations in their nasal secretion which can originate from intranasal macrophages residing on the mucosal surfaces and/or tissues of the upper respiratory tract. Functionally, the slightly more gradual and prolonged sCD14 upregulation compared to IFN-α and CCL2 might represent an alertness stage of the inflamed respiratory epithelium to prevent secondary bacterial colonization after EHV-1 infection. However, the role of sCD14 and the mechanism of its upregulation during the early innate immune response after EHV-1 infection are still unknown and require further analysis.

3.3. Intranasal IFN-α and inflammatory marker secretion correlate with nasal EHV-1 shedding

Importantly, IFN-α and CCL2 expression highly correlated with the amount of infectious EHV-1 virus isolated from nasal secretion on d1–4pi and d2–4pi, respectively, while sCD14 concentrations correlated with virus isolation between d3–5pi (Table 2). During the entire study, IFN-α was undetectable in the nasal secretion of all eight fully protected horses and also in the partially protected horse showing only one day of low viral shedding (10 PFU/ml nasal secretion). The other partially protected horse was shedding low EHV-1 amounts on d1–3pi (10–40 PFU/ml) and had low IFN-α concentrations in the nasal secretion on the same days. This confirms that intranasal inflammatory markers, such as IFN-α, CCL2, and sCD14, represent 'danger signals' occurring simultaneously with nasal shedding of infectious EHV-1. In contrast, full protection from EHV-1 infection was characterized by the absence of nasal shedding and IFN-α secretion together with undetectable or low CCL2 and sCD14 concentrations in nasal secretions 24 h pi and afterwards. The lack of intranasal cytokine upregulation, and especially the absence of IFN-α secretion in protected horses, strongly suggests that EHV-1 did not enter the nasal epithelium in fully protected horses. Notably, infectious Ab4 virus could also not be recovered from the nasal secretions of fully protected horses at 24 h pi or afterwards despite the inoculation of 1×10^7 PFU EHV-1 Ab4 at challenge infection. We next asked which immune parameters prevented the entry of EHV-1 into epithelial cells and simultaneously inactivated the inoculated Ab4 virus in protected horses effectively?

3.4. Protective immunity is characterized by pre-existing EHV-1-specific intranasal IgG4/7 antibodies

In this approach, we measured total antibodies and antibody isotypes against three glycoproteins of EHV-1, gB, gC and gD. Here and as shown previously (Wimer et al., 2018; Schnabel et al., 2018b), antibody responses to all three EHV-1 antigens were highly similar to each other and therefore only the anti-EHV-1 gC responses are shown.

On d-2 prior to EHV-1 infection and compared to the naïve horses in the control/Ab4 group, horses in the Ab4/Ab4 group had increased anti-gC total Ig ($p = 0.014$) composed of some IgG1 ($p = 0.0239$) and high amounts of IgG4/7 antibodies ($p = 0.0071$) in their nasal secretions (Fig. 3A). All three antibody parameters were also increased for several horses in the Ab4ΔORF1/71/Ab4 although this did not reach statistical significance. Interestingly, the partially protected horse in the

Table 2
Correlation (r_{sp}) between virus isolation (PFU) and cytokine concentrations in nasal secretion samples of all horses (n = 15) during the first six days after challenge infection with 1×10^7 PFU EHV-1 Ab4.

Day pi	IFN-α			CCL2			sCD14		
	r_{sp}	95% CI	p-value	r_{sp}	95% CI	p-value	r_{sp}	95% CI	p-value
1	0.64	0.17–0.87	**0.0096**	0.49	− 0.05–0.81	0.0686	0.14	− 0.41–0.62	0.6117
2	0.94	0.82–0.98	< **0.0001**	0.63	0.16–0.87	**0.0138**	0.36	− 0.21–0.74	0.1935
3	0.92	0.76–0.97	< **0.0001**	0.71	0.29–0.90	**0.0043**	0.70	0.28–0.90	**0.0046**
4	0.74	0.35–0.91	**0.0059**	0.61	0.12–0.86	**0.0187**	0.60	0.12–0.86	**0.0189**
5	0.28	− 0.29–0.70	0.1297	0.37	− 0.19–0.75	0.1806	0.55	0.04–0.84	**0.0352**
6	0.44	− 0.11–0.79	0.2000	− 0.06	− 0.57–0.48	0.8667	0.31	− 0.26–0.72	0.4000

r_{sp} = Spearman rank correlation coefficient; 95% CI = 95% confidence interval; PFU = plaque forming units; p-values < 0.05 are highlighted in bold.

Ab4ΔORF1/71/Ab4 group that showed two days of viral shedding together with low intranasal IFN-α secretion had the lowest total Ig, IgG1 and IgG4/7 values in the Ab4ΔORF1/71/Ab4 group (Fig. 3A). In contrast, pre-infection anti-gC IgG3/5 antibodies were of low magnitude in all three groups.

For all 15 horses, pre-infection total Ig and especially IgG4/7 values correlated strongly with protection from fever, clinical signs, viral shedding, and viremia (Table 3). These findings strongly suggested that pre-existing EHV-1-specific IgG4/7 antibodies on the mucosal surface of the upper respiratory tract can effectively and immediately neutralize EHV-1 and completely prevent viral entry into epithelial cells after experimental challenge infection with a high viral dose of neuropathogenic Ab4 virus. The detailed mechanisms of the neutralizing effect(s) of EHV-1-specific intranasal IgG4/7 are still unknown and have to be subject of future studies. For example, neutralization could have occurred by immediate opsonization of the virus, complement activation, induction of antibody-dependent cytotoxicity, blocking the interaction of the virus with its cellular entry receptors, or other mechanisms. The polyclonal antibody response directed against various immunogenic viral glycoproteins including gB, gC and gD suggests effective opsonization of EHV-1 with IgG4/7 which may lead to enhanced phagocytosis of the virus/IgG4/7 complexes by macrophages present in the nasal secretion. Although most work on equine respiratory macrophages has been performed on alveolar macrophages (Joubert et al., 2011; Karagianni et al., 2013), macrophages are also constantly present in the secretion of the upper respiratory tract (data not shown). Overall, the high capacity of IgG4/7 to initiate and perform different antibody effector functions such as complement activation and Fc receptor binding (Lewis et al., 2008), together with the strong correlation of IgG4/7 antibodies to protect from respiratory viral diseases including equine Influenza and EHV-1 (Nelson et al., 1998; Goodman et al., 2006, 2012; Goehring et al., 2010; Soboll Hussey et al., 2011; Wagner et al., 2017; Wimer et al., 2018; Schnabel et al., 2018b) further support the hypothesis of effective neutralizing capacity of EHV-1-specific IgG4/7 at the local infection site.

3.5. *Intranasal EHV-1-specific IgG4/7 increases rapidly after Ab4 challenge infection of protected horses*

After Ab4 challenge infection, susceptible horses in the control/Ab4 group had similar intranasal antibody responses as to those shown during the initial Ab4 infection described by Wimer et al. (2018). These were characterized by increasing anti-gC antibodies starting to be detectable in the nasal secretion by the end of the first week pi and highest intranasal antibody values around d15pi which declined by d22pi (Fig. 3B). The local anti-gC IgG1 response peaked earlier (d9pi) than the IgG4/7 response (d15pi). Horses in the control/Ab4 group mounted a high intranasal anti-gC IgG1 antibody response that was significantly increased compared to the two previously infected groups between d8–10pi (all p-values < 0.01). IgG4/7 antibodies mimicked total anti-gC Ig most closely (Fig. 3C/D). Intranasal IgG3/5 was of overall low magnitude compared to the IgG1 and IgG4/7 antibodies in the control/Ab4 group.

In contrast, horses that were previously infected with EHV-1 (Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups) had pre-existing intranasal antibodies from the initial infection as discussed above. In response to the Ab4 challenge infection, horses in both groups also increased their intranasal antibodies rapidly and more than doubled their anti-gC total Ig and IgG4/7 values by d2pi (Fig. 3B/D). This resulted in significant increases in anti-gC total Ig on d2pi ($p < 0.01$) and IgG4/7 on d2pi ($p < 0.01$) and d3pi ($p < 0.001$) in the Ab4ΔORF1/71/Ab4 compared to the control/Ab4 group. In the Ab4/Ab4 group, anti-gC total Ig was higher on d1pi ($p < 0.05$) and d2pi ($p < 0.01$), and IgG4/7 on d2pi ($p < 0.001$) in longitudinal comparison with the control/Ab4 group. Afterwards, nasal total anti-gC and IgG4/7 antibodies showed a wave-like pattern for the protected groups with a decline by d4pi and a second smaller increase during d7–9pi followed by a gradual decline to d22pi. By d22pi, total Ig and IgG4/7 values were approximately in the same range where they had been on d-2 prior to Ab4 challenge infection. Nasal anti-gC IgG1 and IgG3/5 antibody responses were overall low and short-lasting in the Ab4ΔORF1/71/Ab4 and Ab4/Ab4 groups (Fig. 3C/E). Differences in intranasal antibody values between the Ab4ΔORF1/71/Ab4 and Ab4/Ab4 groups were not observed during this study.

Overall, the rapid increase in intranasal EHV-1-specific IgG4/7 antibodies in protected horses strongly suggests that Ab4 challenge infection provided an immune stimulus despite not inducing disease or viral entry into respiratory epithelial cells. It also suggests that horses in the Ab4ΔORF1/71/Ab4 and Ab4/Ab4 groups had EHV-1-specific memory B-cells originating from the initial infection six months previously. The locally processed EHV-1 antigens likely initiate the rapid increase in antibody production and especially intranasal EHV-1-specific IgG4/7 in the upper respiratory tract. Alternatively, the presence of the virus in the nasal passage could have stimulated an antibody influx from serum *via* local fenestrated capillaries to support virus neutralization at the entry site (Neutra and Kozlowski, 2006). As shown below, protected horses have high pre-existing IgG4/7 antibodies in their circulation prior to challenge. Nevertheless, transfer of IgG from serum to respiratory mucosal surfaces has not yet been confirmed in horses and requires further investigation.

3.6. *EHV-1-specific antibody isotypes in serum reflect intranasal antibody values prior to EHV-1 challenge infection and may be correlates of protection*

Before Ab4 challenge infection (d-2), horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups had high amounts of antibodies in their circulation (Fig. 4A). Their serum antibody response was induced six months prior to this EHV-1 challenge infection when the naïve horses were first infected with Ab4ΔORF1/71 or Ab4 (Wimer et al., 2018). On d-2 pre-challenge infection, horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups had higher serum anti-gC total Ig and IgG4/7 values than those in the control/Ab4 group (all $p < 0.05$; Fig. 4A). Although IgG1

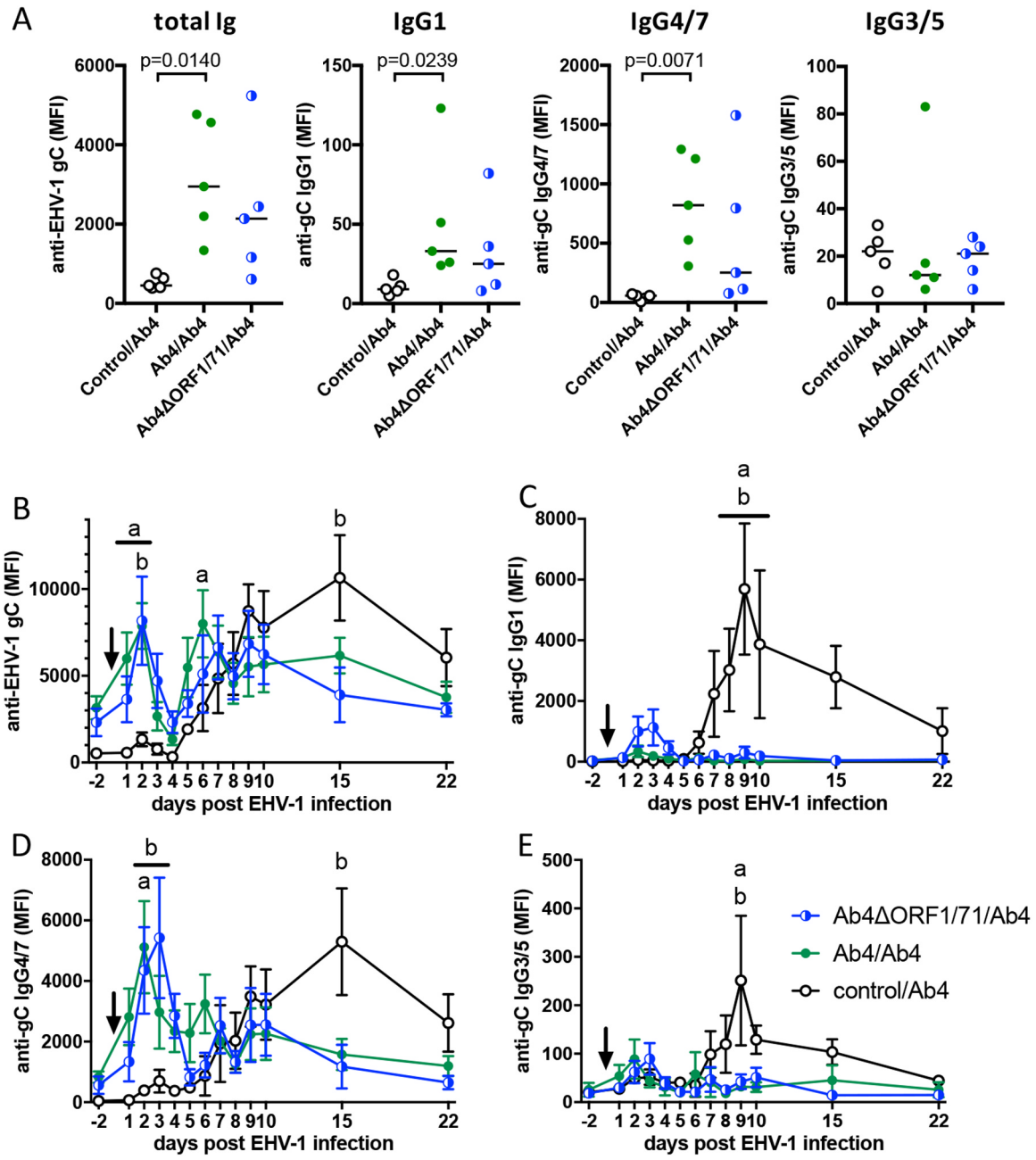

Fig. 3. Anti-EHV-1 gC antibodies in nasal secretions. Total Ig and IgG isotypes were measured by EHV-1 multiplex assays in nasal secretion samples. A) Pre-existing nasal anti-gC antibodies in all 15 horses prior to Ab4 challenge on d-2 by experimental group. (B-E) All horses were then challenged with 1×10^7 PFU Ab4 on day 0 (arrows) and intranasal antibodies were measured until d22pi. The individual graphs show B) total Ig; C) IgG1; D) IgG4/7; and E) IgG3/5 responses post Ab4 challenge. Note differences in the y-axes scales. Significant differences between groups a = Ab4/Ab4 and control/Ab4; b = Ab4ΔORF1/71/Ab4 and control/Ab4.

antibody amounts were mostly low on d-2, horses in the Ab4ΔORF1/71/Ab4 group had also increased anti-gC IgG1 isotypes compared to the control/Ab4 group ($p = 0.0071$), while IgG3/5 values were overall low and not different between groups Fig. 4A). Notably, serum anti-gC total Ig and IgG4/7 antibody values highly correlated with nasal antibody amounts on d-2 and a correlation was also found for the quantitatively much lower serum and nasal IgG1 amounts (Table 3).

Overall, the EHV-1-specific isotype composition in serum, and especially the robust IgG4/7 amounts in protected horses, mirrored the observed antibodies in nasal secretions prior to Ab4 challenge infection. The data support the concept that anti-gC total Ig and IgG4/7 antibodies in serum are robust indicators for and correlate with protection against EHV-1 infection including clinical disease, nasal shedding and viremia (Table 4).

3.7. EHV-1 challenge infection boosts existing humoral immunity in protected horses

Ab4 challenge infection of the control/Ab4 group induced a similar systemic antibody isotype response as previously observed in EHV-1 naïve or susceptible horses with different EHV-1 strains (Goehring et al., 2010; Soboll Hussey et al., 2011; Wagner et al., 2017; Schnabel et al., 2018b; Wimer et al., 2018). Prior to challenge infection, EHV-1-specific serum antibody values in susceptible horses in the control/Ab4 group were overall low or not detectable (Fig. 4). The serum antibody response after challenging these horses was characterized by increasing total anti-EHV-1 gC antibodies detectable on d6–8pi, reaching highest values around d57pi, and starting to decline afterwards. Horses in the control/Ab4 group had lower anti-gC total Ig serum antibodies as those

Table 3
High pre-infection (d-2) intranasal anti-EHV-1 gC IgG4/7 antibodies correlate strongly with the absence of fever, clinical signs, nasal viral shedding and viremia after Ab4 challenge infection of horses (n = 15), as well as with the presence of pre-infection serum antibodies.

		EHV-1 gC specific antibodies in nasal secretion			
		Total Ig	**IgG1**	**IgG4/7**	**IgG3/5**
Fever[a]	r_{sp}	− 0.60	− 0.44	− 0.65	0.07
	95% CI	− 0.86 to − 0.11	− 0.78–0.12	− 0.88 to − 0.20	− 0.47–0.58
	p-value	**0.0203**	0.1055	**0.0098**	0.7929
Clinical signs[b]	r_{sp}	− 0.77	− 0.52	− 0.78	0.09
	95% CI	− 0.92 to − 0.42	− 0.82–0.01	− 0.93 to − 0.44	− 0.46–0.58
	p-value	**0.0011**	**0.0486**	**0.0009**	0.7567
Viral shedding[c]	r_{sp}	− 0.84	− 0.82	− 0.87	0.10
	95% CI	− 0.95 to − 0.56	− 0.94 to − 0.53	− 0.96 to − 0.63	− 0.44–0.60
	p-value	**0.0002**	**0.0003**	< **0.0001**	0.7081
Cell-associated viremia[d]	r_{sp}	0.61	0.55	0.73	0
	95% CI	0.12–0.86	0.04–0.84	0.33–0.91	− 0.52–0.52
	p-value	**0.0186**	**0.0344**	**0.0030**	> 0.9999
serum antibodies[e]	r_{sp}	0.90	0.78	0.86	0.33
	95% CI	0.71–0.97	0.44–0.93	0.62–0.96	− 0.23–0.73
	p-value	< **0.0001**	**0.0009**	< **0.0001**	0.2270

r_{sp} = Spearman rank correlation coefficient; 95% CI = 95% confidence interval.
p-values < 0.05 are highlighted in bold.
[a] body temperature on d2.5pi.
[b] clinical score on d3pi.
[c] nasal viral shedding (PFU) on d3pi.
[d] viremia (CT value) on d5pi.
[e] corresponding pre-infection (d-2) serum antibody isotype.

in the two protected Ab4ΔORF1/71/Ab4 and Ab4/Ab4 groups between d-2 to d6 or d7pi, respectively (all p-values < 0.01; Fig. 4B). Similarly, control/Ab4 group IgG4/7 values were lower than those in the protected groups between d-2 to d6pi (Ab4ΔORF1/71/Ab4) or d8pi, respectively (all p < 0.05). The control/Ab4 group developed anti-gC IgG4/7 isotypes starting on d7pi and remaining high for the duration of the study, thereby mimicking closely the anti-gC total Ig pattern (Fig. 4D). Serum anti-gC IgG1 in the control/Ab4 group increased at the same time (d7pi), peaked on d15pi, and was significantly higher than serum IgG1 in both previously infected groups on d8–15pi (all p-values > 0.01) (Fig. 4C). Anti-gC IgG1 values declined to low values on d57pi and afterwards which was consistent with the decline of EHV-1-specific IgG1 in earlier studies mentioned above. Anti-gC IgG3/5 showed a modest increase in the control/Ab4 group from d7–22pi which was not different from the other groups at any time (Fig. 4E). Anti-EHV-1 gC IgG6 and IgM antibodies in serum were very low in all groups and remained consistent throughout the study period in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups, while some increases were observed in the control/Ab4 group (Suppl. Fig. 2). Anti-gC IgG1/3 antibodies mimicked the IgG1 response (Suppl. Fig. 2) and the IgG1/3 pattern together with the low IgG3/5 response (Fig. 4E) supported the assumption that IgG1/3 responses were mainly composed of IgG1.

The systemic anti-gC antibody response in the Ab4ΔORF1/71/Ab4 group matched the response of the Ab4/Ab4 group for all isotypes and at all times post challenge infection. However, in comparison to the susceptible control/Ab4 group, horses in the two protected groups did not mount an anti-gC IgG1 response after challenge infection (Fig. 4C). Their systemic antibody response was dominated by high pre-existing anti-gC total Ig and IgG4/7 antibodies as discussed above. This, together with the intranasal anti-gC IgG1 and IgG4/7 isotype pattern, confirms that the antibody response to EHV-1 challenge infection in protected horses is not only faster in onset but also characterized by a different isotype pattern. Serum antibody profiles in protected horses are dominated by IgG4/7 showing a consistent IgG4/7^{high}/IgG1^{low} pattern at all sampling points after Ab4 challenge infection. In contrast, EHV-1 susceptible horses change from a IgG4/7^{low}/IgG1^{low} pattern in the first week post challenge infection to IgG4/7^{high}/IgG1^{high} in weeks 2–3, and then finally to IgG4/7^{high}/IgG1^{low}, resembling the profiles seen in protected horses.

Interestingly, systemic anti-gC total Ig and IgG4/7 antibody responses in fully or partially protected horses (Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups) increased only slightly after Ab4 challenge infection instead of showing a major increase as observed in the susceptible control/Ab4 group (Fig. 4B/D). Four months after the initial infection described by Wimer et al. (2018), high anti-gC total Ig (median around 15,000 MFI) and anti-gC IgG4/7 antibodies (median around 4000 MFI) were measured in serum of these horses (Wimer et al., 2018). Two months later on d-2 prior to this challenge infection, the median anti-gC total Ig and IgG4/7 values in both previously infected groups had dropped slightly to around 10,000 MFI and 3000 MFI, respectively, with IgG4/7 antibodies representing the majority of the anti-EHV-1 gC antibodies in serum (Fig. 4). Ab4 challenge infection then boosted the systemic antibody response in all protected horses to maintain and slightly increase the EHV-1 antibody pool in serum. After antigen recognition, B-cells differentiate into memory B-cells and plasma cells. Memory B-cells reside in local and systemic lymphatic tissue to quickly respond to any following contact with the antigen (Kurosaki et al., 2015; Weisel and Shlomchik, 2017; Suan et al., 2017). Long-lived plasma cells reside in the bone marrow and secrete high amounts of antibodies (Nutt et al., 2015; Kometani and Kurosaki, 2015). Here, the IgG4/7 pattern post Ab4 challenge infection further confirm the establishment of a robust EHV-1-specific IgG4/7 memory B-cell population during initial infection with either Ab4 or the Ab4ΔORF1/71 deletion mutant six months ago. The findings also suggest that the antigenic stimulation of these memory B-cells by neutralized Ab4 viral antigens quickly induced additional EHV-1-specific IgG4/7 secreting long-lived plasma cells resulting in stable and slightly increasing serum antibody amounts for at least the next three months after challenge infection (Fig. 4D). Previous results support the decline of EHV-1-specific total Ig or IgG4/7 antibodies by 1–2 months post Ab4 infection of susceptible horses (Wimer et al., 2018; Schnabel et al., 2018b). The starting antibody decline is also shown here for the control/Ab4 group

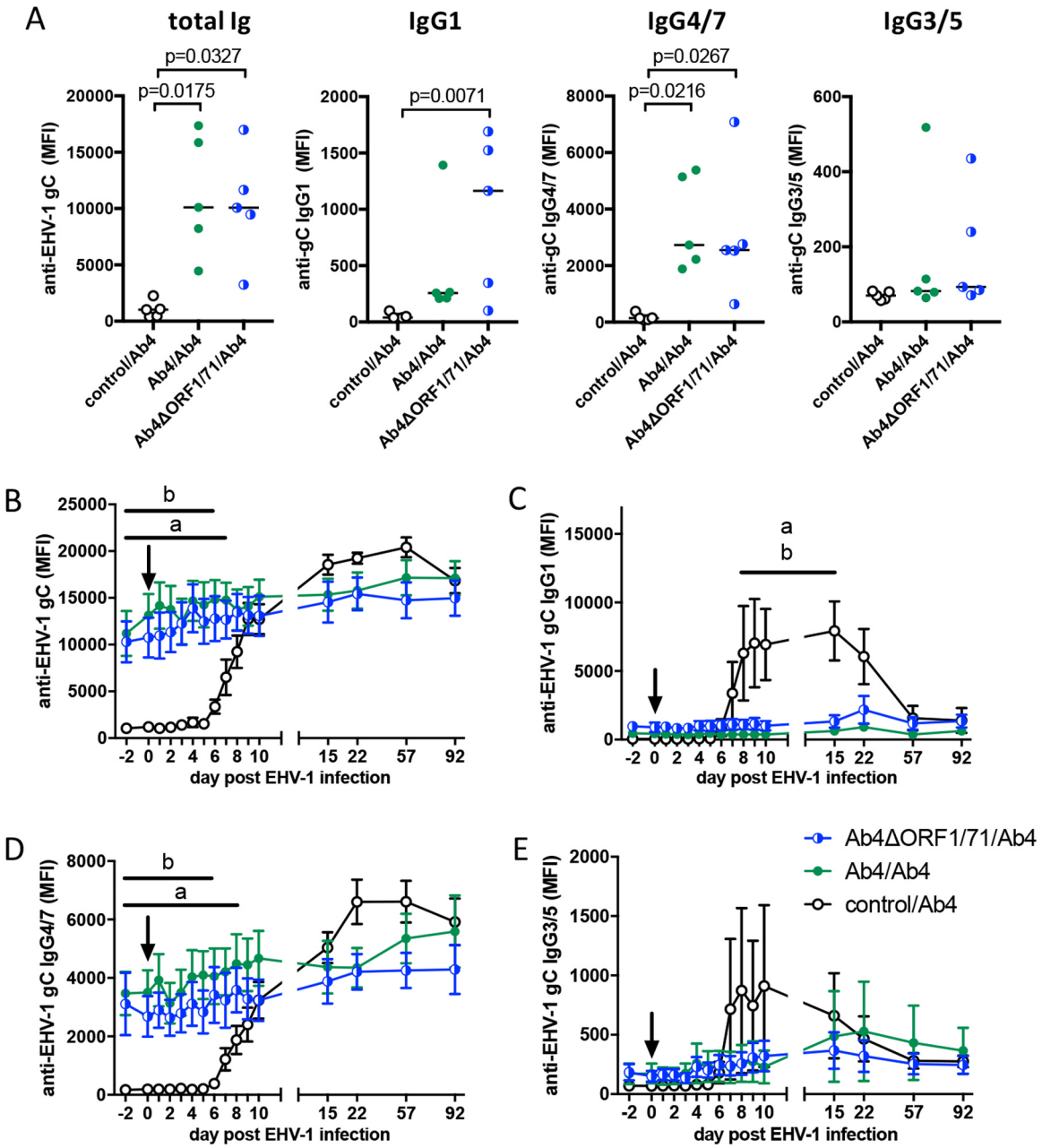

Fig. 4. EHV-1 gC-specific antibodies in serum before and after Ab4 challenge infection. Horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups were initially infected with Ab4 or Ab4ΔORF1/71, respectively. Antibodies in serum were measured by an EHV-1 Multiplex assay. A) Comparison of pre-existing anti-gC antibodies in all 15 horses prior to Ab4 challenge infection on d-2 by experimental group. (B-E) Six months after the initial infection all horses were challenged with Ab4 (arrows). Anti-gC results are shown for B) total Ig, C) IgG1, D) IgG4/7, and E) IgG3/5. Note differences in the y-axes scales. Significant differences between groups: a = Ab4/Ab4 and control/Ab4; b = Ab4ΔORF1/71/Ab4 and control/Ab4.

between d57 and d92 pi (Fig. 4B/D). Based on these observations, it can be assumed that repeated antigenic challenge with EHV-1 in protected horses extends the longevity of EHV-1-specific serum antibody responses and thereby the duration of protective EHV-1 immunity.

3.8. Cellular immunity prior to EHV-1 challenge infection

EHV-1-specific cellular immunity was analyzed by *ex vivo* re-stimulation of PBMC to amplify memory cells. Prior to challenge infection with Ab4, horses in the Ab4/Ab4 or Ab4ΔORF1/71/Ab4 groups had pre-existing cellular immunity against EHV-1 which was characterized by increased IFN-γ secretion from PBMC (Fig. 5A). This was associated with higher percentages of circulating IFN-γ producing EHV-1-specific T-cells compared to the control/Ab4 group (Fig. 5B). In contrast, IL-10 or IL-4 secretion did not differ between the three groups on d-2 (Fig. 5A). EHV-1-specific T-cells were composed of a mixture of $CD8^+$ and $CD4^+$ T-cells. However, their overall percentages in the peripheral blood were very low (Fig. 5B). Most likely the majority of the EHV-1-specific T-cells resides in lymphatic tissues in previously infected horses and only a few of these cells are detectable in the peripheral blood. This makes the detection of EHV-1-specific cellular immunity in PBMC more challenging to perform than the evaluation of the robust systemic EHV-1-specific total Ig and IgG4/7 responses discussed above (Fig. 4).

Nevertheless, pre-challenge infection levels of IFN-γ secretion from PBMC and EHV-1-specific T-cells correlated with protection (Table 4). In particular, IFN-γ secretion from PBMC correlated highly with

Table 4
Adaptive EHV-1-specific immune parameters correlating with protection from disease, nasal viral shedding, and viremia in horses (n = 15).

		Serum antibodies (anti-gC)		Cellular immunity (PBMC)	
		Total Ig	**IgG4/7**	**IFN-γ secretion**	**IFN-γ$^+$ lymphocytes**
Fever[a]	r_{sp}	− 0.63	− 0.61	− 0.62	− 0.69
	95% CI	− 0.87 to − 0.16	− 0.86 to − 0.12	− 0.86 to − 0.14	− 0.89 to − 0.26
	p-value	**0.0130**	**0.0181**	**0.0164**	**0.0055**
Clinical signs[b]	r_{sp}	− 0.87	− 0.79	− 0.47	− 0.57
	95% CI	− 0.96 to − 0.63	− 0.93 to − 0.46	− 0.80–0.08	− 0.84 to − 0.07
	p-value	< **0.0001**	**0.0007**	0.0819	**0.0280**
Viral shedding[c]	r_{sp}	− 0.85	− 0.85	− 0.82	− 0.67
	95% CI	− 0.95 to − 0.58	− 0.95 to − 0.58	− 0.94 to − 0.52	− 0.88 to − 0.23
	p-value	**0.0001**	**0.0001**	**0.0005**	**0.0080**
Cell-associated viremia[d]	r_{sp}	0.72	0.70	0.70	0.79
	95% CI	0.32–0.90	0.28–0.90	0.27–0.89	0.46–0.93
	p-value	**0.0035**	**0.0049**	**0.0054**	**0.0009**

r_{sp} = Spearman rank correlation coefficient; 95% CI = 95% confidence interval.
p-values < 0.05 are highlighted in bold.
[a] body temperature on d2.5pi.
[b] clinical score on d3pi.
[c] nasal viral shedding (PFU) on d3pi.
[d] viremia (CT value) on d5pi.

protection from nasal shedding and (r_{sp} = −0.82) and pre-existing EHV-1-specific IFN-γ producing T-cells with prevention of viremia (r_{sp} = 0.79). The latter is in agreement with the previous observation from Allen (2008) suggesting that pre-existing cytotoxic T-cells prevented viremia and EHM in older mares. In addition, EHV-1-specific IFN-γ producing CD4$^+$ T-cells have been shown to initiate and maintain the EHV-1-specific IgG4/7 antibody response (Wagner et al., 2015).

3.9. Challenge infection with Ab4 results in low systemic cellular immune responses in EHV-1 primed horses

After Ab4 challenge infection, a sharp peak of IFN-γ secretion was observed after EHV-1 re-stimulation of PBMC from control/Ab4 group horses at the end of the first week pi, which was missing in the two protected groups (Fig. 5C). This response was then quickly down-regulated in PBMC of control/Ab4 horses to low IFN-γ secretion levels comparable to the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups. Similar quick onsets followed by rapid down-regulation of *ex vivo* cytokine secretion were detected for IL-10 and IL-4 in control/Ab4 horses, while IFN-α secretion was similar between all three groups and IL-17A was not induced after EHV-1 re-stimulation of PBMC (Suppl. Fig. 3). However, IL-4, IL-10 or IL-17A production was undetectable in T-cells (data not shown) suggesting that the early IL-10 and IL-4 secretion was of innate immune cell origin. Rapid induction of IFN-γ and IL-10 secretion from PBMC followed by immediate downregulation has been described previously after EHV-1 infection of naïve or susceptible horses (Wagner et al., 2017; Wimer et al., 2018; Schnabel et al., 2018b). Interestingly, the up-regulation of secreted cytokines occurred simultaneously with viremia in susceptible horses (Fig. 1D) and both, cytokine secretion from PBMC and viremia were absent in protected horses. The rapid down-regulation of innate cytokine secretion can also be considered as an indicator for the ability of EHV-1 to interfere with the induction of cellular immunity. The mechanistic details on how EHV-1 interacts with immune cells during viremia and how it masters the innate immune down-regulation still need future evaluation.

Previous work has shown that EHV-1 infection of susceptible horses induces cytotoxic T-cell responses (Allen, 2008) as well as IFN-γ producing T-cells (Paillot et al., 2007; Wagner et al., 2017; Wimer et al., 2018; Schnabel et al., 2018b). After initial infection, IFN-γ producing T-cell responses are mainly composed of CD8$^+$ cells and fewer CD4$^+$ cells but are overall of slow onset and low magnitude in PBMC (Wagner et al., 2017; Wimer et al., 2018; Schnabel et al., 2018b) similar to the control/Ab4 group data shown here (Fig. 5D). Challenge infection with EHV-1 Ab4 in the two previously infected groups, Ab4/Ab4 and Ab4-ΔORF1/71/Ab4, only slightly increased the percentages of EHV-1-specific T-cells by d22pi (Fig. 5D) affecting both CD8$^+$ and CD4$^+$ T-cell subsets (Suppl. Fig. 3). However, differences in IFN-γ producing T-cell responses were not observed between the three groups during the entire study. The rather mild increase in EHV-1-specific peripheral T-cells after Ab4 challenge infection in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups is likely a result of the significantly reduced or absent viral entry into nasal epithelial cells due to pre-existing immunity in these horses. In fact, the slow and mild increases in T-cell responses align with the gradual systemic antibodies increases in the protected groups confirming that both humoral and cellular immune responses were boosted by challenging the protected horses with Ab4.

Despite the overall low levels of T-cell immunity that were detectable in PBMC after initial or challenge EHV-1 infection, it can be concluded that T-cell immunity against EHV-1 is established post infection. This is supported by low pre-existing circulating EHV-1-specific T-cell levels in protected horses (Fig. 5B) and high EHV-1-specific IgG4/7 antibody responses which are triggered by EHV-1-specific Th1 cells (Wagner et al., 2015). The rapid increase in local and systemic EHV-1-specific IgG4/7 antibodies further supports the concept that EHV-1-specific T-cell immunity is triggered immediately after challenge infection even if these cells are difficult to detect in the peripheral blood.

4. Conclusions

In comparison to its parent strain Ab4, the potential EHV-1 vaccine candidate Ab4ΔORF1/71 is less virulent, induces similar adaptive immunity after initial infection, but still establishes the same level of cell-associated viremia as described by Wimer et al. (2018). Here, Ab4 challenge infection of previously Ab4ΔORF1/71 infected horses provided less robust protection than challenge infection of horses formerly infected with Ab4. In particular, low levels of nasal viral shedding and viremia were detected in some horses of the Ab4ΔORF1/71/Ab4 group, while all Ab4/Ab4 infected horses were fully protected six months after the initial infection. The initial Ab4ΔORF1/71 or Ab4 infection provoked robust EHV-1-specific antibody responses that highly correlated with protection six months later. Nevertheless, the hypothesized increases in EHV-1-specific T-cell immunity were not observed after using the Ab4ΔORF1/71 deletion mutant *in vivo*. In summary, Ab4ΔORF1/71 will likely not provide any advantages in immune induction compared to existing EHV vaccines, results in cell-associated viremia at the time of initial infection, and, as a genetically modified-live vaccine, will

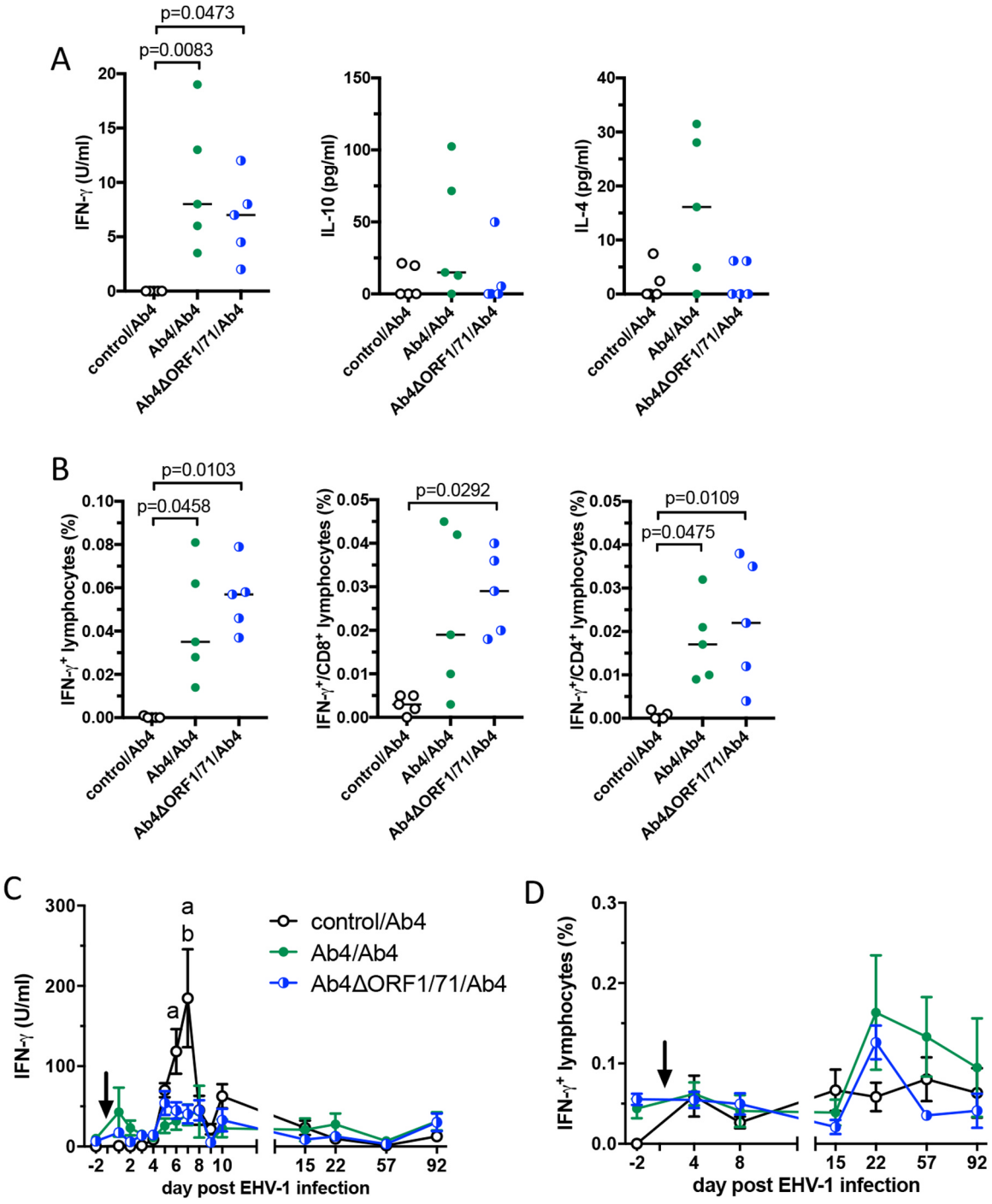

Fig. 5. Systemic cellular immune responses in susceptible and protected horses before and after EHV-1 challenge infection. Horses in the Ab4/Ab4 and Ab4ΔORF1/71/Ab4 groups were infected six months prior to this challenge infection. Control horses were not infected previously. PBMC were re-stimulated with EHV-1 *ex vivo* and cultured for 48 h. A cytokine multiplex assay was used to measure cytokine secretion in cell culture supernatants and IFN-γ producing T-cells were evaluated by flow cytometric analysis. A) Secretion of IFN-γ, IL-10 and IL-4 from PBMC prior to challenge infection (d-2) by group. B) EHV-1-specific IFN-γ producing lymphocytes, IFN-γ^+/$CD8^+$, and IFN-γ^+/$CD4^+$ T-cells on d-2 before challenge. C) IFN-γ secretion and D) IFN-γ producing lymphocytes in PBMC after Ab4 challenge with 1×10^7 PFU Ab4 on d0 (arrows). Significant differences between groups a = Ab4/Ab4 and control/Ab4; b = Ab4ΔORF1/Ab4 and control/Ab4.

likely develop latency and incorporates regulatory challenges.

Nevertheless, the Ab4 challenge infection performed here has revealed new and comprehensive insights into protective immunity against EHV-1. Fully protected horses have high amounts of EHV-1-specific IgG4/7 in their circulation which qualitatively mirror existing antibodies at the mucosa of the respiratory tract. Our findings also support the hypothesis that pre-existing intranasal IgG4/7 antibodies are able to immediately neutralize high amounts of EHV-1 at the local infection site. This mechanism effectively prevents viral entry into the nasal epithelium as indicated by the absent local innate IFN-α and overall low inflammatory responses in fully protected horses. Consequently, all down-stream effects of EHV-1 infection including fever, clinical signs, nasal virus shedding and cell-associated viremia do not occur in fully protected horses. Prevention of cell-associated viremia, the prerequisite of endothelial cell infection, remains the most effective target to counteracts severe disease outcomes such as EHM

and abortion. High EHV-1-specific IgG4/7 antibodies can thus be considered correlates associated with protection from EHV-1 abortions and EHM.

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Competing interest statement

The authors declare that they have no competing interests.

Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.03.014.

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