Chapter 5

In vivo assessment of vaccine improvement by disabling the deubiquitinase activity of equine arteritis virus papain-like protease 2

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ABSTRACT

Arteriviruses are a family of positive-stranded RNA viruses that includes the prototypic equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV), which cause respiratory illness as well as reproductive failure in their respective hosts. Although several vaccines against these viruses are commercially available there is room for improvement, especially in the case of PRRSV, which causes serious economic losses to swine-farming industries worldwide. The ability of arteriviruses to evade or counteract the innate immune response is thought to decrease the efficacy of the current modified live virus vaccines. We have recently shown that the deubiquitinase (DUB) activity of the EAV papain-like protease 2 (PLP2) is important for the inhibition of innate immune activation during infection in cell culture. A vaccine virus lacking PLP2 DUB activity may therefore induce a better immune response and provide improved protection against subsequent challenge than its DUB-competent counterpart. To test this hypothesis, twenty Shetland mares that were seronegative for EAV were randomly assigned to one of three groups. Two groups were vaccinated, either with DUB-positive (Group 1, n=9) or DUB-negative (Group 2, n=9) recombinant virus derived from the pEAV030 infectious cDNA clone of EAV. The third group (Group 3, n=2) was not vaccinated and served as a challenge control. All horses were subsequently challenged with the moderately virulent KY84 strain of EAV. The PLP2 DUB-negative recombinant vaccine virus proved to be replication competent *in vivo*. Furthermore, the data suggested that the DUB-negative vaccine virus provides better protection against a heterologous challenge than its parental counterpart, since viral RNA was more readily detected after challenge in animals vaccinated with the parental virus. Finally, despite a minor decrease in replication efficiency of the DUB-negative vaccine virus compared to the parental virus, no major differences were detected in the induction of two interferon-stimulated genes. Taken together, the data obtained in this study warrant further *in vivo* investigations into the potential of using PLP2 DUB mutant viruses for the improvement of arterivirus vaccines.

INTRODUCTION

Arteriviruses are a family of animal viruses that includes the prototypic equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV). Although the diseases associated with each of these viruses are distinct, both are characterized by the occurrence of abortions in female animals and respiratory illness in young offspring (for reviews, see (1, 2)). Whereas outbreaks of equine viral arteritis (EVA) are only occasionally reported, infections with PRRSV pose a major threat to the swine-farming industries worldwide. Especially, the emergence of highly virulent strains of PRRSV in China since 2006 has been a major concern (3-5). Several vaccines are currently commercially available to prevent the spread of EAV and PRRSV. In the case of EAV, both an inactivated and a modified live virus (MLV) vaccine are commercially available: Artervac and ARVAC, respectively (both produced by Zoetis Animal Health Inc, Kalamazoo, MI, USA). The latter is considered to be safe and efficacious, although some concerns about cross protection against recent field isolates have been reported (1). Furthermore, the MLV vaccine is not recommended for use during the last stages of pregnancy (6), vaccination does not consistently prevent the replication of some field strains, and transient shedding of infectious virus in nasal secretions is sometimes observed in vaccinated animals (7). Thus, current vaccine-based control strategies for EAV are considered adequate, but room for improvement remains. In contrast, even though several MLV vaccines against PRRSV are available, they do not always provide effective protection against the diversity of field strains observed for this virus. The current MLV vaccines for PRRSV generally provide protection against clinical disease, but do not consistently prevent replication of field strains and shedding of infectious virus by vaccinated animals (8, 9). Especially, the limited protection against heterologous field strains provided by current vaccines is a major issue that complicates the control of PRRSV (9). Importantly, PRRSV strains display a considerable genetic heterogeneity, which is exemplified by the existence of two very divergent genotypes (10). The design of improved PRRSV vaccines that provide protection against both homologous and heterologous field strains is therefore of significant importance.

It has been suggested that the immune-evasive capabilities of PRRSV play an important role in reducing vaccine efficacy (9). Rationally decreasing the ability of a (vaccine) virus to interfere with the immune response of the host might therefore provide a means to improve vaccine efficacy. Arteriviruses have been reported to counteract both innate and adaptive immune responses in various ways (for reviews, see (6, 11)). In this study, we will focus in particular on inhibition of innate immune signalling mediated by one of the arterivirus nonstructural protease domains. The innate im-

mune response constitutes the first line of defence against invading pathogens and plays an essential role in activating adaptive immunity (for reviews, see (12-14)). It is extensively regulated through ubiquitination (for reviews, see (15-17)), i.e. the covalent attachment of ubiquitin to target proteins, which can for example mediate protein-protein interactions in signalling cascades. Importantly, ubiquitination can be reversed through the action of deubiquitinases (DUBs) (for reviews, see (18, 19)). Arteriviruses, similar to other mammalian positive-stranded (+) RNA viruses, express their nonstructural proteins as part of two large replicase polyproteins that are co- and posttranslationally cleaved into their respective functional subunits (20-27). One of the viral protease domains involved in this process, papain-like protease 2 (PLP2), has previously been shown to also possess DUB activity, which was suggested to be important for the inhibition of innate immune signalling (28-31). This hypothesis could not be easily verified in the context of an infection due to the essential role of PLP2 in maturation of the replicase polyproteins, which precluded straightforward inactivation of this domain without consequences for virus viability. Nevertheless, based on the crystal structure of EAV PLP2 in complex with ubiquitin, we have recently succeeded in creating protease mutants that are defective in DUB activity yet are still capable of processing the viral replicase polyproteins (32). The rational design of these mutants consisted of substituting up to three of the amino acids in the PLP2 region that are involved in the binding of ubiquitin, thus disturbing the interaction between protease and substrate. Using these mutants, we could show that the DUB activity of EAV PLP2 is indeed important for the evasion of innate immunity in infected host cells (32).

As a follow-up to the work described above, the aim of the current study was to determine whether a recombinant vaccine virus lacking PLP2 DUB activity provides better protection against a subsequent challenge infection than its parental DUB-competent counterpart in horses. Furthermore, we were interested to see whether the effect of such PLP2 mutations on innate immune signalling observed in cell-culture could also be seen *in vivo*. Since currently the concept of structure-based separation of the DUB and polyprotein processing functions of arterivirus PLP2 has only been developed for EAV, we have used this virus for the present animal study.

MATERIALS AND METHODS

Cells and viruses

BHK-21 cells were cultured in Glasgow minimum essential medium (Lonza) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate broth, and 10

mM Hepes (pH 7.4). Primary equine lung fibroblasts (ELFs) were cultured in minimum essential medium (Lonza) supplemented with 10% FBS and grown on collagen-coated plastics for a maximum of 10 passages. Vero cells were cultured in proprietary cell culture medium (MSD Animal Health) supplemented with 1% FBS. All culture media contained 100 U/mL of penicillin and 100 mg/mL of streptomycin or neomycin.

The vaccine viruses were derivatives of an infectious cDNA clone of EAV strain Bucyrus (pEAV030) (33, 34). Specifically, the parental cDNA clone used in this study is pEAN551A/B, which differs from pEAV030 by the absence of a strong-stop sequence for *in vitro* transcription and the addition of a number of silent mutations for the purpose of creating unique restriction sites (32, 35). The DUB-negative virus was produced from the pEAN551A/B T312A/I313V/I353R (numbering based on amino acid sequence of EAV replicase polyprotein 1a) mutant clone described previously (32). Virus stocks were produced essentially as described before (32). Briefly, *in vitro* transcribed RNA was was generated from the linearized full-length cDNA clones using the mMESSAGE Machine T7 kit (Ambion) and electroporated into BHK-21 cells using the AMAXA cell line nucleofector kit T (Lonza). After a 30-h incubation at 39.5°C, virus-containing supernatants were harvested, spun down to remove cellular debris, and stored at -80°C until further use. Viral titers were determined by standard plaque assay on ELFs. For experimental challenge, we used the moderately virulent Kentucky 1984 (KY84) strain of EAV, which has been previously described (36, 37).

To confirm the use of the correct virus for vaccination, the presence or absence of PLP2 mutations was established, both before and after vaccination. Before vaccination, viral RNA was isolated from the produced virus stocks using the QIAamp viral RNA mini kit (Qiagen) and converted to cDNA using RevertAid H Minus reverse transcriptase (Fermentas) and random hexamer primers. The PLP2-encoding region was subsequently PCR amplified using *Pfu* DNA polymerase (Fermentas) and sequenced. After vaccination, viral RNA present in the blood of four horses each from the vaccinated groups at 4 days post vaccination was subjected to sequencing. This was done essentially as described above with the exception that viral RNA in whole blood total RNA (see below) was converted to cDNA using a primer that is specific for the EAV genome. Primer sequences are available upon request.

Experimental vaccination and challenge of horses

The experiment was performed in accordance with European Community guidelines and national laws on animal experiments. The design of the experiment was approved by the MSD Animal Health's Committee on the Ethics of Animal Experiments (*Dierexperimentencommissie*), which is required by national legislation to include both MSD Animal Health employees and independent members, prior to the start of the trial (Permit Number: EXP 12.059). All efforts were made to minimize animal discomfort.

Twenty female Shetland horses (*Equus ferus caballus*; average age 7.5 ±5.1 years) that tested negative for EAV-neutralizing antibodies (titers were determined as described previously (36)) before the start of the experiment, were randomly assigned to one of three treatment groups. After a one-week acclimatization period, horses in Group 1 (n=9) and Group 2 (n=9) received an intramuscular (cervical muscle) vaccination of 1 ml phosphate-buffered saline containing $1x10^7$ plaque-forming units (PFU) of parental or PLP2 DUB-negative EAV551A/B, respectively. Horses in Group 3 (n=2) were not vaccinated and were housed outside the animal facilities until their inclusion in the study approximately one week before challenge. At 34 days post vaccination (dpv), all horses were challenged by intranasal inoculation with $1x10^5$ PFU of EAV KY84 in a total volume of 5 ml phosphate-buffered saline.

Given the fact that the viruses used for vaccination qualify as genetically modified organisms (GMO), vaccinated horses were kept in BSL3 containment during the entire experiment. All horses were housed in groups, but Group 1 horses were kept separate from Group 2 horses to prevent any cross-contamination. Horses from Group 3 were divided among the two stables upon inclusion. Water was provided *ad libitum* and standard feeding procedures were applied.

The general health status of the animals was checked by a veterinarian before vaccination as well as before challenge, and daily by animal care-takers during the entire course of the experiment. In addition, clinical signs were recorded daily from 0 to 14 days post challenge (dpc) and scored according to **Table V-1**. Rectal temperatures were taken daily from 0 to 14, and at 21 and 28 dpv, and daily from 0 to 14, and at 21 and 27 dpc. Blood samples for serum and total RNA isolation were taken every other day between 0 and 14, and at 21 and 28 dpv, and every other day between 0 and 14, and at 21 and 27 dpc. Animals were euthanized according to standard procedures at 61 dpv. For a schematic overview of the experimental set-up see **Figure V-1**.

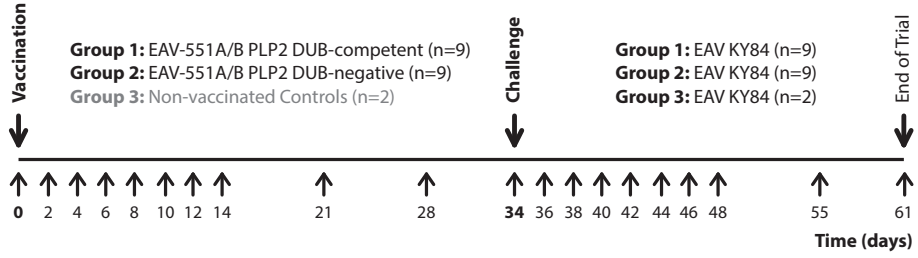

Figure V-1. Schematic representation of the animal trial. Twenty female Shetland horses were randomly assigned to one of three groups. At the start of the experiment, horses from Groups 1 and 2 (n=9 each) were vaccinated with PLP2 DUB-competent or DUB-negative EAV-551A/B, respectively. Horses in Group 3 (n=2) were not vaccinated. At 34 dpv, all horses were challenged with moderately virulent EAV KY84. The experiment ended at 61 dpv. Blood samples for serum and total RNA isolation were taken at the indicated days (lower arrows).

Table V-1. Clinical signs scoring table.

Clinical sign	Score
No abnormality found	0 (daily)
Malaise/depression/normal eating	1 (daily)
Malaise/depression/reduced appetite	2 (daily)
Anorexia	4 (daily)
Dehydration	2 (daily)
Down/unable to stand	30 (daily)
Vomiting	1 (daily)
Diarrhoea	1 (daily)
Moribund	50 (daily)
Dead	100 (once)
Hyperpnoea	2 (daily)
Dyspnoea	4 (daily)
Cough (2-5x in 10 minutes)	1 (daily)
Cough (6-20x in 10 minutes)	2 (daily)
Cough (>20x in 10 minutes)	3 (daily)
Lachrymation	1 (daily)
Mucopurulent eye discharge (mild)	2 (daily)
Mucopurulent eye discharge (marked)	4 (daily)
Conjunctivitis (mild)	2 (daily)
Conjunctivitis (marked)	4 (daily)
Nasal serous dicharge	1 (daily)
Nasal mucopurulent (mild)	2 (daily)
Nasal mucopurulent (marked)	4 (daily)
Sneeze (2-5x in 10 minutes)	1 (daily)
Sneeze (6-20x in 10 minutes)	2 (daily)
Sneeze (>20x in 10 minutes)	3 (daily)
Temperature 38.5-39.0 °C	1 (daily)
Temperature 39.1-39.5 °C	2 (daily)
Temperature 39.6-40.0 °C	3 (daily)
Temperature > 40 °C	4 (daily)

Virus neutralization assay

Blood for serum neutralizing antibody analysis was collected in 8 ml Vacuette Serum Clot Activator Tubes (Greiner Bio-One) and incubated for at least 4 h at room temperature to allow for clotting. Serum samples were subsequently collected by centrifugation at 700x *g* for 15 min at 4°C and stored at -20°C until further use. To determine EAV neutralizing antibody titers in the serum, a virus neutralization assay was performed similar to what was described before (36). Briefly, duplicate two-fold serial dilutions (1:2 to 1:4096) of serum samples were made in 96-well plates and mixed with a 50% tissue culture infective dose ($TCID_{50}$) of 800 of EAV030 (33). After

a 1-h incubation at 37°C, $1.2x10^4$ Vero cells were added to each well. Plates were subsequently incubated for 4 days at 37°C after which each well was scored (positive or negative) for cytopathic effect (CPE) by visual inspection. The EAV neutralizing antibody titer was finally determined as the reciprocal value of the highest serum dilution at which no CPE was observed.

Quantitative reverse transcriptase PCR assay

Total RNA was isolated from whole blood collected in Tempus Blood RNA Tubes (Greiner Bio-One) using the MagMAX for Stabilized Blood Tubes RNA isolation kit (Life Technologies) according to the manufacturer's instructions. Isolated RNA was converted to cDNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo$(dT)_{20}$ primer. Samples were subsequently analysed by quantitative reverse-transcriptase (qRT) PCR on a CFX384 Touch Real-Time PCR detection system (BioRad) using iTaq Sybr Green Supermix (BioRad). Primers (**Table V-2**) targeting mRNAs encoding equine β-actin, ISG15, and MX1, or EAV RNA were designed using Primer3 (38). The EAV-specific primer set amplifies cDNA derived from both genomic and subgenomic viral mRNAs. The real-time PCR was performed in triplicate, included a standard dilution series, and was followed by a melting-curve analysis to confirm the identity of the reaction product.

Table V-2. Primers used for quantitative RT-PCR.

Target (Accession)	Forward primer (5'-3')	Reverse primer (5'-3')
Equine beta-actin mRNA (NM_001081838)	CCACGCCATCCTGCGTCTGG	ACCGCTCGTTGCCGATGGTG
Equine ISG15 mRNA (XM_001496658)	GAATTCCTGGTGCCCCTGAA	CAGTTCTGCACGACAAGCAC
Equine MX1 mRNA (NM_001082492)	CGGCCAGCAGCTGCAGAAGT	GGCCTCCGCTCCCTGGAGAT
EAV RNA (NC_002532)	GGTTCGCGGCAACGGGTACA[1]	GGTGGCGCGCTCCTGTTGAT[2]

[1] Primer position in EAV genome: 12269-12288.
[2] Primer position in EAV genome: 12488-12507.

RESULTS

Vaccine viruses do not differ in the induction of fever or neutralizing antibodies. To assess whether a vaccine virus lacking PLP2 DUB activity would provide better protection against heterologous challenge than its DUB-positive parental counterpart, we performed a vaccination-challenge trial in horses. Twenty female Shetland horses were randomly assigned to one of three groups. Animals from two groups were subsequently vaccinated intramuscularly with cell culture-adapted DUB-competent (Group 1, n=9) or DUB-negative (Group 2, n=9) EAV551A/B. Horses from Group 3 (n=2)

were not vaccinated and served as challenge controls. All horses were intranasally challenged with moderately virulent EAV KY84 at 34 dpv. A schematic representation of the experimental set-up including timing of vaccination, challenge, and sampling is depicted in **Figure V-1**. The identity of the virus used for vaccination was confirmed by sequencing of viral RNA isolated from whole blood at 4 dpv for a random subset of four horses from Groups 1 and 2 (data not shown).

Upon vaccination, animals from Groups 1 and 2 (DUB-competent and DUB-negative, respectively) developed comparable levels of mild fever which reached an average maximum of 38.8°C at 3 dpv and subsided approximately 8 dpv in both groups (**Figure V-2A**). Upon challenge, only the non-vaccinated animals in Group 3 developed a fever, which reached higher levels (average maximum 40.3°C) and lasted approximately two days longer than the fever that was observed after vaccination. Neutralizing antibodies could be detected in both Groups 1 and 2 from 6 dpv onwards and no difference

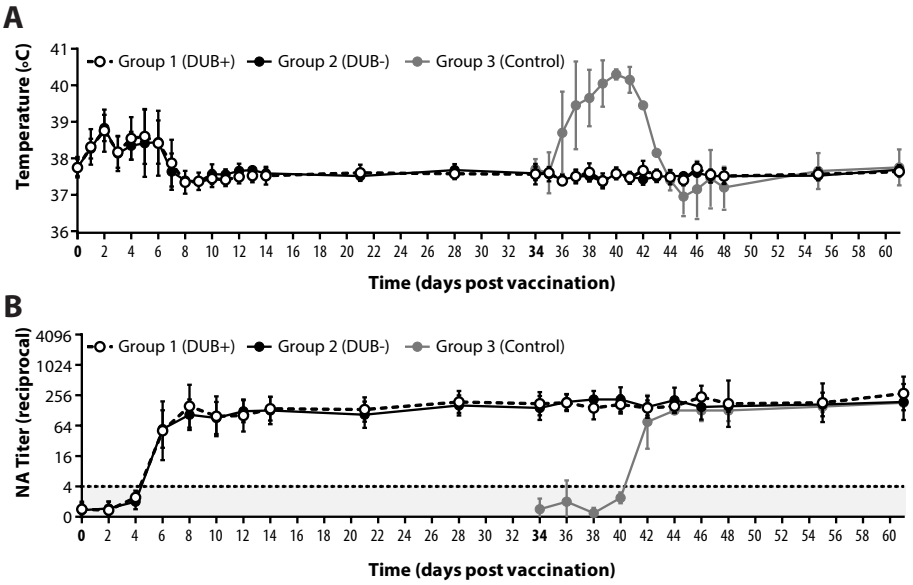

Figure V-2. Rectal temperatures and neutralizing antibody titers. A) Rectal temperatures were taken daily for the first 14 days post vaccination/challenge and at 21, 28, 55, and 61 dpv. **B)** Neutralizing antibody (NA) titers in serum were determined using a virus neutralization assay for samples obtained every other day during the first 14 days post vaccination/challenge and at 21, 28, 55, and 61 dpv. White, black, and grey circles represent data from animals in Group 1, 2, and 3, respectively. The days of vaccination (0) and challenge (34) are depicted in bold face. The dotted line in panel B represents the limit of detection. Measurements/samples taken on the day of vaccination/challenge were obtained before infection and horses in Group 3 were included in the experiment starting at the day of challenge. **Abbreviations:** DUB+, deubiquitinase-competent vaccine virus; DUB-, deubiquitinase-negative vaccine virus; NA, neutralizing antibody.

in titers was observed between the two groups (**Figure V-2B**). Titers remained stable during the course of the experiment and did not show an increase upon challenge. In the unvaccinated controls, neutralizing antibody titers could be detected at 8 days post challenge (dpc) and reached similar titers as observed after vaccination.

From 0 to 14 dpc, clinical signs (including for example fever, nasal secretions, and loss of appetite) were recorded daily for each animal. An overall clinical signs score was subsequently determined for each animal by scoring these clinical signs according to **Table V-1**. For example, a horse that has diarrhoea on day 5, mild conjunctivitis and a temperature of 39°C on day 8, and no abnormalities on any of the other days has an overall clinical signs score of 4 (1+2+1). This resulted in an average score per animal of 4.2 (±3.3, Group 1) and 5.3 (±3.3, Group 2), which did not differ significantly between the two groups (Student's t-test; $p>0.05$; **Table V-3**). The horses in Group 3 reached an average score of 46.5 per animal, which is consistent with the fact that these animals had not been vaccinated. Thus, vaccination with either virus provided a similar degree of protection against clinical disease.

Table V-3. Clinical signs score per animal at 0-14 dpc.

Group 1 (DUB+)		Group 2 (DUB-)	
Horse ID	**Score**	**Horse ID**	**Score**
1	5	11	2
2	2	12	10
3	0	13	4
4	4	14	4
5	0	15	7
6	3	16	3
7	8	17	1
8	9	18	7
9	7	19	10
Average	**4.2 (±3.3)**	**Average**	**5.3 (±3.3)**

PLP2 DUB-negative vaccine virus appears to replicate slightly less efficiently than parental virus *in vivo*. We then assessed viral replication from 0 to 10 dpv by performing real-time qRT-PCR analysis on total RNA isolated from whole blood. The quantification limit of this assay was set to the amount of EAV RNA (10 arbitrary units) that no longer yielded consistent read-outs in PCR triplicates, and this limit corresponded to a threshold cycle (C_T) value of approximately 37. As can be seen in **Figure V-3A** and **Table V-4**, all vaccinated horses showed viral replication which, on average, peaked at 4 dpv in both groups. Notably, the DUB-negative virus (Group 2) appeared to replicate slightly less efficiently than its DUB-competent counterpart (Group 1). Whereas the

Table V-4. Amounts of viral RNA (arbitrary units) as determined by qRT-PCR at 0-10 dpv.

Group	Horse ID	0 dpv	2 dpv	4 dpv	6 dpv	8 dpv	10 dpv
Group 1 (DUB+)	1	ND	- [1]	743	1528	68	19
	2	<10 [2]	20	835	1624	302	205
	3	ND	229	438	142	107	89
	4	ND	41	1440	578	55	61
	5	<10 [2]	321	5367	3849	514	120
	6	ND	43	78	30	12	<10
	7	ND	107	5550	1445	1229	130
	8	ND	105	1587	218	40	74
	9	ND	37	170	16	12	<10
Group 2 (DUB-)	11	ND	150	1257	189	124	15
	12	ND	197	2674	482	107	50
	13	<10 [2]	23	885	63	25	17
	14	ND	23	403	45	20	11
	15	ND	<10	28	<10	ND	<10
	16	ND	24	171	<10	ND	<10
	17	ND	15	246	13	18	<10
	18	<10 [2]	<10	37	<10	17	ND
	19	ND	38	320	12	13	<10

Abbreviations: dpv, days post vaccination; ID, identifier; ND, not detected.

[1] No data available.

[2] Considering that all horses tested negative for EAV neutralizing antibodies prior to the start of the experiment, these signals are likely due to a contamination event during processing of the samples.

amount of parental DUB-competent virus was above the limit of quantification in all Group 1 horses at each of days 2 to 8 post vaccination, several of the horses in Group 2 tested negative for viral RNA or had levels of viral RNA below the limit of quantification at 2, 6, and/or 8 dpv (**Table V-4**). In addition, the average amount of viral RNA at each day post vaccination was lower in Group 2 compared to Group 1, although this difference was statistically significant only at 10 dpv (Student's t-test; $p<0.05$).

Upon challenge with the moderately virulent EAV KY84 strain, both non-vaccinated control horses (Group 3) showed virus replication, with amounts of viral RNA reaching levels comparable to those produced by the vaccine viruses (**Figure V-3A** and **Table V-5**). In contrast, in all vaccinated horses, except for one horse from Group 1 at 8 and 10 dpc, the levels of viral RNA remained below the quantification limit from 2 to 10 dpc (**Figure V-3B** and **Table V-5**). This result was consistent with the observed appearance of neutralizing antibodies after vaccination in all horses and the low clinical sign scores in both vaccinated groups after challenge.

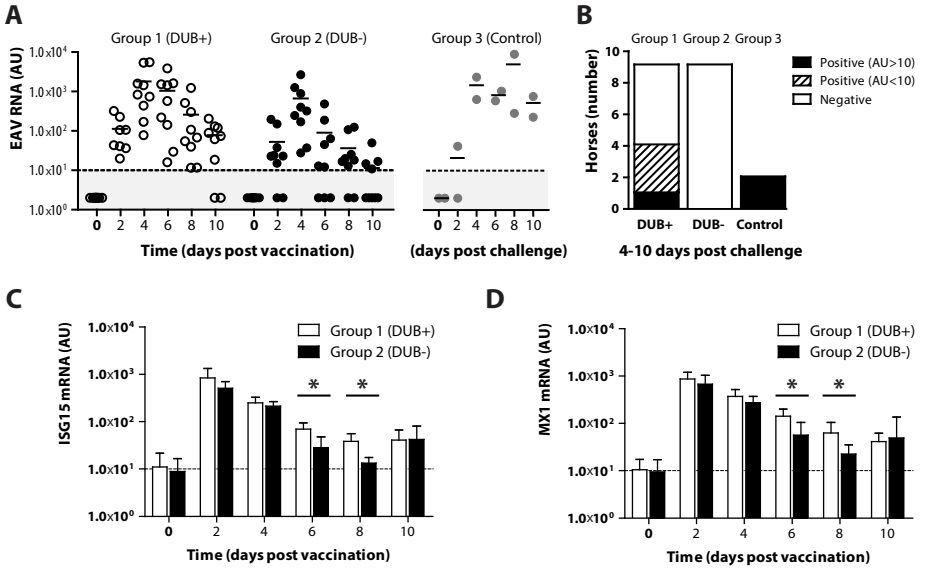

Figure V-3. Quantitative RT-PCR analysis of viral RNA and cellular mRNA encoding ISG15 and MX1. **A)** Viral replication from 0 to 10 dpv (Groups 1 and 2) or dpc (Group 3) was assessed by real-time qRT-PCR analysis on total RNA isolated from whole blood using EAV-specific primers. White, black, and grey circles represent animals from Group 1, 2, and 3, respectively. Horizontal bars represent the mean for all animals on that day and the dotted line represents the limit of quantification, which was set at 10 arbitrary units (AU). **B)** The presence of viral RNA in animals from each group at 4 to 10 dpc was assessed as in panel A. The bars represent the number of animals per group that tested positive (over 10 AU; black bars) or nearly positive (under 10 AU, hatched bars) for viral RNA on at least one of the indicated days, or that remained negative (white bars) for viral RNA on each of the indicated days. Activation of the innate immune response was assessed by real-time qRT-PCR on total RNA isolated from whole blood using primers specific for **C)** ISG15- or **D)** MX1-encoding mRNA. Values obtained for ISG15 and MX1 mRNA levels were normalized to the amount of β-actin mRNA. The dotted lines represent average baseline levels, error bars represent standard deviations, and asterisks indicate statistical significance (Student's t-test; $p<0.05$). **Abbreviations:** AU, arbitrary units; DUB+, deubiquitinase-competent vaccine virus; DUB-, deubiquitinase-negative vaccine virus.

Upon closer examination of the data, we did occasionally observe specific signal representative of viral RNA, as assessed by melting curve analysis, below the quantification limit (i.e. C_T values between approximately 37 and 40) in samples of vaccinated animals between 4 and 10 dpc (**Figure V-3B**). Interestingly, this appeared to be the case only in animals from Group 1 (n=4/9) and not Group 2 (n=0/9). Although this difference was not statistically significant (Fisher's exact test; $p=0.08$), it is suggestive of a trend towards the DUB-negative virus (Group 2) providing better protection against challenge than its parental DUB-competent counterpart (Group 1). It needs to be noted however, that in several horses from both vaccinated groups, signal specific

Table V-5. Amounts of viral RNA (arbitrary units) as determined by qRT-PCR at 0-10 dpc.

Group	Horse ID	0 dpc	2 dpc	4 dpc	6 dpc	8 dpc	10 dpc
Group 1 (DUB+)	1	<10	<10	<10	<10	<10	ND
	2	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	<10
	4	ND	ND	ND	ND	ND	ND
	5	ND	ND	ND	ND	263	20
	6	ND	ND	ND	ND	ND	ND
	7	ND	ND	<10	ND	<10	<10
	8	ND	ND	ND	ND	ND	ND
	9	<10	ND	ND	ND	ND	ND
Group 2 (DUB-)	11	ND	<10	ND	ND	ND	ND
	12	<10	<10	ND	ND	ND	ND
	13	ND	ND	ND	ND	ND	ND
	14	<10	ND	ND	ND	ND	ND
	15	<10	ND	ND	ND	ND	ND
	16	<10	ND	ND	ND	ND	ND
	17	ND	ND	ND	ND	ND	ND
	18	ND	ND	ND	ND	ND	ND
	19	ND	<10	ND	ND	ND	ND
Group 3 (control)	10	ND	ND	2376	592	9037	766
	20	ND	42	642	1041	286	229

Abbreviations: dpc, days post challenge; ID, identifier; ND, not detected.

for viral RNA but below the limit of quantification could also be observed at 0 and 2 dpc (**Table V-5**). Since the 0 dpc samples were taken before inoculation, the signal observed in these samples most likely represents progeny of the vaccine virus.

No major differences in the induction of ISG15 and MX1 mRNA expression could be detected between vaccine viruses. Finally, we assessed the induction of an innate immune response by parental and PLP2 DUB-negative virus from 0 to 10 dpv by means of real-time qRT-PCR. Since we were unable to detect mRNA encoding type I interferons (a hallmark of innate immune activation), we instead focussed on two highly expressed interferon-stimulated genes encoding ISG15 and MX1, respectively. Both of these genes showed an approximately 2-log increase of expression at 2 dpv, which did not significantly differ between animals from Groups 1 and 2 (**Figures V-3C and D**). At 6 and 8 dpv, the expression of ISG15 and MX1 mRNA was significantly lower in the animals vaccinated with the PLP2 DUB-negative virus (Group 2) than in those vaccinated with the parental virus (Group 1) (Student's t-test; $p<0.05$), which might be explained by the slight decrease in replication efficiency of the former virus. How-

ever, this apparently did not adversely affect the levels of protection provided by the DUB-negative vaccine virus. Overall, no major differences between parental and PLP2 DUB-negative virus in the induction of ISG15 and MX1 expression were found.

DISCUSSION

We have previously shown that mutant EAV lacking PLP2 DUB activity induces a more potent innate immune response than its DUB-competent parental virus upon infection in cell culture (32), and hypothesized that this feature might be included in an improved arterivirus vaccine. In order to assess whether a PLP2 DUB-negative vaccine virus provides better protection than its DUB-competent counterpart against challenge with a virulent EAV strain, we performed a vaccination-challenge trial in horses. The data obtained in this study showed that although the PLP2 mutant virus appears to replicate slightly less efficiently than its parental virus, it is definitely replication-competent *in vivo* (**Figure V-3A**). In addition, despite this decrease in replication efficiency, both the antibody response (**Figure V-2B**) and innate immune response (assessed based on the expression of two interferon-stimulated genes; **Figures V-3C and D**) induced by the PLP2 DUB-negative virus was comparable to that induced by the parental virus. Surprisingly, we did not observe an increase in EAV-neutralizing antibody titers upon challenge infection of vaccinated animals (**Figure V-2B**). A possible explanation for this finding is that the virus dose used for challenge was too low, resulting in its efficient clearance before (re-)activation of the antibody response. Finally, although the data hinted at the PLP2 DUB-negative virus providing a better protection than its parental DUB-competent virus, the already high degree of protection that was provided by vaccination with the latter virus made it impossible to substantiate this conclusion.

In contrast to what was previously found in cell culture-based assays (32), we did not detect an increased activation of innate immunity by the PLP2 DUB-negative virus compared to its parental virus *in vivo*. However, it needs to be noted that we have thus far assessed the expression of only two interferon-stimulated genes, whereas many more exist. It therefore remains a possibility that differences do exist in the expression of other interferon-stimulated genes. Similarly, the induction of interferon-stimulated genes is only one of several consequences of the activation of innate immune signalling. It therefore remains possible that the DUB-negative vaccine virus differs from the parental virus in some other respect pertaining to immunity, for example the activation of cell-mediated adaptive immunity. Also, PLP2 DUB activity likely constitutes only one of several innate immune evasion strategies employed by arteriviruses.

For example, nonstructural protein 1 (nsp1) of EAV was recently shown to inhibit the induction of interferon beta in a luciferase reporter assay (39) and the EAV nsp4 main protease was recently shown to cleave the innate immune signalling factor NEMO (40). More studies have been performed with PRRSV, for which it was suggested that in addition to PLP2, also nsp1α (41-47), nsp1β (48-50), nsp4 (48), and nsp11 (48, 51), and the structural nucleocapsid protein (52) are involved in counteracting innate immune activation. It needs to be noted though that the observed effect of nsp11 on the induction of interferon beta was likely due to toxicity of this endoribonuclease (53). Taken together, the PLP2 DUB activity appears to constitute only a part of the total repertoire of arteriviral innate immune evasion strategies, which perhaps cannot be readily detected in the *in vivo* experimental set-up used here. For example, detecting more prominent consequences of its inactivation may depend on the details of the animal study, including specific properties of viruses and horses used. Nevertheless, the apparently limited negative effect on viral replication of PLP2 DUB-mutations does open up possibilities for combining mutations in multiple domains involved in immune evasion, thereby potentially synergistically increasing the overall immunogenicity of the virus.

Furthermore, the data obtained in this study did not allow us to firmly substantiate (or refute) our hypothesis that a PLP2 DUB-negative mutant vaccine provides better protection against subsequent challenge than its parental DUB-competent counterpart. The main reason for this seems to be the fact that the parental virus already provides a very high degree of protection, which as it turned out could not be detectably improved using this experimental set-up. In hindsight, one solution might have been to perform a more severe challenge, for example by using a higher virus dose for inoculation or a more heterologous strain than KY84. As PRRSV vaccines are in general less efficacious than EAV vaccines, another option would be to perform a similar trial with a PRRSV vaccine in pigs, but this will first require the design of viable PRRSV PLP2 DUB-negative mutants.

Notably, arteriviruses are not the only group of viruses that were shown to harbour DUB activity. For example, coronaviruses (including SARS- and MERS-CoV) encode papain-like proteases that display DUB activity (54-61). Additionally, the OTU proteases of nairoviruses have been found to possess DUB activity (28). Like arterivirus PLP2, these viral DUBs have also been implicated in innate immune evasion (28, 57-60, 62-67) and are therefore potential targets for the design of novel vaccines. Taken together, the data obtained in this study definitely warrant further *in vivo* examination of the consequences of knocking out arterivirus PLP2 DUB activity as well as other immune-evasive activities to improve vaccine efficacy. The data obtained in such studies could

subsequently also be used as preliminary proof of principle for the design of novel vaccines for other viruses, including corona- and nairoviruses.

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