Antiviral Research 92 (2011) 292–304

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

An attenuated EIAV vaccine strain induces significantly different immune responses from its pathogenic parental strain although with similar *in vivo* replication pattern

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ARTICLE INFO

Article history:
Received 18 May 2011
Revised 25 July 2011
Accepted 18 August 2011
Available online 25 August 2011

Keywords:
EIAV
Vaccine
Immune response

ABSTRACT

The EIAV (equine infectious anemia virus) multi-species attenuated vaccine $EIAV_{DLV121}$ successfully prevented the spread of equine infectious anemia (EIA) in China in the 1970s and provided an excellent model for the study of protective immunity to lentiviruses. In this study, we compared immune responses induced by $EIAV_{DLV121}$ to immunity elicited by the virulent $EIAV_{LN40}$ strain and correlated immune responses to protection from infection. Horses were randomly grouped and inoculated with either $EIAV_{DLV121}$ (Vaccinees, Vac) or a sublethal dose of $EIAV_{LN40}$ (asymptomatic carriers, Car). Car horses became $EIAV_{LN40}$ carriers without disease symptoms. Two of the four Vac horses were protected against infection and the other two had delayed onset or reduced severity of EIA with a lethal $EIAV_{LN40}$ challenge 5.5 months post initial inoculation. In contrast, all three Car animals developed acute EIA and two succumbed to death. Specific humoral and cellular immune responses in both Vac and Car groups were evaluated for potential correlations with protection. These analyses revealed that although plasma viral loads remained between 10^3 and 10^5 copies/ml for both groups before $EIAV_{LN40}$ challenge, Vac-treated animals developed significantly higher levels of conformational dependent, Env-specific antibody, neutralizing antibody as well as significantly elevated $CD4^+$ T cell proliferation and IFN-γ-secreting $CD8^+$ T cells than those observed in $EIAV_{LN40}$ asymptomatic carriers. Further analysis of protected and unprotected cases in vaccinated horses identified that cellular response parameters and the reciprocal anti-p26-specific antibody titers closely correlated with protection against infection with the pathogenic $EIAV_{LN40}$. These data provide a better understanding of protective immunity to lentiviruses.

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1. Introduction

Among the different types of lentiviral vaccines, the live attenuated formulations demonstrated the best efficacy based on the level of protective immunity by providing complete or near-complete protection from homologous challenges (Koff et al., 2006; Tagmyer et al., 2008), controlling or dramatically reducing the replication of heterologous viral strains (Reynolds et al., 2008), and eliciting long-term protection (Yankee et al., 2009). However, live vaccines are typically not considered viable options in the development of lentiviral vaccines, especially in the context of AIDS vaccines because of the risks that are associated with genomic integration and reverse mutations. Therefore, a better understanding of the protective immune responses that are elicited by attenuated vaccines would assist with the development of vaccines against lentiviral infections (Letvin, 2006; Robb, 2008; Whitney and Ruprecht, 2004).

The equine infectious anemia virus (EIAV) is one of the least complex lentivirus and like other members of the lentivirus genus, such as HIV-1, EIAV has the capacity to change its envelope surface proteins as an immune evasion mechanism. However, after the initial progressive, recurring febrile episodes many infected horses eventually become asymptomatic EIAV carriers. Therefore, defining the nature of the immune response resulting in asymptomatic infections is critical to EIAV vaccine design. Several studies have demonstrated that reduced EIAV replication in asymptomatic horses was a consequence of host immunity and not due to EIAV attenuation over time. For example, adoptive transfer of whole blood from asymptomatic horses to EIAV-negative horses resulted

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doi:10.1016/j.antiviral.2011.08.016

in progression towards equine infectious anemia (EIA) (Craigo et al., 2005; Hammond et al., 1997, 2000; Harrold et al., 2000) and following dexamethasone-mediated immune suppression, plasma viremia in EIAV carriers dramatically increased (Craigo et al., 2002; Newman et al., 1991). These studies suggested that EIAV virulence remained unaltered and that asymptomatic carriage was a consequence of acquired immunity to EIAV (Perryman et al., 1988) thereby providing an ideal model for the study of protective immunity against this pathogen. Several studies characterized immune responses in EIAV-infected horses, including Env-specific antibody maturation, avidity and conformation-dependence, in addition to characterization of EIAV-specific lymphocyte proliferation and cytotoxic T lymphocyte (CTL) activity. These studies suggested that these responses played a role in controlling EIAV *in vivo* (Chung et al., 2004; Hammond et al., 1999, 2000).

However, it remains undefined what specific immune response parameter(s) are most important in mediating resistance (or conferring protection) to lentivirus infections, especially since research in this area is controversial. Some studies demonstrated that controlling EIAV replication requires specific cellular immune responses in combination with high levels of neutralizing antibodies (McGuire et al., 2002; Tagmyer et al., 2007). Conversely, high levels of CTL activity and the presence of neutralizing antibodies were absent from Rhesus macaques (*Macaca mulatta*) resistant to infection with pathogenic SHIV (simian immunodeficiency virus) strains (Mansfield et al., 2008; Reynolds et al., 2008; Yankee et al., 2009). One critical reason for these conflicting results with respect to the role of immunity in containing lentivirus infections is the lack of an appropriate infection model (Harris, 2009). In addition, most data published to date describing lentivirus immunity has been derived from infection studies not designed to examine protective immunity (Craigo et al., 2005; Hammond et al., 2000; Rosenberg et al., 1999). Recently, a series of studies on protective immunity using live attenuated EIAV vaccines were published and revealed important information on the correlation of immunity with protection (Craigo and Montelaro, 2010; Tagmyer et al., 2008).The attenuated EIA vaccine ($EIAV_{DLV121}$) was developed by Shen et al. by serially passing the pathogenic wild-type $EIAV_{LN40}$ strain in donkeys for 110 generations followed by 121 *in vitro* passages in donkey monocyte-derived macrophages (MDM). $EIAV_{DLV121}$ was utilized extensively in China between 1975 and 1990 where 30 million equines were vaccinated to control an EIA pandemic. As a result, the spread of EIA in China, which directly or indirectly (slaughtering of infected animals) resulted in the loss of at least 424,000 horses, mules and donkeys, was under control by the early 1980s as reported by the Chinese Ministry of Agriculture (Shen et al., 1984). Although this attenuated EIA vaccine was the first vaccine that demonstrated effective protection against a lentivirus related diseases, the mechanism resulting in protective immunity remains undefined. More importantly, dexamethasone-mediated immune suppression of $EIAV_{DLV121}$-vaccinated horses did not either significantly elevate plasma viral loads or trigger clinical EIA (Ma et al., 2009), implicating the difference in immune responses between induced by the vaccine strain and resulted by asymptomatic infections.

In this study, we first provided the historic unpublished data on the efficacy of the attenuated $EIAV_{DLV121}$ strain against infections with either homologous or heterologous EIAV pathogenic strains. The immune responses elicited following vaccination with $EIAV_{DLV121}$ or with an $EIAV_{LN40}$ sublethal dose were characterized by comparing humoral and cellular response and characterizing the relationship between the nature of the immune responses and protection. This is the first systematic evaluation of the immune responses elicited by a lentiviral vaccine that effectively prevented infections with either homologous or heterologous EIAV strains.

2. Materials and methods

2.1. EIAV strains

Five EIAV strains were utilized in this study: (i) the virulent $EIAV_{LN40}$ strain initially isolated from an EIA positive horse from the Liaoning Province of China and passed for 16 generations in horses. $EIAV_{LN40}$ infections resulted in a 100% incidence of acute EIA in infected horses when 10^4 $TCID_{50}$ EIAV was used; (ii) the live attenuated $EIAV_{DLV121}$ vaccine strain derived from the virulent $EIAV_{DV117}$ strain by successive MDM passages *in vitro* (Shen et al., 1984); (iii) $EIAV_{FDDV12}$, a strain comprising a second-generation of Chinese attenuated EIAV vaccine that was developed by further passing the $EIAV_{DLV121}$ vaccine strain in fetal donkey dermal cells (FDD) for 12 generations (Shen et al., 2006); (iv) $EIAV_{DLV34}$, a donkey MDM-adapted virulent strain that was passed for 33 generations. Like $EIAV_{LN40}$, infections with $EIAV_{DLV34}$ resulted in acute EIA incidence rates of 100% when horses were infected with 10^4 $TCID_{50}$; (v) the American $EIAV_{Wyoming}$ virulent strain, a gift from Dr. Dawei Shen, Washington State University, and was passed in horses for three generations to increase the virulence.

2.2. Experimental animals, clinical evaluation and longitudinal sample collection

To evaluate the protective efficacy of the attenuated $EIAV_{DLV121}$ vaccine strain, 116 mixed gender, outbread adult horses were randomly selected and hypodermically vaccinated with 4×10^5 $TCID_{50}$ of $EIAV_{DLV121}$ and then challenged with 1×10^4 $TCID_{50}$ of virulent strain $EIAV_{LN40}$ 3, 6, 10 or 12 months post vaccination. Twenty-two horses were immunized with $EIAV_{DLV121}$ for either 6, 9 or 15 months prior to challenge with the $EIAV_{Wyoming}$ strain. Twenty-seven and 13 unvaccinated horses (treated with saline) were infected with either $EIAV_{LN40}$ or with the American $EIAV_{Wyoming}$ strain (1×10^4 $TCID_{50}$). Rectal temperatures for each horse were measured twice daily. Acute EIA was defined as fever >39 °C, thrombocytopenia (<9×10^4 platelets/μl blood) and jaundice.

For experiments comparing immune responses, 16 horses of mixed gender were randomly grouped and inoculated with: (i) the vaccine strain $EIAV_{DLV121}$ (group Vac, four horses); (ii) sublethal dose (1×10^3 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ to develop asymptomatic carriers of EIAV pathogenic strain (group Car, three horses); (iii) lethal dose (1×10^4 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ to develop acute EIA (group Acu, three horses); (iv) the same volume of saline (Mock, three horses). Horses, except for those in the group Acu, were challenged with lethal dose (10^5 $TCID_{50}$) of the virulent strain $EIAV_{LN40}$ 23 weeks post the initial inoculation. In addition, three horses were mock inoculated and challenged with saline as healthy controls (group Hea). Serum samples prior to the start of the experiment were tested twice by the agar gel immunodiffusion assay for the presence of EIAV antiserum to confirm that each animal was uninfected at the beginning of the experiment (Coggins et al., 1972). Serum, plasma, and whole blood samples were collected from horses at indicted intervals and were stored at −20 °C until serological characterization was carried out. A plasma aliquot was stored at −80 °C until plasma viral loads were determined using real-time RT-PCR. Equine peripheral blood mononuclear cells (ePBMC) were isolated from whole blood over a discontinuous density gradient of Ficoll-Histopaque (density = 1.077 g/ml) for evaluating EIAV-specific cellular immune responses. During the course of all experimental process, all animals were clinically monitored daily as described (Zhang et al., 2007). All the above animal studies have been reviewed and approved

by the Institutional Review Committee for Animal Administration and Animal Care.

2.3. Antibody end-point titer assays and determination of anti-EIAV avidity and conformational-dependence

Detection of serum antibodies specific to the EIAV capsid protein p26 was conducted by standard ELISA assay using purified recombinant p26 expressed in *Escherichia coli* (Piza et al., 2007). EIAV envelope protein (Env)-specific serum IgG antibodies were assayed quantitatively (endpoint titer) and qualitatively (avidity index and conformation ratio) using the concanavalin (Con) A antigen-capture ELISA as described (Hammond et al., 1997). EIAV particles were purified from $EIAV_{FDDV12}$ supernatants using gradient centrifugation as described previously (Montelaro et al., 1982).

2.4. Neutralization assay

Serum neutralizing activity to the pathogenic $EIAV_{DLV34}$ was determined using the reverse transcriptase activity-based assay as described (Kwong et al., 2009). Briefly, equine monocytes were isolated from peripheral blood and cultured for 48 h at 37 °C in 96-well flat-bottom microplates containing 200 μl of culture medium consisting of RPMI 1640 supplemented with 20% heat-inactivated horse serum (Hyclone, Logan, UT), 40% fetal bovine serum, 4 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. All serum samples were heat-inactivated at 56 °C for 30 min to inactivate infectious virus and complement factors. The serum samples were serially diluted and incubated with 1000 $TCID_{50}$/ml of indicator virus ($EIAV_{DLV34}$) at 37 °C for 1 h. The serum/virus mixtures were added to cultures of equine monocytes and incubated at 37 °C for 2 h. The wells were then washed and 200 μl of fresh medium added and cultured for 4 days at 37 °C. Virus particles present in respective culture wells were titrated by measuring reverse transcriptase activity using the Reverse Transcriptase Assay Colorimetric kit as described by the manufacturer (Roche, IN, USA). When a reduction in reverse transcriptase activity was greater than 50% of the positive control (virus incubated with EIAV-negative serum) following incubation in $\leqslant$10% horse serum, the sample was defined as neutralization positive. The 50% neutralization titers of samples were determined by linear regression analysis of dilution titers versus optical density (OD) values. These experiments were repeated twice with similar results.

2.5. Measurement of EIAV-specific $CD4^+$ and $CD8^+$ T cell proliferation

EIAV-specific $CD4^+$ and $CD8^+$ T cell proliferation was assessed using CFSE (5-carboxyfluorescin diacetate succinimidyl ester). As the fluorescent intensity of CFSE-stained cells diminishes with each cell division, cells that have undergone cell division are identified by flow cytometry (Gil et al., 2009; Witkowski, 2008). Briefly, ePBMCs (1×10^7/ml) were incubated at 37 °C for 10 min with 5 μM CFSE (Invitrogen, Carlsbad, CA) and then 5 ml culture medium (10% FBS) was added to stop CFSE cell staining. Cells were then washed three times in PBS and resuspended at a concentration of 1×10^7/ml in culture medium. Stained cells were then cultured in 96-well round bottom plates (4×10^5/well) with 1×10^4 pfu, gradient-purified $EIAV_{FDDV12}$. The temperature, time and pH of the incubations were optimized. ePBMCs stimulated with 5 μg/ml PHA-P (Sigma, St. Louis, MO) were used as the positive control. Cells cultured in media containing no EIAV were used as the negative control. After incubating at 37 °C with 6% CO_2 for 4 days, cells from each treatment group were analyzed using a Beckman FC500 flow cytometer. Before the analysis, CFSE-labeled ePBMCs were incubated with monoclonal antibodies specific for equine CD4 or CD8 (VMRD, Pullman, WA) followed by an incubation with a secondary APC-conjugated anti-mouse IgG (SouthernBiotech, Birmingham, AL). Optimal gate settings were determined for each experiment based on single-stained samples. The cell division index (CDI) of $CD4^+$ or $CD8^+$ T cells was calculated using the following equation:

$$\text{EIAV-specific CDI(cell division index)} = \frac{\text{Number of } CD4^+/CD8^+CFSE^{dim}\text{with antigen}}{\text{Number of } CD4^+/CD8^+\text{with antigen}} - \frac{\text{Number of } CD4^+/CD8^+CFSE^{dim}\text{without antigen}}{\text{Number of } CD4^+/CD8^+\text{without antigen}}$$

2.6. Intracellular staining (ICS)

The bovine IFN-γ-specific monoclonal CC302 antibody (AbDSerotec Ltd., Oxfordshire, UK) was used for assessing IFN-γ secretion by individual equine T lymphocytes as described previously (Breathnach et al., 2005). ePBMCs (1×10^7/ml) were isolated from each horse and stimulated with either 1×10^4 pfu of gradient-purified $EIAV_{FDDV12}$ or with the same volume of virus-free medium at 37 °C for 16 h. Brefeldin A was added to the cells at a final concentration of 10 μg/ml for 8 h. Cells stimulated with 25 ng/ml PMA (Sigma) and 1 μM ionomycin (Sigma) were used as positive controls. After an overnight incubation, cells were fixed in 4% formaldehyde (v/v in PBS) for 30 min. Intracellular staining of IFN-γ was performed at room temperature with 1 μg CC302 in permeabilization buffer (PBS with 0.5% bovine serum albumin and 0.25% saponin) for 20 min. $CD4^+$ and $CD8^+$ T cells were also labeled and then analyzed by flow cytometry as described above to determine the percentage of IFN-γ-secreting surface-labeled T cells from EIAV- or medium-stimulated cell cultures. The EIAV-specific IFN-γ response was evaluated by the following equation:

$$\text{EIAV-specific IFN-}\gamma^+CD4^+/CD8^+(\%) = \frac{\text{Number of IFN-}\gamma^+CD4^+/CD8^+\text{with antigen}}{\text{Number of } CD4^+/CD8^+\text{with antigen}} - \frac{\text{Number of IFN-}\gamma^+CD4^+/CD8^+\text{without antigen}}{\text{Number of } CD4^+/CD8^+\text{without antigen}}$$

2.7. Quantitative analysis of viral plasma RNA levels

Quantitative analysis of viral genomic RNA/ml plasma was determined by RT-PCR. Plasma-associated viral RNA from experimentally infected horses was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The following oligonucleotides were used for RT-PCR reactions: EIAV-*gag*-specific forward primer: 5′-CGA TGC CAA ATC CTC CAT TAG-3′, EIAV-*gag*-specific reverse primer: 5′-CTG ATC AAA AGC AGG TTC CAT CT-3′ and the TaqMan probe (supplied by BioSystems Inc., Paris, France) 5′-FAM-CAC CAC AAG GGC CTA TTC CCA TGA CA-TAMRA-3′. RT-PCR was performed using the one-step PrimeScript RT-PCR Kit (Takara, Shiga, Japan) in a 25 μl reaction mixture containing 12.5 μl 2× RT-PCR buffer, 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 μM TaqMan probe, 0.5 μl RT mix, Ex-Taq 0.5 μl ddH_2O 6.3 μl and 4 μl of the RNA sample or RNA standard. The amplification protocol was carried out under the following conditions: 5 min at 42 °C, 10 s at 95 °C followed by 40 cycles at 95 °C for 5 s and 60 °C for 40 s. Linear regression analysis of the standard curve was used to estimate viral genomic RNA/ml of plasma. The standard RNA curve was linear in the range between 10^2 molecules at the lower limit and 10^9 molecules at the upper limit. All samples were measured twice.

2.8. Statistical analysis

Statistical analysis and data presentation were performed using the SAS version 8.1 (SAS Institute Inc., Cary, NC) and Graphpad Prism version 4.0 (GraphPad Software, La Jolla, CA) programs, respectively. Significant differences of humoral and cellular immune responses between experimental groups were determined using the Student's *t* test (two-tailed, confidence intervals of 95%). Significant differences were reported when $p \leqslant 0.05$.

3. Results

3.1. Protection against acute EIA following challenge with either homologous or heterologous virulent EIAV strains

In 1972–1983, a series of experiments were performed to detect the protection of the attenuated EIAV vaccine strain $EIAV_{DLV121}$ against the disease experimentally infected by a Chinese domestic pathogenic strain $EIAV_{LN40}$ and an American pathogenic strain $EIAV_{Wyoming}$. The unpublished historic data obtained by R.X. Shen et al. are first presented in this article to show the efficiency and duration of immune protection against the challenge of homologous and heterologous virulent strains induced by the attenuated EIAV vaccine for a duration between 3 and 30 months post vaccination. Recent analysis revealed that differences between the genomes and the *gp90* sequences between $EIAV_{DLV121}$ and $EIAV_{LN40}$ were 2.8% and 7.1% and between $EIAV_{DLV121}$ and $EIAV_{Wyoming}$ were 23.5% and 37.8% (Wang et al., 2011). Laboratory infection of either $EIAV_{LN40}$ or $EIAV_{Wyoming}$ resulted near 100% death of horses when a high dose (1×10^4 $TCID_{50}$) of virus was used. Results showed that only one survived from acute EIA among 40 non-vaccinated horses challenged with either of these two pathogenic strains. Therefore, the parameter of protection from disease was actually appeared as the protection from death of EIA in the summery of the historic protection test presented below.

As shown in Fig. 1A, 45.5% (5/11) horses were protected from acute EIA 3 months post vaccination with the highest protection observed at 6–12 months post inoculation (79.2%, 61/77). The protection efficiency decreased to 50% (3/6) when animals were challenged 30 months post vaccination. All unvaccinated, $EIAV_{LN40}$-infected animals ($n = 27$) succumbed to acute EIA. More importantly, significant protection was observed in $EIAV_{DLV121}$-vaccinated animals challenged with the heterologous $EIAV_{Wyoming}$ strain. Seventeen of 22 (77.3%) vaccinated horses vaccinated for 6–15 months were protected from acute EIA (Fig. 1B). All 13 unvaccinated horses developed acute EIA and succumbed after challenge.

3.2. Characterization of immune responses in $EIAV_{DLV121}$- and $EIAV_{LN40}$-infected animals

Immune responses elicited by the EIAV vaccine strain $EIAV_{DLV121}$ or domestic virulent strain $EIAV_{LN40}$ were characterized to identify potential differences associated with protection and susceptibility. Healthy horses were inoculated with either $EIAV_{DLV121}$ ($n = 4$) or with sub-lethal ($n = 3$) or lethal doses ($n = 3$) of $EIAV_{LN40}$ as described in Fig. 2A. All four vaccinated horses (Vac 1–4) did not present with any EIA-related symptoms and their circulating viral loads (10^2–10^5 copies/ml plasma) were under the 1×10^7 copies/ml plasma threshold used to diagnose acute EIA. In contrast, the three horses inoculated with a lethal dose of $EIAV_{LN40}$ (Acu group) emerged with characteristic EIA symptoms 2–4 weeks post infection as defined by fever (>39 °C), thrombocytopenia (<6×10^4 platelets/μl blood), jaundice and/or edema. All animals in this group died of severe EIA following the first febrile episode. However, horses (Car 1–3) that received a sub-lethal dose of $EIAV_{LN40}$ did not appear with typical febrile episodes and directly entered into the asymptomatic phase of infection.

To establish a correlation between immunity and protection against EIAV, horses from the Vac and Car groups were challenged with an $EIAV_{LN40}$ lethal dose 23 weeks post primary immunization. Three of four vaccinated animals challenged with $EIAV_{LN40}$ survived and two (Vac 2 and 4) did not present with EIA symptoms following challenge (Fig. 2B, panels B and D). However, two other vaccinated animals (Vac 1 and 3) presented with EIA symptoms at 47 and 29 days post $EIAV_{LN40}$ infection, respectively (Fig. 2B, panels A and C). Vac 3 died of EIA 34 days post challenge. Nucleotide sequence analysis of EIAV *gp90* in the Vac horses revealed that EIAV sequences obtained from Vac 2 and Vac 4 were of the same cluster as the $EIAV_{DLV121}$ vaccine strain, indicating protection against infection of the virulent strain. However, the EIAV *gp90* sequences from Vac 1 and Vac 3 matched the $EIAV_{LN40}$ virulent strain cluster seven and 5 weeks post challenge, respectively, demonstrating that these animals were infected with the virulent strain (Fig. S-1 of the Supplementary Materials).

In contrast, only one of three Car group horses survived and all presented with typical EIA symptoms following challenge with a lethal dose of $EIAV_{LN40}$ (Fig. 2C). The three PBS mock-vaccinated (Moc) positive control animals died of acute EIA after infection with the virulent $EIAV_{LN40}$ strain within 14–28 days post challenge (data not shown).

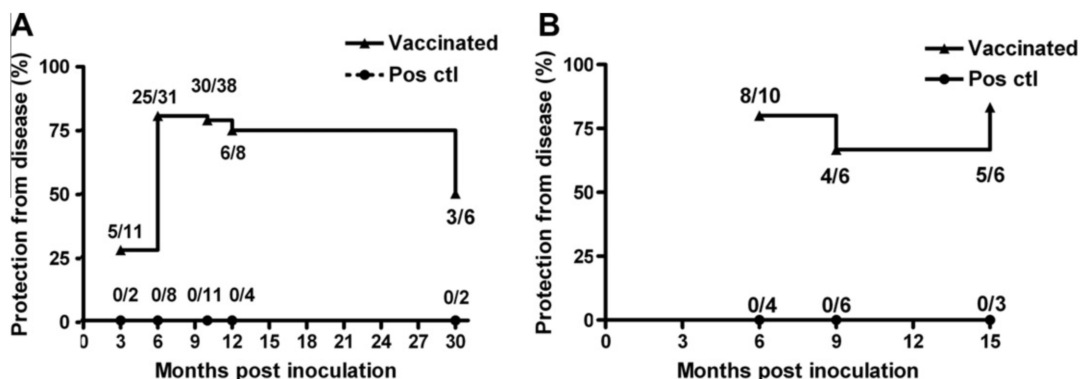

Fig. 1. Protection against acute EIA. Horses of mixed genders were vaccinated with $EIAV_{DLV121}$ for the indicated periods (3–30 months) and subsequently challenged intradermally with 10^4 $TCID_{50}$ of either (A), the Chinese domestic EIAV pathogenic strain $EIAV_{LN40}$ or (B), the heterologous American pathogenic strain $EIAV_{Wyoming}$. Saline-inoculated horses were used as controls. Acute EIA was defined by fever over 39 °C accompanied by thrombocytopenia and jaundice. The numbers in parentheses represent the number of horses protected from EIA at each time point over the number of horses challenged.

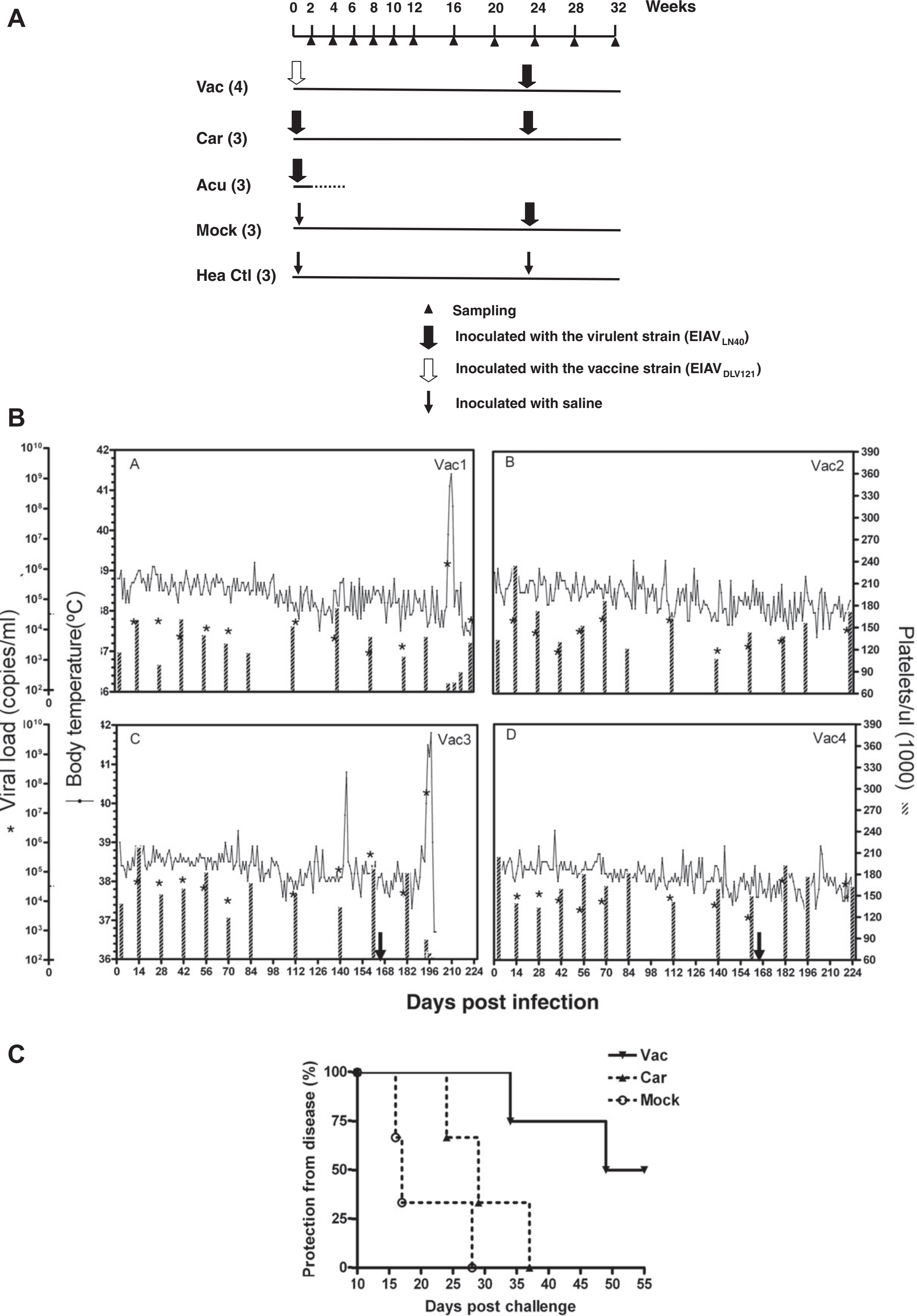

Fig. 2. Differences in immunity resulting from $EIAV_{DLV121}$ vaccination or from a sublethal $EIAV_{LN40}$ infection. (A) Sixteen mixed-gender horses were randomly divided into five groups. The Vac group was immunized with $EIAV_{DLV121}$, the Car group was inoculated with a sublethal $EIAV_{LN40}$ dose resulting in asymptomatic EIAV carriage, the Acu group was inoculated with a lethal dose of $EIAV_{LN40}$ and the Mock group received a saline injection only. Except for the Acu horses, all animals were challenged with 1×10^4 $TCID_{50}$ of $EIAV_{LN40}$ 23 weeks post primary inoculation. In addition, the Hea group was mock vaccinated and challenged with saline and treated as the health control. (B) Body temperature, platelet counts and viral loads of the four Vac group horses after $EIAV_{LN40}$ challenge. (C) Body temperature, platelet counts and viral loads of the three Car group horses after $EIAV_{LN40}$ challenge.

3.3. Characterization of antibody responses elicited by $EIAV_{DLV121}$ and $EIAV_{LN40}$

Detection of serum antibodies specific to the EIAV capsid protein p26 was conducted by standard ELISA assay using purified recombinant p26 expressed in *E. coli* (Piza et al., 2007). The p26 capsid protein is the primary viral core protein making it a major EIAV immunogen. Quantitative measurements of anti-EIAV Env (envelope) glycoprotein specific serum IgG antibodies was carried out by ELISA by immobilizing viral glycoproteins in microtiter wells coated with ConA (Robinson et al., 1990). The EIA diagnostic immuno-agar diffusion assay (Coggin's test) is the standard used for the detection of anti-p26-specific antibodies as defined by the World Organization of Animal Health (OIE).

The endpoint titers for both Env- and p26-specific antibodies in vaccinated horses (Vac group) and virulent strain asymptomatic carriers (Car group) were not statistically different when analyzed as two groups (Fig. S-2A). Chronological examination of the anti-Env and anti-p26 antibodies revealed that anti-Env responses were typically higher in Vac group animals in the early and intermediate stages post inoculation, although statistical significance was only observed at 12 weeks post infection (Fig. S-2B). The anti-p26 response, however, was significantly different in that Vac group animals developed significantly higher anti-p26 titers during the first 8 weeks of infection compared to Car group titers that were significantly lower (10-fold, see Fig. S-2B).

Qualitative properties of Env-specific and p26-specific antibodies were also analyzed. As observed for other lentivirus infection models (including HIV, SIV, SHIV and EIAV) maturation of the humoral response was observed (Cole et al., 1997, 2000; Hammond et al., 1997; Wodarz, 2008). That is, following EIAV infection, Env-specific antibody titers increased, as did their avidity index and conformational dependency over a 6–8 months period post infection, at which point these responses plateaued. In the context of immune response maturity, antibody avidity and conformational dependence correlated with prognosis and vaccine efficacy (Hammond et al., 1999; Montelaro et al., 1998).

Antibody avidity was measured by treating antibody/antigen complexes with given concentrations of chaotropic reagents that dissociate low-avidity antibody/antigen complexes. Generally, if the ratio of remaining antibody (following the incubation with a chaotropic agent to untreated antibody) i.e., the avidity index is less than 0.3 following a 6 M urea wash, it is considered a low avidity response.

The conformational dependency of envelope-specific antibodies was characterized using a ConA antigen-capture ELISA that is based on glycoprotein binding to ConA-coated microplates. If the reactivity ratio of the nondenatured Env ConA-ELISA divided by the ratio of the denatured Env ConA-ELISA was >1.0, Env-specific antibodies primarily recognized conformational Env epitopes. If the ratio was <1.0 the antibodies recognized linear epitopes primarily.

Similar to the endpoint titer results, there were no significant differences between the avidity of the anti-Env or anti-p26 specific antibodies examined between vaccinated animals and asymptomatic horses infected with an $EIAV_{LN40}$ sublethal dose ($p > 0.05$). The median antibody avidity for animals from both of these groups was significantly higher than that of antibodies examined from the untreated healthy controls ($p < 0.01$, Fig. S3-A). The avidity indexes (AI = antibody activity treated with urea/untreated with urea) of both anti-Env and anti-p26 antibodies gradually increased from 0.3 to 0.5 in all vaccinated and asymptomatic horses during the time points tested. However, the avidity of anti-Env-specific antibodies in vaccinated horses increased more rapidly than the avidity observed in asymptomatic $EIAV_{LN40}$ carriers during the first 4 months post inoculation (Fig. S3-B). The median avidity index between these two groups during this initial infection period was statistically different ($p < 0.01$, data not shown).

Significant differences in the maturation of conformational dependent Env-specific antibodies were observed between vaccinated, asymptomatic and $EIAV_{LN40}$ acutely infected animals. As described in Fig. 3A and B, the median conformation index (CI) of Env-specific antibodies examined from Vac group animals was close to 1.5 which was significantly higher than the CI of antibodies examined from either the Car or Acu groups ($p < 0.01$). The maturation pattern of conformationally dependent Env-specific antibodies between Vac and Car group horses was readily discernable. In contrast to the temporal change in the CI of antibodies from Car group animals (which consistently shifted between 0.8 and 1.0 two weeks post inoculation) the ratio of conformational dependent Env-specific antibodies from Vac group animals was as high as 1.2 two weeks post inoculation, at which point it gradually increased and leveled off near 1.5. Challenge with an $EIAV_{LN40}$ lethal dose transiently and modestly increased the CI of antibodies examined from both of these two treatment groups.

3.4. EIAV-specific neutralizing antibody responses

Although the role of neutralizing antibodies in protection from lentivirus infection remains undefined, the importance of anti-lentivirus neutralizing activities has been observed during the course of recent HIV-1 and SIV/SHIV studies (Prado et al., 2009; Wodarz, 2008; Yankee et al., 2009) and the recent RV-144 AIDS vaccine trial (Gilbert et al., 2011). To determine if a correlation existed between

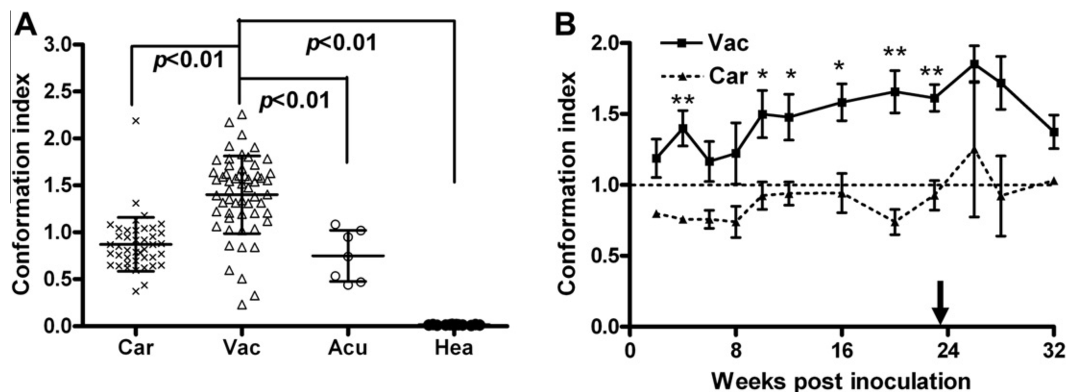

Fig. 3. Conformational dependent maturation of anti-Env glycoprotein antibodies. (A) Comparison of conformation indices of Env glycoprotein-specific antibodies in experimentally infected horses (Vac, Car, Acu and Hea groups) during the experimental period. (B) Conformation indices for Env glycoprotein-specific antibodies in horses inoculated with either $EIAV_{DLV121}$ (Vac) or $EIAV_{LN40}$ (Car). Arrows indicate the time of challenge with a lethal dose of $EIAV_{LN40}$. $^{*}p < 0.05$ using the Student's t test.

neutralizing activity and protection against EIAV infection, neutralizing antibodies from vaccinated horses (Vac group) and from asymptomatic carriers infected with a sublethal $EIAV_{LN40}$ dose (Car group) were measured and compared. Due to *in vitro* growth limitations of the $EIAV_{LN40}$ challenge strain in eMDM, $EIAV_{DLV34}$, an eMDM-adapted strain derived from $EIAV_{LN40}$, was used in antibody neutralization assays. To confirm that $EIAV_{DLV34}$ remained pathogenic, this strain was used to infect two EIA-naive horses using the same dose established for $EIAV_{LN40}$. Both $EIAV_{DLV34}$-infected horses developed EIA 21 and 28 days post infection, respectively, and succumbed to the infection 27 and 42 days post infection (Fig. S-4A).

Phylogenetic comparison of the $EIAV_{DLV34}$ genome to its parental $EIAV_{LN40}$ strain and to the $EIAV_{DLV121}$ and $EIAV_{FDDV12}$ vaccine strains demonstrated that $EIAV_{DLV34}$ clustered with $EIAV_{LN40}$ and not with the two vaccine strains (Fig. S-4B). Antibody reactivity to the gp90 principal neutralizing domain (PND) is considered important to viral neutralization (Shen et al., 2006). Amino acid sequence comparison of the PND sequence from $EIAV_{LN40}$, $EIAV_{DLV34}$, $EIAV_{DLV121}$ and $EIAV_{FDDV12}$ revealed that the $EIAV_{DLV34}$ PND sequence was more closely related to the $EIAV_{LN40}$ parental strain than to the vaccine strain PND sequences (Fig. S-4C).

Serum samples from infected horses from the respective groups were collected to evaluate their capacity to neutralize $EIAV_{DLV34}$. The median neutralization titer of serum samples from vaccinated horses during an 8-month period was significantly higher than the median neutralization titers of antibodies collected from asymptomatic carriers (Car group) or from antibodies collected from horses with acute EIA (Acu group) (Fig. 4A, $p < 0.01$). Furthermore, EIAV neutralizing activity in vaccinated horses emerged at as early as 14 days post infection compared to the neutralization activity observed in serum samples collected from asymptomatic carriers which developed much slower (56 days post infection) (Fig. 4B).

3.5. Characterization of cell-mediated immune responses

Due to the failure of the AIDS STEP vaccine trial, it will be necessary to re-evaluate AIDS vaccine design strategies focused on inducing protective cytotoxic T lymphocyte (CTL)-based responses. However, these responses are still considered essential to the control of lentivirus infections, particularly if neutralizing antibodies cannot completely prevent the entrance of viruses to target cells (Blish et al., 2007). Because the nature of the cell-mediated response is critical to infection outcome, we evaluated these responses in our model system.

Antigen-specific T cell proliferation was examined by harvesting ePBMCs from EIAV-inoculated and control horses, incubating them with purified $EIAV_{FDDV12}$ and measuring the level of antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation. Proliferation data are expressed as cell division index (CDI), defined as the proliferation ratio of antigen-stimulated cells divided by the proliferation of unstimulated cells. CDI values >0.1 are considered positive responses.

$CD4^+$ T cells had the highest median CDI values (compared to $CD8^+$ T cell proliferation), particularly $CD4^+$ cells harvested from $EIAV_{DLV121}$-vaccinated horses (Vac group) and from $EIAV_{LN40}$ asymptomatic carriers (Car group), but not $CD4^+$ cells harvested from horses with acute EIA (Acu group) (Fig. 5A). Furthermore, the CDI of Vac group horses was significantly higher than the median CDI for Car group horses ($p < 0.05$, Fig. 5A). Specifically, $CD4^+$ T cell CDI increased gradually over the first 3 months post the infection. This early CDI increase reached a plateau 16 weeks post the initial inoculation, however, the level of proliferation observed for Vac group animals was significantly higher than the proliferation observed for $CD4^+$ cells harvested from animals in the Car group infected with an $EIAV_{LN40}$ lethal dose (Fig. 5B, top panel). The $CD8^+$ T cell proliferation response over time had a profile similar to that observed for $CD4^+$ T cells (Fig. 5B, lower panel). Specifically, $CD8^+$ cells harvested from Vac group animals proliferated at a higher level than the response observed for $CD8^+$ cells collected from Car group horses 12 weeks post inoculation, but was not statistically significant.

3.6. IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells

Characterization of IFN-γ secretion in response to antigenic stimulation is critical in evaluating the nature of cellular immune responses. The importance of $CD4^+$ T cell-mediated regulation in the context of lentivirus infections e.g., HIV-1 and SIV was highlighted following examination of the data obtained from the failed STEP AIDS vaccine trial (Hanke, 2008; Robb, 2008). For this reason, we examined the EIAV-specific IFN-γ response by both $CD4^+$ and $CD8^+$ cells in our EIAV infection groups. The IFN-γ response post initial inoculation demonstrated that significantly more $CD8^+$ cells secreted IFN-γ than $CD4^+$ cells and that statistically more $CD8^+$ cells from Vac group animals secreted IFN-γ than cells harvested from either the Car, Acu group or health control groups. However, there was no significant difference in IFN-γ-producing $CD4^+$ cells between the Car and Acu groups (Fig. 6A). The temporal profile showed that slightly more $CD4^+$ cells gradually became INF-γ producers in Vac group animals compared to Car group animals 20 weeks post inoculation. Only at 2 weeks post infection were the numbers of INF-γ-producing $CD4^+$ cells significantly greater in the Vac group than in Car group animals. More significant differ-

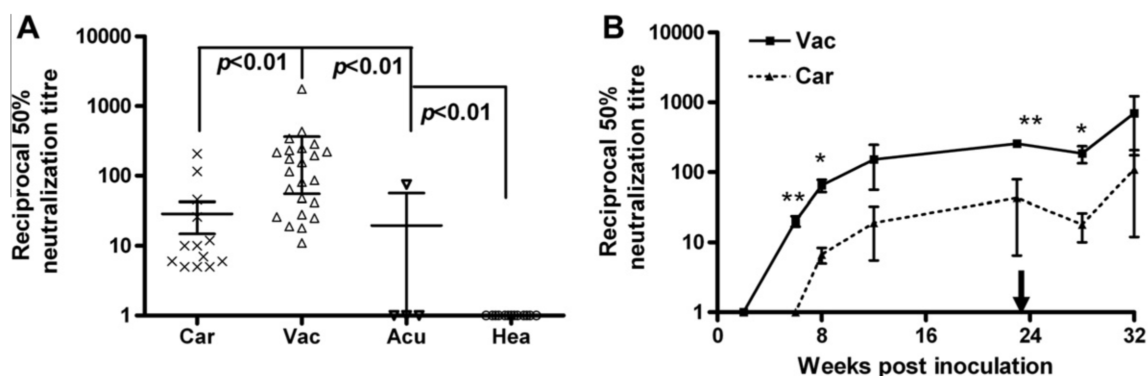

Fig. 4. Anti-EIAV neutralization titers. Serum neutralizing activity to virulent strain $EIAV_{DLV34}$ was measured and expressed as reciprocal 50% neutralization titers. (A) Serum titers from the Vac, Car, Acu and Hea groups. (B) Temporal development of neutralizing activity expressed as reciprocal 50% neutralization titers of $EIAV_{DLV121}$-immunized horses and $EIAV_{LN40}$ asymptomatic carries. $^*p < 0.05$ using the Students *t* test.

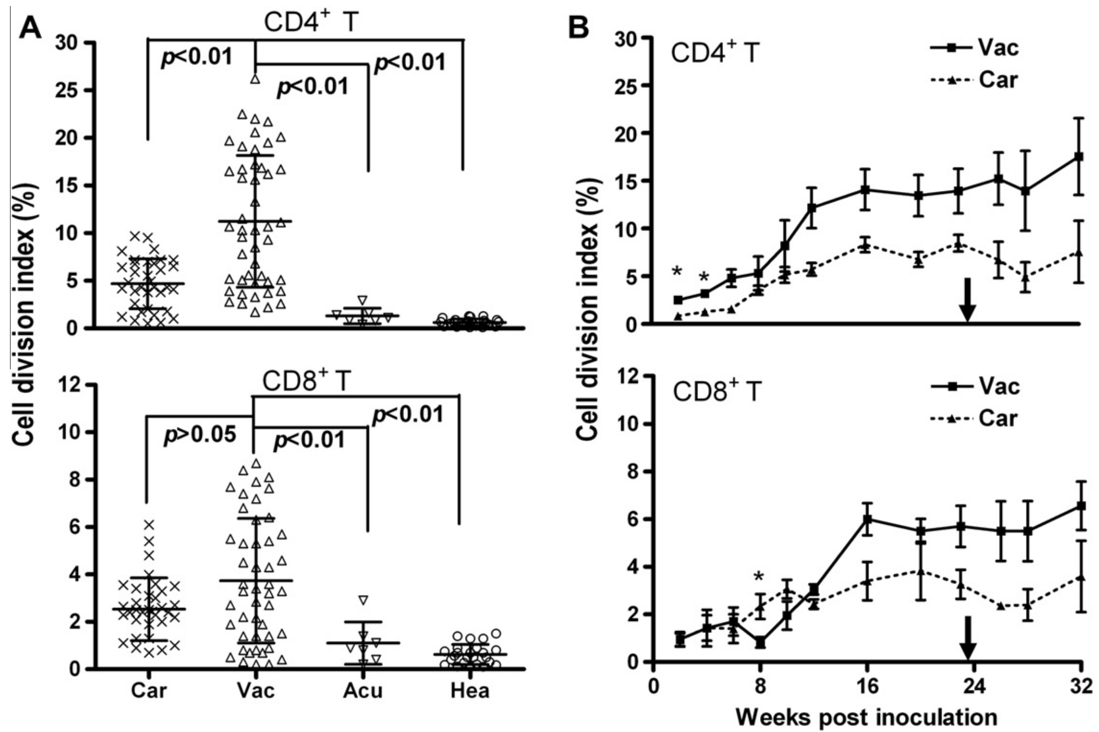

Fig. 5. EIAV-specific CD4+ and CD8+ T cell proliferation. (A) Proliferation of EIAV-specific CD4+ and CD8+ T cells isolated from Vac, Car, Acu and Hea animals during the experimental period was expressed as cell division index (CDI). (B) Temporal development of EIAV-specific CD4+ and CD8+ T cell proliferation from $EIAV_{DLV121}$-immunized horses and $EIAV_{LN40}$ asymptomatic carries. CDIs are expressed as the proliferation in the presence of antigen/proliferation in the absence of antigen. $^{*}p < 0.05$ using the Students *t* test.

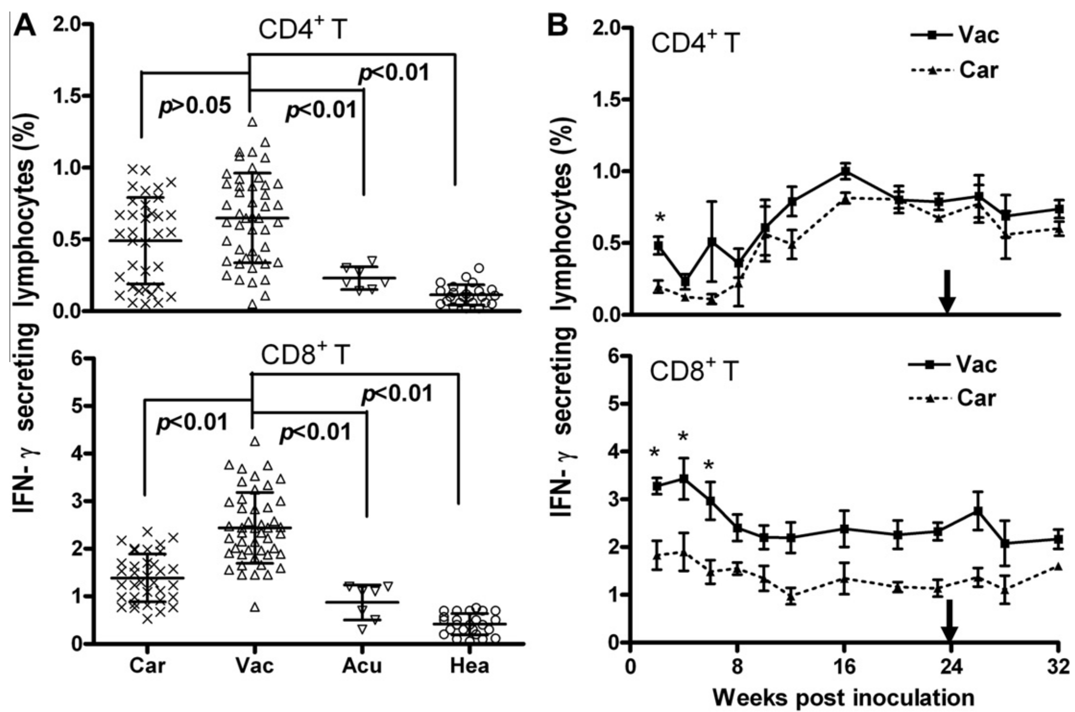

Fig. 6. EIAV-specific CD4+ and CD8+ T cell IFN-γ secretion. (A) Percent CD4+ and CD8+ IFN-γ secreting cells isolated from Vac, Car, Acu and Hea animals during the experimental period. (B) Temporal EIAV-induced IFN-γ secretion by CD4+ and CD8+ T cells from $EIAV_{DLV121}$-immunized horses and $EIAV_{LN40}$ asymptomatic carries. Background IFN-γ secretion from EIAV-unstimulated lymphocytes was subtracted from the antigen stimulated cells. $^{*}p < 0.05$ using the Student's *t* test.

ences were observed in IFN-γ-producing CD8+ cells. There were more IFN-γ-producing cells harvested from Vac group animals at all time points examined compared to the number of IFN-γ producing cells collected from Car group animals (Fig. 6B).

3.7. Plasma viral loads

Because plasma viral levels are often predictive of disease prognosis and are an important factor that determines the type and

magnitude of the host's immune response (Whitney and Ruprecht, 2004), it was essential to determine the EIAV plasma loads in $EIAV_{DLV121}$-vaccinated horses and $EIAV_{LN40}$-infected asymptomatic carriers in the context of the different immune responses elicited in these two treatment groups. Previous studies performed by us and others showed that the EIAV viral loads during the febrile phases were between 10^6 and 10^9 copies/ml of plasma compared to 10^2–10^5 copies/ml of plasma in vaccinated animals and during asymptomatic EIA (Langemeier et al., 1996; Li et al., 2003). In this study, EIAV plasma RNA copy numbers from Vac and Car group horses were determined by quantitative RT-PCR. Results demonstrated that viral loads in both groups were below 1×10^6 copies/ml before challenge with $EIAV_{LN40}$. There were no significant differences in the viral loads between the $EIAV_{DLV121}$-vaccinated and sublethal dose $EIAV_{LN40}$-infected horses (Fig. 7)A and B. However, viral loads in most Vac group horses (3/4) began declining 16 weeks post vaccination (Fig. 7A). In contrast, viral loads in two Car group horses (and one Vac group horse) began increasing at this time point (Fig. 7B).

3.8. Correlation between the immune responses and sterile protection

Since protection from infection failed in two of four vaccinated horses (Vac1 and Vac3), we investigated immune response differences between the protected and non-protected horses. The humoral and cellular immune responses during the early (2–6 weeks post inoculation), intermediate (6–12 weeks post inoculation) and late (12–23 weeks post inoculation) stages post vaccination and post challenge were grouped according to protective efficacy against infection with the virulent $EIAV_{LN40}$ strain by detection of *env* sequences. Although significant differences in the anti-Env antibody conformational dependence and neutralization activity between $EIAV_{DLV121}$-vaccinated horses (Vac group) and $EIAV_{LN40}$ asymptomatic carriers (Car group) were observed, these variations in humoral immune responses were not observed between protected and non-protected animals. However, remarkable differences in the reciprocal end-point anti-p26 antibody-binding titers were observed after the early vaccination stages that showed a significant elevation of this antibody response in protected horses (Fig. 8A). This is the first data that correlated capsid-specific antibody responses with protection against challenge with an EIAV virulent strain (sterile protection).

In contrast, the differences in cellular immune responses between Vac group and Car group animals were easily discernable (Fig. 8B). Differences in the EIAV-specific $CD4^+$ and $CD8^+$ T cell proliferation were apparent during the medium and late time points post vaccination and post challenge, and IFN-γ secretion by these T cells was significantly elevated during late stages post vaccination and challenge. Combined with the differences observed in cellular immune responses between Vac and Car group animals, these results strongly suggested the importance of cell-mediated immunity in the protection induced by the EIAV attenuated vaccine strain.

4. Discussion

Our inability to understand the nature of protective anti-lentivirus infections such as HIV-1 is preventing the development of effective vaccines (Baker et al., 2009; Wodarz, 2008). These unsuccessful attempts further suggest the need for research aimed at characterizing the nature of immune responses with the capacity of inhibiting or at least restricting HIV proliferation (Appay, 2009; Robb, 2008). In this study, we demonstrated for the first time significant protection (77%) conferred by the $EIAV_{DLV121}$ vaccine strain against clinical disease caused by infection with the heterologous $EIAV_{Wyoming}$ strain, which was 23.5% and 37.8% different at the genomic level and sequence of the gp90 glycoprotein, respectively. These results demonstrate that this attenuated EIAV vaccine provides a useful lentivirus vaccine model with the capacity of establishing a correlation between the nature of the immune response and protection from disease and infection. Specifically, we compared humoral and cellular responses in $EIAV_{DLV121}$-vaccinated and sublethally $EIAV_{LN40}$-infected animals since both forms of EIAV had similar circulating viral loads in these two groups of horses but protection was only observed in the $EIAV_{DLV121}$ vaccine group.

Analysis of the humoral response in both $EIAV_{DLV121}$-vaccinated and sublethally $EIAV_{LN40}$-infected animals demonstrated that this response matured over a lengthy period of time similar to previously published studies that were designed to define the nature of immune responses mounted following infections with HIV, SIV or EIAV, which developed over 6–8 months post infection (Cole et al., 2000; Hammond et al., 1997; Montelaro et al., 1998). The gradual increase in both avidity and conformational dependence of Env-specific antibodies in $EIAV_{DLV121}$-vaccinated and asymptomatic $EIAV_{LN40}$ carriers displayed a comparable maturation pattern to that described in SIV-infected monkeys (Cranage et al., 1997; Reynolds et al., 2008; Valentine and Watkins, 2008). Medium to high avidity and conformational-dependent antibodies were observed in $EIAV_{DLV121}$-vaccinated animals early post immunization. However, the envelope-specific antibodies in asymptomatic $EIAV_{LN40}$ carriers were of low avidity and predominantly directed to linear epitopes. A more significant difference in the

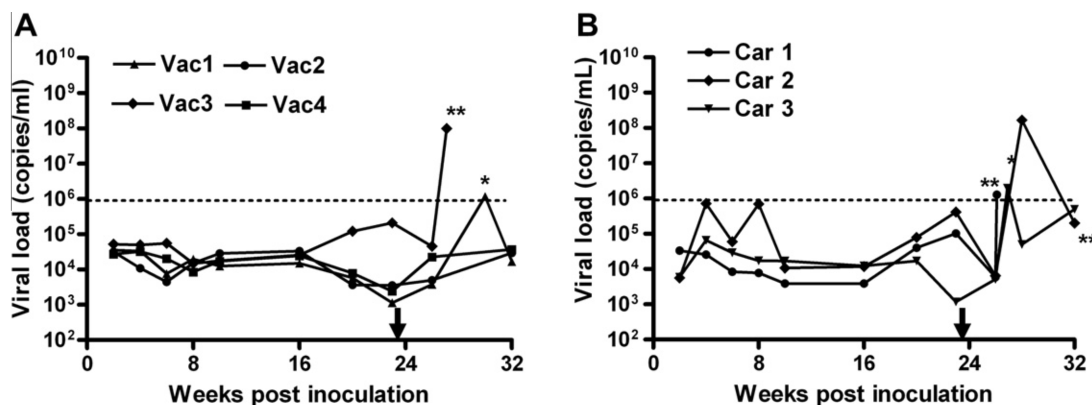

Fig. 7. Replication of $EIAV_{LN40}$ and $EIAV_{DLV121}$ *in vivo*. Viral RNA from (A), $EIAV_{DLV121}$- or (B), $EIAV_{LN40}$-infected horses was measured by quantitative RT-PCR and expressed as copy numbers/ml of plasma. The viral load considered a threshold for clinical EIA is indicated by the dashed line. *Indicates an episode of typical EIA symptoms. **Indicates death as a result of EIA. Arrows indicate the time of the $EIAV_{LN40}$ challenge.

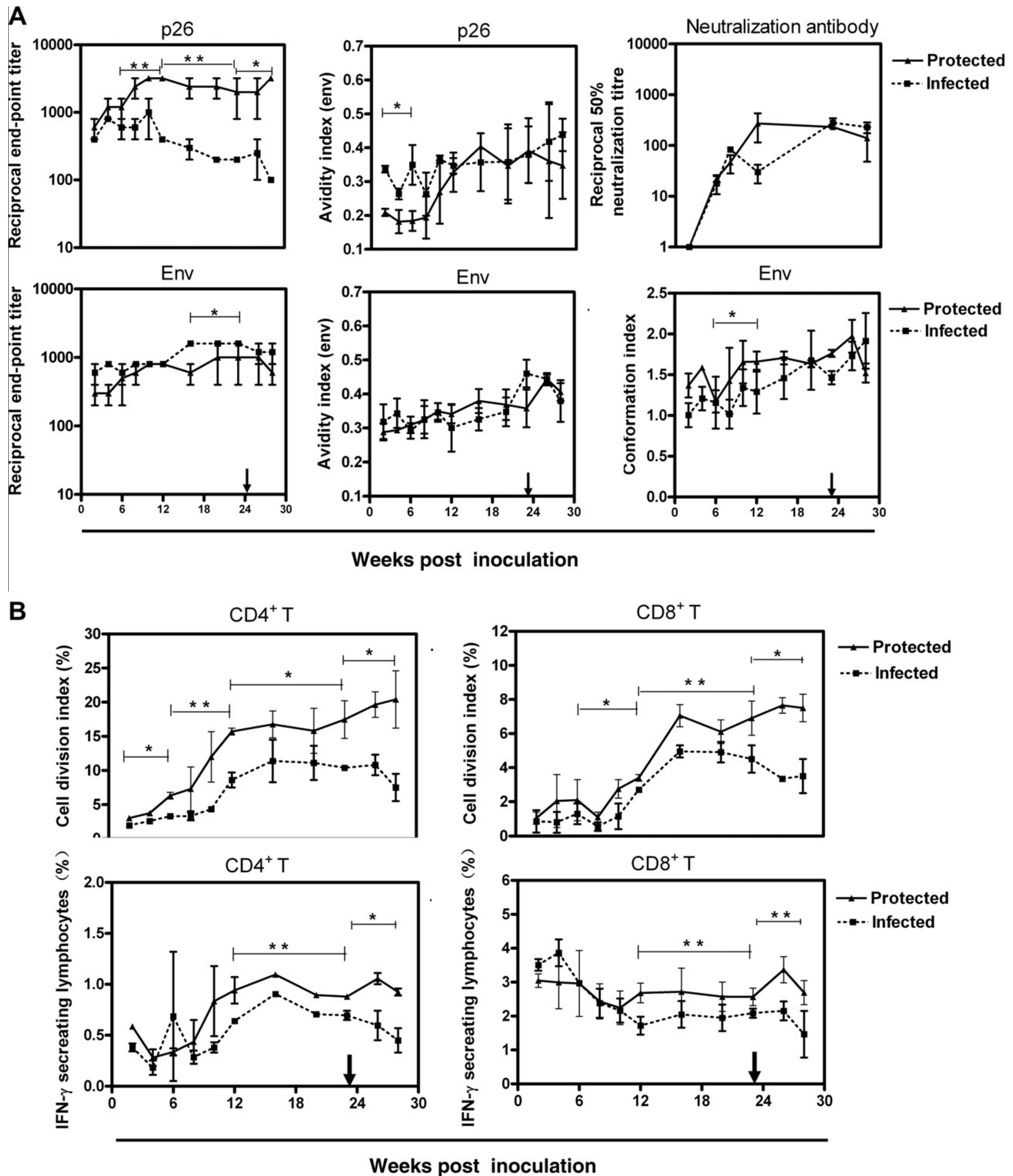

Fig. 8. Correlation between the nature of the immune responses and protection from infection. Humoral (A) and cellular (B) immune responses from Vac group horses reclassified into 'protected' or 'infected' groups according to resistance to virulent $EIAV_{LN40}$ strain infection. Immune response parameters during the early, intermediate and late stages post vaccination and after challenge were grouped as indicated by horizontal bars and analyzed separately for correlation with protection. *p* Values determined using the Student's *t* test.

antibody response between $EIAV_{DLV121}$-vaccinated and asymptomatic $EIAV_{LN40}$ carriers was the development of neutralizing antibody, even though low levels of neutralizing antibodies in asymptomatic $EIAV_{LN40}$ carriers were detected 2–3 months post infection that gradually increased similar to what has been reported previously following infection with either EIAV or HIV-1 (Hammond et al., 1997; Wodarz, 2008). In addition, a previous study showed that neutralizing antibodies could not be detected in horses inoculated with the *S2*-deficient, non-pathogenic $EIAV_{D9}$ strain or with an inactivated EIAV vaccine (Hammond et al., 1999). However, in this study, the neutralization titer in vaccinated horses developed 2–3 weeks post inoculation, increased rapidly and was maintained at high levels throughout the examination period. This difference in the humoral response profile suggested that the response elicited by the $EIAV_{DLV121}$ vaccine correlated with protection.

Our data demonstrated that some aspects of cell-mediated immune responses also developed gradually. In contrast to a number of previously published reports that failed to show a defined increase in T cell response in EIAV-infected horses (Hammond et al., 1997, 2000; Tagmyer et al., 2007), a steady and significant increase in both $CD4^+$ and $CD8^+$ T cell proliferative responses and in the number of IFN–producing $CD4^+$ T cells 3–4 months post infection were observed in our study. In addition, we observed significant differences in the cellular responses mounted by the $EIAV_{DLV121}$-vaccinated and asymptomatic $EIAV_{LN40}$ carriers, particularly in $CD4^+$ T cell proliferation and IFN-γ-producing $CD8^+$ T cells. It should be noted that significant differences between these two parameters were noticeable during the early stages post vaccination. Our cellular response data differed from responses reported in some HIV-1 and SIV vaccination studies. One study reported that SIV-infected Rhesus macaques that developed anti-SIV envelope protein $CD4^+$ T cell proliferative responses in the absence of CTL responses presented with enhanced SIV replication and accelerated progression to AIDS (Liu et al., 2009). A difference between that study and ours is that $CD4^+$ T cells are the target cells for both HIV-1 and SIV (but not of EIAV) and the activation and proliferation of $CD4^+$ cells therefore facilitated HIV-1 and SIV replication (Dyer et al., 2008; Letvin, 2006; Schrier et al., 1996). The consistently lower circulating viral loads in horses with dramatically increased T helper cell responses observed in our study are likely due to the fact that EIAV does not infect lymphocytes (Leroux et al., 2004). Furthermore, it has been reported that the $CD4^+$ T cell response can influence $CD8^+$ T cell effector mechanisms, the production of neutralizing antibodies and the development of immunological memory (Seder and Ahmed, 2003; Seder et al., 2008). The significantly enhanced $CD4^+$ T cell responses observed in $EIAV_{DLV121}$ vaccinated animals likely affected other immune parameters such as antibody affinity maturation and conformational dependence.

To minimize the possible variation in immune responses to different viral strains, analyses correlating immune responses with protection were performed among vaccinated animals even though the sample size was limited. Instead of protection from disease, protection from infection was utilized to more stringently evaluate protection. The significant differences in all four parameters of cellular immune responses between protected and unprotected horses strongly demonstrated the importance of cell-mediated immunity, especially $CD4^+$ T cell-mediated immunity in the protection induced by this attenuated lentivirus vaccine. Although the role of CTLs in an effective AIDS vaccine needs to be re-evaluated after the failure of the STEP II trial, the importance of cellular immune responses are further supported by several recent studies, including the AIDS vaccine trial in Thailand (Appay, 2009; Harris, 2009; Robb, 2008; Watkins, 2010).

In contrast, even though the $EIAV_{DLV121}$ vaccine strain induced significantly higher levels of conformationally dependent and neutralizing Env-specific antibodies compared to the virulent strain, the only difference in humoral immune response between protected and unprotected groups of vaccinated horses was observed in the reciprocal end-point titer of anti-p26-specific antibodies. Published studies on humoral immune responses to EIAV by others did not identify significant correlations between specific antibodies and protection (Hammond et al., 1997, 1999). For the first time, our data suggested that the p26 capsid-specific antibody might be a useful marker for identifying protective immune responses to EIAV. The significance of the differences in the p26 antibody is not clear although it has been reported that anti-p24 HIV capsid-specific antibodies were required for protection (Farzadegan et al., 1992). Higher levels of anti-capsid-specific antibodies could be the result of enhanced levels of viral capsid or its precursor (Gag) protein, which is considered the major source of CTL epitopes (Chung et al., 2004; Zhang et al., 1998). Furthermore, the enhanced $CD4^+$ T cell activity in protected horses may more efficiently regulate the production of p26 antibody responses.

The plasma viral load is considered a major factor that determines the level and quality of immune responses and protection (Guo et al., 2004). The pathogenic and attenuated viral strains that were used in this study replicated at similar levels during the 6 months of the pre-challenge period. However, the strains induced significantly different humoral and cell-mediated immune responses. The precise mechanism that mediates these differences is unknown. Several possibilities may contribute to the specific feature of immunity that is induced by the virulent or attenuated EIAV. Differences in the breadth and severity of EIAV immunogenicity, especially against Env, induce different immune responses, which have been reported in several recent publications (Craigo and Montelaro, 2010, 2011; Tagmyer et al., 2008). The predominant quasispecies of the pathogenic strain $EIAV_{LN40}$ were selected by the pressure of immunity *in vivo* and were less effective in inducing specific immunity compared to the predominant quasispecies of the attenuated strain $EIAV_{DLV121}$, which were developed by continuous passages in cultivated macrophages. The antigenic variation and levels of the attenuated vaccine strain were increased in the *in vitro* environment that lacks the selective pressure of immunity. Sequence analysis revealed that the vaccine strain contained multiple species that consisted of quasispecies with a genomic diversity of $2.6 \pm 0.3\%$, which was 4.3-fold higher than that of the pathogenic strain and contained fewer glycosylation sites in the Env surface protein compared to the Env of the pathogenic strain (data not shown). The loss of these glycosylation sites, which were located near the Principle Neutralizing Domain, may expose epitopes that were covered by oligosaccharide side chains. Therefore, the vaccine strain was predicted to generate more antibodies and to express more CTL and antigen-specific helper T (Th)-cell epitopes, and subsequently stimulating an effective immune response and protection that exceed that elicited by the pathogenic strain. In addition, the strain with attenuated virulence and altered intracellular behavior may act differently from the pathogenic strain in inducing innate immune responses such as the expression of cytokines and chemokines) and the function of target cells (such as cell survival), which has been reported by us and other researchers (Covaleda et al., 2010; Haynes et al., 2006), and should largely influence specific adaptive immunity (Mogensen et al., 2010).

The results of this study indicated that $EIAV_{DLV121}$ vaccination or a sublethal infection with $EIAV_{LN40}$ resulted in persistent viremia with similar plasma viral loads under the threshold required for the development of clinical EIA. However, the immune responses elicited by these two EIAV strains were significantly different. $EIAV_{DLV121}$ stimulated significantly more robust humoral (conformational dependent envelope antibodies and neutralizing antibodies) and cellular ($CD4^+$ T cell proliferation and IFN-γ-secreting $CD8^+$ T cells) immune responses. In the context of protection from infection, cell-mediated immune responses appeared to be essential to preventing EIAV infections. Humoral responses also likely played a role but were not sufficient for sterile protection, however, the correlation of enhanced anti-p26-specific antibodies with the protective immunity to EIAV remains to be investigated. The data presented in this report highlight the importance of both cellular and humoral responses in the context of protection against EIAV infections and further demonstrate the attenuated EIAV vaccine as a unique model for better understanding of protective immunity to lentiviruses.

Acknowledgments

This study was funded by the National Special Found for Control and Treatment of Major Infectious Diseases (Grant 2012ZX10001-

008 to JZ) and the National Natural Science Foundation of China (Grants 30901349 to YL and 30771994 to JZ).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.08.016.

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