The immediate early protein of equine herpesvirus-1 (EHV-1) as a target for cytotoxic T lymphocytes in the Thoroughbred horse

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Summary

CTL are associated with protective immunity against disease caused by equine herpesvirus-1 (EHV-1). However, the EHV-1 target proteins for CTL are poorly defined. This limits the development of vaccine candidates designed to stimulate strong CTL immunity. Here, classical CTL assays using lymphocytes from horses of three defined MHC class I types that experienced natural infection with EHV-1, and a modified vaccinia virus construct containing an EHV-1 gene encoding the Immediate Early (IE) protein are reported. Horses homozygous for the Equine Leukocyte Antigen (ELA) A2 haplotype, but not the ELA-A5 haplotype, made MHC-restricted CTL responses against the IE protein. Previously, horses homozygous for the ELA-A3 haplotype also mounted CTL responses against the IE protein. Both haplotypes are common in major horse breeds, including the Thoroughbred. Thus, the IE protein is an attractive candidate molecule for future studies of T-cell immunity to EHV-1 in the horse.

Short Communication

Equine herpesvirus-1 (EHV-1) can cause late gestation abortion in pregnant mares and respiratory and neurological disease in all equids (Ma *et al.*, 2013; Minke *et al.*, 2004). Current vaccines against EHV-1 contain either live attenuated or killed virus, and both types provide partial clinical and virological protection (Minke *et al.*, 2004). However, periodic abortion storms and outbreaks of neurological disease highlight the need for improved vaccines. Protection against EHV-1 in horses is associated with high titres of neutralising antibody (Hannant *et al.*, 1993; Heldens *et al.*, 2001) and high frequencies of CTLs (Allen, 2008; Kydd *et al.*, 2003; O'Neill *et al.*, 1999). Ideal vaccines might consist of defined antigens which can stimulate protective cellular and humoral immunity in all horses and thus eliminate the negative effects of viral proteins which modulate the host's immune response (Ambagala *et al.*, 2005; Griffin *et al.*, 2010; van der Meulen *et al.*, 2006), including down-regulation of MHC class I (Rappocciolo *et al.*, 2003; Said *et al.*, 2012).

To develop novel vaccines against EHV-1 that stimulate protective cellular immune responses, it is necessary to identify conserved and immune-dominant viral proteins and determine the distribution of MHC class I molecules within and between horse breeds. MHC class I molecules act as restriction elements that present viral peptides to the antigen specific receptors on T-cells. The equine MHC region has been defined using serological assays (Lazary et al., 1988) and molecular techniques that have taken advantage of resources of the Horse Genome Project (Gustafson et al., 2003; Tallmadge et al., 2005; 2010). MHC typing using microsatellites (Tseng et al., 2010) has confirmed earlier serological studies (Antczak et al., 1986) which indicated that within the Thoroughbred breed, a limited number of stable MHC haplotypes (Equine Leukocyte Antigens, ELA-A2, A3, A5 and A10) represent the majority of the genetic diversity in this region of the

genome. The economic importance of the Thoroughbred justifies a closer examination of the genetic basis of immunity in this breed.

To date, although high frequencies of CTL precursors are associated with reduced clinical signs (Allen, 2008; Kydd *et al.*, 2003), the only CTL target protein which has been identified for EHV-1 is the immediate early (IE) protein, which is encoded by gene 64 (Soboll *et al.*, 2003). Epitopes of this protein are presented by the B2 allele of the ELA-A3 haplotype (Kydd *et al.*, 2006), which is also known as ELA-A3.1 (Tallmadge et al., 2005). Vaccination of ELA-A3 ponies which expressed the B2 allele with a construct which expressed the IE protein resulted in the stimulation of interferon gamma^{+} lymphocytes in peripheral blood, which are associated with CTL activity (Paillot *et al.*, 2006) and a reduction in cell associated viraemia (Soboll *et al.*, 2010), indicating partial virological protection.

This study aimed to assess the EHV-1 IE protein as a CTL target in horses of defined MHC haplotypes, which had been exposed to a virulent strain of EHV-1 during a field outbreak. The preliminary data suggests that the IE protein acted as a CTL target in two mares carrying the ELA-A2 MHC haplotype, but not in a mare homozygous for the ELA-A5 haplotype. This data enhances our knowledge of CTL target proteins for EHV-1, a finding critical to the rational development of novel vaccines.

Animals: The mares used in this study (Table 1) were members of an experimental herd at Cornell University, United States of America that experienced a natural outbreak of disease caused by EHV-1. One additional mare (Esther) was located at the Animal Health

Trust, United Kingdom. This mare was hyper-immune to EHV-1 following previous experimental infections with strain Ab4 and was thus used as a donor of positive control lymphocytes, due to the high frequencies of blood CTLs. All mares had known ELA haplotypes, which were common in Thoroughbreds, as determined previously by serological typing: the Cornell mares were all homozygous for the specified haplotype (Table 1). All animals were managed according to their host institution's animal care criteria and national regulations.

Blood samples: PBMCs collected by jugular venepuncture were isolated at Cornell University by density gradient centrifugation over Ficoll®, washed, and re-suspended at 10^8/ml in Dulbecco's modified Eagle's medium supplemented with 10% (v:v) heat inactivated fetal calf serum, 2mM L-glutamine, HEPES buffer, 100u/ml penicillin and 100μg/ml streptomycin. Cells were transported to the United Kingdom at ambient temperature over 48 hours under a government permit. On arrival, cells were re-counted and either used immediately in induction cultures or alternatively cryopreserved in liquid nitrogen as described previously (Allen *et al.*, 1995) and thawed as required. Due to the difficulty of harvesting and transporting such large numbers of cells internationally, each experiment was performed once only. Viability in all samples, as determined by Trypan blue exclusion was $\geq$90%

CTL Assay: An EHV-1 specific assay of CTL activity was performed as described previously (Allen *et al.*, 1995; Kydd *et al.*, 2006). Effector cells were induced with live EHV-1 and screened against a variety of autologous or heterologous target cells that were either EHV-1 infected, mock infected, or infected with a modified vaccinia virus construct (NYVAC) expressing EHV-1 gene 64, which encodes the IE protein (see Kydd *et al.*, 2006;

Paillot *et al.*, 2006 for construct details). The optimum m.o.i. for infection of target cells by the construct was determined by titration (m.o.i. 5, 2 and 1) and screened against effector lymphocytes from a mare (Esther) with an ELA-A3/x haplotype that had been hyper-infected with EHV-1. Results were expressed as percent specific lysis, calculated according to a standard formula (experimental c.p.m – spontaneous c.p.m.) / (c.p.m. total release – c.p.m. spontaneous release) x100. Data were expressed as the mean of 3 replicates.

EHV-1 infection was confirmed in the Cornell horses by the local diagnostic laboratory, based on clinical signs, virus isolation and complement fixing (CF) antibody titres. One mare (AM) displayed transient ataxia. The remaining Cornell mares had high titres of CF antibody ($\geq$1:80), regardless of vaccination status.

The first aim of this study was to identify horses of well-characterized MHC class I types with high levels of EHV-1 specific CTL activity: these were used for future screening against target cells presenting only the EHV-1 IE protein encoded by gene 64. Six of seven horses tested had detectable levels of virus specific CTL activity, with low levels of lysis (<6%) against mock-infected autologous target cells (Fig. 1). One mare (G, ELA-A2) had insufficient effector cells and virus specific lysis of <12% at 100:1 effector to target ratio (data not shown) and so was discarded from future experiments. The remaining six mares, all had high levels of virus specific lysis when tested on infected autologous cells (range $\geq$29 - 80.7% at effector to target ratios of 100:1). The effector CTL were also tested against virus infected target cells from the other mares. High levels of target cell lysis (>20%) were observed only when the CTL and target cells carried the same MHC type, thus demonstrating classical MHC restricted lysis.

Having identified mares with detectable CTL activity, additional PBMC from three mares with representative MHC class I haplotypes (FTM and Y2K both ELA-A2 and FW ELA-A5) were collected and transported as described previously for further experiments designed to determine the role of the IE protein as a CTL target. For logistical reasons, the second sample was collected one year later and thus measurement of EHV-1 specific CTL activity was repeated at that time.

To determine the optimum m.o.i. of NYVAC-gene 64 with which to infect target cells, effector CTL from an experimental mare, Esther, which carried the ELA-A3 haplotype were used. This A3/x mare was hyperimmune to EHV-1 and had CTL activity against the IE protein. This titration demonstrated that an m.o.i. of 2 was sufficient to produce detectable target cell lysis (Fig. 2(a)).

Next, the CTL from three mares, Y2K and FTM (both ELA-A2) and FW (ELA-A5) were tested against target cells infected with NYVAC-gene 64 at an m.o.i. of 2 (Fig. 2). All mares showed CTL activity against autologous virus infected target cells, but not against mock-infected targets. For the NYVAC-gene 64 infection, insufficient autologous target cells were available from the mare Y2K (ELA-A2); therefore Y2K effectors were tested against target cells from FTM (ELA-A2). Effector CTL from the two ELA-A2 mares lysed ELA-$A2^+$ target cells infected with NYVAC-gene 64, but not NYVAC-gene 64 infected targets of the ELA-A3 haplotype. Mare FW (ELA-A5) failed to lyse either ELA-$A3^+$ or ELA-$A5^+$ cells infected with NYVAC-gene 64. Cumulatively, this data suggests that the IE

protein provides peptides recognized by CTL in EHV-1 primed horses of the ELA-A2 MHC class I haplotype but not the ELA-A5 haplotype.

This study provides new data on the viral proteins that stimulate CTL activity in horses after natural infection with EHV-1. The use of a unique herd of MHC homozygous horses permitted association between CTL response and MHC haplotype. Previous studies indicated that the EHV-1 IE protein encoded by gene 64 is the source of peptides that bind to the ELA-A3.1 gene (alias B2) of the ELA-A3 haplotype (Kydd *et al.*, 2006; Soboll *et al.*, 2003). Here the data suggest that the IE protein also contains a peptide(s) that is presented by a MHC class I gene of the ELA-A2 haplotype, but not of the ELA-A5 haplotype. This information adds to our understanding of the targets of cellular immune responses against this important equine viral pathogen. Further study will be required to identify which ELA-A2 MHC class I gene presents peptide(s) from the IE protein. Five MHC class I genes have been identified in the ELA-A2 haplotype, with two showing properties of classical, polymorphic, antigen presenting molecules (Tallmadge *et al.*, 2010).

CTL target proteins have been identified in other herpesviruses. For example, in varicella zoster virus tegument proteins encoded by Open Reading Frames (ORFs) 4, 10, 62, 63 and gI act as CTL targets (Arvin *et al.*, 1991; Bergen *et al.*, 1991; Sadzot-Delvaux *et al.*, 1997). In human cytomegalovirus, phosphoprotein 65, a major late matrix protein, is recognised by CTL from HLA-A2 individuals (Kern *et al.*, 2002; McLaughlin-Taylor *et al.*, 1994; Wills *et al.*, 1996). In bovine herpesvirus type 1 (BHV-1), CTL clones lysed BHV-1 infected target cells in a genetically restricted, virus specific manner (Splitter *et al.*, 1988)

189 although whether gB, gC and gD are the targets is controversial (Hart *et al.*, 2011; Levings
190 & Roth, 2013).
191
192 In the horse, CTL target proteins and their genetic restriction elements have also been
193 identified for equine infectious anaemia virus (EIAV). These include Gag p26 which is
194 presented by the ELA-A5.1 and ELA-A9, and peptides of the Env protein which are ELA-
195 A1 restricted (Zhang *et al.*, 1998). Detailed studies of the interaction between viral
196 epitopes and MHC class I alleles have revealed a remarkable degree of complexity. In
197 horses, there is clearly sub-haplotypic variation (Chung *et al.*, 2003) and this may be a
198 reflection of polymorphism at alleles encoded by classical MHC class I loci. Additionally,
199 horse MHC haplotypes appear to have differing numbers of classical MHC class I genes, a
200 feature not found in humans or mice (Tallmadge *et al.*, 2010). This diversity by genes
201 expressed on classical MHC class I loci is consistent with the host's need to generate
202 immune responses and to retain the capacity for flexibility in defence against attack by
203 pathogens, but complicates vaccine design.
204
205 Despite this diversity of MHC class I loci and alleles, there is also evidence that certain
206 viral proteins behave in an immunodominant fashion and stimulate CTL from horses
207 carrying several different MHC haplotypes. For example, studies of CTL targets in six
208 horses that had been infected with EIAV showed that Gag gene products, which encode
209 matrix and capsid proteins, were consistently recognised by various serological MHC class
210 I haplotypes. However, no identical peptides within these proteins were consistently
211 recognised (Zhang *et al.*, 1998). The elegant work of Mealey *et al.*, (2006) showed that
212 within the ELA-A1 haplotype, a single amino acid difference in the α2 domain between the
213 MHC class I genes 7-6 and 141 resulted in the ineffective presentation of the Gag GW-12

peptide by gene 141. As a consequence, there was a functional alteration in the ability of horses carrying the 141 gene to recognise peptides. Modelling suggested that the mechanism was related to the 114-Gag Gw12 complex not being recognised by the T cell receptor. In the ELA-A3 serological haplotype, two subtypes, A3.1 and A3.2 have been revealed with functional differences, namely only ponies with A3.1 recognised the IE protein as a CTL target (Soboll *et al.*, 2003). These subtle yet important functional differences need further investigation if subunit vaccines are to become practical in an outbred population.

In the current study, the degree of lysis of target cells infected with NYVAC–gene 64 was consistently lower than that of target cells infected with EHV-1 virus. Soboll *et al.* (2003) reported a similar phenomenon using single gene products. In the MHC homozygous horses studied here, the lower CTL activity to the IE protein is probably a reflection of the single target protein presented. The equine CTL response to EHV-1 is undoubtedly complex and will include recognition of peptides derived from different EHV-1 proteins.

In summary, the current data in EHV-1 infected mares with defined MHC class I haplotypes suggests that peptides of the IE protein are presented by an allele(s) of the ELA-A2 serological haplotype, but not ELA-A5. The IE protein therefore acts as a CTL target protein in two MHC class I haplotypes, ELA-A2 and ELA-A3.1, which are common in the Thoroughbred breed, thereby strengthening the argument for considering its inclusion in future novel vaccines.

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Figure Legends

Figure 1. MHC restriction of CTL killing of EHV-1-infected target cells. CTL from 6 MHC homozygous horses (1 ELA-A5 (a), 2 ELA-A2 (b, c), and 3 ELA-A3 (d, e, f) were tested against EHV-1 infected target cells of each of the three MHC haplotypes, plus mock infected autologous control cells. Genetically restricted, virus specific CTL activity was detectable in all mares as demonstrated by lysis of virus infected target cells from mares which shared the same haplotype. Levels of lysis were low in autologous, mock infected and heterologous target cells collected from mares which did not share the same haplotype.

Figure 2. The EHV-1 IE protein is a peptide donor for horses of the ELA-A2 and ELA-A3 haplotypes, but not for ELA-A5. CTL activity in effector lymphocytes tested against NYVAC-gene 64 infected target cells. a) Titration of NYVAC-gene 64 m.o.i. in target cells from a mare (Esther) with A3/x haplotype. b & c) Two ELA-A2 homozygous horses showing CTL effector activity against target cells expressing the IE protein encoded by EHV-1 gene 64. d) An ELA-A5 homozygous horse that showed CTL effector activity against target cells infected with whole EHV-1 virus, but not cells that expressed only the IE protein. Target cells were as follows: inf = EHV-1 infected; m= mock infected; g64 = infected with NYVACC-gene 64 at m.o.i. 2.

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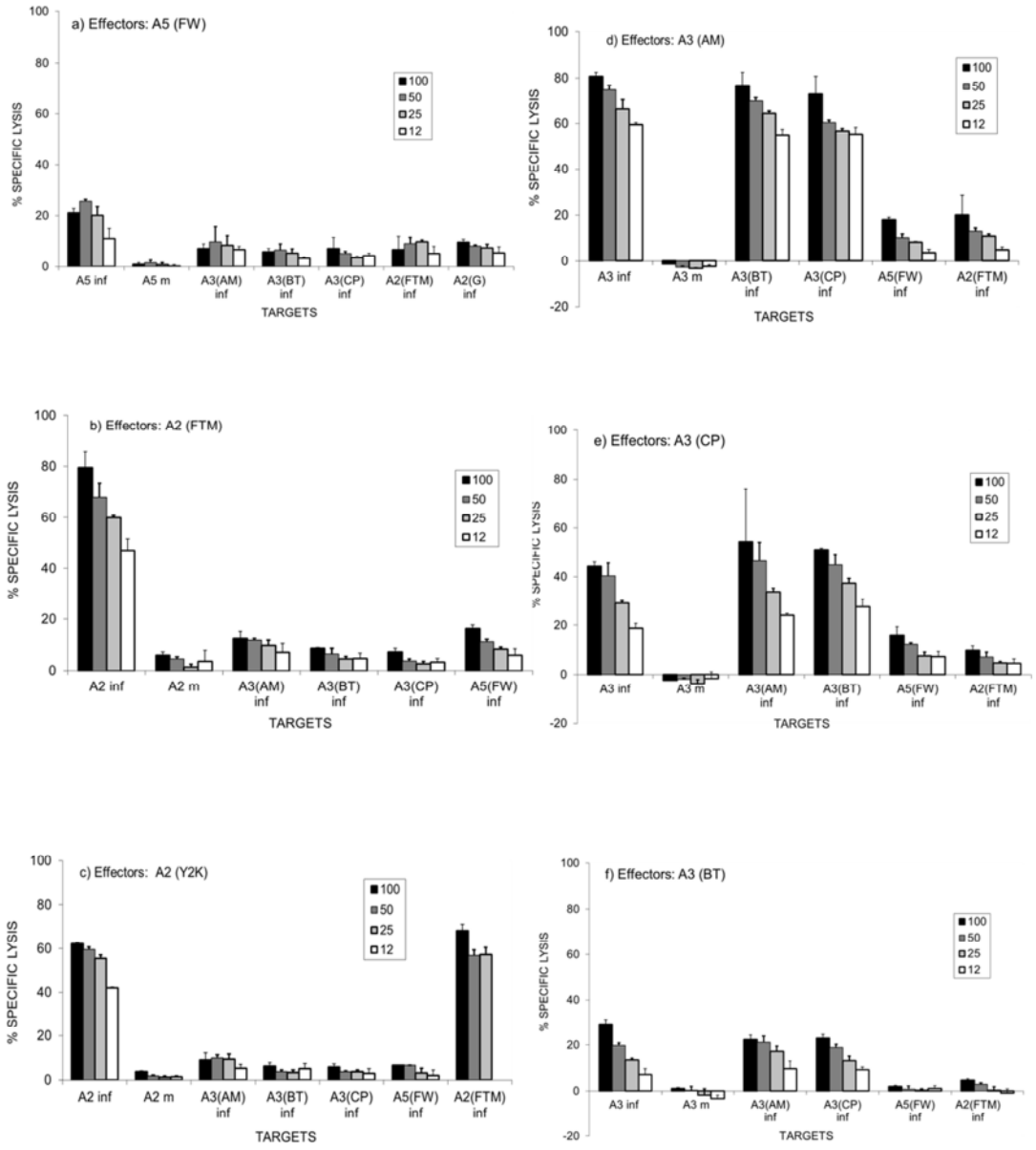
Table 1. Details of horses used as blood donors for CTL assays. ELA = Equine Leukocyte Antigen; CF = Complement Fixing antibody; V= vaccinated; NV = non-vaccinated; n/d = not done.

Horse	Age (years)	ELA serological haplotype*	Vaccination status	**Reciprocal CF antibody titre versus	
				EHV-1	EHV-4
G	13	A2	NV	640	640
Y2K	5	A2	V	320	320
FTM	11	A2	V	80	10
CP	17	A3	NV	80	80
AM	4	A3	V	20	40
BT	12	A3	NV	320	160
FW	14	A5	V	320	160
Esther	13	A3/x	NV	n/d	n/d

*Homozygous unless stated otherwise

**Samples collected on single occasion as part of the outbreak's diagnostic investigation

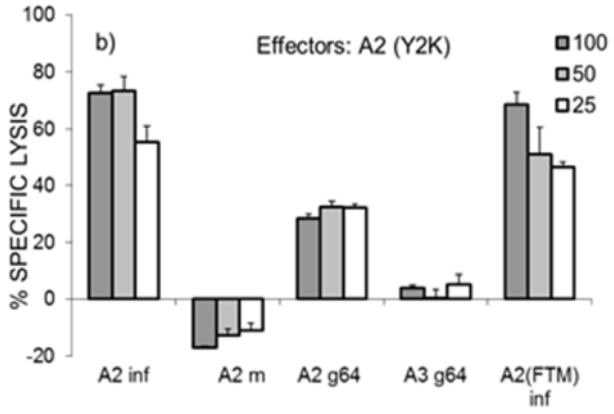
FIGURE 1

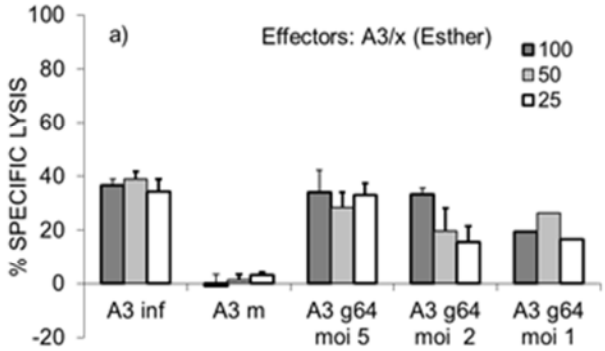


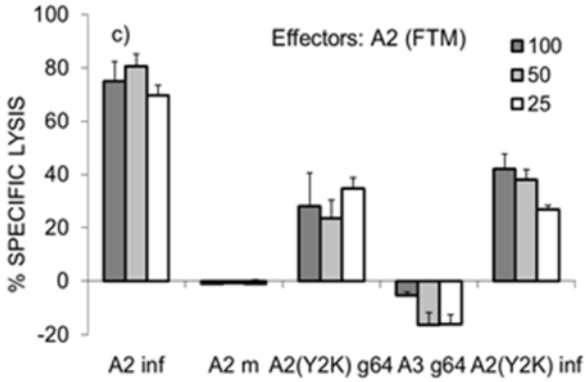
414

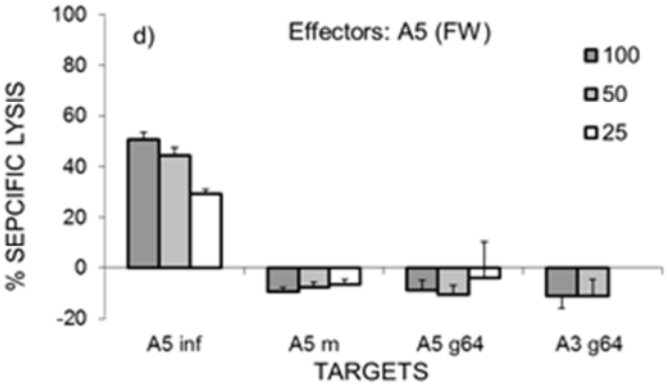
415

FIGURE 2.


b)
Effectors: A2 (Y2K)
100
50
25
% SPECIFIC LYSIS
100
80
60
40
20
0
-20
A2 inf
A2 m
A2 g64
A3 g64
A2(FTM) inf


a)
Effectors: A3/x (Esther)
100
50
25
% SPECIFIC LYSIS
100
80
60
40
20
0
-20
A3 inf
A3 m
A3 g64 moi 5
A3 g64 moi 2
A3 g64 moi 1


c)
Effectors: A2 (FTM)
100
50
25
% SPECIFIC LYSIS
100
80
60
40
20
0
-20
A2 inf
A2 m
A2(Y2K) g64
A3 g64
A2(Y2K) inf


d)
Effectors: A5 (FW)
100
50
25
% SEPCIFIC LYSIS
100
80
60
40
20
0
-20
A5 inf
A5 m
A5 g64
A3 g64
TARGETS