# DIFFERENTIAL CYTOKINE mRNA EXPRESSION INDUCED BY BINDING OF VIRULENT AND AVIRULENT MOLECULARLY CLONED EQUINE INFECTIOUS ANEMIA VIRUSES TO EQUINE MACROPHAGES

A Dissertation

by

WAH-SENG LIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Veterinary Microbiology

# DIFFERENTIAL CYTOKINE mRNA EXPRESSION INDUCED BY BINDING OF VIRULENT AND AVIRULENT MOLECULARLY CLONED EQUINE INFECTIOUS ANEMIA VIRUSES TO EQUINE MACROPHAGES

A Dissertation

by

WAH-SENG LIM

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

| | |
|---|---|
| Judith M. Ball<br>(Chair of Committee) | Ellen W. Collisson<br>(Member) |
| Roger Smith III<br>(Member) | C. Jane Welsh<br>(Member) |
| Ann B. Kier<br>(Head of Department) | |

August 2003

Major Subject: Veterinary Microbiology

# ABSTRACT

Differential Cytokine mRNA Expression Induced by Binding of Virulent and Avirulent Molecularly Cloned Equine Infectious Anemia Viruses to Equine Macrophages.

(August 2003)

Wah-Seng Lim, B.A., University of Kansas

Chair of Advisory Committee: Dr. Judith M. Ball

Equine infectious anemia virus (EIAV) causes rapid development of acute disease followed by recurring episodes of fever, thrombocytopenia and viremia, termed chronic EIA. Most infected horses control the virus by immune mechanisms and become inapparent carriers. To further our understanding of the equine immune response to EIAV, a multi-probe ribonuclease protection assay (RPA) was developed to quantitate equine-specific cytokine mRNAs. Eleven template plasmids specific to ten equine cytokine genes and the β-actin gene were generated, from which radiolabeled anti-sense RNA probes were produced. The RPA simultaneously quantitated mRNA levels of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, interferon (IFN)-γ, transforming growth factor (TGF)-1β and tumor necrosis factor (TNF)-α in equine peripheral blood mononuclear cells and equine monocyte-derived macrophages (EMDM). The assay detected as few as $5\times10^5$ RNA molecules and displayed coefficients of variation of 0.03-0.08 when normalized to β-actin expression. Using this RPA, cytokine expression in EMDM infected with 2 molecularly cloned viruses

(EIAV$_{17}$ and EIAV$_{19}$) was determined. EIAV$_{17}$ varies from EIAV$_{19}$ only in *env*, *rev* and LTR and causes fatal disease in Shetland ponies. When added to EMDM cultures, virulent EIAV$_{17}$ stimulated expression of IL-1α, IL-1β, IL-6, IL-10 and TNF-α. These cytokine mRNAs were significantly elevated by 0.5 to 1 hr post infection (hpi) and returned to basal levels by 12 to 24 hpi, indicating modulation by early event(s), such as receptor binding. In contrast to EIAV$_{17}$, EIAV$_{19}$ is avirulent *in vivo* and failed to induce any of the tested cytokines in EMDM. These data show a direct correlation between the virulence of the EIAV clone and the induction of cytokines. The cytokines stimulated by EIAV$_{17}$ may contribute to EIA-associated symptoms, enhance viral replication in the host, and regulate the host immune response. To determine whether cytokine induction requires EIAV$_{17}$ replication, EMDM cultures were exposed to UV-inactivated EIAV$_{17}$ and cytokine induction was monitored. UV-inactivation did not block cytokine induction by EIAV$_{17}$, suggesting dispensability of viral replication. Given that EIAV$_{17}$ induces cytokines in a rapid and replication-independent manner, the activation of cytokine expression is likely mediated by binding of EIAV$_{17}$ to equine macrophage receptor(s).

This dissertation is dedicated to my beloved family and friends.

## ACKNOWLEDGMENTS

I would like to thank Dr. Judith Ball for serving as my graduate advisor and mentor. I would also like to extend a thank you to the members of my graduate committee: Drs. Ellen Collisson, Roger Smith, and Jane Welsh. In addition, I would like to thank all the members of Dr. Ball's laboratory: Ms. Natalie Boyd, Mrs. Virginia Cox, Ms. Pamela Miller, Dr. DeAnn Mitchell, Dr. Rebecca Parr, Mr. Stephen Storey, Dr. Christina Swaggerty, and Dr. Minglong Zhou.

I am grateful for Dr. Susan Payne for providing the EIAV molecular clones; Drs. John Edwards, Charles Love, and Ronald Martens for providing horse blood; Drs. Noah Cohen and Inyoung Kim for the statistical analyses; Dr. David Horohov (Louisiana State University) for providing the pCREQIL6 and pCREQIL10 plasmids; and Dr. Frederick Fuller (North Carolina State University) for providing the monoclonal antibody I.646.

This work was supported in part by the Texas Equine Research Account administered by Texas Agricultural Experiment Station, United States Department of Agriculture Animal Formula Health Fund, and National Institute of Health.

# TABLE OF CONTENTS

# LIST OF FIGURES

FIGURE Page

# LIST OF TABLES

# CHAPTER I

# INTRODUCTION

## Overview

Equine infectious anemia virus (EIAV) belongs to the lentivirus genus in the *Retroviridae* family. EIAV exclusively infects and replicates in the macrophages of equids, including horses, ponies, donkeys and mules (Ishitani, 1970). The predominant route of EIAV transmission is the interrupted feeding of large arthropods on infected animals (Issel and Foil, 1984). Other transmission routes include transfusion of infected blood and, in rare cases, cross-placental infection of the fetus (Coggins, 1984). EIAV causes acute and chronic disease, but most infected horses ultimately mount an immune response that effectively control viral replication and become inapparent carriers. Although the inapparent carriers are clinically normal, they remain infected for life and are important reservoirs of EIAV in nature (Montelaro et al., 1993). Transmission from inapparent carriers is demonstrated in both experimental and natural conditions (Coggins, 1984).

Since the implementation of mandatory quarantine and surveillance of seropositive horses using an agar gel immunodiffusion (AGID) test (Coggins et al., 1972), spread of EIAV has been controlled in the US. However, all attempts to develop inactivated whole-virus and subunit vaccines have been unsuccessful, and EIAV remains

---

This dissertation follows the style and format of Virology.

an important equid disease with major economic impacts worldwide (Costa et al., 1997; Issel et al., 1992; Wang et al., 1994).

**The Virion**

Electron microscopy reveals that the EIAV virion ranges from round to ellipsoidal in shape and measures 95-125 nm in diameter. A double-layered envelope surrounds a condensed capsid core that is either conical (75-95 nm in length) or tubular (up to125 nm in length) in shape. The outer layer of the envelope is studded with 10-12nm long projections (Weiland et al., 1977; Gonda et al., 1978). The capsid core contains a diploid genome which consists of 2 single-stranded positive RNA held together by base-pairing and a genome-associated $tRNA^{Lys}$ that serves as a primer for the synthesis of the negative strand viral DNA (Cheevers et al., 1977; Charman et al., 1976; Stephens et al., 1986).

**Genomic Organization**

Both ends of the EIAV proviral DNA terminate with the long terminal repeat (LTR) comprised of U3 (unique 3'sequence), R (redundant sequence) and U5, which contain critical transcription regulatory sequences (Fig. 1A) (Maury, 1998; Derse et al., 1987). The 5' LTR is immediately followed by the $tRNA^{Lys}$ primer binding site (PBS) (Derse et al., 1987). As in all lentiviruses, EIAV has *gag*, *pol* and *env* genes that encode the major structural proteins and enzymes (Fig. 1A) (Stephens et al., 1986). In addition, EIAV possesses 3 small open reading frames (ORF), S1, S2 and S3, encoding accessor

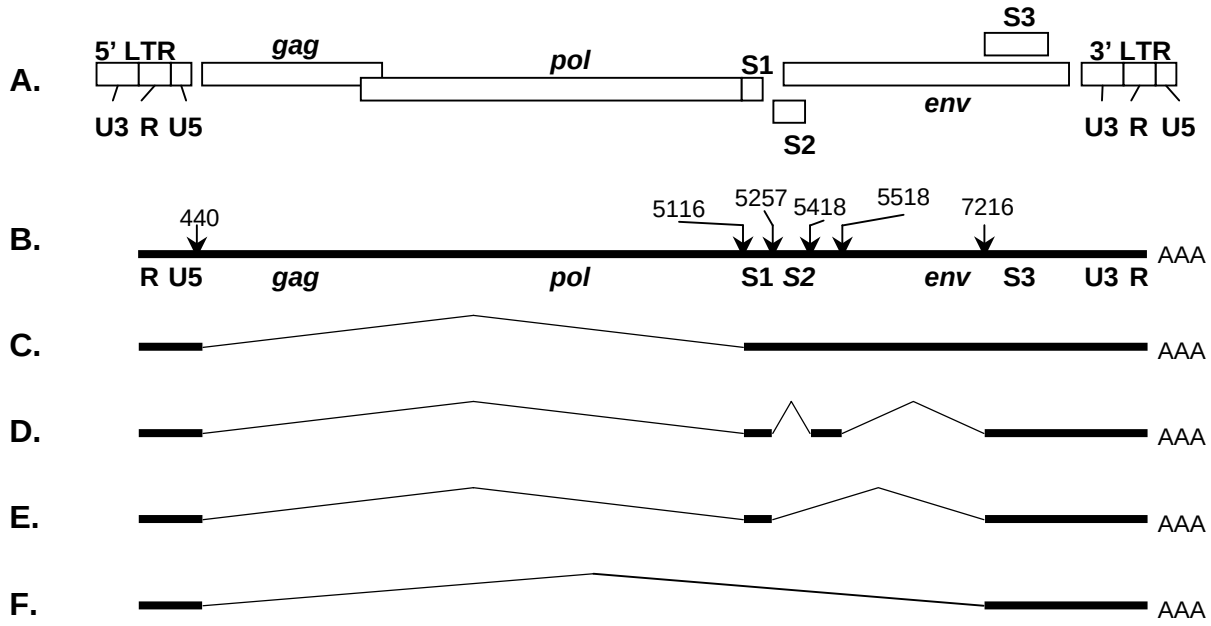


**Fig. 1.** Illustrations of EIAV genomic organization and mRNA transcripts. A schematic of full-length proviral EIAV is illustrated in A, with the long terminal repeats and open reading frames shown as open boxes. The principal mRNA transcripts of EIAV are represented as solid lines in B-F. Genomic positions of the splice sites are indicated by arrows above the solid line in B, numbered according to the previously published EIAV provirus genomic sequence (1,2). Minor and alternative transcripts are not included. Transcript B represents the 8.0kb genomic RNA that encodes Gag and Pol proteins. Transcript C represents the singly spliced polycistronic mRNA that encodes for Env, Tat and S2. Transcripts D and E represent the multiply spliced transcripts that encode Tat and Rev. Transcript F represents the mRNA that encodes the Ttm protein. Illustrations are not drawn to scale.

proteins. S1 is located in the *pol-env* intergenic region. S2 begins in the *pol-env* intergenic region and overlaps with *env* in a different reading frame. S3 is completely contained within *env* as an alternative reading frame (Rushlow et al., 1986). With approximately 8.0 kb, the EIAV genome is the smallest among the lentiviruses because of its short LTR and *pol-env* regions and small number of accessory proteins.

Transcription of EIAV proviral DNA produces three types of mRNAs, unspliced (full-length), singly-spliced and multiply-spliced (Fig. 1B to F). Synthesis of the 8.0-8.2kb full-length mRNA starts at the 5' U3-R junction of the provirus and terminates in the 3' R region (Fig. 1B). This transcript serves as the genomic RNA as well as drives the translation of the Gag and Pol proteins. All spliced EIAV transcripts contain a common leader sequence derived from R, U5 and the region preceding *gag* (Stephens et al., 1990; Maury, 1998). The 3.5-4.0kb singly-spliced transcript is a polycistronic mRNA that encodes for Env, Tat (S1) and S2 (Fig. 1C) (Schiltz et al., 1992). A number of different multiply spliced transcripts have been identified, ranging from 1.2 to 1.5kb in size (Fig. 1D and E). These transcripts encode for the regulatory proteins Tat and Rev (S3) (Stephens et al., 1990; Carroll and Derse, 1993). Beisel et al. reported a small transcript that encodes a fused protein (Ttm) consisting of the Tat leader and the C-terminus of the transmembrane (TM) glycoprotein (Fig. 1F) (Beisel et al., 1993). The function of Ttm is unknown but is likely to contribute to the cytopathic effects of EIAV infection since it contains the putative membrane-destabilizing cytoplasmic TM (Venable et al., 1989). A unique feature of EIAV transcription is that while the multiply spliced mRNAs are readily detectable in liver tissues of EIAV-infected horses and in

EIAV-infected non-equine cell lines, they are rarely detected in infected equine cell lines (Beisel et al., 1993; Rasty et al., 1990; Schiltz et al., 1992; Stephens et al., 1990). Despite the rarity of the multiply spliced mRNAs, Tat activity can be measured in infected equine cell lines (Rasty et al., 1990).

**Gag Proteins**

The *gag* gene encodes structural proteins p15 (matrix, MA), p26 (capsid, CA), p11 (nucleocapsid, NC) and p9. These proteins are arranged as a 55kDa precursor, p15-p26-*-p11-p9, where the asterisk represents a pentapeptide (Henderson et al., 1987). The precursor is cleaved during or after budding of EIAV to yield the 4 individual Gag proteins (Henderson et al., 1987).

MA of retroviruses targets Gag to the plasma membrane during viral assembly and remains associated with the inner membrane leaflet of mature virions to form the outer shell. Membrane binding properties of EIAV MA have been demonstrated by fluorescence and biochemical methods (Provitera et al., 2000). However, EIAV MA lacks the N-terminal myristoylation signal and cluster of positively charged residues that human immunodeficiency virus (HIV)-1 MA utilizes to interact with anionic membranes (Freed, 1998). The recently resolved crystal structure suggests that EIAV MA may interface with membranes through an amphipathic helical structure (Hatanaka et al., 2002). Despite the lack of sequence homology, EIAV MA crystal structure displays overall similarity with those of HIV-1 and simian immunodeficiency virus (SIV) MA. However, EIAV MA forms dimers instead of trimers like HIV-1 and SIV MA (Hatanaka

et al., 2002). In HIV-1, MA also plays important roles in facilitating transport of the viral genome into the nucleus. The nuclear localization signal found in HIV-1 MA is absent in EIAV MA (Hatanaka et al., 2002). However, since nuclear targeting of HIV-1 DNA is dependent on MA serine phosphorylation, the early observation that EIAV MA serine and threonine residues are variably phosphorylated may indicate its involvement in nuclear translocation (Montelaro et al., 1982; Hatanaka et al., 2002).

CA of EIAV forms the core structure that contains the viral genome, nucleocapsid protein and enzymes (Roberts and Oroszlan, 1989). Recombinant EIAV CA forms cubic and hexagonal crystal structures. Crystallography of the protein reveals a novel antiparallel four-helix bundle motif that provides the interface for N-terminal domain dimerization (Jin et al., 1999). Assembly of mature EIAV capsid is believed to depend on this N-terminal interaction in conjunction with the C-terminal domain dimerization analogous to that seen in HIV-1 CA (Jin et al., 1999). It is not clear whether EIAV CA possesses other functions that have been associated with HIV-1 CA such as cyclophilin binding (Freed, 1998). CA is the major protein used in commercial EIA diagnostic tests, including the Coggins' AGID test, because it is abundantly produced and antigenically conserved (Sellon et al., 1994).

The p11 Gag protein is the NC protein which binds to the viral genome to form the ribonucleoprotein complex in the EIAV core (Montelaro et al., 1982). EIAV NC contains two zinc finger motifs, a highly conserved feature among retroviruses (Stephens et al., 1986). In HIV-1 NC, the zinc finger motifs are important for binding RNA, specific encapsidation of full-length genomic RNA and RNA dimerization and stability

(Freed, 1998). Topoisomerase I activity is localized to an 11kDa protein isolated from the EIAV core. This protein is proposed to be the NC protein based on its cross-reactivity with anti-NC antibody, although the identity needs to be confirmed with more definitive evidence such as amino acid sequencing (Matsrafi et al., 1996).

Only EIAV and the primate lentiviruses (HIV, SIV) encode an additional protein following the NC at the C-terminus of Gag (known as p9 in EIAV, p6 in HIV and SIV). EIAV p9 is important for viral budding and indispensable for viral infectivity (Chen et al., 2001; Li et al., 2002; Puffer et al., 1997; Puffer et al., 1998). The budding function of p9 is localized to the L domain of the protein containing a YPDL motif (Puffer et al., 1997). Although the p9 L domain is not absolutely required for viral budding, deletion of the YPDL motif or truncation of 21 or more C terminal amino acids of p9 produces noninfectious EIAV (Chen et al., 2001; Li et al., 2002). The L domain is also shown to interact in vitro with the cellular AP-2 complex, an element of the clathrin-dependent endocytic pathway, which suggests host cell proteins and/or pathways are utilized to perform L domain-mediated functions (Li et al., 2002).

**Pol Proteins**

The *pol* gene overlaps with the *gag* gene by 241 bp. A ribosomal frameshift is required for translation of *pol* gene from the 8.0 kb *gag* - *pol* transcript. The Pol protein is the precursor for 4 viral enzymes: the protease (PR), reverse transcriptase (RT), dUTPase, and integrase (IN) (Montelaro et al., 1993).

PR is a homodimeric protein that is responsible for cleaving the Gag and Pol precursors during the late phases of retroviral replication. EIAV PR cleaves Gag and Pol at specific sites, most of which contain either an aromatic-proline or a hydrophobic-hydrophobic junction (Tozser et al., 1993). EIAV PR also cleaves TM into transmembrane and cytoplasmic proteins, as well as the RNase H domain of the RT to yield the 55kDa RT subunit (Tozser et al., 1993). Interestingly, EIAV PR effectively processes substrates of HIV-1 origin but is resistant to almost all potent inhibitors of HIV-1 PR, thus behaves as a drug-resistant mutant of HIV-1 PR (Powell et al., 1996). This property of EIAV PR has led to interests in biochemical and crystallographic characterizations of the interactions between EIAV PR and inhibitors or substrates, which are potentially useful for design of future anti-AIDS (acquired immune-difficiency syndrome) drugs (Powell et al., 1996).

RT of all retroviruses possess RNA- and DNA-dependent DNA polymerase and ribonuclease (RNase) H activities (Coffin, 1996). The RNA-dependent DNA polymerization (reverse transcription) is initiated by a $tRNA^{Lys}$ base-paired at the PBS of the RNA genome. RNA in the resultant RNA:DNA hybrid is then degraded by the RNase H activity of RT. Finally, the DNA-dependant DNA polymerase function of RT synthesizes the second DNA strand. EIAV RT shares remarkable sequence similarity with other lentiviruses, especially HIV-1 and HIV-2 (Wohrl et al., 1994). EIAV RT is a heterodimer consisting of 66kDa and 51kDa subunits, with the latter lacking the C-terminal RNaseH domain. Studies of recombinant EIAV RT reveal that homodimers of the 51kDa subunit polymerize in an abortive mode which generates short DNA products,

indicating that the RNase H domain is also essential for template processivity of RT (Wohrl et al., 1994). Like other lentiviral RT's, EIAV RT lacks a 3'-5' exonuclease activity (Bakhanashvili and Hizi, 1993). This property of RT leads to high rates of nucleotide misinsertions and efficient mispair extensions during replication of the viral genome and contributes to the high degree of genetic variation seen in EIAV.

dUTPase hydrolyzes dUTP to dUMP, reducing the concentration of dUTP and providing substrate for biosynthesis of dTTP (Dauter et al., 1999). The overall effect is maintaining a low dUTP:dTTP ratio and hence reducing the chances of mis-incorporation of dUTP into DNA. EIAV dUTPase is required for efficient viral replication in the non-dividing equine macrophages which express low levels of cellular dUTPase but is dispensable in permissive cell lines where cellular dUTPase is abundantly expressed (Shao et al., 1997). Crystal structural analyses reveal that the active EIAV dUTPase is a homotrimer (Dauter et al., 1999). The depressions created at the interfaces of the subunits provide the active center and the binding site for a divalent cation that is required for catalysis.

Integration of viral DNA into the host genome is a crucial feature of retrovirus replication. Retrovirus IN cleaves the RT-generated double-strand viral DNA at conserved CA dinucleotides near the 3' end of each strand (Coffin, 1996). The 3'OH created is utilized by IN to attack phosphate bonds of the host genomic DNA during the strand transfer reaction. Purified EIAV IN displays 3' processing and DNA strand-transfer activities and seems to function like other retroviral INs (Engelman, 1996).

EIAV IN bears particular similarity with HIV-1 IN in that they both use water instead of viral DNA 3'OH as the nucleophile in the $Mn^{2+}$-dependent 3' cleavage reaction.

**Env Proteins**

The EIAV Env protein is translated from a singly-spliced, polycistronic mRNA as a 135kDa precursor (Schiltz et al., 1992; Rice et al., 1990). In the synthesis of retrovirus Env proteins, the Env precursor is cotranslationally translocated to the rough endoplasmic reticulum (ER) where the hydrophobic signal peptide is cleaved (Coffin, 1996). However, EIAV Env processing is unique. N-terminal protein sequencing has shown that only 6 residues are cleaved from the EIAV Env N-terminus instead of the predicted 15 amino acid signal peptide (Ball et al., 1988). Retroviral Env precursors undergo oligomerization and glycosylation in the ER (Coffin, 1996). EIAV Env contains 18 potential glycosylation sites, 13 in the SU (surface unit) region and 5 in the TM region (Kawakami et al., 1987). The precursor Env is then transported to the Golgi where it is cleaved by cellular enzymes into SU and TM subunits (Coffin, 1996). Finally, EIAV TM is further cleaved by the viral protease in the virion to yield gp32 and p20 (Rice et al., 1990).

**SU**

Early studies establish that each of the recurring episodes of disease during the chronic phase of EIA is associated with an emergence of new EIAV variants that escape the surveillance of the established immune response (Montelaro et al., 1984) (Fig. 2).

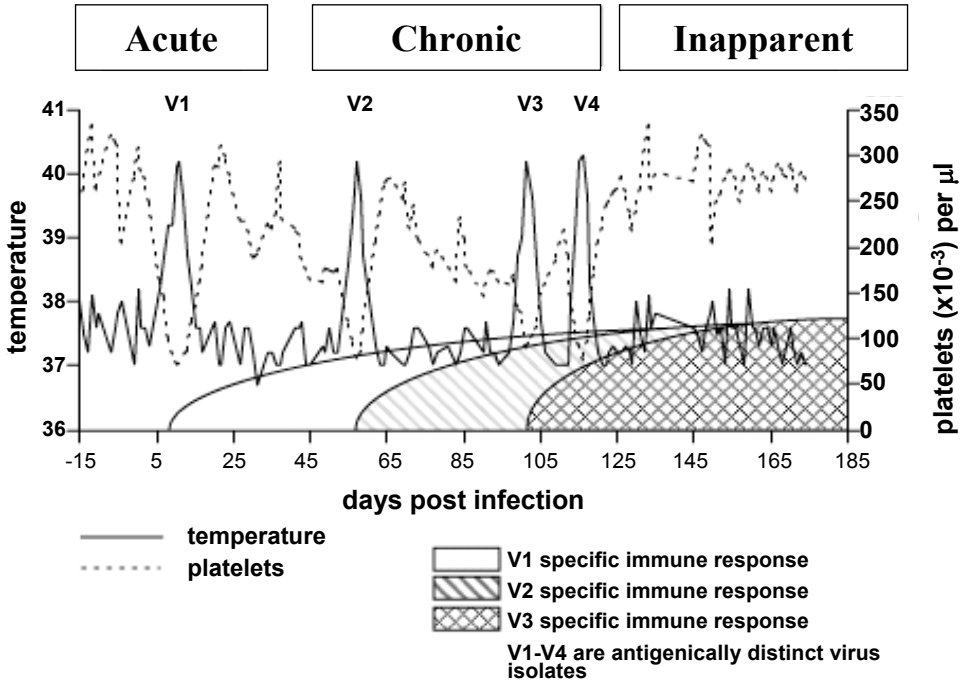


**Fig. 2.** Schematic representation of EIA clinical course and the kinetics of immune response. The chart illustrates the temporal relationship between the recurring episodes of disease, the viremia of antigenically distinct EIAV variants, and the emergence of variant-specific immune response. The x-axis represent days post-infection. The left and right y-axes represent temperatures ($^{o}$C) and platelet counts ($10^3$/ml of whole blood) of infected horses, respectively. (Illustration from S. L. Payne; Issel and Coggins, 1979; Payne et al., 1998)

Immunologic and biochemical assays show that the variations of EIAV are predominantly localized to the Env protein, in particular SU (Salinovich et al., 1986; Montelaro et al., 1984). Since EIAV SU is the major target of neutralizing antibodies, the appearance of escape mutants is attributed to the rapid antigenic alterations within SU that enable new EIAV variants to evade deactivation by pre-existing neutralizing antibodies (O'Rourke et al., 1988; Salinovich et al., 1986; Rwambo et al., 1990). Indeed, the epitopes of all EIAV neutralizing monoclonal antibodies (MAbs) are localized to variable regions of SU (Hussain et al., 1988). Based on nucleotide sequence analyses of EIAV isolates from sequential disease episodes, 2 conserved and 1 variable domains within the EIAV SU are apparent (Payne et al., 1987). The conserved domains are located at the N-terminus ($C_N$, amino acids 1 to 137) and the C-terminus ($C_C$, amino acids 360 to 445). The variable domain (V) lies between $C_N$ and $C_C$ and contains a hypervariable domain ($V_H$, amino acids 300-335). By peptide mapping, Ball et al. localize 3 neutralizing epitopes within the V domain (Ball et al., 1992). A very short segment of the V domain (amino acids 174-207), termed the principal neutralizing domain (PND), contains 2 of the 3 neutralizing epitopes and reacts with 75% of the EIAV-infected horse sera tested. These results reinforce the concept that neutralizing antibodies are directed towards a region of SU that is highly variable. Amino acid alterations within this SU region give rise to new EIAV variants that evade the established humoral immune response and cause recurring disease episodes.

The principal function of retroviral SU proteins is to bind cellular receptors during attachment of the virus to the target cells (Gallaher et al., 1995). Genetic

analyses show that there are substantial differences in the $V_H$ and PND of SU between macrophage-tropic EIAV field strains and EIAV strains adapted to grow in fibroblastic cell lines (Perry et al., 1992; Lichtenstein et al., 1996). However, insertion of SU from a macrophage-tropic EIAV into the cell line-adapted EIAV clone does not restrict the growth of EIAV in cell lines (Perry et al., 1992). These results suggest that $V_H$ and PND are not directly involved in receptor interaction. This is similar to HIV-1 SU, whereby the variable regions do not directly interact with CD4 but form physical "shields" to protect the CD4-binding constant regions from neutralizing antibodies (Coffin, 1996). Such arrangements allow mutations of the neutralizing epitopes in the variable regions without affecting receptor binding. To date, the receptor for EIAV SU has not been identified on the surface of macrophages or fibroblastic cells.

A structural model of EIAV SU has been generated based on computer algorithms and comparison to structures of other lentiviral SU proteins (Ball, 1990; Gallaher et al., 1995). In this model, EIAV SU is an overall globular molecule with randomly distributed positive and negative charges. The proposed model reveals a number of structural features homologous to HIV-1 or other retroviral SU proteins within each region. Functions are assigned to these structures based on the known functions of HIV-1 SU (Ball, 1990; Montelaro et al., 1993; Ball, 1990). The $C_N$ domain contains 3 structural properties that are common among retroviral SU proteins: (1) a predicted amphipathic $\alpha$-helix that is believed to be highly immunogenic; (2) cysteine residues that are potentially involved in disulphide linkages; and (3) a region consisting of strong turn potential followed by N-linked glycosylation sites and an $\alpha$-helix (Ball,

1990). This model predicts that PND is at the most exposed region of the V domain and projects out from the surface of SU (Ball et al., 1992). Based on similarities in antigenic and structural properties, the PND is proposed to be functionally analogous to the V3 loop of HIV-1, which participates in co-receptor utilization and cell tropism. In addition to the PND, the $V_H$ domain is also predicted to protrude from the SU surface since this region contains 2 cysteine residues that may contribute to the formation of a loop structure (Ball, 1990; Ball et al., 1992). The protrusion of the PND and the $V_H$ domain may account for their high antigenicity and variability. In the $C_C$ region, the domain adjacent to the cleavage site is predicted to form a series of amphipathic helices that may facilitate oligomerization of SU (Ball et al., 1992).

**TM**

Like SU, the functions of TM in EIAV replication have not been demonstrated directly. It is assumed that the EIAV TM is functionally homologous to TM of other retroviruses, which mediate the fusion of viral and cellular membranes upon receptor binding to allow release of the viral core into the cytoplasm. A putative structure of EIAV TM has been predicted using computer algorithms and is largely modeled after the known structure of the influenza TM ($HA_2$) (Ball, 1990; Gallaher et al., 1989). As in the model of SU structure, various functions are assigned to the structural features of EIAV TM based on similarities to the HIV-1 TM and the influenza $HA_2$. This model predicts EIAV TM has a fibrous structure with an overall hairpin-like conformation. The N terminus of TM contains a hydrophobic core and is believed to constitute the fusion

peptide, which is inserted into the cellular membrane during membrane fusion. Downstream to the fusion peptide is an extended amphipathic helical structure that gives the protein its fibrous character and facilitates Env oligomerization. The apex of the TM contains 2 vicinal cysteine residues believed to form a disulphide linkage that maintains the hairpin conformation. Near the C terminus, a hydrophobic segment forms the proposed transmembrane domain responsible for anchoring the TM protein to the viral envelope (Ball, 1990; Gallaher et al., 1989). The C terminus of TM is cytoplasmic. The sequence of this region reveals a potential to form membrane-spanning channels that may have cytopathic effects (Venable et al., 1989).

While variations of TM are evident among EIAV isolates, they are more conserved compared to SU (Payne et al., 1987; Leroux et al., 1997). Amino acids are most highly conserved within the proposed fusion peptide and transmembrane domain (Payne et al., 1987). The fusion peptide and the apex of the hairpin structure represent 2 immunodominant regions that react with a high percentage of immune horse sera (Chong et al., 1991). The epitopes within the apex region are highly conserved but do not appear to be neutralizing (Chong et al., 1991; Hussain et al., 1987).

**Accessory Proteins**

The S1 ORF encodes the EIAV Tat protein (Stephens et al., 1990). Translation of Tat utilizes an unusual CTG initiator codon in the leader sequence located in the LTR. Therefore, 29 amino acids derived from the leader sequence are fused to the N-terminus of Tat but are dispensable for Tat activity. Tat activity is required for productive

expression of EIAV proteins from full-length provirus (Carvalho and Derse, 1991; Maury, 1998). Enhancement of EIAV expression from the LTR by Tat is through an interaction with TAR (Tat response element), a stem-loop structure in the nascent transcript formed by the first 24 ntds of the LTR-R. An essential property of EIAV Tat is its ability to bind equine cyclin T1, a component of the positive transcription elongation factor b (P-TEFb), and recruit P-TEFb to TAR (Bieniasz et al., 1999). P-TEFb enhances elongation efficiency of RNA polymerase II and hence promotes viral gene expression. Involvement of additional cellular proteins in Tat activation has been suggested but their identities and roles are unknown (Sune et al., 2000).

The S2 ORF encodes a 7kDa product, termed S2 protein, of undetermined function. S2 seems to be dispensable for EIAV replication in vitro as EIAV that lacks S2 replicates as efficiently as S2-positive EIAV in primary equine macrophages and an equine dermal cell line (Li et al., 1998). In contrast, in vivo data show that EIAV lacking S2 causes less severe thrombocytopenia and displays lower levels of viral replication in ponies compared to S2-positive EIAV (Li et al., 2000). S2 contains distinct functional motifs including a nuclear localization signal, a nucleoporin motif, and an SH3-binding motif (Yoon et al., 2000). The relative genomic location of S2 and its high serine-threonine content spur the concept that S2 is functionally similar to HIV-1 Vpu, an accessory protein that facilitates assembly and release of virions (Li et al., 1998). Supporting this concept, a study demonstrates that, like Vpu, S2 localizes to cytoplasm and associates with subcellular structures (Yoon et al., 2000). In the same

study, S2-Gag precursor interaction is shown by co-immunoprecipitation, which suggests participation of S2 in viral assembly.

EIAV Rev is encoded by at least three different multiply-spliced transcripts (Rosin-Arbesfeld et al., 1993). Two of the transcripts contain 4 exons and generate products consisting of the N-terminus of Env fused to Rev. The third transcript produces a form of Rev that lacks the Env N-terminus. EIAV Rev regulates relative abundance of spliced and unspliced viral RNA in the cytoplasm via a pathway homologous to that of HIV-1 Rev (Belshan et al., 2000). Rev binds viral RNA at the Rev response element (RRE) and shuttles full-length and singly spliced viral RNA out of the nucleus through interactions with the cellular nuclear export protein CRM1 (chromosomal region maintenance) (Otero et al., 1998). In addition to mediating RNA export, EIAV Rev also participates in regulating alternative splicing. Binding of Rev to an exon splicing enhancer (ESE) motif in its own transcript prevents the inclusion of one of the exons in the multiply-spliced mRNA (Belshan et al., 2000).

**EIAV Cell Tropism**

Macrophages in spleen, liver, lymph node, kidney, lung and adrenal gland have been identified as the predominant sites of active EIAV replication based on the analyses of tissues from acutely infected EIA horses by *in situ* hybridization specific for EIAV *gag* (Sellon et al., 1992). Although EIAV DNA is detectable in PBMC of these horses, viral RNA is absent in these cells, suggesting that undifferentiated monocytes are susceptible to EIAV infection but do not support productive viral replication (Sellon et

al., 1992). Tissue macrophages also serve as cellular reservoirs of EIAV during subclinical infection (Sellon et al., 1992; Oaks et al., 1998). In addition, spleen and bone marrow macrophages are shown to be sources of restricted viral transcription in subclinically infected horses, which may be important for viral persistence (Sellon et al., 1992; Oaks et al., 1998). Endothelial cells are the only other cell type that may support productive EIAV replication in acutely infected horses (Oaks et al., 1999). EIAV is also detected in endothelial cell culture isolated from a long term EIAV carrier (Maury et al., 1998).

One of the unique features of EIAV cell tropism is its linkage to the in vivo virulence of the virus. In cell culture, virulent strains or field isolates of EIAV replicate only in equine macrophages and are very restricted for replication in cell lines (Carpenter and Chesebro, 1989). EIAV can be adapted to growth in equine kidney cells or equine, canine or feline fibroblast cell lines through repeated passages (Maury, 1998). However, the cell line-adapted strains are avirulent in vivo (Carpenter and Chesebro, 1989; Maury, 1998). Recently, a canine macrophage-like cell line has been shown to support replication of both macrophage- and fibroblast-tropic EIAV strain (Hines and Maury, 2001). However, this cell line fails to support the growth of the highly virulent Wyoming wild type EIAV ($EIAV_{Wyo}$).

Despite the importance of SU in cell tropism of many retroviruses, it does not seem to be a determinant of EIAV tropism (Perry et al., 1992). Substitution of SU sequences from a fibroblast-tropic proviral clone of EIAV with those derived from the $EIAV_{Wyo}$, which is unable to replicate in fibroblasts, does not affect cell tropism. On the

other hand, the enhancer region (ntds -111 to –31) contained within the U3 domain of EIAV LTR appears to play major roles in EIAV cell tropism. The enhancer region is highly variable and contains binding sites for cellular transcription factors (Maury, 1998). LTR transcription in macrophages and fibroblastic cell lines involves distinct repertoires of transcription factors. In fibroblast, LTR transcription is highly sensitive to point mutations in the PEA-1, octamer, and CRE binding motifs. In primary equine macrophages, however, the *ets* binding sites are critical for LTR transcription and are bound by the myeloid/B cell-specific transcription factor PU.1. Furthermore, changing one of the GTTCC *ets* motifs within the $EIAV_{Wyo}$-derived LTR to a CTTCC *ets* (single ntd substitution), as commonly found in the LTR of fibroblast-adapted EIAV, dramatically increases its transcription in fibroblasts (Payne et al., 1999).

The relationship between in vitro cell tropism and in vivo virulence of EIAV is still not understood. Although the alteration in the transcription factor motifs may affect viral replication and hence the virulence of EIAV in vivo, it has been shown that the sequence of virulent EIAV 3' LTR alone does not impart virulence (Lichtenstein et al., 1996; Cook et al., 1998). EIAV virulence appears to involve combinations of multiple determinants as discussed below.

**Lentiviral Infections and Macrophage Cytokines**

Aside from EIAV, macrophages are also the major targets of infection for all lentiviruses including caprine arthritis encephalitis virus (CAEV), feline immunodeficiency virus (FIV), maedi-visna virus (MVV), HIV and SIV (Gendelman et

al., 1986; Narayan et al., 1983; Meltzer et al., 1990; English et al., 1993; Mori et al., 1992; Onuma et al., 1992). However, with the exception of HIV, studies of cytokines associated with lentiviruses are scant, probably due to the lack of commercially available cytokine reagents for these animal species (Bornemann et al., 1997). Table 1 summarizes the results of select published data that show alteration of macrophage cytokines associated with infection by lentiviruses. Together, these results suggest that dysregulation of cytokine expression is a common outcome of lentiviral infection of macrophages.

To date, there are only 5 studies that report analysis of cytokine activities associated with EIAV infection. Costa et al. report detection of tumor necrosis factor (TNF)-α in plasma of EIAV-infected ponies and shows a positive correlation between plasma TNF-α concentration, severity of symptoms and strain virulence (Costa et al., 1997). TNF-α is again shown to be elevated along with transforming growth factor (TGF)-β and interferon (IFN)-α in serum of ponies infected with $EIAV_{WSU5}$ (Tornquist et al., 1997). The same study detects increased levels of TNF-α, TGF-β and IFN-α in bone marrow biopsies of infected animals. Sera from ponies experimentally infected with $EIAV_{Wyo}$ show increased interleukin (IL)-6 activity, which positively correlates with EIAV replication (Sellon et al., 1999). In the only study that specifically measures macrophage-secreted cytokines, infection of bone marrow-derived macrophages (BMDM) with $EIAV_{WSU5}$ in vitro increases IL-6 but not TNF-α activity in the culture supernatant (Swardson et al., 1997). A recent study investigated the expression of IFN-γ and IL-4 in PBMC of EIAV carrier horses (Fraser et al., 2002). This study shows that

**Table 1**

Dysregulation of macrophage cytokines and chemokines by lentiviruses

| | HIV-1 | | SIV | | FIV | | MVV | | CAEV | | EIAV | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Effects[a] | References | Effects | References | Effects | References | Effects | References | Effects | References | Effects | References |
| GM-CSF | ↑ | Bornemann et al., 1996 | | | | | ↔ | Ebrahimi et al., 2000 | ↑* | | | |
| IFN-α | ↑ | Bornemann et al., 1996 | | | | | | | | | | |
| IFN-γ | ↑↔ | Cicala et al., 2002; Bornemann et al., 1996 | | | ↑ | Ritchey et al., 2001 | | | | | | |
| IL-1β | ↑ | Cicala et al., 2002 | ↑↔* | Sopper et al., 1996; Horvath, et al., 1991 | | | ↔ | Ebrahimi et al., 2000 | ↓* | Lechner et al., 1997 | | |
| IL-4 | ↓ | Bornemann et al., 1996 | | | | | | | | | | |
| IL-6 | ↑ | Bornemann et al., 1996 | ↑↔* | Sopper et al., 1996; Horvath et al., 1991 | ↑ | Ritchey et al., 2001 | ↑ | Ebrahimi et al., 2000 | ↓* | Lechner et al., 1997 | ↑ | Swardson et al., 1997 |
| IL-8 | ↑ | Roux et al., 2000 | ↑↔* | Sopper et al., 1996; Horvath et al., 1991 | | | ↑ | Legastelois et al., 1998 | ↑ | Lechner et al., 1997 | | |
| IL-10 | ↑↓ | Bornemann et al., 1996 | | | ↑ | Ritchey et al., 2001 | ↔ | Ebrahimi et al., 2000 | | | | |
| IL-12 | ↑↓ | Bornemann et al., 1996; Fantuzzi et al., 1996 | | | | | ↔ | Ebrahimi et al., 2000 | ↓* | Lechner et al., 1997 | | |
| IP-10 | ↑ | Cicala et al., 2002 | | | | | | | | | | |
| MIP-1α | ↑ | Choe et al., 2001 | | | | | | | ↑ | Lechner et al., 1997 | | |
| MIP-1β | ↑ | Choe et al., 2001 | | | | | | | | | | |
| NAP2 | ↑ | Cicala et al., 2002 | | | | | | | | | | |
| RANTES | ↑ | Choe et al., 2001 | | | | | | | | | | |
| TGF-β | ↑ | Bornemann et al., 1996 | | | | | ↔ | Ebrahimi et al., 2000 | | | | |
| TNF-α | ↑ | Cicala et al., 2002 | ↑↔* | Sopper et al., 1996; Horvath et al., 1991 | ↑ | Ritchey et al., 2001 | ↑ | Ebrahimi et al., 2000 | ↓* | Lechner et al., 1997 | ↔ | Swardson et al., 1997 |

[a] ↑, increase; ↓, decrease; ↔, no change.

* Viral infection affects LPS-stimulated cytokine expression.

peptides corresponding to Gag epitopes selectively stimulate IFN-γ, whereas the peptide corresponding to a Pol epitope stimulates both IFN-γ and IL-4.

In most lentivirus cytokine studies, in vivo and/or in vitro infection of macrophages result in alterations of the constitutive cytokines levels. For example, both in vitro and in vivo FIV infection causes an increase in the constitutive expression of TNF-α and IL-6 in cat alveolar macrophages (Ritchey et al., 2001). Similarly, increase of IL-8 mRNA expression is detected in sheep alveolar macrophages infected with MVV in vitro and isolated from naturally infected sheep (Legastelois et al., 1997; Legastelois et al., 1998).

Alternatively, lentiviral infection affects the capability of macrophages to produce cytokines upon exposure to exogenous stimulation. Infection of caprine macrophages with CAEV, for instance, does not affect the constitutive expression of IL12 p40 and IL-6 but rather suppresses the lipopolysaccharide (LPS)-stimulated expression of these genes (Lechner et al., 1997). In contrast, microglia (resident tissue macrophages of the brain) isolated from SIV-infected Rhesus monkey display a tendency to produce higher levels of inflammatory cytokines when stimulated with LPS (Sopper et al., 1996).

Discrepant results are often reported in the studies of cytokine alteration in lentivirus-infected macrophages. Effects of a given lentivirus on a cytokine may range from upregulation, no change, to inhibition (Table 1). The factors that contribute to these variations include, among others, origins of macrophages, in vivo versus in vitro infection of macrophages, and timing and methodology of cytokine analysis. For

example, increase in constitutive TNF-α production is detected in microglia cells of SIVmac251-infected monkeys but is absent in peripheral and alveolar macrophages isolated from animals infected with the same strain of SIV (Sopper et al., 1996; Horvath et al., 1991). In another example, macrophages exposed to HIV-1 SU (gp120) produce IL-12 when primed with IFN-γ. However, IL-12 production is impaired in macrophages of HIV-1-infected patients (Fantuzzi et al., 1996). These seemingly paradoxical findings are potentially explained by the ability of HIV-1 to induce IL-10 production, which downregulates IL-12 production by macrophages (Fantuzzi et al., 1996). These results emphasize the complexity of cytokine regulation and the necessity to consider the clinical significance of lentivirus-induced cytokine dysregulation in appropriate physiological contexts.

With the exception of HIV-1, the molecular mechanisms by which lentivirus regulate expression of cytokines in macrophages are largely unknown. HIV-1 gp120 induces cytokines such as IL-1β, IL-6, IL-10, IFN-γ, TNF-α and a number of β chemokines by binding to the CD4 molecules on the surface of macrophages (Chirmule and Pahwa, 1996; Choe et al., 2001). In addition to serving as a receptor for virus entry, CD4 is shown to trigger signal transduction pathways that modulate expression of over 300 genes in monocyte-derived macrophages upon binding to gp120 (Cicala et al., 2002). It is believed some of these signaling events result in activation of transcription factors such as NFAT which are closely associated with regulation of cytokine expression (Cicala et al., 2002). In addition to gp120, exposure of macrophages to HIV-1 Vpr protein also results in upregulation of IL-8, presumably through the activation of

transcription factors NF-IL-6 and NF-kB (Roux et al., 2000). Finally, the HIV-1 Tat protein, which directly participates in transactivation of gene transcription, is shown to enhance the transcription of TGF-β1, TNF-α and IL-6 (Zauli et al., 1992; Lechner et al., 1997). This mechanism may also be employed by CAEV to regulate cytokine expression in macrophages (Lechner et al., 1997). Given the similarity between lentiviruses, these mechanisms may serve as paradigms for understanding mechanisms involved in cytokine induction by EIAV.

**Roles of Macrophage Cytokines in Lentivirus Pathogenesis**

Understanding the alteration of macrophage cytokines associated with lentiviral infection is important as cytokines play central roles in lentiviral pathogenesis. Cytokines produced by macrophages affect disease outcome by directly causing symptoms, regulating lentivirus replication, and modulating both innate and acquired immune responses to lentiviral infection. Unsurprisingly, the pathogenic roles of cytokines have been most comprehensively explored in HIV-1 infection, which will serve as a model for the following discussions.

The direct pathological roles of macrophage cytokines are well-established in the development of neurological disorders commonly associated with late stages of HIV infection (Xiong et al., 1999). Inflammatory cytokines including IL-1 and TNF-α are produced by HIV-1-infected brain macrophages and microglia. These cytokines contribute to degeneration of neuronal functions by both their inherent neurotoxic properties and their ability to induce synthesis of other neurotoxins such as oxygen free

radicals and prostaglandins (Tan and Guiloff, 1998). Many of the macrophage-secreted cytokines and chemokines, such as TGF-β, TNF-α, IL-6, and macrophage inflammatory protein (MIP)-1α, are known as important regulators of the hematopoietic process (Croker and Milon, 1992). Hence, production of macrophage cytokines by HIV-1-infection is believed to have major impacts on development of HIV-1-associated hematological disorders such as anemia and thrombocytopenia (Zauli et al., 1992; Means, , 1997). T cell depletion associated with HIV-1 infection is due to both the death of infected T cells and increase apoptosis of uninfected T cells (Badley et al., 1997). Apoptosis of T cells from HIV-infected individuals is induced by TNF-α in vitro (Badley et al., 1997).

The feature of cytokines in relation to HIV-1 that has attracted the most attention is their ability to regulate HIV-1 replication. Macrophages are a major source of many cytokines that enhance HIV expression in infected cells, including IL-1α, IL-1β, IL-6, IL-12, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (Fauci, 1996). Among these cytokines, TNF-α is the most potent inducer of HIV-1 expression. TNF-α activates the transcription factor NF-kB, which drives HIV-1 transcription by binding to the NF-κB binding site in the LTR of the HIV-1 genome (Fauci, 1996). The molecular mechanisms by which other cytokines enhance HIV-1 replication remain to be elucidated. IL-1α/β appear to increase transcription of HIV-1 without activating the NF-kB pathway, while IL-6 functions at post-transcriptional levels (Vicenzi et al., 1997). In addition to the HIV-1-inducing cytokines, a number of β-chemokines produced by macrophages,

including MIP-1α, MIP-1β and RANTES, suppress HIV-1 replication in T cells (Vicenzi et al., 1997). Since these chemokines are the natural ligands of CCR5 receptors, which serve as co-receptors for the macrophage-tropic HIV-1, they downregulate HIV-1 replication largely by blocking viral entry (Fauci, 1996). A third group of macrophage cytokines exert both inductive and suppressive effects on HIV-1 replication. These cytokines include IL-10, IFN-γ and TGF-β and are shown to affect multiple stages of the HIV-1 life cycle (Vicenzi et al., 1997). The molecular mechanisms of their conflicting roles are unclear.

It is hypothesized that HIV disease progression is due to an imbalanced T helper 1/T helper 2 (Th1/Th2) response (Poli et al., 1995). According to this hypothesis, Th1 repsonse facilitate a protective cytotoxic T lymphocyte (CTL) response that is effective at clearing infected cells early in HIV-1 infection. Over time, decreased production of IL-12 and increased production of IL-10 by macrophages, along with alteration of other Th1- and Th2-associated cytokines, suppress the Th1 response and result in a "shift" towards the Th2 response, which is unable to control the established viral infection (Poli et al., 1995). This hypothesis has not been proven and is both supported and contradicted by results published by different investigators. However, the Th1/Th2 paradigm may be a valuable model to utilize when evaluating the immunologic significance of the various cytokines perturbed by other lentiviruses.

**Clinical Signs of EIAV and Potential Roles of Cytokines in Pathogenesis**

The clinical syndromes experienced by EIAV-infected horses are artificially divided into 3 stages: acute, chronic and inapparent (Fig. 2) (Sellon et al., 1994; Issel and Coggins, 1979). However, these distinctions are not always clear as the clinical course of EIA is variable and depends on viral strains, environment and the horse's immune status. Acute EIA is associated with the initial exposure to the virus. Viremia and clinical signs including fever, thrombocytopenia, hemorrhages, and anorexia become evident in 7-30 days post-infection. Horses in the acute stage are usually seronegative as the immune system has not had enough time to produce antibodies. Only rarely do horses die during the acute stage. Most horses become afebrile for a period of time and then progress to chronic EIA, where the classic EIA symptoms are observed. Chronic EIA is characterized by recurring episodes of fever, thrombocytopenia, anemia, anorexia, hemorrhages, viremia and diarrhea at varying intervals. Most episodes occur within 30 days to one year post-infection, after which about 90% of infected horses become clinically normal and inapparent carriers. In nature most EIA-positive horses detected by the AGID test are inapparent carriers. They are important reservoirs of infection, and recrudescent EIA can be induced in these horses by immune-suppressive drugs or stress. The most marked pathological features of a horse necropsied during a febrile episode include edema, thrombosis, splenomegaly, hepatomegaly, lymphodenopathy, infiltration of lymphocytes and macrophages in many organs and mucosal and visceral hemorrhages (Sellon et al., 1994; Issel and Coggins, 1979).

The pathogenic mechanisms for the EIA symptoms are largely unknown. Because of the robust antibody response to EIAV infection, many of the symptoms are attributed to the detrimental effects of immune complexes. For example, anemia is believed to be mediated by complement-mediated hemolysis and phagocytosis of erythrocytes coated with EIAV antigens or antigen-antibody complexes (Sentsui and Kono, 1987a; Chance et al., 1992). Detection of increased platelet-bound immunoglobulin on platelets of thrombocytopenic horses also supports the hypothesis that deposition of EIAV-specific immune complexes on platelets promotes removal of circulating platelets by phagocytes of the spleen and liver (Clabough et al., 1991). However, the investigators are unable to demonstrate that the platelet-associated immunoglobulins are EIAV-specific (Clabough et al., 1991). Moreover, thrombocytopenia occurs during acute-stage EIA when virus-specific antibody is not yet produced (Issel and Coggins, 1979). EIAV-specific antibodies are also implicated in the glomerulitis and neurologic disorders associated with EIA (Sellon et al., 1994; Montelaro et al., 1993). The conclusion that EIAV-specific antibodies are at best only partially responsible for inducing the symptoms of EIA is made evident by the findings that severe combined immunodeficient (SCID) horses develop fever, anemia and thrombocytopenia as severely as immunocompetent horses when experimentally infected with virulent strains of EIAV (Crawford et al., 1996; Tornquist et al., 1997). SCID horses lack functional T- and B-lymphocytes and are unable to mount antibody and CTL responses. However, these horses have normal macrophage functions.

Accumulating evidence shows that production of cytokines is dysregulated in EIAV-infected horses and might play important roles in EIAV pathogenesis. Biological assays utilizing cytokine-sensitive cell lines reveal increased serum levels of TGF-β, TNF-α and IFN-α in SCID and immunocompetent Arabian foals infected with EIAV (Tornquist et al., 1997). Furthermore, the plasma of EIAV-infected horses collected prior to or at the onset of thrombocytopenia suppresses proliferation of megakaryocyte colonies in culture, and this suppression was partly reversed by antibody neutralization of TGF-β and TNF-α (Tornquist and Crawford, 1997). These results are supported by an earlier study that indicates in vivo suppression of platelet production in EIAV-infected SCID horses (Crawford et al., 1996). Platelets of thrombocytopenic EIAV-infected ponies also show signs of in vivo activation, which could result in aggregation, lysis, or "exhaustion" of platelets, causing accelerated platelet removal from the circulation (Russell et al., 1999). A number of cytokines activate platelets or induce secretion of platelet-stimulating molecules (Oleksowicz et al., 1994; Oleksowicz et al., 1994; Tacchini-Cottier et al., 1998). Taken together these reports suggest that EIAV-associated thrombocytopenia is caused by elevation of cytokines that suppress megakaryocytopoiesis and/or shorten the life span of circulating platelets.

Occurrence of anemia in EIAV-infected SCID horses indicates that this symptom cannot be entirely attributed to immune-mediated destruction of peripheral erythrocytes. Swardson, et al., show that infection of bone marrow mononuclear cells with EIAV in vitro selectively suppresses the proliferation of burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E) cells (Swardson et al., 1997). Since EIAV does not establish a productive infection in erythroid progenitor cells, it is likely that infected macrophages secrete inhibitors of erythropoiesis. IL-1 and TNF-α suppresses

erythropoiesis by downregulating erythropoietin production, impairing response of erythroid progenitors to erythropoietin, and dysregulating iron metabolism (Means, 1997; Means, 2000). Early literature reports reduced serum iron concentration, plasma iron turnover and iron utilization during acute EIA, suggesting suppression of erythropoiesis through a mechanism that is consistent with the activities of IL-1 and TNF-α (Swardson et al., 1997).

In examining the disease enhancement effects of a p26 vaccine, Costa et al. noted a positive correlation between plasma TNF-α of EIAV-infected ponies and disease severity (Costa et al., 1997). In this study, ponies infected with the virulent PV strain of EIAV ($EIAV_{PV}$) develop acute fever, anemia and thrombocytopenia and present higher levels of TNF-α than those inoculated with the avirulent PR strain ($EIAV_{PR}$), which do not develop clinical symptoms. Vaccination enhances the severity of symptoms in both $EIAV_{PR}$- and $EIAV_{PV}$-infected ponies, as well as increases plasma TNF-α activity. These results advance the concept that EIAV infection causes the production of cytokines that contribute to the development of EIAV-associated diseases.

**Virulent EIAV Molecular Clones**

The exclusive tropism of virulent EIAV strains for equine macrophages and the difficulty of culturing primary macrophage have driven numerous efforts to derive virulent viruses that can be propagated in various cell lines. Back-passaging the cell line-adapted avirulent $EIAV_{PR}$ in horses successfully generated the virulent virus stock $EIAV_{PV}$, which retains the ability to grow in cell cultures (Rwambo et al., 1990; Issel et al., 1992). However, genetically defined virulent molecular clones are needed to

manipulate and study EIAV at the molecular level. Initially, molecular clones were directly obtained from the $EIAV_{PV}$ isolates by polymerase chain reaction (PCR) (Payne et al., 1994). Viruses derived from these clones replicate to high titer in cell lines and primary equine macrophages but fail to cause disease in horses. Finally, virulent EIAV molecular clones are successfully generated using chimeric techniques (Payne et al., 1998; Cook et al., 1998). Cook et al. produced infectious molecular clones by inserting a 3' fragment that was PCR-amplified from $EIAV_{PV}$-infected cells into a modified version of the avirulent clone pSPeiav19 (Cook et al., 1998). Virus derived from one of these clones, $EIAV_{UK}$, induces disease with varying severity in horses. This virus has identical 3' LTR, *env*, S3 (with a single amino acid change), S2 and S1 ORFs as the consensus $EIAV_{PV}$ sequence.

In the generation of virulent chimeric molecular clones p19/wenv17 and p19/Wenv16, Payne et al. also utilize pSPeiav19 (Payne et al., 1998). In contrast to the construction of $EIAV_{UK}$, only the LTRs and the 3' end of *env* (excluding the N-terminal 89 amino acids) are replaced, and the inserted sequences are derived from $EIAV_{Wyo}$-infected macrophages (Fig. 3A). Viruses derived from these clones ($EIAV_{17}$ and $EIAV_{16}$) replicate to high titer in equine macrophages and cause EIA in infected Shetland ponies. The symptoms induced by $EIAV_{17}$ are particularly severe. Sequence analyses show that differences in $EIAV_{17}$ and $EIAV_{16}$ from the parental pSPeiav19 are most striking in the SU region (Fig. 3B). These differences concentrate in the principal neutralizing domain and the hypervariable region (Ball et al., 1992). The TM region of *env* is more conserved with only scattered amino acid changes. As the TM region

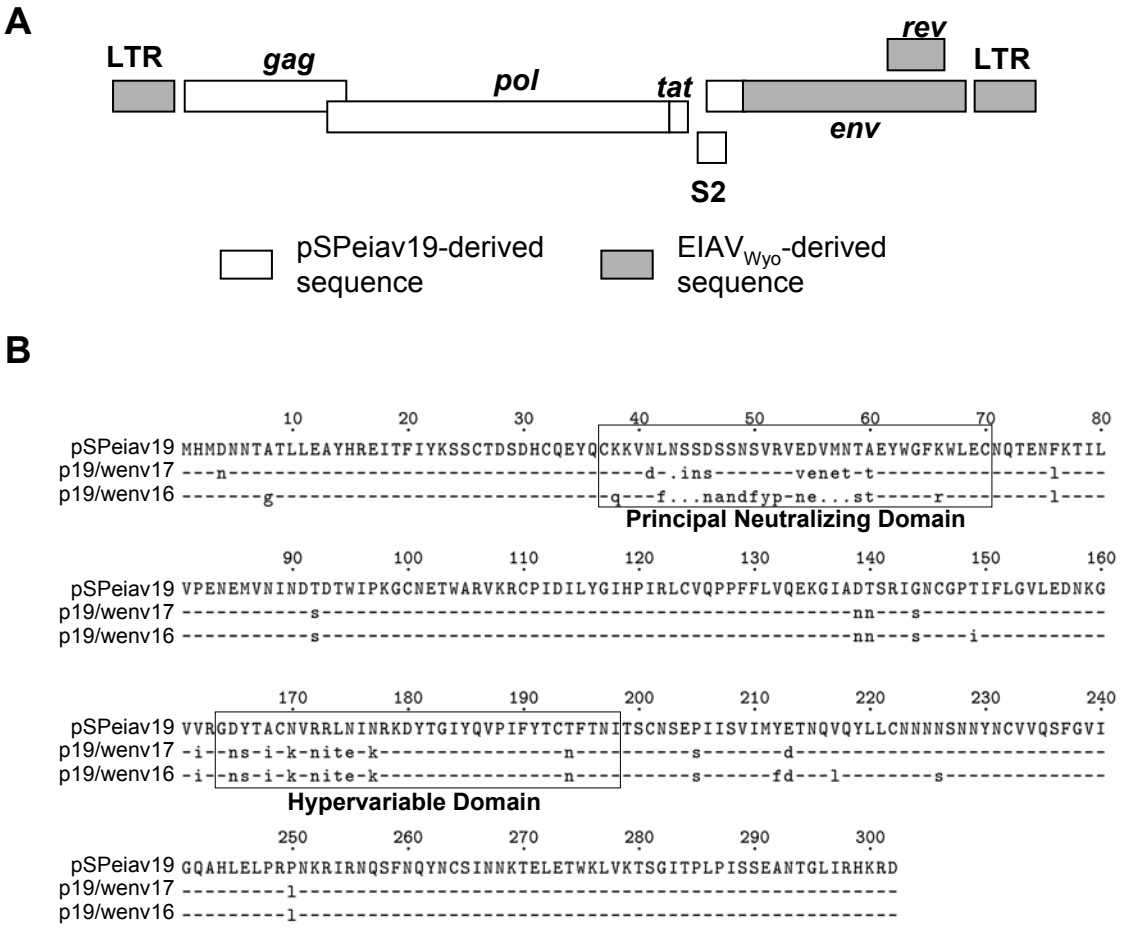


**Fig. 3.** Genomic organization and sequence comparisons of EIAV molecular clones. (A) The schematic diagram indicates the origins of sequences used to construct the different regions of the chimeric virulent molecular clones p19/wenv17 and p19/wenv16. (B) SU amino acid (aa) sequences of the avirulent pSPeiav19 are compared to the virulent p19/wenv17 and p19/wenv16. Sequences shown start at the 144$^{th}$ aa of the SU as the sequences upstream are identical among the clones. Sequences corresponding to the principal neutralizing and hypervariable domains are boxed. Dashes indicate identical aa. Dots indicate aa deletions. (C) The transcription enhancer regions within the 5' U3 of EIAV$_{Wyo}$ and EIAV molecular clones are compared. Transcription factor binding sites are indicated by boxes. The single nucleotide whereby EIAV$_{Wyo}$ and the virulent molecular clones differ is highlighted in grey.

encompasses the second exon of the *rev* gene, substitution with Wyoming *env* also changes the amino acids in the Rev protein. Notably, 1 and 3 amino acid substitutions occur in the Rev effector domain of p19/wenv17 and p19/wenv16, respectively. Due to the cloning strategy, the U3-LTR of p19/wenv17 and p19/wenv16 are identical to the U3 consensus sequence of Wyoming LTR (containing 3 *ets*, 1 CREB and no PEA2 sites) with a single G to C nucleotide substitution at the TATA-proximal *ets* binding site (Fig. 3C). In comparison, the U3-LTR of pSPeiav19 is typical of avirulent EIAV and contains 2 *ets*, 2 CREB and 2 PEA2 sites.

# CHAPTER II

## SIMULTANEOUS QUANTITATION OF EQUINE CYTOKINE mRNAS USING A MULTI-PROBE RIBONUCLEASE PROTECTION ASSAY*

### Introduction

Cytokine functions and interactions have primarily been elucidated in the rodent and human systems, which have provided useful paradigms for understanding other animal cytokines. In contrast, characterization of equine cytokines is at a rudimentary stage. The scarcity of commercially available equine reagents has hindered the study of equine immunology resulting in a limited number of reports of a few equine cytokine responses.

Cell line-based bioassay adaptations have been used to measure equine IL-2, IL-6, IFN, TNF-α, and TGF-β1 (MacKay et al., 1991; Morris et al., 1992; Allen et al., 1993; Ellis et al., 1995; Charan et al., 1997), and antibodies specific to human or other animal cytokines were utilized to analyze equine IL-1β, IL-6, TGF-β, and TNF-α levels (Billinghurst et al., 1995; Charan et al., 1997; Rodriguez et al., 1996). Recently, reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription-competitive polymerase chain reaction (RT-cPCR) and Southern blot techniques were developed to measure equine cytokine mRNA levels (Grunig and Antczak, 1995; Rottman et al., 1996; Byrne et al., 1997; Swiderski et al., 1999; Giguere and Prescott, 1999;

---

* Reprinted with permission from "Simultaneous Quantitation of Equine Cytokine mRNAs Using a Multi-Probe Ribonuclease Protection Assay" by W.-S. Lim et al., from Vet. Immunol. Immunopathol. Volume **91**, 45-51, copyright © 2003 by Elsevier.

Leutenegger et al., 1999). However, very few equine cytokines have been analyzed by the cell line- and antibody-based assays. In addition, none of the currently available techniques allow rapid and simultaneous quantitative analysis of multiple equine cytokines.

The goals of this study were to develop a set of RNA probes to simultaneously quantitate mRNA levels of an array of equine macrophage-specific cytokines and thus provide a useful tool for establishing cytokine profiles in response to equine diseases. Among the equine cytokines that are cloned or sequenced, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1 and TNF-α are produced by macrophages and were included in our multi-probe ribonuclease protection assay (RPA) (Cavaillon, 1994; Gessani and Belardelli, 1998).

## Materials and Methods

### *Cell culture*

Primary cultures of equine peripheral blood mononuclear cells (EPBMC) and equine monocyte-derived macrophages (EMDM) were established by a modification of the methods described by (Raabe et al., 1998). Equine peripheral blood was collected into citrate dextrose solution and centrifuged at 700×*g* for 30 min to separate the buffy coats. EPBMC were extracted from buffy coats by centrifugation through a HybriMax Histopaque (d=1.077 g/cm$^3$) cushion (Sigma, St. Louis, MO); washed 4 times with $Ca^{2+}$, $Mg^{2+}$-free PBS, 5% adult horse serum (HyClone, Logan, Utah); resuspended in MEMα

medium with 10% adult horse serum (GIBCO BRL, Rockville, MD), 25 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (complete MEMα); seeded at $5\times10^6$ cells/$cm^2$; and incubated overnight at 37°C, 5% $CO_2$. Nonadherent cells were removed and adherent monocytes were incubated for 7 days to allow differentiation into macrophages (EMDM).

*Construction of template plasmids*

Equine cytokine sequences published in GenBank database were used to design RT-PCR primer pairs (Table 2 and 3). These primers were chosen to obtain a set of cytokine cDNAs that could then be utilized to generate probes and protected fragments ranging from 100 to 430 nucleotides in length (Table 2), the optimal range for separation on 5% acrylamide gels (Bellin, 1996; Farell, Jr., 1998). A *Hin*d III restriction enzyme site was incorporated into the 5' ends of all the reverse primers to facilitate cloning into the plasmid vector (Table 3). A *Bam*H I site was incorporated into the 5' end of the forward primers for IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TNF-α and β-actin. An *Eco*R I site was incorporated into the 5' ends of the forward primers for IL-1α, IL-1β, and TGF-β1.

EPBMC and equine monocytes were stimulated with 50 μg/ml of ConA (Sigma) for 4 hours at 37°C and total RNA was extracted using the GLASSMAX RNA Microisolation Spin Cartridge System (GIBCO BRL, Rockville, MD) following the manufacture's instructions. Eight 1.5 μg aliquots of total RNA from EPBMC were reverse-transcribed

**Table 2**
cDNAs of cytokine and internal control genes included in the template set and expected sizes of full-length probes and protected fragments

| Cytokine | Nucleotide numbers | Size (bp) | | GenBank accession number |
|---|---|---|---|---|
| | | Full-length probe | Protected fragment | |
| IL-1α | 5-385 | 399 | 381 | U92480 |
| IL-1β | 562-974 | 430 | 413 | U92481 |
| IL-6 | 57-394 | 357 | 338 | U64794 |
| IL-8 | 61-230 | 189 | 170 | AF062377 |
| IL-10 | 377-557 | 196 | 181 | U38200 |
| IL-12 p35 | 34-171 | 150 | 138 | Y11130 |
| IL-12 p40 | 568-681 | 133 | 114 | Y11129 |
| IFN-γ | 204-470 | 285 | 267 | D28520 |
| TGF-β1 | 496-739 | 263 | 244 | AF175709 |
| TNF-α | 1-206 | 223 | 206 | AB035735 |
| β-actin | 861-960 | 119 | 100 | AF035774 |

**Table 3**
Sequences of primers used to generate equine cytokine cDNA fragments

| Cytokine | Forward primer (5'-3') [a] | Reverse primer (5'-3') |
|---|---|---|
| IL-1α | CGGAATTCTTTCACTGGCACTTGAGTCG | CCCAAGCTTAATGTACTGATCGGGGCTTG |
| IL-1β [b] | CGGAATTCGACTGACAAGATACCTGTGGCCT | CCCAAGCTTAGACAACAGTGAAGTGCAGCCT |
| IL-6 | CGGGATCCCCTGGTGATGGCTACTGCTT | CCCAAGCTTCCTTGAACTCGTTCTGGAGG |
| IL-8 | CGGGATCCGAAGCTGCGGTTGTATCAAG | CCCAAGCTTCAGACCTCAGCTCCGTTGAC |
| IL-10 | CGGGATCCGTCATCGATTTCTGCCCTGT | CCCAAGCTTCGTTCCCTAGGATGCTTCAG |
| IL-12 p35 | CGGGATCCTGGTCCTCCTAAACCACCTG | CCCAAGCTTCTGAAGCGTGTTGCTGAC |
| IL-12 p40 | CGGGATCCTACACGGTGGAGTGTCAGGA | CCCAAGCTTGCCGCTGGTGTAGTTTTCAT |
| IFN-γ | CGGGATCCTCAGAGCCAAATCGTCTCCT | CCCAAGCTTTCTGACTCCTCTTCCGCTTC |
| TGF-β1 | CGGAATTCAGTTAAGCGTGGAGCAGCAT | CCCAAGCTTCTGGAACTGAACCCGTTGAT |
| TNF-α | CGGGATCCCAGCTGGACTTGAGCCCCT | CCCAAGCTTGCCGATCACCCCAAAGTG |
| β-actin | CGGGATCCCGACATCCGTAAGGACCTGT | CCCAAGCTTCAGGGCTGTGATCTCCTTCT |

[a] Sequences in *italic* are restriction enzyme cleavage sites: *GAATTC*, *Eco*R I; *GGATCC*, *Bam*H I; *AAGCTT*, *Hin*d III. CG or CCC nucleotides were incorporated into the 5' ends of the primers to increase the efficiency of restriction enzyme digestion.

[b] This pair of primers was derived from primer sequences described by Giguere and Prescott (1999)

into first-strand cDNA for IL-1α, IL-1β, IL-8, IL-12 p35, IFN-γ, TGF-β1, TNF-α and β-actin using the corresponding reverse primers (Table 3) and either M-MLV or AMV reverse transcriptase (Promega Corporation, Madison, WI) following the manufacture's instructions. IL-12 p40 cDNA was synthesized from total RNA of equine monocytes.

The cDNAs for IL-1α, IL-1β, IL-8, IL-12 p35, IFN-γ, TGF-β1, TNF-α and β-actin were amplified by PCR using the primer pairs shown in Table 3 under the following conditions: initial denaturation for 2 minutes at 95°C followed by 35 cycles of 1 minute at 94 °C, 1 minute at 54°C, 1 minute at 72°C, followed by 6 minutes at 72°C. IL-12 p40 cDNA was amplified under the same conditions except the annealing temperature was increased to 60°C to eliminate nonspecific products.

IL-6 and IL-10 fragments were amplified from plasmids (pCREQIL6 and pCREQIL10 respectively) containing the complete equine IL-6 and IL-10 genes (kindly provided by David W. Horohov, Louisiana State University), using primer pairs shown in Table 3 and the PCR conditions described above (with annealing temperature at 54°C). All cDNAs were phenol extracted and subjected to an additional round of PCR to obtain sufficient product for cloning into pGEM-4Z (Promega).

cDNA fragments of IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TNF-α and β-actin were digested with *Hin*d III and *Bam*H I (Promega Corp.) and cloned into *Hin*d III and *Bam*H I digested pGEM-4Z (Promega Corp.). The cDNA fragments of IL-1α, IL-1β, TGF-β1 were digested with *Hin*d III and *Eco*R I (Promega Corp.) and cloned into *Hin*d III and *Eco*R I digested pGEM-4Z. This cloning strategy ensured that the inserted cDNA fragments were oriented such that antisense RNAs were synthesized when the

templates were transcribed with T7 RNA polymerase. Template plasmids were sequenced using pUC/M13 or M13 Universal forward and reverse sequencing primers to confirm orientation and agreement with published sequences.

*Radiolabeled RNA probes*

Plasmids containing IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TNF-α and β-actin were linearized by *Bam*H I digestion, and those containing IL-1α, IL-1β and TGF-β1 were linearized by *Eco*R I digestion. Radiolabeled negative-strand RNA probes were generated using the RiboProbe In vitro Transcription System (Promega Corp.) according to the manufacture's specified protocols. Each linearized template (0.1-0.5 μg) was mixed with 1× Transcription Optimized Buffer, 10 mM DTT, 10 units recombinant Rnasin RNase inhibitor, 2.5 mM each of rATP, rGTP, and rUTP, 100 μM rCTP, 50 μCi [α-$^{32}$P]rCTP (NEN, Boston, MA) and 7.5 units T7 RNA polymerase, and incubated at 37°C for 1 hour. DNA templates were removed by treating each reaction with 0.5 units of RQ1 RNase-free DNase. Full-length probes were gel purified on 5% acrylamide/8 M urea denaturing gels. (5% acrylamide [acrylamide: bis acrylamide = 19 : 1], 8 M urea, 1× Tris-Borate-EDTA buffer, 0.08% ammonium persulfate, 0.1% TEMED). Gel slices containing full-length probes were excised from the gel, submerged in 80 μl of DEPC-treated water, and incubated for 4 hours at 37°C to elute the probes.

*Nonradiolabeled IL-1β RNA*

To synthesize positive-strand IL-1β RNA, the plasmid containing IL-1β was linearized by *Hin*d III digestion and in vitro transcribed using SP6 RNA polymerase in the presence of nonradiolabeled rCTP. Integrity of the RNA was verified by gel electrophoresis and the concentration was estimated by UV spectrophotometry. The positive-strand IL-1β RNA was serially diluted with MA104 (simian kidney, ATCC) cell lysates ($5\times10^6$ cells/ml) to $1.5\times10^9$, $3\times10^8$; $6\times10^7$, $1.2\times10^7$, $2.5\times10^6$; $5\times10^5$; $1\times10^5$, $2\times10^4$ and 0 molecules per 45 μl.

*RPA*

RPA was performed using the Direct Protect Lysate RPA kit (Ambion, Austin, TX). For measurement of EMDM cytokines, EMDM cultured for 7 days were re-seeded onto 6-well plates at $2\times10^5$ cells/well, stimulated with 0 or 10 μg/ml of LPS (from 0111:B4 *E. coli*, Sigma) for 4 hours, and harvested by trypsinization. Pelleted EMDM were lysed with Lysis Solution (Ambion) and stored at –20°C. For each hybridization reaction, 45 μl of cell lysate (from $2\times10^5$ cells) was incubated with 5 μl of probe mixture that contained ~$9\times10^4$ cpm of each of the 11 equine cytokine probes. Negative controls included 5 μl of probe mixture only and 5 μl of probe mixture incubated with 10 μg of yeast tRNA (Ambion). Reaction mixtures were incubated at 37°C overnight, treated with 5 U of RNase A and 200 U of RNase T1, and incubated for 30 min at 37°C in 1×

RNase Digestion Buffer. The probes-only control was treated with 1× RNase Digestion Buffer in the absence of RNase. Digestion was stopped by adding 10 µl of Proteinase K (20 mg/ml) and 20 µl of 10% Sodium Sarcosyl and incubating for 30 min at 37°C. RNA was precipitated from each reaction with isopropanol and pellets were washed with ethanol, air-dried, resuspended in Gel Loading Buffer II (Ambion), heated (95°C, 3 min), and resolved on a 5% acrylamide/8 M urea denaturing gel at 400 V for 3.5 h. An aliquot of the probes-only control containing ~$9\times10^3$ cpm of each probe was applied to the gel as a negative control.

For measurement of EPBMC cytokines, EPMBC isolated from horse blood were seeded onto 12-well plates at $8\times10^6$ cells/well and immediately stimulated with 25 µg/ml ConA for 24 hrs. After the incubation, both the adherent and nonadherent EPBMC were harvested and lysed. Lysates equivalent to $7\times10^5$ EPBMC were analyzed by RPA. Quantitation of EMDM and EPBMC cytokine mRNAs was performed in triplicate using cells maintained in separate cultures.

To evaluate reproducibility, a single flask of EMDM cells was stimulated with 10 µg/ml of LPS for 4 h. Four equivalent aliquots ($7\times10^5$ cells) of the lysate were used to perform four independent RPA.

*Quantification of Cytokine mRNA Levels*

To quantify cytokine mRNA expression levels, the dried gel was exposed to a Fujifilm Imaging Plate and scanned with a Fujifilm Bio-imaging Analyzer BAS-1800II (Kanagawa, Japan). The photo-stimulated luminescence (PSL) released by the radiated

Imaging Plate was analyzed with the ImageGauge™ imaging analysis software (Version 3.12, Science Lab/Fuji Photo Film Co., Ltd.) to generate a graphical representation of the PSL of each lane (Fig. 4). The areas under the curves, calculated as integrated PSL, are directly proportional to the amount of radioactivity of the protected probes. Background (BG) level of each band was automatically calculated and subtracted from the integrated PSL (PSL-BG). To normalize for differences in input RNA and gel loading, cytokine mRNA levels were expressed as a percentage of β-actin mRNA and were calculated as follows:

$$\frac{\text{PSL} - \text{BG of cytokine}}{\text{PSL} - \text{BG of } \beta\text{-actin}} \times 100\%$$

**Results**

All template plasmids were sequenced and shared 100% identity with the published cDNA sequences of these genes. Radiolabeled RNA probes of the expected sizes were generated (Fig. 5A, lane 1). Probes for IL-1α, IL-1β, IL-6, IL-8, IL-10, TGF-β1, TNF-α and β-actin produced protected fragments of the expected size after hybridization with EMDM lysates (Fig. 5A). All probes and protected fragment lengths differed from each other by at least 10 nucleotides allowing simultaneous analysis of all 10 cytokines and the β-actin control. Protection of these probes was sequence-specific since yeast tRNA failed to protect any of the probes from digestion.

To determine whether the lack of IFN-γ detection was due to the absence of IFN-γ mRNA in EMDM or failure of the probe to protect the mRNA, cytokine expression of EPBMC was analyzed by the multi-probe RPA. The IFN-γ probe generated a protected

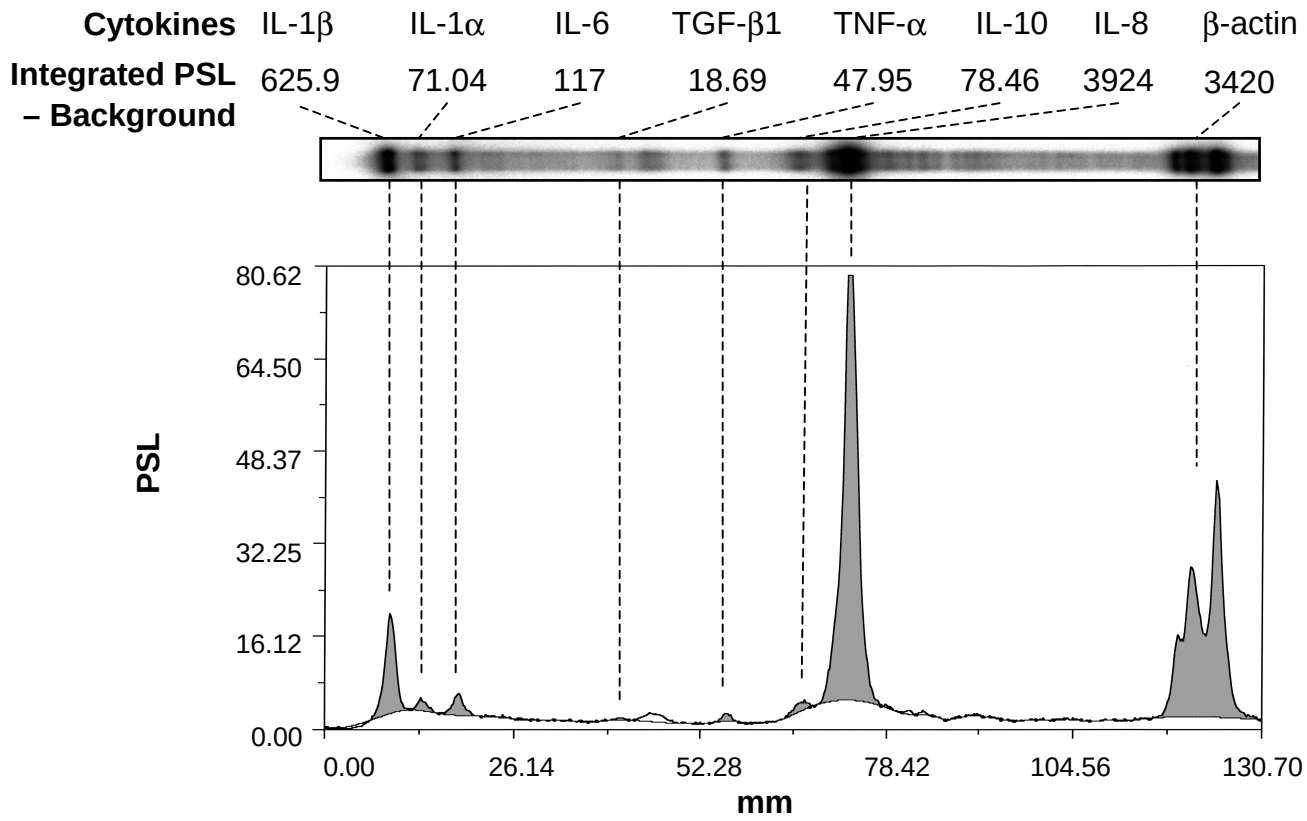


**Fig. 4.** Quantification of equine cytokine mRNAs as measured by phosphor imaging. A Fujifilm Phosphor Imaging Plate was exposed to gels containing the protected fragments of the radioactive RNA probes and scanned with a Fujifilm Bio-imaging Analyzer BAS-1800II. The ImageGauge™ software was employed to generate a graphical representation of the bands in each lane. The x-axis depicts the distance of the bands from the top of the gel. The y-axis shows the photo-stimulated luminescence (PSL) released by the radiated Imaging Plate. Shaded areas under the curve are calculated as integrated PSL, which are directly proportional to levels of radioactivity. Background level s are automatically calculated and subtracted. The names of the cytokine genes and corresponding background-corrected PSL values are indicated across the top of the figure.

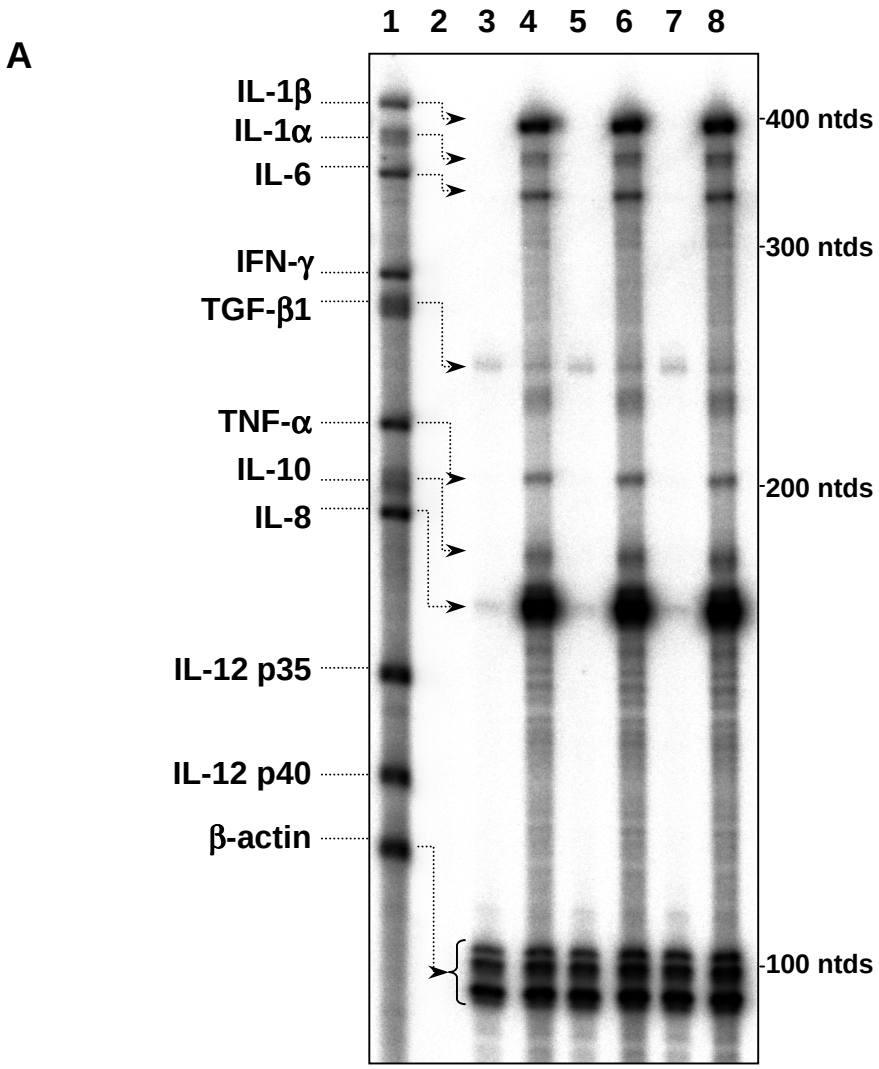


**Fig. 5.** Phosphor image of a 5% acrylamide/8M urea denaturing gel showing the relative migration of full-length RNA probes and protected fragments (Panel A), and the detection of IFN-γ in EPBMC (Panel B). (A) Eleven radiolabeled RNA probes (10 equine-specific cytokines and β-actin control) were hybridized to yeast tRNA (lanes 1 and 2) or EMDM lysates (lanes 3 to 8). Reactions were either undigested (lane 1) or digested with RNases (lanes 2 to 8). The probes were hybridized with EMDM lysates that were stimulated 4 h at 37°C with 0 μg/ml (lanes 3, 5 and 7) or 10 μg/ml of LPS (lanes 4, 6 and 8). The molecular weights of the RNA standards are indicated on the right of the gel. Depicted on the left are the locations of each full-length probe and the corresponding protected fragment(s) when present. The β-actin protected fragments appear as 3 bands in EMDM. (B) To verify that the probe for IFN-γ was correct and could detect the presence of IFN-γ mRNA, EPBMC lysates were examined by RPA. Radiolabeled probes were hybridized to either yeast tRNA (lane 1 and 2), unstimulated EPBMC lysates (lane 3), or EPMBC lysates stimulated with 25 μg/ml of ConA for 24 h at 37°C. With the exception of lane 1, all samples were digested with RNases. RNA molecular weights standards are indicated on the right, and the locations of each full-length probe and the corresponding protected fragment(s) when present are specified on the left. In contrast to EMDM, the β-actin protected fragments are 2 bands in EPBMC. Data shown are representative of experiments done in triplicate.

B

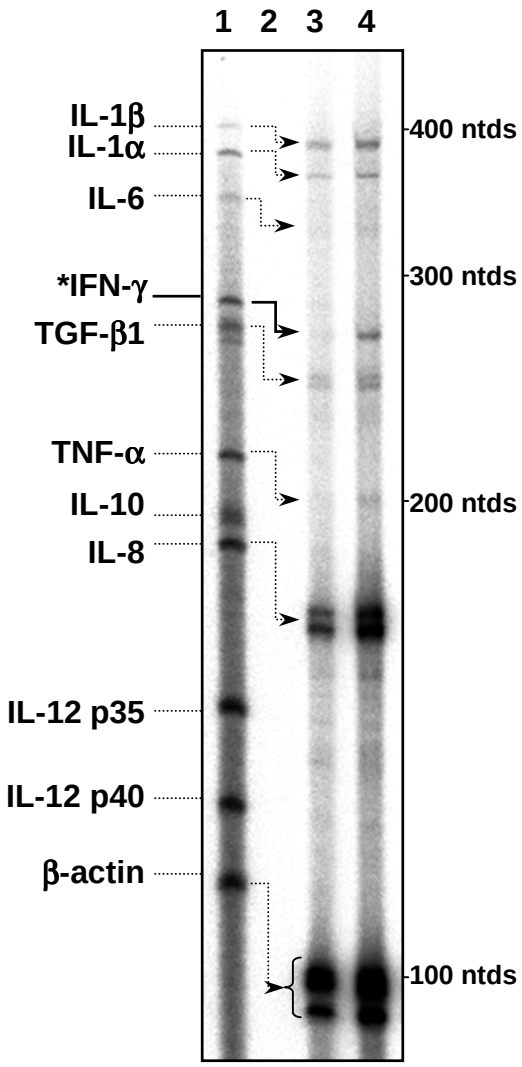


**Figure 5. Continued..**

fragment of the expected size after hybridization with EPBMC lysates (Fig. 5B). IL-1α, IL-1β, IL-6, IL-8, TGF-β1 and TNF-α mRNAs also were detected in EPBMC, however protected fragments of IL-10, IL-12 p35 and IL-12 p40 were not discerned (Fig. 5B). These data demonstrate the multi-probe RPA can detect the expression of IFN-γ mRNA and the assay can be readily adapted to analyze cytokine mRNA expression in EPBMC.

The levels of cytokine mRNAs in EMDM were quantified by phosphor images of the protected probe fragments and normalized to the β-actin mRNA level (Table 4). In unstimulated EMDM, cytokine mRNAs were essentially undetectable with the exception of IL-8 and TGF-β1, which were weakly expressed at 1.53% and 0.67% β-actin levels, respectively. LPS stimulation of EMDM upregulated expression of IL-1α to 1.96%, IL-1β to 18.22%, IL-6 to 3.24%, IL-8 to 116.70%, IL-10 to 2.32% and TNF-α to 1.81%, but slightly downregulated expression of TGF-β1 to 0.61% (Table 4). Expression of IFN-γ mRNA, undetectable in unstimulated EPBMC, was increased by ConA stimulation to 0.59%. Neither IL-12 p35 nor IL-12 p40 were detected in unstimulated or stimulated EMDM or EPBMC. These results demonstrate the capability of the multi-probe RPA to simultaneously quantitate the mRNA levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, IFN-γ, TGF-β1 and TNF-α in a single experiment.

To verify that β-actin mRNA levels in EMDM are not affected by LPS stimulation, 7 day-old EMDM were seeded in 6-well plates at $2\times10^5$ cells/well and incubated with 0 or 10 μg/ml LPS for 4 h. EMDM lysates were hybridized with ~$9\times10^4$ cpm of β-actin probe and analyzed by RPA (Fig. 6A). Comparison of mean β-actin

**Table 4**
Cytokine mRNA levels in EMDM and EPBMC analyzed by multi-probe RPA

| Cytokines[a] | Normalized mRNA levels (% β-actin)[b] | |
|---|---|---|
| | Unstimulated | LPS- or ConA-Stimulated |
| IL-1α | U.D.[c] | 1.96±0.11 |
| IL-1β | U.D. | 18.22±0.32 |
| IL-6 | U.D. | 3.24±0.18 |
| IL-8 | 1.53±0.32 | 116.70±1.71 |
| IL-10 | U.D. | 2.32±0.28 |
| TGF-β1 | 0.67±0.13 | 0.61±0.14 |
| TNF-α | U.D. | 1.81±0.35 |
| IFN-γ | U.D. | 0.59±0.08 |

[a] mRNA levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, TGF-β1 and TNF-α were quantitated in EMDM stimulated with 0 and 10 μg/ml LPS for 4 h. mRNA levels of IFN-γ were quantitated in EPBMC stimulated with 0 and 25 μg/ml of ConA for 24 h.

[b] mRNA levels are expressed as a percentage of the control β-actin mRNA levels. The data shown are means ± standard deviation of triplicate experiments.

[c] Undetectable.

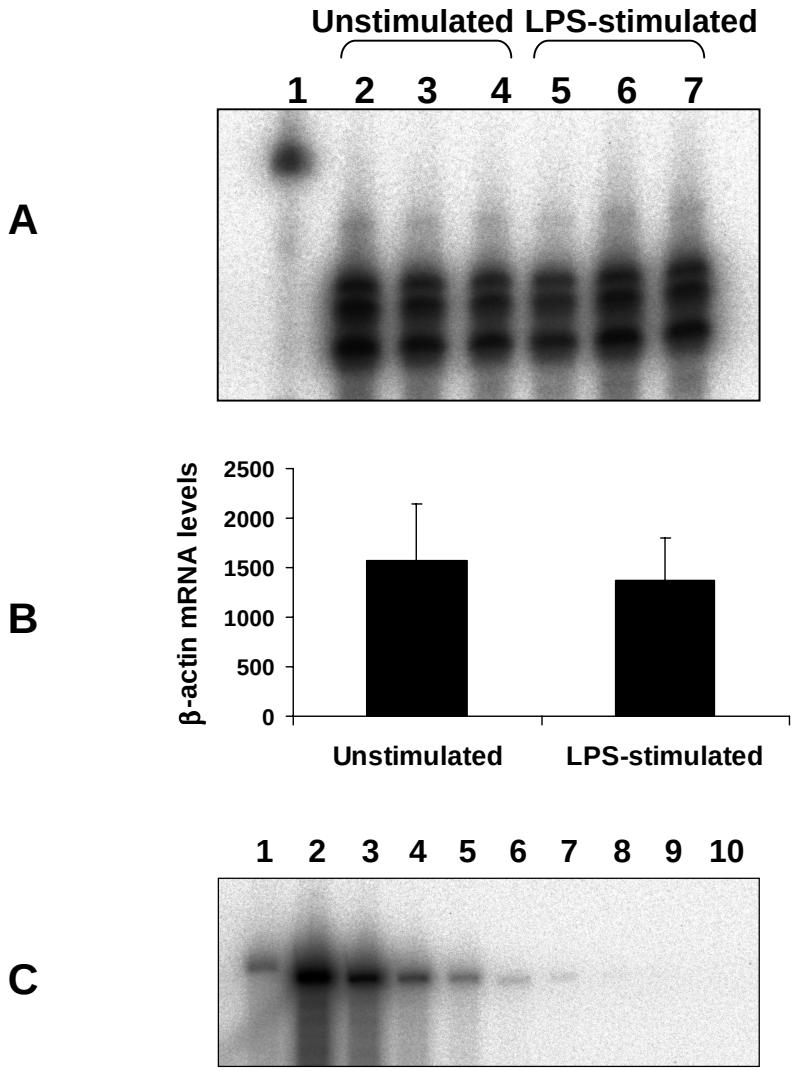


**Fig. 6.** Suitability of the β-actin internal control and sensitivity of the RPA. (A) Expression of β-actin mRNAs in unstimulated and LPS-stimulated EMDM. Radiolabeled β-actin probe was hybridized with EMDM lysates, treated with RNases, and analyzed by gel electrophoresis and phosphor imaging. Lane 1 shows the migration of the full-length β-actin probe. Lanes 2 – 4 show β-actin from unstimulated EMDM; lanes 5-7 show β-actin from LPS-stimulated EMDM. (B) Graphical representation of mRNA levels of β-actin shown in A. The x-axis indicates stimulation of EMDM. The y-axis depicts the β-actin mRNA levels. The data shown are means ± standard deviation of triplicate experiments. (C) Sensitivity of the RPA using IL-1β as target. Positive-strand nonradiolabeled IL-1β was in vitro transcribed and added to MA104 cell lysates. Radiolabeled IL-1β probe was then hybridized with the lysates, treated with RNases, and analyzed by gel electrophoresis and phosphor imaging. Lane 1 shows the full-length IL-1β probe. Based on UV spectrophotometry estimations, the following numbers of target molecules were added to MA104 lysates prior to RPA: lane 2, $1.5\times10^9$; lane 3, $3\times10^8$; lane 4, $6\times10^7$; lane 5, $1.2\times10^7$; lane 6, $2.5\times10^6$; lane 7, $5\times10^5$; lane 8, $1\times10^5$; lane 9, $2\times10^4$. In lane 10, positive-strand IL-1β target was not added to the MA104 cell lysate.

mRNA levels showed that unstimulated and stimulated EMDM expressed equivalent levels of β-actin (Fig. 6B). Thus, β-actin is a suitable internal control for quantitation of LPS-stimulated EMDM cytokines (Suzuki et al., 2000). However, when measuring cytokine responses to other treatments or in other equine cells, it will be necessary to reevaluate the use of β-actin as an apposite internal control.

To assess the sensitivity of the RPA, nonradiolabeled positive-strand IL-1β was generated and used as synthetic targets. Varying numbers of target molecules were diluted in MA104 lysates, hybridized with ~$9\times10^4$ cpm of radiolabeled anti-sense IL-1β probe, and analyzed by RPA. $5\times10^5$ molecules of target were detectable (Fig. 6C), akin to other RPA techniques (Haines and Gillespie, 1992).

The reproducibility of the RPA was evaluated by analyzing 4 equivalent aliquots ($7\times10^5$ cells per aliquot) of the lysate derived from a single culture flask of LPS-stimulated EMDM. The dried gel was quantitated by phosphor imaging as described above (Table 5). The coefficient of variation (CV) of the unnormalized cytokine mRNA levels ranged from 0.11 to 0.17. Since the same amount of a common lysate was used in each assay, the variations were likely due to handling of the samples and gel loading. When the cytokine mRNA levels were normalized by the concentration of β-actin mRNA, the CV was markedly decreased by 1.5-5.7 fold to 0.03-0.08 (Table 5). It is unlikely this level of reproducibility will be attained with experimental samples.

**Table 5**

Reproducibility of the multi-probe RPA and effects of β–actin normalization

| Cytokine | Unnormalized mRNA Levels | | | β-actin Normalized mRNA Levels | | |
|---|---|---|---|---|---|---|
| | Mean[a] | SD[b] | CV[c] | Mean | SD | CV |
| IL-1α | 2580.69 | 437.88 | 0.17 | 2.00 | 0.05 | 0.03 |
| IL-1β | 6097.66 | 873.84 | 0.14 | 4.75 | 0.32 | 0.07 |
| IL-6 | 1823.41 | 219.53 | 0.12 | 1.43 | 0.11 | 0.08 |
| IL-8 | 217812.75 | 26003.42 | 0.12 | 170.20 | 9.47 | 0.06 |
| IL-10 | 11817.41 | 1580.75 | 0.13 | 9.22 | 0.62 | 0.07 |
| TGF-β1 | 2012.91 | 223.21 | 0.11 | 1.27 | 0.08 | 0.05 |
| TNF-α | 9604.38 | 1321.41 | 0.14 | 7.49 | 0.24 | 0.03 |

[a]Mean cytokine mRNA levels measured by 4 individual assays using aliquots of a common LPS-stimulated EMDM lysate.

[b]Standard deviation.

[c]Coefficient of variation.

**Discussion**

This study shows the successful development of a RPA that simultaneously analyzes the mRNA expression of equine IL-1α, IL-1β, IL-6, IL-8, IL-10, IFN-γ, TGF-β1 and TNF-α in EMDM and EPBMC cultures. In the course of developing the RPA, a set of 11 template plasmids were generated from which antisense RNA probes specific to 10 equine cytokine genes and the equine β-actin gene were synthesized by in vitro transcription. By using the equine β-actin to normalize the sample-to-sample differences, the assay had a CV of 0.03-0.08 (or 3%-8%). In a previously reported RPA, a fixed amount of in vitro-transcribed RNA was added as an internal control, resulting in a between-assay CV of 11.3% (Petersen et al., 1995). By comparison, the RPA described in this study is highly precise and reproducible. This assay detected as few as $5\times10^5$ molecules of target RNA, similar to the sensitivity of a previously characterized lysate-based RPA, which detects $10^4$-$10^5$ target mRNAs (Haines and Gillespie, 1992).

The results presented herein show that the transcription of IL-1α, IL-1β, IL-6, IL-8, IL-10, and TNF-α genes in EMDM was induced by LPS. Similarly, LPS upregulates the transcription of these cytokine genes in human macrophages (de Waal et al., 1991; Springs et al., 1992; Zhong et al., 1993). In contrast, mRNA of TGF-β1 was constituitively expressed in EMDM and was slightly downregulated by LPS. In human monocyte cultures, TGF-β1 is also expressed constituitively (Toossi et al., 1995). LPS stimulation accelerates the decay of TGF-β1 mRNA resulting in a decrease of steady state mRNA levels when analyzed by Northern blot (Toossi et al., 1995).

The multi-probe RPA revealed that ConA stimulation of EPBMC increased mRNA levels of IL-1α, IL-1β, IL-6, IL-8, IFN-γ and TNF-α and decreased mRNA level of TGF-β1. Upregulation of IL-1α, IL-1β, IL-6, IFN-γ and TNF-α has been shown in ConA stimulated EPBMC using RT-cPCR. Moreover, in agreement with the RPA results, the RT-cPCR results also show that the increase of IFN-γ mRNA levels is greater than the other cytokines tested after 24 hrs of ConA stimulation (Giguere and Prescott, 1999; Rottman et al., 1996). The report by Giguere and Prescott shows mRNA expression of IL-10, IL-12 p35 and IL-12 p40 at $0.18\text{-}5.74\times10^4$ molecules per μg of total RNA upon stimulation of EPBMC. In contrast, the RPA failed to detect these cytokines. Although it is known that the RT-cPCR technique is more sensitive, RPA allows more rapid evaluation of multiple cytokines.

In summary, the cytokine mRNA profiles of LPS-stimulated EMDM as measured by our multi-probe assay are consistent with those of equivalent human systems. The cytokine mRNA profiles of ConA-stimulated EPMBC are also in general agreement with the EPBMC cytokine mRNA profiles previously analyzed by RT-cPCR assay. Taken together, these results show that the equine-specific multi-probe RPA is highly accurate and dependable, albeit not as sensitive as the RT-cPCR approaches.

This is the first RPA designed to measure mRNA expression of equine cytokines. Although tested in primary EMDM and EPBMC, this assay should be applicable to other equine cells or tissues. The specificity, precision, sensitivity, and simultaneous quantitation capability of this assay make it an extremely useful tool in the analysis of

equine cytokines and will enhance our understandig of equine immunology and disease pathogenesis.

# CHAPTER III

# DIFFERENTIAL EFFECTS OF VIRULENT AND AVIRULENT EQUINE INFECTIOUS ANEMIA VIRUS MOLECULAR CLONES ON PRIMARY EQUINE MACROPHAGE CYTOKINE EXPRESSION

## Introduction

Equine infectious anemia virus (EIAV) belongs to the family Retroviridae, genus lentivirus. The clinical course of EIAV infection is classically divided into acute, chronic, and inapparent carrier phases. The acute phase occurs within 5-30 days post-infection (dpi) and is characterized by viremia, fever, and thrombocytopenia (Sellon et al., 1994; Issel and Coggins, 1979). The acute symptoms vary from mild to severe to fatal depending on the strain of EIAV and the host immune status. Following the acute phase, the infected animal may develop chronic EIA which manifests as recurring cycles of fever, severe anemia, weight loss, thrombocytopenia, hemorrhages, and anorexia (Issel and Coggins, 1979). About 90% of infected horses survive the chronic phase and become clinically normal. Subsequent clinical episodes can be elicited in inapparent carriers by environmental stress or immune-suppressive drugs (Cheevers and McGuire, 1985). As in all retroviral infections, infected horses remain life-long carriers of EIAV (Issel and Coggins, 1979; Kono et al., 1976).

Tissue macrophages are the primary sites for EIAV replication and serve as cellular reservoirs during acute and persistent infection (Sellon et al., 1992; Oaks et al., 1998). EIAV can infect circulating monocytes, but virus expression is limited to differentiated tissue

macrophages. Macrophages are the source of a myriad of cytokines, and perturbation of cytokine expression has been shown in other lentivirus-infected macrophages including human and simian immunodeficiency viruses (HIV, SIV respectively), caprine arthritis encephalitis virus (CAEV) and maedi-visna virus (MVV) (Bornemann et al., 1997; Sopper et al., 1996; Zink et al., 2001; Horvath et al., 1991; Lechner et al., 1997; Adeyemo et al., 1997; Ebrahimi et al., 2000; Legastelois et al., 1998; Legastelois et al., 1997; Legastelois et al., 1996). Cytokines produced by infected macrophages have been shown to impact the outcome of lentiviral infections. For example, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) contribute to the neurological impairment associated with late-stage HIV and SIV infection by facilitating infiltration of macrophages into the CNS and stimulating production of neurotoxic substances by activated macrophages and microglia (Orandle et al., 2001; Sopper et al., 1996; Xiong et al., 2000; Tan and Guiloff, 1998). Macrophage-secreted cytokines and chemokines, including IL-1, IL-6, type I interferons (IFN), TNF-α, RANTES and macrophage inflammatory proteins-1α and 1β (MIP-1α and 1β), are known to regulate the expression and replication of HIV in macrophages and $CD4^+$ T-lymphocytes (Vicenzi et al., 1997; Fauci, 1996). Therefore, investigating the interaction of EIAV with equine monocytes and macrophages is important to the study of EIAV-induced disease.

In this study, viruses derived from two EIAV molecular clones, $pSPEIAV_{19}$ and p19/wenv17, were examined for induction of cytokines in equine monocyte-derived macrophages (EMDM). Virus derived from clone $pSPEIAV_{19}$ ($EIAV_{19}$) causes asymptomatic infection in Shetland ponies (Payne et al., 1994). p19/wenv17 is a chimeric clone generated by replacing the 5' and 3' long terminal repeats (LTR) and the *env* sequences of $pSPEIAV_{19}$

with those derived from the highly virulent Wyoming field strain EIAV ($EIAV_{Wyo}$) (Payne et al., 1998). p19/wenv17 produces a virus, named $EIAV_{17}$, that causes severe EIA in all infected Shetland ponies (Payne et al., 1998). In vitro, however, both viruses replicate to high titer and induce cytopathic effects (CPE) in EMDM, indicating the difference in their clinical manifestations are due to factors other than differential levels of viral expression in macrophages. Cytokine profiles of EMDM infected with $EIAV_{19}$ or $EIAV_{17}$ were determined to evaluate the correlation between cytokine dysregulation and EIAV pathogenesis. We utilized a newly developed set of antisense RNA probes to quantitate the mRNA expression of 10 equine cytokine genes by ribonuclease protection assay (RPA) (Lim et al., 2003a).

**Materials and Methods**

*Cell culture*

Primary EMDM culture was established as previously described (Lim et al., 2003a). Briefly, anticoagulated equine peripheral blood from EIA-negative donor horses was centrifuged to separate the buffy coat, which was then centrifuged through a HybriMax Histopaque cushion (d=1.077 $g/cm^3$; Sigma, St. Louis, MO) to extract equine peripheral blood mononuclear cells (EPBMC). EPBMC were washed 4 times, resuspended in minimum essential medium alpha (MEMα) containing 10% adult horse serum (GIBCO BRL, Rockville, MD), 25 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (complete MEMα), seeded at $3\times10^6$ cells/$cm^2$ into tissue culture flasks and

incubated overnight. Nonadherent cells were removed, and the adherent monocytes were washed twice and incubated for 7 days to allow maturation into macrophages. Purity of the macrophage culture was estimated at 95% by the α-nalphthyl acetate esterase assay (Sigma kit no. 91-A) and by immunofluorescent microscopy using a monoclonal antibody (I.646) specific to a cytoplasmic antigen of equine mononuclear phagocytes (gift of F. J. Fuller; Sellon, 1993). All reagents used in EMDM culture were tested for endotoxin by the *Limulus* amebocyte lysate (LAL) assay (Associates of Cape Cod Inc., Falmouth, MA). Reagents with an endotoxin level of 0.06 EU/ml or lower were considered suitable for our assays. Horse sera containing less than 0.5 EU/ml (as tested by the manufacture) were purchased.

D17 cells (ATCC CCL-183), a cell line derived from canine lung osteosarcoma, were cultured in MEM containing 10% fetal bovine serum (BioWhittaker) in 25-$cm^2$ flasks as previously described (Payne et al., 1998).

*Generation of virus stocks*

$EIAV_{19}$ and $EIAV_{17}$ are derived from $pSPEIAV_{19}$ and p19/wenv17 plasmids, respectively, as reported (Payne et al., 1998). Briefly, D17 cells were plated in 60 mm dishes at $2.5 \times 10^5$ cells per dish 18-24 h prior to transfection (Calcium Phosphate Transfection System, Life Technologies). Each dish of cells was incubated with 10 μg of plasmid for 1.5 hours at 37°C, washed twice with phosphate buffered saline (PBS), and replenished with fresh media. Virus production was monitored by reverse

transcriptase (RT) assay of culture supernatants as previously described except that [$^{3}$H]TTP was used instead of [$^{32}$P]TTP (Gregersen et al., 1988).

Virus stocks were amplified by infecting EMDM at approximately $5\times10^{-3}$ counts per min (cpm) of RT activity per cell. Culture supernatants of infected EMDM were collected when approximately 80% of the cells displayed CPE (7 to 12 dpi). Growth of virus was verified by increases in RT activity. To remove cell-secreted molecules (especially cytokines), 100 ml of infected culture supernatant was sterilely ultra-filtered against 1L of fresh complete MEMα (without horse serum) using a Vivaflow 200 tangential-flow ultra-filtration unit with a MWCO of 100 kDa (Sartorius Corp, Edgewood, New York). The 100-kDa filter was chosen because the molecular weights of cytokines range from 7 to 75 kDa. Ultra-filtration of 100 ml of 50 mg/ml purified bovine serum albumin (66 kDa) solution using these conditions removed 96% of the protein from solution (data not shown). After ultra-filtration, RT activity of the virus was re-measured and infectivity was verified by growth in EMDM. Culture supernatant of mock-infected EMDM was collected and purified under the same conditions, and was termed control medium (CM). Purified CM, $EIAV_{19}$ and $EIAV_{17}$ contained negligible levels of endotoxin (0.12-0.25 EU/ml).

*Western blot*

Aliquots of $EIAV_{19}$ and $EIAV_{17}$ stocks containing 60,000 cpm of RT activity were pelleted by centrifugation at 13,000× g for 2 hrs. Viral pellets were resuspended, separated by SDS-PAGE and analysed by Western Blotting using a mouse monoclonal antibody specific to

EIAV gp90 (EIAV gp90(A)-86) obtained from NIH AIDS Research and Reference Reagent Program (Hussain et al., 1987).

*Infection of EMDM*

Six days post isolation, EMDM were transferred to 6-well plates at $2.35\times10^5$ cells per well and incubated for 24 hrs. $EIAV_{19}$ or $EIAV_{17}$ (containing 30,000 cpm of RT activity in 0.5 ml) or 0.5 ml of CM was added to each well of EMDM, incubated for 1 hr at 37°C, and 0.5 ml of fresh complete MEMα was added. At 0, 0.5, 1, 2, 4, 12, and 24 hpi, EMDM were washed with PBS, harvested by trypsinization and pelleted. EMDM pellets were then lysed with Lysis Solution (Ambion) at $1.6\times10^7$ cells/ml and stored at -20°C.

*RPA*

Cytokine mRNA levels were measured by RPA as previously described (Lim, 2003) using the Direct Protect Lysate RPA kit (Ambion, Austin, TX). Eleven $^{32}$P-labeled antisense RNA probes specific to equine IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, transforming growth factor-β1 (TGF-β1), TNF-α, and β-actin mRNAs were synthesized by in vitro transcription from their respective templates (Lim et al., 2003a). Approximately $9\times10^4$ cpm of each probes were hybridized with EMDM lysate (derived from $2.35\times10^5$ cells) overnight and then digested with RNase A/RNase T1 cocktail. The protected probe fragments were isopropanol precipitated and resolved on a 20 cm (length) × 16 cm (width) 5% acrylamide / 8 M urea denaturing gel. The gels were dried and exposed to a Fuji Film Imaging Plate (Fuji Photo Co., Ltd., Kanagawa, Japan).

Abundance of the protected fragments was quantitated using a Fuji Film Bio-imaging Analyzer BAS-1800II Phosphor Imaging System (Fuji Photo Co., Ltd.). Cytokine mRNA levels were calculated based on the photo-stimulated luminescence of the cytokine bands, which are directly proportional to the radioactivity, and were normalized to the expression levels of β-actin mRNA (expressed as percentage of β-actin mRNA).

*Statistical analysis*

Exploratory, descriptive and inferential methods were used to analyze the data. Exploratory data analyses included examination of graphical displays of the cytokine data by time for each horse. Descriptive methods included examination of summary statistics for various parameters of the cytokine measurements (for example, ratio of mean values at fixed time points). For inferential analyses, linear mixed-effects models were fitted using S-PLUS both for comparisons of changes over time among treatments (ie, temporal changes) and for comparisons among treatments for a given time (Pinheiro and Bates, 2000). For the linear mixed-effects modeling, an individual animal and each replicate within an individual animal were considered as random effects. For comparison of temporal changes, treatment and time were considered as fixed effects. For comparisons among treatments for a given time, treatment was considered as a fixed effect and data from other times were excluded from the model.

Data were modeled using a common slope for all horses and random intercepts. The assumption that a common slope for all horses was appropriate for a given covariate was examined by comparing the likelihood ratio test statistics of a model with differing

(random) slopes and intercepts for each horse with a model with a common slope for all horses but different intercepts for each horse. Goodness-of-fit of the models was graphically assessed in 3 ways. First, the standardized residuals were plotted against the fitted values. These plots were examined for outliers and any evidence of a systematic pattern, and showed that the residuals were distributed symmetrically around zero with an approximately constant variance. Second, the normal quantile-quantile plot of the standardized residuals did not indicate any violations of the normality assumption. For the normality assumption of random effects, we also used a normal quantile-quantile plot even if there were only 3 to 4 horses. The assumptions about the random effects were not violated. Third, the adequacy of the fitted model was better visualized by plotting fitted curves with the observed data. These plots showed that our model fitted well with the observed data. For all analyses, a significance level of $P \leq 0.01$ was used.

## Results

### *Characterization of $EIAV_{17}$ and $EIAV_{19}$*

To verify the infectivity of the virus stocks and compare the replication of $EIAV_{17}$ and $EIAV_{19}$, EMDM were infected with $EIAV_{17}$ or $EIAV_{19}$ stocks containing 3000 cpm RT activity or were mock-infected. Virus replication was monitored by measuring RT activity in culture supernatants. As shown in Fig. 7A, $EIAV_{17}$ and $EIAV_{19}$ replicated to the same levels in EMDM culture.

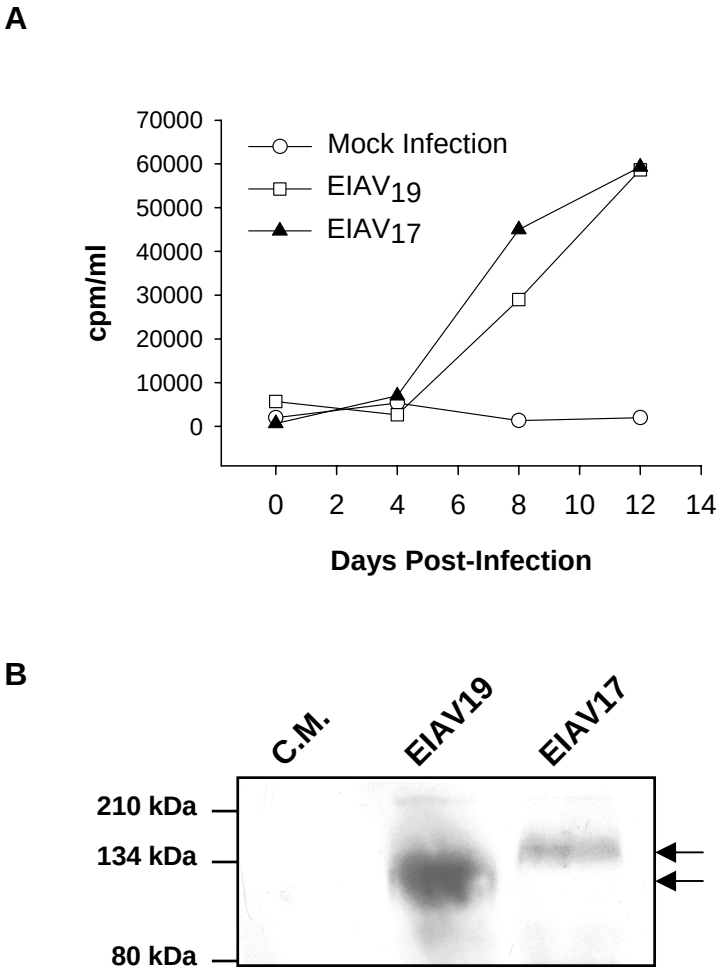


**Fig. 7.** Characterization of $EIAV_{17}$ and $EIAV_{19}$ stocks. (A) Graphical representation of EIAV replication in equine monocyte-derived macrophages (EMDM) as monitored by culture supernatant reverse transcriptase (RT) activity. EMDM cultures were infected with 3000 cpm of $EIAV_{17}$ (▲) or $EIAV_{19}$ (□) based on RT activity, or were mock infected (○). Aliquots of supernatants were removed from the cultures at 0, 4, 8 and 12 dpi and assayed for RT activity. The x-axis indicates time (dpi), and the y-axis indicates RT activity expressed as cpm/ml. (B) Western blot analysis of EIAV SU (gp90). Control media (CM), $EIAV_{17}$ and $EIAV_{19}$ stocks (both containing 60,00 cpm of RT activity) were pelleted, separated by 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and probed with a monoclonal antibody specific to EIAV SU. The migration of molecular weight markers (210, 134 and 80 kDa) is indicated on the left. Arrows on the right indicate the SU migration of $EIAV_{17}$ (top) and $EIAV_{19}$ (bottom).

The SU protein contents of $EIAV_{17}$ and $EIAV_{19}$ stocks containing equal cpm of RT activity were estimated by Western blot analysis using a monoclonal antibody specific to a conserved epitope of EIAV SU (Hussain et al., 1987). The $EIAV_{19}$ stock appeared to contain a higher concentration of SU than the $EIAV_{17}$ stock (Fig. 7B). A difference in electrophoretic mobility between the SU of the 2 viruses was evident, likely due to a difference in their glycosylation since $EIAV_{17}$ SU has 5 more potential N-linked glycosylation sites than $EIAV_{19}$ (Payne et al., 1998).

*$EIAV_{17}$ stimulated expression of IL-1α, IL-1β, IL-6 and TNF-α in EMDM*

Initial experiments were performed to determine the effects of $EIAV_{19}$ and $EIAV_{17}$ on equine macrophage cytokine expression. EMDM were cultured for 7 days and infected with $EIAV_{19}$ or $EIAV_{17}$ or mock-infected with CM. At 2, 4, 12 and 24 hpi, lysates of the infected cells were analyzed with the multi-probe RPA for IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1, TNF-α, and β-actin mRNA expression.

As shown in the phosphor image of a representative RPA (Fig. 8), IL-1α, IL-1β, IL-6 and TNF-α were expressed at barely detectable levels in CM-treated EMDM, whereas TGF-β1, IL-10 and IL-8 mRNAs were readily detected. IFN-γ, IL12 p35 and IL-12 p40 mRNAs were not detected in CM-treated EMDM. $EIAV_{19}$ infection induced a slight increase of IL-1β mRNA expression at 4 hpi and did not appear to affect the expression of other cytokines. In EMDM exposed to $EIAV_{17}$, there was a readily

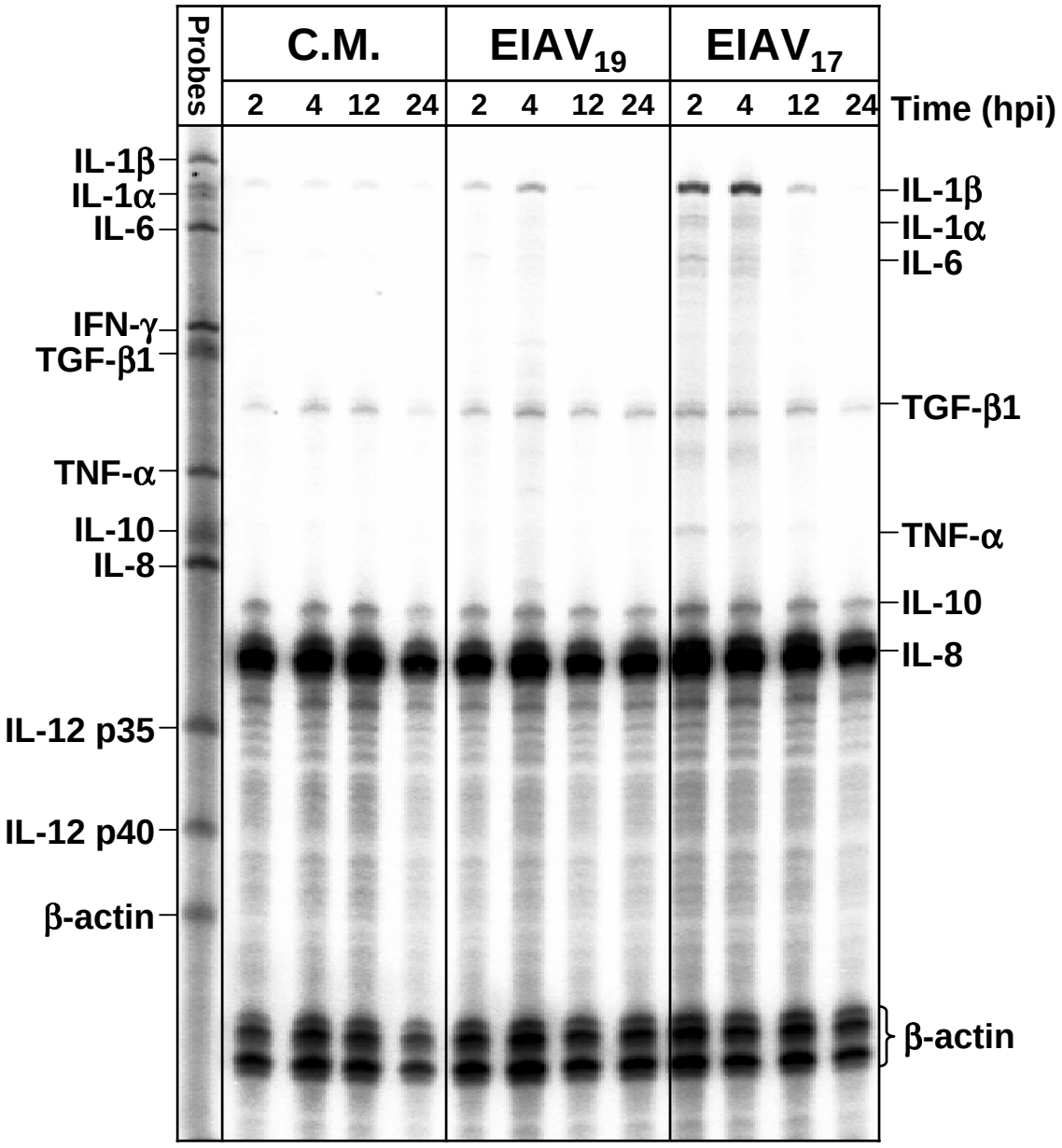


**Fig. 8.** Phosphor image analysis of cytokine expression in EMDM at 2-24 hrs post treatment with control media (CM) or infection with $EIAV_{19}$ or $EIAV_{17}$. In this representative experiment, EMDM derived from donor horse KB were harvested at 2, 4, 12 and 24 hpi, and cytokine mRNA levels monitored by RNAse protection assay (RPA) as described in Materials and Methods. Results are presented as individual protected fragments for IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1, TNF-α and β-actin separated on a 5% acrylamide/8 M urea denaturing gel and indicated on the right. Shown on the left are the positions of the full-length radiolabeled probes.

apparent increase of IL-1α, IL-1β, IL-6 and TNF-α mRNA levels at 2 and 4 hpi. IL-1β mRNA remained elevated up to 12 hrs post $EIAV_{17}$ infection.

To confirm these initial observations, the experiment was performed in duplicate at 2, 4, 12, and 24 hpi using EMDM derived from 4 different horses (total of 8 independent measurements). Cytokine mRNA levels were analyzed by phosphor imaging, normalized to the level of β-actin mRNA and expressed as percentage of β-actin (relative mRNA levels) (Fig. 9). Since out-bred horses were used, there was considerable variation in the levels of cytokine expression by EMDM derived from different animals. Hence, to evaluate the statistical significance of changes in cytokine mRNA expression levels, a linear mixed-effect (LME) model was used in which horse-to-horse variations and variations among duplicate measurements were taken into account by considering them as random effects. Adequacy of the LME models was assessed with graphical methods, as described above, and fit of the models was deemed appropriate.

Changes of relative cytokine mRNA levels in EMDM of the 4 horses were evaluated by LME modeling and descriptive statistics (Table 6). At 2 hpi, the mRNA levels of IL-1α, IL-1β, IL-6 and TNF-α in $EIAV_{17}$-infected EMDM were statistically higher than those of CM-treated EMDM ($p \leq 0.01$). IL-6 and TNF-α mRNA expression remained significantly elevated at 4 hpi in $EIAV_{17}$-infected EMDM, whereas IL-1α and IL-1β returned to that of CM-treated EMDM. By 12 hpi, mRNA levels of IL-6 and TNF-α in $EIAV_{17}$-infected EMDM were also at the same levels as those in CM-treated EMDM. In contrast, infection of EMDM with $EIAV_{19}$ did not increase the expression of

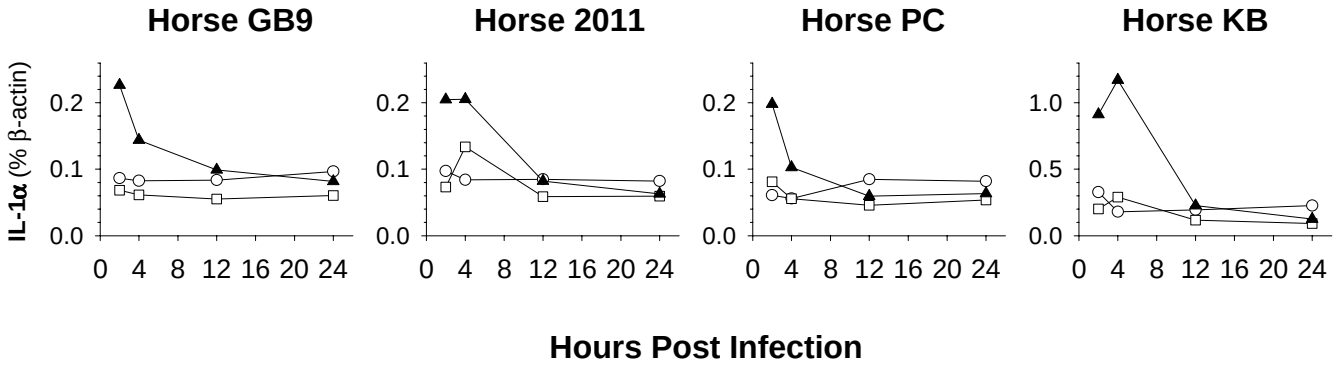


**Fig. 9.** Graphical representation of relative cytokine mRNA levels in EMDM derived from 4 horses and infected with $EIAV_{17}$ (▲) or $EIAV_{19}$ (□), or treated with CM (○). The x-axis shows the time (2, 4, 12 and 24 hpi) when cytokine mRNAs in EMDM were measured by RPA, and the y-axis indicates relative mRNA levels expressed as a percentage of β-actin mRNA. Identities of the donor horses are shown across the top. To quantitate the cytokine mRNA levels, radioactivity of the protected cytokine probe were analyzed with a phosphor imager and normalized to the β-actin mRNA levels. Each data point represents the average of duplicate measurements (total of 8 analyses per cytokine).

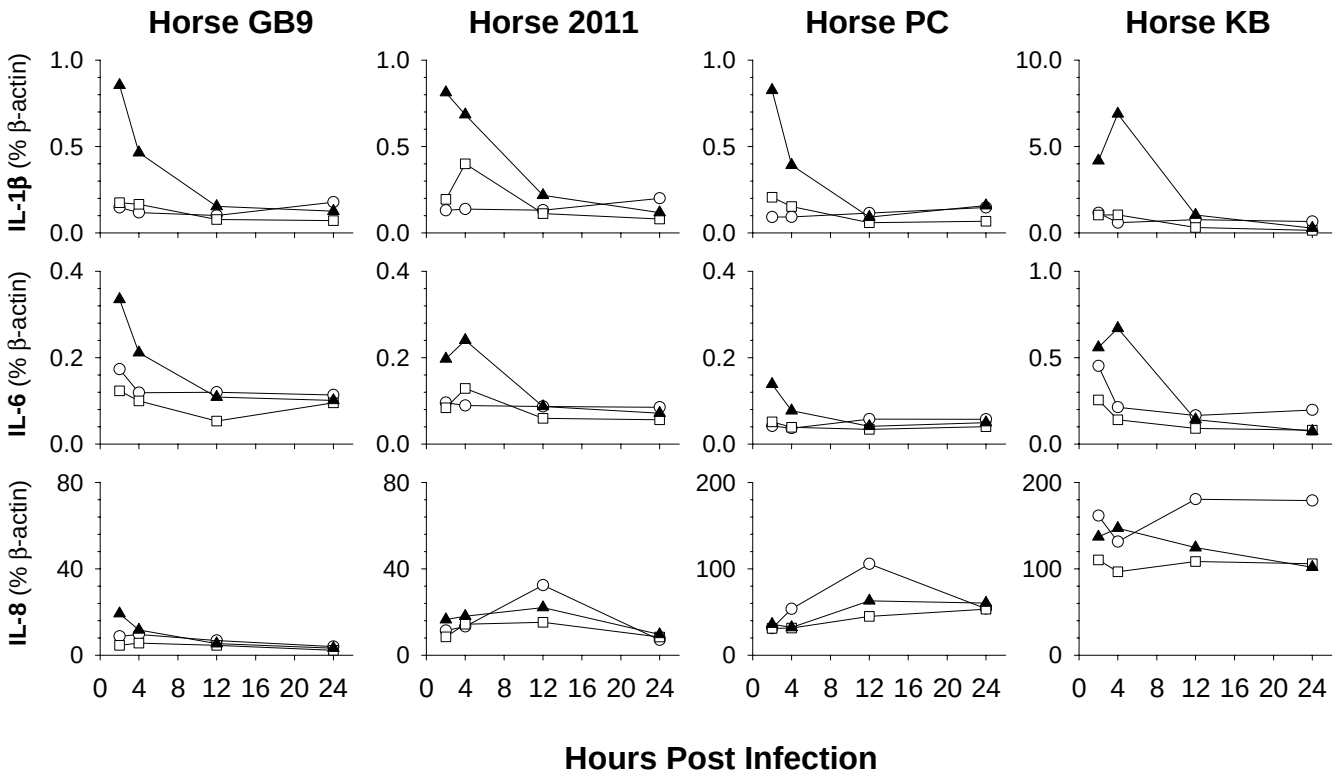


**Fig. 9.** Continued.

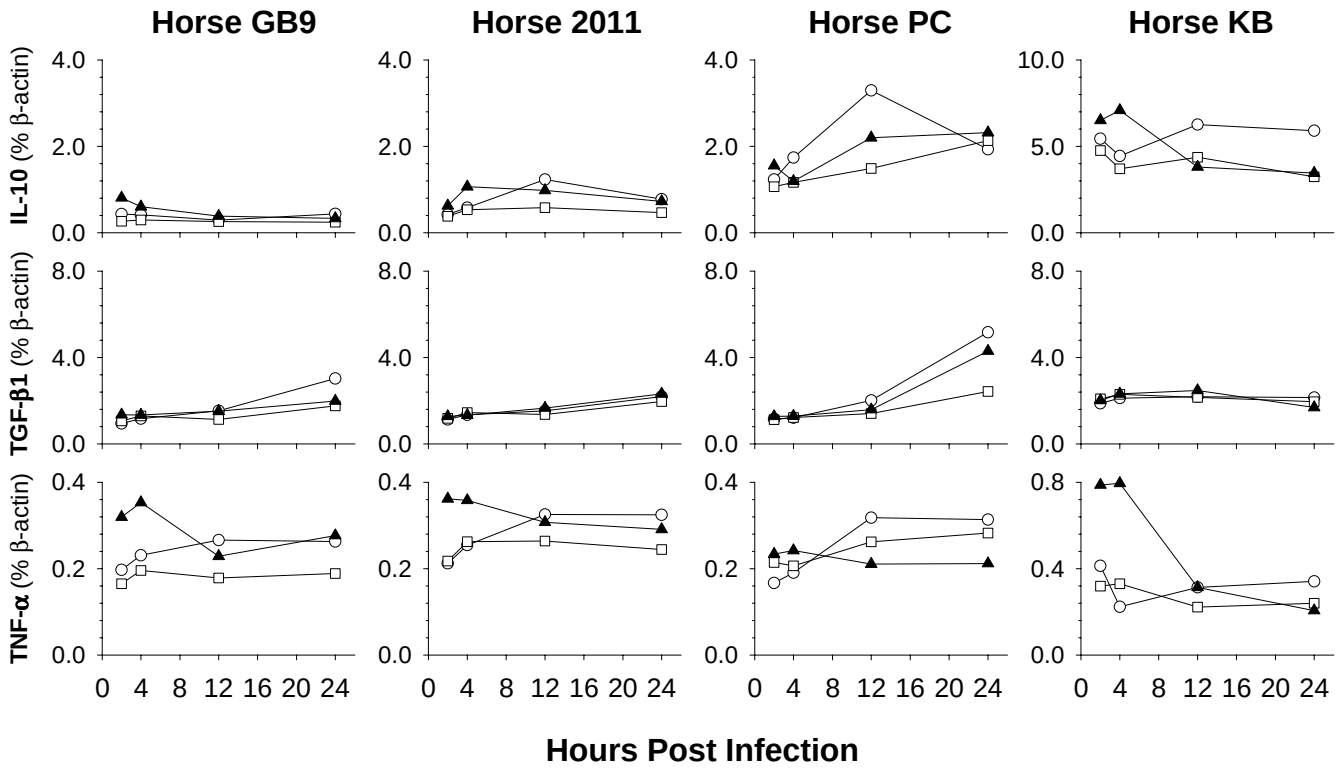


**Fig. 9.** Continued.

**Table 6**
Comparison of cytokine mRNA levels in $EIAV_{17}$- or $EIAV_{19}$-infected EMDM to CM-treated EMDM at 2 to 24 hpi

| | **$EIAV_{17}$ vs. CM** | | | | | | | | **$EIAV_{19}$ vs. CM** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2 hpi** | | **4 hpi** | | **12 hpi** | | **24 hpi** | | **2 hpi** | | **4 hpi** | | **12 hpi** | | **24 hpi** | |
| **Cytokine** | Ratio[a] | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value |
| **IL-1α** | **2.70**[b] | 0.0028 | 4.04 | 0.0107 | 1.04 | 0.7168 | 0.68 | 0.0242 | 0.74 | 0.5891 | 1.34 | 0.7441 | 0.62 | 0.0059 | 0.55 | 0.0027 |
| **IL-1β** | **4.40** | 0.0006 | 9.08 | 0.0153 | 1.35 | 0.2209 | 0.57 | 0.0192 | 1.05 | 0.9446 | 1.90 | 0.7642 | 0.50 | 0.0843 | 0.30 | 0.0007 |
| **IL-6** | **1.61** | 0.0011 | **2.62** | 0.0033 | 0.88 | 0.2232 | 0.57 | 0.0414 | 0.67 | 0.0470 | 0.89 | 0.8154 | 0.55 | 0.0003 | 0.30 | 0.0211 |
| **IL-8** | 0.98 | 0.8639 | 1.00 | 0.9669 | 0.66 | 0.0035 | 0.72 | 0.0849 | 0.73 | 0.0277 | 0.71 | 0.0277 | 0.53 | 0.0003 | 0.70 | 0.0655 |
| **IL-10** | 1.26 | 0.1592 | 1.39 | 0.0851 | 0.66 | 0.0108 | 0.75 | 0.1815 | 0.86 | 0.4336 | 0.80 | 0.3427 | 0.60 | 0.0038 | 0.67 | 0.0805 |
| **TGF-β1** | 1.16 | 0.0378 | 1.07 | 0.1715 | 1.00 | 0.9723 | 0.65 | 0.0891 | 1.07 | 0.3239 | 1.07 | 0.1532 | 0.84 | 0.0257 | 0.60 | 0.0030 |
| **TNF-α** | **1.72** | 0.0028 | **1.94** | 0.0042 | 0.87 | 0.1625 | 0.82 | 0.0622 | 0.93 | 0.7182 | 1.11 | 0.7081 | 0.76 | 0.0180 | 0.65 | 0.0395 |

[a] Ratios of cytokine mRNA levels in $EIAV_{17}$- or $EIAV_{19}$- infected EMDM over cytokine mRNA levels in CM-treated EMDM were derived from linear mixed-effect models.

[b] Bold face indicates that cytokine mRNA level is significantly increased above the CM level ($p < 0.01$).

any cytokine when compared to CM treatment (Table 6). A slight but significant ($p \leq 0.01$) decrease of IL-1α, IL-1β, IL-6, IL-8, IL-10 and TGF-β1 mRNA levels was observed in EMDM at 12 to 24 hrs after $EIAV_{19}$ infection. IFN-γ, IL-12 p35 and IL-12 p40 mRNAs were not detected in EMDM exposed to CM, $EIAV_{17}$ or $EIAV_{19}$ (data not shown).

*Kinetics of cytokine mRNA levels at 0 to 4 hpi*

Since the modulation of cytokine expression only occurred during the early stages of infection (2 and 4 hpi) and was absent at the late stages (12 and 24 hpi), we investigated the response of these cytokine genes to EIAV infection at early times in more detail. EMDM were treated with CM, $EIAV_{19}$ or $EIAV_{17}$, and cytokine mRNA levels were measured at 0.5, 1, 2 and 4 hpi. In these experiments, cytokine mRNA levels were also measured in EMDM prior to addition of EIAV or CM to establish the mRNA expression profile of the tested cytokines in untreated EMDM (considered equivalent to 0 hpi).

The phosphor image of a representative RPA revealed that the mRNA levels of IL-1α, IL-1β, IL-6, TGF-β1 and TNF-α mRNAs in CM-treated EMDM were similar to those in untreated EMDM (0 hpi), whereas mRNA levels of IL-8 and IL-10 appear to vary over time following CM treatment (Fig. 10). The representative RPA indicated IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α mRNA levels were increased in $EIAV_{17}$-infected EMDM as early as 0.5 hpi and 1 hpi. At 4 hpi, IL-1α, IL-1β and IL-6 mRNAs remained elevated in $EIAV_{17}$-infected cells. There were no apparent alterations of TGF-β1 mRNA in

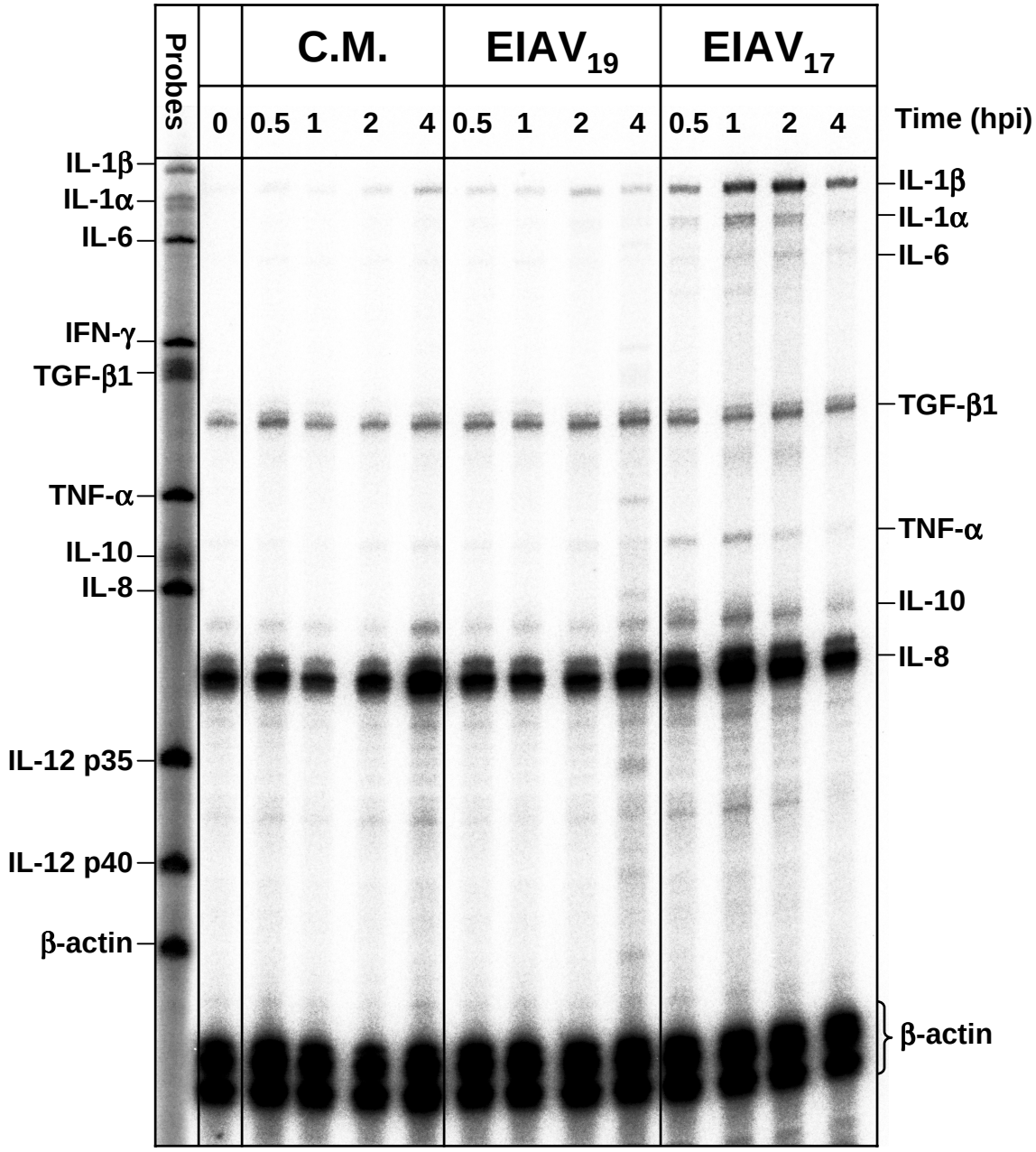


**Fig. 10**. Phosphor image showing a representative experiment of early cytokine mRNA expression in EMDM treated with CM or infected with $EIAV_{19}$ or $EIAV_{17}$ at 0.5 to 4 hpi. EMDM derived from donor horse 2025 were cultured for 7 days, treated with CM (left panel) or infected with $EIAV_{19}$ (center panel) or $EIAV_{17}$ (right panel), and harvested at 0.5, 1, 2, and 4 hpi. Lysates derived from $2.35\times10^5$ EMDM were analyzed for IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1, TNF-α and β-actin mRNA expression by RPA. Shown on the left are the positions of the full-length radiolabeled probes. Indicated on the right are positions of the individual protected fragments.

$EIAV_{17}$-infected EMDM. As previously noted and in contrast to $EIAV_{17}$, $EIAV_{19}$ infection failed to affect cytokine expression in EMDM. mRNAs of IFN-γ, IL-12 p35 and IL-12 p40 were undetected in CM-treated EMDM and were not induced by $EIAV_{17}$ or $EIAV_{19}$ infection at any of the tested times.

To ensure reproducibility, the experiment was repeated in duplicate using EMDM derived from peripheral blood of 3 different horse donors (total of 6 independent measurements). Fig. 11 shows the quantitative results of these experiments with the cytokine mRNA levels normalized to the β-actin mRNA level. These graphical representations also illustrate that the effects of EIAV infection and CM treatment on cytokines were consistent, but the relative mRNA levels were variable among EMDM of different animals (Fig. 11). Therefore, statistical significance of the changes in cytokine mRNA levels was determined by LME modeling as described and is summarized in Table 7. Statistical analyses of results from EMDM of 3 horses confirmed that there were no significant differences in IL-1α ($p = 0.5362$), IL-1β ($p = 0.2239$), IL-10 ($p = 0.3425$), TGF-β1 ($p = 0.9257$) or TNF-α ($p = 0.7205$) mRNA level between CM-treated and untreated EMDM (data not shown). IL-6 and IL-8 mRNA expression was significantly altered by CM treatment ($p = 0.0019$ and $p = 0.0008$, respectively).

In $EIAV_{17}$-infected EMDM, significant elevations of IL-1α and IL-1β expression were detected as early as 0.5 hpi and peaked at 1 hpi (Table 7). IL-1α and IL-1β mRNA levels in $EIAV_{17}$-infected EMDM were 8.7-fold and 6.2-fold, respectively, higher than those in CM-treated EMDM. At 2 and 4 hpi, IL-1α and IL-1β mRNA levels in $EIAV_{17}$-infected EMDM were still significantly higher (2.8-fold to 5.5-fold) than those in CM-treated EMDM.

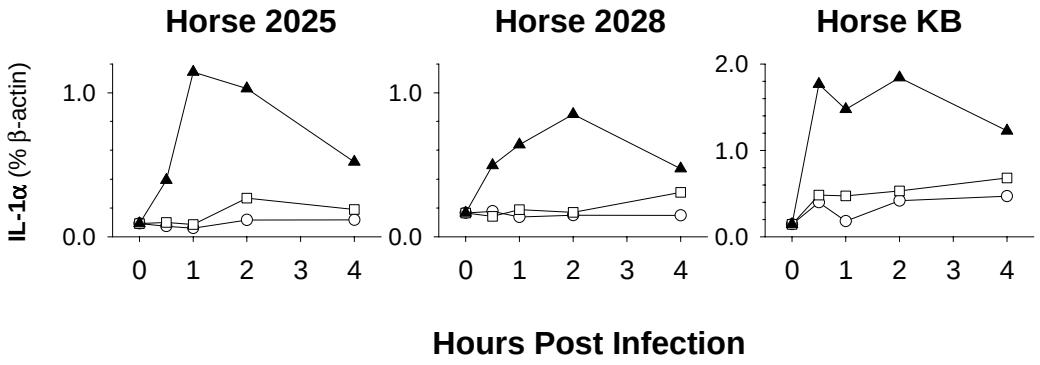


**Fig. 11.** Graphical representation of the relative cytokine mRNA levels induced early (0.5 to 4 hrs) by $EIAV_{17}$ (▲) or $EIAV_{19}$ (□) infection, or CM (○) treatment in EMDM derived from 3 horses. The x-axis shows the time (hpi) when cytokine mRNAs in EMDM were analyzed by RPA and the y-axis indicates relative mRNA levels (expressed as a percentage of β-actin mRNA). To quantitate the cytokine mRNA levels, radioactivity of the protected cytokine probe were analyzed with a phosphor imager and normalized to the β-actin mRNA levels. Identities of the donor horses are indicated across the top. Each data point represents the average of duplicate measurements.

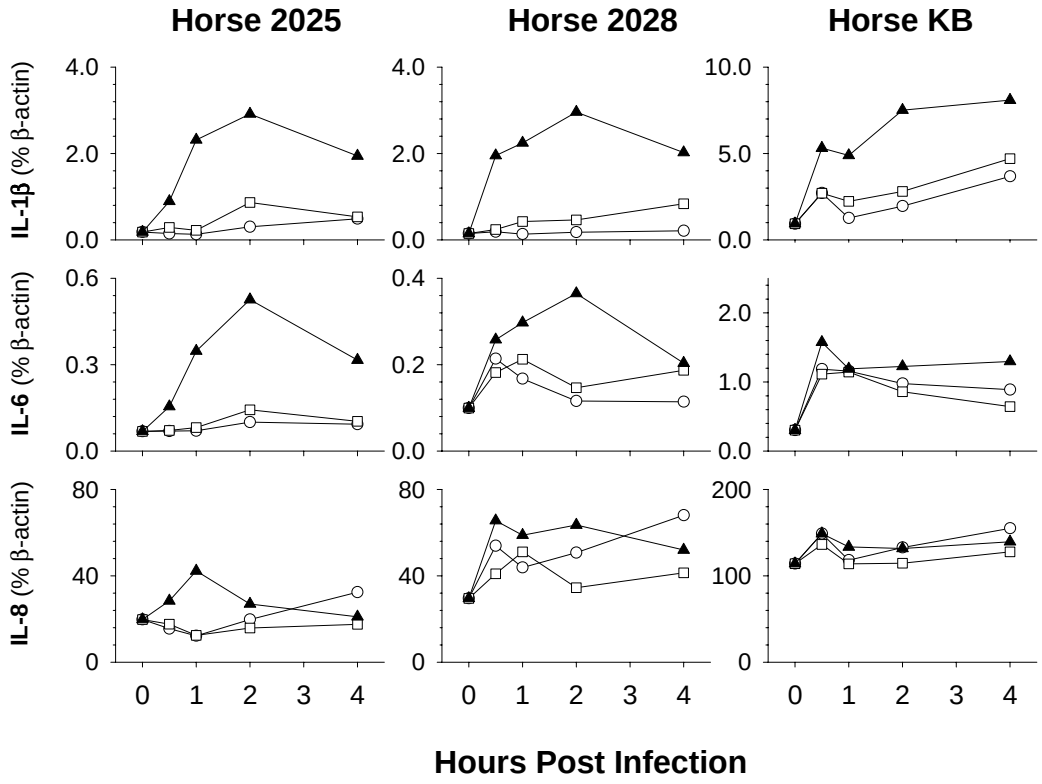


**Fig. 11.** Continued.

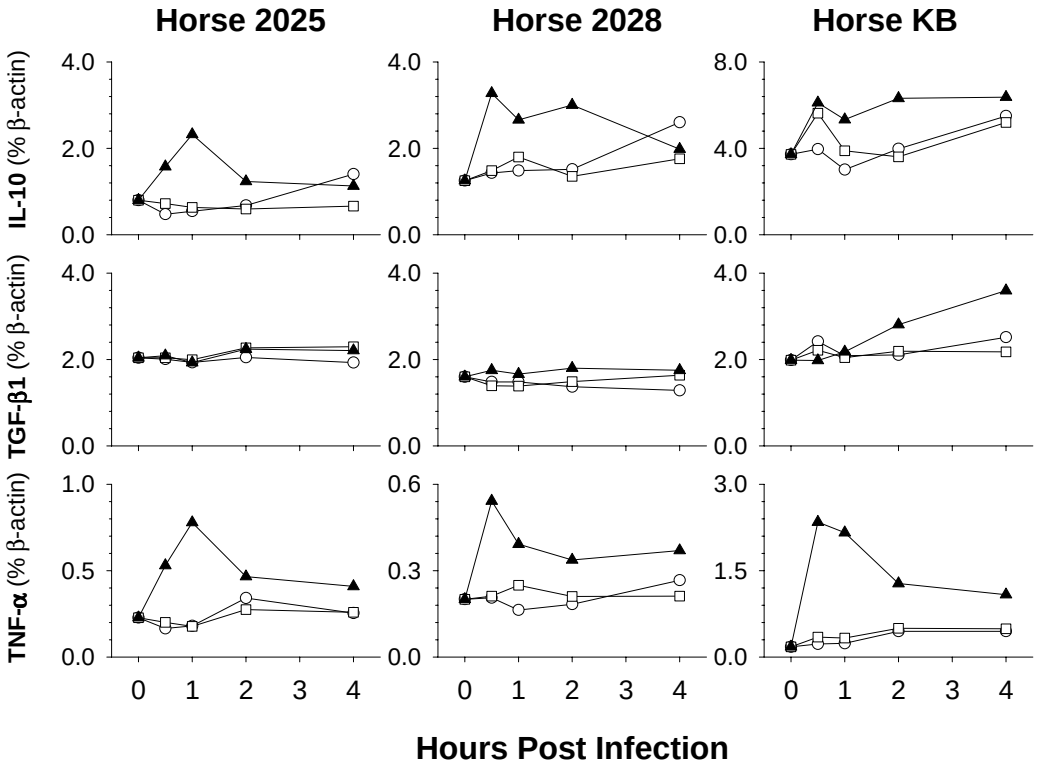


**Fig. 11.** Continued.

**Table 7**
Comparison of cytokine mRNA levels in $EIAV_{17}$- or $EIAV_{19}$-infected EMDM to CM-treated EMDM at 0.5 to 4 hpi

| | **$EIAV_{17}$ vs. CM** | | | | | | | | **$EIAV_{19}$ vs. CM** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0.5 hpi** | | **1 hpi** | | **2 hpi** | | **4 hpi** | | **0.5 hpi** | | **1 hpi** | | **2 hpi** | | **4 hpi** | |
| **Cytokine** | Ratio[a] | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value | Ratio | *P* value |
| **IL-1α** | **4.12**[b] | 0.0025 | **8.68** | 0.0001 | **5.46** | <0.0001 | **3.03** | <0.0001 | 1.12 | 0.8786 | 1.98 | 0.4464 | 1.42 | 0.4762 | 1.60 | 0.0580 |
| **IL-1β** | **2.69** | 0.0006 | **6.21** | <0.0001 | **5.51** | 0.0001 | **2.76** | 0.0003 | 1.06 | 0.8601 | 1.89 | 0.2298 | 1.70 | 0.2962 | 1.39 | 0.2522 |
| **IL-6** | 1.35 | 0.0844 | **1.31** | 0.0050 | **1.77** | 0.0001 | 1.66 | 0.0428 | 0.93 | 0.7154 | 1.03 | 0.7400 | 0.96 | 0.7135 | 0.85 | 0.6148 |
| **IL-8** | 1.11 | 0.2899 | **1.35** | 0.0006 | 1.09 | 0.1840 | 0.83 | 0.0052 | 0.89 | 0.3120 | 1.02 | 0.7997 | 0.81 | 0.0160 | 0.73 | 0.0002 |
| **IL-10** | **1.87** | 0.0004 | **2.05** | 0.0001 | **1.71** | 0.0015 | 1.00 | 0.9774 | 1.34 | 0.0727 | 1.26 | 0.1328 | 0.90 | 0.5458 | 0.80 | 0.0374 |
| **TGF-β1** | 0.99 | 0.8305 | 1.05 | 0.2512 | 1.24 | 0.0171 | 1.32 | 0.0259 | 0.96 | 0.5336 | 0.99 | 0.7365 | 1.08 | 0.3712 | 1.07 | 0.5912 |
| **TNF-α** | **5.74** | 0.0052 | **5.79** | 0.0082 | **2.15** | 0.0079 | **1.93** | 0.0063 | 1.27 | 0.8433 | 1.31 | 0.8348 | 1.02 | 0.9630 | 0.99 | 0.9769 |

[a] Ratios of cytokine mRNA levels in $EIAV_{17}$- or $EIAV_{19}$- infected EMDM over cytokine mRNA levels in CM-treated EMDM were derived from linear mixed-effect models.

[b] Bold face indicates that cytokine mRNA expression is significantly increased above the CM level ($p < 0.01$).

In contrast, IL-1α and IL-1β mRNA expression was only slightly increased (approximately 2-fold above CM) at 0.5 to 4 hpi in $EIAV_{19}$-infected EMDM, but the differences were not statistically significant (Table 7). $EIAV_{17}$-infected EMDM consistently expressed significantly higher levels of IL-1α and IL-1β mRNAs than $EIAV_{19}$-infected EMDM at all of the time points tested (data not shown).

Although an increase of IL-6 mRNA level was detected in $EIAV_{17}$-infected EMDM at 0.5 hpi (Fig. 10), the difference was not significant when compared to CM-treated EMDM until 1 and 2 hpi (Table 7). At 4 hpi, IL-6 mRNA level still appeared to be increased in $EIAV_{17}$-infected EMDM when compared to CM-treated EMDM, but again the levels were not significantly elevated. $EIAV_{17}$-induced IL-6 expression only reached 1.8-fold above CM, even at the peak level (2 hpi). Nonetheless, it is noteworthy that the $EIAV_{17}$-induced upregulation of IL-6 mRNA was significantly higher at 1 and 2 hpi when compared to CM treatment, which itself has an upregulating effect on IL-6 mRNA expression. In $EIAV_{19}$-infected EMDM, IL-6 mRNA level was not significantly different from that in the CM-treated EMDM at any time point (Table 7).

IL-8 mRNA expression was significantly increased in $EIAV_{17}$-infected EMDM at 1 hpi (Table 7). However, since IL-8 mRNA expression was also significantly increased in CM-treated EMDM at 4 hpi, we were unable to determine whether the upregulation of this gene in $EIAV_{17}$-infected EMDM was infection-specific. IL-8 mRNA expression in $EIAV_{19}$-infected EMDM also fluctuated but never significantly varied from CM levels.

IL-10 mRNA was significantly increased by $EIAV_{17}$ at 0.5 hpi, peaked at 1 hpi (2-fold increase compared to CM), and remained significantly higher than that of the CM-treated EMDM through 2 hpi (Table 7). At 4 hpi, $EIAV_{17}$-induced IL-10 expression decreased such that the mRNA level was no longer significantly different from that of CM treatment. The early and transient elevation of IL-10 mRNA expression was absent in $EIAV_{19}$-infected EMDM.

TGF-β1 mRNA was constitutively expressed in CM-treated EMDM and was not affected by infection with either $EIAV_{17}$ or $EIAV_{19}$ (Table 7).

The highest levels of TNF-α mRNA were detected in $EIAV_{17}$-infected EMDM at 0.5 and 1 hpi (Fig. 10). Although the mRNA levels decreased over time, $EIAV_{17}$-infected EMDM continued to express significantly more TNF-α mRNA than CM-treated EMDM, while $EIAV_{19}$ infection did not affect TNF-α mRNA expression (Table 7).

Taken together, these data show that there is a clear distinction between the virulent $EIAV_{17}$ and the avirulent $EIAV_{19}$ in their effects on EMDM cytokines. These differential effects on cytokine expression correlated with the virulence of the 2 molecularly cloned viruses. $EIAV_{17}$ infection of EMDM increased the levels of IL-1α, IL-1β, IL-6, IL-10 and TNF-α mRNAs, whereas $EIAV_{19}$ had no effect on any of the cytokines tested. $EIAV_{17}$-induced cytokine expression occurred early (0.5 to 4 hrs) after infection and abated by 12 hpi.

**Discussion**

This detailed study of cytokine induction by EIAV is enhanced by the used of EMDM, the natural host cells for EIAV, and the well-characterized, biologically distinct EIAV stocks. $EIAV_{17}$ is a chimeric virus contructed by substituting the LTR and *env/rev* regions of $EIAV_{19}$ with those derived from the consensus sequence of the highly virulent $EIAV_{Wyo}$ (Payne et al., 1998). $EIAV_{17}$ and $EIAV_{19}$ share common gag, pol, tat, S2 and the amino terminus of *env*, which consists of the first 89 amino acids of the *env* open reading frame and the first coding exon of *rev* (Payne et al., 1998). The most remarkable differences between $EIAV_{17}$ and $EIAV_{19}$ are found in 2 regions of SU previously identified as the primary neutralizing domain (PND) and the hypervariable domain. The PND region of EIAV SU is proposed to be functionally analogous to the V3 loop of HIV-1 SU, known to be important for macrophage/T cell tropism and receptor-mediated fusion (Ball et al., 1992). In vivo, experimental infection of Shetland ponies with $EIAV_{17}$ causes fatal acute symptoms including sustained febrile episodes and profound thrombocytopenia (Payne et al., 1998). Although $EIAV_{19}$ replicates and causes seroconversion in infected Shetland ponies, it fails to cause disease. Since both $EIAV_{17}$ and $EIAV_{19}$ replicate to high titer in primary horse macrophages in vitro, the distinct clinical manifestations of the 2 clones likely are not attributed to differential levels of viral expression, but changes in other aspects of the virus-host cell interaction (Payne et al., 1998; Payne et al., 1994; Payne et al., 1994).

To our knowledge, this is the first study to investigate the cytokine profiles in equine macrophages infected with EIAV derived from molecular clones. We

demonstrated that in vitro infection of EMDM with a stock of the highly virulent $EIAV_{17}$ upregulated mRNA expression of IL-1α, IL-1β, IL-6, IL-10 and TNF-α. Significant induction of these cytokine mRNAs was detected as early as 0.5 to 1 hr after $EIAV_{17}$ infection. The expression of these cytokines remained elevated for up to 4 hpi and returned to control levels thereafter. In contrast, EMDM infected with a stock of the avirulent $EIAV_{19}$ either showed no effects or slightly decreased cytokine expression.

Only one previous study has investigated the production of cytokines by equine macrophages in vitro in response to EIAV infection (Swardson et al., 1997). This study showed an in vitro infection of bone marrow derived macrophages (BMDM) with the mildly virulent, cell culture-adapted WSU5 strain of EIAV ($EIAV_{WSU5}$) increases IL-6 activity in culture media, which agrees with the findings of the present study. Increased IL-6 activity is also detected in serum of ponies experimentally infected with $EIAV_{Wyo}$ (Sellon et al., 1999). TNF-α activity is elevated in $EIAV_{Wyo}$ -infected Arabian foals, $EIAV_{WSU5}$-infected SCID foals (Tornquist et al., 1997) and ponies infected with PR and PV strains of EIAV ($EIAV_{PR}$ and $EIAV_{PV}$, respectively )(Costa et al., 1997). However, increase of TNF-α production was not detected in $EIAV_{WSU5}$-infected BMDM culture, which may due to the differences in strains of EIAV and/or sources of macrophages (Swardson et al., 1997). Finally, TGF-β (isoform unknown) and IFN-α levels are upregulated in plasma and bone marrow of EIAV-infected foals (Tornquist et al., 1997). As our data failed to detect an increase of TGF-β1 expression in EMDM infected in vitro by either EIAV strain, TGF-β in the infected horse may represent other TGF-β isoforms (TGF-β2 and TGF-β3) or TGF-β1 produced by other cells. Moreover, TGF-β

production by macrophages is often regulated by activation of the latent molecules instead of transcription of the gene (Toossi et al., 1995).

Macrophages are major targets for infection of other lentiviruses and have been evaluated for cytokine induction. In vitro infection of human monocyte-derived macrophages by HIV-1 increases both mRNA expression and protein production of IL-1 and TNF-α (Herbein et al., 1994; Merrill et al., 1989). Elevated levels of IL-6 are found in monocytes derived from HIV-positive donors and are induced by in vitro infection of monocytes with HIV-1 (Breen et al., 1990; Nakajima et al., 1989). However, some investigators report contradictory findings wherein pro-inflammatory cytokines are not induced by HIV infection of macrophages (Molina et al., 1990). Variations such as HIV isolate, macrophage culture methodology and time at which cytokines are analyzed could contribute to the discrepant results. Likewise, FIV infection causes elevation of IL-6 and TNF-α in feline alveolar macrophage (Ritchey et al., 2001). Microglial cells isolated from rhesus monkeys acutely infected with SIVmac produce increased levels of IL-1β, IL-6 and TNF-α, whereas cytokine production by peripheral macrophages is not altered (Sopper et al., 1996). Primary ovine microglial cultures infected with MVV also express elevated levels of IL-6 and TNF-α but not IL-1β (Ebrahimi et al., 2000). In contrast, CAEV infection of primary macrophage culture has suppressive effects on IL-1β, IL-6 and TNF-α expression (Lechner et al., 1997). In summary, upregulation of IL-1α/β, IL-6 and TNF-α expression appears to be a common outcome of lentivirus-infected macrophages with the exception of CAEV.

One of the most notable aspects of the current study is the differential effects of $EIAV_{17}$ and $EIAV_{19}$ on cytokine expression correlate with their ability to cause severe disease. Such correlation is also observed in ponies infected with the virulent $EIAV_{PV}$ and the avirulent $EIAV_{PR}$, where higher levels of TNF-α is detected in sera of $EIAV_{PV}$-infected ponies than that of $EIAV_{PR}$-infected ponies (Costa et al., 1997). In addition, the levels of serum TNF-α positively correlate with viremia, severity of clinical signs and vaccine-induced disease enhancement, suggesting a pathogenic role of TNF-α in acute EIA (Costa et al., 1997). Correlation between cytokine gene expression and viral pathogenicity has been reported in other lentiviral models. For example, monkeys acutely infected with the virulent SIVmac express higher levels of IL-1β, IL-6 and TNF-α in lymph nodes and PBMC than those infected with the avirulent derivatives of SIVmac (Zou et al., 1997; Benveniste et al., 1998). Therefore, we hypothesize that the propensity of $EIAV_{17}$ to upregulate macrophage cytokines contributes, at least in part, to the disease outcome of $EIAV_{17}$ infection.

IL-1α, IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines predominantly produced by macrophages (Murtaugh et al., 1996). IL-1 has a plethora of local and systemic biologic activities, most of which are achieved through modulating expression of the biologically active molecules within and outside the immune system (Dinarello, 1996). For example, IL-1 causes fever, anorexia and lethargy, common symptoms of EIA, by inducing expression of prostanglandins (Dinarello, 1996). IL-1 also induces expression of other cytokines, including IL-6 and TNF-α.

Biological effects of TNF-α are dose dependent, with high doses inducing shock, tissue injury, vascular leakage, hypoxia and other pathological features identical to septic shock. Prolonged exposure to low doses of TNF-α causes cachexia (Tracey and Cerami, 1994). The inflammatory effects of TNF-α are similar to those of IL-1. An abridged list of these effects includes migration of leukocytes, activation of endothelial cells and stimulation of the hypothalamic pituitary axis (HPA) (Tracey and Cerami, 1994).

Although IL-6 shares many functions of IL-1 and TNF-α, the induction of the acute phase response is the most prominent IL-6 activity in vivo, which downregulates certain aspects of the inflammatory response, including inhibition of IL-1 and TNF-α production (Gadient and Patterson, 1999). IL-6 is regarded as a B cell proliferation and differentiation factor due to its ability to promote immunoglobulin (Ig) secretion in vivo (Jego et al., 2001). IL-6 also participates in regulating various steps of hematopoiesis in vivo and is a potent cofactor of hematopoietic progenitor cells in vitro (Heike and Nakahata, 2002).

Proinflammatory cytokines (IL-1, IL-6 and TNF-α) are well-known for their pyrogenic activities (Dinarello, 1999). In addition to fever, these cytokines cause anorexia and hypermetabolism, leading to the wasting diseases commonly seen in HIV AIDS (Chang et al., 1998). Fever, anorexia and wasting also are common clinical signs of acute and chronic EIA and characteristically coincide with viremia (Montelaro et al., 1993), suggesting pro-inflammatory cytokines may play a role in EIA pathogenesis.

A correlation between elevation of plasma TNF-α levels and severity of anemia is found in ponies experimentally infected with EIAV (Costa et al., 1997). IL-1 and

TNF-α may exacerbate EIA anemia by activating macrophages and neutrophils, enhancing phagocytosis of complement-coated erythrocytes and may suppress erythropoiesis by downregulating erythropoietin production and dysregulating iron metabolism (Means, 1997; Means, 2000), both shown to contribute to EIA anemia (Sellon et al., 1994; Sentsui and Kono, 1987a; Sentsui and Kono, 1987b; Swardson et al., 1992).

There is accruing evidence for roles of cytokines in mechanisms of EIA-associated thrombocytopenia. Elevated levels of TNF-α, TGF-β and IFN-α are found in the plasma and bone marrow of EIAV-infected immunocompetent and SCID foals during and prior to the onset of thrombocytopenia (Tornquist et al., 1997). The pre-thrombocytopenic plasma has suppressive effects on proliferation of megakaryocyte colonies in culture, which are at least partially attributed to activities of TNF-α and TGF-β (Tornquist and Crawford, 1997). Human recombinant TNF-α has suppressive effects on megakaryocyte colonies in vitro (Lu et al., 2000). In addition to suppressing production of platelets, injection of purified TNF-α induces severe thrombocytopenia in mice and humans possibly by stimulating secretion of platelet agonists (Michelmann et al., 1997; Tacchini-Cottier et al., 1998). Other studies show that IL-1 and IL-6 are capable of directly activating platelets in vitro (Oleksowicz et al., 1994; Bar et al., 1997). In vivo platelet activation is observed in thrombocytopenic ponies acutely infected with $EIAV_{Wyo}$, such that the accelerated removal of activated platelets may contribute to the net decrease of platelet in circulation (Russell et al., 1999).

IL-10, also induced by $EIAV_{17}$, is regarded as an anti-inflammatory cytokine that downregulates the inflammatory reaction by inhibiting production of pro-inflammatory cytokines and bioactive prostaglandins (Moore et al., 2001). IL-10 also inhibits the antigen-presentation capabilities of monocytes/macrophages.

IL-10 and IL-6 enhance various B cell functions and increase total serum globulin and IgM concentrations, which are commonly seen in EIAV-infected horses (Sellon et al., 1994; Sellon et al., 1999; Russell et al., 1998). Polyclonal B cell activation by IL-6 and IL-10 may exacerbate some of the chronic EIA pathological features mediated by immune complexes, including hemolytic anemia, glomerulitis and hepatitis (Swardson et al., 1997). Although antibody-mediated enhancement of EIAV infection has not been directly demonstrated, vaccination of ponies with either p26 or go90 resulted in enhanced viral replication and disease severity following challenge with heterologous pathogenic strains of EIAV(Issel et al., 1992; Costa et al., 1997). These data suggest a detrimental effect of non-neutralizing antibodies.

The cytokine-inductive properties of $EIAV_{17}$ may also affect EIAV disease outcome by modulating viral replication. In HIV infection, IL-1α, IL-1β, IL-6 and TNF-α are among the cytokines best known to upregulate HIV replication in monocyte-derived macrophages and monocytic cells lines, enhancing HIV expression at the transcriptional (TNF-α) or post-transcriptional (IL-1α, IL-1β and IL-6) level (Fauci, 1996). The concept that cytokines induced by HIV-1 infection of macrophages could in turn modulate viral replication may also apply to EIAV. In experimentally infected Shetland ponies, $EIAV_{17}$ replicates to higher levels than $EIAV_{19}$, however the two viruses

replicate with comparable efficiencies in EMDM culture. We speculate that induction of the pro-inflammatory cytokines IL-6 and TNF-α contributes to the enhanced replication of $EIAV_{17}$ in vivo. This hypothesis is supported by the positive correlation of IL-6 and TNF-α serum levels with EIAV viremia in experimentally infected ponies (Sellon et al., 1999; Costa et al., 1997).

Cytokine mRNAs stimulated by $EIAV_{17}$ were expressed shortly after infection of EMDM. The kinetics of IL-1 and TNF-α mRNA upregulation by $EIAV_{17}$ is remarkably similar to that observed in HIV-1 infection (Herbein et al., 1994), whereby the rapidity of the cytokine response is due to HIV-1 Env binding to its receptor (CD4) on the macrophage surface (Chirmule and Pahwa, 1996). To date, the identity of receptor(s) for EIAV infection is unclear, however the rapid induction of cytokine mRNAs suggests that interactions between EIAV Env and macrophage surface molecules are sufficient to stimulate cytokine expression. Given the marked differences in *env* sequence between $EIAV_{17}$ and $EIAV_{19}$, it is conceivable that the differential effects on cytokine expression are due to differential Env binding and activation of surface molecule(s). $EIAV_{17}$ and $EIAV_{19}$ also have distinct Rev proteins and LTR sequences, but it is highly unlikely either of these regions impact events that occur within 0.5 to 4 hrs after EIAV infection of EMDM.

# CHAPTER IV

## INDUCTION OF EQUINE MACROPHAGE CYTOKINES BY THE VIRULENT MOLECULARLY CLONED EQUINE INFECTIOUS ANEMIA VIRUS ($EIAV_{17}$) IS INDEPENDENT OF VIRAL REPLICATION

p19/wenv17 is a virulent EIAV molecular clone generated by replacing the LTR, *env* and *rev* of the avirulent EIAV clone pSPeiav19 with sequences derived from the Wyoming field strain EIAV ($EIAV_{Wyo}$) (Payne et al., 1998). We previously demonstrated infection of equine monocyte-derived macrophages (EMDM) by virus derived from p19/wenv17 ($EIAV_{17}$) causes an increase of IL-1α, IL-1β, IL-6, IL-10 and TNF-α mRNA expression (Lim et al., 2003b). In contrast, the virus derived from pSPeiav19 ($EIAV_{19}$) fails to stimulate cytokine expression in cultured equine macrophages. Since both viruses replicate to similar levels in EMDM cultures, their differential effects on cytokine expression are not simply due to differences in viral loads (Lim et al., 2003b).

The goal of this study is to begin to elucidate mechanisms by which $EIAV_{17}$ induces cytokine expression. The rapid induction (0.5 to 4 hrs post-infection, hpi) of cytokine expression in $EIAV_{17}$-infected EMDM suggests that cytokines are stimulated by early events following exposure of the virus to the macrophages (Lim et al., 2003b). Since binding of the viral Env glycoprotein to cell surface molecules comprises the earliest interaction between a retrovirus and its host cell (Hunter, 1997), we hypothesized that $EIAV_{17}$ induces cytokines by binding to surface molecules of equine macrophages.

In support of this hypothesis, the most remarkable sequence differences between $EIAV_{17}$ and $EIAV_{19}$ are found in the Env protein (Payne et al., 1998). The divergence in Env may affect the interactions of the 2 viruses with macrophage surface molecules and lead to their differential effects on cytokine expression. Although sequence differences are also found in the Rev proteins and U3 regions of $EIAV_{17}$ and $EIAV_{19}$, they are unlikely to affect the early events of viral infection (Payne et al., 1998). To further demonstrate that cytokine induction is mediated by $EIAV_{17}$ binding, we exposed EMDM to UV-inactivated $EIAV_{17}$ (UV-$EIAV_{17}$) and monitored cytokine induction to test if viral replication is required for upregulation of cytokines.

EMDM cultures were established from peripheral blood of EIA-negative donor horses as previously described (Lim et al., 2003b). EMDM cultures were utilized to amplify $EIAV_{17}$ generated from p19/wenv17-transfected D17 cells (canine lung osteosarcoma, ATCC CCL-183). The amplified $EIAV_{17}$ were ultra-filtered as previously described (Lim et al., 2003b). Supernatants of mock-infected EMDM (termed control media, CM) were collected, ultra-filtered under the same conditions and served as the negative control.

For UV irradiation, 6 ml of $EIAV_{17}$ or CM were layered in a 150×25mm petri dish and kept on ice. The open dish was exposed to a 1330 mW/cc UV bulb at a 15cm distance for 30 min with agitation every 10 min. The effects of UV irradiation on $EIAV_{17}$ infectivity were determined by comparing the growth of $EIAV_{17}$ and UV-$EIAV_{17}$ in EMDM cultures. EMDM were exposed to UV-$EIAV_{17}$ stock containing 3000 cpm of reverse transcriptase (RT) activity or were mock-infected with UV-irradiated CM (UV-

CM), and virus replication was monitored by measuring the RT activity in culture supernatants. As expected, culture supernatants of $EIAV_{17}$-infected EMDM showed a steady increase of RT activity from 4 to 12 days post-infection (dpi), indicating production and release of virions (Fig. 12). In contrast, there was no increase of RT activity in supernatants of EMDM cultures exposed to UV-$EIAV_{17}$ or UV-CM. These results confirm that the UV irradiation protocol employed was sufficient to block the replication of $EIAV_{17}$ in EMDM.

The replication-defective UV-$EIAV_{17}$ was then tested for its effects on IL-1α, IL-1β, IL-6, IL-10 and TNF-α mRNA expression in EMDM, since our previous study shows that $EIAV_{17}$ induces elevation of these cytokines (Lim et al., 2003b). EMDM cultured for 6 days were transferred to 6-well plates at $2.35\times10^5$ cells per well. Twenty-four hrs later, each well of EMDM was exposed to 0.5 ml of UV-$EIAV_{17}$ (containing 30,000 cpm of RT activity) or UV-CM. EMDM cytokine mRNA levels at 0, 0.5, 1, 2 and 4 hrs post exposure (hpe) was analyzed by multi-probe RPA using radiolabeled RNA probes specific to equine cytokine genes followed by phosphor imaging (Lim et al., 2003a).

Fig. 13 shows representative data from an experiment performed using EMDM derived from a single donor horse. Each treatment was replicated in 3 separate wells of EMDM. The relative cytokine mRNA level at each time point represent the mean ± standard error (SE) of 3 parallel measurements. Significant differences between treatments were determined with 2-tailed Student's *t* test. $P < 0.05$ was considered

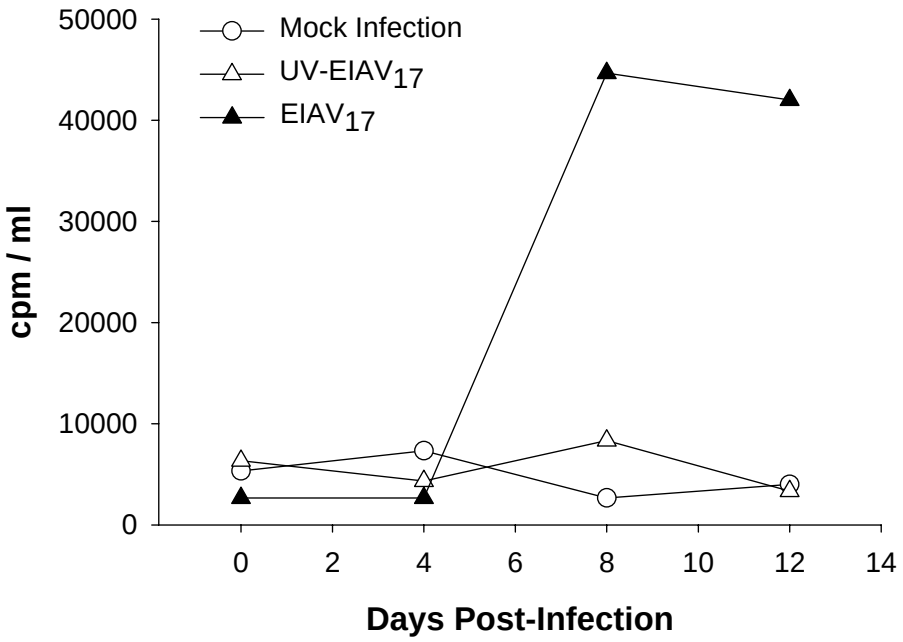


**Fig. 12.** Graphical representation of RT activities in EIAV-infected EMDM culture supernatants. EMDM cultures were infected with 3000 cpm of $EIAV_{17}$ (▲) or $UV\text{-}EIAV_{17}$ (△) or mock-infected (○). Aliquots of supernatants were removed from the cultures at 0, 4, 8 and 12 dpi and analyzed by RT activity assay. The x-axis indicates time, and the y-axis indicates RT activity expressed as cpm/ml.

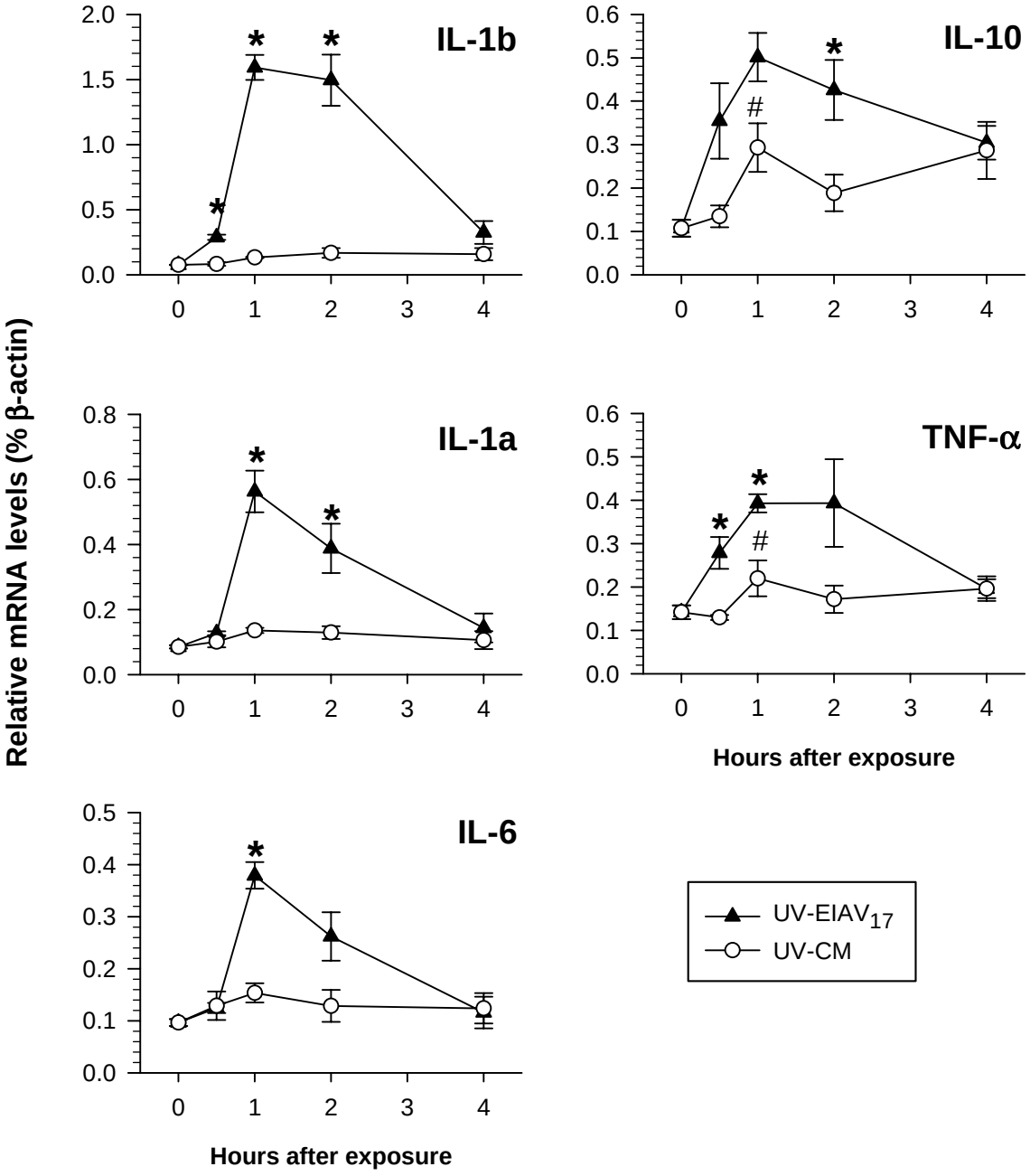


**Fig. 13.** Graphical representation of relative cytokine mRNA levels in EMDM exposed to UV-EIAV$_{17}$. EMDM were exposed to UV-EIAV$_{17}$ (▲) or UV-CM (○), and cytokine expression was analyzed with RPA at 0, 0.5, 1, 2 and 4 hrs after exposure. To quantitate cytokine mRNA levels, the radioactivity of the protected cytokine probe were analyzed with a phosporimager and normalized to the β-actin mRNA levels. The x-axis indicates the time when cytokine mRNAs in EMDM were analyzed (hrs post exposure), and the y-axis indicates relative mRNA levels (expressed as a percentage of β-actin). Each data point represents the means ± SE of triplicate measurements. Asterisks (∗) indicate significant differences ($p < 0.05$) between EMDM exposed to UV-EIAV$_{17}$ and UV-CM. Pound signs (#) indicate significant differences ($p < 0.05$) between EMDM exposed to UV-CM and untreated EMDM (0 hpe).

significantly different. The results described below were reproduced in experiments performed using EMDM derived from 3 other horses.

In untreated EMDM (0 hpe), IL-1α, IL-1β, IL-6, IL-10 and TNF-α mRNAs were expressed at barely detectable levels (Fig. 13). Exposure to UV-CM did not affect the expression levels of IL-1α, IL-1β and IL-6 at any of the tested time points (Fig. 13). IL-10 and TNF-α expression slightly but significantly ($p < 0.05$) increased at 1 hpe of EMDM to UV-CM. These 2 cytokines were not affected by UV-CM at other time points. A slight increase of IL-10 expression was observed in CM-treated EMDM in our previous study (Lim et al., 2003b). The reason for TNF-α induction in EMDM exposed to UV-CM is unclear.

In EMDM exposed to UV-EIAV$_{17}$, IL-1β and TNF-α expression was significantly increased as early as 0.5 hpe when compared to UV-CM treatment (Fig. 13). IL-1β expression in EMDM exposed to UV-EIAV$_{17}$ peaked at 1 hpe (12-fold above UV-CM level) and returned to basal level at 4 hpe. TNF-α expression in UV-EIAV$_{17}$-treated EMDM remained significantly elevated at 1 hpe. At 2 hpe, the mean TNF-α mRNA level did not decrease in UV-EIAV$_{17}$-exposed EMDM but was not significantly different ($p = 0.1043$) from that of UV-CM treatment, likely due to the large variance of UV-EIAV$_{17}$-induced TNF-α expression (Fig. 13).

IL-1α and IL-6 mRNA levels in EMDM were significantly elevated (4.1-fold and 2.5-fold above UV-CM levels, respectively) at 1 hpe to UV-EIAV$_{17}$ (Fig. 13). IL-1α expression remained significantly elevated through 2 hpe and returned to basal level by 4

hpe. Although IL-6 expression in EMDM exposed to UV-EIAV$_{17}$ was noticeably higher than that in UV-CM-treated EMDM at 2 hpe, the difference was not significantly different. Again, this is likely due to the large variance of UV-EIAV$_{17}$-induced IL-6 expression at this time point (Fig. 13).

IL-10 appeared to increase in EMDM exposed to UV-EIAV$_{17}$ for 0.5-2 hrs (Fig. 13). However statistical analysis revealed that the increase was significant only at 2 hpe. Although IL-10 expression peaked at 1 hpe to UV-EIAV$_{17}$, the increase was not significant due to the concomitant increase of IL-10 expression in UV-CM-treated EMDM (Fig. 13).

In summary, exposure of EMDM to UV-EIAV$_{17}$ resulted in increased expression of IL-1α, IL-1β, IL-6, IL-10 and TNF-α, the cytokines shown stimulated by EIAV$_{17}$ infection (Lim et al., 2003b). IL-1α, IL-1β, IL-6, and TNF-α were rapidly induced within 0.5 to 1 hpe to UV-EIAV$_{17}$**,** akin to the kinetics of EIAV$_{17}$-induced cytokine expression (Lim et al., 2003b). Since UV-EIAV$_{17}$ is unable to replicate in EMDM, these results indicate that viral replication is not required for induction of cytokines by EIAV$_{17}$. In addition, UV irradiation does not affect binding of HIV-1 to target cells (Henderson et al., 1992). Taken together, these show that EIAV$_{17}$ activation of cytokine gene expression is replication-independent and suggest binding of EIAV$_{17}$ to equine macrophage surface molecules is sufficient.

It is well established that binding of HIV-1 Env to the CD4 cellular receptor induces expression of a plethora of host-cell genes including various cytokines and chemokines (Chirmule and Pahwa, 1996; Cicala et al., 2002). IL-1, IL-6, IL-10 and

TNF-α are among the cytokines that are induced by binding of HIV-1 Env to human macrophages (Nakajima et al., 1989; Herbein et al., 1994; Merrill et al., 1989; Akridge et al., 1994). Remarkably, the kinetics of cytokine induction by HIV-1 binding bears striking resemblance to that of $EIAV_{17}$ and UV-$EIAV_{17}$. For example, IL-1β and TNF-α mRNA levels increase in human macrophages within 0.5-3 hrs after HIV-1 infection and decrease to control levels thereafter (Herbein et al., 1994). Similarly, elevation of IL-10 mRNA in human macrophages is detected as early as 3 hpe to HIV-1 (Akridge et al., 1994). In agreement with our observation, blocking HIV-1 replication by heat-inactivation or β-propiolactone does not affect the induction of IL-1, IL-6 and TNF-α by HIV-1 binding (Nakajima et al., 1989; Merrill et al., 1989).

While the transmembrane (TM) region of HIV-1 Env induces some cytokines, most of the Env-mediated cytokine activation are the results of CD4 receptor binding by hte SU region (gp120) (Koka et al., 1995; Cicala et al., 2002; Chirmule and Pahwa, 1996). Binding of HIV-1 SU to CD4 transduces intracellular signaling events that activate the transcription factors regulating cytokine expression. A study using truncated proteins and synthetic peptides of HIV-1 SU shows that many different regions of HIV-1 SU induce cytokines (Koka et al., 1995). Among these regions, the third variable domain (termed the V3 loop), which is a major determinant of cell tropism and coreceptor usage, is consistently identified as the critical domain for induction of cytokines in different cell types (Ankel et al., 1994; Koka et al., 1995; Khanna et al., 2000).

In support of the concept that induction of cytokines is mediated by $EIAV_{17}$ binding, the most remarkable differences between $EIAV_{17}$ and $EIAV_{19}$ are found within SU (31 out of 443 amino acids, or 7.0%) (Payne et al., 1998). In addition, the divergent amino acids are concentrated in 2 previously identified variable domains of SU (Payne et al., 1998). One of these regions is termed the principal neutralizing domain and is predicted to contain structural features analogous to the V3 loop of HIV-1 (Ball et al., 1992). In this domain, the amino acid sequences of $EIAV_{17}$ and $EIAV_{19}$ differ by as much as 32.4% (11 out of 34 amino acids). It is possible that these domains are critical for binding of $EIAV_{17}$ to macrophage surface molecules, and the sequence variations result in the inability of $EIAV_{19}$ to induce cytokines. Additional studies are needed to confirm this hypothesis.

Since the receptor for EIAV has not been identified, we were unable to directly assess the roles of macrophage surface receptor(s) in cytokine induction. Recombinant $EIAV_{17}$ SU has been successfully expressed in *Spodoptera frugiperda* (SF9) cells by the baculovirus expression system (S. L. Payne, unpublished data). Investigating the effects of purified $EIAV_{17}$ SU on EMDM cytokines will provide further evidence for the mechanism of $EIAV_{17}$-mediated cytokine induction.

# CHAPTER V

# DISCUSSIONS AND CONCLUSIONS

## Discussion

*Comparison of RPA to other methods used to analyze equine cytokine expression*

Because equine cytokine reagents are not commercially available, the first goal of this study was to develop an assay for quantitating equine cytokine expression. After comparing several methods that have been utilized by other laboratories to analyze specific equine cytokines, we concluded that RPA has the features that best serve our aims to examine a large variety of cytokines.

In the past, investigators adapted cell line-based bioassays to measure the secreted and biologically active equine cytokines, such as IL-2, IL-6, IFN, TNF-α and TGF-β1 (MacKay et al., 1991; Morris et al., 1992; Allen et al., 1993; Ellis et al., 1995; Charan et al., 1997). However, these assays are very cumbersome and obviously not suitable for screening the response of a large variety of cytokines as required by this study.

The lack of appropriate antibodies has hindered analysis of equine cytokines by immunological assays. To date, a monoclonal antibody specific to equine TNF-α and an antiserum against recombinant equine IL-8 have been produced and show reactivity in immunohistochemistry and dot blot assays, respectively (Franchini et al., 2000; Grunig and Antczak, 1995). However, these antibodies are not commercially

distributed. Detection of equine TGF-β using antibodies specific to human or porcine TGF-β has been successful because this gene is highly conserved among different species (Chesters et al., 2000; Rodriguez et al., 1996).

With a rapidly increasing number of published equine cytokines gene sequences, it is now feasible to characterize cytokine responses by DNA- or RNA-based techniques. For example, RT-PCR, RT-cPCR and Southern blot techniques have been developed to measure limited equine cytokine mRNA levels (Grunig and Antczak, 1995; Rottman et al., 1996; Byrne et al., 1997; Swiderski et al., 1999; Giguere and Prescott, 1999; Leutenegger et al., 1999). An important advantage of the RPA over other molecular techniques is that up to 10-12 cytokines can be simultaneously quantitated when a multi-probe system is employed. Although it is possible to detect RNA of multiple genes on a single membrane in Northern blot analysis by stripping and reprobing, it is comparatively laborious and, more importantly, sensitivity is lost during the process. The equine cytokine-specific multi-probe RPA described in this study is capable of simultaneously quantitating 10 equine cytokines in a single reaction. This feature allows screening of a large number of cytokines in a time-, reagent-, and sample-saving manner. In addition, given that cytokines have redundant functions and complex interactions with each other, simultaneous profiling of multiple cytokines is frequently necessary to fully assess the contributions of individual cytokines in a physiologically relevant context. Another advantage of RPA over Northern blot analysis is its superior sensitivity (Davis et al., 1997; Tymms, 1995). The lysate-based RPA used in this study has been shown to detect $10^4$-$10^5$ target mRNAs or less than 0.5 molecules per cell (Haines and Gillespie,

1992). The sensitivity of the RPA is only rivaled by RT-PCR based techniques. However, the RT-PCR techniques lack the capability of simultaneous detection and are best suited for analysis of a limited number of cytokines (Giguere et al., 1999). They are relatively labor-intensive when applied to screening a wide variety of cytokines. Taken together, these features of the RPA make it superior to the other RNA quantitation techniques.

The multi-probe RPA developed in this study simultaneously analyzes the mRNA levels of equine IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IFN-γ, TGF-β1, TNF-α and β-actin (an internal control) in EMDM and EPBMC cultures (Lim et al., 2003a). This assay detects as few as $5\times10^5$ copies of cytokine RNA molecules and has a CV of 0.03-0.08 (or 3%-8%) when using the β-actin mRNA level to normalize the sample-to-sample variations.

*Relationship between EIAV replication and virulence*

In EIAV-infected horses, the disease episodes during the acute and chronic phases coincide with high titers of viremia (Issel and Coggins, 1979). In asymptomatic animals, the viral load is very low and can only be detected by PCR (Oaks et al., 1998). According to a widely accepted model, reduction of EIAV viral load is due to humoral and cellular immune responses that clear the predominant viral population, such that a new episode of viremia reflects the growth of a different quasispecies that temporarily escapes immunologic control (Leroux et al., 1997). Ultimately, an enduring immunity specific to a broad array of EIAV quasispecies is developed, and the animals become

clinically asymptomatic. Upon closer inspection, it is clear that immunity is not the only factor that controls viral replication. For example, the highly virulent $EIAV_{Wyo}$ replicates to greater levels than the less virulent $EIAV_{WSU}$ and $EIAV_{PV}$ in infected horses during both the acute phase, prior to the development of an immune response, and during the subclinical phase (Oaks et al., 1998; Harrold et al., 2000). Studies also show that the reduction of viral load in asymptomatic animals is associated with a restriction of viral transcription (Oaks et al., 1998; Harrold et al., 2000). In agreement with these findings, levels of HIV-1 transcription in PBMC are shown to be predictive of disease progression and severity (Michael et al., 1995; Saksela et al., 1994). Therefore, it appears that not only is EIA disease severity correlated with viral load, but the virulence of a virus is also correlated with its in vivo replicative potential. The replicative potential of a virus is presumably a result of interactions between viral and host factors that are yet to be identified. Viral factors that may affect EIAV expression include the U3-LTR transcription factor binding motifs and the *rev* gene (Maury, 1998; Belshan et al., 2001). In comparing HIV-1 RNA sequences between nonprogessive and progressive infection, a large deletion is found in the predominant U3-LTR sequences during the nonprogressive infection (Fang et al., 2001). The CAT reporter gene assay shows that this deletion causes downregulation of LTR-driven transcription in vitro. At least one host cell factor, the B-cell- and macrophage-specific transcription factor PU.1, has been shown to control the expression of EIAV in primary equine macrophages (Maury, 1994).

Cytokines are among the best known host factors that modulate HIV-1 replication (Fauci, 1996). The current study demonstrates that the virulent $EIAV_{17}$

induces the expression of IL-1α, IL-1β, IL-6, IL-10 and TNF-α, while the avirulent $EIAV_{19}$ has no effect on these cytokines. TNF-α is the most thoroughly studied HIV-inducing cytokine. It induces HIV-1 transcription by activating the cellular transcription factor NF-κB (Fauci, 1996). IL-1α and IL-1β also enhance HIV transcription presumably through NF-κB-independent pathways, and IL-6 upregulates HIV production at post-transcriptional levels (Vicenzi et al., 1997). The effects of IL-10 are equivocal. On the one hand, IL-10 suppresses HIV replication by inhibiting the production of pro-inflammatory cytokines (Breen, 2002). On the other hand, adding IL-10 at low doses has been shown to enhances HIV replication. It is likely that the same cytokines impact the expression of EIAV.

In vitro studies of cytokine-regulated EIAV expression have generated conflicting results. Electropheretic mobility shift and CAT reporter gene assays demonstrate that the myeloid/B cell-specific transcription factor PU.1 is an important regulator of EIAV LTR transcription (Maury, 1994). As PU.1 is an important intermediate for the induction of gene expression by cytokines such as GM-CSF, M-CSF and IFN-γ, EIAV transcription may be susceptible to cytokine modulation (DeKoter et al., 1998; Nguyen and Benveniste, 2002). Stimulation of promonocytic cell line U937 transiently transfected with an EIAV LTR/CAT construct with phorbol myristate acetate (PMA) increases CAT activity (Carvalho et al., 1993). These results support the enhancing effects of cytokines on EIAV replication since PMA is a potent inducer of macrophage cytokines. Other investigators report that stimulation of EMDM by PMA or bacteria endotoxin, which also induces a wide variety of cytokines, inhibits the

production of EIAV (Sellon et al., 1996; Smith et al., 1998). Although TNF-α production is detected following stimulation with PMA or endotoxin, it does not correlate with suppression of EIAV replication in the macrophages (Smith et al., 1998). PMA-induced downregulation of HIV production in chronically infected promonocytic cells is mediated by the activity of IFN-γ, which suppresses HIV maturation and budding (Vicenzi et al., 1997). According to the current study, $EIAV_{17}$ does not replicate at higher levels than $EIAV_{19}$ in primary equine macrophage cultures, despite its ability to induce cytokine expression, suggesting that these cytokines do not directly modulate EIAV expression in infected macrophages.

In contrast to the similar growth rates in culture, $EIAV_{17}$ replicates to a higher titer than $EIAV_{19}$ in infected Shetland ponies (S. L. Payne, personal communication). We speculate that induction of cytokines contributes to the enhanced replication of $EIAV_{17}$ in vivo via paracrine or endocrine actions. In support of this hypothesis, serum levels of two pro-inflammatory cytokines induced by $EIAV_{17}$, TNF-α and IL-6, are shown to positively correlate with EIAV viremia in experimentally infected ponies (Sellon et al., 1999; Costa et al., 1997). Pro-inflammatory cytokines may increase virus load in $EIAV_{17}$-infected animals by recruiting uninfected monocytes to the site of viral replication through stimulation of monocyte-chemotactic factors, such as monocyte chemoattractant protein-1, 2 and 3 (MCP-1, 2, and 3, respectively), macrophage inflammatory proteins 1α (MIP-1α), MIP-1β and RANTES (Wetzel et al., 2002; Tofaris et al., 2002; Loghmani et al., 2002; Mukaida et al., 1998). This mechanism is employed by HIV and SIV to recruit target cells to the brain and lymph nodes (Schmidtmayerova

et al., 1996; Zink et al., 2001; Wetzel et al., 2002). HIV and SIV are shown to directly stimulate production of chemokines by macrophages, including MCP-1, macrophage inflammatory proteins 1α and 1β and RANTES (Wetzel et al., 2002). Hence, investigation of the direct and indirect effects of $EIAV_{17}$ infection on the expression of these cytokines is of great interest. Other mechanisms by which the $EIAV_{17}$-induced cytokines may enhance viral load in vivo include stimulation of adhesion molecules that promote monocyte migration into tissue (Sampson et al., 2002) and expression of EIAV-activating molecules by non-monocytic cells.

This study reveals cytokine induction as a distinguishing property for the virulent versus avirulent EIAV. We speculate that this property plays important roles in the enhanced viral load and disease severity associated with virulent EIAV infection. Conversely, the inability to induce cytokines and reduction of viral expression may be an important mechanism for EIAV persistence of avirulent virus.

*EIAV virulence determinants*

The viral factors that control the clinical manifestations of EIAV infection are unknown. The successful generation of the virulent $EIAV_{17}$ clone used in the current study is due to the insertion of $EIAV_{Wyo}$ LTR, *rev* and *env* into avirulent $EIAV_{19}$ (Payne et al., 1998). The rationale for this modification is that these regions are the "hot spots" in the quasispecies evolution of EIAV (Lichtenstein et al., 1996; Belshan et al., 2001; Leroux et al., 1997) and the same regions show major sequence differences between the virulent EIAV isolates and the avirulent cell culture-adapted viruses (Payne et al., 1994;

Perry et al., 1992). Likewise, the virulent molecular clone ($EIAV_{UK}$) developed by Cook et al. also predominantly differs from its avirulent parental clone in the LTR, *rev* and *env*, although its construction also involves substitution of the integrase, S1 and S2 genes (Cook et al., 1998). Taken together, it is apparent that one or a combination of these factors contains the determinants of EIAV virulence.

LTR is considered a potential EIAV virulence determinant because a clear demarcation between the virulent and avirulent EIAV strains is consistently found in the hypervariable regions of the U3 region of the LTR. Analyses of the consensus LTR sequence of the Wyoming wild type strain ($EIAV_{Wyo}$) reveal that its U3 region contains 3 *ets*, 1 CREB binding sites and a double CAAT-box motif (Payne et al., 1999; Cook et al., 1998). The U3-LTRs of virulent strains of EIAV, including the virulent molecular clones (Payne et al., 1998; Cook et al., 1998), the fibroblast-tropic pathogenic PV strain (Cook et al., 1998) and LTRs PCR-amplified from samples of acutely ill EIA horses (Maury et al., 1997) are highly homologous to the U3 sequence of Wyoming LTR. All of these virulent LTRs contain 3 *ets* sites and 1 CREB site and vary only in the number of PEA-2 and CAAT-box motifs. In comparison, the U3 sequences of avirulent EIAVs are more divergent from the Wyoming U3-LTR, typically having only 1 to 2 *ets* sites, 2 PEA2 sites, 2 CREB site and one CAAT-motif box (Payne et al., 1994; Cook et al., 1998; Maury et al., 1997; Cook et al., 1998). Macrophages are the primary sites of EIAV replication in vivo, and the *ets* motifs have been shown to be crucial for EIAV LTR-driven transcription in primary equine macrophages (Maury, 1994). It follows that variations in the transcription factors may affect EIAV virulence by altering viral load

and/or cell tropism. Nonetheless, insertion of the U3 sequence derived from the virulent PV strain into the LTR of an avirulent EIAV molecular clone fails to generate a virulent virus, indicating that LTR is not the sole virulence determinant (Lichtenstein et al., 1996). $EIAV_{19}$ and $EIAV_{17}$ have U3 LTR sequences that are typical of virulent and avirulent EIAV, respectively (Payne et al., 1998). In particular, $EIAV_{17}$ carries all 3 to the *ets* motifs while $EIAV_{19}$ only has 2. In support of the role of LTR in EIAV virulence, lower levels of viremia were observed in the $EIAV_{19}$-infected animals compared to those infected with $EIAV_{17}$ (F.J. Fuller, unpublished data). However, the current study shows that there is no difference between the growth of $EIAV_{17}$ and $EIAV_{19}$ in primary equine macrophage culture. This contradiction emphasizes the complex in vivo factors affecting LTR transcription that are not reflected in the in vitro system.

Rev modulates the expression of structural genes and virion production by regulating nuclear export of incompletely spliced viral RNA (Belshan et al., 2000). It is therefore likely that variation in Rev may impact virus load and hence pathogenesis of EIAV. To test this hypothesis, Belshan et al. studied genetic and biological changes of *rev* variants amplified from the inoculum and serum samples collected at different times or disease stages of an $EIAV_{Wyo}$-infected horse (Belshan et al., 2001). The Rev quasispecies of the inoculum and the acute and afebrile stages are predominated by the same variant, while novel variants appear and predominate during the recurrent and late febrile episodes. Moreover, the predominant Rev variants associated with the febrile episodes have significantly higher nuclear export activity than those associated with the

acute and afebrile stages, suggesting a correlation between Rev activity and EIA disease progression. Contradictory to the prediction that Rev affects pathogenesis by modulating viral replication, Rev activity does not correlate with in vivo virus load (Belshan et al., 2001). Therefore, the mechanism by which Rev alters EIAV pathogenicity is still unknown.

*Is SU an EIAV virulence determinant?*

We propose that the SU glycoprotein of EIAV is a virulence determinant based on (1) the differential effects of $EIAV_{17}$ and $EIAV_{19}$ on macrophage cytokine expression, as found in the current study; (2) the conferral of virulence to the avirulent $EIAV_{19}$ by insertion of $EIAV_{17}$ SU; and (3) the differences in the enterotoxic activities between purified SU glycoproteins derived from $EIAV_{17}$ and $EIAV_{19}$ (J. M. Ball et al., unpublished data).

The current study demonstrates that $EIAV_{17}$ induces cytokine expression through an early event of the infection process. This process is likely mediated by binding of $EIAV_{17}$ Env protein to the surface molecules of equine macrophages for three reasons. First, $EIAV_{17}$ cytokine induction is replication-independent. Second, the kinetics of induction is strikingly similar to that mediated by binding of HIV-1 gp120 to the CD4 molecule. Finally, the most notable differences between $EIAV_{17}$ and its parental virus $EIAV_{19}$, which fails to induce cytokines, are found in the Env (Payne et al., 1998). In particular, the SU sequences of the 2 viruses diverge by 11 out of 34 amino acids (32.4%) in a region of SU containing neutralizing epitopes (the PND) and predicted to

be structurally analogous to the HIV-1 V3 loop, a strong inducer of cytokines (Ball et al., 1992; Koka et al., 1995). In close parallel to our findings, Khanna et al. show that a molecularly cloned HIV-1 (p125) containing the envelope protein derived from $HIV_{ADA}$, the prototypic macrophage-tropic HIV-1 isolated from an AIDS patient, potently induces TNF-α in PBMC culture (Khanna et al., 2000). Insertion of the V3 region from the avirulent molecular clone HxBc2 into p125 abrogates it TNF-α-inducing activity. In HIV-1, most of the peptides required for cytokine induction are mapped to the SU region(Koka et al., 1995). Therefore, we propose that SU of EIAV determines the ability of the virus to induce cytokines. Since the cytokines induced by $EIAV_{17}$ are capable of inducing many of the cardinal symptoms of EIA and potentially regulate viral expression (as discussed above), it is conceivable that SU may play major roles in the pathogenic characteristics of different EIAV strains.

In a recent study of EIAV virulence determinants, a set of distinct EIAV chimeric clones were constructed based on the $EIAV_{19}$ backbone such that each clone contains a different combination of $EIAV_{17}$ SU, TM and LTR sequences (S. L. Payne et al., unpublished data). Results from infection of Shetland ponies with these viruses showed only the clone that derives its LTR and SU sequences from $EIAV_{17}$ induced EIA. A clone that derives its TM and LTR sequences from $EIAV_{17}$ but SU sequence from $EIAV_{19}$ is avirulent. Therefore, in the presence of $EIAV_{17}$ LTR, SU is more important to EIAV virulence than TM. These results also indicate that the $EIAV_{17}$ *rev* (overlapped by TM) sequence is not sufficient to impart virulence to EIAV.

The notion that the differential abilities of $EIAV_{17}$ and $EIAV_{19}$ in causing disease can be at least in part attributed to the differences in their SU sequences is supported by a study that characterized the enterotoxic activities of EIAV SU (J.M. Ball et al., unpublished data). Due to the recent discovery that SIV SU encodes an enterotoxic domain (Swaggerty et al., 2000), SU derived from $EIAV_{17}$ and $EIAV_{19}$ (termed vSU and avSU, respectively) were expressed in a baculovirus expression system, purified and tested for diarrhea induction in a mouse model. While vSU induced diarrhea in a dose-dependent manner with a diarrhea dose 50 ($DD_{50}$) of 0.14 nmols, the dose of avSU sufficient to induce diarrhea in at least 50% of animals could not be attained. To determine whether the EIAV SU acts as an enterotoxin, vSU and avSU were assessed and compared for their effects on intestinal fluid accumulation in ligated loops, chloride secretory current as measured in Ussing chamber, and intracellular signal transduction. The average vSU-treated intestinal loops were 60.8 mg/cm, compared to the average of 55.4 mg/cm for the avSU-treated loops. Addition of 1.5 μM of vSU or avSU to the basolateral and apical sides of mouse intestines induced a forskolin-potentiated short-circuit chloride current of 2.41 or 1.25 $\mu A/cm^2$, respectively. Finally, treating HT-29 cells (human colon adenocarcinoma) with 150 nM of vSU or avSU increased inositol triphosphate levels by 2.26 or 1.47 fold above basal levels, respectively. In summary, this study reveals that EIAV SU is an enterotoxin and, more importantly, that there are differences in the intrinsic pathogenic properties of vSU and avSU, which may contribute to the difference in clinical outcome of $EIAV_{17}$ and $EIAV_{19}$ infection.

The role of the envelope glycoprotein as a virulence determinant has been shown in simian-human immunodeficiency virus (SHIV), a chimeric SIV containing *tat*, *rev*, *vpu* and *env* of HIV-1 used for studying HIV-1 pathogenesis in simian models (Cayabyab et al., 1999). SHIV-HXBc2, a molecularly cloned SHIV containing *env* of cell line adapted HIV-1 isolate, is avirulent in rhesus monkey. Replacing the *env* of SHIV-HXBc2 with *env* sequences derived from a pathogenic HIV isolate generates a highly virulent virus (SHIV-HXBc2P 3.2) that causes AIDS-like symptoms within 2 years of infection. The two viruses differ only in 12 amino acids in the envelope glycoprotein, 10 of which are located in the SU region. The same study demonstrates that the envelope glycoprotein of SHIV-HXBc2P 3.2 contributes to virulence by enhancing early events during the infection cycle, leading to higher levels of replication both in vivo and in cultured PBMC. It is also proposed that the envelope glycoprotein of SHIV-HXBc2P 3.2 is more efficient at destroying CD4+ T cells (Cayabyab et al., 1999).

Despite the apparent efficacy of virulent SU in mediating disease, we do not rule out the possibility that EIAV virulence is a phenotype that involves multiple viral factors. For example, in the study of EIAV virulence determinants described above, a chimeric clone that derives it *env* from $EIAV_{17}$ and LTR from $EIAV_{19}$ failed to cause disease in Shetland ponies (S. L. Payne, unpublished data). EIAV encoding the virulent SU may not attain the viral load that is sufficient for exerting its pathological effects if the appropriate LTR sequence is absent.

**Pitfalls and Problems**

Since equine cytokine-specific antibodies are unavailable, RPA was employed to to characterize cytokine responses by measuring mRNA transcription levels. Measurement of cytokine mRNA expression by RPA, which is highly sensitive to nucleotide mismatches, may be affected by polymorphisms of cytokine genes. More importantly, production of some cytokine, such as TGF-β1, is regulated by post-transcriptional processes including stabilization of mRNA and activation of precursor proteins by extracellular enzymes (Toossi et al., 1995). This implies that the transcription level of TGF-β1 genes may not directly correlate to the production of biologically active TGF-β1 molecules. Similarly, IL-1β and IL-1α transcripts may accumulate intracellularly in the absence of protein secretion (Zhong et al., 1993). Therefore, the induction of cytokine mRNA is only an indicator of increased cytokine production and should be interpreted with caution. However, this study screened the response of a large number of equine cytokines and identified five cytokines that warrant further investigation. It will now be feasible to produce equine cytokine specific antibodies or perform bioassays to quantitate the secreted molecules with the relevant cytokines known.

The most significant problem in this study is the lack of an equine macrophage cell line. The primary equine macrophage culture is time-consuming, labor-intensive and technically demanding. As macrophages were isolated from different out-bred animals for each experiment, there was a great deal of variability in their viability in culture, ability to produce EIAV and constitutive cytokine expression levels. Occasional

shortage of horse donors caused a delay in research progress. The high degree of variability and low numbers of animals rendered statistical analysis of the data difficult. This difficulty was met by employing the linear-mixed effects model in data analysis, which takes into account the random effects of individual animals. Remarkably, the trends of cytokine responses to $EIAV_{17}$ and $EIAV_{19}$ were highly consistent and reproducible in macrophages that originated from different horses, indicating the generality of the results. Although derivation of an equine macrophage cell line may greatly facilitate future experiments, precautions must be taken when comparing the response to EIAV infection to that of mature equine macrophages as macrophage differentiation apparently has major impact on the life cycle of EIAV.

Another issue related to use of macrophage culture is the inability of the in vitro system to reflect the complexity of the in vivo conditions. This shortcoming was made apparent by the discrepancy between the in vitro and in vivo growth rate of the EIAV molecular clones. More importantly, cytokines are not exclusively produced by macrophages. Other important sources of cytokines such as the lymphocytes, dendritic cells and endothelial cells are not included in the macrophage culture system. Cytokines produced by macrophages influence the synthesis of cytokines by other cells, which may in turn influence production and activities of macrophage cytokines, forming a complex network of interacting molecules. For example, in characterizing the RPA, it was observed that lipopolysaccharide (LPS) failed to stimulate IL-12 p35 and IL-12 p40 expression, which was surprising since macrophages are a main source of IL-12 (Cavaillon, 1994). This discrepancy is likely because LPS-induced expression of IL-12

requires priming by IFN-γ, which is mainly produced by stimulated lymphocytes (Hayes et al., 1995). Therefore, to assess cytokine responses under more physiological conditions, cells and tissue samples collected from EIAV-infected animals should be analyzed. However, the distinct advantage of using primary macrophage culture is that the results represent the response of a defined population of cells to EIAV infection. It is especially important to understand the cytokine profiles of macrophages since they are the predominant targets for EIAV infection. These profiles also help predict potentially affected genes and cellular functions that can be examined in future studies.

One weakness of this study is the lack of evidence for the mechanism of cytokine induction. Based on the rapid kinetics of $EIAV_{17}$-induced cytokine response, I hypothesized that binding of SU to macrophage surface receptor(s) is responsible for $EIAV_{17}$-induced cytokine expression. Providing direct evidence for this hypothesis was difficult in part because the cellular receptor for EIAV is unknown and consequently could not be blocked. I demonstrated that cytokine induction by $EIAV_{17}$ does not require viral replication, as would be expected for effects mediated by receptor binding. The effort to demonstrate the effects of SU on cytokine expression was hampered by the difficulties in maintaining the solubility of purified SU, likely due to protein aggregation. As discussed earlier, a synthetic peptide corresponding to the $V_H$ region of SU did not induce cytokine expression when added to EMDM. At present, different techniques to purify soluble SU and alternative approaches to test the effects of SU are being explored. While more evidence is clearly needed, the existing results indicate the cytokine-inductive activities of EIAV closely parallel those of HIV-1, which is well

established to induce cytokines via binding of SU glycoprotein to its cellular receptor, the CD4 molecule (Cicala et al., 2002).

## Future studies

### *Mechanisms of cytokine induction by EIAV*

Although both rapid kinetics and dispensability of viral replication suggest that $EIAV_{17}$ induction of cytokine expression is mediated by binding of the envelope glycoprotein, direct evidence for the mechanism(s) of cytokine induction is needed. Soluble Env, SU and TM proteins derived from $EIAV_{17}$ and $EIAV_{19}$ could be expressed and tested for their induction of cytokines in equine macrophages. These experiments would provide definitive evidence that cytokines are induced by the viral envelope glycoprotein as well as provide a means to identify the cytokine-inductive subunit. Modifications such as truncation of the proposed transmembrane domain of TM might be necessary to allow secretion of Env and TM. Another method to approach this question is to insert *env*, SU or TM, derived from $EIAV_{17}$ into the non-cytokine-inducing $EIAV_{19}$. Pseudotyped viruses expressing $EIAV_{17}$ *env* genes could also be used. Chimeric and pseudotyped viruses have advantages over purified proteins in mimicking native protein structures. Accurate representation of protein structure is especially important considering that EIAV Env is likely to assemble into trimers, as is the case with HIV-1 Env, and that the trimeric structure is critical for the formation of HIV-1 Env receptor binding sites. To further characterize the cytokine inductive regions of EIAV

Env, truncation or substitution mutants of SU or TM could be generated and compared for cytokine stimulation. Synthetic peptides corresponding to different domains of Env could also be used to determine the sequences involved in cytokine induction. Because peptides are linear and are not post-translationally modified, these experiments would provide insight into the importance of conformation, oligomerization and glycosylation in the cytokine-inductive activities of the envelope proteins. However, this approach could be problematic since macrophages may phagocytose or pinocytose the peptides.

A peptide (EIAV6) corresponding to the $V_H$ region of $EIAV_{17}$ SU was synthesized, HPLC-purified, characterized by mass spectrophotometry and added to primary equine macrophages. Shown below is the sequence of the peptide.

EIAV6: EDNKGVVRGDYTACNVRRLNINRKDYTGIYQVP

This peptide was selected because it was shown to be biologically active in mouse diarrhea experiments (J. M. Ball, unpublished data). Although the peptide failed to stimulate cytokines at 20 and 200 nmol per ml, the importance of this region in cytokine induction cannot be ruled out as the result may reflect that linear amino acid sequence is not sufficient for receptor recognition. Alternatively, the peptide may enter macrophages without binding to the receptor, as shown with the SIV enterotoxic peptide (C. L. Swaggerty et al., unpublished data). Testing the inhibitory effects of this peptide on $EIAV_{17}$- or purified envelope protein-induced cytokine expression would yield interesting information regarding contribution of $V_H$ to cytokine induction. Elucidation of the cytokine induction mechanisms would provide new concepts for design of protective vaccines and reveal potential therapeutic targets and strategies.

*Other cytokines dysregulated by EIAV infection*

This study reveals striking resemblance between EIAV and HIV-1 in the cytokine-pathogenesis relationship. Therefore, analyses of other cytokines known to be important in HIV-1 disease progression are warranted. Among these cytokines, the expression of β-chemokines MIP-1α, MIP-1β, MCP's and RANTES in primary equine macrophage infected by $EIAV_{17}$ and $EIAV_{19}$ deserves immediate investigation. These molecules are believed to downregulate HIV-1 replication by blocking the CCR5 coreceptor (Vicenzi et al., 1997). More importantly, they are monocyte/macrophage chemoattractants that may enhance EIAV viral load in vivo by recruiting target cells to the site of viral replication. Given that transcription of EIAV in primary equine macrophages appear to be regulated by binding of the PU.1 transcription factor to the *ets* sites of U3-LTR (Maury, 1994), it would be interesting to examine the expression of granulocyte-monocyte and monocyte colony-stimulating factors (GM-CSF and M-CSF, respectively), which activate PU.1 upon binding to their cellular receptors (DeKoter et al., 1998). Furthermore, these cytokines regulate the differentiation and maturation of monocytic cells, which are processes known to activate the expression of EIAV (Maury, 1994). Partial or complete cDNA sequences of equine GM-CSF, RANTES, and MCP's are available in Genbank and can be used to generate RNA probes for RPA analysis. Cloning and sequencing of equine MIP-1α, MIP-1β and M-CSF can be performed by designing primers corresponding to the most conserved regions of these genes.

*Roles of cytokines in EIAV immunology*

Both humoral and cellular immunity are involved in the host response to EIAV infection. The humoral immune response to EIAV antigens and the importance of neutralizing antibodies in controlling viral replication have been discussed earlier. In EIAV-infected animals, neutralizing antibodies specific to the infecting strains are not detected until 2-3 months post-infection, whereas virus-specific CTL appears within 3-4 weeks post-infection and coincides with resolution of the initial viremia, emphasizing the importance of CTL in viral clearance during acute infection (Hammond et al., 1997). It is hypothesized that HIV disease progression is the consequence of a "shift" from the protective CTL response that effectively controls viral replication to the potentially harmful humoral immune response (Poli et al., 1995). Analysis and comparison of the horse immune responses involved in managing $EIAV_{17}$ and EAIV19 infection would provide new concepts for EIAV pathogenesis and vaccine design.

According to the mouse Th1/Th2 paradigm, Th1 are $CD4^+$ T cells that promote CTL response by secreting IL-2 and IFN-γ. In contrast, Th2 cells promote humoral immune response by secreting IL-4 and IL-5. Fraser et al. showed that Gag peptides containing broadly recognized CTL epitopes selectively stimulate IFN-γ but not IL-4 expression by PBMC of EIAV-infected horses (Fraser et al., 2002). Thus, it appears that the Th1/Th2 dichotomy exists in the horse immune system, and the cytokine profiles of PBMC can be used as indicators for Th1 versus Th2 immune response to EIAV antigens.

The Th1/Th2 phenotype of $CD4^+$ T cells in horses infected with $EIAV_{17}$ and $EIAV_{19}$ could be characterized by analyzing expression of cytokines that distinguish Th1

(IL-2 and IFN-γ) and Th2 (IL-4, IL-5) responses in PBMC isolated from infected horses. Analyzing PBMC cytokines isolated at different stages of EIA and stimulated with different EIAV antigens would yield interesting information regarding the evolution and mechanisms of immune response to these viruses. I expect PBMC of $EIAV_{17}$- and $EIAV_{19}$-infected horses to display distinct cytokine profiles since the differentiation of the Th1/Th2 subsets are heavily influenced by cytokines secreted by $EIAV_{17}$-infected macrophages. For example, IL-1 and TNF-α stimulate IL-2 production, which is associated with the Th1 phenotype. On the other hand, IL-10 is regarded as a Th2 cytokine since it inhibits the Th1 response. In addition, Env contains epitopes that are frequently recognized by CTL of infected horses, variation of Env sequences between $EIAV_{17}$ and $EIAV_{19}$ may impact these epitopes (McGuire et al., 2000).

*Pathogenic roles of cytokines in EIAV diseases*

I speculate that the cytokines induced by $EIAV_{17}$ in primary equine macrophages contribute to its virulence based on the following three reasons. First, the differential abilities of $EIAV_{17}$ and $EIAV_{19}$ in stimulating these cytokines in vitro mirror their differential abilities to cause diseases in vivo. Second, the pathological changes associated with dysregulation of these cytokines mimic the principal symptoms of EIA such as fever, thrombocytopenia and anemia (Powell et al., 1996). The contributions of cytokines to these EIA symptoms are supported by results of previous studies (Tornquist and Crawford, 1997; Costa et al., 1997). Finally, cytokines have been implicated in

pathogenesis of HIV-1 disease similar to those of EIAV (Chang et al., 1998; Means, 1997; Zauli et al., 1992).

The role(s) of cytokines in EIA could be tested by in vitro and in vivo experiments. Establishment of equine bone marrow culture for assessing the growth of megakaryocyte colonies and erythrocyte progenitors has been described previously (Swardson et al., 1992; Tornquist and Crawford, 1997). Equine macrophage culture could be exposed to $EIAV_{17}$ or $EIAV_{19}$ to stimulate cytokine expression, and the conditioned culture supernatants could be tested for their effects on the proliferation and differentiation of megakaryocyte colonies and erythrocyte progenitors. These experiments would indicate whether the secretory products of EIAV-stimulated macrophages have suppressive effects on platelet and erythrocyte production. Contribution of cytokines to thrombopoiesis and erythropoiesis suppression could be determined by whether addition of cytokine-specific neutralizing antibodies reverses the suppressive activities. Since such equine cytokine-specific antibodies are not commercially available, cross-reactive or pan-specific antibodies would have to be used. Alternatively, equine cytokine-specific antibodies could be generated *de novo*.

Another means to demonstrate that cytokines mediate EIA diseases is to experimentally infect horses with EIAV and examine the correlation between elevation of cytokine expression and development of symptoms. Cytokine mRNA levels should be analyzed in tissue or bone marrow macrophages, the sites of active EIAV replication, instead of peripheral monocytes (Sellon et al., 1992; Swardson et al., 1997). Cytokine expression levels could be compared between horses infected with virulent EIAV versus

those infected with avirulent EIAV. Correlation between cytokines levels and disease severity as well as temporal correlation between cytokine elevation and onset of symptoms could also be examined. However, these data would be suggestive rather than conclusive.

**Advancement of Equine Immunology and EIAV Pathogenesis**

This study has advanced the field of equine immunology by developing a multi-probe RPA that simultaneously quantitates the mRNA levels of 10 equine cytokines, using the equine β-actin gene as an internal control. This assay was carefully validated and shown to be highly specific, reproducible and sensitive. It is a valuable tool for equine cytokine research as equine cytokine reagents are not commercially available. In fact, these probes have been used in a study of neonatal foal immune development by measuring PBMC cytokines over the first few weeks of life (Boyd et al., 2003). This study incorporates two additional cytokine probes to the template set (total of 12 cytokines) and labels the probes by biotin instead of radioactive isotopes, demonstrating the versatility of this assay.

The findings of this study establish cytokine induction as an in vitro biological property that differentiate the virulence of EIAV isolates. Previously, EIA was hypothesized to be largely an immune complex disease. This hypothesis is supported by little or no evidence and is further undermined by the observation of severe EIA in infected SCID horses. The potential roles of cytokines in EIAV virulence provide a new concept for the mechanisms of EIA diseases. However, the proposition of cytokines as

disease mediators does not exclude the involvement of specific immune responses. Rather, the fact that cytokines are essentially immune regulatory molecules bespeaks that this concept will also bring new insight into the roles of the immune response in EIAV pathogenesis.

Finally, the use of molecularly cloned viruses in this study allowed for identification of SU as a virulence determinant. Although more studies are needed to confirm that the virulent SU alone is sufficient to induce cytokines, the reasoning that variations in the SU sequences differentiate the pathogenic properties of the 2 EIAV clones is in line with the results of previous functional and genetic studies on EIAV SU. This finding is valuable for development of therapeutic strategies and design of future vaccines.

# REFERENCES

Adeyemo, O., Gao, R. J., Lan, H. C., 1997. Cytokine production in vitro by macrophages of goats with caprine arthritis-encephalitis. Cell. Mol. Biol. 43, 1031-1037.

Akridge, R. E., Oyafuso, L. K., Reed, S. G., 1994. IL-10 is induced during HIV-1 infection and is capable of decreasing viral replication in human macrophages. J. Immunol. 153, 5782-5789.

Allen, G. K., Green, E. M., Robinson, J. A., Garner, H. E., Loch, W. E., Walsh, D. M., 1993. Serum tumor necrosis factor alpha concentrations and clinical abnormalities in colostrum-fed and colostrum-deprived neonatal foals given endotoxin. Am. J. Vet. Res. 54, 1404-1410.

Ankel, H., Capobianchi, M. R., Castilletti, C., Dianzani, F., 1994. Interferon induction by HIV glycoprotein 120: role of the V3 loop. Virology 205, 34-43.

Badley, A. D., Dockrell, D., Simpson, M., Schut, R., Lynch, D. H., Leibson, P., Paya, C. V., 1997. Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. J. Exp. Med. 185, 55-64.

Bakhanashvili, M. and Hizi, A., 1993. Fidelity of DNA synthesis exhibited in vitro by the reverse transcriptase of the lentivirus equine infectious anemia virus. Biochemistry 32, 7559-7567.

Ball, J. M., 1990. Synthetic peptide analyses of the surface glycoproteins of equine infectious anemia virus. Ph.D. dissertation, Louisiana State University.

Ball, J. M., Payne, S. L., Issel, C. J., Montelaro, R. C., 1988. EIAV genomic organization: further characterization by sequencing of purified glycoproteins and cDNA. Virology 165, 601-605.

Ball, J. M., Rushlow, K. E., Issel, C. J., Montelaro, R. C., 1992. Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infectious anemia virus by using synthetic peptide strategies. J. Virol. 66, 732-742.

Bar, J., Zosmer, A., Hod, M., Elder, M. G., Sullivan, M. H., 1997. The regulation of platelet aggregation in vitro by interleukin-1beta and tumor necrosis factor-alpha: changes in pregnancy and in pre-eclampsia. Thromb. Haemostasis 78, 1255-1261.

Beisel, C. E., Edwards, J. F., Dunn, L. L., Rice, N. R., 1993. Analysis of multiple mRNAs from pathogenic equine infectious anemia virus (EIAV) in an acutely infected

horse reveals a novel protein, Ttm, derived from the carboxy terminus of the EIAV transmembrane protein. J. Virol. 67, 832-842.

Bellin, D., 1996. The RNase Protection Assay. Methods Mol.Biol. 58, 131-136.

Belshan, M., Baccam, P., Oaks, J. L., Sponseller, B. A., Murphy, S. C., Cornette, J., Carpenter, S., 2001. Genetic and biological variation in equine infectious anemia virus Rev correlates with variable stages of clinical disease in an experimentally infected pony. Virology 279, 185-200.

Belshan, M., Park, G. S., Bilodeau, P., Stoltzfus, C. M., Carpenter, S., 2000. Binding of equine infectious anemia virus Rev to an exon splicing enhancer mediates alternative splicing and nuclear export of viral mRNAs. Mol. Cell. Biol. 20, 3550-3557.

Benveniste, O., Vaslin, B., Le Grand, R., Dormont, D., 1998. Comparing IL-6, TNF-alpha, and IL-1beta responses to acute infection with attenuated nef-truncated or pathogenic SIVmac251 in macaque peripheral blood mononuclear cells. J. Acquired Immune Defic. Syndr. 18, 389-390.

Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., Cullen, B. R., 1999. Highly divergent lentiviral Tat proteins activate viral gene expression by a common mechanism. Mol. Cell. Biol. 19, 4592-4599.

Billinghurst, R. C., Fretz, P. B., Gordon, J. R., 1995. Induction of intra-articular tumour necrosis factor during acute inflammatory responses in equine arthritis. Equine Vet. J. 27, 208-216.

Bornemann, M. A., Verhoef, J., Peterson, P. K., 1997. Macrophages, cytokines, and HIV. J. Lab. Clin. Med. 129, 10-16.

Boyd, N. K., Cohen, N. D., Lim, W. S., Martens, R. J., Chaffin, M. K., Ball, J. M., 2003. Temporal changes in cytokine expression of foals during the first month of life. Vet. Immunol. Immunopathol. 92, 75-85.

Breen, E. C., 2002. Pro- and anti-inflammatory cytokines in human immunodeficiency virus infection and acquired immunodeficiency syndrome. Pharmacol. Ther. 95, 295-304.

Breen, E. C., Rezai, A. R., Nakajima, K., Beall, G. N., Mitsuyasu, R. T., Hirano, T., Kishimoto, T., Martinez-Maza, O., 1990. Infection with HIV is associated with elevated IL-6 levels and production. J. Immunol. 144, 480-484.

Byrne, K. M., Davis, W. C., Holmes, M. A., Brassfield, A. L., McGuire, T. C., 1997. Cytokine RNA expression in an equine CD4+ subset differentiated by expression of a novel 46-kDa surface protein. Vet. Immunol. Immunopathol. 56, 191-204.

Carpenter, S. and Chesebro, B., 1989. Change in host cell tropism associated with in vitro replication of equine infectious anemia virus. J. Virol. 63, 2492-2496.

Carroll, R. and Derse, D., 1993. Translation of equine infectious anemia virus bicistronic tat-rev mRNA requires leaky ribosome scanning of the tat CTG initiation codon. J. Virol. 67, 1433-1440.

Carvalho, M. and Derse, D., 1991. Mutational analysis of the equine infectious anemia virus Tat-responsive element. J. Virol. 65, 3468-3474.

Carvalho, M., Kirkland, M., Derse, D., 1993. Protein interactions with DNA elements in variant equine infectious anemia virus enhancers and their impact on transcriptional activity. J. Virol. 67, 6586-6595.

Cavaillon, J. M., 1994. Cytokines and macrophages. Biomed. Pharmacother. 48, 445-453.

Cayabyab, M., Karlsson, G. B., Etemad-Moghadam, B. A., Hofmann, W., Steenbeke, T., Halloran, M., Fanton, J. W., Axthelm, M. K., Letvin, N. L., Sodroski, J. G., 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. 73, 976-984.

Chance, M. R., Sagi, I., Wirt, M. D., Frisbie, S. M., Scheuring, E., Chen, E., Bess, J. W., Jr., Henderson, L. E., Arthur, L. O., South, T. L., 1992. Extended x-ray absorption fine structure studies of a retrovirus: equine infectious anemia virus cysteine arrays are coordinated to zinc. Proc. Natl. Acad. Sci. U. S. A. 89, 10041-10045.

Chang, H. R., Dulloo, A. G., Bistrian, B. R., 1998. Role of cytokines in AIDS wasting. Nutrition 14, 853-863.

Charan, S., Palmer, K., Chester, P., Mire-Sluis, A. R., Meager, A., Edington, N., 1997. Transforming growth factor-beta induced by live or ultraviolet-inactivated equid herpes virus type-1 mediates immunosuppression in the horse. Immunology 90, 586-591.

Charman, H. P., Bladen, S., Gilden, R. V., Coggins, L., 1976. Equine infectious anemia virus: evidence favoring classification as a retravirus. J. Virol. 19, 1073-1079.

Cheevers, W. P., Archer, B. G., Crawford, T. B., 1977. Characterization of RNA from equine infectious anemia virus. J. Virol. 24, 489-497.

Cheevers, W. P. and McGuire, T. C., 1985. Equine infectious anemia virus: immunopathogenesis and persistence. Rev.Infect. Dis. 7, 83-88.

Chen, C., Li, F., Montelaro, R. C., 2001. Functional roles of equine infectious anemia virus Gag p9 in viral budding and infection. J. Virol. 75, 9762-9770.

Chesters, P. M., Hughes, A., Edington, N., 2000. Equid herpesvirus 1: platelets and alveolar macrophages are potential sources of activated TGF-β1 in the horse. Vet. Immunol. Immunopathol. 75, 71-79.

Chirmule, N. and Pahwa, S., 1996. Envelope glycoproteins of human immunodeficiency virus type 1: profound influences on immune functions. Microbiol. Rev. 60, 386-406.

Choe, W., Volsky, D. J., Potash, M. J., 2001. Induction of rapid and extensive beta-chemokine synthesis in macrophages by human immunodeficiency virus type 1 and gp120, independently of their coreceptor phenotype. J. Virol. 75, 10738-10745.

Chong, Y. H., Ball, J. M., Issel, C. J., Montelaro, R. C., Rushlow, K. E., 1991. Analysis of equine humoral immune responses to the transmembrane envelope glycoprotein (gp45) of equine infectious anemia virus. J. Virol. 65, 1013-1018.

Cicala, C., Arthos, J., Selig, S. M., Dennis, G., Jr., Hosack, D. A., Van Ryk, D., Spangler, M. L., Steenbeke, T. D., Khazanie, P., Gupta, N., Yang, J., Daucher, M., Lempicki, R. A., Fauci, A. S., 2002. HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication. Proc. Nat. Acad. Sci. U. S. A. 99, 9380-9385.

Clabough, D. L., Gebhard, D., Flaherty, M. T., Whetter, L. E., Perry, S. T., Coggins, L., Fuller, F. J., 1991. Immune-mediated thrombocytopenia in horses infected with equine infectious anemia virus. J. Virol. 65, 6242-6251.

Coffin, J. M., 1996. Retroviridae: the viruses and their replication, in: Fields, B. N., Knipe, D. M., Howley, P. M. (Eds.), Fundamental Virology, Lippincott-raven Publishers, Philadelphia, pp. 763-843.

Coggins, L., 1984. Carriers of equine infectious anemia virus. J. Am. Vet. Med. Assoc. 184, 279-281.

Coggins, L., Norcross, N. L., Nusbaum, S. R., 1972. Diagnosis of equine infectious anemia by immunodiffusion test. Am. J. Vet. Res. 33, 11-18.

Cook, R. F., Leroux, C., Cook, S. J., Berger, S. L., Lichtenstein, D. L., Ghabrial, N. N., Montelaro, R. C., Issel, C. J., 1998. Development and characterization of an in vivo pathogenic molecular clone of equine infectious anemia virus. J. Virol. 72, 1383-1393.

Costa, L. R., Santos, I. K., Issel, C. J., Montelaro, R. C., 1997. Tumor necrosis factor-alpha production and disease severity after immunization with enriched major core

protein (p26) and/or infection with equine infectious anemia virus. Vet. Immunol. Immunopathol. 57, 33-47.

Crawford, T. B., Wardrop, K. J., Tornquist, S. J., Reilich, E., Meyers, K. M., McGuire, T. C., 1996. A primary production deficit in the thrombocytopenia of equine infectious anemia. J. Virol. 70, 7842-7850.

Croker, P. R. and Milon, G., 1992. Macrophages in the control of haematopoiesis, in: Lewis, C. E., McGee, J. O. (Eds.), The Natural Immune System: The Macrophage, Oxford University Press, Oxford, pp. 115-156.

Dauter, Z., Persson, R., Rosengren, A. M., Nyman, P. O., Wilson, K. S., Cedergren-Zeppezauer, E. S., 1999. Crystal structure of dUTPase from equine infectious anaemia virus; active site metal binding in a substrate analogue complex. J. Mol. Biol. 285, 655-673.

Davis, M. J., Bailey, C. S., Smith, C. K., 1997. Use of internal controls to increase quantitative capabilities of the ribonuclease protection assay. BioTechniques 23, 280-285.

de Waal, M. R., Abrams, J., Bennett, B., Figdor, C. G., de Vries, J. E., 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174, 1209-1220.

DeKoter, R. P., Walsh, J. C., Singh, H., 1998. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. EMBO J. 17, 4456-4468.

Derse, D., Dorn, P. L., Levy, L., Stephens, R. M., Rice, N. R., Casey, J. W., 1987. Characterization of equine infectious anemia virus long terminal repeat. J. Virol. 61, 743-747.

Dinarello, C. A., 1996. Biologic basis for interleukin-1 in disease. Blood 87, 2095-2147.

Dinarello, C. A., 1999. Cytokines as endogenous pyrogens. J. Infect. Dis. 179 Suppl 2, S294-S304.

Ebrahimi, B., Allsopp, T. E., Fazakerley, J. K., Harkiss, G. D., 2000. Phenotypic characterisation and infection of ovine microglial cells with Maedi-Visna virus. J. Neurovirol. 6, 320-328.

Ellis, J. A., Bogdan, J. R., Kanara, E. W., Morley, P. S., Haines, D. M., 1995. Cellular and antibody responses to equine herpesviruses 1 and 4 following vaccination of horses with modified-live and inactivated viruses. J. Am. Vet. Med. Assoc. 206, 823-832.

Engelman, A., 1996. Biochemical characterization of recombinant equine infectious anemia virus integrase. Protein Expression Purif. 8, 299-304.

English, R. V., Johnson, C. M., Gebhard, D. H., Tompkins, M. B., 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J. Virol. 67, 5175-5186.

Fang, G., Burger, H., Chappey, C., Rowland-Jones, S., Visosky, A., Chen, C. H., Moran, T., Townsend, L., Murray, M., Weiser, B., 2001. Analysis of transition from long-term nonprogressive to progressive infection identifies sequences that may attenuate HIV type 1. AIDS Res. Hum. Retroviruses 17, 1395-1404.

Fantuzzi, L., Gessani, S., Borghi, P., Varano, B., Conti, L., Puddu, P., Belardelli, F., 1996. Induction of interleukin-12 (IL-12) by recombinant glycoprotein gp120 of human immunodeficiency virus type 1 in human monocytes/macrophages: requirement of gamma interferon for IL-12 secretion. J. Virol. 70, 4121-4124.

Farell, R. E., Jr., 1998. Quantification of specific messenger RNAs by nuclease protection, in: Farell, R. E. J. (Ed.), RNA Methodologies: A Laboratory Guide ror Isolation and Characterization, Academic Press, San Diego, pp. 385-405.

Fauci, A. S., 1996. Host factors and the pathogenesis of HIV-induced disease. Nature 384, 529-534.

Franchini, M., Gill, U., von Fellenberg, R., Bracher, V. D., 2000. Interleukin-8 concentration and neutrophil chemotactic activity in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease following exposure to hay. Am. J. Vet. Res. 61, 1369-1374.

Fraser, D. G., Oaks, J. L., Brown, W. C., McGuire, T. C., 2002. Identification of broadly recognized, T helper 1 lymphocyte epitopes in an equine lentivirus. Immunology 105, 295-305.

Freed, E. O., 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology 251, 1-15.

Gadient, R. A. and Patterson, P. H., 1999. Leukemia inhibitory factor, Interleukin 6, and other cytokines using the GP130 transducing receptor: roles in inflammation and injury. Stem Cells 17, 127-137.

Gallaher, W. R., Ball, J. M., Garry, R. F., Griffin, M. C., Montelaro, R. C., 1989. A general model for the transmembrane proteins of HIV and other retroviruses. AIDS Res. Hum. Retroviruses 5, 431-440.

Gallaher, W. R., Ball, J. M., Garry, R. F., Martin-Amedee, A. M., Montelaro, R. C., 1995. A general model for the surface glycoproteins of HIV and other retroviruses. AIDS Res. Hum. Retroviruses 11, 191-202.

Gendelman, H. E., Narayan, O., Kennedy-Stoskopf, S., Kennedy, P. G., Ghotbi, Z., Clements, J. E., Stanley, J., Pezeshkpour, G., 1986. Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. J. Virol. 58, 67-74.

Gessani, S. and Belardelli, F., 1998. IFN-gamma expression in macrophages and its possible biological significance. Cytokine Growth Factor Rev. 9, 117-123.

Giguere, S. and Prescott, J. F., 1999. Quantitation of equine cytokine mRNA expression by reverse transcription-competitive polymerase chain reaction. Vet. Immunol. Immunopathol. 67, 1-15.

Giguere, S., Wilkie, B. N., Prescott, J. F., 1999. Modulation of cytokine response of pneumonic foals by virulent Rhodococcus equi. Infect. Immun. 67, 5041-5047.

Gonda, M. A., Charman, H. P., Walker, J. L., Coggins, L., 1978. Scanning and transmission electron microscopic study of equine infectious anemia virus. Am. J. Vet. Res. 39, 731-740.

Gregersen, J. P., Wege, H., Preiss, L., Jentsch, K. D., 1988. Detection of human immunodeficiency virus and other retroviruses in cell culture supernatants by a reverse transcriptase microassay. J. Virol. Methods 19, 161-168.

Grunig, G. and Antczak, D. F., 1995. Horse trophoblasts produce tumor necrosis factor alpha but not interleukin 2, interleukin 4, or interferon gamma. Biol. Reprod. 52, 531-539.

Haines, D. S. and Gillespie, D. H., 1992. RNA abundance measured by a lysate RNase protection assay. BioTechniques 12, 736-741.

Hammond, S. A., Cook, S. J., Lichtenstein, D. L., Issel, C. J., Montelaro, R. C., 1997. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. J. Virol. 71, 3840-3852.

Harrold, S. M., Cook, S. J., Cook, R. F., Rushlow, K. E., Issel, C. J., Montelaro, R. C., 2000. Tissue sites of persistent infection and active replication of equine infectious anemia virus during acute disease and asymptomatic infection in experimentally infected equids. J. Virol. 74, 3112-3121.

Hatanaka, H., Iourin, O., Rao, Z., Fry, E., Kingsman, A., Stuart, D. I., 2002. Structure of equine infectious anemia virus matrix protein. J. Virol. 76, 1876-1883.

Hayes, M. P., Wang, J., Norcross, M. A., 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. Blood 86, 646-650.

Heike, T. and Nakahata, T., 2002. Ex vivo expansion of hematopoietic stem cells by cytokines. Biochim. Biophys. Acta 1592, 313-321.

Henderson, E. E., Tudor, G., Yang, J. Y., 1992. Inactivation of the human immunodeficiency virus type 1 (HIV-1) by ultraviolet and X irradiation. Radiat. Res. 131, 169-176.

Henderson, L. E., Sowder, R. C., Smythers, G. W., Oroszlan, S., 1987. Chemical and immunological characterizations of equine infectious anemia virus gag-encoded proteins. J. Virol. 61, 1116-1124.

Herbein, G., Keshav, S., Collin, M., Montaner, L. J., Gordon, S., 1994. HIV-1 induces tumour necrosis factor and IL-1 gene expression in primary human macrophages independent of productive infection. Clin. Exp. Immunol. 95, 442-449.

Hines, R. and Maury, W., 2001. DH82 cells: a macrophage cell line for the replication and study of equine infectious anemia virus. J. Virol. Methods 95, 47-56.

Horvath, C. J., Desrosiers, R. C., Sehgal, P. K., King, N. W., Ringler, D. J., 1991. Effect of simian immunodeficiency virus infection on tumor necrosis factor-alpha production by alveolar macrophages. Lab. Invest. 65, 280-286.

Hunter, E., 1997. Viral entry and receptors, in: Coffin, J. M., Hughes, S. H., Varmus, H. E. (Eds.), Retroviruses, Cold Spring Harbor Laboratory Press, Plainview, pp. 71-119.

Hussain, K. A., Issel, C. J., Schnorr, K. L., Rwambo, P. M., Montelaro, R. C., 1987. Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies. J. Virol. 61, 2956-2961.

Hussain, K. A., Issel, C. J., Schnorr, K. L., Rwambo, P. M., West, M., Montelaro, R. C., 1988. Antigenic mapping of the envelope proteins of equine infectious anemia virus: identification of a neutralization domain and a conserved region on glycoprotein 90. Arch. Virol. 98, 213-224.

Ishitani, R., 1970. Equine Infectious Anemia. National Instititute of Animal Health Quarterly 10, 1-28.

Issel, C. J. and Coggins, L., 1979. Equine infectious anemia: current knowledge. J. Am. Vet. Med. Assoc. 174, 727-733.

Issel, C. J. and Foil, L. D., 1984. Studies on equine infectious anemia virus transmission by insects. J. Am. Vet. Med. Assoc. 184, 293-297.

Issel, C. J., Horohov, D. W., Lea, D. F., Adams, W. V., Jr., Hagius, S. D., McManus, J. M., Allison, A. C., Montelaro, R. C., 1992. Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. J. Virol. 66, 3398-3408.

Jego, G., Bataille, R., Pellat-Deceunynck, C., 2001. Interleukin-6 is a growth factor for nonmalignant human plasmablasts. Blood 97, 1817-1822.

Jin, Z., Jin, L., Peterson, D. L., Lawson, C. L., 1999. Model for lentivirus capsid core assembly based on crystal dimers of EIAV p26. J. Mol. Biol. 286, 83-93.

Kawakami, T., Sherman, L., Dahlberg, J., Gazit, A., Yaniv, A., Tronick, S. R., Aaronson, S. A., 1987. Nucleotide sequence analysis of equine infectious anemia virus proviral DNA. Virology 158, 300-312.

Khanna, K. V., Yu, X. F., Ford, D. H., Ratner, L., Hildreth, J. K., Markham, R. B., 2000. Differences among HIV-1 variants in their ability to elicit secretion of TNF-alpha. J. Immunol. 164, 1408-1415.

Koka, P., He, K., Camerini, D., Tran, T., Yashar, S. S., Merrill, J. E., 1995. The mapping of HIV-1 gp160 epitopes required for interleukin-1 and tumor necrosis factor alpha production in glial cells. J. Neuroimmunol. 57, 179-191.

Kono, Y., Hirasawa, K., Fukunaga, Y., Taniguchi, T., 1976. Recrudescence of equine infectious anemia by treatment with immunosuppressive drugs. Nat. Inst. Anim. Health Q. 16, 8-15.

Lechner, F., Machado, J., Bertoni, G., Seow, H. F., Dobbelaere, D. A., Peterhans, E., 1997. Caprine arthritis encephalitis virus dysregulates the expression of cytokines in macrophages. J. Virol. 71, 7488-7497.

Legastelois, I., Cordier, G., Cozon, G., Cadore, J. L., Guiguen, F., Greenland, T., Mornex, J. F., 1996. Visna-maedi virus-induced expression of interleukin-8 gene in sheep alveolar cells following experimental in vitro and in vivo infection. Res. Virol. 147, 191-197.

Legastelois, I., Cottin, V., Mornex, J. F., Cordier, G., 1997. Alveolar macrophages from sheep naturally infected by visna-maedi virus contribute to IL-8 production in the lung. Vet. Immunol. Immunopathol. 59, 131-139.

Legastelois, I., Levrey, H., Greenland, T., Mornex, J. F., Cordier, G., 1998. Visna-maedi virus induces interleukin-8 in sheep alveolar macrophages through a tyrosine-kinase signaling pathway. Am. J. Respir. Cell Mol. Biol. 18, 532-537.

Leroux, C., Issel, C. J., Montelaro, R. C., 1997. Novel and dynamic evolution of equine infectious anemia virus genomic quasispecies associated with sequential disease cycles in an experimentally infected pony. J. Virol. 71, 9627-9639.

Leutenegger, C. M., von Rechenberg, B., Huder, J. B., Zlinsky, K., Mislin, C., Akens, M. K., Auer, J., Lutz, H., 1999. Quantitative real-time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. Calcif. Tissue Int. 65, 378-383.

Li, F., Chen, C., Puffer, B. A., Montelaro, R. C., 2002. Functional replacement and positional dependence of homologous and heterologous L domains in equine infectious anemia virus replication. J. Virol. 76, 1569-1577.

Li, F., Leroux, C., Craigo, J. K., Cook, S. J., Issel, C. J., Montelaro, R. C., 2000. The S2 gene of equine infectious anemia virus is a highly conserved determinant of viral replication and virulence properties in experimentally infected ponies. J. Virol. 74, 573-579.

Li, F., Puffer, B. A., Montelaro, R. C., 1998. The S2 gene of equine infectious anemia virus is dispensable for viral replication in vitro. J. Virol. 72, 8344-8348.

Lichtenstein, D. L., Issel, C. J., Montelaro, R. C., 1996. Genomic quasispecies associated with the initiation of infection and disease in ponies experimentally infected with equine infectious anemia virus. J. Virol. 70, 3346-3354.

Lim, W. S., Edwards, J. F., Boyd, N. K., Payne, S. L., Ball, J. M., 2003a. Simultaneous quantitation of equine cytokine mRNAs using a multi-probe ribonuclease protection assay. Vet. Immunol. Immunopathol. 91, 45-51.

Lim, W.-S., Payne, S. L., Edwards, J. F., Kim, I., Ball, J. M., 2003b. Differential effects of virulent and avirulent equine infectious anemia virus molecular clones on primary equine macrophage cytokines. Submitted for publication.

Loghmani, F., Mohammed, K. A., Nasreen, N., Van Horn, R. D., Hardwick, J. A., Sanders, K. L., Antony, V. B., 2002. Inflammatory cytokines mediate C-C (monocyte chemotactic protein 1) and C-X-C (interleukin 8) chemokine expression in human pleural fibroblasts. Inflammation 26, 73-82.

Lu, L., Wang, L. S., Cooper, R. J., Liu, H. J., Turner, K., Weich, N., Broxmeyer, H. E., 2000. Suppressive effects of TNF-alpha, TGF-beta1, and chemokines on megakaryocytic colony formation in CD34+ cells derived from umbilical cord blood

compared with mobilized peripheral blood and bone marrow. J. Hematother. Stem Cell Res. 9, 195-204.

MacKay, R. J., Merritt, A. M., Zertuche, J. M., Whittington, M., Skelley, L. A., 1991. Tumor necrosis factor activity in the circulation of horses given endotoxin. Am. J. Vet. Res. 52, 533-538.

Matsrafi, L., Aflalo, E., Blair, D. G., Priel, E., 1996. Isolation of an 11-kDa protein associated with the topoisomerase I activity from equine infectious anemia virus. Biochem. Biophys. Res. Commun. 220, 1028-1035.

Maury, W., 1994. Monocyte maturation controls expression of equine infectious anemia virus. J. Virol. 68, 6270-6279.

Maury, W., 1998. Regulation of equine infectious anemia virus expression. Journal of Biomed. Sci. 5, 11-23.

Maury, W., Oaks, J. L., Bradley, S., 1998. Equine endothelial cells support productive infection of equine infectious anemia virus. J. Virol. 72, 9291-9297.

Maury, W., Perryman, S., Oaks, J. L., Seid, B. K., Crawford, T., McGuire, T., Carpenter, S., 1997. Localized sequence heterogeneity in the long terminal repeats of in vivo isolates of equine infectious anemia virus. J. Virol. 71, 4929-4937.

McGuire, T. C., Leib, S. R., Lonning, S. M., Zhang, W., Byrne, K. M., Mealey, R. H., 2000. Equine infectious anaemia virus proteins with epitopes most frequently recognized by cytotoxic T lymphocytes from infected horses. J. Gen. Virol. 81, 2735-2739.

Means, R. T., Jr., 1997. Cytokines and anaemia in human immunodeficiency virus infection. Cytokines, Cell. Mol. Ther. 3, 179-186.

Means, R. T., Jr., 2000. The anaemia of infection. Bailliere's Best Prac. Clin. Haematol. 13, 151-162.

Meltzer, M. S., Nakamura, M., Hansen, B. D., Turpin, J. A., Kalter, D. C., Gendelman, H. E., 1990. Macrophages as susceptible targets for HIV infection, persistent viral reservoirs in tissue, and key immunoregulatory cells that control levels of virus replication and extent of disease. AIDS Res. Hum. Retroviruses 6, 967-971.

Merrill, J. E., Koyanagi, Y., Chen, I. S., 1989. Interleukin-1 and tumor necrosis factor alpha can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. J. Virol. 63, 4404-4408.

Michael, N. L., Mo, T., Merzouki, A., O'Shaughnessy, M., Oster, C., Burke, D. S., Redfield, R. R., Birx, D. L., Cassol, S. A., 1995. Human immunodeficiency virus type 1

cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. J. Virol. 69, 1868-1877.

Michelmann, I., Bockmann, D., Nurnberger, W., Eckhof-Donovan, S., Burdach, S., Gobel, U., 1997. Thrombocytopenia and complement activation under recombinant TNF alpha/IFN gamma therapy in man. Ann. Hematol. 74, 179-184.

Molina, J. M., Schindler, R., Ferriani, R., Sakaguchi, M., Vannier, E., Dinarello, C. A., Groopman, J. E., 1990. Production of cytokines by peripheral blood monocytes/macrophages infected with human immunodeficiency virus type 1 (HIV-1). J. Infect. Dis. 161, 888-893.

Montelaro, R. C., Ball, J. M., Rushlow, K. E., 1993. Equine retroviruses, in: Levy, J.A. (Ed.), The Retroviridae, Plenum Press, New York, pp. 257-360.

Montelaro, R. C., Lohrey, N., Parekh, B., Blakeney, E. W., Issel, C. J., 1982. Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. 42, 1029-1038.

Montelaro, R. C., Parekh, B., Orrego, A., Issel, C. J., 1984. Antigenic variation during persistent infection by equine infectious anemia virus, a retrovirus. Journal of Biol. Chem. 259, 10539-10544.

Moore, K. W., de Waal, M. R., Coffman, R. L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683-765.

Mori, K., Ringler, D. J., Kodama, T., Desrosiers, R. C., 1992. Complex determinants of macrophage tropism in env of simian immunodeficiency virus. J. Virol. 66, 2067-2075.

Morris, D. D., Crowe, N., Moore, J. N., Moldawer, L. L., 1992. Endotoxin-induced production of interleukin 6 by equine peritoneal macrophages in vitro. Am. J. Vet. Res. 53, 1298-1301.

Mukaida, N., Harada, A., Matsushima, K., 1998. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 9, 9-23.

Murtaugh, M. P., Baarsch, M. J., Zhou, Y., Scamurra, R. W., Lin, G., 1996. Inflammatory cytokines in animal health and disease. Vet. Immunol. Immunopathol. 54, 45-55.

Nakajima, K., Martinez-Maza, O., Hirano, T., Breen, E. C., Nishanian, P. G., Salazar-Gonzalez, J. F., Fahey, J. L., Kishimoto, T., 1989. Induction of IL-6 (B cell stimulatory factor-2/IFN-beta 2) production by HIV. J. Immunol. 142, 531-536.

Narayan, O., Kennedy-Stoskopf, S., Sheffer, D., Griffin, D. E., Clements, J. E., 1983. Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. Infect. Immun. 41, 67-73.

Nguyen, V. T. and Benveniste, E. N., 2002. Critical role of tumor necrosis factor-alpha and NF-kappa B in interferon-gamma -induced CD40 expression in microglia/macrophages. J. Biol. Chem. 277, 13796-13803.

Oaks, J. L., McGuire, T. C., Ulibarri, C., Crawford, T. B., 1998. Equine infectious anemia virus is found in tissue macrophages during subclinical infection. J. Virol. 72, 7263-7269.

Oaks, J. L., Ulibarri, C., Crawford, T. B., 1999. Endothelial cell infection in vivo by equine infectious anaemia virus. J. Gen. Virol. 80, 2393-2397.

Oleksowicz, L., Mrowiec, Z., Zuckerman, D., Isaacs, R., Dutcher, J., Puszkin, E., 1994. Platelet activation induced by interleukin-6: evidence for a mechanism involving arachidonic acid metabolism. Thromb. Haemostasis 72, 302-308.

Onuma, M., Koomoto, E., Furuyama, H., Yasutomi, Y., Taniyama, H., Iwai, H., Kawakami, Y., 1992. Infection and dysfunction of monocytes induced by experimental inoculation of calves with bovine immunodeficiency-like virus. J. Acquir. Immune Defic. Syndr. 5, 1009-1015.

Orandle, M. S., Williams, K. C., MacLean, A. G., Westmoreland, S. V., Lackner, A. A., 2001. Macaques with rapid disease progression and simian immunodeficiency virus encephalitis have a unique cytokine profile in peripheral lymphoid tissues. J. Virol. 75, 4448-4452.

O'Rourke, K., Perryman, L. E., McGuire, T. C., 1988. Antiviral, anti-glycoprotein and neutralizing antibodies in foals with equine infectious anaemia virus. J. Gen. Virol. 69, 667-674.

Otero, G. C., Harris, M. E., Donello, J. E., Hope, T. J., 1998. Leptomycin B inhibits equine infectious anemia virus Rev and feline immunodeficiency virus rev function but not the function of the hepatitis B virus posttranscriptional regulatory element. J. Virol. 72, 7593-7597.

Payne, S. L., Fang, F. D., Liu, C. P., Dhruva, B. R., Rwambo, P., Issel, C. J., Montelaro, R. C., 1987. Antigenic variation and lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. Virology 161, 321-331.

Payne, S. L., La Celle, K., Pei, X. F., Qi, X. M., Shao, H., Steagall, W. K., Perry, S., Fuller, F., 1999. Long terminal repeat sequences of equine infectious anaemia virus are a major determinant of cell tropism. J. Gen. Virol. 80, 755-759.

Payne, S. L., Qi, X. M., Shao, H., Dwyer, A., Fuller, F. J., 1998. Disease induction by virus derived from molecular clones of equine infectious anemia virus. J. Virol. 72, 483-487.

Payne, S. L., Rausch, J., Rushlow, K., Montelaro, R. C., Issel, C., Flaherty, M., Perry, S., Sellon, D., Fuller, F., 1994. Characterization of infectious molecular clones of equine infectious anaemia virus. J. Gen. Virol. 75, 425-429.

Perry, S. T., Flaherty, M. T., Kelley, M. J., Clabough, D. L., Tronick, S. R., Coggins, L., Whetter, L., Lengel, C. R., Fuller, F., 1992. The surface envelope protein gene region of equine infectious anemia virus is not an important determinant of tropism in vitro. J. Virol. 66, 4085-4097.

Petersen, N. E., Larsen, L. K., Nissen, H., Jensen, L. G., Jensen, A., Hyltoft, P. P., Horder, Gregersen, N., Kristiansen, K., 1995. Improved RNase protection assay for quantifying LDL-receptor mRNA; estimation of analytical imprecision and biological variance in peripheral blood mononuclear cells. Clin. Chem. 41, 1605-1613.

Pinheiro, J. C. and Bates, D. M., 2000. Mixed Effects Models in S and S-Plus. Springer-Verlag, New York.

Poli, G., Vicenzi, E., Ghezzi, S., Lazzarin, A., 1995. Cytokines in the acquired immunodeficiency syndrome and other infectious diseases. Int. J. Clin. Lab. Res. 25, 128-134.

Powell, D. J., Bur, D., Wlodawer, A., Gustchina, A., Payne, S. L., Dunn, B. M., Kay, J., 1996. Expression, characterization and mutagenesis of the aspartic proteinase from equine infectious anaemia virus. Eur. J. Biochem. 241, 664-674.

Provitera, P., Bouamr, F., Murray, D., Carter, C., Scarlata, S., 2000. Binding of equine infectious anemia virus matrix protein to membrane bilayers involves multiple interactions. J. Mol. Biol. 296, 887-898.

Puffer, B. A., Parent, L. J., Wills, J. W., Montelaro, R. C., 1997. Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein. J. Virol. 71, 6541-6546.

Puffer, B. A., Watkins, S. C., Montelaro, R. C., 1998. Equine infectious anemia virus Gag polyprotein late domain specifically recruits cellular AP-2 adapter protein complexes during virion assembly. J. Virol. 72, 10218-10221.

Raabe, M. R., Issel, C. J., Montelaro, R. C., 1998. Equine monocyte-derived macrophage cultures and their applications for infectivity and neutralization studies of equine infectious anemia virus. J. Virol. Methods 71, 87-104.

Rasty, S., Dhruva, B. R., Schiltz, R. L., Shih, D. S., Issel, C. J., Montelaro, R. C., 1990. Proviral DNA integration and transcriptional patterns of equine infectious anemia virus during persistent and cytopathic infections. J. Virol. 64, 86-95.

Rice, N. R., Henderson, L. E., Sowder, R. C., Copeland, T. D., Oroszlan, S., Edwards, J. F., 1990. Synthesis and processing of the transmembrane envelope protein of equine infectious anemia virus. J. Virol. 64, 3770-3778.

Ritchey, J. W., Levy, J. K., Bliss, S. K., Tompkins, W. A., Tompkins, M. B., 2001. Constitutive expression of types 1 and 2 cytokines by alveolar macrophages from feline immunodeficiency virus-infected cats. Vet. Immunol. Immunopathol. 79, 83-100.

Roberts, M. M. and Oroszlan, S., 1989. The preparation and biochemical characterization of intact capsids of equine infectious anemia virus. Biochem. Biophys. Res. Commun. 160, 486-494.

Rodriguez, A., Castano, M., Pena, L., Sanchez, M. A., Nieto, A., Rodriguez, M., 1996. Immunocytochemical detection of growth factors (PDGF and TGF beta) in equine chronic pneumonia. Res. Vet. Sci. 60, 82-87.

Rosin-Arbesfeld, R., Rivlin, M., Noiman, S., Mashiah, P., Yaniv, A., Miki, T., Tronick, S. R., Gazit, A., 1993. Structural and functional characterization of rev-like transcripts of equine infectious anemia virus. J. Virol. 67, 5640-5646.

Rottman, J. B., Tompkins, W. A., Tompkins, M. B., 1996. A reverse transcription-quantitative competitive polymerase chain reaction (RT-qcPCR) technique to measure cytokine gene expression in domestic mammals. Vet. Pathol. 33, 242-248.

Roux, P., Alfieri, C., Hrimech, M., Cohen, E. A., Tanner, J. E., 2000. Activation of transcription factors NF-kappaB and NF-IL-6 by human immunodeficiency virus type 1 protein R (Vpr) induces interleukin-8 expression. J. Virol. 74, 4658-4665.

Rushlow, K., Olsen, K., Stiegler, G., Payne, S. L., Montelaro, R. C., Issel, C. J., 1986. Lentivirus genomic organization: the complete nucleotide sequence of the env gene region of equine infectious anemia virus. Virology 155, 309-321.

Russell, K. E., Perkins, P. C., Hoffman, M. R., Miller, R. T., Walker, K. M., Fuller, F. J., Sellon, D. C., 1999. Platelets from thrombocytopenic ponies acutely infected with equine infectious anemia virus are activated in vivo and hypofunctional. Virology 259, 7-19.

Russell, K. E., Walker, K. M., Miller, R. T., Sellon, D. C., 1998. Hyperglobulinemia and lymphocyte subset changes in naturally infected, inapparent carriers of equine infectious anemia virus. Am. J. Vet. Res. 59, 1009-1015.

Rwambo, P. M., Issel, C. J., Hussain, K. A., Montelaro, R. C., 1990. In vitro isolation of a neutralization escape mutant of equine infectious anemia virus (EIAV). Arch. Virol. 111, 275-280.

Saksela, K., Stevens, C., Rubinstein, P., Baltimore, D., 1994. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes. Proc. Nat. Acad. Sci. U. S. A. 91, 1104-1108.

Salinovich, O., Payne, S. L., Montelaro, R. C., Hussain, K. A., Issel, C. J., Schnorr, K. L., 1986. Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. J. Virol. 57, 71-80.

Sampson, M. J., Davies, I. R., Brown, J. C., Ivory, K., Hughes, D. A., 2002. Monocyte and neutrophil adhesion molecule expression during acute hyperglycemia and after antioxidant treatment in type 2 diabetes and control patients. Arteriosclerosis, Thromb. Vasc. Biol. 22, 1187-1193.

Schiltz, R. L., Shih, D. S., Rasty, S., Montelaro, R. C., Rushlow, K. E., 1992. Equine infectious anemia virus gene expression: characterization of the RNA splicing pattern and the protein products encoded by open reading frames S1 and S2. J. Virol. 66, 3455-3465.

Schmidtmayerova, H., Nottet, H. S., Nuovo, G., Raabe, T., Flanagan, C. R., Dubrovsky, L., Gendelman, H. E., Cerami, A., Bukrinsky, M., Sherry, B., 1996. Human immunodeficiency virus type 1 infection alters chemokine beta peptide expression in human monocytes: implications for recruitment of leukocytes into brain and lymph nodes. Proc. Nat. Acad. Sci. U. S. A. 93, 700-704.

Sellon, D. C., Fuller, F. J., McGuire, T. C., 1994. The immunopathogenesis of equine infectious anemia virus. Virus Res. 32, 111-138.

Sellon, D. C., Perry, S. T., Coggins, L., Fuller, F. J., 1992. Wild-type equine infectious anemia virus replicates in vivo predominantly in tissue macrophages, not in peripheral blood monocytes. J. Virol. 66, 5906-5913.

Sellon, D. C., Russell, K. E., Monroe, V. L., Walker, K. M., 1999. Increased interleukin-6 activity in the serum of ponies acutely infected with equine infectious anaemia virus. Res. Vet. Sci. 66, 77-80.

Sellon, D. C., Walker, K. M., Russell, K. E., Perry, S. T., Fuller, F. J., 1996. Phorbol ester stimulation of equine macrophage cultures alters expression of equine infectious anemia virus. Vet. Microbiol. 52, 209-221.

Sentsui, H. and Kono, Y., 1987a. Complement-mediated hemolysis of horse erythrocytes treated with equine infectious anemia virus. Arch. Virol. 95, 53-66.

Sentsui, H. and Kono, Y., 1987b. Phagocytosis of horse erythrocytes treated with equine infectious anemia virus by cultivated horse leukocytes. Arch. Virol. 95, 67-77.

Shao, H., Robek, M. D., Threadgill, D. S., Mankowski, L. S., Cameron, C. E., Fuller, F. J., Payne, S. L., 1997. Characterization and mutational studies of equine infectious anemia virus dUTPase. Biochim. Biophys. Acta 1339, 181-191.

Smith, T. A., Davis, E., Carpenter, S., 1998. Endotoxin treatment of equine infectious anaemia virus-infected horse macrophage cultures decreases production of infectious virus. J. Gen. Virol. 79, 747-755.

Sopper, S., Demuth, M., Stahl-Hennig, C., Hunsmann, G., Plesker, R., Coulibaly, C., Czub, S., Ceska, M., Koutsilieri, E., Riederer, P., Brinkmann, R., Katz, M., ter, M., V, 1996. The effect of simian immunodeficiency virus infection in vitro and in vivo on the cytokine production of isolated microglia and peripheral macrophages from rhesus monkey. Virology 220, 320-329.

Springs, D. R., Deutsch, S., Kufe, D. W., 1992. Genomic structure, induction, and product of TNF-α, in: Aggarwal, B. B., Vilcek, J. (Eds.), Tumor Necrosis Factors: Structure, Functions, and Mechanism of Action, Marcel Dekker, Inc, New York, pp. 3-34..

Stephens, R. M., Casey, J. W., Rice, N. R., 1986. Equine infectious anemia virus gag and pol genes: relatedness to visna and AIDS virus. Science 231, 589-594.

Stephens, R. M., Derse, D., Rice, N. R., 1990. Cloning and characterization of cDNAs encoding equine infectious anemia virus tat and putative Rev proteins. J. Virol. 64, 3716-3725.

Sune, C., Goldstrohm, A. C., Peng, J., Price, D. H., Garcia-Blanco, M. A., 2000. An in vitro transcription system that recapitulates equine infectious anemia virus tat-mediated inhibition of human immunodeficiency virus type 1 Tat activity demonstrates a role for positive transcription elongation factor b and associated proteins in the mechanism of Tat activation. Virology 274, 356-366.

Swaggerty, C. L., Frolov, A. A., McArthur, M. J., Cox, V. W., Tong, S., Compans, R. W., Ball, J. M., 2000. The envelope glycoprotein of simian immunodeficiency virus contains an enterotoxin domain. Virology 277, 250-261.

Swardson, C. J., Kociba, G. J., Perryman, L. E., 1992. Effects of equine infectious anemia virus on hematopoietic progenitors in vitro. Am. J. Vet. Res. 53, 1176-1179.

Swardson, C. J., Lichtenstein, D. L., Wang, S., Montelaro, R. C., Kociba, G. J., 1997. Infection of bone marrow macrophages by equine infectious anemia virus. Am. J. Vet. Res. 58, 1402-1407.

Swiderski, C. E., Klei, T. R., Horohov, D. W., 1999. Quantitative measurement of equine cytokine mRNA expression by polymerase chain reaction using target-specific standard curves. J. Immunol. Methods 222, 155-169.

Tacchini-Cottier, F., Vesin, C., Redard, M., Buurman, W., Piguet, P. F., 1998. Role of TNFR1 and TNFR2 in TNF-induced platelet consumption in mice. J. Immunol. 160, 6182-6186.

Tan, S. V. and Guiloff, R. J., 1998. Hypothesis on the pathogenesis of vacuolar myelopathy, dementia, and peripheral neuropathy in AIDS. J. Neurol. Neurosurg. Psychiatry 65, 23-28.

Tofaris, G. K., Patterson, P. H., Jessen, K. R., Mirsky, R., 2002. Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. J. Neurosci. 22, 6696-6703.

Toossi, Z., Young, T. G., Averill, L. E., Hamilton, B. D., Shiratsuchi, H., Ellner, J. J., 1995. Induction of transforming growth factor beta 1 by purified protein derivative of Mycobacterium tuberculosis. Infect. Immun. 63, 224-228.

Tornquist, S. J. and Crawford, T. B., 1997. Suppression of megakaryocyte colony growth by plasma from foals infected with equine infectious anemia virus. Blood 90, 2357-2363.

Tornquist, S. J., Oaks, J. L., Crawford, T. B., 1997. Elevation of cytokines associated with the thrombocytopenia of equine infectious anaemia. J. Gen. Virol. 78, 2541-2548.

Tozser, J., Friedman, D., Weber, I. T., Blaha, I., Oroszlan, S., 1993. Studies on the substrate specificity of the proteinase of equine infectious anemia virus using oligopeptide substrates. Biochemistry 32, 3347-3353.

Tracey, K. J. and Cerami, A., 1994. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu. Rev. Med. 45, 491-503.

Tymms, M. J., 1995. Quantitative measurement of mRNA using the RNase protection assay. Methods Mol. Biol. 37, 31-46.

Venable, R. M., Pastor, R. W., Brooks, B. R., Carson, F. W., 1989. Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. AIDS Res. Hum. Retroviruses 5, 7-22.

Vicenzi, E., Biswas, P., Mengozzi, M., Poli, G., 1997. Role of pro-inflammatory cytokines and beta-chemokines in controlling HIV replication. J. Leukocyte Biol. 62, 34-40.

Wang, S. Z., Rushlow, K. E., Issel, C. J., Cook, R. F., Cook, S. J., Raabe, M. L., Chong, Y. H., Costa, L., Montelaro, R. C., 1994. Enhancement of EIAV replication and disease by immunization with a baculovirus-expressed recombinant envelope surface glycoprotein. Virology 199, 247-251.

Weiland, F., Matheka, H. D., Coggins, L., Hatner, D., 1977. Electron microscopic studies on equine infectious anemia virus (EIAV). Arch. Virol. 55, 335-340.

Wetzel, M. A., Steele, A. D., Henderson, E. E., Rogers, T. J., 2002. The effect of X4 and R5 HIV-1 on C, C-C, and C-X-C chemokines during the early stages of infection in human PBMCs. Virology 292, 6-15.

Wohrl, B. M., Howard, K. J., Jacques, P. S., Le Grice, S. F., 1994. Alternative modes of polymerization distinguish the subunits of equine infectious anemia virus reverse transcriptase. J. Biol. Chem. 269, 8541-8548.

Xiong, H., Zeng, Y. C., Lewis, T., Zheng, J., Persidsky, Y., Gendelman, H. E., 2000. HIV-1 infected mononuclear phagocyte secretory products affect neuronal physiology leading to cellular demise: relevance for HIV-1-associated dementia. J. Neurovirol. 6 Suppl 1, S14-S23.

Xiong, H., Zheng, J., Thylin, M., Gendelman, H. E., 1999. Unraveling the mechanisms of neurotoxicity in HIV type 1-associated dementia: inhibition of neuronal synaptic transmission by macrophage secretory products. AIDS Res. Hum. Retroviruses 15, 57-63.

Yoon, S., Kingsman, S. M., Kingsman, A. J., Wilson, S. A., Mitrophanous, K. A., 2000. Characterization of the equine infectious anaemia virus S2 protein. J. Gen. Virol. 81, 2189-2194.

Zauli, G., Davis, B. R., Re, M. C., Visani, G., Furlini, G., La Placa, M., 1992. tat protein stimulates production of transforming growth factor-beta 1 by marrow macrophages: a potential mechanism for human immunodeficiency virus-1-induced hematopoietic suppression. Blood 80, 3036-3043.

Zhong, W. W., Burke, P. A., Hand, A. T., Walsh, M. J., Hughes, L. A., Forse, R. A., 1993. Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. Arch. Surg. 128, 158-163.

Zink, M. C., Coleman, G. D., Mankowski, J. L., Adams, R. J., Tarwater, P. M., Fox, K., Clements, J. E., 2001. Increased macrophage chemoattractant protein-1 in cerebrospinal fluid precedes and predicts simian immunodeficiency virus encephalitis. J. Infect. Dis. 184, 1015-1021.

Zou, W., Lackner, A. A., Simon, M., Durand-Gasselin, I., Galanaud, P., Desrosiers, R. C., Emilie, D., 1997. Early cytokine and chemokine gene expression in lymph nodes of macaques infected with simian immunodeficiency virus is predictive of disease outcome and vaccine efficacy. J. Virol. 71, 1227-1236.

# APPENDIX A

## LETTER OF PERMISSION


Date: April 14, 2003 Ref: TLimTexasA&MNM4-03

Wah-Seng Lim
wslim@cvm.tamu.edu

Dear Dr. Lim:

***PUBLICATION DETAILS: VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, V91(1): 45-51, Lim W-S et al: "Simultaneous quantitation of…" © 2003 Elsevier BV***

As per your letter, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   "Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for ProQuest to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely,

Nicole McIntyre
for Elsevier

**Your future requests will be handled more quickly if you complete the online form at www.us.elsevierhealth.com.**

# VITA

## Wah-Seng Lim

Work:
116 Veterinary Medicine Science Building
Texas A&M University
College Station, TX 77843-4467
(979) 845-8013, wslim@cvm.tamu.edu

Home:
2905 D Silver Spur Circle
Bryan, TX 77801
(979) 775-3689

**EDUCATION:**

Ph.D. in Veterinary Microbiology, 2003, Texas A&M University, College Station, Texas

B.A. in History of Art, 1997, University of Kansas, Lawrence, Kansas

**PUBLICATIONS:**

**Lim, W.-S.**, Edwards, J.F., Boyd, N.K., Payne, S.L., Ball, J.M., 2003. Simultaneous quantitation of equine cytokine mRNA using a multi-probe ribonuclease protection assay. Vet. Immunol. Immunopathol., 91, 45-51.

Boyd. N.K., Cohen, N.D., **Lim, W.-S.**, Martens, R.J., Chaffin, M.K., Ball, J.M., 2003**.** Temporal changes in cytokine expression of foals during the first month of life. Vet. Immunol. Immunopathol., 92, 75-85.

**Lim, W.-S.**, Payne, S.L., Edwards, J.F., Ball, J.M., 2003. Differential effects of virulent and avirulent equine infectious anemia virus on macrophage cytokine expression. Submitted.

Swaggerty, C.L., Huang, H., **Lim, W.-S.**, McArthur, M.J., Compans, R.W., Schroeder, F., and Ball, J.M., 2003. Comparison of SIVmac239$_{(352-382)}$ and SIVPBj41$_{(360-390)}$ enterotoxic synthetic peptides. Submitted.

Ball, J.M., Swaggerty, C.L., Pei, X., **Lim, W.-S.**, Xu, X., and Payne, S.L., 2003. The surface unit glycoprotein of equine infectious anemia virus exhibits enterotoxic activity. Submitted.

**Lim, W.-S.**, Payne, S.L., Ball, J.M., 2003. Induction of equine macrophage cytokines by the virulent molecularly cloned equine infectious anemia virus (EIAV$_{17}$) is independent of viral replication. Manuscript in preparation**.**