DEVELOPMENT AND EVALUATION OF NONRADIOACTIVE METHODS FOR MONITORING T LYMPHOCYTE RESPONSE TO EQUINE ARTERITIS VIRUS (EAV) IN HORSES

Annet Kyomuhangi
University of Kentucky, annet.kyomuhangi@uky.edu
Author ORCID Identifier: https://orcid.org/0000-0003-3608-9249
Digital Object Identifier: https://doi.org/10.13023/etd.2019.027

Click here to let us know how access to this document benefits you.

Recommended Citation
https://uknowledge.uky.edu/gluck_etds/39

This Master's Thesis is brought to you for free and open access by the Veterinary Science at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Veterinary Science by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Annet Kyomuhangi, Student
Dr. Udeni Balasuriya, Major Professor
Dr. Daniel Howe, Director of Graduate Studies
DEVELOPMENT AND EVALUATION OF NONRADIOACTIVE METHODS FOR MONITORING T LYMPHOCYTE RESPONSE TO EQUINE ARTERITIS VIRUS (EAV) IN HORSES

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By
Annet Kyomuhangi
Lexington, Kentucky

Director: Dr. Udeni Balasuriya, Professor of virology
Lexington, Kentucky
2018

Copyright © Annet Kyomuhangi 2018
https://orcid.org/0000-0003-3608-9249
Target cell lysis is the hallmark of immune effector responses of cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and monocytes. The most commonly used assay to measure target cell lysis is the $^{51}$Cr release assay and is considered the ‘gold standard’. However, this assay has many disadvantages that limit its use by most laboratories. Thus, several alternative assays have been developed. Some of these alternative assays are more sensitive, easy to perform and do not use radioactive elements.

In this study, four of these assays were evaluated for their ability to detect antigen-specific CTL responses in equine blood. Three long-term equine arteritis virus (EAV) carrier stallions, two vaccinated stallions and one naïve stallion were included in this study. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected of these stallions to be used as effector cells. The PBMCs were stimulated with EAV in vitro for 7-10 days to generate antigen-specific effector cells. The granzyme B assay, the Carboxyfluorescein succinimidyl ester (CFSE)/7-Aminoactinomycin D (7AAD) assay and the Lactate dehydrogenase (LDH) assay were performed using these effector cells and autologous equine dermal cells (isolated from each stallion) as target cells.

The first two assays (i.e., granzyme B and CFSE/7AAD assays) were difficult to optimize for this study because they work well with non-adherent targets and require immediate flow cytometry analysis. The LDH assay, however detected CTL lysis in one of the two vaccinated stallions at day 99 post vaccination and no response was detected in PBMCs isolated from carrier stallions and control stallion. Based on these findings, the LDH assay is the most suitable assay since it works well with adherent target cells, it produces quantitative data, and is ideal for high-throughput screening.

KEYWORDS: Cytotoxic T lymphocyte assays, Lactate dehydrogenase assay, Equine arteritis virus.

Annet Kyomuhangi

02/20/2019
DEVELOPMENT AND EVALUATION OF NONRADIOACTIVE METHODS FOR MONITORING T LYMPHOCYTE RESPONSE TO EQUINE ARTERITIS VIRUS (EAV) IN HORSES

By
Annet Kyomuhangi

Dr. Udeni Balasuriya
Director of thesis

Dr. Daniel Howe
Director of graduate studies

02/20/2019
Date
TABLE OF CONTENTS

LIST OF TABLES ............................................................................................................. vii

LIST OF FIGURES ......................................................................................................... viii

CHAPTER 1. INTRODUCTION ....................................................................................... 1
  1.1 Background ........................................................................................................... 1
  1.2 Rationale for this study. ....................................................................................... 2

CHAPTER 2. LITERATURE REVIEW ............................................................................ 3
  2.1 Equine arteritis virus (EAV) .................................................................................. 3
  2.2 Major structural proteins of EAV ........................................................................ 4
  2.3 Equine viral arteritis (EVA) .................................................................................. 5
  2.4 Immune response to EAV .................................................................................... 7
  2.5 Immune response to viruses .............................................................................. 8
    2.5.1 Innate immune response to viral infection ..................................................... 8
    2.5.2 Humoral immune response to viral infection ................................................. 8
    2.5.3 Innate immunity and antigen presentation .................................................... 9
    2.5.4 Differentiation of CD4+ T lymphocytes after antigen presentation ............. 9
      2.5.4.1 Role of TH1 cells in viral infections ......................................................... 11
      2.5.4.2 Role of TH2 cells in viral infections ......................................................... 12
      2.5.4.3 Role of TH17 cells in viral infections ....................................................... 13
      2.5.4.4 The role of CD4+ T regulatory cells (Tregs) in viral infections ............... 14
      2.5.4.5 The mechanism of CD4+ T help to CD8+ T cells ................................... 15
      2.5.4.6 CD4+ T follicular helper (T_{FH}) cells help for B cells ......................... 17
      2.5.4.7 Antiviral functions of CD4+ T lymphocytes ........................................... 17
        2.5.4.7.1 Induction of an antiviral state ............................................................ 17
        2.5.4.7.2 Direct cytotoxicity of CD4+ T lymphocytes ...................................... 18
      2.5.4.8 The role of CD4+ T lymphocytes in equine viral infections .................... 18
        2.5.4.9 Summary ............................................................................................... 18
      2.5.5 Role of CD8+ T lymphocytes cells in viral infections .................................. 19
        2.5.5.1 CTL response in hepatitis B Virus (HBV) infections ................................. 20
        2.5.5.2 CTL response in human immunodeficiency virus (HIV) infection .......... 21
        2.5.5.3 CTL response to influenza virus ............................................................ 24
        2.5.5.4 Role of CTL response in equine viral infections ..................................... 25
          2.5.5.4.1 Equine infectious anemia virus (EIAV) ............................................ 25
          2.5.5.4.2 Equine herpesvirus 1 (EHV 1) ......................................................... 26
          2.5.5.4.3 Equine arteritis virus (EAV) ............................................................ 27
    2.5.6 Cytotoxic T Lymphocyte killing mechanisms ............................................... 27
      2.5.6.1 Stages of CTL killing .............................................................................. 30
        2.5.6.1.1 Target cell recognition and formation of the immunological synapse 30
LIST OF TABLES

Table 1: Results of screening stallions for VN antibodies to EAV................................. 51
Table 2: EAV VN antibody findings at different time-points after vaccination.................. 52
Table 3: Virus neutralization test and competitive ELISA (cELISA) results...................... 52
LIST OF FIGURES

Figure 1: Schematic representation of the EAV virion ................................................................. 3
Figure 2: Activation of CD4+ T lymphocytes and their effector functions .............................. 10
Figure 3: Dendritic cell licensing and killing of target cells by CTL ...................................... 16
Figure 4: Fas-FasL and Granzyme pathways ............................................................................ 28
Figure 5: Immunofluorescence microscopy performed on primary EDCs ............................... 53
Figure 6: Flow cytometry data for expression of MHC class I and MHC class II molecules .... 53
Figure 7: Immunofluorescence staining of EDCs infected with EAV VBS ............................... 54
Figure 8: Immunofluorescence staining of EDCs infected with EAV VBS ............................... 54
Figure 9: UV inactivation of EAV VBS ................................................................................... 55
Figure 10: Flow cytometry analysis of EDCs infected with live and UV inactivated EAV VBS . 55
Figure 11: IFA staining of EDCs transfected with EAV IVT RNA ........................................ 56
Figure 12: ELISpot data conducted with PBMCs (isolated from carrier stallions) stimulated with autologous EDCs transfected with EAV IVT RNA ......................................................... 56
Figure 13: Results of LDH assays performed with LAK cells and EQT8888 cells ................. 57
Figure 14: LDH data for carrier stallions ................................................................................... 58
Figure 15: LDH data for vaccinated horses (N122 and L134) and one naïve horse (0114) ....... 59
CHAPTER 1. INTRODUCTION

1.1 Background

Immune defense mechanisms fall into two broad categories: innate immunity and adaptive immunity.\(^1\) Innate immunity is always present and is the first line of defense against invading microorganisms. Adaptive immunity is also referred to as acquired immunity because it requires prior exposure to an antigen. Adaptive immunity is further divided into humoral immunity and cell-mediated immunity. Humoral immunity is mediated by antibodies produced by B cells and is usually directed at extracellular organisms. Cell-mediated immunity is mediated by T lymphocytes and is usually directed at intracellular invaders.\(^1\)

T lymphocytes recognize antigens through their T cell receptor (TCR), in the form of short peptides presented on major histocompatibility complex (MHC) by antigen presenting cells or infected cells.\(^2\) T lymphocytes that express CD8 molecules on their surface are known as CD8\(^+\) T lymphocytes. Upon recognition of antigen, CD8\(^+\) T lymphocytes differentiate into cytotoxic T lymphocytes (CTL) which directly kill microbe infected cells. Another subset of T lymphocytes, the CD4\(^+\) T lymphocytes, are very active at producing cytokines that help B cells make antibodies and activate antigen presenting cells. Studies in mice and human show that CD4\(^+\) T lymphocytes are also required for generation of functional memory CD8\(^+\) T lymphocytes.\(^3\)

Despite the remarkable sensitivity of the immune system at recognizing and eliminating infected cells, chronic infections still exist. This is because some microbes have evolved mechanisms to evade immune system recognition including down regulating MHC expression,\(^4\) antigenic variation,\(^5\) molecular piracy\(^6\) and dysregulation of the
immune system. Hence, there remains a need for novel vaccines that stimulate both antibody and cellular immune responses. While there are a number of methods available to measure antibody responses, accurate and reproducible assays for measuring T cell responses are lacking. Furthermore, T cell assays that closely correlate with host protection are very difficult to develop. The chromium release assay has been successfully used to measure T cell mediated immune response in horses, but this assay uses radioactive material which limits its use by some laboratories. Here, we evaluated three alternative methods to identify a CTL response to a virus.

1.2 Rationale for this study

Measuring T cell response provides valuable information that can be used to develop new vaccines or optimize existing vaccines. Immune system monitoring can also reveal factors that can limit protective immunity. The aim of this project was to develop and evaluate T cells assays that could be used in future studies for monitoring T cell responses to an important viral pathogen of horses, equine arteritis virus (EAV).
CHAPTER 2. LITERATURE REVIEW

2.1 Equine arteritis virus (EAV)

EAV is an enveloped, positive sense single stranded RNA virus with a spherical virion that has a diameter of 40-60nm. The EAV N protein forms the nucleocapsid which encapsulates the positive sense ssRNA genome of 12.7 kilobases. The EAV genome is polycistronic; with two large open reading frames (at 5’ end) that encode the replicase protein (the nonstructural protein of EAV) and a set of eight open reading frames (2a, 2b, 3, 4, 5a, 6 and 7) located downstream of the genome. The eight open reading frames (ORFs) encode structural proteins: E, GP2, GP3, GP4, GP5, M and N respectively (Figure 1). 

Figure 1: Schematic representation of the EAV virion

The GP5 and M are the major envelope proteins while E, GP2, GP3 and GP4 are minor envelope proteins. Translation of the replicase protein yields two large proteins; EAV replicase polyprotein1a and polyprotein1ab. These polypeptides undergo posttranslational cleavage by three viral proteases (nsp1, nsp2, and nsp4) encoded by ORF1a to produce 13 nonstructural proteins (nsp1-nsp6, 7α and 7β, nsp8-nsp12).
Comparison of GP5, M and N sequences of EAV from carrier stallions revealed that M and N proteins are more conserved than GP5. The N protein is the most conserved structural protein of EAV whereas the GP5 is the most variable structural protein and contains major neutralizing determinants of EAV. 26-29

2.2 Major structural proteins of EAV

The EAV nucleocapsid (N) protein is a non-glycosylated protein (14kda;110aa) encoded by ORF 7.30 It is dispensable for genome replication and mRNA synthesis since these processes have been shown to go on with the N protein trapped in the nucleus.31 In infected cells, the N protein constitutes 30 to 45% of the protein moiety making it the most abundant protein.32 It plays an important role in encapsidating the viral genome and in interacting with envelope proteins during virus assembly. Thus N protein is necessary for virus assembly and for production of infectious virus particles.33 A 7 year-long comparative nucleotide sequence analysis of virus isolated from carrier stallion semen showed that ORF7 is the most conserved gene of EAV.26

The non-glycosylated membrane protein (M) and the large envelope glycoprotein GP5 are the two major envelope proteins of the virus, and are encoded by ORF 6 and 5, respectively. The M protein is 16-kDa and 162 amino acid long,30 it is predicted to span the viral envelope three times with its internal trans-membrane segments, leaving a short stretch of 10-18aa exposed at the outside of the virion (ectodomain) and an approximately 72-residue buried inside the virion (endodomain).9,34

The GP5 protein is 42-kDa and 255 amino acids in length.30 It is heterogeneously glycosylated and has an ectodomain (of 19-116aa), three membrane-spanning domains and an endodomain of about 64 amino acids. Comparative analysis of deduced amino acid sequences of the GP5 protein identified three distinct variable regions (V1 [aa 61-121], V2
[aa 141-178], and V3 [aa 202-222]), a putative signal sequence (S[aa 1-18]), and four conserved regions (C1[aa 19-60], C2 [aa 122-140], C3 [aa 179-201], and C4 [aa 223-255]). The GP5 protein expresses the neutralization determinants of the virus, all located on the ectodomain of the GP5 protein. When expressed individually, GP5 and M proteins are retained only in the ER. In contrast, when co-expressed, the M protein localizes both in ER and the Golgi complex and the GP5 protein consistently co-localizes with the M protein in the Golgi complex showing that their transport is dependent upon the formation of a GP5/M heterodimer.

2.3 Equine viral arteritis (EVA)

Majority of EAV infections are subclinical, but occasional sporadic respiratory disease, sudden death of foals and mild clinical symptoms in adult horses have been reported. Abortion in pregnant mares and delivery of weak foals presenting with interstitial pneumonia are the most important clinical manifestations of EVA. EAV is transmitted through the respiratory route (by aerosolization of respiratory secretions in acutely infected animals) or the venereal route. Experimental inoculations of horses reveal that, within a day of infection, the virus invades and replicates in the respiratory epithelium and alveolar macrophages before spreading to bronchial lymph nodes on day 2. On day 3, the virus replicates in bronchopulmonary lymph nodes and circulating monocytes. Viral shedding in nasal secretions typically lasts 7-14 days, although it can extend to 21 days post infection. Systemic distribution of the virus is facilitated by infected monocytes. Cell-associated viremia develops within 3 days of infection and can go on until day 19 post infection. During this period, the virus replicates in macrophages, vascular smooth muscle cells and endothelial cells causing systemic
panvasculitis. EAV replication in endothelial cells damages the endothelium leading to increased permeability and recruitment of inflammatory cells to the endothelium. Increased vascular permeability and leukocyte infiltration results into edema and hemorrhage around the vessels. This severe vascular damage will eventually lead to disseminated vascular coagulation.

Between 10% and 70% of EAV infected stallions become carriers. The carrier state established in the reproductive tract of stallions provides a reservoir of EAV because carrier stallions can transmit the virus to susceptible mares by natural breeding or by artificial insemination. These infected mares can in turn disseminate the virus to susceptible horses. Studies done by Little et al, show that the establishment and maintenance of the carrier state is testosterone dependent, as persistently infected stallions eliminate the virus after castration and those that receive testosterone after castration, continue to shed the virus in semen. The carrier state can be short-term that lasts for a few weeks after clinical recovery, intermediate carrier state that lasts for 3 to 7 months, or long-term carrier state that lasts several years. Long-term carrier stallions may spontaneously cease virus shedding after intervals of 1 to 10 years, with no reversion to the shedding state. EAV has been shown to persist in the ampulla of the stallion reproductive tract.

Recent studies show that horses can be divided into two groups (i.e. susceptible and resistant) based on in vitro susceptibility of their CD3+ T lymphocytes to EAV. Stallions that possess the susceptible phenotype are at increased risk of becoming long-time carriers whereas stallions lacking this phenotype are less likely to become long-time carriers. A genome wide association study (GWAS) revealed that the ability of EAV to infect CD3+ T lymphocytes and establish long-term carrier status in stallions correlated
with a region within equine chromosome 11 (exon 1) that encodes CXCL16 protein. Equine CXCL16 susceptible (CXCL16S) and CXCL16 resistant (CXCL16R) have been shown to differ at positions 40, 49, 50 and 52. Consequently, the in vitro susceptibility is associated with CXCL16S while the resistant phenotype is associated with CXCL16R. Long term EAV persistence has been associated with downregulation of miRNA (eca-mir-128) a putative target of CXCL16 leading to overexpression of this protein.

2.4 Immune response to EAV

EAV has been shown to inhibit type 1 interferon production in endothelial cells. In addition, infection of alveolar macrophages and blood derived microphages with avirulent and virulent strains of EAV showed increased expression of pro-inflammatory cytokines, and the magnitude of this response was greater in virulent strains than avirulent strains. Humoral immune response to EAV also varies with the infecting strain and duration of infection. Sera from EAV infected horses recognize M, N, GP5, nsp2, nsp4, nsp5 and nsp12. The GP5 ectodomain contains the four major EAV neutralizing sites, all located in GP5’s variable region 1 (V1). EAV induces production of Virus Neutralizing (VN) antibodies within 2 weeks of infection, these antibodies peak at 2-4 months and persist for 3 years or more after that. Complement fixing antibodies develop 1-2 weeks post infection and peak 2-3 weeks after infection and they disappear by the eighth month. IgM antibodies have been reported as early as 6 Days Post Infection (DPI), they peak at 10DPI and decline by 21-49 DPI. Foals are protected against EAV infection by passive transfer of VN antibodies in colostrum and these antibodies persist for 6 months. Recently, EAV has been shown to elicit a mucosal antibody response in the reproductive tract of persistently infected horses. Analysis of seminal plasm shows the presence of EAV-specific immunoglobulin isotypes IgA, IgG1,
IgG3/5, and IgG4/7 with IgG1 and IgG4/7 showing virus-neutralizing activity.\textsuperscript{75} Also, significant infiltration of T lymphocytes, macrophages and dendritic cells into the reproductive tract of carrier horses has been reported.\textsuperscript{76} However, the role of these cells is not known since the virus was still persists irrespective of immune cells infiltrating the reproductive tract.

\section*{2.5 Immune response to viruses}

\subsection*{2.5.1 Innate immune response to viral infection}

Innate immune response uses pattern recognition receptors (PRRs) to detect specific viral components such as viral RNA or DNA or viral intermediates. Endosomal Toll like receptors (TLRs) are activated by nucleic acids. These TLRs include: TLR3, TLR7, TLR8 and TLR9.\textsuperscript{77} Activation of these receptors induces the production of type 1 interferons and other proinflammatory cytokines by infected cells or immune cells. Type 1 interferons are the principal cytokines involved in antiviral response. These include INF-\textalpha, INF-\textbeta and others.\textsuperscript{78}

\subsection*{2.5.2 Humoral immune response to viral infection}

Mucosal immune system acts as the first line of defense to limit respiratory viral infection. Secreted IgA and IgM are the major neutralizing antibodies on mucosa to prevent viral entry. During infection with influenza, nasal secretions contain IgA which can neutralize hemagglutinin and neuraminidase of influenza A virus (IAV). Immunoglobulins: IgG, IgM and IgA are present in mucosal secretions during primary IAV infection.\textsuperscript{79} In certain viral infections, serum neutralizing antibodies are associated with reduced viral replication.\textsuperscript{80} All clinically protective vaccines depend on the production of neutralizing antibody response. This is because antibodies not only prevent entry of
viruses, but they also facilitate the removal of infected cells.

2.5.3 Innate immunity and antigen presentation

Cells of the innate immune system use pattern recognition receptors (PRRs) to sense viral pathogens by detecting pathogen-associated molecular patterns (PAMPs) on viruses. PAMPs are conserved moieties expressed by microbes. Majority of viruses are sensed via recognition of their nucleic acids which accumulate during replication. Pattern recognition receptors (PRRs) also respond to signatures such as 5′ triphosphate RNA, which is not normally found in host RNA but present in viruses. Pattern recognition receptors (PRRs) are germ-line encoded receptors expressed by innate immune cells such as macrophages, neutrophils and dendritic cells. There are 3 main categories of virus associated PRRs: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). Toll-like receptors (TLRs) are in the plasma membrane or endosomal vesicles. Plasma membrane TLRs are usually specific for hydrophobic lipids and proteins while those found in endosomes detect nucleic acids. When viruses enter host cells, they uncoat their genomes and release viral components into the cytoplasm. These viral components are detected by retinoic acid-inducible gene I-like receptors (RLRs), the nucleotide oligomerization domain-like receptors (NLRs) and cytosolic DNA sensors such as members of the AIM2 family.

2.5.4 Differentiation of CD4+ T lymphocytes after antigen presentation

At the initial stages of infection, innate immune cells such as APCs sense the invading virus by engaging PRRs. This activates the APCs to upregulate co-stimulatory molecules (e.g. CD40, CD80:CD86), major histocompatibility (MHC) molecules and promotes migration of the APCs to draining lymph nodes. Here, the APCs present virus-derived peptides on MHC class II molecules to naive CD4+ T cells and
deliver co-stimulatory signals.\textsuperscript{55} (Figure 2)

The naïve CD\textsuperscript{4+} T cells then undergo clonal expansion and differentiate into distinct subsets of effector CD\textsuperscript{4+} T cells\textsuperscript{87-89} depending on the cytokine milieu at the time of activation (Figure 2). The CD\textsuperscript{4+} T cell subsets are defined by the cytokines they produce and the expression of specific transcriptional factors.\textsuperscript{90-98}
2.5.4.1 Role of TH1 cells in viral infections

CD4+ effectors generated in response to viral infection are mainly TH1, characterized by strong IFN-γ production, due to exposure to high levels of interleukin-12 (IL-12), interferon gamma (IFN-γ) and type I interferons in the priming milieu. TH1 cells produce antiviral cytokines, such as IFN-γ, tumor necrosis factor alpha (TNFα), and interleukin IL-2 (IL-2) which are involved in generation of cytotoxic T cells, and activation of innate immune cells such as natural killer (NK) cells and macrophages.

IL-2 produced by TH1 cells attracts NK cells to sites of infection and stimulates them to produce IFN-γ. A study conducted by Janssen et al., using C57BL/6 mice immunized with adenovirus 5 E1- transformed Tap−/− mouse embryo cells showed that depletion of CD4+ helper T cells before immunization, blocked the production of antigen-specific cytotoxic T lymphocytes (CTLs) after secondary in vitro re-stimulation. Addition of IL-2 to primary effectors rescued this secondary expansion defect. Thus IL-2 is critical in the development of memory CTLs possibly by sustaining CD8+ T cell proliferation or by enhancing survival of antigen-specific CTLs. Similarly, studies with IL-15 and IL-15 receptor deficient mice show a potent primary CD8+ T cell response to infection with lymphocytic choriomeningitis virus (LCMV) that was phenotypically and functionally similar to CD8+ T cell response present in IL-15+/+ mice. But longitudinal analysis revealed a decrease in virus-specific memory CTLs in IL-15−/− mice due to a severe defect in the
proliferative renewal of antigen-specific memory CD8⁺ T cells. Thus, cytotoxic T lymphocytes generated with CD4⁺ T cell help are long-lived, functional memory cells that can respond quickly to re-challenge with virus. These studies underpin the role of CD4⁺ helper T cells in programming the development of CD8⁺ T memory cells and the application of this knowledge to the development of vaccines and thereuptics that enhance memory T cell responses.

IFN-γ has been shown to upregulate both major histocompatibility complex class I (MHC I) and major histocompatibility complex class II (MHC II) expression, activate macrophages in an antigen-specific fashion, and induce several IFN-inducible antiviral mechanisms. During hepatitis C virus (HCV) infection, ability of T cells to produce IFN-γ is significantly associated with decreased viremia and reduced risk of chronic infection. Hepatitis B virus (HBV) studies show that IFN-γ inhibits hepatitis B virus (HBV) DNA replication in hepatocytes. The critical role of IFN-γ in viral infections was demonstrated in mice lacking IFN-γ and IFN-γ receptor. The mice were highly vulnerable to lethal west Nile virus (WNV) infection with mortality rates rising from 30% (wild type mice) to 90% (IFN-γ⁻/⁻ or IFN-γR⁻/⁻ mice).

The role of TNF-α in viral infections is demonstrated in patients with autoimmune disorders who develop subfulminant hepatitis B infection after infusion with infliximab (a chimeric monoclonal antibody against TNF-α) due to an unrecognized HBs-antigen carrier state.

2.5.4.2 Role of T₃₄2 cells in viral infections

T₃₄2 cell responses were thought to drive optimal humoral immune responses through production of IL-4. However, adoptive transfer of T₃₄1 cells, and T₃₄2 cells,
demonstrated that IFN-γ (produced by T<sub>H1</sub> cells in response to viral infections) mediates IgG2a class-switch responses. In 1987, Jean-Paul et al., infected mice with a panel of 11 RNA and DNA mice viruses. Their data identifies IgG2a as the predominant antiviral isotype in these mice. In fact, T<sub>H2</sub> cell responses are not protective but drive immune mediated injury during infection with several viruses including, influenza virus, respiratory syncytial virus (RSV), herpes simplex virus (HSV) and vaccinia virus.

2.5.4.3 Role of T<sub>H17</sub> cells in viral infections

The role of T<sub>H17</sub> effector responses to viral infections is not well understood, but IL-17-producing CD4<sup>+</sup> T cells responses are generally considered to be detrimental to the host due to immune mediated injury caused by recruitment of neutrophils to the infected site. However, in several viral infections, T<sub>H17</sub> cells could be necessary to prevent disease exacerbation.

Infection with Theiler’s murine encephalomyelitis virus infection induces development of T<sub>H17</sub> cells in vitro and in vivo in an IL-6–dependent manner. The IL-17 cytokine produced by these cells was shown to promote viral persistence by upregulating antiapoptotic molecules and consequently enhancing the survival of virus-infected cells. Also, neutralization of IL-17 augmented virus clearance and enhanced the cytotoxic function of T cells. Contrary to these findings, McKinstry et al., show that T<sub>H17</sub>-polarized CD4<sup>+</sup> T effectors can protect against high doses of flu (in the absence of IL-10) and that this response is IFNγ-, perforin- and IL-17A-independent. This protection could be mediated by recruitment of neutrophils to the site of infection since IL-17 has been shown to promote granulopoiesis (via granulocyte colony-stimulating factor (G-CSF)) and upregulation of CXC-chemokines that promote neutrophil recruitment.
Unique CD8+ T cytotoxic (Tc) 17 cells which are CD8+RORγt+ IL-17-secreting Tc17 cells have been shown to protect mice from lethal influenza virus challenge. The Tc17 cells are unique in that they produce IL-17A and IL-17F, and lack perforin and granzyme B expression and have no cytolytic activity. Tc17 protection is accomplished by great neutrophil influx into the lung of infected mice and production of IFN-γ.

Th17 cells often produce significant levels of IL-21 and IL-22 in addition to IL-17. The role of IL-22 is not extensively studied in viral infections but IL-22 has been shown to promote tissue repair via upregulation of defensins. IL-21 is involved in sustaining CD8+ T cell responses during chronic viral infection.

Th17 cells are necessary for host defense against some extracellular bacteria and fungi. Therefore, viral infections like HIV that deplete Th17 cells allow opportunistic infections in the gut to take hold. In this regard, Th17 are very critical in preventing disease exacerbation that could result into host death.

2.5.4.4 The role of CD4+ T regulatory cells (Tregs) in viral infections

Studies aimed at understanding the role of antigen-specific Tregs have been reported in models of chronic infection. The outcome of Treg responses can be beneficial or detrimental depending on the infecting virus. In a herpes simplex virus model, mice depleted of CD25+ T-cell (nTregs) had enhanced in vivo CD8+ T-cell proliferation and cytotoxicity responses to the immunodominant peptide. This response was three-fold the response observed in animals with intact nTregs. In HCV studies, patients with persistent HCV infection were shown to have a higher frequency of CD4+CD25+ T cells than recovered persons, and these nTregs could directly suppress the HCV-specific reactivity of CD8+ T cells ex vivo. Targeting and disrupting of Tregs by viruses like HIV may
contribute to hyperactivation of conventional T cells which could hasten progression to AIDS.\textsuperscript{140, 141} In most viral infections it seems like nTregs work to dampen the efficacy of protective immunity hence contribute to persistence and perhaps chronic disease.\textsuperscript{142} It is also evident that Tregs profoundly inhibit T cell activation, proliferation and effector function which can limit the immunopathology caused by persistent high level of immune stimulation from chronic viral infections.\textsuperscript{142, 143}

\subsection*{2.5.4.5 The mechanism of CD4\textsuperscript{+} T help to CD8\textsuperscript{+} T cells}

During viral infections, CD4\textsuperscript{+} T helper cells (CD4\textsuperscript{+}TH) promote engagement of CD8\textsuperscript{+} T cells with antigen presenting cells (such as dendritic cells; figure 3). Dendritic cells (DCs) upon cognate interaction with CD4\textsuperscript{+}TH increase their ability to attract/retain (get ‘licensed’) antigen specific CD8\textsuperscript{+} T cells.\textsuperscript{144-147} However, some authors think that this pathway may be of minor importance since APCs can be activated effectively by viruses through PRRs without help from helper cells.\textsuperscript{148-151} Nevertheless, the absence of CD4\textsuperscript{+} T help has been shown to compromise the generation of primary cytotoxic T lymphocyte (CTL) responses to vaccinia virus (VV),\textsuperscript{152} herpes simplex virus (HSV)\textsuperscript{147} and influenza virus.\textsuperscript{153, 154}
Figure 3: Dendritic cell licensing and killing of target cells by CTL

The generally accepted model of APC licensing suggests that CD4⁺TH activate/’license’ DCs to attract naïve CD8⁺ T cells (figure 3). Viruses use two routes to enter host cells: 1) Receptor mediated entry which involves the virus binding to its receptor on DCs. In this route, the virus replicates and viral proteins generated during replication are degraded into peptide fragments and presented by MHC class I molecules to CD8⁺ T cells; 2) Through pinocytosis, DCs capture viral particles or remnants of virus infected cells. These antigens are processed, and cross presented by MHC class II to CD4⁺ TH. CD4⁺TH then upregulate CD40L expression and, through interaction with CD40, activate DCs to upregulate the expressing of CD80/CD86 marker.
CD80/CD86 with CD28 activates CD8⁺ T cells to differentiate into cytotoxic cells that induce apoptosis of virus infected cells by release of cytotoxic granules, and the production of TNF-α and IFN-γ. Thus CD4⁺ T_H provide help to CD8⁺ T cells indirectly by activating APCs through CD40.

2.5.4.6 CD4⁺ T follicular helper (T_{FH}) cells help for B cells

The expression of SLAM-associated protein (SAP) by T_{FH} promotes the formation of germinal center and drives production of memory B cells and long-lived plasma cells. T_{FH} cells provide help to cognate B cells via the expression of CD40L, inducible T cell co-stimulator (ICOS), IL-21, IL-4, and other molecules.

2.5.4.7 Antiviral functions of CD4⁺ T lymphocytes

CD4⁺ T cells have potent protective roles that are independent of their helper functions. First, CD4⁺ T cells produce cytokines (such as IFN-γ and TNFα) that induce an antiviral state at infected sites. Second, CD4⁺ T cells have a direct cytotoxicity function.

2.5.4.7.1 Induction of an antiviral state

IFN-γ and other interferons induce proteins that contribute to the antiviral state. One of these proteins is the double stranded RNA dependent enzyme 2’5’- oligoadenylate synthetase which activates the oligomerization of ATP. The 2’5’ ATP oligomers activate an RNAse that can cleave both cellular and viral RNAs. The second protein is the double stranded RNA dependent protein kinase known as protein kinase R which inhibits virus and host cell protein synthesis by directly phosphorylating the α subunit of the translation initiation factor eIF-2. The third is a group of proteins referred to as Mx proteins, are highly conserved large GTPase that inhibit the trafficking or activity of virus polymerases.
2.5.4.7.2 Direct cytotoxicity of CD4+ T lymphocytes

Cytotoxic CD4+ T cells have been reported in γ-herpesvirus,172 dengue virus,173 lymphocytic choriomeningitis virus174 and friend retrovirus.175 These cells express the degranulation marker, CD107,172 and the cytotoxicity is perforin and FASL mediated.176,177 The transcription factor eomesodermin (Eomes) is crucial in driving the development of cytolytic CD4+ T cells in vivo. Dual costimulation (DCo) through OX40 and 4-1BB is responsible for activating Eomes to program naïve CD4+ T cells into cytotoxic T_H1 cells.178 Low antigen dose and early IL-2 signals have also been shown to play a role in the differentiation of CD4+ T cells into cytotoxic cells.179

2.5.4.8 The role of CD4+ T lymphocytes in equine viral infections

Bovine papillomavirus (BPV) infections of the equine species causes sarcoid. Equine sarcoids have been shown to contain a large number of CD4+ CD8+ dual positive T cells which express FOXP3 a key transcription factor of regulatory T cells. Regulatory cells allow the persistence of lesions by dampening protective antiviral response.180

2.5.4.9 Summary

On activation, naïve CD4+ T cells differentiate into distinct subsets i.e. TH1, TH2, TH17 and TFH, and we have discussed how each of these subsets contribute to viral infections. The common denominator of these effector subsets is to produce cytokines that not only establish an antiviral state but also program the development of memory CD8+ T cytotoxic cells. Another key role is the provision of help to B cells for development of germinal centers and long-lived memory humoral response. CD4+ T cells carry out a variety of functions that could differ depending on the site of infection, the infecting pathogen and the level of infection. Sometimes the different mechanisms utilized by the
effector subsets synergize to produce a robust T cell response.

2.5.5 Role of CD8+ T lymphocytes cells in viral infections

The role of Cytotoxic T lymphocytes (CTLs) in viral infections was first demonstrated by Zinkernagel and Doherty in LCMV infected mice. In this study, spleen and lymph node cells from infected mice lysed mice fibroblasts infected with LCMV in a chromium release assay. A few years later, transfer of CTL clones into influenza A infected mice showed antiviral effects which were mediated by killing of virus-infected cells. Similar results were obtained with studies in respiratory syncytial virus and herpes simplex virus.

CD8+ T cells typically mediate protection against intracellular pathogens and tumor cells. They are the major contributors to antiviral T cell immunity. The role of CD8+ T cells in viral infections has been investigated in various viral infections including HIV, HBV, HCV, LCMV, influenza virus and others. CD8+ T cells recognize cognate antigen presented via major histocompatibility complex class 1 (MHC1). In the presence of co-stimulatory signals, antigen-specific CD8+ T lymphocytes undergo clonal expansion and differentiate into cytotoxic T lymphocytes (CTL) that produce cytolytic granules and other factors (such as IFN-γ and TNF-α).
2.5.5.1 CTL response in hepatitis B Virus (HBV) infections

HBV is a hepatotropic DNA virus in the hepadnaviridae family. It causes both acute and chronic liver infections. Despite significant vaccination and antiviral therapy programs, HBV-related sickness still accounts for about one million deaths annually.

Strong HBV specific CD8\(^+\) T cells responses have been correlated with viral clearance during acute infection.\(^{187}\) CD8\(^+\)- depletion studies confirm that these cells are the main effector cells responsible for viral clearance and disease pathogenesis during acute HBV infection.\(^{188}\) Depletion of CD8\(^+\) T cells prolonged the infection and delayed the onset of viral clearance until CD8\(^+\) T cells reappeared and entered the liver.\(^{188}\) The mechanisms through which CD8\(^+\) T cells mediate viral clearance during HBV infection are both cytolytic and non-cytolytic. The contribution of non-cytolytic effector mechanisms is supported by findings in a cell culture model, where virus-specific CD8\(^+\) T cells were able to inhibit HBV replication with minimal cell lysis. IFN-\(\gamma\) and TNF-\(\alpha\) are the mediators of these effector mechanisms since blocking these cytokines abrogated non-cytolytic functions.\(^{189}\)

The HBV core protein (HBcAg) has an intrinsic ability to activate the immune system since many epitopes generated from the core protein can efficiently crosslink B cell receptors.\(^{190}\) Assessment of liver tissue from acutely infected patients revealed an overwhelming B cell response targeting HBcAg.\(^{191}\) It is hypothesized that small amounts of soluble HBcAg are released into the blood stream following viral particle destruction or cytolysis of infected hepatocytes. These antigens are captured by B cells and presented to CD4\(^+\) T lymphocytes.\(^{192}\) This is supported by Li et al.,\(^{193}\) who demonstrated the presence of HBcAg-specific IL-21-producing CD4\(^+\) T cells (in acute patients) that enhanced the
production of IFN-γ and perforin expression by CD8⁺ T cells from chronic HBV patients. Thus CD4⁺ T cells might contribute to HBV control by sustaining CD8⁺ T cells. These findings show that, HBcAg can induce an immune response involving CD8⁺ T cells, and B cells that produce antiviral antibodies. Based on this knowledge, a novel HBV vaccine was designed, and shown to induce both T and B cell responses in mice. The vaccine formulation comprised of particulate hepatitis B surface (HBsAg) and core antigen (HBcAg). It induced multifunctional HBsAg- and HBcAg-specific CD8⁺ T cells detected by staining for IFN-γ, TNF-α and IL-2, as well as high antibody titers against both antigens.¹⁴

In chronically infected individuals, virus-specific CD8⁺ T cells are rarely detectable in peripheral blood due to functional T cell exhaustion caused by persistent antigen stimulation. Consequently, CD8⁺ T cells have elevated levels of exhaustion markers such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA4), Tim-3 and 2B4.¹⁵ Furthermore, elevated levels of PD-1 ligand have also been reported in hepatocytes during HBV infection.¹⁶ Expression of these exhaustion markers will lead to apoptosis and subsequent deletion of the CD8⁺ T cells. CD4⁺ T cells also display functional exhaustion in chronic HBV. The loss of CD4⁺ T helper cells may prevent the maturation of functionally efficient CD8⁺ T response.

### 2.5.5.2 CTL response in human immunodeficiency virus (HIV) infection

The HIV epidemic is one of the main global health challenges. Design of vaccines and drugs have been complicated by the virus’s rapid evolution to evade the immune system, drug toxicity and drug resistance.
Cytotoxic T lymphocytes were first shown to suppress HIV by two studies in 1994, which showed a strong CTL response (during the acute stage) that correlated with low viremia and this response preceded the production of neutralizing antibodies.\textsuperscript{197, 198} CD8\textsuperscript{+} T cells produce a soluble factor that was shown to suppress human immunodeficiency virus (HIV) long terminal repeat (LTR)-mediated gene expression in human CD4\textsuperscript{+} T cells. This factor is known as CD8\textsuperscript{+} T antiviral factor (CAF).\textsuperscript{199-203} The CAF and β-chemokines mediate the CD8\textsuperscript{+} T cell non-cytotoxic antiviral response (CNAR).\textsuperscript{199, 203} Beta-chemokines suppress HIV replication by binding to their cognate chemokine receptors, thereby blocking HIV from using these receptors for binding and entry into cells.\textsuperscript{199, 204} CNAR occurs early in HIV before antibodies are produced\textsuperscript{202, 205} and thus could be responsible for the initial reduction in viremia observed during acute infection.\textsuperscript{206, 207} Individuals who repeatedly get exposed to HIV but remain uninfected, or individuals who do not progress to AIDS without antiretroviral therapy, have been shown to have a strong CNAR.\textsuperscript{208, 209} Both the cytotoxic and non-cytotoxic pathways have been reported to correlate with delayed disease progression in HIV-infected individuals.\textsuperscript{210-212}

A single individual can express up to six different HLA class I alleles: two from each of the following MHC class I loci HLA-A, HLA-B and HLA-C. HIV positive individuals who are homozygous for one or more HLA loci progress rapidly to AIDS and death, while maximum heterozygosity delayed onset of disease (10 or more years).\textsuperscript{213, 214} This implies that the diversity of HIV- specific CTL epitopes is very important in determining disease outcome. However, recognition of a single epitope has been associated with effective control of viremia.\textsuperscript{215} Thus certain CTL specificities could be more efficacious than others. Indeed, CTL responses directed at conserved viral proteins are
associated with lower viremia than responses directed at variable proteins.\textsuperscript{216, 217} This is why certain HLA alleles are associated with long-term non-progression while others are associated with rapid progression to AIDS.\textsuperscript{218-220}

CTL escape in HIV was first described by Koenig et al.,\textsuperscript{221} who transferred a CTL clone that was specific for the conserved region of negative factor (nef) protein into an HIV patient. Instead of supplementing the CTL response, the infusion caused decline in circulating CD4\(^+\) T cells and a rise in viral load. Furthermore, HIV isolates from plasma or CD4\(^+\) T cells lacked the nef epitope, implying that CTL selected for mutants of the protein being recognized. CTL escape has been shown to occur in acute stage\textsuperscript{197, 222, 223} as well as chronic stage of HIV.\textsuperscript{215} CTL escape was described in two HIV patients who rapidly progressed to AIDS after it occurred.\textsuperscript{215} Besides CTL selection, HIV evades immune recognition by downmodulating surface expression of HLA-A and HLA-B\textsuperscript{224, 225} but not HLA-C or HLA-E.\textsuperscript{226} This does not only reduce killing by HLA-A and HLA-B restricted CTLs, but it also blocks natural killer cells from killing virus infected cells.\textsuperscript{227}

T cell exhaustion is very common in chronic infections inclusive of HIV. Infection with HIV results into chronic immune activation which leads to progressive loss of CD4\(^+\) T and CD8\(^+\) T cells. Exhausted T cells show progressive loss of function characterized by loss of IL-2 production, proliferation and effector functions.\textsuperscript{228} Expression of T cell exhaustion markers such as PD-1 and Tim-3 have been associated with HIV disease progression in cross sectional studies.\textsuperscript{229} The main route through which HIV affects the immune system is by infection of CD4\(^+\) T cells. Diminished numbers and function of CD4\(^+\) T cells impairs CD8\(^+\) T cell function because CD4\(^+\) T cells are required in the maintenance of memory CD8\(^+\) T cells and proliferative renewal.\textsuperscript{230}
2.5.5.3 CTL response to influenza virus

Influenza is an acute respiratory disease caused by RNA viruses belonging to the orthomyxovirus group. The ability of these viruses to constantly and rapidly change genes encoding for their surface proteins has made it quite challenging to develop effective vaccines. The majority of CTLs are directed at conserved epitopes such as epitopes located in the internal portion of the nucleoprotein (NP).\textsuperscript{231, 232} Thus, these CTLs are broadly cross-reactive and recognize all major subtypes. However, CTL response directed at these conserved parts of NP is transient and partially protective.\textsuperscript{233}

Although influenza infection in mice does not simulate the natural infection in higher vertebrates, mouse models have been critical in understanding the T cell response to influenza virus. To investigate the role of CTL response in influenza infection, Bender et al.,\textsuperscript{234} used transgenic mice homozygous or heterozygous for beta 2-microglobulin gene (β2M). The homozygous mice lack MHC class 1 CD8\textsuperscript{+} T cell restriction. Challenge with a virulent strain of influenza virus caused a significantly high mortality in homozygous mice compared to heterozygous mice. Furthermore, a non-lethal challenge caused significant delay in pulmonary viral clearance in β2M (-/-) mice.\textsuperscript{234} In another mouse model, using B-cell deficient mice shows that these mice have a 50-100 fold greater susceptibility to lethal influenza A infection than wild type. Adoptive transfer of influenza A specific CD8\textsuperscript{+} T cell clones promoted recovery from lethal infection.\textsuperscript{235} These studies highlight the protective role of CTL, however, vaccination with influenza dominant proteins confers mild CTL-mediated protection.\textsuperscript{236-239} Thus an efficacious immune response against influenza could be mediated by antibodies. Indeed, using transgenic mice expressing a uniform TCR heterodimer (αβ4/β11; termed F5-Tg) derived from an NP specific T cell clone obtained
from C57BL/10 mice, Moskophidis et al.,\textsuperscript{233} were able to track effector CTL functions \textit{in situ}. Their data indicate that CD8\textsuperscript{+} T cells only conferred protection at low viral dose challenge, at high viral dose they exacerbated viral pathology and increased mortality.

\textbf{2.5.5.4 Role of CTL response in equine viral infections}

\textbf{2.5.5.4.1 Equine infectious anemia virus (EIAV)}

EIAV is a lentivirus that causes disease in horses worldwide. More than 90\% of infected horses progress to the inapparent carrier state during which time they have persistent low viral loads. CTL response is associated with control of primary viremia in EIAV infected horses. This was demonstrated in Arabian SCID foals.\textsuperscript{240} SCID horses lack functional T and B lymphocytes and thus are incapable of launching a functional adaptive response. Indeed, SCID horses failed to eliminate virus from their plasma during primary infection while normal horses eliminated the virus.\textsuperscript{240} Furthermore, immune reconstitution of an SCID foal with virus-specific cytotoxic lymphocytes prevented continuous EIAV infection.\textsuperscript{241} Recurrence of viremia in EIAV infected horses is caused by antigenic variation. Antigenic drift of EIAV has been demonstrated with autologous neutralizing antibodies\textsuperscript{242, 243} and CTL epitopes.\textsuperscript{244} During EIAV infection, neutralizing antibodies are observed after clearance of primary viremia\textsuperscript{245, 246} and the CTL response has been correlated with this initial clearance of viremia.\textsuperscript{247} So, CTL response is a very critical part of the immune response to EIAV.

Antigen-specific CTLs that do not require \textit{in vitro} stimulation have been detected (in \textsuperscript{21}Cr release assay) but disappear 3 months after infection.\textsuperscript{248} CTL memory to EIAV has been observed at least 7 years after infection.\textsuperscript{248} CTL epitopes have been mapped using retroviral vector-transduced target cells expressing different Gag proteins and overlapping
synthetic peptides.249, 250 Other epitopes have been mapped in the Env251 and Rev proteins.251

2.5.5.4.2 Equine herpesvirus 1 (EHV 1)

Equine herpesvirus 1 is a highly prevalent respiratory virus of horses worldwide.252 Infection of horses is characterized by fever, respiratory distress, abortion and severe neurological sequelae.253 EHV-1-specific, CTL response has been detected in equine PBMCs of EHV-1 infected and vaccinated horses.254, 255

A murine model of EHV-1 that closely mimics infection in the natural host showed the generation of primary and memory virus specific CTL response to the attenuated EHV-1 strain KyA. In this model, primary virus-specific CTL response was detected in draining mediastinal lymph nodes 5 days following infection.256 Also, EHV-1 CTL response was detected in the spleen up to 26 weeks after the resolution of the infection. Adoptive transfer of splenocytes (containing high levels of CD4+ and CD8+ cells) from KyA immune mice into irradiated mice infected with EHV-1 RacL11 strain caused a greater than 250-fold reduction of RacL11 in the lung. Depletion of both CD4+ and CD8+ cells from splenocytes abrogated the clearance of RacL11.256 Similarly, transfer of spleen cells from Balb/C mice primed with EHV-1 AB4 conferred protection in recipient mice.257 To confirm that this response was mediated by T cells, recipient mice were tested for delayed hypersensitivity (DTH). Recipients of spleen cells from primed mice had a higher DTH response than controls.257

Pregnant mares that exhibit high frequencies of circulating EHV-1 specific CTL response have an enhanced protection against EHV-1 induced abortion and other clinical
signs. Similarly, ponies with high frequencies of precursor CTLs were protected against EHV-1 challenge showed by reduced or absence of clinical signs.

2.5.5.4.3 Equine arteritis virus (EAV)

Although humoral immunity to EAV has been studied extensively, little is known about the role of T cell response to EAV in both non-carrier and carrier horses. There is only one study showing EAV specific CTL precursors being detected at 4 months post infection and persisting for at least 1-year post infection. Significant infiltration of T lymphocytes, macrophages and dendritic cells into the reproductive tract of carrier horses has been reported. But, the role of these cells is not known since EAV still persists in the presence T lymphocytes.

2.5.6 Cytotoxic T Lymphocyte killing mechanisms

Cytotoxic T Lymphocytes use three pathways to kill infected target cells: the Fas-FasL pathway, granzyme-perforin pathway, and secretion of cytokines such as IFN-γ and TNF-α. TNF-α binds to its receptor on target cells and triggers the caspase cascade, leading to apoptosis. IFN-γ upregulates the expression of MHC class I antigens and Fas molecules in target cells, leading to enhanced presentation of foreign antigens and increased Fas-mediated target-cell lysis.

Fas (also known as CD95 or APO-1) is a type I transmembrane protein, containing a death domain (DD) in its cytoplasmic region. The interaction of Fas with its ligand (FasL) triggers apoptosis through recruitment of the adaptor protein Fas-associated death domain (FADD) and binding of procaspase-8. The interaction of FADD, procaspase 8 and DD results in the formation of the death-inducing signaling complex (DISC) which
finally leads to the activation of effector caspase-3 by active caspase-8 (figure 4). The tumor necrosis factor receptor (TNFR1) also contains a death domain, that utilizes FADD and TRADD (TNFR1-associated death domain protein) to induce apoptosis via activation of caspase-8.263

Figure 4: Fas-FasL and Granzyme pathways
After a CTL recognizes its target, cytolytic granules move to the synapse by exocytosis. The granules are then deposited into the synaptic cleft where they become endocytosed by the target cell. Cytotoxic granules contain a pore-forming protein perforin and a group of serine proteases called granzymes. Perforin polymerizes into transmembrane channels that inflict osmotic stress and facilitate target cell uptake of proapoptotic granzymes. Granzymes can also enter the target cell independent of perforin by endocytosis (figure 4) after initial electrostatic binding to the membrane of the target cell. Mice and humans lacking perforin are severely immunocompromised as evidence by the lack of LCMV clearance in perforin deficient mice and the presence of a potentially fatal immunoregulatory disorder, type 2 familial haemophagocytic lymphohistiocytosis (FHL) in humans with congenital perforin deficiency.

Multiple granzymes (Granzyme A-M) have been reported. The mouse has nine, the rat has eight rat and the human has five granzymes. Granzyme A and B are the most abundant. Granzyme A induces single-stranded DNA damage as well as rapid loss of cell membrane integrity and destroys the nuclear envelope by targeting lamins and unwinds DNA for degradation by targeting histones. Granzyme B mediated apoptosis is both caspase dependent and caspase independent. In cells treated with perforin loaded granzyme, caspases 3 is directly processed by granzyme B. Granzyme B can also cleave BID (BH3-interacting domain death agonist) to activate the mitochondrial apoptotic pathway. It induces DNA damage by degrading the inhibitor of caspase-activated DNAase (ICAD). Caspase-activated DNAase (CAD) exists as a heterodimer with ICAD (figure 4). In this form CAD is inactive.
Granzyme B is the most potent activator of caspase-mediated, as well as caspase-independent, cell death. *In vitro* CTL assays exploit this pathway to study CTL killing by measuring the presence of granzyme B activity in target cells. To increase the precision of these assays, upstream caspases are also incorporated into the assays by using fluorescently labelled inhibitors of caspases to detect active caspases in target cells.

2.5.6.1 Stages of CTL killing

2.5.6.1.1 Target cell recognition and formation of the immunological synapse

Migrating CTLs recognize target cells by engaging the TCR to recognize peptide-MHC complexes on target cells, which initiates signaling to form the immunological synapse (IS). The IS forms at the point of contact between the CTL and the target cell. It forms a characteristic bullseye pattern made up supramolecular activation clusters (SMAC). The center (cSMAC) of the IS is made up of T cell receptor clusters, the periphery (pSMAC) is made up of intergrins and the distal (dSMAC) is made up of phosphatases and actin.

2.5.6.1.2 Polarization of the CTL and degranulation

The formation of the IS induces polarization of the CTL by initiating the movement of the centrosome (microtubule organization center). The centrosome moves from the uropod to the point of TCR signaling i.e. edge of the cSMAC forming the secretory domain. Cytotoxic granules move along the microtubules towards the polarized centrosome and are secreted at the secretory domain of the IS. The granules are formed in membrane bound vesicles when CD8 cells differentiate into CTLs. The
vesicles at the secretory domain fuse with the CTL membrane and are released into the synaptic cleft towards the target cell.\textsuperscript{298}

2.5.6.2 Stages of cell death

2.5.6.2.1 Apoptosis

After delivery of cytotoxic granules by CTLs, the target cell begins to undergo apoptosis, a form of programmed cells death.\textsuperscript{299-302} There are three apoptotic pathways: the extrinsic or death receptor pathway, the intrinsic or mitochondrial pathway and the granzyme/perforin pathway.

The extrinsic pathway is initiated by death receptors which are members of the tumor necrosis factor (TNF) receptor gene family.\textsuperscript{301, 303} The best described models of this pathway are the Fas and TNF-\(\alpha\) described above. The perforin/granzyme pathway is a variant of type IV hypersensitivity since sensitized CD8\(^+\) cells kill antigen baring cells. The intrinsic pathway is initiated by a diverse array of non-receptor-mediated stimuli that leads to opening of the mitochondria permeability transition (MPT) pore, loss of mitochondrial transmembrane potential and release of cytochrome c which binds to Apaf-1 as well as procaspase -9.\textsuperscript{304} Another molecule, apoptosis-inducing factor (AIF) translocates to the nucleus where it causes DNA fragmentation.\textsuperscript{305}

The extrinsic and intrinsic pathways end at the execution phase, where the activation of executioner caspases takes place. Caspases -3, 6 and 7 function as effector or executioner caspases. Caspase -3 is the most important executioner and is activated by any of the initiator caspases i.e. caspase -8, 9 or 10.\textsuperscript{306} Activation of executioner caspases activates cytoplasmic endonucleases that degrade nuclear material, and activates proteases that degrade the nuclear and cytoskeleton proteins.\textsuperscript{300}
As the cell undergoes apoptosis, the chromatin condenses, the membrane blebs and its DNA fragments.\textsuperscript{302} Phosphatidylserine (PS) is normally confined to the cytoplasmic face of the plasma membrane. During apoptosis it translocates to the cell surface. Externalization of PS signals to neighboring cells such as macrophages to remove the dying cells by phagocytosis.\textsuperscript{307} Once PS is externalized, it can be detected by fluorescein-isothiocyanate - labeled annexin V using flow cytometry or fluorescence microscopy.\textsuperscript{307}

2.5.6.2.2 Secondary necrosis

This is a phenomenon referring to progressive loss of plasma membrane integrity of apoptotic cells. It happens when apoptotic cells are not efficiently removed by scavenging cells or when cells are infected by apoptotic inducing pathogens such as vesicular stomatitis virus or encephalomyocarditis virus. Caspase-3 has been shown to trigger necrosis of apoptotic cells.\textsuperscript{308}

Loss of membrane integrity associated with apoptosis leads to unidirectional leakage of molecules. The presence of these molecules (e.g. lactate dehydrogenase) in the culture medium of cell under CTL attack has been used to measure CTL response.

2.6 Assays to measure T cell cytotoxicity

For many years the chromium release and limiting-dilution assays were the only techniques used to measure antigen specific T-cell responses. During the past ten years or so, new methods to analyze T-lymphocyte responses have been developed. These new methods are more sensitive, less hazardous and provide more information on the functionality of T cells. T cell assays used to monitor T cell immunity to infection or vaccination can be divided into three groups: Functional assays, assays based on structural features of the T cell receptor (TCR) and assays that detect precursor T cells.\textsuperscript{309}
2.6.1 Functional assays

These are mainly the interferon gamma (IFN-γ) enzyme-linked immunoSpot (IFN-γ ELISpot) assay and intracellular cytokine staining assay (ICS). The ELISpot assay measures antigen-specific lymphocyte frequency by measuring the secretion of cytokines or other immune proteins that are engaged in the cytolytic pathway. ELISpot assays are conducted in antibody coated plates and the secreted proteins are detected locally (as spots) by the capture antibody. The ELISpot assay offers quantitative data, uses low numbers of effector cells, is easier to perform, and highly specific and sensitive. The IFN-γ ELISpot assay however, on its own is not sufficient to detect cytotoxic cells as T cells with cytotoxic activity do not always secrete IFN-γ. A modification of the IFN-γ ELISpot, the Granzyme B(GrB) ELISpot assay may be better at detecting cell-mediated cytotoxicity, since GrB is a key mediator of target cell death in vivo. However, GrB ELISpot assay may not work in cases where the target cells contain serpin proteinase inhibitor 9 (a GrB inhibitor) or if effector cells are perforin deficient.

For intracellular cytokine staining, cells are treated with golgi inhibitors such as monensin or brefeldin A, so the cytokines accumulate in the cytoplasm of these cells upon antigen activation. After fixation and permeabilization of the lymphocytes, intracellular cytokines can be quantified by flow cytometry. Thus, ICS does not only allow the measurement of cytokine secreting cells but also gives the phenotype of cells that produced these cytokines. The quantity of cytokine produced per cell is also enumerated. Combining this assay with tetramer staining allows the detection of rare cell populations.
2.6.2 Assays that assess the structural features of the TCR

Fluorescently labelled MHC-peptide complexes that bind the T Cell Receptor (TCR) can be used to visualize antigen-specific T cells.\textsuperscript{324-327} Since the interaction between TCR and MHC-peptide complexes is too weak for stable binding, multimers of MHC-peptide complexes are used to increase the overall avidity for the T cell.\textsuperscript{309} To construct these multimers, the heavy and light chains of the MHC are cloned and produced in \textit{Escherichia coli}, solubilized and refolded in the presence of high concentrations of the antigenic peptide. The C-terminals of these refolded complexes are then labelled with a single biotin and the complexes are incubated with fluorescent streptavidin.\textsuperscript{328} Dimers, tetramers or even pentamers of both MHC I and MHC II have been produced and used in flow cytometry to characterize T cell responses.\textsuperscript{329-331} This assay does not provide clues on the functionality of the T cells since it only allows visualization of peptide specific T cells. Thus, it’s important to couple this assay to phenotypic characterization of the T cells to determine if the responding T cells are effectors, functional or anergic.\textsuperscript{332} Also, T cell clones can be isolated using tetramers and enriched without the need for several cycles of \textit{in vitro} stimulation.\textsuperscript{333} Tetrimer analysis is very sensitive,\textsuperscript{283, 298, 308} quick to perform, and uses a small number of cells. However, its reliability varies with cell type as lack of staining for T cell clones has been reported and not all peptides form functional tetramers. Also, tetramer positive T cells are not always cytotoxic and tetramers can only be used to detect T cell responses to known antigens.\textsuperscript{334}

Antigen-specific T cells can also be phenotypically detected by PCR, by sequencing the third complementarity-determining region 3 (CDR3) of the TCR.\textsuperscript{335, 336} This is achieved by using clone-specific primers that flank the CDR3. The CDR3 region
encodes the highly polymorphic portion of the TCR responsible for recognizing peptide-MHC complexes. For the β chain, the CDR3 region encodes the V-D segment and D-J segment junctions, whereas for the α-chain, it encodes the V-J junction. Using V, D, or J region subfamily-specific PCR primers, the development of restricted TCR gene usage can be analyzed.\textsuperscript{337} T cells of the same clone will have the same profile.\textsuperscript{338} This assay is very sensitive, robust and less susceptible to handling or culture methods. TCR CDR3 can also be used in combination with tetramer analysis to confirm the phenotype of tetramer positive cells.

2.6.3 Assays that detect precursors of T cells

2.6.3.1 Proliferation assay

In this assay, purified T cells or PBMCs are mixed with various dilutions of antigen or in the presence of antigen presenting cells. Then the ability of T cells to proliferate is measured after 72-120 hrs of stimulation using $[^3]$H-thymidine by quantifying the amount of radiolabeled thymidine incorporated into the DNA or the amount of bromodeoxyuridine (BrdU) incorporated by dividing cells.\textsuperscript{339-341} Its major advantage is that it can be performed directly on peripheral blood samples without \textit{in vitro} stimulation thus giving data that correlates well with T-cell response \textit{in vivo}. Its major disadvantage is that it can be influenced by the nonspecific immune response of test subjects. Also, the calculated stimulation index does not necessarily correlate with the number of antigen-specific T cells present \textit{in vivo}. For instance, high levels of proliferation by a few cells or low levels of proliferation by many cells would give a similar stimulation index.\textsuperscript{334}
2.6.3.2 Direct cytotoxicity assays: the chromium release assay (CRA)

The CRA is based on the ability of CTL to lyse $^{51}$Cr-labeled target cells loaded with the antigen of interest. After incubation of target cells with the antigen of interest, the target cells are labeled with $^{51}$Cr. Labeled target cells are then incubated (at different effector to target ratios) with effector cells that have been expanded \textit{in vitro} for 7-21 days. \footnote{342} Target cells that are recognized and lysed by the effector cells subsequently release $^{51}$Cr into the supernatant, the chromium is then quantitated by scintillation $\gamma$ counting. \footnote{8} The CRA has been considered ‘the gold standard’ to measure CTL activity, but its many drawbacks have forced researchers to develop less hazardous and more sensitive techniques. Besides the CRA using hazardous material, it’s not very sensitive, sometimes effectors have to be stimulated many times to get a response. This response might not mirror \textit{in vivo} response because the phenotype of effectors can change during these repeated cycles of stimulation. Other disadvantages include: poor labelling of some target cells, high background due to spontaneous release of $^{51}$Cr from certain target cells and it is semiquantitative.

Several nonradioactive CTL assays have been developed as alternatives to the CRA. These assays are based on release of nonradioactive probes, detection of enzymatic activity, analysis of targets cells transfected with foreign enzyme genes, detection of early activation of effectors, or measurement of apoptosis or necrosis in target cells. \footnote{309} Among the assays that measure apoptosis or necrosis in target cells, is the colorimetric measurement of lactate dehydrogenase (LDH) activity in the culture medium. The LDH assay uses the same procedure as the CRA but the target cells are not labelled with $^{51}$Cr because LDH is present in the cytoplasm and is released into the medium when the cell membrane is compromised. LDH release assays correlate with the $^{51}$Cr release assay but
are less sensitive.8,343-345

2.6.3.3 Direct cytotoxicity assays: flow cytometry CTL assays

Another commonly used assay to measure CTL response is based on annexin V binding to apoptotic cells. Upon induction of apoptosis, phosphatidylserine (PS) is externalized, and so Annexin V will bind to the PS. After effector/target cell incubation, effector cells are stained with an effector cell-specific antibody, and annexin V–fluorescein isothiocyanate (FITC) or CFSE is used to stain target cells. To differentiate between early apoptotic and late apoptotic cells, Propidium Iodide (PI) or 7-amino-actinomycin(7-AAD) is added. Then the cells are analyzed with a flow cytometer.346, 347

Kim et al.,348 developed a four-color flow cytometry assay that simultaneously measures effector cell phenotype using K562 or Daudi cells as targets. The targets were labeled with cell tracker orange prior to the addition of effector cells. After effector/target cell coincubation, 7-AAD was added followed by phenotypic markers and finally, GrB antibody. The 7-AAD detected cell death by necrosis and late apoptosis and GrB antibody detected the presence of proapoptotic activity in targets.

Pickard et al.,290,349 developed an assay that involves the incubation of target cells with a GrB substrate. The substrate is designed by inserting a peptide recognition-motif around the granzyme B cleavage site. Covalent labeling with the same fluorophore at both ends of the peptide results in quenching. Cleavage of the peptide relieves the quenching, giving a fluorescent signal. When targets containing the substrate are cocultured with CTL effectors, the GrB released by effector cells into targets cleaves the substrate to give a
fluorescent probe that can be detected by a flow cytometer. The presence of this fluorescence in the cytoplasm of target cells can be used to quantitate and visualize cellular attack by effector cells.

2.6.4 What to consider before choosing a CTL assay

Before choosing a CTL assay, it’s important to consider the performance characteristics of the assay and immunological properties of the subjects you are working with. The assay chosen must have enough sensitivity, specificity, reliability and reproducibility.\textsuperscript{309} It must be able to measure T cell response without distorting the phenotype of effector cells. It is also important that the assay is simple and rapid to perform and uses a small number of cells. To correctly detect T cell responses, the time points after vaccination or infection are very important. For each assay, the time point must be chosen carefully to avoid false negative results since the cells being assayed may have not yet developed or might have already disappeared from peripheral blood into other lymphoid organs. Even when the response is at its maximum, but when this maximum response is below the detection threshold for the assay being used, it will still give a false negative result. Therefore, it is important to choose an assay that is very sensitive. Finally, the T cell immune system is very complex so using just one assay or parameter or cell type may not give a complete analysis of the T cell response, a combination of two or more assays may give a more realistic evaluation.\textsuperscript{334}
CHAPTER 3. MATERIALS AND METHODS

3.1 Cells

Equine pulmonary artery endothelial cells (EECs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Herndon, VA) supplemented with sodium pyruvate, 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 100 U/ml of penicillin, 100 µg/ml streptomycin, and 200 mM L-glutamine. High-passage-number rabbit kidney cells (HP-RK-13 [KY] P399-409 cells; originally derived from CCL-37 cells [American Type Culture Collection, Manassas, VA]) were propagated in Eagle’s minimal essential medium with 10% ferritin-supplemented bovine calf serum (HyClone Laboratories, Inc., Logan, UT), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Gibco, Carlsbad, CA). Primary Equine Dermal Cells (EDCs) were propagated in EDC growth medium [Eagle’s Minimum Essential Medium (MEM) supplemented with 10% fetal equine serum (Biowest, Riverside, Missouri USA), 100 U/ml of penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA)]. The EDCs were used as target cells in CTL assays and as antigen presenting cells in IFN-γ ELISpot assay. Equine Tumor cells (EQT8888) were obtained from the Animal Health Trust, UK. The EQT8888 cells were propagated in a mixture of AIM V serum free medium (Thermo Fisher scientific Inc, Waltham, MA USA) and RPMI 1640 (Gibco, Carlsbad, CA) medium supplemented with 0.05 mM non-essential amino acids, 0.5 mM sodium pyruvate, 55 µM 2-mercaptoethanol, 50 µg/ml gentamicin and 10% fetal bovine serum. Lymphokine activated killer (LAK) cells were generated by incubating PBMCs with 2000 Units/ml of recombinant human IL-2 (R&D Systems Inc., Minneapolis MN) for 4 - 7 days in RPMI 1640 supplemented with 2.5% fetal equine serum, 55 µM 2-mercaptoethanol and 50 µg/ml gentamicin.
3.2 Viruses

Two strains of EAV; the virulent Bucyrus strain (VBS; HP 15 53) and the modified live virus (MLV) vaccine virus were used in this study. Both viruses were propagated in EECs to generate high-titer working stocks. EECs infected with each virus were frozen at -80°C when 90 to 100% cytopathic effect (CPE) was observed. Cell lysates were clarified by centrifugation (1500 \( \times \) g) at 4°C for 20 min, followed by ultracentrifugation (Beckman Coulter, Miami, FL) at 121,600 \( \times \) g through a 20% sucrose cushion in NET buffer (150 mM NaCl, 5 mM EDTA, 50mMTris-HCl, pH 7.5) at 4°C for 4 h to pellet the virus. Purified preparations of each strain of EAV were resuspended in phosphate-buffered saline (PBS; pH 7.4) and frozen at -80°C. Virus stocks were titrated by standard plaque assay in EECs, and titers were expressed as the number of PFU per milliliter. The VBS strain was UV inactivated to investigate its use in target cells. 50µL of partially purified EAV VBS was resuspended in 550µL of Minimum Essential Medium (MEM). 600µl of the virus suspension was aliquoted onto a 6-well plate and exposed to UV light for 5,10,20,30,60 and 120 seconds using the Spectrolinker XL-1000 (Spectronics™ Corporation, Westbury New York). The 6-well plate (without the cover) was placed on wet ice during the exposure to radiation and a distance of 10cm was maintained between the UV tubes and the bottom of the plate. An infectivity assay of the TCID\(_{50}\) was set up to determine the degree of UV inactivation of virus. Flow cytometry analysis for EAV was performed with anti-N (MAb 3E2), anti-GP5 (MAb 6D10) and anti-M (rabbit anti-peptide serum) on primary equine dermal cells cultured with the UV inactivated virus and live VBS. Virus was also isolated from semen of three carrier stallions in accordance with the standard protocol used by the EAV OIE Reference Laboratory at the Maxwell H. Gluck Equine Research Center,
University of Kentucky.\textsuperscript{350-352}

3.3 Horses

Three confirmed long-term EAV carrier stallions were used in the study. These were located at the UK North Farm, University of Kentucky, Lexington, KY. Three EAV seronegative stallions were also included; two of these stallions were vaccinated by the intramuscular route with the modified live virus (MLV) vaccine (ARVAC). These stallions were used as a comparison group to the carrier stallion group. Before vaccination, the stallions were confirmed to be seronegative (<1:4 titer) according to the World Organization for Animal Health (Office International des Epizooties [OIE]) standardized protocol.\textsuperscript{327} Blood was obtained by jugular venipuncture and punch biopsies were collected from all the six horses. Punch biopsies were used to establish primary equine dermal cell lines (EDCs) and Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole venous blood. Whole venous blood without anticoagulant was also collected for serum processing. The serum was either used for culture medium supplementation or for serum neutralization (SNT) and Enzyme-linked immunosorbent assay (ELISA) testing. Blood was collected at 0, 7, 9, 14, 21, 23, 35, 37, 41, 48, 63, 78, 85- and 99-days post vaccination. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The experimental protocol involving horses was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC; protocol number 2015–1347).
3.4 Establishment and characterization of primary EDCs

Primary equine dermal cell lines were established for each stallion used in the study. Skin punch biopsies were collected (under aseptic conditions) from the cervical region of each horse. The epidermal portion of the skin was removed and immersed into 15ml of transport medium (500mL of Hank’s Balanced Salt Solution containing 100 IU/ml penicillin, 10 µg/ml streptomycin, 250µg/ml gentamicin and 2.5mls fungizone). Once at the laboratory, each biopsy sample was cut into small pieces and transferred into 6 well flat-bottomed plates. 22mm round coverslips were gently placed on top of the tissues pieces before adding warm MEM medium containing 20% fetal equine serum, the plates were incubated at 37°C, 5% CO₂. Growth medium was changed every two days until the cells formed a confluent layer. The coverslips and the explants were then removed. The cells were washed with warm Phosphate Buffered Saline (PBS), trypsinized and transferred to T25 flasks. When flasks were fully confluent, the cells were harvested and resuspended in freezing medium (10% DMSO in MEM containing 10% fetal bovine serum) at 2 × 10⁶ cells/ml.

Since these primary EDCs were to be used as target cells in a CTL assay, we were concerned about EAV altering the expression of MHC Class I molecules. So, we obtained a monoclonal antibody (MAb) against equine MHCI (Clone CZ3) from Dr. Antczak (Cornell University) and used it in immunofluorescence staining (IFA) for MHC Class I expression, we also obtained flow cytometry mouse anti-Equine MHC Class I and Class II antibodies (clones: 14A2 and H58A) from Washington State University Antibody Centre. The EDCs were also stained for vimentin, a generic marker for fibroblasts. To determine the m.o.i of EAV and length of time to infect EDCs, the cells were infected with EAV at
different m.o.i, at different time points and then Immunofluorescence microscopy (IFA) was performed. The EDCs were stained with anti-N (MAb 3E2) and anti-nsp1 (MAb 12A4). For flow cytometry, EDCs were cultured in T25 flasks until confluent. One of the flasks was mock infected while the other flask was infected with EAV VBS at m.o.i of 2 for 24hrs. The cells were harvested by trypsinization and fixed (in the dark) with 3% paraformaldehyde for 15 min. on wet ice. The cells were centrifuged at 500 × g for 5 min. and the supernatant removed. The cells were then resuspended in FACS buffer (PBS containing 1% BSA and 0.1% azide) and 100µL of the cell suspension transferred to a 96 well V-bottom plate. The plate was centrifuged at 500 × g for 5 min. and the cells were permeabilized with cold 0.1% saponin for 5 min. Blocking was done with 1% Bovine Serum Albumen (BSA) for 30 min. at room temperature. The cells were washed three times with FACS buffer. A mouse anti-EAV Nsp1 (MAb 12A4) monoclonal antibody conjugated to Alexa Fluor 488 was added and the plates incubated on ice for 30 min. After three rounds of washing, mouse anti-Equine MHC Class I or Class II antibodies conjugated to Alexa Fluor 647 were added. Thirty thousand live events were acquired on the LRSII flow cytometer (BD Biosciences, San Jose CA) and data were analyzed with WinList 3D 8.0 (Verity Software House) software.

3.5 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

Whole blood was collected via venipuncture into sodium heparin tubes (Monoject™ Mansfield, MA). The blood was pooled into 50ml conical tubes and centrifuged at 500 × g for 10 min. The plasma was removed, and the tubes filled with Hank’s Balanced Salt Solution (HBSS; Thermo Fisher scientific Inc, Waltham, MA USA). Twenty five mL of the contents of each tube were gently laid onto 20ml of Ficoll
Paque™ (Ficoll Paque™ Plus, GE Healthcare Bio Sciences AB). The tubes were then centrifuged at 500 × g for 30 min. at 25°C (with the brake off). The PBMCs at the interface between the HBSS and the Ficoll were collected into 50ml conical tubes. Forty mL of HBSS were added and the cell suspensions mixed gently. The tubes were then centrifuged at 500 × g for 10 min. at 25°C. The supernatant was removed, and this step repeated twice. The cell pellet was re-suspended in 10ml of 1X red blood cell lysis buffer (prepared as 10X with sterile distilled water containing 155mM ammonium chloride, 10mM sodium bicarbonate and 0.1mM Ethylenediaminetetraacetic acid (EDTA)) and incubated at room temperature for 5 min. Then 30ml of HBSS were added and tubes were centrifuged as before. The pellet was resuspended in 10ml of RPMI and cells counted using the Vi-CELL XR instrument (Beckman Coulter, Brea CA). Some cells were resuspended in freezing medium (10% DMSO in complete RPMI) while others were saved for in vitro expansion.

3.6 In vitro expansion of in vivo primed effector cells

Since freshly isolated PBMCs contain very few antigen-specific CTLs, there is need to expand these in vivo stimulated cells using the same virus the horses were infected or vaccinated with. Therefore, PBMCs (50-100 × 10⁶ cells) from each horse were aliquoted into two micro-centrifuge tubes. EAV VBS or MLV virus (m.o.i 1) was added to one of the tubes and the other was mock infected. The tubes were then incubated at 37°C for one hour. The cell suspensions were later transferred into two separate T75 flasks and 50ml of induction medium [1:1, v/v, mixture of AIM-V/RPMI 1640 supplemented with 2 mM L-glutamine, 0.5mM minimal essential medium non-essential amino acids, 0.05mM sodium pyruvate, 55µM 2-mercaptoethanol, 50µg gentamicin and 5% equine serum (collected from the stallions before vaccination and inactivated at 56°C for 40 min)] were added. The
PBMCs were incubated at 37°C (upright position), in the presence of 5% CO₂ for 7-10 days. They were later harvested into 50ml tubes, centrifuged at 800 × g for 10 min. and resuspended in 10ml of complete RPMI (without phenol red) and purified with the ClioCell dead cell removal kit (AMSBIO, Abingdon UK).

3.7 Cytotoxic T Lymphocyte assays

3.7.1 Granzyme B assay

After *in vitro* stimulation of PBMCs, a granzyme assay kit (Pan Toxilux; OncoImmune Gaithersburg, MD) was used in accordance with the manufacturer’s instructions. Briefly, 2 × 10⁶ of EAV infected or mock infected EDCs were labeled with 1 µL of TFL4 and 1 µL of NFL1 in the dark at RT for 15 min and then washed twice. The cells were counted and resuspended in wash buffer at a concentration of 1 × 10⁶/ml. Then 100µL of the cell suspension were added to each well of a 96-well round bottomed plate. *In vitro* stimulated and mock stimulated PBMCs were resuspended at 5 × 10⁶/ml and 100µL added to EDCs at different effector/target ratios. 75 µL of PanToxilux substrate (contains substrate for granzyme B and upstream caspases) was added to each tube and wash buffer was added to the controls. The following control wells were included: (1) EDCs target cells alone; (2) EDCs cells alone with Pan Toxilux substrate (3) Effector cells alone (4) Effector cells and Pan Toxilux substrate. Each plate was then incubated at 37 °C and 5% CO₂ for 60 min, washed twice using wash buffer and re-suspended in 200 µL of wash buffer for acquisition. Approximately 5000 TL4 positive events were acquired on the BD LSR II. Data were analyzed with WinList 8.0 software.
3.7.2 Carboxyfluorescein succinimidyl ester (CFSE) /7-Aminoactinomycin D (7-AAD) /Sulforhodamine fluorochrome labeled inhibitor of caspases (SR-FLICA) assay

The CFSE/7-AAD kit (Abcam, Cambridge MA) was used in this assay. To prepare target cells, two T25 flasks of EDCs were used; one flask was infected with 2 m.o.i of EAV VBS and the other was mock infected for 8h at 37°C in the presence of 5% CO2. The targets were harvested onto 15ml tubes, washed once with PBS and resuspended in CFSE at $1 \times 10^6$ cells/ml for 15 min. at room temperature. After staining with CFSE, targets were resuspended in MEM (containing 10% fetal equine serum) and diluted to $4 \times 10^5$ cells/ml. 100µl of this cell suspension was aliquoted into each well of the 96-well round-bottom plate. Effector cells were prepared as described above and then added to the target cells at different Effector (E): Target (T) ratios. The plates were incubated at 37°C, 5% CO2 for 4h. CTL activity was detected by adding SR-FLICA dye to the cells and incubating them at 37°C, 5% CO2 for 1h. This reagent recognizes and binds to active caspases in target cells. It is a marker for apoptotic cells in this assay. To differentiate between necrotic and apoptotic cells, 7-AAD was added and the cells incubated at 4°C for 15 min. Using a CFSE versus side scatter plot, 3000 CFSE+ events were acquired on the iCyt Synergy flow cytometer. Data were analyzed with WinList 3D 8.0 software.

3.7.3 Lactate dehydrogenase (LDH) assay

Initially, the LDH assay was performed with Lymphokine Activated Killer (LAK) cells as effector cells and EQT8888 cells as target cells. LAK cells were generated as describe by Liu et al.,353 and Hormanski et al.,354 PBMCs prepared as described above were re-suspended at a concentration of $1.0 \times 10^6$ cells/ml in 10ml of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 2.5% (v/v) fetal equine serum, 2 mM glutamine (Sigma),
50µg/ml gentamicin, and 55µM 2-mercaptoethanol (GIBCO, Grand Island, NY). The PBMC were incubated (at 37 °C in 5% CO2) for 4-8 days in T25 flasks supplemented with 2000U/ml of recombinant human interleukin 2 (hIL-2; R&D Systems, Minneapolis, MN) to generate LAK cells. The LAK cells were harvested, resuspended in RPMI 1640 (with no phenol red) and counted with the Vi-CELL XR instrument (Beckman Coulter, Miami FL). The cells were then purified by means of ClioCell dead cell removal kit (AMSBIO, Abingdon UK) to remove dead cells. EQT8888 cells were cultured for 24h before the LDH assay was performed. These cells were re-suspended at a concentration of 3 × 10^5 cells/ml in RPMI 1640 (with no phenol red). 50µL of this cell suspension was aliquoted into each well of a 96-well round-bottom plate except for control wells. 50µL of LAK cells was then added at different E:T ratios and the plates incubated at 37°C in the presence of 5% CO2 for 6h. After incubation, plates were centrifuged at 250 × g for 3 min. The supernatants were collected and transferred to a 96-well flat-bottomed plate. 50µL of the LDH substrate (Pierce LDH cytotoxicity assay kit; Rockfold, IL) was added to each well and the plates incubated at room temperature for 30 min. The reaction was stopped by adding 50µL of the stop solution. Absorbance was measured at 490nm and 680nm with a spectrophotometer (BioTek synergy H1 hybrid; Winooski, Vermont).

After optimizing the LDH assay with LAK cells, the above described procedure was then repeated with EDCs as targets and in vitro stimulated PBMCs as effector cells. In this approach, EDC targets for each stallion were cultured in T25 flasks until confluent. Each T25 flask contained approximately 2.5 × 10^6 cells. The cells were trypsinized and resuspended in EDC growth medium (with no phenol red) at a concentration of 3 x 10^5/ml, 50µL of this cell suspension was transferred per well of a 96-well except for control wells.
The plates were incubated at 37°C in the presence of 5% CO₂ for 12h to allow the cells to adhere to the plates. The medium was removed and 100µL of EDC growth medium containing 5% fetal equine serum with or without 10 m.o.i virus were added to the appropriate wells and the plated incubated for another 8h before adding effector cells. The medium was removed, and the plates were washed three times with 300µL of warm sterile PBS (pH 7.0). 50µL of effector cell suspension were added at different E: T ratios in triplicates and the plates were incubated at 37°C in the presence of 5% CO₂ for 6h.

For both scenarios, maximal target cells lysis was achieved by adding 10µL of 10x lysis buffer to triplicate wells containing target cells only. Four groups of controls were also set up: 1) Medium only; 2) Target cells only (target minimum); 3) Effector cells only for each E: T ratio; 4) Volume correction. Percent cytotoxicity was calculated using the following formula:

\[
\% \text{Cytotoxicity} = \frac{OD \text{ Experimental value} - OD \text{ Effector spontaneous control} - OD \text{ Target cell spontaneous control}}{OD \text{ Target cell maximum control} - OD \text{ Target cell minimum}}
\]
3.8 Transfection of EDCs with EAV RNA(IVTRNA)

*In vitro* transcribed RNA was generated for EAV GP5/M, GP5, M and N using the RiboMax large scale RNA production system (Promega, Madison WI). As a control, blue tongue virus (BTV) M5 was also included. *E. coli* cells were transformed with plasmids carrying the above ORFs and the cells plated on LB agar overnight. A single colony was picked and inoculated into 250ml of LB broth and the flasks placed in the incubator shaker overnight. The following day, the plasmids were purified using the QIAgen maxiprep kit (QIAgen, Carlsbad,CA). The plasmids were then linearized using NotI restriction enzyme before chloroform extraction. *In vitro* transcribed RNA was synthesized using the RiboMax kit from Promega following the manufacturer’s instructions. Ten microliters of the IVT RNA was aliquoted into tubes and stored at -80°C. Three microliters from each tube was used for transfection with the LONZA SE 4D transfection kit. Immunofluorescence microscopy was then carried out to confirm that transfection had taken place.

3.9 Interferon-gamma enzyme-linked immune absorbent spot (IFN-γ ELISpot) assay

A crude overlapping peptide library was synthesized (GenScript, Piscataway, NJ) from open reading frame 7 (of the MLV vaccine strain) which encodes N protein. The peptides were 15aa long and overlapped by 11aa. The peptides were dissolved in 50µL of dimethylsulfoxide (DMSO) and 950µL of distilled water were added to each tube, aliquots of each peptide were made and stored at -80°C. The ELISPOT assay was performed with EDCs transfected with M, N, GP5 and GP5/M of EAV. The equine IFN-γ ELISpot kits were obtained from Mabtech (Mabtech,Cincinnati,OH) and used in accordance with the manufacturer’s instructions.
To perform the assay, 3 tubes of PBMCs for each horse were removed from the freezer. They were immediately thawed (in a water bath) and the cells resuspended in 25ml of RPMI containing 20% fetal bovine serum. The tubes were then transferred to an incubator for 10h. The following day, the cells were centrifuged at $500 \times g$ for 10 min. and resuspended into 10ml of complete RPMI containing 2% horse serum and counted. Cell viability was >85%. For transfected EDCs, the cells were placed into an ELISpot plate at approximately $20 \times 10^4$ cells/well and $2.5 \times 10^5$ PBMCs/well were added. For peptides, 50µg/ml or 100µg/ml of each peptide in different peptide pools was added per well. PMA/Ionomycin was added to the positive control wells. The plates were wrapped in aluminum foil and incubated at 37°C for 20h. Live EAV VBS and MLV virus were used in the ELISpot assay at 1 m.o.i. Each virus was mixed with PBMCs and incubated at 37°C for 1 hour before adding the cells to the ELISpot plates. The number of spots was enumerated by the CTL ELISPOT reader. The number of spots in the medium only was subtracted from the number spots in each of the test sample.

3.10 Virus neutralization (VN) test

Antibody determination was conducted with the virus neutralization (VN) test as described in the OIE Manual for Diagnostics and Vaccines for Terrestrial Animals. A total of 16 stallions were screened for EAV neutralizing antibodies to identify the horses to be vaccinated. Stallions that were less than 20 years of age and had a titer of < 1:4 were included in the study. The ELISpot assay was performed on the selected stallions to identify those stallions whose PBMCs had low levels of spontaneous IFN-γ release.
CHAPTER 4. RESULTS

4.1 Virus neutralization (VN) test

The outcome of VN testing of the sera from 16 horses for EAV antibodies before vaccination is presented in Table 1. After vaccination with the MLV vaccine, serum was collected at the time points shown in Table 2 to determine whether the horses had seroconverted or not. Serum was also collected from the three carrier stallions to establish their infective VN titers for EAV (Table 3). All vaccinated horses seroconverted by day 14 post vaccination. They all had a titre of 1:512 by day 23.

Table 1: Results of screening stallions for VN antibodies to EAV

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Sex</th>
<th>Breed</th>
<th>Age (Years)</th>
<th>VN antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 K113</td>
<td>Stallion</td>
<td>QH</td>
<td>18</td>
<td>1:256</td>
</tr>
<tr>
<td>2 L111</td>
<td>Stallion</td>
<td>Pony</td>
<td>24</td>
<td>1:256</td>
</tr>
<tr>
<td>3 L118</td>
<td>Stallion</td>
<td>Pony</td>
<td>ND</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>4 L134</td>
<td>Stallion</td>
<td>TB</td>
<td>9</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>5 N118</td>
<td>Stallion</td>
<td>Paint</td>
<td>7</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>6 N120</td>
<td>Stallion</td>
<td>app</td>
<td>21</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>7 N122</td>
<td>Stallion</td>
<td>TWHX</td>
<td>8</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>8 N123</td>
<td>Stallion</td>
<td>QHX</td>
<td>14</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>9 O114</td>
<td>Stallion</td>
<td>TWHX</td>
<td>13</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>10 O121</td>
<td>Stallion</td>
<td>TB</td>
<td>4</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>11 636</td>
<td>Mare</td>
<td>STB</td>
<td>21</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>12 641</td>
<td>Mare</td>
<td>Arab</td>
<td>ND</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>13 B008</td>
<td>Mare</td>
<td>STBX</td>
<td>15</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>14 B083</td>
<td>Mare</td>
<td>QH</td>
<td>ND</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td>15 G011</td>
<td>Mare</td>
<td>TB</td>
<td>10</td>
<td>1:128</td>
</tr>
<tr>
<td>16 C067</td>
<td>Mare</td>
<td>TB</td>
<td>14</td>
<td>&lt;1:4</td>
</tr>
</tbody>
</table>

ND – Not determined
Table 2: EAV VN antibody findings at different time-points after vaccination

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days post vaccination</th>
<th>VN antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td></td>
</tr>
<tr>
<td>Horse ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>O114</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>L134</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>N122</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Carrier stallion</td>
<td>H069</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Known seronegative horse</td>
<td>O121</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>Horse ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>O114</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>L134</td>
<td>1:256</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>N122</td>
<td>1:128</td>
</tr>
<tr>
<td>Carrier stallion</td>
<td>H069</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Known seronegative horse</td>
<td>O121</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td></td>
<td>Day 23</td>
<td></td>
</tr>
<tr>
<td>Horse ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>O114</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>L134</td>
<td>1:512</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>N122</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Carrier stallion</td>
<td>H069</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Known seronegative horse</td>
<td>O121</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td></td>
<td>Day 37</td>
<td></td>
</tr>
<tr>
<td>Horse ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>O114</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>L134</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>N122</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Carrier stallion</td>
<td>H069</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Known seronegative horse</td>
<td>O121</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td></td>
<td>Day 41</td>
<td></td>
</tr>
<tr>
<td>Horse ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>O114</td>
<td>&lt; 1:4</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>L134</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>N122</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Carrier stallion</td>
<td>H069</td>
<td>&gt; 512</td>
</tr>
<tr>
<td>Known seronegative horse</td>
<td>O121</td>
<td>&lt; 1:4</td>
</tr>
</tbody>
</table>

Table 3: Virus neutralization test and competitive ELISA (cELISA) results for EAV in three carrier stallions

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Horse Breed</th>
<th>VN Titers</th>
<th>VN</th>
<th>cELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I050</td>
<td>Rocky Mountain</td>
<td>1:256</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I070</td>
<td>Tennessee Walker</td>
<td>1:512</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H069</td>
<td>Paint</td>
<td>&gt;1:512</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
4.2 Establishment and characterization of primary EDCs

EDCs for stallion H069 were mock and EAV infected. Expression of MHC class I and class II was detected by IFA (Figure 5) and flow cytometry (Figure 6). The EDCs were also tested for expression of EAV NspI (Figure 6).

![Image of Immunofluorescence microscopy](image)

**Figure 5:** Immunofluorescence microscopy performed on primary EDCs.
A) Primary EDCs stained with mouse anti-Equine MHC Class I antibody;
B) Primary EDCs stained with mouse anti-human vimentin antibody;
C) Secondary antibody alone (anti-mouse Alexa Fluor 488)

Data from IFA and flow cytometry (Figure 5 & Figure 6) show that EDCs constitutively express high levels of MHCI but not MHCII. EDCs were also susceptible to EAV since expression of nspI was detected in EDCs treated with EAV.

![Image of Flow cytometry data](image)

**Figure 6:** Flow cytometry data for expression of MHC class I and MHC class II molecules on Mock infected and EAV infected primary equine dermal cells (EDCs). EDCs were infected (at 2 m. o. i) with EAV VBS for 24hrs.
To determine the optimal m.o.i of virus and length of time to infect, IFA was conducted on EDCs infected with different m.o.i of EAV at different time points. At 8h and 12h, the nsp1 protein is mainly localized to the nucleus (Figure 7) but at 24 hours it is predominant in the cytoplasm. The M protein is mainly expressed in the cytoplasm at all time points studied (Figure 8). From these data it is evident that 2 m.o.i for 24hrs would work well for T cell assays.

Figure 7: Immunofluorescence staining of EDCs infected with EAV VBS and stained with primary antibody mouse anti-nsp1 and secondary antibody anti-mouse Alexa Fluor 488

Figure 8: Immunofluorescence staining of EDCs infected with EAV VBS and stained with primary antibody mouse anti-M and secondary antibody anti-mouse Alexa Fluor 594
### 4.3 Ultraviolet (UV) inactivation of EAV VBS

EAV VBS was UV inactivated to determine if it can be used in CTL assays instead of live virus. Since the CTL assays used in this study rely on target cell lysis induced by the granzyme B pathway, and EAV is known to induce apoptosis, using live virus would create high background.

![Graph showing UV inactivation of EAV VBS](image)

**Figure 9:** UV inactivation of EAV VBS. Red bars show virus titers for live virus while the blue bars show virus titers for UV inactivated virus. These data clearly demonstrate that EAV is rapidly inactivated by UV light.

However, the data in figure 10 indicate that UV inactivated virus is not able to infect EDCs since there was no staining of viral proteins as per flow cytometry data (Figure 10).

![Flow cytometry analysis of EDCs infected with live and UV inactivated EAV VBS](image)

**Figure 10:** Flow cytometry analysis of EDCs infected with live and UV inactivated EAV VBS.

Data from figure 9 shows that the virus is completely inactivated as early as 2 minutes of exposure to UV light (Figure 9).
4.4 Interferon-gamma ELISpot Assay (IFN-γ ELISpot) performed with transfected EDCs

Currently, it’s not possible to predict ELA epitopes using available epitope prediction software. To go around this problem, we performed transient transfection (of EDCs) with EAV viral proteins to produce full length proteins that would probably contain anchor amino acids for ELA of all the horses used in this study.

![Figure 11: IFA staining of EDCs transfected with EAV IVT RNA (24h transfection)](image)

Immunofluorescence microscopy data indicates that transfected EDCs express high levels of viral proteins at 24h post transfection which drops tremendous by 48hrs (data not shown). So, for the IFN-γ ELISpot assay, the 24h time point was used (Figure 11).

![Figure 12: ELISpot data conducted with PBMCs (isolated from carrier stallions) stimulated with autologous EDCs transfected with EAV IVT RNA](image)
Stimulation of PBMCs with transfected EDCs seemed to produce positive results (Figure 12), but this response could not be reproduced with peptide pools or virus.

4.5 Measuring cytotoxicity of in vitro stimulated PBMCs with the LDH assay

Cell mediated response against EAV was measured using PBMCs from vaccinated stallions (n=2), long term carrier stallions (n=3) and one EAV seronegative stallion. The PBMCs were incubated in vitro with or without virus for 7-10 days. The PBMCs were later co-cultured with EDCs that had been mock infected or infected with EAV VBS or MLV virus.

Figure 13: Results of LDH assays performed with LAK cells and EQT8888 cells at different E:T ratios. A) Low E:T ratios B) High E:T ratios

The LDH assay was first optimized with LAK cells and EQT8888 cells. LAK cells were cocultured with EQT8888 cells at 37°C in the presence of 5% CO₂ for 6h at different E:T ratios. A range of E:T ratios was tried, and it was very clear that this assay works well at low E:T ratios (Figure 13). As the E:T ratio increased, so did the number of dead or dying cells thus lowering the signal/noise ratio and leading to reduced or no target lysis signal. After optimizing the LDH assay with LAK cells, the assay was applied to PBMCs isolated from long-term carrier stallions (Figure 14) and vaccinated stallions. Positive data was only observed in one vaccinated stallion (at day 99) and this was obtained after in vitro
re-stimulation of PBMCs with the MLV virus for 10 days (Figure 15).

Figure 14: LDH data for carrier stallions
Figure 15: LDH data for vaccinated horses (N122 and L134) and one naïve horse (0114)
CHAPTER 5. DISCUSSION AND CONCLUSION

5.1 Discussion

In this study, four assays were evaluated for monitoring T cell immunity in EAV infected horses. The flow cytometric assays work well with nonadherent target cells, they have been evaluated in this context and found to be more sensitive and more informative than the chromium release assay.\textsuperscript{355, 356} Since EDCs are adherent cells, it was not possible to optimize these assays. Flow cytometry assays also require immediate analysis to minimize background emanating from dying cells.\textsuperscript{357} This requires that the laboratory have a flow cytometer on-site. For these reasons, we only evaluated the LDH and ELISpot assays.

The data from the ELISpot assay was inconsistent as transfected cells provided some positive results, but peptides and EAV did not. Since frozen PBMCs were used for all ELISpot assays, this could be the reason why peptide and virus stimulated PBMCs did not respond. Very low numbers of monocytes have been reported in frozen PBMCs which is consistent with our findings. In this study, frozen PBMCs were tested for CD14 expression and monocytes could not be detected (data not shown), they were neither seen on the side scatter vs forward scatter plot. This is supported by the study of Castillo-Olivares \textit{et al.}\textsuperscript{260} who failed to detect a CTL response using frozen PBMCs in their \textsuperscript{51}Cr release assay. They also noted that frozen PBMCs were not susceptible to EAV as expression of N protein was not detected in frozen PBMCs exposed to EAV.

The data obtained using EDCs could also be non-specific since blue tongue virus (BTV) M5 transfected EDCs also showed high spot forming units in stallion I050. The carrier stallions used in this study are over 30 years old, and old horses tend to have
spontaneous production of IFN-γ. It should also be noted that EDCs do not express MHC-II antigens, thus are unable to stimulate a CD4+ T cell response. This would result in a lack of “help” for the CD8+ T cells. While other APC in the PBMC culture could present antigen to CD4+ T cells. Equine dermal cells may also lack some of the costimulatory molecules expressed by professional antigen presenting cells. Thus, will fail to stimulate T cell proliferation and cytokine production if exogenous cytokines are not added to the culture medium. Since electroporated EDCs tend to clump, it is very difficult to add an equal number of cells per well; this will result in a lot of variation in number of spots between wells. Equine dermal cells are adherent cells, using them in the ELISpot plate not only introduced artefacts, but it also interfered with the detection antibody binding thus creating a lot of background. Non-adherent cells like dendritic cells are better suited for this kind of analysis.

In mouse models, T cell function is measured using cells obtained from lymphoid tissues such as spleen, bone marrow and lymph nodes. These tissues have a high concentration of antigen specific T cells so do not require in vitro re-stimulation for several days. PBMCs, on the other hand, require in vitro stimulation for 7-21 days. This process can have profound modifying effect on T cell function. Besides, cultured cells tend to have high numbers of dead or dying cells, which can be a source of high background in most CTL assays. The LDH assay has the same working principle as ¹⁵¹Cr release assay but differs from the ¹⁵¹Cr release assay in that it relies on the intrinsic cellular production of lactate dehydrogenase. Its sensitivity is highly affected by high background coming from culturing cells for several days. Consequently, high E: T ratios are not recommended as these will lead to very low signal/noise ratios (Figure 13B). Since PBMCs contain low
concentrations of antigen specific cells, low E:T ratios might under-estimate or fail to capture antigen specific T cell responses especially when they are below the detection threshold of the assay. This could be the reason why we were not able to detect CTL lysis in PBMCs stimulated for 7 days. Even with effectors stimulated for 10 days, only one stallion of the two vaccinated stallions responded. Carrier stallions did not show any CTL response at 7 and 10 days of EAV *in vitro* re-stimulation. Several reasons could account for this: 1) the strain of EAV used for re-stimulation is a laboratory adapted strain and could lack the epitopes recognized by the CTLs of carrier stallions; 2) since these horses have been shedding virus in semen for over ten years, EAV specific CTLs could be sequestered in the reproductive tract and not present in the peripheral circulation; 3) the CTL response could have diminished with time and so not detectable.

Finally, 99 days after vaccination may not be an optimal time to evaluate the LDH assay because the cellular immune response to EAV may not be properly developed and thus could be below the threshold for LDH assay detection given that this assay is not very sensitive. This is supported by the observation of Castillo-Olivares *et al.*,260 who were unable to detect a CTL response before 4 months of infection. This raises the need to further validate this assay with a larger number of vaccinated horses and allow enough time for the immune system to develop before using the LDH assay to measure CTL response. Another approach could be to look at other CTL assays that are more sensitive and specific. One such assay is the granzyme B ELISpot assay which is simple, rapid, very sensitive, reproducible and does not use a lot of cells. The other very sensitive and robust assay is tetramer assay, tetramers have been developed for equine leucocyte antigen A1(ELA-A1).298 The same approach could be used to produce EAV peptide tetramers to avoid the
problems associated with culturing effector cells for days and using assays with low sensitivity.

5.2 Conclusion

The LDH assay discussed in this study works well with cells obtained from lymphoid organs. Because of the reasons discussed above, I wouldn’t recommend it to someone working with PBMCs. In my opinion, equine T cell research should stick with the chromium release assay until a new validated and sensitive assay is introduced e.g. the tetramer, granzyme B ELISpot or TCR phenotyping. Also, the process of choosing horses to include in any T cell study should ensure that the most common ELA haplotypes are properly represented in both the control group and test group. This will improve the probability of identifying epitopes, and there will be no need to use transfected cells as antigen presenting cells.

Because of the reasons mentioned above, naturally infected long-term EAV carrier stallions are a poor model to study T cell immunity to EAV. Unless the stallions are infected with a known EAV strain and followed overtime, there will always be problems working with naturally infected long-term carrier stallions. This is because, the infecting EAV virus is not always known and so will require isolation of the virus, sequencing it and making peptides out of each isolate which is costly and time consuming. Isolation of the virus from semen and using it in the ELISpot assay will still give high background since semen contains bacteria that produce endotoxins. Furthermore, EAV semen isolates cannot be used for in vitro re-stimulation because the viruses are not culture adapted so most of them will not infect PBMCs. Therefore, T cell studies require cautious planning before any experiments can commence.
REFERENCES


13. Snijder, E.J., van Tol, H., Roos, N. & Pedersen, K.W. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the


173. Yauch, L.E. et al. CD4<sup>+</sup> T Cells Are Not Required for the Induction of Dengue Virus-Specific CD8<sup>+</sup> T Cell or Antibody Responses but Contribute to Protection after Vaccination. The Journal of Immunology 185, 5405-5416 (2010).


193. Li, L. et al. HBcAg-Specific IL-21-Producing CD4+ T Cells are Associated with Relative Viral Control in Patients with Chronic Hepatitis B. *Scandinavian Journal of Immunology* 78, 439-446 (2013).


201. Levy, J.A. The search for the CD8+ cell anti-HIV factor (CAF). *Trends in*


213. Carrington, M. *et al.* HLA and HIV-1: heterozygote advantage and B*35-Cw*04


258. Kydd, J.H., Wattring, E. & Hannant, D. Pre-infection frequencies of equine herpesvirus-1 specific, cytotoxic T lymphocytes correlate with protection against


271. Froelich, C.J. *et al.* New Paradigm for Lymphocyte Granule-mediated Cytotoxicity TARGET CELLS BIND AND INTERNALIZE GRANZYME B, BUT AN ENDOSOMALYTIC AGENT IS NECESSARY FOR CYTOSOLIC DELIVERY AND SUBSEQUENT APOPTOSIS. *Journal of Biological*


Vita

A. Educational institutions attended:
   i. Makerere University, awarded a Bachelor of Science degree in Biomedical laboratory technology
   ii. North Dakota State University, awarded a Master of Science degree in Infectious disease management and biosecurity

B. Scholarships awarded:
   i. Research assistantship, University of Kentucky
   ii. United States Agency for Internal Development(USAID) scholarship for a master’s program at North Dakota State University
   iii. Undergraduate summer intern fellowship by Centre for Biomedical Research(COBRE) North Dakota state University

C. Professional positions held: Research laboratory technician, University of Kentucky

D. Name of student: Annet Kyomuhangi