2012-03-13

The Role of Heterologous Immunity in Mediating Natural Resistance to Infection in Human Subjects: A Dissertation

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THE ROLE OF HETEROLOGOUS IMMUNITY IN MEDIATING NATURAL RESISTANCE TO INFECTION IN HUMAN SUBJECTS

A Dissertation Presented

By

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Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 13, 2012

Immunology and Virology

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March 13, 2012

List of Publications

Clute SC, **Watkin LB**, Cornberg M, Naumov YN, Sullivan JL, Luzuriaga K, Welsh RM, Selin LK. Cross-reactive influenza virus-specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. J Clin Invest. 2005 Dec;115(12):3602-12.

Naumov YN, Naumova EN, Clute SC, **Watkin LB**, Kota K, Gorski J, Selin LK. Complex T cell memory repertoires participate in recall responses at extremes of antigenic load. J Immunol. 2006 Aug 1;177(3):2006-14.

Towne CF, York IA, **Watkin LB**, Lazo JS, Rock KL. Analysis of the role of bleomycin hydrolase in antigen presentation and the generation of CD8 T cell responses. J Immunol. 2007 Jun 1;178(11):6923-30.

Clute SC, Naumov YN, **Watkin LB**, Aslan N, Sullivan JL, Thorley-Lawson DA, Luzuriaga K, Welsh RM, Puzone R, Celada F, Selin LK. Broad cross-reactive TCR repertoires recognizing dissimilar Epstein-Barr and influenza A virus epitopes. J Immunol. 2010 Dec 1;185(11):6753-64.

Cornberg M, Clute SC, **Watkin LB**, Saccoccio FM, Kim SK, Naumov YN, Brehm MA, Aslan N, Welsh RM, Selin LK. CD8 T cell cross-reactivity networks mediate heterologous immunity in human EBV and murine vaccinia virus infections. J

Immunol. 2010 Mar 15;184(6):2825-38.

Watkin LB, Aslan N, Gil A, Quigley M, Luzuriaga K, Selin LK. Increased level of lytic, high avidity cross-reactive T-cells in EBV sero-negative adults. Manuscript in preparation

Acknowledgements

I would like to thank my thesis advisor Liisa Selin for the opportunity to perform my thesis research in her laboratory. Her enthusiasm for the concepts of heterologous immunity is contagious and inspiring. I would also like to thank her and my unofficial co-mentor Ray Welsh not only for the immense wealth of knowledge and experience that I have gained through the years in the lab but also for encouraging me to go through with graduate school when I was having doubts of attending.

I would like to thank my thesis committee members Ray Welsh, Katherine Luzuriaga, and Alan Rothman for the guidance they gave me during the course of my graduate studies. I would especially like to thank John Harris and Larry Stern for participating in my thesis defense.

I would like to thank my family for their continued interest and support of my endeavor through graduate school over the years.

Finally I would like to thank my wife Amanda. Had we not endured the time in graduate school together we may not have ever fallen in love and married.

Abstract

Heterologous immunity is a mechanism by which immunological memory within an individual, developed in response to a previous infection, plays a role in the immune response to a subsequent unrelated infection. In murine studies, heterologous immunity facilitated by cross-reactive CD8 T-cell responses can mediate either beneficial (protective immunity) or detrimental effects (e.g. enhanced lung and adipose immunopathology and enhanced viral titers) (Selin et al., 1998; Chen et al., 2001; Welsh and Selin, 2002; Nie et al., 2010; Welsh et al., 2010). Protective heterologous immunity results in enhanced clearance of virus during a subsequent infection with an unrelated pathogen. Such is the case when mice are immunized with lymphocytic choriomeningitis virus (LCMV) and subsequently challenged with Pichinde virus (PV) or vaccinia virus (VACV) (Selin et al., 1998). However, heterologous immunity may also mediate enhanced immunopathology as mice immunized with influenza A virus (IAV) and challenged with LCMV show increased viral titers and enhanced lung immunopathology (Chen et al., 2003).

The role heterologous immunity plays during infection is not limited to the murine system. In fact, there have now been several reports of enhanced immunopathology due to heterologous immunity during human infections, involving viruses such as IAV, Epstein-Barr Virus (EBV), hepatitis C virus (HCV), and dengue virus (DENV) (Mathew et al., 1998; Wedemeyer et al., 2001; Acierno et al., 2003; Nilges et al., 2003; Clute et al., 2005; Urbani et al., 2005).

Interestingly, in all reported cases in humans, heterologous immunity mediated enhanced immunopathology.

Upon infection with EBV the clinical presentation can range from asymptomatic to severe, occasionally fatal, acute infectious mononucleosis (AIM) (Crawford et al., 2006b; Luzuriaga and Sullivan, 2010) which is marked by a massive CD8 lymphocytosis. This lympho-proliferative effect in AIM was shown to be partially mediated by reactivation of cross-reactive IAV-$M1_{58-66}$ (IAV-GIL) specific CD8 memory T-cells in HLA-A2 patients reacting to the EBV-$BMLF1_{280}$ (EBV-GLC) epitope (Clute et al., 2005).

Interestingly, EBV infects ~90% of individuals globally by the third decade of life, establishing a life-long infection (Henle et al., 1969). However, it is unknown why 5-10% of adults remain EBV-sero-negative (EBV-SN), despite the fact that the virus infects the vast majority of the population and is actively shed at high titers even during chronic infection (Hadinoto et al., 2009). Here, we show that EBV-SN HLA-A2+ adults possess cross-reactive IAV-GIL/EBV-GLC memory CD8 T-cells that show highly unique properties. These IAV-GIL cross-reactive memory CD8 T-cells preferentially expand and produce cytokines to EBV antigens at high functional avidity. Additionally, they are capable of lysing EBV-infected targets and show the potential to enter the mucosal epithelial tissue, where infection is thought to initiate, by CD103 expression. This protective capacity of these cross-reactive memory CD8 T-cells may be explained by a

unique T-cell receptor (TCR) repertoire that differs by both organization and CDR3 usage from that in EBV-seropositive (EBV-SP) donors.

The composition of the CD8 T-cell repertoire is a dynamic process that begins during the stochastic positive selection of the T-cell pool during development in the thymus. Thus, upon egress to the periphery a naïve T-cell pool, or repertoire, is formed that is variable even between genetically identical individuals. This T-cell repertoire is not static, as each new infection leaves its mark on the repertoire once again by stochastically selecting and expanding best-fit effectors and memory populations to battle each new infection while at the same time deleting older memory CD8 T-cells to make room for the new memory cells (Selin et al., 1999). These events induce an altered repertoire that is unique to each individual at each infection. It is this dynamic and variable organization of the T-cell repertoire that leads to private specificity even between genetically identical individuals upon infection with the same pathogens and thus a different fate (Kim et al., 2005; Cornberg et al., 2006a; Nie et al., 2010). It is this private specificity of the TCR repertoire that helps explain why individuals with the same epitope specific cross-reactive response, but composed of different cross-reactive T-cell clones, can either develop AIM or never become infected with EBV.

Our results suggest that heterologous immunity may protect EBV-SN adults against the establishment of productive EBV infection, and potentially be the first demonstration of protective T-cell heterologous immunity between

unrelated pathogens in humans. Our results also suggest that CD8 T-cell immunity can be sterilizing and that an individual's TCR repertoire ultimately determines their fate during infection.

To conclusively show that heterologous immunity is actively protecting EBV-SN adults from the establishment of a productive EBV infection, one would have to deliberately expose an individual to the virus. Clearly, this is not an acceptable risk, and it could endanger the health of an individual. A humanized mouse model could allow one to address this question.

However, before we can even attempt to address the question of heterologous immunity mediating protection from EBV infection in humanized mice, we must first determine whether these mice can be infected with, and build an immune response to the two viruses we are studying, EBV and IAV. We show here that these mice can indeed be infected with and also mount an immune response to EBV. Additionally, these mice can also be infected with IAV. However, at this time the immune responses that are made to these viruses in our established humanized mouse model are not substantial enough to fully mimic a human immune response capable of testing our hypothesis of heterologous immunity mediating protection from EBV infection.

Although the immune response in these mice to EBV and IAV infection is not suitable for the testing of our model the data are promising, as the humanized mouse model is constantly improving. Hopefully, with constant improvements

being made there will be a model that will duplicate a human immune system in its entirety.

This thesis will be divided into 5 major chapters. The first chapter will provide an introduction to both general T-cell biology and also to the role of heterologous immunity in viral infection. The second chapter will provide the details of the experimental procedures that were performed to test our hypothesis. The third chapter will describe the main scientific investigation of the role of heterologous immunity in providing natural resistance to infection in human subjects. This chapter will also consist of the data that will be compiled into a manuscript for publication in a peer-reviewed journal. The fourth chapter will consist of work performed pertaining to the establishment of a humanized mouse model of EBV and IAV infection. The establishment of this model is important for us to be able to show causation for protection from EBV infection mediated by heterologous immunity.

Table of Contents

List of Tables

List of Figures

List of Symbols, Abbreviations or Nomenclature

ADAP	adhesion and degranulation-promoting adapter protein
AIM	Acute infectious mononucleosis
APC	Antigen presenting cell
BLCL	B-lymphoblastoid cell line
CDR	Complementarity determinant region
CRAC	Ca^{2+}-release activated Ca^{2+}
CTLA-4	cytotoxic T lymphocyte antigen-4
DAG	diacyl glycerol
DC	Dendritic cell
DP	Double Positive
DPT	Diphtheria Pertussis Tetanus
DENV	Dengue Virus
EBV	Epstein-Barr Virus
EBV-SN	EBV Sero-negative
EBV-SP	EBV Sero-positive
ER	endoplasmic reticulum
GADS	GRB2-related adapter downstream of Shc
GRB2	growth factor receptor-bound protein 2
H&E	Hematoxylin/eosin
HCV	Hepatitis C Virus
HPK1	hematopoietic progenitor kinase 1

HSC	Hematopoietic stem cell
ICOS	Inducible co-stimulator
ICS	Intra-cellular cytokine stain
IFNγ	Interferon-γ
IN	Intra Nasal
IP_3	inositol 1,4,5-triphosphate
IS	Immunological Synapse
ITAM	immune-receptor tyrosine-based activation motifs
ITK	IL-2-induced tyrosine kinase
LAT	linker for the activation of T-cells
LN	Lymph Node
MHC	Major Histocompatibility complex
MPEC	Memory precursor effector cell
NOD	Non-obese diabetic
NSG	NOD SCID IL2rγ KO
PD-1	Programmed death-1
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLCγ-1	phosphor lipase C-γ-1
pMHC	peptide MHC
PTK	Protein tyrosine kinase

PTLD	Post transplantation lymphoproliferative disease
SCID	Severe combined immune deficiency
SH2	Src homology 2
SHP1	SH2 domain-containing protein tyrosine phosphatase
SLEC	Short lived effector cell
SLP-76	SH2 domain-containing leukocyte phospho-protein of 76kDa
TCR	T-cell Receptor
VACV	Vaccinia Virus
ZAP-70	CD3 ζ associated protein of 70kDa

Chapter I: Introduction

Memory CD8 T-cells are an important mediator of resistance to re-infection by previously encountered pathogens, primarily viruses (Welsh et al., 2004). After viral clearance the activated effector CD8 T-cell pool begins its contraction phase and formation of a stable memory pool. Memory CD8 T-cells in general are superior to naïve CD8 T-cells, as they have the ability to recognize cognate peptide-MHC (pMHC) in the peripheral tissues and exert effector functions, while naïve CD8 T-cells that see cognate pMHC without proper costimulation signals undergo peripheral tolerization (Redmond and Sherman, 2005). As compared to naïve CD8 T-cells, memory CD8 T-cells are maintained at a higher frequency due to their previous clonal expansion, mediated by IL-7 and IL-15 with the help of CD4 cells, and can be maintained without the need for antigen (Surh and Sprent, 2008). Memory CD8 T-cells are also rapidly activated due to an open chromatin structure from the previous activation, require less antigen to become activated, and show a varied tissue distribution (Arens and Schoenberger, 2010). Therefore, being maintained at a higher frequency than naïve CD8 T-cells with the ability to exert rapid effector function in response to a lower level of antigen puts memory CD8 T-cells at an advantage when challenged with a recognizable pMHC antigen

Over the years it has become evident that CD8 T-cells can recognize more than one antigen. If a CD8 T-cell can be cross-reactive and recognize more than one antigen, and a memory CD8 T-cell is at an advantage over naïve

CD8 T-cells, then it stands to reason that a memory CD8 T-cell exposed to a cross-reactive epitope from an unrelated virus would preferentially become activated over a naïve CD8 T-cell. This is indeed the case as our lab has demonstrated this phenomenon in both murine and human systems and found it to be a common occurrence (Welsh et al., 2010; Selin et al., 2011). Of the several cross-reactive responses that have been identified there appear to be consequences resulting from them. In the murine system one of the resulting consequences is partial protection, where prior immunity to one virus results in a lower viral titer when challenged with an unrelated virus as compared to naïve controls (beneficial). Another consequence is that of immune-pathology following challenge with an unrelated virus that is distinct from naïve controls challenged with the same virus (detrimental). This phenomenon of altered immunity resulting from cross-reactive CD8-T-cells has been coined heterologous immunity.

Detrimental heterologous immunity has also been described during human infections. The reports of heterologous immunity and cross-reactive CD8 T-cells in humans have coincided with enhanced pathology such as dengue shock syndrome during DENV infection, necrotizing fulminant hepatitis during HCV infection, and AIM during EBV infection where IAV-GIL specific memory CD8 T-cells were shown to cross-react with EBV-GLC peptide (Mathew et al., 1998; Wedemeyer et al., 2001; Acierno et al., 2003; Nilges et al., 2003; Clute et al., 2005; Urbani et al., 2005). Although there are no direct descriptions of beneficial

protective heterologous immunity in humans there is some epidemiological evidence that shows children vaccinated with live measles virus (MV) vaccine or Bacille-Calmette-Guerin (BCG) have unexpectedly lower morbidity and mortality to other pathogens than those not vaccinated (Aaby et al., 1995; Stensballe et al., 2005; Farrington et al., 2009).

The goal of this thesis was to determine if heterologous immunity and cross-reactive CD8 T-cells could mediate protection from viral infection in humans. To determine whether heterologous immunity and cross-reactive CD8 T-cells were mediating protection from viral infection we used EBV as our infection model. Previously our lab had identified a cross-reactive CD8 T-cell response between IAV and EBV. This cross-reactivity was between the HLA-A2 restricted epitopes IAV-GIL and EBV-GLC and was found not only to occur during AIM but also contribute to lymphoproliferation associated with AIM (Clute et al., 2005). Subsequently, we have also identified an additional cross-reactivity between IAV and EBV this time mediated again by IAV-GIL but cross-reacting with EBV-BRLF1 (EBV-YVL) (Aslan et. al. unpublished data). We questioned whether these same cross-reactive patterns between IAV and EBV were capable of mediating protection from EBV infection. To do this we examined EBV-SN adults and assessed their cross-reactive patterns compared to that of EBV-SP adults. Additionally, we also examined the T-cell receptor (TCR) repertoire within the EBV-SN adults to determine whether there were any unique features that might explain why the same cross-reactive epitopes lead to AIM in some

individuals while protecting others from infection. We also set out to establish a humanized mouse model of viral infection so as to recapitulate our finding in a testable model.

This introduction is separated into two major sections. The first section is an overall description of T-cell immunity. The second half more specifically pertains to the biology of heterologous immunity.

1. T-cell Immunity:

A. Generation of the CD8 T-cell pool

The generation of a highly diverse CD8 T-cell receptor (TCR) repertoire within an individual is started by somatic rearrangement of the germline encoded V-, D-, and J-regions within the *tcrb* locus and V- and J-regions of the *tcra* locus (Taghon and Rothenberg, 2008). Mathematically it is theorized that there can be up to 10^{15} different TCR heterodimer pairs in mice. Though following negative and positive thymic selection which results in a massive die off of cells that either can not react to pMHC (positive selection) or react to strongly pMHC (negative selection) the actual number is estimated to be in the area of 10^{6}-10^{8}, much lower than theorized (Arstila et al., 1999; Casrouge et al., 2000; Nikolich-Žugich et al., 2004). The complementarity determinant regions (CDRs), CDR1 and CDR2, within the V region of the TCR have natural affinity for the major histocompatibility complex (MHC) molecule, while the CDR3, the region covering the somatic rearrangement, recognizes peptide bound to the MHC (pMHC) (Huseby et al., 2005; Dai et al., 2008).

T-cells develop in the thymus where they undergo a process known as positive selection. Cells that can bind the MHC with low affinity survive the process by receiving survival signals, while cells that cannot bind the MHC undergo death by neglect (Starr et al., 2003). At this stage of development T-cells express both CD4 and CD8 and are referred to as double positive (DP) thymocytes. DP thymocytes that have a lower affinity for pMHC-I, lose CD4 expression and retain CD8 expression, while DP thymocytes with a higher affinity for pMHCII retain the expression of CD4. This later results in the expression of the CD4 lineage commitment transcription factor ThPOK (Singer et al., 2008; Collins et al., 2009).

DP thymocytes that survive positive selection go on to negative selection. During this process thymocytes that demonstrate high affinity binding to self pMHC are selectively deleted by receiving signals to undergo apoptosis (Hogquist et al., 2005). This selection event results in the deletion of cells that could cause autoimmune disease in the host if they were allowed access to the periphery.

After thymocytes finish negative selection they leave the thymus and become recent thymic emigrants with a naïve phenotype of $CD62L^{high}$, $CCR7^{high}$, $CD44^{low}$, and $CD24^{+}$ (Tough and Sprent, 1994). As they migrate through the secondary lymphoid tissues they increase the levels of their markers slightly and lose CD24 expression to become mature naïve T-cells (Makaroff et al., 2009). In humans naïve T-cells have a half-life of 4-6 years and require IL-7 for their

survival (Link et al., 2007; Vrisekoop et al., 2008). An additional necessity for survival of CD8 T-cells is interaction with self-pMHC-I in the periphery (Tanchot et al., 1997; Polic et al., 2001; Surh and Sprent, 2008; Takada and Jameson, 2009).

The MHC-I heavy chains are a set of 3 separate genes all of which are highly polymorphic. These molecules present small peptides derived primarily from proteins synthesized within the cell. The high degree of polymorphism is manifest within the peptide-binding groove of the MHC-I (Trowsdale, 2005). This alteration of the binding groove results in variations in the anchor residues responsible for peptide binding and consequently a more diverse peptide pool. With the combination of a highly diverse peptide pool and a highly diverse TCR repertoire CD8 T-cells can recognize virtually any foreign protein.

B. T-cell Activation

Activation of T-cells by ligation of TCR/pMHC results in an array of downstream signaling events. The first event is the activation of the src family of protein tyrosine kinases (PTKs), primarily lck (Samelson et al., 1986; Straus and Weiss, 1992). These kinases then phosphorylate the homo- and hetero-dimeric CD3-ζ, -ε, -δ, and -γ proteins within their immune-receptor tyrosine-based activation motifs (ITAMs). These phosphorylation events then result in the recruitment of the CD3 ζ associated protein of 70kDa (ZAP-70) which subsequently activates the trans-membrane adapter protein linker for the activation of T-cells (LAT) by phosphorylation. ZAP-70 also recruits and activates the cytosolic adapter protein

Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76kDa (SLP-76) by phosphorylation (Bubeck Wardenburg et al., 1996; Zhang et al., 1998). The recruitment of these two molecules builds the backbone of the signaling complex by organizing the signaling effector molecules in the correct spatiotemporal order to begin the multiple signaling cascades (Smith-Garvin et al., 2009).

When LAT becomes phosphorylated and activated by ZAP-70 it then recruits the kinases phospholipase C-γ-1 (PLCγ-1) and phosphoinositide 3-kinase (PI3K). Additionally, it recruits the adapter molecules growth factor receptor-bound protein 2 (GRB2) and GRB2-related adapter downstream of Shc (Gads) (Sommers et al., 2004). SLP76 is then recruited to LAT by their mutual binding partner Gads and itself recruits Vav1, Nck, IL-2-induced tyrosine kinase (Itk), adhesion and degranulation-promoting adapter protein (ADAP) and hematopoietic progenitor kinase 1 (HPK1). SLP76 also binds to PLCγ-1, which has already been recruited to LAT (Koretzky et al., 2006). While LAT and SLP76 mediate the nucleation of the signaling complex, the effector molecules that are recruited help to stabilize the complex (Reynolds et al., 2002; 2004; Dombroski et al., 2005). The LAT/SLP76 complex ultimately results in the activation of PLCγ-1 which hydrolyses phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to inositol 1,4,5-triphospate (IP_3) and diacyl glycerol (DAG). The release of IP_3 to the cytosol results in release of Ca^{2+} from the endoplasmic reticulum (ER), which in turn activates Ca^{2+}-release activated Ca^{2+} (CRAC) channels to produce a flux in

Ca^{2+} (Lewis and Cahalan, 1995). This increase in Ca^{2+}, coupled with DAG mediated activation of protein kinase C (PKC) (which also activates NFκB), results in the activation and translocation to the nucleus of NFAT where it is free to associate with AP-1 and induce transcription of target genes, most notably IL-2 (Ullman et al., 1990; Jain et al., 1995; Karin et al., 1997).

The increase in Ca^{2+} flux is also linked to cytoskeleton restructuring (Burkhardt et al., 2008). Cytoskeleton restructuring is an important part of T-cell activation, as treatment with actin polymerization inhibitors impedes T-cell/APC interaction and proper signaling (Henney and Bubbers, 1973; Holsinger et al., 1998). One of the primary events in the cytoskeleton restructuring is the polarization of the microtubular organizing complex toward the TCR/pMHC contacts (Kupfer et al., 1987). This event is crucial for the formation of the immunological synapse (IS) where TCR and costimulation signals are thought to be amplified (Cemerski et al., 2008; Yokosuka et al., 2008). Also there is a formation of the distal pole complex which may be important for shuttling away inhibitory regulatory factors from the IS (Burkhardt et al., 2008) and also may contribute to the polarization of signaling molecules responsible for memory versus effector fate decisions (Chang et al., 2007). These signaling events are summarized in **figure 1.1**.

In addition to TCR engagement and signaling there is also a need for costimulation to ensure proper T-cell activation. The best characterized of the costimulation molecules is CD28. Signaling through CD28 ultimately results in

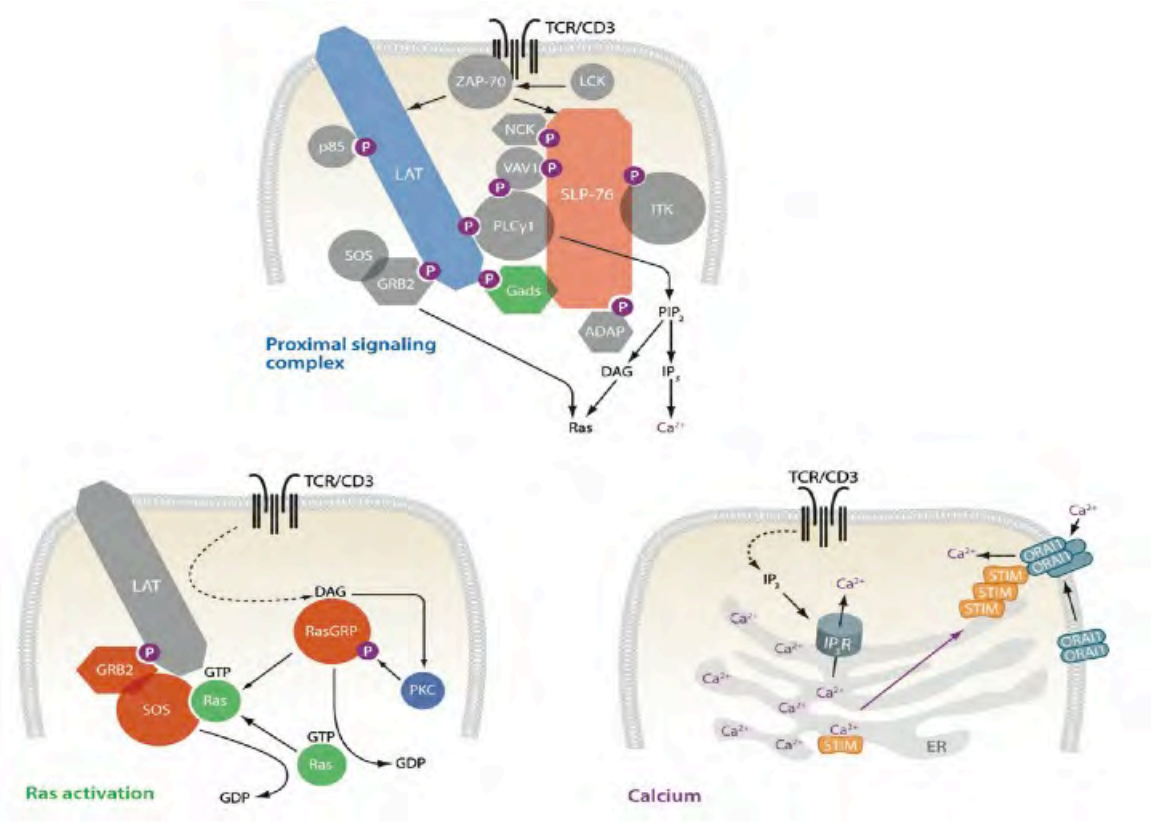

Figure 1.1 Schematic diagram of TCR signaling showing the separate arms and players involved. The first panel depicts the links between adapter protein tyrosine kinases involved in the initial signaling events and the downstream events of Ca^{++} flux and Ras activation. The bottom two panels represent events involved in Ras activation and Ca^{++} flux. Figure adapted from Smith-Garvin et. al. T-cell Activation. Annu. Rev. Immunol. 27, 591-619 (2009)

the activation of Akt which is responsible for many activities, most notably NFκB activation and IL-2 production (Acuto and Michel, 2003; Narayan et al., 2006; Qiao et al., 2008). In addition to CD28 there are other molecules involved in costimulation. Inducible costimulator (ICOS) is a molecule which is related to CD28 and performs many of the same functions, but it is not involved in IL-2 production, and it is not constitutively expressed (Hutloff et al., 1999; Coyle et al., 2000). Two additional costimulation molecules in the TNF receptor family are 41BB and OX40, which both result in enhanced Akt and NFκB activation. While CD28 and ICOS are important for activation, 41BB and OX40 seem to be important for memory formation (Watts, 2005).

In contrast to costimulation there are also important negative regulators of TCR signaling. Both CD45 and SH2 domain-containing protein tyrosine phosphatase (SHP1) are phosphatases which act to limit the phosphorylation state of the signaling complex (Hermiston et al., 2003; Stefanová et al., 2003). Two additional inhibitory molecules, cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1), are involved in dampening TCR signaling and have been shown to recruit and activate SHP1. Mice with either of these molecules deleted develop autoimmunity (Waterhouse et al., 1995; Nishimura et al., 2001). CTLA-4 has the added property of binding to the CD28 ligands CD80 and CD86 with a high affinity potentially sequestering them away from the costimulation molecule.

Up to this point I have been discussing CD4 and CD8 T-cells interchangeably. Although CD4 cells are very important players in the immune system the rest of this dissertation will focus on CD8 T-cells.

C. Acquisition of Effector Functions and Memory Formation

Naive CD8 T-cells survey the body for infection by migrating to secondary lymphoid tissues and sampling antigen presenting cells (APCs), primarily dendritic cells (DCs), for their cognate antigen. During an infection naïve CD8 T-cells first come into contact with primed antigen bearing DCs within the sub-capsular sinus or the interfollicular region of the draining lymph node (LN) (Hickman et al., 2008; John et al., 2009). Under non-inflammatory conditions antigen recognition results in anergy or peripheral tolerance, but in the context of inflammation or infection antigen recognition from an APC results in a clonal expansion and rapid acquisition of effector functions by antigen specific T-cells (Steinman et al., 2003; Masson et al., 2008; Arens and Schoenberger, 2010). After clonal expansion and at the peak of the primary CD8 T-cell response there is a heterogeneous population of short lived effector cells (SLEC) defined by $KLRG1^{+}$ $CD127^{-}$ which mostly die off during contraction, and a population of memory precursor effector cells (MPEC) defined by a $KLRG1^{-}$ $CD127^{+}$ phenotype which go on to form the memory pool (Mescher et al., 2006; Parish and Kaech, 2009). Single cell transfer studies have shown that a single naïve CD8 T-cell can differentiate into a diverse population of both effector and memory cells (Stemberger et al., 2007; Gerlach et al., 2010), although this

phenomenon has been questioned as naïve CD8 T-cells with differing amounts of interferon-γ (IFN-γ) transcript have been shown to maintain their differing transcript levels even after many rounds of division (Beuneu et al., 2010). Another observation during initial CD8 T-cell activation is the phenomenon of asymmetric cell division that ultimately results in an unbalanced segregation of protein degradation machinery and the T-box transcription factor T-bet between the two daughter cells (Chang et al., 2011). Since it is known that an increased amount of T-bet forces CD8 T-cells to be more of a SLEC (Badovinac et al., 2007a) it is thought that this asymmetric division results in a differing lineage decision (Chang et al., 2007). This idea has been questioned as CD8 T-cells with signaling deficiencies have been shown to split T-bet evenly and still develop MPECs (Smith-Garvin et al., 2010).

For a single naïve CD8 T-cell to undergo many rounds of division with one division occurring every 2-6 hours (Yoon et al., 2010) there would have to be a large need for nutrients. It has been shown that with antigen priming CD8 T-cells enhance the uptake of nutrients like glucose, amino acids, and iron and also switch from oxidative phosphorylation to aerobic glycolysis (Vander Heiden et al., 2009; Michalek and Rathmell, 2010). CD28 costimulation and activation of mammalian target of rapamycin (mTOR) is responsible for this metabolic shift (Macintyre et al., 2011). More recently it has been thought that Erk signaling may also contribute (Carr et al., 2010; Marko et al., 2010).

To attain maximal clonal expansion naïve CD8 T-cells need to receive signals 1 (TCR/pMHC engagement), 2 (CD28 costimulation), and 3 (inflammatory cytokine stimulation) (Zhang and Bevan, 2011). The inflammatory cytokine IL-12 effectively promotes effector cell differentiation through mTOR-dependent T-bet induction (Takemoto et al., 2006; Badovinac et al., 2007a; Rao et al., 2010). It was originally thought that IL-2 was the major player in clonal expansion based on in vitro studies. However, recent in vivo studies have noted that clonal expansion upon initial naïve T-cell priming is only slightly decreased in CD8 T-cells that lack the high affinity IL-2 receptor CD25, but they do show functional and phenotypic alterations (Williams et al., 2006; Bachmann et al., 2007). These CD25 KO effectors trended towards an MPEC and T-central memory (T_{cm}) phenotype with increased CD62L and CD127 and decreased KLRG1. Recently, it has become more apparent that IL-2 may be more important for terminal effector cell generation. It has been shown that activated CD8 T-cells cultured in the presence of high IL-2 concentrations acquire superior effector functions and that CD8 T-cells deficient in CD25 are defective in killing ex vivo with a decrease in granzyme B and perforin expression (Carrio et al., 2004; Pipkin et al., 2010). In general CD8 T-cells are $CD25^{high}$ during early infection, but by day 3.5-5 the effector pool starts to take on a bimodal distribution, with $CD25^{high}$ cells expressing higher KLRG1 and granzyme B and lower CD62L and IL-2 expression (Kalia et al., 2010). The end of the peak of the CD8 T-cell response brings on the contraction phase. CD8 T-cells with a SLEC

phenotype that lack CD25 show a drastic decline during contraction as compared to WT SLECs (Mitchell et al., 2010) suggesting that IL-2 is important for the maintenance of SLECs during the contraction phase. Additionally, it has been shown that low IL-2 signaling results in $CD62L^{hi}$ memory cells (Decaluwe et al., 2010). These data hint that IL-2 will be found to be important for the maintenance of $CD62L^{-}$ $CCR7^{-}$ effector memory cells (T_{EM}).

IL-2 signaling through CD25 results in the activation of the transcriptional repressor Blimp-1, the protein product encoded by the *Prdm1* gene, which in turn creates a negative feedback loop by inhibiting IL-2 production (Malek and Castro, 2010). Blimp-1 is primarily expressed by SLECs and decreases as memory is formed (Kallies et al., 2009; Rutishauser et al., 2009). Blimp-1's higher association with effector cells is consistent with the fact that there is a higher level of Blimp-1 expression during chronic infections (Shin et al., 2009). $Prdm1^{-/-}$ CD8 T-cells exhibit normal clonal expansion, but there is a defect in their effector functions and SLEC differentiation (decrease in granzyme B, perforin, and KLRG1) resulting in an increase in MPEC markers (increase of CD127, CCR7, CD62L, CD27, and IL-2) (Rutishauser et al., 2009; Zhang and Bevan, 2011). These $Prdm^{-/-}$ CD8 T-cells also have a decrease in exhaustion markers PD-1, LAG3, CD160, and 2B4 during chronic infection (Shin et al., 2009). **Figure 1.2** illustrates effector T-cell generation.

After there has been sufficient activation and expansion of the effector subsets the effectors need to leave the secondary lymphoid tissue and enter the

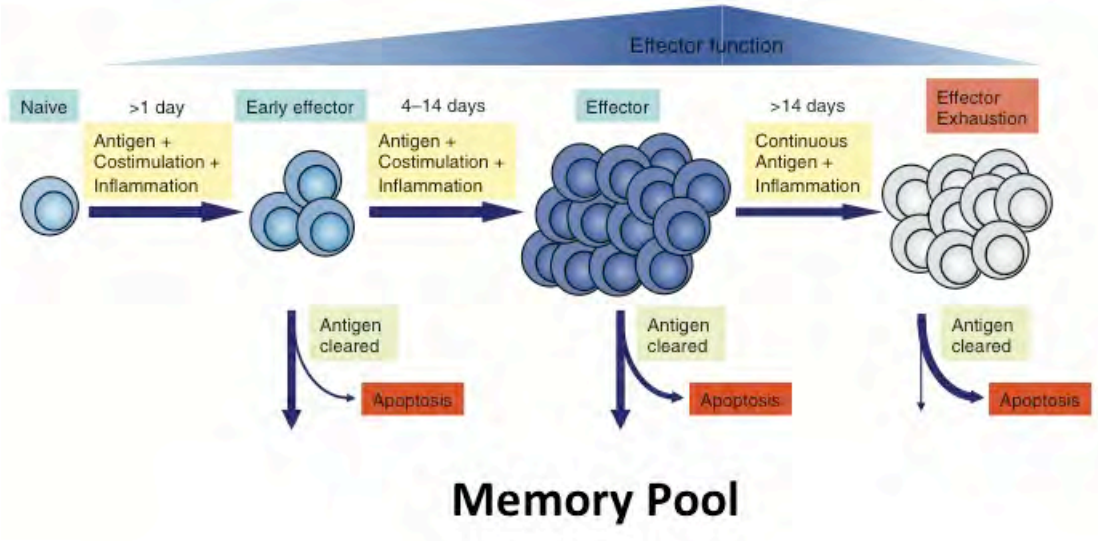

Figure 1.2. Effector T-cell generation. Naïve T-cells seeing their antigen in addition to costimulation and in an inflammatory environment differentiate into effector cells. After antigen clearance some effector cells (SLECs) undergo apoptosis while some effector cells (MPECs) go on to form the memory pool. Duration of antigen exposure has consequences on effector function as long exposure results in exhaustion with high apoptosis and little to no memory pool formation. Adapted from Arens, R & Schoenberger, S. P. Plasticity in programing of effector and memory CD8 T-cell formation. Immunol. Rev. 235, 190-205 (2010)

peripheral tissues. This entry into peripheral tissues is mediated by an up regulation of the chemokine receptor CXCR3 (Groom and Luster, 2011). It is believed that CD8 cells are recruited to the sites of infection by CXCL9 and CXCL10 expressed by epithelial cells that have been activated by CD4 effector cells which have arrived previously and secreted IFNγ in the area of infection (Bevan, 2004; Nakanishi et al., 2009). Interestingly, after arriving at the site of infection in the periphery effector CD8 T-cells continue to undergo antigen-specific interactions that drive further proliferation and cytokine release (McGill and Legge, 2009; Bedoui and Gebhardt, 2011). This phenomenon appears to require the recruitment of monocyte-derived DCs which continue to present antigen and costimulation signals (Hufford et al., 2011).

While CD8 T-cell recognition of cognate antigen and engagement of effector function at the site of infection are very important aspects of immunity, they create a highly inflammatory environment, which could cause collateral damage to surrounding tissues. Some interesting observations have been made lately that suggest that CD8 T-cells may self-regulate by producing the immune-suppressive cytokine IL-10 to help limit this collateral damage. CD8 T-cells have been shown to produce IL-10 at the site of infection, but IL-10^+ CD8 T-cells disappear after the infection is controlled (Sun et al., 2009; Palmer et al., 2010; Trandem et al., 2011; Zhang and Bevan, 2011). These IL-10^+ CD8 T-cells at the site of infection are generally better killers with higher levels of granzyme B, IFNγ, and TNFα. Additionally these IL-10^+ CD8 T-cells do not appear to be a

different lineage of effector cell, just a transient reversible state that seems to wane after antigen is cleared (Trandem et al., 2011). This IL-10 production is activated by a combination of IL-2 from CD4 effector cells and IL-27 from an innate cell, most likely a neutrophil. Blimp has also been found to be essential for IL-10 induction (Sun et al., 2011). Strong and continuous TCR/MAPK signaling may be important for this IL-10 production as many IL-10^{+} CD8 T-cells are also $CD69^{+}$, suggesting recent TCR stimulation, and this ability to produce IL-10 seems to revert after viral clearance (Trandem et al., 2011).

After viral clearance the activated effector CD8 T-cell pool begins its contraction phase and formation of a stable memory pool. Memory CD8 T-cells in general are superior to naïve CD8 T-cells. As compared to naïve CD8 T-cells, memory CD8 T-cells are maintained at a higher frequency due to their previous clonal expansion, mediated by IL-7 and IL-15 with the help of CD4 cells, and can be maintained without the need for antigen (Surh and Sprent, 2008). Memory CD8 T-cells are also rapidly activated due to an open chromatin structure from previous activation and are no longer restricted to peripheral blood and secondary lymphoid tissues as they can enter peripheral tissues (Arens and Schoenberger, 2010).

The CD8 memory cells that survive the contraction phase are divided into two general groups know as T_{EM} and T_{CM}. These two groups are based on their phenotypic expression of CD62L and CCR7, two molecules that are required for entry into the high endothelial venule of the secondary lymphoid organs (Sallusto

et al., 1999). T_{EM} cells are defined by their low expression of CD62L and CCR7 and primarily reside in the peripheral tissues while T_{CM} have high expression of these two molecules and are primarily found within, and circulating to, lymphoid organs, but both can be found in the blood and spleen (Sallusto et al., 1999; Masopust et al., 2001). These two populations of memory cells also vary in their mediation of effector functions. T_{EM} cells have the ability to produce effector functions such as cytotoxicity much more rapidly than their T_{CM} counterparts, but their ability to proliferate is markedly decreased as compared to T_{CM} cells. Conversely, T_{CM} have an increased ability to proliferate when they encounter their antigen as compared to T_{EM}. A complication to the field is the debate of whether T_{EM} and T_{CM} cells are distinct lineages or whether they can undergo inter-conversion. Independent laboratories have shown conflicting results. When a physiological level of naïve precursors are primed during an infection it appears that there is seldom T_{EM} to T_{CM} conversion, but when there are a higher level of precursors T_{EM} are able to convert to T_{CM} (Wherry et al., 2003; Badovinac et al., 2007b; Sarkar et al., 2007). Other groups also suggest that T_{CM} cells are able to convert to T_{EM} (Huster et al., 2006; Marzo et al., 2007).

Both of these populations have protective capacity when re-exposed to pathogen, but the context of the infection is what determines their protective ability. For instance, a peripheral VACV infection requires a T_{EM} population for protection while a systemic LCMV infection requires T_{CM} (Wherry et al., 2003; Bachmann et al., 2005).

D. CD8 T-cell Immunity to EBV

EBV infects 95% of the world population and is essentially ubiquitous (Henle et al., 1969). Infection occurs by salivary contact, and the virus establishes infection in cells within the oropharynx (Hislop and Sabbah, 2008). The initially infected cell type is unclear but it is probably an epithelial cell or B-cell residing in the oropharynx. Productive infection results in the establishment of latent infection and long term shedding of the virus and is established in B-cells that have been transformed by the virus. Latent viral proteins induce cell proliferation and replication of the episomal viral genome. The genome is maintained in memory B-cells, which transit between blood and oropharyngeal tissue where the virus can reactivate to the lytic cycle and resume viral shedding and infect additional B-cells (Laichalk et al., 2002).

EBV has been associated with many malignancies of B-cells and epithelial cells such as Burkett's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. EBV has also been associated with post transplant lymph-proliferative disorder (PTLD). Each of these malignancies expresses a differing repertoire of viral proteins that could be used as targets for therapeutic intervention (Hislop and Sabbah, 2008).

Primary infection can result in asymptomatic infection or AIM, which is characterized by pharyngitis and lymphadenopathy, but can range from mild symptoms to very severe disease with splenomegaly, hepatomegaly, and even result in death. Within the adolescent and young adult population 25% of primary

infections result in AIM, and, although it is rare, young children have been reported to come down with AIM (Chan et al., 2003; Crawford et al., 2006b). The marked expansion of CD8 T-cells during AIM was at one point thought to be mediated by a superantigen, as a selective expansion of Vβ6.1-3 and Vβ7 CD8 T-cells resembled that of a S. aureus enterotoxin B mediated expansions (Smith et al., 1993). This extensive Vβ restricted CD8 T-cell activation has now been shown to be primarily antigen specific, as only certain clones within the Vβ6.1-3 and Vβ7 families were expanded and shown to react to EBV antigens (Callan et al., 1996; Maini et al., 2000). Additionally, the majority of the activated CD8 T-cells were found to be antigen specific by tetramer and intra-cellular cytokine staining for IFNγ (Callan et al., 1998; Hoshino et al., 1999; Catalina et al., 2001; Hislop et al., 2002). The CD8 T-cell response reacts to both lytic and latent epitopes at the height of the disease. Initial studies limited their investigation to peptide epitopes from the EBNA3 family and LMP2 family and concluded that they accounted for the majority of the CD8 T-cell response (Steven et al., 1996), but we now know that they account for only about 3% of the response (Hislop et al., 2002). The major contributors of epitopes are the lytic proteins (Steven et al., 1997; Callan et al., 1998; Catalina et al., 2001; Hislop et al., 2002; Woodberry et al., 2005b; Cameron et al., 2006) with the immediate early proteins BZLF1 and BRLF1 encoding peptides across a range of HLA alleles. There are also strong T-cell responses to the early proteins, such as BMLF1, but only to a subset of

these viral products that are expressed early during infection (Pudney et al., 2005).

Upon resolution of the acute symptoms the expanded CD8 T-cell effector pool shrinks with the contraction of EBV-specific responses (Catalina et al., 2001; Hadinoto et al., 2007). An interesting but un-deciphered observation is the differing levels of contraction of the different antigen-specific populations within the expanded CD8 T-cell pool, with antigen-specific populations that had a high frequency completely retracting while low level populations survive (Hislop et al., 2002). For example EBV-YVL, an epitope derived from a lytic protein, is immune-dominant during primary infection yet retracts to near depletion in the resolved infection, while EBV-CLG, an epitope derived from the latent protein LMP2, is absent from primary infection yet dominates in convalescence.

The activated EBV-specific CD8 T-cells show an effector phenotype with up-regulation of CD38 and CD45RO, while being in cycle and showing cytolytic capabilities (Callan et al., 2000; Catalina et al., 2002; Dunne et al., 2002; Hislop et al., 2002; Soares et al., 2004). Again the lytic versus latent protein reactive CD8 T-cells show differing characteristics. Lytic protein reactive CD8 T-cells show a down-regulation of co-stimulation markers CD27 and CD28 while latent protein reactive CD8 T-cells show intermediate expression of these molecules (Soares et al., 2004). There is also a switch back to CD45RA expression on the lytic protein reactive CD8 T-cells while the latent protein reactive CD8 T-cells retain CD45RO expression (Catalina et al., 2002; Hislop et al., 2002). It has also

been shown that lytic reactive cells are more resistant to apoptosis, are not in cycle, and can be easily reactivated (Dunne et al., 2002). It is unclear why this occurs. It may be that the CD8 T-cells reactive against lytic epitopes are a more central memory cell and have not seen antigen since the initial infection, while the CD8 T-cells recognizing the latent epitopes are continually exposed to antigen and are in more of an effector state.

Most EBV studies are performed on samples that have come from the peripheral blood. However, lytic replication and B-cell transformation will most likely occur at the oropharyngeal epithelial surfaces or nearby lymphoid tissues (Pegtel et al., 2004). Therefore, tonsillar tissues will be particularly informative. During infection there appears to be a poor recruitment of lytic reactive cells into the tonsillar tissues whereas latent reactive cells appear to be in the tonsil at similar proportions as in the blood (Hislop et al., 2005). After resolution of infection 20% of the CD8 T-cells in the tonsil are reactive to EBV and there appears to be a 10-fold increase in latent reactive cells and a 3-fold increase in lytic reactive cells as compared to the blood, with most expressing CD103, an integrin required for entry into mucosal epithelial tissues (Hislop et al., 2005; Woodberry et al., 2005a).

The question still remains why do some people get AIM and others appear to be asymptomatic? Some findings have suggested that polymorphisms in the IL-10 promoter result in an altered outcome of disease (Helminen et al., 1999). One allele that resulted in higher levels of IL-10 production was found to correlate

with EBV-SN adults and patients with asymptomatic disease. The other allele, which results in lower IL-10 production, correlated with the occurRance of AIM and highly correlated with severe symptoms. Our group has shown that heterologous immunity and CD8 T-cell cross-reactivity between IAV and EBV may mediate increased pathology (Clute et al., 2005).

E. Characteristics of IAV infection and HLA-A2 restricted immunodominant IAV-GIL response

Most of the world's population has been infected with IAV while very young and have virus-specific memory CD8 T-cells. IAV-specific memory T-cells can comprise 0.06-1.6% of the CD8 T-cell pool found in the blood as determined by whole virus stimulation (Boon et al., 2002; He et al., 2003). After primary infection an increase in the number of CD8 T-cells can be found in the peripheral blood between days 6-14 post infection returning to baseline 21-28 days after infection (Wright, 2001). The CD8 T-cell response is known to target all the viral proteins, but the immunodominant response is mediated against matrix 1 (M1), the most abundant viral protein and most highly conserved (Gotch et al., 1987; Man et al., 1995; Jameson et al., 1998). All individuals who are HLA-A2 positive present and mount an immunodominant response to IAV-$M1_{58-66}$ (IAV-GIL) (Gotch et al., 1987; Bednarek et al., 1991; Lehner et al., 1995; Lalvani et al., 1997; Lawson et al., 2001; Pittet et al., 2001). The frequency of IAV-GIL restricted cells averages out to about 0.1% of the CD8 T-cells in the blood (Lehner et al., 1995; Lalvani et al., 1997; Jameson et al., 1998; Pittet et al.,

2001). By making point mutations in the IAV-GIL peptide it was determined that the central portion of the peptide was important for its recognition by IAV-GIL specific T-cells. When the crystal structure of IAV-GIL bound to HLA-A2 was solved it was evident that IAV-GIL was unusual, as the amino acid (aa) side chains did not face the TCR with the AA backbone facing the solvent (Madden et al., 1993). Perhaps this structure would require the TCR to interact with more of the MHC rather then the generic solvent exposed backbone. It is possible that this unique structure is responsible for the IAV-GIL TCR repertoire being restricted primarily by Vβ 19 Jβ 2.7 and Vα 27 Jα 42 while having an x-arginine-serine-x (xRSx) CDR3β motif and a CDR3α that consists of multiple glycines (Moss et al., 1991; Lehner et al., 1995; Naumov et al., 1998; Lawson et al., 2001). The IAV-GIL/HLA-A2/TCR crystal structure revealed that the TCR bound to the pMHC in a unique fashion (Stewart-Jones et al., 2003). As opposed to the previously solved crystal structures where TCR/pMHC interactions show diagonal binding of the TCR, the IAV-GIL reactive TCR binds perpendicular to the pMHC. Additionally, the IAV-GIL specific CD8 TCR was found to predominantly use the CDR3β to cover the peptide rather than a 50:50 split with the CDR3α. Despite the dominant selection for Vβ 19 and Vα 27 the repertoire can be described as polyclonal. Many Vβ 19 clones exist based on nucleotide sequences that encode the xRSx motif (Naumov et al., 2006). There are also clones that express different Jβ families resulting in variability in the length of the CDR3 loop (Naumov et al., 2006). By maintaining a large clonal distribution the

immune system is better able to adapt to any changes in the properties of the ligand over time and thus decrease the chances of developing viral escape mutants (Naumov et al., 2006).

2. Heterologous T-cell Immunity

A. Definition of Heterologous Immunity

Heterologous immunity can be broadly defined as the impact of immunity from a previous infection on the outcome of a subsequent unrelated infection. Thus each new infection we experience is potentially influenced by our past histories of infection. The concept was first described to help explain the differences observed in protective immunity and immunopathology in C57Bl/6 mice that had been immunized with one virus and later challenged with another virus anywhere from 6 weeks to 2 years later (Selin et al., 1998; Chen et al., 2001; 2003). This heterologous immunity was ultimately found to be mediated by cross-reactive T-cells (Brehm et al., 2002; Kim et al., 2005; Cornberg et al., 2010a; Nie et al., 2010)(Chen et. al. in press Plos Pathogen). However, there may be other mechanisms contributing to heterologous immunity such as macrophage activation by bacterial infection creating a short lived protection from other bacteria (Mackaness, 1964). There is also the concept of original antigenic sin, which suggests that previous exposure to one strain of IAV diverts the antibody response after exposure to a second IAV strain to epitopes that are shared between the two strains (Fazekas de St Groth and Webster, 1966).

Heterologous immunity may not be as effective as homologous immunity, but it can help to facilitate a much attenuated infection as measured by decreased viral loads and increased survival rates from lethal dose (Selin et al., 1998; Chen et al., 2001; Nie et al., 2009). Using an adoptive transfer of immune splenocytes it was originally observed that both CD4 and CD8 T-cells were required for mediating protective immunity in LCMV immune mice challenged with VACV (Selin et al., 1998). However, a selective expansion of some, but not all, LCMV epitope-specific CD8 T-cells during a vaccinia virus infection suggested that cross-reactive CD8 T-cell responses were responsible (Chen et al., 2001; Kim et al., 2002). It was later found that these cells are responsible for mediating protective heterologous immunity by the reduction of viral titers (Cornberg et al., 2010a).

Heterologous immunity can be beneficial by mediating partial protective immunity, but it can also lead to detrimental immunity that can manifest itself as greatly enhanced immunopathology, higher viral loads, or increased morbidity and mortality (Selin et al., 1998; Walzl, 2000; Chen et al., 2003). These differential effects likely occur through multiple different mechanisms. One possible mechanism is the activation of lower avidity cross-reactive memory CD8 T-cells which may hamper the development of a new cognate high avidity CD8 T-cell response from naïve precursors (Selin et al., 2011). A high avidity agonist (cognate peptide) TCR interaction induces full activation potential of a CD8 T-cell including proliferation, cytokine production, and cytotoxicity. A low avidity partial

agonist (cross-reactive peptide) TCR interaction requires approximately 10-100 fold more ligand to induce similar effector functions (Sloan-Lancaster and Allen, 1996; Hemmer et al., 1998; Kersh et al., 1998; Ding et al., 1999; Zehn et al., 2009). Low avidity TCR interactions may also produce different cytokines, altered cytokine amounts, or could be less cytolytic or proliferative (Selin et al., 2011). Recent studies have found that CD8 T-cells responding to low affinity partial agonist peptides initially undergo clonal expansion at the same rate as CD8 T-cells undergoing activation by agonist interactions; however, the CD8 T-cells responding to the partial agonist leave the lymph node sooner and lose sustained expansion, causing them to eventually be out competed by agonist reactive CD8 T-cells (Zehn et al., 2009). These differences in reactivity to agonist and partial agonist peptide are most likely related to differences in TCR signal strength perhaps mediated by ITAM usage. Developmental studies have shown that negative selection is influenced by the number and specificity of the TCR-CD3 ITAMS activation state, suggesting that the TCR is capable of scalable signaling (Holst et al., 2008).

B. Examples of heterologous immunity in a murine infection model

One of the first models developed that identified heterologous immunity was LCMV-immune C57Bl/6 mice challenged with VACV. From this experimental induction of heterologous immunity there was an observation of both partial immune protection, as measured by a 2-log reduction in viral titer, and also some induction of immunopathology, as exhibited by paniculitis of the

visceral fat (Yang et al., 1985; Selin et al., 1998). VACV was shown to activate LCMV-specific memory CD8 T-cells specific to the LCMV epitope NP_{205} (Chen et al., 2001). Based on these results the VACV proteome was screened for sequence homology to LCMV $NP_{205,}$ and peptide sequences from two viral proteins, e7r and a11r, were found (Welsh et al., 2004; Cornberg et al., 2006b; 2010a). Both VACV epitope-specific responses provide protection to VACV infection, but only one, a11r, was cross-reactive with LCMV NP_{205} (Cornberg et al., 2006b; Moutaftsi et al., 2009). Interestingly, the expansion of LCMV NP_{205} specific memory CD8 T-cells cells due to VACV infection occurred only half of the time, while the other half of the time other LCMV-specific responses were expanding, including GP_{34} and GP_{118} which are K^b restricted (Kim et al., 2005; Cornberg et al., 2010a). In fact, there are multiple cross-reactive CD8 T cell responses in LCMV-immune mice infected with VACV. VACV infection sometimes expands LCMV NP_{205}-specific T cells, but other times LCMV GP_{34}- or GP_{118}-specific T cells expand upon adoptive transfer of LCMV-immune splenocytes into naïve mice (Kim et al., 2005). This variability in responses is not due to random stochastic events but instead reflects the private specificity of the LCMV-immune T cell repertoire in individual mice. This was demonstrated by adoptive transfer of LCMV immune splenocytes into 3 recipients from the same donor, which was shown to generate the same specificity of outgrowth of LCMV epitope-specific T cells. However, recipients of splenocytes from a different donor demonstrate a different specificity. These results indicate that the private

specificities of the LCMV memory T-cell population dictate which cross-reactive epitope would be recognized.

This cross-reactivity between LCMV and VACV has proven to provide partial protective immunity. Interestingly, many immunizing viruses provide partial protective immunity to VACV infection including LCMV, PV, IAV, and MCMV, and also the bacterium BCG (Selin et al., 1998; Chen et al., 2001; 2003; Mathurin et al., 2009). It is proposed that the cross-reactive T-cells that are mediating this protection. CD8 T-cell lines generated from LCMV-immune mice and stimulated with a11r peptide were transferred into naïve mice and challenged with VACV. Transfer of cell lines which demonstrated the most functional cross-reactive response, as measured by EC50, a measure of functional avidity, to both LCMV and VACV epitopes, resulted in the greatest level of protective immunity based on VACV titers (Cornberg et al., 2010a).

One aspect left to determine is what type of cross-reactive response leads to beneficial protective versus harmful detrimental heterologous immunity. One potential mediator of these differences is differential production of cytokines to the cross-reactive ligands. It has been shown that IFNγ can be responsible for both partial protection and immunopathology (Selin et al., 1998; Chen et al., 2001). Additionally, it is known that TNFα is responsible for mediating paniculitis but not partial protection in a LCMV-immune VACV infection model (Selin et al., 2011).

In order to examine a more natural route of infection an intra-nasal infection model was used. Mice were immunized with LCMV and subsequently challenged with VACV. This model also showed partial protection by reduction in viral loads and altered immunopathology in the lung (Chen et al., 2001; 2003). Again, pathologies did vary from mouse to mouse consistent with the idea of private specificity.

A second model of heterologous immunity in the murine system is that of LCMV immune mice challenged with the distantly related PV. This model preferentially shows partial protection, as LCMV immune mice present with a log lower viral titer without significant immune-pathology (Selin et al., 1998; Brehm et al., 2002). The heterologous immunity associated with this model has been shown to be mediated by cross-reactive CD8 T-cells, more specifically LCMV NP_{205} reactive cells recognizing PV NP_{205}, as immunization with a LCMV NP_{205} mutant virus did not mediate protection (Cornberg et al., 2006a)(Chen et. al. 2012).

The two NP_{205} cross-reactive peptides have 6 of 8 amino acids in common and only differ in the MHC anchor site (Brehm et al., 2002). As seen in the overlaid crystal structure the overall conformations of the LCMV- NP_{205} and PV-NP_{205} peptides bound to $H2K^b$ are similar. The respective $H2K^b$ binding clefts adopt similar conformations with the largest difference in a specific region of the α2-helix. The presence of the tyrosine hydroxyl group in position 5 of the LCMV peptide, instead of the phenylalanine found in the PV peptide, accounts for this

perturbation of the α2-helix (Chen et. al. in press Plos Pathogens). Most of the CD8 T-cells that are generated to one NP_{205} epitope will recognize the other but with differing binding affinities as suggested by differing tetramer staining patterns (Cornberg et al., 2006a). Surprisingly, the TCR repertoire usage between these two epitope specific populations is quite different. LCMV NP_{205} almost exclusively uses Vβ 16 while PV NP_{205} uses Vβ 5 and Vβ 16, and the Vβ CDR3 region is very different from that usually seen in LCMV NP_{205} response (Cornberg et al., 2006a). The perturbation of the α2-helix most likely accounts for the differing TCR repertoire. The TCR repertoire that is available for cross-reactive expansions is established during the first infection and is unique between individuals and accounts for private specificity (Cornberg et al., 2006a; Selin et al., 2011). During the second infection a narrow subset of the antigen specific CD8 T-cell memory pool is stimulated to proliferate when exposed to the cross-reactive antigen (Haanen et al., 1999; Cornberg et al., 2006a). Infections with these two heterologous viruses, encoding very similar cross-reactive epitopes, induce a high frequency of an oligo-clonal set of T-cell clones that dominate the CD8 T-cell response. This results in a narrowing of the LCMV/PV NP_{205} restricted TCR repertoire (Cornberg et al., 2006a; Selin et al., 2011).

Heterologous immunity and CD8 T-cell cross-reactivity are frequently not reciprocal. For instance, mice that have been immunized with VACV and then re-challenged with LCMV, PV, IAV, or MCMV demonstrate no protective

immunity or increased immunopathology (Selin et al., 1998). This may be due to differences in the private specificity of the TCR repertoire.

These data taken together begin to show a pattern or network of cross-reactive T-cell responses to the epitopes encoded by LCMV, PV, and VACV as shown in **figure 1.3**. It is these networks of cross-reactivities that are the underlying mediators of heterologous immunity. Additionally, it is also the private specificity of the response to this network of cross-reactivities that determines one's fate when encountering a new infection (Kim et al., 2005; Cornberg et al., 2010b).

The final murine model of heterologous immunity I will discuss is that of a respiratory intra-nasal (i.n.) model of IAV-immune mice challenged with LCMV. In this model there are increased virus titers and greatly enhanced pathology. This infection is characterized by massive lung pathology demonstrating consolidating mononuclear pneumonia and bronchiolization instead of a mild lymphocytic pneumonitis commonly seen during intranasal LCMV infection (Chen et al., 2003). It was found that the increased viral titers were dependent on IAV-specific memory CD4 T-cells and the increased immunopathology was dependent on IAV-specific memory CD8 T-cells. IAV-$PB1_{703}$- and -PA_{224} specific cells were cross-reactive with LCMV-GP_{34} and –GP_{276} (Wlodarczyk et. Al. unpublished data). These two populations were found to be the mediators of the pathology. IAV strains with these epitopes mutated, peptide tolerization of IAV-$PB1_{703}$- and -PA_{224} specific memory cells (Redmond et al., 2005), or anti-IFNγ

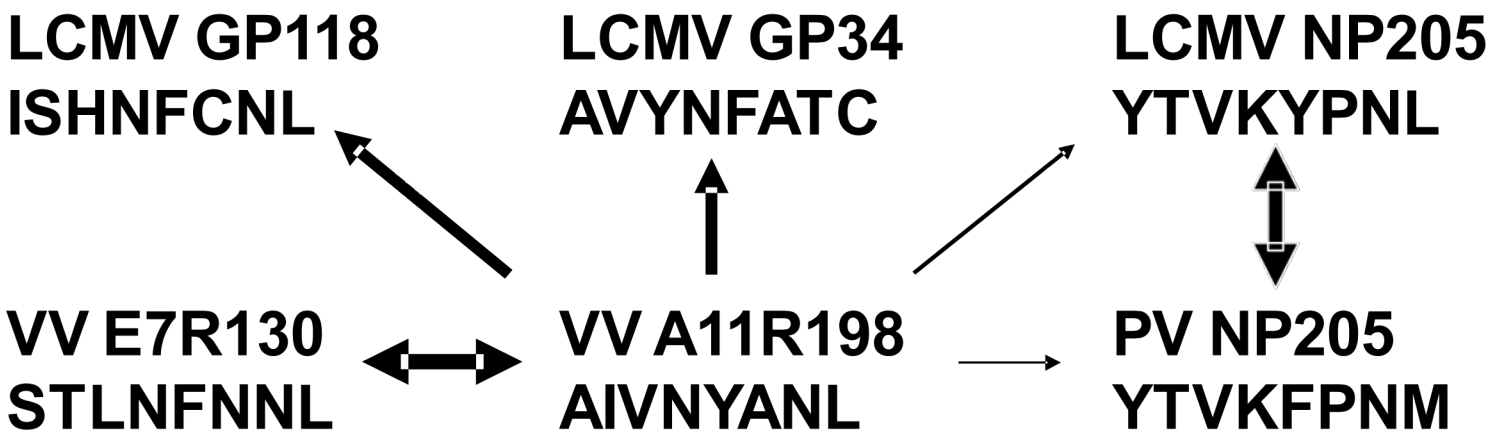

Figure 1.3. The network of cross-reactivity seen in the mouse model. Summary of the cross-reactive networks between epitope specific $H2K^b$ restricted responses found within C57/Bl6 mice infected with LCMV, VACV, or PV. Direction of arrows indicates a memory population capable of responding to peptide. Arrow thickness is a relative indication of the frequency of detection of the response.

therapies alleviated the enhanced pathologies. These two IAV epitopes, IAV-$PB1_{703}$- and -$PA_{224,}$ could potentially be thought of as “pathogenic” epitopes. These results strongly support the usefulness of studying heterologous immunity in order to develop new therapies to prevent viral immunopathogeniesis and improve vaccine design.

C. Heterologous immunity during human infection

Ultimately we as scientists want our work to translate to a better understanding of human disease to help advance human health. However, studying disease directly in humans can be very difficult and at times frustrating, due to ethical considerations and patient non-compliance.

The study of protective beneficial heterologous immunity in humans can be particularly difficult, as a protective effect would more than likely go unnoticed. However, the study of enhanced immunopathology during human infection is easier than studying protective immunity because of its overt nature. If cross-reactive T cell responses are harmful to the host, their influence on the outcome of an infection may become more evident, perhaps resulting in an altered disease state. Some infections have very different pathological outcomes perhaps being mediated by differences in the private specificity of their TCR repertoire. These infections provide strong hints as to where one could investigate the roles of heterologous immunity. Some specific examples include viral infections such as EBV with the associated disease AIM where it is more severe in young adults than in younger children. Another example is the

variation in pathology within HCV infected patients. Some patients that acquire HCV infection are capable of clearing the infection. Within others the disease progresses to a chronic state where the virus is never cleared, and pathologies range from asymptomatic to fulminant necrotizing hepatitis and even death. Another example of an infectious disease with differing symptoms is dengue virus (DENV) infection. Interestingly, all of these infections have had CD8 T-cell cross-reactive responses associated with their course of disease, as I will discuss below.

DENV is divided up into four distinct serotypes (DENV 1-4) (Halstead, 1989; Morens, 1994) which serologically cross-react but do not provide neutralizing antibody. DENV has also been shown to encode variable CD8 T-cell epitopes that cross-react between the sero-types (Spaulding et al., 1999; Zivny et al., 1999; Mongkolsapaya et al., 2003; Bashyam et al., 2006; Beaumier et al., 2008). DENV infection can cause a wide array of disease presentations ranging from asymptomatic upward to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. The more severe forms of the disease occur when an individual is first infected with one serotype and then later infected with an alternate serotype. This phenomenon was originally thought to be mediated by non-neutralizing antibodies binding virus and delivering the virus to Fc receptor bearing cells, a process known as antibody-dependent enhancement (Halstead, 1989), but this may be only a portion of the phenomenon. It is believed that heterologous immunity and cross-reactive CD8 T-cells are contributing to this

pathology as well (Mathew et al., 1998). It has been found that cross-reactive memory CD8 T-cells responding to the second DENV infection have a higher avidity to the primary antigen than the secondary antigen. These cross-reactive cells expand at the expense of the secondary cognate epitope-reactive cells and mediating pathology (Mongkolsapaya et al., 2003; Friberg et al., 2011). One of the important implications of these studies is that there may be a problem designing a pan-dengue serotype vaccine since primary DENV infection ultimately results in an increased possibility for pathogenesis upon subsequent cross-serotype infection.

A second example of CD8 T-cell cross-reactivity comes from the field of hepatitis C virus. Many HCV sero-negative individuals have reactivity against the HCV HLA-A2 restricted epitope HCV-$NS3_{1073}$ and a portion of the CD8 T-cells reactive to this epitope cross-react with an IAV-epitope, IAV-NA_{231} (Wedemeyer et al., 2001). While most HCV patients with mild disease have a diverse CD8 T-cell pool recognizing epitopes across the HCV proteome, two patients that developed a CD8 T-cell response that was highly focused to only this cross-reactive HCV-$NS3_{1073}$ epitope developed a rare fulminant necrotizing hepatitis upon HCV infection (Urbani et al., 2005). This example of human heterologous immunity and cross-reactive CD8 T-cells is similar to the murine system in that there appears to be private specificity as only a portion of patients developed this focused detrimental cross-reactive response and adverse immunopathology.

Heterologous immunity also may play a role during EBV infection. EBV is a gamma herpes virus that infects up to 95% of the population. It falls into the category of viral infections that are more severe in young adults, who theoretically have more experienced large complex T-cell memory populations due to previous viral exposures, than in young children. EBV infection in younger children usually results in an asymptomatic response. Infection in young adults more often results in acute infectious mononucleosis (AIM), which can vary greatly in severity from mild pharyngitis and lymphadenopathy, to severe hepatomegaly, splenomegaly, and even death. The main characteristic of AIM is an acute lymphocytosis consisting primarily of activated CD8 T-cells. These cells are often referred to as atypical lymphocytes, which are cytotoxic lymphocytes (CTLs) responding to infected B-cells and epithelial cells of the oropharynx. There is no evidence of increased viral load in AIM patients as compared to asymptomatic patients (Silins et al., 2001). Therefore the major pathological feature of AIM is a massive CD8 T-cell response. Our group has shown that HLA-A2 positive AIM patients have an increase in IAV-GIL reactive CD8 T-cells and that these cells are cross-reactive with the EBV-GLC peptide in some individuals (Clute et al., 2005). Recently, our group has found that IAV-GIL specific cells can also be cross-reactive to another lytic EBV epitope EBV-BRLF1 (EBV-YVL) and, interestingly, that EBV-YVL and EBV-GLC responses also show intra-viral cross-reactivity (Cornberg et al., 2010b). Additionally, we have observed a direct correlation between the frequency of cells cross-reactive

between IAV-GIL/EBV-GLC and the severity of AIM, while EBV viral load does not correlate (Aslan et. al. unpublished data) suggesting that this particular cross-reactive response is important in mediating the severity of AIM (**Fig 1.4**). IAV-GIL and EBV-GLC have only three of nine amino acids in common, and IAV-GIL and EBV-YVL share only one amino acid. Interestingly, the TCR repertoire of these cross-reactive cells in AIM patients is devoid of highly dominant clonotypes as compared to the non-cross-reactive repertoire and is in fact rather broad (Clute et al., 2010). This may be explained by less similar epitopes not having any high affinity clones which might dominate and narrow the response as appears to happen with more similar cross-reactive epitopes as described earlier (Cornberg et al., 2006a).

In addition to the cross-reactive responses seen between IAV-GIL and EBV-GLC and –YVL we have noted other cross-reactive responses between these two viruses that also occur, but less frequently. These include inter-viral cross-reactive responses between EBV-GLC and influenza B virus (IBV) IBV-NP_{85} (IBV-KLG) and also between IAV-GIL and EBV-$EBNA3a_{596}$ (EBV-SVR), and also intra-viral cross-reactive responses between EBV-GLC and EBV-YVL and EBV-GLC also reacting to EBV-$LMP2_{329}$ (EBV-LLW). Again these cross-reactive patterns begin to form networks of cross-reactivity between the antigen specific responses, and each individual will have a different cross-reactive pattern, based on the private specificity of their TCR repertoire. Thus the private specificity of each individual's TCR repertoire will determine their reaction to

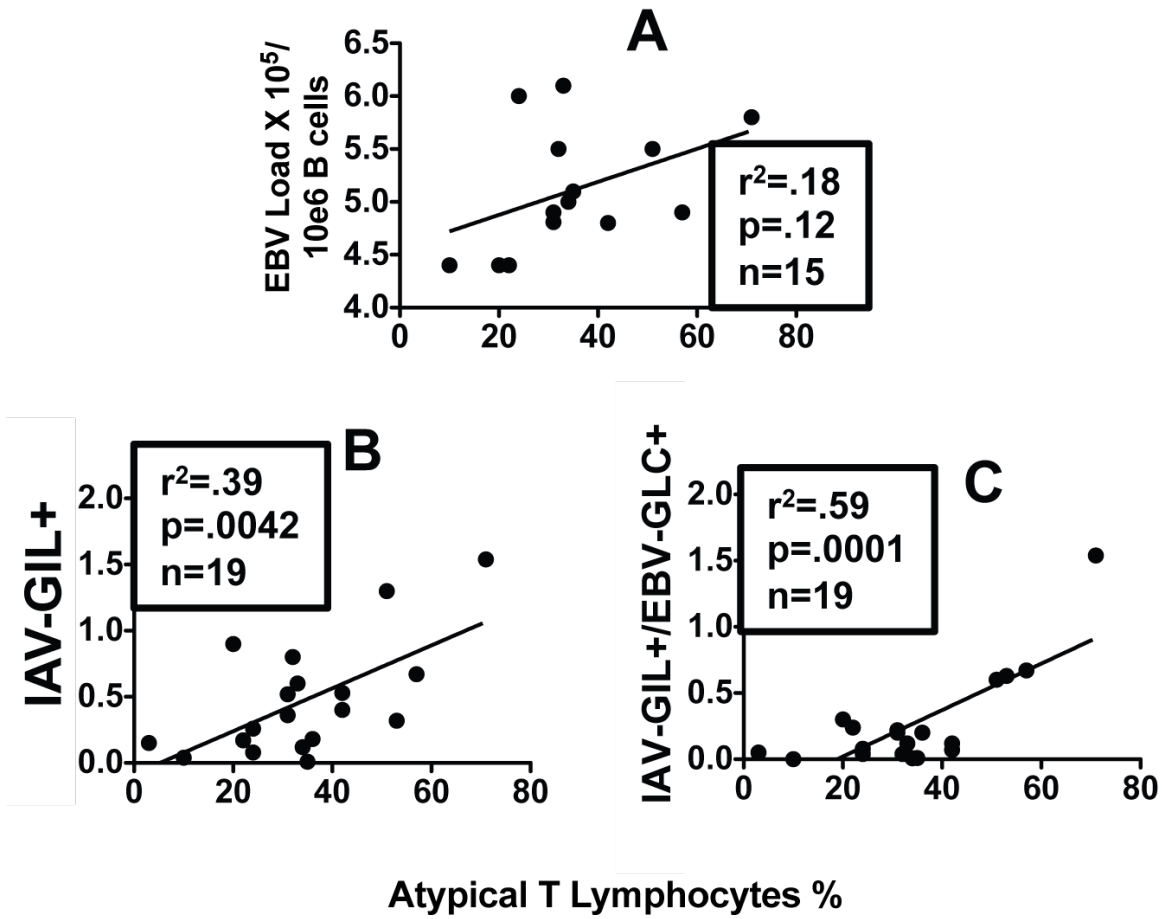

Figure 1.4. Correlations of viral load and T-cell responses to % atypical lymphocytes. Atypical lymphocytes are a useful measure of disease severity in EBV infection as we found they directly correlated with severity of adenopathy and inversely correlated with the CD4:CD8 ratio. These data show a significant positive correlation between IAV-GIL specific cells and IAV-GIL/EBV-GLC cross-reactive cells as measured by tetramer staining directly ex vivo in the peripheral blood with % atypical lymphocytes while viral load does not correlate.

these antigens and will ultimately decide the outcome of the infection (Kim et al., 2005; Cornberg et al., 2010b) (**Fig 1.5**).

These data taken together suggest that heterologous immunity and cross-reactive CD8 T-cells may be a common event during human infections. Additionally, these data further help explain the variability in disease outcome that is seen between individuals infected with the same pathogen often previously attributed to host genetics, pathogen dose and physiological conditions of the host.

On the other hand studying protective beneficial heterologous immunity in humans can be particularly difficult as a protective effect would more than likely go unnoticed. To study this one must rely on epidemiological studies. One such study found that BCG or live measles vaccine offered decreased mortality to rotaviral diarrhea and other infectious pneumonias in African children (Aaby et al., 1995; Stensballe et al., 2005; Farrington et al., 2009). Interestingly, subsequent vaccination with killed diphtheria, pertussis, and tetanus vaccine (DPT) can reverse these beneficial effects and also increase mortality (Aaby et al., 2007; Benn et al., 2009).

An additional case where heterologous immunity may be mediating protection is the case of Kenyan female sex workers who are consistently HIV sero-negative despite the fact that they are continuously exposed to the virus (Fowke et al., 1996). Intriguingly, this protection waned if the individuals left the trade (Jennes et al., 2006). This may indicate that constant exposure may be a

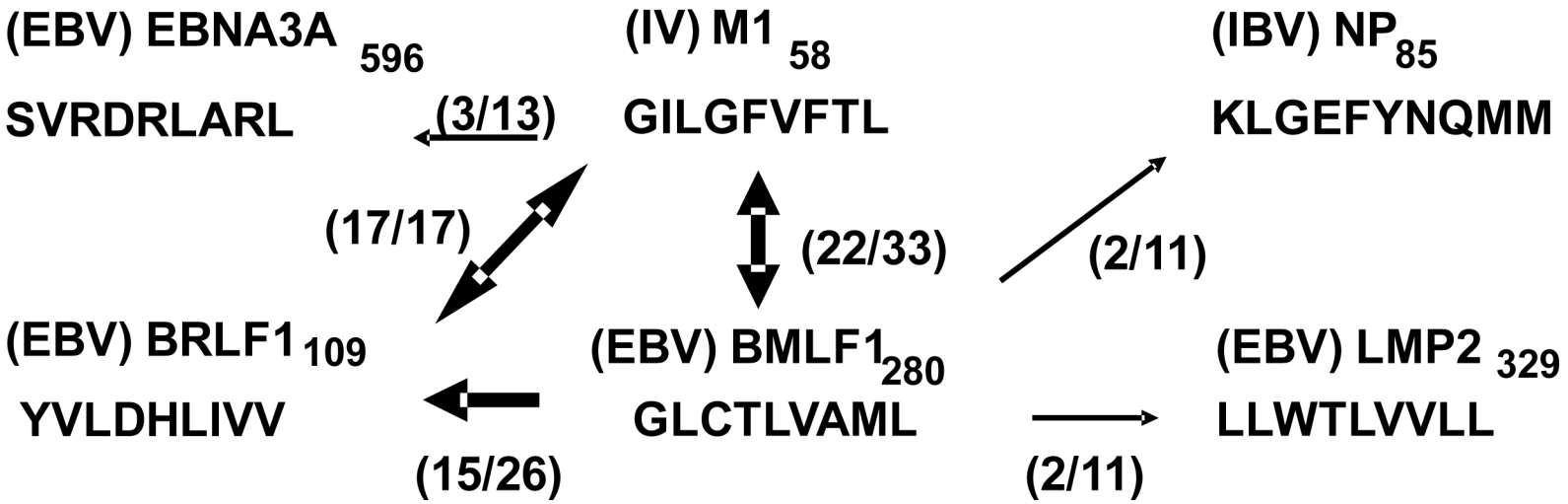

Figure 1.5. The network of cross-reactivity seen in EBV infection. Summary of the cross-reactive networks seen within HLA-A2+ individuals between EBV and IAV. Direction of arrow indicates a memory population capable of responding to the epitope. Arrow thickness is a relative indicator of detection frequency. Numbers in brackets indicate the number of times the cross-reactive response was detected and the number of patients that were examined.

protective factor. Interestingly, there has been a report of cross-reactive CD8 T-cells amongst HIV sero-negative individuals between IAV-GIL (IAV-$M1_{58-66}$) and HIV-gp17-GAG_{77-85} (Acierno et al., 2003). Perhaps this is what is mediating protection from HIV infection.

D. The use of humanized mice to study human infection

Due to the limitations in conducting research on humans there is a need for the development of an animal model, which can mimic human immunity. The model of humanized mice may be able to fill this gap. Humanized mice are mice that have either had human genetic elements transgenicly inserted into their genome or mice that have received an implantation of human tissues. The first model of human cell implantation in mice came in 1983 with the discovery of a severe combined immunodeficiency (SCID) mutation in CB17 mice. Human hematopoietic cells were able to engraft in these mice, but engraftment levels were low primarily because of high levels of NK cells (Shultz et al., 2011). Later, SCID mice were crossed to non-obese diabetic (NOD) mice and these mice exhibited enhanced engraftment due to decreased levels of NK cells (Shultz et al., 2007; 2011). The next major step forward was the cross of NOD/SCID mice to IL2 receptor common gamma chain knockout (*IL2rγ*^{null}) mice to create NOD/SCID *IL2rγ*^{null} (NSG) mice. These mice showed even greater engraftment of human hematopoietic cells and also development of a fully functional human immune system when engrafted with hematopoietic stem cells (HSC) (Shultz et al., 2007). While there are other base models of humanized mice, this model

seems to provide the best engraftment. Importantly, there are even more improvements being made onto this base model. Two in particular are the development of mice expressing the human MHC molecule HLA-A2 and a model that incorporates fetal human tissues. One of the caveats of the NSG model is the lack of a human MHC molecule for CD8 T-cell selection. By crossing the NSG mice to an HLA-A2 transgenic mouse CD8 T-cell development is improved. **Figure 1.6** summarizes the history of the development of the humanized mouse model. Even more critical for the proper development of T-cells is the presence of human thymic tissue. In the BLT (bone marrow, liver, and thymus) mouse model NSG-HLA-A2 transgenic mice have had fetal organs placed under the kidney capsule followed by injection of HSCs. A major advantage to this last humanized mouse model is the development of a mucosal immune system and an extremely high level of engraftment. These BLT mice that show marked increases in hematopoietic engraftment and increased immune function are a good model to study human viral infections and in fact have been used to examine HIV, DENV, malaria, EBV, and Salmonella infections (Shultz et al., 2007; Jaiswal et al., 2009; Libby et al., 2010; Shultz et al., 2010). As a part of my thesis work I will be testing the resilience of these humanized mouse models upon infection with EBV and IAV to determine whether they are feasible to study human heterologous immunity.

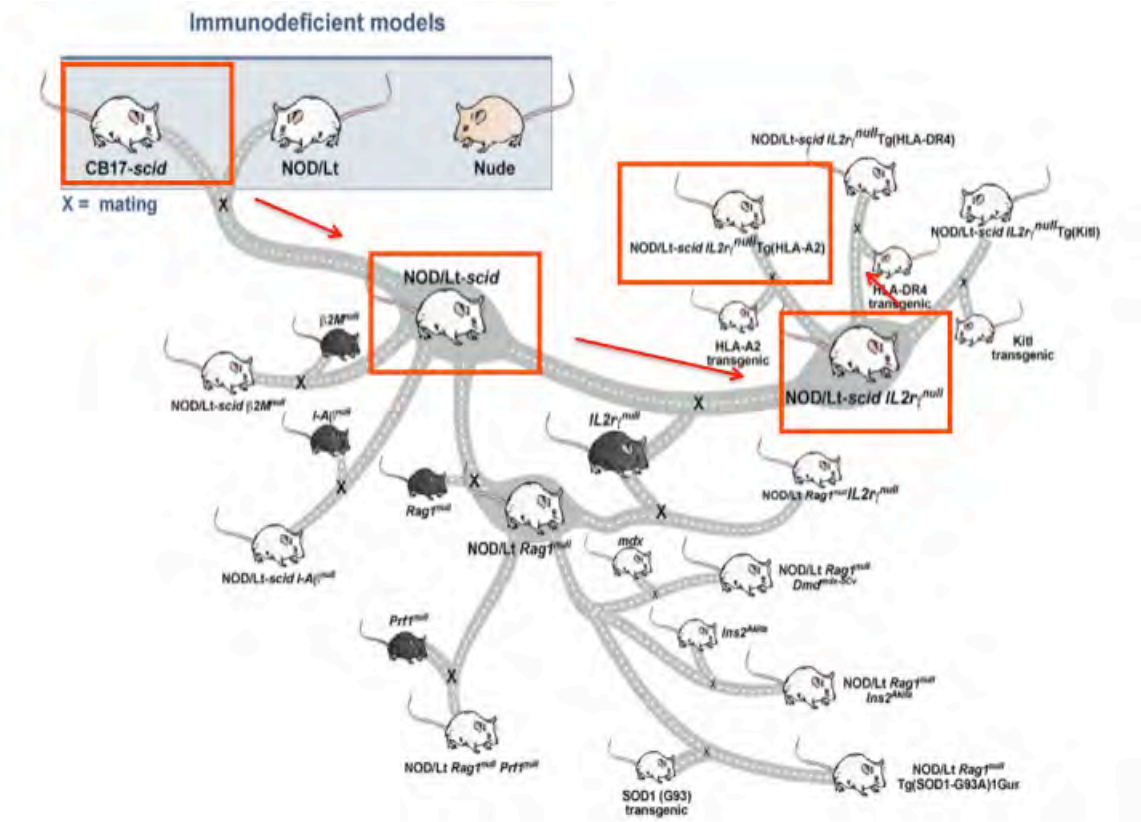

Figure 1.6. History and family tree of the development of the humanized mouse models. Highlighted mice and arrows show the lineage of the models used in this study. The initial SCID mouse was crossed to the NOD mouse to create NOD/SCID. NOD/SCID then crossed to IL2rγ$^{-/-}$ to create the NSG mouse. NSG mice were then wascrossed to HLA-A2 transgenic mice. Figure adapted by Mike Brehm and Levi Watkin from Shultz et. al. Nature Reviews Immunology 7:118-130, 2007

E. Thesis Objectives

The gamma-herpes virus EBV infects ~95% of individuals globally, establishing a life-long infection (Henle et al., 1969). However, it is unknown why 5-10% of adults remain EBV-SN, despite the fact that the virus infects the vast majority of the population and is actively shed at high titers even during chronic infection (Hadinoto et al., 2009). Additionally, we have previously identified cross-reactive responses from IAV-GIL memory CD8 T-cells directed against two EBV epitopes EBV-GLC and EBV-YVL. **We hypothesize that heterologous immunity mediated by IAV-GIL specific memory CD8 T-cells cross-reactive against the EBV lytic epitopes EBV-GLC and EBV-YVL can protect EBV-SN adults from the establishment of productive EBV infection and sero-conversion.** This thesis will be presented in two main parts:

Chapter III: Demonstration and characterization of IAV-GIL memory CD8 T-cells cross-reactive to EBV lytic epitopes EBV-GLC and EBV-YVL in EBV-SN adults.

- **a.** What are the frequencies of the IAV-GIL cross-reactive population in the EBV-SN adults and how do they differ from EBV-SP adults?
- **b.** What are the effector functions that are responsible for mediating this protection?
- **c.** Are there any unique features of the TCR repertoire that could be mediating this protection?

Chapter IV: Characterization of viral infection in humanized mice for the establishment of a murine model to test protection from EBV infection mediated by heterologous immunity.

a. Can these mice become infected with EBV and IAV?

b. Can these mice mount an efficient immune response comparable to a human infection?

Chapter II: Materials and Methods

Heterologous Immunity Within EBV-SN Adults

EBV-SN Donors: The 3 male and 2 female IAV-immune, EBV-SN patients ages 32-55 were consented volunteers. Positive staining with HLA-A2-tetramers loaded with IAV-GIL was used as an indication that these individuals had been exposed to influenza A virus in the past. For this study, a 50 mL blood sample was provided from patients. All 5 patients were seronegative for IgG antibodies to EBV EBNA and EBV viral capsid antigen. EBV genomic DNA was not detected in peripheral blood using quantitative PCR performed as previously described (Hislop et al., 2005). The IRB committee at UMass Medical School approved this study.

Blood preparation and bulk T-cell culture. PBMC were isolated using Ficoll-paque plus (Amersham Bioscience, Uppsala, Sweden). CD8 cells were isolated using the Miltenyi Biotech (Auburn, CA) MACS system and were cultured using our published protocol (Clute et al., 2005). Briefly, CD8 lymphocytes were plated at a 5:1 ratio with 1mM peptide-pulsed irradiated T2 cells (ATCC #CRL-1992), which were washed before combining with CD8 T-cells. They were used to re-stimulate the T-cell lines weekly. Cell lines were given fresh media 3-4 days after stimulation.

HLA-A2-restricted peptides and MHC-Class I tetramers and MHC-Class I pentamers. The following peptides were synthesized to >90% purity by Biosource (Camarillo, CA): EBV-BMLF1$_{280\text{-}288}$ (**GLC**TLVAML),

EBV-BRLF1$_{109-117}$ (**YVL**DHLIVV), EBV-EBNA3A$_{596-604}$ (**SVR**DRLARL), EBV-LMP2$_{426-434}$ (**CLG**GLLTMV), IAV-M1$_{58-66}$ (**GIL**GFVFTL), IBV-NP$_{85-94}$ (**KLG**EFYNQMM), measles virus M$_{50-58}$ (**FMY**MSLLGV), and human endogenous tyrosinase$_{369-377}$ (**YMN**GTMSQV). A detailed description of the protocol used by the tetramer facility at UMass Medical School has been previously published (Catalina et al., 2001). Tetramers, a tetrameric complex of pMHC molecules bound to streptiavidin, were assembled using the above peptide sequences and were conjugated to APC (Caltag, Burlingame, CA). MHC-Class I pentamers, a pentameric complex of pMHC molecules, were purchased from Proimmune (Oxford, UK). Both tetramers and pentamers are reagents capable of detecting antigen specific CD8 T-cell populations.

Extracellular/Intracellular staining and cell sorting. Cells were plated at 10^6 per well and washed with FACS buffer (PBS, 2% FCS, 1% sodium azide). Tetramers were incubated at room temperature for 20 min, followed by an additional 20 min incubation with monoclonal antibodies specific for CD3 (clone UCHT1 BD), CD4 (clone RPA-T4 BD), CD8 (clone HIT8a bio legend), or CD103 (clone Ber-ACT8 bio legend) according to the manufacturer's protocols. Samples were fixed in FixPerm (BD) and read on an LSRII (BD).

Cells stained for intracellular IFNγ and MIP1β were permeabilized with cytofix/cytoperm reagent (BD) and incubated 30 min at 4^{o}C with anti-IFNγ

clone B27 (BD) and anti-MIP1β clone D211351. Samples were read on a LSRII (Beckman Coulter, Fullerton, CA). Cells for sorting were incubated with tetramer for 40 min at room temperature in 2% FCS/PBS buffer and were immediately isolated unfixed using the FACS Aria cell sorter (Beckman Coulter, Fullerton, CA).

CDR3 clonotype analysis. Clonotype analysis was performed as previously described (Cornberg et al., 2010a). Briefly RNA was isolated from tetramer-sorted cells using the Qiagen Oligotex Direct mRNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using the SMART RACE cDNA amplification kit from Clontech using the manufacturer's protocol. In place of the kit provided power-script for cDNA synthesis, Superscript III was used. To amplify the TCR-β and -α specific genes the Advantage2 system was used according to the manufacturer's protocol (Clonetech, Mountain View, CA). For the 5' forward primer the Universal Primer Mix from the SMART RACE cDNA amplification kit was used. To amplify the genes of interest a 3' reverse primer specific to the TCR-β or -α region was used as reported previously (Brochet et al., 2008; Lefranc et al., 2009). The PCR amplification program used was provided in the manufacturer's protocol. The resulting PCR product of appropriate size (~500-700 bp) was then gel purified using NucleoSpin Extract II kit according to the manufacturer's protocol (Clonetech, Mountain View, CA). Purified PCR product was then ligated

into the pCR4 vector from the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and transformed into DH5α *E. coli* from One Shot TOP10 chemically competent cells (Invitrogen, Carlsbad, CA), using the manufacturer's procedures. Colonies were then selected and amplified overnight for sequencing. Amplified colonies were then preserved in 20% glycerol on dry ice and sent to Genewiz for sequencing. Resulting sequences were then aligned using sequencher (Gene Codes Co. Ann Arbor, MI) and analyzed using IMGT/V-quest (Brochet et al., 2008; Lefranc et al., 2009).

^{51}Chromium release assay. Autologous BLCLs created with either wild type EBV or a BZLF1 KO virus (Feederle et al., 2000) were used as target cells. Cells were either untreated or treated with PMA at 10ng/ml overnight to induce the EBV lytic cycle. Targets were labeled with 100mCi ^{51}Cr per 10^6 cells and the assay was performed as previously described (Brehm et al., 2002). Target cells were washed and plated at $2.5x10^4$ cells/ml. Effector cells from CD8 T-cell lines were plated with targets at 8 different effector-to-target ratios as indicated using 2-fold serial dilutions. Supernatants were then harvested and read on a MicroBeta TriLux scintillation counter (Perkin Elmer). Lytic units were determined as previously described (F Pross and Maroun, 1984). Briefly, lytic units are defined as the number of cells needed to kill x% of target cells. For this study we used 15% killing of our target cells. Additionally, a portion of

untreated BZLF1 KO target cells were pulsed with 100mM of peptide for 1 hour at 37 ^{o}C and the assay was conducted as stated above.

Statistical Analysis: Statistical analysis was performed using Prism version 5 (Graphpad Software Inc.). Individual tests are indicated in figure legends. Tests include Student's T test and Simpson's diversity index $D=\Sigma n(n-1)/N(N-1)$ where n is the number of individual clonotypes and N is the number of unique clonotypes (Venturi et al., 2007).

Humanized Mice for the Study of Human Viral Infection

Mice. Three mouse models were used during this study. The first model was of NOD.Cg-*Prkdc*^{SCID}*IL2rg*^{tmlWjl}/Sz (NSG) mice that were developed at the Jackson Laboratory by crossing an *IL2rg* null mutation to the NOD.Cg-*Prkdc*^{SCID} mouse (Shultz et al., 2005). For the second model NOD.Cg-*Prkdc*^{SCID}*IL2rg*^{tmlWjl}Tg(HLA-A2/H2-D/B2M) 1Dvs/Sz (NSG-HLA-A2/HHD) mice were made by backcrossing the (HLA-A/H2-D/B2M) transgene (Pascolo et al., 1997) from the NOD/ShiLtDvs-Tg(HLA/H2-D/B2M)1Dvs/J strain (Takaki et al., 2006) onto the NSG background (Shultz et al., 2010). In the third model, BLT mice were generated by implanting 1mm^3 pieces of human fetal liver and thymus under the kidney capsule of NSG-HLA-A2/HHD mice. Mice used from all three models were female.

Purification of Human HSCs and Xenogeneic Transplantation. For NSG mice HLA-A2+ cord blood samples were enriched for CD34$^+$

HSCs by using anti-hCD34 micro-beads (Miltenyi Biotech). For NSG-HLA-A2/HHD mice cord blood PBMCs were depleted of T-cells using anti-hCD3 micro-beads (Miltenyi Biotech) and $CD34^+$ numbers were calculated from precursor frequencies. Newborn (2-3 day old) mice were given total body irradiation of 150cGy followed by intra-cardiac injection of ~$3x10^4$ $CD34^+$ HSCs. Mice were allowed to reconstitute for 12-20 weeks before infection.

Virus Preparation and Infections. The gastric carcinoma cell line AGS, harboring a GFP-expressing EBV, was induced to express virus as previously described (Borza and Hutt-Fletcher, 2002). Briefly EBV-harboring AGS cells were treated with 30ng per ml of 12-o-tetra-decanoylphorbol-13-acetate and 2.5mM sodium butyrate overnight and allowed to secrete virus for 4 days. Supernatants were collected and filtered through 0.2mm filter and stored at -80^{o}C. Viral titrations were preformed as previously described (Strowig et al., 2009). Briefly $1x10^4$ Raji cells were plated and infected with two-fold dilutions of virus stock. After two days Raji cells were examined for GFP expression by flow cytometry. One unit was defined as the amount of virus needed to induce 50% maximal GFP expression. IAV x31 was cultivated in embryonated chicken eggs (Szretter et al., 2006). Mice were infected with 100 units of EBV intra-peritoneally (i.p.) and sacrificed 6-8 weeks after infection. IAV infected mice were anesthetized with

methoxyflurane (Metafane™) and given 50 pfu in 50µL intra-nasally. Mice were then harvested 4 weeks after infection.

Flow Cytometry Analysis. Splenocytes were plated at a concentration of $1x10^6$ cells/mL and washed and stained with monoclonal antibodies specific for hCD45 (clone HI30 BD), hCD3 (clone UCHT1 BD), hCD4 (clone RPA-T4 BD), hCD8 (clone HIT8a bio legend), and HLA-DR (clone TU36 BD) according to the manufacturer's protocols. Samples were fixed in FixPerm (BD) and read on an LSRII (BD).

Intracellular Cytokine Production. Intracellular cytokine staining was performed as previously described (Shultz et al., 2010). Briefly splenocytes were plated at a concentration of $1x10^6$ cells/mL in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10mg/mL brefeldin A, and 10 units/mL of IL-2. Cells were either untreated or stimulated with 25ng phorbol 12-myristate 13-acetate and 10mg/mL of Ionomycin for 5 hours at 37^oC and stained with monoclonal antibodies mentioned above and then permeabilized with Cytofix/Cytoperm (BD). Cells were then stained intracellularly with monoclonal antibodies specific to IFNγ (clone 4S.B3 ebiosciences) and granzyme B (clone 16G6 E??ebiosciences). Samples were read on an LSRII (BD).

Histological Staining. Hematoxylin/eosin (H&E) staining was performed on fixed sections of liver and lung tissue from EBV and IAV

infected animals, respectively. Staining was performed using standard procedures.

Chapter III: Increased frequency of high avidity, lytic, cross-reactive CD8 T-cells in EBV sero-negative adults

The gamma-herpes virus Epstein-Barr virus (EBV) infects ~90% of individuals globally, establishing a life-long infection (Henle et al., 1969). The clinical presentation of EBV infection can range from asymptomatic to severe, occasionally fatal, acute infectious mononucleosis (AIM) (Crawford et al., 2006b; Luzuriaga and Sullivan, 2010). There is also a strong causal relationship between EBV infection and malignancies, including carcinomas, and Burkitt's lymphoma (Luzuriaga and Sullivan, 2010). However, it is unknown why 5-10% of adults remain EBV-SN, despite the fact that the virus infects the vast majority of the population and is actively shed at high titers even during chronic infection (Hadinoto et al., 2009). Here, we show that EBV-SN HLA-A2+ adults possess cross-reactive IAV-GIL/EBV-GLC memory CD8 T-cells that show highly unique properties. These IAV-GIL cross-reactive cells preferentially expand, and produce cytokines to, EBV antigens with high functional avidity. Additionally they are capable of lysing EBV-infected targets and show the potential to enter the mucosal epithelial tissue where infection is thought to initiate. They also possess a TCR repertoire that differs by both organization and CDR3 usage from that in EBV-SP donors. Our results imply that heterologous immunity may protect EBV-SN adults against the establishment of productive EBV infection, and thus are the first demonstration of protective heterologous immunity between unrelated pathogens in sero-negative human subjects

Here we question whether heterologous immunity mediated by cross-reactive CD8 T-cells can protect EBV-SN adults from EBV infection and sero-conversion. In murine studies, heterologous immunity facilitated by cross-reactive CD8 T-cell responses can mediate either beneficial (protective immunity) or detrimental effects (e.g., enhanced lung and adipose immunopathology) (Selin et al., 1998; Chen et al., 2001; Welsh and Selin, 2002; Welsh et al., 2010). In this study we sought to determine if EBV-SN adults had an IAV-GIL/EBV-GLC cross-reactive T-cell response with the potential to protect against the establishment of productive infection with EBV.

A. Characterization of EBV-SN adult donors

For our study we procured blood samples from 5 HLA-A2+ healthy adult donors that were documented to be EBV sero-negative. Three of the 5 donors were male and 2 were female. The 5 donors ranged in age from 32-55 years of age. One male and one female donor tested positive for CMV IgG. Due to the possibility that these individuals could be EBV-SN yet still be infected with EBV, quantitative PCR for EBV DNA was performed on CD8 depleted PBMCs and found to be negative **(table 1)**.

To determine frequencies of IAV-GIL, EBV-GLC, and EBV-YVL reactive CD8 T-cells ex vivo, tetramer staining was performed on sorted CD8 T-cells from peripheral blood mononuclear cells (PBMCs). IAV-GIL frequencies ranged from 0.07-1.2% of the CD8 T-cell pool, with a mean of 0.41+/-0.42% and an MFI of 1164+/- 23 while EBV-GLC and EBV-YVL tetramer staining, if present, was very

dim **(Fig 3.1)**. Dim EBV-GLC tetramer staining frequencies ranged from 0-0.27% of the CD8 T-cell pool, with a mean of 0.13+/-0.11% and an MFI of 180+/- 135. Dim EBV-YVL tetramer staining frequencies ranged from 0-0.08%s of the CD8 T-cell pool, with a mean of 0.05+/-0.03% with an MFI of 384+/-542 **(table 1)**. Control tyrosinase tetramer staining exhibited no staining **(Fig 3.1)**.

B. Increased frequencies of IAV-GIL tetramer positive cells detected in EBV-SN adults

Immunological memory is the mechanism by which an individual is protected from secondary infection. In heterologous immunity immunological memory developed in response to one pathogen has an impact on the response to a secondary unrelated pathogen. Previously we have reported that IAV-GIL memory CD8 T-cells have the ability to recognize EBV-GLC and participate in mediating AIM (Clute et al., 2005). Here we wanted to determine the frequency of IAV-GIL reactive cells in EBV-SN adults directly ex vivo. To determine the frequencies of IAV-GIL reactive CD8 T-cells, tetramer staining was performed on sorted CD8 T-cells from donor PBMCs. Within the EBV-SN adults there was a significant 7-fold higher frequency of IAV-GIL tetramer+ cells detected directly ex vivo as compared to EBV-SP donors (EBV-SN: 0.42 +/-0.4 vs. EBV-SP: 0.06 +/-0.03: p=0.003) **(Fig 3.1)**.

This increase in frequency of IAV-GIL reactive cells, some of which have been previously shown to be cross-reactive to EBV-GLC, suggests that the EBV-

Donors	D01	D02	D03	D04	D05
Age	37	43	32	55	55
Sex	M	M	M	F	F
EBV EBNA IgG	-	-	-	-	-
EBV VCA IgM	-	-	-	-	-
EBV VCA IgG	-	-	-	-	-
EBV Genome PCR	-	-	NP	NP	NP
CMV IgG	-	-	+	-	+
IAV-GIL	0.5	0.07	1.19	0.14	0.17
EBV-GLC	0.03	0	0.21	0.27	0.13
EBV-YVL	0	0.05	0.04	0.08	0.08

Table 3.1: EBV-SN donor characterization. EBV-SN donor sex, age, sero-status, and indicated tetramer frequencies within the CD8 T-cell pool determined *ex vivo*. EBV genome PCR performed on both peripheral blood and throat wash. NP Not

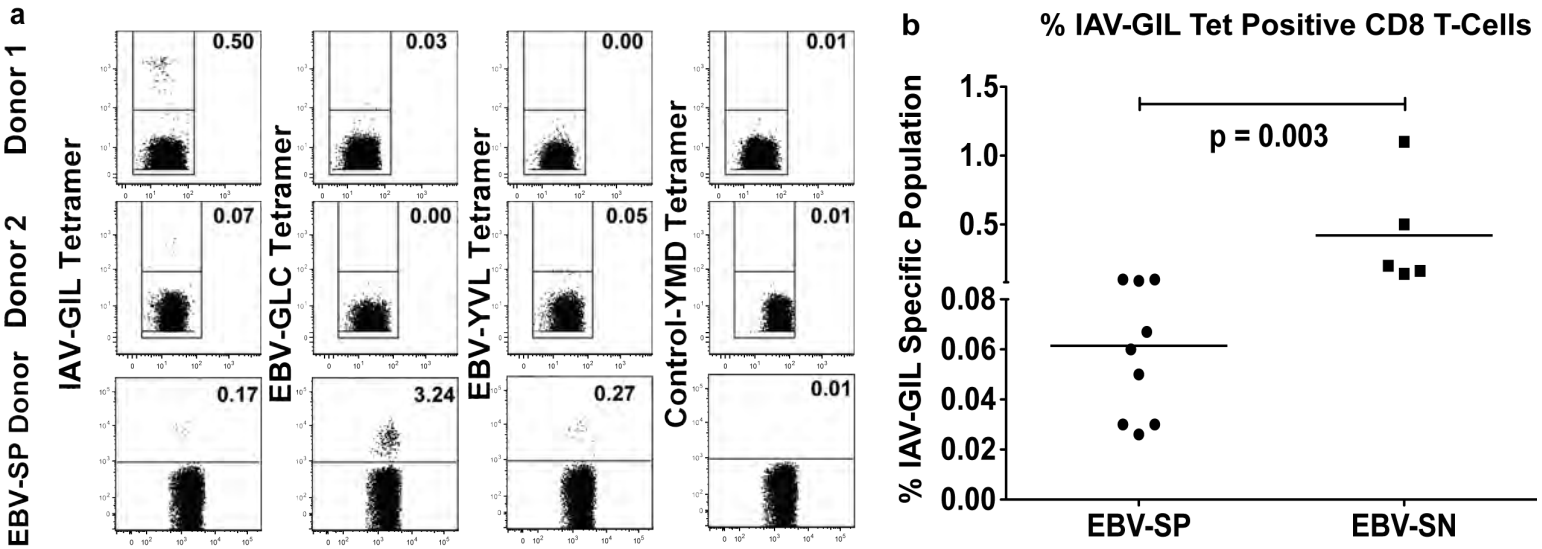

Figure 3.1: Increased frequency of IAV-GIL-specific CD8 cells directly ex vivo in EBV-SN adults. CD8 T-cells were isolated from the PBMCs of EBV-SN and EBV-SP donors by MACS separation. Cells were stained with live/dead violet viability kit, anti-CD3 and anti-CD8 and indicated tetramers and analyzed by flow cytometry. **a)** Representative examples of IAV-GIL, EBV-GLC and -YVL tetramer binding in EBV-SN and -SP adults (gated on live CD3+CD8+ cells). **b)** Significantly increased mean frequency of IAV-GIL-specific tetramer+ cells in EBV-SN versus EBV-SP adults.

SN donors may have a heightened sensitivity to the early EBV antigen EBV-GLC.

C. IAV-GIL tetramer positive cells preferentially expand when cultured in the presence of EBV antigens

Now that we had observed that there is an increase in the precursor frequency of the potentially cross-reactive IAV-GIL restricted CD8 T-cells, we next wanted to start investigating their effector functions. Even though there was a relatively high frequency of IAV-GIL restricted cells within EBV-SN donors, the corresponding number of cells that can be recovered *ex vivo* is too small to be able to conduct functional experiments. In order to procure enough cells we used an *in vitro* system.

Sorted CD8 T-cells were plated at a concentration of $2.5x10^5$ cells per ml with irradiated T2 feeder cells at a concentration of $5x10^4$ cells per ml. Feeder cells were coated with 1µM of indicated HLA-A2 restricted peptides. Media were replaced every 3-4 days and cells were re-stimulated every 7 days. After 3 stimulations cells had expanded enough to use in functional assays.

Interestingly, when sorted CD8 cells from EBV-SN adults were cultured in the presence of EBV lytic peptides, EBV-GLC or –YVL, the same frequency of IAV-GIL tetramer+ cells expanded as compared to CD8 cells cultured in the presence of IAV-GIL. Importantly, feeder cells only, an irrelevant control tyrosinase peptide, or an unrelated viral epitope CMV-pp65 (CMV-NLV), did not induce expansion of IAV-GIL specific cells **(Fig 3.2)**. Cross-reactive IAV-GIL

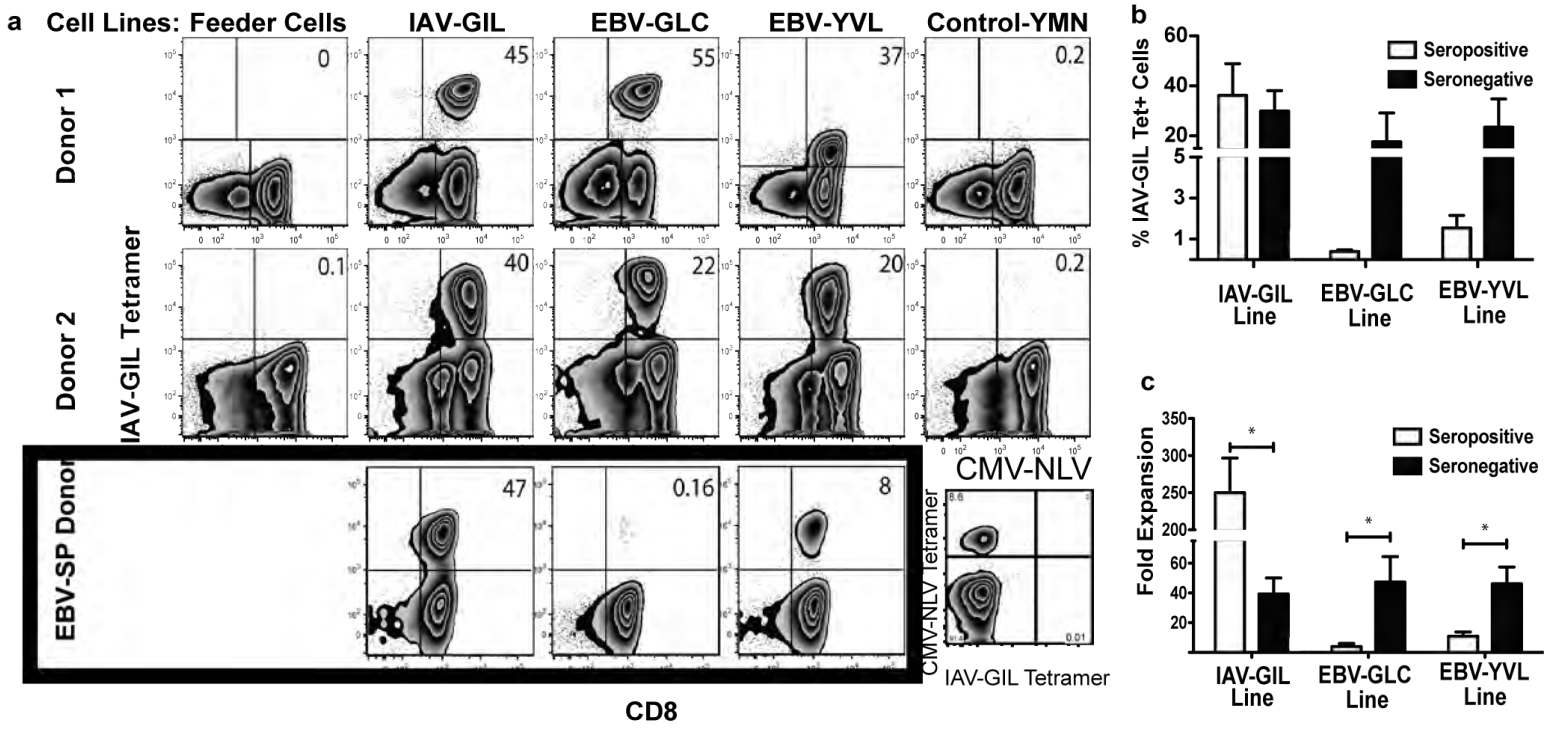

Figure 3.2: Increased expansion of IAV-GIL-specific cells in response to EBV lytic antigens in EBV-SN adults. CD8 T-cells were stimulated with peptide-loaded T2 cells 3 times for a period of 3 wks. After resting for 1-wk cell lines were stained for viability, anti-CD3 and -CD8 and IAV-GIL tetramer. **a)** Representative examples of cell cultures from EBV-SN and EBV-SP adults demonstrating dramatic increase in IAV-GIL tetramer+ cells after not only IAV-GIL, but also EBV-GLC and -YVL stimulation in culture in EBV-SN adults (gated on live CD3+ cells) and CMV-NVL in EBV-SN donor 5 (who is CMV-SP). Data for representative EBV-SP donor is in black box **b)** Mean frequency of IAV-GIL-specific tetramer+ cells in IAV-GIL, EBV-GLC and -YVL cell lines of EBV-SN and –SP adults (gated on live CD3+ cells). **c)** Significant fold increased expansion of IAV-GIL-specific cells in EBV lytic antigen stimulated cultures of EBV-SN vs EBV-SP adults. None of the EBV-SN cell lines stained with EBV-GLC or –YVL tetramers (See figure 3.5).

tetramer+ cells growing in EBV-GLC or -YVL peptide-stimulated cell lines of EBV-SP donors are frequently observed (Clute et al., 2005; 2010; Cornberg et al., 2010a) (**Fig 3.2a,b**). However, the IAV-GIL-specific cells from EBV-SN adults expanded 7- and 4-fold greater than in EBV-GLC or -YVL peptide-stimulated cell lines from EBV-SP donors, respectively **(Fig 3.2c)**. Notably, IAV-GIL specific cells had a statistically significant higher fold expansion within EBV-SP donors than EBV-SN donors when cultured in the presence of IAV-GIL. This is an artifact of the calculation due to the significantly lower starting frequency of IAV-GIL reactive cells present *ex vivo* as compared to EBV-SN donors **(Fig 3.2c)**.

D. IAV-GIL memory cells are necessary for EBV antigen mediated expansion

To show that this cross-reactive EBV induced expansion of IAV-GIL restricted CD8 T-cells is a special feature of EBV-SN adult donors, and not an inherent reactivity of all IAV-GIL CD8 T-cells, CD8 T-cells from immunologically naïve HLA-A2+ cord-blood PBMCs were examined. CD8 T-cells were subjected to the same in vitro procedure as described above.

Antigen experienced IAV-GIL-specific CD8 T-cells appeared to be required for EBV-GLC or -YVL induced expansion, as expansion did not arise from immunologically naïve (never exposed to IAV or EBV) HLA-A2+ cord-blood CD8 T-cells **(Fig 3.3)**. As previously reported, there was a small expansion of IAV-GIL reactive cells from the cord blood but only in cell lines grown in the

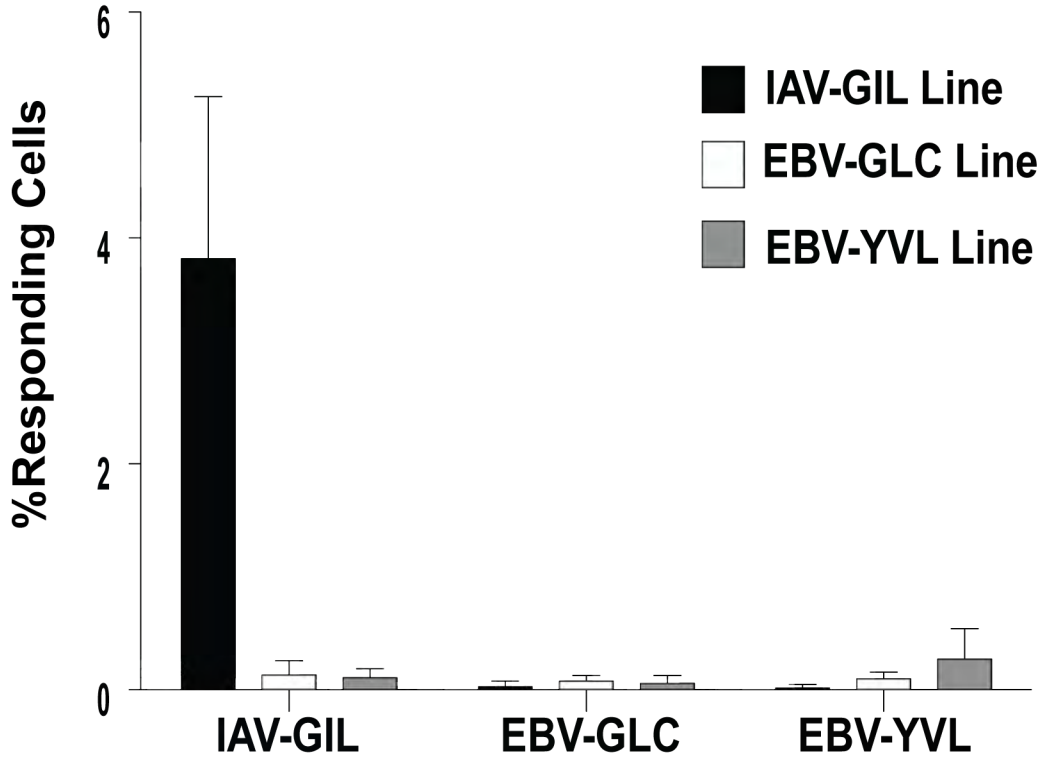

Figure 3.3: IAV-GIL memory cells are necessary for EBV antigen mediated expansion No expansion of IAV-GIL-specific CD8 T-cells in response to EBV lytic antigens in immunologically naïve cord blood. CD8 cells from immunologically naïve cord blood were prepared and grown as stated for EBV-SN and EBV-SP donors.

presence of IAV-GIL peptide and only in response to IAV-GIL peptide and not to EBV-GLC or –YVL (Lawson et al., 2001). These results imply that this strong proliferation in response to EBV-GLC or -YVL peptide is not an inherent property of all IAV-GIL reactive T-cells, but is unique to the IAV-GIL memory population in these EBV-SN adults.

E. IAV-GIL tetramer positive cells are functionally cross-reactive as demonstrated by cytokine production

An additional ability of CD8 T-cell effector function is the ability to secrete antiviral cytokines. To further elucidate the cross-reactive effector function of the IAV-GIL restricted CD8 T-cells expanded by EBV lytic epitopes from EBV-SN adult donors, an intracellular cytokine-staining (ICS) assay was performed.

The majority of the IAV-GIL tetramer+ cells in the IAV-GIL, EBV-GLC and -YVL stimulated CD8 lines from EBV-SN adults produced IFNγ and MIP1β in response to IAV-GIL in (ICS) assays **(Fig 3.4a,b)**. Remarkably, as seen with donor 1 **(Fig 3.4a)**, 37% of the IAV-GIL tetramer+ cells from the IAV-GIL peptide-stimulated line produced IFNγ in response to EBV-GLC stimulation. In the EBV-GLC and -YVL peptide-stimulated lines an even greater proportion, 83-88% of the IAV-GIL tetramer+ cells produced IFNγ in response to EBV-GLC stimulation **(Fig 3.4a)**. EBV-YVL peptide-stimulated IAV-GIL tetramer+ cells produced IFNγ, in the IAV-GIL, EBV-GLC and -YVL peptide-stimulated lines, however it was a lower proportion of cells **(Fig 3.4a)**. This cross-reactivity was specific and

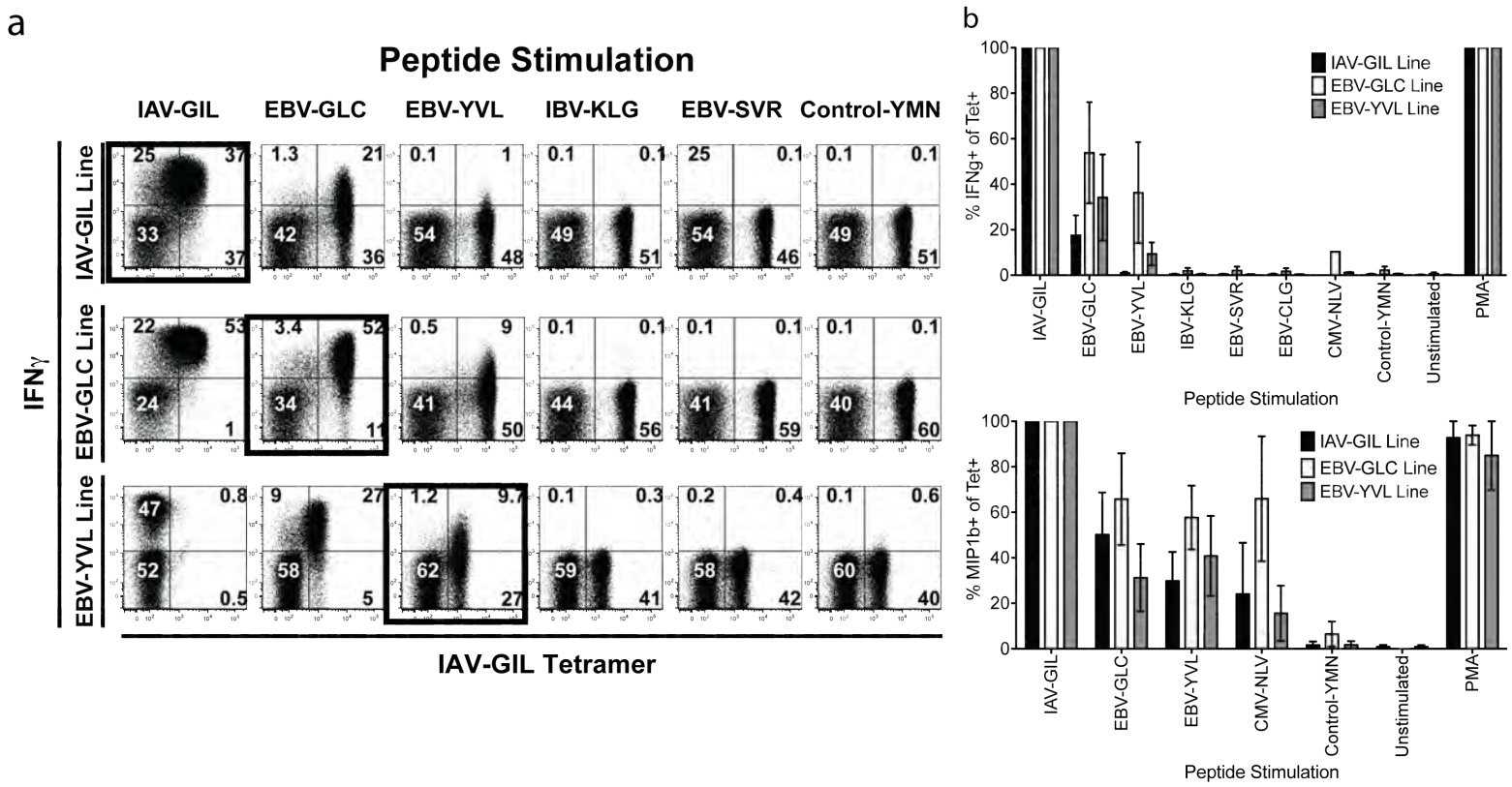

Figure 3.4: Functional anti-viral cross-reactive responses between IAV-GIL and EBV lytic antigens in EBV-SN adults: a) CD8 T-cells from the indicated cell lines were subjected to an intra-cellular cytokine-staining (ICS) assay measuring IFNγ production upon stimulation with indicated cognate, cross-reactive and control peptides for 5 hours at 37 degrees (gated on live CD3+CD8+ cells). Representative results from donor 1 demonstrated IAV-GIL tetramer+ cells from all 3 lines produced IFNγ in response to not only IAV-GIL peptide but also both EBV lytic peptides. Boxes indicate cognate stimulation. **b)** Mean percentage of IFNγ and MIP1β producing IAV-GIL tetramer+ cells in response to indicated peptides of all 5 EBV-SN donors.

restricted to these 3 epitopes, as peptide stimulation with other viral and self-epitopes did not induce cytokine production **(Fig 3.4a,b)**.

These data demonstrate that IAV-GIL-specific cells from EBV-SN adults expanded by cognate (IAV-GIL) or cross-reactive (EBV-GLC, or –YVL) peptides could produce antiviral cytokines in response to EBV epitopes and were functionally cross-reactive. These results also suggest that the IAV-GIL-specific TCR repertoires in the 3 cultures may differ, as different proportions of the population were able to produce IFNγ to the cross-reactive ligands, EBV-GLC and -YVL.

F. IAV-GIL restricted cells have high avidity to IAV-GIL and EBV-GLC

An additional characteristic of memory CD8 T-cells is an enrichment of high functional avidity cells. CD8 T-cells with higher avidity usually result in increased effector function (Alexander-Miller, 2005).

To decipher the functional avidity of these cross-reactive responses, these cell lines were stimulated with 10-fold serial dilutions of IAV-GIL or EBV-GLC peptides, and IFNγ production was measured. An effective concentration that resulted in 50% maximal IFNγ production (EC50) was then calculated. Unexpectedly, the cognate IAV-GIL functional avidity as measured by EC50 in the IAV-GIL lines from the EBV-SN adults was 10-fold higher ($10^{-8.5 \pm 0.4}$M, n=3) than in the EBV-SP adult lines ($10^{-7.3 \pm 0.3}$M, n=4) (p=0.05) **(Fig 3.5a)**. The EBV-GLC functional avidity as measured by EC50 in the EBV-GLC lines from the EBV-SN adults was nearly 100-fold higher ($10^{-8.6 \pm 0.3}$M, n=3) as compared to the

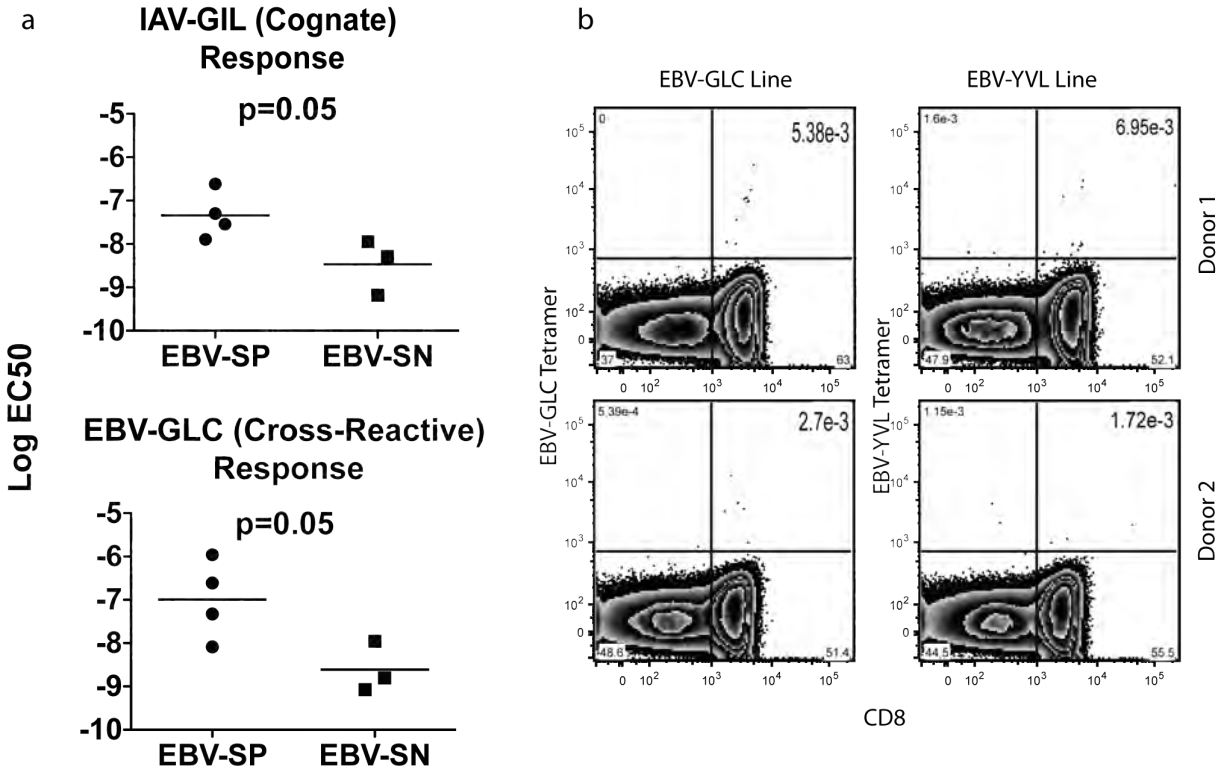

Figure 3.5: IAV-GIL restricted cells have high avidity to IAV-GIL and EBV-GLC Functional avidity was measured by ICS examining IFNγ production in response to 10-fold serial dilutions of IAV-GIL or EBV-GLC peptide. The percent of IFNγ-producing cells was measured across the peptide dilutions and EC50 was determined (n=3-5 EBV-SP; 3-4 EBV-SN adults). b) **EBV-GLC and –YVL tetramers do not stain cells expanded by EBV lytic antigens in EBV-SN adults.** Representative examples of EBV-GLC and –YVL tetramer staining on CD8 T-cells stimulated with peptide coated T2 cells once a week for 3 weeks. After resting cells 1-week cells were stained for viability, CD3, CD8, and indicated tetramers. Shown population gated on live CD3+ cells.

EBV-SP adult lines ($10^{-7.0\pm0.5}$M, n=4)(p=0.05) **(fig 3.5a)**. It is surprising that CD8 T-cells with this high functional avidity did not bind EBV-GLC or EBV-YVL tetramer **(Fig 3.5b)**. However, similar findings have been shown in which antigen specific cells from in vitro generated cell lines are capable of producing cytokines in response to peptide ligands, but are unable to bind tetramers (Spencer and Braciale, 2000; Sabatino et al., 2011). There may be a possibility that the EBV-GLC and –YVL peptides are being presented by an alternate MHC. While unlikely due to their HLA-A2 restriction this could be tested for by blocking EBV-GLC pMHC-TCR interactions with a HLA-A2 blocking antibody. If the responses remained then that would indicate that there may be another mechanism involved. This result would not rule out heterologous immunity and cross-reactive memory CD8 T-cells as the mechanism mediating these events, as it is primarily the IAV-GIL tetramer positive cells that produce cytokine in response to EBV-GLC and –YVL peptides (**Fig 3.4**).

These data show that EBV-SN adults have EBV-GLC reactive cells with significantly higher functional avidity to EBV-GLC than do EBV-SP individuals, perhaps selected for by low dose exposure to antigen. These data suggest that these EBV-GLC reactive cells may be able to protect against the establishment of productive EBV infection.

G. Cross-reactive CD8 T-cells in EBV-SN adults lyse EBV-infected and peptide-coated autologous BLCL targets

The most important hallmark of CD8 T-cell function is the ability to kill tumor cells and virus infected cells. To do this a CD8 T-cell must see its antigen in the context of a peptide-MHC (pMHC) complex. CD8 T-cells must be educated to recognize the MHC of the individual during thymic development. CD8 T-cells that pass thymic selection then migrate to the periphery and join the naïve T-cell pool. If a CD8 T-cell interacts with a MHC other than the ones on which it has been selected on the cells can exert their effector function in response to an alloantigen.

In order to test whether these cross-reactive IAV-GIL-specific cells from these EBV-SN adults could kill EBV-infected targets, and to avoid any allo-reaction, autologous B lympho-blastoid cell lines (BLCLs) were generated from two of the donors. The fact that we were able to generate BLCL is evidence that B-cells from EBV-SN individuals can be infected with EBV. As a control, infecting autologous B cells with BZLF1 KO EBV created autologous BLCLs incapable of presenting lytic antigens. BZLF1 is required for reactivation from latent to lytic cycle and leads to the expression of the lytic proteins BMLF1 and BRLF1 (Feederle et al., 2000). By using these target cells and also HLA-A2-restricted peptide-coated autologous BZLF1-KO BLCL targets we demonstrate that these cross-reactive CD8 T-cells are capable of killing EBV-infected targets in a lytic antigen-dependent manner. CD8 T cell lines grown in the presence of IAV-GIL, EBV-GLC, or -YVL peptides were able to lyse WT autologous BLCL targets but not the BZLF1 KO autologous BLCL targets **(Fig 3.6a-b)**. These CD8

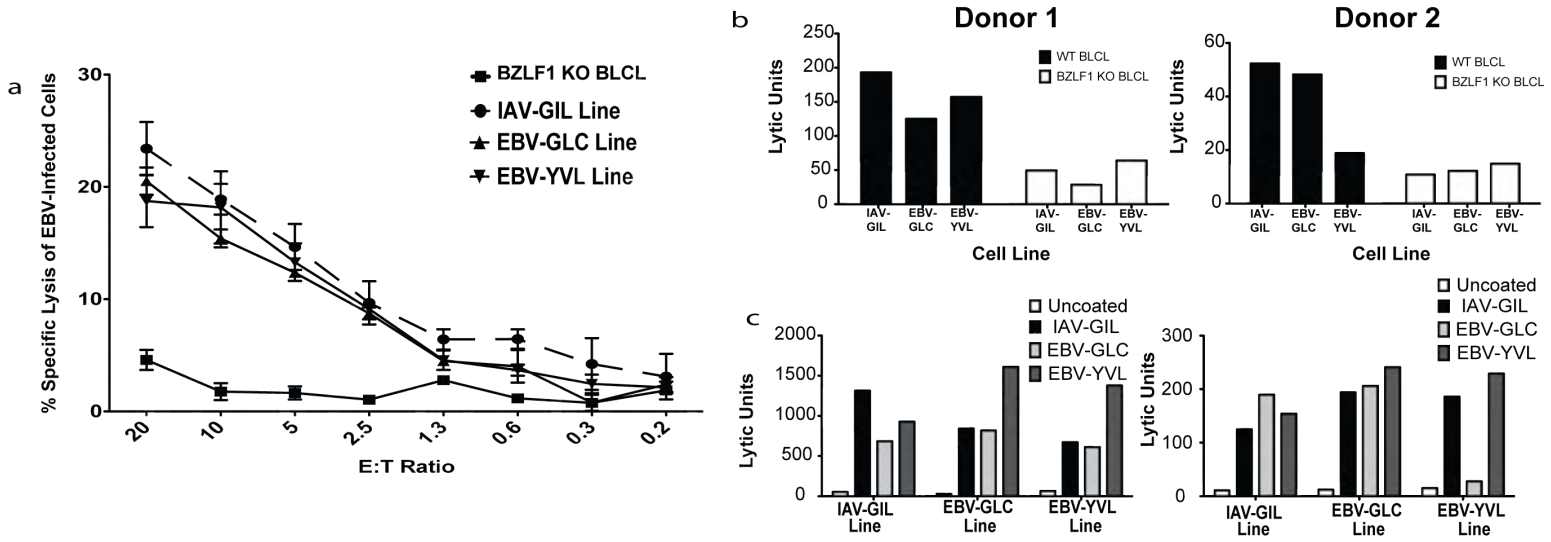

Figure 3.6: Cross-reactive CD8 T-cells in EBV-SN adults demonstrated potential to protect against EBV by their ability to lyse EBV-infected and peptide-coated autologous BLCL targets a) Representative example of increased lysis of EBV-infected targets by IAV-GIL-specific cells in all 3 cells lines from donor 1. In a ^{51}Cr-release assay CD8 T-cells from the indicated cell lines were used as effector cells and incubated with target cells for 5 hrs at 37^{o}C at indicated effector:target ratios to measure cytotoxicity. Targets used were either autologous WT or BZLF1 KO BLCLs (■) stimulated with PMA to induce EBV lytic cycle. The lack of BZLF1 results in the inability of the cells to enter lytic cycle and express lytic cross-reactive antigens BMLF1 (EBV-GLC) and BRLF1 (EBV-YVL). **b)** Lytic unit (LU) representation of increased specific lysis of EBV-infected targets. One LU is defined as the number of effector cells capable of killing 15% of 5x10^{3} target cells during the 5-hour assay. **c)** Increased lysis of cognate IAV-GIL, and cross-reactive EBV-GLC and -YVL, vs control peptide-coated autologous BZLF1 KO BLCL targets from all 3 lines of donor 1 and 2

lines also lysed IAV-GIL, EBV-GLC, or -YVL peptide-loaded targets but not control targets **(Fig 3.6c)**. This ability of EBV-SN adult CTL to kill EBV-infected and EBV-peptide-loaded targets suggests that these IAV-GIL cross-reactive cells have the potential to protect against EBV infection.

H. IAV-GIL tetramer positive cells show potential to enter the mucosal epithelium

Recent findings have suggested that effector cells in the initial site of infection can mediate protection from the establishment of infection, particularly in the mucosa (Hansen et al., 2011). While it was long thought that EBV only infects memory B-cells, recent findings have shown that EBV can also infect epithelial cells of the tonsillar mucosa (Arvin et al., 2007). Here we wished to determine whether this unique population of IAV-GIL specific cells from EBV-SN adults have the ability to enter the mucosal epithelium.

To determine whether these EBV-specific cross-reactive cells could migrate into the site of initial viral replication, such as the linguinal and tonsillar mucosal epithelial tissues, tetramer+ cells were stained directly *ex vivo* for the expression of CD103, an integrin molecule associated with migration into these tissues (Schön et al., 1999). There was a significant 4-fold higher frequency of CD103 expressing (6.3±1.3%, n=3) IAV-GIL tetramer+ cells in EBV-SN adults as compared to EBV-SP adults (1.5±1.0%, n=5) **(Fig 3.7)**. Also, there was a significant 40-fold higher frequency of CD103-expressing dim EBV-GLC tetramer+ cells directly *ex vivo* in the EBV-SN adults at 10.1±2.2% (n=3), as

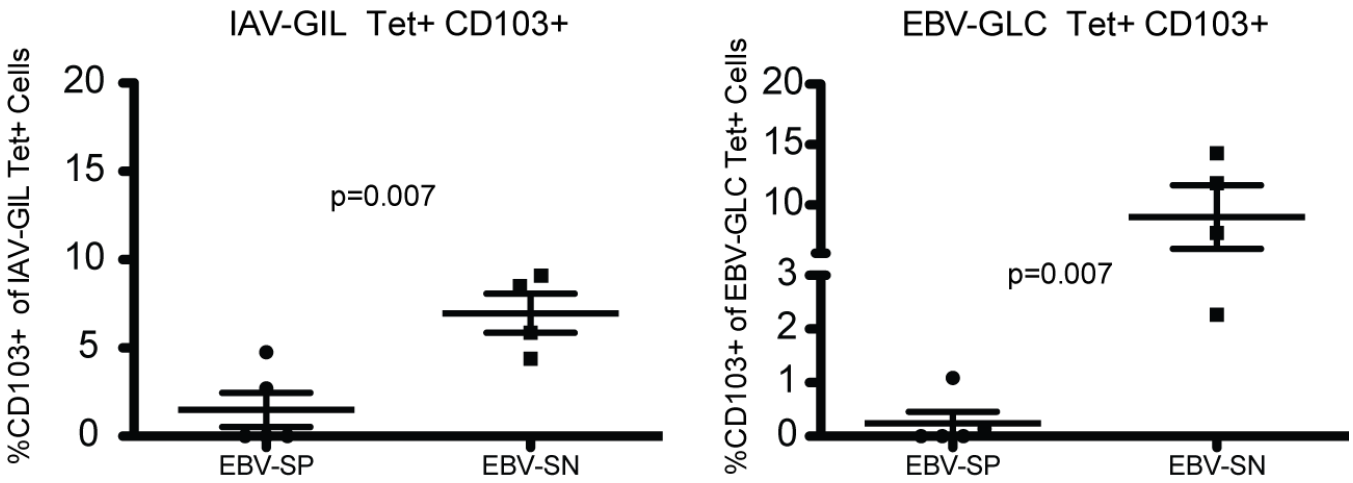

Figure 3.7: Cross-reactive CD8 T-cells in EBV-SN adults demonstrated potential to protect against EBV by their potential ability to enter mucosal epithelial tissues: CD8 T-cells isolated from PBMCs from either EBV-SP or EBV-SN adults were stained with IAV-GIL or EBV-GLC tetramers and anti-CD103, an integrin molecule important for entry into mucosal epithelium. The percentages of CD103+ cells from IAV-GIL or EBV-GLC tetramer+ population are shown.

compared to only 0.25±0.21% (n=5) of the bright EBV-GLC tetramer+ cells in EBV-SP adults **(Fig 3.7)**. These data suggest that the IAV-GIL and the dim EBV-GLC tetramer+ cells from EBV-SN adults have the appropriate antigen display to enter mucosal epithelial tissues. Antigen exposure is required for CD103 expression of CD8 T-cells (Lee et al., 2011). This would suggest that these EBV-GLC reactive cells that express CD103 could be seeing continuous low level antigen exposure.

I. IAV-GIL restricted CD8 T-cells within EBV-SN adult use a different TCR β chain

Due to the unique features of the IAV-GIL restricted CD8 T-cells within EBV-SN adult donors we next questioned whether there were any unique features in the IAV-GIL-specific TCR repertoire that could explain why these EBV-SN adults might have protective immunity. **Figure 3.8a** shows a representative example of the well characterized IAV-GIL-specific TCR repertoire in a middle-aged EBV-SP donor (Lehner et al., 1995; Lawson et al., 2001; Naumov et al., 2008; Clute et al., 2010). This and other EBV-SP donors typically have a polyclonal response restricted by a public Vβ19 usage, primarily expressing xRSx complementarity determining region 3 (CDR3) motif (~90% of clonotypes), that predominantly utilizes Jβ2.7 (Naumov et al., 2006). Within EBV-SN donors, Vβ usage by IAV-GIL tetramer+ cells in the lines grown in the presence of IAV-GIL, EBV-GLC or -YVL antigen primarily used Vβ19, as demonstrated by antibody staining (data not shown) and CDR3β sequencing **(Fig**

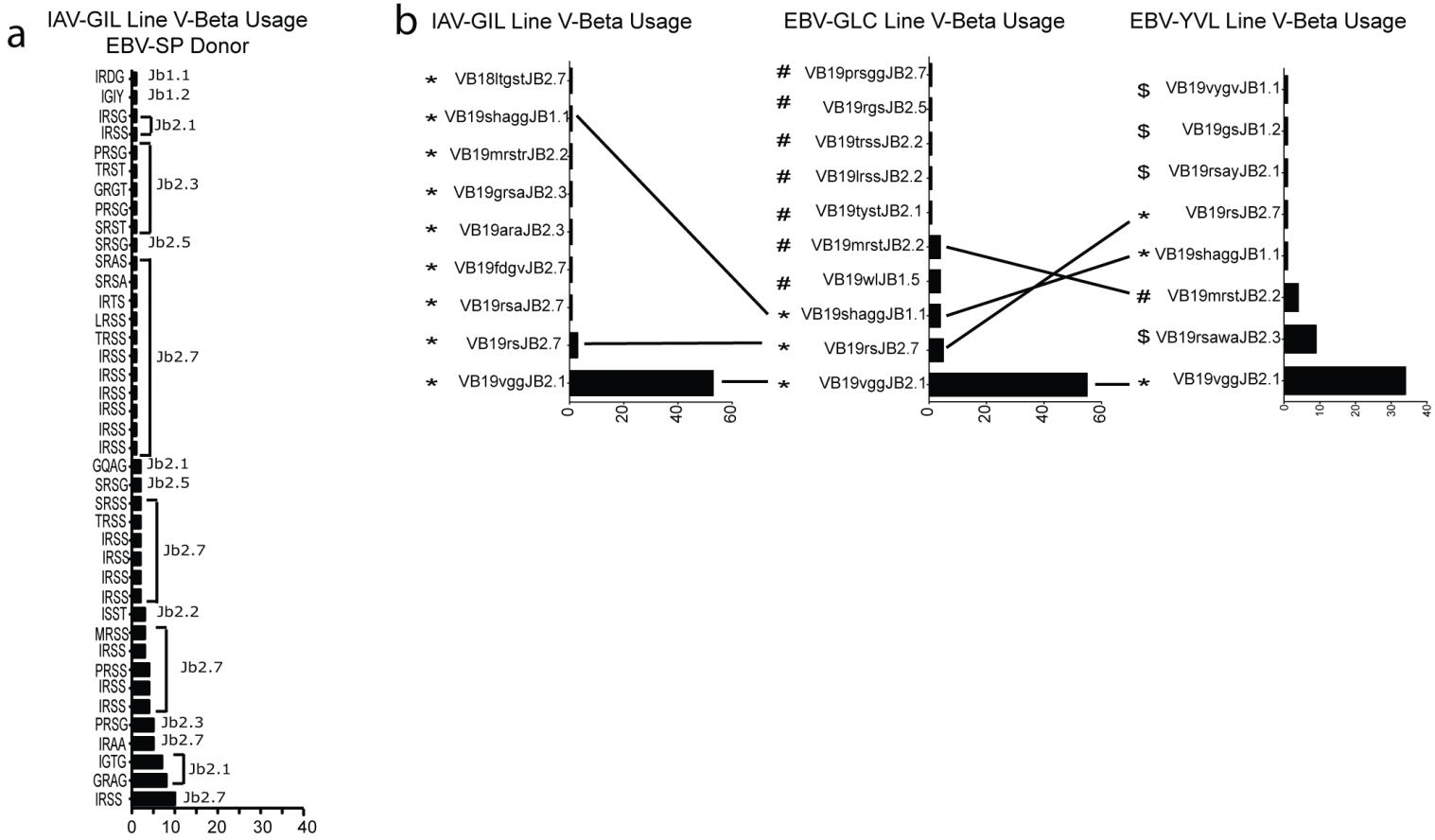

Figure 3.8: EBV-SN adults display an altered oligoclonal IAV-GIL repertoire as compared to EBV-SP adults: a) Typical clonal distribution of a representative IAV-GIL-specific Vβ19 TCR repertoire in EBV-SP adults showing high diversity with increased usage of the signature xRSx CDR3 motif and Jβ2.7 with no dominant clonotypes. **b)** Representative clonal distribution (Donor 1) of an IAV-GIL-specific Vβ19 and Vβ2.1 dominated TCR repertoire in EBV-SN adults showing a highly oligoclonal response. The same clone dominates in the IAV-GIL and EBV-GLC lines. The symbol beside each clonotype indicates whether a clonotype is present in a specific culture: * for IAV-GIL line, # for EBV-GLC line, and $ for EBV-YVL line. The lines drawn between cultures indicate the presence of that same clonotype in different cultures

3.8, Table 3.2). The dominant clonotype in donor 1, rather than containing the common xRSx motif used by EBV-SP donors, contained a non-canonical IVGG motif and used an uncommon Jβ2.1 instead of the common Jβ2.7 used by EBV-SP adults **(Fig 3.8)**. Unique Vβ19 TCR repertoires were observed in the other EBV-SN donors **(Table 3.2)**.

J. EBV-SN donors have a different IAV-GIL specific TCR repertoire organization

In contrast to EBV-SP donors, the CDR3β repertoire within the IAV-GIL-restricted response from the IAV-GIL, EBV-GLC and -YVL lines from the EBV-SN adults was less diverse and oligo-clonal. To quantitate the diversity of the TCR repertoire Simpson's diversity index was used (Venturi et al., 2007). The Vβ19 CDR3β repertoire of the IAV-GIL response was significantly less diverse within EBV-SN adults as compared to EBV-SP adults **(Fig 3.9)**.

K. IAV-GIL restricted CD8 T-cells within EBV-SN adult use different Vα chain

The Vα repertoire organization in the IAV-GIL restricted T-cell population of EBV-SP donors was similar to that of the Vβ repertoire, in that it was highly diverse with no dominating clonotypes (Clute et al., 2010). The Vα repertoire predominantly used Vα27, with some additional usage of Vα10, 8.6, and 34, most often combined with Jα42. However, like the Vβ repertoire organization, in all 3 representative EBV-SN adults, the Vα repertoire was not polyclonal but instead was dominated by one or two clonotypes **(Fig 3.10)**. Interestingly, the

Table 3.2. Clonal composition of the IAV-GIL TCR beta repertoire within EBV-SP adults. 5 EBV-SP donors showing the distribution of IAV-GIL reactive clones showing V-β family and J-β family usage, CDR3 amino acid compositions, and number of clones detected.

EBV-SP IAV-GIL Line VB Usage

Donor 4 (Codja D 012)

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2.5	CASSSRSGWTQYFGPG	50
BV19	BJ2.7	CASSIRSSYEQYFGPG	11
BV19	BJ2.7	CASSIRSSYEQYFGPG	11
BV19	BJ1.5	CASSYYSNQPQHFGDG	7
BV19	BJ2.7	CASSIRSAYEQYFGPG	3
BV19	BJ2.7	CASSIRSAYEQYFGPG	3
BV19	BJ2.7	CASSIEQYYEQYFGPG	3
BV19	BJ2.3	CASSTRSTDTQYFGPG	3
BV19	BJ2.7	CASSIRSSYEQYFGPG	2
BV19	BJ2.7	CASSIRSSYEQYFGPG	2
BV19	BJ2.3	CASSSRSTDTQYFGPG	2
BV19	BJ2.3	CASETTSTDTQYFGPG	2
BV19	BJ1.4	CASSIRSTEKLFFGSG	2
BV19	BJ1.1	CASSIRSDAEAFFGQG	2
BV19	BJ1.1	CASTHSAISEAFFGQG	2
BV19	BJ2.7	CASSIRSSYEQYFGPG	1
BV19	BJ2.7	CASSIRSSYEQYFGPG	1
BV19	BJ2.7	CASSIRSAYEQYFGPG	1
BV19	BJ2.7	CASSIRSAYEQYFGPG	1
BV19	BJ2.7	CASSILASYEQYFGPG	1
BV19	BJ2.7	CASSTLASYEQYFGPG	1
BV19	BJ2.7	CASSTGTSWEQYFGPG	1
BV19	BJ2.5	CASSSCSGWTQYFGPG	1
BV19	BJ2.3	CASSIRSTDTQYFGPG	1
BV19	BJ2.3	CASSIFQTDTQYFGPG	1
BV19	BJ2.2	CASSITHTGELFFGEG	1
BV19	BJ2.1	CASSIRSGFEQFFGSG	1
BV19	BJ2.1	CASSIDGGNEQFFGPG	1
BV19	BJ1.2	CASSNQGPVGYTFGSG	1
BV19	BJ1.1	CASSIGSATEAFFGQG	1
	Clonotypes :30		
	Sequences:120		120

Donor 6 (Selli D 002)

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2.7	CASSIRSSYEQYFGPGT	10
BV19	BJ2.1	CASSGRAGVEQFFGPGT	8
BV19	BJ2.1	CASSIGTGEQFFGPGT	7
BV19	BJ2.7	CASSIRAAYEQYFGPGT	5
BV19	BJ2.3	CASSPRSGDTQYFGPGT	5
BV19	BJ2.7	CASSIRSSYEQYFGPGT	4
BV19	BJ2.7	CASSIRSSYEQYFGPGT	4
BV19	BJ2.7	CASSPRSSYEQYFGPGT	4
BV19	BJ2.7	CASSIRSSYEQYFGPGT	3
BV19	BJ2.7	CASSMRSSYEQYFGPGT	3
BV19	BJ2.2	CASSISSTGELFFGPGT	3
BV19	BJ2.7	CASSIRSSYEQYFGPGT	2
BV19	BJ2.7	CASSIRSSYEQYFGPGT	2
BV19	BJ2.7	CASSIRSSYEQYFGPGT	2
BV19	BJ2.7	CASSIRSSYEQYFGPGT	2
BV19	BJ2.7	CASSTRSSYEQYFGPGT	2
BV19	BJ2.7	CASSSRSSYEQYFGPGT	2
BV19	BJ2.5	CASSSRSGETQYFGPGT	2
BV19	BJ2.1	CASSGQAGVEQFFGPGT	2
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSTRSSYEQYFGPGT	1
BV19	BJ2.7	CASSLRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRTSYEQYFGPGT	1
BV19	BJ2.7	CASSSRSAYEQYFGPGT	1
BV19	BJ2.7	CASSSRASYEQYFGPGT	1
BV19	BJ2.5	CASSSRSGETQYFGPGT	1
BV19	BJ2.3	CASSSRSTDTQYFGPGT	1
BV19	BJ2.3	CASSPRSGDTQYFGPGT	1
BV19	BJ2.3	CASSGRGTDTQYFGPGT	1
BV19	BJ2.3	CASSTRSTDTQYFGPGT	1
BV19	BJ2.3	CASSPRSGDTQYFGPGT	1
BV19	BJ2.1	CASSIRSSVEQFFGPGT	1
BV19	BJ2.1	CASSIRSGGEQFFGPGT	1
BV19	BJ1.2	CASSIGIYGYTFGPGT	1
BV19	BJ1.1	CASSIRDGVNTEAFFGPGT	1
	Clonotypes :40		
	Sequences :93		93

Donor 3 (Fliam)

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2.2	CASSSRSTGELFFGEGS	23
BV19	BJ2.7	CASSVRSSYEQYFGPGT	14
BV19	BJ2.7	CASSSRSAYEQYFGPGT	13
BV19	BJ1.5	CASSIPTVSVGAQHFGDGT	12
BV19	BJ2.7	CASSTRSGYEQYFGPGT	9
BV19	BJ2.1	CASSIRSTHEQFFGPGT	6
BV19	BJ2.2	CASSIRSTGELFFGEGS	4
BV19	BJ2.1	CASSSRSGHEQFFGPGT	4
BV19	BJ1.5	CASSFYSNQPQHFGDGT	3
BV19	BJ2.7	CASSIRSAYEQYFGPGT	2
BV19	BJ2.7	CASSTRSSYEQYFGPGT	2
BV19	BJ2.7	CASSIRSAYEQYFGPGT	2
BV19	BJ1.1	CASSIRAATEAFFGQGT	2
BV19	BJ2.7	CASSIRSAYEQYFGPGT	1
BV19	BJ2.7	CASSIRAGVEQYFGPGT	1
BV19	BJ2.7	CASSVQEGPTYEQYFGPGT	1
BV19	BJ2.7	CASSLRASGEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSAYEQYFGPGT	1
BV19	BJ2.7	CASSPASGSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.3	CASSIRSTDTQYFGPGT	1
BV19	BJ2.3	CASSIRSTDTQYFGPGT	1
BV19	BJ2.3	CASSTRSADTQYFGPGT	1
BV19	BJ2.3	CASSTIAGGTDTQYFGPGT	1
BV19	BJ2.2	CASSSRSAGELFFGEGS	1
BV19	BJ2.2	CASSRRSTGELFFGEGS	1
BV19	BJ2.1	CASSIFGLNEQFFGPGT	1
BV19	BJ2.1	CASSISAGPYNEQFFGPGT	1
BV19	BJ2.1	CASSILGLHEQFFGPGT	1
BV19	BJ2.1	CASSMGLVSFGQFFGPGT	1
BV19	BJ2.1	CASSIRSSYNEQFFGPGT	1
BV19	BJ2.1	CASSIKSSYNEQFFGPGT	1
BV19	BJ1.6	CASSIGNSPLHFGNGT	1
BV19	BJ1.6	CASSASRGEVGSPLHFGNGT	1
BV19	BJ1.5	CASSVGLGQPQHFGDGT	1
BV19	BJ1.5	CASSIPTVSVGAQHFGDGT	1
BV19	BJ1.5	CASSPRSSEPQHFGDGT	1
BV19	BJ1.5	CASSPGQDYQPQHFGDGT	1
BV19	BJ1.3	CASSTPDRGTISGNTIYFGEGS	1
BV19	BJ1.2	CASSPPFVGGHGYTFGSGT	1
BV19	BJ1.1	CASSIPATEAFFGQGT	1
	Clonotypes:42		
	Sequences:125		125

Donor 2 (Gilko)

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2.1	CASSIRAGYEQFFGPGT	40
BV19	BJ1.5	CASSIRSNQPQHFGDGT	16
BV19	BJ2.7	CASSIRSSYEQYFGPGT	6
BV19	BJ1.5	CASSRRSTQPQHFGDGT	6
BV19	BJ2.3	CASSTSSTDTQYFGPGT	5
BV19	BJ2.2	CASSLRSTGELFFGEGS	5
BV19	BJ2.2	CASSQRSTGELFFGEGS	3
BV19	BJ1.3	CASSMRSGNTIYFGEGS	3
BV19	BJ2.7	CASSIRSGYEQYFGPGT	2
BV19	BJ2.7	CASSMRSSYEQYFGPGT	2
BV19	BJ2.3	CASSIRSTDTQYFGPGT	2
BV19	BJ2.2	CASSITGGEGGELFFGEGS	2
BV19	BJ2.7	CASSTRSTYEQYFGPGT	1
BV19	BJ2.7	CASSIYRLGYEQYFGPGT	1
BV19	BJ2.3	CASSTWSTDTQYFGPGT	1
BV19	BJ2.2	CASSGRSAGELFFGEGS	1
BV19	BJ2.2	CASSLRSTGELFFGEGS	1
BV19	BJ1.5	CASSRRSTQPQHFGDGT	1
	Clonotypes :18		
	Sequences :98		98

Donor 1 (Gilan)

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2.7	CASSIRSAYEQYFGPGT	13
BV19	BJ1.5	CASSIFSNQPQHFGDGT	10
BV19	BJ1.2	CASSIGHYGYTFGSGT	10
BV19	BJ1.1	CASSIRSVAEAFFGQGT	9
BV19	BJ1.1	CASSIRSGNTEAFFGQGT	7
BV19	BJ2.3	CASSIVHAADTQYFGPGT	6
BV19	BJ1.1	CASSIRSVAEAFFGQGT	6
BV19	BJ1.5	CASSTLTGGHQPQHFGDGT	5
BV19	BJ2.3	CASSVRSGDTQYFGPGT	4
BV19	BJ2.7	CASSIRSSYEQYFGPGT	3
BV19	BJ1.2	CASSQGFYGYTFGSGT	3
BV19	BJ2.7	CASSIRSAYEQYFGPGT	2
BV19	BJ2.1	CASSIFSAGNEQFFGPGT	2
BV19	BJ2.1	CASSILGASYNEQFFGPGT	2
BV19	BJ1.5	CASSIRSGEPQHFGDGT	2
BV19	BJ1.2	CASSMGSGYGYTFGSGT	2
BV19	BJ1.1	CASSIHSGGNTEAFFGQGT	2
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSIRSAYEQYFGPGT	1
BV19	BJ2.7	CASSIRSSYEQYFGPGT	1
BV19	BJ2.7	CASSTRSAYEQYFGPGT	1
BV19	BJ2.7	CASSARVGNEQYFGPGT	1
BV19	BJ2.7	CASSMRSAYEQYFGPGT	1
BV19	BJ2.5	CASSTRSQETQYFGPGT	1
BV19	BJ2.5	CASSARSAETQYFGPGT	1
BV19	BJ2.3	CASSIRSTDTQYFGPGT	1
BV19	BJ2.3	CASSALGAGGDTQYFGPGT	1
BV19	BJ2.3	CASSMRSTDTQYFGPGT	1
BV19	BJ2.2	CASSTRAAGELFFGEGS	1
BV19	BJ2.1	CASSIRSSYNEQFFGPGT	1
BV19	BJ2.1	CASSASGRYNEQFFGPGT	1
BV19	BJ1.5	CASSVYSNQPQHFGDGT	1
BV19	BJ1.2	CASSIGLYGYTFGSGT	1
	Clonotypes :35		
	Sequences :106		106

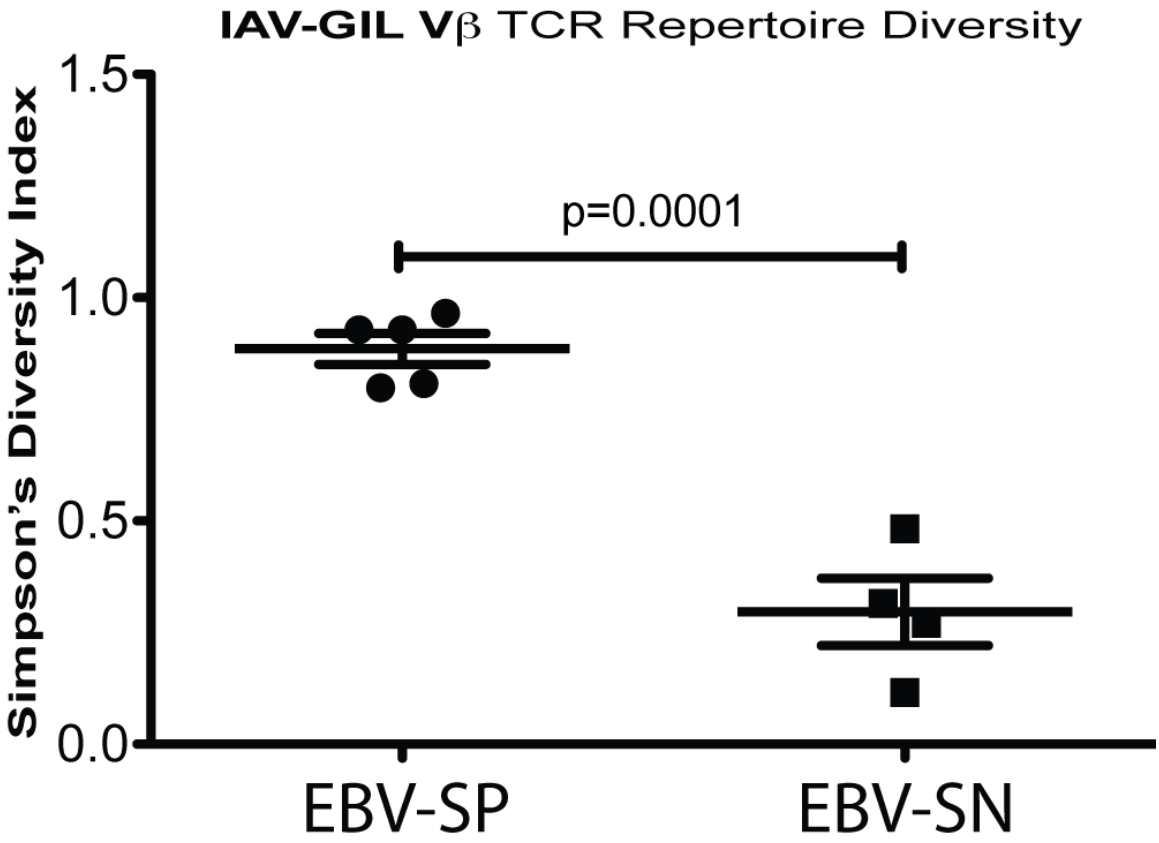

Figure 3.9: Significantly decreased diversity of the IAV-GIL specific V TCR repertoire in EBV-SN as compared to EBV-SP donors as demonstrated by using the Simpson Diversity Index. For these studies IAV-GIL tetramer+ cells were sorted from each cell line, and subjected to SMART-Race PCR to identify individual clonotypes by sequencing the CDR3 as shown in tables 3.2 and 3.3.

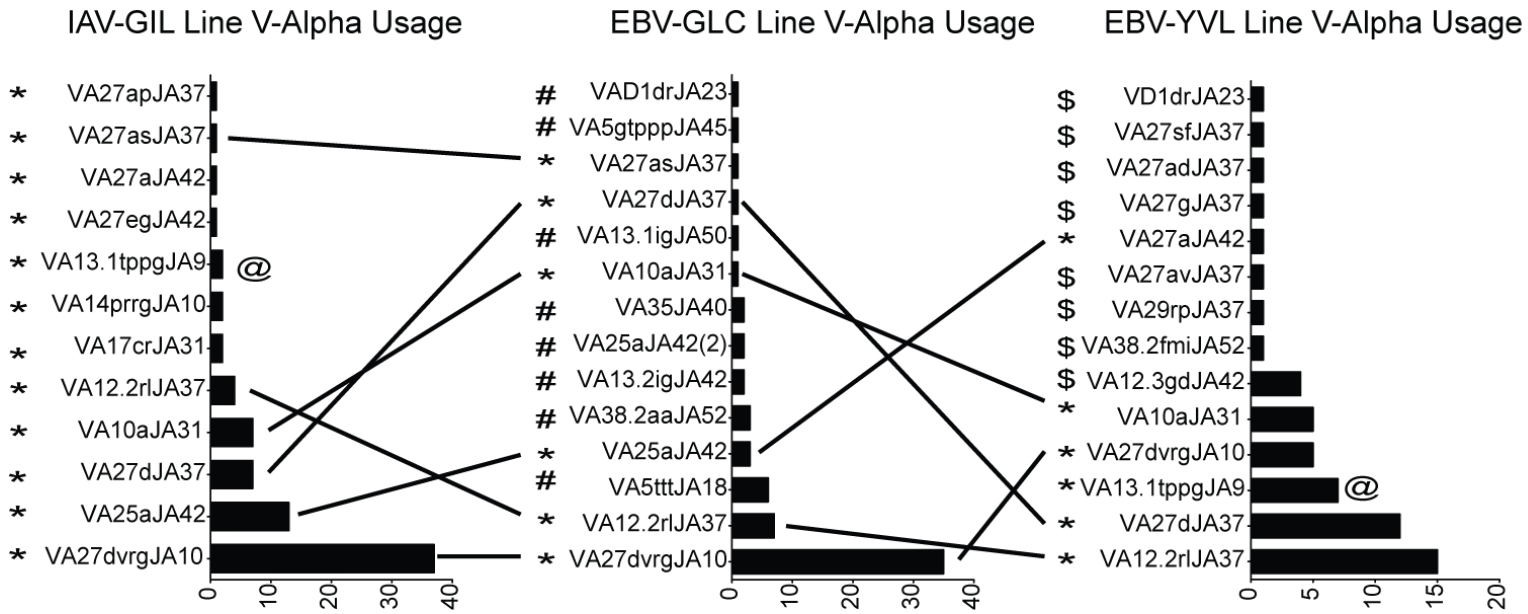

Figure 3.10: EBV-SN adults display an altered oligoclonal IAV-GIL specific TCRα repertoire: Representative clonal distribution (Donor 1) of an IAV-GIL-specific Vα27 dominated TCR repertoire in EBV-SN adults showing a highly oligoclonal response. The same clone dominates in the IAV-GIL and EBV-GLC lines. The symbol beside each clonotype indicates whether a clonotype is present in a specific culture: * for IAV-GIL line, # for EBV-GLC line, and $ for EBV-YVL line. The lines drawn between cultures indicate the presence of that same clonotype in different cultures. The @-symbol represents a clone found in the IAV-GIL and EBV-YVL lines but not the EBV-GLC line.

dominant clonotypes contained either Vα27 or Jα42, but not both together as was usually observed in the EBV-SP. Donor 1 T-cells used predominantly Vα27Jα10, whereas the other two donors used Jα42 but not Vα27 **(Table 3.3)**.

L. Repertoire organization coincides with differential cytokine production

When we compared both the Vα and β TCR repertoires of the IAV-GIL-specific cells between the 3 different epitope stimulated lines from donor 1 it was evident that the most dominant clonotype was identical in each line **(Fig 3.8, & 3.10)**. However, the EBV-YVL line did have a second dominant Vα clonotype that was present only at low frequency in the other two cultures. The sub-dominant IAV-GIL-specific Vα and β clonotypes differed significantly between the cultures, suggesting that each epitope could select a different group of T-cell clones. These differences in TCR repertoire would be consistent with the different patterns of functional cytokine responses in each culture upon stimulation with the 3 different cross-reactive ligands **(Fig 3.4)**.

M. Summary

I have therefore demonstrated that the IAV-GIL-restricted memory pool in EBV-SN adults possessed a highly functional cross-reactive response that reacted against EBV lytic antigens. These cross-reactive T-cell responses were shown by their ability to proliferate and secrete protective antiviral cytokines such as IFNγ and MIP-1β in response to EBV lytic antigens. Additionally, CD8 T-cells from these EBV-SN adults were able to kill EBV-infected targets in a lytic antigen dependent manner, and they also killed EBV lytic antigen peptide-loaded targets.

EBV-SN IAV-GIL Line VB Usage

EBV-SN Donor 1

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2-1	**CASSIVGGNEQFF**	49
BV19	BJ2-7	**CASSIRSSYEQYF**	3
BV19	BJ2-7	**CASSIRSAYEQYF**	1
BV19	BJ2-7	**CASSFDGVYEQYF**	1
BV19	BJ2-3	**CASSARATDTQYF**	1
BV19	BJ2-3	**CASSGRSADTQYF**	1
BV19	BJ2-2	**CASSMRSTRELFF**	1
BV19	BJ2-1	**CASSIIGGYEQFF**	1
BV19	BJ2-1	**CASSIVGGNEQFF**	1
BV19	BJ2-1	**CAGSIVGGNEQFF**	1
BV19	BJ2-1	**CASSIVGGNGQFF**	1
BV19	BJ1-1	**CASSSHAGGNTEAFF**	1
BV18	BJ2-7	**CASSLTGSTYEQYF**	1
	Clonotyes:13		
	Sequences:63		**63**

EBV-SN Donor 2

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ1-4	**CASSIFGEKLFF**	32
BV19	BJ2-7	**CASSIRSAYEQYF**	12
BV7-2	BJ2-7	**CASSLVGTGPYEQYF**	2
	Clonotyes:3		
	Sequences:46		**46**

EBV-SN IAV-GIL Line VA Usage

EBV-SN Donor 1

VA family	JA family	CDR3 amino acid sequence	# of copies
AV27	AJ10	**CADVRGTGGGNKLTF**	37
AV25	AJ42	**CAANYGGSQGNLIF**	13
AV27	AJ37	**CAGDGSSNTGKLIF**	7
AV10	AJ31	**CVVSANNNARLMF**	7
AV12-4	AJ37	**CAVRLSNTGKLIF**	4
AV14	AJ10	**CAMREPRRGGGGNKLTF**	2
AV13	AJ9	**CAASTPPGTGGFKTIF**	2
AV17	AJ31	**CATCRNNNARLMF**	2
AV27	AJ42	**CAGEGGGSQGNLIF**	1
AV27	AJ42	**CAAGGSQGNLIF**	1
AV27	AJ37	**CAGASGSSNTGKLIF**	1
AV27	AJ37	**CAGAPGSSNTGKLIF**	1
AV12-1	AJ52	**CVPPEAGGTSYGKLTF**	1
	Clonotyes:13		
	Sequences:79		**79**

EBV-SN Donor 2

VA family	JA family	CDR3 amino acid sequence	# of copies
AV26-2	AJ42	**CILRDLTLGGSQGNLIF**	39
AV27	AJ42	**CAGGGSQGNLIF**	8
AV25	AJ42	**CAGNYGGSQGNLIF**	5
AV36	AJ43	**CAVDPSYNNNDMRF**	4
AV27	AJ37	**CAGARGSSNTGKLIF**	3
AV13-2	AJ42	**CAENGGGGSQGNLIF**	3
AV38-2	AJ23	**CAYRGLFYNQGGKLIF**	2
AV13-2	AJ42	**CAASGGGSQGNLIF**	2
AV12-3	AJ42	**CAMSGDGGSQGNLIF**	1
	Clonotyes:9		
	Sequences:67		**67**

EBV-SN EBV-GLC Line VB Usage

EBV-SN Donor 1

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2-1	**CASSIVGGNEQFF**	51
BV19	BJ2-7	**CASSIRSSYEQYF**	5
BV19	BJ1-1	**CASSSHAGGNTEAFF**	4
BV19	BJ1-5	**CASSWLSNQPQHF**	4
BV19	BJ2-1	**CAGSIVGGNEQFF**	4
BV19	BJ2-2	**CASSMRSTGELFF**	4
BV19	BJ2-1	**CASSTYSTNEQFF**	1
BV19	BJ2-2	**CASSLRSSGELFF**	1
BV19	BJ2-2	**CASSTRSSGELFF**	1
BV19	BJ2-5	**CASSIRGSETQYF**	1
BV19	BJ2-7	**CASSPRSGGEQYF**	1
	Clonotyes:11		
	Sequences:77		**77**

EBV-SN Donor 2

VB family	JB family	CDR3 amino acid sequence	# of copies
BV19	BJ2-2	**CASSIRSTGELFF**	31
BV19	BJ2-7	**CASSIRSSYEQYF**	5
	Clonotyes:2		
	Sequences:36		**36**

EBV-SN EBV-GLC Line VA Usage

EBV-SN Donor 1

VA family	JA family	CDR3 amino acid sequence	# of copies
AV27	AJ10	**CADVRGTGGGNKLTF**	35
AV12-4	AJ37	**CAVRLSNTGKLIF**	6
AV5	AJ18	**CAETTTDRGSTLGRLYF**	6
AV25	AJ42	**CAANYGGSQGNLIF**	3
AV38-2	AJ52	**CAYAANAGGTSYGKLTF**	3
AV13-2	AJ42	**CAENIGGGSQGNLIF**	2
AV25	AJ42	**CAANYGGSQGNLIF**	2
AV35	AJ40	**CAGQPGTYKYIF**	2
AV10	AJ31	**CVVSANNNARLMF**	1
AV12-4	AJ37	**CAVRLSNTGKLIF**	1
AV13-1	AJ9	**CAASTPPGTGGF**	1
AV13-2	AJ50	**CAASIGTSYDKVIF**	1
AV27	AJ37	**CAGDGSSNTGKLIF**	1
AV27	AJ37	**CAGASGSSNTGKLIF**	1
AV5	AJ45	**CAGTPPPGGGADGLTF**	1
AV9-2	AJ10	**CALSTGGGNKLTF**	1
DV1	AJ23	**CALGDRYNQGGKLIF**	1
	Clonotyes:17		
	Sequences:68		**68**

EBV-SN Donor 2

VA family	JA family	CDR3 amino acid sequence	# of copies
AV26-2	AJ42	**CILRDLTLGGSQGNLIF**	22
AV36	AJ43	**CAVDPSYNNNDMRF**	14
AV27	AJ37	**CAGTHGSSNTGKLIF**	8
AV12-3	AJ42	**CAMSGHGGSQGNLIF**	2
AV27	AJ42	**CAGGGSQGNLIF**	2
	Clonotyes:5		
	Sequences:48		**48**

Table 3.3. Clonal composition of the IAV-GIL specific TCR alpha and beta repertoire within EBV-SN adults. Two EBV-SN donors showing the distribution of IAV-GIL reactive clones showing V-β family and J-β family usage, CDR3 amino acid compositions, and number of clones detected.

These cells also showed the potential to enter the mucosal epithelium where EBV infection initially occurs, thus potentially mediating resistance to infection upon exposure to EBV.

These data strongly suggest that the characteristics of an individual's TCR repertoire determines the outcome of disease when exposed to a new pathogen. In EBV-SP donors the IAV-GIL-specific response, while being restricted to the Vβ19 family, is quite diverse without dominant clones and maintains a xRSx CDR3B motif (Lehner et al., 1995; Lawson et al., 2001; Naumov et al., 2006; 2008; Clute et al., 2010). In contrast, the EBV-SN adults also maintained a Vβ19 family restriction, but had an oligo-clonal response dominated by one clonotype with unique CDR3β motifs, which may have a better structure to recognize both antigens with higher avidity and contribute to a potent antiviral response.

Chapter IV: The use of humanized mice to study human EBV and IAV infections

Many experimental models of infection are set up in a murine system. Mice are relatively inexpensive, they are genetically malleable, and there are many reagents available for use in their study. However, mice are not always susceptible to human pathogens, and also findings in mice do not necessarily transfer over to be relevant to human disease. Therefore, a model system is needed that has the clinically relevant human tissues and also the flexibility of an animal model. By using a humanized mouse system one would potentially be able to conduct clinically relevant studies using pathogens that infect humans in a less restrictive environment without putting patients at risk.

To conclusively show that heterologous immunity is actively protecting EBV-SN adults from the establishment of a productive EBV infection, one would have to deliberately expose an individual to the virus. Clearly this is not an acceptable risk and could endanger the health of an individual. A humanized mouse model could allow one to address this question.

There have been many models of humanized mice, as was discussed earlier in the introduction (**Fig1.6**). Here, we will be discussing mice that have been engrafted with human hematopoietic stem cells. In particular we will be examining NOD SCID Il2rg$^{-/-}$ mice. Three different models were used. First was a straight NOD SCID Il2rg$^{-/-}$ (NSG) that was reconstituted with human CD34+ hematopoietic stem cells. These mice engraft fairly well yet lack human MHC,

eliminating the possibility of detecting clinically relevant antigen specific T-cells. Additionally the T-cells that develop in these mice are not educated on a human MHC. This would result in a human T-cell pool that is educated on murine MHC. This generates many complications.

The second model was a NOD SCID il2γ$^{-/-}$ HLA-A2 (NSG-HLA-A2/HHD) transgenic mouse that was reconstituted with human CD34+ hematopoietic stem cells. These mice have a human MHC which will allow the human T-cells to be educated on human MHC, however there are no human tissues such as the human thymus which is important for T-cell development and appropriate positive and negative selection. This model assumes that human T-cells can migrate to the murine thymus and develop properly.

The third model was a NOD SCID il2γ$^{-/-}$ HLA-A2 transgenic mouse that was reconstituted with human CD34+ hematopoietic stem cells from the fetal liver and also was implanted with fetal thymus and fetal liver (BLT mouse). These mice show the most promise. They have human tissues for the T-cells to be educated on and also human MHC molecules systemically for the T-cells to recognize.

Since EBV is known to only infect human B-cells and mucosal epithelial cells we must first determine whether these mice can be infected with EBV. Since these mice are reconstituted with a human immune system, and should have human B-cells, we hypothesized that these mice would develop an infection and a functional immune response to the virus. To determine the establishment

of infection we will be looking for an immune response. This will also show that these mice are capable of mounting an immune response to EBV. Additionally we also have to determine whether these mice can develop memory to IAV. There are mouse-adapted strains of influenza that should ensure the infectibility of these mice. However, we need to determine whether they can mount an immune response to the virus, resolve the infection and also generate a memory response.

A. Humanized mice demonstrate pathology from viral infection by weight loss.

One indicator of the establishment of an active infection and immune response is the onset of weight loss. In all three murine models (NSG, NSG-HLA-A2/HHD, and BLT) mice infected intraperitoneally (i.p.) with EBV lost weight as compared to uninfected controls **(Fig 4.1)**. In the NSG model a virus dose of either 100U of EBV supernatant in 100 µL or 400U of EBV supernatant in 400µL resulted in weight loss. Additionally, control mice that did not receive donor cells, but had been infected with EBV did not lose weight. These mice would have lacked B-cells, the primary EBV reservoir. These results suggest that an infection has occurred and the cells necessary for infection by EBV must be present. EBV infection resulted in a slow progression of weight loss with only ~10% reduction in weight. IAV infection on the other hand, showed a rapid weight loss of ~20%. These differences are probably attributable to the difference in viral and immune kinetics between the two viruses. IAV is known to

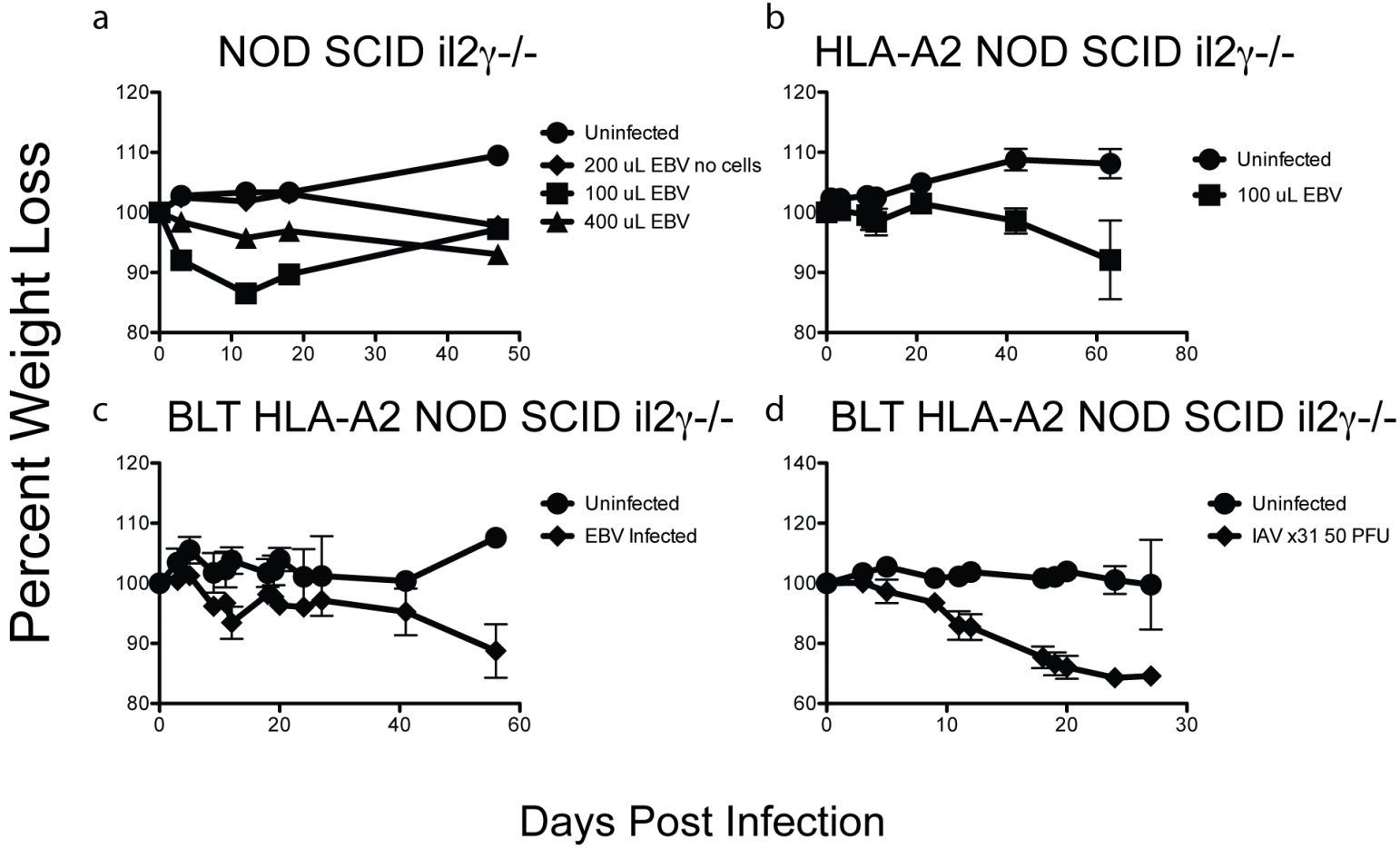

Figure 4.1: Humanized mice infected with either EBV or IAV lose weight in response to infection. a) NSG, b) NSG-HLA-A2/HHD, and c) BLT mice were infected with 100 units of EBV i.p. d) BLT mice were infected with 50 PFU IAV-x31 in 50μL PBS i.n. while anesthetized with metafane. Weights were monitored over the indicated time periods. Weights were averaged and normalized to day 0 to determine percent weight loss.

replicate rapidly while little is known about the rate of EBV replication, particularly early in infection as patients with AIM are thought to have been exposed 4-6 weeks before presentation with symptoms (Luzuriaga and Sullivan, 2010).

B. Splenomegaly and T-cell expansions in viral infection

A classic hallmark of a viral infection is the expansion of lymphocytes in response to the infection. EBV in particular shows an expansion of activated CD8 T-cells. To determine whether our three models of humanized mice are producing an effective immune response to EBV infection we sought to examine the expansion of lymphocytes within these mice.

Figure 4.2a shows that in NSG mice there was an onset of splenomegaly at 7 weeks post infection with EBV. Note the increase in physical size of the spleen from a representative from each group of mice. Next we wanted to determine which cell types were responsible for this increase in spleen size. Monoclonal antibodies directed against human and murine CD45 were used to identify human cells within the splenocytes. Additionally monoclonal antibodies specific for CD3, CD4, CD8, and either CD19 or CD20 were used to distinguish T-cells from B-cells and helper T-cells from cytotoxic T-cells. Frequencies were then compared to cell numbers to determine population specific cell numbers. As shown in **Fig 4.2b** mice that were infected with EBV showed an increase in human lymphocytes, particularly T-cells. These data again suggest that these three humanized mouse models were successfully infected with EBV and were mounting an immune response to the infection.

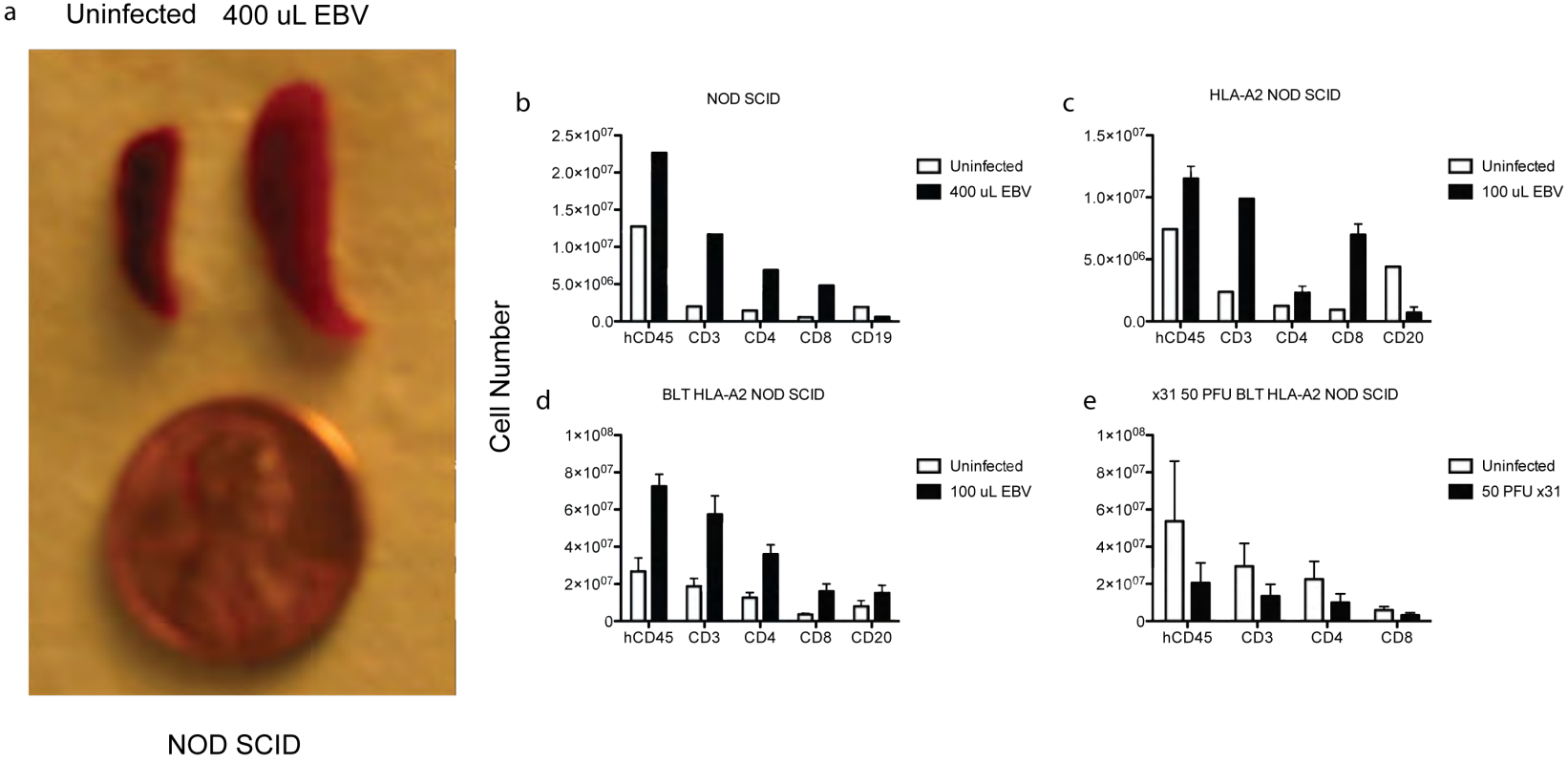

Figure 4.2. Humanized mice show an increase in cell numbers and splenomegly in response to viral infection. a) Humanized mice show an increase in spleen size upon infection with EBV. A spleen from a NSG mouse taken at 7 weeks post infection with EBV showed an increase in overall size. NSG-HLA-A2/HHD mice showed a similar increase in size (not shown). BLT mice had a larger increase in spleen size upon EBV infection (not shown). Infection with IAV-x31 showed a decrease in spleen size on day 28 of infection (not shown). b-d) NSG, NSG-HLA-A2/HHD, and BLT mice demonstrated an expansion of human lymphocytes upon infection with EBV primarily consisting of T-cell expansions at 7 weeks post infection. e) BLT mice infected with IAV showed a decrease in human cells 28 days post infection

In contrast to EBV infection in the humanized mice, IAV infection results in a lymphopenic state within the mice. **Figure 4.2e** shows that there was a general decrease in human cells within mice infected with 50 PFU of IAV x31. This also resulted in overall lower T-cell numbers in infected mice. Although we lack viral titers to say for certain it appears these mice were unable to control the infection, as these mice had lost 20% of their weight by day 28 and appeared moribund, requiring euthanasia.

C. Increased frequency of activated CD8 T-cells during infection

During an immune response T-cells get activated and develop effector functions in order to control infection. While gaining these functions they also develop phenotypic markers, which enable one to identify their generation. To this end we sought to identify effector functions and phenotypes in the T-cells within the humanized mice infected with EBV and IAV.

In **figure 4.3a** we have gated on live human CD3+ cells and then looked at various effector markers. In addition, we also examined the frequencies of CD4 and CD8 cells within the T-cell pool. During an infection there is an increase in the frequency of CD8 cells as compared to CD4 cells, resulting in a skewed ratio of CD4 to CD8 T-cells. As shown in **figure 4.3a,** during the EBV infection there was a shift to an increased frequency of CD8 cells as compared to CD4 cells at 8 weeks post infection. Additionally, there was also an increased frequency of cells expressing the effector marker HLA-DR, an increased level of granzyme-B+ cells, and an increase of antigen-experienced cells as

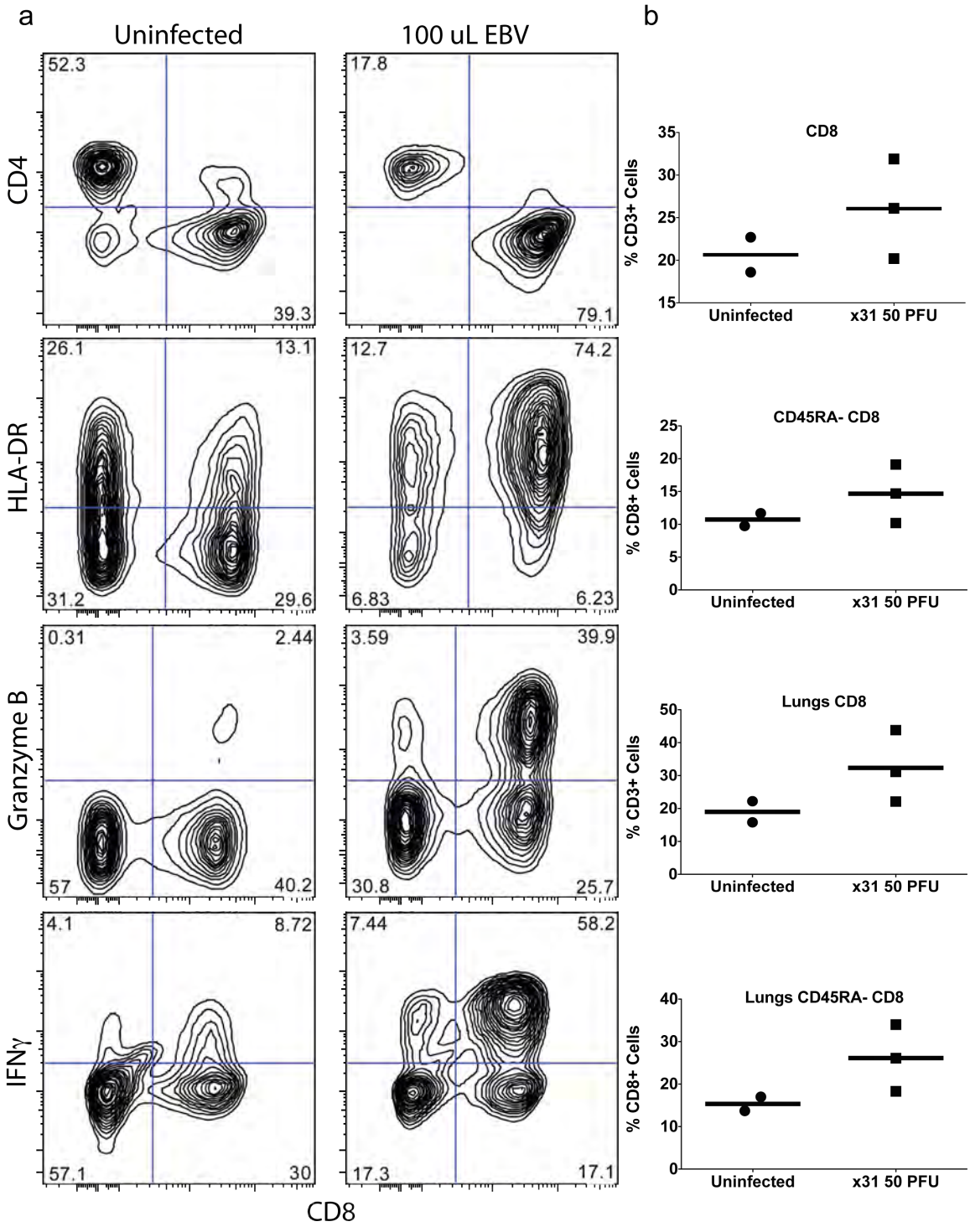
a
Uninfected
100 uL EBV
CD4
HLA-DR
Granzyme B
IFNγ
CD8
52.3
39.3
17.8
79.1
26.1
13.1
31.2
29.6
12.7
74.2
6.83
6.23
0.31
2.44
57
40.2
3.59
39.9
30.8
25.7
4.1
8.72
57.1
30
7.44
58.2
17.3
17.1
b
CD8
% CD3+ Cells
35
30
25
20
15
Uninfected
x31 50 PFU
CD45RA- CD8
% CD8+ Cells
25
20
15
10
5
0
Lungs CD8
50
40
Lungs CD45RA- CD8

Figure 4.3. Humanized mice show an increase in activated T-cells when infected. a) NSG mice either uninfected (left column) or infected with 100 U EBV IP (right column) at 7 weeks post infection. Population gated on live human CD3+ cells. CD8 cells show activation by inversion of CD4:CD8 ratios, increased expression of HLA-DR, granzyme B, and IFN production when stimulated with PMA and Ionomycin. b) Day 28 post infection of BLT mice either uninfected or infected with 50 PFU IAV-x31 i.n. The first and third graphs show the frequencies of CD8 cells gated on live human CD3+ cells from either the spleen or lung as indicated. The second and fourth graphs show the frequencies of activated CD8 cells as measured by their lack of CD45RA expression within the spleen and lung as indicated.

measured by IFNγ staining in response to PMA stimulation. **Figure 4.3b** shows an increase in the frequency of CD8 cells in IAV infected mice within the spleen and lung at day 28 post infection. Additionally, there was an increase in the frequency of effector CD8 cells (CD45RA negative cells) within the spleen and lungs of IAV infected mice.

To determine whether there was an active infection and evidence of immune-mediated pathology in these mice histological H and E staining was performed on histological sections of lungs and liver of these mice. The top row of **figure 4.4** shows a focal mononuclear lymphocytic infiltrate within the liver of EBV infected mice. Mice infected with IAV also showed pathology. Uninfected mice have clear open airways, whereas mice infected with IAV show enhanced pathology with pneumonic consolidation, hemorrhaging, and bronchiolization. These pathologies show us that there was an active infection with evidence of immune-mediated pathology occurring within these mice.

D. Summary

From the evidence put forth in this thesis it appears that the humanized mice were indeed becoming infected with EBV and IAV. The mice lost weight in response to infection, showed an increase in T-cell activation, and showed an increase in immune-mediated pathology. While the IAV infected mice showed no increase in total cell numbers, presumably because of an uncontrolled infection, the EBV infected mice did show an increase in total cell number in the spleen.

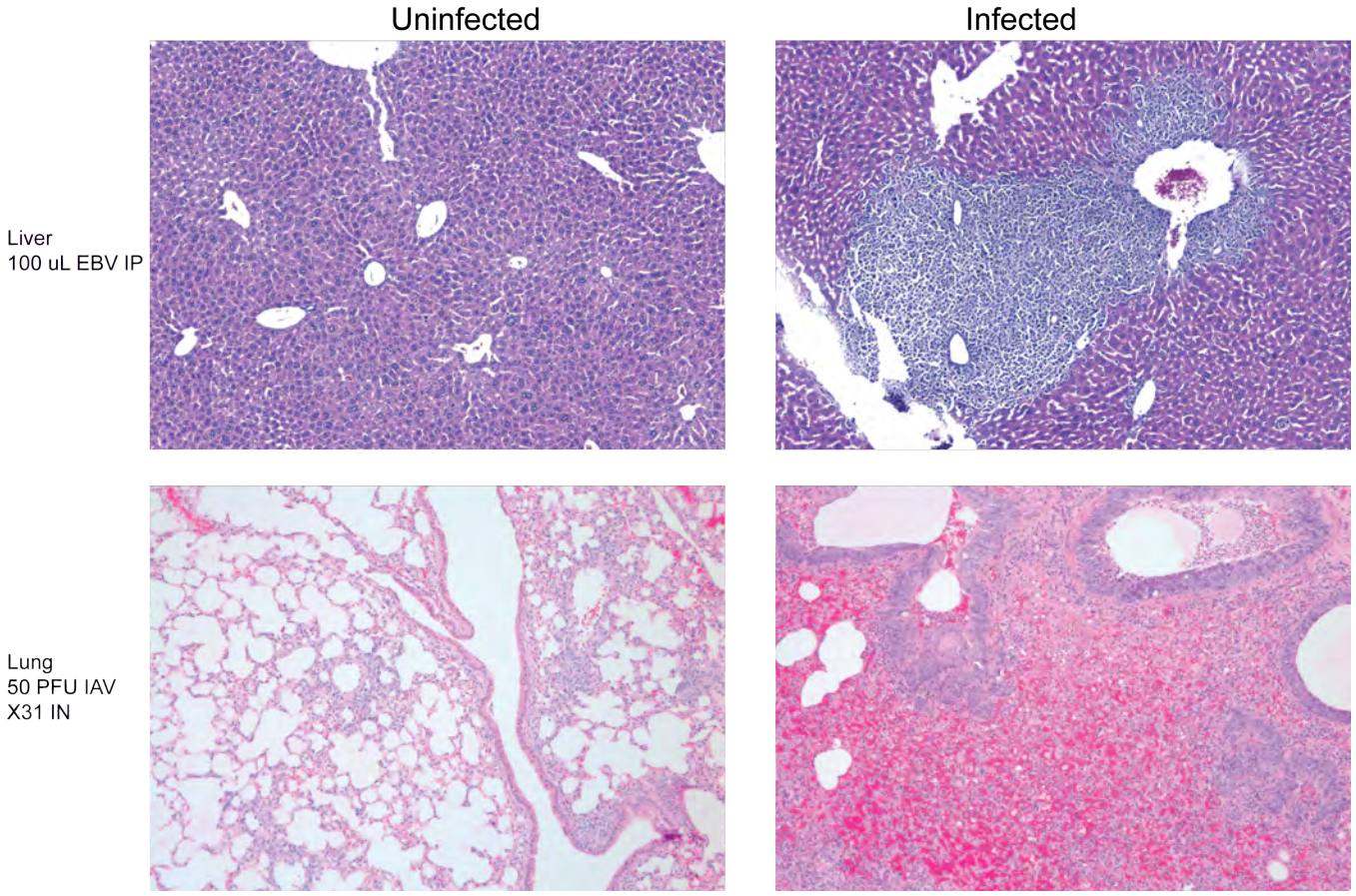

Figure 4.4. Increased pathology in infected humanized mice. Histological Hematoxylin/eosin (H&E) staining was performed on sections of liver and lung tissues from EBV and IAV-x31 infected animals, respectively. Staining was performed using standard procedures. The top row shows liver from NSG mice infected with 100U EBV IP 7 weeks later. The infected samples show focal mononuclear cell infiltrates. The bottom row shows lung from BLT mice infected i.n. with 50 PFU IAV-x31 at day 28 post infection. The uninfected sample shows normal lung with open airways. The infected lung shows massive pathology with pneumonic consolidation, hemorrhaging and bronchiolization.

However, using our present models the quality of the response and similarity to human disease observed in the humanized mouse model are not optimum. For the EBV infection it would be more desirable to see a larger CD8 T-cell response within the mice during infection. For the IAV infection model it would be nice to see the mice be able to mount an immune response and clear the virus, but instead they succumb to the infection. Thus, at this time we have not developed the IAV model to the point that we could test our hypothesis of IAV-GIL specific T-cell mediated protection from EBV infection. These data are promising as the humanized mouse model is constantly improving. Hopefully, with constant improvements being made there will be a model that will duplicate the human immune system more completely.

Chapter V: Discussion

Previous reports of heterologous immunity during human infections have focused on detrimental heterologous immunity, resulting from an altered immune response leading to increased and altered immunopathology (Mathew et al., 1998; Clute et al., 2005; Urbani et al., 2005). It is much easier to identify and study overt pathology than to examine the role of heterologous protective immunity in human infections, as a person is unlikely to come to medical attention when they have an asymptomatic infection. However, many infections have variable outcomes ranging from asymptomatic to severe pathology, even death. These severe outcomes provide hints as to where one could investigate the role of heterologous immunity during human infection. Some specific examples include viral infections such as EBV where AIM develops in young adults who have large complex memory populations, while younger children are relatively asymptomatic (Clute et al., 2005). Another example is the variation in severity of hepatitis in HCV infected patients, or the variation in outcome from mild fever to severe dengue hemorrhagic fever upon DENV infection (Mathew et al., 1998; Urbani et al., 2005). Interestingly, these infections have had T-cell cross-reactive responses associated with their disease outcome. However, in this thesis I demonstrated a high frequency of highly functional EBV cross-reactive IAV-specific memory CD8 T-cells in EBV-SN individuals. I believe this could be, for the first time, a description of beneficial protective heterologous immunity in humans, where heterologous immunity could prevent the

establishment of a productive EBV infection. These results also suggest that memory CD8 T-cells may be able to mediate sterilizing immunity and that an individual's unique TCR repertoire determines his or her fate.

We have demonstrated that the IAV-GIL-specific memory T-cell pool in EBV-SN adults possesses a highly cross-reactive response that reacts against EBV lytic antigens. These cross-reactive responses are shown by the ability to proliferate and secrete protective, antiviral cytokines such as IFNγ and MIP-1β in response to EBV lytic antigens. Additionally, CD8 T-cells from these EBV-SN adults are able to kill EBV-infected targets in a lytic antigen-dependent manner, and they also kill EBV lytic antigen peptide-loaded targets. These cells, by expressing CD103, also showed the potential to enter the mucosal epithelium where EBV infection initially occurs, thus potentially mediating resistance to infection upon exposure to EBV.

These data strongly suggest that the characteristic of an individual's TCR repertoire determines the outcome of disease when exposed to a new pathogen. In EBV-SP donors the IAV-GIL-specific response, while being restricted to the Vβ19 family, is quite diverse, often including over 200 TCR Vβ clonotypes, without dominant clones and maintains a xRSx CDR3β motif (Lehner et al., 1995; Clute et al., 2005; Naumov et al., 2006; 2008; Clute et al., 2010). In contrast, the EBV-SN adults also maintain a Vβ19 family restriction, but have an oligo-clonal response dominated by one clonotype with unique CDR3β motifs that may be better structured to recognize both antigens with higher avidity and contribute to

a potent antiviral response. Additionally, the IAV-GIL specific TCR repertoire from AIM patients exhibits similar properties of the EBV-SP donors in that their IAV-GIL specific TCR repertoire is broad and very diverse (Clute et al., 2010). We believe it is this difference in the organization of the IAV-GIL memory CD8 T-cell repertoire that is responsible for protecting these EBV-SN donors from the establishment of a productive EBV infection.

This body of work demonstrates that heterologous immunity has the potential to protect individuals from infection, and it appears that it is the memory CD8 T-cell repertoire that is responsible for the protection. If we could understand how to manipulate or control the memory CD8 T-cell repertoire, we could design vaccination strategies that would select for high avidity cross-reactive memory CD8 T-cells that could protect an individual from an infection that he or she had never encountered. However, great care would have to be taken, as a T-cell repertoire with high avidity to an antigen most likely would be narrow and oligo-clonal. This would leave open the possibility for a viral escape mutant, which would render the vaccination strategy obsolete such as has been described (Cornberg et al., 2006a). The study by Cornberg et. al. used the model of PV-immune mice subsequently challenged with LCMV. This model results in the cross-reactive activation and expansion of memory CD8 T-cells specific for a relatively similar epitope (6 of 8 amino acids in common) shared between the two viruses, NP_{205}. Upon subsequent infection there was a drastic narrowing of the NP_{205} specific memory CD8 T-cell TCR repertoire. PV immune

mice were infected with LCMV clone 13, a strain of LCMV that causes persistent infection, and 8 months after LCMV challenge, after being put under pressure by NP_{205} reactive memory cells, a viral escape mutant emerged that showed a mutation within the NP_{205} epitope. Thus, an artificially altered repertoire could allow for viral escape mutants.

From the work presented in this thesis we have shown that the IAV-GIL specific response within EBV-SN donors is quite distinctive when compared to that of EBV-SP donors. Although the EBV-SN donor group included only five individuals, the demographic characteristics of this group does not appear to differ from our EBV-SP donors. The EBV-SN donor's geographical locations were also varied ranging from Europe to the southwestern United States, showing there is no environmental bias. Thus, there are no obvious unintended biases within the donor pool that might suggest a genetic or geographic component to explain the differences in their EBV infection rate.

The observation of an increased frequency of IAV-GIL specific memory cells cross-reactive with EBV was the primary indication that there might be a unique cross-reactive response in these individuals. This nearly ten-fold increase in IAV-GIL reactive memory cells suggested that this population might have been reactivated on more than one occasion. However, there is no obvious reason why they should have been reactivated any more than EBV-SP donors by exposure to IAV. Perhaps, it is due to frequent and recent encounters with the

cross-reactive antigen, EBV, in the absence of a diverse EBV-specific memory population.

These IAV-GIL reactive cells from EBV-SN donors can proliferate in response to EBV lytic antigens EBV-GLC and EBV-YVL (**Fig 3.2**). These data imply that cross-reactive IAV-GIL memory cells from EBV-SN donors can become activated and divide so as to increase the size of the effector pool reactive against EBV antigens and go on to participate in an immune response to EBV infection. Importantly, a truly naïve population of CD8 T-cells from cord blood does not exhibit this phenomenon of IAV-GIL specific expansion when cultured with EBV lytic antigens.

These IAV-GIL cross-reactive CD8 T-cells from the EBV-SN donors can exert the effector function of cytokine production when stimulated with EBV antigens (Fig 3.4). This is important in mediating protection from infection, as cytokine production is one of the most important aspects of T-cell effector function as cytokines manipulate the environment on which they act, rendering it inhospitable to virus.

After we saw that these IAV-GIL cross-reactive cells could produce cytokines we next naturally wanted to know if they had a similar avidity for IAV-GIL and EBV-GLC. If these cross-reactive IAV-GIL memory cells from EBV-SN donors had a difference in functional avidity as compared to EBV-SP donors this may account for their unique abilities to expand and produce cytokines to the extent they did when grown in the presence of EBV antigens. Remarkably, the

IAV-GIL cross-reactive cells from EBV-SN donors had a 1 and 2 log higher functional avidity to IAV-GIL and EBV-GLC, respectively than T-cells from EBV-SP subjects. This high functional avidity to EBV antigens may be what enables these cross-reactive IAV-GIL specific memory cells to mediate protection against EBV, and in fact mediate sterilizing immunity. Interestingly, two independent groups using two different models have shown that T_{EM} CD8 T-cells can mediate sterilizing protection. One showed that T_{EM} CD8 T-cells were generated after immunization of mice with radiation-attenuated Plasmodium sporozites and that these T_{EM} populations correlated with protection from Plasmodium re-challenge, preventing a chronic infection, while Plasmodium specific Ig levels did not correlate with protection (Schmidt et al., 2010). The second group showed that a SIV vaccine that includes rhesus cytomegalovirus (RhCMV) vectors established persistent, high frequency, SIV-specific T_{EM} responses at potential sites of SIV replication in rhesus macaques and controlled SIV infection early after mucosal challenge before it establishes a chronic infection (Hansen et al., 2011). Taken together these results suggest that T-cell vaccines against chronic infections such as EBV, malaria, and HIV may be feasible if designed correctly.

Curiously though the IAV-GIL cross-reactive cells, despite having high functional avidity to EBV-GLC, do not seem to bind tetramer (**Fig 3.5**). Since TCR/pMHC affinities are ratio of on rates and off rates, it may be that this interaction has a very high on rate, which could be responsible for the increased functional avidity, while an equally high or higher off rate could be responsible for

the lack of tetramer staining. Interestingly, there have been other reports of antigen-specific T-cells that do not bind tetramer while being able to exert effector functions. One group showed that CD8 T-cells reactive to a subdominant IAV epitope could not bind its tetramer when grown in the presence of whole virus infected stimulators, but with continued in vitro stimulation ultimately gained enhanced effector functions and low levels of tetramer staining (Spencer and Braciale, 2000). A second group, using a complex biophysical 2D-binding assay, demonstrated that a polyclonal MOG_{35-55} restricted CD4 T-cell population contained cells with a large range of affinities. Even though only 8% of the population stained with tetramer, over 60% were reactive as measured by the 2D-binding assay and thus ~70% of the tetramer negative cells were antigen specific as measured by 2D-binding assay (Sabatino et al., 2011). Thus, the tetramer, though a useful tool for defining antigen-specific T-cells, may under estimate some populations of T-cells, particularly cross-reactive T-cells as cross-reactive T-cells may have a different affinity or avidity for its cross-reactive ligands. Additionally a cross-reactive cell may see its alternate ligand in a different orientation than its cognate ligand. Any of these may contribute to the different activating profiles of a cross-reactive T-cell.

In order for CD8 T-cells to eliminate virus from the infected host they usually need to kill virally infected cells. That is exactly what we see in the case of EBV-SN donors (**Fig 3.6**). Cross-reactive cell lines generated by growth in the presence of either cognate IAV-GIL peptide or cross-reactive EBV-GLC peptide

were capable of killing EBV infected autologous BLCLs in a lytic antigen dependent manner. Again this is a hallmark of CD8 T-cell effector function and could be what is mediating protection from infection.

If an effector CD8 T-cell cannot make it to the site of infection than its abilities to kill virus-infected cells are wasted. EBV-SN subjects' IAV-GIL specific cells and cells that dimly stained with EBV-GLC tetramer ex vivo showed a higher level of CD103 staining as compared to EBV-SP donors. CD103 is an integrin molecule that has been shown to be necessary for entry into mucosal epithelial tissues, which is where EBV infection is thought to initiate. We believe these dim EBV-GLC tetramer staining cells to be the IAV-GIL/EBV-GLC cross-reactive CD8 T-cells because when both IAV-GIL and EBV-GLC tetramers are used to stain the CD8 T-cells, the dim EBV-GLC cells disappear, suggesting that there is a competition for the tetramers (**Fig 5.1**). Presumably, these cells no longer stain with tetramer after in vitro stimulation due to the exposure to large amounts of antigen, which can cause down regulation of the TCR complex. These CD103 data imply that IAV-GIL specific cells from EBV-SN donors have the potential to enter the mucosal areas where infection initially occurs.

Finally, we showed that the TCR repertoire within the IAV-GIL reactive cells is oligo-clonal. This differs from EBV-SP donors who have a highly diverse TCR repertoire in response to IAV-GIL (Naumov et al., 2006). This oligo-clonal structure may be a result of the high functional avidity to the cross-reactive antigens IAV-GIL and EBV-GLC that this population demonstrates, as this clone

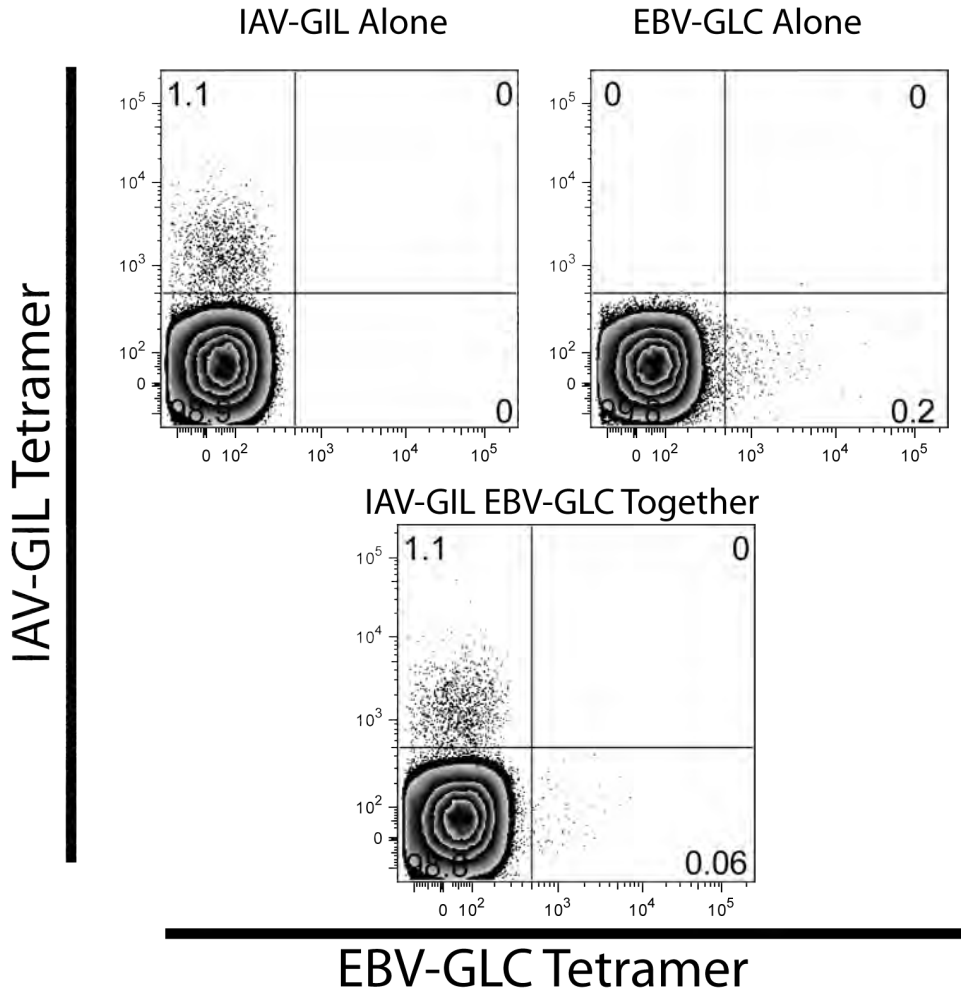

Figure 5.1. Competition for tetramer binding. CD8 sorted T-cells from PBMCs were stained with a viability marker, anti CD3, anti CD8, and tetramers loaded with IAV-GIL and EBV-GLC. Cells were gated on live CD3+ CD8+ cells and tetramer frequencies were examined. Note how the EBV-GLC population frequency decreases when co-stained with IAV-GIL as compared to staining alone.

may have out competed others to dominate the IAV-GIL specific memory pool. This is also the first evidence that the TCR repertoire to a certain antigen can determine disease outcome in humans. If the cross-reactive IAV-GIL specific TCR repertoire from AIM patients are compared to that of EBV-SN donors it becomes clear that there is a difference in organization. This may be why the AIM patients get sick and the EBV-SN donors show protection. It may be that in AIM patients the broad TCR repertoire has a lower avidity to the EBV antigens. This could cause a broad CD8 T-cell expansion and recruitment, as these rather ineffective cross-reactive memory CD8 T-cells try to clear EBV, while EBV-SN donors with their oligo-clonal repertoire and high functional avidity are able to exert effector functions rapidly completely eliminating EBV before it is able to establish a chronic infection. It is important to determine the composition of the T-cell TCR repertoire to decipher the differences that can account for disease progression, or in this case, the mediators of protective immunity. If we could develop the technology to manipulate the T-cell repertoire we could use it to steer the repertoire away from a disease-mediating repertoire, such as during AIM, towards a more protective repertoire such as is seen within these EBV-SN adults.

Another possibility that could explain the difference between protective heterologous immunity, as shown in this thesis, and detrimental heterologous immunity, as in AIM patients, may be that the IAV-GIL cross-reactive cells in the EBV-SN do not show a high expansion when encountering either cognate IAV-

GIL antigen or cross-reactive EBV-GLC, -YVL antigens (**Fig 3.2c**). Perhaps it is the ability to exert effector functions, which is seen more in T_{EM} cells, over that of proliferation, which is seen more in T_{CM} cells, that is important in mediating protection from infection. Interestingly, a recent study demonstrated that this is indeed the case (Jiang et al., 2012). In the study by Jiang et. al., mice with a localized VACV skin infection were surgically attached to uninfected mice to form parabiotic mice. This allowed for circulating T_{CM} cells to migrate to the uninfected mouse while resident T_{EM} cells would remain in the tissues. After eight weeks of attachment mice were surgically separated and allowed 2 weeks to recover. Mice were then given a skin VACV infection and viral loads within the skin were examined at 6, 14, and 26 days after infection. Remarkably, the mice that received the primary skin infection retainined the resident T_{EM} cells, showed significantly lower viral titers than the parabiotic mouse that had only the circulating T_{CM} cells. The data generated from this study show us that it is the T_{EM} cell population that is primarily responsible for protection from reinfection at epithelial surfaces, and not $T_{CM,}$ which are most likely more important in combating systemic infections such as during LCMV infection.

Because of the oligo-clonal nature of the α and β TCR repertoire within the IAV-GIL populations, which show high cross-reactive effector functions, we believe that the dominating clonotype encodes a highly cross-reactive TCR. From this study we may have defined the first cross-reactive T-cell receptor. As a bonus this cross-reactive TCR may be protective in nature. Potentially this

TCR, due to its high functional avidity, could be used to generate transgenic human T-cells for the treatment of EBV related diseases such as post transplant lympho-proliferative disorder (Bollard et al., 2003).

The initial events in EBV infection are controversial. It is generally accepted that EBV can infect both B-cells and also epithelial cells (Arvin et al., 2007). Data have shown that EBV generated from a B-cell, rather than from an epithelial cell, is better at infecting epithelial cells and that EBV generated from an epithelial cell, rather than from a B-cell is better at infecting B-cells (Borza and Hutt-Fletcher, 2002). This was performed by isolating virus from either Akata-Burrkit lymphoma cells (B-cell) or from AGS gastric carcinoma cells (epithelial cells). These viruses were then used to infect either epithelial cells or B-cells. Viral infection was measured by western blot for B-cells or by colony transformation on epithelial cells. It was found that this difference in tropism was being mediated by the difference in the viral attachment complex. Virus with B-cell tropism had trimeric attachment complex consisting of viral proteins gH-gL-gp42, while virus with epithelial tropism had a dimeric complex consisting of gH-gL. This suggests that there is a cycle of EBV replication and infection between these two cell types. This also suggests that the virus may need to ramp itself up to make the switch in tropism. Since there is no in vivo experimental model to conclusively show the early events of infection we are left to speculate that perhaps EBV needs to first ramp up its B-cell tropism by initially infecting epithelial cells and then make the jump over to the B-cell pool. Therefore, we

can say that B-cells are the primary reservoir of virus in persistently infected individuals and it is likely, although not certain, that the first cell infected in vivo is an epithelial cell (Arvin et al., 2007).

When we consider all of the data we can begin to form a comprehensive model of what may be happening within these EBV-SN donors. During an EBV infection the virus first comes and infects the tonsilar epithelial tissues (Arvin et al., 2007). The virus then multiplies and accumulates, eventually managing to establish infection within the B-cell pool and go on to either cause disease in the form of AIM, or quietly slip into latency (**Fig 5.2**). We propose that when EBV-SN donors are exposed to the virus their unique cross-reactive IAV-GIL memory CD8 T-cells can enter, or are already present in, the tonsillar epithelium and immediately kill the initially infected epithelial cells not allowing for the establishment of productive infection (**fig 5.3**).

When one considers the fact that EBV infects over 95% of the population (Henle et al., 1969), and at any given time an infected individual is shedding large quantities of viral genome, (Hadinoto et al., 2009) the chances of never being exposed to the virus are slim, yet EBV-SN middle aged adults exist. Due to the obvious ethical considerations of challenging an individual with virus, studying EBV-SN adults is a useful alternative to assess whether these donors possess unique cross-reactive responses that have properties that may protect them from EBV infection or from the establishment of productive infection. This observation of potentially protective cross-reactive responses in EBV-SN adults

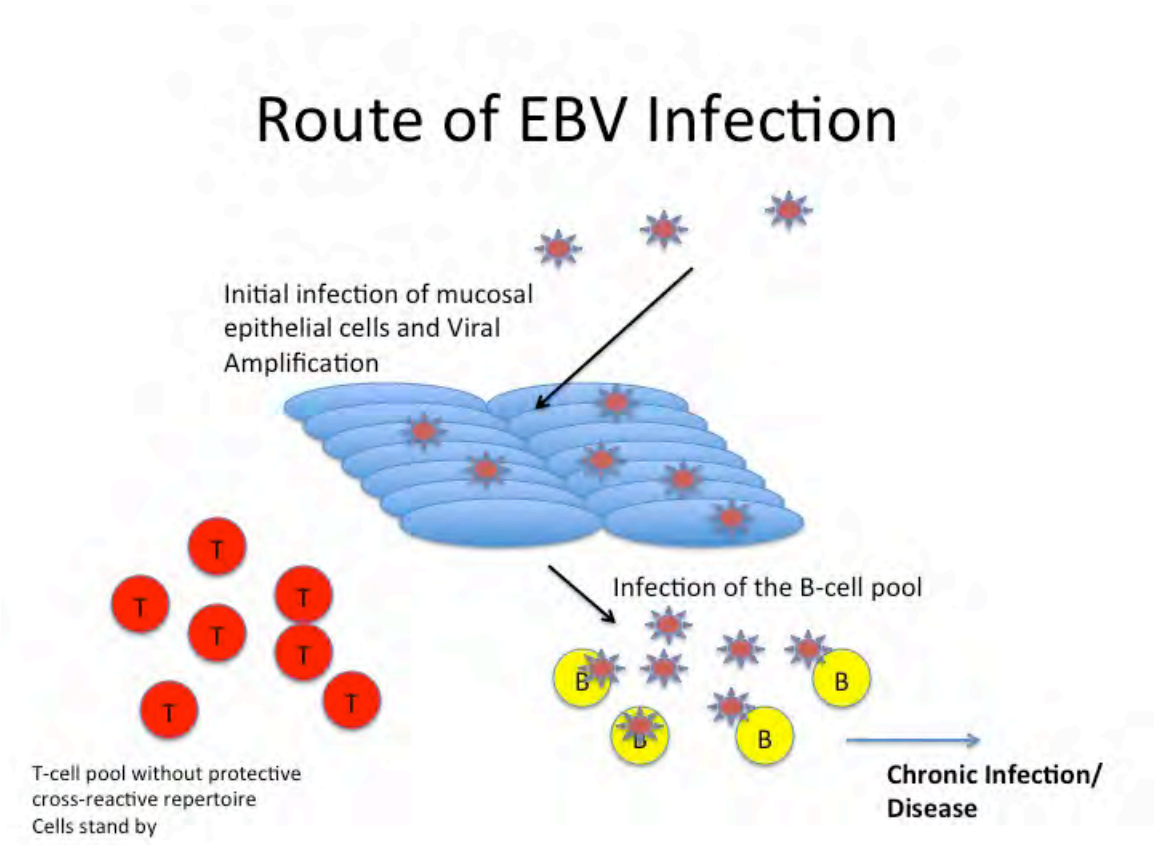

Figure 5.2. Route of EBV infection. The route of EBV infection begins with viral infection of the tonsiallar epithelial cells (blue) where the virus amplifies ultimately to high enough levels to then infect the B-cell pool (yellow) and establish infection. Normally T-cells (Red) are not yet specific to EBV antigens and do not participate until later in infection.

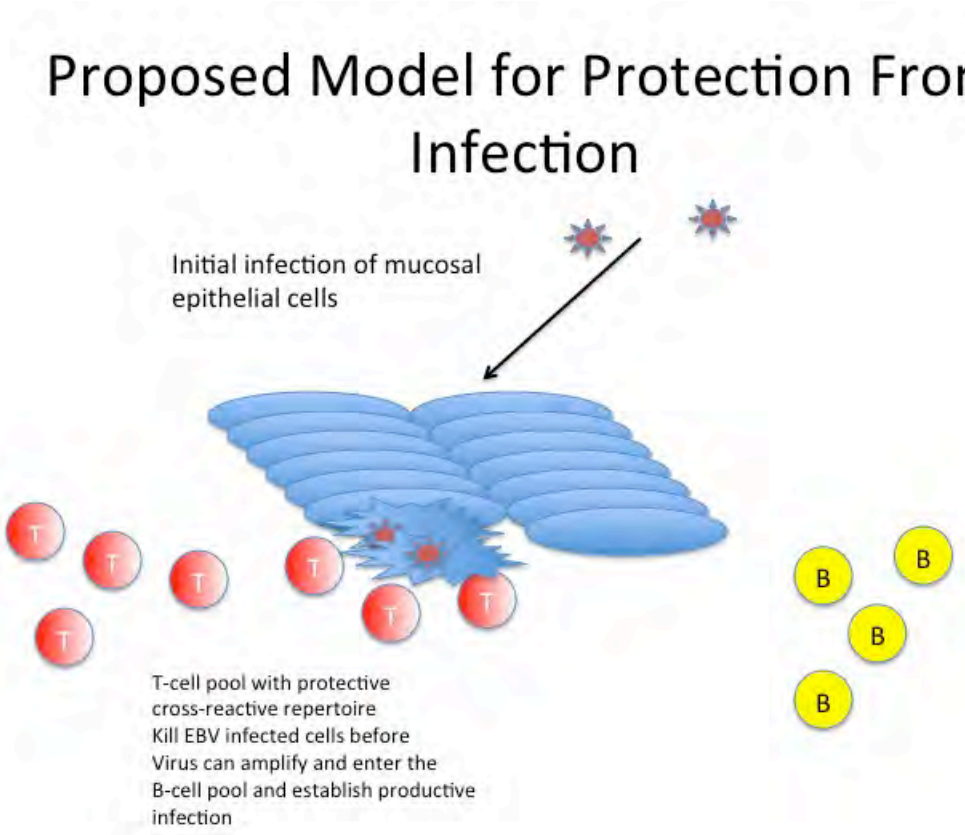

Figure 5.3. Proposed model for protection from EBV infection. The route of EBV infection begins with viral infection of the tonsiallar epithelial cells (blue); however, in EBV-SN adults the cross-reactive T-cell pool (shaded red) is capable of killing infected cells not allowing the amplification of virus and the establishment of productive infection within the B-cell pool.

leads to the questions of whether this is an example of protective heterologous immunity and how common is protective heterologous immunity in humans? If cross-reactive responses are beneficial in humans they may easily go undetected. It is much more likely that cross-reactive responses would be more likely detected in a pathological state, where they would be more evident. Interestingly, the HIV and HCV fields have noted that there appears to be natural resistance (as demonstrated by sero-negativity) to infection in certain high-risk groups that are continuously exposed to these viruses (Clerici et al., 1992; Langlade-Demoyen et al., 1994; Barcellini et al., 1995; Fowke et al., 1996; Mazzoli et al., 1997; Kamal et al., 2004; Roque-Cuéllar et al., 2011). The HIV field has noted that a cohort of female sex workers from Kenya had a surprisingly high level of HIV sero-negativity considering the fact that they were a high-risk group. Interestingly, if a worker left the trade for a time and then returned the worker would contract the virus. This would suggest that the constant exposure to the virus was contributing to the immunity. In fact, constant exposure may be the key, as the HCV studies mentioned above showed HCV specific responses in HCV sero-negative adults whose partner was HCV sero-positive. Interestingly, cross-reactive epitopes have been identified in HCV and HIV sero-negative individuals (Wedemeyer et al., 2001; Acierno et al., 2003). Perhaps this may be the mechanism by which these high-risk individuals are resisting infection. These findings would also suggest that if a cross-reactive memory T-cell population is protecting these individuals it requires continuous antigen exposure

to maintain a high enough frequency and to maintain a T_{EM} phenotype so that it can immediately lyse virus infected cells.

An alternative approach to further test our hypothesis of heterologous immunity mediated protection by cross-reactive IAV-GIL memory CD8 T-cells would be to perform a prospective study. To do this we would enroll a large number of individuals into the study and follow them over a long period of time. A perfect sample population would be to enroll incoming college freshmen. EBV sero-status would initially be evaluated at enrollment and monitored over their course of college studies. This population is ideal as 75% of the individuals are EBV-SP, leaving 25% EBV-SN (Crawford et al., 2006a). As these students are mixed together the EBV-SN students would become exposed to the virus. For our study we would evaluate the same metrics used through out this thesis such as IAV-GIL frequencies ex vivo, the ability to respond to EBV antigens by cytokine production and cytotoxicity, and examine the IAV-GIL TCR repertoire structure. We could then group the EBV-SN subjects by patterns of cross-reactive strength and repertoire organization and potentially predict, based on data from this thesis, which individuals would sero-convert or remain EBV-SN.

In human studies we are dependent on associations and correlations as demonstrated here. The humanized mouse model would be an ideal alternative to directly address mechanistic questions. It would offer the flexibility of a murine system with the relevance of a human immune system. The humanized mouse system as it stands now allows for infection with the viruses, IAV and EBV, that

we need to use to study the protective role of IAV memory against EBV infection, but in our hands the immune response that is generated is not quite up to the physiological standards that need to be met. For our studies we would need to be able to generate a CD8 T-cell epitope specific response. Other groups have demonstrated EBV infection within the humanized mouse system using the same models we have used and generated an epitope specific response. However, in their systems they used a much higher dose of virus, which most likely accounts for the difference (Melkus et al., 2006; Yajima et al., 2008; Shultz et al., 2010). Additionally other infection systems have been developed in these humanized mouse models including HIV, DENV, Malaria, and *Salmonella typhi*. One aspect that will be difficult to overcome will be the establishment of IAV memory and to get antigen specific cells with a correct TCR repertoire, especially with the murine MHC still present in the mice. As shown above the humanized mice were not able to control IAV and ultimately died even at low doses with a low virulence IAV-strain. While not included in this thesis, prior immunization with the peptide, IAV-GIL in Freud's complete adjuvant was not enough to protect the mice from IAV x31 challenge. One possible reason that the mice used in these studies succumbed to IAV is that our particular HLA-A2 transgenic mouse strains are known to be poor presenters of HLA-A2 on their cell surfaces. This may also help explain why we have difficulty developing HLA-A2 restricted responses. On the other hand infection with an avirulent non-mouse adapted strain was attempted but that elicited no response suggesting there was no infection. As of

now we cannot use the humanized mouse model of infection to test our hypothesis of IAV-GIL mediated protection from EBV infection. However, there have been rapid advances in the field of humanized mice as of late. One such advance is the development of NSG mice with the transgenic expression of membrane-bound human stem cell factor (SCF) which results in HSC engraftment without the need for irradiation (Brehm et al., 2012). I am confident that soon we will be able to test out our theories in a humanized mouse model.

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