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IMMUNITY, PATHOGENESIS, AND PREVENTION OF POXVIRUS
INFECTIONS

A Dissertation Presented

By

MINA O. SEEDHOM

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

December 15, 2010

Immunology and Virology

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Acknowledgements

First I would like to thank my mentor Raymond M. Welsh for his seemingly never-ending patience and guidance during my graduate career. His hands-on, hands-off approach allowed me to learn on my own without falling too hard into the “one more experiment” trap. There was always a new way of looking at information with Ray, and I hope his enthusiasm for science is something I can continue to cultivate in myself as I move on to other adventures.

I also am also very fortunate to have had Eva Szomolanyi-Tsuda as a mentor. While working with Eva, I was encouraged to ask questions, take classes, attend seminars and just drop in for a talk about anything of interest. I can still remember discussions with her where a previously foreign immunological concept suddenly dropped into place, and I will always be grateful to her for the constant support she has given me throughout my time at UMass Medical School. I thank you Eva for giving me the opportunity.

I would also like to thank my mother, father, brother and fiancée, Kate. Without their constant love and support throughout, I would not be who I am today.

I would also like to thank the members of my thesis advisory committee Hardy Kornfeld, Kenneth L. Rock, and Liisa K. Selin, for their helpful advice throughout my graduate work, and would also like to thank Francis Chan and Shane Crotty for taking the time to serve on my dissertation committee.

ABSTRACT

Vaccinia virus (VAC) is the prototypical member of the orthopoxvirus genus of the poxvirus family and the virus used for smallpox vaccinations. The following describes the testing of VAC variants designed to have similar immunoprotective profiles with decreased pathogenicity, examines the immune response to VAC after lethal infection in wild type and lupus-prone mice, and describes a method that allows for the enumeration of VAC-specific CD8⁺ T in naïve and VAC-immune mice.

The first part describes work examining VAC Wyeth (VAC-Wy) variants engineered to be less pathogenic *in vivo*. VAC-Wy variants included genes that code for three immunomodulatory proteins, an interferon- γ (IFN γ) binding protein (B8R), an interleukin 18 (IL-18) binding protein (C12L), and a complement binding protein (C3L, or C21L) or various combinations of the three knockouts, and a triple knockout (VAC-Wy -/-/-) in which all three genes were knocked out of a variant virus.

The immunomodulatory effects of other IFN γ binding proteins on VAC-Wy pathogenesis in the mouse were also examined. Virus recombinants where the B8R gene was replaced with a truncated mouse IFN γ receptor gene or a gene that encodes a B8R/IFN γ fusion that allows for dimerization of the secreted IFN γ receptor were studied.

As the knockouts and variants were made in the current vaccine VAC-Wy strain, only high dose (1×10^7 PFU's) intra nasal (I.N.) infection of mice reliably

resulted in detectable virus in the lungs. Further testing revealed that all knockout and variant viruses grew to similar levels after high dose I.N. infections.

Protection induced by vaccination with the VAC-Wy variants was studied in comparison to immunizations with the VAC-Wy parental strain. Mice were immunized by tail skin scarification to mimic human immunizations, and this was followed months later by I.N. challenge with 20 LD50's of VAC-WR. All VAC-Wy recombinants tested, including the VAC-Wy -/-, provided similar levels of protection as the parental VAC-Wy strain from a lethal VAC-WR I.N. infection. Mice immunized with the VAC-Wy -/- induced similar amounts of neutralizing antibody and similar numbers of CD8⁺ T cells specific to a subdominant determinant as VAC-Wy.

While examining high dose, normally lethal, VAC-WR I.N. infections, a profound splenic CD8⁺ T cell immune suppression was noted that might have been caused by Fas dependent activation induced cell death (AICD). Using high dose intra-peritoneal (I.P.) and I.N. models of VAC-WR infection, decreased weight loss, decreased virus titers, and increased T cell numbers were found in Fas mutant (B6.MRL-*Fas*^{*lpr*}/J) mice in comparison to B6 wild type mice on day 6. It would be expected that Fas-deficient CD8⁺ T cells from B6.MRL-*Fas*^{*lpr*}/J mice (B6-*lpr*) would survive a high dose VAC-WR infection better than CD8⁺ T cells that could express Fas if T cells were being eliminated by Fas-dependent AICD, but co-adoptive transfer experiments using splenocytes from B6-*lpr* and B6.Cg-

IgH^aThy-1^aGPI-1^a/J (IgH^a) wild type counterparts found no difference in the numbers or proliferation of donor CD8⁺ T cells at day 6.

As the B6-*lpr* mice were better protected from VAC-induced weight loss early after lethal VAC-WR infections, it was possible that B6-*lpr* mice might be protected early in infection. In fact, Fas mutant mice had decreased virus loads in the fat pads, livers, and spleens in comparison to B6 wild type mice at days 2 and 3. In addition to the decreased virus titers, the severe splenic lymphocyte deficiency noted in B6 wild type mice as early as day 2 after high dose I.P. infection was ameliorated in B6-*lpr* mice. Further experiments demonstrated that uninfected B6-*lpr* mice had increased numbers of memory phenotype (CD44⁺) CD4⁺, CD8⁺ and $\gamma\delta$ ⁺ T cells, with an increased number of $\gamma\delta$ ⁺ T cells and NK cells in splenic lymphocytes in comparison to wild type B6 mice. Uninfected B6-*lpr* mice also had increased numbers of IFN γ ⁺ CD8⁺ T cells after polyclonal stimulation with an antibody against CD3 ϵ . In lymphocyte depletion experiments performed at day 3, antibody depletion of CD4, CD8, or NK or treatment with an antibody that was specific to the $\gamma\delta$ ⁺ TCR did not significantly alter virus loads in B6-*lpr* mice. In co-adoptive transfer experiments, splenocytes from wild type or B6-*lpr* mice survived high dose VAC-WR challenge similarly suggesting that B6-*lpr* splenocytes were not intrinsically better protected from lymphocyte depletion by lack of the Fas protein. On day 2 after high dose I.P. VAC-WR infection, B6-*lpr* mice had increased numbers of IFN γ ⁺ NK cells, IFN γ ⁺ CD8⁺ T cells, and IFN γ ⁺ CD4⁺ T cells. B6-*lpr* and B6 mice treated with an antibody against IFN γ had

significantly increased virus titers in the spleens and livers. Interestingly, there was no significant difference in liver or spleen virus titers when comparing anti-IFN γ antibody treated B6 mice or anti-IFN γ antibody treated B6-*lpr* mice. These results suggest that multiple leukocyte populations co-operatively or redundantly provide B6-*lpr* mice with increased protection from high dose VAC-WR infections through increased production of IFN γ .

The third part of this work describes the enumeration of total numbers of pathogen-specific CD8⁺ T cells in a mouse through use of an *in vivo* limiting dilution assay (LDA). The extensive proliferation of virus-specific CD8⁺ T cells that occurs after virus infection was used to enumerate numbers of virus-specific CD8⁺ T cells in a naïve mouse. By transferring limiting amounts of carboxyfluorescein succinimidyl ester (CFSE)-labeled Thy1.1⁺Ly5.2⁺ heterogeneous CD8⁺ T cells into Thy1.2⁺Ly5.1⁺ hosts, CD8⁺ T cell precursor frequencies to whole viruses can be calculated. The calculations are based on finding the number of donor CD8⁺ T cells that results in CFSE⁰ (i.e. proliferated) donor CD8 T cells in 50% of the hosts. Using probit or Reed and Muench 50% endpoint calculations, CD8⁺ T cell precursor determinations were made for naïve and immune states to a virus challenge. It was found that in naïve B6 mice, 1 in 1444 CD8⁺ T cells proliferated in response to VAC-WR (~13,852 VAC-WR-specific CD8⁺ T cells per mouse) and 1 in 2956 proliferated in response to lymphocytic choriomeningitis virus (LCMV) (~6,761 LCMV-specific CD8⁺ T cells per mouse). In mice immune to VAC-WR, the number of VAC-WR-specific LDA

precursors, not surprisingly, dramatically increased to 1 in 13 (~1,538,462 VAC-WR-specific CD8⁺ T cells per mouse) consistent with estimates of VAC-WR-specific memory T cells. In contrast, precursor numbers to LCMV did not increase in VAC-WR-immune mice (1 in 4562, ~4384 LCMV-specific CD8⁺ T cells in a VAC-WR-immune mouse) consistent with the fact that VAC-WR provides no heterologous immunity to LCMV. Using H-2D^b-restricted LCMV GP33-specific P14 transgenic T cells it was found that, after accounting for take of donor T cells, approximately every T cell transferred underwent a full proliferative expansion in response to an LCMV infection and a high efficiency was also seen in memory populations. This suggests that most antigen-specific T cells will proliferate in response to infections at limiting dilution. These results, which are discussed in comparison to other methods, show that naïve and memory CD8⁺ T cell precursor frequencies to whole viruses can be remarkably high.

In total this work further advances knowledge of the immunity, pathogenesis, and prevention of poxvirus infections. This was accomplished by studying VAC-Wy recombinants as improved vaccines, by examining the mechanisms and cell types important in early protection from high dose poxvirus infections in B6 and B6-*lpr* mice, and by describing a method to enumerate total numbers of virus-specific CD8⁺ T cells in a mouse.

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A chapter of this dissertation has appeared in the following publication:

Seedhom MO, Jellison ER, Daniels KA, Welsh RM. 2009. High frequencies of virus-specific CD8⁺ T-cell precursors. *J Virol.* Dec;83(24):12907-16.

Publications completed during thesis research not included in this manuscript.

Welsh RM, Seedhom MO. Lymphocytic choriomeningitis virus (LCMV): propagation, quantitation, and storage. *Curr Protoc Microbiol.* 2008 Feb;Chapter 15:Unit 15A.1.

List of Abbreviations

ALN	axillary lymph node
ALPS	autoimmune lymphoproliferative disorders
B6	C57BL/6J
BID	BH3 interacting domain death agonist
BP	base pair
CD40L	CD40 ligand, also CD154
cDNA	complementary deoxyribonucleic acid
CDR3	complement determining region 3
C1	complement component 1
C2	complement component 2
C3	complement component 3
C3H	C3H/HeJ
C3bINA	C3b inactivator (Factor I)
C4	complement component 4
C4BP	complement component 4 binding protein
CAPS	cytotoxicity-dependent APO-1 associated proteins
CFSE	carboxyfluorescein succinimidyl ester
conA	concanavalin A
CPE	cytopathic effect
CTL	cytotoxic T cell
CVF	cobra venom factor

DD	death domain
DDAO-SE	9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt succinimidyl ester
DEAE	diethylaminoethyl
DISC	death-inducing signaling complex
dsDNA	double stranded DNA
EGF	epidermal growth factor
EMCV	encephalomyocarditis virus
ETn	early transposable element
FACS	fluorescence-activated cell sorting
FasL	fas ligand
FLICE	FADD-homologous ICE/CED-3-like protease
FLIP	FLICE inhibitory protein
G-CSF	granulocyte colony stimulating factor
<i>gld</i>	generalized lymphoproliferative disorder, mouse mutation
GM-CSF	granulocyte macrophage colony-stimulating factor
GP	glycoprotein
HSA	human serum albumin
HLA	human leukocyte antigen
IAV	influenza A virus
IFN γ	interferon gamma
IFN γ R	interferon gamma receptor

IFN γ BP	interferon gamma binding protein
Ig	immuno-globulin
IgH ^a	B6.Cg-IgH ^a Thy-1 ^a Gpi-1 ^a /J
IL	interleukin
IL-18BP	interleukin-18 binding protein
IL-18R	interleukin-18 receptor
I.N.	intra-nasal
IGIF	interferon gamma inducing factor
I.P.	intra-peritoneal
JNK	c-Jun N-terminal kinase
LCMV	lymphocytic choriomeningitis virus
LDA	limiting dilution assay
LM	<i>Listeria monocytogenes</i>
<i>lpr</i>	lymphoproliferative disorder, mouse mutation
<i>lpr^{cg}</i>	<i>lpr</i> complementing <i>gld</i> , mouse mutation
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACH	MORT1-associated CED-3 homolog
MASP 1	mannose binding lectin serine protease 1
MASP 2	mannose binding lectin serine protease 2
MBL	mannose binding lectin
MHC	major histocompatibility complex

MHV	mouse hepatitis virus
MLN	mediastinal lymph node
MRL	MRL/MpJ
MVA	modified vaccinia Ankara
NP	nucleoprotein
NDV	Newcastle disease virus
NF κ B	nuclear factor kappa B
NK	natural killer
NYCBH	New York City Board of Health strain of vaccinia virus
<i>P. acnes</i>	<i>Propionibacterium acnes</i>
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC	peritoneal exudate cells
PFU	plaque forming unit
PLN	peribronchial lymph nodes
pMHC	peptide major histocompatibility complex
RAG	recombinase activating gene
SCID	severe combined immunodeficiency
sIL-18R	soluble interleukin-18 receptor
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus

ssDNA	single stranded DNA
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
TNF α	tumor necrosis factor alpha
TNF-R	tumor necrosis factor receptor
VAC	vaccinia virus
VAC-WR	vaccinia virus Western reserve strain
VAC-Wy	vaccinia virus Wyeth strain (also known as Dryvax®)
VAC-Wy-/-	vaccinia virus Wyeth strain lacking genes encoding B8R, C12L, and C21L (C3L) proteins
vsmIFN γ R	virus expressing soluble version of the murine interferon gamma receptor, vaccinia virus variant
VSV	vesicular stomatitis virus
VV	vaccinia virus

CHAPTER I: INTRODUCTION

A. Variolation

The devastation and death that smallpox caused throughout the millennia were profound, but the observations that survivors of the infection were rarely re-infected gave hope that there might be a way to fight or even prevent the disease. There are descriptions of infections with smallpox material in controlled circumstances to prevent later more severe smallpox infections as far back as 8th century India by the author Madhav in a chapter of the Nidana (a book describing diseases and symptoms) that focused on smallpox (Holwell 1767; Hopkins 2002). It would seem that this practice was not widely adopted until the Sung Dynasty (960 to 1280 A.D.) and interestingly was only first practiced in southwestern China near the border with India (Hopkins 2002). The purposeful infection procedure was only well described later by Yu Chang in his book Notes on my Judgment published in 1643 (Temple 1986). The procedure was eventually widely described to Europeans by physician Emmanuel Timoni. Dr. Timoni characterized the infection as an inoculation, which seems to be the first time this word is used to label infections (Timonius and Woodward 1714). He explains...

“They make choice of some boy, or young lad, of a found healthy Temperament, that is seized with the common Smallpox...on the twelfth or thirteenth day from the beginning of his sickness: they with a needle prick the Tubercles (chiefly those on the shins and hands) and press out the matter coming from them into

some convenient vessel...” then to the inoculation “The patient therefore being in a warm chamber, the operator is to make several little wounds with a needle in one, two or more places of the skin, till some drops of blood follow, and immediately drop out some drops of the matter in the glass, and mix it well with the blood effusing out; one drop of the matter is sufficient for each prick’d. These punctures are made indifferently in any of the fleshy parts, but succeed best in the muscles of the arm or Radius”

This publication describes the outcome of this controlled infection as being less harmful than contracting smallpox in the usual way, and while it does mention individuals that succumb to the inoculation as might happen during a natural infection, explains the inoculation procedure as wholly protective against smallpox infection.

Inoculation, or variolation, began to become somewhat widespread in Europe after small demonstrations of the relative safety of the procedure. A demonstration in 1721 on six inmates of Newgate prison set up by the then Prince and Princess of Wales and witnessed by twenty-five members of the Royal Society showed the successful inoculations and subsequent protection against smallpox challenge (Oldstone 2000). This incident was reported on publicly in newspapers, and while the inoculation itself was associated with a 2% death rate, this was much better than the threat imposed by natural smallpox infection.

B. Vaccination and the Elimination of Smallpox from The Human

Population

While variolation was proving to be a successful preventive measure against smallpox, there was still concern as to the high death rate associated with the procedure. Discussion was ongoing as to ways that this procedure might be made less lethal and publications on the procedure questioned how attenuation of variolation might be accomplished. An interesting phenomenon noticed at the time was the relative resistance of dairymaids to smallpox infection. As dairymaids would often interact with cows, one interesting thought was that the contraction of cowpox by dairymaids might provide some kind of cross-protection from smallpox infection. In fact a farmer in 1764 named Benjamin Jesty inoculated his wife and two sons with pustulous material taken from the lesion of a cow as protection against smallpox infection, noting that his two servant girls who had contracted cowpox seemed to be resistant to smallpox (Oldstone 2000). A more rigorous testing of the hypothesis that cowpox could protect against smallpox infections came from a country physician named Edward Jenner (Jenner 1800). Dr. Jenner began by attempting to variolate individuals with past histories of cowpox infection, and in every case described he found that the people who had been previously infected with cowpox had symptoms that were much less severe than what would normally occur with variolation. Jenner remarks *"It is remarkable that variolous matter, when the system is disposed to reject it, should excite inflammation on the part to which it*

is applied more speedily than when it produces the Small Pox”, seeming to remark on the quick response in these individuals to the variolation procedure. The individuals had described being infected with cowpox anywhere from a year to up to fifty-two years previously, and so he felt whatever protection the cowpox infection provided was long lasting. He then describes a more controlled examination of the phenomenon in which he inoculates an eight year-old boy with material from the sore of a dairymaid. The boy fell ill to the infection, but recovered, and a year later Jenner variolated the boy and found that the boy was protected from the smallpox infection that normally occurred after variolation. This and two similar experiments described in the publication, along with the rest of his studies seemed to solidify the phenomenon for Jenner. With Jenner’s publication of these observations, and later experiments that confirmed the results, there seemed to at last be a method to protect populations from the scourge of smallpox. The purposeful infection of a human being with material that typically makes them sick (and was lethal in about 1 to 100,000 to 1 in a 1,000,000 people) protected them from an infection that killed anywhere from 20-40% of those infected. Jenner deemed the procedure vaccination (from the Latin *vaccinus*, “from cows”) in 1803.

Smallpox vaccination began to be practiced throughout the world, and by the 1950’s many countries had contained the spread of smallpox. It was suggested (actually all the way back to Jenner and some others of his time) that this procedure might be able to rid the world of smallpox, but it took a few pushes

before a worldwide effort to eliminate the virus from the human population as a whole gained momentum (Oldstone 2000). Individual countries would eradicate the disease from their soil, but it wasn't until 1967, when a measure passed by two votes at the World Health Organization, that there became an explicit mission to eradicate smallpox from the human population. Dr. Donald Ainslie Henderson was put into the position of Chief Medical Officer of the Smallpox Eradication Program and remained director until smallpox was officially declared eradicated by the WHO in 1980 (Fenner, Henderson et al. 1988).

C. Concerns Today

The successful eradication of smallpox through a worldwide vaccination program was a great biomedical achievement of the 20th century. Smallpox, the causative agent being eventually named variola virus, was a devastating disease, with two variants being described, one variola major with an estimated mortality rate of 5-35%, with the related variola minor designation having a mortality rate of 0.5-2% (Fenner, Henderson et al. 1988). Subsequent genomic analysis has been successful in identifying discrete genetic sequences allowing for the identification of the two variola strains, although no mutation described was able to explain the reduced mortality of variola minor (Shchelkunov, Totmenin et al. 2000). While the eradication of the disease within the human population was a resounding success, the threat of accidental or purposeful reintroduction of variola virus remains a concern. There has also been concern regarding recent monkeypox outbreaks (Di Giulio and Eckburg 2004).

Monkeypox, while seemingly less lethal than variola major, is still associated with a 10% mortality rate, and it is thought that smallpox vaccination would provide some protection from monkeypox infection (Hammarlund, Lewis et al. 2005). It is therefore important to continue to learn of the immune mechanisms important in fighting poxvirus infections, and to develop vaccines that elicit protection equal to or better than the current vaccine, while lowering the incidence of harmful side effects associated with smallpox vaccination.

The vaccine used to elicit protective immunity to smallpox was found to be a live virus inoculation with the closely related virus, vaccinia virus (VAC). It is not known if the vaccination procedure early on used cowpox as stated, or whether the inoculum given was actually VAC, but it is known that the virus now given to immunize from smallpox infection is not cowpox. VAC infection seems to provide long-term protective immunity from infection with variola virus, but the inoculation is not without side-effects. Milder harmful effects include fever and body aches, as had been described by Jenner, but there are other risks from vaccination, including accidental exposure and generalized rash on the skin that historically occurred in about 1 in 2,000 vaccinated, a generalized spread of the virus on the skin termed *eczema vaccinatum* which occurred in about 1 in 20,000 primary vaccinations, and in more severe cases vaccinia necrosum and postvaccinial encephalitis which can lead to death (Copeman and Wallace 1964; Fulginiti, Papier et al. 2003). Other side effects are still being described, as demonstrated in the more recent vaccination efforts. Cardiac complications such

as myocarditis and myopericarditis were noted, with the rate of myopericarditis being about 1 in 2000, much higher than had previously been seen with smallpox immunization (Nalca and Zumbrun 2010). In 14-52 out a million people, the vaccination itself may prove life threatening, with about 1 in a million people dying from the vaccine (Cono, Casey et al. 2003).

Unfortunately, the threat of the smallpox vaccination is also increased in individuals that have skin conditions such as eczema or atopic dermatitis (AD). The vaccine is also not recommended for individuals with immune systems compromised by immunosuppressive drugs or infections, as the live virus inoculum might overwhelm the suppressed immune system (Redfield, Wright et al. 1987; Edghill-Smith, Bray et al. 2005) (Fulginiti, Papier et al. 2003).

Unfortunately, the HIV epidemic has most likely led to an increased number of immuno-compromised individuals within the population and with the continued success of organ transplantation, which typically require immunosuppressive drug regimens, it is important to identify vaccinations that offer similar protective immunity, with decreased pathogenic effects.

D. Better Smallpox Vaccination Through VAC Modification

VAC variants used as smallpox vaccines have been characterized as 1st generation (original vaccines grown in many different ways Ex. Dryvax®), 2nd generation (the 1st generation vaccines plaque purified and adapted to grow in cell culture Ex. ACAM2000™), 3rd generation (passaging of virus in cell culture to attenuate Ex. MVA) and 4th generation (VAC recombinants with targeted

mutations to enhance immunity or decrease pathogenicity) (Kennedy, Ovsyannikova et al. 2009). The 1st generation vaccines were typically a mix of VAC strains. The previously licensed smallpox vaccine in the United States, Dryvax® (also known as VAC-Wyeth), was a mix of New York City Board of Health (NYCBH) strains grown on calf or sheep skin (Nalca and Zumbrun 2010), and while the vaccination was effective, the sterility of the vaccination material was questionable (Fenner, Henderson et al. 1988). The newly licensed smallpox vaccine in the United States is ACAM2000™, a 2nd generation preparation made from plaque purified Dryvax® propagated in cell culture (Weltzin, Liu et al. 2003; Monath, Caldwell et al. 2004). This vaccination was shown to be as protective as Dryvax® in multiple animal models, and to induce similar amounts of immunological memory as Dryvax® if given at similar doses (Nalca and Zumbrun 2010). While ACAM2000™ looks to have a similar immuno-protective profile with the benefit of being propagated in a more controlled fashion, it remains important to develop smallpox vaccines which are as immuno-protective, with decreased adverse reactions, especially in light of the recent realization that the new smallpox vaccine may be associated with previously rare cardiac side effects (Nalca and Zumbrun 2010). The recent renewed interest in the highly attenuated modified vaccinia Ankara (MVA) as a smallpox vaccine strain is therefore not surprising. MVA was originally generated by serial passage (>500) in chicken embryo fibroblasts of material from a poxvirus lesion on a horse (Hochstein-Mintzel, Huber et al. 1972; Mayr, Hochstein-Mintzel et al. 1975). MVA was

demonstrated to have decreased pathogenesis in many models early on, and in more recent analyses, MVA has been shown to grow to lower titers *in vitro*, and to lack many genes that code for immuno-modulatory proteins that increase VAC pathogenesis (Blanchard, Alcami et al. 1998). Also, MVA, when given at multiple doses, as it replicates poorly in comparison with Dryvax®, is able to protect rhesus macaques from lethal infections with monkeypox (Earl, Americo et al. 2004). These results are promising, but the requirement for an increased virus dose, and the recent revelation that introduction of the six major genomic deletions in MVA into the parental VAC strain did not recapitulate the MVA phenotype (Meisinger-Henschel, Spath et al. 2010), suggests that a more directed approach might help in generating viruses with similar immuno-protective phenotypes and reduced pathogenicity.

Discovering variants of VAC that are as protective but less pathogenic may not only be important in developing better smallpox vaccines. VAC variants have been engineered to express proteins of non-vaccinia origin are currently being considered as a potential platform for immunizations against other pathogens, with success already being described with a recombinant VAC expressing the rabies glycoprotein (Pastoret and Brochier 1996). It has also been described that some poxvirus infections are directly oncolytic, possibly revealing a new mode of cancer treatment (Thorne, Hwang et al. 2007). As these stories unfold, it becomes even more important to understand how the

immune system responds to VAC infection and to develop less pathogenic vaccinations.

E. Early Studies Examining Correlates of Protection In Acute and Immune States

Studies examining immunological smallpox correlates of protection typically compared smallpox-vaccinated (immunized with VAC) and unvaccinated patients. An early study described that vaccinated individuals responded more quickly to contact with smallpox, with increased levels of neutralizing antibody, vaccinia antihaemagglutinin activity, and complement-fixing antibody when compared to unvaccinated individuals (Downie and Mc 1958). They characterize this result as not surprising, as the smallpox infection would be considered a second antigenic stimulus, but this result confirms, as Jenner had illustrated, the robust smallpox-specific response in VAC-immune individuals. A later study of 142 individuals found that neutralizing antibody levels of greater than 1/32 as detected by plaque inhibition on a monkey kidney cell line (Cutchins, Warren et al. 1960), were protected from subsequent smallpox infections (Mack, Noble et al. 1972). Later research described 57 contacts of 6 smallpox cases where all vaccinated individuals, as determined by note of a pock, did not develop clinical smallpox and 6 of 42 with titers under 1/20 (6 of 14 unvaccinated patients) developed smallpox (Sarkar, Mitra et al. 1975). Although the presence of poxvirus-induced antibody was demonstrated to be important, and experiments had demonstrated that transfer of neutralizing antibody helped to prevent

smallpox disease in individuals in close contact with smallpox patients (Kempe, Bowles et al. 1961; Marennikova 1962) there was an indication that neutralizing antibody was not the only immune mechanism providing protection from poxvirus infections. Other investigations revealed that agammaglobulinemic patients could sometimes be vaccinated without side effects (Kempe 1960) and mouse experiments with ectromelia (mousepox) virus and VAC described a cellular poxvirus-specific antiviral activity that was not neutralizing antibody or interferon.

Original experiments describe increases in mortality and VAC spread in anti-thymocyte sera treated mice (Hirsch, Nahmias et al. 1968). Later experiments demonstrated a non-interferon, non-neutralizing antibody, cellular activity in day 6 infected mice, which was neutralized by anti-thymus sera that reduced ectromelia virus titers (Blanden 1970; Blanden 1971). These experiments also described the passive transfer of this cellular anti-ectromelia activity to other mice. It was further described that this activity helped mononuclear cell invasion of infected foci, and it was suspected that this activity would help clear an ectromelia virus infection (Blanden 1971). The potential role of thymus-derived cells was further solidified by use of fowlpox-infected thymectomized or bursectomized chickens, and found that at week 2, thymectomized chickens had increased virus loads in skin lesions (Morita 1973). Eventually it was demonstrated that splenocytes from mice infected 4-6 weeks previously, after *in vitro* culture with the same pathogen and cells, could act much like the protective cell-mediated effectors that had been described to appear

starting at day 6, and that this protection was H-2 and pathogen-restricted (Kees and Blanden 1977; Dunlop 1978). Further, much of the cytotoxic activity in mice could be removed by treatment with an anti-sera against Ly2.1 (later designated CD8 α) both in primary and secondary ectromelia immune response (Pang, McKenzie et al. 1976). The combination of these works seems to be the first time that the role of thymus-derived CD8⁺ cells (CD8⁺ T cells) was examined in poxvirus infections, with the initial findings suggesting that CD8⁺ T cells were important in control of poxvirus infections. Later studies examined intra-dermal infections of β 2-microglobulin knockout mice (lack CD8⁺ T cells) with VAC-WR, and found no difference in lesion size when compared to wild-type mice, suggesting that CD8⁺ T cells did not participate in protection against acute infections (Spriggs, Koller et al. 1992). These results seemed to contrast with the earlier studies with ectromelia, but actually serve to highlight the important differences that result when examining VAC infections or ectromelia infections in mice. Although the WR strain of VAC was generated by 18 intra-cerebral passages of the NYCBH strain in mice, followed by a passage in rabbit skin (Parker, Bronson et al. 1941) and is therefore a mouse-adapted neurovirulent strain of VAC, ectromelia, as a mouse poxvirus is more lethal. Therefore the immune mechanisms necessary to protect against either may be different.

F. Recent Work Examining Correlates of Protection In Acute and Immune States

More recent studies have also examined the importance of different cell subsets in protection from acute poxvirus infections and have tried to determine what mechanism in immune mice offers protection from lethal challenge. In one study, CD4 or CD8 cells were dispensable in protecting intra-muscularly immunized MVA-immune mice from VAC-WR challenge, but in mice without B cells, both CD4 and CD8 cells were necessary to protect immune mice from lethal challenge, and CD8 cells were found to be important in acute intra-nasal (I.N.) VAC-WR challenge (Belyakov, Earl et al. 2003). The observation that CD8⁺ T cells helped protect mice from lethal VAC-WR challenge was consistent with results in the ectromelia infection model, with B cells, and most likely neutralizing antibody the main protection mechanism against subsequent lethal poxvirus infections. Later experiments examined protection afforded by different components of immunized mice, and found that naïve mice that received 2×10^7 CD8⁺ T cells or 300 μ l sera from an immunized mouse, had comparable decreases in virus titers in the ovaries 6 days after I.P. VAC-WR challenge, and suggested that both cellular and humoral immunity could provide protection against poxvirus infections (Xu, Johnson et al. 2004).

A later study examined footpad injections with ectromelia, and suggested that both CD8⁺ T cell and B cell responses were important in resolving ectromelia infections by examining CD8^{-/-} mice and CD40^{-/-} mice, and suggested that B cell

responses were important in later control with CD8⁺ T cells playing an earlier role in controlling ectromelia virus infection (Fang and Sigal 2005). These studies complemented the early studies done with ectromelia, and the more recent VAC-WR studies in that acute resolution of poxvirus infection seemed to require CD8⁺ T cells, while later time points required a B cell response. Other studies found that early control of poxvirus infection could be mediated by $\gamma\delta^+$ T cells for I.P. VAC-WR infections (Selin, Santolucito et al. 2001) and natural killer (NK) cells for ectromelia (Parker, Parker et al. 2007) and VAC-WR (Bukowski, Woda et al. 1983; Martinez, Huang et al. 2008) infections. The results generated by poxvirus infections in mice seem to support the importance of multiple leukocyte types in protection from poxvirus infections. More recent primate studies reinforce some of these observations.

Early studies in rhesus macaques had described that lethal monkeypox infection could be prevented by three methods of vaccination, either MVA followed by MVA, MVA followed by Dryvax[®], or Dryvax[®] alone, with the macaques immunized with MVA/MVA developing poxvirus lesions that eventually cleared (Earl, Americo et al. 2004). In studies published soon thereafter, it was found that MVA/MVA/Dryvax[®], NYVAC/NYVAC/Dryvax[®], or a single immunization with Dryvax[®] could not protect simian immunodeficiency virus (SIV)-infected macaques that had CD4⁺ T cell counts of $<300/\text{mm}^3$ from monkeypox (12 of 13), in contrast to SIV-infected macaques with CD4⁺ T cell counts of $>300/\text{mm}^3$ (5 of 5) (Edghill-Smith, Bray et al. 2005). Further analysis

demonstrated that there was a deficient switch to IgG antibody in macaques with CD4⁺ T cell counts of <300/mm³, and this was probably the determining factor in susceptibility. These studies pointed to the important role of CD4⁺ T cells in developing protective immunity to poxvirus immunizations. Later experiments building on this study found that depletion of B cells with anti-CD20 antibody during Dryvax vaccination, but not depletion of CD8 cells during monkeypox infection, resulted in death by day 13 of 3 of 4 macaques, with the remaining macaque shown to not have efficient depletion of B cells (Edghill-Smith, Golding et al. 2005). In other experiments in this study, depletion of CD4 or CD8 cells immediately before monkeypox infection in vaccinated macaques had no effect on protection, but passive transfer of immune sera was able to protect macaques from death. These studies support the idea that, in immune hosts, VAC-specific neutralizing antibody is probably the best correlate of protection against smallpox infection, although these studies did not rule out a role of memory CD8⁺ T cells in protection, and never examined acute infections.

Examining the immune mechanisms and leukocyte types important for protection from poxvirus infections is important, but it is also important to know how long poxvirus-specific immunity after smallpox vaccination might last, and recent studies have described promising results.

G. Immunological Memory Induced By Smallpox Vaccination

Recent investigations of poxvirus-specific immunity in individuals that had been immunized against smallpox up to 75 years previously found that the half-

life of VAC-specific CD4⁺ T cells was 8-12 years as detected by an intracellular cytokine assay and also described long lived antibody responses as detected by ELISA which directly correlated with neutralizing antibody titers (Hammarlund, Lewis et al. 2003). The half-life of VAC-specific CD8⁺ T cells was about 8-15 years as detected by an intracellular cytokine assay. Paradoxically, the VAC-specific CD8⁺ T cell response was more variable in that it seemed that 50% of individuals had VAC-specific CD8⁺ T cells no matter the time since vaccination, although this result was not discussed. These results were quickly followed by another study examining poxvirus immunity in humans. This study also demonstrated that VAC-neutralizing activity in the sera is detectable in most individuals throughout life, and this activity directly correlated with the number of VAC-specific B cell memory, as detected by a B cell memory assay (Crotty, Felgner et al. 2003). Analysis of VAC-specific CD4⁺ T cells detected by ELISPOT demonstrated a drop occurring within a few years of vaccination, with VAC-specific CD4⁺ T cells slowly declining throughout life, with a measured half-life of about 14 years. These results are promising, in that VAC-induced B cell memory seems to be maintained throughout life, and that antibody seems to correlate most strongly with protection in the immune state. Recent studies have suggested that previous immunization against smallpox provides some protection against monkeypox infection (Hammarlund, Lewis et al. 2005), although infection with monkeypox may generate a decreased poxvirus-specific response as detected by assays using VAC (Sivapalasingam, Kennedy et al. 2007).

Promisingly, more recent work has suggested that the levels of immunity induced by variola virus infection or smallpox vaccination were indistinguishable, further strengthening the idea that smallpox vaccination may provide a similar immunoprotective profile as smallpox infection (Hammarlund, Lewis et al. 2010).

H. Description of T Cell Epitopes

In many studies, it has been demonstrated that T cells are important in resolving pathogenic poxvirus infections in mice, (Pang, McKenzie et al. 1976; Belyakov, Earl et al. 2003; Xu, Johnson et al. 2004; Fang and Sigal 2005), rhesus macaques (Edghill-Smith, Bray et al. 2005), and in humans (Redfield, Wright et al. 1987), and so it was important to determine what regions within poxviruses would encode T cell epitopes in order to more closely examine immune responses within animal models and to determine what vaccinations would induce strong T cell responses.

The first description of an epitope encoded by a poxvirus was done by screening early VAC nonstructural gene products by an epitope prediction algorithm for human leukocyte antigen A*0201 (HLA) (Drexler, Staib et al. 2003). This was quickly followed by screening early and early/late gene VAC peptides predicted to have high binding affinity to HLA-A*0201 in VAC-specific CD8⁺ T cell lines established from Dryvax® immunized HLA-A*0201 individuals, and this defined two more HLA-A*0201 restricted VAC epitopes (Terajima, Cruz et al. 2003). As this HLA type is quite common in the human population, the description of these epitopes enhances the ability to follow VAC-specific CD8⁺ T

cell responses, and the eventual description of many human VAC-specific CD4⁺ and CD8⁺ T cell epitopes soon followed, which are reviewed here (Sette, Grey et al. 2009).

The first mouse major histocompatibility complex (MHC)-restricted epitopes encoded within VAC was identified by molecular mimicry. Earlier studies had demonstrated that VAC-WR infection of LCMV-immune mice would often result in expansion of LCMV nucleoprotein (NP)205 epitope specific CD8⁺ T cells, and this result suggested that an epitope presented after VAC-WR infection mimicked the LCMV NP205 peptide (Kim, Cornberg et al. 2005). By searching for nucleotide and amino acid homologies to LCMV NP205 in VAC, and then screening the resulting 115 peptides that were predicted to bind H-2K^b against VAC-specific CD8⁺ T cells, the VAC determinants A11R₁₉₈ and E7R₁₃₀ were found, the first mouse MHC-restricted VAC epitopes (Welsh, Selin et al. 2004; Cornberg, Sheridan et al. 2007). This was soon followed by the identification of five other CD8⁺ T cell epitopes, B8R₂₀ (B8R), A19L₄₇, A47L₁₃₈ (A47L), A42R₈₈, and K3L₆ (K3L), by expressing plasmids encoding each of the predicted 258 open reading frames of VAC in cells expressing H-2D^b or H-2K^b, and screening these against splenocytes from day 7 VAC-infected C57BL/6J (B6) mice (Tscharke, Karupiah et al. 2005), and the same group used a similar methodology to describe epitopes in BALB/c mice (Tscharke, Woo et al. 2006). The description of these epitopes was followed by description of many other CD8⁺ T cell epitopes by a consensus epitope prediction algorithm (Moutaftsi,

Peters et al. 2006), and other broad screening methods were used to find other VAC-specific CD8⁺ T cell epitopes (Mathew, Terajima et al. 2005) and CD4⁺ T cell epitopes (Moutaftsi, Bui et al. 2007).

The description of these T cell determinants not only allows for the tracking of T cell responses in humans after different vaccination protocols, but also allows for the testing of various poxvirus vaccines in animal models, and allows for improved vaccine testing and characterization.

I. In Search of a Better Vaccine

There are many strategies to overcome the potential harmful effects of smallpox vaccination, including using a different immunization protocol altogether that avoids infecting humans with live VAC. While this might seem attractive, the striking success of the smallpox eradication effort and the protection the immunization affords against further smallpox infection, some say lifelong protection, would suggest that the vaccinia platform, while flawed, is a beginning and not an end. It has already been described that infection with VAC offers long-term VAC-specific immunological memory. This memory has been most extensively described in the form of virus specific CD4⁺ T cells, CD8⁺ T cells, and B cells, and it might therefore be more beneficial to work on ways to limit the pathogenicity of VAC while retaining the immunological memory induced by infection with VAC.

As VAC is a large double stranded DNA virus that encodes many proteins that seek to evade or shut down the immune response, one strategy to limit

pathogenicity is to create viruses that lack genes which code for proteins that have been shown to limit the host immune response to the virus. To this end, the pathogenicity of and protection provided by VAC-Wy knockouts that lack one, two, or three genes that code for previously described pathogenic immunomodulatory proteins, and some other VAC recombinants, is described using a mouse model. The immunomodulatory proteins targeted for knockout included an interferon- γ (IFN γ) binding protein B8R, an interleukin 18 (IL-18) binding protein C12L, and a complement binding protein (C3L).

J. The Discovery of Interferon- γ and the Interferon- γ Receptor

The description of soluble effectors induced by virus stimulation of cells interfering with virus infectivity was first described as interferon when investigations were made into the inhibition of influenza hemagglutinin activity by a membrane soaked in a buffer containing heat-inactivated influenza virus (Isaacs and Lindenmann 1957). This designation of interferon was subsequently used to describe a virus-inhibitor that had been detected in the media of leukocytes treated with phytohemagglutinin (Wheelock 1965). In these experiments it was demonstrated that inhibition of Sindbis virus cytopathic effect (CPE) was probably not a byproduct of phytohemagglutinin and was inducible in cells only at 37°C, but not at 4°C, suggesting that the product was of cellular origin. Interestingly, when comparing it to the interferon effect previously described to be induced by virus infection of cells, this factor was unstable at pH 2 or when heated to 56°C, unlike the previously described interferon effect. This

virus-inhibitor product produced by leukocytes seems to have been described in earlier studies with mouse leukocytes stimulated with Newcastle disease virus (NDV) or VAC (Glasgow and Habel 1963; Glasgow 1965), but before the resolution of the activities of interferon into discrete factors, it was difficult to make sense of the phenomenon. Even after the description of discrete interferon activities, the interferon effect was still discussed as being attributed to a single heterodimeric protein (Colby and Morgan 1971). Later studies confirmed the classification of the interferons into at least two separate factors by examining the differences in size, stability at acidic pH (pH of 2), and the biochemical properties of the interferon factors induced by either NDV or anti-lymphocyte globulin and divalent F(ab')₂ fragment stimulation of human peripheral lymphocytes from a single donor (Falcoff 1972).

As evidence for multiple interferon types began to grow, the separation of the interferons into two types was proposed. Type I interferon would be distinguished by their ability to be induced by various pathogens or components of pathogens, and Type II interferon would be defined as interferon produced by activated immunocytes through mitogenic or some other stimulation (Ho and Armstrong 1975). A further distinction was later made, and Type I interferons were given the designation IFN α and IFN β and the only known Type II interferon would be named IFN γ (Stewart and Blalock 1980). Further studies were made on the actions of both types of interferon, but the eventual cloning of IFN γ from a cDNA library, and the ability to produce large quantities of pure protein now

simplified the biological characterization of this protein (Gray, Leung et al. 1982). Subsequent experiments demonstrated that IFN γ could up-regulate MHC class I and II on macrophages and B cells (Wong, Clark-Lewis et al. 1983), was the lymphokine that had been previously identified to enhance hydrogen peroxide activity in macrophages and stimulate the macrophage's ability to kill intracellular pathogens (Nathan, Murray et al. 1983), and was also found to enhance production of IgG2A and inhibit production of IgG1 and IgE in an *in vivo* mouse model of infection with *Brucella abortus* (Finkelman, Katona et al. 1988). These and many other findings demonstrated the important role that IFN γ played in the immune response. The major producers of IFN γ were found to be CD4⁺ and CD8⁺ T cells after mitogenic stimulation (Sandvig, Laskay et al. 1987) and NK cells which produce IFN γ after culture in IL-2 (Handa, Suzuki et al. 1983).

Early studies had suggested that the receptor for IFN γ and the receptors for the known Type I interferons did not share a common receptor (Branca and Baglioni 1981), and a realization eventually arose that the IFN γ receptor (IFN γ R) would only function biologically as a heterodimer. Initial experiments that looked to identify the chromosomal location of the IFN γ R used hamster-human or mouse-human cell hybrids, as human IFN γ does not bind or activate mouse or hamster cells (Finbloom, Hoover et al. 1985), and demonstrated that only hybrids that contained human chromosome 6 were able to bind IFN γ (Rashidbaigi, Langer et al. 1986). Interestingly, this binding activity did not result in protection of these hybrids from vesicular stomatitis virus (VSV)-induced cytotoxicity, and it

was later found by the same group that hybrids containing both chromosomes 6 and 21 were required to be able up-regulate MHC in response to IFN γ , suggesting that both chromosomes were needed to respond to IFN γ binding (Jung, Rashidbaigi et al. 1987). It would prove necessary to clone and transfect two separate genes into cells to confer biological responsiveness to IFN γ . The first gene described was found to express a protein that was demonstrated to be the IFN γ binding subunit (later IFN γ R α), and discovery of this gene was accomplished by screening of a cDNA library of mRNA products with polyclonal rabbit anti-sera directed against highly purified human IFN γ R (Aguet, Dembic et al. 1988). Expressing this gene in L1210 mouse lymphocytic leukemia cells allowed binding of radioactive human IFN γ , but it did not result in MHC up-regulation upon human IFN γ treatment, further confirming the necessity of a second human protein (at least) to confer biological activity. The identification and cloning of the gene that expressed the protein that bound IFN γ in the human was soon followed by the cloning of the homologous gene in the mouse (Gray, Leong et al. 1989; Hemmi, Peghini et al. 1989; Munro and Maniatis 1989). Further biochemical analysis suggested that an IFN γ dimer bound to two molecules of IFN γ R, suggesting that IFN γ binding to its receptor would induce dimerization (Fountoulakis, Zulauf et al. 1992), and a crystal structure would eventually confirm the this stoichiometry (Walter, Windsor et al. 1995).

The identification and cloning of the second factor necessary to confer biological responsiveness to IFN γ was described through use of cDNA

complementation studies, using hamster cells expressing the human IFN γ binding component to screen for the human counterpart (Soh, Donnelly et al. 1994), and monkey cells expressing the mouse IFN γ binding component to screen for the mouse counterpart (Hemmi, Bohni et al. 1994).

Many cell types were demonstrated to respond to treatment with IFN γ , and early experiments in mice provided evidence that IFN γ R expression was widespread, as daily I.P. injection of IFN γ for 6-9 days induced heightened expression of MHC class I and II in multiple cell types and organs (Skoskiewicz, Colvin et al. 1985). Later experiments confirmed these results by use of immunohistochemistry and fluorescence-activated cell sorting (FACS) performed with anti-IFN γ R purified rabbit polyclonal antibodies and found IFN γ R expression in multiple lymphocytes and organs (Valente, Ozmen et al. 1992). Therefore, it was likely that IFN γ would play an important role in immune responses to infection.

K. The Role of IFN γ in Virus Infections

The first *in vivo* demonstration of the role of IFN γ in virus infections was performed using sheep anti-sera specific for IFN γ . It was found that B6 mice infected with lymphocytic choriomeningitis virus (LCMV, strain Aggressive) treated daily with anti-IFN γ sera had increased virus loads in the livers and spleens, with decreased cytotoxic killer cell activity, and, paradoxically increased survival rates when compared to mice treated with normal sera (Leist, Eppler et al. 1989). Subsequent experiments demonstrated that treatment with anti-IFN γ

sera one day before ectromelia infection, followed by daily treatment with anti-IFN γ sera, resulted in a typical nonlethal footpad dose now becoming lethal in the anti-IFN γ treated mice (Karupiah, Fredrickson et al. 1993). The treatment of mice with anti-IFN γ also significantly increased viral titers in the livers, spleens and ovaries. When other investigators instead used VAC infections, they found similar results. In this case continuous treatment with a monoclonal antibody (mAb) against IFN γ transformed a sub-lethal dose into a lethal dose, and these experiments further went on to demonstrate that protection afforded by adoptive transfer of CD8⁺ T cells from a mouse that had been previously infected with VAC, could be neutralized when mice were treated with a mAb against IFN γ (Ruby and Ramshaw 1991). Further evidence that IFN γ played an important role in limiting pathogen spread was described in IFN γ R gene knockout mice (Huang, Hendriks et al. 1993). There was increased mortality in IFN γ R knockout mice intra-venously infected with VAC-WR in comparison to wild type mice, and increases in the amount of virus per gram of tissue in the spleens, lungs, testes, and ovaries in IFN γ R knockout mice when compared to wild type. The enhanced amount of VAC in IFN γ R knockout mice was later repeated (Muller, Steinhoff et al. 1994), and similar results were found in mice that lacked the gene that encodes IFN γ (Cantin, Tanamachi et al. 1999). Other experiments suggested that IFN γ might have a direct negative effect on poxvirus replication and determined that both infected primary macrophages and RAW264.7 mouse leukemic monocyte macrophage cell lines, when treated with recombinant IFN γ

released decreased amounts of two poxviruses, ectromelia virus and VAC (Karupiah, Xie et al. 1993). Competitive inhibition of nitric oxide activity by N^w-methyl-L-arginine (L-NMA) would negate the virus-inhibition effect of IFN γ in these studies. Subsequent studies using VAC-WR-expressing luciferase under a late promoter, found decreased DNA synthesis, and determined that in the J774.G8 monocyte/macrophage cell line, there was a dose dependent decrease of VAC late gene expression and total VAC DNA when macrophages were treated with recombinant IFN γ , and this decrease in VAC gene expression seemed to correlate with increased production of nitric oxide, as detected by examining nitric oxide intermediates in IFN γ -treated or untreated VAC-infected macrophages (Melkova and Esteban 1994). In total, it was found that IFN γ was a protein with many immune modulating activities that could inhibit virus spread both directly and indirectly and might therefore be an interesting target to inhibit virus spread or increase immune reactivity.

L. Poxvirus IFN γ Binding Proteins

Interestingly, poxviruses were already targeting the IFN γ pathway. Analysis of a major secretory protein encoded by the myxoma poxvirus revealed a protein that was found to have 20% amino acid identity to the extracellular domain of the IFN γ R with homologs in many other poxviruses including VAC (Upton, Mossman et al. 1992). Further analysis demonstrated that this protein (T7) was able to bind IFN γ , and when T7 was mixed with IFN γ , it was able to block the IFN γ -mediated inhibition of VSV-induced CPE. Later studies confirmed

that multiple poxviruses, including VAC-WR, cowpox, camelpox (Alcami and Smith 1995), and in a separate study Shope fibroma, VAC (WR and IHDW), ectromelia, cowpox, and rabbitpox viruses (Mossman, Upton et al. 1995) all encoded proteins that could bind IFN γ . Human, bovine and rat IFN γ bound the soluble IFN γ receptor encoded by VAC-WR, cowpox, or camelpox with similar affinities, but the IFN γ -binding protein encoded by VAC (B8R) had lower affinity for mouse IFN γ (Alcami and Smith 1995). In a separate study, ectromelia T7 was found to bind to human, murine, and rabbit IFN γ , but, as described above, B8R had lower relative affinity to mouse IFN γ than to human or rabbit IFN γ (Mossman, Upton et al. 1995). It is known that human IFN γ will not bind cells that express murine IFN γ R (Finbloom, Hoover et al. 1985), and that murine IFN γ will not bind to cells that express either hamster IFN γ R or human IFN γ R (Langer, Rashidbaigi et al. 1986), so the finding that B8R did not bind to mouse IFN γ may not be surprising.

To more closely examine the role of the B8R protein, many groups created VAC variants in which the gene encoding B8R was knocked out. The first description of B8R knockout VAC was done through homologous recombination of VAC-WR, and compared the virus to the parental strain, and also created a recombinant B8R knockout in an already recombinant VAC that expressed VSV for easier analysis of the antibody response (Verardi, Jones et al. 2001). In these experiments, supernatants from B8R knockout (B8R $^{-/-}$) VAC-infected HeLaS3 epithelial carcinoma cells could not block IFN γ -mediated

inhibition of encephalomyocarditis virus (EMCV)-induced CPE as could supernatant from VAC wild type infected cells, thereby confirming the lack of B8R protein activity. The knockout viruses grew as well as the wild type VAC *in vitro* on A459 lung adenocarcinoma cells and L929 cells. Interestingly, there was decreased weight loss and lethality in CB6F₁ mice I.N. infected with B8R^{-/-} VAC when compared to wild type VAC-infected mice, and decreased lethality in nude mice I.P. infected with B8R^{-/-} VAC when compared to infection with wild type VAC, with similar amounts of VAC-specific neutralizing antibody induced by intramuscular infection of any of the four viruses. A second group created two B8R^{-/-} VAC variants (WR and PrHa strain) and found that knocking out the B8R gene eliminated the capacity of supernatant from VAC-infected CV-1 African Green monkey kidney cells to crosslink to human and mouse IFN γ , confirming the knockout VAC (Sroller, Ludvikova et al. 2001). Furthermore, they showed that intra-dermal inoculation of B8R^{-/-} VAC induced smaller lesions than wild type VAC when infecting on either side of the dorsal spine in rabbits. In a third study, B8R^{-/-} VAC-WR were generated alongside a recombinant that instead of B8R, expressed the mouse IFN γ R α -chain truncated after Ser-257 so as to be secreted as an IFN γ binding protein, and named the virus “virus that expressed a soluble version of the mouse IFN γ R” (vsmIFN γ R) (Symons, Tschärke et al. 2002). The two recombinant VAC’s, the revertant VAC, and the parental strain were found to replicate similarly on African Green monkey kidney BSC-1 cells, but only the supernatant from vsmIFN γ R VAC-infected L929 cells was shown to

be able to bind mouse IFN γ . I.N. infection of mice with vsmlIFN γ R VAC increased signs of illness when compared to the B8R $^{-/-}$, revertant, and parental VAC's. In an intra-dermal rabbit model of infection, B8R $^{-/-}$ VAC induced greater infiltration of inflammatory cells and this infection had decreased amounts of VAC antigen when compared to infection with the revertant or parental VAC's. The most recent study used an attenuated strain of VAC, VAC Lister, and generated B8R knockout viruses via a homologous recombination method (Denes, Gridley et al. 2006). This study found no difference in induction of humoral or cellular immunity induced by the knockout virus infection when compared to wild type infection. In I.P. infection of nude BALB/c mice, the revertant VAC infection resulted in decreased survival, increased weight loss, and increased pock lesion appearance as compared to the B8R $^{-/-}$ VAC infection.

The negative influence of VAC spread by IFN γ has consistently been demonstrated, but the effect of deleting the B8R gene from VACV, and its effect on virus spread is still a matter of debate. This is most likely due to the fact that many of these experiments are performed in the mouse model, and B8R has been shown to have low relative affinity to mouse IFN γ . Interestingly, deletion of B8R from VAC does consistently attenuate the virus in rabbit models of infection, and studies that have examined the immune response to B8R knockout viruses have not found differences in poxvirus-specific immunity induced by infection. These results support the further examination of this knockout as a strategy to

decrease VAC pathogenicity, while maintaining the same immuno-protective phenotype of VAC infection

M. The Discovery of Interleukin-18 and the Interleukin-18 Receptor

Interleukin-18 (IL-18) was first described as a co-stimulator of IFN γ production in *Mycobacterium bovis* *Bacillus Calmette-Guérin*-infected mice treated I.P. with *Escherichia coli* 0127:B8 lipopolysaccharide (LPS), and was originally termed IFN γ inducing factor (IGIF) (Nakamura, Okamura et al. 1989). Later reports described the purification of this activity from mouse serum, described it as a co-stimulator of IFN γ production and proliferation in T cells when combined with concanavalin A (conA), IL-2, or a mAb against CD3, and in this same study ruled out interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and TNF as possible candidates of this activity by use of neutralization antibody assays (Nakamura, Okamura et al. 1993). Further work described the same activity in livers of mice challenged with *Propionibacterium acnes* (*P. Acnes*) (Okamura, Nagata et al. 1995), and IGIF protein induced by this model was used to clone and identify IGIF (Okamura, Tsutsui et al. 1995). Briefly, IGIF was subjected to tryptic digestion to allow sequencing of peptide fragments, which were then used to create a DNA probe that allowed for cloning from IGIF-expressing cells, and this probe was finally used to screen a cDNA library which allowed for identification of IGIF. These experiments also described an interesting synergistic effect of interleukin-12 (IL-12) and IL-18 in inducing IFN γ . Further analysis described structural homology of murine IL-1 α and IL-1 β

to murine IGIF (12 and 19% respectively) when compared by the fold recognition method, and when amino acid sequence homologies were compared between human IGIF and human IL-1 α and IL-1 β , the similarity was 15% and 18% respectively (Bazan, Timans et al. 1996). The cloning of the human IGIF protein led to the designation of IL-18, and also led to the observation that conA stimulated peripheral blood mononuclear cells (PBMC's) cultured with IL-18 not only had increased production of IFN γ when compared to cultures without IL-18, but also had increased production of Granulocyte-macrophage colony-stimulating factor (GM-CSF), and decreased production of IL-4 and interleukin-10 (IL-10). It was also described that if IL-18 were combined with interleukin-2 (IL-2), there was an increase in NK cell mediated cytotoxicity in comparison to IL-2 treated cultures alone.

Later work found that the IL-18 receptor (IL-18R) was a heterodimeric complex composed of an orphan receptor of the IL-1R family called IL-1Rrp (later IL-18R α) (Parnet, Garka et al. 1996) and a novel member of the IL-1R family (later IL-18R β). IL-18R α was identified by first screening multiple cell lines with a radioactively labeled IL-18 (^{125}I -IL-18) to identify a line with constitutively high levels of the protein that bound IL-18. The line chosen, the L428 Hodgkins disease cell line was then used to immunize BALB/c mice to make a mAb to purify this protein, which was then subjected to tryptic digest and sequenced, and in sequence analysis it was discovered that the protein that bound IL-18 was actually an orphan receptor of the IL-1R family called IL-1Rrp (Torigoe, Ushio et

al. 1997). Transfection of this protein (IL-18R α) into COS-1 SV40-immortalized African green monkey kidney cells showed low-affinity binding of IL-18 (Kd46nM) and allowed for nuclear factor kappa β (NF- κ B) activation after addition of IL-18. IL-18R α (then identified as IL-1R ρ) mRNA was detected in the spleen, thymus, leukocytes, liver, lung, heart, small and large intestine, prostate, and placenta but not in the brain, skeletal muscle, kidney or pancreas (Parnet, Garka et al. 1996). IL-18R β was identified based on its homology to IL-1R AcP, and after demonstrating this protein had no identifiable role in IL-1 or IL-8 signaling, signaling through IL-18 was examined as IL-18 had been classified as a member of the IL-1 family of cytokines (Born, Thomassen et al. 1998). It was then demonstrated that IL-R AcP (later IL-18R β) was not necessary for binding to IL-18, but was instead found to be important in activation of NK- κ B and c-Jun N-terminal kinase (JNK), and thus important in downstream signaling once IL-18 binds to its receptor. IL-18R β mRNA was detected in the lung, spleen, leukocytes, and colon, but not in the heart, kidney or muscle, mimicking many of the expression patterns of IL-18R α expression. IL-18R protein was expressed on a large number of cell lines including B cell, T cell, NK cell, myeloid cell, monocytoic cell, erythroid cell, and megakaryocytic cell lines (Nakamura, Otani et al. 2000). IL-18R was also found on T helper 1 (Th1), but not T helper 2 (Th2) cells in a separate study (Xu, Chan et al. 1998).

While originally identified as a co-stimulator of IFN γ production, especially when combined with IL-12, IL-18 was eventually found to have a broader role

than originally described. IL-18 could not only work as a co-stimulator of IFN γ production, but IL-18 has also been shown to increase the cytotoxic capacity of NK cells (Tsutsui, Nakanishi et al. 1996) and Th1 cells (Dao, Ohashi et al. 1996) through up-regulation of FasL. Later work demonstrated that IL-18 could have a more complicated role in that CD4⁺ T cells cultured in IL-18 produced interleukin-13 (IL-13) and GM-CSF, especially when IL-2 was added (Hoshino, Wiltrot et al. 1999), and found that naïve CD4⁺ T cells cultured in IL-2 and IL-18 without T cell receptor (TCR) engagement for 4 days expressed CD40 ligand (CD40L) and produced large amounts of IL-13 and IL-4, and that anti-CD3 stimulation of these cells now allowed them to develop into Th2 cells (Yoshimoto, Mizutani et al. 2000). If this same protocol was repeated, but anti-IL-4 was added to the culture, Th1 cells developed, suggesting that IL-18 might play a complicated role in differentiation of T helper subsets, depending on conditions available.

N. Interleukin-18 Binding Protein, and An Interleukin Binding Protein Encoded By Poxviruses

While trying to identify the IL-18R, one group was able to purify a protein that bound IL-18 from human urine and named this protein sIL-18R for soluble IL-18 receptor. In an early review, there was speculation that the IL-18R might be a heterodimer with the newly identified sIL-18R as the major determinant of IL-18 binding in the heterodimer, as the IL-18R identified at that time did not have high affinity to IL-18 (Dinarello, Novick et al. 1998). The then named sIL-18R was able to prevent LPS-induced IFN γ production as did neutralizing antibodies to IL-

18, but did not affect IFN γ production after stimulation with the mitogen conA, once again much like IL-18 neutralizing antibodies. They also found that sIL-18R did not possess species specificity, as it neutralized both human and murine IL-18. It was later found that this factor was not the IL-18R or even a component of the receptor, but was instead a soluble IL-18 binding protein (IL-18-BP) (Aizawa, Akita et al. 1999; Novick, Kim et al. 1999). Further work described the ability of this protein to block the binding of IL-18 to the IL-18R, although the IL-18BP shares no amino acid or structural homology to either IL-18R component. It is now thought that the IL-18BP works as a soluble decoy receptor, analogous to the membrane associated IL-1R2 for Type 1 interferon signaling, and might work as a soluble regulator of IL-18.

In examining poxviruses, a number of proteins were found that seemed to have a similar function as the IL-18BP. Originally, two proteins, MC53L and MC54L, from the human poxvirus molluscum contagiosum were tested and found to both bind IL-18 and inhibit IL-18's IFN γ stimulatory capacity, with MC53L having slightly higher affinity to IL-18 and a greater ability to inhibit IL-18's IFN γ stimulatory capacity (Xiang and Moss 1999). A later study described a protein in ectromelia virus that acted as an IL-18 decoy receptor (p13) (Born, Morrison et al. 2000). This viral protein was also shown to bind IL-18, and inhibit IL-18-induced NF κ B reporter activity as determined by a luciferase assay. It was also demonstrated that peritoneal exudate cells (PEC's) of mice infected with a p13 knockout ectromelia virus had increased NK cell activity as measured by a YAC

killing assay with no increase in NK cell numbers at days 2 and 3 when compared to mice infected with a wild type ectromelia virus. Subsequent analysis also revealed that the p13 deletion virus exhibited decreased levels of infectivity at day 3 when compared to wild type ectromelia virus. Finally, a later study provided evidence confirming the existence of the two IL-18BP's in molluscum contagiosum, but importantly went further and also described proteins that bound IL-18 in cowpox, VAC Ankara, VAC Lister, VAC-WR, and MVA, with a deletion in VAC Copenhagen (Smith, Bryant et al. 2000). The VAC IL-18 binding activity was subsequently mapped to the C12L open reading frame by other researchers, who then described the creation of a C12L knock out (C12L^{-/-}) in VAC-WR (Symons, Adams et al. 2002). The C12L^{-/-} VAC-WR had similar virus growth curves when compared to wild type or knock-in viruses when grown on BS-C-1 African green monkey kidney cell, but the knockout virus resulted in less weight loss and signs of illness when BALB/c mice were infected I.N.. In later work, the same group once again demonstrated the decreased weight loss and signs of illness when mice were infected I.N. with the C12L^{-/-} VAC-WR as compared to wild type or revertant, but also went on to show decreased viral titers in the lungs and brains of mice infected with the C12L^{-/-} VAC-WR (Born, Morrison et al. 2000). Further experiments found that mice infected with the C12L^{-/-} VAC-WR had increased NK and T cell cytotoxicity measured by chromium release assays, with greater amounts of IFN γ in bronchiolar lavage

exudates and increased IFN γ production in lung CD4⁺ T cells and CD8⁺ T cells when compared to mice infected with the wild type or revertant virus.

Work that had occurred shortly before identification of poxvirus-encoded IL-18-BP's had demonstrated that I.P. treatment of BALB/c mice with IL-18 two hours after intra-venous VAC-WR inoculation limited pock formation. These studies also described increased NK and cytotoxic T lymphocyte (CTL) killing activity in mice I.P. treated with IL-18 after infection, so it had been known that IL-18 treatment could limit the pathogenicity of a poxvirus infection before an IL18-BP was described (Tanaka-Kataoka, Kunikata et al. 1999).

This work thus demonstrates that infection with VAC in mice is attenuated by treatment with IL-18, and that IL-18BP knockout virus variants of the poxviruses ectromelia and VAC are significantly attenuated in mouse models of infection. This supports the further examination of this knockout as a strategy to decrease VAC pathogenicity, while maintaining the same immuno-protective phenotype.

O. The Complement Cascades

The observation that blood did not seem to decompose as quickly as other organic material, and that it might have some bactericidal effect had been suggested early on, but experimental investigations of this effect and therefore of the complement system only started in the late 1800's. The work is commonly described to have begun with the demonstration that defibrinated but not heat inactivated blood of *Anthrax bacilli* inoculated animals, when mixed with *Anthrax*

bacilli, had a measurable destructive effect on the bacteria (Nuttall 1888). In work that quickly followed with *Clostridium tetani* and *Corynebacterium diphtheria*, the specific anti-bacterial activity was confirmed, the observation that this activity could be passively transferred to other animals was described, and the bactericidal factor was named “alexin” (von Buchner 1889) (from reviews (Zinsser 1918; Crist and Tauber 1997; Cooper 2006). This work was extended upon in what was eventually termed “Pfeiffer’s Phenomenon”, wherein lysis of *Vibrio cholera* occurred in the peritoneal fluids of animals injected with attenuated *Vibrio cholera*, and also re-demonstrated that the activity could be passaged to other animals and was pathogen-specific, and also that the effect was not heat labile. Further examinations of the phenomenon demonstrated that this effect could be separated into two activities in the sera, one that would agglutinate the blood and was not heat labile (55 or 56°C for half hour), and the other component was found to be heat labile but was the component responsible for the lytic activity (Bordet 1896; Bordet 1898) (from reviews (Zinsser 1918; Crist and Tauber 1997; Cooper 2006), and this heat labile component was subsequently termed complement (Ehrlich and Morgenroth 1899).

As more work was done on the complement phenomenon, it became clear that there was more than one factor at work. This was evidenced early on by separation of the complement activity into two components, a water soluble and insoluble fraction neither having the complement activity alone (von Buchner 1889). Later work confirmed this and demonstrated that the insoluble fraction

bound antibody with the soluble fraction imparting lytic activity (Ferrata 1907). Eventually a third heat stable component of complement was described by the removal of complement activity by yeast cells followed by addition of a heat-inactivated guinea serum (Flexner and Noguchi 1902; A.F. 1914). Further experiments built on these observations, and described inactivation of the third component more reliably using zymosan-treated sera, and this third component became known as the third component or heat stable component of complement (Whitehead, Gordon et al. 1925). This same group later demonstrated that treatment with ammonia could destroy another heat stable component, and that zymosan-treated and ammonia-treated sera could complement one another, and therefore demonstrated a fourth component of the complement cascade (Gordon, Whitehead et al. 1926). The complement components were numbered in order of their discovery, but the biological order of action of the four components of complement was eventually biochemically worked out to be C1, C4, C2, C3 (Bier, Leyton et al. 1945). It was eventually determined through chromatography on diethylaminoethyl (DEAE) cellulose that the first component of complement was made up of three separate proteins (C1q, C1s, and C1r), and that these individual three components were necessary for the C1 factor to function. Further biochemical characterizations describe C1 (C1q) as the early binder of antibody-antigen complexes (Augener, Grey et al. 1971), and once bound, the complex now had proteolytic activity (C1s) (Becker 1956). This cleaves C4 (Borsos and Rapp 1963) and C2 (Polley and Muller-Eberhard 1968)

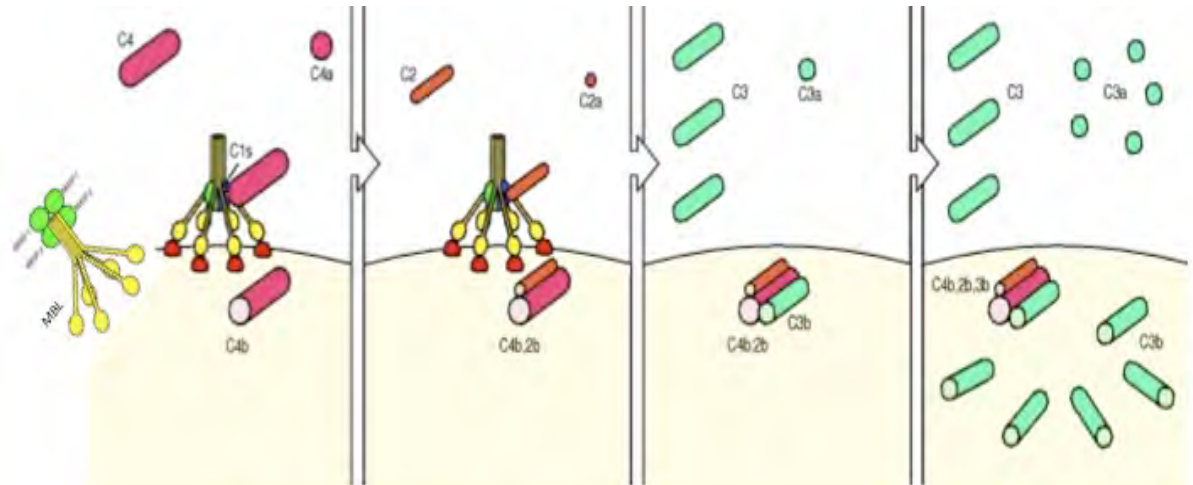
into components C4b and C4a and C2b and C2a. C4b then binds C2a and cleaves C3 (Muller-Eberhard, Polley et al. 1967), thus activating it, and the C4bC2a complex acts as a C3 convertase, enzymatically cleaving C3 to C3b and C3a, and C3b then binds to the surface of pathogens or cells allowing for opsonization or lysis (Figure 1.1A). Thus, the early cascade of the main mediator of lysis of antibody treated pathogens or cells, either directly or through opsonization, had now been worked out. The latter part of the pathway which leads to formation of a membrane attack complex allowing for direct pathogen or cellular lysis is reviewed here (Muller-Eberhard 1986).

The story becomes more interesting with the identification of two other pathways that activate the complement cascade. The first was originally described as a factor dubbed properdin that would activate the complement cascade, resulting in lysis of certain red cells, bacteria (*Shigella dysenteria*) and inactivation of infectivity of viruses (Pillemer, Blum et al. 1954). This original observation was soon suggested to instead be an artifact caused by residual natural antibody (Nelson 1958). Subsequent studies indirectly suggested that there was an alternative way of inducing the complement cascade, as edema and hemorrhage to injection of two purified anti-human serum albumin (HSA) antibodies along with purified HSA was detected in C4 deficient guinea pigs (Ellman, Green et al. 1970), and this reaction in previous studies had been demonstrated to be complement dependent (Maillard and Zarco 1968; Cochrane, Muller-Eberhard et al. 1970). Other *in vitro* work demonstrated that endotoxin

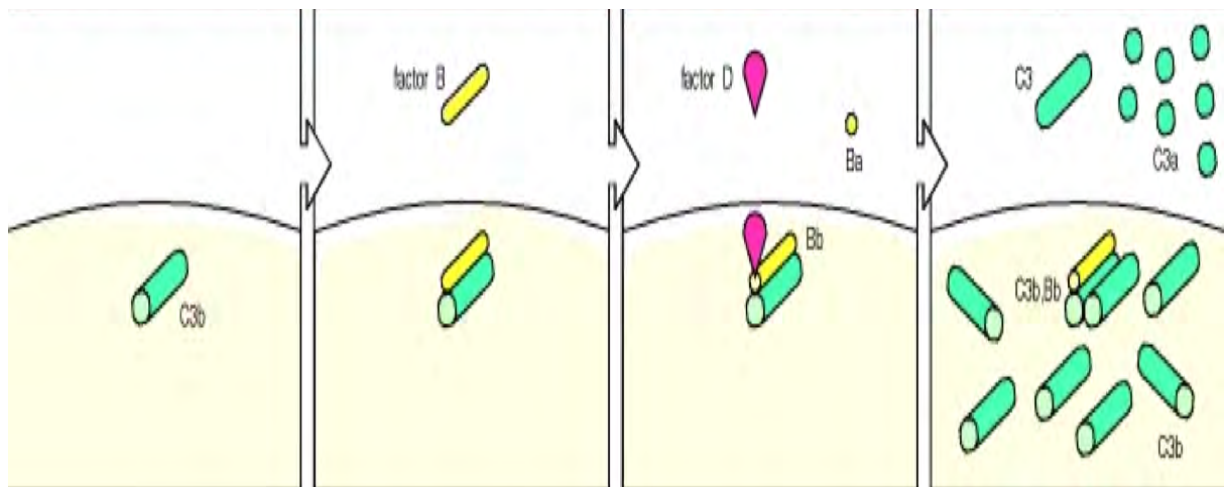
and antigen-antibody complexes could induce the late activities of the complement cascade (Frank, May et al. 1971). As evidence mounted, the isolation of the initiator protein of this alternate system was described (Gotze and Muller-Eberhard 1971) and finally purified and described as a β - pseudoglobulin. This initiator protein was eventually named Factor B. A second factor was found to be important in this alternate pathway (Muller-Eberhard and Fjellstrom 1971), and its subsequent isolation and naming as Factor D happened soon afterwards (Hunsicker, Ruddy et al. 1973). In this pathway, C3 undergoes spontaneous hydrolysis that allows Factor B to bind. Factor D can now cleave Factor B into two portions, Bb and Ba, and this becomes a positive feedback loop as the C3Bb can now cleave more Factor B. Thus an alternative manner to activate the complement cascade was described and named the alternative complement pathway (Figure 1.1B).

The second description of an alternative manner in which to activate complement started with the description of a mannose binding lectin (MBL). MBL recognizes sugars normally only exposed on envelopes of non-vertebrate origin (Robinson, Phillips et al. 1975). Further work described the action of MBL in conjunction with proteins later named MBL serine proteases (MASP's 1 and 2) (Takayama, Takada et al. 1994) that acted similarly to C1s and C1r. This MBL/Masp1,2 complex was found to activate the classical pathway of complement by activating C4, C2 and finally C3 as was demonstrated in the classic model of complement activation, and so this was described as a separate

way to activate the classic complement pathway without need of antibody-antigen complexes. As the complement pathway can lead to direct cell lysis, there are many proteins that serve to control initial activation and serve to shut down the pathway. One of these inhibitor pathways involves a C4 binding protein (C4BP) that works with a second protein, the C3b inactivator (C3bINA, later named Factor I) to degrade C4b and to a lesser extent C3b to limit complement activation (Gigli, Fujita et al. 1979), and evidence suggests that poxviruses have taken advantage of the this pathway to limit complement activation and virus spread after infection (Figure 1.1A).



A. (Adapted from Figure 2.11, 2.13 (Janeway, Travers et al. 2005)).



B. (Adapted from Figure 2.17 (Janeway, Travers et al. 2005)).

Figure 1.1. The complement cascade.

Figure 1.1. The complement cascade.

(A) C1q or MBL binds antigen, with C1q binding antigen/antibody complexes, releasing restriction by C1r of C1s, (or MBL binding pathogen activating MASP's). These cleave and activate C4 to C4b and C4a. C4b binds C2 allowing cleavage of C2 to C2b and C2a, C4bC2a acts as a C3 convertase cleaving C3 to C3b and C3a. (Adapted from Figure 2.11, 2.13 (Janeway, Travers et al. 2005)).

(B) Auto-hydrolyzed C3b binds surface of pathogen, C3b binds Factor B, bound Factor B is cleaved by Factor D, producing Bb and Ba. C3bBb acts as C3 convertase. (Adapted from Figure 2.17 (Janeway, Travers et al. 2005)).

P. A Poxvirus Encoded Inhibitor of the Complement Cascade

Early work examining the *in vivo* effect of complement on poxvirus infections was done using fowlpox, and it was found that the alternative complement cascade worked to inhibit fowlpox virus spread in chicken embryo cells (Ohta, Kai et al. 1983). A follow-up using cobra venom factor (CVF) to inhibit the complement cascade, found that chickens treated for three days with CVF following sub-lethal fowlpox all died by day 10, with significant virus dissemination and a 2 log increase in virus in poxvirus lesions (Ohta, Yoshikawa et al. 1986). This early work demonstrated the importance of complement in limiting poxvirus pathogenicity. In work examining the major proteins secreted from VAC-WR-infected RK-13 rabbit kidney cell, two important immune modulators were discovered. One of these secreted proteins had previously been identified as a protein with structural similarity to epidermal growth factor (EGF) (Blomquist, Hunt et al. 1984; Brown, Twardzik et al. 1985; Reisner 1985) and was subsequently shown to behave similarly to EGF *in vitro* (Twardzik, Brown et al. 1985; King, Cooper et al. 1986) and *in vivo* (Buller, Chakrabarti et al. 1988). The other was found to have structural similarity to the superfamily of complement control proteins. This protein was predicted to be most similar to the C4BP component of this superfamily and was later found to act similarly to C4BP in that virus supernatant from wild type infected RK-13 cells but not knockout virus-infected cells could inhibit the classical complement cascade as assessed by inhibition of human complement-mediated lysis of IgM sensitized red blood

cells, but not the alternative complement pathway (Kotwal and Moss 1988). A more purified isolation of the proteins did the same and was also found to bind to C4b-coated red blood cells and block binding of these coated cells to human erythrocytes, which have a C3b/C4b receptor on their cell surface (Kotwal, Isaacs et al. 1990). Interestingly, a later study using inhibition of virus growth to measure VAC-specific antibody-enhanced complement inhibition found that not only was the classical complement pathway inhibited but now also found that the alternative complement pathway was inhibited by the complement binding protein (Isaacs, Kotwal et al. 1992). This work also mapped and named the VAC complement binding protein (C21L, or C3L), and after generating a knockout VAC-WR variant, found that intra-dermal infection with the C21L knockout VAC-WR on the shaved back of rabbits resulted in decreased lesion size when compared to VAC-WR wild type inoculation, and this difference was noted in both wild type and C4-deficient rabbits starting at about Day 5. These *in vivo* results are consistent with the idea that the knockout virus has decreased virus spread, leading to decreased inflammation, after an antibody response begins to be generated due to the lack of a protein that inhibits complement, and suggests that both the classical and alternative complement cascade are inhibited. As it was found that both the classical and alternative pathway of the complement cascade was inhibited, it did not seem likely that C21L only inhibited complement by binding C4b. Later experiments indeed confirmed that not only did C21L bind C4b allowing for degradation by Factor I, but also bound C3b, which also

increased Factor I's degradation of C3b (McKenzie, Kotwal et al. 1992; Sahu, Isaacs et al. 1998). These results suggest that C21L limits inflammation and poxvirus spread, and this was later confirmed using a knockout cowpox virus infection of mice (Miller, Shchelkunov et al. 1997), and the results in total suggest that C21L might be an attractive knockout target to limit poxvirus spread while inducing a comparable or stronger immune response when designing recombinant VAC.

Q. The Discovery and Cloning of Fas and Fas Ligand

The control of an infecting pathogen by a host immune response requires the tightly coordinated action of many cell subtypes and soluble mediators that work in tandem to eliminate or contain the infecting pathogen. This response must be swift, specific and tightly regulated so as not to cause more harm than necessary to the host than required to fend off the pathogen. Unfortunately, in some individuals there are mutations that predispose them to autoimmune disorders where an immune response will develop and result in cellular or humoral attack against the host's own tissues.

One family of genetic mutations that can often lead to autoimmune disorders in individuals are mutations in the genes encoding the Fas and FasL proteins and genes encoding proteins in the Fas and FasL signaling pathways. Fas was first described when the screening of a library of >20,000 hybridoma clones revealed a clone which produced an antibody that had cytolytic activity against a variety of human cell lines (Yonehara, Ishii et al. 1989). The cytolytic

activity of the anti-Fas antibody was found to be similar in many ways to the cytolytic effect that had been previously noted when cells were treated with recombinant tumor necrosis factor α (TNF α), in that the cytolytic effect of the antibody was enhanced after treatment with IFN γ , mitomycin C, a DNA cross-linker that induces cell death, actinomycin D, a transcriptional inhibitor, or cycloheximide, a protein synthesis inhibitor, and also seemed to induce cell death on most cell types that TNF α worked on as well. Interestingly, there were human cell lines where the anti-Fas antibody, but not TNF α , induced cytotoxicity. Further results suggested that the anti-Fas antibody worked on a protein that did not bind TNF α . Pull-down experiments with the anti-Fas antibody revealed a protein of a different size than the TNF receptor (TNF-R). It was found that in cells that were responsive to anti-Fas but not TNF α , there was no detectable down-regulation of TNF-R after treatment with the anti-Fas antibody, and that TNF α treatment did not down-regulate Fas, but did down-regulate the TNF-R. In parallel, a separate cytolytic mAb was described that reacted with a cell surface protein named Apo-1, and this interaction also induced apoptosis, but in this study the protein was described as being expressed on activated lymphocytes as well as malignant cells lines of leukemic origin (Trauth, Klas et al. 1989). Further investigations into the cell surface protein that the anti-Fas antibody bound led to the identification and cloning of the Fas gene (Itoh, Yonehara et al. 1991). To confirm that the gene cloned was indeed necessary for anti-Fas antibody-apoptosis, it was then demonstrated that recombinant expression of this gene in

a cell that is not normally sensitive to anti-Fas antibody induced apoptosis rendered the cell susceptible to apoptosis induced by the antibody. Characterization of the cell surface receptor that bound the anti-Apo-1 antibody revealed that anti-Fas and anti-Apo-1 antibody were binding to the same protein (Oehm, Behrmann et al. 1992). It was also demonstrated that the Fas protein belonged to the same family as both the TNF-R and the low-affinity nerve growth factor receptor. The subsequent isolation of the gene that encoded the Fas protein in mice allowed for the examination of mRNA expression in different tissues, and it was found that Fas mRNA was detected in the liver, ovaries, thymus, and heart, but not in mouse spleens or brain, and that IFN γ up-regulated Fas mRNA expression in L929 and macrophage BAM3 cell lines (Watanabe-Fukunaga, Brannan et al. 1992).

The identification of the protein that bound Fas was done by use of a Fas-Ig antibody to enrich high expressors of Fas in a d10S-2 murine Th2 cell line from AKR/J mice, which then allowed for creation of a cDNA library and the eventual cloning of the FasL protein (Suda, Takahashi et al. 1993). This protein was a type II transmembrane protein of the TNF family, and further experiments demonstrated that expression of FasL on COS cells conferred the cells with the ability to kill Fas-expressing cells, and that the concentrated supernatant of FasL-expressing COS cells was able to induce apoptosis in Fas-expressing cells. The biochemical processes that led to apoptosis still needed to be worked out.

R. Fas/Fas Ligand Signaling in Inducing Apoptosis

It is clear that FasL can induce cells expressing Fas to undergo apoptosis, and one of the first actions observed after FasL interacts with Fas was aggregation of Fas, first demonstrated using apoptosis-inducing antibodies (Dhein, Daniel et al. 1992). This aggregation was later demonstrated to quickly (within 1 second) recruit a set of four factors termed CAPS (cytotoxicity-dependent APO-1 associated proteins) to form a DISC (death-inducing signaling complex), but only with apoptosis inducing antibodies, whose formation was shown to be required to induce cell death by use of truncated Fas proteins (Kischkel, Hellbardt et al. 1995). These studies also identified CAP1 and CAP2 as “phosphorylated mediator of receptor induced toxicity 1”/ “Fas associated protein with death domain” (MORT1/FADD), which had recently been described as proteins that interacted with Fas through homologous domains known as death domains (DD) (Boldin, Varfolomeev et al. 1995; Chinnaiyan, O'Rourke et al. 1995). These studies were soon followed by the identification of CAP3 and CAP4. In one study, this was accomplished through nanospray mass spectrometry, and the CAP3 and CAP4, which were found to be the same protein, was named FADD-homologous ICE/CED-3-like protease (FLICE) (Muzio, Chinnaiyan et al. 1996). In a second study, the same protein was identified through two-hybrid screening with FADD, and the protein was named MORT1-associated CED-3 homolog (MACH) (Boldin, Goncharov et al. 1996). The identification of these binding partners of FADD, MACH and FLICE which

were eventually identified as the same protein, caspase 8, and of an inhibitor of the apoptosis inducing complex termed FLICE inhibitory protein (FLIP) that was found to bind and inhibit caspase 8 and FADD (Irmeler, Thome et al. 1997) resolved the major components of the DISC. Caspase 8 activation was eventually shown to initiate apoptosis through two pathways, one involving activation of caspase 3 by caspase 8 through high level DISC formation, and the other through activation of a protein named BH3 interacting domain death agonist (BID) to activate the mitochondrial apoptosis pathway directly (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Scaffidi, Fulda et al. 1998). (Figure 1.2) Interestingly, in examining *in vitro* proliferation of human T cells, Fas co-stimulation was described to increase levels of proliferation in comparison to CD3 stimulation alone (Alderson, Armitage et al. 1993). This proliferation was eventually shown to depend on caspase activation (Kennedy, Kataoka et al. 1999), and in murine transgenic CD4⁺ T cells, also seems to be controlled by NK κ B signaling (Maksimow, Soderstrom et al. 2006).

The description of the Fas and Fas L interaction, signaling pathways and the cloning of the genes, allowed for the identification of the cause of two mouse lupus-like lymphoproliferative disorders that had previously been of unknown etiology.

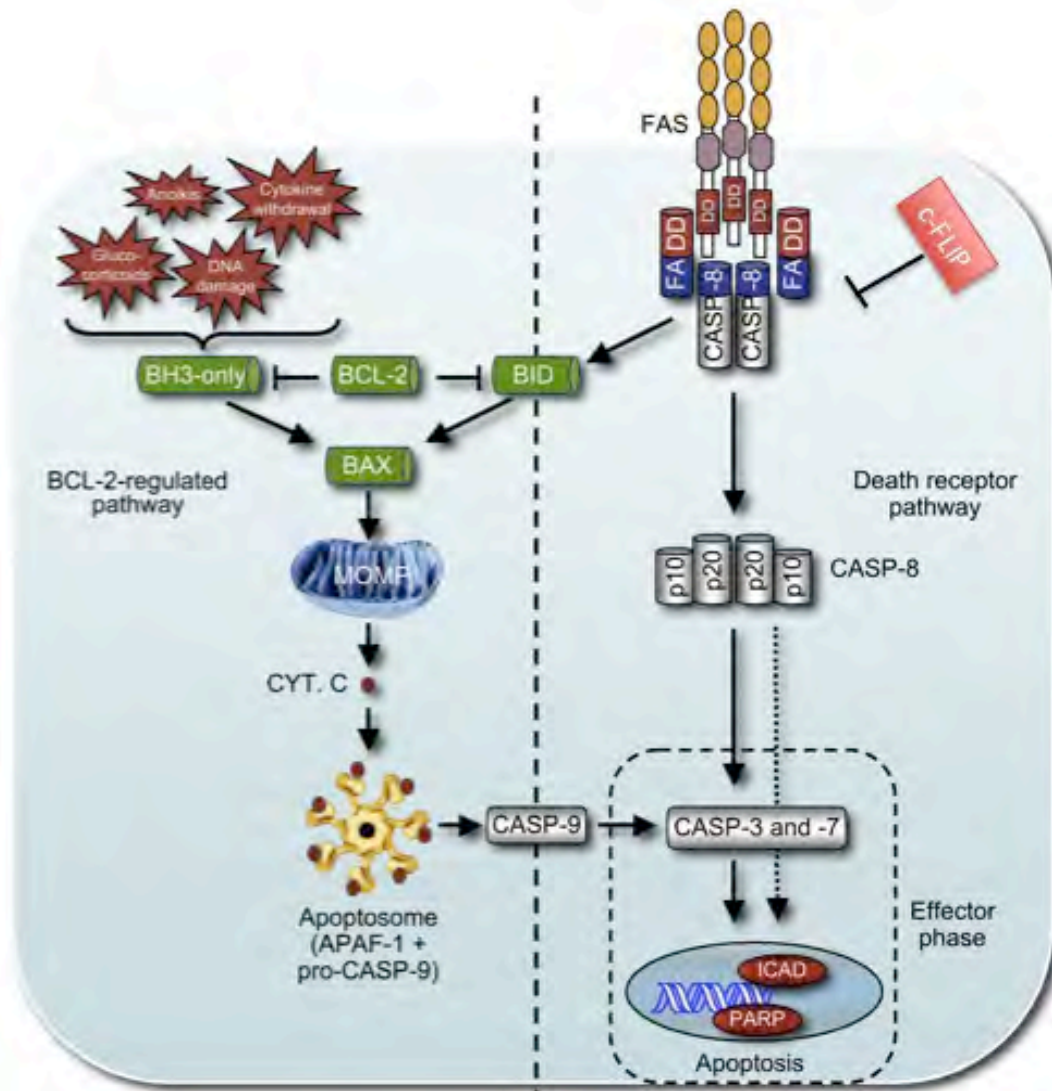


Figure 1.2. Fas-FasL signaling. (Adapted from Figure 1 (Strasser, Jost et al. 2009)).

Figure 1.2. Fas-FasL signaling.

FasL interacts with Fas inducing trimerization. This leads to the formation of the DISC, which includes two FADD proteins and two caspase 8 proteins that interact through DD. cFLIP can inhibit this interaction through binding of caspase 8 or FADD. Activation of caspase 8 in high amounts allows cleavage of caspase 3, leading to apoptosis. Alternatively BID is cleaved, which can be inhibited by Bcl-2, and BID cleavage cleaves Bax allowing for cytochrome C release from the mitochondria, leading to apoptosis through induction of the apoptosome.

(Adapted from Figure 1 (Strasser, Jost et al. 2009)).

S. The *lpr* and *gld* Mouse Models of Lupus

The first of these lymphoproliferative disorders had been dubbed *lpr*, and the disorder had been originally described during derivation of the MRL/MpJ mouse strain, which was a cross of the AKR/J, B6, C3H/Di, and LG/J strains (Murphy and Roths 1978). This disorder (*lpr*) was found to mimic mouse models of lupus such as the NZW/B mice in that, at 6 months of age, there was an increase in the number of immune complexes in the sera, in the amount of anti-double stranded and anti-single stranded DNA activity in the sera, and in total and anti-nuclear antibody in the sera when compared to BALB/c mice (Andrews, Eisenberg et al. 1978). There was also a marked increase in mouse mortality in these lupus prone animals, with mice typically succumbing to glomerulonephritis, with a severe lymph node hyperplasia noted at the end of life. These symptoms in many ways mimicked symptoms of humans with systemic lupus erythematosus (SLE). When first describing the MRL/Mp *lpr* (MRL-*lpr*) mouse there was some question as to whether the lupus-like disorder was multigenic, or derived from a single mutation. Further studies that examined B6 mice crossed to MRL-*lpr* mice (B6-*lpr*) suggested that there might be a single region change in *lpr* mice that had a profound role in the lupus-like disorder. It was found that B6-*lpr* mice had increased levels of antibody against double stranded DNA (dsDNA) as did MRL-*lpr* mice, although the disorder occurred at a later time point than in MRL-*lpr* mice (Pisetsky, Caster et al. 1982). Subsequent studies where the *lpr* mutation was backcrossed to C3H/HeJ (C3H), B6, and AKR/J mice at least 8

times confirmed the observation that the *lpr* region belayed a large part of the lupus-like disorder. C3H and B6 mice with the *lpr* mutation had a significantly reduced lifespan when compared to their wild type counterparts, although life span could not be examined in AKR/J mice as these mice spontaneously develop and die of thymic lymphoma. All three *lpr* strains had increased lymphadenopathy at four months of age when compared to their counterpart wild type mice (Izui, Kelley et al. 1984). All three *lpr* strains also had increased amounts of antibody against single stranded DNA (ssDNA), increased amounts of anti-IgG antibody, and increased levels of IgM and IgG immune complexes by six months of age when compared to their respective wild type counterparts. These studies provided strong evidence that a single genetic region imparted a significant portion of the *lpr* phenotype.

Further work included the description of a new strain of mice designated *lpr^{cg}* (*lpr* complementing *gld*) that had a similar lymphoproliferative disorder, that by backcrossing studies was identified to very likely be a mutation in the same region as the original *lpr* mutation (Matsuzawa, Moriyama et al. 1990). The same group that had cloned Fas in the mouse next examined MRL-*lpr* and C3H/HeJ *lpr* (C3H-*lpr*) strains of mice and found that there was no Fas mRNA detected in the *lpr* strains tested, even after cDNA conversion and amplification by polymerase chain reaction (PCR), and this loss of Fas mRNA was attributed by Southern blots to a re-arrangement of exon 2 in the Fas gene (Watanabe-Fukunaga, Brannan et al. 1992). When analysis of the *lpr^{cg}* mutation was examined, it was

found that Fas mRNA was expressed from cells that contained the *lpr^{cg}* mutation, but that there was a single point mutation in the Fas gene, and when this gene was expressed in L929 cells, which are not sensitive to Fas-antibody induced apoptosis, it did not impart sensitivity to Fas-antibody induced apoptosis while the wild type gene did. Later studies from the same laboratory determined that the *lpr* mutation was likely due to insertion by retrotransposition of the RMg2 early transposable element (ETn) (Shell, Collins et al. 1990) into intron 2 of the Fas gene, and while no Fas mRNA could be detected in the *lpr* mice, experiments inserting the RMg2 ETn into an intron of an unrelated gene encoding granulocyte colony stimulating factor (G-CSF) suggested that the insertion might only severely depress expression of functional protein, and therefore the *lpr* mutation might not behave like a full Fas-null mutation (Adachi, Watanabe-Fukunaga et al. 1993). Later results would support this hypothesis, with some of the strongest evidence resulting from the Fas knockout mouse. It was discovered that B6 Fas-null mice developed lymphadenopathy, splenomegaly, and increased Ig levels much sooner than even the most severe strain of *lpr*, MRL-*lpr* mice (Adachi, Suematsu et al. 1995; Adachi, Suematsu et al. 1996).

The second murine lymphoproliferative disorder described by the identification of the Fas and FasL pair was originally discovered in C3H mice after the *lpr* mutation was suggested to be generated by a single mutation, and was termed *gld* (generalized lymphoproliferative disorder) (Roths, Murphy et al.

1984). C3H/HeJ *gld* (C3H-*gld*) mice seemed similar to MRL-*lpr* mice in many ways, in that they had an early onset lymphoid hyperplasia, increased amounts of all Ig classes tested, increased amounts of antibodies that bound dsDNA, decreased life spans, and increased amounts of anti-nuclear antibodies when compared to the normal C3H wild type mice. Although there were many similarities, it was determined through linkage studies that the *lpr* and *gld* were not allelic, and initial results suggested that disease onset in C3H-*gld* mice was different from MRL-*lpr* mice in that the C3H-*gld* lymphoproliferation was followed by a regression of lymph node size before the lymphoproliferation set back in. Furthermore, the life span of C3H-*gld* mice was increased when compared to MRL-*lpr* mice. MRL-*lpr* typically died of glomerulonephritis and C3H-*gld* died of chronic interstitial pneumonitis, and the marked lymphoid infiltrates of the kidney, liver and other organs normally found in MRL-*lpr* mice were not found in the C3H-*gld* mice. Further analysis of the *lpr* or *gld* mutations on the same background, C3H, suggested that the mutations might be in a similar pathway (Izui, Kelley et al. 1984; Davidson, Holmes et al. 1985), with some suggesting that the *lpr* and *gld* mutations might be in the same metabolic system (Davidson, Holmes et al. 1985). Later studies directly comparing C3H-*lpr* and C3H-*gld* mice demonstrated the outgrowth of a very similar abnormal Thy1.1+B220+CD4-CD8- cell population in both sets of mice (Davidson, Dumont et al. 1986). Further studies demonstrated similar increased total IgM and IgG serum levels, ssDNA binding IgM and IgG in sera, and dsDNA binding IgM and IgG in the sera of C3H-

lpr and C3H-*gld* mice when compared to wild type C3H mice, supporting the idea that the *lpr* and *gld* mutations were different mutations in a single pathway (Seldin, Reeves et al. 1987). Studies examining the phenotype of *lpr*^{cg} mice crossed with *gld* mice described an ameliorated lymphoproliferative phenotype when compared to either the *lpr*^{cg} mice or *gld* mice, further supporting the idea that *lpr* and *gld* mutations were in the same metabolic pathway (Matsuzawa, Moriyama et al. 1990). Interestingly, when bone marrow chimera experiments were performed, it was postulated that the *lpr* and *gld* mutations might not be in the same metabolic pathway, but that the *lpr* and *gld* mutations might be individual mutations on interacting proteins (Allen, Marshall et al. 1990).

The cloning and description of Fas, and shortly thereafter FasL, and the evidence that the *lpr* and *gld* mutations might be individual mutations on interacting molecules helped lead to the discovery that the *gld* mutation was an inactivating mutation of FasL (Takahashi, Tanaka et al. 1994). Initial analysis indicated no gross realignments in the FasL gene in *gld* mice, and no differences in the levels of FasL mRNA as had been observed with Fas mRNA in *lpr* mice, but further analysis through sequencing described a point mutation in *gld* FasL DNA, and described that expressing this variant of FasL into COS cells did not confer the ability to kill Fas expressing cells as would expression of the non-*gld* FasL gene.

Later experiments suggested, that as with the *lpr* mutation, the *gld* mutation might also be leaky (Adachi, Suematsu et al. 1995), and the eventual

description of a mouse with the FasL gene floxed, and then ubiquitously knocked out provided evidence that this was the case (Karray, Kress et al. 2004). These experiments demonstrated that B6 FasL-null (B6-*FasL*^{-/-}) mice developed hypergammaglobulinemia, splenomegaly, and lymphadenopathy at earlier time points than B6-*gld* mice, with residual ability to induce Fas-mediated cytotoxicity in splenocytes from B6-*gld* as compared to B6-*FasL*^{-/-} splenocytes.

T. Deleterious Mutations In Fas and FasL In Humans Are Associated With Autoimmune Lymphoproliferative Disorders (ALPS)

The identification of Fas and FasL as the mutations that led to the severe lymphoproliferative disorders in the *lpr*, *lpr*^{cg}, and *gld* mutations, suggested that mutations in this gene family might also explain some autoimmune lymphoproliferative disorders (ALPS) in humans. In publications quickly following the identification of Fas as the mutant gene in *lpr* mice and *lpr*^{cg} mice, mutations in Fas that led to either decreased protein levels, or decreased activity were found in patients with ALPS. In the first example, three children with ALPS were described to have two separate mutations in the Fas gene (Rieux-Laucat, Le Deist et al. 1995). All three patients had a significant population of CD3⁺CD4⁻CD8⁻ cells (as seen in *lpr* and *gld* mice) and the patient with the most severe phenotype (hemolytic anemia, neutropenia, and thrombocytopenia) had a 290 nucleotide base pair (BP) knockout in the Fas gene, with very little Fas protein detected on the surface of activated cells, and little apoptosis induced by mAb to Fas. The other two patients were found to have a 2BP frame shift in one copy of

the Fas gene that when cloned and tested *in vitro*, led to a decrease in Fas mediated apoptosis. This gene was found to be from the mother, and a second unknown mutation outside of the Fas gene was suspected to derive from the father, as his cells were also less susceptible to Fas mediated apoptosis, although the mechanism was not described. A study published very soon afterward also described deleterious Fas mutations in five children diagnosed with ALPS, also characterized by autoimmune hemolytic anemia, thrombocytopenia, and neutropenia, with four of the five children requiring removal of enlarged spleens, and one patient requiring dialysis for glomerulonephritis (Fisher, Rosenberg et al. 1995). All five patients were found to have increased percentages of CD3⁺CD4⁻CD8⁻ cells, and all were found to have mutations in the Fas gene, two mutations leading to little cell surface expression of the Fas protein, one mutation led to Fas that could not interact with anti-Fas antibody, and two mutations that could not transmit a death signal through Fas. Further analysis revealed that co-expression of four of the mutant Fas genes with wild type Fas resulted in a dominant negative effect, where expression of the mutant Fas gene resulted in decreased Fas expression on the cell surface and decreased anti-Fas- antibody mediated apoptosis. Subsequent studies associated heterozygous Fas mutations, in this case predicted to impair signal transduction, with Canale-Smith ALPS (Drappa, Vaishnav et al. 1996) and in a broader study in kindred of ALPS patients, found a 14 and 51 times greater fold risk of non-Hodgkin's and Hodgkin's lymphomas respectively, with all

patients that had lymphomas having deleterious Fas mutations resulting in decreased Fas mediated lymphocyte apoptosis (Straus, Jaffe et al. 2001). A more recent study described an ALPS disorder in a patient caused by a homozygous FasL gene mutation (Del-Rey, Ruiz-Contreras et al. 2006). This disorder, as were the Fas mutant disorders, was initially characterized by increased numbers of CD3⁺CD4⁻CD8⁻ cells in blood, but in this case Fas induced apoptosis was normal and AICD was impaired, which suggested a mutation in FasL. Further results demonstrated that the cells transfected with the FasL gene from the patient could not induce apoptosis in Fas expressing cells, while transfection of wild type FasL did, and subsequent analysis described a point mutation in the FasL gene that seemed to effect function and FasL protein expression.

The recent observation that individuals with dual hypoactive mutations of Fas or FasL genes have quicker recovery of their CD4⁺ T cell counts after antiretroviral therapy may suggest a different phenomenon that could be occurring (Nasi, Pinti et al. 2005). A hypoactive mutation in a pathway many times thought to be involved in killing infected cells, or leading to an autoimmune disorder, may be leading to increased protection against certain high dose virus infections.

U. Measuring T Cell Diversity

The immune response to a viral infection often involves the rapid proliferation of CD8⁺ effector T cells that recognize virus-infected targets

expressing 8-11 amino acid long peptides on class I MHC molecules. This recognition is mediated by membrane-bound TCR's that are generated through largely random DNA recombination events of the many TCR α and β genes, whose encoded polypeptide chains heterodimerize to form the recognition structure of T cells. The recombination of the segments also involves addition or deletion of nucleotides during the joining process, causing even greater diversity, and these processes allow for a very broad range of T cell specificities, with a calculated theoretical diversity of $\sim 10^{15}$ T cell receptors in the mouse (Davis and Bjorkman 1988). Using PCR, complement determining region 3 (CDR3) spectratyping, and sequencing techniques, it was estimated that there are approximately 2×10^6 distinct TCR specificities in a mouse spleen (Casrouge, Beaudoin et al. 2000; Baum and McCune 2006). This is far below the theoretical T cell diversity, but, considering estimates of T cell degeneracy that propose that a single TCR can recognize up to 10^6 pMHC (peptide MHC) complexes (Mason 1998; Wilson, Wilson et al. 2004), it is likely that the functional diversity is much greater than the number of individual TCR's.

It has been of interest to calculate the number of T cells that would either recognize or respond to a pathogen or to a specific peptide-MHC (pMHC) complex. Early estimates of the number of CD8⁺ T cells that are specific to a single virus, i.e. precursor frequencies, took advantage of an *in vitro* limiting dilution assay (LDA), and calculated CD8⁺ T cell virus-specific precursor frequencies to be on the order of 1 in 100,000 in naïve mice and predicted that

these cells needed to undergo about 15 divisions to reach the higher precursor frequencies found at day 8 post-infection (Selin, Nahill et al. 1994; Selin, Vergilis et al. 1996). The efficiency of such assays, however, is relatively poor. Later studies estimated the number of pMHC-specific CD8⁺ T cells in a naïve mouse by CDR3 sequencing. H-2K^d-restricted HLA 170-179 specific T cells were sorted by tetramer from human tumor immunized mice, 6 amino acid long CDR3 regions of the dominant responding V β chain amplified using V β specific primers, and then sequenced. After reaching a plateau, suggesting that the majority of the TCR's in the response had been sequenced, the same primers and exhaustive sequencing was used to identify the frequency of these sequences in naïve mice. These studies found that there were about 600 CD8⁺ T cells specific for the K^d-restricted epitope from HLA-A2 in naïve mice (Bousoo, Casrouge et al. 1998). A second strategy used an *in vivo* competition assay with H-2D^b-restricted LCMV glycoprotein (GP)33-specific P14 transgenic T cells to estimate the number of GP33-specific CD8 T cells in naïve mice and calculated the number to be between 100-200 cells per mouse (Blattman, Antia et al. 2002) and this same technique was subsequently used to determine the number of IA^b-restricted GP61 specific CD4 T cells in naïve mice and calculated ~100 cells per mouse (Whitmire, Benning et al. 2006). A modification of this technique has recently been used to determine the precursor frequency of self antigen-specific CD8 T cells. In these studies, mice that had received a sublethal dose of irradiation were adoptively constituted with heterogeneous naïve CD8⁺ T cells along with

varying concentrations of pmel-1 transgenic T cells that are specific for a tumor-encoded epitope derived from a melanosomal membrane glycoprotein. In this instance, the CD8⁺ T cell precursor frequency to a self-specific epitope, H-2D^b mgp100 25-33, was estimated to be about 1 in 1,000,000 (Rizzuto, Merghoub et al. 2009). Others estimated numbers of pMHC specific T cells by sequencing the CDR3 β regions of antigen-specific T cells that had expanded during an acute infection. By calculating a measure of CDR3 diversity, and then assuming a logarithmic distribution of diversity, they extrapolated the number of T cell clones that responded to an acute infection. This technique was used to examine responses in the central nervous system after I.N. inoculation of the neurotropic JHM strain mouse hepatitis virus (MHV). Three to five hundred H-2D^b-restricted MHV encoded S510 clonotypes were calculated to be in the central nervous system (CNS) of acutely infected mice, with ~100-900 clonotypes calculated to be in chronically infected mice (Pewe, Heard et al. 1999) and later studies by the same group used I.P. infection with MHV and an IFN γ capture assay instead of tetramer sorting, and estimated 1100-1500 H-2D^b-restricted S510-specific clonotypes and 600-900 clonotypes of the subdominant H-2K^b-restricted S598 peptide-specific T cells in the spleens of acutely infected mice (Pewe, Netland et al. 2004). In this later study it was also estimated that there were 1100-1200 different H-2D^b restricted GP33-specific clonotypes that could respond to an LCMV infection.

More recent assays have taken advantage of magnetic tetramer binding enrichment and double tetramer staining of cells from the spleen and lymph nodes of naïve mice to determine pMHC precursor frequencies, with the assumption that most CD8⁺ T cells in a naïve mouse reside in lymphoid organs and will react with tetramers. This technique was first described by Moon et al. for CD4⁺ T cells, and it detected ~190 I-A^b 2W1S I-A^b 52-68 specific T cells, ~20 I-A^b *Salmonella typhimurium* FLiC 427-441 specific T cells, and ~16 I-A^b chicken ovalbumin (OVA) 323-339 specific T cells per mouse (Moon, Chu et al. 2007). This same technique was then used to determine numbers of pMHC-specific CD8⁺ T cells to epitopes derived from multiple viruses (LCMV, influenza, and VSV) and the model antigen OVA and found ~80-1200 pMHC specific CD8⁺ T cells per mouse, depending on the specificity of the pMHC tetramer used for the pull down (Kotturi, Scott et al. 2008; Obar, Khanna et al. 2008). Further experiments using tetramer-based enrichment have recently been used to examine the phenotype of pMHC-specific CD8⁺ T cells in unimmunized mice, finding that there are both phenotypically naïve and memory like CD8⁺ T cells in unimmunized mice, suggesting a process whereby some pathogen-specific CD8⁺ T cells in unimmunized mice become memory-like (Haluszczak, Akue et al. 2009). In another recent publication, limiting dilutions of T cells were transferred into T cell-deficient T cell receptor $\beta/\delta^{-/-}$ mice followed by influenza A virus (IAV) infection to look for T cell responses in the lungs of mice of different ages. The results demonstrated a loss of certain pMHC specificities as mice aged (Yager,

Ahmed et al. 2008). Determinations of CD8⁺ T cell precursor frequencies in humans are currently not experimentally attainable, but exhaustive sequencing of an HLA-A2.1-restricted IAV M1 58-66 specific T cell response has suggested that there are at least 141 different clonotypes that can grow out in response to an *in vitro* stimulation with peptide, providing a possible floor for the number of T cells that can respond to a pMHC complex in human beings (Naumov, Naumova et al. 2003).

Most of the assays described above estimate the number of T cells specific to single peptides in individual mice. These assays, therefore, do not determine the numbers of CD8⁺ T cells that can proliferate in response to an entire virus, especially if the virus is known to have many epitopes, or if epitopes for the virus have not been described. It has been estimated using *in vitro* LDA that a CD8⁺ T cell can divide up to 15 times after *in vivo* activation by a foreign antigen (Welsh and Selin 2002). Further, assuming that all pMHC-specific CD8⁺ T cells detected in naïve mice proliferate in response to an infection, and that all progeny resulting from each division survive, it can be similarly calculated that naïve pMHC-specific CD8⁺ T cells divide approximately 13-14 times after virus infection, this number being calculated by examining the average number of pMHC-specific CD8⁺ T cells in a naïve mouse, and comparing this to the number of pMHC-specific CD8⁺ T cells that are in a mouse at the peak of the T cell response (Obar, Khanna et al. 2008). Considering that the progeny of one precursor after only 12 divisions can result in just over 4000 cells, and since

recent experiments using H-2K^b-restricted chicken OVA 257-264-specific OT-1 transgenic T cells have confirmed that the progeny from a single cell can be detected in a mouse after infection (Stemberger, Huster et al. 2007), an *in vivo* LDA was set up to take advantage of the extensive division and proliferation of virus-specific CD8⁺ T cells in order to determine virus-specific CD8⁺ T cell precursor frequencies.

CHAPTER II: MATERIALS AND METHODS

A. Mice and Mouse Procedures

B6.SJL (Ly5.1⁺Thy1.2⁺ host) mice were used between 6-20 weeks of age and were either from Taconic Farms (Germantown, NY) or bred in our own mouse-breeding colony. B6.Cg-IgH^aThy-1^aGPI-1^a/J (Ly5.2⁺Thy1.1⁺ donor) and IFN- $\alpha\beta$ receptor knockout mice were between 6-32 weeks of age and bred in our own mouse-breeding colony. Transgenic TCR-LCMV-P14 mice were used between 6-20 weeks of age and bred in our own mouse-breeding colony. B6.MRL-*Fas*^{lpr}/J were used between 5 and 7 weeks of age and were bred in our own mouse-breeding colony. B6Smn.C3-*Fas*^{gld}/J (B6-*gld*) mice were between 5 and 7 weeks of age and were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN $\alpha\beta$ R knockout mice (129 strain) were used between 4-12 weeks of age and were bred in our own mouse-breeding colony. SCID (B6 strain) mice were used between 7-12 weeks of age and were bred in our own mouse-breeding colony. B6-*rag1*^{-/-} and B6-*rag1*^{-/-}*lpr* mice were used between 5-7 weeks of age, were kindly provided by Dr. Merav Socolovsky, and were bred in our own mouse-breeding colony. All experiments were done in compliance with the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Worcester, MA).

B. Viruses and Viral Infections

LCMV strain Armstrong was propagated in BHK21 baby hamster kidney cells (Welsh, Lampert et al. 1976; Yang, Dundon et al. 1989). VAC-WR was

propagated on L929 cells (Yang, Dundon et al. 1989). All VAC recombinants and VAC-Wy were kindly provided by Pei Wu as part of a collaboration with Elliot Kieff (Harvard University). Mice were inoculated as indicated. Mice were infected I.P. with 5×10^4 PFU's of LCMV Armstrong or 1×10^6 or 4×10^6 PFU's of VAC-WR in 0.2ml for acute viral infections, and some of these mice were tested for memory T cell responses 2-8 months later. I.N. delivery of VAC-WR (1×10^4 or 2×10^5 PFU's) first required anesthetization by methoxyflurane (Anafane) purchased from IVESCO™ (Iowa Falls, Iowa). Mice were anesthetized in a fume hood by placement in a covered beaker that contained a small amount of tissue paper soaked in a solution of methoxyflurane. Once mice ceased most movement, 50 μ l of virus diluted in PBS (phosphate buffered saline) was applied directly onto the nostril with a 27½ gauge needle on a 1ml syringe. Skin scarification of VAC-Wy or VAC-WR was done by first immobilizing the mouse in a mouse holder, and then applying approximately 10 μ l (one drop) of virus properly diluted in PBS on to a mouse's tail. Then using, a 27½ gauge needle, the region of the tail which contained the drop of virus was lightly scratched 15-30 times until a small amount of blood appeared.

C. Neutralizing Antibody Assay

1ml of Vero cells were resuspended in complete MEM at 2×10^5 per ml and plated into the individual wells of 12 well plates and placed into a 37°C incubator overnight. On the following day, ½ ml of media was removed from each well and discarded and the plates were then placed back into the incubator. Dilutions of

virus in complete MEM (on ice) were prepared so that there were approximately 100-120 plaques per 100µl complete MEM. Typically, a large stock of diluted virus is prepared and frozen down ahead of time, as this limits assay-to-assay variability. Next, heat inactivated serum (56°C for ½ hour) was diluted in complete MEM in a 96 well plate in a cold tray to the appropriate dilutions for the assay (done in duplicate). The sera dilutions were eventually mixed 1:1 with the virus, so the sera was at 2x the desired starting dilution. Next, 100µl of the virus dilution was added to each of the diluted serum samples and mixed well (200µl total per well, with each well containing 50-60 PFU's of virus per 100µl). The 96 well plates were then incubated at 37°C for one hour. After 1 hour, 100µl of each sera/virus mixture were added to a well of the 12 well plate. After gently rocking the plate to distribute the virus/sera mixtures the, 12 well plate was placed into the 37°C incubator. After 1 ½ hours, 2mls of a 1:1 mixture of 1% Seakem agarose (Lonza, 50014, Rockland, ME) and complete 2x EMEM (Lonza 12-668E, Walkersville, MD) was gently added to each well of the 12 well plate. The mixture was prepared by first microwaving a 1% Seakem agarose mixture in ultra pure dH₂O (Invitrogen/Gibco, 10977, Grand Island NY) until it began to bubble, and this was then mixed 1:1 with cold complete 2x EMEM. The mixture was allowed to cool, and 2ml was gently added to each well of the 12 well plate. The plate was then placed in the 37°C virus incubator for two days. On day two, 1 ml of a neutral red staining solution was added to each well. A 1:1 mixture of 1% Seakem agarose and complete 2x EMEM was made, and to this 16-18ul of 1%

neutral red (Sigma-Aldrich, 861251-256, Natick, MA) solution was added per ml Seakem Agarose/2xEMEM mixture. This was then mixed well, and 1ml of this was added to each well. This was placed in the 37°C virus incubator for one day. Plaques were counted the next day, if there was neutralizing activity in the serum, there will be fewer plaques when compared to the wells containing just virus or uninfected sera. 50% endpoints were calculated if neutralizing activity diluted out completely, otherwise the serum was re-titered. Serum from an uninfected mouse was used as a negative serum control, serum from a VAC-immune mouse was used as a positive serum control, and a media control (straight MEM) was also used in all assays.

D. Plaque Assay

Plaque assay of VAC was done as previously described (Selin, Varga et al. 1998). Briefly, organ homogenate was diluted 1:10 in complete MEM, and 100µl of this homogenate was plated on 6 well plates containing Vero monolayers. This was overlaid 1 ½ hours later with a 1:1 Seakem agarose/complete 2xEMEM mixture. Two days later a neutral red stain was added in a 1:1 Seakem agarose/complete 2xEMEM mixture, and a day later plaques were counted. Values expressed are log₁₀ transformations, and titers are calculated as PFU/ml of whole liver, spleens, or both fat pads.

E. Antibody Depletion and Antibody Blocking Experiments

All treatments were given I.P. either one, or two days before, or on the day of the virus infection, and the antibody was diluted in PBS (see individual Figures for

time of antibody treatment). Mouse mAb antibodies were purchased from BioXCell (10 Technology Dr., Unit 2B, West Lebanon, NH, 03784-1671) and were as follows: Anti-CD4 clone GK1.5 (BE0003-1) made in rat, and the control isotype rat IgG2b clone LTF-2 (BE0090), anti-CD8 α clone 2.43 (BE00061) made in rat, and the control isotype rat IgG2b (as above), anti- $\gamma\delta$ clone UC7-13D5 (BE0070) made in hamster, anti-NK1.1 antibody clone PK136 made in mouse (BE0036), and control isotype mouse IgG2a clone Cl.18.4 (BE0085), and anti-IFN γ clone XMG1.2 made in rat, and control isotype rat IgG1 clone HRPN (BE0088).

F. CFSE and DDAO-SE Label of Mouse Splenocytes

CFSE (carboxyfluorescein succinimidyl ester) and DDAO-SE (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt succinimidyl ester) (Molecular Probes, C34553, Carlsbad CA) labeling of splenocytes was performed as previously described (Lyons and Parish 1994; Gudmundsdottir, Wells et al. 1999). Briefly, a single cell suspension was prepared from the spleen, red blood cells were lysed in an 0.84% NH $_4$ Cl solution, and splenocytes were washed in cold HBSS (Hank's balanced salt solution) (Invitrogen, Carlsbad CA) and resuspended in HBSS for counting. Spleen leukocytes were then resuspended in HBSS at 2×10^7 per ml and labeled in a solution of 2 μ M CFSE for 15 minutes in a 37°C water bath, with mixing every 5 minutes. After CFSE labeling, cells were again washed twice with cold HBSS, and counted immediately before transfer. An aliquot of splenocytes was used for a surface

stain, and the rest of the splenocytes were diluted in HBSS for adoptive transfer. DDAO-SE labeling of splenocytes was the same, except that the DDAO-SE final working concentration was 0.5 μ M.

G. FACS Staining

Single cell suspensions of the spleen, lymph nodes, bone marrow, blood, PEC's or lung (minced finely with razorblade and filtered) were prepared, red blood cells were lysed in an 0.84% NH_4Cl solution, and the leukocytes were then washed in RPMI 1640 media (Sigma-Aldrich, 11875-093, St. Louis MO). Cells were then counted by hemocytometer and resuspended in FACS buffer for staining. Fc receptors were blocked with antibody to CD16/CD32 (Fc γ III/II Receptor, BD Biosciences 553142, San Diego CA), and cells were then stained in 96 well plates. For the *in vivo* LDA, the single cell suspension from each whole spleen was divided into 8-16 wells of a 96 well plate for staining, and later recombined for analysis on an LSRII flow cytometer. After the surface stain with indicated antibodies, cells were either fixed using Cytotfix (BD, 554655) and resuspended in FACS buffer for analysis, or for intracellular assays, permeabilized using Cytotfix/Cytoperm (BD, 554722), and stained intracellularly with the indicated antibodies as per manufacturer's instructions.

H. Antibodies and Peptides

CD3 ϵ PE-Cy7 (BD 552774), CD8 α Pacific Blue (BD 558106), Thy1.1PE (BD 554898), Ly5.2PerCP-Cy5.5 (BD 552950), and V α 2 APC (eBioscience 17-5812-80) were used for *in vivo* LDA's. For surface stains and intracellular cytokine

assay, mAb to CD3 ϵ FITC (BD 555274), CD3 ϵ PE (BD 555275), CD3 ϵ Ly5.2PerCP-Cy5.5 (BD 551163), CD3 ϵ PE-Cy7 (BD 552774), $\gamma\delta$ TCR PE (BD 553178), $\gamma\delta$ TCR PE (BD 553177), CD8 α PE (BD 55303), CD8 α APC (BD 553035), CD8 α V500 (BD 556077), CD8 α PE-Cy7 (BD 552877), CD8 α Alexa Fluor 700 (BD 557959), CD8 β (eBioscience 12-0083083), CD44 PE-Cy7 (eBioscience 25-0441-82), Thy1.1PE (BD 554898), Ly5.2PerCP-Cy5.5 (BD 552950), CD127 APC (eBioscience 17-1271-82), CD62L Pacific Blue (eBioscience 57-0621-82), CD44 PerCP Cy.5 (eBioscience 45-0441-82), Thy1. AF 700 (BioLegend 105319, San Diego CA), Thy1.2 FITC (BD 553004), Thy1.2 PE (BD 553006), Thy1.2 APC (BD 553007), Ly5.1 PE (BD 553776), Ly5.1 PE-Cy7 (eBioscience 25-0453-82), Ly5.1 PB (BioLegend 110772), Ly5.1 PerCP-Cy5.5 (eBioscience 45-0453-82), Thy 1.1 APC (eBioscience 17-0900-82), Thy1.1 PE-Cy7 (eBioscience 25-0900-82), Thy1.1 PerCP-Cy5.5 (eBioscience 45-0900-80), Thy1.1 APC (eBioscience 48-0900-80), CD4 FITC (BD 553047), CD4 PE (BD 553653), CD4 APC (BD 553051), CD4 AF700 (BD 557956), CD4 PB (BD 558107), CD44 FITC (BD 553133), CD44 PE (BD 553134), CD44 APC (BD 559250), CD44 PE-Cy7 (eBioscience 25-0441-82), CD44 AF700 (BioLegend 103026), CD44 PerCP-Cy5.5 (eBioscience 45-0441-82), NK1.1 PB (BioLegend 108722), NK1.1 PE (BD 553165), NK1.1 APC (BD 550627), NK1.1 PerCP-Cy5.5 (BD 551114), NK1.1 APC (BD 550627), DX5 PE-Cy7 (eBioscience 25-5771-82), DX5 APC (eBioscience 17-5971-81) and NKp46 PE (12-3351-82) were used for surface stain, and mAb to IFN γ APC (BD 554413), IFN γ AF700 (BD 557998),

IFN γ eFluor450 (eBioscience 48-7311-80) and IFN γ PerCP-Cy5.5 (eBioscience 45-7311-80) was used for the intracellular stain. Peptides for stimulation were purchased from 21st Century Biochemicals (Marlboro, MA).

I. Peptide Specific Stimulations

Peptide stimulations were performed as previously described (Varga and Welsh 1998). Briefly, single cell suspensions of lymphocytes were cultured for 4.5-5 (depending on the assay) hours in the presence of 3 μ M of the indicated peptides (21st Century Biochemicals) or purified mAb to CD3 ϵ (1 μ g/ml) (BD 553058) for a polyclonal stimulation, with human recombinant IL-2 (10 U/ml) and GolgiPlugTM (BD 555029).

J. *In Vivo* Limiting Dilution Assay (LDA)

Splenocytes were labeled with CFSE as described above and diluted in HBSS to appropriate concentrations. To determine the number of surviving CD8⁺ T cells after transfer, the following formula was used: (number of donor splenocytes transferred into host mouse * (1 / %CD8⁺ T cells in donor population) * donor take (in our experiments, and from results from (Cose, Brammer et al. 2006; Ganusov and De Boer 2007) calculated to be 0.038). Pilot experiments gave an indication as to the limiting number of T cells that would need to be in host mice to respond to each viral infection. To determine precursor frequencies, 2-fold dilutions of splenocytes were made in HBSS. Each dilution was transferred into 4-6 (normally 5) host mice. In each experiment, one mouse at each dilution was left uninfected to serve as a negative control. An infected mouse was scored as

a responder if donor Thy1.1⁺Ly5.2⁺ CFSElo cells were detected after FACS analysis above a determined threshold as described below. If there were Thy1.1⁺Ly5.2⁺ CFSElo cells in any dilution of any of the uninfected animals, this number of cells was multiplied by three (with a correction for number of cells collected), and this would serve as a responder cutoff. In instances where there was no background detected by FACS in the uninfected mice of the individual experiment, a cutoff of 10 CFSElo cells was used. The background value of 10 CFSElo cells was used because it was three times the average number of CFSElo cells detected in all uninfected mice in all experiments, and it was also the average number of CFSElo cells in uninfected mice that had detectable CFSElo cells plus one standard deviation. After calculating the number of surviving CD8⁺ T cells after transfer, and determining responders versus non-responders, probit and Reed and Muench analyses were done to determine precursor frequencies for each experiment (Bliss 1934; Reed and Muench 1938). These two analyses resulted in comparable, but not identical precursor numbers. Almost all individual experiments also included two control host mice that received adoptive transfers of a large number of donor splenocytes, with one mouse infected and one uninfected, serving as positive and negative controls.

CHAPTER III: VACCINIA VIRUS WYETH IFN γ BINDING PROTEIN RECOMBINANTS, PATHOGENICITY AND PROTECTION

The role of VAC-Wy encoded IFN γ binding proteins in VAC-Wy pathogenesis was examined in the mouse. The protection afforded to mice by immunization with three VAC-Wy recombinants encoding three different IFN γ binding proteins, a VAC-Wy recombinant encoding no IFN γ binding protein, or the VAC-Wy parental strain to subsequent lethal I.N. infection with VAC-WR was also examined.

Previous experiments have found conflicting roles for the gene encoding the B8R protein in the pathogenesis of VAC infections in the mouse. In two studies it was demonstrated with two different strains of VAC (VAC-WR and VAC Lister) that B8R knockout viruses were less pathogenic than the revertant or parental strain in mice (Verardi, Jones et al. 2001; Denes, Gridley et al. 2006). In a separate study B8R knockout VAC's had no difference in pathogenicity in mice, while a virus where the IFN γ R α -chain truncated after Ser-257 so as to be secreted as an IFN γ binding protein was found to be more pathogenic in mice (Symons, Tschärke et al. 2002).

To more closely examine this phenomenon, four VAC-Wy recombinants were tested for pathogenicity and virus spread in various mouse models. The recombinants were made in VAC-Wy, as this live virus was the licensed smallpox vaccine in the United States. The four recombinants tested were a B8R knockout (B8R $^{-/-}$) virus, a B8R revertant virus (B8R-R), and two knock-in viruses.

One knock-in virus encoded a truncated version of the mouse IFN γ R that would serve as a decoy receptor (mIFN γ R) in place of the B8R gene. The second knock-in virus instead expressed a recombinant B8R/IFN γ R fusion protein that dimerized the mouse IFN γ receptor (DmIFN γ R). The B8R/IFN γ R fusion protein was expected to have increased avidity to mouse IFN γ as compared to the mouse IFN γ R protein as it is known that the IFN γ dimer induces dimerization of the IFN γ R, but the IFN γ R protein itself does not dimerize (Walter, Windsor et al. 1995).

The ability of each of these recombinants to protect mice against a lethal I.N. VAC-WR infection was also examined, as testing VAC-Wy variants with reduced pathogenicity but a similar immuno-protective phenotype was an important goal in examining the VAC-Wy variants.

A. Virus Pathogenesis of VAC-Wy Parental Strain and VAC-Wy Recombinant Strains In Multiple Mouse Backgrounds By Two Different Inoculation Routes

Table 3.1. Virus titers and weight loss observed after infection of multiple mouse backgrounds with parental VAC-Wy or VAC-Wy B8R recombinants.

	Mouse Type	# Mice	Age	Dose	Route	Day		Titers, PFU	Wyeth	B8R-/-	B8R-R	mIFN γ R	DmlFN γ R
Experiment 1	C57Bl/6J male	5 mice	7 weeks	5x10 ³	intra-nasal	Day 6	Experiment 1	Livers Lungs	<100 <10	<100 <10	<100 <10	<100 <10	<100 <10
Experiment 2	C57Bl/6J male	5 mice	6 weeks	5x10 ⁴	intra-nasal	Day 7	Experiment 2	Livers Lungs	<100 <10	<100 <10	<100 <10	<100 <10	<100 <10
Experiment 3	BALB/c 129SVE male	2 mice	8 weeks	5x10 ⁴	intra-nasal	Day 3	Experiment 3	Livers Lungs Spleens	n/a n/a n/a	n/a n/a n/a	<100 <10 <10	n/a n/a n/a	n/a n/a n/a
Experiment 4	129SVE male	2 mice	4 weeks	5x10 ⁴	intra-nasal	Day 3	Experiment 4	Livers Lungs Spleens	<100 <10 <10	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a
Experiment 5	C57Bl/6J male	2 mice	4 weeks	1x10 ⁵	intra-nasal	Day 4	Experiment 5	Livers Lungs Spleens	<100 <10 <10	n/a n/a n/a	<100 <10 <10	n/a n/a n/a	n/a n/a n/a
Experiment 6	C57Bl/6J female	2-3 mice	10 weeks	5x10 ⁴	intra-nasal	Day 4	Experiment 6	Livers Lungs Spleens Ovaries	<100 <10 <10 <10	n/a n/a n/a n/a	<100 <10 <10 <10	n/a n/a n/a n/a	n/a n/a n/a n/a
Experiment 7	C57Bl/6J male	3 mice	6 weeks	5x10 ⁵	intra-nasal	Day 3	Experiment 7	Livers Lungs Spleens	<100 760 <10	n/a 80 n/a	<100 100 <10	n/a 113 n/a	n/a 24 n/a
Experiment 8	C57Bl/6J male	4 mice	7 weeks	5x10 ⁵	intra-nasal	Day 6	Experiment 8	Livers Lungs	<100 <10	<100 <10	<100 <10	<100 <10	<100 <10
								Weight Loss	no loss	no loss	no loss	no loss	no loss
Experiment 9	C57Bl/6J female	4 mice	6 weeks	2.5x10 ⁶	intra-peritoneal	Day 4	Experiment 9	Livers Lungs Fat Pads Ovaries	<100 <10 <10 2/4	<100 <10 <10 1/4	<100 <10 <10 1/4	<100 <10 <10 1/4	<100 <10 <10 0/4
								Weight Loss	no loss	no loss	no loss	no loss	no loss
Experiment 10	C57Bl/6J male	3 mice	8 weeks	1x10 ⁷	intra-nasal	Day 4	Experiment 10	Livers Lungs Spleens	<100 5.8x10 ⁴ <10	<100 1.4x10 ⁴ <10	<100 7.2x10 ⁴ <10	<100 4.6x10 ⁴ <10	<100 2.3x10 ⁴ <10
								Weight Loss	5%	no loss	no loss	no loss	no loss
Experiment 11	C57Bl/6J male	5-6 mice	8 weeks	1x10 ⁷	intra-nasal	Day 5	Experiment 11	Livers Lungs Spleens	<100 5.4x10 ⁴ <10	<100 3.3x10 ⁴ <10	<100 1.5x10 ⁴ <10	n/a n/a n/a	n/a n/a n/a
								Weight Loss	5%	no loss	no loss	n/a	n/a
Experiment 12	SCID male	2-3 mice	8 weeks	1x10 ⁷	intra-nasal	Day 20	Experiment 12	Livers Lungs Spleens	n/a n/a n/a	<100 <10 <10	n/a n/a n/a	n/a n/a n/a	<100 <10 <10
								Weight Loss	n/a	no loss	n/a	n/a	1/2, 20%
Experiment 13	SCID male	1-2 mice	12 weeks	1x10 ⁷	intra-nasal	Day 34	Experiment 13	Livers Lungs Fat Pads Brains Spleens Kidneys	1.5x10 ³ <10 <10 <10 <10 <10	<100 <10 <10 <10 <10 <10	<100 <10 <10 <10 <10 <10	n/a n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a n/a
								Weight Loss	40%	20%,40%	30%	no loss	no loss
Experiment 14	SCID male	3 mice	7 weeks	4.5x10 ⁷	intra-peritoneal	Day 16	Experiment 14	Livers Lungs Spleens	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a
								Weight Loss	dead	no loss	n/a	n/a	n/a
Experiment 15	Type 1 IFN ko female	2 mice	12 weeks	1.5x10 ⁷	intra-nasal	Day 10	Experiment 15	Livers Lungs Kidneys Brains Spleens	<100 6.5x10 ⁵ <10 <10 <10	<100 <10 <10 <10 <10	<100 <10 <10 <10 <10	n/a n/a n/a n/a n/a	<100 <10 <10 <10 <10
								Weight Loss	1 dead, 30%	15%	15%	no loss	15%

As seen in Table 3.1, VAC-Wy recombinant infection done in multiple backgrounds of mice by many different routes reveals that the VAC-Wy grows poorly in the mouse and is only reliably detectable in the lung after high dose I.N. infection.

Table 3.1 led us to the conclusion that the best model to examine virus spread and pathogenicity was a high dose ($1-2 \times 10^7$) I.N. infection, and so the rest of our experiments examining VAC-Wy recombinant pathogenicity and virus spread were done using this high dose I.N. infection of mice.

B. Pathogenicity of VAC-Wy IFN γ binding protein recombinants in mice

To more closely examine the virus spread and pathogenicity of the four recombinant VAC-Wy viruses in comparison to the parental VAC-Wy, five sets of B6 mice were infected with 1×10^7 PFU's I.N.

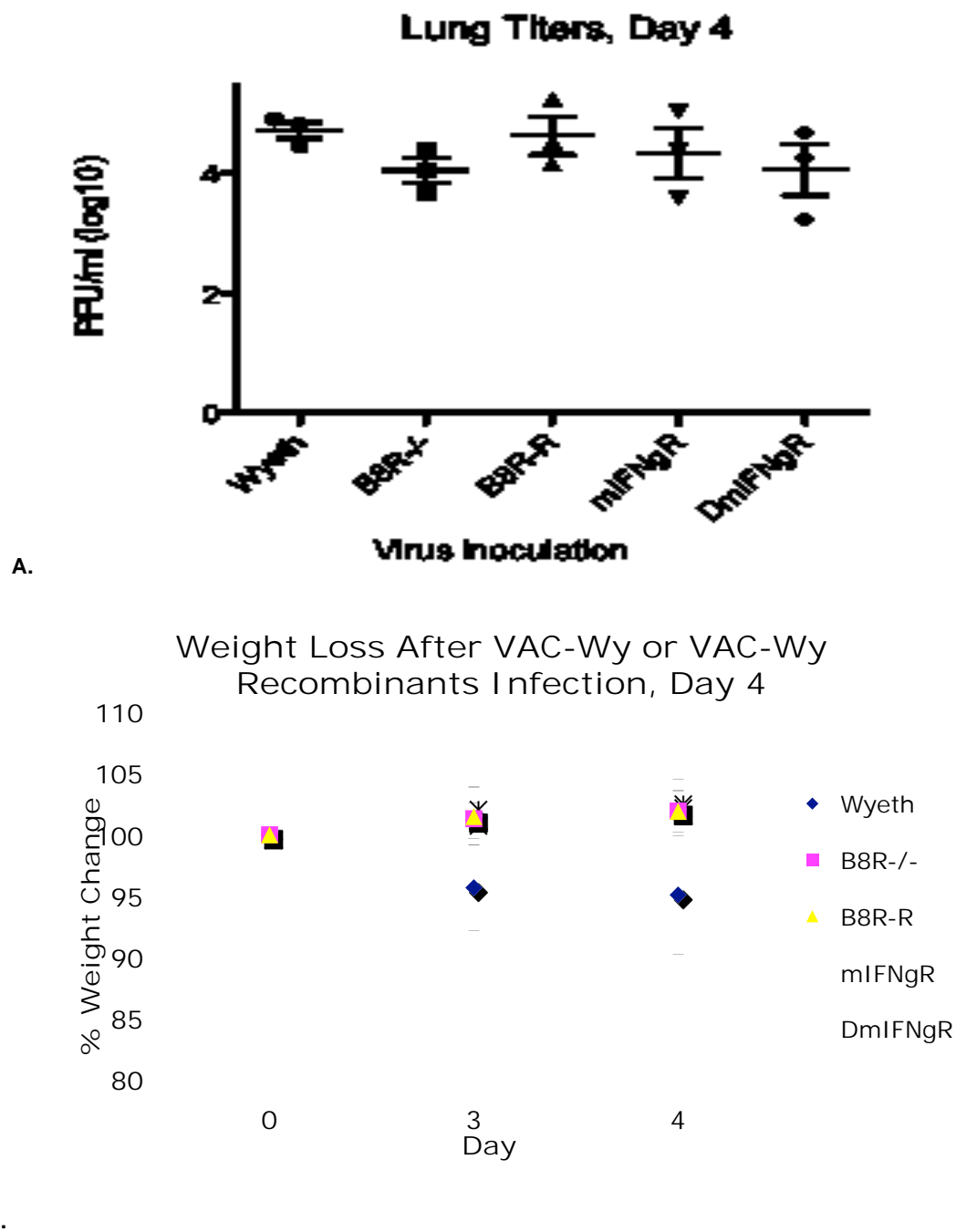


Figure 3.1. At 1×10^7 PFU's I.N., VAC-Wy and VAC-Wy B8R variant viruses grow to similar titers in the mouse lung, with only the parental VAC-Wy causing weight loss.

Figure 3.1 At 1×10^7 PFU's I.N., VAC-Wy and VAC-Wy B8R variant viruses grow to similar titers in the mouse lung, with only the parental VAC-Wy causing weight loss.

B6 mice were infected with 1×10^7 of each of the indicated viruses I.N. (A) On Day 4, mice were sacrificed and lung virus titers were determined by plaque assay. (B) Mice were weighed each day, and the average and standard deviations of each group are plotted as percent of initial weight lost. Wyeth is VAC-Wy, B8R-/- is VAC-Wy with the gene encoding the B8R protein knocked out, B8R-R is a revertant of the B8R-/- virus, mIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a truncated variant of the gene encoding the mouse IFN γ R, and DmIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a recombinant B8R/IFN γ R dimer that allows dimerization of the protein before IFN γ protein binding.

No profound differences in viral titer were found after infection with any of the recombinants or the parental VAC-Wy strain (Figure 3.1A). Mice were also weighed as a measure of pathogenicity, expecting that mice would lose weight if sick. Only mice that were infected with the VAC-Wy parental strain lost weight, with no weight loss differences noted when mice were infected with any of the recombinant viruses (Figure 3.1B). To more closely examine the growth of the B8R-/- VAC-Wy recombinant, experiments were repeated with the same virus load, but with an increased number of mice. In this experiment, day five titers were examined instead.

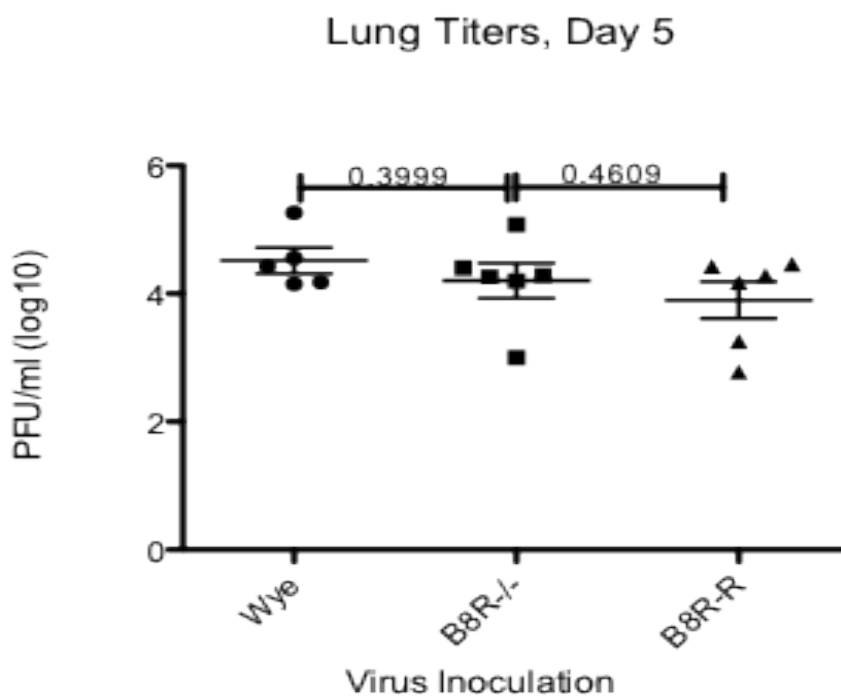


Figure 3.2. B8R^{-/-} VAC-Wy grows to similar titers as B8R revertant VAC-Wy and VAC-Wy parental strain.

Figure 3.2. B8R^{-/-} VAC-Wy grows to similar titers as B8R revertant VAC-Wy and VAC-Wy parental strain.

B6 mice were infected with 1×10^7 of each of the indicated viruses I.N. On Day 5, mice were sacrificed and lung virus titers were determined by plaque assay. P values were determined by Student's t-test. Wye is VAC-Wy, B8R^{-/-} is VAC-Wy with the gene encoding the B8R protein knocked out, B8R-R is a revertant of the B8R^{-/-} virus.

There was no significant difference in day 5 lung virus titers after infection with the B8R-/- VAC-Wy, B8R-R VAC-Wy, or the parental VAC-Wy in experiments that included a larger number of mice (Figure 3.2). Combined with other results with the other recombinants (described later in the manuscript, and in Table 3.1), it was likely that the mouse may not be the best model to measure VAC-Wy pathogenicity as VAC-Wy grows poorly in the mouse, but might still be a good model to examine protection induced by the recombinants. This conclusion was reached as it was found that even at doses where virus was only barely detectable in the lung after recombinant VAC-Wy infection at Day 3, with no detectable virus at Day 6 (see Table 3.1, Experiments 7 and 8), there was a detectable VAC-specific CD8⁺ T cell response.

C. VAC-specific CD8⁺ T cell response induced by infection of mice with parental VAC-Wy or VAC-Wy IFN γ binding protein recombinants

Interestingly, the B8R protein contains the peptide that encodes the immunodominant epitope in B6 mice, and so using an intracellular cytokine assay after peptide stimulation, confirmation of B8R knockout viruses is demonstrated.

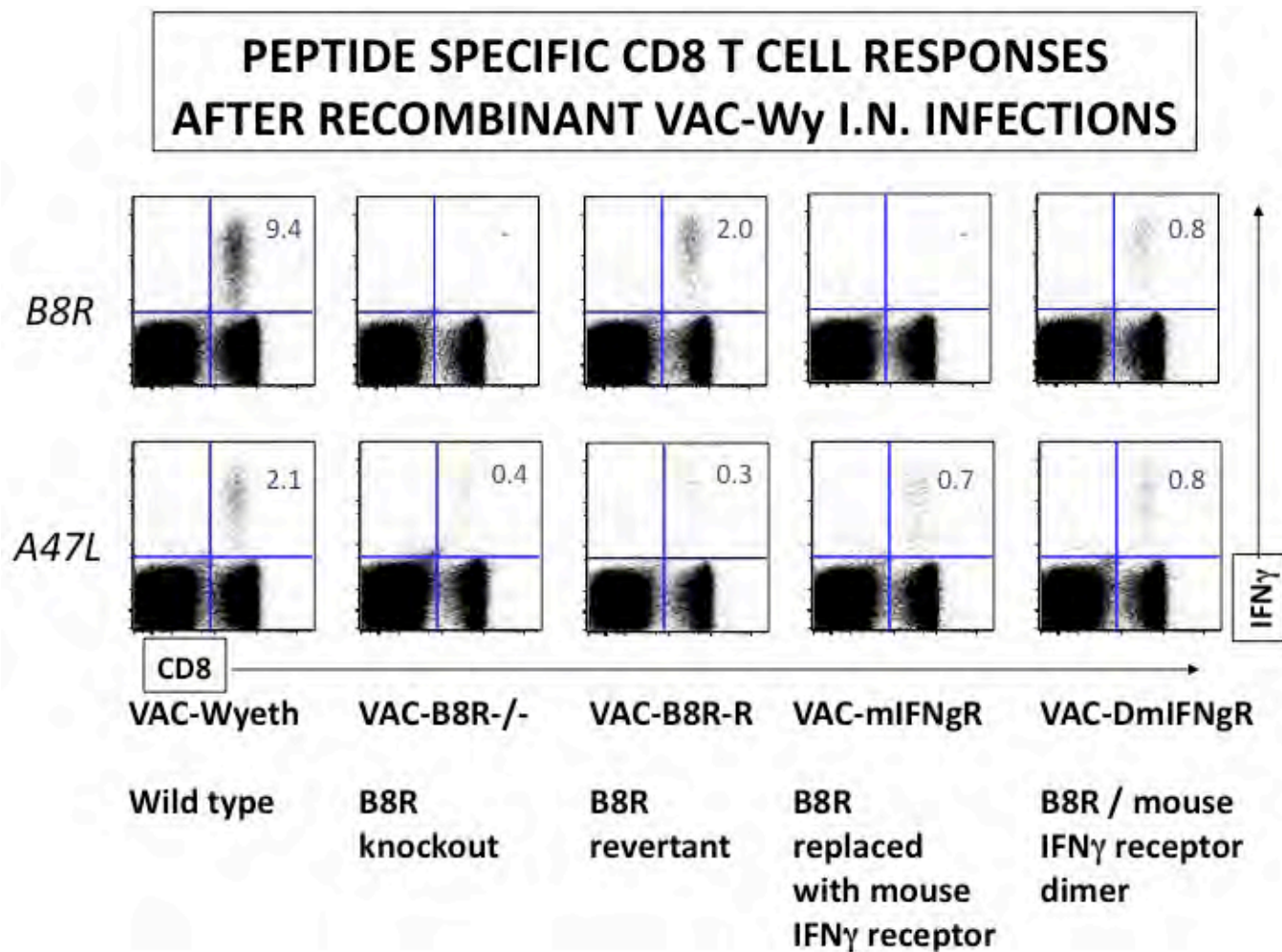


Figure 3.3. VAC-specific CD8⁺ T cell response after I.N. infection.

Figure 3.3. VAC-specific CD8⁺ T cell response after I.N. infection.

B6 mice were infected with 5×10^5 of each of the indicated viruses I.N. On Day 6, mice were sacrificed and splenocytes were cultured for five hours with the B8R peptide or A47L peptide from VAC in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. All plots are gated on CD8⁺ cells.

All viruses were able to induce a detectable VAC-specific CD8⁺ T cell response, as stimulation with A47L resulted in IFN γ ⁺ CD8⁺ T cells (Figure 3.3). When splenocytes were stimulated with the B8R peptide instead, IFN γ ⁺ CD8⁺ T cells were only detected in mice that had been infected with viruses that encode the B8R gene. It was also observed that infection with the parental strain of the virus resulted in greater CD8⁺ T cell responses when compared with all recombinant viruses at this dose. This may be because at this dose, the Wyeth parental strain grew to higher titers *in vivo*, although at higher doses the titers of the parental strain and recombinants were the same (see Table 3.1 and Figure 3.1A and 3.2). As a mimic of smallpox vaccination, mice were infected with VAC-Wy parental strain and the revertant B8R recombinant VAC-Wy strain by skin-scarification.

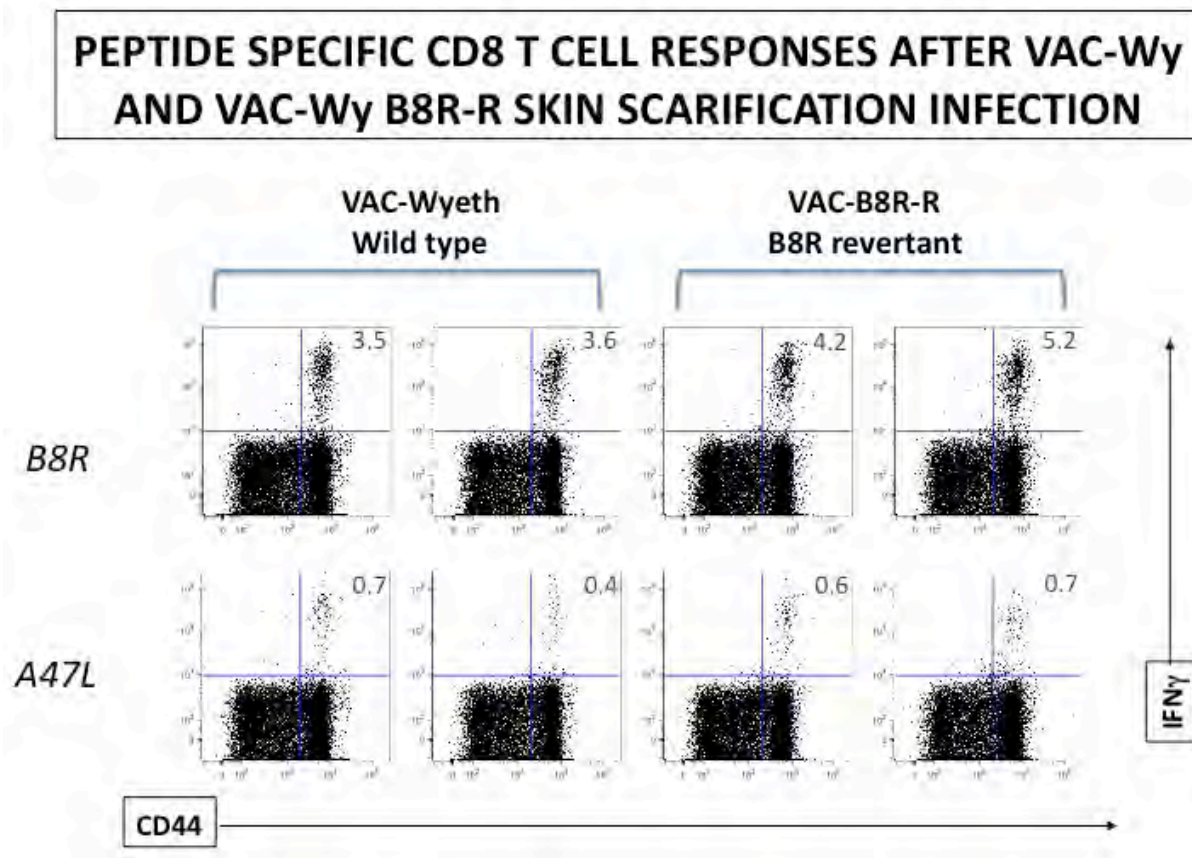


Figure 3.4. VAC-specific CD8⁺ T cell response after skin-scarification.

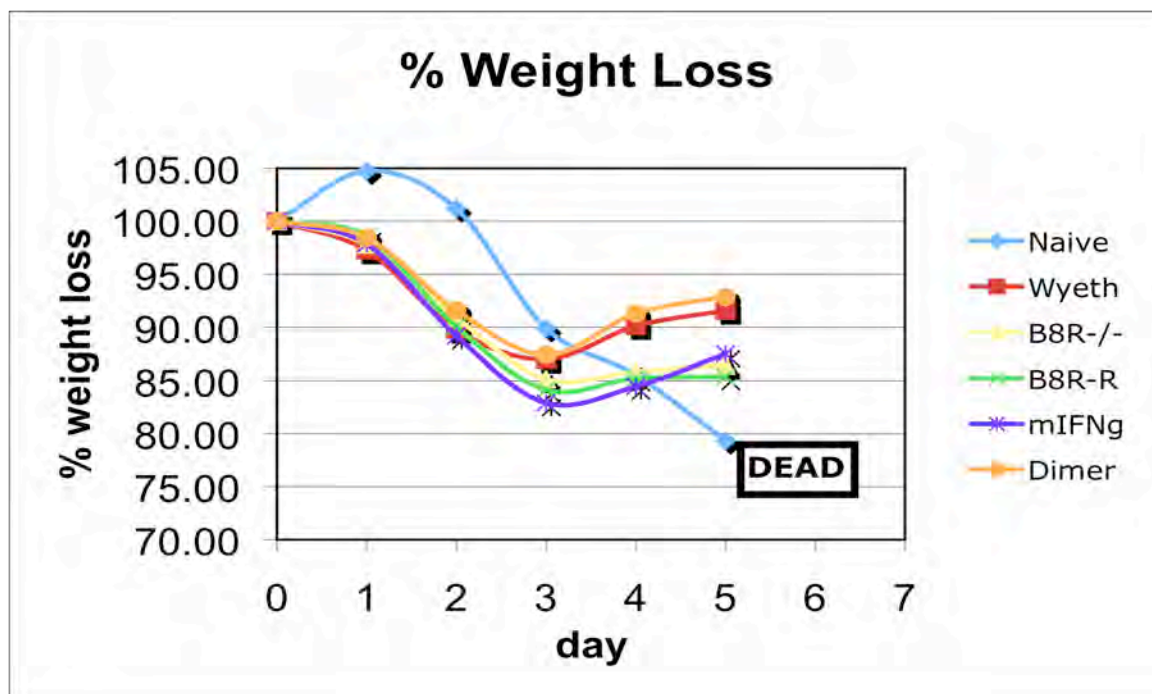
Figure 3.4. VAC-specific CD8⁺ T cell response after skin-scarification.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 17, mice were sacrificed and splenocytes were cultured for five hours with the B8R peptide or A47L peptide from VAC in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. All plots are gated on CD8⁺ cells. Stimulations were with the B8R peptide and A47L peptide from VAC.

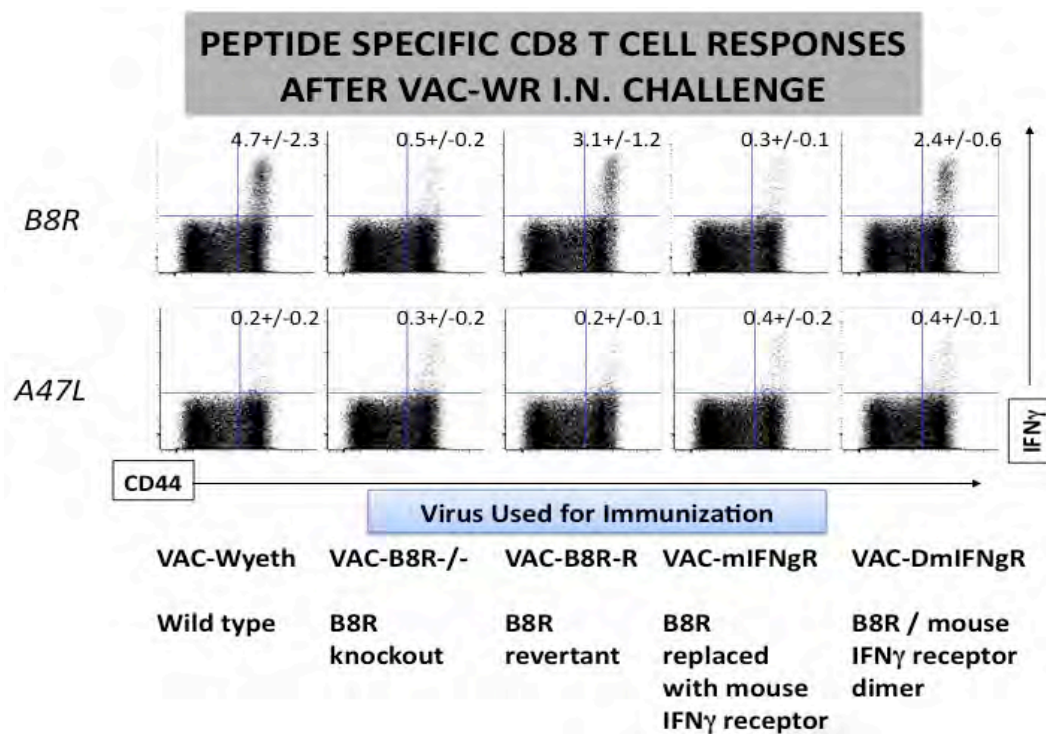
Skin-scarification with the parental VAC-Wy or the recombinant VAC-Wy revertant was able to induce similar numbers of VAC-specific IFN γ ⁺ CD8⁺ T cells as detected by an intracellular cytokine assay with either the B8R or A47L peptide (Figure 3.4).

D. VAC-Wy IFN γ Binding Protein Recombinants Protect Mice as Well as VAC-Wy Against A Lethal Dose of VAC-WR, and the VAC-Specific CD8⁺ T Cell Responses After Challenge

The ability of each of the recombinants and the parental strain of VAC-Wy to provide protection against a lethal dose (20 LD₅₀'s, 2x10⁵ PFU's) VAC-WR was next examined, and the VV-specific CD8⁺ T cell response in the surviving mice was next examined.



A.



B.

Figure 3.5. Weight loss and VV-specific CD8⁺ T cell response after 20 LD₅₀'s VAC-WR challenge of VAC-Wy or B8R recombinant VAC-Wy immune mice.

Figure 3.5. Weight loss and VV-specific CD8⁺ T cell response after 20 LD50's VAC-WR challenge of VAC-Wy or B8R recombinant VAC-Wy immune mice.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 71, mice were infected I.N. with 20 LD50's of VAC-WR (2×10^5). (A) Mice were weighed each day, and the average of each group is plotted as percent of initial weight lost (5-6 mice per group) Immunizing virus designations are as described in (B). (B) 6 days later the surviving mice were sacrificed and splenocytes were cultured for five hours with the B8R peptide or A47L peptide from VAC in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. All plots are gated on CD8⁺ cells. Numbers displayed are the average +/- standard deviations IFN γ ⁺CD44⁺.

Immunization with any of the recombinants or the parental strain by skin-scarification was able to provide protection against a lethal I.N. dose of VAC-WR given on day 71, as all non-immunized mice died by day 6, while all mice in the immunized groups, after an initial drop in weight, began to gain weight by day 3, and no immunized mouse died from the high dose infection (Figure 3.5A). The VAC-specific CD8⁺ T cell response in the remaining mice was also examined. Mice immunized with any VAC-Wy recombinant that encoded the B8R protein had detectable B8R-specific CD8⁺ T cells, while mice immunized with VAC-Wy recombinants that did not encode the B8R protein had very few B8R-specific CD8⁺ T cells after I.N. infection with 20 LD50's of VAC-WR (Figure 3.5B). The challenge virus, VAC-WR, normally induced expansion of B8R-specific CD8⁺ T cells, so this result suggests that the CD8⁺ T cell response in an immunized host will largely be of memory origin when the challenge virus is largely homologous. All immunized surviving animals had similar levels of A47L-specific CD8⁺ T cells (Figure 3.5B).

The immunization was then repeated, and VAC-WR challenged mice were instead sacrificed on day 5 after lethal viral challenge, to allow measurement of virus load after challenge in immunized and un-immunized hosts, and to examine day 5 VAC-specific memory CD8⁺ T cell recall responses.

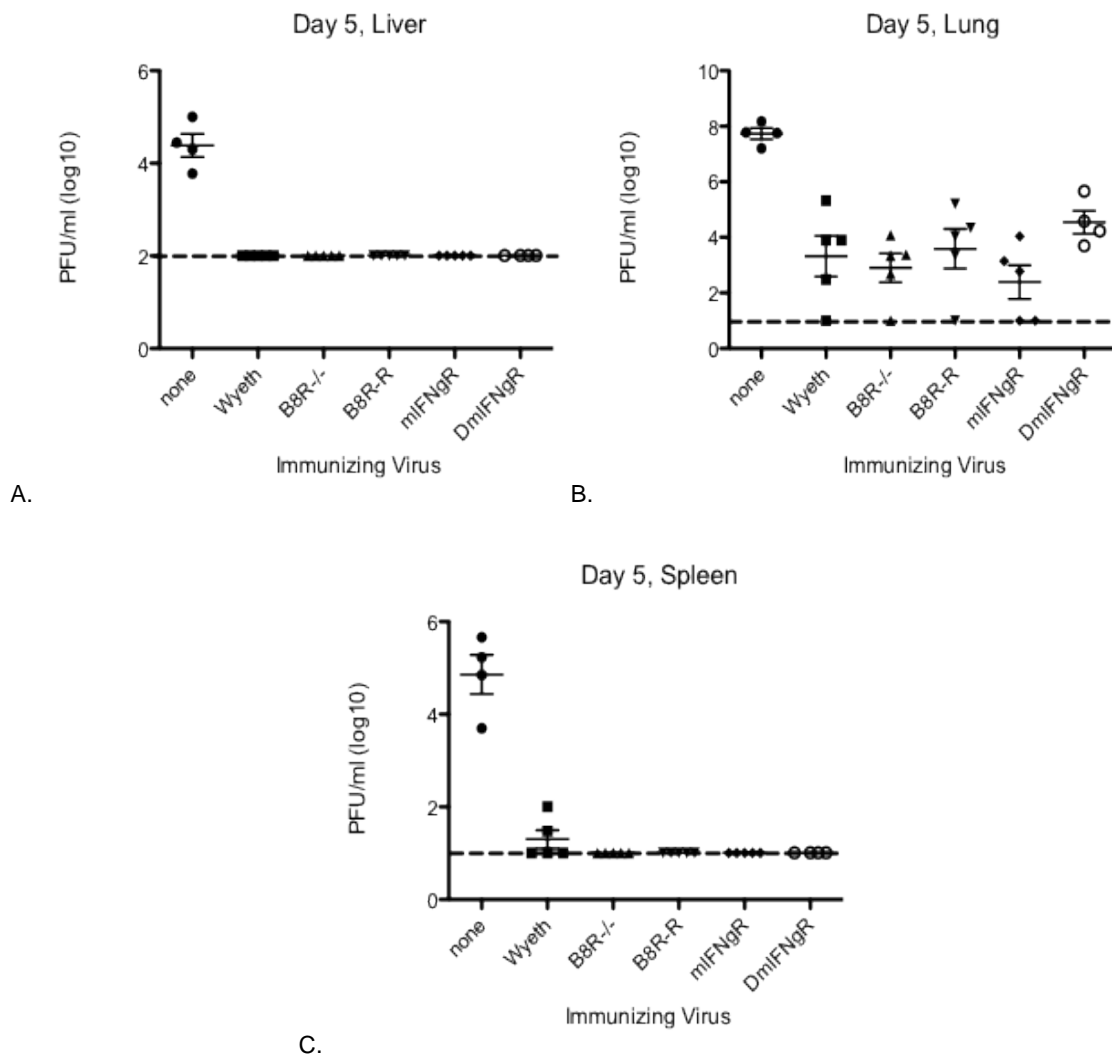


Figure 3.6. Skin-scarification immunization with either VAC-Wy or the B8R VAC-Wy recombinants resulted in decreased virus loads after VAC-WR challenge.

Figure 3.6. Skin-scarification immunization with either VAC-Wy or the B8R VAC-Wy recombinants resulted in decreased virus loads after VAC-WR challenge.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 162, mice were infected I.N. with 20 LD₅₀'s of VAC-WR (2×10^5). Mice were sacrificed on day 5 after the challenge, and viral titers in the (A) livers (B) lungs and (C) spleens were determined by plaque assay (the dotted line represents the limit of detection of the assay). Wyeth is VAC-Wy, B8R^{-/-} is VAC-Wy with the gene encoding the B8R protein knocked out, B8R-R is a revertant of the B8R^{-/-} virus, mIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a truncated variant of the gene encoding the mouse IFN γ R, and DmIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a recombinant B8R/IFN γ R dimer that allows dimerization of the protein before IFN γ protein binding.

Mice that had been immunized by skin-scarification with any of the recombinant VAC-Wy's or the parental VAC-Wy had approximately 4 logs lower virus in the lungs after 20 LD50's VAC-WR challenge in comparison to unimmunized mice (Figure 3.6B). No virus was found in the livers of immunized mice, while unimmunized mice had almost five logs of virus in the livers, and only two VAC-Wy-immunized mice had detectable virus in the spleens while the non-immunized mice had almost five logs of virus in the spleen (Figure 3.6A and 3.6C). The VAC-specific CD8⁺ T cell response in the spleen after 20 LD50's VAC-WR was next examined by intracellular cytokine stain.

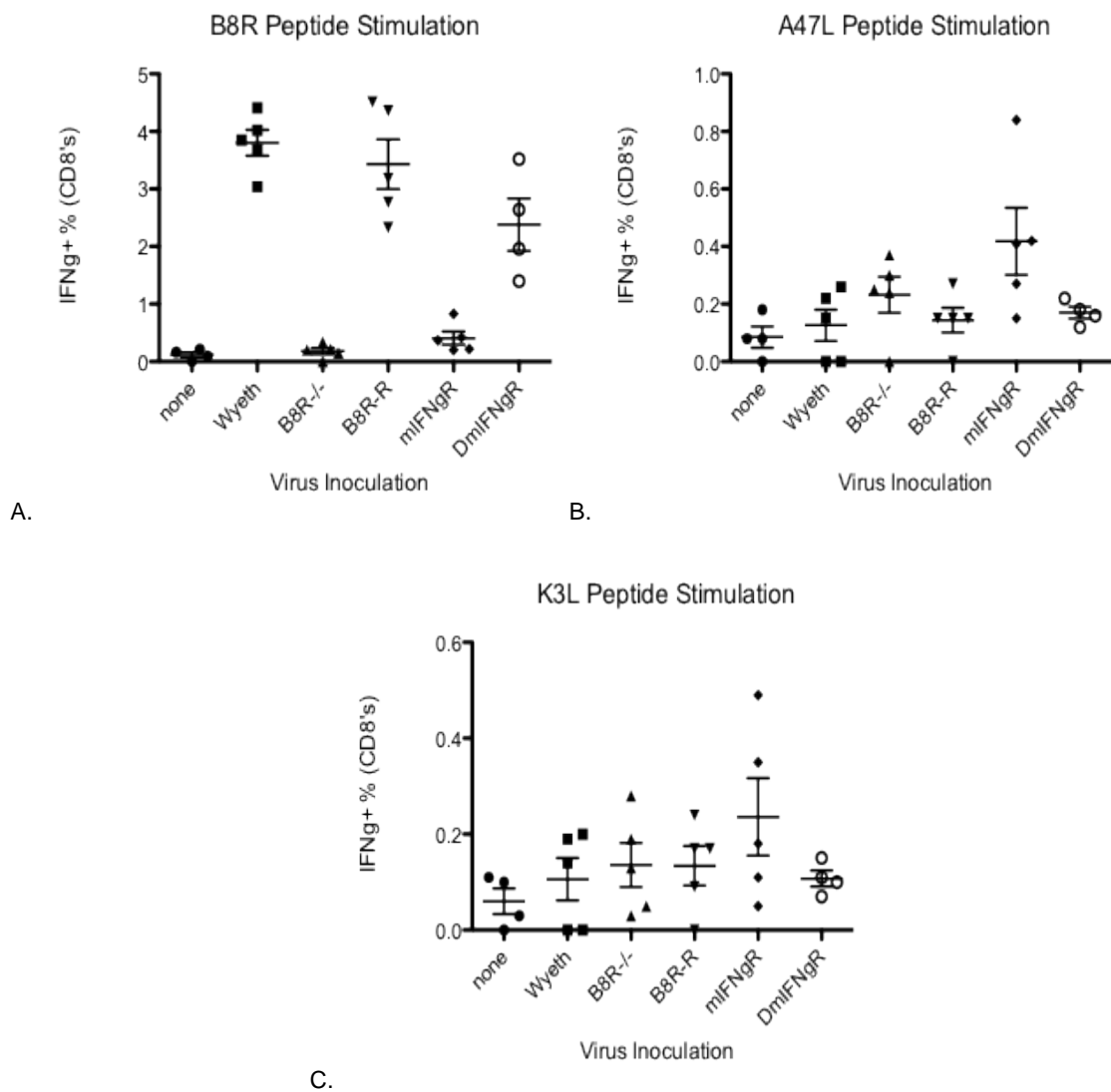


Figure 3.7. VAC-specific CD8 $^+$ T cell response in skin-scarified mice on day 5 after VAC-WR challenge.

Figure 3.7. VAC-specific CD8⁺ T cell response in skin-scarified mice on day 5 after VAC-WR challenge.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 162, mice were infected I.N. with 20 LD50's of VAC-WR (2×10^5), and 5 days later mice were sacrificed and splenocytes were cultured for five hours with the (A) B8R peptide, (B) A47L peptide, or (C) K3L peptide from VAC in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. Wyeth is VAC-Wy, B8R $^{-/-}$ is VAC-Wy with the gene encoding the B8R protein knocked out, B8R-R is a revertant of the B8R $^{-/-}$ virus, mIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a truncated variant of the gene encoding the mouse IFN γ R, and DmIFN γ R is VAC-Wy with the gene encoding the B8R protein replaced with a recombinant B8R/IFN γ R dimer that allows dimerization of the protein before IFN γ protein binding.

After I.N. challenge with 20 LD50's of VAC-WR, only mice immunized with a virus that encoded B8R had CD8⁺ T cells that produced IFN γ upon stimulation with the B8R peptide, even though the challenge virus was not a B8R knockout virus (Figure 3.7A). This again suggests that in immunized mice, the CD8⁺ T cell response was entirely of memory origin, as infection of mice that had been immunized with a virus that did not contain the B8R gene, had no detectable B8R-specific CD8⁺ T cell response. This was even in light of the decreased virus loads in the mice that had been immunized with the B8R knockout viruses (Figure 3.7). VAC-specific CD8⁺ T cell responses could be detected when the peptide stimulation was with two peptides that are part of subdominant CD8 T cell epitopes, A47L (Figure 3.7B) and K3L (Figure 3.7C).

E. Chapter Conclusion

Although no difference in pathogenicity or virus growth was found using these IFN γ binding protein VAC-Wy recombinants, all recombinants protected mice as well as the Wyeth parental strain. The apparent lack of pathogenicity differences with the different IFN γ binding protein VAC-Wy recombinants may have been due to the poor growth of VAC-Wy in mice, and so it might be interesting to instead examine these knockout and knock-in variants in a mouse adapted strain of VAC such as VAC-WR. Interestingly, all recombinants provided similar protection to a lethal I.N. challenge with VAC-WR, even though B8R knockout immunization could not stimulate the normally immunodominant CD8⁺ T cell response.

CHAPTER IV: VACCINIA VIRUS WYETH VACCINE CANDIDATES DELETED FROM B8R, C12L, AND C3L(C21L) GENES

Recombinant VAC-Wy viruses were tested for differences in virus growth in our I.N. mouse model of infection. These recombinant VAC-Wy were knockouts of one, two, or three genes that code for immunomodulatory proteins. The protection afforded to mice by immunization with VAC-Wy or a VAC-Wy recombinant where all three genes were knocked out (VAC-Wy-/-/-), to a typically lethal I.N. infection with VAC-WR was also examined.

Both C12L knockout VAC's (Born, Morrison et al. 2000; Symons, Adams et al. 2002) and C21L (C3L) knockouts have been shown to be less pathogenic in *in vivo* animal models (Isaacs, Kotwal et al. 1992). These knockout viruses as well as double and triple knockout viruses were tested for pathogenicity differences in our mouse I.N. model. The immunological memory induced by skin-scarification of the VAC-Wy-/-/- in comparison to the VAC-Wy parental strain, as well as the ability of VAC-Wy-/-/- inoculation by skin-scarification to protect against a lethal VAC-WR challenge, in comparison to immunization with the VAC-Wy strain or PBS immunization was also examined.

Pathogenicity differences between the single knockout, double knockout, or triple knockout VAC-Wy recombinants and the parental VAC-Wy were first examined in a high dose I.N. mouse model.

A. Virus Growth of Multiple VAC-Wy Recombinants After I.N. Infection of Mice

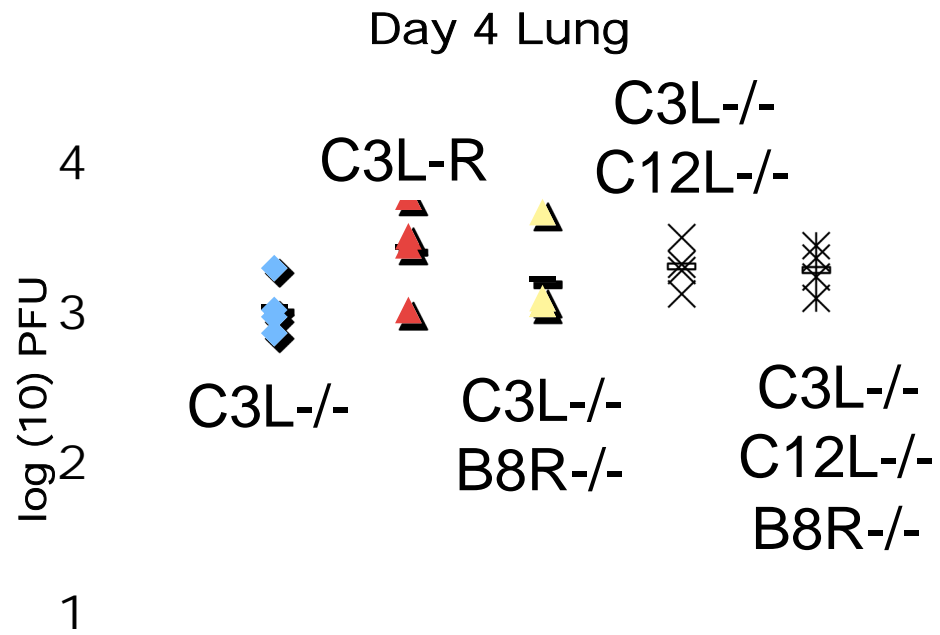


Figure 4.1. Knockout VAC-Wy variants and revertant VAC-Wy grow to similar titers in mouse lung.

Figure 4.1. Knockout VAC-Wy variants and revertant VAC-Wy grow to similar titers in mouse lung.

B6 mice were infected with 1×10^7 of each of the indicated viruses I.N. On Day 4, mice were sacrificed and lung virus titers were determined by plaque assay. C3L^{-/-} is VAC-Wy with the gene encoding the C3L protein knocked out, C3L-R is a revertant of the C3L^{-/-} virus, C3L^{-/-}B8R^{-/-} is a double knockout VAC-Wy with the genes encoding the C3L and B8R proteins knocked out, C3L^{-/-}C12L^{-/-} is another double knockout VAC-Wy with the genes encoding the C3L and C12L proteins knocked out, and C3L^{-/-}C12L^{-/-}B8R^{-/-} is a triple knockout VAC-Wy with the genes encoding the C3L, C12L, and B8R proteins knocked out.

No significant difference in day 4 lung virus titers was found when comparing any of the recombinants, probably owing to the poor growth of Wyeth in the mouse (Figure 4.1).

B. VAC-specific Immune Response Induced by VAC-Wy-/- and VAC-Wy Parental Strain Immunization, and the Protection the Immunizations Provide Against Lethal VAC-WR Challenge.

Mice were next inoculated with PBS, the VAC-Wy parental strain, or the VAC-Wy-/- by skin-scarification, and the VAC-specific immunological memory induced by each was examined.

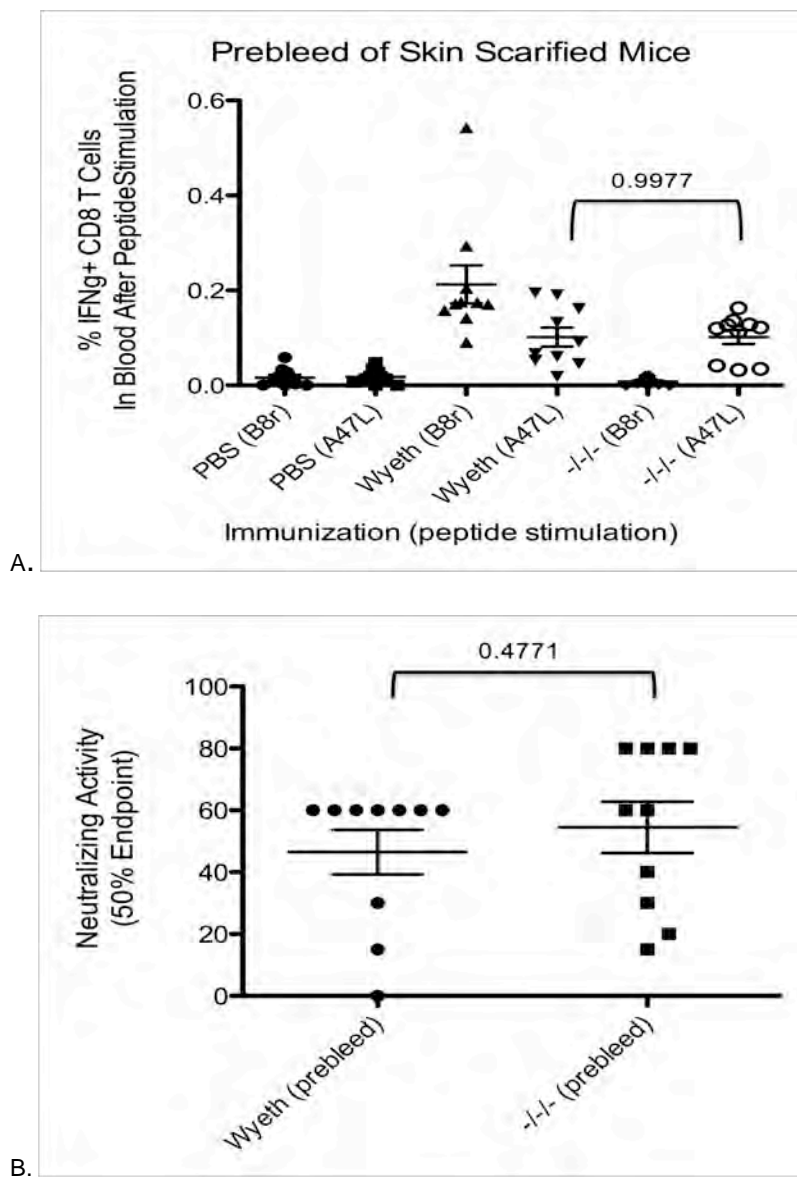


Figure 4.2. Similar numbers of VAC-specific CD8⁺ T cells and amount of neutralizing antibody in VAC-Wy and VAC-Wy-/- immune mice.

Figure 4.2. Similar numbers of VAC-specific CD8⁺ T cells and amount of neutralizing antibody in VAC-Wy and VAC-Wy-/- immune mice.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. (A) On day 207, mice were bled, and blood lymphocytes were cultured for five hours with the B8R peptide, A47L peptide, or K3L peptide from VAC in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. All plots are gated on CD8⁺ cells. (B) Heat inactivated sera from the mice were used to measure neutralizing antibody activity against VAC-WR. P values were determined by Student's t-test.

Only mice immunized with the parental VAC-Wy strain had CD8⁺ T cells that produced IFN γ upon stimulation with the B8R peptide, as was expected as the VAC-Wy-/- does not express B8R (Figure 4.2A). Interestingly, there were similar numbers of CD8⁺ T cells that produced IFN γ upon stimulation with the A47L stimulation (Figure 4.2A), with similar titers of neutralizing antibody in both the VAC-Wy immunized and VAC-Wy-/- immunized mice (Figure 4.2B). These mice were next I.N. infected with 20 LD50's of VAC-WR (2×10^5 PFU).

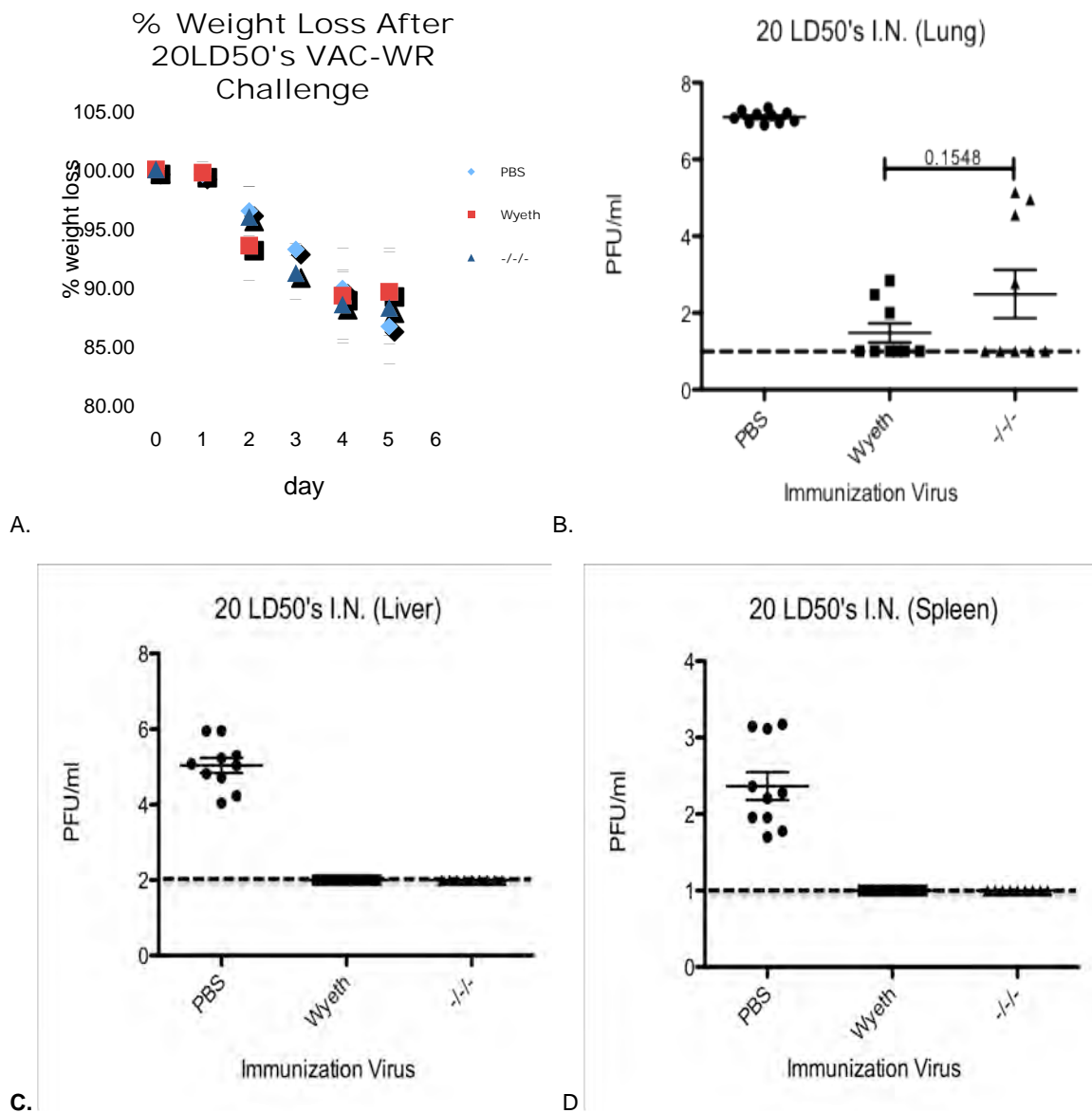


Figure 4.3. Weight loss and virus titers after 20 LD50's I.N. VAC-WR challenge in VAC-Wy, VAC-Wy-/-, or PBS skin-scarified mice.

Figure 4.3. Weight loss and virus titers after 20 LD50's I.N. VAC-WR challenge in VAC-Wy, VAC-Wy-/-, or PBS skin-scarified mice.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 210, mice were infected I.N. with 20 LD50's of VAC-WR (2×10^5). (A) Mice were weighed each day, and the average and standard deviations of each group are plotted as percent of initial weight lost (9-10 mice per group). Mice were sacrificed on day 5 after the challenge, and viral titers in the (B) livers, (C) lungs, and (D) spleens were determined by plaque assay (the dotted line represents the limit of detection of the assay). P values were determined by Student's t-test. PBS is phosphate buffered saline, Wyeth is parental VAC-Wy, and -/- is a VAC-Wy with the genes encoding the C3L, C12L, and B8R proteins knocked out.

In this case, the immunization with VAC-Wy or the VAC-Wy-/- might have stopped weight loss by day 5, but this result was not significant (Figure 4.3A) and was not as striking as had been seen in Figure 3.5A. This result may be because of the increased time between immunization and challenge (71 days after immunization in Figure 3.5, 210 days after immunization in Figure 4.3A).

Next, on day 5 after the 20 LD50's VAC-WR challenge, as it has been previously demonstrated that un-immunized mice succumb to this virus dose at day 6 (Figure 3.5A), mice were sacrificed to determine virus titers. Mice that had been immunized with either VAC-Wy-/- or the parental VAC-Wy had approximately five logs less virus in the lungs (Figure 4.3B), with no virus detectable virus in the livers or the spleens in comparison to approximately 2.5 logs of virus in the spleen and 5 logs of virus in the livers of the PBS-immunized group (Figure 4.3C and 4.3D). There was an increased titer on average in the mice that had detectable virus in mice immunized with Wy-/- (Figure 4.3B). This result might be due to the lack of the immunodominant CD8⁺ T cell epitope in the immunization, and although the result did not differ significantly from the VAC-Wy immunization, further experiments might reveal a role of memory CD8⁺ T cells in protection against VAC-WR challenge. The VAC-specific CD8⁺ T cell response after challenge with 20 LD50's of VAC-WR was next measured by an intracellular cytokine stain after peptide stimulation.

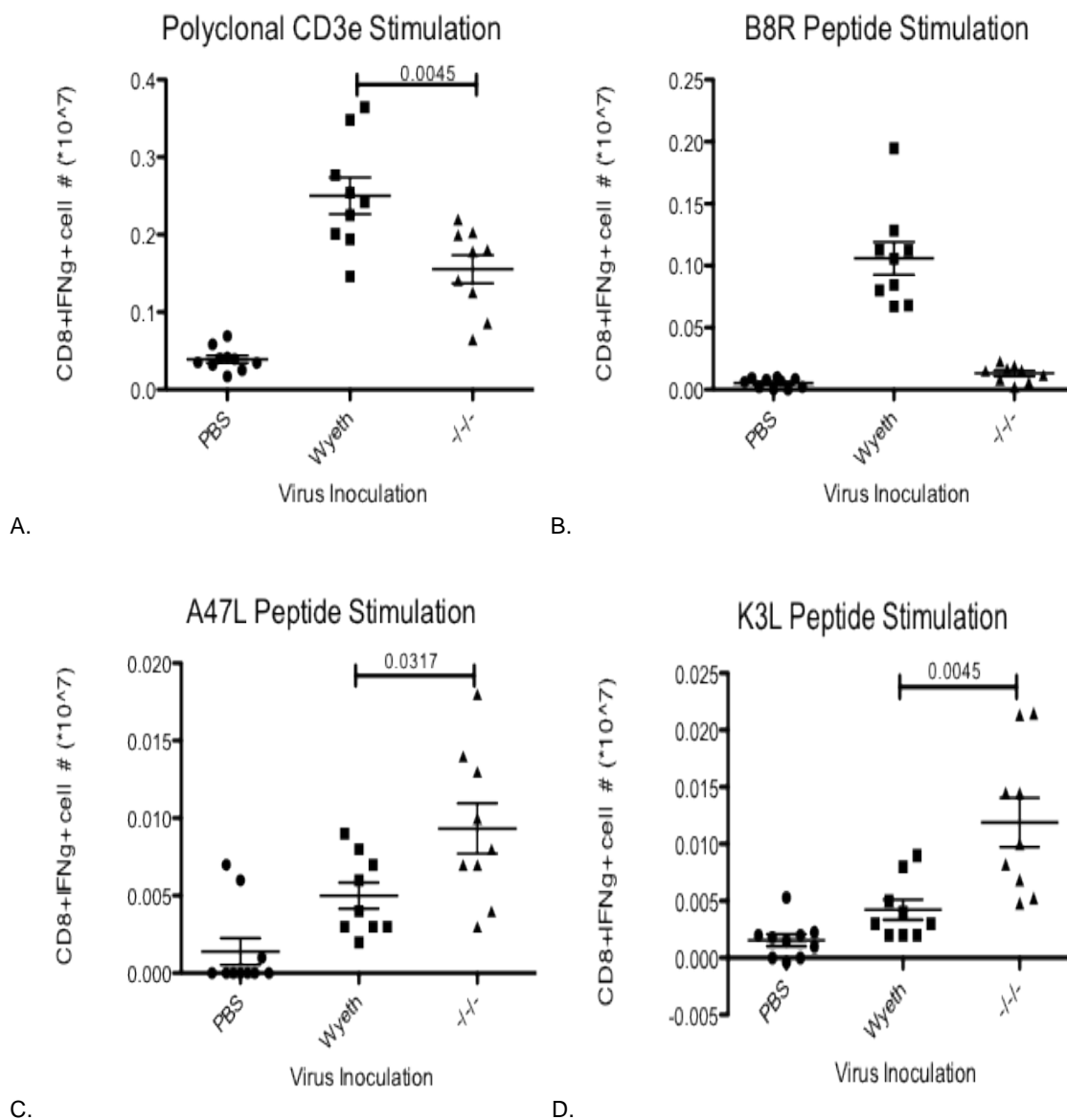


Figure 4.4. VAC-specific CD8⁺ T cell response after 20 LD₅₀'s VAC-WR challenge in PBS, VAC-Wy, or VAC-Wy-/- skin-scarified mice.

Figure 4.4. VAC-specific CD8⁺ T cell response after 20 LD50's VAC-WR challenge in PBS, VAC-Wy, or VAC-Wy-/- skin-scarified mice.

B6 mice were infected with 2×10^5 of each of the indicated viruses by skin-scarification. On Day 210, mice were infected I.N. with 20 LD50's of VAC-WR (2×10^5), and 5 days later mice were sacrificed and splenocytes were cultured for five hours with the B8R peptide, A47L peptide, or K3L peptide from VAC, or with a mAb against CD3 ϵ in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. All plots are gated on CD8⁺ cells. (A) Polyclonal CD3 ϵ stimulation (B) B8R peptide stimulation (C), A47L peptide stimulation (D), or K3L peptide stimulation. P values were determined by Student's t-test. PBS is phosphate buffered saline, Wyeth is parental VAC-Wy, and -/- is a VAC-Wy with the genes encoding the C3L, C12L, and B8R proteins knocked out.

After challenge with 20 LD50's of VAC-WR, only mice immunized with the parental VAC-Wy, had CD8⁺ T cells that produced IFN γ upon stimulation with the B8R peptide, even though the challenge virus was not a B8R knockout virus (Figure 4.4B). This again suggests that in immunized mice at this time point, the CD8⁺ T cell response was entirely of memory origin. Interestingly, fewer CD8⁺ T cells produced IFN γ after a polyclonal stimulation with an antibody against CD3 ϵ in mice that had been immunized with the VAC-Wy-/-/- when compared to mice that had been immunized with the parental VAC-Wy (Figure 4.4A). This decrease might be due to the lack of the gene that encodes the B8R protein from the VAC-Wy-/-/-, and therefore does not contain the peptide that forms the immunodominant epitope in B6 mice. Furthermore, there were increases in the number of CD8⁺ T cells that produce IFN γ after either K3L (Figure 4.4D) or A47L (Figure 4.4C) peptide stimulation, possibly compensating for the lack of B8R-specific CD8⁺ T cells.

C. CHAPTER CONCLUSION

Although virus pathogenesis was not affected by the lack of these three immunomodulatory proteins, the skin-scarification immunization with the VAC-Wy-/-/- protected mice as well as the parental VAC-Wy strain to 20 LD50's I.N. challenge with VAC-WR. The apparent lack of pathogenicity differences could be due to the poor growth of VAC-Wy inside of the mouse, as we noted poor growth of the B8R recombinants made in VAC-Wy in the mouse in our earlier study. Interestingly, the lack of a B8R-specific CD8⁺ T cell response from the

skin-scarification immunization did not have a significant effect on virus titers, although we did find increased numbers of CD8⁺ T cells that produced IFN γ upon stimulation with two subdominant determinants, and this might be a compensation mechanism, and is discussed further in the Discussion.

CHAPTER V: INCREASED PROTECTION FROM HIGH DOSE VACCINIA
VIRUS INFECTION IN MICE GENETICALLY PRONE TO
LYMPHOPROLIFERATIVE DISORDERS

Individuals that have mutations that code for hypoactive versions of either the Fas or Fas ligand protein are more susceptible to many autoimmune disorders. It is also possible that these mutations might lead to increased protection from some infections as the immune systems in these individuals might be considered to be in a continuously activated state. To examine this possibility, the immune response to high dose VAC-WR infections was examined in Fas mutant mice.

Fas/FasL interactions have been shown to lead to AICD in T cells repeatedly stimulated through their TCR's. Early studies done in our lab had demonstrated that lethal I.N. infection of mice with VAC-WR resulted in few VAC-specific CD8⁺ T cells detectable in spleens. As this high dose infection might be driving cells into AICD, it was possible that mice that had mutations in Fas or Fas ligand that result in decreased Fas/FasL signaling might have increased T cell responses as the mechanism of the decreased T cell response might be Fas induced AICD.

Interestingly, concurrent with the time of my graduate studies, observations in one animal model suggested that hosts that have mutations in this pathway may be better able to fight off certain infections. Specifically, it had been demonstrated that BALB/*c-gld* mice lose less weight and had better survival

when compared to their BALB/c wild type counterparts after high dose influenza virus infections, and this had been attributed to the increased CD8⁺ T cell response in the BALB/c-*g/d* mice (Legge and Braciale 2005). In this model, it was described that as the influenza dose was increased to lethal levels, FasL would be up-regulated on DC's, and this FasL up-regulation would then cause activated CD8⁺ T cells to be eliminated through apoptosis, resulting in a hampered influenza-virus specific CD8⁺ T cell response. It has also been demonstrated that HIV-infected individuals with combined hypoactive mutations in Fas or FasL performed better after anti-retroviral therapy than those with no mutations (Nasi, Pinti et al. 2005). The observation that hosts that have mutations that often lead to autoimmune disorders might be able to fight off or survive certain infections more readily may explain why these mutations continue to be seen in the population. To more closely study this phenomenon using VAC-WR, the immune response and control of lethal VAC-WR infections was examined with Fas or Fas ligand mutant mice.

A. B6-*lpr* Mice Have Reduced Weight Loss, Have Reduced Virus Loads, And Have Increased Numbers Of CD8⁺ T Cells On Day 6 In Response To A Normally Lethal I.N. or I.P. VAC-WR Challenge

To examine if Fas-induced AICD was leading to decreased T cell responses after lethal VAC-WR infection, B6 and B6-*lpr* mice were infected I.N. with 1×10^4 VAC-WR.

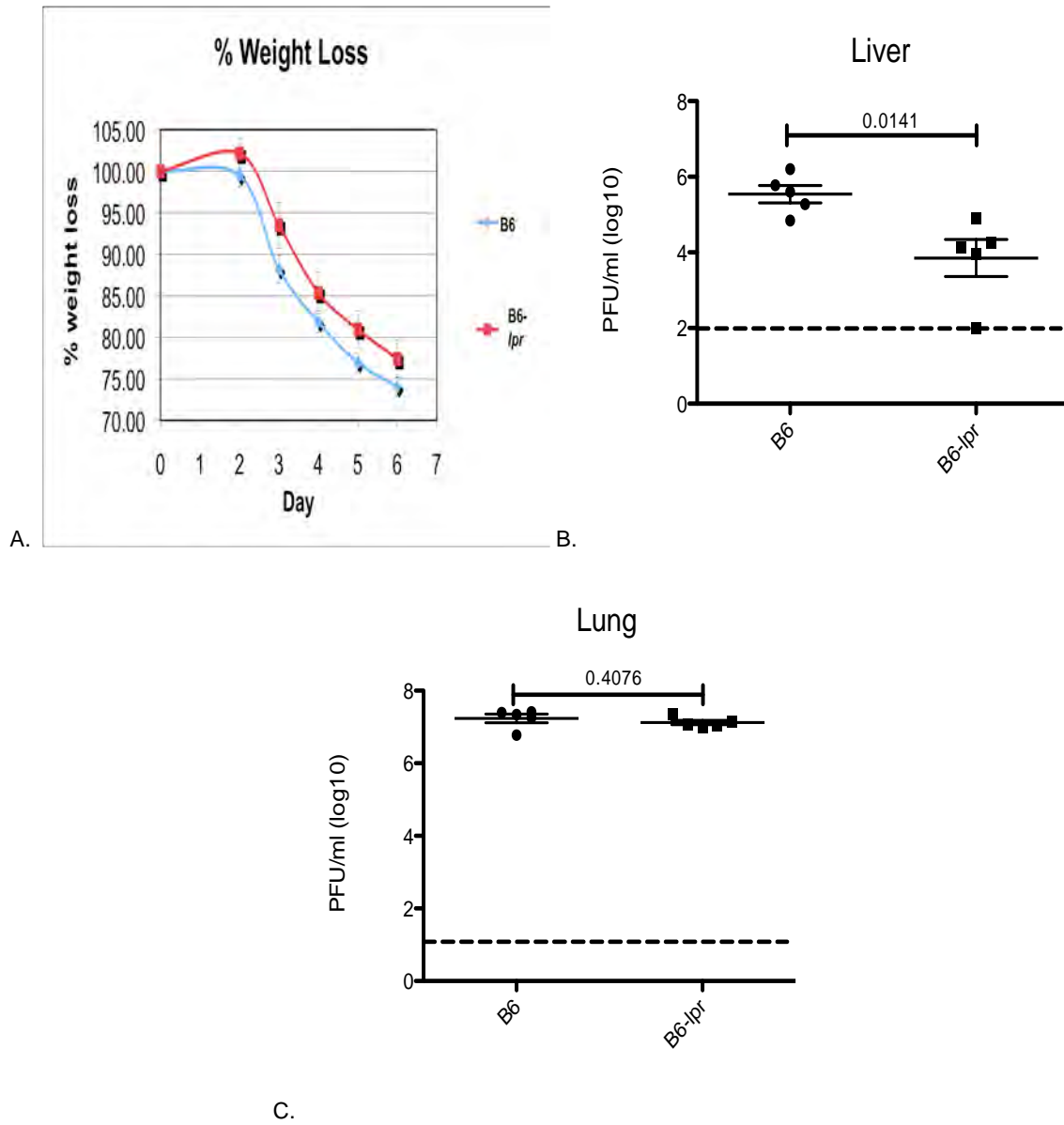


Figure 5.1. Decreased weight loss and liver virus titers on day 6 after lethal I.N. VAC-WR challenge in B6-*lpr* mice.

Figure 5.1. Decreased weight loss and liver virus titers on day 6 after lethal I.N. VAC-WR challenge in B6-Ipr mice.

*B6 or B6-Ipr mice were infected with 1×10^4 PFU's of VAC-WR by I.N. inoculation. Mice were weighed each day, and the average and standard deviations of each group are plotted as percent of initial weight lost (5 mice per group, * = $p < 0.05$) (A) Mice were sacrificed on day 6 after the challenge, and viral titers in the livers (B) and lungs (C) were determined by plaque assay (the dotted line represents the limit of detection of the assay) P values were determined by Student's t-test.*

B6-*Ipr* mice lost less weight (Figure 5.1A) and had almost 2 logs less virus in the livers (Figure 5.1B) when compared to B6 wild type mice after I.N. infection with an amount of VAC-WR that typically causes death, with no difference in lung virus titers (Figure 5.1C). Interestingly, B6-*Ipr* mice weighed more throughout the time course of the infection when compared to the B6 wild type mice, even though both sets of mice weighed the same at the time of infection (data not shown). The lymphocyte subsets and VAC-specific CD8⁺ T cell responses in the mediastinal lymph nodes (MLN) after I.N. infection were next examined.

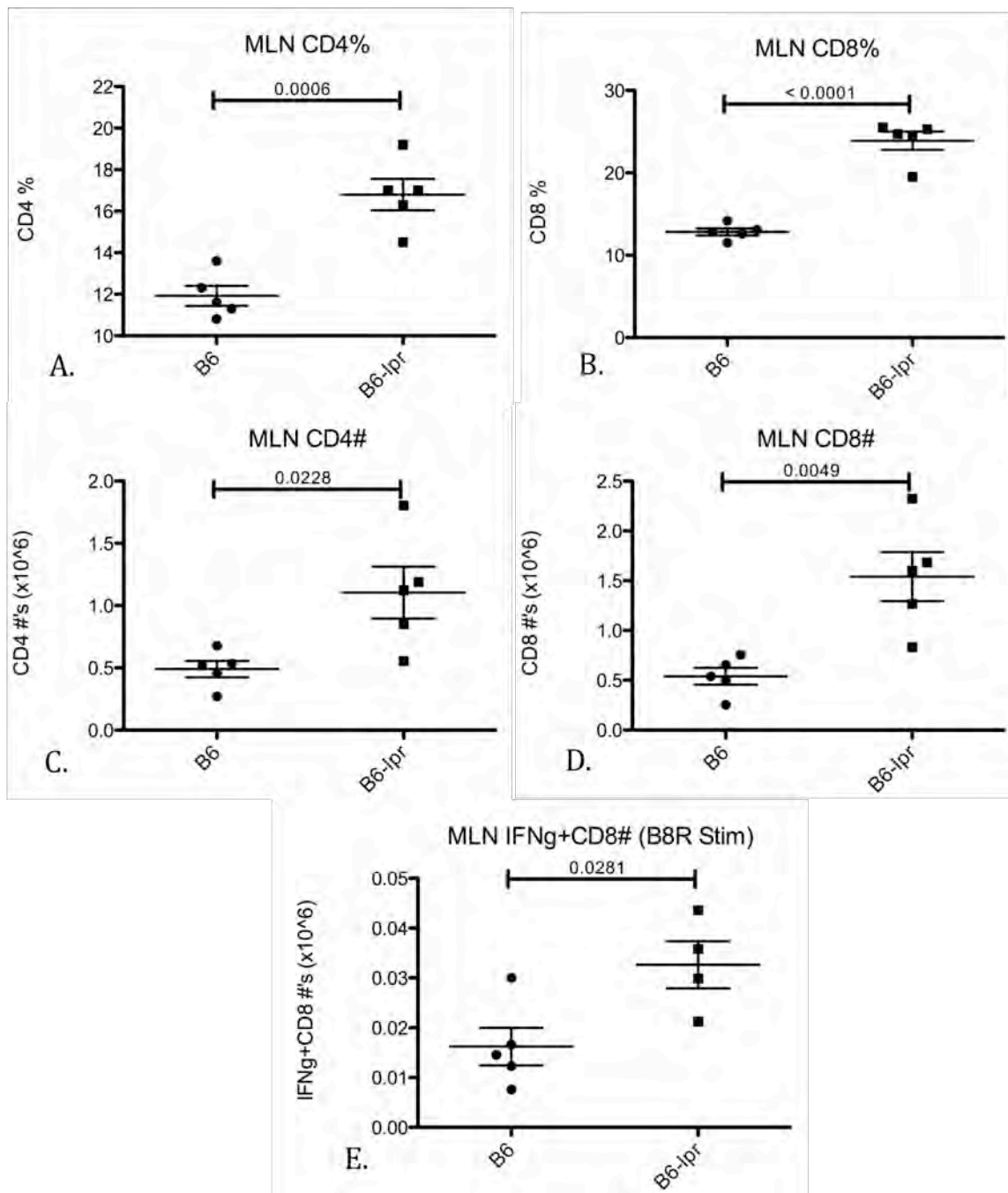


Figure 5.2. Increased percentage and number of T Cells and B8R-specific CD8⁺ T cells in MLN after lethal VAC-WR I.N. infection in B6-*Ipr* mice.

Figure 5.2. Increased percentage and number of T Cells and B8R-specific CD8⁺ T cells in MLN after lethal VAC-WR I.N. infection in B6-lpr mice.

B6 or B6-lpr mice were infected with 1×10^4 PFU's of VAC-WR by I.N. inoculation.

On day 6 after infection, the MLN was prepared and lymphocytes were cultured

for five hours with the B8R peptide in the presence of GolgiPlug. This was

followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS

analysis. The average and standard errors of the mean are shown. (A) CD4⁺

percentage (B) CD8⁺ percentage (C) total CD4⁺ numbers (D) total CD8⁺ numbers

(E) and number of IFN γ ⁺ CD8⁺ T cells after B8R peptide stimulation are shown.

P values were determined by Student's t-test.

There was an increased percentage and number of CD4⁺ and CD8⁺ T cells on day 6 in the MLN's of B6-*Ipr* mice in comparison to B6 wild type mice after VAC-WR infection (Figure 5.2A, 5.2B, 5.2C and 5.2D). There was also an increased number of IFN γ ⁺CD8⁺ T cells after B8R peptide stimulation in the MLN's of B6-*Ipr* mice in comparison to B6 wild type mice (Figure 5.2E). B6 or B6-*Ipr* mice were next infected with a lethal dose of VAC-WR I.P. to determine if a separate inoculation route with a lethal dose of virus would also result in an increased number of T cells in B6-*Ipr* mice in comparison to B6 mice, and to find if B6-*Ipr* mice were better protected from high dose I.P. infections as well.

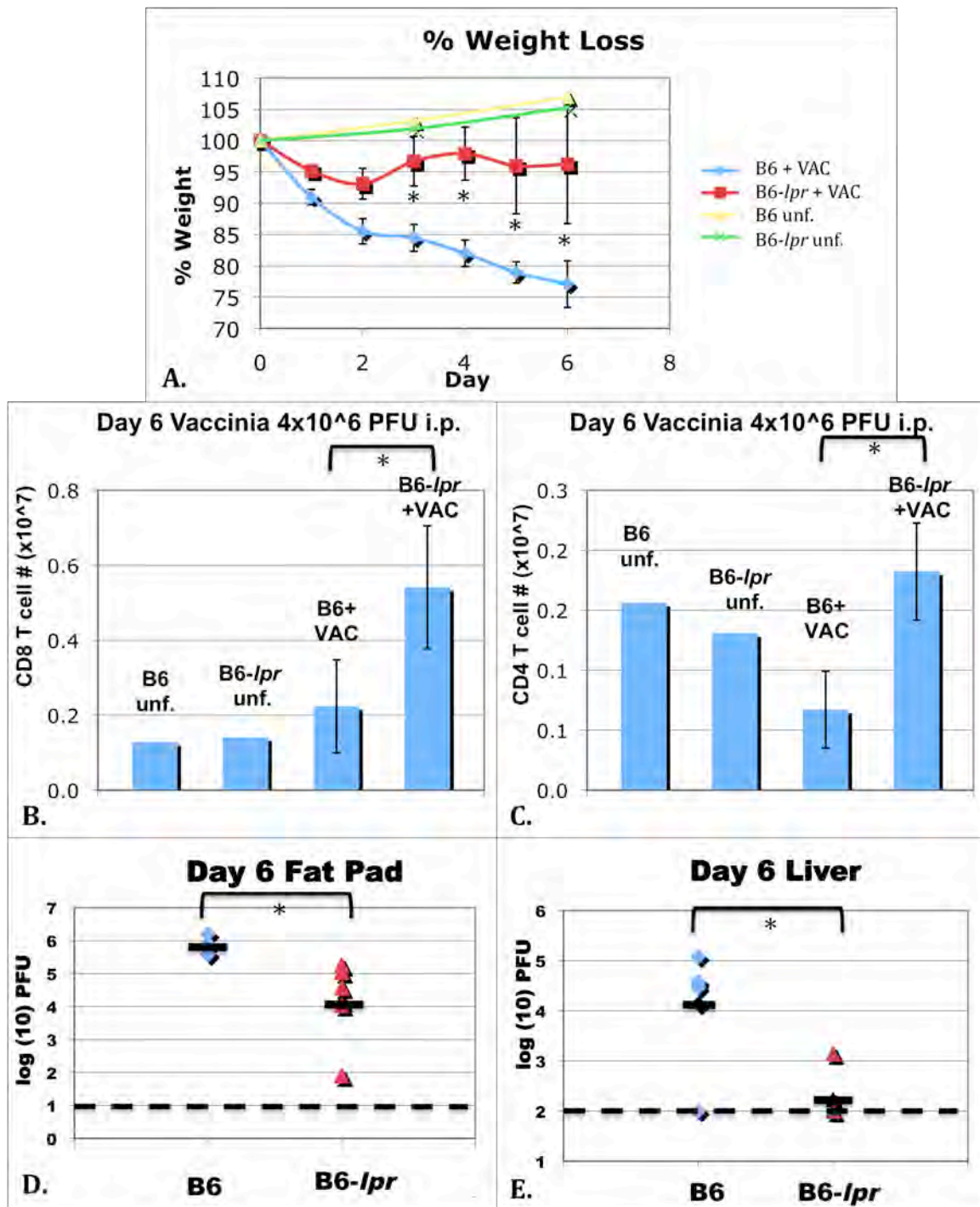


Figure 5.3. Decreased weight loss and virus titers on day 6, and increased numbers of T cells in day 6 spleens after lethal I.P. VAC-WR challenge in B6-*lpr* mice.

Figure 5.3. Decreased weight loss and virus titers on day 6, and increased numbers of T cells in day 6 spleens after lethal I.P. VAC-WR challenge in B6-lpr mice.

B6 or B6-lpr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. (A) Mice were weighed each day, and the average and standard deviations of each group are plotted as percent of initial weight lost. On day 6 after infection, splenocytes were cultured for five hours with the B8R peptide in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis. (B) CD4⁺ numbers and (C) CD8⁺ numbers with the average and standard deviation are shown. Viral titers in the (D) fat pads, and (E) livers were determined by plaque assay. All P values were determined by Student's t-test. (5 mice per group, * = $p < 0.05$). Unf. are uninfected mice.

After 4×10^6 VAC-WR I.P. infection, B6-*lpr* mice lost less weight (Figure 5.3A) when compared to B6 wild type mice, and B6-*lpr* mice also had almost 2 logs less virus in the fat pads and livers at day 6 in comparison to the B6 wild type mice (Figure 5.3D and 5.3E). There was an increased number of CD4⁺ and CD8⁺ T cells in the spleens of B6-*lpr* mice in comparison to B6 wild type mice (Figure 5.3B and Figure 5.3C), although there was not an increase in the number of VAC-specific CD8⁺ T cells as detected by an intracellular IFN γ assay (data not shown). In a lethal influenza model of virus infection, there was an increased number of CD8⁺ T cells responding to an influenza virus infection when adoptively transferred into mutant BALBc-*gld* mice in comparison to wild type BALB/C mice, and this was assumed to be because of the decreased apoptosis of activated CD8⁺ T cells (Legge and Braciale 2005). Adoptive transfer experiments were next set up to determine if CD8⁺ T cells that lack Fas would proliferate more extensively in comparison to CD8⁺ T cells that normally express Fas. Splenocytes from B6-*lpr* (Ly5.2⁺Thy1.2⁺) or IgH^a wild type (Ly5.2⁺Thy1.1⁺ donor) were mixed 1:1 and adoptively transferred into B6.SJL (Ly5.1⁺Thy1.2⁺ host) mice, and one day later, mice were infected with 4×10^6 VAC-WR I.P.

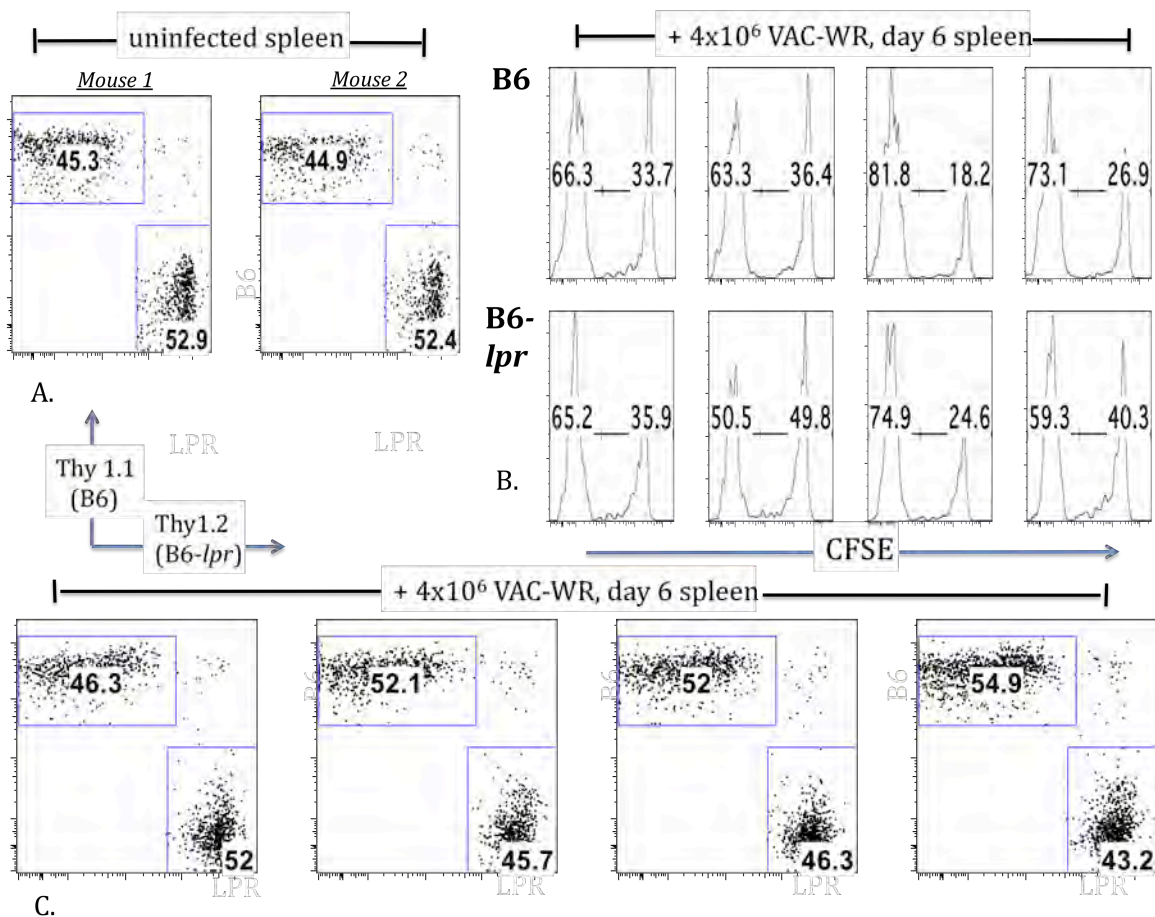


Figure 5.4. Adoptively transferred B6-*lpr* CD8⁺ T cells do not have increased proliferation or survival in comparison with wild type CD8⁺ T cells at day 6 after lethal I.P. VAC-WR infection.

Figure 5.4. Adoptively transferred B6-lpr CD8⁺ T cells do not have increased proliferation or survival in comparison with wild type CD8⁺ T cells at day 6 after lethal I.P. VAC-WR infection.

0.75x10⁷ B6-lpr (Ly5.2⁺Thy1.2⁺ donor) and 0.75x10⁷ IgH^a (Ly5.2⁺Thy1.1⁺ donor) splenocytes were CFSE labeled mixed 1:1 and adoptively transferred into B6.SJL (Ly5.1⁺Thy1.2⁺ host) mice, and one day later, some of the mice were infected with 4x10⁶ VAC-WR I.P. On day 6 after infection, splenocytes were stained with the appropriate fluorescent antibodies, and FACS analysis was performed. All analyses shown is gated on CD8⁺ T cells. (A) Donor subset of uninfected mice. (B) CFSE dilution of each donor type after 4x10⁶ VAC-WR I.P. (C) % of donor CD8⁺ T cells after 4x10⁶ VAC-WR I.P.

Similar percentages of donor B6-*Ipr* (Ly5.2⁺Thy1.2⁺) and IgH^a (Ly5.2⁺Thy1.1⁺ donor) wild type CD8⁺ T cells were found in uninfected mice, confirming the 1:1 mix of donor splenocytes (Figure 5.4A). After infection with 4x10⁶ VAC-WR I.P., similar percentages of CFSElo B6-*Ipr* (Ly5.2⁺Thy1.2⁺) and CFSElo IgH^a (Ly5.2⁺Thy1.1⁺ donor) wild type CD8⁺ T cells were detected in the host mice (Figure 5.4B). There was also a similar percentage of B6-*Ipr* (Ly5.2⁺Thy1.2⁺) and IgH^a (Ly5.2⁺Thy1.1⁺ donor) wild type CD8⁺ T cells in the host mice (Figure 5.4C), suggesting that CD8⁺ T cells that lack Fas do not survive high dose VAC-WR I.P. infection better than CD8⁺ T cells that can normally express Fas. Similar experiments were performed using the high dose VAC-WR I.N. infection model, and it was found that CD8⁺ T cells that lack Fas do not survive high dose VAC-WR I.N. infection better than CD8⁺ T cells that can normally express Fas (data not shown). This suggests that the increased protection of B6-*Ipr* from high dose VAC-WR infection did not occur by the same mechanism as had been noted after high dose influenza virus infection (Legge and Braciale 2005). It was noted that weight loss induced by normally lethal doses of VAC-WR was significantly ameliorated in B6-*Ipr* mice in both the I.N. and I.P. infection model in comparison to B6 wild type mice at early time points (Figure 5.1A and Figure 5.3A), and there was also an increased NK% (0.84 +/- 0.48 in B6 vs. 1.63 +/- 0.22 in B6-*Ipr*) and $\gamma\delta$ T cell% (0.21 +/- 0.04 in B6 vs. 0.44 +/- 0.05) found in day 6 infected B6-*Ipr* mice. These observations pointed to

increased protection of B6-*lpr* mice at earlier time points, and so virus titers at early time points after high dose VAC-WR infection was examined.

B. Fas Mutant Mice Have Reduced Virus Loads At Day 2 And Day 3 Time Points in Response To A Normally Lethal I.P. VAC-WR Challenge

B6 or B6-*lpr* mice were infected with a typically lethal dose of VAC-WR I.P., and virus titers were examined at day 2 or day 3. B6 or B6-*gld* mice were also infected with a typically lethal dose of VAC-WR I.P., and virus titers were examined on day 2.

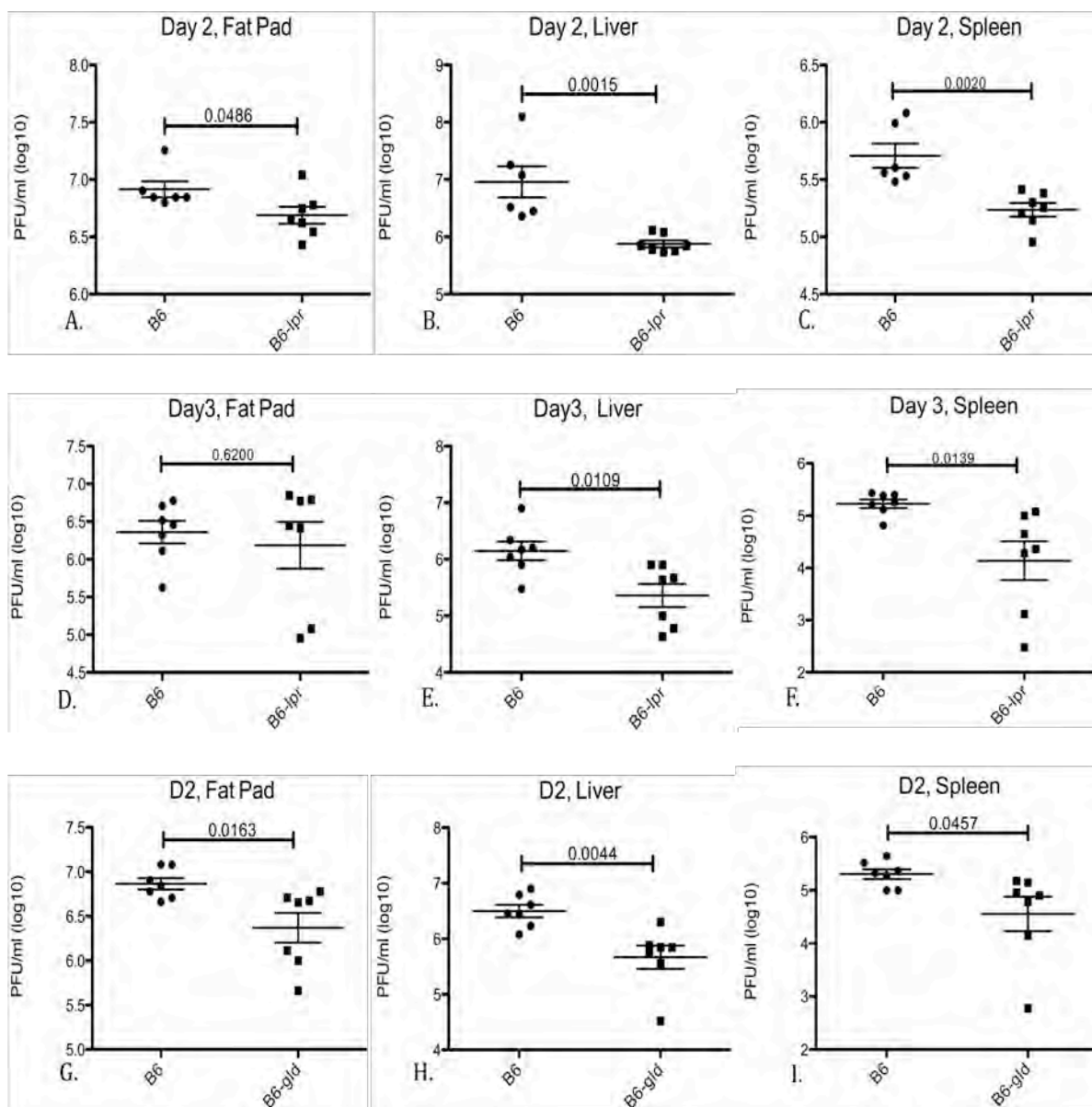


Figure 5.5. Fas mutant mice have decreased amounts of virus at early time points after lethal I.P. VAC-WR infection.

Figure 5.5. Fas mutant mice have decreased amounts of virus at early time points after lethal I.P. VAC-WR infection.

B6 or B6-lpr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 2 (A,B,C) or day 3 (D,E,F) after infection, viral titers in the (A, D) fat pads, (B,E) livers, and (C,F) spleens were determined by plaque assay. B6 or B6-gld were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 2 viral titers in the (G) fat pads, (H) livers, and (I) spleens were determined by plaque assay. All P values were determined by Student's t-test.

After 4×10^6 VAC-WR I.P. infection, B6-*Ipr* mice had slightly less virus in the fat pads (Figure 5.5A), approximately one log less virus in livers (Figure 5.5B), and about $\frac{1}{2}$ a log less virus in spleens (Figure 5.5C) at day 2 in comparison to the B6 wild type mice and at day 3, B6-*Ipr* mice had $\frac{3}{4}$ of a log less virus in the livers (Figure 5.5E), and about a log less virus in the spleens (Figure 5.5F) than B6 wild type mice. B6-*gld* mice had $\frac{1}{2}$ a log less virus in the fat pad (Figure 5.5G), and about a log less in the livers (Figure 5.5H) and spleens (Figure 5.5I) when compared to B6 wild type mice. In light of these data suggesting that B6-*Ipr* mice were better protected than B6 wild type mice as early as day 2, and because earlier data suggested that the NK cell % and $\gamma\delta$ T cell % were increased in B6-*Ipr* mice infected as compared to B6 wild type infected mice, uninfected B6-*Ipr* and B6 mice were examined for increases in NK and $\gamma\delta$ T cell numbers, as these lymphocytes are thought to be able to exert effector functions early in virus infections.

C. Uninfected B6-*Ipr* Mice Have More Innate Like Leukocytes, and More Memory Like T Cells than B6 Mice, But Antibody Depletions of Individual Subsets in B6-*Ipr* Mice Does not Increase Virus Titers.

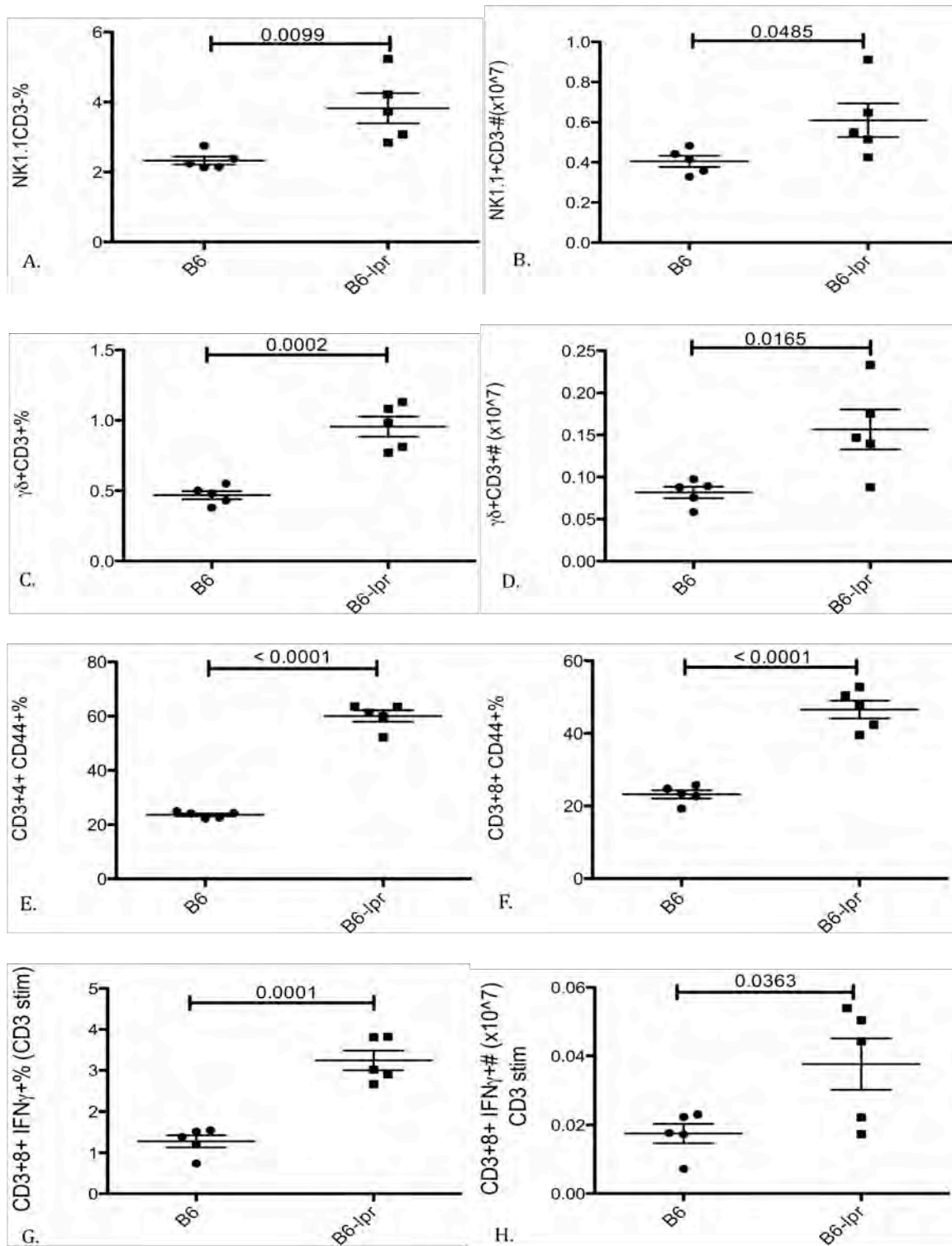


Figure 5.6. Increased NK, $\gamma\delta$ T cells, and memory phenotype T cells, and IFN γ ⁺ CD8⁺ T cells after polyclonal stimulation in B6-*lpr* mice.

Figure 5.6. Increased NK, $\gamma\delta^+$ T cells, and memory phenotype T cells, and $IFN\gamma^+$ CD8⁺ T cells after polyclonal stimulation in B6-lpr mice.

Six week old uninfected B6 or B6-lpr mice were sacrificed, and splenocytes were cultured for five hours with a mAb against CD3 ϵ in the presence of GolgiPlug.

This was followed by an intracellular $IFN\gamma$ assay with fluorescent antibodies, and FACS analysis. (A) NK1.1⁺CD3⁻%, (B) NK1.1⁺CD3[#]'s, (C) $\gamma\delta^+$ CD3⁺%, (D) $\gamma\delta^+$ CD3[#]'s, (E) CD3⁺CD4⁺ CD44⁺%, (F) CD3⁺CD8⁺ CD44⁺%, and the (G) $IFN\gamma^+$ CD8⁺%, (H) $IFN\gamma^+$ CD8[#]'s after CD3 ϵ stimulation. All P values were determined by Student's t-test.

Increases in the NK1.1⁺CD3⁻% (Figure 5.6A), NK1.1⁺CD3⁻#s (Figure 5.6B), $\gamma\delta^+$ CD3⁺% (Figure 5.6C), $\gamma\delta^+$ CD3⁺#s (Figure 5.6D), and increases in the number of CD44⁺ CD4⁺ and CD44⁺ CD8⁺ T cells were demonstrated in 6 week old uninfected B6-*lpr* mice as compared to 6 week old uninfected B6 wild type mice (Figure 5.6E and 5.6F). There was also an increased percentage and number of CD8⁺ T cells that were IFN γ ⁺ after a polyclonal CD3 ϵ stimulation (Figure 5.6G and 5.6H). It has been demonstrated that $\gamma\delta^+$ T cell knockout mice have increased VAC titers when compared to wild type mice, and that treatment with an antibody against the $\gamma\delta^+$ TCR exacerbates VAC infection (Selin, Santolucito et al. 2001). To examine if the increased $\gamma\delta^+$ T cell number resulted in increased protection in B6-*lpr* mice, $\gamma\delta$ antibody blocking experiments were performed in B6-*lpr* mice.

It has also been suggested that NK cells have a role in controlling VAC infections, as depletion of NK cells exacerbates VAC infection (Bukowski, Woda et al. 1983; Kawakami, Tomimori et al. 2009). As there were more NK cells in uninfected B6-*lpr* mice as compared to B6 mice, NK depletion experiments were also performed in B6-*lpr* mice.

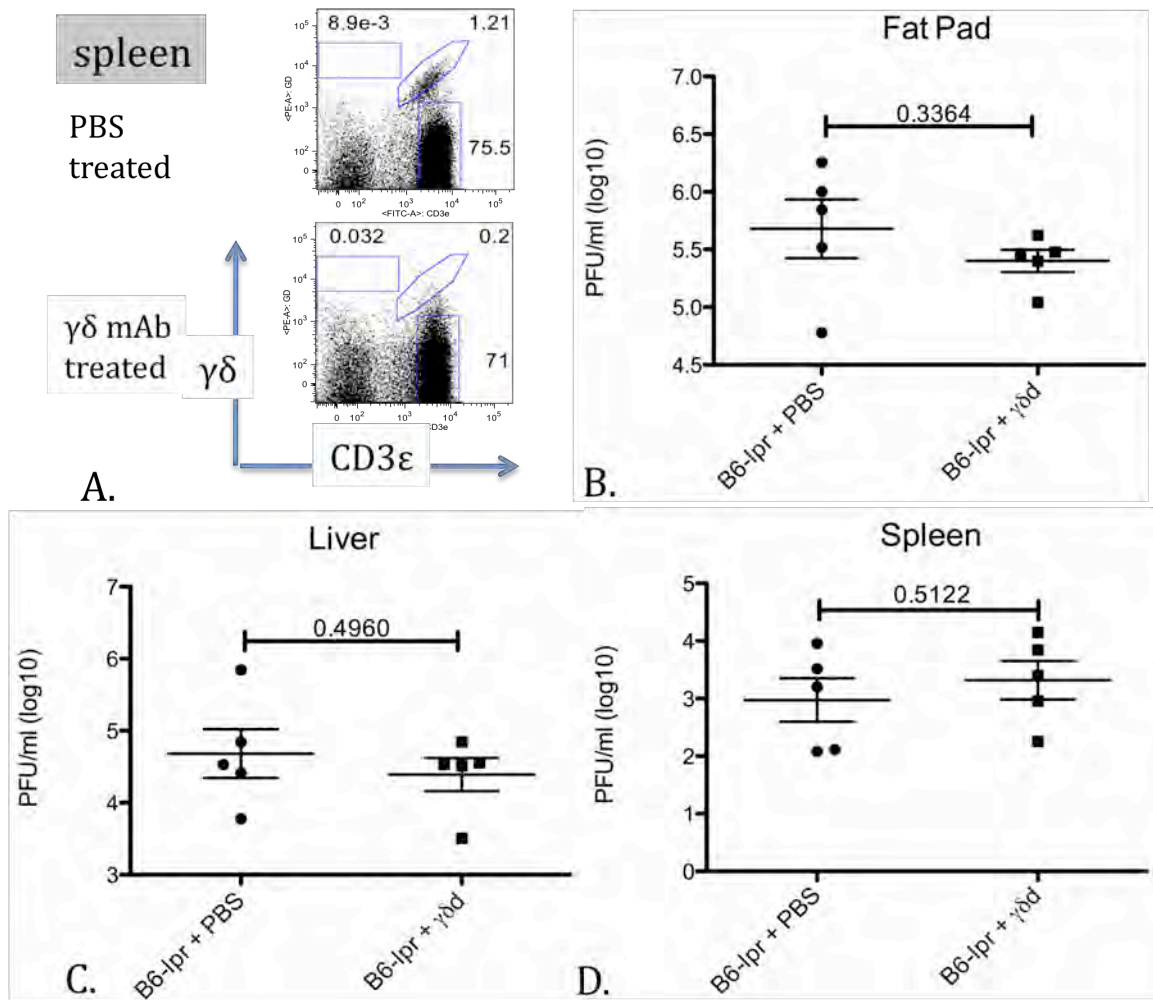


Figure 5.7. $\gamma\delta$ TCR antibody treatment does not result in increased virus titers in B6-*lpr* mice.

Figure 5.7. $\gamma\delta$ TCR antibody treatment does not result in increased virus titers in B6-lpr mice.

B6-lpr mice were treated with 200 μ l PBS or 200 μ l PBS containing 100 μ g of $\gamma\delta$ TCR-blocking antibody. One day later both groups of mice were I.P. infected with 4×10^6 PFU's of VAC-WR. On day 3 splenocytes were stained with the appropriate fluorescent antibodies, and (A) FACS analysis was performed to ensure of the antibody blocking effect. Viral titers in the (B) fat pads, (C) livers, and (D) spleens were determined by plaque assay. All P values were determined by Student's t-test. $\gamma\delta$ is $\gamma\delta$ TCR monoclonal antibody treatment, and PBS is phosphate buffer saline treated mice.

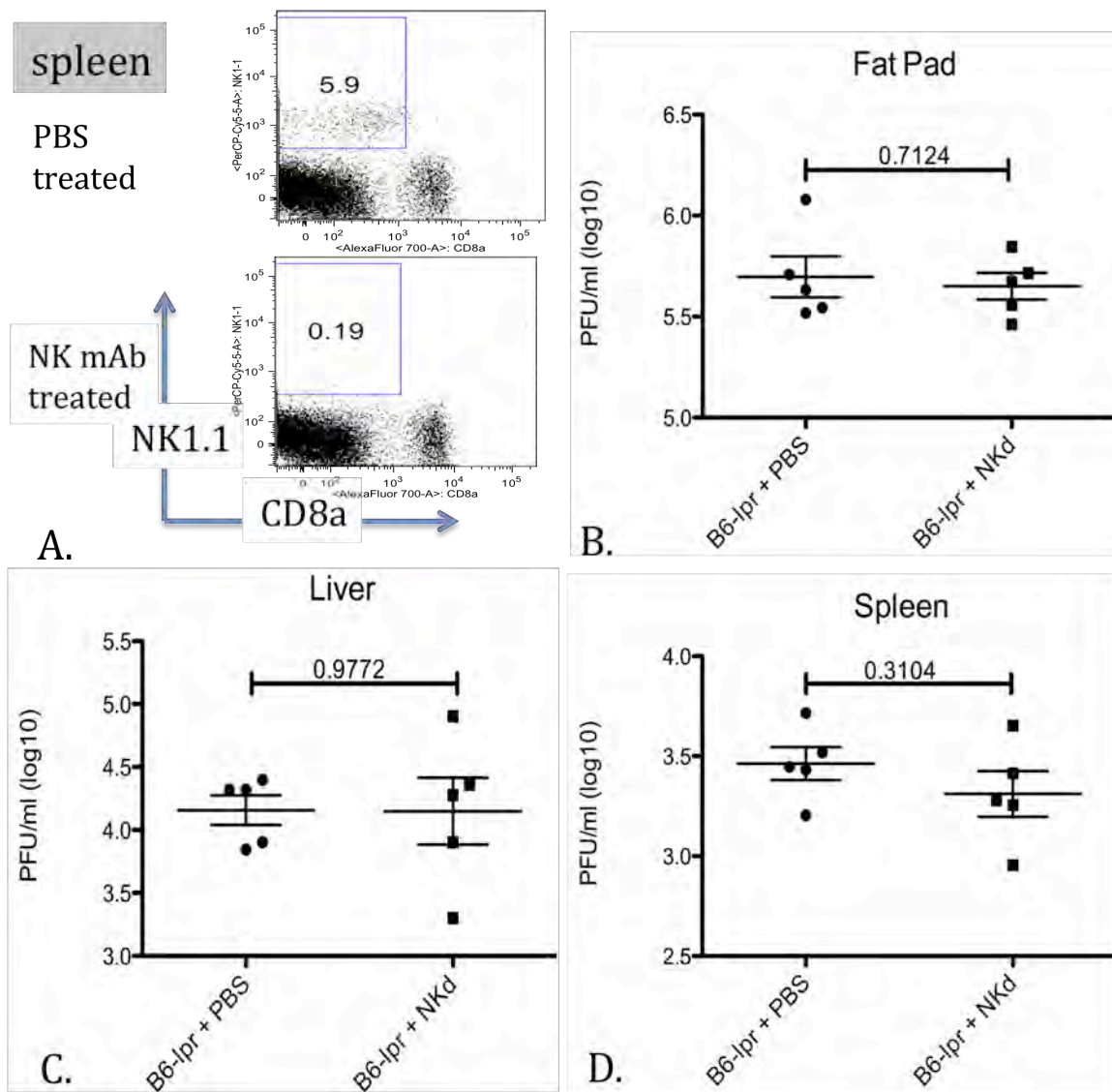


Figure 5.8. NK antibody treatment does not result in increased virus titers in B6-*lpr* mice.

Figure 5.8. NK antibody treatment does not result in increased virus titers in B6-Ipr mice.

B6-Ipr mice were treated with 200 μ l PBS or 200 μ l PBS containing 50 μ g of NK-depleting antibody. One day later both groups of mice were I.P. infected with 4x10⁶ PFU's of VAC-WR. On day 3 splenocytes were stained with the appropriate fluorescent antibodies, and (A) FACS analysis was performed to ensure of the NK depletion. Viral titers in the (B) fat pads (C) livers and (D) spleens were determined by plaque assay. All P values were determined by Student's t-test. NKd is NK monoclonal antibody treatment, and PBS is phosphate buffer saline treatment.

Splenocytes from B6-*Ipr* mice treated with a $\gamma\delta$ TCR-blocking antibody did not stain with an antibody against the $\gamma\delta$ TCR (Figure 5.7A), confirming the blocking effect, but treatment with the $\gamma\delta$ TCR-blocking antibody had no effect on virus titer in the fat pad (Figure 5.7B), liver (Figure 5.7C) or spleen (Figure 5.7D).

B6-*Ipr* mice treated with an NK depleting antibody did not have any detectable NK cells in the spleen (Figure 5.8A), confirming the NK depletion, but there were no differences in viral titers in the fat pads (Figure 5.8B), livers (Figure 5.8C), or spleens (Figure 5.8D) of NK depleted vs. undepleted B6-*Ipr* mice.

Increases in the percentage of memory-like CD4⁺ and CD8⁺ T cells (CD44^{hi}) had also been demonstrated in uninfected B6-*Ipr* mice (Figure 5.6E-H). It is possible that the increase in the number of memory phenotype T cells might allow T cells to act earlier in B6-*Ipr* mice. This might then allow T cells to have an early immuno-protective role in high dose VAC-WR infections in B6-*Ipr* animals, even though the antiviral properties of these populations are thought to occur after T cell expansion in B6 mice, so depletions experiments using CD4 and CD8 antibodies were performed in B6-*Ipr* mice.

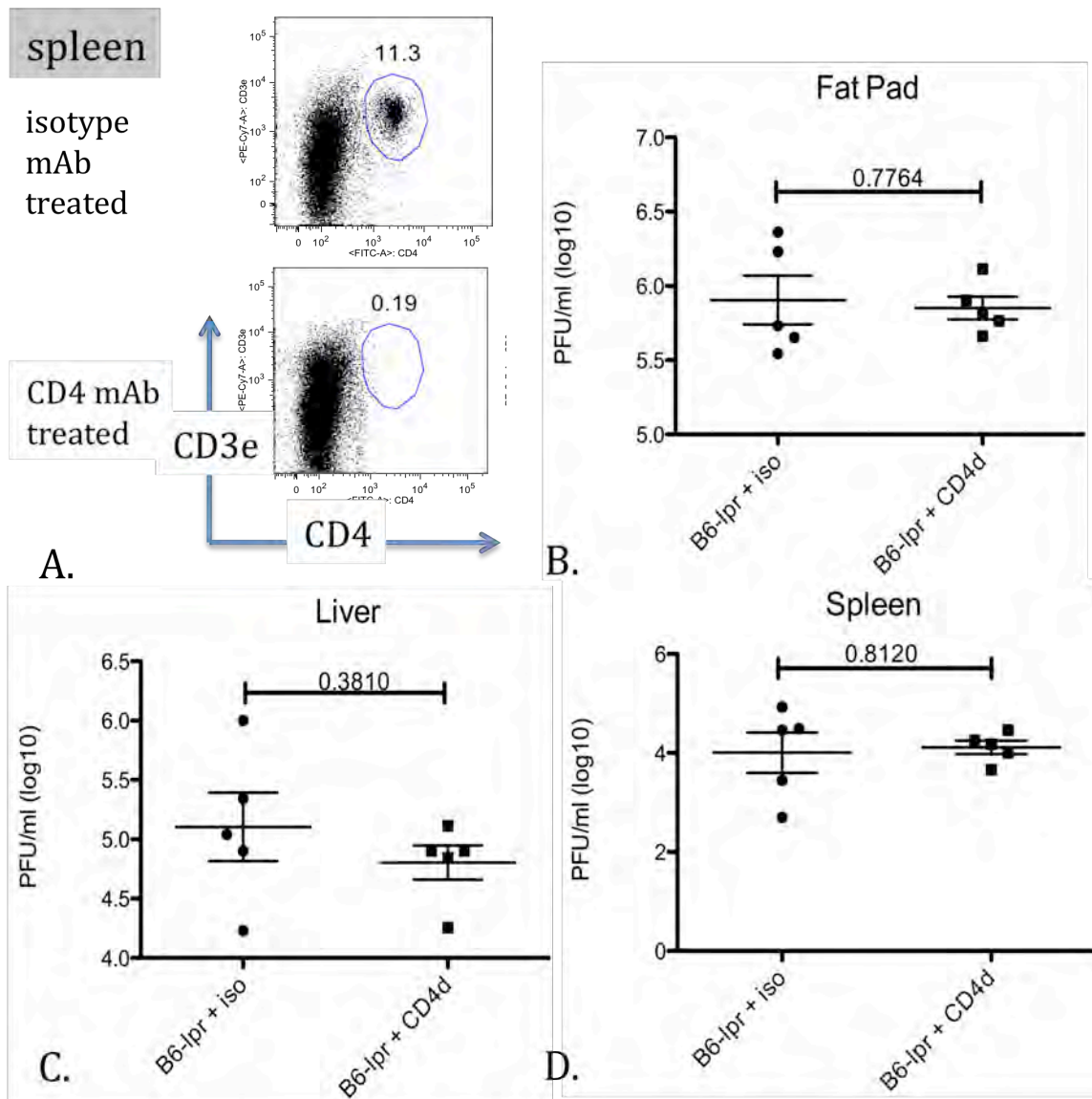


Figure 5.9. CD4 antibody treatment does not result in increased virus titers in B6-*lpr* mice.

Figure 5.9. CD4 antibody treatment does not result in increased virus titers in B6-Ipr mice.

B6-Ipr mice were treated with 200µl PBS containing 25µg of the isotype antibody or 200µl PBS containing 25µg of the CD4-depleting antibody. One day later both groups of mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 3 splenocytes were stained with the appropriate fluorescent antibodies, and (A) FACS analysis was performed to ensure of the CD4 depletion. Viral titers in the (B) fat pads, (C) livers, and (D) spleens were determined by plaque assay. All P values were determined by Student's t-test. CD4d is CD4 monoclonal antibody treatment, and iso is isotype control monoclonal antibody treatment.

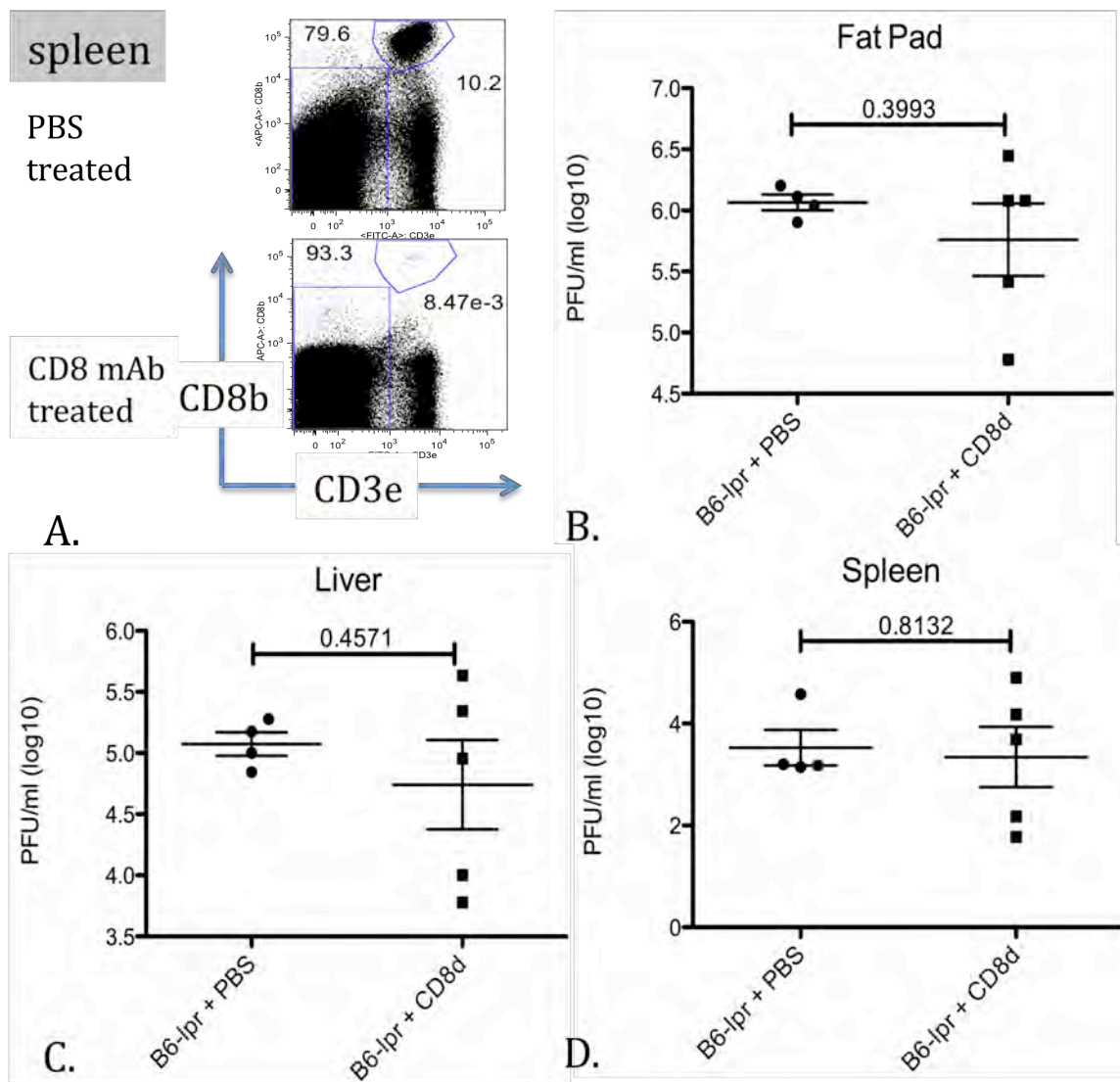


Figure 5.10. CD8 antibody treatment does not result in increased virus titers in B6-*lpr* mice.

Figure 5.10. CD8 antibody treatment does not result in increased virus titers in B6-lpr mice.

B6-lpr mice were treated with 200 μ l PBS containing 25 μ g of the isotype antibody or 200 μ l PBS containing 25 μ g of the CD8-depleting antibody. One day later both groups of mice were infected with 4x10⁶ PFU's of VAC-WR by I.P. inoculation. On day 3 splenocytes were stained with the appropriate fluorescent antibodies, and (A) FACS analysis was performed to ensure of the CD8 depletion. Viral titers in the (B) fat pads (C) livers and (D) spleens were determined by plaque assay. All P values were determined by Student's t-test. CD8d is CD8 monoclonal antibody treatment, and PBS is phosphate buffer saline treatment.

B6-*lpr* mice treated with a CD4 depleting antibody did not have any detectable CD4⁺ T cells in the spleen (Figure 5.9A), confirming the CD4 depletion, but no difference in virus titers in the fat pads (Figure 5.9B), livers (Figure 5.9C), or spleens (Figure 5.9D) of depleted vs. non-depleted mice were noted.

B6-*lpr* mice treated with a CD8 depleting antibody did not have any detectable CD8⁺ T cells in the spleen (Figure 5.10A), confirming the CD8 depletion, but no difference in virus titers in the fat pads (Figure 5.10B), livers (Figure 5.10C), or spleens (Figure 5.10D) in CD8 depleted vs. non-depleted B6-*lpr* mice were found.

As none of the individual depletions was able to explain the decreased virus titers in B6-*lpr* mice, the cellular immune response induced at early time points after high dose VAC-WR I.P. infection was examined, as this might give an indication to effector mechanisms that might be involved in the increased protection of B6-*lpr* from high dose I.P. VAC-WR infections.

D. Increased Numbers of Splenocytes in B6-*lpr* Mice After High Dose VAC-WR Infection, With More IFN γ ⁺ Leukocytes, But Not An Inherent Protection of B6-*lpr* Leukocytes From VAC-WR Induced Depletion

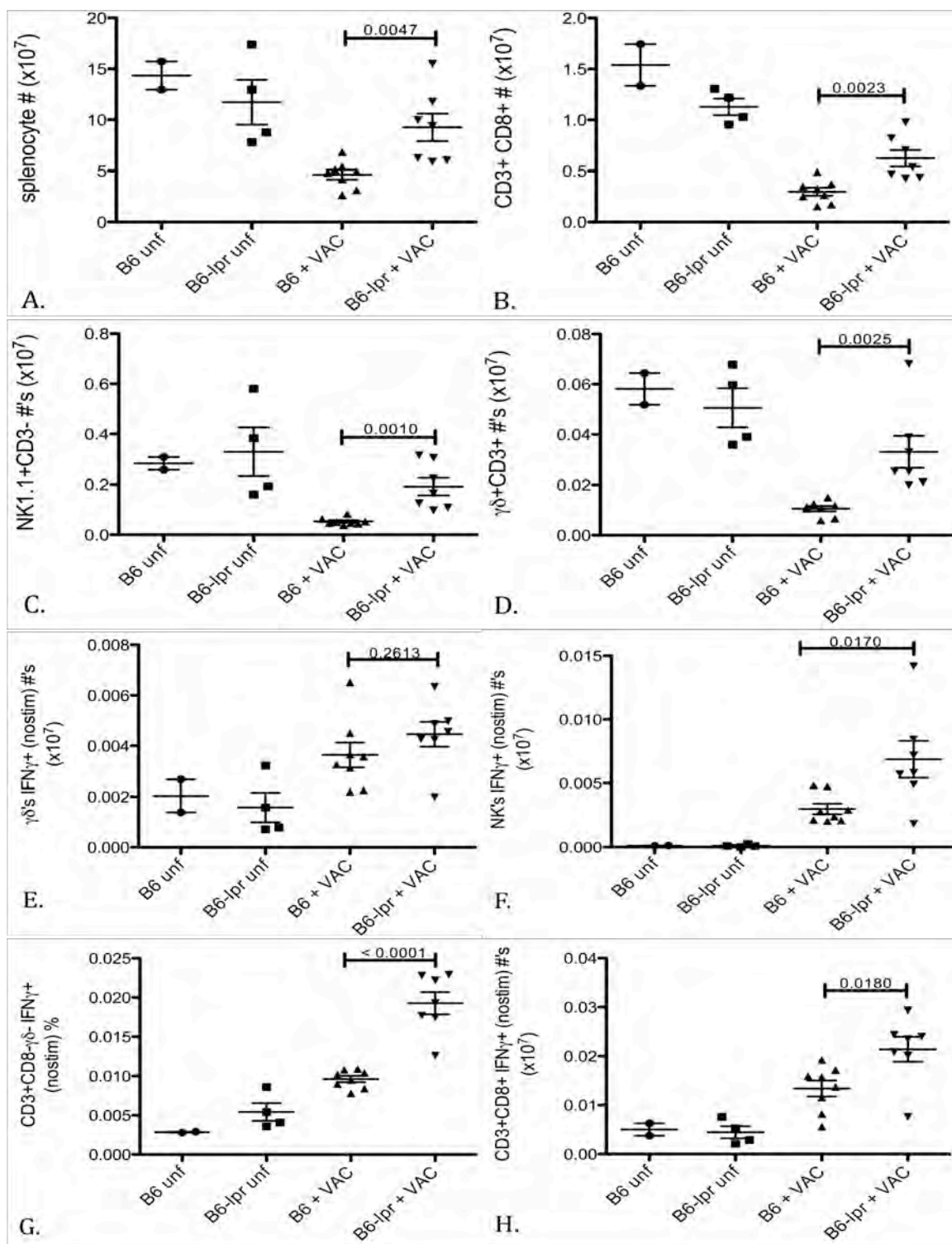


Figure 5.11. Increased number of splenocytes, IFN γ producing NK, CD4 $^+$, and CD8 $^+$ T cells in B6-*lpr* mice after lethal VAC-WR I.P. infection on day 2.

Figure 5.11. Increased number of splenocytes, IFN γ producing NK, CD4 $^+$, and CD8 $^+$ T cells in B6-lpr mice after lethal VAC-WR I.P. infection on day 2.

B6 or B6-lpr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 2 after infection, splenocytes were cultured for 4.5H in the presence of GolgiPlug. This was followed by an intracellular IFN γ assay with fluorescent antibodies, and FACS analysis.. The average and standard errors of the mean are shown. (A) Splenocyte numbers (B) CD3 $^+$ CD8 $^+$ numbers ($\times 10^7$) (C) NK1.1 $^+$ CD3 $^-$ numbers ($\times 10^7$) (D) CD3 $^+$ $\gamma\delta^+$ numbers ($\times 10^7$) (E) and IFN γ^+ CD3 $^+$ $\gamma\delta^+$ numbers (F) IFN γ^+ NK1.1 $^+$ CD3 $^-$ numbers (G) IFN γ^+ CD3 $^+$ CD8 $^-$ $\gamma\delta^-$ numbers (G) and IFN γ^+ CD3 $^+$ CD8 $^+$ numbers. P values were determined by Student's t-test.

After 4×10^6 VAC-WR I.P. infection, B6-*lpr* mice had increased numbers of splenocytes (Figure 5.11A), increased numbers of CD8⁺ T cells (Figure 5.11B), increased number of NK cells (Figure 5.11C), and increased number of $\gamma\delta^+$ T cells (Figure 5.11D) when compared to B6 wild type mice. B6-*lpr* mice also had increased numbers of IFN γ^+ NK cells (Figure 5.11F), increased numbers of IFN γ^+ CD3⁺CD8⁻ $\gamma\delta^-$ cells (Figure 5.11G), and increased numbers IFN γ^+ CD8⁺ T cells (Figure 5.11H) when compared to B6 wild type mice. The increase in CD8⁺ T cells was due to the increase in splenocyte numbers (CD8 % in B6 6.34 +/- 0.36, in B6-*lpr* 6.93 +/- 0.46), while increases in NK cells and $\gamma\delta^+$ T cells was due to the increased splenocyte number and the increased percentage of these subsets within the splenocyte population (B6 NK% in B6 1.33 +/- 0.23, in B6-*lpr* 2.03 +/- 0.15, $\gamma\delta^+$ T % in B6 0.23 +/- 0.01 in B6-*lpr* 0.35 +/- 0.02). The increased spleen numbers after high dose VAC-WR infection of B6-*lpr* mice when compared to B6 wild type mice suggested that there might be an inherent defect in splenocyte death in B6-*lpr* mice, possibly through a decreased AICD dependent mechanism, and this protection from death might allow for increased protection from high dose virus infections. To examine this hypothesis, adoptive transfer experiments at day 2 time points were examined.



Figure 5.12. Adoptively transferred B6-*lpr* CD8⁺ T cells do not have increased proliferation or survival in comparison with wild type CD8⁺ T cells at day 2 after lethal I.P. VAC-WR infection.

Figure 5.12. Adoptively transferred B6-lpr CD8⁺ T cells do not have increased proliferation or survival in comparison with wild type CD8⁺ T cells at day 2 after lethal I.P. VAC-WR infection.

4.5x10⁷ B6-lpr (Ly5.2⁺Thy1.2⁺) splenocytes were labeled with DDAO-SE and 4.5x10⁷ IgH^β wild type (Ly5.2⁺Thy1.1⁺ donor) splenocytes were CFSE labeled and mixed 1:1 and adoptively transferred into B6.SJI (Ly5.1⁺Thy1.2⁺) mice, and one day later, some mice were infected with 4x10⁶ VAC-WR I.P. On day 2 after infection, splenocytes were stained with the appropriate fluorescent antibodies, and FACS analysis was performed. Donor % of two uninfected mice is on the far left in all rows, infected with 4x10⁶ VAC-WR I.P. in three mice on the right in all rows. (A) Total donor % in splenocytes (B). $\gamma\delta^+CD4^-CD8^-$ donor % (C), $NK^+CD4^-CD8^-$ donor % (D) $CD4^+$ and $CD8^+$ cells in CFSE⁺ and DDAO⁺ splenocytes.

Similar percentages of donor DDAO⁺ B6-*Ipr* (Ly5.2⁺Thy1.2⁺) and CFSE⁺ IgH^a wild type (Ly5.2⁺Thy1.1⁺ donor) splenocytes cells were detected in uninfected mice, confirming the 1:1 mix of donor splenocytes (Figure 5.12A). After infection with 4x10⁶ VAC-WR I.P., similar percentages of DDAO⁺ B6-*Ipr* (Ly5.2⁺Thy1.2⁺) and CFSE⁺ IgH^a wild type (Ly5.2⁺Thy1.1⁺ donor) splenocytes cells were detected in host mice (Figure 5.12A). In further analysis $\gamma\delta^+$ CD4⁻CD8⁻ cells (Figure 5.12B), NK⁺CD4⁻CD8⁻ cells (Figure 5.12C), CD4⁺ cells and CD8⁺ cells (Figure 5.12D) survived 4x10⁶ VAC-WR I.P. infection similarly if adoptively transferred from B6 or B6-*Ipr* mice. This suggests that Fas dependent AICD mechanisms are most likely not the reason for increased splenocyte number after infection in B6-*Ipr* mice, and that no single lymphocyte subset from the B6-*Ipr* is protected from high dose I.P. VAC-WR challenge.

Lymphocyte depletion experiments in multiple combinations were also performed on day 2, but it was found that no depletion combination consistently resulted in reducing protection of B6-*Ipr* mice in comparison with B6 wild type mice (data not shown). While depletion experiments were not able to identify a single lymphocyte subset or combination of subsets that resulted in the increased early protection of B6-*Ipr* mice from high dose VAC-WR infection, it did provide us with a large number of B6 wild type and B6-*Ipr* mice infected with two stocks of VAC-WR that replicated to the same extent *in vivo*. This cohort of mice allowed for a closer examination of early correlates of protection early in high dose VAC-WR I.P. infection. In all experiments B6 wild type and B6-*Ipr* mice

were age and weight matched, but to determine whether a difference in weight would make a difference in virus titers, virus loads in the fat pad, livers, and spleens were plotted against the initial weights of the mice.

E. Correlates of Reduced Virus Load In B6 wild type and B6-*lpr* mice

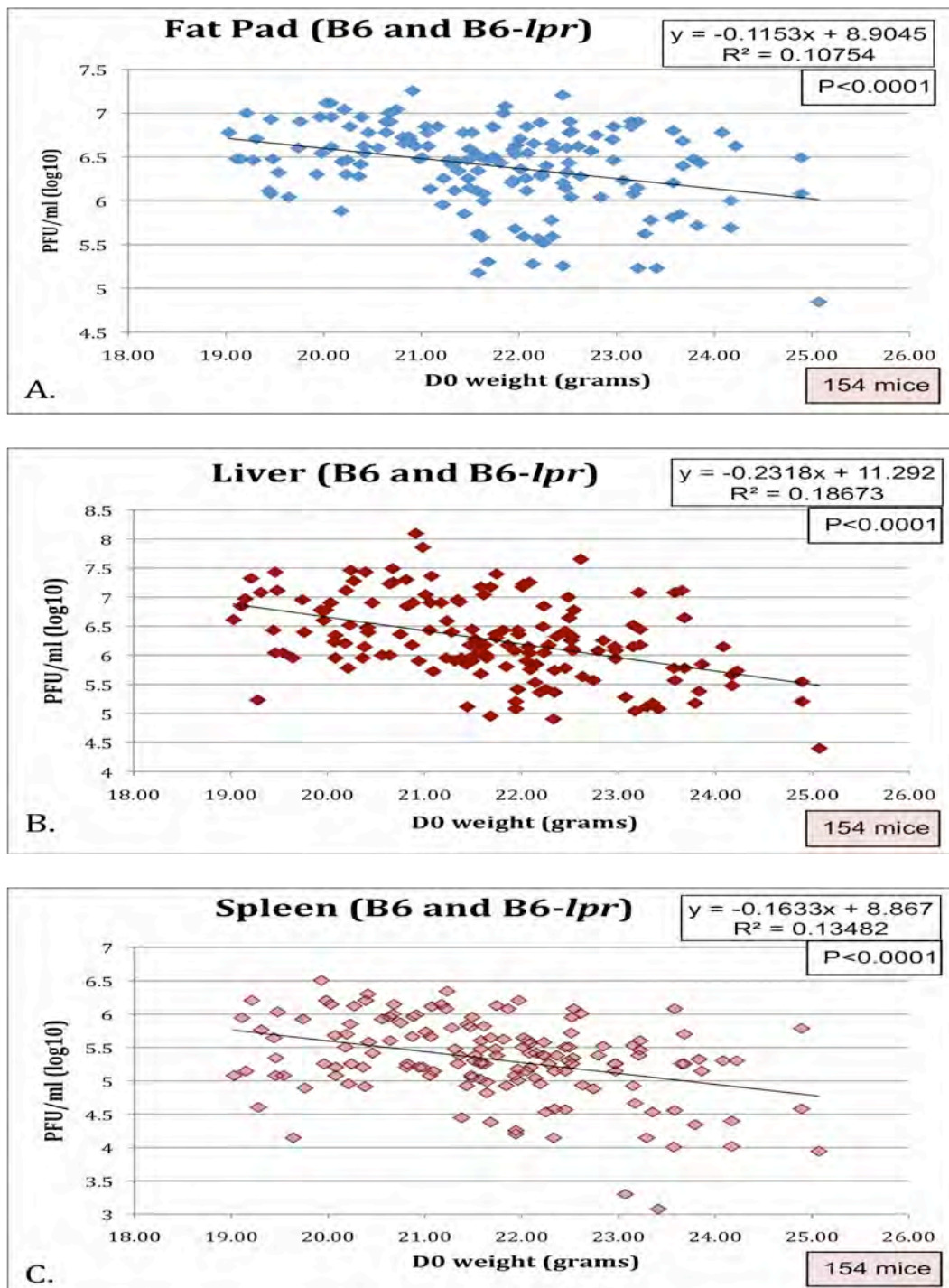


Figure 5.13. Initial mouse weight negatively correlates with virus titers in B6 and B6-*lpr* mice.

Figure 5.13. Initial mouse weight negatively correlates with virus titers in

B6 and B6-Ipr mice.

B6 or B6-Ipr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation.

Weights at time of infection are plotted against D2 viral titers in the (A) fat pad,

(B) liver, and (C) spleen.

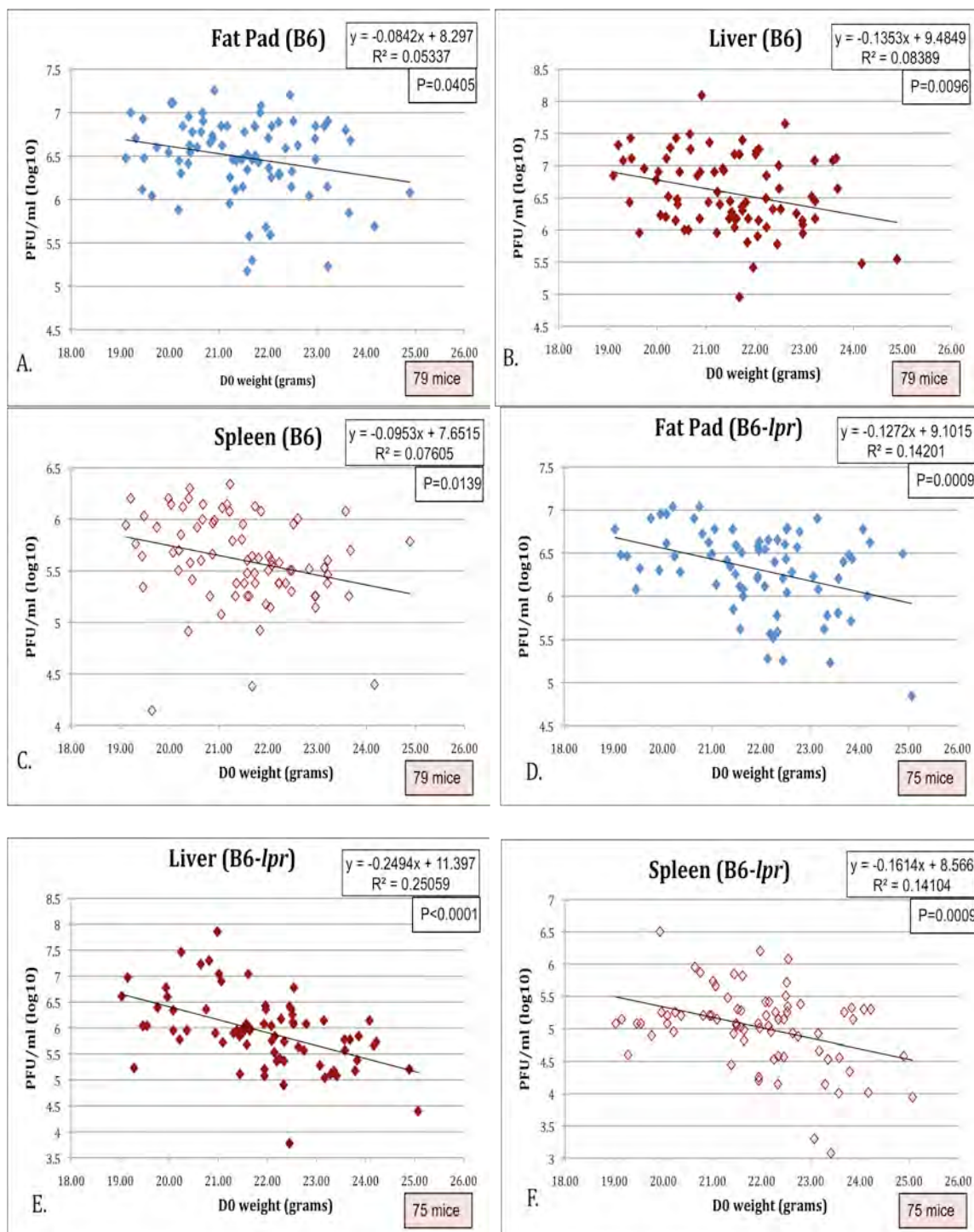


Figure 5.14. Initial mouse weight negatively correlates with virus titers in B6 or B6-*lpr* mice.

Figure 5.14. Initial mouse weight negatively correlates with virus titers in B6 or B6-lpr mice.

B6 or B6-lpr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation.

Weights at time of infection are plotted against D2 viral titers in the (A) B6 fat pad, (B) B6 liver, (C) B6 spleen, (D) B6-lpr fat pad, (E) B6-lpr liver, or (F) B6-lpr spleen.

It was found that initial weight negatively correlated with viral titers in B6 wild type combined with B6-*lpr* mice (Figure 5.13). The more the mice weighed the lower the amount of virus in all three organs. The initial weight vs. day 2 virus titers in the individual sets of B6 wild type or B6-*lpr* mice was next plotted.

Initial weight again negatively correlated with viral titers in B6 wild type or B6-*lpr* mice (Figure 5.14). The more mice weighed the lower the amount of virus in all three organs. It was found that this correlation was typically strongest in the liver (Figure 5.13B), in the B6 wild type (Figure 5.13B) or B6-*lpr* mice (Figure 5.13D). Each experiment was carefully age and weight matched, and when all the initial weights of the B6 or B6-*lpr* were compared, there was no significant difference in the weights of the B6 or B6-*lpr* groups ($P=0.12$). Although, this data suggested the B6-*lpr* mice were not better protected because of increased weight, initial weight was divided by virus titers and these results are shown to confirm that increased weight was not the reason for decreased titers in B6-*lpr* mice.

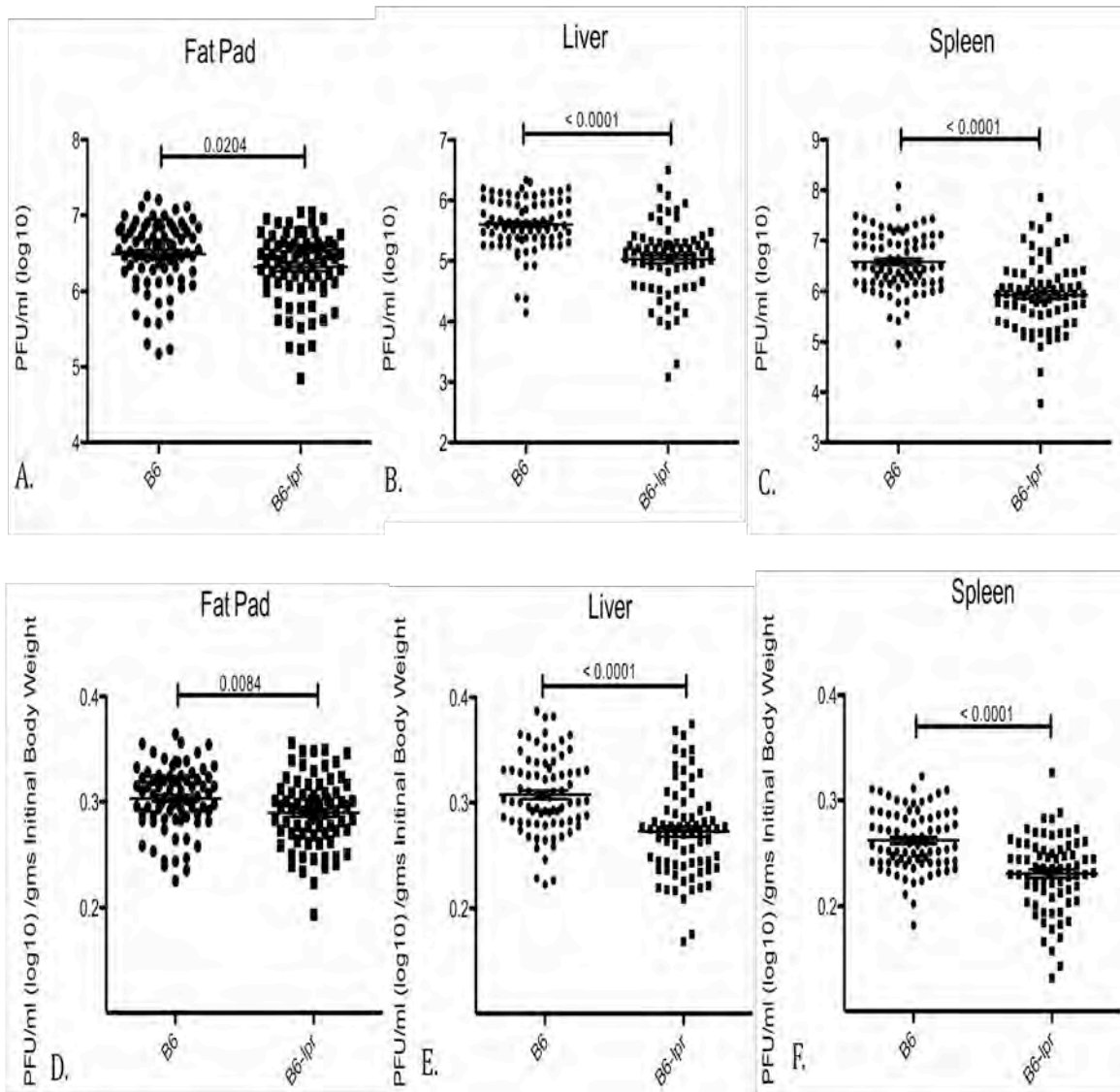
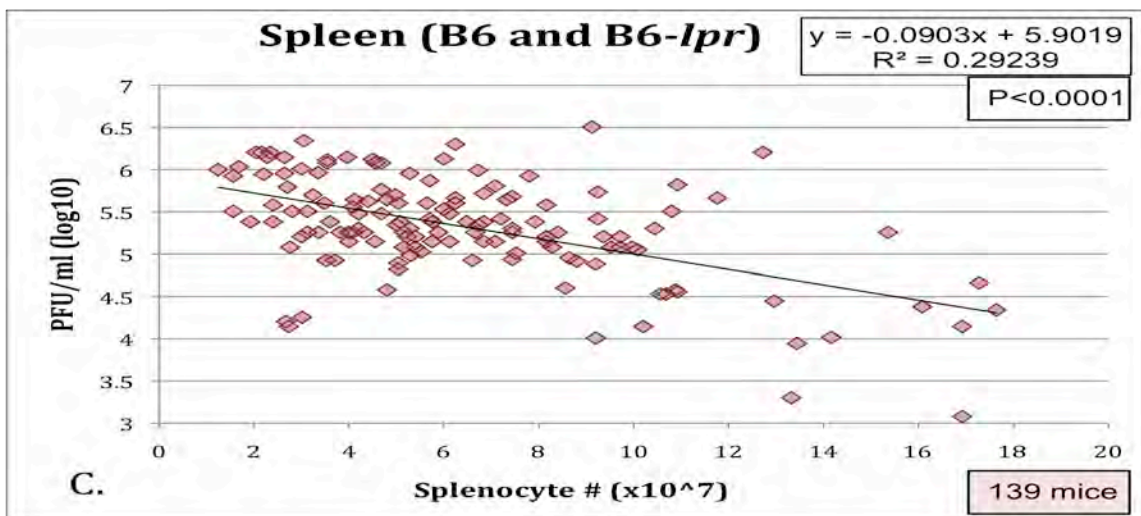
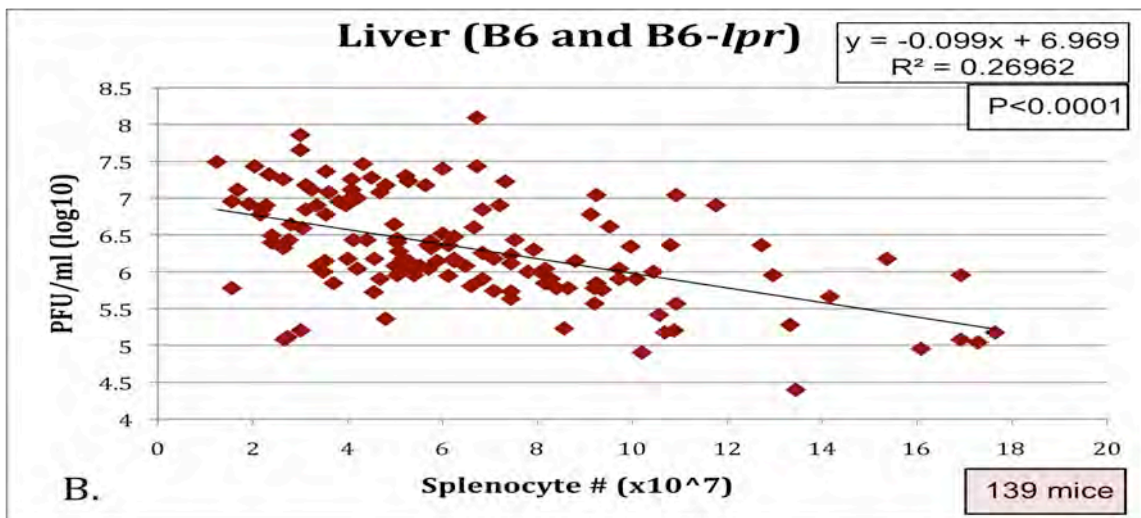
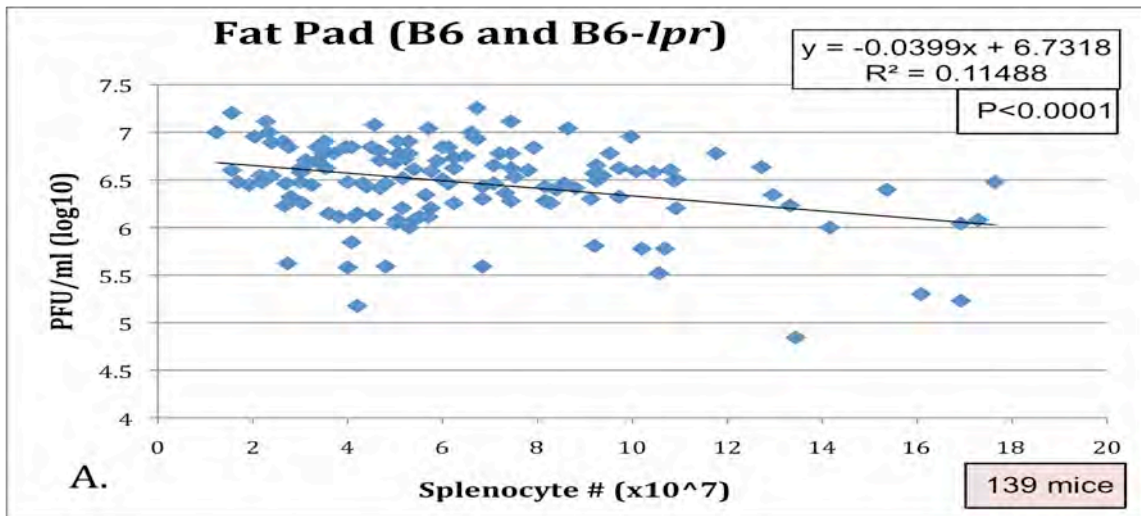


Figure 5.15. B6-*lpr* mice have decreased virus loads on day 2, even if initial weight is taken into consideration.

Figure 5.15. B6-Ipr mice have decreased virus loads on day 2, even if initial weight is taken into consideration. B6 or B6-Ipr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 2 after infection, viral titers in the (A) fat pads, (B) livers, and (C) spleens were determined by plaque assay. In (D) (E) and (F), the viral titers are first divided by the initial weights, and the result is then plotted. All P values were determined by Student's t-test. 13 grouped experiments.

When including all mice, B6-*Ipr* mice had less virus in the fat pads (Figure 5.15A), livers (Figure 5.15B), and spleens (Figure 5.15C) after 4×10^6 PFU's of VAC-WR I.P. when compared to B6 wild type mice. Even after accounting for the discrepancy that initial weight might cause in viral titers by dividing the day 2 titers by the initial weight, B6-*Ipr* mice still had less virus in the fat pads (Figure 5.15D), livers (Figure 5.15E), and spleens (Figure 5.15F) per gram of initial weight when compared to the B6 wild type mice.

Although splenocytes from B6-*Ipr* mice were not inherently more resistant to lymphocyte depletion by high dose VAC-WR I.P. infection (Figure 5.12) and no single cell depletion or combination of depletions consistently resulted in loss of protection in B6-*Ipr* mice, it still seemed likely that the increased splenocyte number in B6-*Ipr* (Figure 5.11) mice might negatively correlate with virus loads, and so this correlation was next examined.



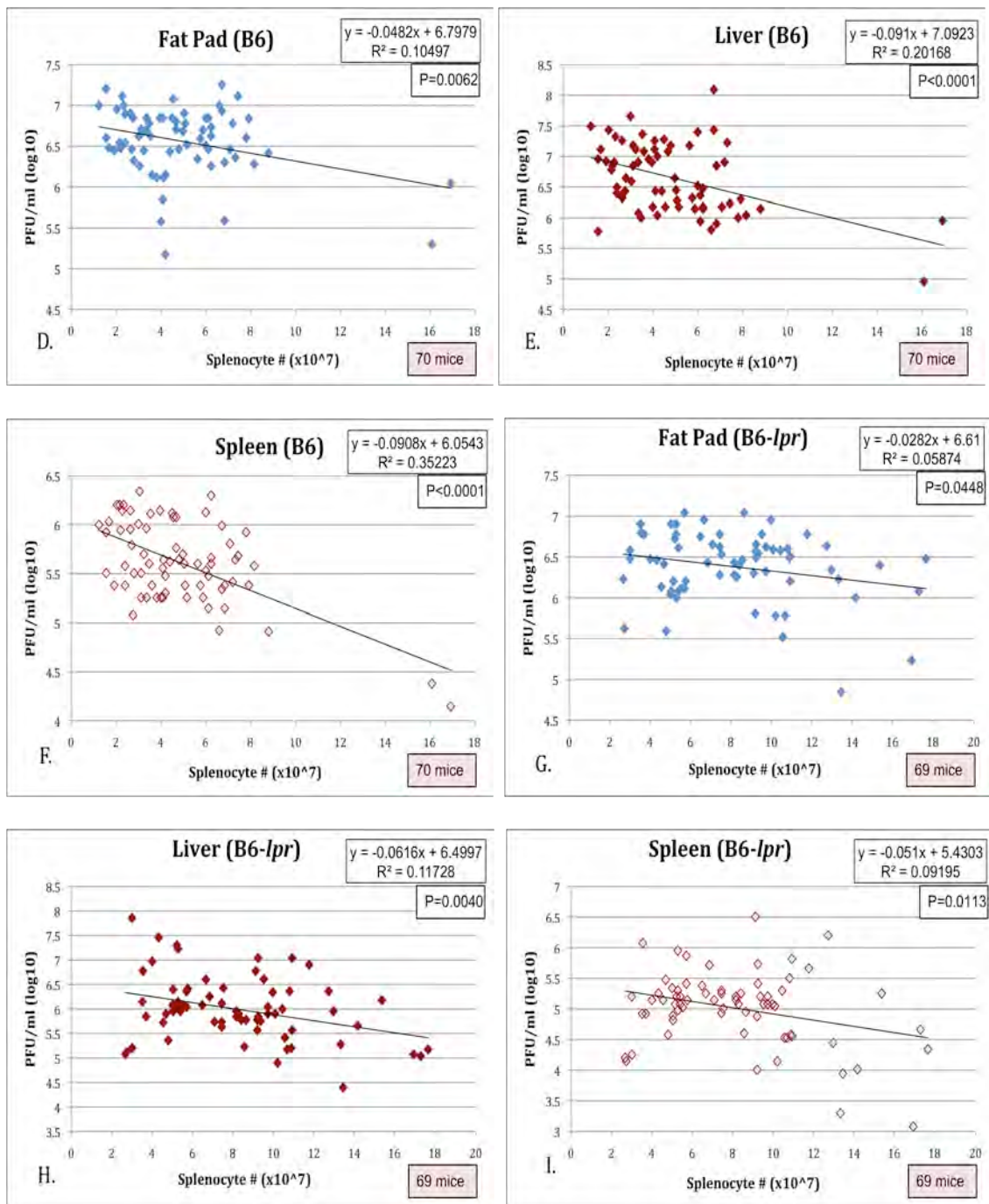


Figure 5.16. Day 2 splenocyte number negatively correlates with virus titers in B6 and/or B6-*lpr* mice.

Figure 5.16. Day 2 splenocyte number negatively correlates with virus titers in B6 and/or B6-lpr mice.

B6 or B6-lpr mice were infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. D2 splenocyte counts are plotted against D2 viral titers in the (A) B6 and B6-lpr fat pad, (B) B6 and B6-lpr liver, (C) B6 and B6-lpr spleen, (D) B6 fat pad, (E) B6 liver, (F) B6 spleen, (G) B6-lpr fat pad, (H) B6-lpr liver, and (I) B6-lpr spleen.

Day 2 splenocyte number negatively correlated with fat pad (Figure 5.16 A,D,G), liver (Figure 5.16 B,E,H), and spleen (Figure 5.16 C,F,I) viral titers in B6 wild type, B6-*lpr* mice, or the combination of the two groups of mice. Although this negative correlation was expected in the combination group as B6-*lpr* had decreased virus loads and increased splenocyte numbers when compared to B6 mice, the observation that the negative correlation was apparent in the individual groups suggested that the negative correlation was not just due to two individual overlapping observations.

In further correlation analyses such as this, but instead focusing on lymphocyte subsets, it was found that $\gamma\delta^+$ T cell percentage significantly negatively correlated with VAC titers in the fat pad ($P=0.0560$, $R^2=0.02913$), liver ($P<0.0001$, $R^2=0.12827$) and spleen ($P<0.0001$, $R^2=0.20836$) in the combination of B6 wild type and B6-*lpr* group, and in the fat pad ($P=0.0520$, $R^2=0.0615$), spleen ($P=0.0096$, $R^2=0.10649$), but not the liver ($P=0.1311$, $R^2=0.03758$) of B6 wild type group, but not in the B6-*lpr* group. It was also found that CD8⁺ T cell percentage significantly negatively correlated with VAC titers in the fat pad ($P<0.0001$, $R^2=0.33935$), liver ($P<0.0001$, $R^2=0.26592$), and spleen ($P<0.0001$, $R^2=0.26027$) in the combination of B6 wild type and B6-*lpr* group, in the fat pad ($P<0.0001$, $R^2=0.32216$), liver ($P<0.0001$, $R^2=0.2768$), and spleen ($P=0.0006$, $R^2=0.17849$) B6 wild type group, and in the fat pad ($P<0.0001$, $R^2=0.3078$) and spleen ($P=0.00136$, $R^2=0.09419$) of B6-*lpr* mice, and suggestive ($P=0.0756$, $R^2=0.05003$) in the liver. This might suggest that $\gamma\delta^+$ T cells and CD8⁺ T cells are

protective at a day 2 time point in response to high dose I.P. VAC-WR infection, but none of the depletion studies gave a clear answer as to lymphocyte subsets involved in protection. Although NK depletion studies in B6-*lpr* mice did not affect virus titers (Figure 5.8), it was possible that NK cells might still play some role in VAC-WR virus control as has previously been suggested with poxviruses (Bukowski, Woda et al. 1983; Parker, Parker et al. 2007; Martinez, Huang et al. 2008). To more closely examine if NK cells played any role in VAC-WR virus control in high dose I.P. VAC-WR infection, we next performed experiments in B6 recombinase activating gene 1 (RAG) knockout (B6-*rag1*^{-/-}) and B6-*lpr* recombinase-activating gene 1 knockout (B6-*rag1*^{-/-}*lpr*) mice.

F. B6-*rag1*^{-/-}*lpr* Uninfected Mice Have More Splenocytes and NK Cells When Compared to B6-*rag1*^{-/-} Mice, and Depletion of NK Cells Increases Virus Loads In Both Groups of Mice

Experiments in uninfected B6 and B6-*lpr* mice had already demonstrated that B6 mice with the *lpr* mutation had an altered immune makeup with increased NK cell numbers when compared to mice without the mutation (Figure 5.6). The *lpr* mutation might also result in an altered immune makeup in B6-*rag1*^{-/-} mice as well, and to examine this possibility, splenocyte number and NK cell numbers were examined in B6-*rag1*^{-/-} and B6-*rag1*^{-/-}*lpr* mice.

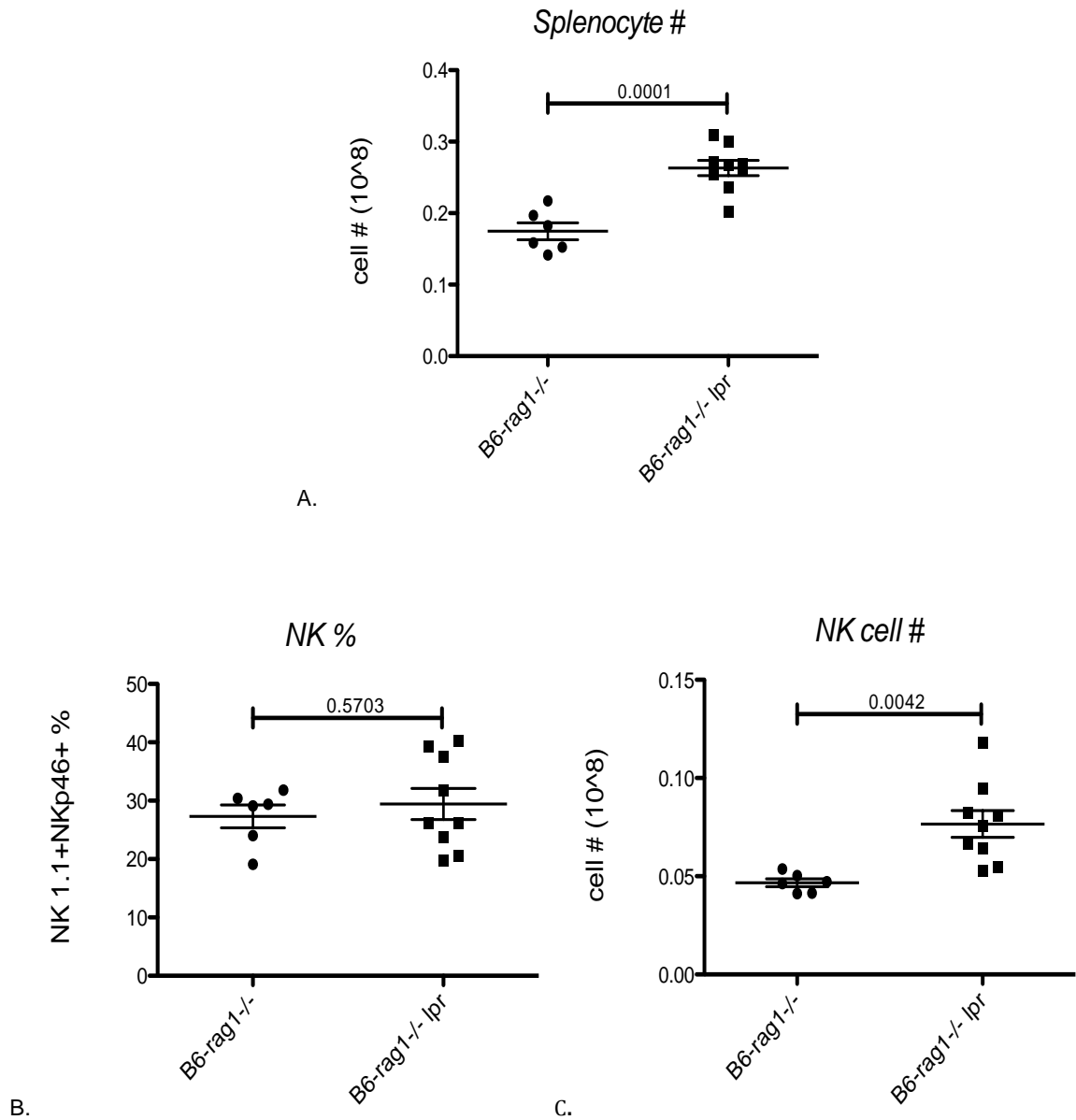


Figure 5.17. Increased splenocyte # and NK cell # in uninfected B6-*rag1*^{-/-lpr} in comparison to B6-*rag1*^{-/-} mice.

Figure 5.17. Increased splenocyte # and NK cell # in uninfected B6-*rag1*^{-/-}-*lpr* in comparison to B6-*rag1*^{-/-} mice.

Six week old uninfected B6-*rag1*^{-/-} and B6-*rag1*^{-/-}-*lpr* mice were sacrificed, and splenocytes were stained with the appropriate fluorescent antibodies followed by FACS analysis. (A) Splenocyte #'s (B) NK1.1⁺CD3⁻%, (C) NK1.1⁺CD3⁻#'s. All P values were determined by Student's t-test.

Six week old uninfected B6-*rag1*^{-/-}*lpr* mice had increased numbers of splenocytes (Figure 5.17A) and NK1.1⁺NKp46⁺ cells in the spleen (Figure 5.17C) in comparison to six week old uninfected B6-*rag1*^{-/-} mice. Although there was an increase in the number of NK cells in the spleen, the NK1.1⁺NKp46⁺ % was the same in B6-*rag1*^{-/-}*lpr* and B6-*rag1*^{-/-} mice (Figure 5.17B).

To examine if the increased NK cell number in B6-*rag1*^{-/-}*lpr* in comparison to B6-*rag1*^{-/-} resulted in decreased virus titers after infection, and whether NK depletion in B6-*rag1*^{-/-}*lpr* and B6-*rag1*^{-/-} mice results in increased virus loads, B6-*rag1*^{-/-}*lpr* and B6-*rag1*^{-/-} were either depleted of NK cells by a mAb against NK or left untreated or treated with an isotype control mAb, and then infected with 4x10⁶ PFU's of VAC-WR I.P. All experiments done had similar trends, so the experimental results were next combined and plotted.

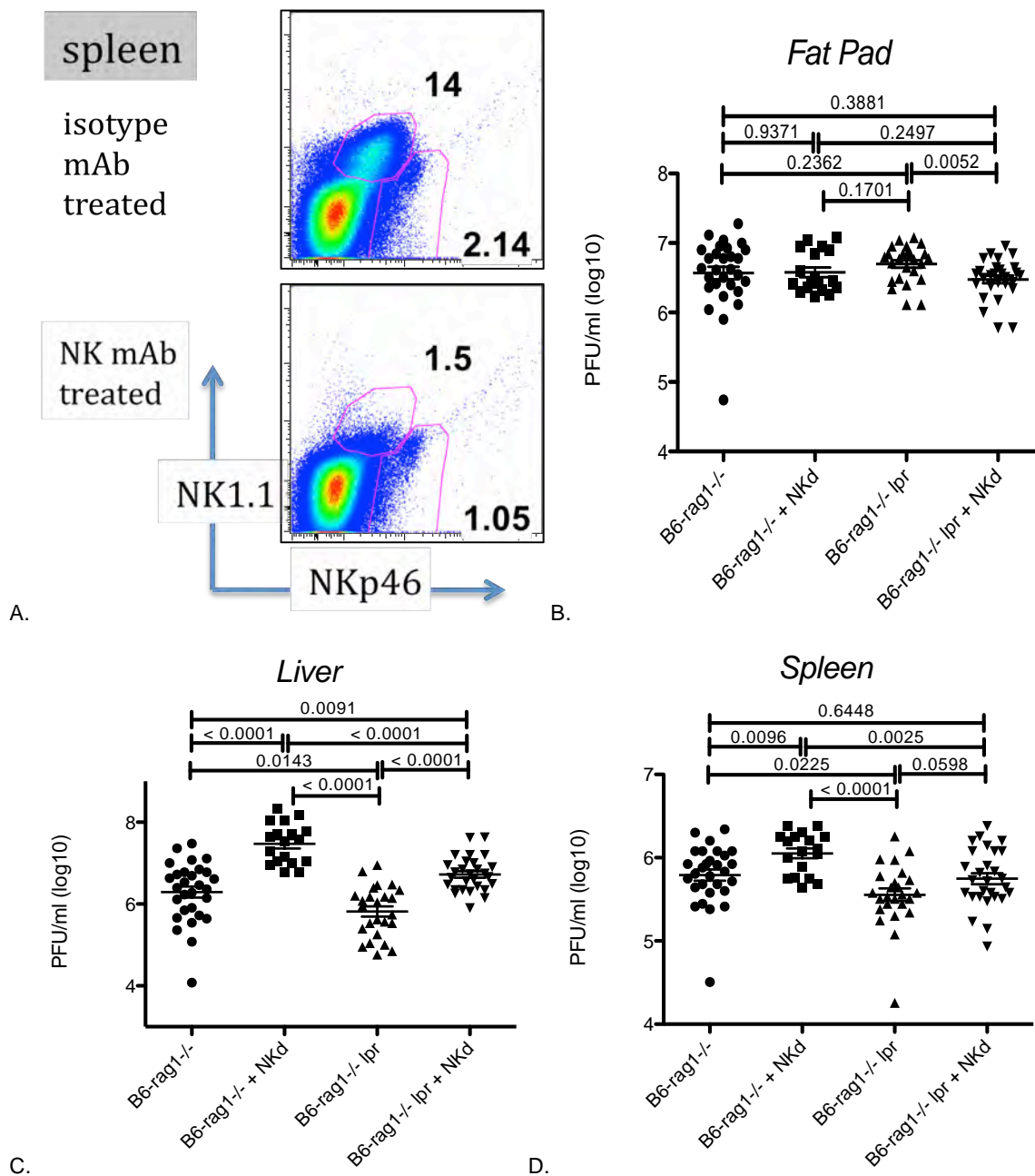


Figure 5.18. B6-*rag1*^{-/-}*lpr* have decreased amounts of virus in the livers and spleens when compared to B6-*rag1*^{-/-}, and treatment with anti-NK antibody results in increased virus titers in B6-*rag1*^{-/-}*lpr* spleens and B6-*rag1*^{-/-} and B6-*rag1*^{-/-}*lpr* livers.

Figure 5.18. B6-raq1^{-/-}lpr have decreased amounts of virus in the livers and spleens when compared to B6-raq1^{-/-}, and treatment with anti-NK antibody results in increased virus titers in B6-raq1^{-/-}lpr spleens and B6-raq1^{-/-} and B6-raq1^{-/-}lpr livers. B6-raq1^{-/-} or B6-raq1^{-/-}lpr mice were treated with 200µl PBS (or 200µl PBS containing 100µg IgG2A isotype antibody) or 200µl PBS containing 100µg of NK-depleting antibody. One day later the mice were I.P. infected with 4x10⁶ PFU's of VAC-WR. On day 2 splenocytes were stained with the appropriate fluorescent antibodies, and (A) FACS analysis was performed to ensure of the NK depletion and viral titers in the (B) fat pads (C) livers and (D) spleens were determined by plaque assay. All P values were determined by Student's t-test. NKd is NK monoclonal antibody treatment, and iso is isotype control monoclonal antibody treatment.

B6-rag1^{-/-lpr} had about $\frac{1}{2}$ a log less virus in the livers (Figure 5.18C) and $\frac{1}{4}$ a log less virus in the spleens (Figure 5.18D) when compared to *B6-rag1^{-/-}* mice, with a comparable amount of virus in the fat pad (Figure 5.18B) after I.P. infection with 4×10^6 VAC-WR. *B6-rag1^{-/-}* and *B6-rag1^{-/-lpr}* mice treated with a mAb to NK1.1 did not have any detectable NK1.1⁺ cells in the spleen (*B6-rag1^{-/-lpr}* shown in Figure 5.18A), confirming the NK depletion. Treatment with the NK depleting antibody resulted in an approximate 1 log increase in liver virus titers in *B6-rag1^{-/-}* and *B6-rag1^{-/-lpr}* mice after 4×10^6 VAC-WR I.P. infection (Figure 5.18C). NK depleting antibody also increased spleen virus titers significantly in *B6-rag1^{-/-}* and suggestively in *B6-rag1^{-/-lpr}* mice by approximately $\frac{1}{4}$ of a log after infection (Figure 5.18D), with small but significant decreases in fat pad virus titers in *B6-rag1^{-/-lpr}* after NK depletion, and no difference in fat pad titers noticed in any other comparisons (Figure 5.18B). These results were obtained by the combination of 6 different experiments, so while it seems that NK cells play a protective role after this high dose I.P VAC-WR challenge, it seems likely that the antiviral role might be limited.

It has been demonstrated that blocking IFN γ increases VAC titers and decreased survival after infection, and that mice that are deficient in either the IFN γ gene or IFN γ R gene have higher titers of VAC and decreased survival after infection in comparison to wild type controls ((Ruby and Ramshaw 1991; Huang, Hendriks et al. 1993; Karupiah, Fredrickson et al. 1993; Cantin, Tanamachi et al. 1999). In light of these studies demonstrating the importance of IFN γ in control of

poxvirus spread and survival of poxvirus infections, and the observation that there were increased numbers of IFN γ ⁺ NK cells, IFN γ ⁺ CD8⁺ T cells, and IFN γ ⁺ CD4⁺ T cells in B6-*Ipr* mice when compared to B6 mice, B6 and B6-*Ipr* mice were next treated with a mAb against IFN γ , and infected with high dose VAC-WR I.P. In three individual experiments, a trend was demonstrated that antibody against IFN γ increased virus titers in B6 mice, and more so in B6-*Ipr* mice. The experimental results were next combined, and plotted.

G. B6 or B6-*Ipr* Mice Treated With an Antibody Against IFN γ Have Increased Viral Loads in Livers and Spleens, with Virus Titers no Longer Being Decreased In B6-*Ipr* Mice In Comparison To B6 Mice

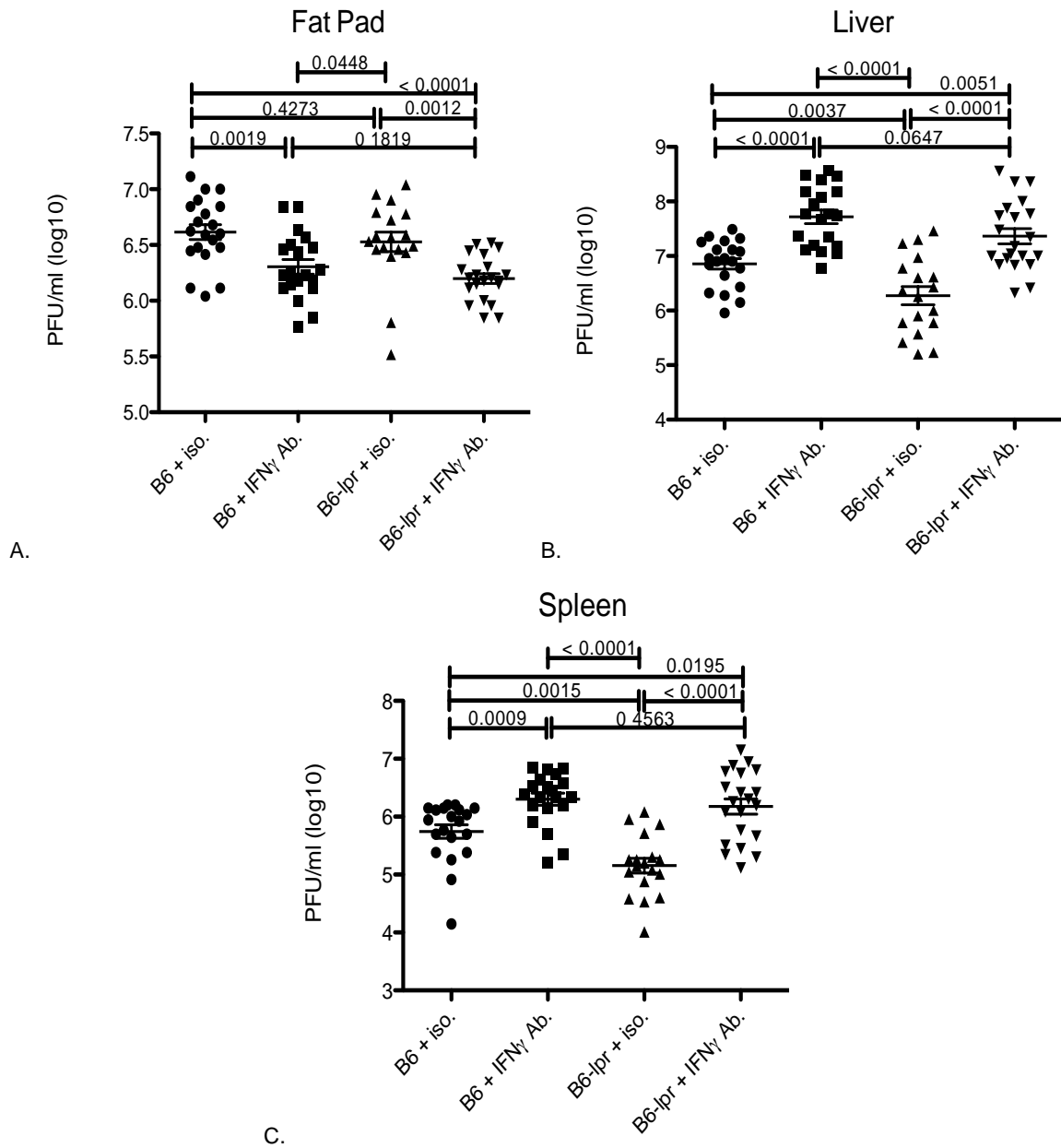


Figure 5.19. IFN γ antibody treatment increases virus titers in livers and spleens of B6 and B6-lpr mice, and decreases virus titer in fat pad, with treatment making virus titers similar in both groups.

Figure 5.19. IFN γ antibody treatment increases virus titers in livers and spleens of B6 and B6-lpr mice, and decreases virus titer in fat pad, with treatment making virus titers similar in both groups.

B6 or B6-lpr mice were treated with 200 μ l PBS containing 350 μ g of the isotype antibody or 200 μ l PBS containing 350 μ g of an IFN γ blocking antibody two days before infection, and on the day of infection. Both groups of mice were then infected with 4×10^6 PFU's of VAC-WR by I.P. inoculation. On day 2 viral titers (A) in the fat pads (B) livers (C) and spleens were determined by plaque assay. All P values were determined by Student's t-test. 3 grouped experiments.

B6 or B6-*lpr* mice treated with an IFN γ blocking antibody had significantly increased virus in the livers and spleen when compared to their genetic counterparts treated with an isotype control antibody (Figure 5.19B and 5.19C). Interestingly, in the fat pad, treatment with an IFN γ blocking antibody significantly decreased virus titers in the B6 or B6-*lpr* mice (Figure 5.19A), possibly because of decreased infiltration of VAC-infected leukocytes into the fat. Another interesting observation was that B6 mice treated with an antibody against IFN γ had similar virus amounts in comparison to B6-*lpr* mice treated with an antibody against IFN γ in the spleen (Figure 5.19C), with lower virus titers in the livers of B6-*lpr* mice treated with an antibody against IFN γ that were close to, but not significantly different (Figure 5.19B, P=0.065). This would suggest that IFN γ plays an important role in protection against VAC infection in the spleen and liver, as has been previously demonstrated, and may play an increased role in B6-*lpr* mice in comparison to B6 wild type mice.

H. Chapter Conclusion

These experiments demonstrate an altered immune makeup in B6-*lpr* before the onset of a full lymphoproliferative disorder that might lead to increased protection from high dose virus infections. These results do not suggest that decreased AICD during the course of infection of B6-*lpr* mice leads to increased protection, but the increased number of NK and $\gamma\delta^+$ T cells, already present and perhaps due to reduced apoptosis, and the increased numbers of IFN γ -producing

cells of multiple subsets might lead to an immuno-protective phenotype from high dose viral infections.

CHAPTER VI: HIGH FREQUENCIES OF VIRUS SPECIFIC CD8⁺ T CELL PRECURSORS

An *in vivo* LDA was designed to estimate the CD8⁺ T cell precursor frequencies to entire viruses. This was achieved by adoptive transfer of donor splenocytes into host mice that differed by two congenic markers (Ly5, Thy1) to decrease the fluorescent background when staining for donor CD8⁺ T cell populations, and by increasing the detection limit of resultant T cell progeny by counting only CD8⁺ events by FACS. By ignoring non-CD8⁺ events, we increase the total number of CD8⁺ events we are able to collect, and we are able to collect just over 4×10^6 CD8⁺ events, about 1/5 of the total number of CD8⁺ T cells from an uninfected animal (assuming 2×10^7 CD8 T cells per mouse) (Ganusov and De Boer 2007), and because of T cell proliferation, approximately 5% of all CD3⁺CD8⁺ events in an LCMV or VAC-infected mouse. This allows reliable detection of donor CD8⁺ T cell progeny at limiting dilutions.

A. The Phenotype of Adoptively Transferred Donor CD8⁺ T Cells Is Naïve And Shows Linear “Take” After Transfer

To set up the *in vivo* LDA, it was first verified that the adoptive transfer of donor cells into host mice did not alter the phenotype of donor cells and that adoptive transfer of decreasing numbers of T cells resulted in a linear decrease of donor T cells in host mice.

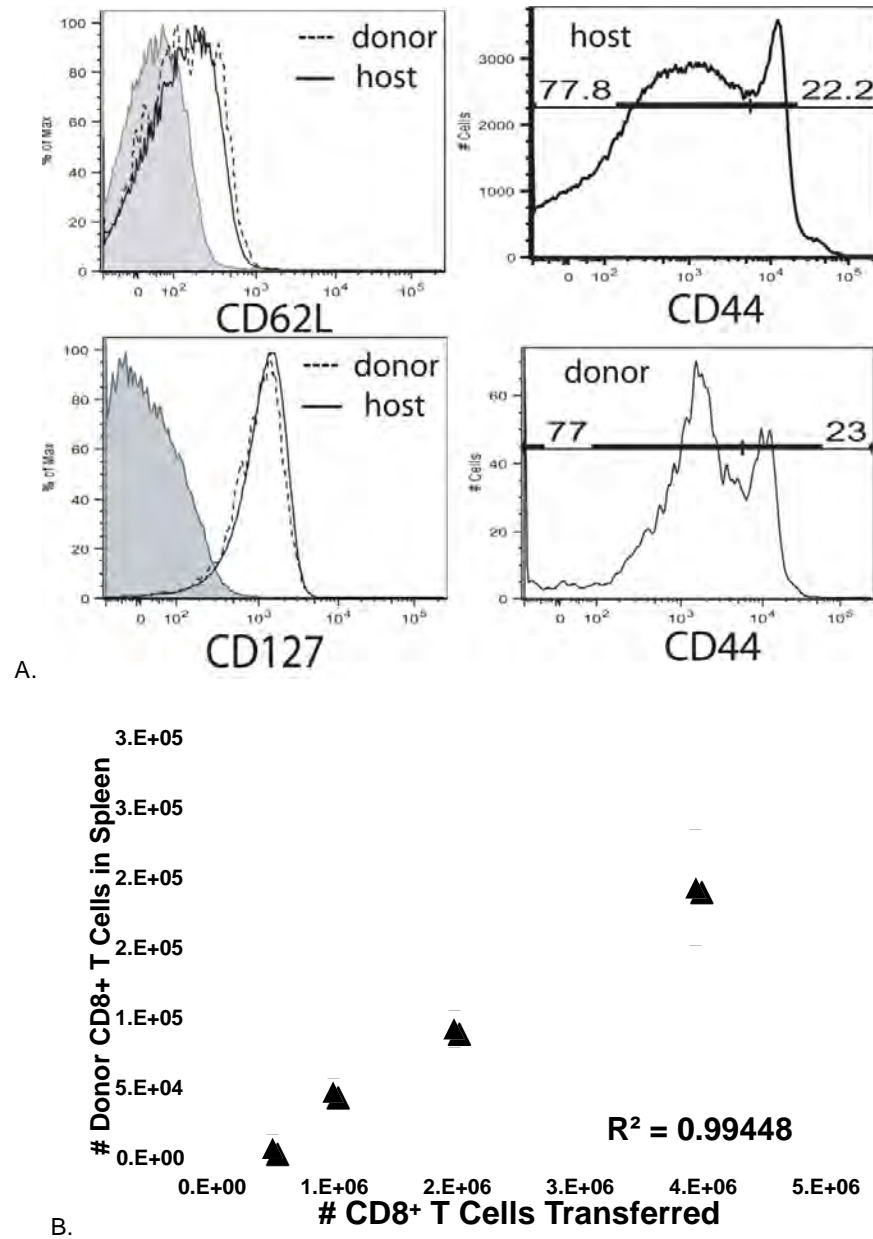


Figure 6.1. Adoptively transferred donor CD8⁺ T cells dilute linearly in host mice and have a phenotype that is similar to host CD8⁺ T cells.

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IgH^a (Ly5.2⁺Thy1.1⁺ donor) splenocytes were labeled with CFSE, three two-fold dilutions were made (mice at highest dilution received 5×10^7 splenocytes), and each dilution was adoptively transferred into four B6.SJL (Ly5.1⁺Thy1.2⁺ host) mice. On day 5, the mice were sacrificed and FACS analysis performed on splenocytes. (A) Immuno-phenotyping (CD62L, CD127, and CD44) of donor and host CD8⁺ T cells (B). Graph plots the number of donor CD8⁺ T cells transferred into mice against the number of donor CD8⁺ T cells detected in the spleens of host mice. Mean +/- standard deviation is shown, 4 mice per dilution.

Donor CD8⁺ T cells had phenotypes that remained largely naïve, CD127^{hi}, CD62L^{hi}, and largely CD44^{lo}, and similar to the phenotype of host CD3⁺CD8⁺ T cells (Figure 6.1A). As decreasing numbers of T cells were transferred into host mice, the number of donor CD8⁺ T cells detected in the spleen linearly decreased with an R² value of 0.994 (Fig. 6.1B).

B. Adoptively Transferred CD8⁺ T Cells Traffic To Lymphoid Organs and Peripheral Sites at Similar Frequencies

It was next tested whether adoptively transferred T cells would traffic normally throughout the body.

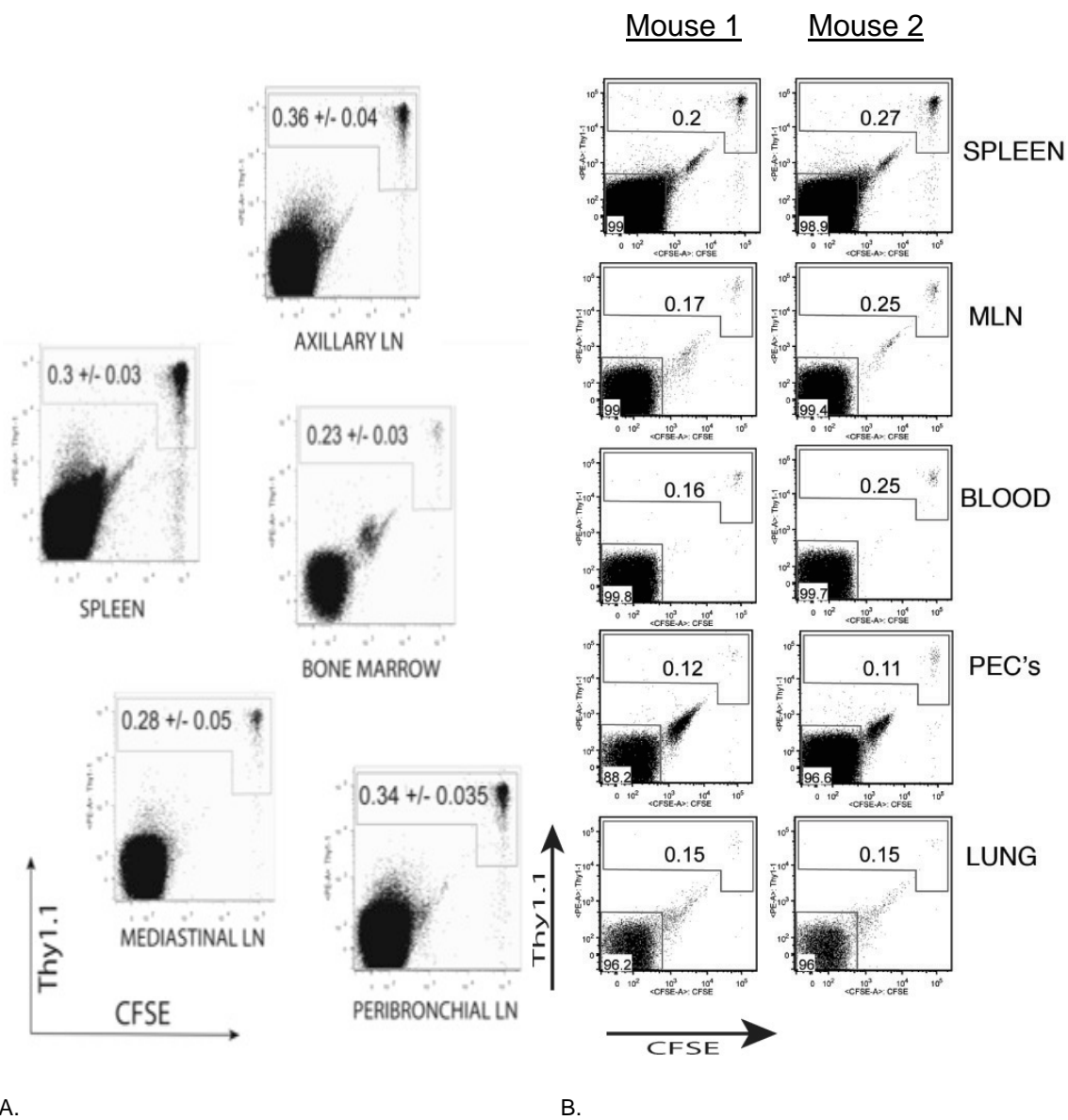


Figure 6.2. Adoptively transferred donor CD8⁺ T cells traffic to similar frequencies to lymph organs and to peripheral sites.

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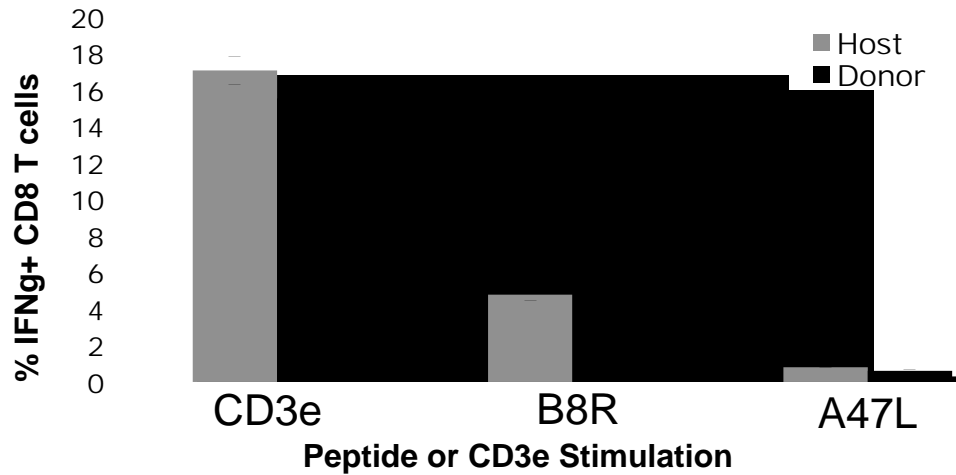
Ly5.2⁺Thy1.1⁺ donor splenocytes were labeled with CFSE and 1.35×10^7 splenocytes were adoptively transferred into Ly5.1⁺Thy1.2⁺ host mice. (A) Five days later, FACS analysis was performed on lymphocytes from the bone marrow, spleen, peribronchial lymph nodes, axillary lymph nodes, and MLN's to examine donor CD8⁺ T cell take in lymph nodes of host mice (B). Ly5.2⁺Thy1.1⁺ donor splenocytes were labeled with CFSE, adoptively transferred into Ly5.1⁺Thy1.2⁺ host mice and FACS analysis was performed on lymphocytes isolated from the spleen, blood, peritoneal cavity, and lungs to examine donor CD8⁺ T cell take in peripheral sites of host mice.

CD8⁺ T cells trafficked at similar frequencies to the MLN, axillary lymph nodes (AXLN), peribronchial lymph nodes (PLN), spleen, and to some extent, the bone marrow (Figure 6.2a). When total numbers of recovered Ly5.2⁺Thy1.1⁺ donor CD8⁺ T cells were counted from these tissues, 65% of the recovered donor CD8⁺ T cells were in the spleen. In a separate similar experiment, peripheral sites were examined. There was a reproducibly detectable number of donor T cells in peripheral sites such as the PEC's and lungs, although the "take" in such peripheral sites was lower when compared to lymphoid sites such as the spleen or MLN (Figure 6.2B).

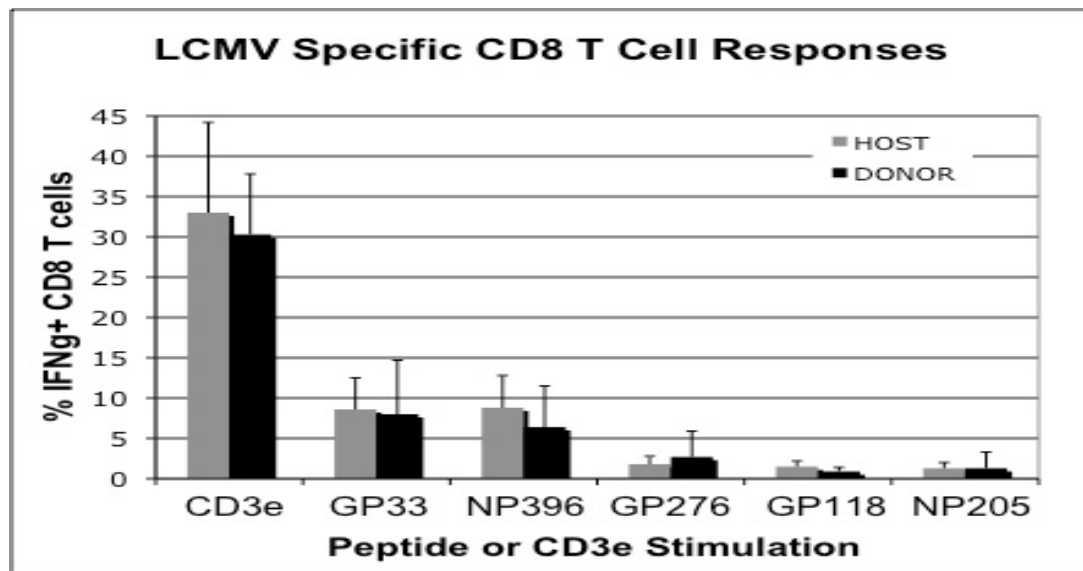
C. Immunodominance Hierarchies of Host and Donor CD8⁺ T cells Are Similar After LCMV or VAC Infection.

To ensure that transferred T cells had immunodominance hierarchies comparable to the ones observed in normal B6 mice, large numbers ($\sim 5 \times 10^7$) of CFSE-labeled Ly5.2⁺Thy1.1⁺ donor splenocytes were transferred into Ly5.1⁺Thy1.2⁺ host mice, and after three days, the mice were infected with LCMV or VAC.

VAC Specific CD8 T Cell Responses



A.



B.

Figure 6.3. Transferred donor and host CD8⁺ T cells have similar immunodominance hierarchies to LCMV and VAC infections.

Figure 6.3. Transferred donor and host CD8⁺ T cells have similar immunodominance hierarchies to LCMV and VAC infections.

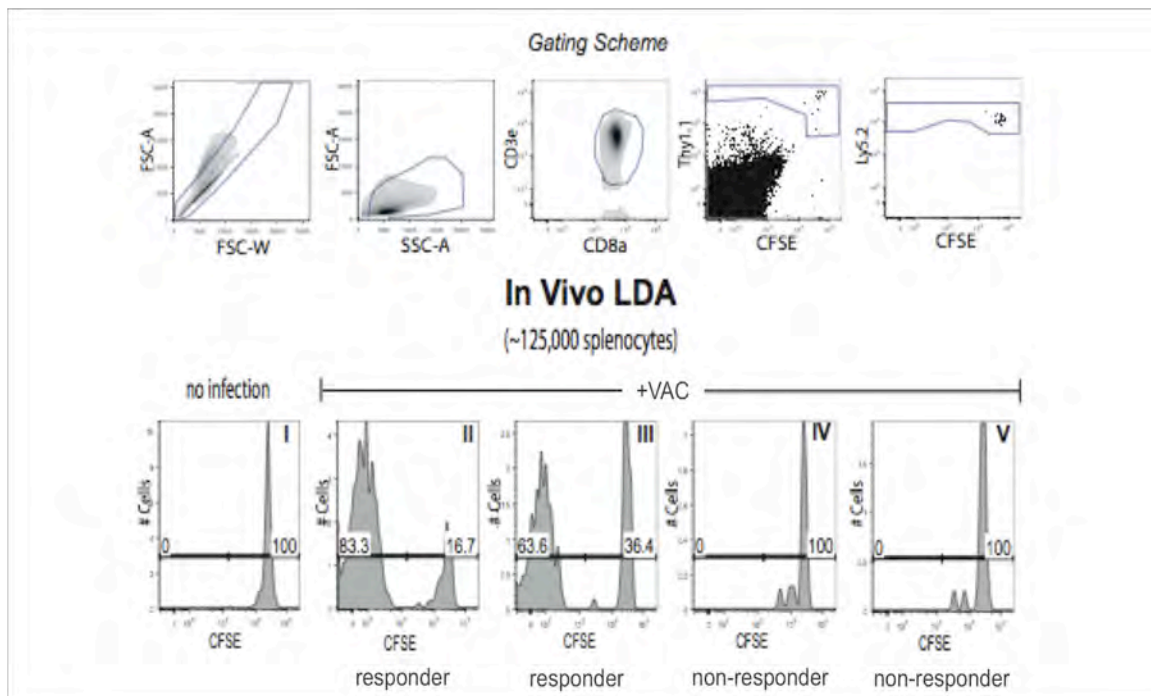
5 x 10⁷ Ly5.2⁺Thy1.1⁺ donor splenocytes were labeled with CFSE and adoptively transferred into Ly5.1⁺Thy1.2⁺ host mice, which were subsequently I.P. infected with LCMV or VAC. Six (VAC) or seven (LCMV) days later, mice were sacrificed, splenocytes isolated, and peptide and polyclonal stimulations performed, followed by an intracellular cytokine stain for IFN γ . FACS analysis was then performed. Results are plotted as the percentage of donor or host CD8⁺ T cells that are IFN γ ⁺. (A) Host and donor IFN γ ⁺ after VAC-WR infection. (B) (A) Host and donor IFN γ ⁺ after LCMV infection.

On Day 6 of VAC-WR infection, intracellular IFN γ assays were performed by stimulating spleen cells with VAC peptides or by polyclonal stimulation with mAb to CD3 ϵ . The percentages of host and donor CD8 $^+$ T cells that produced IFN γ when stimulated with mAb to CD3 ϵ or with VAC-specific peptides B8R or A47L was similar (Figure 6.3A). On Day 7 of LCMV infection, intracellular IFN γ assays were performed by stimulating spleen cells with LCMV peptides or by polyclonal stimulation with mAb to CD3 ϵ . The percentages of host and donor CD8 $^+$ T cells that produced IFN γ when stimulated with mAb to CD3 ϵ or with LCMV-specific peptides GP33, NP396, GP276, GP118, and NP205 were the same in both donor and host CD8 $^+$ T cells (Figure 6.3B).

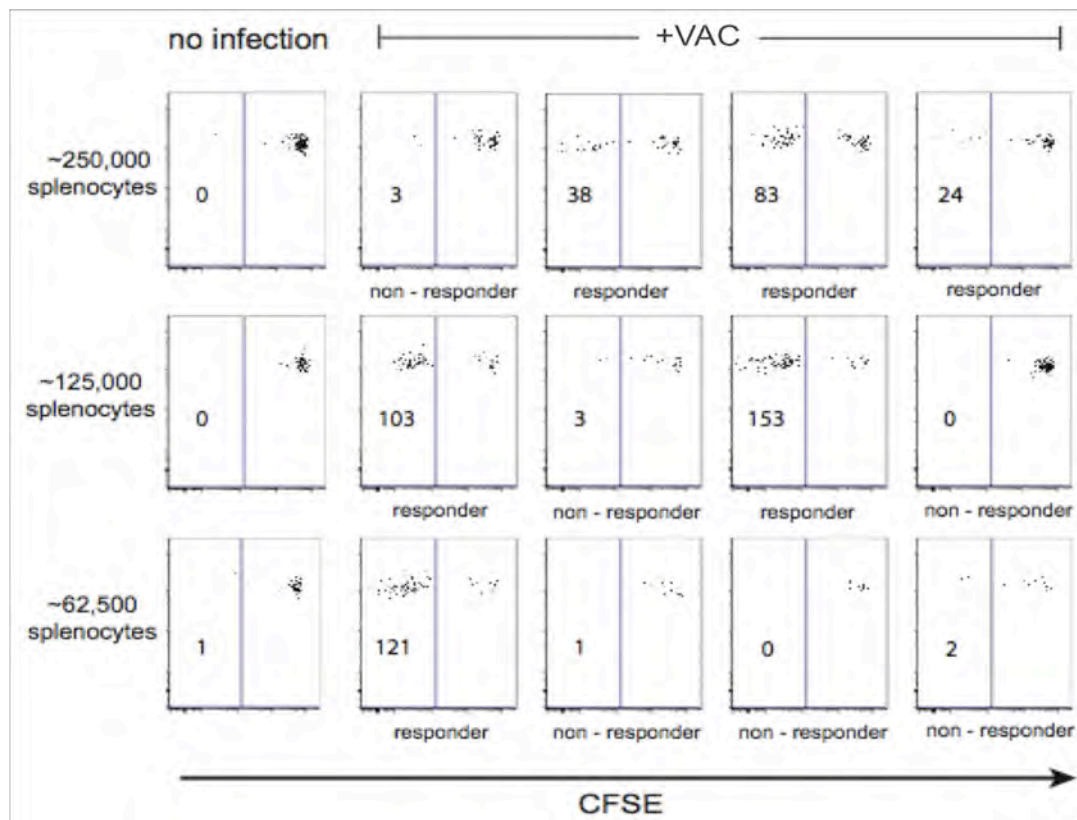
D. *In Vivo* LDA for Virus-Specific T Cells

To determine virus specific CD8 $^+$ T cell precursor frequencies, graded amounts of splenocytes were transferred into host mice at limiting dilution numbers that result in proliferated donor CD8 $^+$ T cells in ~50% of hosts. Ly5.2 $^+$ Thy1.1 $^+$ donor splenocytes from uninfected mice were labeled with CFSE and transferred at decreasing numbers (5×10^6 , 2.5×10^5 , 1.25×10^5 , 0.625×10^5) into Ly5.1 $^+$ Thy1.2 $^+$ hosts. Pilot experiments had demonstrated that in all host mice at the 5×10^6 dose, some donor CD8 $^+$ T cells proliferated, as shown by a CFSE elo peak in response to a VAC infection, so this dilution was used in subsequent experiments as a positive control. The 2.5×10^5 , 1.25×10^5 , and 0.625×10^5 splenocyte dilutions resulted in responders and nonresponders, so these dilutions were used for *in vivo* LDA calculations. Typically, five hosts

received each *in vivo* LDA dilution, with one host from each dilution being left uninfected as a negative control for background staining (see Materials and Methods). A small aliquot of the transferred splenocytes was stained, and FACS analysis was performed to determine the exact number of CD8⁺ T cells transferred into host mice.



A.



B.

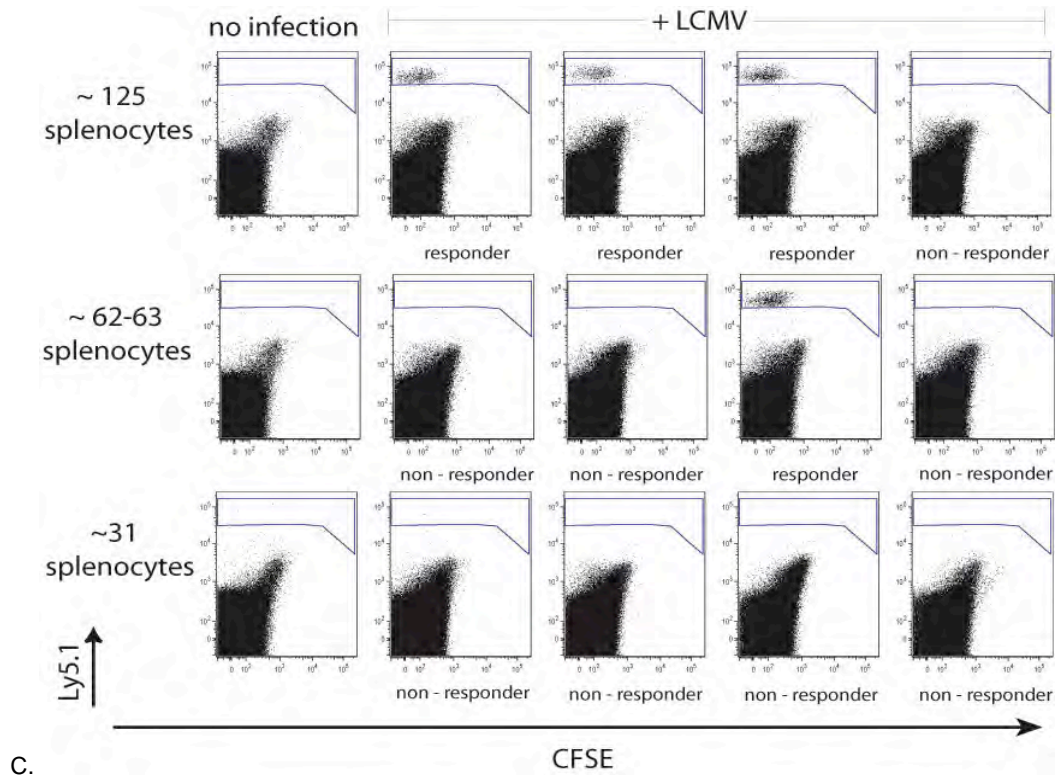


Figure 6.4. *In vivo* limiting dilution assay.

Figure 6.4. In vivo limiting dilution assay.

1.25 x 10⁵ Ly5.2⁺Thy1.1⁺ donor splenocytes were labeled with CFSE and adoptively transferred into Ly5.1⁺Thy1.2⁺ host mice, which were subsequently I.P. infected with VAC. Six days later, mice were sacrificed, and stained for FACS analysis. (A) Gating scheme is shown for the in vivo LDA. Analysis was done, from left to right, gating on singlets by gating on FSC-A vs. FSC-W, lymphocytes by FSC-A vs. SSC-A, CD8⁺ T cells by gating on CD3⁺CD8⁺ cells, and donor cells by Thy1.1⁺ and Ly5.2⁺ gates. In I is an uninfected mouse at a limiting dilution dose (1.25 x 10⁵ splenocytes) of donor CD8⁺ T cells. In II and III are examples of responders at this same dose and IV and V examples of non-responders (B). Ly5.2⁺Thy1.1⁺ donor splenocytes were labeled with CFSE, two two-fold dilutions made, and each dilution was adoptively transferred into five Ly5.1⁺Thy1.2⁺ host mice. At each dilution, one mouse was left uninfected, the other mice were infected with VAC-WR I.P., and FACS analysis was performed on splenocytes as described as in Figure 6.4A. Responders vs. non-responder determinations were as described in Materials and Methods (Cose, Brammer et al. 2006; Ganusov and De Boer 2007) (C) Splenocytes from Ly5.1⁺ H-2D^b-restricted LCMV GP33-specific P14 transgenic animals were labeled with CFSE, and two two-fold dilutions were adoptively transferred into five B6 (Ly5.2⁺Thy1.2⁺ host) mice. Gating scheme, and responder non-responder determinations as in (A).

Previous experiments (data not shown) indicated that VAC-specific CD8⁺ T cell responses peaked at day 6, so on day 6 mice were sacrificed and their splenocytes analyzed by FACS under two conditions. For each spleen a small aliquot was run, analyzing all events to determine the CD3⁺CD8⁺ percentage, and then, to allow detection of the small number of donor CD3⁺CD8⁺ progeny at limiting dilutions, the rest of the spleen was analyzed with the threshold set to only collect CD8⁺ events. Figure 6.4A is an example of a transfer at limiting dilution in VAC-infected or uninfected mice. Responders vs. non-responders were scored as described in Materials and Methods. Figure 6.4B is an example of a full *in vivo* LDA for VAC-WR, where the number of responders per concentration is 3 of 4 at the high concentration, 2 of 4 at the intermediate concentration, and 1 of 4 at the low concentration.

This assay also allowed for a rough estimate of the number of divisions a single virus-specific CD8⁺ T cell by the time of harvest. These results suggested that a CD8⁺ T cell at limiting dilution underwent an average of 11 divisions by day 6 of VAC infection, which is in line with what can be calculated from experiments performed by others (Obar, Khanna et al. 2008). This number can be calculated from tetramer pull-down assays by calculating the number of divisions it requires to get from the naïve precursor number CD8⁺ T cell number to the peak CD8⁺ T cell number. For the *in vivo* LDA this is a rough calculation where the total number of donor CFSE^{lo} events in a responder is divided by the total number of CD8⁺ events collected to get a CFSE^{lo} donor frequency, this is then multiplied by

the total number of CD8⁺ T cells in the spleen, and the dilution that this responder occurred at is then multiplied or divided to get the dilution that is expected to give a single CD8⁺ precursor, and this resulting value is then multiplied by 1 1/3 with the assumption that approximately 67% of CD8 T cells are present in the spleen (Cose, Brammer et al. 2006; Ganusov and De Boer 2007). These calculations assume no cell death during division. So, instead of a true calculation for the number of divisions a T cell has undergone, the calculations instead estimate the least number of divisions a T cell must have undergone to result in the number of progeny detected.

As a validation for the efficiency of the *in vivo* LDA, Ly5.1⁺ H-2D^b-restricted LCMV GP33-specific P14 transgenic T cells (P14 transgenic T cells) were transferred into B6 (Ly5.2⁺) mice at limiting dilution numbers, and the mice were subsequently infected with LCMV

In this experiment, the number of responders per concentration was 3 of 4 at the high concentration, 1 of 4 at the intermediate concentration, and 0 of 4 at the lowest concentration (Figure 6.4C).

E. Determination of Donor “Take”

One caveat of determination of precursor frequencies using an adoptive transfer method is the donor “take”. It has been known that after adoptive transfer, not all donor CD8⁺ T cells survive in the host, and the percentage that do survive in a host mouse has been referred to as donor “take”.

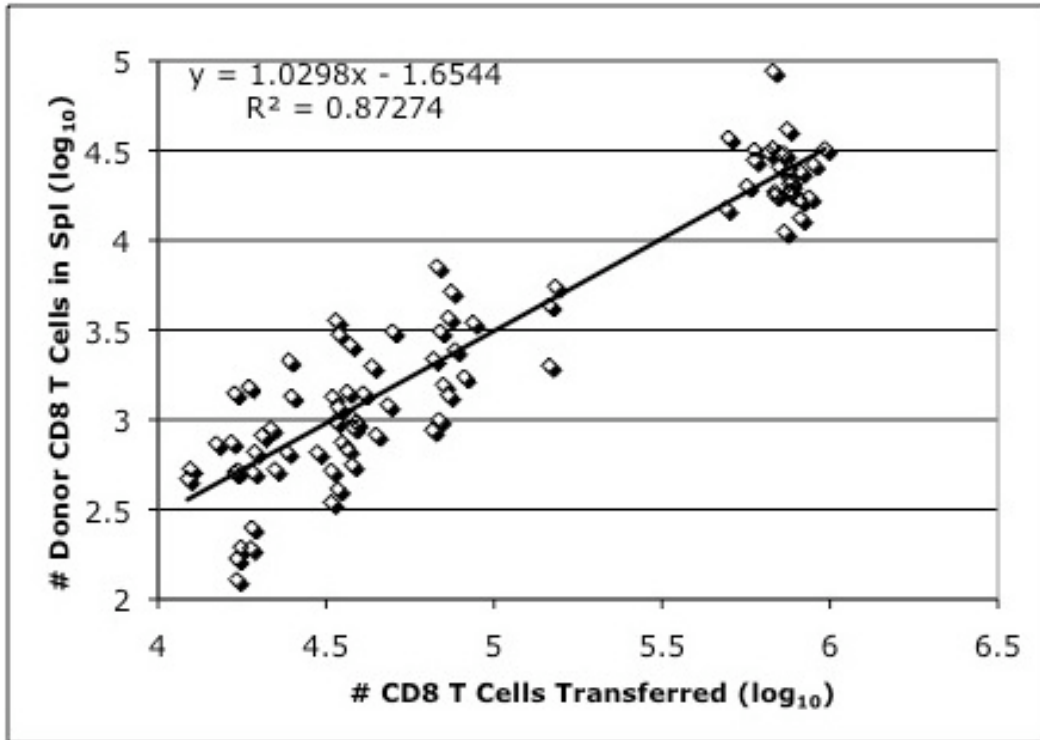


Figure 6.5. Determination of donor take.

Figure 6.5. Determination of donor take.

The number of donor CD8⁺ T cells transferred was plotted against the number of donor CD8⁺ T cells detected in host spleens after transfer. Graph is from 91 uninfected mice used in 26 different experiments. Determination of donor take was calculated by using the equation generated, and assuming that splenic CD8⁺ T cells account for 67% of CD8⁺ T cells.

Figure 6.5 is a graph of 91 uninfected mice and plots the number of Ly5.2⁺Thy1.1⁺ donor CD8⁺ T cells transferred into Ly5.1⁺Thy1.2⁺ hosts against the number of Ly5.2⁺Thy1.1⁺ donor CD8⁺ T cells found in the spleen. The “take” of Ly5.2⁺Thy1.1⁺ donor CD3⁺CD8⁺ cells in Ly5.1⁺Thy1.2⁺ host mice was determined by plotting the log₁₀ of the number of donor CD3⁺CD8⁺ cells transferred by the log₁₀ of the number of CD3⁺CD8⁺ donor cells recovered in the spleens of uninfected host mice. Mice that received less than 1.25 x 10⁵ splenocytes were not included in the analysis because at this number transferred, donor CD3⁺CD8⁺ cells were not reproducibly detectable in uninfected hosts. The resulting formula was then used to calculate a percent take in the spleen. Then using the assumption that 67% of all CD3⁺CD8⁺ cells in a naïve uninfected mouse reside in the spleen (Cose, Brammer et al. 2006; Ganusov and De Boer 2007) (this may be a slight overestimate when compared to the results above, that suggest that less than 65% of donor CD3⁺CD8⁺ cells reside in the spleen (Figure 6.2)), the calculation of a total donor take is possible. The CD8⁺ T cell “take” was calculated to be 3.8%.

F. Precursor Frequency Determination

Using multiple *in vivo* LDA's, the above take value, and probit analysis (Bliss 1934) T cell precursor frequencies in naïve and immune mice can be calculated as shown in Table 3 and described in Materials and Methods. There were about 1 in 2958 +/- 392 CD8⁺ T cells in naïve mice that proliferated in response to LCMV, while there were almost twice as many, 1 in 1444 +/- 171

CD8⁺ T cells that proliferated in response to VAC ($P < 0.0001$ in comparison to LCMV precursors). The number of CD8⁺ T cells in VAC-immune mice that proliferated in response to VAC was, as expected, greatly increased, with 1 in 13 +/- 2 CD8⁺ T cells able to proliferate in response to this homologous infection ($P < 0.0001$ in comparison to naïve immune state). As expected, the LCMV-specific CD8⁺ T cell precursor frequency was not elevated in VAC-immune mice; in fact it was slightly, although significantly, decreased, with about 1 in 4425 +/- 1705 CD8⁺ T cells proliferating ($P < 0.05$ in comparison to naïve immune state).

P14 cells are monoclonal transgenic T cells that recognize the H2K^b LCMV GP33 peptide, and this monoclonal T cell population allows us to perform an *in vivo* LDA with T cells of a single specificity. The number of P14 transgenic T cells that responded to an LCMV infection was next calculated by the *in vivo* LDA, and the precursor frequency by probit analysis was calculated to be 1 in 0.93 +/- .04. This suggests a virtually 100% efficiency in the outgrowth of the transgenic T cells and supports the calculations made concerning T cell "take" after transfer.

The commonly used Reed and Muench 50% endpoint analysis was also employed to determine precursor frequencies (Reed and Muench 1938), and these calculations result in precursor frequencies comparable but not identical to the probit method. By Reed and Muench analysis, naïve mice had 1 in 3121 +/- 291 CD8⁺ T cells specific to LCMV and 1 in 1615 +/- 409 CD8⁺ T cells specific to VAC, while in VAC-immune mice, 1 in 3956 +/- 787 were specific to LCMV, and 1

in 13 +/- 1 were specific to VAC in these immune mice. Using the Reed and Muench method it was calculated that 1 in 1.22 +/- 0.11 P14 transgenic T cells responded to an LCMV infection, again a figure close to 100%.

A summary of B6 mouse precursor frequency determinations by different methods is given in Table 3, and is discussed further below.

G. Chapter Conclusion

The *in vivo* LDA now allows for the enumeration of the number of pathogen specific CD8⁺ T cell in naïve mice with no knowledge of the CD8⁺ T cell determinants encoded within the pathogen. This technique also allows for the calculation of the total number of CD8⁺ T cells within a mouse that will proliferate in response to a pathogen.

**Table 6.1. Precursor frequency determination of naïve and immune states
by *in vivo* LDA.**

<u>Immune State</u>	<u>Virus Infection</u>	<u>Precursor Frequency</u> <i>(Reed and Muench)</i>	<u>Precursor Frequency</u> <i>(Probit)</i>
naïve	LCMV	1 in 2880	1 in 3143
		1 in 3092	1 in 2832
		1 in 2899	1 in 3427
		1 in 3461	1 in 2852
		1 in 3601	1 in 3204
		1 in 2965	1 in 3047
		1 in 2946	1 in 2202
		<hr/>	<hr/>
	1 in 3121 +/- 291	1 in 2958 +/- 392	
naïve	VV	1 in 1081	1 in 1142
		1 in 2010	1 in 1534
		1 in 1280	1 in 1492
		1 in 1811	1 in 1492
		1 in 1892	1 in 1559
		<hr/>	<hr/>
	1 in 1615 +/- 409	1 in 1444 +/- 171	
VV-immune	LCMV	1 in 3708	1 in 6721
		1 in 3917	1 in 6456
		1 in 5519	1 in 4997
		1 in 3606	1 in 2918
		1 in 3622	1 in 2985
		1 in 3362	1 in 3294
		<hr/>	<hr/>
	1 in 3956 +/- 787	1 in 4562 +/- 1824	
VV-immune	VV	1 in 11	1 in 15
		1 in 14	1 in 14
		1 in 12	1 in 13
		1 in 14	1 in 10
		<hr/>	<hr/>
	1 in 13 +/- 1	1 in 13 +/- 2	
P14	LCMV	1 in 1.34	1 in 0.92
		1 in 1.19	1 in 0.98
		1 in 1.12	1 in 0.89
		<hr/>	<hr/>
	1 in 1.22 +/- 0.11	1 in 0.93 +/- 0.04	

Table 6.2. Precursor frequency determination in B6 mice by many techniques.

<u>Background & Immune State</u>	<u>Specificity</u>	<u>Total # Precursors Per Mouse</u>	<u>Ref.</u>	<u>Technique</u>
naïve	H2Db LCMV GP33-41	100-200	(Blattman, Antia et al. 2002)	T cell transgenic competition
	H2Db LCMV GP33-41	1100-1200**	(Pewe, Netland et al. 2004)	diversity estimate by CDR3 sequencing
	H2Db LCMV GP33-41	~287	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Db LCMV GP33-41	~449	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Db LCMV NP396-404	~151	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Db LCMV NP396-404	~117	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Kb LCMV L2062-2069	~90	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Kb LCMV NP205-212	~57	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Kb LCMV GP118-125	~43	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Kb LCMV L156-163	~24	(Kotturi, Scott et al. 2008)	tetramer pull-down
	H2Db LCMV L338-346	~15	(Kotturi, Scott et al. 2008)	tetramer pull-down
	IAb LCMV GP61-80	~100	(Whitmire, Benning et al. 2006)	T cell transgenic competition
naïve VV-immune	LCMV	~6760	(our results)	<i>in vivo</i> LDA
	LCMV	~4384	(our results)	<i>in vivo</i> LDA
naïve	H2Db MHV-JHM S510-518	300-500*	(Pewe, Heard et al. 1999)	diversity estimate by CDR3 sequencing
	H2Db MHV-JHM S510-518	1100-1500**	(Pewe, Netland et al. 2004)	diversity estimate by CDR3 sequencing
	H2Kb MHV-JHM S598-605	600-900**	(Pewe, Netland et al. 2004)	diversity estimate by CDR3 sequencing
	H2Db IAV NP366-374	50-300**	(Kedzierska, Day et al. 2006)	diversity estimate by CDR3 sequencing
	H2Db IAV PA224-233	350-600**	(Kedzierska, Day et al. 2006)	diversity estimate by CDR3 sequencing
	H2Db IAV PA224-233	~120	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Kb VV B8R20-27	~1070	(Haluszczak, Akue et al. 2009)	tetramer pull-down
naïve VV-immune	VV	~13850	(our results)	<i>in vivo</i> LDA
	VV	~1538500	(our results)	<i>in vivo</i> LDA
naïve	IAb chicken OVA 323-339	~16	(Moon, Chu et al. 2007)	tetramer pull-down
	H2Kb chicken OVA 257-264	~130	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Kb chicken OVA 257-264	~170	(Haluszczak, Akue et al. 2009)	tetramer pull-down
	IAb <i>Salmonella typhimurium</i> FLiC 427-441	~20	(Moon, Chu et al. 2007)	tetramer pull-down
	H2Db MCMV M45 985-993	~603	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Kb VSV N52-59	~166	(Obar, Khanna et al. 2008)	tetramer pull-down
	H2Kb HSV-1 gB498-505	~489	(Haluszczak, Akue et al. 2009)	tetramer pull-down
	H2Db mgp100 25-33	~20	(Rizzuto, Merghoub et al. 2009)	T cell transgenic competition
	IAb 2W1S I 52-68	~190	(Moon, Chu et al. 2007)	tetramer pull-down

* clonotypes per infected CNS

** clonotypes per infected spleen

CHAPTER VII: DISCUSSION

The World Trade Center attacks on September 11, 2001 and the anthrax letters sent through the mail soon after revitalized the national will to protect against future terrorist attacks, one of these being a biological attack with a poxvirus agent. This renewed interest brought about increased funding of program projects whose ultimate goal was to improve smallpox vaccines so that the new vaccines would have the same immuno-protective profile but have decreased pathogenicity when given to a large population. This led to a collaboration of our group with a consortium at Harvard to first, work on generating vaccines with improved prophylactic profiles, to second, understand the pathogenesis of high dose poxvirus infections and understand the immune mechanisms that can provide protection from these infections, and third, to develop a better basic understanding of the immune response against poxvirus infections and develop tools that might aid in discovering improved vaccines.

The recent publications of the long lived nature of smallpox vaccination using VAC as the immunization (Crotty, Felgner et al. 2003; Hammarlund, Lewis et al. 2003), especially considering the fact that most individuals tested have not been re-challenged, provided great incentive to further work on VAC as a vector to improve smallpox vaccine efficacy. This work might also advance the effort to establish VAC as a vector for immunization against other pathogens, and this approach has already proved effective for rabies (Pastoret and Brochier 1996) and for rinderpest using a capripox recombinant (Ngichabe, Wamwayi et al.

2002), and it could be imagined this might be extended to human vaccinations. This strategy may become even more attractive as the population ages, as the percentage of individuals that have been vaccinated against smallpox decreases with time as the originally vaccinated population succumbs to old age. The inclusion of proteins that might provide protection against other pathogens might actually be seen as doubly beneficial, as it would be expected, as has been described (Harrington, Most et al. 2002), that poxvirus specific immunity would be retained if genes encoding other proteins are included in the genome.

VAC variants used as smallpox vaccines have been characterized as 1st generation (original vaccines originally grown in many different ways Ex. Dryvax®), 2nd generation (the 1st generation vaccines adapted to grow in cell culture, ACAM2000™), 3rd generation (passaging of virus in cell culture to attenuate Ex. MVA) and 4th generation (VAC recombinants with targeted mutations to enhance immunity or decrease pathogenicity) (Kennedy, Ovsyannikova et al. 2009). One of the hopes for the fourth generation vaccines would be to knock out regions of the VAC genome that have already been shown to increase virus spread, thereby possibly accelerating the process of discovery of improved variants. The three proteins targeted were an IFN γ BP (B8R), IL-18BP (C12L), and CBP (C3L, C21L). Using poxviruses strains that did not include VAC-Wy, which is used for smallpox vaccination, infections with all three of these gene knockout variants have been demonstrated to have decreased pathogenicity (Miller, Shchelkunov et al. 1997; Sroller, Ludvikova et al. 2001;

Symons, Adams et al. 2002) with two in various animal models demonstrating decreased pathogenicity (Isaacs, Kotwal et al. 1992; Sroller, Ludvikova et al. 2001), and with their respective activities being described in various poxviruses, suggesting that the proteins they encode would be widely pathogenic (Spriggs, Hruby et al. 1992; Alcamí and Smith 1995; Miller, Shchelkunov et al. 1997; Smith, Bryant et al. 2000). Promisingly, one of the B8R-/- VAC variants has been demonstrated to reduce pathogenicity in an attenuated strain of VAC (VAC Lister) (Denes, Gridley et al. 2006).

Single knockout, double knockout, and triple knockout VAC variants along with IFN γ binding protein variants were tested for differences in virus spread and pathogenicity, with no difference being detected in various knockout mouse models, strains, or through the use of different infection routes (Table 3.1, Figures 3.1 and 3.2, Figure 4.1). This may have been due to the attenuated character of the VAC-Wy strain inside of mice. It took 50x the normally lethal I.N. dose of VAC-WR to even begin to detect virus in the lungs of infected mice. It was promising to note that all recombinants tested grew at similar rates and to similar titers *in vitro* (communicated by Pei Wu), but this outcome precluded the study of the pathogenesis of the different IFN γ BP knock-in and knock out viruses.

The relative poor growth of VAC-Wy in the mouse model had already been illustrated (Hayasaka, Ennis et al. 2007), but the generation of these knockouts within VAC-Wy was done to allow for quick transition to human vaccination. In light of the fact that the parental virus grows poorly in mice, and

that these gene knockout variants have demonstrated reduced pathogenicity in other animal models and poxvirus strains, it still seems likely that testing the knockout recombinants in an appropriate animal model would demonstrate reduced virus spread and pathogenicity.

However, I.N. infection of the recombinants at doses that just barely allowed virus detection by plaque assay (Table 3.1), still induced a VAC-specific CD8⁺ T response (Figure 3.3), and skin-scarification induced similar amounts CD8⁺ T cells by the VAC recombinant infection as the VAC-Wy infection (Figure 3.4). The comparable immune responses induced by the skin-scarification route was promising as recent studies have demonstrated the increased efficacy of the skin-scarification immunization with VAC in comparison with intra-dermal, intramuscular, or sub-cutaneous inoculation in providing protection against either cutaneous, or lethal I.N. VAC challenge (Liu, Fuhlbrigge et al. 2006; Liu, Zhong et al. 2010)

The identity of the B8R knockout was also confirmed, as the immunodominant CD8⁺ T cell epitope in the B6 mouse is encoded within the B8R protein. Mice infected with viruses that did not contain the B8R gene did not have a B8R-specific response, but still had CD8⁺ T cells that responded to peptide stimulation with a peptide from a subdominant epitope in VAC (Figure 3.3).

Promisingly, immunization with the Wyeth parental or with the four B8R recombinant VAC's was able to protect mice from death induced by 20 LD50's of

VAC-WR (Figure 3.5A), with all groups of immunized mice gaining weight starting at day 3. Further experiments examining viral loads after 20 LD₅₀'s of VAC-WR, demonstrated that immunization with any of the B8R recombinants or the Wyeth parental strain resulted in almost no virus being detected in the livers or spleens of the immunized mice, as compared to the almost 5 logs of virus being detected in the livers and spleens of non-immunized mice, with significant reductions of virus also found in the lung in immunized mice (Figure 3.6). These results suggest that the protection provided by the recombinants was similar to the protection induced by the parental VAC-Wy strain. Previous studies have examined the pathogenicity (Sroller, Ludvikova et al. 2001; Verardi, Jones et al. 2001; Symons, Tschärke et al. 2002; Denes, Gridley et al. 2006) and VAC-specific immune responses (Verardi, Jones et al. 2001; Denes, Gridley et al. 2006) induced by B8R^{-/-} VAC variants, but none have examined immunoprotective profiles, and these results establish that skin-scarification with any of the IFN γ BP recombinants or the parental strain provide protection against lethal VAC-WR challenge. The similar protection induced by the B8R knockout and the dimer mIFN γ knock-in is also important to point out. These two viruses might be thought to encode two opposite ends of a spectrum, one encodes a secreted protein with high affinity to IFN γ , and the other no longer encodes a protein that binds IFN γ , and the fact that both are able to induce similar amount of protection to a VAC-WR challenge was promising.

These viruses also allow another unique opportunity, as it is possible to immunize with a virus that lacks the protein that encodes the immunodominant epitope, and challenge with a strain that encodes this epitope. Interestingly, lack of the B8R epitope from the immunization virus had no effect on protection from the 20 LD₅₀'s of VAC-WR challenge, and in mice immunized with a B8R knockout virus 167 day previously, there was almost no detectable B8R-specific CD8⁺ T cell response on day 5 after VAC-WR challenge, even though the challenge virus contained the epitope (Figure 3.7B) or on day 6 (Figure 3.5B). This suggests that the CD8⁺ T cell response to a homologous infection is almost entirely of memory origin (Figure 3.7 and Figure 3.5B). This may simply be an issue of numbers. From tetramer-pulldown assays it has been estimated that in a naïve mouse, about 1 in 10,000 naïve CD8⁺ T cells are B8R-specific (Haluszczak, Akue et al. 2009), while in an immunized mouse, about 1 in 1,000 CD8⁺ T cells were specific to A47L as shown by intracellular cytokine assay (Figure 4.2A). Memory CD8⁺ T cells are also not suspected to be necessary in recall responses against VAC in the mouse, as previous depletion studies have described them as dispensable in a VAC-WR challenge model after vaccination (Belyakov, Earl et al. 2003). These results in some ways support this study, as the lack of an immunodominant CD8⁺ T cell epitope from immunization does not change the immunoprotective phenotype when examining near homologous virus challenge.

The VAC-specific memory induced by the VAC-Wy^{-/-} (B8R^{-/-}, C3L^{-/-}, C12L^{-/-}) immunization was also examined, and it was found that the triple VAC-Wy^{-/-} and the VAC-Wy parental strain were able to induce the same level of neutralizing antibody and VAC-specific CD8⁺ T cells to a subdominant epitope (Figure 4.2A). Once again, although no pathogenic difference was demonstrated in mice (Figure 4.1), it was of interest to note that the VAC-Wy^{-/-} recombinant with three genes that encode for immunomodulatory proteins knocked out was able to induce the same level of VAC-specific immunological memory as the Wyeth parental strain from which it was generated. Either vaccination with VAC-Wy or the VAC-Wy^{-/-} provided significant protection from I.N. challenge of 20 LD50's VAC-WR, as there was no detectable virus in livers or spleens of these immunized mice with about 2.5 logs of virus detected in the lungs. There was an increased, but not statistically different amount of virus in the lungs of VAC-Wy^{-/-} immunized mice in comparison to Wyeth-immunized mice. In the PBS - immunized mice on the other hand, 5 logs of virus was detected in the livers, 7 logs of virus in the lungs, and about 2 1/2 logs of virus in the spleen (Figure 4.3). It is known that each of these immunomodulatory proteins contribute to pathogenesis in other mouse and rabbit poxvirus infection models (Isaacs, Kotwal et al. 1992; Symons, Adams et al. 2002; Denes, Gridley et al. 2006). Interestingly, the MVA virus contains a fragmented version of B8R with both C3L and C12L being completely deleted (Antoine, Scheifflinger et al. 1998), but a recombinant VAC encoding the six major deletions of MVA which include C3L

and C12L deletions but not a B8R deletion was not as attenuated as MVA (Meisinger-Henschel, Spath et al. 2010). This result then gives rise to the question of, what else is attenuating in MVA, and why does it take increased virus to induce responses that are as protective as the usual smallpox vaccination. The observation that the VAC-Wy-/- recombinant is able to induce similar immune responses as the VAC-Wy parental strain in a skin-scarification infection with similar protection as the VAC-Wy parental strain supports the continued investigation of targeted gene knockouts of VAC as a method to develop improved smallpox vaccines.

Further evidence of a CD8⁺ T cell response to a homologous infection being almost entirely of memory origin (Figure 3.7) is demonstrated again as immunizing mice with a VAC-Wy-/- that lacks the protein B8R, results in a CD8⁺ T cell response to VAC-WR I.N. challenge that includes almost no B8R-specific CD8⁺ T cells (Figure 4.4B). Further results from these experiments demonstrate a decrease in the number of CD8⁺ T cells that respond to a polyclonal stimulation in the VAC-Wy-/- immune mice challenged with 20 LD50's VAC-WR as compared to the VAC-Wy immune mice challenged with 20 LD50's VAC-WR I.N. (Figure 4.4A). This decrease is most likely due to the lack of the peptide that encodes the immunodominant epitope in the immunization virus, but in this experiment that included a larger number of mice, there were increases in the number of CD8⁺ T cells responding to subdominant epitopes in the VAC-Wy-/- immune mice as compared to the VAC-Wy vaccination and

challenge, and this might represent a compensation mechanism (Figure 4.4C and 4.4D). This compensation mechanism does not look to be complete, however, as the response to a polyclonal stimulation was lower. However, these experiments cannot rule out that other non-tested or cryptic epitopes combine to make up for the lack of the B8R-specific response, as it has been demonstrated in HSV mouse models that deletion of an immunodominant epitope can result in detection of other epitopes that normally cannot be detected with a parental virus infection (Wallace, Keating et al. 1999).

These results in total are supportive of further study of these recombinants, both as a vaccine against smallpox, and possibly as a vector for vaccines to other infections. Further analysis in other models might yet demonstrate that these viruses are less pathogenic than the Wyeth parental strain. The MVA virus that is deficient in these three genes, among many others requires multiple inoculations to be as protective as Wyeth (Earl, Americo et al. 2004), while in our hands the VAC-Wy-/-/- recombinant provides similar protection as VAC-Wy to a lethal I.N. VAC-WR challenge in the mouse model, and this supports the continued investigation of this variant of VAC-Wy as a new smallpox vaccine.

The description of improved vaccines is an important step in the study of immunology, but improved understanding of acute infections, and what factors are important in resolving them allows for better therapeutic intervention and prognostic markers. It is also important to study acute infection in models that

mimic human disease, such as SLE, as these investigations may provide insight into the infection, and to the disorder.

Many cell types have been implicated in resolution of acute poxvirus infections, B cells (Morita 1973; Belyakov, Earl et al. 2003; Fang and Sigal 2005), CD8⁺ T cells (Belyakov, Earl et al. 2003; Fang and Sigal 2005), CD4⁺ T cells (Xu, Johnson et al. 2004), $\gamma\delta$ ⁺ T cells (Selin, Santolucito et al. 2001), and NK cells (Bukowski, Woda et al. 1983; Fang, Lanier et al. 2008; Martinez, Huang et al. 2008), but how resolution of a typically lethal infection might occur is not as well studied.

Early studies in our lab had demonstrated that lethal I.N. doses of VAC-WR in mice would result in few VAC-specific CD8⁺ T cells detectable in the mouse spleens. As this decreased number of VAC-specific CD8⁺ T cells seemed to occur only after high dose lethal I.N. infections, it was thought that VAC-specific CD8⁺ T cells might be being eliminated by AICD. AICD is a phenomenon whereby continued stimulation of the TCR's of T cells results in their apoptosis (Takahashi, Maecker et al. 1989; Ucker, Ashwell et al. 1989; Russell, White et al. 1991), and it has been demonstrated with acute LCMV infection and with LCMV that grow to high titers inside of mice, that continued stimulation of CD8⁺ T cells can result in their apoptosis (Moskophidis, Lechner et al. 1993; Razvi and Welsh 1993). AICD has, in some cases, also been linked to Fas expression, as T cells from B6-*lpr* (Russell, Rush et al. 1993) and B6-*gld* have defects in *in vitro* induced AICD (Russell and Wang 1993). This Fas

dependent AICD might not be a primary mechanism *in vivo* though, as apoptosis of T cells during the contraction phase of the response after acute LCMV infection does not seem to be exclusively dependent on Fas (Razvi, Jiang et al. 1995) and the T cell apoptosis (exhaustion) induced after infection with an LCMV variant that grows to high titers inside of a mouse also seems not to be exclusively dependent on Fas (Lohman and Welsh 1998).

Although this mechanism did not seem to be the primary mechanism of AICD in LCMV infections, it was still a possibility that Fas might be contributing to an AICD phenomenon after high dose I.N. VAC-WR infection. B6-*Ipr* were indeed found to have increased percentages and numbers of CD4⁺ and CD8⁺ T cells in the MLN's with an increased number of B8R-specific CD8 T cells after high dose I.N. VAC-WR infection (Figure 5.2). In fact, even after high dose I.P. VAC-WR infection, there was an increased number of CD4⁺ and CD8⁺ T cells in the B6-*Ipr* spleens (Figure 5.3B and 5.3C). Interestingly, B6-*Ipr* mice infected with a high dose of VAC-WR I.N. lost less weight, and had a decrease of almost 2 logs of virus in the livers (no difference in lungs) when compared to B6 wild type (Figure 5.1). B6-*Ipr* mice infected with a lethal dose of VAC-WR I.P. also lost less weight over the infection period and had almost 2 logs less virus in fat pads and spleens (Figure 5.3). These results seem to be consistent with the idea that AICD was leading to increased apoptosis of T cells, and in the B6-*Ipr* mice, the decreased Fas-dependent AICD was allowing increased T cell responses allowing for decreased virus titers and less weight loss.

Intriguingly, work had recently been published that suggested a mechanism by which high dose virus infection might be causing CD8⁺ T cell immuno-suppression by Fas/FasL interactions. In this influenza model of infection, lethal doses of influenza virus up-regulated FasL on dendritic cells, which would then kill off activated CD8⁺ T cells, thereby limiting the influenza virus specific CD8⁺ T cell response (Legge and Braciale 2005). This model could explain the observations noted with high dose VAC-WR infections, but further experiments would needed to be performed to test the hypothesis that high dose infection was up-regulating FasL on cells leading to increased apoptosis of virus specific T cells.

In competitive adoptive transfer experiments, in which CFSE- labeled B6-*lpr* and CFSE- labeled wild type IgH^a cells were transferred into B6.SJL mice, which were then infected with a lethal dose of VAC-WR I.P., CD8⁺ T cells from B6-*lpr* mice did not survive or proliferate more extensively than CD8⁺ T cells from a B6 wild type mouse (Figure 5.4). There was no difference in either the percentage of CD8⁺ T cells that were CFSE^{lo} when comparing the IgH^a and B6-*lpr* donor cells (Figure 5.4B), nor was there a difference in total numbers of donor cells surviving (Figure 5.4C). These results were not consistent with the hypothesis that increased FasL was killing off activated CD8⁺ T cells.

As the protection from weight loss in B6-*lpr* mice in comparison to B6 mice was seen early in both I.N. and I.P. infections (Figure 5.1A and 16A), and FACS staining revealed increases in NK and $\gamma\delta^+$ T cell percentage in B6-*lpr* infected

mice it was possible that there was an early mechanism of protection occurring in B6-*Ipr* mice. Both cell types have been demonstrated to provide protection from poxvirus infections (Bukowski, Woda et al. 1983; Selin, Santolucito et al. 2001; Fang and Sigal 2005; Martinez, Huang et al. 2008), and both NK cells and $\gamma\delta^+$ T cell might work early in the infection to provide B6-*Ipr* mice protection against hi-dose VAC-WR infection.

To further examine the protection in B6-*Ipr* mice, virus titers after lethal VAC-WR I.P. infection at days 2 and 3 in B6-*Ipr* mice in comparison to B6 wild type mice were examined, and day 2 titers of B6-*gld* mice and B6 wild type mice were compared. These experiments demonstrated that Fas mutant mice had decreased virus titers in the livers and spleens in comparison to B6 wild type mice (Figure 5.7), with decreases in fat pad titer only noted at day 2 in the Fas mutant mice. This result suggested there was some early mechanism at work that either decreased virus loads, or did not allow the virus to grow in Fas mutant mice. Interestingly, although Fas/FasL interactions are typically described as important mechanisms by which T cells can eliminate infected cells (Price, Huang et al. 2005; Chen, Hsu et al. 2006), it has been shown that B6-*Ipr* mice and mice receiving an antibody that blocks FasL (DcR3) subcutaneously throughout an experiment in comparison to untreated B6 mice have decreased amounts of bacteria in the lung at day 2 after an I.N. *Streptococcus pneumoniae* infection (Matute-Bello, Liles et al. 2005), although the mechanisms of protection in these experiments were not detailed.

As the mechanism of protection seemed to act very early, uninfected B6-*lpr* mice were examined. There were about 1.5-2x as many NK cells and $\gamma\delta^+$ T cells, with increases in the number of memory like (CD44⁺) CD4⁺ and CD8⁺ T cells in B6-*lpr* mice as compared to B6 wild type mice (Figure 5.6 A,B,C,D). There was also an increased percentage and number of CD8⁺ T cells that were IFN γ ⁺ (Figure 5.6E and 5.6F) after a polyclonal stimulation. All subsets mentioned had been described to provide protection against acute poxvirus infections (Selin, Santolucito et al. 2001; Belyakov, Earl et al. 2003; Xu, Johnson et al. 2004; Martinez, Huang et al. 2008), so these subsets were depleted or blocked individually in B6-*lpr* mice through use of mAb. None of the single depletion treatments significantly changed virus titers at day 3 in the fat pads, livers, or spleens ($\gamma\delta$ block Figure 5.7, NK depletion Figure 5.8, CD4 depletion Figure 5.9, CD8 depletion Figure 5.10). While no one subset was demonstrated to be the subset providing protection against VAC-WR in B6-*lpr* mice, it was still a possibility that multiple subsets were acting together or redundantly to provide protection.

Indeed, when examining splenocytes in day 2 infected mice, it was found that B6-*lpr* mice had increased numbers of splenocytes, and increased number of NK, CD4⁺, and CD8⁺ T cells that spontaneously produced IFN γ after a five hour culture when compared with B6 mice (Figure 5.11). As it had been demonstrated that IFN γ can play a significant role in resolving VAC infections,

this result was intriguing and merited further follow-up (Huang, Hendriks et al. 1993; Karupiah, Fredrickson et al. 1993).

Another interesting observation noted in Figure 5.11A was the protection of B6-*lpr* mice from the VAC-WR induced lymphocyte deficiency in the spleen, and one explanation for this result would be the lack of an early Fas dependent AICD mechanism. This hypothesis was tested by high dose (4×10^6 PFU I.P.) VAC-WR infection of B6.SJL mice that had received a 1:1 mix of DDAO labeled B6-*lpr* splenocytes and CFSE labeled IgH^a splenocytes. The adoptive transfer results did not support this conclusion, as both splenocytes populations survived equally well (Figure 5.12A), with no increased survival in $\gamma\delta^+$ T cells (Figure 5.12B), NK cells (Figure 5.12C), or CD4⁺ or CD8⁺ T cells (Figure 5.12D) in B6-*lpr* donor cells versus IgH^a wild type donor cells.

At this point there had been no specific subset that could be depleted that removed the increased protection in B6-*lpr*, and adoptive transfer experiments had suggested that both B6-*lpr* splenocytes and IgH^a wild type splenocytes were as susceptible to VAC-WR induced lymphocyte depletion.

In examining all day 2 mice, it was confirmed that B6-*lpr* mice had decreased virus loads when compared to B6 wild type mice (Figure 5.15A, 5.15B, and 5.15C), and if viral titers were divided by initial weight, B6-*lpr* still had decreased titers when compared to B6 mice, thus suggesting that weight was not the reason behind the increased protection in B6-*lpr* mice (Figure 5.15D, 5.15E, and 5.15F).

In the correlation studies, CD8⁺ T cells and $\gamma\delta$ ⁺ T cells were found to negatively correlate with virus titers and in B6-*rag1*^{-/-} and B6-*rag1*^{-/-}/*lpr*, depletion of NK cells was found to increase virus titers in most organs (Figure 5.18). These results, in combination with the observation that no single depletion decreased virus titers in B6-*lpr* mice makes it seem likely that multiple leukocyte types were working together or redundantly to provide the increased protection from high dose VAC-WR infection in B6-*lpr* mice.

The observation in Figure 5.11 of increased numbers of IFN γ ⁺ NK, IFN γ ⁺ CD4⁺, and IFN γ ⁺ CD8⁺ T cells in B6-*lpr* mice compared to B6 wild type mice, and the knowledge that IFN γ was important in resolving poxvirus infections (Ruby and Ramshaw 1991; Huang, Hendriks et al. 1993; Karupiah, Fredrickson et al. 1993; Cantin, Tanamachi et al. 1999), left open the possibility that several different types of leukocytes could be cooperatively or redundantly producing IFN γ in response to this high dose infection in B6-*lpr* mice and thereby provide increased protection. This possibility was studied by using treatment with an anti-IFN γ antibody, and it was found that after combining three studies, as each study demonstrated similar trends, anti-IFN γ antibody increased VAC titers in B6-*lpr* and B6 mice in the livers and spleens, with a decrease in virus titers in fat pads (Figure 5.19). The increased titer in fat pad was unexpected, but the block of IFN γ in the fat pad may instead work to impede infected leukocyte cell migration into the fat. It may also be possible that different mechanisms are required for virus clearance in the fat pad versus the liver or spleen as has been

demonstrated with MCMV, where production of IFN γ by NK cells, is more important in clearance of MCMV in the livers of mice, while perforin seems more important in virus clearance in the spleen (Tay and Welsh 1997; Selin, Varga et al. 1998). Interestingly, anti- IFN γ treatment of B6-*lpr* and B6 mice resulted in spleen and liver virus titers that were not significantly different between the groups,. This seems to suggest that IFN γ may play a heightened role in the increased early protection of B6-*lpr* mice when compared to B6 mice.

These results suggest a paradox, where mice that are prone to develop an autoimmune disorder are better protected against high dose virus infections. A similar phenomenon has been described in HIV-infected patients, in that patients with two hypoactive mutations in Fas or FasL recovered their CD4⁺ T cell counts more quickly after anti-retroviral treatment (Nasi, Pinti et al. 2005). Thus, the altered immune system in mice, months before they develop pathogenic signs of an auto-lymphoproliferative disorder, suggests that mutations in the Fas pathway may significantly alter the way in which they respond to high dose infections, and further suggests that there might be a more vigorous early immune response to high dose virus infections in those with Fas hypoactive mutations.

The generation of protective, non-pathogenic vaccines, and the identification of correlates of protection after acute infection or re-challenge provide a lot of information about how the different components of the immune system are acting together to fight off a pathogen. One of the most fascinating

things about the immune system as a whole, however, is the complexity of pathogens to which it can respond and eliminate.

The broad possible pMHC reactivity generated by random gene rearrangements of α and β TCR chains on T cells would seem to ensure reactivity against a diverse array of pathogens, but this broad diversity then begs the question of how many T cells in a host would respond to a specific pMHC complex or against an entire pathogen. Many determinations of CD8⁺ T cell precursor frequencies have now been used to calculate the number of pMHC-specific CD8⁺ T cells within a mouse (Table 3), but the determination of the total number of CD8⁺ T cells that could respond to a viral infection would be possible with these methods only if all of the epitopes of the virus were known. These assays are useful for viruses that have a strongly immunodominant epitope or a very limited number of epitopes, but the numbers of epitopes found to stimulate T cell responses are now becoming quite large, as means for detecting them have become more sensitive. A virus like VAC is now reported to encode close to 50 H-2K^b and H-2D^b-restricted epitopes (Moutaftsi, Peters et al. 2006), a number that would make it very difficult, if not prohibitive, if the precursor frequency to the entire virus was to be determined with the usual methods. The *in vivo* LDA allows for the determination of the number of virus-specific CD8⁺ T cells within a mouse, and so we next compare our results with other methods of precursor frequency determination.

It is interesting to note the differences calculated for T cell precursor frequencies depending on the method used (Table 3). Extrapolation of clonotype number per spleen by calculation of a measure of diversity by examining the CDR3 sequences of pMHC-specific populations as done in (Pewe, Heard et al. 1999; Pewe, Netland et al. 2004) and (Kedzierska, Day et al. 2006) gives precursor frequencies on the high end of most estimates, with the highest number of H-2D^b LCMV GP33-specific CD8⁺ T cells calculated among all methods. This is interesting in that this method is assumed to be an underestimate, because it does not include TCR α diversity, and it also does not account well for redundancy in T cell populations, i.e. there may be more than one cell of a single clone in a naïve mouse. However, there is some uncertainty in these numbers because they rely on extrapolations of the numbers and diversities of sequenced molecular clones to estimate T cell precursor frequencies. When compared to the above results, this extrapolation method would probably assume the above results to be underestimates. If there are 1100-1200 GP33-specific CD8⁺ T cells per naïve mouse spleen, and the GP33-specific CD8⁺ T cell response is approximately 10% of the total LCMV-specific response, it might be expected that there would be about twice as many LCMV-specific precursors. Instead of the 6760 per mouse as calculated, 11,000-12,000 LCMV-specific CD8⁺ T cells would be expected. If precursor frequencies are instead calculated by transgenic T cell competition, where transferred monoclonal transgenic T cells compete against heterogeneous endogenous T

cell populations to determine precursor frequencies, these results look to be an overestimate. Assuming a 10% take and 2×10^7 CD8⁺ T cells per mouse, this method estimates ~100 H-2D^b LCMV GP33-specific CD8⁺ T cells (Blattman, Antia et al. 2002). This take is much higher than we have calculated, although we remain confident in our value as the P14 transgenic T cell transfer seems to confirm the 3.8% figure in our hands. This result, however, would put the above LCMV-specific CD8⁺ T cell precursor determination on the high end. The tetramer-based enrichment assay, which makes use of pMHC tetramers, magnetic bead enrichment and double tetramer FACS staining of spleens and lymph nodes to identify pMHC-specific CD8⁺ T cells (assuming that most naïve T cells reside in lymph organs) seems to yield numbers that are in line with results determined by the *in vivo* LDA. Depending on the individual determination, there are ~287 (Obar, Khanna et al. 2008) or ~449 (Kotturi, Scott et al. 2008) H-2D^b LCMV GP33-specific CD8⁺ T cells in all lymph organs of a naïve mouse, and this result would be on the low end, yet still compatible with what the above results might predict for a GP33-specific CD8⁺ T cell naïve precursor frequency. For VAC-WR, the *in vivo* LDA calculates about 13,850 responsive CD8⁺ T cells per B6 mouse. The tetramer-based enrichment assay estimated 1200 CD8⁺ T cells specific for the H-2K^b VAC B8R epitope in the spleen, lymph nodes, and ovaries (Haluszczak, Akue et al. 2009), and those results would seem consistent with the results described, considering that the B8R peptide response may represent about 10% of the VAC-specific response.

About twice as many T cells were responsive to VAC-WR than to LCMV ($P < 0.0001$). This might in part reflect the observations that the T cell response to VAC-WR peaks earlier than that of LCMV. Having more CD8⁺ T cells that are specific to VAC-WR may increase the likelihood that VAC specific CD8⁺ T cells interact with stimulating antigen-presenting cells earlier, allowing peak T cell proliferation to occur earlier. VAC also encodes more proteins than LCMV, with almost twice as many VAC epitopes described in the B6 mouse, and consistent with almost twice as many VAC-specific precursor T cells than LCMV-specific CD8⁺ T cells (Moutaftsi, Peters et al. 2006; Kotturi, Peters et al. 2007).

The *in vivo* LDA results also estimate that 8% of CD8⁺ T cells in VAC-immune mice are VAC-responsive, and these data are supported by results from VAC-immune mice using peptide stimulations and intracellular cytokine stains that estimate that anywhere from 2-11% of CD8⁺ T cells are specific to the VAC-encoded immunodominant B8R epitope in D21 or D40 VAC-immune mice (Tschärke, Karupiah et al. 2005; Salek-Ardakani, Moutaftsi et al. 2008), and my results from intracellular cytokine assays estimate that 0.5-2% of CD8⁺ T cells in VAC-immune mice are specific to the VAC B8R epitope at 3-8 months post-infection. This increase in CD8⁺ T cell precursor frequency to VAC in VAC-immune animals by greater than two orders of magnitude ($P < 0.0001$) demonstrates the expected considerable increase of VAC-specific CD8⁺ T cell precursor frequency after VAC infection. As expected, there was no increase in the number of CD8⁺ T cells that respond to LCMV in VAC-immune mice, and this

helps to validate the specificity of the *in vivo* LDA assay. The small, but significant decrease in LCMV CD8⁺ T cell precursor frequency in VAC-immune mice is interesting and may suggest that memory cells may displace some naïve cells in the immune response. This has been not systematically addressed as there is a high degree of heterologous immunity in this virus sequence, and the immunity, due to private specificities in the immune repertoire, has such high variability that the *in vivo* LDA would likely suffer from reproducibility issues (Kim, Cornberg et al. 2005).

It is possible to make an approximation of the number of divisions a CD8⁺ T cell undergoes after stimulation by examining the burst size, or recovered cell number at limiting dilution. By determining the frequency of CFSE^{lo} donor cells among all CD8⁺ events collected, multiplying that frequency by the total number of CD8⁺ T cells found in the spleen, and then multiplying that number using the assumption that 67% of all CD8⁺ T cells are present in the spleen during infection, a calculation of the approximate number of divisions a CD8⁺ T cell undergoes after virus infection can be made. The number of divisions a VAC-specific precursor undergoes by day 6 (~11 divisions) or an LCMV-specific precursor undergoes by day 7 (~12-13 divisions) (P=0.07) fall within predicted ranges. However, it can be calculated that a P14 transgenic T cell undergoes ~14 divisions by day 7 of an LCMV infection, and this is significantly different (P=0.023) when compared to the ~12-13 divisions that a naïve CD8⁺ T cell from a heterogeneous population of T cells undergoes. This may reflect differences in

avidity between the transgenic T cells and the heterogeneous populations as a whole, or may instead be related to the examination of a monoclonal T cell population that responds to a highly expressed immunodominant epitope versus a heterogeneous population of CD8⁺ T cells that contains T cells responding to immunodominant and subdominant epitopes.

My calculated “take” of CD8⁺ T cells is not the normally quoted 10% figure. If a 10% value is instead used for take, my calculated precursor frequencies would be decreased, with a naïve mouse having about 1 in 7,805 +/- 1,034 CD8⁺ T cells specific for LCMV, 1 in 3,809 +/- 452 CD8⁺ T cells specific for VAC, and a VAC-immune mouse would have about 1 in 34 +/- 6 and 1 in 12,036 +/- 4812 CD8 T⁺ cells specific for VAC or LCMV respectively. The rough estimates from results above (Figure 6.2), would suggest that less than 67% of CD8⁺ T cells reside in the spleen, in contrast to what has been suggested (Ganusov and De Boer 2007). This would lower the take value, but given that my analysis of cell recovery in different tissues was not exhaustive, and that the take would probably only change by at most a 20-30% value, it is probable that the calculations are within a reasonable range of total virus specific CD8⁺ T cell precursor frequencies. Further, experiments using P14 transgenic T cells also strongly support the estimation of take, because the calculations of numbers of precursors equals the number of transgenic T cells using my 3.8% take value. Further, this *in vivo* LDA calculated at a 3.8% take gives frequencies with high concordance with the anticipated number of VAC-specific memory cells, which

can be measured directly by intracellular cytokine assays. Considering the large amount of data in generating the 3.8% figure (Figure 6.5), this estimate of take is reasonably accurate in these experiments.

Whereas tetramer-based enrichment assays measure the number of cells that are reactive to a particular pMHC complex, the *in vivo* LDA requires CD8⁺ T cell division and proliferation, measuring instead the number of CD8⁺ T cells that do proliferate in response to a viral infection. It might seem likely that not all virus-specific T cells would react with tetramer, and not all tetramer-specific T cells would be capable of proliferating in response to their cognate antigen. Remarkably though, while the *in vivo* LDA described here tells us something different than the tetramer-based enrichment assays described recently, it gives reasonable concordance with those techniques.

In closing, this thesis describes VAC-Wy recombinants engineered to be less pathogenic *in vivo* that have the same immuno-protective profile as the VAC-Wy parental strain from which they were generated to a lethal poxvirus challenge in a mouse model. One of these recombinants lacks three genes that code for immuno-modulatory proteins that have previously been shown to increase pathogenesis in other models, but still induces similar amounts of immunological memory when given at equal doses as the parental strain, and protects mice as well as the parental VAC-Wy to a lethal poxvirus infection. This supports the further analysis of this recombinant as a potential smallpox vaccine and vector for other immunizations. This work also describes a paradox, where

mice that have mutations in the Fas/FasL system that predispose them to lymphoproliferative disorders, are better protected at early time points to high dose VAC-WR infections. These mutant mice were found to have increases in multiple leukocyte types before infection, and it seems the protection from high dose VAC-WR infection was largely due to the co-operative or redundant production of IFN γ by multiple leukocyte populations. This might suggest that hypoactive Fas mutations might provide protection against high dose virus infections. Finally, we describe a technique, the *in vivo* LDA, that allows for enumeration of the total number of virus-specific CD8⁺ T cells within a mouse. We also describe results of experiments with the *in vivo* LDA, and find large numbers of LCMV and VAC-specific CD8⁺ T cell in naïve mice, with 100x increases being noted in VAC-immune mice when measuring the number of VAC-specific precursors. This technique can now be used to determine CD8 T cell precursor frequencies to entire pathogens, and may also be useful in testing immunization protocols.

CHAPTER VIII: REFERENCES

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