HIV-1 Vaccine Development: Evaluating Mechanisms of CD8$^+$ T cell Avidity

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Statement of Declaration

I, Danushka Kumara Wijesundara, confirm that all the data presented in this thesis, except where indicated, were obtained entirely from my experiments under the supervision of Dr. Charani Ranasinghe at The John Curtin School of Medical Research (JCSMR), Canberra, Australia. The individuals who prepared the virus stocks and liposomes used in this PhD project are acknowledged in chapter 2 (**section 2.4 and 2.5**). The flow cytometry analysis in chapter 5 was done in collaboration with Dr. Ben Quah. This thesis is under 100, 000 words and has not been submitted for examination elsewhere.

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Dedicated to My Beloved Parents

Publications

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❖ Quah BJ, **DK Wijesundara**, C Ranasinghe and CR Parish. Fluorescent target array killing assay: a multiplex cytotoxic T-cell assay to measure detailed T-cell antigen specificity and avidity in vivo. Cytometry A 2012; **81**: 679-90.

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Conferences and Public Forums

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IL-4 and IL-13 regulate CD8 co-receptor expression to dampen the quality of anti-viral CD8^{+} T cell immunity following HIV-1 prime-boost vaccination

Wijesundara DK[1], RJ Jackson[1], DC Tscharke[2], and C Ranasinghe[1]

❖ **Poster presenter** at the 2013 Keystone Symposium HIV Vaccines (X2) Conference, Colorado, USA

Dynamic regulation of IL-4 receptor alpha (IL-4Rα) following viral infections and HIV-1 vaccination correlates with anti-viral CD8^{+} T cell quality

❖ **Poster presenter** at the 2012 Australasian Society of Immunology Annual Scientific Meeting, Melbourne, Australia

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Wijesundara DK[1], DC Tscharke[2], RJ Jackson[1], and C Ranasinghe[1]

❖ **Oral presenter** at the 2012 Australasian Society for HIV/AIDS Medicine Conference, Melbourne, Australia

Dynamic regulation of Interleukin-4 receptor alpha (IL-4Rα) following viral infections and modulation of CD8^{+} T cell avidity

Wijesundara DK[1], DC Tscharke[2], RJ Jackson[1], and C Ranasinghe[1]

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Interleukin (IL)-4/IL-13 receptor distribution on immune cells following vaccinia virus infection: implications on CD8^{+} T cell functional quality

Wijesundara DK[1], DC Tscharke[2], RJ Jackson[1], and C Ranasinghe[1]

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  Interleukin (IL)-4/IL-13 receptor distribution on immune cells following vaccinia virus infection: implications on CD8^{+} T cell functionality

  Wijesundara DK[1], DC Tscharke[2], RJ Jackson[1], and C Ranasinghe[1]

- ❖ **Oral presenter** at the 2011 University of Third Age Public Forum, Canberra, Australia

  The long road to an elusive HIV vaccine: an example of the difficulties of medical research in resolving a global health crisis

  Wijesundara DK

- ❖ **Oral presenter** at the 2011 Australian Society for HIV and Hepatitis Virology Research Conference, Brisbane, Australia

  Do IL-4Rα and IL-13Rα2 receptor expression levels on CD8^{+} T cells play a role in avidity?

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
Ad5	Adenovirus serotype 5
AEEC	The ANU Animal Experimentation and Ethics Committee
AIDS	acquired immunodeficiency syndrome
ANU	The Australian National University
APCs	antigen presenting cells
ART	anti-retroviral therapy
AUC	area under the curve
aSFV	avirulent Semliki Forest virus
A52R	KYGRLFNEI (K^dA52_{75-83}) epitope
BD	Becton Dickinson
BRF	The Biomolecular Resource Facility
cART	combined anti-retroviral therapy
CCR5	C-C chemokine receptor 5
CCR5Δ32	homozygous 32-base-pair deletion of CCR5
CFSE	carboxyfluorescein succinimidyl ester
CPD	cell proliferation dye eFluor®670
CTV	cell trace violet
DOCK2	dedicator of cytokinesis 2
DCs	dendritic cells
DiI	dialkylcarbocyanine lipophilic tracer dye
EC$_{50}$	peptide concentrations giving half maximal % specific killing
ELISPOT	enzyme-linked immunospot
Env	K^d-restricted RGPGRAFVTI epitope
FACS	fluorescence activated cell sorting

FCS	foetal calf serum
FPV	fowlpox virus
FPV-HIV	recombinant FPV encoding HIV-1 AE clade Gag, Pol and Env
FTA	fluorescent target array
F2L	SPYAAGYDL (L^dF2_{26-34}) epitope
F2L mut	L^d-restricted SP$\underline{G}$AAGYDL epitope
γc	common γ chain
Gag	AMQMLKETI ($K^dGag_{197-205}$) epitope
Gag mut	K^d-restricted AMQMLK$\underline{D}$TI epitope
Gag T_H	H-2^d-restricted PVGEIYKRWIILGLN epitope
GzmB	granzyme B
HIV	human immunodeficiency virus
HIV neg	K^d-restricted AMQML$\underline{EK}$TI negative control epitope
IFN	interferon
IFN-I	type I interferons
ICS	intracellular cytokine staining
Ig	Immunoglobulin
IL	interleukin
IL-4Rα	IL-4 receptor α
IL-13Rα1	IL-13 receptor α1
IL-13Rα2	IL-13 receptor α2
IL-13Rα2Δ10	soluble IL-13Rα2
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous

JCSMR	The John Curtin School of Medical Research
lck	lymphocytic-specific protein tyrosine kinase
LCMV	lymphocytic choriomenangitis virus
LPS	lipopolysaccharides
MAIT cells	mucosal-associated invariant T cells
MFI	mean (geometric) fluorescent intensity
MVA	modified vaccinia Ankara
NK cells	natural killer cells
NKT cells	natural killer T cells
NYVAC	Copenhagen strain of New York VV
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming units
p.i.	post-infection
pMHC	peptides-major histocompatibility complexes
pMHC-I	peptides-major histocompatibility complexes class I
pMHC-II	peptides-major histocompatibility complexes class II
Pol	K^d-restricted VGPTPVNII epitope
PRRs	pattern recognition receptors
RBC	red blood cell
RPMI	Roswell Park Memorial Institute
SEM	standard error of the mean
sIL-4Rα	soluble IL-4Rα
SIV	simian immunodeficiency virus
STAT6	signal transducer and activator of transcription 6

TCR	T cell receptor
T$_H$	T helper
TNF	tumour necrosis factor
VV	vaccinia virus
VV-HA	VV encoding hemagglutinin
VV-HA-IL-4	VV encoding murine IL-4 and hemagglutinin
VV-HIV	recombinant VV encoding HIV-1 AE clade Gag and Pol
VV-OVA$_{257-264}$	recombinant VV expressing the ovalbumin peptide SIINFEKL
VV-WR	VV Western Reserve
VV337	recombinant VV encoding HIV AE clade Env
WT	wild-type

Abstract

$CD8^+$ T cells play pivotal roles in anti-viral immunity. In particular, $CD8^+$ T cells that make high avidity interactions with virus-infected cells are extremely efficient in controlling virus infections including human immunodeficiency virus (HIV)-1. Therefore, induction of high avidity anti-viral $CD8^+$ T cells following vaccination is expected to be beneficial for conferring protection against HIV-1.

Our laboratory has shown that mucosal HIV-1 recombinant pox viral prime-boost vaccination can induce HIV-specific $CD8^+$ T cells with reduced interleukin (IL)-4 and IL-13 cytokine expression and higher avidity compared to systemic vaccine delivery. To understand how these cytokines regulated $CD8^+$ T cell avidity, the PhD studies initially evaluated the expression of receptors for these cytokines on immune cells using flow cytometry following a range of viral infections (e.g. pox viruses and influenza virus) in mice. Results indicated that unlike other IL-4/IL-13 receptor subunits, IL-4 receptor α (IL-4Rα) was significantly down-regulated on anti-viral $CD8^+$ T cells in a T cell receptor (TCR) and cognate antigen dependent manner. These studies also showed that up-regulation of IL-4Rα on naïve $CD8^+$ T cells most likely resulting from signal transducer and activator of transcription 6 (STAT6) signaling correlated with poor avidity anti-viral $CD8^+$ T cell cytokine responses. Poor avidity anti-viral $CD8^+$ T cells that developed in this instance as well as following systemic HIV-1 recombinant pox viral prime-boost vaccination expressed lower CD8 co-receptor densities. Interestingly, mucosal and IL-13 inhibitor HIV-1 recombinant pox viral prime-boost vaccination strategies prevented significant down-regulation of CD8 densities and enhanced avidity of anti-viral $CD8^+$ T cells. Collectively, data suggest that enhancing responsiveness of naïve $CD8^+$ T cells to IL-4 and IL-13 (i.e. up-regulation of IL-4Rα) primes poor avidity

anti-viral CD8$^+$ T cells with reduced CD8 densities during virus infection and recombinant HIV-1 pox viral prime-boost vaccination.

Given the poor capacity of current assays to evaluate CD8$^+$ T cell avidity *in vivo* following HIV-1 vaccination, the current PhD studies also evaluated the use of a fluorescent target array (FTA) for this purpose. The FTA assay involves measuring *in vivo* T cell responses against peptide-pulsed splenocytes (targets) that have unique fluorescent signatures (e.g. over 200 signatures) after injection into vaccinated mice. This assay was utilized to effectively screen for 24 HIV-1 recombinant pox viral vaccination regimens for the capacity to induce high avidity and epitope variant cross-reactive CD8$^+$ T cells as well as T helper (T$_H$) responses *in vivo*. Overall, the FTA was found to be an extremely versatile assay for screening vaccines that could induce high quality T cell responses *in vivo* using pre-clinical models.

Currently, the lack of knowledge regarding mechanisms that affect CD8$^+$ T cell avidity and methods that evaluate CD8$^+$ T cell avidity *in vivo* is a major barrier for developing efficacious HIV-1 vaccines. Therefore, the findings from the PhD research studies regarding how IL-4 and IL-13 regulate avidity and the use of a FTA to measure avidity *in vivo* could be exploited to foster future development of more efficacious HIV-1 vaccines.

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Chapter 1

Introduction

1.1 Context of the study

HIV-1, the causative agent of the acquired immunodeficiency syndrome (AIDS), has to date claimed over 25 million lives and over 34 million people are thought to be currently infected with this virus globally [UNAIDS World AIDS Day Report, 2012]. Although numerous care and treatment programmes including the use of anti-retroviral therapy (ART) are currently in place to suppress this global epidemic, the development of a vaccine is deemed essential to effectively eradicate HIV-1 [McElrath, 2010]. The development of a rationale HIV-1 vaccine is mainly impeded by the fact that an exact correlate of protection against this virus is unknown owing mainly to the capacity of HIV-1 to mutate rapidly and maintain latency [McMichael *et al.*, 2010]. However, several studies have isolated immune correlates that could provide effective resistance against HIV-1 [Critchfield *et al.*, 2007; Walker *et al.*, 2009; Haynes *et al.*, 2012; Julien *et al.*, 2013; Liao *et al.*, 2013; Turk *et al.*, 2013]. In particular as will be described in detail later, developing vaccination strategies that facilitate the development of high avidity anti-viral CD8$^+$ T cells are promising given that these cells are extremely efficient at controlling virus infections [Virgin and Walker, 2010].

Our laboratory findings collectively suggest that mucosal compared to systemic HIV-1 recombinant pox viral prime-boost vaccinations promote the development of high avidity anti-viral CD8$^+$ T cells most likely due to lesser induction of avidity dampening cytokines IL-4 and IL-13 [Ranasinghe *et al.*, 2007; Ranasinghe *et al.*, 2009]. This is also in agreement with Ranasinghe *et al* [2013] recent study where mucosal vaccinations with novel HIV-1 recombinant pox viral vectors that co-express an IL-13 inhibitor protein (soluble IL-13 receptor α2 (IL-13Rα2Δ10) were shown to enhance anti-viral CD8$^+$ T cell avidity. Recombinant pox virus vaccine vectors are commonly incorporated in HIV-1 prime-boost vaccination regimens in human clinical trials.

Therefore, understanding how IL-4 and IL-13 regulate anti-viral CD8$^+$ T cell avidity following pox viral infections and vaccinations is expected to be important for developing effective HIV-1 prime-boost vaccination regimens. Furthermore, engineering screening tools that can effectively evaluate avidity *in vivo* is also important for this purpose especially for HIV-1 vaccination regimens that aim to induce high avidity anti-viral CD8$^+$ T cells *in vivo*.

1.2 The immune system: an overview of innate and adaptive immunity

1.2.1 Overview

Many infectious microorganisms (i.e. pathogens) such as viruses need to infect a certain host in order to survive. Once pathogens circumvent the numerous host physical barriers of infection (e.g. epithelial surfaces, skin, mucus, saliva, tears, etc.), the functioning of the immune system is critical for limiting/eliminating pathogen infections from the host. In vertebrates, the immune system is comprised of innate and adaptive components; the roles of these components in controlling pathogen infections will be overviewed in this section.

1.2.2 Innate immunity: the first line of immune defense against pathogens

All cells of the blood including the immune system are derived from hematopoietic stem cells in the bone marrow through a process referred to as hematopoiesis [Sabin *et al.*, 1936]. During hematopoiesis, various cell types with the potential of exerting effector functions and/or initiating adaptive immune responses against pathogens emerge (**table 1.1**). In innate immunity, macrophages, dendritic cells (DCs), neutrophils, basophils, mast cells, natural killer (NK) cells, mucosal-associated invariant T (MAIT) cells, nuocytes, γδ T cells and NK T (NKT) cells respond immediately (i.e.

Table 1.1. Key functions of major cell types involved in the immune system.

Cell type	Key functions in immunity	Reference
Macrophages	❖ Destroy pathogens via phagocytosis, nitric oxide production or anti-microbial peptide production ❖ Present antigens to T cells to help initiate adaptive immunity ❖ Induce inflammation in response to pathogen infections to recruit other immune cells	∂
DCs	❖ Present antigens to T cells to help initiate adaptive immunity	Ø
Neutrophils	❖ Trap and destroy bacteria via extrusion of chromatin fibers and phagocytosis ❖ Induce inflammation in response to pathogen infections to recruit other immune cells	Π
Basophils, eosinophils and mast cells	❖ Produce toxic proteins (e.g. histamines) and enzymes to destroy parasitic worm infections ❖ Induce allergic inflammatory reactions in some instances such as asthma	Ω
NK cells	❖ Recognize and mediate cytolysis of pathogen-infected or tumor cells to control infection or tumor growth	Δ
NKT cells	❖ Produce immunoregulatory cytokines to help clear bacterial/parasitic infections and tumors ❖ Lysis of tumors	¶
MAIT cells	❖ Recognize conserved bacterial-derived ligands and protect hosts against bacterial infections	η
Nuocytes	❖ Produce type-2 cytokines such as IL-13 to help expel helminth infections	π
$\gamma\delta$ T cells	❖ Produce cytokines such as IFN-γ and IL-17 and exert cytotoxic responses to help resolve tumors and pathogen infections	ß
Tregs	❖ Remove T cell clones that can react against self so as to prevent autoimmunity ❖ Shut down immune responses to prevent immunopathology	§
CD8^{+} T cells	❖ Adaptive recognition and cytolysis of pathogen-infected cells or tumors to control infection or tumor growth	†
CD4^{+} T cells	❖ Produce cytokines that help activate and regulate the function of other immune cells such as B cells, macrophages and CD8^{+} T cells	€
B cells	❖ Produce antibodies that prevent infection of cells by pathogens ❖ Produce antibodies that can opsonize pathogens for phagocytosis	¥

∂ [Evans *et al.*, 1914; Murray and Wynn, 2011]

Ø [Steinman and Cohn, 1973; Steinman and Witmer, 1978]

Π [Page and Good, 1958; Kaplan and Radic, 2012]; Ω [Rothwell and Dineen, 1972; Akdis, 2012]

Δ [Kiessling *et al.*, 1975; Jost and Altfeld, 2013]; ¶ [Sumida *et al.*, 1984; Godfrey *et al.*, 2000]

η [Le Bourhis *et al.*, 2010]; π [Neill *et al.*, 2010]; ß [Born *et al.*, 2006]

§ [Sakaguchi, 2004]; † [Cerottini *et al.*, 1970; Freedman *et al.*, 1972; Zinkernagel *et al.*, 1974]

€ [Cantor and Boyse, 1975; Abbas *et al.*, 1996]; ¥ [Andersson *et al.*, 1972; Ahmed and Gray, 1996]

within hours) following pathogen exposure to either destroy or limit systemic spread of pathogens (**table 1.1; figure 1.1**). Macrophages and DCs cells are also professional antigen presenting cells (APCs) [Mosier, 1967; Steinman and Witmer, 1978]. Following pathogen ingestion, these APCs undergo maturation to express costimulatory molecules/danger signals (i.e. CD80/CD86) and present pathogen derived peptides on major histocompatibility complexes (pMHC) to CD4$^+$ and CD8$^+$ T cells to initiate adaptive immune responses (**figure 1.1**) [Hathcock *et al.*, 1994]. Furthermore, a group of plasma protein forming the complement cascade as well as circulating natural immunoglobulin (Ig)M antibodies also function in innate immunity (**figure 1.1**). These proteins help to opsonize pathogens for destruction, induce inflammation to recruit other immune cells and induce antibody responses during adaptive immunity against pathogens (**figure 1.1**) [Baugmarth *et al.*, 1999; Sprong *et al.*, 2004].

A seminal feature of all immune responses is the ability to distinguish self from non-self pathogen-associated components. Cells of the innate immune system use pattern recognition receptors (PRRs) for this purpose [Janeway and Medzhitov, 2002]. PRRs recognize evolutionary conserved pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides (LPS), bacterial flagellin, unmethylated CpG dinucleotide motifs, viral single/double-stranded RNA and viral DNA [Kawai and Akira, 2010]. Recognition of PAMPs in this instance triggers innate immune cells to exert effector functions (**table 1.1**) and induce inflammation to recruit other immune cells such as B and T cells of adaptive immunity to sites of infection [Iwasaki and Medzhitov, 2010].
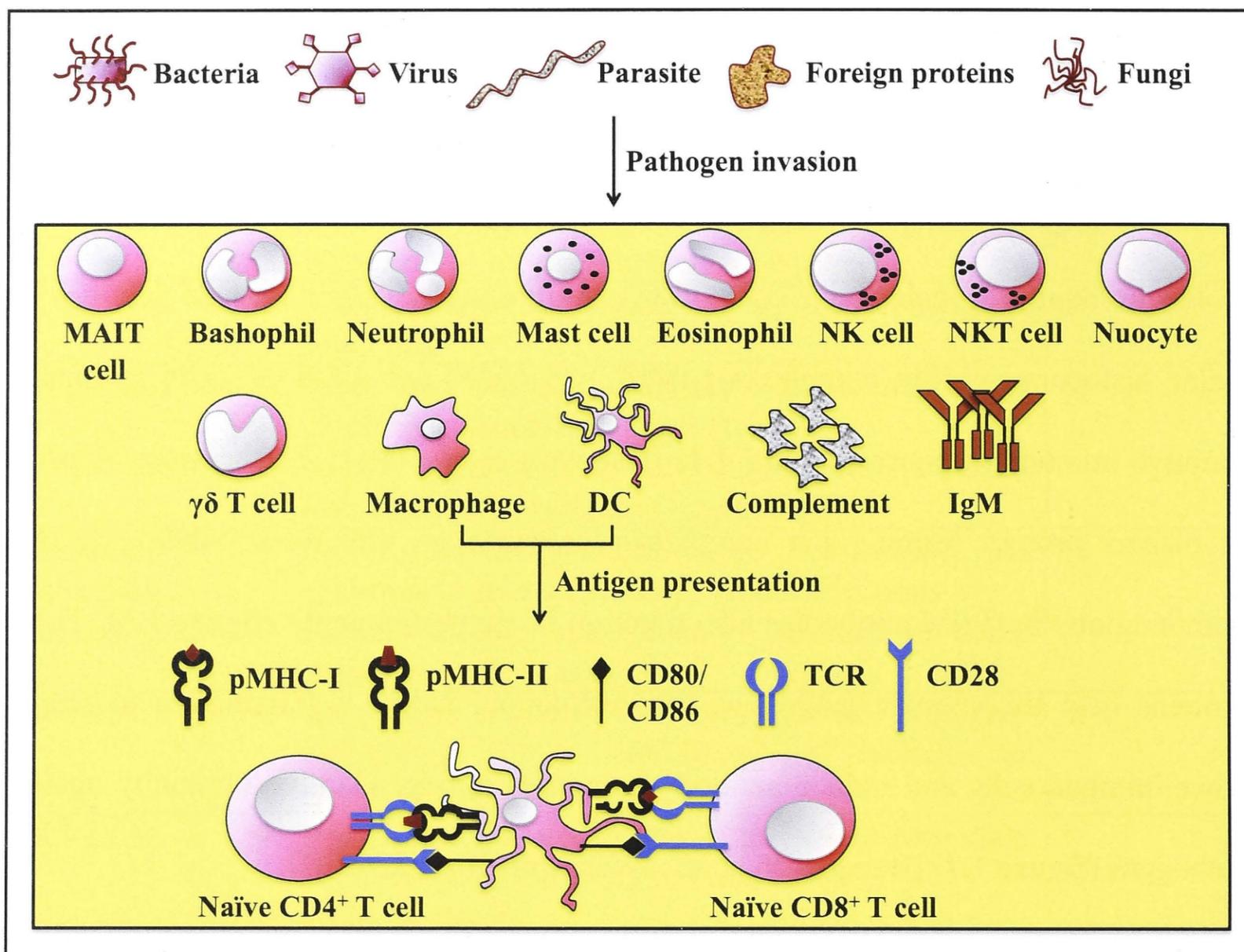
Figure 1.1. Innate immune barriers to pathogen invasion. Once pathogens (i.e. bacteria, virus, parasite and fungi) and/or foreign proteins invade a host, the innate immune system acts immediately to combat these foreign invaders. Numerous cell types illustrated above (also see **table 1.1**) together with plasma proteins (i.e. complement and IgM) collaboratively act to destroy pathogens and induce inflammation to recruit more immune cells. Furthermore, macrophages and DCs also present costimulatory molecules (CD80/CD86) and peptides from ingested pathogens on MHC-II or MHC-I to activate naïve CD4^{+} T cells or naïve CD8^{+} T cells respectively. Presentation of peptide antigens in this instance is important for initiating adaptive immune responses.

1.2.2 Adaptive immunity: lymphocyte selection and T cell activation

Innate immunity is sufficient to clear most pathogen infections, but a second line of immune defense known as adaptive immunity is sometimes required for pathogens to be efficiently cleared (**table 1.2**). Adaptive immunity is mainly comprised of lymphocytes such as B cells, helper CD4$^+$ T (T$_H$) cells and CD8$^+$ T cells (**figure 1.2; table 1.1**). Similar to PRRs of innate immune cells, B cells use the B cell receptor (BCR) or T cells use the TCR to recognize pathogens [Bentley and Mariuzza, 1996; Schamel and Reth, 2000]. However, each B or T cell clone has a receptor with a unique specificity to antigens and the assembly process of these receptors allow a broader array of antigens to be recognized compared to PRRs [Burnet, 1959; Schatz and Swanson, 2011]. To eliminate self-reactive clones with the potential of causing autoimmunity BCRs or TCRs that recognize self-antigens with high avidity undergo apoptosis during development in a process referred to as central tolerance [Burnet, 1959; Miller, 1961; Derbinski et al., 2001; Pieper et al., 2012]. Naïve (i.e. foreign antigen inexperienced) B and T cell clones that have bypassed central tolerance then circulate in the periphery and drain into secondary lymphoid organs (i.e. spleen and lymph nodes) where mature APCs present antigens derived from pathogens.

For naïve T cells to clonally expand and differentiate into effector cells at least two activation signals are required [Lafferty and Cunningham, 1975]. The first signal is derived following engagement of the TCR with cognate pMHC class I (pMHC-I) in the case of CD8$^+$ T cells or cognate pMHC class II (pMHC-II) in the case of CD4$^+$ T cells (**figure 1.1**). The second signal is derived following engagement of the CD28 co-receptor on T cells with costimulatory molecules (CD80/CD86) on APCs, which is usually up-regulated on APCs following recognition of PAMPs (**figure 1.1**) [Hathcock et al., 1994].

Table 1.2. Key features that distinguish innate and adaptive immunity.

Feature	Innate immunity	Adaptive immunity
Major cell types involved in pathogen defense	Mast cells, neutrophils, macrophages, DCs, basophils, eosinophils, NK cells, NKT cells, MAIT cells, nuocytes and $\gamma\delta$ T cells	T (CD4$^+$ and CD8$^+$) cells and B cells
Lifespan of cells involved	Short	Long
Pathogen recognition and adaptability	Recognition is fixed (evolutionary conserved features of pathogens (i.e. PAMPs) recognized through PRRs)	Recognition is highly adaptive (specific components of pathogens recognized through TCR (T cells) or BCR (B cells))
Immunological memory	Absent	Present
Speed of pathogen specific responses	Shortly after pathogen encounter (i.e. within hours)	Delayed in the absence of immunological memory (i.e. 4-7 days)
Tolerance to prevent self reactivity	Absent	Present
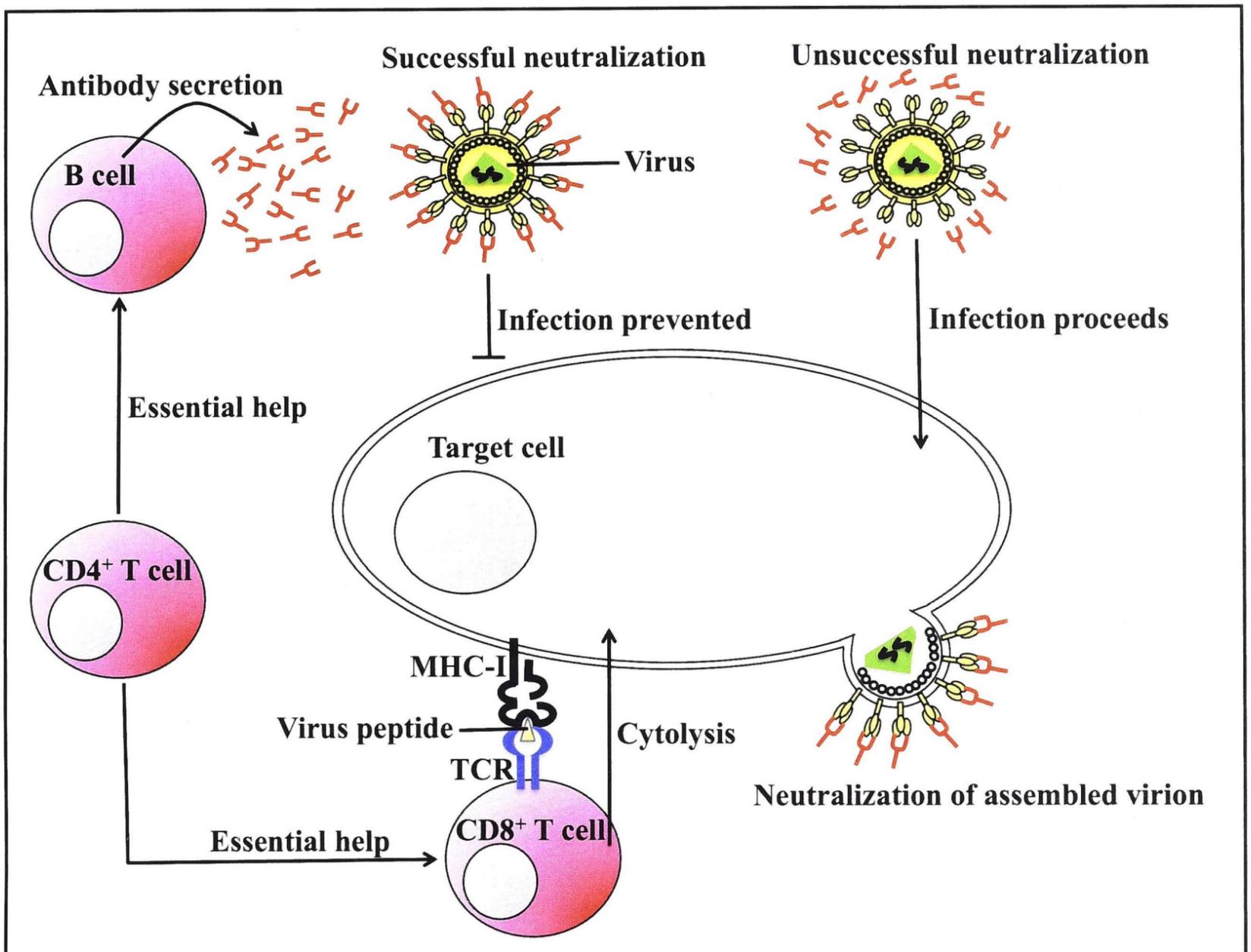
Figure 1.2. Key functions of major cell types involved in adaptive immunity against virus (HIV-1) infection. CD4^{+} T cells, CD8^{+} T cells and B cells have all been known to play an important role in either preventing or limiting HIV-1 infection. B cells secrete antibodies with the potential to neutralize this virus. Successful neutralization requires antibodies to bind to the protruding viral envelope glycoproteins that are crucial for mediating viral entry into target cells. Failure to do so allows HIV-1 to infect target cells. Further infections with HIV-1 can be prevented or limited either via antibodies that can effectively neutralize newly assembled virions or via effector CD8^{+} T cells that can mediate cytolysis of infected cells following TCR mediated recognition of virus pMHC-I complexes. Effector CD4^{+} T cells provide help for efficient mobilization of B cell and CD8^{+} T cell mediated immune responses against HIV-1.

The costimulatory second signal (i.e. danger signal) appears to be required for naïve T cells to up-regulate bcl-x_L expression and survive during T cell activation [Boise et al., 1995]. Interestingly, there are also studies to suggest that a distinct third signal (IL-1 in case of CD4^{+} T cells and type I interferons (IFN-I) or IL-12 in case of CD8^{+} T cells) is also required for optimal clonal expansion and effector differentiation of naïve T cells [Curtsinger et al., 1999; Curtsinger et al., 2007].

Once naïve T cells develop into armed effectors, only signal one (cognate pMHC) is required for triggering of effector functions (**figure 1.2**). Some effector T cells survive long after pathogen is cleared from the infected hosts and are referred to as memory T cells [Flynn et al., 1998; Reinhardt et al., 2001]. These cells can respond immediately after re-exposure with the same pathogen unlike naïve T cells allowing for even more rapid clearance of the pathogen compared to the initial pathogen exposure.

Overall, the immune system is comprised of a highly specialized network of cells that collaborate efficiently and exert various effector functions to eliminate foreign invaders (e.g. pathogens). This system is also specialized to distinguish self from non-self components allowing infected hosts to specifically destroy pathogens.

1.3 Adaptive immunity against HIV-1

1.3.1 Overview

The hallmark of rationale vaccine design is to identify adaptive immune responses that can be protective against a pathogen and induce these responses using safe/attenuated vaccine vectors. In this section, the importance of adaptive immune responses involving

B cells (antibodies), CD8$^+$ T cells and CD4$^+$ T cells in the control of HIV-1 infections is discussed.

1.3.2 Importance of antibodies against HIV-1

All known viruses including HIV-1 need to infect cells in order to replicate and survive. The successful prevention of HIV-1 infections or *trans*-infection of target cells (e.g. DCs and CD4$^+$ T cells) is mainly reliant on the availability of HIV-1 neutralizing antibodies [McDonald *et al.*, 2003; Yu *et al.*, 2008; Mascola and Montefiori, 2010]. To infect cells, HIV-1 envelope proteins need to interact with the CD4 receptor and the co-receptor C-C chemokine receptor 5 (CCR5)/CXC-chemokine receptor-4 of cells [Dalgleish *et al.*, 1984; Deng *et al.*, 1996; Bleul *et al.*, 1996]. Neutralizing antibodies that bind to the viral surface envelope protein usually prevent this interaction and infection of cells (**figure 1.2**) [Lasky *et al.*, 1986; Weiss *et al.*, 1986]. Binding of antibodies to the viral envelope of viruses about to bud out from infected cells could also neutralize viruses through activation of NK cell antibody-dependent cellular cytotoxicity (ADCC) machinery [Chung *et al.*, 2011; Haynes *et al.*, 2012; Tomaras *et al.*, 2013]. Although HIV-1 neutralizing antibodies are known to occur following natural infection with this virus, these antibodies are largely ineffective owing to extensive mutations, glycosylation and conformational masking of the envelope protein [Kwong *et al.*, 2002; Dhillon *et al.*, 2007; Mascola and Montefiori, 2010]. Given that the ancestor of HIV-1, simian immunodeficiency virus (SIV), can be transmitted as a cell-associated form, HIV-1 might also be transmitted in a similar manner in which case neutralizing antibodies could be ineffective [Sodora *et al.*, 1998]. Nonetheless, numerous research groups have recently identified monoclonal antibodies even during natural infections that bind to conserved regions of HIV-1 envelope and neutralize a broad spectrum of HIV-1 quasi-species [Zhou *et al.*, 2010; Walker *et al.*, 2011; Huang

et al., 2012; Julien et al., 2013; Liao et al., 2013]. The discovery of these broadly neutralizing antibodies has generated much enthusiasm and hope for the development of HIV-1 vaccines.

1.3.3 Importance of CD8$^+$ T cells against HIV-1

If antibodies fail to prevent HIV-1 infection then effector CD8$^+$ T cells play a crucial role in limiting further infections (**figure 1.2**). Upon recognition of cognate pMHC-I, effector CD8$^+$ T cells mediate apoptosis of virus-infected cells either through the interaction of Fas ligand with Fas on target cells or through the release of cytolytic granules (e.g. perforin and granzymes) [Lieberman, 2003]. Lysis of infected cells prevents further viral replication and results in the release of immature HIV-1 virions, which are rapidly degraded in the extracellular milieu [Walker and Burton, 2008]. To date, the best vaccines that confer protection against SIV in simian models are live attenuated SIV vaccines [Daniel et al., 1992]. A recent study by Fukuzawa et al [2012] suggests that protection of macaques that were vaccinated with various live attenuated vaccines was dependent on effector-differentiated CD8$^+$ T cell responses in the lymph nodes. A few studies from the same group where macaques were vaccinated with a persistent recombinant rhesus cytomegalovirus prior to SIV challenge also suggest that CD8$^+$ T cell responses could protect against SIV challenge [Hansen et al., 2011; Hansen et al., 2013]. Data from simian models suggest that CD8$^+$ T cells could play pivotal roles in protection against HIV-1. The effectiveness of CD8$^+$ T cells in suppressing HIV-1 replication appears to be primarily due to responses against internal viral epitopes (e.g. Gag) that are well conserved within and across viral subtypes [Yusim et al., 2002; Robinson, 2002; Kunwar et al., 2013]. Indeed, as will be discussed in **section 1.4** numerous studies have shown that elite controllers or long-term non-progressors

who are resistant to HIV-1 following infection exhibit enhanced HIV Gag-specific CD8$^+$ T cell activity (e.g. cytotoxicity, avidity and/or polyfunctionality) compared to more susceptible individuals [Pontesilli *et al.*, 1998; Betts *et al.*, 2006; Almeida *et al.*, 2007; Critchfield *et al.*, 2007; Ferre *et al.*, 2010; Berger *et al.*, 2011; Turk *et al.*, 2013].

1.3.4 Importance of CD4$^+$ T cells against HIV-1

HIV-specific CD4$^+$ T cells appear to be particularly susceptible for HIV-1 infection and memory CD4$^+$ T cells given their long life-span are ideal reservoirs for viral latency [Douek *et al.*, 2002; Bosque and Planelles, 2009; Perreau *et al.*, 2013]. Despite this, CD4$^+$ T cell activation may yet be more favourable than detrimental to the HIV-1 infected host [Virgin and Walker, 2010]. This is especially true given that T$_H$ cells help activate B cells and appear to provide essential help (e.g. IL-21 secretion, sustaining CD8$^+$ T cell cytotoxicity and mucosal homing) to CD8$^+$ T cells responding against HIV-1 [Virgin and Walker, 2010; Chevalier *et al.*, 2011]. The requirement of CD4$^+$ T cells for combating HIV-1 is still a controversial issue given that the presence of CD4$^+$ T cells at sites of HIV-1 infections may fuel virus replication, but CD4$^+$ T cell help is known to be required for optimizing B cell and CD8$^+$ T cell responses.

1.4 CD8$^+$ T cell avidity: definition, mechanisms and importance in HIV-1 control

CD8$^+$ T cell avidity or functional avidity is classically defined by the amount/strength of cognate pMHC-I interaction required to trigger effector responses with high avidity cells capable of responding to lower amounts of cognate pMHC-I than low avidity counterparts (**figure 1.3**) [Alexander-Miller *et al.*, 1996; Zeh *et al.*, 1999; Derby *et al.*, 2001]. High avidity anti-viral CD8$^+$ T cells also appear to produce higher amounts of anti-viral cytokines interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-2
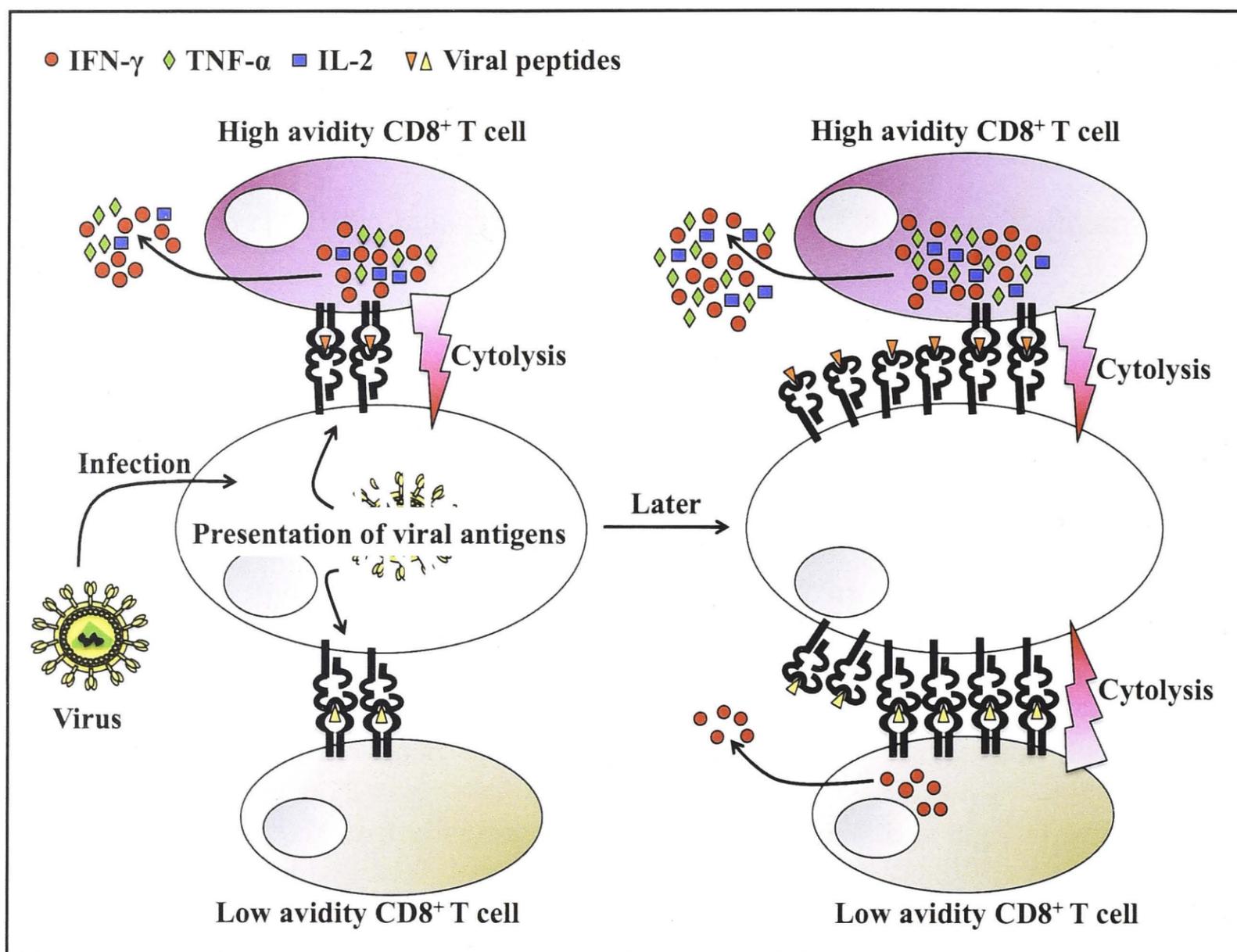
Figure 1.3. High avidity CD8⁺ T cells control virus infections more efficiently than low avidity CD8⁺ T cells. Early on following viral infection of a target cell, lower levels of viral proteins are produced and presented on MHC-I molecules compared to a infected target cell at a later phase. Therefore at early stages of infection high avidity CD8⁺ T cells respond against infected cells given that these cells require lower amounts of cognate pMHC-I than low avidity CD8⁺ T cells to trigger effector functions (i.e. cytolysis and production/secretion of cytokines). High avidity CD8⁺ T cells are also polyfunctional and produce/secrete TNF-α, IFN-γ and IL-2. Low avidity CD8⁺ T cells can only mediate cytolysis of infected target cells at a later phase where these cells express greater amounts of cognate viral pMHC-I than early-infected cells. Hence, low avidity CD8⁺ T cells are poor controllers of virus infection. Low avidity CD8⁺ T cells are also thought to be poorly polyfunctional and produce low levels of IFN-γ, but not significant levels of TNF-α and IL-2.

compared to low avidity anti-viral CD8$^+$ T cells [Almeida *et al.*, 2007; Almeida *et al.*, 2009]. Thus, high avidity anti-viral CD8$^+$ T cells are expected to respond against virus-infected cells much earlier during infection and control viral infections more efficiently than low avidity anti-viral CD8$^+$ T cells.

There are several cell-associated components that could regulate the functional avidity of CD8$^+$ T cells. Components/molecules that have been reported to enhance the functional avidity include: 1) increasing TCR affinity to pMHC-I [Busch and Pamer, 1999], 2) enhancing expression levels of TCR, CD8 $\alpha\beta$ co-receptor and adhesion molecules such as CD2 and CD11a [Springer *et al.*, 1987; Alexander *et al.*, 1991; Viola and Lanzavecchia, 1996], 3) increasing cholesterol content to allow the formation of lipid rafts [Fahmy *et al.*, 2001], and 4) amplifying intracellular signalling through enhanced expression or activity of molecules such as lymphocytic-specific protein tyrosine kinase (lck) [Bachmann *et al.*, 1999; Slifka and Whitton, 2001]. The expression levels, affinity or activity of these molecules could also be down-regulated to reduce the functional avidity of CD8$^+$ T cells. There are also several inhibitory molecules that could serve to reduce the functional avidity of CD8$^+$ T cells. These include CD5 mediated inhibition of TCR signalling [Azzam *et al.*, 1998; Perez-Villar *et al.*, 1999] and expression of molecules that can exhaust the function of CD8$^+$ T cells such as programmed death-1 and cytotoxic T lymphocyte-associated antigen-4 [Nakamoto *et al.*, 2009].

Despite the knowledge regarding molecules that can regulate functional avidity, the mechanisms involved in modulating functional avidity of anti-viral CD8$^+$ T cells are not thoroughly understood and appear to be dependent on the virus infection. Following lymphocytic choriomenangitis virus (LCMV) infections, enhancing lck expression is a feature of LCMV-specific CD8$^+$ T cells undergoing avidity maturation [Slifka and

Whitton, 2001]. During pox virus infections enhancement in CD8αβ co-receptor densities have been predictive of avidity maturation of anti-viral CD8+ T cells [Oh *et al.*, 2004; Xiao *et al.*, 2007]. Furthermore, qualitative differences in TCR assembly/clonotypes (i.e. use of Vβ7) rather than enhancement in expression of molecules such as TCR, CD8, lck, CD11a and CD2 are predictive of avidity on influenza-specific CD8+ T cells [Kedzierska *et al.*, 2005; La Gruta *et al.*, 2006]. There are also various other CD8+ T cell extrinsic factors that could regulate anti-viral CD8+ T cell avidity. These include T_{regs} [Pace *et al.*, 2012], cytokines (e.g. IL-4, IL-12, IL-13, IL-15 and IFN-I) [Xu *et al.*, 2003; Oh *et al.*, 2004; Xiao *et al.*, 2007; Ranasinghe *et al.*, 2009], DC costimulation [Oh *et al.*, 2003; Hodge *et al.*, 2005] and DC subsets [Ranasinghe *et al.*, 2013]. Overall, regulation of anti-viral CD8+ T cell avidity is dependent on the context of virus infection and is multifactorial.

During HIV-1 infections, high avidity HIV-specific CD8+ T cells appear to be important to minimise or prevent the spread of HIV-1 from early-exposed sites (e.g. genito-rectal mucosa) to the gut where the greatest CD4+ T cells depletion occurs [Wijesundara *et al.*, 2011]. Several studies in macaques suggest that high avidity anti-viral CD8+ T cells can protect against SIV [Belyakov *et al.*, 2001; Belyakov *et al.*, 2006; Belyakov *et al.*, 2007]. Furthermore, numerous studies have suggested that resistance to HIV-1 infection in elite controllers correlates with enhanced high avidity and polyfunctional HIV-1 Gag-specific CD8+ T cell responses [Pontesilli *et al.*, 1998; Betts *et al.*, 2006; Almeida *et al.*, 2007; Critchfield *et al.*, 2007; Ferre *et al.*, 2010; Berger *et al.*, 2011; Turk *et al.*, 2013]. High avidity HIV-specific CD8+ T cells also have the capacity to recognize mutant viral epitopes and are therefore highly cross-reactive [Mothe *et al.*, 2012]. Overall, high avidity HIV-specific CD8+ T cells appear to play critical roles in protection against HIV-1.

1.5 Strategies for developing a functional cure against HIV-1

1.5.1 Overview

Worldwide HIV-1 currently has infected over 60 million people and claims 2.5 million new infections every year [UNAIDS Special Report, 2013]. Given the latency capacity of this virus it is extremely difficult to develop a complete cure against HIV-1. However, therapeutic or prophylactic approaches that can reduce viral loads to undetectable levels and maintain an infected individual's health similar to that of an uninfected individual are deemed achievable. These approaches are referred to as strategies for developing a functional HIV-1 cure [Deeks *et al.*, 2012]. Here the prospects of the three major approaches (stem cell therapy, ART and vaccination) for developing a functional cure against HIV-1 are briefly reviewed.

1.5.2 Stem cell therapy against HIV-1

Some Caucasians express a truncated version of CCR5 (homozygous 32-base-pair deletion of CCR5 (CCR5Δ32)) that allow them to effectively resist HIV-1 infections [Samson *et al.*, 1996]. This knowledge was used by a team of transplant surgeons in Germany to treat a patient (referred to as the "Berlin Patient") who was suffering from leukaemia and HIV-1 infection [Hütter *et al.*, 2009]. Subsequently, the first ever HIV-1 cure was announced due to the Berlin Patient receiving allogenic hematopoietic stem cell transplantation from a CCR5Δ32 donor [Hütter *et al.*, 2009]. However, grand-scale therapeutic application of this curative strategy is expected to be limited due to the lack of MHC-matched donors, toxicity and cost. To circumvent these issues, researchers are currently developing cost-effective gene therapy strategies to knockout CCR5 expression on T cells and hematopoietic stem cells for autologous transplantation purposes [Cannon and June, 2011].

1.5.3 Anti-retroviral therapy against HIV-1

The use of drugs such as ART or combined ART (cART) for inhibiting various stages of the virus life cycle (e.g. inhibiting viral reverse transcriptase, protease and integrase activity) have been the most successful strategy to treat HIV-infected individuals [Volberding and Deeks, 2010]. These strategies under strict patient adherence can be used to functionally cure HIV-1 infected individuals: reduce HIV-1 viral loads to undetectable levels, improve $CD4^+$ T cell counts and survival of HIV-1 infected patients [UNAIDS World AIDS Day Report, 2012]. A recent clinical trial (HPTN 052) also showed that early (350-550 $CD4^+$ T cells/mm^3 blood) compared to late (<250 $CD4^+$ T cells/mm^3 blood) cART treatment of HIV-1 infected individuals significantly reduces HIV-1 transmission in serodiscordant couples [Cohen *et al.*, 2011]. Early diagnosis and treatment with ART drugs (e.g. Raltegravir) that inhibit viral DNA integration into the genome of host $CD4^+$ T cells could also be important in preventing significant loss of $CD4^+$ T cells during acute phase infections [Cooper *et al.*, 2013]. Thus, immunodeficiency related deaths or morbidity resulting from opportunistic co-infections such as tuberculosis, hepatitis, malaria and cryptococcosis might also be averted from early cART treatment [Chang *et al.*, 2013].

Despite the enormous potential and success of cART, its use to treat about 34 million HIV-1 infected individuals is deemed to be too costly and difficult to sustain especially given that drugs in cART regimens need to be taken on a daily basis [Andrieux-Meyer *et al.*, 2012]. Ideally, a true functional cure will also not require life-long treatment and an immune intervention following cART treatment to bolster durable suppressive anti-viral immune responses might be required to improve the feasibility of using cART as a functional cure. There have also been reports of HIV-1 drug resistance [Masimba *et al.*, 2013], lack of patient adherence to cART regimens [Charurat *et al.*, 2010] and adverse

side-effects associated with the use of cART [Rather *et al.*, 2013]. Furthermore, cART cannot be used to deplete latent reservoirs of HIV-1 [Vandergeeten *et al.*, 2013].

1.5.4 Vaccination against HIV-1

Since Edward Jenner pioneered vaccination during the 19th century, vaccines have been used to eradicate smallpox and prevent human infections such as measles, polio and yellow fever [Fenner *et al.*, 1988; Rueckert and Guzmàn, 2012]. For HIV-1, vaccines can be used for immunoprophylaxis and are also expected to be much more cost-effective than ART especially given that vaccines have the potential to induce long-lasting protective immune responses. However, historically the use of vaccines in phase IIb (STEP trial) and phase III (VAX003, VAX004 and RV144) HIV-1 human clinical trials have been met with failure to induce significant protective outcomes [Flynn *et al.*, 2005; Pitisuttithum *et al.*, 2006; Buchbinder *et al.*, 2008; Rerks-Ngarm *et al.*, 2009]. The lack of natural immunity and immune correlates of protection against HIV-1 have also impeded progress for developing a HIV-1 vaccine [Wijesundara *et al.*, 2011; Sanou *et al.*, 2012]. Furthermore, the reasons for poor translatability of findings in animal models and the failures of HIV-1 vaccines tested in human clinical trials are unclear. For instance, it was thought that the Adenovirus serotype 5 (Ad5) vaccine vector used in the STEP trial induced protective CD8^{+} T cell responses in macaques, but not in humans due to pre-existing immunity against Ad5 in humans [Cohen, 2013]. However, the recent failure of the HVTN 505 trial suggests that this was not the case and the use of Ad5 for vaccination in humans could increase the risk of HIV-1 acquisition [Cohen, 2013; Hammer *et al.*, 2013]. Furthermore, the vaccine used in this trial was ineffective in inducing Gag-specific CD8^{+} T cell responses and predominantly induced CD8^{+} T cell responses against the highly variable HIV-1 envelope protein [Hammer *et al.*, 2013].

Less than 1% of HIV-infected individuals, referred to as elite controllers, have the capacity to control HIV-1 effectively to undetectable levels (usually <50-75 viral RNA copies per ml of plasma) [Deeks and Walker, 2007]. Therefore, understanding immune responses that allow elite controllers to resist HIV-1 infections and developing vaccines that will induce these responses is a rationale path for developing a HIV-1 vaccine. As discussed in **section 1.3 and 1.4**, the responses elucidated thus far that correlate with resistance include HIV-1 broadly neutralizing antibodies and high avidity/polyfunctional $CD8^+$ T cell responses particularly against HIV-1 Gag epitopes.

Apart from elite controllers understanding immune correlates of protection in the RV144 clinical trial, which reported a 30% protective efficacy is another attractive approach for developing a HIV-1 vaccine [Rerks-Ngarm *et al.*, 2009]. In the RV144 trial the induction of IgG, but not IgA antibodies against HIV-1 envelope that help mediate ADCC in vaccinated individuals have correlated with protection [Haynes *et al.*, 2012; Tomaras *et al.*, 2013]. Overall, mucosal (e.g. transmission sites like genito-rectal mucosa) and systemic induction of broadly neutralizing antibodies, ADCC antibodies and high avidity $CD8^+$ T cells that respond against conserved HIV-1 antigens are all promising targets when designing prophylactic HIV-1 vaccines.

The use of therapeutic vaccines to boost HIV-specific immune responses and reduce viral loads in HIV-1 infected individuals could be difficult due to several reasons: 1) pre-existing immunity and/or high viral loads could reduce lymphocyte proliferation [Moss *et al.*, 2002], 2) continuous immune stimulation can lead to anergy and immune exhaustion [Day *et al.*, 2006; Carcelain and Autran, 2013], 3) the existence of escape mutants of immune surveillance [Geels *et al.*, 2003], and 4) latent viral reservoirs can be found in immunologically privileged sites and expansion of $CD4^+$ T cells could increase

the number of viral reservoirs [Pierson *et al.*, 2000]. Passive transfer of broadly neutralizing antibodies and vector-based immunoprophylaxis are emerging novel approaches that could be more successful for therapeutic purposes [Nishimura *et al.*, 2003; Balazs *et al.*, 2012; Diskin *et al.*, 2013]. Given that neutralizing antibodies can be engineered to have a long half-life *in vivo* [Zalevsky *et al.*, 2010], these approaches could also be cost-effective surrogates for cART treatment.

The use of vaccines for prophylactic and therapeutic purposes is expected to be more cost-effective and sustainable for developing a functional cure against HIV-1 than cART. Rationale vaccine design against HIV-1 will require vaccines that will effectively induce immune responses that correlate with HIV-1 resistance in elite controllers and protection in the RV144 clinical trial. However, this is not straightforward and requires detailed understanding of mechanisms that facilitate the induction of high avidity CD8$^+$ T cells, broadly neutralizing antibodies and ADCC.

1.6 Poxviruses: why are they ideal vaccine vectors?

Since Edward Jenner inoculated individuals with cowpox virus to confer protection against smallpox, the use of pox viruses for developing vaccines against numerous deadly pathogens such as malaria, influenza and HIV-1 is currently commonplace. Significant efforts have also been devoted for the use of recombinant Ad5 vectors for HIV-1 vaccination in humans, but recent clinical trials suggest that Ad5 vectors could increase the risk of HIV-1 acquisition and are not safe for human use [Buchbinder *et al.*, 2008; Cohen, 2013]. The characteristics of pox viruses are described thoroughly in McFadden [2005] and summarized in **table 1.3**. Amongst the two subfamilies, species belonging to the *Chordopoxvirinae* (**table 1.3**) are used for vaccine development and the features that make them ideal for this purpose are listed below:

1) These viruses have large genome and packaging flexibility allowing for deletion of large amounts of viral DNA (at least 25 kb) for insertion of multiple antigens/adjuvants of interest [Smith and Moss, 1983].

2) They induce robust long-lasting T and B cell responses even after a single inoculation [Gherardi and Esteban, 1999].

3) Recombinants of these viruses can be easily grown and stored freeze-dried making them easy to manufacture and administer [Collier, 1955].

4) Pox viruses are safe or can be engineered (e.g. deletion of virulent genes) to be safe for use in humans.

    ❖ Recombinant Avipox viruses such as fowlpox virus (FPV) and canarypox virus do not replicate in human cells and are safe for use in humans [Boyle *et al.*, 2004; Pitisuttithum, 2005; Kelleher *et al.*, 2006].

    ❖ Thymidine kinase deficient strains of vaccinia virus (VV) and attenuated strains of VV such as Copenhagen strain of New York VV (NYVAC) and modified vaccinia Ankara (MVA) that do not replicate in human cells are safe for use in humans [Moss, 1996].

5) Pox viruses when used as vaccines have rendered protective outcomes against human and animal diseases. Few examples are listed below:

    ❖ VV was used for the global eradication of smallpox [Fenner *et al.*, 1988].

    ❖ Recombinant VV vaccines were used to vaccinate wild foxes to reduce rabies incidences in Europe [Pastoret *et al.*, 1988].

Table 1.3. Characteristics of pox viruses.

Characteristic	Description
Subfamilies	Two subfamilies: 1. *Chordopoxvirinae* (infects vertebrates) 2. *Entopoxviridae* (infects invertebrates/insects)
Genera of *Chrodopoxvirinae*	Eight genera for *Chordopoxvirinae*: 1. Orthopox (e.g. ectromelia, monkeypox, vaccinia and variola) 2. Parapox (e.g. pseudocowpox and somatitis papulosa) 3. Avipox (e.g. canarypox, fowlpox and quailpox) 4. Capripox (e.g. goatpox and sheep pox) 5. Leporipox (e.g. myxoma) 6. Suipox (e.g. swinepox) 7. Molluscipox (e.g. molluscum contagiosum) 8. Yatapox (e.g. tanapox)
Genome	Linear double stranded DNA genome (130-300 kb) with a hairpin loop at each end
Size	~300 nm
Morphology	Ovoid or brick-shaped enveloped viruses
Replication	Exclusively in the cytoplasm of cells
Host range	Host range depends on the species of virus (e.g. ectromelia virus (mice), FPV (chicken), variola virus (humans) and VV (natural host unknown, but infects human and mice)
Transmission	Most commonly transmitted through inhalation of virus droplets, but can also be transmitted through direct skin contact
Genetic similarities	Considerable genetic similarities between different virus species: ❖ ~50-75% of genes are conserved between all species of *Chordopoxvirinae* ❖ ~25% of genes are conserved between all species of *Chordopoxvirinae* and *Entopoxviridae*

❖ Recombinant FPV have been used to protect Australian poultry against infectious bursal disease virus [Boyle and Heine, 1994].

❖ In the RV144 Thai trial where protection against HIV-1 was reported, HIV-1 recombinant canarypox virus was used to vaccinate human participants [Rerks-Ngarm *et al.*, 2009].

1.7 Prime-boost vaccination to generate high avidity HIV-specific CD8$^+$ T cells

1.7.1 Overview

Prime-boost vaccination is a consecutive immunization strategy used to amplify the number of antigen-specific effector/memory lymphocytes responding against encoded vaccine antigens (**figure 1.4**). The use of heterologous vectors in this instance is desirable to minimise the effects of anti-vector immunity that could occur if genetically identical (i.e. homologous) vectors are used for the prime and the booster vaccinations [Leong *et al.*, 1995; Ramsay *et al.*, 1997; Kent *et al.*, 1998; Wijesundara *et al.*, 2012]. Since its inception, heterologous prime-boost vaccination strategies have been used in an attempt to generate protective immunity against diseases such as malaria, tuberculosis, influenza and HIV-1 [Wijesundara *et al.*, 2012]. However, there are various parameters that have to be optimized for developing efficacious heterologous prime-boost vaccination regimens [Wijesundara *et al.*, 2012]. Here, ways to manipulate vaccination route, vector choice and molecular adjuvants such that heterologous prime-boost vaccination regimens can generate high avidity anti-viral CD8$^+$ T cells are briefly reviewed.
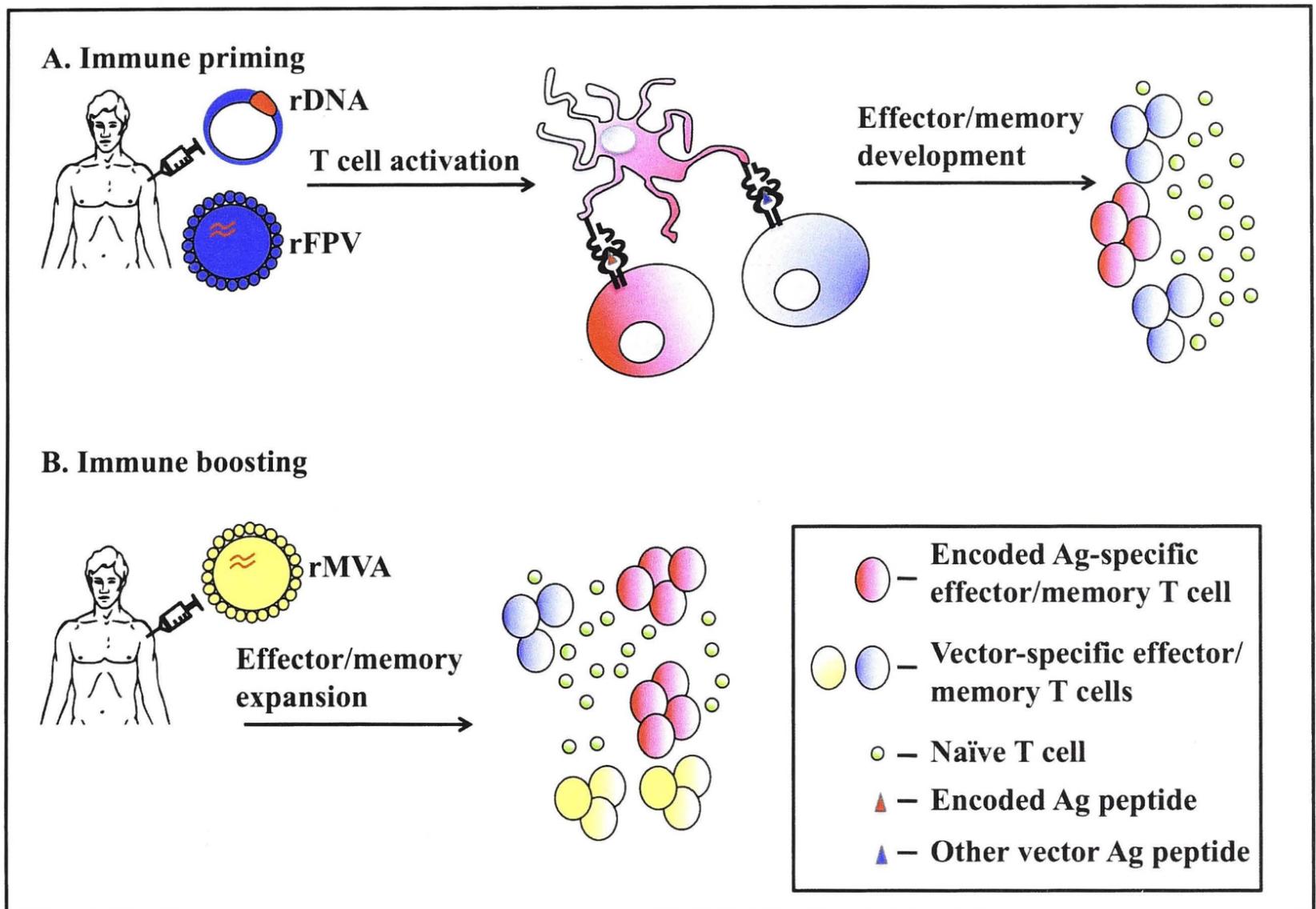
Figure 1.4. Heterologous prime-boost vaccination for generating T cell immunity. Heterologous prime-boost vaccination involves immune priming and at least one immune boost to amplify immune responses of interest. A, In immune priming, antigens of interest (red) encoded in a recombinant DNA (rDNA) vector or a live virus vector (i.e. recombinant FPV (rFPV)) is used for immunization. Subsequently, vector encoded antigens gets presented by APCs to naïve T cells, which facilitates naïve T cell activation and differentiation into effector/memory T cells. Following immune priming, the peripheral T cell pool is expected to comprise primarily of naïve T cells (green) and relatively small proportion of effector/memory T cells specific to the encoded vaccine antigens of interest (red) or T cells specific to other vector components (blue). B, In immune boosting, same vaccine antigens (red) are delivered in a heterologous viral vector such as recombinant MVA (rMVA) to minimise the anti-vector immunity. Hence, following heterologous booster immunization the number of effector and memory T cells against the desired vaccine antigens gets further expanded.

1.7.2 Route of vaccine delivery: mucosal versus systemic

Most pathogens including HIV-1 are encountered at the mucosa and generating protective mucosal immunity is crucial for preventing the systemic spread of pathogens like HIV-1. Mucosal vaccination strategies are extremely effective in generating protective mucosal immunity against various mucosal pathogen infections [Belyakov and Ahlers, 2009]. This could be partially due to the capacity of mucosal vaccination regimens to generate high avidity anti-viral CD8$^+$ T cells at mucosal surfaces. Indeed, several prime-boost vaccination studies have shown that mucosal (e.g. intranasal (i.n.) or intrarectal) compared to systemic (e.g. subcutaneous or intramuscular (i.m.)) vaccine delivery particularly during the prime is better at generating high avidity mucosal HIV/SIV-specific CD8$^+$ T cells and protection [Belyakov *et al.*, 2001; Belyakov *et al.*, 2006; Belyakov *et al.*, 2007; Ranasinghe *et al.*, 2007; Belyakov *et al.*, 2008; Ranasinghe *et al.*, 2011]. The exception here is when recombinant DNA vectors are used where i.m. priming is required for enhanced expression of vector encoded antigens [Wolff *et al.*, 1990; Ranasinghe *et al.*, 2011]. Purely systemic vaccinations have failed to protect against HIV-1 in human clinical trials and are also poor at inducing mucosal homing marker (i.e. integrin $\alpha_4\beta_7$ and C-C chemokine receptor 9) expression and mucosal homing of T cells [Wijesundara et al., 2011]. However, Ranasinghe et al [2007] have shown that combined mucosal/systemic (i.n. FPV-HIV prime/VV-HIV boost) compared to purely mucosal (i.n. FPV-HIV/i.n. VV-HIV) regimens are more effective in generating high magnitude of HIV-specific CD8+ T cells. This was shown to occur in both mucosal and systemic compartments without significantly compromising T cell avidity [Ranasinghe et al., 2007]. Overall, mucosal prime-boost vaccination regimens exhibit great potential for inducing high avidity HIV-specific CD8+ T cells and protection against HIV-1.

1.7.3 Vaccine vector choice

The choice of vectors for the prime and the boost could be a critical determinant for the avidity outcomes of anti-viral CD8$^+$ T cells induced following prime-boost vaccination. For immune priming, non-replicating vaccine vectors (e.g. recombinant DNA or FPV) in mammalian cells that do not express high doses of vector-encoded antigens are desirable for obtaining protective outcomes and high avidity CD8$^+$ T cells [Kent *et al.*, 1998; Ramsay *et al.*, 1999; Ranasinghe *et al.*, 2011]. This is likely because the avidity of effector CD8$^+$ T cells is expected be enhanced when their naïve precursors are primed with low doses of cognate antigens [Alexander-Miller *et al.*, 1996; Zeh *et al.*, 1999]. However, a caveat is that using extremely low doses of antigen could be ineffective in priming T cell responses [Ranasinghe *et al.*, 2006]. For immune boosting, the use of recombinant live viral vectors is extremely effective in expanding desired anti-viral CD8$^+$ T cell populations [Ramsay *et al.*, 1999; Ranasinghe *et al.*, 2006]. In particular, the use of recombinant VV for i.m. boosting especially following an i.n. prime with recombinant FPV have been established in our laboratory to be excellent at expanding high avidity anti-viral CD8$^+$ T cells [Ranasinghe *et al.*, 2006; Ranasinghe *et al.*, 2007]. Overall, *in vivo* immune priming with vectors that express low levels of cognate antigens (e.g. recombinant DNA or FPV) prior to immune boosting with recombinant live viral vectors could be extremely effective in generating high avidity anti-viral CD8$^+$ T cells.

1.7.4 Vaccine adjuvants

Historically adjuvants have been incorporated to enhance the immunogenicity of vaccines, but very few adjuvants are known to enhance the avidity of CD8$^+$ T cells given that mechanisms dictating T cell avidity are poorly understood [Wijesundara *et al.*, 2012]. A few studies suggest that enhancing costimulatory capacity and maturation

of DCs through immunization with recombinant pox viruses containing T cell costimulatory molecules and/or granulocyte-macrophage colony stimulating factor are effective in inducing high avidity CD8$^+$ T cells that can protect against tumors [Oh *et al.*, 2003; Hodge *et al.*, 2005]. Belyakov *et al* [2001; 2006] suggest that helper-CD8$^+$ T cell peptide vaccination strategies adjuvanted with *E.coli* labile toxin can also enhance avidity of anti-viral CD8$^+$ T cells and protection of macaques against SIV. In our laboratory, Ranasinghe *et al* [2013] showed that i.n./i.m. prime-boost vaccination with HIV-1 recombinant pox viral vaccines adjuvanted with IL-13 inhibitor (IL-13Rα2Δ10) can enhance the avidity of Gag-specific CD8$^+$ T cells and protection. In Ranasinghe *et al* [2013] mucosal (i.n.) priming with recombinant IL-13 inhibitor FPV vectors were also shown to enhance the recruitment of a distinct subset of CD11b$^+$ CD11c$^+$ DCs into the lung, which is currently being evaluated for the capacity to prime high avidity CD8$^+$ T cells. Thus, given that naïve T cell priming conditions can dictate avidity outcomes adjuvants can be used to alter the priming milieu such that high avidity anti-viral CD8$^+$ T cells develop.

1.8 IL-4 and IL-13: regulation of T cell avidity and cellular signaling

IL-4 and IL-13 share many biological functions due to their ability to signal through a unique network of complex receptors (**table 1.4; figure 1.5**). IL-4 can signal through the type I IL-4 receptor (heterodimer of IL-4Rα and common γ (γc) chains) and the type II IL-4 receptor (heterodimer of IL-4Rα and IL-13 receptor α 1 (IL-13Rα1) chains), but IL-13 only signals through the type II IL-4 receptor [Tabata *et al.*, 2007; Wills-Karp and Finkelman, 2008]. IL-4 and IL-13 cytokine responses can be completely abrogated on cells lacking IL-4Rα expression as it is a component of both type I and type II IL-4 receptors [Barner *et al.*, 1998; Mohrs *et al.*, 1999]. IL-13Rα2 binds with higher affinity to IL-13 than IL-13Rα1, but appears to inhibit IL-13 signaling [Kawakami *et al.*, 2001;

Table 1.4. Common biological effects of IL-4 and IL-13.

Biological effect	Reference
↑ MHC-II and low affinity IgE receptor expression on B cells	ω
↑ mammary cell development	ξ
↑ clearance of nematode infections	π
↑ pulmonary fibrosis	σ
↓ CD8$^+$ T cell avidity	υ
↑ eosinophilia, neutrophilia and mucus secretion in asthma	λ

ω [Defrance *et al.*, 1987; Punnonen *et al.*, 1993]

ξ [Khaled *et al.*, 2007]

π [Urban *et al.*, 1991; Urban *et al.*, 1998]

σ [Rankin *et al.*, 1996; Zhu *et al.*, 1999]

υ [Ranasinghe *et al.*, 2009]

λ [Grünig *et al.*, 1998; Wills-Karp *et al.*, 1998; Tomkinson *et al.*, 2001].

↑ indicates up-regulation or enhancement of the indicated effect(s)

↓ indicates down-regulation or dampening of the indicated effect(s)
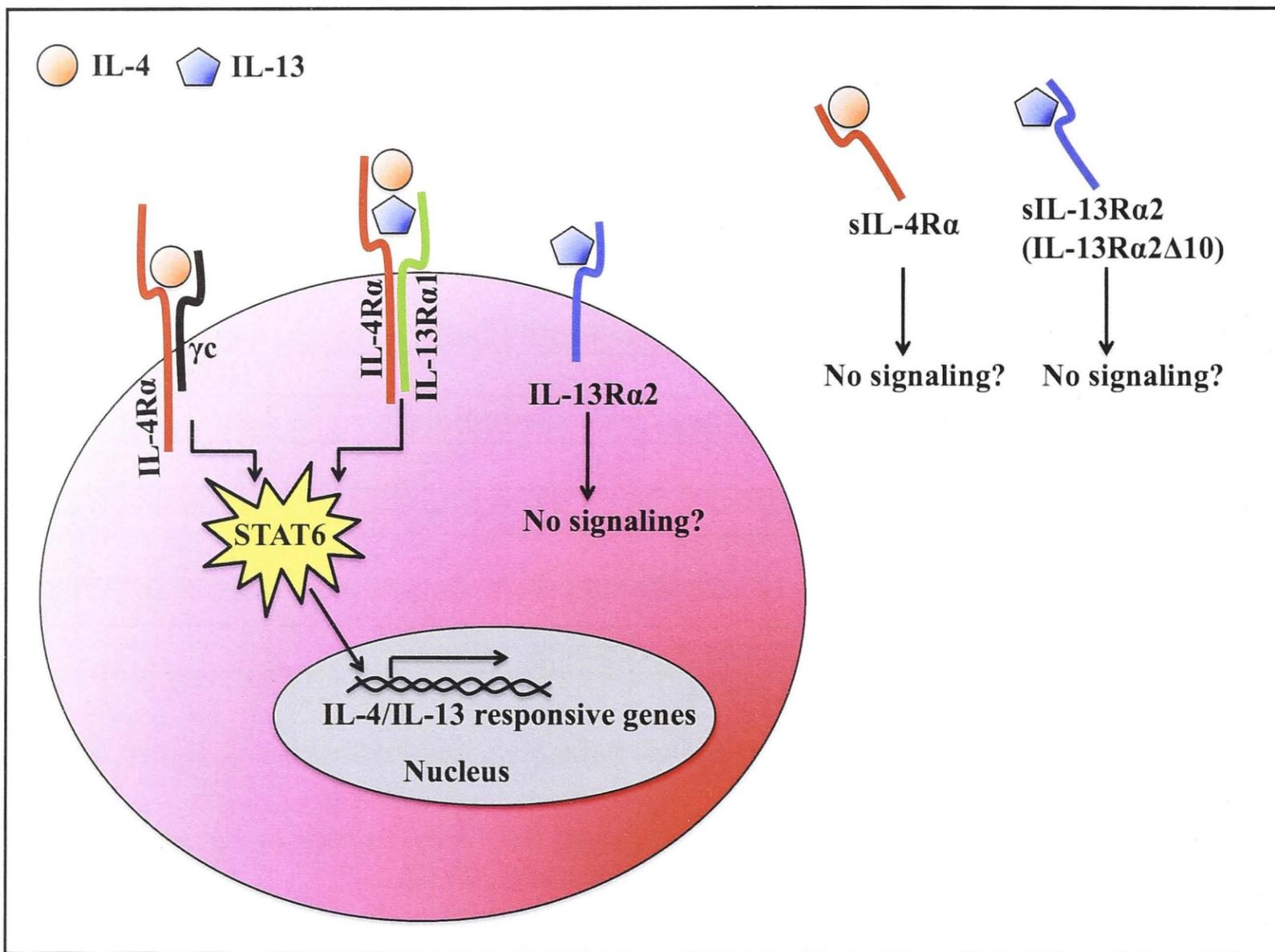
Figure 1.5. IL-4/IL-13 receptor complex. IL-4 can signal through type I (heterodimer of IL-4Rα and γc) or type II (heterodimer of IL-4Rα and IL-13Rα1) IL-4 receptors. IL-13 can only signal through the type II IL-4 receptor. Signaling through these cytokines/receptors usually leads to activation of STAT6, which translocates into the nucleus to activate transcription of IL-4/IL-13 responsive genes. IL-4 can also bind to sIL-4Rα or IL-13 can bind to IL-13Rα2 or IL-13Rα2Δ10, which is thought to not result in signaling. Hence, sIL-4Rα and IL-13Rα2 are referred to as decoy/inhibitors of IL-4/IL-13 signaling.

Sivaprasad *et al.*, 2010]. This decoy/inhibitory receptor can be membrane associated or soluble (IL-13Rα2Δ10) (**figure 1.5**). Similarly, a soluble form of IL-4Rα (sIL-4Rα) has been shown to inhibit the biological effects of IL-4 [Mosley *et al.*, 1989; Gessner *et al.*, 1994; Borish *et al.*, 2001]. IL-4 and IL-13 cellular signaling via the type I or type II IL-4 receptors mainly leads to activation of STAT6 although, activation of STAT1, STAT3 and STAT5 has also been reported [Hou *et al.*, 1994; Takeda *et al.*, 1996; Acacia de Sa Pinheiro *et al.*, 2007]. Following activation, STAT6 translocates into the nucleus to activate transcription of genes (e.g. CD23, MHC-II and IL-4Rα) [Nelms, 1999].

As described previously, our laboratory has identified that CD8^{+} T cell avidity can be influenced based on the route of vaccine delivery or the presence of IL-4 and IL-13 during vaccination of wild-type (WT) and gene knockout mice (**table 1.5**). As shown in **table 1.5** (**model II**) following HIV-1 recombinant pox viral prime-boost vaccination, the avidity of HIV (K^dGag$_{197-205}$)-specific CD8^{+} T cells inversely correlated with their IL-4 and IL-13 production capacity [Ranasinghe *et al.*, 2007]. Enhancement in avidity in these studies favoured mucosal prime-boost vaccination regimens in the following manner: i.n./i.n. > i.n./i.m. > i.m./i.m.. Ranasinghe *et al* [2009; 2013] then established that IL-4 and IL-13 dampen the avidity of HIV-specific CD8^{+} T cells, which led to development of novel IL-13Rα2Δ10 adjuvanted recombinant pox viral vaccines (**table 1.5 (model I and III)**). These novel vaccines as hypothesized enhanced protection and Gag-specific CD8^{+} T cell avidity following vaccination [Ranasinghe *et al.*, 2013]. Overall, our laboratory has established that mucosal HIV-1 recombinant pox viral prime-boost vaccination likely through reduced induction of IL-4 and IL-13 can enhance the avidity of HIV-specific CD8^{+} T cells.

Table 1.5. Prime-boost models of avidity regulation in our laboratory.

Model	Prime-boost vaccination regimen	Vaccines used	Mouse genotype	HIV-specific CD8$^+$ T cell avidity
I*	i.n./i.m.	FPV-HIV/VV-HIV	IL-13$^{-/-}$	+ + + + +
	i.n./i.m.	FPV-HIV/VV-HIV	IL-4$^{-/-}$	+ + + + +
	i.n./i.m.	FPV-HIV/VV-HIV	STAT6$^{-/-}$	+ + + +
	i.n./i.m.	FPV-HIV/VV-HIV	WT	+ + +
II$^\psi$	i.n./i.n.	FPV-HIV/VV-HIV	WT	+ + + + +
	i.n./i.m.	FPV-HIV/VV-HIV	WT	+ + +
	i.m./i.m.	FPV-HIV/VV-HIV	WT	+ +
III$^\xi$	i.n./i.m.	FPV-HIV-IL-13Rα2Δ10/ VV-HIV-IL-13Rα2Δ10	WT	+ + + + +
	i.n./i.m.	FPV-HIV/VV-HIV	WT	+ + +

+ is a relative incremental indicator of avidity (i.e. more '+' = higher avidity)

* [Ranasinghe *et al.*, 2009]

ψ [Ranasinghe *et al.*, 2007]

ξ [Ranasinghe *et al.*, 2013]

1.9 FTA assay for measuring CD8^{+} T cell avidity *in vivo*

Various *in vitro* based methods are commonly used to measure various aspects of CD8^{+} T cell avidity (**table 1.6**). Surface plasmon resonance and pMHC-I tetramer staining/dissociation assays are sensitive assays that can be used to define the affinity of the TCR or the overall avidity of TCR and CD8 co-receptors of a CD8^{+} T cell clone of interest (**table 1.6**). Tetramer technology is also extremely advantageous to further scrutinize the avidity profiles of antigen-specific CD8^{+} T cells of interest. In fact, a few studies have used this technology to examine expression levels of various markers that can regulate avidity and the TCR signatures (clonotypes) of high and low avidity antigen-specific CD8^{+} T cells of interest [Kedzierska *et* al., 2005; La Gruta *et al.*, 2006]. However, the major shortcoming of surface plasmon resonance and tetramer technologies are that they need to be complemented with assays that provide direct information regarding the effector functions of CD8^{+} T cells. Defining avidity profiles based on the effector function capacity/sensitivity of CD8^{+} T cells to the availability of co-receptor molecules (i.e. CD8) or titrated amounts of peptide pulsed target cells have classically been described using ^{51}Cr release, enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) assays [Alexander-Miller *et al.*, 1996; Slifka and Whitton, 2001; Draenert *et al.*, 2004]. These assays provide direct evidence regarding the functional avidity of CD8^{+} T cells, but they are usually performed *in vitro*.

Ideally, avidity is best measured *in vivo* and will not require any *in vitro* manipulation of CD8^{+} T cells. Previously developed *in vivo* CD8^{+} T cell killing assays [Oehen *et al.*, 1997; Barchet *et al.*, 2000] are only able to measure the magnitude of the killing response in a single animal without providing any detailed measurements on functional avidity and epitope variant cross-reactivity in a single host animal. These limitations are significant obstacles for vaccine strategies that aim to induce high avidity CD8^{+} T cells

Table 1.6. Common *in vitro* methods used to determine CD8⁺ T cell avidity fates.

Method	Aspects of CD8⁺ T cell avidity measured	References
Surface Plasmon resonance	❖ Technique used to determine the affinity between one TCR and one pMHC-I in real-time based on ligand binding to surface immobilized receptors	δ
pMHC-I tetramer staining and dissociation	❖ Flow cytometry based assay used to infer avidity of multiple TCRs and CD8 co-receptors to cognate pMHC-I based on the ability of CD8⁺ T cells to interact with fluorochrome conjugated tetramerized pMHC-I ❖ Flow cytometry based cell sorting of tetramer bound CD8⁺ T cells can be used to characterize TCR chains (clonotypes) that characterize high and low avidity cells	φ
CD8 blocking	❖ Anti-CD8 antibodies or CD8 null tetramers are used to block CD8 co-receptor involvement in cognate pMHC-I recognition and effector functions	λ
Marker expression analysis	❖ Flow cytometry based measurements made to determine the expression levels of various candidate markers that correlate with CD8⁺ T cell avidity	θ
Peptide pulsing	❖ Using ELISPOT, ICS or ^{51}Cr release assays, the ability to respond (e.g. kill or produce IFN-γ) against various target cells pulsed with titrated amounts of cognate antigens (peptides) can be used to determine the antigen sensitivity (overall functional avidity) of CD8⁺ T cells	€

δ [Holler *et al.*, 2001; Stone *et al.*, 2009]

φ [Kedzierska *et al.*, 2005; La Gruta *et al.*, 2006]

λ [Wooldridge *et al.*, 2003; Choi *et al.*, 2003]

θ [Slifka and Whitton, 2001; La Gruta *et al.*, 2006]

€ [Alexander-Miller *et al.*, 1996; Slifka and Whitton, 2001; Draenert *et al.*, 2004]

in vivo. However, a novel FTA technology has been recently developed and can be used to overcome these limitations [Quah *et al.*, 2012; Quah *et al.*, 2013]. The FTA comprises of lymphocyte target cells with unique fluorescent signatures (>200) based on labeling cells with a combination of carboxyfluorescein succinimidyl ester (CFSE), cell trace violet (CTV) and cell proliferation dye eFluor®670 (CPD) [Quah *et al.*, 2012; Quah *et al.*, 2013]. The FTA can then be pulsed with various titrated concentrations of MHC-I and MHC-II binding peptides and injected into HIV-1 vaccinated mice to evaluate T_H cell responses and $CD8^+$ T cell avidity (based on killing responses) *in vivo* [Quah *et al.*, 2012; Quah *et al.*, 2013]. Consequently, this assay can be used thoroughly evaluateavidity and epitope variant cross-reactivity of $CD8^+$ T cells as well as T_H cell responses *in vivo* in the same vaccinated animal.

1.10 Pre-clinical models for testing putative HIV-1 vaccines

In 1940, Howard Florey and Ernst Chain demonstrated that penicillin could be administered safely to protect mice against deadly *Streptococci* infections [Chain *et al.*, 1940], which later translated to saving millions of human lives. This landmark study showed the enormous potential of using pre-clinical models for developing safe yet protective substances for use in humans. There are three major pre-clinical animal models used for HIV-1 vaccine development or as 'gatekeepers' for advancement of HIV-1 vaccines into humans: mice (including humanized mice), macaques and chimpanzees. Mice genomes are easy to manipulate and their immune responses are well characterized making them ideal models for testing the immunogenicity and protective mechanisms of putative HIV-1 vaccines. However, all known mice strains apart from humanized mice cannot be used to test the efficacy of HIV-1 vaccines given that HIV-1 does not infect mice. HIV-1 is only known to naturally infect humans and

chimpanzees [Gao *et al.*, 1999], which makes chimpanzees an ideal HIV-1 challenge model. However, successful outcomes of HIV-1 vaccine regimens tested in chimpanzees have not been translated in human clinical trials [Bailey, 2008]. This has pushed researchers to use macaques and humanized mice models to determine the protective potential of HIV-1 vaccines [Mosier, 1996; Shedlock *et al.*, 2009]. Macaques are a natural host of SIV and humanized mice can be infected with HIV-1 given that they are engrafted with human peripheral blood mononuclear cells. However, HIV-1 transmission in humanized mice does not occur and the protective effects associated with the use of SIV vaccines in macaques so far have also not been translated in human clinical trials [Buchbinder *et al.*, 2008; Van Duyne *et al.*, 2009]. Macaques and humanized mice are also difficult to maintain/access and are expensive to use.

The reasons for the poor translational potential of putative HIV-1 vaccines tested in pre-clinical animals models is not clear, but it is probably not exclusively due to the challenge model chosen. It is very likely due to a significant lack in understanding of vaccine induced protective immune mechanisms in animals and whether induction of these protective responses is feasible in humans following vaccination [Shapiro, 2013]. Resolving these issues will help researchers understand the validity of the animal models chosen for developing efficacious HIV-1 vaccines for use in humans.

1.11 Basis and scope of this PhD thesis

The PhD research project predominantly aimed towards understanding how IL-4 and IL-13 regulate anti-viral CD8$^+$ T cell avidity/quality following virus infection (**chapter 3**) and HIV-1 recombinant pox-viral prime-boost vaccination (**chapter 4**). The ability of FTA assays to screen for effective HIV-1 recombinant pox viral prime-boost vaccination regimens that generated high quality T cell responses *in vivo* were also

examined (**chapter 5**). Overall, the data generated from these studies provided more insights as to how CD8$^+$ T cell avidity can be regulated following virus infection and HIV-1 recombinant pox viral prime-boost vaccination. The rationales for each of these studies are outlined below:

1. Cytokines require receptors to mediate signaling and no studies have evaluated how cytokine receptors for IL-4 and IL-13 are regulated following pox virus infection. Therefore, in the first study (**chapter 3**) it was investigated how receptors for IL-4 and IL-13 are regulated on immune cells following predominantly pox virus infections and whether regulation of these receptors influenced the avidity of anti-viral CD8$^+$ T cells.

2. In the second study (**chapter 4**), how cytokines IL-4 and IL-13 act on anti-viral CD8$^+$ T cells to regulate avidity was investigated using a range of HIV-1 recombinant pox viral prime-boost vaccination strategies (**table 1.5**). For this purpose, whether differential regulation of avidity molecules (i.e. lck, CD8αβ, TCR, lck, CD11a and CD2) played a role in modulating HIV-specific CD8$^+$ T cell avidity and polyfunctionality was investigated.

3. Current assays that measure avidity of CD8$^+$ T cells following HIV-1 vaccination are usually performed *in vitro*, which can limit the interpretation of the effectiveness of vaccines in inducing high quality (i.e. avidity) T cell responses *in vivo*. Therefore, in the final study (**chapter 5**) the utility of a novel FTA assay (**section 1.9**) to determine the efficacy of vaccine vectors and routes of vaccine delivery for the induction of high avidity and epitope variant cross-reactive CD8$^+$ T cells as well as T$_H$ cell responses *in vivo* was evaluated.

Chapter 2

Materials and Methods

2.1 Mice

Pathogen-free 6-10 weeks old WT, IL-4 $^{-/-}$, IL-13 $^{-/-}$, STAT6 $^{-/-}$ BALB/c mice were bred and maintained under The ANU Animal Experimentation and Ethics Committee (AEEC) guidelines. C57BL/6, C57BL/6 IFN-γ $^{-/-}$, C57BL/6.SJL (CD45.1) and TCR transgenic C57BL/6 OT-I (CD45.2) mice were all purchased from the Australian Phenomics Facility, ANU.

2.2 Ethics statement

All animals were maintained and experiments were performed in accordance with The Australian National Health and Medical Research Council guidelines within The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals were also maintained in accordance with guidelines approved by AEEC, protocol numbers JIG 74.09 and A2011/018. Animals used for experimentation were monitored daily and infected mice were scored for signs of illness and weight loss. Animals were ethically sacrificed using cervical dislocation in accordance with the above AEEC approved protocols.

2.3 Peptide and tetramer synthesis

All peptides and tetramers used in this study were synthesized at The BRF, JCSMR. Peptides included the MHC-I-binding peptides, SPYAAGYDL (F2L, immunodominant VV epitope L^dF2_{26-34}), KYGRLFNEI (A52R, subdominant VV epitope K^dA52_{75-83}), SPGAAGYDL (F2L mut, a MVA epitope homologous to F2L), AMQMLKETI (HIV Gag, Gag epitope $K^dGag_{197-205}$), AMQMLKDTI (HIV Gag mut, HIV Gag subtype C variant [Earl *et al.*, 2009]), AMQMLEKTI (HIV neg), VGPTPVNII (HIV Pol, K^d-restricted HIV Pol epitope [Wild *et al.*, 2009]), RGPGRAFVTI (HIV Env, K^d-restricted

HIV Env epitope [Takeshita *et al.*, 1995], and the MHC-II-binding peptide PVGEIYKRWIILGLN (Gag T_H, H-2^d-restricted HIV Gag epitope [Mata and Paterson, 1999]). Tetramers used in this study include allophycocyanin conjugated K^dGag$_{197-205}$ or K^dA52$_{75-83}$ tetramers.

2.4 Viruses

MVA, avirulent Semliki Forest virus (aSFV), FPV, recombinant VV encoding the ovalbumin peptide SIINFEKL (VV-OVA$_{257-264}$) or A/PR8 (H1N1) influenza virus stocks used in this study were gifts from Associate Professor Guna Karupiah, Dr. Mohammed Alsharifi, Dr. David Boyle, Dr. David Tscharke and Dr. Yoichi Furuya, respectively. All FPV and MVA stocks were grown and titrated in chick embryonic epithelial cells. aSFV stocks were grown and titrated in BHK cells. A/PR8 influenza virus stocks were prepared as described in Alsharifi *et al* [2009]. Briefly, one hemmagglutinin unit of virus in 100 μl of saline was injected into 10-days old embryonated chicken eggs, incubated for 48 hours at 37 °C, and then stored at 4 °C overnight. Subsequently, the allantoic fluids from these eggs were then harvested and the virus purified using hemagglutinin based binding of chicken red blood cells. The purified virus was titrated using Madin-Darby canine kidney cells to determine the virus stock concentration. VV Western Reserve (VV-WR) strain and recombinant VV stocks were grown and titrated in 143B cells. Recombinant VV encoding murine IL-4 and hemagglutinin (VV-HA-IL-4) and the VV encoding hemagglutinin (VV-HA) were prepared as described in Sharma *et al* [1996]. These stocks were kindly provided by Professor Alistair Ramsay. The recombinant FPV encoding HIV-1 AE clade Gag, Pol and Env (FPV-HIV; FPV117a) and recombinant VV encoding HIV-1 AE clade Gag and Pol (VV-HIV; VV336) used in the current study were exactly as those used

previously in Ranasinghe *et al* [2006]. Recombinant VV encoding HIV AE clade Env (VV337) was prepared as described in Coupar *et al* [2006]. Recombinant FPV-HIV and VV-HIV encoding the murine IL-13Rα2Δ10 (IL-13 inhibitor vaccines) were constructed exactly as describe in Ranasinghe *et al* [2013].

2.5 Liposome preparation

Jason Price at Lipotek kindly synthesized the liposome constructs used in this project and the liposome construction process is briefly described here. Liposome doses were encapsulated with a 35 mg bolus of HIV Gag mut peptide and a 1 mg bolus of LPS. Liposomes were formed by suspending a desiccated thin film of lipids composed of DOPC:DOPG:DSPE-PEG750:DOPE:cholesterol (Avanti Polar Lipids) at a w:w ratio of 10:4:2:1:2 respectively, in water. HIV Gag mut and LPS were incorporated into the liposomes by vortexing for 30 seconds, freezing on dry ice and lyophilisation, before stepwise rehydration in water. Liposomes were then sized by sequential extrusion through 0.4 mm, 0.2 mm and 0.1 mm PC membranes using a Northern Lipids 'Lipex' extruder with a 10 ml thermobarrel warmed to 50 °C. Unencapsulated peptide was removed by dialysis using 300 kDa MWCO tubing (Spectrapore). Peptide content of the liposomes was analyzed by RP-HPLC (Waters HPLC system) and concentration determined by comparison with peptide standards. The Prepared liposomes were then administered intravenously (i.v.) in a 200 μl bolus.

2.6 Virus infection and HIV-1 prime-boost vaccination

All virus infections in age- and sex-matched mice were conducted intraperitoneally (i.p.) at a dose of 3×10^6 plaque forming units (PFU)/mouse or 5×10^6 PFU/mouse for a period of 7 days unless otherwise stated.

For all HIV-1 prime-boost vaccinations except where indicated, pathogen free 6-10 weeks old BALB/c mice were primed i.n. with 1 x 10^7 PFU of FPV-HIV (FPV117a) followed by i.m. booster vaccination with 1 x 10^7 PFU of VV-HIV (VV336) 14 days apart as described in Coupar *et al* [2006] and Ranasinghe *et al* [2006]. In chapter 5, VV-HIV immunizations were done using i.n. or i.m. routes with a mixture of 2.5 x 10^6 PFU of VV336 and 2.5 x 10^6 PFU of VV337 in an attempt to generate Env-specific T cell responses. T cell responses were evaluated 7 or 14 days post booster vaccination.

2.7 Adoptive cell transfer of OT-I cells

Red blood cell (RBC)-depleted splenocytes from 8 weeks old C57BL/6 OT-I mice were injected i.v. (10 x 10^6 cells in 200 µl of phosphate buffer saline (PBS)) into a lateral tail vein of 8 weeks old recipient C57BL/6.SJL mice. Subsequently, the recipient mice were rested overnight (12-18 hours) and infected i.p. with 5 x 10^6 PFU/mouse of VV-WR control or VV-OVA$_{257-264}$.

2.8 Flow cytometry

2.8.1 Antibodies

The following monoclonal antibodies against mouse antigens and the respective isotype controls for CD8α, CD4, CD45R (B220), CD124 (IL-4Rα), CD132 (γc), CD49b (DX5), CD44, CD69, CD25, CD45.1, CD62L, CD11c and TNF-α were all obtained from Becton Dickinson (BD) Biosciences, USA. Monoclonal antibodies against mouse granzyme B (GzmB), CD45.2, CD2, CD11a, TCRβ, IFN-γ, H-2K^d and CD8β.2 (clone 53-5.8) and I-A^d were obtained from BioLegend, USA. Monoclonal antibodies against mouse CD213a (IL-13Rα1) and IL-2 were obtained from eBioscience, USA. These

antibodies were used as purified, fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, pacific blue, alexa fluor 700, horizon V500, brilliant violet 605, PE-cyanine, allophycocyanin or allophycocyanin-eFluor 780 conjugates. Secondary fluorescein isothiocyanate conjugated anti-goat IgG (Jackson ImmunoResearch) was used to detect purified polyclonal goat IgG anti-mouse IL-13Rα2 (R&D systems) binding to splenocytes. Cell viability was assessed with the dye Hoechst 33258 (1 mg/ml, Calbiochem-Behring Corp.) as described in Quah *et al* [2004].

2.8.2 Cell surface and intracellular staining procedures

In all flow cytometry-based studies RBC-depleted splenocytes were used. Cell surface staining and intracellular staining were performed using the respective methods described in Ranasinghe *et al* [2006]. Briefly, for cell surface staining $1-4 \times 10^6$ cells were incubated 30 minutes at 4 °C in the presence of purified or fluorochrome conjugated antibodies diluted in PBS containing 1% foetal calf serum (FCS). Subsequently, samples were washed twice using PBS containing 1% FCS, fixed in 0.5% paraformaldehyde, prior to flow cytometry analysis.

In all the experiments described here, intracellular GzmB staining of cell surface stained samples was conducted using freshly isolated RBC-depleted splenocytes that were not stimulated with peptides. For IFN-γ, TNF-α and IL-2 ICS, $1-4 \times 10^6$ cells in 200 μl of Roswell Park Memorial Institute (RPMI) culture medium (Invitrogen, Australia) containing 10% FCS were seeded in 96 well U-bottom plates (BD Biosciences) in the presence or absence of 0.1 μg/ml of $K^d A52_{75-83}$, $L^d F2_{26-34}$ or $K^d Gag_{197-205}$ peptides. When CD8 blocking was performed, cells were incubated with 1 μg/ml of purified anti-mouse CD8β.2 prior to and during $K^d Gag_{197-205}$ peptide

stimulation. Subsequently, peptide stimulated or unstimulated cultures were left for 1 hour at 37 °C + 5% CO_2 prior to addition of 1x Brefeldin A (eBioscience, USA). The cultures were then left for further 4 hours at 37 °C + 5% CO_2 prior to conducting cell surface staining as described before.

For intracellular staining, cell surface stained samples were fixed and permeabilized using commercial intracellular fixation and permeabilization buffers according to the manufacturer's protocol (eBioscience, USA). Subsequently, permeabilized cells were incubated for 30 minutes at 4 °C in the presence of purified or fluorochrome conjugated antibodies diluted in 1x permeabilization buffer (eBioscience, USA). Intracellular stained samples were washed twice using PBS containing 1% FCS, fixed in 0.5% paraformaldehyde prior to flow cytometry analysis. All cell surface and intracellular stained samples were analyzed using fluorescence activated cell sorting (FACS) Calibur (BD Biosciences, USA) or BD LSR II (BD Biosciences, USA) flow cytometry machine. For each sample $2 \times 10^5 - 1 \times 10^6$ events were acquired. Flow cytometry plots of the analyzed data were constructed using the FlowJo Tree Star software (version 8.7.1).

2.9 Tetramer staining and dissociation assays

Tetramer staining was done using RBC-depleted splenocytes from vaccinated mice as described in Ranasinghe *et al* [2007]. For blocking CD8 engagement with $K^dGag_{197-205}$ tetramer, cells (2×10^6 cells/25 μl PBS) were incubated with varying concentrations of purified anti-mouse CD8β.2 for 30 minutes at 4 °C prior to tetramer staining. When tetramer dissociation was performed, tetramer stained cells were incubated for 60 minutes at 37 °C + 5% CO_2 in the presence of 25 μg/ml of anti-mouse H-2K^d (2×10^6 cells/40 μl PBS) to prevent tetramer re-binding to cells. After tetramer staining and/or

dissociation, cells were washed twice using PBS + 1 % FCS prior to conducting a cell surface stain.

2.10 FTA preparation

FTA was constructed by labeling spleen cells with combinations of the dyes CFSE, CTV and CPD as described previously [Quah *et al.*, 2012]. Briefly, splenocytes supplemented with RPMI + 10% FCS at 20 °C, were labeled with 0-66000 nM of each dye in 1-2 ml aliquots for 5 minutes and then washed at least three times. For FTA constructs, aliquots of splenocytes from BALB/c WT mice were initially labeled with several concentrations of CTV. Cells were then split equally and labeled with several concentrations of CFSE, washed once and then incubated with MHC-I and MHC-II binding peptides for 1 hour at 37 °C. Cells were then washed through a FCS cushion and subsequently washed twice more. Replicate samples were then labeled with CPD. After washing the cell samples twice, all aliquots were pooled and washed again and then labeled with 14 mM dialkylcarbocyanine lipophilic tracer dye (DiI) from Invitrogen. The FTA was then resuspended at up to 25×10^7 cells/ml for injection into mice. 5×10^7 of prepared FTA target cells were injected into a mouse and left *in vivo* for 18 hours prior to assaying for FTA target cell help and killing.

2.11 *In vitro* anti-TCR stimulation of splenocytes

Splenocytes from naïve BALB/c mice were cell surface stained using fluorochrome conjugated antibodies against mouse CD44 and CD8α for purification of CD44$_{lo}$ CD8$^+$ splenocytes using the FACSAria II (BD Biosciences). 2×10^4 of FACS sorted CD44$_{lo}$ CD8$^+$ splenocytes in 100 μl of RPMI + 10% FCS were then cultured in U-bottom well plates coated with 3 μg/ml of anti-CD3ϵ or PBS (mock) in the presence or absence of

0.1 ng/ml of recombinant murine IL-4 (R&D systems). Prior to seeding of cells, 96 well U-bottom plates were coated with 3 μg/ml of purified mouse anti-CD3ε (BioLegend) or PBS (30 μl/well) for 18 hours at 4 °C and washed three times with PBS (200 μl/well). The plates containing FACS purified cells were then incubated for further 8 hours at 37 °C + 5% CO_2 prior to cell surface staining and flow cytometry analysis.

2.12 Quantitative real-time polymerase chain reaction (PCR)

IL-4Rα_{lo} and IL-4Rα_{hi} CD8$^+$ splenocytes (see **supplementary figure 3A**) from BALB/c mice infected with VV-WR for 7 days or total CD8$^+$ splenocytes from unimmunized control BALB/c mice were sorted using FACSAria II machine. RNA extracted from sorted cells was used to generate cDNA as described in Ranasinghe *et al* [2007]. Quantitative real-time PCR with specific primers (**table 2.1**) and Power SYBR Green PCR Master Mix (Applied Biosystems) were used to amplify cDNA for 40 cycles according to the manufacturer's guidelines of the Applied Biosystems SDS 7900 real-time PCR machine.

Primer melting curves were analyzed to ensure that target-specific amplification had occurred. NCBI Primer-BLAST database was used to verify that the primers used in this study did not anneal to any unintended products in the mouse genome. mRNA encoding ribosomal protein L32 was amplified as the house keeping reference control to measure fold change in mRNA transcript levels (see **section 2.13.3**).

Table 2.1. Primers used for quantitative real-time PCR.

Primer	Sense primer 5'→3'	Anti-sense primer 5'→3'
IL-4Rα	ACACTACAGGCTGATGTTCTTCG	TGGACCGGCCTATTCATTTCC
GzmB	CCACTCTCGACCCTACATGG	GGCCCCCAAAGTGACATTTAT
L32	GCTGGAGGTGCTGCTGATGTG	CGTTGGGATTGGTGACTCTGATGG

2.13 Statistical analysis

2.13.1 Net fold reduction in IL-4Rα expression calculation

Net fold reduction of cell surface IL-4Rα expression was calculated using mean (geometric) fluorescent intensity (MFI) obtained from flow cytometry on gated CD8$^+$ splenocytes from unimmunized or VV-WR infected mice as follows: ((MFI (IL-4Rα)$_{unimmunized}$ – MFI (IL-4Rα)$_{VV-WR}$) / MFI (IL-4Rα)$_{unimmunized}$).

2.13.2 FTA statistical analysis

The % specific killing was assessed as previously described [Quah *et al.*, 2012] using the following formula:

$$
\% \text{ specific killing} = \left[1 - \left(\frac{Targets^{+peptide}_{primed} / Targets^{+nil}_{primed}}{Targets^{+peptide}_{naive} / Targets^{+nil}_{naive}} \right) \right] \times 100
$$

T cell help was assessed on the basis of CD69 up-regulation of FTA B cell by antibody labeling and flow cytometry. Expression of CD69 was calculated by subtracting the MFI (CD69) on FTA B cells from naïve mice from that of FTA B cells from the vaccinated mice as previously described in Quah *et al* [2013].

Statistic values, including area under the curve (AUC = peptide (μM) x % specific killing response) and EC$_{50}$ (peptide concentrations giving half maximal % specific killing) were calculated using GraphPad Prism Software. To calculate EC$_{50}$ values the peptide concentrations in response curves were transformed to log$_{10}$ (μM), specific killing normalized to 100% and data modeled to a sigmoidal dose-response curve.

2.13.3 Fold change in mRNA transcript levels calculation

The fold change in mRNA transcript levels was calculated using the $2^{-\Delta\Delta CT}$ method. $\Delta\Delta CT$ was calculated as follows: $((CT_{target} - CT_{reference})$ of sorted IL-4Rα_{lo}/ IL-4Rα_{hi} CD8$^+$ T cells) $- (CT_{target} - CT_{reference})$ of naïve CD8$^+$ T cells.

2.13.4 Statistical significance testing

All the data presented in this study have been reproduced in at least two independent experiments unless otherwise stated. The data plotted in all the graphs shown except where indicated represent the mean and the error bars depict the standard error of the mean (SEM). Statistical significance of the data and the p values were calculated using the Graph InStat software (version 3.10). In all statistical significance analysis, a student's unpaired t-test, Mann-Whitney nonparametric two-tailed t-test or one-way ANOVA was used.

Chapter 3

The Role of IL-4/IL-13 Cytokine Receptors in Modulating Anti-Viral CD8$^+$ T Cell Quality

3.1 Introduction

Protection against intracellular pathogens (e.g. viruses and parasites) and tumors often requires the activation of effector $CD8^+$ T cells, which usually mediate cytolysis upon recognition of non-self pMHC-I complexes presented on the surface of malignant cells or virus-infected cells [Zinkernagel *et al.*, 1974; Harty *et al.*, 2000; Dudley *et al.*, 2002]. In HIV-1 infections, increased susceptibility of virus-infected $CD4^+$ T cells to $CD8^+$ T cell mediated cytolysis has been associated with superior virus control in elite controllers [Buzon *et al.*, 2014]. However, a collection of $CD8^+$ T cell effector functions, not exclusively cytolysis, appears to be important in controlling virus infections. These include the ability to mount high avidity interactions with virus-infected cells, produce multiple anti-viral cytokines (i.e. IFN-γ and TNF-α), induce a high clonal turnover rate, and/or the ability to produce chemoattractants (e.g. macrophage inflammatory protein-1β) to recruit immune cells to virus-infected sites [Derby *et al.*, 2001; Almeida *et al.*, 2007; Seder *et al.*, 2008]. These effector functions define the quality of effector $CD8^+$ T cell responses against viruses.

The use of IL-4Rα $^{-/-}$, IL-13Rα1 $^{-/-}$, γc $^{-/-}$ and IL-13Rα2 $^{-/-}$ mice have shown that these receptors are indeed important for controlling functions of IL-4 and IL-13 in allergic diseases and parasitic infections [Mohrs *et al.*, 1999; Sivaprasad *et al.*, 2010, Junttila *et al.*, 2008]. However, these studies provide limited insights as to how these receptors are regulated at a cellular level *in vivo* during the course of a pathogenic infection. A study

by Tanaka *et al* [2007] reported that cell surface IL-4Rα expression is down-regulated on activated CD4$^+$ T cells *in vivo* following *L. major* infection. This was found to be due to degradation of IL-4Rα in intracellular compartments of activated CD4$^+$ T cells in a TCR and dedicator of cytokinesis 2 (DOCK2) dependent manner [Tanaka *et al.*, 2007]. In another study, Perona-Wright *et al* [2010] have also shown that cell surface IL-4Rα expression was down-regulated on activated CD4$^+$ T cells following *H. polygyrus* infection of mice, which was thought to render these cells refractory to further stimulation with IL-4. On the contrary, naïve bystander CD4$^+$ T cells in this instance were found to up-regulate IL-4Rα making them more responsive to IL-4 [Perona-Wright *et al.*, 2010]. These studies suggest that IL-4Rα plays a critical role in tuning responsiveness of CD4$^+$ T cells to IL-4 and/or IL-13 during infection with pathogens.

Despite recent studies showing the importance of regulating IL-4Rα expression on CD4$^+$ T cells following parasitic infection *in vivo*, only few studies have addressed how the cytokine receptors for IL-4/IL-13 are regulated on CD8$^+$ T cells following virus infection *in vivo*. The current study initially investigated how IL-4Rα, γc, IL-13Rα1 and IL-13Rα2 were regulated on CD8$^+$ T cells and other immune cells predominantly following VV infection of mice. After obtaining results showing that only IL-4Rα expression was differentially regulated on CD8$^+$ T cells as a consequence of VV infection of mice, the current study explored the mechanisms involved in this process and whether differential regulation of IL-4Rα affected the quality of anti-viral CD8$^+$ T cell immunity.

3.2 Results

3.2.1 Regulation of IL-4/IL-13 receptor components during VV infection

To examine whether VV infection induced differential regulation of IL-4/IL-13 receptor subunits on immune cells known to be important for clearance of viral infections, splenocytes from unimmunized or VV-WR strain infected BALB/c WT mice were analyzed using flow cytometry during the peak (i.e. 7 days) of anti-viral immunity. Cell surface IL-13Rα1, γc and intracellular IL-13Rα2 expression levels on gated B220$^+$, CD4$^+$, CD8$^+$, DX5$^+$ (NK cells) and CD11c$_{high}$ I-A$^d_{high}$ (DCs)) splenocytes from VV-WR infected mice and unimmunized mice were similar (**figure 3.1**). Cell surface IL-13Rα2 expression was not detectable above background expression levels in the cell subsets shown in **figure 3.1** (data not shown). Interestingly, cell surface IL-4Rα expression was up-regulated on CD11c$_{high}$ I-A$^d_{high}$ cells and down-regulated on CD4$^+$ and CD8$^+$ T cells as a consequence of VV infection (**figure 3.1**). Down-regulation of IL-4Rα was not uniformly observed on all CD4$^+$ or CD8$^+$ T cells following VV infection with some cells retaining similar IL-4Rα expression levels to that observed in unimmunized mice.

3.2.2 Down-regulation of IL-4Rα expression correlates with the magnitude of effector CD8$^+$ T cells

To clearly understand the mechanisms responsible for mediating down-regulation of cell surface IL-4Rα expression on CD8$^+$ T cells following VV infection, the kinetics of down-regulation of this receptor on CD8$^+$ T cells was monitored. Using ICS and GzmB expression assays, data revealed that VV-specific effector CD8$^+$ T cells emerged on day 5 post-infection (p.i.), peaked on day 7 p.i. and gradually declined from days 14-28 p.i. with VV-WR (**figure 3.2A and 3.2B**). Similarly, reduction in cell surface IL-4Rα
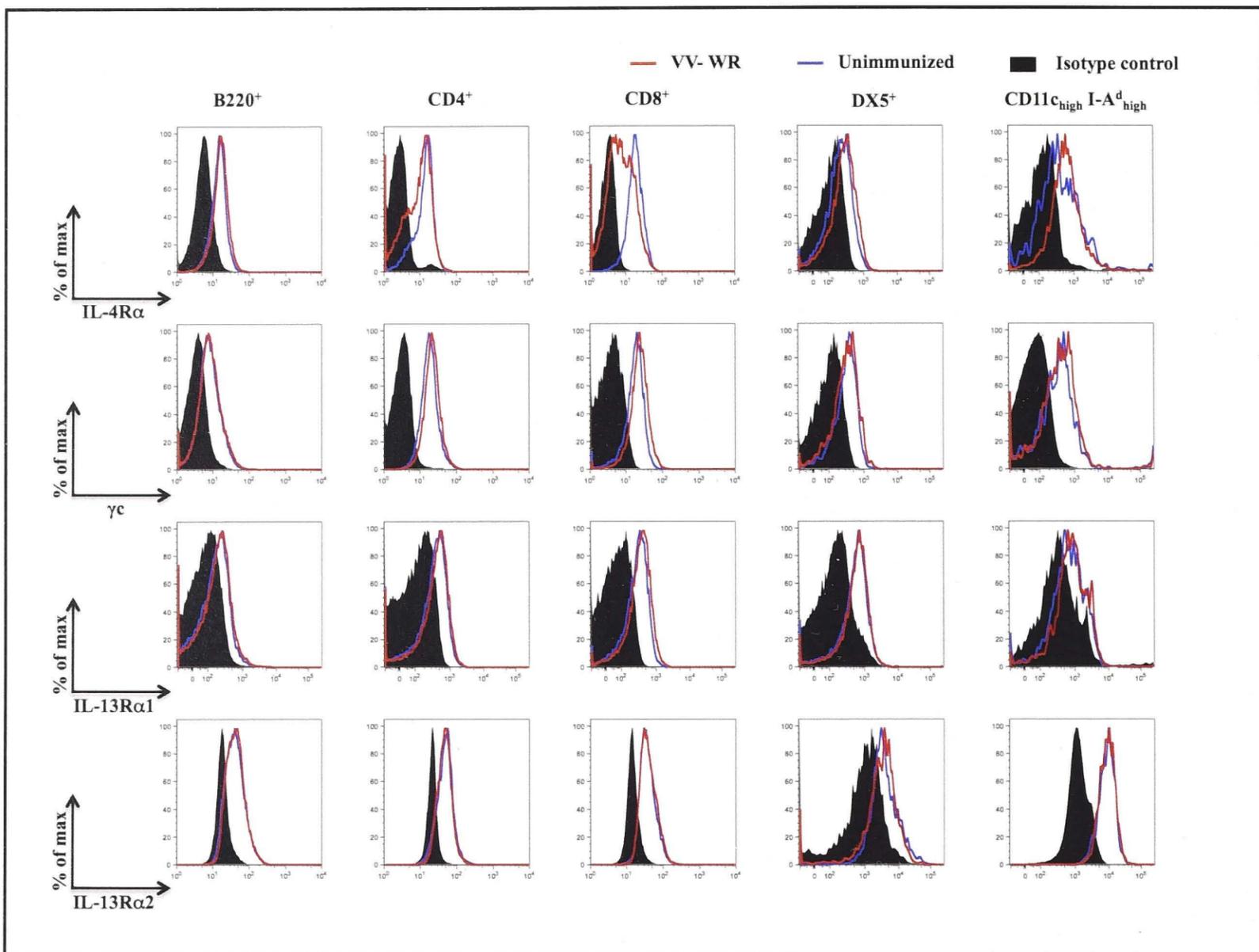
Figure 3.1. Distribution of IL-4Rα, γc, IL-13Rα1 and IL-13Rα2 on immune cells following VV infection. BALB/c WT mice infected for 7 days with VV-WR or unimmunized were sacrificed and splenocytes harvested for analysis using flow cytometry. The histogram plots show cell surface expression of IL-4Rα, γc, IL-13Rα1 and intracellular expression of IL-13Rα2 on gated B220^{+}, CD4^{+}, CD8^{+}, DX5^{+}, and CD11c$_{high}$ I-A$^d_{high}$ splenocytes from a representative VV-WR infected mouse (red lines) and an unimmunized mouse (blue lines). The plots are representative of at least 12 mice tested in at least three independent experiments.
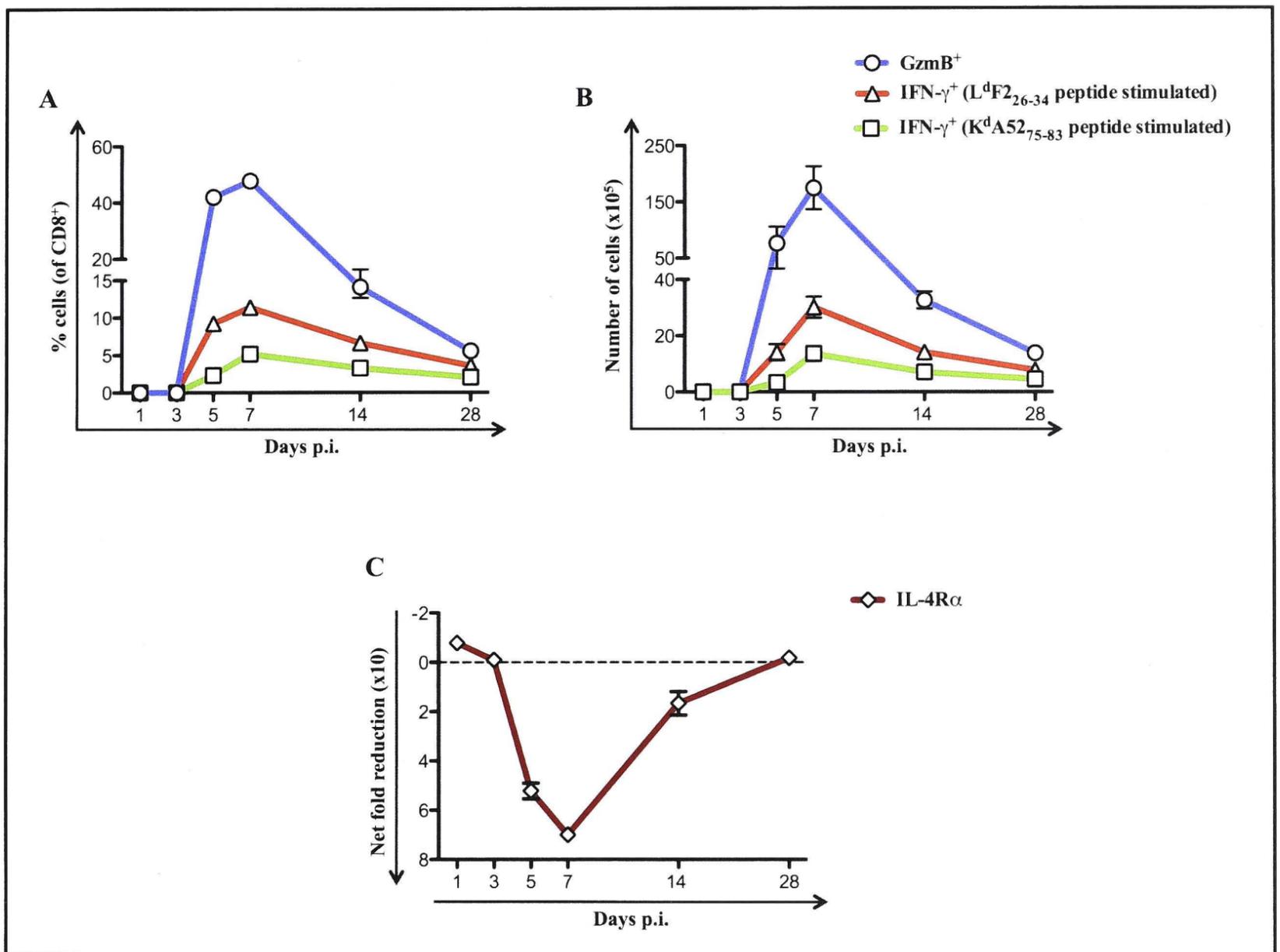
Figure 3.2. Reduction in IL-4Rα expression correlates with magnitude of anti-viral effector responses on CD8⁺ T cells. Unimmunized or VV-WR infected BALB/c WT mice were sacrificed at the indicated time points and splenocytes used for flow cytometry analysis or for ICS following K^dA52_{75-83} or L^dF2_{26-34} *in vitro* peptide stimulation of splenocytes as described in **section 2.8.2**. A and B, The mean (n = 4) percentage of CD8⁺ splenocytes (A) and the total number of CD8⁺ splenocytes (B) from VV-WR infected mice that expressed GzmB or IFN-γ following *in vitro* peptide stimulation. C, The kinetics of the mean (n = 4) net fold reduction in cell surface expression of IL-4Rα on gated CD8⁺ splenocytes from VV-WR infected mice relative to unimmunized mice. Net fold reduction was calculated using MFI values as described in **section 2.13.1**. The data shown is representative of at least two independent experiments and the error bars depict the SEM.

expression on CD8$^+$ T cells following VV-WR infection was detected on day 5 p.i., peaked on day 7 p.i. (**figure 3.2C**). The expression of this receptor on CD8$^+$ T cells then increased from days 14-28 p.i. with VV-WR (**figure 3.2C**). Thus, the magnitude of down-regulation of IL-4Rα expression on CD8$^+$ T cells correlated with the increase in the magnitude of the anti-viral effector CD8$^+$ T cell responses following VV infection.

3.2.3 Down-regulation of IL-4Rα is TCR dependent and restricted to virus-specific CD8$^+$ T cells

Next the OT-I TCR transgenic system was used to show whether IL-4Rα down-regulation was restricted to CD8$^+$ T cells responding to virus. Splenocytes from OT-I mice (CD45.2$^+$) were transferred i.v. into congenic C57BL/6.SJL (CD45.1$^+$; CD45.2$^-$) recipient mice prior to infection of these mice with VV-OVA$_{257-264}$ or VV-WR, which does not express K^{b}OVA$_{257-264}$ (SIINFEKL) epitope. CD8$^+$ T cells from OT-I mice almost exclusively recognize K^{b}OVA$_{257-264}$ and so should only be primed by VV-OVA$_{257-264}$ and not VV-WR. This was confirmed using GzmB as a marker of activation (**figure 3.3A**). IL-4Rα levels were also measured (**figure 3.3B and 3.3C**), and down-regulation of this receptor on OT-I cells (CD45.2$^+$) was only seen in mice infected with VV-OVA$_{257-264}$. By contrast IL-4Rα levels were reduced on recipient (CD45.2$^-$) CD8$^+$ T cells irrespective of the strain of virus. Thus, IL-4Rα levels were only reduced on activated virus-specific CD8$^+$ T cells.

FACS sorted polyclonal naïve (CD44$_{lo}$) CD8$^+$ T cells were also cultured *in vitro* with or without anti-TCR stimulation to determine whether IL-4Rα down-regulation was TCR dependent. IL-4Rα down-regulation was observed on CD8$^+$ T cells as a consequence of plate-bound anti-CD3ϵ stimulation suggesting that this phenomenon was also TCR dependent (**supplementary figure 1**).

3.2.4 Down-regulation of IL-4Rα is a general property of activated anti-viral CD8$^+$ T cells

To evaluate whether down-regulation of cell surface IL-4Rα expression on activated CD8$^+$ T cells is specific to VV infections, IL-4Rα expression was analyzed on effector (GzmB$^+$ CD62L$^-$) and naïve (GzmB$^-$ CD62L$^+$) CD8$^+$ T cells as described in Yuen *et al* [2010] following infection of BALB/c WT mice with VV-WR, MVA, aSFV, FPV or A/PR8 influenza virus (**figure 3.4A**). Cell surface IL-4Rα expression on effector CD8$^+$ T cells compared to naïve bystander CD8$^+$ T cells was significantly lower in all the mice infected with the different viruses (**figure 3.4B and 3.4C**). There were no significant differences between the levels of cell surface IL-4Rα expression on effector or naïve CD8$^+$ T cells that developed following VV-WR infection and other viral infections (**figure 3.4B and 3.4C**). Therefore, down-regulation of cell surface IL-4Rα expression on activated CD8$^+$ T cells is a general feature of virus infections *in vivo*.

3.2.5 Importance of IL-4, IL-13, IFN-γ and STAT6 in regulating IL-4Rα expression on CD8$^+$ T cells following virus infection

IL-4, IL-13 and IFN-γ are cytokines that play a role in regulating IL-4Rα expression on cells [Serpier *et al.*, 1997; Wills-Karp and Finkelman, 2008]. Thus, it was next determined whether down-regulation of cell surface IL-4Rα expression on anti-viral effector CD8$^+$ T cells was dependent on IL-4, IL-13, STAT6 or IFN-γ. For this purpose, IL-4Rα expression was analyzed on effector (GzmB$^+$ CD62L$^-$) and naïve (GzmB$^-$ CD62L$^+$) CD8$^+$ T cells from relevant gene knockout and littermate WT control mice infected with VV-WR. Cell surface IL-4Rα expression on naïve CD8$^+$ T cells, but not effector CD8$^+$ T cells was significantly lower in IL-4 $^{-/-}$, IL-13 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice compared to BALB/c WT control mice (**figure 3.5A and 3.5B**). Interestingly,

Figure 3.3. Cell surface down-regulation of IL-4Rα specifically occurs on activated CD8$^+$ T cells. Naïve C57BL/6.SJL (CD45.1$^+$; CD45.2$^-$) mice that received 10 x 10^6 C57BL/6 OT-I splenocytes (CD45.2$^+$) i.v. were kept unimmunized or infected i.p. with 5 x 10^6 PFU of VV-WR or VV-OVA$_{257-264}$ for 7 days prior to sacrifice and flow cytometry analysis. A, Representative contour plots showing cell surface CD45.2 and intracellular GzmB expression on gated CD8$^+$ splenocytes from a recipient mouse of the indicated group. B, Representative histogram plots showing cell surface IL-4Rα expression on gated CD8$^+$ CD45.2$^-$ (left column of plots) or CD8$^+$ CD45.2$^+$ (right column of plots) splenocytes from a recipient mouse kept unimmunized, infected with VV-WR or VV-OVA$_{257-264}$. C, MFI (n = 6) representing cell surface IL-4Rα expression on gated CD8$^+$ CD45.2$^-$ or CD8$^+$ CD45.2$^+$ splenocytes from recipient mice of the indicated group. One-way ANOVA (Tukey's Multiple Comparison) was used for testing significance of the data (*** - p < 0.001). Similar results have been obtained in three independent experiments and the error bars depict the SEM.

A

Transferred + VV-WR   Transferred + VV-OVA$_{257-264}$ Transferred only

GzmB (y-axis), CD45.2 (x-axis)

Transferred + VV-WR: 34.6 | 0.39 | 63.4 | 1.64
Transferred + VV-OVA$_{257-264}$: 28.2 | 20.1 | 42.5 | 9.16
Transferred only: 0.54 | 0.27 | 96.7 | 2.52

B

CD8$^+$ CD45.2$^-$ CD8$^+$ CD45.2$^+$

% of max (y-axis), IL-4Rα (x-axis)

VV-WR
Unimmunized
Isotype control

VV-OVA$_{257-264}$
Unimmunized
Isotype control

C

Transferred + VV-WR
Transferred + VV-OVA$_{257-264}$
Transferred + unimmunized

CD8$^+$ CD45.2$^-$ CD8$^+$ CD45.2$^+$

MFI (IL-4Rα) (y-axis)

*** ***   *** ***
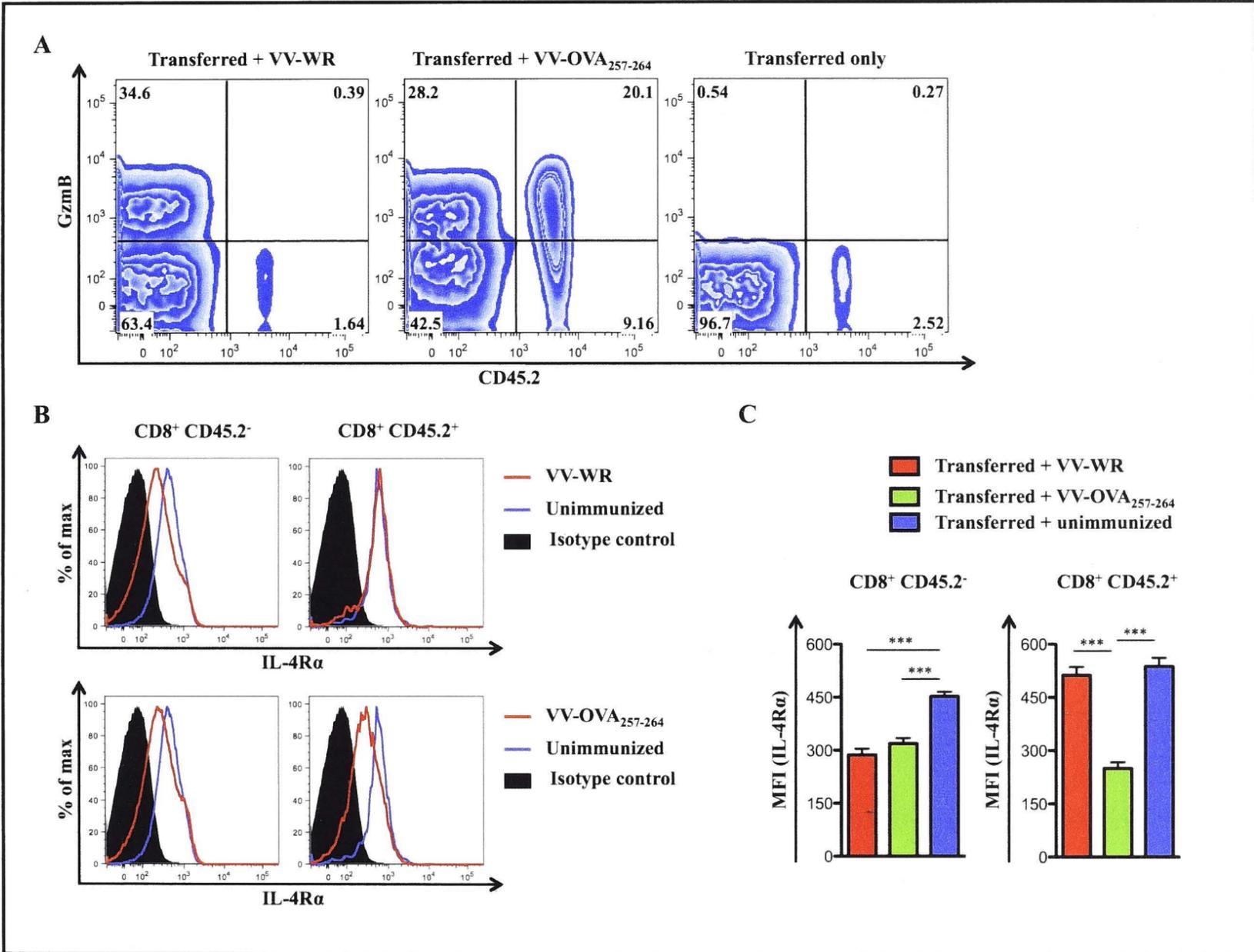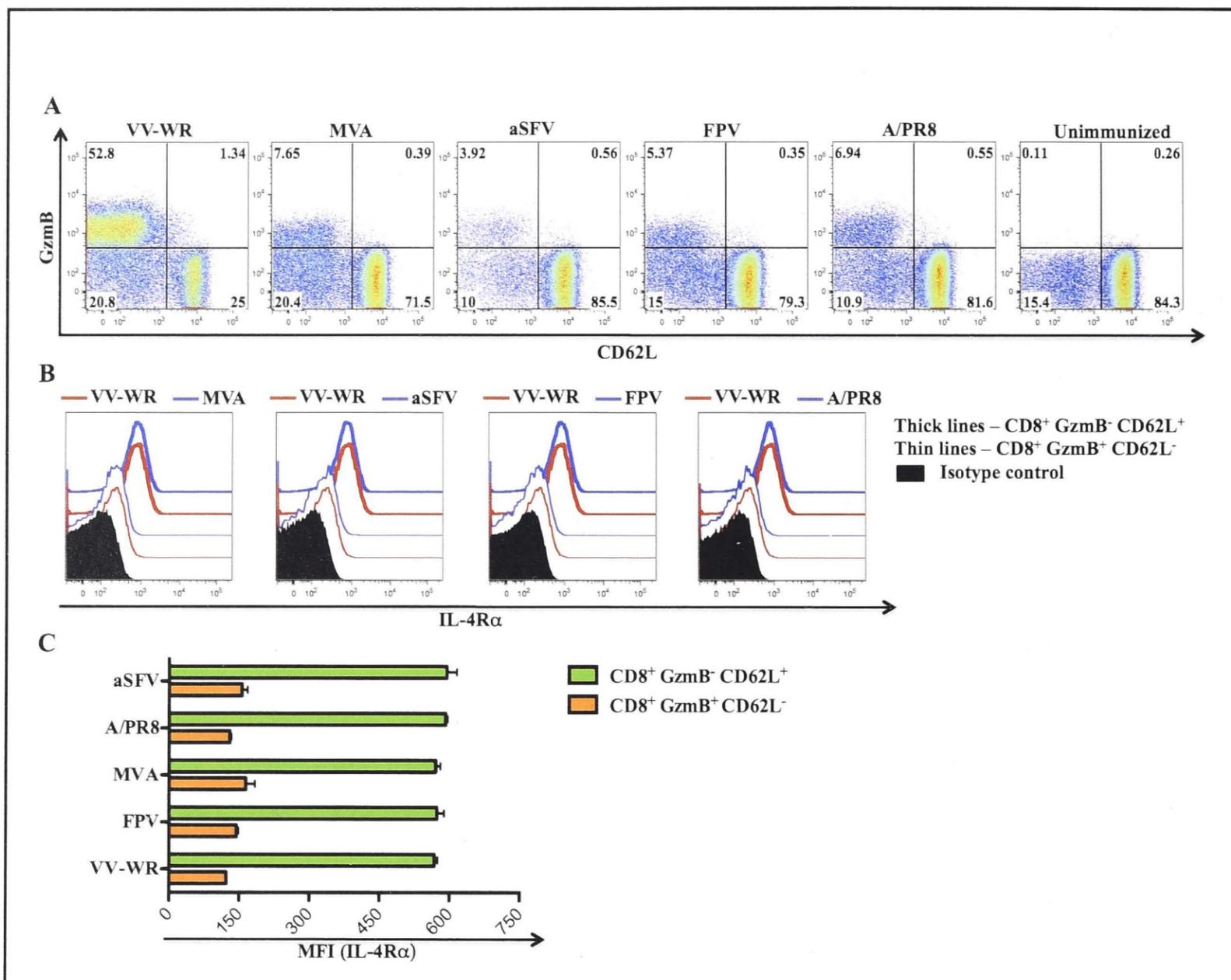
Figure 3.4. Down-regulation of IL-4Rα is a general feature of activated CD8⁺ T cells following virus infection. BALB/c WT mice were infected i.p. with 3×10^6 PFU of the indicated viruses or kept unimmunized for 7 days prior to sacrifice and flow cytometry analysis. A, Dot plots showing cell surface CD62L expression and intracellular GzmB expression on gated CD8⁺ splenocytes from a representative mouse of the indicated group. B, Representative histogram plots showing cell surface IL-4Rα expression on the indicated CD8⁺ splenocyte subset from a representative mouse infected with the indicated virus. C, MFI (n = 5) representing cell surface IL-4Rα expression on the indicated splenocyte subset from mice infected with the indicated viruses. Similar results have been obtained in three independent experiments and the error bars shown depict the SEM.

60
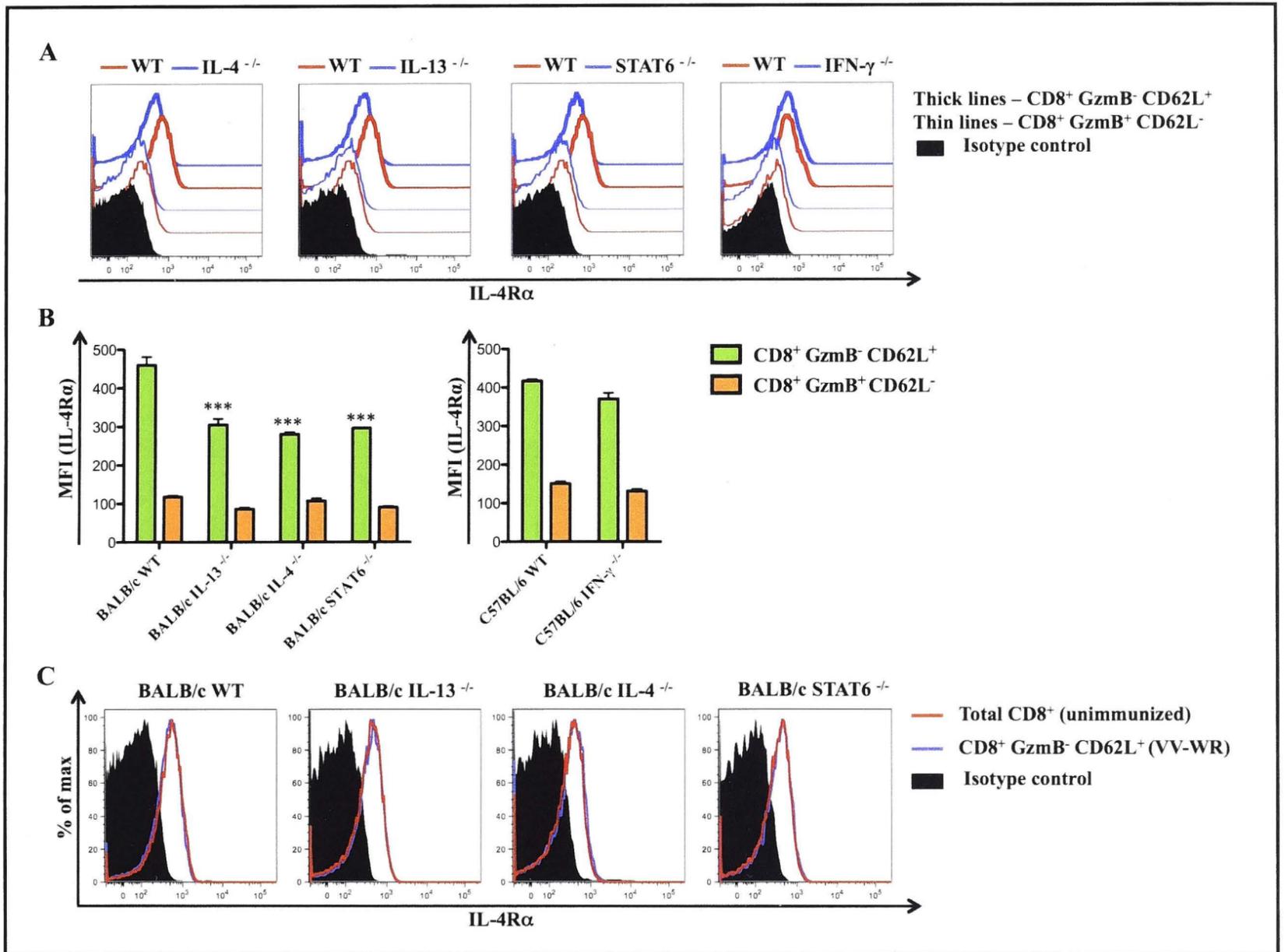
Figure 3.5. IL-4, IL-13 and STAT6 regulate IL-4Rα expression on CD8$^+$ T cells following VV-WR infection. Gene knockout mice and respective littermate WT control mice were infected with VV-WR for 7 days or kept unimmunized prior to sacrifice and analysis using flow cytometry. A, Representative histogram plots showing cell surface IL-4Rα expression on the indicated CD8$^+$ splenocyte subset from a gene knockout mouse or a littermate WT control mouse. B, MFI (n = 5) representing cell surface IL-4Rα expression on the indicated CD8$^+$ splenocyte subset from mice of the indicated genetic background infected with VV-WR. C, Representative histogram plots showing cell surface IL-4Rα expression on the indicated CD8$^+$ splenocyte subset from an unimmunized mouse or VV-WR infected mouse belonging to the indicated genetic background. All the results shown are representative of at least two independent experiments. Error bars when shown depict the SEM and one-way ANOVA (Dunnett Multiple Comparison) was used to determine the statistical significance of the data relative to WT control mice (*** - p < 0.001).

naïve B cells, CD4$^+$ T cells, NK cells and DCs did not express lower levels of IL-4Rα in naïve gene knockout mice for IL-4, IL-13 and STAT6 relative to WT controls (**supplementary figure 2**).

In these experiments, IL-4Rα expression levels on naïve CD8$^+$ T cells from VV-WR infected mice and unimmunized mice were also measured. This was done to investigate the possibility that VV-WR infection induced IL-4 and/or IL-13 to up-regulate IL-4Rα expression on naïve bystander CD8$^+$ T cells similar to what has been reported on naïve bystander CD4$^+$ T cells responding to IL-4 following parasitic infection [Perona-Wright et al., 2010]. However, naïve CD8$^+$ T cells from VV-WR infected IL-13 $^{-/-}$, IL-4 $^{-/-}$, STAT6 $^{-/-}$ or WT control BALB/c mice expressed similar levels of cell surface IL-4Rα to that of naïve CD8$^+$ T cells from unimmunized mice belonging to the respective genetic background (**figure 3.5C**). This suggests that VV-WR infection was not inducing significant levels of IL-4 and/or IL-13 to up-regulate IL-4Rα expression on naïve CD8$^+$ T cells. In contrast to the knockout mice described above, no difference in IL-4Rα expression levels were seen on any CD8$^+$ T cells from C57BL/6 IFN-γ $^{-/-}$ compared to C57BL/6 WT controls (**figure 3.5A and 3.5B**). Collectively, data suggest that IL-4, IL-13 and STAT6 are required for maintaining high levels of IL-4Rα expression on naïve CD8$^+$ T cells.

3.2.6 Increased IL-4 during virus infection induces higher levels of IL-4Rα on CD8$^+$ T cells

Next whether IL-4Rα levels on CD8$^+$ T cells were fixed/unchanged, or responsive to IL-4 during infection was investigated. For these studies, VV-HA-IL-4 and the control VV-HA were used for infection. Strikingly, IL-4Rα expression was higher in BALB/c

WT mice infected with VV-HA-IL-4 relative to VV-HA infection when comparing naïve (GzmB$^-$ CD62L$^+$) or effector (GzmB$^+$ CD62L$^-$) CD8$^+$ T cells (**figure 3.6**). Similarly, up-regulation of IL-4Rα was also observed following IL-4 exposure of FACS sorted naïve CD8$^+$ splenocytes from BALB/c WT mice *in vitro* stimulated or unstimulated with plate-bound anti-CD3ε (**supplementary figure 1**). When the VV-HA and VV-HA-IL-4 infection experiments were performed using BALB/c STAT6$^{-/-}$ mice, no difference in IL-4Rα expression was observed on CD8$^+$ T cells unlike that observed in BALB/c WT mice (**figure 3.6**). Data indicate that the amount of IL-4 available during virus infection can regulate the expression of IL-4Rα on CD8$^+$ T cells in a STAT6 dependent manner.

3.2.7 Transcriptional control is not predictive of protein IL-4Rα expression on effector CD8$^+$ T cells following VV-WR infection

Whether lower production of IL-4Rα mRNA transcripts was responsible for down-regulating protein expression of IL-4Rα on CD8$^+$ T cells following VV infection was also evaluated. RNA extracted from FACS sorted IL-4Rα$_{lo}$ and IL-4Rα$_{hi}$ CD8$^+$ T cells (as shown in **supplementary figure 3A**) were used in these studies to synthesize cDNA for a quantitative real-time PCR analysis as described in **section 2.12**. The data indicate that there were no significant differences in the IL-4Rα mRNA transcript levels between IL-4Rα$_{lo}$ and IL-4Rα$_{hi}$ CD8$^+$ T cells despite the differences in IL-4Rα protein expression between these two cell populations (**supplementary figure 3B**). GzmB mRNA transcript levels were measured as a positive control given that GzmB mRNA transcript levels have been reported to be enhanced on VV-specific effector/memory CD8$^+$ T cells compared to naïve CD8$^+$ T cells [Yoshida *et al.*, 2006]. Data showed that IL-4Rα$_{lo}$ CD8$^+$ T cells clearly expressed much higher (~50 fold) GzmB mRNA
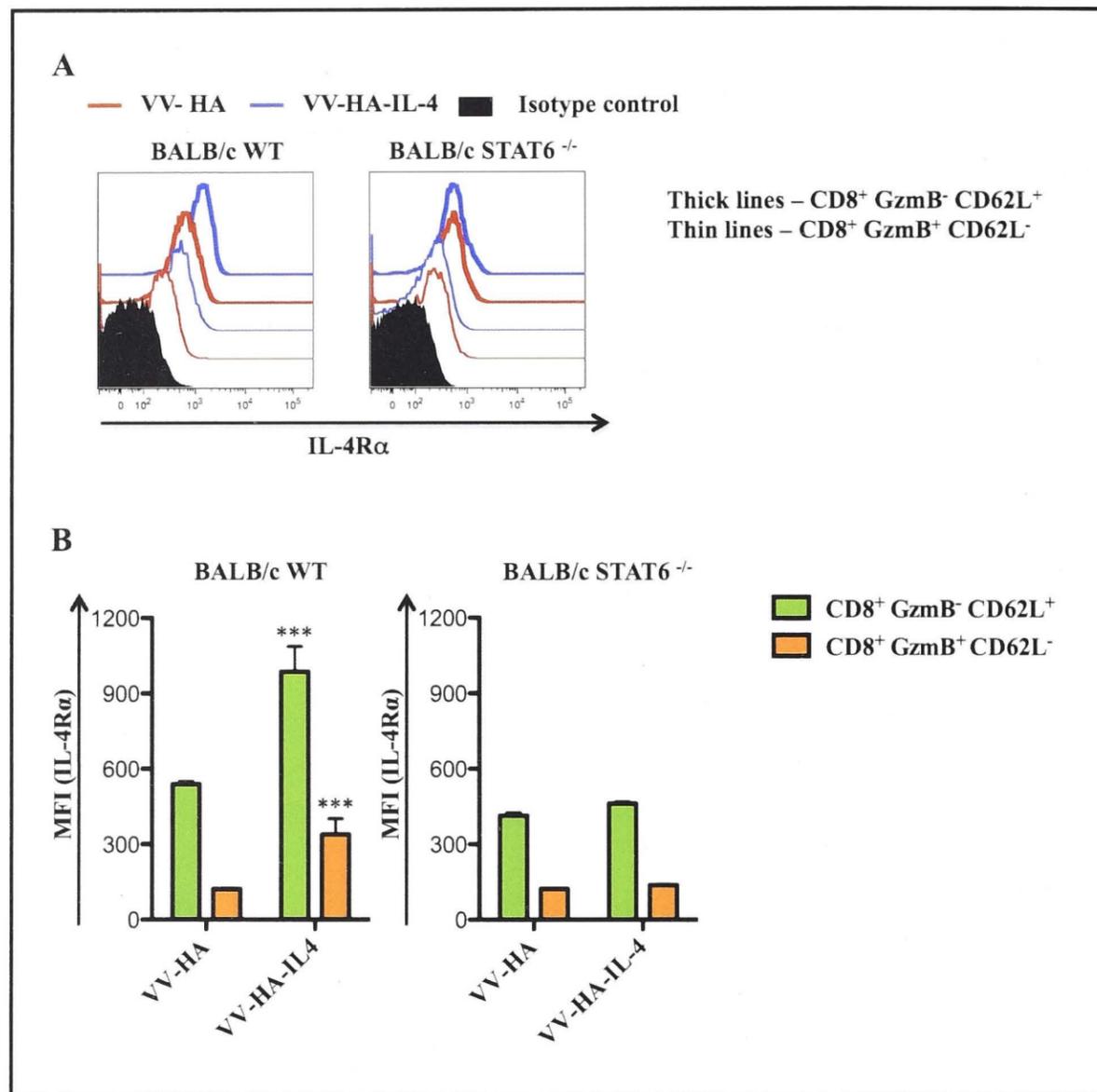
Figure 3.6. IL-4Rα is up-regulated on CD8$^+$ T cells in a STAT6 dependent manner following VV-HA-IL-4 infection. WT or STAT6 $^{-/-}$ BALB/c mice were infected with 5 x 10^6 PFU of VV-HA control, VV-HA-IL-4 or kept unimmunized for 7 days prior to sacrifice and analysis using flow cytometry. A and B, Representative histogram plots showing cell surface IL-4Rα expression (A) and the MFI (n = 5) of cell surface IL-4Rα expression (B) on the indicated CD8$^+$ splenocyte subset from infected mice of the indicated genetic background. The data shown is representative of two independent experiments and the error bars depict the SEM. One-way ANOVA (Dunnett's Multiple Comparison) was used to determine the statistical significance of the data relative to respective cell subset from VV-HA infected mice (*** - p < 0.001).

transcript levels compared to IL-4Rα_{hi} CD8$^+$ T cells (**supplementary figure 3B**).

3.2.8 IL-4 and IL-13 regulate the establishment of IFN-γ$^+$ TNF-α$^+$ producing effector CD8$^+$ T cells following virus infection

High avidity virus-specific CD8$^+$ T cells express elevated levels of both IFN-γ and TNF-α [La Gruta *et al.*, 2006; Ranasinghe *et al.*, 2007]. To evaluate whether differential regulation of cell surface IL-4Rα expression on CD8$^+$ T cells may play a role in regulating CD8$^+$ T cell functional quality, IFN-γ and TNF-α production were measured using ICS following *in vitro* K^{d}A52$_{75\text{-}83}$ or L^{d}F2$_{26\text{-}34}$ peptide stimulation of splenocytes from VV infected WT and gene knockout BALB/c mice. Amongst the gene knockout mice examined only BALB/c IL-4$^{-/-}$ mice developed greater numbers of IFN-γ expressing K^{d}A52$_{75\text{-}83}$ or L^{d}F2$_{26\text{-}34}$ specific CD8$^+$ T cells compared to BALB/c WT control mice following VV-WR infection (**figure 3.7B**). However, the enhancement in cell numbers in this instance was not robust enough to reach statistical significance (**figure 3.7B**). The number of IFN-γ$^+$ TNF-α$^+$ K^{d}A52$_{75\text{-}83}$ or L^{d}F2$_{26\text{-}34}$ specific CD8$^+$ T cells and the proportion of K^{d}A52$_{75\text{-}83}$ or L^{d}F2$_{26\text{-}34}$ specific CD8$^+$ T cells that expressed TNF-α in addition to IFN-γ was higher in VV-WR infected IL-13$^{-/-}$, IL-4$^{-/-}$ and STAT6$^{-/-}$ BALB/c mice compared to BALB/c WT control mice (**figure 3.7**). Given that IL-4Rα expression on naïve CD8$^+$ T cells was lower in IL-13$^{-/-}$, IL-4$^{-/-}$ and STAT6$^{-/-}$ BALB/c mice compared to BALB/c WT mice (**figure 3.5**), in these studies, lower IL-4Rα expression on naïve CD8$^+$ T cells correlated with the enhancement in the anti-viral IFN-γ$^+$ TNF-α$^+$ CD8$^+$ T cell responses.

Figure 3.7. IL-4 and IL-13 dampen polyfunctional (IFN-γ^+ TNF-α^+) CD8$^+$ T cell numbers following VV-WR infection. IL-13 $^{-/-}$, IL-4 $^{-/-}$, STAT6 $^{-/-}$ and WT BALB/c mice were infected with 3 x 10^6 PFU of VV-WR or kept as unimmunized controls for 7 days prior to sacrifice and analysis using ICS after *in vitro* peptide stimulation. A, Representative dot plots showing IFN-γ and TNF-α expression on gated CD8$^+$ splenocytes from VV-WR infected mice of the indicated genetic background following *in vitro* stimulation of splenocytes with the indicated peptides. B, Mean (n = 6) total number of K^{d}A52$_{75-83}$ or L^{d}F2$_{26-34}$ specific CD8$^+$ IFN-γ^+ or CD8$^+$ IFN-γ^+ TNF-α^+ splenocytes from the indicated mice infected with VV-WR. C, Mean (n = 6) proportion of K^{d}A52$_{75-83}$ or L^{d}F2$_{26-34}$ specific CD8$^+$ IFN-γ^+ splenocytes that also produced TNF-α from the indicated mice infected with VV-WR. The data presented in all panels are representative of at least two independent. Error bars depict the SEM and statistical significance was determined using a one-way ANOVA (Dunnett's Multiple Comparison) relative to WT control mice (* - $p < 0.05$; *** - $p < 0.001$).

A

BALB/c WT   BALB/c IL-13 $^{-/-}$ BALB/c IL-4 $^{-/-}$ BALB/c STAT6 $^{-/-}$

K^dA52_{75-83}

L^dF2_{26-34}

IFN-γ

TNF-α

B

☐ BALB/c WT   ☐ BALB/c IL-13 $^{-/-}$ ☐ BALB/c IL-4 $^{-/-}$ ☐ BALB/c STAT6 $^{-/-}$

K^dA52_{75-83}

L^dF2_{26-34}

Number of CD8$^+$ IFN-γ$^+$ cells (x10^5)

Number of CD8$^+$ IFN-γ$^+$ TNF-α$^+$ cells (x10^5)

C

% TNF-α$^+$ (of CD8$^+$ IFN-γ$^+$)

K^dA52_{75-83}

L^dF2_{26-34}

67
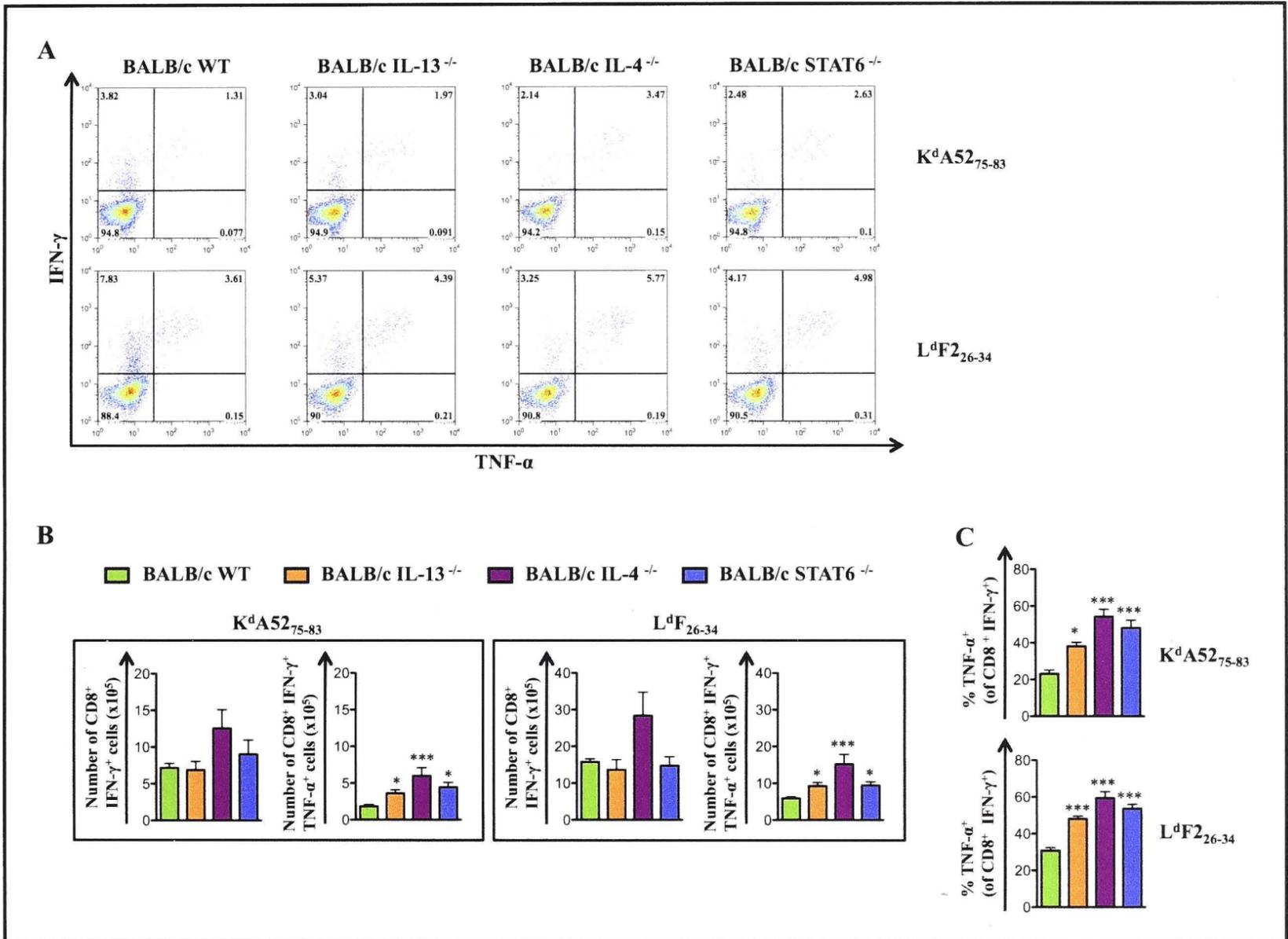
Figure 3.8. STAT6 is required for IL-4 mediated attrition of VV-specific CD8$^+$ T cell responses. WT and STAT6 $^{-/-}$ BALB/c mice were infected with 5 x 10^6 PFU of VV-HA, VV-HA-IL-4 or kept unimmunized for 7 days prior to sacrifice and analysis using ICS after *in vitro* peptide stimulation. A and B, Representative dot plots showing IFN-γ and TNF-α expression on gated CD8$^+$ splenocytes from infected BALB/c WT (A) or BALB/c STAT6 $^{-/-}$ (B) mice following *in vitro* stimulation of splenocytes with the indicated peptides. C, The mean (n = 4-5) number of cytokine producing K^{d}A52$_{75-83}$ or L^{d}F2$_{26-34}$ specific CD8$^+$ splenocytes and the mean (n = 4-5) proportion of CD8$^+$ splenocytes that produced TNF-α in addition to IFN-γ from WT (top row of plots) or STAT6 $^{-/-}$ (bottom row of plots) BALB/c mice infected with the indicated virus. The data are representative of two independent experiments and the error bars depict the SEM. One-way ANOVA (Dunnett's Multiple Comparison) was used to determine statistical significance of the data relative to VV-HA infected mice (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$).

A

VV-HA   VV-HA-IL-4

IFN-γ

| 3.37 | | 1.85 |
| 94.4 | | 0.4 |

| 2.64 | | 1.08 |
| 96.1 | | 0.21 |

K^dA52_{75-83}

| 8.18 | | 3.55 |
| 87.8 | | 0.42 |

| 6.12 | | 2.1 |
| 91.4 | | 0.45 |

L^dF2_{26-34}

TNF-α

B

VV-HA   VV-HA-IL-4

IFN-γ

| 3.03 | | 2.77 |
| 93.9 | | 0.33 |

| 2.37 | | 3 |
| 94.2 | | 0.41 |

K^dA52_{75-83}

| 6.37 | | 5.71 |
| 87.6 | | 0.35 |

| 5.93 | | 5.56 |
| 87.7 | | 0.84 |

L^dF2_{26-34}

TNF-α

C

Number of CD8$^+$ IFN-γ$^+$ cells (x10^5)

Number of CD8$^+$ IFN-γ$^+$ TNF-α$^+$ cells (x10^5)

% TNF-α$^+$ (of IFN-γ$^+$)

■ VV-HA
■ VV-HA-IL-4

BALB/c WT

BALB/c STAT6 $^{-/-}$

69
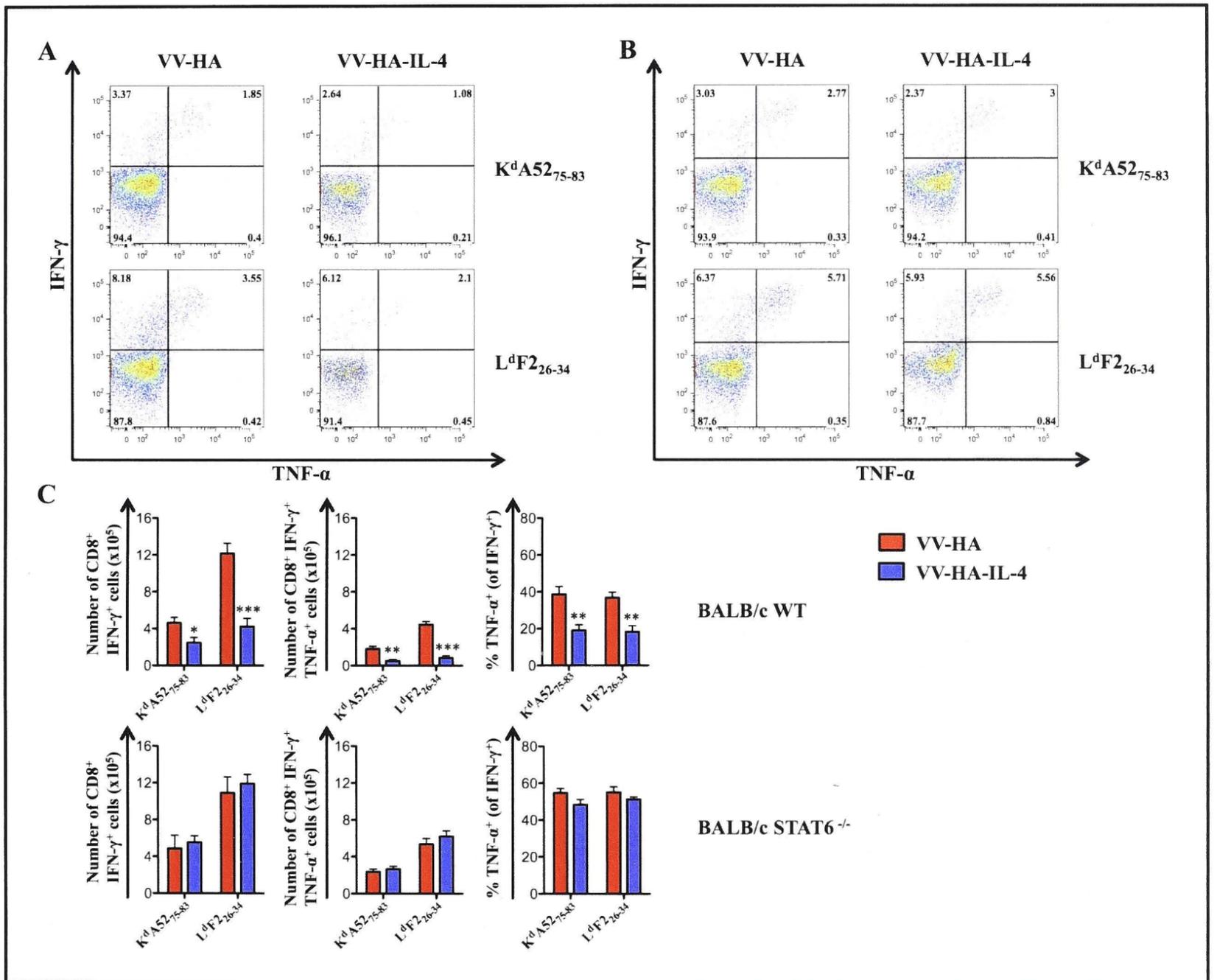
Consistent with the above findings, infection of BALB/c WT mice with VV-HA-IL-4 compared to VV-HA control infection significantly reduced the numbers of IFN-γ^+ and IFN-γ^+ TNF-α^+ K^dA52_{75-83} or L^dF2_{26-34} specific CD8$^+$ T cells (**figure 3.8A and 3.8C**). The proportion of K^dA52_{75-83} or L^dF2_{26-34} specific CD8$^+$ T cells that expressed TNF-α in addition to IFN-γ was also significantly reduced in BALB/c WT mice infected with VV-HA-IL-4 compared to VV-HA (**figure 3.8A and 3.8C**). Unlike that observed with BALB/c WT mice, infection of BALB/c STAT6 $^{-/-}$ mice with VV-HA-IL-4 compared to VV-HA control did not impair the IFN-γ and TNF-α cytokine production capacity of K^dA52_{75-83} or L^dF2_{26-34} specific CD8$^+$ T cells (**figure 3.8B and 3.8C**). Thus, elevation of IL-4Rα expression on CD8$^+$ T cells during virus infection strongly correlated with the reduction of anti-viral IFN-γ^+ TNF-α^+ CD8$^+$ T cell responses.

3.3 Discussion

Results clearly indicated that amongst the different IL-4/IL-13 receptor components only cell surface IL-4Rα expression was significantly down-regulated on activated CD8$^+$ T cells following virus infection. IL-4, IL-13 and STAT6 were required to elevate IL-4Rα expression on naïve CD8$^+$ T cells, but not IFN-γ. VV-HA-IL-4 infection studies showed that IL-4 and STAT6 were required to up-regulate IL-4Rα expression on naïve and effector CD8$^+$ T cells. In all these studies higher IL-4Rα expression on CD8$^+$ T cells strongly correlated with the reduction in polyfunctional or IFN-γ^+ TNF-α^+ anti-viral CD8$^+$ T cells. Thus, it is plausible to propose that regulation of IL-4Rα expression during virus infection plays an important role in regulating the quality of anti-viral CD8$^+$ T cell immunity.

Contrary to suggestions from other reports [Graber *et al.*, 1998; Wills-Karp and Finkelman, 2008], data in the current study showed that IL-13Rα1 is ubiquitously expressed on the cell surface of T cells as well as B cells, NK cells and DCs. The reason for this discrepancy is unclear, but it is most likely due to the lack of specific monoclonal antibodies used to detect surface expression of this receptor [Graber *et al.*, 1998]. Similar ubiquitous expression on immune cells was also observed with respect to IL-4Rα, γc, and IL-13Rα2. However, following virus infection only IL-4Rα was notably differentially regulated on certain immune cell subsets (i.e. CD4$^+$ T cells, CD8$^+$ T cells and DCs). The mechanisms responsible for regulating IL-4Rα expression on CD4$^+$ T cells and DCs following virus infection were not investigated in this study and warrants further investigation especially given the importance of these cell subsets in driving immune responses that help control intracellular pathogens [Seder *et al.*, 2008; Kroger *et al.*, 2008].

In an acute LCMV adoptive transfer model, Wherry *et al* [2007] have reported changes in many cellular markers, including IL-4Rα expression on transferred LCMV DbGP33-specific transgenic P14 CD8$^+$ T cells. In their model, IL-4Rα expression was down-regulated on LCMV DbGP33-specific CD8$^+$ T cells following acute and chronic LCMV infection. These findings are consistent with the findings of the current study. However, unlike the current study, Wherry *et al* [2007] did not investigate the factors responsible for regulating IL-4Rα expression on polyclonal CD8$^+$ T cells or the implications of regulating this receptor on CD8$^+$ T cell functionality following virus infection in WT mice.

IL-4 can induce the activation of STAT1, STAT3, STAT5 and STAT6 on naïve and activated CD8$^+$ T cells *in vitro* [Acacia de Sa Pinheiro *et al.*, 2007], but the current

infection studies with VV-HA-IL-4 suggest that STAT6 was indispensible for IL-4 mediated up-regulation of cell surface IL-4Rα expression on naïve and effector CD8$^+$ T cells. Even in naïve gene knockout mice STAT6 signaling, which appeared to be optimal in the presence of both IL-4 and IL-13, was required to maintain high levels of cell surface IL-4Rα expression on CD8$^+$ T cells. Thus, IL-4 and/or IL-13 signaling through STAT6 appears to be important in maintaining high levels of IL-4Rα expression on naïve CD8$^+$ T cells even when no pathogen is encountered. Furthermore, this signaling mechanism appears to also be important in elevating IL-4Rα expression on naïve and effector CD8$^+$ T cells *in vivo* following virus infection. However, a caveat in this interpretation is that deficiency of STAT6 in other cells may play a role in regulating IL-4Rα expression levels on CD8$^+$ T cells.

Previous infection studies with VV-HA-IL-4 or recombinant ectromelia virus encoding IL-4 suggest that IL-4 dampened the effector function capacity of anti-viral CD8$^+$ T cells and enhanced viral pathogenesis [Sharma *et al.*, 1996; Jackson *et al.*, 2001]. In chronic HIV progressors, HIV-specific CD8$^+$ T cells with reduced cytolytic capacity have been reported to express IL-4 [Maggi *et al.*, 1994]. In our laboratory, following HIV-1 recombinant pox-viral prime-boost vaccination, both IL-4 and IL-13 were found to dampen the avidity of HIV-specific CD8$^+$ T cells [Ranasinghe *et al.*, 2009]. However, none of the above studies have monitored the expression of IL-4Rα in conjunction with the anti-viral effector functions on CD8$^+$ T cells. This was addressed this in the current study and the data suggest that enhancing CD8$^+$ T cell responsiveness to IL-4 and/or IL-13 via STAT6 dependent up-regulation of IL-4Rα expression can exacerbate the quality (IFN-γ and TNF-α cytokine production) of anti-viral CD8$^+$ T cell immunity.

Paradoxical to these studies, our laboratory findings have also shown that HIV-specific CD8$^+$ T cells that developed in IL-4Rα $^{-/-}$ mice following HIV-1 recombinant prime-boost vaccination expressed high levels of IL-4 and IL-13, but low levels of IFN-γ [Ranasinghe *et al.*, 2009]. Other studies using IL-4Rα $^{-/-}$ mice have also shown that IL-4Rα is required for the maintenance of optimal CD8$^+$ T cell cytotoxicity, IFN-γ production and memory responses [Marsland *et al.*, 2005; Morrot *et al.*, 2005]. It is highly likely that in IL-4Rα $^{-/-}$ mice, other compensatory mechanisms may play a role in dampening CD8$^+$ T cell functionality as Mohrs *et al* [2000] have shown that IL-4 producing T$_H$ cell (T$_H$2) differentiation can still occur in IL-4Rα $^{-/-}$ mice *in vivo*. Identification of these compensatory mechanisms may not only help understand mechanisms important for regulating T cell quality, but also help reconcile the paradoxical findings discussed above.

The exact intracellular mechanisms that facilitate down-regulation of IL-4Rα on CD8$^+$ T cells are not clear. The findings from this study suggest that mRNA transcriptional regulation is not involved in this process. Given that DOCK2 is important for down-regulating IL-4Rα expression on CD4$^+$ T cells [Tanaka *et al.*, 2007], it would be intriguing to investigate whether DOCK2 also plays a similar role in CD8$^+$ T cells. The current study also did not investigate how IL-4 and/or IL-13 mediated elevation of IL-4Rα expression on CD8$^+$ T cells act to dampen IFN-γ and TNF-α cytokine production by anti-viral CD8$^+$ T cells. It is possible that activation of suppressor of cytokine signaling-1 and -3 on anti-viral CD8$^+$ T cells is important for this effect as IL-4 and IL-13 mediated activation of these transcription factors have been shown to dampen IFN-γ and TNF-α cytokine production by keratinocytes [Albanesi *et al.*, 2007]. IL-4 has also been shown to down-regulate the CD8 co-receptor expression levels on antigen-specific CD8$^+$ T cells, which dampens the functionality of these cells [Erard *et al.*, 1993;

Kienzle *et al.*, 2005; Apte *et al.*, 2010]. Therefore, IL-4 and/or IL-13 mediated regulation of the CD8 co-receptor expression levels on anti-viral CD8$^+$ T cells may also play a role in regulating anti-viral cytokine production and this will be addressed in chapter 4.

To my understanding, this is the first study to evaluate how the different cellular receptor components for IL-4 and IL-13 are regulated on CD8$^+$ T cells following virus infection. The data from this study suggest that differential regulation of IL-4Rα, unlike other IL-4/IL-13 receptor components (i.e. γc, IL-13Rα1 and IL-13Rα2) plays a more critical role in determining the responsiveness of CD8$^+$ T cells to IL-4 and/or IL-13 in order to regulate the quality of anti-viral CD8$^+$ T cell immunity. This is consistent with our laboratory studies where IL-4 and IL-13 were shown to play an important role in modulating the avidity of HIV-specific CD8$^+$ T cell responses following prime-boost vaccination [Ranasinghe *et al.*, 2007; Ranasinghe *et al.*, 2009]. Thus, the current findings could be exploited to design more effective pox viral-vectored vaccines against chronic infections such as HIV-1 where robust CD8$^+$ T cell immunity is required for protection. Furthermore, given that IL-4Rα is constitutively expressed on T cells, it may easily be used as a novel biomarker to assess T cell quality following vaccination.

Chapter 4

IL-4 and IL-13 Modulation of Anti-Viral CD8$^+$ T Cell Avidity Following HIV-1 Recombinant Pox Viral Prime-Boost Vaccination

4.1 Introduction

Even though effective anti-retroviral drugs are currently available against HIV-1 [Thompson *et al.*, 2010], developing a vaccine still remains a major priority for slowing down the progression of HIV-1 incidences worldwide. Numerous studies have shown that elite controllers despite being infected with HIV-1 typically have undetectable HIV-1 in the plasma (<50 RNA copies/ml plasma), which correlates with the presence of high avidity and polyfunctional HIV-specific CD8$^+$ T cells [Betts *et al.*, 2006; Almeida *et al.*, 2007; Critchfield *et al.*, 2007; Harari *et al.*, 2008]. Recombinant pox viruses are extensively used for HIV-1 vaccine development. Therefore, understanding factors that could dictate avidity outcomes of anti-viral CD8$^+$ T cells following HIV-1 recombinant pox viral vaccination could be important for rationale design of effective HIV-1 vaccines in the future.

In the previous chapter, it was discussed that the expression levels of receptors for cytokines IL-4 and IL-13, particularly IL-4Rα was important for modulating anti-viral CD8$^+$ T cell avidity. To gain more insight into the roles of IL-4 and IL-13 in modulating avidity, the current chapter investigates whether these cytokines regulated the expression of T cell associated avidity markers (i.e. TCR, CD8, CD11a, CD2 and lck; **section 1.4**) to affect anti-viral CD8$^+$ T cell avidity. These investigations were conducted predominantly using published HIV-1 recombinant pox viral prime-boost

vaccination strategies in our laboratory (**table 1.5**) to help understand how these strategies generated high avidity anti-viral CD8$^+$ T cells.

4.2 Results

4.2.1 IL-4 and IL-13 selectively dampen CD8 densities on HIV-specific CD8$^+$ T cells

Initially it was examined whether IL-4 and IL-13 regulated the expression of various T cell associated molecules, which could dampen the avidity of anti-viral CD8$^+$ T cells induced following HIV-1 recombinant pox viral prime-boost vaccination. For this purpose, the expression of a representative panel of T cell avidity markers (i.e. TCR, CD8, CD11a, CD2 and lck) were measured on K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells that were induced following i.n. FPV-HIV/i.m. VV-HIV vaccination of WT, IL-13 $^{-/-}$, IL-4 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice (**figure 4.1**).

Following HIV-1 recombinant prime boost vaccination, only CD8α and CD8β levels were found to be consistently enhanced on K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells obtained from gene knockout mice relative to the WT controls (**figure 4.1B**). Although at least one report has suggested that high avidity anti-viral CD8$^+$ T cells can enhance the expression ratios of CD8β:CD8α [Kroger *et al.*, 2007], these ratios were similar on K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells from all vaccinated animals (**figure 4.2A**). IL-4 and IL-13 mediated reduction of CD8 densities was also observed on K^{d}A52$_{75-83}$ specific CD8$^+$ T cells 7 days following VV-WR infection (**figure 4.2B**). This suggested that this phenomenon was not specific to K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells or the time point (i.e. 14 days post booster vaccination) designated to evaluate immunity following prime-boost vaccination.
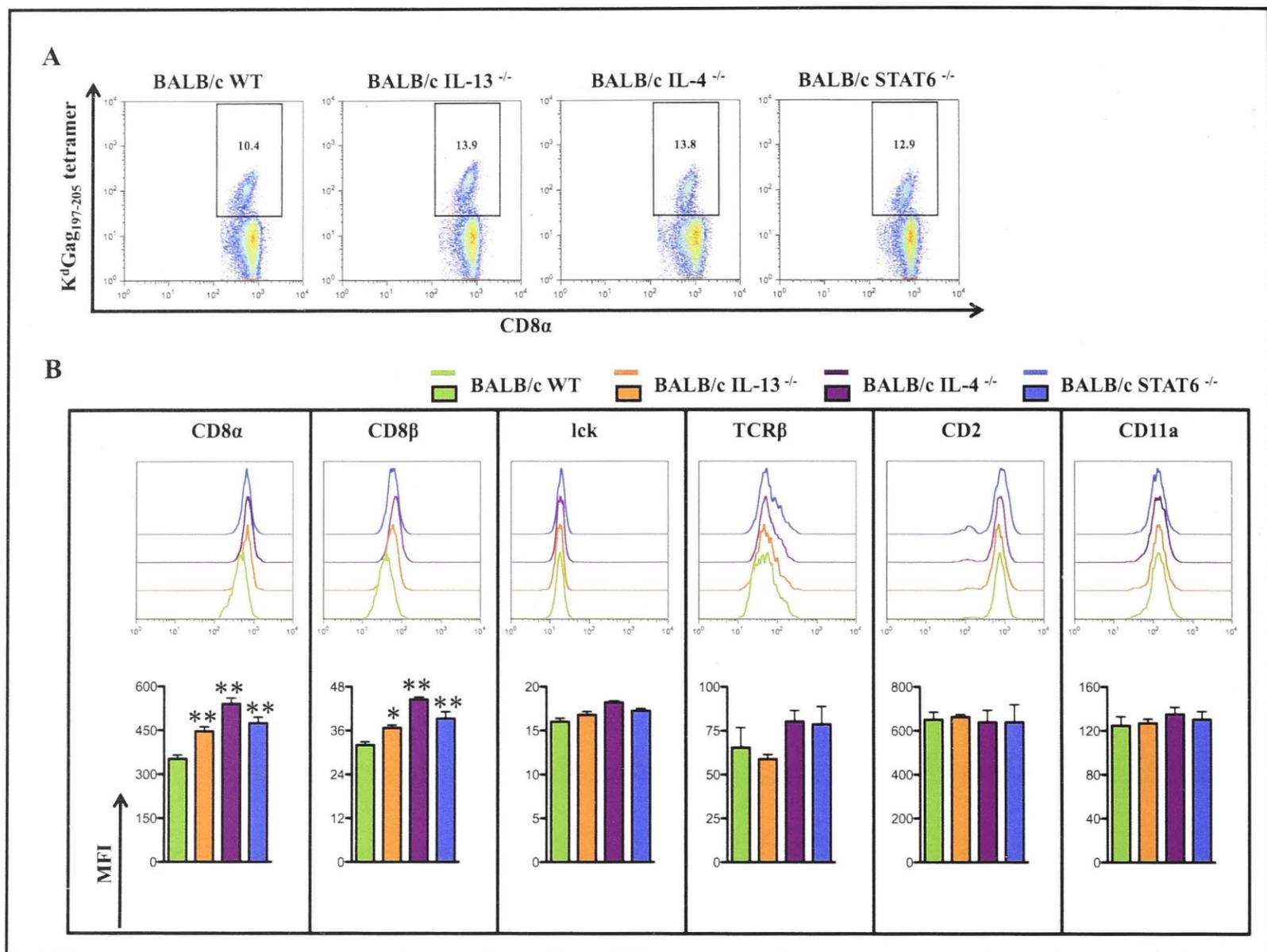
Figure 4.1. IL-4 and IL-13 dampen CD8 densities on $K^dGag_{197-205}$ specific $CD8^+$ T cells following HIV-1 recombinant pox viral prime-boost vaccination. A, Representative dot plots showing $CD8\alpha$ and $K^dGag_{197-205}$ tetramer expression on gated $CD8^+$ splenocytes from mice of the indicated genetic background vaccinated i.n FPV-HIV/i.m. VV-HIV 14 days apart. The number in the gate of each plot represents the percentage of $CD8^+$ splenocytes that recognize the $K^dGag_{197-205}$ tetramer. B, Representative histogram plots and the MFI (n = 4) representing the expression of the indicated T cell avidity markers on $K^dGag_{197-205}$ tetramer specific $CD8^+$ splenocytes obtained from vaccinated mice in A. Statistical significance of the data is shown relative to BALB/c WT mice using one-way ANOVA (Dunnett's Multiple Comparison). The data are representative of at least two independent experiments and the error bars depict the SEM. * - $p < 0.05$; ** - $p < 0.01$.
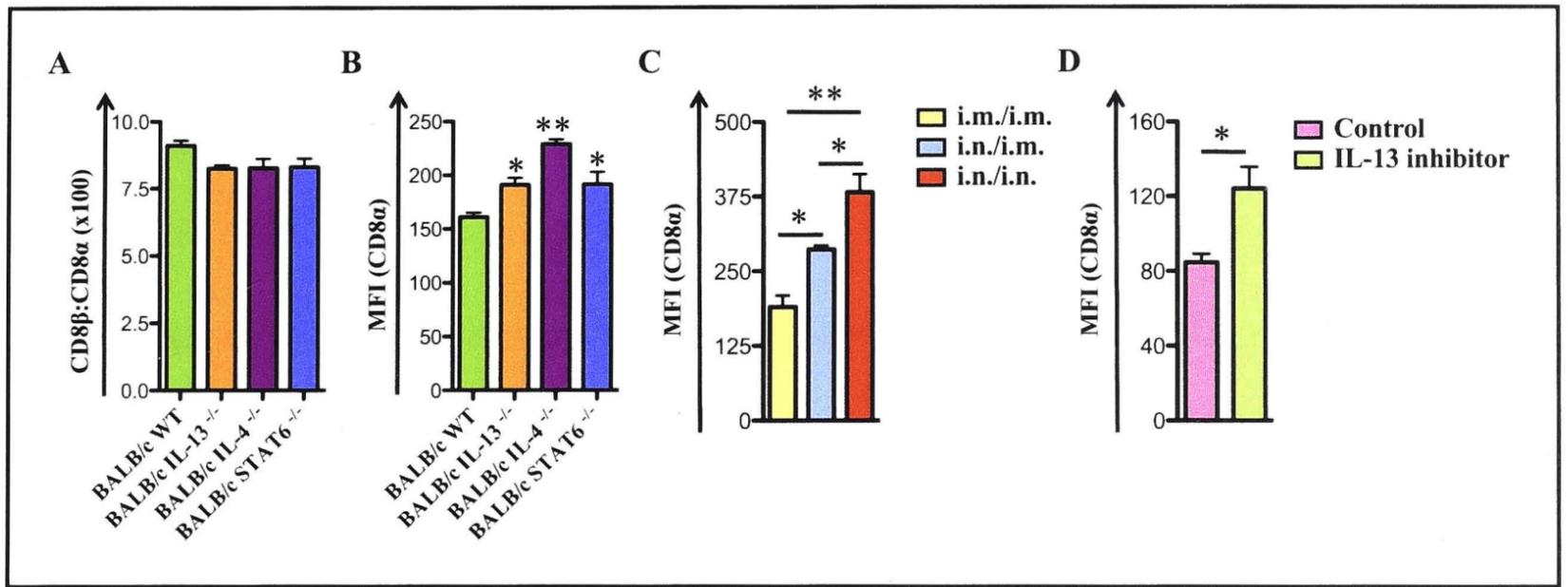
Figure 4.2. Up-regulation of CD8 densities, but not CD8β:CD8α ratios are a feature of high avidity anti-viral CD8$^+$ T cells following pox virus infection and vaccination. A, Mean (n = 4) CD8β:CD8α expression ratios calculated using MFI values on K^{d}Gag$_{197-205}$ tetramer specific CD8$^+$ splenocytes from mice prime-boost vaccinated in **figure 4.1**. B, MFI (n = 6) representing CD8α expression on K^{d}A52$_{75-83}$ tetramer specific CD8$^+$ splenocytes from mice of the indicated genetic background infected for 7 days with 3 x 10^6 PFU of VV-WR. Statistical significance of the data is shown relative to BALB/c WT mice using one-way ANOVA (Dunnett's Multiple Comparison). C, MFI (n = 4) representing CD8α expression on K^{d}Gag$_{197-205}$ tetramer specific CD8$^+$ splenocytes from BALB/c WT mice prime-boost vaccinated i.n./i.n., i.n./i.m. or i.m./i.m. 14 days apart with FPV-HIV/VV-HIV. Statistical significance of the data is calculated using one-way ANOVA (Tukey's Multiple Comparison). D, MFI (n = 4) representing CD8α expression on K^{d}Gag$_{197-205}$ tetramer specific CD8$^+$ splenocytes from BALB/c WT mice prime-boost vaccinated i.n./i.m. with FPV-HIV/VV-HIV (control) or FPV-HIV-IL-13Rα2Δ10/VV-HIV-IL-13Rα2Δ10 (IL-13 inhibitor vaccine). Statistical significance of the data is calculated using a student's unpaired t-test. Data presented in this entire figure is representative of at least two independent experiments and the error bars depict the SEM. * - p < 0.05; ** - p < 0.01.

Our laboratory findings have demonstrated that the avidity of $K^dGag_{197-205}$ specific CD8$^+$ T cells can be regulated depending on the route of vaccine delivery (i.e. avidity: i.n./i.n. > i.n./i.m. > i.m./i.m.) [Ranasinghe *et al.*, 2007]. The avidity hierarchy from this published report correlated with the CD8 expression levels on $K^dGag_{197-205}$ specific CD8$^+$ T cells (i.e. CD8 expression: i.n./i.n. > i.n./i.m. > i.m./i.m.) in the current study (**figure 4.2C**). Furthermore, our laboratory has recently shown that vaccines that transiently inhibit IL-13 activity at the vaccination site can induce high avidity CD8$^+$ T cells with better protective efficacy (i.e. avidity and protection: IL-13Rα2Δ10 adjuvanted IL-13 inhibitor vaccine > control vaccine) [Ranasinghe *et al.*, 2013]. In the current study, compared to the control vaccination (i.n. FPV-HIV/i.m. VV-HIV), IL-13 inhibitor vaccination strategy (i.n. FPV-HIV-IL-13Rα2Δ10/i.m. VV-HIV-IL-13Rα2Δ10) also facilitated the development of $K^dGag_{197-205}$ specific CD8$^+$ T cells with elevated CD8 expression levels (**figure 4.2D**). Collectively, data suggest that IL-4 and/or IL-13 can down-regulate CD8 densities, which correlate with reduced avidity on anti-viral CD8$^+$ T cells following VV-WR infection and HIV-1 recombinant pox viral prime-boost vaccination.

4.2.2 HIV-specific CD8$^+$ T cells require CD8 for optimal cognate pMHC-I engagement

Previous studies using CD8 null tetramers and anti-CD8 blocking antibodies have shown that TCR engagement alone on some anti-viral CD8$^+$ T cell clones is sufficient for effector functions and maintaining a high avidity interaction with cognate pMHC-I [Choi *et al.*, 2003; Wooldridge *et al.*, 2003]. Therefore, it was next examined whether the availability of CD8 on $K^dGag_{197-205}$ specific CD8$^+$ T cells was important in maintaining a high avidity interaction with cognate pMHC-I and for effector functions (i.e. cytokine production).

In order to achieve the above, splenocytes obtained from i.n. FPV-HIV/i.m. VV-HIV vaccinated mice were incubated with CD8 blocking antibodies (i.e. anti-CD8β.2) prior to $K^dGag_{197-205}$ tetramer staining or prior to and during $K^dGag_{197-205}$ peptide stimulation for ICS (**figure 4.3**). Data demonstrated a dose dependent reduction of $K^dGag_{197-205}$ tetramer engagement with increasing concentrations of anti-CD8β.2 (**figure 4.3A**). The production of cytokines IFN-γ, TNF-α and IL-2 was also significantly reduced on $CD8^+$ T cells due to CD8 blocking during *in vitro* $K^dGag_{197-205}$ peptide stimulation (**figure 4.3B**). Interestingly, in each vaccinated mouse the dependence of CD8 co-receptor on $K^dGag_{197-205}$ specific $CD8^+$ T cells for cytokine production was as follows: IL-2 > TNF-α > IFN-γ (**figure 4.3B**). Overall, our data indicated that the availability of CD8 co-receptor on $K^dGag_{197-205}$ specific $CD8^+$ T cells was crucial for maintaining a high avidity interaction with cognate pMHC-I and for effector functions.

4.2.3 Regulation of CD8 densities is a feature of $CD8^+$ T cell polyfunctionality

Given that there was greater dependence of CD8 co-receptor for production of cytokines associated with polyfunctionality (**figure 4.3B**; e.g. TNF-α and IL-2), this study next examined how CD8 densities were regulated on different polyfunctional subsets of HIV-specific $CD8^+$ T cells. Therefore, the CD8 expression levels on $IFN-γ^+$ $TNF-α^+$ $IL-2^+$ (highly polyfunctional), $IFN-γ^+$ $TNF-α^+$ $IL-2^-$ (moderately polyfunctional) and $IFN-γ^+$ $TNF-α^-$ $IL-2^-$ (poorly polyfunctional) $K^dGag_{197-205}$ specific $CD8^+$ T cells were measured following i.n. FPV-HIV/i.m. VV-HIV vaccination (**figure 4.4**). IFN-γ was monitored (**figure 4.4B and 4.4C**) as a positive control given that highly polyfunctional T cells have been reported to produce more IFN-γ on a per cell basis compared to poorly polyfunctional T cells [Precopio *et al.*, 2007]. There was a modest yet statistically insignificant enhancement in CD8 expression levels on
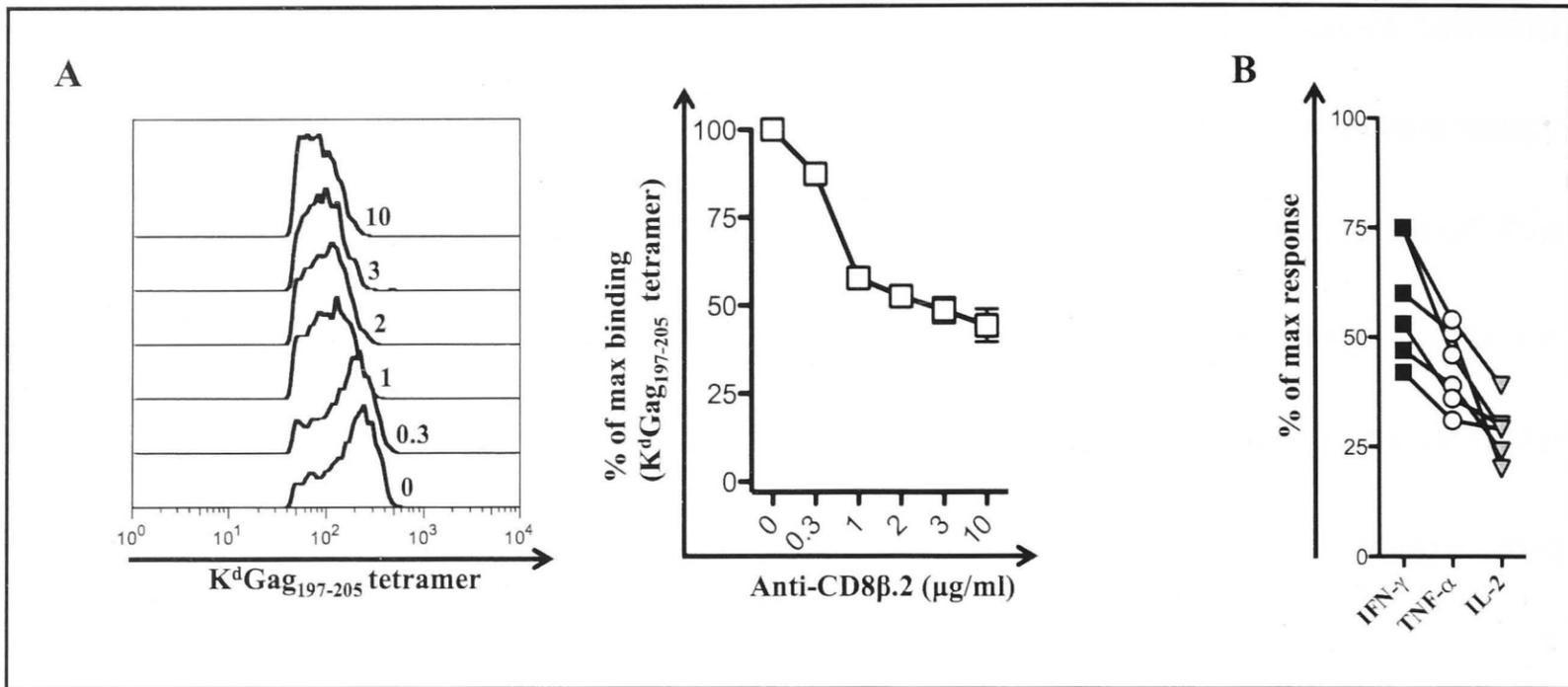
Figure 4.3. CD8 is required by $K^dGag_{197-205}$ specific $CD8^+$ T cells to optimally engage with $K^dGag_{197-205}$ tetramer and for effector functions. Splenocytes obtained from i.n. FPV-HIV/i.m. VV-HIV (14 days apart) vaccinated BALB/c WT mice (n = 4-6) were incubated with 1 μg/ml of purified anti-CD8β.2 prior to (and also during stimulation when peptide stimulated) staining with $K^dGag_{197-205}$ tetramer or *in vitro* stimulation with 0.1 μg/ml of $K^dGag_{197-205}$ peptide for ICS. A, Representative histogram plot (left panel) or mean (n = 4) percentage of maximum $K^dGag_{197-205}$ tetramer binding (right panel) on $K^dGag_{197-205}$ tetramer specific $CD8^+$ splenocytes is shown. The numbers in the histogram plot represents the concentration (μg/ml) of anti-CD8β.2 used for CD8 blocking. B, Percentage of maximum cytokine producing $CD8^+$ splenocytes after CD8 blocking of splenocytes during *in vitro* $K^dGag_{197-205}$ peptide stimulation. Each line is representative of the responses from a single vaccinated mouse and the maximum response (100%) is the response resulting in the absence of CD8 blocking. The data is representative of at least three independent experiments and the error bars when shown depict the SEM.

moderately polyfunctional cells compared to poorly polyfunctional cells (**figure 4.4B and 4.4C**). However, greatly elevated IFN-γ, CD8α and CD8β densities were detected in highly polyfunctional compared to poorly/moderately polyfunctional cells (**figure 4.4B and 4.4C**). On the contrary, elevation of lck expression was not a feature of moderately or highly polyfunctional cells suggesting that there was no generic up-regulation of avidity markers supporting enhancement in polyfunctionality (**figure 4.4B and 4.4C**).

Given that IL-4 and IL-13 reduced CD8 densities on anti-viral CD8$^+$ T cells (**figure 4.1**), this study next examined whether this reduction also affected the establishment of highly polyfunctional CD8$^+$ T cells following prime-boost vaccination. It was observed that greater proportion of IFN-γ producing K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells expressed TNF-α and IL-2 in IL-13 $^{-/-}$, IL-4 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice compared to WT controls (**figure 4.5A and 4.5B**). Furthermore, the absolute numbers of IFN-γ^+ TNF-α^+ IL-2$^+$ K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells were also enhanced in the gene knockout mice (**figure 4.5C**). Overall, increasing CD8 densities appeared to be a feature of anti-viral CD8$^+$ T cells that were highly polyfunctional following HIV-1 recombinant pox viral prime-boost vaccination.

4.2.4 IL-4 and IL-13 mediated CD8 density regulation on HIV-specific CD8$^+$ T cells modulate T cell avidity/polyfunctionality

This study next examined whether IL-4 and IL-13 mediated reduction of CD8 densities could also dampen the avidity of HIV-specific CD8$^+$ T cells from WT controls compared to IL-13 $^{-/-}$, IL-4 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice. For this purpose, splenocytes from i.n. FPV-HIV/i.m. VV-HIV vaccinated mice were incubated with or without anti-CD8β.2 prior to conducting an ICS for measuring polyfunctionality or tetramer

Figure 4.4. Enhancement of CD8 densities correlates with enhancement in cytokine polyfunctionality of HIV-specific CD8$^+$ T cells from HIV-1 recombinant pox viral prime-boost vaccinated mice. Splenocytes from i.n. FPV-HIV/i.m. VV-HIV (14 days apart) vaccinated BALB/c WT mice (n = 4) were stimulated *in vitro* with 0.1 μg/ml of K^{d}Gag$_{197-205}$ peptide and analyzed for expression of CD8α, CD8β, lck and IFN-γ on cytokine producing CD8$^+$ T cells. A, Representative dot plots from a peptide unstimulated or stimulated culture where IFN-γ$^+$ TNF-α$^-$ IL-2$^-$, IFN-γ$^+$ TNF-α$^+$ IL-2$^-$ and IFN-γ$^+$ TNF-α$^+$ IL-2$^+$ CD8$^+$ T cells were gated for analysis. B, Representative histogram plots and the MFI showing the expression of CD8α, CD8β, lck and IFN-γ on different polyfunctional populations of K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells. Each line is representative of an individual mouse from a total of four mice examined. Data presented in this figure is representative of at least two independent experiments. A paired t-test was used to determine the statistical significance of the data. ns – p > 0.05; * - p < 0.05; ** - p < 0.01; *** - p < 0.001.
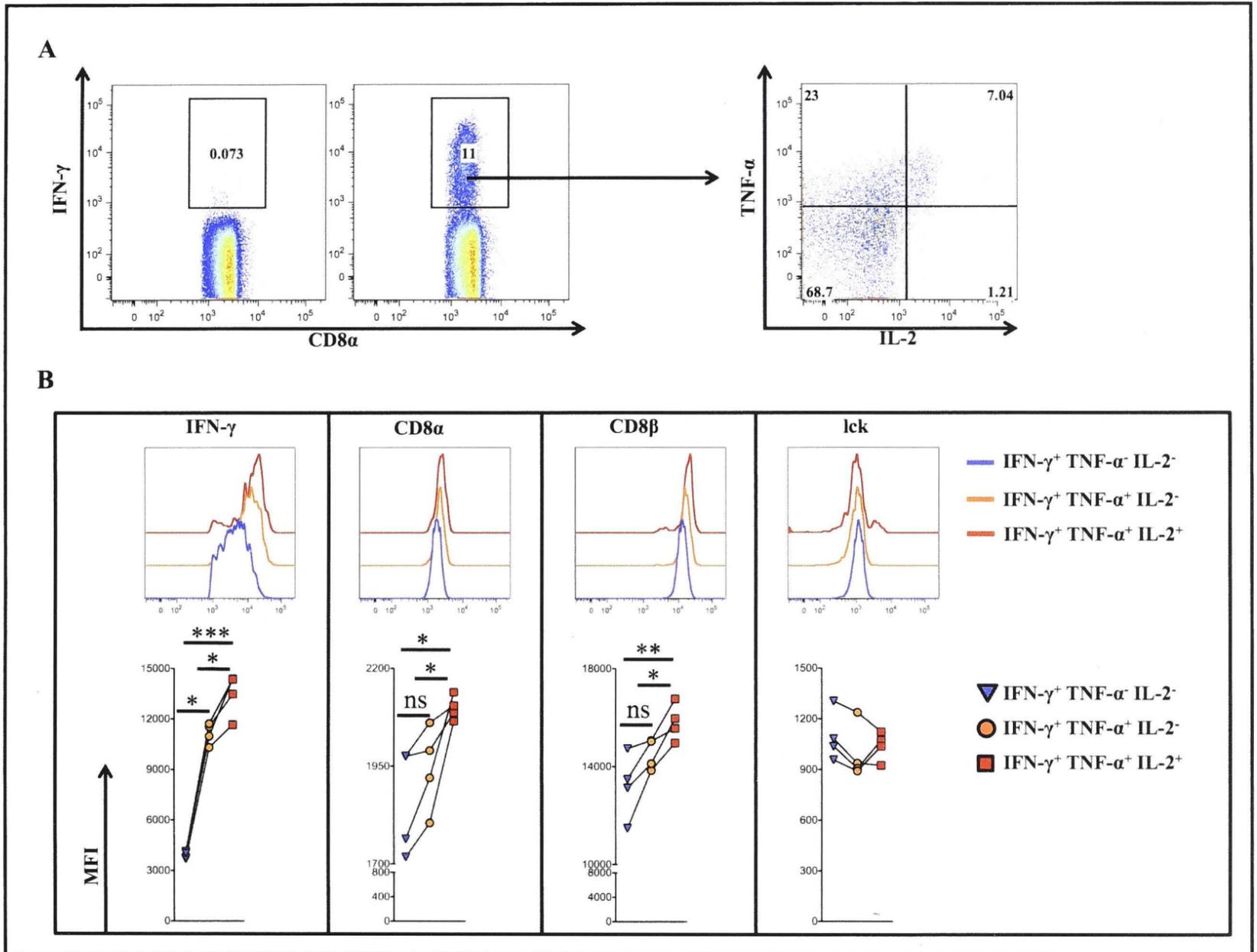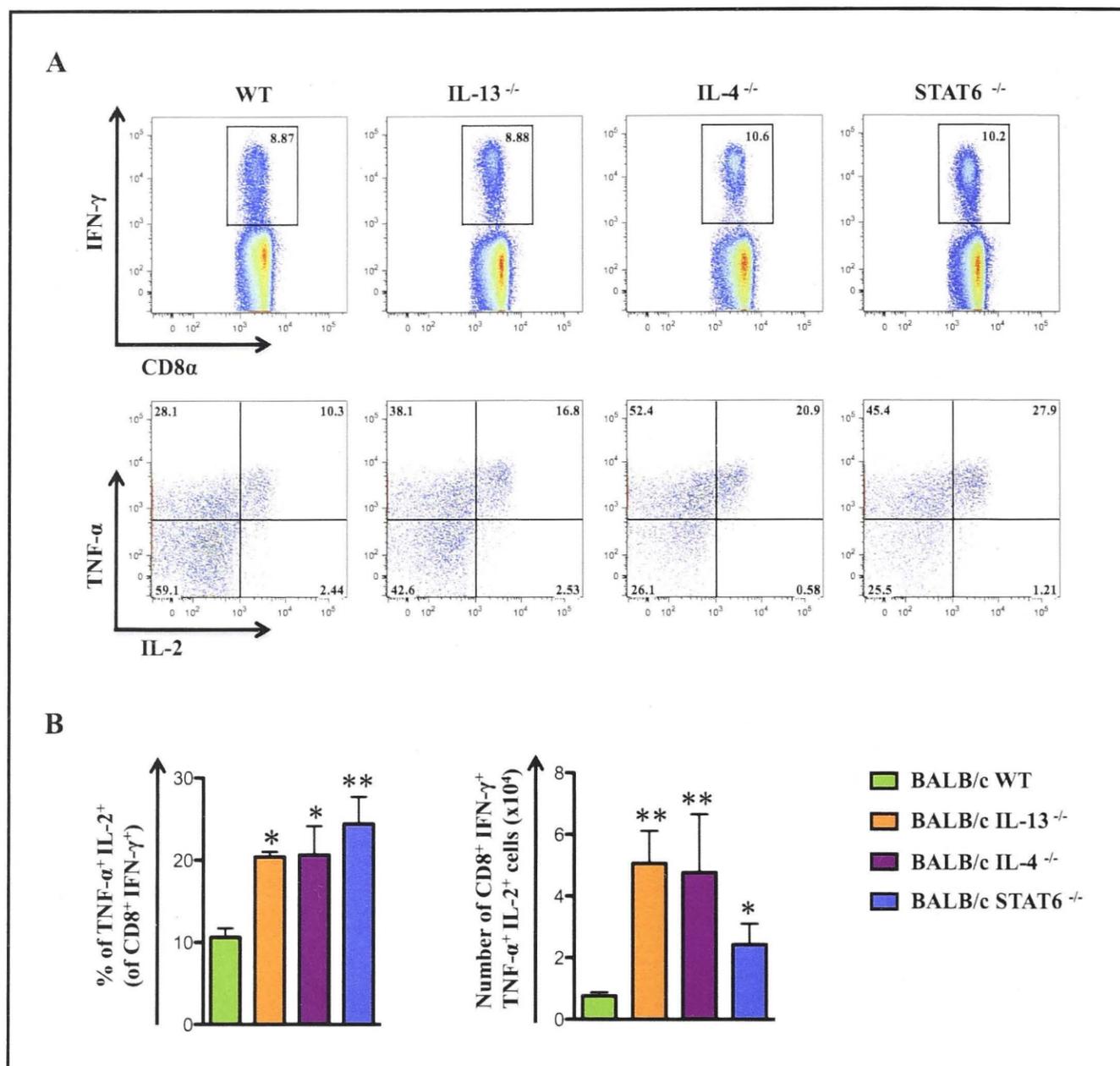
Figure 4.5. IL-4 and IL-13 dampen the establishment of highly polyfunctional K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells following HIV-1 recombinant pox viral prime-boost vaccination. Splenocytes from i.n. FPV-HIV/i.m. VV-HIV (14 days apart) vaccinated mice were stimulated *in vitro* with 0.1 µg/ml of K^{d}Gag$_{197-205}$ peptide for ICS analysis. A, Representative dot plots showing IFN-γ and CD8α expression (top row of plots) on gated CD8$^+$ splenocytes or TNF-α and IL-2 production (bottom row of plots) on gated CD8$^+$ IFN-γ$^+$ splenocytes from vaccinated mice of the indicated genetic background. B, The mean (n = 5) proportion of TNF-α and IL-2 producing CD8$^+$ IFN-γ$^+$ splenocytes and the mean (n = 5) absolute numbers of CD8$^+$ IFN-γ$^+$ TNF-α$^+$ IL-2$^+$ splenocytes for the mice vaccinated in A. Statistical significance of the data is shown relative to BALB/c WT mice using one-way ANOVA (Dunnett's Multiple Comparison) * - p < 0.05; ** - p < 0.01.

dissociation assay for measuring avidity. The rationale here was that if reduction of CD8 densities on HIV-specific CD8$^+$ T cells was important then blocking CD8 availability for tetramer engagement and cytokine production should abrogate avidity differences observed between IL-13 $^{-/-}$, IL-4 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice and WT controls.

CD8 blocking of splenocytes from all vaccinated mice reduced the amount of K^{d}Gag$_{197-205}$ tetramer engagement following tetramer dissociation and polyfunctionality of K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells during *in vitro* K^{d}Gag$_{197-205}$ peptide stimulation (**figure 4.6**). Furthermore, statistically significant reduction in K^{d}Gag$_{197-205}$ tetramer engagement of CD8$^+$ T cells from WT mice was not observed relative to the gene knockout mice when CD8 was blocked on splenocytes during tetramer dissociation (**figure 4.6A**). Statistically significant reduction in the proportions of IFN-γ^+ K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells that produced TNF-α and IL-2 was also not observed in vaccinated WT mice relative to gene knockout mice when CD8 was blocked on splenocytes during K^{d}Gag$_{197-205}$ peptide stimulation (**figure 4.6B**). Thus, IL-4 and IL-13 mediated down-regulation of CD8 densities appears to be important in reducing avidity and polyfunctionality of K^{d}Gag$_{197-205}$ specific CD8$^+$ T cells.
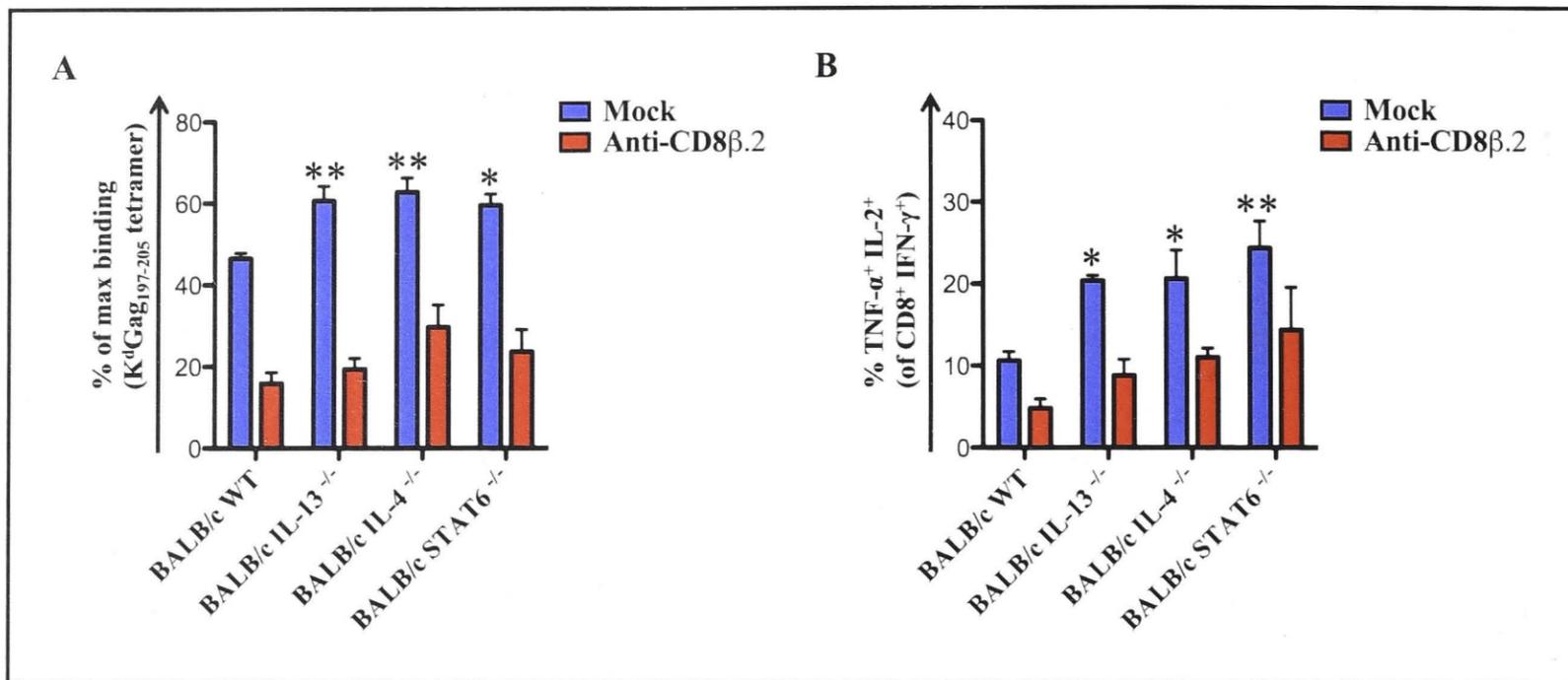
Figure 4.6. IL-4 and IL-13 can regulate CD8 expression levels and modulate T cell quality of $K^d Gag_{197-205}$ specific CD8$^+$ T cells following HIV-1 recombinant pox viral prime-boost vaccination. WT, IL-13 $^{-/-}$, IL-4 $^{-/-}$ and STAT6 $^{-/-}$ BALB/c mice (n = 5-8) were i.n. FPV-HIV/i.m. VV-HIV (14 days apart) vaccinated. Splenocytes from these mice with (1 μg/ml anti-CD8β.2) or without (mock) CD8 blocking were stimulated *in vitro* using 0.1 μg/ml of $K^d Gag_{197-205}$ peptide for ICS or stained using $K^d Gag_{197-205}$ tetramer for tetramer dissociation. A, Mean (n = 8) percentage of maximum $K^d Gag_{197-205}$ tetramer binding of CD8$^+$ T cells following tetramer dissociation for 60 minutes in the absence or presence of CD8 blocking. B, The mean (n = 5) proportion of CD8$^+$ IFN-γ^+ splenocytes that also produced TNF-α and IL-2 in the absence or presence of CD8 blocking of splenocytes during $K^d Gag_{197-205}$ peptide stimulation. The data in this figure is representative of 2-3 independent experiments and the error bars depict the SEM. Statistical significance of the data was calculated using a one-way ANOVA relative to WT mice (Dunnett's Multiple Comparison). * - $p < 0.05$; ** - $p < 0.01$.

4.3. Discussion

Due to the disappointing outcomes of numerous HIV-1 vaccine trials conducted thus far, development of more efficacious HIV-1 vaccines is a necessity for slowing the global progression of HIV-1. This requires the fundamental understanding of mechanisms that generate favourable immune outcomes following vaccination. Our laboratory has previously shown that following HIV-1 recombinant pox viral prime-boost vaccination, IL-4 and IL-13 can dampen the avidity of HIV-specific CD8$^+$ T cells [Ranasinghe *et al.*, 2009; Ranasinghe *et al.*, 2013]. The current study reports that IL-4 and IL-13 can dampen CD8 densities on anti-viral CD8$^+$ T cells following HIV-1 recombinant pox viral prime-boost vaccination. Furthermore, reduced expression levels of CD8 were a feature of low avidity anti-viral CD8$^+$ T cells that developed in various pox virus infection and vaccination settings.

Data in the current study suggest that IL-4 and IL-13 selectively regulated the expression levels of CD8, but not other T cell avidity markers such as TCR, CD2, CD11a and lck on anti-viral CD8$^+$ T cells following HIV-1 recombinant pox viral prime-boost vaccination. This is also the first report to show the involvement of IL-13 in regulating CD8 densities on anti-viral CD8$^+$ T cells. Previous reports have shown that IL-4 exposure *in vitro* and *in vivo* can down-regulate CD8 expression levels to reduce the cytolytic capacity of effector CD8$^+$ T cells [Kienzle *et al.*, 2005; Apte *et al.*, 2008]. Furthermore, IFN-I have also been shown to reduce CD8 expression levels and avidity of anti-viral CD8$^+$ T cells [Xiao *et al.*, 2007]. These reports are consistent with the findings of the current study where reduction of CD8 densities also appeared to reduce the avidity and functional capacity of anti-viral CD8$^+$ T cells. It would be of great interest for future investigations to also examine whether IFN-I cooperate

synergistically with IL-4 and/or IL-13 in down-regulating CD8 densities on anti-viral CD8$^+$ T cells following pox virus infection or vaccination.

Belyakov *et al* [2007; 2008] have shown that mucosal vaccination strategies are efficient in inducing high avidity anti-viral CD8$^+$ T cells particularly at tissues closer to the sites of immunization. Several other studies suggest that mucosal vaccination strategies can induce high avidity CD8$^+$ T cells that can protect macaques against SIV challenge [Belyakov *et al.*, 2001; Kent *et al.*, 2005; Belyakov *et al.*, 2006]. In the current study mucosal HIV-1 recombinant pox viral prime-boost vaccination strategies were found to enhance CD8 densities on HIV-specific CD8$^+$ T cells, which correlated with T cell avidity. Ranasinghe *et al* [2013] and the current study collectively show that mucosal vaccination strategies that temporarily inhibit IL-13 function (i.e. IL-13Rα2Δ10 adjuvanted vaccines) can enhance CD8 densities and avidity of HIV-specific CD8$^+$ T cells with better protective outcomes. These findings are consistent with Ahlers *et al* [2002] study showing that immunization of mice with peptide vaccine constructs in conjunction with granulocyte macrophage colony stimulating factor and IL-13Rα2-Fc antibodies can enhance the cytotoxic activity of HIV-specific CD8$^+$ T cells and protection. Interestingly, Isakov *et al* [2011] have shown that IL-15 plays an important role in enhancing avidity of anti-viral CD8$^+$ T cells following mucosal immunization, which is consistent with Oh *et al* [2004] where IL-15 was shown to enhance CD8 densities and avidity of anti-viral CD8$^+$ T cells. Therefore, development of vaccines that prevent significant down-regulation of CD8 densities on antigen-specific CD8$^+$ T cells offer exciting prospects for developing efficacious vaccines against intracellular mucosal pathogens such as HIV-1.

Even though polyfunctionality correlates with avidity of anti-viral CD8$^+$ T cells [La Gruta *et al.*, 2006; Almeida *et al.*, 2007], regulation of molecules that can affect both T cell avidity and polyfunctionality is not well understood. In the current study, enhancement in CD8 densities highly correlated with enhancement in avidity and polyfunctionality of HIV-specific CD8$^+$ T cells following pox viral vaccination. Furthermore, blocking CD8 availability significantly reduced the polyfunctional capacity and avidity of HIV-specific CD8$^+$ T cells. Thus, monitoring CD8 expression levels could be used as a predictor of both avidity and polyfunctional capacity of anti-viral CD8$^+$ T cells especially for CD8 dependent T cell clones following pox viral vaccination. The caveat here is that IFN-γ, TNF-α and IL-2 production by LCMV-specific monoclonal P14 transgenic CD8$^+$ T cells can occur independently of CD8, especially during later stages of LCMV infection [Slifka and Whitton, 2001; Kerry *et al.*, 2005]. Therefore, it is likely that according to different viruses and/or the infection state (i.e. acute or chronic) multiple redundant mechanisms may be involved in regulating polyfunctionality of anti-viral CD8$^+$ T cells. These mechanisms may include regulation of lipid raft formation [Cawthon *et al.*, 2004], TCR clonotypes [Kedzierska *et al.*, 2005], IL-4Rα (**chapter 3**), lck [Slifka and Whitton, 2001] and IL-15 receptor α [Oh *et al.*, 2004] on CD8$^+$ T cells.

The current study did not examine whether IL-4 and IL-13 reduced CD8 densities on anti-viral CD8$^+$ T cells during long-term memory phases even though effector phase data suggests that this phenomenon is not specific to an epitope or a particular time point following immunization. However, whether this phenomenon operates during long-term memory phases to regulate the quality of anti-viral CD8$^+$ T cell responses requires further investigation. Another caveat in the current study is that anti-CD8 antibodies were used to determine the dependence of avidity and functional quality on

the CD8 co-receptor molecules of CD8⁺ T cells. There have been no reports to suggest that the anti-CD8β.2 antibodies (clone 53-5.8) used in the current study can sterically hinder other interactions (i.e. TCR with pMHC-I) of CD8⁺ T cells. In fact, several publications have used this particular antibody to determine the dependence of survival and functionality on CD8 co-receptor molecules of CD8⁺ T cells [Slifka and Whitton, 2001; La Gruta *et al.*, 2006; Takeda and Jameson, 2009; Loi *et al.*, 2013]. In an ideal scenario, however, these experiments are best performed using CD8 null tetramers as additional controls given that these tetramers specifically do not allow CD8 co-receptor engagement with pMHC-I [Choi *et al.*, 2003].

Most HIV-1 vaccination strategies to date have mainly focused on the induction of either neutralizing antibodies or CD8⁺ T cell responses against HIV-1. However, as discussed in **section 1.5.4** the induction of heterosubtypic immunity (both neutralizing antibody and CD8⁺ T cell responses) is expected to be beneficial in generating optimal protective immunity against HIV-1. The findings from the current study suggest that HIV-1 recombinant pox viral vaccination strategies that inhibit IL-13 function can enhance the quality of anti-viral CD8⁺ T cell immunity most likely through up-regulation of CD8 densities on anti-viral CD8⁺ T cells [Ranasinghe *et al.*, 2013]. Such vaccination strategies could also enhance the quality of antibody immunity, as IL-4 and IL-13 are known to mediate isotype switching of antibodies to IgE; enhanced IgE production has correlated with enhanced pathogenesis of HIV-1 [Vigano *et al.*, 1995; Ouaaz *et al.*, 1996; Oettgen *et al.*, 2000].

In conclusion, the current study suggest that IL-4/IL-13 could mediate down-regulation of CD8 expression levels as a likely mechanism to dampen the avidity/polyfunctionality of anti-viral CD8⁺ T cells. Furthermore, recombinant HIV-1 pox viral mucosal

vaccination strategies that can transiently inhibit IL-13 activity at the vaccination site can enhance CD8 densities and avidity of anti-viral CD8$^+$ T cells. It is plausible to propose that in the absence of IL-4 and IL-13 signalling, anti-viral CD8$^+$ T cells express higher amounts of CD8 allowing these cells to build high avidity interactions with cognate pMHC-I complexes presented on virus-infected cells. This should facilitate high avidity CD8$^+$ T cells to maintain more durable interactions with virus-infected cells allowing them to exert effector functions efficiently. Thus, vaccine strategies that inhibit IL-4/IL-13 activity offer great promise for future vaccines against many chronic infections including HIV-1 that require high avidity CD8$^+$ T cells for protective immunity.

Chapter 5

The FTA Assay for Assessing T Cell Magnitude, Avidity and Epitope Cross-Reactivity *In Vivo*

5.1 Introduction

The induction of high avidity, polyfunctional and epitope variant cross-reactive anti-viral $CD8^+$ T cell responses particularly against conserved HIV-1 epitopes (e.g. Gag) have been associated with superior HIV-1 control in elite controllers [Pontesilli *et al.*, 1998; Betts *et al.*, 2006; Almeida *et al.*, 2007; Critchfield *et al.*, 2007; Ferre *et al.*, 2010; Berger *et al.*, 2011; Mothe *et al.*, 2012; Turk *et al.*, 2013]. Hence, it can be postulated that measuring and inducing high avidity $CD8^+$ T cell responses *in vivo* are necessary for effective and rationale HIV-1 vaccine design. As described in **section 1.9**, many of the current T cell based-assays provide limited capacity to measure avidity and epitope variant cross-reactivity, which led us to develop the FTA technology to make these measurements *in vivo* [Quah *et al.*, 2012]. Furthermore, this technique also allows for the measurements of T_H cell responses based on the ability of T_H cells to activate (e.g. up-regulate CD69 expression) FTA B cells pulsed with peptides [Quah *et al.*, 2013].

In previous studies Ranasinghe *et al* [2006; 2007; 2011] have established that the combination of vaccine route and the order in which HIV-1 recombinant pox viral vaccine vectors are delivered in prime-boost regimens can alter HIV-specific $CD8^+$ T cell immunity and avidity. In the current study, the FTA assay was used to further clarify which vaccine vector combinations induced the best T cell immune responses (e.g. avidity, cross-reactivity and helper capacity) *in vivo* following HIV-1 recombinant

pox viral prime-boost vaccination. The primary aim of the current study was to test the feasibility of using the FTA assay as a high-throughput tool to screen for the most effective HIV-1 vaccination strategies that can induce desired T cell responses *in vivo*.

5.2 Results

5.2.1 Utility of the FTA Assay in measuring T cell responses in vivo

To establish the utility of the FTA assay as a screening tool to measure T cell effector responses in a reproducible manner, six BALB/c mice were immunized with VV-WR and *in vivo* CD8$^+$ T cell killing responses were measured 7 days p.i. using the FTA. Responses were assessed against immunodominant VV F2L (L^dF2_{26-34}), subdominant VV A52R (K^dA52_{75-83}) and F2L mut (MVA homologue of F2L; **section 2.3**) epitopes. A generic schematic representation of the steps involved in this technique is depicted in **figure 5.1**. As anticipated killing responses were not detected against the HIV neg control epitope, but were obvious against F2L and A52R epitopes (**Figure 5.2A-B**). Despite not being expressed by VV-WR, epitope variant cross-reactive CD8$^+$ T cell killing responses against the F2L mut epitope were detected in all VV-WR infected animals (**Figure 5.2A**). Furthermore, the magnitude of the killing responses against the epitopes were as follows: F2L > A52R > F2L mut. In addition to the AUC measurements describing the magnitude of the killing responses (**figure 5.2B**), the EC$_{50}$ measurements were also made to determine the avidity of the CD8$^+$ T cell responses (**figure 5.2C**). This analysis revealed that CD8$^+$ T cells required 10 times the amount of A52R peptide and 70 times the amount of F2L mut peptide to generate half maximal killing compared to F2L (**Figure 5.2C**). Overall, the above data provide an example of the utility of the FTA assay in reproducibly measuring magnitude, avidity and epitope variant cross-reactivity of CD8$^+$ T cell killing responses *in vivo*.

5.2.2 Screening FPV- and VV–based prime-boost vaccine regimens using the FTA assay

Having established the reproducibility and utility of the FTA assay, we then applied the technique to screen various HIV-1 recombinant pox virus prime-boost vaccination regimens for their ability to induce T cell responses. To assess this we generated a vaccine regimen matrix based on all the combinations (i.e. 24 combinations) of two vectors FPV-HIV and VV-HIV given either i.n. or i.m. (**table 5.1**). To comprehensively assess these regimens, immune responses to seven different T cell epitopes were investigated. This included CD8$^+$ T cell epitopes from VV (F2L, F2L mut) as positive controls and HIV (Gag (K^{d}Gag$_{197-205}$), Pol, Env and Gag mut) as described in **section 2.3**. Also a HIV Gag T$_H$ eptiope (**section 2.3**) that allow activation of B cell targets in the FTA was included to measure T$_H$ cell responses (**figure 5.1C**) [Quah *et al.*, 2013].

A representative experiment from three independent experiments is shown in **supplementary figure 4 and 5** and includes data from six intra-animal replicates (6048 data points per experiment). Due to the large amount of data generated in this instance, AUC values were calculated for responses against each epitope and depicted as a heat map to allow trends to be revealed more easily (**figure 5.3A**). Killing responses from several vaccine regimens were clearly detectable against the Gag and even the Gag mut epitopes, but only negligible killing responses were detected against Pol and Env epitopes (**figure 5.3**). Interestingly, the magnitude of the killing responses against Gag, Gag mut, F2L and F2L mut epitopes were greatest in the heterologous FPV-HIV/VV-HIV prime-boost regimens (**figure 5.3**). The responses against the Gag mut are also indicative of Gag epitope variant cross-reactive CD8$^+$ T cell responses following vaccination. Furthermore, FPV-HIV/VV-HIV regimens also gave robust T$_H$ cell responses against the Gag T$_H$ epitope (**figure 5.3**).

Figure 5.1. Schematic representation of a 252-parameter FTA assay. 5×10^7 splenocytes from mice were labeled with combinations of CTV (0 nM, 350 nM, 1295 nM, 4792 nM, 17729 nM and 65595 nM), CFSE (0 nM, 79 nM, 315 nM, 1106 nM, 3859 nM, 13505 nM and 47269 nM) and CPD (0 nM, 106 nM, 690 nM, 2738 nM, 10262 nM and 38506 nM) to generate 252 discernable cell clusters. Cell clusters were pulsed with MHC-binding peptides (as outlined in **figure 5.3**) to generate a panel of 42 peptide pulsed clusters and this repeated six times to generate six intra-animal repeats (i.e., 252 target cell clusters in total). Target cells were also labeled with DiI for discrimination from host splenocytes (not shown). A, FTA cells were injected i.v. into host mice that had 6 days earlier been vaccinated with VV-HIV or left unimmunized as a control. 18 hours after FTA injection, splenocytes were collected and target cells delineated from host splenocytes by DiI label using flow cytometry (not shown). B, 2D plots of the fluorescence intensities of a panel of 42 VV F2L peptide-pulsed clusters from unimmunized (left plots) and vaccinated (right plots) mice and an associated histogram analysis of clusters pulsed with titrated amounts of VV F2L peptide. The disappearance of target cells in these clusters in vaccinated mice relative to unimmunized controls reveals specific killing of F2L peptide pulsed targets. C, An example of histogram analysis of the FTA T helper assay where $B220^+$ FTA cells pulsed with the Gag T_H cell peptides were assessed for CD69 up-regulation in vaccinated and unimmunized mice.

A Peptide-pulsed FTA

CFSE
CPD
CTV

Inject FTA into mice

18 hours *in vivo*

B

Unimmunized control   Vaccinated mouse

CFSE

CTV

Pulsed peptide amount

CFSE

120
90
60
30
0

CTV

Killing assay

C FTA B cell activation

— Unimmunized
Vaccinated

Counts

CD69

Pulsed peptide amount

T_H assay
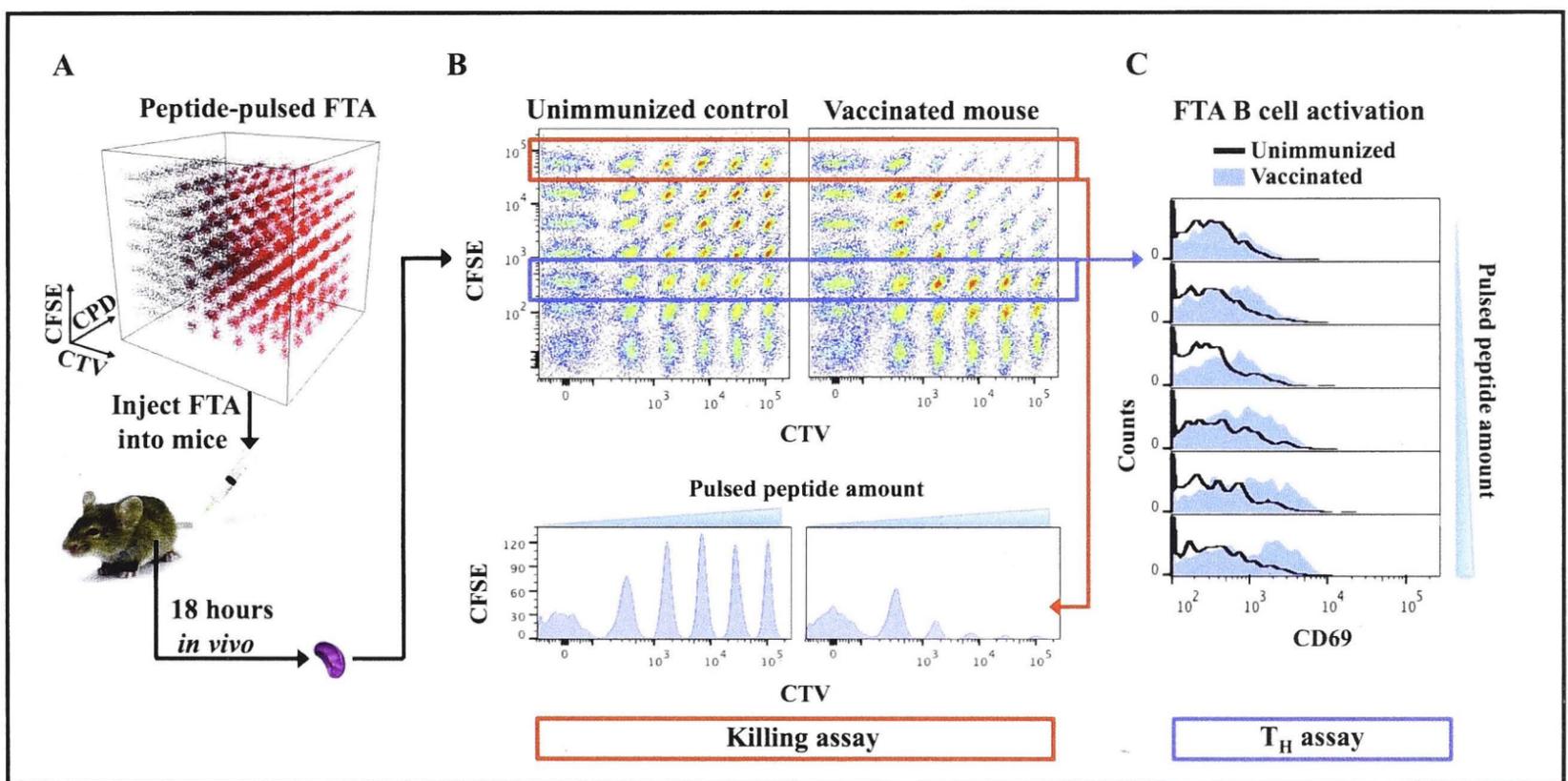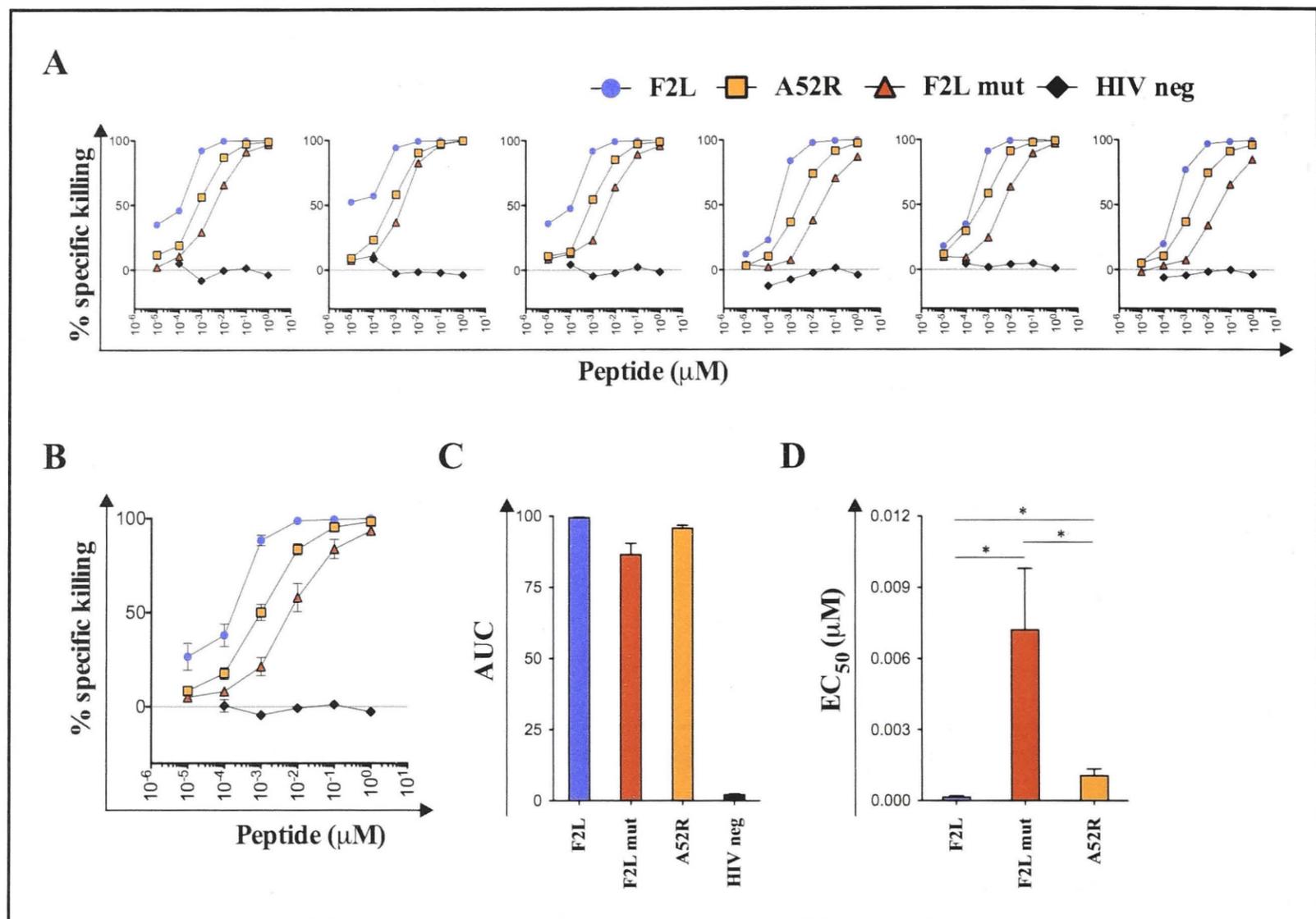
Figure 5.2. The FTA assay can reproducibly measure magnitude, functional avidity and epitope variant cross-reactivity of CD8^{+} T cells *in vivo*. Six BALB/c mice were immunized i.p. with 5×10^6 PFU of VV-WR. FTA was constructed using mouse splenocytes and comprised of FTA cells pulsed with six different concentrations of the MHC-I binding peptides F2L, F2L mut, A52R, and HIV neg (as a negative control). FTA target cells were injected i.v. into infected mice 6 days p.i. and after 18 hours *in vivo* percent specific killing calculated for FTA target cells from harvested spleens. A, *In vivo* killing responses from six infected mice where each plot represents an individual mouse. B, Summary of responses depicted in A from all mice with means (n = 6) of percent specific killing. C, Mean (n = 6) AUC measurements from percent specific killing response curves in A. D, Mean (n = 6) EC_{50} of the killing response in C. The error bars depict the SEM and the p values were calculated using a one-way ANOVA (Tukey's Multiple Comparison). Data is representative of several independent experiments. * - $p < 0.05$.

Table 5.1. Prime-boost vaccination regimens tested in the FTA.

1 Nill	2[ν] i.n. FPV-HIV	3 i.m. FPV-HIV	4 i.n. VV-HIV	5 i.m. VV-HIV
6 i.n. FPV-HIV	7[Ψ] i.n. FPV-HIV/ i.n. FPV-HIV	8 i.m. FPV-HIV/ i.n. FPV-HIV	9 i.n. VV-HIV/ i.n. FPV-HIV	10 i.m. VV-HIV/ i.n. FPV-HIV
11 i.m. FPV-HIV	12 i.n. FPV-HIV/ i.m. FPV-HIV	13 i.m. FPV-HIV/ i.m. FPV-HIV	14 i.n. VV-HIV/ i.m. FPV-HIV	15 i.m. VV-HIV/ i.m. FPV-HIV
16 i.n. VV-HIV	17 i.n. FPV-HIV/ i.n. VV-HIV	18 i.m. FPV-HIV/ i.n. VV-HIV	19 i.n. VV-HIV/ i.n. VV-HIV	20 i.m. VV-HIV/ i.n. VV-HIV
21 i.m. VV-HIV	22 i.n. FPV-HIV/ i.m. VV-HIV	23 i.m. FPV-HIV/ i.m. VV-HIV	24 i.n. VV-HIV/ i.m. VV-HIV	25 i.m. VV-HIV/ i.m. VV-HIV

[ν]Yellow and blue shaded boxes represent the prime and boost only controls

[Ψ]Green shaded boxes represent the different prime-boost combinations tested

Figure 5.3. Heat map screen of the magnitude of *in vivo* killing of the indicated peptide-pulsed targets. BALB/c WT mice were vaccinated with 24 different vaccine regimens as shown in **table 5.1** and T cell responses were assessed using a 252-parameter FTA comprised of cells pulsed with six concentrations of F2L, F2L mut, Gag, Gag mut, Pol, Env and Gag T_H. FTA target cells were injected i.v. into vaccinated mice 6 days post vaccination and responses assessed after 18 hours *in vivo* using harvested spleens for analysis using flow cytometry. AUC were then calculated and portrayed as a heat map where darkest shading indicates highest magnitude and lightest shading indicates lowest magnitude T cell responses. Data is representative of three independent experiments.

	Env	F2L mut	F2L	Gag mut	Gag	Pol		Gag T_H
Prime i. n. FPV-HIV								
Boost i. n. FPV-HIV								
i. n. FPV-HIV/ i. n. FPV-HIV								
i. n. FPV-HIV/ i. m. FPV-HIV								
i. n. FPV-HIV/ i. n.VV-HIV								
i. n. FPV-HIV/ i. m. VV-HIV								
Prime i. m. FPV-HIV								
Boost i. m. FPV-HIV								
i. m. FPV-HIV/ i. n. FPV-HIV								
i. m. FPV-HIV/ i. m. FPV-HIV								
i. m. FPV-HIV/ i. n.VV-HIV								
i. m. FPV-HIV/ i. m. VV-HIV								
Prime i. n.VV-HIV								
Boost i. n. VV-HIV								
i. n. VV-HIV/ i. n. FPV-HIV								
i. n. VV-HIV/ i. m. FPV-HIV								
i. n. VV-HIV/ i. n.VV-HIV								
i. n. VV-HIV/ i. m. VV-HIV								
Prime i. m.VV-HIV								
Boost i. m. VV-HIV								
i. m.VV-HIV/ i. n. FPV-HIV								
i. m. VV-HIV/ i. m. FPV-HIV								
i. m. VV-HIV/ i. n.VV-HIV								
i. m.VV-HIV/ i. m. VV-HIV								

From the above analysis, it was also apparent that FPV-HIV/VV-HIV and VV-HIV/FPV-HIV regimen clusters gave strong killer and helper T cell responses against Gag epitopes (**figure 5.3 and 5.4A**). When evaluating the avidity for the killer responses against Gag and Gag mut epitopes, FPV-HIV/VV-HIV prime-boost vaccination cluster had the lowest EC_{50} and therefore the highest avidity killing responses (**figure 5.4B**). There appeared to be no vaccination route dependent trends in the killing responses for the FPV-HIV/VV-HIV cluster (**figure 5.4B**). Interestingly, there was evidence to suggest that the VV-HIV/FPV-HIV heterologous prime-boost vaccination strategy benefited from the i.m. boost with FPV-HIV as this regimen gave a comparable avidity killing response against Gag epitopes to that of the FPV-HIV/VV-HIV cluster. Whilst the FPV-HIV/VV-HIV and VV-HIV/FPV-HIV generated T_H cell responses against Gag T_H epitope, negligible EC_{50} changes were apparent with T_H cell responses in these clusters (**figure 5.4**). Overall, the best vaccine strategy in terms of inducing high avidity killer and helper T cell responses appeared to be regimens comprised of FPV-HIV priming followed by VV-HIV booster vaccination.

5.2.3 Liposome boosting improves the magnitude, functional avidity and epitope variant cross-reactivity of CD8^{+} T cell responses

FPV-HIV/VV-HIV vaccination generated robust killing responses against Gag and Gag mut epitopes, but these responses were consistently lower compared to the responses against immunodominant VV F2L epitope (**figure 5.5A and 5.5B**). This was also observed with respect to the avidity of CD8^{+} T cell responses. For example, whilst the i.n. FPV-HIV/i.m. VV-HIV regimen typically generated a 10-fold and 40-fold avidity enhancement against Gag epitope and Gag mut epitope respectively compared to vaccination with VV-HIV alone, the avidity of these responses was still lower than
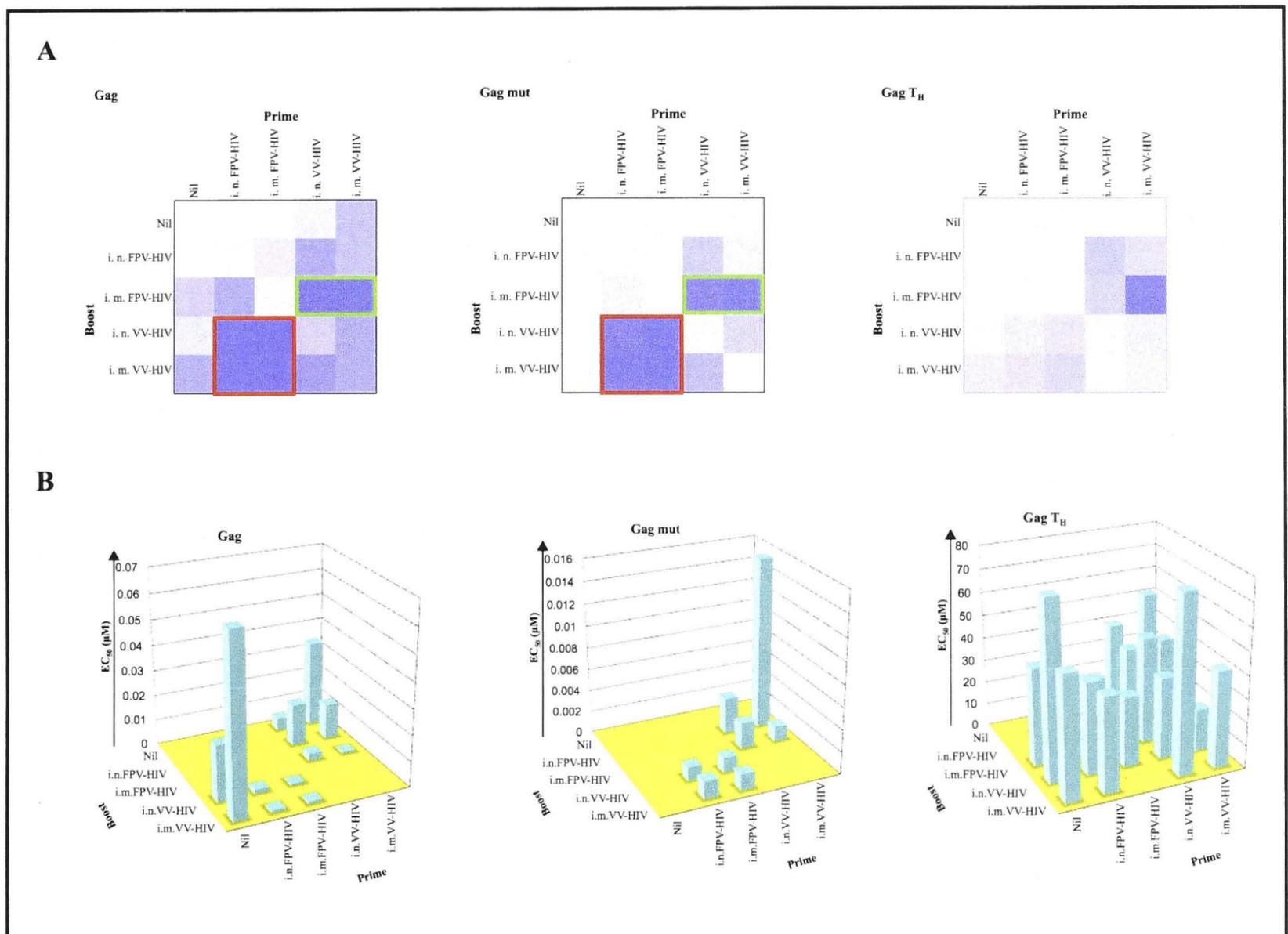
Figure 5.4. Magnitude, avidity and cross-reactivity of Gag-specific T cell responses for the experiment described in figure 5.3. Mice vaccinated from **figure 5.3** were further analyzed for magnitude (A) and avidity (B) to determine vaccine clusters that were robust at inducing high avidity and cross-reactive Gag-specific T cell responses *in vivo*. Two separate clusters (FPV-HIV/VV-HIV (red box)) and VV-HIV/FPV-HIV (green box)) were identified as the most effective vaccine regimens in generating high avidity and cross-reactive Gag-specific CD8^{+} T cell responses *in vivo*.

Figure 5.5. Tertiary vaccination with liposomes improves the magnitude, functional avidity and epitope variant cross-reactivity of CD8$^+$ T cell responses following prime-boost vaccination. Mice were vaccinated i.n. with FPV-HIV, i.m. with VV-HIV, and/or i.v. with liposomes (containing 35 mg of Gag mut and 1 mg of LPS per dose). Liposome booster vaccinations were given 4 weeks post the i.m. VV-HIV and 6 weeks post the i.n. FPV-HIV vaccinations. T cell responses were assessed using 252-parameter FTAs as in **figure 5.3.** A, Percent specific killing of FTA *in vivo* by CD8$^+$ T cells induced by prime, boost, and/or tertiary liposome vaccination regimens showing killing responses to F2L, F2L mut, HIV Gag and HIV Gag mut epitopes. B, Mean (n = 6) for the killing responses against the epitopes shown in A. C, Mean (n = 6) AUC values for the killing responses shown in B. D, Mean (n = 6) EC$_{50}$ values for the killing responses shown in A. The error bars depict the SEM and the p values were calculated using Mann-Whitney nonparametric two-tailed t-test. ** - p < 0.01.
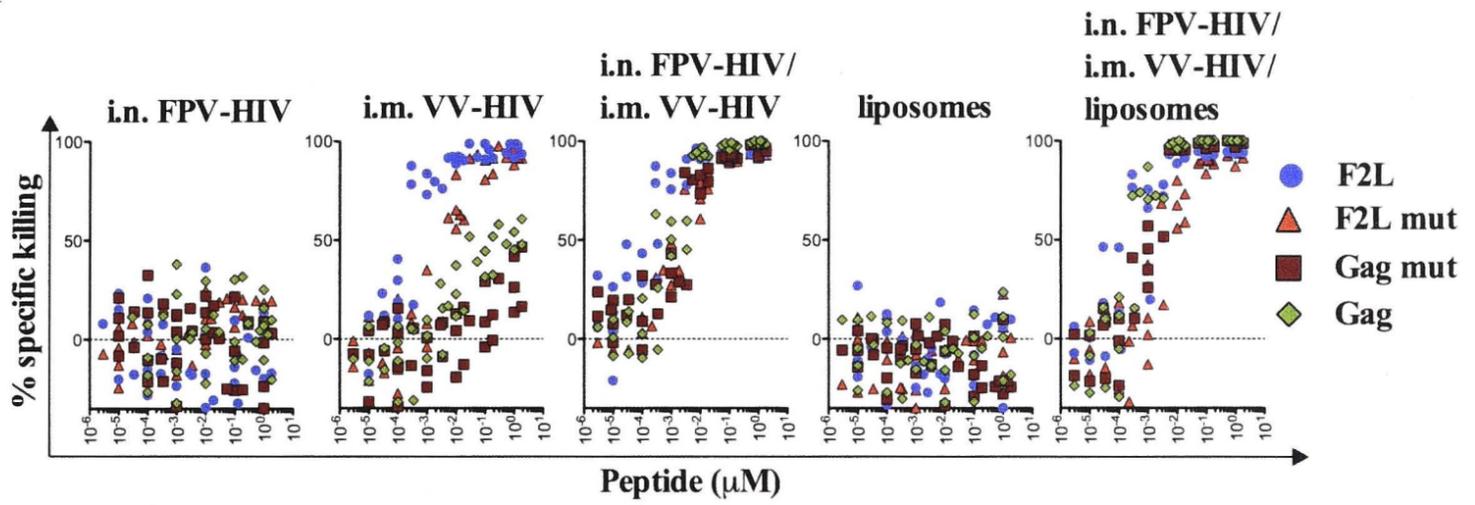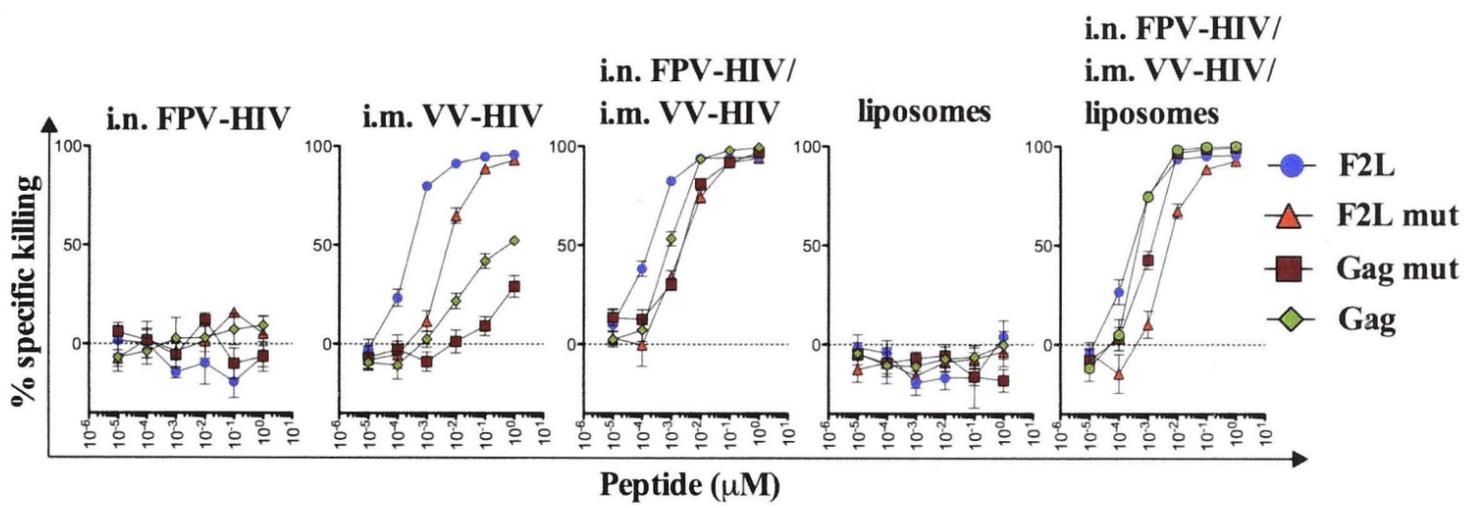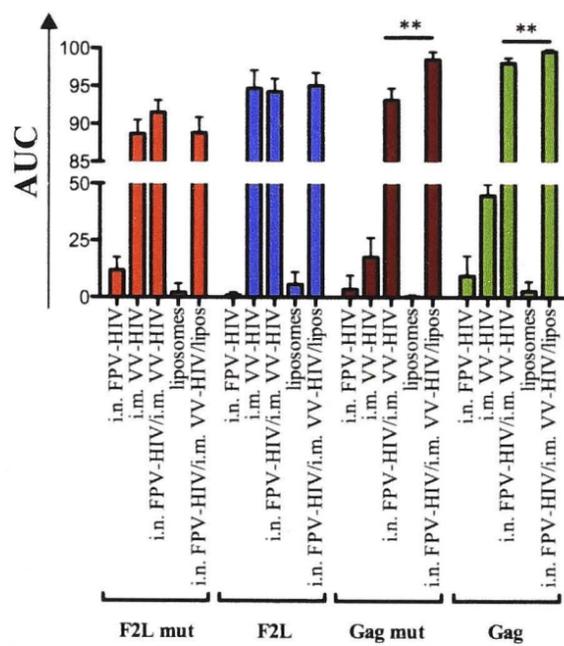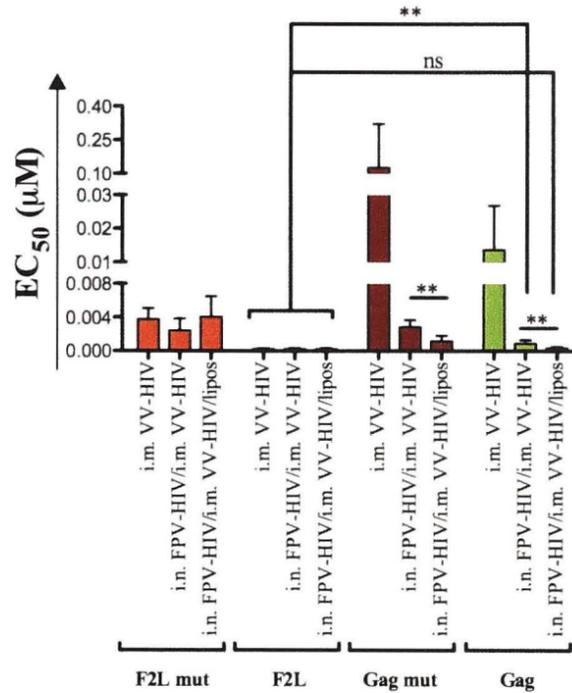
responses against F2L epitope in seven independent experiments (**representative example give in figure 5.5D**). Therefore, the use of a peptide (HIV Gag mut)-liposome (**section 2.5**) construct for vaccination was investigated to determine whether the avidity of HIV Gag-specific CD8^{+} T cell responses could be improved beyond that of F2L-specific CD8^{+} T cells. Gag mut peptide was chosen such that the highest avidity and cross-reactive Gag-specific CD8^{+} T cells could be expanded.

Peptide-liposome constructs and LPS were administered i.v. 6 weeks post i.n. FPV-HIV vaccination and 4 weeks post i.m. VV-HIV booster vaccination. T cell responses were assessed using the FTA T cell assay 7 days post peptide-liposome booster vaccination (**figure 5.5**). As controls, mice were also vaccinated with liposome constructs alone, which generated negligible killing responses against all epitopes (**figure 5.5A-B**). When liposome constructs were included as a tertiary vaccination for the i.n. FPV-HIV/i.m. VV-HIV regimen, the magnitude of the killing responses against Gag and the Gag mut epitopes was higher than that of the i.n. FPV-HIV/i.m. VV-HIV vaccination alone (**figure 5.5C**). Interestingly, following i.n. FPV-HIV/i.m. VV-HIV/liposomes vaccination the magnitude and the avidity (EC_{50}) of the killing responses against Gag and F2L epitopes were similar (**figure 5.5**). The EC_{50} values of the killing responses against Gag and Gag mut epitopes revealed a three-fold enhancement in the i.n. FPV-HIV/i.m. VV-HIV/liposomes regimen relative to the i.n. FPV-HIV/i.m. VV-HIV regimen (**figure 5.5D**). Collectively, data suggests that a liposomal booster vaccination can be used to further enhance the magnitude, avidity and epitope variant cross-reactivity of desired (e.g. Gag-specific) CD8^{+} T cell responses.

5.3 Discussion

Here the use of a high-throughput multi-parameter *in vivo* assay with the capacity to measure magnitude, avidity and epitope variant cross-reactivity of T cell responses *in vivo* following HIV-1 vaccination was described. This assay is unique in that it allows the simultaneous measurement of killing and helper activity against numerous target cells pulsed with a broad concentration range of several different MHC-I/II binding peptides *in vivo*. Furthermore, previously developed *in vivo* CD8^{+} T cell killing assays [Oehen *et al.*, 1997; Barchet *et al.*, 2000] can only measure the magnitude of killing responses in a single animal whereas the FTA can also provide detailed measurements of avidity and epitope variant cross-reactivity in a single animal. *In vitro* assays such as the ^{51}Cr-release, ICS and ELISPOT peptide dilution assays, typically require *ex vivo* stimulation of host effector T cells. Such stimulation may also result in changes in avidity at a population level due to preferential outgrowth of high avidity effector T cells [Yerly *et al.*, 2008]. Thus, the FTA assay has an excellent capacity with distinct advantages to measure multiple parameters of T cell responses in a single animal *in vivo*.

The versatility of the FTA assay allowed the comprehensive screening of T cell responses generated from 24 different vaccination regimens against 7 distinct viral epitopes (Gag, Gag T_H, Gag mut, Pol, Env, F2L and F2L mut). From this analysis, heterologous prime-boost vaccination regimens, particularly FPV-HIV/VV-HIV regimens were found to be the most effective strategy in generating high magnitude of anti-viral CD8^{+} T cell responses with high avidity and epitope variant cross-reactivity *in vivo*. The FTA assay cannot be used to quantify the absolute numbers of responding CD8^{+} T cells, but our laboratory using *in vitro* assays such as tetramer staining/dissociation assays and ICS have addressed this aspect. The findings from these

assays also suggest that the FPV-HIV/VV-HIV vaccination regimen was most effective in generating high avidity Gag-specific CD8^{+} T cell responses [Ranasinghe *et al.*, 2006; Ranasinghe *et al.*, 2007; Ranasinghe *et al.*, 2011]. These findings also demonstrate that vector combinations in pox viral prime-boost vaccination regimens play an important role in determining vaccine efficacy and CD8^{+} T cell avidity. Despite the consisting of findings with respect to the choice of vector combinations, the FTA assay did not indicate avidity differences on Gag-specific CD8^{+} T cells when FPV-HIV was delivered i.n. or i.m. in the FPV-HIV/VV-HIV regimen. This is in contrast to Ranasinghe *et al* [2007] where data from tetramer dissociation assays demonstrated that i.n. compared to i.m. priming with FPV-HIV was more efficient in generating high avidity Gag-specific CD8^{+} T cells in the FPV-HIV/VV-HIV regimen [Ranasinghe *et al.*, 2007]. The reasons for this discrepancy are unclear and it could suggest that data from *in vitro* assays such as tetramer dissociation do not always correlate with the data from FTA assays. However, it remains to be seen whether this discrepancy is due to the fact that killing responses using the FTA assay were measured 18 hours post target cells transfer instead of an earlier time point where high avidity CD8^{+} T cells more actively participate in killing than low avidity CD8^{+} T cells.

More and more studies are indicating that vaccines can induce T cell responses to cross-reactive epitopes and techniques to evaluate these parameters are of great importance when dealing with diseases like HIV-1. Broad epitope variant cross-reactivity of CD8^{+} T cells have been observed in elite controllers [Mothe *et al.*, 2012]. Similar findings have been reported in hepatitis C virus patients, where clearance of the virus was associated with increased epitope variant cross-reactivity and heightened avidity of CD8^{+} T cells [Yerly *et al.*, 2008]. In the current study, the FPV-HIV/VV-HIV vaccination regimen was also extremely efficient in generating highly cross-reactive

CD8$^+$ T cell responses against Gag mut epitope. Collectively, the current study demonstrates that FPV-HIV/VV-HIV heterologous prime-boost vaccination regimens have the hallmarks of an efficacious vaccination strategy that could induce robust protective immunity.

Another important finding from the current study was that CD8$^+$ T cell killing responses generated by FPV-HIV/VV-HIV vaccination were further enhanced when liposomes carrying HIV-1 epitopes were used as a tertiary vaccination. Specifically, this allowed the avidity of the killing responses against Gag and Gag mut to be increased by about three fold from the FPV-HIV/VV-HIV vaccination alone. In various prime-boost vaccination studies, a tertiary booster has been shown to enhance both T and B cell immunity [Stambas et al., 2005]. Also, in the recent Thai RV144 trial, recombinant HIV-1 canary pox priming followed by a HIV-1 protein booster vaccination yielded immune outcomes with a 30% protective efficacy [Rerks-Ngarm et al., 2009]. Therefore, there the use of liposome vectors could be extremely beneficial for vaccine regimens requiring multiple booster vaccinations, especially when anti-vector immunity is of concern. Liposomes are also safe in humans and can be engineered to express tailor-made epitopes, danger signals and targeting motifs to amplify immune responses generated previously using recombinant DNA or viral vectors [Immordino et al., 2006]. However, the use of safer immune adjuvants other than LPS is recommended for application in humans.

There are few caveats with the FTA assay in its current form for evaluating HIV-1 vaccine efficacy. One caveat of the FTA assay is that it relies on target cells that are pulsed with designated viral peptides rather than target cells infected with HIV-1 where the relative expression of these designated peptides could be significantly different.

Furthermore, in the current assay killing responses were measured 18 hours post-transfer of target cells. Given that high avidity CD8$^+$ T cells are expected to kill virus-infected target cells early compared to low avidity counterparts (**figure 1.3**), it will also be important to determine whether the kinetics of the killing responses can change the avidity and magnitude measurements made using this assay. These caveats could be better addressed if the FTA was optimised for use in MHC-I matched macaques instead of mice where the killing responses against SIV-infected target cells could be monitored. Along with relevant SIV protective studies, this is expected to improve the translatability of the findings from the FTA assay regarding the efficacy of vaccination regimens in facilitating the development of high avidity CD8$^+$ T cells with a great potential to control HIV-1 infections.

In conclusion, using the novel FTA assay a combination of HIV-1 recombinant pox viral prime-boost regimens were evaluated for their ability to generate robust anti-viral T cell responses *in vivo*. In this study, it was found that the magnitude, avidity and epitope variant cross-reactivity of CD8$^+$ T cells *in vivo* are dependent on the vaccine vector combination used for vaccination. Out of the 24 vaccine combinations tested, FPV-HIV/VV-HIV vaccination strategies induced the best immune outcomes. Moreover, liposome tertiary vaccination further enhanced the magnitude, avidity and epitope cross-reactivity of CD8$^+$ T cell immunity, which offers great prospects for future vaccine development. Overall, data suggest that the FTA assay is an extremely valuable and cost-effective tool that can be used for large scale screening of vaccine combinations *in vivo* for pre-clinical testing.

Chapter 6

General Discussion

6.1 Synopsis

After over three decades since the discovery of HIV-1, a vaccine against this deadly virus is currently not available. Rationale HIV-1 vaccine design is a difficult prospect given the rapid mutation rate and latency capabilities of this virus [Chomont *et al.*, 2009; McMichael *et al.*, 2010]. However, developing HIV-1 vaccination strategies that can induce broadly neutralizing antibodies, ADCC and high avidity anti-viral CD8$^+$ T cells offer hope especially given that these immune components correlate with HIV-1 resistance (e.g. in elite controllers) [Walker *et al.*, 2009; Haynes *et al.*, 2012; Julien *et al.*, 2013; Liao *et al.*, 2013; Critchfield *et al.*, 2007; Turk *et al.*, 2013]. Our laboratory has previously shown that systemic (i.m./i.m.) HIV-1 vaccination strategies promote greater induction of IL-4 and IL-13 to dampen anti-viral CD8$^+$ T cell avidity compared to mucosal (i.n./i.n. and i.n./i.m.) HIV-1 vaccination strategies [Ranasinghe *et al.*, 2007; Ranasinghe *et al.*, 2009]. Based on these findings our laboratory also constructed novel and protective IL-13Rα2Δ10 adjuvanted HIV-1 pox viral vaccines that transiently inhibit IL-13 function and enhance the avidity of anti-viral CD8$^+$ T cells following mucosal vaccination [Ranasinghe *et al.*, 2013]. To understand how our mucosal HIV-1 vaccinations strategies generated favourable immune outcomes, the current PhD investigated how IL-4 and IL-13 dampened anti-viral CD8$^+$ T cell avidity. Furthermore, the use of a novel FTA assay as a screening tool to select HIV-1 pox viral vaccine strategies that can induce high avidity T cell responses *in vivo* was also evaluated. The key findings from these studies and their ramifications will be discussed in this chapter.

6.2 IL-4Rα is an important regulator of anti-viral CD8$^+$ T cell avidity

In order to understand how IL-4 and IL-13 dampened avidity of anti-viral CD8$^+$ T cells, we initially evaluated the expression of receptors for these cytokines on immune cells

following virus infection. Given that cytokines require receptors to exert their biological effects, it was hypothesized that regulation of receptors in a manner that allow CD8$^+$ T cells to reduce the responsiveness to IL-4 and/or IL-13 can enhance the avidity of CD8$^+$ T cells. In support of this hypothesis, our findings suggest that down-regulation of IL-4Rα, the common signalling receptor subunit of IL-4 and IL-13 [Wills-Karp and Finkelman, 2008], on naïve CD8$^+$ T cells was important for priming high avidity and polyfunctional anti-viral CD8$^+$ T cell responses. This conclusion is consistent with previous *in vitro* studies showing that IL-4 during polyclonal stimulation primes naïve CD8$^+$ T cells to become poorly functional effector cells [Erard *et al.*, 1993; Kienzle *et al.*, 2005]. The PhD studies also showed that even on effector CD8$^+$ T cells greater down-regulation of IL-4Rα expression following cognate antigen encounter correlated with significant enhancement in IFN-γ and TNF-α production during virus infection. These findings are in agreement with previous reports where heightened IL-4 and IL-13 responses in the context of tumour and virus infections were shown to dampen functional capacity and avidity of effector CD8$^+$ T cells [Sharma *et al.*, 1996; Jackson *et al.*, 2001; Apte *et al.*, 2008; Apte *et al.*, 2010; Ranasinghe *et al.*, 2009; Ranasinghe *et al.*, 2013]. Thus, down-regulating IL-4Rα expression on naïve CD8$^+$ T cells appeared to be important for priming high avidity anti-viral CD8$^+$ T cells and for established anti-viral effector CD8$^+$ T cells to maintain high avidity anti-viral responses during the course of a virus infection.

Elevation of IL-4Rα expression on CD8$^+$ T cells could also play an important role in immune evasion of HIV-1 during natural infections. There are reports showing that enhanced IgE levels are featured in patients with poor HIV-1 prognosis [Israël-Biet *et al.*, 1992, Vigano *et al.*, 1995, Rancinan *et al.*, 1998]. Similarly anti-viral IgE responses have been associated with enhanced pathogenesis of respiratory syncytial virus [Russi *et*

al., 1993; Dakhama *et al.*, 2009]. IL-4 and IL-13 are required for IgE synthesis in humans and IL-4 producing CD8$^+$ T cells are enriched in HIV-1 infected patients [Maggi *et al.*, 1994; Punnonen *et al.*, 1997]. Therefore, it is possible that IL-4 and IL-13 induced following HIV-1 infection could in turn elevate IL-4Rα expression on CD8$^+$ T cells and reduce CD8$^+$ T cell avidity as a mechanism to evade anti-viral CD8$^+$ T cells immunity.

6.3 IL-4 and IL-13 regulate CD8 densities to dampen anti-viral CD8$^+$ T cell avidity

The above observations suggest that reducing the responsiveness to IL-4 and IL-13 via down-regulation of IL-4Rα is important for enhancing the avidity of anti-viral CD8$^+$ T cell responses. It was next evaluated whether these cytokines affected the expression of T-cell associated molecules (e.g. CD11a, CD2, TCR, CD8 and lck) on anti-viral CD8$^+$ T cells that participate in maintaining high avidity cell-cell interactions with APCs. Ranasinghe *et al* [2007; 2009] have identified differences in CD8$^+$ T cell avidity following HIV-1 vaccination using tetramer dissociation assays; these assays distinguish avidity based on the strength of tetramer engagement with the TCR and/or CD8 co-receptor. This led to the hypothesis that IL-4 and IL-13 can reduce the affinity and/or the expression levels of TCR and/or CD8 co-receptor for down-regulating avidity of HIV-specific CD8$^+$ T cells. Indeed, it was found that IL-4 and IL-13 selectively dampen CD8 expression levels, which also dampen the avidity and polyfunctionality of HIV-specific CD8$^+$ T cells following i.n. FPV-HIV/i.m. VV-HIV vaccination.

A few reports following influenza virus infections have suggested that the usage of certain TCR clonotype (e.g. TCR Vβ7) is characteristic of high avidity CD8$^+$ T cells

[Kedzierska *et al.*, 2005; Kedzierska *et al.*, 2008]. Although in the current study TCR clonotypes were not evaluated, data suggest that enhancement in CD8 densities was sufficient to enhance avidity of HIV-specific CD8$^+$ T cells that developed in vaccinated IL-4 $^{-/-}$, IL-13 $^{-/-}$ and STAT6 $^{-/-}$ mice relative to WT controls. Previous reports are also in agreement with the above findings where anti-viral CD8$^+$ T cell avidity was shown to be up-regulated due to enhancement in CD8, but not TCR densities following pox viral infection [Oh *et al.*, 2004; Xiao *et al.*, 2007]. Overall, data suggest that IL-4 and IL-13 mediated regulation of CD8 densities is an important determinant of CD8$^+$ T cell avidity following pox viral infection and HIV-1 recombinant pox viral prime-boost vaccination.

6.4 How does IL-4 and IL-13 responsiveness affect anti-viral CD8$^+$ T cell avidity?

The data obtained from the virus infection and vaccination studies can be used to propose a tentative model where anti-viral CD8$^+$ T cell avidity could be regulated based on IL-4 and IL-13 responsiveness. Previously, Xiao *et al* [2007] have shown that CD8 densities are down-regulated on anti-viral CD8$^+$ T cells following cognate antigen encounter during pox virus infections. In chapter 3, it was shown that naïve CD8$^+$ T cells in the absence of IL-4 and IL-13 signalling (i.e. in IL-4 $^{-/-}$, IL-13 $^{-/-}$ and STAT6 $^{-/-}$ mice) expressed lower levels of IL-4Rα making them less responsive to these cytokines during T cell priming. Therefore, it is plausible to propose that IL-4 and IL-13 signalling through activation of STAT6 promotes greater down-regulation of CD8 densities on naïve CD8$^+$ T cells following cognate antigen encounter during pox virus infection and vaccination. Consequently, anti-viral effector CD8$^+$ T cells that develop with reduced CD8 densities have poor polyfunctionality and avidity. It has been shown that heterologous mucosal HIV-1/SIV prime-boost vaccination regimens are most effective in preserving avidity and protective capacity of anti-viral CD8$^+$ T cells [Kent *et al.*, 2005; Belyakov *et al.*, 2006; Belyakov *et al.*, 2008; Ranasinghe *et al.*, 2007;

Ranasinghe *et al.*, 2011; Ranasinghe *et al.*, 2013]. Therefore, vaccines (e.g. IL-13 inhibitor) that minimise down-regulation of CD8 densities on HIV-specific CD8$^+$ T cells even following mucosal vaccination hold great potential in facilitating protective CD8$^+$ T cell immunity against HIV-1.

6.5 FTA assay is a valuable screening tool for assaying CD8$^+$ T cell avidity *in vivo*

Previously, it was discussed that identifying mechanisms that can regulate anti-viral CD8$^+$ T cell avidity is important for developing more efficacious HIV-1 vaccines. In particular, the importance of regulating IL-4Rα and CD8 co-receptor expression levels on anti-viral CD8$^+$ T cells was discussed. Another important aspect for developing more efficacious HIV-1 vaccines is to develop assays that evaluate CD8$^+$ T cell avidity *in vivo* in pre-clinical models. Therefore, as the final study during the PhD project the use of a novel FTA assay was evaluated for this purpose.

Interestingly, the FTA assay efficiently isolated clusters of HIV-1 recombinant pox viral vaccination strategies that were most effective in generating high avidity T cell responses *in vivo* (e.g. heterologous FPV-HIV/VV-HIV vaccination strategies). The high avidity Gag-specific CD8$^+$ T cell killing responses induced following FPV-HIV/VV-HIV vaccination regimens also exhibited superior epitope cross-reactivity. Various T cell responses (avidity, epitope cross-reactivity and helper activity) using the FTA were also measured against a range of peptide pulsed target cells (>200) in the same HIV-1 vaccinated animal allowing for thorough screening of large number of vaccine strategies in a cost-effective manner. Overall, the development of the FTA assay is a significant advancement in the field of HIV-1 vaccine development given that this is the only assay that can be used to comprehensively measure functional avidity of anti-viral CD8$^+$ T cell responses *in vivo* following HIV-1 vaccination.

A few caveats for using this assay include that HIV-1 infected target cells could not be used in mice and that the kinetics of killing are also expected to provide useful information regarding how efficient high avidity CD8$^+$ T cells are in clearing virus-infected targets. Overall, the studies conducted clearly demonstrated the utility and effectiveness of the FTA assay in measuring various T cell quality parameters that are representative hallmarks of protective immunity against viral infections *in vivo*. Furthermore, the FTA assay could be utilized to develop effective prime-boost vaccination strategies for novel vaccines/vectors that aim to induce high avidity T cell responses *in vivo* using pre-clinical models.

6.6 Significance of the PhD

Effective HIV-1 prophylactic vaccines will require the induction of high avidity HIV-specific CD8$^+$ T cells and robust antibody responses. Given that HIV-1 transmissions occur mainly through a single founder virus [Keele *et al.*, 2008], it is important to have these responses at transmission sites (e.g. genito-rectal mucosa in case of sexual transmissions and systemic compartments in case of blood transfusions). This should serve to minimise systemic viral spread and establishment of latent reservoirs. The findings from our laboratory thus far suggest that vaccination strategies, which minimise IL-4/IL-13 responses and maintain high CD8 densities on anti-viral CD8$^+$ T cells are beneficial for this purpose. In particular, i.n. FPV-HIV/i.m. VV-HIV and mucosal IL-13 inhibitor vaccination strategies were extremely effective at inducing high avidity HIV-specific CD8$^+$ T cells at both mucosal and systemic compartments [Ranasinghe *et al.*, 2007; Ranasinghe *et al.*, 2013]. Preliminary findings from our laboratory also suggest that mucosal IL-13 inhibitor vaccines promote anti-viral IgG

responses. Matthew Worley, current student in the laboratory, is evaluating whether these IgG responses are similar to those that have correlated with ADCC responses and protection against HIV-1 [Haynes *et al.*, 2012; Tomaras *et al.*, 2013]. Therefore, mucosal HIV-1 recombinant pox viral vaccination strategies that inhibit IL-13 responses have provided promising outcomes for developing prophylactic HIV-1 vaccines.

Currently, about 34 million people are infected with HIV-1 globally and therapeutic approaches to cure these individuals are desperately in need. Mucosal HIV-1 recombinant pox viral vaccination strategies that induce high avidity HIV-specific CD8$^+$ T cells may also be used for this purpose. Shan *et al* [2012] have shown that autologous Gag-specific CD8$^+$ T cells are required to efficiently clear latently-infected CD4$^+$ T cells following re-activation *in vitro*. Recently, histone deacetylase inhibitors such as vorinostat and panobinostat have emerged to be promising drugs for reactivating latency on CD4$^+$ T cells and thereby exposing these cells to the immune system [Archin *et al.*, 2012; Rasmussen *et al.*, 2013]. Therefore, vaccination of HIV-infected individuals with i.n./i.m. HIV-1 IL-13 inhibitor vaccines prior to treatment with histone deacetylase inhibitors might facilitate the rapid clearance of latently infected CD4$^+$ T cell reservoirs. A caveat with this strategy is the presence of immune exhausted CD8$^+$ T cells in HIV-1 infected patients, but evaluation of these approaches should help develop a complete cure for HIV-1 infected patients.

6.7 Future directions

It is intriguing why up-regulation of IL-4Rα, but not down-regulation of this receptor on CD8$^+$ T cells was dependent on STAT6 following virus infection *in vivo*. Down-regulation of IL-4Rα on CD4$^+$ T cells has previously been described to be dependent on

DOCK2 [Tanaka *et al.*, 2007]. Even though the dependence of IL-4Rα down-regulation on DOCK2 has not been explored on CD8$^+$ T cells, future investigations could benefit from examining whether DOCK2 and STAT6 play antagonizing roles in regulation of IL-4Rα expression on CD8$^+$ T cells. This will provide more insight as to how IL-4Rα expression and CD8$^+$ T cell avidity are regulated.

Whether reduced responsiveness to IL-4 and IL-13 in a CD8$^+$ T cell autonomous manner is sufficient to enhance anti-viral CD8$^+$ T cell avidity was not evaluated in the current PhD project. This is important for identifying whether cell types other than CD8$^+$ T cells can contribute to regulating CD8 expression levels and avidity of anti-viral CD8$^+$ T cells. To evaluate this initially, infection or vaccination of mice where IL-4Rα is mainly or exclusively deficient only in CD8$^+$ T cells is required. These mice could be generated using bone marrow chimeras (i.e. chimeras between CD8α $^{-/-}$ and IL-4Rα $^{-/-}$ mice) or cre-lox system as done in Dewals *et al* [2009].

Given the ethical, technical and sampling limitations it is more difficult to conduct elaborate functional assays using human specimens compared to laboratory animals. Therefore, there is a constant requirement for identifying novel yet reliable biomarkers that are descriptive of lymphocyte functions especially CD8$^+$ T cell avidity in the context of HIV-1 infections. Monitoring cell surface IL-4Rα expression levels on naïve CD8$^+$ T cells might be ideal and descriptive for predicting avidity of HIV-specific CD8$^+$ T cells in HIV-1 infected patients. To address this possibility, initially IL-4Rα expression levels on naïve CD8$^+$ T cells from elite controllers (where elite control is associated with enhanced HIV-specific CD8$^+$ T cell avidity) and HIV-1 progressors should be monitored. It can be hypothesized that IL-4Rα expression levels will be lower

on naïve CD8$^+$ T cells or at least on naïve precursors of HIV-specific CD8$^+$ T cells from elite controllers relative to HIV-1 progressors.

Studies in our laboratory have demonstrated that mucosal (i.n./i.m.) IL-13 inhibitor vaccination strategies can protect mice against a lethal recombinant influenza-HIV virus mucosal challenge [Ranasinghe *et al.*, 2013]. It is also important to determine whether such mucosal vaccination strategies can be protective in the context of SIV infections in macaques in order to thoroughly evaluate the translational prospects of these vaccines. It will be intriguing to determine whether regulation of IL-4Rα and CD8 co-receptor levels on naïve and SIV-specific CD8$^+$ T cells following IL-13 inhibitor vaccination can be predictive of protective outcomes and avidity of SIV-specific CD8$^+$ T cells. Our laboratory is currently constructing vaccines for use in macaques for this purpose, which will hopefully allow for the full evaluation of our vaccines' translational potential for testing in human clinical trials in the near future.

Supplementary Figures
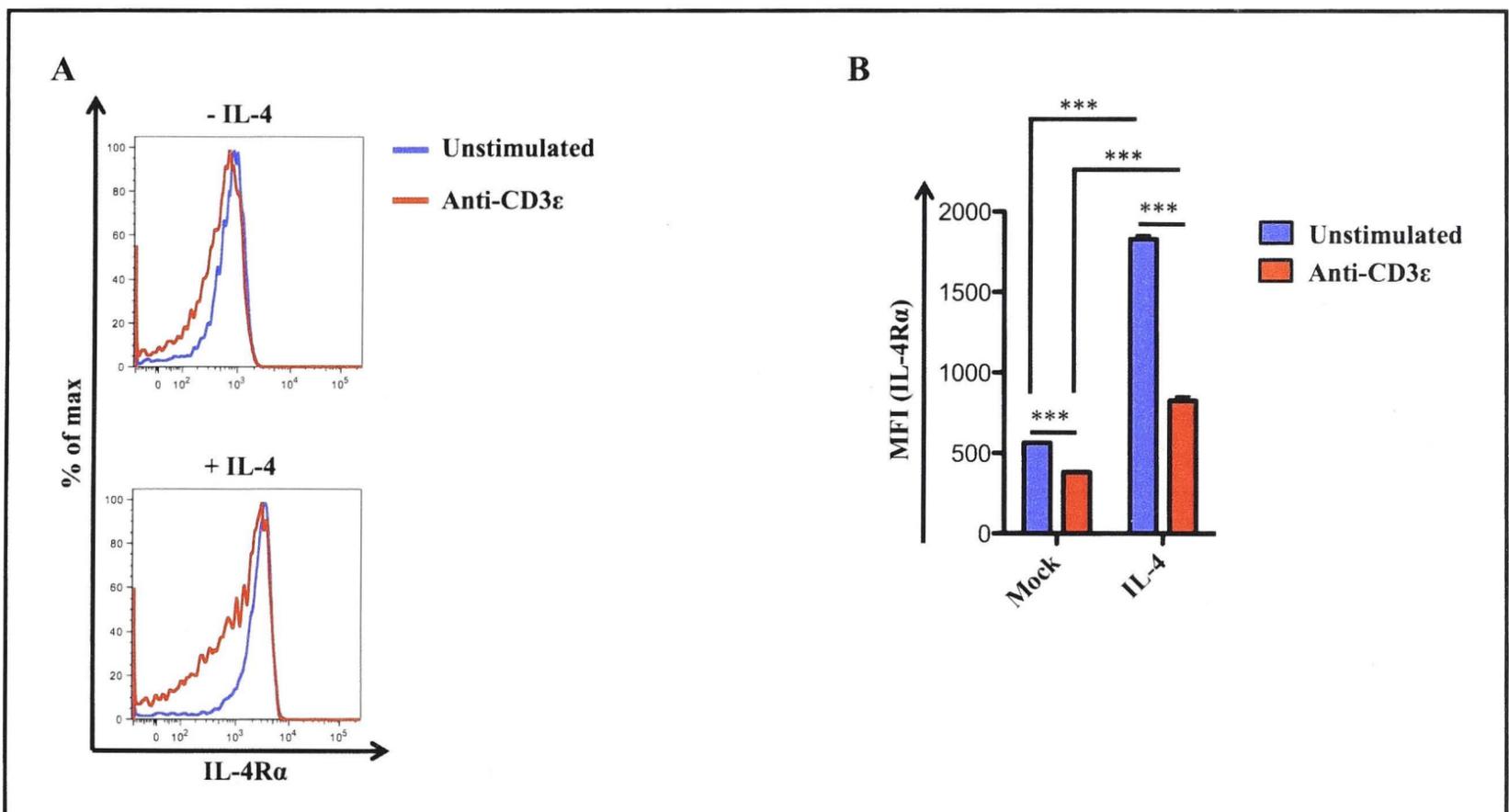
Supplementary figure 1. The importance of TCR signaling and IL-4 in regulating IL-4Rα expression of CD8^{+} T cells *in vitro*. FACS sorted $CD44_{lo}$ CD8^{+} splenocytes from naïve BALB/c mice were cultured for 8 hours in presence or absence of 0.1 ng/ml of recombinant murine IL-4 using microwells (2 x 10^4 cells/well) coated with anti-CD3ε or not (unstimulated). Subsequently, cultured splenocytes were analyzed using flow cytometry for IL-4Rα expression. A, Representative histogram plots showing IL-4Rα expression on CD8^{+} splenocytes cultured in the presence or absence of anti-CD3ε ± IL-4. B, MFI (n = 4) representing IL-4Rα expression on CD8^{+} splenocytes cultured in the presence or absence of anti-CD3ε ± IL-4. The data presented is representative of two independent experiments and the error bars depict the SEM. One-way ANOVA (Tukey's Multiple Comparison) was used to determine statistical significance of the data (*** - p < 0.001).
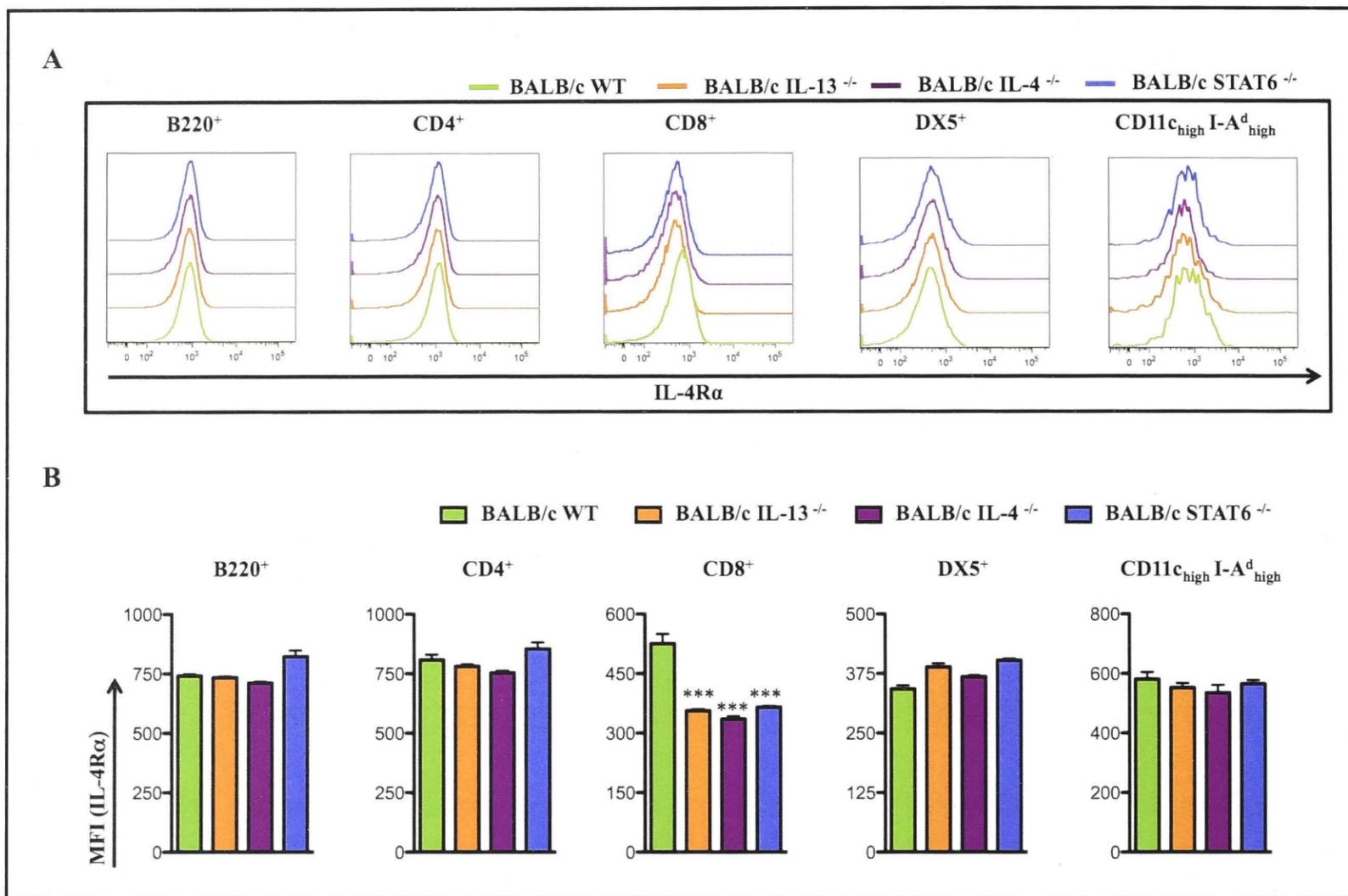
Supplementary figure 2. The absence of IL-4 and IL-13 does not affect the *in vivo* IL-4Rα expression on naïve B cells, CD4^{+} T cells, NK cells and DCs. Splenocytes from naïve mice were isolate and analyzed using flow cytometry for IL-4Rα expression. A, Representative histogram plots showing IL-4Rα expression on the indicated immune cell subset. B, MFI (n = 3) representing IL-4Rα expression on the indicated immune cell subset. The data presented is representative of at least two independent experiments and the error bars depict the SEM. One-way ANOVA (Dunnett's Multiple Comparison) was used to determine statistical significance of the data relative to WT mice (*** - $p < 0.001$).
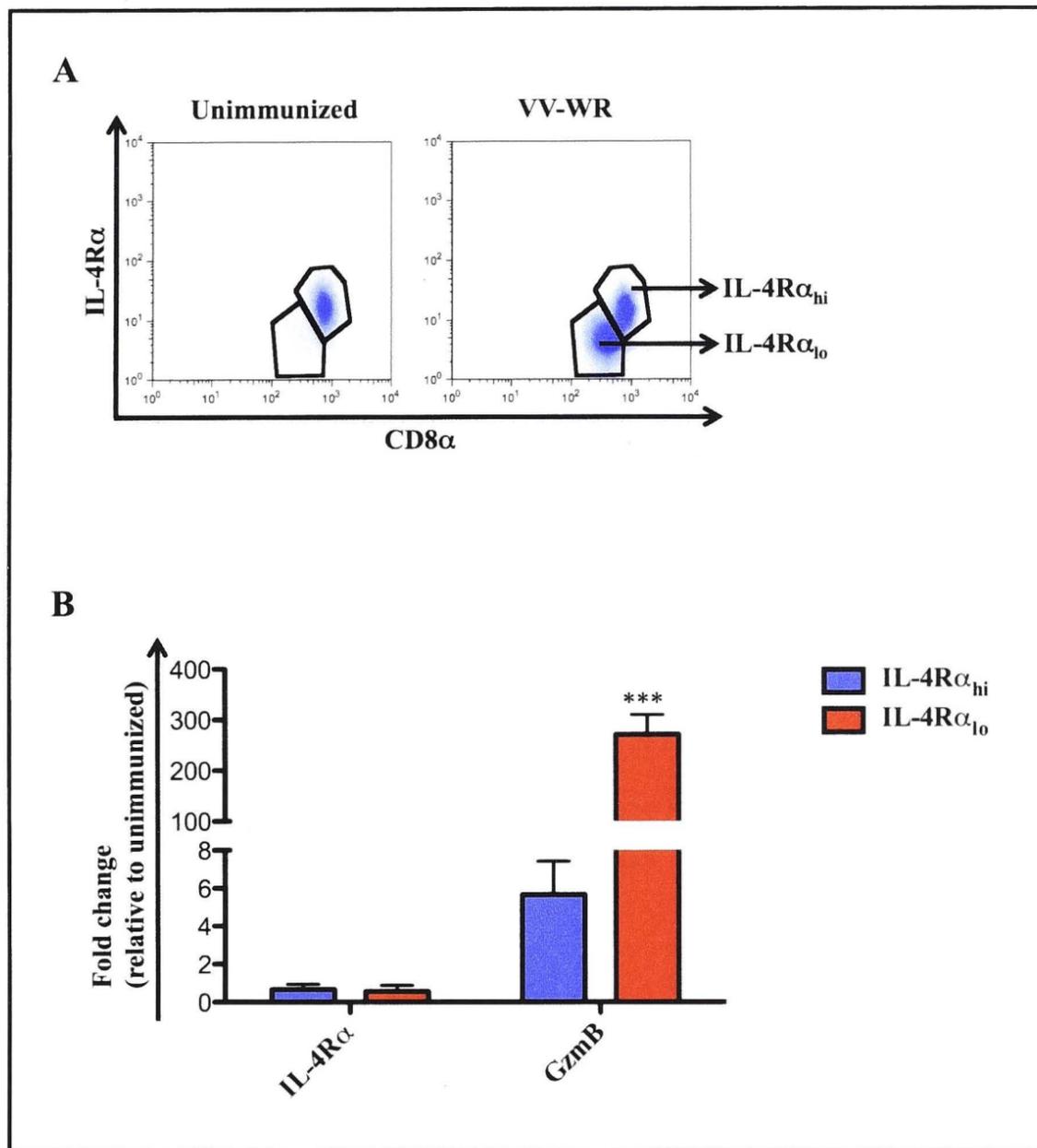
Supplementary figure 3. Down-regulation of IL-4Rα expression on CD8$^+$ T following VV infection is not due to transcriptional regulation. Splenocytes from 7 days VV-WR infected (3 x 10^6 PFU/mouse) or unimmunized BALB/c WT mice were FACS sorted for quantitative real-time PCR analysis as described in the materials and methods. A, Representative density plots showing IL-4Rα and CD8α expression on gated CD8$^+$ splenocytes from mice infected with VV-WR or kept as unimmunized. B, Mean (n = 4-6) fold change in mRNA transcript levels of IL-4Rα and GzmB on FACS sorted IL-4Rα$_{lo}$ and IL-4Rα$_{hi}$ cells from VV-infected mice with respect to the mRNA transcript levels of FACS sorted CD8$^+$ T cells from unimmunized mice. The data shown is representative two independent experiments and the error bars depict the SEM. Statistical significance of the data was determined relative to IL-4Rα$_{hi}$ cells using a student's unpaired t-test (*** - p < 0.001).
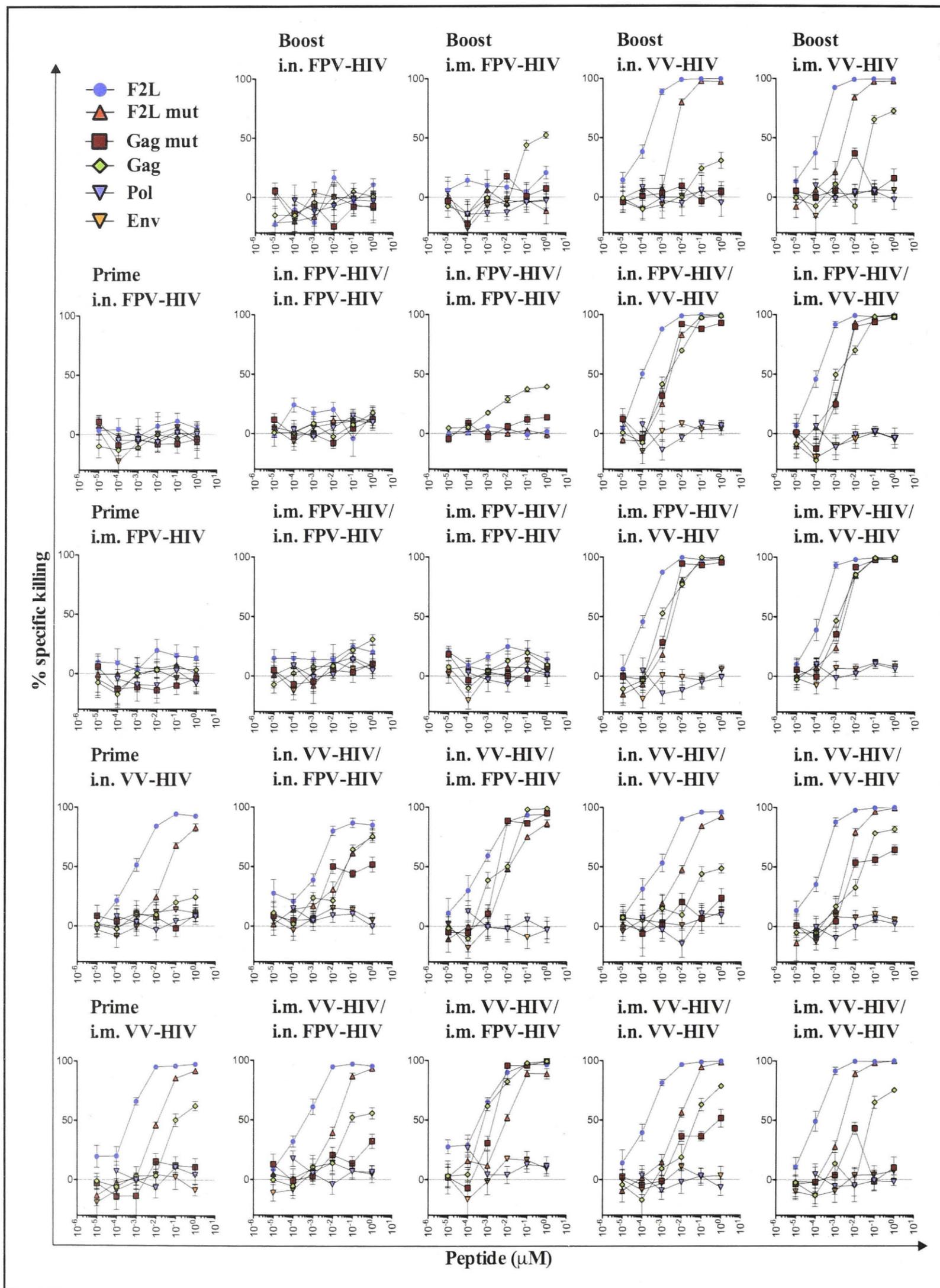
Supplementary figure 4. Killing responses data used to construct the heat map shown from the experiment in figure 5.3.
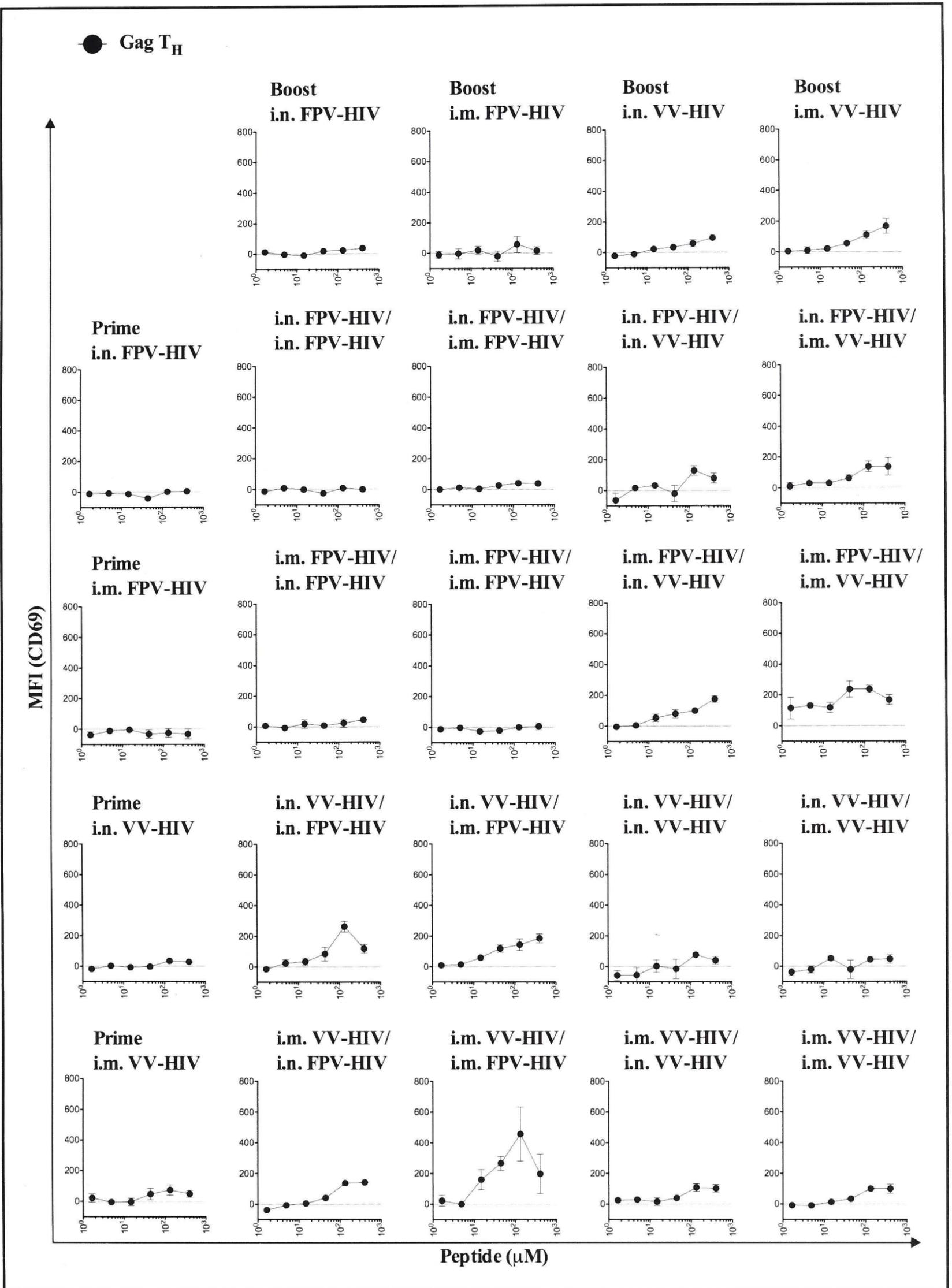
Supplementary figure 5. Gag T$_H$ data used to construct the heat map shown from the experiment in figure 5.3.

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