Cytotoxic T-Cells in HIV-1: “The Good” and “The Bad”



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Abstract

CD8+ T-cell antigen sensitivity is critical for optimal control of persistent viral infections, including HIV-1. The devastating HIV-1 pandemic may be countered by development of a cytotoxic T lymphocyte based vaccine if qualities associated with protection can be defined at the molecular level. However, the heterogeneity of the total viral-specific CTL response confounds identification of protective correlates and T-cell sensitivity is no exception and remains controversial. To address this issue we reduced the heterogeneity of the HIV-1 CTL response to single units, generating 19 CTL clones that recognise the same HIV-1 derived epitope restricted by HLA B*08. Correlation of functional assays directly with the ability of each clone to control HIV-1 replication *in vitro*, the "viral suppression assay," identified antigen sensitivity as a key quality for anti-viral efficacy. Remarkably, four clones from this panel, isolated from one individual, a long-term non-progressor, all used an identical TCR yet had distinct antigen sensitivity and suppressive activity. Two of these clones were characterised in detail, and had distinct cytokine profiles, regulated by epigenetic mechanisms, and differential expression of a group of cell surface receptors with the potential to modulate the signalling threshold to antigen. Expression of the TNFα locus of the high sensitivity clone with "Good" suppression was repressed by DNA methylation. Understanding how CTL qualities required for optimal control of HIV-1 replication differentiate and are then enriched in the total CTL response, and if repression of TNFα contributes to this process, will contribute to rational vaccine design. This is the first evidence that avidity maturation in CD8+ T cells with the same TCR affinity occurs in viral infections in humans as reported in the mouse. This suggests the induction of high sensitivity CTL will be critical for an effective HIV-1 vaccine, but offers hope that this can be achieved even in individuals without protective HLA alleles, by further exploration of peripheral avidity maturation and epigenetic regulation of the HIV-1 specific CD8+ T-cell response.

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Declaration

I declare that the work presented in this DPhil thesis entitled “Cytotoxic T cells in HIV-1:”The Good” and “The Bad” is entirely my own work, except for where the contributions of collaborators have been clearly acknowledged. No part of my thesis has been submitted for any other degree or other qualification in this University or elsewhere.

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Publications and Awards

Publications

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Awards

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2nd prize winner for oral presentation "HIV-1 suppression beyond the TCR" from over 30 talks at the annual Weatherall Institute of Molecular Medicine Student Day 2011.

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
ART	antiretroviral treatment
BSA	Bovine Serum albumin
B_2m	Beta-2-microglobulin
CCL	C-C motif ligand
CD	cluster of differentiation
CDR	complementarity determining region
CTL	cytotoxic T lymphocyte
CXCL	cysteine-cysteine linked chemokine ligand
CXCR	cysteine-cysteine linked chemokine receptor
DC	Dendritic Cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunoabsorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL8	FLKEKGGL Nef $_{(90\text{-}97)}$ epitope, HLA B*08 restricted
Gag	group associated antigen
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICS	Intracellular cytokine staining
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif

IW9	ISPRTLNAW Gag p24 $_{(147\text{-}155)}$ epitope, HLA B*57 restricted
KIR	killer cell immunoglobulin-like receptor
KK10	KRWIILGLNK Gag p24 $_{(263\text{-}272)}$ epitope, HLA B*27 restricted
KF11	KAFSPEVIPMF Gag p24 $_{(162\text{-}172)}$ epitope, HLA B*57 restricted
LTNP	long term nonprogressors
LTR	long terminal repeat
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
Nef	negative factor
NK	natural killer cell
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohaemaglutinin
PMA	phorbol myristate acetate
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
RT	reverse transcriptase
SD	standard deviation
SL9	SLYNTNATL Gag p17 $_{(77\text{-}85)}$ epitope, HLA A*02 restricted
SIV	simian immunodeficiency virus
TW10	TSTLQEQIAW Gag p24 $_{(162\text{-}172)}$ epitope, HLA B*57 restricted
TCR	T cell receptor
TRA/B	T cell receptor alpha/beta chain
T_{EM}	Effector memory T cell
T_{CM}	Central memory T cell
TNF	tumour necrosis factor
VSA	Viral suppression assay

AMINO ACID LETTER CODES

A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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1 INTRODUCTION

1.1 Cytotoxic T cells

CD8+ Cytotoxic T lymphocytes (CTL) use the T cell receptor to sense 8-11 amino acid peptides presented by class I major histocompatibility (MHC) molecules to trigger a highly specific effector response that can cause elimination of virus infected cells and change the immediate tissue milieu by the secretion of cytokines and chemokines. All cells in the body display class I MHC molecules, enabling total CTL immunosurveillance. CTL can be distinguished by the expression of the co-receptor CD8+, and a unique effector response upon TCR activation that occurs by expression of key transcription factors and epigenetic modifications that create a characteristic transcriptional signature. CTL functional diversity is only just being unearthed, driven to a large extent by persistent viral infections, particularly the devastating HIV-1 pandemic. Defining components that characterise CD8+ CTL with optimal control of HIV-1 replication is an essential step towards the development of a successful HIV-1 vaccine and is the objective of this thesis.

1.2 The Human Leucocyte Antigen Locus

The MHC locus spans at least 7 million base pairs (bp) on the short arm of chromosome 6p21.31, and contains over 200 genes involved in antigen presentation, antigen processing and the immune response. The MHC locus contains the most polymorphic genes of the human genome, known as human leucocyte antigens (HLA). First identified to encode the genetic determinants of "self" in transplantation

studies, it became clear that immune response genes to foreign antigens also mapped within this locus (Benacerraf and McDevitt, 1972). This was resolved with the landmark discovery that CD8+ cytotoxic T-cells were restricted in recognition of foreign viral antigens to targets that shared the same self-MHC class I antigen (Zinkernagel and Doherty, 1974) (McMichael *et al.*, 1977). Survival following infection was proposed to select for advantageous HLA genes and drive the extreme polymorphism of the MHC locus (Doherty and Zinkernagel, 1975). Outcome following HIV-1 infection has been linked to single nucleotide polymorphisms (SNP) within the MHC class I locus in large genome-wide association studies (GWAS) (Carrington and Walker, 2012).

There are three gene families, MHC class -I, -II, and -III. MHC class I gene loci, with the number of functional gene products in parentheses, (Class Ia: HLA-A (1448 proteins), -B (1998 proteins) and –Cw (1119 proteins)) encode the polymorphic structural heavy chain of the MHC class I molecule (Robinson *et al.*, 2000). This loci also contains genes that encode the non-classical (HLA-E and HLA-G) MHC class I molecules, and the polymorphic MHC class I-related chain A (MICA) (84 alleles) and B (MICB) (35 alleles). The MHC class I loci gene products are ligands for the two main cytotoxic cell populations, natural killer (NK) cells and CD8+ T-cells. CD8+ T-cells recognise antigen presented by classical MHC-I and non-peptide antigens presented by CD1-restricted antigens (Barral and Brenner, 2007). NK cells use non-antigen specific receptors that recognise classical and non-classical MHC-I molecules (Lanier, 2005). Some NK receptors are expressed by CD8+ T-cells and can provide co-stimulation or co-inhibition to the antigen specific αβ TCR. HLA-A, -B, -C and –E are expressed by all somatic cells, and HLA-G is expressed on the human placenta,

monocytes and T-cells (Huang *et al.*, 2010). MICA and MICB expression is induced by cell stress.

MHC class II gene loci (HLA –DRA, -DRB, -DQA1, -DQB1, –DPA1 and DPB1) are expressed on activated T cells, B cells, macrophages, and dendritic cells and form the α and β structural chains of MHC class II molecules. Each chain contributes to the peptide-binding groove, but the DRB1 locus is the most polymorphic, with 860 proteins described (Robinson *et al.*, 2000). The HLA DR β-chain has up to three additional loci, DRB3, DRB4 and DRB5 on different haplotypes. The peptide binding groove is similar to MHC class I, but remains open at either end, tolerating longer peptides of 11-17 amino acids in length, derived from extracellular proteins, processed and loaded within endosomes. MHC class II molecules present antigen to T-helper cells, and bind the CD4+ co-receptor. MHC class II region includes genes required for antigen processing, for example, transporter associated with antigen processing (TAP) -1 and -2. MHC class III region contains genes for proteins of the complement cascade, tumor necrosis factor (TNF) alpha, lymphotoxins alpha (LTA) and beta (LTB).

1.3 Peptide-MHC Class I molecules

Classical MHC class I molecules (HLA –A, -B and –C) are non-covalently associated heterodimers composed of a polymorphic glycosylated heavy chain encoded by *HLA –A, -B* or *–C* genes located within the (MHC) class I locus, that bind peptide and non-covalently associate with beta-2 microglobulin (β_2m) (chromosome 15) (Cresswell *et al.*, 1973) (Grey *et al.*, 1973). The heavy chain has three extracellular domains, α1, α2 and α3. Structural resolution of the first MHC class I crystals demonstrated that the two membrane distal domains, α1 and α2, fold to form a groove, composed of two anti-parallel alpha helices set on a floor of eight anti-parallel beta pleated sheets, and

revealed a bound peptide within the groove (Bjorkman *et al.*, 1987a). This confirmed that CTL recognise virus infection by the proteolytic cleavage of viral proteins in the cytosol of the cell, which are bound to MHC class I receptors and displayed on the cell surface (Townsend *et al.*, 1986). This first HLA A*02 structure identified that the sites of greatest sequence variability between the polymorphic alleles were located within the peptide binding groove, identifying six pockets (A-F) that determine the specificity of the peptide binding motif for that HLA allele (Bjorkman *et al.*, 1987b). The peptide is held in an elongated conformation, and "anchored" by amino acid side chains that fill complementary pockets, with exposed amino-acid side chains that are able to interact with the T-cell receptor (Madden *et al.*, 1992). The CTL co-receptor $CD8_{\alpha\beta}$+ binds to residues 223-227 of the α_3 domain of MHC class I (Salter *et al.*, 1989) (Gao *et al.*, 1997).

1.4 MHC Class I antigen processing

The classical MHC class I antigen pathway determines how viral proteins in the cytoplasm are cleaved into peptide fragments and presented by MHC class I alleles for CD8+ T-cell recognition. Each cell expresses up to six different MHC class I antigens (HLA –A, -B and –C) (Mouse H-2K, D and L) and has an estimated 100 000 MHC class I alleles on the surface. Each proteolytic step confers specificity and determines that only a few fragments from any given protein can be presented. Cytosolic proteins are often degraded by the proteasome, a cylindrical catalytic chamber composed of four stacked heptameric rings (Murphy *et al.*, 2008). Interferons induce the expression of sub-units that alter the composition of the two inner rings to form the immunoproteasome. Target proteins are marked for degradation by polyubiquitination by E3-ubiquitin ligases. These proteins diffuse through the cytosol until encountering the proteasome cap, and are unfolded and

cleaved within the catalytic chamber into peptides 2-25 amino acids in length. The proteasome determines the COOH-terminal end αα residue of the peptide. The N-terminus can be trimmed in the cytosol by amino-peptidases, endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), or translocated into the endoplasmic reticulum (ER) and trimmed by endopeptidases, such as tripeptidylaminopeptidase II (Yewdell and Haeryfar, 2005). Peptides are actively translocated into the ER by TAP-1 and TAP-2 that form a heterodimer. Retrotranslocation of ER proteins into the cytosol may allow the cross-presentation of exogenous antigens for CD8+ T-cell recognition. TAP transports cytosolic peptides of 8-16 amino acids length, with hydrophobic or basic COOH-terminus αα residues, that are the preferred residues for MHC I allele binding (Murphy *et al.,* 2008). The MHC class I heavy chain is cotranslationally translocated into the ER where it is folded, and glycosylated in association with the chaperone calnexin. The chain then associates with β_2 m and dissociates from calnexin to form a "loading complex" with other MHC class I molecules, TAP, tapaisin, calreticulin, and protein disulfide isomerase and ERp57. The peptide is an integral component of the MHC class I chain and binding stabilises the peptide-MHC I complex for transport to the cell surface.

1.5 The T-Cell Receptor

Each CD8+ CTL pre-cursor and its progeny can be defined by its clonotypic TCR (Infante *et al.*, 1982). The αβ TCR is a heterodimer of two glycosylated polymorphic polypeptide chains, the alpha chain (TRA) (chromosome 14q11.2) and the beta chain (TRB) (chromosome 7q34) covalently linked by a disulphide bond (Meuer *et al.*, 1983) (Rabbitts *et al.,* 1985) (Collins *et al.*, 1984). The αβ TCR non-covalently associates with the non-polymorphic CD3 proteins to form a functional TCR complex

on the cell surface (Acuto *et al.*, 1983a). Peptide mapping and molecular sequencing of the TCR chains highlighted the close resemblance of the TCR to an immunoglobulin, with two extracellular domains per chain, a membrane proximal constant (C) region, and a membrane distal variable (V) region (Reinherz *et al.*, 1983; Acuto *et al.*, 1983b) (Seidman *et al.*, 1979; Early *et al.*, 1980). Sequence variation between TCR chains was focused within three hypervariable loops in the V region that form the antigen recognition site and are known as complementarity determining regions -1, -2, -3 (CDR1-3). Extraordinary DNA template diversity is generated for the CDR3 region by the somatic rearrangement of two (TRA) or three (TRB) gene segments, a variable (V), diversity (D) (TRB chain only), and joining (J) gene, randomly selected from multiple copies of each gene in the *TRA* or *TRB* locus. Somatic DNA rearrangement requires expression of Recombination Activating Gene 1 (RAG1) and RAG2 (Schatz *et al.*, 1989). Combinatorial diversity has the potential to generate TRA chains (44-46 TRAV x 52 TRAJ functional gene segments) and TRB chains (56-65 TRBV x 2 TRBD x 12-13 TRBJ) (Lefranc, 2003). Junctional diversity is introduced during DNA recombination, as the gene segments are imprecisely joined, introducing variable numbers of DNA template palindromic "P"-nucleotides, insertion of non-template "N"-nucleotides by the enzyme terminal deoxynucleotidyl transferase, and base pair editing by exonuclease activity (Oettinger, 2004). CDR1 and CDR2 are germline encoded within the V gene segment, and form distinct TCR receptor families. Unique pairings of α and β chains increase the total number of possible different TCRs to $>10^{18}$ (Venturi *et al.*, 2008). The peripheral TCR repertoire is selected *in vivo* by rigorous thymic positive and negative selection and limited by the finite composition of the total number of peripheral naïve and memory CTL, estimated at ($\sim 10^{12}$) that is tightly regulated by homeostatic

mechanisms (Arstila *et al.*, 2000). Despite this enormous diversity, T cells bearing identical TCRs can dominate the CD8+ T-cell response to the same antigen-MHC I complex in multiple individuals, and these TCRs are known as "Public" TCRs (Venturi *et al.*, 2008). The production of public TCRs may arise from more "germline-like" V(D)J recombination events, convergent recombination or structural properties and may have important contributions to viral control (Stewart-Jones *et al.*, 2003) (Venturi *et al.*, 2006; Iglesias *et al.*, 2011) (Berger *et al.*, 2011) (Price *et al.*, 2009) (Chen *et al.*, 2012) (Kjer-Nielsen *et al.*, 2003; Turner *et al.*, 2006).

1.6 Antigen Recognition

The structure of the first human TCR-viral peptide-MHC class I complex identified a diagonal alignment of the TCR threading between the "peaks" of the peptide-binding groove, to centre the CDR3α and CDR3β in direct contact with the exposed amino acid side-chains of the peptide (Garboczi *et al.*, 1996). Maximal sequence diversity is thus focused on the coding joint that forms the CDR3 loop that is central to recognition of potential pathogen peptides. TCR affinity for cognate epitope-MHC is several orders of magnitude lower than for the B cell receptor, ranging between 10^{-4} to 10^{-7} M, with a rapid dissociation rate (Matsui *et al.*, 1991) (Weber *et al.*, 1992; Corr *et al.*, 1994), yet CTL sensitively recognise as few as 100 pMHC complexes from a sea of 100 000 irrelevant pMHC (Davis *et al.*, 2007). The TCR is also inherently cross-reactive as TCRs are selected on self peptide-MHC I complexes (Nanda *et al.*, 1995). Cross-reactivity may be a structural feature of the TCR, or be secondary to high antigen sensitivity (Davenport *et al.*, 2007). Structural mechanisms of cross-reactivity reported include a flexible CDR3 loop, and thermodynamically favourable conformational change within the peptide (Reiser *et al.*, 2003) (Lee *et al.*, 2004). TCR can be engineered with increased affinity for the peptide-MHC complex, which

also increased cross-recognition of viral variants (Varela-Rohena *et al.*, 2008; Bennett *et al.*, 2010).

1.7 TCR signalling

Antigen recognition by the αβ TCR is communicated to the CTL by the phosphorylation of tyrosine residues on CD3 subunits (εγ, εδ, ζζ) that are integral components of the TCR complex (Weiss and Stobo, 1984) (Weissman *et al.*, 1988). This process is known as "TCR triggering" (Smith-Garvin *et al.*, 2009). The tyrosine residues are located in immunoreceptor tyrosine-based activation motifs (ITAMs) (YXXL/I X_{6-8} YXXL/I) (Reth, 1989) and are phosphorylated by Lck, a T-cell specific Src-family kinase associated with the CD8 receptor (Goldman *et al.*, 1998) (Barber *et al.*, 1989). Lck activity is regulated by phosphorylation of two tyrosine residues, autophosphorylation of the catalytic domain tyrosine residue required for full activity, and phosphorylation of an inhibitory tyrosine residue by the tyrosine kinase carboxy-terminal src kinase (Csk) (Figure 1-1). CD45, a haematopoietic cell surface glycoprotein with tyrosine phosphatase activity, dephosphorylates both the inhibitory and activating tyrosines to act as a positive and negative regulator of TCR signalling (Koretzky *et al.*, 1990) (Hermiston *et al.*, 2003). The phosphorylated ITAMs on CD3 chains recruit ZAP-70 (ζ associated protein of 70 kDa), that activates two scaffold proteins, linker for the activation of T cells (LAT) and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) that nucleate the proximal signalling complex required for TCR signal propagation (Smith-Garvin *et al.*, 2009) (Figure 1.1).

Figure 1-1: TCR Signaling

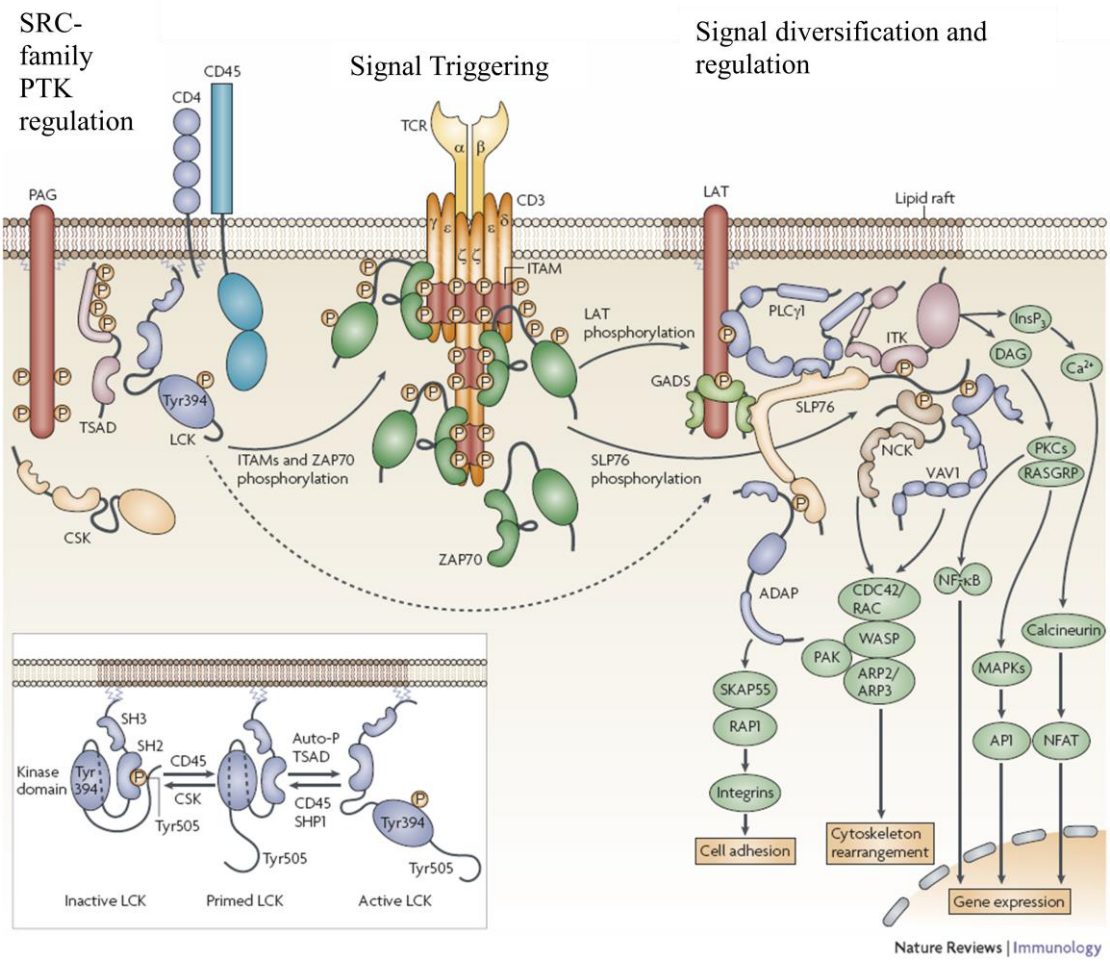


Figure 1-1: TCR Signalosome modules and their connections. The SRC-family protein tyrosine kinase (PTK) regulation (and insert) involves LCK, PAG, CSK, CD45 and TSAD. LCK is associated with the co-receptor CD4 or CD8. PAG (phosphoprotein associated with glycolipid-enriched membrane domains) recruits CSK (C-terminal SRC kinase). CSK phosphorylates an inhibitory residue on LCK (Tyr505). CD45 and the binding of T-cell-specific adaptor protein (TSAD) activate and positively regulate LCK. LCK phosphorylates the tyrosine residues on CD3 chains, to recruit ζ-chain-associated protein kinase of 70 kDa (ZAP70). ZAP-70 phosphorylates the scaffold proteins that form the backbone of the proximal signalling complex, linker for activation of T cells (LAT) and SRC homology 2 (SH2)-domain-containing leukocyte protein of 76 kDa (SLP-76). Phospholipase Cγ1 (PLCγ1) and interleukin 2 inducible T-cell kinase (ITK) regulate calcium and diacylglycerol (DAG) production. Non-catalytic region of tyrosine kinase (NCK) and VAV mediate actin polymerisation. Adhesion- and degranulation promoter adapting protein (ADAP) regulate integrin activation. These control cell adhesion, cytoskeletal rearrangements and gene expression (Acuto *et al.*, 2008).

1.7.1 TCR Down-stream signalling pathways

Calcium is one of the key secondary messengers on TCR activation. Calcium ions activate degranulation and alter gene transcription, including activation of calcineurin that leads to nuclear translocation of nuclear factor of activated T cells (NFAT) (Lyubchenko *et al.*, 2001) (Figure 1-1). Phospholipase C γ 1 (PLCγ1) hydrolyses $PI(4,5)P_2$ (phospholipid phosphatidylinositol 4,5 bisphosphate) to generate IP_3 (inositol triphosphate) and DAG (diacylglycerol). IP_3 binds the IP_3 receptor (IP_3R) to release calcium stored in the ER and trigger an influx of extracellular calcium ions across the plasma membrane (Imboden and Stobo, 1985). DAG activates two pathways, Ras-Raf 1 mediated mitogen activated protein kinase (MAPK) pathway, which activates extracellular signal related kinase (ERK) and regulates transcription factor activator protein -1 (AP-1) (Fos/Jun heterodimer), and protein kinase C theta (PKCΘ) that regulates NF-κB (Smith-Garvin *et al.*, 2009). ERK is also reported to protect Lck from the action of SH2-domain containing tyrosine phosphatase (SHP-1) (Altan-Bonnet and Germain, 2005).

1.7.2 The Actin and Cytoskeletal Response

TCR signals induce changes in the actin cytoskeleton that convert the CTL, one of the most motile of the tissue cells, from a form structurally adapted for amoeboid locomotion, into a form with polarised mechanical activity focused towards the target cell, with an accumulation of filamentous actin (F-actin) at the stimulatory interface (Dustin, 2008). Cytoskeletal rearrangement occurs on TCR signalling downstream of Vav-1, a guanine nucleotide exchange factor (GEF) and non-catalytic region of tyrosine kinase (Nck) (Figure 1-1). TCR plasma membrane fluidity increases by Vav1-dependent dephosphorylation of ezrin, radixin, and moesin (ERM), which

uncouples the plasma membrane links to the actin cytoskeleton (Smith-Garvin *et al.*, 2009). F-actin accumulates at the T cell/APC interface by localised TCR induced activation of multiple actin regulatory and polymerising pathways, of which actin-related proteins 2/3 complex (Arp 2/3) is the best described.

1.7.3 Integrins

Integrins are heterodimers composed of non-covalently associated alpha and beta subunits. Lymphocytes express three families of integrin receptors, defined by their common beta chain, β_2 (CD18), β_1 (CD 29) and β_7 (Luo *et al.*, 2007). Integrins mediate cell:cell and cell:extracellular tissue adhesion and are essential components of the CTL immune response and tissue homing and migration to inflamed tissues. The integrin lymphocyte function associated-1 (LFA-1) (α_L β_2, CD11a:CD18) binds intercellular adhesion molecules (ICAM -1, -2 and -3). The expression of distinct integrin subunits is regulated during CTL activation, with upregulation of LFA-1 and the adhesion molecule CD2 (ligand CD58) that facilitate cell:cell interactions. The adhesiveness of the extracellular domains is dynamically regulated by intracellular signals from the TCR or chemokine receptors, known as "inside-out" signalling. Inside-out signalling changes the conformation of the inserted or "I" domain in the extracellular region of the αL subunit from "closed" to "open", increasing the affinity for ICAM-1 9000 fold (Shimaoka *et al.*, 2001). This mediates "tight" adhesion for CTL:target cell conjugates and tissue homing, endothelial extravasation and migration.

1.7.4 The Immune Synapse

T cell activation leads to the formation of a SupraMolecular Activation Cluster (SMAC) or immunological synapse (IS) (Monks *et al.*, 1998) (Stinchcombe *et al.*, 2001; Smith-Garvin *et al.*, 2009). The CD8+ T-cell SMAC is composed of a central (c-SMAC) signalling domain and secretory domain, surrounded by a peripheral (p-SMAC) ring of adhesion proteins, including LFA-1 and talin (Stinchcombe *et al.*, 2001). The c-SMAC signalling domain contains the TCR-CD3 complex, CD8 co-receptor, CD28, CD2, p56 Lck, and PKCΘ. Submicron TCR and LFA-1 microclusters form in the distal-SMAC (d-SMAC) that is rich in CD45, and are transported by retrograde F-actin flow to the c-SMAC where they are endocytosed (Dustin and Long, 2010). The symmetry of the IS depends on PKCθ-Wiskott Aldrich Syndrome protein (WASP) signalling and may determine the IS stability and influence the efficiency of lytic granule release through the secretory domain into the secretory cleft (Beal *et al.*, 2008).

1.8 CTL Effector Response: A Diverse Armamentarium

The functional hallmark of CD8 T cells *in vitro* is cell contact dependent cytolytic activity, but it is debated if lytic activity, the secretion of soluble factors, or a combination of both, are the predominant effector mechanisms against viral infections *in vivo* (Kagi and Hengartner, 1996). Both cytotoxicity and the secretion of soluble factors are MHC class I restricted.

1.8.1 Lytic Granule Exocytosis

CTL are highly efficient serial killers. Cytotoxic T cells can induce cell death in target cells within 2-10 minutes (Stinchcombe *et al.*, 2001) (Matter, 1979) (Rothstein *et al.*, 1978), and then disengage to locate and kill further targets only 6 minutes later (Poenie *et al.*, 1987). CTL scan the target cell, randomly crawling over the target cell surface, until recognition of a cognate pMHC complex triggers CTL activation, firm adhesion and polarisation towards the target cell. The microtubule organising center (MTOC), golgi apparatus and lytic molecules polarise to lie immediately beneath the secretory domain (Geiger *et al.*, 1982), (Yannelli *et al.*, 1986) (Stinchcombe *et al.*, 2001). Lytic granules are released into the secretory cleft and cause target cell death indicated by membrane blebbing characteristic of apoptosis by 4-5 minutes of conjugate formation (Figure 1.2). The CTL disengages and the cycle is repeated (Stinchcombe *et al.*, 2001).

Figure 1-2: CD8+ Cytotoxic T cells in action

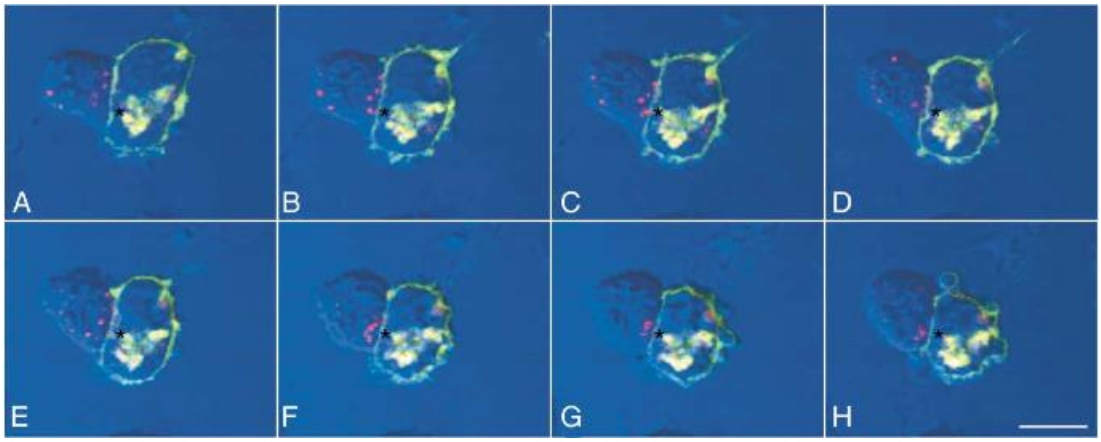


Figure 1.2: The dynamics of CTL exocytosis and target cell death: Lytic granules polarise towards the target cell (right), are released into the secretory cleft and induce target cell membrane blebbing characteristic of apoptotic cell death in (G) and (H). Single images from live cell time-lapse video microscopy at 0, 40, 60, 90, 120, 180, 240 and 300 (A-H) second intervals after cell:cell contact. Nomarski differential interference contrast (blue) and superimposed 1 uM confocal fluoresence (CTL lytic granules-red; cell membrane P815 target cell-green). Scale bar represents 10uM. Adapted from (Stinchcombe *et al.*, 2001).

1.8.2 Perforin and Granzymes

Perforin was first isolated in 1984 (Henkart *et al.*, 1984) and has a central role in CTL activity against viral infections (Kagi *et al.*, 1994a). Familial haemophagocytic lymphohistiocytosis (FHL) is caused by impaired synthesis, function or release of perforin (Janka, 2012). Perforin acts in a two-step process to deliver granule serine proteases (granzymes) to the target cell where they induce programmed cell death. Perforin forms transient pores in the cell membrane that triggers endocytosis of perforin and granzymes by the target cell and then forms pores in the endosome to deliver granzymes to the cytosl (Thiery *et al.*, 2011). Granzyme B (Chr 14q11) is one of the most abundant of a family of granzymes (A, B, H, K and M), which induce target cell apoptosis by activation of aspartic acid specific cysteine proteases (caspase) dependent and non-caspase dependent pathways, and can have redundant actions (Chowdhury and Lieberman, 2008). Granzyme B directly activates the effector caspase, caspase 3. On TCR ligation, newly synthesised perforin and granzyme B can be detected at two hours, and can be secreted directly through the immune synapse without prior storage in lytic granules (Isaaz *et al.*, 1995) (Makedonas *et al.*, 2009). Degranulation can be blocked with little or no effect on target cell lysis, and lysis can proceed in the absence of calcium that is required for perforin polymerisation (Ostergaard *et al.*, 1987) (Trenn *et al.*, 1987).

1.8.3 Death Ligand Mediated Cytotoxicty

Following the study of perforin knock-out mice, it was found that CTL that lacked perforin had substantial residual killing activity *in vitro* that was MHC class I restricted (Kagi *et al.*, 1994b). This killing activity was attributable to expression of Fas ligand (CD178) that induces apoptosis in the target cell on ligation of Fas (CD 95)

(Lowin *et al.*, 1994). FasL expression is tightly regulated by the TCR, and occurs in two waves, the first from fusion of vesicles containing preformed FasL, and the second from newly synthesised FasL at two hours (He *et al.*, 2010). Mutations within Fas cause autoimmune lymphoproliferative syndrome (ALPS) (Oliveira *et al.*, 2010). Other death receptors, also members of the tumour necrosis factor (TNF) receptor family, expressed by CD8+ T-cells include TNF-related apoptosis inducing ligand (TRAIL) (also known as TNFSF10) and TNF.

1.8.4 Tumour Necrosis Factor alpha

TNFα is a pleiotropic cytokine that contributes to defence against viruses and tumour growth. There are two forms of TNF, homotrimeric membrane protein (mTNF) that can be proteolytically cleaved by TNF alpha converting enzyme (TACE) to release the second form, a soluble homotrimer (sTNF), which rapidly dissociates into inactive 17 kDa monomers (Wajant *et al.*, 2003). There are two TNF receptors, TNF-R1 (also known as TNFRSF1A; CD120a; p55/60) and TNF-R2 (also known as TNFRSF1B; CD120b; p75/80). Most cells constitutively express TNF-R1, but lymphoid cells inducibly express TNR-R2. TNF-R1 and TNF-R2 can be cleaved to soluble forms that neutralise TNF activity and engineered TNF inhibitors formed from TNF-R2:dimeric IgG fusion proteins are used clinically (Etanercept) (Taylor, 2001; Wallach *et al.*, 1991).

TNF-R1 has a cytoplasmic death-domain (DD) and TNF-R2 is a member of the TNF receptor associated factor (TRAF)-interacting receptors. TNF-R1 interacts with DD containing proteins to activate caspases and induce apoptosis, and non-directly recruits TRAF2 that alters gene expression (Figure 1.3). As in TCR signalling, TNF can regulate transcription factors NF-κB, JNK, AP-1 and p38 MAPK. TNF-R2

signals via TRAF2 but can also induce apoptosis by driving endogenous mTNF expression, which activates TNF-R1 mediated apoptosis (Figure 1.4) (Wu and Zhou, 2010) (Bigda *et al.*, 1994; Grell *et al.*, 1993; Grell *et al.*, 1999). The action of TNF on TNF-R1 to induce apoptosis is enhanced by TNF-R2 signalling and requires TNF-R2 induced recruitment and proteasomal degradation of TRAF2 to prevent expression of anti-apoptotic factors (Wajant *et al.*, 2003) (Li *et al.*, 2002).

TNF is induced in macrophages in response to lipopolysaccharide, and by CD8+ T-cells on activation by high antigen density-pMHC complexes (Almeida *et al.*, 2009). TNFa transcription is controlled by the TNFα enhanceosome, a unit of transcription factors and co-activating proteins that binds a highly conserved 200 bp core promoter region (Falvo *et al.*, 2010). The distal promoter extends 1.5 kb upstream of the transcription start site and encodes a SNP that modulates the transcription level (Abdou *et al.*, 2010). TNFα was first described for its cytotoxic action against tumours and viruses (Sugarman *et al.*, 1985) (Biswas *et al.*, 1992). However, TNFα induces NF-κB that binds to the virus long terminal repeat to promote HIV-1 transcription and HIV-1 proteins Nef, Vpr and Tat imitate the action of TNFα signalling to enhance HIV-1 replication (Duh *et al.*, 1989) (Herbein and Khan, 2008) (Chan and Greene, 2012). It remains debated whether TNFα controls or enhances HIV-1 replication because its function is dictated by the timing, duration, concentration, cellular context and tissue compartment that TNFα acts within.

Figure 1-3: Down-stream TNFα signalling pathways

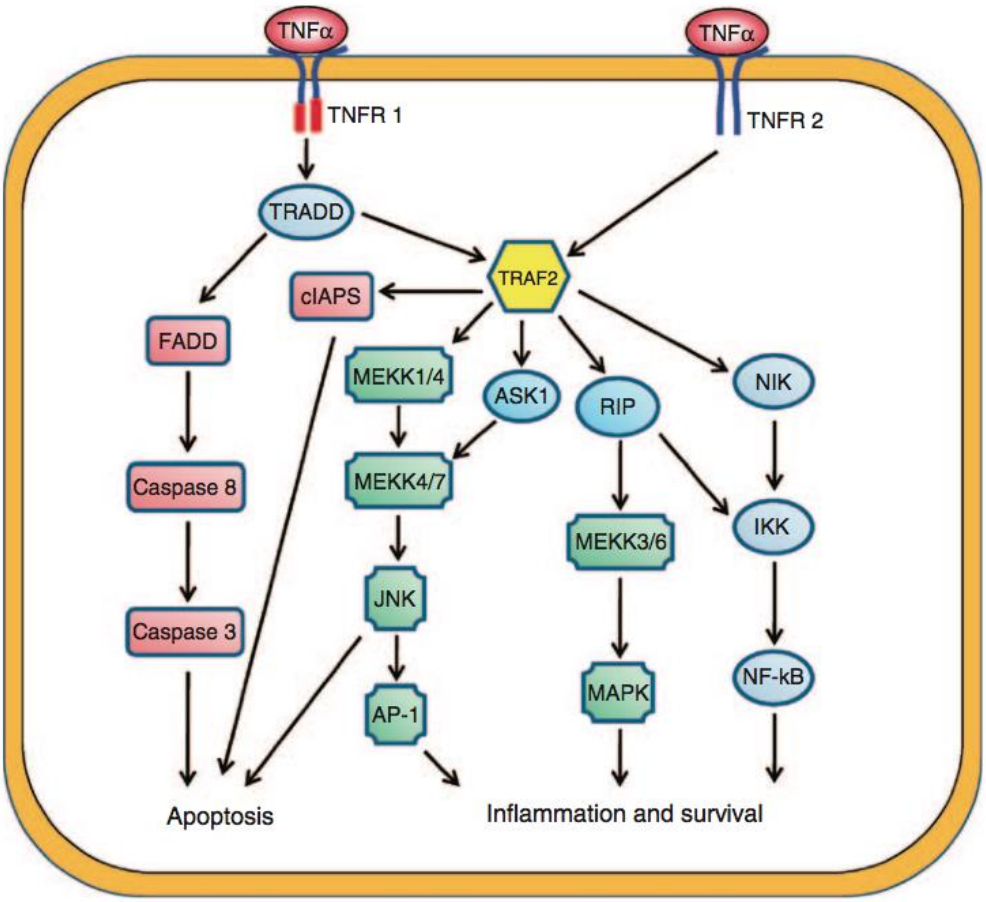


Figure 1.3: Down-stream TNFα signalling pathways. TNF-α can induce signalling pathways to induce apoptosis, cell survival or inflammation (Wu and Zhou, 2010). Apoptosis is induced by TNF-Receptor-1 Associated Death Domain (TRADD) recruitment of Fas-associated death domain (FADD) that leads to the activation of Caspase 8 and Caspase 3. TNF-receptor associated factor 2 (TRAF2) activates C-Jun-N-terminal kinase (JNK), AP-1, p38 MAPK, NF-kB and cIAPS, inhibitor of apoptosis protein. cIAPs promotes inactivation of caspases and proteasomal degradation of TRAF2. TRAF2, RIP and IKK form a signalling complex. RIP activates IKK that phosphorylates inhibitor of kappa beta (I-κβ) (not shown), targeting proteasomal degradation of I-κβ and exposure of nuclear localisation motifs on NF-κβ. MEKK, Mitogen activated protein kinase/Erk kinase kinase; RIP, receptor interacting protein; ASK1, apoptosis signal related kinase 1; NIK, NF-κβ inducing kinase; IKK, IkB kinase; mitogen activated protein kinase (MAPK).

Figure 1-4: TNF-R2 induced apoptosis

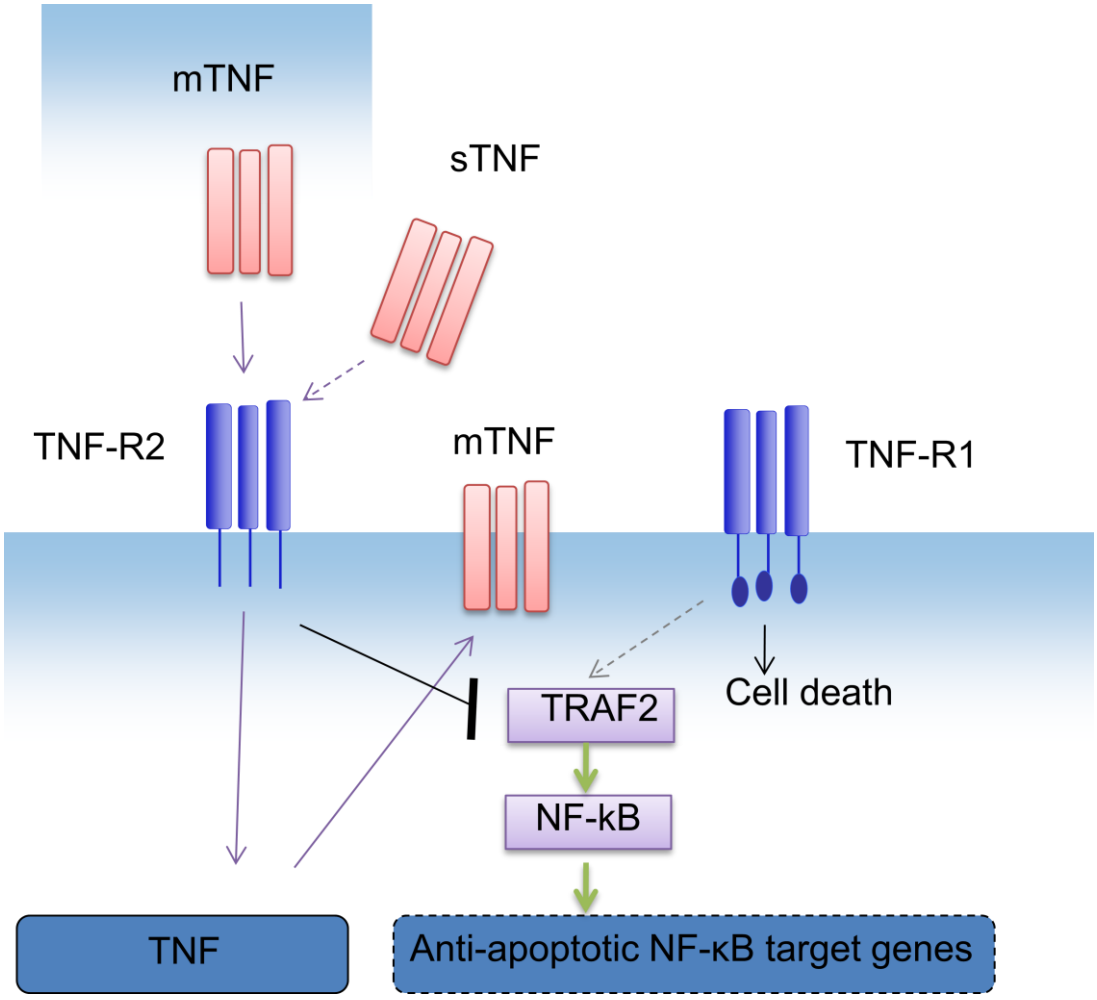


Figure 4: TNF-R2 induced apoptosis. TNF-R2 is activated by membrane TNF and weakly by soluble TNF, and recruits and degrades TRAF2 and induces expression of mTNF. Newly synthesised mTNF is expressed and binds TNF-R1 in an autocrine or paracrine pathway that induces apoptosis. This is enhanced as TNF-R2 inhibits TRAF2 mediated NK-κB activation of anti-apoptotic target genes by TNF-R1. Adapted from (Wajant *et al.*, 2003).

1.8.5 IFN gamma

Interferon gamma (IFNγ) is the only type II interferon, and was first discovered by its ability to interfere with viral replication (Isaacs and Lindenmann, 1957). IFNγ is a homodimer in solution and mediates its biological effects by binding to the IFNγ receptor, a heterodimer composed of IFNGR1 and IFNGR2 (Schroder *et al.*, 2004). This signals via the Janus tyrosine kinase- signal transducer and activator of transcription 1 (JAK-STAT 1) pathway. IFNγ has direct non-cytopathic anti-viral effects and is required for to clear measles virus infection from neurons (Stubblefield Park *et al.*, 2011). Activation of the JAK-STAT pathway induces expression of genes that regulate antigen processing, (for example sub-units of the immunoproteasome, PA28, MHC class I alleles, and TAP), and intrinsic retroviral defense factors (eg TRIM5α, APOBEC and Tetherin (See below)) (Schroder *et al.*, 2004). HIV-1 infected cells induce IFNγ, in addition to TNFα, which like TNFα, has been reported to enhance HIV-1 replication *in vitro* (Vyakarnam *et al.*, 1990) (Poli and Fauci, 1992).

1.9 T-cell ontogeny

The earliest precursor T lymphocytes are bone marrow haematopoietic stem cell derived progenitors that migrate to the thymus and commit to T cell differentiation following Notch 1-Notch ligation. These first T-cell progenitors do not express the co-receptors CD4 or CD8, “double negative” (DN) thymocytes, and migrate to the outer cortex while simultaneously rearranging the TCR beta, gamma and delta chains. Successful expression of a β-chain with the pre-T cell receptor alpha chain (pTα) commits lineage differentiation to an αβ T cell rather than a γδ T cell, and rescues the thymocyte from apoptosis, known as β-selection (von Boehmer *et al.*, 1998). The expression of the pre-TCR is sufficient to initiate constitutive signalling without a ligand, which triggers cell cycle entry, and allelic exclusion of the β-locus, preventing

further β chain rearrangement. Thus all T-cells have a unique TCR β chain. After proliferation, CD4 and CD8 are both expressed and "double positive" thymocytes start to rearrange the alpha chain locus (Kreslavsky *et al.*, 2012). If successful, DP thymocytes express the TCR at low level on the cell surface. A single β chain can therefore associate with several different α chains in distinct progeny cells. Allelic exclusion does not occur at the *TRA* locus, and a proportion of T-cells will express both *TRA* genes (Padovan *et al.*, 1993).

The mature TCR will dictate a preference for binding to class I or class II MHC molecules. This preference determines co-receptor expression and dictates lineage differentiation into single positive cells, either CD8+ cytotoxic T-cells or CD4+ helper or regulatory T-cell lineage (Germain, 2002). The TCR is extensively tested and selected to enable recognition of self-MHC molecules but tolerate self- antigen (Zinkernagel *et al.*, 1978). This process is known as positive and negative selection respectively. A critical parameter may be the TCR signal strength, known as the "differential avidity model" of T cell selection (Ashton-Rickardt *et al.*, 1994). If a TCR signal threshold is surpassed, apoptosis may be induced, or negative selection to prevent autoimmunity. A TCR signal below this threshold may promote survival, known as positive selection. The lower affinity range is set by the minimum TCR signal required to rescue the thymocyte from death by neglect. Positive and negative selection occur following exposure to several types of antigen presenting cells (Starr *et al.*, 2003). Thymic cortical epithelial cells contribute to positive selection. Bone marrow derived macrophages and dendritic cells are particularly effective at mediating negative selection. DCs and macrophages migrate to the thymus from the tissues, where they naturally sample and present host antigen. In addition, thymic medullary stromal cells express the gene *AIRE*, which encodes autoimmune regulator,

which elongates transcripts of tissue-restricted proteins (Anderson *et al.*, 2002). The MHC genotype modulates the TCR repertoire and may impact viral control, for example HLA B*57 may promote positive selection of HLA B*57-restricted TCRs that then form a larger fraction of the naïve T-cell repertoire, and are more cross-reactive (Kosmrlj *et al.*, 2010). Polymorphisms within self-proteins or the expression of human endogenous retro-viral elements may also modulate the naïve T-cell repertoire, as reported in the mouse (Young *et al.*, 2012). Naïve T cells exit the thymus and re-circulate through the bloodstream and peripheral lymphoid tissue.

1.9.1 Co-stimulatory and cytokine signals

Naïve CD8+ T-cells are primed by encounter with mature dendritic cells that display the appropriate epitope-MHC class I complex in the peripheral lymphoid tissue (Bousso, 2008). The CD8+ T-cell signal for activation is integrated from three signals, signal 1: the TCR-epitope-MHC class I complex, signal 2: co-stimulatory (or inhibitory) receptors and signal 3: cytokine receptors. CD28 is the best characterised co-stimulatory molecule, a member of the immunoglobulin superfamily of receptors that is already expressed on naïve T-cells. Ligation of CD80 and CD86, expressed on mature antigen presenting cells, promotes T cell proliferation, cytokine production, cell survival and cell metabolism (Acuto and Michel, 2003). Ligation of CD28 activates phosphatidyl inositol 3 kinase (PI3K) leading to activation of Akt (Smith-Garvin *et al.*, 2009). CD28 ligation promotes expression of the high affinity IL-2 receptor α chain (CD25), prolonged activation of transcription factors NFAT, AP-1 and NK-κB, and enhances IL-2 transcription and mRNA stability. IL-2 production is required to prevent anergy and drive proliferation. CTLA-4 is an inhibitory member of the CD28 family of receptors, which binds CD80 and CD86 with higher avidity than CD28, and acts to limit proliferation. The TNF family of receptors also deliver

co-stimulatory signals, including CD27 (ligand CD70), 4-1BB (CD137) (ligand 4-1BBL), OX-40 (OX40L) and CD40 (CD40L). CD27, OX40 and 4-1BBL ligation leads to activation of PI3K/Akt, NF-κB, JNK and p38 MAPK via TRAF (Smith-Garvin *et al.*, 2009). The expression of co-stimulatory molecules not only regulate priming, but also have critical roles in modulating CD8+ T-cell effector function (Nolte *et al.*, 2009).

1.9.2 Priming the HIV-1 specific CD8+ T-cell response

Conventional DC (cDC), prime HIV-1 specific CD8+ T-cells although the expression of co-stimulatory molecules and IL-12 production is impaired in acute HIV-1 infection (McMichael *et al.*, 2010). The predominant mechanism of activating naïve CD8+ T-cells in HIV-1 infection is by cross-priming of viral antigens by uninfected cDCs, where the innate sensors for HIV-1 remain undefined (Iwasaki, 2012). pDC are also activated by HIV-1, by pathways that converge on Toll like receptor 7, and produce Type I interferons (IFNα and IFNβ) (Beignon *et al.*, 2005; Iwasaki, 2012).

1.9.3 Effector and Memory CD8+ T-Cell Differentiation

HIV-1 infection triggers expansion of effector CD8+ T-cells, and although the antigen exposure is persistent, development of memory CD8+ T-cells. There is incredible heterogeneity in the HIV-1 CD8+ T-cell population in terms of effector function, gene and protein expression, migratory patterns, proliferative capacity and long-term fate (Kaech and Cui, 2012). There are at least three memory T-cell sub-sets, defined by phenotypic markers, anatomical location and function (Sallusto *et al.*, 1999). T central memory (T_{CM}) cells are defined by CD62Lhi, CCR7hi phenotype, migrate through the secondary lymphoid organs and maintain the capacity to produce IL-2 and proliferate. T effector memory (T_{EM}) cells are defined by CD62Llow, CCR7low

phenotype, migrate in non-lymphoid tissue and release cytotoxic proteins. Tissue-resident memory T (T_{RM}) cells defined by CD103hi, CD69hi, CD27low, GZMB hi phenotype, reside in the brain and mucosal tissues, and express tissue specific homing markers for example $\alpha_4\beta_7$ and CCR9 for the GALT (Masopust *et al.*, 2010).

It is known that a single naïve CD8+ T-cell can give rise to diverse effector and memory populations (Stemberger *et al.*, 2007) (Gerlach *et al.*, 2010). This has led to several models to explain how effector and memory differentiation arise (Kaech and Cui, 2012). Repetitive encounter with antigen can augment the effector properties of memory CD8+ T-cells and increase the frequency of T_{EM} (Nolz and Harty, 2011) (Badovinac *et al.*, 2005) (D'Souza and Hedrick, 2006). The decreasing-potential model suggests that recurrent stimulation with antigen and pro-inflammatory cytokines can drive proliferation of the activated T-cells and differentiation into terminal effectors that have enhanced cytolytic capacity but lose memory cell properties of longevity and proliferative capacity. The signal strength model suggests that the composite signal (1: antigen; 2: co-stimulatory receptors and 3:cytokines) received during priming specifies different cell fates early in the viral infection (Lanzavecchia and Sallusto, 2002). The asymmetric cell fate model proposes that signal strength can determine effector/memory differentiation by repetitive encounter with antigen, due to asymmetric division of a single naïve T-cell (Chang *et al.*, 2007). The daughter cell in close contact with the APC is proposed to inherit the immune synapse and be exposed to more IFNγ and IL-12, leading to a stronger TCR and co-stimulation signal that drives differentiation signals and leads to terminal differentiation. The distal daughter cell is preserved in a less differentiated state due to a limited capacity for TCR signal transduction, maintains memory cell potential and may even have stem cell like qualities (Kaech and Cui, 2012). Early migratory

patterns of activated T cells may also influence their exposure to pro-inflammatory cytokines, particularly type I interferons, IL-2, IL-12, IL-27 and IL-33, that enhance proliferation, differentiation and CTL survival (Obar *et al.*, 2011) (Mescher *et al.*, 2006) (Pipkin *et al.*, 2010). Chemokine receptors that mediate cellular location are crucial in determining cellular differentiation, for example the sub-set of CD4+ T-cells that express CXCR5 traffic to B cell follicles and develop into T follicular helper cells (Kaech and Cui, 2012).

1.9.4 "Exhaustion"

Repetitive encounter with antigen can alter the function and gene expression of virus specific CD8+ T-cells (Kaech and Wherry, 2007). In chronic viral infection, maintenance of viral specific CD8 T-cells is less dependent on cytokines IL-7 and IL-15, and more dependent on antigen (Shin *et al.*, 2007). Hierarchical loss of IL-2, TNF, IFNγ, impaired proliferation and decreased cytotoxicity can occur with repetitive antigen exposure (Shin and Wherry, 2007). Loss of function can be associated with expression of inhibitory receptors, programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG3), CD244 and CD160, resulting is a state of dysregulated T-cell signalling that has been called "exhaustion" (Day *et al.*, 2006) (Trautmann *et al.*, 2006) (Blackburn *et al.*, 2009). Exhausted CD8+ T-cells have a distinct transcriptional state from effector and memory CD8+ T cells, characterised by B lymphocyte-induced maturation protein 1 (BLIMP1) that promotes expression of inhibitory receptors, loss of T-bet that leads to upregulation of PD-1, and PD-1 induced expression of basic leucine zipper transcriptional factor ATF-like (BATF) (Shin *et al.*, 2009) (Quigley *et al.*, 2010) (Kao *et al.*, 2011). Repetitive stimulation of CD8+ T-cells by antigen does not lead to the expression of phenotypic markers of exhaustion (Wirth *et al.*, 2010) (Kaech and Cui, 2012).

1.10 AIDS

In 1981 the first cases of generalised lymphadenopathy, opportunistic pathogen infections and malignancies, Kaposi sarcoma and unusual B-cell lymphomas, were reported in previously healthy individuals who had acquired low CD4+ cell counts. The causative pathogen of acquired immunodeficiency syndrome (AIDS) was first isolated in 1983 (Barre-Sinoussi *et al.*, 1983) (Gallo *et al.*, 1983) and later named human immunodeficiency virus (HIV)-1 following the isolation of a related retrovirus from patients with AIDs in Western Africa, HIV-2 (Clavel *et al.*, 1986). Retroviruses were also isolated from non-human primates, and named simian immunodeficiency virus (SIV), as although the natural primate host is usually asymptomatic, inoculation into a different primate species caused an AIDs-like syndrome (Letvin *et al.*, 1985; Beaumier *et al.*, 2009). Inoculation of SIV_{mac}, a strain of SIV from sooty mangabeys that circulated in African macaques in captivity, into Asian macaques (rhesus monkeys) is one of the most useful animal models for HIV-1.

1.10.1 The HIV-1 pandemic

It is estimated that 33.3 million people are living with HIV-1 infection, most of whom, 22.5 million, are in Sub-Saharan Africa (UNAIDS, 2010). 1.8 million new infections were reported in Sub-Saharan Africa in 2009, and 41% were in 15 to 24 year olds. The prevalence of HIV-1 infection in girls aged 21 yrs increased from 4% at 16 yrs to 31% in 2005, highlighting the predominant route of infection by sexual transmission (Pettifor *et al.*, 2005). It is estimated that of the 15 million people who require treatment, 5.2 million have access. These figures indicate the importance of developing a vaccine but the greatest challenge to vaccine development is the extraordinary genetic diversity of HIV-1.

1.10.2 Origin of the HIV-1 pandemic and genetic diversity

The HIV-1 pandemic may have ignited from a single transmission of SIV in chimpanzees (*Pan troglodytes troglodytes*) (SIV_{cpz}) to humans in Central Africa (Gao *et al.*, 1999) estimated to have occurred between 1908-1941 (Korber *et al.*, 2000) (Worobey *et al.*, 2008). This correlates with the earliest isolated sequence identified in a serum sample stored in Kinshasa, Democratic Republic of Congo in 1956 (Zhu *et al.*, 1998). It was soon appreciated that no two HIV-1 viruses isolated shared the same sequence, and numerous viral variants termed "quasi-species" could be isolated even from one individual (Meyerhans *et al.*, 1989). This extraordinary genetic diversity results from reverse transcription errors (Roberts *et al.*, 1988), mutations induced by host restriction factors (Harris *et al.*, 2003), and is amplified by the prolific rates of virion production per individual (Coffin, 1995; Wei *et al.*, 1995) (Ho *et al.*, 1995). In addition, template switching between the two RNA genomes in each virion creates circulating recombinant forms (CRF) (Robertson *et al.*, 1995). The ability to escape detection from the immune system by variation in the coding sequence leading to adaptation to the human population is the greatest challenge to vaccine design (Barouch *et al.*, 2005) (Kiepiela *et al.*, 2004). Phylogenetic analysis clusters HIV-1 isolates into four groups, main (M), outlier (O), new (N) and P (http://www.hiv.lanl.gov/). Most isolates of the global pandemic are from group M and are classified into at least 11 clades (A1, A2, B, C, D, F1, F2, G, H, J and K). Inter-clade and intra-clade amino acid sequence diversity averaged 17-35%, and 8-17% in 2001, and these distances continue to increase and evolve over time (Korber *et al.*, 2001; Rambaut *et al.*, 2001) (Figure 1.5).

Figure 1-5: HIV-1 Pandemic

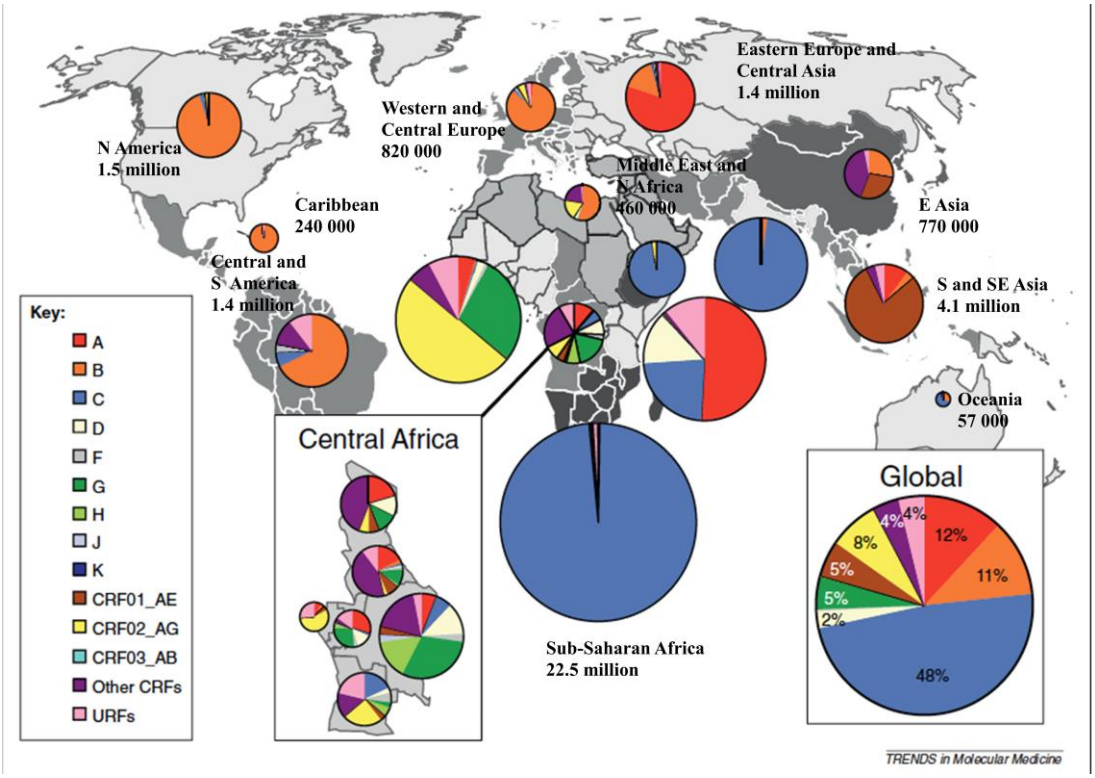


Figure 1.5: HIV-1 pandemic. Estimated number of individuals living with HIV-1 by region, with pie charts illustrating the proportion of infections caused by each clade and CRF. Countries forming each region are shaded on the map. The pie chart size represents the estimated number of individuals living with HIV-1 2004-2007 in each region, with the statistics for 2009 in text (UNAIDS 2010). Each pie chart segment represents a Group M clade or CRF as listed in the left hand panel. CRF, circulating recombinant form. URF,unclassifiable recombinant form. Adapted from (Hemelaar, 2012).

Dual infections, inter-clade and intra-clade from co-infection and super-infection, are reported in 10-20% of infected individuals in Africa (Powell *et al.*, 2009) (Gottlieb *et al.*, 2004). There are at least 15 circulating recombinant forms (CRF) (01-15) worldwide. Clade C is dominant in the epicentre, Sub-Saharan Africa, and accounts for the majority of individuals infected, but clade B predominates in North America and Europe and has been most studied (Figure 1-5). Three other groups of HIV-1 infect fewer individuals, Group N (new) from a separate SIV_{CPZ} transmission in Cameroon, Group P, arising from transmission of SIV gorilla (SIV_{GOR}), and Group O (outlier) in West Central Africa (Keele *et al.*, 2006; Plantier *et al.*, 2009) (Van Heuverswyn *et al.*, 2006).

1.11 HIV-1 structure, proteins and genome

HIV-1 particles have reverse transcriptase activity, the defining quality of the family *Retroviridae,* and the distinctive conical capsid of the lentivirus genus (Barre-Sinoussi *et al.*, 1983) (Figure 1-6). Molecular cloning and sequencing of the 9.7 kilobase genome identified both typical lentivirus *gag, pol* and *env* genes, and distinct accessory genes *vif, vpr, vpu, tat, rev* and *nef* in three open reading frames (Ratner *et al.*, 1985). *Gag* is transcribed as a 55-kd Gag precursor polypeptide that is cleaved into four proteins in the mature virion, p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6 protein. *Pol* encodes three enzymes, protease (Pro), reverse transcriptase (RT) and integrase (IN). The 160-kd glycosylated Env precursor is cleaved into gp120 surface (SU) and gp41 transmembrane (TM) proteins. Tat and rev are essential viral regulatory factors, and Vif, Vpr, Vpu and Nef are viral accessory proteins. Vpu is unique to HIV-1 (Table 1-1).

Table 1-1: Genes and proteins encoded within the HIV-1 genome

Gene	Protein	Function
tat	Transactivator of HIV gene expression (Tat)	Transcription factor binds to transactivation response region (TAR) RNA element to activate initiation and elongation of the viral genome
rev	Regulator of expression of viral proteins (Rev)	Transports unspliced mRNA from the nucleus to the cytoplasm by binding to the rev response element (RRE)
nef	Negative factor (Nef)	Enhances viral infectivity Downregulates MHC class I: HLA-A and –B, but not –C or -E Promotes cellular activation Inhibits Ig isotype switching in B cells
gag	Group associated antigen (Gag) P17 Matrix P24 Capsid P7 Nucleocapsid P6	Pr55 Gag precursor, assembling, orchestrates virion assembly Structural protein of the outer shell, attaches to lipid bilayer Highly conserved structural protein of the capsid RNA encapsidation, packaging and reverse transcription initiation Incorporation of Vpr into the virion, essential for budding and release
pol	Polymerase (Pol) Viral protease (Pro) Reverse transcriptase (RT) Viral integrase (IN)	160 kD gag-pol polyprotein Cleaves viral precursor proteins Reverse transcription of viral RNA genome Integration of DNA provirus into the host genome
env	Envelope (Env) gp 120 surface (SU) gp 41 transmembrane (TM)	Glycosylated 160 kD Env precursor (gp160) Trimer binds CD4 and chemokine co-receptor for viral entry Trimer mediates fusion of the viral envelope and plasma membrane
vif	Viral infectivity factor (Vif)	Prevents action of apoplipoprotein B mRNA editing , enzyme-catalytic, polypeptide- like (APOBEC)
vpr	Viral protein R (Vpr)	Promotes cell cycle arrest
vpu	Viral protein U (Vpu)	Degradation of CD4, Counteracts tetherin

Figure 1-6: The HIV-1 Virion and Genome

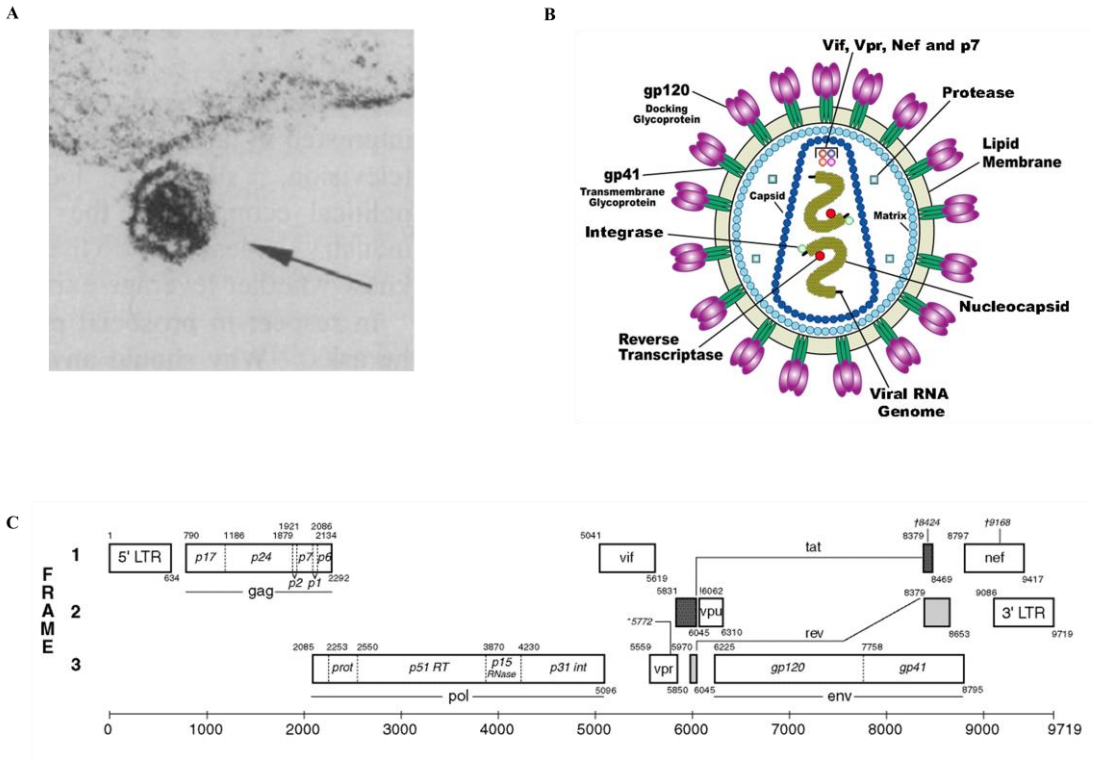


Figure 1.6: HIV-1 Virion and Genome: A) The first electromagnetic (EM) pictures of an HIV-1 virion isolated from an asymptomatic patient with lymphadenopathy (Barre Sinoussi 1983, Marx JL, 1993) B) The HIV-1 virion, illustrating the constituent proteins (www.niaid.nih.gov/) and two RNA genomes. C) Landmarks of the HIV-1 genome, HXB2 strain. Open reading frames are indicated by rectangles, numbers in the top left corner mark the position of the start codon, and numbers in the bottom right corner indicate the last position of the stop codon. (www.hiv.lanl.gov)

1.12 The HIV-1 lifecycle, host restriction factors and anti-retroviral therapy

The HIV-1 lifecycle is unique to the Retroviridae family and is illustrated with the sites of action of selected host restriction factors and anti-retroviral drugs (Figure 1-7) (Table 1-2).

Table 1-2: HIV-1 Anti-Retroviral Therapy

Target	**Antiretroviral Class (Abbreviation)**	**Generic Name**
Reverse transcription (RT)	Nucleoside analogue reverse transcriptase inhibitors (NRTI)	Abacavir,Emtricitabine, Didanosine, Lamivudine, Stavudine, Zidovudine
	Nucleotide analogue reverse transcriptase inhibitor (NtRTI)	Tenofovir
	Non-nucleoside reverse transcriptase inhibitor (NNRTI)	Rilpivirine, Delavirdine, Efavirenz, Nevirapine, Eltravirine
Viral protease	Protease inhibitor	Atazanavir, Darunavir, Indinavir, Nelfinavir, Saquinavir, Ritonavir
Viral integrase	Integrase inhibitor	Raltegravir
Gag p24	Maturation inhibitor	Interferon alpha, Bevirimat, Vivecon
CCR5	Entry inhibitor	Maraviroc
Gp41 fusion peptide	Fusion inhibitor	Enfuviritide (T-20)
Histone deacetylase (HDAC) inhibitor	Latent provirus activation	Vorinostat

Figure 1-7: The HIV-1 Life Cycle, Host Restriction Factors and Anti-Retroviral Drug Targets

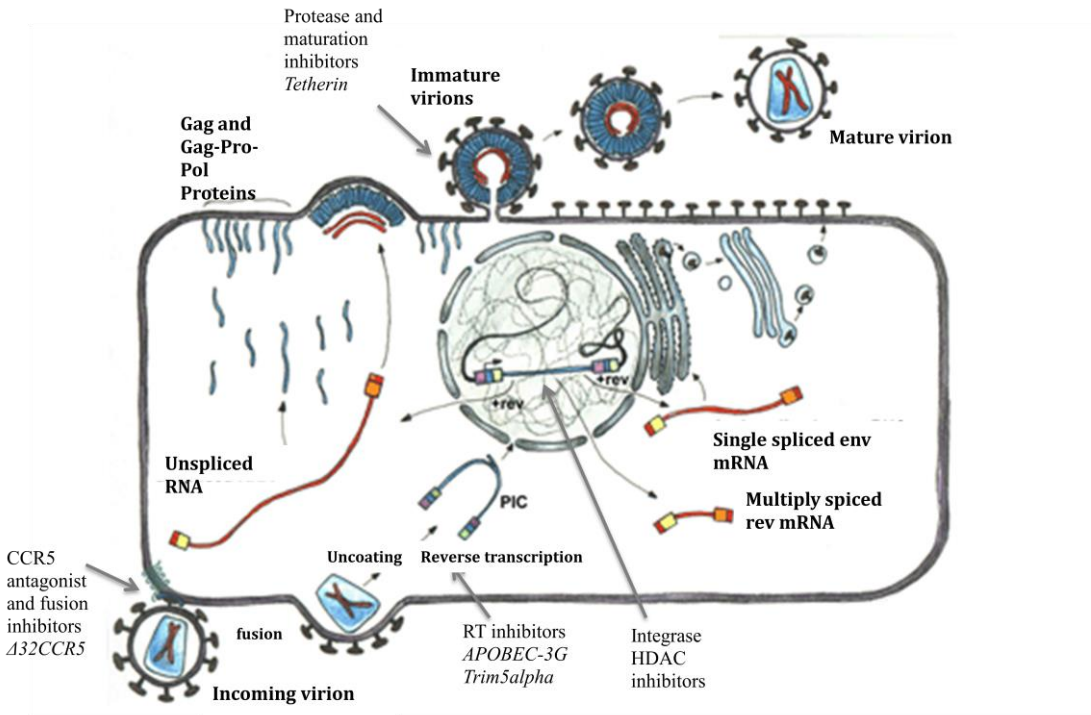


Figure 1.7: The HIV-1 Life Cycle and sites of action of host restriction factors (italics) and anti-retroviral drugs. HIV-1 binds CD4 and a chemokine receptor CCR5 or CXCR4, to fuse with the cell membrane and release the core into the cytoplasm. This is the site of action of entry and fusion inhibitors, and a protective 32 bp deletion in CCR5. Uncoating releases the RNA genome for reverse transcription, and is vulnerable to apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (APOBEC)-3G, Trim5α and reverse transcription (RT) inhibitors. The preintegration complex (PIC) is imported to the nucleus for integration into the host chromosome, targeted by integrase inhibitors. The provirus may remain latent or be actively transcribed, a target of HDAC inhibitors, into mRNAs that are spliced and exported for translation of tat, rev and nef. Rev enables the nuclear export of partially spliced and unspliced (genomic) mRNAs. Genomic RNA is encapsidated, and assembled during budding. Virion release is countered by tetherin. Particle maturation is targeted by protease and maturation inhibitors. Adapted from (Dyana Terri Saenz, www.mayoresearch.edu).

HIV-1 cellular tropism is determined by CD4+ and chemokine co-receptor, CCR5 or CXCR4, binding sites in the envelope trimeric glycoprotein, gp120 (SU) (Dalgleish *et al.*, 1984; Alkhatib *et al.*, 1996) (Choe *et al.*, 1996) (Feng *et al.*, 1996) (Hu *et al.*, 2004). The majority of CCR5+CD4+ T-cells are activated effector memory CD4+ T-cells. Individuals with a homozygous or heterozygous 32 base pair deletion (Δ32) in CCR5 are protected from HIV-1 transmission and progression respectively, and CCR5 antagonists are a new class of anti-retroviral (Liu *et al.*, 1996) (Dean *et al.*, 1996a) (Dean *et al.*, 1996b) (Walker *et al.*, 2005). Gp120 binding to CD4 leads to the dissociation of transmembrane gp41 (TM) and exposes a hydrophobic fusion peptide, which melds the viral envelope with the plasma membrane to insert the core into the cytoplasm (Freed *et al.*, 1990; Kilby *et al.*, 1998). Viral uncoating exposes the RNA genome, and may be disrupted by a host factor, TRIM5α (Stremlau, 2007). The RNA genome is used as a template to transcribe DNA and then double-stranded DNA from the RNA:DNA heteroduplex, by the enzyme reverse transcriptase. The host restriction factor apolipoprotein B mRNA-editing enzyme, catalytic polypeptide (APOBEC) -3G and -3F are incorporated into the infecting virion, and deaminate cytosines to uracil during first strand DNA synthesis, causing G to A hypermutation, increased stop codons and defective viral progeny (Sheehy *et al.*, 2002; Harris *et al.*, 2003). Reverse transcriptase was the target of the first anti-retroviral, zidovudine, (Yarchoan *et al.*, 1986; Weissman, 1987), and the first of the class of nucleoside and nucleotide inhibitors (NRTI). Non-nucleoside RT inhibitors (NNRTI) are allosteric inhibitors of the RT active site (Merluzzi *et al.*, 1990) (Esnouf *et al.*, 1995). Combination therapy of a "cocktail" of three drugs, including an NRTI, NNRTI and protease inhibitor, revolutionised HIV-1 treatment nearly a decade later, and became

known as highly active anti-retroviral therapy (Hammer *et al.*, 1997) (Ho *et al.*, 1995) (Perelson *et al.*, 1997; Richman, 1996). The preintegration complex is transported to the nucleus in resting cells, and inserted into host DNA by viral integrase. This is the site of action viral integrase inhibitors (Cahn and Sued, 2007) (Grinsztejn *et al.*, 2007). The provirus may remain latent, or may be actively transcribed to produce viral progeny. Histone deactylase inhibitors (HDAC) are a new class of drugs that activate latently infected cells (Archin *et al.*, 2012). Active transcription requires cellular activation and the induction of transcription factor nuclear factor-kappa B (NF-κB) (Nabel and Baltimore, 1987). Initial transcription in the early stage of viral replication is slow, and mRNA is multiply spliced prior to nuclear export and translation of the early proteins Tat, Rev and Nef. Nef mRNA accounts for 80% of viral mRNA at 8-12 hrs following infection (Klotman *et al.*, 1991). As Rev accumulates, partially spliced and unspliced (genomic) mRNA transcripts are exported from the nucleus. These are translated into late gene products, the precursor polyproteins of env, gag and gag-pol. The gag-pol precursor polyprotein is translated from genomic mRNA by a -1 ribosomal frame shift at a hepatmeric (U) "slippery sequence" in gag that occurs in a ratio of 1:20 (Tsomides *et al.*, 1994) (Shehu-Xhilaga *et al.*, 2001). Gag P55 precursor protein, assemblin, orchestrates virion packaging and budding and is cleaved during particle maturation into the structural proteins by viral protease to create the infective virus.

1.13 Structural and biological factors that contribute to the HIV-1 CD8+ T-cell response

Gag (495 αα), Pol (1001αα) and Nef (206αα) are the most immunogenic antigens for cell-mediated immunity (Goulder and Watkins, 2008). Cells infected with virus are recognised by CD8+ CTL by the presentation of short 8-11 αα residue peptides by MHC class I molecules. CD8+ CTL responses occur within a dynamic range that can be triggered by different p-MHC densities (Valitutti *et al.*, 1996). If an infected cell can be recognised and suppressed by a CD8+ T-cell prior to the release of new virions, the infectious cycle will be broken. A conserved epitope sequence, timing and abundance of peptide-MHC I expression is therefore likely to be crucial to triggering an effective CTL response. Each mature virion has 1000 to 1500 capsid (p24) molecules (Briggs *et al.*, 2004), 50 to 75 polymerase molecules and 60 to 200 Nef molecules (Freed and Martin, 2001). Proteins derived from the infecting virion, particularly Gag, may be rapidly presented (Sacha *et al.*, 2007) (Sacha *et al.*, 2008) (Payne *et al.*, 2010). Antigen presentation is a controversial area, but evidence now supports that some antigenic peptides are derived from newly synthesised polypeptides, known as DRiPs (defective ribosomal proteins) (Yewdell and Haeryfar, 2005). 40% of Gag was degraded within minutes of synthesis in one study (Schubert *et al.*, 2000). This finding links the translation of neo-antigens to rapid presentation favouring the presentation of viral epitopes, particularly proteins synthesised early in the life cycle, over more abundant but stable host proteins.

The extensive sequence diversity of HIV-1 variants is more conserved in some viral antigens than in others. Gag is one of the most conserved antigens, both between clades and within clades (Korber *et al.*, 2001) and Gag-p24 Capsid is one of the most conserved of all the HIV-1 proteins (Goulder and Watkins, 2008). This high level of

conservation may be due to structural and functional constraints of the primary, secondary or tertiary protein structure. As sequence variation is a great challenge to vaccination, this is considered in the next section for a few of the CTL immunodominant epitopes derived from Gag and Nef.

1.13.1 Gag p24 Capsid

Capsid (Gag residues 133 to 363) forms the conical shell of the core, packaging the RNA viral genome. It consists of two alpha helical domains connected by a flexible linker. Uncoating and initiation of reverse transcriptase following cell entry is tightly regulated, and requires the binding of cyclophilin A (Cyp A) to the Cyp A binding loop of p24 located within the N-terminal alpha helical domain (Gag residues 133-283). Two protective HLA class I-alleles restrict CTL responses that target epitopes within the p24 capsid protein, KRWIILGLNK, (KK10), $gag_{(263-272),}$ restricted by HLA B*27(Nixon *et al.*, 1988), and three HLA B*57 restricted epitopes, TSTLQEQIGW (TW10) $gag_{(240-249)}$, KAFSPEVIPMF (KF11) $gag_{(162-172),}$ and ISPRTLNAW (IW9) $gag_{(147-155).}$ The TW10 epitope lies within the N-terminal domain and defines the start and stability of helix 6, a structural motif involved in polymerisation of capsid monomers and binding of cyclophilin A, and although viral variants that escape CTL detection are selected early in infection, there is an incurred fitness cost to the rate of viral replication (Martinez-Picado *et al.*, 2006) (Leslie *et al.*, 2004). KK10 also lies within the N-terminal domain, on helix 7, a region required for conformational multimerisation of the capsid (Schneidewind *et al.*, 2007). This epitope remains highly conserved and emergence of late escape variants has been associated with progression to AIDs (Goulder *et al.*, 2004).

1.13.2 Nef

Nef is a myristoylated 27-35 kDa phospho-protein, which has three major functions, the downregulation of cellular receptors, modulation of cellular activation pathways, and enhanced virulence. These functions have been mapped to several highly conserved motifs, the glycine residue (G_2) of the myristoylation site, a $PxxP_{75}$ motif that binds signalling molecules with an SH3 domain and is required for MHC I downmodulation, and an acidic $EEEE_{65}$ motif that mediates MHC class I downregulation (Arien and Verhasselt, 2008). Nef downregulates MHC class I molecules (Schwartz *et al.*, 1996). HLA-A and HLA-B complexes are preferentially targeted for degradation while expression of HLA-C complexes is maintained which may facilitate escape from both cytotoxic T cells and natural killer cells (Collins *et al.*, 1998) (Cohen *et al.*, 1999). Nef-deleted HIV-1 strains have been associated with delayed progression to AIDs (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995). The immunodominant CTL epitope FLKEKGGL, (FL8), Nef $_{(90\text{-}98)}$, restricted by HLA B*0801 is not within one of these highly conserved functional motifs, and yet the sequence is conserved across several consensus sequences for HIV-1 Group M clades promoting consideration of this region for inclusion into an HIV-1 vaccine (Korber *et al.*, 2001).

1.14 HIV-1 Infection

Most HIV-1 transmission occurs across the genital or rectal mucosa epithelium following sexual exposure (UNAIDS, 2010). The earliest transmission events are particularly challenging to study in vivo but may determine the course of infection and provide “a window of opportunity” to intervene by vaccination, as suggested from the Rhesus macaque model of SIV-1 transmission (Figure 1-8) (McMichael *et al.*, 2010) (Haase, 2010).

Figure 1-8: The Rhesus Macaque Model of SIV Transmission and Dissemination

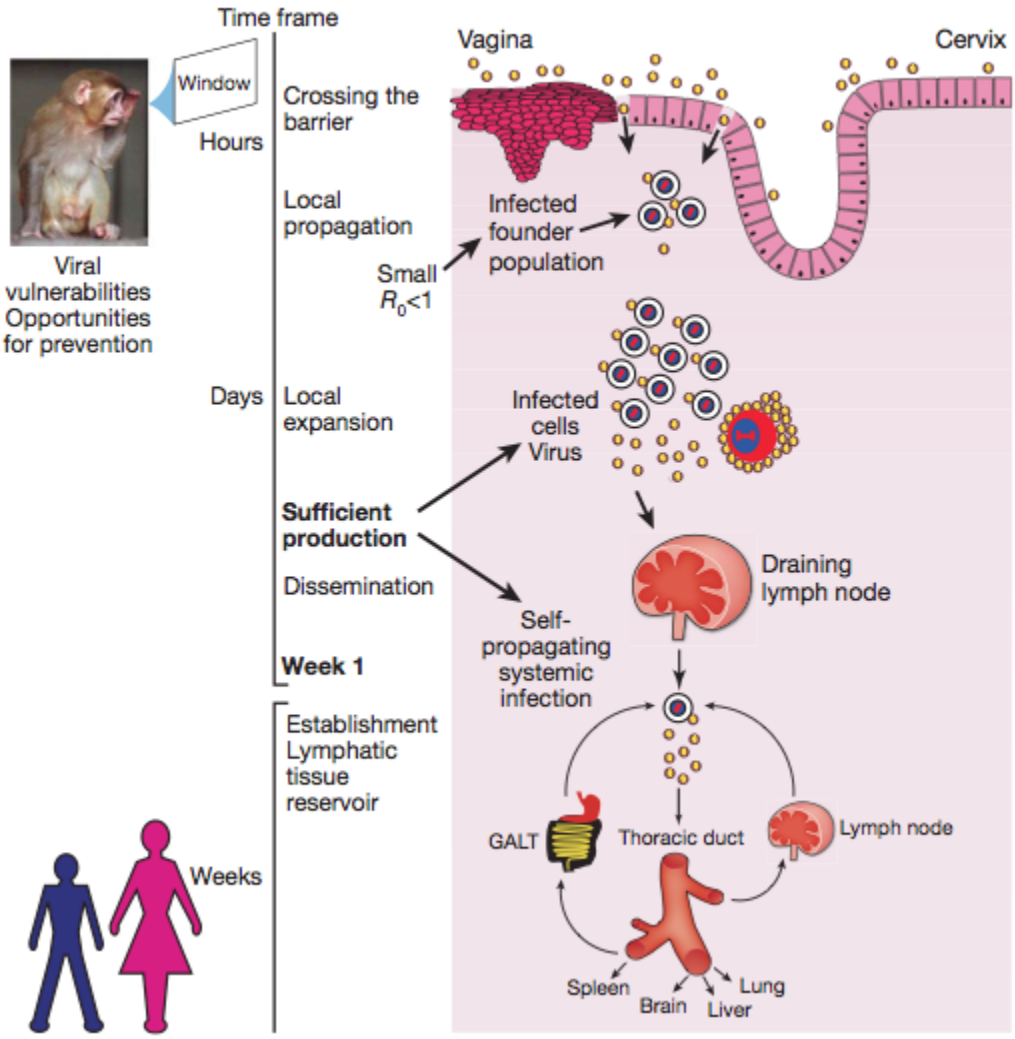


Figure 1.8: The Rhesus Macaque Model of SIV transmission and dissemination. The timeframe of transmission events of SIV are depicted in the time line on the left. The cervix may provide a route of entry for one or two SIV variants to cross the mucosal barrier and locally propagate prior to spread to the draining lymph node. Further propagation is followed by systemic seeding of lymphoid tissue, particularly the GALT, gut-associated lymphoid tissue, leading to very high systemic viral load, massive depletion of CD4+ T-cells and the establishment of a latent reservoir (Haase, 2010).

The mucosa of the genital tract is an effective barrier to sexual transmission, with relatively low rates of transmission from a single exposure (Royce *et al.*, 1997). Genome amplification and ultra-deep sequencing of the first viral isolates from plasma suggest that over 80% of infections arise from a single founder virus despite sexual exposure to a swarm of viruses (Keele *et al.*, 2008) (Fischer *et al.*, 2010). Rhesus macaques inoculated vaginally with high-dose SIV_{mac} first establish foci of HIV-1 replication in resting mucosal CD4+ T cells, prior to spread to activated CD4+CCR5+ T cells in the draining lymph node (Haase, 2010). These earliest foci of infection were particularly vulnerable to extinction and are proposed to be an important site to boost immune cells by vaccination (Reynolds *et al.*, 2008). Transmission to the draining node may be enhanced by HIV-1 binding to uninfected dendritic cells via mannose residues on gp120 to cell surface receptor DC specific ICAM3-grabbing non-integrin (DC-SIGN) (CD209) (Geijtenbeek *et al.*, 2000). The lymph node supports rapid viral replication and systemic spread via the thoracic duct to other sites of lymphoid tissue, particularly the gut-associated lymphoid tissue (GALT) where over 80% of the lamina propria CD4+ T cells are activated CCR5+ memory T cells (Mehandru *et al.*, 2004). Seeding the GALT fuels the exponential phase of viral replication that peaks within the first month of infection, and may establish a stable viral reservoir of latently infected "resting" CD4+ T-cells and intact HIV-1 virions in immune complexes on follicular dendritic cells (Chun *et al.*, 1997) (Dinoso *et al.*, 2009) (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). High viraemia can be accompanied by symptoms of acute HIV-1 infection, with fever, lymphadenopathy and skin rashes but is often asymptomatic. Peripheral blood and lymph node CD4+ T cells are depleted but there is a profound and persistent depletion of CD4+ T cells in the GALT (Veazey *et al.*, 1998; Brenchley *et al.*, 2004)

(Mehandru *et al.*, 2004). Damage to the GALT provokes microbial translocation across the gastrointestinal tract that contributes to immune activation (Brenchley *et al.*, 2006). Preventing HIV-1 replication in the draining lymph node and GALT are critical sites for vaccine-mediated immunity (McMichael *et al.*, 2010). From peak viraemia, plasma viral load gradually declines to "set-point," which marks the transition into the chronic phase of infection. During the period of clinical latency, the predominant site of CD4 T cells with active HIV-1 replication is in lymphoid tissue, GALT, spleen, lymph nodes, adenoids and tonsils (Pantaleo *et al.*, 1993) (Embretson *et al.*, 1993) (Cheynier *et al.*, 1994) (Mehandru *et al.*, 2004). The chronic or asymptomatic phase is characterised by progressive decline in the peripheral CD4+ T cell lymphocyte count, until progression to AIDs intervenes, defined by a CD4+ T cell count <200 cells/mm^3 or development of an opportunistic infection or malignancy. Without treatment, survival from AIDS diagnosis is a median of 15 months.

1.15 Viral "Set-point"

One of the most sensitive assays to monitor HIV-1 infection is by detecting plasma viral RNA (Lefrere *et al.*, 1997). The time until HIV-1 RNA is first detected in the plasma, known as the Eclipse phase, is approximately 10 days (McMichael *et al.*, 2010). Plasma viraemia then exponentially increases to peak at over one million copies/ml between 21-28 days after infection. This peak is followed by a decline in plasma viral load over the next 10–20 weeks that plateaus to a stable "set point" at a median of 10,000 copies/ml by 3 to 6 months post infection (Figure 1-9).

Figure 1-9: Natural Course of Plasma Viraemia in Acute and Early Chronic HIV-1 Infection

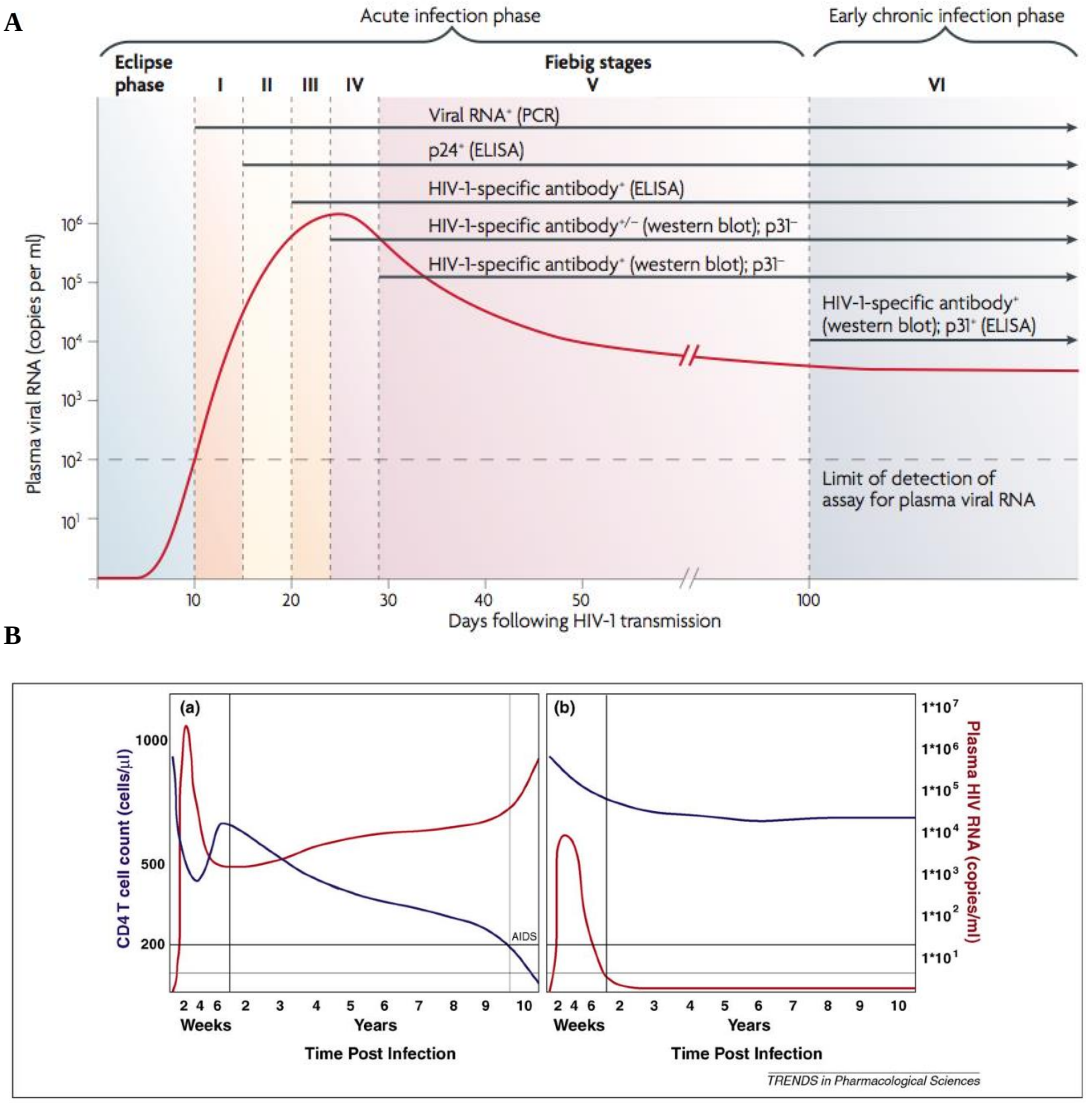


Figure 1-9: Natural history of plasma viraemia in HIV-1 infection.

A) Viral RNA can be first isolated from plasma approximately ten days post transmission, marking the end of the eclipse phase. There is then an exponential rise in viraemia following systemic inoculation of lymphoid tissue, particularly the gut-associated mucosal tissue, which peaks at 21-28 days post transmission. Viral load then declines until "set-point" at 3 to 6 months post infection. Acute events have been defined by Fiebig Stages (I-VI) (Fiebig *et al.*, 2003). Fiebig stages are classified by the sequential gain of positive diagnostic assays (Plasma viral RNA by PCR, p24 and p31 antigen and HIV-1 specific antibodies by enzyme-linked immunosorbent assay (ELISA), and HIV-1 specific antibodies by western blot). Figure from (McMichael *et al.*, 2010). **B**) Representative plasma viral load (red) and CD4 T cell counts (blue) in a progressor (a) and elite controller (b)(O'Connell *et al.*, 2009).

At "set-point" viral replication is contained to some degree by the host immune response, and HIV-1 particles are cleared from the bloodstream at the same rate they are produced. Not all individuals infected with HIV-1 follow the same clinical course. The rate of CD4+ T cell attrition and therefore time to progression to AIDs and survival is highly variable. Within adults of the same age, the reported median time to progression to AIDs is 10 years (Rutherford *et al.*, 1990), but 20% of individuals develop AIDs in under 5 years, known as "rapid progressors", and 10-12% remained asymptomatic with a CD4 count >500 cells/mm^3 for at least 8 to 10 years and were defined as long-term non-progressors (LTNP) (Buchbinder *et al.*, 1994; Pantaleo *et al.*, 1995). The critical factor that was found to correlate with time to progression and survival was the viral load at set-point (Ho, 1996) (Mellors *et al.*, 1996). Rare individuals (0.8%) were found to have undetectable plasma viraemia, even though qualitative tests were positive suggesting continuous HIV-1 replication. These individuals had exceptionally durable spontaneous control of HIV-1 infection and were variously named elite controllers, HIV-1 controllers or LTNP (Lefrere *et al.*, 1997) (Migueles and Connors, 2010).

The decline in plasma viral load and establishment of "set-point" is thought to result from the interaction of the infecting founder virus, and host immune response (Goonetilleke *et al.*, 2009). The innate immune response to HIV-1 can be detected systemically just following detection of plasma viraemia, and is characterised by a strong interferon alpha response and expansion of natural killer cells (Alter *et al.*, 2009) (Li *et al.*, 2009b) (Borrow, 2011). The level of immune activation is extremely high, creating a "cytokine storm" and can persist into chronic infection and may contribute to the development of AIDS, as the CD8 T cell activation marker CD38, correlates with HIV disease progression (Deeks *et al.*, 2004) (Stacey *et al.*, 2009).

The peak of the first detectable HIV-1 specific CD8+ T-cells coincides with the decline in peak viraemia, and is followed by the late development of neutralising antibodies several months later (Borrow *et al.*, 1994) (Koup *et al.*, 1994).

1.15.1 Host Genetic Factors Associated with HIV-1 Control

The HLA B genotype is one of the strongest genetic associations with viral load at set-point and protective alleles, HLA B*5701, HLA B*5703, HLA B*27 and HLA B*51 are enriched in cohorts of HIV-1 controllers (Carrington and O'Brien, 2003) (Fellay *et al.*, 2007) (Kiepiela *et al.*, 2004) (Migueles and Connors, 2010). HLA homozygosity and HLA B*35 are associated with poor outcome (Carrington *et al.*, 1999). This association could be related to MHC class I allele- epitope presentation to CD8+ CTL, the KIR-NK cell interaction, or linkage with other polymorphic genes, eg TNFa, or human endogenous retrovirals (HERVs) elements. A GWAS comparing HIV-1 controllers and progressors, identified specific amino acids within the peptide binding groove of HLA-B molecules that associated with HIV-1 control (Pereyra *et al.*, 2010). This association with HIV-1 control remained significant for some of the residues, for example position 97 located in the floor of the HLA peptide binding groove and part of the “C” pocket, even following correction for known protective alleles HLA B*57 and HLA B*27(Carrington and O'Brien, 2003; Stewart-Jones *et al.*, 2005b) (Goulder and Walker, 2012). However, there is no common pattern of preferred residues in HLA B*27 and HLA B*57, and each have distinct anchor residues, motifs and Bw4 epitopes. The mechanism by which HLA alleles protect against HIV-1 is a matter of intense investigation, particularly as this is the ligand for the T-cell receptor on CD8+ cytotoxic T lymphocytes. KIR-MHC I binding can be modulated by the amino acid sequence of the bound peptide (Stewart-Jones *et al.*,

2005a) (Brackenridge *et al.*, 2011) (Alter *et al.*, 2011). Correlation of amino acid residues within the peptide-binding groove with control of HIV-1 suggests a predominant role for epitope-HLA interaction that is essential in the antigen recognition for the CD8+ T-cell response.

1.15.2 Natural Killer Cells

NK cells are innate immune lymphocytes that recognise viral infected cells using germline encoded non-variant polymorphic receptors. NK receptors include members of the Killer-cell immunoglobulin-like receptor (KIR) family, and the C-type lectin-like proteins known as killer lectin-like receptors (KLRs) encoded by genes in the NK receptor complex on chromosome 12, that can also be expressed by CD8+ cytotoxic T-lymphocytes (Lanier, 2005). These include NKG2A, and NKG2C that form heterodimers with CD94 and recognise HLA-E. NKG2A transmits an inhibitory signal by an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic domain. NKG2C transmits an activating signal by association with DAP12 (also known as TYRO protein tyrosine kinase-binding protein (TYROBP)). NKG2D is a homodimer that induces activating signals by association with DAP 10 (also known as Haematopoietic Cell Signal Transducer (HCST)) on binding ligands MICA and MICB.

1.15.3 Killer cell immunoglobulin-like receptors (KIRs) and HIV-1

Killer-cell immunoglobulin receptors (KIR, CD158), a family of polymorphic germ-line encoded receptors are expressed on NK cells and CD8+ T cells, and can interact with MHC class I receptors and other as yet unidentified ligands to inhibit or activate cytolysis and cytokine secretion. KIRs can have two (2D) or three (3D) extracellular

immunoglobulin domains and can be either activating (S), where the cytoplasmic domain is "short" and signal is transduced by association with an adapter protein, TYROBP (also known as DAP12), or inhibitory (L), where signal is transduced by the long cytoplasmic domain directly (Lanier, 2008). The KIR gene locus (LRC, Chr 19q13.4) is polygenic and polymorphic. One gene locus can encode an activating or inhibitory allele (KIR3DL1/KIR3DS1), and KIR2DL4 (CD 158d) can be both activating and inhibitory (Miah *et al.*, 2008). There are 15 KIR genes, KIR2DL 1-4, 5A, 5B, KIR3DL1-3, KIR2DS1-5 and KIR3DS1. KIR genes are stochastically expressed by NK cells and following activation of CD8 T cells (Li *et al.*, 2009a).

Some inhibitory KIR bind a motif (amino acids 77-83) on the soluble surface of the alpha helix of the peptide binding groove on MHC class I alleles, known as the Bw4 epitope. KIR3DL1 binds Bw4, most commonly but not exclusively found on HLA-B alleles, that is further defined by threonine (T) or isoleucine (I) at position 80. KIR2DL receptors also recognise the alpha helix towards the COOH-terminal end of the peptide-binding groove. HLA C alleles can be classified into two classes (C1 and C2) by the amino acid at residues 77 and 80. KIR2DL1 binds to C2 (Asn 77 and Lys 80), and KIR2DL2 and KIR2DL3 bind C1 (Ser 77 and Asn 80) (Cw1, 3, 7, 8, 13 and 14) (Long, 2008).

Nef downregulates HLA –A and HLA-B alleles but relatively spares HLA-C alleles which has been proposed to protect HIV-1 infected cells from NK mediated cell lysis (Cohen *et al.*, 1999). A GWAS identified a protective SNP 35 kb upstream of HLA-C (rs9264942, termed -35) that has been linked to high surface HLA-C expression (Fellay *et al.*, 2011) (Thomas *et al.*, 2009). HIV-1 variants may be selected by NK cell mediated immune pressure, although in one study the selected variant enhanced KIR2DL2 binding (Alter *et al.*, 2011). Selection of viral variants with altered amino

acid residues within the HLA B*57 restricted TW10 epitope that abrogate KIR3DL1 ligand binding have also been reported (Brackenridge *et al.*, 2011). Individuals with the 80 Ile Bw4 motif variant and expression of KIR3DS1/KIR3DL1 are reported to have delayed progression to AIDs (Martin *et al.*, 2002) (Martin *et al.*, 2007). NK cells expressing these receptors expand in acute infection in Bw4 80Ile individuals, and KIR3DS1 NK cells can control HIV-1 replication in Bw4 80Ile target cells *in vitro* (Alter *et al.*, 2009) (Alter *et al.*, 2007). NK receptor-HLA allele interactions are therefore extremely complex, particularly as inhibitory MHC class I alleles are involved in NK cell maturation and licensing of NK effector function and NK receptors can be expressed by CTL and modulate cytotoxicity and IFNγ secretion (Kim *et al.*, 2005).

1.15.4 HIV-1 Broadly Neutralising Monoclonal Antibodies

HIV-1 infection induces HIV-1 specific antibodies that bind gp41 and gp 120 in all infected individuals from 3-4 weeks post infection (Fiebig stage III). Antibodies that have the capacity to neutralise a broad spectrum of viral variants only develop in 20% of HIV-1 infected individuals, and arise after two to four years of HIV-1 infection (Kwong and Mascola, 2012). Inducing broadly neutralising antibodies by vaccination is a considerable challenge, as the antibodies are highly specialised with long or hydrophobic heavy-chain variable CDR 3 loop or extensive somatic hypermutation (Burton *et al.*, 2012). The trimeric envelope glycoprotein spike evades antibody binding by sequence variable loops, a glycan shield and conformational masking of conserved regions including the CD4 receptor and co-receptor CCR5 and CXCR4 binding site on gp120 (Pantophlet and Burton, 2006). Potent and broadly neutralising antibodies have been found that target the CD4 binding site, variable loop regions 1

and 2 (V1V2) site and Glycan-V3 site on gp120, and the membrane proximal external region (MPER) on gp41, (for example b12 and VRC01; PG9 and PG16; 2G12; 2F5, 4E10, and 10E8 respectively) (Burton *et al.*, 1994; Wu *et al.*, 2011; Walker *et al.*, 2009) (Stiegler *et al.*, 2001; Trkola *et al.*, 1996; Huang *et al.*, 2012). Although rapid viral evolution means that even potent naturally arising broadly neutralising antibodies do not confer clinical benefit on the individual, these antibodies can confer protection in SIV infected macaques on passive transfer (Mascola and Montefiori, 2010).

1.16 AIMS of this study: CD8+ T-cells associated with HIV-1 control: Defining the "Good" and the "Bad"

Detection of the first HIV-1 specific CD8+ CTL temporally coincides with the decline in acute viraemia and selects for viral variants within known CD8+ T-cell epitopes, even with a cost to replication fitness (Goonetilleke *et al.*, 2009; Koup *et al.*, 1994) (Borrow *et al.*, 1994) (Pantaleo *et al.*, 1994). Escape from HIV-1 specific CD8+ CTL in chronic infection can correlate with progression to AIDS (Goulder *et al.*, 1997) (Almeida *et al.*, 2007). GWAS link protection to amino acid residues in the peptide binding groove of HLA class I alleles, and cohorts of elite controllers are highly enriched in individuals with protective HLA-alleles, including HLA B*27 and HLA B*57 (Gillespie *et al.*, 2006) (Pereyra *et al.*, 2010) (Carrington and Walker, 2012). However, attributes that define protective CTL remain elusive due to the extraordinary heterogeneity of the total HIV-1 specific CD8+ T-cell response. We aim to address this question by limiting these variables by generating a panel of CD8+ T-cell clones restricted by the same HLA allele, HLA B*08, that target the same epitope, FLKEKGGL (Nef 90-97), and define optimal attributes that correlate directly with the ability of each CD8+ T-cell clone to control of HIV-1 replication *in vitro*.

2 MATERIALS AND METHODS

2.1 Study cohorts and healthy donors

2.1.1 London Riverside cohort, London, UK

The London Riverside cohort, established in 1995, consists of 165 HIV-1 infected individuals enrolled in a nested case-control study of the behavioural and biological correlates of HIV-1 non-progression from volunteers attending clinics in London, UK (Easterbrook *et al.*, 1999). Non-progressors were defined as individuals infected with HIV-1 for at least 10.5 years, who remained asymptomatic with a CD4 cell count > 500 x 10^6/L without treatment. The majority were white homosexual or bisexual men infected with HIV-1 clade B viral strains.

2.1.2 San Diego Acute Cohort, San Diego, USA.

Individuals with acute HIV-1 clade B infection ("Pre-seroconversion" defined as symptomatic viral illness, recent high-risk exposure, detectable plasma HIV-1 RNA and a non-reactive ELISA for HIV-1 antibody or negative/indeterminate Western blot assay; and "Post-seroconversion" defined as diagnosis within 180 days of infection) were recruited into a study of early anti-retroviral therapy from volunteers attending the San Diego AIDs Treatment Centre, USA. Patients in the acute cohort were treated with anti-retroviral therapy following diagnostic and research sample collection.

The studies obtained local ethical approval from the Central Oxford Regional Ethics Committee, the Ethics Review Committee of King's College Hospital, and The University of California, San Diego, Institutional Review board. All subjects were fully informed and gave written consent. Healthy control subjects were recruited

anonymously and thus did not require ethical consent. All healthy subjects were fully informed and gave written consent to donate blood.

2.2 HLA Typing

HLA Class I and II molecular typing was performed by the amplification refractory mutation systems PCR (ARMS-PCR) by Tim Rostron (WIMM, Oxford, UK), using sequence specific primers (Bunce *et al.*, 1995). 144 allele specific primer pairs simultaneously detect all known HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 and DQB1 specificities. PCR products from each individual reaction including the internal positive control, a 796 bp fragment from the third intron of HLA DRB1, are visualised following electrophoresis on a 2% agarose gel, in a process known as "phototyping". This gives absolute HLA typing to 2 digits and a high probability resolution to 4 digits.

2.3 Peripheral blood mononuclear cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by Ficoll-Hypaque (Nycomed, Oslo, Norway) density gradient centrifugation. Blood was layered into Leucosep tubes (Greiner-Bio-One, Germany) prepared with 16mls of Lymphoprep (Axis-Shield, USA) and fractionated by centrifuging at 2000 rpm for 20 minutes without the brake. PBMCs were harvested from the plasma interface and washed in R10 (RPMI 1640 (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal calf serum (FCS; Sigma), 100 IU of penicillin-L, 100mg of streptomycin/L and 2mM L-Glutamine) by centrifugation at 1500 rpm for 5 minutes to remove the platelets. PBMCs were resuspended in H10 (RPMI 1640, 10% heat-inactivated human AB serum (National Blood Service), 100 IU of penicillin, 100mg of streptomycin, and 2 mM L-Glutamine), counted using trypan blue exclusion to

ensure only live cells were counted (Invitrogen, California, USA) and either used immediately or cryo-preserved in 10% dimethylsulphoxide (DMSO) /FCS in vapour-phase liquid nitrogen.

2.4 Preparation and maintenance of cell lines

2.4.1 CTL clones

Samples were obtained from four study subjects selected from two well characterised cohorts based on HLA B*08 typing and previous studies that indicated a significant HLA B*08-FL8 tetramer-staining CD8+ CTL population in peripheral blood (Papagno *et al.*, 2002; Dong *et al.*, 2004). The clinical details at the time of sample collection for the four individuals (Patient ID: 005, E833, G713 and G916) are documented in Table 1. CTL clones specific for the HIV-1 epitopes FLKEKGGL (FL8) Nef_{90-97}, restricted by HLA B*0801, were generated by limiting dilution by Dr Tao Dong. PBMCs from study subjects were stained using PE-labelled B8-FL8 MHC class I tetramers and sorted using magnetic anti-PE MACS microbeads (Miltenyi Biotec, Paris, France). 3-5 million PBMCs were incubated with B8-FL8 tetramers for 20 minutes at 37°C. Cells were washed in cold buffer (0.05% bovine serum albumin/phosphate buffered saline) and resuspended in 40 ul of magnetic anti-PE microbeads for 15 minutes at 4°C. PBMCs were washed, resuspended in 500 ul cold phosphate buffered saline (PBS), and separated by positive selection on magnetic columns according to the manufacturers instructions. Eluted cells were counted by trypan blue exclusion, diluted to 0.3 cells per well on U-bottom 96-well plates in mixed heterologous feeder cells (Irradiated PBMCs from a minimum of three healthy donors, suspended at 2 x 10^6 cells/ml in H10 supplemented with 40ug/ml phytohaemagglutinin (PHA)) and incubated at 37°C in 5% CO_2/air. CTL clones were

maintained in 200 U/ml recombinant IL2 (Preptech) in H10 media added on day three, and either cryopreserved or re-stimulated to proliferate every three weeks. All CTL clones were screened for purity (>98%) and specificity on day 10 following restimulation by staining with anti-CD8 and B8-FL8 tetramer. All experiments were performed on day 12 to 14 post stimulation.

2.4.2 CD4+ T-cell line

The C8166 cell line (human T-lymphoblastoid cell, reported to carry but not express HTLV-1 (Salahuddin *et al.*, 1983)) stably expresses HLA B*0801 and was obtained at no charge from the National Institute for Biological Standards and Control (NIBSC) and maintained in R10 at 37°C in a 5% CO_2 /air incubator. All cell lines were routinely screened for mycoplasma (negative).

2.5 HIV-1 clade B viral isolates and propagation of viral stock

Unattenuated HIV-1 X4 laboratory strain viruses IIIB and MN were obtained at no charge from the EU Programme EVA centre for AIDs Reagents, National Institute for Biological Standards and Control, UK. High titre stocks of each virus were propagated by inoculating the CD4+ T-cell line (5x 10^6 C8166) with 200ul of virus, followed by incubation for 90 minutes at 37°C/5% CO_2 and re-suspension in 5mls R10. Supernatant containing virus was harvested at days 2,4, 7 and 12 post-infection and assayed for p24 concentration using the quantitative p24 antigen enzyme linked immunosorbent assay (ELISA) (Immunodiagnostics, Woburn, MA) as per the manufacturers instructions.

2.6 Antigens and antibodies

2.6.1 Peptides

The FLKEKGGL (FL8) peptide, a previously defined optimal CTL epitope (Korber and T, 2000), was synthesised (WIMM, Oxford, UK) using FMOC protection on an automated synthesiser (Zinsser, California, USA). Peptide purity >90% and mass were confirmed by HPLC and mass spectroscopy.

2.6.2 Monoclonal Antibodies

Directly conjugated antibodies were obtained from the following commercial sources and used in different combinations (the fluorochrome and volume used is indicated in brackets): Beckman Coulter (Hialeah, FL): anti-TCR Vβ 13.2 (phycoerythrin (PE) 1ul), anti-CD94 (PE, 2ul), anti-p24 antigen (KC57 RD1) (PE, 3ul), anti-NKG2A (Allophycocyanin (APC, 4ul); BD Bioscience (San Jose, CA): anti-CD8 (PE-cyanine dye (Cy^7) tandem conjugate (PE-Cy^7), 3ul), anti-CD8 (Pacific Blue (PB), 6ul), anti-CD8 (Fluorescein isothiocyanate (FITC), 3ul), anti-CD4 (APC, 1ul), anti-CD27 (APC, 2ul), anti-MIP1β (PE, 3ul), anti-CD107α (FITC, 1ul), anti-IFNγ (PE-Cy^7, 4ul), anti-TNFα (APC, 5ul), anti-CD279 (PD-1) (APC, 1ul), anti-CD28 (FITC, 4ul), anti-Granzyme A (PE, 3ul), anti-Cd8 HIT 8a (PE, 1ul), anti-NKG2C (Peridinin-chlorophyll-protein complex (PerCP, 4ul), anti-ITG-α5 (APC, 3ul), anti-CXCR3 (PE, 2ul); Diaclone (San Diego, CA): anti-Perforin (B-D48) (FITC, 3ul); Biolegend (San Diego, CA): anti-CD57 (FITC, 4ul); R and D Systems (Minneapolis, MN): anti-KIR2DL4 (APC, 3ul), anti-KIR2DL3 (PE, 3ul).

2.6.3 Peptide/HLA class I tetramers

The HLA-B*0801-FLKEKGGL and HLA A*0201-SLYNVATL monomers used were synthesised and biotinylated by Yanchung Peng. In brief, the HLA molecule heavy chain cDNAs were obtained from EBV-transformed B-cell-lines from HLA B*08 and HLA A*02 healthy donors. Specific primers were used to amplify the extracellular region of the HLA heavy chain and incorporate a biotinylation signal sequence for enzymatic addition of biotin to the C-terminus of the heavy chain. The PCR product was cloned and expressed using heavy chain transformed *Escherichia coli* (Novagen, Merck, Darmstadt, Germany). The heavy chain protein was purified and refolded with β_2m and the CTL epitope to form a monomer complex. The refolded product was concentrated, enzymatically biotinylated, and purified by gel filtration using an Fast Protein Liquid Chromatography (FPLC) machine (Pharmacia) and Superdex-75 column. The monomer complex was concentrated and protease inhibitors added. Tetrameric complexes were formed by the slow addition of 1 molar phycoerythrin (PE) coupled extravidin (Sigma) to four molars of monomer complex using a simplified working ratio of 0.312mg of extravidin PE for 1 mg biotinylated monomer.

2.7 Flow Cytometry

CD8+ T cell clones and the CD4+ T cell target (C8166 T-cell line) were characterised by flow cytometry. Titrated tetramers (PE conjugated) were incubated with each CD8+ CTL clone for 15 minutes at 37°C in the dark. Titrated surface antibodies were added for 20 minutes at 4°C in the dark. Cells were washed in FACS buffer (PBS; 1% BSA; 0.5 mM EDTA), centrifuged (5 minutes at 1500 rpm), the supernatant discarded and fixed for two hours prior to analysis in PBS with 1% formaldehyde. If intracellular staining was required, following cell surface staining, cells were fixed

and permeabilised using BD cytofix/cytoperm Kit (BD Biosciences). This was the optimised method for intracellular staining that preserved more live cells after experiments that compared fixing with lysolecithin (20ug/ml) in 1% paraformaldehyde and permeabilisation with 50% methanol and 0.1% Nonidet P-40. Cells were acquired using a CYAN (Beckman Coulter) flow cytometer after compensation using single stained samples with bright signal and analysed with FlowJo software (Treestar, Ashland, OR).

2.8 Viral Suppression Assay

The ability of each population of CD8+ T-cell clone to suppress p24 production in an HLA-matched HIV-1 infected CD4+ T-cell line (C8166) was assayed by co-culture in carefully optimised conditions. A cell pellet of the CD4+ T-cell line (C8166) was infected with a pre-titrated dose of HIV-1_{MN} at high or low multiplicity of infection (MOI) for 90 minutes at 37° C/5% CO_2, washed to remove free virus and re-suspended in H10. Using C8166 in the exponential phase of growth improved inter-assay reliability in infectivity rates. Infected C8166 (10^5/well) were aliquotted into a 96 or 24 well flat-bottomed plate. CD8+ T-cell clones at different CD4:CD8 ratios were added to the plate in triplicate wells. Positive (infected C8166 alone) and negative controls (uninfected C8166) in triplicate, and an HLA mismatched clone (isolated from LTNPs in the London Riverside Cohort by Dr T Dong) were added. All suppression assays were carried out in the presence of IL2 (200U/ml). After 96 hours of co-culture at 37°C 5% Co_2, viral suppression was measured by determining the p24 antigen concentration in 50ul of neat supernatant using a p24 ELISA Kit (ImmunoDiagnostics Inc., USA) as per the manufacturers instructions. All viral titrations used were optimised to maximise the discriminatory range of the ELISA assay sensitivity (5-300 pg/ul). The CD4/CD8 co-culture was analysed by flow

cytometry using surface markers CD4 (APC), CD8 (FITC) (BD Biosciences, San Jose, CA) and intracellular Gag p24-RD1 mAb (PE) (KC57, Becton-Dickinson). All compensations were performed using additional wells of co-culture included on the assay plate. Live single cells were acquired. CD4+ cells were gated to assess the p24 positive population using uninfected CD4+ T-cell line (C8166) cells to set the negative population. CD8 T-cell mediated suppression was expressed as the percentage reduction in the average p24 content of day 4 co-cultures compared to the average p24 content of C8166 infected cells alone (positive control).

2.9 Cytokine Bead Array (Luminex system)

CD4+ T-cell line (C8166) cells infected with differing MOI of HIV-1_{MN} for 72 hours or exogenously pulsed with epitope (FL8) were used as targets to compare the cytokine, chemokine and growth factor secretion of epitope specific CD8+ CTL clones using a BioRad Bio-Plex Human Cytokine 17-Plex Panel assay (BioRad). CD8+ CTL clones (10^5) were co-cultured for 24 hrs 37°C/5%CO_2 at a 10:1 ratio (adjusted for the percentage of infected cells as appropriate) with the CD4+ target cell of interest in triplicate and were also activated using PHA or phorbol myris-tate acetate (PMA) and ionomycin. Negative controls in triplicate of CD8+ CTL clone alone, infected C8166 alone, or co-cultures with non-infected C8166 or pulsed with 0uM peptide were included. 70ul of supernatant was harvested and frozen immediately at -80°C. Cytokine secretion was analysed using 50ul thawed CTL supernatant using a BioRad Bio-Plex Human Cytokine 17-Plex Panel assay (IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL 17, G-CSF, GM-CSF, IFN gamma, MCP-1, MIP-1Beta, and TNF alpha) (Bio-Rad Laboratories) according to the manufacturers instructions. This is a flow cytometry based assay that couples a capture sandwich immunoassay for each molecule to a unique fluorescently dyed

bead allowing simultaneous detection of up to 100 molecules. In brief, antibody against the molecule of interest is coupled to an internally dyed bead. Coupled beads were incubated with the supernatant sample, standards (Range 0 to 32 000 pg/ml) and blanks in a 96 well filtration plate (Millipore, USA) for 30 minutes at room temperature on a plate shaker at 300rpm. The beads were washed to remove unbound protein using a vacuum manifold and incubated with biotinylated anti-cytokine-detection antibodies. A streptavidin-PE reporter complex was added for 10 minutes to bind the detection antibody on the bead surface. Beads were washed and fixed in 2% paraformaldehyde for two hours prior to data collection and analysis on a Luminex 100 IS System (Bio-Rad Laboratories).

2.10 Intracellular Cytokine Staining: "Polyantigen sensitivity"

ICS performed at three epitope dilutions was used to detect the population of each CD8+ CTL clone responding with simultaneous upregulation of four parameters CCL4, IFNγ, TNFα, and surface expression of CD107a. CD4+ T-cell line (C8166) cell pellet was pulsed with epitope (FL8) (10uM to 0.001uM) or media (negative control) for one hour at 37°C/5% CO_2, washed to remove unbound peptide, and used as targets. CD8+ T-cell clone (10^5 cells) were mixed with target cells (10:1 E:T ratio) and CD107a mAb (FITC) (1ul) in FACs tubes and incubated for 1 hour at 37 °C/5% CO_2. Monensin (6 ug/ml final concentration; BD Bioscience) was added to block protein transport. Cells were left for 5 hours at 37°C then stained with surface markers against CD8 (Pacific Blue) and CD4 (PerCp) and for intracellular expression of cytokine (CCL4 (MIP 1b) (PE), IFNγ (PE-Cy7), and TNFα (APC)(All mAb, BD Bioscience). Compensation was performed using fluorochromes in each channel conjugated to anti-CD8+ mAb. Gates were set to acquire live, single, CD8+ cells. Gates for each parameter were allocated following identification of the resting

population (Negative control). Simultaneous effector responses were calculated using the Boolean gate function (FlowJo Software).

2.11 Tetramer Avidity Assay

The concentration of FL8-HLA B*0801 tetramer (PE) was measured using the BioRad protein assay (BioRad, Hemel Hempstead, Hertfordshire). CD8+ CTL clones (5×10^4 /well) in a U-bottomed 96 well plate were stained with an HLA mismatched tetramer (PE) (SL9-HLA *A2), or FL8-HLA B*08 tetramer at serial 2 fold dilutions from 4ug/ml to 62.5 ng/ml, and anti-CD8 mAb (APC). The proportion of cells that stained for tetramer was calculated for each peptide concentration using the HLA mismatched tetramer to allocate the negative population. The normalised percentage of positive cells for each clone was calculated defining tetramer staining at 4ug/ml as 100%, and 62.5 ng/ml as 0%.

2.12 Standard Chromium-release Assay

CD4+ T-cell line (C8166) cells were used as targets in a standard four-hour ^{51}Chromium (Cr)-release assay. C8166 were labelled for one hour with 200 ul (200 μCi) $Na^{51}CrO_4$ (Perkin Elmer, Massachusetts, USA), washed extensively and aliquotted into a U-bottomed 96 well plate at 5×10^3 cells per well. Media alone or peptide epitope (FL8) was added to experimental wells (final concentrations 10uM to 10^{-3} uM in 10 fold dilutions) prior to the addition of CTL clones (optimised E:T ratio of 3:1) in triplicate. Control wells contained media only (to measure non-specific lysis) or 5% Triton X-100 detergent only (to measure maximal lysis) in quadruplicate. The plate was incubated for four hours at 37°C/ 5% CO_2. 25ul supernatant was harvested into a microbeta plate (Perkin Elmer, Bridgeville, Pensylvania), mixed with scintillation cocktail (PerkinElmer) and counted using a MicroBeta Liquid

Scintillation Counter (MicroBeta-Jet, Perkin Elmer). Spontaneous release of ^{51}Cr ((media control)/ (maximal lysis) x 100 %) was required to be less than 20%. Specific lysis was calculated with the formula: 100% x (Experimental Lysis– Media control)/ (Maximal lysis – Media Control). Results were normalised by declaring specific lysis at 10uM as 100%, and at 0uM as 0%.

2.13 TCR Sequencing

Total RNA was extracted from CD8+ T-cell clones (2-5 x 10^6) 15 days post stimulation using an RNeasy Mini Kit (Qiagen, Crawley, UK). To maximise the yield of cDNA with full length 5' sequence, a modified SMART (Switching Mechanism At 5' end of RNA Template) technique was used (Zhu *et al.*, 2001). cDNA was synthesised from an optimised quantity of RNA template (30 ng) using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and a 3' poly T primer (Biotech). At the 3' end of full-length first strand cDNA the RT has terminal transferase activity and adds 3-5 non-template guanine (G_n) residues. An undisclosed RNA/DNA hybrid 5' Template Switching Oligo primer (BD Bioscience) anneals to the G_n residues on the newly synthesised DNA strand by three RNA Cs. The RT switches template from the mRNA to the oligonucleotide primer. The DNA sequence of the oligo primer is transcribed to incorporate a defined DNA sequence to the new cDNA strand. Reaction conditions were 42° C for 90 minutes, followed by incubation in Tricine EDTA buffer (10mM Tricine-KOH, 1.0 mM EDTA, pH 8.5.) at 72° C for 7 minutes. cDNA template was used in a Rapid Amplification cDNA Ends (RACE) PCR reaction to amplify TRA or TRB chain gene products containing the full length V region using primers specific for the Constant region of each TCR chain. To decrease background, a combination of suppression and step-out PCR techniques were employed using the

following primers 5'- CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA 3' (Universal primer long, forward, 0.02ul) (first round PCR amplification), 5'- CTA ATA CGA CTC ACT ATA GGG C 3' (Universal primer short, forward, 0.08ul) and 5'- GTC CAT AGA CCT CAT GTC TAG CAC AG 3' (TRA constant region, reverse, 1ul) or 5'- ATT CAC CCA CCA GCT CAG CTC CAC G 3' (TRB constant region, reverse, 1ul). Advantage 2 PCR kit (Clontech, Mountain View, CA) was used for high fidelity long distance PCR in the following reaction conditions: hot start, 95°C 2min, 10 cycles at each of the four touchdown annealing temperatures 68°C, 65°C, 60°C and 55°C for 3min, preceded by denaturing at 95°C for 25 s and followed by extension at 68°C for 3 min, with a final extension step at 68°C of 6 min. The PCR product was gel-purified using QIAquick PCR purification kit (Qiagen) and sequenced by the dideoxy chain termination method on a 48 capillary ABI-3730 DNA analyser (Applied Biosciences Inc., USA) using TRA or TRB constant primers. Sequences were edited using 4Peaks (Version 1.7) and ClustalW (Version 2.0) and TRA and TRB sequences identified from alignment with genomic sequences in the human TCR gene database (http://imgt.cines.fr:8104/home.html) using IMGT/V-QUEST (Brochet *et al.*, 2008) (Brochet *et al.*, 2008) (Giudicelli *et al.*, 2011).

2.14 TcLandscape

Total RNA was extracted by Dr T. Dong from PE-labelled HLA B*08-FL8 tetramer staining populations sorted using magnetic anti-PE MACS microbeads (Miltenyi Biotec) from PBMCs isolated from three individuals (E833, G713, G961) selected from the San Diego Acute Cohort as reported (Dong *et al.*, 2004). RNA was sent to TcLandscape Service (TcLand-biotech); Institut National de la Sante et de la Recherche Medicale, Paris, France for analysis. In brief, T cell landscape plots

display integrated qualitative and quantitative analysis of the CDR3 length distribution for each TRBV family. Total RNA was used to synthesise cDNA using the Boehringer cDNA synthesis kit (Boehringer Mannheim, Meylan, France). cDNA product was amplified with 24 TRBV family specific forward primers and a reverse TRBC primer. PCR products were resolved on a 6% polyacrylamide 8M urea sequencing gel to provide the precise size of the CDR3 length in nucleotide base pairs for each TRBV family, and the distribution was computed using Immunoscope and Reperturb software (Sebille *et al.*, 2001). Each TRBV family was quantified by real time PCR on an ABI PRISM 7700 Sequence Detection System PerkinElmer machine (Applied biosytems) by comparison to a TRBC standard curve and standardised against the level of transcripts of a standard DNA, hydroxyphosphoribosyltransferase (HPRT). MatLab software was used to compute and display the integrated quantitative PCR data for a given CDR3 size in the qualitative landscape plot for each TRBV family.

2.15 Gene Expression Arrays

2.15.1 RNA extraction

Gene expression was determined for two populations of CD8+ CTL clone using HumanHT-12 v 4 Expression BeadChips (Ilumina, San Diego, USA) in collaboration with Dr Dilair Baban, Genomics Group, Wellcome Trust Centre for Human Genetics, Oxford. CD4+ T-cell line (C8166) infected with high MOI HIV-$1_{MN,}$ or pulsed for one hour at 37°C with 0uM or 2uM epitope (FL8) was used as targets. CD8+ CTL clones (3 x 10^6) from three separate batches of re-stimulation (biological replicates) were mixed with targets at 3:1 E:T ratio in U-14 ml falcon tubes and either separated

either immediately (T=0), or after two hours (T=2) or 6 hours (0uM and 2uM) of co-culture at 37°C/5% CO_2. The supernatant was harvested and frozen immediately at -80°C for cytokine analysis. CD8+ CTL clones were negatively selected from the co-culture by depleting the CD4+ cell fraction using magnetic anti-CD4 microbeads (Miltenyi Biotec) and MACS technology (Miltenyi Biotech) as per the manufacturers instructions. The eluate was passed through a second MS column to enhance the purity of the CD8+ CTL fraction (65% to 95%). Isolated CD8+ cells were counted and RNA extracted from equal cell numbers using Mir-vana miRNA isolation kit (Applied Biosystems, Austin, Texas) or RNeasy Mini Kit (Qiagen, Crawley, UK) with an on-column DNase digestion step using the RNase-free DNase set (Qiagen). RNA quantity and quality was measured using the Agilent Bioanalyser 2100 (Agilent, Santa Clara, USA).

2.15.2 Gene expression profiling

Samples were analysed for gene expression using Human Bead Chip HT12 chips (Ilumina) by Dr Dilair Baban. Samples were amplified using the Illumina "totalPrep-96 RNA Amplification Kit" (Applied Biosystems) prior to hybridisation onto Chips. Chips were scanned with Illumina BeadArray Reader and GenomeStudioV2010.1 (Illumina Inc) was used for data extraction.

2.15.3 Data analysis

Data was analysed by Dr Dilair Baban. Data was imported to GeneSpring GX 11.0.2 (Agilent Technologies), normalised with Shift to 75 percentile and baseline transformed to median of all samples to identify significantly differentially expressed

genes which were then interrogated using Protein Analysis Through Evolutionary Relationships (PANTHER) (Thomas *et al.*, 2003). Data was also analysed by Dr Steve Taylor in the Computational Biology Research Group (CBRG), WIMM, University of Oxford. Normalisation was achieved using the lumi package in R (Du *et al.*, 2008). Genes with altered expression were detected using an empirical Bayes analysis approach, as implemented in the "limma' (Linear Models for Microarray Analysis) package of the R programming language (Smyth, 2004). Hierarchical clustering was performed in R and heat maps were drawn using heatmap.2. Venn diagrams were drawn using Venny (Oliveros J.C 2007, VENNY).

2.16 Quantitative Real-Time PCR

RNA was isolated as above (RNeasy minikit and on column DNase Digestion step) from CTL clones (A3 and A20) day 14 post stimulation that had been sorted to >99% purity by fluorescence activated cell cytometry using FL8- HLA B*0801 tetramer and anti-CD8 (APC) antibodies. TaqMan Gene Expression Assays (Applied Biosystems) were used to validate target genes of interest in a two-step RT-PCR reaction using random oligonucleotide primers for first strand cDNA synthesis. cDNA was produced from total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosytems). Multiple reactions containing 500ng total RNA per 20ul reaction volume were run at the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and then pooled by sample for TaqMan assay analysis. Each TaqMan gene expression assay consists of two sequence specific primers and a hybridising probe, with a 5' fluorescent reporter, FAM, and a 3' non-fluorescent quencher, which is cleaved by the exonuclease activity of the AmpliTaq Gold DNA polymerase during extension of the complementary strand. Primers are designed from

in the Applied Biosystems Genome Aided Assay Design Pipeline (Applied Biosystems 2004) with transcripts obtained from the NCBI Reference sequence Project database (RefSeq). Optimal regions of the transcript for primer/probe pair design are identified by first masking regions of repeats and SNPs, and then identifying exon-exon boundaries by alignment with the genomic sequence. Primers are optimised for amplification efficiency at universal cycling conditions (%GC content, T_m, secondary structure, length) and interrogated *in silico* using the public genome and transcripts database (NCBI) through BLAST searches to ensure gene specificity. Primers and probes from within a single exon have a "s" suffix (Gene expression assay for CXCR6 only). Controls containing either no RT, or no template (H_2O only) were included for all primers. The assay was carried out in triplicate, using 25ng total cDNA (as total input RNA) in 20ul for each sample and run on an ABI 7500 Fast PRISM spectrofluorometric thermal cycler (Applied Biosytems, Inc.) using universal cycling conditions (2 min at 50°C, 10 min at 95°C; 15 s at 95°C, 1 min at 60°C, 40 cycles). Fluorescence above background was detected at a predetermined threshold (C_T) during exponential amplification. Results were analysed using a modification of the Pfaffel method that assumes 100% efficiency for each primer/probe pair. The delta C_T is calculated for each gene of interest using GAPDH, a housekeeping gene with similar levels of expression in the two CD8+ CTL clone populations, as an endogenous control gene. The delta delta C_T is calculated by subtracting the delta C_T A3 from the delta C_T for A20, and expressed as a log fold change. A SD of C_T of 0.167 gives a 99.72% confidence for measuring 2-fold discrimination.

2.17 Micro RNA profiling

MicroRNA profiling was performed on total RNA extracted from four biological replicates of each CD8+ CTL clone population following fluorescence activated cell sorting (>99% purity) using FL8-HLA B*08 tetramer (PE) on day 14 post-restimulation. CD8+ CTL clone (106) was re-suspended in 1 ml TRIZOL LS reagent (Invitrogen) for RNA isolation and mircoRNA profiling, performed by QuantoBio (Beijing, China). In brief, 0.25 ml chloroform (Beijing chemical works) was added to each sample, which was then vigorously mixed and incubated at room temperature for 10 minutes. Samples were centrifuged (12,000 g for 15 minutes at 4°C), the aqueous phase was harvested, and then 6.25mls isopropyl alcohol (Beijing chemical works) added followed by incubation at room temperature for 10 minutes. Precipitated RNA was centrifuged at 12,000g (15 minutes, 4°C), washed in 2mls 75% ethanol, air-dried, and dissolved in RNase-free water.

Quantobio microRNA profiling uses a three-step quantitative PCR method, with specific forward primers designed to screen 370 miRNAs related to the T-cell. In brief, first strand cDNA was synthesised at 37°C for 1h using A-Plus bacterial polyA polymerase to add a polyA tail to pre-and mature forms of miRNA. The RT primer is added, incubated at 70° C for 5 mins, and placed on ice for two minutes to disrupt the secondary structure of the RNA and primer. Second strand cDNA was synthesised using M-MLV RT at 42° C for 1h. QPCR using 1ng cDNA per 20ul reaction, was run on an ABI 7500 instrument (Applied Biosystems) using the following cycling conditions: 95°C for 5min, 40 cycles of 95°C for 30s and 60°C for 1 min, followed by a dissociation stage of 95°C for 15s, 60°C for 1 min and 95°C for 15s. A set of multiple invariant internal control probes was used to normalise fluorescence intensity across each plate and for the whole array. Following normalisation, the intensity log

fold change for each sample was calculated using multiple invariant internal control probes. Hierarchical clustering and dendrogram analysis was performed using GeneSpring GX (Agilent Technologies). Further analyses to identify candidate microRNAs were performed using BRB-ArrayTools (NCBI) developed by Dr Richard Simon and BRB-Array Tools Development Team. Target genes and potential microRNA pairing sites in candidate genes of interest were identified using miRecords (Xiao *et al.*, 2009), miRanda and an unpublished computer program (J.C. and C.-Z.C., unpublished data) that considers the secondary RNA structure, mismatch and G:U base pairing in the seed region, and coding and untranslated regions (UTRs) of candidate targets (Li *et al.*, 2007). Gene expression data from clones in the resting state was reviewed with microRNA expression data using the cellular pathways tool in GeneGo software (Thomson Reuters, St Joseph's, MI, USA).

2.18 Bisulfite PCR

The methylation status of CpG dinucleotides in the TNFα core promoter was assayed by bisulfite conversion of genomic DNA and PCR amplification and sequencing by Dr G Gillespie and P Simpson (Simpson *et al.*, 2012). Genomic DNA was extracted from each population of CTL clone using PureGene DNA purification kit (Gentra System, Minneapolis, USA). 500ng of genomic DNA was treated with bisulfite to convert unmethylated cytosines into uracil using Methylcode^{TM} Bisulfite Conversion Kit (Invitrogen, Corp., Carlsbad, USA) according to the manufacturer's instructions. PCR fragments were amplified using the following primers: 5'-TTT GTA TTT TGT TTG GAA GTT AGA AGG AAA TAG ATT A-3' and 5'-AAA ACT TCC TTA ATA AAA AAA CCC ATA AAC TCA TC-3'. PCR products were subcloned into

the pCR4 TOPO TA cloning vector (Invitrogen, Corp., Carlsbad, USA) and 8 – 10 colonies sequenced.

2.19 Statistical Analysis

Statistical analysis of the data was performed using Prism version 4.0b (Graphpad, San Diego, USA). Statistical differences between groups were determined using the non-paired Student's t test. Correlations were determined using linear regression. Significantly altered expression of genes was analysed using the LIMMA package for R (Wettenhall and Smyth, 2004) and corrected for multiple comparisons. P values below 0.05 were considered as significant. The LIMMA package analyses Empirical Bayes values to determine gene-wise residual variance. B value < 3.0 was considered significant. Statistically significant differential gene expression was also performed in GeneSpring GX using one-way Analysis of variance (ANOVA) and corrected for multiple comparisons. P values below 0.01 were considered as significant.

3 DEFINING ATTRIBUTES OF OPTIMAL HIV-1 SPECIFIC CD8+ T-CELL CLONES

3.1 Introduction

Cytotoxic T cells (CTL) are critical in the control of HIV-1 infection. The emergence of the first HIV-1 specific CD8+ CTL immediately precedes the decline in peak viraemia, prior to the detection of neutralising antibodies, and is associated with the resolution of acute symptomatic HIV-1 infection (Koup *et al.*, 1994) (Borrow *et al.*, 1994). In the macaque model of AIDs, depletion of CD8+ lymphocytes prevents control of acute HIV-1 infection, and causes a rise in plasma viraemia in chronic infection for the duration of action of the CD8+ monoclonal antibody (Schmitz *et al.*, 1999; Jin *et al.*, 1999). The first HIV-1 specific CD8+ CTL rapidly select viral variants, exerting sufficient immune pressure to select variants with mutations within CTL epitopes or the flanking regions that escape CTL recognition, despite incurring a cost to replication fitness (Goonetilleke *et al.*, 2009) (Borrow *et al.*, 1997) (Leslie *et al.*, 2004) (Martinez-Picado *et al.*, 2006). Escape from an immunodominant CTL response in chronic infection has been associated with progression to AIDs (Goulder *et al.*, 1997) (Koenig *et al.*, 1995).

MHC class I alleles have been consistently associated with time to progression to AIDs (Carrington and O'Brien, 2003) (Kaslow *et al.*, 1996). Genome wide association studies have identified single nucleotide polymorphisms that correlate with viral set-point or were enriched in HIV-1 controllers only within the HLA class I region of the MHC locus on chromosome 6 (Fellay *et al.*, 2007) (Pereyra *et al.*, 2010). HIV-1 control was further correlated to specific polymorphic amino acid residues located

within the peptide binding groove of HLA class I alleles (Pereyra *et al.*, 2010) suggesting a critical role for HLA-viral peptide interaction in immune mediated HIV-1 suppression. HLA B*2705 and HLA B*5701 are consistently associated with delayed progression to AIDS and are highly enriched in cohorts of rare individuals who spontaneously suppress HIV-1 replication to undetectable levels (Migueles and Connors, 2010) (Migueles *et al.*, 2000) (Emu *et al.*, 2008) (Goulder and Walker, 2012). Although this implies that a CTL response restricted by particular MHC class I molecules may be more protective than that restricted by other alleles, a clear mechanism has yet to be defined. HLA B*27 and HLA B*57 both restrict immunodominant CTL responses in acute and chronic HIV-1 infection, that can account for up to 65% of the total HIV-1 specific CTL response (Scherer *et al.*, 2004; Altfeld *et al.*, 2006) (Altfeld *et al.*, 2003). HLA B*27 and B*57 restricted CTL target epitopes presented from gag, an epitope specificity widely associated with HIV-1 control (Kiepiela *et al.*, 2007) (Saez-Cirion *et al.*, 2007) (Emu *et al.*, 2008) (Migueles *et al.*, 2000). HLA B*27-restricted CTL target a single epitope in Gag p24 $_{(263\text{-}272)}$ KK10 (Nixon *et al.*, 1988) that is slow to mutate due to structural constraints (Kelleher *et al.*, 2001) (Schneidewind *et al.*, 2007) and associated with progression to AIDS once escape occurs (Goulder *et al.*, 1997). This KK10 specific CTL response is unusual in that the magnitude of the CTL response to this epitope inversely correlates with viral load in untreated slow progressors, previously only reported for HLA-A2 restricted gag SLYNVATYL (SL9)-specific CTL by tetramer and cytolysis assays (Almeida *et al.*, 2007; Ogg *et al.*, 1998). More commonly, the magnitude of the HLA B*57 and HLA B*27 restricted CTL response in controllers and chronic progressors has been reported to be the same (Migueles *et al.*, 2000) (Migueles *et al.*, 2002) (Chen *et al.*, 2012). T-cell quality in HIV-1 controllers is distinct, as CD8+ T-cells

immediately ex vivo from HIV-1 controllers but not chronic progressors, suppress HIV-1 *in vitro* (Saez-Cirion *et al.,* 2007). This suggests that the CTL quality of small sub-populations of the total HIV-1 specific CTL define HIV-1 control, yet correlates of protection have been challenging to identify. One barrier is the heterogeneity of the HIV-1 specific CTL response, which differs in activation status (Oxenius *et al.*, 2001) (Saez-Cirion *et al.*, 2007), state of proliferation, cytokine profile, killing efficiency, state of effector/memory differentiation (arrested maturation of terminal effectors CD45RA+/CD27-) (Champagne *et al.*, 2001) (Appay *et al.*, 2002), and even exhaustion from chronic antigen exposure (Day *et al.,* 2006) (Trautmann *et al.,* 2006). For these reasons, the field has focused on defining attributes of the HLA B*57 and HLA B*27-restricted CTL response in HIV-1 controllers. CTL restricted by HLA B*27 had higher antigen sensitivity and were more functional, assessed by simultaneous degranulation and secretion of cytokines and chemokines, than CTL restricted by other HLA alleles in slow progressors (Berger *et al.*, 2011; Almeida *et al.*, 2007) (Almeida *et al.*, 2009). Enrichment of HIV-1 specific CTL with enhanced function, known as "polyfunctionality", was first described in nine elite controllers, with a strong HLA B*57 background (Betts *et al.,* 2006). CTL from HIV-1 controllers in contrast to HLA B*57 progressors maintain a high proliferative capacity in response to antigen, and high killing efficiency, with enhanced lytic granule loading and release of granzyme B and perforin (Migueles *et al.,* 2002) (Chen *et al.*, 2012) (Migueles *et al.,* 2008) (Hersperger *et al.,* 2011). In addition, HIV-1 controllers may select more "germline" TCR that are cross-reactive, and may respond earlier and recognise viral variants (Chen *et al.*, 2012). Recently in an animal model of HLA B*27 mediated elite control, protection of Indian Rhesus macaques against high viral loads of SIVmac239 was associated with early, high frequency, cytotoxic CD8+ T-

cells responding to Mamu B*08 restricted NefRL10epitope (residues 137 to 146) (Mudd *et al.*, 2012). Few of these protective phenotypes can be broadly characterised to the total HIV-1 CTL response. HLA B*08 is a common North European haplotype (*HLA A1-Cw7-B8-DR3-DQ2*) associated with autoimmunity, as is HLA B*27 and HLA B*57, but is associated with poor outcome following HIV-1 infection (Carrington and O'Brien, 2003) (Steel *et al.*, 1988) (Kaslow *et al.*, 1990). Over 70% of HLA B*08 individuals infected with HIV-1 respond to a highly conserved region of *Nef*, epitope FLKEKGGL (FL8), amino acid residues 90-97 (Altfeld *et al.*, 2006) (Korber *et al.*, 2001). The HLA B*08 restricted CTL response is distinct as the only HLA genotype included in a study of acute HIV-1 infection that restricted a high magnitude of HIV-1 specific CTL that did not correlate with the viral load at set-point (Streeck *et al.*, 2009). Although viral variants emerge during acute infection, the FL8-specific CTL response is also immunoprevalent in HLA B*08 individuals in chronic HIV-1 infection (Price *et al.*, 1997; Papagno *et al.*, 2002).

The devastating HIV-1 pandemic may be countered by development of a cytotoxic T cell based vaccine if these protective qualities can be defined at the molecular level, and extended to CTL restricted by a breadth of HLA types. This important question is addressed in this chapter.

3.2 Results

3.2.1 HIV-1 specific CD8+ T-cell clones that recognise the same epitope have "Good" and "Bad" *in vitro* viral suppression

Defining attributes of HIV-1 specific CD8+ T cells associated with durable control of HIV-1 replication is confounded *in vivo* by the heterogeneity of the total HIV-1 specific CD8+ T cell response. The *in vitro* viral suppression assay is widely regarded as the most relevant ex vivo assay to assess the quality of CD8+ T cell function (Watkins *et al.,* 2008). In order to identify intrinsic or ontogenetic factors that define optimal CD8+ T cell function, we reduced the *in vitro* viral suppression assay to its most simple, removing the heterogeneity of the CTL response and controlling for extrinsic factors such as antigen density, kinetics of antigen presentation, and intra assay variability in infection rates, by using a panel of HLA B*0801 restricted CD8+ T cell clones specific for one epitope, FLKEKGGL in NEF $_{(90\text{-}97)}$. FL8 is highly immunogenic, and is a conserved region in Nef between HIV-1 clades (Korber *et al.*, 2001). Clones were generated from three individuals with acute infection (Volunteers E833, G713 and G961) and from one long-term nonprogressor (LTNP005, Clones A3, A9, A17 and A20) by Dr Tao Dong (Table 3-1, Figure 3-1). We compared the ability of each clone to suppress p24 antigen production in an HLA matched (HLA B*08) CD4+ T-cell line (C8166), infected with X4-tropic laboratory strain virus, HIV-1_{MN}.

Table 3-1: Samples used to generate HLA B*08 restricted-FL8 specific CD8+ T-cell clones

Cohort	Patient ID	CLONES ID	Dx (Yr)	Date of Sample	Tetramer (% CD8+)	HLA Type	CD4+ Count (cells/ul)	Viral Load (Copies/ml)	HAART
London Riverside	LTNP005	A3, A9, A17, A20	1985	2001	1.29	A2, A24, B8, B57, Cw6, Cw7	572	52, 000	No
San Diego Acute	E833	-10, -11, -13, -18, -30, -38	1998	1998	0.64	A1, A1, B8, B38, Cw7, Cw12	320	600,100	No
Infection	G713	-4, -6, -16, -20	1999	1999	0.54	A3, A30, B8, B13, Cw6, Cw7	236	283,554	No
	G961	-48, -51, -56, -64, -71	1999	1999	0.74	A11, A30, B8, B55, Cw3, Cw7	378	1037	No

Table 3.1: Clinical information on four HIV-1 infected individuals who donated samples used to generate a panel of 19 HLA B*08 restricted-FL8 specific CD8+ T-cell clones. Year of diagnosis (Dx), Tetramer = percentage of FL8/HLA B*08 tetramer staining population of the total CD8+ population.

Figure 3-1: CD4+ T-cell count in donor LTNP 005

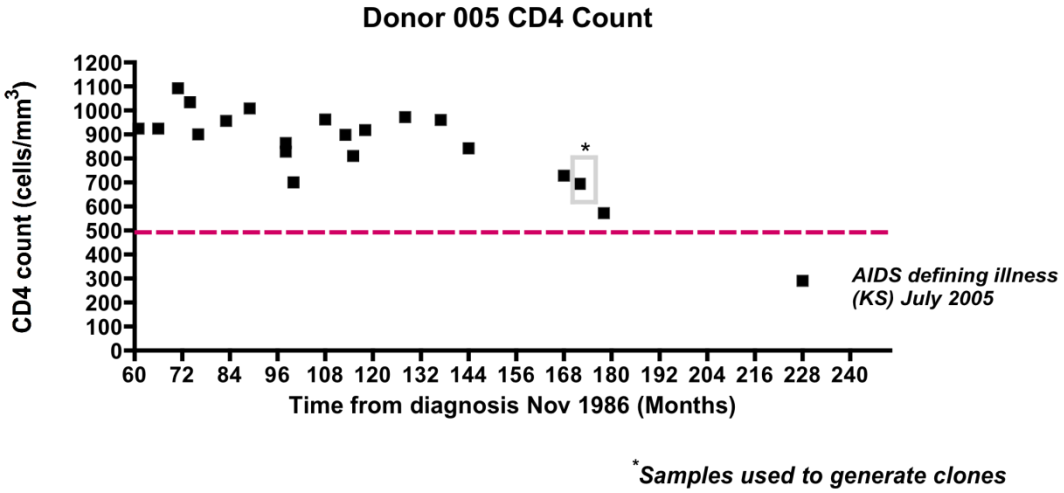


Figure 3.1: Donor 005 was defined as a long-term non-progressor (Papagno *et al.* 2002). Four HLA B*08-restricted FL8-specific CD8+ T-cell clones (A3, A9, A17 and A20) were cultured from PBMCs collected in 2000 (*), 14 years following the diagnosis of HIV-1 when the CD4 cell count was >500 cells/mm.[3] HIV-1 infection then gradually progressed with a decline in CD4+ T-cell count until the diagnosis of Kaposi Sarcoma (KS) in July 2005.

HLA B*08-FL8 clones prepared from PBMC taken in acute infection from one individual, Donor G961 (Clones G961-48, -51, -56, -64 and -71) suppressed p24 production similarly, from 51.2% (Clone G961 51, 1:16 ratio) to 68.8% (Clone G961 48, 1:16 ratio) (p=0.3 by t-test) (Figure 3-2). The panel of clones isolated from Donors G713 (G713-4, -6, -16 and -20) and Donor E833 (E833-10, -13, -18, 30 and -38) were all highly effective at suppressing p24 production, so the stringency of the VSA was enhanced by reducing the E:T ratio to 1:128 (Figure 3-2B). Others have also found that HIV-1 specific CD8+ T cell clones can effectively suppress at high MOI and low E:T target ratios (1:1000) (Yang *et al.*, 1997). No difference in suppression could be detected between clones E833 -13 and -30, but clone G713-20 was more suppressive than G713-4 at low E:T ratio (1:128 E:T ratio, 90% v 5% suppression, p=0.0014 by t-test).

This thesis focuses on the four clones, A3, A9, A17 and A20, isolated from LTNP005 during chronic infection. A20 exhibits "good" HIV-1 suppression, reducing mean p24 production by 92.00% (+/- 3.05), in comparison to 59.08% (+/- 5.72) in clones A3 and A9 (p=0.0005 by t-test), and 17.72% (+/- 4.95) (p=<0.0001) in clone A17 at 1:16 ratio (Figure 3.2C). In this viral suppression assay, efficacy of viral suppression correlates with elimination of p24 positive cells (A20 96.1% to 17.95% at 1:16 ratio) (Figure 3-3). Consistent with other reports, p24 suppression is dependent on classical MHC-restricted CD8+ T cell activation, as even at high E:T ratio, 1:2, G-10, the HLA mis-matched clone does not suppress p24 production (Figures 3-2 and 3-3) (Yang *et al.*, 1997; Saez-Cirion *et al.*, 2007).

Figure 3-2: In vitro viral suppression of HLA B*08-restricted FL8-specific CD8+ T-cell clones isolated during acute infection

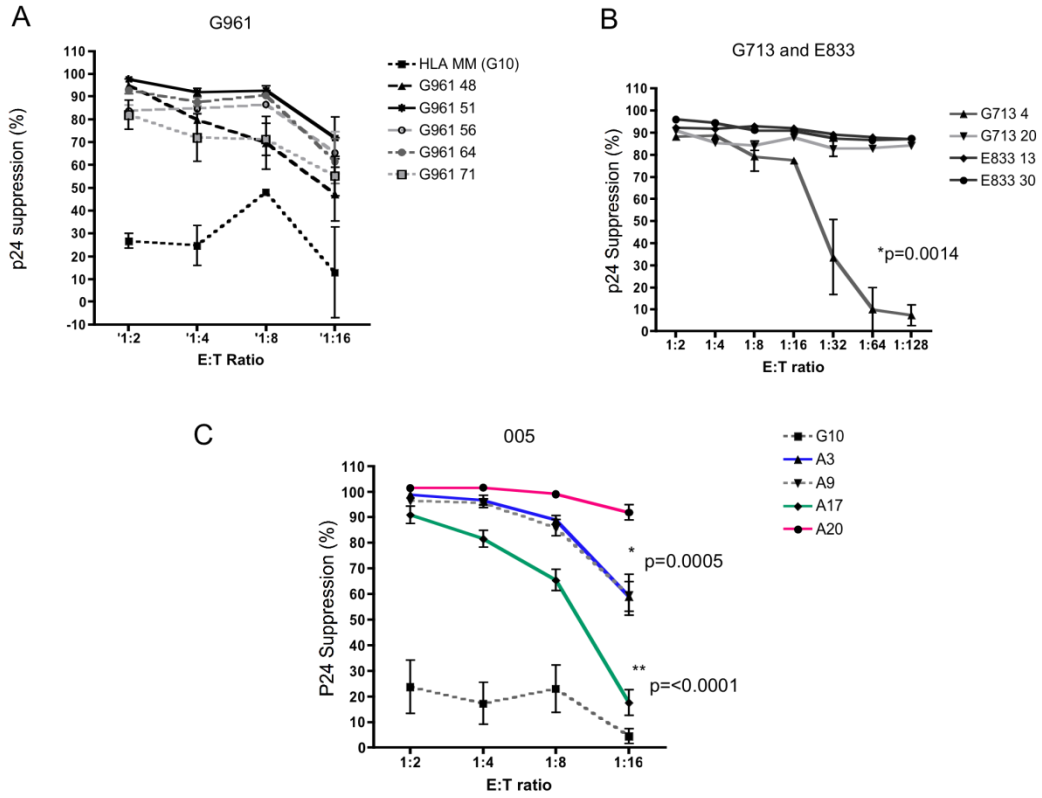


Figure 3.2: HLA B*08-restricted FL8- specific CD8+ T-cell clones were compared in an *in vitro* viral suppression assay. Clones were cultured with HIV-1_{MN} infected HLA B*0801 CD4+ T-cell line (C8166) cells at E:T ratios 1:2, 1:4, 1:8 and 1:16 (y-axis). p24 antigen concentration was determined by ELISA in the supernatant on day 4, and used to calculate the percentage of p24 suppression (x-axis). **A)** B8-FL8 clones isolated during acute infection from donor G961 (G961-48, -51, -56, -64 and -71), ((G961-51 v G961-48 (p=0.3)(t-test) (E:T ratio 1:16)). **B)** B8-FL-8 clones isolated during acute infection in donors G713 (G713 -4, and -20) and E833 (-13, and -30) at E:T ratio extending to 1:128 (G713-4 v G713-20 (*p=0.0014) (t-test) (E:T ratio 1:128)). Beyond these dilutions results became non-reproducible. **C)** B8-FL8 clones isolated during chronic infection from donor LTNP005 (A-3, -9, -17 and -20) (A20 v A3 (*p=0.0005) and A20 v A17 (**p<0.0001) (t-test) (E:T ratio 1:16)). G10 is an HLA-mismatched control clone, HLA A*02-restricted gag $p17_{(77-85)}$ SLYNTVATL (SL9)-specific (Lee *et al.*, 2004). Results are shown from at least two independent experiments, with errors bars indicating the S.D.

Figure 3-3: Intracellular p24 staining of HIV-1MN infected CD4+ T-cell line cells on day 4 of the viral suppression assay

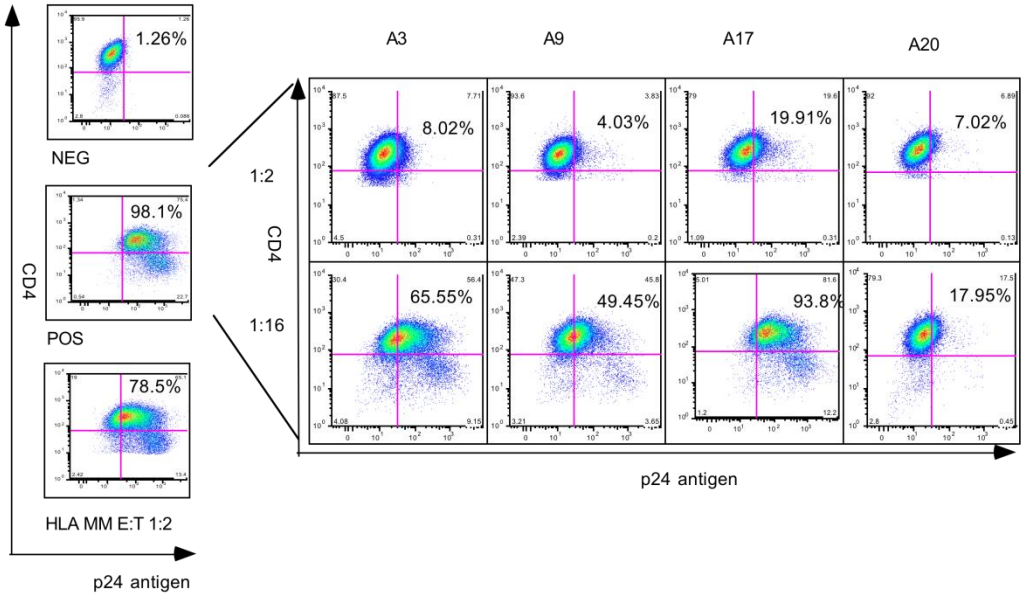


Figure 3.3: Intracellular P24 antigen staining in HIV-1$_{MN}$ infected CD4+ C8166 on day 4 of *the in vitro* viral suppression assay. Flow cytometry data from a representative *in vitro* viral suppression assay to show the percentage of p24 antigen+ CD4+ T-cell line (C8166) cells following co- culture for 96 hours alone (Positive control (POS)), with an HLA mismatched clone (G10) (HLA MM), uninfected C8166 (Negative control, NEG), and four clones isolated from Donor 005 (A3, A9, A17 and A20) at the indicated E:T ratio (1:2 or 1:16). Data was acquired on single, live CD4 positive cells.

In conclusion, CD8+ T cell anti-viral efficacy was not defined by the timing of isolation of HLA B*08-restricted FL8-specific CD8+ T-cell clones during acute or chronic HIV-1 infection. *In vitro* efficacy of the clones isolated from each individual could not be correlated to *in vivo* HIV-1 control, as individuals with acute HIV-1 were treated with HAART immediately post-diagnosis. A panel of four HLA B*08-restricted FL8-specific CD8+ T-cell clones (A-3, A-9, A-17 and A-20) were isolated from one long-term non-progressor that had distinct anti-viral efficacy. Clone A20 was a "good" viral suppressor in comparison to clones A3, A9 and A17.

3.2.2 HLA B*08-FL8 CD8+ T-cell clones isolated from chronic infection with distinct HIV-1 suppression use identical T-cell receptors

The T cell receptor is the key structure that determines antigen recognition. HIV-1 controllers may select CTL that use more "germline" TCR that are cross-reactive (Chen *et al.*, 2012). A high sensitivity TCR repertoire with broad cross-reactivity may enhance control of persistent viral infections (Davenport *et al.*, 2007). To investigate if the TCR could account for the observed differences in viral suppression, the junctional region of the T cell receptor (TRA and TRB chains) of all the HLA B*08-FL8 specific CD8+ T-cell clones was sequenced (Table 3-2). Surprisingly, all clones from LTNP005 (A3, A9, A17 and A20) that had distinct anti-viral efficacy *in vitro*, had identical TCR sequences at the nucleotide level in the CDR3 region of both TRA and TRB chains (Figure 3-4). This finding suggests that populations of CD8+ T-cell clones that use the same TCR can have unique *in vitro* viral suppression.

Table 3-2: Junctional sequences (CDR3) of the TRA and TRB chains of HLA B*08-restricted FL8 CD8+ T-cell clones

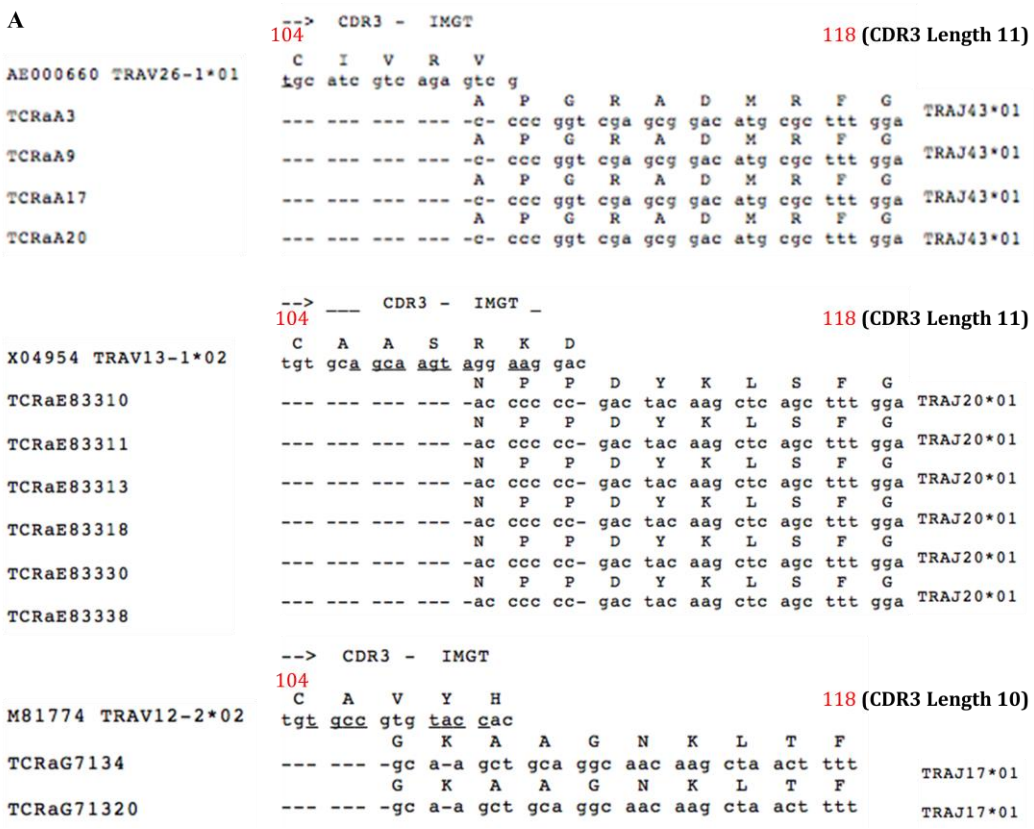
Patient ID	CLONE ID	TRAV	TRAJ	CDR3 AA JUNCTION	TRBV	TRBD	TRBJ	CDR3 AA JUNCTION
LTNP005	A3	26-1*01	43-1*01	CIVRAPGRADMRF	6-2*01	1*01	1-5*01	CASSYLPQGDHYSNQPQHF
	A9	26-1*01	43-1*01	CIVRAPGRADMRF	6-2*01	1*01	1-5*01	CASSYLPQGDHYSNQPQHF
	A17	26-1*01	43-1*01	CIVRAPGRADMRF	6-2*01	1*01	1-5*01	CASSYLPQGDHYSNQPQHF
	A20	26-1*01	43-1*01	CIVRAPGRADMRF	6-2*01	1*01	1-5*01	CASSYLPQGDHYSNQPQHF
G713	G713 4	12-2*02	17*01	CAGKAAGNKLTF	6-2*01	1*01	1-5*01	CASSFQPGQGLFFSNQP
	G713 6			ND	11-2*01	2*01	2-3*01	CASSLVEGLPDTQYFGPGTRL
	G713 16	13-1*02	7*01	CAASAPGGNNRLAF	7-8*01	2*01	2-1*01	CASSLVQGLPNEQFF
	G713 20	12-2*02	17*01	CAGKAAGNKLTF	6-2*01	1*01	1-5*01	CASSFQPGQGLFFSNQP
E833	E833 10	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 11	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 13	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 18	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 30	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 38	13-1*02	20*01	CAASNPPDYKLSF	6-2*01	2*02	2-1*01	CASSYNTPGNEQFF
	E833 48	8.3*02	54*01	CAVGAPYSIQGAQKLVF	4-3*01	1*01	2-7*01	CASSQDVGQGSYEQYF
G961	G961	5*01	18*01	CAEGALGRLYF	27*01	1*01	2-7*01	CASSSGQGLLSYEQYF
	G961 48			ND				ND
	G961 51	17*01	24*02	CAGRKTTDSWGKLQF	18*01	1*01	2-2*01	CASSPPERTDANTGELFF
	G961 56			ND	30*01	1*01	2-7*01	CAWSARQGYEQYF
	G961 64	8-6*02	29*01	CAVRMNSGNTPLVF	2*01	2*01	2-7*01	CASSPFGASVYEQYF
	G961 71	5*01	18*01	CAEGALGRLYF	27*01	1*01	2-7*01	CASSSGQGLLSYEQYF

Table 3.2: Junctional sequences (CDR3) of the TRA and TRB chains of a panel of HLA B*08-restricted FL8 CD8+ T-cell clones. HLA B*08-FL8 clones isolated from one individual may share an identical TCR. Clones from one individual that use the same TCR are highlighted in colour. TRAV, TRAJ, TRBV, TRBD, TRBJ usage and CDR3 amino acid sequence are shown for each clone.

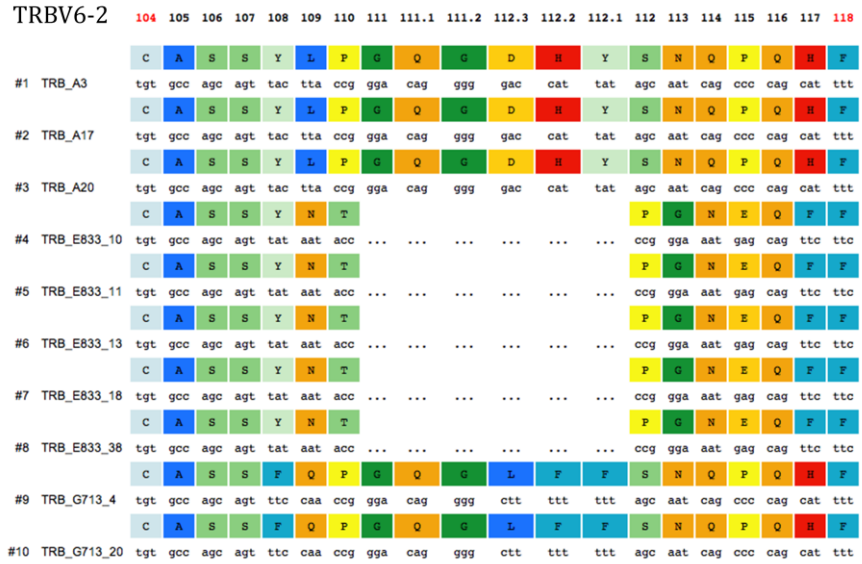
Figure 3.4 (Next Page): Junctional sequences (CDR3) of the TRA and TRB chains of HLA B*08-restricted FL8 CD8+ T-cell clones. A) TRA junctions (CDR3) and **B)** TRB junctions (CDR3) were sequenced for each HLA B*08-restricted FL8- specific CD8+ T-cell clone (see Table 1). TRA sequences were aligned with genomic TRA sequences using IMGT/V-QUEST (Giudicelli and Lefranc, 2011).

Figure 3-4: TRA and TRB complementarity determining region 3 (CDR3) sequences for HLA B*08-FL8 CD8+ T cell clones

A



B



From the panel of acute clones, six out of seven clones isolated from donor E833 used the same TCR (E833-10, -11, -13, -18, 30 and -38) and two clones isolated from donor G713 used the same TCR (G713 -4, and -20) (Figure 3-4). T cell receptor landscaping was performed on the TRB chain of the FL8-HLA B*08 tetramer positive population from individuals E833, G713 and G961 (Figure 3.5) to explore if the clones isolated were representative of the in vivo HLA B*08-FL8-specific TCR repertoire. This was the case as TRBV6-2 was dominant in individual E833, co-dominant in individual G713 and non-dominant in individual G961. Staining of PBMC from LTNP005 by Dr Tao Dong confirmed 51% of the B8-FL8 tetramer response in donor LTNP005 used TRBV 6-2 (Dong *et al.*, 2004).

In conclusion, the panel of clones isolated from donor LTNP 005 all use an identical TCR yet have distinct anti-viral efficacy *in vitro.*

3.2.3 HLA B*08-restricted FL8-specific CD8+ T-cell clones with the same TCR have distinct antigen sensitivity that correlates with control of HIV-1 replication

We observed MHC class I restricted elimination of infected cells correlated with viral suppression, suggesting that killing is an important mechanism of anti-viral efficacy. In addition, we sought to determine if antigen sensitivity correlates with anti-viral efficacy. Functional avidity refers to the concentration of antigen required to elicit half-maximal effector function (EC_{50}), usually measured by cytokine release or cell lysis (Yang *et al.*, 2003). Functional avidity will be referred to as T cell antigen sensitivity (sensitivity). TCR affinity is a central component of TCR sensitivity, yet controversially, sensitivity maturation without selection of high affinity clonotypes, and mechanisms to maintain clonal diversity by compensating for lower affinity TCR

Figure 3-5: Tc landscape plots on FL8-HLA B*08 tetramer staining population in acute infection

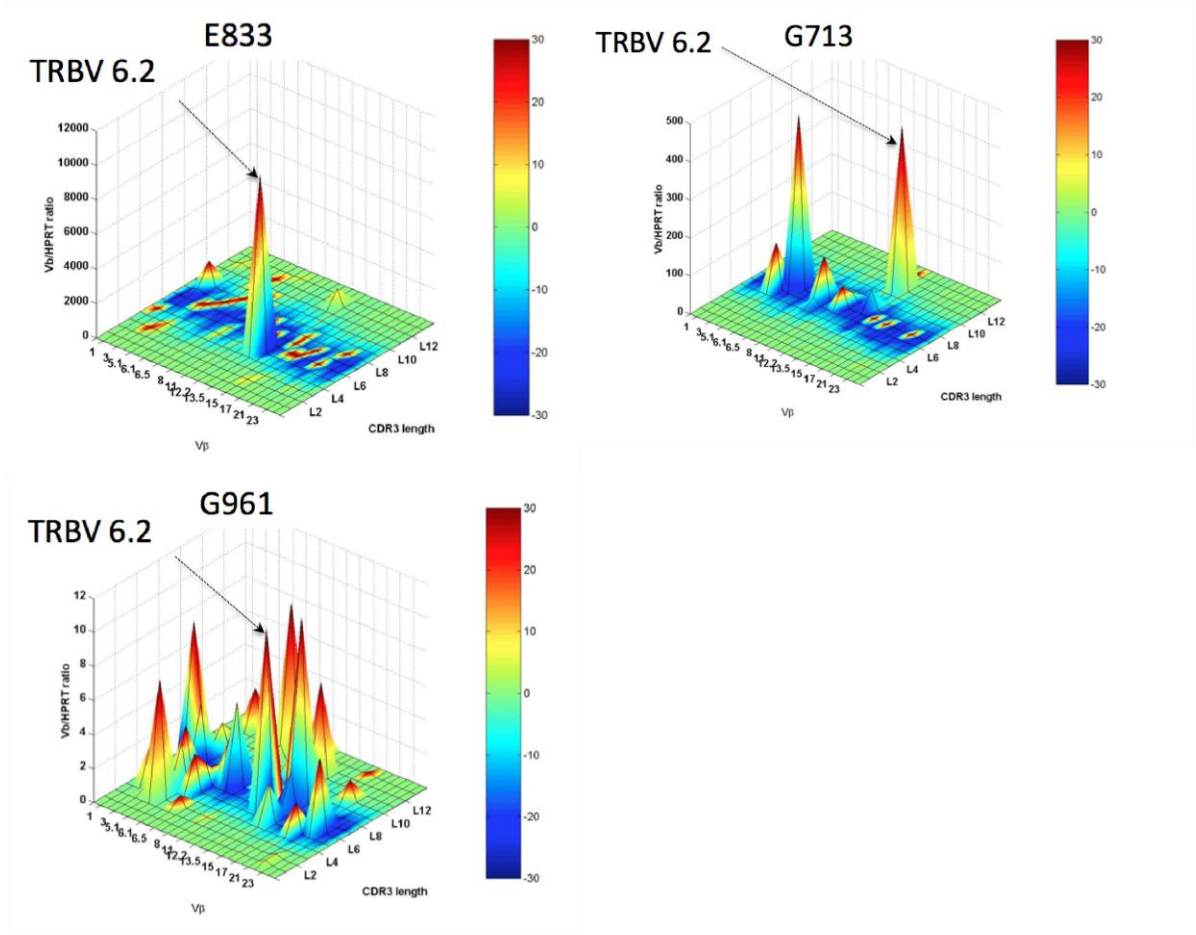


Figure 3.5: Tclandscape graphic representation of the TRBV repertoire of HLA B*0801-FL8 MHC class I tetramer populations from three individuals (E833, G713 and G961) with acute HIV-1. TRBV 6.2 is indicated (arrows). Total RNA was isolated from three HLA B*08-FL8 tetramer populations, amplified by PCR and sequenced to provide a combined quantitative TRBV usage and qualitative CDR3 length distribution for each TRBV family. TRBV (Vβ) families are indicated on the x-axis in Arden TCR nomenclature (Arden, B., Clark, S. P., Kabelitz, D. & Mak, T. W. Human T-cell receptor variable gene segment families. Immunogenetics 42, 455-500 (1995)), the y-axis shows the CDR3 lengths and the z-axis the Vβ-HPRT transcript ratio. The colour of each peak indicates the percentage alteration and is indicated on the right of each plot as a colour code from deep blue to dark red.

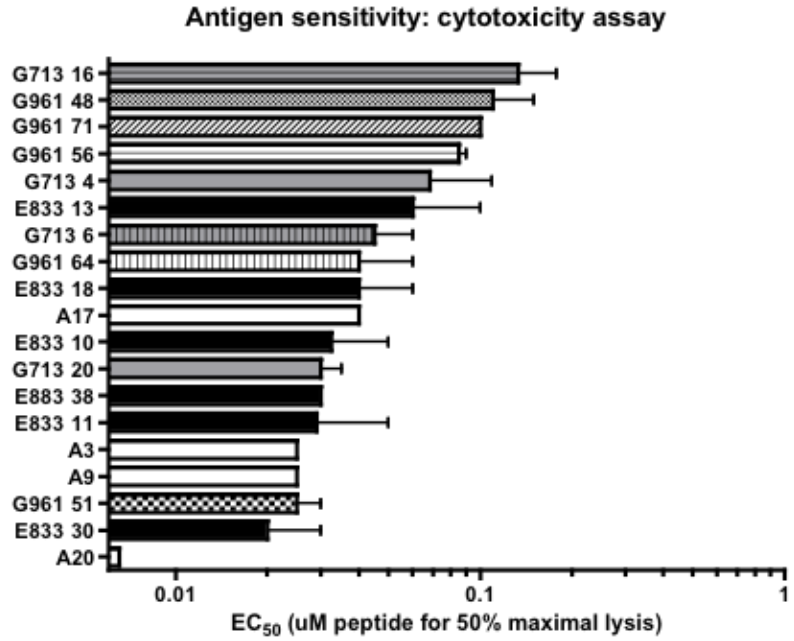
have been reported (Slifka and Whitton, 2001; Busch and Pamer, 1999; Kedzierska *et al.*, 2005) (Price *et al.*, 2005; van Gisbergen *et al.*, 2011). To assess if antigen sensitivity, or specific killing correlate with elimination of infected target cells as determined in the viral suppression assay, all 19 clones were assessed in a standard ^{51}Cr release assay (Figure 3-6).

It was found that HLA B*08-restricted FL8-specific CD8+ T-cell clones with the same TCR can indeed have distinct antigen sensitivity. The four clone populations isolated from donor LTNP005 during chronic infection that all use the same TCR, have a distinct range of antigen sensitivity (EC_{50}: A20 0.0065uM v A3 and A9 0.025uM and A17 0.04uM) (Figure 3-6B). Clone A20 is more potent at killing at 10uM peptide: this is the 'good' suppressor, and has the highest antigen sensitivity. Of note, at peptide concentrations thought to be close to the level of epitope presented during HIV-1 infection (10^{-8}M = 0.01uM) (Varela-Rohena *et al.*, 2008) enhanced antigen sensitivity of clone A20 also results in higher specific cell lysis (normalised results: A20 60%, A3 and A9 25%, and A17 15%).

The finding that populations of clones with the same TCR can have distinct antigen sensitivity was extended to two additional TCRs in the panel of clones isolated from individuals with acute infection. The five clones isolated from donor E833 (-10, -11, -13, -18 and -30), that all use the same TCR, also TRBV 6-2 that was dominant on Tc landscaping, demonstrate a range of sensitivity (EC_{50} 0.02uM to 0.06uM) (Figure 3-7). This range of sensitivity was confirmed in a second assay, using CD107a staining (EC_{50} 0.3 uM-0.07 uM) (Figure 3-7B).

Figure 3-6: Antigen Sensitivity of a Panel of HLA B*08-FL8 CD8+ T-cell Clones

A



B

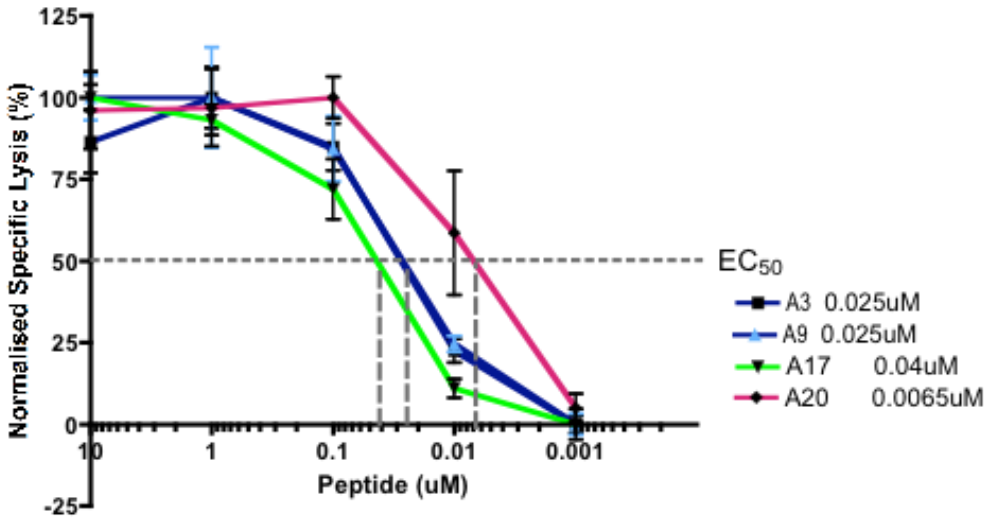


Figure 3-6: Antigen sensitivity of 19 HLA B*08-restricted FL8-specific CD8+ T cell clones.

A) Standard four-hour chromium release assay was used to determine the sensitivity (EC_{50}= peptide concentration (uM) for 50% maximal lysis) for 19 CD8 T cell clones. Effector to target (E:T) ratio 3:1. Antigen sensitivity (EC_{50}= peptide concentration (uM) for 50% maximal lysis). Clones that use the same TCR are indicated in the same colour. A minimum of two experiments was performed for each clone, with each data point in duplicate. (Error bars, s.d.) **B)** Antigen sensitivity of clones isolated from donor LTNP 005 (A20, A3, A9 and A17). Data from five independent experiments performed on day14 post-stimulation on five generations of clones (error bars, s.d.)

Figure 3-7: Antigen sensitivity by Standard ^{51} Cr-release in HLA B*08-restricted FL8-specific CD8+ T-cell Clones Isolated from an individual with acute HIV-1 (E833)

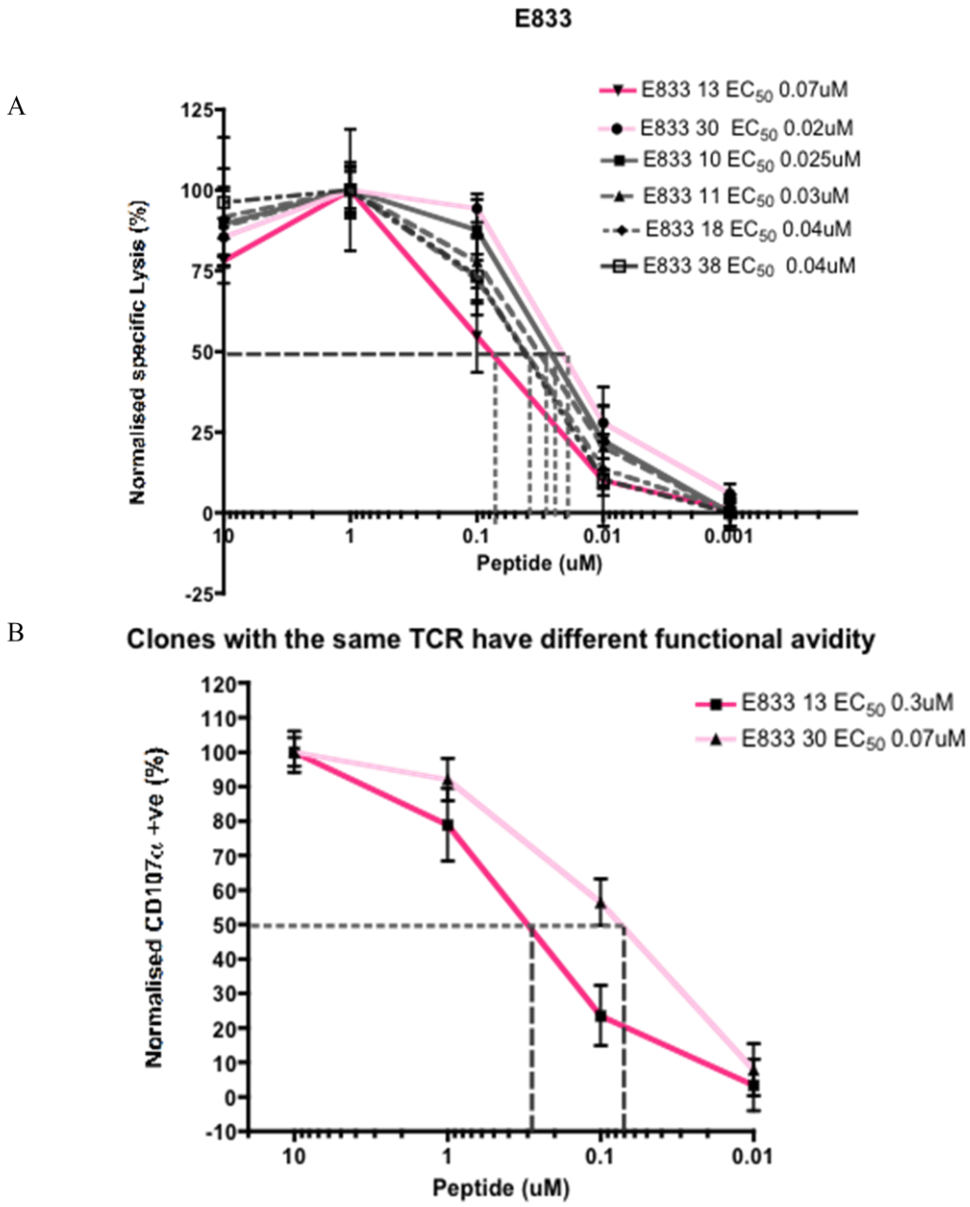


Figure 3.7: Antigen sensitivity of HLA B*08-restricted FL8-specific CD8+ T cell clones that use the same TCR from individual E833. A) Antigen sensitivity for clones (E833-10, -11, -13, -18, -30 and -38) determined by ^{51}Cr release assay. **B)** Antigen sensitivity of two clones from acute infection (E833-13, and -30) in a second assay, determined by 50% maximal anti- CD107a mAb staining. Results are from three experiments, (error bars s.d.).

Two clones isolated from individual G713 (G713 -4 and -20) that used the same TCR also had distinct sensitivity (EC_{50} 0.8uM and 0.2uM). In the above viral suppression assay, the higher sensitivity clone, G713-20 had enhanced anti-viral efficacy at low E:T ratio, compared with the lower sensitivity population, G713-4.

In fact, antigen sensitivity could be demonstrated to correlate with viral suppression in HLA B*08-restricted FL8-specific CD8+ T-cell clones isolated during acute (regression analysis R^2=0.09 (p=0.047)) and chronic infection (R^2=0.95, p=0.0229)(Figure 3-8).

In conclusion, populations of HLA B*08-FL8 CD8+ T-cell clones that use the same TCR can have distinct antigen sensitivity. Antigen sensitivity correlates with good control of HIV-1 replication *in vitro.* The range of antigen sensitivity in the panel of clones generated during acute infection is broader than in the chronic panel (0.02uM-0.1uM acute clones v 0.00625uM-0.04uM chronic clones). Of note, clone A20, isolated during chronic infection has the highest sensitivity of all of the clones (EC_{50} 6.5 ng), which correlates with enhanced viral suppression compared to clone A3, A9 and A17, and results in a higher percentage of specific target cell lysis at physiological peptide stimulus.

Figure 3-8: Viral suppression correlates with antigen sensitivity

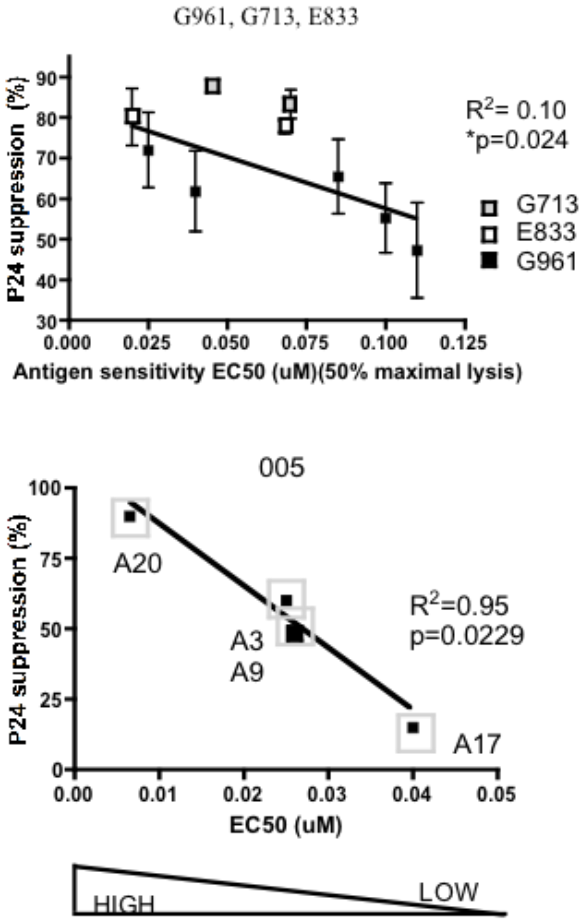


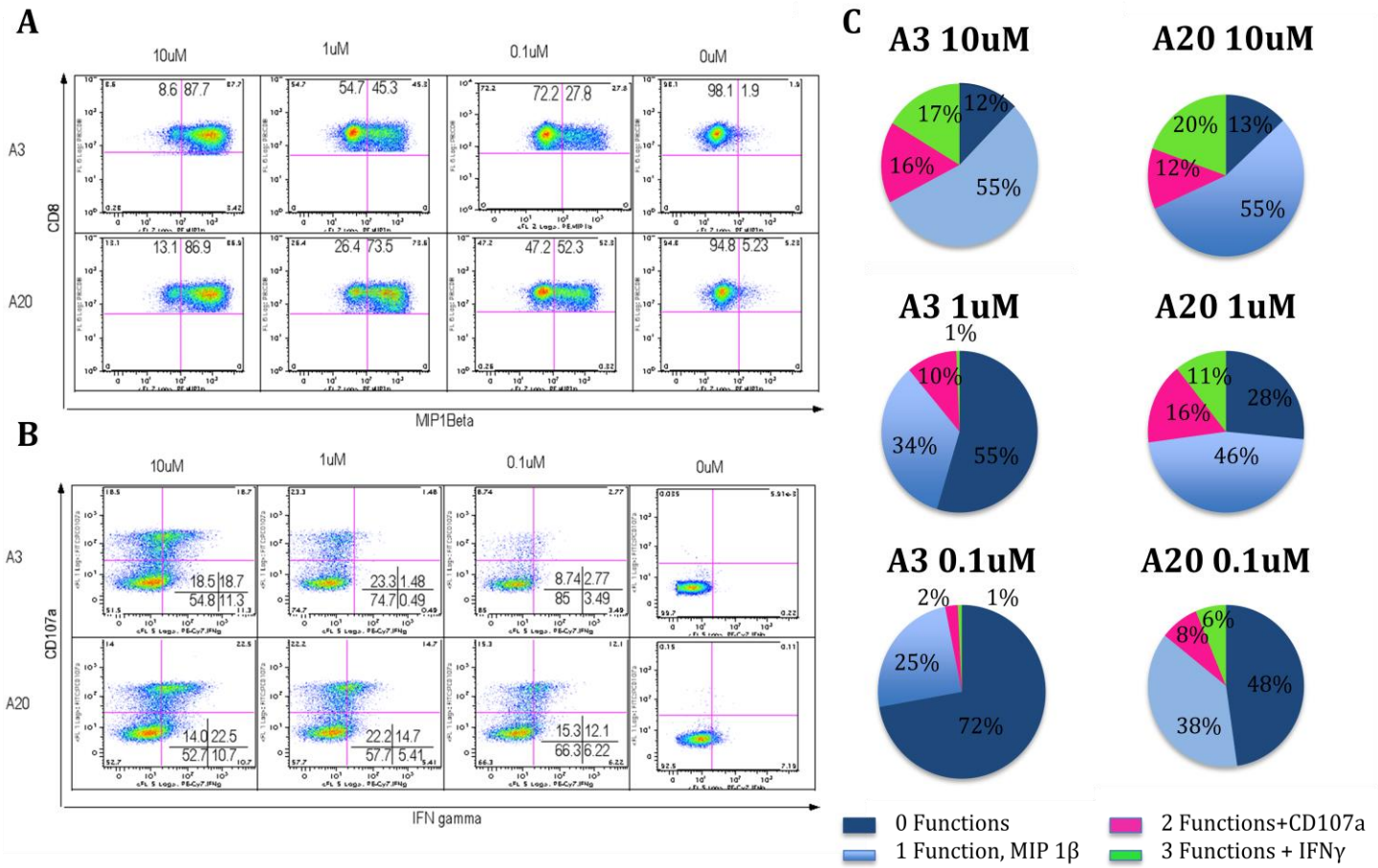
Figure 3.8: High antigen sensitivity correlates with viral suppression. A) Linear regression of sensitivity and p24 suppression (1:16 ratio) for a panel of 9 acute HLA B*08-restricted FL8-specific CD8+ T cell clones that use different TCR (black squares) and two pairs of clones (G713-4 and G713-20 (grey); E833-13 and E833-30 (white)) that each use the same TCR as indicated. **B)** There is a strong correlation of viral suppression and antigen sensitivity in clones A3, A9, A17 and A20 (as indicated) that use the same TCR isolated from chronic infection.

3.2.4 "Polyfunctional Sensitivity" Correlates with *in vitro* Viral Suppression

Cytotoxic T cells also secrete chemokines CCL3, CCL4 and CCL5 that compete with R5 tropic HIV-1 strains for the chemokine co-receptor CCR5 to prevent viral entry (Cocchi *et al.*, 1995; Walker *et al.*, 1991), and cytokines IL2, IFNγ and TNFα. It is reported that effector T cell functions are acquired in an ordered succession depending on different thresholds of antigen density and TCR occupancy (Valitutti *et al.*, 1996). In HIV-1 specific clones, it was shown that depending on the strength of TCR stimulus, first CCL3 (MIP1α), second degranulation (CD107a), third IFNγ ≥ TNFα, and finally IL2 are induced (Almeida *et al.*, 2009). In order to investigate if clone A20, with "good" viral suppression also has a higher sensitivity in a second experimental system by broader cytokine secretion profile at lower peptide stimulus, "polyfunctional sensitivity" was assessed. Our polyfunctional sensitivity assay demonstrated that at lower peptide stimulus (0.1uM), 52% of clone A20 responded with at least one function, in comparison to 28% in clone A3. 6% of clone A20 responded with all three possible parameters in comparison to 1% of clone A3 (Figure 3-9).

Figure 3.9 (Next Page): **Intracellular cytokine staining of two HLA B*0801-FL8 CD8+ T-cell clones, A3 and A20, isolated from LTNP005.** ICS was performed following 6 hour incubation (10:1 ratio) with peptide pulsed CD4+ T-cell line C8166 cells (10uM, 1uM and 0.01uM). Cells were stained for surface CD107a and intracellular MIP1β (CCL4), IFNγ and TNFα, and gated on live, single CD8+ cells. **a**) Flow cytometry plots of MIP1β staining. The percentage of negative and positive populations are indicated on each plot. (**b**) Flow cytometry plots for CD107a and IFNγ staining. Cells are gated on MIP1β positive cells from Fig 3A. Percentage of cells that stain for MIP1β only, MIP1β and CD107a, MIP1β, CD107a and IFNγ and MIP1β and IFNγ is given in figures in the quadrant on each plot. (**c**) Pie charts representing the percentage of each clone responding with 0, 1, 2 and 3 simultaneous effector responses for a given peptide concentration (Blue= 0 functions, light blue= MIP1β, pink= MIP1β and CD107a+, green= MIP1β, CD107a and IFNγ).

Figure 3-9: Intracellular cytokine staining to measure "poly sensitivity" of two HLA B*08-FL8 CD8+ T-cell clones, A3 and A20, isolated from LTNP 005



In conclusion, clone A20 is more sensitive in two experimental systems, which at low physiological peptide stimulus results in enhanced simultaneous expression of the available repertoire of effector functions, enhanced killing, as determined in the ^{51}Cr release assay, and confirmed in this assay by the higher percentage of cells that degranulate (CD107a+).

3.2.5 HLA B*08-FL8 CD8+ T-cell Clones have Distinct Cytokine Profiles

In the above experiment, Clone A20 did not secrete either TNFα or IL2 at all peptide concentrations (10uM to 0.1uM). This observation was confirmed using illumina bead array technology to analyse the supernatant from co-cultures of CD8+ T-cell clones (A3, A9, A17 and A20) activated using epitope (10uM) pulsed HLA B*08+ CD4+ T-cell line (C8166) cells (Figure 3-10). Clone A9 was excluded from subsequent analysis due to high spontaneous IFNγ secretion. Clone A3 was the only population of CD8+ T-cell clone to secrete TNFα and IL2 in small quantities in addition to IFNγ and CCL4 (MIP1β), a profile that has been previously described as "polyfunctional" and reported to be preferentially maintained in HIV-1 non-progressors (Betts *et al.*, 2006). However, Clone A3 is less effective than clone A20 at HIV suppression *in vitro* as reported above. This suggests that it is not the extended cytokine repertoire *per se* that is required for effective viral suppression.

Figure 3-10: Cytokine and chemokine profile of HLA B*08-FL8 CD8+ T-cell clones isolated from LTNP 005

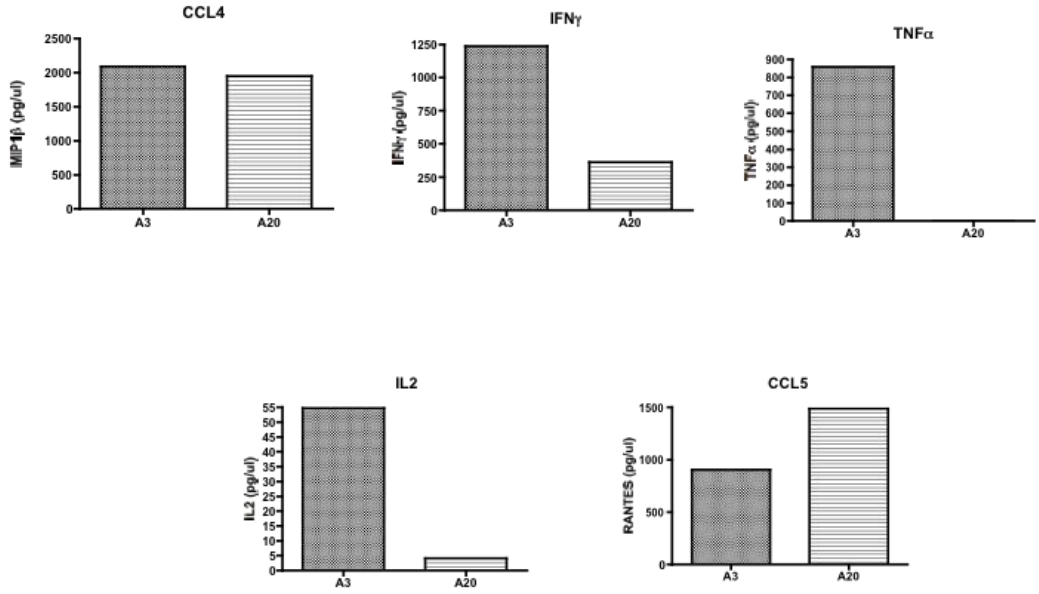


Figure 3-10: Populations of CD8+ T-cell clones isolated from individual 005 have distinct cytokine profiles. Illumina bead array technology was used to analyse cytokines and chemokines in the supernatant harvested following 24 hours of co-culture of CD8+ T-cell clones (A3 and A20) and epitope (10uM) pulsed CD4+ HLA B*08+ T-cell line (C8166) cells at 10:1 ratio. Clone A9 was excluded from subsequent analysis due to high spontaneous IFNγ secretion. Clone A20 does not secrete TNFα. Each assay was performed in triplicate, with the mean concentration depicted.

Figure 3-11: Cytokine and chemokine secretion of HLA B*08-FL8 CD8+ T-cell clones activated for 24 hours

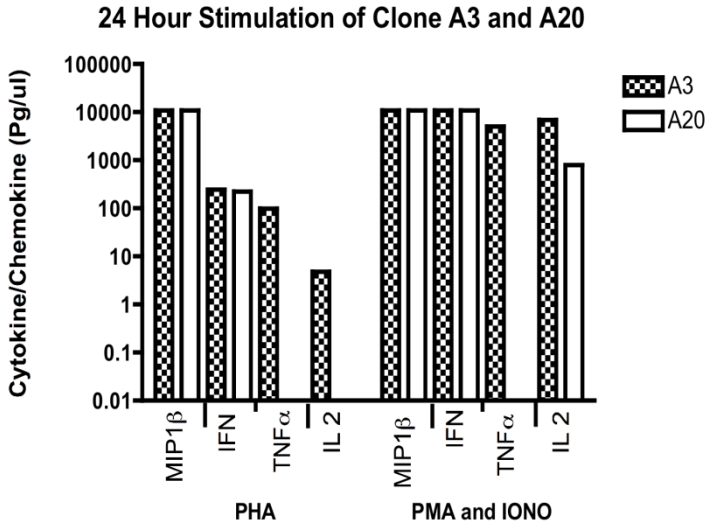


Figure 3-11: Clone A20 does not secrete TNFα in contrast to clone A3. Clones were stimulated in duplicate for 24 hours with phytohaemagglutinin (PHA) or phorbol-myristate (PMA) and ionomycin (IONO) and soluble factors in the supernatant assayed using luminex bead technology. IL2 secretion can be induced in clone A20 by activating the clone below the TCR.

3.2.6 Clone A20 does not secrete TNFα despite maximal TCR stimulation

To determine if clone A20 was capable of TNFα production, clones were stimulated through the TCR with phytohaemagglutinin (PHA) and by using ionomycin and phorbol myristate acetate (PMA). TNFα could not be induced in clone A20 (Figure 3-11). TNFα secretion is therefore modulated not only by TCR activation but by additional mechanisms downstream of maximal TCR signal strength. This finding is in contrast to that for IL-2 that was induced in clone A20 by agents that activate the T-cell downstream of the proximal TCR signalling complex.

In conclusion, a panel of four populations of HLA B*08 restricted-FL8 specific CD8+ T-cell clones that use the same TCR isolated from an individual with chronic HIV-1 infection, LTNP005, have distinct cytokine profiles, and distinct ability to suppress HIV-1 replication *in vitro*, that correlates with antigen sensitivity. The observation that clones with the same TCR have distinct antigen sensitivity was extended to two additional T-cell receptors on populations of CD8+ T-cell clones isolated from acute infection.

3.3 Discussion

In a panel of four populations of HLA B*08-FL8 CD8+ T-cell clones that use the same TCR isolated from a long term non-progressor, we found distinct ability to suppress HIV replication *in vitro.* The T cell quality most closely linked to viral suppression was high antigen sensitivity. The mechanism of viral suppression was MHC class I restricted, required recognition of a conserved epitope, and appeared to correlate with elimination of p24 positive cells, suggesting a predominant role for killing. Of note, the optimal suppressor clone, A20, did not secrete TNFα or IL2, which have been reported to activate viral replication directly or activate CD4+ T cells that enhances HIV-1 replication respectively.

3.3.1 Antigen Sensitivity and Control of Persistent Viral Infections

CTL with high antigen sensitivity are more effective at clearing viral infections in mice and in clearing acute hepatitis C in man (Alexander-Miller *et al.*, 1996) (Sedlik *et al.*, 2000; Yerly *et al.*, 2008). High antigen sensitivity has been associated with durable HIV-1 control, as high sensitivity gag-specific CTL were preferentially reported in HIV-1 controllers and not chronic progressors (Mothe *et al.*, 2012) (Berger *et al.*, 2011). The HLA genotype may contribute to the antigen sensitivity of the HIV-1 CTL response (Berger *et al.*, 2011) (Messaoudi *et al.*, 2002) (Iglesias *et al.* 2011). The MHC restriction of high sensitivity CTL may be one mechanism of epitope-HLA associated protection, as HLA-B*27-restricted KK10-specific CTL were of higher sensitivity than CTL restricted by other HLA alleles in slow progressors (Almeida *et al.* 2007) (Berger *et al.*, 2011). Evidence that high sensitivity CTL can directly limit HIV-1 replication has been reported *in vitro,* as high sensitivity HLA B*27 CTL clones are more suppressive than low sensitivity CTL clones (Almeida *et al.*, 2009). Our findings extend this observation to HLA B*08 restricted

CTL clones targeting Nef. Conflicting data on the role of sensitivity and HIV-1 control have been published, in assays confounded by the kinetics of epitope presentation, or with small numbers of HIV-1 specific CTL clones (Chen *et al.*, 2012) (Bennett *et al.*, 2007) (Yang *et al.*, 2003) (Vojnov *et al.*, 2010) (Chen *et al.*, 2009). Our observation that sensitivity maturation and rather than selection of high affinity TCR is critical to HIV-1 suppression *in vitro* is not previously reported.

3.3.2 Antigen Sensitivity and Enhanced Cytolysis

It is not clear how high sensitivity leads to enhanced viral suppression. One factor supported by our data is early recognition of the infected cell and a potent CTL function triggered in response to only a few epitope-MHC I alleles (Derby *et al.*, 2001) (Messaoudi *et al.*, 2002). Our data support the role of MHC class I restricted cytotoxicity as the predominant mechanism of suppression *in vitro* (Saez-Cirion *et al.*, 2007). A caveat is that our experiments were performed in media, and cytolysis within a 3D tissue may require soluble factors to optimise the tissue environment. Cytotoxicity requires recognition of only three peptide-MHC complexes (Purbhoo *et al.*, 2004), and may not be enhanced by increasing the epitope density beyond a specific threshold (Sykulev *et al.*, 1996). Killing efficiency may however be enhanced downstream of the TCR, as TCR signal strength and quality dictates the route of granule polarisation, and the efficiency of delivery of granule contents to the target cell is enhanced if a complete peripheral supra-molecular activation complex or immune synapse forms (Purbhoo *et al.*, 2004) (Beal *et al.*, 2008) (Jenkins *et al.*, 2009; Beal *et al.*, 2009). One interpretation of our observed different viral suppression in CTL clones that use the same TCR is that the fine modulation of the threshold of TCR signal that triggers cytotoxicity in response to small numbers of epitope-MHC

complexes is crucial. TCR signal strength may also regulate the transcription of perforin and granzyme B that could be required for effective serial killing, either modulating the kinetics or quantity of lytic molecule expression (Makedonas *et al.*, 2009) (Makedonas *et al.*, 2010). TCR sensitivity may modulate proliferation that has been linked to perforin expression (Migueles *et al.*, 2002) (Jenkins *et al.*, 2008) (Almeida *et al.*, 2007). Our data support a critical role for enhanced cytotoxicity that arises by modulation of signalling dynamics downstream of the TCR and a lower threshold for cytotoxicity in CTL that control HIV-1 infection.

We find that killing efficiency becomes critical if the peptide or CTL concentration is limiting. HIV-1 specific CTL are likely to encounter infected cells within the lymphoid tissues, particularly the GALT (Eisele and Siliciano, 2012) (Mehandru *et al.*, 2004). Encounter at sufficient concentrations will therefore require effective migration, and proliferation, requiring chemokines and cytokines. Soluble factors secreted by CTL following activation by epitope-MHC complexes have been reported to contribute to viral suppression (Cocchi *et al.*, 1995) (Walker *et al.*, 1986). In contrast to previous reports that link polyfunctionality, or the simultaneous secretion of chemokines and cytokines, to viral control, we find that the CTL clone that is most suppressive *in vitro* does not secrete TNFα or IL-2. This result indicates that neither TNFα nor IL-2 are required for HIV-1 suppression *in vitro* and may even enhance HIV-1 replication and immune activation. Polyfunctionality has been linked to high sensitivity, due to the ascending TCR signal that triggers thresholds for several functions simultaneously (Almeida *et al.*, 2009). Our data support this finding, as although the highest sensitivity clone is not polyfunctional, the repertoire of potential functions are more efficiently elicited at the lowest peptide concentrations, resulting in a higher percentage of cells with three simultaneous functions, and a greater

proportion of cells responding with at least one function. This finding suggests that high sensitivity rather than multiple soluble factors may explain the observed correlation of polyfunctionality and HIV-1 control. In addition, the balance between cell activation, proliferation, differentiation and resistance to apoptosis may be a mechanism of viral control in highly avid CTL.

The viral suppression mediated by CTL clones that use the same TCR isolated from individual E833 was so efficacious that the impact of sensitivity could not be fully determined. This TCR was dominant in that individual. Narrow TCR repertoires, and even selection of public TCR, have been linked to HIV-1 control, although this was not possible in our acutely infected patients who were all treated with HAART at diagnosis (Berger et al., 2011) (Iglesias et al., 2011). It is possible that the T-cell receptor or cell intrinsic qualities account for the marked viral suppression. Selection of cross-reactive and more germ-line like TCRs has been linked to control of HIV-1 replication (Chen *et al.* 2012) (Dong *et al.,* 2004). Optimal CTL function is likely to be incremental, and sensitivity is one important component.

3.3.3 Sensitivity Maturation within TCR Clonotypes

What is fascinating is that we find distinct sensitivity in clones that use the same TCR. Although it is possible that this arose in vitro, as has been reported (Kroger and Alexander-Miller, 2007), our clones were generated from tetramers and not using peptide. Experiments were performed on subsequent generations of clones, with stable phenotype, and a difference in sensitivity was already apparent in the earliest generations. Our data suggest that sensitivity maturation may occur in chronic viral infection. A range of sensitivities was already apparent in a panel of clones isolated during acute infection that used the same TCR, but the highest sensitivity CTL clone,

A20, was isolated from chronic infection. It will be interesting to explore the timing of sensitivity maturation, and if maturation is enhanced during persistent viral infection. Our observations provide a unique circumstance in which to explore the mechanisms of sensitivity maturation in two clones with the same TCR, A3 and A20, which will be pursued in the next chapter.

3.3.4 Antigen Sensitivity and Viral Mutation

CTL responses to HIV-1 antigens are reported to be oligoclonal, and are frequently dominated by a single clonotype (Wilson *et al.*, 1998) (Chen *et al.*, 2012) (Iglesias *et al.* 2011). Escape from the dominant TCR in a high sensitivity HLA B*27 restricted-KK10 speicifc CTL population has been associated with progression to AIDs (Almeida *et al.*, 2007). High sensitivity (1-10nm in IFNG ELISPOT) has been reported to conserved epitopes in chronic infection (Draenert *et al.*, 2004). Although these CTL may be "passenger" CTL (Bangham, 2009) and not sufficiently potent to select for viral variants, it is possible that high sensitivity CTL may act to contain the emergence of viral variants despite the high viral load and high viral replication necessary for escape to occur (Kelleher *et al.*, 2001). A public TCR, *TRBV4-3/TRBJ1-3* was associated with a highly sensitive HLA B*27-KK10 CTL-response, and enhanced *in vitro* viral suppression (Iglesias *et al.*, 2011). Of note, individuals with this public TCR were all wild type at the TCR contact residue in the KK10 epitope, $L_{268}M$. Although our data set is very limited, one interpretation is that sensitivity maturation that first arises during priming, as reported for mice (Slifka and Whitton, 2001), is enhanced by repetitive exposure to a conserved epitope. The T cell receptor used by our panel of HLA B*08-FL8-specific CTL clones has been reported to be broadly cross-reactive to viral variants (Dong *et al.*, 2004). Cross-reactivity may be essential to preserve the high sensitivity clonotype by maintaining stimulation, and

conserve the sequence of the epitope preventing the emergence of escape variants. Thus HIV-1 control may require high sensitivity, and broad TCR cross reactivity that initiate a self-sustaining situation that conserves the CTL epitope. 24% of epitopes targeted by the first CTL response were conserved into chronic infection (Goonetilleke *et al.*, 2009). FL8 does accumulate escape mutations in acute infection (Price *et al.*, 1997), yet is commonly immunodominant in chronic infection. It is possible that an HIV-1 vaccine using this epitope and methods of priming that generate and sustain a high sensitivity broadly cross reactive CTL response may be protective.

4 Tuning Viral Suppression Downstream of the TCR: Exploring Molecular Correlates of Sensitivity

4.1 Introduction

A CD8+ T cell response of high antigen sensitivity is an important determinant for optimal control of persistent viral infections, including HIV-1 (Alexander-Miller *et al.*, 1996) (Gallimore *et al.*, 1998) (Almeida *et al.*, 2007; Almeida *et al.*, 2009) (Mothe *et al.*, 2012) (Kattan *et al.*, 2009; Neveu *et al.*, 2008; Sedlik *et al.*, 2000). The frequency of high sensitivity CTL specific for single HIV epitopes or that target the HIV-1 Gag antigen have been reported to correlate with low HIV-1 viral load (Bihl *et al.*, 2006) (Almeida *et al.*, 2009) (Almeida *et al.*, 2007; Mothe *et al.*, 2012). High avidity CTL may respond more rapidly and with greater potency to viral infected cells *in vivo*, containing the spread of infection (Derby *et al.*, 2001). This more potent response may result from enhanced lytic efficiency or enhanced secretion of IFNγ on a per cell basis, and may be linked to a polyfunctional profile although this is controversial (Almeida *et al.*, 2007) (Kattan *et al.*, 2009) (La Gruta *et al.*, 2004) (Harari *et al.*, 2007; Ferrari *et al.*, 2011). The success of the first CTL response is often undermined by rapid emergence of viral variants. High antigen sensitivity may enhance CTL recognition of these variants (Bennett *et al.*, 2010) (Varela-Rohena *et al.*, 2008). In addition, the HLA class I genotype has been linked to HIV-1 control, and may impact the sensitivity of the responding CTL response, by influencing the TCR repertoire from which CTL are selectively expanded during priming (Kosmrlj *et al.*, 2010; Messaoudi *et al.*, 2002;). However the heterogeneity of the total viral-specific CTL response in chronic infection can confound identification of protective

correlates (Zafiropoulos *et al.*, 2004) and antigen sensitivity is no exception (Draenert *et al.*, 2004) (Harari *et al.*, 2007) (Yang *et al.*, 2003) (Bennett *et al.*, 2007). Although the selective expansion of CTL with high-affinity T cell receptors is one approach to generating CTL of high sensitivity specific for a single epitope, we have found that antigen sensitivity and viral suppression *in vitro* can be distinct in a panel of four HLA B*08-restricted FL8-specific CD8+ T-cell clones that target a single epitope in Nef and use the same TCR. These clones were isolated from one individual, from a single sample, and were not generated ex vivo using peptide stimulation that can generate high and low avidity populations from the same T-cell clone but by limiting dilution of the FL8/HLA B*08 tetramer population (Kroger and Alexander-Miller, 2007a; Almeida *et al.*, 2009). Avidity maturation of naïve CTL with the same TCR affinity and in TCR transgenic animals is reported to occur during viral infection, and following peptide encounter in the periphery (Slifka and Whitton, 2001) (Kroger and Alexander-Miller, 2007a) (Kroger *et al.*, 2008). The modulation of antigen sensitivity as a result of CTL encounter with antigen in cells with the same TCR affinity may occur by changes in the number of TCR that must engage to cross the activation threshold, and by tuning of the TCR signalling cascade. Modulating effects reported include the level and isoform of CD8 co-receptor expression (Park *et al.*, 2007; Cawthon *et al.*, 2001) (Kroger and Alexander-Miller, 2007b), optimal co-localisation of the TCR and CD8 (Demotte *et al.*, 2008; Cawthon and Alexander-Miller, 2002), level of TCR expression linked to activation state (San Jose *et al.*, 2000; Ueno *et al.*, 2004) (De Mattia *et al.*, 1999) (Demotte *et al.*, 2002) (Drake *et al.*, 2005), ratio of monovalent to multivalent TCR stoichiometry (Schamel *et al.*, 2005), expression of accessory molecules including CD2 and LFA-1, level of expression of ZAP-70 and Lck, and augmentation of membrane proximal signal transduction (Sharma and

Alexander-Miller, 2011; Amoah *et al.*, 2012) (Slifka and Whitton, 2001) (Li *et al.*, 2007).

T cell responsiveness to antigen is modulated during thymic ontogeny, by the induction of microRNAs (miRNA) (Li *et al.*, 2007). MiRNAs are short 19-22 nt non-coding RNA molecules that regulate gene expression post-transcriptionally (Lee *et al.*, 2004b). There are now over 1000 human miRNAs reported to regulate 60% of human genes (Friedman *et al.*, 2009). MiRNAs bind the 3' UTR of target messenger RNA (mRNA) to cause mRNA degradation or block translation without mRNA degradation. The expression of mir-181a in CD4+CD8+ mouse thymocytes enhances antigen sensitivity by regulation of membrane proximal signalling events (Li *et al.*, 2007). Antigen sensitivity may also influence ontogenetic factors of mature T cells in the periphery. Progeny of a single naïve T-cell that developed into distinct effector and memory sub-sets are reported (Stemberger *et al.*, 2007). Differentiation into terminal effector or memory phenotypes may be determined by the antigen sensitivity of viral specific CD8+ T-cells following priming, as TCR signal strength is associated with differentiation into either short lived effector cells (SLEC) or long lived memory cells (Amoah *et al.*, 2012). Ontogenetic fate of viral specific CD8+ CTL has been reported to influence viral control by modulating effector function (Cellerai *et al.*, 2010; Appay *et al.*, 2002) (Champagne *et al.*, 2001).

TCR activation may also modulate T-cell responsiveness to antigen, by acute downregulation of the TCR and CD8 and more mature induction of regulatory pathways that feedback to modulate the TCR signal. This was first described for one inhibitory receptor of the CD28 family, programmed death receptor 1 (PD-1), that is upregulated following activation and persistently expressed at high levels on "exhausted" cells (Sauce *et al.*, 2007; Day *et al.*, 2006) (Trautmann *et al.*, 2006)

(Wherry *et al.*, 2007). Exhaustion can be defined as the accumulation of multiple inhibitory receptors and co-stimulatory receptors that actively signal to regulate progressive immune dysfunction by modulation of the TCR signal (Blackburn *et al.*, 2009) (Wang *et al.*, 2007).

Defining molecular mechanisms that modulate the antigen sensitivity of primed CTL to enhance the differentiation of highly sensitive CTL that efficiently recognise infected cells and respond with enhanced cell lysis, but without triggering autoimmunity, and identifying new correlates of high sensitivity CTL by surface phenotype are important contributions to development and monitoring of an HIV-1 vaccine. The identification of populations of HLA B*08-resticted FL8-specific CD8+ T-cell clones that use the same TCR but have distinct antigen sensitivity provides a unique opportunity to examine these mechanisms at the level of the TCR, accessory molecule expression and cell intrinsic and ontogenetic events downstream of the TCR. In order to explore molecular correlates of high antigen sensitivity we compared the attributes of two HLA B*08-restricted FL8-specific CD8+ T cell clones of high and intermediate sensitivity that use the same TCR by surface phenotype and resting transcriptome analysis. We identified a group of cell surface receptors with diverse biological processes and interesting candidate molecules that may fine-tune TCR signalling sufficiently to reduce the threshold of antigen stimulus required to trigger a particular effector function.

4.2 Results

4.2.1 CD8+ T-cell Clones with the Same TCR but distinct antigen sensitivity have similar TCR and CD8 surface expression

Ultimately, antigen sensitivity is a measurement of virus specific CD8 T cell effector function that results from the integration of three signals: TCR/pMHC ligation, co-stimulatory and inhibitory receptors and cytokine receptors. We first sought to confirm that TCR surface expression was identical for all four HLA B*08-restricted FL8-specific CD8+ T-cell clones isolated from one individual, LTNP005, that use the same TCR (Clones A3, A9, A17 and A20) by surface staining with Vβ6.2 antibody. CD8 co-receptor binding enhances the affinity of the TCR for pMHC complex 1 million fold (Garcia *et al.*, 1996). Although we did not determine the co-localisation of the TCR and CD8 (Cawthon and Alexander-Miller, 2002) or surface expression of CD8β (Cawthon *et al.*, 2001), surface expression of the TCR and CD8α was the same for all four clones (Figure 4-1).

4.2.2 Comparison and stability of surface markers in clones with the same TCR but distinct antigen sensitivity

Co-stimulation is required for activation of naïve T cells to differentiate into effector T-cells. Important co-stimulatory molecules include CD28 and the TNF-receptor family member CD27 (Nolte *et al.*, 2009) (Alegre *et al.*, 2001). Co-stimulatory receptors CD27 and CD28, and the protein tyrosine phosphatase CD45RA have been used to identify a state of skewed maturation in HIV-1 antigen experienced CD8+ T-cells (Appay *et al.*, 2002; Champagne *et al.*, 2001).

Figure 4-1: Surface Expression of the TCR and CD8+ Molecules in HLA B*08 restricted-FL8 specific CD8+ T-cell Clones that use the Same TCR

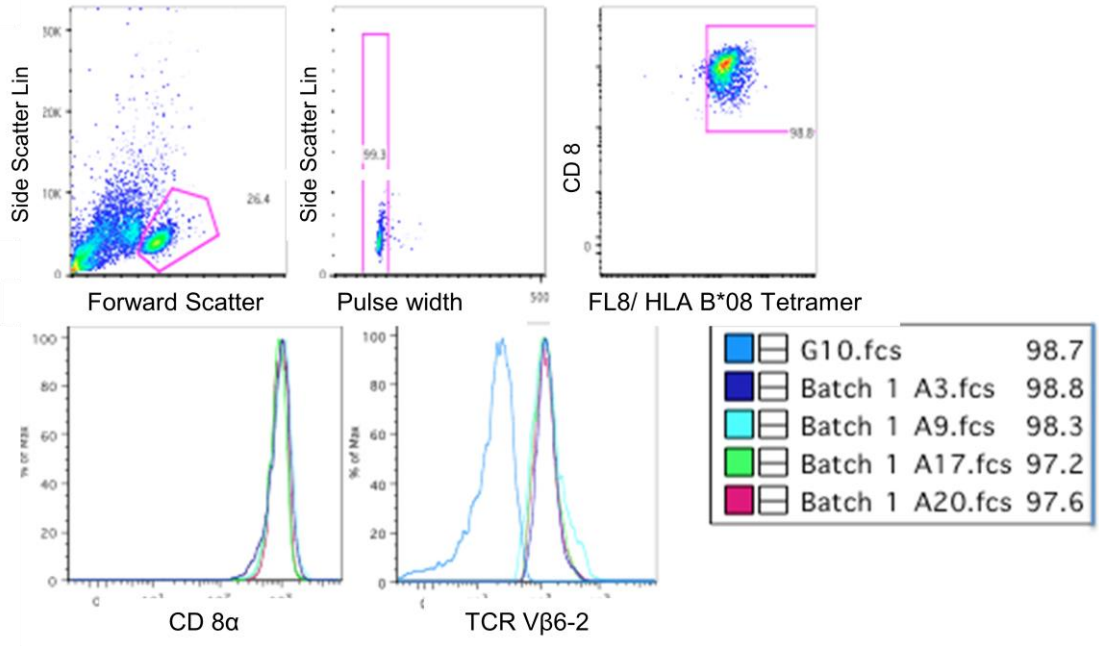


Figure 4.1: Purity, CD8 and TCR expression of HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR (A3, A9, A17 and A20). Clones are >97% pure and have comparable surface expression of TcR and CD8α on day 14 post stimulation. Flow cytometry plots were gated on live, single cells, to determine the purity of each clone using FL8-HLA B*08 tetramer, and surface expression of CD8α and TCRVB6-2. Clone G10, used as a negative control (HLA mismatch) for the Vβ6-2 staining, is specific for the HIV-1 Gag epitope SLYNVATYL restricted by HLA *02 (SL9-HLA A2) (Lee *et al.*, 2004a).

Inhibitory signalling molecules such as Programmed Death 1 (PD1) are also upregulated in HIV-1 in individuals with high viral load (Day *et al.*, 2006). CD57 is a marker for replicative senescence, indicating a history of numerous cell divisions and correspondingly short telomeres (Brenchley *et al.*, 2003). We sought to characterise phenotypic markers associated with HIV-1 control that could modulate the TCR signal in detail for two clone populations with the same TCR, A3 and A20, and assess the stability of the phenotype with successive rounds of re-stimulation (Figure 4-2). Our panel of markers included differentiation markers (CD28, CD27, CD45RA), inhibitory receptor (PD1), markers of replicative senescence (CD57), and function (perforin and granzyme A). Clones A3 and A20 do not express CD28 or CD57 and have comparable expression of CD27 (CD28+ve: A3 0.26 v A20 0.1%; CD27+ve: 35.48 v 35.14%), perforin and granzyme A. The intermediate avidity clone population, A3, expresses CD45RA in contrast to the high sensitivity population, A20. PD-1 is expressed in 34.6% of A3 and 13.3% of A20. This surface expression is very low in comparison to that found in studies examining PD-1 expression in exhausted HIV-1 specific CTL by tetramer (PD-1 80-95%) (Day *et al.*, 2006).

In conclusion, the higher antigen sensitivity of clone population A20 cannot be explained by TCR affinity, surface expression of the TCR or the CD8α co-receptor, co-stimulatory molecules CD28 or CD27, or expression of CD45RA (Hermiston *et al.*, 2003). Clone A3 has a higher expression of inhibitory receptor PD-1 than A20, but overall expression is low compared with that of exhausted ex vivo HIV-1 specific CD8+ T-cells from chronic infection.

Figure 4-2: Phenotype of Successive Generations of HLA B*08-restricted FL8-specific CD8+ T-cell clone populations (A3 and A20) from LTNP005 that use the same TCR

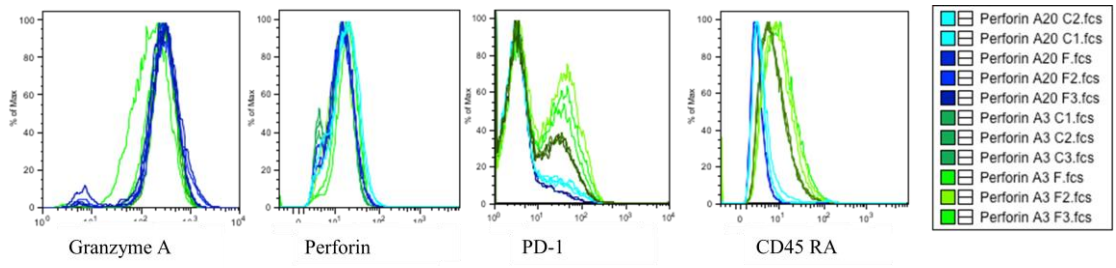


Figure 4.2: Distinct stable phenotype for HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR (A3 and A20). Clones are armed effectors with a stable phenotype between rounds of re-stimulation. Representative data from flow cytometry of A3 and A20 clones from 2 different generations grown from early frozen stock (G1) and following serial culture (G4). Each generation is represented in biological triplicates and gated on live, single TRVβ+CD8α+ cells, to show the fluoresence intensity of intracellular granzyme A and perforin, cell surface PD-1 and CD45RA.

4.2.3 CD8+ T-cell clones with the same TCR but distinct antigen sensitivity have the same tetramer binding avidity

The binding of peptide-HLA tetramer complexes is altered following activation of viral specific CD8+ T-cells *in vitro* and is associated with a change in surface TCR organisation, reduced signalling through the TCR, and diminished effector responses (Drake *et al.*, 2005). We therefore determined the tetramer-binding for a range of concentrations for clones A3, A17 and A20 that use the same TCR but have distinct antigen sensitivity. There was no difference in tetramer-binding at all concentrations of FL8/HLA B*08 tetramer tested (Figure 4-3). This assay was performed on a further panel of HLA B*08-restricted FL8-specific CD8 T-cell clones isolated from acute infection that use a second TCR from individual E833 (Clones E833-10, -13, and -30). Again, no difference in tetramer-binding could be demonstrated in clones that use the same TCR (Figure 4.3B). Differential tetramer-binding was observed in this assay for five CTL that differ in the amino acid sequence of the TCR, and therefore affinity for the p-MHC complex (Figure 4.3C). This suggested that events downstream of the TCR modulate antigen sensitivity in two panels of clones that use the same TCR.

Figure 4-3: Tetramer Binding Assay for HLA B*08-restricted FL8-specific CD8+ T-cell Clones

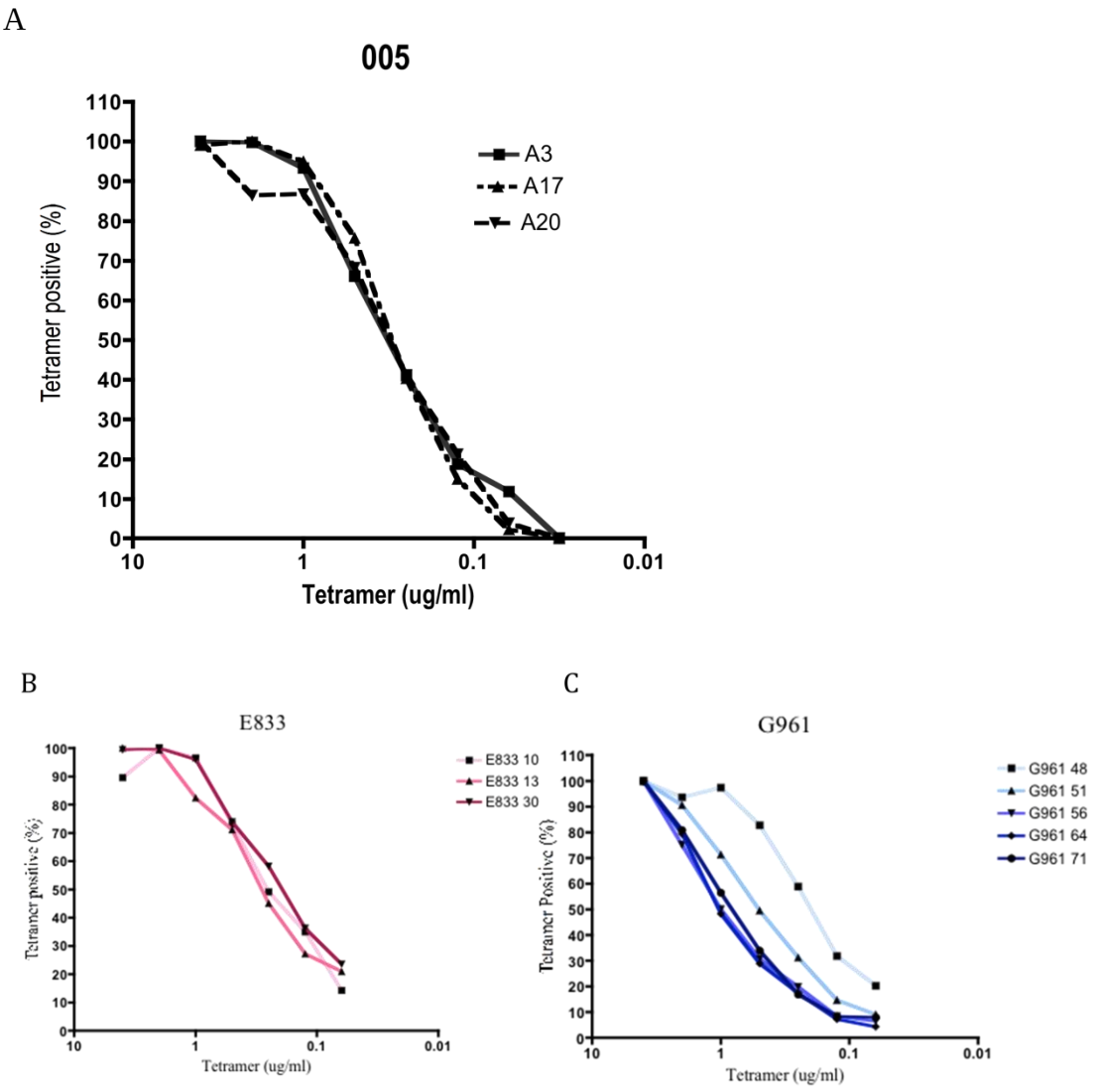


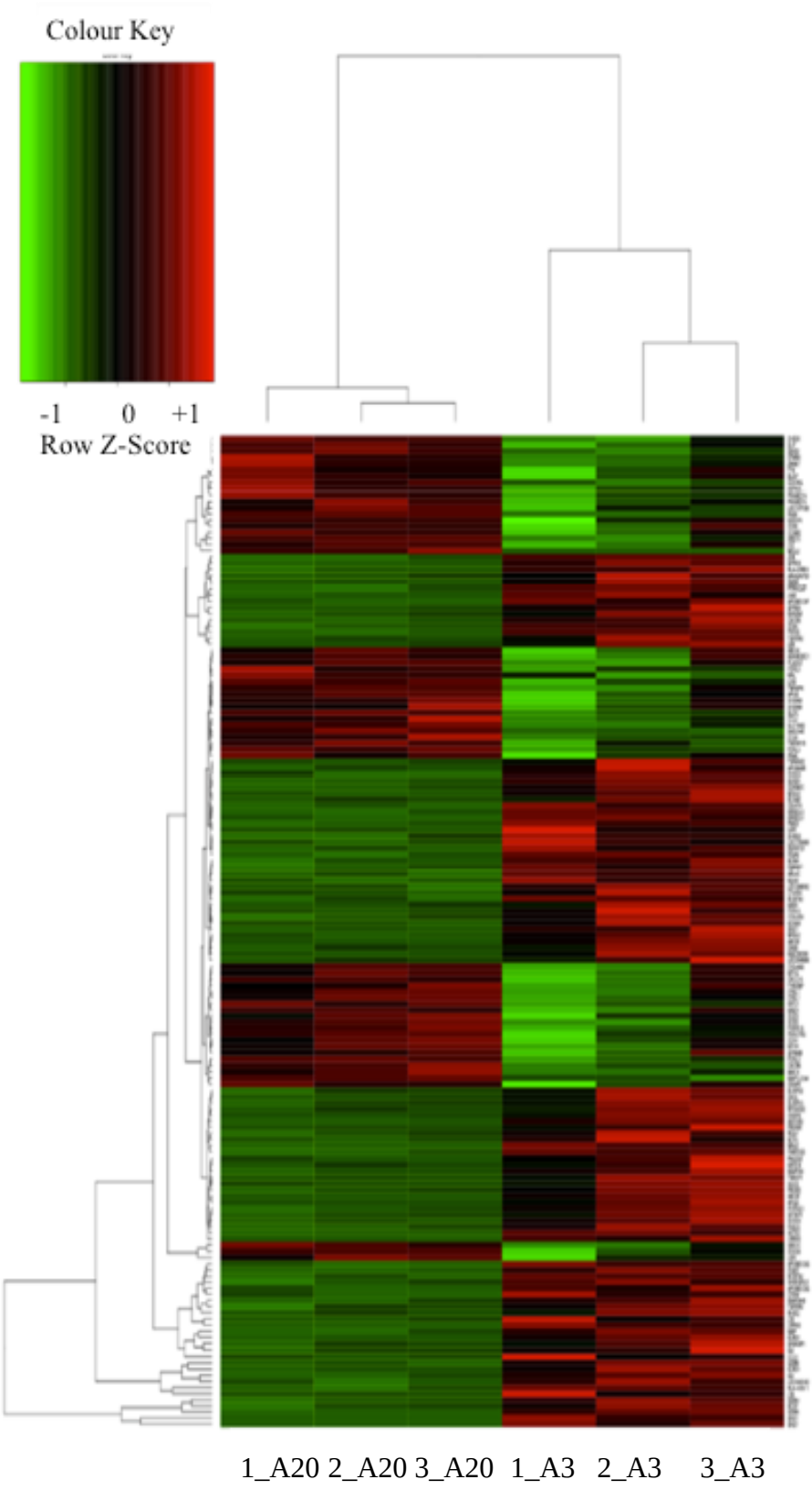
Figure 4.3: **HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR have the same tetramer avidity.** (A) Tetramer avidity assay for clones A3, A17 and A20 that use the same TCR. The results of one experiment are illustrated, but comparable data was obtained from three experiments. B) Tetramer avidity assay for three clones that use the same TCR isolated from individual E833 (Clones E833-10, -13, and -30). C) Tetramer avidity assay demonstrating a panel of five HLA B*08-restricted FL8-CD8+ T cell clones from one individual (G961) with different TCR sequences (G961-48, -51, -56, -64, and -71). The assay was performed by flow cytometry by gating on live single cells, CD8+, and by using an HLA mismatched tetramer at 4ug to set the negative gate.

4.2.4 Comparison of the resting transcriptomes of high and moderate sensitivity CD8+ T-cell clone populations identified 140 differentially expressed genes

In an endeavour to determine how the antigen sensitivity was enhanced in clone A20, from the abundance of potential mechanisms ranging from surface expression of adhesion molecules, CD2 and LFA-1 (Contento *et al.*, 2010), cytokine milieu (Apte *et al.*, 2008) (Soudja *et al.*, 2012; Markiewicz *et al.*, 2009; Oh *et al.*, 2004), expression of co-stimulatory and inhibitory molecules, maturation and poise of the TCR signalling cascade and ontogenetic factors, we compared the gene expression of clone A20 (high antigen sensitivity) and clone A3 (intermediate antigen sensitivity) in the resting state using illumina HT12 Gene Expression Arrays. HLA B*08-restricted FL8-specific CD8+ T-cell clones were co-cultured with HLA-matched CD4+ T-cells (no epitope was added) for 24 hrs and then negatively selected using anti-CD4+ magnetic beads for total RNA extraction. 140 genes had distinct expression in clone population A20 compared to that in population A3 (absolute fold change at least two fold up or down in A20 v A3, (one way ANOVA p=0.01)) (Figure 4-4) (all candidate genes are listed in the Appendix, Table 8-1).

Figure 4: (NEXT PAGE) Hierarchical clustering of gene expression data from two HLA B*08-restricted FL8-specific CD8+ T cell Clones that use the same TCR (A3 and A20). Each column represents a separate mRNA sample for each clone from three replicate experiments. Each row represents a probe from the bead array that maps to a gene in the Ensembl database. The colour represents the relative quantity of complementary RNA directly hybridised to each probe for each sample, and is scaled for each row, to range from -1 (green) to +1 (red). Each gene is represented by more than one probe. 140 genes were differentially expressed by an absolute fold difference of <2>, at p=0.01 (One way ANOVA). HLA B*08-restricted FL8-specific CD8+ T-cell clones were co-cultured with HLA-matched CD4+ T-cells (no epitope was added) for 24 hrs and then negatively selected using anti-CD4+ magnetic beads for total RNA extraction. Whole genome expression was analysed on Illumina HT12v4 Expression BeadChip arrays.

Figure 4-4: Hierarchical Clustering of Gene Expression Data from Two Resting HLA B*08-restricted FL8-specific CD8+ T cell Clones that use the Same TCR (A3 and A20)



Biological processes showing significant enrichment included immunity and defense ($p=2.87 \times 10^{-17}$), natural killer cell mediated immunity ($p=3.24 \times 10^{-10}$), cytokine and chemokine mediated signalling pathway ($p=6.83 \times 10^{-8}$), signal transduction ($p=1.16 \times 10^{-6}$), T-cell mediated immunity ($p=2.32 \times 10^{-6}$), cell cycle ($p=2.17 \times 10^{-5}$) and cell surface receptor mediated signal transduction ($p=2.24 \times 10^{-3}$) (Figure 4-5).

4.2.5 Classification of genes with altered expression by molecular and cellular function identified a large category of cell surface markers

Of the 140 genes with significant differential expression (ANOVA $p<0.01$), 57 genes were at least two-fold more highly expressed in the high sensitivity population, A20, and 87 genes were at least two fold more highly expressed in the intermediate sensitivity population, A3. The molecular and cellular function of each gene was analysed (Huang da *et al.*, 2009) (Figure 4.6). A high proportion of genes with altered expression were assigned to four categories of function, predominantly cell surface receptors (A20: 11/57=20%; A3: 28/87=33%), signal transduction (A20: 7/57=13%; A3: 11/87=13%), metabolism, transport and survival, or immune defense. The high sensitivity population, A20, had differential expression of more cell cycle genes than population A3 (A20: 11/57= 20%; A3: 3/87=3%), and less transcriptional regulators (A20 0/57; A3 8/87=9%). In conclusion, the genes with altered expression in populations of clone with distinct avidity could be categorised into six cellular and molecular functions and indicated a small difference in cell cycle genes between the two clones (14/140 genes =11%). The largest category of genes identified consisted of cell surface receptors (39/140 genes = 27.9%).

Figure 4-5: Classification of Genes with Distinct Expression in Two Resting HLA B*08-resticted FL8-specific CD8+ T-cell Clones (A3 and A20) with the same TCR into Biological Process

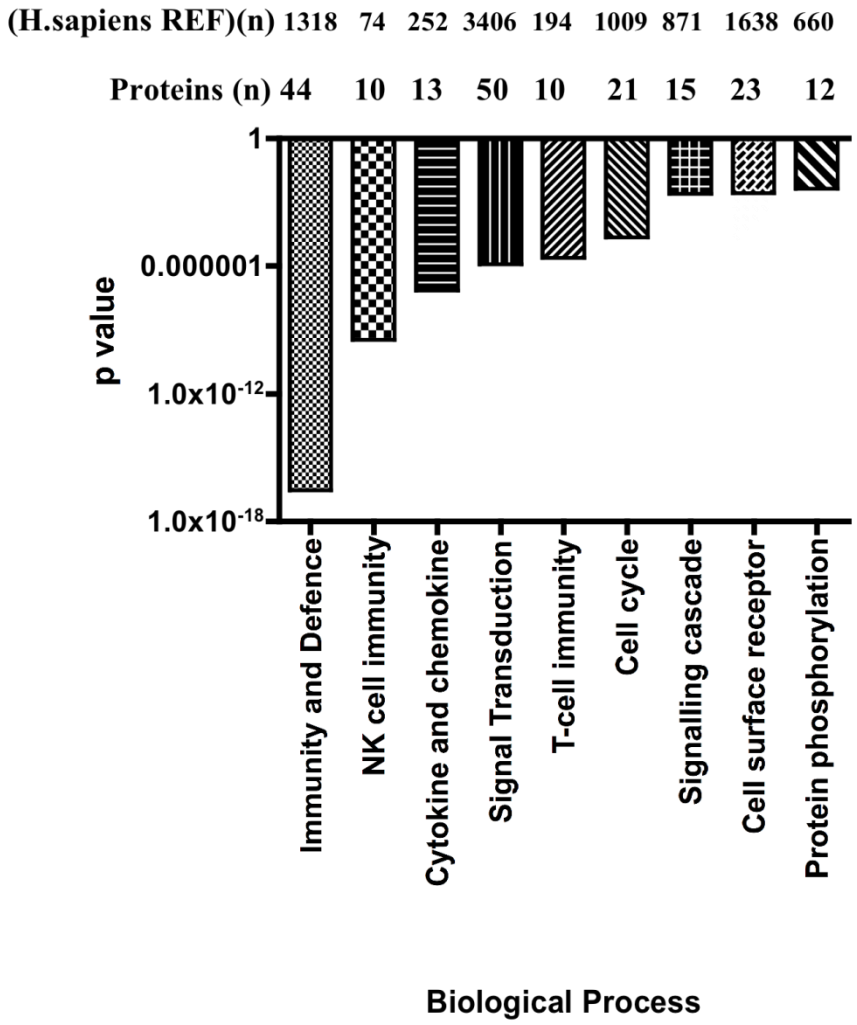


Figure 4-5: Classification of genes with significant differential expression by biological process. Bar chart of the biological pathways and processes identified within the 140 genes with altered expression by gene set enrichment analysis in PANTHER (Thomas *et al.*, 2003)**.** P-value (vertical axis) indicates the probability that the biological pathway is indicated by chance alone. The number (n) of *H.sapiens* genes in the reference group (REF) and number of observed genes (protein) with altered expression (absolute up or down regulation in A20 compared to A3 of at least two fold changes, $p<0.01$ by ANOVA) is indicated for each pathway.

Figure 4-6: Classification of Genes with Distinct Expression in the Resting Clone Populations by Molecular and Cellular Function

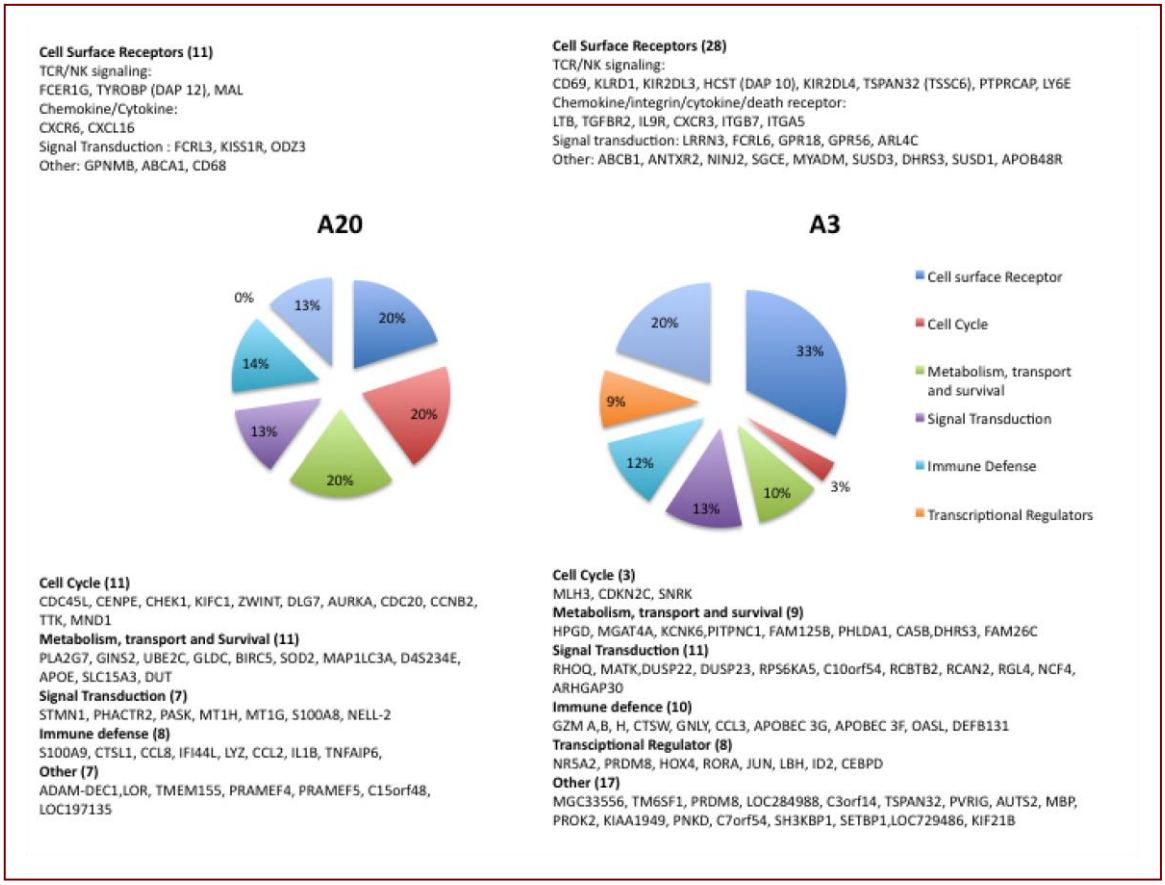


Figure 4.6: **Classification into general functional categories of 140 genes with altered expression in the transcriptome comparison of two clones that use the same TCR**. Pie charts illustrate genes with altered expression (Left pie chart (A20): 57 genes with at least 2 fold higher expression than clone A3 ANOVA $p<0.01$; Right pie chart (A3): 87 genes with at least 2 fold higher expression than clone A20) that were assigned to categories of function by analysis in DAVID (Huang da *et al.*, 2009). The size of each segment is proportional to the number of genes within the assigned category. All gene names, entrez ID and regulation (A20 v A3) can be found in the appendix.

4.2.6 Cell surface molecules identified include receptors with known integration in TCR signalling and receptors with immunoreceptor tyrosine based motifs but unknown function in CTL signalling

To further explore the cell surface receptors with altered expression in the transcriptome analysis of populations of high (A20) and moderate (A3) sensitivity HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR, all potential cell surface proteins were identified by Uniprot classification and manual assignment by review of the literature. Candidate genes were further selected by comparison with published gene expression data sets to identify genes restricted to the CD8+ T-cell or NK cell lineage (Evans *et al.*, 2003). This identified 34 genes encoding cell surface receptors (24.6% of candidate genes). Nine genes were more highly expressed in clone A20 (Table 4-1, Figure 4-7) and 25 genes were more highly expressed in clone A3 (Table 4-2, Figure 4-8). Three of the genes encoding cell surface molecules in the high sensitivity clone, A20, have cytoplasmic domains that contain an immunoreceptor tyrosine-activating motif (ITAM), *FCRL3* (Fold change A20 v A3 (FC) 3.58), *FCER1G* (FC 3.33) and *TYROBP* (also known as DAP 12) (FC 2.71). Four of the genes identified with higher expression in the intermediate avidity clone, A3, have cytoplasmic domains that contain an immunoreceptor tyrosine-inhibitory motif (ITIM), *LRRN3* (FC -19.39), *FCRL6* (FC -4.32), *KIR2DL3* (FC -3.04) and *KIR2DL4* (FC -2.25). *FCRL3, FCER1G* and *FCRL6* are encoded within the Fc-receptor superfamily on Chr1q21-23 that includes *CD3ζ*. FCER1G, TYROBP, KIR2DL3 and KIR2DL4 are key determinants of NK cell signalling. HCST (also called DAP 10) (FC -2.53) is an adaptor protein, involved in activation of NK cells but was more highly expressed by the intermediate avidity population, A3. CD69 (FC -2.4), a TCR co-stimulatory receptor encoded within the NK complex on Chr12 p13-

12, was also upregulated in A3, suggesting a higher state of activation than that of A20. Four genes with known roles in modulating CTL signalling were identified (Upregulated A20: MAL; Downregulated in A20: LY6E (FC-2.33), TSPAN32 (also called TSSC6) (FC -2.19), and PTPRCAP (FC-2.05) (Anton *et al.*, 2008; Schoggins *et al.*, 2011; Tarrant *et al.*, 2002; Gartlan *et al.*, 2010). Five receptors have signalling capacity but unknown roles in CTL (Upregulated A20; GPNMB (FC 2.16); Downregulated A20; MYADM (FC -7.52), GPR18 (FC -2.51) and GPR56 (FC -2.29)).

Table 4-1: Genes encoding cell surface molecules with higher expression in A20 compared to A3

Entrez ID	Symbol	Name	Fold Change [A20] v [A3]
10663	**CXCR6**	chemokine (C-X-C motif) receptor 6	3.74/2.66
115352	**FCRL3**	Fc fragment of IgE, high affinity I, receptor for gamma polypeptide	3.58/2.67
2207	**FCER1G**	Fc receptor-like 3	3.33
7305	**TYROBP**	TYRO protein tyrosine kinase binding protein	2.71
58191	**CXCL16**	chemokine (C-X-C motif) ligand 16	2.76
4118	**MAL**	mal, T-cell differentiation protein	2.48
10457	**GPNMB**	glycoprotein (transmembrane) nmb	2.16/2.06
55714	**ODZ3**	Teneurin 3	2.1
19	**ABCA1**	ATP-binding cassette, sub-family A (ABC1), member 1	2.06

Table 4.1: Cell surface molecules more highly expressed in populations of clone A20 (at least two fold higher expression than clone A3, p<0.01). Cell surface molecules were identified by Uniprot classification, manual review of the literature and previous reports of gene expression within T-cells or NK cells. Cell surface molecules reported to contain immunoreceptor activating motifs are indicated in red. Further information is provided for each gene in Figure 4.7.

Figure 4-7: Genes Encoding Cell Surface Molecules More Highly Expressed in A20 compared to A3

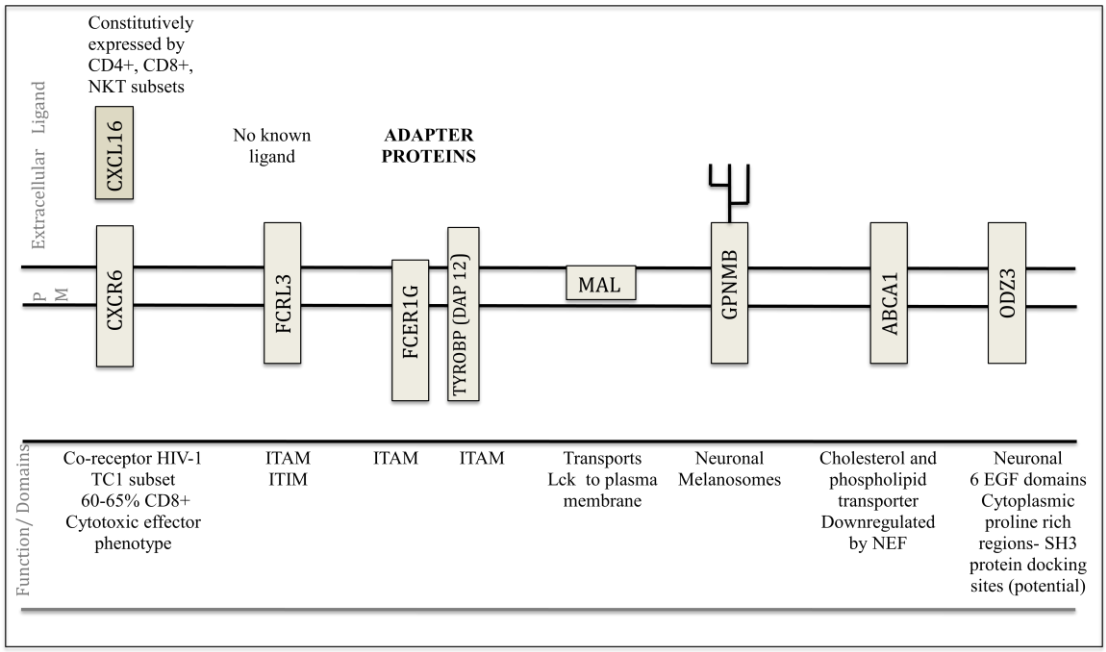


Figure 4-7: Cartoon depicting cell surface molecules with higher expression (at least 2 fold upregulation compared to clone A3, $p<0.01$) in populations of clone A20. Only one ligand is known for these molecules, CXCL16, and is also highly expressed by clone A20. A brief description of functional domains or motifs are indicated. PM=plasma membrane.

Table 4-2: Genes Encoding Cell Surface Molecules with Higher Expression in A3 compared to A20

Entrez ID	Symbol	Name	Fold Change [A3] v [A20]
54674	**LRRN3**	leucine rich repeat neuronal 3	19.39/10.37
5243	**ABCB1**	ATP-binding cassette, sub-family A (ABC1), member 1	5.45/2.75
3824	**KLRD1**	killer cell lectin-like receptor subfamily D, member 1	5.25/2.04
91663	**MYADM**	myeloid-associated differentiation marker	7.52/2.62
343413	**FCRL6**	Fc receptor-like 6	4.32
118429	**ANTXR2**	anthrax toxin receptor 2	4.23
4815	**NINJ2**	ninjurin 2	3.43/2.41
3804	**KIR2DL3**	killer cell immunoglobulin-like receptor, two domains, long cytoplasm	3.04
10870	**HCST**	hematopoietic cell signal transducer	2.53
2841	**GPR18**	G protein-coupled receptor 18	2.51
969	**CD69**	CD69 molecules	2.4
7048	**TGFBR2**	transforming growth factor, beta receptor II (70/80kDa)	2.34
4061	**LY6E**	lymphocyte antigen 6 complex, locus E	2.33
2833	**CXCR3**	chemokine (C-X-C motif) receptor 3	2.32
3581	**IL9R**	interleukin 9 receptor	2.29
9289	**GPR56**	G protein-coupled receptor 56	2.29/2.21
3805	**KIR2DL4**	killer cell immunoglobulin-like receptor, two domains, long cytoplasm	2.25
203328	**SUSD3**	sushi domain containing 3	2.24
3695	**ITGB7**	integrin, beta 7	2.23
10077	**TSPAN32**	tetraspanin 32	2.19
9249	**DHRS3**	dehydrogenase/reductase (SDR family) member 3	2.18
5790	**PTPRCAP**	protein tyrosine phosphatase, reeptor type, C-associated protein	2.05
356	**FASLG**	Fas ligand)TNF superfamily, member 6)	2.01
3678	**ITGA5**	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	2.21/2.00
64420	**SUSD1**	sushi domain containing 1°	2.38

Table 4-2: Cell surface molecules more highly expressed in populations of clone A3 (at least two fold higher expression than clone A20, p<0.01). Cell surface molecules were identified by Uniprot classification, manual review of the literature and previous reports of gene expression within T-cells or NK cells. Cell surface molecules reported to contain immunoreceptor inhibitory motifs are indicated in blue. The ITIM motif in LRRN3 has not yet been reported (pale blue). Further information is provided for each gene in Figure 4-8.

Figure 4-8: Genes Encoding Cell Surface Molecules more Highly Expressed in A3 compared to A20

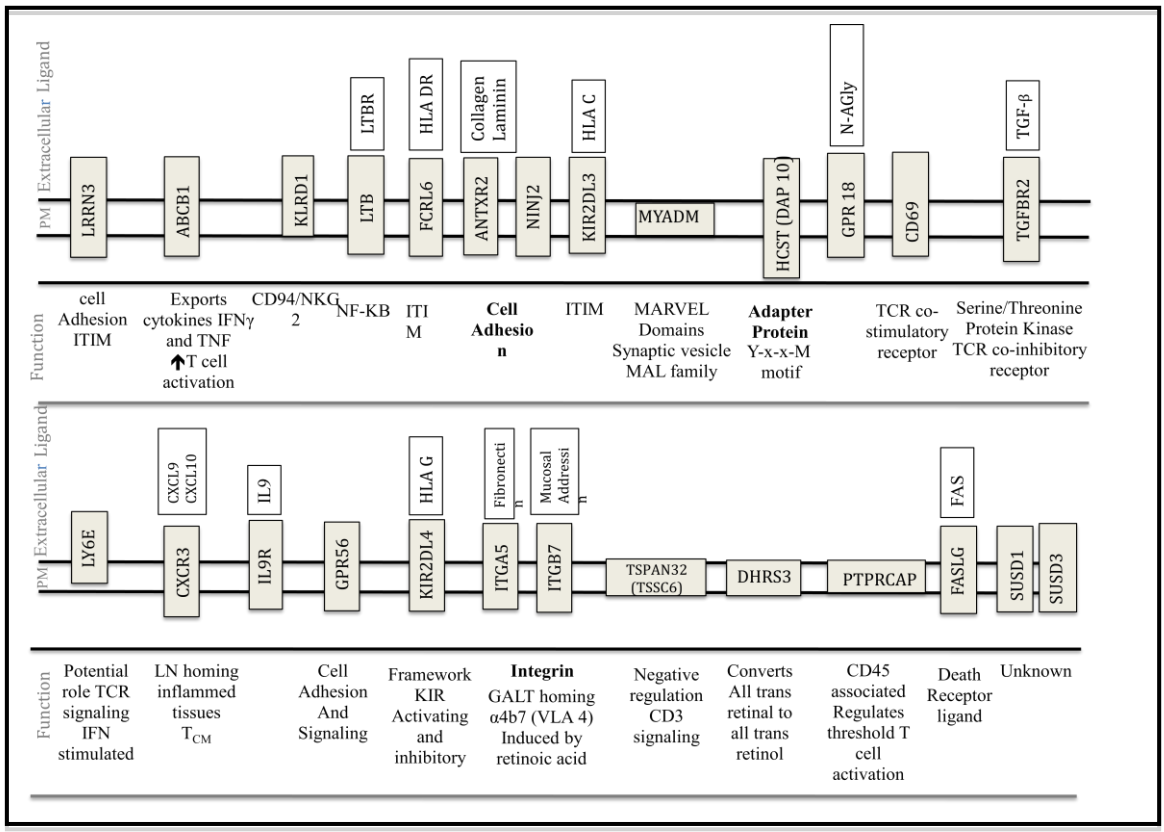


Figure 4-8: Cartoon depicting cell surface molecules with higher expression (at least 2 fold upregulation compared to clone A20, $p<0.01$) in populations of clone A3. Ligands and a brief description of functional domains or motifs are indicated. PM=plasma membrane.

4.2.7 Clone populations with distinct antigen sensitivity have differential expression of adhesion molecules and tissue trafficking chemokines and integrins

The gene most highly expressed in the high sensitivity population, A20, compared to the intermediate population, A3, was the chemokine, *CXCR6* (Fold change A20 v A3 (FC) 3.74). A20 also expressed the *CXCR6* ligand, *CXCL16* (FC 2.76). CXCR6 expression is reported in TC-1 polarised CTL with enhanced cytotoxicity with enhanced homing to inflamed tissues. By contrast, the intermediate population, A3, expressed *CXCR3* (FC -2.32). CXCR3 expression is reported on CTL subsets that home to lymphoid tissue, and has been controversially associated with both T central memory phenotypes and short-lived effector cells (Flatz *et al.*, 2011) (Guarda *et al.*, 2007) (Hu *et al.*, 2011). Integrins have integral roles in trafficking, adhesion, and regulation of cell signalling and the cytoskeleton (Kanchanawong *et al.*, 2010) (Springer and Dustin, 2012). Integrin chains *ITGβ7* (FC -2.23) and *ITGα5* (FC -2.21) were expressed by the intermediate avidity A3 population. α4β7 integrin binds MAdCAM-1 to mediate GALT homing. A3 also had altered expression of cytokine receptors associated with tolerance in the GALT, *TGFBR2* (-2.34), *IL9R* (-2.29) and the cell surface enzyme *DHRS3* (-2.18) that catalyses conversion of all trans retinal to all trans retinol. Very late activation antigen alpha 5 (VLA-5), α5β1 (CD49e/CD29) binds fibronectin (Luo *et al.*, 2007). In conclusion, genes that regulate localisation of the CTL and interaction with neighbouring cells were differently expressed in populations of clone with distinct sensitivity.

4.2.8 Candidate genes were validated by Quantitative Real-time PCR and at the protein level

To validate the expression array data, mRNA transcripts with the highest fold difference were first confirmed by real time PCR (13/15 (88.6%)) and subsequently for three additional resting populations of each clone A3 and A20, after sorting to >99% purity in the resting state by fluorescence activated cell sorting using FL8-HLA B*08 tetramer (Figure 4-9).

Where possible, distinct expression was explored at the protein level, with higher surface expression of KLRD1 (MFI: A3 28.7;A20 19.65), ITGα5 (A3 38%, A20 15.9%), and CXCR3 (MFI A3 260; A20 188) in clone A3 than clone A20 (Figure 4-9), lower secretion of CCL2 (A3 10 pg/ul; A20 30 pg/ul in supernatant at 24hrs), and higher secretion of CCL3 confirmed. KLRD1 can form homodimers, and heterodimers with NKG2A and NKG2C. Surface expression of these receptors and NKG2D, KIR2DL3 and KIR2DL4 was the same for both populations (Figure 4-10).

Figure 4-9: Quantitative Analysis of mRNA Expression in A20 and A3 in Real-time by PCR

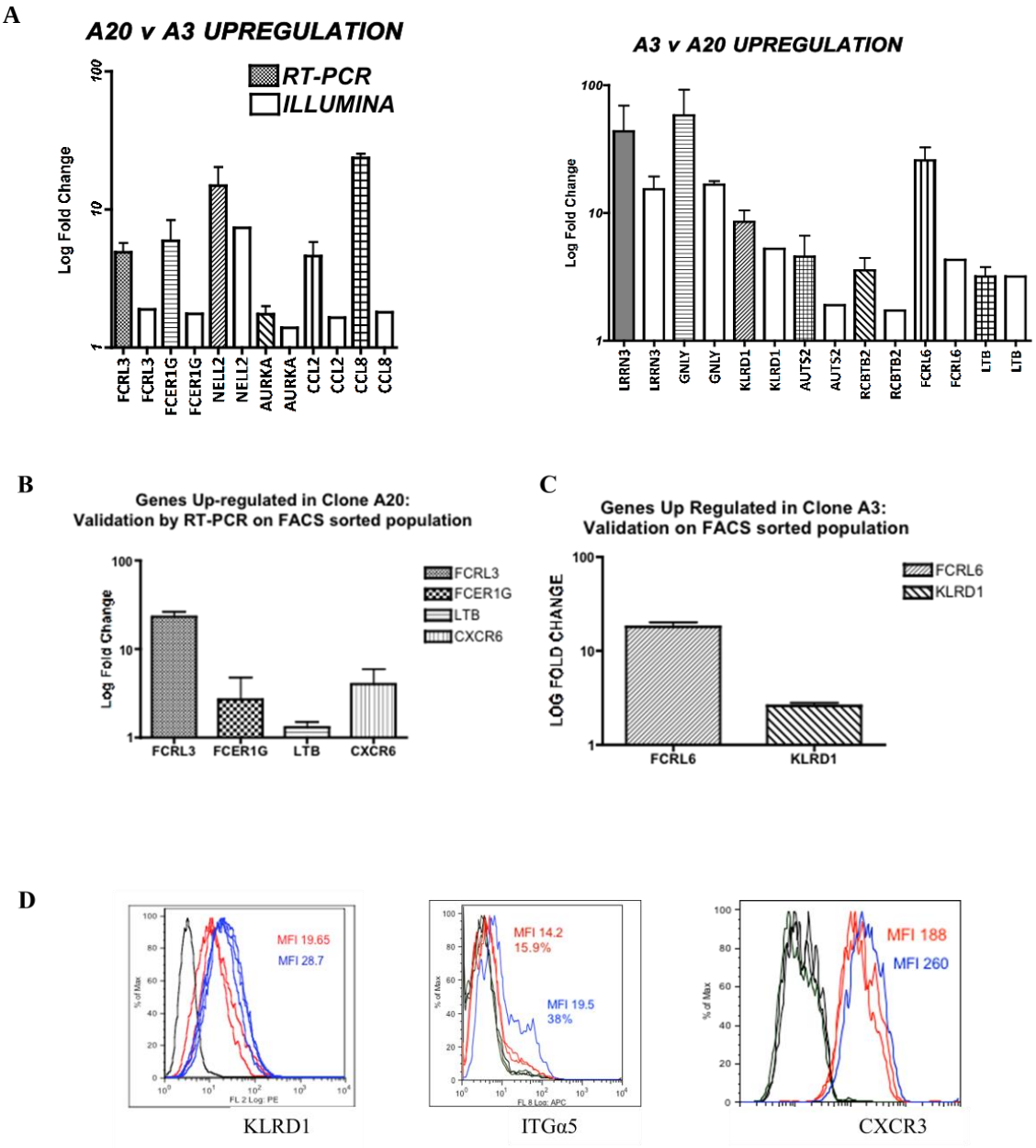


Figure 4.9: Quantitative analysis of mRNA expression in clones A20 and A3 in real-time by PCR to validate altered expression of selected genes identified on illumina microarray HT12v4 BeadChips. **A)** Genes with altered expression in the resting transcriptome of HLA B*08-restricted FL8-specific CD8+ T-cell clones A3 and A20 were selected for validation by quantitative PCR. **B)** Populations of HLA B*08-restricted FL8-specific CD8 T cell clones A3 and A20 were sorted to >99% purity by fluoresence activated cell sorting, mRNA was extracted and used for PCR analysis in real time. **C)** Flow cytometry histograms, gated on live, single, CD8+ cells, to indicate surface staining of KLRD1 (CD94), ITGα5 and CXCR3 for populations of clone A3 (blue) and A20 (red). A full list of all gene names for each gene symbol is listed in the supplementary data.

Figure 4-10: Cell surface expression of NK receptors on A3 and A20

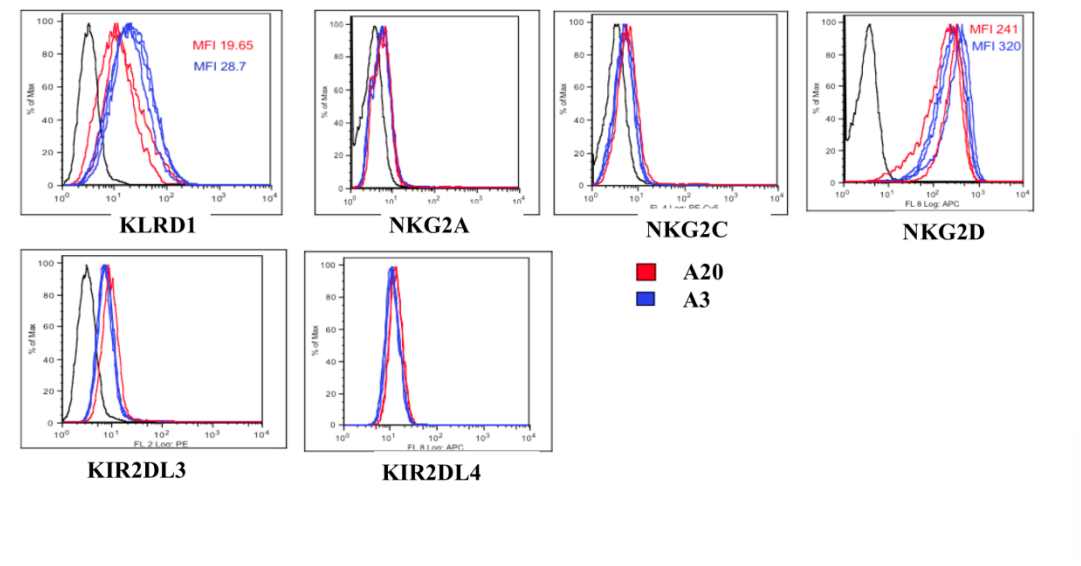


Figure 4.10: Cell surface expression of NK receptors on A3 and A20. Flow cytometry histograms, gated on live, single, CD8+ cells, to indicate surface staining of KLRD1 (CD94), NKG2A, NKG2C, NKG2D, KIR2DL3 and KIR2DL4 for populations of clone A3 (blue) and A20 (red).

4.2.9 Clones with the same TCR but distinct antigen sensitivity have differential expression of micro-RNAs

Micro-RNAs have been reported to modulate T cell sensitivity during the selection of thymocytes (Li *et al.*, 2007). MiRNA expression of the high and intermediate avidity resting clone populations A20 and A3 was compared (Figure 4-11).

The top 100 microRNAs with differential expression (at least five-fold difference in regulation between clone A3 and A20) were selected for further analysis, 53 with higher expression in clone A20 and 47 with higher expression in clone A3 (Figure 4-12, Table 4-3 and 4-4). miRNAs bind and degrade the target mRNA. Computational screening of miRNAs with low expression in the high sensitivity clone population, A20, identified target-binding sites for five genes of interest with high mRNA expression in A20 (Table 4-5). Computational screening of miRNAs with high expression in A20 identified nine microRNAs (miR-106b, miR-520b, miR-302a, let-7g, let 7a, let 7d, miR-24-2, miR-363 and miR-92q) that potentially pair with mRNA from the *FASLG* gene that has lower expression in population A20. This pathway was identified following analysis of microRNA and mRNA expression in GeneGo (Figure 4-13). No pathways with several targets were identified.

In conclusion, the miRNA profiling of the resting transcriptome of high and low avidity populations of HLA B*08-restricted FL8-specific CD8+ T cell identified several potential pairing sites in target genes of interest, but no global pathways by GeneGo analysis. Mir-181a, previously reported to augment membrane proximal signalling is more highly expressed by the high sensitivity population A20.

Figure 4-11: Hierarchichal clustering of micro RNAs with low expression in A20

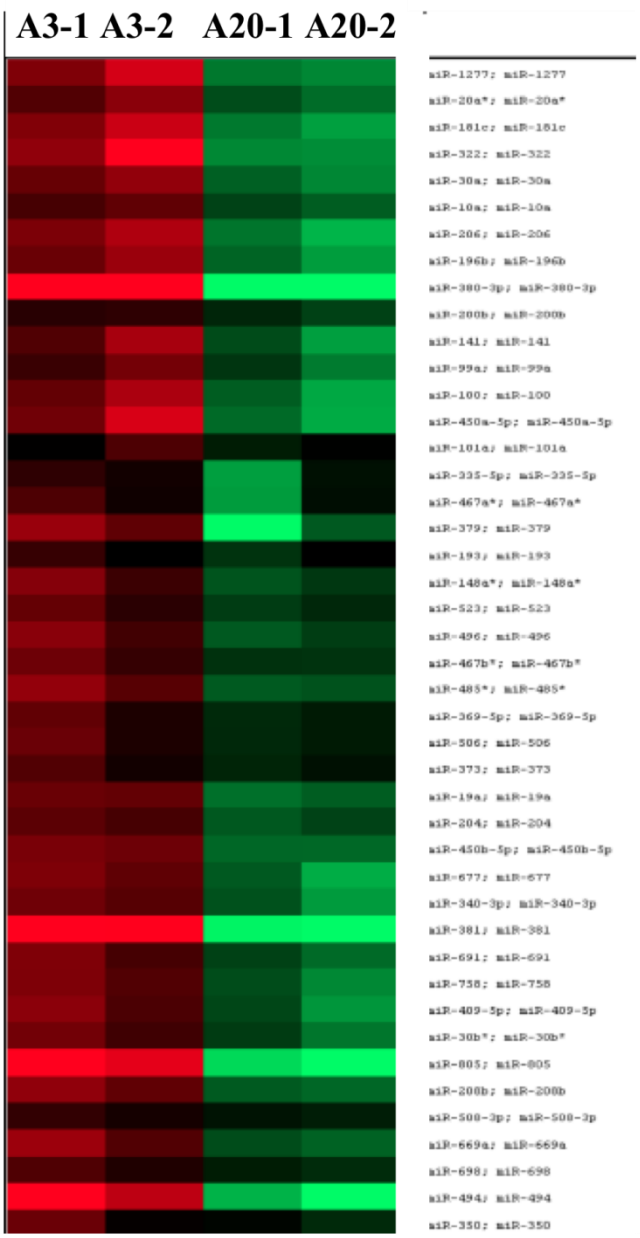


Figure 4.11: Heat map of microRNAs with low expression (Less than five fold lower expression than clone A3) in clone A20. Total RNA was extracted from two populations of clone A3 and A20 and sent for microRNA profiling by QuantoBio (Beijing, China). Hierarchical clustering was performed using GeneSpring GX.

Figure 4-12: Top 100 microRNAs with altered expression in A3 and A20

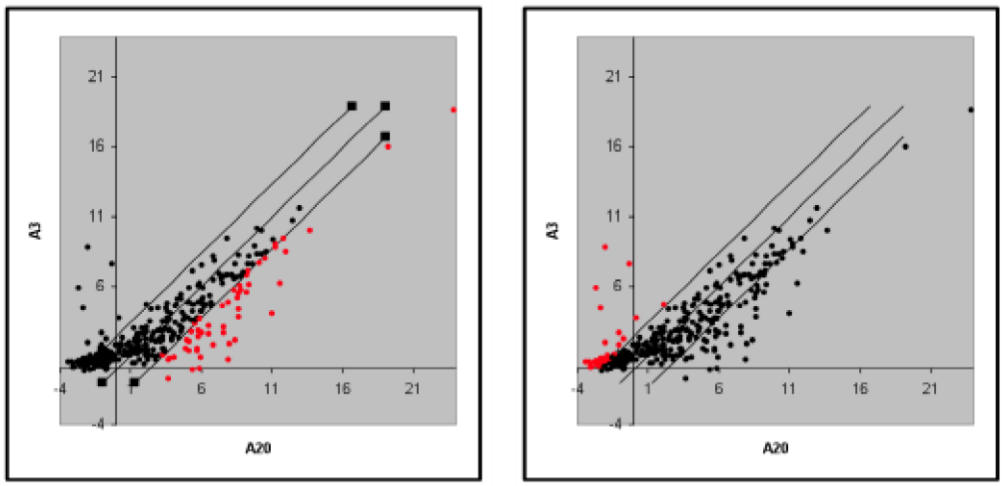


Figure 4.12: Top 100 microRNAs with altered expression in A3 and A20 (at least five fold change in expression between clones A3 and A20) were identified using BRB-array tools (NCBI). These are listed in tables 3 and four below.

Table 4-3: Top 53 microRNAs with High Expression in A20 Compared to A3

Unique id	mean of log intensities for A20	mean of log intensities for A3	FoldDiff [A20] v [A3]	Unique id	mean of log intensities A20	mean of log intensities A3	FoldDiff [A20]v[A3]
miR-520b	7.9427	0.6517	156.61	mir-214*	8.67541	5.4105	9.61
miR-16-2*	11.08563	3.9199	143.58	miR-17*	5.16492	1.9118	9.53
miR-24-2*	8.436	1.9909	87.13	miR-425	8.86117	5.6133	9.50
miR-187	8.04373	1.7789	76.90	miR-155	7.96377	4.7586	9.22
miR-18b	5.89507	-0.0075	59.82	miR-363	5.82886	2.6677	8.95
miR-27b*	5.44398	-0.1494	48.28	miR-200c	3.80298	0.7258	8.44
let-7g*	23.89001	18.6658	37.38	miR-374	5.04724	1.9749	8.41
miR-688	5.97401	0.7787	36.64	miR-30a*	3.69826	0.645	8.30
miR-27a*	7.67586	2.4854	36.52	miR-423-3p	7.52315	4.4761	8.27
miR-302a	6.89337	1.7127	36.27	let-7d	8.79496	5.9319	7.28
miR-674	8.69917	3.6604	32.87	miR-378	5.39018	2.5835	7.00
miR-34b-3p	7.6458	2.9494	25.93	miR-191	10.56754	7.8616	6.52
miR-181a	3.6929	-0.80473	22.59	miR-484	8.33163	5.6434	6.45
miR-666-5p	5.75498	1.2715	22.37	miR-30c	10.19381	7.5342	6.32
miR-17	8.73563	4.2551	22.32	miR-92a	11.30381	8.6653	6.23
miR-298	5.73688	1.4214	19.91	miR-22	9.26176	6.6691	6.03
miR-26b*	6.54595	2.444	17.17	miR-423-5p	11.91279	9.3502	5.91
miR-451	5.15719	1.2977	14.52	miR-34a	9.37212	6.9386	5.40
miR-504	13.71784	9.9122	13.98	miR-324-5p	5.57332	3.1431	5.39
miR-223	9.27775	5.4885	13.83	miR-26a-1*	3.34458	0.9231	5.36
miR-770-5p	12.02037	8.3178	13.02	mir-106b*	5.89792	3.4863	5.32
miR-744	6.08141	2.4853	12.09	miR-342-3p	11.30947	8.9355	5.18
let-7a	8.64106	5.0589	11.98	miR-143	3.4938	1.1303	5.15
miR-129-3p	4.96825	1.4001	11.86	miR-30e*	5.896	3.5594	5.05
miR-181b	5.85356	2.2887	11.83				
miR-132	6.58298	3.0398	11.66				
miR-212	4.15336	0.762	10.49				
miR-130b	9.43432	6.076	10.26				
miR-34c	19.30021	15.9629	10.11				

Table 4-4: Top 47 microRNAs with high expression in A3 compared to clone A20

Unique id	mean of log intensities A20	mean of log intensities A3	Log FoldDiff [A20]v[A3]	Unique id	mean of log intensities A20	mean of log intensities A3	Log FoldDiff [A20]v[A3]
miR-494	-1.98105	8.7006	0.00	miR-758	-2.53767	0.1656	0.15
miR-380-3p	-2.63184	5.777	0.00	miR-485*	-2.35428	0.3488	0.15
miR-381	-0.24575	7.485	0.00	miR-100	-2.18226	0.5141	0.15
miR-805	-2.31883	4.3288	0.01	miR-340-3p	-2.24034	0.4388	0.16
miR-106a	-1.95779	1.8882	0.07	miR-205	-0.94778	1.7117	0.16
miR-379	-3.37339	0.4165	0.07	miR-20a	-2.0689	0.5648	0.16
miR-721	-3.08332	0.4719	0.09	miR-340-5p	-2.14208	0.4773	0.16
miR-615-5p	-0.96344	2.5463	0.09	miR-450b-5p	-2.03506	0.5722	0.16
miR-411	-3.01416	0.3976	0.09	miR-1197	-1.81456	0.788	0.16
miR-322	0.2555	3.5399	0.10	miR-10b	-2.08748	0.486	0.17
miR-206	-2.70505	0.481	0.11	miR-30a	-2.19141	0.3555	0.17
miR-181c	-2.60463	0.5394	0.11	miR-524-5p	-1.96515	0.5313	0.18
miR-677	-3.00052	-0.0039	0.13	miR-691	-2.33303	0.1498	0.18
miR-335-3p	-2.30592	0.6753	0.13	miR-496	-1.97879	0.4986	0.18
miR-1277	-2.95197	0	0.13	miR-19a	2.16752	4.6017	0.19
miR-450a-5p	-2.20182	0.7127	0.13	miR-95	-1.92328	0.5084	0.19
miR-297a*	-2.44595	0.4591	0.13	miR-32	-1.88845	0.5035	0.19
miR-409-5p	-2.61495	0.2749	0.13	miR-30b*	-1.6509	0.7274	0.19
miR-669a	-2.1241	0.6842	0.14	miR-144	-1.74938	0.6248	0.19
miR-208b	-2.49479	0.2916	0.14	miR-148a*	-2.10266	0.2657	0.19
miR-302c	-1.83787	0.9484	0.14	miR-487b	-1.30305	1.0602	0.19
miR-196b	-2.23442	0.4965	0.15	miR-101b	-1.93676	0.4057	0.20
miR-1	-2.24036	0.4678	0.15	miR-141	-2.15572	0.1814	0.20
				miR-196a	-2.10799	0.2261	0.20

Table 4-3 and 4-4: The Top 100 miRNAs with altered expression (at least five fold change in expression between A20 and A3).

Table 4-5: Potential pairings of microRNAs and mRNA in clone A20

Target Gene [A20 > A3]	miR	Log Fold Diff [A20] v [A3]
FCRL3	**miR 485-5p**	0.15
FCER1G	**miR-30b**	0.19
CXCR6	**miR-101**	0.2
TYROBP	**miR-496**	0.18
MAL	**miR-196b**	0.15
	miR-196a	0.2
	miR-208b	0.14

Table 4-5: Target genes and potential miRNA pairing sites for high avidity CD8+ T-cell clone population A20. Potential microRNA pairing sites in candidate genes of interest were identified using miRecords (Xiao *et al.*, 2009), miRanda and an unpublished computer program (J.C. and C.-Z.C., unpublished data) that considers the secondary RNA structure, mismatch and G:U base pairing in the seed region, and coding and untranslated regions (UTRs) of candidate targets and compares predicted pairings using 11 programs (diana, microinspector, miranda, mirtarget 2, mitarget, nbmitar, pictar, pita, rna22, rnahybrid, and target scan) to give a final enrichment score (Li *et al.*, 2007). miRNA with altered expression in A20 (at least five fold lower expression than the intermediate avidity CD8+ T-cell clone A3) are listed in the table as the log fold difference.

Figure 4-13: GeneGo pathway analysis of mRNA and microRNA profiling of two populations of HLA B*08-restricted FL8-specific CD8+ T-cell clones (A3 and A20) that use the same TCR

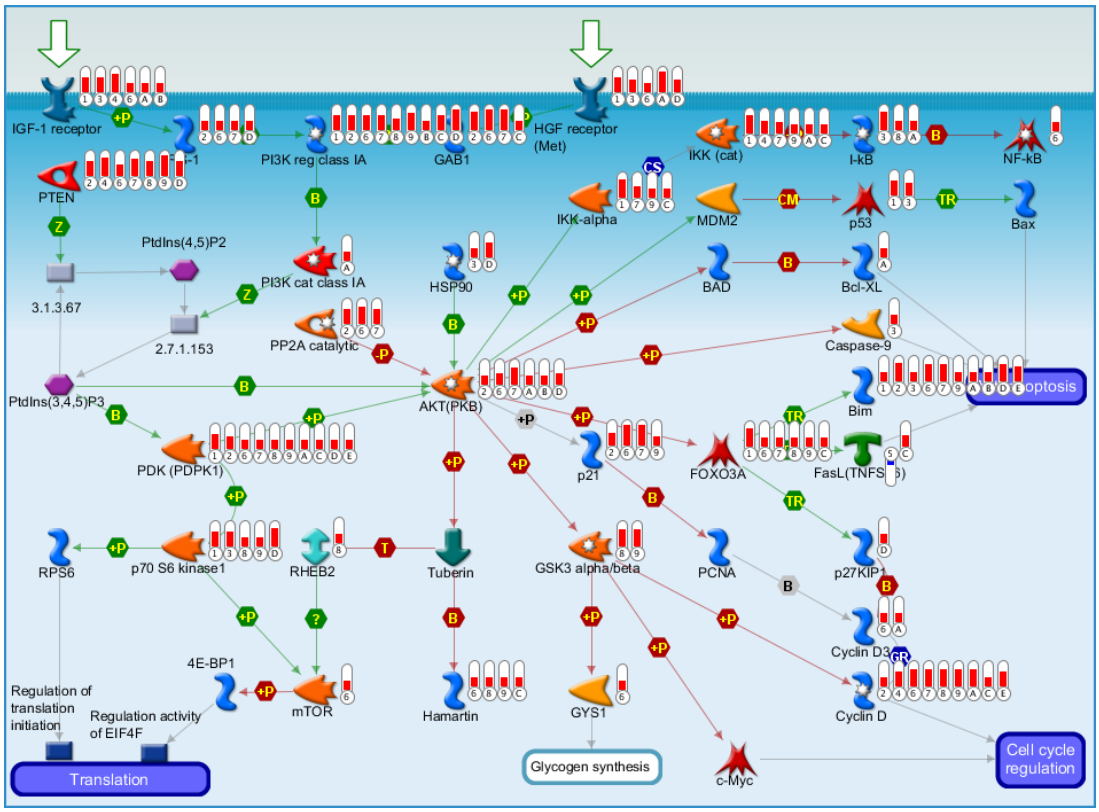


Figure 4-13: The Akt signalling pathway.

Gene expression data from A3 and A20 in the resting state was reviewed with microRNA expression data using the cellular pathways tool in GeneGo software (Thomson Reuters, St Joseph's, MI, USA). mRNA for *FASL* has reduced expression in clone A20 compared to clone A3 (Blue bar, "5") but there are seven (1, 6-9, C) miRNA targets that are more highly expressed in A20 compared with A3 (Red bars).

4.3 Discussion

The phenomenon of avidity maturation in CD8+T-cells that bear identical T cell receptors has previously been confined to animal models or mouse CD8+ T cell clones with distinct avidity generated following re-exposure to peptide ex vivo (Sharma and Alexander-Miller, 2011) (Kroger and Alexander-Miller, 2007a) (Cawthon *et al.*, 2001) (Cawthon and Alexander-Miller, 2002) (Slifka and Whitton, 2001) (Amoah *et al.*, 2012). These elegant studies highlight the importance of high sensitivity CTL in the control of viral infections, and implicate the role of subsequent antigen encounter in the periphery as the driving force behind avidity maturation.

The HIV-1 specific CD8+ T-cell clones from LTNP005 were all isolated from one blood sample, with a predominant (51%) usage of TRVB6-2 within the FL8/HLA B*08 tetramer positive population. All four clones used exactly the same TCR, suggesting a common origin from one naïve T cell. These four human HIV-1 specific CD8+ T-cell clones were generated by limiting dilution of the FL8/HLA B*08 tetramer positive population without exposure to peptide *in vitro.* Of note, the phenotype of each clone population was stable throughout subsequent rounds of re-stimulation *in vitro* and was evident in the earliest expanded CTL clones (also see Chapter 3). This raises the possibility of sensitivity maturation of the CD8+ T cell response in human viral infections as reported in mice. Critically, the high antigen sensitivity CD8+ T-cell clone displays optimal viral suppression *in vitro* and is a highly potent killer, with high magnitude of IFNγ secretion, qualities previously associated with control of retroviral infections (Almeida *et al.*, 2007) (Kattan *et al.*, 2009).

Various mechanisms of avidity maturation have been reported in CD8+ T cells bearing TCR of the same affinity but with distinct antigen sensitivity, all of which

occurred by subsequent encounter with antigen. Recently, the differentiation of a single naïve T-cell into short-lived effector cells (SLEC) or long-lived memory effector cells (MPEC) has been correlated with antigen sensitivity in mice (Amoah *et al.*, 2012). The gene most highly expressed in the high sensitivity population, A20, was CXCR6 and its ligand CXCL16. CXCR6 was first identified as a co-receptor for HIV-1 (Matloubian *et al.*, 2000), and is now known to be expressed by particular sub-sets of effector CD8+ T-cells, that are polarised toward secretion of type I cytokines, display an activated effector CD8 T cell phenotype and preferentially migrate into inflamed non-lymphoid tissues (Matsumura *et al.*, 2008) (Billerbeck *et al.*, 2010) (Sato *et al.*, 2005) (Freeman *et al.*, 2007) (Kim *et al.*, 2001). By contrast, the intermediate sensitivity clone population, A3, had high expression of CXCR3, that regulates trafficking of effector CD8+ T-cells to lymphoid tissue, including the lymph nodes, the spleen and discrete sites within the CNS that are induced by infection to secrete the ligands CXCL9 and CXCL10 (Guarda *et al.*, 2007) (Hu *et al.*, 2011) (Zhang *et al.*, 2008) (Stiles *et al.*, 2006).

Differential homing to inflamed tissues or lymphoid sites may reflect the opportunity for distinct encounter of each clone with antigen, HIV-1 viral variants, inflammatory cytokine milieu (Soudja *et al.*, 2012), and interaction with other immune cells that may all influence sensitivity maturation (Hu *et al.*, 2011) (Kohlmeier *et al.*, 2011) (Kurachi *et al.*, 2011). Analysis of the transcriptome of single CD8+ T-cells following HIV-1 vaccines identified *CXCR3* as a potential phenotypic marker (Flatz *et al.*, 2011). Our data would support the inclusion of CXCR3 into a panel of markers to be validated for monitoring HIV-1 vaccine outcome.

Mechanisms of sensitivity maturation in mice are reported to involve TCR and CD8 expression (Viola and Lanzavecchia, 1996) (Cawthon *et al.*, 2001) (Park *et al.*, 2007)

and mRNA levels of CD8β (Cawthon and Alexander-Miller, 2002), which were the same in our two clone populations. This suggested events downstream of the TCR regulated antigen sensitivity and viral suppression. Augmented membrane proximal T-cell receptor signal transduction at the earliest event of CD3ζ phosphorylation, and the level of phosphorylated ZAP-70 and Lck has been reported to tune antigen sensitivity in TCR transgenic mice (Slifka and Whitton, 2001) (Amoah *et al.*, 2012) (Sharma and Alexander-Miller, 2011). The expression of mir-181a in CD4+CD8+ mouse thymocytes enhances antigen sensitivity by the negative regulation of multiple membrane proximal signalling phosphatases, increasing steady state levels of phosphorylated intermediates and reducing the TCR signalling threshold (Li *et al.*, 2007).

The most interesting finding of this study is that the three next most highly expressed genes by the high avidity population of clone A20 are *FCRL3*, *FCER1G* and *TYROBP* that all have an ITAM motif in the cytoplasmic domain. FCRL3, an orphan receptor of unknown function, is a member of the Fc-receptor like family (Davis, 2007). This family of Fc-like receptors are located within a gene locus that includes an extended family of immune cell signalling molecules, including the CD3ζ chain and FCER1G.

FCRL3 is a type 1 cell surface glycoprotein receptor with 6 extracellular immunoglobulin domains, an uncharged transmembrane region and a cytoplasmic tail that contains an unconventional ITAM in addition to an orthodox ITIM. The FCRL3 ITAM motif differs from the conventional ITAM motif, (E/D)XXYXX(L/I)-X_{6-9}-YXX(L/I) (Reth, 1989), with a V rather than L or I residue at the +3 position in relation to the first tyrosine. This should not be a barrier to signalling, as unconventional ITAM motifs with signalling function are seen in other receptors, for example FCRL1, a member of the same gene family, is an activating co-receptor in B

cells (Leu *et al.*, 2005). Consistent with the two motifs, FCRL3 has been shown to associate with tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1) and SHP-2, and Syk and ZAP-70 (Xu *et al.*, 2002; Swainson *et al.*, 2010).

p56 Lck, a Src family tyrosine kinase phosphorylates the two tyrosine residues within ITAM motifs on the cytoplasmic tails of the CD3 complex, the first biochemical event of TCR signalling (Smith-Garvin *et al.*, 2009) (Veillette *et al.*, 1988). Phosphorylation of the ITAM provides a docking site for ZAP-70, a src-family tyrosine kinase. ZAP-70 is activated by Lck, and in turn phosphorylates a number of downstream signalling and adaptor molecules that nucleate the proximal signalling complex leading to the activation of multiple downstream pathways. Lck also phosphorylates the tyrosine residue of the ITIM on co-receptors that are reported to negatively regulate the TCR signal, by recruiting protein tyrosine phosphatases, Src homology region 2 domain-containing phosphatase -1 (SHP-1) and SHP-2. The balance of phosphatases and tyrosine kinases associated with the CD3 complex may regulate the phosphorylation of the CD3ζ chain, Lck, Zap-70 and signalling and adapter molecules of the proximal signalling complex (Hermiston *et al.*, 2002; Stefanova *et al.*, 2003). FCRL3 may therefore act as a key regulatory molecule in T cell function.

FCER1G and TYROBP, adaptor proteins with ITAM motifs, are more highly expressed in the high sensitivity clone population A20. These adaptor proteins are coupled to distinct ligand-binding partners according to cell type, for example NK cells, γδ T cells, CD8+ T cells and myeloid lineage cells and play key roles in the cross-regulation of calcium signalling (Ivashkiv, 2009). FCER1G is associated with the high affinity IgE receptor on mast cells, triggering downstream signalling events

that lead to mast cell degranulation. FCER1G knock out mice have defects in NK cell mediated ADCC but the T cells are normal (Davis *et al.*, 2002). It is however possible, that FCER1G has a distinct role in human T cells.

It is intriguing that we observe differential expression of cell surface receptors with ITAM motifs on the high sensitivity population of clone, and expression of inhibitory receptors with ITIM motifs on the intermediate sensitivity clone population, *LRRN3, FCRL6, KIR2DL3* and *KIR2DL4*. NK receptors can integrate with TCR signalling to modulate effector and memory CD8 CTL cytokine secretion, cytotoxicity and enhance survival of memory CTL (Henel *et al.*, 2006) (Junevik *et al.*, 2007) (Li *et al.*, 2009) (Mingari *et al.*, 2005). KIR2DL3, has a cytoplasmic ITIM motif, and KIR2DL4, a framework KIR gene has a cytoplasmic ITIM and a charged transmembrane residue that associates with ITAM bearing adaptors. KIR genes are differentially expressed on the progeny of a single naïve CD8 T (Vely *et al.*, 2001). This is controlled epigenetically, and once expressed, expression is stable through subsequent rounds of proliferation.

FCRL6 is also a member of the Fc-receptor like family but contains an orthodox ITIM motif. Intriguingly the ligand was recently described to be the MHC class II allele HLA DR (Schreeder *et al.*, 2010) but little is known regarding the biochemical aspects of how it signals. FCRL6 is expanded in HIV-1 and CLL on exhausted CD8+ T-cells, and is associated with dysregulated cellular proliferation (Schreeder *et al.*, 2008) (Wilson *et al.*, 2007). Exhausted viral specific CD8+ T-cells are actively maintained in a state of progressive immune dysfunction by signalling of both activating and inhibitory receptors. This was first described for PD-1, but a further 6 receptors have now been identified (Blackburn *et al.*, 2009) (Day *et al.*, 2006). A20 has a limited cytokine profile, IFNγ and MIP1β, but does not express PD-1. A3 has a

higher expression of PD-1 although this may be the result of its higher activation status, indicated by CD69 even when "resting" (Sauce *et al.*, 2007) (Salisch *et al.*, 2010). Selective blockade of multiple inhibitory receptors or stimulation of activating receptors may convert A3 into a population of clone with similar sensitivity and anti-viral potential as A20 (Trautmann *et al.*, 2006).

We also observe differential expression of several molecules involved with the regulation of Lck between our two cell populations. The activity of Lck is tightly regulated by phosphorylation of two tyrosine residues, autophosphorylation of the catalytic domain tyrosine residue required for full activity, and phosphorylation of an inhibitory C-terminal tyrosine residue by carboxyl-terminal src kinase (Csk) (Hermiston *et al.*, 2003). CD45, a receptor-like tyrosine phosphatase abundantly expressed on the T-cell surface, dephosphorylates the inhibitory and activating tyrosines to act as both a positive and negative regulator of TCR signalling (Hermiston *et al.*, 2003).

The expression of CD45 isoforms by alternative splicing is modulated during naïve, memory and terminal effector T-cell differentiation, with a short isoform, CD45RO, expressed by memory CTL (Zikherman and Weiss, 2008). The short isoform carries less negatively charged sialic acid residues, permitting more efficient dimerisation, that is reported to block the phosphatase active site inhibiting the TCR signal (Xu and Weiss, 2002). Of note, the most sensitive population of HLA B*08-restricted FL8-specific CD8+ CTL clone expresses the CD45RO isoform (CD45RA negative, Figure 4-2). Unravelling this phenotype is therefore complex given the poise of positive and negative regulation exhibited by CD45. Many studies of HIV-1 control have not found a consistent correlation of CD8+ T-cell function and expression of CD45RA or CD45RO alone (Almeida *et al.*, 2007) (Saez-Cirion *et al.*, 2007) (Betts *et al.*, 2006).

Two genes that regulate CD45 and Lck were also identified, A3 had higher expression of *PTPRCAP,* that encodes protein tyrosine phosphatase receptor type C-associated protein, also known as CD45 associated protein, that is reported to act as both a positive and negative regulator of the TCR signal, and A20 had higher expression of *MAL,* (also known as T-cell differentiation protein) that delivers Lck to the plasma membrane (Takeda *et al.,* 2004) (Leitenberg *et al.,* 2007) (Kung *et al.,* 1999) and could augment TCR proximal signalling events.

This is the first report of human derived T-cell clones that use the same TCR but have distinct antigen sensitivity. The impact of antigen sensitivity on the control of persistent viral infections underlies the importance of understanding the mechanism of functional maturation and ontogeny in response to recurrent antigen encounter in the periphery, and particularly exposure to different cytokine milieu. Our data supports the key role of the regulation of the membrane proximal signalling complex in antigen sensitivity. Highly sensitive CTL are more potent killers. It will be important to explore if this is due to differences in TCR strength alone, or if the expression of the receptors we identified, KIR2DL3, KIR2DL4, FCRL3, FCRL6, LRRN3, FCER1G and TYROBP, change the quality of the signal to enhance degranulation or the induction of transcripton of lytic molecules perforin and granzyme B. Our work suggests a panel of cell surface markers including CXCR6, FCRL3, FCER1G and TYROBP ("Good" CTL) and CXCR3, FCRL6, and LRRN3 ("Bad" CTL) to validate in HIV-1 infected cohorts to determine if this could lead to a meaningful assessment of the CTL quality induced by an HIV-1 vaccine.

5 CD8 T cell response to HIV-1; epigenetic distinctions between seeing and acting

5.1 Introduction

HIV-1 specific CD8+ T-cells of high antigen sensitivity may contribute to the durable control of HIV-1 infection (Mothe *et al.*, 2012; Almeida *et al.*, 2007). Factors that have been associated with high antigen sensitivity and enhanced viral suppression include rapid recognition of the infected cell and efficient cell lysis in response to only a few epitope-MHC I alleles (Derby *et al.*, 2001; Kattan *et al.*, 2009), the balance between cell activation, proliferation and resistance to apoptosis (Alexander-Miller, 2000), ontogenetic factors including differentiation into either short lived effector cells (SLEC) or long lived memory cells (Amoah *et al.*, 2012) (Harari *et al.*, 2009) and enhanced cross-recognition of viral escape variants (Varela-Rohena *et al.*, 2008).

The role of CD8+ T-cell function "killing or 'kines" (cytokines and soluble factors) remains controversial for optimal suppression of HIV-1 (Borrow *et al.*, 1994; Plata *et al.*, 1987; Cocchi *et al.*, 1995; Walker *et al.*, 1986). Antigen sensitivity may enhance lytic efficiency, secretion of IFNγ on a per cell basis, and may be linked to a polyfunctional profile that simultaneously examines killing and release of soluble factors (Almeida *et al.*, 2007) (Kattan *et al.*, 2009) (La Gruta *et al.*, 2004).

CTL use two mechanisms to induce apoptosis in a target cell displaying a cognate pMHC complex. The predominant pathway, degranulation, is the exocytosis of lytic granules that release a myriad of pre-formed anti-microbial molecules into the synaptic cleft, particularly perforin (Kagi *et al.*, 1994), and granzyme B, a member of the family of granule serine proteases (Heusel *et al.*, 1994). Newly synthesised

perforin and granzymes can also be directly secreted at the immune synapse (Isaaz *et al.*, 1995) (Makedonas *et al.*, 2009). CTL that efficiently kill by effective delivery of granzyme B and perforin, not by exocytosis of preformed lytic proteins marked by CD107a, but by de novo synthesis of lytic proteins has been previously correlated with HIV-1 protection (Wolint *et al.*, 2004; Migueles *et al.*, 2002) (Betts *et al.*, 2006) (Hersperger *et al.*, 2011) (Hersperger *et al.*, 2010) (Makedonas *et al.*, 2009). The second lytic mechanism is mediated by the ligation of death receptors, FasL (Walsh *et al.*, 1994) (Anel *et al.*, 1994), TNF and TNFR10 (also known as TRAIL) (Chavez-Galan *et al.*, 2009). Cytotoxicity requires cell-to-cell contact, is exquisitely sensitive to class I MHC restriction, and is the predominant mechanism of HIV-1 suppression reported *in vitro* (Saez-Cirion *et al.*, 2007) (Zinkernagel and Doherty, 1974). Whether cytotoxicity and antigen sensitivity directly correlate is highly controversial, as killing can be triggered by recognition of only a few peptide-MHC complexes (Purbhoo *et al.*, 2004), and may not be enhanced by increasing the epitope density beyond a specific threshold (Sykulev *et al.*, 1996) (Bennett *et al.*, 2007), although TCR signal strength enhances killing efficiency by directing the route of granule polarisation, and delivery of lytic molecules (Beal *et al.*, 2008) (Beal *et al.*, 2009) (Jenkins *et al.*, 2009).

CTL have an armament of soluble factors that may contribute to HIV-1 suppression, the chemokines CCL3, CCL4, and CCL5 that competitively inhibit viral entry (Cocchi *et al.*, 1995) (Alkhatib *et al.*, 1996) (Feng *et al.*, 1996), and cytokines interferon gamma (IFN-γ), critical for non-cytolytic suppression of measles (Stubblefield Park *et al.*, 2011), but that might activate HIV-1 replication as does tumour necrosis factor alpha (TNFα) (Poli *et al.*, 1990) (Warfel *et al.*, 1995). Based on multiparameter flow cytometry it has been argued that the "robustness" of the

effector response by small "polyfunctional" CTL sub-populations, as measured by the simultaneous detection of five (CCL3 or CCL4, CD107a, IFNγ, TNFα and IL2) or six functions (+perforin) following ex vivo antigen stimulation, correlates with protection (Betts *et al.*, 2006) (Hersperger *et al.*, 2010). Polyfunctionality has been linked to sensitivity, due to the ascending TCR signal that triggers thresholds for several functions simultaneously (Almeida *et al.*, 2009) but high avidity CTL with limited cytokine 'functionality' are reported (Harari *et al.*, 2007) and ontogenetic factors may confound the correlation of polyfunctionality and antigen sensitivity (Rehr *et al.*, 2008) (Streeck *et al.*, 2008) with progressive loss of cytokines reported on repeated exposure to antigen, anergy, senescence and exhaustion(Shin and Wherry, 2007; Henson *et al.*, 2009; Blackburn *et al.*, 2009). Indeed, only a minority of all CD8+ T-cells are able to respond to even high dose antigen stimulation by the simultaneous secretion of IFNγ, TNFα and IL2. The capacity of CD8+ T-cells to secrete the cytokine IFNγ, and lytic molecules granzyme B and perforin upon TCR engagement is induced during the first encounter with antigen, and has been reported to be coupled to cellular proliferation and epigenetic changes that activate these genes for transcription (Migueles *et al.*, 2002) (Araki *et al.*, 2008) (Juelich *et al.*, 2009; Araki *et al.*, 2009).

Antigen sensitivity may therefore modulate the cytokine and chemokine profile, both quantitatively and qualitatively, of CD8 CTL that may alter anti-viral efficacy. We aimed to detect this by functional assays, transcriptome analysis and to determine if epigenetic regulation contributed to the transcriptional signature. Epigenetic modification of the genome permits the expression of patterns of genes, known as a transcriptional signature, that determine the specific functions that define the cell, for example the polarisation of CD4 Th1/2 cells. Cellular DNA is ravelled around

histones to form chromatin in the nucleus. The nucleosome is the basic structural subunit of chromatin, and is composed of approximately 156 base pairs of DNA wrapped around an octamer of histones (Wilson *et al.*, 2009). Epigenetic processes that affect gene expression include methylation of cytosine residues in CpG dinucleotides in DNA, post-translational modification of histone proteins and changes in the position and compaction of chromatin structure, that is tightly coiled in heterochromatin or unravelled in transcriptionally accessible euchromatin. Histone proteins can be modified by enzymes that catalyse the addition or removal of acetyl, methyl, phosphate, ubiquitin, sumoyl or ADP ribose groups (Wilson *et al.*, 2009). Little is known regarding how epigenetic modification of the CD8+ T-cell transcriptome in anti-viral CD8+ T-cells impact HIV-1 control.

We have identified two populations of HLA B*08 restricted-FL8 specific CD8+ T-cell clone, with distinct cytokine profiles, antigen sensitivity and ability to control HIV-1 replication *in vitro.* Critically, the high antigen sensitivity CD8+ T-cell clone displayed optimal viral suppression *in vitro*, despite a more limited cytokine profile, as TNFα and IL2 were not secreted, and was a highly potent killer, with high magnitude of IFNγ secretion, qualities previously associated with control of retroviral infections (Almeida *et al.*, 2007) (Kattan *et al.*, 2009). Comparison of the resting transcriptome of these two HLA B*08-restricted FL8-specific T-cell clones identified that the high sensitivity population expressed higher levels of cell surface receptors containing the immunoreceptor activating motif (ITAM) receptor and the intermediate sensitivity population more highly expressed receptors containing the immunoreceptor inhibitory motif (ITIM), consistent with reported mechanisms that "fine-tune" TCR signalling at the proximal signalling complex (Sharma and Alexander-Miller, 2011; Amoah *et al.*, 2012) (Slifka and Whitton, 2001) (Li *et al.*,

2007; Hermiston *et al.*, 2002; Stefanova *et al.*, 2003). The high sensitivity CTL had higher expression of CXCR6 and FCRL3, and the intermediate sensitivity CTL had higher expression of CXCR3, FCRL6, LRRN3, KIR2DL3, and KIR2DL4. NK receptors can integrate with TCR signalling to modulate effector and memory CD8 CTL cytokine secretion, cytotoxicity and enhance survival of memory CTL (Henel *et al.*, 2006) (Junevik *et al.*, 2007) (Li *et al.*, 2009) (Mingari *et al.*, 2005). KIR genes are differentially expressed on the progeny of a single naïve CD8 T cell (Vely *et al.*, 2001). This is controlled epigenetically, and once expressed, expression is stable through subsequent rounds of proliferation.

High antigen sensitivity may result in a quantitatively enhanced response that is triggered earlier, "seeing", or a qualitatively distinct effector response, "acting". In order to explore how the expression of effector function can differ between genetically identical CD8+ T-cell populations that have the same TCR but distinct antigen sensitivity we compared the activated transcriptomes of HLA B*08-restricted FL8-specific CD8+ T-cell clone populations of high sensitivity, A20, and intermediate sensitivity A3. We then asked if these differences in gene expression could be explained by epigenetic modifications.

5.2 Results

5.2.1 T-cell sensitivity or distinct effector program for optimal viral suppression: activation with 2uM peptide for 6 hours

Thus far, we have found that HLA B*08-restricted FL8-specific CD8+ T-cell clones with the same T-cell receptor had distinct T-cell sensitivity. It has been reported that CD8+ T-cells with high antigen sensitivity respond more rapidly and more potently to viral infected cells with enhanced killing and functionality (Almeida *et al.*, 2009) (Kattan *et al.*, 2009; Derby *et al.*, 2001). In fact, we found that the more sensitive population of clone, A20, has enhanced *in vitro* control of viral replication, predominantly mediated by the elimination of p24 positive CD4+ T-cells, and does not secrete TNFα or IL2, in contrast to the intermediate sensitivity population, A3. To address whether the high sensitivity A20 CD8+ T-cell clone is more effective at viral suppression *in vitro,* because of a more potent response when the epitope-MHC complex density is limiting, “seeing”, or due to the triggering of a distinct effector program upon activation ”acting”, we analysed the transcriptome of A20 and A3 following activation with a limiting antigen stimulus. Ex vivo activation, with the simultaneous analysis of multiple parameters of HIV-1 specific CD8+ T-cells with 2uM peptide for six hours has been reported to correlate with viral load (Betts *et al.*, 2006). This has been used to monitor vaccine efficacy as one of the best available assays (McElrath *et al.*, 2008).

We re-created these activation conditions using an HLA-matched CD4+ T-cell line (C8166) pulsed with 2uM epitope to compare simultaneously the secretion of soluble factors in the supernatant (Figure 5-1) and the activated transcriptome of the high and intermediate sensitivity CD8+ T-cell clone populations, A20 and A3, following six hours of co-culture. High magnitude of IFNγ secretion by high avidity HLA B*27-restricted KK10 specific CD8+ T-cell clones has been previously reported with a higher IFNγ median fluorescence intensity by intracellular cytokine staining (Almeida *et al.*, 2007). IFNγ secretion may therefore act as a rheostat to indicate TCR signal strength (Betts *et al.*, 2004). The high sensitivity population, A20, secreted a significantly higher magnitude of IFNγ (A20: 3524 +/- 145.9 pg/ul v A3: 379.6 +/- 7.836 pg/ul, $p<0.0001$ (t-test)) and IL13 (A20 1786+- 24.79 pg/ul v A3 1786+- 24.79 pg/ul, $p<0.001$ (t-test)) than the intermediate sensitivity population, A3 (Figure 5-1). Confirming our earlier reports (Chapter 3) population A20 secreted significantly less TNFα (A20 79.97+/- 4.035 v A3 440.1+/- 24.4 pg/ul, p value 0.001 (t-test)) and IL2 (A20 53.47 v A3 64.76 pg/ul, $p=0.036$ (t-test)) than A3. Transcriptome analysis identified 206 genes that were differentially expressed (Absolute fold change (FC) of at least 2.0 in A20 compared with A3, $p<0.01$ (ANOVA)) in the high sensitivity population, A20, compared to the intermediate population, A3 (Appendix, Table 8.2). Biological processes showing significant enrichment included signal transduction ($p=9.49 \times 10^{-13}$), cell surface receptor signal transduction ($p=5.90 \times 10^{-11}$), immune response ($p=1.19 \times 10^{-7}$), cell motion ($p=9.02 \times 10^{-6}$), signalling cascade ($p=2.32 \times 10^{-5}$), cell to cell adhesion ($p=4.09 \times 10^{-5}$) and apoptosis ($p=2.09 \times 10^{-4}$) (Figure 5-2).

Figure 5-1: Cytokine and chemokine secretion of HLA B*08-restricted FL8-specific CD8+ T-cell clones activated for six hours with 2uM epitope

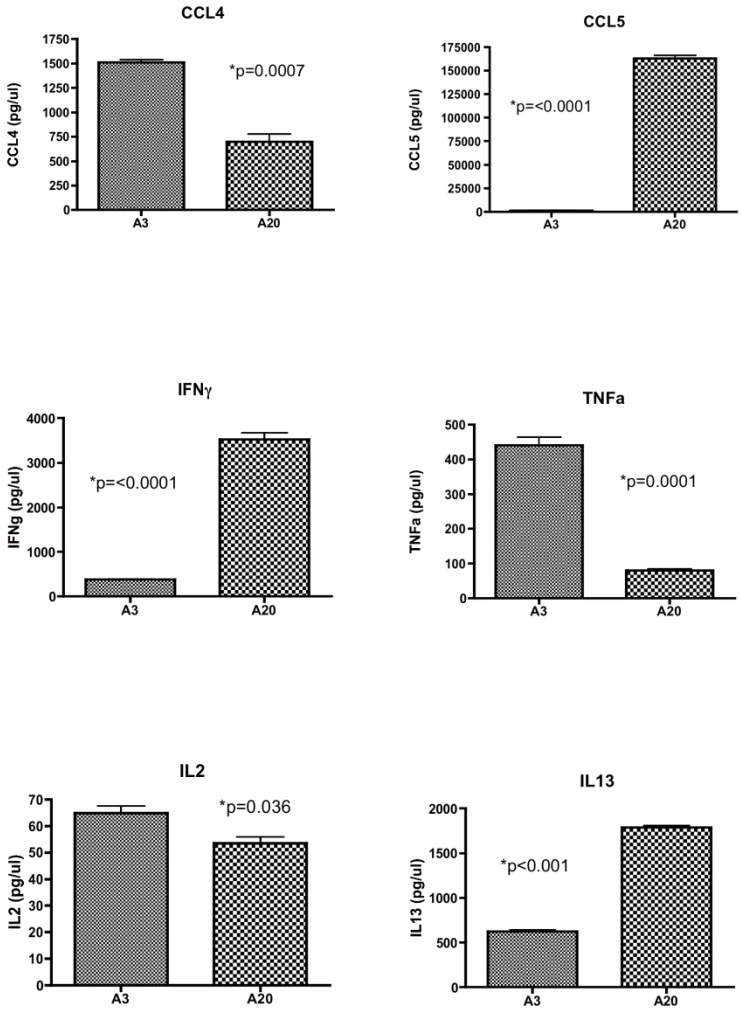


Figure 5.1: HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR have distinct effector function on activation with the same stimulus, 2uM peptide. HLA B*08-restricted FL8-specific CD8+ T-cell clones A20 (high sensitivity) and A3 (intermediate sensitivity) were co-cultured with HLA B*08 CD4+ T-cell line (C8166) pulsed with 2uM peptide at 10:1 E:T ratio. At 6 hours, the supernatant was harvested and analysed by illumina bead technology for CCL4 (MIP1β), IFNγ, TNFα, IL2, CCL5 (RANTES) and IL13. Each CD8+ T-cell clone population was sorted by negative selection using magnetic beads and total RNA extracted for transcriptome analysis on HT112v4.0 illumina expression arrays (See figure 2). Each clone was analysed in duplicate in three experiments. P values were calculated using the student t-test.

Figure 5-2: Classification of genes differentially expressed in HLA B*08-restricted FL8-specific CD8+ T-cell clones (A20 and A3) with the same TCR into biological process

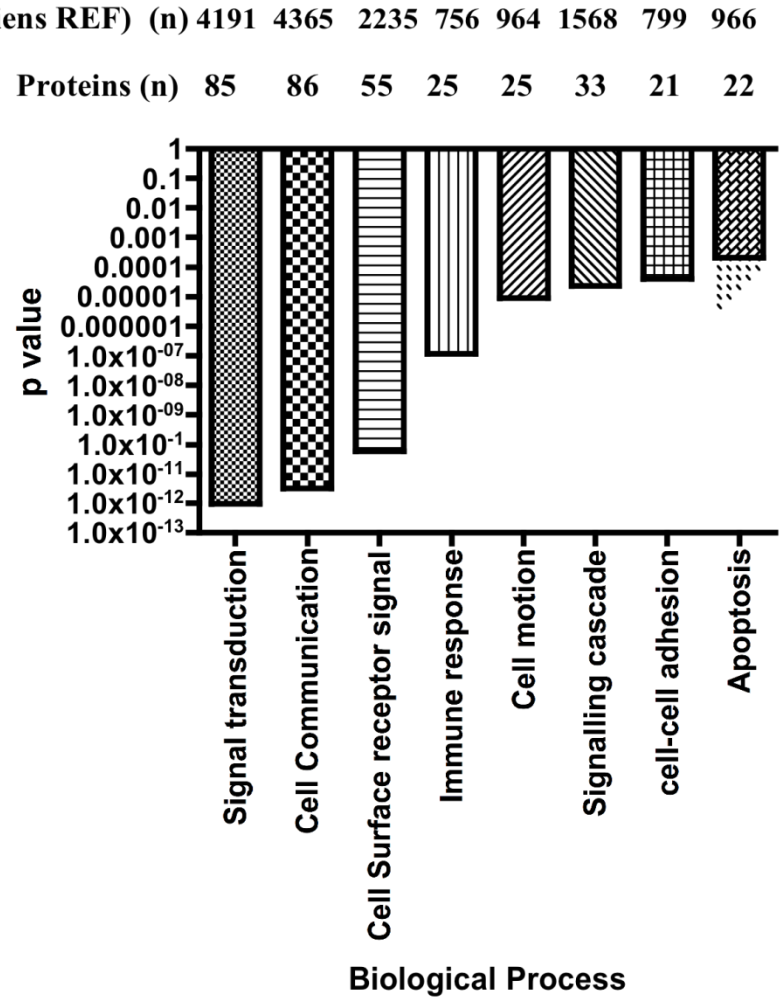


Figure 5-2: Classification of differentially expressed genes by biological process. Bar chart of the biological pathways and processes identified within the 206 genes differentially expressed (Absolute FC A20 [2uM] v A3 [2uM] <2>, p<0.01(ANOVA) following activation with 2uM peptide for six hours by gene set enrichment analysis in PANTHER (Thomas *et al.*, 2003). P-value (vertical axis) indicates the probability that the pathway is indicated by chance alone. The number (n) of *H.sapiens* genes in the reference group (REF) and number of observed genes (protein) within the candidate genes of interest (Absolute up or down regulation in A20 compared to A3 of at least two fold changes, p<0.01 by ANOVA) is indicated for each pathway

However, a significant difference at the mRNA expression level for *IFNG* was not detected. The transcriptome data was therefore re-analysed by applying an empirical Bayes method, implemented within the linear models for microarray data "limma' package in the R programming language (Smyth, 2004) that models the variation profiles of all genes, and then uses this to better estimate the variance of gene expression (Figure 5-3). In this more stringent analysis, both HLA B*08-restricted FL8-specific CD8+ T-cell clones had distinct expression (base 2 logarithmic (log FC) of at least 1.5 fold difference (=absolute fold change of 2.83), $p<0.05$ (LIMMA)) of 32 genes (Table 5-1 and 5-2, Figure 5-4 and 5-5). A significant difference (adjusted P value = 6.54×10^{-7}, B=10.53) in the expression level of *IFNG* was detected, at a log FC of 0.73 in A20 compared to A3. This suggested that quantitative differences directly resulting from distinct TCR signal strength in mRNA expression between the two clone populations might be below the level of sensitivity of detection for the transcriptome analysis, as although clearly statistically significant (*IFNG:* adjusted P value = 6.54×10^{-7}), a log-fold cut-off of at least 1.0 is commonly applied. We therefore focused on the molecular and biological process of the 32 candidate genes that were identified. Candidate genes more highly expressed in the high sensitivity population, A20, compared with the moderate sensitivity population, A3, following co-culture with CD4+ T-cell line (C8166) cells pulsed with 2uM epitope, are listed in Table 5-1: A20 > A3 [2uM], and their function is illustrated in Figure 5-4. Candidate genes with lower expression in A20 compared with A3 are listed in Table 5-2: A20 < A3 [2uM], and their function is illustrated in Figure 5-5.

Figure 5-3: Heat map of transcriptome analysis of HLA B*08-restricted FL8-specific CD8+ T-cell clones of high and intermediate sensitivity activated for six hours with CD4+ T-cell line pulsed with 2uM peptide

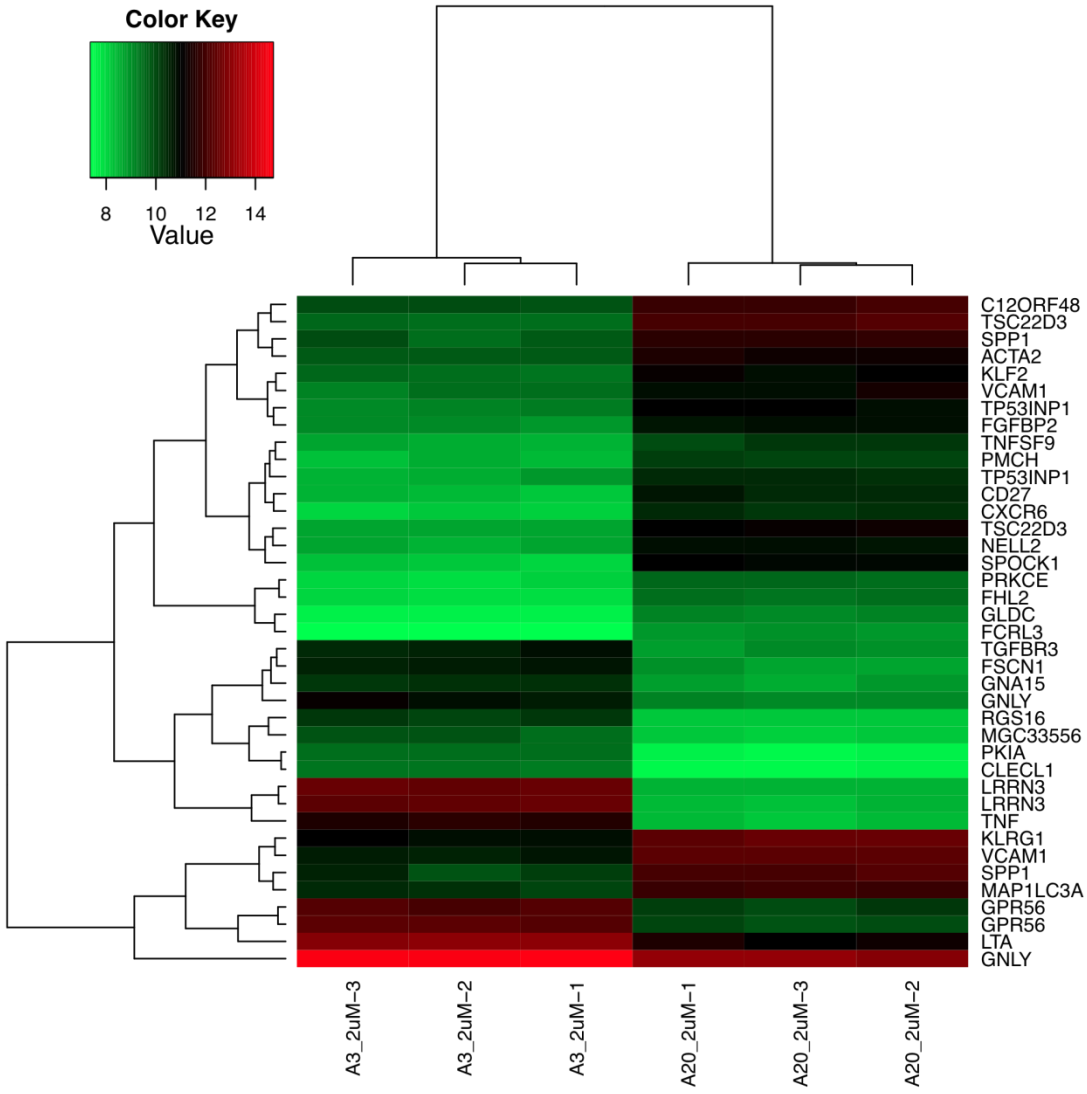


Figure 5-3: Hierarchical clustering of gene expression data from two HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR (A20 and A3). Each column represents a separate mRNA sample for each clone from three replicate experiments. Each row represents a probe from the bead array that maps to a gene in the Ensembl database. The colour scale represents the normalised intensity of complementary RNA directly hybridised to each probe for each sample. Each gene may be represented by more than one probe. 32 genes had significant differential expression (Log_2 FC of at least 1.5 up or down in A20 compared to A3, $p<0.05$ (LIMMA)) and are listed below (Table 1 and 2) with function illustrated (Figure 5 and 6). Heatmap was generated using Heatmap2.

Table 5-1: A20 >A3 [2uM]

logFC	Adj.P.Val	B	Symbol	Name	Entrez_ID
2.83	2.78E-19	40.77	**SPOCK1**	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	6695
2.55	1.98E-12	25.08	**TSC22D3**	TSC22 domain family, member 3	1831
2.26	1.57E-08	14.76	**CXCR6**	Chemokine (C-X-C motif) receptor 6	10663
2.18	1.91E-13	28.27	**NELL2**	NEL-like 2 (chicken)	4753
2.16	6.18E-12	23.82	**CD27**	CD27 molecule	939
2.15	1.13E-12	25.81	**PARPBP**	PCNA-interacting partner	55010
1.98	2.12E-06	8.84	**SPP1**	Secreted phosphoprotein 1(Osteopontin)	6696
1.94	8.22E-11	20.93	**FGFBP2**	fibroblast growth factor binding protein 2	83888
1.81	1.55E-07	12.06	**TP53INP1**	tumor protein p53 inducible nuclear protein 1	94241
1.77	7.82E-13	26.41	**PMCH**	Pro-melanin-concentrating hormone	5367
1.67	1.23E-11	23.00	**VCAM1**	Vascular cell adhesion molecule 1	7412
1.65	4.65E-09	16.20	**MAP1LC3A**	Microtubule-associated protein 1 light chain 3 alpha	84557
1.57	3.04E-09	16.71	**TNFRSF9**	Tumor necrosis factor (receptor) superfamily, member 9	21942
1.57	1.91E-13	28.22	**PRKCE**	Protein kinase C, epsilon	5581
1.55	2.20E-13	28.00	**ACTA2**	Actin, alpha 2, aortic smooth muscle	59
1.53	8.76E-07	9.97	**KLRG1**	Killer cell lectin-like receptor subfamily G, member 1	10219
1.52	6.36E-15	31.67	**GLDC**	Glycine dehydrogenase (decarboxylating)	2713
1.52	6.08E-15	31.85	**FHL2**	Four and a half LIM domains 2	2274
1.51	3.59E-10	19.13	**KLF2**	Kruppel-like factor 2 (lung)	10365
1.51	4.45E-13	27.12	**FCRL3**	Fc receptor-like 3	115352

Figure 5-4: A20 >A3 [2uM] Cartoon of gene function

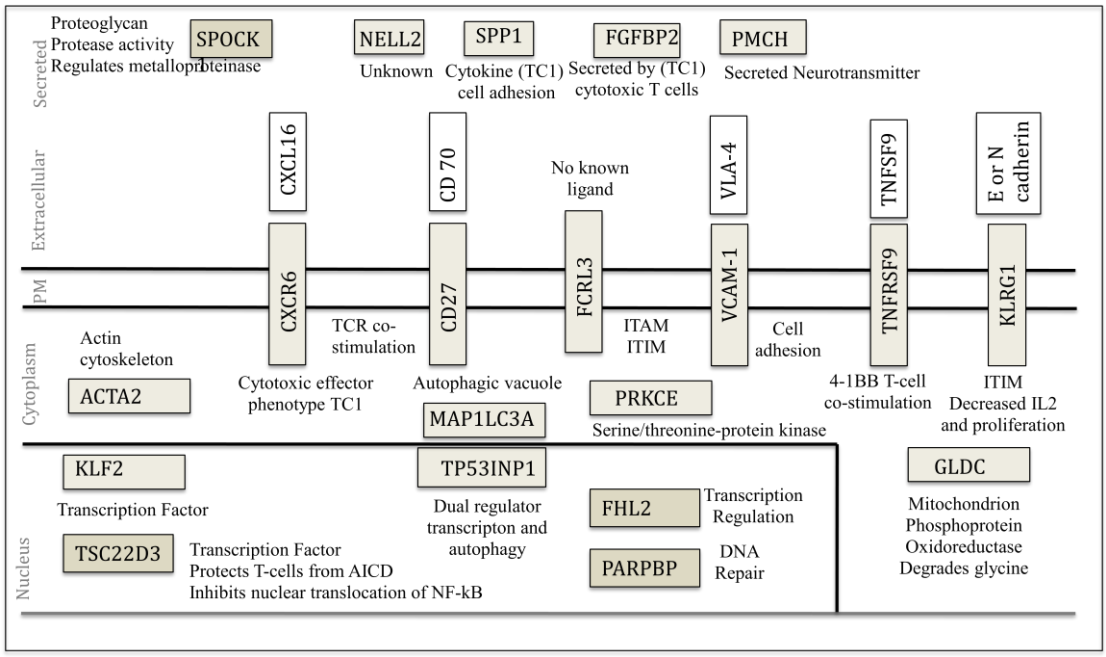


Table 5-1 and Figure 5-4: Genes more highly expressed by the high sensitivity population, A20 compared to population, A3 (logFC 1.5 A20>A3, p<0.05 (LIMMA). Function and cellular location is illustrated in the cartoon. logFC= [A20 v A3] 2uM, adj.P.Val=adjusted P value (LIMMA), B=Bayes.

Table 5-2: A3 >A20 [2uM]

logFC	Adj.P.Val	B	Symbol	Name	Entrez_ID
-4.06	5.46E-22	44.85	**LRRN3**	Leucine rich repeat neuronal 3	54674
-3.18	2.95E-13	27.65	**TNF**	Tumor necrosis factor (TNF superfamily, member 2)	7124
-2.31	2.52E-16	35.01	**GPR56**	G protein-coupled receptor 56	9289
-2.04	1.14E-12	25.80	**RGS16**	Regulator of G-protein signalling 16	6004
-1.89	9.50E-13	26.09	**PKIA**	Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	5569
-1.87	3.55E-11	21.81	**FSCN1**	Fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratas)	6624
-1.77	6.08E-15	32.00	**CLECL1**	C-type lectin-like 1	160365
-1.74	5.41E-11	21.36	**LTA**	Lymphotoxin alpha (TNF superfamily, member 1)	4049
-1.74	6.18E-12	23.83	**GNLY**	granulysin	10578
-1.68	1.99E-12	25.10	**TGFBR3**	Transforming growth factor, beta receptor III	7049
-1.57	2.17E-10	19.76	**GNA15**	Guanine nucleotide binding protein (C/EBP), delta	2769
-1.57	1.44E-10	20.23	**MGC33556**	Hypothetical LOC339541	339541

Figure 5-5: A3 >A20 [2uM] Cartoon of gene function

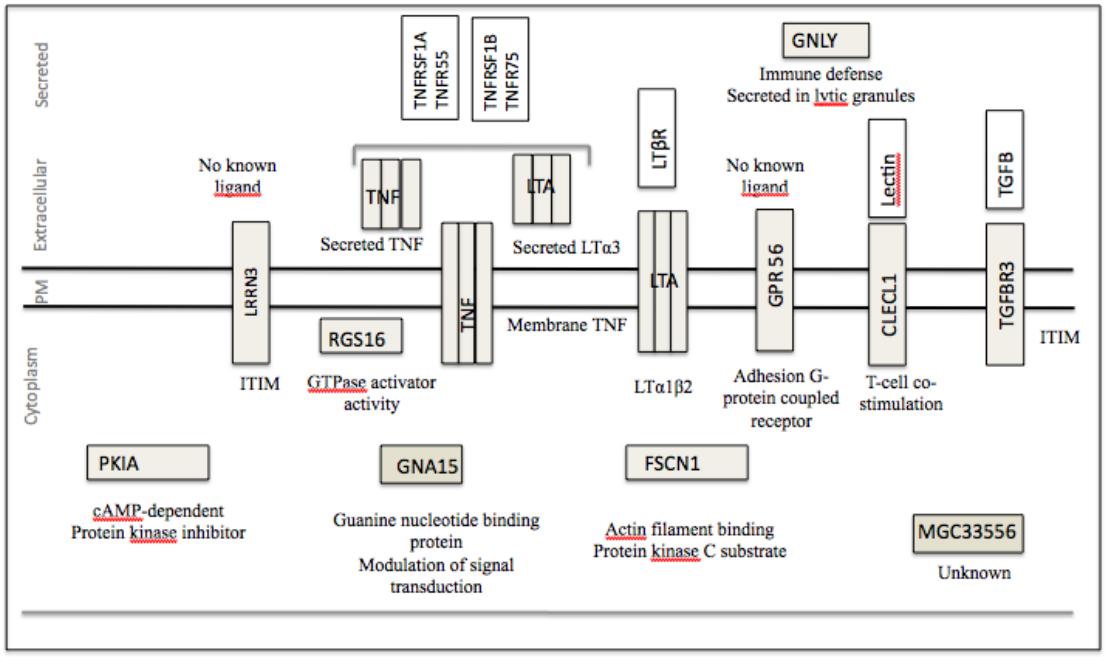


Table 5-2 and Figure 5-5: Genes more highly expressed by the low sensitivity population, A3 compared to population, A20 (logFC 1.5 A20>A3, p<0.05 (LIMMA). Function and cellular location is illustrated in the cartoon. logFC= [A20 v A3] 2uM, adj.P.Val=adjusted P value (LIMMA), B=Bayes.

The high sensitivity population, A20, had higher expression of five genes in comparison to population A3, that encoded secreted proteins (at least 1.5 log FC A20>A3, $p<0.05$ (LIMMA)), *SPOCK1* (log FC 2.83), *NELL2* (log FC 2.18), *SPP1* (log FC 1.98), *FGFBP2* (log FC 1.94) and *PMCH* (log FC 1.77) (Figure 5-4). *SPP1* encodes secreted phosphoprotein 1 (SPP1) (also known as osteopontin), a highly phosphorylated cytokine that modulates protection against influenza infection in mice by regulation of NK cells and effector/memory differentiation of CD8+ T-cells (Morimoto *et al.*, 2011). SPP1 has been studied in HIV-1 infection but not found to correlate with viral load (Saitoh *et al.*, 2012) (Brown *et al.*, 2011) (Li *et al.*, 2005). *FGFBP* encodes fibroblast growth factor-binding protein 2 (also known as 37 kDa killer-specific secretory protein) that is specifically secreted by Type 1 specific cytotoxic T-cells but has not been studied further (Ogawa *et al.*, 2001). The A20 population had higher expression of six cell surface receptors, *CXCR6* (log FC 2.26), *FCRL3* (log FC 1.51), as reported previously on comparison of the resting transcripomes (Chapter 4), and *CD27* (log FC 2.15), *VCAM-1* (log FC 1.65), *TNFRSF9* (log FC 1.57) and *KLRG1* (log FC 1.53). CD27 expression has been reported to modulate antigen sensitivity to maintain a clonally diverse CTL response by enhancing TCR signal strength of low affinity TCR receptors (Nolte *et al.*, 2009; van Gisbergen *et al.*, 2011). No difference in CD27 had been previously detected on surface staining of the resting clone populations (CD27+ve: A20 35.48% v A3 35.14%). *TSC22D3* (log FC 2.55) is the second most highly expressed gene by the high sensitivity population A20 in contrast to A3. This encodes Glucocorticoid-induced leucine zipper (GILZ) (also known as Tsc22 domain member 3), that protects T-cells from activation induced cell death and is reported to inhibit nuclear transport of NFκB and reduce TNFα and IFNγ secretion in CD4 T-cells in GILZ-knock out

mice (D'Adamio *et al.*, 1997; Cannarile *et al.*, 2009). *TNF* mRNA expression is significantly lower in clone A20 than clone A3 upon activation with 2uM epitope (Log FC -3.18 A20 v A3 [2uM], adjusted P value = 2.95 x 10^{-13} (LIMMA)) (Table 5.2). Comparison of the normalised fluorescence intensity (NFI) of the TNF transcript indicated very low absolute expression in A20 (NFI: A20 [2uM] 8.327 +/- 0.048 v A3 [2uM] 11.510 +/- 0.062).

In conclusion, the transcriptome analysis identified that both antigen sensitivity (Biological processes: signal transduction, cell surface signal transduction and signalling cascade) and the immune response (Biological processes: immune response, cell motion, cell to cell adhesion and apoptosis) were distinct in these populations of clone on activation. Antigen sensitivity may enhance anti-viral efficacy at low antigen levels, and modulate the level of expression of effector molecules, such as IFNγ but this was below the sensitivity of detection for the transcriptome analysis. The cytokine profile was also qualitatively distinct between the two clone populations. A20 did not secrete TNFα and did not upregulate *TNFα* mRNA in comparison to A3.

5.2.2 Activation with HIV-1_{MN} infected targets

Thus far, experiments were confined to using peptide as a source of antigen. To investigate if similar results could be obtained in a system using live virus, we infected a CD4+ T-cell line (C8166) at three HIV-1_{MN} viral titrations (0.1 TCID50: 5.22% p24 positive; 0.5 TCID50: 25.22% p24 positive; 1.0 TCID50: 68.3% p24 positive). It was found that results obtained using peptide were reproducible using HIV-1 infected target cells. The high sensitivity population of clone, A20, secreted more IFNγ than A3 at low antigen stimulation (6ul: A20 2240.73 pg/ul v A3 824.04 pg/ul) (Figure 5-6).

Figure 5-6: Cytokine and chemokine secretion of HLA B*08-restricted FL8-specific CD8+ T-cell clones activated for two hours with HIV-1 infected targets

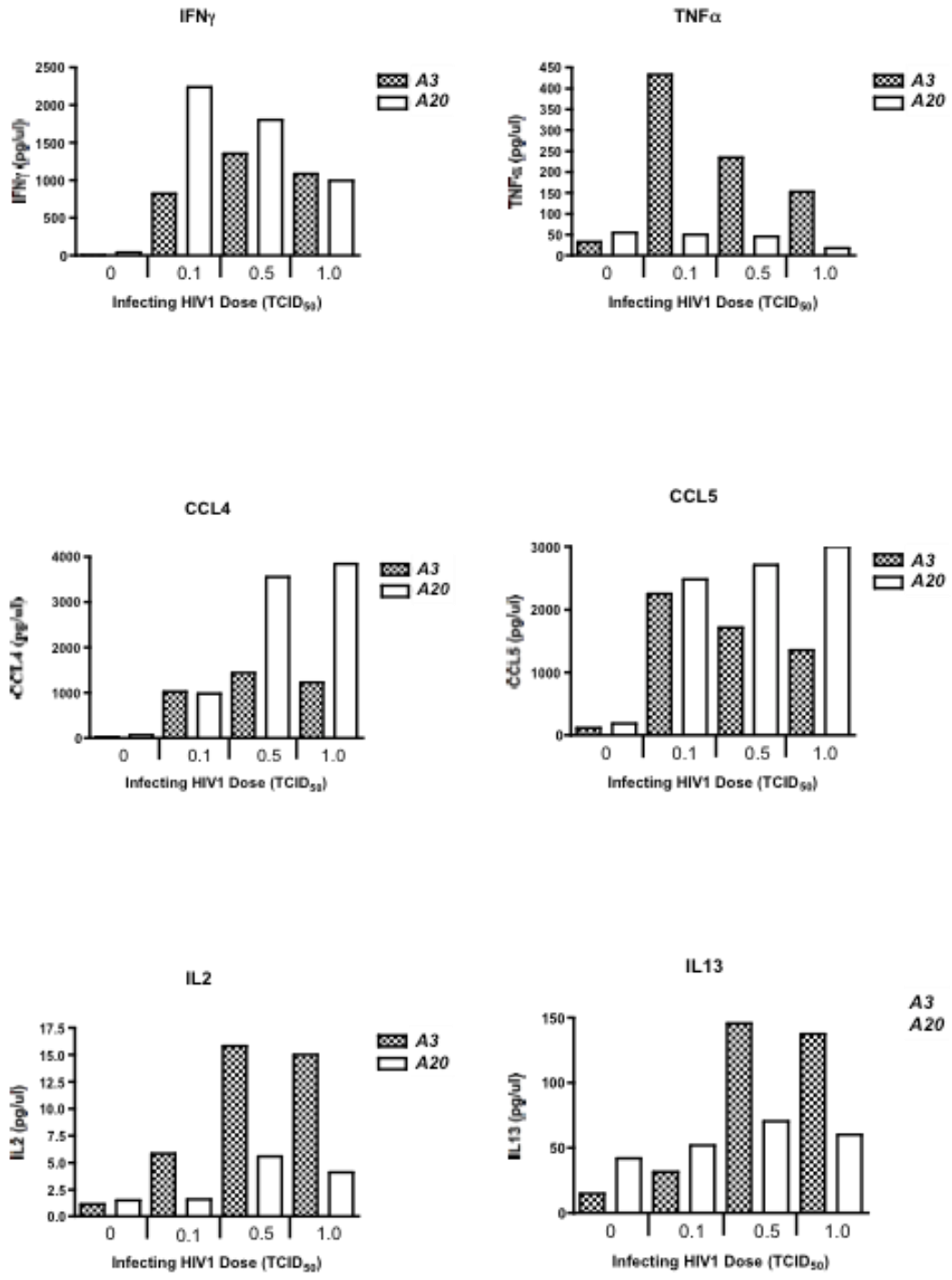


Figure 5.6: HLA B*08-restricted FL8-specific CD8+ T-cell clones that use the same TCR have distinct effector function on activation with the same stimulus, HIV-1 infected targets. HLA B*08-restricted FL8-specific CD8+ T-cell clones A20 (high sensitivity) and A3 (intermediate sensitivity) were co-cultured with HLA B*08 CD4+ T-cell line (C8166) infected with three titrations of HIV-1 (0.1 x TCID50, 0.5 x TCID50 and 1.0 x TCID50). At 24 hours, the supernatant was harvested and analysed by illumina bead technology for CCL4 (MIP1β), IFNγ, TNFα, IL2, IL13 and CCL5 (RANTES) .

There was no induction of TNFα in A20 at all three viral titrations, and secretion of TNFα by A3 decreased as target MOI increased (A3: 6ul 432.98 pg/ul, 17ul 234.42 pg/ul, 50ul 152.41 pg/ul). A decline in effector function when optimal stimulation is exceeded has been reported (Sykulev *et al.*, 1996; Valitutti *et al.*, 1996). The low avidity population A3 secreted more IL13 than A20 when activated with high MOI targets (50ul: A3 137.54 pg/ul v 60.19 pg/ul) but consistent with our findings using 2uM peptide, at low MOI targets the IL13 production by the high sensitivity population, A20, exceeded A3 (6ul: A20 52.12 pg/ul, A3 31.68 pg/ul) (Figure 5-6). Differences in CD8+ T-cell avidity in mice have been previously reported to correlate with the cytokines they produce, with type 2 cytokines IL4 and IL13 associated with low avidity CTL (Kienzle *et al.*, 2004) (Ranasinghe and Ramshaw, 2009) (Ranasinghe *et al.*, 2007) (Kelso and Groves, 1997).

As the secretion of soluble factors was so dynamic and sensitive to the level of stimulation, activation of the HLA B*08-restricted FL8-specific CD8+ T-cell clone populations A20 and A3 for transcriptome analysis using the CD4+ T-cell line (C8166) pre-infected for 72 hrs at the high titration (50ul) HIV-1_{MN} as targets. The time of co-culture prior to transcriptome analysis was reduced to two hours. This provided a "maximal" stimulation to detect qualitative rather than quantitative changes in the context of physiological antigen presentation, co-stimulatory receptor expression and cytokine milieu induced by HIV-1 infection (Figure 5-7).

Figure 5-7: Comparison of the transcriptome of two HLA B*08-restricted FL8-specific CD8+ T-cell clone populations following activation by HIV-1MN infected CD4+ T-cells for two hours

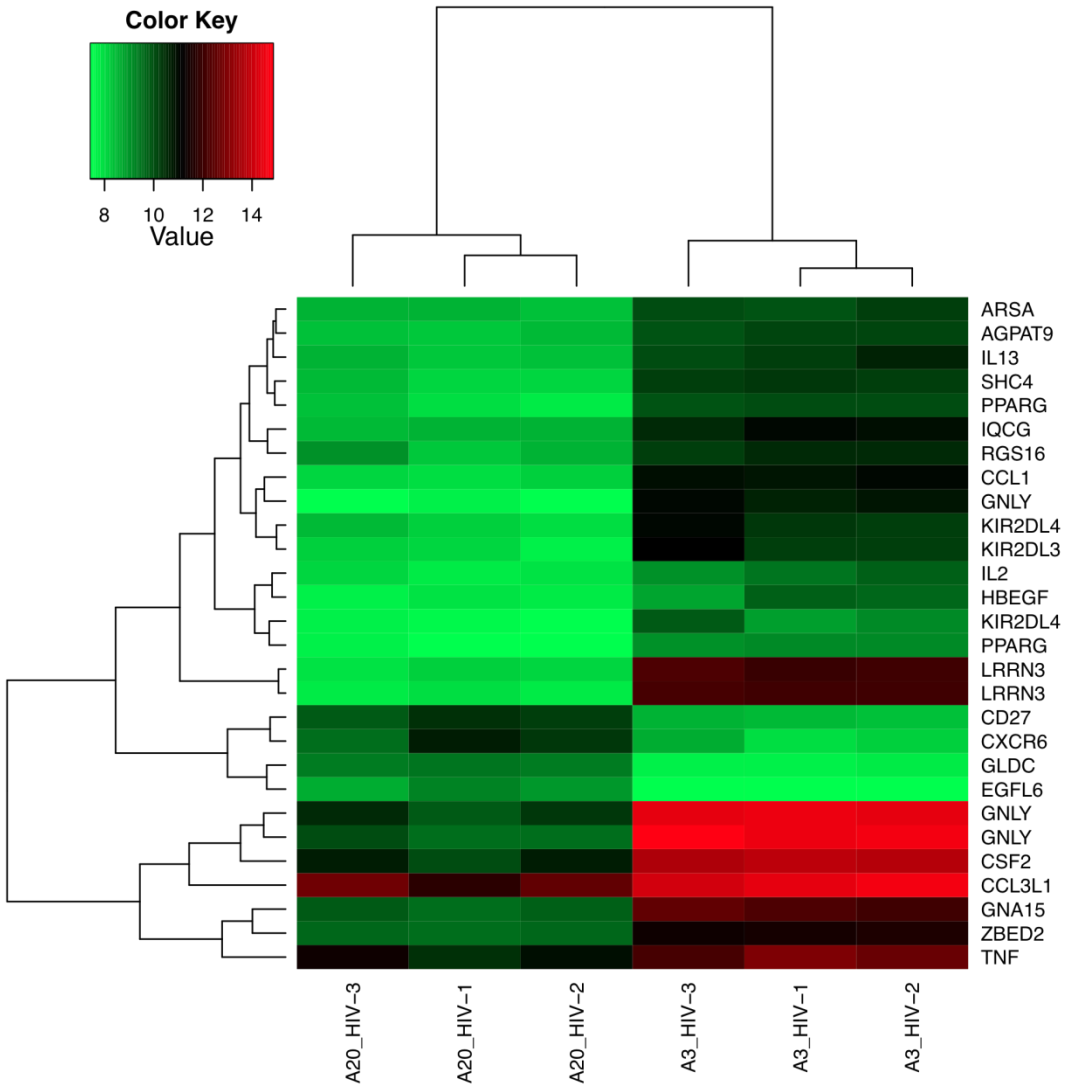


Figure 5.7: Hierarchical clustering of transcriptome analysis of populations of HLA B*08-restricted FL8-specific CD8+ T-cell clone (A20 and A3) activated for two hours with HIV-1$_{MN}$ infected targets. HLA B*08-restricted FL8-specific CD8+ T cell clones were stimulated with a CD4+ T-cell line (C8166) previously infected with X4 HIV-1$_{MN}$ for 72 hrs. The CD8 T cell clone was sorted by negative selection from the CD4+ C8166 cells using magnetic following two hours of co-culture [HIV]. Total RNA was extracted and the transcriptome analysed on HT12v4.0 illumina expression arrays. Heatmap was generated using Heatmap2. The colour scale represents the normalised intensity of complementary RNA directly hybridised to each probe for each sample. 23 genes had significant differential expression (Log FC of at least 1.5 up or down in A20 compared to A3, $p<0.05$ (LIMMA)) and are listed below (Table 5.3) with function illustrated (Figure 5.8).

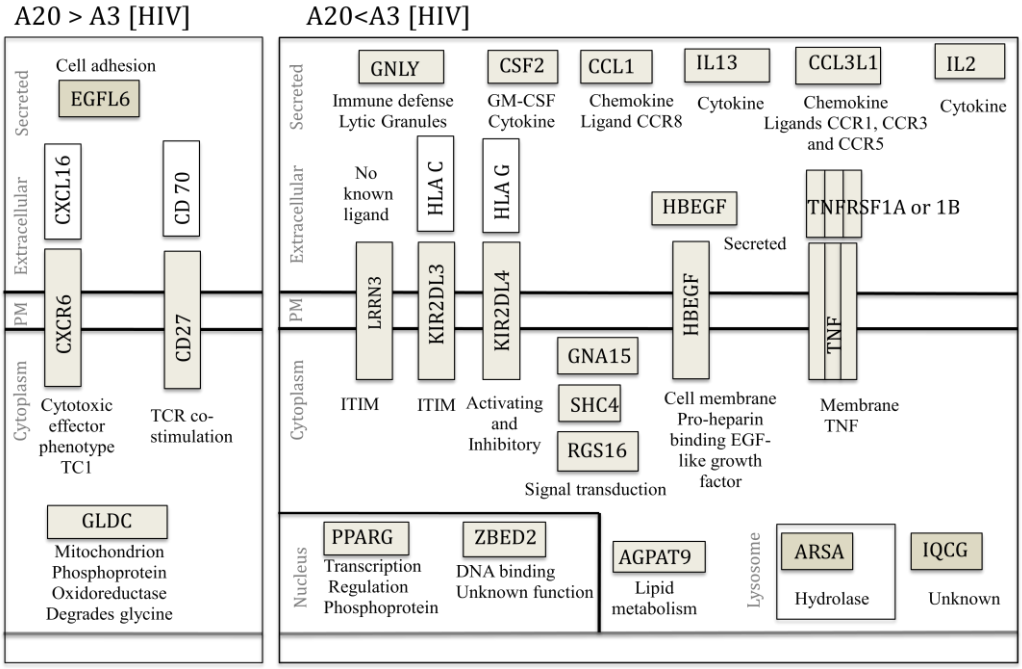
23 genes with significantly different expression (at least a log FC of 1.5 in A20 compared to A3 following activation for two hours with HIV-1 infected targets (A20 [HIV] v A3 [HIV]), $p<0.05$ (LIMMA)) were identified (Table 5.3: A20 > A3 [HIV] and Figure 5.9). Four genes had increased expression in the high sensitivity population A20, *CXCR6* (log FC 1.94) as reported for the resting and activation (2uM) transcriptome analysis, *CD27* (Log FC 1.72) also identified above, *GLDC* (Log FC 1.68), and *EGFL6* (Log FC 1.52). *GLDC* encodes a mitochondrial enzyme for glycine metabolism (Zhang *et al.*, 2012). *EGFL6* transcript has been cloned, and may encode a secreted protein involved in cell adhesion (Yeung *et al.*, 1999). Nineteen genes had decreased expression in the high sensitivity population A20. The cell surface protein LRRN3 (Log FC -4.20) has been consistently identified in all three transcriptome analyses to be one of the most highly expressed mRNA in the intermediate avidity population, A3, compared to A20. Distinct expression of KIR2DL3 (Log FC -2.57) and KIR2DL4 (Log Fc -2.35) was also identified. The intermediate sensitivity population with less anti-viral efficacy had higher expression of eight secreted factors that modulate immune defense, *GNLY* (Log FC -4.93), *CSF2* (Log FC -3.23), *CCL1* (Log FC -2.88), *CCL3L1* (Log FC -2.03), *IL13* (Log FC -1.91), *HBEGF* (Log FC -1.62), *TNF* (Log FC -1.61) and *IL2* (Log FC -1.48).

In conclusion, consistent differences in the mRNA expression of cell surface receptors and cytokines, predominantly TNFα and IL2, have been identified in the transcriptome analysis of two HLA B*08-restricted FL8 specific CD8+ T-cell clone populations A20 and A3. The high sensitivity population, A20, has higher expression of mRNA encoding the cell surface receptors CXCR6 and FCRL3 and does not secrete TNFα or IL2, with significantly lower expression of *TNFα* mRNA in contrast

Table 5-3: A20 v A3 [HIV]

LogFC A20 v A3 [HIV]	Adj.P. Value	B	Gene symbol	Gene Name	Entrez ID
1.94	3.40E-07	11.78	**CXCR6**	Chemokine (C-X-C motif) receptor 6	10663
1.72	6.58E-10	18.96	**CD27**	CD27 molecule	939
1.68	5.64E-16	33.40	**GLDC**	Glycine dehydrogenase (decarboxylating)	2731
1.52	1.52E-10	20.62	**EGFL6**	EGF-like-domain, multiple 6	25975
-4.93	4.13E-21	43.02	**GNLY**	granulysin	10578
-4.20	2.46E-22	45.37	**LRRN3**	Leucine rich repeat neuronal 3	54674
-3.23	3.36E-15	31.59	**CSF2**	Colony stimulating factor 2 (granulocyte-macrophage)	1437
-2.88	1.56E-19	40.21	**CCL1**	Chemokine (C-C motif) ligand 1	6346
-2.57	3.87E-11	22.16	**KIR2DL3**	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	3804
-2.54	1.47E-14	30.01	**GNA15**	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	2769
-2.35	4.03E-10	19.53	**KIR2DL4**	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	3805
-2.26	1.07E-15	32.75	**IQCG**	IQ motif containing G	84223
-2.11	7.66E-17	35.18	**SHC4**	SHC (Src homology 2 domain containing) family, member 4	399694
-2.03	1.75E-08	15.20	**CCL3L1**	Chemokine (C-C motif) ligand 3-like 1	6349
-2.01	6.24E-15	30.90	**PPARG**	Peroxisome prolifererator-activated receptor gamma	5468
-1.91	1.98E-12	25.36	**IL13**	Interleukin 13	3596
-1.84	1.12E-11	23.53	**RGS16**	Regulator of G-protein signalling 16	6004
-1.78	3.02E-15	31.76	**ZBED2**	Zinc finger, BED-type containing 2	79413
-1.71	1.01E-14	30.40	**AGPAT9**	1-acylglycerol-3-phosphate O-acyltransferase 9	84803
-1.62	1.06E-08	15.79	**HBEGF**	Heparin-binding EGF-like growth factor	1839
-1.61	1.02E-07	13.14	**TNF**	Tumor necrosis factor (TNF superfamily, member 2)	7124
-1.60	1.47E-11	23.23	**ARSA**	arylsulfatase	410
-1.48	3.87E-11	22.17	**IL2**	Interleukin 2	3558

Figure 5-8: A20 v A3 [HIV] Cartoon of Gene Function



to the intermediate population, A3, that has higher expression of cell surface receptors LRRN3, KIR2DL3 and KIR2DL4.

5.2.3 Constitutive expression or TCR induced?

In vitro viral suppression has been demonstrated to require cell:cell contact and depend on MHC class I restriction (Saez-Cirion *et al.*, 2007) (Walker *et al.*, 1986) (Plata *et al.*, 1987). However, it is also reported that constitutively expressed soluble factors (non –MHC restricted), known as CD8 cell anti-HIV factor (CAF), may suppress HIV-1 replication at low input CD8+: CD4+ ratios (Levy, 2003). Soluble factors that compete with R5 HIV-1 strains for the CCR5 chemokine have been identified, but factors that restrict X4 HIV-1 strains, as in our assay, HIV-1_{MN}, remain elusive (Cocchi *et al.*, 1995). Cell surface receptors that modulate the TCR-pMHC signal may also be constitutively expressed or induced on activation. To explore which of the factors identified thus far were induced on TCR stimulation, total RNA was extracted from each clone population, A20 and A3, immediately [T0=time zero] and after two hours of co-culture with HIV-1_{MN} infected targets [HIV] for transcriptome analysis (Figure 5-9).

125 genes had altered expression (at least 1.5 log FC increased or reduced expression upon activation (A20 [HIV] v [T0]), $p<0.05$ (LIMMA)) in the high sensitivity population, A20, and 165 genes had altered expression in the intermediate sensitivity population, A3, of which 106 genes had altered expression in both A20 and A3. All genes identified are listed in the appendix, with the alteration in expression for the other CD8+ T-cell clone population listed for comparison (Appendix Table 8-3).

Figure 5-9: Transcriptome analysis of HLA B*08-restricted FL8-specific CD8+ T-cell clones activated by HIV-1 infected CD4+ T-cell line (C8166) cells

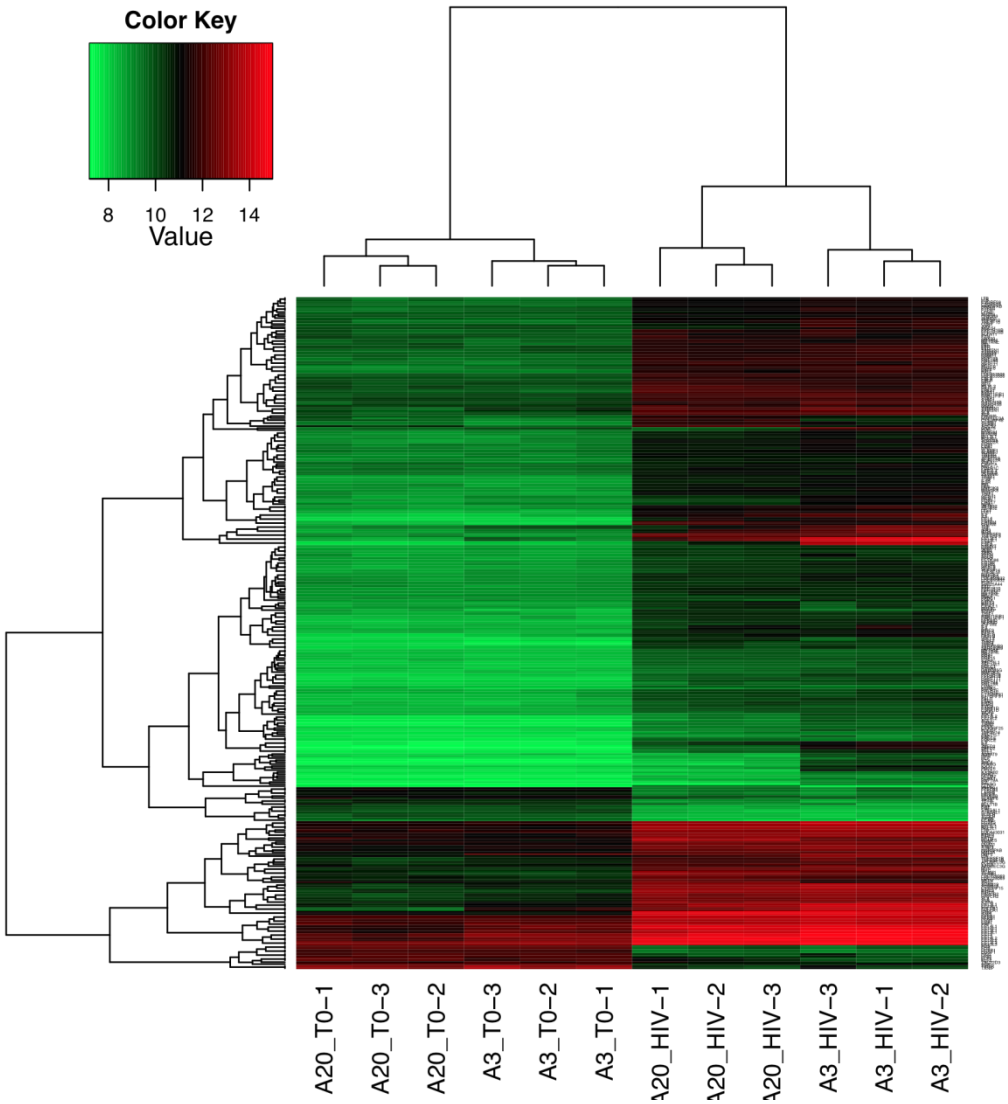
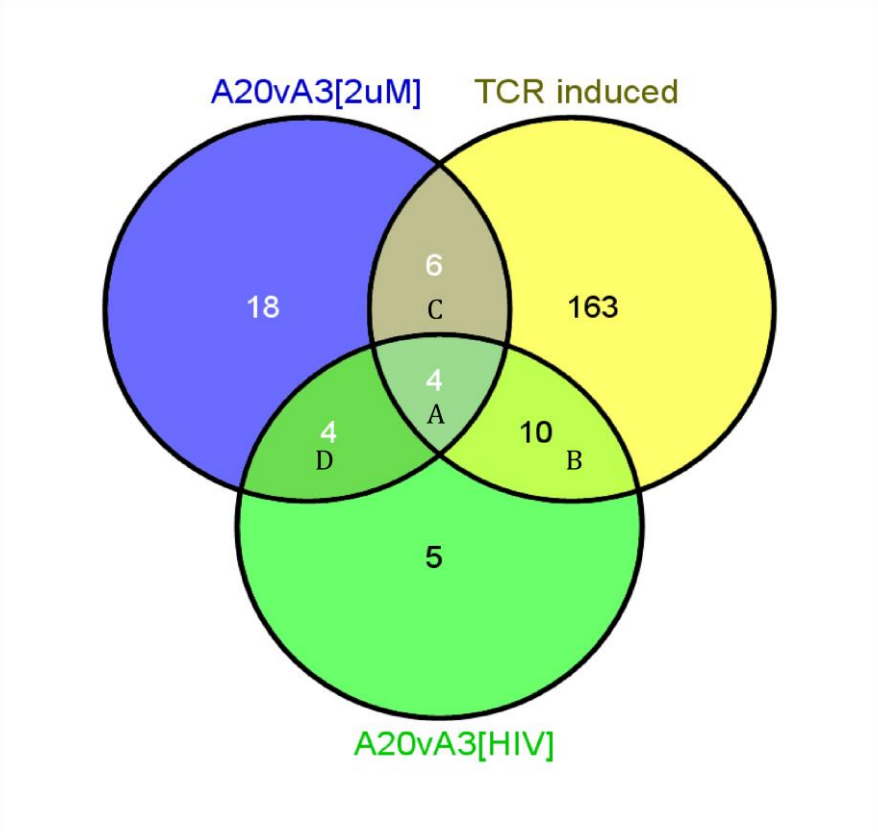


Figure 5.9: Heatmap of transcriptome analysis of CD8+ T-cell clone populations, A20 and A3, activated with HIV-1 infected targets. HLA B*08-restricted FL8-specific CD8+ T cell clones were stimulated with a CD4+ T-cell line (C8166) previously infected with X4 HIV-1_{MN} for 72 hrs. The CD8 T cell clone was sorted by negative selection from the CD4+ cells using magnetic beads immediately [T0] and following two hours of co-culture [HIV]. Total RNA was extracted and the transcriptome analysed on HT12v4.0 illumina expression arrays. Altered gene expression (at least 1.5 log FC in the activated [HIV] v resting [T0] transcriptome $p<0.05$ (LIMMA)) is illustrated. The normalised fluorescence intensity of mRNA binding to each probe is indicated on the colour key from green to red. All candidate genes are listed in the appendix.

For the candidate genes identified in the activated transcriptome analysis described above (A20 v A3 [HIV] and A20 v A3 [2uM]) the alteration of gene expression upon TCR stimulation is summarised in tables (Appendix Table 8.4 and Table 8.5).

These data-sets A20 v A3 [HIV] and A20 v A3 [2uM]) could then be integrated with TCR inducible genes (Genes differently expressed in clone A20 or clone A3 after two hours co-culture with HIV (Time = 2hrs [T2]) compared to resting (Time = 0 hrs [T0]) to identify genes with altered expression on activation that were significantly different between the high sensitivity clone population, A20, and the intermediate sensitivity clone population, A3 (Figure 5-10). This highlighted *TNFα* as one of four genes that is induced by TCR stimulation but that was qualitatively distinct in A20 compared to A3 both after peptide and after HIV-1 activation (Section A on Figure 10). The other three genes, *GLDC, RGS16* and *GNA15* encode a mitochondrial enzyme for glycine metabolism, and signalling proteins with no previously reported role in T-cell signalling. Of note, *LTA* (Figure 5.10; Section C) is just below the log FC cut-off (1.5) for inclusion in the above category, as after exposure to HIV, A20 has significantly lower *LTA* expression than A3 (A20 v A3 [HIV]: log FC of 1.37, $p=7.30 \times 10^{-9}$). TNFα is a known CD8+ T-cell effector protein with pleiotropic effects from cellular differentiation, to induction of apoptosis, cellular activation and induces the activation of multiple signalling pathways. We had also validated that TNFα could be not be detected in the supernatant harvested from clone A20 following activation with peptide pulsed or HIV infected targets. The absolute mRNA transcript was very low in population A20 following activation with 2uM peptide (NFI A20 [2uM] 8.327, Scale 8-14) (Figure 5.7). Surprisingly TNFα mRNA was upregulated in clone population A20 following exposure to HIV-1 infected target cells which is difficult to

Figure 5-10: Pie chart illustrating the integrated analysis of transcriptome data-sets on two HLA B*08-restricted FL8-specific CD8+ T-cell clone populations



A	**GLDC, TNF, RGS16, GNA15**
B	**IQCG, AGPAT9, CSF2, IL2, ZBED2, IL13, PPARG, CCL3L1, SHC4, CCL1**
C	**TSC22D3, VCAM1, MAP1LC3A, PRKCE, KLF2, LTA**
D	**CXCR6, CD27, LRRN3, GNLY**

Figure 5.10: Pie-chart summarising the genes that are significantly different in two populations of HLA B*08-restricted FL8-specific CD8+ T-cell clones after six hours peptide and two hours HIV infected cells exposure, that alter on TCR activation. Genes with significantly different expression (log fold change of at least 1.5, $p<0.05$ (LIMMA) were identified in three transcriptome analysis: "TCR induced" = Genes that altered after HIV exposure in clone A3 and A20, Genes that were differently expressed in A20 compared to A3 after HIV "A20 v A3 [HIV]" and after peptide "A20 v A3 [2uM]". Some segments of the pie-chart is labelled and matched in the table below that indicates which genes are represented in each category. Genes in red are more highly expressed by population A20 (high senstitivity). Genes in blue are more highly expressed by population A3 (intermediate sensitivity). The function of each gene is illustrated in Figures 4, 5 and 8.

reconcile with the other experimental data and may represent contamination of the HIV-1 infected target cells remaining following separation. However, when the CD8+ T-cell clone population was separated from the CD4+ T-cell line using CD4+ magnetic beads, the co-culture was passed twice through MACs columns to enhance purity by removal of HIV-1 infected and dead cells, and real-time PCR using primers for both CD4 and CD8 indicated no difference between the pre- stimulation (Time =0 (T=0)) and post-stimulation (Time=2 (T=2)). We therefore focused on TNFα, particularly on defining how TNFα expression was regulated in A20.

5.2.4 TNFα expression is regulated by epigenetic regulation

In the previous assay where we measured the supernatant following co-culture of CD8+ T-cell clones with HIV-1 infected targets, TNFα could not be detected for clone A20 in contrast to clone A3, at all stimulating HIV-1_{MN} titrations. TNFα secretion could not be induced by phytohaemagglutinin or bypassing the proximal TCR signalling cascade, using ionomycin and phorbol-myristate acetate (Chapter 3). TNFα secretion is tightly regulated by specific sets of transcription factors, mRNA stability, mRNA splice variants, translation, protein transport and cleavage from the cell surface by metalloproteinase disintegrin (Tsytsykova and Goldfeld, 2002). The epigenetic regulation of TNFα by the remodelling of histone proteins during clonal expansion of TCR transgenic CD8+ T-cells exposed to antigen has been studied (Denton *et al.*, 2011) but TNFα can be silenced by methylation of the distal promoter in T cell lymphoma (Zhang *et al.*, 2009). In order to determine if the methylation status of the TNFα core promoter can moderate TNFα expression in CD8+ T-cell clone populations, DNA was extracted from pure A20 and A3 clone populations (>99%), treated with bisulfite to convert unmethylated cytosines to uracil, amplified by PCR and sequenced (Simpson *et al.*, 2012). Clone A20 was heavily methylated in

the core TNFα promoter in contrast to clone A3 (Figure 5.11). This suggests that TNFα can be epigenetically regulated in human CD8+ T-cells, and even suggests that the progeny of a single naïve CTL can selectively acquire the loss of TNFα as an effector function by alternative pathways that delineate epigenetic control.

In conclusion, the activation of two HLA B*08-restricted FL8-specific CD8+ T-cell clones, with distinct antigen sensitivity has confirmed some of the fundamental differences between the clones in terms of cell surface receptor expression, with the high sensitivity clone expressing CXCR6 and FCRL3, and the intermediate sensitivity clone expressing LRRN3 and KIR2DL3 and KIR2DL4. We find that anti-viral efficacy may depend both on enhanced "seeing" and "acting", although counter-intuitively the highly sensitive population, A20, with optimal anti-viral efficacy is characterised by a more limited effector response, in that this population does not secrete TNFα, which is most probably silenced by DNA methylation of the TNFα core promoter.

Figure 5-11: Methylation of CpG dinucleotides in the TNFα core promoter of population A20

A

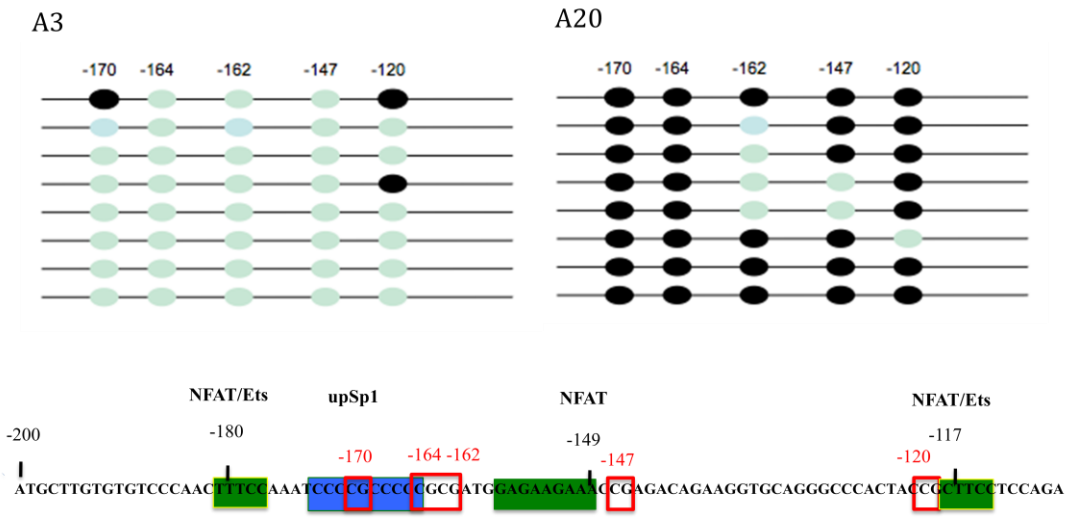


Figure 5.11: Methylation of the TNFα core promoter in HLA B*08-restricted FL8-specific CD8+ T-cell clone populations A3 and A20. DNA isolated from two populations of CD8+ T-cell clone sorted to >99% purity by FACS using FL8/HLA B*08 tetramers, A3 and A20, was bisulfite converted, amplified by PCR, and sequenced to assess the methylation status of CpG dinucleotides in the core TNF alpha promoter from amino acid residues -200 to -117 (red boxes). A) A representative sequencing screen is illustrated for each population, with the frequency of methylated cytidines denoted by black circles. B) Raw sequencing data from the representative screen for residues -171 to –119 is illustrated for CD8+ T-cell clone population A3 and C) A20. Bisulfite sequencing was performed by Dr Gerry Gillespie.

Figure 5.11 : Methylation of CpG dinucleotides in the TNFα core promoter of population A3

B

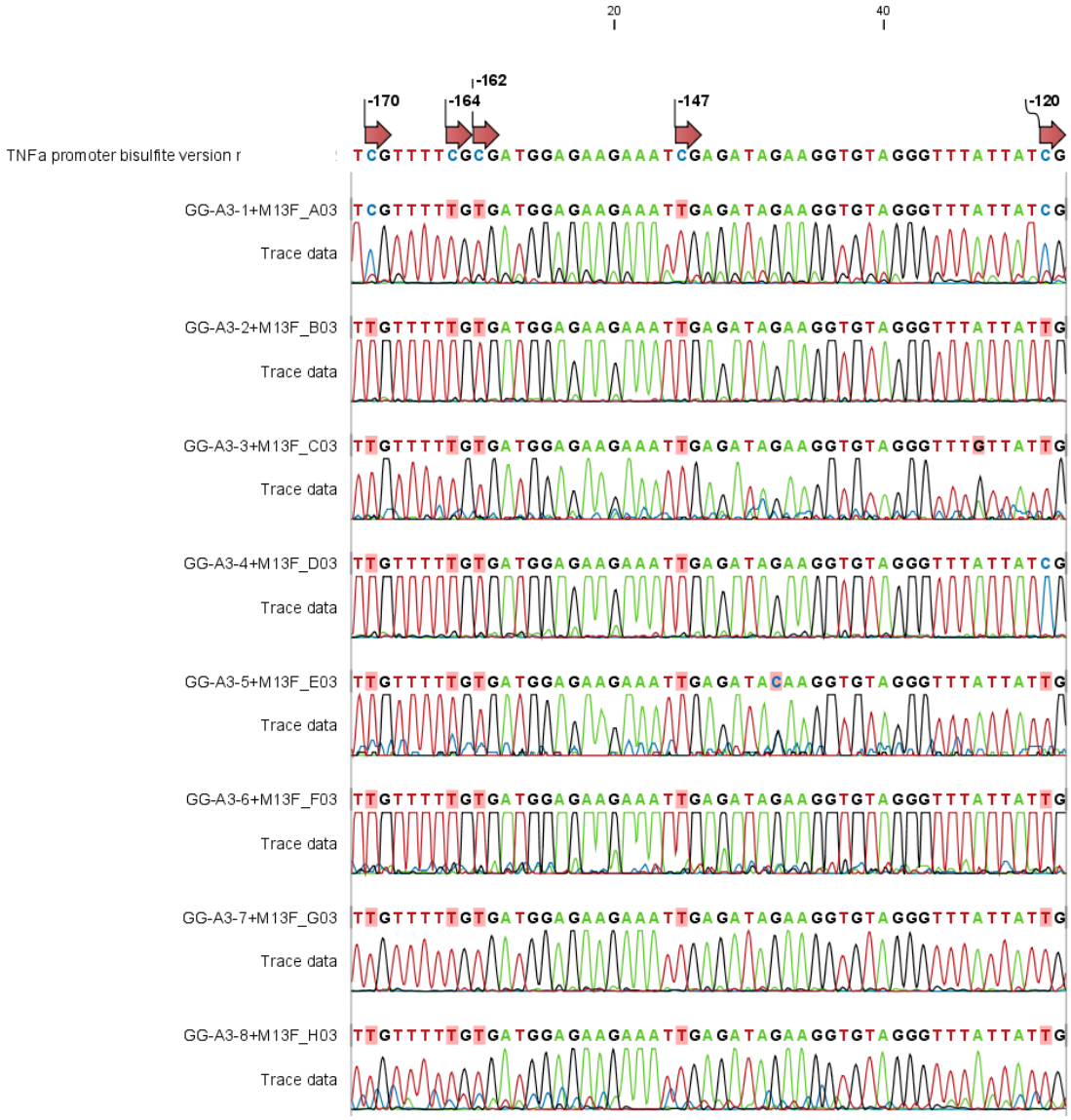
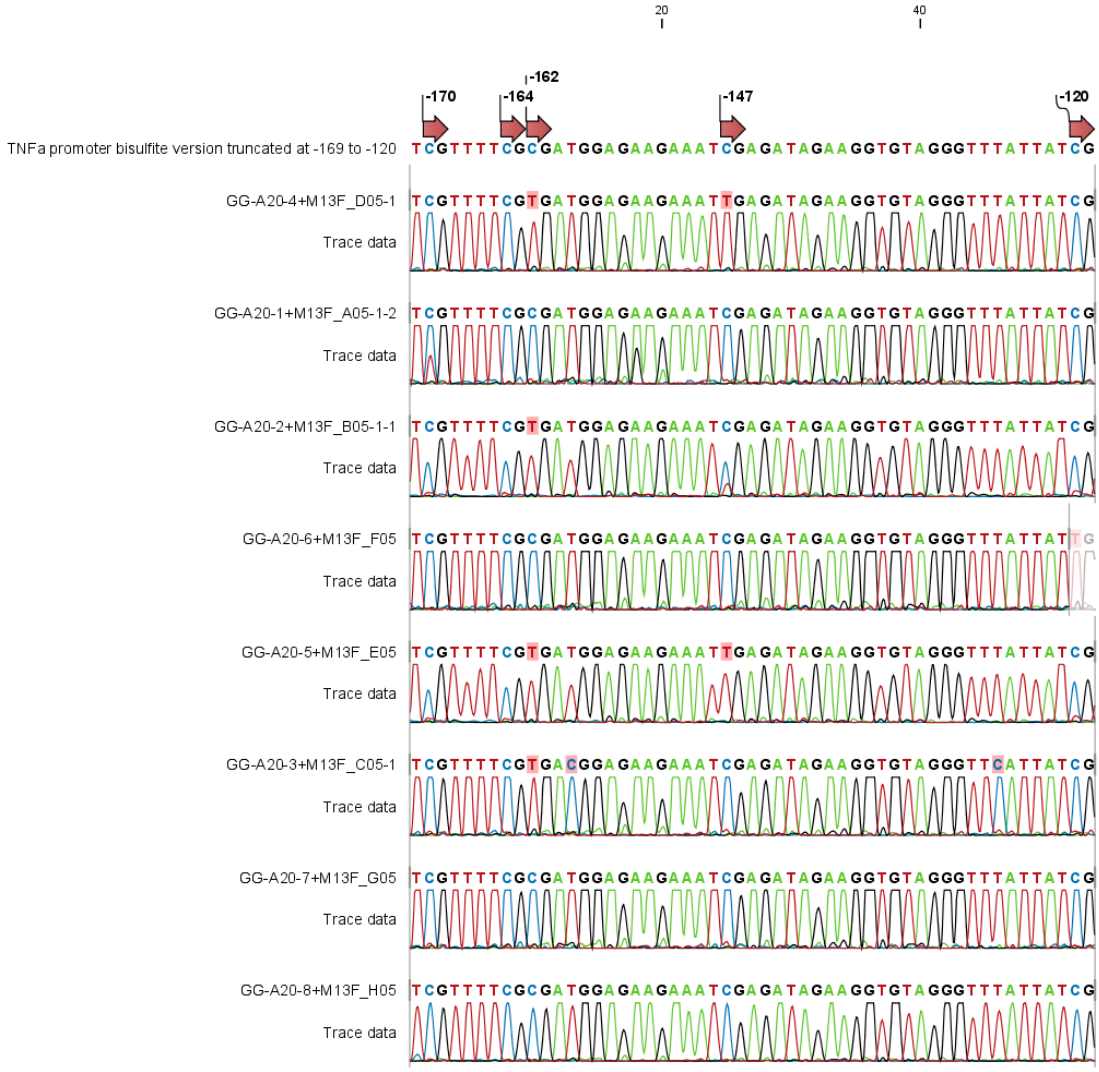


Figure 5.11: Methylation of CpG dinucleotides in the TNFα core promoter of population A20

C



5.3 Discussion

We have compared the activated transcriptomes of two HLA B*08-restricted FL8-specific CD8+ T-cell clone populations, a high sensitivity population, A20, with "good" anti-viral efficacy, and an intermediate sensitivity population, A3, with moderate anti-viral efficacy, to explore attributes that might contribute to superior HIV-1 suppression, downstream of the TCR. The differentially expressed genes identified between the two populations when activated with a limiting dose of antigen were enriched within cell signalling and immune defense biological processes supporting evidence that both rapid recognition of antigen and enhanced effector function contribute to the protective effect (Derby *et al.*, 2001). We find that the DNA of the TNFα promoter in the high sensitivity population, A20, is heavily methylated. To our knowledge, this is the first report of epigenetic regulation of the TNFα locus in a human CD8+ T-cell clone by DNA methylation. The evidence supporting this conclusion is that the high sensitivity population, A20, failed to secrete TNFα and the DNA of the TNFα gene promoter was heavily methylated, previously reported to silence TNFα mRNA expression in CD4+ T-cell derived tumours (Zhang *et al.*, 2009). Methylation of CpG islands within this region of the promoter (-170 bp to start site) have also been reported to silence TNFα secretion in an acute monocytic leukaemia derived cell line, which was reversed by 5'-azacytidine treatment, a DNA demethylation agent (Sullivan *et al.*, 2007). The mRNA for TNFα was expressed at very low concentration within the high sensitivity population, A20, following activation with 2uM peptide, in contrast to population A3. It was surprising that TNFα expression appeared to be upregulated in clone A20 after HIV exposure. This may be due to some variation within methylation at the core promoter between individual cells in the population of clone A20, leading to a small production of the

TNFα mRNA transcript from some cells with incomplete methylation, when using highly sensitive techniques such as gene array platforms (Figure 5.11). Chromatin remodelling tends to occur in areas of euchromatin and it is interesting to observe that the gene expression of lymphotoxin alpha (LTA), the gene immediately downstream of the TNFα locus, was also significantly decreased in the high sensitivity population A20.

This data suggests that a single naïve CD8+ T-cell can give rise to progeny that differ in TNFα effector function due to discrete regulation of epigenetic events. The distinct cytokine expression of tetramer positive populations had previously been thought to be due to differences in TCR affinity, although the heterogeneity in cytokine profile reported in TCR transgenic mice and the lack of preferential TCRβ usage by polyfunctional (IL2+) CTL within CD8+ T-cell populations responding to immunodominant influenza epitopes, implicated other mechanisms, including cell cycle and activation status (Moffat *et al.*, 2010; Kristensen *et al.*, 2004). Mechanisms that influence the epigenetic silencing of TNFα in some of the progeny of a single naïve CD8+ T-cell but not others, will be important to investigate further, particularly as we find that the clone population that does not secrete TNFα has enhanced CD8+ T-cell anti-HIV-1 activity. DNA methylation represses gene transcription by binding specific proteins, eg methyl-CpG binding proteins, that block the access of transcription factors to the gene promoter (Lopez-Serra *et al.*, 2008) (Watt and Molloy, 1988). DNA methylation is performed by DNA methyltransferases (DNMT). DNMT1 is a maintenance DNA methyltransferase that recognises hemi-methylated DNA strands generated during cellular proliferation and regenerates a fully methylated locus. De novo DNA methylation is performed by DNMT3A and DNMT3B, that is targeted to genomic regions by repressive histone modifications and

the absence of activating histone modifications respectively (Okano *et al.*, 1999) (Carey *et al.*, 2011). This has led to a model where gene transcription is initially repressed via more transient histone modifications, then silenced by DNA methylation. DNA demethylation has also been reported, both by passive mechanisms, such as the failure of DNMT1 to copy methylation status to the daughter strand, and by active mechanisms, although this is very controversial (Ooi and Bestor, 2008). Interestingly, DNA demethylation of the IL2 promoter following activation of naïve CD4+ T-cells has been reported, and was not prevented by blocking DNA replication, suggesting an active enzyme mechanism (Bruniquel and Schwartz, 2003). Enzymes that may catalyse active DNA demethylation include members of the TET family and Apolipoprotein B mRNA editing enzyme 1(APOBEC). APOBEC3G expression was induced on TCR ligation in both clone populations. DNA methylation in the high sensitivity clone population at the TNFα locus may therefore arise from the *de novo* action of DNMT3A or DNMT3B by recruitment to sites with repressive histone modifications or may arise in the intermediate clone population by the action of DNA demethylases. It is known that during myeloid differentiation the DNA of the TNFα locus is demethylated very early at the haematopoietic stem cell stage, followed by progressive migration from tightly coiled heterochromatin, to more accessible "open" euchromatin (Sullivan *et al.*, 2007). This suggests that population A20 has acquired epigenetic modifications that have silenced TNFα during the activation, expansion and differentiation of the progeny of the naïve CD8+ T-cell. Epigenetic regulation of the TNFα locus has been previously studied in mice (Denton *et al.*, 2011). Naïve CD8+ T-cells in TCR transgenic mice can already secrete TNFα on TCR ligation, associated with permissive histone modifications, histone 3 lysine 9 acetylation (H3K9ac) and histone 3 lyine 4 trimethylation (H3K4me3) (Denton *et al.*,

2011). In this study, the emergence of TNFα negative CD8+ T-cell effector subsets was reported to peak after four or five rounds of cellular division, and was associated with acquired repressive histone marks, histone 3 lysine 27 trimethylation (H3k27me3). This suggests that the repressive histone marks that might lead to DNA methylation at the TNFα locus may be acquired during the initial rounds of replication that occur in the lymph node following priming and be modulated by antigen encounter or cytokine milieu in the periphery. It is known that transcription factors determine epigenetic processes at genetic loci, for example the upregulation of GATA-3 drives IL4 expression and silences IFNγ that leads to the differentiation of the CD4+ Th2 sub-set (Chang and Aune, 2007; Kanno *et al.*, 2012). Antigen encounter and the cytokine milieu are important factors that moderate the activity of transcription factors. Transcription factors can be regulated by encounter with antigen, and the CD8+ T-cell clones differ in antigen sensitivity. TCR signal strength contributes to CD4+ T-cell differentiation, as mice that select CD4+ T-cells with high TCR affinity develop polarised secretion of IFN-γ, a type 1 cytokine, and protection against leishmania infection, whereas susceptible mice select CD4+ T-cells with low affinity TCRs with polarised secretion of predominantly IL4 (Malherbe *et al.*, 2000). The cytokine environment is also critical, and not only do the clones express different chemokine receptors, CXCR6 (A20, high sensitivity) and CXCR3 (A3, intermediate sensitivity), and may migrate through distinct tissue niches and cytokine milieu, also associated with effector and central memory phenotypes respectively, but TNFα signalling through TNF-R1 and TNF-R2 can itself regulate gene expression (Wajant *et al.*, 2003; Kim *et al.*, 2001) (Flatz *et al.*, 2011) (Koch *et al.*, 2009). This raises the question, could the methylation and inactivation of TNF lead to the phenotypic and

functional differences observed between A20 and A3 in terms of viral suppression and antigen sensitivity.

We have also reported that the high sensitivity population that does not secrete TNFα has optimal control of HIV-1 replication *in vitro.* Experiments thus far indicate that this is due to enhanced elimination of infected cells by cytolysis. Interestingly, the high sensitivity population, A20, had significantly higher magnitude of IFNγ secretion at low antigen exposure, and significantly higher expression of the transcription factor eomesodermin, (EOMES) (log FC A20 v A3 [HIV] 1.21, $p=1.09 \times 10^{-7}$), the major regulator of IFNγ production in CD8+ T-cells, that is also required for epigenetic remodelling of perforin and granzyme B (Araki *et al.,* 2008). Surprisingly, no significant difference in the total quantity of mRNA following activation was seen for PRF, perhaps due to the high background constitutive expression (Makedonas *et al.,* 2009). This suggests that other mechanisms may be in action. If CD8+ T-cell subsets differ in their cytotoxic capacity related to the lack of TNFα expression, or if the lack of TNFα modulates the antigen sensitivity of the clone populations are important questions that we are actively exploring. Experiments are currently in progress to explore if blocking TNFα using Recombinant Human Tumor Necrosis Factor Receptor Fc Fusion Protein (TNFR:Fc) (Enbrel, also known as Etanercept) can enhance HIV-1 suppression in the intermediate population clone A3 or if silencing TNFα in A3 converts the phenotype to a more "A20" like profile.

The impact of TNFα on HIV-1 infection has been intensively studied. Protective HLA class I haplotypes, for example HLA B*57:01, are in linkage disequilibrium with single nucleotide polymorphisms (SNPs) of the TNFa gene promoter, located within the MHC class III region, that lower TNFα expression and may contribute to the causal effect of the B*57 haplotype (Simpson *et al.,* 2012). Serum TNFα is elevated

in patients with AIDS (Lahdevirta *et al.*, 1988). TNFα production by the infected cell is known to activate HIV-1 replication *in vitro* (Vyakarnam *et al.*, 1990) (Griffin *et al.*, 1989) (Folks *et al.*, 1989; Osborn *et al.*, 1989) (Duh *et al.*, 1989) and induce expression of CXCR4 and CCR5, HIV-1 co-receptors that modulate the spread of infection (Juffermans *et al.*, 2000). TNFα production by monocytes exposed to lipopolysaccharide may contribute to HIV-1 associated immune activation by microbial translocation from reduced gut integrity (Brenchley *et al.*, 2006). Reduced immune activation in Sooty mangabeys may be mediated by Simian Immunodeficiency Virus mediated inhibition of TNFα production (Mir *et al.*, 2012). The lack of TNFα secretion by the CD8+ T-cell clone with optimal suppression may therefore contribute to reduced HIV-1 replication and generalised immune inflammation. TNFα may also indirectly impede HIV-1control by autocine affects on the CD8+ T-cell. TNFα is a pleiotropic cytokine that induces cellular activation, apoptosis and differentiation. TNFα is a homotrimeric cell surface receptor, or can be cleaved to a soluble form by TNF-α converting enzyme (TACE). Secretion is not focused towards the area of target cell contact as for IFNγ, but occurs in a multidirectional manner (Huse *et al.*, 2006) (Sullivan *et al.*, 2007). Rapid on-off cycling of cytokine production is reported for CD8+ effector T-cells (Slifka *et al.*, 1999), suggesting a tight coupling between TCR activation and TNFα expression. The active homotrimeric soluble form rapidly dissociates so is only locally effective, suggesting cell:cell contact and TCR-pMHC ligation would be essential. Ligation of the TNFα receptors, TNF-R1 and TNF-R2, expressed by the CD8+ T-cells, can induce cell death, and is a mechanism of activation induced cell death (AICD) (Chavez-Galan *et al.*, 2009) (Wajant *et al.*, 2003). The induction of apoptosis in high avidity CD8+ T-cells has been reported to occur by autocrine TNF-TNFR1

engagement (Alexander-Miller *et al.*, 1996b). The HLA B*08-restricted FL8-specific CD8+ T-cells clones studied are induced to express TNF-R2 (Also known as receptor superfamily member 1b (TNFRSF1B) on TCR ligation. TNFα may therefore act in an autocrine manner by ligating TNF-R1 or TNF-R2, and induce AICD in the intermediate sensitivity population, A3, that would reduce the effector to target ratio that is a critical component for efficient cytotoxicity (Derby *et al.*, 2001). Lymphoma cells derived from CD4+ T-cells are reported to evade cell death by apoptosis by epigenetic silencing of TNFα (Zhang *et al.*, 2009). Epigenetic regulation of TNFα may even permit the survival of CD8+ T-cell clone populations to undergo avidity maturation in the periphery.

TNFα signalling regulates nuclear factor-kappa B (NF-κB), AP-1 and can upregulate expression of PD-1 (Herbein and Khan, 2008). CD8+ T-cell secretion of TNFα is also reported to de-differentiate tumour cell populations (Landsberg *et al.*, 2012). Longer term culture of population A3 with TNFα inhibitors are underway in order to examine if blocking TNFα signalling can modify ontogenetic factors that alter the general state of activation of the T-cell and expression of other genes that may moderate sensitivity or viral suppression.

In summary, we describe two HLA B*08-restricted FL8-specific CD8+ T-cell clone populations with distinct TNFα expression, due to epigenetic regulation by DNA methylation. The TNFα silenced A20 clone demonstrated high antigen sensitivity and optimal control of HIV-1 replication, and whether this superior anti-viral effect relates to TNFα silencing is currently under investigation.

6 SUMMARY

Engagement of the TCR by epitope-MHC complexes initiates a diverse CD8+ cytotoxic T-cell response that includes cytotoxicity, secretion of chemokines and cytokines, and balances cell activation, proliferation, resistance to apoptosis and state of differentiation that tightly regulates the frequency and function of viral specific CD8+ T-cell effectors. The total HIV-1 specific CTL response is so dynamic that this heterogeneity has confounded the identification of protective CD8+ T-cell attributes. CD8+ T-cells are known to be critical for control of HIV-1 infection (Goonetilleke *et al.*, 2009) (Goulder *et al.*, 1997), and vaccine induced CD8+ T-cells can control AIDS virus replication in non-human primate models of HIV-1 (Mudd *et al.*, 2012). Genome wide association studies and cohorts of HIV-1 controllers have revealed the key role of certain HLA alleles in protection, particularly HLA B*27 and B*57, and studies ex vivo, *in vitro* and *in silico* implicate that the properties of the CD8+ T-cells that interact with these specific peptide-MHC complexes may be optimal for control of HIV-1 (Carrington and Walker, 2012) (Migueles and Connors, 2010) (Appay and Iglesias, 2011) (Kosmrlj *et al.*, 2010).

In order to define anti-viral attributes most tightly associated with direct control of HIV-1 replication that are broadly applicable to other HLA allele-epitope complexes, we generated a panel of HLA B*08-restricted FL8-specific CD8+ T-cell clones and compared their ability to suppress HIV-1 *in vitro*. Our predominant finding was that *in vitro* suppression correlated with high antigen sensitivity, extending the observation that HLA B*27 restricted KK10-specific CD8+ T-cell clones with high sensitivity have enhanced viral control to a second HLA class I– HIV-1 epitope complex (Almeida *et al.*, 2009). CD8 + T-cells of high sensitivity have been reported to be

more effective at viral clearance in animal models (Alexander-Miller *et al.*, 1996a) (Gallimore *et al.*, 1998) (Sedlik *et al.*, 2000) and in control of persistent viral infections in man, including hepatitis C, HTLV and HIV-1 (Neveu *et al.*, 2008) (Yerly *et al.*, 2008) (Kattan *et al.*, 2009) (Mothe *et al.*, 2012) (Berger *et al.*, 2011) (Almeida *et al.*, 2007) (Almeida *et al.*, 2009) (Bihl *et al.*, 2006).

Exactly how highly sensitive antigen recognition leads to enhanced CTL anti-viral efficacy remains debated, but the rapid recognition of viral infected cells, more efficient CTL killing in response to only a few peptide-MHC complexes, high magnitude of IFNγ secretion, the ability to trigger cytolysis without TCR downregulation or cytokine production, and a polyfunctional profile, have all been demonstrated or proposed (Derby *et al.*, 2001) (Kattan *et al.*, 2009) (Almeida *et al.*, 2009) (Betts *et al.*, 2004). Cross-reactivity and high avidity CTL was an important component of HCV control (Yerly *et al.*, 2008). Enhanced recognition of HIV-1 variants by high sensitivity CTL may limit immune escape particularly if low entropy epitopes are targeted that have structural constraints on variation to balance the high selection pressure (Varela-Rohena *et al.*, 2008) (O'Connor *et al.*, 2002) (Schneidewind *et al.*, 2008) (Davenport *et al.*, 2002).

One of the limitations of the natural CTL response to the AIDS-virus is the delay in reaching the peak effector response at sufficient concentration within the gut-associated lymphoid tissue (GALT) that can be overcome by vaccination induced high avidity CD8+ T-cells in the mucosa (Reynolds *et al.*, 2005) (Belyakov *et al.*, 2007). We find that antigen sensitivity is most critical if the effector to target ratio or antigen concentration is limiting as occurs at the earliest time points of HIV-1 infection (Budhu *et al.*, 2010). AIDS-virus control has been associated with the rapid recognition of Gag epitopes (Sacha *et al.*, 2007) (Sacha *et al.*, 2008) (Payne *et al.*,

2010). We demonstrate that a Nef epitope can also be targeted effectively by high sensitivity CTL that overcome Nef mediated down-regulation of HLA class I molecules possibly as HLA-B alleles are partially spared to control HIV-1 replication *in vitro* (Rajapaksa *et al.*, 2012) (Cohen *et al.*, 1999). A protective CTL response to a Nef epitope (RL10) restricted by Mamu B*08 was recently reported (Mudd *et al.*, 2012).

We found that viral suppression correlated with the elimination of infected cells, was MHC class I restricted and required the recognition of a conserved epitope, supporting evidence that killing is the predominant mechanism of HIV-1 control *in vitro* and that high antigen sensitivity may enhance killing efficiency in HIV-1 as reported for HTLV-1 (Saez-Cirion *et al.*, 2007) (Kattan *et al.*, 2009). The correlation of enhanced killing efficiency and antigen sensitivity that our data suggests is controversial in the light of conflicting reports that have used smaller numbers of clones or been confounded by the kinetics of epitope presentation but particularly critical given the body of evidence that is accumulating that supports a protective effect of HIV-1 specific CTL that can efficiently deliver perforin and granzyme B to infected target cells (Mudd *et al.*, 2012; Chen *et al.*, 2012) (Migueles *et al.*, 2002) (Migueles *et al.*, 2008) (Hersperger *et al.*, 2010) (Bennett *et al.*, 2007) (Yang *et al.*, 2003) (Vojnov *et al.*, 2010) (Chen *et al.*, 2009). One mechanism of cell lysis is the direct secretion of perforin and granzyme B through the immune synapse toward the target cell, without prior storage in granules (Makedonas *et al.*, 2009) (Isaaz *et al.*, 1995). The upregulation of perforin has been reported to occur as early as two hours post TCR ligation (Makedonas *et al.*, 2009), and can only be detected by some monoclonal perforin antibodies (Hersperger *et al.*, 2008). As TCR signal strength may dictate the timing and magnitude of perforin expression, it was surprising not to detect

a difference in mRNA expression between the two HLA B*08-restricted FL8-specific clone populations that had distinct anti-viral efficacy. This may be due to the high constitutive expression of perforin mRNA rather than detecting newly synthesised protein. We did report that the highly sensitive CTL population had a high magnitude of IFNγ secretion at low cognate-MHC stimulus, and higher expression of the transcription factor EOMES. EOMES epigenetically regulates the expression of perforin and granzyme B (Araki *et al.*, 2008).

High sensitivity CTL may secrete high magnitudes of IFNγ that correlates with rapid expression of perforin and granzymes due to the poise of the epigenetic modifications at these gene loci. Magnitude of IFNγ secretion has also been used as a "rheostat" to indicate TCR signal strength, and in our data, the high sensitivity CD8+ T-cell clone population secreted a higher magnitude of IFNγ on stimulation with low density cognate peptide-MHC complexes (Betts *et al.*, 2004). Killing efficiency can be modulated by the quality and strength of the TCR signal, which directs the rapid re-assembly and polarisation of the cytoskeleton, dictates the route of granule mobilisation, and enhances efficient delivery of lytic molecules by forming a complete peripheral supra-molecular activation complex (Beal *et al.*, 2009) (Beal *et al.*, 2008) (Jenkins *et al.*, 2009). Our investigations would not have captured these early events. Further studies using imaging techniques to compare the high and low antigen sensitivity CTL clones to investigate if a strong TCR signal enhances the dynamics of serial killing by more flexible control of the CTL cytoskeleton and sustained, complete immune synapse formation would be interesting. CTL killing has been studied in vivo using two-photon imaging in mice (Kim *et al.*, 2009). This highlighted the importance of a strong "STOP" signal to enable sustained interaction of CTL with the cognate- peptide MHC complex. Of note, in this in vivo model of

killing, the only single CTL effector molecule that increased survival was the knock-out of TNFα (Kim *et al.*, 2009). This is consistent with our observation that the high sensitivity population with optimal viral control did not secrete TNFα. This was the most robust finding, even following comparison of the transcriptomes of the high sensitivity population and intermediate sensitivity populations on activation with a limiting concentration of peptide and a maximal stimulation with high MOI infected CD4+ T-cell line. We found that TNFα was silenced in the high sensitivity clone by DNA methylation of the core promoter. These findings suggest that silencing TNFα may enhance CD8+ T-cell anti-viral efficacy through a cytotoxic mechanism or lead to the differentiation of CTL with high antigen sensitivity. One mechanism we propose is that silencing of TNFα avoids activation induced cell death reported for other high sensitivity CTL populations (Alexander-Miller *et al.*, 1996b). We have provisional evidence that suggests that blocking TNFα using Recombinant Human Tumor Necrosis Factor Receptor Fc Fusion Protein (TNFR:Fc) (etanercept), enhances CD8+ T-cell efficacy in p24 suppression (Figure 6-1). This is the most convincing of four experiments, and work is actively in progress to confirm or refute this result in a system with clear MHC class I restricted viral suppression and determine the mechanism. One focus will be on using RNA interference (RNAi) silencing methods to suppress TNFα expression in the intermediate sensitivity clone to examine if this enhances viral suppression or antigen sensitivity by modulating gene expression.

Figure 6-1: CD8+ T-cell clone anti-HIV-1 efficacy is enhanced by blocking TNFα

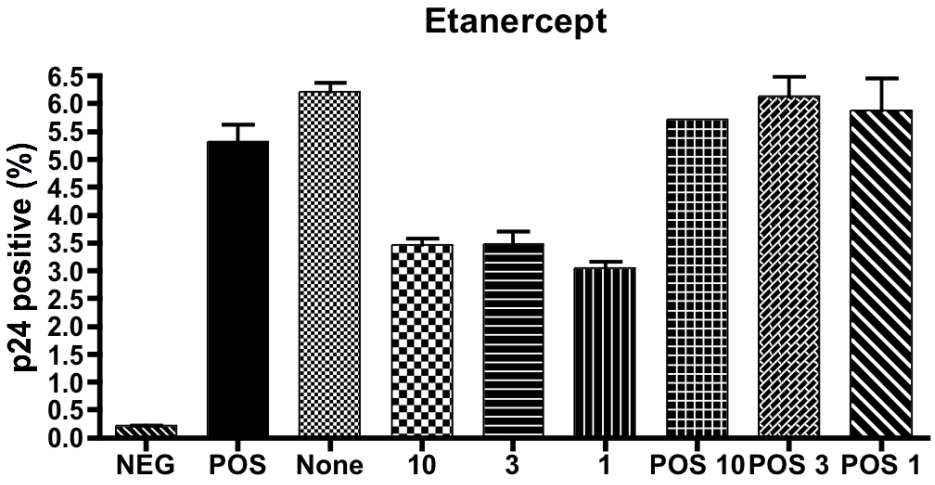


Figure 6-1: TNFa inhibition enhances the elimination of p24 positive target cells. Bar chart to illustrate the percentage of p24 positive CD4+ T-cell line (C8166) cells previously infected with HIV-1_{MN} for 72 hours (positive=POS) and following six hours co-culture with HLA B*08-restricted FL8-specific CD8+ T-cell clone A3 at the effector to target ratio of 3:1, in the absence (None) and presence (10 ug/ml, 3 ug/ml and 1ug/ml) of a fusion protein inhibitor of TNFα, Etanercept. The infected CD4+ T-cell line was also cultured alone without the CD8+ T-cell clone A3, in the TNFα inhibitor (POS 10ug/ml, POS 3ug/ml and POS 1ug/ml). The graph illustrates the percentage of p24 suppression at each concentration of Etanercept. Results of one out of four experiments, each data point is in duplicate (experiment 1) (error bars=s.d.).

Perhaps the most unexpected and interesting finding was the observation that *in vitro* viral suppression, antigen sensitivity and regulation of TNFα secretion by epigenetic mechanisms could be distinct in a population of CD8+ T-cell clones that were not only genetically identical, isolated from one long-term non-progressor, but that also used the same T cell receptor. This uncovers two of the "unknown factors" that mediate the intraclonal heterogeneity in effector function observed in a population of CD8+ T-cells that stain with one peptide/MHC class I tetramer (Betts *et al.*, 2004). These clones were not generated using differing concentrations of peptide that can give rise to high or low sensitivity clones ex vivo (Almeida *et al.*, 2009) (Kroger and Alexander-Miller, 2007b) but by the limiting dilution of the HLA B*08-FL8 tetramer population. The phenotype was stable between rounds of re-stimulation. This suggests that a single naïve CTL can give rise to progeny that undergo distinct avidity maturation, and epigenetic modification that alters their anti-viral efficacy.

Avidity maturation without the selection of high affinity TCR of the CTL response has been previously reported to occur in animal models of viral infection or mouse CD8+ T-cell clones (Slifka and Whitton, 2001) (Sharma and Alexander-Miller, 2011) (Kroger and Alexander-Miller, 2007a) (Cawthon *et al.*, 2001) (Cawthon and Alexander-Miller, 2002) (Amoah *et al.*, 2012). In our high and intermediate sensitivity CD8+ T-cell clones that have the same TCR affinity, we found similar expression of the CD8 co-receptor, CD8α to CD8β mRNA expression ratio, and co-localisation of the TCR and CD8, as the clones had the same expression level of CD8, the TCR and the same tetramer avidity (Park *et al.*, 2007), (Cawthon *et al.*, 2001) (Cawthon and Alexander-Miller, 2002). This suggested a mechanism of avidity maturation downstream of the TCR, consistent with reports that highlight augmented membrane proximal TCR signal transduction, with the degree of CD3ζ, ZAP-70 and

lck phosphorylation, increasing with more antigen sensitive populations (Slifka and Whitton, 2001) (Amoah *et al.*, 2012) (Sharma and Alexander-Miller, 2011). The negative regulation of phosphatases has also been reported to enhance antigen sensitivity (Li *et al.*, 2007). We observed a high expression of receptors with ITAM motifs in the high sensitivity population, FCRL3, TYROBP and FCER1G, and a high expression of receptors with ITIM motifs in the intermediate sensitivity population, FCRL6, LRRN3, KIR2DL3 and KIR2DL4. We also observed high expression of PD-1 by the intermediate sensitivity population at the protein level, which carries an ITIM motif. This supports a model of TCR signal strength that is regulated by the balance of kinases and phosphatases recruited to the proximal signalling complex following engagement of a cognate p-MHC (Figure 6-2) (Hermiston *et al.*, 2002).

We found a range of functional avidity already apparent in CD8+ T-cell clones with the same TCR isolated during acute infection, but the highest sensitivity CD8+ T-cell clone was isolated during chronic infection. We speculate that avidity maturation may arise during priming, but require encounter with a conserved antigen in the periphery (Kroger and Alexander-Miller, 2007a). It has been observed that functional avidity actually decreases in HIV-1 specific CTL on commencement of HAART (Berger *et al.*, 2011).

Figure 6-2: Theoretical model of augmented membrane proximal signalling

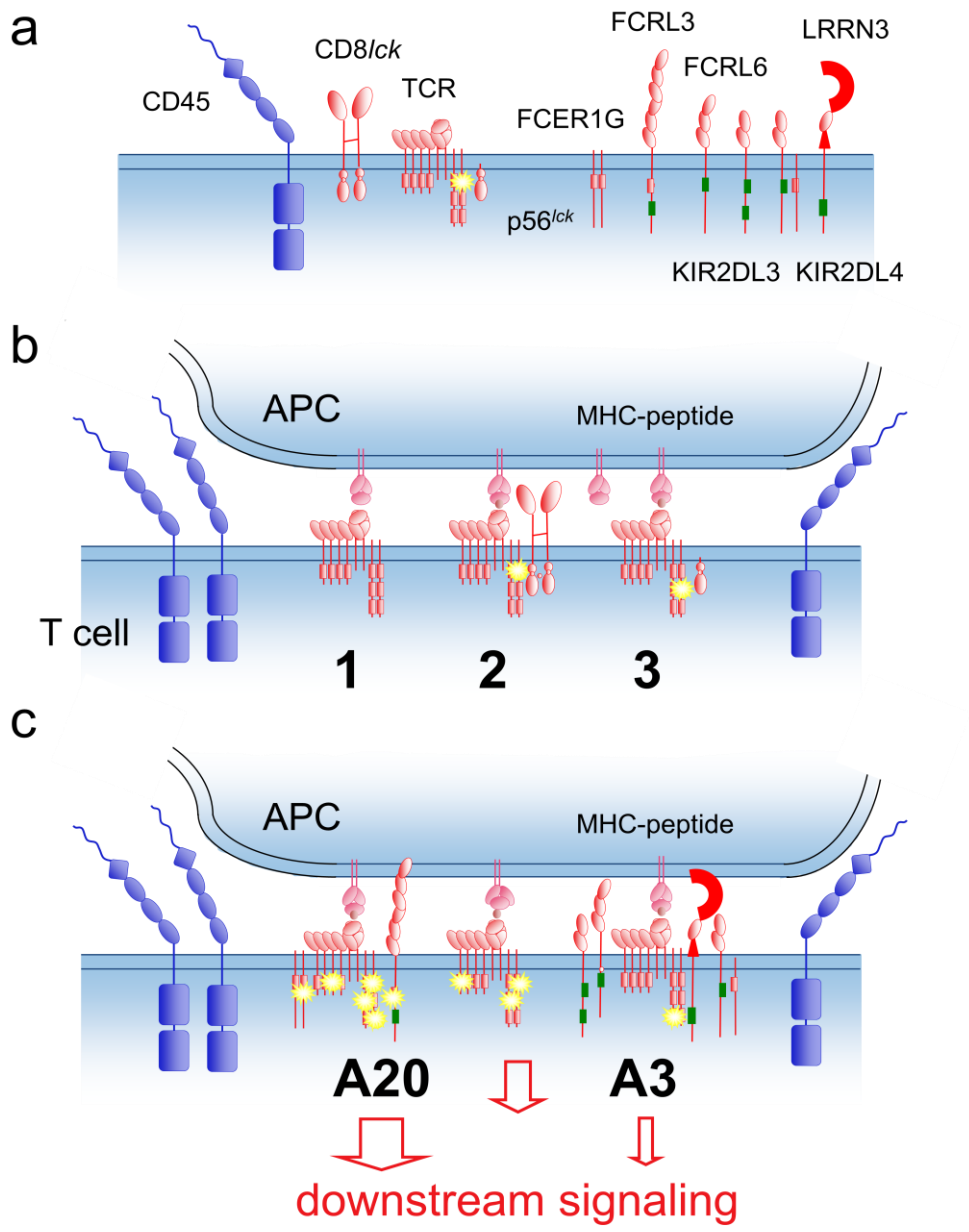


Figure 6.2: Theoretical model of augmented membrane proximal signalling

A) Cartoon illustrating the TCR complex, CD8, CD45, FCER1G, FCRL3, FCRL6, KIR2DL3, KIR2DL4 and LRRN3, with ITAM motifs (red boxes), and ITIM motifs (green boxes), and phosphorylation (yellow). B) Lck phosphorylates the TCR complex C) High expression of receptors with ITAM motifs in the high sensitivity clone, A20, augments the TCR signal strength. In the intermediate sensitivity clone, A3, high expression of receptors with ITIM motifs leads to the recruitment of phosphatases that reduced ITAM phosphorylation, resulting in a low TCR signal.

Antigen sensitivity has been linked to memory/effector cell differentiation (Amoah *et al.*, 2012) with proliferation and differentiation of effectors associated with loss of TNFα and gain of IFNγ, perforin and granzyme B in mice (Denton *et al.*, 2011). Memory cells with "stem-cell" like properties have been described, and the asymmetrical division of a single cell into daughter populations that differ in effector function is reported (Chang *et al.*, 2007) (King *et al.*, 2012).

Epigenetic programming of gene expression fundamentally defines the cell and is tightly regulated during CD8 T-cell ontogeny and during priming as naïve CD8+ T-cells gain expression of effector molecules (Chiu *et al.*, 2007) (Araki *et al.*, 2008) (Juelich *et al.*, 2009) (Bruniquel and Schwartz, 2003). Little is understood about how these programs might differ to modulate CD8 T cell effector function although this could be critical to the control of HIV-1 infection. We find that TNFα can be epigentically silenced by DNA methylation of the core promoter, and studies in mice have implicated that repressive histone marks are acquired at the TNFα promoter during the first four to five rounds of proliferation (Denton *et al.*, 2011). These findings in T-cells with the same TCR emphasise the role that encounter with antigen can have on the phenotype and function of the progeny of a single naïve CTL. Each clone expressed a different set of homing molecules, CXCR6 (High sensitivity population, inflamed tissues) and CXCR3, ITGα5 and ITGβ7 expression (Intermediate sensitivity, lymphoid tissue and GALT), that would lead to migration to distinct tissue niches, and exposure to different cytokine milieu (Matsumura *et al.*, 2008) (Sato *et al.*, 2005) (Kim *et al.*, 2001) (Flatz *et al.*, 2011). Evaluating the panel of cell surface receptors, for "good" CTL FCRL3 and CXCR6 and "Bad" CTL LRRN3, FCRL6, and CXCR3 may help to identify the most efficacious vaccine candidates.

One of the great challenges facing the rational design of a successful HIV-1 vaccine, is to expedite the early expansion of CD8+ T-cells, that have optimal effector function, and migrate or locally expand to sufficient magnitude within the lymphoid tissue and GALT (Reynolds *et al.*, 2005) (Haase, 2011). We report that the induction of high sensitivity CTL will be critical, but offer hope that this can be achieved even in individuals without protective HLA alleles, by further exploration of peripheral avidity maturation and epigenetic regulation of the HIV-1 specific CD8+ T-cell response.

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8 APPENDIX

Table 8-1: Genes with distinct expression (Absolute fold change at least 2.0, p<0.01 (ANOVA) in the high sensitivity clone population A20, compared with the moderate sensitivity population A3 in the resting state

Entrez ID	Gene Symbol	Gene Name	Absolute FC A20vA3
4753	NELL2	NELL-like 2(chicken)	7.40
27065	D4S234E	DNA segment on chromosome 4 (unique) 234 expressed genes	4.48
4069	LYZ	Lysozyme	4.10
1514	CTSL1	Cathepsin L1	3.79
10663	CXCR6	C-X-C receptor 6	3.75
115352	FCRL3	Fc receptor-like 3	3.58
6355	CCL8	C-C motif chemokine 8	3.43
2207	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	3.33
6347	CCL2	C-C motif chemokine 2	3.22
84419	C15orf48	Chromosome 15 open reading frame 48	3.20
197135	LOC197135	protein associated with topoisomerase II homolog (yeast)	3.14
4495	MT1G	Metallothionein 1G	2.85
4496	MT1H	Metallopthionein 1H	2.76
2731	GLDC	Glycine dehydrogenase	2.73
7305	TYROBP	TYRO protein tyrosine kinase binding protein	2.72
132332	TMEM155	Transmembrane protein 155	2.71
58191	CXCL16	C-X-C motif chemokine 16	2.65
10964	IFI44L	Interferon-induced protein 44 like	2.55
51296	SLC15A3	solute carrier family 15, member 3	2.53
4118	MAL	mal, T-cell differentiation protein	2.49
6790	AURKA	aurora kinase A	2.46
968	CD68	CD68 molecule	2.43
11065	UBE2C	Ubiquitin-conjugating enzyme E2	2.41
9133	CCNB2	Cyclin B2	2.40
332	BIRC5	baculoviral IAP repeat-containing 5	2.39
991	CDC20	cell division cycle 20 homologue (S.cerevisiae)	2.34
9787	DLG7	discs, large (Drosophila) homolog-associated protein 5	2.30
23178	PASK	PAS domain containing serine/threonine kinase	2.25
9749	PHACTR2	Phosphatase and actin regulator 2	2.25
27299	ADAMDEC1	ADAM-like, decysin 1	2.25
3553	IL1B	Interleukin 1, beta	2.23
929	CD14	CD14 molecules	2.23
11130	ZWINT	ZW10 interactor	2.22
7130	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	2.21
84557	MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha	2.20
400735	PRAMEF4	PRAME family member 4	2.19
7941	PLA2G7	Phospholipase A2, group VII	2.18
10457	GPNMB	Glycoprotein (transmembrane) nmb	2.17
4014	LOR	Loricin	2.16
51659	GINS2	GINS complex subunit 2 (Psf2 homolog)	2.16
343068	PRAMEEF5	PRAME family member 5	2.14
6648	SOD2	superoxide dismutase 2, mitochondrial	2.13
8318	CDC45L	CDC45 cell division cycle 45-like (S.cerevisiae)	2.13
6279	S100A8	S100 calcium binding protein A8	2.12

Entrez ID	Gene Symbol	Gene Name	Absolute FC A20vA3
348	APOE	Apolipoprotein E	2.12
55714	ODZ3	odz, odd Oz/ten-m homolog 3 (Drosophila)	2.11
3833	KIFC1	Kinesin family member C1	2.11
6280	S100A9	S100 calcium binding protein A9	2.07
19	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	2.07
84634	KISS1R	KISS 1 receptor	2.07
7272	TTK	TTK protein kinase	2.06
3925	STMN1	stathmin 1	2.04
1111	CHEK1	CHK1 checkpoint homolog (S.pombe)	2.03
1062	CENPE	centromere protein E, 312kDa	2.03
84057	MND1	Meiotic nuclear divisions 1 homolog (S.cerevisiae)	2.02
1854	DUT	deoxyuridine triphosphate	2.02
10578	GNLY	Granulysin	-15.69
54674	LRRN3	Leucine rich repeat neuronal 3	-11.49
3248	HPGD	hydroxyprostaglandin dehydrogenase 15	-6.76
339541	MGC33556	chromosome 1 open reading frame 228	-6.47
5243	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	-5.35
3824	KLRD1	Killer cell lectin-like receptor subfamily D, member 1	-5.26
2494	NR5A2	Nuclear receptor subfamily 5, group A, member 2	-4.51
4050	LTB	Lymphotoxin beta	-4.47
343413	FCRL6	Fc receptor-like 6	-4.33
118429	ANTXR2	Anthrax toxin receptor 2	-4.33
26053	AUTS2	autism susceptibility candidate 2	-3.93
1102	RCBTB2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	-3.50
4815	NINJ2	Ninjurin 2	-3.43
1052	CEBPD	CCAAT/enhancer binding protein (C/EBP), Delta	-3.28
8910	SGCE	sarcoglycan, epsilon	-3.04
53346	TM6SF1	Transmembrane 6 superfamily member 1	-2.86
23433	RHOq	ras homolog gene family, member Q	-2.75
4145	MATK	Megakaryocyte associated tyrosine kinase	-2.75
11320	MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	-2.73
60675	PROK2	Prokineticin 2	-2.67
56978	PRDM8	PR domain containing 8	-2.65
3804	KIR2DL3	Killer cell Immunoglobulin-like receptor, two domains, long cytoplasmic, tail, 3	-2.62
91663	MYADM	Myeloid-associated differentiation marker	-2.53
10870	HCST	Haematopoietic cell signal transducer	-2.51
27030	MLH3	mutL homolog 3 (E.coli)	-2.46
3002	GZMB	Granzyme B	-2.42
2841	GPR18	G protein-coupled receptor 18	-2.40
1521	CTSW	Cathepsin W	-2.40
79037	PVRIG	Poliovirus receptor related immunoglobulin domain containing	-2.39
9252	RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	-2.39
3221	HOXC4	homeobox C4	-2.39
64420	SUSD1	sushi domain containing 1	-2.38
284988	LOC284988	Hypothetical LOC284988	-2.38
4155	MBP	Myelin Basic Protein	-2.34
57415	C3orf14	Chromosome 3 ORF 14	-2.34
969	CD69	CD69 molecule	-2.34
7048	TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	-2.33
9424	KCNK6	Potassium channel, subfamily K, member 6	-2.33
6095	RORA	RAR-related orphan receptor A	-2.33
4061	LY6E	Lymphocyte antigen 6 complex, locus E	-2.32

Entrez ID	Gene Symbol	Gene Name	Absolute FC A20vA3
64115	C10orf54	Platelet receptor Gi24	-2.29
2833	CXCR3	Chemokine (C-X-C motif) receptor 3	-2.29
3581	IL9R	Interleukin 9 receptor	-2.29
1031	CDKN2C	Cyclin dependent kinase 4 inhibitor C	-2.28
26207	PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	-2.26
89853	FAM125B	Family with sequence similarity 125, member B	-2.26
9289	GPR56	G protein-coupled receptor 56	-2.25
81606	LBH	Limb bud and heart development homologue (mouse)	-2.25
3905	KIR2DL4	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	-2.24
203328	SUSD3	sushi domain containing 3	-2.23
60489	APOBEC3G	Apolipoprotein B, mRNA editing enzyme, catalytic polypeptide-like 3F	-2.19
170954	KIAA1949	KIAA1949	-2.23
54935	DUSP23	Dual specificity phosphatase 23	-2.22
22822	PHLDA1	Pleckstrin homology-like domain, family A, member 1	-2.22
3125	HLA-DRB3	major histocompatibility complex, class II, DR beta 3	-2.21
25953	PNKD	Paroxysmal nonkinesigenic dyskinesia	-2.21
3398	ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-2.20
11238	CA5B	Inactivation escape 2 (non-proetin coding); carbonic anhydrase VB, mitochondrial	-2.20
3001	GZMA	Granzyme A	-2.20
4689	NCF4	Neutrophil cytosolic factor 4, 40kDa	-2.20
3695	ITGB7	Integrin, beta 7	-2.19
200316	APOBEC3F	Apolipoprotein B, mRNA editing enzyme, catalytic polypeptide-like 3F	-2.19
644414	DEFB131	similar to defensin, beta 131; defensin beta 131	-2.19
10077	TSPAN32	Tetraspanin 32	-2.18
9249	DHRS3	Dehydrogenase/reductase (SDR family) member 3	-2.18
27099	C7orf54	Chromosome 7 ORF 54	-2.17
3725	JUN	jun oncogene	-2.17
6348	CCL3	C-C motif chemokine 3	-2.15
83442	SH3BGRL3	SH3 domain binding glutamic acid rich-protein like 3	-2.14
10231	RCAN2	Regulator of calcineurin 2	-2.13
54861	SNRK	SNF related kinase	-2.12
26040	SETBP1	SET binding protein 1	-2.12
266747	RGL4	ral guanine nucleotide dissociation stimulator-like 4	-2.12
8638	OASL	2'-5' oligoadenylate synthetase-like	-2.07
729486	LOC729486	IL 9 receptor pseudogene 3	-2.06
5790	PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein	-2.01
55911	APOB48R	Apolipoprotein B48 receptor	-2.03
30011	SH3KBP1	SH3-domain kinase binding protein 1	-2.03
255022	FAM26C	calcium homeostasis modulator 1	-2.03
56940	DUSP22	dual specificity phosphatase 22	-2.03
23046	KIF21B	Kinesin family member 21B	-2.02
257106	ARHGAP30	Rho GTPasw activating protein 30	-2.01
10123	ARL4C	ADP-ribosylation factor-like 4C	-2.01
356	FASLG	Fas ligand	-2.01
3678	ITGA5	Integrin, alpha 5	-2.01
2999	GZMH	Granzyme H	-2.00

Table 8-2: Genes with distinct expression (Absolute fold change at least 2.0, p<0.01 (ANOVA) in the high sensitivity clone population A20, compared with the moderate sensitivity population A3 following activation with 2uM epitope pulsed target cells for six hours.

Entrez_ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20 v A3 [2uM]
6695	SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	1.21E-06	10.12
115352	FCRL3	Fc receptor-like 3	2.41E-05	6.78
10663	CXCR6	chemokine (C-X-C motif) receptor 6	1.07E-05	6.75
1831	TSC22D3	TSC22 domain family, member 3	4.78E-05	6.73
939	CD27	CD27 molecule	1.59E-04	5.89
4753	NELL2	NEL-like 2 (chicken)	5.71E-06	5.67
25975	EGFL6	EGF-like-domain, multiple 6	3.09E-04	5.17
2731	GLDC	glycine dehydrogenase (decarboxylating)	1.28E-05	4.77
55010	C12orf48	chromosome 12 open reading frame 48	4.03E-05	4.30
6696	SPP1	secreted phosphoprotein 1	3.93E-05	4.27
5581	PRKCE	protein kinase C, epsilon	3.32E-06	4.19
2274	FHL2	four and a half LIM domains 2	9.10E-06	4.19
83888	FGFBP2	fibroblast growth factor binding protein 2	3.37E-05	4.15
94241	TP53INP1	tumor protein p53 inducible nuclear protein 1	1.37E-06	4.14
5367	PMCH	pro-melanin-concentrating hormone	2.44E-05	4.10
10253	SPRY2	sprouty homolog 2 (Drosophila)	9.77E-05	3.91
825	CAPN3	calpain 3, (p94)	8.36E-05	3.74
8744	TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	3.81E-05	3.74
7412	VCAM1	vascular cell adhesion molecule 1	1.14E-04	3.46
2882	GPX7	glutathione peroxidase 7	2.75E-05	3.39
59352	LGR6	leucine-rich repeat-containing G protein-coupled receptor 6	5.54E-04	3.35
84557	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha	9.80E-06	3.26
10365	KLF2	Kruppel-like factor 2 (lung)	1.82E-06	3.22
59	ACTA2	actin, alpha 2, smooth muscle, aorta	8.17E-05	3.20

Entrez_ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20 v A3 [2uM]
27065	D4S234E	DNA segment on chromosome 4 (unique) 234 expressed sequence	1.41E-05	3.17
10219	KLRG1	killer cell lectin-like receptor subfamily G, member 1	7.06E-04	3.15
7292	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4	3.02E-05	3.12
3005	H1F0	H1 histone family, member 0	1.95E-05	3.11
4118	MAL	mal, T-cell differentiation protein	5.40E-06	3.07
970	CD70	CD70 molecule	2.33E-06	3.05
326624	RAB37	RAB37, member RAS oncogene family	9.51E-04	3.04
2203	FBP1	fructose-1,6-bisphosphatase 1	2.98E-05	2.95
9124	PDLIM1	PDZ and LIM domain 1	5.59E-05	2.92
5336	PLCG2	phospholipase C, gamma 2	3.62E-05	2.90
1232	CCR3	chemokine (C-C motif) receptor 3	7.32E-05	2.89
6235	RPS29	ribosomal protein S29	6.20E-06	2.87
23612	PHLDA3	pleckstrin homology-like domain, family A, member 3	8.89E-06	2.85
929	CD14	CD14 molecule	1.03E-05	2.84
8530	CST7	cystatin F (leukocystatin)	1.78E-04	2.84
1647	GADD45A	growth arrest and DNA-damage-inducible, alpha	3.30E-05	2.82
9214	FAIM3	Fas apoptotic inhibitory molecule 3	3.44E-05	2.81
27333	GOLPH4	golgi phosphoprotein 4	0.002	2.76
760	CA2	carbonic anhydrase II	3.15E-05	2.76
25999	CLIP3	CAP-GLY domain containing linker protein 3	2.66E-05	2.75
6279	S100A8	S100 calcium binding protein A8	1.61E-05	2.72
8795	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	5.45E-04	2.72
221188	GPR114	G protein-coupled receptor 114	7.28E-05	2.71
10172	ZNF256	zinc finger protein 256	4.34E-05	2.70
79961	DENND2D	DENN/MADD domain containing 2D	0.005	2.65
4495	MT1G	metallothionein 1G	6.08E-05	2.64
64393	ZMAT3	zinc finger, matrin type 3	3.54E-04	2.62
1643	DDB2	damage-specific DNA binding protein 2, 48kDa	7.20E-06	2.61
90843	TCEAL8	transcription elongation factor A (SII)-like 8	0.001	2.57
11046	SLC35D2	solute carrier family 35, member D2	0.001	2.55
116841	SNAP47	synaptosomal-associated protein, 47kD	0.0019	2.55

Entrez-ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20vA3[2uM]
953	ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1	1.08E-05	2.54
55423	SIRPG	signal-regulatory protein gamma	2.84E-04	2.52
199746	U2AF1L4	U2 small nuclear RNA auxiliary factor 1-like 4 .	0.0014	2.44
27244	SESN1	sestrin 1	4.62E-05	2.44
9476	NAPSA	napsin A aspartic peptidase	0.0051	2.43
11040	PIM2	pim-2 oncogene	2.57E-04	2.43
4496	MT1H	metallothionein 1H	2.39E-05	2.40
2268	FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	2.36E-04	2.36
388403	YPEL2	yippee-like 2 (Drosophila)	2.10E-04	2.35
388	RHOB	ras homolog gene family, member B	2.93E-05	2.32
820	CAMP	cathelicidin antimicrobial peptide	1.10E-04	2.32
2149	F2R	coagulation factor II (thrombin) receptor	7.65E-04	2.30
1471	CST3	cystatin C	1.24E-04	2.28
10875	FGL2	fibrinogen-like 2	8.57E-04	2.28
120425	AMICA1	adhesion molecule, interacts with CXADR antigen 1	0.0031	2.28
345778	MTX3	metaxin 3	1.60E-04	2.28
201633	TIGIT	T cell immunoreceptor with Ig and ITIM domains	6.76E-04	2.27
7169	TPM2	tropomyosin 2 (beta)	7.55E-05	2.26
944	TNFSF8	tumor necrosis factor (ligand) superfamily, member 8	4.18E-05	2.26
2710	GK	glycerol kinase	1.05E-04	2.25
900	CCNG1	cyclin G1	2.25E-04	2.25
653381	LOC653381	PREDICTED: similar to Sorbitol dehydrogenase (L-iditol 2-dehydrogenase)	6.77E-06	2.25
56521	DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	4.54E-04	2.25
132332	TMEM155	transmembrane protein 155	1.34E-04	2.24
130589	GALM	galactose mutarotase (aldose 1-epimerase)	0.0081	2.24
64776	C11orf1	chromosome 11 open reading frame 1	3.05E-06	2.23
581	BAX	BCL2-associated X protein	1.55E-05	2.23
3983	ABLIM1	actin binding LIM protein 1	4.88E-05	2.23
27299	ADAMDEC1	ADAM-like, decysin 1	7.87E-05	2.23
56253	CRTAM	cytotoxic and regulatory T cell molecule	6.10E-06	2.22
5763	PTMS	parathymosin	0.0022	2.20

Entrez ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20vA3[2uM]
3075	CFH	complement factor H	1.07E-04	2.20
54072	C21orf42	chromosome 21 open reading frame 42	3.49E-06	2.20
92017	SNX29	sorting nexin 29	5.39E-04	2.19
56978	PRDM8	PR domain containing 8	2.14E-04	2.18
4033	LRMP	lymphoid-restricted membrane protein	7.05E-04	2.17
140691	TRIM69	tripartite motif-containing 69	7.85E-05	2.17
157627	LOC157627	hypothetical LOC157627	2.15E-05	2.16
80162	ATHL1	ATH1, acid trehalase-like 1 (yeast)	7.21E-05	2.16
10161	P2RY5	purinergic receptor P2Y, G-protein coupled, 5	1.02E-04	2.16
50852	TRAT1	T cell receptor associated transmembrane adaptor 1	1.27E-04	2.16
4258	MGST2	microsomal glutathione S-transferase 2	3.75E-06	2.16
53637	S1PR5	Sphingosine-1-phosphate receptor 5	1.13E-05	2.15
2219	FCN1	ficolin (collagen/fibrinogen domain containing) 1	0.0017	2.13
6354	CCL7	chemokine (C-C motif) ligand 7	1.13E-05	2.13
6652	SORD	sorbitol dehydrogenase	1.13E-04	2.13
5996	RGS1	regulator of G-protein signaling 1	3.06E-04	2.12
389	RHOC	ras homolog gene family, member C	5.24E-04	2.12
713	C1QB	complement component 1, q subcomponent, B chain	3.06E-05	2.12
29083	GTPBP8	GTP-binding protein 8 (putative)	0.0063	2.10
5140	PDE3B	phosphodiesterase 3B, cGMP-inhibited	0.0050	2.10
901	CCNG2	cyclin G2	7.11E-06	2.10
10346	TRIM22	tripartite motif-containing 22	2.12E-04	2.10
51065	RPS27L	ribosomal protein S27-like	7.07E-05	2.09
8875	VNN2	vanin 2	2.14E-04	2.09
55769	ZNF83	zinc finger protein 83	5.85E-05	2.08
137872	ADHFE1	alcohol dehydrogenase, iron containing, 1	1.34E-04	2.08
401124	FLJ16686	PREDICTED: FLJ16686 protein	0.001	2.07
9749	PHACTR2	phosphatase and actin regulator 2	2.70E-04	2.07
2081	ERN1	endoplasmic reticulum to nucleus signalling 1	1.77E-04	2.07
196743	PAOX	polyamine oxidase (exo-N4-amino)	3.77E-04	2.07
80723	TMEM22	transmembrane protein 22	3.48E-05	2.06
401124	FLJ16686	PREDICTED: FLJ16686 protein	0.001	2.07

Entrez ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20 v A3 [2uM]
6280	S100A9	S100 calcium binding protein A9 (calgranulin B)	1.07E-04	2.06
692084	SNORD13	small nucleolar RNA, C/D box 13	8.57E-05	2.06
643031	LOC643031	PREDICTED: similar to NADH dehydrogenase subunit 5	1.32E-05	2.05
3902	LAG3	lymphocyte-activation gene 3	3.75E-04	2.05
8611	PPAP2A	phosphatidic acid phosphatase type 2A	9.36E-04	2.04
80325	ABTB1	ankyrin repeat and BTB (POZ) domain containing 1	6.72E-05	2.03
118932	ANKRD22	ankyrin repeat domain 22	0.010	2.03
64864	RFX7	regulatory factor X, 7	7.08E-04	2.02
22806	IKZF3	IKAROS family zinc finger 3 (Aiolos)	0.005	2.02
83605	CCM2	cerebral cavernous malformation 2	1.02E-04	2.01
23521	RPL13A	ribosomal protein L13a	9.55E-05	2.01
55615	PRR5	proline rich 5 (renal)	0.004	2.01
5527	PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	9.04E-04	2.01
26118	WSB1	WD repeat and SOCS box-containing 1	3.36E-04	2.01
54674	LRRN3	leucine rich repeat neuronal 3	1.11E-06	-19.19
7124	TNF	tumor necrosis factor (TNF superfamily, member 2)	1.29E-04	-10.67
5569	PKIA	protein kinase (cAMP-dependent, catalytic) inhibitor alpha	1.43E-05	-7.15
160365	CLECL1	C-type lectin-like 1	7.11E-07	-6.57
6004	RGS16	regulator of G-protein signalling 16	1.72E-05	-5.29
9289	GPR56	G protein-coupled receptor 56	2.75E-05	-4.42
57415	C3orf14	chromosome 3 open reading frame 14	1.10E-04	-4.18
4915	NTRK2	neurotrophic tyrosine kinase, receptor, type 2	2.64E-05	-3.91
6624	FSCN1	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	2.32E-06	-3.79
339541	MGC33556	hypothetical LOC339541	8.70E-06	-3.78
6361	CCL17	chemokine (C-C motif) ligand 17	6.58E-05	-3.65
5891	RAGE	renal tumor antigen	1.76E-05	-3.46
10578	GNLY	granulysin	3.83E-07	-3.26
7049	TGFBR3	transforming growth factor, beta receptor III	3.12E-04	-3.25
4609	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.27E-05	-3.15
2769	GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	9.87E-05	-3.13

Entrez ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20 v A3 [2uM]
1052	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	1.47E-06	-3.11
81618	ITM2C	integral membrane protein 2C	3.26E-05	-2.97
1236	CCR7	Chemokine (C-C motif) receptor 7	2.58E-05	-2.94
4049	LTA	lymphotoxin alpha (TNF superfamily, member 1)	1.45E-04	-2.93
2041	EPHA1	EPH receptor A1	7.00E-04	-2.92
7462	LAT2	linker for activation of T cells family, member 2	4.76E-05	-2.79
5307	PITX1	paired-like homeodomain transcription factor 1	0.001	-2.70
6367	CCL22	chemokine (C-C motif) ligand 22	1.64E-06	-2.67
4316	MMP7	matrix metallopeptidase 7	0.004	-2.65
51522	TMEM14C	transmembrane protein 14C	0.001	-2.65
1437	CSF2	colony stimulating factor 2 (granulocyte-macrophage)	2.44E-06	-2.61
8809	IL18R1	interleukin 18 receptor 1	1.00E-04	-2.59
5361	PLXNA1	plexin A1	0.003	-2.54
51332	SPTBN5	spectrin, beta, non-erythrocytic 5	4.34E-04	-2.54
23189	KANK1	KN motif and ankyrin repeat domains 1	3.22E-06	-2.54
3627	CXCL10	chemokine (C-X-C motif) ligand 10	5.92E-07	-2.52
3892	KRT86	keratin 86	5.89E-06	-2.49
6520	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.17E-04	-2.48
2494	NR5A2	nuclear receptor subfamily 5, group A, member 2	4.14E-06	-2.46
5733	PTGER3	prostaglandin E receptor 3 (subtype EP3)	0.003	-2.46
347733	TUBB2B	tubulin, beta 2B	0.004	-2.45
3516	RBPJ	recombination signal binding protein for immunoglobulin kappa J region	1.18E-05	-2.43
3581	IL9R	interleukin 9 receptor	7.42E-05	-2.42
6723	SRM	spermidine synthase	1.28E-04	-2.41
1627	DBN1	drebrin 1	2.88E-04	-2.40
64375	IKZF4	IKAROS family zinc finger 4 (Eos)	2.55E-05	-2.39
6925	TCF4	transcription factor 4	6.19E-05	-2.39
3221	HOXC4	homeobox C4	1.51E-05	-2.38
3898	LAD1	ladinin 1	0.001	-2.38

Entrez ID	Gene Symbol	Gene Name	Corrected p-value	Absolute FC A20vA3[2uM]
928	CD9	CD9 molecule	1.19E-07	-2.35
1031	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	2.00E-05	-2.30
203328	SUSD3	sushi domain containing 3	1.06E-04	-2.28
4283	CXCL9	chemokine (C-X-C motif) ligand 9	5.32E-05	-2.28
6809	STX3	syntaxin 3	1.15E-04	-2.26
3804	KIR2DL3	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	9.02E-04	-2.24
81831	NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	7.40E-05	-2.22
339512	C1orf110	chromosome 1 open reading frame 110	5.21E-05	-2.22
29796	UCRC	ubiquinol-cytochrome c reductase complex (7.2 kD)	2.69E-04	-2.21
8323	FZD6	frizzled homolog 6 (Drosophila)	1.67E-04	-2.21
4645	MYO5B	myosin VB	2.64E-04	-2.19
27074	LAMP3	lysosomal-associated membrane protein 3	1.08E-04	-2.19
8857	FCGBP	Fc fragment of IgG binding protein	7.52E-04	-2.18
8651	SOCS1	suppressor of cytokine signaling 1	1.03E-04	-2.18
5445	PON2	paraoxonase 2	2.41E-06	-2.16
26472	PPP1R14B	protein phosphatase 1, regulatory (inhibitor) subunit 14B	6.73E-05	-2.16
80326	WNT10A	wingless-type MMTV integration site family, member 10A	2.20E-05	-2.15
51678	MPP6	membrane protein, palmitoylated 6	0.002	-2.15
284944	DNHL1	dynein heavy chain-like 1	1.71E-04	-2.13
3805	KIR2DL4	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	7.62E-04	-2.12
1803	DPP4	dipeptidyl-peptidase 4	0.001	-2.11
22846	VASH1	vasohibin 1	8.42E-04	-2.10
642477	FLJ39632	PREDICTED: misc_RNA (FLJ39632)	0.005	-2.09
8140	SLC7A5	solute carrier family 7 member 5	4.55E-06	-2.09
1880	EBI2	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	1.27E-05	-2.09
4082	MARCKS	myristoylated alanine-rich protein kinase C substrate	7.61E-06	-2.07
399967	PATE2	prostate and testis expressed 2	3.42E-05	-2.04
221613	HIST1H2AA	histone cluster 1, H2aa	7.74E-05	-2.03
26053	AUTS2	autism susceptibility candidate 2	2.58E-04	-2.01

Table 8-3: TCR inducible genes (Log_2 FC at least 1.5, p<0.05(Limma)) in HLA B*08-restricted CD8+ T-cell clone populations following two hours activation with HIV-1 infected targets (Red=Only in population A20, Black= Both A20 and A3, Grey=Only in population A3).

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_021006.4	CCL3L1	chemokine (C-C motif) ligand 3-like 1	3.76	2.34E-14	28.77	**1**	4.85	9.47E-17	34.73	**2**
NM_019604.2	CRTAM	cytotoxic and regulatory T cell molecule	3.62	4.64E-20	44.98	**2**	3.39	3.33E-20	43.84	**9**
NM_001561.4	TNFRSF9	tumor necrosis factor receptor superfamily, member 9	3.43	7.19E-20	44.00	**3**	3.59	1.63E-20	45.24	**6**
NM_000879.2	IL5	interleukin 5 (colony-stimulating factor, eosinophil)	3.41	7.51E-17	35.76	**4**	3.98	2.05E-18	39.44	**4**
NM_000619.2	IFNG	interferon, gamma	3.26	1.20E-19	43.19	**5**	3.42	2.16E-20	44.46	**8**
NM_002984.1	CCL4	chemokine (C-C motif) ligand 4	3.16	2.34E-14	28.77	**6**	3.76	4.72E-16	32.83	**5**
NM_007350.3	PHLDA1	pleckstrin homology-like domain, family A, member 1	3.14	6.54E-18	38.55	**7**	2.33	1.23E-15	31.73	**32**
NM_014059.2	C13ORF15	chromosome 13 open reading frame 15	3.03	7.76E-19	41.05	**8**	3.15	1.66E-19	42.13	**11**
NM_001165.3	BIRC3	baculoviral IAP repeat-containing 3	2.94	7.65E-19	41.27	**9**	3.14	7.91E-20	42.93	**12**
NM_003897.3	IER3	immediate early response 3	2.89	1.48E-11	21.12	**10**	3.50	2.46E-13	25.48	**7**
NM_032782.3	HAVCR2	hepatitis A virus cellular receptor 2	2.82	7.66E-17	35.68	**11**	2.32	1.78E-15	31.25	**35**
NM_001001435.2	CCL4L1	chemokine (C-C motif) ligand 4-like 1	2.80	6.99E-16	33.09	**12**	1.99	3.15E-13	25.15	**65**
NM_000639.1	FASLG	Fas ligand (TNF superfamily, member 6)	2.80	2.56E-15	31.38	**13**	2.63	5.60E-15	29.97	**22**
NM_207007.2	CCL4L2	chemokine (C-C motif) ligand 4-like 2	2.78	3.12E-16	34.17	**14**	2.24	4.01E-16	33.01	**47**
NM_001005736.1	LYST	lysosomal trafficking regulator	2.75	9.42E-19	40.71	**15**	2.85	2.35E-19	41.71	**17**
NM_000758.2	CSF2	colony stimulating factor 2	2.70	3.27E-14	28.28	**16**	5.53	2.05E-20	44.78	**1**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_033274.2	ADAM19	ADAM metallopeptidase domain 19	2.66	5.72E-16	33.36	**17**	2.94	3.98E-17	35.80	**15**
NM_153453.1	VGLL2	vestigial like 2 (Drosophila)	2.53	8.86E-16	32.82	**18**	2.16	1.10E-14	29.24	**55**
NM_000417.1	IL2RA	interleukin 2 receptor, alpha	2.49	1.20E-14	29.55	**19**	2.17	1.15E-13	26.37	**53**
NM_001078.2	VCAM1	vascular cell adhesion molecule 1	2.46	1.23E-15	32.27	**20**	2.25	4.35E-15	30.24	**42**
NM_172037.2	RDH10	retinol dehydrogenase 10 (all-trans)	2.46	3.59E-18	39.34	**21**	2.28	8.85E-18	37.74	**39**
NM_006748.1	SLA	Src-like-adaptor	2.45	9.83E-16	32.62	**22**	2.41	1.23E-15	31.69	**28**
NM_014383.1	ZBTB32	zinc finger and BTB domain containing 32	2.41	1.36E-13	26.58	**23**	2.77	6.80E-15	29.75	**19**
NM_001430.3	EPAS1	endothelial PAS domain protein 1	2.40	2.61E-17	36.90	**24**	2.15	1.22E-16	34.45	**56**
NM_024873.3	TNIP3	TNFAIP3 interacting protein 3	2.39	1.35E-17	37.68	**25**	2.69	6.80E-19	40.55	**20**
NM_024508.3	ZBED2	zinc finger, BED-type containing 2	2.37	3.87E-18	39.15	**26**	4.08	5.24E-23	51.08	**3**
NM_172348.1	IL4	interleukin 4	2.35	7.87E-11	19.18	**27**	2.50	1.64E-11	20.54	**25**
NM_022136.3	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	2.34	3.66E-14	28.13	**28**	1.94	1.04E-12	23.77	**71**
NM_015435.3	RNF19A	ring finger protein 19A	2.32	3.66E-14	28.12	**29**	2.35	2.33E-14	28.35	**29**
NM_005442.2	EOMES	eomesodermin homolog (Xenopus laevis)	2.32	7.72E-14	27.27	**30**	1.32	2.61E-09	14.6	0
NM_013308.2	GPR171	G protein-coupled receptor 171	2.25	3.98E-11	19.96	**31**	2.24	2.98E-11	19.83	**46**
NM_006399.2	BATF	basic leucine zipper transcription factor, ATF-like	2.24	5.72E-16	33.37	**32**	2.50	3.29E-17	36.05	**24**
NM_139239.1	NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta	2.21	4.16E-16	33.85	**33**	2.47	2.45E-17	36.48	**27**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_004155.3	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	2.21	4.21E-16	33.79	**34**	2.24	1.51E-16	34.22	**44**
NM_002983.1	CCL3	chemokine (C-C motif) ligand 3	2.18	4.83E-15	30.59	**35**	1.87	6.99E-14	27.01	**77**
NM_005658.3	TRAF1	TNF receptor-associated factor 1	2.17	1.35E-17	37.75	**36**	2.25	3.82E-18	38.65	**43**
NM_025151.3	RAB11FIP 1	RAB11 family interacting protein 1 (class I)	2.11	1.07E-15	32.47	**37**	2.04	3.39E-17	35.99	**62**
NM_005204.2	MAP3K8	mitogen-activated protein kinase kinase kinase 8	2.09	2.83E-14	28.52	**38**	2.32	2.32E-15	30.97	**34**
NM_181509.1	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha	2.06	7.45E-12	21.97	**39**	1.28	3.01E-08	11.68	0
NM_170695.2	TGIF1	TGFB-induced factor homeobox 1	2.01	4.01E-15	30.81	**40**	2.27	2.35E-16	33.59	**40**
NM_003575.2	ZNF282	zinc finger protein 282	2.00	2.83E-14	28.49	**41**	2.30	1.23E-15	31.74	**36**
NM_003975.2	SH2D2A	SH2 domain protein 2A	2.00	2.02E-14	28.98	**42**	2.04	9.34E-15	29.41	**63**
NM_138578.1	BCL2L1	BCL2-like 1	1.99	2.97E-16	34.27	**43**	2.29	9.92E-18	37.58	**38**
NM_145058.1	RILPL2	Rab interacting lysosomal protein-like 2	1.98	6.09E-14	27.56	**44**	1.75	4.92E-13	24.65	**94**
NM_002467.3	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.96	3.22E-15	31.09	**45**	2.49	2.06E-17	36.69	**26**
XM_927769.1	LOC653506	PREDICTED: similar to meteorin, glial cell differentiation regulator-like	1.94	9.68E-16	32.67	**46**	1.77	2.97E-15	30.64	**91**
NM_005263.2	GFI1	growth factor independent 1 transcription repressor	1.93	1.00E-14	29.78	**47**	1.60	2.43E-13	25.50	**121**
NM_002995.1	XCL1	chemokine (C motif) ligand 1	1.93	8.74E-14	27.10	**48**	3.05	8.85E-18	37.75	**13**
NM_018664.1	BATF3	basic leucine zipper transcription factor, ATF-like 3	1.92	2.09E-12	23.43	**49**	1.57	7.07E-11	18.83	**135**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_000418.2	IL4R	interleukin 4 receptor	1.92	1.08E-14	29.68	**50**	2.24	2.74E-16	33.40	**45**
NM_016391.3	HSPC111	hypothetical protein HSPC111	1.91	2.13E-14	28.89	**51**	2.20	8.07E-16	32.26	**50**
NM_004419.3	DUSP5	dual specificity phosphatase 5	1.87	2.61E-17	36.98	**52**	2.23	4.25E-19	41.07	**49**
NM_001001870.1	C17ORF91	chromosome 17 open reading frame 91	1.86	8.80E-13	24.44	**53**	1.58	1.48E-11	20.68	**128**
NM_005746.2	NAMPT	nicotinamide phosphoribosyltransferase	1.86	3.27E-12	22.92	**54**	2.26	5.09E-14	27.38	**41**
XM_001128421.1	LOC728069	PREDICTED: hypothetical LOC728069	1.85	3.41E-12	22.87	**55**	1.65	2.01E-11	20.32	**112**
NM_017915.2	C12ORF48	chromosome 12 open reading frame 48	1.85	9.48E-13	24.34	**56**	-0.76	01.48E-7	9.79	0
NM_002716.4	PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform	1.84	2.62E-15	31.34	**57**	2.18	5.98E-17	35.34	**51**
NM_013390.1	TMEM2	transmembrane protein 2	1.84	1.11E-13	26.81	**58**	1.70	3.59E-13	24.99	**104**
NM_002996.3	CX3CL1	chemokine (C-X3-C motif) ligand 1	1.82	8.10E-12	21.86	**59**	1.35	4.25E-11	15.25	0
NM_001001437.3	CCL3L3	chemokine (C-C motif) ligand 3-like 3	1.81	1.26E-15	32.23	**60**	1.63	6.35E-15	29.83	**117**
NM_005400.2	PRKCE	protein kinase C, epsilon	1.80	1.78E-15	31.81	**61**	1.59	1.49E-14	28.84	**126**
NM_015568.2	PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B	1.80	2.04E-11	20.75	**62**	1.75	2.47E-11	20.07	**92**
NM_003877.3	SOCS2	suppressor of cytokine signaling 2	1.80	8.03E-12	21.87	**63**	1.49	1.9E-10	17.68	0
NM_000594.2	TNF	tumor necrosis factor (TNF superfamily, member 2)	1.80	2.31E-09	15.16	**64**	2.51	3.30E-12	22.45	**23**
NM_138373.3	MYADM	myeloid-associated differentiation marker	1.79	7.72E-14	27.27	**65**	1.84	3.53E-14	27.83	**81**
NM_014210.2	EVI2A	ecotropic viral integration site 2A	1.77	1.10E-11	21.47	**66**	1.27	2.44E-11	20.09	0

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_170662.3	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	1.77	9.68E-16	32.69	**67**	1.67	1.71E-15	31.31	**109**
NM_001977.3	ENPEP	glutamyl aminopeptidase (aminopeptidase A)	1.77	3.86E-12	22.72	**68**	0.45	0.000828	-0.21	0
NM_001010927.2	TIAM2	T-cell lymphoma invasion and metastasis 2	1.77	2.53E-15	31.42	**69**	2.18	2.86E-17	36.29	**52**
NM_020651.2	PELI1	pellino homolog 1 (Drosophila)	1.76	6.17E-13	24.86	**70**	2.09	1.49E-14	28.85	**59**
NM_002908.2	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	1.75	7.35E-14	27.35	**71**	2.34	1.58E-16	34.15	**31**
NM_004430.2	EGR3	early growth response 3	1.74	2.85E-11	20.35	**72**	1.74	2.05E-11	20.29	**96**
NM_001066.2	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	1.74	1.60E-11	21.03	**73**	1.58	6.75E-11	18.90	**127**
NM_015675.2	GADD45B	growth arrest and DNA-damage-inducible, beta	1.73	1.11E-11	21.45	**74**	1.95	7.87E-13	24.09	**70**
NM_002714.2	PPP1R10	protein phosphatase 1, regulatory (inhibitor) subunit 10	1.72	1.14E-14	29.61	**75**	1.58	4.43E-14	27.56	**132**
NM_003998.2	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.72	1.44E-15	32.07	**76**	1.93	9.19E-17	34.81	**72**
NM_001098200.1	GPR18	G protein-coupled receptor 18	1.70	3.03E-14	28.37	**77**	1.89	2.63E-15	30.80	**75**
NM_000170.2	GLDC	glycine dehydrogenase (decarboxylating)	1.70	1.84E-16	34.79	**78**	0.4	1.73E-5	4.22	0
NM_003151.2	STAT4	signal transducer and activator of transcription 4	1.69	8.74E-14	27.10	**79**	1.69	6.18E-14	27.15	**106**
NM_032796.2	SYAP1	synapse associated protein 1, SAP47 homolog (Drosophila)	1.68	2.06E-11	20.73	**80**	1.88	1.81E-12	23.16	**76**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_014795.2	ZEB2	zinc finger E-box binding homeobox 2	1.68	4.82E-13	25.14	**81**	1.69	3.01E-13	25.22	**107**
NM_005041.4	PRF1	perforin 1 (pore forming protein)	1.68	1.13E-10	18.74	**82**	1.37	3.33E-09	14.32	0
NM_001097635.1	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	1.67	1.07E-10	18.80	**83**	2.15	6.09E-13	24.38	**57**
NM_025079.1	ZC3H12A	zinc finger CCCH-type containing 12A	1.67	2.53E-15	31.43	**84**	1.62	2.76E-15	30.73	**119**
NM_002756.3	MAP2K3	mitogen-activated protein kinase kinase 3	1.67	4.45E-15	30.69	**85**	1.92	1.62E-16	34.10	**73**
NM_007053.2	CD160	CD160 molecule	1.63	3.65E-14	28.15	**86**	1.83	2.35E-15	30.94	**82**
NM_001784.3	CD97	CD97 molecule	1.62	2.28E-12	23.32	**87**	1.86	1.08E-13	26.46	**79**
NM_020226.3	PRDM8	PR domain containing 8	1.61	6.31E-15	30.29	**88**	1.26	5E-13	24.62	0
NM_003810.2	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1.61	1.61E-10	18.34	**89**	2.30	1.13E-13	26.41	**37**
NM_005461.3	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	1.59	6.12E-09	14.00	**90**	1.35	7.02E-08	10.69	0
NM_012428.2	NPTN	neuroplastin	1.58	1.83E-11	20.87	**91**	1.13	1.87E-09	14.99	0
NM_015908.4	ARS2	arsenate resistance protein 2	1.57	4.37E-14	27.92	**92**	0.8	1.07E-08	12.93	0
NM_005066.1	SFPQ	splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)	1.57	6.18E-10	16.75	**93**	1.83	2.69E-11	19.97	**84**
NM_003037.1	SLAMF1	signaling lymphocytic activation molecule family member 1	1.56	1.55E-13	26.42	**94**	1.96	1.23E-15	31.70	**68**
NM_003797.2	EED	embryonic ectoderm development	1.56	1.98E-12	23.50	**95**	1.95	1.86E-14	28.59	**69**
NM_178496.2	C3ORF59	chromosome 3 open reading frame 59	1.55	4.54E-14	27.86	**96**	1.83	1.23E-15	31.69	**83**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_080588.1	PTPN7	protein tyrosine phosphatase, non-receptor type 7	1.55	1.38E-11	21.20	**97**	1.60	5.47E-12	21.85	**124**
NM_053064.3	GNG2	guanine nucleotide binding protein (G protein), gamma 2	1.54	1.78E-09	15.47	**98**	1.4	7.89E-09	13.31	0
NM_139062.1	CSNK1D	casein kinase 1, delta	1.54	2.73E-15	31.27	**99**	1.54	1.66E-15	31.36	**140**
NM_004120.3	GBP2	guanylate binding protein 2, interferon-inducible	1.53	1.55E-13	26.42	**100**	0.61	1.41E-6	7.13	0
NM_003807.2	TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	1.53	2.51E-09	15.06	**101**	1.75	1.62E-10	17.87	**93**
NM_003003.2	SEC14L1	SEC14-like 1 (S. cerevisiae)	1.53	4.51E-13	25.23	**102**	1.80	1.30E-14	29.02	**87**
NM_015169.3	RRS1	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)	1.52	2.86E-13	25.73	**103**	1.67	3.66E-14	27.78	**110**
NM_005911.4	MAT2A	methionine adenosyltransferase II, alpha	1.52	8.22E-13	24.53	**104**	1.4	3.39E-12	22.41	0
XM_939919.2	LOC650832	PREDICTED: similar to mitogen-activated protein kinase kinase 3 isoform A	1.51	1.12E-12	24.15	**105**	1.78	3.50E-14	27.84	**90**
NM_203290.1	POLR1C	polymerase (RNA) I polypeptide C, 30kDa	1.51	3.52E-11	20.12	**106**	1.75	5.15E-12	21.92	**95**
NM_002341.1	LTB	lymphotoxin beta (TNF superfamily, member 3)	1.51	8.86E-12	21.75	**107**	1.86	1.07E-13	26.50	**80**
NM_001004431.1	METRNL	meteorin, glial cell differentiation regulator-like	1.51	1.57E-13	26.38	**108**	1.79	1.32E-13	26.20	**89**
NM_004289.5	NFE2L3	nuclear factor (erythroid-derived 2)-like 3	1.50	1.30E-13	26.64	**109**	1.52	8.11E-14	26.84	**147**
NM_024098.1	CCDC86	coiled-coil domain containing 86	1.5	3.04E-12	23	**110**	1.64	3.63E-13	24.98	**115**
NM_003722.3	TP73L	tumor protein p73-like	-1.54	6.58E-12	22.12	**111**	-1.55	3.72E-12	22.30	**151**
NM_153047.1	FYN	FYN oncogene related to SRC, FGR, YES	1.50	3.79E-09	14.59	**112**	1.54	1.58E-09	15.18	**139**
NM_002632.4	PGF	placental growth factor	-1.56	8.14708E-13	24.56	**113**	-1.56	5.36E-13	24.53	**152**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_016270.2	KLF2	Kruppel-like factor 2 (lung)	-1.56	5.08E-11	19.68	**114**	-2.11	1.01E-13	26.57	**162**
NM_003032.2	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	-1.56	1.48E-12	23.81	**115**	-1.94	1.53E-14	28.81	**160**
NM_031479.3	INHBE	inhibin, beta E	-1.56	2.95E-14	28.43	**116**	-1.36	2.92E-13	25.27	0
NM_001018056.1	VLDLR	very low density lipoprotein receptor	-1.63	8.74E-14	27.10	**117**	-1.55	1.74E-13	25.90	**150**
NM_005627.2	SGK	serum/glucocorticoid regulated kinase	-1.73	1.73E-11	20.95	**118**	-1.44	3.88E-11	16.84	0
NM_018357.2	LARP6	La ribonucleoprotein domain family, member 6	-1.74	1.55E-13	26.41	**119**	-1.67	2.50E-13	25.46	**156**
NM_004567.2	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	-1.78	1.23E-12	24.03	**120**	-1.86	3.22E-13	25.12	**159**
NM_013332.3	HIG2	hypoxia-inducible protein 2	-2.28	1.76E-11	20.92	**121**	-2.03	1.13E-10	18.28	**161**
NM_004417.2	DUSP1	dual specificity phosphatase 1	-2.48	6.57E-16	33.19	**122**	-2.65	9.19E-17	34.78	**163**
NM_005252.2	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	-2.56	2.83E-14	28.49	**123**	-2.68	8.60E-15	29.50	**164**
NM_006472.2	TXNIP	thioredoxin interacting protein	-2.65	1.15E-11	21.39	**124**	-2.86	1.85E-12	23.14	**165**
NM_021822.1	APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	1.49	6.97E-12	21.8	0	1.83	5.80E-13	24.44	**85**
NM_019886.2	CHST7	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	1.49	3.63E-14	28.17	0	2.17	1.82E-17	36.85	**54**
NM_020230.4	PPAN	peter pan homolog (Drosophila)	1.48	2.27E-12	23.34	0	1.98	4.75E-15	30.14	**66**
NM_004499.3	HNRNPAB	heterogeneous nuclear ribonucleoprotein A/B	1.47	8.71E-13	24.46	0	1.72	3.06E-14	28.04	**100**
XM_035299.8	ZSWIM6	PREDICTED: zinc finger, SWIM-type containing 6	1.46	2.72E-13	25.79	0	1.57	5.11E-14	27.37	**136**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_152270.2	SLFN11	schlafen family member 11	1.45	6.79E-10	16.64	0	1.52	2.09E-10	17.56	**143**
NM_016052.3	RRP15	ribosomal RNA processing 15 homolog (S. cerevisiae)	1.45	1.26E-12	24.01	0	1.73	2.89E-14	28.10	**98**
NM_005966.3	NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)	1.44	1.17E-10	18.7	0	1.98	1.92E-13	25.80	**67**
NM_001033714.1	NOL1	nucleolar protein 1, 120kDa	1.41	1.06E-10	18.82	0	1.53	1.55E-11	20.62	**142**
NM_014655.1	SLC25A44	solute carrier family 25, member 44	1.41	4.54E-13	25.22	0	1.56	4.65E-14	27.49	**138**
NM_013322.2	SNX10	sorting nexin 10	1.4	4.58E-10	17.1	0	1.59	2.88E-11	19.89	**125**
NM_005018.1	PDCD1	programmed cell death 1	1.4	1.16E-12	21.41	0	1.87	3.09E-14	27.99	**78**
NM_021035.2	ZNFX1	zinc finger, NFX1-type containing 1	1.39	1.12E-13	26.8	0	1.72	1.23E-15	31.70	**102**
NM_001040280.1	CD83	CD83 molecule	1.39	1.47E-13	26.5	0	2.23	2.99E-17	36.21	**48**
NM_144599.3	NIPA1	non imprinted in Prader-Willi/Angelman syndrome 1	1.36	2.8E-12	20.28	0	1.73	1.96E-13	25.77	**97**
NM_004741.1	NOLC1	nucleolar and coiled-body phosphoprotein 1	1.34	2.24E-11	20.64	0	1.64	3.01E-13	25.22	**114**
NM_004973.2	JARID2	jumonji, AT rich interactive domain 2	1.3	8.29E-13	24.52	0	1.79	1.18E-15	31.82	**88**
XM_931068.1	C13ORF25	PREDICTED: chromosome 13 open reading frame 25	1.29	5.78E-13	24.93	0	1.70	1.78E-15	31.26	**105**
NM_005475.1	SH2B3	SH2B adaptor protein 3	1.27	9.06E-12	21.71	0	1.57	1.17E-13	26.35	**137**
NM_003234.1	TFRC	transferrin receptor (p90, CD71)	1.27	2.77E-14	28.56	0	1.60	1.90E-16	33.90	**123**
NM_015062.3	PPRC1	peroxisome proliferator-activated receptor gamma, coactivator-related 1	1.27	1.33E-12	23.93	0	1.60	1.12E-14	29.19	**122**
NM_033661.3	WDR4	WD repeat domain 4	1.26	2.29E-12	23.22	0	1.52	4.45E-14	27.54	**144**
NM_001002019.1	PUS1	pseudouridylate synthase 1	1.22	1.28E-11	21.3	0	1.69	1.66E-14	28.71	**108**
NM_005044.2	PRKX	protein kinase, X-linked	1.19	1.79E-7	9.95	0	1.58	1.01E-09	15.71	**129**
NM_182543.1	NSUN6	NOL1/NOP2/Sun domain family, member 6	1.18	4.19E-11	19.89	0	1.63	6.14E-14	27.16	**116**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_032830.1	CIRH1A	cirrhosis, autosomal recessive 1A	1.15	2.56E-12	20.49	0	1.57	4.07E-14	27.66	**134**
NM_012328.1	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.12	1.23E-10	18.64	0	1.52	2.36E-13	25.53	**145**
NM_172212.1	CSF1	colony stimulating factor 1 (macrophage)	1.1	2.22E-07	9.68	0	1.73	6.50E-11	18.94	**99**
NM_002188.2	IL13	interleukin 13	1.03	2.78E-08	12.25	0	2.92	5.17E-17	35.51	**16**
NM_000595.2	LTA	lymphotoxin alpha (TNF superfamily, member 1)	1.02	1.51E-06	10.16	0	2.33	3.08E-14	28.01	**33**
NM_006540.2	NCOA2	nuclear receptor coactivator 2	0.96	3.34E-08	12.03	0	1.58	2.80E-12	22.65	**130**
NM_002126.4	HLF	hepatic leukemia factor	0.9	1.91E-06	7.7	0	2.01	5.36E-13	24.52	**64**
NM_003740.3	KCNK5	potassium channel, subfamily K, member 5	0.84	1.02E-06	7.86	0	1.58	1.42E-11	20.73	**131**
NM_203349.2	SHC4	SHC (Src homology 2 domain containing) family, member 4	0.83	2.15E-09	15.24	0	2.98	2.16E-20	44.37	**14**
NM_002228.3	JUN	jun oncogene	0.8	8.95F-07	8.02	0	1.71	9.45E-13	23.89	**103**
NM_002245.2	KCNK1	potassium channel, subfamily K, member 1	0.74	5.88E-09	11.34	0	1.51	1.01E-13	26.58	**149**
NM_002999.2	SDC4	syndecan 4	0.7	3.87E-06	6.3	0	1.57	2.91E-12	22.61	**133**
NM_002928.2	RGS16	regulator of G-protein signalling 16	0.67	3.38E-06	3.79	0	2.66	8.44E-16	32.19	**21**
NM_003745.1	SOCS1	suppressor of cytokine signaling 1	0.65	1.32E-05	4.88	0	1.51	7.81E-12	21.41	**148**
NM_001559.2	IL12RB2	interleukin 12 receptor, beta 2	0.64	2.9E-08	12.2	0	1.67	2.35E-16	33.57	**111**
NM_004728.2	DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.60	9.85E-10	16.21	0	1.65	1.06E-11	21.05	**113**
NM_005665.4	EVI5	ecotropic viral integration site 5 (EVI5), mRNA.	0.6	0.002547	-1.21	0	2.09	2.45E-11	20.08	**60**
NM_032263.2	IQCG	IQ motif containing G	0.54	0.000029	3.95	0	2.80	3.82E-18	38.73	**18**

Gene ID	Gene Symbol	Gene Name	logFC A20 [HIV]v [T0]	adj.P. Val	B	RANK A20	logFC A3 [HIV][T0]	adj.P. Val	B	RANK A3
NM_032717.3	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	0.52	4.77E-06	6.06	0	1.91	2.30E-16	33.65	**74**
NM_015869.4	PPARG	peroxisome proliferator-activated receptor gamma	0.52	3.46E-05	3.76	0	2.34	6.19E-17	35.28	**30**
NM_004165.1	RRAD	Ras-related associated with diabetes	0.47	1.03E-05	5.16	0	1.62	2.59E-15	30.83	**120**
NM_002163.2	IRF8	interferon regulatory factor 8	0.47	0.000267	1.38	0	2.07	2.66E-15	30.78	**61**
NM_002981.1	CCL1	chemokine (C-C motif) ligand 1	0.41	0.000138	2.15	0	3.24	1.63E-20	45.32	**10**
NM_001384.4	DPH2	DPH2 homolog (S. cerevisiae) (DPH2), transcript variant 1	0.4	5.74F-05	3.17	0	1.72	8.78E-16	32.13	**101**
NM_000586.3	IL2	interleukin 2	0.37	0.003542	-1.59	0	1.82	7.15E-14	26.98	**86**
NM_001040167.1	LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.2	0.11	-5.46	0	1.52	2.54E-14	28.25	**146**
NM_002068.1	GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	0.2	0.2	-6.08	0	2.12	1.08E-13	26.47	**58**
NM_139235.3	NOL6	nucleolar protein family 6 (RNA-associated	0.14	0.18	-5.94	0	1.54	2.63E-14	28.21	**141**
NM_006705.2	GADD45G	growth arrest and DNA-damage-inducible, gamma	-0.09	0.297	-6.47	0	1.62	8.15E-13	24.05	**118**
NM_001029835.1	CCM2	cerebral cavernous malformation 2	-0.49	0.000782	0.14	0	-1.70	9.00E-11	18.55	**157**
NM_004089.3	TSC22D3	TSC22 domain family, member 3	-0.58	0.001652	-0.723	0	-1.66	4.84E-10	16.56	**155**
NM_015864.2	FAM65B	family with sequence similarity 65, member B	-1.19	1.63E-10	18.32	0	-1.61	3.09E-13	25.17	**154**
NM_052931.3	SLAMF6	SLAM family member 6	-1.22	5.76E-07	8.55	0	-1.56	8.27E-09	13.24	**153**
NM_138576.2	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)	-1.38	5.40E-06	5.92	0	-1.76	1.03E-07	10.22	**158**

Table 8-4: Summary of TCR stimulation on genes with altered expression between high and intermediate sensitivity HLA B*08-restricted FL8-specific T-cell clones following two hours activation with HIV-1 infected CD4+ T-cell line (C8166).

logFC A20vA3 [HIV]	Entrez ID	Symbol	logFC A20 [T2][T0]	adj.P. Val	B	ΔTCR	logFC A3 [T2][T0]	adj.P. Val	B	ΔTCR	Cell Surface
1.94	10663	**CXCR6**	1.42	5.73E-06	5.85	UP	0.5	0.06	-5.05	-	Y
1.72	939	**CD27**	0.3	0.07	-4.86	-	0.14	0.44	-7.04	-	Y
1.68	2731	**GLDC**	1.7	1.84E-16	34.78	UP	0.4	1.73E-05	4.22	UP	N
1.52	25975	**EGFL6**	0.45	0.0014	-0.57	-	0.09	0.57	-7.27	-	N
-4.93	10578	**GNLY**	0.5	0.00084	0.04	-	0.26	0.07	-5.18	-	N
-4.20	54674	**LRRN3**	-0.13	0.32	-6.54	-	1.42	2.61E-13	25.4	UP	Y
-3.23	1437	**CSF2**	2.7	3.28E-14	28.28	UP	5.53	2.05E-20	44.78	UP	N
-2.88	6346	**CCL1**	0.41	0.00014	2.15	-	3.24	1.64E-20	45.32	UP	N
-2.57	3804	**KIR2DL3**	0.26	0.26	-6.33	-	0.65	3.00E-03	-1.58	-	Y
-2.54	2769	**GNA15**	0.2	0.2	-6.08	-	2.12	1.08E-13	26.47	UP	N
-2.35	3805	**KIR2DL4**	0.24	0.31	-6.53	-	0.64	1.30E-03	-0.69	-	Y
-2.26	84223	**IQCG**	0.54	2.94E-05	3.95	UP	2.8	3.82E-18	38.72	UP	N
-2.11	399694	**SHC4**	0.82	2.15E-09	15.2	UP	2.97	2.17E-20	44.37	UP	N
-2.03	6349	**CCL3L1**	2.67	9.51E-15	29.87	UP	4.85	9.47E-17	34.73	UP	N
-2.01	5468	**PPARG**	0.52	3.46E-05	3.76	UP	2.34	6.19E-17	35.28	UP	N
-1.91	3596	**IL13**	2.91	5.18E-17	35.51	UP	1.03	2.78E-08	12.25	UP	N
-1.84	6004	**RGS16**	0.67	3.38E-05	3.79	UP	2.66	8.45E-16	32.19	UP	N
-1.78	79413	**ZBED2**	2.37	1.34E-21	39.15	UP	4.08	5.25E-23	51.07	UP	N
-1.71	84803	**AGPAT9**	0.52	4.77E-06	6.06	UP	1.9	2.30E-16	33.65	UP	N
-1.62	1839	**HBEGF**	-0.02	9.40E-01	-7.51	-	0.76	1.15E-04	2.04	UP	Y
-1.61	7124	**TNF**	1.8	2.31E-09	15.16	UP	2.51	3.30E-12	22.45	UP	Y
-1.60	410	**ARSA**	-0.19	1.40E-01	-5.67	-	-0.21	1.90E-02	-3.71	-	N
-1.48	3558	**IL2**	0.37	3.54E-03	-1.59	-	1.82	7.16E-14	26.98	UP	N

Table 8-5: Summary of TCR stimulation on genes with altered expression between high and intermediate sensitivity HLA B*08-restricted FL8-specific T-cell clones following six hours activation with 2uM epitope pulsed CD4+ T-cell line (C8166).

logFC [2uM]	Gene Symbol	logFCA20 [T2][T0]	adj.P.Val	B	ΔTCR	logFCA3 [T2][T0]	adj.P.Val	B	ΔTCR
2.83	**SPOCK1**	-0.039	0.76	-7.38	-	-0.03	0.8	-7.53	-
2.55	**TSC22D3**	-0.59	0.002	-0.72	-	-1.66	4.84E-10	16.56	DOWN
2.26	**CXCR6**	1.42	5.73E-06	5.85	UP	0.5	0.06	-5.05	-
2.18	**NELL2**	-0.7	0.69	-7.3	-	-0.13	0.4	-6.88	-
2.15	**CD27**	0.3	0.07	-4.86	-	0.14	0.44	-7.04	-
1.98	**C12orf48**	1.85	9.48E-13	24.34	UP	1.2	2.07E-09	14.9	UP
1.94	**SPP1**	-0.04	0.93	-7.51	-	-0.27	0.43	-7.01	-
1.81	**FGFBP2**	-0.12	0.53	-7.07	-	-0.26	0.1	-5.54	-
1.77	**TP53INP1**	0.65	0.000644	0.36	-	0.47	0.048	-4.74	-
1.67	**PMCH**	1.48	1.46E-12	23.83	UP	0.71	4.13E-07	8.58	-
1.65	**VCAM1**	2.46	1.24E-15	32.27	UP	2.25	4.36E-15	30.24	UP
1.57	**MAP1LC3A**	2.06	7.45E-12	21.97	UP	1.28	3.02E-08	11.68	UP
1.57	**TNFSF9**	1.32	1.55E-08	12.92	UP	1.05	4.47E-07	8.48	UP
1.57	**PRKCE**	1.8	1.78E-15	31.81	UP	1.59	6.10E-17	28.84	UP
1.55	**ACTA2**	-0.61	6.14E-07	8.47	-	-0.79	8.04E-09	13.28	-
1.53	**KLRG1**	-0.17	2.20E-01	-6.14	-	-0.61	8.90E-03	-2.88	-
1.52	**GLDC**	1.7	1.84E-16	34.78	UP	0.4	1.73E-05	4.22	UP
1.52	**FHL2**	-1.21	8.74E-14	27.12	DOWN	-0.61	2.15E-08	12.1	-
1.51	**KLF2**	-1.56	5.09E-11	19.68	DOWN	-2.11	1.02E-13	26.57	DOWN
1.51	**FCRL3**	0.12	2.20E-01	-6.15	-	-0.09	3.80E-01	-6.88	-
-4.06	**LRRN3**	-0.13	0.32	-6.54	-	1.42	2.61E-13	25.4	UP
-3.18	**TNF**	1.8	2.31E-09	15.16	UP	2.51	3.30E-12	22.45	UP
-2.31	**GPR56**	-0.95	1.56E-09	15.65	DOWN	-0.47	4.88E-05	3.02	-
-2.04	**RGS16**	0.67	3.38E-05	3.79	UP	2.66	8.45E-16	32.19	UP
-1.89	**PKIA**	-0.74	2.96E-06	6.62	-	0.63	8.35E-06	5.06	-
-1.87	**FSCN1**	-0.53	1.50E-03	-0.6	-	-0.56	6.70E-04	0.02	-
-1.77	**CLECL1**	-0.54	1.78E-06	7.22	-	-0.35	4.30E-04	0.53	-
-1.74	**LTA**	1.02	1.51E-07	10.16	UP	2.32	3.09E-14	28.01	UP
-1.74	**GNLY**	0.5	0.000847	0.04	-	0.26	0.07	-5.18	-
-1.68	**TGFBR3**	0.43	8.00E-04	0.086	-	-0.11	4.30E-01	-7.01	-
-1.57	**GNA15**	0.2	0.2	-6.08	-	2.12	1.08E-13	26.47	UP
-1.57	**MGC33556**	-0.08	6.50E-01	-7.25	-	0.11	5.10E-01	-7.17	-