NK cell and T-cell immunity and TLR responsiveness during chronic HIV-1 infection

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Declaration of Originality

I declare that all work presented and contained within this thesis was completed by me (Adam Robert Coleman) and that all else is appropriately referenced.

Abstract

This project investigates the potential of Natural Killer (NK) cells to respond to pathogen associated molecular patterns (PAMP) as model adjuvants and establishes the potential of PAMP to aid the recovery of antigen specific T-cell responses in chronic HIV-1 infection.

Innate immunity is mediated by through the recognition of conserved PAMP recognised by receptors such as the toll-like receptors (TLR), expressed by accessory cells including blood DC, which are responsible for initiating adaptive immune responses, but also activate NK cells. NK cells are no longer seen solely as killers, but instead produce cytokines, including IFN-γ, and participate in the generation of adaptive immune responses with DC.

HIV-1 infection potentially chronically activates TLR pathways, via recognition of the viral genome and as a result of damage to mucosal surfaces leading to microbial translocation and systemic recognition of PAMP including bacterial lipopolysaccharides. NK cell activation, cytokine production, differentiation and proliferation in response to PAMP have therefore been examined. NK cell responsiveness to certain TLR agonists was refractory in HIV-1 infected individuals although responses were maintained within $CD56^{+}CD16^{-}$ NK cells, potentially providing help for antigen specific T-cell responses. The effects of HIV-1 infection on NK cell maturation were also investigated.

T-cell activation by TLR agonists was also refractory in HIV-1 infected individuals. The impact of TLR ligation on antigen specific T-cell responses was investigated, combining TLR agonists with peptide pools (influenza, Epstein-Barr virus and cytomegalovirus or HIV-1 gag antigens). Of the TLR agonists tested, only CpG DNA resulted in enhanced frequencies of IFN-γ producing T-cells in HIV-1 infected individuals, whereas LPS demonstrated consistently reduced IFN-γ production.

In conclusion, maintenance of responsiveness of $CD56^{+}CD16^{-}$ NK cells and enhancement of HIV-1 antigen specific T-cell responses by CpG DNA have implications for both repopulation of the NK cell compartment and reconstitution of acquired immunity during HIV-1 infection.

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List of abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell or Allophycocyanin
APOBEC	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like
ART	Anti-retroviral therapy
BHIVA	British HIV-association
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CLIP	Class II-associated invariant chain peptide
CMV	Cytomegalovirus
CpG ODN	cytidine-phosphate-guanosine oligodeoxynucleotide
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T-Lymphocyte Antigen
CXCR	CXC-chemokine receptor
DC	Dendritic cell
mDC	Myeloid DC
pDC	Plasmacytoid DC
iDC	immature DC
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot

ER	Endoplasmic reticulum
ESN	Exposed, seronegative
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable (constant region)
FCS	Foetal Calf Serum
FEC	Flu, EBV, CMV
FITC	Fluorescein isothiocyanate
FSL-1	Synthetic diacylated lipoprotein
GALT	Gut associated lymphoid tissue
GI	Gastro-intestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HAART	Highly active anti-retroviral therapy
HC	Healthy control
HIV	Human immunodeficiency virus
HKLM	Heat-killed *Listeria monocytogenes*
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
ICCS	Intracellular cytokine staining
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IMQ	Imidazoquinoline

IRF	IFN Regulatory Factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer immunoglobulin-like receptor
LBP	LPS-binding protein
LFA	Lymphocyte function associated antigen
LIR	Leukocyte Ig-like inhibitory receptor
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTNP	Long-term non-progressor
MACS	Magnetic-activated cell sorting
MALT	Mucosa-associated lymphoid tissue
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	MHC class I-like molecule
MIP	Macrophage inflammatory protein
MyD88	Myeloid differentiation factor 88
NCR	Natural cytotoxicity receptor
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NIBSC	National Institute for Biological Standards and Control
NK	Natural Killer
NNRTI	Non- nucleoside reverse-transcriptase inhibitor
NRTI	Nucleoside reverse-transcriptase inhibitor

PAMP	Pathogen-associated molecular pattern
Pam3CSK4	Synthetic triacylated lipoprotein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Programmed-death
PerCP	Peridinin chlorphyll protein
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PI	Protease inhibitor
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RNA	Ribonucleic acid
ssRNA	single-stranded RNA
dsRNA	double-stranded RNA
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SCF	Stem cell factor
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
SLT	Secondary lymphoid tissue
TAP	Transporter associated with antigen processing
TCM	Central memory T-cell, or tissue culture medium
TCR	T-cell receptor
TD	Treatment-experienced
TEM	Effector memory T-cell

TEMRA	Terminally differentiated effector memory T-cell
TGF	Tumour growth factor
Th	T-helper
TIR	Toll/IL-1R homology
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TN	Treatment-naïve
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
ULBP	UL16-binding protein

List of publications and presentations

Publications

- Goodier MR, Coleman AR. (2009) Innate natural killer cell phenotype and function during HIV-1 infection: Potential avenues for modulation. *HIV Therapy,* 3(2), 161-170
- Wahren, B., Biswas, P., Borggren, M., Coleman, A., Da Costa, K., De Haes, W., Dieltjens, T., et al. (2010) Correction: Rational design of HIV vaccine and microbicides: report of the EUROPRISE annual conference. *Journal of Translational Medicine,* 8 (1), 82.
- Al-Hubeshy, Z. B., Coleman, A., Nelson, M. & Goodier, M. R. (2011) A rapid method for assessment of natural killer cell function after multiple receptor crosslinking. *Journal of Immunological Methods.* 366 (1-2), 52-59.

Submitted manuscripts and manuscripts under preparation

- Coleman AR, McLaughlin L, Nelson, M & Goodier MR. (submitted 20/12/2011) Preservation of CD56hiCD16- NK cell responsiveness to TLR ligands during HIV-1 infection. Submitted: *Journal Infectious Diseases.*
- Norris S, Coleman A, Bower M, Nelson M & Goodier MR. (under preparation) Low frequency of PD-1 expression on Natural Killer cells during HIV-1 infection. Being prepared for *Viral Immunology*
- Coleman AR, McLaughlin L, Nelson, M & Goodier MR. (under preparation) Loss of c-kit on peripheral NK cells during chronic HIV-1 infection is not reversed by HAART.

Professional presentations

- Innate Immune Responses to TLR Agonist Stimulation (poster). Presented at the Europrise Annual Conference, Malta, 2008. Authors: A. Coleman, F. Gotch, M. Bower, B. Wahren and M.R. Goodier
- Natural Killer Cell and $CD8^+$ T Cell Responses to TLR Agonists in $HIV\text{-}1^+$ Individuals Receiving HAART (poster) Presented at the Conference on Retroviruses and Opportunistic Infections, Montreal, Canada, 2009 Authors: A.Coleman, M. Bower, M. Nelson, B. Wahren, B. Gazzard, F. Gotch and M.R. Goodier
- Innate Immune Responses to TLR Agonists. (poster). Presented at the Europrise Annual Conference, Budapest, Hungary, 2009. Authors: A. Coleman, M. Bower, M. Nelson, B. Wahren, B. Gazzard, F. Gotch and M. Goodier
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- Elevated PD-1 expression on NK cells during HIV-1 infection. (poster). Presented at the 17th annual conference of the British HIV Association (BHIVA), Bournemouth, UK, 2011. Authors: Norris S, Coleman AR, Gazzard B, Nelson M & Goodier MR

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- Gamma/delta T cell responsiveness to zoledronic acid and adjuvant potential in HIV-1 infection. (poster). Presented at the 17th annual conference of the British HIV Association (BHIVA), Bournemouth, UK, 2011. Authors: Begom S, Coleman AR, Gazzard B, Nelson M, Imami N & Goodier MR

1. Introduction

1.1. Natural killer cells

Natural killer (NK) cells are large granular lymphocytes that were first described by Kiessling *et al.* (1975b) for their ability to spontaneously kill target cells without prior exposure. It was later discovered that NK cells were killing targets that failed to express class I MHC molecules and so the 'missing self' hypothesis was proposed by Kärre and coworkers (1986 & 1990), which described how NK cells could kill transformed tumour cells or virus-transformed cells without previous encounter. The 'missing self' hypothesis was further clarified by the discovery of the predicted inhibitory receptors in the mouse (Karlhofer, Ribaudo & Yokoyama, 1992) and in humans (Ciccone et al., 1992) that could recognise self MHC-I molecules and inhibit a cytotoxic response. NK cells are now known to function according to complex inhibitory and activatory interactions with a number of different ligands and to have an expanded range of functions impacting on many aspects of both the innate and acquired immune response (Lotze & Thompson, 2010). The spatial distribution of NK cells throughout the body gives an indication to their function and is discussed in detail below.

1.1.1. NK cell phenotype and distribution

NK cells in humans are usually phenotypically described as $CD3^{-}CD56^{+}$, and represent 5-15% of all lymphocytes (Timonen, Ortaldo & Herberman, 1981). Walzer *et al.* (2007) have suggested that, because CD56 is expressed on some T-cells and is not found in other mammals, a different marker should be used to identify true NK cells. NKp46 is proposed as an alternative marker for NK cells as it has been found to be present on peripheral blood NK cells in all mammals (Walzer et al., 2007). NKp46 is a natural cytotoxicity receptor (NCR) and is largely restricted to NK cells, but has been found at low concentrations on some human T-cells and so is not entirely NK cell specific (Caligiuri, 2008). Beyond this basic phenotype, NK cells express a variety of other receptors useful for their identification, the distribution of which are important in describing their functional capacity and maturational states.

NK cells are broadly split into two major subsets based on their expression of CD56 and the FcγRIIIa receptor CD16, which are $CD3^{-}CD56^{hi}CD16^{-}$ and $CD3^{-}CD56^{lo}CD16^{+}$. For consistency the $CD56^{lo}$ nomenclature is used throughout this thesis although other authors have used $CD56^{dim}$ or $CD56^{low}$, additionally $CD56^{hi}$ is used throughout this thesis, but others have used $CD56^{bright}$ or $CD56^{high}$ to describe the same populations. The $CD56^{lo}CD16^{+}$ NK cells classically contain high levels of perforin and granzymes and are the subset primarily responsible for killing infected and/or transformed target cells (Caligiuri, 2008, Walzer et al., 2007). These cells also predominate in the peripheral blood where they make up 85-95% of the total NK cell population. In contrast the $CD56^{hi}CD16^{-}$ subset only represent 5-15% of NK cells in peripheral blood, but represent the majority of NK cells in the secondary lyphoid tissue (SLT) (Ferlazzo et al., 2004). $CD56^{hi}CD16^{-}$ NK cells predominantly do not express granzyme or perforin and are therefore not naturally cytotoxic, instead these are cells that specialise in cytokine production, particularly IFN-γ, and have been described as 'helper' NK cells as they have been implicated as important for modulating and shaping adaptive immune responses toward a Th1-type response (Poli et al., 2009, Hanna & Mandelboim, 2007, Mailliard et al., 2005, Martin-Fontecha et al., 2004, Mailliard et al., 2003, Cooper et al., 2001, Jacobs et al., 2001).

1.1.2. NK cell receptors

Activation of NK cells is reliant on complex interactions between various families of receptors. Initially the 'missing self' hypothesis (Karre et al., 1986) postulated the need for only inhibitory receptors, imagining the default NK cell setting to be set to kill. It is now known, however, that both inhibitory and activating receptors are necessary for NK cell killing and/or cytokine production to take place. Studies have shown that NK cells do not spontaneously kill cells expressing mismatched MHC-I, such as in bone marrow transplantation, and that killing requires positive activating signals (Bryceson et al., 2006a, Ruggeri et al., 2002). It is also of note that inhibitory signals dominate over activating signals to prevent cytotoxicity even in the presence of ligands for activating NK cell receptors, and this is has been shown to be exploited by tumours and viruses such as human cytomegalovirus (HCMV) which upregulates non-classical HLA-I molecules (Caligiuri, 2008, Bryceson et al., 2006a, Tomasec et al., 2000, Ulbrecht et al., 2000). NK cell receptors associated with activation and inhibition are presented in Table 1.1.

Activation of NK cell effector function therefore relies on a balance of inhibitory versus activating signals, and the major receptor families are discussed below.

Table 1.1 Natural Killer Cell Inhibitory and Activating Receptors

Receptor	Inhibitory	Activating	Ligand
KIR	KIR2DL1	KIR2DS1	HLA-C2
	KIR2DL2	KIR2DS2	HLA-C1
	KIR2DL3		HLA-C1
		KIR2DS4	HLA-Cw4
	KIR3DL1	KIR3DS1	HLA-Bw4
	KIR3DL2		HLA-A3/-A11
Heterodimeric C-type Lectin Receptors	CD94/NKG2A/B	CD94/NKG2C	HLA-E
		NKG2D	MIC-A/-B, ULBP-1/-2/-3
NCR		NKp30	HCMV tegument protein pp65, HLA-B-associated transcript 3
		NKp46	Influenza Haemaglutinin
		NKp44	Influenza Haemaglutinin
Ig-like transcript	LIR-1 (ILT-2, CD85j)		HLA-class I
Activating Co-Receptors		FCγRIII (CD16)	IgG - Fc portion
		CD2	CD58 (LFA-3)
		LFA-1	ICAM-1
		2B4	CD48
		NKp80	Activation-induced C-type lectin
		CD40L	CD40

NB. Activating KIR have the same extracellular domain as Inhibitory KIR, but it has not necessarily been demonstrated they have the same Ligand

Inhibitory receptors

As mentioned above the major ligands for NK cell inhibitory receptors are MHC class I molecules. These receptors fall into two classes, firstly the immunoglobulin superfamily receptors, which include the killer immunoglobulin (Ig)-like receptors (KIR) and the leukocyte Ig-like inhibitory receptor 1 (LIR1, also known as ILT2 and CD85j). The second family consists of the C-type lectin receptors and is represented by the heterodimer formed between CD94 and NKG2A (Stern-Ginossar & Mandelboim, 2010).

KIR proteins are expressed on NK cells (Colonna & Samaridis, 1995, Moretta et al., 1995), $CD8^+$ and $\gamma\delta^+$ T-cells (Ferrini et al., 1994) and can be either inhibitory or activating

depending on the cytoplasmic tail of individual molecules; inhibitory KIR have an immunoreceptor tyrosine based inhibitory motif (ITIM) tail, as do the majority of inhibitory receptors expressed by NK cells (Stern-Ginossar & Mandelboim, 2010). KIR are a major receptor family responsible for recognition of the classical MHC-I molecules HLA-A, -B and -C. They are highly polymorphic and have been shown to bind with different allelic specificity (Stern-Ginossar & Mandelboim, 2010, Bryceson et al., 2006a, Lanier, 2005, Mandelboim et al., 1996).

LIR1 is expressed on NK cells, T-cells, B-cells, monocytes, macrophages and dendritic cells (Colonna et al., 1997) and, similar to KIR, acts as an inhibitory receptor on NK cells signalling through an ITIM cytoplasmic tail. Unlike KIR, LIR1 molecules can bind to various class-I MHC molecules as they bind the conserved α3 region, albeit with low affinity (Colonna, Nakajima & Cella, 1999). Many viruses, such as HCMV, downregulate MHC-I to avoid detection by T-cells, but in doing so become targets for destruction by NK cell mediated lysis. HCMV encodes for a protein, UL18, which binds LIR1 thus transducing the inhibitory signal (Cosman et al., 1997), demonstrating the importance of LIR1 and other inhibitory receptors for the control of viruses.

The C-type lectin heterodimer CD94/NKG2A is the third major inhibitory receptor expressed on NK cells and, unlike KIR and LIR1, recognises the non-classical HLA-E molecule (Carretero et al., 1998). HLA-E expresses leader peptides from other MHC molecules and, as such, CD94/NKG2A is thought to be responsible for the surveillance of general downregulation of MHC-I that might occur in viral infection or tumour-transformed tissues (Braud et al., 1998, Lee et al., 1998). HLA-E is another protein whose expression is modified by HCMV, which encodes for the protein UL40 which upregulates the expression of HLA-E on infected cells (Tomasec et al., 2000).

Activating receptors

As discussed above NK cell effector functions are generally induced when there is a lack of inhibition and activating signals present, and are not normally induced purely through the lack of inhibition (Caligiuri, 2008, Lanier, 2003). Activating receptors have cytoplasmic tails that associate with a variety of adapter proteins, which contain an immunodominant tyrosine-based activation motif (ITAM). These proteins signal to downstream effectors, leading to various mechanisms of NK cell mediated lysis and or cytokine production

(Lanier, 2003). The major activating receptors including the NKG2D receptor, the natural cytotoxicity receptors (NCR), CD16 and the activating KIR are discussed below.

The activating NKG2D receptor is expressed by virtually all NK cells, $CD8^+$ T-cells and $\gamma\delta^+$ T-cells and, when stimulated, have been shown to induce strong cytolytic responses against target cells (Bauer et al., 1999, Wu et al., 1999). Unlike other NKG2 molecules, NKG2D forms a homodimer and does not associate with CD94 or propagate inhibitory signals (Wu et al., 1999). The first ligand described for NKG2D was the stress-induced molecule MHC-class-I-polypeptide-related sequence A (MIC-A), which has MHC homology (Bauer et al., 1999). Subsequently MIC-B and the CMV UL16-binding proteins 1-5 (ULBP1-5) were also described as ligands (2007). NKG2D ligands have been shown to be upregulated in response to stress induced by viral infection, DNA damage and as a result of toll like receptor (TLR) agonist engagement (Stern-Ginossar & Mandelboim, 2010, Eagle & Trowsdale, 2007, Nedvetzki et al., 2007). The CMV ULBP proteins hint at a close relationship with the virus, which encodes the protein UL16 capable of binding, and therefore sequestering, MIC-B and ULBP-1 and -2 (Dunn et al., 2003). NKG2D function is also disrupted by human immunodeficiency virus 1 (HIV-1) infection, as discussed later (Section 1.6.4).

The NCR family of receptors consist of three different proteins; NKp46, NKp44 and NKp30, which all belong to the Ig superfamily and are capable of inducing natural cytotoxicity (Pegram et al., 2011). NKp46 is ubiquitously expressed on NK cells almost exclusively, although some reports suggest a low frequency of NKp46 on $CD3^+$ T-cells (Caligiuri, 2008). NKp46 was identified through cross-linking experiments as an activation marker that elicited both cytokine and cytotoxic responses in NK cells (Sivori et al., 1997).

NKp44 is expressed on activated NK cells and is not known to be expressed by other cell types, and has therefore been implicated as an exclusive NK cell activation marker (Vitale et al., 1998). NKp30 is expressed on most NK cells in peripheral blood and tissues (Pende et al., 1999) with the exception of expression in the lymph nodes (LN) (Ferlazzo et al., 2004) or endometrium (Manaster et al., 2008). It is also NKp30 that is proposed as the receptor responsible for the deletion of immature DC (Ferlazzo et al., 2002), which is discussed later (Section 1.3).

CD16, the FcγRIIIa, is the receptor responsible for mediating antibody dependant cellular cytotoxicity (ADCC) (Lanier, Yu & Phillips, 1991, Vivier et al., 1991). ADCC is mediated by

the binding to the constant region (Fc) on IgG molecules and leads to potent cytotoxic responses (Caligiuri, 2008, Bryceson et al., 2006a).

Activating KIR possess different cytoplasmic tails to inhibitory KIR, which interact with ITAM containing the adapter molecule DAP12 (Biassoni et al., 1996, Moretta et al., 1995). Although little structural difference is observed in the extracellular portion of the activating KIR compared to inhibitory molecules, it was found that activating KIR bind their MHC-I ligands at a lower affinity than their inhibitory counterparts. Alternatively, putative non-MHC ligands expressed by tumours have been postulated as a method of activation through the activating KIR2DS4 (Katz et al., 2004).

1.1.3. NK cell licensing

As discussed above NK cell activation occurs primarily in the absence of inhibitory signals through MHC class I receptors, indicating that inhibitory signalling is dominant. A hyporesponsive NK cell state has been described where NK cells are unable to kill MHC class-I-devoid target cells in both mouse models and in individuals with bare lymphocyte syndrome (Furukawa et al., 1999, Bix et al., 1991, Hoglund et al., 1991).

The concept of NK cell licensing or education was therefore devised, whereby NK cells are first made tolerant to self MHC class I molecules in order to become functionally competent (Elliott & Yokoyama, 2011). Kim *et al*. (2005) demonstrated that NK cells from mice lacking MHC class I expression did not produce IFN-γ in response to activating receptor cross-linking, whereas they did in cells from mice that expressed MHC class I. A similar reliance on MHC class I expression for the generation of functionally competent NK cells has since been described in human NK cells and the requirement for KIR in the development of mature functional NK cells was also demonstrated (Kim et al., 2008, Anfossi et al., 2006).

NK cell licensing normally occurs under non-inflammatory conditions, however recovery of mature hyporesponsive NK cells has been demonstrated in mice and in humans in response to cytokine stimulation although responses are general and not directed to specific target cells (Juelke et al., 2009, Kim et al., 2002). Finally it is noteworthy that a similar hyporesponsive or anergic NK cell phenotype is observed in murine studies where NKG2D is artificially chronically stimulated (Oppenheim et al., 2005, Wiemann et al., 2005).

1.1.4. NK cell development and maturation

NK cells originate from bone marrow haematopoietic stem cells (HSC), developing first into the common lymphoid progenitor, which gives rise not only to NK cells, but also the T-cell and B-cell lineages (Kondo, Weissman & Akashi, 1997). NK cells are traditionally considered cells of the innate immune system and posses many qualities and functions to place them in this category, although developmentally NK cells undergo processes more akin to those seen for adaptive T-cells and B-cells. NK cell development is generally discussed according to sequential changes in the phenotypic makeup of cells and, as such, mouse and human NK cells must be described separately as they have different phenotypic characteristics, although parallels can be made.

Kim *et al*. (2002) demonstrated five distinct populations of murine NK cells representing different developmental stages. First, NK cell precursors that express the IL-2/IL-15Rβ chain (CD122) and lack the expression of the murine NK markers Ly49 or NK1.1 become $CD122^{+}NK1.1^{+}CD94/NKG2A/C/E^{+}$. These express high levels of α_v integrin, low levels of DX5 (CD49b) and Mac-1 (CD103) integrins and low CD43 expression. The next stage of development sees the expression of the murine NK marker Ly49 and c-Kit (CD117) before the expansion stage where α_v integrin is downregulated, DX5 upregulated and cells undergo rapid proliferation and expansion before becoming mature NK cells. Murine NK cell maturity is associated with a gradual decline in c-Kit expression, the loss of α_v integrin and the upregulation of Mac-1 and CD43. CD27 expression has also been shown as an important marker on mature murine NK cells, further sub-dividing them into $CD27^{hi}$ or $CD27^{lo}$ populations (Hayakawa & Smyth, 2006). $CD27^{lo}$ NK cells had a higher stimulation threshold and were controlled by inhibitory receptors whereas $CD27^{hi}$ NK cells showed greater responsiveness to chemokines (Hayakawa & Smyth, 2006). Interestingly CD27 is also found on human NK cells and its expression parallels that of CD56, as such defining functional NK cell subsets (Silva et al., 2008).

Human NK cell development can be divided into similar stages to that of murine NK cells following a linear model of development (Freud & Caligiuri 2006), although it is worth noting that alternative non-linear models have been proposed (Mailliard et al. 2005) (Figure 1.1). Human NK cells develop from $CD34^{+}$ progenitor cells present in the bone marrow (Freud & Caligiuri, 2006, Freud et al., 2005). IL-15 is present in the bone marrow stroma and has been shown to stimulate the differentiation of $CD34^{+}$ cells toward the $CD3^{-}$

$CD56^+$ NK cell lineage (Mrozek, Anderson & Caligiuri, 1996). $CD34^{dim}$ cells expressing CD45RA and the integrin α4β7 have been isolated from SLT and have been suggested to originate in the bone marrow and migrate to LN where they give rise to $CD56^{hi}$ NK cells (Freud et al., 2005). As a result, the LN have been proposed as the major site of maturation for NK cells, although specific populations of NK cells possibly mature at different sites such as the intestinal epithelia (Lynch et al., 2006), thymus (Vosshenrich et al., 2006) and uterine decidua (Ashkar et al., 2003).

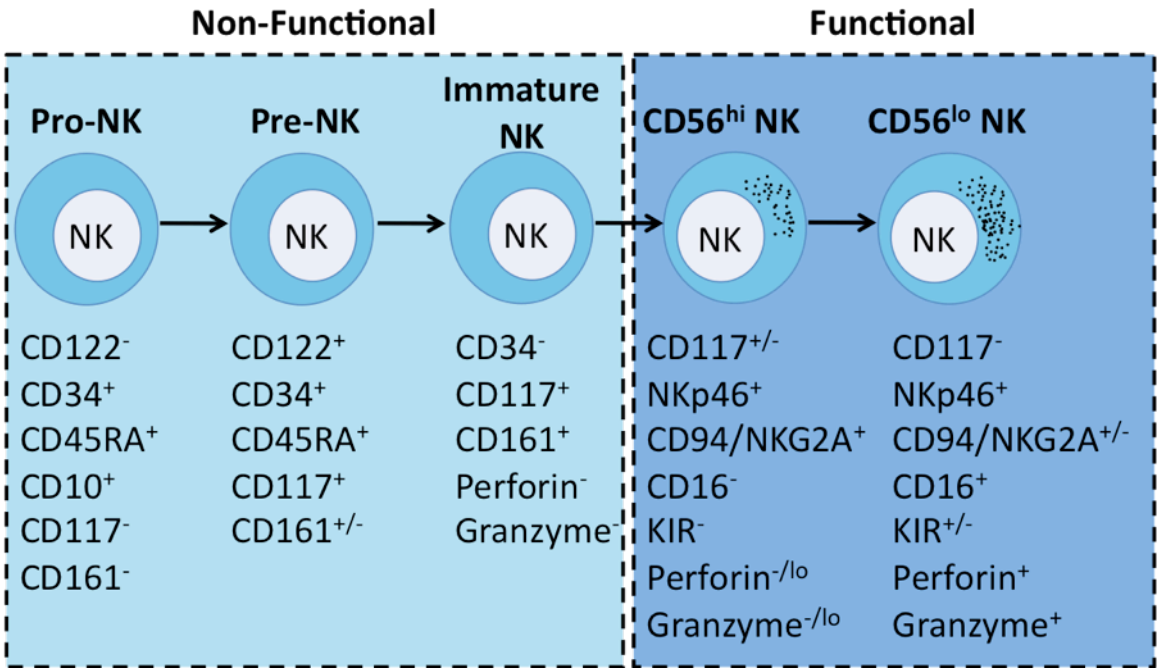


Figure 1.1 Linear model of human NK cell development. *In vivo* model of NK cell development illustrating the different stages proposed by Freud & Caligiuri (2006). Adapted from Freud & Caligiuri 2006, and Caligiuri 2008.

$CD122^-CD34^+CD45RA^+$ pro-NK cells located within SLT are thought to be committed to the NK lineage and progress to pre-NK cells defined as $CD122^+CD34^+CD45RA^+CD117^+CD161^{+/-}$ (Freud & Caligiuri, 2006). Pre-NK cells then give rise to the immature NK cell population defined as $CD34^-CD117^+CD161^+$, which in turn develops into $CD56^{hi}CD117^{+/-}CD94^+$ NK cells, expressing functional markers such as NKp30, NKp46, CD94/NKG2A and NKG2D, but lacking CD16 and KIR expression and therefore capable of a restricted set of NK cell functions (Grzywacz et al., 2006, Ferlazzo et al., 2004). $CD56^{hi}$ NK cells can transition to $CD56^{lo}CD117^-CD94^{+/-}CD16^+$ NK cells, and

also gain KIR expression and increased cytotoxic potential through perforin and grazyme production (Freud & Caligiuri, 2006, Ferlazzo et al., 2004, Jacobs et al., 2001).

As mentioned previously, alternative pathways for human NK cell development have been proposed, specifically suggesting non-linear progression between $CD56^{hi}$ and $CD56^{lo}$ subsets (Mailliard et al., 2005, Loza & Perussia, 2004). The linear model, however, although incomplete, arguably represents the most likely route of NK cell maturation considering that: 1) $CD34^{+}$ cells stimulated *in vitro* first give rise to $CD56^{hi}$ NK cells (Freud et al., 2005); 2) phenotypic progression from $CD56^{hi}$ to $CD56^{lo}$ NK cells with their corresponding phenotypes has been directly observed (Chan et al., 2007, Ferlazzo et al., 2004); and 3) $CD56^{hi}$ NK cells have been shown to have longer telomeres than $CD56^{lo}$ NK cells (Ouyang et al., 2007, Romagnani et al., 2007). Additionally, recent reports have demonstrated functional intermediates between $CD56^{hi}$ and $CD56^{lo}$ NK cells based on both expression of CD16 on $CD56^{hi}$ NK cells and the declining expression of CD94 by $CD56^{lo}$ NK cells (Beziat et al., 2011, Yu et al., 2010).

1.1.5. c-Kit and SCF biology with respect to NK cells

The stem cell factor receptor c-Kit or CD117 is a tyrosine kinase receptor that is predominantly expressed on progenitor haematopoietic cells in bone marrow, where the majority of $CD34^{+}$ cells express c-Kit (Ashman, 1999). Whereas many different cell lineages have been demonstrated to express c-Kit, $CD56^{hi}$ NK cells are the only mature lymphocyte to express this receptor (Ashman, 1999, Matos et al., 1993). SCF has been described for its ability to act on haematopoietic cells to promote cell survival, and to stimulate proliferation, differentiation, adhesion and activation. It is found in two forms, soluble and membrane associated (Ashman, 1999, Broudy, 1997). The experiments by Matos *et al*. (1993) demonstrated that co-stimulation of NK cells with SCF enhanced IL-2 mediated NK cell proliferation such that very low (pM) concentrations of IL-2 were able to induce cellular proliferation. Additionally Carson *et al.* (1994) demonstrated *in vitro* that ligation of c-Kit with SCF on $c\text{-}Kit^{+}$ NK cells induced the upregulation of the anti-apoptotic Bcl-2 protein thereby promoting the survival of these cells. The authors claim there *in vitro* findings could translate to an *in vivo* scenario as soluble SCF is detected in human serum (Carson et al., 1994, Langley et al., 1993). The fact that c-Kit is expressed predominantly on immature undifferentiated cells (Borge et al., 1997) adds further evidence to the theory that $CD56^{hi}$ NK cells are less mature and mature into $CD56^{lo}$ cells. In further agreement

with this finding, it is also known that stimulation of c-Kit$^+$ cells with growth factors and cytokines such as TGF-β, granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ down regulate c-Kit expression on a range of different cell types (Ashman, 1999).

1.1.6. NK cell function

Natural killer cells were first described for their ability to lyse target cells without prior sensitisation, hence their name, and as such their cytotoxic capabilities were the first function described (Kiessling, Klein & Wigzell, 1975). Since the early observations of NK cells as killers it has become clear that they are a cell type with multiple effector functions. NK cell killing is elicited through a number of different effector mechanisms and not solely though the lack of self-MHC class I molecules expression by target cells, as first described. NK cells respond to chemokines and cytokines and themselves also secrete cytokines that are important for immunoregulatory activities, shaping adaptive responses through reciprocal interactions with DC (Mailliard et al., 2003, Biron, 1999). One more recently described function for NK cells indicates a role in limiting excessive and non-specific inflammation through killing immature DC (iDC), mediated via the CD94/NKG2A Receptor complex (Della Chiesa et al., 2003, Ferlazzo et al., 2003, Ferlazzo, Semino & Melioli, 2001).

As described above NK cells can be split into two broad functional categories based on the level of CD56 expression with the CD56^{hi} NK cells thought to mature first before maturing themselves to become CD56^{lo} NK cells (Freud & Caligiuri, 2006, Freud et al., 2005). Functionally, the CD56^{hi} NK cells appear to be specialist cytokine-producing cells with a lower capacity for cytotoxic effector functions than CD56^{lo} cells, although the latter are also capable of cytokine production (Ferlazzo et al., 2004, Cooper et al., 2001, Jacobs et al., 2001, Fehniger et al., 1999).

CD56^{hi} NK cells are abundant in SLT, but only represent approximately 10% of the NK cells in the peripheral blood, where they express chemokine receptors such as CCR7 and adhesion molecules such as CD62L that enable circulating NK cells to efficiently migrate and enter SLT in response to chemokines (Fehniger et al., 2003, Campbell et al., 2001). NK cytokine production is stimulated by interactions with other cells present in the same parafollicular regions of SLT, such as DC, which produce a variety of cytokines shown to

stimulate NK cytokine production including IL-12, IL-15, IL18, IL-1 and others (Cooper et al., 2001). $CD56^{hi}$ NK cells lack expression of perforin (Jacobs et al., 2001), CD16 and KIR molecules, and it has been demonstrated that cells must be stimulated with IL-2 before they acquire cytotoxic functions (Fehniger et al., 2003). $CD56^{hi}$ NK cells also express c-Kit, which, when stimulated by its ligand stem cell factor (SCF), enhances IL-2 mediated NK cell proliferation even at very low IL-2 concentrations (Matos et al., 1993).

Cytokines produced by NK cells include IFN-γ, TNF-α, TNF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10 and IL-13 (Poli et al., 2009, Cooper et al., 2001). The combination of cytokines stimulating the NK cells influences the exact NK cytokines they produce, for example, stimulation with IL-12 combined with IL-18 has been shown as the most efficient combination to stimulate IFN-γ production, whereas IL-15 in combination with IL-18 was the most efficient combination for GM-CSF production (Cooper et al., 2001). Cytokines produced by NK cells, particularly IFN-γ, function in various ways promoting macrophage maturation and IL-12 production, stimulating an increase in MHC class-I and class-II expression and thereby enhancing antigen presentation and promoting antiviral T-helper cell type 1 responses (Th1) (Schoenborn & Wilson, 2007, Martin-Fontecha et al., 2004). IFN-γ has also been implicated in stimulating the production of APOBEC enzymes such as APOBEC3G, which has direct anti-viral activity against HIV-1 (Casartelli et al., 2010, Peng et al., 2006). $CD56^{hi}$ NK cells have been described as 'helper' cells by some as a way of describing their ability to enhance DC activity and promote Th1 polarisation (Mailliard et al., 2005).

The $CD56^{lo}$ NK cells are the cells primarily responsible for cytolytic activity mediated through one of two different mechanisms. Firstly, these NK cells possess high levels of intracellular granzymes and perforin, cytolytic components which directly induce apoptosis and cellular lysis through a process of degranulation (Jacobs et al., 2001). Secondly, $CD56^{lo}$ cytotoxic cells respond to stimulation by presenting Fas ligand and TNF-related apoptosis inducing ligand (TRAIL), which stimulate apoptotic pathways on target cells. TRAIL expression by $CD56^{hi}$ NK cells has been demonstrated in response to IFN-α stimulation and during acute hepatitis C infection (Stegmann et al., 2010). In order for cytotoxic responses to occur, signals must be elicited through activating receptors, but activation only occurs when inhibitory signals are not present (Caligiuri, 2008, Bryceson et al., 2006a, Moretta et al., 2000). Figure 1.2 presents a generalised diagram of NK cell

mediated cytotoxicity in the absence of self-MHC class I molecules (Adapted from Fauci, Mavilio & Kottilil, 2005)

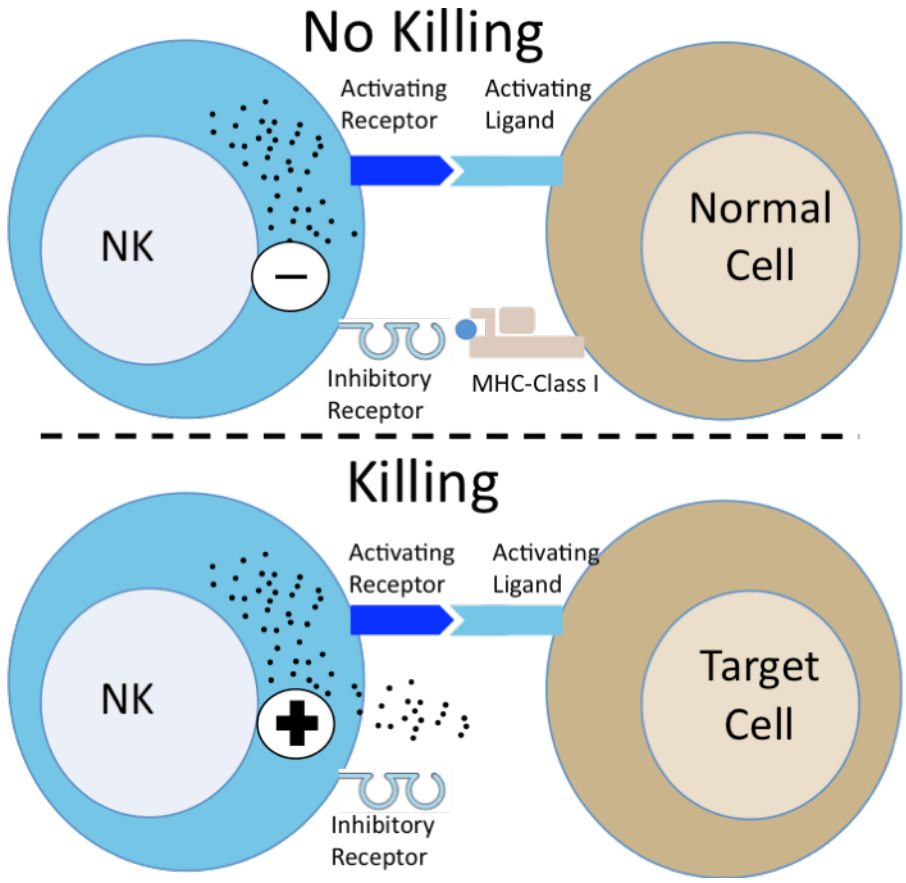


Figure 1.2 NK cell recognition and lysis of target cells. Generalised diagram of NK cell killing via the 'missing self' mechanism. In this model NK inhibitory signals received via inhibitory receptors such as KIR are dominant and NK cell cytotoxicity will not be triggered. In order for NK cell mediated cytotoxicity to occur a lack of inhibitory signal must be detected, allowing activating signals to predominate, triggering release of perforin and granzyme and the destruction of the target cell. Adapted from Fauci *et al.* 2005

For degranulation to occur, appropriate activating receptors must be engaged. With the exception of CD16, multiple receptors, such as the NCR, NKG2D, NKG2A must be cross-linked in order for degranulation to occur in resting NK cells. However, signalling through a single activating receptor is enough to trigger degranulation in NK cells that have been previously activated, with for example IL-2 (Al-Hubeshy et al., 2011, Bryceson et al., 2006b). Following initial engagement of activating receptors, either LFA-1 adhesion to ICAM-1 on target cells, or CD16 adhesion to IgG in combination with 2B4 co-receptor engagement with its ligand CD48, leads to increased binding affinity and avidity between NK cells and their target cell (Moretta et al., 2008, Bryceson et al., 2006b, Bryceson et al., 2005). Following adhesion, calcium-dependant cytoskeletal rearrangement occurs. This

polarises the secretory lysosomes, which contain perforin and granzyme, directing them to the site of engagement with the target cell prior to degranulation (Moretta et al., 2008, Bryceson et al., 2005). Perforin acts by creating pores in the plasma membrane of target cells, whereas granzyme is internalised by the target cell, where it acts by activating caspase enzymes, which in turn activate DNAse enzymes and subsequently triggers apoptosis through DNA cleavage (Bryceson et al., 2006a).

Degranulation is also stimulated in NK cells triggered through CD16 stimulation in antibody-dependent cellular cytotoxicity (ADCC) or when the NCR are triggered. NK cells also trigger surface receptors on target cells that are associated with death domains that promote apoptosis. The Fas receptor and the TRAIL receptors are both TNF super family receptors which transmit signals through their death domains, activating various caspases (cysteine proteases) and leading to the induction of apoptosis (Smyth et al., 2005). NK cells can be induced to express the ligands (FasL or TRAIL) for Fas and TRAIL receptors when stimulated with inflammatory cytokines such as IL-2, IL-15 and both type I and II interferons (Smyth et al., 2005).

1.2. Toll-like receptors

TLR are pattern recognition receptors (PRR), which are germline encoded Type I integral membrane glycoproteins, adapted for the innate recognition of pathogen associated molecular patterns (PAMP) found in viruses, bacteria, protozoa and funghi (Akira, 2011, Kawai & Akira, 2010, Akira, Uematsu & Takeuchi, 2006). TLR are expressed on a variety of cells, including B-cells, certain types of T-cell as well as some epithelial cells such as airway and intestinal cells. Arguably, however, TLR expression in DC and macrophages is most significant, as these cells are responsible for the generation of and shaping of the adaptive immune responses (Akira, Uematsu & Takeuchi, 2006). Through recognition of their ligands, TLR expressing DC and macrophages are responsible for the priming and establishment of adaptive immune responses and as such have been proposed as vaccine adjuvants (Lahiri, Das & Chakravortty, 2008, Iwasaki & Medzhitov, 2004, Kaisho & Akira, 2002).

1.2.1. TLR ligands and distribution

There are 9 known human TLR with defined ligands and function that recognise different PAMP. TLR1, 2, 4, 5 and 6 are expressed on, and encounter their ligands at, the cell

surface whereas TLR3, 7, 8 and 9 are expressed within acidified endosomes, lysosomes and endolysosomes, where they encounter their nucleic acid ligands (Kawai & Akira, 2010). Table 1.2 summarises known TLR ligands and their origins and the DC subset that given ligands are associated with.

Table 1.2 TLR, TLR ligands and DC subset distribution.

Receptor	DC Subset Distribution	Ligand(s)	Origin of Ligand
TLR1/2	**Myeloid**	Triacyl lipopeptides	Bacteria & Mycobacteria
		Soluble factors	*Neisseria meningitidis*
TLR2	**Myeloid**	Lipoprotein/lipopeptides	Various pathogens
		Peptidoglycan/Lipoteichoic acid	Gram-positive bacteria
		Lipoarabinomannan	Mycobacteria
		Phenol-soluble modulin	*Staphylococcus epidermidis*
		Glycoinositolphospholipids	*Trypanosoma cruzi*
		Glycolipids	*Treponema maltophilum*
		Porins	*Neisseria*
		Atypical lipopolysaccharide	*Leptospira interrogans/Porphyromonas gingivalis*
		Zymosan	Fungi
TLR3	**Myeloid**	Double-Stranded RNA	Viruses
TLR4	**Myeloid**	Lipopolysaccharide	Gram-negative bacteria
		Fusion protein	Respiratory syncytial virus
TLR5	**Myeloid**	Flagellin	Bacteria
TLR6/2	**Myeloid**	Zymosan	Fungi
		Diacyl lipopeptides	Mycoplasma
		Lipoteichoic acid	Gram-positive bacteria
TLR7	**Plasmacytoid**	Single Stranded RNA	Viruses
TLR8	**Myeloid**	Single Stranded RNA	Viruses
TLR9	**Plasmacytoid**	CpG-containing DNA	Bacteria and Viruses
TLR10	**-**	-	-
TLR11	**-**	-	Uropathogenic Bacteria

TLR1 and TLR6 form heterodimers with TLR2; the TLR2/TLR1 heterodimer recognises triacylated lipopeptides of gram-negative bacteria and the TLR2/TLR6 heterodimer recognises diacylated lipopeptides of gram-positive bacteria (Kang et al., 2009, Jin et al., 2007). TLR2 has also been implicated in the recognition of other ligands including lipopolysaccharides (LPS), lipoteichoic acids (LTA) and various lipoproteins, glycoproteins, zymosan and peptidoglycan (Zahringer et al., 2008). TLR4 is the receptor for bacterial LPS, but requires other molecules to recognise and respond to LPS. LPS binding protein (LBP) is present in plasma and binds LPS, after which CD14 binds and brings LPS to the

surface receptor molecule MD-2, which subsequently associates with and stimulates TLR4 (Shimazu et al., 1999). TLR5 recognises flagellin, a component of the flagellum from flagellated bacteria (Hayashi et al., 2001). TLR5 expression on DC is particularly found at potential sites of infection such as in the intestinal epithelium or lung, but is expressed basolaterally and therefore only encounters pathogens that have crossed the epithelium during infection and so do not encounter commensal gut bacteria (Akira, Uematsu & Takeuchi, 2006).

The nucleic acid-sensing TLR specialise in recognising viral components and not only trigger inflammatory cytokines, but also induce anti-viral type I IFNα and β (Akira, 2006). Additionally, TLR7 and TLR9 are exclusively expressed on plasmacytoid DC (pDC) which produce very high levels of type I IFN (Kawai & Akira, 2010). TLR3 recognises dsRNA genomes of viruses and dsRNA stages of ssRNA virus replication phases, and can also be triggered by the synthetic compound polyinosinic-polycytidylic acid Poly(I:C) (Kawai & Akira, 2010, Alexopoulou et al., 2001). TLR7 and TLR8 in humans have very close homology and are responsible for the sensing of viral ssRNA (Akira, Uematsu & Takeuchi, 2006, Heil et al., 2004). Self ssRNA is generally degraded before it can reach the appropriate compartments to generate an autoimmune response (Akira, 2006). TLR9 is responsible for the recognition of unmethylated 2'deoxyribo(cytidine-phosphate-guanosine) CpG DNA that is characteristic of bacterial DNA and some viral genomes (Kawai & Akira, 2010, Akira, 2006, Hemmi et al., 2000). pDC exclusively express TLR7 and TLR9 whereas other myeloid cells including myeloid DC (mDC) and macrophages express a broader range of TLR (Akira, 2011).

1.2.2. TLR structure and signalling

TLR recognise PAMP via their extracellular domains, which contain leucine-rich repeats (LRR) (Kawai & Akira, 2010, Akira, Uematsu & Takeuchi, 2006). Engagement of TLR with their ligands induces a signalling cascade through their cytoplasmic Toll/IL-1R homology (TIR) domain, which engages with TLR-specific TIR domain-containing adapter molecules leading, ultimately, to the release of cytokines and the maturation of cells (2011, Akira, 2006). The adapter molecules include: myeloid differentiation factor-88 (MyD88), TIR domain-containing adapter-inducing interferon-β (TRIF), TIR domain-containing adapter protein (TIRAP) and TRIF related adapter molecule (TRAM) (Akira, 2006). There are two broad signalling pathways; the MyD88-dependent pathway leads to the activation of the

transcription factor NF-κB and the release of inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-α; alternatively, the TRIF-dependent pathway activates IFN Regulatory Factor (IRF) family transcription factors and leads to the release of type I IFN molecules (Kawai & Akira, 2010, Lahiri, Das & Chakravortty, 2008, Akira, 2006). The adapter molecules utilised vary according to which TLR is engaged, leading to different specific maturation profiles and, in turn, affecting which type of adaptive response is primed by TLR activated cells. TLR3 is the only TLR to utilise only the TRIF dependant pathway, eventually activating both the NF-κB transcription factor for inflammatory cytokine release and the IRF3 transcription factor for type I IFN release (Kawai & Akira, 2010). TLR4 uses both the MyD88 pathway and the TRIF pathway. First TLR4 transduces conventional MyD88 signals leading to the release of inflammatory cytokines before the receptor complex is internalised into an endosome and TRIF is recruited leading to IRF3 activation and release of type I IFN (Kawai & Akira, 2010). TLR7 and TLR9 expressed on pDC utilise the MyD88 and not the TRIF pathway, ultimately activating both NF-κB and IRF7, leading to the release of inflammatory cytokines and high concentrations of type I IFN (Kawai & Akira, 2010, Akira, 2006).

1.2.3. TLR as adjuvant receptors

TLR agonists posses many of the qualities that are potentially useful for stimulating the robust and long term memory responses required for vaccination. TLR allow the recognition of non-self organisms that express highly conserved molecular patterns required for survival of the organism. By recognising these products, TLR-expressing cells, especially DC, provide a link to the adaptive immune response so that future encounter with a specific pathogen generates a rapid and potent response that prevents further infection and pathology. TLR ligation of mDC predominantly occurs at the epithelial surface. Following TLR engagement, mDC are induced to mature. They upregulate CCR7 and migrate to the LN, MHC class I and II expression are increased, as are co-receptors such as CD40, CD80, CD86 and CD70, and inflammatory cytokines are released. All this helps DC to engage with T-cells and stimulate adaptive responses (Gnjatic, Sawhney & Bhardwaj, 2010, Lahiri, Das & Chakravortty, 2008, Ida et al., 2006).

pDC are more likely come into contact with their TLR7 and TLR9 agonists in circulating blood, following which they also express CCR7 and migrate to the LN, express high levels of type I IFN, and release chemokines with the ability to induce NK cell and T-cell

migration (Ida et al., 2006, Fonteneau et al., 2004, Megjugorac et al., 2004). pDC do not express co-stimulatory molecules to the same level as mDC, however, and it has been suggested that they are able to stimulate mDC through a bystander mechanism in HIV-1 infection, and that mDC are primarily responsible for antigen presentation (Fonteneau et al., 2004).

TLR agonists represent an attractive proposition for vaccine adjuvants as they mimic natural infection and modulate adaptive responses allowing for the manipulation towards certain types of immune response. A number of studies and trials have been undertaken using various TLR agonists, (Gnjatic et al. 2010 and Lahiri et al. 2008). In order for vaccines to be successful it has been suggested that they should enhance the expansion phase of T-cell proliferation and/or reduce the contraction phase, thereby providing a larger memory T-cell pool able to respond rapidly and robustly following infection (Pulendran & Ahmed, 2006). The actions of TLR agonists on DC help to fulfil these aims as demonstrated in HIV-1 infection by Wille-Reece *et al*. (2005b, Wille-Reece et al., 2005a) who demonstrated the generation of HIV-1 specific $CD8^+$ T-cells was enhanced when using a TLR7/8 adjuvant when vaccinating mice and non-human primates.

The engagement of TLR leads to the release of cytokines at the site of infection or vaccination, recruiting other DC to the area before migrating to LN where they will encounter antigen-specific T-cells. By attracting other DC to the site of infection they will take up further antigen and also migrate to the LN, increasing the chances of interacting with specific T-cells (Pulendran & Ahmed, 2006). By increasing the expression of MHC and therefore presenting more antigen, DC are more likely to interact with specific T-cells (Pulendran & Ahmed, 2006). As DC mature they also form their characteristic dendrites, which provide a greater surface area to interact with potential antigen-specific cells. The expression of co-stimulation molecules such as CD80 and CD86 also augments the MHC-TCR interaction and, through binding of CD28 on T-cells, upregulates the expression of the CD40 ligand (CD40L). Engagement of CD40L to CD40 leads to the release of IL-12 and IL-18, which reciprocally induce the release of IFN-γ by T-cells, enhancing the generation of a Th1-type response (Lanzavecchia & Sallusto, 2001). The release of type I IFN, TNF and IL-6 by pDC has also been implicated in stimulating mDC to increase the expression of co-stimulatory molecules (Fonteneau et al., 2004, Le Bon et al., 2003).

Through all of these actions TLR agonists enhance DC survival, the production of co-stimulatory molecules and the release of cytokines, which in turn induces T-cell expansion (Pulendran & Ahmed, 2006). There is also some evidence that cytokines produced by DC including type I IFN, IL-2, IL-4, IL-7 and IL-15 also enhance T-cell survival and therefore possibly reduce the contraction phase of memory T-cell formation (Marrack & Kappler, 2004).

1.3. NK cell-DC interactions

Following infection, DC interact with NK cells, either at the site of inflammation or within SLT (Moretta et al., 2008, Degli-Esposti & Smyth, 2005, Walzer et al., 2005, Dalbeth et al., 2004, Martin-Fontecha et al., 2004, Mailliard et al., 2003). Activated DC produce NK cell-stimulating cytokines, and, in a reciprocal loop, are aided in maturation and activation by NK-produced cytokines such as IFN-γ, granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNF (Wesa & Mailliard, 2010, 2008, Moretta, 2002). Figure 1.3 illustrates basic DC-NK cell interactions leading to reciprocal activation and NK cell-mediated killing of immature DC (iDC) (adapted from Degli-Esposti & Smyth, 2005). The interactions between NK cells and DC are complex, and there is evidence that direct cell-cell contact and communication through the production of soluble factors is required for reciprocal maturation and activation to take place (Wesa & Mailliard, 2010, Nedvetzki et al., 2007, Vitale et al., 2004, Gerosa et al., 2002, Fehniger et al., 1999). DC produce IL-10 and TGF-β following their activation, providing negative feedback signals to the NK cell population, limiting their activation and proliferation (Wesa & Mailliard, 2010, Caligiuri, 2008). IFN-γ is known to direct DC to produce more IL-12 and direct a Th1 type T-cell response and this system has therefore been described as a link between the innate and the adaptive immune system (Caligiuri, 2008, Mocikat et al., 2003, Gerosa et al., 2002, Moretta, 2002).

As discussed above, $CD56^{hi}CD16^{-}$ NK cells are present in the SLT and also at low concentrations in the peripheral blood (Caligiuri, 2008); it is this NK cell subset that is predominantly responsible for cytokine production upon encounter with DC (Dalbeth et al., 2004, Vitale et al., 2004). Direct cell contact between NK cell and DC is mediated by a number of surface receptors responsible for the activation of NK cells. NK cell cytotoxic activity is induced by activation through NKG2D, NKp46 and NKp30 (Eissmann et al., 2010, Draghi et al., 2007, Nedvetzki et al., 2007, Jinushi et al., 2003, Ferlazzo et al., 2002). DC have been shown to bind IL-15 by the IL-15 receptor-α-chain and trans-present

IL-15 to NK cells, inducing proliferation and survival of NK cells (Lucas et al., 2007, Ferlazzo et al., 2004). CD80 and CD48 expression by DC have been shown to stimulate NK cell IFN-γ production and proliferation through their respective CD28 and 2B4 ligands (Wesa & Mailliard, 2010, Newman & Riley, 2007). It has also been shown that IL-12 is efficiently delivered from DC to NK cells when immune synapses form between the two cell types, allowing IL-12 polarisation and release directly to NK cells. IL-18 has also been shown to be secreted at immune synapses (Semino et al., 2005) although IL-12 is the most efficient inducer of NK IFN-γ (Nedvetzki et al., 2007, Borg et al., 2004, Ferlazzo et al., 2004).

Although IL-12 is the most potent co-stimulator of IFN-γ, IL-18, acts in synergy with a number of cytokines, including IL-2, IL-15, IL-10 and type I IFN, to enhance both NK cell IFN-γ production and cytotoxicity (Wesa & Mailliard, 2010, Agaugue et al., 2008, Mailliard et al., 2005). Type I IFN acts on NK cells by directly inducing cytotoxic effector functions, inducing the upregulation of FASL, perforin and NKG2D, and has been shown to directly enhance NK cell degranulation and killing (Benlahrech et al., 2009, Gerosa et al., 2005, Jinushi et al., 2003).

NK cells respond to stimulation, either by induction of cytotoxic responses or through the production of pro-inflammatory cytokines, which has led to the description of NK cells as 'helper' cells. Cytokines produced by NK cells such as IFN-γ, TNF-α, GM-CSF, MIP1-α/β and RANTES are involved in the recruitment and activation of DC and the induction of maturation (Agaugue et al., 2008, Gerosa et al., 2005, Mailliard et al., 2005, Dalbeth et al., 2004, Martin-Fontecha et al., 2004). In the majority of circumstances NK cell release of IFN-γ and other cytokines has the effect of enhancing DC IL-12 production, leading to a Th1-polarised adaptive T-cell response (Newman & Riley, 2007, Mailliard et al., 2005, Martin-Fontecha et al., 2004, Fehniger et al., 2003).

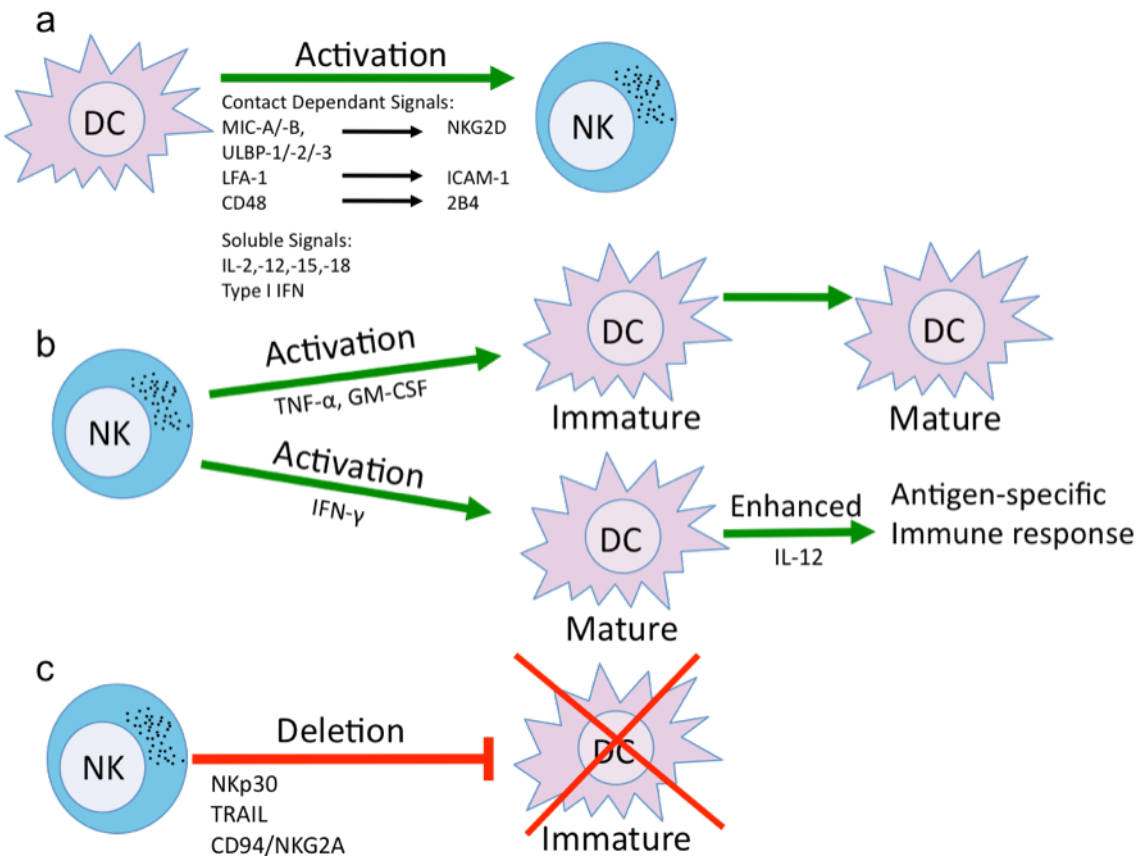


Figure 1.3 Interactions between NK cells and DC illustrating a) DC-mediated activation of NK cells via both contact-dependant signals and soluble signals stimulates NK cells, inducing cytokine production and/or cytotoxicity and promoting proliferation and NK cell survival. b) Activated NK cells are then capable of inducing DC maturation and activation including the production of IL-12, enhancing the generation of antigen-specific immune responses. c) NK cells can kill iDC that add to inflammation without presenting appropriate levels of MHC via the activating NKp30. Adapted from Degli-Esposti & Smyth 2005.

DC also produce anti-inflammatory cytokines which limit the activation and therefore potential immunopathology of cells such as NK at the site of inflammation. It has also been shown that NK cell killing of iDC acts as a mechanism to limit excessive infiltration of DC that either are not maturing or are maturing too slowly to be useful. This would ensure selection of mature DC fully capable of eliciting effective T-cell responses and has been termed immune 'editing' (Della Chiesa et al., 2006, Moretta et al., 2006, Ferlazzo et al., 2003). iDC express low levels of HLA-E and are recognised by NK cells that express the CD94/NKG2A receptor, but lack KIR expression preventing NK cell inhibitory signals (Moretta et al., 2006, Della Chiesa et al., 2003). The NCR NKp30 is the activating receptor responsible for the induction of NK cell killing of iDC and is mediated through the TRAIL killing pathway (Hayakawa et al., 2004, Ferlazzo et al., 2002).

1.4. Antigen-specific T-cell activation

Unlike NK cell responses, antigen-specific immunity requires prior sensitization. Adaptive, antigen-specific, immune responses once generated provide robust and appropriate defence against specific pathogens. Unlike cells of innate immunity, activated T-cells can develop into long-lived memory cells able to proliferate and respond rapidly and appropriately, with minimal inflammation upon reencounter with the same pathogen (Janeway et al., 2005b). Educating the immune system to respond in this way to pathogens is the goal of most vaccination strategies.

1.4.1. Antigen recognition

Antigen recognition is mediated through recognition of peptide sequences complexed to MHC class I or class II molecules presented to the T-cell receptor (TCR) expressed by T-cells. Any one individual expresses a huge array of TCR such that a single individual is capable of potentially recognising a huge number of MHC-presented foreign antigens. Encounter of a TCR with its specific antigen leads to the activation and proliferation of that antigen-specific T-cell in a process termed clonal expansion, generating many thousands of armed effector cells, all bearing that one TCR and capable of clearing the infection.

MHC class I and class II are recognised by $CD8^+$ T-cell and $CD4^+$ T-cells respectively and this has consequences for the type of immune response generated. CD8 and CD4 are themselves co-receptors for MHC class I and class II respectively, and are useful immunological markers for defining these two different T-cell subsets. Another important aspect of TCR engagement with MHC:peptide complexes is that TCR bind directly to the MHC molecule as well as recognising the specific peptide sequence. As there are an enormous array of different MHC class I and class II, and each unique TCR recognises only one, the TCR is said to be MHC restricted i.e. it recognises a specific MHC molecule in combination with a specific peptide.

MHC class II is expressed on specialist immune cells that are considered to be professional antigen presenting cells (APC); these are macrophages, DC and B-cells. APC actively take up antigen either by receptor-mediated endocytosis, the principal uptake method for B-cells, or through the sampling of the extracellular environment by phagocytosis (macrophages) or macropinocytosis (DC) (Janeway et al., 2005a). Both the type of cell presenting the antigen and the soluble factors present are important for

determining the type of CD4 mediated T-helper cell generated. Generally B-cells and IL-4 stimulate a Th2, humoral-type response whereas DC and IFN-γ stimulate a Th1, cellular response. MHC class II normally presents peptides of 13-17 amino acids in length to $CD4^+$ T-cells.

Unlike MHC class II, class I molecules are ubiquitously expressed and, as has been discussed, the lack of MHC class I expression is recognised by NK cells and can lead to NK cell-mediated killing. MHC class I is responsible for presenting antigens for intracellular pathogens, including viruses and intracellular bacteria which are capable of infecting most cell types, and therefore all cells express MHC class I. The majority of cells, however, do not express the necessary co-stimulatory factors needed to activate naïve $CD8^+$ T-cells; for this, APC, mainly DC, are required (Janeway et al., 2005a). Memory $CD8^+$ T-cell activation however requires less co-stimulation and can be activated by cells expressing antigen from intracellular pathogens. MHC class I presents peptide sequences of 8-10 amino acids in length at the cell surface.

1.4.2. Antigen processing and presentation

The origins of peptide antigens displayed by MHC class I and class II are different. As discussed, MHC class I presents antigens from intracellular pathogens and MHC class II presents antigens derived from the extracellular environment. MHC molecules posses binding grooves in which they bind peptides with certain anchor residues at specific points in the peptide sequence. Different MHC molecules have different peptide binding grooves that bind different amino acid side chain residues at different positions on the peptide sequence. There is, therefore, specificity, as certain peptides bind to certain MHC molecules.

Antigens presented on MHC class I are degraded in the cytosol and actively transported into the endoplasmic reticulum (ER) by the TAP 1/2 heterodimer and loaded onto waiting MHC class I molecules. Proteins are constantly degraded in the cytosol through the ubiquitin-dependant pathway by the proteosome, which not only acts to recycle misfolded and non-functional proteins, but also allows the production of suitable length (8-16 amino acids) peptides that can be loaded onto MHC class I molecules (Janeway et al., 2005a). Stimulation by IFN-γ, such as that produced by NK cells, has been shown to upregulate proteosome activity and also the synthesis of MHC class I molecules (Rosa & Fellous,

1988). Peptides with amino acid sequences longer than 8-10 are able to bind to MHC class I, but are cleaved by aminopeptidase enzymes before they can be transported to the cell surface. Only stable MHC:peptide complexes are released from the ER and transported to the cell surface.

Extracellular antigens endocytosed by the specialist APC are kept within acidified membrane bound endosomes; these fuse with lysosomes containing cathepsins, cysteine proteases, which are activated at low pH. Pathogenic material is then degraded in the endolysosomes, providing the source of antigenic peptides for MHC class II molecules. MHC class II molecules are, similarly to class I molecules, assembled and trafficked through the ER, but in order to prevent them binding peptides here they are complexed with an invariant chain (Ii) protein, which occupies the binding groove. The MHC:Ii complex is then loaded onto vesicles capable of transporting the complexes to endolysosomes, fusion with which removes the majority of the Ii protein, leaving behind a class II-associated invariant chain peptide (CLIP), which occupies the binding groove. The MHC-II-like molecule HLA-DM mediates CLIP removal, which catalyses its removal from the binding groove whilst loading peptide sequences. HLA-DM also removes peptide that is too weakly bound to MHC class II, as only stable MHC:peptide complexes can be expressed on the cell surface (Janeway et al., 2005a).

1.4.3. T-cell activation

DC are considered the most potent of APC and, upon activation by encounter with pathogen, they migrate to local LN and present antigen to naïve T-cells, which are continuously circulating between the blood and lymph system (Janeway et al., 2005b). As discussed earlier, activation of DC leads to a process of maturation that sees the upregulation of MHC molecules and the co-stimulatory molecules necessary to engage with and activate naïve T-cells. Activated naïve T-cells in LN undergo a process of clonal expansion, which lasts for several days and generates a large population of antigen-specific T-cells (Janeway et al., 2005b). Toward the end of the clonal expansion process, T-cells develop into effector cells and it is these cells that are released from the LN and migrate to the site of infection or inflammation and efficiently combat and clear infections (Janeway et al., 2005a).

Interaction between T-cells and DC occurs in the T-cell region of the LN and is initially mediated by integrins such as lymphocyte function-associated antigen-1 (LFA-1) on DC with intercellular adhesion molecules-1 and -2 (ICAM-1 and -2) on T-cells. Without engagement of the TCR with its specific peptide:MHC complex, this initial interaction is transient and the bonds are quickly broken as the T-cell continues through the LN, interacting with other APC as it does so. When a TCR does bind its specific ligand there is a conformational change in the LFA-1 molecule such that binding affinity to ICAM-1 and -2 is increased maintaining cell contact. Interaction of the co-stimulatory CD80 and CD86 molecules on DC with CD28 on T-cells is necessary for naïve T-cell activation. The CD40 ligand (CD40L) is upregulated on activated T-cells and interacts with CD40 on DC, eliciting the expansion of T-cells and the eventual differentiation into effector T-cells. Activated T-cells upregulate the expression of the IL-2Rα chain (CD25) and produce IL-2, which acts in an autocrine fashion inducing rapid proliferation (Janeway et al., 2005b).

1.4.4. T-cell differentiation

Following primary infection, the multitude of antigen-specific effector cells mostly die, but some cells remain as memory cells. Memory T-cells do not require co-stimulation for activation and respond rapidly, proliferating to high numbers at a much faster rate. Additionally memory T-cells are divided into different groups, some of which, termed the effector memory (TEM) cells, are primed to carry out effector functions immediately, but do not proliferate when activated (Wherry et al., 2003, Champagne et al., 2001). Central memory T-cells (TCM) cells express LN homing markers such as CCR7 and CD62L and are the cells which undergo rapid proliferation and differentiate into effector cells before migrating to the site of infection (Wherry et al., 2003, Champagne et al., 2001).

1.5. Human immunodeficiency virus

The continued spread of HIV-1 and acquired immune deficiency syndrome (AIDS) remains a problem of global importance, with approximately 34 million people living with HIV/AIDS at the end of 2010, and estimates of 2.7 million people newly infected with HIV-1 and 1.8 million AIDS-related deaths during 2010 (UNAIDS, 2011). There is considerable effort being made in the distribution of antiretroviral drugs to low and middle income countries, with around 3 million people receiving therapy in these countries at the end of 2007 (UNAIDS, 2008) and an estimated 6.6 million people receiving therapy at the end of 2010 (UNAIDS, 2011). Current drugs are, however, expensive and their availability limited, and

much work has been undertaken in the search for a vaccine for HIV-1 (McMichael, 2006). There is also a need for improved treatment for those already infected with HIV-1 that would serve not only to suppress virus activity, as current anti-retroviral drugs do, but also to restore hosts' own immune capabilities. Immune-based therapies could enable the host immune system to limit opportunistic infections better, and could be used in conjunction with anti-retroviral therapy as further defense against HIV-1 infection. Despite these successes much more work is still needed, if we are to fully understand, control and prevent HIV infections. Work is being undertaken with people who are described as either 'slow progressors', 'elite controllers' or 'long-term non-progressors' (LTNP) who control viral loads for many years without the need for therapy and without progressing, or progressing exceptionally slowly to AIDS (Theze et al., 2011, Pantaleo & Fauci, 1996). The ability to induce this kind of state in normal progressors is one aim for immunotherapy, as the disease would be well tolerated and, because of sustained low viral loads, the risk of transmission would be greatly reduced (Autran et al., 2011).

1.5.1. Basic HIV lifecycle and generalised course of infection

The HIV-1 retrovirus is a lentivirus with a ssRNA genome which encodes nine genes that, through the generation of polyproteins, produce 15 separate proteins (Frankel & Young, 1998). There are three distinct genetic groups of HIV-1; M, O and N ('main', 'outlier' and 'non-main, non-outlier'). The M group is further subdivided into 11 clades A-K, which are observed to form geographical clusters, for example clade B virus predominates in Europe, Australia and North America and clade C is the most common virus found in sub-Saharan Africa, India and Ethiopia (McMichael, 2006). Different clades, however, have the ability to recombine in individuals infected with multiple clades of virus (McMichael, 2006).

Figure 1.4 illustrates the basic lifecycle of HIV infection and indicates the targets of action of various available anti-retroviral drugs (Modified from NIAD, 2012). HIV-1, the principal causative agent of AIDS, binds to and infects cells that express CD4 and either of the chemokine receptors CXCR4 or CCR5 (Levy, 2009, Simon, Ho & Abdool Karim, 2006, Gallo et al., 1984, Montagnier et al., 1984, Barre-Sinoussi et al., 1983). The HIV-1 glycoprotein gp120, expressed on the exterior of the virion, attaches to CD4 and then to CXCR4 or CCR5 before fusion takes place and the virus is released into the cell (Simon, Ho & Abdool Karim, 2006). Once the virus emerges in the cytoplasm the viral core

disassembles and the virus's own reverse transcriptase molecules transcribe the viral RNA genome to DNA before another viral protein, integrase, inserts viral DNA in host chromosomal DNA, irreversibly transforming the cell (Simon, Ho & Abdool Karim, 2006). Once integrated, HIV can produce the proteins necessary for production of new infectious virions, which, together with the ssRNA genome, are able to exit the cell by exploiting the host cell's own vesicular sorting pathway to bud from the cell surface (Simon, Ho & Abdool Karim, 2006).

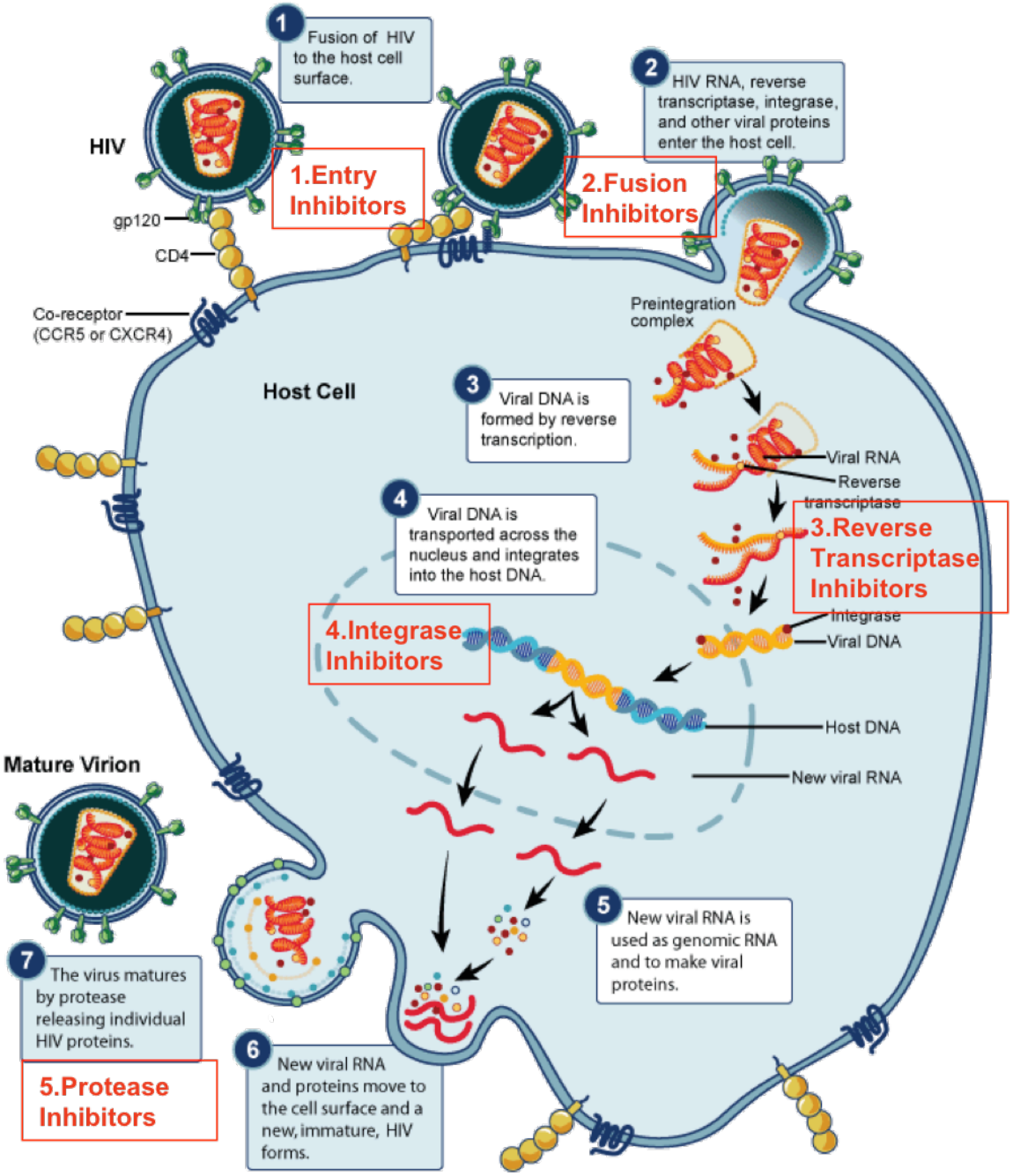


Figure 1.4 Basic HIV lifecycle from online resources available online for the National Institute of Allergy and Infectious Disease, modified to include sites of anti-retroviral drug action (summarised in Table 1.3). (NIAID, 2012).

Following initial infection, virus replicates rapidly and disseminates to become a systemic infection detectable in several compartments throughout the body (Levy, 2009). Cells of the innate immune system detect HIV-1 infection, and there is a corresponding rapid and large inflammatory cytokine response as viral load continues to increase (Stacey et al., 2009). Following this initial response, other cells of the innate system, particularly NK

cells, are recruited before some viral control is initiated and viral load begins to fall (Alter & Altfeld, 2009). The induction of acquired immune responses are detected later, after about two weeks, and the viral load then drops to what is described as the viral set point where $CD8^+$ T-cells are thought to be the predominant cells controlling viral replication; this marks onset of the chronic phase of infection (Levy, 2009, Borrow et al., 1994). The chronic phase typically lasts for around 10 years when untreated, during which time there is a constant slow decrease in $CD4^+$ T-cell numbers (Levy, 2009). Once the peripheral blood $CD4^+$ T-cell count drops below around 200 cells μl^{-1} there is significant loss of immune activity and patients become susceptible to opportunistic infections, which are characteristic of the onset of AIDS (Weiss, 2008).

1.5.2. HIV-1 treatment

Treatment for HIV infection is typically referred to as highly active anti-retroviral therapy (HAART). Current HAART drugs target different aspects of the HIV lifecycle. In the UK the British HIV Association (BHIVA) sets out guidelines for treatment of patients infected with HIV and up-to-date versions of these guidelines can be accessed at http://www.bhiva.org/ClinicalGuidelines.aspx (BHIVA, 2012). Currently therapy is generally initiated based on the absolute numbers of $CD4^+$ T-cells detected in peripheral blood and is begun when numbers drop below 350 cells μl^{-1}, but may be initiated at other times if, for example, a high viral load is detected or if other opportunistic infections indicative of an immunocomprimised host are experienced. HAART has two main outcomes, the gradual recovery of $CD4^+$ T-cell numbers and the eradication of detectable virus (usual detection limit is 50 copies of viral genome per ml peripheral blood (BHIVA, 2012)) (Jayasuriya, Robertson & Allan, 2007). The outcome of these effects act to reduce pathogenesis and improve resistance to opportunistic infection. Full immune reconstitution is not, however, seen, especially within the gut-associated lymphoid tissue (GALT) of the gastrointestinal (GI) tract, where a rapid and irreversible loss of immune cells, particularly $CD4^+$ T-cells, is observed. The consequences of this on chronic immune stimulation will be discussed later (Section 1.6.4) (Brenchley et al., 2004, Mehandru et al., 2004). Complete viral clearance is also not achieved and HIV-1 particles continue to replicate at low numbers within a viral reservoir made up predominantly of quiescent $CD4^+$ T-cells (Finzi et al., 1999, Wang et al., 1999, Chun et al., 1997). A list of currently approved HIV-1 medicines is listed in Table 1.3.

Cells of the innate immune system, such as DC and NK cells, are reduced in number and function during HIV-1 infection but show recovery in numbers and function following HAART. Early treatment has been shown to more fully restore these cells (Azzoni et al., 2007, Chehimi et al., 2007, Guadalupe et al., 2003). Initiation of HAART earlier and at higher $CD4^+$ T-cell numbers has thus been shown to lead to faster, more complete recovery of immune function, but there are problems with drug toxicity and, perhaps more seriously, the generation of drug-resistant virus which may be exacerbated by poor treatment adherence.

Table 1.3 Current licensed anti-retroviral drugs for HIV-1 treatment approved by the US Food and Drug Administration (FDA), accessible at: http://www.fda.gov/forconsumers/byaudience/forpatientadvocates/hivandaidsactivities/ucm118915.htm

Class	Trade Name	Generic name
Nucleoside Reverse Transcriptase Inhibitor	Combivir	lamivudine and zidovudine
	Emtriva	emtricitabine, FTC
	Epivir	lamivudine, 3TC
	Epzicom	abacavir and lamivudine
	Retrovir	zidovudine, azidothymidine, AZT, ZDV
	Trizivir	abacavir, zidovudine, and lamivudine
	Truvada	tenofovir disoproxil fumarate and emtricitabine
	Videx EC	enteric coated didanosine, ddI EC
	Videx	didanosine, dideoxyinosine, ddI
	Viread	tenofovir disoproxil fumarate, TDF
	Zerit	stavudine, d4T
	Ziagen	abacavir sulfate, ABC
Non-Nucleoside Reverse Transcriptase Inhibitor	Edurant	rilpivirine
	Intelence	etravirine
	Rescriptor	delavirdine, DLV
	Sustiva	efavirenz, EFV
	Viramune	nevirapine, NVP
Protease Inhibitor	Agenerase	amprenavir, APV
	Aptivus	tipranavir, TPV
	Crixivan	indinavir, IDV,
	Invirase	saquinavir mesylate, SQV
	Kaletra	lopinavir and ritonavir, LPV/RTV
	Lexiva	Fosamprenavir Calcium, FOS-APV
	Norvir	ritonavir, RTV
	Prezista	darunavir
	Reyataz	atazanavir sulfate, ATV
	Viracept	nelfinavir mesylate, NFV
Fusion Inhibitor	Fuzeon	enfuvirtide, T-20
Entry Inhibitor - CCR5 Antagonist	Selzentry	maraviroc
HIV Integrase Strand Transfer Inhibitor	Isentress	raltegravir

Current standards for HIV therapy in the West utilise multiple drugs, usually a combination of three (triple therapy), to avoid generation of resistant viral strains. Standard regimes will use two different nucleoside reverse transcriptase inhibitors (NRTI) and a single non-nucleoside reverse transcriptase inhibitor (NNRTI). Other drugs are available, including protease inhibitors and nucleoside analogues and other drugs such as viral entry inhibitors are being developed to target other stages of the viral life-cycle (BHIVA, 2012).

1.5.3. Requirement for prophylactic vaccination and immunotherapies

The limitations of HAART to completely clear viral infection and the risk of developing drug-resistant strains of virus means there is still a need for both a prophylactic vaccine and effective immunotherapies that have the ability to restore immunocompetence and prevent the spread of infection by controlling viral load without being toxic or promoting viral resistance. There is a known mutation of the CCR5 gene, a 32 base-pair deletion, which prevents CCR5 tropic virus (the most commonly transmitted form of virus) from entering $CD4^+$ cells in individuals homozygous for the mutation. Furthermore, if infection is established in individuals heterozygous for the mutation, they are more likely to be slow progressors (Dean et al., 1996). Although individuals with the CCR5 mutation are relatively rare, they demonstrate that blocking viral entry is an effective barrier to infection. It has been suggested that in order for a vaccine or immunotherapy to establish a high frequency of antigen-specific T-cells, such that would be required for prevention or control of infection, optimal antigenic priming conditions will have to be met (McMichael, 2006). Vaccination requires the recognition of particular epitopes by cells of the acquired immune system in order to generate a high frequency of antigen-specific T-cells (McMichael, 2006, Kaisho & Akira, 2002). To achieve optimal conditions for antigenic priming, adjuvants should be selected that activate innate immune functions capable of modulating the magnitude and quality of adaptive immune response generated (Kaisho & Akira, 2002). TLR agonists have been proposed as possessing the qualities desirable for adjuvanticity, and their use as adjuvants in vaccination strategies has previously been proposed and is summarised by Kaisho and Akira (2002) and Lahiri *et al*. (2008).

1.6. Immune responses and HIV-1 infection

HIV-1 infects $CD4^+$ T-cells (and other immune cells expressing CD4 and CCR5 or CXCR4) and this presents the obvious problem of eliciting an immune response to these cells, ultimately removing not only cells responsible for controlling HIV infection, but also other infections (Levy, 2009, Pantaleo & Fauci, 1996). Additionally there is direct pathology associated with acute viral infection, as demonstrated by high cytokine responses and damage to intestinal epithelium (Stacey et al., 2009, Mela et al., 2007, Mehandru et al., 2004). The eventual outcome of HIV-1 infection is AIDS, which makes patients susceptible to opportunistic infections that would not normally be able to establish in an immunocompetent host (Pantaleo & Fauci, 1996). There is evidence that both innate and adaptive immune responses can provide responses beneficial to infected individuals in controlling HIV-1, and there is also evidence of resistance to infection, as seen, for example, in discordant couples (Ghadially et al., 2011, Lederman et al., 2010, Ballan et al., 2007, Montoya et al., 2006, Scott-Algara et al., 2003). The known factors driving protection and resistance are largely genetic and are discussed in the following sections.

1.6.1. Acute HIV-1 infection

Early events during HIV-1 infection are represented in a generalised model where an initial peak in viraemia is associated with a rapid decline in $CD4^+$ T-cells, particularly in the gut associated lymphoid tissue (GALT). An initial innate immune response precedes the development of an antigen-specific acquired $CD8^+$ T-cell responses, leading to a rapid decrease in viral load and a partial recovery in $CD4^+$ T-cell numbers (Centlivre et al., 2007, Pantaleo & Fauci, 1996). A recent study by Stacey *et al.* (2009) measured the levels of 30 different innate cytokines at multiple time points during the acute/early phase of HIV-1 infection. Innate cytokines were found to be produced in a particular sequence, thus providing insight to the likely cell populations that are responding to HIV-1 at a given time point following initial infection. IFN-α, IL-15 and TNF-α are present in the initial wave of cytokine production, first detected at day six, followed by IL-6, IL-8, IL-10, IL-18 and IFN-γ in a second wave detected between days seven to 8.5. A third wave of cytokine production involving IL-4, IL-12(p70) and IL-22 occurs between days 9.5 and 12.5 (Stacey et al., 2009). The high peak in IFN-α indicates that pDC initially detect HIV-1 following uptake of virus particles and/or ligation of TLR7 (Baenziger et al., 2009, Chang & Altfeld, 2009, Alter et al., 2007a, Fonteneau et al., 2004). Fonteneau *et al.* (2004) showed, *in*

vitro, that $CD11c^+$ DC (mDC) were not directly activated by HIV-1 infection and that they are activated in a bystander fashion by TNF-α and IFN-α produced by HIV-1 activated pDC. The results from this study are in agreement with the Stacey study (Stacey et al., 2009) where IL-12 production was first detected 12.5 days after infection and 6.5 days after IFN-α and TNF-α are detected.

The rapid, high and early production of IFN-α and the delayed IL-12 production would have consequences as to the type of adaptive response generated, as IL-12 promotes a $CD4^+$ Th1-type response (Hsieh et al., 1993). IL-12 also promotes IFN-γ release by NK 'helper' cells, which is important for DC activation and maturation, and therefore for the priming of acquired immune responses, particularly Th1 polarised responses (Caligiuri, 2008, Trinchieri, 1995). The relatively late peak in the immunoregulatory cytokines IL-10 and IL-4 may act to slow the development of acquired immune responses by respectively inhibiting Th1-type responses or by enhancing unsuitable Th2-type responses, as suggested by the authors (Stacey et al., 2009).

1.6.2. Adaptive immune responses to HIV-1 infection

Following acute infection resulting in the rapid loss of $CD4^+$ T-cells and high viraemia, an adaptive immune response is mounted, mediated predominantly by $CD8^+$ cytotoxic T-lymphocytes (CTL) (Streeck & Nixon, 2010, Borrow et al., 1994). This antigen-specific response reduces the viral load and allows $CD4^+$ T-cell recovery, although complete eradication of virus has not been demonstrated (Yang et al., 1997, Walker et al., 1987). Viral set point is reached after the initial CTL response and is correlated with disease progression; a low viral set point, demonstrating good control of virus, correlates with slow progression to AIDS (Levy, 2009, Streeck et al., 2009). Early and irreversible damage to the gut mucosa is observed and there is a particularly severe loss of $CD4^+$ T-cells from this compartment (Brenchley et al., 2004). The loss of integrity that follows damage to the gut mucosal surface has been implicated in chronic non-specific immune activation (Brenchley et al., 2006).

Shortly after adaptive immune responses are mounted, viral escape mutants appear (Borrow et al., 1997), to which cross-reactive or new responses are not generated (Streeck et al., 2009), and future antigen-specific responses are impaired. It has, however, been demonstrated in so called 'HIV-1 controllers' (HIC), who control viral replication efficiently

below levels detectable by routine viral load measurement (50 copies ml^{-1} blood), that $CD8^+$ T-cell continue to mount effective responses against virus, maintaining all aspects of functionality despite very low, undetectable, viral loads (Saez-Cirion et al., 2007a, Migueles et al., 2002).

Although it has been demonstrated that HIV-1 preferentially infects HIV-1-specific $CD4^+$ T-cells (Brenchley et al., 2004, Douek et al., 2002), there is evidence from cohorts of patients who control viral replication that HIV-1-specific $CD4^+$ T-cells are maintained and fully functional, although it is unclear if this is a cause or effect of viral control (Rosenberg et al., 1997). Further work demonstrated that central memory $CD4^+$ T-cells, the main viral reservoir, and effector memory cells are functionally preserved in a different cohort of individuals with proven long-term viral suppression (Potter et al., 2007). Further evidence that $CD4^+$ T-cell responses are important in control of HIV-1 infection are demonstrated through the requirement for $CD4^+$ T-cell production of IL-2 for $CD8^+$ T-cell maintenance, CTL function and proliferative responses (Lichterfeld et al., 2004). $CD4^+$ antigen-specific T-cells have also been shown to be polyfunctional in asymptomatic HIV-2 infection (Duvall et al., 2006) demonstrating production of both IL-2 and IFN-γ.

In addition to the loss of overall numbers of $CD4^+$ T-cells, early functional defects have also been observed that occur prior to the loss of $CD4^+$ T-cells in peripheral blood (Miedema et al., 1988). It has been shown that HIV-1-specific memory $CD4^+$ T-cells have defective proliferative responses to antigen stimulation (Clerici et al., 1991). The impairment of function of T-helper cells, particularly dysfunctional production of cytokines such as IL-2, has severe knock on effects, particularly toward CTL responsible for eliminating virus-infected cells (Lichterfeld et al., 2004).

Early adaptive immune responses against HIV-1 infection mounted predominantly by effector $CD8^+$ T-cells are able to drastically reduce viraemia, but escape mutants are rapidly generated and loss of control ensues (Borrow et al., 1997, Borrow et al., 1994). There are, however, genetic determinants that are associated with enhanced CTL function and maintenance of responsiveness against virus, with cross reactivity demonstrated against escape mutants continuing into chronic infection (Saez-Cirion et al., 2007b). Individuals who express the HLA-B alleles HLA-B27 and HLA-B57 commonly show sustained viral control and these alleles are frequently present in individuals who are either LTNP or efficient viral controllers (Flores-Villanueva et al., 2001, Migueles et al., 2000).

The exact mechanism underpinning protection is unknown, but it has been speculated that these MHC molecules might present antigens in such a way that enhances TCR engagement, or that the MHC molecule itself demonstrates differential binding avidity to TCR such that more appropriate T-cell responses are generated (Saez-Cirion et al., 2007b). The HLA-B27 and –B57 molecules are involved in the presentation of HIV-1 gag protein epitopes, which are particularly important for immune recognition and CTL activity, and it has been demonstrated that responses to gag peptides generate the most active anti-HIV-1 responses that are most capable of lowering plasma viral load (Saez-Cirion et al., 2009, Kiepiela et al., 2007, Edwards et al., 2002).

HIV-1, through a number of mechanisms, modulates or downregulates $CD8^+$ T-cell activity to prevent elimination. The HIV-1 derived protein nef downregulates surface MHC class I expression, particularly classical HLA-A and HLA-B molecules, by disrupting the trafficking of molecules to the cell surface, preventing epitope presentation to $CD8^+$ T-cells and therefore preventing CTL-mediated killing of infected cells (Kasper & Collins, 2003, Williams et al., 2002, Cohen et al., 1999, Schwartz et al., 1996). Chronic HIV-1 infection upregulates the expression of inhibitory KIR on $CD8^+$ T-cells, which are normally expressed at low levels on $CD8^+$ T-cells and are more usually associated with NK cells and $CD8^+$ T-cells in elderly individuals (Alter et al., 2008). KIR^+CD8^+ T-cells in HIV-1 infected individuals were found to be severely functionally impaired following antigen-specific stimulation with pools of HIV-1 peptides, demonstrating defects in cytokine production, degranulation responses and proliferation (Alter et al., 2008). Interestingly though, KIR^+CD8^+ T-cells from HIV-1 seropositive individuals were shown to be sensitive to non-antigenic stimulation and defects were not KIR-specific, but instead represented a broader dysfunction in TCR mediated activation (Alter et al., 2008).

HIV-1-specific $CD8^+$ T-cells demonstrate skewed maturation with an accumulation of cells in the TEM subset that are non-terminally differentiated and this has been associated with HIV-1-specific $CD8^+$ T-cell dysfunction seen in chronic HIV-1 infection (Champagne et al., 2001). Chronic antigen-specific stimulation, 'antigenemia', ultimately leads to the generation of exhausted $CD8^+$ T-cells, which in some chronic infections can lead to the deletion of exhausted cells (Wherry, 2011, Wherry et al., 2003). T-cell exhaustion occurs during chronic infections and is associated with a loss of effector functions including defects in cytokine production, proliferative responses and cytotoxic functions (Wherry, 2011). Combined with the loss of effector function, there is an increase in the expression

of inhibitory receptors, particularly PD-1 or KIR, and a loss of some activating receptors such as NKG2D, further disrupting normal T-cell function (Blackburn et al., 2009, Alter et al., 2008). Blockade of the PD-1 pathway enhanced T-cell responsiveness in chronic simian immunodeficiency virus (SIV) infection (Velu et al., 2009) and similar findings for other receptors are discussed in a review by Wherry (2011).

Experimental addition of IL-2 has been shown to restore T-cell proliferation and function (Blattman et al., 2003). Clinical trials using IL-2, however, in chronic HIV-1 infection in combination with HAART increased overall cell numbers, but they did not improve the function of HIV-1-specific T-cells or reduce viral load any faster than HAART alone (Michaelsson et al., 2008). Furthermore, administering IL-2 has also been associated with the generation of regulatory T-cells (T_{Reg}), which inhibit anti-HIV activity (Velilla et al., 2008, Shah et al., 2006). Maintenance of IL-21 production during chronic HIV-1 infection by $CD4^+$ T-cells maintains $CD8^+$ T-cells and prevents them becoming exhausted and is associated with better viral control (Chevalier et al., 2011); $CD8^+$ T-cells in a cohort of elite controllers have also been demonstrated to produce IL-21 (Williams et al., 2002). Expanded populations of $FoxP3^+CD25^+$ T_{Reg} cells are associated with HIV-1 progression (Andersson et al., 2005). T_{Reg} cells produce the immunosuppressive cytokines TGF-β and IL-10 and function by suppressing immune activity, reducing inflammation and antigen-specific immune responses (Elsaesser, Sauer & Brooks, 2009, Brooks et al., 2006).

Finally, the minor $\gamma\delta^+$ T-cell subset expressing the γδ-TCR may have a minor role in HIV-1 pathogenesis (Boullier et al., 1997). The γδ-T-cells are an important subset of T-cells with innate cell-like properties and the γδ-TCR cannot match the αβ-TCR of $CD4^+$ or $CD8^+$ T-cells in terms of antigen-specific responses (Casetti et al., 2009). Instead of peptide-specific recognition the γδ-TCR recognises unprocessed phosphoantigens not associated with MHC-molecules and can respond potently, displaying strong proliferative responses and cytotoxic as well as cytokine-producing properties (Poccia et al., 1999, Haas, Pereira & Tonegawa, 1993). Upon activation γδ-T-cells also stimulate and boost $CD8^+$ T-cell responses, and this property has led to the idea that γδ-T-cells are a useful target for adjuvants. Drugs targeting the γδ-TCR are already used in cancer therapy (Roelofs et al., 2009, Kunzmann et al., 2000). Other activating receptors such as TLR and NKG2D are also expressed on γδ-T-cells, further suggesting their potential use as adjuvant targets (Casetti & Martino, 2008).

Chronic HIV-1 infection leads to the increase in number, in peripheral blood, of the Vδ1 class of γδ-T-cells, switching the normal Vδ1:Vδ2 balance (Poccia et al., 1998, Wallace, Malkovsky & Carding, 1995). Furthermore clonal expansion of peripheral blood Vδ1 cells is not observed, and it is believed that these cells emigrate from tissue compartments, where they are the predominant cell type, in response to the cytokine environment created by HIV-1 infection (Wallace, Malkovsky & Carding, 1995). The Vδ1 cells that traffic to the blood display an activated phenotype and are thought to contribute to the immunopathogenesis seen in HIV-1 infection (Boullier et al., 1997). One γδ-T-cell subset, the Vγ9Vδ2 cells, have been demonstrated to have anti-HIV-1 cytotoxic activity towards HIV-1 infected cells, but is also functionally impaired in chronic infection (Wallace et al., 1997, Poccia et al., 1996).

1.6.3. Dendritic cells in HIV-1 infection

DC located in the skin and in the mucosa encounter virions early in infection. DC express CCR5 and CXCR4 making them a viable target for HIV infection and pDC, which express CD4, are particularly susceptible (Borrow & Bhardwaj, 2008, Donaghy et al., 2003, Patterson et al., 2001). Infected DC have been shown to transfer infectious virions directly to T-cells (Yu, Reuter & McDonald, 2008) without releasing virus into the extracellular environment and this has been proposed as a model for dissemination of the virus to the secondary lymphoid tissue, prior to systemic infection and viraemia (Piguet & Steinman, 2007). HIV-1 infection of DC prevents normal activation and leads to dysregulation, interfering with their ability to promote appropriate innate and adaptive immune responses (Donaghy et al., 2003).

The single stranded RNA genome of HIV-1 is innately recognised by and activates pDC via TLR7 and TLR8 ligation in endosomal compartments and is likely responsible for the high levels of IFN-α produced early in infection. IFN-α enables the activation of $CD4^+$ and $CD8^+$ T-cells in the short term, but they become unresponsive and unable to proliferate as infection progresses (Stacey et al., 2009, Boasso et al., 2008, Borrow & Bhardwaj, 2008, Alter et al., 2007a). pDC have been shown to produce the enzyme indoleamine 2,3-dioxygenase (IDO) following exposure to HIV, which prevents the proliferation of T-cells during HIV-1 infection (Boasso et al., 2008, Boasso et al., 2007). HIV-1 infection has also been shown to block DC maturation and enhance IL-10 production, causing immunosuppression during chronic infection (Patterson et al., 2005, Granelli-Piperno et al.,

2004). It has been shown that the total number of pDC and mDC in peripheral blood are reduced during HIV-1 infection (Donaghy et al., 2001); and in lymphoid tissues in SIV-infected rhesus macaques (Brown, Trichel & Barratt-Boyes, 2007). TLR responsiveness of DC is reduced in chronically HIV-1 infected patients, who produce less IL-12 and IFN-α and more immunosuppressive IL-10 (Martinson et al., 2007). The normal responses of DC to infection are altered during acute and chronic HIV-1 infection, which effects the production and maintenance of appropriate innate and adaptive immune responses.

pDC and mDC frequency in peripheral blood recovers with HAART, although the rate of recovery and the full extent is correlated to baseline $CD4^+$ T-cell number at initiation of treatment and/or viral set point; higher $CD4^+$ T-cell frequencies and a lower viral set point correlate with more rapid and full recovery of cell frequency (Azzoni et al., 2007, Chehimi et al., 2007). IFN-α production is recovered in pDC following successful treatment, as demonstrated by responsiveness to both CpG 2216 motifs and inactivated Influenza virus (Azzoni et al., 2007). mDC show similar functional recovery in seropositive individuals receiving HAART and produce IL-12 in response to LPS at equivalent levels to seronegative individuals (Azzoni et al., 2007). A reduction in MHC class I expression is also observed on both DC subsets indicating reduced chronic activation (Azzoni et al., 2007). It is noteworthy that other studies have demonstrated incomplete pDC recovery, but it is likely that individuals used in this study had more progressive disease at the initiation of therapy (Chehimi et al., 2002).

1.6.4. NK Cells in HIV-1 Infection

NK cells display functions that demonstrate a protective effect against HIV-1 infection when normal anti-viral function is maintained and additionally certain genetic determinants are associated with enhanced viral control and slow disease progression. A subset of NK cells directly kill virus-infected $CD4^+$ T-cells; NK cells lacking the KIR2DL1, -DL2 and CD94 inhibitory receptors were able to kill HIV-1 infected $CD4^+$ T-cells whose HLA-A and –B alleles had been downregulated by HIV-1 nef modulation, which prevents CTL responses (Bonaparte & Barker, 2004). CD85j is an inhibitory NK cell receptor that usually functions by binding MHC-class I molecules, but has been shown to control HIV-1 replication *in vitro* within monocyte-derived DC via an unknown ligand and inhibitory pathway (Scott-Algara et al., 2003). A further mechanism by which NK cells directly lyse HIV-1 infected cells is through ADCC, mediated by $CD16^+$ NK cells in model systems using HIV-1-peptide-

specific antibodies, demonstrating that non-neutralising antibodies directed toward infected cells represent a potential target for prophylactic vaccine and immunotherapeutic strategies (Stratov, Chung & Kent, 2008, Forthal et al., 2006, Forthal et al., 2005, Gupta et al., 2005).

NK cells also indirectly inhibit HIV-1 infection by producing the CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES, which can successfully compete with HIV-1 for the CCR5 receptor, thereby inhibiting viral entry of target cells (Kottilil et al., 2003). Inhibition via this route, however, is diminished in viraemic infection and the ability of NK cells to inhibit is inversely correlated to viral load.

Increasing evidence is emerging for a link suggesting that patients with the HLA-B Bw4-80I allele (also described as the *B58Ile80* allele) are slow progressors to AIDS and that this effect is associated with its interaction with the activating KIR3DS1 receptor expressed on NK cells (Alter et al., 2007, Martin et al., 2002). HLA-B Bw4-80I is the putative ligand for KIR3DS1 and it has been shown that KIR3DS1$^+$ (and other activating KIR) NK cells are significantly upregulated during acute HIV infection in patients who are HLA-B Bw4-80I$^+$. It has also been shown that these individuals have increased inhibitory KIR3DL1 expression during chronic HIV-1 infection (Alter et al., 2007b, Martin et al., 2002). KIR3DS1$^+$ KIR3DL1$^+$ NK cells preferentially expanded during acute HIV-1 infection *in vitro* in the presence of HLA-Bw4-80I (Alter & Altfeld, 2009, Alter et al., 2007). Furthermore, NK cells expressing KIR3DS1 were shown to lyse HIV-1 infected cells expressing the HLA-B Bw4-80I allele and efficiently restricted viral replication (Alter et al., 2007).

During acute HIV-1 infection CD56^{lo}CD16$^+$ cells are rapidly expanded whereas CD56^{hi}CD16$^-$ NK cells are not, and the proceeding responses are predominantly cytotoxic, demonstrating a defect in CD56^{hi}CD16$^-$ cytokine production that is important for the generation of adaptive immune responses (Alter et al., 2005). The production of IFN-γ remains impaired in chronic HIV-1 infection despite effective treatment that increases NK cell numbers and improves their cytotoxic potential (Azzoni et al., 2002). Early initiation of treatment during acute infection better restores the NK cell compartment, delaying the onset of functional impairment, and a similar situation is observed in non-progressor patients who preserve NK cell cytotoxic and cytokine functions without treatment (Alter et al., 2005).

NK-derived IFN-γ impacts on maturation and activation of DC further influencing the formation of an appropriate adaptive response, and NK activity and IFN-γ production has been shown to be important in the generation of antigen-specific immune responses directed against HIV-1 infection (Loo et al., 2009, Alter et al., 2007b). It has been suggested that the apparent lack of expansion of $CD56^{hi}CD16^{-}$ NK cells in peripheral blood may be attributed to the known capacity of these cells to migrate to SLT where T-cell activation occurs (Borrow & Bhardwaj, 2008). IFN-γ production by NK cells is impaired in chronic HIV-1 infection and this impairment has in part been attributed to reduced IL-12 stimulation, which in turn is attributed to reduced numbers of mDC (Yonkers et al., 2009). Furthermore it has been shown that NK cell IFN-γ production is maintained in LTNP and that the maintenance of DC numbers and DC-NK interactions, particularly the production of IL-12 by mDC, is a major determining factor in the control of infection (Saez et al., 2007). Higher levels of NK IFN-γ production are observed in individuals who have been exposed to HIV-1 infection through sexual contact, but remain seronegative, termed exposed seronegative (ESN) individuals, compared to both HIV-1 infected individuals and to healthy control individuals (Montoya et al., 2006). A different cohort of ESN individuals exposed to HIV-1 through intravenous drug use demonstrated high activation and cytotoxic capacity directed toward MHC class I-devoid K562 target cells compared to either HIV-1 infected individuals or seronegative controls (Scott-Algara et al., 2003). Further studies with ESN individuals reveal that NK cells in these individuals may have a lower threshold for activation and increased ability to recognise HIV-1 transformed cells due to specific receptor repertoires on NK cells (Ravet et al., 2007). The ESN in this study had high expression of activating KIR and the activating NKG2C receptor with lower proportions of the inhibitory NKG2A receptor and displayed high background levels of activation and degranulation as measured by CD69 and CD107a expression (Ravet et al., 2007).

Viral infections are known to subvert immune responses in order to survive in their host; HIV-1 expresses genes that prevent immune responses that would otherwise have a negative effect on the virus. Subversion of normal NK activity occurs during HIV-1 infection. As described above, the HIV-1 nef protein downregulates the cell surface expression of the MHC class I molecules HLA-A/B, potently restricting $CD8^{+}$ T-cell responses whilst retaining the expression of HLA-C/E alleles, downregulation of which normally leads to activation of NK cells and target cell killing (Alter & Altfeld, 2009, Borrow

& Bhardwaj, 2008). HIV-1 nef also prevents the upregulation of surface expression of MIC-A and ULBP1, 2 and 3 (MHC class I homologues recognised by the NKG2D NK cell activating receptor) thereby reducing the ability of infected cells to activate NK cells (Cerboni et al., 2007). The impairment of normal NK cell function strongly implies that NK cells have a role in the control of viral infection, either through direct killing of target cells or through the cross talk with DC that enhances antigen-specific immune responses. The downregulation of NK cell activity by HIV-1 implies these mechanisms have been established as it is beneficial for virus survival and transmission.

Overall numbers of $CD3^-CD56^+$ NK cells are reduced in chronic HIV-1 infection and are displaced in long-term infected treatment-naïve individuals by a dysfunctional $CD3^-CD56^-$ $CD16^+$ fraction with severely impaired cytotoxic and cytokine producing functions (Mavilio et al., 2003). The mechanism driving the generation of the dysfunctional $CD56^-$ NK cell population is not clear, although HIV-1-associated chronic immune activation may have some impact (Alter et al., 2005, Mavilio et al., 2005, Goodier et al., 2003). Phenotypically, a high frequency of $CD3^-CD56^-CD16^+$ cells express inhibitory KIR, including KIR2DL1, KIR2DL2 and KIR3DL1, and have low surface expression of the activating natural cytotoxicity receptors (NCR) NKp30 and NKp46 (Mavilio et al., 2005).

In contrast to the above changes in NK cell receptors, the expression of inhibitory NKG2A/CD94 receptor is reduced in $CD56^+$ NK cells from HIV-1 infected individuals and there is an increase in the activatory NKG2C/CD94 heterodimer that further disrupts NK cell function during HIV-1 infection (Mela et al., 2005). The increase in the NKG2C at the expense of NKG2A has been linked to chronic infection and co-infection with human cytomegalovirus. This may contribute to the decline in $CD4^+$ T-cells expressing HLA-E and could also potentially be a mechanism driving NK cell exhaustion (Goodier & Coleman, 2009, Guma et al., 2006, Mela et al., 2005).

NK cells have also been demonstrated to be responsible for the removal of HIV-1 infected $CD4^+$ T-cells (Vieillard, Strominger & Debre, 2005). The HIV-1 protein, gp41, was shown to induce the presentation on $CD4^+$ T-cells of a ligand for the NCR NKp44, which is upregulated on peripheral blood NK cells during chronic HIV-1 infection and NK-mediated cytotoxicity of infected cells has been demonstrated in an NKp44-dependant fashion (Vieillard, Strominger & Debre, 2005). Furthermore, in a Simian human immunodeficiency virus (SHIV) model of HIV infection, macaques immunised with an anti-gp41 antibody

demonstrated maintenance of $CD4^+$ T-cells by preventing the expression of the NKp44 ligand and subsequent NK cell-mediated cytotoxicity (Vieillard et al., 2008).

Functional impairment of NK cells and alteration of NK cell phenotype in chronic HIV-1 infection is associated with chronic stimulation of NK cells, as demonstrated by the activated phenotype in NK cells from HIV-1 seropositive individuals, which, though partially activated are functionally deficient (Fogli et al., 2004). The NK cells in the study by Fogli *et al* (2004) were demonstrated to have increased expression of CD69, CD25 and HLA-DR, but had reduced cytotoxic capabilities.

As described in Section 1.6.3, DC numbers, particularly pDC, are reduced in chronic HIV-1 infection (Donaghy et al., 2001) and responsiveness to TLR agonist stimulation is also reduced (Martinson et al., 2007). The HIV-1 ssRNA genome is recognised by DC (Alter et al., 2007a), and is a potential mechanism of chronic, non-specific, immune stimulation of DC leading to exhausted responses and reduced innate responsiveness (Martinson et al., 2007). An additional mechanism of potential chronic innate stimulation has been demonstrated, whereby early pathology seen in GALT allows microbial translocation to occur and microbial products to enter the body, causing chronic, systemic immune activation demonstrated by increased plasma LPS in chronic HIV-1 infection (Brenchley et al., 2006, Brenchley et al., 2004). It has further been demonstrated that NK cells from individuals with raised plasma LPS are refractory to further LPS stimulation, indicating that chronic stimulation, most likely through TLR4, is also responsible for DC and subsequent NK impairment. This has also been associated with dementia due to increased monocyte activation in the brain (Gregson et al., 2009, Ancuta et al., 2008).

In healthy individuals the NK cell population in the gut is predominantly $CD56^{hi}CD16^-$ and therefore has relatively low cytotoxic potential, and a population of NK cells in mucosa associated lymphoid tissue (MALT) produce the cytokine IL-22 involved in promoting anti-inflammatory responses at mucosal sites, thereby limiting inflammation and damage (Cella et al., 2009). Chronic HIV-1 infection reduces NK cell numbers in the gut, but this is partially reversed by HAART, however the NK cells that remain in the colonic lamina propria are $CD56^{lo}CD16^+$ NK cells, which potentially contribute further to HIV-1 pathogenesis observed in the gut (Goodier & Coleman, 2009, Mela et al., 2007).

The lack of complete functional and numerical recovery of NK cells with HAART and the fact that NK cell recovery is slower than T-cell and DC recovery implies that for NK

recovery to take place factors produced by these cells are required and demonstrates a lack of full immune control, despite recovery in $CD4^+$ T-cell numbers and reduction of viraemia (Azzoni et al., 2007).

The loss of cytokine stimulation during HIV-1 infection impacts on NK cell differentiation, proliferative capacity and function (Goodier & Coleman, 2009). IL-2, -7, -15, -21 and -12 production is reduced in HIV-1 infection and is partially recovered with HAART (Sirskyj et al., 2008, Marshall et al., 1999). Different cytokine stimulation affects NK cells differently, but can promote differentiation, proliferation, NK cell-mediated killing of HIV-1 infected $CD4^+$ T-cells and the production of cytokines, chemokines and perforin (Goodier & Coleman, 2009 and references therein).

1.7. Aims of thesis

The aims of this thesis are to provide a better understanding of innate immune responsiveness in chronic HIV-1 infection. Using TLR agonists as model innate stimulants, work undertaken within this thesis will investigate the impact of chronic anti-retroviral treated and untreated HIV-1 infection on both the NK cell compartment and the T-cell compartment and seek to further resolve functional abnormalities. Furthermore peripheral blood NK cell maturation will be investigated in the context of chronic immune activation during HIV-1 infection. Studies will be conducted examining the innate responsiveness of different memory T-cell subsets and, finally, using TLR agonists as model adjuvants, the ability to modulate antigen-specific T-cell responses in HIV-1 infected individuals will be explored.

1.8. Hypothesis

Responsiveness to certain classes of TLR agonists are maintained better than others during chronic HIV-1 infection and these TLR agonists represent potential model adjuvants for the recovery of HIV-1-specific T-cell responses in chronically infected individuals. Furthermore NK cell function is intrinsically linked to successful strategies aimed at improving appropriate T-cell priming conditions for effective anti-HIV-1 adaptive immunity.

2. Materials and methods

2.1. Overview

All laboratory work was carried out in the Department of Immunology, Chelsea and Westminster Hospital, London, UK, and was completed in accordance with local health and safety rules. All work with known infectious samples was performed in a containment level three facility. Full training for the use of this facility was completed, including 30 hours observation and 30 hours supervised use by registered users before being granted authorisation by the facility manager. All other work with human samples was carried out in containment level two laboratories.

2.2. Ethical approval

The Riverside Research Ethics Committee and the National Research Ethics Committee provided permission for all the studies conducted and presented in this thesis. Informed, written consent was obtained from all individuals who provided peripheral blood samples for the studies conducted.

2.3. Recruitment

HIV-1 seropositive patients attending outpatient clinics at Chelsea and Westminster Hospital were recruited and peripheral blood samples collected. HIV-1 seronegative samples were obtained with informed consent from local healthy volunteers.

2.4. Sample collection

Up to 60ml peripheral blood was collected into 6ml lithium heparin (green-capped) vacutainers (Becton Dickinson, Oxford, UK). Trained phlebotomists carried out the sample collection, and all samples were processed within two hours of being obtained. Recruiting clinicians provided patient viral load and $CD4^+$ T-cell quantification information.

2.5. PBMC isolation from peripheral blood

Blood samples were pooled into 50ml screw-top tubes before up to 30ml blood was gently layered over 15ml hystopaque-1077 (Sigma-Aldrich, St. Louis, USA). Samples were then spun in a centrifuge at 2000 RPM for 30 minutes without the use of a brake, which could disturb separated layers, causing mixing. After separation was complete at least 2ml of the upper, plasma layer was removed and split into 500µl aliquots for storage at -80°C for

use in future assays. PBMC were then transferred to 50ml centrifuge tubes and washed with cell culture media containing: RPMI-1640 cell culture medium supplemented with 100IU ml^{-1} penicillin, 100µg ml^{-1} streptomycin and 2mM L-glutamine (all Sigma-Aldrich). Cell suspensions were first spun for seven minutes at 1800 RPM, supernatant poured out into virkon and then washed again with culture media before cells were enumerated. To count cells, 10µl cell suspension was removed and mixed 1:1 with trypan blue (Sigma-Aldrich) before viable cells were counted using disposable haemocytometers (Hycor, California, USA). Once cells had been counted, they were spun for 7 minutes at 1500 RPM, supernatant was discarded and cells were resuspended in culture media with 10% sterile foetal calf serum (FCS) or 10% pooled screened human AB sera (both Sigma-Aldrich) at a concentration of either 1×10^6 cells ml^{-1} for innate stimulation assays (unless otherwise stated) or at 2.5×10^6 cells ml^{-1} for ELISpot (described in section 2.10) assays. FCS and human AB sera were both heat inactivated prior to use by incubating in a water bath at 56°C for 30 minutes.

PBMC were then stimulated as described below and cultured in 5ml round-bottomed polypropylene tubes (Becton Dickinson), 96-well U-bottomed cell culture plates (Greiner Bio-One, Stonehouse, UK), or 96-well plates with Immobilon-P membranes (Millipore, Tullagreen, Ireland) for ELISpot assays. Stimulated cell cultures were incubated at 37°C and 5% CO_2.

2.6. *In vitro* stimulations

The majority of assays presented in this thesis involve *in vitro* stimulation of PBMC. PBMC were prepared as described above and stimulants were added directly to cell cultures. All assays included a negative control of cells cultured in media alone without additional stimulation.

2.6.1. TLR agonist preparation and stimulation

Lyophilized TLR agonists were prepared according to manufacturers' instructions using sterile, endotoxin-free water. Pam3CSK, Poly(I:C), Flagellin, Imiquimod R837 and CpG ODN2216 & ODN2006 (all InVivoGen, San Diego, USA) were all dissolved and stored in aliquots at -20°C. Heat-killed *Listeria monocytogenes* (HKLM), LPS, FSL-1 and ssRNA40 & ssRNA41 were all dissolved and stored at 4°C. LPS and both ssRNA molecules were used within two weeks of reconstitution and HKLM and FSL-1 were stable for six and 12

months, respectively (All InVivoGen except LPS, Sigma-Aldrich). TLR agonists were diluted in cell culture media before being directly applied to cell cultures. Concentrations of agonists used are described in individual results sections.

2.6.2. Cytokine preparation and stimulation

For many of the assays presented in this thesis a combination of IL-12 and IL-15 was used as a control for stimulation. Both cytokines were reconstituted from lyophilised preparations using sterile PBS and stored in aliquots at -80°C (IL-12) and -20°C (IL-15) (Both Peprotech, London, UK). Cytokines were thawed and diluted in cell culture media before being applied to cell cultures at 1ng ml^{-1} for IL-12 and 20ng ml^{-1} for IL-15.

2.6.3. Peptide preparation

In some assays peptide pools were used to stimulate PBMC. Three different pools were used. 1) a lyophilised pool of 32 8-12mer peptide fragments from influenza, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (FEC) was obtained in conjunction with the European Vaccines and Microbicides Network from the Centre for AIDS Reagents held at the National Institute for Biological Standards and Controls (NIBSC) (Appendix Table A.11). 2) 22 separate lyophilised HIV-1 gag p24 20mer peptide fragments with 10 amino acid overlaps, also obtained from the European Vaccines and Microbicides Network from the Centre for AIDS Reagents at NIBSC (Appendix Table A.12). 3) 36 8-11mer HIV-1 gag p24 (majority 9mer and referred to as '9mer pool') peptide fragments (dissolved in cell culture media and stored at -80°C) (HIV-1 gag 9mer peptides kindly provided by Dr N. Imami, Department of Immunology, Imperial College London). An additional six individual lyophilised HIV-1 gag p24 9mer peptide fragments were obtained from the European Vaccines and Microbicides Network at NIBSC (Appendix Table A.13).

The lyophilised FEC peptide pool was dissolved in sterile PBS at 1mg ml^{-1} and stored in aliquots at -80°C. Lyophilised HIV-1 gag p24 20mer and 9mer peptides were dissolved in cell culture medium before being pooled and diluted at 20µg ml^{-1} for storage in aliquots at -20°C.

Peptides were thawed, and where necessary diluted further in cell culture medium, before being applied directly to cell cultures at 5µg ml^{-1}, unless otherwise stated.

2.6.4. Phytohaemagglutinin preparation

Phytohaemagglutinin (PHA) (Sigma-Aldrich) was used as a positive mitogen control in ELISpot assays. This was diluted in cell culture medium to 10µg ml^{-1} and stored in aliquots at -20°C. PHA was thawed and applied directly to cell cultures at a final concentration of 5µg ml^{-1}.

2.7. Antibody staining for flow cytometry assays

Many of the assays presented in this thesis involve the use of flow cytometry involving various different staining techniques. Individual monoclonal antibody cocktails for various assays are listed in Appendix Section A.1. Antibodies were titrated for optimal staining.

2.7.1. Surface staining

Supernatant was first removed without disturbing cells, and was stored at -80°C for future assays. PBMC for staining were transferred to 4.5ml round-bottomed polystyrene FACS tubes (Becton Dickinson), 2ml of FACS buffer was added to tubes before being spun for four minutes at 1200 RPM. FACS buffer contained: 500ml PBS + 1% FCS (both Sigma-Aldrich) + 1% EDTA (Invitrogen, Paisley, UK) + 0.05% NaN_3 (Sigma-Aldrich). Excess buffer and culture media were then poured off into virkon and fluorochrome-conjugated monoclonal antibody cocktails were added to each tube and mixed thoroughly before incubation for 30 minutes at 4°C. Tubes were then washed again with 2ml FACS buffer and spun for four minutes at 1200 RPM. Depending on the assay, excess FACS buffer and antibodies were then poured off into virkon and cells were fixed in an appropriate volume of 2% paraformaldehyde (PFA) prior to acquisition by flow cytometry or further processed and stained as described below.

2.7.2. Intracellular cytokine staining

Intracellular cytokine staining (ICCS) of IFN-γ required further processing than for surface staining alone. Firstly, prior to the surface stain procedure described above, GolgiPlug (Becton Dickinson) (containing Brefeldin A) was incubated with cell cultures for four hours at a final volume of 1µl per ml of culture. Brefeldin A is a protein transport inhibitor, which prevents the release of cytokines such as IFN-γ, resulting in their accumulation within the cell. After four hours, surface staining was carried out as described, and prior to fixation in PFA cells were incubated with 500µl of FACS fixation/permeabilising solution (Perm2 –

Becton Dickinson) diluted 1:10 with distilled water, vortexed, and incubated at room temperature for 10 minutes. Permeabilisation creates holes in the cell membrane, allowing antibodies targeting intracellular components to pass into cells and bind to their targets. Following permeabilisation, cells were washed again in FACS buffer and spun for five minutes at 2000 RPM and the excess was poured off. Cells are then incubated for 30 minutes at room temperature in the dark with anti-IFN-γ monoclonal antibodies before being washed in FACS buffer and spun for five minutes at 2000 RPM. Cells were then fixed in 2% PFA prior to acquisition.

2.7.3. Degranulation; CD107a staining

CD107a is used as a marker for lymphocyte activation and degranulation for some assays (Alter et al., 2004b, Betts et al., 2003). Following stimulation and culture of PBMC, cells are incubated for one hour with anti-CD107a monoclonal antibody at 37°C and 5% CO_2. Following incubation with antibody, GolgiPlug (Becton Dickinson) was incubated with cells (1µl per ml of culture, as for ICCS) for four hours at 37°C and 5% CO_2 before surface staining was carried out (as described in 2.8.1).

2.7.4. MHC class I pentamer staining

Antigen-specific $CD8^+$ T-cells expressing specific MHC class I-peptide complexes were stained using fluorochrome-conjugated MHC class I pentamers (ProImmune, Oxford, UK). PBMC were washed in FACS buffer and centrifuged for four minutes at 1200 RPM. Cells were then incubated with pentamers for 10 minutes at room temperature before washing with FACS buffer and centrifugation for four minutes at 1200 RPM. Surface staining was then carried out as described in section 2.8.1, except that this was performed on ice for 20 minutes.

2.8. Isolation or purification of lymphocyte subsets by magnetic cell separation

A number of assays have been carried out using magnetic cell separation to either remove a particular lymphocyte population or isolate a single lymphocyte population. For all assays, a sample of cells was taken before and after separation to determine the purity of recovered populations.

2.8.1. Isolation of untouched total peripheral blood NK cells

NK cell isolation was carried out using an NK cell isolation kit (Miltenyi-Biotec, Bergisch Gladbach, Germany), according to the manufacturers instructions. In order to isolate an untouched pure NK cell population, PBMC isolated as described in section 2.6, were enumerated and resuspended in MACS buffer (sterile PBS containing 1% FCS (both Sigma-Aldrich) + 1% EDTA (Invitrogen, Paisley, UK)). The required volume of cells was transferred to 15ml centrifuge tubes and MACS buffer was added to a total volume of 10ml before tubes were spun for five minutes at 1300RPM. Excess MACS buffer was poured off into virkon before cells were mixed with a biotinylated antibody cocktail that specifically recognised non-NK cells within PBMC suspensions (Miltenyi-Biotec, Bergisch Gladbach, Germany). The cocktail was added at a volume of 10µl per 1×10^7 PBMC per 40µl MACS buffer and incubated for 15 minutes at 4°C. Streptavidin conjugated Macs™ microbeads (Miltenyi-Biotec) were subsequently added at a volume of 20µl per 1×10^7 cells alongside an additional 30µl MACS buffer and incubated for 15 minutes at 4°C. Cells were then washed in 10ml MACS buffer and spun for five minutes at 1300 RPM and resuspended in 500µl MACS buffer.

Cells were then applied to pre-rinsed (3ml MACS buffer) LS-sized MACS™ depletion columns (capacity up to 3 x 10^7 bound cells) in a magnetic MACS™ separator (Miltenyi-Biotec). NK cells that passed through the column were collected in 15ml centrifuge tubes. After cells were applied the column, they were flushed through three times with a further 3ml MACS buffer; each 3ml was allowed to run through completely before further application. MACS buffer was added to the collection tubes to give a total volume of 10 ml before cell suspensions were spun for five minutes at 1300 RPM. Cells were resuspended in 10ml cell culture medium and enumerated before being washed again as above and resuspended at 1×10^6 cells ml^{-1} in culture medium for stimulation with TLR agonists or cytokines as described in Section 2.7.1 and 2.7.2.

2.8.2. $CD56^+$ cell depletion

CD56 depleted PBMC were either used in add-back assays in conjunction with $CD56^{hi}CD16^-$ NK cells, the isolation of which is described below in section 2.9.3, or they were stimulated directly in antigen-specific ELISpot assays described in section 2.11.

$CD56^+$ cell depletion was carried out using a MACS kit (Miltenyi-Biotec). The procedure followed was similar to that described above for NK cell isolation (2.9.1) with some amendments. 2×10^7 PBMC were diluted in 2ml of MACS buffer in 4ml round bottomed tubes before being spun for four minutes at 1200 RPM. Excess buffer was discarded and cells were mixed with 20µl MACS™ beads conjugated to anti-CD56 antibodies and 80µl MACS buffer and incubated for 15 minutes at 4°C before further washing and resuspension in 500µl MACS buffer.

Cells were then applied to an MS-sized MACS™ column (capacity up to 1 x 10^7 bound cells) attached to a MACS™ separator. The column was pre-rinsed with 500µl MACS buffer before cells were added. Cells were collected in a 15ml centrifuge tube and the column was flushed through three times with 500µl MACS buffer. The tube was then spun for four minutes at 1200 RPM and cells were resuspended in 10ml cell culture medium. Cells were enumerated and washed once more and spun for seven minutes at 1500 RPM. NK cell-depleted PBMC were resuspended in medium at a concentration of 1×10^6 cell ml^{-1}, for assays where they were recombined with purified NK cell populations, or at 2.5×10^6 cells ml^{-1} for ELISpot assays.

2.8.3. Isolating $CD56^{hi}CD16^-$ NK cells for add-back

To study the maturation of NK cells it was necessary to firstly remove lineage $marker^+$ (including CD3, CD14, CD19) and $CD16^+$ NK cells by negative selection to facilitate the isolation of $CD56^{hi}CD16^-$ NK cells by positive selection (with anti-CD56). Enriched $CD56^{hi}CD16^-$ NK cells were subsequently added back to CD56-depleted PBMC generated as described above in Section 2.9.2. This cell enrichment was performed using the $CD56^+CD16^-$ NK cell isolation kit (Milteyi-Biotec).

8×10^7 PBMC were prepared from 80ml of blood (HIV-1 seronegative control donors only), washed as described above and resuspended in 400µl MACS buffer in a 15ml centrifuge tube. Cells were then incubated for 10 minutes at 4°C with 100µl of biotin-antibody cocktail (Miltenyi-Biotec), which labels all cells within PBMC except for those expressing CD56 in the absence of other lineage markers such as CD3, CD14 and CD16. Cells were then washed in 10ml MACS buffer, spun for five minutes at 1300 RPM, resuspended in 800µl buffer and then incubated for 15 minutes at 4°C with 200µl micro bead cocktail (Miltenyi-Biotec). Washing was repeated and cells were resuspended in 500µl MACS

buffer. Labeled cells were then passed through an LS size MACS column as described in section 2.9.1, and the eluent was washed as described and resuspended in 400µl MACS buffer in a 15ml centrifuge tube.

Following initial enrichment of $CD56^+CD16^-$ NK cells, these were purified further by incubation for 15 minutes at 4°C with CD56 Micro Beads (Mitenyi-Biotech). Cells were then washed and resuspended in 500µl prior to being flushed through an MS-sized MACS column (described in 2.9.2). In this positive selection step, the flow-through was discarded, the column was then removed from the MACS separator magnet and positioned above a 5ml round-bottomed tube, 1ml MACS buffer was added and then the column flushed through using a plunger. Cells were then washed twice in 2ml cell culture medium, spun each time for seven minutes at 1500 RPM before being resuspended in 2ml culture media.

$CD56^+CD16^-$ NK cells were then added to 2×10^7 CD56 depleted PBMC generated from the same sample at a frequency of 1-5%, depending on cell recovery. The $CD56^+CD16^+$ depleted PBMC were then spun down once more and resuspended at 1×10^6 cells ml^{-1} in media. Cells were aliquoted into 5ml polypropylene cell culture tubes (Becton Dickinson) and stimulated with cytokines or TLR agonists for maturation assays described in Section 4.2.

2.8.4. $CD19^+$ B-cell depletion

B-cells were removed for some antigen-specific ELISpot assays in order to determine the role played by this cell type in observed results. CD19 microbeads (Miltenyi-Biotec) were used to negatively select for non-$CD19^+$ cells. 2×10^7 PBMC were prepared as described for CD56 depletion in section 2.9.2 and incubated with 20µl CD19 microbeads for 15 minutes at 4°C. PBMC were then washed before being passed through an MS-sized MACS column (as described in Section 2.9.2) and washed again before being resuspended in cell culture medium at 2.5×10^6 cells ml^{-1} for ELISpot assays.

2.8.5. $BDCA\text{-}4^+$ plasmacytoid dendritic cell depletion

Plasmacytoid dendritic cells were removed for the same ELISpot assays as described in 2.9.4. BDCA-4 microbeads were used to negatively select non-$BDCA\text{-}4^+$ cells. 2×10^7 PBMC were prepared as described above in Sections 2.9.2 and 2.9.4 and incubated with

20µl of FcR blocking reagent and 20µl of BDCA-4 microbeads and incubated for 15 minutes at 4°C. PBMC were then washed before being passed through an MS-sized MACS column as described in Section 2.9.2 and washed again before being resuspended in cell culture medium at 2.5×10^6 cells ml^{-1} for ELISpot assays.

2.9. Enzyme-linked immunosorbent assay (ELISA) assays for c-Kit and stem cell factor

ELISA assays were carried out on stored frozen plasma samples obtained from samples at the time of PBMC isolation to quantify c-Kit and stem cell factor (SCF) concentrations.

2.9.1. c-Kit ELISA

c-Kit was quantified according to the manufacturers protocol using a specific ELISA kit (R&D Systems, Abingdon, UK). Capture antibody (R&D Systems) was diluted in PBS (Sigma-Aldrich) to 4µg ml^{-1} and 100µl was added per well to 96-well flat-bottomed ELISA plates (Nunc, Roskilde, Denmark). Plates were then sealed and incubated overnight at room temperature. Following incubation plates were washed three times with wash buffer consisting of PBS with 0.05% Tween 20 (Sigma-Aldrich); each well was filled using a wash bottle, then the contents discarded. After the final wash remaining buffer was blotted out onto clean paper towels. Blocking was achieved by adding 300µl reagent diluent, consisting of PBS with 1% FCS (Sigma-Aldrich) to each well and incubated at room temperature for one hour.

Whilst plates were being blocked, samples and standards were prepared. Samples were thawed and diluted at a ratio of 1:50 in diluent. Standards were diluted in diluent and a seven point serial dilution was carried out starting at 2000pg ml^{-1}. Reagent diluent was used as a negative control sample.

After blocking plates were washed three times as described, before 100µl of sample or standards was added to each well. Samples and standards were added in triplicate. Plates were incubated for two hours at room temperature before being washed three times. Streptavidin-HRP was diluted to working concentration in reagent diluent, 100µl was added per well, plates were incubated for 20 minutes at room temperature in the dark and then washed three times. Substrate solution was made containing a 1:1 mixture of hydrogen peroxide (H_2O_2) and tetramethylbenzidine (both Becton Dickinson) and incubated for approximately 15 minutes at room temperature in the dark. The plate was

checked occasionally and when the lowest standard concentration appeared to have changed colour compared to the negative control, 50µl stop solution (2 N Sulphuric acid (H_2SO_4)) was added per well and plates were immediately analysed on a microplate reader at 450 nm.

2.9.2. SCF ELISA

SCF was quantified using an ELISA kit (Peprotech) and assays were carried out according to manufacturers instructions. Capture antibody was first dissolved in sterile water to 100µg ml^{-1} and then further diluted in PBS to a working concentration of 1µg ml^{-1}. 100µl capture antibody was added per well to 96-well flat-bottomed ELISA plates (Nunc) and incubated overnight at room temperature. Plates were then washed four times as described in 2.10.1 and 300µl block buffer (as in 2.10.1) was added to each well and incubated for one hour at room temperature.

Whilst plates were being blocked, samples and standards were prepared. For SCF ELISA the samples were diluted at a ratio of 1:3 in diluent (PBS containing 0.1% FCS and 0.05% tween-20 – all Sigma-Aldrich). Standards were reconstituted in sterile water to 1µg ml^{-1} before being further diluted in diluent and a seven point serial dilution was carried out starting at 2000pg ml^{-1}. Reagent diluent was used as a negative control sample.

After blocking, plates were washed four times before 100µl of sample or standards was added per well. Samples and standards were added in triplicate. Plates were incubated for two hours at room temperature before being washed four times. The Avidin-HRP conjugate was diluted 1:2000 in diluent and 100µl was added per well, incubated for 30 minutes and then plates were washed four times. 100µl of ABTS substrate was then added to each well and incubated at room temperature while colour change was monitored. When the lowest standard concentration became obviously different to negative controls, the plates were analysed immediately at 405nm.

2.10. IFN-γ ELISpot assays

Antigen-specific T-cell responses to specific peptide pools in combination with TLR agonists were quantified by ELISpot analysis. 96-well polyvinylidene difluoride-backed ELISpot plates (Millipore, Billerica, Massachusetts, USA) were coated with 100µl anti-IFN-γ antibody diluted in PBS to 10µg ml^{-1} (Mabtech, Stockholm, Sweden) and incubated

overnight at 4°C. Plates were washed six times with 200µl per well with cell culture media before being blocked for one hour at room temperature with PBS containing 10% human AB serum (Sigma-Aldrich).

PBMC were prepared at a concentration of 2.5×10^6 cells ml^{-1} in culture medium with 20% human AB serum. Peptide pools, cytokines and TLR agonists were prepared as described in Sections 2.7.1, 2.7.2 and 2.7.3 at four times the final concentration required in cell culture medium. PHA aliquots were also thawed. After plates were blocked, the blocking buffer was tapped out of the plates and 100µl cells were immediately added per well. 50µl of peptide, TLR agonists or cytokines was added to wells as required or 100µl PHA was added. Wells that did not already have 200µl total volume were topped up using cell culture medium; all conditions were carried out in duplicate. Plates were incubated overnight at 37°C in 5% CO_2.

After incubation the contents of the plates were shaken off into virkon and the plates washed six times with 200µl PBS using a plate washer (Tricontinent, Grass Valley, USA). Excess PBS was shaken off and 100µl of 1µg ml^{-1} biotinylated anti-IFN-γ antibody (Mabtech) was then added to each well and the plate incubated for two hours at room temperature. Plates were then washed again as described and 100µl of 1:1000 streptavidin-alkaline phosphatase (Mabtech) diluted in PBS was added per well and plates were incubated for one hour at room temperature. Plates were washed a final time, leaving the last wash in the plate. Chromogen was prepared using the alkaline phosphatase conjugate substrate kit, by mixing development buffer at a 1:25 ratio with distilled water and adding 100µl of both chromogen reagents A and B per 10ml (BioRad, Hemel Hempstead, UK) and 100µl was added per well. Plates were then incubated at room temperature and the formation of spots was monitored. When clearly defined spots were visible in positive control wells and/or peptide-stimulated wells, plates were washed thoroughly with tap water three times and left to air-dry before being analysed with a plate-reading microscope.

2.11. DNA isolation for HLA tissue-typing

Cells were frozen at -80°C at 5×10^6 cells ml^{-1} in freezing medium containing cell culture medium with 20% FCS and 10% DMSO (Sigma-Aldrich). Cells were initially frozen in a 'Mr Frosty' container (isopropanol coolant), before being transported to normal freezer boxes for later processing.

DNA isolation was performed using a commercially available kit (Qiagen, Crawley, UK). Frozen PBMC samples were thawed in a 37°C heat block, transferred to 15ml centrifuge tubes and washed twice in PBS, spun each time for five minutes at 1400 RPM, then resuspended in 300µl PBS and transferred to 1.5ml sterile tubes. 900µl of RBC lysis solution was added, and the tube was inverted 10 times and incubated for five minutes at room temperature. Tubes were then spun in a microcentrifuge at maximum speed for 20 seconds and the excess discarded leaving approximately 10µl liquid in tubes, which were then vortexed vigorously. 300µl of the cell lysis solution was then added and was drawn up and down for 10 seconds using a pipette. 1.5µl RNAse A solution was then added and tubes inverted 25 times before incubation for 15 minutes at 37°C. Sample tubes were then placed on ice for one minute after which 100µl protein precipitation solution was added and the sample vortexed for 20 seconds. Samples were spun at maximum speed in a microcentrifuge for one minute, the supernatant was then carefully removed, avoiding the visible protein pellet and added to 300µl isopropanol prepared in a clean 1.5ml tube. The tube was then inverted 50 times until threads or clumps of DNA were visible, the sample was then spun at maximum speed for one minute. The supernatant was poured off and the remainder was carefully discarded by inverting the tube onto clean tissue. 300µl ethanol was then added to the sample and the DNA washed by inverting the tube several times before being spun again for one minute at maximum. Supernatant was then discarded and carefully drained by inverting onto tissue before being left to air dry for five minutes. 100µl hydration solution was then added and the tube vortexed for five seconds. Tubes were then incubated for five minutes at 65°C on a heat block before being left on a shaker overnight at room temperature. DNA yield was determined using a Nanodrop (Thermo Scientific, Waltham, USA). A minimum of 2µg DNA at 20µg ml^{-1} was sent for tissue-typing to ProImmune (Oxford, UK).

2.12. Flow cytometry

All flow cytometry was carried out using either a FACSCalibur (four colours) or an LSR II cytometer (six colours, both Becton Dickinson), which was upgraded during this project to include an additional UV laser. Data was acquired using Cell Quest Pro software (Becton Dickinson) for the FACSCalibur and FACSDiva software (Becton Dickinson) for the LSR II. All data presented was analysed using FlowJo software (Tree Star, Oregon, USA). Example dot plots and gating strategies are presented throughout this thesis. All samples were stored at 4°C and acquired no more than 24 hours after fixation.

2.13. Graphs and statistical analysis

All graphs shown in this thesis have been prepared using GraphPad Prism Software (GraphPad Software Inc, California, USA).

ELISA data were analysed using Microsoft Excel software (Microsoft Corporation, Redmond, Washington, USA).

All other statistical analysis was carried out using GraphPad Prism software using non-parametric tests; Wilcoxon's signed rank test was used to analyse differences between paired data and the Mann-Whitney U test was used to examine differences between non-paired data, such as comparison of responses between groups of HIV-1 seropositive and seronegative individuals.

3. Innate immune responsiveness to TLR agonists during chronic HIV-1 infection

Aim

In this first results chapter, the impact of chronic HIV-1 infection on innate cell responsiveness and activation in response to pathogen-associated molecular patterns (PAMP) will be determined. The effects of chronic HIV-1 infection on responses will be explored in an attempt to better understand how this disrupts innate immunity, with the goal of identifying non-exhausted immune pathways. Assays were carried out to identify PAMP capable of eliciting and enhancing innate immune responses for potential use in both prophylactic and immunotherapeutic settings.

Introduction

Chronic HIV-1 infection affects many aspects of both innate and adaptive immunity. In this chapter we have explored innate immune responses in chronic infection with a particular focus on NK cell responsiveness. It has been shown that NK cells interact with TLR-activated cells such as DC and that these interactions ultimately shape the adaptive immune response (Moretta et al., 2008, Gerosa et al., 2002). It is also known that both NK cells and DC are both reduced in numbers and driven toward a dysfunctional state in HIV-1 infection (Mavilio et al., 2005, Fogli et al., 2004, Donaghy et al., 2003, Mavilio et al., 2003, Donaghy et al., 2001). We have therefore investigated which innate TLR-driven NK cell functions are preserved during chronic HIV-1 infection.

PAMP stimulating TLR were compared for their effect on innate immune activation and function within $CD8^+$ T-cells and NK cells, and the extent of accessory cell involvement in NK cell activation was explored. NK cell functional subset responsiveness to innate stimulation was subsequently examined in chronic HIV-1 infection, and the influence of viral suppression under anti-retroviral therapy investigated.

3.1. Natural killer cells and $CD8^+$ T-cells are sensitive to TLR agonist stimulation

In order to establish the capacity of NK cells and $CD8^+$ T-cells to respond to TLR agonists alone, preliminary stimulation assays were carried out to measure activation marker

expression in response to LPS and CpG ODN2216. PBMC from healthy donors were isolated from peripheral blood and cultured overnight in the presence of the TLR4 agonist LPS (100ng ml^{-1}) and the TLR9 agonist CpG ODN2216 (2.5µM). The expression of the early activation marker CD69 was determined on both $CD3^+CD8^+$ T-cells and $CD3^-CD56^+$ NK cells (see appendix table A.1 for antibody staining panel). Figure 3.1.1 demonstrates the gating strategy used for analysis, including a lymphocyte gate defined by forward and side scatter, and examples of CD69 expression in response to LPS and CpG ODN2216 stimulation of gated $CD8^+$ T-cells and NK cells. Both NK cells and $CD8^+$ T-cells are sensitive to *in vitro* stimulation with LPS and CpG ODN2216, and showed a clear increase in expression of CD69 compared to unstimulated PBMC (left in tissue culture media alone).

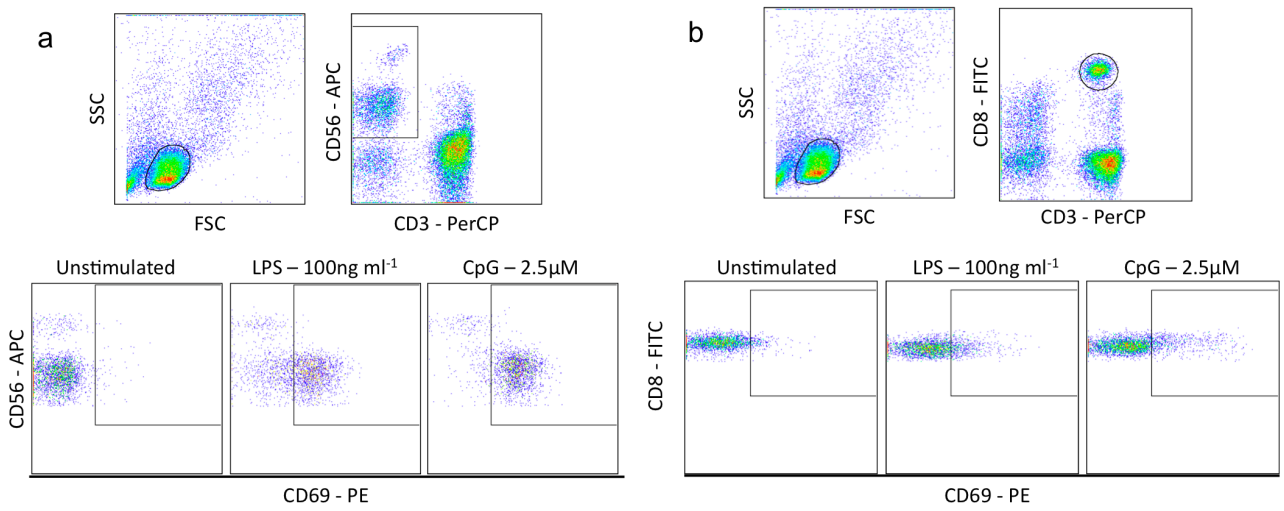


Figure 3.1.1 Both NK cells and $CD8^+$ T-cells are sensitive to TLR stimulation. Representative, healthy control, flow cytometry dot plots, demonstrating a) NK cell or b) $CD8^+$ T-cell gating strategies from PBMC and subsequent expression of the early activation marker CD69 by total NK and $CD8^+$ T-cells following overnight culture with or without TLR agonist stimulation *in vitro*.

3.2. High frequency of NK cells respond to TLR stimulation; varying sensitivity of NK and $CD8^+$ T-cells to different agonists

The optimum conditions for *in vitro* stimulation of PBMC were then investigated in order to establish the extent to which both NK cells and $CD8^+$ T-cells could be activated by representative examples of different human TLR agonists. A full list of TLR agonists and the concentrations used is shown in Table 3.2.1. PBMC isolated from 12 healthy donors

were cultured overnight in the presence of different TLR agonists at varying concentrations (see Table 3.2.1).

Agonist	TLR Ligated	Units	Concentration				
			1	2	3	4	5
Pam3CSK4	1/2	ng ml^{-1}	1000	100	10	1	-
HKLM	2	cells ml^{-1}	10^8	10^6	10^4	10^2	-
Poly (I:C)	3	µg ml^{-1}	10	5	1	0.1	-
LPS	4	ng ml^{-1}	200	20	2	0.2	-
Flagellin	5	ng ml^{-1}	1000	100	10	1	-
FSL-1	6/2	ng ml^{-1}	1000	100	10	1	0.1
Imiquimod R837	7	µg ml^{-1}	5	2.5	1	0.5	0.1
ssRNA40 LyoVec	8	µg ml^{-1}	5	2.5	1	0.5	0.1
CpG ODN2216	9	µM	2.5	1	0.5	0.1	-

Table 3.2.1 TLR agonists and concentrations used for titration experiments.

Figure 3.2.1 shows histograms for CD69 expression by NK cells and $CD8^+$ T-cells. The graphs reveal variation in the sensitivity of NK and $CD8^+$ T-cells to different TLR agonists. The TLR1/2 agonist Pam3CSK4 and the TLR5 agonist flagellin stimulated little CD69 expression on NK cells except for relatively small increases at high agonist concentrations. FSL-1 (a TLR6/2 agonist) did not stimulate significant increases in CD69 expression by NK cells compared with unstimulated cells at any of the concentrations tested. Flagellin and FSL-1 were also unable to stimulate significant CD69 expression by $CD8^+$ T-cells at any concentration tested. All other agonists generated significant increases in the frequency of CD69 expression by both NK cells and $CD8^+$ T-cells, compared to unstimulated cells.

The TLR3 agonist Poly(I:C), the TLR4 agonist LPS, the TLR8 agonist ssRNA40 and the TLR9 agonist CpG ODN2216 all generated consistently high frequency CD69 expression by NK cells and increased expression by $CD8^+$ T-cells. Both the TLR2 agonist HKLM and the TLR7 agonist Imiquimod R837 generated high frequency CD69 expression by NK cells in PBMC from some individuals, however these responses were inconsistent and showed great inter-individual variation.

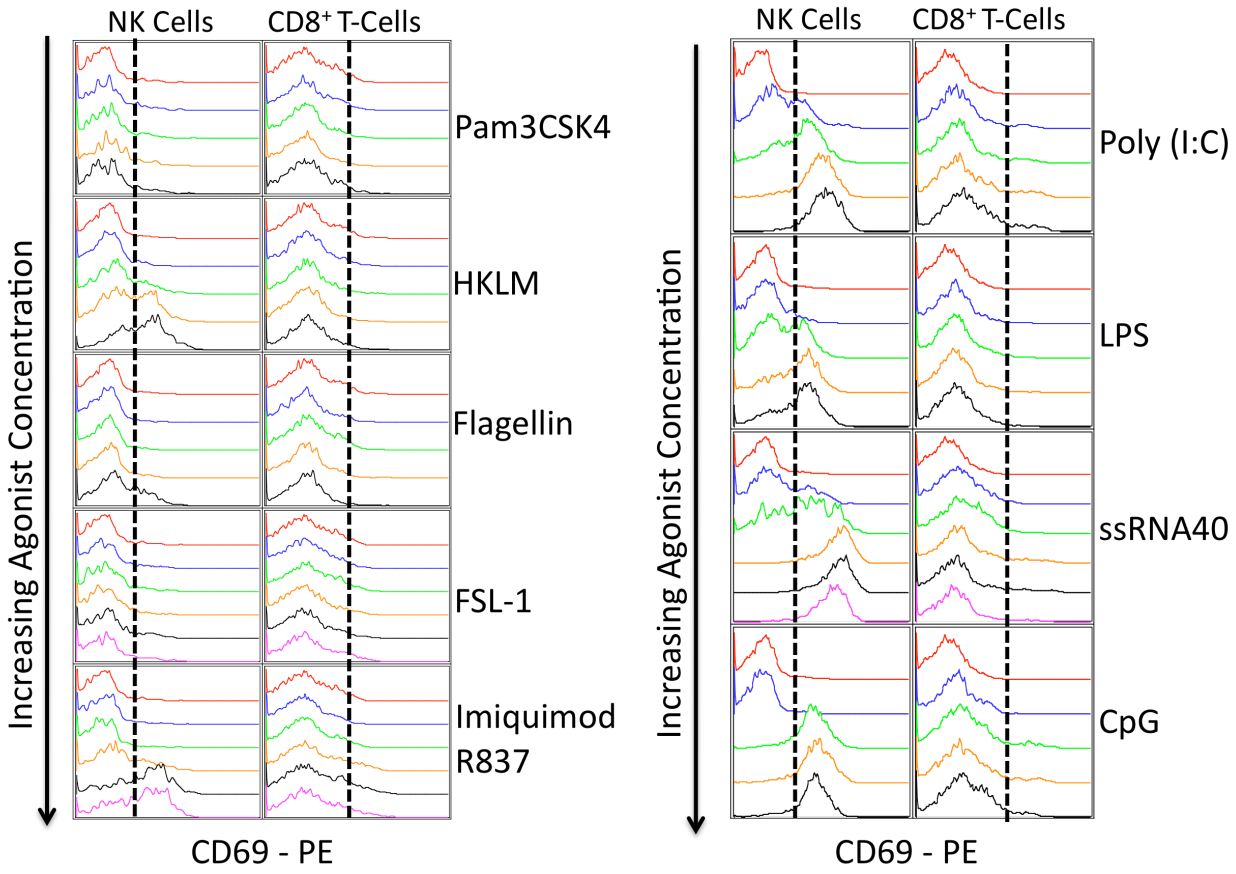


Figure 3.2.1 NK and CD8$^+$ T-cells are stimulated to express CD69 at varying intensities and frequencies following *in vitro* stimulation of PBMC by different TLR agonists. Histogram plots from a single representative healthy control individual (n=27) demonstrating CD69 expression by total NK cells (left columns) and CD8$^+$ T-cells following overnight stimulation with different TLR agonists at different concentrations (see table 1). Dashed lines illustrate positive thresholds and are for illustration purposes.

3.3. Lyovec and CpG ODN2006 activate NK and CD8$^+$ T-cells at far lower frequency than ssRNA40 and CpG ODN2216

The HIV-1 derived ssRNA sequence present in the TLR8 agonist ssRNA40 is complexed with lyovec, a cationic lipid that protects the ssRNA sequence and enhances cellular uptake needed for TLR8 stimulation (InVivoGen, 2012). ssRNA41 has the same ssRNA sequence as ssRNA40, but has uridine molecules in place of adenosine molecules and is also complexed to lyovec; this has been shown to not stimulate production of type 1 interferons and is therefore considered to be a suitable negative control for NK cell and CD8$^+$ T-cell activation (InVivoGen, 2012). In order to assess the impact of the lyovec component on NK cell and CD8$^+$ T-cell responses, PBMC were stimulated with ssRNA40

in parallel with the negative control agent ssRNA41 and NK cell and $CD8^+$ T-cell activation was assessed. Expression of the early activation marker CD69 and the expression of the IL-2Rα chain (CD25) were measured using flow cytometry. Figure 3.3.1 compares CD69 and CD25 expression by NK and $CD8^+$ T-cells following overnight stimulation of healthy donor PBMC with ssRNA40 and ssRNA41. ssRNA41 weakly stimulated expression of both CD69 and CD25, but does so at a reduced frequency compared to ssRNA40 at the same concentration.

Three different classes of CpG ODN molecules, types A, B and C are recognised by TLR9 exhibiting distinct stimulatory properties. Type A CpG ODN molecules, including CpG ODN2216, interact with TLR9 on plasmacytoid DC (pDC) and stimulate IFN-α production. Type B CpG ODN molecules, such as CpG ODN2006, activate B-cells whilst stimulating negligible IFN-α responses. Type C CpG ODN molecules stimulate both pDC and B-cell responses (Marshall et al., 2005, Krug et al., 2001, Krieg et al., 1995). CpG ODN2216 has been used exclusively throughout these studies because it activates pDC and induces IFN-α production, a known pathway for NK cell activation. Type B CpG ODN2006 was therefore tested as a negative control for type A CpG ODN stimulated pDC in NK and $CD8^+$ T-cell activation. PBMC from healthy donors were stimulated overnight with either CpG ODN2006 or ODN2216 and expression of CD69 and CD25 by NK cells and $CD8^+$ T-cells was measured by flow cytometry. Figure 3.3.1 reveals that CpG ODN2006 stimulated a low frequency of CD69 expression in NK cells, although CpG ODN2216 led to a higher frequency of responding NK cells. CpG ODN2216 induced a low frequency of NK cells to express CD25 (8.37%), with little or no induction observed in response to CpG ODN2006 (1.1%) (Figure 3.3.1). CD69 and CD25 expression was induced in $CD8^+$ T-cells following *in vitro* stimulation of PBMC with CpG ODN2216, and both activation markers were also expressed following stimulation with CpG ODN2006, albeit at a lower frequency (Figure 3.3.1b). These data indicate that pDC may play an accessory role in the responses of NK cells to ODN2216.

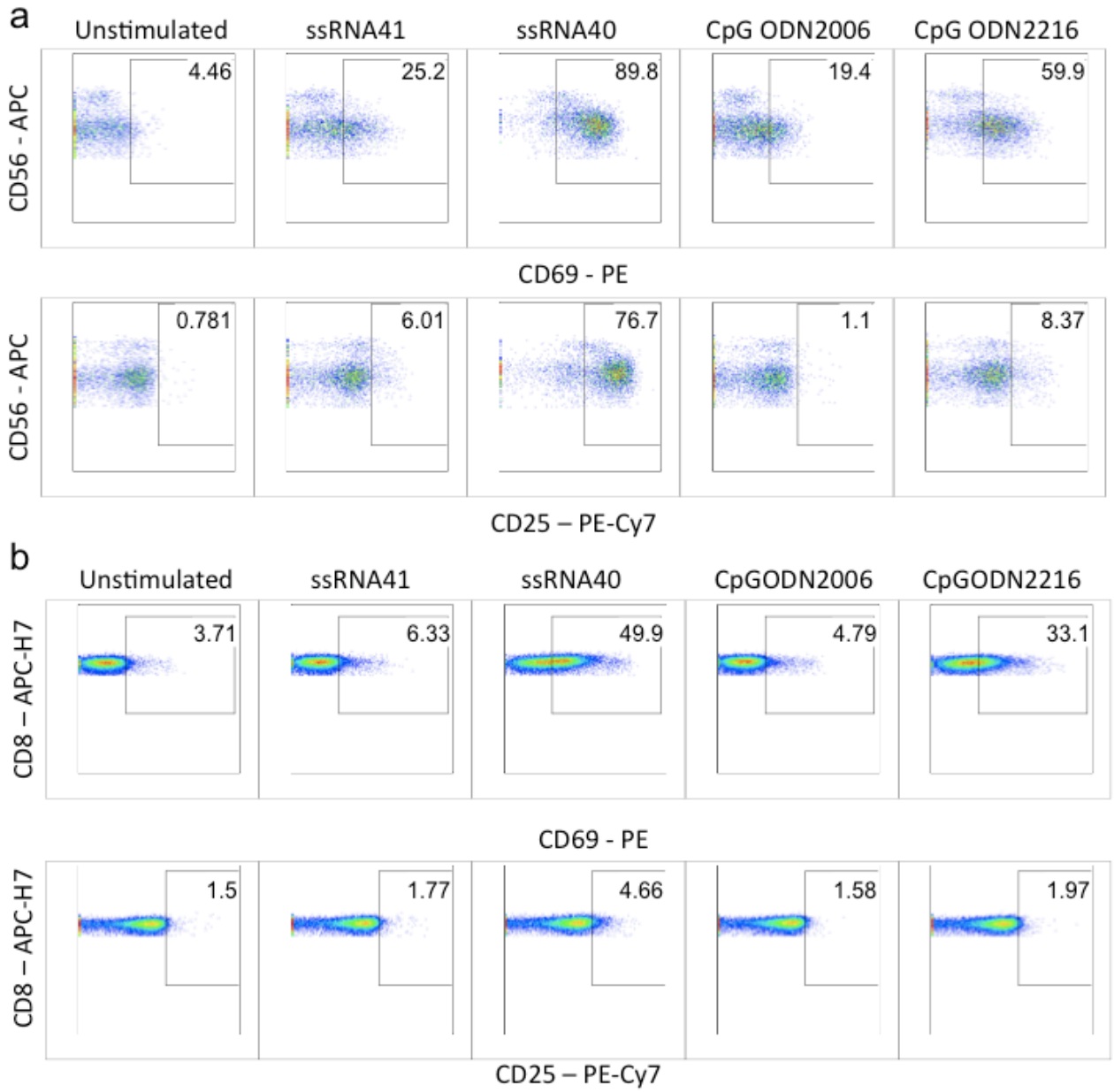


Figure 3.3.1 ssRNA41 and CpG ODN2006 do stimulate NK and CD8$^+$ T cells *in vitro*, at reduced frequency compared to ssRNA40 and CpG ODN2216 respectively. Dot plots demonstrating the induced expression of CD69 and CD25 on a) NK and b) CD8$^+$ T cells from a single healthy control individual and are representative of 2 separate experiments following overnight stimulation with the TLR agonists shown. Both types of ssRNA were used at 5μg/ml and both types of CpG were used at a

3.4. Isolated NK cells remain sensitive to cytokine stimulation, but are unresponsive to TLR agonist stimulation

In order to confirm the requirement of accessory cells for NK cell activation, untouched NK cells were purified from four healthy donor PBMC by negative selection on magnetic

beads. This isolation protocol allowed highly pure NK cell cultures to be generated reaching a minimum of 89% purity in all experiments as illustrated in Figure 3.4.1a.

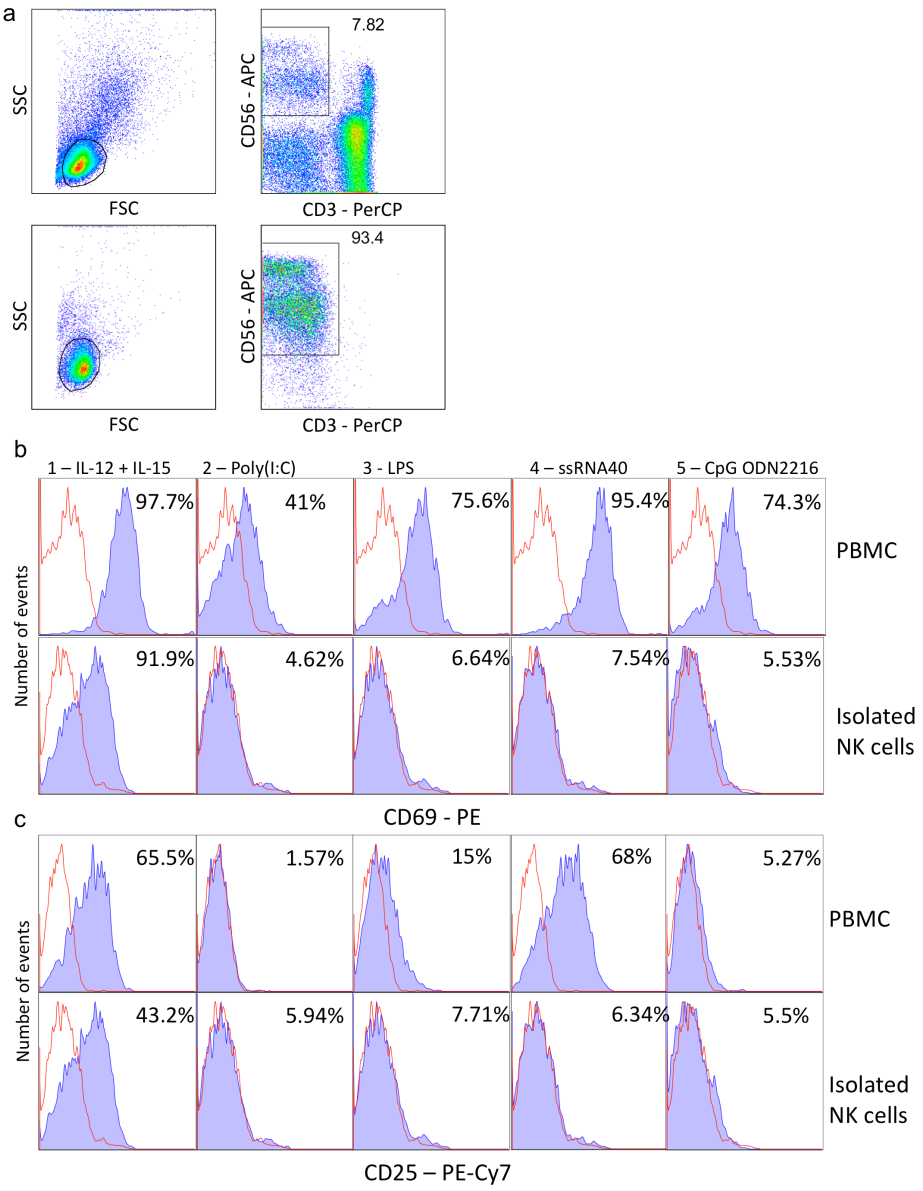


Figure 3.4.1 NK cells become unresponsive to TLR agonist stimulation in the absence of accessory cells. NK cells were isolated from PBMC using magnetic cell separation and the resulting, negatively selected, NK cells were cultured *in vitro* with TLR agonists in parallel with total PBMC and the resulting activation compared by flow cytometry. a) Representative dot plots from a healthy control individual demonstrating the efficiency of the negative selection technique. The top row is plots from total PBMC and the bottom row are plots from isolated NK cells. Overlay histograms comparing b) CD69 or c) CD25 expression by NK cells in response to cytokine or TLR agonist stimulation of PBMC or isolated NK cells (filled lines) compared to unstimulated PBMC or isolated NK cells (solid, unfilled line). Percentages displayed are frequency of b) $CD69^+$ or c) $CD25^+$ stimulated $CD3^-CD56^+$ NK cells. Results demonstrated are from a single individual and are representative of 4 isolation experiments using PBMC from HIV-1 seronegative individuals. These results also show b) CD69 expression is detected on NK cells stimulated with all TLR agonists tested here and c) CD25 expression is detected in NK cells following stimulation of PBMC with LPS or ssRNA, but very low expression is detected with Poly(I:C) or CpG.

Overlay histograms clearly demonstrate an increased frequency of CD69 expression by

NK cells stimulated within unfractionated PBMC in response to TLR ligands or cytokines (unfilled lines), compared to unstimulated cultures (filled blue lines) (Figures 3.4.1b&c). Furthermore, IL-12 + IL-15, LPS and ssRNA40 stimulated NK cells within PBMC beyond early activation to express CD25. Isolated NK cells cultured with the TLR agonists tested did not become activated, demonstrating a clear requirement for accessory cells, either through direct contact or through soluble mediators or a combination of both. Of the stimuli, tested only the combination of cytokines IL-12 (1ng ml^{-1}) and IL-15 (20ng ml^{-1}) could drive the activation of NK cells in the absence of accessory cells, confirming that the cells are intrinsically functionally competent (Figure 3.4.1).

3.5. Confirmation of DC sensitivity to TLR agonist stimulation

In view of the lack of activation of purified NK cells in response to TLR agonists, a brief study was performed to confirm the relative ability of different TLR agonists to activate mDC and pDC. TLR-activated DC produce pro-inflammatory cytokines, such as IL-12, IL-18, IL-15 and type I interferons IFN-α and IFN-β, the combination of which depends on the subset of DC activated, which is, in turn, dependant on the TLR ligated (Pulendran & Ahmed, 2006). pDC specialise in recognition of intracellular pathogens, viruses and intracellular bacteria, which are innately recognised by TLR7 and TLR9 and stimulate high levels of type I interferons, which in turn activate Th1 T-cells and lead to the release of IFN-γ (Pulendran & Ahmed, 2006). Alternatively, extracellular pathogens may be recognised by different TLR agonists and promote Th2 responses. These pro-inflammatory signals are involved in activating other immune cell populations such as NK cells; direct cell-to-cell contact has also been implicated in DC-induced NK cell activation. The co-stimulatory molecule CD40 is expressed on activated DC and has been shown to interact with its ligand CD40L on NK cells, leading to NK cell activation and as such has been used as an activation marker for DC in this assay (Carbone et al., 1997).

Isolated PBMC from healthy donors (n=2) were stimulated for either four hours or overnight with the TLR4 agonist LPS (100ng ml^{-1}) or the TLR9 agonist CpG ODN2216 (2.5μM). PBMC were then stained for $CD123^+$ pDC or $CD11c^+$ mDC (see Appendix Tables A.2 and A.3 for antibody panels). Activation was analysed by measuring CD40 expression on Lin-1^-HLA-DR^+CD123^+ pDC or Lin-1^-HLA-DR^+CD11c^+ mDC (see Figure 3.5.1 for gating strategy).

Figure 3.5.1b shows that an increase in expression of CD40 was detected on pDC after four hours of stimulation with CpG ODN2216. After overnight stimulation, upregulation of CD40 expression on pDC and also upregulation on mDC is observed. Reciprocally, the TLR4 agonist LPS, induced the expression of CD40 on mDC after four hours of stimulation, and both DC subsets express CD40 following overnight stimulation.

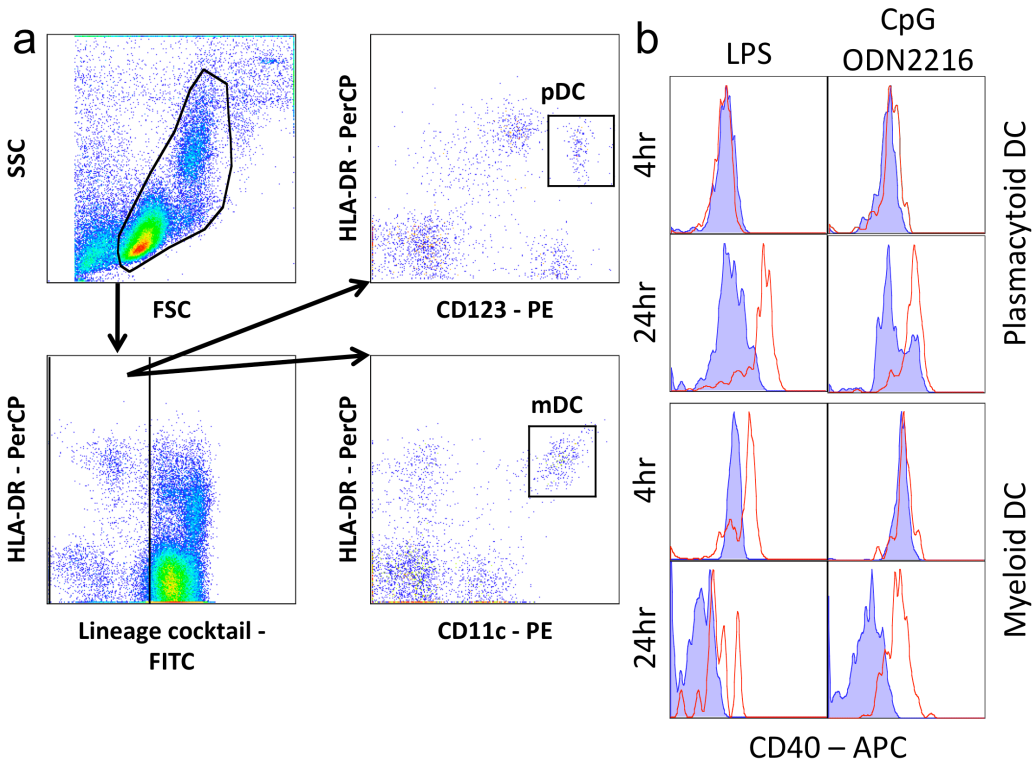


Figure 3.5.1 TLR agonists activate mDC and pDC. PBMC were stimulated for four hours and overnight with LPS (100ng/ml) and CpG ODN2216 (2.5μM) before dendritic cell activation was determined by flow cytometry. CD40 expression was used as a marker of activation for both plasmacytoid and myeloid DC. a) Representative gating strategy from a healthy control individual. b) Overlay histograms showing CD40 expression by unstimulated (filled blue lines) or TLR agonist stimulated (unfilled red line) PBMC. After overnight stimulation CD40 expression by both DC populations is induced. Graphs are representative of two healthy control individuals.

3.6. Peripheral blood NK and CD8$^+$ T-cells from HIV-1+ individuals are refractory to *in vitro* TLR agonist stimulation

Once it was established that TLR agonists could activate NK cells and CD8$^+$ T-cells, and that the activation was accessory cell dependant, the potential changes in these responses were examined in PBMC cultures from HIV-1+ individuals. Two different patient groups were studied, those who were treatment experienced, receiving HAART

and controlling virus levels below detectable levels (plasma viral load <50 copies ml^{-1} blood), and individuals who were treatment naïve, displaying detectable viraemia. Comparison of these two patient groups revealed the effect of active viraemia and the effect of treatment on innate responsiveness to TLR agonist stimulation. All HIV-1+ individuals were recruited from clinics at Chelsea and Westminster Hospital, London. Assays were also carried out using samples from HIV-1- individuals as 'healthy control' responses. Treatment guidelines for HIV-1 patients are constantly changing and it was difficult to match patients for age. The BHIVA guidelines are moving towards treating patients earlier in the course of disease progression, while they still have relatively high $CD4^+$ T-cell counts (BHIVA, 2012). The current suggested time to treat when no other complications exist is when $CD4^+$ T-cells fall below 350 cells μl^{-1}; clinical guidelines for HIV-1 treatment can be found at http://www.bhiva.org/ClinicalGuidelines.aspx. This early treatment strategy is likely to explain why the median age of the group of treatment-naïve patients was lower than that of treatment-experienced patients (see patient characteristics tables). The influence median age difference may have on the results obtained are considered in the interpretation of data.

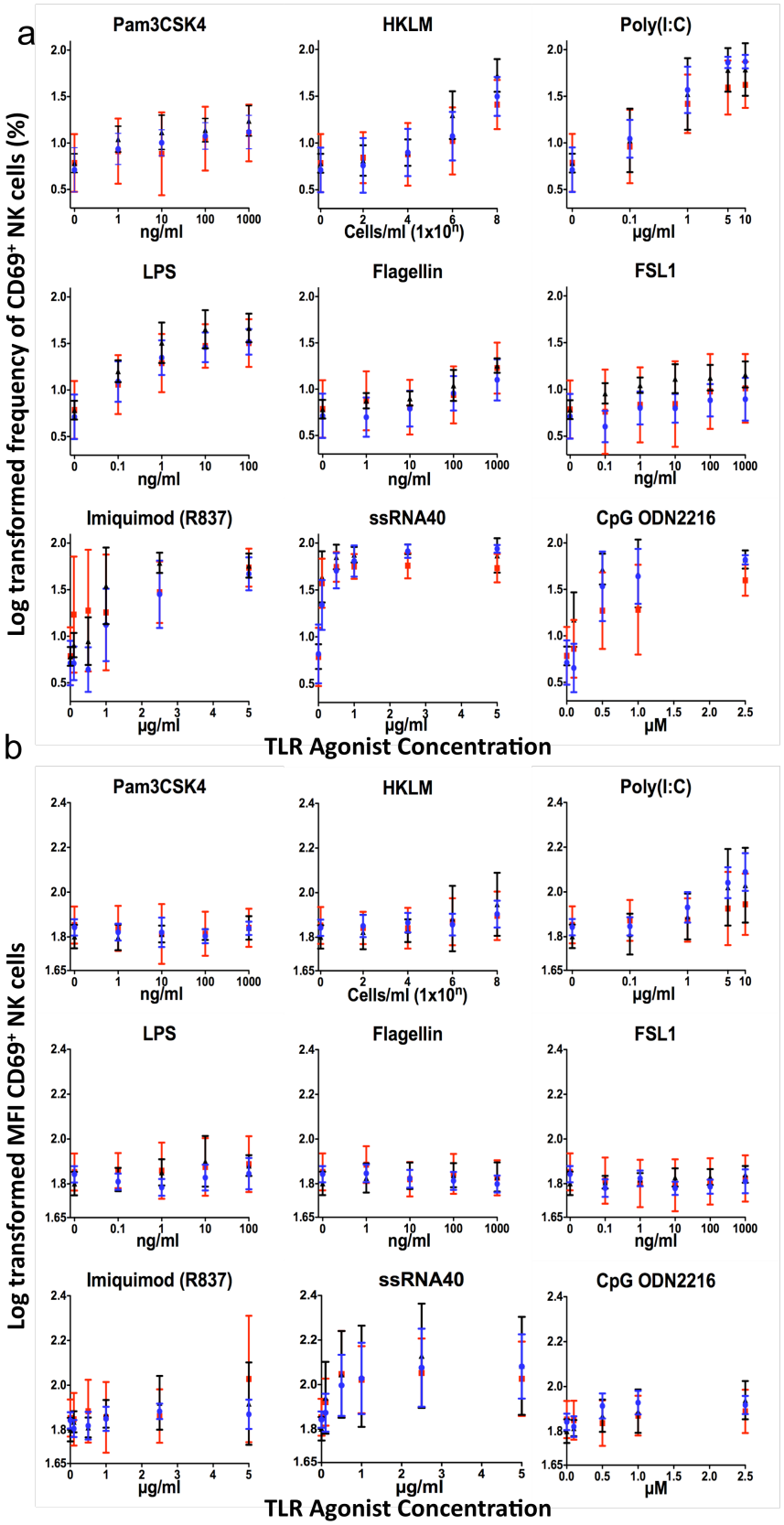
Cultures of patient PBMC were stimulated with a range of TLR agonists at varying concentrations as described in section 3.2. An initial study was carried out in order to establish if there are defects in responsiveness to TLR agonist stimulation within the NK cell or the $CD8^+$ T-cell population. Patient characteristics for this study are shown in Table 3.6.1.

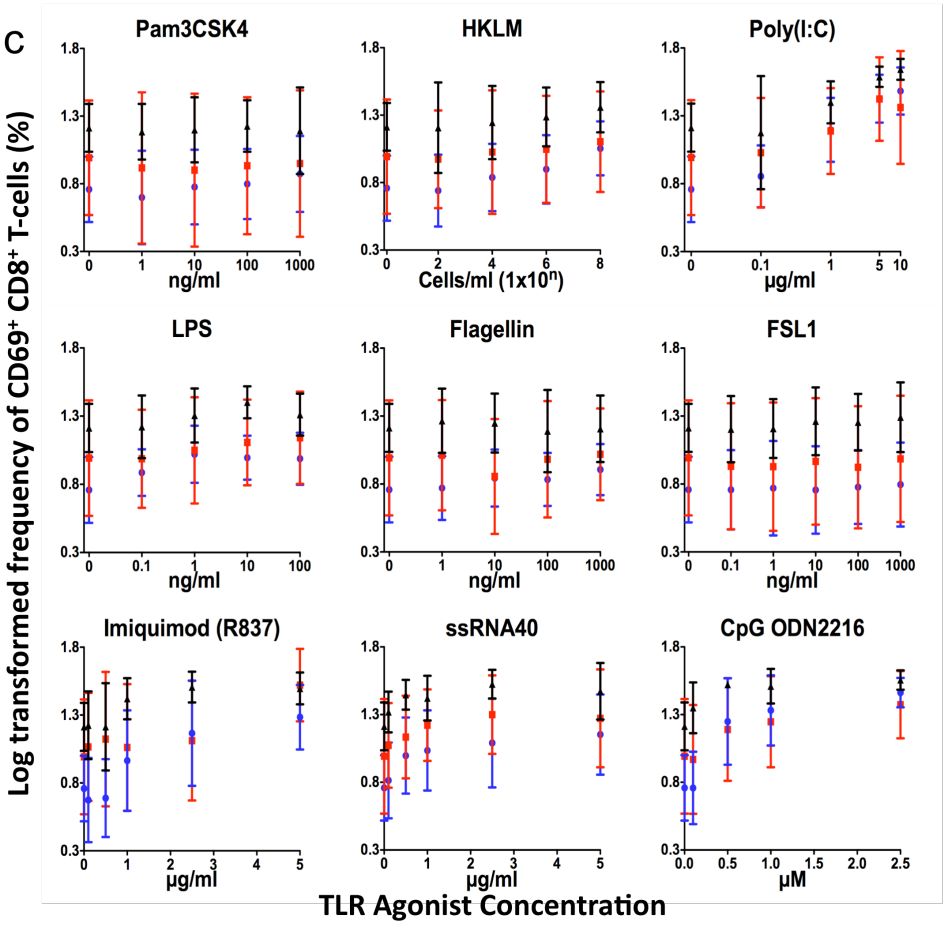
Table 3.6.1 Patient Characteristics.

Median Values	Healthy Controls (n=12)	HIV-1+ treatment-naïve (n=7)	HIV-1+ treatment-experienced (n=6)
Age (range)	27 (23-46)	35 (19-48)	40 (33-55)
CD4 T-Cell Count (cells μl^{-1} blood)	n/a	498 (293-1039)	260 (65-905)
Plasma viral load (copies ml^{-1} blood)	n/a	1775 (284-391572)	0 (0-101)

Figure 3.6.1 shows dose response curves for frequency and mean fluorescence intensity (MFI) of CD69 expression by total NK cells and total $CD8^+$ T-cells following stimulation with TLR agonists as shown. Appendix Table A.14 summarises the responses to optimal TLR agonist concentrations, comparing CD69 frequencies (median and range) by both

unstimulated cells from the same subject group, and HIV-1+ responses with HIV-1- control individuals.




c
Log transformed frequency of CD69+ CD8+ T-cells (%)
Pam3CSK4
HKLM
Poly(I:C)
LPS
Flagellin
FSL1
Imiquimod (R837)
ssRNA40
CpG ODN2216
ng/ml
Cells/ml (1x10n)
µg/ml
µM
TLR Agonist Concentration

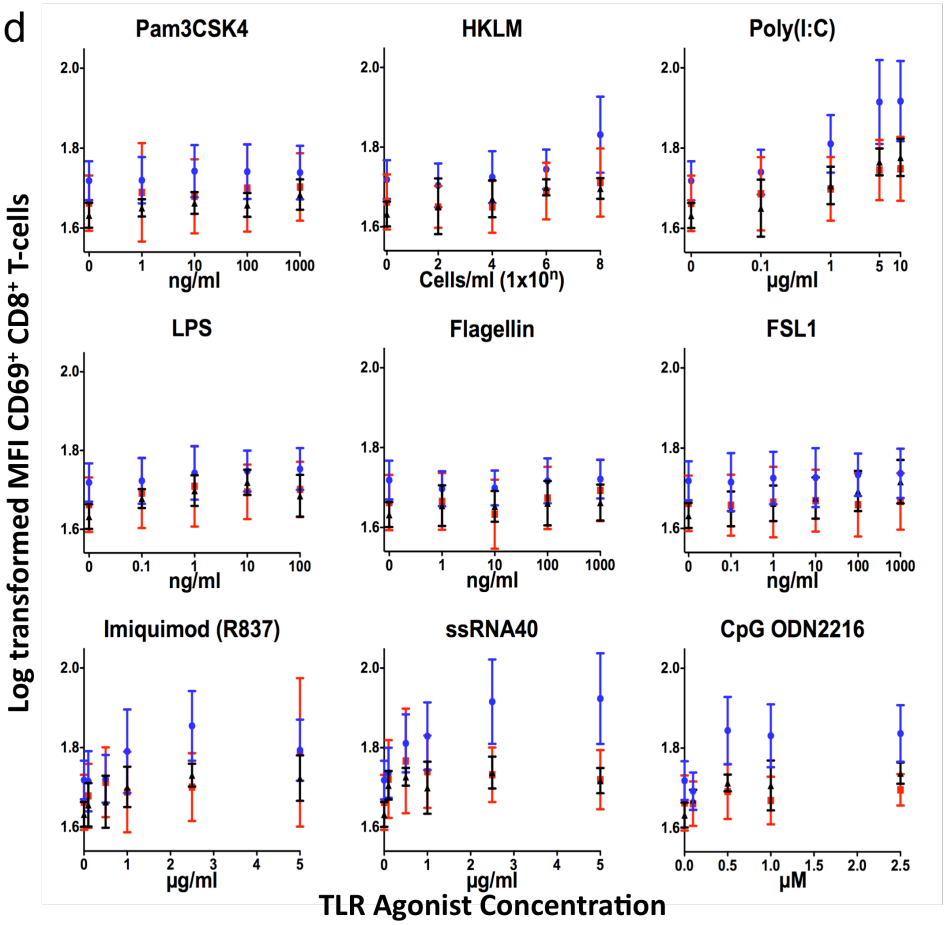

d
Log transformed MFI CD69+ CD8+ T-cells
Pam3CSK4
HKLM
Poly(I:C)
LPS
Flagellin
FSL1
Imiquimod (R837)
ssRNA40
CpG ODN2216
ng/ml
Cells/ml (1x10n)
µg/ml
µM
TLR Agonist Concentration

Figure 3.6.1 Early activation dose response curves. PBMC from HIV-1+, treatment-naïve & experienced and HIV-1- control individuals were cultured *in vitro* overnight with TLR agonists at varying concentrations. Early activation (CD69) of a&b) NK cells and c&d) $CD8^+$ T cells was measured using flow cytometry following overnight stimulation. Data has been log transformed and the mean with 95% confidence intervals have been plotted. Differences are considered significant when error bars do not overlap. Transformed frequency of CD69 expression is shown in a) & c) and the MFI are shown in b) and d). HIV-1- individuals (n=12) are represented by blue lines, HIV-1+ treatment-naïve individuals (n=7) are represented by black lines and treatment-experienced individuals (n=6) are represented by red lines.

Pam3CSK4, flagellin and FSL-1 stimulated very little *in vitro* response by either NK cells or $CD8^+$ T-cells in either HIV-1+ group or HIV-1- control individuals (described in section 3.2 and shown in Figure 3.6.1). Data also revealed significantly higher levels of basal activation (frequency of CD69 expression) in $CD8^+$ T-cells from PBMC from treatment-naïve individuals in the absence of *in vitro* stimulation (Figure 3.6.1c). Higher levels of background CD69 expression were observed, consistent with ongoing immune activation in HIV-1+ individuals reported by other groups (Fogli et al., 2004). These data also revealed, however, that despite this higher background, the proportion of $CD8^+$ T-cells responding to TLR ligand is significantly higher in HIV-1+ treatment-naïve individuals, but not in treatment-experienced individuals, compared to HIV-1- individuals. No significant difference was observed in the MFI for CD69 expression by $CD8^+$ T-cells from HIV-1+ treatment-naïve individuals compared to the other subject groups (Figure 3.6.1d).

Stimulation of PBMC with HKLM activated NK cells and $CD8^+$ T-cells in all subject groups tested, particularly at high concentrations (1×10^8 cells ml^{-1}) (Figure 3.6.1). NK cell activation (frequency of CD69 expression) indicates lower responses (not statistically significant) in the treatment-experienced HIV-1+ individuals (Figure 3.6.1a). These data also revealed that responsiveness (as assessed by frequency of CD69 expression) in treatment-naïve HIV-1+ individuals is maintained following HKLM stimulation, and tends to be greater (although not statistically significant) than in HIV-1- control individuals (Figure 3.6.1a). Examination of $CD69^+$ MFI levels or $CD8^+$ T-cell responsiveness in cultures stimulated with HKLM revealed no further differences between sample groups other than higher CD69 frequencies on $CD8^+$ T-cells as described above (Figure 3.6.1) and a significantly higher MFI on $CD8^+$ T-cells from treatment experienced individuals compared to healthy controls when stimulated with 10^8 cells ml^{-1} HKLM.

Stimulation of PBMC with Imiquimod R837 activates both NK cells and $CD8^+$ T-cells, although a large variation of responsiveness was seen in all sample groups (Figure 3.6.1). No significant differences were observed at low agonist concentration in the frequency or

MFI for CD69 expression on NK cells from different sample groups, and the increased overall frequency of expression on $CD8^+$ T-cells at low agonist concentrations is likely due to higher background activation (Figure 3.6.1). An increase in CD69 MFI of $CD8^+$ T-cells from HIV-1- control individuals compared to treatment-experienced HIV-1+ individuals was observed at 2.5µg ml^{-1} Imiquimod R837 (Figure 3.6.1d). This demonstrates that higher quantities of CD69 on a per cell basis are expressed by $CD8^+$ T-cells from HIV-1- control individuals (fig 3.6.1d).

Stimulation of PBMC with Poly(I:C), ssRNA40 or CpG ODN2216 activated NK cells to express CD69 at high frequency *in vitro*. The frequency of NK cells expressing CD69 was significantly greater on NK cells from HIV-1- control individuals compared to treatment-experienced HIV-1+ individuals for ssRNA40 (at 5µg ml^{-1}) and CpG ODN2216 (at 2.5µM) (Figure 3.6.1a). Conversely, activation of total NK cells from samples was maintained in samples from treatment-naïve HIV-1+ individuals relative to HIV-1- control individuals (Figure 3.6.1a). The pattern of NK cell activation was maintained when examining $CD69^+$ MFI levels although no significant differences were observed (Figure 3.6.1b). Stimulation with all three TLR agonists activated $CD8^+$ T-cells *in vitro,* inducing a relatively high frequency of CD69 expression in all sample groups (Figure 3.6.1c). Examination of the $CD69^+$ MFI levels for $CD8^+$ T-cells revealed that responses from both treatment-naïve and treatment-experienced HIV-1+ individuals were significantly reduced compared to seronegative samples stimulated with ssRNA40 and CpG ODN2216, but only compared to treatment-experienced when stimulated with Poly(I:C) (fig 3.6.1d).

Optimal concentrations of TLR agonists were therefore selected for use in future assays and, because of low or variable levels of NK cell responsiveness, use of Pam3CSK4, HKLM, flagellin, FSL-1 and Imiquimod R837 was discontinued in further assays. The optimal TLR agonist concentrations used in further assays are listed in Table 3.6.2.

Agonist	TLR Ligated	Concentration Used
Poly (I:C)	3	10µg ml^{-1}
LPS	4	200 ng ml^{-1}
ssRNA40 LyoVec	8	5 µg ml^{-1}
CpG ODN2216	9	2.5 µM

Table 3.6.2 TLR agonists and concentrations used for titration experiments.

3.7. Responsiveness to TLR agonist stimulation is maintained in the $CD56^{hi}CD16^{-}$ NK cell subset despite chronic HIV-1 infection

Having determined that NK cell responses in treatment-experienced HIV-1+ individuals were refractory to stimulation with some TLR agonists, further studies were performed. Samples from newly recruited groups of individuals were examined to elucidate how each of the two principal functional NK cell subsets ($CD56^{hi}CD16^{-}$ and $CD56^{lo}CD16^{+}$) were affected during chronic HIV-1 infection. $CD56^{hi}CD16^{-}$ NK cells are stimulated to produce high levels of cytokines on a per cell basis and are involved in immune modulation by interacting with DC and helping to shape the type and magnitude of immune response generated (Poli et al., 2009). $CD56^{lo}CD16^{+}$ NK cells however are associated with cytotoxicity through innate recognition of damaged or non-self cells or adaptive recognition by ADCC (Caligiuri, 2008). By examining the responsiveness of these two distinct NK cell subsets to TLR agonists in HIV-1 seropositive samples it was possible to interpret how NK cell functionality was affected by chronic infection.

Samples obtained from treatment-naïve and treatment-experienced HIV-1+ individuals were again used for this study. Patient characteristics of the different sample groups are shown in Table 3.7.1. Absolute $CD4^{+}$ T-cell counts and plasma viral loads are shown, in addition to data on the relative frequencies of DC, NK cells and NK cell subsets (Table 3.7.1). Consistent with previous studies, the frequency of pDC and NK cells from treatment-naïve patient samples were significantly reduced compared to seronegative samples and the frequencies of mDC tended to be lower (Donaghy et al., 2001). There was also a trend towards a significant increase in KIR^{+} NK cells and a reciprocal decrease in KIR^{-} NK cells in samples from HIV-1+ treatment-naïve individuals compared to HIV-1- controls (Table 3.7.1). The frequency of peripheral blood DC and NK cells in samples from treatment experienced-individuals did not differ significantly to the frequencies seen

in seronegative samples, indicating effective immune cell recovery after antiretroviral treatment.

Table 3.7.1 Patient Characteristics

	Healthy Control (n=17)	HIV-1+ treatment-naïve (n=11)	Vs Healthy control (p-value)	HIV-1+ treatment-experienced (n=13)	Vs Healthy control (p-value)	Treatment-naïve Vs treatment-experienced (p-value)
Age	26 (21-64)	34.5(26-45)	-	54(39-64)	-	-
CD4 (cells μl^{-1})	N/A	397.5(267-554)	-	488(363-810)	-	-
Plasma Viral Load (copies ml^{-1} blood)	N/A	10978.5(174-388894)	-	N/A	-	-
Plasmacytoid Dendritic Cells (% total lymphocytes)	0.31(0.17-0.68)	0.22(0.39-0.06)	0.02*	0.34(0.11-0.52)	0.832	0.097
Myeloid Dendritic Cells (% total lymphocytes)	0.38(0.23-1.25)	0.28(0.13-0.68)	0.076	0.55(0.11-0.61)	0.832	0.380
Total NK Cells (% $CD3^{-}CD56^{+}$ lymphocytes)	9.45(4.72-25.7)	6.12(1.84-18.1)	0.027*	8.58(1.98-20.4)	0.513	0.381
$CD56^{hi}CD16^{-}$ NK cells (% total NK)	3.62(1.17-11.9)	4.09(0.71-9.83)	0.963	5.89(0.91-15.1)	0.797	0.396
$CD56^{lo}CD16^{+}$ NK cells (% total NK)	91.9(78.4-97.3)	89.2(79.1-97.7)	0.347	90.55(78.1-97.6)	0.578	1.000
KIR^{-} NK cells (% Total NK)	66.15(32.7-82.2)	46.8(27.8-88.3)	0.072	58.4(14.7-81.4)	0.177	0.239
KIR^{+} NK cells (% total NK)	35.1(17.8-67.3)	53.2(11.7-72.2)	0.072	41.6(18.6-85.3)	0.177	0.239

For this study, both CD69 and CD25 were studied as activation markers. CD25, the IL-2Rα chain, contributes to the high affinity IL-2 receptor and mediates growth and proliferation in T-cells and NK cells (Janeway et al., 2005b). Expression of CD25 is therefore required for optimal proliferation in response to low IL-2 concentrations, and its expression demonstrates that a cell is ready to proliferate should the appropriate adaptive signals (IL-2) be generated.

PBMC from HIV-1 seronegative individuals and seropositive individuals, both treatment-naïve and treatment-experienced, were cultured overnight in the presence of control cytokines (IL-12 + IL-15) or TLR agonists Poly(I:C) (5μg ml^{-1}), LPS (200ng ml^{-1}), ssRNA40 (5μg ml^{-1}) or CpG ODN2216 (2.5μM). These TLR agonists were selected as they were consistently shown to activate both NK cells and $CD8^{+}$ T-cells (Sections 3.2 and 3.6). NK cell activation was studied by flow cytometry following overnight culture (Methods Section

2.7 and antibody panel in Appendix A.4). The expression of CD69 and CD25 was first examined on total $CD3^{-}CD56^{+}$ NK cells using the gating strategy shown in Figure 3.1.1.

3.7.1. Total $CD56^{+}$ NK cell responses

Figure 3.7.1a shows the expression of CD69 on NK cells in stimulated PBMC cultures from HIV-1 seropositive and seronegative control individuals. Consistent with previous data, NK cells in HIV-1+ individuals were sensitive to cytokine stimulation with little or no loss of responsiveness compared to seronegative individuals (Section 3.6). NK cell responses to the TLR agonists tested were lower in HIV-1+ treatment experienced individuals compared to HIV-1- controls; the differences did not however reach significance when adjustment for multiple testing was applied. With the exception of ssRNA40 stimulation the frequency of CD69 was less in treatment-experience individuals compared to treatment-naïve individuals (figure 3.7.1a).

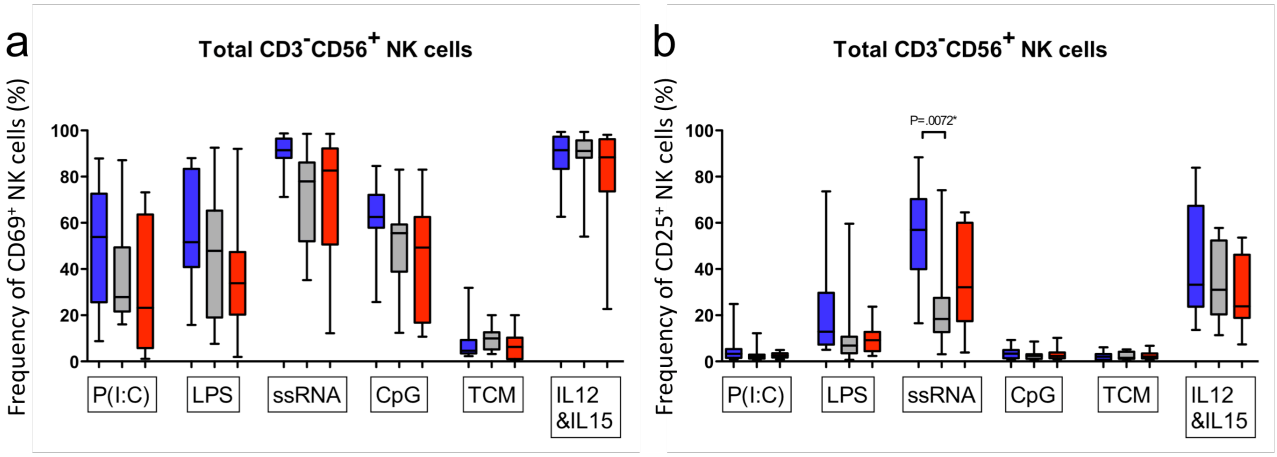


Figure 3.7.1 NK cell responsiveness to TLR agonist stimulation in HIV-1 infected versus seronegative individuals. Graphs summarising total $CD3^{-}CD56^{+}$ NK cell a) $CD69^{+}$ frequency & b) $CD25^{+}$ frequency to overnight TLR agonist stimulation in PBMC from HIV-1+, treatment-naïve (n=11, grey bars) or treatment-experienced (n=13, red bars) or HIV-1- control individuals (n=17, blue bars). Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out with adjustment for multiple testing.

Stimulation of PBMC with LPS or ssRNA40 induced the expression of CD25 on NK cells from HIV-1- control individuals (median, 12.85%, range 5.0-73.6% and median, 56.95%, range 16.5-88.4% respectively) although, little or no CD25 expression was, observed following stimulation with either Poly(I:C) or CpG (Figure 3.7.1b). A significant reduction in the frequency of $CD25^{+}$ NK cells was observed within PBMC from HIV-1+ treatment-

experienced individuals stimulated with ssRNA40 compared to HIV-1- controls (56.95%, 16.5-88.4%) (p=0.0072).

3.7.2. Different effects on $CD56^{hi}CD16^{-}$ and $CD56^{lo}CD16^{+}$ NK cell subset responses

Recent studies have suggested that functional subsets of NK cells are developmentally related and that $CD56^{hi}CD16^{-}$ 'helper' type NK cells differentiate into mature cytotoxic $CD56^{lo}CD16^{+}$ NK cells (Beziat et al., 2011, Yu et al., 2010). A potential linear relationship and differences in the maturity between these subsets raises the possibility that chronic viral infection could have a differential impact on their activation and functional capacity. In order to test the influence of chronic untreated or treated HIV-1 infection the ability of these distinct functional subsets within NK cells to respond to TLR agonists was tested. Figure 3.7.2a shows the gating strategy used to identify the different NK cell subsets discussed above.

ssRNA40 stimulation of PBMC from HIV-1+, treatment-naïve individuals, resulted in a significantly lower proportion of $CD56^{lo}CD16^{+}$ NK cells expressing CD69 (77.4%, 42.9-98.6%) compared to HIV-1- control individuals (91.8%, 73.2-97.4%) (p=0.0252) (Figure 3.7.2b). A similar pattern was also observed for ssRNA40 stimulated CD25 expression, which is significantly reduced within $CD56^{lo}CD16^{+}$ NK cells from HIV-1+, treatment-naïve individuals (14.75%, 1.5-54.9%), compared to HIV-1- controls (43.8%, 7.1-71.2%) (p=0.0252) (Figure 3.7.3). Similar to the responses observed within total NK cells, analysis of CD69 expression by the $CD56^{lo}CD16^{+}$ subset stimulated with ssRNA40 revealed a trend toward refractory NK cell responses in treatment experienced HIV-1+ individuals (80.0%, 25.6-99.3%) compared to seronegative samples (91.8%, 73.2-97.4%) (Figure 3.7.2b).

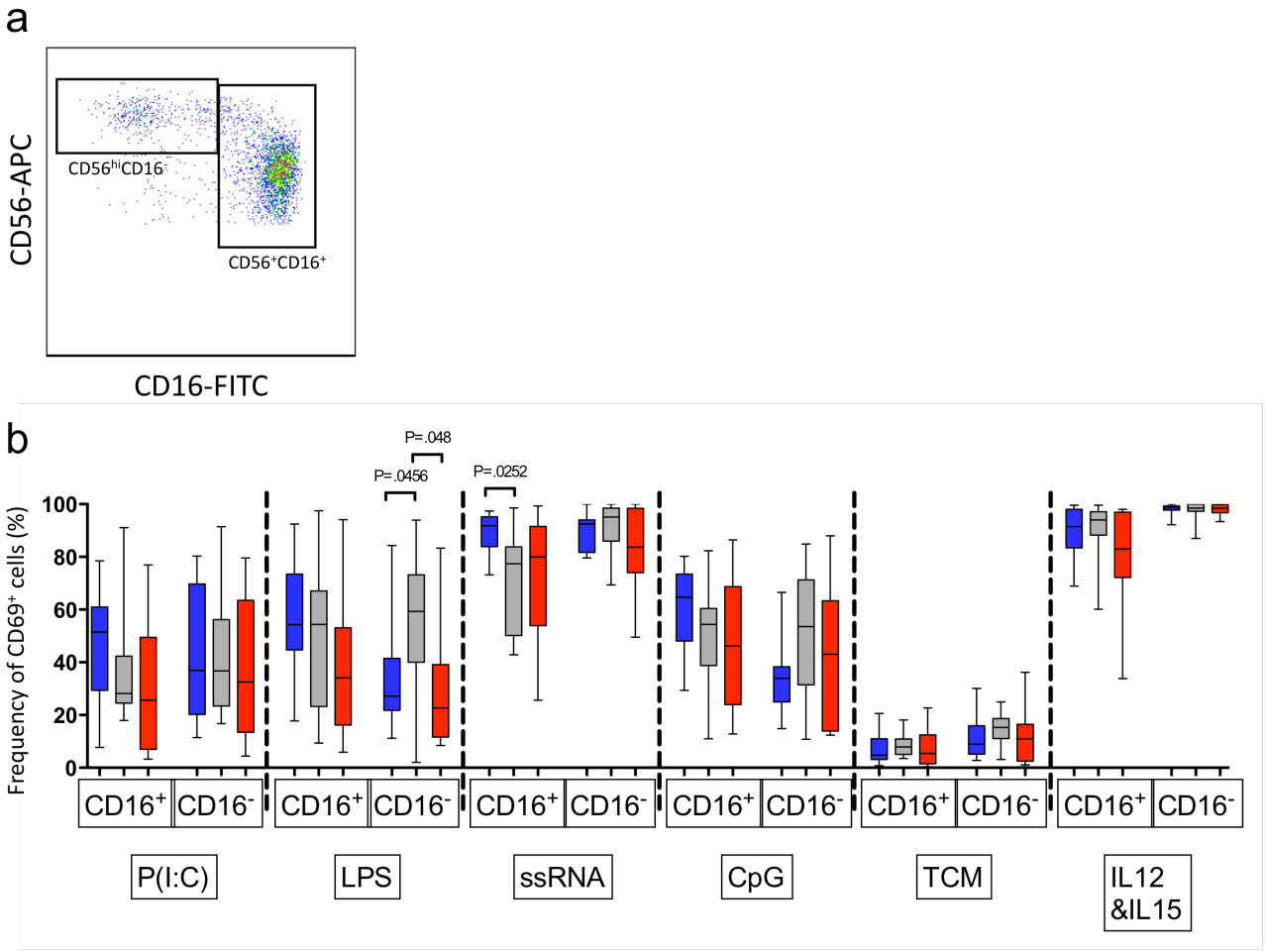


Figure 3.7.2 Functional NK cell subset responsiveness (CD69) in HIV-1 infected versus seronegative individuals following PBMC stimulation with TLR agonists. a) Gating strategy for the identification of the $CD3^{-}CD56^{+}CD16^{+}$ and $CD3^{-}CD56^{+}CD16^{-}$ NK cell subsets from total $CD3^{-}CD56^{+}$ NK cells. b) Graph showing frequency of CD69 expression by $CD3^{-}CD56^{+}CD16^{+}$ NK cell and $CD3^{-}CD56^{+}CD16^{-}$ NK cells from PBMC stimulated with TLR agonists in HIV-1+, treatment-naïve (n=11, grey bars) or treatment-experienced (n=13, red bars) or HIV-1- control individuals (n=17, blue bars). Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out with adjustment for multiple testing.

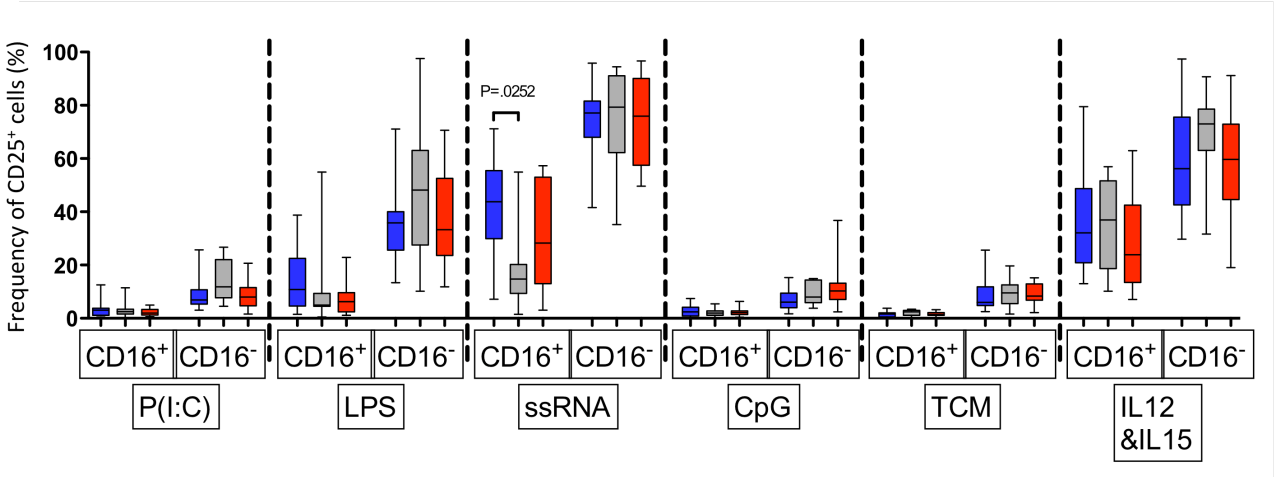


Figure 3.7.3 Functional NK cell subset responsiveness (CD25) in HIV-1 infected versus seronegative individuals following PBMC stimulation with TLR agonists. Graph showing frequency of CD25 expression by $CD3^-CD56^+CD16^+$ NK cell and $CD3^-CD56^+CD16^-$ NK cells from PBMC stimulated with TLR agonists in HIV-1+, treatment-naïve (n=11, grey bars) or treatment-experienced (n=13, red bars) or HIV-1- control individuals (n=17, blue bars). Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out with adjustment for multiple testing.

Stimulation of PBMC from treatment-experienced, HIV-1+ individuals with LPS resulted in a reduced frequency of CD69 expression on $CD56^{lo}CD16^+$ NK cells (34.1%, 5.9-94.1%) compared to HIV-1- control individuals (54.3%, 17.8-92.5%) (not significantly different), no obvious or significant differences were observed in CD25 expression (Figure 3.7.2b and Figure 3.7.3). There was lower CD69 induction on $CD56^{lo}CD16^+$ NK cell responses in HIV-1+ treatment-naive individuals stimulated with Poly(I:C) (25.65%, 3.2-76.9%) compared to HIV-1- control individuals (51.5%, 7.7-78.5%), also with no effect on CD25 expression (Figure 3.7.2b and Figure 3.7.3). No reduction in either CD69 or CD25 induction in response to LPS or Poly(I:C) was observed in $CD56^{lo}CD16^+$ NK cells from HIV-1+ treatment-naïve individuals (Figure 3.7.2b and Figure 3.7.3).

Analysis of the $CD56^{hi}CD16^-$ NK cell subset revealed that the frequency of $CD56^{hi}CD16^-$ NK cells expressing CD69 was similar or higher in both treatment-naïve and treatment-experienced HIV-1+ individuals compared to HIV-1- control individuals for all agonists tested (Figure 3.7.2b). Remarkably, LPS stimulation of the $CD56^{hi}CD16^-$ NK cell subset from treatment-naïve HIV-1+ individuals led to a significantly higher frequency of CD69 expression (median, 59.3%; range, 2.0-93.9%) compared to HIV-1- control individuals (median, 27.15%; range, 11.2-84.3%) ($p=0.0456$) and also HIV-1+ treatment-experienced individuals (22.7%,8.4-83.3%) ($p=0.048$) (Figure 3.7.2b). The frequency of CD69 expression was higher on $CD56^{hi}CD16^{lo}$ NK cells from HIV-1+ treatment-naïve individuals stimulated with CpG ODN2216 (53.5%, 10.7-84.8%) compared to HIV-1- controls (33.9%, 14.8-66.5%) (not significantly different) (Figure 3.7.2b).

TLR-induced expression of CD25 on $CD56^{hi}CD16^-$ NK cells was either maintained or higher in chronic HIV-1 infection compared to seronegative controls (Figure 3.7.3). Differences in CD25 expression following stimulation with the TLR agonists tested here did not, however, achieve significance after adjustment for multiple testing was applied.

These data indicate that the loss of responsiveness to TLR agonists observed in samples from HIV-1 seropositive individuals occurs exclusively within the major $CD56^{lo}CD16^+$ NK

cell subset whilst those of the minor $CD56^{hi}CD16^{-}$ NK cell subset are preserved, irrespective of treatment status. It is also noteworthy that the maintenance of responsiveness in the $CD56^{hi}CD16^{-}$ NK cell subset from treatment-naïve individuals persists despite reduced frequencies of plasmacytoid and myeloid DC (Table 3.7.1). $CD56^{hi}CD16^{-}$ NK cells do not express inhibitory KIR, which are increased in frequency on $CD56^{lo}CD16^{+}$ NK cells from HIV-1 infected individuals, therefore maintenance of responsiveness is potentially achieved through lack of inhibition.

3.8. ssRNA40 induces IFN-γ production, which is maintained within $CD56^{hi}$, but not $CD56^{lo}$ NK cells from HIV-1 infected individuals

$CD56^{hi}CD16^{-}$ NK cells are known potent producers of IFN-γ, secreting higher levels of this cytokine per cell than their $CD56^{lo}CD16^{+}$ counterparts. IFN-γ production in NK cells was therefore measured by intracellular cytokine staining of PBMC following stimulation with TLR agonists. PBMC were stimulated as described in Section 3.7 and NK cell IFN-γ production was studied by flow cytometry (Methods Section 2.7 and antibody panel in Appendix A.5). In these experiments NK subsets were gated according to $CD56^{hi}$ or $CD56^{lo}$ expression. Of the TLR agonists studied, only ssRNA40 stimulation was able to consistently stimulate IFN-γ production in NK cells from HIV-1- control individuals (median, 12.2% and range, 23.0-51.8%) (Figure 3.8.1). IFN-γ production was reduced within total NK cells from HIV-1+ treatment-experienced individuals (median, 5.2%, range, 2.4-40.0%) compared to HIV-1- controls (not significantly different) (Figure 3.8.1b). Stimulation with cytokines (IL-12 + IL-15) induced low frequency IFN-γ production in NK cells from all sample cohorts with no significant differences in the observed responses between these groups (Figure 3.8.1b).

Differences in IFN-γ production within the different NK cell subsets as well as between the different cohorts were also determined (Figure 3.8.1c). ssRNA40 induced IFN-γ production in $CD56^{lo}$ NK cells was lower in both treatment-naïve (median, 3.19%, range, 1.83-39.9%) and treatment-experienced (median, 4.7%, range, 0.8-27.9%) HIV-1+ individuals compared to HIV-1- individuals (median, 10.6%, range, 2.1-49.3%) although differences did not reach significance when adjusted for multiple testing (Figure 3.8.1c). Similar to the expression of CD69 and CD25, IFN-γ production was maintained within the $CD56^{hi}$ NK cell subset. Comparable proportions of $IFN\text{-}\gamma^{+}$ cells were detected in both

treatment-naïve and treatment-experienced HIV-1+ individuals compared to HIV-1- controls (Figure 3.8.1c).

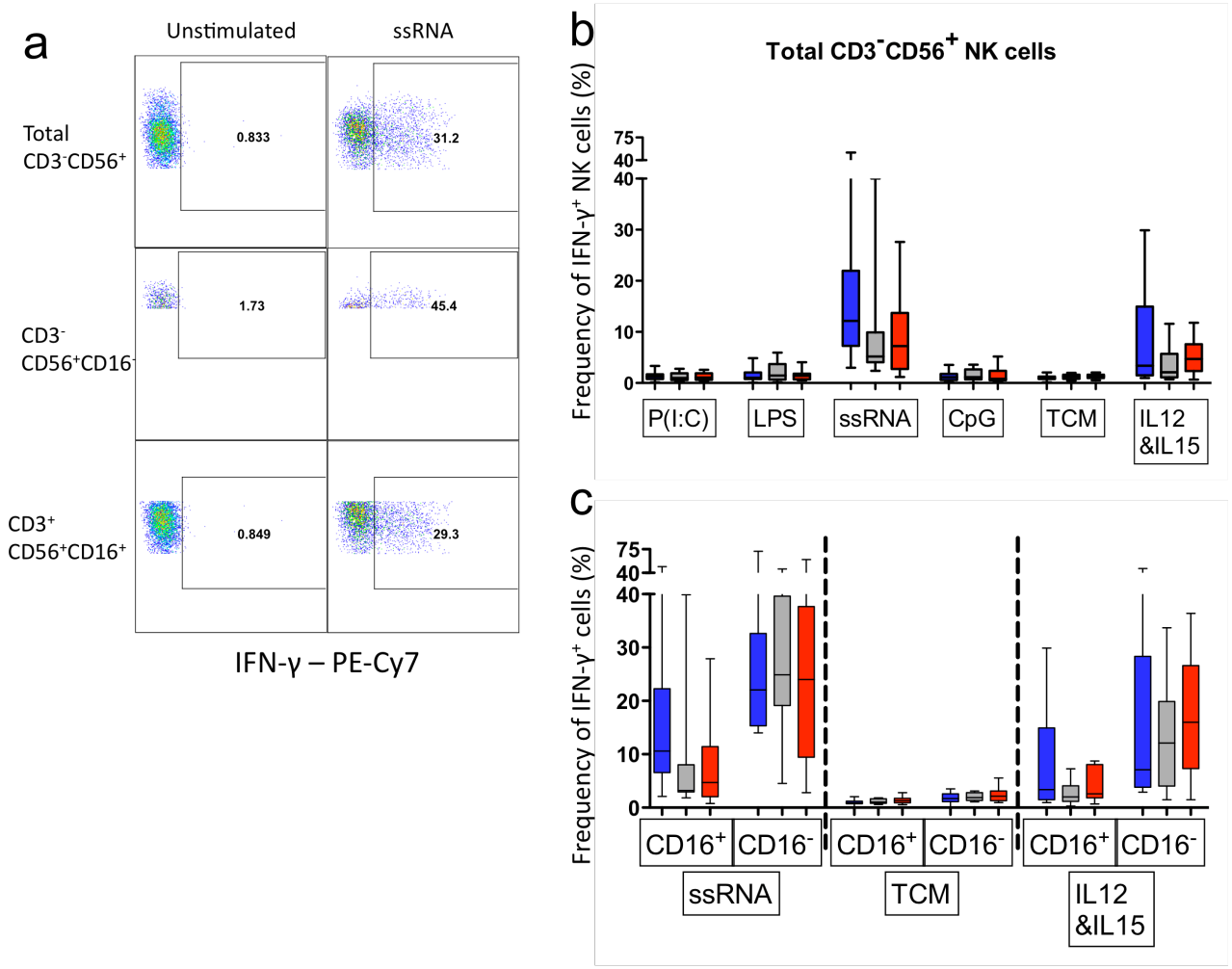


Figure 3.8.1 NK cells produce IFN-γ following ssRNA stimulation and responsiveness is selectively maintained in the CD56$^+$CD16$^-$ NK cell population during chronic HIV-1 infection. ssRNA stimulation of PBMC generates production of IFN-γ in NK cells and can also be detected in both CD56$^+$CD16$^+$ and CD56$^+$CD16$^-$ NK cells in HIV-1- control individuals. a) Flow cytometry dot plots demonstrating IFN-γ production in total NK cells and subsets, either cultured overnight in tissue culture medium alone or in the presence of ssRNA (example shown is an HIV-1- control individual). b) IFN-γ production is detected in NK cells following stimulation with cytokines (IL-12 & IL-15) or ssRNA and is only produced at very low levels following stimulation with other TLR agonists. c) Graph displaying frequency of IFN-γ expression by CD3$^-$ CD56$^+$CD16$^+$ NK cell and CD3$^-$CD56$^+$CD16$^-$ NK cells NK cells from HIV-1+, treatment-naïve (n=11, grey bars) or treatment-experienced (n=13, red bars) or HIV-1- control individuals (n=17, blue bars). Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out with adjustment for multiple testing.

3.9. KIR$^+$ NK cells from HIV-1 seropositive individuals maintain early activation *in vitro*, whilst IFN-γ responses are reduced

As $CD56^{lo}CD16^{+}$ NK cells are enriched for various KIR (Jacobs et al., 2001), the relationship between KIR expression and TLR agonist responsiveness was examined. Expression of KIR has been shown to be important in HIV-1 infection, displaying properties that enhance immunological responses against HIV-1 infection in individuals expressing specific HLA-B alleles (Alter et al., 2007, Martin et al., 2002). Increased expression of inhibitory KIR has, however, been identified on dysfunctional NK cell subsets associated with chronic HIV-1 infection (Mavilio et al., 2005). Increased KIR expression has also been found on $CD8^{+}$ T-cells in HIV-1 infected individuals and is associated with non-responsiveness to TCR-dependant stimulation (Alter et al., 2008). Inhibitory KIR expression and interaction with their self HLA ligands is a proposed mechanism for NK cell education or 'licensing' allowing NK cells to respond to cells not expressing MHC molecules (Anfossi et al., 2006, Kim et al., 2005, Kim et al., 2002). It is therefore possible that alteration of KIR expression will have knock on effects on overall NK cell responsiveness.

Results presented demonstrate a trend for higher KIR^{+} NK cell frequencies in treatment-naïve HIV-1+ individuals compared to seronegative samples (p=0.072) (Table 3.7.1). The frequency of KIR^{+} NK cells is increased in treatment-experienced HIV-1+ individuals, although not statistically significant, indicating some recovery with HAART (Table 3.7.1). Although not examined here, it is likely that the increase in KIR expression on NK cells is in part due to the generation of dysfunctional NK cells found in HIV-1 infection, which are known to express higher frequencies of inhibitory KIR than $CD56^{+}$ NK cells (Mavilio et al., 2005).

Early activation (CD69) and IFN-γ expression were analysed in $KIR^{+/-}CD56^{+}$ NK cells in response to TLR agonist stimulation, comparing HIV-1 seropositive and seronegative samples. PBMC stimulated with Poly(I:C) and CpG ODN2216 demonstrated significantly greater CD69 expression on KIR^{+} compared to KIR^{-} NK cells from HIV-1 seronegative samples. Poly(I:C) KIR^{-} median 20.9% (range 6.8-66.6%) and KIR^{+} 29.3% (6.6-79.5%) (p=0.0057). CpG ODN2216 KIR^{-} 30.3% (11.8-63.4%) and KIR^{+} 30.9% (15.7-73.9%) (p=0.0067) (Figure 3.9.1). CD69 expression on KIR^{+} NK cells from HIV-1 seropositive individuals stimulated with Poly(I:C) or CpG ODN2216 was not significantly different to KIR^{-} NK cells, and no other significant differences were observed between $KIR^{+/-}$ NK cells for any stimulation in any of the three sample cohorts.

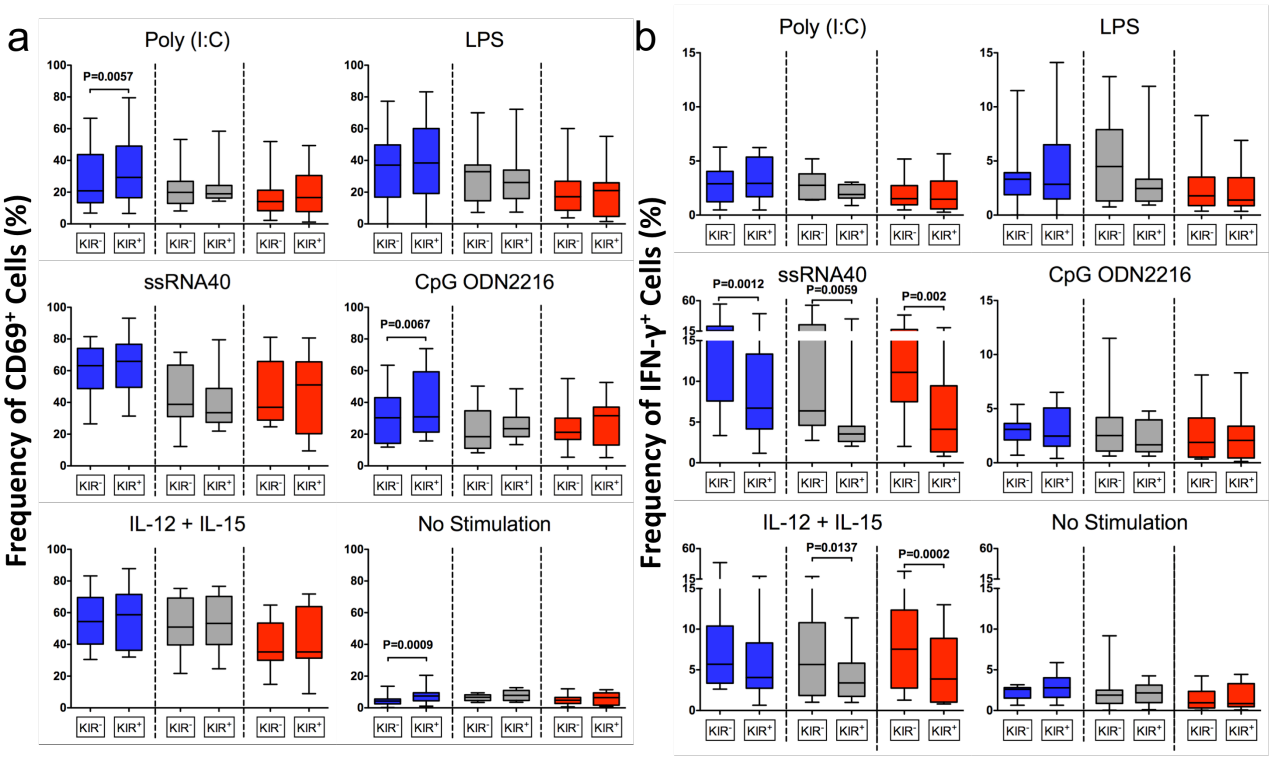


Figure 3.9.1 KIR$^+$ NK cells maintain early activation (CD69) responses to TLR agonist stimuli, but KIR$^+$ NK cells show reduced IFN-γ production in response to stimuli. Graphs display frequency of a) CD69 expression and b) IFN-γ production from KIR$^{+/-}$ NK cells from HIV-1+, treatment-naïve (n= 11, grey bars) or treatment-experienced (n=13, red bars) or HIV-1- control individuals (n=17, blue bars). CD69 expression by KIR$^+$ cells is equal to or greater than expression by KIR$^-$ cells. IFN-γ production in the KIR$^+$ NK cell population, where there is a relatively high frequency response, is reduced compared to KIR$^-$ NK cells. Box plots display median, interquartile range and range. Wilcoxon's matched pairs test has been applied to this data.

In contrast to early activation, ssRNA40 or IL-12 + IL-15 stimulated a lower frequency of IFN-γ production within KIR$^+$ NK cells compared to KIR$^-$ NK cells. Stimulation with IL-12 + IL-15 induced significantly greater IFN-γ in KIR$^-$ compared to KIR$^+$ NK cells in samples from HIV-1+ treatment-naïve and treatment-experienced individuals but not in HIV-1- controls. Treatment naïve: KIR$^-$ 5.7% (1.0-18.7%) and KIR$^+$ 3.4% (1.0-11.4%) (p=0.0137). Treatment experienced: KIR$^-$ 7.5% (1.3-26.4%) and KIR$^+$ 3.9% (0.8-13%) (p=0.0002). Stimulation with ssRNA40 induced significantly greater IFN-γ production in KIR$^-$ NK cells in all three different sample groups. Seronegative: KIR$^-$ 15.8% (3.3-55.0%) and KIR$^+$ 6.7% (1.2-40.9%) (p=0.0012). Seropositive treatment-naïve: KIR$^-$ 6.4% (2.8-53.3%) and KIR$^+$ 3.5 (2.0-33.2%) (p=0.0059). Seropositive treatment-experienced: KIR$^-$ 11.1% (2.0-38.9%) and KIR$^+$ 4.1% (0.8-21.0%) (p=0.002).

These data indicate that KIR^+ NK cells are able to respond to *in vitro* stimulation with TLR leading to CD69 expression and early activation, but are less able to produce IFN-γ than KIR^- cells.

3.10. Discussion

Data presented in this chapter, consistent with other studies (Wesa & Mailliard, 2010, Moretta et al., 2008, Iwasaki & Medzhitov, 2004, Moretta, 2002) has shown that NK cells and $CD8^+$ T-cells are sensitive to TLR agonist stimulation and that stimulation is, in most cases, sufficient to induce early activation responses in these cells. TLR agonists for intracellular TLR (Poly(I:C), Imiquimod R837, ssRNA40 and CpG ODN2216) were particularly potent inducers of NK cell and $CD8^+$ T-cell early activation. This likely reflects the requirement for cellular acquired immune response against infections of the kind associated with these PAMP. LPS also induced a strong early activation response whereas other agonists (Pam3CSK, HKLM, flagellin and FSL-1) stimulated weaker or no activation in NK cells or $CD8^+$ T-cells.

NK cells have been reported to express functional TLR, notably TLR2, 3, 7, 8 and 9 (Martinez, Huang & Yang, 2010, Sivori et al., 2010, Hart et al., 2005, Schmidt et al., 2004, Sivori et al., 2004). It is worth noting that, with the exception of TLR3 (which is unusually expressed on the cell surface and not associated with lysosomal membranes (Hart et al., 2005)), none were shown to be functional without accessory cell involvement or prior exposure to cytokines such as IL-2, IL-12 or IFN-α, although the data do not conclusively rule out the presence of functional TLR on NK cells. Assays presented here demonstrate the inability of TLR agonists to activate isolated NK cell. Furthermore, isolated NK cells are functional and retain the ability to respond to cytokine stimulation. NK cell activation by TLR agonists is therefore reliant on accessory cells and NK cells are activated via bystander mechanisms, including the release of soluble agents by DC subsets, including IFN-α, IL-12, IL-15 and IL-18, which are potent activators of NK cells (Moretta et al., 2008, Fehniger et al., 1999) and also physical cell-to-cell interactions (Nedvetzki et al., 2007, Vitale et al., 2004). Further work is necessary to fully determine whether refractory NK cell responses observed in these studies are due to dysfunctional NK cells and/or because of reduced numbers and function of dendritic cells in different patient groups. Dendritic cell numbers however appear to have recovered in treatment experienced individuals relative

to treatment naïve individuals and therefore parallel DC functional assays would need to be carried out to fully solve this issue.

Chronic innate immune stimulation is thought to occur during HIV-1 infection through stimulation of both TLR4 and TLR8 either as a result of HIV-1 induced damage to the gut leading to systemic microbial translocation, or through direct recognition of the HIV-1 genome (Alter et al., 2007a, Brenchley et al., 2006). Data presented here shows that LPS and ssRNA40 are both highly potent NK cell activators inducing not only high CD69 expression *in vitro,* but also expression of the IL-2Rα chain, CD25, in HIV-1 seronegative individuals. NK cell proliferation was, however, not observed following stimulation with LPS or ssRNA alone (data not shown), indicating that TLR stimulation was therefore not sufficient to induce IL-2 or growth factor production in NK cells or other lymphocytes *in vitro*. Nevertheless, the induction of CD25 expression indicates that the cells have become potentially more responsive to IL-2 and would therefore be 'primed' to proliferate under the appropriate conditions, such as those occurring during antigen specific T-cell responses.

The data further demonstrate that chronic HIV-1 infection renders NK cells less responsive to TLR activation *in vitro*. Interestingly, refractory NK cell responses in HIV-1 infected individuals were principally observed against agonists that stimulate intracellular TLR. Poly(I:C), ssRNA40 and CpG ODN2216 all stimulated TLR associated with endosomal vesicles and all demonstrated significant refractory NK cell activation as measured by CD69 expression in HIV-1+ treatment-experienced individuals. Examination of the magnitude of CD69 expression (MFI) on $CD8^+$ T-cells revealed the same pattern as for NK cell activation, showing refractory responses in samples from treatment-experienced individuals compared to responses in treatment-naïve individuals and seronegative healthy control individuals. This is interesting since treatment-naïve individuals are likely to be exposed to chronic stimulation by the HIV ssRNA genome and are, in addition, more likely to have virally induced damage to the gut, and thus potentially ongoing microbial translocation (Gregson et al., 2009, Alter et al., 2007a, Brenchley et al., 2006). Higher baseline activation of $CD8^+$ T-cells was, however, observed in unstimulated cultures from treatment-naïve individuals, which could partially account for the apparent maintenance of responsiveness. The MFI values for TLR-mediated stimulation of $CD8^+$ T-cells revealed that although cells from treatment-naïve individuals express a higher frequency of CD69,

they do so at a lower magnitude than in healthy control samples and so might have impaired functionality on a per cell basis.

Continued examination of NK cell activation in HIV-1 infection further revealed responsiveness to LPS and ssRNA40 as being deficient compared to healthy controls. NK cells from treatment-naïve individuals were refractory to stimulation with ssRNA40, displaying refractory responses in both CD69 and CD25 expression. The lack of responsiveness following stimulation with ssRNA40 in these treatment-naïve individuals indicates impairment; possibly due to ongoing chronic stimulation of TLR8 by the HIV-1 viral genome as these individuals have active viraemia. It must be considered that the refractory responsiveness could simply stem from the reduced function and frequency of peripheral blood DC populations. If it is the case that ongoing chronic viraemia reduces the ability of innate immune cells to respond to PAMP, it would have implications for the immunodeficiency of patients and also, therefore, the potential of using TLR agonists as adjuvants in immunotherapies.

Examination of NK cell subset activation responses demonstrated that the refractory responses seen during HIV-1 infection discussed above were found exclusively within the $CD56^{lo}CD16^{+}$ NK cell subset and that responsiveness was, by contrast, maintained in the $CD56^{hi}CD16^{-}$ subset. The significant decrease in responsiveness of $CD56^{lo}CD16^{-}$ NK cells from treatment-naïve individuals following ssRNA40 stimulation indicated that these cells had been chronically stimulated prior to *in vitro* stimulation and that the functional capacity of these cells may be impaired. Preservation of responsiveness in $CD56^{hi}CD16^{-}$ NK cells from treatment-naïve individuals was observed despite a reduction in frequency of peripheral blood DC populations. This indicates that these NK cells have a lower threshold for DC-dependent activation, likely due to the lack of inhibitory KIR expression, or that other accessory cells in human peripheral blood are involved. These possibilities were investigated for certain TLR agonists in subsequent chapters 4 and 5, particularly section 4.2 (which examines the differentiation of $CD56^{hi}CD16^{-}$ NK cells) and section 5.7 (which demonstrates a partial requirement of DC in the recovery of recall of antigen specific immune responses). Stimulation with LPS led to greater activation of $CD56^{hi}CD56^{-}$ NK cells from treatment-naïve individuals compared to healthy controls. It could be speculated that these cells are already partially activated *in vivo* by endotoxin as a result of microbial translocation and that responses are boosted by further recruitment of activated cells *in-vitro*.

The refractory responses occurring in the $CD56^{lo}CD16^{+}$ NK cell subset might also be associated with chronic stimulation; it has been suggested that this subset is developmentally more mature (this is discussed in the following chapter) and as such is more likely to have become exhausted (Beziat et al., 2011, Yu et al., 2010, Freud & Caligiuri, 2006). Activation of the $CD56^{lo}CD16^{+}$ NK cell subset did not recover, even after decreases in patient NK cell and DC frequencies were reversed post-therapy. This again raises the possibility that a proportion of NK cells remain functionally deficient, or that accessory cell populations have not functionally recovered, despite successful treatment and recovery of normal cell frequencies. Anti-retroviral treatment itself may also affect responsiveness, which could explain the differences observed between treatment experienced and treatment naïve individuals.

Of the agonists tested, only stimulation of PBMC with ssRNA40 or cytokines was able to induce IFN-γ production in NK cells at a high frequency. This again demonstrated the potency of ssRNA as a TLR agonist, and the impact that refractory responses may have on NK cell function. A similar pattern of responsiveness was observed when PBMC from HIV-1 seropositive individuals are stimulated with ssRNA40 as has previously been described. Total NK cells from treatment-experienced samples were refractory to stimulation, and $CD56^{lo}$ NK cell responses from both treatment-experienced and treatment-naïve individuals were refractory. Refractory IFN-γ production could have consequences for the development of a Th1-type acquired immune response, as NK derived IFN-γ is known to feedback to DC and promote a cellular immune response (Andoniou, Coudert & Degli-Esposti, 2008). Encouragingly the $CD56^{hi}$ NK cell subset from HIV-1 samples remained responsive to ssRNA40 stimulation *in vitro* and produced IFN-γ at a higher frequency than $CD56^{lo}$ NK cells.

KIR expression on $CD56^{lo}CD16^{+}$ NK cells was increased in chronic HIV-1 infection, in keeping with previous reports (Mavilio et al., 2005, Alter et al., 2004b, Mavilio et al., 2003), with partial recovery observed in treatment-experienced individuals. Interestingly, early activation (CD69 expression) was maintained following TLR agonist stimulation in KIR^{+} NK cells from HIV-1 seropositive individuals, although it has previously been shown that there is increased activation of NK cells in HIV-1 infected individuals (Alter et al., 2004b). Examination of more functional IFN-γ responses revealed KIR^{+} NK cells were, however, less responsive than KIR^{-} cells. As discussed above this is unsurprising given that

specialist cytokine producing $CD56^{hi}CD16^{-}$ NK cells were found exclusively within the KIR^{-} NK subset and that KIR^{+} NK cells are inhibited in an MHC-I sufficient environment. There did, however, appear to be a knock on effect of HIV-1 infection, as there were higher frequencies of inhibitory KIR^{+} NK cells observed in chronic HIV-1 infection. Combined with the decreased levels of NCR also observed in $CD56^{lo}CD16^{+}$ NK cells, this indicates these cells are less responsive and contribute to pathology in HIV-1 infection as previously reported (Mavilio et al., 2005). The frequency and responsiveness $CD56^{-}CD16^{+}$ NK cells was not examined here, but they are also known to have increased levels of inhibitory KIR expression and reduced levels of NCR and are unresponsive to stimulation (Mavilio et al., 2005).

3.11. Summary

This chapter has explored innate immune activation in response to TLR agonist stimulation, with particular focus on NK cell activation. $CD8^{+}$ T-cell activation was also demonstrated, and the requirement for accessory cells to generate these responses was shown.

Refractory innate cell responses to TLR agonist stimulation in chronic HIV-1 infection were observed in NK cells and $CD8^{+}$ T-cells. Refractory responses were particularly evident in the treatment-experienced HIV-1 seropositive sample group, despite all samples having undetectable viraemia and recovering $CD4^{+}$ T-cell frequencies. Refractory responses observed following LPS and ssRNA40 stimulation were particularly revealing as both have been implicated to have a role in chronic immune activation seen in HIV-1 infection (Alter et al., 2007a, Brenchley et al., 2006). Further break down of NK cell responses into the two functionally distinct subsets based on CD56 and CD16 expression revealed that refractory responses were confined exclusively to the $CD56^{lo}CD16^{+}$ NK cell subset, and that responses within the $CD56^{hi}CD16^{-}$ subset were comparable to (or were in some cases higher than) those observed in seronegative control samples.

4. Effect of chronic HIV-1 infection on natural killer cell maturation and cell survival

Aim

Data presented in the previous chapter established a normal range for NK cell and $CD8^+$ T-cell activation responses to TLR agonist stimulation and the effect of chronic HIV-1 infection. In this chapter, experiments to investigate the effect of infection on the maturational stages of peripheral blood NK cells, and the effect of treatment on these subsets are described. Assays were carried out with the aim of demonstrating how chronic HIV-1 infection affected NK cell maturation and survival *in vitro*. Furthermore, the capacity of PAMP to affect maturation in NK cells from HIV-1 negative healthy control individuals was determined through the expression of NK cell maturation markers. Comparisons were then made with expression of the same markers in HIV-1 infected individuals.

Introduction

It is known that dysfunctional NK cells are generated in chronic HIV-1 infection, preventing normal activation and full responsiveness to stimuli (Mavilio et al., 2005). It is also well established that $CD8^+$ T-cells are skewed toward more mature and exhausted phenotypes in HIV-1 infection (Champagne et al., 2001). Given the parallels between $CD8^+$ T-cell development and NK cell development (Sun & Lanier, 2011), it is likely that NK cells are also skewed to a more mature state during chronic HIV-1 infection.

Current models of differentiation of human blood NK cells suggest that $CD56^{hi}CD16^-$ NK cells are replenished from the bone marrow from $CD56^{hi}CD16^-$c-$Kit^+CD62L^+CD94/NKG2A^+$ precursors (which also lack perforin or KIR expression) initially migrating to peripheral lymphoid tissues (Chan et al., 2007, Romagnani et al., 2007, Farag & Caligiuri, 2006). Activation of $CD56^{hi}CD16^-$ cells in vitro leads to the loss of c-Kit and the acquisition of CD16 and other markers of cytotoxic NK cells (Romagnani et al., 2007, Farag & Caligiuri, 2006). Such a lineage relationship is supported by recent studies demonstrating that CD94 defines three peripheral blood NK cell populations with distinct functional maturity, with $CD94^{high}CD56^{bright}CD16^-$ cells being the least mature, $CD94^{low}CD56^{dim}CD16^+$ the most highly differentiated, and a population of

$CD94^{bright}CD56^{dim}$ NK cells showing intermediate expression and functional capacity (Yu et al., 2010). Another recent study by Béziat *et al* (2011) demonstrated a different intermediate NK cell population, defined as $CD56^{bright}CD16^{+}$, that are capable of producing large quantities of cytokine and also demonstrate potent cytotoxic capabilities. Further evidence that $CD56^{dim}CD16^{+}$ NK cells represent a mature population has been demonstrated through their shorter telomere length compared to $CD56^{bright}CD16^{-}$ cells (Chan et al., 2007, Romagnani et al., 2007).

Previous studies have indicated that the $CD56^{lo}CD16^{+}$ NK cell subset is preferentially reduced in frequency during chronic HIV-1 infection, leading to the emergence of a dysfunctional pre-apoptotic, $CD56^{-}CD16^{+}$ subset (Mavilio et al., 2005, Mela et al., 2005). This raises the possibility that chronic immune activation during HIV-1 infection leads to the accelerated differentiation of $CD56^{hi}CD16^{-}$ NK cells into $CD56^{lo}CD16^{+}$ NK cells and eventually into the $CD56^{-}CD16^{+}$ subset. $CD56^{hi}CD16^{-}$ NK cells can be considered the least mature of NK cell subsets within peripheral blood and are likely to be the least exposed to chronic activation, being constantly replenished from the bone marrow (Romagnani et al., 2007, Farag & Caligiuri, 2006, Ferlazzo et al., 2004). Such a replenishment of $CD56^{hi}CD16^{-}$ NK cells is consistent with the maintenance of the activation and cytokine responses to TLR ligands demonstrated in Chapter 3.

Utilising the phenotypic markers of functional maturation described by Yu *et al* and Béziat *et al*, the potency of TLR agonists or cytokines for promoting maturation of $CD56^{hi}CD16^{-}$ NK cells *in vitro* was examined here (Beziat et al., 2011, Yu et al., 2010). The frequency of different maturational stages of NK cells was also assessed during HIV-1 infection, and the ability of PAMP to further influence maturation or survival in these samples studied. Finally, factors involved in cellular maturation were measured in the plasma from control and HIV-1 seropositive individuals.

4.1. Phenotypic analysis of maturational markers on freshly isolated and *in vitro* cultured NK cells

There is evidence that chronic immune activation likely impacts on NK cell maturation and function (discussed in Section 1.6.4), particularly with regard to the generation of the dysfunctional $CD3^{-}CD56^{-}CD16^{+}$ NK cell population (Mavilio et al., 2005, Mela et al., 2005). c-Kit, the receptor for stem cell factor, is expressed by $CD56^{hi}CD16^{-}$ NK cells, but is

absent on the more cytotoxic $CD56^{lo}CD16^{+}$ subset. It is therefore postulated that the $CD56^{hi}CD16^{-}$ NK subset represents a less mature cell population, potentially giving rise to $CD56^{lo}CD16^{+}$ NK cells as they mature (Caligiuri, 2008, Romagnani et al., 2007, Freud et al., 2005, Ferlazzo et al., 2004, Carson, Fehniger & Caligiuri, 1997). CD94, which associates with NKG2A, B, C, E and H (whose ligands are self HLA-E molecules) (Caligiuri, 2008), represents a further marker useful for NK cell maturation studies (Yu et al., 2010). NK cell maturation was therefore examined *ex vivo* using a model for human NK cell maturation based on the expression of c-Kit on NK cell subsets, defined by CD56 and CD16 expression. Data were corroborated using CD94 and CD56 expression to define NK cell maturational stages. These NK cell populations were compared between healthy control individuals (n=12) and HIV-1 seropositive individuals from both treatment-naïve (n=9) and treatment-experienced individuals (n=12). Patient characteristics for this study are shown in Table 4.1.1.

Table 4.1.1 Patient Characteristics

	HIV-1- Healthy Control (n=12)	HIV-1+ Treatment Naïve (n=9)	HIV-1+ Treatment Experienced (n=12)
Age	28 (24-40)	45 (28-45)	43 (37-52)
CD4 (cells μl^{-1})	N/A	417 (228-622)	584 (236-1592)
Plasma Viral Load (copies ml^{-1} blood)	N/A	93277 (13526-401605)	N/A

PBMC from healthy donor peripheral blood were stained *ex vivo* for CD3, CD56, CD16, CD94 and c-Kit (CD117) (see Appendix Table A.6 for full details). For analysis of c-Kit on freshly isolated cells, NK cell populations were firstly gated into four subsets based on CD56 and CD16 expression within a $CD3^{-}$ gate on lymphocytes (Figure 4.1.1a). $CD56^{hi}CD16^{-}$, $CD56^{hi}CD16^{lo}$, $CD56^{lo}CD16^{+}$ and $CD56^{-}CD16^{+}$ subset were included to avoid any bias when comparing HIV-1 seronegative with seropositive samples, the latter of which is potentially enriched for these NK cells. c-Kit expression was then examined on these subsets using the gating strategy described by Béziat *et al.* (2011).

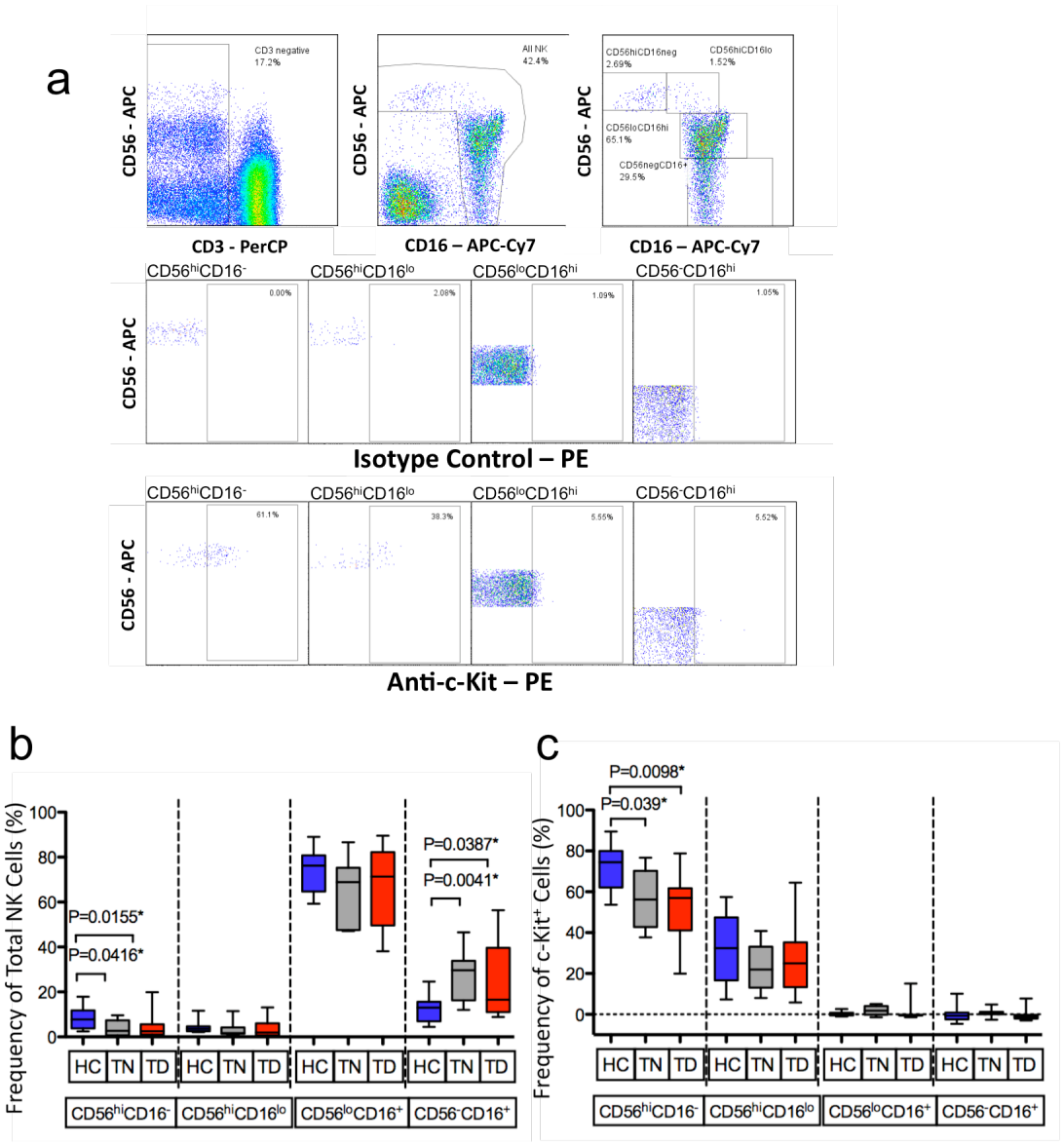


Figure 4.1.1 NK cell maturation – c-Kit expression. a) Gating strategy demonstrating the four NK cell populations based on CD56 and CD16 expression within $CD3^-$ lymphocytes; $CD56^{hi}CD16^-$, $CD56^{hi}CD16^{lo}$, $CD56^{lo}CD16^+$ and $CD56^-CD16^+$ and the c-Kit expression within each subset. b) Graph displaying the frequency of NK cell subsets as a proportion of total NK cells in HIV-1- control and HIV-1+ individuals, both treatment-experienced and naive. c) Graph displaying frequency of c-Kit expression as a percentage of the NK cell subsets shown in in HIV-1- control and HIV-1+ individuals, both treatment-experienced and naïve. HIV-1- control (HC, n=12, blue bars), HIV-1+ treatment-naïve (TN, n=9, grey bars) and experienced (TD, n=12, red bars) individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out.

Analysis of $CD56^{hi}CD16^-$ NK cell frequencies revealed that they were significantly reduced in HIV-1+ treatment-naïve (median 2.7%, range 0.4-9.6%, p=0.04) and treatment-experienced (median 2.5%, range 0.6-19.9%, p=0.02) individuals compared to seronegative controls (median 7.8%, range 2.5-17.9%) (Figure 4.1.1b). This finding was likely influenced by the fact that the analysis included the dysfunctional $CD56^-CD16^+$ NK

cell population, the frequency of which was significantly less in HIV-1- controls (median 13.0%, range 4.4-24.6%) compared with HIV-1+ treatment-naïve (median 29.7%, range 12.0-46.5%, p=0.004) and treatment-experienced individuals (median 16.6%, range 8.9-56.4% p=0.039) in (Figure 4.1.1b). There was no significant difference in frequency between sample groups for either the $CD56^{hi}CD16^{lo}$ or the $CD56^{lo}CD16^{+}$ subsets although a reduction in the median percentage was observed for the latter in treatment-naïve HIV-1+ individuals (not significant), consistent with previously published data (healthy controls: median 76.2%, range 59.3-89.0%; treatment-naive: median 68.9%, range 47.1-86.6%) (Mavilio et al., 2003).

c-Kit expression was analysed on each of the four NK cell subsets defined above (Figure 4.1.1c). For analysis of this data, isotype control staining using a phycoerythrin-conjugated mouse $IgG1^{k}$ antibody was subtracted from the values for c-Kit. In addition to observing a lower frequency of $CD56^{hi}CD16^{-}$ NK cells in HIV-1+ individuals, it was found that there was a significantly lower frequency of c-Kit expressed by this subset both in HIV-1+ treatment-naïve (median 56.2%, range37.7-76.7%) and treatment-experienced (median 56.9%, range 19.8-78.7%, p=0.039) individuals compared to HIV-1- control individuals (median 74.5%, range 53.7-89.5%, p=0.0098). Lower frequencies of $c\text{-}Kit^{+}$ cells were observed within the $CD56^{hi}CD16^{lo}$ NK cell subset in all three groups of individuals, compared to that observed within $CD56^{hi}CD16^{-}$ NK cells, consistent with a loss of this marker on maturation (HIV-1- controls: median 32.4%, range 7.3-57.4%; HIV-1+ treatment-naïve: median 22.0%, range 8.0-40.8%; treatment-experienced: median 24.0%, range 5.8-64.5%). Although the HIV-1 seropositive patient groups had a lower median proportion of c-Kit expressing cells within $CD56^{hi}CD16^{lo}$ cells, there was no significant difference in the frequency of c-Kit expression between groups of individuals, consistent with this being an intermediary subset that is replenished from the $CD56^{hi}CD16^{-}$ subset. It is clear from this data that neither $CD56^{lo}CD16^{+}$ NK cells nor $CD56^{-}CD16^{+}$ NK cells expressed any significant level of c-Kit, consistent with the relative maturity of these subsets.

Figure 4.1.2a shows the gating strategy used to examine NK cell maturation in three subsets defined by CD94 expression (Yu et al., 2010): $CD56^{hi}CD94^{+}$, $CD56^{lo}CD94^{+}$ and $CD56^{lo}CD94^{-}$ (although Yu *et al* describe these subsets as $CD94^{high}CD56^{bright}$, $CD94^{high}CD56^{dim}$ and $CD94^{low}CD56^{dim}$ respectively). Yu *et al.* (2010) describe the $CD56^{lo}CD94^{+}$ subset as an intermediary subset with the ability to produce high levels of

cytokine in response to stimuli and also to carry out cytotoxic effector functions. No significant differences were found between groups of individuals in any of the three CD94-defined subsets. However, consistent with the data for c-Kit expression, a reduction in the median proportion of NK cells within the $CD56^{hi}CD94^{+}$ subset and a reciprocal increase in the median proportion of cells in the $CD56^{lo}CD94^{-}$ subset was observed in HIV-1 seropositive treatment-experienced individuals whilst the frequency of $CD56^{lo}CD94^{+}$ varied little between groups (Figure 4.1.2b).

c-Kit and CD94 expression are both useful determinants of NK cell maturation and were used to determine changes in maturation brought about by TLR agonist stimulation discussed in the following sections.

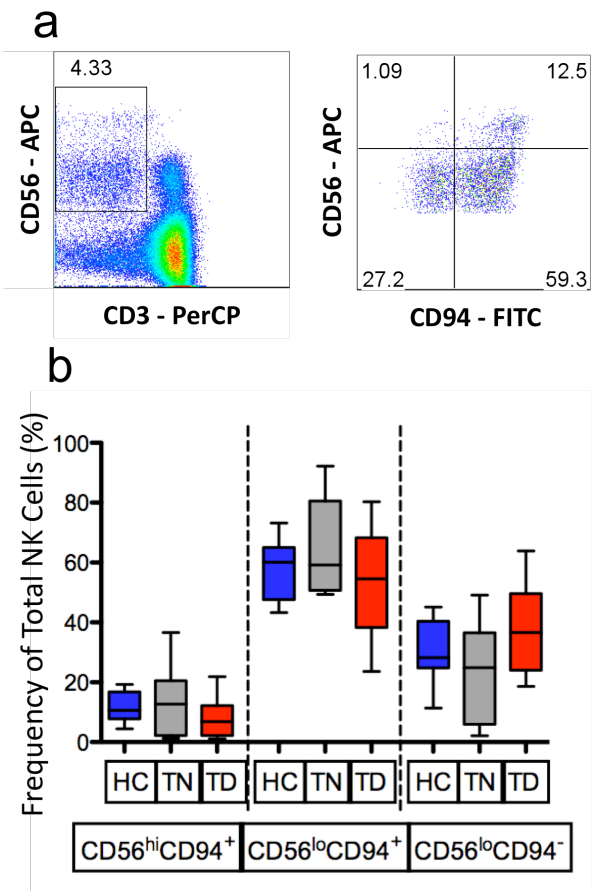


Figure 4.1.2 NK cell maturation – CD94 expression. a) Gating strategy demonstrating NK cell populations based on CD56 and CD94 expression within $CD3^{-}CD56^{+}$ NK cells; $CD56^{hi}CD94^{+}$, $CD56^{lo}CD94^{+}$, $CD56^{lo}CD94^{-}$. b) Graph displaying the frequency of the three NK cell subsets shown as a proportion of total NK cells in HIV-1- control and HIV-1+ individuals, both treatment-experienced and naive. HIV-1- control (HC, n=12, blue bars), HIV-1+ treatment-naïve (TN, n=9, grey bars) and experienced (TD, n=12, red bars) individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out.

4.2. $CD56^{hi}CD16^{-}$ NK cells lose expression of CD94 and c-Kit when stimulated *in vitro* with cytokines and TLR agonists.

In order to determine whether $CD56^{+}CD16^{-}$ NK cells could progress toward $CD56^{lo}CD16^{+}$ cells, NK cells were depleted from healthy donor PBMC (n=2) and then reconstituted for culture with autologous isolated $CD56^{hi}CD16^{-}$ NK cells as described in Sections 2.9.2 and 2.9.3. Figure 4.2.1 demonstrates the different steps in the procedure, showing the phenotype of PBMC before (upper panels) and after (middle panels) NK cell depletion and reconstitution with $CD3^{-}CD56^{hi}CD16^{-}$ NK cells (lower panels). Cells were then cultured for six days in medium alone or with the cytokines IL-12 (1ng ml^{-1}) and IL-15 (20ng ml^{-1}) or the TLR agonists LPS (200ng ml^{-1}) or ssRNA40 (5µg ml^{-1}). Figure 4.2.2 shows the results from a single representative individual of two tested. When cultured in medium alone 90.4% of reconstituted NK cells remain $CD56^{hi}CD16^{-}$ (for the individual shown) compared to 88.6% in the equivalent freshly reconstituted (day 0) sample and 90.3% of NK cells remain $CD56^{hi}CD94^{+}$ compared to 91% in the freshly reconstituted sample. Interestingly, the frequency of c-Kit^{+} NK cells increased from 42.7% compared to 74.2% in the *ex vivo* reconstituted sample shown after overnight culture in medium alone (Figure 4.2.2, centre panels).

In the example shown, stimulation of cells with cytokines or with the TLR agonists LPS or ssRNA40 reduced the frequency of $CD56^{hi}CD16^{-}$ NK cells. Conversely, an increase in the frequency of $CD56^{lo}CD16^{+}$ cells was observed in the presence of cytokines, LPS or ssRNA40, consistent with a redistribution of NK cell subsets towards a more mature phenotype (Figure 4.2.2).

A reduction in the frequencies of c-Kit^{+} and $CD56^{hi}CD94^{+}$ NK cells was observed when stimulated with cytokines (IL-12 and IL-15) and the TLR agonists LPS and ssRNA40. A reciprocal rise was observed in the frequency of the most mature $CD56^{lo}CD94^{-}$ subset after stimulation, again consistent with progressive differentiation (Figure 4.2.2 and Tables 4.2.1 and 4.2.2).

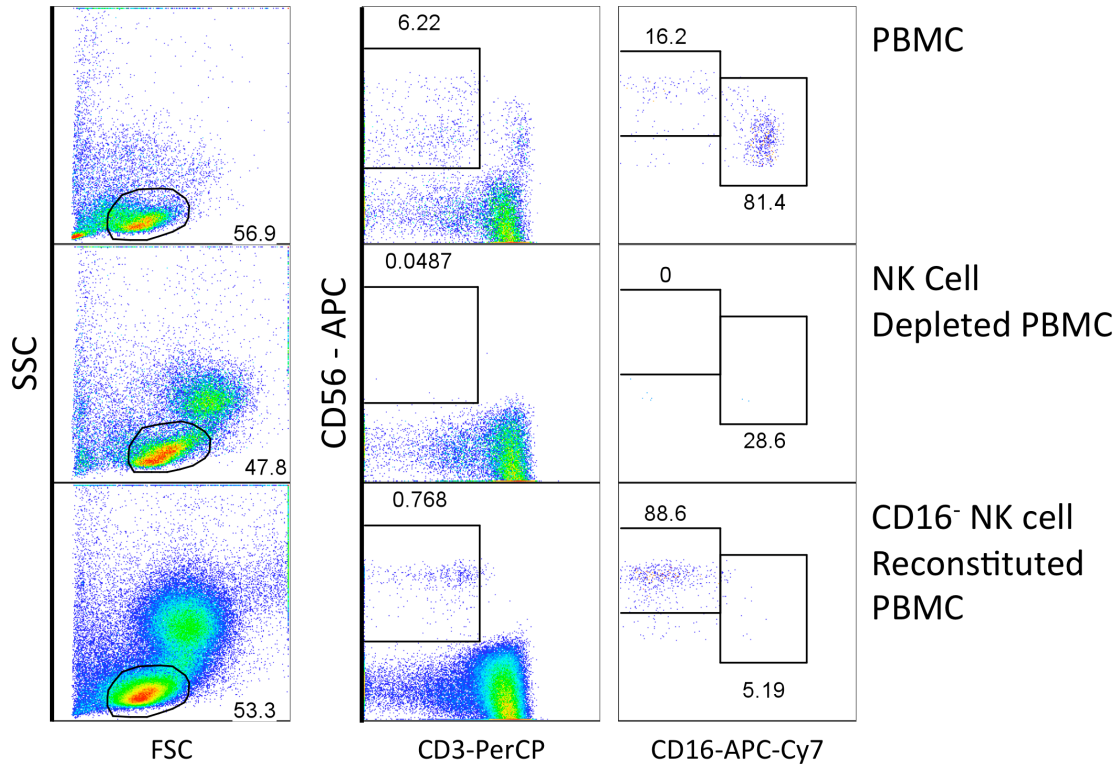


Figure 4.2.1 NK cell depletion and $CD56^{hi}CD16^{-}$ NK cell reconstitution. Dot plots demonstrating the various stages for removing $CD56^{lo}CD16^{+}$ NK cells. Total NK cells were removed from PBMC and in parallel $CD56^{hi}CD16^{-}$ cells were isolated and then added back into NK depleted PBMC.

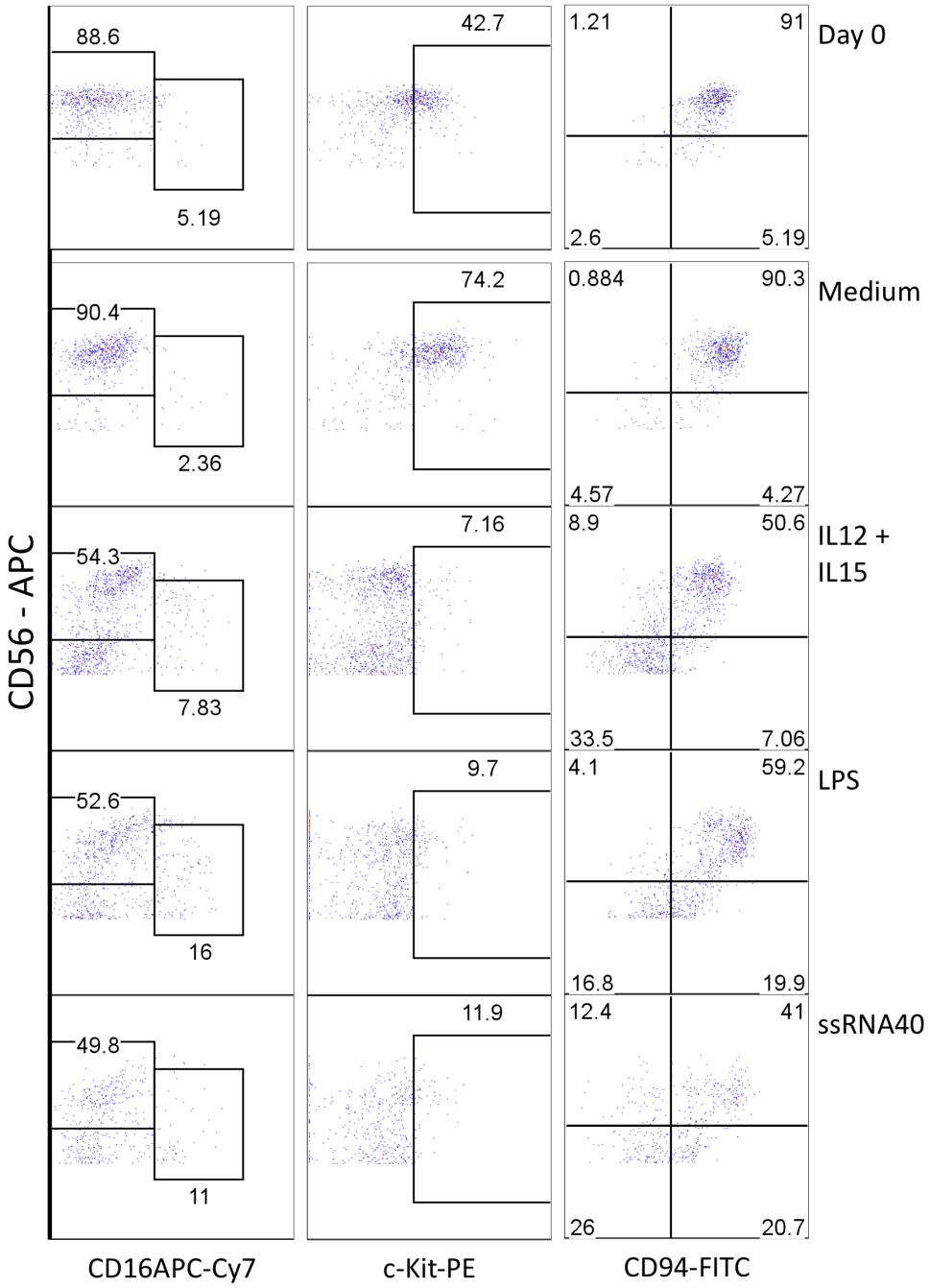


Figure 4.2.2 CD56^{hi}CD16^{-} NK cells lose CD56^{hi}, CD94 and c-Kit expression and gain CD16 expression following TLR agonist stimulation. CD56^{lo}CD16^{+} NK cell depleted PBMC were cultured in the presence of different TLR agonists and cytokines for 6 days and the resulting changes in differentiation markers was measured by flow cytometry. Dot plots demonstrating that some CD56^{hi}CD16^{-} NK cells, which express both CD94 and c-Kit become CD56^{lo}CD16^{+} NK cells and that those NK cells lose CD94 expression. Furthermore c-Kit expression is being lost from all NK cells following stimulation. Dot plot from a single healthy control individual and representative of 2 separate individuals.

A full summary of data from two individuals is shown in Tables 4.2.1 and 4.2.2. Data from sample one clearly demonstrated NK cell maturation in response to stimulation and is

described above. Sample two followed a similar pattern to sample 1, but data were skewed for cytokine and ssRNA40 stimulation where a higher intensity stain for CD56 was observed. c-Kit frequency was, however, reduced in stimulated cultures for sample two and LPS stimulation did not appear to affect CD56 expression, demonstrating that NK cells were induced to mature as was seen in sample one. A small increase in the proportion of $CD16^{+}$ cells was observed in both individuals tested after stimulation of reconstituted cultures with cytokines, LPS or ssRNA40, although this did not account for the observed loss in $CD56^{hi}$c-Kit^{+} cells. It is likely however that the six-day assays did not permit sufficient time for full differentiation and for CD16 expression to occur. Others have demonstrated induced CD16 expression after stimulation for longer periods of 10 or 14 days (Beziat et al., 2011, Chan et al., 2007). However, a reduction was observed in both individuals in the proportion of $CD56^{hi}CD94^{+}$ cells with reciprocal increases in the proportions of $CD56^{lo}CD94^{+}$ and $CD56^{lo}CD94^{-}$ cells, indicating that an early progression of differentiation may be occurring. Assays such as this serve as a useful model to examine how chronic stimulation with PAMP such as endotoxin and viral RNA affects NK cell maturation *in vivo* in HIV-1 infection.

Day 0		Sample	
	Frequency (%)	1	2
PBMC	$CD56^{hi}CD16^{-}$	16.2	1.59
	$CD56^{lo}CD16^{+}$	81.4	97.3
	c-Kit^{+}	6.97	1.21
	$CD56^{hi}CD94^{hi}$	38.4	1.52
	$CD56^{lo}CD94^{hi}$	32.4	35.5
	$CD56^{lo}CD94^{lo}$	22.7	62.8
Reconstituted	$CD56^{hi}CD16^{-}$	88.6	79
	$CD56^{lo}CD16^{+}$	5.19	12.3
	c-Kit^{+}	42.7	56.8
	$CD56^{hi}CD94^{hi}$	91	42
	$CD56^{lo}CD94^{hi}$	5.19	46.9
	$CD56^{lo}CD94^{lo}$	2.6	11.1

Table 4.2.1 Frequencies of NK cell differentiation groups from freshly isolated PBMC and NK cell depleted $CD56^{hi}CD16^{-}$ NK cell reconstituted PBMC.

Table 4.2.2 Frequencies of NK cell differentiation groups from PBMC and NK cell depleted $CD56^{hi}CD16^{-}$ NK cell reconstituted PBMC cultured for 6 days in the conditions shown.

Day 6		Individual	
	Frequency (%)	1	2
Med	$CD56^{hi}CD16^{-}$	90.4	54.9
	$CD56^{lo}CD16^{+}$	2.36	4.88
	c-Kit^{+}	74.2	65.9
	$CD56^{hi}CD94^{hi}$	90.3	47.6
	$CD56^{lo}CD94^{hi}$	4.27	34.1
	$CD56^{lo}CD94^{lo}$	4.57	14.6
IL12 + IL15	$CD56^{hi}CD16^{-}$	54.3	74.3
	$CD56^{lo}CD16^{+}$	7.83	1.34
	c-Kit^{+}	7.16	8.35
	$CD56^{hi}CD94^{hi}$	50.6	64.2
	$CD56^{lo}CD94^{hi}$	7.06	21.1
	$CD56^{lo}CD94^{lo}$	33.5	4.43
LPS	$CD56^{hi}CD16^{-}$	52.6	34.6
	$CD56^{lo}CD16^{+}$	16	11.3
	c-Kit^{+}	9.7	32.1
	$CD56^{hi}CD94^{hi}$	59.2	32.1
	$CD56^{lo}CD94^{hi}$	19.9	39.6
	$CD56^{lo}CD94^{lo}$	16.8	25.2
ssRNA40	$CD56^{hi}CD16^{-}$	49.8	61.1
	$CD56^{lo}CD16^{+}$	11	3.13
	c-Kit^{+}	11.9	22.2
	$CD56^{hi}CD94^{hi}$	41	57.2
	$CD56^{lo}CD94^{hi}$	20.7	27.9
	$CD56^{lo}CD94^{lo}$	26	10.4

4.3. Reduced frequencies of CD94^{+} and c-Kit^{+} NK cells are maintained in PBMC cultures from HIV-1 infected individuals.

In order to establish the impact of chronic HIV-1 infection on NK cell maturation, *in vitro* culture assays using PBMC were carried out. PBMC from HIV-1 seropositive individuals who were treatment-naïve and viraemic, or treatment-experienced with undetectable viraemia, were cultured for six days in culture medium. The frequencies of the different maturational groups were examined and compared to the frequencies of the same populations in HIV-1 seronegative individuals. Patient characteristics for this study were shown previously in Table 4.1.1. As it was demonstrated in Section 4.1.1 that c-Kit is only expressed on CD56^{hi} NK cells, CD56^{hi}c-Kit^{+} cells were compared with CD56^{lo}c-Kit^{-} NK cells in this analysis. A lower frequency of total CD56^{hi}c-Kit^{+} cells was observed in treatment-naïve (median 3.2%, range 0.5-12.0%) and treatment-experienced (median 1.9%, range 0.5-7.3%) HIV-1+ individuals compared to HIV-1 seronegative controls (median 5.0%, range 2.1-14.2%), although this did not reach significance (Figure 4.3.1).

After six days *in vitro* culture in medium alone, *ex vivo* differences persisted, becoming statistically significant in samples from both treatment-experienced and treatment-naïve HIV-1 seropositive compared to seronegative individuals. There was a significantly higher frequency of c-Kit^{+} NK cells from seronegative samples after six days (median 7.1%, range 3.6-39.6%) compared to both treatment-naïve (3.7%; 0.4-10.3%) and treatment-experienced (3.5%; 0.2-13.4%) seropositive samples (p=0.0251 and p=0.0209 respectively) (see Figure 4.3.1). Correspondingly, there was a significantly greater frequency of c-Kit negative NK cells in both treatment-naïve (93.6%; 82.8-98.5%) and treatment-experienced (94.3%; 78.5-99.2%) compared to seronegative (76.4%; 43.7-93.0%) samples (p=0.006 and p=0.004 respectively) (see Figure 4.3.1). The differences observed appeared to be as a result of a spontaneous enrichment of CD56^{hi}c-Kit^{+} NK cells in healthy control individuals. This observation was consistent with data presented in Section 4.2 where a spontaneous rise in CD56^{hi}c-Kit^{+} was also observed. The mechanism driving this phenomenon is not clear, but it is possible that loss of mature cells was seen as an enrichment of this less mature subset. Alternatively, there is a possibility that the CD56^{hi}c-Kit^{+} NK cells were proliferating and this could account for the increased frequency of this population.

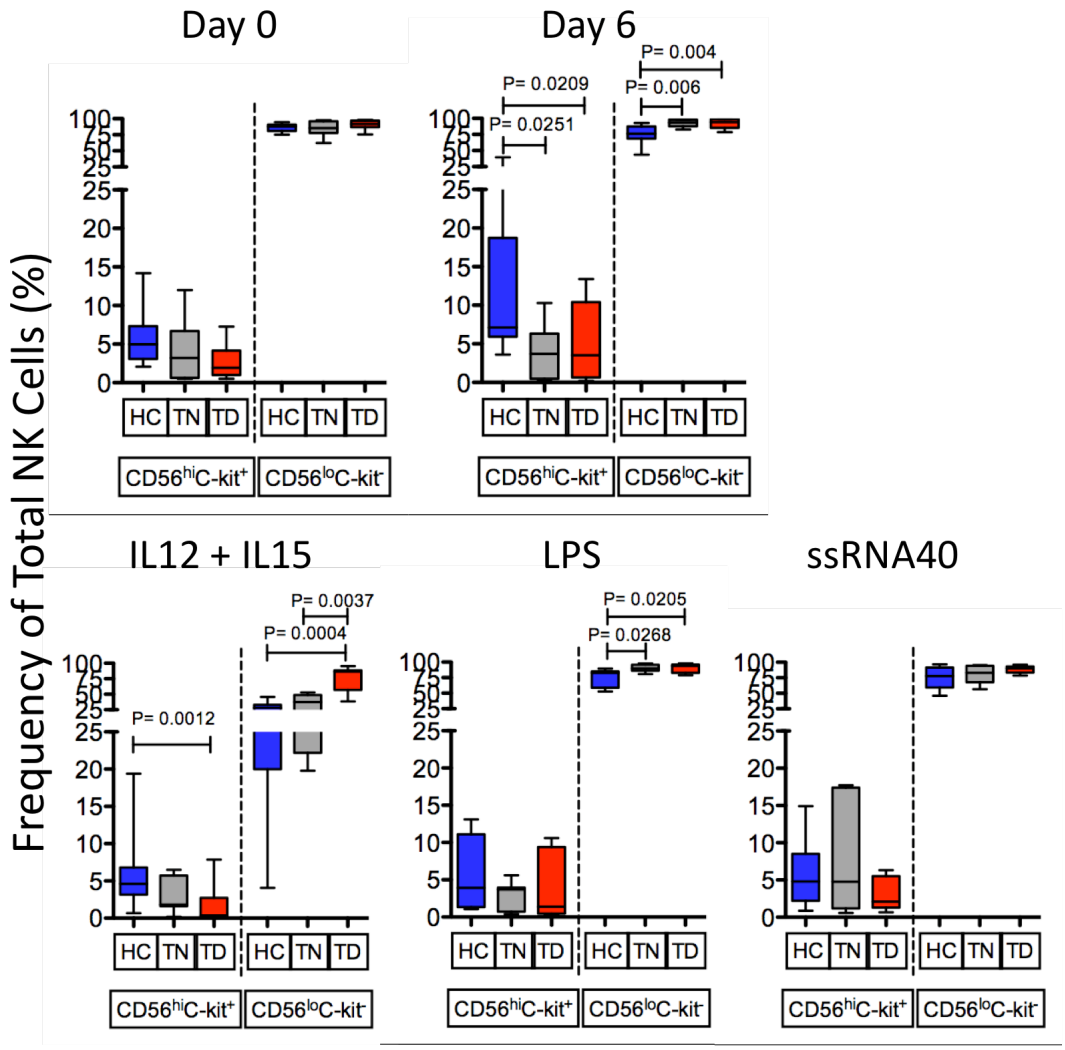


Figure 4.3.1 Spontaneous increase in c-Kit$^-$ NK cells from HIV-1+ individuals following 6 days unstimulated culture of PBMC and stimulation with cytokines or TLR agonists does not further reduce c-Kit$^+$ NK cell frequencies in HIV-1+ patient groups. PBMC cultured in the presence of cytokines and TLR agonists for 6 days does not lead to further decreases in CD56^{hi}c-Kit$^+$ NK cell frequency expression as those observed after culture in medium alone. Blue bars (HC-Healthy Control, n=12) – HIV-1- control; grey bars (TN, n=9) – HIV-1+, treatment-naïve; red bars (TD, n=12) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out.

Similar changes were observed in the expression of CD94 on NK cells during *in vitro* culture (Figure 4.3.2). A reduction in the median frequency of CD56^{hi}CD94$^+$ cells was also observed within NK cells from treatment-experienced HIV-1+ individuals compared to seronegative controls and HIV-1+ treatment-naïve individuals, although this difference did not reach significance (Figure 4.3.2 and see Table 4.3.1 for medians and ranges). A reciprocal increase in the median frequency of CD56^{lo}CD94$^-$ NK cells was observed in HIV-1+ treatment-experienced individuals compared to HIV-1 seronegative and HIV-1+ treatment-naïve individuals. The median frequency of CD56^{lo}CD94$^+$ NK cells remained

consistent between groups (Figure 4.3.2 and see Table 4.3.1 for medians and ranges). Similar to the effects observed on c-Kit$^+$ NK cells, leaving cells unstimulated for six days *in vitro* culture resulted in significant differences between sample groups. The frequency of CD56^{hi}CD94$^+$ NK cells in HIV-1+ treatment-naïve and treatment-experienced individuals was significantly less than in HIV-1- controls (p=0.0086 and p=0.0185 respectively) (Figure 4.3.2 and Table 4.3.1). No significant difference was observed between sample groups for the intermediate CD56^{lo}CD94$^+$ NK cell group in either freshly isolated PBMC or after six days culture across the different sample groups. A significantly higher frequency of CD56^{lo}CD94$^-$ NK cells were observed in samples from treatment-experienced HIV-1+ individuals after six days *in vitro* culture compared to seronegative controls (p=0.0062) (Figure 4.3.2). Data for spontaneous change in frequency of CD94 populations is shown in full in Table 4.3.1.

This data either indicates a shift from CD56^{hi}c-Kit$^+$ to CD56^{lo}c-Kit$^-$ NK cells and a shift from CD56^{hi}CD94$^+$ to CD56^{lo}CD94$^-$ NK cells or preferential survival or proliferation of the CD56^{hi}c-Kit$^+$ and CD56^{hi}CD94$^+$ populations or the loss of CD56^{lo}c-Kit$^-$ and CD56^{lo}CD94$^-$ NK cells with no apparent influence on the intermediate, CD56^{lo}CD94$^+$ population.

	CD56^{hi}CD94$^+$			CD56^{lo}CD94$^+$			CD56^{lo}CD94$^-$		
Samples	**Day 0**	**Day 6**	**Day 0 Vs 6 (P-value)**	**Day 0**	**Day 6**	**Day 0 Vs 6 (P-value)**	**Day 0**	**Day 6**	**Day 0 Vs 6 (P-value)**
HIV-1- control n=12	10.6 (4.4-19.3)	12.5 (4.1-46.4)	0.0942	60.1 (43.3-73.2)	47.9 (31.9-66.3)	0.0215*	28.2 (11.4-45.1)	30.5 (13.3-46.8)	0.946
treatment-naïve HIV-1+ n=9	12.7 (1.3-36.6)	5.17 (0.9-10.6)	0.0156*	59.2 (49.4-92.2)	51.2 (41.1-93.5)	0.2188	24.9 (2.1-49.1)	43.5 (1.7-50.5)	0.2969
treatment-experienced HIV-1+ n=12	6.905 (1.1-21.9)	5.76 (0.3-15.7)	0.1641	54.55 (23.6-80.3)	39.6 (12.7-70.1)	0.0391*	36.65 (18.6-63.9)	54 (29.5-82.7)	0.0195*

Table 4.3.1 Median frequencies and ranges (%) of CD56^{hi}C94$^+$, CD56^{lo}C94$^+$ and CD56^{lo}C94$^-$ NK cells from total CD3$^-$CD56$^+$ NK cell populations on freshly isolated PBMC and on PBMC cultured for six days without stimulation.

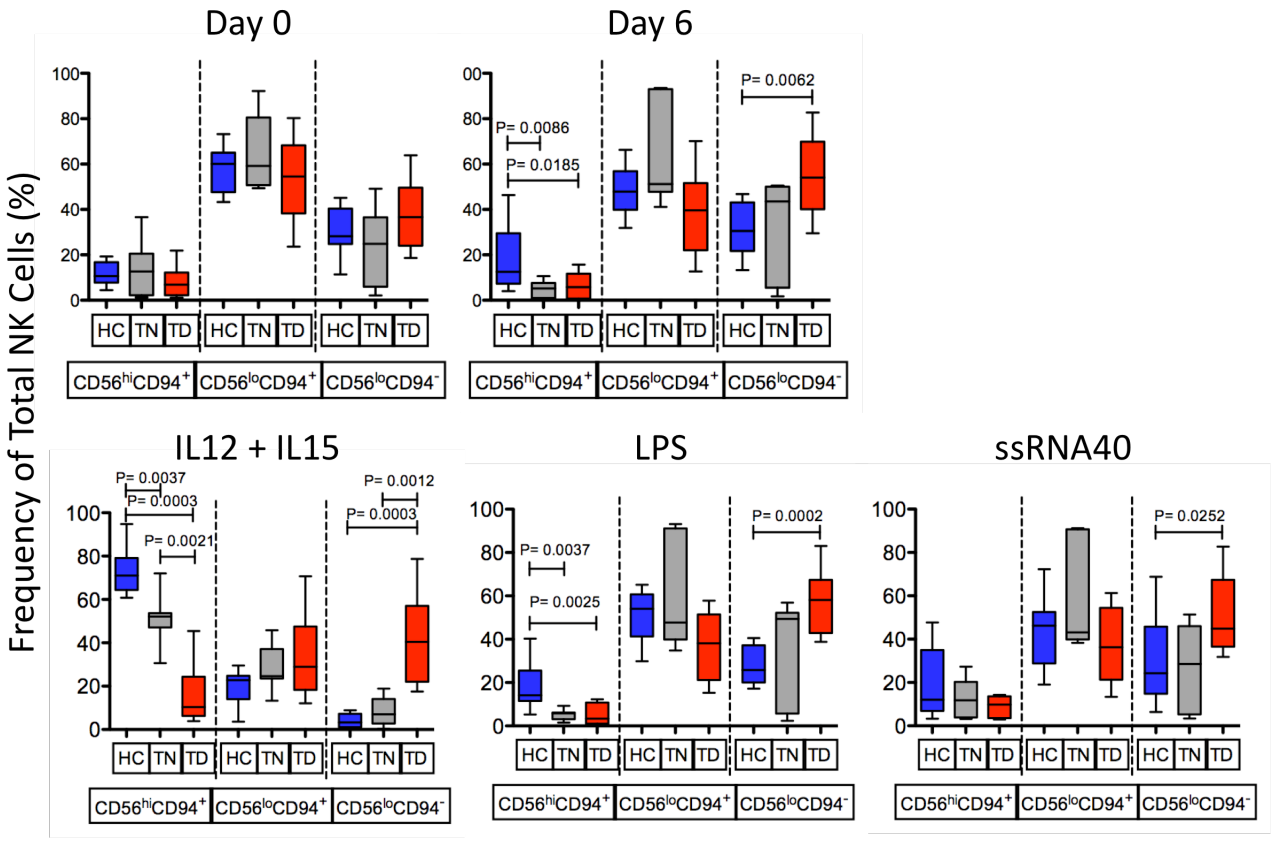


Figure 4.3.2 Spontaneous decrease in frequency of CD94^{+} NK cells and corresponding increase in CD94^{-} NK cells from HIV-1+ individuals following 6 days unstimulated culture of PBMC and Stimulation with cytokines and LPS further reduces CD94 frequencies in HIV-1+ individuals. Stimulation with cytokines and LPS further reduces CD94 frequencies in HIV-1+ individuals. PBMC cultured in the presence of cytokines or LPS for 6 days leads to further decreases in CD94 expression as those observed after culture in medium alone and the pattern is maintained with ssRNA stimulation. Blue bars (HC-Healthy Control, n=12) – HIV-1- control; grey bars (TN, n=9) – HIV-1+, treatment-naïve; red bars (TD, n=12) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out. Day 0 graph repeated from figure 4.1.2b.

4.4. Cytokine and TLR agonist stimulation does not further reduce low c-Kit frequencies observed in HIV-1 sample groups

In Addition to establishing that frequencies of NK cells from HIV-1 infected individuals were spontaneously changing *in vitro*, the effects of stimulation with the cytokines IL-12 (1ng ml^{-1}) and IL-15 (20ng ml^{-1}) and the TLR agonists LPS (200ng ml^{-1}) and ssRNA40 (5µg ml^{-1}) were also examined. Appendix Table A.15 shows a full summary of the median frequencies and ranges of CD56^{hi}c-Kit^{+} and CD56^{lo}c-Kit^{-} NK cell populations following stimulation.

There was a significantly lower frequency of $CD56^{hi}$c-Kit^{+} NK cells after six days culture with IL-12 and IL-15 in PBMC from treatment-experienced individuals compared to seronegative individuals (p=0.0012) (Figure 4.3.1), however no such difference is observed for treatment-naïve individuals. A significantly greater frequency of $CD56^{lo}$c-Kit^{-} NK cells was observed in HIV-1+ treatment-experienced individuals compared to seronegative controls (p=0.0004), but samples from treatment-naïve individuals were not significantly different to seronegative controls, but did have a significantly lower frequency of $CD56^{lo}$c-Kit^{-} NK cells than treatment-experienced individuals (p=0.0037) (Figure 4.3.1).

Interestingly, stimulation with LPS revealed no significant differences in frequency of $CD56^{hi}$c-Kit^{+} NK cells across the different sample groups. The same pattern of significantly higher frequencies of $CD56^{lo}$c-Kit^{-} NK cells in both treatment-naïve and treatment-experienced HIV-1+ individuals compared to HIV-1- controls (p=0.0268 and p=0.0205 respectively) (Figure 4.3.1) was observed as was seen for spontaneous changes observed in unstimulated cultures seen in Section 4.3. Stimulation of PBMC with ssRNA40 for six days did not induce changes in c-Kit expression that were significantly different between sample groups in either the $CD56^{hi}$c-Kit^{+} or $CD56^{lo}$c-Kit^{-} NK cells subsets (Figure 4.3.1).

4.5. $CD56^{hi}CD94^{+}$ NK cell populations are higher or preserved in HIV-1- and HIV-1+ treatment-naïve individuals when stimulated with cytokines and LPS

Continuing from section 4.4, frequencies of NK cell maturation populations based on CD56 and CD94 expression in response to six day *in vitro* stimulation with the cytokines IL-12 and IL-15 and the TLR agonists LPS and ssRNA40 was studied.

Stimulation with cytokines led to an increased frequency of $CD56^{hi}CD94^{+}$ NK cells in samples from HIV-1 seronegative individuals such that the frequency was significantly greater than in either treatment-naïve (p=0.0037) or treatment-experienced (p=0.0003) HIV-1+ individuals (Figure 4.3.2). The Frequency of this population in HIV-1+ treatment naïve individuals has also increased such that it is significantly higher than in treatment-experienced individuals (P=0.0021) (Figure 4.3.2). A significantly higher frequency of $CD56^{lo}CD94^{-}$ NK cells from seropositive treatment-experienced individuals compared to

both seronegative and seropositive treatment-naïve individuals was also observed (p=0.0003 and p=0.0012 respectively) (Figure 4.3.2).

The cause of the change in frequency is unclear, however increased intensity of CD56 staining has previously been observed with prolonged cytokine stimulation. It is however interesting that the phenomenon occurs to a lesser degree in samples obtained from HIV-1+ individuals suggesting either proliferation of the subset or the loss of other populations such that the frequency of this population is increased. Appendix Table A.16 shows a full summary of the median frequencies and ranges of $CD56^{hi}CD94^{+}$, $CD56^{lo}CD94^{+}$ and $CD56^{lo}CD94^{-}$ NK cell populations following stimulation.

A similar pattern of expression was found for LPS stimulation except significant differences did not arise between the two HIV-1 seropositive sample groups. Furthermore, the changes observed in the presence of LPS or ssRNA40 mirrored those observed in cultures left in medium alone for six days, although differences between groups achieved greater statistical significance (Figure 4.3.2).

A significantly higher frequency of $CD56^{hi}CD94^{+}$ NK cells was observed in LPS stimulated HIV-1- individuals compared to seropositive treatment-naïve and treatment-experienced individuals (p=0.0037 and p=0.0025 respectively) (Figure 4.3.2). A significantly higher frequency of $CD56^{lo}CD94^{-}$ NK cells was present within NK cells from treatment-experienced seropositive compared to seronegative samples (p=0.0002) (Figure 4.3.2). No differences in frequency between $CD56^{hi}CD94^{+}$ NK cells between sample groups were observed in ssRNA40 stimulated cultures, a greater frequency of mature $CD56^{lo}CD94^{-}$ NK cells was, however, observed in samples from HIV-1+ treatment-experienced individuals compared to HIV-1- controls (p=0.0252) (Figure 4.3.2).

These data and the data presented in Section 4.4 provide evidence that LPS and ssRNA40 stimulation resulted in the preservation of, or increase in, NK cell populations with a less mature phenotype in HIV-1- individuals compared with HIV-1+ individuals. Without determining the extent of cell proliferation and apoptosis, within each subset, it is difficult to fully interpret this data.

4.6. Spontaneous and stimulation induced changes in HIV-1 infected individuals

Data re-analysis was carried out to compare the change in maturational subsets, in the same individuals, between freshly isolated PBMC and PBMC cultured for six days with and without TLR agonist stimulation. The $CD94^{+/-}$ NK cell subsets were used for this analysis and paired tests were used to compare changes (Figure 4.6.1).

HIV-1 seronegative PBMC cultured in medium alone showed no significant differences in either $CD56^{hi}CD94^{+}$ or $CD56^{lo}CD94^{-}$ NK cell expression when comparing the frequencies at day 0 and day 6, although a significant decrease in frequency was observed in the intermediate $CD56^{lo}CD94^{+}$ subset (p=0.0205). Samples from HIV-1+ treatment-naïve individuals cultured in medium alone did not show any significant frequency difference in either the $CD56^{lo}CD94^{+}$ or $CD56^{lo}CD94^{-}$ subsets, but there was a spontaneous and significant decrease in frequency of the $CD56^{hi}CD94^{+}$ subset over six days (p=0.0156). Samples from HIV-1+ treatment-experienced individuals showed no difference in $CD56^{hi}CD94^{+}$ NK cell frequency after six days *in vitro* culture, but a significantly higher frequency of $CD56^{lo}CD94^{+}$ NK cells was observed after six days and a corresponding reduction in the $CD56^{lo}CD94^{-}$ subset was also found (p=0.0391 and p=0.0195 respectively) (Figure 4.6.1).

No significant changes in frequency occurred between the NK cell subsets discussed above in samples from either HIV-1- or HIV-1+ treatment-naïve individuals when stimulated for six days with either LPS or ssRNA40. The same pattern of decreasing $CD56^{lo}CD94^{+}$ and reciprocal increasing $CD56^{lo}CD94^{-}$ frequency that was observed in medium alone cultures in samples from HIV-1 seropositive treatment-experienced individuals was maintained in LPS (p=0.0195 and p=0.0195 respectively) and ssRNA40 (p=0.0273 and p=0.0273 respectively) stimulated cultures (Figure 4.6.1).

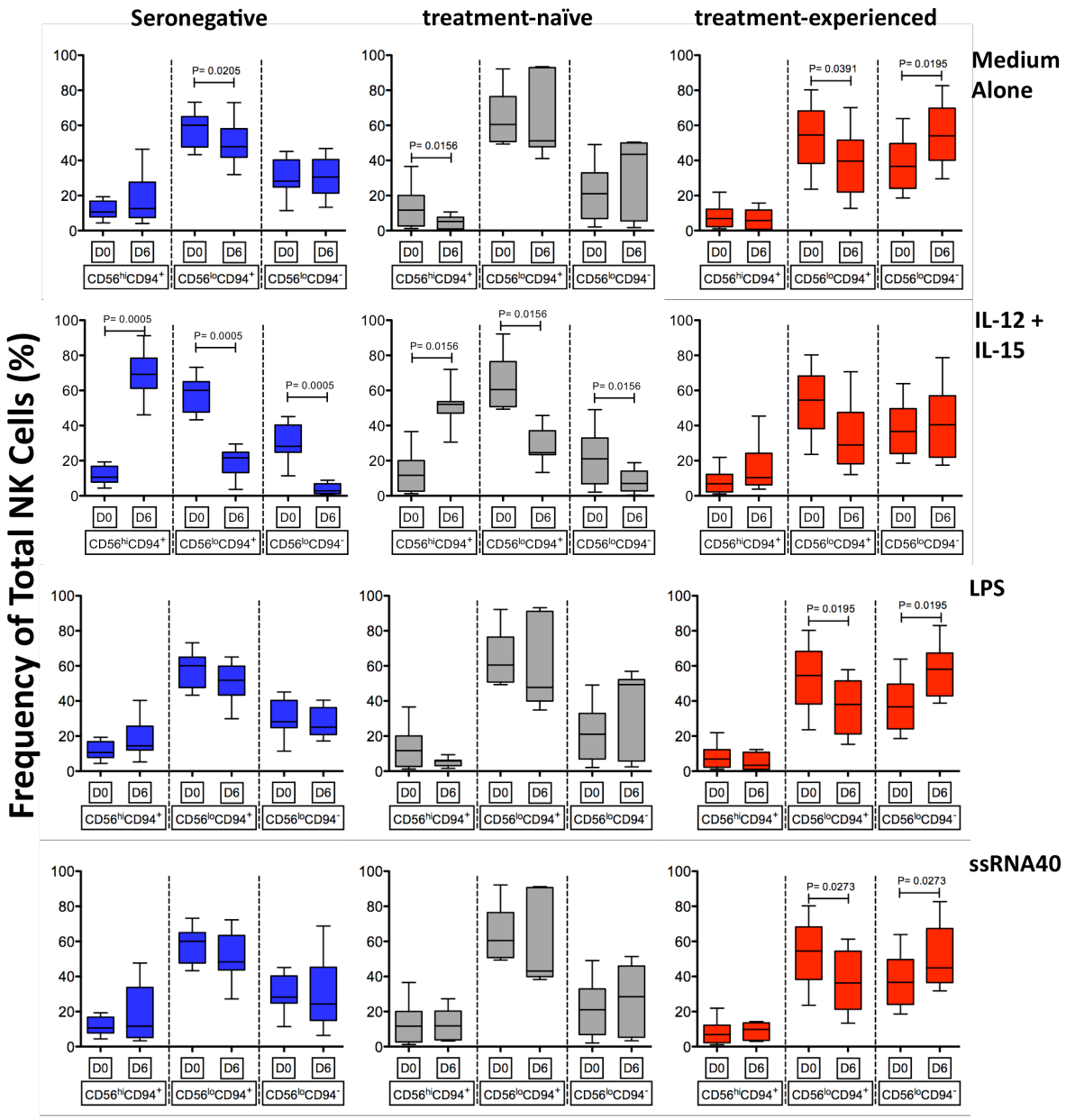


Figure 4.6.1 Side by side comparison of NK cell CD94 expression on freshly isolated PBMC or following 6 days in culture with TLR agonists. There is a spontaneous increase in $CD94^-$ NK cells at the expense of $CD56^{lo}CD94^+$ NK cells in PBMC from HIV-1+ treatment-experienced individuals (red bars) and this pattern is maintained when PBMC are stimulated with both LPS and ssRNA. Blue bars (HC-Healthy Control, n=12) – HIV-1- control; grey bars (TN, n=9) – HIV-1+, treatment-naïve; red bars (TD, n=12) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Wilcoxon's matched pairs tests have been carried out.

Data presented here confirms that NK cells from HIV-1 seropositive individuals have a more mature phenotype prior to *in vitro* culture and that this is exacerbated in NK cells cultured *in vitro* without further stimulation. This phenomenon is particularly evident in samples from treatment-experienced individuals, where the increase in $CD56^{lo}CD94^-$ cells occurs with a corresponding decrease in $CD56^{lo}CD94^+$ NK cells. This undoubtedly has an

impact on the function of these cells, as CD94 is part of the CD94/NKG2A inhibitory receptor complex (Borrego et al., 2006) and $CD56^{lo}CD94^{-}$ cells have also been shown to be $NKG2A^{-}$ (Yu et al., 2010), therefore these cells are considered less responsive to stimuli. It is probable that the HIV-1+ treatment-experienced individuals enrolled in studies presented throughout this thesis have been infected for longer than treatment-naïve individuals, leading to greater terminal differentiation and exhaustion within the NK cell compartment; this will be discussed further in discussion at the end of this chapter (Section 4.8) and the final discussion (Section 6.1).

4.7. Reduced levels of soluble c-Kit and its ligand stem cell factor are detected in plasma of HIV-1 seropositive samples

ELISA assays were carried out on plasma samples collected at the time of PBMC isolation and stored at -80°C. This assay was carried out to assess whether plasma levels of c-Kit (and its ligand stem cell factor (SCF)) reflected the changes observed in NK cells. It could be expected that, if ongoing maturation from $CD56^{hi}$c-Kit^{+} to $CD56^{lo}$c-Kit^{-} NK cells is occurring *in vivo*, there would be a higher concentration of c-Kit in plasma from HIV-1 seropositive samples as NK cells shed c-Kit. There was, however, a significantly reduced concentration of soluble c-Kit in plasma from both HIV-1+ treatment-naïve and treatment-experienced individuals compared to HIV-1 seronegative controls ($p=0.0098$ and $p=0.0012$ respectively) (Figure 4.7.1). Furthermore, a lower concentration of SCF was observed in plasma samples from treatment-naïve HIV-1+ individuals compared to HIV-1- control individuals ($p=0.0474$). Appendix Table A.17 summarises the quantities of both c-Kit and SCF detected in plasma samples from the different groups.

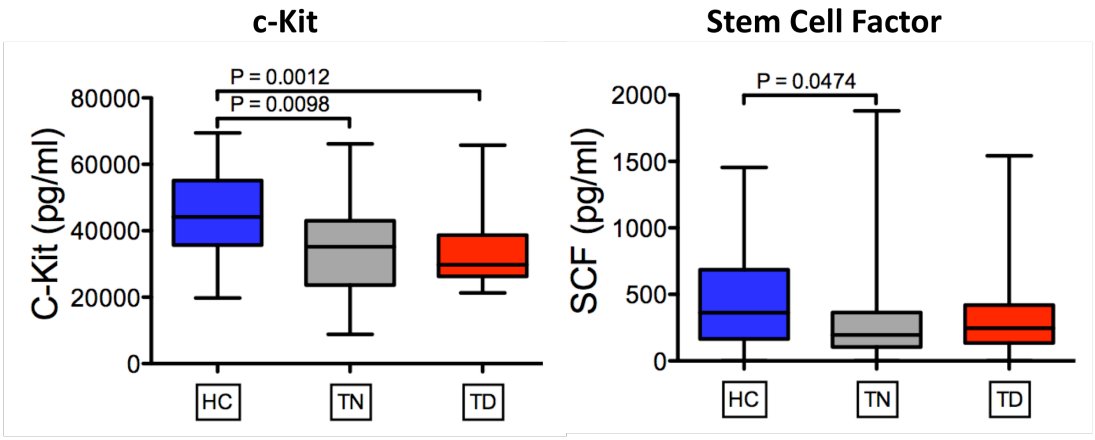


Figure 4.7.1 c-Kit and its ligand, stem cell factor, are detected in plasma and are reduced in HIV-1 seropositive individuals. ELISA were carried out on stored plasma samples to quantify the levels of c-Kit and SCF. Blue bars (HC-Healthy Control, n=12) – HIV-1- control; grey bars (TN, n=9) – HIV-1+, treatment-naïve; red bars (TD, n=12) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out.

4.8. Discussion

Analysis of NK cell maturation was performed using two established phenotypic strategies (Beziat et al., 2011, Yu et al., 2010). Data presented, consistent with previous studies (Beziat et al., 2011, Carson, Fehniger & Caligiuri, 1997), showed that c-Kit^{+} NK cells are almost exclusively CD56^{hi} NK cells and that c-Kit^{-} NK cells are CD56^{lo}. We have also confirmed the presence of the minor, intermediate, CD56^{hi}CD16^{lo} NK cell subset described by Béziat *et al.* (2011).

Examination of CD56 versus CD94 expression on NK cells, in agreement with Yu *et al.* (2010), revealed three distinct NK cell populations; CD56^{hi}CD94^{+}, CD56^{lo}CD94^{+} and CD56^{lo}CD94^{-}. Recently it has been shown that the CD56^{lo}CD94^{+} NK cell subset has functional properties attributed to both CD56^{hi}CD16^{-} and CD56^{lo}CD16^{+} NK cells, namely that they are capable of cytokine secretion and of cytotoxicity (Yu et al., 2010). Using these models of NK cell maturation, a skewed population of NK cells was demonstrated in chronic HIV-1 infection. A lower frequency of CD56^{hi}CD16^{-} NK cells was observed in both treatment-naïve and treatment-experienced HIV-1 seropositive individuals compared to seronegative when the dysfunctional CD56^{-}CD16^{+} NK cell subset previously described in HIV-1 seropositive individuals by Mavilio *et al.* (2005) was also included in the analysis. This finding further demonstrated that NK cells in chronic infection are driven towards a dysfunctional population and that less mature cell populations that are still capable of responding potently to stimuli, are reduced in frequency.

Assays were carried out to determine if CD56^{hi}CD16^{-} NK cells expressing c-Kit and CD94 could be driven to mature towards CD56^{lo}CD16^{+} cells on *in vitro* stimulation. Isolated CD56^{hi}CD16^{-} NK cells were added back to PBMC depleted of NK cells and stimulated with cytokines or TLR agonists. The results demonstrated that CD56^{hi}CD16^{-} NK cells expressed both c-Kit and CD94 and that after six days *in vitro* culture with either LPS or ssRNA40, the phenotype of these cells shifted towards more mature phenotypes. CD56 expression declined and some cells began to express CD16; furthermore c-Kit expression was largely lost and there was an increase in frequency of CD94^{-} NK cells. Interestingly CD56^{lo}CD94^{+} 'intermediate' NK cells were also induced by TLR agonist stimulation. The observed shift in maturational phenotype of NK cells in the presence of LPS and ssRNA40 may have relevance for HIV-1 infection, as these stimuli are systemically elevated during chronic infection as a result of bacterial translocation or viral replication respectively

(Brenchly *et al.* 2006, Gregson *et al.* 2009 and Alter *et al.* 2007). This assay is therefore a useful model, demonstrating the ability of these ligands to drive NK cell maturation, which has important implications for chronic HIV-1 infection pathology and the generation of exhausted and dysfunctional NK cells.

A surprising apparent increase in c-Kit expression was observed after *in vitro* culture of PBMC from HIV-1 seronegative control individuals without stimulation for six days (and also after overnight culture, data not shown). It is possible, although unlikely, that c-Kit expression would increase in these cultures, however such an effect may be due to result from an uncharacterised inhibitory effect of the PBMC isolation procedure, seemingly preventing effective staining of c-Kit on freshly isolated PBMC and which is subsequently reversed during culture. Alternatively a selective loss of cells with a more mature phenotype could account for the apparent rise in frequency of c-Kit expressing cells or that these cells are proliferating. This phenomenon aside, it was clear that PBMC from healthy control individuals, cultured without stimulation did not alter in their expression of maturation markers, and after six days culture showed no reduction in CD94 or CD56 expression or any increase in CD16 expression.

In order to determine the effect of chronic HIV-1 infection on the frequencies of NK cell populations according to maturation markers, assays were carried out on samples from both treatment-naïve and treatment-experienced HIV-1 infected individuals and compared to responses from seronegative individuals. Significant differences were observed in c-Kit expression on freshly isolated cells that were consistent with models for continued turnover and replenishment of NK cells, loss of cells with a mature phenotype or proliferation of less mature cells. Similar trends were observed using CD94-based differentiation staging.

After six days *in vitro* culture, peripheral blood NK cells from both HIV-1+ patient groups clearly showed a greater frequency of mature subsets and lower frequencies of less mature subsets. This spontaneous effect was seen in both patient groups and therefore it is possible that elevated LPS levels, previously observed in patient plasma from both viraemic and aviraemic individuals (Gregson et al., 2009, Brenchley et al., 2006), could play a significant a role in driving the changes in proportion of the populations seen here. Viral ssRNA, only being present in very low/undetectable levels in treatment-experienced individuals, is less likely to contribute to the changes observed. The possibility that low

copy recrudescence of viral replication in the presence of anti-retroviral therapy contribute to accelerated immune maturation in HIV-1 infected individuals receiving anti-retroviral therapy cannot be excluded. Data on changes in maturational phenotype in the presence of the positive control (stimulation with IL-12 and IL-15) proved difficult to interpret as these cytokines induced additional confounding changes in NK cell markers, these changes could be attributed to selective proliferation or loss of specific populations. Previous reports have demonstrated that $CD56^{lo}$ NK cells stimulated with cytokines become transiently $CD56^{hi}$ (Loza & Perussia, 2004), but these cells are functionally distinct from $CD56^{hi}CD16^{-}$ NK cells and instead represent highly activated NK cells. Data presented here also shows that cells from HIV-1 seronegative and seropositive treatment-naïve samples stimulated with cytokines had an increased frequency of $CD56^{hi}CD94^{+}$ NK cells, indicating that a proportion of the $CD56^{lo}$ NK cells that become $CD56^{hi}$ are excluded in the analysis for the intermediate $CD56^{lo}CD94^{+}$ NK cell population. $CD56^{lo}$ NK cells from treatment-experienced samples did not show the same pattern, implying that either cytokine responsiveness is compromised in these patients, or that the observed cells are derived from more mature $CD56^{lo}CD94^{-}$ NK cells which, on activation, become $CD56^{hi}CD94^{-}$ NK cells. The frequency of such activated $CD56^{hi}CD94^{-}$ cells was not included in our analysis.

Changes in the maturation phenotype of NK cells was partially induced in ssRNA40 stimulated HIV-1 seronegative individuals, and the differences in the proportions of different maturational stages between these and HIV-1 seropositive individuals became less pronounced. This indicates that *in vitro* stimulation of samples from seronegative individuals achieves a similar phenotype to the spontaneous maturation observed in seropositive individuals.

Interestingly, there were no significant differences in frequency of 'immature' $CD56^{hi}$c-Kit^{+} NK cells between sample groups after stimulation. This indicates that *in vitro* stimulation of NK cells from HIV-1 seronegative control individuals resulted in similar changes in phenotype of NK cells similar to that observed spontaneously for HIV-1 seropositive. Perhaps unsurprisingly, it appears that *in vitro* stimulation with LPS was insufficient to drive phenotypic changes seronegative samples to the same degree as that resulting from HIV-1 infection, there being significantly more $CD56^{lo}$c-Kit^{-} NK cells in both groups of seropositive samples after culture. In contrast, ssRNA40 stimulation resulted in higher frequencies of mature populations of NK cells in seronegative control individuals to a

similar level the same level to that observed for HIV-1 infected individuals, indicating a hierarchy of potency for different innate signals. Differences in the patterns of maturational stages observed according to CD94 expression corroborated the above findings. ssRNA40 was more potent than LPS at lowering the frequency of $CD56^{hi}CD94^{-}$ cells in HIV-1 seronegative control individuals and significant spontaneous changes were observed in NK cells from HIV-1 seropositive treatment-experienced individuals. It is likely that, prior to treatment, NK cells from HIV-1 seropositive individuals have been chronically exposed to both ssRNA and systemic LPS and have therefore undergone continuous activation affecting the changes in phenotype observed.

Analysis of HIV-1 seropositive NK cell subsets following *in vitro* culture provided further evidence for a shift in maturation phenotype. A spontaneous decrease in the proportion of $CD56^{hi}CD94^{+}$ NK cells was observed in treatment-naïve samples and, although not significant, there was both a reduction in $CD56^{lo}CD94^{+}$ and an increase in $CD56^{lo}CD94^{-}$ NK cell frequencies in unstimulated and stimulated cultures. Examination of results from treatment-experienced samples revealed significant reductions in the frequency of $CD56^{lo}CD94^{+}$ NK cells and a corresponding increase in $CD56^{lo}CD94^{-}$ NK cells in stimulated and unstimulated cultures.

Quantification of soluble c-Kit and SCF concentrations in plasma samples revealed lower levels of both in HIV-1 seropositive compared to seronegative control individuals. This assay, however, was not capable of distinguishing the origins of soluble c-Kit, and it is likely that it derived from multiple cells and not only NK cells. It is also a possibility that lower amounts of c-Kit protein is synthesised and released during chronic HIV-1 infection, or that c-Kit simply has a shorter half-life and is degraded more quickly in HIV-1 seropositive individuals. This data does not support a model whereby NK cells in HIV-1 seropositive individuals are continually maturating due to chronic stimulation with either LPS or viral ssRNA.

Assays presented in this chapter are unable to determine the mechanisms driving the changes in frequency of the different populations studied. In order to better determine whether maturation is occurring or if populations are expanding or being lost, assays, such as fate mapping, with the capability of identifying how cells have changed need to be carried out. The survival and proliferative potential of the different populations should be

examined and use of proliferation markers such as CFSE should be utilised and changes in absolute cell numbers should also be examined.

Data presented here contrast with the early activation studies presented in Chapter 3, which demonstrated NK cells from HIV-1 seronegative individuals responded more vigorously to stimulation than the same cells in HIV-1 seropositive samples, albeit within a shorter time frame (overnight). It is likely that low-level stimulation, which occurs in chronic infection, drives exhaustion and therefore weaker activation responses, whilst maturation toward senescent or dysfunctional populations is ongoing. This seems likely given that the activation data demonstrated that refractory responses were confined to the more mature $CD56^{lo}CD16^{+}$ NK cell subset, which would have been exposed to either viral ssRNA or LPS stimulation for longer, leading to exhaustion and therefore a lower capacity to respond to further stimulation. Conversely, the data presented here also provided support for the hypothesis that $CD56^{hi}CD16^{-}$ NK cells are less mature and as such have had a lower exposure to stimulatory signals, permitting more robust activation responses. It is also interesting that samples from HIV-1 seropositive, treatment-experienced individuals undergo spontaneous frequency changes in maturation phenotypes to a greater extent than samples from treatment-naïve individuals despite successful anti-retroviral treatment and undetectable viral loads. This indicates that extremely low levels of viral ssRNA, below current diagnostic detection limits, are potentially capable of chronically stimulating through TLR pathways, or that elevated microbial translocation and raised LPS concentrations are more important in driving the *in vitro* population changes observed. Furthermore, treatment-experienced individuals are likely to have been exposed to chronic infection for longer periods than those within the treatment-naïve group. Treatment-experienced patients will have commenced therapy as a result of diagnosis of AIDS-defining symptoms, high plasma viral loads or low levels of $CD4^{+}$ T-cells (current guidelines are for treatment to begin at counts below 350 cells ul^{-1} blood (BHIVA, 2012)). Longer duration of infection before effective viral suppression will likely result in increased immune exposure to stimuli promoting chronic immune activation and exhaustion.

4.9. Summary

In this chapter it has been demonstrated that both c-Kit and CD94 are useful NK cell maturation markers when used in combination with CD56. $CD56^{hi}CD16^{lo}$c-Kit^{+} and $CD56^{lo}CD94^{+}$ populations are particularly useful for the identification of intermediate

transitional NK maturation states. Additionally, increased expression of CD16, coincident with the loss of c-Kit and CD94 provided further evidence of progressive maturation *ex vivo* and *in vitro*.

Examination of NK cell maturation markers in HIV-1 seropositive samples revealed that different NK cell populations spontaneously change *in vitro* without additional stimulation. This was the case in both treatment-naïve individuals with detectable viraemia and treatment-experienced individuals with undetectable viraemia. It was also shown that further stimulation with TLR agonists did not cause further changes in HIV-1 seropositive individuals, but population changes were observed in NK cells from seronegative individuals, similar to those observed spontaneously in patients, when stimulated with ssRNA40 or LPS. These data provide evidence that ongoing innate stimulation in chronic HIV-1 infection affects the different maturation states of NK cell populations, such that higher frequencies of mature NK cells dominate *in vitro.*

5. Enhancement of antigen specific T-cell responses using TLR agonist adjuvants

Aim

The previous chapter focused on innate NK cell activation and maturation, and the impact of TLR driven changes in HIV-1 seronegative and chronically infected HIV-1 seropositive individuals. This chapter will focus on how different TLR agonists influence innate and antigen-specific T-cell activation and maturation and the potential influence of different accessory cell populations, including NK cells.

Introduction

It has been shown that TLR agonist stimulated DC activation leads to reciprocal NK cell interactions and that these interactions have been shown to modulate the type and magnitude of adaptive immune response generated (Gerosa et al., 2005, 2002, Moretta, 2002). There is also evidence that NK cells are involved in triggering antigen-specific effector $CD8^+$ T-cell responses and enhancement of antigen presentation to memory T-cells (Jennings & Yuan, 2009, Krebs et al., 2009). HIV-1 infection, as discussed in Chapter 1, has been shown to interfere with the normal generation of adaptive immune responses in many different ways, leading to ineffective or unnecessary immune activity. Chronic immune activation is observed during ongoing HIV-1 infection, leading to T-cell exhaustion and an accumulation of HIV-1 specific $CD8^+$ T-cells that are skewed towards a pre-terminally differentiated phenotype and have been shown to be dysfunctional (Alter et al., 2008, Champagne et al., 2001). Additionally HIV-1 preferentially infects HIV-1-specific $CD4^+$ T-cells, rendering them targets for CTL; this is the fundamental mechanism for the loss of $CD4^+$ T-cells that defines HIV disease (Appay & Sauce, 2008, Douek et al., 2002).

Understanding how different adjuvants activate and prime T-cells prior to the generation of adaptive responses is therefore important for the design of vaccines and therapeutic strategies that aim to overcome the evasion and exhaustion of antigen-specific immunity characteristic of acute and chronic HIV-1 infection, respectively.

Building on data presented in Chapter 3, innate activation of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells in response to TLR agonist stimulation was evaluated and assessed further by examination of innate responses within different memory T-cell subsets. Data from innate stimulation

studies was used to inform the selection of TLR ligands for use in assays to determine their potential for enhancement of antigen-specific immune responses to peptides representing HIV-1 antigens.

5.1. TLR agonist stimulated innate T-cell activation

An initial study was carried out to determine the ability of the TLR agonists Poly(I:C) and CpG ODN2216 to stimulate activation in different T cell subsets, namely $CD8^+$, $CD4^+$ and $\gamma\delta^+$ T-cells. Responses from HIV-1 seronegative control subjects were compared to treatment-experienced and treatment-naïve HIV-1+ individuals. Early activation (CD69 expression) was examined in stimulated PBMC cultures after one and four days (see Appendix Section A.7 for antibody panel).

Figure 5.1.1 demonstrates the gating strategy used to analyse activation responses of the three T-cell subsets after stimulation. $\gamma\delta^+$ T-cells, despite only accounting for a small fraction of T-cells overall, were highly sensitive to Poly(I:C) and CpG ODN2216 driven activation, responding with high frequency of CD69 expression in the individual shown. Early activation was also observed in $CD4^+$ and $CD8^+$ T-cell cultures stimulated with TLR agonists.

Table 5.1.1 shows the patient characteristics for the samples used and Figure 5.1.2 summarises the findings. The graphs show that there was a relatively high frequency of background CD69 expression in $\gamma\delta^+$ T-cells in all sample groups. This was particularly evident in cells from HIV-1+ treatment-naïve individuals and was significantly higher in this cohort compared to HIV-1- controls after overnight culture in medium alone ($p=0.0098$). Stimulation for four days with IL-12 and IL-15 resulted in a significantly higher frequency of CD69 on $\gamma\delta^+$ T-cells from treatment-naïve compared with seronegative individuals ($p=0.0072$). Conversely, a significantly lower frequency of CD69 expression was observed within $\gamma\delta^+$ T-cells from treatment-experienced compared to seronegative individuals ($p=0.0063$). After four days of cytokine stimulation, significantly higher frequencies of CD69 were observed in $CD4^+$ and $CD8^+$ T-cells in HIV-1+ treatment-naïve individuals compared to seronegative individuals, possibly due to differences in response kinetics ($p=0.0234$ and $p=0.0037$ respectively).

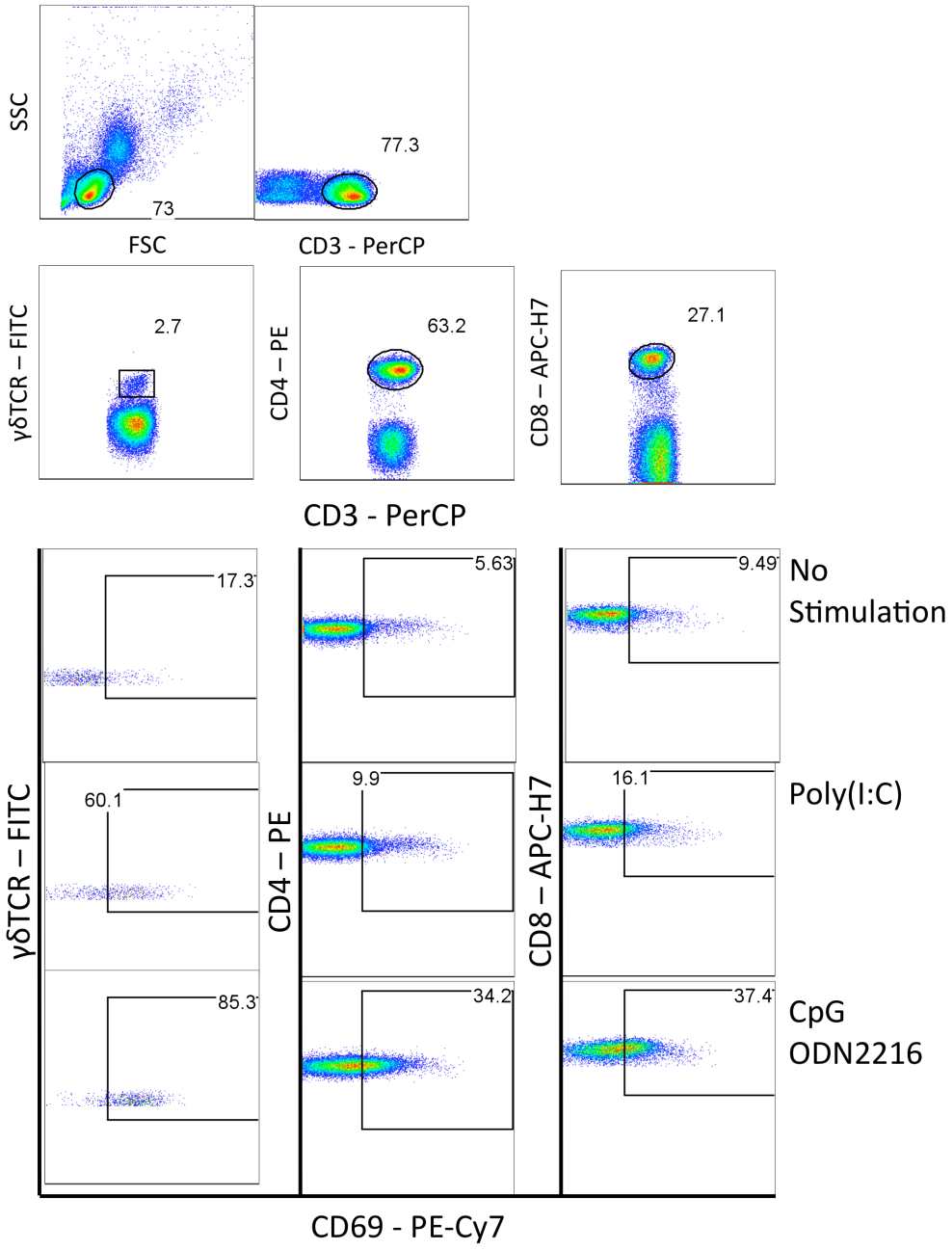


Figure 5.1.1 T cells are activated in PBMC cultures stimulated with TLR agonists. PBMC were cultured in the presence of TLR agonists and the expression of CD69 and HLA-DR on γδTCR$^+$, CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry. Dot plots show gating strategy for different T cell groups and expression of CD69 following overnight culture. Representative HIV-1- control individual shown.

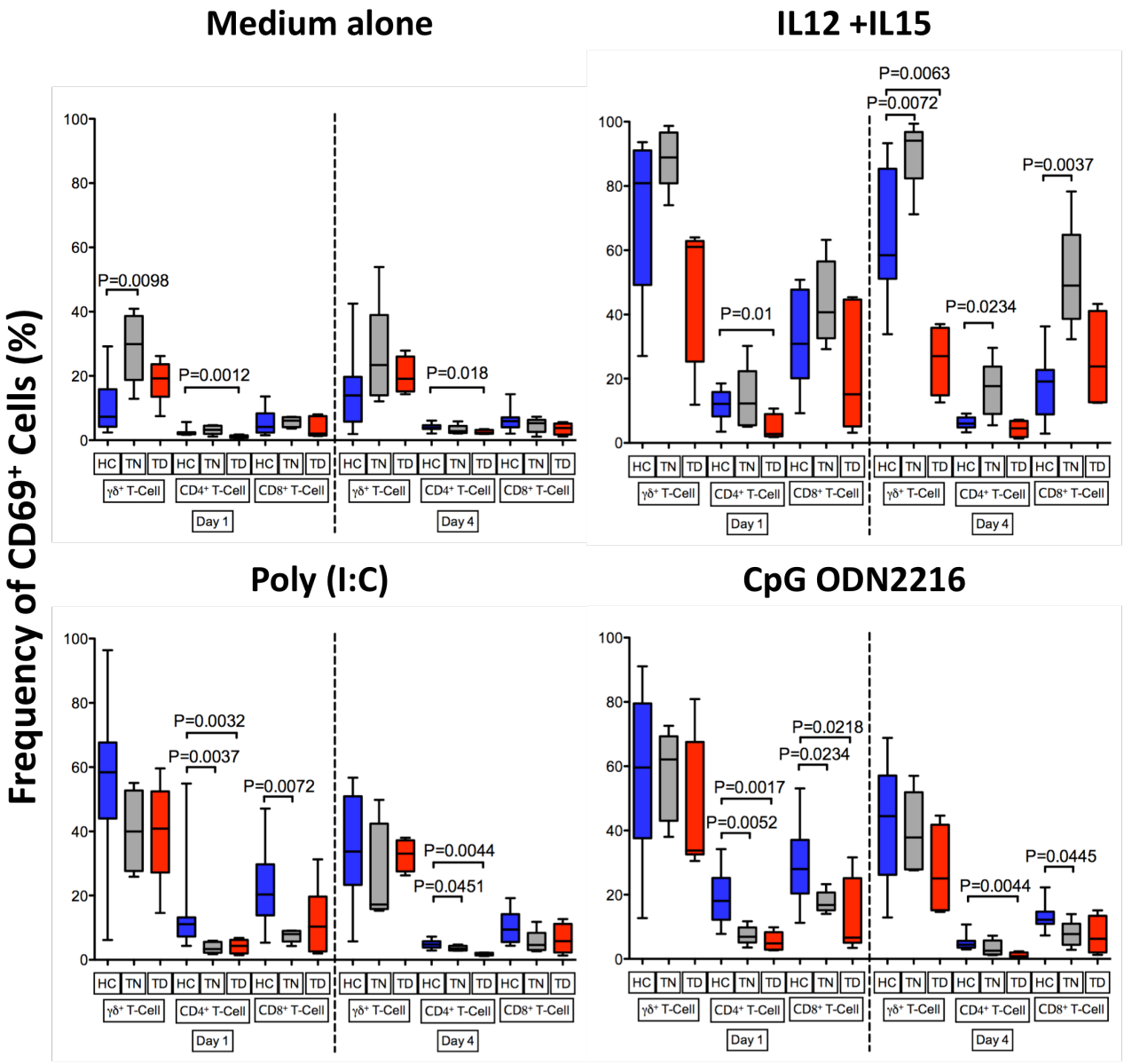


Figure 5.1.2 T-cell responsiveness after 1 or 4 days of culture with TLR agonists. $\gamma\delta TCR^+$ T cells are the principal T-cell group responding to TLR agonist stimulation. A high frequency of CD69 expression on $\gamma\delta TCR^+$ T cells from HIV-1+, treatment-naïve, individuals is observed particularly following stimulation with cytokines and is significantly greater than expression on HIV-1- control individuals, however background expression is also greater and stimulation with TLR agonists reveals a deficit in the magnitude of activation. Blue bars (HC-Healthy Control, n=12) – HIV-1- control; grey bars (TN, n=5) – HIV-1+, treatment-naïve; red bars (TD, n=6) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Wilcoxon's matched pairs tests have been carried out.

No differences in early activation were observed in $\gamma\delta^+$ T-cells between patient groups after Poly(I:C) or CpG ODN2216 treatment, despite the higher levels of background CD69 expression in HIV-1+ individuals. A significantly higher frequency of CD69 expression was observed within $CD4^+$ T-cells from HIV-1- controls compared to both HIV-1+ treatment-naïve and treatment-experienced individuals stimulated with Poly(I:C) after one day

(p=0.0037 and p=0.0032 respectively) and four days (p=0.0451 and p=0.0044 respectively). Similar responses were observed for $CD4^+$ T cells after stimulation with CpG ODN2216: HIV-1- control individuals had a significantly higher frequency of CD69 expression than HIV-1+ treatment-naïve or treatment-experienced individuals after one day (p=0.0052 and p=0.0017, respectively) and a significantly higher frequency than treatment-experienced individuals after four days of culture (p=0.0044). A significantly higher frequency of $CD69^+CD8^+$ T-cells was observed in HIV-1- control individuals compared to HIV-1+ treatment-experienced individuals stimulated with Poly(I:C) for one day (p=0.0072), however no differences were observed after four days. A significantly higher frequency of $CD69^+CD8^+$ T-cells was observed in HIV-1- controls compared to both HIV-1+ treatment-naïve and treatment-experienced individuals after one day of stimulation with CpG ODN2216 (p=0.0234 and p=0.0218 respectively) and a significantly higher frequency was observed for treatment-naïve patients after four days (p=0.0445).

Table 5.1.1 Patient Characteristics

	Healthy Control (n=12)	HIV-1+ treatment-naïve (n=5)	HIV-1+ treatment-experienced (n=6)
Age	27.5 (22-46)	38 (30-46)	43.5 (36-59)
CD4 (cells µl-1)	N/A	374 (171-465)	462 (323-668)
Plasma Viral Load (copies ml-1 blood)	N/A	8616.5 (8140-70094)	N/A

5.2. Impact of TLR agonist stimulation on distinct $CD8^+$ T cell maturation stages

Chronic HIV-1 infection results in the polarisation of $CD8^+$ T cells towards a senescent pre-terminally differentiated effector memory cell functional phenotype (TEM), with a consequent loss of cells from within the central memory pool (TCM) (Alter et al., 2008, Appay & Sauce, 2008, Champagne et al., 2001). Early activation of total $CD8^+$ T-cells has previously been demonstrated in response to TLR agonist stimulation (Chapter 3). In depth analysis of $CD8^+$ T-cell memory subset activation was therefore performed to establish which $CD8^+$ T-cell subsets could be potentially recruited into the effector T-cell response by TLR agonists. Figure 5.2.1 displays the gating strategy used to identify the four different, naïve ($CD8^+CCR7^+CD45RA^+CD62L^+$), TCM ($CD8^+CCR7^+CD45RA^-CD62L^+$),

TEM ($CD8^+CCR7^-CD45RA^-CD62L^-$) and terminally differentiated effector memory RA^+ (TEMRA) ($CD8^+CCR7^-CD45RA^+CD62L^-$) subsets based on protocols by Champagne *et al.* (2001) and Wherry *et al.* (2003) (see Appendix Section A.8 for antibody panel).

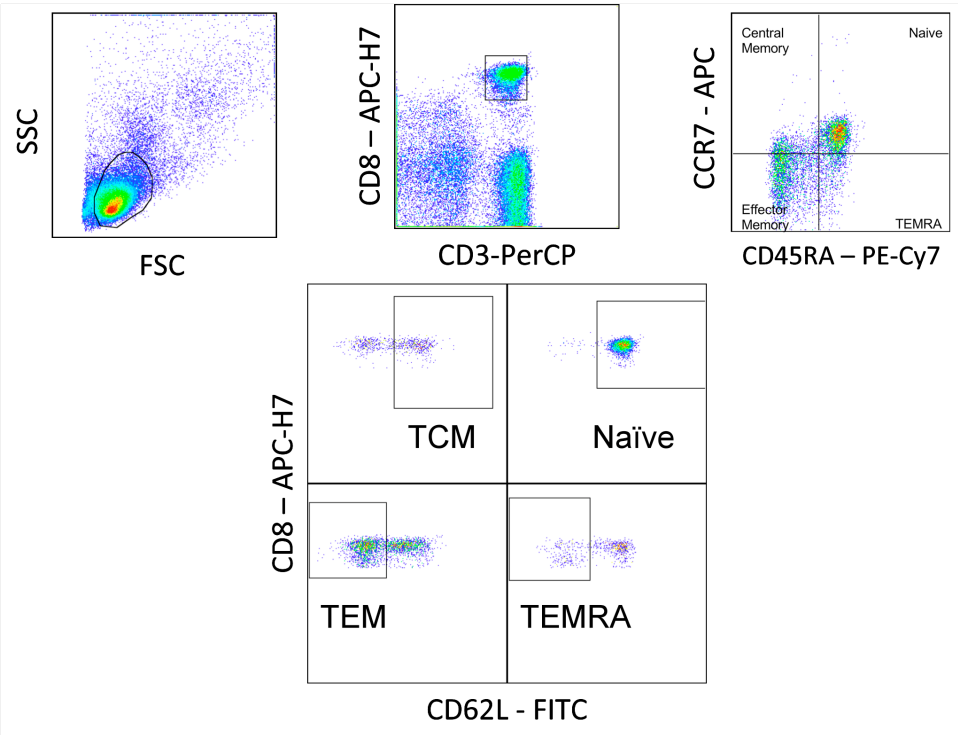


Figure 5.2.1 Gating strategy for $CD8^+$ T-cell memory subsets. Dot plots from a single HIV-1- control individual demonstrating the gating strategy to identify the four memory subsets: naïve, central memory (TCM), effector memory (TEM) and terminally differentiated effector memory $CD45RA^+$ (TEMRA) $CD8^+$ T-cells.

PBMC from healthy controls (n=17) were cultured overnight without stimulation, with the cytokines IL-12 and IL-15 or with the TLR agonists Poly(I:C), LPS, ssRNA40 and CpG ODN. CD69 expression was measured on distinct memory $CD8^+$ T-cell subsets and the results are presented in Figure 5.2.2 and Table 5.2.1. Naïve and TCM $CD8^+$ T-cells displayed high frequencies of $CD69^+$ expression without stimulation compared with the more mature TEM and TEMRA (see table 5.2.1 for values).

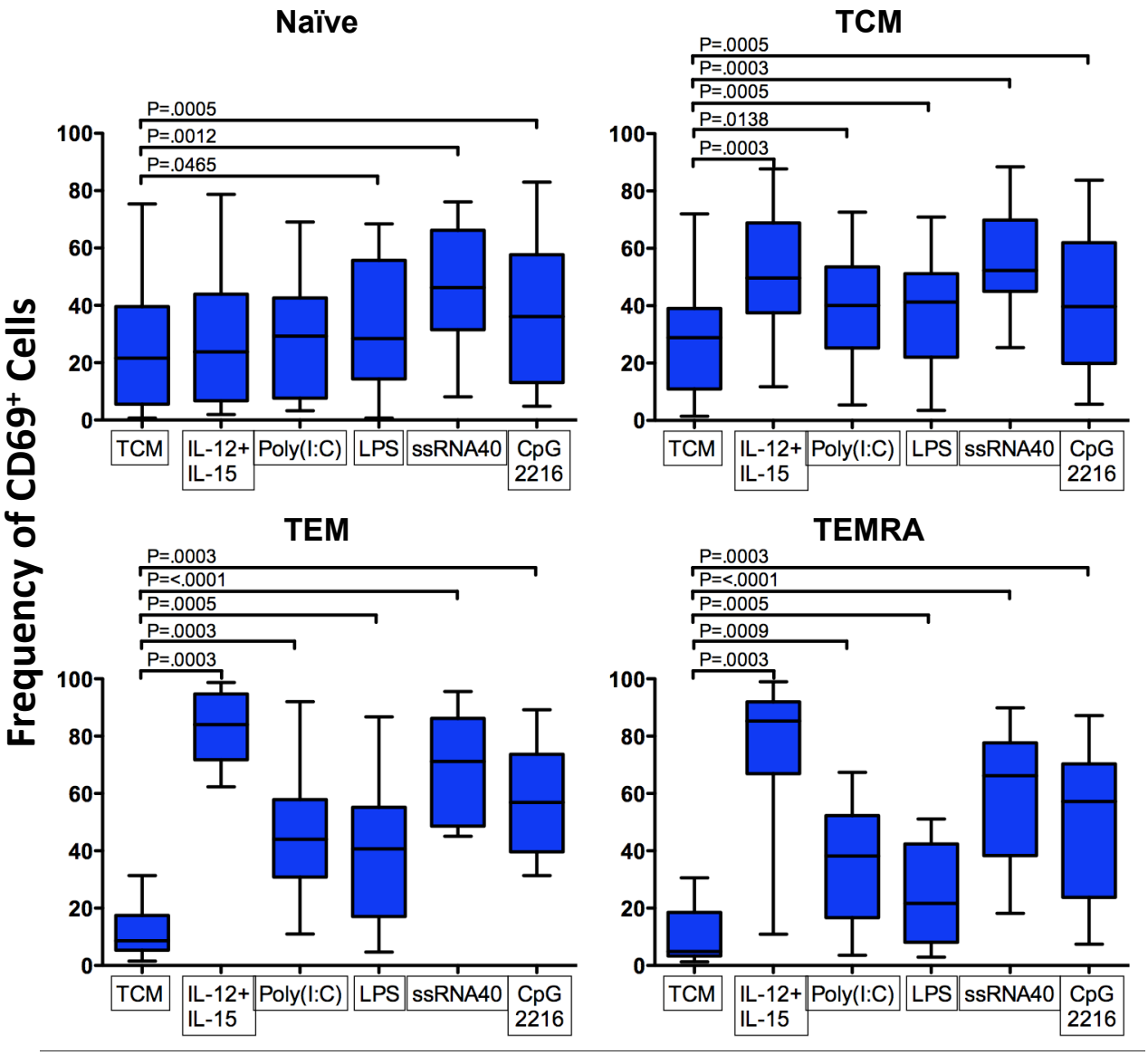


Figure 5.2.2 Memory CD8$^+$ T-cell early activation responses to TLR agonist stimulation. PBMC from 17 HIV-1- control individuals were cultured overnight with cytokines or TLR agonists as shown and the CD69 expression on the four memory CD8$^+$ T-cell subsets was measured by flow cytometry. Graphs show the frequency of CD69$^+$ cells with and without the stimuli shown. Box plots display median, interquartile range and range. Wilcoxon's matched pairs test has been applied to this data.

Stimulation with cytokines induced robust increases in CD69 expression within both TEM and TEMRA CD8$^+$ T-cells with lower increases seen in the less mature naïve and TCM subsets, particularly in naïve cells stimulated with cytokines (Figure 5.2.2 and Table 5.2.1). A similar pattern was seen following stimulation with different TLR agonists, where the more mature TEM and TEMRA CD8$^+$ T-cells demonstrated a greater increase in CD69 frequency above background compared to naïve and TCM cells (Figure 5.2.2 and Table

5.2.1). Interestingly, neither IL-12 & IL-15 nor poly(I:C) induced significantly higher CD69 frequency on naïve $CD8^+$ T-cells compared to unstimulated cells (Table 5.2.1).

Stimulation with LPS, ssRNA40 and CpG ODN2216 induced activation in all $CD8^+$ T cell subsets, with mature TCM and TEMRA achieving higher overall CD69 expression than observed in either naïve or TEM subsets when stimulated with ssRNA40 and CpG ODN2216 (Figure 5.2.2 and Table 5.2.1). These data demonstrate that innate TLR agonist stimulation preferentially promotes activation in mature $CD8^+$ T-cell subsets.

Table 5.2.1 HIV-1- control individuals $CD8^+$ T-cell memory early activation responses to cytokine or TLR agonist stimulated PBMC culture.

	Naïve			Central Memory			Effector Memory			TEMRA		
Stimuli	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**
Medium Alone	**21.6**	0.7-75.4	--	**28.9**	1.5 – 72.0		**8.7**	1.6-31.4	-	**4.9**	1.3-30.6	-
IL12+IL15	**23.8**	1.9-79	0.2184	**49.7**	11.8-87.7	0.0003*	**84.0**	62.3-98.7	0.0003*	**85.3**	10.9-99.0	0.0003*
Poly(I:C)	**29.3**	3.3-69.1	0.2763	**40.1**	5.4-72.6	0.0138*	**44.0**	11.0-92.0	0.0003*	**38.2**	3.6-67.4	0.0009*
LPS	**28.4**	0.7-68.4	0.0465*	**41.3**	3.5-70.9	0.0027*	**40.7**	4.7-86.7	0.0005*	**21.7**	3.0-51.1	0.0005*
ssRNA40	**46.2**	8.1-76.1	0.0012*	**52.3**	25.4-88.4	0.0003*	**71.2**	45.1-95.6	<0.0001*	**66.2**	18.2-89.9	<0.0001*
CpG ODN2216	**36.1**	4.8-83.0	0.0005*	**39.7**	5.7-83.8	0.0005*	**56.9**	31.4-89.2	0.0003*	**57.2**	17.4-87.2	0.0003*

5.3. Innate activation responses of $CD8^+$ T-cells are reduced within mature cell populations in HIV-1+ individuals

Having determined responses to innate stimulation of different memory $CD8^+$ T-cell subsets in HIV-1- controls, assays were undertaken to identify the subsets impacted during chronic HIV-1 infection. PBMC from HIV-1 seropositive treatment-naïve and treatment-experienced samples were stimulated as described in Section 5.2, and CD69 expression from different memory $CD8^+$ T-cell subsets were compared to responses from seronegative samples. Patient characteristics for this study are shown in Table 5.3.1.

Table 5.3.1 Patient Characteristics.

	Healthy Control (n=17)	HIV-1+ treatment-naïve (n=11)	Vs Healthy control (p-value)	HIV-1+ treatment-experienced (n=13)	Vs Healthy control (p-value)	Treatment-naïve Vs treatment-experienced (p-value)
Age	26 (21-64)	34.5(26-45)	-	54 (39-64)	-	-
CD4 (cells μl^{-1})	N/A	397.5(267-554)	-	488 (363-810)	-	-
Plasma Viral Load (copies ml^{-1} blood)	N/A	10978.5(174-388894)	-	N/A	-	-
$KIR^{-}CD8^{+}$ T-cells (% Total $CD8^{+}$ T-cells)	95.95(90.4-98.6)	92.15(87.8-96.9)	0.0152*	94.15(84.2-98.3)	0.118	0.135
KIR^{+} T-cells (% Total $CD8^{+}$ T-cells)	4.05(1.37-9.59)	7.85(3.07-12.3)	0.0165*	5.87(1.72-15.8)	0.119	0.143

No significant differences in background unstimulated CD69 expression were observed between different sample populations for the different $CD8^{+}$ T-cell memory subsets (Figure 5.3.1). Stimulation of cells with IL-12 and IL-15 induced significantly higher frequencies of CD69 expression by HIV-1 seronegative TEM and TEMRA $CD8^{+}$ T-cells compared to seropositive treatment-experienced samples (p=0.021 and p=0.0439 respectively) (Figure 5.3.1 and Tables 5.3.1 and 5.3.2). Interestingly, no differences were observed in the activation of naïve and TCM subsets between groups. Naïve and TCM $CD8^{+}$ T-cells from HIV-1 infected individuals, similar to seronegative samples, showed no significant increase in CD69 frequency after stimulation with IL-12 & IL-15 (Table 5.3.2).

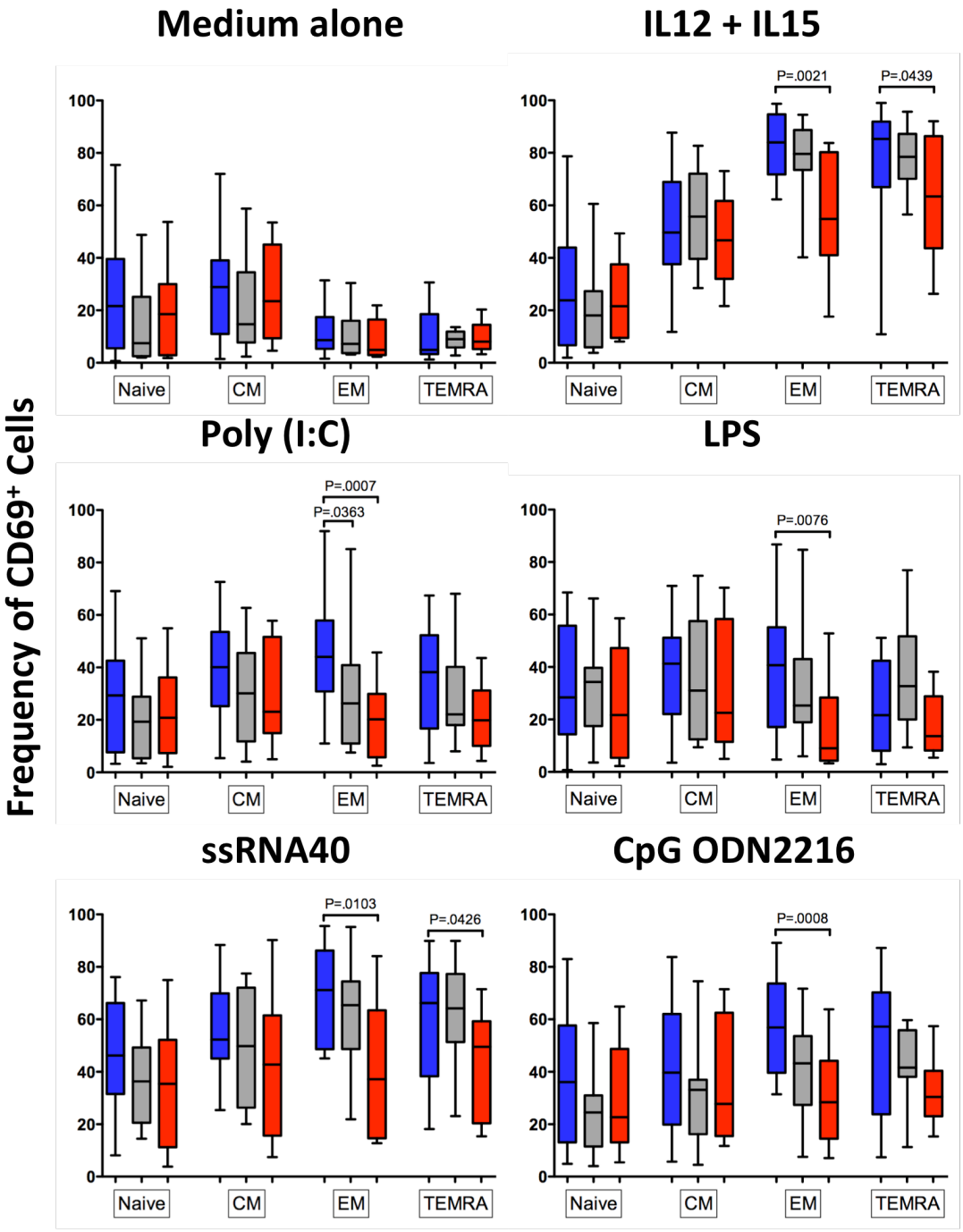


Figure 5.3.1 Memory CD8$^+$ T-cell early activation responses to TLR agonist stimulation in HIV-1 seronegative and seropositive individuals. Graphs show CD69 frequency on memory CD8$^+$ T-cells after PBMC were cultured overnight with cytokines or TLR agonists as shown. Blue bars (Healthy Control, n=17) – HIV-1- control; grey bars (n=11) – HIV-1+, treatment-naïve; red bars (n=13) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Mann-Whitney tests have been carried out.

Stimulation with any of the TLR agonists tested revealed no significant differences in CD69 expression by either the naïve or TCM CD8$^+$ T-cell subsets between different sample groups, although HIV-1- controls tended to have a higher frequency of expression

(Figure 5.3.1). In contrast, significant differences occurred in the more mature TEM and TEMRA subsets. A significantly lower frequency of CD69 was induced on TEMRA cells from HIV-1+ treatment-naïve individuals compared to HIV-1- control individuals stimulated with ssRNA40 (p=0.0426). There was a significantly lower frequency of $CD69^+$ TEM cells from HIV-1+ treatment-experienced individuals compared with HIV-1- control individuals after stimulation with Poly(I:C) (p=0.0007), LPS (p=0.0076), ssRNA40 (p=0.0426) and CpG ODN2216 (p=0.0080) (Figure 5.3.1). With the exception of Poly(I:C) stimulation (p=0.0363), the frequency of CD69 expression within TEM cells from HIV-1+ treatment-naïve individuals was not significantly lower than observed in HIV-1- controls.

$CD8^+$ T-cell subsets with a more advanced maturational status were therefore observed to be more readily activated than naïve or memory subsets using innate TLR stimulation alone. These assays also revealed substantial defects in responsiveness of effector memory and terminally differentiated effector memory $CD8^+$ T-cell subsets during chronic HIV-1 infection, particularly in individuals receiving successful anti-retroviral treatment.

Table 5.3.2 HIV-1+ treatment-naïve individuals, $CD8^+$ T-cell memory early activation responses to cytokine or TLR agonist stimulated PBMC culture.

	Naïve			Central Memory			Effector Memory			TEMRA		
Stimuli	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**
Medium Alone	**7.5**	2.0-48.8	-	**14.7**	2.4-58.8	-	**7.2**	3.1-30.4	-	**9.0**	2.8-13.6	-
IL12+IL15	**18.1**	3.8-60.6	0.001*	**55.7**	28.5-82.7	0.001*	**79.6**	40.2-94.5	0.001*	**78.5**	56.5-95.7	0.001*
Poly(I:C)	**19.3**	3.5-51.1	0.001*	**30.1**	4.1-62.7	0.0049*	**26.3**	7.6-85.1	0.001*	**22.1**	8.0-68.1	0.001*
LPS	**34.3**	3.6-661	0.001*	**31.0**	9.4-74.8	0.001*	**25.3**	5.9-84.7	0.001*	**32.7**	9.3-76.9	0.001*
ssRNA40	**36.4**	14.5-67.2	0.002*	**49.8**	20.1-77.5	0.002*	**65.4**	21.9-95.2	0.002*	**64.2**	23.1-89.9	0.002*
CpG ODN2216	**24.5**	4.0-58.6	0.049*	**33.1**	4.5-74.5	0.0049*	**43.2**	7.6-71.7	0.001*	**41.6**	11.3-59.7	0.001*

Table 5.3.3 Table 5.3.3. HIV-1+ treatment-experienced individuals, $CD8^+$ T-cell memory early activation responses to cytokine or TLR agonist stimulated PBMC culture.

	Naïve			Central Memory			Effector Memory			TEMRA		
Stimuli	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**	**Median $CD69^+$ (%)**	**Range**	**P-Value (Vs medium alone)**
Medium Alone	**18.6**	1.8-53.7	-	**23.5**	4.6-54.5	-	**4.9**	2.3-21.9	-	**8.1**	3.3-20.3	-
IL12+IL15	**21.6**	8.2-49.3	0.1763	**46.7**	21.6-73.1	0.0024*	**54.8**	17.6-83.8	0.005*	**63.4**	26.3-92.1	0.005*
Poly(I:C)	**20.8**	2.1-54.9	0.3013	**23.1**	5.0-57.8	0.1294	**20.2**	2.5-45.7	0.0024*	**19.8**	4.4-43.6	0.0005*
LPS	**21.7**	2.2-58.6	0.0093*	**22.5**	4.9-70.2	0.0093*	**9.0**	3.3-52.8	0.0024*	**13.7**	5.4-38.2	0.001*
ssRNA40	**35.4**	3.8-75.0	0.0039*	**42.7**	7.4-90.2	0.0091*	**37.2**	12.8-84.1	0.0039*	**49.5**	15.4-71.5	0.0039*
CpG ODN2216	**22.7**	5.4-64.9	0.0029*	**27.8**	11.7-71.5	0.0049*	**28.4**	7.1-63.8	0.0005*	**30.4**	15.3-57.4	0.0005*

5.4. KIR^+ $CD8^+$ T-cells are sensitive to innate stimulation despite a higher frequency observed in HIV-1 infection

An increased frequency of KIR expression on $CD8^+$ T-cells during chronic HIV-1 infection has been shown to correlate with plasma viral load (Alter et al., 2008). This study also demonstrated that such KIR^+CD8^+ T-cells are unresponsive to antigen-specific stimulation but remain responsive to TCR independent stimuli, raising the possibility of recovery of functional competence. In view of the reduced responsiveness of TEM and TEMRA $CD8^+$ T-cells to TLR ligands, assays were performed to determine whether KIR expression also influences TLR ligand-induced activation.

Consistent with previously published data (Alter et al., 2008) a significantly higher frequency of KIR^+CD8^+ T-cells was detected in HIV-1+ treatment-naïve individuals with detectable viraemia compared to HIV-1- controls (p=0.0165) (Table 5.3.1). Interestingly, samples from treatment-experienced individuals without detectable viraemia showed no significant difference in frequency of KIR^+CD8^+ T-cells compared to seronegative controls, indicating a reversion to normal levels.

In order to assess the responsiveness of KIR^+CD8^+ and KIR^-CD8^+ T-cells to TLR agonists, PBMC were stimulated *in vitro* as described previously (Section 3.7). Frequencies of CD69 and IFN-γ producing cells were then quantified using flow cytometry (Figure 5.4.1).

KIR^+CD8^+ T-cells were clearly responsive to TLR agonist stimulation, and exhibited a higher frequency of both CD69 and IFN-γ expression than KIR^- cells. This trend was maintained across all three different patient groups (Figure 5.4.1). It is noteworthy that a statistically significantly higher frequency of CD69 and IFN-γ expression was observed on KIR^+CD8^+ T-cells following overnight *in vitro* culture without stimulation. This indicates that these cells were already partially activated. Also, TLR-mediated stimulation did not significantly influence the frequency of KIR^+CD8^+ T-cells (data not shown). TLR agonist stimulation alone was therefore unable to induce KIR expression on $CD8^+$ T-cells *in vitro* over the time period studied.

KIR expression on $CD8^+$ T-cells is confined to memory subsets, particularly TEM (Alter et al., 2008, Anfossi et al., 2004). It is therefore not surprising that KIR^+CD8^+ T-cells demonstrated a higher degree of responsiveness to TLR agonist stimulation (Figure 5.4.1) and showed higher frequencies of mature TEM and TEMRA cells that were responsive to innate stimulation (Section 5.2 and 5.3). In addition to a higher frequency of KIR^+CD8^+ T cells observed during chronic HIV-1 infection, responses specific for HIV-1 antigens were found to be deficient in this subset (Alter et al., 2008). The ability of KIR^+CD8^+ T-cells to respond to innate signals, including TLR ligands, indicates that appropriate adjuvanting systems could potentially be used to recover antigen-specific function in this subset.

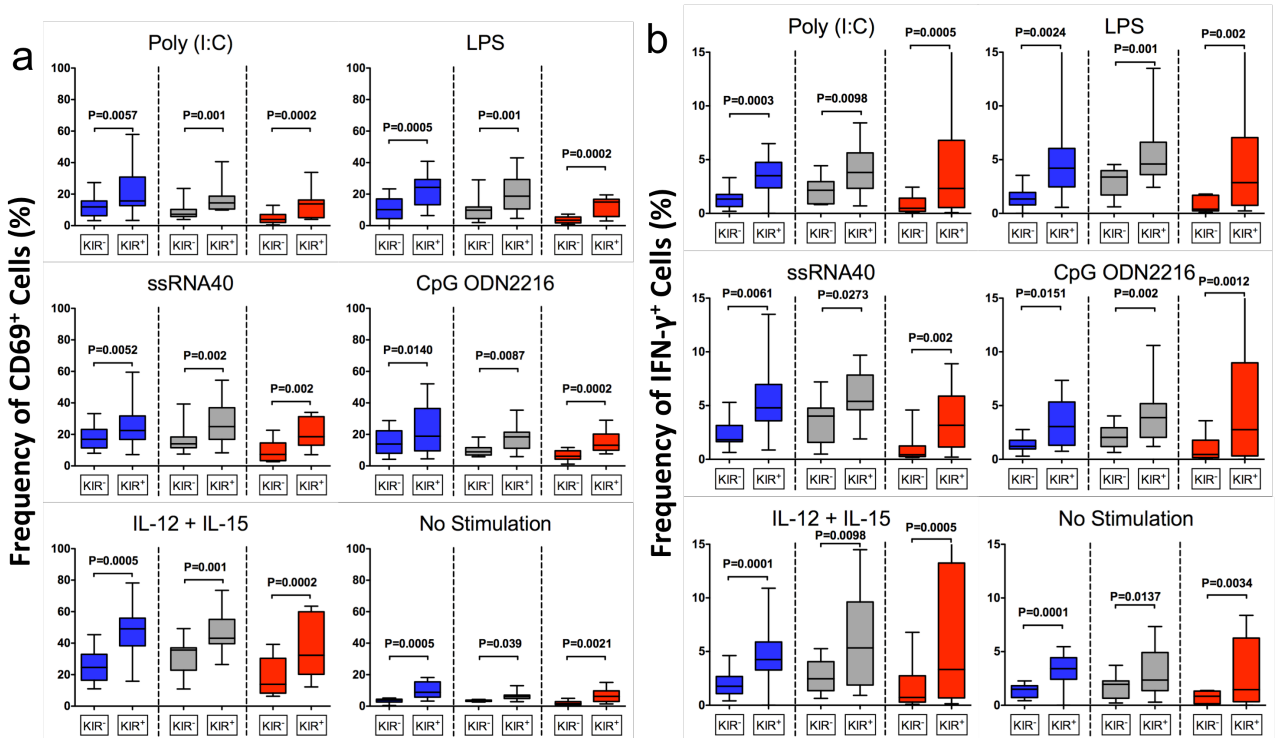


Figure 5.4.1 KIR$^+$ CD8$^+$ T cells maintain responsiveness to TLR agonist stimuli. Graphs display frequency of a) CD69 expression and b) IFN-γ production from KIR$^{+/-}$ CD8$^+$ T cells from. Blue bars (n=17) – HIV-1- control; grey bars (n=11) – HIV-1+, treatment-naïve; red bars (n=13) – HIV-1+, treatment-experienced individuals. Box plots display median, interquartile range and range. Wilcoxon's matched pairs tests have been carried out.

5.5. Effect of TLR agonist co-stimulation on antigen specific T-cell responses

Chronic stimulation in HIV-1 infection results in the generation of senescent, exhausted antigen specific CD8$^+$ T-cells. This has been attributed to either chronic activation leading to irreversible loss of function in these cells (Appay & Sauce, 2008, Brenchley et al., 2003) or reversible exhaustion of CD8$^+$ T-cell expressing the programmed death-1 (PD-1) receptor (Sauce et al., 2007). Furthermore, PD-1 expression and CD38 upregulation are associated with cellular activation, and are seen on HIV-1 specific CD8$^+$ T-cells and are reduced with HAART, implying functional recovery (Appay & Sauce, 2008, Sauce et al., 2007). Having demonstrated the ability of TLR agonists to preferentially stimulate memory CD8$^+$ T-cell responses, co-stimulation assays were performed to assess whether TLR agonists could enhance antigen-specific recall responses in CD4$^+$ and CD8$^+$ T-cells. Preliminary experiments were performed using the FEC peptide pool (described in Section 2.6.3 and Appendix Table A.11) to stimulate PBMC from healthy donors (n=4; Table

5.5.1), either with or without TLR agonists in IFN-γ ELISpot assays (for details see Section 2.10). Co-stimulations were carried out using Poly(I:C), LPS, ssRNA40 and CpG ODN2216, which were titrated at the concentrations shown in Table 5.5.2. TLR agonists were titrated in these assays to determine whether very low concentrations, such as might be observed *in vivo*, would have any effect on antigen-specific responses. IFN-γ ELISpot assays were employed in this study as they are highly sensitive and enable accurate determination of antigen-specific T-cell frequency.

Table 5.5.1 Patient Characteristics

	HIV-1 seronegative (n=4)	HIV-1+ treatment-naïve (n=13)	HIV-1+ treatment-experienced (n=13)
Age	36 (24-64)	37 (26-45)	54 (36-64)
CD4 T-cell count (cells/µl Blood)	N/A	320 (171-758)	462 (285-668)
Plasma Viral Load (copies/ml Blood)	N/A	40686 (6495-568080)	N/A

Figure 5.5.1 shows the number of IFN-γ spot forming cells (SFC) per million PBMC cultured. The black dotted line at 50 SFC per million cells represents the chosen threshold of responsiveness; responses below this level are considered as background. Data is presented as background subtracted and for TLR agonist co-stimulated co-cultures, TLR agonist alone responses are also subtracted so that any responses observed are above those, which would be achieved by simply adding the responses to peptide and TLR ligand stimuli alone. Background subtracted values shown to be below 0 SFC were rounded to 0 for analysis. Background values where cultures were stimulated with TLR agonists alone are not presented here or for the following sections (5.6 and 5.7) as they were zero in the vast majority of cases and only rarely induced a response. Additionally, when a response was observed it was only seen in a very low number of cells, and the highest recorded value was 84 SFC per million PBMC.

FEC stimulation resulted in responses in all samples above background levels, albeit with varying frequency, with one individual responding more vigorously than the others. Both Poly(I:C) and ssRNA40 co-stimulation had little effect on responses, except at very low concentrations (1ng ml^{-1}) of Poly(I:C), when all four samples showed reduced

responsiveness compared to FEC stimulation alone, reducing the mean SFC per million to 383 (range 66-1212) from 425 (76-1248).

Co-stimulation with FEC peptides and LPS also revealed an overall reduction in responsiveness, particularly at a concentration of 10ng ml^{-1}, when the mean SFC per million cells was reduced in three out of four samples tested, from 425 (76-1248) stimulated with FEC alone to 274 (86-770) (Figure 5.5.1). Both samples co-stimulated with 100ng ml^{-1} LPS, showed decreased responses below the 50 SFC per million threshold, from 130 to 36 and 76 to 26 respectively. Reduction of responsiveness to antigen-specific stimulation may have relevance for HIV-1 infection where it is known that plasma LPS levels are elevated (Gregson et al., 2009, Brenchley et al., 2006). This will be explored further in the next section (5.6).

Co-stimulation of FEC peptide responses with higher concentrations of CpG ODN2216 (10nM) caused a reduction in overall responsiveness in all four samples tested, reducing the mean SFC per million cells to 362.5 (24-1122) compared to 425 (76-1248) with FEC alone, and reducing one sample to below cut-off threshold levels. Although this reduction is not as dramatic as that observed in LPS co-stimulation, it does suggest that, at higher concentrations, TLR agonist co-stimulation can have a negative effect on antigen-specific responses. However, in contrast to these findings, co-stimulation of PBMC with FEC and lower concentrations (1nM) of CpG ODN2216 resulted in enhanced IFN-γ production in three out of four samples tested, increasing the mean SFC per million cells for these individuals from 425 (76-1248) for FEC alone to 474 (94-1412) for FEC plus CpG ODN2216. Of all the combinations tested, CpG ODN2216 at a concentration of 1nM was the only one that enhanced responses, providing evidence that antigen-specific responses may, in some circumstances, be boosted using TLR agonists.

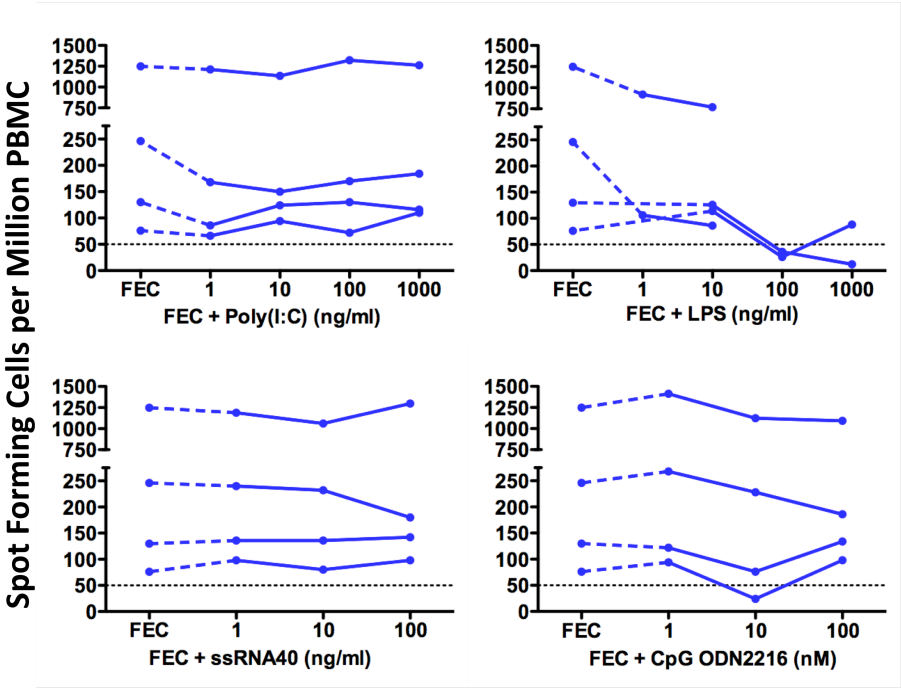


Figure 5.5.1 IFN-γ ELISpot responses of PBMC from HIV-1- control individuals in response to a FEC peptide pool in combination with TLR agonists. PBMC were cultured in the presence of a mixed peptide pool containing peptides from Influenza, EBV and CMV alone or in the presence of TLR agonists. Each line represents a single individual; dotted lines were used to compare between FEC alone and FEC with the lowest TLR agonist concentration used for each individual. Dotted line at 50 spots per million cells represents the threshold considered responsive to peptide. All peptide + TLR agonist points have had TLR agonist alone responses subtracted. Wilcoxons matched pairs analysis has been carried out on all samples where FEC alone responses were above 50 spots per million cells and p values are only shown where they are <0.05 (none of the changes in response were significant for these assays).

Table 5.5.2 TLR agonist concentrations used in titration co-stimulation assays.

TLR Agonist	Concentration			
Poly(I:C) (ng ml^{-1})	1	10	100	1000
LPS (ng ml^{-1})	1	10	100	1000
ssRNA40 (ng ml^{-1})	1	10	100	-
CpG ODN2216 (nM)	1	10	100	-

5.6. Effect of TLR agonist co-stimulation on HIV-1 antigen specific T-cell responses

Further to experiments described above in section 5.5, similar assays were undertaken using samples from both treatment-naïve (n=13) and treatment-experienced (n=13) HIV-1+ individuals. Patient characteristics for this study are displayed in Table 5.5.1. Two concentrations of each TLR agonist were used in these assays (based on results from seronegative studies in Section 5.5) and results are shown in Figures 5.6.1, 5.6.2 and 5.6.3. For this study three different peptide pools were used to stimulate PBMC for the IFN-γ ELISpot assays: the control FEC peptide pool used above, and two different HIV-1 gag peptide pools referred to as the 20mer and the 9mer pools (Section 2.6.3 and Appendices Tables A.11, A.12 and A.13). In the 20mer peptide pool, peptides overlap by 10 amino acids, encompassing the possibility of stimulating both $CD4^+$ and $CD8^+$ T-cells. The latter pool contained different 9mer peptides with varying sequence overlaps that have previously been demonstrated to be immunogenic for the majority of HIV-1+ individuals, stimulating antigen-specific $CD8^+$ T-cell responses. A value below 50 SFC per million PBMC in cultures stimulated with peptide alone, was considered below the threshold of a specific response. Individuals unresponsive to the peptide pools were excluded from analysis of TLR co-stimulatory effects.

5.6.1. TLR agonist co-stimulation of T cell responses to FEC peptides in HIV-1 infected individuals

Figure 5.6.1 summarises *in vitro* co-stimulation responses of HIV-1 seropositive PBMC responses using the FEC peptide pool. The majority of individuals made responses to FEC peptide pool alone above threshold levels; only one HIV-1+ treatment-naïve individual displayed a response below threshold.

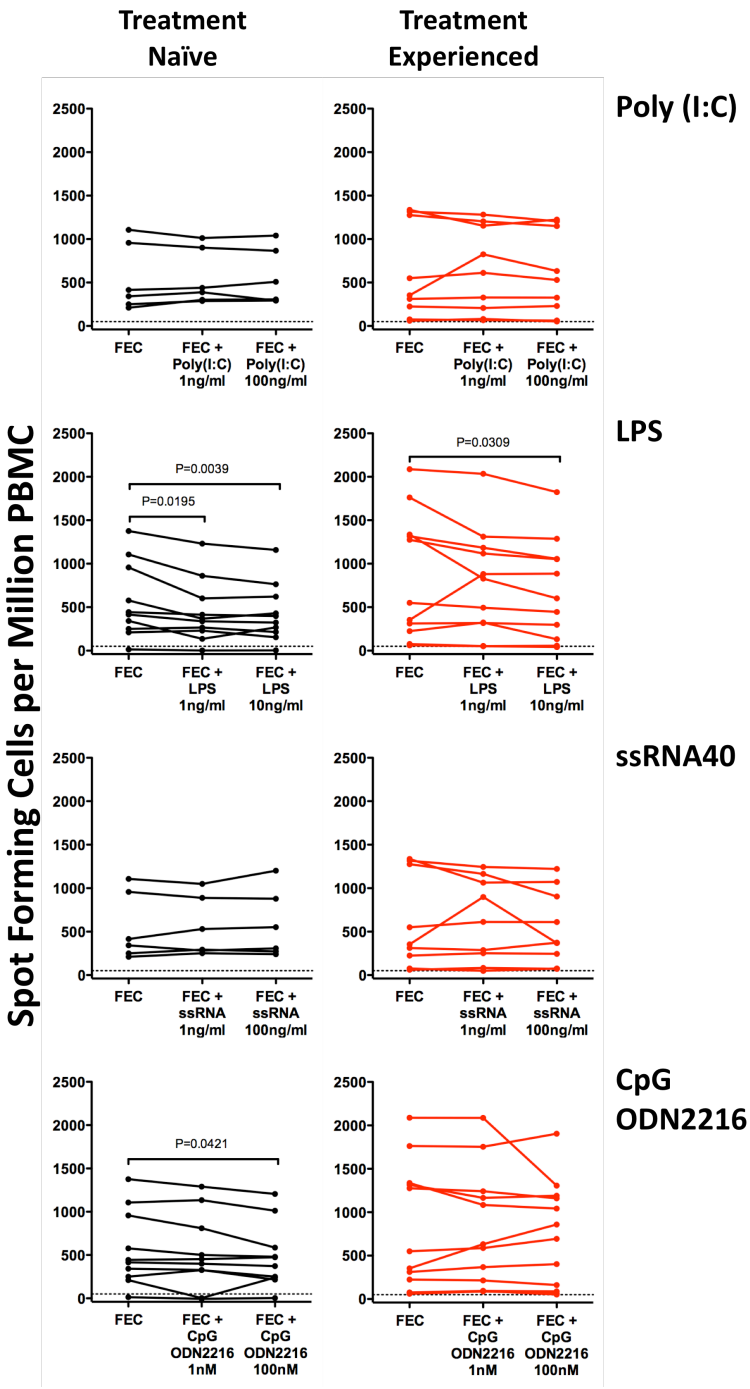


Figure 5.6.1 IFN-γ ELISpot responses of PBMC from HIV-1+ individuals in response to a FEC peptide pool in combination with TLR agonists. PBMC from HIV-1+ individuals, either treatment-naïve (black lines) or treatment-experienced (red lines) were cultured in the presence of FEC. Each line represents a single individual. Dotted line at 50 spots per million cells represents the threshold considered responsive to peptide. All peptide + TLR agonist points have had TLR agonist alone responses subtracted. Wilcoxons matched pairs analysis has been carried out on all samples where FEC alone responses were above 50 spots per million cells and p values are only shown where they are <0.05

Co-stimulation with Poly(I:C) revealed some minor changes in responsiveness compared to FEC alone. There was a modest increase in mean SFC per million using low concentrations of Poly(I:C) (1ng ml^{-1}) compared to peptide alone in four out of six treatment-naïve individuals tested. Four out of ten treatment-experienced individuals also demonstrated an increase in the mean frequency of IFN-γ producing cells in response to FEC in the presence of Poly(I:C) (1ng ml^{-1}), with the mean SFC per million increasing from 558.1 (range 61-1336) to 583 (range 68-1282) for the entire group. Less consistent results were obtained using the higher concentration of Poly(I:C) (100ng ml^{-1}). An increase in SFC was observed in three out of six treatment-naïve individuals, with no difference in the mean for the group, and only three out of ten treatment-experienced individuals demonstrated an increase in IFN-γ SFC.

LPS co-stimulation showed a clear change in response compared to FEC alone. Cells co-stimulated with LPS and antigen consistently showed decreased IFN-γ production in both treatment groups at different LPS concentrations (1 and 10ng ml^{-1}) (Figure 5.6.1). Treatment-naïve individuals displayed a significant reduction in mean SFC per million with LPS co-stimulated cultures, from 631 (210-1376) in FEC alone to 494.2 (136-1232) (1ng ml^{-1} LPS) and 481.3 (154-1158) (10ng ml^{-1} LPS) ($p=0.0195$ and $p=0.0039$ respectively). Seven out of nine (1ng ml^{-1}) and nine out of nine (10ng ml^{-1}) LPS co-stimulated cultures from treatment-naïve individuals had a lower frequency of IFN-γ stimulated cells compared to cultures stimulated with FEC alone. Cells from treatment-experienced individuals followed a similar reduction on co-stimulation of FEC responses with increasing concentrations of LPS: 9/12 and 11/12 individuals showed a reduction with 1ng ml^{-1} and 10ng ml^{-1} LPS, respectively. The mean SFC per million was reduced from 785.9 (61-2088) with FEC alone to 720.8 (52-2034) (1ng ml^{-1} LPS; not significant) and significantly reduced to 643.7 (42-1824) (10ng ml^{-1} LPS; $p=0.0309$). Variable responses were observed in cultures from HIV-1 seropositive samples co-stimulated with FEC and ssRNA40. No difference in mean IFN-γ production was observed following ssRNA40 (1ng ml^{-1}) co-stimulation in treatment-naïve individuals compared to cultures stimulated with FEC alone (FEC alone mean 547, range 210-1106; FEC + ssRNA40 mean 549.3, range 252-1050), with 3/6 individuals showing increased IFN-γ production. Similarly, cells from treatment-experienced individuals co-stimulated with FEC and ssRNA40 (1ng ml^{-1}) had a similar frequency of IFN-γ producing cells (mean 570.4, range 50-1244) compared to FEC alone (mean 558.1, range 61-1336), with 4/10 individuals showing increases in IFN-γ production.

Co-stimulation with 100ng ml^{-1} ssRNA40 generated a modest increase in the frequency of IFN-γ production in treatment-naïve samples (mean 575.3, range 240-1202) compared to cultures stimulated with FEC alone (mean 547, range 210-1106), with four out of six individuals increasing IFN-γ production. Samples from treatment-experienced individuals, however generated a lower frequency of IFN-γ spots when co-stimulated with ssRNA40 (100ng ml^{-1}) (mean 501.7, range 71-1222) compared to cultures stimulated with FEC alone (558.1, range 61-1336), although five out of ten individual samples showed increased in IFN-γ production.

Interestingly, CpG ODN2216 co-stimulation with FEC generated lower or stable production of IFN-γ in HIV-1+ seropositive samples in contrast to the observed enhancement of IFN-γ production in HIV-1- control individuals (Section 5.5). PBMC from treatment-naïve individuals had a reduced frequency of IFN-γ SFC when co-stimulated with the lower concentration (1nM) of CpG ODN2216 (mean 582.9, range 4-1290) compared to cultures stimulated with FEC alone (mean 631, range 210-1376) (Figure 5.6.1). This was reduced further, and significantly, at the higher concentration (100nM mean 537.3, range 216-1206) ($p=0.0421$). Six out of nine treatment-naïve individuals had lower SFC per million counts compared to FEC stimulated cultures at both concentrations of CpG ODN2216. Co-stimulation of PBMC from treatment-experienced individuals with lower concentrations of CpG ODN2216 (1nM) had no effect on the frequency of IFN-γ SFC, (FEC alone mean 785.9, range 61-2088; FEC + ODN2216 mean 784.2, range 88-2086), but was reduced at the higher concentration of 100nM (mean 743.6, range 52-1904). The frequency of IFN-γ SFC was reduced in 6/12 HIV-1+ treatment-experienced individuals in the presence of CpG ODN2216 at both concentrations compared to cultures stimulated with FEC alone.

5.6.2. TLR agonist co-stimulation of the HIV-1 gag 20mer peptide responses

HIV-1+ individuals are known to better maintain antigen-specific T-cell responses to viruses other than HIV-1 itself (Komanduri et al., 2001, Villacres et al., 2001). In particular, a high frequency of IFN-γ producing, CMV-specific effector memory $CD8^+$ T-cells can be found during HIV infection. It is therefore perhaps unsurprising that such responses proved difficult to enhance further in the presence of TLR agonists. The potential of TLR agonists to influence T-cell responses to HIV-1 derived antigen was therefore tested *in*

vitro to establish whether such responses could be recovered from the partial anergy caused by chronic stimulation *in vitro*. PBMC from treatment-naïve and treatment-experienced individuals were stimulated with peptides derived from HIV-1 and co-stimulated with the range of TLR agonists described above.

Responses to the 20mer peptide pool alone were not as vigorous as those observed in response to the FEC peptide pool with five out of seven from the treatment-naïve and 6/11 from the treatment-experienced group responding above the threshold level of 50 SFC per million cells. Data from this experiment is presented in Figure 5.6.2.

Co-stimulation of gag 20mer responses with Poly(I:C) were variable. Co-stimulation of PBMC from treatment-naïve individuals with 1ng ml^{-1} Poly(I:C) had no effect on the mean frequency of IFN-γ SFC compared to stimulation with peptide alone (mean peptide + Poly(I:C) 246.8, range 130-368; mean peptide only 245.2, range 124-310) and this was reduced when PBMC were co-stimulated with 100ng ml^{-1} Poly(I:C) to 213.6 (2-350). The frequency of IFN-γ SFC increased in two out of five HIV-1+ treatment-naive individuals when co-stimulated with 1ng ml^{-1} and in one out of five increased with 100ng ml^{-1} Poly(I:C). By contrast, co-stimulation of PBMC from HIV-1+ treatment-experienced individuals reduced the frequency of SFC when co-stimulated with both 1ng ml^{-1} (mean 236.4, range 24-538) and 100ng ml^{-1} (mean 242, range 14-572) Poly(I:C) compared to stimulation with peptide alone (mean 262, range 68-508).

Co-stimulation of 20mer peptides with LPS displayed a similar reduction in the frequency of IFN-γ SFC. In treatment-naïve samples, mean IFN-γ SFC per million PBMC were reduced from 245.2 (range 124-310) to 116.4 (range 0-218) for 1ng ml^{-1} and 160.8 (range 60-272) for 100ng ml^{-1} LPS. Five out of five and four out of five individuals had reduced SFC frequencies respectively. Treatment-experienced individuals had a reduced mean IFN-γ SFC per million PBMC of 198.4 (0-530) for 1ng ml^{-1} and 175.6 for 100ng ml^{-1} LPS compared to 262 SFC per million PBMC for cultures stimulated with 20mer peptides alone. Four out of five and five out of five individuals had reduced SFC per million PBMC, respectively compared to peptide alone.

ssRNA40 had little impact on the responses to HIV-1 Gag 20mer peptides in HIV-1+ treatment-naïve individuals. Co-stimulation with 1ng ml^{-1} ssRNA40 increased mean IFN-γ SFC from 245.2 (peptide alone) in four out of five individuals to 258.8 (range 166-292), but

decreased the mean SFC at 100ng ml^{-1} to 225.2 (range 142-280). Four out of five and two out of five individuals had increased SFC per million PBMC, respectively, compared to peptide alone.

In contrast, a marked decrease in the mean IFN-γ SFC per million PBMC was observed in treatment-experienced individuals when co-stimulated with 100ng ml^{-1} ssRNA40 (mean 166.8, range 40-472) compared to PBMC stimulated with peptide alone (262, range 68-508). Little change, however, was observed at lower concentration (1ng ml^{-1}) (mean 246, range 0-610). The frequency of SFC per million PBMC was reduced in five out of five (100ng ml^{-1} ssRN40) and three out of five individuals (1ng ml^{-1} ssRNA40) respectively.

Co-stimulation of PBMC with HIV-1 gag 20mer peptides and CpG ODN2216 showed stable or reduced antigen-specific responses. The mean SFC per million cells reduced from 245.2 when treatment-naïve samples were stimulated with 20mer alone to 209.6 (range 118-306) when co-stimulated with 1nM CpG ODN2216, but remained relatively stable (mean 242.8, range 148-290) when co-stimulated with 100nM. One out of five and two out of five individual treatment-naïve samples had increased IFN-γ SFC frequencies when co-stimulated with 1nM and 100nM CpG ODN2216 respectively. Co-stimulation of PBMC from treatment-experienced individuals with 20mer and CpG ODN2216 reduced the mean IFN-γ SFC per million cells from 262 with peptide alone to 238.4 (range 12-516) with 1nM and 235.2 (range 16-524) with 100nM.

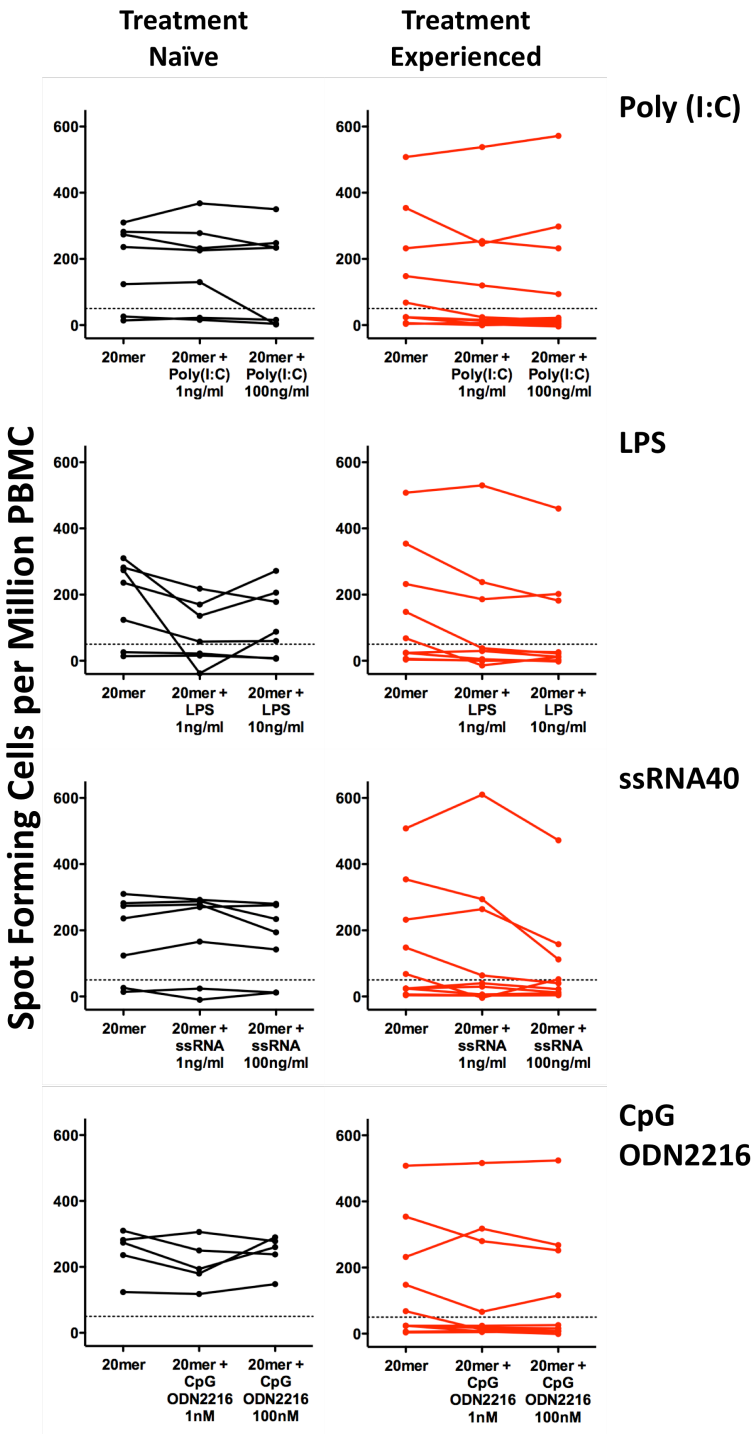


Figure 5.6.2 IFN-γ ELISpot responses of PBMC from HIV-1+ individuals in response to an HIV-1 gag 20mer peptide pool in combination with TLR agonists. PBMC from HIV-1+ individuals, either treatment-naïve (black lines) or treatment-experienced (red lines) were cultured in the presence of an HIV-1 gag 20mer peptide pool. Each line represents a single individual. Dotted line at 50 spots per million cells represents the threshold considered responsive to peptide. All peptide + TLR agonist points have had TLR agonist alone responses subtracted. Wilcoxons matched pairs analysis has been carried out on all samples where 20mer alone responses were above 50 spots per million cells and p values are only shown where they are <0.05 (none of the changes in response were significant for these assays).

5.6.3. TLR agonist co-stimulation using the HIV-1 gag 9mer peptide pool

MHC class I-restricted HIV-1 gag 9mer pooled peptides were also used in co-stimulation assays with TLR agonists. $CD8^+$ T-cells and PBMC from HIV-1+ treatment-naïve individuals responded more frequently to the 9mer peptides alone, with 9/12 responding compared to 6/13 treatment-experienced individuals. The data for these assays is shown in Figure 5.6.3.

Co-stimulation of PBMC from HIV-1+ samples with Poly(I:C) led to modest increases in IFN-γ SFC per million cells in both treatment-naïve and treatment-experienced samples at 1 and 100ng ml^{-1}. Co-stimulation of treatment-naïve samples increased the mean SFC per million cells from 175.2 (96-336) when stimulated with peptide alone to 195.5 (96-366) with 1ng ml^{-1} and 188.5 (104-352) with 100ng ml^{-1} Poly(I:C). Two out of four individuals increased the frequency of SFC at both concentrations. A similar pattern was observed in PBMC from treatment-experienced individuals, where the mean IFN-γ SFC per million cells was 233.5 (range 58-558) when stimulated with peptide alone, 261.5 (range 26-624) with 1ng ml^{-1} Poly(:IC), and 247 (36-644) with 100ng ml^{-1} Poly(:IC), with three out of four and two out of four individuals showing an increase in SFC respectively.

Surprisingly, low concentrations of LPS (1ng ml^{-1}) increased the frequency of IFN-γ producing cells in treatment-naïve samples when combined with 9mer peptides. The mean IFN-γ SFC per million was raised from 419.6 (range 96-1880) when stimulated with peptide alone to 466.4 (range 26-2236) when co-stimulated with 1ng ml^{-1} LPS. Increases were observed in six out of nine individuals. Conversely, the mean SFC per million PBMC dropped to 359.1 (range 38-1618) at higher (100ng ml^{-1}) LPS concentration, with increases observed in only three out of nine individuals respectively. Reduced frequencies of IFN-γ SFC were observed from treatment-experienced individuals in response to gag 9mer peptides when co-stimulated with LPS (peptide alone mean 230, range 58-558; peptide + LPS 1ng ml^{-1} mean 166.7, range 0-484; peptide + LPS 10ng ml^{-1} mean 182, range 18-470). An increased frequency of IFN-γ SFC was only observed in a single individual at both concentrations of LPS.

Co-stimulation with ssRNA40 increased the mean frequency of IFN-γ SFC in PBMC from treatment-naïve individuals from 175.2 (range 96-336) when stimulated with peptide alone,

to 220 (range 54-538) with 1ng ml^{-1}, and to 192 (range 160-368) with 100ng ml^{-1} ssRNA40. The frequency increased in two out of four and three out of four individuals. Modest increases were also observed on co-stimulation of cells from treatment-experienced individuals, where the mean SFC per million cells increased from 233.5 (range 58-558) when stimulated with peptide alone, to 261 (range 30-634) with 1ng ml^{-1}, and to 247 (range 10-562) with 100ng ml^{-1} ssRNA40, with increases observed in two out of four and three out of four individuals respectively.

CpG ODN2216 co-stimulation of gag 9mer responses demonstrated a consistent increase in responsiveness in treatment-naïve individuals at low concentrations (1nM), with six out of nine individual samples increasing the number of IFN-γ SFC. The mean number of SFC per million cells increased from 419.6 (range 96-1880) with peptide alone to 478.9 (range 62-2146) with 1nM CpG ODN2216. At higher concentration (100nM) CpG ODN2216 reduced the mean SFC per million to 397.8 (82-1796); increases were observed in only two out of nine individuals. Co-stimulation of PBMC from treatment-experienced individuals with CpG ODN2216 and 9mer peptides in combination did not substantially alter IFN-γ production compared to that observed with peptide alone. IFN-γ SFC per million PBMC, after co-stimulation with peptides and 1nM CpG ODN2216, was 238.3 (range 28-616) compared to 230 (range 58-558) with peptide alone and 226.7 (30-632) when co-stimulated with 100nM CpG ODN 2216.

Data presented for co-stimulation of antigen-specific responses using different peptides and TLR agonists has revealed consistent inhibitory effects with certain TLR ligands whereas others, when used at an appropriate concentration, enhance peptide-stimulated responses. LPS consistently decreased IFN-γ responses compared to peptide alone in both treatment-naïve and treatment-experienced HIV-1 infected individuals at low and high concentrations, and with all three different peptide pools studied. Exceptionally, IFN-γ responses to gag 9mer were enhanced within PBMC from HIV-1+ treatment-naïve individuals when co-stimulated 1ng ml^{-1} LPS.

The most consistent enhancement of IFN-γ responses was observed when co-stimulating peptide induced responses with CpG ODN2216. This was the case both for PBMC from HIV-1- control individuals stimulated with FEC peptides and from HIV-1+ treatment-naïve individuals stimulated with HIV-1 gag 9mer in combination with CpG ODN2216 at low concentration (1nM).

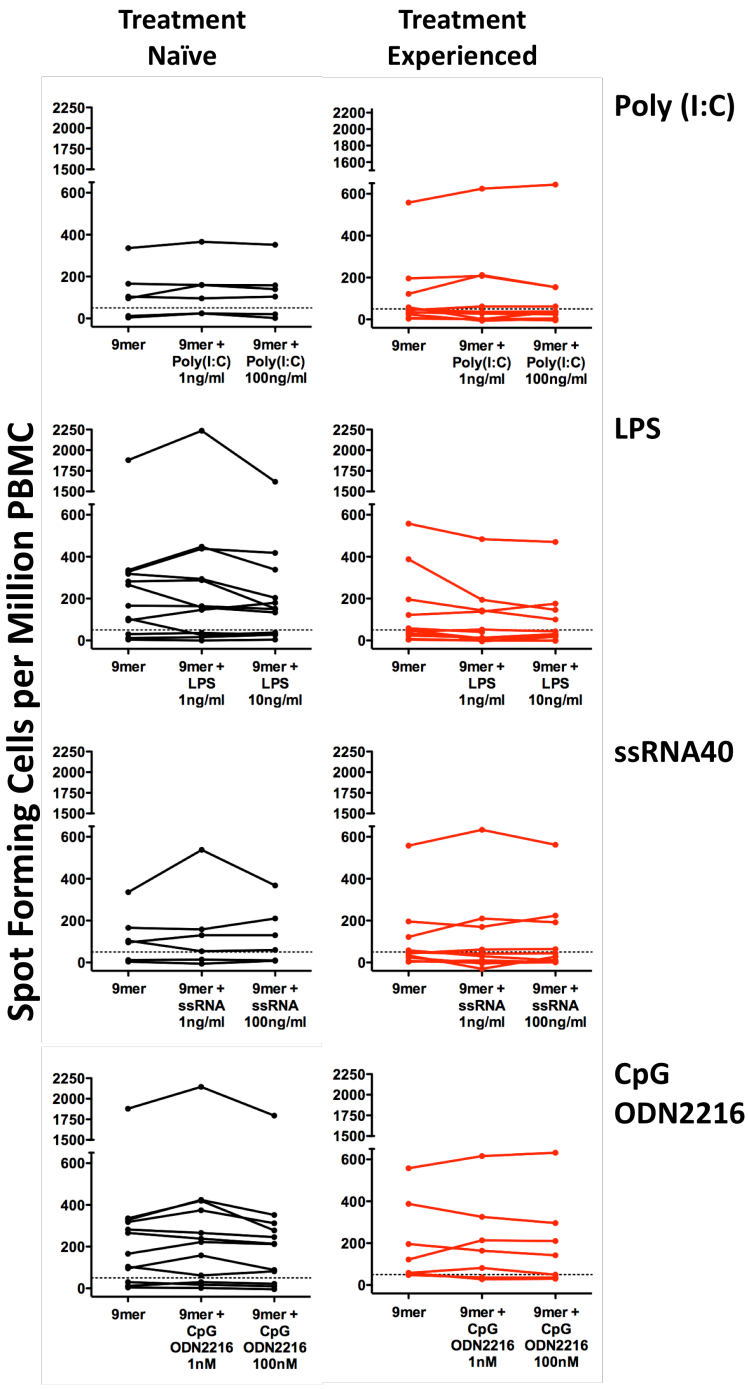


Figure 5.6.3 IFN-γ ELISpot responses of PBMC from HIV-1+ individuals in response to an HIV-1 gag 9mer peptide pool in combination with TLR agonists. PBMC from HIV-1+ individuals, either treatment-naïve (black lines) or treatment-experienced (red lines) were cultured in the presence of an HIV-1 gag 9mer peptide pool. Each line represents a single individual. Dotted line at 50 spots per million cells represents the threshold considered responsive to peptide. All peptide + TLR agonist points have had TLR agonist alone responses subtracted. Wilcoxons matched pairs analysis has been carried out on all samples where 9mer alone responses were above 50 spots per million cells and p values are only shown where they are <0.05 (none of the changes in response were significant for these assays).

5.7. NK cell and BDCA4$^+$ DC depletion reduces IFN-γ production in response to antigen-specific stimulation

In order to establish which cell populations provide accessory help for antigen-specific stimulation of IFN-γ production in PBMC, a small experiment was carried out using PBMC depleted of specific cell types and stimulated with the FEC peptide pool. For these assays three HIV-1- control samples were depleted of B-cells, NK cells and BDCA4$^+$ pDC (pDC as described by Dzionek *et al*. (2000)), using magnetic cell depletion as described in Section 2.8, before being stimulated overnight with FEC in IFN-γ ELISpot assays. Table 5.7.1 shows the efficiency of the different depletions for the three samples. The frequency of pDC is displayed as frequency of CD123$^+$ cells in the gated lineage cocktail$^-$ (Lin-1$^-$) HLA-DR$^+$ population. NK cells and B-cells were efficiently depleted beyond 75% for all three samples. Depletion of pDC was less complete with sample two only depleted by 37% of the total lineage$^-$HLA-DR$^+$ pDC population (see Figure 3.5.1 for pDC gating strategy).

Table 5.7.1 Frequency of NK cells, B-Cells and pDC before and after magnetic cell separation from total PBMC from 3 HIV-1- control individuals. BDCA-4 was used as a marker to remove pDC from PBMC.

Depletion	Frequency (%)	Sample 1	Sample 2	Sample 3
PBMC	B-Cells	4.37	11.3	5.8
	NK cells	3.78	15.5	4.86
CD19 Depleted	B-Cells	0.0428	0.497	1.06
CD56 Depleted	NK cells	0.884	1.99	1.08
PBMC pDC	pDC (frequency Lin-1$^-$HLA-DR$^+$)	6.9	12.6	12.6
BDCA-4 Depleted	pDC (frequency Lin-1$^-$HLA-DR$^+$)	1.84	7.91	4.1

NB. Frequency of pDC is frequency of Lin$^-$HLA-DR$^+$ cells.

Table 5.7.2 summarises the ELISpot data for the depletion assays. All three individuals had strong responses to FEC peptide alone, with responses above the 50 SFC per million PBMC threshold of activation. The SFC per million cells for the depleted samples is listed and the proportion compared to total PBMC is shown. Depletion of B-cells only reduced IFN-γ production in one of the individuals by 16%, and actually increased production in the other two individuals. The increase in IFN-γ seen in samples 1 and 3 is potentially caused by the slightly higher frequency of IFN-γ producing cells that would be present without B-

cells. From this data, it appeared that B-cells did not contribute to antigen-specific IFN-γ production.

Table 5.7.2 IFN-γ ELISpot Responses to FEC peptide pool of NK cell, B-cell and pDC depleted PBMC from 3 HIV-1- control individuals. Spot forming cells per million (SFC) shown for 3 individuals in response to a FEC peptide pool cultured overnight with PBMC or PBMC depleted of $CD19^+$, $CD56^+$, or BDCA-4^+ cells by magnetic cell separation. Proportion of SFC per million cells from depleted versus total PBMC also shown.

Stimulation	Depletion	Individual					
		1 (SFC/million)	Proportion compared to PBMC	2 (SFC/million)	Proportion compared to PBMC	3 (SFC/million)	Proportion compared to PBMC
FEC	No Depletion	121.33	-	573.33	-	161.33	-
	CD19	133.33	1.1	469.33	0.84	190.67	1.18
	CD56	101.33	0.84	436	0.76	113.33	0.70
	BDCA-4	74.68	0.62	298.33	0.52	109.33	0.68

Depletion of NK cells led to a modest reduction of stimulated IFN-γ production in the three samples (16%, 24% and 30%, respectively). This reduction indicates that NK cells might produce IFN-γ in response to antigen-specific stimuli, however further studies are needed to confirm this. This is examined further in section 5.8.3.

Depletion of pDC led to a larger decrease (38%, 48% and 32% respectively) in stimulated IFN-γ production than B-cells or NK cells, despite the less efficient depletion. This is not surprising as DC are specialist antigen presenting cells that express high levels of MHC class I and II, and therefore reduced DC frequencies will lead to less peptide presentation and less T-cell stimulation. This experiment confirmed a requirement for pDC to generate optimal responses to antigen-specific stimulation *in vitro*. In addition, a potential contribution of NK cells in promoting optimal antigen-specific recall responses is indicated. The interaction of NK cells with DC is well established (Moretta et al., 2008, Gerosa et al., 2005) and it is possible that without NK cell feedback, DC are unable to fully stimulate T-cell activation. It is unclear however whether adjuvant systems such as TLR agonist stimulation are further stimulating antigen-specific T-cells or rather providing a mechanism to enhance bystander, non-specific T-cell responses.

5.8. CpG ODN2216 enhancement of T-cell responses is not restricted to antigen-specific T-cells

Preliminary assays were carried out in an attempt to identify which cell populations were responsible for the enhanced IFN-γ production observed in ELISpot assays presented in Section 5.6. In order to identify antigen-specific T-cell populations, MHC class I pentamer staining was used to identify antigen-specific $CD8^+$ T-cells and activation (CD69) and degranulation (CD107a) responses were measured. Pentamer staining allows the identification of very small populations of antigen-specific $CD8^+$ T-cells in flow cytometric assays, and was employed here to establish the levels of activation and degranulation of cells recognising particular CMV and HIV-1 gag epitopes (CMV and HIV-1 gag epitopes are listed in Appendix Table A.10) (HIV-1 gag epitope pentamers are referred to as HIV-1 gag pentamer (1) or pentamer (2)). Degranulation was assessed using CD107a expression as described previously by Betts *et al* (2003) and by Alter *et al* (2004a). Methods for pentamer staining and degranulation are described in Section 2.7.4. The preliminary nature of these assays combined with the technical difficulties involved with evaluating very low numbers of pentamer stained cells and the low numbers of individuals tested mean that conclusions are more based on qualitative descriptions and firm conclusions could not be made.

In order to identify antigen-specific cells, the HLA-A and HLA-B profiles of responding individuals were established in order to use appropriate HLA-specific pentamers. DNA was isolated from stored cell samples (see Section 2.11 for method) and HLA-typed (ProImmune, Oxford, UK). Individuals were selected for testing if co-stimulation of CpG ODN2216 and the HIV-1 gag 9mer peptide pool had enhanced their cells' IFN-γ production in ELISpot assays (Section 5.6). Experiments focused on the treatment-naïve sample group, as this is where the most consistent (seven out of nine samples) enhancement of antigen-specific responses was observed. Of seven individuals showing enhanced responses, samples from six were tissue-typed, the results of which are shown in Table 5.8.1. Of these, five were found to have HLA-A*02:01 (highlighted in Table 5.8.1). Pentamers specific for this allele were therefore selected (see Appendix Table A.10). From the five suitable individuals it was possible to obtain a fresh peripheral blood sample from three individuals when they were visiting clinics for routine appointments. The patient

characteristics of these individuals are presented in Table 5.8.2. A single HIV-1- control individual was also used in this study (Table 5.8.2).

Table 5.8.1 HLA typing results for 6 HIV-1+ individuals selected on the basis they showed increased IFN-γ SFC per million when cultured with HIV-1 peptide + CpG ODN2216 compared to peptide alone. Samples marked between * were used in used in assays described in this section.

Sample		1	2
W325	HLA-A	*02:01/01L/07/09/15N/ 18/20/22/24/25/29/30/31/32N/ 33/34/42/43N/53N/59/62/66/67	*68:01/07/08/11N/12/16/17/21/24
	HLA-B	*57:01/06	*35:01/07/30/40N/41/42
P671	HLA-A	*02:01/01L/07/09/15N/ 18/20/24/29/30/31/33/ 42/43N/49/53N/ 59/66/67	*33:01/03/04/05/06
	HLA-B	*14:02	*37:01/03N
A405	HLA-A	*02:01/01L/06/07/09/15N/ 18/20/24/29/30/31/ 33/42/43N/53N/ 59/66/67	*25:01/03
	HLA-B	*07:02/04/10/21/22/23/30	*44:02/02S/19N/23N/27/28/33
P110	HLA-A	*02:01/01L/07/09/15N/ 18/20/24/26/29/30/31/ 33/34/35/42/43N/49/53N/59/62/66/67	*03:01/01N/04/07/09/11N/13/14
	HLA-B	*14:02/03	*35:01/07/08/30/40N/41/42/45
M696	HLA-A	*02:01/01L/07/09/15N/ 18/20/24/29/30/31/33/ 40/42/43N/ 49/53N/59/66/67	*32:01/05/06/07
	HLA-B	*15:01/01N/28/33/34/57/60/70/78/79N	*40:02/29/35/37
D036	HLA-A	*25:01	*29:01/01N/02/04/06/08N/10/11
	HLA-B	*44:02/03/07/13/26/30/32/35	*40:01/22N

Table 5.8.2 Individual patient characteristics

	HIV-1- control Sample	HIV-1+ Sample 1	HIV-1+ Sample 2	HIV-1+ Sample 3
Age	28	39	44	38
CD4 T-cell count (cells/µl Blood)	N/A	882	362	330
Plasma Viral Load (copies/ml Blood)	N/A	U/D	50585	53163

PBMC were cultured overnight either in medium alone or in the presence of IL-12 (1ng ml^{-1}) and IL-15 (20ng ml^{-1}), PHA (10µg ml^{-1}), FEC peptides (5µg ml^{-1}) or HIV-1 gag 9mer peptides (5µg ml^{-1}) with or without CpG ODN2216 (1nM). An appropriate volume of cells was removed for intracellular cytokine staining assays before degranulation and pentamer and cell surface marker immunostaining was carried out, all as described in Section 2.7. Antibody panels and pentamer details for these assays can be seen in Appendix tables A.9 and A.10. Example dot plots and the gating strategy for these assays are shown in Figure 5.8.1 and 5.8.2. These demonstrate the large number of events that were acquired (10^6 cells within the lymphocyte gate), as is necessary for examination of responses within the minor $CD8^+pentamer^+$ subset of cells. Responses within the total $CD8^+$ T-cell population and within maturational subsets were also examined (Figures 5.8.1 and 5.8.2). IFN-γ could not be examined in parallel with pentamer staining, for technical reasons, but was examined on NK cells (gating not shown, see Sections 3.7 and 3.8 for NK gating) and is included in analysis shown in Table 5.8.3. The dot plots demonstrated in Figure 5.8.1 and 5.8.2 represent results from a single individual of three tested, and demonstrate stimulation with HIV-1 9mer peptides with and without CpG ODN2216 co-stimulation, or for CMV pentamer staining after stimulation with the FEC peptide pool. Gating of $pentamer^+CD8^+$ T-cells was set according to negative pentamer staining for each individual. Results from this individual indicate increased CD69 expression and CD107a expression within total $CD8^+$ T-cells when co-stimulated with CpG ODN2216 compared to 9mer alone (Figure 5.8.1). Furthermore, the increase is more pronounced in the more mature TEM and TEMRA subsets (Figure 5.8.2). Surprisingly, a decrease was observed in IFN-γ production in total $CD8^+$ T-cells as well as a decrease in degranulation within the $pentamer^+$ subset. Conversely, a small increase in CD69 expression was observed within this subset. Data from these assays is displayed in full in Tables 5.8.3, 5.8.4 and 5.8.5.

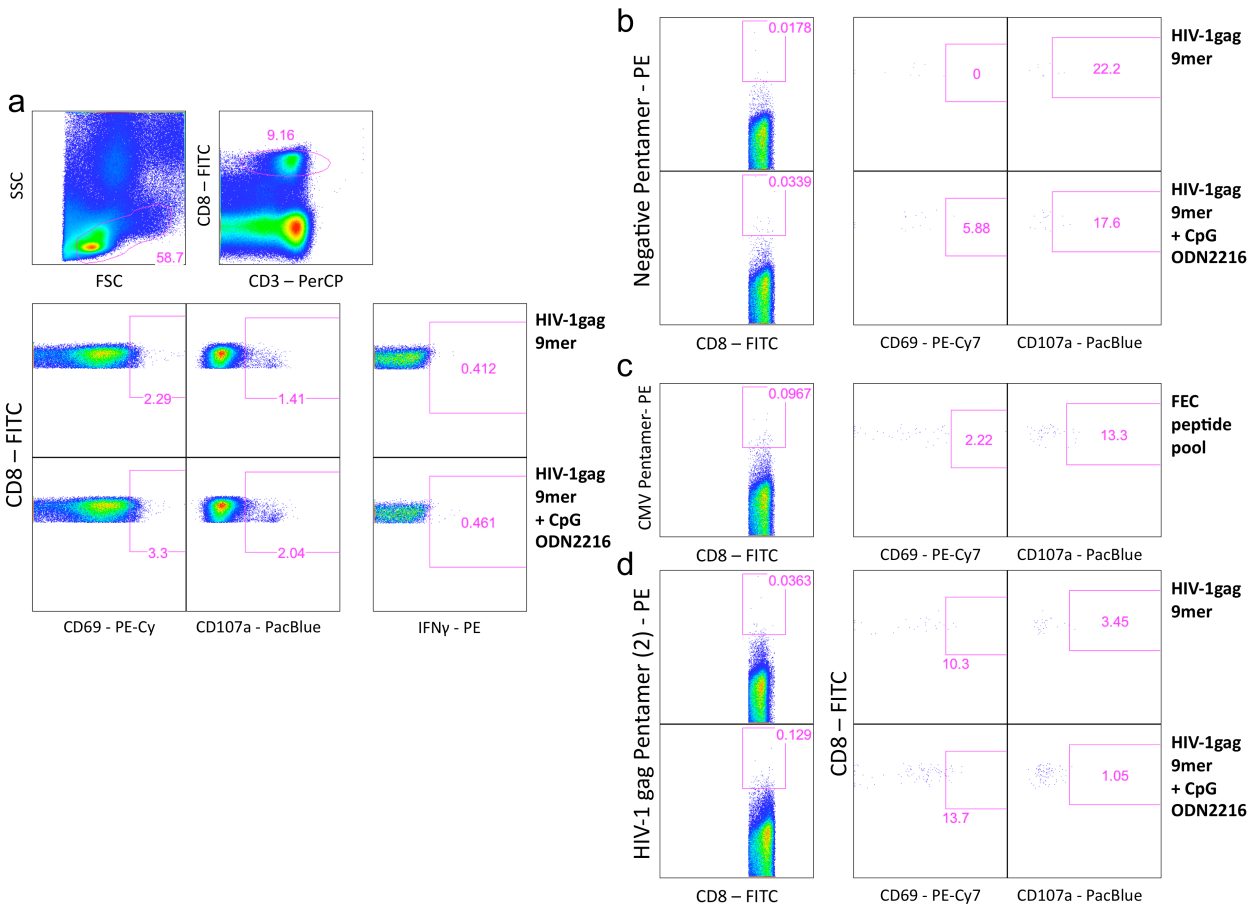


Figure 5.8.1 TLR agonist co-stimulation of antigen-specific T-cells. Gating strategy demonstrating the CD69 (activation) expression or CD107a (degranulation) expression by a) total $CD8^+$ T-cells (IFN-γ expression is also shown on total $CD8^+$ T-cells), b) negative control $pentamer^+$ $CD8^+$ T-cells, c) CMV $pentamer^+$ $CD8^+$ T-cells and d) HIV-1 gag $pentamer^+$ in response to overnight stimulation of PBMC with either the HIV-1 gag 9mer peptide pool alone or in combination with 1nM CpG ODN2216 or in the case of CMV pentamer staining to the FEC peptide pool.

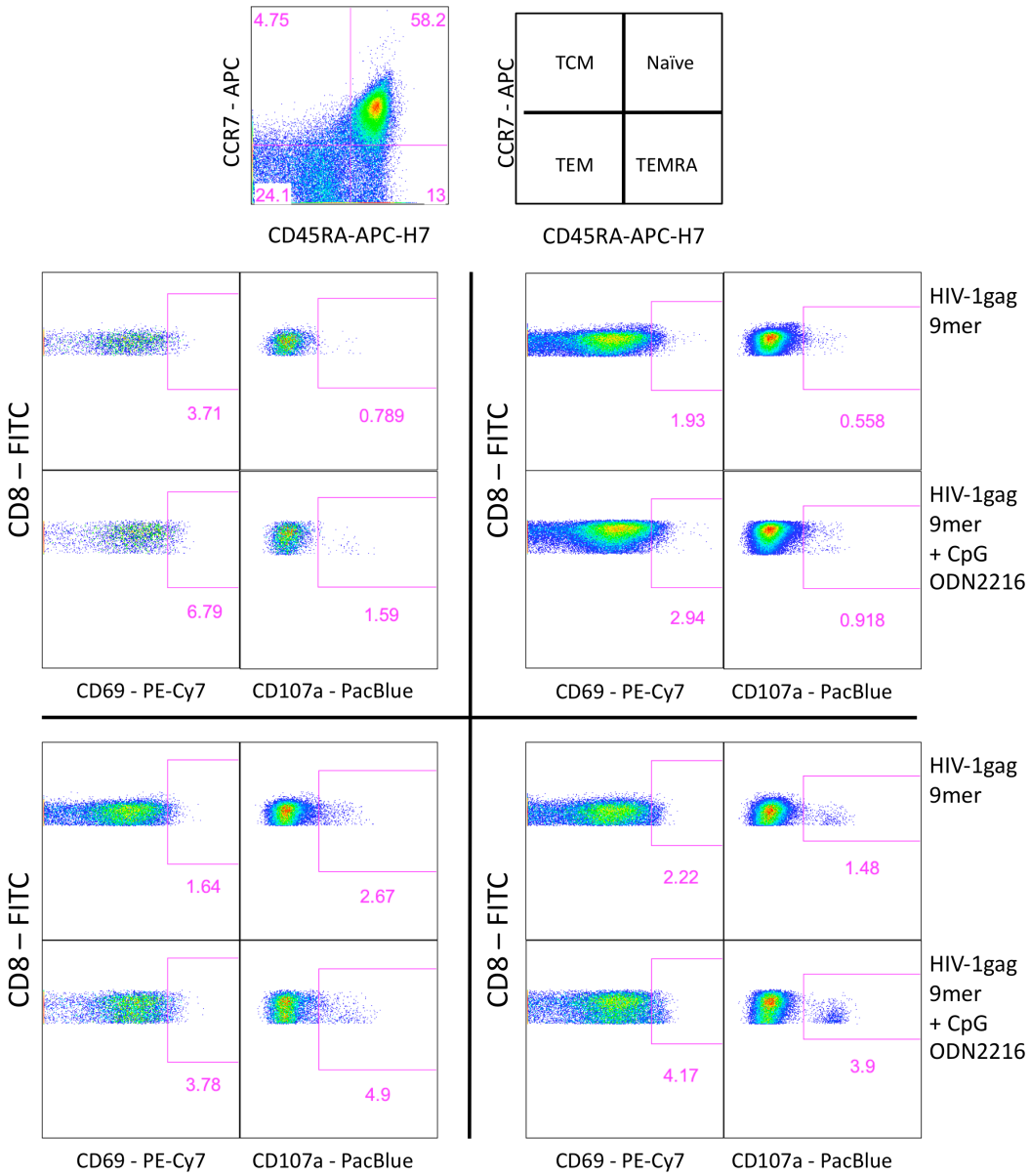


Figure 5.8.2 TLR agonist co-stimulation of memory T-cells. Gating strategy demonstrating responsiveness of the 4 different memory $CD8^+$ T-cell subsets in response to either HIV-1 gag 9mer peptide stimulation alone or in combination with 1nM CpG ODN2216. Early activation (CD69) and degranulation (CD107a) expression are shown for all cell types and IFN-γ was only measurable in this assay on total $CD8^+$ T-cells.

5.8.1. Early activation

Early activation responses (CD69 expression) were examined within total $CD8^+$ T-cells and $CD8^+$ T-cell memory subsets as well as antigen-specific cells (Table 5.8.3). As mentioned above, analysis of this data is qualitative and statistics have not been carried out. Overnight stimulation of PBMC from the HIV-1- control individual with IL-12 and IL-15 increased CD69 expression only on TCM and TEMRA cells. All three HIV-1+ individuals

had increased responses within CMV pentamer$^+$CD8$^+$ T-cells, two out of three had increased responses in TEM cells, one out of three had increased responses in naïve and TEMRA cells, and all three individuals had reduced responses in total CD8$^+$ T-cells, TCM cells and in both HIV-1 gag pentamer$^+$CD8$^+$ T-cells subset cells.

Overnight stimulation with PHA decreased CD69 expression on all subsets except TEMRA cells in the HIV-1- control individual compared to unstimulated cultures. All three HIV-1+ individuals had increased CD69 expression in response to PHA in CMV pentamer$^+$CD8$^+$ T-cells. Two out of three individuals had increased expression in both HIV-1 gag pentamer$^+$CD8$^+$ T-cells and in naïve, TCM and TEMRA cells. Only one out of three of the individuals increased CD69 expression in total CD8$^+$ T-cells and in TEM cells.

CD69 expression in the HIV-1- control individual in response to overnight FEC peptide stimulation compared to unstimulated cultures was reduced in all cell subsets except TEMRA cells. All three HIV-1+ individuals had reduced CD69 expression in total CD8$^+$ T-cells, naïve, TCM and TEMRA cells and only one out of three individuals had an increase in CD69 expression in TEM cells. All three individuals, however, had increased expression in CMV pentamer$^+$CD8$^+$ T-cells.

Two out of three HIV-1+ individuals had reduced expression of CD69 when stimulated with the HIV-1 gag 9mer peptides compared to unstimulated cultures in all subsets except naïve CD8$^+$ T-cells, where all three individuals had reduced expression. Although the same pattern is largely seen in CpG ODN2216 co-stimulated cultures compared to unstimulated cells, improvements were seen compared to cultures stimulated with 9mer alone. All three samples had higher CD69 expression in total CD8$^+$ T-cells and in the naïve, TEM and TEMRA subsets, and two out of three samples had higher expression in both the HIV-1 pentamer$^+$CD8$^+$ T-cell subsets and in TCM cells.

Table 5.8.3 Early activation (CD69) responses of total CD8$^+$ T-cells, pentamer$^+$ CD8$^+$ T-cells and memory subset CD8$^+$T-cell subsets from different individuals to the stimulations shown.

		Individual			
Stimulation	Cell Type	HC	HIV-1+ 1	HIV-1+ 2	HIV-1+ 3
No Stimulation	Total CD8$^+$ T-cell	0.497	2.860	1.798	4.185
	CMV Pentamer$^+$ CD8$^+$ T-cell	1.370	0.000	0.000	7.440
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	20.000	11.100	9.370
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	8.890	4.550	12.000
	Naïve CD8$^+$ T-cell	0.817	2.913	1.995	7.458
	Central Memory CD8$^+$ T-cell	2.615	6.603	4.825	7.133
	Effector Memory CD8$^+$ T-cell	0.568	3.023	2.345	3.410
	TEMRA CD8$^+$ T-cell	0.442	2.698	1.838	6.820
IL-12 + IL-15	Total CD8$^+$ T-cell	0.432	2.585	1.845	3.425
	CMV Pentamer$^+$ CD8$^+$ T-cell	0.000	8.000	4.440	9.180
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	4.760	6.380	7.580
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	8.850	0.000	7.560
	Naïve CD8$^+$ T-cell	0.543	2.293	2.073	5.900
	Central Memory CD8$^+$ T-cell	3.110	5.958	4.605	5.910
	Effector Memory CD8$^+$ T-cell	0.438	3.120	2.355	2.748
	TEMRA CD8$^+$ T-cell	0.544	2.683	1.955	5.700
PHA	Total CD8$^+$ T-cell	0.331	2.835	1.668	4.415
	CMV Pentamer$^+$ CD8$^+$ T-cell	1.000	3.330	4.650	12.500
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	0.000	11.400	12.500
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	9.840	5.260	5.630
	Naïve CD8$^+$ T-cell	0.437	2.870	2.273	9.990
	Central Memory CD8$^+$ T-cell	1.455	5.940	5.080	8.083
	Effector Memory CD8$^+$ T-cell	0.345	2.675	1.870	3.330
	TEMRA CD8$^+$ T-cell	0.491	2.820	1.918	7.425
FEC	Total CD8$^+$ T-cell	0.470	2.710	1.335	2.900
	CMV Pentamer$^+$ CD8$^+$ T-cell	0.000	2.220	5.880	13.000
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	-	-	-
	Naïve CD8$^+$ T-cell	0.580	2.370	1.550	5.565
	Central Memory CD8$^+$ T-cell	2.490	6.195	3.520	5.710
	Effector Memory CD8$^+$ T-cell	0.499	3.355	1.685	2.095
	TEMRA CD8$^+$ T-cell	0.718	2.940	1.470	5.495
HIV-1 gag 9mer	Total CD8$^+$ T-cell	-	2.997	1.100	3.597
	CMV Pentamer$^+$ CD8$^+$ T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	12.000	0.000	19.100
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	10.300	0.000	9.200
	Naïve CD8$^+$ T-cell	-	2.650	1.320	5.390
	Central Memory CD8$^+$ T-cell	-	6.510	2.633	8.540
	Effector Memory CD8$^+$ T-cell	-	3.383	1.387	2.893
	TEMRA CD8$^+$ T-cell	-	3.053	1.206	6.367
HIV-1 gag 9mer + CpG ODN 2216	Total CD8$^+$ T-cell	-	4.213	1.507	3.773
	CMV Pentamer$^+$ CD8$^+$ T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	21.400	7.140	19.100
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	13.800	0.000	10.200
	Naïve CD8$^+$ T-cell	-	3.663	1.557	6.477
	Central Memory CD8$^+$ T-cell	-	8.237	3.683	7.580
	Effector Memory CD8$^+$ T-cell	-	4.730	1.907	3.017
	TEMRA CD8$^+$ T-cell	-	4.573	1.697	6.763

5.8.2. Degranulation

Expression of CD107a was used as an indicator of degranulation (Table 5.8.4). It was possible to measure the degranulation response in pentamer stained $CD8^+$ T-cells and in the four maturational subsets. As mentioned above, analysis of this data is qualitative and statistics have not been carried out. The degranulation response to cytokine stimulation in the HIV-1- control individual was reduced in total $CD8^+$ T-cells, naïve, TCM and TEM T-cell subsets, but was increased in CMV $pentamer^+CD8^+$ T-cells and TEMRA cells compared to unstimulated cultures. The response to cytokines in HIV-1+ individuals showed that all three had increased degranulation in the CMV $pentamer^+$ population. Two out of three samples showed increased degranulation responses in total $CD8^+$ T-cells, HIV-1 gag $pentamer^+$ (1) $CD8^+$ T-cells and within all maturational subsets when stimulated with cytokines. Two out of three individuals had decreased degranulation responses in the HIV-1 gag $pentamer^+$ (2) cell subset when stimulated with cytokines.

Stimulation of HIV-1 seropositive samples and the seronegative sample with PHA increased CD107a frequency in all cell subsets measured, with two exceptions. Degranulation was reduced in TCM cells in the HIV-1- control sample and in HIV-1 gag $pentamer^+$ (1) cells from the first HIV-1+ individual, although this is likely due to the high background degranulation observed in this cell subset in this individual. Peptide stimulation of the HIV-1- individual with the FEC peptide pool showed an increase or no change in degranulation in all cell subsets except TCM cells, which were reduced. All three HIV-1+ individuals had increased degranulation responses in total $CD8^+$ T-cells, CMV $pentamer^+$ cells, TEM and TEMRA cells when stimulated with FEC peptides. Two out of three individuals had increased degranulation in naïve cells and two out of three individuals had reduced responses in TCM.

All three HIV-1+ individuals had increased degranulation responses in total $CD8^+$ T-cells, TCM and TEM cells in response to the HIV-1 gag 9mer peptide pool alone compared to unstimulated cells. Two out of three individuals had decreased degranulation in both HIV-1 gag $pentamer^+CD8^+$ T-cell subsets and in TEMRA cells, and all three individuals had a decreased degranulation response in naïve $CD8^+$ T-cells. CpG ODN2216 co-stimulation enhanced degranulation responses in some cell subsets in HIV-1+ individuals also stimulated with HIV-1 gag 9mer peptides. All three individuals had an increased CD107a frequency in total $CD8^+$ T-cells, and interestingly in naïve, TEM and TEMRA cells

compared to stimulation with 9mer alone, but none of the individuals had an increased response in TCM cells. One out of three and two out of three individuals had increased degranulation responses in HIV-1 gag pentamer^{+} (1) and (2) CD8^{+} T-cells respectively. These data indicate that non-specific T-cells are recruited and or that cells maintain functional for longer when co-stimulated with CpG ODN2216, as responses above background were observed in co-stimulated cultures even though they were not in cultures stimulated with peptide alone. These findings are similar to the early activation data presented above, in that it appears that co-stimulation with CpG ODN2216 might help sustain responses for longer and, as discussed in the next section, the chosen time point may miss peak responses.

Table 5.8.4 Degranulation (CD107a) responses of total CD8$^+$ T-cells, pentamer$^+$ CD8$^+$ T-cells and memory subset CD8$^+$T-cell subsets from different individuals to the stimulations shown.

		Individual			
Stimulation	Cell Type	HC	HIV-1+ 1	HIV-1+ 2	HIV-1+ 3
No Stimulation	Total CD8$^+$ T-cell	0.217	1.513	0.716	2.428
	CMV Pentamer$^+$ CD8$^+$ T-cell	6.850	0.000	4.290	6.790
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	40.000	3.700	12.500
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	0.000	2.270	9.570
	Naïve CD8$^+$ T-cell	0.203	0.774	0.776	3.490
	Central Memory CD8$^+$ T-cell	0.396	0.667	0.276	3.755
	Effector Memory CD8$^+$ T-cell	0.085	0.316	0.276	1.743
	TEMRA CD8$^+$ T-cell	0.396	3.555	2.508	3.528
IL-12 + IL-15	Total CD8$^+$ T-cell	0.181	2.073	0.997	1.928
	CMV Pentamer$^+$ CD8$^+$ T-cell	9.460	16.000	5.190	14.100
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	23.800	6.380	13.600
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	5.310	0.000	5.230
	Naïve CD8$^+$ T-cell	0.167	0.894	0.983	2.923
	Central Memory CD8$^+$ T-cell	0.000	0.845	0.488	2.918
	Effector Memory CD8$^+$ T-cell	0.069	0.429	0.406	1.278
	TEMRA CD8$^+$ T-cell	0.434	5.800	3.653	2.935
PHA	Total CD8$^+$ T-cell	0.342	9.985	16.625	30.550
	CMV Pentamer$^+$ CD8$^+$ T-cell	12.000	23.300	4.650	26.000
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	33.300	20.000	28.600
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	7.380	10.500	17.600
	Naïve CD8$^+$ T-cell	0.254	3.633	2.773	12.525
	Central Memory CD8$^+$ T-cell	0.247	9.573	7.800	51.050
	Effector Memory CD8$^+$ T-cell	0.295	14.250	20.625	31.550
	TEMRA CD8$^+$ T-cell	0.674	15.250	11.625	26.875
FEC	Total CD8$^+$ T-cell	0.306	1.830	1.196	2.925
	CMV Pentamer$^+$ CD8$^+$ T-cell	7.550	13.300	8.820	37.500
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	-	-	-
	Naïve CD8$^+$ T-cell	0.205	1.108	0.724	3.640
	Central Memory CD8$^+$ T-cell	0.239	1.605	0.698	3.735
	Effector Memory CD8$^+$ T-cell	0.304	1.005	0.888	2.130
	TEMRA CD8$^+$ T-cell	0.795	5.250	2.860	4.295
HIV-1 gag 9mer	Total CD8$^+$ T-cell	-	1.903	1.447	12.563
	CMV Pentamer$^+$ CD8$^+$ T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	24.000	0.000	19.100
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	3.450	0.000	8.620
	Naïve CD8$^+$ T-cell	-	0.739	0.572	3.320
	Central Memory CD8$^+$ T-cell	-	1.136	1.143	39.400
	Effector Memory CD8$^+$ T-cell	-	2.453	1.437	12.433
	TEMRA CD8$^+$ T-cell	-	3.320	2.297	14.147
HIV-1 gag 9mer + CpG ODN 2216	Total CD8$^+$ T-cell	-	2.433	1.510	13.733
	CMV Pentamer$^+$ CD8$^+$ T-cell	-	-	-	-
	HIV-1 gag pentamer$^+$ (1) CD8$^+$T-cell	-	14.300	0.000	21.300
	HIV-1 gag pentamer$^+$ (2) CD8$^+$T-cell	-	1.060	8.330	11.900
	Naïve CD8$^+$ T-cell	-	1.054	0.596	4.667
	Central Memory CD8$^+$ T-cell	-	1.680	1.079	26.950
	Effector Memory CD8$^+$ T-cell	-	3.493	1.500	13.633
	TEMRA CD8$^+$ T-cell	-	5.027	2.493	15.500

5.8.3. IFN-γ production

Table 5.8.5 describes the observed frequencies of IFN-γ production and as before the low numbers of individuals examined dictates the qualitative nature of analysis and the lack of statistics applied. Stimulation of HIV-1- PBMC with cytokines increased IFN-γ frequency in NK cells, but reduced the frequency in $CD8^+$ T-cells. In HIV-1+ individuals, IFN-γ frequency was reduced in both $CD8^+$ T-cells and NK cells when PBMC were stimulated with cytokines compared to unstimulated PBMC. The second positive control, PHA, also revealed reduced responses across both $CD8^+$ T-cells and NK cells in HIV-1+ individuals. This is unusual and contradicts the ELISpot data, which showed very strong IFN-γ responses to mitogen. It is possible that the overnight time point for stimulation was too long and that peak IFN-γ production had passed and therefore would not have been detected by this assay. The situation seen in the HIV-1- control individual was less clear with small increases observed in total NK cells and $CD56^{lo}CD16^+$, but decreases in $CD56^{hi}CD16^-$ NK cells or $CD8^+$ T-cells.

Stimulation with FEC increased IFN-γ frequency in $CD8^+$ T-cells, total NK cells and both NK subsets in the HIV-1- control individual tested compared to unstimulated cultures. More varied results were seen in the HIV-1+ individuals, where one out of three samples had an increased IFN-γ frequency in total $CD8^+$ T-cells and $CD56^{hi}CD16^-$ NK cells. All three individuals had a reduced IFN-γ frequency in total NK cells, and the frequency was either the same or reduced in $CD56^{lo}CD16^+$ NK cells stimulated with the FEC peptide pool.

IFN-γ responses to HIV-1 gag 9mer peptides were examined in HIV-1+ individuals (Figure 5.8.1 and Table 5.8.5). IFN-γ frequency was reduced in two out of three individuals stimulated with 9mer peptides alone compared to unstimulated cultures in $CD8^+$ T-cells, and $CD56^{lo}CD16^+$ NK cells, but was increased in two out of three total NK cell populations and in $CD56^{hi}CD16^-$ NK cells was increased in all three individuals. Co-stimulation with CpG ODN2216 increased the IFN-γ frequency in $CD8^+$ T-cells in two out of three individuals compared to cells stimulated with peptide alone. Two out three of the samples also had reduced IFN-γ frequency in total NK and both NK subsets compared to stimulation with peptide alone.

Table 5.8.5 IFN-γ responses of total $CD8^+$ T-cells, total NK cells and NK cell subsets from different individuals to the stimulations shown.

		Individual			
Stimulation	**Cell Type**	**HC**	**HIV-1+ 1**	**HIV-1+ 2**	**HIV-1+ 3**
No Stimulation	**$CD8^+$ T-cell**	0.434	0.636	0.235	0.333
	Total NK cell	0.562	0.322	0.278	2.51
	$CD56^{hi}CD16^-$ NK cell	1.2	0.726	1.57	5.38
	$CD56^{lo}CD16^+$ NK cell	0.476	0.286	0.197	0
IL-12 + IL-15	**$CD8^+$ T-cell**	0.403	0.474	0.0609	0.138
	Total NK cell	1.91	0.147	0.183	0.763
	$CD56^{hi}CD16^-$ NK cell	5.2	0.285	0.457	1.92
	$CD56^{lo}CD16^+$ NK cell	0.634	0.114	0.0927	0
PHA	**$CD8^+$ T-cell**	0.398	0.513	0.034	0.158
	Total NK cell	0.608	0.126	0.18	1.72
	$CD56^{hi}CD16^-$ NK cell	0.742	0.118	0.277	3.15
	$CD56^{lo}CD16^+$ NK cell	0.477	0.128	0.148	0
FEC	**$CD8^+$ T-cell**	0.502	0.455	0.101	0.41
	Total NK cell	0.715	0.301	0.244	2.37
	$CD56^{hi}CD16^-$ NK cell	1.66	0.447	1.78	5
	$CD56^{lo}CD16^+$ NK cell	0.588	0.286	0.117	0
HIV-1 gag 9mer	**$CD8^+$ T-cell**	-	0.412	0.174	0.381
	Total NK cell	-	0.219	0.295	4.79
	$CD56^{hi}CD16^-$ NK cell	-	1.04	1.91	7.35
	$CD56^{lo}CD16^+$ NK cell	-	0.155	0.175	2.56
HIV-1 gag 9mer + CpG ODN 2216	**$CD8^+$ T-cell**	-	0.461	0.139	0.477
	Total NK cell	-	0.214	0.208	6.09
	$CD56^{hi}CD16^-$ NK cell	-	0.911	0.959	11.1
	$CD56^{lo}CD16^+$ NK cell	-	0.143	0.145	2.86

5.9. Discussion

Chapters 3 and 4 explored innate immune responses to TLR agonists, focusing particular attention on NK cell activation and maturation. TLR agonist stimulation of DC and NK cell responsiveness affects adaptive immune responses, effectively shaping the type of response generated and the magnitude of that response. Experiments have been carried out in this chapter to quantify the innate responsiveness of various T-cell populations, specifically $CD8^+$ T-cell memory subsets, and to investigate how responsiveness is affected by chronic HIV-1 infection. From this point, further studies were carried out to assess the *in vitro* effects of a range of TLR agonists on antigen-specific T-cell responses in both HIV-1 seronegative and seropositive individuals.

Initial assays were carried out looking at the activation of $CD4^+$ T-cells, $CD8^+$ T-cell and $\gamma\delta^+$ T-cells in response to Poly(I:C) and CpG ODN2216 stimulation. These assays revealed $\gamma\delta^+$ T-cells to be the primary responsive subset activated by innate immune stimulation. It was also clear from these assays that early activation in the form of CD69 expression could easily be observed on $CD8^+$ T-cells and $\gamma\delta^+$ T-cells.

Bystander T-cell responses were also examined in HIV-1+ individuals and these assays, in agreement with NK cell data, revealed that bystander T-cell responses were refractory compared to seronegative control responses. Both treatment-naïve and treatment-experienced individuals displayed significantly reduced responsiveness, despite treatment-experienced individuals having received successful anti-retroviral therapy. Interestingly there was an exception to this trend: T-cells from treatment-naïve individuals responded at a higher frequency compared to those from seronegative control individuals. This is likely due to the ongoing viraemia and therefore chronic immune stimulation these individuals are experiencing, which was indicated by the higher background activation of T-cells, particularly $\gamma\delta^+$ T-cells, observed.

In order to better understand which T-cell memory subsets were activated by innate stimulation, activation (as measured by CD69 expression) of these subsets was examined. These assays demonstrated that, despite having a higher background level of activation, unsurprisingly naïve and TCM cells did not respond to stimulation as vigorously as the more mature TEM and TEMRA subsets. Although not necessarily reaching as high a frequency of expression as naïve and TCM cells when stimulated with Poly(I:C) or LPS,

TEM and TEMRA cells had a higher relative increase in expression. It therefore appears that TLR agonist stimulation preferentially stimulates the more mature TEM and TEMRA subsets. It is also noteworthy that Poly(I:C) was unable to significantly increase activation in naïve cells, and LPS only managed to activate these cells by a relatively small amount, which contraindicates their potential use as adjuvants.

The same responses were then compared in cells from HIV-1 seropositive individuals, and revealed that the refractory responses previously observed in total $CD8^+$ T-cells were confined to the more mature and most responsive TEM and TEMRA subsets. This is perhaps unsurprising as these cells are more mature and, as such, are likely to have been subjected to chronic stimulation and the associated fatigue. Furthermore, it is known that these cells are increased in frequency and are dysfunctional in chronic HIV-1 infection (Champagne et al., 2001). A further interesting observation was that responses in treatment-naïve individuals trended to be lower than seronegative ones, although none were individually significantly reduced.

The expression of KIR on $CD8^+$ T-cells was determined on HIV-1 seronegative and seropositive individuals. In agreement with findings by Alter *et al.* (2008), it was shown that there was a significantly higher frequency of KIR^+CD8^+ T-cells in HIV-1+ treatment-naïve individuals, but not so in treatment-experienced samples indicating recovery with treatment. The responsiveness of the KIR^+CD8^+ T-cells was then examined in response to TLR agonist stimulation and they were found to be more sensitive to stimulation, expressing higher levels of CD69 and IFN-γ than KIR^- cells. This situation was maintained across all the sample groups. No change in KIR expression in response to TLR agonist stimulation was found after overnight stimulation (data not shown).

Having established the characteristics of innate immune activation of $CD8^+$ T-cells, attention was shifted to antigen-specific immune responses and the effects of TLR agonist stimulation on them. Much lower concentrations of TLR agonist were used in this assay to better represent potential *in vivo* scenarios, and also as the primary stimulants in these assays were antigen-specific peptide pools.

Initial IFN-γ ELISpot assays were conducted using samples from HIV-1- control individuals and the FEC peptide pool as a stimulant, in order to accurately establish IFN-γ production on a per cell basis over a prolonged period. This was a small study conducted for

optimisation of conditions for studies using samples obtained from HIV-1+ individuals. This study revealed neither Poly(I:C) nor ssRNA40 co-stimulation consistently altered IFN-γ production either positively or negatively. LPS was found to have a consistent negative effect on IFN-γ production, and there was an indication that CpG ODN2216 provided some enhancement of IFN-γ production at low concentrations in three out of four samples tested. These data provided evidence that antigen-specific responses could be enhanced using some TLR agonists, but also revealed potential negative or inhibitory effects of other TLR agonists on IFN-γ production using LPS. The potential for negative effects of adjuvants emphasises the need for studies such as these with the ability to determine the functional consequences of using such systems.

Further IFN-γ ELISpot assays were carried out using samples from HIV-1 seropositive individuals to determine if there was any potential for TLR agonists to enhance antigen-specific responses in chronically infected individuals. These assays were also able to reveal negative factors that inhibited maximum IFN-γ production. For these assays HIV-1 specific peptide pools were used, specifically gag 20mer and 9mer peptides (as these had previously been used within our group and were known to elicit high frequency IFN-γ responses; data not shown) as well as the FEC peptide pool. Responses to the different peptide pools alone varied: consistent IFN-γ responses were observed against FEC peptides but there were less individual responders to both 20mer and 9mer gag peptides in treatment-naïve samples and even less responders in treatment-experienced samples.

As seen for seronegative samples co-stimulation of antigen-specific responses with Poly(I:C) did not induce any consistent changes in IFN-γ production in seropositive samples for any of the peptide pools tested. ssRNA40 co-stimulation did not noticeably affect FEC or 9mer stimulated cultures, but proved to have some modulating effects with 20mer stimulation. A small but consistent enhancement of IFN-γ production was seen in treatment-naïve samples co-stimulated with low concentrations (1ng ml^{-1}) of ssRNA40. A substantial drop in IFN-γ production was, however, observed when treatment-experienced cultures were co-stimulated with higher concentrations (100ng ml^{-1}) of ssRNA40.

The co-stimulation effects of LPS were the most consistent of responses observed, demonstrating substantially reduced IFN-γ production in the majority of samples across all combinations of peptide and LPS concentration. One exception to this was observed in cells from treatment-naïve individuals co-stimulated with HIV-1 gag 9mer peptides and low

LPS concentrations, which demonstrated increases in IFN-γ production in six out of nine samples. This finding demonstrates further the potentially damaging pathology brought about by HIV-1 infection, which has been associated with elevated bacterial endotoxin levels, which have been shown to persist despite successful treatment (Gregson et al., 2009, Brenchley et al., 2006).

Finally, co-stimulation with CpG ODN2216 provided interesting, but mixed results. The effects of CpG ODN2216 co-stimulation on PBMC from treatment-experienced individuals demonstrated even responses with no substantial or consistent changes in IFN-γ production. Co-stimulation of cells from treatment-naïve individuals with both FEC and HIV-1 gag 20mer peptides led to stable or slightly reduced IFN-γ responses, as did co-stimulation with 9mer peptides and a higher concentration of CpG ODN2216. Co-stimulation of treatment-naïve samples with 9mer peptides and the lower CpG ODN2216 concentration (1nM) revealed a consistent and substantial, but not statistically significant, increase in IFN-γ production, indicating enhancement of antigen-specific responses is possible given the right conditions.

A brief experiment was conducted to confirm the roles of different accessory cells in antigen-specific responses where IFN-γ was produced. Depletion ELISpot assays confirmed that B-cells were not involved and NK cells played a minor role in stimulating an antigen-specific IFN-γ response. Removal of $BDCA4^+$ pDC revealed the biggest defect, confirming pDC as the major accessory cell type required for the generation of IFN-γ in an antigen-specific immune response.

The final study undertaken attempted to identify which specific cell types were involved in antigen-specific immune responses and furthermore whether CpG co-stimulation was simply enhancing antigen-specific $CD8^+$ T-cell responses, or if other non-specific cells were being recruited. Flow cytometry assays utilising TCR-specific pentamer staining and degranulation staining were carried out to achieve this. It must be stressed that these assays were preliminary in nature and due to the low numbers tested statistical tests could not be applied and the data presented were qualitative in nature.

Because of the technical difficulties involved, intracellular IFN-γ staining was carried out separately to pentamer staining of cells. IFN-γ could not, therefore, be looked at directly on antigen-specific cells. Unlike ELISpot or ELISA assays, intracellular cytokine staining is

unable to determine total IFN-γ produced, and will only reveal cells that have produced IFN-γ within the final four hour time frame that brefeldin A was applied to cultures. It was therefore difficult to accurately measure total IFN-γ production in some of these assays, as it is likely that peak production may have been rapid and therefore have been missed in some of the conditions tested after an overnight stimulation. This also seems likely given the fact that a number of responses, such as those against the PHA positive control, were lower than those observed in unstimulated cultures suggesting that IFN-γ production had been down regulated. It is also likely that, due to ongoing viraemia and bacterial translocation, T-cells in HIV-1 samples were already activated and therefore unstimulated cultures were not truly unstimulated. In this assay it was also surprising that cytokine stimulation with IL-12 and IL-15 was unable to stimulate IFN-γ production in $CD8^+$ T-cells as would be expected.

Examination of the degranulation responses did provide some interesting data despite suffering a similar problem to IFN-γ staining whereby peak expression could have been missed. An increase in degranulation was observed in the mature TEM and TEMRA $CD8^+$ T-cell subsets when stimulated with FEC peptides, but the same results were not observed in cultures stimulated with HIV-1 gag 9mer peptides alone. Co-stimulation with CpG ODN2216 demonstrated improved responses over stimulation with peptide alone, not only in total $CD8^+$ T-cells, TEM and TEMRA cell, but interestingly also in the naïve cell subset, indicating recruitment of non-specific cells compared to stimulation with HIV-1 gag 9mer peptide alone.

The final measure of responsiveness examined was CD69 expression. In the HIV-1 seronegative individual tested, mostly negative responses were observed in response to all stimuli, which indicates that peak expression of CD69 might have been missed. Interestingly, however, in the HIV-1- control sample tested there was an increase in CD69 expression in the TEMRA subset, a phenomenon also observed in degranulation responses, indicating that TEMRA cells in HIV-1- individuals remain activated and functional for longer than less mature cells. Responses in HIV-1+ individuals also indicated that peak expression of CD69 had been missed, as the majority of responses in the various subsets were consistently reduced following stimulation. There was, however, one exception: CMV pentamer$^+$$CD8^+$ T-cell activation remained high regardless of the stimulation. Despite CD69 expression being lower than unstimulated cultures, CpG

ODN2216 co-stimulated cultures revealed higher CD69 frequencies than cultures stimulated with peptide alone. This indicates that, rather than simply boosting antigen-specific responses, co-stimulation might be stimulating functional responses such that they are maintained for longer.

Further assays exploring the potential of TLR agonists to disrupt or improve antigen specific T-cell responses are required and the mechanisms involved in such scenarios would need to be elucidated if such materials were to be used as adjuvants in an HIV vaccine or immunotherapeutic treatment.

5.10. Summary

This chapter has examined bystander immune responses within T-cell populations and then progressed to examine the effect of TLR-agonist stimulation on antigen-specific immune responses. $\gamma\delta^+$ T-cells were shown to be the primary innate responsive T-cells activated to high frequencies after stimulation. It was demonstrated that TLR agonists were only capable of stimulating early activation in T-cells. It was then shown that, similarly to NK cells, T-cell activation in response to TLR-agonist stimulation was refractory in chronic HIV-1 infection. Activation within different maturational $CD8^+$ T-cell subsets was then explored, and it was demonstrated that mature TEM and TEMRA cells were most sensitive to TLR agonist stimulation and that it was responses within these subsets that were refractory in HIV-1 seropositive samples.

Investigations focusing on the effects of TLR agonist co-stimulation on antigen-specific T-cell responses revealed their ability to modulate IFN-γ production. LPS was shown to consistently down regulate IFN-γ production, which may have implications for HIV-1 pathology. Enhancement of antigen-specific IFN-γ production was seen with CpG ODN2216 under certain circumstances. This provides at least some evidence of the potential to enhance responses in a manner that would be of benefit in both prophylactic vaccinations and immunotherapeutic strategies. Assays designed to identify the cell types affected by TLR agonists yielded some interesting results and demonstrated that a range of non-antigen-specific T-cells were potentially recruited. Further work is however needed to fully establish which cells are involved and the mechanisms by which they are operating.

6. Discussion

6.1. Final Discussion

The work presented in this thesis investigated innate NK cell and T-cell immunity during chronic HIV-1 infection and specifically characterised innate immune responsiveness toward TLR agonist stimulation. The potential properties of TLR agonists as model adjuvants in antigen-specific immune responses were also investigated.

NK cell and $CD8^+$ T-cell immune responses were demonstrated at relatively low concentrations of TLR agonist. These responses were largely accessory cell dependent, in contrast to other studies, which have found direct NK cell stimulation in the absence of accessory cellular support (Sivori et al., 2010, Marcenaro et al., 2008, Hart et al., 2005, Chalifour et al., 2004, Sivori et al., 2004).

Sivori *et al*. (2004) demonstrated NK cell responsiveness to both CpG ODN (type A and B) (5μg ml^{-1}) and Poly(I:C) (50μg ml^{-1}), but only in the presence of cytokines (IL-2, IL-12 or IL-8) and state that no responses were observed in the absence of cytokines. The presence of TLR3 and TLR9 mRNA in NK cells, but not protein, was also demonstrated. A further study by Sivori and colleagues demonstrated TLR9 protein in endosomal NK compartments through co-localisation assays. Furthermore trafficking of CpG molecules was demonstrated to occur via interaction with and internalisation of KIR3DL2, but again IL-12 was required for this interaction to occur (Sivori et al., 2010). Hart and colleagues demonstrated TLR3, TLR7 and TLR8 expression on NK cell lines, but not in freshly isolated NK cells. NK cell activation in response to TLR agonists (Poly(I:C) (10μg ml^{-1}) or R848 (3μg ml^{-1})) was demonstrated, but described as accessory cell dependant, as activation was inhibited by anti-IL-12 antibodies (Hart et al., 2005). Chalifour and colleagues demonstrated NK responsiveness to the TLR2 and TLR5 agonists KpOmpA (membrane protein A of *Klebsiella pneumoniae*) and flagellin, both at a range of concentrations, but only found substantial IFN-γ production when co-stimulated with cytokines (Chalifour et al., 2004). Finally, Marcenaro and colleagues demonstrated NK cell activation in response to BCG (at a 1:1 ratio of bacterial particles to NK cells) via TLR2, which they showed was expressed at low levels on the surface of NK cell. This study also demonstrated that responses were enhanced by IL-12, but were not reliant on its presence (Marcenaro et al., 2008).

These studies, however, use either high concentrations of agonists and/or co-stimulate NK cells with cytokines (Sivori et al., 2010, Marcenaro et al., 2008, Hart et al., 2005, Chalifour et al., 2004, Sivori et al., 2004). It is also noteworthy that these studies used different TLR agonists to those presented in this thesis. Assays presented here, therefore, cannot rule out functional expression of TLR on NK cells, although it is shown that TLR agonists used at the stated concentrations were not directly stimulatory. It is also likely that resting NK cells do not express functional TLR. However, it would be interesting to explore TLR expression by NK cells in the context of chronic HIV-1 viraemia as HIV-1 genomic ssRNA has been shown to stimulate TLR7 and TLR8, and these receptors have been found to be expressed in NK cell lines (Alter et al., 2007a, Hart et al., 2005).

NK cells and $CD8^+$ T-cells from HIV-1 infected individuals were shown to be refractory to TLR agonist-mediated stimulation. Particularly interesting was the finding that treatment-experienced individuals demonstrated refractory responses more frequently than did treatment-naïve individuals. It should be noted, however, that individuals in the treatment-naïve cohort were generally younger than individuals in the treatment-experienced cohort (as illustrated by patient characteristics tables throughout). Therefore, a generalised impact of ageing on immune competence in treatment-experienced individuals compared to treatment-naïve individuals studied must be considered a possibility. Additionally, although precise information regarding time since diagnosis and initiation of treatment was not available, it is possible that consistent refractory responses in treatment-experienced individuals may reflect a longer period of infection in this group, leading to greater overall burden of concomitant infection and immune exhaustion, as compared to the treatment-naïve group, who are likely to be more recently infected. Alternatively, the impact of periodic viral recrudescence, low virus copy number (<50 copies ml^{-1} of blood) and previous treatment failures cannot be ruled out in treatment-experienced individuals.

Innate responsiveness to TLR agonist stimulation within $CD56^{hi}CD16^-$ NK cells was entirely maintained and refractory responsiveness was observed only in $CD56^{lo}CD16^+$ NK cells. This is interesting data, as $CD56^{hi}CD16^-$ NK cells are known to produce cytokines involved in the generation of adaptive immune response and indicates that these cells might have an important role in potential anti-HIV-1 immunotherapeutic approaches. It should be noted, however, that the frequency of $CD56^{hi}CD16^-$ NK cells are reduced in peripheral blood from chronically infected patients (Mavilio et al., 2003). Therefore approaches seeking to utilise the cytokine-producing potential of these cells might also

need to seek to induce their proliferation and enhance their survival. Interestingly, clinical trials with ultra-low dose IL-2 in combination with SCF have demonstrated recovery of $CD56^{hi}CD16^{-}$ NK cell subsets, but also stimulate the growth of immunosuppressive regulatory T-cells, which in turn might suppress HIV-1 specific immunity (Goodier & Coleman, 2009, Shah et al., 2006).

The study of NK cell maturational subsets in Chapter 4 further investigated the ability of $CD56^{hi}CD16^{-}$ NK cells from HIV-1 infected individuals to mature into effector NK cells in the presence or absence of TLR ligands. Specifically, the variable expression of c-Kit, CD94, CD16 and CD56, used previously by other groups as markers of human NK cell maturation, provided useful information (Beziat et al., 2011, Yu et al., 2010). *In vitro* stimulation assays clearly demonstrated a shift in frequencies of the different NK cell subsets, reducing $CD56^{hi}CD16^{-}$ cells and increasing $CD56^{lo}CD16^{+}$ cells, potentially indicating a linear progression for innately stimulated NK cells. Our data did not exclude other models for NK cell maturation and we did observe, as others have an, increase in CD56 expression, especially in longer term cultures stimulated with IL-12 and IL-15, although this was not seen in cultures stimulated with TLR agonists (Loza & Perussia, 2004). It could be that this represented an alternative maturation pathway, however it seems that only robust *in vitro* cytokine stimulation, over a prolonged time period, induced this phenotype indicating that it perhaps represents a hyper-activated state, not representative of normal maturation.

Alternatively the data could demonstrate proliferation or loss of specific cell populations during *in vitro* culture as well as preferential survival. Fate mapping and proliferation assays need to be carried out in order to fully appreciate the mechanisms causing the shift in populations observed. Further work using assays such as the $CD56^{+}CD16^{-}$ NK cell reconstitution assay would also prove useful to further study how NK cells mature during HIV-1 infection.

After demonstrating that $CD56^{hi}CD16^{-}$ NK cells cultured *in vitro* matured upon stimulation with TLR agonists, maturation of NK cells cultured within whole PBMC populations from healthy and HIV-1 seropositive individuals in the presence or absence of TLR agonist stimulation, was investigated. NK cells from HIV-1 seropositive individuals that were cultured within whole PBMC in the absence of stimulation showed signs of spontaneous changes in the frequency of maturation phenotypes above that seen in healthy controls.

This suggests that chronic stimulation in HIV-1 infection is sufficient to drive NK cell changes when the cells are cultured *ex vivo,* it is likely that cell loss due to apoptosis is also a possibility here and this possibility should also be further examined. It is interesting to note that this was also significant in treatment-experienced individuals with suppressed viraemia, indicating that multiple factors are involved in driving chronic immune stimulation and that detectable HIV-1 viraemia alone is not wholly responsible. TLR agonist stimulation of PBMC from healthy individuals revealed similar phenotypic changes observed in HIV-1 seropositive individuals. This raises the possibility that TLR agonist stimulation is a driver of ongoing NK cell maturation in chronic HIV-1 infection.

The exact mechanisms driving changes have not been determined. Assays used to study human NK cell maturation directly *ex vivo* do, however, require longer periods of cell culture, which may limit the availability of the required accessory cells for TLR stimulation. The *in vitro* assays used here thus have technical limitations in this respect. Addition of exogenous factors, including cytokines, to substitute for accessory cell-dependence may overcome these limitations, but this in itself is likely to skew NK cell maturation *in vitro*, as observed after IL-12 and IL-15 stimulation.

T-cell responses to TLR agonist stimulation were transient and declined after four days of stimulation (Chapter 5). The γδ-T-cell subset was highly sensitive to innate stimulation, indicating that adjuvant studies should also monitor their activation and function, although further investigation of this was beyond the scope of this thesis. Activation in response to TLR agonist stimulation was skewed towards mature memory $CD8^+$ T-cell phenotypes in healthy control individuals. Stimulation of PBMC from HIV-1 infected individuals revealed that refractory responses were confined mostly to the mature TEM subset, consistent with skewed maturation of HIV-1 specific T-cells towards this subset demonstrated by Champagne *et al*. (2001). This indicates that HIV-1-specific $CD8^+$ effector memory T-cells are exhausted, accounting for their poor responsiveness. Again, treatment-experienced individuals show the biggest reduction compared to healthy control responses and, given that these individuals are likely to have been exposed to infection for longer than treatment-naïve individuals this further supports the notion that their TEM cells are exhausted. Detailed longitudinal studies examining changes in responsiveness of memory $CD8^+$ T-cell subsets might better define loss of function in these cells, for example, by looking at the relationship between the acquisition of inhibitory molecules (such as CTLA-4, PD-1 and TIM-3) and TLR ligand-mediated activation.

It was also shown that KIR^+CD8^+ T-cells, which are expanded in chronic HIV-1 infection and are an indicator of reduced HIV-1-specific activation, maintained innate responsiveness compared to KIR^-CD8^+ T-cells in the same individuals and expressed the activation marker CD69 at a higher frequency than KIR^- cells. This analysis did not examine responsiveness between HIV-1 infected and healthy control cohorts, but it is likely that KIR^+CD8^+ T-cells represent more mature memory subsets given the higher frequency of CD69 observed and that antigen-specific KIR^+CD8^+ T-cells have been shown to be unresponsive (Alter et al. 2008). Data presented here demonstrated that KIR^+CD8^+ T-cells responded to TLR agonist stimulation as potently as KIR^-CD8^+ T-cells in HIV-1 infected individuals (both treatment-experienced and treatment-naïve) indicating that although these cells may be unresponsive to TCR engagement, they are not exhausted (Alter et al., 2008).

Studies were undertaken with the aim of investigating the potential of TLR agonist stimulation to modulate antigen-specific $CD8^+$ T-cell responses. Interestingly LPS was shown to have a negative effect on antigen-specific IFN-γ production in healthy individuals in response to the FEC peptide pool and this trend was also seen in HIV-1 infected individuals in response to FEC and HIV-1 peptide pools. The mechanism leading to this observation is unclear, but increased LPS levels are seen in HIV-1 infection, particularly acute infection, and not only add to immunopathology but also have implications for the generation of acquired immune responses (Brenchley et al., 2006, Mehandru et al., 2004). By contrast, co-stimulation with CpG ODN2216 was shown to have a positive effect, though not statistically significant, on antigen-specific IFN-γ production, particularly in HIV-1 seropositive treatment-naïve individuals. The mechanism involved here is also unclear, but TLR9 stimulation is known to stimulate pDC to produce high levels of IFN-α and potently activates other immune cells. TLR9 and TLR7 are both expressed by pDC, TLR7, however, has been implicated in the recognition of the HIV-1 ssRNA genome and is likely to contribute to chronic stimulation and exhaustion. Therefore TLR9 agonists might prove a more attractive target as an adjuvant, as it is not involved in chronic stimulation during HIV-1 infection. The exact parameters that led to enhanced responses need to be more fully established, however, as certain combinations of CpG ODN2216 and peptide led to downregulation of IFN-γ production, and this also raises the possibility that other agonists, either those tested here or others, might also have beneficial effects when used in the appropriate combination.

An attempt was made to further characterise antigen-specific co-stimulation responses using flow cytometry assays to monitor activation and functional responses of memory $CD8^+$ T-cells and antigen specific $CD8^+$ T-cells. The results of these assays were however difficult to interpret, and it is likely that peak $CD8^+$ T-cell activation, IFN-γ production and degranulation responses occurred early following peptide stimulation and were therefore missed after overnight culture. Detailed time course studies would help to identify optimal conditions. Ideally this study would need to be extended to larger numbers of individuals, and encompassing different MHC class I haplotypes would achieve more robust data that may lead to clearer elucidation of events.

Although the responses observed in these assays did not convincingly demonstrate enhanced responses using CpG ODN2216 above those seen for negative control individuals, they were consistently higher than responses observed for cultures stimulated with peptide alone. It is possible that CpG ODN2216 stimulation might have enhanced responses by maintaining them longer than stimulation with peptide alone. A time course assay would, again, address this hypothesis.

Evidence presented in this thesis has demonstrated the importance of innate responsiveness in chronic HIV-1 infection. The principle focus of the early chapters was on NK cell activity, and it was demonstrated that although NK cells have been shown to be dysfunctional in chronic HIV-1 infection, some subsets, particularly the immature immunoregulatory $CD56^{hi}CD16^-$ NK cells, remain functional. Recent work by others has further illustrated NK cell immune-modulating abilities in chronic viral infection, still further demonstrating that immunotherapies for HIV-1 infection should seek to promote healthy NK cell activity (Waggoner et al., 2011). Furthermore, work presented in this thesis described how the use of TLR agonists used as adjuvants in model stimulations could boost antigen-specific immune responses in HIV-1 infection, demonstrating that innate immune responses should be more thoughtfully considered when designing both vaccines and immunotherapies.

6.2. Future work

Although this thesis has demonstrated much in terms of the basic functional properties associated with innate agonist stimulation of 'helper' type NK cell function, larger scale studies are warranted to determine how knowledge found here could be applied to clinical use in the future.

Parallel longitudinal studies following responsiveness of individuals to the various stimulants beginning at acute infection would greatly aid the understanding of the progression of the generation of dysfunctional responses. Such studies would reveal the kinetics of dysfunction and would provide further insight into the effects of treatment regimes to control immune disruption. A limitation in designing such studies is, however, the difficulty in identifying and recruiting individuals in the early acute phase of HIV infection.

Animal models of the effects of chronic viral infection on NK cell maturation are needed to fully explain data presented within this thesis. As HIV does not infect mice, other models of chronic viral infections such as murine CMV (MCMV) or lymphocytic choriomeningitis (LCMV) have been used to demonstrate the long-term effects of chronic infection on NK cells (Waggoner et al., 2011). Studies such as these provide very useful data, but interpretation from mouse to human, particularly within NK cell compartments, must be cautious as, despite the genetic similarities between species, many major differences still persist. Mouse models, however, provide data that can be obtained under controlled conditions that cannot be investigated in human systems.

Similar investigations to those presented in this thesis using tissue samples instead, where the dominant NK cell population is $CD56^{hi}CD16^{lo}$, would provide information regarding responsiveness of cell populations in the context of where the interactions being investigated would likely be occurring *in vivo*. Either tonsillar tissue or gut biopsy tissue samples could be obtained from clinical settings for use in such studies.

Clinical investigations or trials should be the ultimate goal of any such research in order to provide better adjuvant systems for vaccines or immunotherapeutic treatments. It is becoming ever more clear that modulation of adaptive immune responses through innate immune manipulation is capable of efficiently priming appropriate immune responses and immune memory.

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Appendices

Antibody panels used for Flow Cytometry Assays

A.1 Total NK and $CD8^+$ T-cell activation panel

Basic innate NK & $CD8^+$ T-cell activation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	FITC	B9.11, IgG_1 κ	2.5	BC - A07756
CD56	APC	N901, IgG_1 κ	5	BC - IM2474
CD69	PE	FN50, IgG_1 κ	10	BD - 555531

A.2 Plasmacytoid DC activation panel

pDC activation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
Lin-1	FITC	Various*	2.5	BD - 340546
CD123	PE	9F5, IgG_1 κ	5	BD - 555644
HLA-DR	PerCP	TÜ36, IgG_{2b} κ	2.5	BD - 555561
CD40	APC	5C3, IgG_1 κ	10	BD - 555591

*Lin-1 markers & Clones

CD3 - SK7 CD16 - 3G8 CD19 - SJ25C1 CD20 - L27	IgG_1 κ
CD14 - MϕP9 CD56 - NCAM16.2	IgG_{2b} κ

A.3 Myeloid DC activation panel

mDC activation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
Lin-1	FITC	Various*	2.5	BD - 340546
CD11c	PE	B-ly6, IgG_1 κ	5	BD - 555644
HLA-DR	PerCP	TÜ36, IgG_{2b} κ	2.5	BD - 555561
CD40	APC	5C3, IgG_1 κ	10	BD - 555591

A.4 NK cell subset activation panel

NK subset panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	SK1, IgG_1 κ	1.25	BD - 560179
CD56	APC	N901, IgG_1 κ	5	BC - IM2474
CD16	FITC	LNK16, IgG_1 κ	10	MCA1193F
CD69	PE	FN50, IgG_1 κ	10	BD - 555531
CD25	PE-Cy7	M-A251, IgG_1 κ	2.5	BD - 557741

A.5 NK cell functional activity and KIR panel

NK functional activation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	SK1, IgG_1 κ	1.25	BD - 560179
CD56	APC	N901, IgG_1 κ	5	BC - IM2474
KIR:				
CD158a		143211, IgG_1 κ	5	R&D FAB1844F
CD158b	FITC	CH-L, IgG_{2b} κ	10	BD – 559784
NKB1		DX9, IgG_1 κ	10	BD - 555960
IFN-γ	PE-Cy7	4S.B3 IgG_1 κ	1:80 ➔ 20	BD - 557844

A.6 NK cell maturation panel

NK cell maturation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD56	APC	N901, IgG_1 κ	5	BC - IM2474
CD16	APC-H7	3G8, IgG_1 κ	1	BD - 560195
CD94	FITC	HP-3D9, IgG_1 κ	10	BD - 555888
c-Kit(CD117)	PE	104D2, IgG_1 κ	10	BD - 332785
CD25	PE-Cy7	M-A251, IgG_1 κ	2.5	BD - 557741

A.7 Innate T-cell activation panel

Innate T-cell activation

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	SK1, IgG_1 κ	1.25	BD - 560179
CD4	PE	SK3, IgG_1 κ	2.5	BD - 345769
γδ TCR	FITC	B1, IgG_1 κ	1	BD - 559878
CD69	PE-Cy7	FN50, IgG_1 κ	2.5	BD - 557745

A.8 $CD8^+$ T-cell maturation and activation panel

$CD8^+$ T-cell maturation panel

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	SK1, IgG_1 κ	1.25	BD - 560179
CD45RA	PE-Cy7	L48, IgG_1 κ	2.5	BD - 337186
CCR7	APC	150503, IgG_{2a} κ	5	R&D - FAB197A
CD62L	FITC	DREG56, IgG_1 κ	10	BD - 555543
CD69	PE	FN50, IgG_1 κ	10	BD - 555531

A.9 $CD8^+$ T-cell/NK cell antigen specific activation panel

Peptide-TLR activation

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	SK1, IgG_1 κ	1.25	BD - 560179
CD69	PE-Cy7	FN50, IgG_1 κ	2.5	BD - 557745
CD56	APC	N901, IgG_1 κ	5	BC - IM2474
CD16	APC-H7	3G8, IgG_1 κ	1	BD - 560195
IFN-γ	PE	4S.B3 IgG_1 κ	10	BD - 559326

A.10 Antigen specific $CD8^+$ T-cell activation panel

Pentamer stain

Marker	Fluorochrome	Clone & Isotype	Volume per test (µl)	Manufacturer & Cat. No.
CD3	PerCP	SK7, IgG_1 κ	2.5	BD - 345766
CD8	APC-H7	LT8, IgG_1 κ	1	ProImmune A003-3B-G
CD69	PE-Cy7	FN50, IgG_1 κ	2.5	BD - 557745
CD45RA	APC-Cy7	H100 IgG_{2b} κ	2.5	BD - 560674
CCR7	APC	150503, IgG_{2a} κ	5	R&D - FAB197A
CD107a	Pacific Blue	H4A3, IgG_1 κ	1:50 (RPMI)➔20µl/ml	Biolegend - 328624
Pentamer	PE	MHC specific*	10	ProImmune (various*)

*Pentamer	Sequence	ProImmune cat. No.
Negative	-	FN01-2A-G
CMV	NLVPMVATV	F008-2A-G
HIV-1 gag (1)	SLYNTVATL	F010-2A-G
HIV-1 gag (2)	TLNAWVKVV	F174-2A-G

Peptide pool sequences

A.11 FEC peptide pool

CFAR FEC Peptides

Peptide	Sequence	Antigen
FEC1	GILGFVFTL	Flu M
FEC2	FMYSDFHFI	Flu
FEC3	SIIPSGPLK	Flu M
FEC4	RVLSFIKGTK	Flu NP
FEC5	KTGGPIYKR	Flu NP
FEC6	LPFDKTTVM	Flu NP
FEC7	ELRSRYWAI	Flu NP
FEC8	SRYWAIRTE	Flu NP
FEC9	ASCMGLIY	Flu M
FEC10	CLGGLLTMV	EBV
FEC11	GLCTLVAML	EBV
FEC12	AVFDRKSDAK	EBV
FEC14	DYCNVLNKEF	EBV
FEC15	RAKFKQLL	EBV
FEC16	FLRGRAYGL	EBV
FEC17	QAKWRLQTL	EBV
FEC18	RRIYDLIEL	EBV
FEC19	YPLHEQHGM	EBV
FEC20	NLVPMVATV	CMV
FEC21	TPRVTGGGAM	CMV
FEC22	SDEEEAIVAYTL	CMV
FEC23	IPSINVHHY	CMV
FEC24	VSDGGPNLY	Flu
FEC25	CTELKLSDY	Flu
FEC26	ILRGSVAHK	Flu
FEC27	RVRAYTYSK	EBV
FEC28	RLRAEAQVK	EBV
FEC29	IVTDFSVIK	EBV
FEC30	ATIGTAMYK	EBV
FEC31	RPPIFIRRL	EBV
FEC32	EENLLDFVRF	EBV
FEC33	EFFWDANDIY	CMV

A.12 HIV-1 gag p24 20mer peptide pool

CFAR HIV-1 gag p24 20mer peptides

Peptide	Sequence
ARP788.1	PIVQNLQGQMVHQAISPRTL
ARP788.2	VHQAISPRTLNAWVKVVEEK
ARP788.3	NAWVKVVEEKAFSPEVIPMF
ARP788.4	AFSPEVIPMFSALSEGATPQ
ARP788.5	SALSEGATPQDLNTMLNTVG
ARP788.6	DLNTMLNTVGGHQAAMQMLK
ARP788.7	GHQAAMQMLKETINEEAAEW
ARP788.8	ETINEEAAEWDRVHPVHAGP
ARP788.9	DRVHPVHAGPIAPGQMREPR
ARP788.10	IAPGQMREPRGSDIAGTTST
ARP788.11	GSDIAGTTSTLQEQIGWMTN
ARP788.12	LQEQIGWMTNNPPIPVGEIY
ARP788.13	NPPIPVGEIYKRWIILGLNK
ARP788.14	KRWIILGLNKIVRMYSPTSI
ARP788.15	IVRMYSPTSILDIRQGPKEP
ARP788.16	LDIRQGPKEPFRDYVDRFYK
ARP788.17	FRDYVDRFYKTLRAEQASQD
ARP788.18	TLRAEQASQDVKNWMTETLL
ARP788.19	VKNWMTETLLVQNANPDCKT
ARP788.20	VQNANPDCKTILKALGPAAT
ARP788.21	ILKALGPAATLEEMMTACQG
ARP788.22	LEEMMTACQGVGGPGHKARV

A.13 HIV-1 gag 9mer peptide pool

CFAR HIV-1 gag 9mer peptides

Peptide	Sequence	Epitope
EVA7083.1	SPRTLNAWV	p24 148-156
EVA7083.2	TPQDLNTML	p24 180-188
EVA7083.3	AEWDRLHPV	p24 210-218
EVA7083.4	ILGLNKIVR	p24 267-275
EVA7083.5	RMYSPTSIL	p24 275-283
EVA7083.6	ILDIKQGPK	p24 282-290

Sigma-Genosys (Sigma-Aldrich) HIV-1 gag 9mer peptides

Peptide	Sequence	Epitope
DR9	DRWEEKIRLR	p17 14-22
RK9	RLRPGGKKK	p17 20-28
GL9	GGKKKKYKL	p17 24-31
KI9	KKKYKLKHI	p17 26-34
KW9	KYKLKHIVW	p17 28-36
WF9	WASRELERF	p17 36-44
GY9	GSEELRSLY	p17 71-79
EV9	ELRSLYNTV	p17 74-82
SL9	SLYNTVATL	p17 77-85
TI9	TLYCVHQRI	p17 84-92
IL10	IEIKDTKEAL	p17 92-101
EL9	EIKDTKEAL	p17 93-101
QW11	QAISPRTLNAW	p24 13-23
IW9	ISPRTLNAW	p24 15-23
SW8	SPRTLNAW	p24 16-24
KF11	KAFSPEVIPMF	p24 30-40
FF9	FSPEVIPMF	p24 32-40
EL9	EVIPMFSAL	p24 35-43
SL9	SEGATPQDL	p24 44-52
GL9	GHQAAMQML	p24 61-69
EW10	ETINEEAAEW	p24 71-80
VA10	VHPVHAGPIA	p24 83-92
HA9	HPVHAGPIA	p24 84-92
TW10	TSTLQEQIGW	p24 108-117
TN10	TSTLQEQIGN	p24 108-117
PY9	PPIPVGDIY	p24 122-130
GI9	GEIYKRWII	p24 127-135
EL9	EIYKRWIIL	p24 128-136
KK10	KRWIILGLNK	p24 131-140
AW11	AEQASQVDKNW	p24 174-184
QW9	QASQVDKNW	p24 176-184
DL9	DCKTILKAL	p24 197-205
AK11	ACQGVGGPGHK	p24 217-227
VV9	VLAEAMSQV	p24 230-231 / p2 1-7
YL9	YPLTSLRSL	p6 36-44
RI9	RQANFLGKI	p7 52-55 / p1 1-5

Chapter 3 Items

A.14

Comparison of early activation (CD69 frequency) responses between HIV-1 seronegative and seropositive, both treatment-naïve and experienced, individuals using optimal TLR agonist concentrations shown. Comparisons between unstimulated controls and stimulated PBMC are also shown for each subject group (Wilcoxons matched pairs test was used for this analysis) *denotes statistically significant differences.

		NK Cells				$CD8^+$ Cells			
TLR [Concentration]	Sample Goup	Median	Range	Vs Unstimulated (p-value)	Vs Healthy Control (p-value)	Median	Range	Vs Unstimulated (p-value)	Vs Healthy Control (p-value)
Unstimulated	HC	5.58	1.17 - 28.65	-	-	7.67	1.75 - 27.7	-	-
	TN	5.83	4.48 - 8.69	-	0.6734	17.1	9.89 - 29	-	0.01*
	TD	7.8	1.85 - 15.2	-	0.6421	17.7	25.1 - 28.5	-	0.2539
Pam3CSK [1µg ml^{-1}]	HC	10.3	3.53 - 16.8	0.0078*	-	6.73	2.63 - 37.9	0.0117*	-
	TN	11.55	7.18 - 15.4	0.0313*	0.3884	16.7	5 - 42.1	0.8438	0.0675
	TD	6.84	3.47 - 23.1	0.0313*	0.8639	8.54	2.49 - 41.2	0.5625	0.9546
HKLM [1×10^8 cells ml^{-1}]	HC	40.1	6.17 - 86.1	0.0005*	-	12.9	2.85 - 43.4	0.0005*	-
	TN	53.25	28.9 - 87.7	0.0313*	0.1223	22.95	13.8 - 41.9	0.0625	0.0441*
	TD	23.6	8.3 - 61.7	0.0156*	0.5828	16	3.84 - 43.6	0.375	0.6119
Poly (I:C) [5µg ml^{-1}]	HC	78.35	47.1 - 96.1	0.0005*	-	28.95	6.87 - 62.8	0.0005*	-
	TN	73.65	24 - 91.7	0.0313*	0.7431	41.4	28 - 43.2	0.0313*	0.2061
	TD	45.9	13.7 - 93.4	0.0156*	0.0251*	30.7	5.67 - 55.2	0.0156*	0.9663
LPS [100ng ml^{-1}]	HC	32.8	16.6 - 92.7	0.0005*	-	11.5	3.62 - 30.7	0.0005*	-
	TN	45.7	32.6 - 69.4	0.0313*	0.1112	20.15	12.5 - 33	0.0313*	0.0245*
	TD	40	11 - 67.6	0.0156*	0.8991	15.7	4.85 - 38.4	0.0781	0.3103
Flagellin [1µg ml^{-1}]	HC	13.75	1.95 - 48.3	0.0068*	-	6.73	3.11 - 36.8	0.0771	-
	TN	18.75	14.2 - 22.3	0.0313*	0.2417	16.6	6.74 -27.4	0.6875	0.0441*
	TD	17.7	7.27 - 47.2	0.0156*	0.6421	14.1	2.86 - 34	1	0.7037
FSL-1 [100ng ml^{-1}]	HC	9.02	3.49 - 16.3	0.0742	-	4.22	2.26 - 33.8	0.0977	-
	TN	12.6	9.45 - 23.1	0.0313*	0.036*	19.2	9.44 - 33.3	0.0938	0.0176*
	TD	8.665	2.89 - 27.1	0.0313*	0.7234	10.6	2.33 - 25.7	0.4375	0.4559
Imiquimod R837 [2.5µg ml^{-1}]	HC	52.7	4.24 - 69	0.0195*	-	20.4	1.73 - 55.7	0.0195*	-
	TN	60.3	47.6 - 93.3	0.0313*	0.3277	32.15	22.3 - 43	0.0313*	0.2238
	TD	32.25	13.6 - 64.1	0.0313*	0.8639	14.91	3.14 - 38	0.0625	0.8639
ssRNA40 [5µg ml^{-1}]	HC	88.35	69.5 - 98.9	0.0078*	-	17.75	1.46 - 55.6	0.0024*	-
	TN	82.7	41.3 - 97.2	0.0625	0.6216	33.75	15.5 - 50.2	0.0625	0.1223
	TD	58.4	35.6 - 92.4	0.0156*	0.0093*	29.6	4.2 - 43.6	0.0156*	0.5262
CpG ODN2216 [2.5µM]	HC	66.5	49.2 - 82	<0.0001*	-	28.95	16.8 - 58.8	0.0001*	-
	TN	67.25	52.5 - 87.3	0.0022*	0.9626	37.9	29.3 - 41.5	0.0022*	0.1112
	TD	35.6	25.6 - 77.3	0.0006*	0.0088*	29.8	7.04 - 43.5	0.0728	0.9663

Chapter 4 Items

A.15

Median frequencies and ranges (%) of CD56hiC-Kit+ and CD56loC-Kit- NK cells from total CD3-CD56+ NK cell populations following six days in vitro culture of PBMC with the cytokines or TLR agonists (as shown).

	IL-12 + IL-15		LPS		ssRNA40	
	$CD56^{hi}c\text{-}Kit^{+}$	**$CD56^{lo}c\text{-}Kit^{-}$**	**$CD56^{hi}c\text{-}Kit^{+}$**	**$CD56^{lo}c\text{-}Kit^{-}$**	**$CD56^{hi}c\text{-}Kit^{+}$**	**$CD56^{lo}c\text{-}Kit^{-}$**
HIV-1- control	4.61 (0.66-19.40)	28.20 (4.07-45.60)	3.91 (1.06-13.10)	82.60 (52.70-89.70)	4.82 (0.88-14.90)	78.00 (46.20-96.60)
treatment-naïve HIV-1+	1.81 (0.20-6.50)	37.60 (19.80-52.20)	3.71 (0.41-5.61)	89.50 (80.30-97.50)	4.79 (0.59-17.70)	83.10 (56.70-95.40)
treatment-experienced HIV-1+	0.35 (0.20-7.87)	86.40 (38.60-95.40)	1.37 (0.33-10.60)	94.10 (78.80-98.10)	2.10 (0.67-6.31)	90.20 (78.60-95.90)

A.16

Median frequencies and ranges (%) of CD56hiC94+, CD56loC94+ and CD56loC94- NK cells from total CD3-CD56+ NK cell populations following six days in vitro culture of PBMC with the cytokines or TLR agonists (as shown).

	IL-12 + IL-15			LPS			ssRNA40		
	$CD56^{hi}CD94^{+}$	**$CD56^{lo}CD94^{+}$**	**$CD56^{lo}CD94^{-}$**	**$CD56^{hi}CD94^{+}$**	**$CD56^{lo}CD94^{+}$**	**$CD56^{lo}CD94^{-}$**	**$CD56^{hi}CD94^{+}$**	**$CD56^{lo}CD94^{+}$**	**$CD56^{lo}CD94^{-}$**
HIV-1- control	71.00 (60.80-94.80)	22.80 (3.62-29.50)	3.30 (0.53-8.84)	14.15 (5.29-40.30)	54.05 (29.90-65.10)	25.75 (17.20-40.50)	12.05 (3.33-47.40)	46.20 (19.00-72.30)	24.25 (6.37-68.80)
treatment-naïve HIV-1+	52.10 (30.60-72.00)	24.60 (13.30-45.80)	7.02 (0.36-18.90)	5.75 (1.57-9.32)	47.70 (34.80-93.10)	49.40 (2.41-56.90)	11.80 (3.20-27.30)	43.10 (38.30-91.20)	28.50 (3.38-51.40)
treatment-experienced HIV-1+	10.32 (3.76-45.40)	28.95 (12.10-70.70)	40.45 (17.50-78.70)	3.38 (0.36-12.3)	38.10 (15.30-57.80)	58.10 (38.80-83.00)	9.82 (2.97-14.30)	36.30 (13.40-61.30)	44.90 (31.80-82.70)

A.17

Median (and range) quantities of C-Kit and stem cell factor (SCF) detected in plasma from HIV-1- control individuals and HIV-1+, both treatment-naïve and experienced individuals. u/d – undetectable.

	c-Kit (pg ml^{-1})	SCF (pg ml^{-1})
HIV-1 seronegative (n=29)	44184 (19756-69434)	362 (u/d-1454)
HIV-1 seropositive treatment-naïve (n=29)	35188 (8812-66145)	194 (u/d-1880)
HIV-1 seropositive treatment-experienced (n=32)	29773 (21283-65752)	245 (u/d-1543)