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Abstract

HIV-specific interleukin-10 responses and immune modulation

Genevieve Tyndale Clutton, Merton College, Hilary Term 2012

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Interleukin-10 (IL-10) helps to limit the duration of potentially harmful inflammatory responses but has also been implicated in the persistence of a number of chronic viral infections. This thesis aimed to investigate the phenotype and function of HIV-specific IL-10-producing cells in chronic HIV-1 infection, and the effect of IL-10 blockade on responses to candidate HIV-1 vaccines.

A cytokine capture assay was used to determine the HIV-specific cellular sources of IL-10 in PBMC from 55 chronically infected individuals. A rare subset of CD8+ T cells was found to be the major HIV-1 Gag-specific IL-10-producing population; these cells were restricted to ART-naïve individuals and did not express the regulatory T cell markers CD25 or FoxP3 but could co-express IFN-γ. A proportion of the population (median 48% and 9% respectively) expressed the β7 chain of the gut-homing integrin α4β7 and the chemokine receptor CXCR3, which mediates lymphocyte migration to sites of inflammation.

Experimental depletion of Gag-specific IL-10+ CD8+ T cells did not affect T cell activation, or the production of cytokines such as IL-2 or IFN-γ during short-term culture. However, depletion was associated with a significant increase in CD38 expression on CD14+ monocytes, a trend towards increased HLA-DR expression on the same cells, and a significant increase in the concentration of the pro-inflammatory cytokine IL-6 in culture supernatants. There was also a significant increase in the number of HIV-infected (p24 antigen+) CD4+ T cells in cultures depleted of Gag-specific IL-10+ CD8+ T cells after 3 days, indicating that this population may contribute to control of viral replication.

In order to determine the effect of IL-10 blockade on vaccine immunogenicity, IL-10R blocking antibody was administered to BALB/c mice prior to immunisation with two HIV-1 candidate vaccines, HIVA and HIVconsv. IL-10R blockade resulted in a trend towards increased IFN-γ production by CD8+ T cells in response to the dominant H (Env) and P (Pol) epitopes of HIVA, and a significant increase in IFN-γ ELISPOT responses to the subdominant G1 (Gag) epitope of HIVconsv *in vitro*.

Collectively, these data suggest that IL-10-producing cell populations may play critical but different roles in chronic infection and vaccination. Further research into how the timing of IL-10 responses affects disease outcome may allow IL-10 blockade to be explored as a therapeutic strategy in humans.

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A unique HIV-specific IL-10 producing CD8+ T cell subset modulates pro-inflammatory immune responses and viral replication during chronic infection.
Submitted, under revision.

H Yang, H Wu, G Hancock, **G Clutton**, N Sande, X Xu, H Yan, X Huang, B Angus, K Kuldanek, S Fidler, T Denny, J Birks, A McMichael & L Dorrell.
The antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ cell decline in HIV-1 infection.
Journal of Infectious Diseases, in press.

List of abbreviations

aa	Amino acid
Ab	Antibody
Ad5	Adenovirus 5
ADCC	Antibody-dependent cellular cytotoxicity
AEC	3-Amino-9-ethylcarbazole
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	Allophycocyanin
APOBEC	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like
ART	Antiretroviral therapy
bcl-2	B cell lymphoma 2
BNAb	Broadly neutralizing antibody
BSA	Bovine serum albumin
C	Constant chain
CCR5	Cysteine-cysteine linked chemokine receptor 5
CD	Cluster of differentiation
CDR	Complementarity-determining regions
CFSE	Carboxyfluorescein succinimidyl ester
CLIP	Class II associated invariant-chain peptide
CMTMR	(5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine
CMV	Cytomegalovirus
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CXCR3	Cysteine-x-cysteine linked chemokines receptor 3
CXCR4	Cysteine-x-cysteine linked chemokines receptor 4
D	Diversity chain
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DNTPs	Deoxynucleotide triphosphates
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
ELISPOT	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FcγR	Fragment crystallisable region receptor gamma
FEC	Influenza virus, Epstein Bar Virus, Cytomegalovirus optimal epitopes
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate, green

FoxP3	Forkhead box p3
Gag	Group antigen
GALT	Gut associated lymphoid tissue
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HCL	Hydrochloride
HCV	Hepatitis C Virus
HESN	HIV-1 exposed seronegative
HIV-1	Human immunodeficiency syndrome type 1
HIV-2	Human immunodeficiency syndrome type 2
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
ICS	Intracellular cytokine staining
IDU	Intravenous drug user
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IL	Interleukin
IL-10	Interleukin-10
IL-10R	Interleukin-10 receptor
IRF	Interferon regulatory factor
iNKR	Inhibitory natural killer cell receptor
ITAM	Immunoreceptor tyrosine-based activation motif
J	Joining chain
Jak	Janus kinase
KIR	Killer cell immunoglobulin like receptor
LCMV	Lymphocytic Choriomeningitis Virus
LEAF	Low endotoxin azide free
LPS	Lipopolysaccharide
LTNP	Long term non-progressor
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAPK	Mitogen-activated protein kinase
MDDC	Monocyte-derived dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-alpha
MIP-β	Macrophage inflammatory protein–beta
M-CSF	Macrophage colony-stimulating factor
µl	Microlitre
ml	Millilitre
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
NAb	Neutralising antibody
ND	No data
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa B

NHP	Non-human primate
NIAID	National Institute for Allergy and Infectious Diseases
NIH	National Institutes for Health
NK	Natural killer cell
NO	Nitric oxide
NP-40	Nonidet P-40
OXREC	The Oxford Research Ethics Committee
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death
PDL-1	Programmed death ligand
PE	Phycoerythrin
PFU	Plaque forming units
PHA	*Phaseolus vulgaris* (red kidney bean) Phytohemagglutinin
PI	Phosphatidylinositol
PMA	Phorbol myristate acetate
pMHC	Peptide-major histocompatibility complex
RANTES	Regulated upon activation of normal T cells, expressed and secreted
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RT	Reverse transcriptase
SC	Stem cell
SEB	Staphylococcus enterotoxin B
SFU	Spot forming units
SFU/10^6	Spot forming units per million cells
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
Tat	Transactivating protein
TCM	Central memory T cell
TCR	T cell receptor
TEM	Effector memory T cell
TFH	T follicular helper cell
Th	T helper cell
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
TRIM5α	Tripartite motif-containing protein 5
Tyk	Leukocyte receptor tyrosine kinase
V	Variable chain
Vif	Virion infectivity factor
Vpu	Viral protein U

Chapter 1: Introduction

Despite its identification more than 25 years ago as the etiological agent responsible for acquired immunodeficiency syndrome (AIDS) (1, 2), the global epidemic caused by human immunodeficiency virus type 1 (HIV-1) continues unabated. Today, approximately 34 million people, predominantly in sub-Saharan Africa, are infected with HIV-1, and 1.8 million people died of AIDS-related illnesses in 2010 alone (UNAIDS report 2010). The advent of anti-retroviral therapy (ART) has transformed HIV-1 infection from a fatal illness to a chronic condition in wealthier countries; however, attempts to develop a vaccine or cure have been largely unsuccessful (3-5). One of the key challenges to surmount is the ability of HIV-1 to evade and subvert host immune responses in order to establish a persistent infection, but the mechanisms by which this is achieved are still incompletely understood. This thesis will examine the potential role of the immunomodulatory cytokine interleukin-10 (IL-10) in HIV-1 pathogenesis and control.

1.1 HIV-1 pathogenesis

Acquisition and establishment of infection

HIV-1 is most commonly transmitted by either homosexual or heterosexual intercourse (6). It is unclear whether transmission is mediated by free virions or cell-associated virus; studies of simian immunodeficiency virus (SIV) infection in rhesus macaques initially suggested that the intact vaginal mucosa was penetrable by free but not cell-associated virus (7) but more recently it has been demonstrated that cell-associated SIV could also penetrate the vaginal mucosa (8). *In vitro* experiments using human cervical

explants suggested that cell-associated and free virus had comparable rates of transmission (9). It is therefore probable that HIV-1 can enter the human mucosal tissue in either form. The passage of virus across the mucosal epithelium may involve transcytosis through epithelial cells (10, 11), direct penetration, or the infection of Langerhans cells, which may be the primary cellular targets of HIV-1 (12, 13). However, the intact mucosa presents a formidable barrier, and despite large numbers of circulating viruses in the quasispecies within the infected host, 80% of heterosexually transmitted HIV-1 infections are caused by a single virion, or "founder virus" (14, 15). In contrast, where HIV-1 is acquired by a non-mucosal route, for example through intravenous drug injection, productive infection can be established by ten or more separate viral strains (16).

Once HIV-1 has crossed the mucosal barrier, it can enter CD4+ T cells in the immediate vicinity via binding of the viral envelope glycoprotein gp120 to CD4 and one of two co-receptors, either CXCR4 (in the case of X4-tropic viruses) or CCR5 (for R5-tropic viruses). Dendritic cells (DCs) and macrophages that express CD4 and CXCR4 and/or CCR5 can also be infected. Most sexually transmitted virus utilizes the CCR5, rather than the CXCR4 co-receptor, and individuals homozygous for a 32 base pair deletion in CCR5 that results in a loss of expression of the receptor at the cell surface (known as CCR5 Δ32) are highly resistant to HIV-1 infection (17-19). The binding of HIV-1 to CD4 and one or other co-receptor triggers a conformational change in another viral envelope protein, gp41, leading to fusion with and penetration of the host cell membrane (20). Initial entry into mucosal CD4+ T cells is followed by an "eclipse phase" of one to three weeks, where HIV-1 replicates in the local mucosal tissue but is not readily detected in the peripheral blood (21, 22). At this stage, the

virus appears to preferentially infect resting CD4+ T cells that express the integrin α4β7 and low levels of CCR5 (23-26). Some of these cells may revert to a resting state after HIV-1 DNA integration, forming a long-lived latent viral reservoir that is extremely resistant to killing either by host immune responses or ART (27, 28). Once replication reaches a "threshold" level, the virus enters draining lymph nodes and the gut-associated lymphoid tissue (GALT), where the plentiful supply of CD4+ T cells allows rapid viral replication and dissemination around the body (29-31).

Acute HIV-1 infection

Once HIV-1 has succeeded in entering the lymphoid tissue, it is able to replicate rapidly due to the presence of a large population of activated CCR5+ CD4+ T cells in this tissue. As infection progresses, the gut mucosal barrier is damaged, compromising its integrity and allowing leakage of gut microbial products into the bloodstream (32-34). Indeed, studies in macaques and humans have demonstrated that up to 80% of gut CD4+ T cells may be lost during the first three weeks of HIV-1 infection (30, 35), due to both direct cytopathy of infected CD4+ T cells and bystander activation-induced apoptosis of uninfected cells. Plasma viral RNA, which is detectable around 10 days after transmission, peaks at 10^7-10^8 copies/ml during acute infection (21, 22).

Chronic infection and AIDS

After peaking around 20-30 days after transmission, plasma viral load declines by 10-200 fold to a relatively steady state, or "set point". This decline occurs in parallel with the emergence of HIV-specific CD8+ T cells (36, 37). There is also a partial recovery in the number of CD4+ T cells, although not to pre-infection levels (38). Following the establishment of the set point, there is a period of clinical latency where viral load

remains relatively stable due to a dynamic equilibrium between viral replication and turnover and anti-viral immune responses. This period of persistent infection varies in length between individuals, but generally lasts between two and fifteen years (39). Host genetic factors play a role in determining the length of the latent phase, and a lower set point after acute infection has also been shown to correlate with slower disease progression (39-42). A very small percentage of HIV-infected individuals are able to maintain a low or undetectable viral load in the absence of ART for many years. However, in the majority of untreated patients, CD4+ T cell number gradually declines during the latent phase until a threshold is reached where the patient becomes susceptible to a multitude of opportunistic infections, including bacterial pneumonia, tuberculosis, and rare cancers such as Karposi's sarcoma. Clinically, HIV-1+ patients are considered to have progressed to AIDS when they have a CD4+ count of < 200/μl and/or have contracted an AIDS-defining illness such as those listed above (US Centres for Disease Control Classification System for HIV-infected adults and adolescents).

1.2 The immune response to HIV-1 infection

1.2.1 Innate immune responses

After infection with HIV-1, the host immune system rapidly initiates attempts to eliminate or control the virus. This early response is mediated primarily by the various branches of the innate immune system.

Soluble factors

One of the earliest responses to viral infection is the production of type I interferons (IFNs), which are subdivided in humans into IFN-α (which consists of 13 related

subtypes), IFN-β and IFN-ω (43). These proteins can be secreted by almost any cell in the body in response to the binding of microbial components such as nucleic acids or bacterial polysaccharides to transmembrane, or more commonly cytosolic receptors such as retinoic acid inducible gene I (RIG-I). However, the major producers of type I IFNs are plasmacytoid dendritic cells (pDCs), as these cells constitutively express the intracellular receptors TLR7 and TLR9, allowing them to respond to viral ssRNA and ssDNA respectively (44). Type I IFNs have a number of antiviral functions, including the enhancement of antibody-dependent cellular cytotoxicity (ADCC) by macrophages, the activation of natural killer (NK) cells and the upregulation of IL-12R on CD4+ T cells, which enhances their differentiation into IFN-γ-producing Th1 cells (45-47). Type I IFNs are rapidly produced by pDCs in response to HIV-1 infection (48), but the virus is able to prevent IFN induction in other cell types, such as macrophages and T cells, by inhibiting the activity of the transcription factor interferon regulatory factor 3 (IFR-3) (49, 50). Furthermore, type I IFN production by pDCs may drive T cell activation in HIV-infected individuals, a phenomenon that has been associated with accelerated disease progression (51).

In addition to type I IFNs, other cytokines and chemokines are secreted during the early acute phase of HIV-1 infection. Using plasma samples collected from acutely infected individuals, Stacey *et al* demonstrated that IFN-α, IL-5 and TNF-α levels were elevated within six days of the detection of viraemia, followed by IFN-γ and IL-6 and then by IL-12 (52). The induction of the anti-inflammatory cytokine IL-10 was delayed compared to that of pro-inflammatory cytokines such as IFN-α and TNF-α but occurred prior to upregulation of IL-12. Similar studies have been conducted in non-human primate (NHP) models, which have the advantage of allowing a more precise

determination of the timing of cytokine induction following infection. In rhesus macaques, elevations in IFN-α and IFN-β are detectable in the endocervical epithelium within 24 hours of mucosal challenge with SIV (53). TNF-α and IL-6 mRNA increases are detected in the vaginal mucosa three to five days post infection, whilst in the lymph nodes IL-12 and IFN-γ mRNA is detectable by days three to five and IL-10 protein by day seven (54, 55). Plasma levels of IL-15 protein peak at day ten after intravenous challenge (56). The cellular sources of cytokines during acute HIV infection are yet to be elucidated, but in acute SIV infection include epithelial cells and pDCs (53). Following viral spread to the draining lymph nodes, T cells, monocytes and pDCs all contribute to the cytokine cascade (54, 55).

MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) are amongst the earliest chemokines to be induced as a result of HIV/SIV infection (53). These chemokines, which can be produced by monocytes, DCs and natural killer (NK) cells as well as T cells, are capable of binding to CCR5 and therefore compete with HIV-1 for access to this receptor, which is critical for the entry of R5-tropic HIV-1 virions into host cells (57). However, they are unable to block cellular entry of X4-tropic viruses that use CXCR4 rather than CCR5 as a co-receptor.

It has recently been suggested that the cytokine expression profile during acute HIV/SIV infection plays a role in determining the viral set point and subsequent progression of disease (58, 59). Higher plasma levels of IL-12 and IFN-γ during acute HIV infection have been associated with lower viral load twelve months post infection, whilst higher IL-7 and IL-15 plasma levels are associated with higher viral load (59). Furthermore, subcutaneous treatment with IL-15 during acute SIV infection increased

viral set point by 1000-fold in rhesus macaques, whilst administration of IL-12 lowered the set point by 100-fold (60, 61). CD4+ T cells that secrete higher amounts of MIP-1α, MIP-1β and RANTES exhibit a greater degree of resistance to HIV-1 infection *in vitro* compared to cells producing lower levels of these chemokines (62), and polymorphisms in MIP-1α, MIP-1β and RANTES that increase their expression have been associated with delayed progression to AIDS (63, 64). The manipulation of early cytokine and chemokine responses therefore represents a potential therapeutic strategy to improve the subsequent course of disease.

Intrinsic cellular factors

In addition to cytokines and chemokines, the immune system has evolved a collection of restriction factors that can inhibit retroviral replication by disrupting the life cycle at various stages. APOBEC3G (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) is an mRNA-editing enzyme that induces extensive mutation of nascent HIV-1 cDNA during reverse transcription, thereby inhibiting elongation of viral reverse transcripts, whilst tetherin, a membrane-bound protein, blocks HIV-1 virion release from infected cells (65-67). The potent antiviral activity of these factors, along with the observation that APOBEC3G is expressed at elevated levels in the PBMC of HIV-1 exposed seronegative (HESN) individuals, has prompted suggestions that they could be utilised in therapeutic interventions against HIV/AIDS (65, 67).

A further restriction factor, TRIM-5α (tripartite motif-containing protein 5), promotes the rapid, premature disassembly of viral capsids (68). This protein is part of a larger family of TRIMs comprising approximately sixty members in humans (plus "TRIM-like" molecules), and many have been demonstrated to possess antiviral functions such

as the inhibition of viral entry, transcription, assembly and particle release (69-71). TRIMs are also involved in the post-translational modification and regulation of a number of proteins, including transcription factors such as NF-κB and interferon regulatory factor (IRF), often via the ubiquitination either of the protein itself, or proteins that regulate its expression (72, 73). Whilst TRIM-5α has some anti-HIV activity, it is much less potent than its counterpart in rhesus macaques (rhTRIM-5α) (74). It is thought that the loss of activity is due to a single point-mutation in the C-terminal region involved with binding to the viral capsid (75-77).

Complement

The complement system primarily targets bacterial pathogens, initiating a protease cascade that can result in direct lysis of the bacterial membrane (via the formation of a membrane attack complex) or enhanced phagocytosis of the pathogen (78). HIV-1 is also capable of activating the complement system; however, rather than resulting in viral killing, the binding of proteins of the complement system to the surface of the virus appears to facilitate its entry into monocytic cell lines via the complement receptors expressed by these cells (79, 80). Nevertheless, complement lysis activity has been found to correlate inversely with plasma viral load during the acute phase of infection and so the complement system may play an important role in the resolution of acute HIV-1 infection, although the precise mechanisms have yet to be elucidated (81).

Cellular responses

Phagocytic cells

One of the major weapons of the innate immune system is the phagocytosis of pathogens, which are then destroyed inside the phagocytic cell. This process is

conducted primarily by macrophages and neutrophils that recognise either pathogen-associated molecular patterns (PAMPs), opsonising antibodies or complement bound to the pathogen surface (82-86). Once the infectious agent has been phagocytosed it can be killed by reactive oxygen intermediates, reactive nitrogen intermediates or antimicrobial peptides called defensins released into the phagosome. Myeloperoxidase released by neutrophils can react with hydrogen peroxide and chloride to produce reactive oxygen intermediates that are highly viricidal to HIV-1 (87). However, neutrophils from HIV-infected individuals release lower levels of superoxide compared to those from uninfected controls, suggesting that the ability of neutrophils to kill phagocytosed pathogens may be impaired in HIV-1 infection (88, 89). In addition to phagocytosing pathogens, neutrophils and macrophages can produce a variety of cytokines in response to infection, including IL-1α, IL-1β and IL-12, which can enhance T cell activation, NK cytotoxicity and IFN-γ production (90-92). Neutrophils also produce chemokines, such as IL-8, MIP-1α and MIP-1β, that can aid the chemotaxis of other neutrophils, monocytes, immature DCs and T cells to sites of inflammation (93-95). However, in HIV-infected individuals, production of IL-8 and IL-12 by macrophages and neutrophils is dysregulated (96, 97). Production of the anti-inflammatory cytokine IL-10 is also elevated in the macrophages of HIV-infected individuals compared to those from uninfected controls (97).

Cytotoxic cells

If HIV-1 succeeds in evading phagocytosis by neutrophils and macrophages and infecting CD4+ T cells, these infected cells can be killed by another member of the innate immune system, the NK cell. NK cells possess inhibitory receptors (iNKRs) that recognise antigen presenting major histocompatibility complex (MHC) class I

molecules and activating receptors that recognise molecules upregulated on cancerous or infected cells (98-100). When the NK cell encounters a cell that has downregulated MHC class I molecules or is expressing molecules recognised by the activating receptors, apoptosis is induced in the target cell via one of two mechanisms. The NK cell can either upregulate "death ligands" that bind to their receptors on target cells, initiating an apoptotic cascade, or release cytotoxic granules that mediate proteolysis in the target cell, triggering a similar apoptotic cascade. NK cells can also kill pathogens coated with opsonising antibodies that bind to receptors for the constant region of the antibody (Fc receptors) in a process known as antibody-dependent cellular cytotoxicity (ADCC). Studies using HIV-infected tumour cells have shown that NK cells are capable of killing HIV-infected cells by both direct lysis and ADCC *in vitro* (101-103). In addition to directly killing HIV-infected host cells, NK cells produce cytokines such as IFN-γ that enhance both innate and adaptive responses to infection (104). They also secrete MIP-1α, MIP-1β and RANTES and so can inhibit the entry of R5-tropic HIV-1 into host cells by this means (105). However, NK cells may also have a negative impact on the pathogenesis of HIV-1 infection. A recent study suggested that IL-10 induced by HIV-1 infection rendered mature DCs more susceptible to aberrant killing by NK cells, whilst immature DCs survived (106). This skewing of the NK response resulted in the accumulation of poorly immunogenic, immature DCs.

Natural killer T cells, which can be regarded as bridging the innate and adaptive immune systems, express a semi-invariant TCR that recognises glycolipids presented in the context of the non-polymorphic MHC Class I-like molecule CD1d (reviewed in (107, 108)). Following signalling via the TCR, NKT cells can induce apoptosis in target cells, as well as producing cytokines such as IL-2 that can activate NK cells and

T cells (109). However, a subset of NKT cells expressing both CD4 and CCR5 is highly susceptible to infection with R5-tropic HIV-1 (110), and the frequency of these cells in PBMC is dramatically reduced in HIV-1-infected individuals compared to healthy donors (111, 112).

Antigen-presenting cells

In addition to directly controlling HIV-1 replication through phagocytosis and cytotoxicity, cells of the innate immune system play a vital role in the initiation of adaptive immune responses by presenting antigen to T lymphocytes. The process of presenting antigen to T cells is mediated predominantly by DCs, macrophages and monocytes, which are described in this context as professional antigen-presenting cells (APCs). However, the priming of naïve T cells is conducted exclusively by DCs, due to the requirement for costimulatory molecules (such as B7 molecules) that are expressed by mature DCs. Peptide antigens are presented to T cells in complex with one of two classes of molecule: MHC Class I molecules present antigens to CD8+ T cells, whilst MHC Class II molecules present antigen to CD4+ T cells.

MHC Class I molecules comprise a 44 kDa heavy polypeptide α chain linked to a 12 kDa polypeptide called β2-microglobulin. The region distal to the membrane of the APC forms a distinctive groove occupied by peptides that are to be presented to CD8+ T cells. These peptides usually arise from endogenous antigen (for example, viral protein) that has been cleaved by the immuno-proteosome and cytosolic proteases into polypeptides eight to ten amino acids long and transported into the endoplasmic reticulum (ER), where they encounter and form a complex with MHC Class I molecules. The MHC-peptide (pMHC) complex is then transported via the Golgi to the

surface of the APC (113). However, approximately 25% of MHC Class I molecules present antigen of exogenous origin. This occurs when phagocytosed or endocytosed antigen escapes into the cytosol from the vacuole in which it was engulfed, is degraded by the immuno-proteosome and thus enters the Class I presentation pathway. This phenomenon is known as cross-presentation (114).

MHC Class II molecules consist of two polypeptide chains, α and β. As with MHC Class I molecules, there is a peptide binding groove at the distal surface, but as it is open at both ends it is less restricted than the Class I groove, with the result that peptides presented by MHC Class II can be rather longer, typically around fifteen amino acids long (115). These peptides are usually of exogenous origin, taken up by the APC by endocytosis and degraded by lysosomal proteins in the late endosome. Meanwhile, MHC Class II molecules are assembled in the ER in association with an invariant chain (Ii) that inhibits premature binding of peptides to the groove. This complex is transported through the Golgi and packaged into vacuoles. Fusion between these vacuoles and the late endosomes containing exogenous peptide exposes the MHC class II – Ii complex to proteases that degrade Ii, leaving just a small fragment in the binding groove called CLIP (class II – associated invariant chain peptide). Removal of CLIP by the MHC-related molecule HLA-DM allows the binding of exogenous peptide in the groove, forming MHC-peptide complexes that are then transported to the cell surface to be presented to CD4+ T cells (116, 117). MHC Class II molecules can also present endogenous antigens. This occurs when portions of the cytosol containing proteosomal products and other endogenous proteins are engulfed into autophagosomes, which can then fuse with MHC Class II compartments, resulting in the loading of the endogenous peptide onto MHC Class II molecules (118).

The human MHC, or HLA (human leukocyte antigen) genes are polygenic. There are three different types of MHC Class I α chains, HLA-A, HLA-B and HLA-C, and three different types of MHC Class II α and β chains, HLA-DQ, HLA-DP and HLA-DR. Due to differences in the structure of the binding groove, each presents a different range of peptides to T cells. Further variation arises between individuals thanks to the considerable polymorphism of the HLA locus, with at least 86 HLA-A locus, 184 HLA-B locus and 45 HLA-C locus alleles described to date (119); these polymorphisms mean that the spectrum of HIV-1 peptides presented to T cells varies from one infected individual to another. This phenomenon is highly relevant to HIV-1 pathogenesis, as different MHC Class I alleles have been associated with different rates of disease progression. HLA-B*27 and B*57 alleles, for example, are associated with delayed progression to AIDS, whilst HLA-B*08, certain B*35 alleles, Cw*04 and Cw*07 have been linked to rapid progression (120-127). The reasons for the slower disease progression seen in individuals with particular HLA alleles have yet to be fully elucidated; however, patients who possess "protective" alleles such as B*27 and B*57 exhibit specific and strong CD8+ T cell responses towards highly conserved areas of the HIV-1 Gag protein, which the virus cannot mutate without suffering a fitness cost (128-130). In addition, recent evidence suggests that CD8+ T cells restricted by HLA-B*27 and HLA-B*57 allele groups are less resistant to suppression by regulatory T cells (Tregs) compared to CD8+ T cells restricted by "non-protective" alleles (131).

1.2.2 Adaptive immune responses

Humoral responses

HIV-1 specific antibody responses are detectable within eight to ten days after plasma virus detection in the form of immune complexes. The first free plasma anti-HIV-1 antibodies, which appear around five days later, are IgA, IgG and IgM antibodies directed against envelope gp41 (132). IgM class antibodies are potent activators of the classical complement pathway but bind antigen with relatively low affinity (133). Activated B cells may swap to the production of higher-affinity IgA and IgG antibodies in a process known as class switching but this phenomenon is inhibited by HIV-1 Nef (134). The early antibody response fails to neutralise virus, and neutralising antibodies (NAb) do not emerge until months after transmission, long after the resolution of acute infection (135-139). The delay in the development of NAbs is partly due to the dysregulation and loss of CD4+ T cell help (mediated in part by the extensive depletion of CD4+ T cells during acute infection) that is necessary for the full activation of B cells following their encounter with antigen in the lymph nodes (35, 140, 141). Damage to germinal centres, the sites of B cell development, during early HIV-1 infection may also contribute towards the delay in plasma antibody responses (142).

Infection with HIV-1 leads to the induction of persistent antibody responses to both Gag and Env, although antiviral activity is restricted to antibodies recognising the viral envelope proteins gp41 and gp120 (143). The most prominent mechanism of anti-HIV-1 antibody activity is neutralisation; by binding to gp41 and gp120, antibodies can prevent viral binding to CD4 and subsequent entry into host cells (144-146). Anti-HIV-1 antibodies can also promote viral lysis via the classical complement pathway (81), via

phagocytosis by phagocytes bearing antibody Fc receptors on their surfaces (147, 148), or via ADCC by NK cells (149).

Although NAbs can be identified in most HIV-infected individuals during some, if not all, stages of infection, most of these antibodies recognise a narrow range of viral isolates and may not provide protection against heterologous viral strains (150). Broadly neutralising antibodies (BNAbs) targeting regions on gp41 and gp120 can be detected in up to 25% of individuals with chronic, untreated HIV-1 infection (151-153) but attempts to elicit such antibodies through vaccination have proved largely unsuccessful (137, 154, 155). It is hoped that vaccination strategies able to elicit these BNAbs would provide a greater degree of protection against acquisition than has currently been achieved in prophylactic vaccine trials.

Cellular responses

T cell responses appear to play a critical role in immune control of HIV-1 infection. These responses are initiated by the presentation of viral peptide in complex with MHC molecules to the T cell receptor (TCR).

The T cell receptor

The TCR consists of two transmembrane polypeptide chains, α and β (or, more rarely, γ and δ) (156). Each chain is encoded by gene segments that are recombined during T cell development to form a continuous sequence. In the case of the β chain, the sequence is formed by recombination between variable (V), diversity (D), joining (J) and constant (C) segments, whilst α chains are formed from recombined V, J and C segments. There are multiple copies of each segment (for example, there are around 60

different Jα genes), with the result that recombination generates a considerable number of different VDJ or VJ sequences. Further diversity is created by the generation of palindromic sequences arising from the formation of DNA hairpins during the recombination process, the random insertion of nucleotides by terminal deoxynucleotidyl transferases, and the removal of nucleotides at exposed junctions by nucleases. The result is an enormous diversity of sequences, creating a TCR repertoire capable of recognising an almost unlimited array of peptide-MHC complexes. However, the number of different TCRs present in an individual is limited by both space constraints and thymic selection; it has been estimated that the human TCR repertoire in an individual comprises approximately 2.5×10^7 clonotypes (157). Recognition is mediated by three hypervariable complementarity determining regions (CDRs), CDR1, CDR2 and CDR3. CDR1 and CDR2 are germline encoded, whilst CDR3 is generated by V(D)J recombination and therefore contains substantial non-germline variability (158). These CDRs determine the precise specificity of the TCR for a particular peptide-MHC combination.

T cell development and thymic selection

T cells, like B cells, are derived from bone marrow hematopoietic stem cells. Unlike B cells, however, they exit the bone marrow and travel via the blood to the thymus, where they develop into mature T cells (reviewed in (159)). On entering the thymus, immature thymocytes, which are described as double negative (DN) as they express neither CD4 nor CD8, undergo slow but robust proliferation and upregulate CD25. At this stage the TCR β chain locus is undergoes rearrangement and the expressed protein forms an association with an invariant pre-TCR α chain to form the pre-TCR complex; cells that have not achieved productive TCR β rearrangement undergo apoptosis. Those

that survive downregulate CD25, undergo rapid proliferation and express both CD4 and CD8 to become double positive (DP). Following the acquisition of CD4 and CD8, DP cells withdraw from the cell cycle and undergo TCR α chain rearrangement, with the rearranged α chain replacing the invariant pre-TCR α chain in the TCR complex. The DP cells now undergo a process called positive selection, where they are selected for their ability to bind self peptides presented by MHC molecules on cortical epithelial cells. DP cells that are capable of binding self pMHC with low affinity receive survival signals that protect them from apoptosis. Thymocytes that do not bind self peptide-MHC do not receive this signal and die by a process known as "death by neglect", whilst those that bind with high affinity undergo activation-induced apoptosis. The positively selected cells thus have the ability to recognise antigens only when they are associated with self-MHC molecules. At this point thymocytes commit to either the CD4+ or CD8+ lineage: those that recognise MHC Class I downregulate CD4 synthesis to become CD8+ T cells, whilst those that recognise MHC Class II downregulate CD8 synthesis and become CD4+ T cells. In order to prevent the circulation of potentially harmful self-reactive T cells, the single positive thymocytes then undergo negative selection by medullary thymic epithelial cells expressing a wide variety of tissue-specific self-antigens, with T cells that bind strongly to these antigens eliminated from the population. Thymocytes that survive this final selection process undergo terminal differentiation and exit the thymus, travelling to lymphoid tissues where they may encounter viral antigen (160, 161).

T cell activation

Upon entering lymph nodes, naïve T cells with the correct specificity may encounter viral antigen presented by APCs in complex with MHC Class I or II molecules that bind

to the TCR complex. The TCR complex consists of the αβ TCR closely associated with the CD3 coreceptor complex and either CD4 or CD8. However, pMHC-TCR binding is insufficient to activate naïve T cells; a second signal is required otherwise the T cell becomes anergic. This signal is provided by the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) on APCs that bind to CD28 on the naïve T cell. In the presence of this costimulatory signal, binding of the pMHC complex by the TCR and CD4 (in the case of MHC Class II molecules) or CD8 (MHC Class I molecules), results in the phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) contained within CD3 by kinases associated with CD4 or CD8. This provokes a signalling cascade via pathways including the Ras/MAPK pathway and the phosphatidylinositol pathway that culminates in the transcription of genes involved in proliferation, such as IL-2, as well as genes for other cytokines and effector molecules (162).

In an acute infection where the pathogen is rapidly cleared, the initial burst of T cell proliferation is followed by a contraction phase, with widespread apoptosis of antigen-specific T cells. A subpopulation of these cells survive and form a memory compartment capable of mounting a more rapid and efficient response to the same pathogen upon subsequent exposure. Memory cells can be subdivided into central memory cells (TCM), which differentiate from naïve T cells, and terminally differentiated effector memory cells (TEM) that are thought to subsequently arise from TCM (163, 164). These cells play a major role in protective immunity against a variety of pathogens after initial exposure and infection; however, in HIV-1 infection the memory T cell population is highly compromised. CD4+ TCM are rapidly depleted in acute HIV-1 infection and are all but absent during the chronic stage of disease (9, 165,

166). The CD8+ T cell response, meanwhile, appears to be skewed towards the generation of short-lived effector cells, with limited generation of a TCM population (167-169).

Whilst necessary for the initiation of effector responses, T cell activation is increasingly recognised as a factor in both an individual's susceptibility to HIV-1 infection and prognosis after acquisition (170). HIV-1 replicates most efficiently in activated CD4+ T cells (171, 172), and a high level of T cell activation is associated with rapid disease progression independently of viral load (173). T cell activation can therefore be regarded as a double-edged sword in HIV-1 infection, simultaneously helping to control viral replication and rendering target cells more vulnerable to infection.

The role of CD4+ T cells in HIV-1 infection

CD4+ T cells are often referred to as helper T cells (or Th cells), reflecting their role in enabling and enhancing responses to pathogens by other cells of the immune system. Th cells were initially subclassified, in mice and then in humans, into Th1 and Th2 subsets, according to their function and cytokine output (174-176). Th1 cells secrete IFN-γ, IL-2 and TNF-α and coordinate responses to intracellular bacterial and viral infections by activating macrophages (leading to enhanced killing of phagocytosed pathogens) and assisting the expansion of cytotoxic CD8+ T cells (177). Th2 cells, on the other hand, produce IL-4, IL-5, IL-6, IL-10 and IL-13 and typically mediate responses to extracellular pathogens by supporting B cell differentiation and proliferation (176, 178, 179). Subsequently, the identification of other functionally distinct subsets, such as Th17 cells, T follicular helper (TFH) cells and regulatory T cells (Tregs) has further expanded the CD4+ T cell family (180-182). Th17 cells

promote inflammatory responses at mucosal interfaces through the production of IL-17A, IL-17F and IL-22, playing an important role in the immune response to extracellular bacterial and fungal infections (183-185). The maintenance of Th17 function has been associated with delayed progression in both HIV and SIV infection; Th17 cells may help to maintain the integrity of the mucosal barrier, decreasing the leakage of microbial products into the bloodstream and thereby limiting immune activation (186-189).

Tregs have been described as CD4+ CD25+ FoxP3+ cells with suppressive functions (183). These thymus-derived or "natural" Tregs can suppress effector T cell function via a number of mechanisms, including direct lysis of effector T cells, the inhibition of proliferation via competition for IL-2 and the production of immunomodulatory cytokines such as TGF-β and IL-10 (190). It has subsequently become clear that a number of other Treg subsets exist, including Tr1 cells and Th3 cells, that can mediate their suppressive effects through both contact-dependent and independent means (191, 192). Since Tregs limit T cell activation, it has been speculated that they may help to limit viral replication in HIV-1 infection. However, Treg numbers and frequencies are similar in HIV-infected non-progressors and progressors, although a study of asymptomatic HIV-infected individuals showed that those with suppressive Treg responses had lower viral loads than individuals where Treg responses were impaired (193, 194).

The role of CD8+ T cells in HIV-1 infection

CD8+ T cells possess the ability to kill host cells infected with HIV-1 and other viruses, and for this reason are often referred to as cytotoxic T lymphocytes (CTL). Activation

of CTLs requires help from Th cells, either directly or via DCs that interact with the CTL. Upon recognition of pMHC Class I on the surface of an APC, CTLs release cytotoxic granules containing perforin and granzymes, whose respective functions are the lysis of the infected target cell and the induction of apoptosis in that cell. Alternatively, the apoptotic cascade can be initiated by binding of Fas ligand (FasL) on the CTL to the Fas receptor on the target cell. The importance of the CTL response in controlling acute HIV-1 infection can be inferred from the observation that the emergence of HIV-specific CD8+ T cell responses coincides with the decline in viraemia (36, 37). Further evidence emerged with the discovery that depletion of CD8+ cells from acutely SIV-infected rhesus macaques abrogated viral control, resulting in the maintenance of viral load at peak levels (195, 196). In addition to direct killing of HIV-infected cells, CTLs can inhibit viral replication by secreting cytokines and the viral entry inhibitors MIP-1α, MIP-1β and RANTES (57, 197, 198).

In summary, the immune system is able to mount a broad spectrum of responses, both innate and adaptive, against HIV-1. In order to survive, the virus must employ a sophisticated set of evasion mechanisms. These mechanisms allow it to persist and promote a chronic infection.

1.3 Why does the immune response fail to control HIV-1?

1.3.1 Viral escape mechanisms

One of the greatest challenges facing the immune system in its attempt to clear HIV-1 is the extraordinary genetic diversity of the virus. The virus's reverse transcriptase (RT)

enzyme is highly error-prone, yielding approximately 0.2-2 mutations per genome per cycle (199). Thus, whilst the majority of sexually transmitted infections are established by a single "founder virus" (15, 200, 201), genetic variation within an individual is established rapidly following infection (15, 202). The early emergence of HIV-specific CTL responses leads to rapid escape in regions containing known CTL epitopes, with near total loss of the founder sequence as little as two weeks after initial screening (203-205). Selection of escape mutants continues during chronic infection (129, 206-208). Whilst escape may sometimes involve a fitness cost to the virus, the ongoing requirement for the CTL response to evolve may contribute to its failure to adequately control viral replication.

The requirement for Th cell assistance in B cell activation means that NAbs to HIV-1 are not detectable until several weeks after acquisition, by which time antibodies are confronted with a formidable array of viral variants. The antibody response is thus left to play "catch up" with a rapidly evolving virus (209). Neutralisation is further hindered by the degree of variability of the envelope proteins gp120 and gp41, which is much higher than that observed in internal structural proteins such as Gag p24 (210-212). Rare conserved regions are often masked by highly variable loops, and antibody binding is further impeded by the extensive glycosylation of both proteins (213, 214). Whilst experimental depletion of B cells in SIV-infected rhesus macaques suggests that B cell responses are important for control of viraemia (215), broadly neutralising antibody responses in HIV-1 infected humans are generally not associated with viral control (216-218).

HIV-1 has also evolved mechanisms that enable it to escape from innate immune responses. The virus is able to subvert antigen presentation pathways by gaining entry to DCs via the C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-grabbing nonintegrin). The DC-SIGN-bound HIV-1 is internalised into non-lysosomal organelles and can thus "hide" in DCs without being degraded, enabling it to recycle back to the cell surface and infect any CD4+ T cells in the proximity of the DC (219-221). HIV-1 can inhibit phagocytosis by macrophages via downregulation of FcR on the macrophage membrane, or through Nef-dependent inhibition of phagosome formation (222, 223). The virus can evade NK cell killing by maintaining the expression of HLA-C and HLA-E MHC molecules on infected cells, thereby preserving the inhibitory signal to NK cells, whilst at the same time downregulating HLA-A and HLA-B in order to escape killing by CD8+ T cells (224). There is also evidence that ADCC by NK cells is impaired in HIV-1-infected individuals (225). Finally, HIV-1 has evolved mechanisms to overcome inhibition of the viral life cycle by intrinsic cellular factors such as APOBEC and tetherin. Viral infectivity protein (Vif) degrades APOBEC3G, whilst tetherin is antagonised by Viral protein U (Vpu) (67, 226).

1.3.2 Latent viral reservoir formation

Another major obstacle to the eradication of HIV-1 after infection, either by sterilising immunity or ART, is the early formation of latent, persistent viral reservoirs in various locations of the body (227). Potential reservoirs include macrophages or microgial cells in the central nervous system (CNS) and infected cells in the male urogenital tract (228, 229). However, the most worrisome reservoir consists of latently infected resting memory CD4+ T cells carrying integrated viral DNA that is replication competent if the cells undergo antigen-induced activation (27, 28). The half-life of this CD4+ T cell

compartment has been estimated at 44 months, meaning that eradication would require more than 60 years of treatment (230). It therefore seems unlikely that viral clearance will be achievable by conventional treatment or vaccination approaches. This has lead some researchers to investigate mechanisms of reactivating latently infected CD4+ T cells to enable their eradication, using agents such as protein kinase c-activating particles, histone deacetylase inhibitors and antibiotics (231, 232). However, it remains unclear whether such an approach could enable complete viral eradication *in vivo*.

1.3.3 T cell dysregulation

Whilst HIV-1 infection elicits a robust immune response in the majority of individuals, the capacity of this response to control viral replication is limited. One of the major reasons for this dysfunction is the impaired capability of CD4+ and CD8+ T cells to mount appropriate responses to viral antigen. The profound depletion of CD4+ T cells in acute HIV-1 infection results in a considerable reduction in T cell help for B cell development, CD8+ T cell expansion and phagocytosis (35, 233). The establishment of chronic infection sees T cells lose the ability to proliferate, secrete antiviral and proliferative cytokines or lyse infected cells in response to antigenic stimulation. This phenomenon is known as T cell exhaustion.

T cell exhaustion was first described in the murine lymphocytic choriomeningitis (LCMV) model, where in the context of chronic infection LCMV-specific CD8+ T cells were unable to kill virally-infected target cells (234, 235). Subsequently, CD4+ and CD8+ T cell exhaustion has also been demonstrated in chronic HIV-1 infection (236, 237). During chronic infection, T cell exhaustion is associated with the heightened expression of negative regulators of T cell activation such as programmed death (PD-1)

and cytotoxic T lymphocyte antigen-4 (CTLA-4). PD-1, which dampens TCR-induced cytokine production following binding to its ligands PDL-1 and PDL-2, is upregulated on CD8+ T cells during chronic HIV-1 infection, and its expression correlates with both viral load and the reduced capacity of HIV-specific CD8+ T cells to produce cytokines and proliferate (236, 238, 239). Long term non-progressors exhibit markedly lower expression levels of PD-1 on CD8+ T cells compared to progressors (240). CTLA-4, which is structurally related to CD28 but binds CD80 and CD86 on APCs with much higher affinity, suppresses TCR signalling and is upregulated on CD4+ T cells of HIV-infected individuals (241). In chronic infection, the blockade of CTLA-4 increased production of IL-2 and IFN-γ by HIV-specific CD4+ T cells *in vitro* (242).

T cell exhaustion can be further compounded by the elevated expression of immunosuppressive cytokines during both acute and chronic HIV-1 infection. One such cytokine, IL-10, is produced by monocytes in HIV-infected individuals in response to PD-1 signalling and can also be secreted by HIV-specific Tregs (243, 244). CD4+ CD25+ IL-10+ Tregs from HIV-infected individuals suppressed the ability of HIV-specific CD4+ $CD25^-$ cells to proliferate in response to viral antigen (243). However, promoter polymorphisms associated with higher expression of IL-10 have been associated with reduced mortality and CD4+ T cell loss in HIV-infected individuals (245, 246). Before T cell dysregulation can be addressed therapeutically, it may be necessary to elucidate the precise function of IL-10 in HIV-1 pathogenesis and control.

1.4 Interleukin-10 and the immune response to infection

With its pleiotropic effects on cells of both the innate and adaptive immune systems, IL-10 is critical to the balance between effector responses and the prevention of immune pathology in a multitude of diseases (reviewed in (247)).

1.4.1 IL-10 structure and signalling

Human IL-10 is encoded by a 5-exon gene on chromosome 1, with the resulting polypeptide forming noncovalent homodimers of approximately 39 kDa (248-252). Whilst it was initially described in mice as a product of Th2 cells, IL-10 is expressed by a multitude of cells of the immune system, including T cells, B cells, monocytes and macrophages, as well as non-immune cells such as keratinocytes, epithelial cells and tumour cells (253-258).

The IL-10 receptor (IL-10R) is a heterotetramer, consisting of two molecules of the IL-10R1 and two of the IL-10R2 (259-261). The cytoplasmic domains of the receptor are constitutively associated with two protein kinases, Jak1 and Tyk2 which, upon binding of IL-10, phosphorylate tyrosine residues in the cytoplasmic regions of IL-10R1, allowing recruitment and docking of the transcription factor STAT3 (signal transducer and activator of transcription 3) (262-264). STAT1 and STAT5 can also be recruited to phosphorylated tyrosine residues on IL-10R1, but their precise roles in IL-10 signalling are unclear (265). Homodimerisation of STAT3 results in its release from the receptor and translocation to the nucleus, where it can bind to STAT-binding elements in the

promoters of various genes. One of these genes is SOCS3 (suppressor of cytokine signalling 3), which can inhibit the expression of IFN-α and IFN-γ-induced genes by suppressing phosphorylation of STAT1 (266). IL-10 can inhibit NF-κB-dependent expression of TNF-α either by inhibiting binding to its DNA motif or by preventing the degradation of IκB, which is essential for the translocation of NF-κB to the nucleus (267-270). However, IL-10 can also enhance NF-κB activity in CD8+ T cells (271). IL-10 may also activate the PI-3 kinase signalling pathway, although Bhattacharyya *et al* demonstrated that pre-treatment of bone marrow-derived DCs with IL-10 actually inhibited LPS-induced PI-3 kinase induction (267, 272-274).

1.4.2 Biological activities of IL-10

Effects of IL-10 on antigen-presenting cells

One of the most important mechanisms by which IL-10 modulates T cell function is the inhibition of APC function. IL-10 downregulates MHC Class II on the surface of monocytes by inhibiting the exocytosis and recycling of MHC-containing vesicles (275, 276). The expression of the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) on monocytes, macrophages and DCs is also negatively regulated by IL-10 (277-279); by these means, it can induce a state of anergy in CD4+ T cells (280, 281).

IL-10 can also inhibit the production of cytokines and chemokines by APCs. It downregulates the expression of IL-1α, IL-1β, IL-6, IL-10 itself, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF and PAF by activated monocytes and/or macrophages, MIP-1α, MIP-1β and RANTES (which inhibit HIV-1 entry into CD4+ T cells) by activated monocytes, and IL-12 by DCs (282-291). Since these cytokines and chemokines are implicated in amplifying inflammatory immune responses and

recruiting effector cells to sites of inflammation, IL-10 can have potent anti-inflammatory effects. Furthermore, IL-10 can upregulate antagonists of pro-inflammatory effectors, such as the soluble TNF receptors p55 and p75 (292, 293).

Effects of IL-10 on phagocytes

IL-10 can modulate the phagocytic response to infection in a number of ways. The expression of CD16 and CD64 FcγR on macrophages is increased by IL-10, leading to enhanced ADCC (294, 295). However, in neutrophils IL-10 can inhibit ADCC (296). IL-10 also reduces production of superoxide anion (O_2^-) and nitric oxide (NO) and thus impairs the capacity of neutrophils and macrophages to kill phagocytosed pathogens (297-299).

The effects of IL-10 on cytokine and chemokine production by neutrophils are similar to those observed in monocytes and macrophages, with TNF, IL-1α, IL-1β, MIP-1α and MIP-1β all downregulated (90, 95). Neutrophil expression of IL-8, which is involved in chemotaxis, is also downregulated by IL-10 (300). By restricting the production of these inflammatory mediators, IL-10 may help to limit the duration and harmful pathology of inflammatory responses.

Effects of IL-10 on NK cells

IL-10 can mediate both stimulatory and suppressive effects on NK cells. It inhibits the production of IFN-γ by NK cells in mice (301), but can also enhance their cytotoxic activity, proliferation and migration, particularly in combination with IL-18 (302-304).

Effects of IL-10 on lymphocytes

IL-10 can increase the survival of B cells (depending on their activation state), possibly via induction of the anti-apoptotic protein bcl-2 (305, 306), as well as inducing the differentiation of both immature B cells and memory B cells into antibody-producing plasma cells (307, 308). Proliferation of B cell precursors and mature B cells is also enhanced by IL-10 (179, 309). Both B cell-derived and exogenous IL-10 can mediate class switching, the process where isotype-committed B cells switch to the production of a different antibody isotype; for example, it can induce production of IgG1 and IgG3 by activated tonsillar B cells (310). Class switching to IgA and IgG, which is important for antibody effector function, is impaired by HIV-1 Nef, but it is unclear whether this phenomenon can be reversed by IL-10 (134).

Whilst IL-10's effects on T cells are mostly mediated by APCs, it can also directly modulate T cell function. IL-10 can inhibit IL-2 and IL-5 production by CD4+ T cells, as well as the expression of CXCR4, a crucial co-receptor for X4-tropic strains of HIV-1 (291, 311, 312). Further evidence of IL-10's capacity to affect T cells directly has been delivered using transgenic mice expressing a dominant negative IL-10 receptor specifically in T cells (313, 314). Adoptive transfer of these CD4+ T cells into Rag1 knockout mice induced colitis, whereas transfer of wild type CD4+ T cells did not. Since IL-10 knockout mice also develop spontaneous colitis, this study demonstrated the importance of IL-10 signalling via T cells in the prevention of autoimmunity. In contrast to its inhibitory effects on CD4+ T cells, IL-10 can directly promote the recruitment and proliferation of CD8+ T cells, but these positive effects may be outweighed by IL-10-mediated anergy caused by the downregulation of costimulatory molecules on APCs (315, 316).

The pleiotropic functions of IL-10 and its diverse range of cellular targets enable it to play a crucial role in the balance between achieving pathogen clearance and preventing excessive immune pathology. However, if the response is mistimed or misdirected, it may help to facilitate persistent infection. This notion has lead to IL-10 signalling being implicated in a number of chronic viral infections.

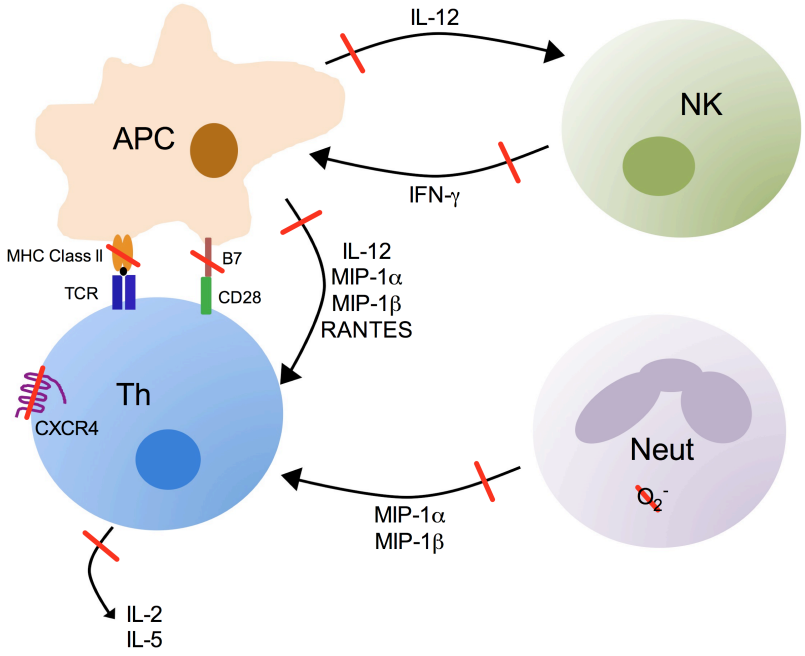


Figure 5.1 The inhibitory effects of IL-10 on cells of the innate and adaptive immune systems. Red lines indicate functions that are inhibited by IL-10. APC, antigen-presenting cell; Th, T helper (CD4+) cell; Neut, neutrophil; NK, natural killer cell.

1.4.3 IL-10 in chronic viral infections and other pathological settings

IL-10 is upregulated in a number of chronic human viral infections, including hepatitis C virus (HCV), Epstein Barr virus (EBV) and HIV-1 (52, 317-321). Elevated levels of IL-10 are detectable in the plasma of HCV-infected individuals approximately ten days after the initial detection of viraemia (52), whilst in chronic infection monocytes and antigen-specific CD8+ T cells have been identified as sources of the cytokine (318, 319, 322). The higher frequency of HCV-specific IL-10-producing CD8+ T cells in healthier regions of the liver compared to areas with active necroinflammatory disease suggests that these cells may play a protective role in chronic HCV (319). Indeed, subcutaneous administration of IL-10 has been found to reduce liver fibrosis in HCV-infected patients who were either untreated or did not respond to conventional interferon-based therapy (323). However, long term IL-10 therapy led to increased virus burden and a reduction in HCV-specific IFN-γ-secreting CD4+ and CD8+ T cells (323). It seems likely that IL-10 plays a complex role in chronic HCV, helping to limit immune pathology but also impairing viral control. The idea that IL-10 can enable viral persistence is given further credence by the observation that both the EBV and CMV genomes encode an IL-10 homologue (324-326). These homologues share many characteristics with the ancestral human gene, although their bioactivity is somewhat diminished, and it seems likely that capture of the human variant was an important event in the evolution of these viruses as persistent, predominantly benign parasites (297, 327).

Whilst the cellular sources of IL-10 in many bacterial, viral and protozoal infections have yet to be fully elucidated, a number of recent studies have suggested that the relative levels of IL-10 and IFN-γ produced by T cells may help to determine whether a

pathogen is successfully cleared or is able to persist in the host (328, 329). In mice infected with *Leishmania major*, Th1 cells that co-produced IL-10 and IFN-γ were found to be responsible for IL-10-mediated immune suppression and failure to control parasite burden (330). However, production of IL-10 by IFN-γ-producing T cells may also be a means to limit the duration of potentially harmful responses. IL-10+ IFN-γ+ Th1 cells were necessary for the prevention of lethal immune pathology in *Toxoplasma gondii*-infected mice, with IL-10 blockade resulting in death (331). The balance between the production of IL-10 and IFN-γ by CD4+ T cells may also determine the effectiveness of anti-tumour T cell responses. It has been demonstrated that the TLR-7 agonist imiquimod, a candidate breast cancer therapeutic agent, induces IL-10 secretion by IL-10 and IFN-γ dual secreting CD4+ T cells in mice, which in turn limits the efficacy of the agent. Blockade of IL-10 using an anti-IL-10 antibody enhanced the antitumour effect of imiquimod and significantly prolonged survival time compared to mice treated with imiquimod alone (332).

IL-10's potential role in the pathogenesis of immunodeficiency diseases has been studied in the SIV model, where the timing of anti-inflammatory responses during acute infection may influence the subsequent course of disease. Estes and colleagues observed a dramatic early increase in SIV-specific FoxP3+ Tregs (the majority of which produced IL-10) in rhesus macaques that correlated positively with SIV RNA+ cells and inversely with the magnitude of the SIV-specific CTL response. It was thus proposed that the anti-inflammatory response during acute infection plays a detrimental role by delaying the SIV-specific CTL response to the point that it is "too little, too late" (55). However, a contemporary study found that SIV-infected African green monkeys that developed a chronic but non-pathogenic infection experienced an earlier

upregulation of IL-10 compared to SIV-infected rhesus macaques that subsequently developed immunodeficiency (333). The authors suggested that this early anti-inflammatory response in African green monkeys was instrumental in preventing chronic immune hyperactivation, which is a feature of HIV-1 and pathogenic SIV but not non-pathogenic SIV. Due to the conflicting findings of these studies, it remains far from clear whether IL-10 plays a protective or detrimental role in SIV.

The role of IL-10 in chronic viral infections is clearly both complex and incompletely understood. However, an improved understanding of IL-10 signalling may allow it to be manipulated in order to improve the immune response to both infection and vaccination.

1.5 Manipulation of the IL-10 pathway as a therapeutic intervention

Since IL-10 can downregulate antigen presentation to T cells, IL-10 blockade may represent a means of enhancing antiviral T cell responses. Indeed, the manipulation of IL-10 signalling has already been used in animal models of disease and vaccination, with some noteworthy results (reviewed in (334)).

1.5.1 IL-10 manipulation in existing infection, therapeutic and vaccination models

Blockade of IL-10 signalling, both alone and in combination with vaccination, has proved an efficacious therapeutic strategy in mouse models of bacterial, protozoal and viral infections. In the mouse Leishmaniasis vaccination model, a high level of CD4+ T cell-derived IL-10 prior to challenge with *Leishmania major* predicted the failure of the vaccine, whereas low pre-challenge IL-10 levels were observed in mice that were

protected by the vaccine (335). Moreover, ablation of IL-10 signalling, either by gene knockout or blockade of IL-10R promoted rapid parasitic clearance after challenge with *L. donovani*, whilst in wild type and IL-10 transgenic mice infection persisted (336). Taken together, these observations suggest strongly that IL-10 influences the outcome of *Leishmania* infection in mice. IL-10 also inhibits killing of *L. major* by human macrophages, and production of the cytokine correlates with pathology in human *L. donovani* infections (337, 338). Antibody-mediated blockade of IL-10 signalling in cultures of splenic cells from patients with visceral Leishmaniasis resulted in enhanced IFN-γ and TNF-α production by these cells, and a significant reduction in the number of parasites present in the culture (339). It is therefore possible that IL-10 blockade may represent a valuable therapeutic strategy for tackling human Leishmaniasis disease

Therapeutic blockade of IL-10 signalling has also been used in the mouse *Listeria monocytogenes* model. Both IL-$10^{-/-}$ and anti-IL-10 antibody-treated mice demonstrated enhanced innate and adaptive immune responses to *Listeria*, leading to rapid bacterial control and prevention of fatality after a high dose challenge (340, 341).

IL-10 blockade has been used to good effect in cancer models. As discussed previously, anti-IL-10 antibodies have been used to enhance the efficacy of imiquimod as a treatment in the mouse model of breast cancer (332). In a separate study, a combination of an IL-10R blocking antibody and the TLR9 agonist CpG restored tumour-associated antigen presentation by DCs in a mouse tumour model, leading to enhanced anti-tumour immunity (342). These findings suggest that blockade of IL-10 signalling may represent a promising means of increasing the efficacy of current cancer treatments.

The mouse model that has perhaps yielded the most relevant information about the role of IL-10 in chronic viral pathogenesis and control is the lymphocytic choriomeningitis virus (LCMV) model. Infection of mice with a persistent strain of LCMV, LCMV Clone 13 (Cl 13), resulted in a marked upregulation of IL-10 that was up to 26 times higher than that seen in mice infected with a rapidly cleared strain (343, 344). Genetic removal of IL-10 or antibody blockade of the IL-10R resulted in the induction of robust effector T cell responses, rapid clearance of virus and the development of anti-viral memory T cell responses. Furthermore, IL-10R blockade enhanced the efficacy of a therapeutic vaccine administered to mice infected with LCMV Cl 13 (345). The vaccine was ineffective on its own, but when administered in the context of IL-10R blockade it elicited robust anti-viral CD4+ and CD8+ T cell responses and accelerated viral control.

These findings suggest that blockade of IL-10 signalling may help to alleviate T cell exhaustion in chronic viral infection, allowing the restoration of anti-viral immunity and boosting responses to therapeutic vaccines. If such a strategy could be translated into HIV-infected humans, it may be possible to achieve long-term control of viraemia in the absence of ART.

1.5.2 Could IL-10 manipulation be used to improve the efficacy of vaccines for HIV-1?

Since the identification of HIV-1 as the causative agent of AIDS in 1983, the development of a prophylactic vaccine to halt the spread of the virus has been one of the major goals of scientists in the field. In recent years, two high-profile HIV-1

vaccine trials have been conducted, with mixed results. The vaccine used in the first of these trials, the STEP trial, contained Gag, Pol and Nef epitopes delivered by Adenovirus 5 (Ad5) vectors with the aim of eliciting cell-mediated immunity. It stimulated strong IFN-γ production by antigen-specific T cells in both pre-clinical testing and in vaccinees but did not reduce either the risk of acquisition of HIV-1 or the set point viral load after acquisition (346). Indeed, the trial was prematurely halted due to a slightly higher rate of acquisition in vaccinated individuals with pre-existing Ad5 immunity compared to placebo-treated controls. The more recent Rv144 trial yielded more positive results, with a 26% lower incidence of infection in vaccinees compared to placebo-treated controls according to the per-protocol analysis (347). This vaccine contained Gag and Env epitopes and elicited both cell-mediated and humoral responses, with IgG antibodies directed against the V1 and V2 variable loops of gp120 associated with 43% protection from acquisition (B Haynes, AIDS Vaccine 2011, Bangkok, Thailand). These trials and other studies suggest that to provide sterilising immunity, a HIV-1 vaccine would need to elicit both T cell responses and neutralising antibodies (3, 4). However, due to the extensive variability and glycosylation of the Envelope protein, it has proven extremely difficult to design a vaccine that can elicit antibodies capable of neutralising a broad spectrum of viral isolates (348). These difficulties have lead some experts to believe that it will be extremely difficult to design a prophylactic vaccine for HIV-1 given what is currently known (349).

In light of the pessimism surrounding the prospects for a prophylactic vaccine, and the difficulties in eliciting BNAbs, some researchers have focussed on attempting to develop a T cell-based therapeutic vaccine. Rather than protecting uninfected individuals against acquisition of HIV-1, the aim of such a vaccine would be to enhance

T cell responses to conserved regions of the viral proteome in HIV+ subjects, enabling them to clear the virus, or at least to maintain long-term control of viraemia in the absence of ART. However, definitive proof of the efficacy of this approach has yet to be established. One of the challenges associated with the development of a T cell-based therapeutic vaccine is the need to elicit protective T cell responses in patients where, by definition, the immune response has already failed to achieve sustained viral control (350). Thus far, vaccines tested in phase I and II clinical trials have succeeded in eliciting antigen-specific CD4+ and CD8+ T cell responses, which in some cases persist for a number of years, but have failed to confer long-term control of viraemia in the majority of individuals (351-354) (van Lunzen, AIDS Vaccine 2011, Bangkok, Thailand). The ability to respond appropriately to vaccine antigen may be further compromised by the widespread T cell exhaustion observed in chronic HIV-1 infection (205). In a study conducted by our group, the failure of a therapeutic vaccine to control viraemia following treatment interruption was associated with a rapid induction of IL-10, which declined after the reintroduction of ART (355). Whilst *in vitro* blockade of IL-10 signalling did not restore HIV-specific proliferative responses in PBMC collected from vaccinees during the viral rebound phase, Brockman and colleagues have demonstrated that IL-10R blockade can augment HIV-specific cytokine production in chronically infected, ART-naïve individuals (356). Together, these data suggest that in individuals who are either treatment-naïve or who have had their treatment interrupted, circulating IL-10 may limit the capacity of vaccine-induced T cells to control viraemia. Manipulation of IL-10 signalling should therefore be explored as a means of increasing the efficacy of therapeutic HIV-1 vaccines.

1.6 IL-10 and HIV-1 infection

As Stacey and colleagues observed, IL-10 is upregulated during acute HIV-1 infection, and plasma IL-10 concentration remains elevated in ART-naïve chronically infected individuals (52, 356). HIV-1 is capable of inducing IL-10 production directly in monocytes *in vitro* via binding of its transactivating (Tat) protein to an undefined receptor, triggering a signalling cascade that culminates in transcription mediated by CREB-1, Sp-1 or NF-κB (316, 317). HIV-1 negative regulatory factor (Nef) protein can also induce *in vitro* IL-10 production by human monocytic cell lines via the calcium/calmodulin-dependent phosphodiesterase signal transduction pathway (357). In addition to these direct inductive effects, HIV-1 infection may promote the expansion of HIV-specific T cells that produce IL-10 in response to antigenic stimulation (358, 359).

Whilst IL-10 can be produced by a multitude of cell types, there is debate as to its predominant cellular source in chronic HIV-1 infection. Elrefaei *et al* defined a subset of HIV-specific CD8+ T cells as the major producers of IL-10, whilst Torheim and colleagues reported the presence of both CD4+ and CD8+ T cells producing IL-10 in viraemic, chronically infected individuals (358-360). However, Brockman *et al* observed an upregulation of IL-10 mRNA in multiple cell subsets in ART-naïve individuals, particularly T cells, B cells and NK cells (356). It remains unclear, therefore, whether IL-10 production is generally induced or restricted to a particular cellular subset in chronic HIV-1 infection.

The effect of IL-10 in chronic HIV-1 infection has been studied using two methods: the depletion of IL-10+ cells from patient PBMC and the blockade of IL-10 signalling

using antibodies directed against IL-10R. Using the first method, the depletion of IL-10-producing cells was shown to restore proliferation, cytolysis and IL-2 production by HIV-specific CD4+ and CD8+ T cells in vitro, although there was some evidence that this process was contact-dependent and so was unlikely to be mediated solely by IL-10 (359, 360). However, using the second method, Brockman *et al* demonstrated that antibody blockade of IL-10R restored the *in vitro* proliferative capacity of HIV-specific CD4+ and CD8+ T cells, suggesting that IL-10 signalling alone was enough to inhibit proliferation (356). A key unresolved question arising from these studies is whether the effects observed resulted from the removal of all IL-10 signalling or the depletion of a particular HIV-specific IL-10-producing cellular subset. Furthermore, the effect of ablation of IL-10 signalling on the activation and antigen-presenting capacity of APCs, which could have crucial implications for T cell functionality, has not been investigated.

Whilst the functions of IL-10-producing cells isolated from HIV-1+ individuals have begun to be elucidated, it remains far from clear whether these cells play a detrimental or protective role in chronic infection. By inhibiting the effector functions of HIV-specific CD4+ and CD8+ T cells, IL-10-producing cells may limit the effectiveness of the antiviral response and contribute to viral persistence. However, since HIV-1 replicates more efficiently in activated CD4+ T cells (171, 172), and immune activation is associated with disease progression (173), IL-10 responses may help to protect the host from pathogenesis. This is a very important question to address, as it may influence future therapeutic strategies to control viraemia in chronically infected individuals.

1.7 Questions to be addressed in this thesis

This thesis aims to address some of the uncertainty regarding the role of IL-10 in HIV-1 infection, specifically:

- What are the cellular sources of IL-10 in chronically infected HIV-1+ individuals? Do these sources vary according to the stage of disease?

- What are the phenotypic characteristics of HIV-specific IL-10+ cells? Do they possess the capacity to migrate to key sites of HIV-1 pathogenesis such as the GALT?

- What effects do specific IL-10-producing populations have on immune function during chronic HIV-1 infection? Which cellular populations respond to HIV-specific IL-10+ cells?

- What are the consequences of IL-10 blockade *in vitro* and *in vivo*?

Chapter 2: Materials and Methods

2.1 Study subjects and ethical approval

Adults with chronic HIV-1 infection were recruited from clinics at the Churchill Hospital, Oxford, UK. Buffy coats from healthy HIV-uninfected donors were obtained via the Oxford Radcliffe Hospitals Blood Transfusion Service. The study was approved by the Oxfordshire Research Ethics Committee (OXREC) (Ref. 08/H0607/51) and all subjects gave written informed consent. Additional patients were recruited from HIV clinics in Oxford, Slough and Reading with approval from OXREC (Ref. 10/H0604/95). Further samples from HIV-infected, ART-naïve donors were obtained from the SCOPE cohort based at the Center for AIDS Research, University of California San Francisco, USA, with study protocols approved by the Institutional Review Board, University of California–Davis, and the Committee on Human Subjects Research, University of California–San Francisco (361).

Adults with chronic HCV infection who were not receiving treatment were recruited from the John Radcliffe Hospital, Oxford, UK and Barts and the London NHS Trust, London, UK with approval from OXREC (Ref. 04.OXA.010).

Immunisation experiments were conducted using female BALB/c mice aged between five and eight weeks. Animals were housed in the Biomedical Services unit at the John Radcliffe Hospital, Oxford. Experiments were carried out under Personal License number 40/1027 held by Genevieve Clutton and Project License number 30/2833 held by Prof. Tomáš Hanke. All animal procedures and care conformed strictly to the

United Kingdom Home Office Guidelines under the Animals (Scientific Procedures) Act, 1986.

2.2 Reagents and buffers

R0: 500 ml RPMI (Sigma-Aldrich, UK) with 5 ml Penicillin/Streptomycin (Sigma-Aldrich) and 5ml L-glutamine (Sigma-Aldrich).

R10: 500 ml RPMI with 50 ml FCS (Gemcell^{TM}, Gemini Bio Products, USA), 5 ml Penicillin/Streptomycin and 5 ml L-glutamine

H10: 500 ml RPMI with 50 ml heat inactivated human AB serum (Sigma-Aldrich), 5 ml Penicillin/Streptomycin and 5ml L-glutamine

MACS buffer: 500 ml PBS with 33 ml 7.5% BSA (Sigma-Aldrich) and 2 ml 0.5M EDTA (Sigma-Aldrich)

PBS tween: 100 ml PBS with 50 μl Tween 20 (Sigma-Aldrich)

20 μg/ml lysolecithin in 1% paraformaldehyde: 10 g paraformaldehyde (Sigma-Aldrich) was dissolved in 100 ml 1M NaOH (Sigma-Aldrich) and titrated to pH 7.2 using 4M HCl (Sigma-Aldrich). PBS was added to bring the total volume to a litre and 20 mg lysolecithin (Sigma-Aldrich) was dissolved in the solution.

NP-40: 499.5 ml PBS with 0.5 ml Nonidet P-40 (Sigma-Aldrich)

FACS buffer I: 500 ml PBS with 5 ml FCS

FACS buffer II: 180 ml FACS buffer I with 20 ml Perm/Wash Buffer (BD Biosciences, USA)

Mouse IFN-γ ELISPOT substrate solution: A 20 mg AEC tablet (Sigma-Aldrich) was dissolved in 2.5 ml dimethyformide (DMF). 333.3 μl of this solution was added to

10 ml 0.1M acetate solution, which was then filtered before the addition of 5 μl 30% H_2O_2 (Sigma-Aldrich) immediately prior to development of the plate.

Cell fix: 1 ml Cytofix (BD Biosciences) with 9 ml distilled water

2.3 Peptides for human and mouse experiments

HIV-1 subtype B Gag peptides were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. A total of 123 15-mer peptides overlapping by 11 amino acids provided complete coverage of the HIV-1 subtype B Gag p55 protein. Peptides were dissolved in 20% DMSO/PBS to generate 10 mg/ml stock solutions. From these stock solutions a working stock solution containing 40 μg/ml of each peptide was generated. The sequences of the peptides are shown in the Appendix.

FEC and CMV peptides restricted by common HLA class I alleles were synthesised in house, dissolved in 20% DMSO/PBS and pooled to create a 40 μg/ml working stock. The sequences of the peptides used are shown in the Appendix.

HCV peptides obtained from the Biodefense and Emerging Infections Research Resources Repository (NIAID, NIH, USA) were dissolved in 20% DMSO/PBS to create a 40 μg/ml working stock. The sequences of the peptides used are shown in the Appendix.

For mouse experiments, 9 and 10-mer peptides (ProImmune, Oxford, UK) were resuspended in 20% DMSO/PBS at a stock concentration of 10 mg/ml. The sequences of the peptides used are shown in the Appendix.

2.4 Identification and characterisation of IL-10-producing cells

2.4.1 Isolation and freezing of PBMC

PBMC were isolated from whole blood within 4 hours of sampling by Ficoll-Hypaque density centrifugation. The plasma layer was removed and stored at -80°C. PBMC were washed and resuspended in R10 medium for counting. They were then cryopreserved at a density of 10^7/ml in 10% DMSO/FCS and stored in liquid nitrogen.

2.4.2 Cytokine capture assay

Cryopreserved PBMC were thawed, washed and resuspended in H10 medium and rested overnight at 37°C and 5% CO_2. The following day, PBMC were resuspended in H10 at a density of 10^7/ml and stimulated for 3 hours at 37°C with a 0.05% DMSO/PBS control or with a pool of 123 overlapping 15-mer peptides (2.5 μg/ml) based on the HIV-1 clade B Gag p55 protein sequence (Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; see section 2.3 and Appendix), then labelled with a bispecific capture antibody that binds both CD45 on the surface of leukocytes and secreted IL-10 (Miltenyi Biotec, Germany) for 45 minutes at 37°C. Cells were incubated with a PE-conjugated anti-IL-10 antibody, followed by anti-PE antibody-coated microbeads, and enriched by passage through a magnetic field. The

enriched cell fraction was stained with CD3-APC-Cy7, CD4-FITC, CD8-APC, CD14-Pacific Blue, CD19-PerCP (BD Biosciences, UK) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen). Cells were fixed for 15 minutes with 2% paraformaldehyde before analysis by flow cytometry.

2.4.3 Phenotyping of IL-10+ CD8+ T cells

Cryopreserved PBMC were thawed and subjected to the cytokine capture assay as above, but labelled with a bispecific IFN-γ capture antibody in addition to the IL-10 capture antibody, and a FITC-conjugated anti-IFN-γ antibody in addition to the PE-conjugated anti-IL-10 antibody (Miltenyi Biotec). Following enrichment using anti-PE microbeads, the enriched cell fraction was stained with CD3-APC-Cy7, CD8-PerCP, β7-PE-Cy5 (BD Biosciences), CD25-Alexa Fluor 700, CXCR3-Pacific blue or FoxP3-Pacific blue (Biolegend, UK) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen). Cells were fixed as above and analysed by flow cytometry.

2.4.4 Luminex analysis of plasma IL-10 levels

Plasma IL-10 levels were measured using a Bio-Plex Pro (Bio-Rad, UK) human IL-10 cytokine assay, according to the manufacturer's instructions. Briefly, frozen plasma samples were thawed and added to a 96 well plate that had been pre-treated with magnetic beads coupled to an anti-IL-10 antibody and incubated at room temperature for 1 hour. The plate was washed and incubated with a biotinylated anti-IL-10 antibody, followed by PE-conjugated streptavidin. After removal of excess PE-conjugated streptavidin, the plate was analysed immediately using a Luminex-100 system and Bio-Plex Manager™ software version 4.1. The concentration of IL-10 for

each sample was estimated based on fluorescence values obtained from a standard curve constructed using 4-fold serial dilutions of the IL-10 standard of known concentration supplied by the manufacturers.

2.5 Phenotyping of PBMC from chronically HIV-infected individuals

2.5.1 Analysis of expression of T cell activation markers

Cryopreserved PBMC were thawed, washed and resuspended in H10 and rested overnight at 37°C and 5% CO_2. The following day PBMC were washed, resuspended in PBS and stained with CD3-FITC, CD4 or CD8-PerCP, CD38-PE, HLA-DR-APC (BD Biosciences) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen). Cells were fixed and analysed by flow cytometry.

2.5.2 Analysis of β7 expression

Cryopreserved PBMC were thawed, washed and resuspended in H10 and rested overnight at 37°C and 5% CO_2. The following day PBMC were washed, resuspended in PBS and stained with CD3-APC-Cy7, CD4-FITC, CD8-PerCP, β7-PE-Cy5 (BD Biosciences) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen).

2.6 Functional analysis of Gag-specific IL-10+ CD8+ T cells

2.6.1 Depletion of Gag-specific IL-10+ CD8+ T cells from PBMC

Removal of IL-10-producing CD8+ T cells was achieved in two steps. First, cryopreserved PBMC were thawed and rested in H10 medium overnight, after which CD8+ cells were isolated to >90% purity by anti-CD8 multi-sort microbead selection followed by enzymatic removal of the microbeads (Miltenyi Biotec). The CD8+ and $CD8^-$ fractions were stimulated separately with HIV-1 Gag peptides for 6 hours, after which the $CD8^-$ fraction was maintained at 4°C. The CD8+ fraction was split into two aliquots and IL-10-producing cells were depleted from one aliquot by cytokine capture and magnetic separation, as described in section 2.4.2. The other aliquot was treated identically apart from addition of the IL-10 capture antibody. The CD8+ fractions containing or depleted of IL-10+ cells were each recombined with $CD8^-$ cells and incubated either overnight, for 1 day or for 3 days in H10 medium. These cultures were analysed by ELISPOT and flow cytometry in order to assess the effect of depleting Gag-specific IL-10+ CD8 T cells (see below). Supernatants were harvested and stored at -20°C to enable subsequent determination of the effect of removing Gag-specific IL-10+ CD8 T cells on the level of T cell- and monocyte-derived cytokines and Gag p24 protein (see below).

2.6.2 IFN-γ ELISPOT assay

PBMC subjected to the depletion procedure detailed in 2.6.1 were plated at a density of 1 x 10^6/ml or 5 x 10^5/ml, depending on cell numbers, onto a 96 well filter plate

(Millipore Corporation, USA) pre-coated with anti-IFN-γ antibody 1-DIK (Mabtech, Sweden) and incubated overnight at 37°C and 5% CO_2. The following day, cells were removed for antibody staining and flow cytometry (see below) and the plate was washed 6 times with PBS/0.05% Tween20. A secondary biotinylated anti-IFN-γ antibody (Mabtech) was added and the plate incubated at room temperature for 3 hours. After a further 6 washes with PBS/Tween the plate was incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 1 hour at 37°C and 5% CO_2 and then washed. Finally, alkaline phosphatase substrate (Bio-Rad, USA) was added, allowing the direct visualisation of the captured IFN-γ. Spots were counted using an automated ELISPOT plate reader (AID Systems, Germany) and the frequencies of IFN-γ-producing cells were expressed as IFN-γ spot-forming units (SFU)/ 10^6 PBMC.

2.6.3 CD38 and HLA-DR staining

PBMC subjected to the depletion procedure detailed in 2.6.1 were harvested from ELISPOT plates that had been incubated overnight at 37°C and 5% CO_2 (see previous section). The cells were washed, resuspended in PBS and stained with CD3-FITC, CD8-PerCP, CD14-Pacific blue, CD38-PE, HLA-DR-APC (BD Biosciences) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen). Cells were fixed and analysed by flow cytometry.

2.6.4 CD80 and CD86 staining

PBMC subjected to the depletion procedure detailed in 2.6.1 were incubated either for 1 day or for 3 days at a density of 1 x 10^6/ml or 5 x 10^5/ml at 37°C and 5% CO_2. Following incubation, cells were washed, resuspended in PBS and stained with CD3-

APC-Cy7, CD14-Pacific blue, CD38-PE (BD Biosciences), CD80-FITC, CD86-APC (Biolegend) and LIVE/DEAD® fixable aqua dead cell stain (Invitrogen). Cells were fixed and analysed by flow cytometry.

2.6.5 Luminex analysis of cytokines in cell culture supernatant

The concentration of IL-2, IL-4, IL-5, IL-10, IL-12 p70, IL-13, GM-CSF, IFN-γ and TNF-α was measured in supernatants (collected as described in 2.6.1) using a Bio-Plex Pro (Bio-Rad) human Th1/Th2 cytokine panel assay. The concentration of IL-6 in supernatants was quantified in a separate experiment, using the same Bio-Plex assay. Both experiments were conducted according to the manufacturer's instructions. Briefly, supernatants were thawed, added to a 96 well plate that had been pre-treated with magnetic beads conjugated to antibodies against the cytokines of interest, and incubated at room temperature for 1 hour. The plate was washed and incubated with a cocktail of biotinylated antibodies against the cytokines of interest, followed by PE-conjugated streptavidin. After removal of excess PE-conjugated streptavidin, the plate was analysed immediately using a Luminex-100 system and Bio-Plex Manager™ software version 4.1. Cytokine concentrations for each sample were estimated based on fluorescence values obtained from a standard curve constructed using 4-fold serial dilutions of cytokine standard supplied by the manufacturers.

2.6.6 Intracellular staining for HIV Gag p24 antigen

CD8+ cells were isolated and stimulated for 6 hours with the HIV-1 subtype B Gag peptide pool to enable removal of IL-10+ CD8+ T cells, as described in 2.6.1. $CD8^-$ PBMC were incubated with 20 U/ml IL-2 (BD Biosciences) for 6 hours and then

maintained on ice. CD8+ cell aliquots, either containing or depleted of IL-10+ cells, were recombined with $CD8^-$ fractions at a density of 10^6 PBMC/ml and cultured in H10 supplemented with IL-2 (20 U/ml) for 7 days. After 3 and 7 days, supernatants were harvested and stored at -20°C for subsequent determination of the concentration of extracellular HIV-1 Gag p24 antigen (see below). PBMC were stained with LIVE/DEAD® fixable aqua dead cell stain (Invitrogen) and then permeabilised by incubation in 20 μg/ml lysolecithin in 1% paraformaldehyde at room temperature for 2 minutes. PBMC were incubated on ice in 50% methanol for 15 minutes, followed by 0.1% NP-40 on ice for 5 minutes. Cells were washed, resuspended in PBS and stained with CD3-APC-Cy7, CD4-PerCP, CD8-APC (BD Biosciences) and KC-57-FITC (Beckman Coulter) to detect intracellular HIV-1 Gag p24 antigen. They were then analysed by flow cytometry.

2.6.7 HIV Gag p24 ELISA

Supernatants collected from the experiments detailed in 2.6.1 and 2.6.6 were thawed and the concentration of HIV-1 Gag p24 antigen was measured by ELISA (Cell Biolabs, Inc.), according to the manufacturer's instructions. Briefly, supernatants were added to an ELISA plate pre-coated with anti-p24 antibody and incubated overnight at 4°C. The following day, the plate was washed and incubated, shaking, with FITC-conjugated anti-p24 antibody for 1 hour at room temperature. After further washing, HRP-conjugated anti-FITC monoclonal antibody was added and the plate incubated, shaking, for 1 hour at room temperature. The plate was washed and substrate solution added until a blue colour appeared in the wells, at which point stop solution was added.

The concentration of p24 antigen was determined immediately using a Model 680 Microplate Reader (Bio-Rad).

2.7 IL-10 receptor (IL-10R) blockade in a mouse vaccination model

2.7.1 Administration of αIL-10R antibody and HIVA or HIVconsv vaccine

Mice were injected intraperitoneally with 200 μg low endotoxin azide free (LEAF) anti-IL-10R antibody (Biolegend) or isotype control antibody. For vaccination, mice were injected intramuscularly with 50 μl per hindleg of 1mg/ml pTHr.HIVA or pTHR.HIVconsv vaccine, or with 50 μl per hindleg (10^7 plaque-forming units (PFU) in total) of MVA.HIVconsv under general anaesthesia. Mice were monitored throughout the day to ensure full recovery from anaesthesia. At the end of the experiment, mice were killed by cervical dislocation. Spleens were immediately harvested for analysis (see below).

2.7.2 Collection of peripheral blood and harvesting of spleens

During the experiment, blood was collected from the lateral tail vein on a maximum of 2 occasions into tubes containing 50mM EDTA. The erythrocytes were lysed by incubation with red cell lysis buffer (Qiagen, UK) at room temperature for 30 minutes. Cells were resuspended in R10, counted and immediately used for intracellular cytokine staining (see below). At the end of the experiment, spleens were removed immediately from mice killed as described in 2.7.1 and placed in R10. The spleen was homogenised by passage through a cell strainer and the erythrocytes lysed by incubation with red cell

lysis buffer at room temperature for 5 minutes. Splenocytes were resuspended in R10, counted, and used immediately for intracellular cytokine staining and IFN-γ ELISPOT (see below).

2.7.3 Intracellular cytokine staining for IFN-γ, TNF-α and surface staining for CD107a

Splenocytes or peripheral blood leukocytes were resuspended in R10, aliquoted into a 96 well plate (at a density of 2 x 10^6/ml for peripheral blood leukocytes and 2 x 10^7/ml for splenocytes) and stimulated with 2 μg/ml H, P or G1 peptides for 90 minutes at 37°C and 5% CO_2. Golgistop solution (BD Biosciences) was added and the plate incubated at 37°C and 5% CO_2 for 5 hours, followed by incubation overnight at 4°C. The following day, samples were blocked using anti-CD16/CD32 and stained with LIVE/DEAD® fixable aqua dead cell stain (Invitrogen) for 20 minutes at 4°C. The cells were washed with FACS buffer I and stained with CD8-PE-Texas red (Abcam) and CD19-Pacific blue (ebioscience) for 30 minutes at 4°C. After a further wash step, cells were permeabilised by incubating with Fix/Perm solution (BD Biosciences) for 20 minutes at 4°C. The cells were washed with FACS buffer II and stained with IFNγ-APC and TNF-α-PE (ebioscience) for 20 minutes at 4°C. After a further wash, cells were incubated in Cell Fix (BD Biosciences) for 30 minutes at 4°C and then analysed by flow cytometry.

2.7.4 IFN-γ ELISPOT assay

Splenocytes were resuspended in R10 at a density of 1.5 x 10^6/ml and added to an ELISPOT plate (Millipore Corporation, USA) that had been pre-coated with anti-IFN-γ antibody AN18 (Mabtech). Cells were stimulated with 2 μg/ml H, P or G1 peptides

overnight at 37°C and 5% CO_2. The next day, the cells were discarded and the plate was washed with PBS and incubated with a biotinylated anti-IFN-γ antibody for 2 hours at room temperature. Following a further wash step, the plate was incubated with HRP-conjugated streptavidin at room temperature for 1 hour. The streptavidin was washed off and substrate solution added for approximately 30 minutes, or until spots appeared. Spots were counted using an automated ELISPOT plate reader (AID Systems, Germany) and the frequencies of IFN-γ-producing cells were expressed as IFN-γ spot-forming units (SFU)/ 10^6 PBMC.

2.7.5 *In vivo* killing assay

Splenocytes from non-immunised female BALB/c mice were harvested and isolated as previously and divided into four equal aliquots. Three of the aliquots were pulsed with 10 μg/ml H, P or G1 peptide at room temperature for 90 minutes, with the fourth aliquot remaining unpulsed. The splenocytes were washed and then incubated in 5-fold serial dilutions of carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) at room temperature for 10 minutes. The H-pulsed cells were incubated in 400 nM CSFE, the P-pulsed cells in 80 nM CSFE, the G1-pulsed cells in 16 nM CFSE and the unpulsed cells in PBS. FCS was added to the splenocytes, which were incubated on ice for five minutes and then washed in R10. The unpulsed cells were then incubated in 8 μM (5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine (CMTMR, Invitrogen), and the H-, P- and G1-pulsed cells in R10, at 37°C for 12 minutes, followed by a wash and a second incubation at 37°C for 15 minutes. After three further washes, the splenocytes were counted, resuspended in PBS and an equal number from each aliquot combined to give a concentration of 5 x 10^7/ml. 200 μl of the

resulting cell suspension was immediately injected into the lateral tail vein of each immunised mouse. After 15 hours the immunised mice were killed by cervical dislocation and the splenocytes harvested, isolated as previously and resuspended in PBS. The cells were incubated in cell fix for 30 minutes and then analysed by flow cytometry.

2.8 Statistical analyses

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software (GraphPad Software). P values < 0.05 were considered significant. All data sets were analysed using the D'Agostino & Pearson omnibus normality test to determine whether they followed a Gaussian (normal) distribution. Normally distributed data sets were then analysed using parametric tests (ANOVA, t test, Pearson's product moment correlation), whilst data sets that were not normally distributed were analysed using non-parametric tests (Kruskal-Wallis test, Wilcoxon matched-pairs signed rank test, Friedman test, Spearman's rank correlation).

Chapter 3: Cellular sources of IL-10 in chronic HIV-1 infection and the relationship with immune activation

3.1 Introduction

The immunomodulatory cytokine IL-10 is upregulated in both acute (52) and chronic (356) HIV-1 infection but its cellular sources remain poorly defined. Previous studies have either examined mRNA expression (356), which may not correlate with protein production, or have focussed exclusively on T cells (358-360) without taking into account the production of IL-10 by other cell types. In this study, a cytokine capture assay, using a bispecific antibody that binds the cytokine to the surface of the cell that secretes it, was used to examine IL-10 production by CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes in patients with chronic HIV-1 infection. The advantage of this assay over methods such as ELISPOT or intracellular cytokine staining is that IL-10+ cells can be enriched from the cell population as a whole and remain intact, allowing them to be used for phenotyping and functional assays (see chapters 4 and 5). Both constitutive and HIV-1 Gag-specific IL-10 production were examined. The Gag protein was chosen as a model antigen because it is one of the more highly conserved and immunodominant proteins in the HIV-1 proteome (212, 362). An overlapping peptide set based on the entire Gag clade B sequence was chosen on the basis that this clade, along with Clade C, is the most common virus clade seen in HIV-infected people in the UK (363, 364). IL-10 secretion was compared in patients receiving ART with undetectable peripheral blood viral loads, ART-naïve patients with detectable circulating viraemia and HIV-negative controls to determine whether HIV-

specific production of the cytokine was restricted to a particular patient group and/or cell population.

3.2 Study subjects

62 HIV-positive patients were enrolled from two clinics at the Churchill Hospital, Oxford. Patients receiving treatment (designated "ART") had maintained undetectable viral loads (<40 copies/ml) for 12 months or longer. Patients designated "ART-naïve" had never received treatment and had detectable plasma viraemia (>40 copies/ml) except for one patient who had never received treatment but had undetectable plasma viraemia and was thus designated as an elite controller. All subjects displayed CD4+ T cell counts of >200/μl at the time of sampling. Clinical characteristics of both groups are summarised in Table 3.1. Due to sample availability, not all the assays in this chapter were conducted on all the patients listed.

	ART	**ART-naïve**
Number of patients	24	38
Median age (years)	46	34
Male:female ratio	15:9	21:17
Median time since diagnosis, when known (years)	7	2
Median CD4 count (cells/μl) at time of sampling	395	450
Median viral load (copies/ml) at time of sampling	<40	21493

Table 3.1 Clinical characteristics of ART and ART-naïve patients in the cohort.

3.3 Constitutive IL-10 production in HIV-infected subjects and uninfected controls

In order to determine whether chronic HIV-1 infection increases constitutive production of IL-10 by PBMC, the cytokine capture assay was performed in the absence of peptide stimulation on cryopreserved PBMC from 20 ART, 23 ART-naïve and 5 uninfected subjects. Constitutive IL-10 production in CD8+ T cells only was examined in a further 12 ART-naïve subjects. After IL-10 capture, IL-10+ cells were enriched by magnetic separation, with 10% of the cells retained as an unenriched fraction, and examined by flow cytometry. The gating strategy for the identification of CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes (using the unenriched fraction) is shown in Figure 3.1. For each cell subset, the frequency of IL-10+ cells was determined using the following calculation:

$$\%\ \text{producing IL-10} = \frac{\text{number of IL-10-producing cells in enriched sample}}{(\text{total number of cells in unenriched fraction} \times 9)} \times 100\%$$

A minimum of 10 000 live events per unenriched sample were collected, with result that a minimum of 90 000 cells were enriched for IL-10+ cells. The SEB-based superantigen Cytostim (Miltenyi Biotec, Germany), which stimulates IL-10 production, particularly by CD4+ T cells, in the majority of individuals, was used as a positive control, and only patients who mounted a response to Cytostim were included in the analysis (Figure 3.2).

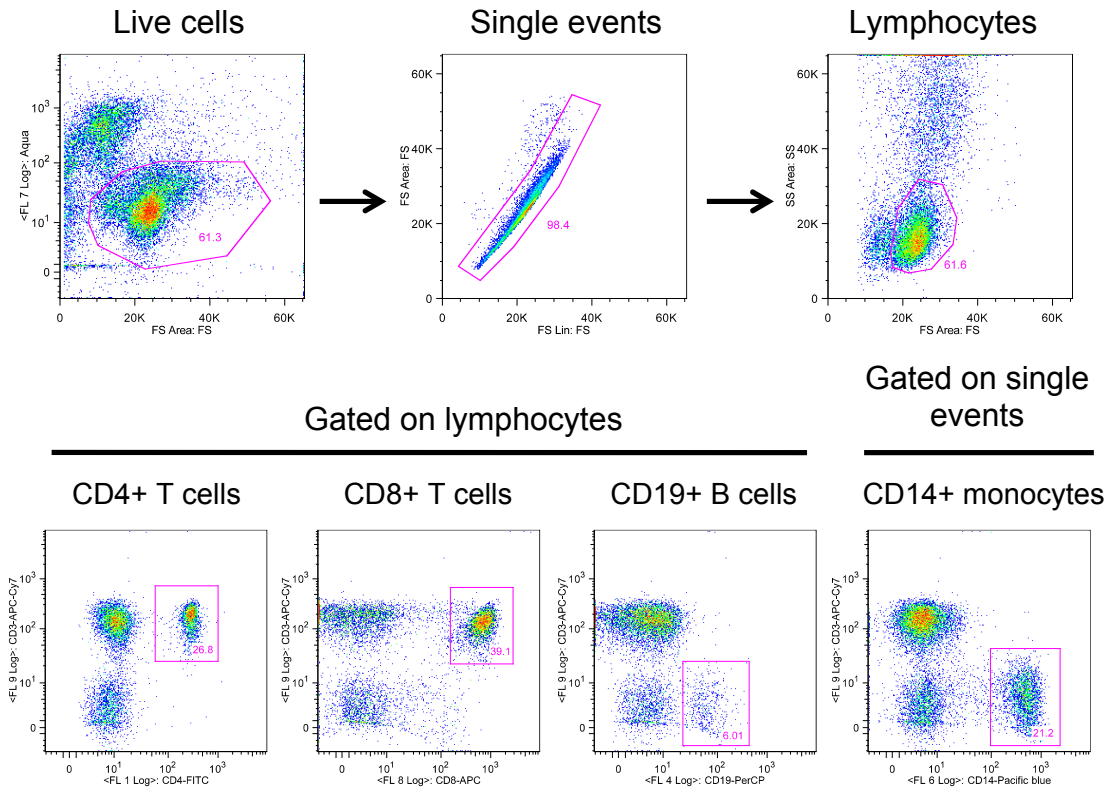


Figure 3.1 Gating strategy for the identification of CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes. PBMC were stained with the following antibody panel: CD3-APC-Cy7; CD4-FITC; CD8-APC; CD19-PerCP; CD14-Pacific blue; IL-10-PE and Aqua live/dead cell stain.

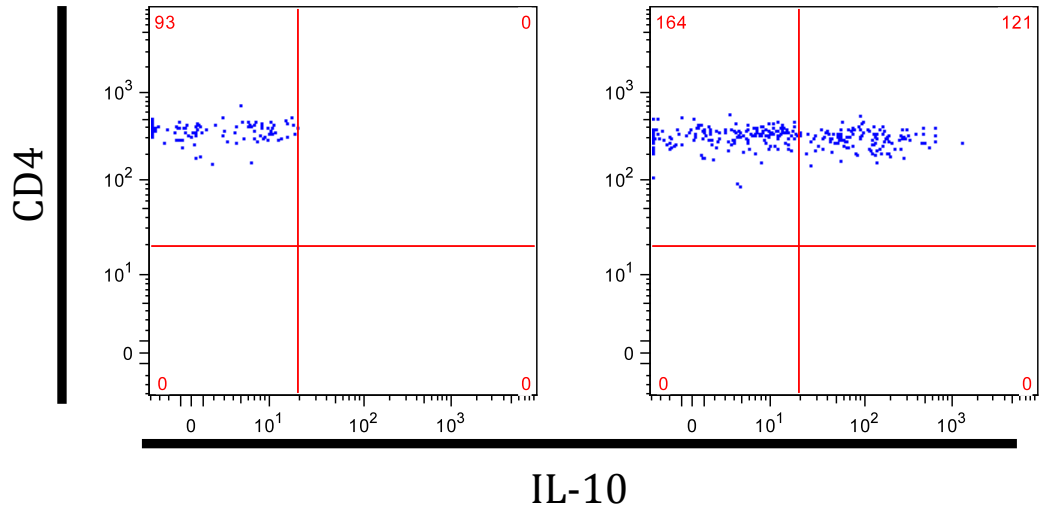


Figure 3.2 Representative flow cytometric plots from a subject showing constitutive *ex vivo* IL-10 production (left) and IL-10 production after stimulation with Cytostim (right) by CD4+ T cells. Only subjects who mounted a response to Cytostim (≥ 0.05% of CD4+ T cells IL-10+) were included in subsequent analyses.

Figure 3.3 shows representative flow cytometric plots of constitutive IL-10 production by an ART patient, ART-naïve patient and uninfected control. IL-10+ B cells were found in the ART (median frequency 0.068%), ART-naïve (0.086%) and untreated (0.040%) groups (Figure 3.4). There was no significant difference in the frequencies of IL-10+ B cells between any of the groups, as determined by the Kruskal-Wallis test. IL-10+ monocytes were also detected in all three groups (ART median = 0.041%; ART-naïve = 0.050%; uninfected = 0.028%) but again there was no statistically significant difference between the groups. Very few IL-10+ CD4+ T cells (ART median = 0.003%; ART-naïve = 0%; uninfected = 0%) or CD8+ T cells (ART median = 0.001%; ART-naïve = 0.001%; uninfected = 0%) were detected in any of the groups.

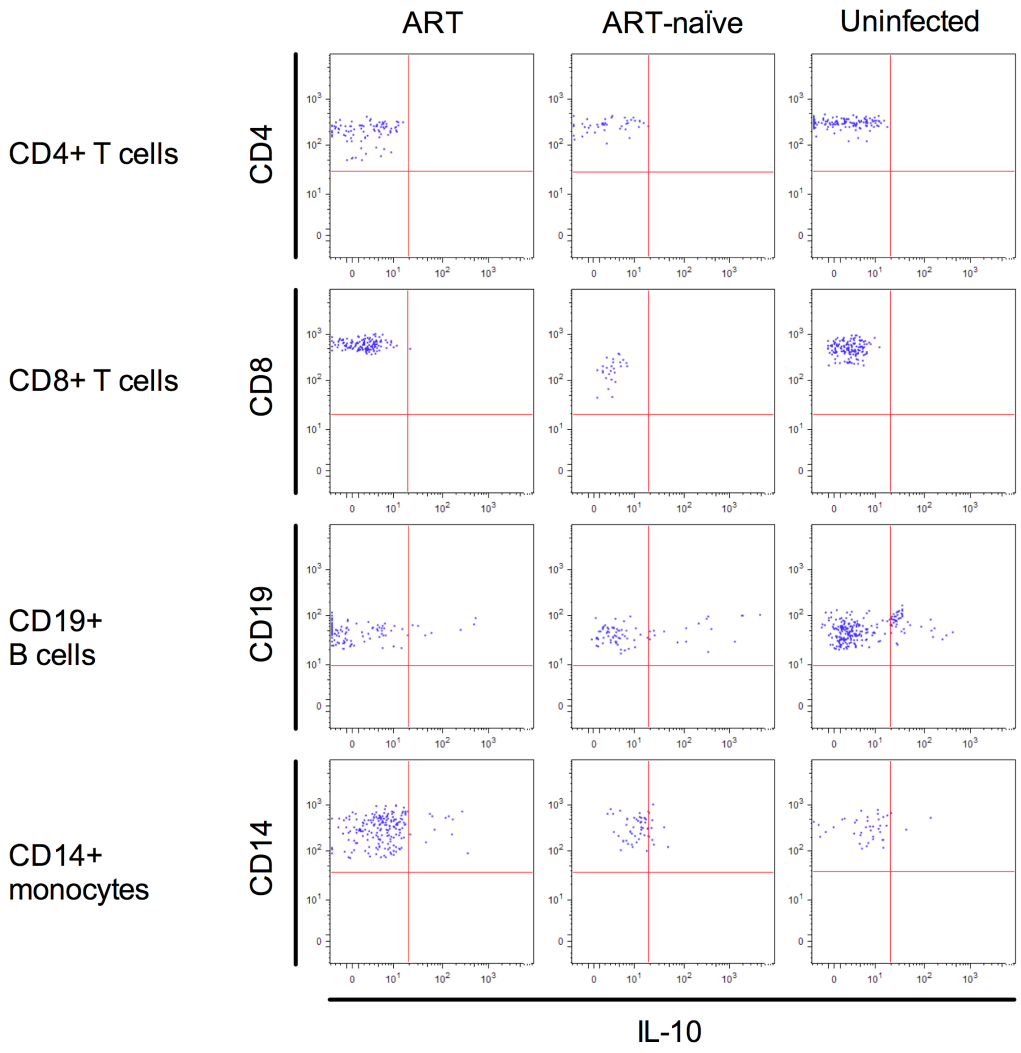


Figure 3.3 Representative flow cytometric plots showing constitutive *ex vivo* IL-10 production by CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes in a HIV-infected subject on ART, ART-naïve HIV-infected subject and uninfected control. Cells were incubated with an IL-10 capture antibody, anti-IL-10-PE and anti-PE magnetic beads and then passed through a magnetic field in order to isolate IL-10+ cells.

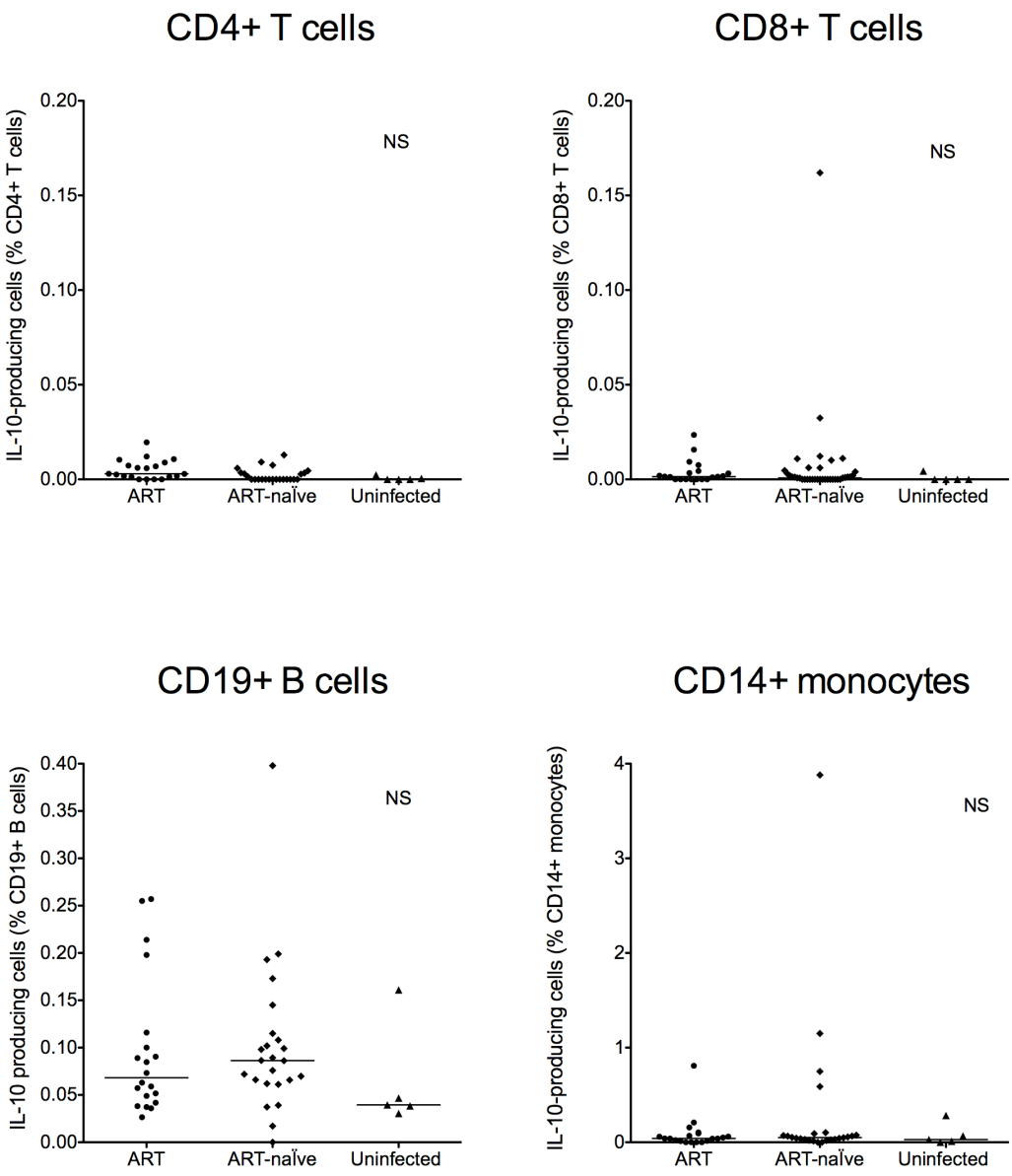


Figure 3.4 Constitutive *ex vivo* IL-10 production by CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes in HIV-infected subjects on ART, ART-naïve HIV-infected subjects and uninfected controls. For each cell subset, the frequency of IL-10+ cells was determined by multiplying the total number of cells of that subset in the unenriched fraction by 9 and then dividing the number of IL-10+ cells of that subset in the enriched fraction by this number. IL-10+ cells were enriched from a minimum of 90 000 live cells. Graph scales are conserved between this figure and figure 3.7 for consistency. Kruskal-Wallis test used for statistical analysis.

To further characterise total constitutive production of IL-10, levels of IL-10 in the plasma of 23 ART, 19 ART-naïve and 2 uninfected subjects were examined by luminex assay. Plasma IL-10 levels were below the limit of detection of the assay (1 pg/ml) in the majority of subjects (median 0.45 pg/ml for ART subjects, 0.79 pg/ml for ART-naïve and 0.50 pg/ml for uninfected) and no significant differences were found between the groups (Figure 3.5).

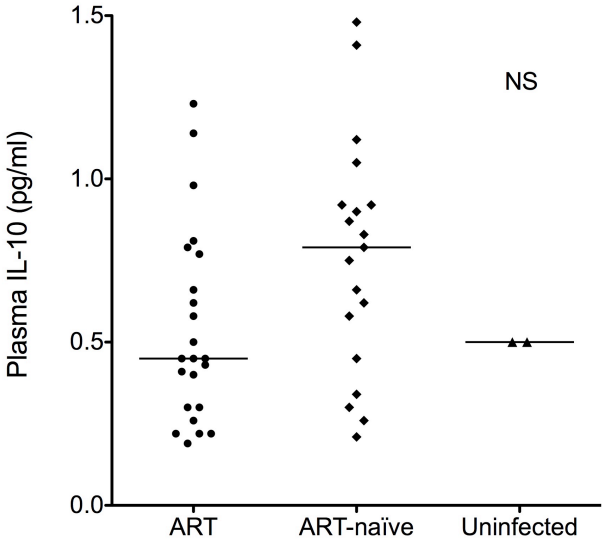


Figure 3.5 Levels of IL-10 in the plasma of HIV-infected subjects on ART, ART-naïve HIV-infected subjects and uninfected controls. Statistical analysis was conducted using Kruskal-Wallis test.

3.4 HIV-1 Gag-specific IL-10 production by HIV-infected subjects and uninfected controls

Since constitutive IL-10 production did not appear to vary between HIV-infected and uninfected individuals, the cytokine capture assay was next used to examine IL-10 production in response to a three hour stimulation with HIV-1 Clade B Gag peptides (2.5 μg/ml) in the same subjects. The same gating strategy was used and the same cell types were analysed.

Figure 3.6 shows representative flow cytometric plots of IL-10 production after HIV-1 Gag stimulation by an ART patient, ART-naïve patient and uninfected control. For each cell type, responses more than double the mean constitutive response were considered positive. When constitutive IL-10 production was subtracted, there was no significant difference between any of the groups in terms of IL-10 production by CD4+ T cells, B cells or monocytes ($p > 0.05$; Kruskal-Wallis test) (Figure 3.7). However, the frequency of Gag-specific IL-10+ CD8+ T cells was significantly elevated in ART-naïve subjects (median 0.011%) compared to both ART subjects (median 0.001%; $p < 0.01$ by Kruskal-Wallis test) and uninfected controls (median 0%; $p < 0.05$). Very little Gag-specific IL-10 production was detected in either the ART or uninfected groups. In a minority of ART and ART-naïve subjects Gag-specific IL-10+ CD4+ T cells were detected, but the median frequency of these cells was less than 0.003% (the cut off for positive responses) in both groups.

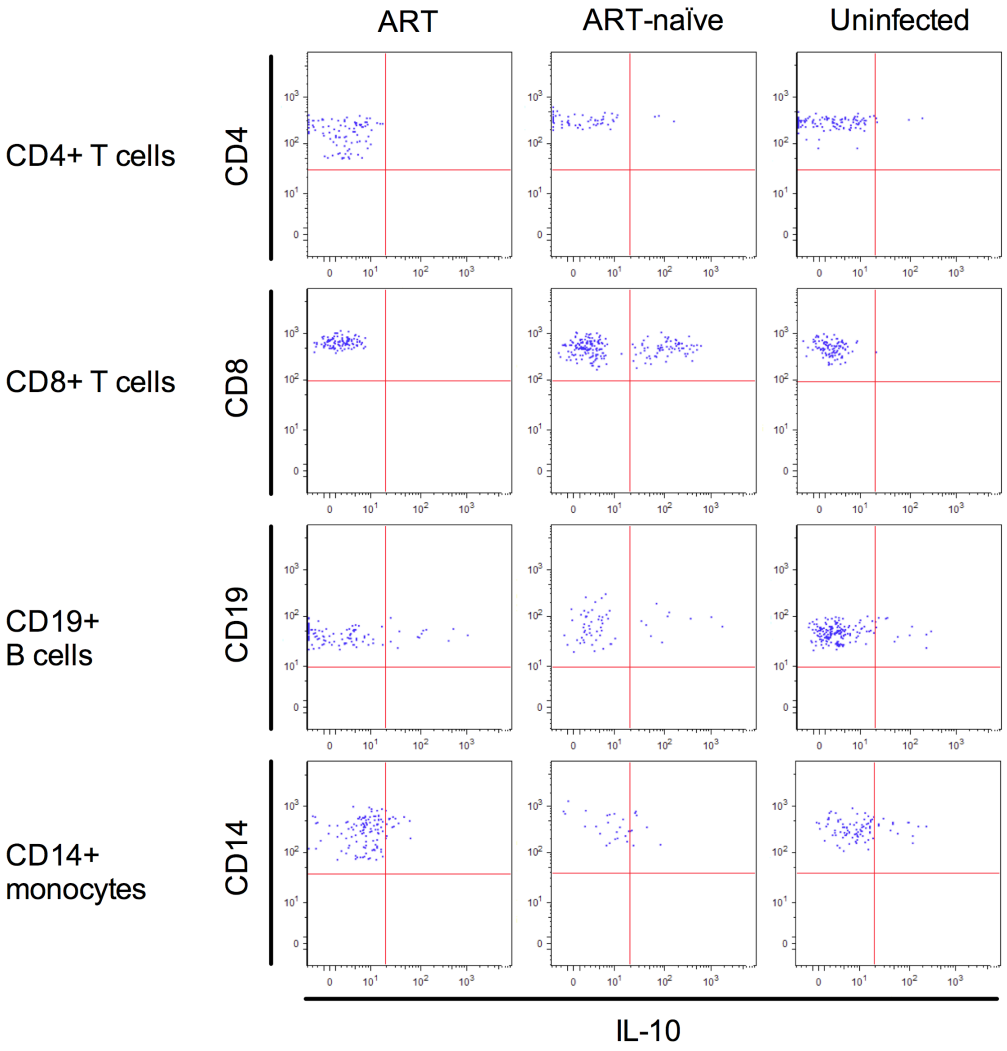


Figure 3.6 Representative flow cytometric plots showing IL-10 production after a three hour stimulation with HIV-1 Gag by CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes in a HIV-infected subject on ART, ART-naïve HIV-infected subject and uninfected control.

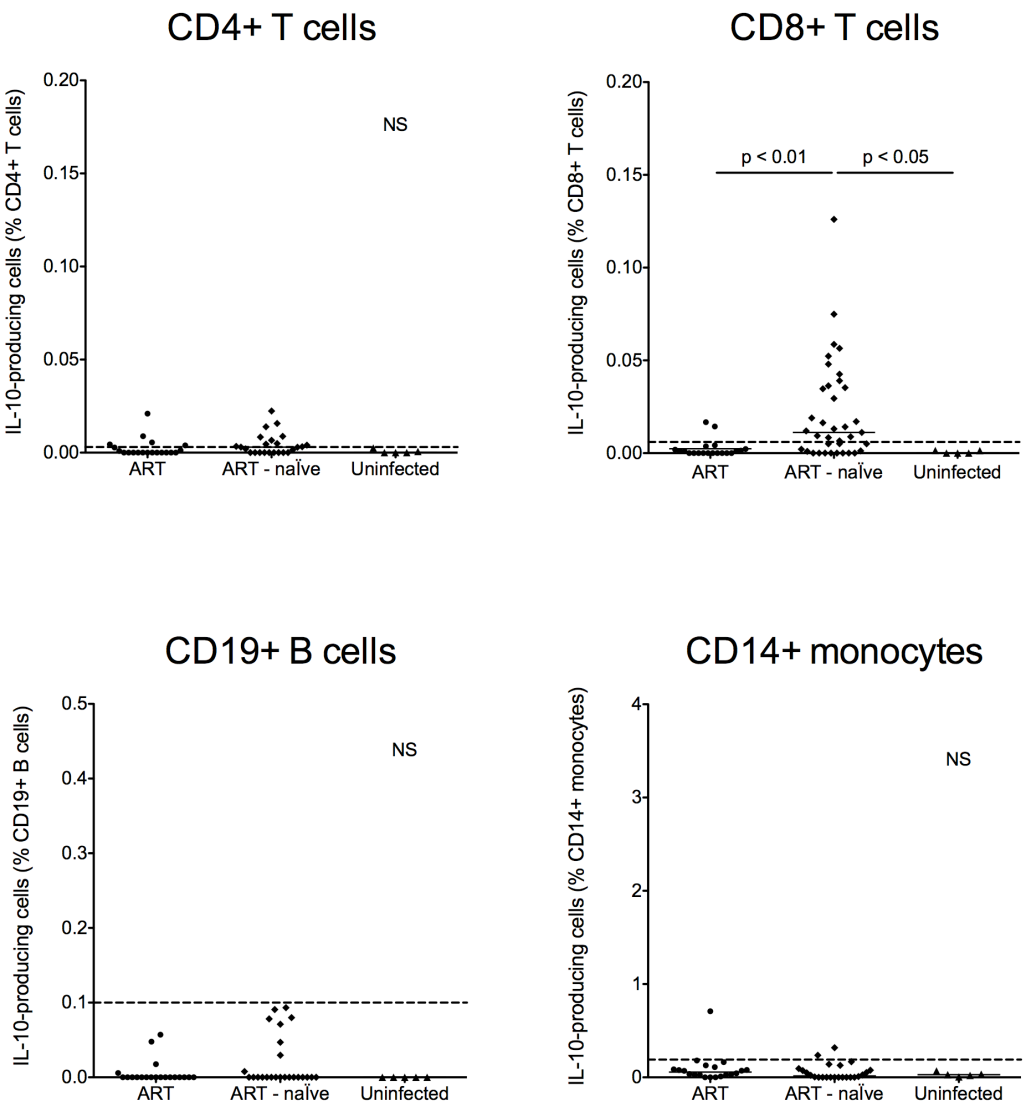


Figure 3.7 HIV-1 Gag-specific IL-10 production by CD4+ T cells, CD8+ T cells, B cells and monocytes in HIV-infected subjects receiving ART, ART-naïve HIV-infected subjects and uninfected controls. PBMC were stimulated for three hours with 2.5 µg/ml overlapping Clade B Gag peptides. IL-10+ cells were enriched from a minimum of 90 000 live cells. Constitutive IL-10 levels from figure 3.4 have been subtracted and graph scales are conserved between this figure and figure 3.4 for consistency. The dashed lines show the cut off point for positive responses for each cell type (equal to double the mean constitutive expression by that cell type). Kruskal-Wallis test used for statistical analysis.

Analysis of the contribution of each cell type to total IL-10 production can be confounded by the fact that some cell types are found in greater numbers in the peripheral blood than others, for example B cells typically comprise 15-30% of peripheral blood lymphocytes, whereas T cells account for 65-85% (365). Reporting the frequency of IL-10+ cells of each subtype can therefore distort this analysis. In order to overcome this, the absolute number of IL-10+ cells of each type was quantified in PBMC obtained from each patient. This analysis showed that for all three subject groups, CD19+ B cells were the predominant constitutive producers of IL-10. However, when Gag-specific IL-10 production in ART-naïve subjects was examined, the majority of IL-10-producing cells were CD8+ T cells (Figure 3.8). Gag-specific IL-10 production in ART-treated patients and uninfected subjects was not included in this analysis since the majority of these individuals had no detectable IL-10 production in response to Gag stimulation. Collectively, these results indicate that the majority of HIV-1 Gag-specific IL-10 production was mediated by CD8+ T cells, and that these cells are selectively enriched in ART-naïve viraemic individuals.

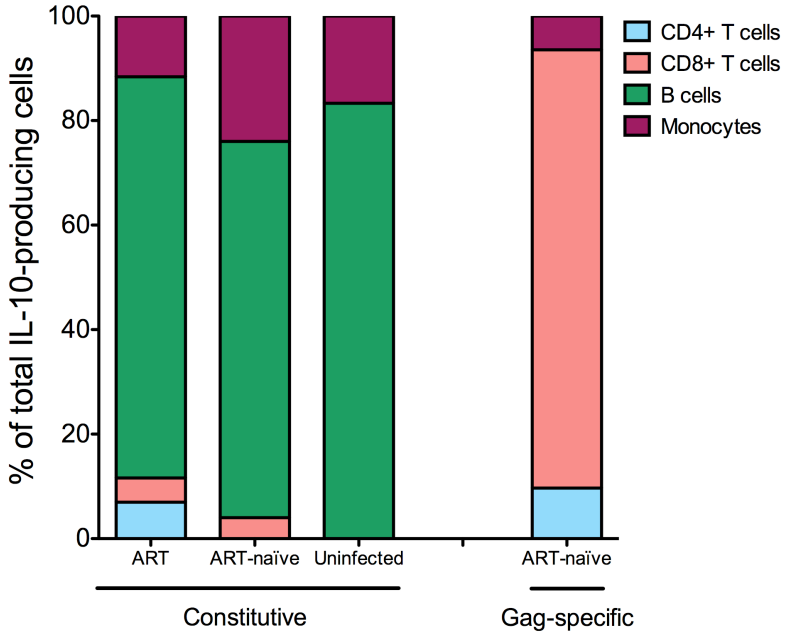


Figure 3.8 Contribution of each cell type to constitutive IL-10 production (determined by the median number of IL-10+ cells of each type in each patient group) compared to Gag-specific IL-10 production in ART-naïve HIV-infected subjects. Gag-specific IL-10 production was quantified by subtracting the number of IL-10+ cells found in unstimulated cultures from the number of IL-10+ cells found in Gag-stimulated cultures. Gag-specific IL-10 production by ART and uninfected subjects was omitted from this analysis as in the majority of these individuals no Gag-specific IL-10 production was detected.

The observation that Gag-specific IL-10+ CD8+ T cells were detected almost exclusively in ART-naïve subjects suggested that they may arise in response to ongoing viral replication; therefore the relationship between the frequency of these cells and viral load was examined (Figure 3.9). When ART subjects were included there was a significant positive correlation between frequency of Gag-specific IL-10+ CD8+ T cells and viral load (Spearman's rank correlation; $r = 0.48$; $p = 0.0006$). However, this correlation was not maintained when ART subjects were excluded from the analysis ($p > 0.05$).

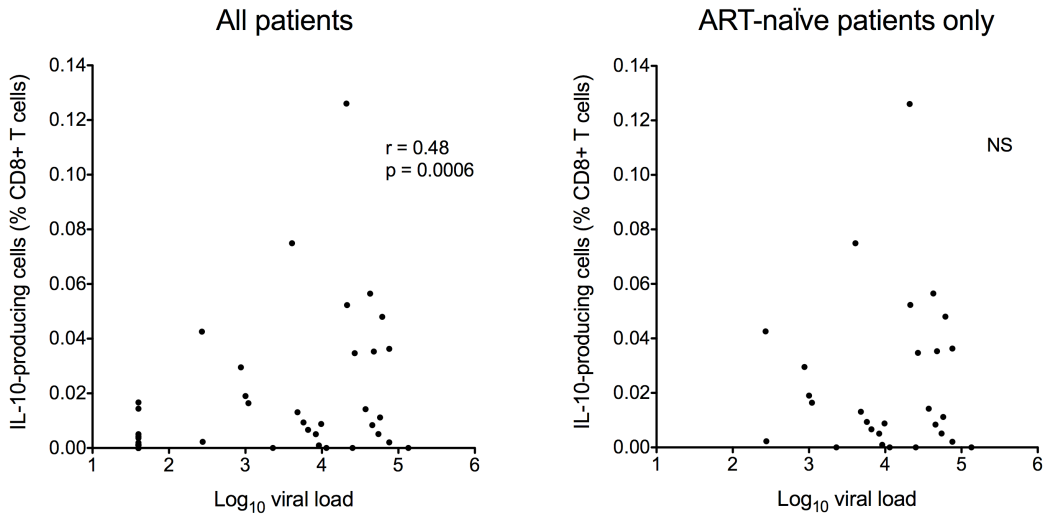


Figure 3.9 Correlation between the frequency of Gag-specific IL-10+ CD8+ T cells and viral load in HIV-infected subjects when ART patients are included in the analysis (left) and when they are excluded from the analysis (right). Spearman's rank correlation used for statistical analysis.

3.5 T cell activation in HIV infection

IL-10-producing T cells are induced in settings of both acute and chronic infection or inflammation and may help to limit damage to the host caused by immune activation (366, 367). Elevated T cell activation, defined by increased expression of the ectoenzyme CD38 and the MHC Class II molecule HLA-DR, has been associated with accelerated disease progression in HIV-1 infection (368, 369), with anti-retroviral therapy reducing T cell activation, but not to pre-infection levels (370). It is therefore possible that Gag-specific IL-10+ CD8+ T cells arise in response to chronic immune activation in HIV-infected individuals. In order to investigate this possibility, the constitutive *ex vivo* expression of CD38 and HLA-DR on CD4+ T cells and CD8+ T cells of patients from the same cohort was examined by flow cytometry. Expression of the markers was examined in 22 ART, 20 ART-naïve and 6 uninfected subjects. The gating strategy used for the identification of $CD38^{hi}$ $HLA\text{-}DR^{hi}$ CD4+ and CD8+ T cells is shown in Figure 3.10.

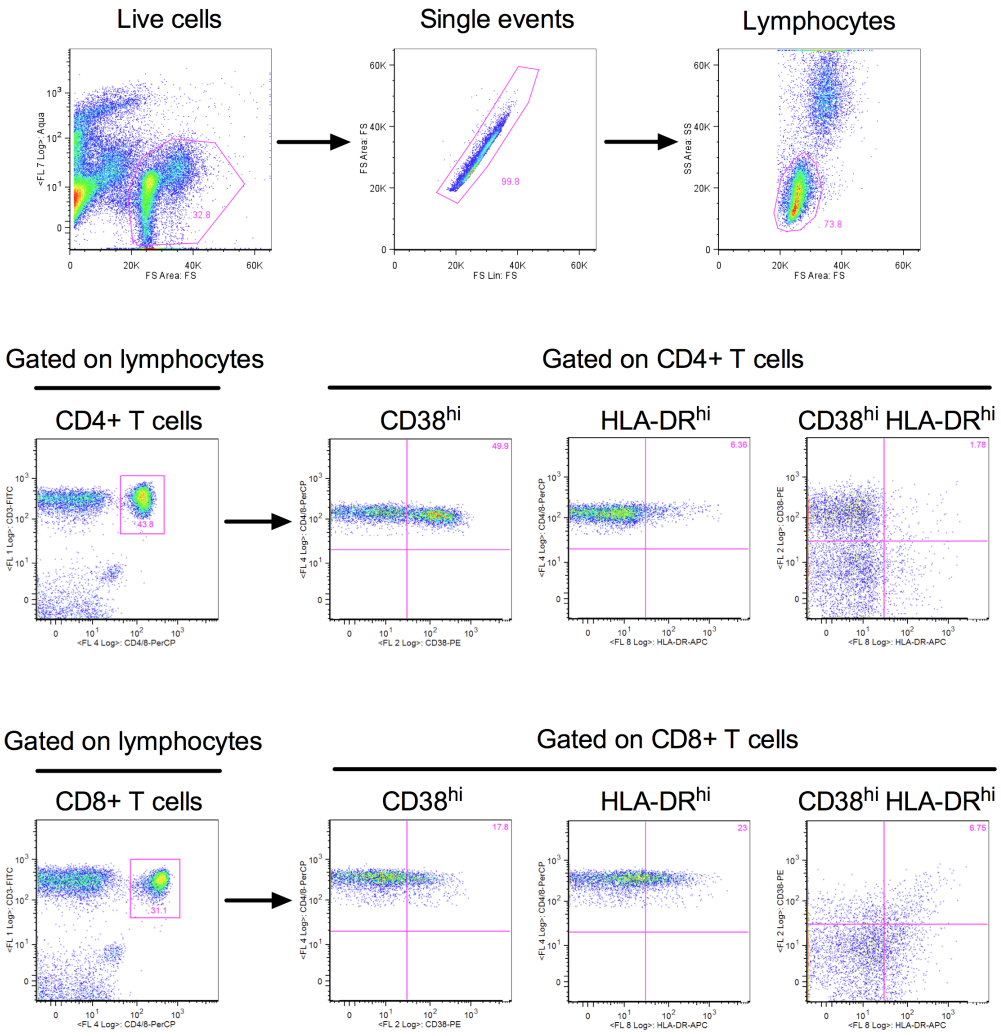


Figure 3.10 Gating strategy for the identification of activated ($CD38^{hi}$ $HLA\text{-}DR^{hi}$) CD4+ T cells and CD8+ T cells. PBMC were stained with the following antibody panel: CD3-FITC; CD4-PerCP or CD8-PerCP; CD38-PE; HLA-DR-APC and Aqua live/dead cell stain. CD14+, CD16+ and CD19+ cells were gated out using Pacific blue antibodies for each of these molecules. $CD38^{hi}$ and $HLA\text{-}DR^{hi}$ cells were gated individually and this information was then used to gate cells that expressed high levels of both markers.

Figure 3.11 shows representative flow cytometric plots of CD38^{hi} HLA-DR^{hi} CD4+ and CD8+ T cells from an ART patient, an ART-naïve patient and an uninfected control. The frequency of CD38^{hi} HLA-DR^{hi} CD4+ T cells and CD8+ T cells and was significantly elevated in ART-naïve subjects compared to both ART subjects and uninfected controls, with a p value <0.001 in all cases (Kruskal-Wallis test) (Figure 3.12). There was no significant difference in CD4+ T cell or CD8+ T cell activation between ART subjects and uninfected controls ($p > 0.05$).

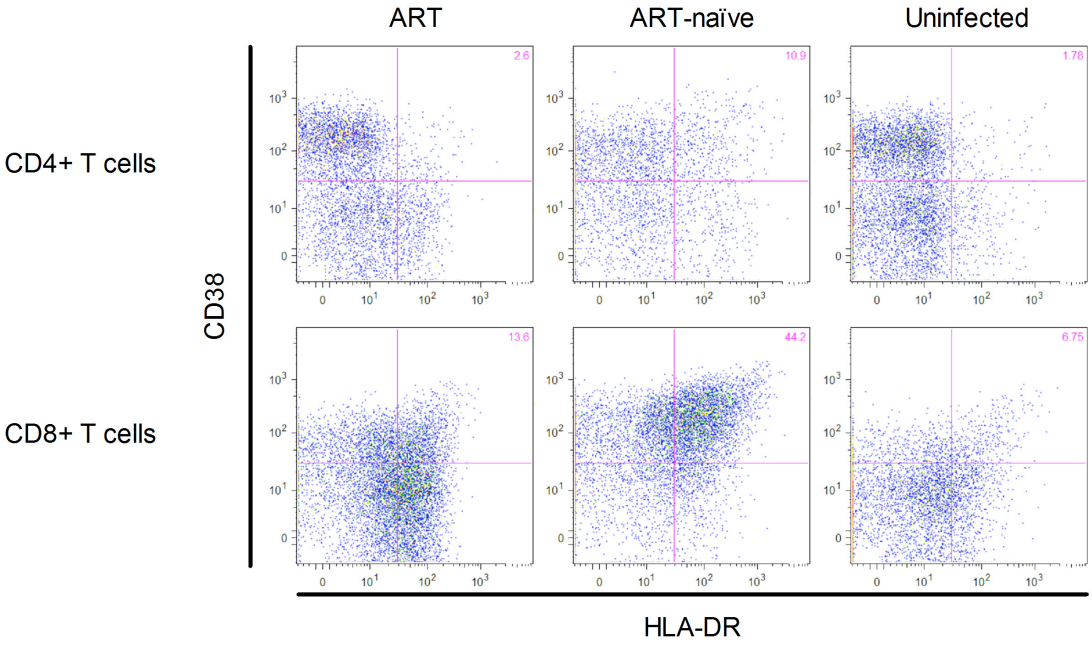


Figure 3.11 Representative flow cytometric plots showing the percentage of activated (CD38^{hi} HL-DR^{hi}) CD4+ T cells and CD8+ T cells sampled *ex vivo* in a HIV-infected subject receiving ART, an ART-naïve HIV-infected subject and an uninfected control.

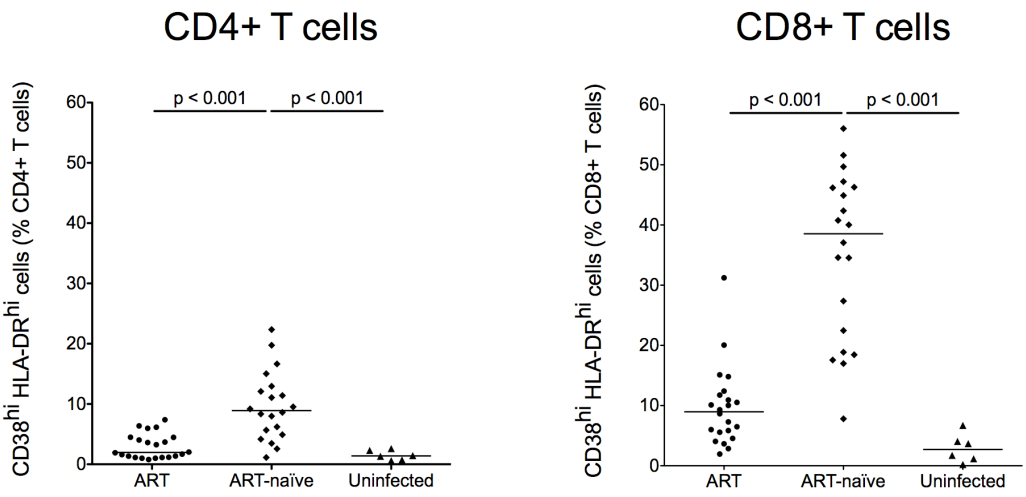


Figure 3.12 Frequency of activated ($CD38^{hi}$ $HLA\text{-}DR^{hi}$) CD4+ T cells and CD8+ T cells in HIV-infected subjects receiving ART, ART-naïve HIV-infected subjects and uninfected controls. A minimum of 10 000 lymphocytes were collected for analysis from each subject. Kruskal-Wallis test used for statistical analysis.

Since the frequencies of activated CD4+ and CD8+ T cells were increased in ART-naïve subjects, the correlation between the percentage of $CD38^{hi}$ $HLA\text{-}DR^{hi}$ CD4+ T cells and CD8+ T cells and viral load was investigated in ART-naïve subjects. There was a significant positive correlation between viral load and the percentage of activated CD4+ T cells (Pearson's product moment correlation; $r = 0.45$; $p = 0.046$) and CD8+ T cells ($r = 0.45$; $p = 0.046$) (Figure 3.13).

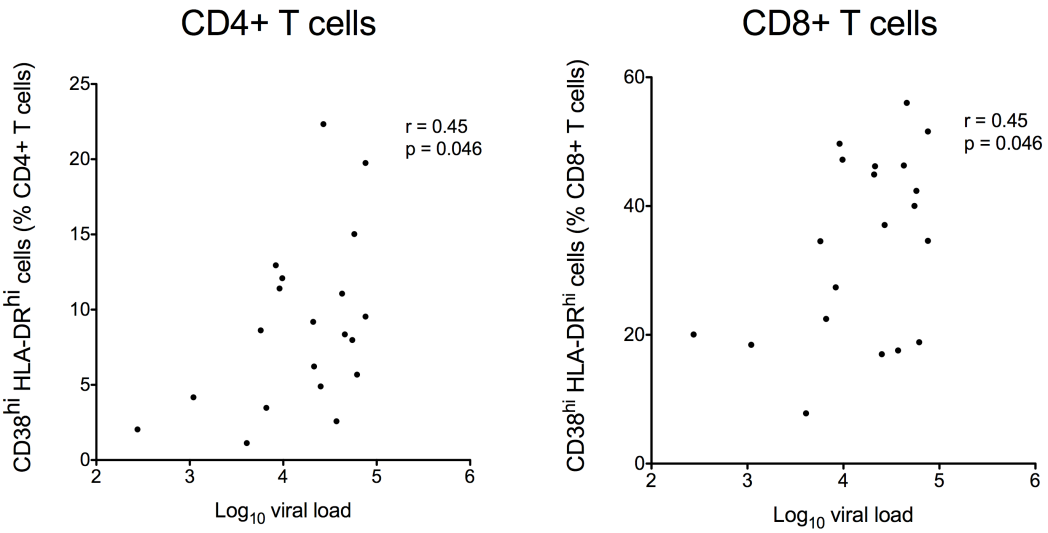


Figure 3.13 Correlation between activated ($CD38^{hi}$ $HLA\text{-}DR^{hi}$) CD4+ T cells and CD8+ T cells and viral load. Pearson's product moment correlation used for statistical analysis.

3.6 Relationship between gag-specific IL-10+ CD8+ T cells and T cell activation

To further investigate the possibility that Gag-specific IL-10+ CD8+ T cells arise in response to T cell activation, the correlation between these two variables was examined in ART-naïve individuals. There was no significant correlation between the frequency of Gag-specific IL-10+ CD8+ T cells and CD4+ T cell activation or CD8+ T cell activation (Spearman's rank correlation; $p > 0.05$).

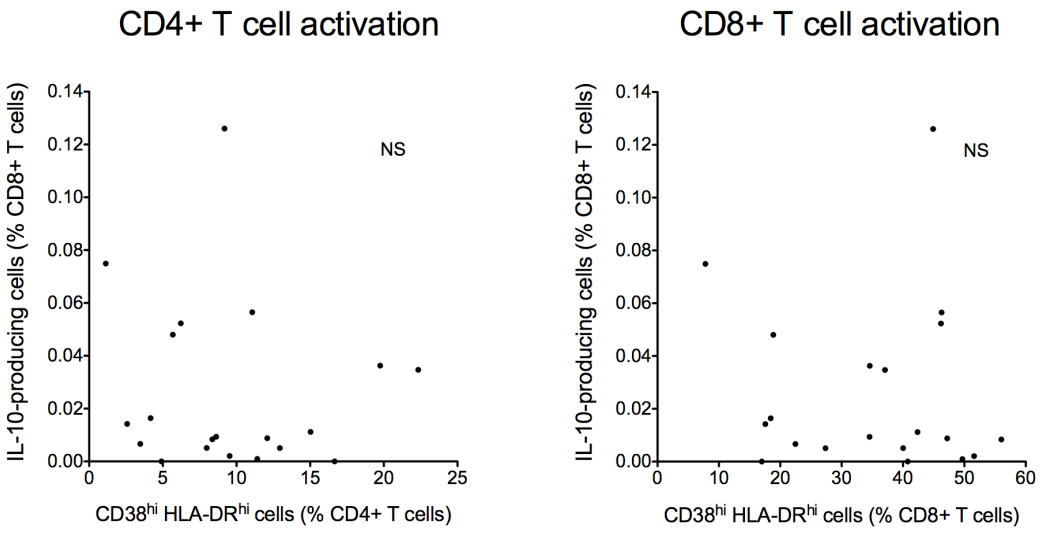


Figure 3.14 The relationship between the frequency of HIV-1 Gag-specific IL-10+ CD8+ T cells and CD4+ T cell activation (left) or CD8+ T cell activation (right) in ART-naïve patients. Spearman's rank correlation; $p > 0.05$.

3.7 Discussion

The experiments described in this chapter sought to identify the cellular sources of IL-10, both constitutive and HIV-specific, in chronic HIV-1 infection. Studies seeking to address this question have previously been conducted but have either examined mRNA rather than protein expression (356) or focussed purely on T cells (358-360), without taking into account other potential sources of IL-10. Moreover, the studies examining IL-10 protein expression have focussed exclusively on HIV-specific IL-10 production without addressing whether IL-10 is upregulated constitutively in HIV-infected individuals.

A cytokine capture assay was used to examine constitutive production of IL-10 by both HIV-infected and uninfected individuals. This technique allows frequencies of IL-10+ cells as low as 0.001% to be detected. Furthermore, the cells are left intact and functional, and can be enriched for further study. Constitutive IL-10 production was detected predominantly in B cells and monocytes, rather than CD4+ or CD8+ T cells, in ART, ART-naïve and uninfected subjects. There was no significant difference in IL-10 production in any cell type between the three subject groups, indicating that chronic HIV-1 infection does not appear to increase constitutive production of IL-10 in the cell types studied. Previously, an increase in constitutive IL-10 mRNA expression has been reported in multiple cell subsets in HIV-infected individuals, including CD4+ T cells, CD8+ T cells, B cells and monocytes (356). The discrepancy between these results and those in the current study highlights the benefit of studying protein rather than mRNA, as IL-10 expression is subjected to post-transcriptional regulation, including mRNA destabilising mechanisms that may rapidly terminate production of the cytokine (371, 372). In support of this, there was also no significant difference between the groups in

the level of plasma IL-10. However, the levels of plasma IL-10 were lower than has been reported in previous studies, where median plasma and serum IL-10 concentrations in the range of 5-25 pg/ml have been observed (356, 373-375). In some of these studies patients with advanced disease (CD4+ counts below 200/μl or an AIDS-defining illness) were included, whilst in the current study only asymptomatic patients with CD4+ counts above 200/μl were enrolled. This factor may partially account for the discrepancy observed. However, it would be necessary to repeat the experiment with a larger cohort of subjects with more varied CD4+ counts in order to confirm these findings.

HIV-1 Gag-specific IL-10 production was mediated predominantly by CD8+ T cells and was almost entirely restricted to ART-naïve subjects. Such a population of CD8+ T cells has previously been identified in HIV-infected individuals (358-360). However, in addition to not examining constitutive IL-10 responses, these studies focussed exclusively on IL-10 production by T cells. Furthermore, Torheim *et al* restricted their investigation to subjects not receiving ART. The current study broadened the analysis of HIV-specific IL-10-production by examining both treatment-naïve patients and patients receiving ART and included monocytes and B cells as well as T cells. Due to the limited number of fluorochromes that were available for flow cytometry it was not possible to investigate IL-10 production by cell subsets such as natural killer cells, dendritic cells and neutrophils, although it has previously been demonstrated that all these cells can produce IL-10 (376-378).

The observation that HIV-specific IL-10+ CD8+ T cells were almost entirely restricted to ART-naïve subjects suggests that circulating virus is necessary for the maintenance

of this population. However, when ART-naïve subjects alone were considered there was no correlation between the frequency of Gag-specific IL-10+ cells and viral load. Gag was chosen as a relatively conserved and immunodominant protein (212, 362) but it is possible that HIV-specific IL-10+ populations with different antigen specificities also exist and contribute to total HIV-specific IL-10 production. Due to the cell numbers that would be required, it was not feasible to analyse IL-10 responses to the entire HIV-1 proteome but it is possible that this would produce a better correlation with viral load rather than focussing on a single antigen. Since it is unlikely that all the patients were infected with a Clade B virus it is also possible that some Gag-specific responses have been missed, although variation in the Gag sequence between different clades is low (typically under 10%) compared with variation in other viral proteins such as Env, which can vary by up to 35% between clades (210-212). Since it was not possible to examine cell subsets such as NK cells, DCs and neutrophils, the possibility that these cells are contributing to IL-10 production cannot be ruled out. Finally, if HIV-specific IL-10+ CD8+ T cells are indeed induced in response to circulating viraemia, a longitudinal study may be required in order to observe this phenomenon.

T cell activation in the cohort was investigated by examining the expression of CD38 and HLA-DR. Both molecules are upregulated in advanced HIV-1 infection and their expression has been found to correlate with accelerated disease progression (368, 369). T cell activation was increased in ART-naïve compared to ART subjects and correlated positively with viral load. These findings agree with those of a number of previous studies investigating T cell activation in HIV infection (368, 369, 379, 380). There was no significant difference between T cell activation in ART subjects and uninfected controls. However a previous investigation has demonstrated that whilst anti-retroviral

therapy reduces T cell activation it does not return to pre-infection levels (370). With a larger cohort it may have been possible to detect differences between ART subjects and uninfected controls.

IL-10 has a range of immunosuppressive functions: it downregulates the production of pro-inflammatory cytokines such as TNF-a, IL-1 and IL-12 by monocytes and macrophages (288, 290), reduces MHC Class II expression by antigen-presenting cells (275, 276) and can also inhibit T cell proliferation (291). It could therefore be speculated that the induction of a Gag-specific IL-10+ CD8+ T cell population is a response to immune activation, but when ART-naïve subjects were considered alone there was no significant correlation between the size of this population and the frequency of activated T cells. However, the frequency of total, rather than HIV-specific, activated T cells was examined in this study; it is possible that a stronger correlation exists between the frequency of activated HIV-specific T cells and Gag-specific IL-10+ CD8+ T cells. This possibility could be partially addressed by using tetramers to identify HIV-specific T cells. However, the tetramers currently available do not span the entire HIV genome and so it is likely that certain populations of HIV-specific T cells would be missed with this approach. The low frequency of IL-10+ cells in the peripheral blood may not reflect their frequency in sites of inflammation such as the gut and other lymphoid compartments. A longitudinal study might also cast more light on the relationship between IL-10 production and T cell activation, as the kinetics of change of these two phenomena may be different.

In summary, in this patient cohort there was no evidence of enhanced constitutive IL-10 production in chronic HIV-1 infection. However, a population of HIV-1 Gag-specific

IL-10-producing CD8+ T cells was identified in the majority of ART-naïve subjects. These findings raise questions about the ontogeny and function of this cell population that will be addressed in the following chapters.

Chapter 4: Phenotypic analysis of HIV-1 Gag-specific CD8+ T cells

4.1 Introduction

In the previous chapter, a population of HIV-1 Gag-specific IL-10+ CD8+ T cells was identified in HIV-1 seropositive ART-naïve subjects. Since IL-10 has a range of immunosuppressive functions, including the downregulation of antigen presentation (275, 276) and inflammatory cytokine production (288-290), it is possible that these cells represent a regulatory T cell (Treg) population.

The existence of T cell subsets with regulatory functions was proposed as early as the 1970s (381, 382). Since this time, both CD4+ and CD8+ Treg populations have been identified, but CD4+ Tregs have been more extensively characterised. "Natural" CD4+ Tregs develop in the thymus and perform a range of functions, including the induction of apoptosis in effector T cells and the inhibition of T and B cell proliferation, differentiation and effector function (183, 383-386). The suppressive effects of CD4+ Tregs can be mediated by direct contact with their cellular targets, the secretion of immunomodulatory cytokines such as IL-10 and TGF-β or by out-competing naïve T cells for the growth factor IL-2. In the latter mechanism, Tregs are aided by their constitutive expression of the IL-2R alpha chain, CD25. Natural CD4+ Tregs also express the forkhead transcription factor FoxP3, which suppresses the expression of cytokines such as IL-2 and IFN-γ, and can thus be delineated as FoxP3+ CD25+ CD4+ T cells with immune regulatory functions (387, 388). In addition to natural Tregs, phenotypically diverse populations of "adaptive" CD4+ Tregs can be induced in response to antigenic stimulation in the periphery (389, 390). Th3 cells secrete IL-10

and TGF-β and may play an important role in oral tolerance (391), whilst Tr1 cells are induced by antigen in the presence of IL-10 and mediate immunosuppressive functions partly via the secretion of further IL-10 (351). Under certain conditions, Tr1 cells may also secrete IFN-γ (392).

CD8+ Tregs have been identified in both healthy individuals (393, 394) and in individuals suffering from diseases such as cancer and inflammatory bowel disease (395, 396). However, the phenotype of these cells is much more heterogeneous, with only certain studies demonstrating expression of FoxP3 and CD25, and the mechanism of suppression was often poorly defined. In order to clarify the phenotypic characteristics of IL-10-producing CD8+ T cells in HIV-1 infection, Gag-specific IL-10+ CD8+ T cells from ART-naïve patients (detected by cytokine capture) were examined by flow cytometry for expression of FoxP3, CD25 and IFN-γ. In addition, to determine whether Gag-specific IL-10+ CD8+ T cells possess the ability to locate to sites of inflammation in HIV-1 infection, such as the lymph nodes and gut-associated lymphoid tissue (GALT), the expression of the chemokine receptor CXCR3, which is guided to inflamed tissues by binding its ligands CXCL9 (MIG) CXCL10 (IP-10) and CXCL11 (IP-9) (397-399), and the β chain of the gut-homing integrin α4β7 were examined.

IL-10-producing CD8+ T cells have previously been described in subjects with chronic hepatitis C virus (HCV) (319) and Epstein-Barr virus (EBV) (320) but their phenotypic characterisation has been limited. To determine whether IL-10-producing CD8+ T cells with a conserved phenotypic profile are a common feature of chronic viral infections, CD8+ T cells from patients with chronic HCV and cytomegalovirus (CMV) infections

were examined by cytokine capture and flow cytometry for expression of IL-10, IFN-γ, FoxP3 and CD25.

In summary, this chapter explores the phenotypic characteristics of Gag-specific IL-10+ CD8+ T cells and whether similar populations are found in other chronic viral infections.

4.2 Study subjects and phenotyping antibody panel

Fifteen ART-naïve subjects identified in Chapter 3 as having a detectable population of Gag-specific IL-10+ CD8+ T cells were selected for phenotypic analysis. Additional patients that had been screened for detectable Gag-specific IL-10+ CD8+ T cells were recruited from the University of Oxford Centre for Clinical Magnetic Resonance Research (samples kindly provided by Cameron Holloway – these patients have ID numbers beginning with HD in Table 4.1). Due to sample availability, not all phenotypic analyses were performed on all patients. A minimum of 15 Gag-specific IL-10+ CD8+ T cells were collected for analysis, since due to the extremely low frequency of such cells it was not possible to enrich a greater number from some subjects. E Barnes and A von Delft of the Medawar Institute, Oxford University kindly provided samples from ten subjects with chronic HCV infection for the analysis of HCV-specific IL-10 responses. HCV infection was confirmed by PCR and serology and only patients with an IFN-γ response of 40 spot-forming units (SFU) per million or higher to at least one peptide were used in the study. HCV peptides were selected on the basis of having provoked an IFN-γ response of 40 SFU per million or higher by ELISPOT. CMV-specific responses were analysed using PBMC from HIV-infected

ART-naïve subjects who were also infected with CMV. All subjects were seropositive for CMV but none had active CMV disease and all displayed CD4+ counts of >200/μl at the time of sampling. A pool of CMV peptides known to be associated with common HLA alleles was used to elicit responses (see Appendix).

Subject PBMC were stained with the following antibody panel: CD3-APC-Cy7; CD8-PerCP; Aqua live/dead cell stain; IL-10-PE; IFN-γ-FITC; CD25-Alexa Fluor 700; either CXCR3-Pacific blue or FoxP3-Pacific blue and β7-PE-Cy5. Fluorescence minus one (FMO) analysis was conducted to ensure there was no spectral overlap between the fluorochromes (Figure 4.1).

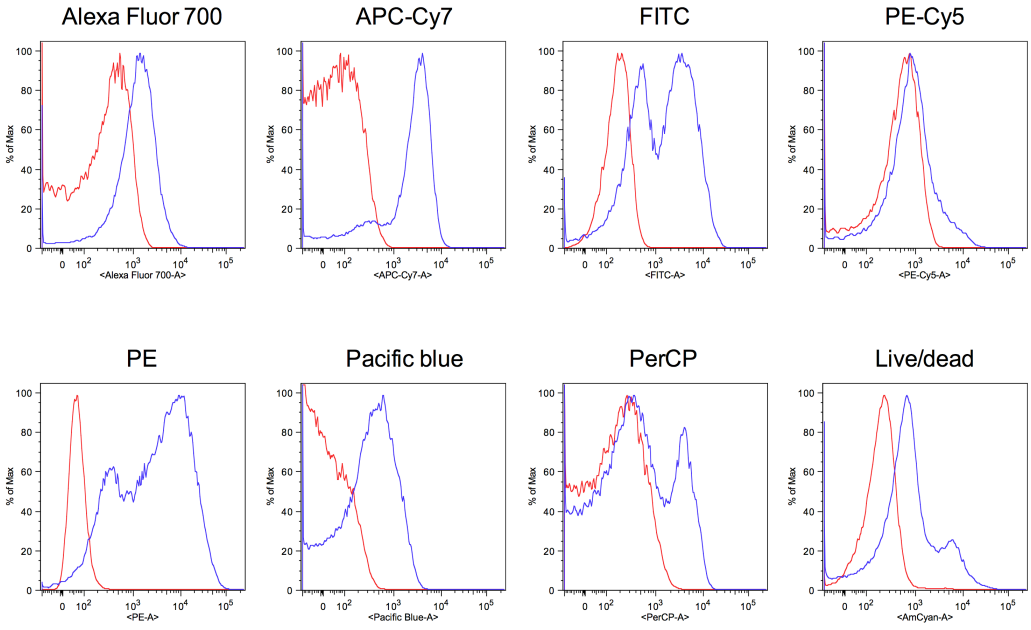


Figure 4.1 Fluorescence minus one (FMO) analysis to check for spectral overlap between fluorochromes. In each histogram, the level of fluorescence in the relevant channel when that flurochrome is omitted is shown in red, whilst the level of fluorescence when the fluorochrome is included is shown in blue. PBMC from a HIV-negative subject were stimulated for 3 days with 1μg/ml PMA before staining. Due to the low frequency of IL-10+ and IFN-γ+ cells, CD38-PE and CD4-FITC, rather than IL-10-PE and IFN-γ-FITC, were used for staining.

4.3 HIV-1 Gag-specific IL-10+ CD8+ T cells do not express FoxP3 or CD25

To determine whether Gag-specific IL-10+ CD8+ T cells share phenotypic properties with natural CD4+ Tregs, their expression of FoxP3 and CD25 was analysed. PBMC from ART-naïve patients were stimulated for 3 hours with HIV-1 Clade B Gag peptides, after which IL-10+ cells were isolated using bead capture. The isolated cells were then stained with Aqua live/dead cell stain and anti-CD3, CD8, IL-10, FoxP3 and CD25 antibodies. Figure 4.2 shows the gating strategy used for the identification of FoxP3+ and CD25+ cells.

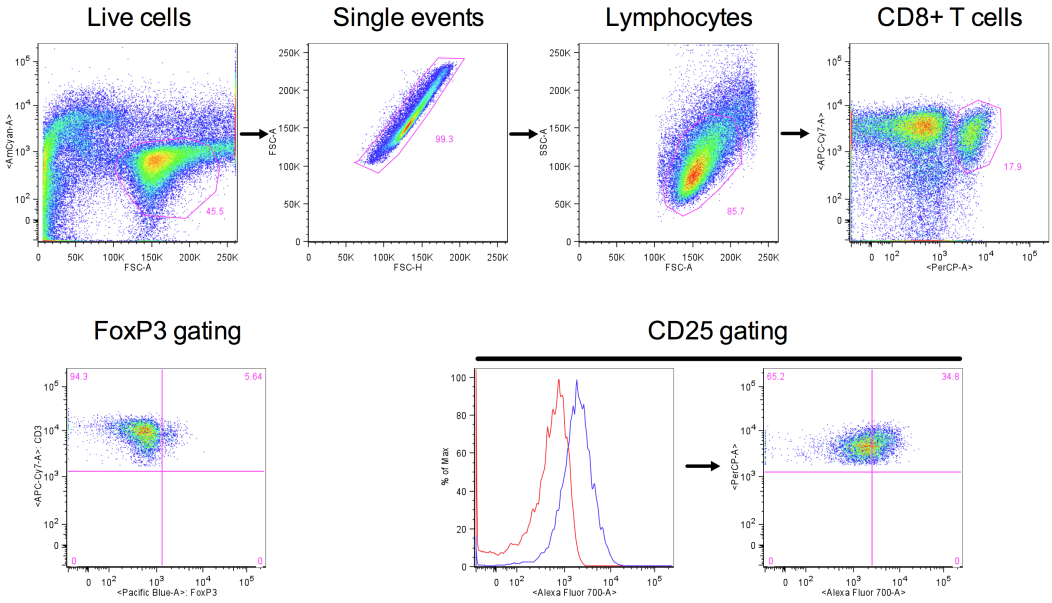


Figure 4.2 Gating strategy for the identification of FoxP3+ and CD25+ cells. In order to enhance expression of CD25, PBMC were stimulated for 3 days with 1µg/ml phorbol myristate acetate (PMA). They were then stained with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain, Fox-P3-Pacific blue and CD25-Alexa Fluor 700. Since FoxP3+ cells are found at higher frequencies in CD4+ T cells than CD8+ T cells, FoxP3 gating was performed on CD3+ cells (including both CD4+ and CD8+ T cells). CD25 gating was performed on CD8+ T cells. Since it can be difficult to define positive expression of CD25, an isotype control antibody was used. In the histogram, the red line indicates the fluorescence intensity of the isotype control, whilst the blue line indicates the fluorescence intensity of the anti-CD25 antibody. This fluorescence intensity information was then used to gate CD25+ cells among CD8+ T cells. Gating was performed on a minimum of 5000 cells.

Figure 4.3 shows representative flow cytometric plots of FoxP3 and CD25 expression by Gag-specific IL-10+ CD8+ T cells from an ART-naïve patient. The median percentage of Gag-specific IL-10+ CD8+ T cells expressing FoxP3 was 1.4%, and in 3 of the 6 patients studied none of the cells expressed FoxP3 (Figure 4.4). The median percentage of Gag-specific IL-10+ CD8+ T cells expressing CD25 was 1.9%, with a total lack of CD25+ cells in 5 out of the 10 patients studied. This expression pattern was similar to that observed in IL-10$^-$ CD8+ T cells after Gag stimulation ($p > 0.05$; Wilcoxon matched-pairs signed rank test) (Figure 4.4), indicating that Gag-specific IL-10+ CD8+ T cells did not differ significantly from the general CD8+ T cell population in the expression of these molecules.

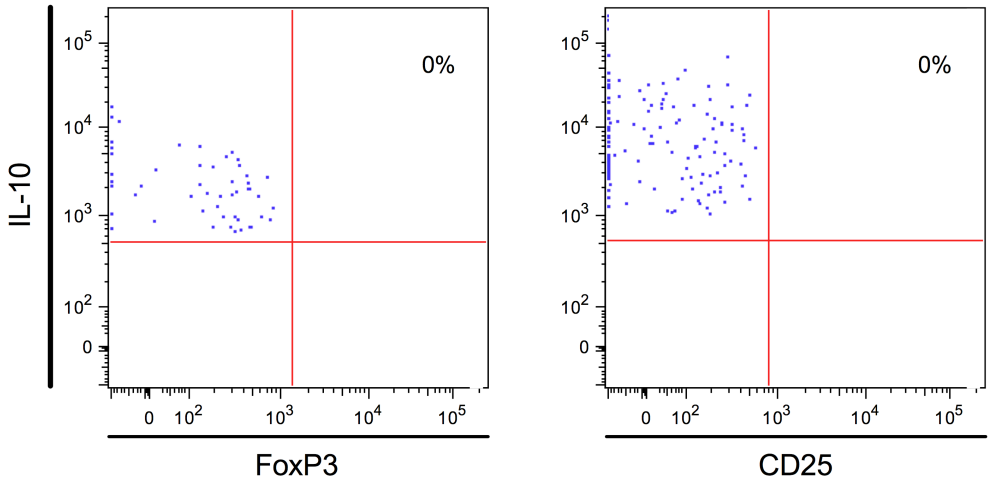


Figure 4.3 Representative flow cytometic plots showing the expression of FoxP3 (left) and CD25 (right) by Gag-specific IL-10+ CD8+ T cells from an ART-naïve subject. IL-10-producing cells were identified as in chapter 3 (section 3.3), and FoxP3 and CD25+ cells as shown in figure 4.2.

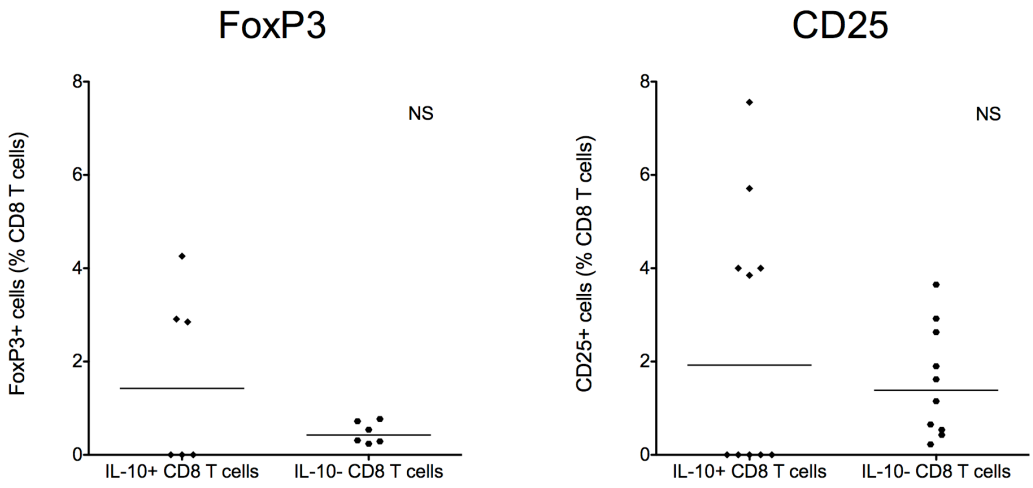


Figure 4.4 Expression of FoxP3 and CD25 by Gag-specific IL-10+ CD8+ T cells, compared to Gag-stimulated IL-10- CD8+ T cells, in ART-naïve subjects. Wilcoxon matched-pairs signed rank test; $p < 0.05$.

4.4 HIV-1 Gag-specific IL-10+ CD8+ T cells can co-produce IFN-γ

Since most Gag-specific IL-10+ CD8+ T cells do not express the natural Treg markers FoxP3 or CD25, their ability to secrete IFN-γ, a cytokine that can be produced by Tr1 regulatory cells (392) was assessed. IL-10+ cells were isolated as described in section 4.3 and stained with Aqua live/dead cell stain and anti-CD3, CD8, IL-10 and IFN-γ antibodies. Figure 4.5 shows the gating strategy used for the identification of IFN-γ+ cells.

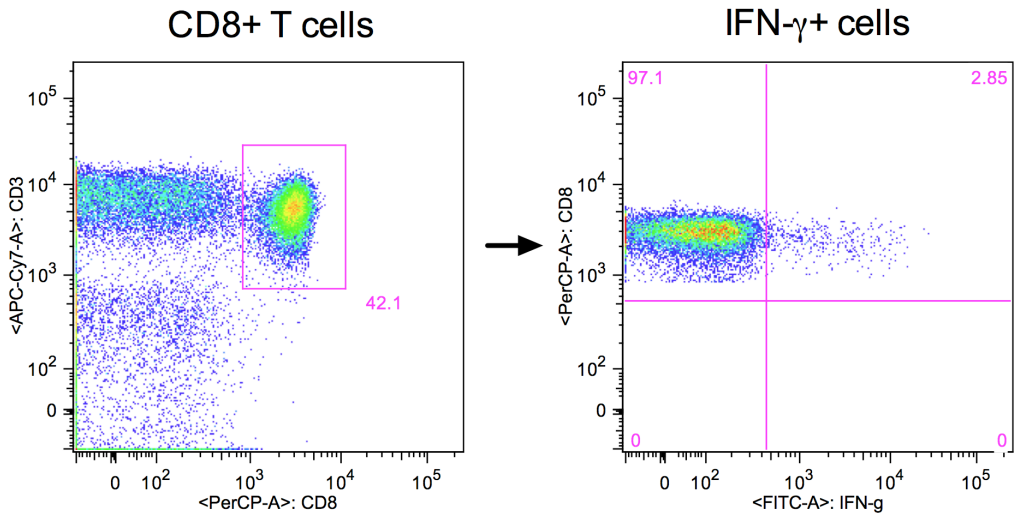


Figure 4.5 Gating strategy for the identification of IFN-γ+ cells. PBMC were stimulated with Gag peptides for 3 hours, followed by cytokine capture of IFN-γ+ cells, and staining with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain and IFN-γ-FITC. CD8+ T cells were identified as in figure 4.2. A minimum of 5000 CD8+ T cells were collected for gating. This gating was then used to identify IFN-γ+ Gag-specific IL-10+ CD8+ T cells (see figure 4.6).

Having optimised the staining panel, Gag-specific IL-10+ CD8+ T cells from 15 ART-naïve patients were examined for co-expression of IL-10 and IFN-γ. Figure 4.6 shows a representative flow cytometric plot of IFN-γ production by Gag-specific IL-10+ CD8+ T cells. The majority of the cells (median 59.0%) produced IFN-γ (Table 4.1).

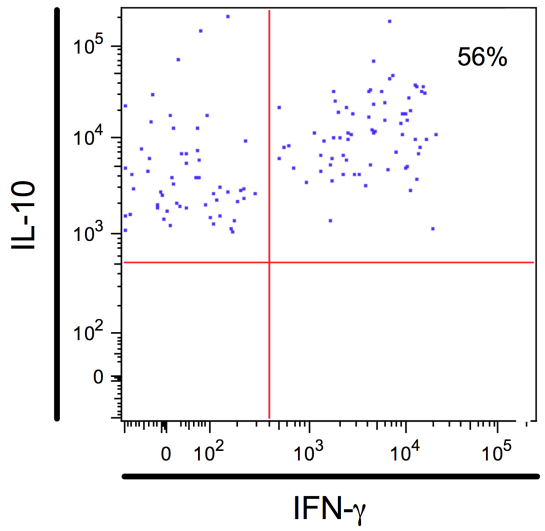


Figure 4.6 Representative flow cytometric plot showing expression of IFN-γ by Gag-specific IL-10+ CD8+ T cells from an ART-naïve subject. IL-10-producing cells were identified as in chapter 3 (section 3.3), and IFN-γ+ cells were identified as shown in figure 4.5.

Patient ID	Gag-specific IL-10+ CD8+ T cells (% CD8+ T cells)	% Gag-specific IL-10+ CD8+ T cells that co-produce IFN-γ
HD59	0.017	71.4
HD100	0.012	58.6
HD139	0.014	10.3
HD141	0.011	88.5
IC-104	0.048	72.7
IC-116	0.015	59
IC-125	0.021	59
IC-130	0.012	47.1
IC-137	0.021	63.5
IC-145	0.034	75.6
IC-148	0.018	48.6
IC-150	0.047	56
IC-154	0.030	50.5
IC-157	0.013	24
IC-161	0.059	73.7

Table 4.1 The frequency of Gag-specific IL-10+ CD8+ T cells that co-produce IFN-γ. A minimum of 15 Gag-specific IL-10+ CD8+ T cells were collected for analysis.

Since Gag-specific IL-10+ CD8+ T cells are restricted to ART-naïve HIV-infected subjects (see Chapter 3) and the majority also produce IFN-γ, it is possible that these cells originated as IFN-γ-producing effector or memory cells and had acquired the ability to produce IL-10 in response to ongoing antigenic stimulation (400). A higher frequency of Gag-specific IL-10+ CD8+ T cells might therefore be expected in subjects with a higher frequency of Gag-specific IFN-γ+ CD8+ T cells. In order to investigate this possibility, IFN-γ responses to overlapping peptides spanning the entire HIV-1 proteome were determined by ELISPOT using PBMC collected from the same clinical visit as the PBMC used in the cytokine capture assays (ELISPOT assays performed by Gemma Hancock, University of Oxford). There was a trend towards a positive correlation between the frequency of HIV-specific IFN-γ+ T cells and Gag-specific IL-10+ CD8+ T cells (Figure 4.7) but this did not reach statistical significance (Spearman's rank correlation; $r = 0.40$; $p = 0.051$). However, there was a highly significant positive correlation between the frequency of Gag-specific IFN-γ+ CD8+ T cells, as determined by ELISPOT, and the frequency of Gag-specific IL-10+ CD8+ T cells (Figure 4.7; Spearman $r = 0.74$; $p < 0.0001$). To confirm this correlation IFN-γ responses to Gag were examined by cytokine capture. Once again, there was a significant positive correlation between the frequency of Gag-specific IFN-γ-producing CD8+ T cells and the frequency of Gag-specific IL-10+ CD8+ T cells (Figure 4.8; Spearman $r = 0.56$; $p = 0.02$).

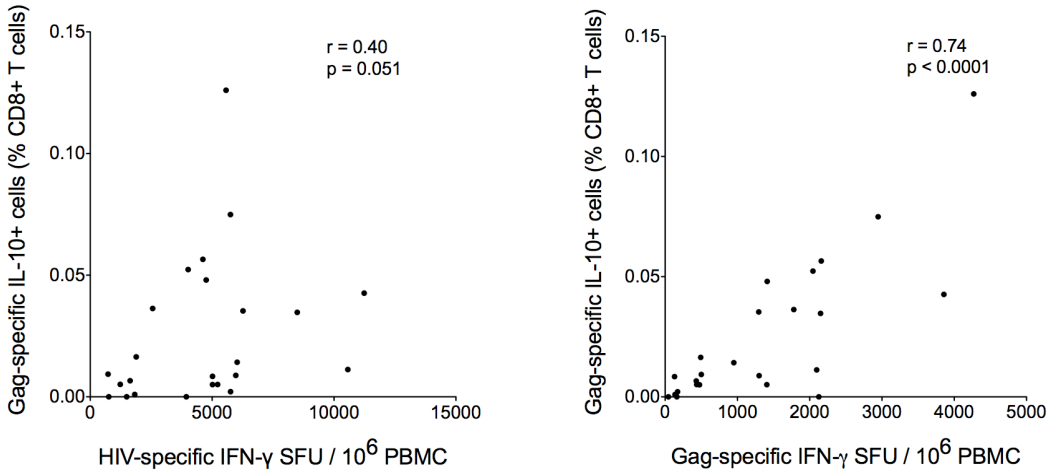


Figure 4.7 Correlation between the frequency of HIV-specific IFN-γ+ T cells (left) or Gag-specific IFN-γ+ T cells (right), determined by ELISPOT, and the frequency of Gag-specific IL-10+ CD8+ T cells, determined by cytokine capture. Statistical analysis carried out using Spearman's rank correlation.

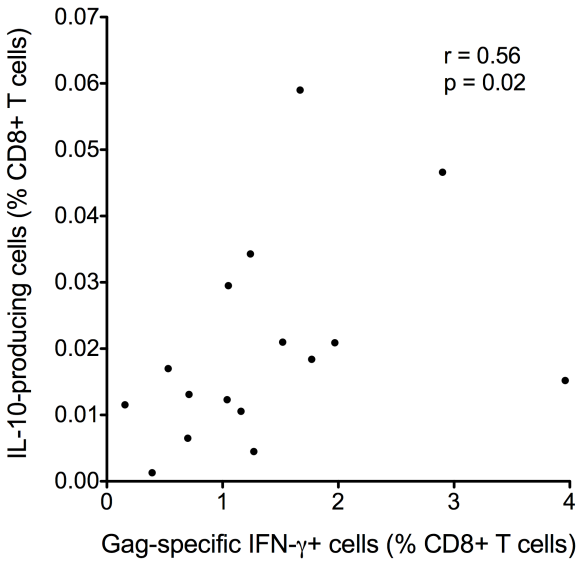


Figure 4.8 Correlation between the frequency of Gag-specific IL-10+ CD8+ T cells (calculated as described in chapter 3, section 3.3) and the frequency of Gag-specific IFN-γ+ CD8+ T cells, determined by cytokine capture after a 3 hour stimulation with HIV-1 Gag peptides. Statistical analysis carried out using Spearman's rank correlation.

4.5 HIV-1 Gag-specific CD8+ T cells express CXCR3 and β7

To determine whether Gag-specific IL-10+ CD8+ T cells possess the capacity to migrate to the lymph nodes and GALT, key sites of inflammation and pathogenesis in HIV-1 infection, the cells were examined for expression of CXCR3 and β7 integrin. IL-10+ cells were isolated as described in section 4.3 and stained with Aqua live/dead cell stain and anti-CD3, CD8, CXCR3 and β7 antibodies. Figure 4.9 shows the gating strategy used for the identification CXCR3+ and β7+ cells. In order to determine the proportion of β7-expressing CD8+ T cells that also expressed α4, CD8+ T cells from 4 ART-naïve individuals were co-stained with α4 and β7 antibodies in a separate experiment. Over 95% of β7+ CD8+ T cells also expressed α4 (Figure 4.10). An investigation of the expression of the gut homing chemokine receptor CCR9 on Gag-specific IL-10+ CD8+ T cells was curtailed due to the inability of the commercially available antibody (R & D Systems) to bind CCR9 on subject PBMC (data not shown).

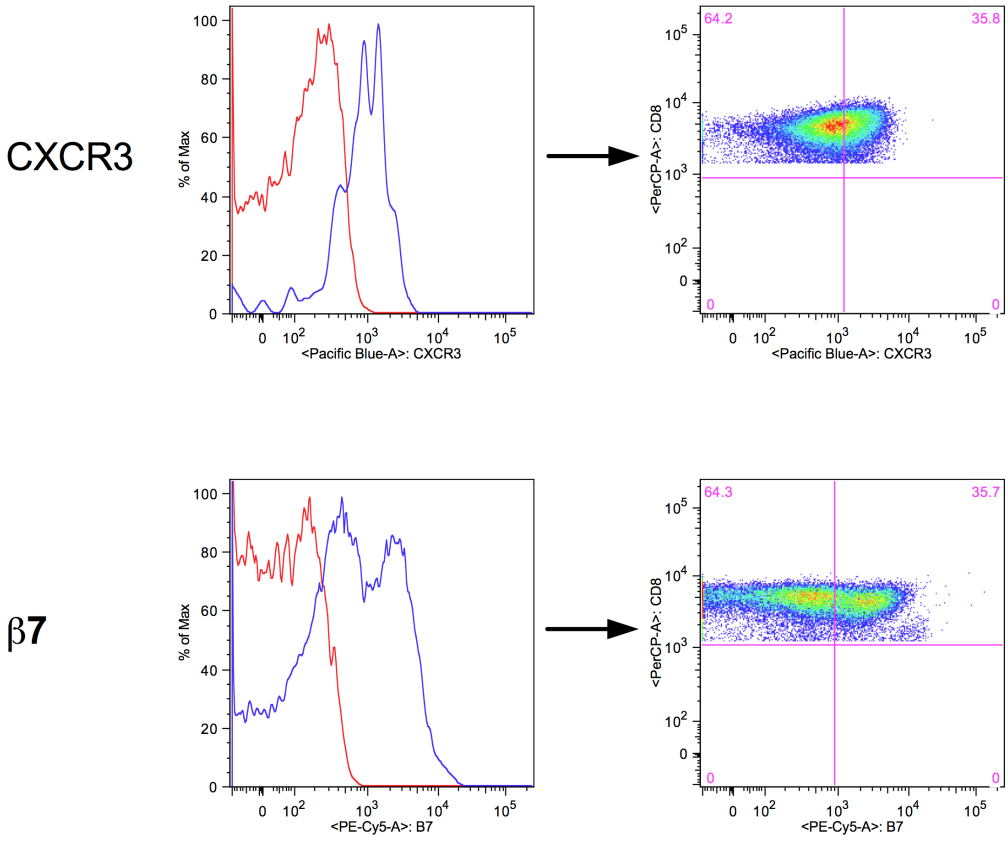


Figure 4.9 Gating strategy for the identification of CXCR3+ and β7+ cells. PBMC were stained with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain, CXCR3-Pacific blue and β7-PE-Cy5. CD8+ T cells were identified as in figure 4.2. In order to accurately identify positive expression of these molecules, isotype control antibodies were used. The left hand panels show CD8+ T cells stained with the relevant isotype control (red line) compared to CD8+ T cells stained with the relevant antibody (blue line). This expression pattern was then used to gate CXCR3+ and β7+ CD8+ T cells (right hand panels). A minimum of 5000 CD8+ T cells were collected for gating. This gating was then used to identify CXCR3+ and β7+ Gag-specific IL-10+ CD8+ T cells (see figure 4.12).

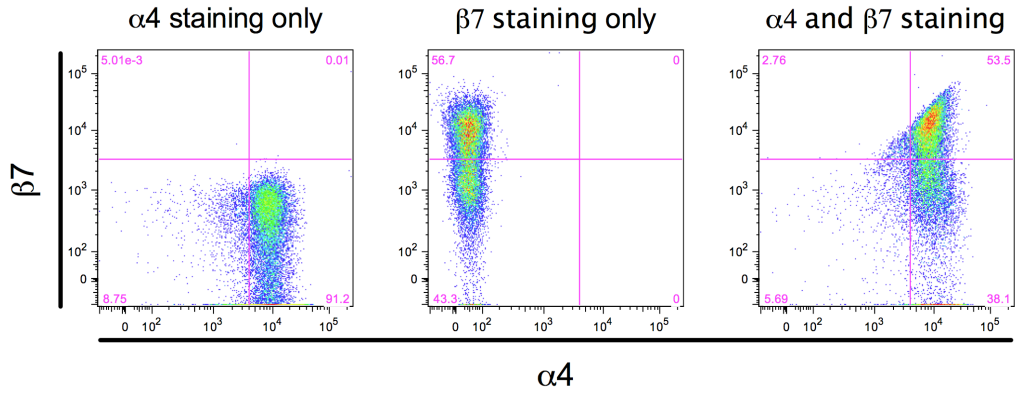


Figure 4.10 Representative flow cytometric plots of α4β7 expression by CD8+ T cells from an ART-naïve subject. PBMC were stained with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain, α4-PE and β7-PE-Cy5.

Figure 4.11 shows representative flow cytometric plots of CXCR3 and β7 expression by Gag-specific IL-10+ CD8+ T cells. A median of 8.6% of the cells expressed CXCR3, with CXCR3 expression detectable in the population in all 7 patients studied (Figure 4.12). A median of 48.0% of the cells expressed β7, and β7 expression was detected in the Gag-specific IL-10+ CD8+ T cell population of all patients studied. This expression pattern was similar to that observed in IL-10$^-$ CD8+ T cells after Gag stimulation (Figure 4.12), indicating that Gag-specific IL-10+ CD8+ T cells did not differ significantly from the general CD8+ T cell population in the expression of CXCR3 or β7 ($p > 0.05$; Wilcoxon matched-pairs signed rank test).

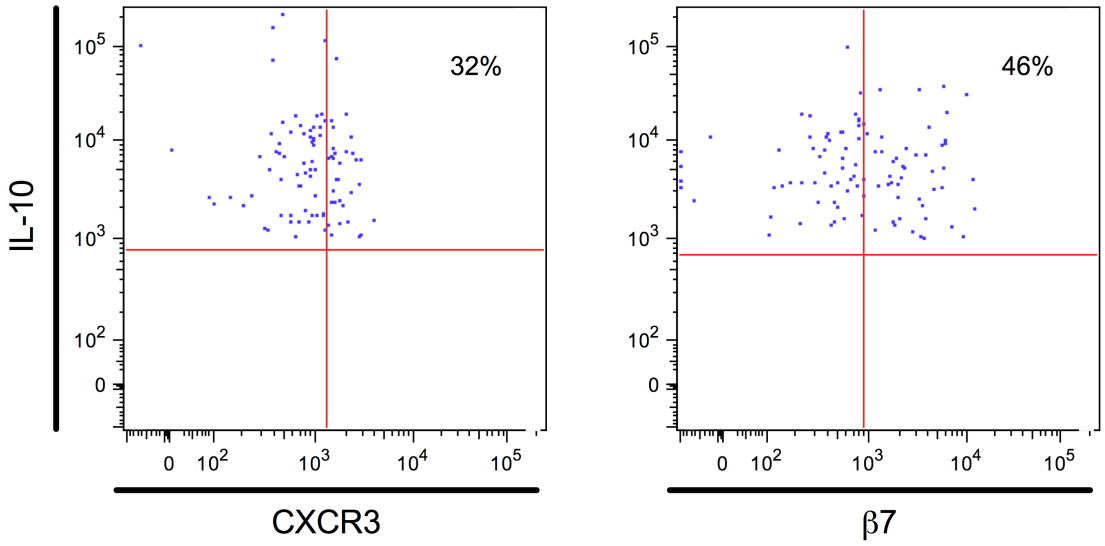


Figure 4.11 Representative flow cytometric plots showing expression of CXCR3 and β7 by Gag-specific IL-10+ CD8+ T cells from an ART-naïve subject. IL-10+ cells were identified as in chapter 3, and CXCR3+ and β7+ cells were identified as shown in figure 4.10.

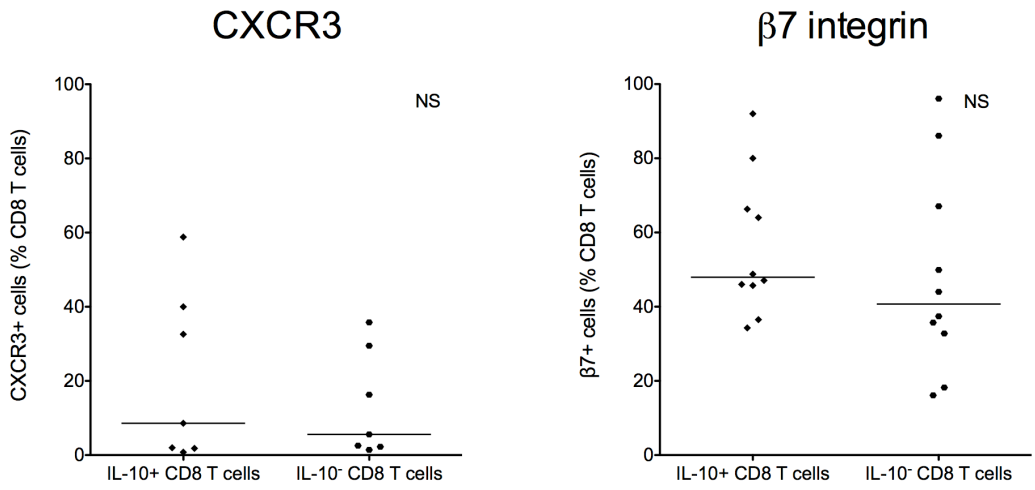


Figure 4.12 Expression of CXCR3 and β7 by Gag-specific IL-10+ CD8+ T cells, compared to Gag-stimulated IL-10$^-$ CD8+ T cells, in ART-naïve subjects. Wilcoxon matched-pairs signed rank test; $p > 0.05$.

4.6 Antigen-specific IL-10+ CD8+ T cells are found in other chronic viral infections but are phenotypically distinct from HIV-specific IL-10+ CD8+ T cells

Having established a detailed phenotypic profile for Gag-specific IL-10+ CD8+ T cells in ART-naïve HIV-infected individuals, PBMC from CMV and HCV-infected subjects were studied to see if similar populations could be identified in individuals chronically infected with these viruses. CD8+ T cells were examined by cytokine capture for production of IL-10 after a 3-hour stimulation with CMV or HCV viral peptides (see Materials and Methods chapter and Appendix). IL-10+ cells were identified as described in Chapter 3 (section 3.3). Figure 4.13 shows representative flow cytometric plots of CMV and HCV-specific IL-10+ CD8+ T cells. CMV-specific IL-10+ CD8+ cells were identified in 8 out of the 10 subjects studied, at a median frequency of 0.004% (Figure 4.14). HCV-specific IL-10+ CD8+ T cells were detected in 6 out of the 10 subjects studied, at a median frequency of 0.005%. These frequencies were lower than the median frequency of Gag-specific IL-10+ CD8+ T cells (which was 0.011%; see Chapter 3), but the difference was not statistically significant ($p > 0.05$; Kruskal-Wallis test).

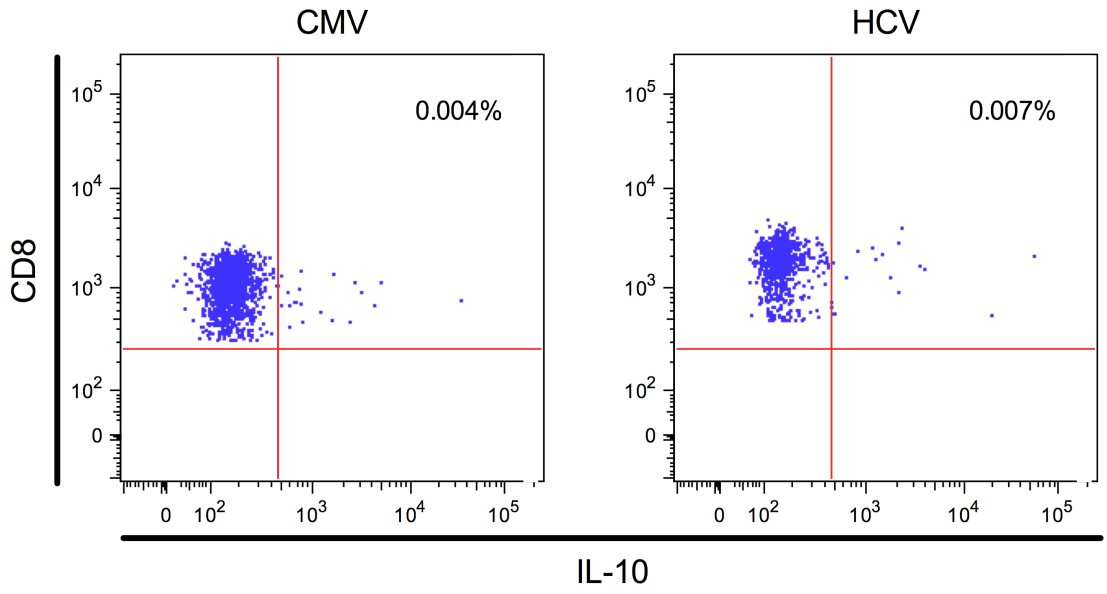


Figure 4.13 Representative flow cytometric plots showing IL-10 production after a 3 hour stimulation with CMV or HCV peptides by CD8+ T cells in a subject with CMV (left) and a subject with HCV (right). PBMC were stained with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain and IL-10-PE. The percentages of CD8+ T cells that secrete IL-10 after stimulation with CMV or HCV peptides (minus background) are shown.

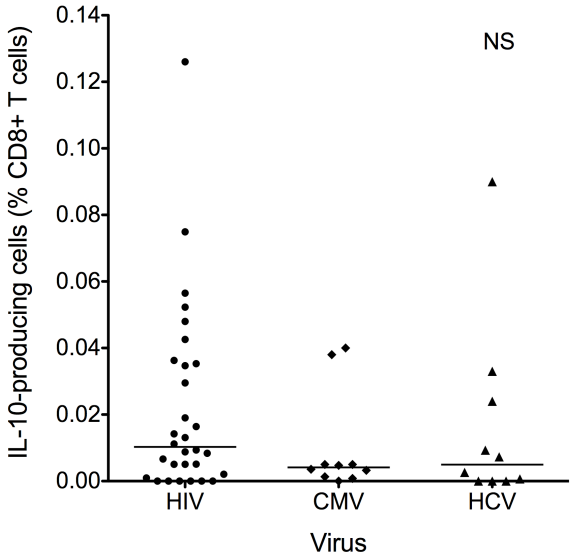


Figure 4.14 Antigen-specific IL-10 production by CD8+ T cells in ART-naïve HIV-infected subjects, CMV-infected subjects and HCV-infected subjects. Kruskal-Wallis test; $p < 0.05$.

In subjects with a detectable virus-specific IL-10+ CD8+ T cell population, these cells were examined by cytokine capture and flow cytometry for expression of FoxP3, CD25 and IFN-γ. Both CMV and HCV-specific IL-10+ CD8+ T cells displayed a more heterogeneous pattern of FoxP3 expression (range 0 - 63.6% in CMV and 0 - 76.5% in HCV, compared to 0 - 4.3% in HIV) and CD25 expression (range 0 - 77.8% in CMV and 0 - 57.1% in HCV compared to 0 - 7.56% in HIV) compared to Gag-specific IL-10+ CD8+ T cells (Figure 4.15). Significantly fewer HCV-specific IL-10+ CD8+ T cells produced IFN-γ compared to both Gag-specific IL-10+ CD8+ T cells ($p < 0.05$; Kruskal-Wallis test) and CMV-specific IL-10+ CD8+ T cells ($p < 0.05$).

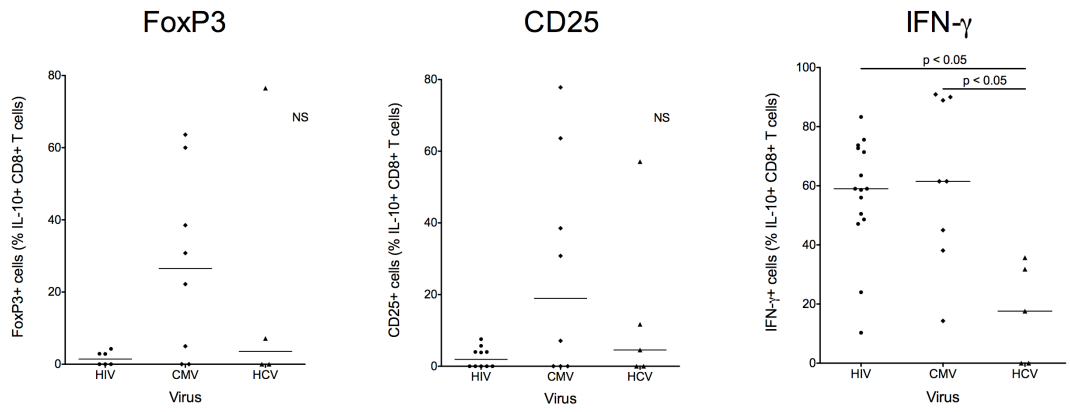


Figure 4.15 Expression of FoxP3, CD25 and IFN-γ by antigen-specific CD8+ T cells in ART-naïve HIV-infected subjects, CMV-infected subjects and HCV-infected subjects. PBMC were stimulated with HIV-1 Gag, CMV or HCV peptides for 3 hours. Following this, IL-10+ cells were enriched by cytokine capture and stained with CD3-APC-Cy7, CD8-PerCP, Aqua live/dead cell stain, IL-10-PE, IFN-γ-FITC, FoxP3-Pacific blue and CD25-Alexa Fluor 700. Statistical analyses performed using Kruskal-Wallis test.

4.7 β7 expression by CD4+ T cells and CD8+ T cells in chronic HIV infection

The analysis in section 4.5 demonstrated that Gag-specific IL-10+ CD8+ T cells retain expression of the gut-homing integrin β7, suggesting that they maintain the ability to migrate to this important site of HIV-1 pathogenesis. However, since the population is HIV-specific it might have been expected that increased expression of gut-homing molecules would be observed. Since there is substantial depletion of gut CD4+ T cells during acute HIV infection, with only partial restoration after the initiation of ART, it is possible that cells expressing gut-homing molecules such as β7 integrin are preferentially lost due to infection or bystander activation and apoptosis, which could explain the lack of enriched β7 expression in the Gag-specific IL-10+ CD8+ T cell fraction. To determine whether α4β7 expression on T cells in human peripheral blood declines as disease progresses, and whether it can be restored by ART, expression of the integrin on CD4+ and CD8+ T cells was examined by flow cytometry in 19 HIV-infected subjects receiving ART, 19 ART-naïve HIV-infected subjects and 5 uninfected controls. ART-naïve patients were further subdivided into viraemic controllers (subjects who maintained a viral load of 5000 copies/ml or lower for at least 12 months) and non-controllers (subjects with a viral load of greater than 5000 copies/ml). Analysis of β7 expression on CD4+ T cells was performed on samples from a further 10 ART-naïve viraemic controllers and 2 ART-naïve non-controllers recruited from the SCOPE cohort (Centre for AIDS Research, UCSF, USA) (samples provided by N Goonetilleke, University of Oxford). β7+ cells were identified as shown in Figure 4.10. For both CD4+ and CD8+ T cells there was no statistically significant difference in the frequency of β7+ cells compared to uninfected controls in the ART treated, ART-naïve

controller or ART-naïve non-controller groups ($p > 0.05$; Kruskal-Wallis test) (Figure 4.16).

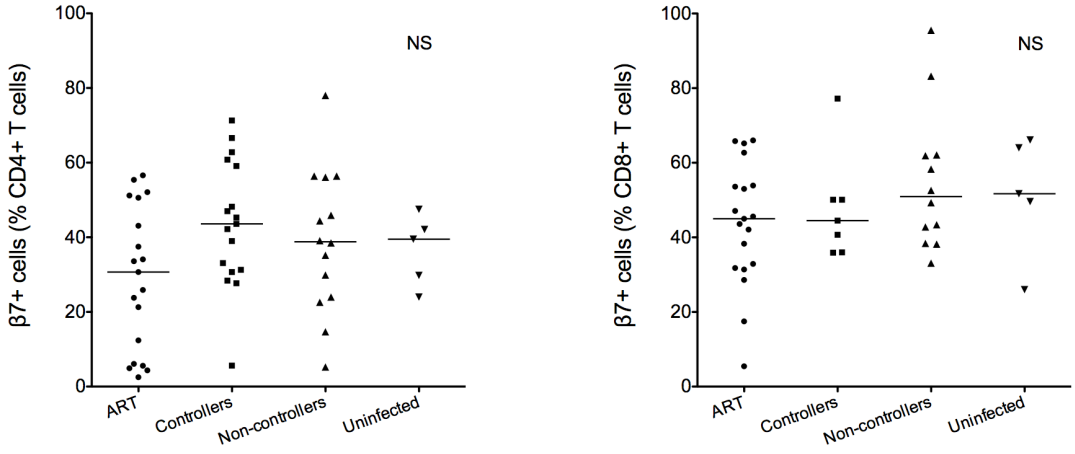


Figure 4.16 Expression of β7 by CD4+ T cells and CD8+ T cells in HIV-infected subjects receiving ART, ART-naïve viraemic controllers (subjects maintaining a viral load of ≤ 5000 copies/ml for at least 12 months), ART-naïve non-controllers (with viral loads of > 5000 copies/ml) and uninfected controls. PBMC were stained with CD3-APC-Cy7, CD4-FITC, CD8-PerCP, Aqua live/dead cell stain and β7-PE-Cy5. Sample processing performed by Genevieve Clutton and Gemma Hancock. Statistical analyses conducted using Kruskal-Wallis test.

4.8 Discussion

HIV-specific IL-10-producing CD8+ T cells have previously been identified in chronically HIV-infected individuals (358-360) but the detailed phenotypic profile of these cells, particularly with regard to the expression of tissue homing markers, has not been established. Since effector and regulatory T cell populations are often delineated on the basis of their phenotype, the expression profile of Gag-specific IL-10+ CD8+ T cells may provide an important indication of their functional role.

Both natural and adaptive Tregs can produce IL-10 (392, 401) and certain suppressive functions mediated by these cells may be due to secretion of the cytokine (396), so it could be postulated that Gag-specific IL-10+ CD8+ T cells have regulatory functions. Natural CD4+ Tregs, in particular, constitutively express the IL-2R alpha chain CD25 and the forkhead transcription factor FoxP3. Additionally, CD8+ Tregs expressing FoxP3 and CD25 have been identified in the thymus of healthy humans (394). However, in the present study the majority of Gag-specific IL-10+ CD8+ T cells did not express either FoxP3 or CD25. HIV-specific CD8+ T cells have previously been characterised as $FoxP3^-$ (358-360) and $CD25^-$ (358, 360) so the results of this study are supported by earlier findings. Gag-specific IL-10+ CD8+ T cells do not, therefore, share the phenotypic characteristics of natural CD4+ Tregs. This does not, however, rule out a regulatory role for these cells, as $FoxP3^-$ $CD25^-$ CD8+ T cells with regulatory function have previously been described in HIV-1 infection, although these cells were described as secreting TGF-β rather than IL-10 (402). However, the number of molecules that could be studied was limited by the number of fluorochromes that could be used simultaneously for flow cytometric analysis, and even if it had been possible to

use more fluorochromes, the low cell number available from some patients would have made it extremely difficult to confidently divide the population into many different phenotypic subpopulations. These considerations meant that it was not possible to investigate the expression of molecules, such as TGF-β, CD28 and CTLA-4, that have previously been identified on CD8+ Tregs (394, 402-404). Whilst multiple parallel analyses could have been conducted, this would not have confirmed or ruled out the existence of a population expressing all these markers simultaneously.

Since the problem of examining many phenotypic markers simultaneously on a rare cell population is difficult to overcome using traditional flow cytometric methods, other approaches could have been used. One alternative would have been to carry out transcriptional analysis, but again the low number of Gag-specific IL-10+ CD8+ T cells available may have hampered this approach. A more desirable solution would have been to utilise the NanoString nCounter gene expression system, a relatively recent innovation that uses capture probes and fluorescently coded reporter probes that hybridise to target mRNA sequences at a 1:1 ratio (405). Each fluorescent signature corresponds to a different mRNA target. The hybridised probe-target complexes are then visualised using a microscope and camera in order to determine the quantity of each transcript present in the sample. The system has the sensitivity to detect mRNA at concentrations as low as 0.1 fM and can detect up to 800 different gene transcripts simultaneously (405). With this approach it would have been possible to perform a much more comprehensive phenotypic analysis of Gag-specific IL-10+ CD8+ T cells, although, as outlined in the Chapter 3, some mRNA transcripts are degraded before translation can occur (371, 372) and so a transcriptional profile does not always present an accurate reflection of the protein output of a cell. The final alternative would have

been to clone the cells in order to obtain more for flow cytometric analysis. However, there would be multiple problems with this approach. Firstly, when starting with such small numbers of cells it might be difficult to successfully generate clones. Secondly, if cloning was only successful from one or two of the original cells then the phenotype of the clones would only reflect the phenotype of these cells, and may not be representative of the original population. Thirdly, the phenotype of cells cultured for long periods can change over time and with different culture conditions (406) so the phenotype at the time of analysis might not reflect the phenotype of the original patient cells.

Since Gag-specific IL-10+ CD8+ T cells do not express natural Treg markers, they may be more phenotypically similar to other regulatory T cell populations such as CD4+ Tr1 cells. These cells have previously been described as $FoxP3^-$ and $CD25^-$ and secrete both IL-10 and IFN-γ under conditions of persistent antigenic stimulation (392). Furthermore, they suppress naïve and memory T cell proliferation in a partially IL-10-dependent manner. Although similar cells had previously been generated *in vitro* (407, 408), this was the first description of an *ex vivo* Tr1 cell population in human blood, and it remains to be determined whether comparable CD8+ T cell populations exist in humans. When IFN-γ production was investigated, more than half the Gag-specific IL-10+ CD8+ T cells were able to co-produce this cytokine. Given the phenotypic similarity of Gag-specific IL-10+ CD8+ T cells to the Tr1 cells previously described (392), and the fact that they are found in treatment-naïve subjects where there is an environment of persistent antigenic stimulation, it is possible that these cells also have IL-10-mediated regulatory functions. A previous study described Gag-specific IL-10+ CD8+ T cells as $IFN\text{-}\gamma^-$, based on the observation that less than 0.01% of CD8+ T cells

co-produced IL-10 and IFN-γ after Gag stimulation (359). However, this analysis was based on an unenriched cell population where only 0.01% of the cells were IL-10+. In using a more sensitive method, examining cells that were enriched on the basis of IL-10 secretion, the current study has demonstrated that Gag-specific IL-10+ CD8+ T cells do indeed possess the capacity to co-express IFN-γ. To my knowledge, this is the first identification of HIV-specific IL-10 and IFN-γ co-producing CD8+ T cells.

The ability to migrate to sites of inflammation and pathogenesis is an important feature of antigen-specific B and T cells. In HIV-infected individuals the secondary lymphoid organ, including the gut-associated lymphoid tissue (GALT), is a major site of HIV-1 replication (409-411). Since it was not possible to obtain samples from the GALT or lymph nodes for analysis, Gag-specific IL-10+ CD8+ T cells were instead examined for the expression of molecules that would assist their migration to these sites. The binding of the chemokine CXCR3 to its ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (IP-9) leads to the migration of activated T cells to sites of inflammation (397, 398), which in HIV-1 infection includes the lymph nodes, whilst the integrin α4β7 guides lymphocytes to the gut and other mucosal tissues by binding mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (412). The majority of Gag-specific IL-10+ CD8+ T cells did not express CXCR3, but in every subject studied a minority of cells (median 8.6%) did express the chemokine receptor, suggesting that a proportion of the cells are able to migrate to the lymph nodes and other inflamed tissues. Interestingly, CXCR3+ CD8+ T cells that produce IL-10 and inhibit IFN-γ production have previously been described (413); however, to my knowledge, CXCR3 expressing IL-10+ CD8+ T cells have not previously been reported in HIV-1 infection. Just under half the cells (median 48.0%) expressed β7, indicating that a substantial proportion were primed in and

capable of homing to the GALT. Gag-specific IL-10+ CD8+ T cells may, therefore, migrate to and function in these key sites of pathogenesis. The chemokine receptor CCR9 also plays an important role in guiding certain T cell populations to mucosal tissues (414, 415) but due to the difficulty in obtaining an effective anti-CCR9 antibody it was not possible to determine whether Gag-specific IL-10+ CD8+ T cells express the receptor. Transcriptional or NanoString analysis may have helped to resolve this issue.

FoxP3, CD25, CXCR3 and β7 were expressed by similar frequencies of IL-10$^-$ CD8+ T cells (which comprised the majority of the CD8+ population) and IL-10+ CD8+ T cells after stimulation by peptides spanning the HIV-1 Clade B Gag p55 protein. The lack of a phenotypic distinction between these CD8+ populations, plus the fact that the majority of Gag-specific IL-10+ CD8+ T cells produced IFN-γ, suggests that, rather than developing as a distinct population, Gag-specific IL-10+ CD8+ T cells may have originated as IFN-γ-producing effector or memory T cells that subsequently acquired the ability to produce IL-10 as a result of continuous antigenic stimulation. This phenomenon has been demonstrated in acute respiratory infection in mice, where virus-specific IFN-γ producing effector cells can acquire the ability to produce IL-10 after entry into the infected lung (416). Furthermore, IL-10-secreting CD8+ Tregs expressing CCR7 and CD45RO have been described in the tumour environment in human ovarian cancer and may have arisen from a central memory T cell population (417).

CD8+ Tregs that mediate their suppressive effects in an IL-10-dependent manner have been described in a number of clinical settings, including cancer and transplantation (396, 404). Pathogen-specific IL-10+ CD8+ T cells have also been described in chronic

Epstein-Barr virus (EBV) (320), HCV (318, 319) and Herpes Simplex Virus (HSV-2) (418), but the detailed phenotype and mechanism of suppression of these populations has yet to be fully elucidated. To determine whether phenotypically similar IL-10+ CD8+ T cells are found in other chronic viral infections, cytokine capture and flow cytometry was performed on PBMC from individuals with chronic CMV and HCV. Antigen specific IL-10+ CD8+ T cells were detected in both groups, at frequencies that were not significantly different to those seen in ART-naïve HIV-infected individuals. However, whereas expression of FoxP3 and CD25 was consistently low in HIV-specific IL-10+ CD8+ T cells, the expression of these markers varied considerably in CMV and HCV-specific IL-10+ CD8+ T cells. These data suggest that whilst antigen-specific IL-10+ CD8+ T cells may be found in a number of viral infections, the phenotype of Gag-specific IL-10+ CD8+ T cells may be unique to HIV-1. Significantly fewer HCV-specific IL-10+ CD8+ T cells secreted IFN-γ compared to CMV and HIV-specific IL-10+ CD8+ T cells. Compared to responses found in chronic HIV-1, where up to 4% of the total CD8+ T cell population may secrete IFN-γ in response to Gag (see Figure 4.9), HCV-specific IFN-γ+ CD8+ T cells are found at low frequencies, typically around 0.01% (419). This may explain the lower frequency of IL-10 and IFN-γ co-producing HCV-specific CD8+ T cells, particularly if these cells originated as IFN-γ-producing effector cells. It should be noted, however, that the majority of HCV-specific IL-10+ CD8+ T cells may be found in sites such as the liver (319), where their phenotype may be different from that observed in the peripheral blood. Furthermore, CMV encodes its own homologue of the IL-10 gene (325) and so some of the IL-10 production observed in CMV+ individuals may have been virus rather than host-derived.

Expression of the β7 integrin chain by CD4+ and CD8+ T cells was examined in HIV-infected subjects receiving ART, ART-naïve viraemic controllers, ART-naïve non-controllers and uninfected controls. Since the GALT is a major site of HIV-1 replication, it might be expected that the frequency of β7-expressing T cells would be increased in HIV-infected individuals, as elevated numbers of T cells are recruited to the site of infection. However, an elevation in β7 expression was observed in neither CD4+ nor CD8+ T cells in HIV-infected individuals compared to uninfected controls. Furthermore, there was no difference between ART-treated and ART-naïve HIV-infected subjects, nor between ART-naïve viraemic controllers and non-controllers. The latter finding was particularly surprising, as it might be expected that a greater degree of viral control would be associated with increased migration of effector T cells to the GALT. A possible explanation is that when α4β7-expressing cells migrate to the gut in HIV-infected individuals they become infected (in the case of CD4+ T cells) and/or rapidly undergo apoptosis (31). This effect could balance out any upregulation in α4β7 expression. Alternatively, α4β7-expressing cells may be retained in the gut for longer in HIV-infected individuals. However, the expression of αEβ7, the integrin responsible for lymphocyte retention in the GALT, has previously found to be decreased in the CD8+ T cells of the GALT of HIV-infected individuals (420). As patient samples were only available from one time point it was only possible to carry out a cross-sectional analysis of the cohort. Monitoring individual subjects over time, preferably both before and after HIV-1 infection, would provide a clearer indication of any changes in α4β7 expression as disease progresses.

In summary, Gag-specific IL-10+ CD8+ T cells do not express the natural Treg markers FoxP3 or CD25 but can co-produce IFN-γ, meaning that phenotypically they more

closely resemble Tr1 cells. They can also express markers associated with homing to key sites of HIV-1 pathogenesis, CXCR3 and β7. Antigen-specific IL-10+ CD8+ T cells are also found in CMV and HCV-infected individuals but these populations are more phenotypically diverse in terms of expression of CD25 and FoxP3 so the phenotype of the Gag-specific cells may be unique to HIV-1. In the next chapter, the function of Gag-specific IL-10+ CD8+ T cells will be investigated.

Chapter 5: Functional analysis of HIV-1 Gag-specific IL-10+ CD8+ T cells

5.1 Introduction

In the previous chapter, the phenotype of HIV-1 Gag-specific IL-10+ CD8+ T cells found in ART-naïve, HIV-infected subjects was examined. These cells expressed CXCR3 and the β7 integrin, suggesting that they could home to important sites of HIV-1 pathogenesis. Since they lacked expression of FoxP3 and CD25, and the majority co-produced IFN-γ, Gag-specific IL-10+ CD8+ T cells did not appear to represent a population of thymus-derived "natural" Tregs; however, the population may still play a regulatory role. HIV-specific IL-10+ CD8+ T cells that did not express FoxP3 have previously been reported to suppress T cell proliferation (359) and IL-2 production (360). Furthermore, exogenous or recombinant IL-10 can suppress the expression of cytokines such as IL-2 and IL-5 by T cells (291, 312). It is possible, therefore, that Gag-specific IL-10+ CD8+ T cells modulate the T cell cytokine profile in chronically infected individuals.

HIV-1 infection is associated with an increase in T cell activation, and the upregulation of the activation markers CD38 and HLA-DR is associated with disease progression (368, 369). Activated CD4+ T cells also upregulate CCR5, a co-receptor for HIV-1 entry, upon stimulation with IL-2 and IL-12 (421). Since Gag-specific IL-10+ CD8+ T cells are detected almost exclusively in ART-naïve subjects who have elevated expression of CD38 and HLA-DR on T cells, it is possible that the IL-10-producing population is induced in response to T cell activation.

Most previous studies concerned with the functions of IL-10 producing T cell populations have focussed on their potential effect on the proliferation, cytokine production and effector function of other T cells (320, 359, 360). However, since the IL-10 receptor is also expressed by antigen-presenting cells (APCs) (104, 261), and IL-10 is known to modulate antigen presentation and cytokine production by monocytes, macrophages and DCs (289, 290, 422), it is possible that Gag-specific IL-10+ CD8+ T cells influence the response of APCs to HIV-1.

In chronic viral infections such as HIV-1 and HCV, it has been proposed that virus-specific IL-10+ CD8+ T cells have a negative effect on viral control, based on the observation that virus-specific effector T cell functions such as proliferation, cytolysis and cytokine production are enhanced when these cells are experimentally depleted (320, 359, 360). However, given the anti-inflammatory properties of IL-10, HIV-specific IL-10-producing cells may play a role in limiting immune pathology, a possibility that has not been explored by previous studies. Furthermore, since HIV-1 replicates more efficiently in activated CD4+ T cells, IL-10 responses may help to limit viral replication (171, 172).

Previously, the effect of IL-10 in chronic HIV-1 has been studied either through the depletion of all IL-10-producing cells or the global blockade of IL-10 signalling using antibodies directed against IL-10R (356, 359, 360). Given the diverse cellular sources of IL-10, it is unclear whether the effects observed in these studies were due to a general effect of IL-10 signalling or to the removal of a specific IL-10+ population. In order to determine the functional characteristics of Gag-specific IL-10+ CD8+ T cells, a

novel method was used whereby CD8+ T cells were separated from other PBMC prior to Gag stimulation and removal of IL-10+ cells. IL-10-depleted CD8+ T cells were subsequently recombined with the rest of the PBMC (see Materials and Methods section 2.6.1 for further details). This approach ensured that only Gag-specific IL-10+ CD8+ T cells were removed from PBMC.

In summary, the primary aim of this chapter was to explore the effect of Gag-specific IL-10+ CD8+ T cells on putative target cell populations.

5.2 Study subjects

Ten ART-naïve subjects with a minimum Gag-specific IL-10 response of 0.01% of CD8+ T cells, as determined by cytokine capture analysis, were selected for further functional study. Due to sample availability not all analyses were performed on all individuals. Subjects were recruited either from the Oxford cohort described in Chapter 3 section 3.2 or from the University of Oxford Centre for Clinical Magnetic Resonance Research, which recruited patients from HIV clinics in Oxford, Reading and Slough (samples kindly provided by C Holloway – cohort data presented in Materials and Methods section 2.1).

5.3 IL-10R expression is maintained in HIV-infected individuals

In order to investigate whether peripheral blood cell populations from HIV-infected individuals retain normal expression of the IL-10R, expression of the receptor by CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes was examined by flow cytometry in nine HIV-infected subjects receiving ART, six ART-naïve HIV-infected subjects and eight uninfected controls. The gating strategy used to identify IL-10R expression on these cells is shown in Figure 5.1.

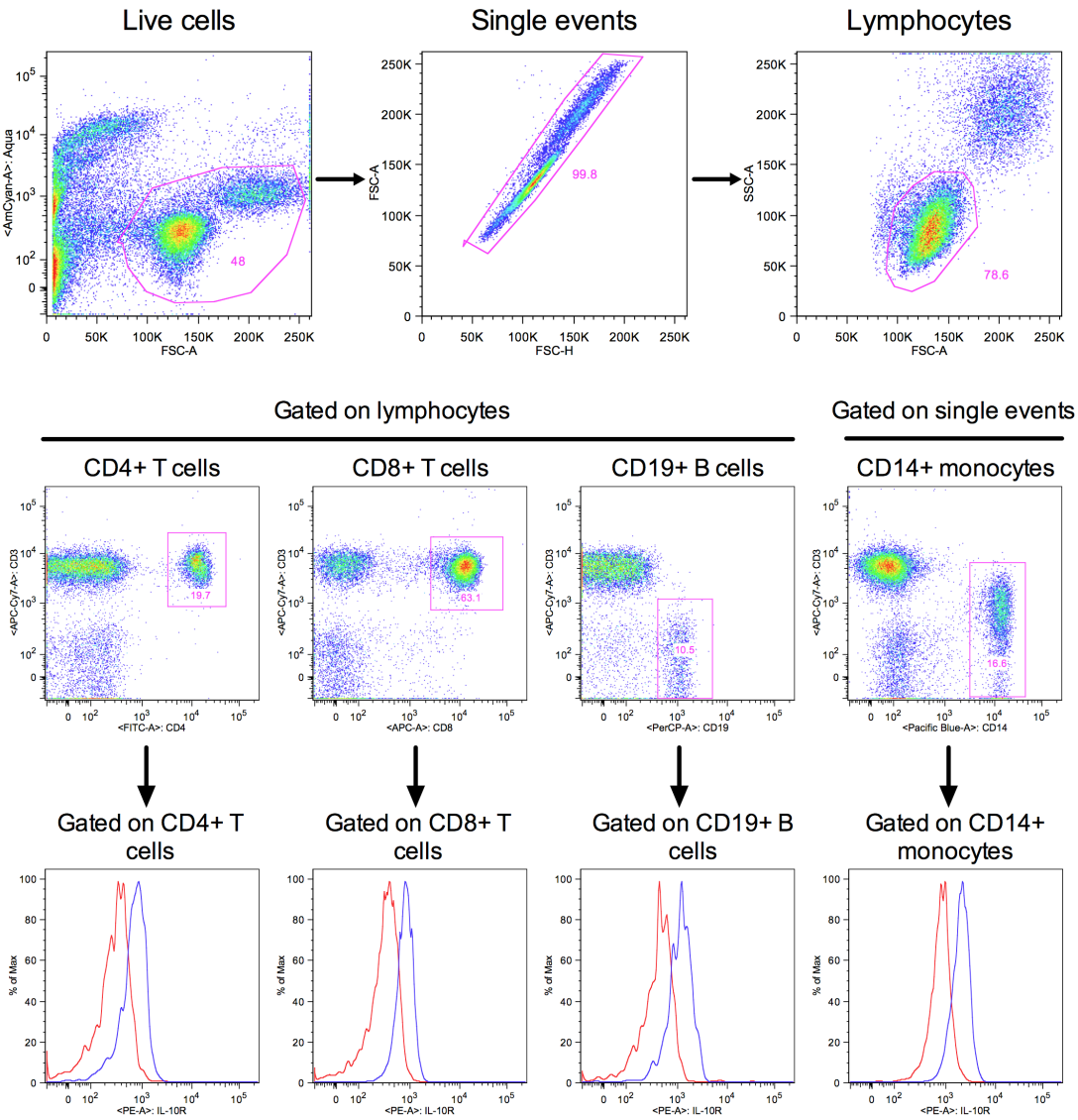


Figure 5.1 Gating strategy for the identification of IL-10R expression on CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes. PBMC were stained with CD3-APC-Cy7, CD4-FITC, CD8-APC, CD19-PerCP, CD14-Pacific blue, IL-10R-PE and Aqua live/dead cell stain. IL-10R mean fluorescence intensity (MFI) was calculated by subtracting the MFI when an isotype control antibody was used (red line in bottom panels) from the MFI when the anti-IL-10R antibody was used (blue line in bottom panels).

Figure 5.2 shows IL-10R mean fluorescence intensity (MFI) on CD4+ T cells, CD8+ T cells, B cells and monocytes in the three subject groups. There was a significant increase in IL-10R MFI on all the cell types in ART-naïve HIV-infected subjects compared to uninfected controls, as determined by Kruskal-Wallis test. Furthermore, there was a significant increase in IL-10R MFI on CD4+ T cells and CD8+ T cells in ART-naïve subjects compared to HIV-infected subjects on ART. However, there was no difference in IL-10R expression between HIV-infected subjects on ART and uninfected controls on any of the cell types ($p > 0.05$). These data indicate that IL-10R expression was increased in ART-naïve HIV-infected subjects compared to uninfected controls.

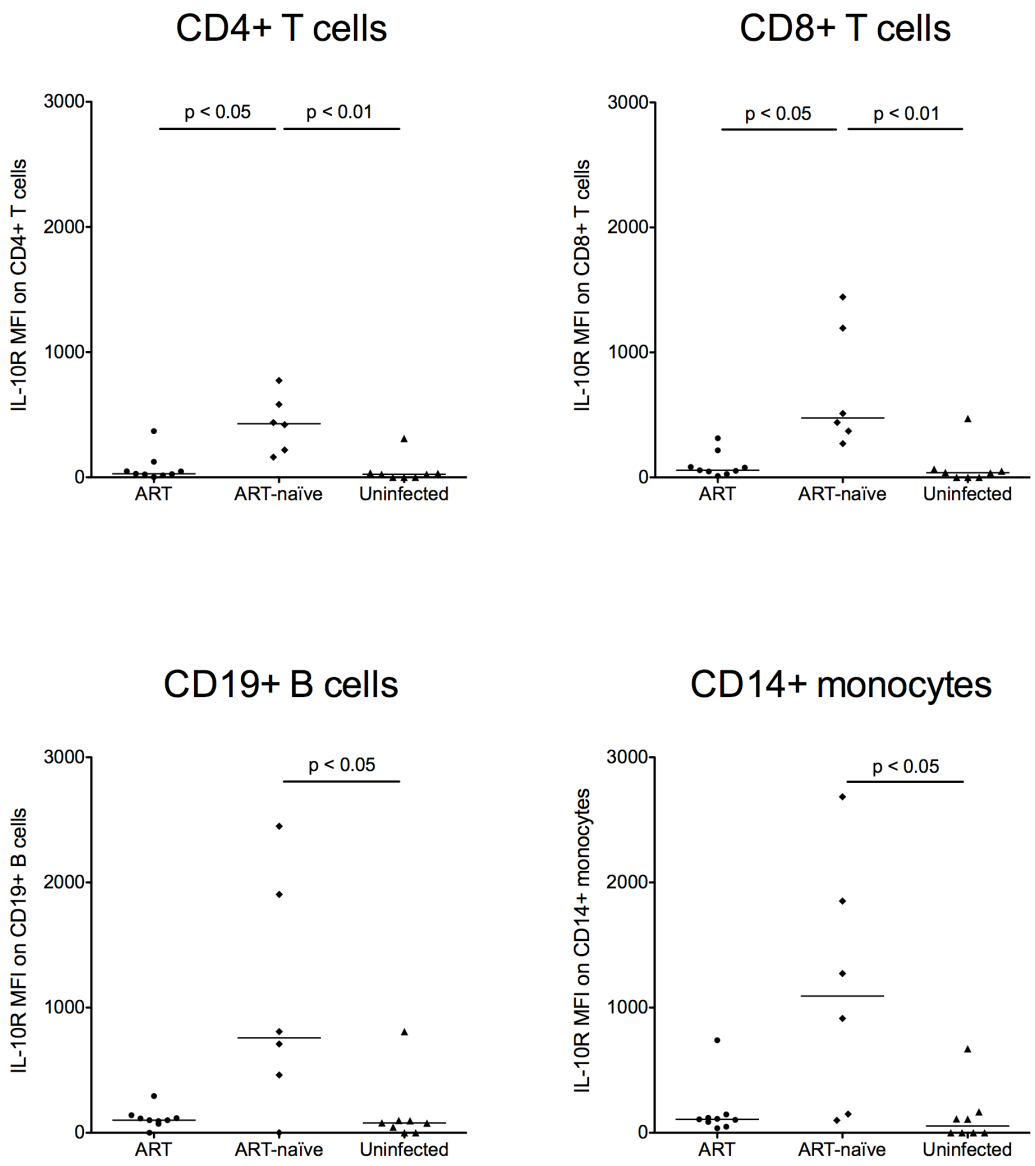


Figure 5.2 IL-10R MFI on CD4+ T cells, CD8+ T cells, CD19+ B cells and CD14+ monocytes. Statistical analyses were carried out using Kruskal-Wallis test.

5.4 Development of a protocol for the depletion of Gag-specific IL-10+ CD8+ T cells

In order to determine the function of Gag-specific IL-10+ CD8+ T cells, these cells were depleted from patient PBMC by magnetic separation (detailed in Materials and Methods section 2.6.1), followed by culture of the depleted PBMC. As described in the introduction to this chapter, depletion of IL-10+ cells was performed on CD8+ T cells in isolation to ensure that other cells producing IL-10 remained in the PBMC. However, it was possible that CD8+ T cells in isolation might not present antigen to each other efficiently, or respond to peptides presented by other CD8+ T cells. In order to address this possibility a comparative cytokine secretion assay was carried out, comparing IFN-γ production by CD8+ T cells that were stimulated in isolation with IFN-γ production by CD8+ T cells that were stimulated in the presence of other PBMC. IFN-γ production, rather than IL-10 production, was chosen as an indicator of the capacity of CD8+ T cells to respond to peptide presented by other CD8+ T cells because cells that produce this cytokine in response to antigenic stimulation are more numerous than those that produce IL-10 (see Figure 4.9). The frequency of IFN-γ+ CD8+ T cells was slightly higher when CD8+ T cells were stimulated in isolation compared to when CD8+ T cells were stimulated in the presence of other PBMC, indicating that CD8+ T cells can efficiently present peptide to one another in the absence of other PBMC (Figure 5.3). This finding demonstrated that it was feasible to perform Gag stimulation followed by depletion of IL-10+ cells on CD8+ T cells alone and then recombine the depleted cells with the other PBMC.

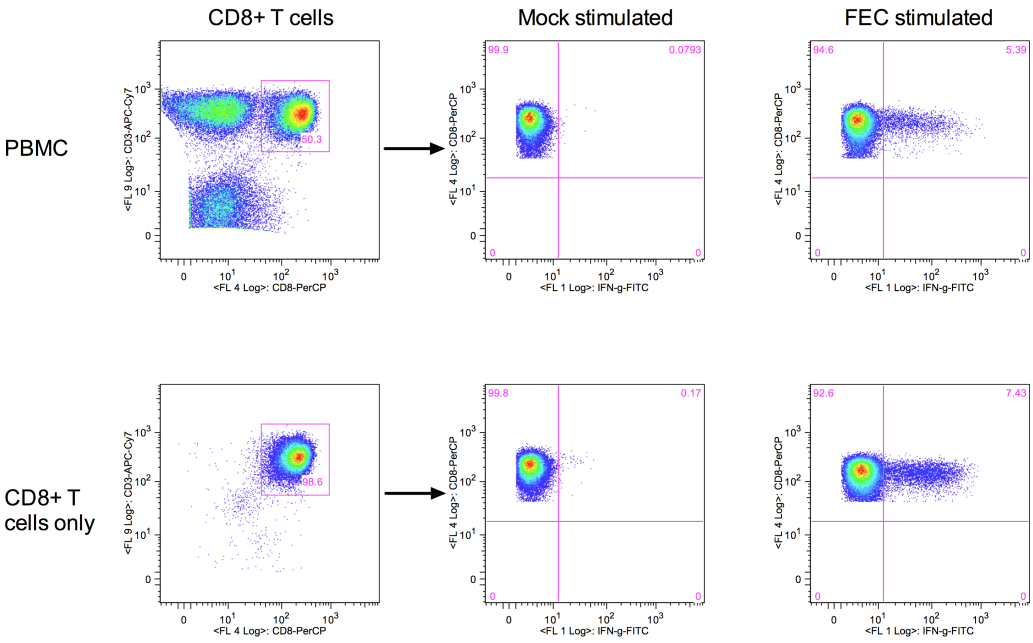


Figure 5.3 IFN-γ-producing CD8+ T cells are detected at comparable frequencies when CD8+ T cells are cultured either alone or with other PBMC. Unfractionated PBMC and purified CD8+ T cells were stimulated for 3 hours with a pool of Influenza, EBV and CMV peptides (FEC) and stained with CD3-APC-Cy7, CD8-PerCP, IFN-γ-FITC and Aqua live/dead cell stain. CD8+ T cells were gated as shown in Figure 5.1.

Having determined that stimulation of CD8+ T cells alone did not affect their ability to present or respond to peptide, IL-10+ CD8+ T cells were depleted from PBMC in two steps, described in detail in the Materials and Methods chapter. Briefly, CD8+ T cells were first isolated from PBMC by magnetic separation and stimulated with HIV-1 Gag peptides for 6 hours, during which time the remaining CD8-depleted fraction was also stimulated with Gag peptides. Next, IL-10+ CD8+ T cells were depleted from the CD8+ fraction using a second round of magnetic separation. The IL-10-depleted CD8+ T cells were added back to the $CD8^-$ PBMC and these cells were cultured for 16 hours. In the undepleted control the cells were treated identically except that IL-10 capture antibody was not added to CD8+ T cells, so that the HIV-specific IL-10-producing

CD8+ T cells remained in the PBMC. The schema for the depletion of Gag-specific IL-10+ CD8+ T cells is shown in Figure 5.4.

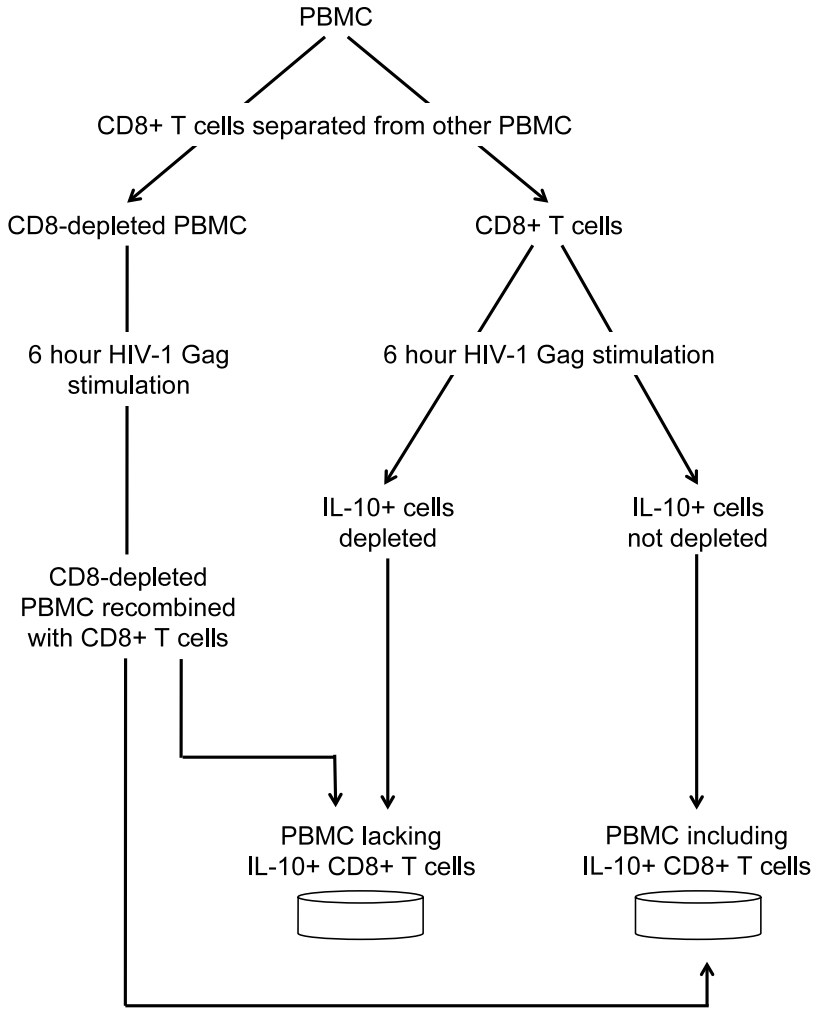


Figure 5.4 Schema showing the protocol that was used to deplete Gag-specific IL-10+ CD8+ T cells from PBMC.

To ensure that depletion of Gag-specific IL-10+ CD8+ T cells reduced IL-10 levels, supernatants from PBMC cultured for 16 hours in the presence or absence of these cells were examined for IL-10 by Luminex. Depletion of Gag-specific IL-10+ CD8+ T cells reduced the level of IL-10 in the supernatant after 16 hours of subsequent culture in all

6 of the ART-naïve individuals studied (p = 0.031; Wilcoxon matched-pairs signed rank test; Figure 5.5).

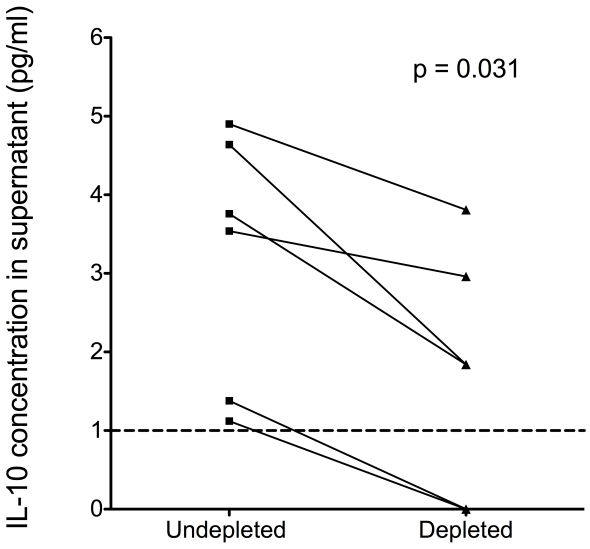


Figure 5.5 Depletion of Gag-specific IL-10+ CD8+ T cells from PBMC resulted in decreased IL-10 concentration in supernatants after 16 hours of culture, compared to PBMC containing IL-10+ CD8+ T cells. Supernatants were collected from cultures containing 10^6 PBMC/ml. The dashed line shows the limit of detection of the assay. Statistical analysis was carried out using Wilcoxon matched-pairs signed rank test.

5.5 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells does not increase IFN-γ production, as measured by ELISPOT

HIV-specific IL-10+ CD8+ T cells have previously been reported to suppress IL-2 but not IFN-γ production by HIV-specific effector CD8+ T cells (360). However, Brockman *et al* observed that antibody blockade of the IL-10R enhanced IFN-γ secretion by HIV-specific CD4+ T cells *in vitro* (356). To determine whether the Gag-specific IL-10+ CD8+ T cells identified in this cohort suppressed HIV-specific IFN-γ production, these CD8+ T cells were depleted from the PBMC of 8 ART-naïve subjects as described previously and an IFN-γ ELISPOT was conducted. The number of IFN-γ-producing cells did not differ significantly between cultures from which Gag-specific IL-10+ CD8+ T cells had been depleted and cultures containing these cells (p > 0.05;

Wilcoxon matched-pairs signed rank test; Figure 5.6), indicating that Gag-specific IL-10+ CD8+ T cells did not affect the ability of other T cells to produce IFN-γ in response to Gag over this period of time.

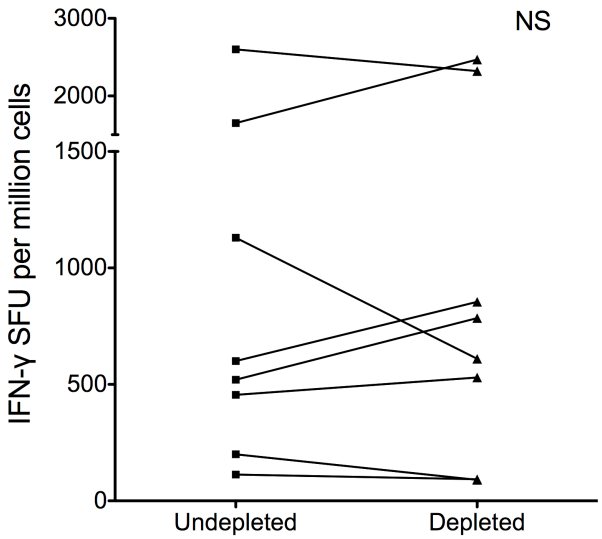


Figure 5.6 IFN-γ production by Gag-stimulated T cells in the presence or absence of Gag-specific IL-10+ CD8+ T cells. PBMC (10^5/well) were incubated on ELISPOT plates for 16 hours. Statistical analysis was performed using Wilcoxon matched-pairs signed rank test.

5.6 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells does not alter levels of Th1-type cytokines in culture supernatants

IL-10 has been reported to suppress the production of a number of cytokines, including IL-2, IL-12, GM-CSF, TNF-α and IFN-γ by human monocytes, macrophages and T cells (288, 290, 291). These cytokines can all either be produced by Th1 cells, or, in the case of IL-12, are required for their development (176, 423). Furthermore, monocyte-derived IL-10 can suppress Th1 responses to HCV (424). In order to determine whether Gag-specific IL-10+ CD8+ T cells influence the production of cytokines by Th1 cells, and to confirm the results of the ELISPOT assay with respect to IFN-γ, supernatants from PBMC from 6 ART-naïve subjects cultured for 16 hours in the presence or absence of these IL-10+ cells were examined by Luminex for levels of IL-2, IL-12 p70, GM-CSF, IFN-γ and TNF-α. The level of IL-12 p70 was below the limit of detection of the assay in 5 out of the 6 the subjects and so it was not possible to determine the effect of depletion on the production of this cytokine. Depletion of Gag-specific IL-10+ CD8+ T cells did not significantly affect the level of any of the other cytokines, indicating that this cell population does not influence the production of Th1-related cytokines over the period of time studied ($p > 0.05$; Wilcoxon matched-pairs signed rank test; Figure 5.7).

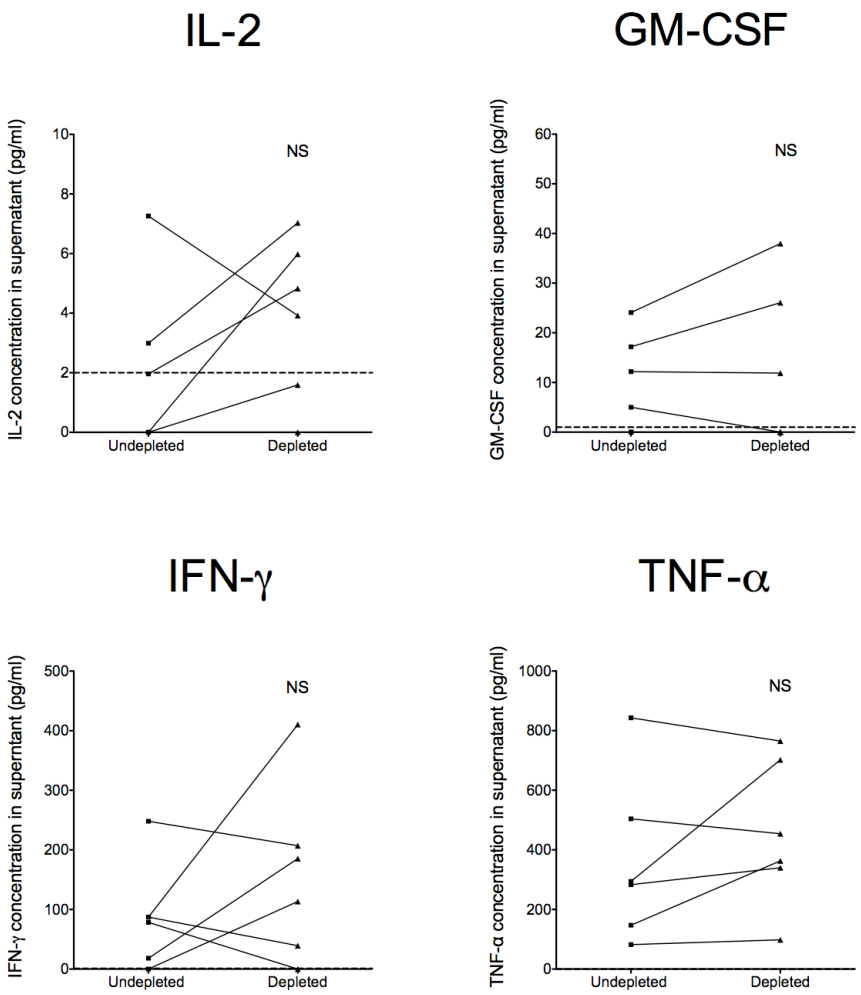


Figure 5.7 Concentrations of IL-2, GM-CSF, IFN-γ and TNF-α in supernatants from Gag-stimulated PBMC (10^6/ml) cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. The limits of detection of the Luminex assay are shown by the dashed lines. Statistical analyses were performed using Wilcoxon matched-pairs signed rank test.

5.7 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells does not alter levels of Th2-type cytokines in culture supernatants

Gag-specific IL-10+ CD8+ T cells did not appear to affect the production of Th1-type cytokines during a 16 hour culture period. However, as IL-10 has previously been shown to inhibit the production of IL-5 by human and mouse cells (312), these IL-10+ cells may instead have an effect on Th2-mediated responses. In order to investigate this possibility, Luminex was used to examine the levels of IL-4, IL-5 and IL-13, all of which can be secreted by Th2 cells (176, 425), in supernatants from PBMC from 6 ART-naïve subjects cultured for 16 hours in the presence or absence of Gag-specific IL-10+ CD8+ T cells. The levels of IL-5 and IL-13 were below the limit of detection of the assay in 6 out of 6 and 5 out of 6 respectively of the subjects studied and so it was not possible to determine the effect of depletion on the production of these cytokines. Depletion of Gag-specific IL-10+ CD8+ T cells did not significantly affect the level of IL-4 in supernatants after 16 hours culture, suggesting that these cells do not influence the production of this cytokine over this period of time ($p > 0.05$; Wilcoxon matched-pairs signed rank test; Figure 5.8).

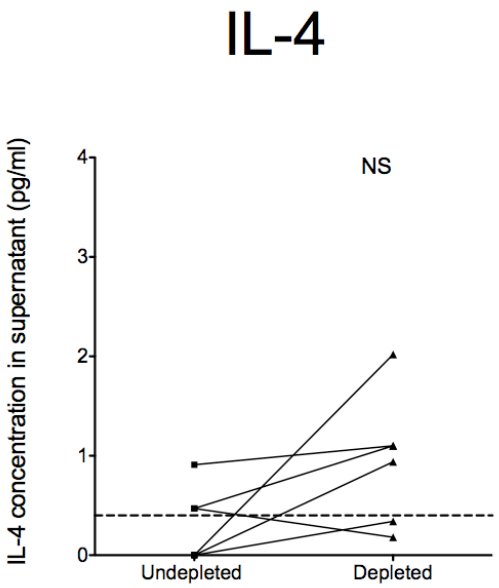


Figure 5.8 The concentration of IL-4 in supernatants from Gag-stimulated PBMC (10^6/ml) cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. The dashed line shows the limit of detection of the assay. Statistical analysis was performed using Wilcoxon matched-pairs signed rank test.

5.8 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells does not increase T cell expression of CD38 or HLA-DR

To determine whether Gag-specific IL-10+ CD8+ T cells affect T cell activation, these cells were depleted from PBMC of 8 ART-naïve HIV-infected subjects as previously described, after which PBMC were cultured for 16 hours, either in the presence or absence of these IL-10+ cells, and then examined by flow cytometry for the expression of CD38 and HLA-DR by T cells. Depletion of Gag-specific IL-10+ CD8+ T cells had no statistically significant effect on CD38 or HLA-DR expression on either CD4+ or CD8+ T cells ($p > 0.05$; paired t test; Figure 5.9), indicating that these cells do not influence T cell activation over this period of time.

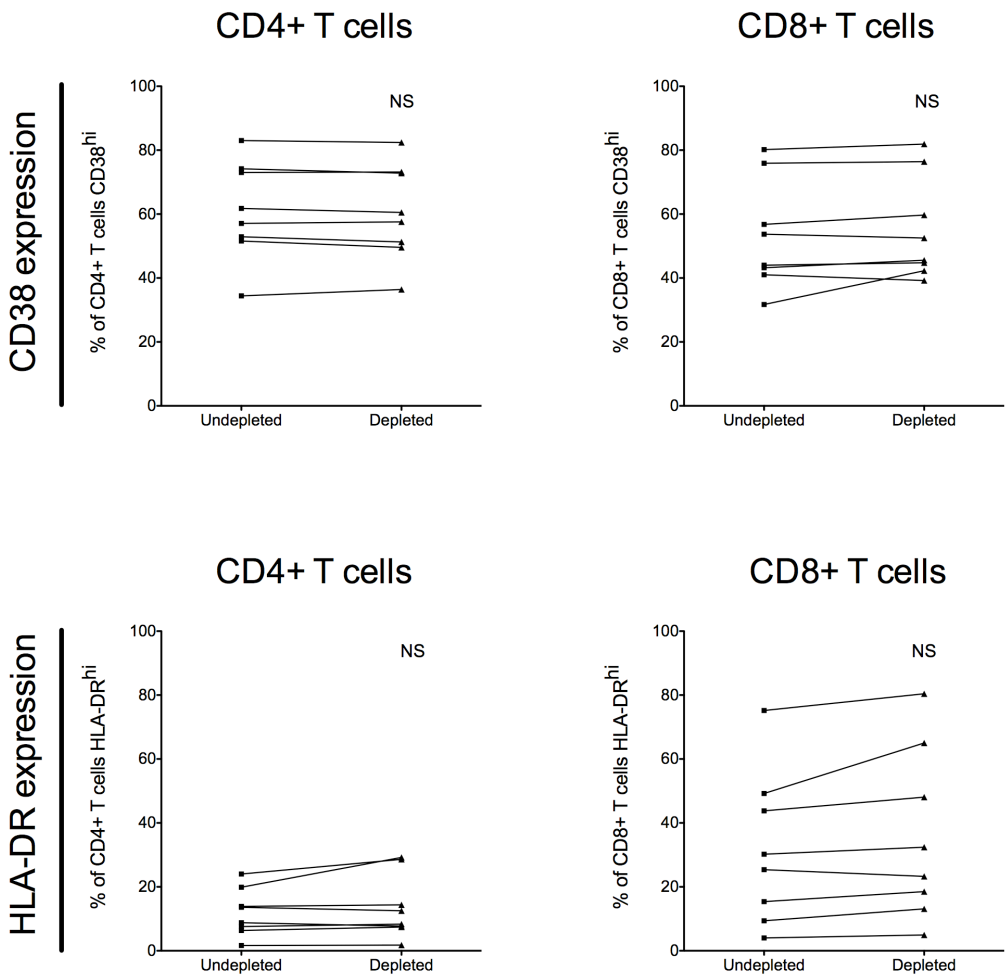


Figure 5.9 Expression of CD38 and HLA-DR by CD4+ and CD8+ T cells in Gag-stimulated PBMC cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. PBMC were stained with CD3-FITC, CD4 or CD8-PerCP, CD38-PE, HLA-DR-APC and Aqua live/dead cells stain. CD38+ and HLA-DR+ cells were identified as shown in Figure 3.10. Statistical analyses were performed using the paired t test.

5.9 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells increases monocyte expression of CD38 and HLA-DR, but not CD80 or CD86

The data presented in the preceding sections of this chapter suggests that Gag-specific IL-10+ CD8+ T cells do not act directly on other T cells to induce changes in cytokine production or activation, at least over a 16-hour period. Given the ability of IL-10 to downregulate MHC Class II molecules, it is possible that Gag-specific IL-10+ CD8+ T cells act on APCs (276). To determine whether this was the case, IL-10+ CD8+ T cell-depleted PBMC were cultured for 16 hours as before, but this time CD14+ monocyte expression of CD38 and HLA-DR was examined by flow cytometry, using the same cytometer and the same settings for every experiment. Since monocytes constitutively express a high level of CD38 and HLA-DR compared to T cells (Figure 5.10), the MFI of these markers on monocytes was determined. Depletion of Gag-specific IL-10+ CD8+ T cells led to a small but reproducible increase in CD38 expression on CD14+ monocytes, which achieved statistical significance ($p = 0.011$; paired t test; Figure 5.11) and there was a trend towards increased HLA-DR expression on monocytes ($p = 0.078$; paired t test). There was a significant positive correlation between the frequency of Gag-specific IL-10+ CD8+ T cells and the change in CD38 mean fluorescence intensity (MFI) on monocytes when these IL-10+ cells were removed ($r = 0.95$; $p = 0.0004$; Spearman's rank correlation; Figure 5.12). Taken together, these data indicate that Gag-specific IL-10+ CD8+ T cells modulate monocyte activation by reducing CD38 and HLA-DR expression on these cells.

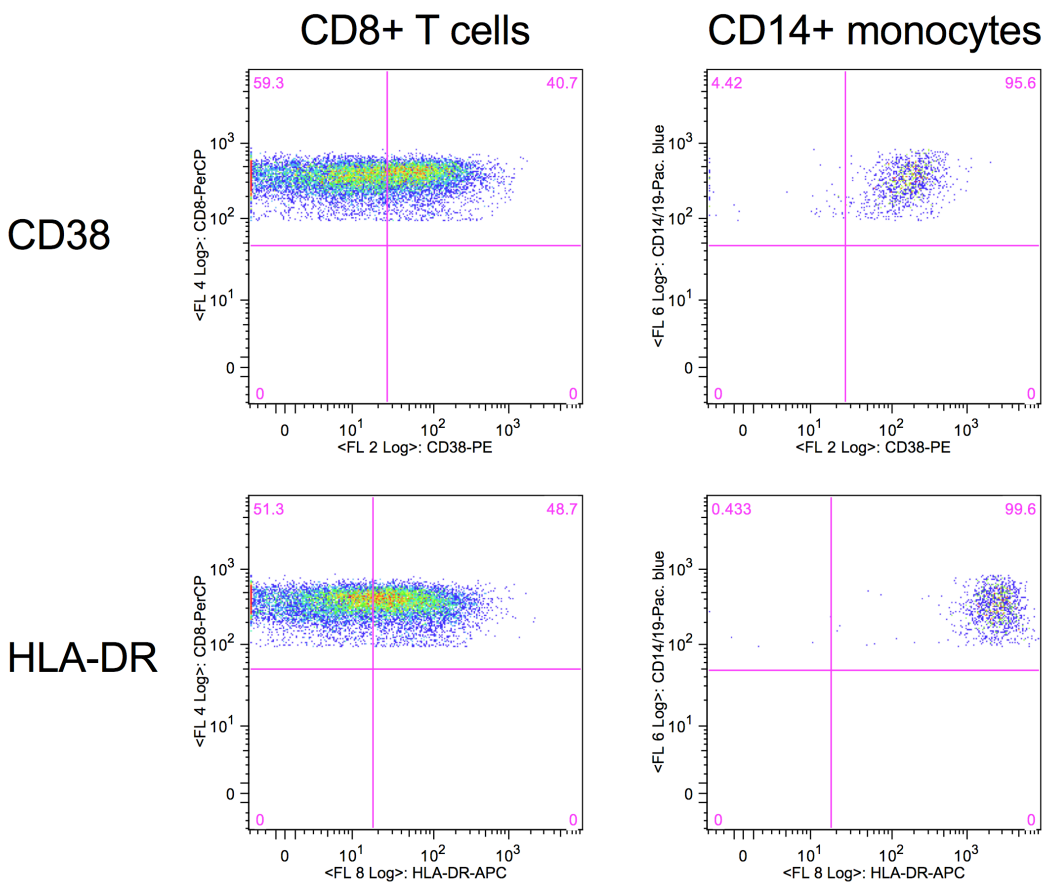


Figure 5.10 Representative flow cytometric plots comparing the expression of CD38 and HLA-DR on CD8+ T cells and CD14+ monocytes. CD8+ T cells and monocytes were identified as in Figure 5.1. CD38+ and HLA-DR+ cells were identified as in Figure 3.10.

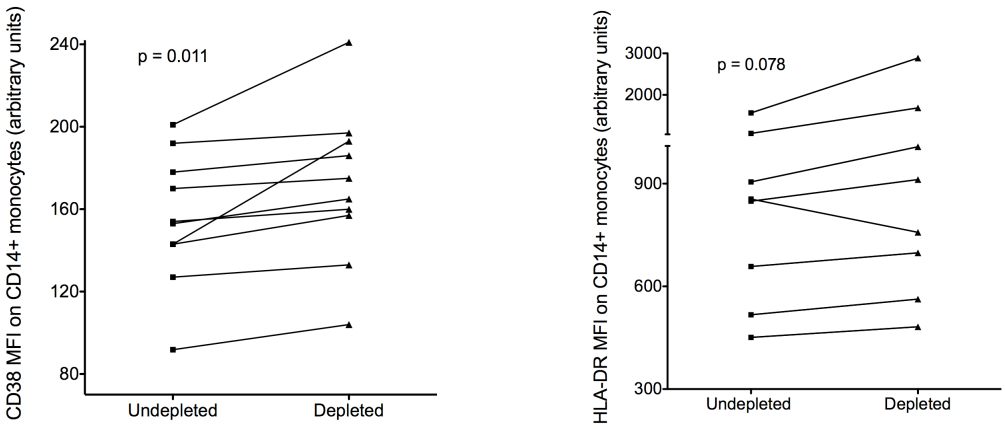


Figure 5.11 Expression of CD38 and HLA-DR by CD14+ monocytes in Gag-stimulated PBMC cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. PBMC were then stained with CD3-APC-Cy7, CD14-Pacific blue, CD38-PE, HLA-DR-APC and Aqua live/dead cells stain. CD14+ monocytes were identified as shown in Figure 5.1. Statistical analyses were performed using the paired t test.

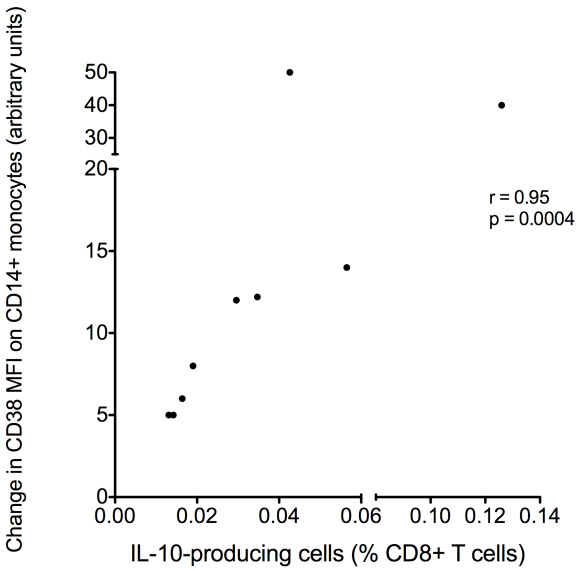


Figure 5.12 Correlation between the frequency of Gag-specific IL-10+ CD8+ T cells and the change in monocyte CD38 MFI. Statistical analysis performed using Spearman's rank correlation.

Since Gag-specific IL-10+ CD8+ T cells appeared to have an effect on the expression of CD38 by monocytes, the effect of depleting these cells on monocyte expression of CD80 (B7.1) and CD86 (B7.2) was next examined in 5 ART-naïve individuals. The gating strategy used to identify CD80 and CD86 expression on CD14+ monocytes is shown in Figure 5.13. CD80 and CD86 are costimulatory ligands that bind to CD28 on T cells to augment the effect of signalling through the TCR following binding to its cognate peptide-MHC complex (426-428). When stimulated in the absence of these costimulatory molecules, naïve T cells become anergic (428). Freshly isolated human monocytes express CD86 but not CD80 *ex vivo* (429); however monocyte differentiation into monocyte-derived dendritic cells (MDDCs) is accompanied by the upregulation of CD80 and CD86 (430). IL-10 has previously been shown to downregulate the expression of CD80 and CD86 on monocytes, suggesting that the cytokine may impair the differentiation of monocytes into MDDCs (278, 279). However, depletion of Gag-specific IL-10+ CD8+ T cells in the present study did not significantly alter the expression of CD80 or CD86 on CD14+ monocytes after a 16 hour culture period ($p > 0.05$; Wilcoxon matched-pairs signed rank test; Figure 5.14), and there was no detectable expression of CD80 above the level of the isotype control in 3 of the 5 subjects studied.

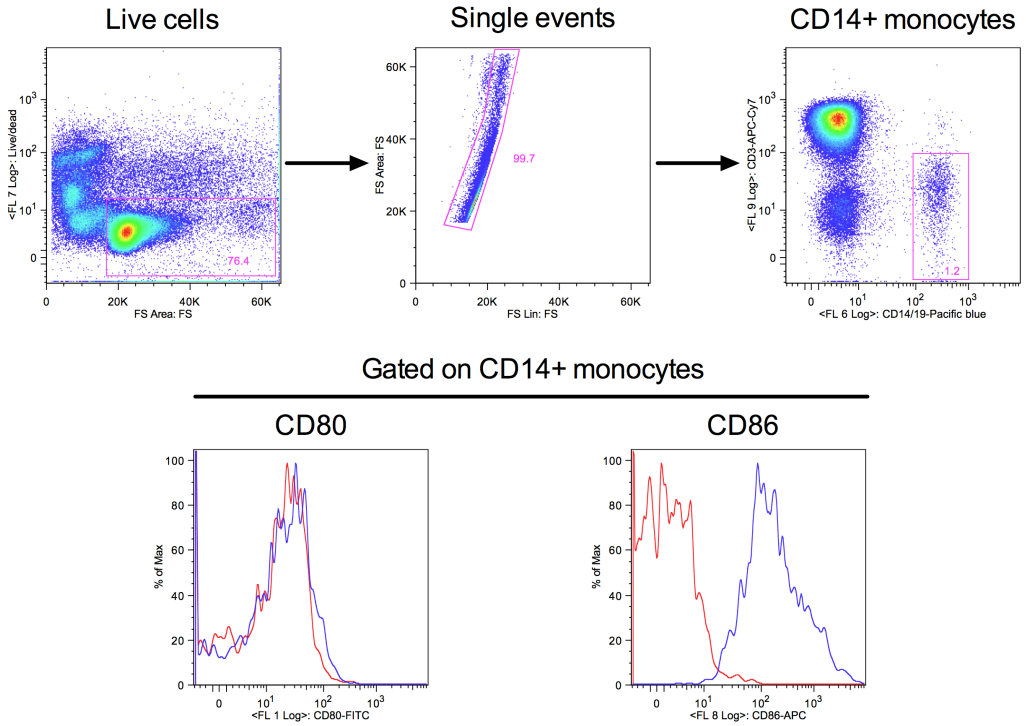


Figure 5.13 Gating strategy for the determination of CD80 and CD86 expression on CD14+ monocytes. PBMC were stained with CD3-APC-Cy7, CD14-Pacific blue, CD80-FITC, CD86-APC and Aqua live/dead cell stain. Since the expression of CD80 is consistently low and the expression of CD86 is consistently high compared to the isotype control, expression of both molecules was quantified by subtracting the MFI of the isotype control (shown in red in the lower panels) from the MFI of the anti-CD80 or CD86 antibody (shown in blue in the lower panels).

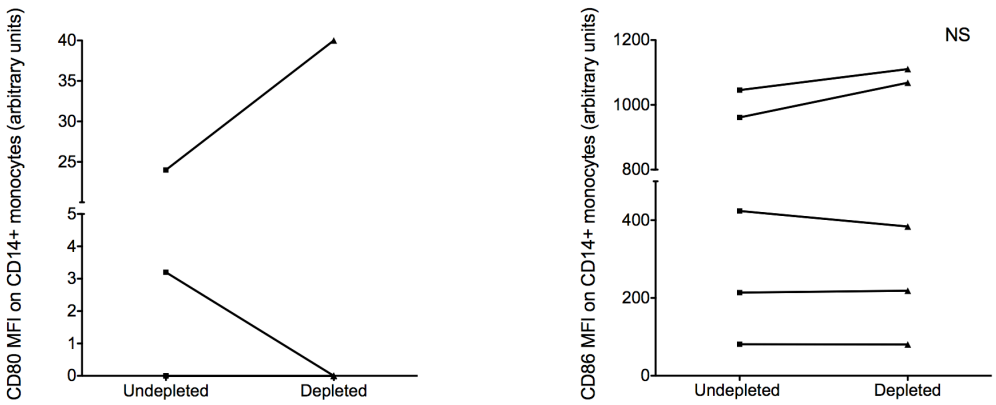


Figure 5.14 Expression of CD80 and CD86 on CD14+ monocytes in Gag-stimulated PBMC cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. Statistical analysis was conducted using Wilcoxon matched-pairs signed rank test.

In summary, the depletion of Gag-specific IL-10+ CD8+ T cells did not seem to affect T cell activation or cytokine production over a 16 hour culture period, but lead to an upregulation of CD38 on CD14+ monocytes.

5.10 Infection of PBMC with HIV-1_{BaL} leads to upregulation of CD38 on monocytes, but this upregulation is not reversed by adding exogenous IL-10

It is well established that CD4+ and CD8+ T cells upregulate CD38 in chronic HIV-1 infection, and that this upregulation is reversed by suppression of viral replication during successful ART (368, 369, 379) but the role of CD38 expressed on monocytes is less clearly defined. The changes in monocyte CD38 expression after the depletion of Gag-specific IL-10+ CD8+ T cells were small, therefore to determine the significance of changes in monocyte CD38 expression in HIV-1 infection, CD8-depleted PBMC from four HIV-uninfected individuals were activated for three days with PHA and then infected with HIV-1_{BaL} at a multiplicity of infection (MOI) calculated to achieve a typical level of infection of 5% of CD4+ T cells (as indicated by the expression of HIV-1 Gag p24 antigen, measured by intracellular staining). After 3 and 5 days culture PBMC were examined by flow cytometry to determine the number of p24+ CD4+ T cells and monocytes and examine whether HIV-1 infection had increased surface expression of CD38 by monocytes. The gating strategy used to identify p24+ CD4+ T cells and CD14+ monocytes is shown in Figure 5.15.

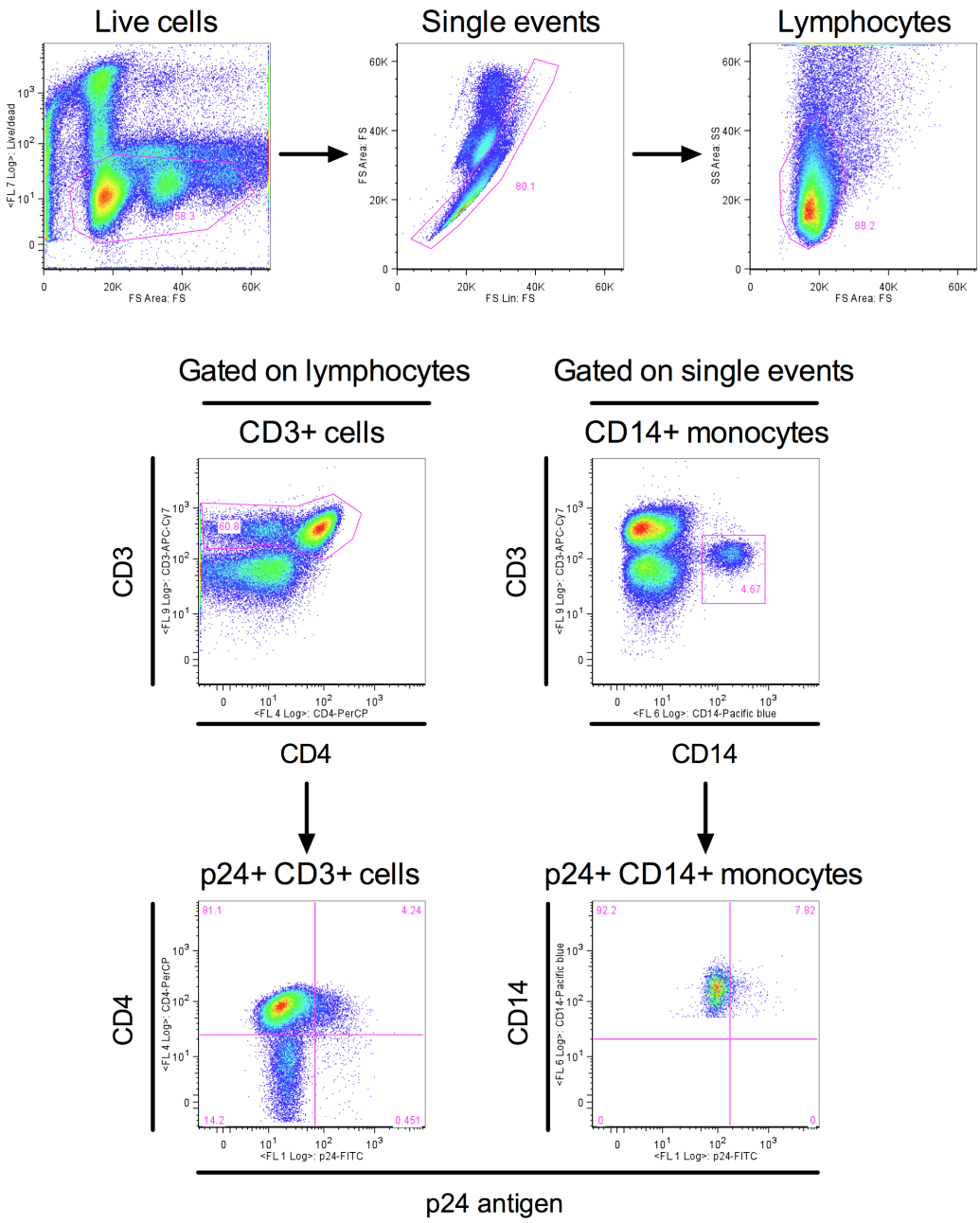


Figure 5.15 Gating strategy for the identification of p24+ CD4+ T cells and CD14+ monocytes. CD8-depleted cells were infected with HIV-1_{BaL} and then stained 3 and 5 days later with CD3-APC-Cy7, CD4-PerCP, CD8-APC, CD14-Pacific blue, KC-57(p24)-FITC, CD38-PE and Aqua live/dead cell stain. p24+ cells that have downregulated CD4 are included in the percentage of HIV-infected CD4+ T cells.

Figure 5.16 shows representative flow cytometric plots of p24+ CD4+ T cells and CD14+ monocytes in mock-infected and HIV-1_{BaL}-infected cultures after 3 days and the expression of CD38 on monocytes from the same cultures. CD38 MFI on monocytes was higher in HIV-1_{BaL}-infected cultures than in mock-infected cultures in all four subjects studied after both 3 and 5 days culture (Figure 5.17). Infected (p24+) monocytes ("exposed infected", or EI) expressed the highest level of CD38, followed by $p24^-$ monocytes from HIV-1_{BaL}-infected PBMC cultures ("exposed uninfected", or EU), with the lowest CD38 expression found on $p24^-$ monocytes from mock-infected PBMC cultures ("unexposed uninfected", or UU; Figure 5.18). This suggests that exposure to HIV is sufficient to upregulate CD38 expression on monocytes and that direct infection augments the level of expression still further.

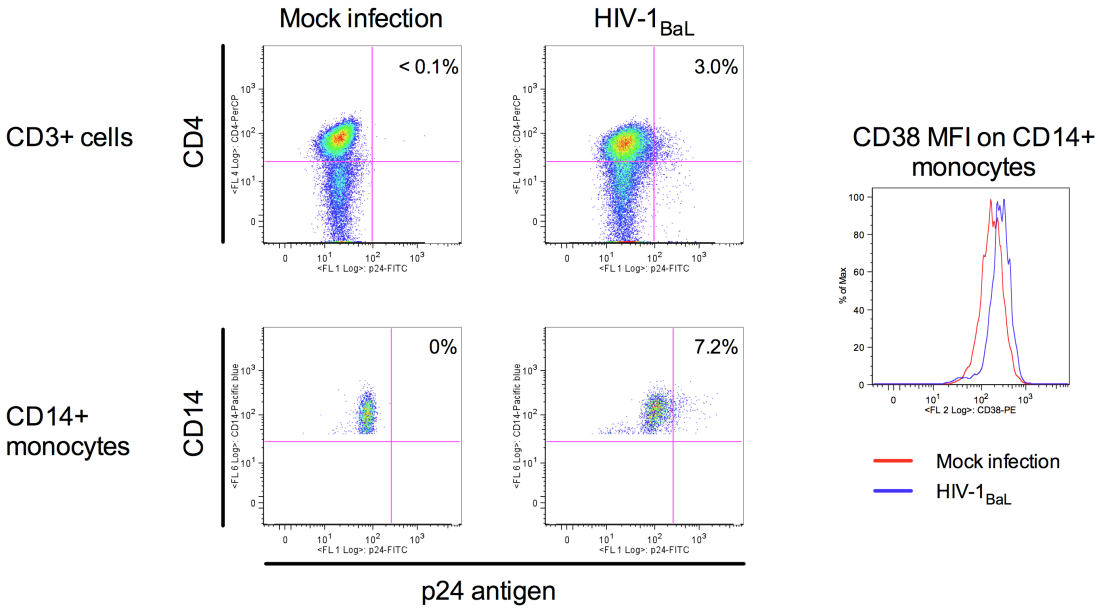


Figure 5.16 Representative plots of HIV-infected (p24+) CD4+ T cells and CD14+ monocytes and CD38 expression on CD14+ monocytes in mock-infected and HIV-1_{BaL}-infected PBMC cultures.

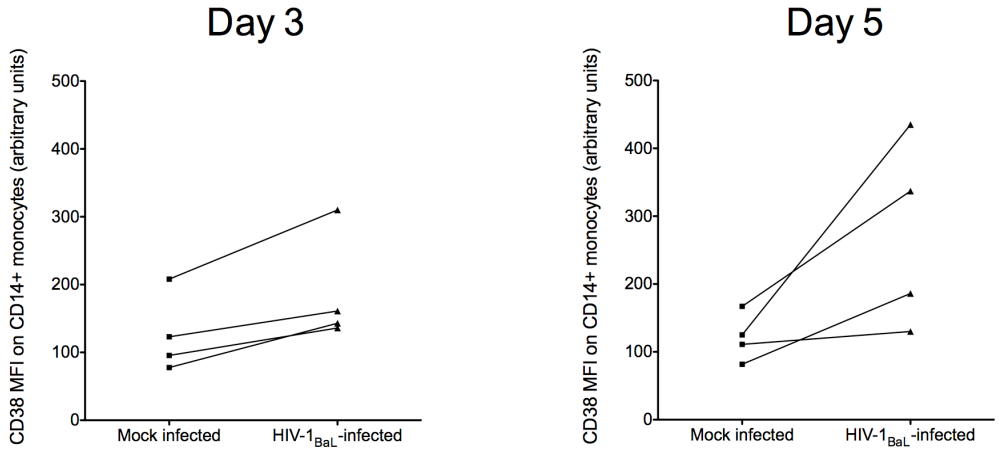


Figure 5.17 Expression of CD38 by CD14+ monocytes in mock-infected and HIV-1_{BaL}-infected PBMC cultures 3 and 5 days after infection.

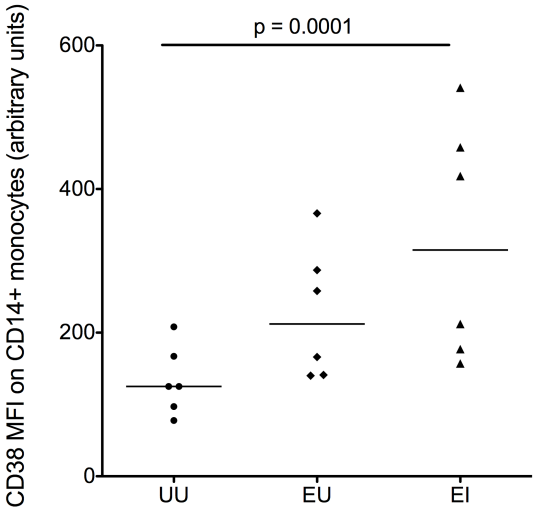


Figure 5.18 Expression of CD38 by $p24^-$ monocytes in mock-infected cultures ("unexposed uninfected", or UU), $p24^-$ monocytes in HIV-1_{BAL}-infected cultures ("exposed uninfected", or EU) and p24+ monocytes in HIV-1_{BaL}-infected cultures ("exposed infected", or EI). Statistical analysis conducted using Friedman test.

Since HIV-1 infection of CD14+ monocytes appeared to increase their expression of CD38, the increase in monocyte CD38 expression that was observed after depletion of Gag-specific IL-10+ CD8+ T cells (Figure 5.11) suggested that the latter may be suppressing the HIV-induced upregulation of CD38, possibly via IL-10. To determine whether IL-10 could reverse the increase in monocyte CD38 expression seen after infection with HIV-1_{BaL}, PBMC from the same 4 uninfected subjects were infected with HIV-1_{BaL} in either the presence or absence of 10 ng/ml exogenous IL-10 and then stained as before with anti-p24 and CD38 antibodies on days 3 and 5 of culture. Surprisingly, the addition of exogenous IL-10 further increased CD38 MFI on the monocytes of all 4 subjects after 3 days and on the monocytes of 3 of the 4 subjects after 5 days (Figure 5.19). This observation suggests that either the reduction of monocyte CD38 expression by Gag-specific IL-10+ CD8+ T cells was not mediated by IL-10 or that IL-10 has different effects on healthy PBMC in short term culture compared to PBMC from individuals with chronic HIV-1 infection.

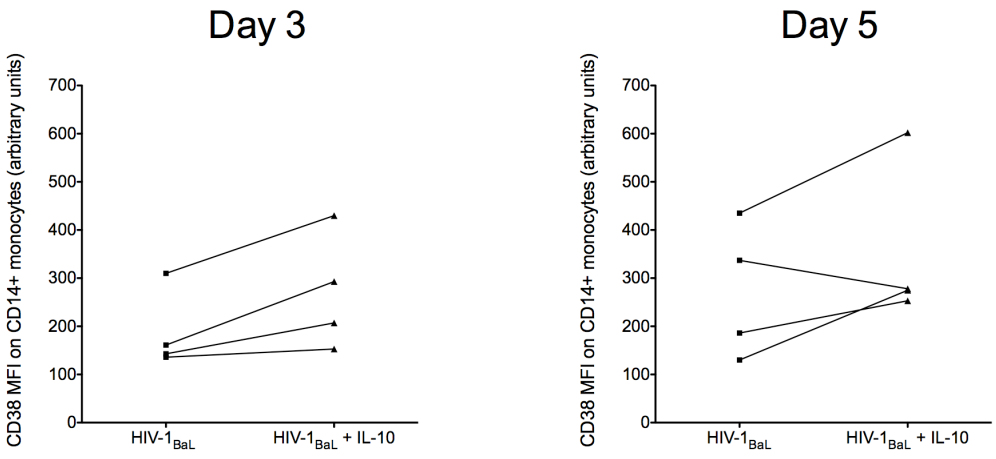


Figure 5.19 Expression of CD38 on CD14+ monocytes in PBMC cultures infected with HIV-$1_{BaL,}$ in the presence or absence of 1ng/ml exogenous IL-10, 3 and 5 days after infection.

5.11 Depletion of HIV-1 Gag-specific IL-10+ CD8+ T cells from PBMC increases IL-6 production in response to Gag peptide stimulation

The results presented in the previous sections demonstrate that Gag-specific IL-10+ CD8+ T cells appeared to have effects on monocytes rather than on other T cells during short-term culture. It might be expected, therefore, that these cells would modulate cytokine production by monocytes. In HIV-infected individuals, monocytes are the predominant source of the pro-inflammatory cytokine IL-6 (431). Additionally, ligation of CD38, a molecule that appears to be downregulated by Gag-specific IL-10+ CD8+ T cells, induces production of IL-6 in resting human monocytes (432). In order to investigate whether Gag-specific IL-10+ CD8+ T cells could influence IL-6 production, a Luminex assay was used to examine the cytokine concentration in supernatants from PBMC from 5 ART-naïve subjects that had been cultured for 16 hours in the presence of absence of these IL-10+ cells. Depletion of Gag-specific IL-10+ CD8+ T cells was accompanied by a significant increase in the concentration of IL-6 in culture supernatants after 16 hours ($p = 0.018$; paired t test; Figure 5.20).

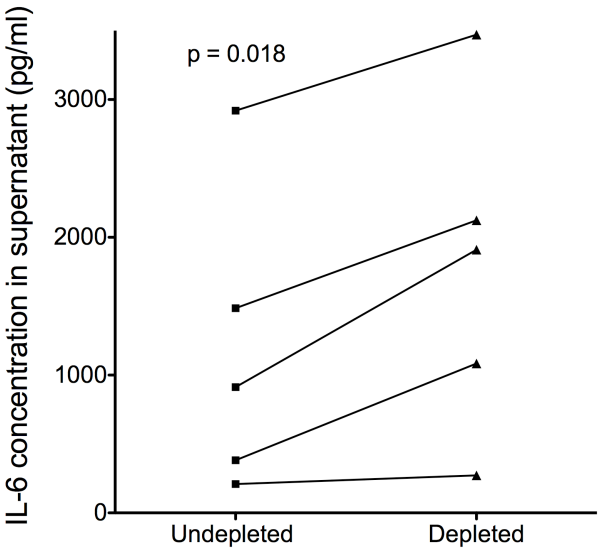


Figure 5.20 Concentration of IL-6 in supernatants from Gag-stimulated PBMC (10^6/ml) cultured for 16 hours in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. Statistical analysis was conducted using the paired t test.

5.12 Depletion of Gag-specific IL-10+ CD8+ T cells increases the number of HIV-1 Gag p24+ CD4+ T cells after 3 or 7 days culture

The effects of Gag-specific IL-10+ CD8+ T cells on the production of the inflammatory cytokine IL-6 suggested that these cells may play a protective role *in vivo*, helping to limit immune activation that might be harmful to the host. Indeed, IL-6 levels in the genital tract have been found to correlate positively with HIV-1 mRNA in the same region (433) and IL-6 has been used to reactivate HIV-1 replication in a latently infected promonocytic cell line *in vitro* (434). In order to determine the effect of Gag-specific IL-10+ CD8+ T cells on endogenous viral replication *in vitro*, these cells were depleted from the PBMC of 5 ART-naïve subjects using the same method as previously (see Figure 5.4). The CD8+ fraction, either including or depleted of IL-10+ cells, was recombined with the $CD8^-$ fraction and the cells were cultured for 3 days (see Materials

and Methods section 2.6.1 for further details). A separate aliquot of cells, consisting of unfractionated unstimulated PBMC, was included as a "null control". After 1 and 3 days, supernatants were harvested and the level of p24 antigen was quantified by ELISA. Cell recovery after 3 days was poor, probably due to the lack of cytokines such as IL-2 and IL-7 in the culture media, so supernatants were not collected after this point. There was no significant increase in the supernatant concentration of p24 antigen above the level of the null control in either the culture where Gag-specific IL-10+ CD8+ T cells had been depleted or the culture in which they had not been depleted ($p > 0.05$; Friedman test; Table 5.1), and in all samples but one (from subject IC-109 on day 3) the level of p24 antigen was below the limit of detection of the assay (which was 1 ng/ml).

Patient ID	**Gag p24 concentration in supernatant (ng/ml)**					
	Day 1			**Day 3**		
	Null	Undepleted	Depleted	Null	Undepleted	Depleted
IC-109	0.22	0.00	0.00	0.00	0.10	1.53
IC-130	ND	0.12	0.05	ND	0.06	0.00
IC-154	0.19	0.00	0.59	0.00	0.00	0.00
IC-157	0.13	0.00	0.00	0.99	0.24	0.00
IC-160	0.00	0.00	0.00	0.00	0.00	0.59

Table 5.1 The concentration of HIV-1 Gag p24 antigen in supernatants from cultures containing unfractionated unstimulated PBMC ("Null"), and Gag-stimulated PBMC cultured in the presence ("Undepleted") or absence ("Depleted") of Gag-specific IL-10+ CD8+ T cells. In the Undepleted and Depleted cultures $CD8^-$ PBMC were stimulated with Gag peptides for 6 hours before CD8+ T cells (either including or depleted of IL-10+ cells) were added back. PBMC (10^6/ml) were then cultured for 3 days. Supernatants were harvested after 1 and 3 days culture. Values represent the mean from duplicate cultures. ND, no data.

As only a small fraction of circulating CD4+ T cells harbouring HIV-1 are productively infected (28), the experiment was repeated, using a method designed to boost endogenous viral replication (see Materials and Methods section 2.6.6), with PBMC from 7 ART-naïve individuals. Gag-specific IL-10+ CD8+ T cells were isolated from CD8+ T cells as before, but this time the $CD8^-$ PBMC were cultured for 6 hours in the presence of 20 U/ml IL-2 rather than Gag peptides, in order to encourage proliferation and thus viral replication in CD4+ T cells. The CD8+ fraction, either containing or depleted of IL-10+ cells, was recombined with the $CD8^-$ fraction and the cells were cultured in the presence of IL-2 for 7 days. After 3 and 7 days CD4+ T cells were stained for intracellular p24 antigen (Table 5.2). The kinetics of viral replication varied between individuals, with a greater frequency of p24+ CD4+ T cells detected at day 3 in 5 of the patients and at day 7 in the remaining 2 patients. In order to account for this difference, the data were combined: for each individual the data from the day on which the frequency of p24+ CD4+ T cells was higher was used for subsequent analysis (Figure 5.21). In cultures of unfractionated PBMC, p24 antigen+ CD4+ T cells were detectable at very low frequencies (median 46 p24+ CD4+ T cells per 2 x 10^5 cells in culture); in comparison, in cultures from which all CD8+ T cells had been depleted there was a median 17-fold increase in p24+ CD4+ T cells (Figure 5.21). These findings suggested that the CD8+ T cells were suppressing endogenous viral replication. In cultures in which the CD8+ T cell fraction containing IL-10+ cells was added back after 6 hours, there was an increase in the number of p24+ CD4+ T cells compared with cultures of unfractionated PBMC, but this did not reach statistical significance ($p > 0.05$; Friedman test; Figure 5.21). In contrast, in cultures in which only CD8+ T cells depleted of IL-10+ cells had been added back after 6 hours there was a significant increase in the number of p24+ CD4+ T cells detected compared with

cultures of unfractionated PBMC (median 7-fold change; $p < 0.05$; Friedman test; Figure 5.21). This observation suggested that Gag-specific IL-10+ CD8+ T cells contribute to control of HIV-1 replication during short-term culture.

Patient ID	Number of Gag p24+ CD4+ T cells / 2 x 10^5 PBMC							
	Day 3				Day 7			
	PBMC	All CD8s added back	IL-10^- CD8s added back	No CD8s added back	PBMC	All CD8s added back	IL-10^- CD8s added back	No CD8s added back
HD59	27	225	305	ND	10	90	170	ND
HD65	30	59	90	118	77	600	955	70
HD100	59	50	34	ND	223	139	130	ND
HD124	46	57	96	ND	ND	ND	ND	ND
HD139	44	23	53	ND	2	2	5	ND
HD141	18	614	1489	798	8	240	230	448
IC-161	384	554	566	1322	6	490	455	825

Table 5.2 The number of p24+ CD4+ T cells in unfractionated PBMC ("PBMC"), cultures in which all the CD8+ T cells (including IL-10+ cells) were added back after 6 hours, cultures in which only IL-10-depleted CD8+ T cells were added back after 6 hours, and cultures in which all CD8+ T cells were permanently removed. $CD8^-$ PBMC were cultured with 20 U/ml IL-2 to stimulate viral replication for 6 hours before CD8+ T cells (either including or depleted of IL-10+ cells) were added back. All 4 cultures were then incubated in the presence of IL-2 for 7 days. After 3 and 7 days cells were stained with CD3-APC-Cy7, CD4-PerCP, CD8-APC, KC-57 (p24)-FITC and Aqua live/dead cell stain. p24+ cells were identified as shown in Figure 5.14. ND, no data.

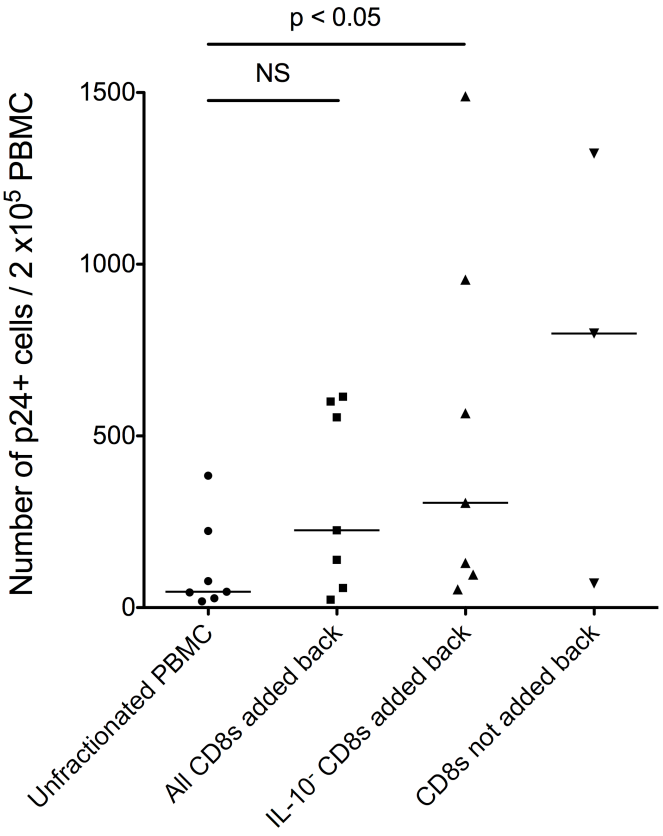


Figure 5.21 The number of p24+ CD4+ T cells in unfractionated PBMC, cultures in which all the CD8+ T cells (including IL-10+ cells) were added back after 6 hours, cultures in which only IL-10-depleted CD8+ T cells were added back after 6 hours, and cultures in which all CD8+ T cells were permanently removed. $CD8^-$ PBMC were cultured with 20 U/ml IL-2 to stimulate viral replication for 6 hours before CD8+ T cells (either including or depleted of IL-10+ cells) were added back. All 4 cultures were then incubated in the presence of IL-2 for 7 days. After 3 and 7 days cells were stained with CD3-APC-Cy7, CD4-PerCP, CD8-APC, KC-57 (p24)-FITC and Aqua live/dead cell stain. p24+ cells were identified as shown in Figure 5.14. For each individual the data from the day on which the frequency of p24+ CD4+ T cells was higher was selected for analysis

The supernatants from cells cultured for 3 and 7 days in the presence of IL-2 were also examined for p24 antigen by ELISA (Table 5.3). Unfortunately, due to assay constraints it was not possible to examine supernatants from cultures where CD8+ T cells were not added back in this experiment. At neither day 3 nor day 7 was there a significant difference in the level of p24 between the null fraction, the fraction where undepleted CD8+ T cells had been added back and the fraction where only IL-10-depleted CD8+ T cells had been added back, and once again the level of p24 antigen was below the limit of detection of the assay (1 ng/ml) in all samples but one (from subject HD100 at day 3).

Patient ID	**Gag p24 concentration in supernatant (ng/ml)**					
	Day 3			**Day 7**		
	Unfractionated PBMC	All CD8s added back	IL-10$^-$ CD8s added back	Unfractionated PBMC	All CD8s added back	IL-10$^-$ CD8s added back
HD59	0.00	0.00	0.00	0.20	0.00	0.16
HD65	0.06	0.66	0.62	0.09	0.17	0.29
HD100	1.09	0.00	0.00	0.66	0.00	0.07
HD124	0.48	0.36	0.66	ND	ND	ND
HD139	0.00	0.00	0.16	1.22	0.00	0.00
HD141	0.00	0.48	0.00	0.00	0.14	0.09
IC-161	0.72	0.00	0.00	0.58	0.15	0.00

Table 5.3 The concentration of HIV-1 Gag p24 antigen in supernatants from unfractionated PBMC, supernatants from cultures in which all the CD8+ T cells (including IL-10+ cells) were added back after 6 hours and supernatants from cultures in which only IL-10-depleted CD8+ T cells were added back after 6 hours. PBMC were cultured at a density of 10^6/ml. Supernatants were harvested after 3 and 7 days culture. Values represent the mean from duplicate cultures. ND, no data.

5.13 Discussion

In this chapter, the effects of Gag-specific IL-10+ CD8+ T cells on T cell and CD14+ monocyte function and on viral replication were investigated. In order to address the limitations of previous studies, which involved the removal of all IL-10-producing populations or total blockade of IL-10 signalling, a novel assay was developed whereby Gag-specific IL-10+ CD8+ T cells could be selectively depleted from patient PBMC. This study revealed for the first time the capacity of HIV-specific IL-10+ CD8+ T cells to influence monocyte function and viral replication.

In order to ensure that the ability to respond to IL-10 is retained in chronic HIV-1 infection, expression of the IL-10R was examined. The elevation in IL-10R expression on T cells, B cells and monocytes of ART-naïve HIV-infected subjects suggests that responses to IL-10 may be enhanced in these individuals. It has been proposed that the profound depletion of gut CD4+ T cells that occurs during acute HIV-1 infection may compromise the integrity of the mucosal barrier, leading to increased translocation of bacterial products into the peripheral blood (435, 436). Of note, one of these bacterial products, endotoxin, has been shown to increase IL-10R expression (437), and so this phenomenon could explain the increase in expression of the receptor seen in ART-naïve HIV-infected individuals. Expression of a receptor does not prove that it is functional; one way to ascertain this would have been to look for expression of a downstream signalling target of the IL-10R such as Jak1 or Tyk2 (264) in cells from these subjects, although these targets can also be phosphorylated by other receptors. However, the expression of IL-10R, plus the observation that the depletion of IL-10-producing cells increases monocyte activation and cytokine production is consistent with the maintenance of IL-10 signalling in individuals with chronic HIV-1 infection.

Gag-specific IL-10+ CD8+ T cells were depleted from patient PBMC by two rounds of magnetic separation, selecting first CD8+ T cells, and then Gag-specific IL-10+ cells from the CD8+ population. This technique allowed the selective depletion of a very rare subset of cells from PBMC. However, in practice it is not possible to obtain a 100% pure population of CD8+ T cells using anti-CD8 microbeads (see Figure 5.3) and so a small minority of the IL-10+ cells that were removed in the second round of selection may not have been CD8+ T cells. One alternative would have been to sort the cells by fluorescence-activated cell sorting (FACS), a technique that divides a population of cells into subpopulations based on their fluorescent labelling (438). However, it might be challenging to successfully sort such a rare population by FACS. Another approach would have been to expand the population *in vitro*, thus overcoming the problem of low cell numbers, and then add these cells back to PBMC. Such a method has previously been used to demonstrate the suppressive effect of human CD25+ CD4+ Tregs on T cell proliferation (439) but typically involves the use of extremely high ratios of suppressor cell to target, in some cases as high as 1:1. Since Gag-specific IL-10+ CD8+ T cells are found at such low frequencies in the peripheral blood, the physiological relevance of such an experiment would be questionable.

Depletion of Gag-specific IL-10+ CD8+ T cells did not affect the concentration of cytokines such as IL-2, TNF-α and IFN-γ in culture supernatants, suggesting that these cells did not affect T cell cytokine production. However, it was not possible to determine the cellular sources of the cytokines using this method; it is possible that changes in cytokine production by T cells were masked by production of the same cytokines by other PBMC. This issue could have been overcome using intracellular

cytokine staining (ICS), a method previously used by Elrefaei and colleagues to demonstrate that depletion of HIV-specific IL-10+ CD8+ T cells increased IL-2 production by HIV-specific effector T cells (360). However, the number of cytokines that could be examined using this technique could be limited, depending on the flow cytometer used. The Luminex assay, on the other hand, allowed the analysis of multiple cytokines simultaneously, therefore generating as much data as possible from limited samples.

In addition to having no discernible effect on T cell cytokine production, Gag-specific IL-10+ CD8+ T cells did not appear to influence T cell activation (assessed by expression of CD38 and HLA-DR) over a short-term culture period. This finding was perhaps surprising, given the ability of IL-10 to decrease the expression of the MHC Class II molecule HLA-DR and costimulatory molecules on antigen-presenting cells (275-279). Rather than acting directly on other T cells, Gag-specific IL-10+ CD8+ T cells may mediate their effects indirectly via antigen-presenting cells, in which case a longer culture period might be necessary to determine whether modulation of APC function could ultimately influence T cell responses. Furthermore, the majority of Gag-specific IL-10+ CD8+ T cells also produced IFN-γ, which can enhance MHC Class II expression (440) and so may have obscured any effect of IL-10. Alternatively, the function of Gag-specific IL-10+ CD8+ T cells may not be mediated primarily by IL-10, as the function of a similar population has previously been shown to be contact-dependent (360). It was beyond the scope of the current study to establish whether the effects of Gag-specific IL-10+ CD8+ T cells were mediated solely or partially via IL-10, or whether they were IL-10-independent. This could have been addressed using an IL-10R blocking antibody, although this would also block IL-10 from other cellular

sources. Alternatively, RNA interference (RNAi) could have been used to prevent IL-10 expression in Gag-specific IL-10+ CD8+ T cells; this method has previously been used to block IL-10 production in human dendritic cells (441, 442). However, in practice it might be difficult to target RNAi to such a rare population.

Unexpectedly, depletion of Gag-specific IL-10+ CD8+ T cells resulted in a significant increase in monocyte expression of CD38 and a trend towards increased expression of HLA-DR. Whilst CD38 has been characterised as a marker of T cell activation in HIV-infected individuals (368, 369), its effects on monocytes have been less extensively studied. However, ligation of CD38 has been shown to promote IL-6 and IL-12 production by monocytes (432, 443). Furthermore, in HIV-infected individuals monocyte CD38 expression declines after the initiation of ART (444), suggesting that elevated expression of this ectoenzyme by monocytes is related to viral replication. This notion was supported by the observation that infection of PBMC from HIV-negative donors with HIV-1_{BaL} increased the expression of CD38 on monocytes, particularly on those monocytes that themselves became infected. However, the fact that addition of exogenous IL-10 did not reverse this upregulation suggests that the effect of Gag-specific IL-10+ CD8+ T cells on monocyte CD38 expression is not primarily IL-10-mediated. On the other hand, since IL-10 has previously been shown to decrease monocyte expression of MHC Class II molecules (276), it is possible that the observed effect on HLA-DR was mediated by the cytokine itself. A decrease in the antigen-presenting capacity of monocytes, in terms of reduced MHC Class II expression, would be likely to have a downstream effect on CD4+ T cell responses to antigen, suggesting that over a longer culture period it might have been possible to see effects on T cell responses due to the removal of Gag-specific IL-10+ CD8+ T cells.

Whilst they appeared to exert effects on monocytes, depletion of Gag-specific IL-10+ CD8+ T cells did not alter the expression of CD80 or CD86 by these cells over a subsequent 16 hour culture period, despite the fact that IL-10 has previously been found to downregulate the expression of these costimulatory molecules on monocytes and macrophages (279). Since IL-10 can also inhibit the expression of CD80 and CD86 on DCs (282), and their upregulation accompanies DC maturation (445), it may have been beneficial to investigate the effect of Gag-specific IL-10+ CD8+ T cells on DC function in terms of antigen presentation and cytokine production. However, the low frequency of DCs in the peripheral blood would have made it difficult to examine this using the samples that were available. Alternatively, the effect of Gag-specific IL-10+ CD8+ T cells on the expression of monocyte activation markers such as IL-1R agonist could have been examined (446).

Since depletion of Gag-specific IL-10+ CD8+ T cells increases monocyte expression of CD38, and CD38 ligation has been reported to induce IL-6 production by monocytes (432), the effect of depleting these IL-10-producing cells on IL-6 was examined. The level of IL-6 in the supernatant of PBMC cultures was significantly increased when Gag-specific IL-10+ CD8+ T cells were depleted. Since monocytes are the predominant source of IL-6 in HIV-infected individuals (431), this finding adds further weight to the hypothesis that these IL-10-producing cells act on antigen-presenting cells such as monocytes, rather than directly on T cells. However, an effect on the production of IL-6 by other cell types, such as macrophages and Th2 cells, cannot be ruled out; ICS analysis could have been used to determine whether the influence of Gag-specific IL-10+ CD8+ T cells on IL-6 production was restricted to monocytes or

whether it also applied to other cellular sources. Since CD38 ligation can also induce IL-12 production by monocytes (443), and IL-10 can reduce TNF-α production by monocytes, it is possible that depletion of Gag-specific IL-10+ CD8+ T cells also influenced the production of these cytokines by monocytes. However, the Luminex assay was not sensitive enough to detect IL-12 in 5 out of 6 subjects, and any change in TNF-α may have been masked by cytokine production from other sources such as T cells.

In addition to T cells and monocytes, the IL-10R is expressed by most haematopoietic cells, including dendritic cells (DCs), B cells, neutrophils and natural killer (NK) cells (261, 282, 447), and so it is possible that Gag-specific IL-10+ CD8+ T cells can modulate the activities of these cells. In HIV-infected individuals, IL-10 can downregulate the expression of MHC Class I molecules by mature DCs, increasing their susceptibility to NK cell killing and resulting in the accumulation of an immature DC population (106). Furthermore, CD8+ Tregs can tolerise DCs by upregulating their expression of the inhibitory receptors ILT3 and ILT4 (448), although this effect was not shown to be dependent on IL-10. It would therefore have been interesting to investigate whether Gag-specific IL-10+ CD8+ Tregs affect the function of DCs during chronic HIV infection, but due to limited sample availability, and the low frequency of DCs in the peripheral blood, the decision was made to focus on possible effects on T cells and monocytes in this study. Taken together, the data presented here suggest that during short-term culture, Gag-specific IL-10+ CD8+ T cells act to reduce monocyte activation and pro-inflammatory cytokine production.

Depletion of Gag-specific IL-10+ CD8+ T cells led to an increase in the frequency of p24+ CD4+ T cells in PBMC cultured in the presence of IL-2 for 3 or 7 days, suggesting that these cells contribute to control of viral replication. Since the IL-10+ CD8+ T cells removed comprise only a small fraction of the total CD8+ population, it is unlikely that the effect observed was the result of removing CD8+ T cells with antiviral suppressive capacity (449, 450). However, the increase was relatively modest (median 7-fold increase above the frequency of p24+ CD4+ T cells in cultures from which CD8+ T cells had not been depleted at any stage), and no increase in p24 antigen was detected in supernatants from PBMC cultured for 1, 3 or 7 days, in either the presence or absence of IL-2. One way to enhance the level of endogenous viral replication might be to culture the cells in the presence of IL-7 as well as IL-2, as IL-7 has previously been used to induce expression of latent HIV-1 in primary human cells (451). Since IL-7 also enhances the survival of naïve and memory T cells, its use would allow PBMC to be cultured for a longer period, which might be necessary for the optimal enhancement of viral replication (452). Alternatively, the level of p24 antigen could have been increased by superinfection with exogenous virus or by activating the cells with PHA or anti-CD3/anti-CD28. However, this could have provoked a cytokine storm that would override the effects on removing the Gag-specific IL-10+ CD8+ T cell population, and in order to ensure that any differences observed were due solely to the depletion of this IL-10+ population it was important to manipulate the culture as little as possible. Despite the relatively modest increase in the number of p24+ CD4+ T cells, it was possible to detect an increase in endogenous viral antigen after removing a rare IL-10+ CD8+ T cell population.

Since IL-6 has previously been used to reactivate HIV-1 replication in a latently infected promonocytic cell line in vitro (434), and Gag-specific IL-10+ CD8+ T cells can suppress IL-6 production, it is possible that depletion of these cells might increase viral replication in monocytes. However, it was not possible to recover sufficient numbers of CD14+ monocytes after 3 or 7 days culture to address this question. If the monocytes were indeed increasingly susceptible to infection it is possible that they were rapidly dying in culture and so had been substantially depleted even after 3 days. It may therefore have been necessary to set up an assay with a shorter culture period to address this possibility.

IL-10-producing cell populations can inhibit viral clearance *in vivo* (343, 344) but may also protect the host from immune activation-induced damage (453, 454). The results presented in this chapter demonstrate the capacity of Gag-specific IL-10+ CD8+ T cells to limit viral replication *in vitro*, possibly by downregulating monocyte activation and pro-inflammatory cytokine production. Contrary to previous reports, therefore, IL-10-producing cell populations may actually play a beneficial role in chronic HIV-1 infection. However, the net positive or negative effect of IL-10 signalling may depend on the setting in which it is induced. In the next chapter, the effect of IL-10 blockade on HIV-1 vaccine efficacy in a mouse model will be investigated.

Chapter 6: IL-10R blockade as a strategy to improve the immunogenicity of HIV-1 vaccination in a murine model

6.1 Introduction

Almost thirty years after HIV-1 was isolated, the search for a universally protective vaccine continues. The most successful human vaccine tested to date, in the Rv144 trial in Thailand, yielded a modest degree of protection, with 26.2% fewer infections (according to the per-protocol analysis) in the vaccinated compared to the control arm of the trial (347), but uncertainty remains regarding immune responses associated with protection in vaccinated individuals in this trial. In the earlier STEP trial, vaccination induced a limited breadth of T cell responses and failed to provide protection from acquisition of HIV-1 (346). Whether a vaccine capable of inducing a greater breadth of T cell responses might confer improved protection is a crucial area for investigation.

One of the major hurdles to successful HIV-1 vaccine development is the extensive degree of variation between viral isolates (455). An ideal vaccine candidate would elicit humoral and cellular immune responses towards highly conserved epitopes in order to provide protection against a broad range of viral isolates (456). Current approaches designed to overcome the variability between isolates include vaccination with a cocktail of immunogens derived from different viral clades, the construction of multi-clade immunogens incorporating the most conserved regions of the proteome, and the use of computational methods to assemble immunogens that provide optimal coverage of T cell epitopes (457-461). In spite of this, studies in humans and macaques have demonstrated that, in naïve individuals, T cell responses to HIV-1 vaccine

candidates are often dominated by responses to the highly variable Env protein, rather than more highly conserved proteins such as Gag and Nef (462).

Mouse models of HIV-1 vaccination, whilst not predictive of the level of protection that can be achieved in humans, can be used to determine the immunodominance hierarchy of the epitopes present in candidate vaccines. Im and colleagues observed that the murine response to the first generation HIV-1 immunogen HIVA was dominated by the response to a highly variable Env epitope, designated epitope H, and an epitope from Pol that is not processed from its natural context, designated epitope P (463). Sequential deletion of these epitopes from HIVA, creating an immunogen designated HIVA dHmP, resulted in an enhanced response to a conserved, subdominant Gag epitope, G1, and a comparable degree of protection from a model viral challenge to the wild type immunogen. These results suggested that, when not dominated by responses to variable epitopes, conserved epitopes can be highly immunogenic *in vivo*. A key unanswered question is whether responses to subdominant epitopes can be enhanced without the deletion of dominant epitopes, thereby increasing the breadth of response to vaccination.

Separate lines of enquiry in mouse models have suggested that modulation of IL-10 signalling during vaccination could influence the effectiveness of vaccine-induced cellular responses to chronic viral infections. IL-10R blockade has been used as a strategy to enhance the efficacy of a therapeutic vaccine for lymphocytic choriomeningitis virus (LCMV) in mice (345). When administered to mice already infected with a persistent strain of LCMV, IL-10R blockade augmented the activity of a previous ineffective DNA vaccine, leading to the induction of robust effector T cell

responses, rapid clearance of virus and the development of anti-viral memory T cell responses. Since IL-10 can inhibit antigen presentation to T cells by downregulating MHC Class II and costimulatory molecules on APCs, blockade of IL-10 signalling may allow a broader range of vaccine epitopes to be presented. In this way it might be possible to increase the breadth of responses to vaccination and achieve enhanced responses to subdominant epitopes without removal of the dominant epitopes. HIVA is an attractive immunogen for such a study, as it has a clearly defined immunodominance hierarchy (463). The use of IL-10R blockade as an adjunct to HIV-1 vaccination, however, has not previously been explored.

In this chapter, the effect of IL-10R blockade on responses to the dominant and subdominant epitopes of HIVA, as well as to a second-generation immunogen, HIVconsv, was investigated in a murine model.

6.2 Methods and ethical approval

Female BALB/c mice aged between five and eight weeks were used in all experiments. Animals were housed in the Biomedical Services unit at the John Radcliffe Hospital, Oxford. Experiments were carried out under Personal License number 40/1027 held by Genevieve Clutton and Project License number 30/2833 held by Prof. Tomáš Hanke. All animal procedures and care conformed strictly to the United Kingdom Home Office Guidelines under the Animals (Scientific Procedures) Act, 1986.

Two vectors were used in the experiments: a recombinant DNA plasmid known as pTHr and the attenuated poxvirus Modified vaccinia virus Ankara (MVA), both of

which were prepared by Dr Anne Bridgeman (Jenner Institute, Oxford, UK). The pTHr vector contains an expression efficient enhancer/promoter/intron A cassette of the human cytomegalovirus strain AD169 genome, followed by the pRc/CMV-derived poly-linker and polyadenylation signal of the bovine growth hormone gene (464). This construct does not contain an origin for replication in mammalian cells. HIVA DNA was inserted into the pTH poly-linker and the resulting pTHr.HIVA was propagated using the repressor-titration system developed by Cobra Pharmaceuticals (Keele, UK). Mice were immunised intramuscularly, with 50 μg pTHr.HIVA per hindleg (see Materials and Methods section 2.7.1).

MVA was originally created by more than 570 passages of chorioallantoid vaccinia virus Ankara, a Turkish smallpox vaccine, in primary chicken embryo fibroblasts (465). For these experiments, chicken embryo fibroblast (CEF) cells were infected with parental MVA at a multiplicity of infection (MOI) of 1 and transfected using Superfectin (Qiagen) with 3 μg of the transfer plasmid pSC11.HIVconsv (457). Mice were immunised intramuscularly with 10^7 plaque-forming units (PFU) MVA.HIVconsv per hindleg.

6.3 IL-10R blockade prevents binding of a secondary αIL-10R antibody *in vitro*

Blockade of IL-10R was performed using a commercially available azide-free antibody (Biolegend). In order to test the ability of the antibody to bind to the IL-10R, a competitive inhibition assay was performed *in vitro* on freshly isolated mouse splenocytes. Splenocytes were incubated first with either the IL-10R blocking antibody or an irrelevant isotype control and then with a second, PE-conjugated, anti-IL-10R antibody. Pre-treatment with the isotype control did not affect the ability of IL-10R-PE to bind to the receptor, as indicated by the level of PE fluorescence. However, pre-treatment with the IL-10R blocking antibody reduced PE fluorescence to background levels, indicating that the blocking antibody was binding to IL-10R and preventing subsequent binding of the PE-conjugated antibody (Figure 6.1).

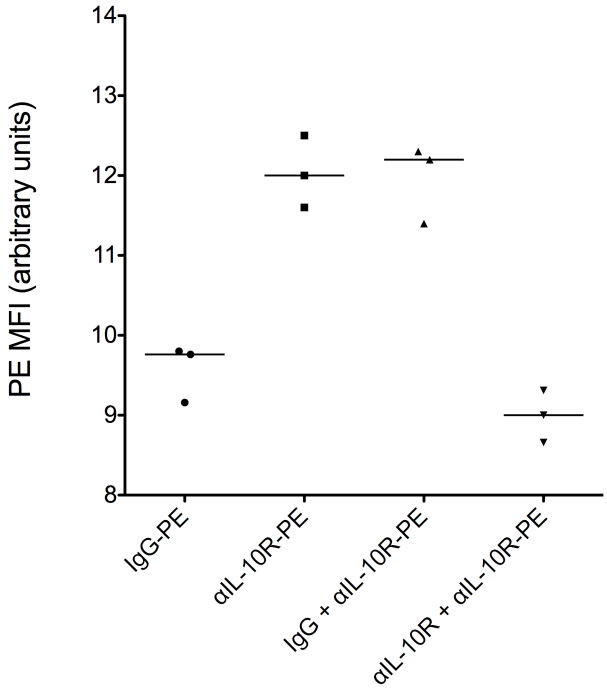


Figure 6.1 IL-10R blocking antibody prevents subsequent binding of a PE-conjugated anti-IL-10R antibody. Splenocytes were incubated with either an irrelevant isotype control or IL-10R blocking antibody prior to the addition of IL-10R-PE.

6.4 Effect of IL-10R blockade on the immunogenicity of a DNA.HIVA prime, DNA.HIVA boost regimen

6.4.1 Experimental design and the HIVA immunogen

Female BALB/c mice were immunised twice with pTHr.HIVA. It has previously been demonstrated that this regimen elicits cytolytic activity by antigen-specific CD8+ T cells (466), but these responses are weak, transient and dominated by responses to the variable H epitope. In order to determine whether IL-10R blockade could increase the immunogenicity of pTHr.HIVA, mice were injected intraperitoneally with either an irrelevant isotype control antibody or IL-10R blocking antibody the day before the first vaccination. In order to control for any non-specific effect of the antibody, separate mice were injected intraperitoneally with either the isotype control or the IL-10R blocking antibody but not subsequently vaccinated. The vaccination schedule is illustrated in Figure 6.2, with the regimen received by each group shown in Table 6.1.

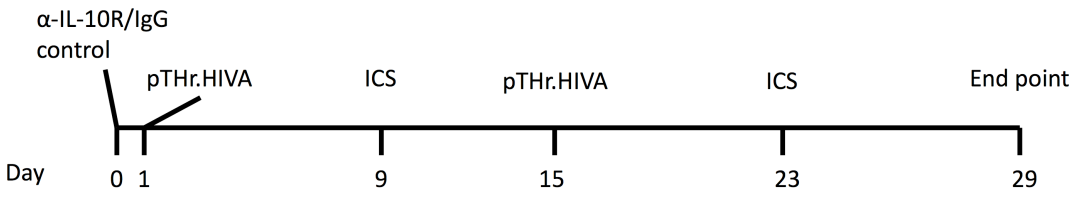


Figure 6.2 Schedule for the vaccination of mice with pTHr.HIVA. Mice were injected intraperitoneally with either an irrelevant isotype control antibody or IL-10R blocking antibody 1 day prior to the first vaccination. At days 9 and 23 blood was collected from the lateral tail vein for analysis of CD8+ T cell responses by intracellular cytokine staining (ICS). At the end point mice were sacrificed and splenocytes harvested for further analysis.

Group	Number of mice	Antibody	Vaccine
1	5	Isotype control	None
2	5	αIL-10R	None
3	5	Isotype control	2 x pTHr.HIVA
4	5	αIL-10R	2 x pTHr.HIVA

Table 6.1 Regimen received by each experimental group.

The HIVA immunogen consists of HIV-1 Gag p24 and HIV-1 Gag p17 fused to a series of 25 partially overlapping CTL epitopes (Figure 6.3) (464). The construct is expressed under the control of the Rous Sarcoma Virus (RSV) promoter, which has been shown to provide high levels of expression of foreign genes in a variety of cell types (467-469). The positions of the H, P and G1 epitopes are indicated in Figure 6.3.



Figure 6.3 Structure of the HIVA immunogen. Gag p24 and p17 are fused to a series of 25 overlapping CTL epitopes and the entire construct is under the control of the Rous Sarcoma Virus (RSV) promoter.

6.4.2 Peripheral blood CD8+ T cell responses to a single vaccination with pTHr.HIVA, with and without IL-10R blockade

As a preliminary, qualitative test of vaccine immunogenicity, peripheral blood was collected from the lateral tail vein of all mice nine days after the injection with IL-10R blocking or isotype control antibody (and eight days after the first immunisation with pTHr.HIVA in the case of mice from groups 3 and 4) and CD8+ T cells were examined by ICS for the expression of IFN-γ, TNF-α and the degranulation marker CD107a in response to H, P and G1 peptide stimulation. Due to the small volume of blood that could be collected, peripheral blood cells from mice from the same group (n = 5) were pooled. The gating strategy for the identification of IFN-γ+, TNF-α+ and CD107a+ CD8+ T cells is shown in Figure 6.4.

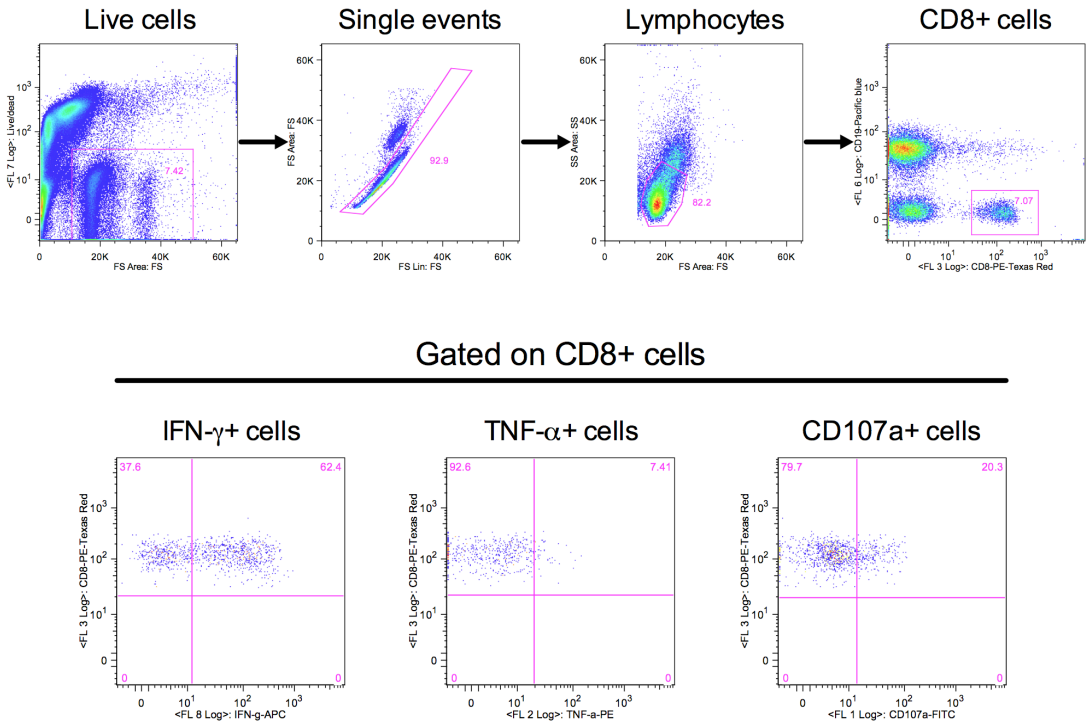


Figure 6.4 Gating strategy for the identification of IFN-γ+, TNF-α+ and CD107a+ CD8+ T cells. Peripheral blood cells were isolated, stimulated with PMA/ionomycin for 6.5 hours (to stimulate cytokine production and degranulation) and then stained with Aqua live/dead cell stain, CD8-PE-Texas Red, CD19-Pacific blue, IFN-γ-APC, TNF-α-PE and CD107a-FITC.

Figures 6.5, 6.6 and 6.7 show the expression of IFN-γ, TNF-α and CD107a by CD8+ T cells in response to H, P and G1 peptide stimulation in pooled blood from the four groups. Responses to the positive control, PMA ionomycin, were detected in all four groups, but responses to the three vaccine epitopes were low in both the unvaccinated and vaccinated mice. For example, the frequency of H-specific IFN-γ+ CD8+ T cells in pooled blood was 0.2% for group 1, 0% for group 2, 0% for group 3 and 0.2% for group 4 (after the subtraction of background IFN-γ production). Constitutive surface expression of CD107a was detected on a proportion of CD8+ T cells in all four groups, confirming observations from a previous study (470), but the level of expression was not boosted by vaccination in any of the groups (Figure 6.7).

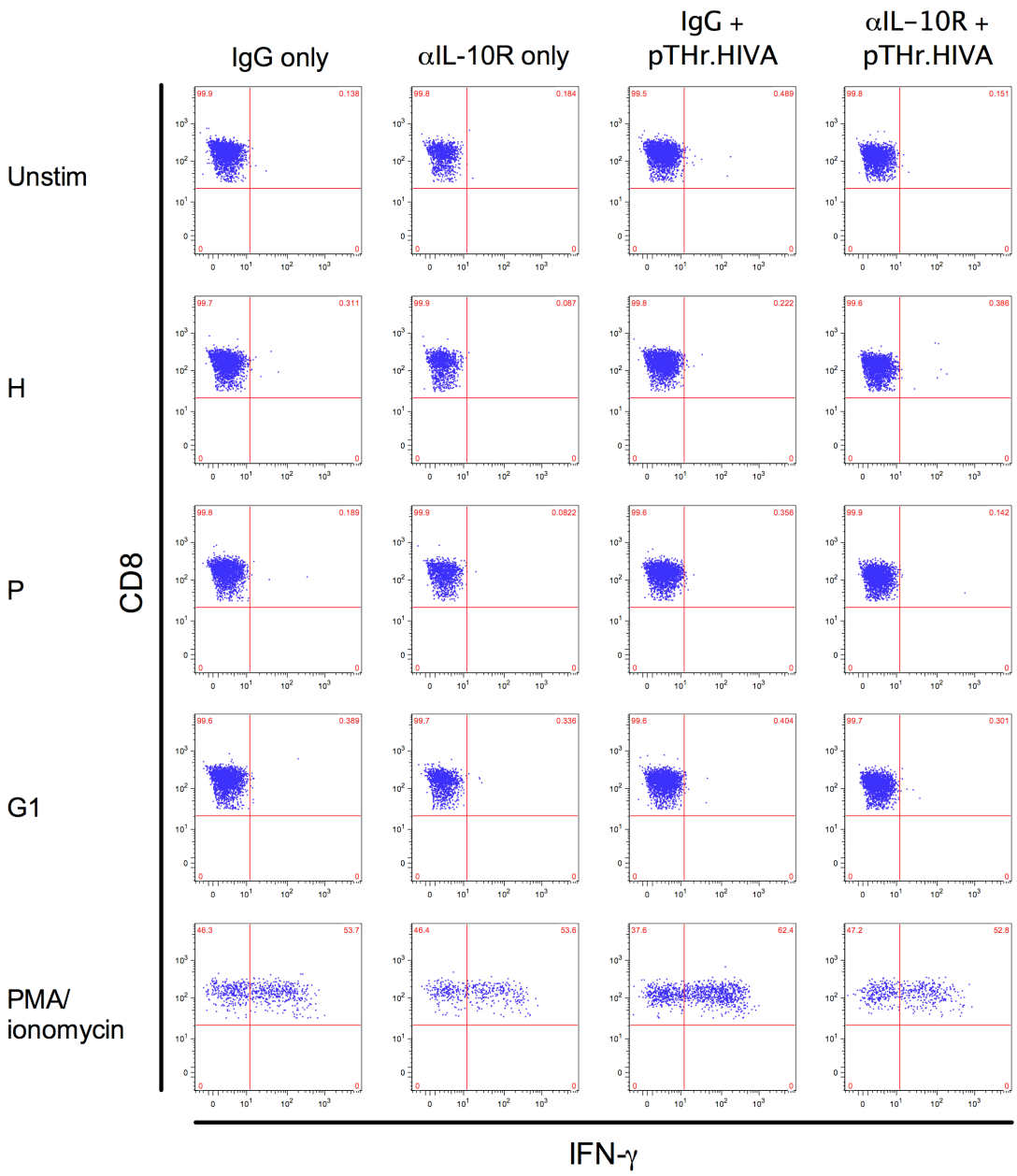


Figure 6.5 Flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

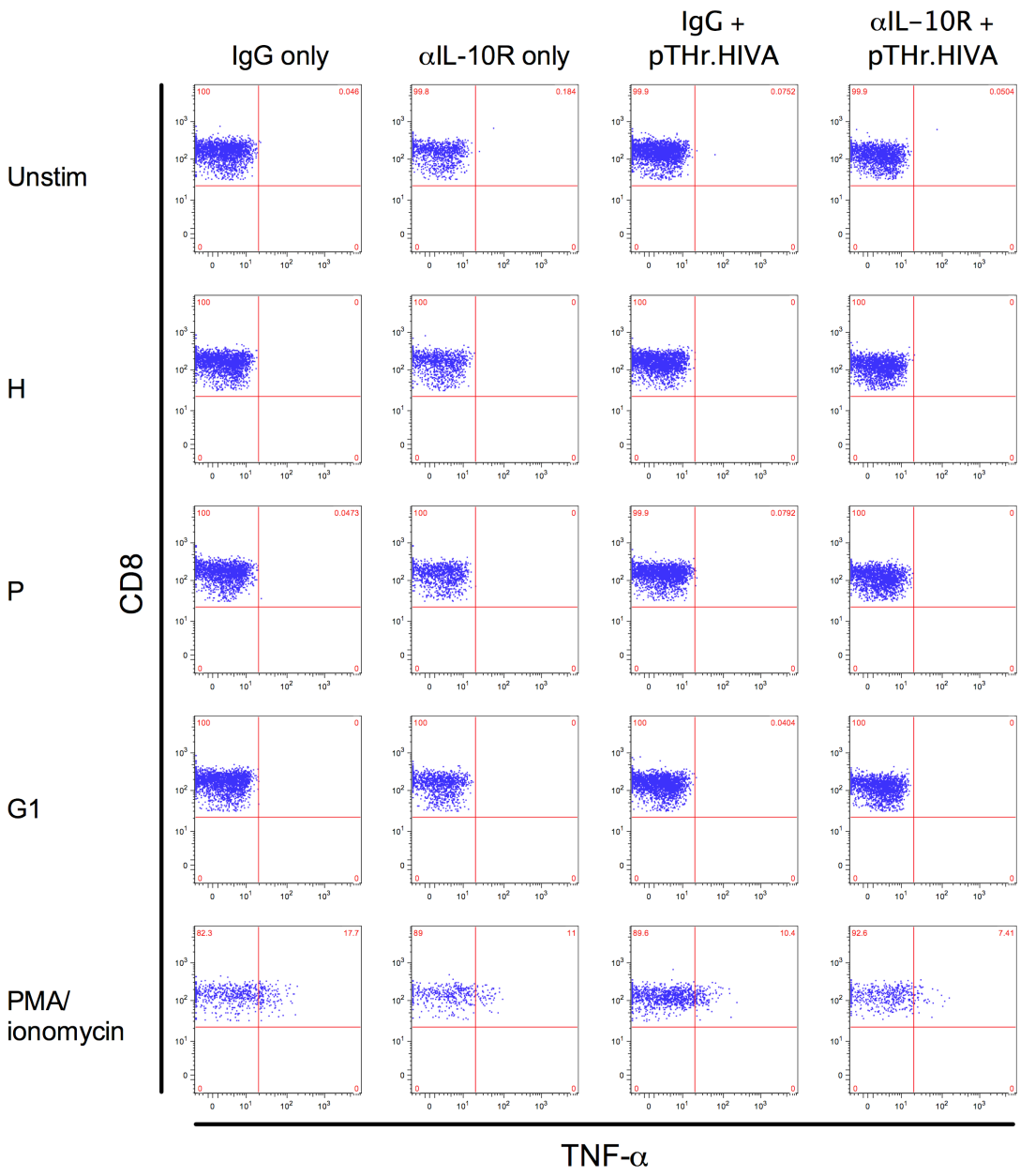


Figure 6.6 Flow cytometric plots of TNF-α production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

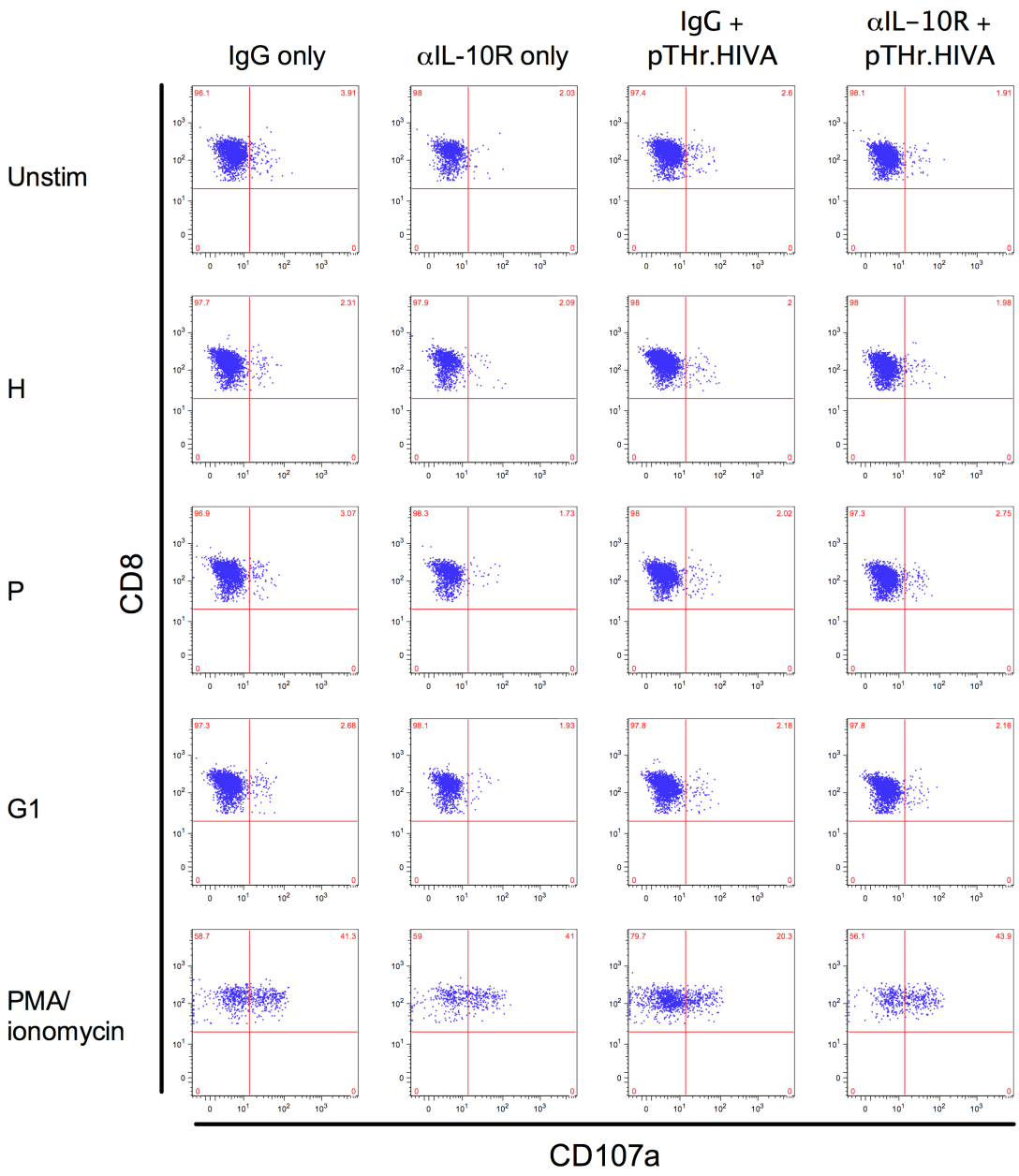


Figure 6.7 Flow cytometric plots of CD107a surface expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

6.4.3 Peripheral blood CD8+ T cell responses after a second vaccination with pTHr.HIVA, with and without IL-10R blockade

Following the lack of responses to H, P and G1 after a single vaccination, peripheral blood was collected from the lateral tail vein of all mice eight days after the mice in groups 3 and 4 had received a second immunisation with pTHr.HIVA. Once again, blood from mice of the same group was pooled together so that there were sufficient cells for this preliminary analysis. Figures 6.8, 6.9 and 6.10 show the production of IFN-γ, TNF-α and CD107a by CD8+ T cells in response to H, P and G1 peptide stimulation in pooled blood from the four groups. The frequency of H, P and G1-specific CD8+ T cells expressing IFN-γ, TNF-α or CD107a did not exceed background levels in the unvaccinated mice (groups 1 and 2). In contrast, mice that were vaccinated after receiving isotype control antibody (group 3) displayed IFN-γ production in response to H (0.5% of CD8+ T cells after the subtraction of background) and P (1% of CD8+ T cells). These responses were elevated further in the mice that were vaccinated after receiving αIL-10R, with 4.8% of CD8+ T cells producing IFN-γ in response to H and 1.8% in response to P (Figure 6.8). The frequency of antigen-specific TNF-α CD8+ T cells did not exceed background in group 3, but in group 4, 0.7% of CD8+ T cells expressed TNF-α in response to stimulation with peptide H (Figure 6.9). Antigen-specific surface expression of CD107a was evident in groups 3 and 4. In group 3, 1.4% of CD8+ T cells expressed CD107a in response to peptide H (after subtraction of background), 1.8% in response to P and 1.8% in response to G1. In group 4, 4.0% of CD8+ T cells expressed CD107a in response to peptide H, 1.4% in response to P and 0.3% in response to G1 (Figure 6.10).

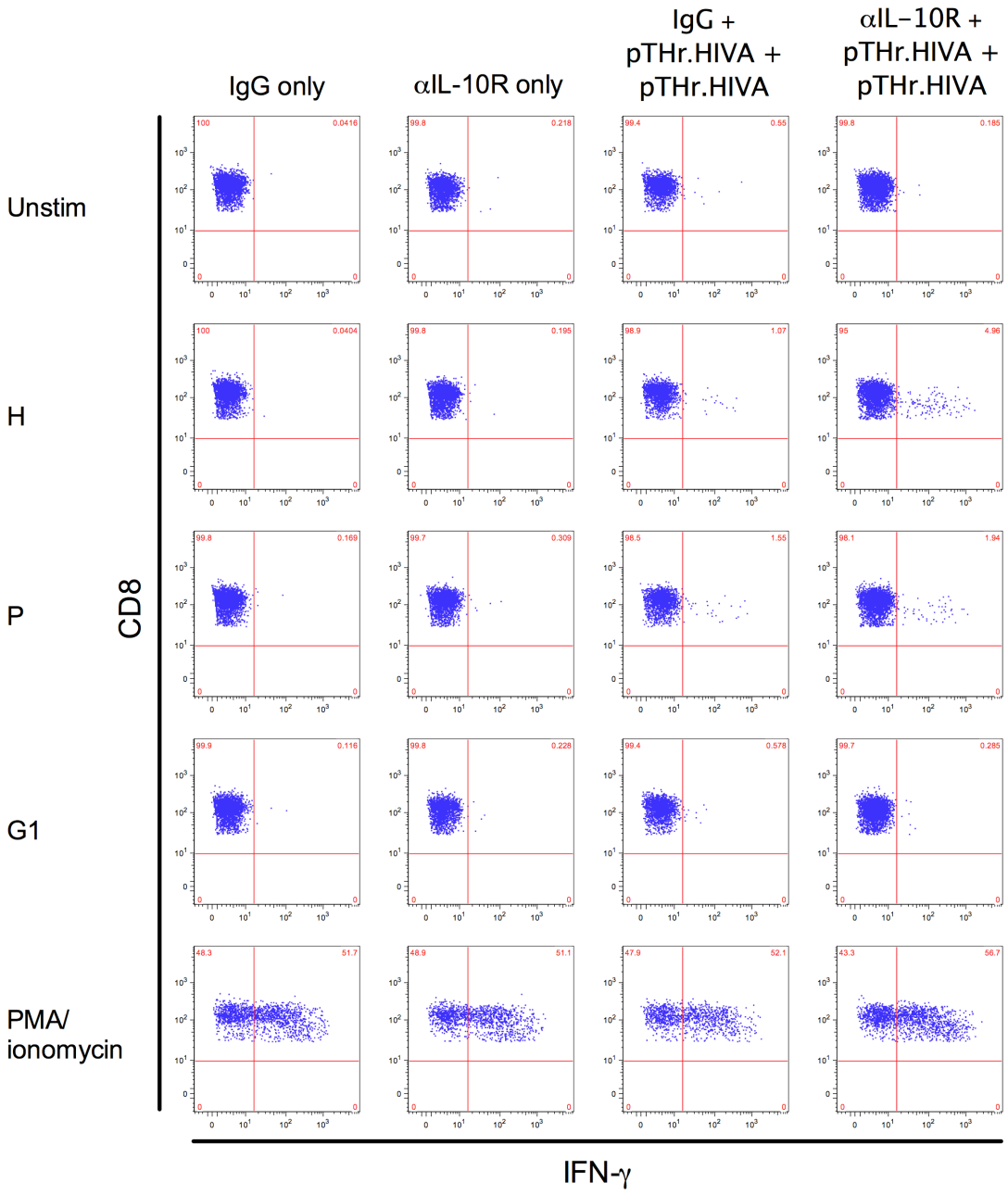


Figure 6.8 Flow cytometric plots of IFN-γ expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

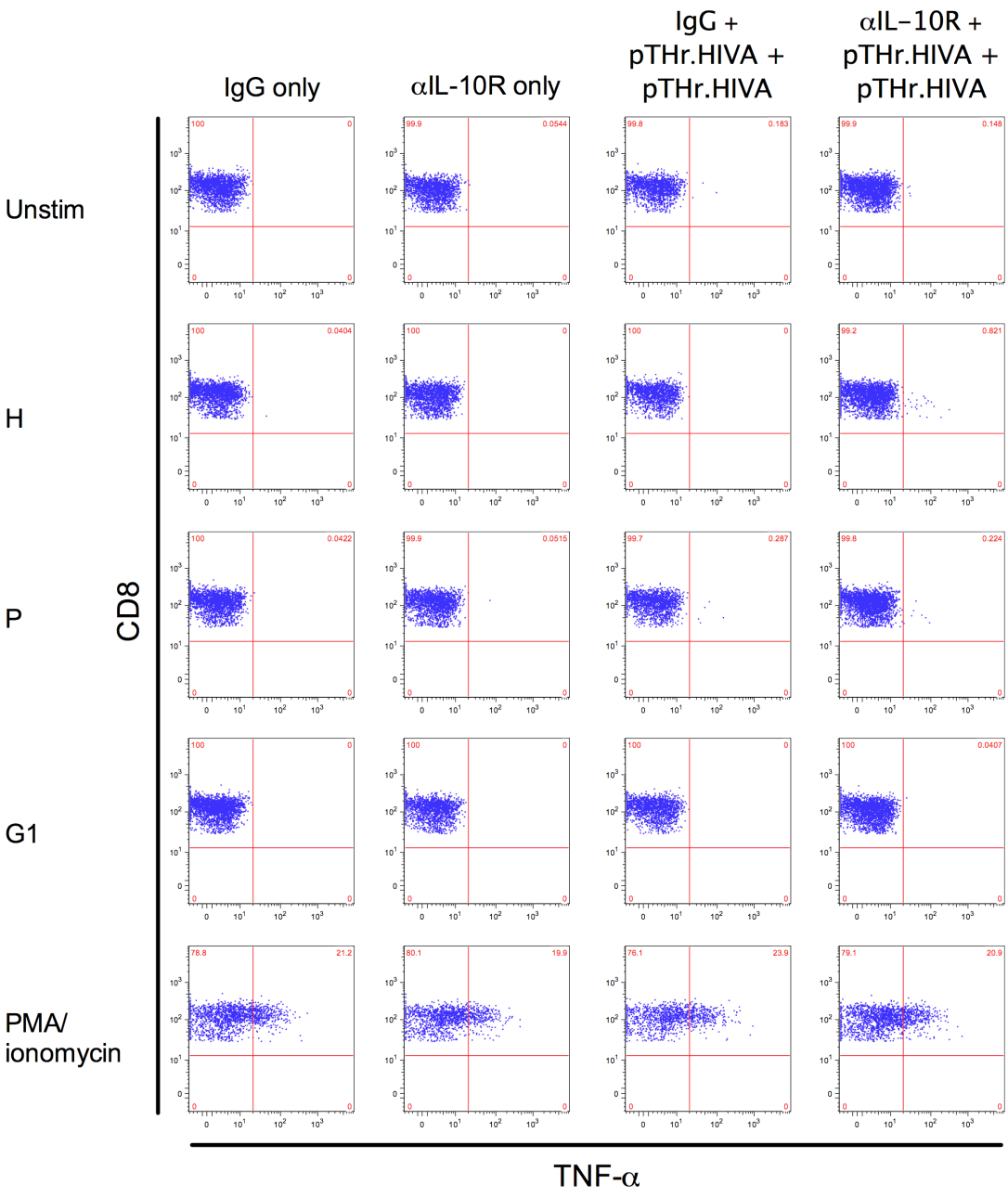


Figure 6.9 Flow cytometric plots of TNF-α expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

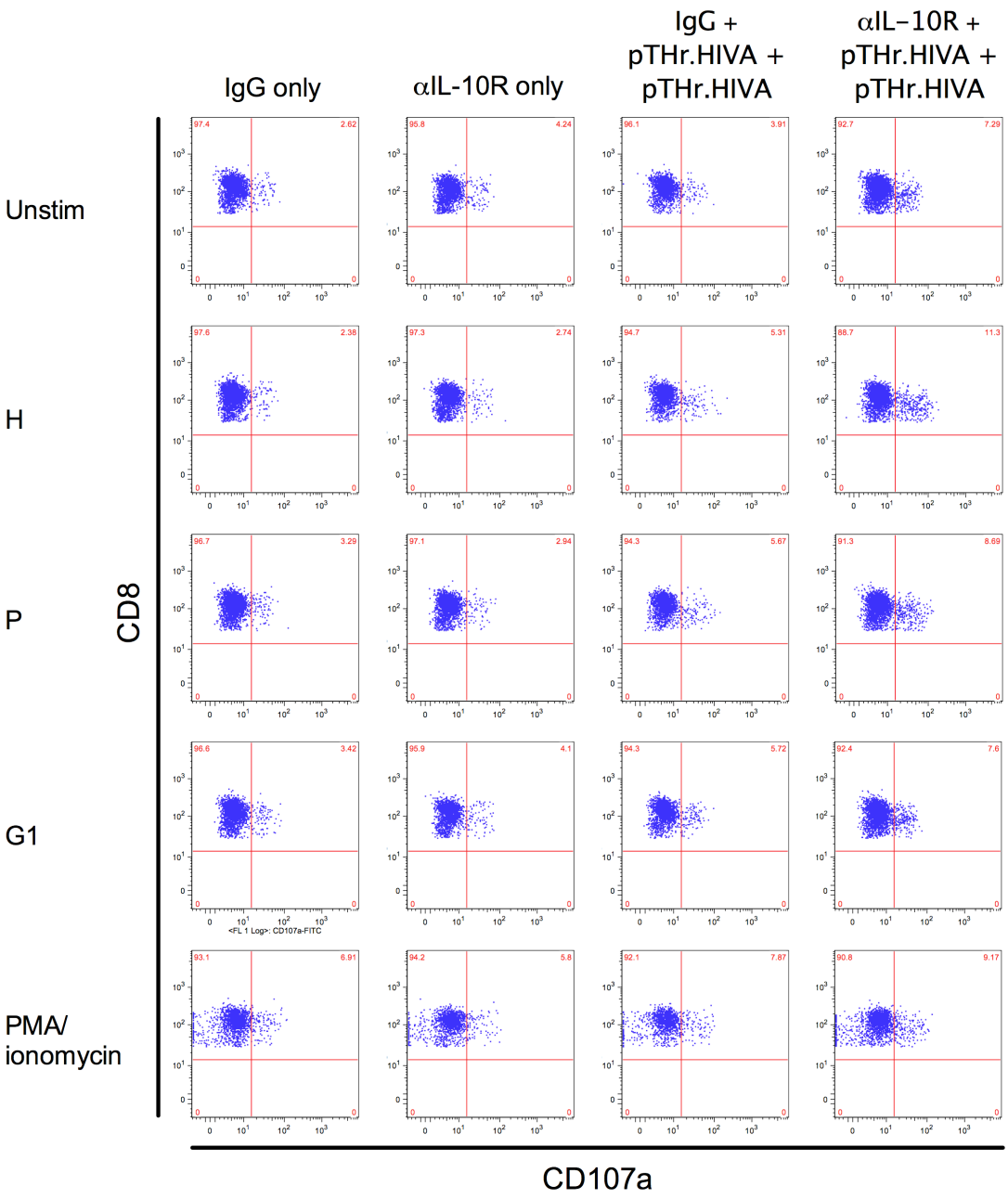


Figure 6.10 Flow cytometric plots of CD107a surface expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

6.4.4 End point responses to pTHr.HIVA prime, pTHr.HIVA boost, with and without IL-10R blockade

The analysis of CD8+ T cell responses in pooled peripheral blood is a useful early indicator of the immunogenicity of a vaccination regimen, but does not provide information about responses by individual mice. In order to obtain this information, all mice were sacrificed 14 days after the mice in groups 3 and 4 had received their second immunisation with pTHr.HIVA. Splenocytes from individual mice were isolated and examined by ICS for the expression of IFN-γ, TNF-α and CD107a in response to H, P and G1 by CD8+ T cells. Representative flow cytometric plots of IFN-γ, TNF-α and CD107a expression are shown in Figures 6.11, 6.12 and 6.13. Since CD8+ T cells constitutively expressed CD107a, the mean background frequency of CD107a+ CD8+ T cells was used as a cut-off; only responses to H, P or G1 that still exceeded this cut-off after the subtraction of background (i.e. were more than double the mean background value) were considered positive.

Figure 6.14 shows IFN-γ, TNF-α and CD107a production by CD8+ T cells in response to stimulation with the H peptide. The frequency of H-specific IFN-γ+ CD8+ T cells was significantly higher in mice vaccinated after receiving αIL-10R (group 4) compared to the unvaccinated groups 1 and 2 ($p < 0.05$; Kruskal-Wallis test). There was also a trend towards a higher frequency of H-specific IFN-γ+ CD8+ T cells in mice from group 4 compared to mice who were vaccinated after receiving isotype control antibody (group 3), but this did not reach statistical significance ($p > 0.05$; Kruskal-Wallis test). TNF-α production by CD8+ T cells in response to H was not elevated in the vaccinated mice compared to the unvaccinated mice. H-specific CD107a expression was increased in the vaccinated (groups 3 and 4) compared to the

unvaccinated mice (groups 1 and 2), but this difference did not reach statistical significance, and the level of expression was below the cut-off in the majority of mice.

CD8+ T cell responses to the P epitope are shown in Figure 6.15. There was a significant increase in the frequency of P-specific IFN-γ+ CD8+ T cells in mice from group 4 compared to those in group 1 ($p < 0.05$; Kruskal-Wallis test), and a trend towards an increase in the frequency in group 4 compared to groups 2 and 3 that did not reach statistical significance ($p > 0.05$; Kruskal-Wallis test). Once again, there was no elevation in TNF-α production in vaccinated groups 3 and 4 compared to unvaccinated groups 1 and 2, and whilst CD107a expression was elevated in groups 3 and 4, this increase did not reach statistical significance, and the level of expression was below the cut-off in the majority of mice.

When responses to the subdominant G1 epitope were examined, IFN-γ, TNF-α and CD107a expression were not found to elevated in either of the vaccinated groups compared to the unvaccinated control groups ($p > 0.05$, Kruskal-Wallis test) (Figure 6.16).

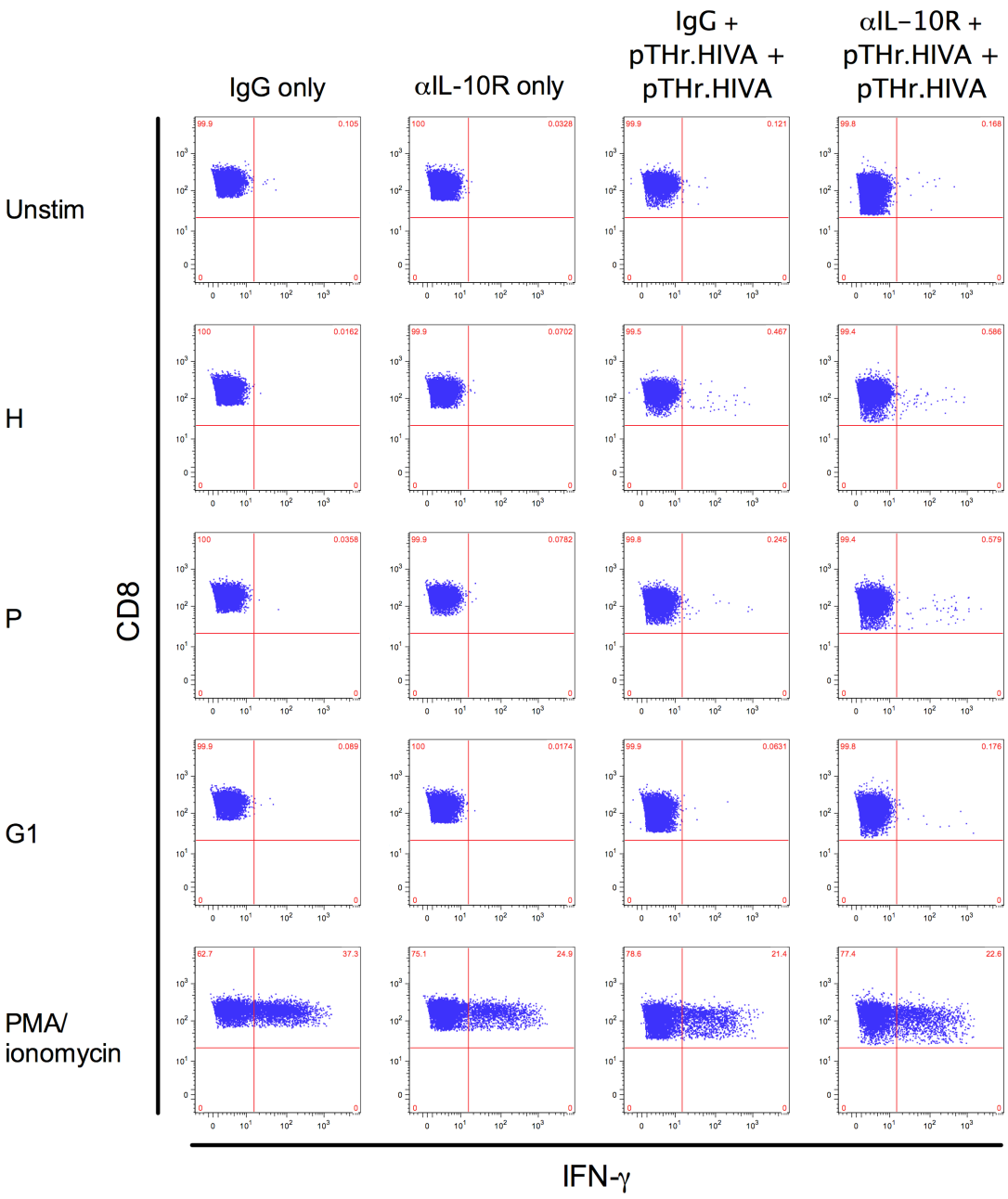


Figure 6.11 Representative flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

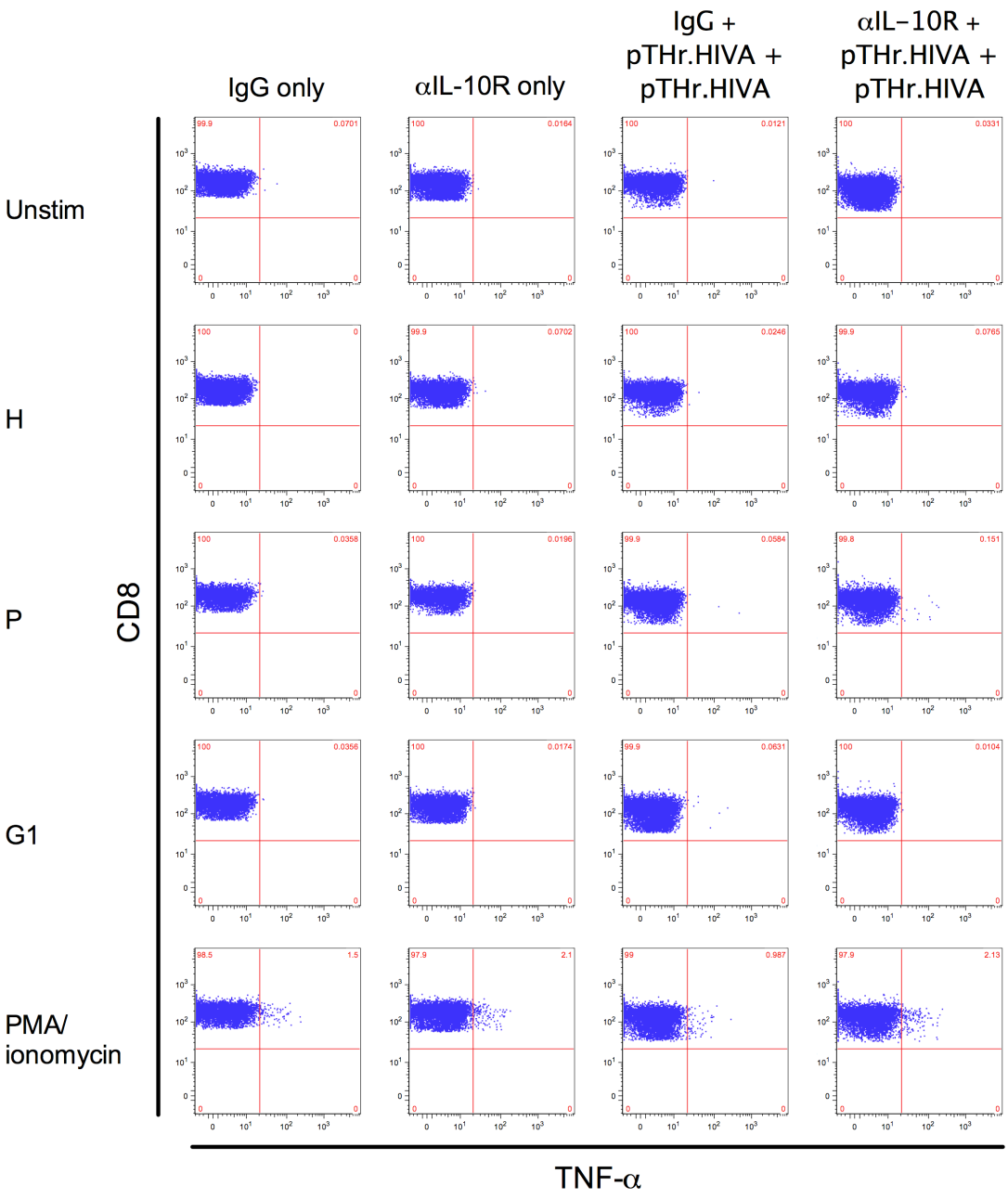


Figure 6.12 Representative flow cytometric plots of TNF-α production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

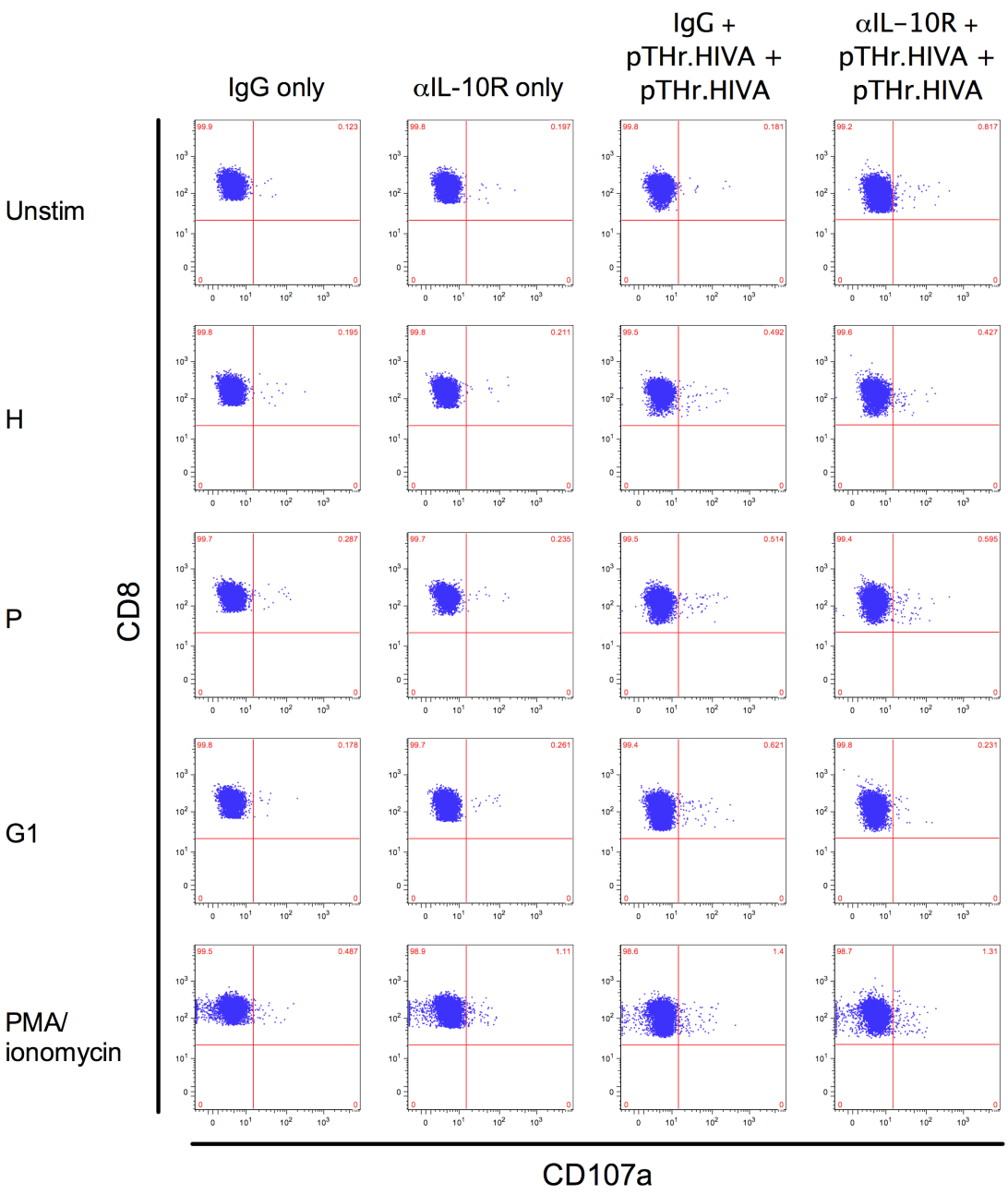


Figure 6.13 Representative flow cytometric plots of CD107a expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and treated as described in Figure 6.4.

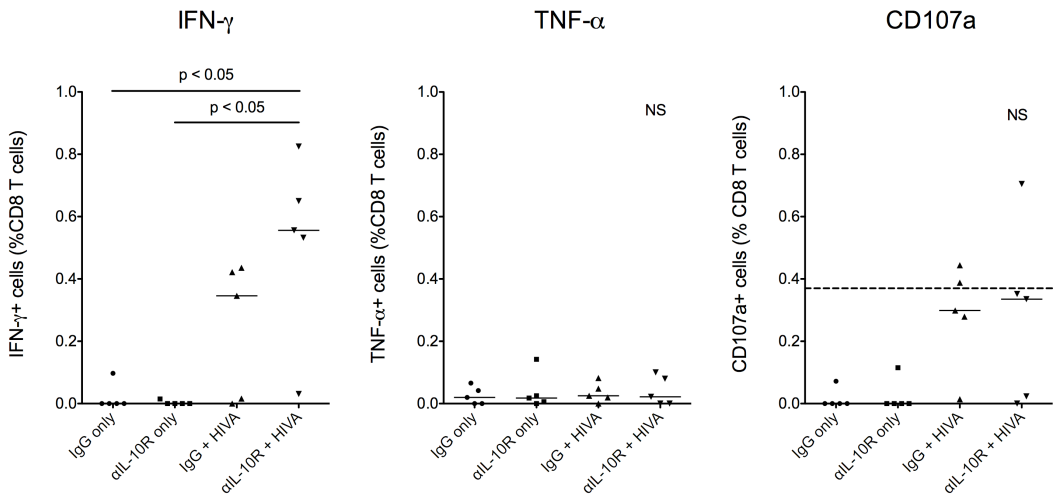


Figure 6.14 The frequencies of IFN-γ+, TNF-α+ and CD107a+ CD8+ T cells after stimulation with the H peptide for 6.5 hours. The dashed line shows the cut-off point below which CD107a responses were not considered positive. Statistical analyses were conducted using Kruskal-Wallis test.

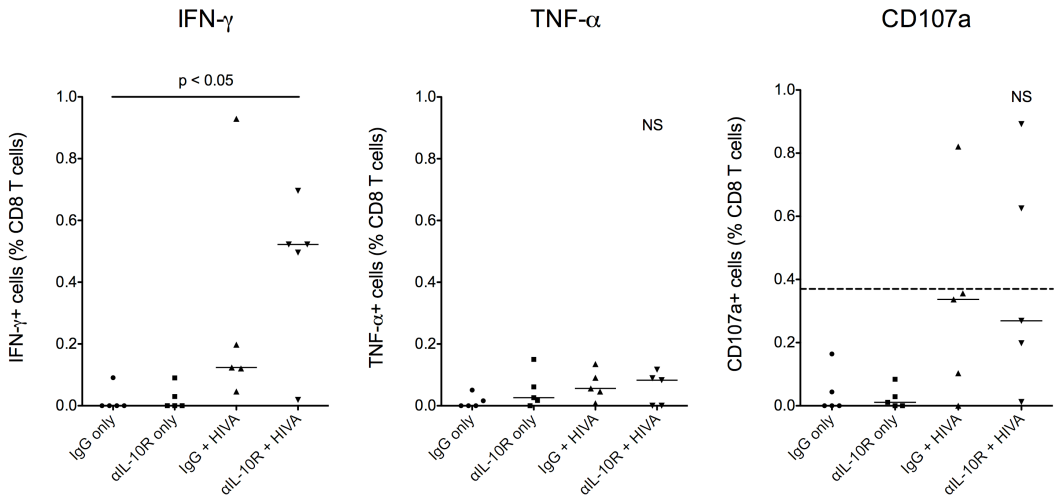


Figure 6.15 The frequencies of IFN-γ+, TNF-α+ and CD107a+ CD8+ T cells after stimulation with the P peptide for 6.5 hours. The dashed line shows the cut-off point below which CD107a responses were not considered positive. Statistical analyses were conducted using Kruskal-Wallis test.

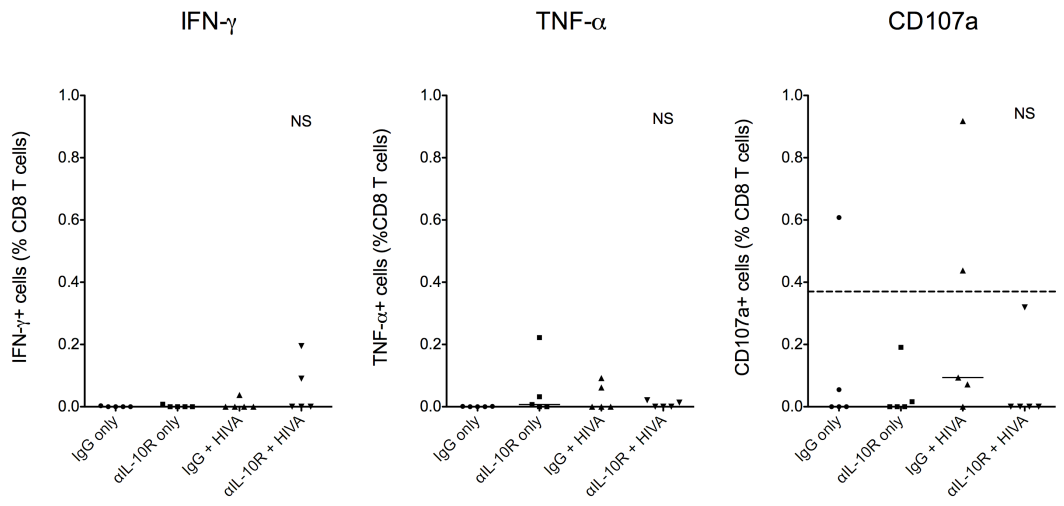


Figure 6.16 The frequencies of IFN-γ+, TNF-α+ and CD107a+ CD8+ T cells after stimulation with the G1 peptide for 6.5 hours. The dashed line shows the cut-off point below which CD107a responses were not considered positive. Statistical analyses were conducted using Kruskal-Wallis test.

In order to confirm the observations from the ICS with respect to IFN-γ responses to vaccination, an IFN-γ ELISPOT was performed on splenocytes from the same mice, using the H, P and G1 peptides for stimulation. IFN-γ production in response to the H and P epitopes was significantly elevated in mice that were vaccinated after receiving IL-10R-blocking antibody (group 4) compared to the unvaccinated groups 1 and 2 ($p < 0.05$, Kruskal-Wallis test) (Figure 6.17). There was also a trend towards an increase in IFN-γ production in response to these epitopes in mice from group 4 compared to mice who were vaccinated after receiving isotype control antibody (group 3), but this difference was not statistically significant ($p > 0.05$). There was no elevation in IFN-γ production in response to G1 stimulation in the vaccinated mice compared to the unvaccinated mice.

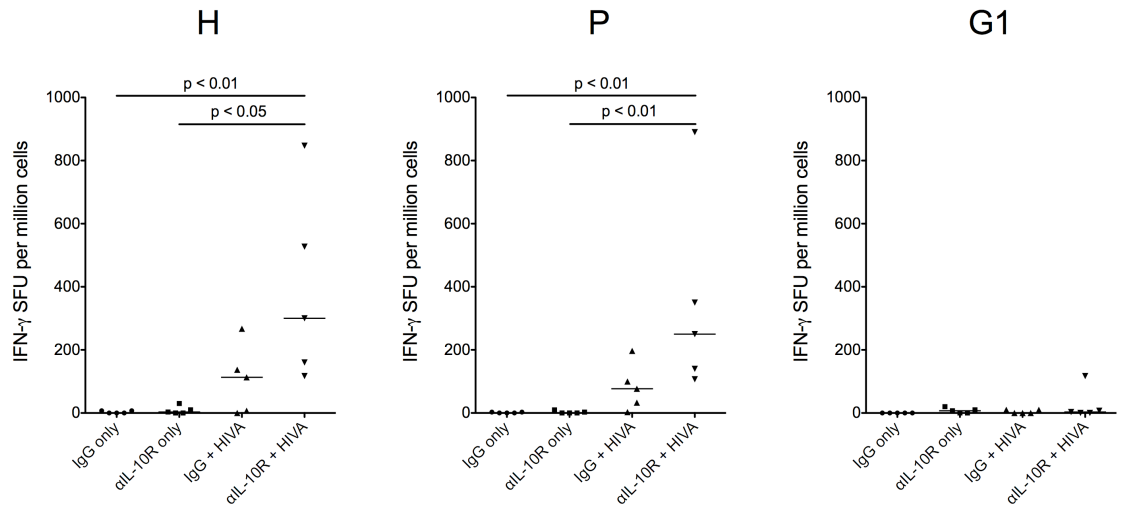


Figure 6.17 IFN-γ ELISPOT responses to H, P and G1. Splenocytes were isolated and stimulated for 16 hours with the relevant peptide. Statistical analyses were conducted using Kruskal-Wallis test.

Collectively, these data indicated that blockade of IL-10 signalling increased the frequency of H- and P-specific IFN-γ+ CD8+ T cells to some degree after vaccination, but did not enhance responses (in terms of IFN-γ, TNF-α or CD107a expression) to the subdominant G1 epitope. The logical next step, therefore, was to determine whether IL-10R blockade could enhance responses to an immunogen designed to target conserved regions of the HIV-1 proteome.

6.5 Effect of IL-10R blockade on the immunogenicity of a pTH.HIVconsv prime, MVA.HIVconsv boost regimen

6.5.1 Immunogen and experimental design

The HIVconsv immunogen incorporates the 14 most invariable segments of the HIV-1 proteome, including Gag, Pol, Env and Vif epitopes, with alternating individual regions among the 4 major clades (A, B, C and D). Its purpose is to refocus T cells to subdominant, invariant epitopes that the virus cannot mutate without a fitness cost (457). The structure of the immunogen is depicted in Figure 6.18.

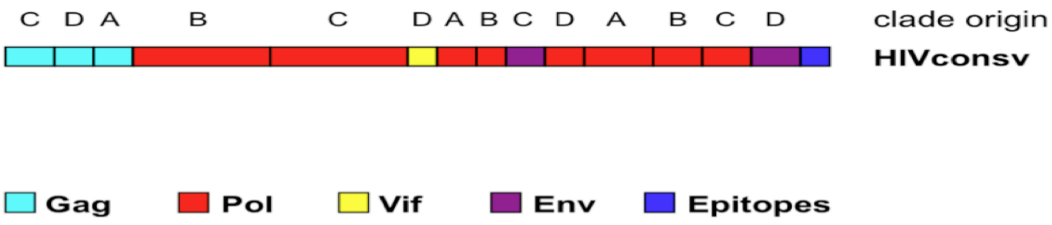


Figure 6.18 Structure of the HIVconsv immunogen.

Female BALB/c mice were injected intraperitoneally with either an irrelevant isotype control antibody or IL-10R blocking antibody 1 day prior to intramuscular immunisation with pTH.HIVconsv. 21 days after the first immunisation the mice received a second intramuscular immunisation with MVA.HIVconsv. The vaccination

schedule is illustrated in Figure 6.19 and the regimen received by each group is described in Table 6.2.

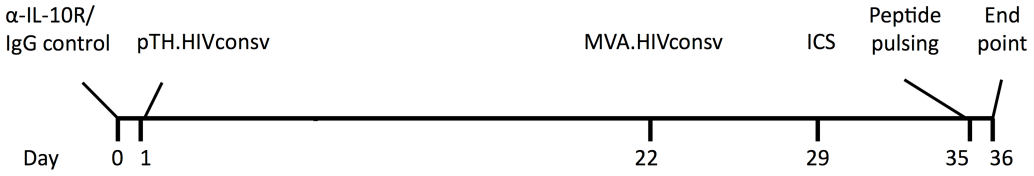


Figure 6.19 Schedule for the vaccination of mice with HIVconsv. Mice were injected intraperitoneally with either an irrelevant isotype control antibody or IL-10R blocking antibody 1 day prior to the first vaccination. At day 29 blood was collected from the lateral tail vein for analysis of CD8+ T cell responses by intracellular cytokine staining (ICS). At the end point mice were sacrificed and splenocytes harvested for further analysis.

Group	**Number of mice**	**Antibody**	**Vaccine**
1	15	Isotype control	1 x pTH.HIVconsv, 1 x MVA.HIVconsv
2	15	αIL-10R	1 x pTH.HIVconsv, 1 x MVA.HIVconsv

Table 6.2 Regimen received by each experimental group.

6.5.2 Peripheral blood CD8+ T cell responses after immunisation with pTH.HIVconsv and MVA.HIVconsv, with and without IL-10R blockade

Since a single immunisation with pTHr.HIVA failed to elicit detectable CD8+ T cell responses in the first experiment, ICS was not performed on pooled peripheral blood cells until after the second immunisation with MVA.HIVconsv in this experiment. Figures 6.20 and 6.21 show the frequencies of H, P and G1-specific IFN-γ+ and CD107a+ CD8+ T cells. Peripheral blood from mice immunised after receiving isotype control antibody (group 1) exhibited a strong IFN-γ response to the H epitope (26.4% of CD8+ T cells were IFN-γ+), whereas the frequency of H-specific IFN-γ+ CD8+ T cells in the blood from mice immunised after receiving IL-10R blocking antibody (group 2) was slightly lower (16.0% of CD8+ T cells were IFN-γ+) (Figure 6.20). IFN-γ responses to the P and G1 epitopes did not exceed background levels, except the response to G1 in group 2, which was very low (0.009%). High constitutive surface expression of CD107a was detected in both groups (44.2% of CD8+ T cells in group 1 and 37.8% of CD8+ T cells in group 2 were CD107a+). CD107a expression was detected in response to the H epitope (15.4% of CD8+ T cells in group 1 and 7.4% in group 2 after the subtraction of background) but did not increase above the level of background in response to the P and G1 epitopes (Figure 6.21).

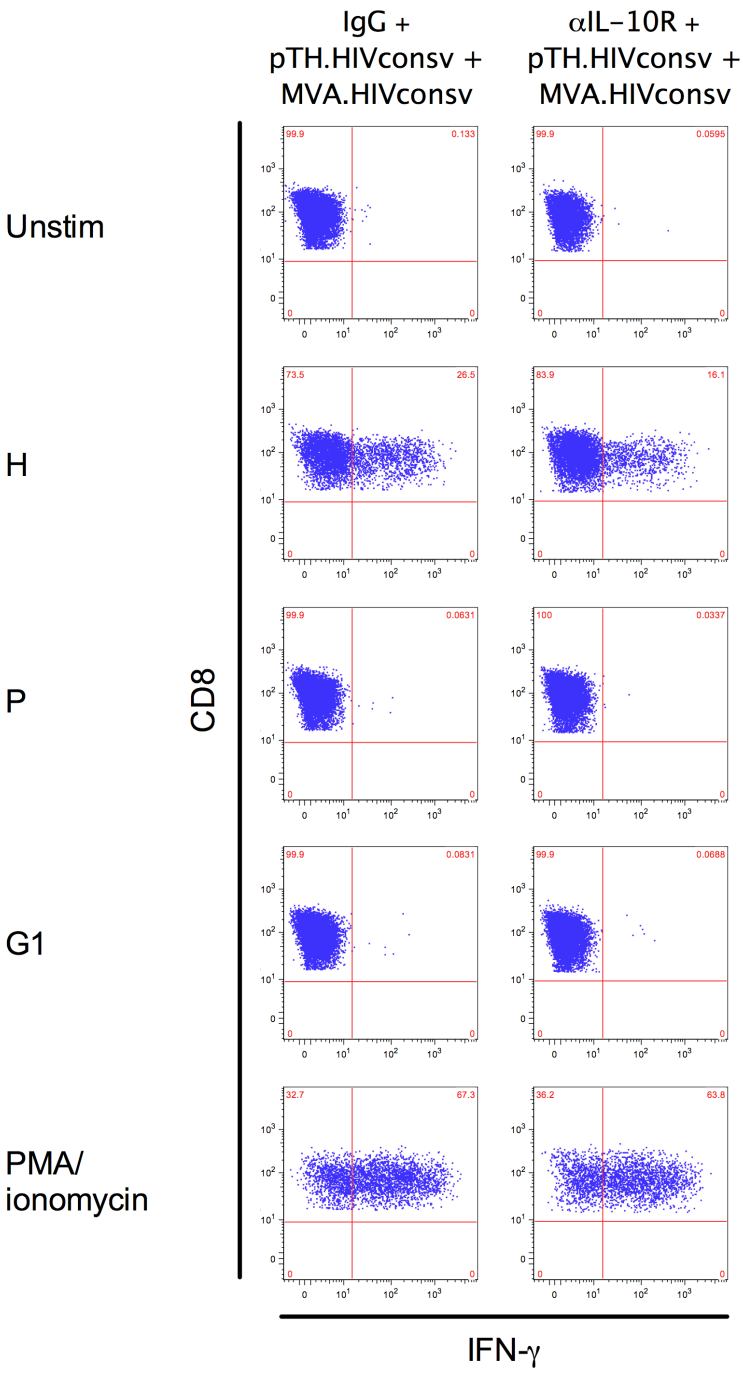


Figure 6.20 Flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and stimulated with the relevant peptide for 6.5 hours, then stained with Aqua live/dead cell stain (Invitrogen), CD8-PE-Texas Red, CD19-Pacific blue, IFN-γ-APC and CD107a-FITC (ebioscience). IFN-γ+ CD8+ T cells were identified as shown in Figure 6.4.

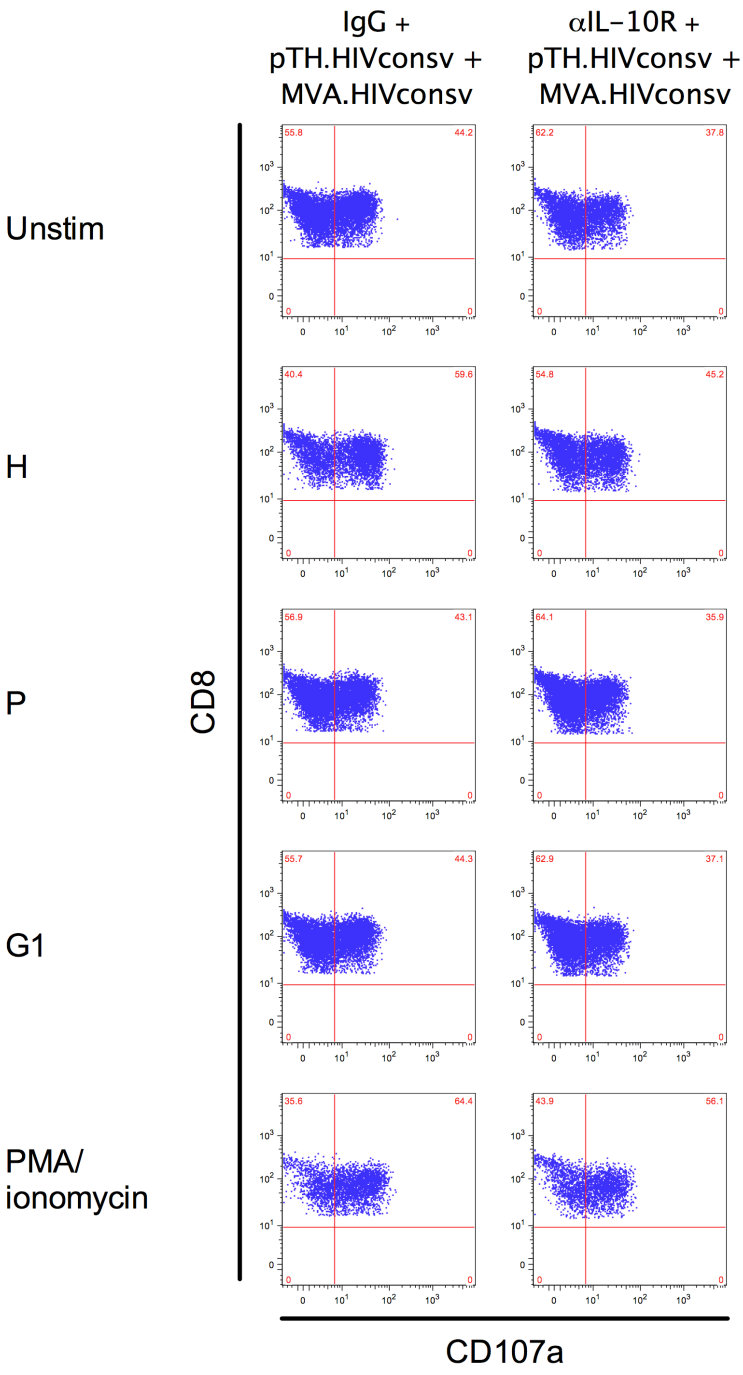


Figure 6.21 Flow cytometric plots of surface CD107a expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were treated as described in Figure 6.20.

6.5.3 End point responses to pTH.HIVconsv prime, MVA.HIVconsv boost

In order to determine the effect of IL-10R blockade on the immunogenicity of HIVconsv in individual mice, ICS was performed on the splenocytes of ten mice from each group. Representative flow cytometric plots of IFN-γ and CD107a responses to the H, P and G1 epitopes are shown in Figures 6.22 and 6.23. As previously, only frequencies of CD107a+ CD8+ T cells that were more than double the background level were considered positive. IFN-γ+ CD8+ T cells were detected after stimulation with H, P and G1 peptides, with the H epitope provoking the strongest response, followed by P and then G1 in both groups (Figure 6.24). There was no difference in the frequency of IFN-γ+ CD8+ T cells in response to any of the three epitopes between mice that had been vaccinated after receiving isotype control antibody (group 1) and mice that had been vaccinated after receiving IL-10R blocking antibody (group 2) ($p > 0.05$, Mann Whitney test). CD107a expression by CD8+ T cells followed a similar hierarchy, with the highest frequency of CD107a+ CD8+ T cells in response to the H epitope, followed by P and then G1. Once again, there was no difference in the frequency of CD107a+ CD8+ T cells in response to any of the epitopes between mice in groups 1 and 2 ($p > 0.05$, Mann Whitney test) and most responses did not exceed the cut-off (Figure 6.24).

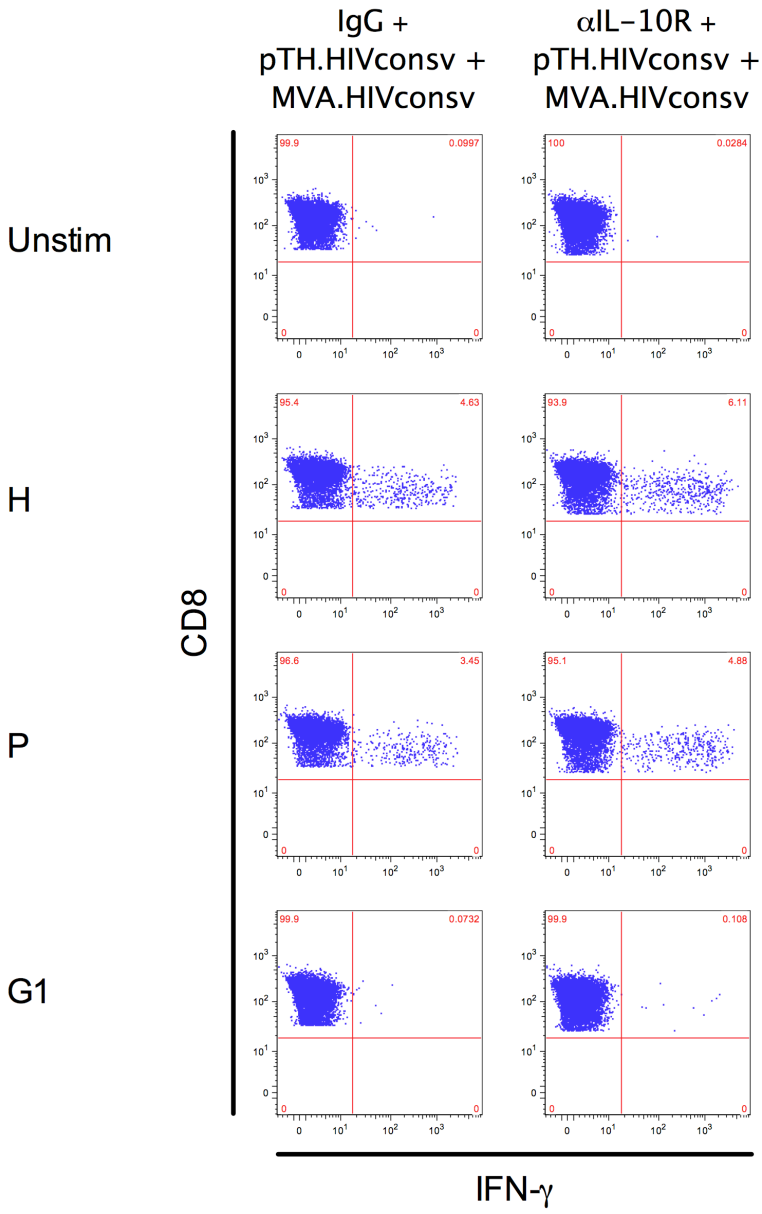


Figure 6.22 Representative flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P or G1. The PMA ionomycin positive control was omitted due to the strong response to the H epitope. Cells were treated as described in Figure 6.20.

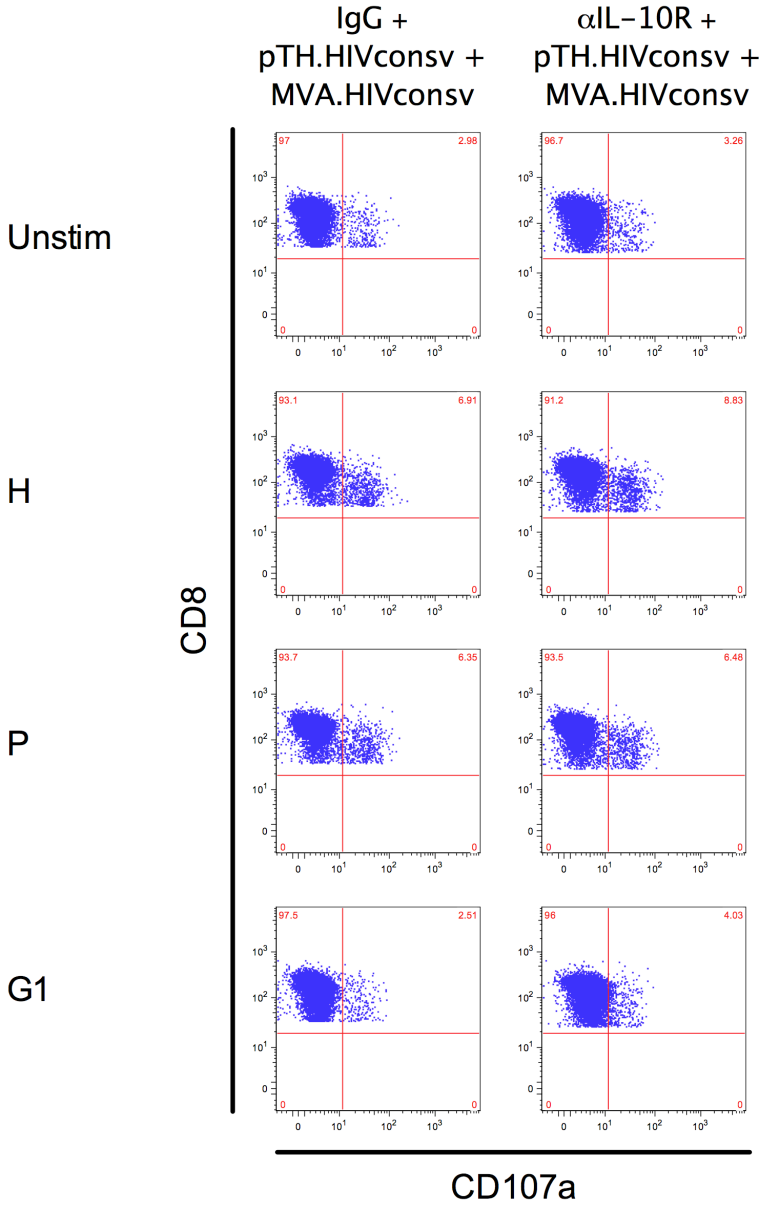


Figure 6.23 Representative flow cytometric plots of CD107a surface expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P or G1. Cells were treated as described in Figure 6.20.

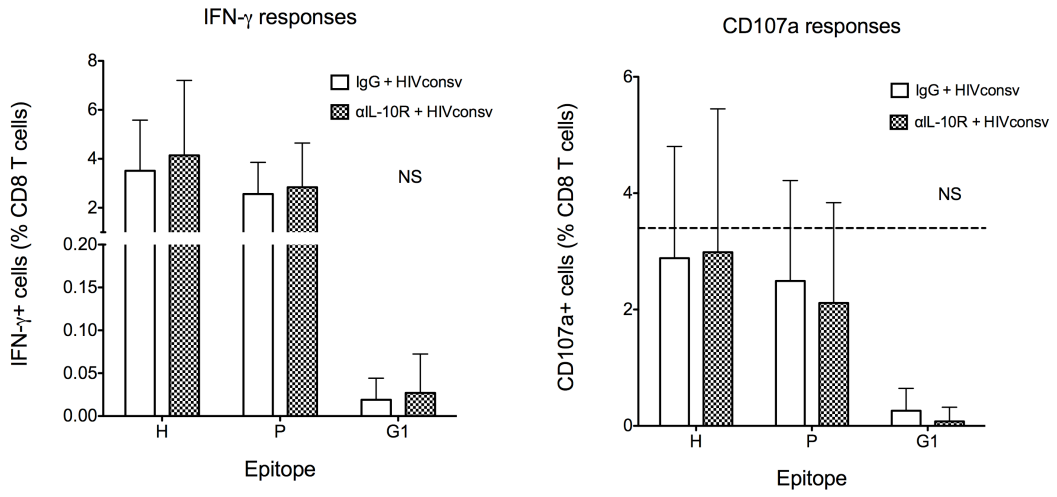


Figure 6.24 IFN-γ and CD107a expression by CD8+ T cells in response to H, P and G1 in mice immunised with HIVconsv after receiving isotype control antibody and mice immunised with HIVconsv after receiving IL-10R blocking antibody. The dashed line shows the cut-off point below which CD107a responses were not considered positive. Statistical analyses were conducted for individual epitopes using Mann Whitney test.

Once again, in order to confirm the observations from the ICS with respect to IFN-γ responses to vaccination, an IFN-γ ELISPOT was performed on splenocytes from the same mice, using the H, P and G1 peptides for stimulation. The same hierarchy of responses was observed, with H the most immunogenic epitope in terms of IFN-γ production and G1 the least (Figure 6.25). There was no difference in the number of IFN-γ spot-forming units (SFU) per million cells in response to the H or P epitopes between mice vaccinated after receiving isotype control antibody (group 1) and mice vaccinated after receiving IL-10R blocking antibody (group 2). However, the number of IFN-γ SFU per million cells in response to the G1 epitope was significantly higher in mice from group 2 than in mice from group 1 ($p = 0.0048$; Mann Whitney test) (Figure 6.25).

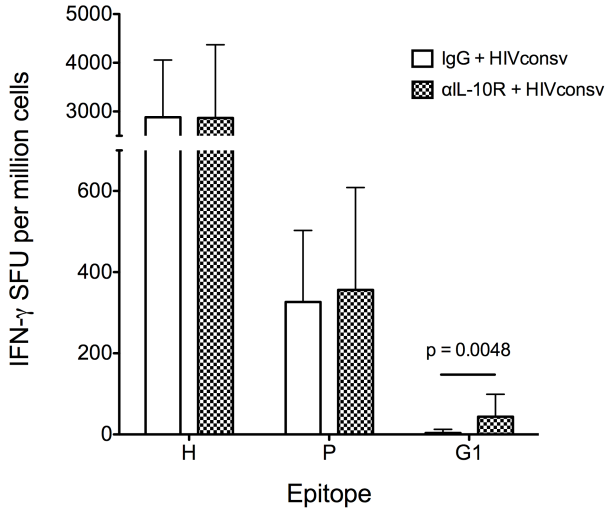


Figure 6.25 IFN-γ ELISPOT responses to H, P and G1. Splenocytes were isolated and stimulated for 16 hours with the relevant peptide. Statistical analyses were conducted for individual epitopes using Mann Whitney test.

ICS and ELISPOT assays have been widely used to assess the immunogenicity of diverse vaccine candidates but rely on the production of cytokines, which is not always indicative of anti-viral function (471-474). In order to assess the cytolytic capacity of vaccine-induced T cells, splenocytes from unvaccinated mice were pulsed with H, P and G1 peptides and then labelled with varying concentrations of CFSE, H-pulsed cells receiving the highest concentration and G1-pulsed cells the lowest. A separate pool of unpulsed cells was labelled with CMTMR. These fluorescently labelled cells were then injected into the lateral tail vein of five vaccinated mice from each group. The following day the vaccinated mice were sacrificed and splenocytes examined for the frequencies of each of the populations of fluorescent cells. The gating strategy for the identification of CFSE- and CMTMR-labelled cells is shown in Figure 6.26. The immunodominance hierarchy with respect to immunogenicity was maintained in terms of *in vivo* killing, with H-pulsed cells being lysed most frequently, followed by P-pulsed cells and then by G1-pulsed cells. There was a trend towards increased killing

of P- and G1-pulsed cells in mice from group 2 compared to mice from group 1, but this did not reach statistical significance ($p > 0.05$, Mann Whitney test) (Figure 6.27).

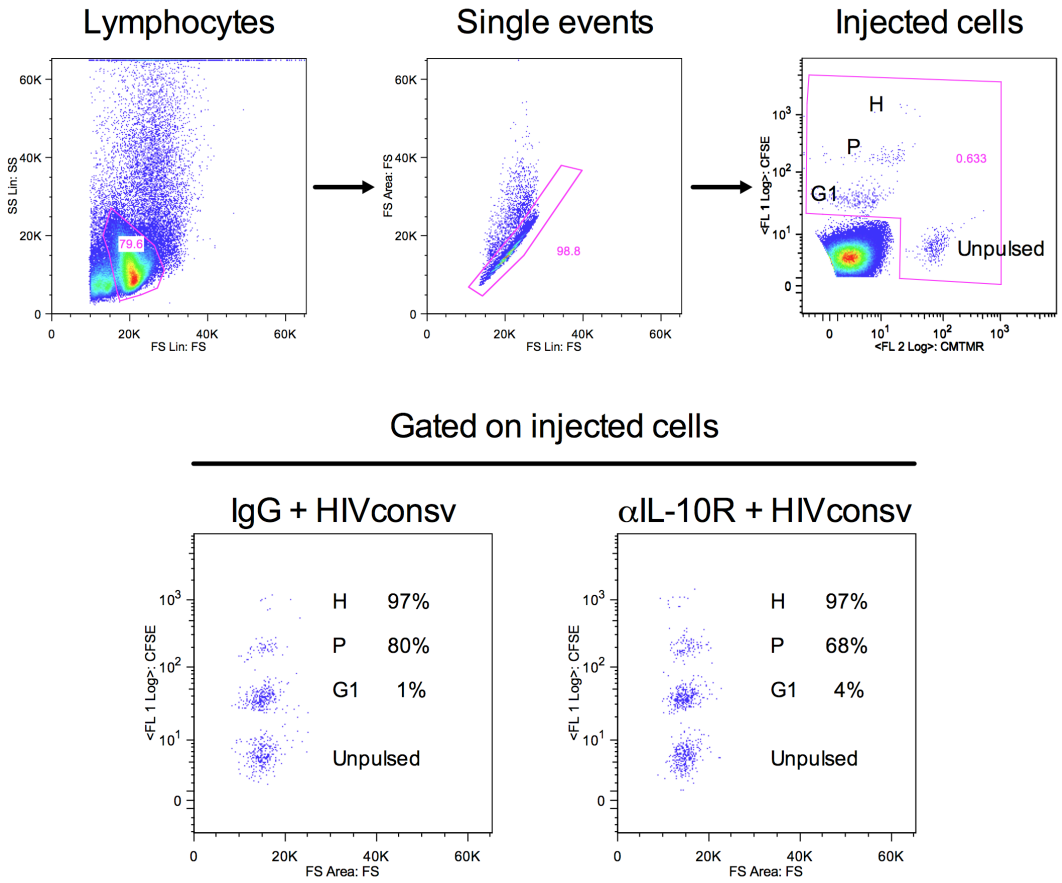


Figure 6.26 Gating strategy for the identification of CFSE- and CMTMR-labelled cells and representative flow cytometric plots showing the percentage of the cells that had been lysed after their recovery from immunised mice. Peptide pulsed splenocytes from unvaccinated mice were labelled with CFSE or CMTMR and injected into vaccinated mice, which were sacrificed the following day. For each epitope, the percentage of pulsed cells lysed was calculated using the following formula: ((unpulsed cells – pulsed cells)/ unpulsed cells) x 100%.

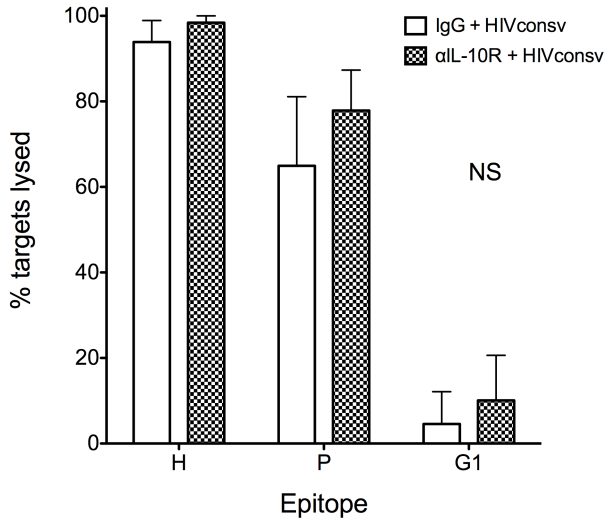


Figure 6.27 The percentage of H, P and G1-pulsed target cells lysed in mice vaccinated after receiving isotype control antibody and in mice vaccinated after receiving IL-10R blocking antibody. Statistical analyses were conducted for individual epitopes using Mann Whitney test.

Collectively, the data from this immunisation regimen revealed a clear hierarchy of responses to three epitopes from HIVconsv, with H provoking the greatest response in terms of both cytokine production and *in vivo* killing and G1 the lowest response. Furthermore, the results of the ELISPOT and *in vivo* killing assays suggested that blockade of IL-10R may increase the IFN-γ response to the subdominant G1 epitope.

6.6 Effect of IL-10R blockade on a single immunisation with MVA.HIVconsv

Since one of IL-10's main functions is the downregulation of antigen presentation, blockade of the IL-10R may improve the initial priming response to vaccination. However, IL-10R blockade failed to enhance CD8+ T cell responses after a single immunisation with pTHr.HIVA (see Figures 6.5 – 6.7). In order to investigate whether IL-10R blockade could enhance responses when a more immunogenic vector was used, female BALB/c mice were injected intraperitoneally with either isotype control antibody or IL-10R blocking antibody and then immunised intramuscularly with MVA.HIVconsv. The vaccination schedule is illustrated in Figure 6.28 and the regimen received by each group is described in Table 6.3.

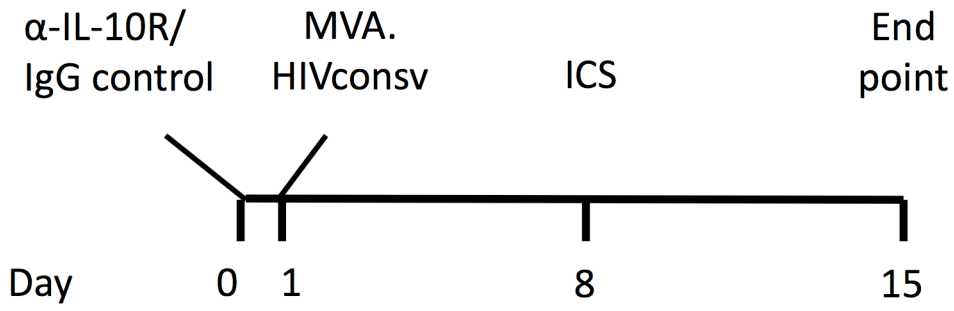


Figure 6.28 Schedule for the vaccination of mice with MVA.HIVconsv. Mice were injected intraperitoneally with either an irrelevant isotype control antibody or IL-10R blocking antibody 1 day prior to vaccination. At day 8, blood was collected from the lateral tail vein for analysis of CD8+ T cell responses by intracellular cytokine staining (ICS). At the end point mice were sacrificed and splenocytes harvested for further analysis.

Group	Number of mice	Antibody	Vaccine
1	5	Isotype control	1 x MVA.HIVconsv
2	5	αIL-10R	1 x MVA.HIVconsv

Table 6.3 Regimen received by each experimental group.

6.6.1 Peripheral blood CD8+ T cell responses to a single immunisation with MVA.HIVconsv, with and without IL-10R blockade

Seven days after immunisation with MVA.HIVconsv, blood was collected from the lateral tail vein of each mouse, pooled according to group and CD8+ T cell responses to the H, P and G1 epitopes examined by ICS. IFN-γ production by CD8+ T cells in response to the dominant H epitope was detected in blood from both the mice vaccinated after receiving the isotype control antibody (group 1) and the mice vaccinated after receiving the IL-10R blocking antibody (group 2), although the frequency of IFN-γ+ CD8+ T cells was slightly higher in group 1 than group 2 (5.5% compared to 3.3%). The IFN-γ response to the P and G1 epitopes did not exceed background levels in either group (Figure 6.29). CD107a expression above the level of background was not detected in response to the H, P or G1 epitopes in either group (Figure 6.30).

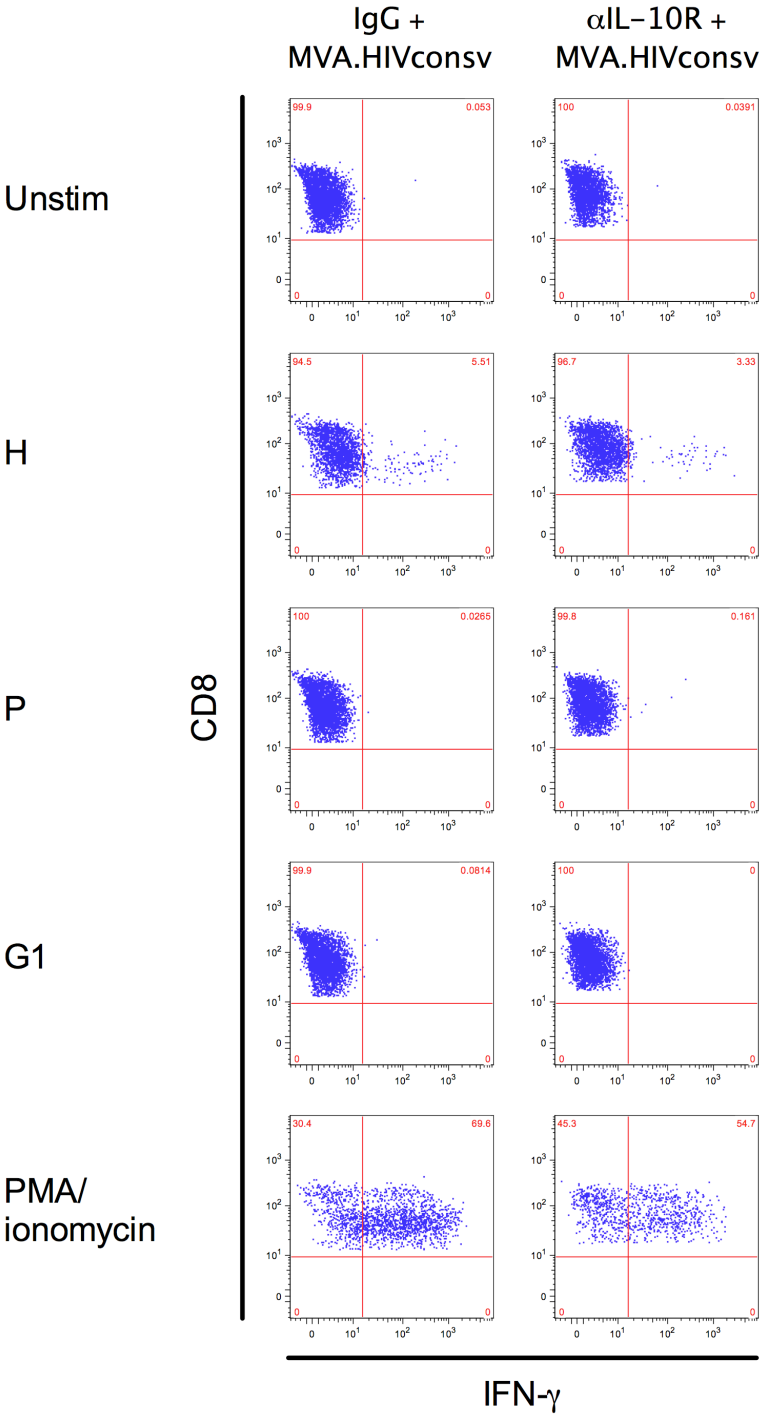


Figure 6.29 Flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were isolated and stimulated with the relevant peptide for 6.5 hours, then stained with Aqua live/dead cell stain (Invitrogen), CD8-PE-Texas Red, CD19-Pacific blue, IFN-γ-APC and CD107a-FITC (ebioscience). IFN-γ+ CD8+ T cells were identified as shown in Figure 6.4.

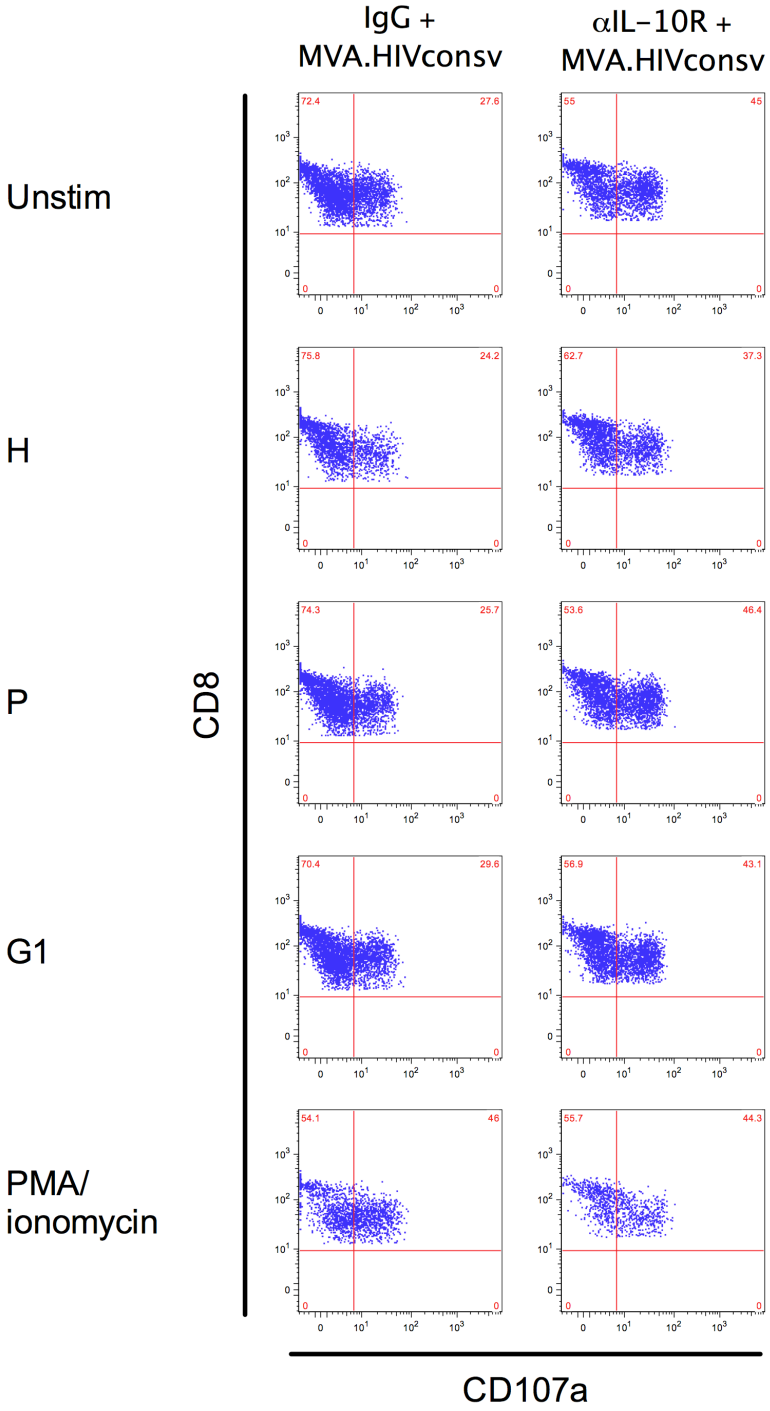


Figure 6.30 Flow cytometric plots of surface CD107a expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P, G1 or PMA/ionomycin. Cells were treated as described in Figure 6.29.

6.6.2 End point responses to MVA.HIVconsv

In order to examine responses to a single immunisation with MVA.HIVconsv in individual mice, splenocytes were harvested fourteen days after immunisation and examined for expression of IFN-γ and CD107a in response to the H, P and G1 epitopes. Only CD107a responses that were more than double the background response were considered positive. Representative flow cytometric plots of IFN-γ and CD107a responses to the H, P and G1 epitopes are shown in Figures 6.31 and 6.32. Similarly to when MVA.HIVconsv was administered as a boost following priming with pTH.HIVconsv, a single immunisation with MVA.HIVconsv elicited IFN-γ production by CD8+ T cells in response to the H, P and G1 epitopes, with H eliciting the highest frequency of IFN-γ+ CD8+ T cells (mean 0.89% for group 1 and 0.83% for group 2) and G1 the lowest frequency (mean 0.03% for group 1 and 0.01% for group 2) (Figure 6.33). There was no difference in the frequency of IFN-γ producing CD8+ T cells in response to any of the epitopes in mice that were vaccinated after receiving isotype control antibody (group 1) compared to mice that were vaccinated after receiving IL-10R blocking antibody (group 2) ($p > 0.05$, Mann Whitney test). CD107a expression by CD8+ T cells in response to H, P and G1 did not reach the cut-off in any of the mice from either group (Figure 6.33).

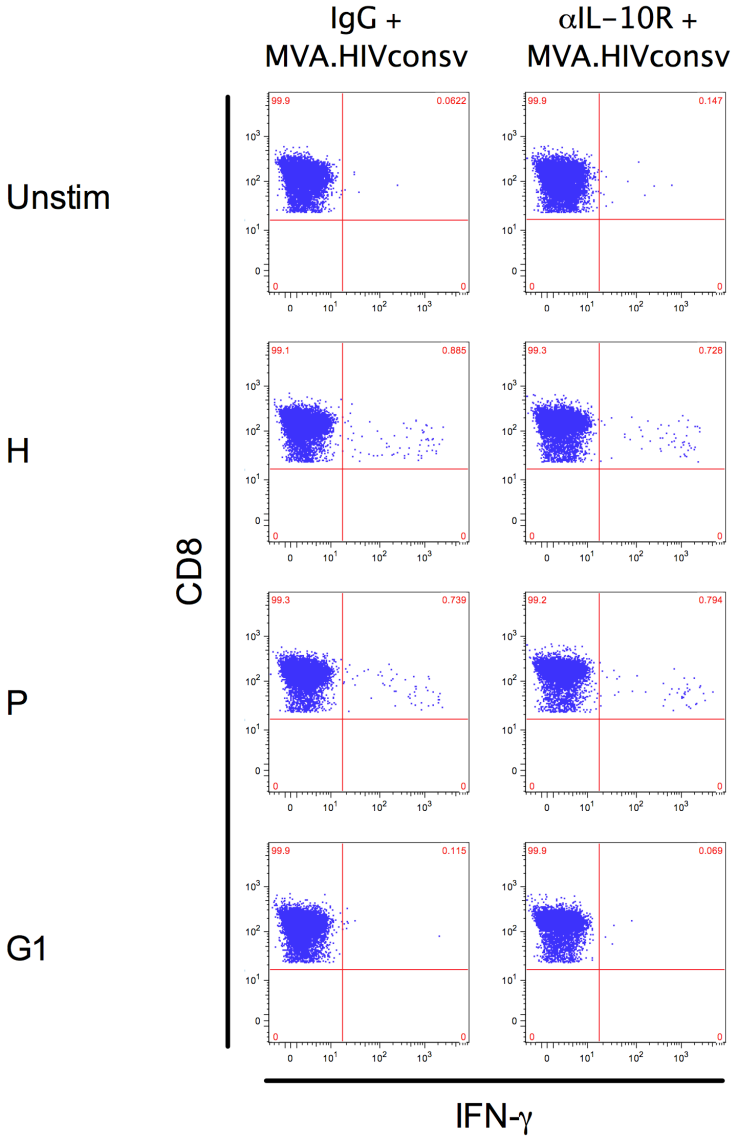


Figure 6.31 Representative flow cytometric plots of IFN-γ production by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P or G1. The PMA ionomycin positive control was omitted due to the strong response to the H epitope. Cells were treated as described in Figure 6.29.

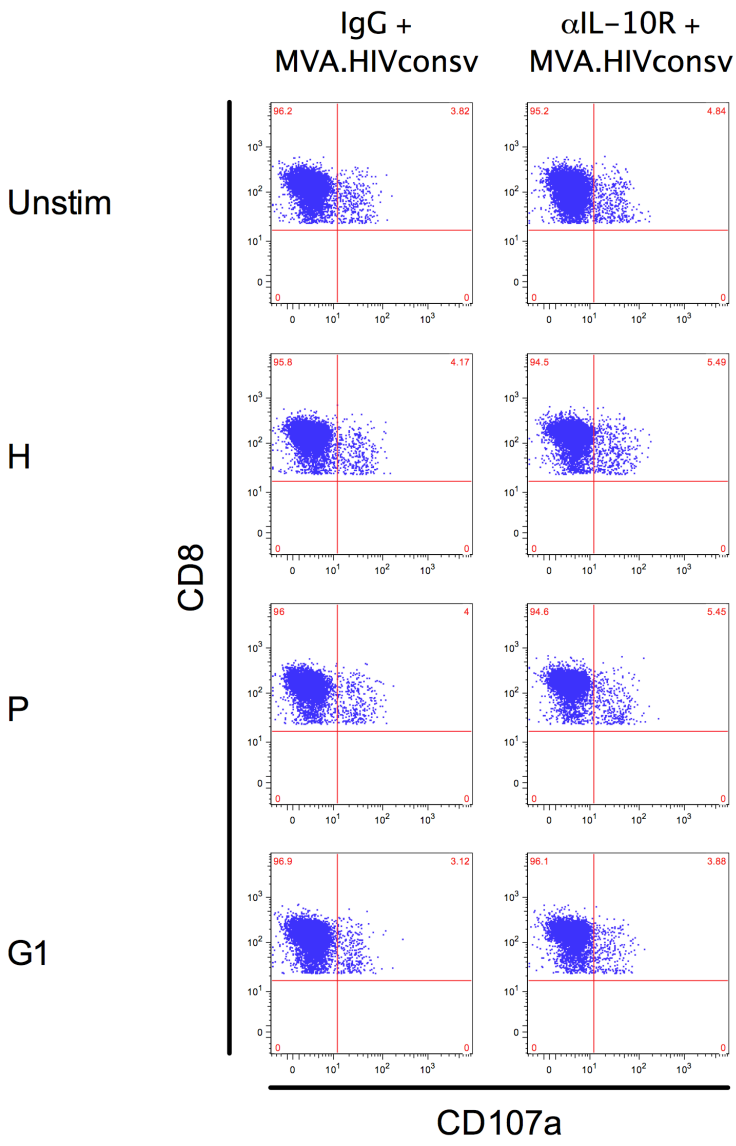


Figure 6.32 Representative flow cytometric plots of CD107a surface expression by CD8+ T cells in response to stimulation with PBS ("Unstim"), H, P or G1. Cells were treated as described in Figure 6.29.

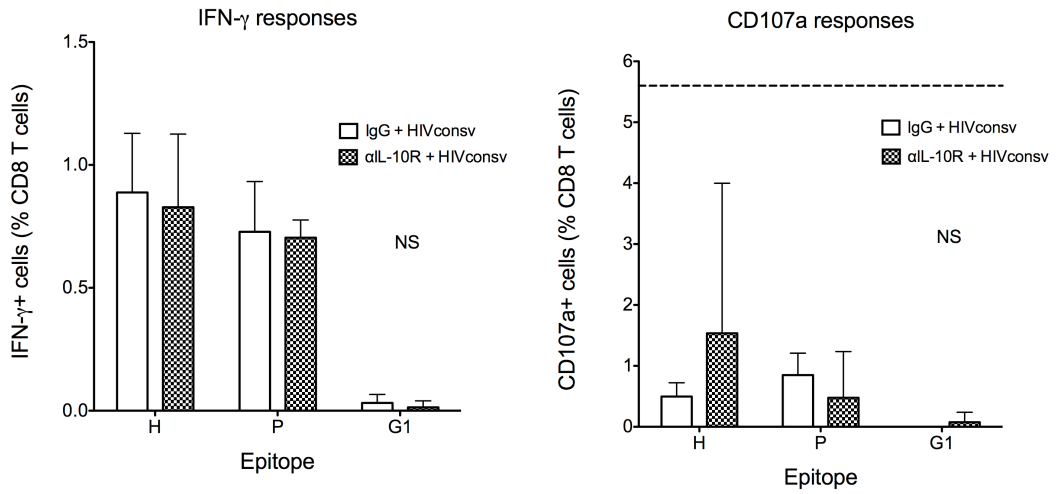


Figure 6.33 IFN-γ and CD107a expression by CD8+ T cells in response to H, P and G1 in mice immunised with HIVconsv after receiving isotype control antibody and mice immunised with HIVconsv after receiving IL-10R blocking antibody. Statistical analyses were conducted for individual epitopes using Mann Whitney test.

IFN-γ responses to H, P and G1 were also quantified using ELISPOT. Once again, the H epitope provoked the largest response in terms of IFN-γ production (mean 1371 SFU per million cells for group 1 and 1081 SFU per million cells for group 2) and the G1 epitope provoked the lowest response (mean 3 SFU per million cells for group 1 and 14 SFU per million cells for group 2) (Figure 6.34). There was a trend towards a decrease in the number of IFN-γ SFU per million cells in response to the H epitope in mice from group 2 compared to mice from group 1, but this did not reach statistical significance ($p > 0.05$, Mann Whitney test). Conversely, there was a trend towards an increase in the number of IFN-γ SFU per million cells in response to the P and G1 epitopes in mice from group 2 compared to mice from group 1, but again this difference did not reach statistical significance ($p > 0.05$, Mann Whitney test).

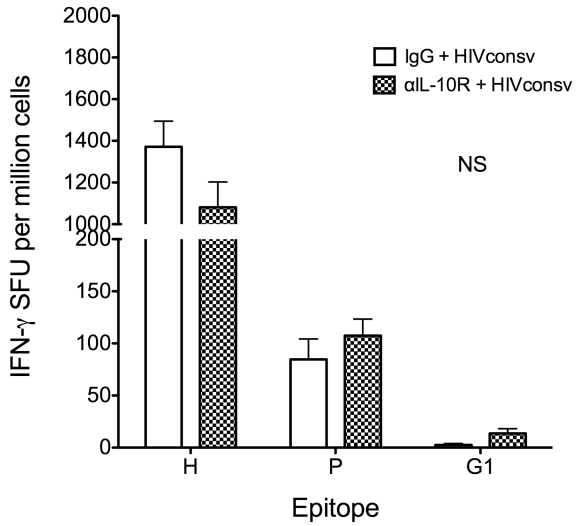


Figure 6.34 IFN-γ ELISPOT responses to H, P and G1. Splenocytes were isolated and stimulated for 16 hours with the relevant peptide. Statistical analyses were conducted using Mann Whitney test.

The data from this experiment demonstrates that a single immunisation with MVA.HIVconsv can elicit IFN-γ production by CD8+ T cells in response to the H and P epitopes. However, the immunogenicity of the vaccine was not enhanced by blockade of IL-10 signalling.

6.7 Discussion

Blockade of IL-10 signalling has been used to enhance the efficacy of a DNA-vectored vaccine for LCMV in a murine model (345) but has not previously been investigated in a HIV-1 vaccine model. In the first experiment, the effect of IL-10R blockade on the immunogenicity of pTHr.HIVA was examined. IL-10R blockade enhanced the frequency of CD8+ T cells producing IFN-γ in response to the dominant H and P epitopes of the HIVA immunogen but this difference did not reach statistical significance, and immunisation did not boost responses to the subdominant G1 epitope in terms of IFN-γ production, TNF-α production or CD107a expression by CD8+ T cells, thereby reinforcing the existing immunodominance hierarchy. However, it is possible that the response to the G1 epitope in mice vaccinated with the modified dHmP immunogen (where H and P are removed) might be enhanced by IL-10R blockade. This possibility will be investigated in future studies.

Even the mice that received IL-10R blocking antibody prior to vaccination exhibited relatively weak responses to HIVA, with less than 1% of CD8+ T cells producing IFN-γ in response to the dominant H epitope 8 days after the second immunisation. Previously, Castro *et al* observed that blockade of IL-10 signalling did not enhance antigen-specific T cell responses unless a TLR agonist such as LPS or CpG was co-administered, suggesting a requirement for APC stimulation (475). In support of this hypothesis, Vicari *et al* reported that IL-10R blockade in combination with a CpG immunostimulatory sequence enhanced tumour antigen-specific DC responses, whereas IL-10R blockade alone was ineffective (342). These observations may explain why IL-10R blockade alone did not significantly enhance T cell responses to the DNA immunogen. An enhanced response might also have been achieved by simultaneously

blocking other pathways associated with T cell dysfunction, such as the programmed death (PD-1) pathway. Upregulation of PD-1 on HIV-specific CD8+ T cells is associated with exhaustion, and blockade of the IL-10 and PD-1 pathways simultaneously was significantly more effective in enhancing anti-viral T cell responses in mice infected with LCMV than blockade of either alone (239, 476).

In contrast to the current study, Brooks *et al* observed that 4% of CD8+ T cells produced IFN-γ in response to LCMV-GP_{33-41} in mice that were immunised with a DNA LCMV vaccine in combination with IL-10R blockade (345). Notably, unlike HIV-1 infection, LCMV infection in mice induces robust T cell responses that are able to clear infection in the absence of intervention after around 60-150 days (477), and so strategies that enhance LCMV-specific T cell responses may not work in HIV-1 infection, where the naturally-induced T cell response is much less effective at controlling viral load. Another likely explanation for the discrepancy is that the mice in Brooks *et al's* experiment were infected with LCMV and so exhibited enhanced responses to LCMV-GP_{33-41} even in the absence of vaccination. LCMV infection induces extensive innate immune activation, including the production of type I IFN and IL-12 by pDCs, whereas immunisation of uninfected mice would not have this effect (478, 479). It is possible that, in order to enhance vaccine-induced T cell responses, IL-10R blockade needs to be combined with agents that stimulate the innate immune system, as suggested by Vicari *et al*'s work on tumour antigen-specific DC responses (342). Circulating IL-10 levels would also be likely to be higher in chronically infected mice, compared to the uninfected mice used in the current study and so IL-10R blockade would have more of an effect. In order to ablate IL-10 signalling for the duration of the experiment, Brooks *et al* administered IL-10R blocking antibody every

three days, whereas in the current experiment IL-10R blocking antibody was only administered once, the day before the initial vaccination. The rationale behind this decision was that firstly, due to its effects on antigen presentation, IL-10R blockade would have the greatest effect during the priming step of vaccination, and secondly, continuous blockade should not be necessary in healthy, uninfected mice where circulating IL-10 levels should be relatively low (480). However, it is possible that a more substantial increase in immunogenicity could be achieved with repeated administrations of the antibody, and this is one avenue that will be investigated in future studies. One potential pitfall would be the risk of eliciting antibodies to the blocking antibody itself; this may limit the number of times the blocking antibody could be administered. Nevertheless, the use of αIL-10R before the boost as well as before the prime may be a feasible way to further increase the immunogenicity of pTHr.HIVA. Another potential strategy would be to increase the number of immunisations; however, Roshorm and colleagues have previously demonstrated that a third immunisation did not increase the immunogenicity of a pTH-vectored HIV vaccine in mice (481).

One way to increase the response to an immunogen is to use different vectors for the prime and boost immunisations (482). In order to investigate whether IL-10R blockade could increase the immunogenicity of a heterologous prime-boost regimen, using a vaccine designed to target the conserved regions of the HIV-1 proteome, mice were immunised with pTH.HIVconsv followed by MVA.HIVconsv. As expected, this regimen was more immunogenic, in terms of IFN-γ responses to the dominant H epitope, than the homologous pTHr.HIVA prime-boost regimen. However, IL-10R blockade did not increase the response to the dominant H and P epitopes, either in terms of IFN-γ and CD107a production by CD8+ T cells or *in vivo* killing of peptide-pulsed

target cells. IFN-γ production in response to G1, as measured by ELISPOT, was significantly increased in mice that were vaccinated after receiving IL-10R blocking antibody, although this result was not corroborated by the data from the ICS, and there was a trend towards increased killing of G1 peptide-pulsed cells in these mice. These observations suggest that IL-10R blockade may enhance the immunogenicity of subdominant epitopes in a pTH.HIVconsv prime, MVA.HIVconsv boost regimen. However, IL-10R blockade failed to enhance the response, in terms of IFN-γ production and CD107a expression by CD8+ T cells, to a single immunisation with MVA.HIVconsv, suggesting that the ablation of IL-10 signalling does not enhance the immunogenicity of vaccine regimens where MVA is the sole vector. One possible explanation is that IL-10 is not induced in response to vaccination with MVA: in a previous study, immunisation of HIV-1-infected patients with MVA.HIVA did not result in an increase in plasma IL-10 levels (355). Measuring the concentration of IL-10 in plasma following immunisation would be one way to determine whether this was the case in the vaccinated mice. However, this would not explain the failure of IL-10R blockade to enhance immunogenicity when pTHr.HIVconsv was used as the prime. Another potential factor is the expression by MVA of a soluble receptor for IL-1β, an inflammatory cytokine that mediates cell proliferation, differentiation and apoptosis (483). Since IL-10 inhibits the production of IL-1β by monocytes and macrophages (290), it is possible that the boosting effect of IL-10R blockade on the immunogenicity of pTHr.HIVA was mediated by IL-1β, but that when MVA is used this effect is negated by the soluble receptor. Finally, since IL-10 exerts effects primarily on APCs, it may have been beneficial to examine the effect of IL-10R blockade on these cells in the vaccination setting, perhaps by analysing the production of IL-12, which has previously been reported to be inhibited by IL-10 following immunisation in the mouse

Leishmania major model (484), and the expression of MHC Class II and costimulatory molecules.

Collectively, these results show that IL-10R blockade has potential promise as an adjunct to HIV-1 vaccination but that a considerable degree of optimisation is required. In particular, the choice of vector may be critical to the success or failure of the strategy. Given the lack of immunogenicity of pTHr, and the seeming inability of IL-10R blockade to significantly enhance responses to MVA.HIVconsv alone, other vectors will need to be tested. Two potentially attractive candidates are human adenovirus 5 (Ad5) and chimpanzee adenovirus (ChAdV63), which have demonstrated a high degree of immunogenicity in mice and rhesus macaques (485-487). Although Ad5 is highly immunogenic, the high prevalence of pre-existing immunity to this vector in the human population may limit its efficacy in human clinical trials (346, 488). For this reason, ChAdV63.HIVconsv, which is currently under evaluation in clinical trials (clinicaltrials.gov/ct2/show/NCT01151319), may be the more promising immunogen for the long-term development of this strategy. It may also be beneficial to explore responses to other epitopes in HIVconsv that have not yet been characterised, using a set of overlapping peptides spanning the entire immunogen, as it is possible that IL-10R blockade could enhance responses to these epitopes.

Another aspect of the IL-10 blocking strategy that needs to be optimised is the timing, route of administration and nature of the blocking agent. Since the repeated use of a receptor-blocking antibody may elicit an antibody response to itself, an alternative would be to use a soluble IL-10R that would bind secreted cytokine rather than the receptor. Such a receptor is currently under development in the laboratory of Professor

Simon Davis (Weatherall Institute of Molecular Medicine, Oxford). One disadvantage of a soluble receptor is that it would probably hamper attempts to measure the level of IL-10 in plasma after vaccination using assays such as ELISA. Nevertheless, the aim of future experiments is to compare the efficacy of the soluble receptor and the commercially available antibody, as well as the effect of multiple administrations of IL-10 blocking agent compared to a single blockade. The intraperitoneal route of administration of αIL-10R was chosen as this method elicited an enhanced response to vaccination in the LCMV model (345). However, intravenous injection may enable a more efficient transfer of the antibody to IL-10R expressing cells in the peripheral blood, spleen and lymphoid architecture. As IL-10R blocking antibody has previously been administered intravenously to mice without adverse effects (489), it may be useful to investigate this route in the HIV-1 vaccine model.

The murine model is commonly used as a cost-effective, rapid means of pre-clinical evaluation of HIV-1 vaccine candidates; however, there are limitations to this model. Since HIV-1 is unable to gain entry to murine cells, any viral challenge following immunisation has to be performed using chimaeric viruses such as EcoHIV, which incorporates the ecotropic murine leukaemia virus glycoprotein gp80 in the place of the HIV-1 envelope (463). One solution to this problem has been the development of "humanised" mouse models for HIV-1 infection and vaccination (reviewed in (490)). These transgenic mice lack one or more genes, for example the recombination activating gene Rag2, that are essential for the development of lymphocytes and so do not develop a functioning immune system. Transplantation with human PBMC or stem cells (SC) allows the reconstitution of a miniature human immune system in the mouse, which can then be infected with HIV-1. Using such a model, it might be possible to

investigate the effect of IL-10R blockade on vaccination in mice already infected with HIV-1. Since, IL-10 is upregulated in humans during both acute and chronic HIV-1 infection, but not following immunisation with MVA.HIVA, IL-10R blockade may hold more promise as an adjunct to therapeutic, rather than prophylactic, vaccination (52, 321, 355, 356).

Some of the pathological effects of HIV-1 infection in humans, for example the loss of CD4+ T cells, can be reproduced in humanised mice (491, 492). However, the decline in viraemia to a set point that is observed in HIV-infected humans does not occur in humanised mice (492). A further limitation of both humanised and non-humanised mouse models is their small size compared to humans. A key component of the human immune system is the movement or trafficking of different immune cells throughout the body, and this is not reproducible in the mouse. A more suitable model to investigate the effect of IL-10 signalling blockade on vaccination might involve using non-human primates (NHP) that are not natural hosts for SIV, such as rhesus macaques. The disease course of SIV in macaques closely parallels that of HIV-1 in humans; however, vaccine candidates that have shown great promise in macaques have not subsequently conferred protection in subsequent human trials (346, 493, 494). Given this consideration, and the costs associated with NHP studies, it may be preferable to continue the evaluation of IL-10 blockade in the mouse model until the strategy has been optimised.

The development of a universally protective HIV-1 vaccine is one of the greatest challenges facing the current generation of immunologists. It is likely that an improved understanding of the nature of protective immune responses to both infection and

vaccination will be required in order to meet this challenge. In this chapter, the manipulation of cytokine signalling as a means of influencing the response to vaccination has been investigated. Whilst further optimisation is required, this strategy could present a novel means to elicit protective responses, thereby enhancing the efficacy of vaccines for HIV-1.

Chapter 7: Concluding remarks and future studies

The work presented in this thesis was conducted with the aim of elucidating the role of IL-10 in modulating the immune response to HIV-1 infection and vaccination. I conducted a detailed phenotypic and functional study of the cellular sources of IL-10 in chronic HIV-1 infection, and studied the effect of IL-10R blockade on the immunogenicity of two candidate HIV-1 vaccines in a murine model. My study differed from previous investigations into the role of IL-10 in HIV-1 infection in a number of ways. Firstly, I identified the cellular subset that dominated HIV-specific IL-10 production and investigated the effect of removing only this subset, rather than blocking all IL-10 signalling. Secondly, I explored the effect of depleting this IL-10-producing subset on the function of a population of antigen presenting cells (peripheral blood monocytes), as well as the effect on endogenous viral replication *in vitro*. To my knowledge, these phenomena have not previously been studied in HIV-infected individuals. Finally, whilst IL-10R blockade has previously been used as an adjunct to mouse LCMV vaccination (345), this is the first time such a strategy has been used in a mouse HIV-1 vaccination model.

A cross sectional analysis of IL-10 production in 55 individuals with chronic HIV-1 infection revealed a rare subset of CD8+ T cells, which were restricted to ART-naïve, viraemic individuals, to be the major HIV-1 Gag-specific producers of IL-10 in the peripheral blood. These Gag-specific IL-10+ CD8+ T cells did not express the Treg markers FoxP3 or CD25 but did express IFN-γ. Approximately half the population expressed the β7 chain of the gut-homing integrin α4β7, and a minority expressed the

chemokine receptor CXCR3, which is involved with the trafficking of lymphocytes to sites of inflammation (397, 398). Depletion of Gag-specific IL-10+ CD8+ T cells from patient PBMC did not affect T cell activation or the production of cytokines such as IL-2, TNF-α or IFN-γ, suggesting that these cells do not act directly on other T cells. Instead, they appeared to act on monocytes, reducing their expression of CD38 and HLA-DR, and the production of the predominantly monocyte-derived pro-inflammatory cytokine IL-6. Depletion also boosted the number of HIV-1 Gag p24+ CD4+ T cells in PBMC cultured for 3 or 7 days in the presence of IL-2, suggesting that Gag-specific IL-10+ CD8+ T cells help to limit viral replication *in vivo*. In mice, blockade of the IL-10R prior to vaccination resulted in a trend towards an increase in IFN-γ production by CD8+ T cells in response to the dominant H (Env) and P (Pol) epitopes after two immunisations with pTHr.HIVA and a significant increase in the IFN-γ ELISPOT response to the subdominant G1 (Gag) epitope after immunisation with pTH.HIVconsv followed by MVA.HIVconsv.

Collectively, these results suggest that in chronic infection Gag-specific IL-10+ CD8+ T cells, acting on antigen presenting cells rather than on T cells directly, play an anti-inflammatory role and may help to limit viral replication. However, in uninfected mice, IL-10R blockade enhanced T cell responses to vaccination. Taken together with the observation that Gag-specific IL-10+ CD8+ T cells were largely absent in patients who had progressed sufficiently to require therapy, these observations suggest that the effects of IL-10-producing cell populations are highly dependent on disease stage and status. Interestingly, Naicker *et al* observed that IL-10 promoter polymorphisms linked to increased expression of the cytokine were associated with significantly higher median plasma viral loads during acute HIV-1 infection but lower median plasma viral

loads during chronic infection (245). These observations suggest that the effects of manipulating IL-10 signalling could be dependent on the timing, which may therefore be critical in determining whether IL-10R blockade would be helpful or harmful *in vivo*. Another factor to consider is that it was not possible to investigate the extent to which the effects of Gag-specific IL-10+ CD8+ T cells were mediated by the cytokine, and so depletion of these cells may not have the same effect as IL-10R blockade.

Whilst IL-10 upregulation has been observed in acute HIV-1 infection, the cells that produce the cytokine during this phase have not been defined (52, 321). If Gag-specific IL-10+ CD8+ T cells are present during acute infection, they may influence the level of the immune activation set point, which is associated independently of viral set point with subsequent CD4+ T cell loss (173). Whilst the results of the current study suggested that Gag-specific IL-10+ CD8+ T cells do not affect T cell activation directly, they may still help to control general immune activation by restricting the production of pro-inflammatory cytokines such as IL-6. However, since Gag-specific IL-10+ CD8+ T cells could also secrete IFN-γ, which promotes inflammation, an alternative possibility is that they originated as Gag-specific IFN-γ-producing effector cells and acquired the ability to produce IL-10 due to the environment of persistent antigenic stimulation present in chronically infected ART-naïve individuals. In support of this idea, Sun and colleagues have observed that infiltrating IFN-γ+ effector CD8+ T cells are a major source of IL-10 in the infected lung of influenza-infected mice (400). However, to my knowledge, a similar phenomenon has not been demonstrated in HIV-infected humans. A longitudinal study following individuals from acute to chronic infection would help to determine the stage of disease at which Gag-specific IL-10+ CD8+ T cells are first detectable in the peripheral blood, their ontogeny, and whether

there is an association between the frequency of these cells during acute infection and the immune activation set point. In addition, it would be important to examine the effects of this population on the production of other pro-inflammatory mediators such as IL-1 and IL-8 by monocytes and neutrophils during acute infection (290, 300).

Another factor that may influence the function of Gag-specific IL-10+ CD8+ T cells is their location. The expression of β7 and CXCR3 suggests that these cells can migrate to key sites of HIV-1 pathogenesis such as the GALT and other sites of inflammation, and therefore may be found at higher frequencies in these areas than they are in the peripheral blood. However, it would be necessary to perform gut biopsies on HIV-infected individuals to confirm this hypothesis. An important outstanding question is whether the migration of IL-10-producing CD8+ T cells to the gut would have a positive or negative impact on HIV-1 pathogenesis. Elevated levels of IL-10 in the intestinal mucosa of HIV-infected individuals have previously been associated with higher viral load in this region (495). However, the induction of IL-10 by commensal bacteria has been shown to be necessary for protection against experimentally induced colitis in a mouse model and so by limiting pro-inflammatory responses IL-10 may help to preserve the integrity of the mucosal barrier (496). This could be important in HIV-1 infection, as it has been proposed that microbial products that have entered the circulation as a result of damage to the mucosal barrier contribute to the elevated immune activation exhibited by infected individuals (497). An understanding of the effect of IL-10-producing cells in the GALT during HIV-1 infection will be crucial in deciding whether IL-10 modulation strategies should be targeted to this tissue.

Despite almost three decades of research, a universally effective HIV-1 vaccine remains elusive. During this period it has become clear that the nature and timing of host immune responses to immunodeficiency viruses, in both humans and NHPs, play a crucial role in determining the course of infection. Studies in NHPs have suggested that early induction of IL-10 during acute infection may contribute to a favourable, non-pathogenic, outcome, but it remains unclear whether such a response would also be protective in HIV-infected humans. If manipulation of the IL-10 response is to be used for therapeutic benefit, an improved understanding of the way in which the timing and location of this response affects the outcome will be crucial. This could provide investigators with an invaluable tool to enhance the efficacy of future vaccine candidates.

Future work

The next studies using the murine HIV-1 vaccination model will aim to determine the optimal vaccination strategy to be used with IL-10R blockade. These experiments will utilise the HIVconsv immunogen, which is currently being tested in human clinical trials (clinicaltrials.gov/ct2/show/NCT01151319). The chimpanzee adenovirus vector, ChAdV63 will be used, either alone or following priming with pTH.HIVconsv. This vector has been rendered replication incompetent through deletion of the E1, E3 and E4 regions of the viral genome, with the HIVconsv open reading frame inserted under the control of the human CMV intermediate/early promoter (473). ChAdV63 has proven highly immunogenic in rhesus macaque trials (473) and has been selected for clinical development due to the low level of pre-existing immunity in the human population compared to the level of immunity to vectors such as human Ad5 (488, 498, 499). Parallel tests will be conducted to determine whether a second administration of IL-10R

blocking antibody before the boosting immunisation results in superior immunogenicity compared to IL-10R blockade before the priming step alone. If a second blockade led to the induction of more potent responses to the vaccine in mice this strategy could be further evaluated in a NHP model, firstly to assess immunogenicity and secondly to determine the effect of blockade on vaccine-induced control of a pathogenic SIV challenge. The ultimate goal would be to translate this approach into human studies; however one caveat is the potential immunogenicity of repeated administrations of a blocking antibody. In order to address this issue, Professor Simon Davis's laboratory (Weatherall Institute of Molecular Medicine, Oxford) is developing a soluble IL-10R as an alternative to the commercially available blocking antibody. Since it lacks an Fc fragment, this receptor would be less immunogenic *in vivo*. It is hoped that the receptor will be available for testing in future trials.

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Appendix

HIV-1 Clade B Gag overlapping 15-mer peptides

NIH peptide name	Sequence	NIH peptide name	Sequence
7872	MGARASVLSGGELDR	7934	WMTNNPPIPVGEIYK
7873	ASVLSGGELDRWEKI	7935	NPPIPVGEIYKRWII
7874	SGGELDRWEKIRLRP	7936	PVGEIYKRWIILGLN
7875	LDRWEKIRLRPGGKK	7937	IYKRWIILGLNKIVR
7876	EKIRLRPGGKKKYKL	7938	WIILGLNKIVRMYSP
7877	LRPGGKKKYKLKHIV	7939	GLNKIVRMYSPTSIL
7878	GKKKYKLKHIVWASR	7940	IVRMYSPTSILDIRQ
7879	YKLKHIVWASRELER	7941	YSPTSILDIRQGPKE
7880	HIVWASRELERFAVN	7942	SILDIRQGPKEPFRD
7881	ASRELERFAVNPGLL	7943	IRQGPKEPFRDYVDR
7882	LERFAVNPGLLETSE	7944	PKEPFRDYVDRFYKT
7883	AVNPGLLETSEGCRQ	7945	FRDYVDRFYKTLRAE
7884	GLLETSEGCRQILGQ	7946	VDRFYKTLRAEQASQ
7885	TSEGCRQILGQLQPS	7947	YKTLRAEQASQEVKN
7886	CRQILGQLQPSLQTG	7948	RAEQASQEVKNWMTE
7887	LGQLQPSLQTGSEEL	7949	ASQEVKNWMTETLLV
7888	QPSLQTGSEELRSLY	7950	VKNWMTETLLVQNAN
7889	QTGSEELRSLYNTVA	7951	MTETLLVQNANPDCK
7890	EELRSLYNTVATLYC	7952	LLVQNANPDCKTILK
7891	SLYNTVATLYCVHQR	7953	NANPDCKTILKALGP
7892	TVATLYCVHQRIEVK	7954	DCKTILKALGPAATL
7893	LYCVHQRIEVKDTKE	7955	ILKALGPAATLEEMM
7894	HQRIEVKDTKEALEK	7956	LGPAATLEEMMTACQ
7895	EVKDTKEALEKIEEE	7957	ATLEEMMTACQGVGG
7896	TKEALEKIEEEQNKS	7958	EMMTACQGVGGPGHK
7897	LEKIEEEQNKSKKKA	7959	ACQGVGGPGHKARVL
7898	EEEQNKSKKKAQQAA	7960	VGGPGHKARVLAEAM
7899	NKSKKKAQQAAADTG	7961	GHKARVLAEAMSQVT
7900	KKAQQAAADTGNSSQ	7962	RVLAEAMSQVTNSAT
7901	QAAADTGNSSQVSQN	7963	EAMSQVTNSATIMMQ
7902	DTGNSSQVSQNYPIV	7964	QVTNSATIMMQRGNF
7903	SSQVSQNYPIVQNLQ	7965	SATIMMQRGNFRNQR
7904	SQNYPIVQNLQGQMV	7966	MMQRGNFRNQRKTVK
7905	PIVQNLQGQMVHQAI	7967	GNFRNQRKTVKCFNC
7906	NLQGQMVHQAISPRT	7968	NQRKTVKCFNCGKEG
7907	QMVHQAISPRTLNAW	7969	TVKCFNCGKEGHIAK
7908	QAISPRTLNAWVKVV	7970	FNCGKEGHIAKNCRA
7909	PRTLNAWVKVVEEKA	7971	KEGHIAKNCRAPRKK

NIH peptide name	Sequence	NIH peptide name	Sequence
7910	NAWVKVVEEKAFSPE	7972	IAKNCRAPRKKGCWK
7911	KVVEEKAFSPEVIPM	7973	CRAPRKKGCWKCGKE
7912	EKAFSPEVIPMFSAL	7974	RKKGCWKCGKEGHQM
7913	SPEVIPMFSALSEGA	7975	CWKCGKEGHQMKDCT
7914	IPMFSALSEGATPQD	7976	GKEGHQMKDCTERQA
7915	SALSEGATPQDLNTM	7977	HQMKDCTERQANFLG
7916	EGATPQDLNTMLNTV	7978	DCTERQANFLGKIWP
7917	PQDLNTMLNTVGGHQ	7979	RQANFLGKIWPSHKG
7918	NTMLNTVGGHQAAMQ	7980	FLGKIWPSHKGRPGN
7919	NTVGGHQAAMQMLKE	7981	IWPSHKGRPGNFLQS
7920	GHQAAMQMLKETINE	7982	HKGRPGNFLQSRPEP
7921	AMQMLKETINEEAAE	7983	PGNFLQSRPEPTAPP
7922	LKETINEEAAEWDRL	7984	LQSRPEPTAPPEESF
7923	INEEAAEWDRLHPVH	7985	PEPTAPPEESFRFGE
7924	AAEWDRLHPVHAGPI	7986	APPEESFRFGEETTT
7925	DRLHPVHAGPIAPGQ	7987	ESFRFGEETTTPSQK
7926	PVHAGPIAPGQMREP	7988	FGEETTTPSQKQEPI
7927	GPIAPGQMREPRGSD	7989	TTTPSQKQEPIDKEL
7928	PGQMREPRGSDIAGT	7990	SQKQEPIDKELYPLA
7929	REPRGSDIAGTTSTL	7991	EPIDKELYPLASLRS
7930	GSDIAGTTSTLQEQI	7992	KELYPLASLRSLFGN
7931	AGTTSTLQEQIGWMT	7993	PLASLRSLFGNDPSS
7932	STLQEQIGWMTNNPP	7994	LRSLFGNDPSSQ
7933	EQIGWMTNNPPIPVG		

CMV peptides

Peptide name	Sequence	Protein	HLA restriction
FEC 20	NLVPMVATV	CMV pp65	A0201
FEC 21	TPRVTGGGAM	CMV	B0702
FEC 22	SDEEEAIVAYTL	CMV	B18
FEC 23	IPSINVHHY	CMV pp65	B35

FEC (Influenza, EBV and CMV) peptides

Peptide name	Sequence	Pathogen	Protein	HLA restriction
FEC 1	GILGFVFTL	Flu	M	A2
FEC 2	FMYSDFHFI	Flu		A2
FEC 3	SIIPSGPLK	Flu		A3, A11, A*6805
FEC 4	RVLSFIKGTK	Flu	NP	A3
FEC 5	KTGGPIYKR	Flu	NP	A68
FEC 6	LPFDKTTVM	Flu	NP	B7
FEC 7	ELRSRYWAI	Flu	NP	B8
FEC 8	SRYWAIRTE	Flu	NP	B27
FEC 9	ASCMGLIY	Flu	M	B27
FEC 10	CLGGLLTMV	EBV	LMP2A	A2
FEC 11	GLCTLVAML	EBV	BMLFE	A2
FEC 12	AVFDRKSDAK	EBV	EBNA4NP	A11
FEC 14	DYCNVLNKEF	EBV	RTA	A24
FEC 15	RAKFKQLL	EBV	B2LF-1	B8
FEC 16	FLRGRAYGL	EBV	EBNA3A	B8
FEC 17	QAKWRLQTL	EBV	EBNA3	B8
FEC 18	RRIYDLIEL	EBV	EBNA3C	B27
FEC 19	YPLHEQHGL	EBV	EBNA3A	B35
FEC 20	NLVPMVATV	CMV	pp65	A*0201
FEC 21	TPRVTGGGAM	CMV		A*0702
FEC 22	SDEEEAIVAYTL	CMV		B18
FEC 23	IPSINVHHY	CMV	pp65	B35

HCV overlapping 16-mer - 19-mer peptides

Genotype 1b pool A (core)

Peptide	Sequence	Peptide	Sequence
1	MSTNPKPQRKTKRNTNRR	15	AGWLLSPRGSRPSWGPTD
2	PQRKTKRNTNRRPQDVKF	16	RGSRPSWGPTDPRRRSR
3	NTNRRPQDVKFPGGGQIV	17	WGPTDPRRRSRNLGKVI
4	DVKFPGGGQIVGGVYLL	18	RRRSRNLGKVIDTLTCGF
5	GGQIVGGVYLLPRRGPRL	19	GKVIDTLTCGFADLMGYI
6	VYLLPRRGPRLGVRATRK	20	TCGFADLMGYIPLVGAPL
7	GPRLGVRATRKASER	21	MGYIPLVGAPLGGAARAL
8	GVRATRKASERSQPRGRR	22	GAPLGGAARALAHGVRVL
9	ASERSQPRGRRQPIPKAR	23	ARALAHGVRVLEDGVNYA
10	RGRRQPIPKARRPEGRAW	24	VRVLEDGVNYATGNL
11	PKARRPEGRAWAQPGYPW	25	EDGVNYATGNLPGCSFSI
12	GRAWAQPGYPWPLYGNEGL	26	TGNLPGCSFSIFLLALL
13	YPWPLYGNEGLGWAGWLL	27	CSFSIFLLALLSCLTIPA
14	NEGLGWAGWLLSPRGSR	28	LALLSCLTIPASA

Genotype 1b pool B (E1)

Peptide	Sequence	Peptide	Sequence
1	YEVRNVSGIYHVTNDCSN	15	CGSIFLVSQLFTFSPRRH
2	GIYHVTNDCSNSSIVYEA	16	SQLFTFSPRRHETVQDCN
3	DCSNSSIVYEAADVIMH	17	PRRHETVQDCNCSIYPGH
4	IVYEAADVIMHTPGCV	18	VQDCNCSIYPGHVSGHRM
5	ADVIMHTPGCVPCVQEGN	19	IYPGHVSGHRMAWDMMM
6	PGCVPCVQEGNSSRCWVA	20	SGHRMAWDMMMNWSPTTA
7	VQEGNSSRCWVALTPTLA	21	DMMMNWSPTTALVVSQLL
8	RCWVALTPTLAARNASV	22	PTTALVVSQLLRIPQAVV
9	TPTLAARNASVPTTTIRR	23	SQLLRIPQAVVDMVAGAH
10	NASVPTTTIRRHVDLLV	24	PQAVVDMVAGAHWGVLA
11	TTIRRHVDLLVGTAAF	25	MVAGAHWGVLAGLAYYSM
12	HVDLLVGTAAFCSAMYV	26	GVLAGLAYYSMVGNWAKV
13	GTAAFCSAMYVGDLCGSI	27	YYSMVGNWAKVLIVALLF
14	AMYVGDLCGSIFLVSQLF	28	WAKVLIVALLFAGVDG

Genotype 1b pool M (NS5B)

Peptide	Sequence	Peptide	Sequence
46	SCGNTLTCYLKATAACRA	69	EKALDCQIYGACYSIEPL
47	CYLKATAACRAAKLQDCTM	70	IYGACYSIEPLDLPQII
48	CRAAKLQDCTMLVNGDDL	71	SIEPLDLPQIIERLHGL
49	DCTMLVNGDDLVVICESA	72	LPQIIERLHGLSAFTLH
50	GDDLVVICESAGTQEDAA	73	RLHGLSAFTLHSYSPGEI
51	CESAGTQEDAAALRAF	74	FTLHSYSPGEINRVASCL
52	TQEDAAALRAFTEAMTRY	75	PGEINRVASCLRKLGV
53	LRAFTEAMTRYSAPPGDP	76	RVASCLRKLGVPPLRTWR
54	MTRYSAPPGDPPQPEYDL	77	KLGVPPLRTWRHRARSVR
55	PGDPPQPEYDLELITSCS	78	RTWRHRARSVRAKLL
56	EYDLELITSCSSNVSVAH	79	HRARSVRAKLLSQGGRAA
57	TSCSSNVSVAHDASGKRV	80	AKLLSQGGRAATCGRYLF
58	SVAHDASGKRVYYLTR	81	GRAATCGRYLFNWAVRTK
59	ASGKRVYYLTRDPTTPLA	82	RYLFNWAVRTKLKLTPI
60	YLTRDPTTPLARAAWETA	83	AVRTKLKLTPIPAASQL
61	TPLARAAWETARHTPI	84	KLTPIPAASQLDLSGWFV
62	AAWETARHTPINSWLGNI	85	ASQLDLSGWFVAGYSGGDI
63	HTPINSWLGNIIMYAPTL	86	WFVAGYSGGDIYHSLSRA
64	LGNIIMYAPTLWARMILM	87	GGDIYHSLSRARPRWFPL
65	APTLWARMILMTHFFSIL	88	LSRARPRWFPLCLLLLSV
66	MILMTHFFSILLAQEQL	89	WFPLCLLLLSVGVGIYLL
67	FFSILLAQEQLEKAL	90	LLSVGVGIYLLPNR
68	LLAQEQLEKALDCQIYGA		

Genotype 3a pool A (core)

Peptide	Sequence	Peptide	Sequence
1	MSTLPKPQRKTKRNTIRR	16	LSPRGSRPNWAPNDPRRR
2	PQRKTKRNTIRRPQDVKF	17	PNWAPNDPRRRSRNLGKV
3	NTIRRPQDVKFPGGGVIY	18	PRRRSRNLGKVIDTL
4	DVKFPGGGVIYVGVYVL	19	SRNLGKVIDTLTCGFADL
5	GGVIYVGVYVLPRRGPRL	20	IDTLTCGFADLMGYIPLV
6	VYVLPRRGPRLGVRATRK	21	FADLMGYIPLVGAPLGGA
7	GPRLGVRATRKTSER	22	IPLVGAPLGGAARALAH
8	GVRATRKTSERSQPRGRR	23	PLGGAARALAHGVRAL
9	TSERSQPRGRRKPIPKAR	24	ARALAHGVRALEDGINFA
10	RGRRKPIPKARRSEGRSW	25	VRALEDGINFATGNL
11	PKARRSEGRSWAQPGYPW	26	EDGINFATGNLPGCSFSI
12	GRSWAQPGYPWPLYGNEG	27	TGNLPGCSFSIFLLALF
13	GYPWPLYGNEGCGWAGWL	28	CSFSIFLLALFSCLIHPA
14	GNEGCGWAGWLLSPRGSR	29	LALFSCLIHPAAS
15	AGWLLSPRGSRPNWA		

Genotype 3a pool G (NS3)

Peptide	Sequence	Peptide	Sequence
50	LTVLATATPPGSITVPH	74	GTYRYVTPGERPSGMF
51	ATPPGSITVPHSNIEEVA	75	VTPGERPSGMFDSVVL
52	TVPHSNIEEVALGSEGEI	76	RPSGMFDSVVLCECYDA
53	EEVALGSEGEIPFYGKAI	77	DSVVLCECYDAGCSWYDL
54	EGEIPFYGKAIPIACIK	78	CYDAGCSWYDLQPAETTV
55	YGKAIPIACIKGGRHLIF	79	WYDLQPAETTVRLRAYL
56	ACIKGGRHLIFCHSKKK	80	AETTVRLRAYLSTPGLPV
57	RHLIFCHSKKKCDKMASK	81	RAYLSTPGLPVCQDHLDL
58	SKKKCDKMASKLRGMGL	82	GLPVCQDHLDLWESVF
59	KMASKLRGMGLNAVAYYR	83	CQDHLDLWESVFTGLTHI
60	GMGLNAVAYYRGLDVSVI	84	WESVFTGLTHIDAHFL
61	AYYRGLDVSVIPTTGDVV	85	TGLTHIDAHFLSQTKQA
62	VSVIPTTGDVVVCATDAL	86	DAHFLSQTKQAGLNFSYL
63	GDVVVCATDALMTGF	87	TKQAGLNFSYLTAYQATV
64	VCATDALMTGFTGDFDSV	88	FSYLTAYQATVCARAQA
65	MTGFTGDFDSVIDCNVAV	89	YQATVCARAQAPPPSW
66	FDSVIDCNVAVEQYVDF	90	CARAQAPPPSWDETWKCL
67	CNVAVEQYVDFSLDPTF	91	PPSWDETWKCLVRLKPTL
68	EQYVDFSLDPTFSIETCTA	92	WKCLVRLKPTLHGPTPLL
69	DPTFSIETCTAPQDAVSR	93	KPTLHGPTPLLYRLGPV
70	TCTAPQDAVSRSQRRGR	94	PTPLLYRLGPVQNEICL
71	DAVSRSQRRGRTGRGRL	95	RLGPVQNEICLTHPITKY
72	SQRRGRTGRGRLGTYRYV	96	EICLTHPITKYVMACMSA
73	GRGRLGTYRYVTPGER	97	ITKYVMACMSADLEVTT

Genotype 3a pool H (NS4A and NS4B)

Peptide	Sequence	Peptide	Sequence
NS4A 1	STWVLLGGVLAAVAAYCL	NS4B 17	THLAGPQASSAFVVSGLA
NS4A 2	GVLAAVAAYCLSVGCVVI	NS4B 18	ASSAFVVSGLAGAAIGGI
NS4A 3	AYCLSVGCVVIVGHIEL	NS4B 19	SGLAGAAIGGIGLGRVLL
NS4A 4	GCVVIVGHIELGGKPALV	NS4B 20	IGGIGLGRVLLDILAGY
NS4A 5	HIELGGKPALVPDKEVLY	NS4B 21	GRVLLDILAGYGAGVSGA
NS4A 6	PALVPDKEVLYQQYDEM	NS4B 22	LAGYGAGVSGALVAFKIM
NS4A 7	KEVLYQQYDEMEEC	NS4B 23	VSGALVAFKIMGGEPPTT
NS4B 1	SQARPYIEQAQVIAHQFK	NS4B 24	FKIMGGEPPTTEDMVNLL
NS4B 2	EQAQVIAHQFKEKVLGLL	NS4B 25	PPTTEDMVNLLPAIL
NS4B 3	HQFKEKVLGLLQRATQQQA	NS4B 26	EDMVNLLPAILSPGALVV
NS4B 4	GLLQRATQQQAVIEPIVV	NS4B 27	PAILSPGALVVGVICAAI
NS4B 5	TQQQAVIEPIVVSNWQKL	NS4B 28	ALVVGVICAAILRRHV
NS4B 6	EPIVVSNWQKLEVLWHKH	NS4B 29	VICAAILRRHVGPGEGPV
NS4B 7	WQKLEVLWHKHMWNFV	NS4B 30	RRHVGPGEGPVQWMNRLI
NS4B 8	VLWHKHMWNFVSGIQYLA	NS4B 31	EGPVQWMNRLIAFASR
NS4B 9	WNFVSGIQYLAGLSTL	NS4B 32	WMNRLIAFASRGNHVSPA
NS4B 10	GIQYLAGLSTLPGNPAVA	NS4B 33	FASRGNHVSPAHYVPESDA
NS4B 11	LSTLPGNPAVASLMAFTA	NS4B 34	SPAHYVPESDAAARVTAL
NS4B 12	PAVASLMAFTASVTSPL	NS4B 35	ESDAAARVTALLSSLTV
NS4B 13	MAFTASVTSPLTTNQTMF	NS4B 36	RVTALLSSLTVTSLLRRL
NS4B 14	TSPLTTNQTMFFNILGGW	NS4B 37	SLTVTSLLRRLHQWI
NS4B 15	NQTMFFNILGGWVATHLA	NS4B 38	TSLLRRLHQWINEDY
NS4B 16	ILGGWVATHLAGPQASSA	NS4B 39	RRLHQWINEDYPSPC

HIVconsv peptides used in mouse experiments

Peptide name	Sequence	Protein	MHC restriction
H	RGPGRAFVTI	gp120	H-2D^{d} , H-2L^{d}
P	IFQSSMTKI	Pol	H-2K^{d}
G1	AMQMLKDTI	p24	H-2K^{d}