Mechanisms Underlying Immunomodulation of HIV-1-Specific T-cell Responses

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Declaration of Originality

I, Anna Herasimtschuk, declare that this thesis is my own work, and that I have appropriately acknowledged any outside contributions to the data presented here. The information derived from the published and unpublished work of others has been fully acknowledged in the text and in the list of references.

Abstract

Highly active antiretroviral therapy (HAART) improves morbidity and mortality in HIV-1-infected patients yet reversal of immune dysfunction remains incomplete. The study objective was to determine the impact of immunomodulatory agents on T-cell function and phenotype in treated, chronic HIV-1 infection. Recombinant human growth hormone (rhGH) was administered daily to patients at a pharmacological dose for 12 weeks. Proliferative CD4 and IFN-γ-producing CD8 HIV-1-specific T-cell responses were induced, however these responses declined with less frequent dosing. In a further study, patients that received a lower, physiological dose of rhGH for 40 weeks demonstrated increased IFN-γ ELISpot responses to pools of both MHC Class I and MHC Class II restricted HIV-1 Gag peptides at week 40. T-cell phenotypes showed a trend toward a reduction in the expression of activation markers. A randomised, open-label, phase I clinical trial investigated the effects of therapeutic immunisation, interleukin (IL)-2, granulocyte macrophage colony stimulating factor (GM-CSF) and rhGH in HIV-1^{+} subjects on HAART. Immunisation plus IL-2, GM-CSF and rhGH administration resulted in increased IFN-γ, IL-2 and perforin production in response to peptide pools of Gag, elevated CD4 T-cell counts and improved CD4/CD8 T-cell ratios at week 48. Subjects that received cytokine and hormone therapy (with or without immunisation) showed reduced immune activation and senescence, and for all patients, exhaustion markers were downregulated by week 48. Distinct Gag- and Nef- specific responses were not found to correlate with particular immunophenotypes. Further analysis of T-cell phenotypes from healthy controls showed immune defects to persist in HIV-1 infection despite HAART, and notwithstanding that, duration of HAART positively correlated with CD4 T-cell count. Together, the data presented here demonstrate the beneficial effects of immunotherapy in treated chronic HIV-1 infection and illustrate the potential to reverse T-cell dysfunction, furthermore highlighting the necessity for the induction and maintenance of HIV-1-specific immune responses.

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List of Abbreviations

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APC	Allophycocyanin
APOBEC3G	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
ART	Antiretroviral therapy
AZT	Zidovudine or azidothymidine
Bcl-2	B-cell lymphoma 2
BLIMP-1	B-lymphocyte-induced maturation protein 1
BPV	Bovine papilloma virus
CAD	Caspase-activated deoxyribonuclease
CAF	$CD8^+$ T-cell antiviral factor
CCL5	Chemokine (C-C motif) ligand 5
CCR5	CC-chemokine receptor 5
CD	Cluster of differentiation
CFSE	Carboxyfluoroscein succinimidyl ester
CM	Central memory
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DRV	Darunavir
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra-acetic acid
EFV	Efavirenz
ELISpot	Enzyme-Linked Immunosorbent Spot
EM	Effector memory
FACS	Fluorescence activated cell scanner
Fas-L	Fas-ligand
FCS	Foetal calf serum
FEC	Flu, EBV, and CMV peptides
FITC	Fluorescein isothiocyanante
FoxP3	Forkhead box P3
FTC	Emtricitabine
GALT	Gut-associated lymphoid tissue
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone-releasing hormone
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HALS	HIV-1-associated lipodystrophy syndrome
HC	Healthy controls or seronegative controls
HCV	Hepatitis C virus
HESN	Highly exposed seronegative
HIV-1	Human immunodeficiency virus-1
HIC	HIV-1 controller
HLA	Human leukocyte antigen
IBT	Immune-based therapy
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
ISP	Intermediate single positive
JAK	Janus kinase

KIR	Killer-cell immunoglobulin-like receptor
Lag-3	Lymphocyte-activation gene 3
LAK	Lymphokine-activated killer
LFA	Lymphocyte function associated antigen
LOP	Lopinavir
LPS	Lipopolysaccharide
LTNP	Long-term non-progressor
LTR	Long-terminal repeat
MAPK	Mitogen-activated protein kinases
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NK	Natural killer
NNRTI	Non-nucleoside RTI
NRTI	Nucleoside RTI
NtRTI	Nucleotide RTI
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cells
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death molecule 1
PD-L1	Programmed death molecule 1-ligand
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PI	Protease inhibitor
PrEP	Pre-exposure prophylaxis
PTK-7	Protein tyrosine kinase 7
PVDF	Polyvinylidene difluoride
pVL	Plasma viral load
RANTES	Regulated upon activation, normal T-cell expressed and secreted
Ras	Rat sarcoma
rhGH	Recombinant human growth hormone
rhIGF-1	Recombinant human insulin-like growth factor 1
RM	Rhesus macaque
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma t
rpm	Revolutions per minute
RSV	Rous Sarcoma Virus
RT	Reverse transcriptase
RTI	Reverse transcriptase inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
RTV	Ritonavir
rVV	Recombinant vaccinia virus
SIV	Simian immunodeficiency virus
SP	Single positive
SQV	Saquinavir
STAT	Signal transducers and activators of transcription
T-bet	T-box expressed in T cells
T_{CM}	T central memory
TCM	Tissue culture medium
TCR	T-cell receptor
T_{EM}	T effector memory
T_{EMRA}	$CD45RA^{+}$ effector memory cell
T_{FH}	T-follicular helper
TFV	Tenofovir
TGF	Transforming growth factor
T_H	T-helper lymphocyte
Tim-3	T-cell immunoglobulin and mucin domain-containing molecule 3
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TREC	T-cell receptor excision circle
Treg	T-regulatory
TRIM5α	Tripartite motif-containing protein 5 alpha
VAT	Visceral adipose tissue
γc	Gamma chain
γδ	Gamma delta
3TC	Lamivudine

Publications Arising From This Thesis

Manuscripts

Herasimtschuk AA, Westrop SJ, Moyle GJ, Downey JS, Imami N. Effects of recombinant human growth hormone on HIV-1-specific T-cell responses, thymic output and proviral DNA in patients on HAART: 48-week follow-up. J Immune Based Ther Vaccines 6:7 (2008).

Herasimtschuk A, Hansen BR, Langkilde A, Moyle GJ, Andersen O, Imami N. Low-dose recombinant human growth hormone for 40 weeks induces HIV-1-specific T-cell responses in patients on effective combination antiretroviral therapy (manuscript submitted).

Herasimtschuk A, Downey JS, Nelson M, Stanescu I, Adojaan M, Sikut R, Gotch F, Imami N. Interleukin-2, GM-CSF and recombinant human growth hormone induced immunotherapeutic restoration of immune function in treated HIV-1 infection (manuscript in preparation; research work completed).

Herasimtschuk A, Thaventhiran T, Nelson M, Moyle G, Gazzard B, Gotch F, Imami N. Correlations between distinct HIV-1 Gag- and Nef- specific responses and immunophenotype in treated chronic HIV-1 infection (manuscript in preparation; research work completed).

Abstracts

Herasimtschuk A, Nelson M, Moyle G, Bower M, Imami N. Impact of recombinant human growth hormone on T-cell phenotype and function *in vitro* and *in vivo* during treated HIV-1 infection. (Abstract). HIV Medicine 10 Supplement 1, 5-6 (2009).

Herasimtschuk A*, Downey J*, Nelson M, Higgs C, Sikut R, Stanescu I, Gazzard B, Gotch F, Imami N. Impact of IL-2/GM-CSF, rhGH and therapeutic immunisation in treated HIV-1 infection: a randomised, open label, phase I immunotherapeutic study. **Equal contribution.* HIV Med 12 Supplement 1, 27-27 (2011).

Herasimtschuk A, Thaventhiran T, Nelson M, Moyle G, Gazzard B, Gotch F, Imami N. Distinct HIV-1 Gag- and Nef- specific responses correlate with immunophenotype in treated chronic HIV-1 infection. HIV Medicine 13 Supplement 1, 32-32 (2012).

Presentations

Downey J, Herasimtschuk A, Gotch F, Imami N. Novel interventions in HIV-1 infection. MRC Showcase 'Vaccines and Immunotherapies'. Cambridge, September 2008.

Herasimtschuk A, Downey J, Moyle G, Gazzard B, Gotch F, Imami N. Effects of recombinant human growth hormone on T-cell phenotype and function *in vitro* and *in vivo* during treated HIV-1 infection. MRC Showcase 'Vaccines and Immunotherapies'. Cambridge, September 2008.

Herasimtschuk A, Nelson M, Moyle G, Bower M, Imami N. Impact of recombinant human growth hormone on T-cell phenotype and function *in vitro* and *in vivo* during treated HIV-1 infection. 15th Annual Conference of the British HIV Association (BHIVA). Liverpool, April 2009.

Herasimtschuk A, Hansen BR, Langkilde A, Moyle G, Andersen O, Imami N. Impact of low-dose recombinant human growth hormone on T-cell function during treated HIV-1 infection. HIV Symposium, Barts & the London School of Medicine, London, June 2010.

Downey J, Herasimtschuk A, Randell P, Stanescu I, Nelson M, Gotch F, Imami N. Therapeutic restoration of immune function in HIV-1 infection. HIV Symposium, Barts & the London School of Medicine, London, June 2010.

Gotch F, Downey J, Herasimtschuk A, Randell P, Nelson M, Sikut R, Stanescu I, Imami N. Can novel rational immunotherapy lead to long-term non-progressor status in chronically infected HIV^+ persons receiving antiretroviral therapy? AIDS Vaccine 2010 Conference, Atlanta, 28th September – 1st October 2010.

Herasimtschuk A, Hansen BR, Langkilde A, Moyle G, Andersen O, Imami N. Low-dose recombinant human growth hormone induces HIV-1-specific T-cell responses in patients on effective antiretroviral therapy. 18th Conference on Retroviruses and Opportunistic Infections (CROI 2011), Boston, February 27th – March 2nd 2011.

Herasimtschuk A, Downey J, Nelson M, Higgs C, Sikut R, Stanescu I, Gazzard B, Gotch F, Imami N. Impact of IL-2/GM-CSF, rhGH and therapeutic immunisation in treated HIV-1 infection: a randomised, open label, phase I immunotherapeutic study. 17th Annual Conference of the British HIV Association (BHIVA). Bournemouth, April 2011.

Herasimtschuk A, Thaventhiran T, Nelson M, Moyle G, Gazzard B, Gotch F, Imami N. Distinct HIV-1 Gag- and Nef- specific responses correlate with immunophenotype in treated chronic HIV-1 infection. 18th Annual Conference of the British HIV Association (BHIVA). Birmingham, April 2012.

Chapter 1 Introduction

1.1 T cells

1.1.1 Early T-cell development

T-cell-mediated immunity is an essential component of the immune response directed against viruses and other intracellular pathogens. T lymphocytes develop from haematopoietic progenitor cells, which migrate from the bone marrow to the thymus, where they differentiate into fully functional, self-tolerant T cells before moving to the periphery [Blom & Spits, 2006; Ciofani & Zuniga-Pflucker, 2007], as shown in Figure 1.1 and Figure 1.2.

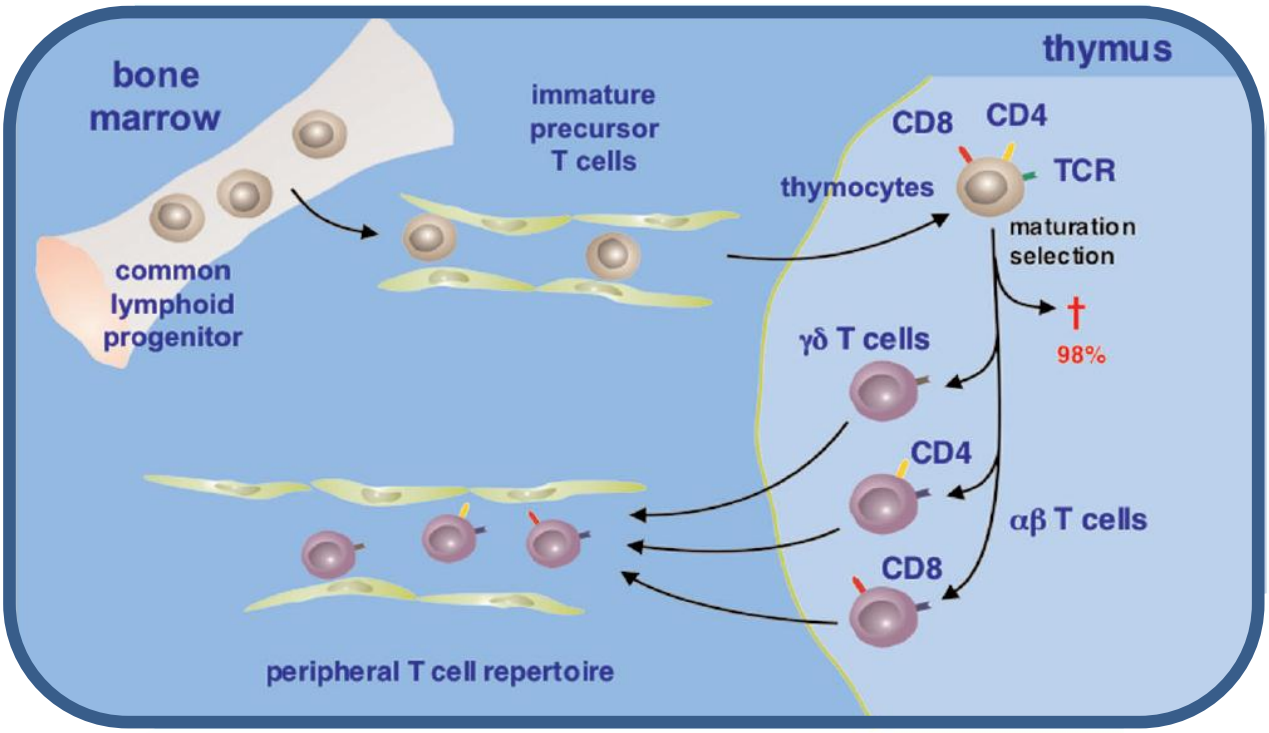


Figure 1.1 Schematic representation of T-cell development.
Originating from a common lymphoid progenitor in the bone marrow, T cells migrate as immature precursors into the thymus via the blood stream. The thymocytes then undergo a series of maturation steps and changes in cell surface receptors take place. These include the CD3 signalling complex (not shown), CD4 and CD8 co-receptors and rearrangement of TCR genes. During maturation, thymocytes undergo positive selection (for their TCR's compatibility with self-MHC molecules) and negative selection against TCRs reactive to autoantigenic peptides; during this stage more than 98% die by apoptosis (†). The majority of peripheral blood T cells in humans are αβ T cells and a small group of peripheral T cells are γδ T cells. The expression of CD4 or CD8 is lost on the αβ T cells that survive thymic selection, the level of the TCR increases and these cells leave the thymus and go to make up the peripheral T-cell repertoire. Taken from Skapenko *et al*., 2005 [Skapenko *et al*., 2005].

The thymus generates two lineages of T cells, αβ and γδ, characterised by the expression of distinct αβ– or γδ– T-cell receptor (TCR) complexes [Ciofani & Zuniga-Pflucker, 2007]. Early T-cell precursors are termed double negative (DN) as they do not express the co-receptors CD4 or CD8, nor do they express the CD3/TCR complex [Sebzda *et al*., 1999; Abbey & O'Neill, 2008] and it is at this stage that commitment to the αβ or γδ T-cell lineage occurs [Petrie *et al*., 1992; Ciofani *et al*., 2006]. The DN thymocytes give rise to a $CD4^+CD8^+$ double positive population from which mature $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) cells differentiate [Ciofani & Zuniga-Pflucker, 2007; D'Acquisto & Crompton, 2011]. In mice, cell surface expression of CD25 and CD44 can be used to subdivide the immature, TCR-lacking, and DN population [Zuniga-Pflucker & Lenardo, 1996; D'Acquisto & Crompton, 2011]. $CD44^+CD25^-$ (DN1) cells differentiate into $CD44^+CD25^+$ (DN2) cells and then $CD44^-CD25^+$ and this population gives rise to $CD44^-CD25^-$ (DN4). The DN4 subset proliferates rapidly to generate the DP population and this is typically via an immature intermediate single positive (ISP) CD8 cell, as detailed in Figure 1.2 [D'Acquisto & Crompton, 2011]. Mouse models have provided invaluable evidence about the pathways and phenotypes involved in thymic development, but it is important to consider that differences exist between humans and mice, in terms of the ontogeny and expression of markers on the cell surface during various transitional phases of development, as well as differences with regard to thymic involution [Spits, 2002].

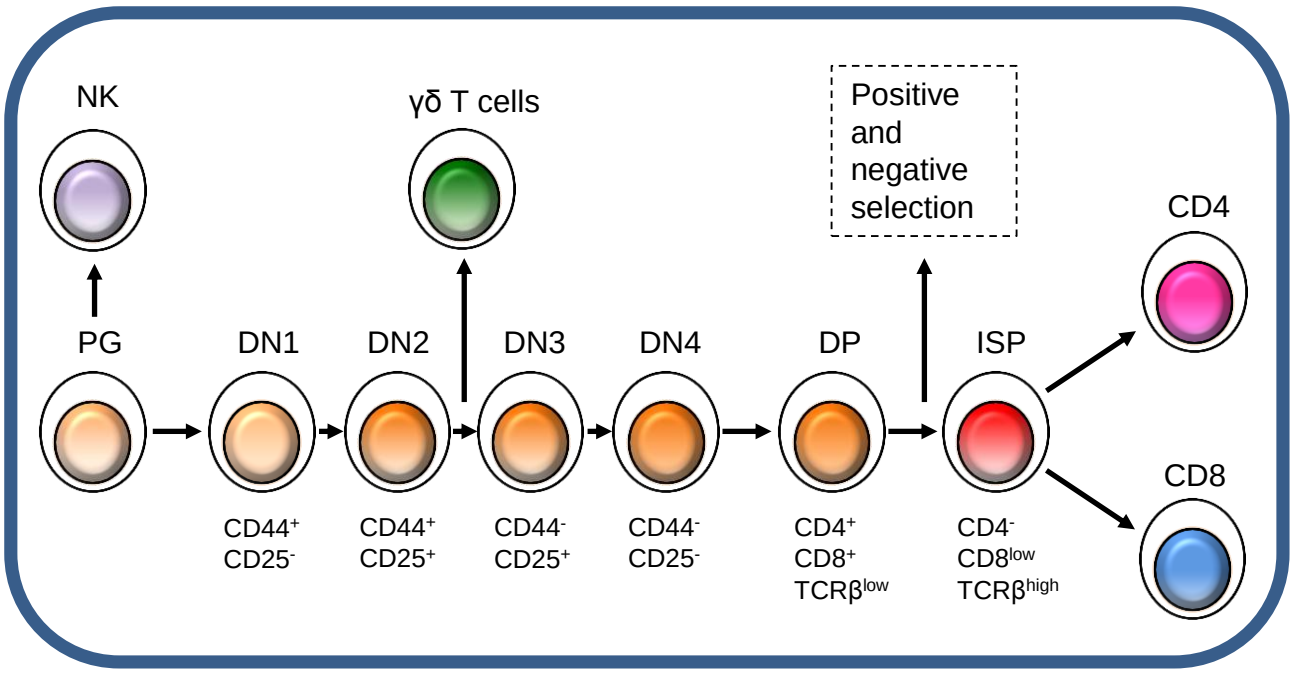


Figure 1.2 Schematic representation of T-cell development in the thymus: stages of DN thymocyte differentiation. From the bone marrow, progenitor (PG) cells move to the thymus where they follow a natural killer cell (NK) or T-cell lineage. If they follow the latter, progressive differentiation occurs from double negative (DN) to double positive (DP) and finally to single positive CD4 or CD8 thymocytes. The expression of CD44 and CD25 distinguishes the immature DN thymocytes into DN1 to DN4 stages. At the DN2 / DN3 stage, the γδ lineage diverges from the αβ lineage. The immature CD8 single positive (ISP) is an intermediate through which progression from the DP to SP stage can occur. Adapted from D'Acquisto & Crompton, 2011 [D'Acquisto & Crompton, 2011].

A diverse repertoire of αβ TCR is generated in the thymus as T cells undergo rearrangement of the TCR α and β gene segments [Sebzda *et al*., 1999; Abbey & O'Neill, 2008]. The expression of the CD4 and CD8 co-receptors on the DP cells arise from signalling through the pre-TCR and these are found in the thymic cortex [Aspinall, 2006]. During the DP stage, rearrangement of the TCR α chain gene segments leads to expression of the mature αβ TCR and these DP T cells are selected through positive and negative selection as they migrate from the cortex to the medulla of the thymus [Aspinall, 2006; Huseby *et al*., 2008; Bosco *et al*., 2009]. The strength and duration of the TCR signal broadly determines the fate of the DP cell with the strongest signals leading to negative selection and apoptosis (for example, when TCR recognises self antigens), intermediate signals resulting in positive selection and very weak signals (or lack of a TCR signal) leading to death by neglect of the DP cell [Starr *et al*., 2003; von Boehmer & Kisielow, 2006; Weinreich & Hogquist, 2008; D'Acquisto & Crompton, 2011]. It has been suggested that TCR signal duration and strength influence the positive selection of DP thymocytes into CD4 and CD8 T cells, with stronger TCR

signals tending to result in CD4 SP cells and weaker or more transient signals more likely to lead to a CD8 T-cell fate [Bommhardt *et al*., 1999]. T cells with TCR that respond to MHC Class I peptides become CD8 cytotoxic T lymphocytes (CTL) while those that respond to MHC Class II molecules become CD4 T-helper lymphocytes (T_H) [Sebzda *et al*., 1999; Stone *et al*., 2009]. Of the DP lymphocytes, approximately 98% die during selection in the thymus and approximately 2% bear the appropriate TCR and are exported from the thymus to the periphery [Skapenko *et al*., 2005].

1.1.2 CD8 T cells

CD8 T cells, when activated, differentiate into CTL, which are vital in the control of infection by viruses and other intracellular pathogens [Zhang *et al*., 2009]. They recognise foreign peptides presented on MHC Class I molecules [Prlic *et al*., 2007], and travel to the site of infection with the ability to destroy infected cells by lysis and by the secretion of antiviral factors [Williams & Bevan, 2007]. Naïve T cells circulate around the body after exiting the thymus until they encounter their specific antigen. Following this encounter, the CTL response to infection can be divided into several phases. At first, antigen-specific T cells clonally expand and as they do so, they acquire various effector functions allowing them to eliminate infected cells. Following neutralisation of the pathogen, the expanded antigen-specific T-cell pool contracts via apoptosis leaving 5-10% of the original expansion size as long-lived memory cells [Williams & Bevan, 2007]. These cells are maintained for many years and their response is rapid and effective if the same pathogen is re-encountered [Williams & Bevan, 2007; Kaech & Wherry, 2007; Wiesel *et al*., 2009]. The clonal expansion of T cells is mediated by many factors, including antigen availability, inflammatory stimuli, co-stimulatory molecules and secreted growth factors. It is thought that a relatively short exposure to antigen can lead CD8 T cells to divide and differentiate in addition to programming the development of a long-lived memory response [Kaech & Ahmed, 2001; van Stipdonk *et al*., 2003; Masopust *et al*., 2004]. CD8 T lymphocytes express a TCR that recognises a specific antigen peptide bound to an MHC class I molecule [Solheim, 1999; Krogsgaard & Davis, 2005] on the antigen-presenting cell. The TCR is a heterodimer consisting of an alpha and beta chain in the

majority of peripheral T cells, and a gamma and delta chain in 1-10% of circulating T cells [Beetz *et al*., 2008]. Engagement of the TCR with antigen and MHC results in activation of the T-cell [Stone *et al*., 2009; Smith-Garvin *et al*., 2009]. The TCR associates with other molecules, such as CD3, to form the TCR complex [Gil *et al*., 2005]. The TCR/MHC complex signal is enhanced by simultaneous binding of the MHC molecule to a specific co-receptor on the T-cell [Rudd *et al*., 2009]. On CD8 T cells, this is the CD8 glycoprotein, which is attracted to the non-variable region of the MHC class I molecule (their affinity keeps the CD8 T-cell and the target cell bound closely together during antigen-specific activation). On CD4 T_H, the co-receptor is CD4, which specifically binds to MHC class II molecules [Smith-Garvin *et al*., 2009].

A second signal is required, in addition to the TCR/MHC binding, on CD8 T cells in order for them to differentiate into effector cells [Smith-Garvin *et al*., 2009]. This signal is provided by a variety of co-stimulatory molecules present on dendritic cells (DC) and other antigen presenting cells (APC) which interact with those expressed on the CD8 T cells. The most well understood co-stimulatory pathway involves B7/CD28 interactions. DC express B7 which is up-regulated upon activation [Wang & Chen, 2004; Keir *et al*., 2008; Kaufmann & Walker, 2009]. B7 binds to its receptor, CD28 (expressed on T cells), promoting, in conjunction with the TCR signal, activation, expansion, survival and the production of IL-2 [Sharpe & Freeman, 2002; Fife & Bluestone, 2008]. Other important co-stimulatory molecules include 4-1BB (CD137) [Tan *et al*., 1999] and CD27 [Hendriks *et al*., 2000]. Signalling through one or more of these pathways is essential for CD8 T-cell expansion and differentiation [Bertram *et al*., 2004]. Which pathway is used and when varies depending on the pathogen as well as the TCR stimulation, and it is possible that the different pathways can elicit distinct functional responses [Williams & Bevan, 2007]. Further, it is thought that CD4 T-cell help is required to generate a memory CTL response [Wodarz & Jansen, 2003; Shedlock & Shen, 2003]. The role of direct CD4 T-lymphocyte help remains to be fully elucidated but could be required for the programming and differentiation of CD8 T-cell during a primary response [Shedlock & Shen, 2003], or for the maintenance of functionality after antigen has been

cleared [Sun *et al*., 2004]. It has been suggested that CD4 T cells interact with DC to activate CD8 T-lymphocyte responses [Zhang *et al*., 2009; Castellino & Germain., 2006]. Although the exact mechanism is not known, it is clear that without CD4 T-cell help an effective CTL memory response is not generated [Janssen *et al*., 2003]. The optimal development of CTL responses also requires signalling from IL-12 [Curtsinger *et al*., 1999] and type I interferons [Zhang *et al*., 2009; Marrack *et al*., 1999].

Preformed effector proteins within the CTL can be rapidly released upon recognition of the target cell, triggering cell-mediated cytotoxicity and consequently apoptosis [Russell & Ley, 2002]. An example of such an effector protein is perforin, found in modified lysosomes, which when released polymerises to form a pore in the membrane of the target cell [Pipkin & Lieberman, 2007]. Perforin damages the integrity of the cell membrane and upsets the osmotic and salt equilibria, as well as facilitating the entry of serine proteases called granzymes (other CTL effector proteins) into the target cell where they trigger apoptosis [Lieberman *et al*., 2002; Gupta *et al*., 2008; Chattopadhyay *et al*., 2009]. A perforin-independent pathway can also cause the death of infected target cells by the ligation of the Fas-ligand (Fas-L), expressed on CTL, with Fas, expressed on the target cell. Death domains within the cytoplasm of the target cell are brought together, activating caspases which remove caspase-activated deoxyribonuclease (CAD) from an inactive complex with the inhibitory protein I-CAD, allowing CAD to enter the nucleus of the target cell where it cleaves DNA [Russell & Ley, 2002; Gupta *et al*., 2008]. CTL can regulate the immune response by means other than direct cytotoxicity. They can produce antiviral cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), chemokines such as macrophage inflammatory proteins (MIP-1α and MIP-1β) and RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted; CCL5), and CD8 T-cell antiviral factors (CAF) [Jassoy *et al*., 1993; Yang & Walker, 1997; Geiben-Lynn, 2002]. Following elimination of the pathogen, most CTL generated in the initial 'burst' die through apoptosis, leaving memory cells [Prlic *et al*., 2007; D'Cruz *et al*., 2009].

1.1.3 Memory CD8 T cells

Mouse models have given insight into the dynamics of CTL responses [Zimmerman *et al*., 1996], however, longitudinal analysis in humans is more difficult. The phenotype and function of human peripheral blood subsets have been examined and several models have been proposed for the differentiation of naïve CD8 T cells. Three distinct populations of CD8 T cells were described in 1997; in addition to naïve cells, memory- and effector- type T cells with distinct functional and phenotypic properties were defined [Hamann *et al*., 1997]. The memory cells had a $CD45RA^-$ $CD45RO^+$ phenotype, and this subset was found to have the ability to produce IL-2, IFN-γ, TNF-α and IL-4, in addition to having a low cytolytic activity *ex vivo* [Hamann *et al*., 1997]. The terms central memory (CM) and effector memory (EM) have since been used to describe memory T-lymphocyte subsets [Sallusto et al., 2004; Romero *et al*., 2007]. According to this model, in response to an encounter with antigen, effector memory T lymphocytes (T_{EM}) migrate to inflamed peripheral tissue and perform immediate effector functions, whereas central memory T cells (T_{CM}) home to secondary lymphoid organs where they rapidly proliferate and differentiate into effector cells [Sallusto *et al*., 1999]. T_{CM} and T_{EM} can be further defined based on their phenotype and effector functions. T_{CM} are $CD45RO^+$ cells that constitutively express chemokine receptor-7 (CCR7) and CD62L [D'Cruz *et al*., 2009]. These molecules, also expressed by naïve T cells, are required for homing to T-cell compartments of secondary lymphoid organs. T_{CM} have a higher sensitivity to antigenic stimulation than naïve cells and following TCR ligation they produce mainly IL-2 but then proliferate and differentiate into cells that produce large amounts of IFN-γ or IL-4. T_{EM} do not express CCR7 and are heterogeneous in their expression of CD62L; they are characterised by their rapid effector function and CD8 T_{EM} contain large amounts of perforin. Both CD8 and CD4 T_{EM} produce IFN-γ, IL-4 and IL-5 rapidly after antigenic stimulation [D'Cruz *et al*., 2009]. A proposed vaccine strategy is one in which T_{EM} responses are prolonged at the viral entry site, with the hopes of halting viral replication in its initial stages [Hansen & Bouvier, 2009]. There has been some question as to the use of CCR7 to distinguish between T_{CM} and T_{EM}, and studies

have found no functional differences between $CCR7^+$ and $CCR7^-$ cells [Unsoeld *et al*., 2002], and it has been shown that CCR7 can be induced upon activation [Sallusto *et al*., 1999]; nevertheless, CCR7 remains a widely used marker in defining T-cell memory subsets.

The expression of CD27 and CD28 have also been used to define memory T-cell subsets [Hamann *et al*., 1997], and these studies have shown sequential down-regulation of, first, CD28 and then CD27 (on CD8 T cells) during differentiation from naïve to memory [Romero *et al*., 2007; Downey and Imami, 2010]. Thus, CD8 T-cell differentiation may be described in terms of naïve ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$), T_{EM} ($CCR7^-CD45RA^-$), and terminally differentiated ($T_{EMRA;}$ $CCR7^-CD45RA^+$) stages, or early ($CD27^+CD28^+$), intermediate ($CD27^+CD28^-$) and late ($CD27^-CD28^-$) differentiation states [Downey & Imami, 2010]. A transitional memory phenotype (T_{TM}) that is intermediary between T_{CM} and T_{EM} has been described in the literature, classified according to the markers used to define this transitional memory state for CD4 T cells (thus, $CCR7^-CD45RA^-CD27^+$) [Killian *et al*., 2011], and also categorised using a different panel ($CCR7^-CD45RO^+CD28^+$) [Freel *et al*., 2010].

Although the nomenclature may differ depending on the study and the surface markers used, Figure 1.3 summarises the expression of a number of markers that have been found to be associated with different CD8 T-lymphocyte differentiation/maturation states, and these include the expression of CD57, CD11a, and granzymes A and B [Appay & Rowland-Jones, 2004; Appay *et al*., 2008; Wood *et al*., 2009], in addition to those already described. It is important to note that the descriptions of the memory subsets in Figure 1.3 may not apply to cells actively responding to antigen, only those in a resting state. In addition, the response to different viruses will vary in terms of phenotype, frequency and function of responders [Appay *et al*., 2002]. The level of antigen has also been shown to influence the differentiation of CD8 CTL [Tussey *et al*., 2003; Papagno *et al*., 2004].

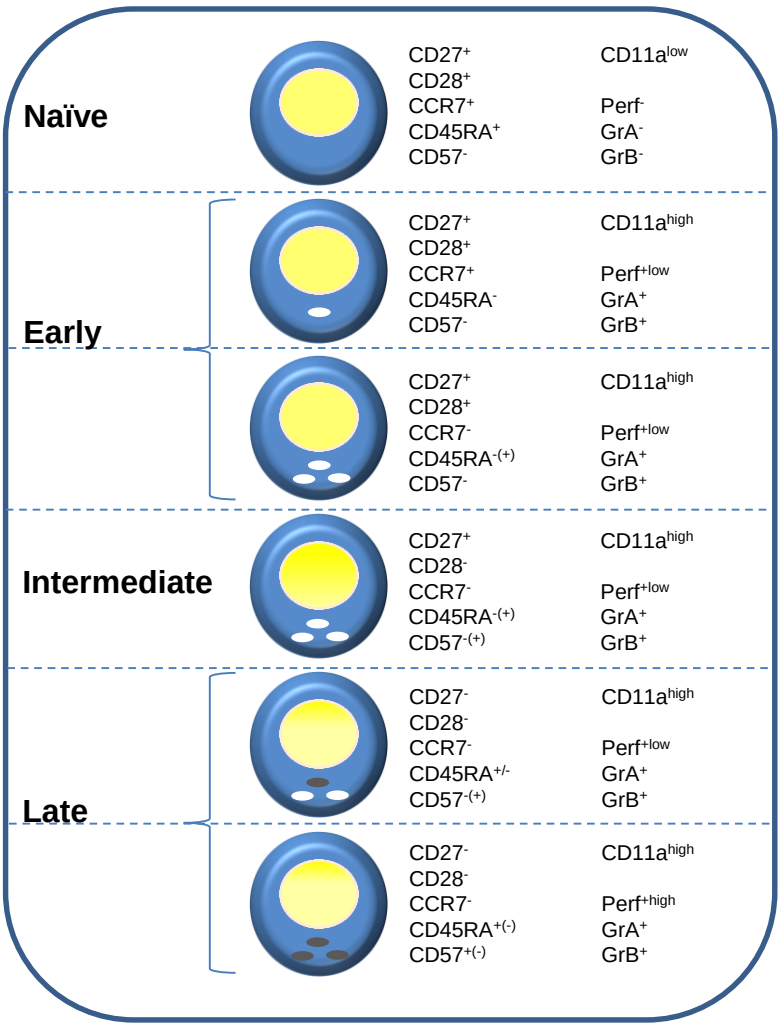


Figure 1.3 CD8 T-cell differentiation/maturation subsets.
The post-thymic development of six subsets of CD8 T cells are shown here, characterised by the expression of CD27, CD28, CCR7, CD45RA, CD57, CD11a, perforin, and granzymes A and B. Adapted from [Appay & Rowland-Jones, 2004; Appay *et al*., 2008].

1.1.4 CD4 T cells

CD4 T cells have the ability to orchestrate an immune response; they can mobilize and co-ordinate a variety of other cell types leading to the clearance of a pathogen [Zhu & Paul 2008]. They express the TCR/CD3 complex, like all T cells, but the TCR on CD4 T lymphocytes is specific for MHC class II [Munz, 2009]. Similarly to CD8 T cells, following maturation, the naïve CD4 T cells leave the thymus and circulate the body. Activation occurs when they recognise the antigen that they are specific for, in the context of presentation on MHC class II [Munz, 2006]. Class II molecules are found on the cell surface of professional APC such as DC, macrophages and B cells [Crotzer & Blum, 2009]. In addition to the TCR/Class II interactions, CD4 T lymphocytes require further

signals through the binding of CD28 with its ligand CD80 or CD86 [Dubey *et al*., 1995], and signalling by specific cytokines for activation [O'Garra, 1998]. The differentiation of the CD4 T-cell is determined by the strength of the signal [Obst *et al*., 2005; Lanzavecchia & Sallusto, 2002]. Once a CD4 T-lymphocyte has been activated, it can differentiate into different functional subsets such as T_H1 and T_H2 [Mosmann *et al*., 1986; Mosmann *et al*., 2005]; others include T_H17, T_H9, T-follicular helper (T_{FH}) and T-regulatory cells (Treg) [Luckheeram *et al*., 2012; Tan & Gery, 2012]. T_H1 cells secrete IL-2, IFN-γ, TNF-α and -β, while T_H2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13 [Agnello *et al*., 2003]. T_H1 cells are thought to be protective against intracellular infection and T_H2 responses are associated with extracellular immunity and act as more potent helpers for T-cell dependent antibody production [O'Garra *et al*., 1998]. More recently, the T_H17 subset was identified; these cells produce IL-17 and are highly pro-inflammatory [Zhu & Paul, 2008; Dong, 2006]. As previously described (in section 1.1.2), CD4 T-helper cells assist in the development of CTL memory responses. CD4 T lymphocytes can provide help to other cells of the immune system including APC and B cells via cell-bound receptors such as CD40 [Zhang *et al*., 2009; Wodarz & Jansen, 2001], and by the secretion of cytokines such as IFN- γ.

Despite similar post-thymic differentiation pathways between CD4 and CD8 T cells (namely, the loss of CD27, CD28 and CCR7 expression, the gain of CD57 and perforin expression, and reduced IL-2 production and proliferative capacity) [Appay & Rowland-Jones, 2002], and the ability to sub-divide CD4 T-cell subsets into early, intermediate and late differentiated states, it is the sequence of these events that differ between the two populations [Appay *et al*., 2008; Amyes *et al*., 2003]. CD4 T cells lose expression of CD27 before CD28 (whereas the opposite is true for CD8 T cells), thus the differentiation states of CD4 T cells using these markers are as follows: early ($CD27^+CD28^+$), intermediate ($CD27^-CD28^+$) and late ($CD27^-CD28^-$) [Appay *et al*., 2008; Downey & Imami, 2010]. More recently, however, a transitional memory (T_{TM}) T-cell subset has been described that has an intermediate phenotype between T_{CM} and T_{EM} and is characterised based on the expression of CCR7, CD45RA and CD27 (where T_{CM} are $CCR7^+CD45RA^-CD27^+$, T_{TM} are $CCR7^-CD45RA^-$

$CD27^+$, and T_{EM} are $CCR7^-CD45RA^-CD27^-$) [Riou *et al*., 2007; Chomont *et al*., 2009]. Only highly differentiated CD4 T cells begin to express perforin whereas for CD8 T cells, acquisition of lytic capacity and expression of perforin occurs early after the priming of naïve cells [Appay & Rowland-Jones, 2004]. The differentiation of CD4 T lymphocytes as compared to CD8 T lymphocytes is otherwise very similar with regard to markers such as CCR7, CD45RA and CD57 [Appay *et al*., 2008]. Figure 1.4 illustrates the differentiation pattern of CD4 T cells. Functional differences between CD4 and CD8 T cells seem to be focused in the early differentiation states and eventually highly differentiated cytotoxic CD4 T cells have a remarkably similar phenotype to late differentiated CD8 T cells. This similar differentiation status seems more likely to occur in instances of chronic activation, such as during HIV-1 infection [Appay & Rowland-Jones, 2002; Appay *et al*., 2008].

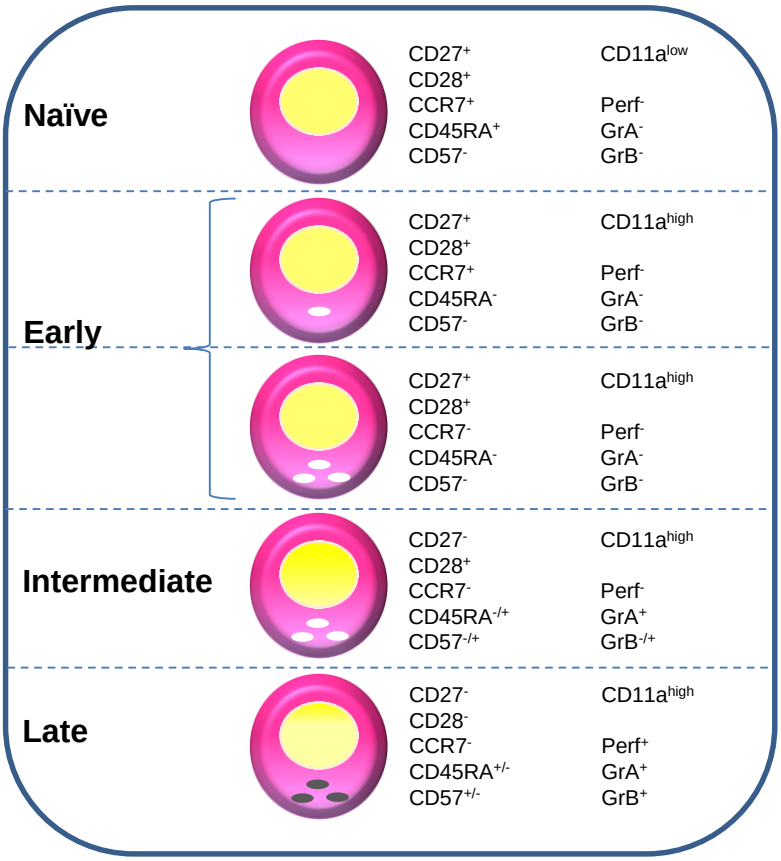


Figure 1.4 CD4 T-cell differentiation/maturation subsets.
The post-thymic development of five subsets of CD4 T cells are shown here, characterised by the expression of CD27, CD28, CCR7, CD45RA, CD57, CD11a, perforin, and granzymes A and B. Adapted from [Appay *et al*., 2008].

1.2 Human Immunodeficiency Virus

1.2.1 HIV-1 epidemiology

Human immunodeficiency virus (HIV)-1 is a retrovirus belonging to the *Retroviridia* family and *Lentivirinea* subfamily. In 1983, it was shown to be the agent which causes acquired immunodeficiency syndrome (AIDS) [Barre-Sinoussi *et al*., 1983]. At the end of 2009, it was estimated that 33.3 million people worldwide were living with HIV, with 2.6 million new infections and 1.8 million AIDS-related deaths that year [UNAIDS Global Report 2010; http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf]. Worldwide, about 7,000 people are infected with HIV each day [IAVI, International AIDS Vaccine Initiative; http://www.iavi.org/Pages/home.aspx]. Typically, life-saving effective antiretroviral therapy (ART) is required 7-10 years after becoming infected. However, at the end of 2009, only 36% (approximately 5.2 million) of the 15 million people in need in low- and middle- income countries were receiving ART [UNAIDS Global Report 2010], primarily due to its high cost, and the search for an effective vaccine continues [IAVI, International AIDS Vaccine Initiative; http://www.iavi.org/Pages/home.aspx]. In addition to the expense of current ART, there is also the associated high pill burden, toxicity and complex undesirable side effects [Gazzard *et al*., 2008]. An improvement in morbidity and mortality can be seen with ART, however it is not a cure and lifelong treatment is required [Siliciano *et al*., 2003]. Additionally, although ART may control viral replication, alternative measures are needed to address persistent viral reservoirs [Shen & Siliciano, 2008].

1.2.2 Infection with HIV-1

Infection with HIV-1 can occur via the mucosal route, via contaminated blood products or needles and through mother-to-child transmission. Infection with HIV-1 results in a brief acute phase (typically lasting around 14 days) and is associated with a high viral load, a decrease in peripheral blood CD4 T-cell counts [Pantaleo & Fauci, 1996], immune activation [Grossman *et al*., 2006], and

a massive depletion of CD4 T cells in the gastrointestinal tract [Brenchley & Douek, 2008]. A latent reservoir of infected CD4 T cells is established and an HIV-1-specific immune response is generated. This phase is followed by an asymptomatic phase, where the viral load drops and a viral set point is reached and is associated with a cellular immune response [Andrews & Koup, 1996]. On average, this chronic stage lasts for seven years during which time the peripheral CD4 T-cell count falls slowly, mucosal CD4 T-cell numbers remain low, and both immune activation and viraemia continue to increase [Grossman *et al.*, 2006]. When peripheral CD4 T-cell counts decline to less than 200 cells per µl of blood and the number of mucosal CD4 T cells becomes very small, opportunistic infections and tumours that characterise AIDS occur, leading to mortality in most individuals [Grossman *et al.*, 2006]; the stages of untreated HIV-1 disease progression are illustrated in Figure 1.5.

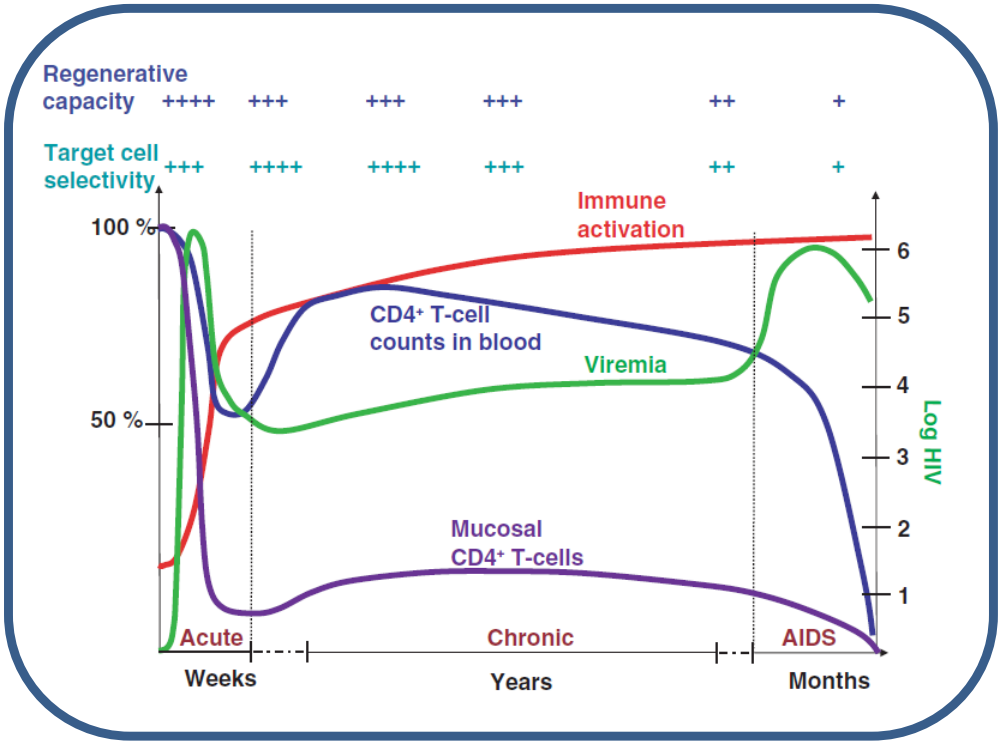


Figure 1.5 The course of HIV-1 disease progression, shown in terms of both quantitative and qualitative measures. Quantitative parameters illustrated include: alterations in blood and mucosal CD4 T-cell counts and levels of viraemia and qualitative measures include: levels of immune activation, 'regenerative capacity' and 'target-cell selectivity'. Taken from [Grossman *et al.*, 2006].

HIV-1 infects mainly T_H cells and macrophages, and to a lesser extent, microglial cells and DC [Dunfee *et al*., 2009]. CD4 is used as the primary receptor for entry into the cells and the co-receptor depends on the strain of the virus. R5 strains of HIV-1, the strain that is usually transmitted, use the CC-chemokine receptor 5 (CCR5) as their co-receptor and can enter macrophages, DC and T cells. X4 strains of HIV-1, which tend to emerge later in infection, use CXCR4 as a co-receptor and can only infect T cells [Doms & Trono, 2000; Kuritzkes, 2009]. Although the dynamics of the initial infection are not well understood [Goonetilleke *et al*., 2009], it is clear that HIV-1 establishes a life-long infection in the host, in long-lived memory T cells [Shen & Siliciano, 2008] causing immunodeficiency and AIDS.

1.2.3 Highly Active Antiretroviral Therapy

The introduction of Highly Active Antiretroviral Therapy (HAART) in 1996 resulted in a substantial decline in mortality and morbidity of AIDS-related symptoms in infected patients [Gulick *et al*., 1997; Hammer *et al*., 1997; Palella *et al*., 1998]. Treatment with HAART controls plasma viraemia to below the limit of detection of standard assays, and leads to an increase in CD4 T-cell counts [Perelson *et al*., 1997; Shen & Siliciano, 2008], however, only 50% of the normal numbers of T cells in the gut are recovered, even after three years of fully suppressive HAART [Talal *et al*., 2001; Effros *et al*., 2008]. HIV-1 viraemia rapidly rebounds if therapy is interrupted, suggesting the persistence of viral reservoirs, and the requirement for life-long therapy [Burton *et al*., 2005; Shen & Siliciano, 2008]. This viral reservoir is thought to reside in long-lived, latently-infected memory CD4 T cells; a model supported by the presence of replication-competent virus in peripheral blood lymphocytes and by the lack of evolution of viral cDNA [Sedaghat *et al*., 2007; Buzon *et al*., 2010]. The latently-infected population is thought to have a long half-life, such that it would take over 60 years of HAART to eliminate this population [Finzi *et al*., 1999; Edelstein *et al*., 2009]. The suggestion of low-level viral replication is also supported by the abnormal levels of immune activation and inflammation in HAART-treated HIV-1-infected individuals [Jiang *et al*., 2009]. In patients who initiate therapy before CD4 T-cell counts fall below 350 cells/μl blood, as is

currently recommended by international guidelines, AIDS-associated malignancies and infections are infrequent, and it is thought that future guidelines will recommend initiation of ART even earlier [Kitahata *et al*., 2009; Sterne *et al*., 2009]. In fact, the latest reports from the USA recommend treatment for all HIV-1-infected persons, particularly those with CD4 T-cell counts below 500 cells/µl blood, those at risk of transmitting the virus to their partners, pregnant women, and those with HIV-1-related kidney disease or hepatitis B co-infection [Alcorn, 2012]. A recent study described a very small number of individuals (five out of thirty two) who maintained very low and stable levels of cell-associated HIV-1 DNA following treatment discontinuation; these patients had received early and prolonged treatment with HAART and did not have characteristics associated with elite controllers and long-term non-progressors (LTNP); none had HLA class I B*27 or B*57 alleles or homozygous Delta 32 deletion for the CCR5-encoding gene [Hocqueloux *et al*., 2010]. Although the authors underline the importance of determining the underlying mechanisms of this immunovirological control, they also reiterate that HAART treatment interruptions are not recommended [Hocqueloux *et al*., 2010; El-Sadr *et al*., 2006].

Improvements in HIV-1-specific CD8 T-cell functionality have been found to be slow and incomplete in progressors who initiate HAART during the chronic stage of infection, even when therapy is given for a prolonged period of time [Migueles *et al*., 2009; Rehr *et al*., 2008]. Similarly, HAART initiated during chronic infection does not recover polyfunctionality, proliferative capacity or cytotoxic ability to the extent that these functions are found in LTNP [Migueles *et al*., 2009; Hersperger *et al*., 2010]. (Long-term non-progressive HIV-1 infection is discussed in detail in section 1.3.6.) The initiation of HAART has been found to rapidly reduce the numbers of HIV-1-specific CD8 T cells [Janbazian *et al*., 2012]. A study investigating the residual CD8 T-cell population revealed antigen decay (either by administration of HAART or by the emergence of epitope escape mutations) to be accompanied by alterations in the TCR repertoire, increased expression of CD45RA and CD127, decreased expression of programmed death-1 (PD-1), and the emergence of polyfunctional HIV-1-specific CD8 T cells [Janbazian *et al*., 2012]. Initiation of

HAART in early HIV-1 infection has been associated with significant reductions in proviral DNA to levels comparable to those found in LTNP, suggesting early HAART may lessen the viral reservoir [Pires *et al*., 2004a; Cellerai *et al*., 2011]. Early and prolonged HAART has been associated with an HIV-1-specific CD4 and CD8 T-cell polyfunctional profile (as assessed by IFN-γ, IL-2, TNF-α production, and perforin expression) that is similar to that found in LTNP, indicating early HAART may preserve these virus-specific responses [Cellerai *et al*., 2011]. Furthermore, early initiation of HAART is thought to lower the activation state of monocytes and macrophages, as measured by a decrease in CD163 shedding [Burdo *et al*., 2011]. In a study of serodiscordant couples (HPTN 052), early initiation of HAART reduced the rate of sexual transmission to the HIV-1 negative partner by 96%, as compared with delayed therapy, highlighting the benefit of early treatment both for the individual and in terms of public health [Cohen *et al*., 2011].

In 1987, the nucleoside reverse transcriptase inhibitor (NRTI) Azidothymidine/zidovudine (AZT) was introduced as a monotherapy for HIV-1 [Broder, 2010] until the introduction of HAART in 1996 [De Clercq, 2010; Ray *et al*., 2010]. To date, there are over twenty antiretroviral drugs available, which target different steps of the HIV-1 life cycle, and can be distributed into six major classes: 1) nucleoside-analog reverse transcriptase inhibitors (NRTI), 2) non-nucleoside reverse transcriptase inhibitors (NNRTI), 3) protease inhibitors, 4) fusion inhibitors, 5) entry inhibitors – co-receptor antagonists, and 6) integrase inhibitors [De Clercq, 2010; Lobritz *et al*., 2010], as detailed in Table 1.1, and as illustrated in Figure 1.6. Combinations of antiretroviral therapy drugs can thus be optimised for the HIV-1-infected individual providing, in most cases, viral suppression, a degree of immune reconstitution, and almost normal life-expectancy [Hawkins, 2010; Ries *et al*., 2012].

There is evidence that not all HIV-1-infected persons benefit from HAART and a portion of treated patients do not see restoration of CD4 T-cell counts after five to seven years of treatment [Moore & Keruly, 2007; Kaufmann *et al*., 2003]. Significant increases in non-AIDS –associated morbidities are found in patients with persistently low CD4 T-cell counts and the mechanisms for incomplete

CD4 T-cell recovery are thought to include thymic dysfunction, failure of naïve T-cell expansion and persistent immune activation [Jiang *et al*., 2009]. Even for individuals that do not have complications, in terms of CD4 T-cell recovery, immune inflammation and activation persist at abnormal levels and long-term HAART-treated patients still have an increased risk of death as a result of non-AIDS complications that are usually associated with aging, such as cardiovascular disease, osteoporosis, and end-organ failure [Deeks, 2011]. In these individuals, the prevalence of non-AIDS-associated malignancies is also rising, and these include Epstein-Barr virus-associated Hodgkin lymphoma and human papilloma virus-associated anal neoplasia [Palefsky, 2008; Ries *et al*., 2012].

Table 1.1 FDA approved therapeutics for the treatment of HIV-1 infection.
NRTI – nucleoside reverse transcriptase inhibitor, NtRTI – nucleotide reverse transcriptase inhibitor; NNRTI – non-nucleoside reverse transcriptase inhibitor, PI – protease inhibitor. Taken from [Lobritz *et al*., 2010]. * Single asterisks indicate where drugs are also licensed in the European Union (EU), or where they have a different brand/trade name.

Brand/Trade Name	Generic Name	Class	Approval year
Emtriva*	emtricitabine, FTC	NRTI	2003
Epivir*	Lamivudine, 3TC	NRTI	1995
Hivid*	Zalcitabine, ddC	NRTI	1992
Retrovir*	Zidovudine, AZT	NRTI	1987
Videx*	Didanosine, ddI	NRTI	1991
VidexEC*	Didanosine, ddI (extended release)	NRTI	2000
Viread*	Tenofovir, TFV/TDF	NtRTI	2001
Zerit*	Stavudine, d4T	NRTI	1994
Ziagen*	Abacavir, ABC	NRTI	1998
Edurant*	Rilpivirine	NNRTI	2011
Intelence*	Etravirine, ETV/ETR	NNRTI	2008
Rescriptor	Delavirdine, DLV	NNRTI	1997
Sustiva*	Efavirenz, EFV	NNRTI	1998
Viramune*	Nevirapine, NVP	NNRTI	1996
Viramune XR* (extended release)	Nevirapine, NVP	NNRTI	2011
Combivir*	3TC/AZT	NRTI	1997
Kivexa* Epzicom	3TC/abacavir	NRTI	2004
Trizivir*	3TC/abacavir/AZT	NRTI	2000
Truvada*	FTC/tenofovir	NRTI/NtRTI	2004
Atripla*	FTC/tenofovir/efavirenz	NRTI/NtRTI/NNRTI	2006
Complera Eviplera*	FTC/TDF/rilpivirine	NRTI/NtRTI/NNRTI	2011
Agenerase*	Amprenavir, APV	PI	1999
Aptivus*	Tipranavir, TPV	PI	2005
Crixivan*	Indinavir, IDV	PI	1996
Invirase*	Saquinavir, SQV	PI	1995
Kaletra Aluvia*	Lopinavir, LOP/ritonavir	PI	2000
Lexiva Telzir*	Fosamprenavir, APV	PI	2003
Norvir*	Ritonavir, RTV	PI	1996
Prezista*	Darunavir, DRV	PI	2006
Reyataz*	Atazanavir, ATV/ATZ	PI	2003
Viracept*	Nelfinavir, NFV	PI	1997
Fuzeon*	Enfuviritide, T-20	Fusion inhibitor	2003
Selzentry Celsentri*	Maraviroc, MVC	CCR5/entry inhibitor	2007
Isentress*	Raltegravir, RTV	Integrase inhibitor	2007

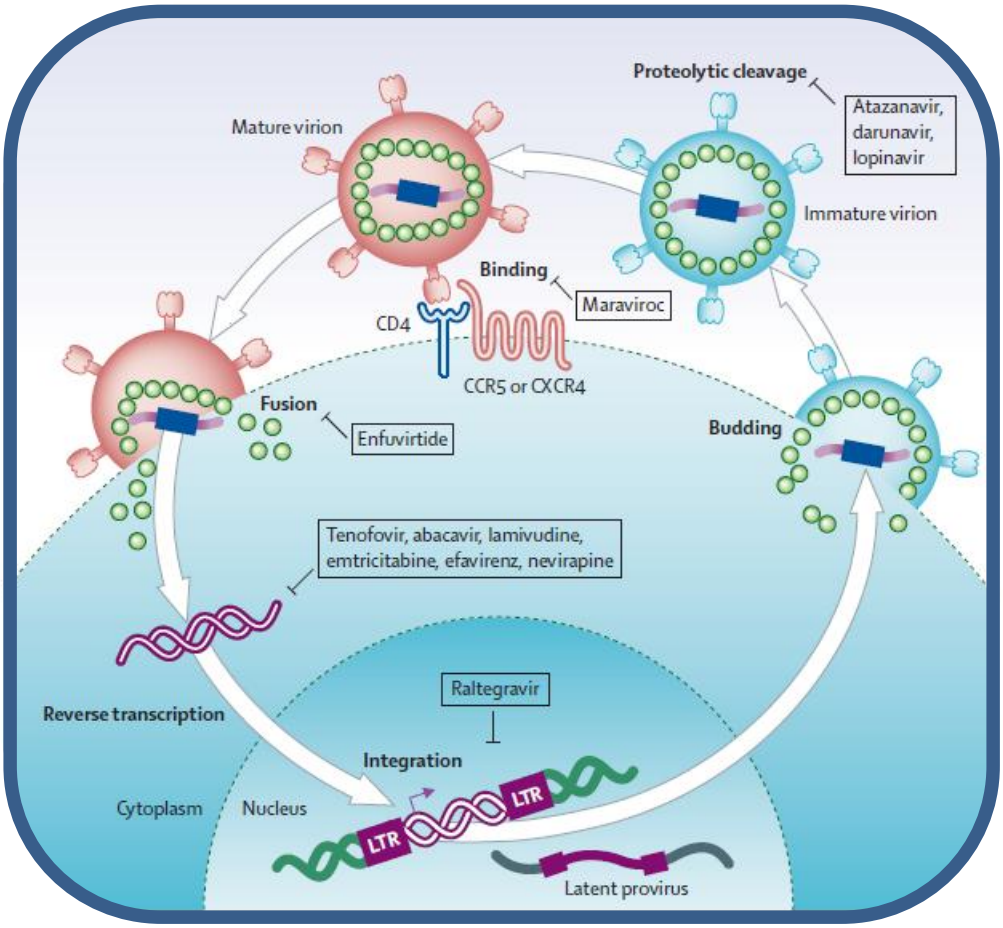


Figure 1.6 HIV-1 viral life cycle and antiretroviral drug targets.
There are currently six classes of antiretroviral drugs that target five steps of the HIV-1 life cycle (binding, fusion, reverse transcription, integration, and proteolytic cleavage). The drugs shown here are those most commonly used in resource-rich settings. The complex three-step process, by which extracellular virions enter the target cell are: 1) attachment to the CD4 receptor, 2) binding to the CCR5 or CXCR4 co-receptors, or both, and 3) membrane fusion. Maraviroc blocks CCR5 binding and enfuviritide blocks fusion. The HIV-1 reverse transcriptase enzyme catalyses the transcription of HIV-1 RNA into double stranded HIV-1 DNA, and nucleoside analogues, and non-nucleoside reverse transcriptase inhibitors (NNRTIs) interfere with this step. The HIV-1 integrase enzyme facilitates the incorporation of the HIV-1 DNA into the host chromosomes and raltegravir and other integrase inhibitors obstruct this process. Following transcription and translation of the HIV-1 genome, immature virions are produced and bud from the cell surface. The HIV protease enzyme cleaves polypeptide chains, allowing the virus to mature, and protease inhibitors prevent this final stage. Taken from [Volberding & Deeks, 2010].

Topical and oral tenofovir-based pre-exposure prophylaxis (PrEP) has been shown in four recent clinical trials to be efficacious, and greater adherence to PrEP has been associated with greater efficacy [Celum, 2011; van der Straten *et al*., 2012]. The studies were as follows: 1) the CAPRISA 004 trial showed a 39% efficacy in reducing the risk of HIV acquisition in young women following pericoital administration of a 1% tenofovir gel, 2) the iPrEx study demonstrated a 44% efficacy in reducing the risk of HIV acquisition among high-risk men who have sex with men (MSM)

subsequent to daily oral emtricitabine/tenofovir, 3) the Partners PrEP Study in African HIV serodiscordant couples where the HIV-seronegative partner received daily oral tenofovir or emtricitabine/tenofovir showed 62% and 73% efficacy, respectively, and 4) the TDF2 trial found 62% efficacy after daily administration of oral emtricitabine/tenofovir in young heterosexual men and women in Botswana [Celum, 2011].

1.3 Immune Response to HIV-1

The immune system controls most viral infections, including those which establish chronic infection. In HIV-1 infection, the immune system appears to respond appropriately to prevent viral spread. There is evidence that antibodies, CD8 CTL and CD4 T_H responses all play a role in containing HIV-1. However, HIV-1, unlike other persistent viruses, progressively destroys the immune system resulting in AIDS and death. HIV-1 has evolved a number of strategies to evade the host immune system [Alcami & Koszinowski, 2000; Kawashima *et al*., 2009]; however, the mechanisms by which immune function is lost remain highly controversial. Factors that may contribute to the loss of immune function include: continuous adaptive mutation of the virus [Barouch & Letvin, 2002], destruction or impairment of cells necessary for optimal immune response (CD4 T cells and APC) [Kalams & Walker, 1998], decreased thymic output [Douek *et al*., 2001], continuous production of virus from stable reservoirs [Shen & Siliciano, 2008], and chronic immune activation [Appay & Sauce, 2008].

The deterioration of the T-cell response during HIV-1 infection follows a characteristic pattern: proliferative capacity, the ability to produce IL-2 and cytotoxic capacity are lost early, whereas the production of IFN-γ is more long-lasting. Eventually, even the ability to produce IFN-γ is impaired as the majority of T cells continually exposed to HIV-1 antigens enter into a state of dysfunction, resulting in disease progression [Appay *et al*., 2000; Kostense *et al*., 2002; Roos *et al*., 2000; Gamadia *et al*., 2002; Ostrowski *et al*., 2000; Jones *et al*., 2008].

Although this thesis focuses on the impact of HIV-1 infection on CD4 and CD8 T cells, and methods to reverse T-cell dysfunction, it is important to note that pathogenic HIV-1 infection also

disrupts other immune subsets, such as DC, B cells, macrophages, natural killer cells, and γδ T cells [Grossman *et al*., 2006; Brenchley *et al*., 2006].

1.3.1 CTL response

HIV-1 infection results in virus-specific CTL responses against both structural and regulatory genes of HIV-1 [Walker *et al*., 1987; Addo *et al*., 2001]. These responses may control HIV-1 by more than one mechanism [Gupta *et al*., 2008; Yang & Walker, 1997]. CTL may recognise and lyse cells which present viral peptides on MHC I [Yang *et al*., 1996] in addition to secreting β chemokines which bind to HIV-1 co-receptors on target cells, thus blocking HIV-1 entry [Hadida *et al*., 1998; Wagner *et al*., 1998].

Evidence exists that CTL do control HIV-1 infection. In acute infection, clearance of primary viraemia correlates with the appearance of HIV-1-specific CTL [Andrews & Koup, 1996; Borrow *et al*., 1994]. *In vitro* experiments have shown that CTL taken from infected patients are able to inhibit viral replication in autologous CD4 T cells [Yang *et al*., 1996]. This role for CD8 T-cell control is supported by studies in rhesus macaques (RM) infected with simian immunodeficiency virus (SIV). In animals with depleted CD8 T cells, there was an increase in viral load and as CD8 T cells were restored, viraemia was controlled [Lieberman *et al*., 2002]. Despite high frequencies of HIV-1-specific CTL present during infection (as determined by tetramer staining), it is possible that such CD8 T cells either are or become defective [Lieberman *et al*., 2001]. In addition, it has been found that HIV-1 specific CTL contain less perforin and have diminished cytotoxicity compared to other virus specific CTL [Appay *et al*., 2000]. There is also the possibility that the maturation of HIV-1 specific memory CD8 T cells is skewed, which may lead to an ineffective response [Champagne *et al*., 2001; Garber *et al*., 2004].

1.3.2 T-cell differentiation and memory subsets during infection

The T-cell differentiation and maturation subset patterns described in section 1.1.3 are skewed in HIV-1 infection. In Figure 1.7A, three models of CD8 T-cell differentiation in the human system

are shown, and classifications differ according to the surface markers used to characterise the subsets [Garber *et al*., 2004]. The top row shows naïve ($CCR7^+CD45RA^+$) CD8 T cells which differentiate into T_{CM} ($CCR7^+CD45RA^-$), then into T_{EM} ($CCR7^-CD45RA^-$), and finally into terminally differentiated effectors or $CD45RA^+$ effector memory cells (T_{EMRA}; $CCR7^-CD45RA^+$) [Champagne *et al*., 2001]. Appay *et al*., use CD28 and CD27 to characterise these subsets and the terminology differs; early- ($CD28^+CD27^+$), intermediate- ($CD28^-CD27^+$), and late- ($CD28^-CD27^-$) primed CD8 T-lymphocyte stages are depicted in the second row [Appay *et al*., 2002]. In the third row, naïve CD8 T cells differentiate into several stages of memory cells before becoming effector T cells, and this process is defined using a combination of CD28, CD27, CCR7, and CD45RA/RO [van Baarle *et al*., 2002]. Figure 1.7B illustrates the defects in maturation and differentiation that occur in HIV-1-specific CD8 T cells in HIV-1-infected individuals, according to each of the three models. The bold arrows indicate increased or excessive production, and broken arrows indicate defective differentiation/maturation [Garber *et al*., 2004]. Regardless of the markers used to characterise these subsets, all three models depict a disproportionate shift from central memory or early-primed CD8 T cells (shown in green) to an effector memory or intermediate primed (shown in yellow) state, with further defects in the differentiation pathway to T_{EMRA}/late-primed/effectors (shown in orange and red; Figure 1.7B).

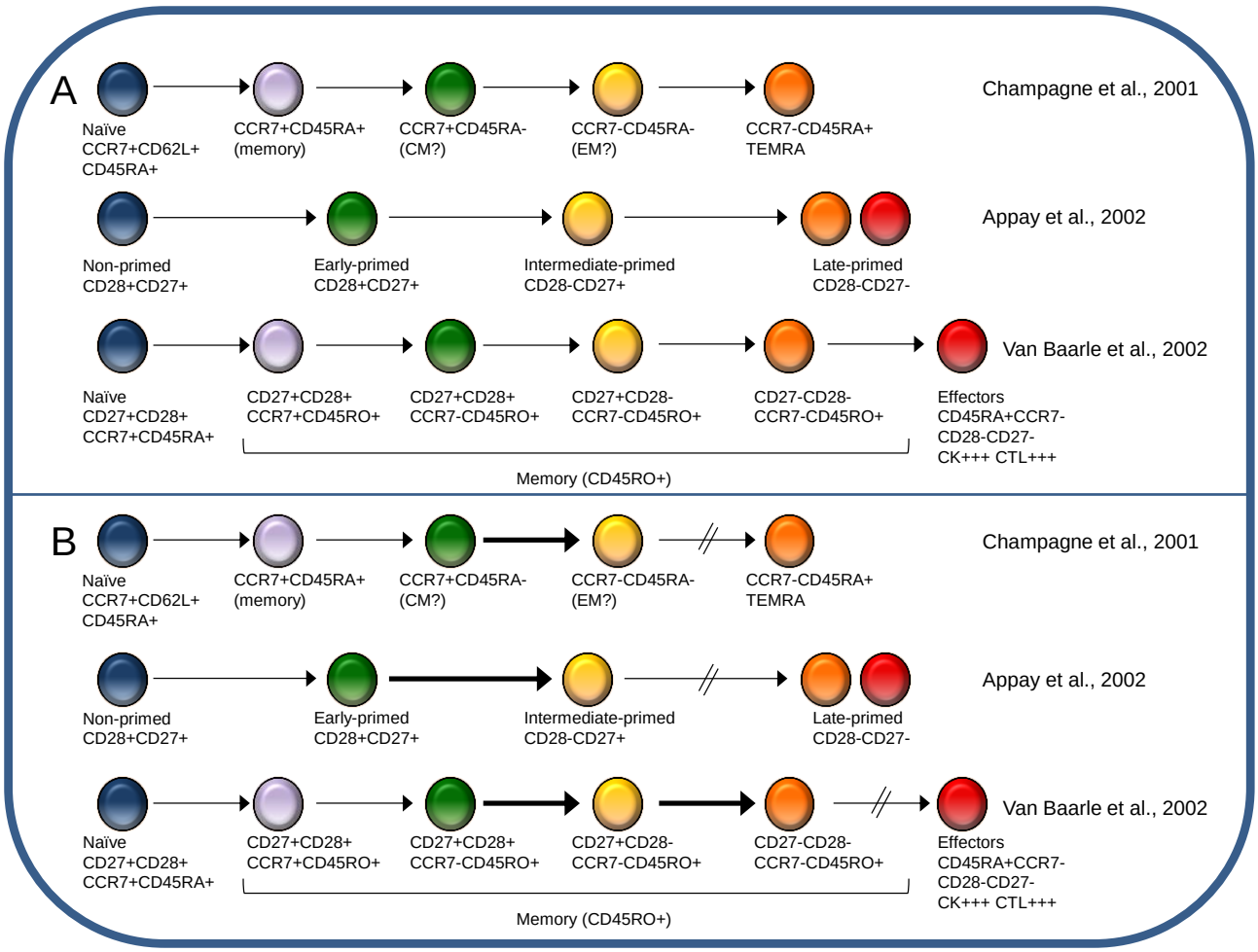


Figure 1.7 CD8 T-cell differentiation/maturation pathways compromised during chronic viral infection.
A) Several models of CD8 T-cell differentiation have been described in the human system. In the first row, naïve CD8 T cells differentiate into central memory (CM), then effector memory (EM) T cells, and finally into terminally differentiated effectors/CD45RA^{+} effector memory cells (TEMRA). In the second row, naïve (non-primed) CD8 T cells differentiate into early-primed, intermediate-primed, and late-primed cells. In the third row, naïve CD8 T cells differentiate into several stages of memory cells, and finally into effector T cells. B) Defective maturation/differentiation of HIV-1-specific CD8 T cells in HIV-1-infected individuals. Bold arrows indicate increased/excessive production; broken arrows indicate defective differentiation/maturation; CK –cytokine production. Taken from [Garber *et al*., 2004].

CD8 T_{CM} preferentially localise in the lymph nodes, secrete IL-2 following antigen stimulation, yet show reduced cytotoxicity and limited immediate effector capabilities [Sallusto *et al*., 1999]; whereas T_{EM} preferentially localise in non-lymphoid peripheral tissue, have reduced proliferative potential, yet possess cytolytic capabilities and can exhibit immediate effector functions, such as IFN-γ production [Ahlers & Belyakov, 2010].

Similar panels of markers have been used to characterise differentiation and memory subsets for CD4 T lymphocytes, although, as described in section 1.1.3, these differ slightly, particularly with regard to the expression of CD27 and CD28. As CD4 T cells progress from early to intermediate

and late stages of differentiation, they lose CD27 first [Downey & Imami, 2010]. Experiments in a mouse model have suggested that CD4 T_{CM} have a superior capacity to reconstitute the memory T-cell pool and to mediate protective immunity compared to T_{EM}, and this was due to a greater proliferative capacity and ability to persist *in vivo* [Wu *et al*., 2002; Zaph *et al*., 2004]. The induction of CD4 T_{CM} following SIV challenge in non-human primates has been found to correlate with prolonged survival [Letvin *et al*., 2006]. Furthermore, CD4 T_{CM} analysed *ex vivo* demonstrated the capacity to resist apoptosis and to persist in a stable manner in the host, thus providing protective immunity against re-infection [Riou *et al*., 2007]. It is of utmost importance to consider how these populations are skewed or dysregulated in HIV-1 infection and therapies to target and reverse these defects are warranted.

A transitional memory phenotype (T_{TM}; $CCR7^{-}CD45RA^{-}CD27^{+}$) has been described for the CD4 T-lymphocyte compartment which was shown to have functional and transcriptional characteristics intermediary between those of T_{CM} and T_{EM} cells [Riou *et al*., 2007]. CD4 T_{CM} and T_{TM} were found to be the major cellular reservoirs for HIV-1, with this viral persistence mainly in the T_{CM} compartment for patients that show CD4 T-cell reconstitution with HAART, and mainly in the T_{TM} for aviraemic subjects with low CD4 T-lymphocyte counts [Chomont *et al*., 2009]. The T_{TM} phenotype has also been described for the CD8 T-cell compartment using the same markers ($CCR7^{-}$ $CD45RA^{-}CD27^{+}$) [Killian *et al*., 2011], and using a slightly different panel ($CCR7^{-}$ $CD45RO^{+}CD28^{+}$) [Freel *et al*., 2010]. More recently, a long-lived memory cell population was described that has an enhanced capacity for self-renewal and from which central memory, effector memory and effector T cells can be derived [Gattinoni *et al*., 2011]. These cells were characteristic of naïve T cells ($CD45RO^{-}CCR7^{+}CD45RA^{+}CD62L^{+}CD27^{+}CD28^{+}$IL-7R$\alpha^{+}$) but showed functional characteristics typically attributed to memory cells and expressed large amounts of CD95, IL-2Rβ, CXCR3, and LFA-1 [Gattinoni *et al*., 2011]. The relevance of this T-cell subset in HIV-1 infection remains to be elucidated but may play a crucial role in vaccine development or T-cell therapies.

A possible explanation for the CD8 T-cell defects seen in HIV-1 infection (described above) could be a lack of CD4 T-cell help [Williams & Bevan, 2007]. Depletion of CD4 T-cell counts is a hallmark of HIV-1 infection; however the mechanisms by which this occur remains elusive [Rowland-Jones, 1999; McCune, 2001]. It is clear that HIV-1 infects and kills CD4 T cells [Lenardo *et al*., 2002], however, this cytopathicity alone does not account for CD4 T-cell loss and the progress of the disease. In late stage AIDS, this decline is associated with a decline in CTL activity [Wherry *et al*., 2007]. Even in early stages of HIV-1 infection, specific CD4 T cells have been shown to be functionally defective [Rosenberg *et al*., 1997; Musey *et al*., 1999; Sun *et al*., 2007].

1.3.3 Regulatory T cells and T_H17 cells

Regulatory T (Treg) cells are important immunoregulators; they control excessive immune activation which may reduce immune-mediated tissue damage but which may also suppress antigen-specific immune responses against pathogens [Angin *et al*., 2012]. To this effect, reports on the role of Treg cells in HIV-1 infection have been mixed. Some studies have found increased frequencies [Andersson *et al*., 2005; Nilsson *et al*., 2006], which normalise with effective ART [Montes *et al*., 2011; Presicce *et al*., 2011], while others have documented decreased frequencies in HIV-1-infected individuals [Tsunemi *et al*., 2005; Eggena *et al*., 2005; Burton et al., 2011]. HIV-1 infection and subsequent PD-1 up-regulation and increased immune activation was determined to be associated with decreased Treg cell proliferation [Sachdeva *et al*., 2010].

The possibility that these Treg cells might be detrimental in HIV-1 infection was supported by data from a phase III clinical trial which showed that IL-2 therapy preferentially expanded the Treg cell population in HIV-1^+ subjects, and that the individuals with the greatest expansion of this subset were more likely to progress to AIDS [Abrams *et al*., 2009; Weiss *et al*., 2010]. Further to this finding, a recent study has demonstrated that CTL that are restricted by protective HLA-alleles (HLA-B*27 and HLA-B*B57) up-regulate less TIM-3 and more granzyme B upon recognition of their cognate epitopes; these cells are less susceptible to Treg cell-mediated suppression, instead

killing the Treg cells that they encounter [Elahi *et al*., 2011]. The authors postulate that in contrast, the majority of HIV-1-specific CD8 CTL will up-regulate TIM-3 when they encounter their cognate epitopes, which leads to suppression by Treg cells [Elahi *et al*., 2011].

However, it has also been suggested that Treg cells may have the ability to directly inhibit HIV-1 replication in activated T cells [Moreno-Fernandez *et al*., 2011]. One study has recently reported that Treg cell frequencies and numbers in elite controllers are not different to uninfected controls, but that in chronically infected subjects, the absolute number of Treg cells in the blood and tissue are diminished [Angin *et al.*, 2012]. Furthermore, the *ex vivo* analysis of Treg cells from chronically infected HIV-1^+ patients found preservation of suppressive function which did not differ from that of Treg cells from elite controllers or seronegative donors [Angin *et al*., 2012]. A role for Treg cells in the reduction of residual immune activation in patients on suppressive ART has been proposed [Weiss *et al*., 2010]. The proportion of Treg cells was found to negatively correlate with the proportion of $CD8^+$HLA-DR^+ T cells in treated HIV-1^+ patients, and following treatment interruption, while the proportion of Treg cells increased, absolute numbers decreased [Weiss *et al*., 2010]. Following treatment interruption, the numbers of activated cells ($CD8^+CD38^+$HLA-DR^+) also significantly increased, but the original correlation with Treg cells was lost, suggesting an insufficient increase in the proportion and/or decrease in the absolute number of Treg, leading to an inability to control immune activation after ART is interrupted [Weiss *et al*., 2010].

Treg cells are capable of suppressing the immune response through competition with proliferating T cells for IL-2, which enhances the growth and differentiation of Treg cells. Thus the use of IL-2 immunotherapy could expand the Treg cell population and blunt the anticipated immune benefits [Macatangay & Rinaldo, 2010]. HIV-1-infected subjects that received IL-2 in addition to ART were found to have an increased Treg cell population compared to patients that received ART alone and modest but significant decreases in Gag-specific CD4 and CD8 T-cell counts following IL-2 therapy [Ndhlovu *et al*., 2010]. IL-7 immunotherapy, however, was not associated with an increase in Treg cell frequencies, but with an increase in T-cell activation [Sereti *et al*., 2009]. It is therefore

crucial to consider the impact of immunotherapies, such as cytokine (IL-2, IFN-α, IL-7) administration, inhibitory receptor blockade, and therapeutic vaccination, on the induction of Treg cells, and strategies to control this cell type may be warranted [Macatangay & Rinaldo, 2010].

One potential confounding factor in such approaches, and in fact, a possible reason for such conflicting data on the role of Treg cells in HIV-1 infection may be the different markers that are used to identify these cells. Commonly used markers such as CD25 and FoxP3 are also found on other T-cell subsets [Macatangay & Rinaldo, 2010]. Abnormal expression of CD27 and CD127 have been described during HIV-1 disease, rendering these markers unreliable to identify Treg cells, and the $CD3^{+}CD4^{+}CD25^{high}CD45RO^{+}$ phenotype was found to correlate with FoxP3 expression in both HIV-1^{+} patients and uninfected individuals [Burton *et al*., 2011].

CD4 T_H17 cells are effector cells which secrete interleukin-17A (IL-17A), and may also secrete IL-17F, IL-22, and CCL20; they have been shown to induce and regulate inflammatory responses, thus playing a crucial role in host defence [Ndhlovu *et al*., 2008; Yue *et al*., 2008; Milner *et al*., 2008; Steinman, 2008; Guillot-Delost *et al*., 2012]. During HIV-1 infection, the frequency of T_H17 cells is found to be decreased in both the peripheral blood and gut-associated lymphoid tissue (GALT); a depletion which appears to be unfavourable in terms of HIV-1 control [Macal *et al*., 2008; Prendergast *et al*., 2010; Ndhlovu *et al*., 2010]. The depletion of T_H17 cells from the gut mucosa after HIV-1 infection may lead to microbial translocation and increased immune activation [Brenchley & Douek, 2008; Aujla *et al*., 2007]. IL-2 has been found to induce the proliferation of T_H17 cells, and in combination with IL-1β thought to drive $CD4^{+}CD25^{+}$ Treg cells into a T_H17 phenotype [Deknuydt *et al*., 2009], although when used alone, IL-2 has also been reported to block IL-17 production and inhibit T_H17 cell development [Laurence *et al*., 2007; Downey & Imami, 2010]. In one study, administration of IL-2 to HIV-1^{+} patients on ART did not alter the numbers of peripheral blood T_H17 cells [Ndhlovu *et al*., 2010]. Another group reported T_H17 numbers to be greater in long-term non-progressors (LTNP) compared to typical progressors, and HIV-1 RNA inversely correlated with IL-17 receptor expression and T_H17 cell numbers [Salgado *et al*., 2011].

CD4 T_H17 cells have been described as having a $CD45RO^+CCR6^+CD161^+$ phenotype, and IL-17 producing cells have been detected in a minor population of CD8 T cells; these were found to be $CD27^{+/-}CD28^+CD45RA^-CCR6^+CCR5^+$ and to produce IFN-γ [Guillot-Delost *et al*., 2012]. CD90 was recently described to be a marker of a subset of T_H17 cells (both CD4 and CD8) that are depleted during HIV-1 infection [Guillot-Delost *et al*., 2012]. Thus, T_H17 cells represent an important cell type to consider, particularly in the case of immune-based therapies in the context of treated HIV-1 infection.

1.3.4 Chronic immune activation

Increasing evidence shows that high levels of immune activation are associated with disease progression in HIV-1 infection [Hazenberg *et al*., 2003; Giorgi, 1993; Sousa *et al*., 2002], although the precise mechanisms for this remain unclear [Hunt *et al*., 2003]. One study reported an association between the level of activation and the differentiation state of T cells; HIV-1 induces the activation of cells, both directly and indirectly, resulting in the differentiation of CD8 T cells toward replicative senescence [Papagno *et al*., 2004]. During HIV-1 infection, the immunosuppression that occurs may lead to increased replication of other viruses present in the host such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV), leading to further immune activation [Doisne *et al*., 2004]. One study found significantly higher levels of CMV-specific effector CD8 T cells in HIV-1^+ patients compared to HIV-1 seronegative, CMV seropositive controls, and these levels were comparable to those typically found in much older individuals [Naeger *et al*., 2010]. A recent study of ART-treated HIV-1^+ CMV seropositive patients with low CD4 T-cell counts reported that daily administration of valganciclovir for eight weeks resulted in CMV DNA levels becoming undetectable and significant reductions in CD8 T-cell activation (as measured by co-expression of CD38 and HLA-DR) compared to placebo-treated patients [Hunt *et al*., 2011]. Moreover, hepatitis C virus (HCV) co-infection has been associated with progression to AIDS/death during HAART [Greub *et al*., 2000; Kovacs *et al*., 2010]. The translocation of microbial products from the damaged gut may be another mechanism for increased immune

activation [Brenchley *et al*., 2006; Douek *et al*., 2009]. The elevated and chronic stimulation induced by HIV-1 may result in the exhaustion of the capacity to generate new T cells [Hazenberg *et al*., 2003]. In addition to a direct effect of HIV-1 on the thymus, decreased thymic output and T-cell renewal may originate from thymic involution [Kalayjian *et al*., 2003], as well as the failure of the bone marrow and a reduction of primitive haematopoietic stem cell subsets [Marandin *et al*., 1996; Moses *et al*., 1998], as observed in HIV-1^+ individuals.

CD8 T-cell activation levels, in terms of CD38 and HLA-DR co-expression, decline with lower levels of viral replication, with even further decreases seen with ART-mediated viral suppression; however, even in aviraemic, treated HIV-1^+ patients, abnormal levels of T-cell activation persist (compared to levels found in HIV-1 seronegative controls) [Hunt *et al*., 2003; Bartovska *et al*., 2011; Hunt *et al*., 2008]. Additionally, high levels of CD8 T-cell activation have been shown to correlate with incomplete CD4 T-cell recovery following initiation of ART [Hunt *et al*., 2003]. Microbial translocation has been shown to correlate with immune activation, and to have an inverse correlation with immune reconstitution following the initiation of HAART [Jiang *et al*., 2009]. Additionally, microbial translocation (as measured by levels of plasma LPS) decreases with HAART but remains significantly higher than uninfected controls [Jiang *et al*., 2009]. Microbial translocation is thought to have a profound impact on both immune activation and inflammation, which may result in HIV-1 disease progression, non-AIDS defining co-morbidities, and early aging [Volberding & Deeks, 2010]. Non-AIDS events were found to be more common in HIV-1 disease, even after adjustment for age, HAART exposure, and traditional risk factors, further indicating persistent inflammation and activation may play a role in the premature aging of the immune system of HIV-1-infected individuals [Deeks & Phillips, 2009]. Activation levels on cells of the innate immune system are also important to consider; levels of soluble CD163 in the plasma of HIV-1-infected subjects have been associated with disease progression, and HAART does not normalise these levels to those seen in healthy persons [Burdo *et al*., 2011]. One immunomodulatory agent that has been shown to reduce levels of immune activation in therapy-

naïve HIV-1^+ patients is atorvastatin (a drug typically used to lower cholesterol levels); subjects that received atorvastatin for eight weeks demonstrated significant reductions in both CD4 and CD8 T-cell activation compared to placebo-treated patients, although HIV-1 RNA levels were unaffected [Ganesan *et al*., 2011].

Key insights into HIV-1 pathogenesis may be derived from studies investigating non-progressive SIV infection in natural hosts such as sooty mangabeys or African green monkeys [Pandrea *et al.*, 2008]. In contrast with pathogenic HIV-1 infection and SIV infection in rhesus macaques, the hallmarks of non-progressive SIV infection include lower levels of immune activation and apoptosis, a lack of excessive T-cell proliferation, and normal levels of LPS during acute and chronic infection, suggestive of an intact intestinal barrier, despite massive CD4 T-cell loss and viral replication during primary infection [Silvestri *et al*., 2003; Pandrea *et al*., 2008].

1.3.5 Co-inhibitory receptors and immune exhaustion

A balance between co-stimulatory signals that activate T cells and inhibitory signals that minimise harmful inflammatory responses is crucial in terms of regulating virus-specific memory CD4 and CD8 T cells that are necessary to control viral replication [Pitcher *et al*., 1999; Addo *et al*., 2007; Chen, 2004; Crawford & Wherry, 2009; Kassu *et al*., 2010]. T-cell activation, proliferation and differentiation occur following binding of the TCR to the cognate MHC-peptide complex and simultaneous binding of CD28 to CD80/CD86; whereas cell cycle arrest, decreased function and death occur when ligation of an inhibitory receptor accompanies the TCR MHC-peptide interaction [Chen, 2004; Crawford & Wherry, 2009]. The balance between excitatory and inhibitory signals is skewed in chronic viral infections toward a negative regulatory pathway, in such a way that virus-specific T-cell function is compromised and ongoing viral replication and/or persistence occurs [Crawford & Wherry, 2009; Virgin *et al*., 2009; Kaufmann & Walker, 2008; Kaufmann & Walker, 2009; El-Far *et al*., 2008]. Virus-specific CD4 T cells become exhausted during HIV-1 infection and lose effector function, and HIV-1-specific CD8 T cells have impaired cytolytic function and skewed differentiation patterns, leading to ineffective T-cell responses [Crawford & Wherry, 2009;

Virgin *et al*., 2009; El-Far *et al*., 2008; Addo *et al*., 2007]. It has been suggested that signalling through inhibitory receptors, such as programmed death 1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) may play a role in the T-cell dysfunction seen in HIV-1 infection, contributing to what has been termed T-cell exhaustion [Kaufmann & Walker, 2008; Kaufmann & Walker, 2009; El-Far *et al*., 2008; Day *et al*., 2006; D'Souza *et al*., 2007; Jones *et al*., 2008; Kaufmann *et al*., 2007; Petrovas *et al*., 2006; Trautmann *et al*., 2006].

PD-1 was first described as a surface receptor involved in apoptosis [Ishida *et al*., 1992]; it is a negative regulator of T cells, and engagement of PD-1 with its ligands PD-L1 and PD-L2 leads to an inhibition of T-cell proliferation and cytokine production [Greenwald *et al*., 2005]. PD-1 expression on CTL has been found to correlate with viral load and disease progression in untreated HIV-1-infected subjects [Kaufmann & Walker, 2009]. Control of viral load following the initiation of ART resulted in reduced PD-1 expression on HIV-1-specific CTL, and *in vitro* blockade of the PD-1 pathway with an anti-PD-L1 antibody leads to enhanced HIV-1-specific T-cell proliferation [Day *et al*., 2006; Trautmann *et al*., 2006; Kaufmann & Walker, 2009]. PD-1 was found to be similarly up-regulated on the total CD4 T-lymphocyte population in HIV-1-infected subjects, and levels of PD-1 correlated directly with viral load and inversely with CD4 T-cell count [Day *et al*., 2006]. CD4 T-cell proliferative responses were found to be improved when the PD-1 pathway was blocked *in vitro* with an anti-PD-L1 antibody, and a strong correlation was described between PD-1 levels in CD4 and CD8 Gag-specific T cells [Day *et al*., 2006; D'Souza *et al*., 2007]. HIV-1 controllers and LTNP were found to have lower PD-1 expression compared to patients with high levels of viral replication and disease progression [Zhang *et al*., 2007]. Recently, PD-1 has been proposed as a marker to identify individuals failing reconstitution despite viral suppression with ART and a reduction in the expression of markers associated with immune activation [Grabmeier-Pfisterhammer *et al*., 2011]. Examination of PBMC from HIV-1-infected persons found PD-L1 expression to be significantly elevated on monocytes and B cells compared with healthy controls,

and PD-L1 levels correlated directly with viral load and inversely with CD4 T-cell count [Trabattoni *et al*., 2003]. Although PD-1 expression was found to be normalised in aviraemic patients on ART, levels of PD-L1 remained elevated compared to healthy seronegative controls [Rosignoli *et al*., 2007].

CTLA-4 binds to B7 molecules with a higher affinity than does CD28 [Curran *et al*., 2010]; it is rapidly up-regulated following T-cell activation, and its inhibitory mechanism works by reducing IL-2 production and by arresting cell cycle progression [Greenwald *et al*., 2005]. CTLA-4 expression is increased on total and virus-specific CD4 T cells during chronic HIV-1 infection [Kaufmann *et al*., 2007; Zaunders *et al*., 2006; Leng *et al*., 2002], although it is not highly expressed on HIV-1-specific CTL [Kaufmann & Walker, 2009]. Even after four years of ART, CD4 T cells from HIV-1^{+} individuals have raised levels of CTLA-4 expression, in addition to a higher number of cells that contain low levels of CD28, suggestive of a population of cells that are unable to respond to antigen [Downey & Imami, 2010]. Moreover, increased HIV-1-specific CD4 T-cell proliferative responses were reported following inhibition of CTLA-4 *in vitro* [Kaufmann & Walker, 2009].

TIM-3 was identified as a cell surface marker of mouse Th1 CD4 T cells [Monney *et al*., 2002]; mouse TIM-3 interacts with its ligand, galectin-9, and promotes the death of IFN-γ-producing Th1 cells, thus regulating this response [Zhu *et al*., 2005]. TIM-3 expression was found to be increased on CD8 T cells from treatment naïve HIV-1-infected persons compared to healthy seronegative controls, and levels correlated with HIV-1 viral load and CD38 expression and inversely with CD4 T-cell count [Jones *et al*., 2008]. Furthermore, initiation of ART only reduced TIM-3 expression in some individuals [Jones *et al*., 2008]. Progressive HIV-1 infection was shown to be marked by increased expression of TIM-3 on HIV-1-specific CD8 T cells, and *in vitro* blockade of the TIM-3 signalling pathway restored proliferative capacity and increased cytokine production to virus-specific T cells [Jones *et al*., 2008]. A recent report postulates that CD8 T cells that are restricted by protective HLA alleles, such as HLA B*27 and B*57, up-regulate less TIM-3 but more granzyme B

upon recognition of their cognate epitopes, and are thus less susceptible to regulatory T-cell-mediated suppression and instead kill the regulatory T cells that they encounter, and subsequently control HIV-1 replication [Elahi *et al*., 2011].

One study used a combination of these co-inhibitory markers and found that ART-treated HIV-1-infected patients had significantly lower percentage expression of PD-1, CTLA-4 and TIM-3–expressing, IFN-γ-producing CD4 T cells compared with untreated viraemic subjects [Kassu *et al*., 2010]. Further elucidation of the mechanisms of action of these negative regulatory molecules may lead to a better understanding of the role that these receptors and their ligands play in HIV-1-specific T-cell dysfunction. This may provide a novel therapeutic target to reverse HIV-1 induced anergy and enhance immune reconstitution after HAART [Kaufmann & Walker, 2009].

1.3.6 Long-term non-progressors, HIV-1-controllers and exposed seronegatives

Long-term control of HIV-1 infection in the absence of therapy is only achieved by a small number of infected individuals, and the mechanisms by which this control is achieved remain debatable [Dembek *et al*., 2010]. HIV-1^{+} patients who are able to maintain stable CD4 T-lymphocyte counts within the normal healthy range for a prolonged period of time, and remain asymptomatic without ART, are termed long-term non-progressors (LTNP), and account for approximately 5% of individuals infected with HIV-1 [Mandalia *et al*, 2012; Okulicz *et al*., 2009]. HIV-1 controllers (HIC), or elite controllers, make up less than 1% of the infected population, and are additionally able to supress HIV-1 replication below the limit of detection of most standard clinical assays (<50-75 HIV-1 RNA copies per ml plasma), again in the absence of ART [Deeks & Walker, 2007]. Insights into the mechanisms of control in the absence of therapeutic interventions may be gleaned from the study of such patients [Imami *et al*., 2002]. Reports have suggested that disease progression in LTNP is inevitable and the rate of disease progression may be associated with virological, immunological and genetic factors [Easterbrook, 1994; Lefrere *et al*., 1997; Mandalia *et al*., 2012]. These same factors are believed to be responsible for the control exhibited by these individuals, and both LTNP and HIC have demonstrated durable and broad HIV-1-specific CD4

and CD8 T-cell responses [Imami *et al*., 2002; Deeks & Walker, 2007]. Particular MHC class I alleles have been shown to correlate with delayed disease progression in HIV-1^+ subjects [Migueles *et al*., 2000; Carrington *et al*., 1999]; in Europeans, HLA-alleles B*5701, B*2701, B*14/Cw*0802, B*52 and A*25 have been associated with protection, and B*35 and B*07 associated with more rapid HIV-1 disease progression [Carrington & Walker, 2012]. Many LTNP possess the HLA-B*57 allele which has been associated with enhanced peptide presentation on the surface of CD4 T cells to cytotoxic CD8 T cells [Pereyra *et al*., 2010; Migueles *et al*., 2008]. HLA-B*5701^+ LTNP/elite controllers were found to have oligoclonal HLA-B*5701-restricted p24-specific CD8 T-cell responses with similar levels of diversity to HLA-matched progressors, and both groups had few public clonotypes [Mendoza *et al*., 2012]. Thus, immunologic control is unlikely to be mediated by variations in the TCR repertoire of immunodominant HIV-1-specific CD8 T-cell populations [Mendoza *et al*., 2012]. Furthermore, high levels of HLA-C expression have been suggested to be advantageous in terms of host genetic control [Carrington & Walker, 2012], and recently, the MHC class III sub-region has been described as a major marker of the LTNP phenotype [Guergnon *et al*., 2012].

HIV-1-specific CD8 T-cell proliferation and perforin production have been described in non-progressors; attributes which have been found to be lacking in aviraemic patients on suppressive ART [Migueles *et al*., 2002]. Non-progressors have also been demonstrated to have enhanced CD8 T-cell polyfunctionality in the blood [Betts *et al*., 2006; Zimmerli *et al*., 2005], and in the mucosa [Ferre *et al*., 2009], with the ability to suppress HIV-1 infection *ex vivo* [Saez-Cirion *et al*., 2007]. In HLA-B*27/B*57^+ LTNP, cell-associated HIV-1 DNA was found to be lower in the CD4 T_{CM} cells compared to levels in the same T-cell compartment for LTNP without these protective alleles [Descours *et al*., 2012]. Further, T_{CM} counts were preserved in patients with these protective alleles, and this correlated positively with the magnitude of the HIV-1 Gag-specific CD8 T-cell response [Descours *et al*., 2012]. In terms of immune activation, elite controllers were found to have lower frequencies of activated CD8 T cells (in terms of CD38 and HLA-DR co-expression) compared to

progressors, yet these levels were still significantly higher than those seen in uninfected persons [Brenchley *et al*., 2006; Kamya *et al*., 2011]. One study found activation levels in such patients to be associated with slow CD4 T-cell depletion [Hunt *et al*., 2008], while another reported that elevated activation levels in elite controllers did not correlate with a faster rate of CD4 T-cell decline [Kamya *et al*., 2011].

Another group of individuals from which important insights may be garnered, particularly in terms of correlates of protection against HIV-1, are individuals that do not get infected despite high exposure to HIV-1. These individuals are referred to as highly exposed seronegatives (HESN) and have been identified among intravenous drug users, children born to seropositive mothers, discordant couples, and commercial sex workers [Songok *et al*., 2012]. Only a fraction of these HESN have genotypes associated with protective HLA-alleles, TRIM5α, specific KIR-HLA associations, and APOBEC3G [MacDonald *et al*., 2000; Carthagena *et al*., 2008; Boyton & Altmann, 2007; Valcke *et al*., 2006]. Microarray analysis suggests that HIV-1 resistance occurs as a result of a down-regulation of genes that are involved in key signalling pathways that HIV-1 depends on for infection, such as natural killer cell cytotoxicity and TCR signalling [Songok *et al*., 2012]. Examination of samples from the genital mucosa of HESN commercial sex workers implicated lower T-cell activation/recruitment at the mucosal compartment as a means of reducing the number of HIV-1 target cells, which may play a role in natural protection from HIV-1 infection [Lajoie *et al*., 2012]. Moreover, it was found that the protective HLA-allele A*01:01 only recognises three Gag epitopes whereas B*07:02, an allele associated with susceptibility, binds thirty epitope variants, and it is this binding of more epitopes that is thought to be associated with susceptibility in HIV-1 infection [Luo *et al*., 2012].

1.4 Immunotherapy in treated HIV-1 infection

Immune-based therapeutic interventions in chronic treated HIV-1 infection have the potential to reverse T-cell dysfunction and modify immune responses to mirror those seen in LTNP or HIC [Imami *et al.,* 2001; Imami *et al*., 2007]. Ideal immunotherapeutic approaches would induce and

maintain HIV-1-specific responses, increase polyfunctionality, enhance thymic function, reduce levels of immune activation, reverse T-cell anergy/exhaustion, and improve skewed differentiation/maturation pathways [Imami *et al*., 2007]. A number of immunotherapeutic strategies in HIV-1 infection are described in the literature; however, this section will focus primarily on the immunomodulatory agents that are investigated in this thesis, namely recombinant human growth hormone (rhGH), the cytokines interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and therapeutic immunisation.

1.4.1 Recombinant human growth hormone

Growth hormone (GH) secretion and subsequent insulin-like growth factor-1 (IGF-1) release is essential for human growth and development, and it has been implicated to play a role in lymphocyte development and function [Welniak *et al*., 2002]. Early research indicated a role for GH in the improvement of a variety of immune functions, including B-cell responses [Kimata & Yoshida, 1994; Kimata & Fujimoto, 1994], macrophage activity and T-cell functions [Stephenson *et al*., 1991]. *In vitro* studies in mice have demonstrated the ability of GH to enhance T-cell functions, stimulate thymic growth, and improve the function of thymic epithelial cells [Snow *et al*., 1981; Knyszynski *et al*., 1992; Yamada *et al*., 1994]. GH administration in humans has also been linked with improvements in immune function, especially in elderly men and women [Khorram *et al*., 1997]. The role of the thymus in immune function is critical [Ho Tsong Fang *et al*., 2008], yet the mechanism by which GH affects the thymus in humans is unclear. It is possible that there is a direct effect, or an effect through IGF-1, which has been shown to have anti-apoptotic properties [Kooijman, 2006; Sabharwal & Varma, 1996]. It is also possible that GH could promote the production of IL-7 or stem cell factor from thymic epithelial cells. GH deficiency in humans, however, is not associated with thymic defects or T-cell deficiencies [Welniak *et al*., 2002], possibly representing redundancy.

In addition to the effects on the thymus, GH can affect T cells directly in the periphery; GH and IGF-1 have been shown to increase T-cell functions *in vitro* [Taub *et al*., 1994]. In accordance with

this effect on the periphery, up to 10% of peripheral blood lymphocytes secrete active GH [Varma *et al*., 1993; Hattori *et al*., 1990], and B cells contain the majority of GH mRNA [Hattori *et al*., 2001], indicating that GH can be produced and act locally. The GH receptor has been found to be expressed on activated T cells [Thellin *et al*., 1998] and at varying levels on peripheral T cells, B cells and neutrophils [Hattori *et al*., 2001]. IGF-1 and its receptor are also expressed by leukocytes; human leukocytes are capable of producing IGF-1 on stimulation with GH [Baxter *et al*., 1991], the main source of which are macrophages [Kirstein *et al*., 1992]. It is currently unclear which memory subsets of T cells express GH or IGF-1 receptors, and the direct effect that GH has on T cells.

During HIV-1 infection, naïve T cells are progressively depleted [Roederer *et al*., 1997] and their maintenance may depend on the thymus [Douek *et al*., 2003]. However it is likely that thymic function is impaired during HIV-1 infection [Douek *et al*., 2001; Dion *et al*., 2004]. It has been suggested that a role for GH in the immune response could be to counteract the immnosuppressive effects of immunological stress [Welniak *et al*, 2002]. As HIV-1 infection puts the immune system under stress, GH can potentially be used to enhance the effects of HAART on thymic recovery. A study in HIV-1 infected children indicated a role for GH in thymic and post-thymic pathways; growth hormone-deficient children show different percentages of effector and memory T-cell subsets, as well as smaller thymic volume and lower IL-7 levels in serum [Vigano *et al*., 2004]. Recombinant human growth hormone (rhGH) has been used to treat HIV-1-associated wasting and HIV-1-associated lipodystrophy [Gelato *et al*., 2007; Sivakumar *et al*., 2011], and was found to restore lean body mass and improve physical function, body weight, body composition and quality of life for HIV-1^{+} patients with HIV-1-associated wasting [Moyle *et al.,* 2004]. Furthermore, HIV-1-infected individuals with abdominal fat accumulation who received daily, low-dose rhGH demonstrated significant reductions in visceral fat, truncal obesity, and triglycerides [Lo *et al*., 2008]. A number of studies have shown that *in vivo* administration of rhGH to HIV-1-infected subjects may be associated with thymopoietic effects; circulating naïve CD4 T cells were found to be increased [Pires *et al*., 2004b; Napolitano *et al*., 2002], recent thymic emigrants (as measured by

T-cell receptor excision circle (TREC) content in PBMC) were found to be elevated [Napolitano *et al*., 2008; Smith *et al*., 2010], and thymic involution was shown to be reversed [Napolitano *et al*., 2002; Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010]. Moreover, rhGH has been shown to promote the restoration of T-cell responses against HIV-1 [Pires *et al*., 2004b; Herasimtschuk *et al*., 2008], and to reduce the levels of T-cell surface markers that are associated with immune activation and senescence [Napolitano *et al*., 2008]. rhGH has not been associated with reductions in levels of HIV-1 proviral DNA [Herasimtschuk *et al*., 2008], although this parameter has not been widely assessed in such studies. Additionally, rhGH has been shown to reverse NK cell dysfunction that is seen in treated chronic HIV-1 infection [Goodier *et al*., 2003].

1.4.2 Interleukin-2

Interleukin-2 (IL-2) was discovered in 1975 as a growth factor for bone marrow derived T cells and has since been extensively studied and found to have diverse effects on different cell types of the immune system [Oppenheim, 2007]. IL-2 belongs to a family of cytokines (that includes IL-4, IL-7, IL-9, IL-15 and IL-21), and signals through a receptor complex consisting of a specific IL-2 receptor alpha (CD25), IL-2 receptor beta (CD122) and a common gamma chain (γc) which is shared by all members of this family of cytokines [Wang *et al*., 2009]. Binding of IL-2 activates the Ras/MAPK, JAK/Stat and PI 3-kinase/Akt signalling molecules [Ellery & Nicholls, 2002]. Antigen binding to the TCR stimulates the secretion of IL-2, and the expression of IL-2 receptors and production of IL-2 is normally seen during an immune response [Smith *et al*., 1988]. IL-2 promotes the differentiation, growth and survival of antigen-selected T cells by the activation of specific genes [Beadling *et al*., 1993; Beadling & Smith, 2002], allowing the rapid selective expansion of naïve T cells, and the development of memory T cells [Smith, 1988; Hoyer *et al*., 2008]. This proliferation occurs via both pro-proliferative and anti-apoptotic signals [Ellery & Nicholls, 2002; Nelson *et al*., 1994].

Levels of IL-2 fall during HIV-1 infection, and low levels have been associated with disease progression [Johnson & Parkin, 1997]. The use of IL-2 as an immunotherapy in HIV-1 infection has

been widely studied and found to increase the number of circulating CD4 T cells, however despite sustained elevated levels of CD4 T cells, no clinical benefit was found following the administration of IL-2 in addition to ART in HIV-1-infected patients [Marchetti *et al*., 2008; Abrams *et al*., 2009]. In one study, IL-2 immunotherapy was not found to alter the number of peripheral T_H17 cells in ART-treated HIV-1^+ patients, but it did expand the Treg cell population, which might suppress HIV-1-specific responses [Ndhlovu *et al*., 2010], although reports on the effect of Treg cells in HIV-1 infection have been mixed [Elahi *et al*., 2011]. Furthermore, IL-2 therapy was not associated with reductions in the HIV-1 viral reservoir [Pett *et al*., 2010], nor was it found to alter the proportion or activation state of CD4 T cells in the gut mucosa [Read *et al*., 2011]. IL-2 has been suggested as a candidate molecule to use in conjunction with therapeutic immunisation to increase HIV-1-specific responses and to control viral load, but results to date have been conflicting [Levy *et al*., 2005; Hardy *et al*., 2007]. These divergent reports may be due in part to differences in the patient cohorts studied, and the stage of HIV-1 disease, length of time on ART, CD4 T-cell count and nadir CD4 T-cell count are important parameters to consider in such trials [Hardy *et al*., 2007].

1.4.3 Granulocyte macrophage colony stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a haematopoeitic growth factor and immune modulator; it promotes the differentiation and mobilisation of myeloid cells *in vivo* [King *et al*., 2010]. It was initially defined by its ability to generate granulocytes and macrophage colonies from precursor cells [Burgess & Metcalf, 1980], and it can enhance the number and function of various cell types including neutrophils, monocytes and lymphocytes [Shi *et al*., 2006]. It is primarily produced by activated T and B cells but also by macrophages, fibroblasts, eosinophils, epithelial, and endothelial cells [Griffin *et al*., 1990]. GM-CSF production is minimal under normal conditions and one study found only low levels in the sera of half of individuals tested [Cebon & Layton, 1994]. GM-CSF acts in an autocrine and paracrine fashion on target cells expressing its receptor. The receptor is expressed on neutrophils, eosinophils, monocytes, macrophages, as well as granulocyte-macrophage progenitors and signalling through the receptor

induces changes in the activation state and function of cells [Hansen *et al*., 2008]. In addition to its function as a growth factor, it has been suggested that GM-CSF is a pro-inflammatory cytokine [Hamilton, 2002]. It has been found at raised levels in sera in response to LPS [Sheridan & Metcalf, 1972] and in the joints of patients with arthritis [Williamson *et al*., 1988]. It may have a role in various human inflammatory diseases such as rheumatoid arthritis and pathological changes follow its over-expression [Hamilton & Anderson, 2004]. mRNA for the GM-CSF receptor has been found on T cells indicating a possibility that it is expressed on T lymphocytes [al-Aoukaty *et al*., 1994]. *In vitro* studies have shown that GM-CSF can augment the induction of a lymphokine-activated killer (LAK) response to IL-2 and also IL-2 induced proliferation [Stewart-Akers *et al*., 1993; Santoli *et al*., 1988]. *In vitro* studies have shown GM-CSF to enhance the activity of antiretroviral agents, suggesting a role for GM-CSF in the purging of viral reservoirs when used in conjunction with HAART [Hammer *et al*., 1990], while *in vivo* studies have indicated a lower frequency of antiretroviral drug-resistant HIV-1 mutants in subjects receiving GM-CSF in addition to zidovudine (AZT) [Brites *et al*., 2000]. It is unknown whether administration of GM-CSF results in a Th1 or Th2 T-cell response, although it has been suggested that it is capable of inducing both, depending on the conditions [Shi *et al*., 2006]. A mouse model study using a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF reported a greater than 7-fold increase in the CD4 T-cell response in terms of IFN-γ production [Barouch *et al*., 2002]. Another study in mice showed complete protection from viral challenge with a recombinant vaccinia virus expressing HIV-1 gp160 co-administered with GM-CSF, IL-12, and TNF-α; and in these experiments, GM-CSF was found to enhance antigen presentation [Ahlers *et al*., 2001]. Thus, GM-CSF has been shown to alter T-cell responses directly, as well as indirectly, and to provide a link between innate and adaptive immune responses [Shi *et al*., 2006].

The potential for the use of GM-CSF as an immune-based therapy in HIV-1 infection has been considered and it has been the subject of a number of phase III clinical studies [Brown & Angel, 2005]. GM-CSF production is significantly reduced in HIV-1 infection [Esser *et al*., 1996; Esser *et*

al., 1998], and replacement therapy in treated HIV-1^+ individuals has been shown to significantly increase the number of CD4 T cells [Angel *et al*., 2000], reduce viral load and improve clinical outcome [Brites *et al*., 2000], although these results have not been universally observed [Brown & Angel, 2005]. Clinical benefit has been attributed to the co-administration of IL-2 and GM-CSF in HIV-1-infected patients on effective ART with mycobacterial immune reconstitution inflammatory syndrome (IRIS), suggesting the potential for such therapy in later stages of HIV-1 disease [Imami *et al*., 1999; Pires *et al*., 2005].

1.4.4 Therapeutic immunisation

Further to the cytokines and hormones mentioned above, HIV-1-specific T-cell responses can be induced by therapeutic immunisation [Heath & Kilby, 2006]. The introduction of exogenous HIV-1 antigens promotes protective immune responses by steering T cells to react to conserved HIV-1 epitopes [McMichael, 2006]. Antigen-specific immunity can be achieved through HIV-1-specific DNA immunisation: one or multiple HIV-1 genes are cloned into a mammalian expression plasmid, which is delivered to the host. The host in turn expresses the DNA encoded antigen within its own cells [Ulmer *et al*., 1993]. As genetic vaccines are endogenously processed through the cellular machinery they are capable of producing CTL clones as well as inducing neutralizing antibodies [Donnelly *et al*., 2005].

Therapeutic vaccinations in rhesus macaques have shown that immunity can be stimulated and viral loads reduced [Lu *et al*., 2003], and SIV-specific responses were enhanced when a DNA vaccine encoding SIV structural proteins was given in conjunction with plasmids expressing IL-12 or IL-15 to chronically infected rhesus macaques treated with anti-retrovirals [Halwani *et al*., 2008]. Studies in HIV-1-infected individuals have shown reductions in plasma viraemia and evidence of improved HIV-1-specific T-cell responses following immunisation [Lu *et al*., 2004; Levy *et al*., 2005; MacGregor *et al*., 2005; Moss *et al*., 2000]. In a recent study, therapeutic vaccination with LFn-p24C, a detoxified anthrax-derived polypeptide fused to the subtype C HIV Gag p24 protein, was shown to be well-tolerated with HIV-1 RNA levels remaining undetectable after three

immunisations, increased CD4 T-cell counts in the vaccine group compared to the placebo-treated group after 12 months, and HIV-1-specific responses associated with higher CD4 T-cell gains [Kityo *et al*., 2011]. Following a 30-day treatment interruption, eight out of twenty four individuals showed no evidence of viral rebound during this period, and for all subjects, viral load was quickly suppressed once ART was resumed [Kityo *et al*., 2011].

The vaccine used in the study (GTU®-MulltiHIV B Clade) was provided by FIT Biotech (Finland). It is comprised of both regulatory and structural HIV-1 proteins (Rev, Nef, Tat, Gag p17, Gag p24 and a stretch of 11 cytotoxic T lymphocyte (CTL) epitope clusters from Pol and Env), representing the B clade, cloned into a DNA vector termed the Gene Transport Unit (GTU®). The GTU® also codes for a nuclear anchoring protein (bovine papilloma virus E2) in order to maintain the vector and to keep the expression level of the selected immunogens high [Krohn *et al*., 2005; Blazevic *et al*., 2006]. E2 is a transcriptional regulator derived from bovine papilloma virus (BPV) [Ustav *et al*., 1991] which has a dual function within the vector: it enhances the transcription and expression of the cloned MultiHIV antigen, and also binds simultaneously to stretches of BPV origin present in the vector, and to chromatin, thus assuring that the plasmid is not lost during mitosis in the dividing cells [Ilves *et al*., 1999]. The stability of the vector is enhanced and prolonged expression of the cloned antigen is facilitated. Figure 1.8 shows a schematic representation of the plasmid vector. This construct is expected to induce a broad immune response against several HIV proteins, producing a vaccine that might be effective in treated HIV-1^{+} individuals with high baseline CD4 T-cell counts (400 cells/µl blood) in conjunction with immune-based therapy (IBT) [Imami *et al*., 2007; Krohn *et al*., 2005].

The investigational vaccine has been tested in Phase I clinical trials in non-infected individuals and HAART-treated individuals in Finland and was found to be safe and well tolerated [Malm *et al*., 2005; Blazevic *et al*., 2006]. A Phase II clinical trial in treatment naïve HIV-1 infected individuals in South Africa is currently ongoing.

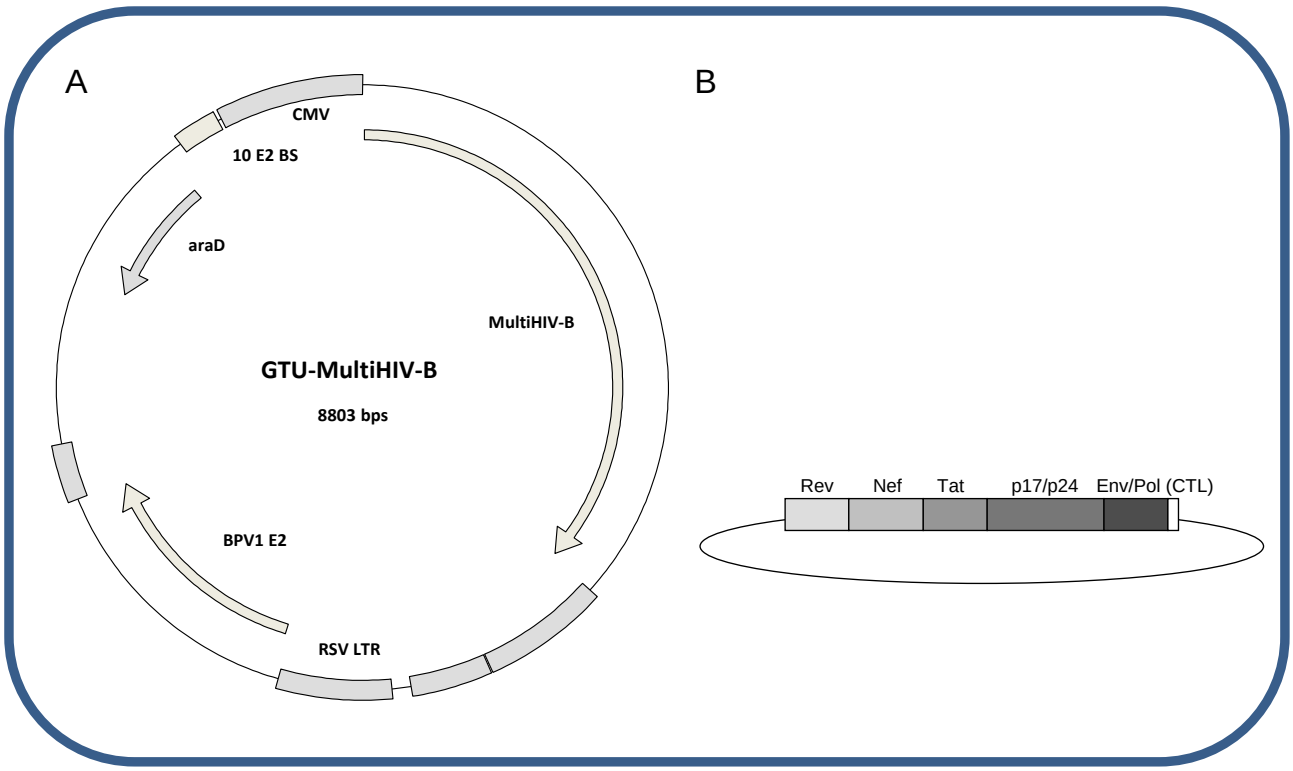


Figure 1.8 The GTU®-MultiHIV-B (B-clade specific) plasmid construct.
(A) The construct consists of a strong cytomegalovirus promoter, the intron/poly A sequence and an additional expression cassette for E2 with an additional viral promoter (such as RSV LTR or a CMV promoter). Also present are multimeric E2 protein binding sites (typically ten copies of BPV1 binding sites are added to the vector). In the Auxo-GTU® variant, the antibiotic resistance gene has been replaced with the Ara D gene thus allowing production in bacterial cells without an antibiotic selection system (adapted from [Krohn *et al*., 2005]. (B) The MultiHIV-B antigenic insert consists of a fusion of the full-length regulatory proteins Rev, Nef and Tat, the structural proteins p17 and p24 and stretches of T helper (T_H) and cytotoxic T lymphocyte (CTL) epitopes from the Pol and Env proteins (adapted from [Malm *et al*., 2005]).

1.5 Aims and hypotheses

The overall hypothesis, based on a large amount of preliminary data, was that rational immunotherapy designed to induce the types of immune responses seen in LTNPs, and to reverse the anergic immunological state characteristic of chronic progression, should improve long-term cellular functional memory responses, which would result in greater virologic control and slower disease progression in treated chronic HIV-1 infection. Efficacious immune-based therapy may eventually enable chronically infected individuals to stop, or at least interrupt, HAART for prolonged periods of time. The central questions that I wished to address were ‘how can we induce or enhance protective HIV-1-specific immune responses, such as those observed in LTNP, in chronic progressors?’, and ‘how can we reverse persistent disrupted immunophenotypes that are

seen in treated HIV-1-infected subjects compared to healthy seronegative controls?' Studies described in this thesis aim to pursue immune-based therapeutic approaches in chronically infected, HAART-treated HIV-1^+ subjects, in order to evaluate both the quality and the quantity of the T-cell response. The main hypotheses for each chapter were as follows:

1. Chapter 3
    a) Daily dosing with rhGH in addition to suppressive ART was hypothesised to boost HIV-1-specific T-cell responses, and these responses subsequently maintained following cessation of rhGH therapy.
    b) Low-dose, longer term treatment with rhGH in ART-treated HIV-1^+ patients was predicted to similarly enhance HIV-1-specific T-cell responses, and reduce the expression of T-cell markers associated with chronic immune activation and exhaustion.
    c) *In vitro* culture of PBMC with rhGH was hypothesised to result in improved immunophenotype, and characterisation of the GH receptor expected to reveal down-regulation with HIV-1 infection (as compared to seronegative donors).
2. Chapter 4
    a) Administration of therapeutic immunisation was hypothesised to result in boosted HIV-1-specific T-cell responses, while concomitant administration of IL-2, GM-CSF and rhGH postulated to maintain these responses, and the administration of cytokines and hormone alone (or with therapeutic immunisation) was predicted to result in increased CD4 T-cell counts, improved functionality and phenotype at the final study time point compared to baseline.
    b) It was also hypothesised that this treatment schedule would be safe and well-tolerated.
3. Chapter 5
    a) It was hypothesised that immune defects would persist in HIV-1^+ patients despite suppressive ART compared to healthy control subjects, in terms of T-cell surface expression of markers of immune activation, exhaustion and senescence.

b) HIV-1 Gag- and Nef-specific functional responses were predicted to correlate with particular immunophenotpyes and successfully treated patients with low responses to these peptides predicted to have elevated levels of markers associated with immune activation, exhaustion, senescence and apoptosis on CD4 and CD8 T cells.

To this effect, rhGH was administered to HIV-1-infected patients on suppressive ART, both at a supraphysiological/pharmacological dose and at a lower physiological dose. Additionally, therapeutic immunisation of HAART-treated HIV-1^{+} patients in conjunction with the administration of the cytokines IL-2, GM-CSF and rhGH was assessed. The primary aim was to investigate the effects of these immunomodulators and HAART on the immune system. The clinical implications of such immune-based therapeutic approaches in terms of patient benefit are immense, and would ideally result in slower disease progression, improved quality of life, and the potential for maintained virologic control during HAART interruption. Harnessing the plethora of current detailed scientific knowledge of the immune system and its role in controlling viral infections including HIV-1, and translating such knowledge into useful immunotherapy for patient benefit is extremely challenging; it is of immediate importance and addresses a substantial unmet clinical need. Although viral eradication from the infected host must remain the ultimate goal; enabling the host to co-exist with the virus may be a more realistic immediate goal.

Chapter 2 Materials and Methods

2.1 Overview

All work with HIV-1^+ samples was carried out in the Containment Level III laboratory in the Department of Immunology at the Chelsea and Westminster Hospital, London, UK. All blood samples were processed immediately after collection and all phenotypic and functional assays carried out on fresh PBMC, unless otherwise detailed.

2.2 Seronegative controls

HIV-1 seronegative control individuals were recruited from the Department of Immunology following Imperial College London Occupational Health guidelines on sampling procedures.

2.3 Patient cohort and ethical approval

Patient samples acquired for the *in vitro* studies described in this report were obtained from the >5000 HIV-1^+ cohort from the St Stephen's Centre at the Chelsea and Westminster Hospital [Mandalia *et al*., 2012]. Informed consent was obtained and all *in vitro* work was carried out under the ethical approval of the Riverside Research Ethics Committee (RREC1108).

The rhGH study was approved by the RREC (REC reference number: 04/Q0406/119) and by the Chelsea and Westminster Hospital NHS Trust, and the patients' informed consent was obtained. The low-dose rhGH study was registered at ClinicalTrials.gov (NCT 00119769) and the trial was approved by the regional scientific ethical committee, the Danish Medicines Agency, and the Danish Data Protection Agency 2004. Good Clinical Practice (GCP) standards were monitored and approved by the GCP Unit at the Copenhagen University Hospital, Denmark, and the Danish Medicines Agency. Ethics committee approval for the shipment of samples to the UK and approval from the Data Protection Agency was obtained prior to the shipment of the cryopreserved PBMC.

The IMIRC 1003 study was registered with EudraCT (reference number 2008-000575-24) and experiments carried out on *ex vivo* clinical trial samples were approved by the relevant regulatory

authorities, including local and national ethical committees: the Genetic Therapy Advisory Committee (GTAC; reference number 145); the Medicines and Healthcare products Regulatory Agency (MHRA; reference number 19174/0262/001-0004); and the Research and Development office at the Chelsea and Westminster Healthcare NHS Trust (Research and Development study reference number 08IMM001). This study was also registered at ClinicalTrials.gov (NCT01130376).

2.4 Sample collection

Venous blood was drawn into Vacutainer™ 6ml lithium heparin (ref. 367885) and 10ml EDTA (ref. 367873) blood collection tubes (Becton Dickinson, Oxford, UK).

2.5 Viral load quantification

Plasma viral load was determined by the use of the Versant HIV-1 RNA 3.0 bDNA assay (Siemens Healthcare Diagnostics, Camberley, UK). The assay was carried out according to the manufacturer's instructions and had a detection range of 50 to 500,000 copies/ml plasma. Monitoring of viral load was performed as part of the patients' routine care by St Mary's Hospital, Department of Virology, Imperial College London Healthcare NHS Trust. A substantial protocol amendment for the IMIRC 1003 study was approved by GTAC and the MHRA (on the 13th April 2010) to change the laboratory which processed the HIV-1 viral load from St Mary's Hospital, Imperial College London Healthcare NHS Trust to Barts and the London NHS Trust (for economic reasons within the Chelsea and Westminster Hospital NHS Trust).

2.6 Lymphocyte subset analysis

Murine anti-human monoclonal antibodies to CD3, CD4, CD8, CD56, CD19 and CD45 (Tetra One, Beckman Coulter, High Wycombe, UK) were used to mark lymphocytes within whole blood. Lymphocyte numbers were then evaluated using a Cytomics FC 500 flow cytometer (Beckman Coulter) and Tetra CXP (version 2.2) software [Burton *et al*., 2011]. This was carried out routinely

within the Clinical Laboratory in the Department of Immunology at the Chelsea and Westminster Hospital.

2.7 Preparation of human male AB serum

Frozen human male AB serum (50ml, Sigma Aldrich, Poole, UK, catalogue number H4522), screened for HIV-1 and -2, Hepatitis B and C, and CMV by the supplier, was heat inactivated by heating to 56˚C in a water bath for 30 minutes, inverting every ten minutes. This procedure inactivated complement but did not affect other essential components within the AB serum. The resulting 50ml of heat inactivated human male AB serum was then separated into 4ml aliquots in a sterile environment and stored at -20°C until required.

2.8 Preparation of media and solutions

Standard tissue culture medium (TCM) was made by adding Penicillin/Streptomycin (final concentration 100IU/ml and 100μg/ml respectively) and L-glutamine (final concentration 2mM) (+PSG) to RPMI-1640 (all from Sigma), and mixing gently. Freezing medium was prepared by supplementing foetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO) (both from Sigma).

2.9 Separation of plasma

Blood samples collected in EDTA or heparinised Vacutainers^{TM} were centrifuged at 1500rpm for ten minutes to separate the plasma. Plasma was aspirated and stored in 1ml aliquots at -80°C for future use. The volume of plasma removed was replaced with the same volume of Dulbecco's Phosphate Buffered Saline (PBS; Sigma) and the sample processed further.

2.10 Separation of peripheral blood mononuclear cells (PBMC) from whole blood

Whole blood was diluted in a 1:1 ratio with PBS and mixed gently. Generally, heparinised whole blood was used for functional studies and EDTA whole blood used for phenotypic and genetic studies unless otherwise specified.

Density gradient centrifugation utilising Histopaque (Sigma) was used to separate PBMC from whole blood. In a sterile 50ml tube 20ml of the whole blood/PBS mix was overlaid onto 20ml of Histopaque and centrifuged at 2000rpm for 20 minutes at room temperature without the use of centrifuge brakes. A sterile Pasteur pipette was used to aspirate the resulting interface layer (PBMC) into a new sterile 50ml tube. The PBMC were washed once with RPMI-1640+PSG (for functional experiments) or PBS and centrifuged at 1800rpm for 10 minutes at room temperature with brakes on full to remove any Histopaque. The PBMC were then washed two more times with RPMI-1640+PSG (for functional experiments) or PBS and centrifuged at 1500rpm for 10 minutes at room temperature.

Cell count and viability was measured using 25µl of the 50ml cell suspension stained with 25µl of 0.4% Trypan blue (Sigma) . A KOVA Glasstic® slide (Hycor Biomedical Inc., Edinburgh, UK) and microscope with magnification x 40 was used to count the cells. Live cells (those that did not stain blue) in two of the large squares were counted and the average number of cells per large square was used.

The total number of cells present in the sample was calculated using the following formula:

Total number of cells:

Average number of cells in large square **x** chamber factor **x** dilution factor **x** total volume

= Average number of cells in large square **x** 10^4 **x** 2 **x** 50

Only cell samples with cell viability greater than 70% were used. This was particularly important with HIV-1^+ samples as virus-specific PBMC may be disproportionately lost. In one study, PBMC preparations from HIV-1^+ subjects with viability greater than or equal to 70% demonstrated consistent lympoproliferative responses and were found to be suitable for functional analysis [Weinberg *et al.*, 2000].

2.11 Cryopreservation and thawing of PBMC

Freezing medium and a Nalgene® box (Nalge Nunc International, Hereford, UK) filled with isopropanol (Sigma) were cooled to 4°C. Cryotubes (Nalge Nunc International) were labelled and

placed in the box. PBMC were resuspended in the cold FCS/DMSO freezing medium to give a concentration of 5 x 10^6 – 1 x 10^7 cells/ml. One ml of the cell suspension was transferred immediately to each cold cryotube and kept at 4°C. The Nalgene® box containing the cryotubes was then placed at -80°C for 24 hours. After 24 hours the cells were quickly transferred to liquid nitrogen (-170°C) for long-term storage.

Cells were thawed as rapidly as possible at 30°C and resuspended in 50ml ice cold PBS (Sigma), added dropwise initially, then at a quicker rate. They were then immediately centrifuged at 1800rpm for 10 minutes, the supernatant discarded and centrifugation repeated to wash the cells. Thawed cell number and viability was then established using the method described in section 2.10.

2.12 Surface staining of PBMC for flow cytometric analysis

Specific antibodies for surface staining were aliquoted into labelled FACS tubes (Greiner Bio-One Ltd., Stonehouse, UK) as shown in the original panel (Table 2.1), in the expanded panel (Table 2.2) and in the panel used to characterise growth hormone receptor (GHR) expression (Table 2.3). 7µl of fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophyocyanin (APC) and phycoerythrin (PE)-Cy7 – conjugated antibody were added to each tube. The volumes of peridinin chlorophyll protein (PerCp)-Cy5.5 and allophyocyanin (APC)-H7 were 5µl and 3µl respectively per tube. Table 2.4 details the antibody, the fluorochrome conjugate, the clone, and the source, for all antibodies used in the experiments described in this thesis.

The washed pellet of 1 x 10^7 PBMC was resuspended in 600µl of PBS and this was divided between the tubes on the panel in Table 2.2 (giving approximately 100µl and 1.7 x 10^6 cells per tube). Incubation was carried out in the dark, at room temperature for 30 minutes. Cells were then washed by adding 2ml PBS to each tube and centrifuging at 1500rpm (600g) for 5 minutes. The supernatant was discarded and the cells resuspended in 200µl of BD Stabilizing Fixative (buffered solution containing 3% paraformaldehyde; prepared by diluting the 3x concentrate 1:2 with deionized water) and stored in the dark at 4°C.

Acquisition was carried out on a BD six-colour LSRII flow cytometer (BD Biosciences, Oxford, UK). Fifty thousand gated lymphocyte events were acquired per sample (ten thousand for the isotype controls) and these were analysed using FACSDiva™ Software version 6.1.2 (BD Biosciences, San Jose, CA, USA).

Table 2.1 Original antibody combinations designed for six-colour immunophenotyping.
The panel consists of one isotype control tube (1) and three experimental tubes (2-4). Each antibody and its conjugated fluorochrome are detailed. All antibodies in the panel were obtained from BD Biosciences (Oxford, UK) except CCR7 which was sourced from R&D Systems (Abingdon, UK).

Tube	FITC	PE	APC	PE-Cy7	PerCP-Cy5.5	APC-H7
1	IgG_1	IgG_1	IgG_1	IgG_1	CD3	CD8
2	CD57	CD38	CD95	HLA-DR	CD3	CD8
3	CD27	CD25	CD28	CD45RO	CD3	CD8
4	PD-1	CCR7	CD45RA	PD-L1	CD3	CD8

Table 2.2 Expanded antibody panel used for six-colour immunophenotyping.
The panel consists of one isotype control tube (1) and five experimental tubes (2-6). Each antibody and its conjugated fluorochrome are detailed. All antibodies in the panel were obtained from BD Biosciences (Oxford, UK) except CCR7 and TIM-3 which were sourced from R&D Systems (Abingdon, UK) and PTK-7 which was obtained from Miltenyi Biotech Ltd (Bisley, UK).

Tube	FITC	PE	APC	PE-Cy7	PerCP-Cy5.5	APC-H7
1	IgG_1	IgG_1	IgG_1	IgG_1	CD3	CD8
2	CD57	CD38	CD95	HLA-DR	CD3	CD8
3	CD27	CD25	CD28	CD45RO	CD3	CD8
4	PD-1	CCR7	CD45RA	PD-L1	CD3	CD8
5	HLA-DR	CD31	PTK-7	CD45RA	CD3	CD8
6	CD4	TIM-3	CTLA-4	CD69	CD3	CD8

Table 2.3 Antibody panel for assessment of growth hormone receptor (GHR) on T cells.
The panel consists of one isotype control tube and one experimental tube. Three different GHR antibodies were tested and these are detailed in Table 2.4. The GHR antibodies and FITC control are from Santa Cruz Biotechnology (Heidelberg, Germany), CCR7 is from R&D Systems (Abingdon, UK) and all other antibodies are from BD Biosciences (Oxford, UK).

Tube	FITC	PE	APC	PE-Cy7	PerCP-Cy5.5	APC-H7
1	IgG_1	IgG_1	IgG_1	IgG_1	CD3	CD8
2	GHR	CCR7	CD45RA	HLA-DR	CD3	CD8

Table 2.4 Monoclonal antibodies used in six-colour flow cytometric analysis.
This table shows the antibody, its fluorochrome, clone, and isotype. All antibodies in the panel were obtained from BD Biosciences (Oxford, UK) except CCR7 and TIM-3 which were sourced from R&D Systems (Abingdon, UK), PTK-7 which was obtained from Miltenyi Biotech Ltd (Bisley, UK) and the GHR antibodies, and respective isotype control, which were from Santa Cruz Biotechnology (Heidelberg, Germany).
NA – not aplicable; the company does not have a specific clone name for this product.

Antibody	**Fluorochrome**	**Clone**	**Isotype**
IgG_1	FITC	MOPC-21	IgG_1, κ
IgG_1	PE	G18-145	IgG_1, κ
IgG_1	APC	MOPC-21	IgG_1, κ
IgG_1	PE-Cy7	X40	IgG_1, κ
CD3	PerCP-Cy5.5	SK7	IgG_1, κ
CD8	APC-H7	SK1	IgG_1, κ
CD57	FITC	HNK-1	IgM, κ
CD38	PE	HB7	IgG_1, κ
CD95	APC	DX2	IgG_1, κ
HLA-DR	PE-Cy7	L243	IgG_{2a}, κ
CD27	FITC	M-T271	IgG_1, κ
CD25	PE	2A3	IgG_1, κ
CD28	APC	CD28.2	IgG_1, κ
CD45RO	PE-Cy7	UCHL1	IgG_{2a}, κ
PD-1	FITC	MIH4	IgG_1, κ
CCR7	PE	150503	IgG_{2a}, κ
CD45RA	APC	HI100	IgG_{2b}, κ
PD-L1	PE-Cy7	MIH1	IgG_1, κ
HLA-DR	FITC	L243 (G46-6)	IgG_{2a}, κ
CD31	PE	WM59	IgG_1, κ
PTK-7	APC	188B	IgG_{2a}, κ
CD45RA	PE-Cy7	L48	IgG_1, κ
CD4	FITC	SK3	IgG_1, κ
TIM-3	PE	344823	IgG_{2a}, κ
CTLA-4	APC	BNI3	IgG_{2a}, κ
CD69	PE-Cy7	FN50	IgG_1, κ
IgM	FITC	G255-228	IgM, κ
GHR	FITC	MAB1	IgG_1, κ
GHR	FITC	3H2186	IgG_1, κ
GHR	FITC	MAB263	IgG_1, κ
IgG_1	FITC	NA	IgG_1, κ

2.13 Gating strategies for flow cytometric analysis

Forward scatter (FSC) and side scatter (SSC) properties were used to determine the lymphocyte population. A dot plot displaying lymphocytes was then used to define the $CD4^+$ T cells (these were $CD3^+CD8^-$) and $CD8^+$ T cells ($CD3^+CD8^+$). Further characterisation of the $CD4^+$ and $CD8^+$ T cells was carried out by looking at these populations on dot plots with percentage expression of each of the markers determined using quadrant gates. The quadrants were set based on logical gating of distinct populations (confirmed with density plots), CD3 back-gating and verified with isotype matched controls that were run for each tube (as described in section 2.12, and Table 2.1 and Table 2.2). Histograms were also plotted for each marker to elucidate the expression of each of the markers in terms of mean fluorescence intensity (MFI). A representative example of the gating strategy, looking at the expression of the activation markers CD38 and HLA-DR on CD4 and CD8 T cells is shown in Figure 2.1. In order to determine the differentiation/maturation state of both T-cell subsets, surface expression of CD45RA and the lymph-node homing CCR7 were used (Figure 2.2). Cells positive for both markers were defined as naïve T cells ($CCR7^+CD45RA^+$), with the loss of CD45RA expression resulting in a central memory phenotype (T_{CM}; $CCR7^+CD45RA^-$), double negatives were the effector memory T cells ($CCR7^-CD45RA^-$; T_{EM}), and terminally differentiated T cells were those re-expressing CD45RA ($CCR7^-CD45RA^+$; T_{EMRA}). This was done for both CD4 and CD8 T cells (Figure 2.2B and Figure 2.2C, respectively). The histograms represent expression of PD-1 on each of the T-cell subsets; this gating strategy and analysis was also used to determine PD-L1 expression according to differentiation/maturation status.

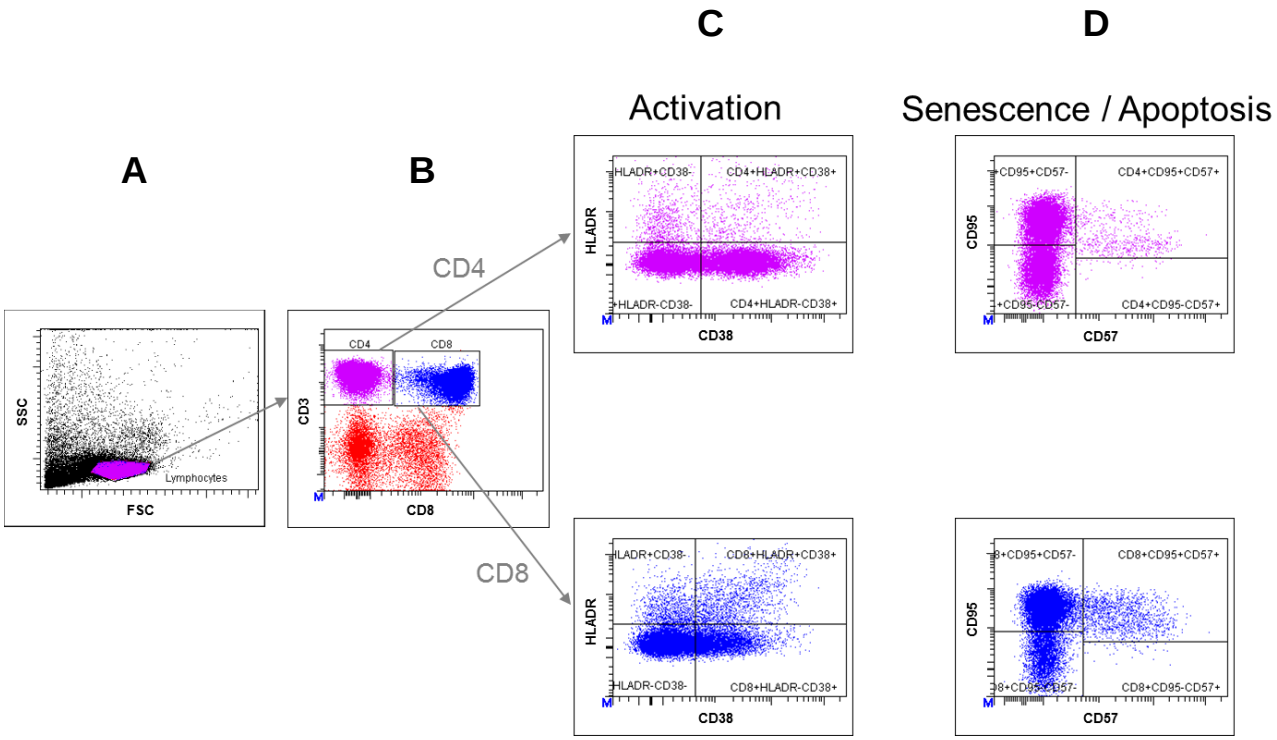


Figure 2.1 Gating strategy for flow cytometric analysis.
Forward and side scatter properties were used to gate tightly around the lymphocyte population (A). $CD4^+$ and $CD8^+$ T cells were defined based on their expression of CD3 and CD8, with CD4 T cells $CD3^+CD8^-$ and CD8 T cells $CD3^+CD8^-$ (B). In this example, the activation markers CD38 and HLA-DR were quantified on both the CD4 and CD8 T-cell populations (C), as was the expression of the senescence marker CD57 and the apoptosis marker CD95 (D). Other surface markers were assessed similarly with percentage expression in the quadrant gates noted. These plots show the PBMC profile from an HIV-1 infected individual (O523, screen visit).

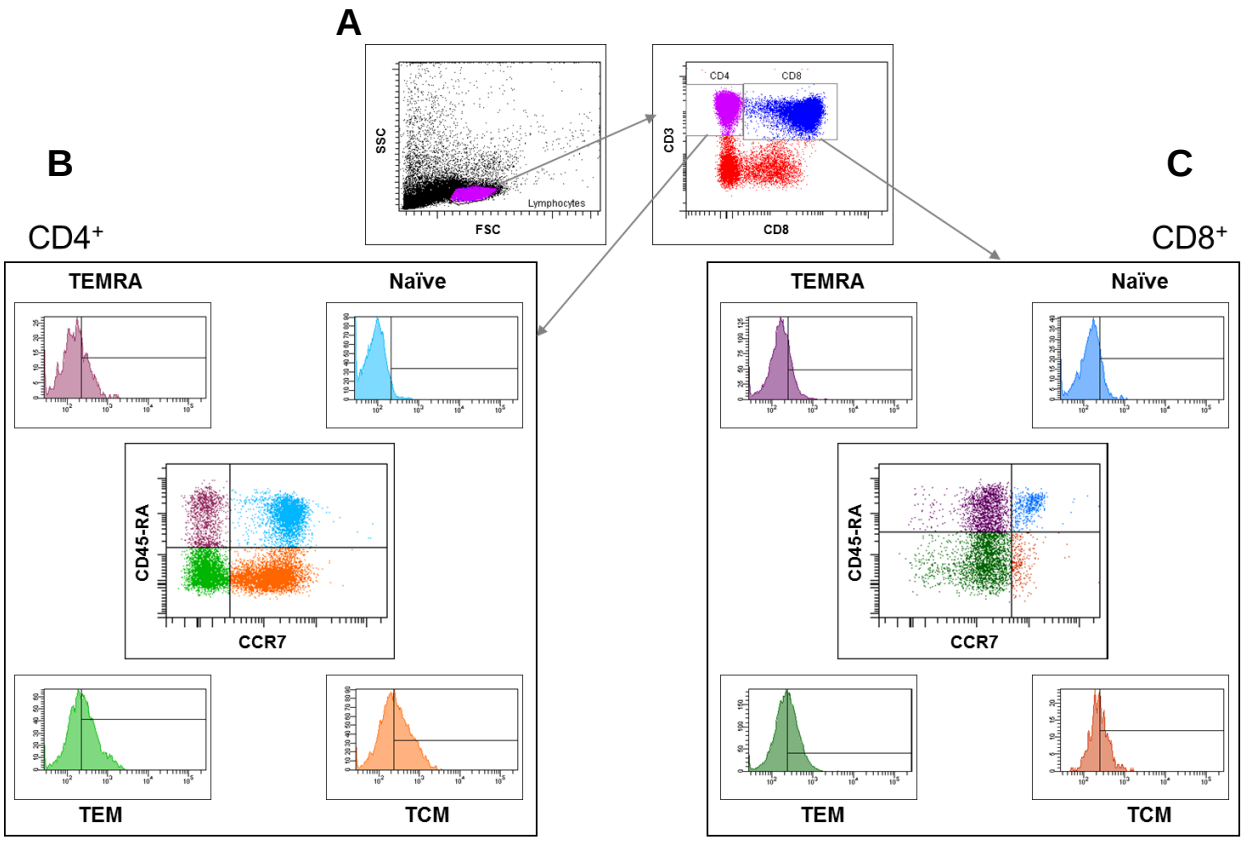


Figure 2.2 Gating strategy for differentiation/maturation of T-cell subsets.
A lymphocyte gate was set and used to assess $CD3^+CD8^-$ (CD4) and $CD3^+CD8^+$ (CD8) T cells (A). The differentiation/maturation state of the CD4 (B) and CD8 (C) T cells was assessed using surface expression of the markers CCR7 and CD45RA. Double positives are naïve T cells ($CCR7^+CD45RA^+$), $CCR7^+CD45RA^-$ are central memory (T_{CM}), double negatives ($CCR7^-CD45RA^-$) are effector memory (T_{EM}), and $CCR7^-CD45RA^+$ are terminally differentiated T cells (T_{EMRA}). The histograms represent PD-1 expression in each of the subsets and this gating was also used to determine PD-L1 expression. These plots show the PBMC profile from an HIV-1^+ patient (K087).

2.14 PBMC culture

Isolated and washed PBMC were cultured in duplicate in a 24-well, flat-bottom plate at a concentration of 2 x 10^6 cells/ml in RPMI-1640+PSG and 10% human male AB serum. rhGH (ImmunoTools, Friesoythe, Germany) re-suspended in TCM was added at a final concentration of 1µg/ml. Duplicate wells were also run in parallel with TCM without rhGH. Cultures were incubated at 37°C and 5% CO_2 for 72 hours. Following culture, the cells (and TCM) were collected into sterile 15ml tubes. The wells were rinsed with 2ml PBS and this was transferred to the tubes prior to washing. Cells were counted and viability was assessed. After two washes with PBS, the pelleted cells were resuspended in 500µl PBS and phenotyping was carried out as described in section 2.12.

Preliminary experiments were also set up using recombinant human insulin-like growth factor 1 (rhIGF-1; ImmunoTools, Friesoythe, Germany). The protocol was carried out as detailed above, testing the following concentrations of rh-IGF-1: 1ng, 10ng, 100ng and 1µg/ml. Cultures were left for 72 hours and phenotypic analysis was carried out, as previously described.

2.15 Lymphoproliferation assays

Triplicate cultures of PBMC (10^5 per well) in 10% human male AB serum/RPMI-1640+PSG were set up in 96-well round-bottomed microtiter plates (Greiner, Stonehouse, UK). These were stimulated with HIV-1 recombinant antigens and peptides obtained from the Medical Research Council Centralised Facility for AIDS Reagents (NIBSC, Potters Bar, UK), and used as previously described [Imami *et al*., 2002]. On day five, each well was pulsed with 1µCi ^{3}H-methyl thymidine (^{3}H-TdR; Amersham International, Amersham UK) and after 16 hours, the cells were harvested onto glass fibre filtermats (Wallac Oy, Turku, Finland). Liquid scintillation spectroscopy using a 1205 Betaplate counter (Wallac) was used to evaluate proliferation, as measured by ^{3}H-TdR incorporation. Wells containing PBMC in AB serum and TCM only were used as a control to calculate background activity. Results are expressed as stimulation index (SI) scores; calculated by dividing the experimental result by the background result. An SI score of five or more is defined as a positive response.

2.16 Preparation of peptides and antigens

2.16.1 Preparation of HIV-1 antigens, peptides and recombinant vaccinia vectors (rVV)

Cells were cultured with recombinant antigen at a final concentration of 10µg/ml [Imami *et al*., 1999; Herasimtschuk *et al*., 2008]. Inactivated, gp120-deleted, HIV-1 immunogen (Remune™), and its constituent Gag p24 antigen ('native' p24: clade G; both from Immune Response Corp. Carlsbad, San Diego, CA) were used at 3µg/ml [Moss *et al*., 1999]. Gag p24 peptides were used at a final concentration of 4µg/ml each in a pool of 22; these were 20mers with a 10 amino acid overlap covering p24 Gag (aa 133-363 of HIV-1 SF2, ARP 788.1-.22). Pol reverse transcriptase (RT)

peptides were used at a final concentration of 1µg/ml each in a pool of 110. These peptides were 15mers overlapping by 10 amino acids and covered RT (HIV-1 LAI, ARP 7010.1-.110). The recombinant vaccinia virus (rVV) constructs encoded Gag, Pol or PB2 (influenza A virus basic polymerase 2 subunit as a negative control; kindly gifted by G. Smith) proteins were obtained through the NIH AIDS Research and Reference Reagent Program (Rockville, MD, USA) and from the MRC AIDS reagent project (NIBSC).

2.16.2 Preparation of peptide pools for low-dose rhGH study

Pools of overlapping Gag 20mer and Gag 9mer peptides were prepared at a concentration of 10µg/ml in culture medium (RPMI-1640+PSG), and used at a final concentration of 5µg/ml. Table 2.5 details the Gag 20mer peptides with a 10 amino acid overlap (from the National Institute for Biological Standards and Control [NIBSC], Potters Bar, Herts, UK) that were used in these experiments, and Table 2.6 shows the Gag 9mer peptides used (obtained from both Sigma Genosys and NIBSC).

Table 2.5 Sequences of the NIBSC (Potters Bar, Herts, UK) Gag 20mer peptides (with a 10 amino acid overlap).

Rep. Ref.	Protein	Peptide	Sequence	Length	Virus Strain
ARP788.1	Gag p24	g1	PIVQNLQGQMVHQAISPRTL	20	1SF2
ARP788.2	Gag p24	g2	VHQAISPRTLNAWVKVVEEK	20	1SF2
ARP788.3	Gag p24	g3	NAWVKVVEEKAFSPEVIPMF	20	1SF2
ARP788.4	Gag p24	g4	AFSPEVIPMFSALSEGATPQ	20	1SF2
ARP788.5	Gag p24	g5	SALSEGATPQDLNTMLNTVG	20	1SF2
ARP788.6	Gag p24	g6	DLNTMLNTVGGHQAAMQMLK	20	1SF2
ARP788.7	Gag p24	g7	GHQAAMQMLKETINEEAAEW	20	1SF2
ARP788.8	Gag p24	g8	ETINEEAAEWDRVHPVHAGP	20	1SF2
ARP788.9	Gag p24	g9	DRVHPVHAGPIAPGQMREPR	20	1SF2
ARP788.10	Gag p24	g10	IAPGQMREPRGSDIAGTTST	20	1SF2
ARP788.11	Gag p24	g11	GSDIAGTTSTLQEQIGWMTN	20	1SF2
ARP788.12	Gag p24	g12	LQEQIGWMTNNPPIPVGEIY	20	1SF2
ARP788.13	Gag p24	g13	NPPIPVGEIYKRWIILGLNK	20	1SF2
ARP788.14	Gag p24	g14	KRWIILGLNKIVRMYSPTSI	20	1SF2
ARP788.15	Gag p24	g15	IVRMYSPTSILDIRQGPKEP	20	1SF2
ARP788.16	Gag p24	g16	LDIRQGPKEPFRDYVDRFYK	20	1SF2
ARP788.17	Gag p24	g17	FRDYVDRFYKTLRAEQASQD	20	1SF2
ARP788.18	Gag p24	g18	TLRAEQASQDVKNWMTETLL	20	1SF2
ARP788.19	Gag p24	g19	VKNWMTETLLVQNANPDCKT	20	1SF2
ARP788.20	Gag p24	g20	VQNANPDCKTILKALGPAAT	20	1SF2
ARP788.21	Gag p24	g21	ILKALGPAATLEEMMTACQG	20	1SF2
ARP788.22	Gag p24	g22	LEEMMTACQGVGGPGHKARV	20	1SF2

Table 2.6 Sequences of the Gag 9mer MHC Class I restricted peptides obtained from Sigma, Genosys (peptides 1-29) and NIBSC (peptides 30-35).

Peptide number	HLA restriction	Protein & Position	Amino Acid Sequence	Short Code	Antigen
1	B*0702 (B7)	p24 (16-24)	SPRTLNAWV	SV9	Gag
2	A*0101 (A1)	p17 (71-79)	GSEELRSLY	GY9	Gag
3	B*5701 (B57)	p24 (108-117)	TSTLQEQIGW	TW10	Gag
4	B*5501 (B55)	p24 (83-92)	VHPVHAGPIA	VA10	Gag
5	A*2402 (A24)	p17 (28-36)	KYKLKHIVW	KW9	Gag
6	B*5701 (B57)	p24 (176-184)	QASQEVKNW	QW9	Gag
7	B*5701 (B57)	p24 (30-40)	KAFSPEVIPMF	KF11	Gag
8	B*5701 (B57)	p24 (32-40)	FSPEVIPMF	FF9	Gag
9	B*5701 (B57)	p24 (15-23)	ISPRTLNAW	IW9	Gag
10	A*2601 (A26)	p24 (35-43)	EVIPMFSAL	EL9	Gag
11	B*0801 (B8)	p24 (197-205)	DCKTILKAL	DL9	Gag
12	B*0801 (B8)	p17 (72-82)	ELRSLYNTV	EV9	Gag
13	B*0801 (B8)	p17 (93-101)	EIKDTKEAL	EL9	Gag
14	B*0801 (B8)	p17 (24-32)	GGKKKYKLK	GK9	Gag
15	B*4402 (B44)	p24 (174-184)	AEQASQDVKNW	AW11	Gag
16	A*0201 (A2)	p17 (17-85)	SLYNTVATL	SL9	Gag
17	B*3501 (B35)	p17 (36-44)	WASRELERF	WF9	Gag
18	B*3501 (B35)	p17 (124-132)	NSSKVSQNY	NY9	Gag
19	B*3501 (B35)	p24 (122-130)	PPIPVGDIY	PY9	Gag
20	B*2705 (B27)	p24 (131-140)	KRWIILGLNK	KK10	Gag
21	A*1101 (A11)	p24 (217-227)	ACQGVGGPGHK	AK11	Gag
22	A*2501 (A25)	p24 (71-80)	ETINEEAAEW	EW10	Gag
23	A*2501 (A25)	p24 (13-23)	QAISPRTLNAW	QW11	Gag
24	B*3901 (B39)	p24 (61-69)	GHQAAMQML	GL9	Gag
25	B*5701 (B57)	p24 (108-117)	TSTLQEQIGN	TN10	Gag
26	A*1101 (A11)	p17 (84-92)	TLYCVHQRI	TI9	Gag
27	B*4001 (B60)	p17 (92-101)	IEIKDTKEAL	IL10	Gag
28	B*4001 (B60)	p24 (44-52)	SEGATPQDL	SL9	Gag
29	A*0301 (A3)	p17 (20-28)	RLRPGGKKK	RK9	Gag
30	B*0801 (B8)	p17 (24-31)	GGKKKYKL	GL8	Gag
31	B*0702 (B7)	p24 (48-56)	TPQDLNTML	TL9	Gag
32	(A2)	p24 (78-86)	AEWDRLHPV	AV9	Gag
33	(A3, A11)	p24 (135-143)	ILGLNKIVR	IR9	Gag
34	(A2)	p24 (143-151)	RMYSPTSIL	RL9	Gag
35		p24 (150-158)	ILDIKQGPK	IK9	Gag

Peptides were identified from the NIH HIV Molecular Immunology Database website (http://www.hiv.lanl.gov). References for each peptide are available at this website.

2.16.3 Preparation of peptide pools for IMIRC 1003 clinical trial

Pools of Gag p24, Gag p17, Rev, Tat and Nef peptides from NIBSC (9mers, 15mers overlapping by 5 and 20mers overlapping by 10) were prepared at a concentration of 10µg/ml in culture medium (RPMI+PSG) and used at a final concentration of 5µg/ml. The lyophilised peptides were originally resuspended in RPMI+PSG to a concentration of 1mg/ml before being pooled. Pooled peptides were carefully aliquoted, 1.8ml into 2ml cryotubes and stored at -80°C.

A second set of peptides was obtained from FIT Biotech (Tartu, Estonia; kindly provided by Rein Sikut) and consisted of: Gag p17/p24, Rev, Tat, Nef and CTL epitope-stretch peptides (15mers with

an 11 amino acid overlap) for the MultiHIV-B consensus antigen [Molder *et al*., 2009]. Additional peptides needed to cover the MultiHIV-Han2 antigen were custom synthesised by ProImmune (Oxford, UK). These were also 15mers overlapping by 11 and pooled with the FIT Biotech peptides to a concentration of 20µg/ml. As per the manufacturer's instructions, peptides were diluted to a concentration of 4µg/ml and used at a final concentration of 2µg/ml. Peptides were aliquoted and stored as described for the NIBSC peptide pools above.

The sequences of the overlapping peptides from both sources can be found in Table 2.7 and Table 2.8.

Phytohaemagglutinin (PHA) (Sigma) was diluted in RPMI-1640+PSG to a concentration of 10µg/ml to be used at a final concentration of 5µg/ml as a mitogen positive control.

Table 2.7 Peptides obtained from NIBSC (Potters Bar, Herts, UK).
The sequences of the overlapping Gag, Rev, Tat and Nef peptides used in pools to stimulate T cells are detailed.

Rep. Ref.	Protein	Peptide	Sequence	Length	Virus Strain
ARP703.1	Gag p17	m1	MGARASVLSGGELDK	15	SF2
ARP703.2	Gag p17	m2	GELDKWEKIRLRPGG	15	SF2
ARP703.3	Gag p17	m3	LRPGGKKKYKLKHIV	15	SF2
ARP703.4	Gag p17	m4	LKHIVWASRELERFA	15	SF2
ARP703.5	Gag p17	m5	LERFAVNPGLLETSE	15	SF2
ARP703.6	Gag p17	m6	LETSEGCRQILGQLQ	15	SF2
ARP703.7	Gag p17	m7	LGQLQPSLQTGSEEL	15	SF2
ARP703.8	Gag p17	m8	GSEELRSLYNTVATL	15	SF2
ARP703.9	Gag p17	m9	TVATLYCVHQRIDVK	15	SF2
ARP703.10	Gag p17	m10	RIDVKDTKEALEKIE	15	SF2
ARP703.11	Gag p17	m11	LEKIEEEQNKSKKKA	15	SF2
ARP703.12	Gag p17	m12	SKKKAQQAAAAAGTG	15	SF2
ARP703.13	Gag p17	m13	AAGTGNSSQVSQNY	15	SF2
ARP789.1	Gag p17	m3b	LRPRGKKRYKLKHIV	15	RF
ARP789.2	Gag p17	m3c	LRPGGKKQYRLKHIV	15	CD41
ARP789.3	Gag p17	m3d	LRPGGKKKYQLKHIV	15	MAN
ARP789.4	Gag p17	m3e	LRPGGKKKYALKHLI	15	NDK
ARP788.1	Gag p24	g1	PIVQNLQGQMVHQAISPRTL	20	1SF2
ARP788.2	Gag p24	g2	VHQAISPRTLNAWVKVVEEK	20	1SF2
ARP788.3	Gag p24	g3	NAWVKVVEEKAFSPEVIPMF	20	1SF2
ARP788.4	Gag p24	g4	AFSPEVIPMFSALSEGATPQ	20	1SF2
ARP788.5	Gag p24	g5	SALSEGATPQDLNTMLNTVG	20	1SF2
ARP788.6	Gag p24	g6	DLNTMLNTVGGHQAAMQMLK	20	1SF2
ARP788.7	Gag p24	g7	GHQAAMQMLKETINEEAAEW	20	1SF2
ARP788.8	Gag p24	g8	ETINEEAAEWDRVHPVHAGP	20	1SF2
ARP788.9	Gag p24	g9	DRVHPVHAGPIAPGQMREPR	20	1SF2
ARP788.10	Gag p24	g10	IAPGQMREPRGSDIAGTTST	20	1SF2
ARP788.11	Gag p24	g11	GSDIAGTTSTLQEQIGWMTN	20	1SF2
ARP788.12	Gag p24	g12	LQEQIGWMTNNPPIPVGEIY	20	1SF2
ARP788.13	Gag p24	g13	NPPIPVGEIYKRWIILGLNK	20	1SF2
ARP788.14	Gag p24	g14	KRWIILGLNKIVRMYSPTSI	20	1SF2
ARP788.15	Gag p24	g15	IVRMYSPTSILDIRQGPKEP	20	1SF2

Table 2.7 continued

ARP788.16	Gag p24	g16	LDIRQGPKEPFRDYVDRFYK	20	1SF2
ARP788.17	Gag p24	g17	FRDYVDRFYKTLRAEQASQD	20	1SF2
ARP788.18	Gag p24	g18	TLRAEQASQDVKNWMTETLL	20	1SF2
ARP788.19	Gag p24	g19	VKNWMTETLLVQNANPDCKT	20	1SF2
ARP788.20	Gag p24	g20	VQNANPDCKTILKALGPAAT	20	1SF2
ARP788.21	Gag p24	g21	ILKALGPAATLEEMMTACQG	20	1SF2
ARP788.22	Gag p24	g22	LEEMMTACQGVGGPGHKARV	20	1SF2
EVA7046.18	Gag p24	g23	TLRAEQATQEVKNWMTETLL	20	Subtype A
EVA7080.51	Gag p24	Cg204	TINEEAAEWDRLHPV	15	C/B(CN54)
EVA7080.52	Gag p24	Cg208	EAAEWDRLHPVHAGP	15	C/B(CN54)
EVA7080.69	Gag p24	Cg276	MYSPTSILDIKQGPK	15	C/B(CN54)
ARP7074.1	Nef	n1	GGKWSKSSVVGWPTVRERMR	20	BRU
ARP7074.2	Nef	n2	GWPTVRERMRRAEPAADGVG	20	BRU
ARP7074.3	Nef	n3	RAEPAADGVGAASRDLEKHG	20	BRU
ARP7074.4	Nef	n4	AASRDLEKHGAITSSNTAAT	20	BRU
ARP7074.5	Nef	n5	AITSSNTAATNAACAWLEAQ	20	BRU
ARP7074.6	Nef	n6	NAACAWLEAQEEEEVGFPVT	20	BRU
ARP7074.7	Nef	n7	EEEEVGFPVTPQVPLRPMTY	20	BRU
ARP7074.8	Nef	n8	PQVPLRPMTYKAAVDLSHFL	20	BRU
ARP7074.9	Nef	n9	KAAVDLSHFLKEKGGLEGLI	20	BRU
ARP7074.10	Nef	n10	KEKGGLEGLIHSQRRQDILD	20	BRU
ARP7074.11	Nef	n11	HSQRRQDILDLWIYHTQGYF	20	BRU
ARP7074.12	Nef	n12	LWIYHTQGYFPDWQNYTPGP	20	BRU
ARP7074.13	Nef	n13	PDWQNYTPGPGVRYPLTFGW	20	BRU
ARP7074.14	Nef	n14	GVRYPLTFGWCYKLVPVEPD	20	BRU
ARP7074.15	Nef	n15	CYKLVPVEPDKVEEANKGEN	20	BRU
ARP7074.16	Nef	n16	KVEEANKGENTSLLHPVSLH	20	BRU
ARP7074.17	Nef	n17	TSLLHPVSLHGMDDPEREVL	20	BRU
ARP7074.18	Nef	n18	GMDDPEREVLEWRFDSRLAF	20	BRU
ARP7074.19	Nef	n19	EWRFDSRLAFHHVARELHPE	20	BRU
ARP7074.20	Nef	n20	HHVARELHPEYFKNC	15	BRU
EVA7081.2	Nef	Cn842	KRQEILDLWVYHTQG	15	C/B(CN54)
EVA7081.44	Nef	Cn1010	GFPVRPQVPLRPMTY	15	C/B(CN54)
EVA7086.6	Nef	Nef 137-145	LTFGWCFKL	15	C/B(CN54)
EVA779.1	Tat	t1	EPVDPRLEPWKHPGSQPKTA	20	LAI
EVA779.2	Tat	t2	KHPGSQPKTACTTCYCKKCC	20	LAI
EVA779.3	Tat	t3	CTTCYCKKCCFHCQVCFTTK	20	LAI
EVA779.4	Tat	t4	FHCQVCFTTKALGISYGRKK	20	LAI
EVA779.5	Tat	t5	ALGISYGRKKRRQRRRPPQG	20	LAI
EVA779.6	Tat	t6	RRQRRRPPQGSQTHQVSLSK	20	LAI
EVA779.7	Tat	t7	SQTHQVSLSKQPTSQPRGD	19	LAI
EVA779.8	Tat	t8	QPTSQPRGDPTGPKE	15	LAI
EVA7084.1	Tat	Tat aa24-32	NCYCKKCCL	9	HXB2
EVA7084.2	Tat	Tat aa35-43	QVCFTRKGL	9	HXB2
EVA7084.3	Tat	Tat aa61-69	DSQTHQVSL	9	HXB2
EVA780.1	Rev	r1	AGRSGDSDEDLLKAVRLIKF	20	LAI
EVA780.2	Rev	r2	LLKAVRLIKFLYQSNPPPNP	20	LAI
EVA780.3	Rev	r3	LYQSNPPPNPEGTRQARRNR	20	LAI
EVA780.4	Rev	r4	EGTRQARRNRRRRWRERQRQ	20	LAI
EVA780.5	Rev	r5	RRRWRERQRQIHSISERILS	20	LAI
EVA780.6	Rev	r6	IHSISERILSTYLGRSAEPV	20	LAI
EVA780.7	Rev	r7	TYLGRSAEPVPLQLPPLERL	20	LAI
EVA780.8	Rev	r8	PLQLPPLERLTLDCNEDCGT	20	LAI
EVA780.9	Rev	r9	TLDCNEDCGTSGTQGVGSPQ	20	LAI
EVA780.10	Rev	r10	SGTQGVGSPQILVESPTVLE	20	LAI
EVA780.11	Rev	r11	ILVESPTVLESGTKE	15	LAI
EVA7085.1	Rev	Rev aa14-22	KTVRLIKFL	9	HXB2
EVA7085.2	Rev	Rev aa56-64	SERILSTFL	9	HXB2
EVA7085.3	Rev	Rev aa67-75	PAEPVPLQL	9	HXB2

Table 2.8 Peptide sub-pools supplied by FIT Biotech and ProImmune.
Overlapping sequences (15mers with an 11 amino acid overlap) are detailed. Asterisks * indicate where peptide synthesis failed.

Sub-pool	Peptide Code	Sequence	Peptide Code	Sequence
Gag p17/p24	HAN-106	GARASVLSGGELDKW	FIT.1-152	TMLNTVGGHQAAMQM
	HAN-107	SVLSGGELDKWEKIR	FIT.1-153	TVGGHQAAMQMLKET
	HAN-108	GGELDKWEKIRLRPG	FIT.1-154	HQAAMQMLKETINEE
	HAN-109	DKWEKIRLRPGGKKK	FIT.1-155	MQMLKETINEEAAEW
	HAN-110	KIRLRPGGKKKYQLK	FIT.1-156	KETINEEAAEWDRLH
	HAN-111	RPGGKKKYQLKHIVW	FIT.1-157	INEEAAEWDRLHPVHA
	HAN-112	KKKYQLKHIVWASRE	FIT.1-158	AEWDRLHPVHAGPIA
	HAN-113	QLKHIVWASRELERF	FIT.1-159	RLHPVHAGPIAPGQM
	FIT.1-114	IVWASRELERFAVNP	FIT.1-160	VHAGPIAPGQMREPR
	FIT.1-115	SRELERFAVNPGLLE	FIT.1-161	PIAPGQMREPRGSDI
	FIT.1-116	ERFAVNPGLLETSEG	FIT.1-162	GQMREPRGSDIAGTT
	FIT.1-117	VNPGLLETSEGCRQI	FIT.1-163	EPRGSDIAGTTSTLQ
	HAN-118	LLETSEGCRQIMGQL	FIT.1-164	SDIAGTTSTLQEQIG
	HAN-119	SEGCRQIMGQLQPSL	FIT.1-165	GTTSTLQEQIGWMTN
	HAN-120	RQIMGQLQPSLQTGS	FIT.1-166	TLQEQIGWMTNNPPI
	FIT.1-121	GQLQPSLQTGSEELR	FIT.1-167	EQIGWMTNNPPIPVGE
	FIT.1-122	PSLQTGSEELRSLYN	FIT.1-168	MTNNPPIPVGEIYKR
	FIT.1-123	TGSEELRSLYNTVAT	FIT.1-169	PPIPVGEIYKRWIIL
	FIT.1-124	ELRSLYNTVATLYCV	FIT.1-170	VGEIYKRWIILGLNK
	FIT.1-125	LYNTVATLYCVHQKI	FIT.1-171	YKRWIILGLNKIVRM
	FIT.1-126	VATLYCVHQKIEVKD	FIT.1-172	IILGLNKIVRMYSPT
	FIT.1-127	YCVHQKIEVKDTKEA	FIT.1-173	LNKIVRMYSPTSILD
	HAN-128	HQKIEVKDTKEALDKV	HAN-174	VRMYSPTSILDIKQG
	HAN-129	VKDTKEALDKVEEEQ	HAN-175	SPTSILDIKQGPKEP
	HAN-130	KEALDKVEEEQNNSK	HAN-176	ILDIKQGPKEPFRDY
	HAN-131	DKVEEEQNNSKKKAQ	HAN-177	KQGPKEPFRDYVDRF
	HAN-132	EEQNNSKKKAQQEAA	FIT.1-178	KEPFRDYVDRFYKTL
	HAN-133	NSKKKAQQEAADAGN	FIT.1-179	RDYVDRFYKTLRAEQ
	HAN-134	KAQQEAADAGNRNQV	HAN-180	DRFYKTLRAEQATQE
	HAN-135	EAADAGNRNQVSQNY	HAN-181	KTLRAEQATQEVKNW
	HAN-136	AGNRNQVSQNYPIVQ	HAN-182	AEQATQEVKNWMTET
	FIT.1-137	SQVSQNYPIVQNLQG	HAN-183	TQEVKNWMTETLLVQ
	FIT.1-138	SQNYPIVQNLQGQMVH	FIT.1-184	KNWMTETLLVQNANP
	FIT.1-139	IVQNLQGQMVHQAIS	FIT.1-185	TETLLVQNANPDCKT
	FIT.1-140	LQGQMVHQAISPRTL	FIT.1-186	LVQNANPDCKTILKA
	FIT.1-141	MVHQAISPRTLNAWV	FIT.1-187	ANPDCKTILKALGPA
	FIT.1-142	AISPRTLNAWVKVVE	FIT.1-188	CKTILKALGPAATLE
	FIT.1-143	RTLNAWVKVVEEKAF	FIT.1-189	LKALGPAATLEEMMT
	FIT.1-144	AWVKVVEEKAFSPEV	FIT.1-190	GPAATLEEMMTACQG
	FIT.1-145	VVEEKAFSPEVIPMF	FIT.1-191	TLEEMMTACQGVGGP
	FIT.1-146	KAFSPEVIPMFSALS	FIT.1-192	MMTACQGVGGPGHKA
	FIT.1-147	PEVIPMFSALSEGAT	FIT.1-193	CQGVGGPGHKARVLP
	FIT.1-148	PMFSALSEGATPQDL		
	FIT.1-149	ALSEGATPQDLNTML		
	FIT.1-150	GATPQDLNTMLNTVG		
	FIT.1-151	PQDLNTMLNTVGGHQA		

Table 2.8 continued

Sub-pool	Peptide Code	Sequence	Sub-pool	Peptide Code	Sequence
Nef	HAN-30	TSVGKWSKCSGWPTV	Tat	FIT.1-81	EPVDPRLEPWKHPGS
	HAN-31	KWSKCSGWPTVRERM		HAN-82	PRLEPWKHPGSQPRT
	HAN-32	CSGWPTVRERMKQAE		HAN-83	PWKHPGSQPRTPCTN
	HAN-33	PTVRERMKQAEPEPA		HAN-84	PGSQPRTPCTNCYCK
	HAN-34	ERMKQAEPEPAADGV		HAN-85	PRTPCTNCYCKKCCL
	HAN-35	QAEPEPAADGVGAAS		HAN-86	CTNCYCKKCCLHCQV
	HAN-36	EPAADGVGAASRDLE		HAN-87	YCKKCCLHCQVCFTR
	HAN-37	DGVGAASRDLEKHGA		HAN-88	CCLHCQVCFTRKGLG
	HAN-38	AASRDLEKHGAITSS		HAN-89	CQVCFTRKGLGISYG
	HAN-39	DLEKHGAITSSNTAT		HAN-90	FTRKGLGISYGRKKR
	HAN-40	HGAITSSNTATNNAA		FIT.1-91	GLGISYGRKKRRQRR
	HAN-41	TSSNTATNNAACAWL		FIT.1-92	SYGRKKRRQRRRAPQ
	HAN-42	TATNNAACAWLEAQE		FIT.1-93	KKRRQRRRAPQDSQT
	FIT.1-43	CAWLEAQEEEEVGFP		FIT.1-94	RQRRRAPQDSQTHQVS
	HAN-44	AWLEAQEEEEVGFPV		HAN-95	APQDSQTHQVSLPKQ
	HAN-45	AQEEEEVGFPVRPQV		HAN-96	SQTHQVSLPKQPSSQ
	HAN-46	EEVGFPVRPQVPLRP		HAN-97	HQVSLPKQPSSQQRGD
	HAN-47	FPVRPQVPLRPMTYK		HAN-98	PKQPSSQQRGDPTGP
	HAN-48	PQVPLRPMTYKGALD		HAN-99	SSQQRGDPTGPKKSK
	HAN-49	LRPMTYKGALDLSHF		HAN-100	RGDPTGPKKSKKKVE
	HAN-50	TYKGALDLSHFLKEK		HAN-101	TGPKKSKKKVERETE
	HAN-51	ALDLSHFLKEKGGLE		HAN-102	KSKKKVERETEADPF
	HAN-52	SHFLKEKGGLEGLIY		HAN-103	KVERETEADPFDAAV
	HAN-53	KEKGGLEGLIYSPKR			
	HAN-54	GLEGLIYSPKRQEIL			
	HAN-55	LIYSPKRQEILDLWV			
	HAN-56	PKRQEILDLWVYHTQ			
*	FIT.1-56	**RQDILDLWVYHTQGYF**			
	HAN-58	LWVYHTQGYFPDWQN			
	HAN-59	HTQGYFPDWQNYTPG			
	HAN-60	YFPDWQNYTPGPGVR			
	HAN-61	WQNYTPGPGVRYPLT			
	HAN-62	TPGPGVRYPLTFGWC			
	HAN-63	GVRYPLTFGWCFKLV			
	HAN-64	PLTFGWCFKLVPVEP			
	HAN-65	GWCFKLVPVEPDEEE			
	HAN-66	KLVPVEPDEEENSSL			
*	HAN-67	VEPDEEENSSLLHPA			
*	HAN-68	EEENSSLLHPASLHG			
	HAN-69	SSLLHPASLHGTEDT			
	HAN-70	HPASLHGTEDTEREV			
	HAN-71	LHGTEDTEREVLKWK			
	HAN-72	EDTEREVLKWKFDSH			
*	FIT.1-74	**EVLVWKFDSRLAFHH**			
	HAN-74	KWKFDSHLAFHHKAR			
	HAN-75	DSHLAFHHKARELHP			
	HAN-76	AFHHKARELHPEYYK			
	HAN-77	KARELHPEYYKDCKL			

Table 2.8 continued

Sub-pool	Peptide Code	Sequence	Sub-pool	Peptide Code	Sequence
Rev	FIT.1-1	AGRSGDSDEELLKTV	CTL	FIT.1-197	ITLWQRPLVTIKIGG
	FIT.1-2	GDSDEELLKTVRLIK	Epitope	HAN-203	ALIEICTEMEKEGKIS
	FIT.1-3	EELLKTVRLIKFLYQ	Stretch	FIT.1-204	CTEMEKEGKISKIGP
	FIT.1-4	KTVRLIKFLYQSNPP		HAN-207	AGLKKKKSVTVLDVG
	HAN-5	LIKFLYQSNPPPSNE		HAN-208	KKKSVTVLDVGDAYF
	HAN-6	LYQSNPPPSNEGTRQ		FIT.1-209	VTVLDVGDAYFSVPL
	HAN-7	SNPPPSNEGTRQARRN		FIT.1-210	DVGDAYFSVPLDKDF
	HAN-8	SNEGTRQARRNRRRR		FIT.1-211	AYFSVPLDKDFRKYT
	FIT.1-9	TRQARRNRRRRWRER		FIT.1-212	VPLDKDFRKYTAFTI
	FIT.1-10	RRNRRRRWRERQRQI		FIT.1-213	KDFRKYTAFTIPSIN
	FIT.1-11	RRRWRERQRQIRSIS		FIT.1-218	WKGSPAIFQSSMTKI
	HAN-12	RERQRQIRSISERIL		FIT.1-223	KQNPDIVIYQYMDDL
	HAN-13	RQIRSISERILSTFL		FIT.1-224	DIVIYQYMDDLYVGS
	HAN-14	SISERILSTFLGRPA		FIT.1-228	TVQPIVLPEKDSWTV
	HAN-15	RILSTFLGRPAEPVP		FIT.1-233	LVGKLNWASQIYAGI
	FIT.1-16	TYLGRPAEPVPLQLP		FIT.1-234	LNWASQIYAGIKVKQ
	FIT.1-17	RPAEPVPLQLPPLER		FIT.1-240	REILKEPVHGVYYDP
	FIT.1-18	PVPLQLPPLERLTLD		FIT.1-244	EKEPIVGAETFYVDG
	HAN-19	LQLPPLERLTLDCSED		FIT.1-245	IVGAETFYVDGAANR
	HAN-20	LERLTLDCSEDCGNS		HAN-250	AGNLWVTVYYGVPV
	HAN-21	TLDCSEDCGNSGTQG		FIT.1-251	LWVTVYYGVPVWKEA
	HAN-22	CSEDCGNSGTQGVGSP		FIT.1-252	VYYGVPVWKEATTTL
	HAN-23	GNSGTQGVGSPQVLV		HAN-258	VERYLRDQQLLGIWGCA
	HAN-24	TQGVGSPQVLVESPA		FIT.1-263	INQMLRGPGRAFVTI
	HAN-25	GSPQVLVESPAVLEP			
	HAN-26	VLVESPAVLEPGTKE			

2.17 IFN-γ, IL-2, IL-4 and perforin ELISpot assays for virus-specific T cells

ELISpot assays were carried out to detect IFN-γ (IL-2, IL-4 and perforin) by first coating a 96 well Polyvinylidene Difluoride (PVDF) backed plate (Millipore, catalogue no. MAIP S45 10) with 100µl per well of capture antibody (MabTech AB, Nacha Strand, Sweden) diluted to a concentration of 10µg/ml in sterile PBS followed by overnight incubation at 4°C. The wells were then washed six times with 200µl of sterile PBS. The PVDF membrane-backed plate was then blocked by adding 100µl of TCM supplemented with 10% human male AB serum and incubated for 60 minutes at room temperature. After removal of the blocking AB medium, 2.5 x 10^5 PBMC (suspended in 100µl of 20% human male AB serum/TCM; at a concentration of 2.5 x 10^6 cells/ml), were added per well.

100µl of the relevant peptide pool from either NIBSC or FIT Biotech (at a final concentration of 5µg/ml or 2µg/ml respecively), PHA (positive control; at a final concentration of 5µg/ml) or TCM

(negative control) was added to each well in duplicate as shown in Figure 2.3. The ELISpot plate was then incubated at 37˚C in 5% CO_2 for 24 hours.

Following the removal of the contents of the plate, each well was washed six times with 200µl of PBS. All non-sterile washes were carried out using an ELISpot plate washer (Multiwash Advantage, TriContinent, California, USA). 100µl of detection antibody (Ab); biotinylated anti-human IFNγ Ab (MabTech), in PBS at a concentration of 1µg/ml was added per well. The plate was then incubated for 24 hours at 4°C (or for 2 hours at room temperature).

The detection antibody was then removed and the plate washed, as described above, six times with 200µl PBS per well. Streptavidin-alkaline phosphatase conjugate (MabTech) was diluted in PBS, in the dark, to a concentration of 1/2000, 100µl of which was then added per well, followed by a 1 hour incubation at room temperature.

The plate was then washed six times with 200µl of PBS per well immediately followed by addition of 100µl of freshly prepared chromogen to each well. 10ml of the chromogen was prepared (per plate) by using the AP Conjugate Substrate Kit (BioRad, Catalogue no. 170-6432) and mixing 9.6ml deionised water with 400 µl Development Buffer™, followed by the addition of 50µl of Reagent A™ and 50µl of Reagent B™.

The plate was then left to develop in the dark for approximately 15 minutes (dependent on ambient temperature) and then washed with water. The plate was left to dry overnight and stored at room temperature, away from direct sunlight, until reading was carried out on a Zeiss KS ELISpot system (KS Elispot, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Responses in the IFN-γ, IL-2 and IL-4 ELISpot assays were considered significant if more than 50 spot forming cells (SFC) were present per million PBMC after background (TCM stimulation) was subtracted, and the results were at least twice the background. Responses in the perforin ELISpot assay were considered significant if the result was more than 100 SFC per million PBMC and at least twice the background (TCM stimulation) after the background was subtracted (this higher cut-off is due to higher background in perforin ELISpot assays).

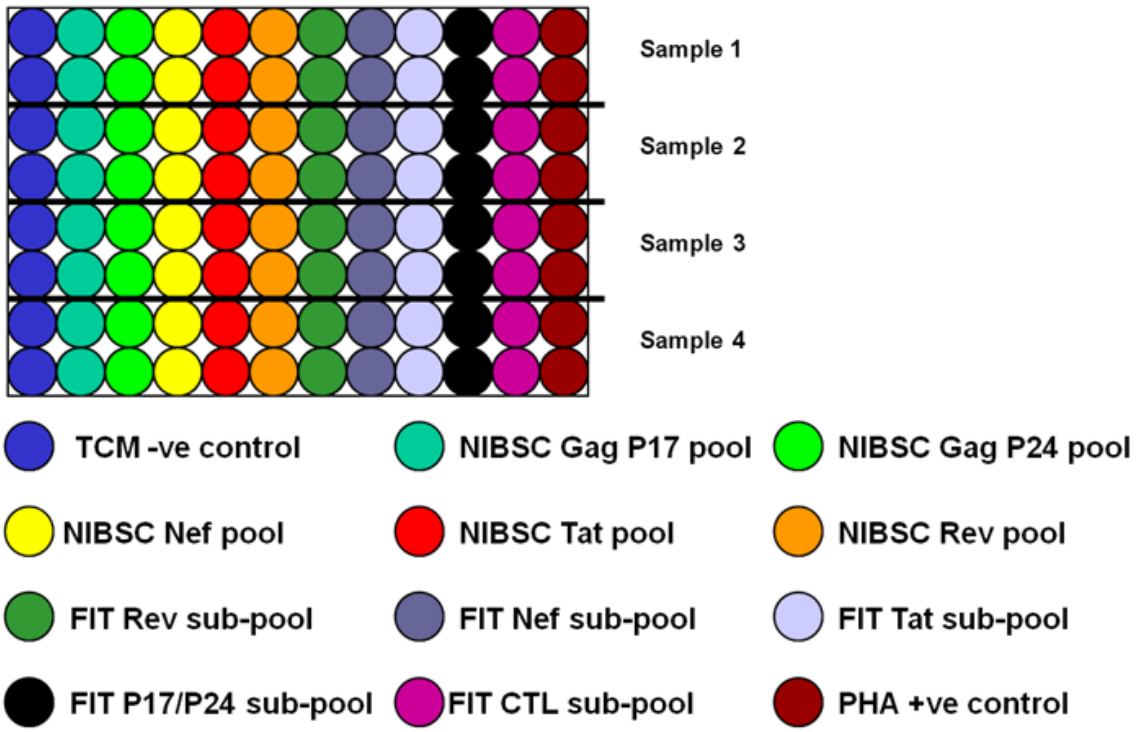


Figure 2.3 ELISpot plate layout for the IMIRC 1003 clinical trial samples.

2.18 Recombinant vaccinia virus construct based ELISpot assay for enumeration of IFN-γ-producing HIV-1-specific CD8 T cells

PBMC were infected with recombinant vaccinia (rVV) virus constructs expressing either HIV-1 Gag or Pol protein. It has been previously demonstrated that CD8 T cells produce >90% of the IFN-γ release in response to rVV stimulation of PBMC [Larsson *et al.*, 1999]. rVV Gag, rVV Pol and rVVPB2 were used to infect PBMC for one hour at 37°C in 5% AB plasma using 20 infectious units per cell. Cells were washed twice in RPMI-1640+PSG containing 5% AB plasma before being resuspended in 10% AB plasma/RPMI-1640+PSG and plated out in duplicate with 2.5 x 10^5 PBMC per well directly onto ELISpot plates. rVVPB2 was used as a negative control and PHA at 5µg/ml as a positive control. Detection and enumeration of SFC was carried out as described in the previous section. A positive result is defined as at least twice the background, and a score of 5 or more above background, which was always <20 SFC/10^6 PBMC. Data represent mean values of duplicate wells at each point with variation among duplicates <10%.

2.19 Viral sequencing

The HIV-1 viral subtypes of the patients enrolled on the IMIRC 1003 clinical trial were determined by Maarja Adojaan at FIT Biotech LTD, Tartu, Estonia. Two proviral DNA regions, *gag*/*pol*/*pr* and *gp41*, were sequenced and genotyped from cryopreserved PBMC (5×10^6). Briefly, samples were shipped and proviral DNA was extracted from PBMC (Quattromed HTI Laborid OÜ) before nested PCR of gp41, gag-pol and pr regions of HIV-1 was performed according to the laboratory's standard operating procedure (SOP). Samples were prepared for sequencing according to the SOP and sequencing electrophoresis was run in Estonian Biocentre Sequencing Centre with ABI 3730x1 DNA Analyzer. Two proviral genomic regions were analysed: gag/pol/pr and envelope (gp41); the sequencing of two regions located in different parts of the viral genome allows possible recombination events to be traced. Both regions contain nucleotide divergence that is sufficient for subtype determination, yet both are conserved enough to allow the design of consensus primers and to create the DNA sequence alignments. In order to overcome HIV-1 variability, a set of different primers was prepared which enabled amplification of samples from various subtypes. Positive control samples of different viral subtypes (A, B, C, D, G, CRF-06.cpx) were successfully amplified and sequenced to test primers.

BioEdit Sequence Alignment Editor version 7.0.9.0 software, Clone Manager 9 Professional Edition software and subtyping tools available at HIV database websites were used to perform the sequencing analysis [Adojaan *et al*., 2005]. Two genomic regions were sequenced in all 12 samples and if sequence data was available for both of these regions, it was merged as amplified fragments of gag/pol and protease partly overlap. Details on the methodology and analysis and interpretation of the results were taken from a study report kindly generated by Maarja Adojaan.

2.20 Genomic DNA extraction

Genomic DNA extraction was carried out on $3 \times 10^6 – 1 \times 10^7$ PBMC using the Nucleon™ BACC2 Genomic DNA Extraction Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Cryopreserved PBMC from IMIRC 1003 patient screen visits were thawed as described previously

(in section 2.11). The thawed cells were washed and collected by centrifugation at 600g and 4°C for 5 minutes. Once the supernatant was discarded, the pellet was resuspended in 1.0ml of Reagent A and left on ice for 5 minutes. A further centrifugation was carried out at 1300g for 5 minutes at 4°C and the supernatant discarded.

For cell lysis and RNA digestion, 2ml of Reagent B was added to the pellet (ensuring the detergent was fully dissolved) and this was vortexed briefly to resuspend the pellet. The resulting suspension was transferred to a screw-cap polypropylene centrifuge tube (max internal diameter 12mm) and 150µl/ml RNAse A solution was added and this was incubated for 30 minutes at 37°C.

For the deproteinisation steps, 500µl of sodium perchlorate was added and this was mixed by inverting ten times by hand. Following this, 2ml of chloroform was added and this was inverted 10 times by hand to emulsify the phases. This was centrifuged for 1 minute at 1300g. Once it was ensured that the Nucleon resin was well suspended, 300µl was added to the tube. Without re-mixing the phases, this was centrifuged at 1300g for 4 minutes.

To precipitate the DNA, the upper phase was first transferred to a fresh (at least 10ml) tube, without disturbing the [orange] Nucleon resin layer. If any resin was carried over, a brief centrifugation was carried out at 1300g to pellet the resin and the upper phase again transferred to a fresh tube. Two volumes of cold (-20°C) absolute ethanol were added and the tube inverted several times to precipitate the DNA. The precipitated DNA was ‘hooked out’ and placed directly into an eppendorf tube containing 200µl of RNAse/DNAse free water. Where ‘hooking out’ was not possible, the DNA was washed as described below.

The DNA washing required centrifugation at 5000g for 5 minutes to pellet the DNA. The supernatant was discarded and 2ml of cold (-20°C) absolute ethanol was added and the tube inverted several times to wash. Re-centrifugation was carried out as above and the supernatant discarded. The pellet was allowed to air dry for ten minutes, ensuring all the ethanol was removed and the sample was resuspended in 200µl of RNAse/DNAse free water.

2.21 HLA-typing

HLA-typing for each patient was carried out on baseline samples at the Clinical Immunology Laboratory, Department of Histocompatibility and Immunogenetics, Hammersmith Hospital. Sequencing of HLA was performed using Invitrogen SeCore® kits. The class I (A, B and Cw Locus) kits use primers that sequence exons 2, 3 & 4, whilst the DRB1 locus kit uses primers that sequence exon 2 only as these are significant sites of HLA polymorphism. The sequencing involves locus specific amplification for class I and group specific amplification for class II, in conjunction with the use of Taq DNA Polymerase and sample genomic DNA. Prior to carrying out sequencing reaction all unincorporated primers and free nucleotides are treated with ExoSAP-ITTM. The nucleotides and resulting HLA subtype is determined using multicolour sequence analyser. Based on the sequence data, the HLA type is assigned using SBT software [Turner *et al*., 1998]. Details on the methodology were kindly provided by Ruhena Sergeant.

2.22 Study design

2.22.1 Impact of rhGH on HIV-1-specific T-cell responses

Twelve chronically infected, ART-treated, HIV-1^+ individuals with lipodystrophy were enrolled onto a randomised, double-blinded, placebo-controlled study to receive 4mg rhGH (Serostim, Serono International, Geneva, Switzerland) daily for 12 weeks. The patients were then randomised into one of three groups to receive: (A) placebo, (B) alternate-day dosing, or (C) twice-per-week dosing of rhGH for a further 12 weeks, after which the subjects went back to receiving ART alone with no immunotherapy. Samples were collected at baseline, at weeks 12 and 24 of the study, and at a 48-week follow up visit.

Patients were eligible to participate in the study if they had CD4 T-cell counts greater than 200 cells/μl blood and if they had been on stable highly active ART (HAART) for more than a year. A schematic of the study design and the treatment schedule is illustrated in Figure 2.4.

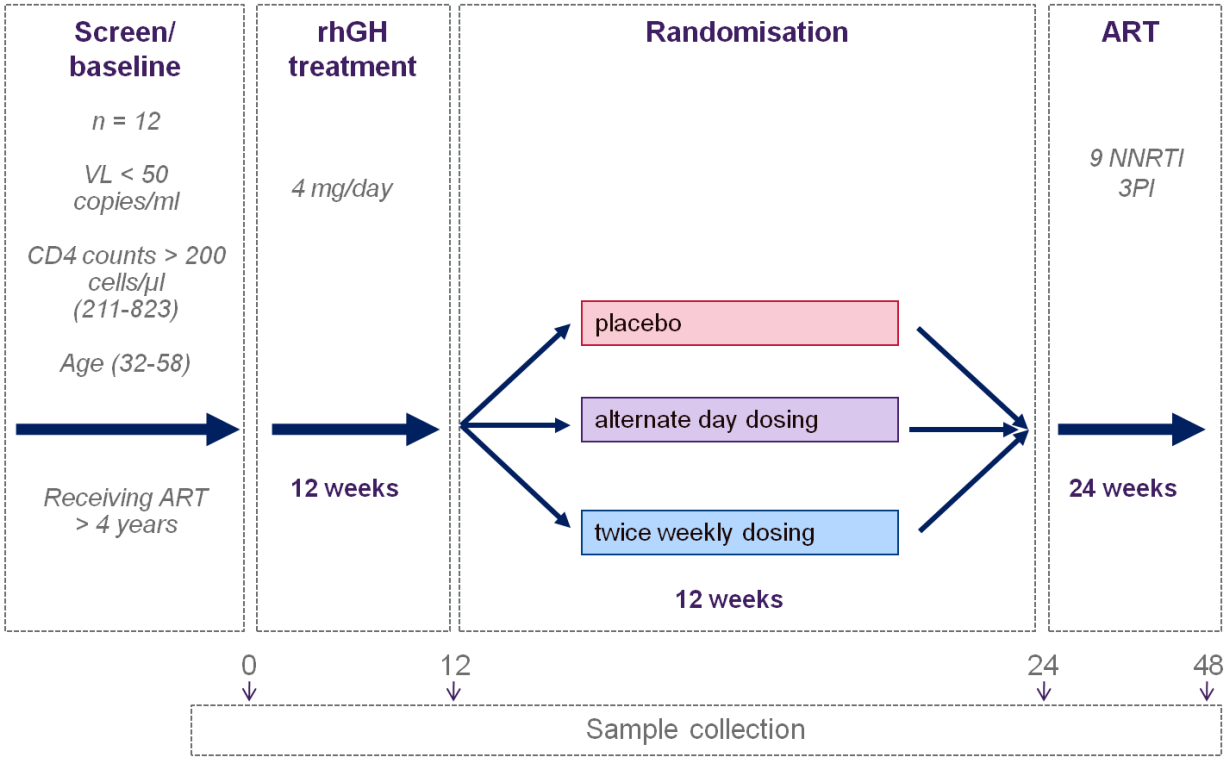


Figure 2.4 Schematic representation of study design and treatment schedule.
PBMC from 12 HIV-1-infected individuals with lipoatrophy, on long-term successful ART receiving rhGH (Serostim, Serono International, Geneva, Switzerland), were assessed for CD4 T-cell proliferation and IFN-γ production by CD8 T cells. rhGH administration: given at 4mg/day for 12 weeks. After 12 weeks, patients were randomised into three groups: placebo; alternate day dosing; or twice weekly dosing for a further twelve weeks. Weeks 24-48, patients received ART alone (no immunotherapy). Samples were collected at baseline and weeks 12, 24 and 48 of the study. ELISpots were carried out at each time point to assess the ability of CD8 T cells to recognise HIV-1 peptides or proteins and produce IFN-γ.

2.22.2 Low-dose rhGH study

Samples were obtained from a randomised, placebo-controlled, double-blinded, clinical, single-centre study (carried out at the Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre, Denmark). Caucasian, male subjects on effective ART received either 0.7mg/day rhGH (n = 21) or placebo (n = 15) subcutaneously for 40 weeks (Genotropin or placebo obtained from Pfizer A/S, DK2750 Ballerup, Denmark).

IFN-γ ELISpot assays were carried out (as described in section 2.17) on cryopreserved PBMC from baseline (week 0) and week 40, stimulated with peptide pools of HIV-1 Gag 20mers and Gag 9mers (as detailed section 2.16.2, in Table 2.5 and Table 2.6, respectively) to assess the virus-specific CD4 and CD8 T cells recognising antigenic peptide in the context of MHC class II or class I, respectively.

Flow cytometric, phenotypic analysis was carried out on T cells from the most robust responders (n = 4). The panel included markers used to determine maturation/differentiation (CCR7, CD45RA, CD27, CD28), activation (CD25, HLA-DR, CD38), exhaustion (PD-1, PD-L1), senescence (CD57) and apoptosis (CD95) as well as assessing markers associated with regulatory T cells ($CD4^+CD45RO^+CD25^{high}$). The panel of antibodies was prepared as described in section 2.12, Table 2.1.

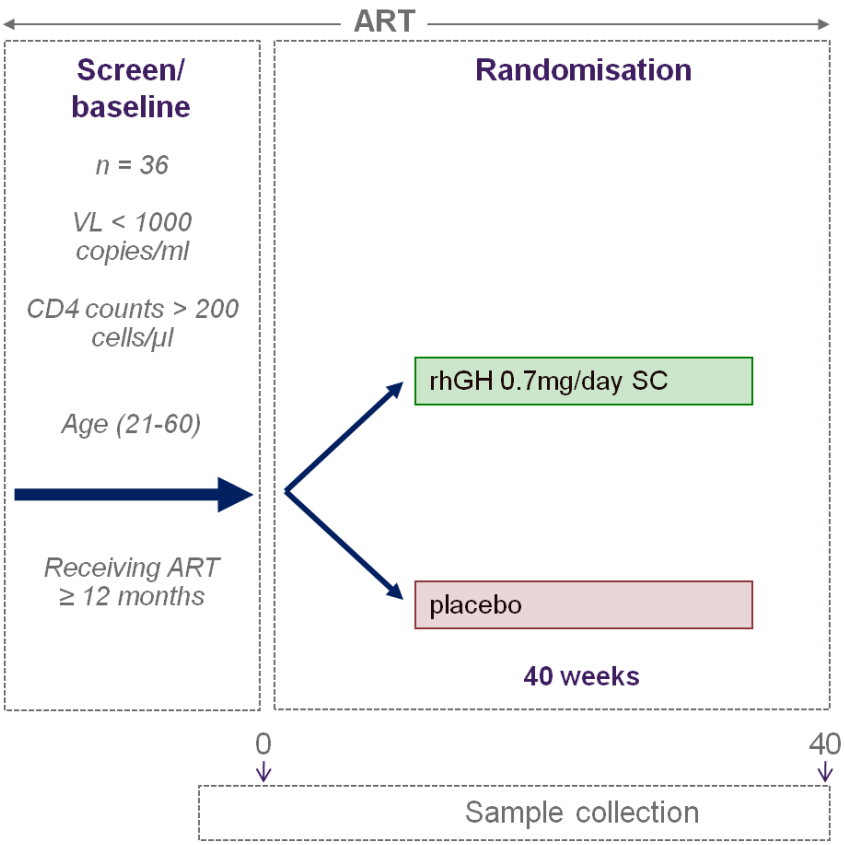


Figure 2.5 Schematic of low-dose rhGH treatment schedule.
From a study of ART-treated HIV-1-infected men, samples were investigated from subjects randomised to receive either 0.7mg/day rhGH (n=21) or placebo (n=15) (Genotropin or placebo, obtained from Pfizer A/S, DK-2750 Ballerup, Denmark), administered as daily subcutaneous injections for 40 weeks. Patients were 21-60 years of age, on ART for at least 12 months, with less than 1000 copies/ml HIV-1 RNA and greater than 200 $CD4^+$ T cells. IFN-γ ELISpot assays were performed on these samples at baseline and at week 40 to determine responses to overlapping Gag 20mer and Gag 9mer peptides following treatment with rhGH or placebo.

2.22.3 Novel interventions in HIV-1 infection – IMIRC 1003 clinical trial

Out of 93 patient referrals and 21 screen visits, 12 patients that met the eligibility criteria were enrolled onto the trial. Patients had to be chronically infected with HIV-1, on stable ART (for at least 6 months), have plasma viral loads <50 copies/ml, with nadir CD4 T-cell counts >200 cells/µl, current CD4 T-cell counts >400 cells/µl, and could not be receiving or have received

immunomodulatory drugs. Patients were randomised into one of three arms of the trial: in the first, patients received the GTU®-MultiHIV DNA Clade B vaccine (FIT Biotech, Tampere, Finland) at baseline, followed by administration of IL-2 (Aldesleukin, Proleukin) and GM-CSF (Sargramostim, Leukine™) for five days during week 1 of the trial, and rhGH (Somatropin, Saizen™) for five days during week 2 of the trial, with vaccine further administered at weeks 6 and 12 of the trial (n = 3); in the second arm, patients received vaccine alone (n = 4); and in the third arm, patients received cytokine and hormone alone (n = 5), all at the aforementioned time points. Blood was drawn at two screen visits, at baseline (week 0) and at weeks 1, 2, 4, 6, 8, 12, 24 and 48, and the treatment schedule is detailed in Table 2.9, with a more detailed patient visit schedule shown in Table 2.10. The vaccine was administered at a dose of 1mg/ml as 10 intradermal injections (five 100µl injections per arm); the IL-2 given twice daily, 5 x 10^6 Units, administered by subcutaneous injection, 8 hours apart; the GM-CSF, 150µg, was administered subcutaneously once daily, 4 hours from the IL-2; and the rhGH self-administered subcutaneously daily at a dose of 4mg/day. Nurses provided training on how to use the Saizen® click.easy® cartridges and the cool.click® needle-free rhGH delivery device (Merck Serono Ltd).

For each patient visit and blood draw, 10ml EDTA blood and 30ml heparinised (lithium heparin; LH) blood was obtained. The samples were centrifuged, the plasma collected and frozen at -80°C and the PBMC separated by density gradient centrifugation over Histopaque as described in section 2.10.

IFN-γ, IL-2, IL-4 and perforin ELISpots were carried out at each time point to assess functional responses to peptide pools of Gag p17, Gag p24, Nef, Rev, and Tat (as detailed in section 2.17).

Phenotypic analysis of the CD4 and CD8 T cells was also carried out and the panel included markers for activation (CD38, HLA-DR), exhaustion (CTLA-4, TIM-3, PD-1, PD-L1), senescence (CD57), and differentiation (CCR7, CD45RA, CD27, CD28), as well as markers associated with regulatory T cells ($CD3^+CD4^+CD45RO^+CD25^{high}$), and the panel is shown in Table 2.2.

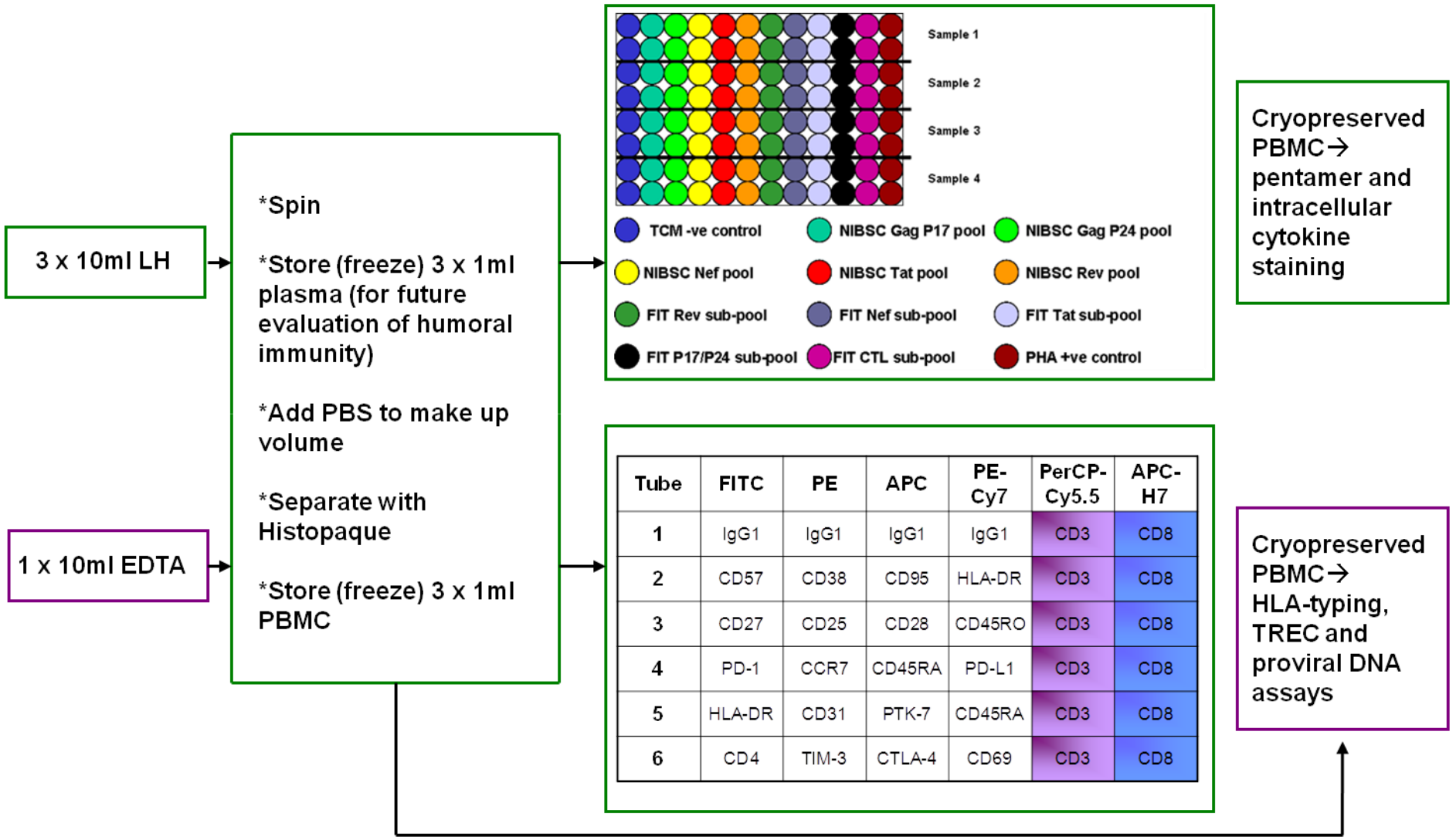
Table 2.9 IMIRC 1003 overview of treatment schedule.
Patients were randomised into one of three arms of the trial: to receive therapeutic immunisation (GTU®-MultiHIV DNA Clade B vaccine), in addition to IL-2, GM-CSF and rhGH (Arm 1); vaccine alone (Arm 2); or cytokines and hormone alone (Arm 3).

Week	Screen X 2	0	1 Days 8-12	2 Days 14-18	4	6	8	12	16	24	48
Arm 1		FIT Vaccine	IL-2 + GM-CSF	rhGH		FIT Vaccine		FIT Vaccine			
Arm 2		FIT Vaccine				FIT Vaccine		FIT Vaccine			
Arm 3			IL-2 + GM-CSF	rhGH							

Table 2.10 IMIRC 1003 Patient visit schedule.
The study ran for 52 weeks with two screen visits, two weeks apart, followed by 48 weeks of the study.

	Screening phase weeks from baseline		Post randomisation phase weeks from baseline									
	-4	-2	0	1	2	4	6	8	12	16	24	48
Blood sampling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Informed consent	✓											
Medical history	✓											
Physical examination*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Laboratory tests**	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Functional*** immunology	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Vaccine†			Groups 1 and 2 only				Groups 1 and 2 only		Groups 1 and 2 only			
Cytokine‡				Groups 1 and 3 only								
Hormone‡‡					Groups 1 and 3 only							

*Pregnancy test (women of child bearing potential only). **When recommended by the physician, with the exception of the first screen when compulsory. ***HIV-1 viral load, lymphocyte subsets, biochemistry (urea, creatinine, glucose, total bilirubin, alkaline phosphatise, AST (SGOT), ALT (SGPT), albumin, globulin, fasting cholesterol triglycerides, sodium, potassium, amylase, calcium and phosphate), and haematology (haemoglobin, platelets, total WBC, and neutrophils). †Vaccine, GTU®-MultiHIV B (Han-2 isolate), FIT Biotech Plc (1mg/ml) administered at 0, 6 and 12 weeks as ten intradermal injections (100µl/injection volume) with five injections per each arm. ‡Cytokines: Aldesleukin (IL-2), Proleukin, Novartis, UK (BD; 8 hours apart) 5 x 10^6 Units administered for five days by SC injection on days 7, 8, 9, 10 and 11 after first vaccination. Sargramostim (GM-CSF), Leukine™, Berlex, Seattle, USA 150µg SC once daily four hours from rhIL-2 injections. ‡‡Hormone, rhGH (Serono International, Geneva, Switzerland) 4mg/day self-administered by SC injection for five days on days 14, 15, 16, 17 and 18 following first vaccination.



Tube	FITC	PE	APC	PE-Cy7	PerCP-Cy5.5	APC-H7
1	IgG1	IgG1	IgG1	IgG1	CD3	CD8
2	CD57	CD38	CD95	HLA-DR	CD3	CD8
3	CD27	CD25	CD28	CD45RO	CD3	CD8
4	PD-1	CCR7	CD45RA	PD-L1	CD3	CD8
5	HLA-DR	CD31	PTK-7	CD45RA	CD3	CD8
6	CD4	TIM-3	CTLA-4	CD69	CD3	CD8

Figure 2.6 Trial sample flow chart.
The steps in the immediate processing of the IMIRC 1003 clinical trial samples are detailed. All experiments were carried out in accordance with standard operating procedures, as described in full in this chapter of the thesis.

2.23 Data and statistical analysis

Data analysis was performed using Microsoft Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism Software version 5.0 (GraphPad Software, San Diego, CA, USA). A professional on-site statistician, Ms Sundhiya Mandalia (Imperial College London, Kobler Clinic St Stephen's AIDS Trust) was consulted regarding statistical analysis. Where data sets were not normally distributed, inter-group statistical significance was calculated using a Mann-Whitney U test and intra-group statistical significance was calculated by comparing the medians of the paired data using the Wilcoxon signed rank test. Results are presented as p values, and considered significant where $p \leq 0.05$.

For the IMIRC 1003 clinical trial analysis, a random intercept model using MIXED procedure in SAS was generated by fitting all functional, phenotypic, immunological and virological results as dependent variables by study time point visits, stratified by the three randomised treatment arms. Mean changes from baseline in all functional, phenotypic, immunological and virological results are presented as point estimates, presented with a 95% confidence interval (CI). For the graphs, where the 95% CI does not cross the line of origin, a statistically significant difference can be inferred (above – a statistically significant increase and below – a statistically significant decrease). Statistically significant differences ($p \leq 0.05$) are indicated with asterisks (*) in the figures, and trends ($0.05 < p > 0.1$) described in the text. All p values and 95% CI limits for this analysis can be found in Appendix 2.

Chapter 3 Impact of rhGH on T-Cell Phenotype and Function in Treated HIV-1 Infection

Human growth hormone (GH) produced by the anterior pituitary gland [Clark, 1997], mediates its effects either directly or through insulin-like growth factor-1 (IGF-1), and plays an important role in T-cell development and function [Welniak *et al*., 2002]. Impaired GH secretion has been described in HIV-1-infected individuals with visceral or central adiposity [Rietschel *et al*., 2001; Vigano *et al*., 2003], and recombinant human growth hormone (rhGH) has been used to successfully treat HIV-1-associated wasting syndrome and visceral adiposity in patients on antiretroviral therapy (ART) [Cominelli *et al*., 2002; Moyle *et al*., 2004; Grunfeld *et al*., 2007] (reviewed in [Gelato *et al*., 2007]). Prolonged treatment with ART has been shown to improve morbidity and mortality associated with HIV-1 infection, reduce HIV-1 replication and normalise CD4 T-cell counts, as well as stabilising naive/memory cell ratios and reducing levels of activated cells [Guihot *et al*., 2011]. However, ART does not eradicate HIV-1 or facilitate immune control of the virus, and immune restoration is functionally incomplete with levels of activation markers remaining elevated in a significant subset of individuals [Kauffman *et al*., 2011]. While GH is not believed to exert direct effects on the peripheral T cells, it is critical for T-cell development in the thymus [Welniak *et al*. 2002; Napolitano *et al*., 2002]. There appears a role for rhGH as an immunomodulator, complimentary to the benefits of effective combination ART in HIV-1 infection. Studies of rhGH in treated HIV-1 infection have shown a reversal of thymic involution, increased numbers of peripheral naive CD4 T cells [Napolitano *et al*., 2002, Pires *et al*., 2004b], increased numbers of total CD4 T cells [Napolitano *et al*., 2008; Smith *et al*., 2010], and a reduction in the expression of markers associated with immune activation (CD38, HLA-DR) and apoptosis (CD95) [Napolitano *et al*., 2008].

This chapter explores the effects of rhGH in treated HIV-1 infection using *ex vivo* samples from two clinical trials: one investigating the impact of short term administration of a ‘supra-physiological’

dose of rhGH (4mg/day), and the other the effect of a 'physiological' low dose of rhGH (0.7mg/day) given for 40 weeks, according to the schematics in Figures 2.4 and 2.5 respectively. *In vitro* PBMC cultures were set up to assess the impact of rhGH directly on CD4 and CD8 T cells and experiments set up to elucidate the expression of the growth hormone receptor (GHR) on both T-cell subsets.

3.1 Effect of rhGH on HIV-1-specific T-cell responses in HIV-1$^+$ individuals on ART

Twelve chronically HIV-1-infected individuals with lipodystrophy were enrolled onto a randomised, double-blind, placebo-controlled study to receive 4mg/day rhGH subcutaneously (Serostim, Serono International, Geneva Switzerland) for 12 weeks. Patients were then randomised into one of three groups: to receive placebo (A), to receive alternate day dosing (B), or to receive twice-per-week dosing of rhGH (C), and this continued for a further 12 weeks. At week 24 of the study, patients went back to receiving ART alone (with no immunotherapy) for 24 weeks. HIV-1-specific proliferative CD4 and IFN-γ-producing CD8 T-cell responses were assessed at baseline, weeks 12 and 24 of the study and at a follow-up week 48 visit. Patients' informed consent and Ethics Committee approval were obtained for this study [Herasimtschuk *et al*., 2008]. Patients were eligible to participate in the study if they had a CD4 T-cell count >200 cells/μl blood and if they had been receiving stable HAART for >1 year.

3.1.1 Patient characteristics

The twelve HIV-1-infected individuals enrolled on the trial had all been receiving ART for >4 years, median age was 41 years (IQR 40-47 years), viral load was undetectable (<50 copies/ml plasma) in ten out of twelve patients, and median baseline CD4 and CD8 T-cell counts were 467 (IQR 313-626) and 1068 (IQR 675-1251) cells/μl blood, respectively (Table 3.1). CD4 and CD8 T-cell numbers and viral load did not change significantly between the sample time points. Viral load remained detectable in only one individual throughout the course of the study, and for all patients and study time points, CD4 and CD8 T-cell counts and viral load remained significantly unchanged. HIV-1-specific CD4 T-cell responses were measured using the conventional lymphoproliferative

assay, while pools of overlapping peptides and rVV constructs were used in ELISpot assays to evaluate HIV-1-specific CD8 T-cell responses.

3.1.2 HIV-1-specific CD4 and CD8 T-cell responses following daily administration of rhGH

In eleven out of twelve individuals, HIV-1-specific CD4 and CD8 T-cell responses were absent at baseline despite stable numbers of both T-cell subsets. Following 12 weeks of daily administration of rhGH in combination with successful HAART, HIV-1-specific CD4 T-cell proliferative responses increased significantly (anti-recombinant p24 $p = 0.0059$, anti-native p24 $p = 0.014$, anti-Remune immunogen $p = 0.0090$; Figure 3.1). This increase was focussed on Gag-specific and whole HIV-1 antigen (Remune)-specific CD4 T-cell responses and these were found to be positive in nine out of the twelve patients. These HIV-1-specific T-cell responses were not maintained at week 24 following less frequent dosing of rhGH and by week 48 follow-up, they were undetectable in the majority of individuals.

Daily administration of rhGH in the context of HAART-treated HIV-1 infection resulted in a significant increase in HIV-1-specific CD8 T-cell responses to rVV constructs (Gag rVV $p = 0.059$, Pol rVV $p = 0.049$) and whole peptide pools of Gag and Pol proteins ($p = 0.049$ and $p = 0.0025$, respectively). At week 24, these virus-specific CD8 T-cell responses were maintained in all patients, including those that did not receive further rhGH. At the week 48 follow-up visit, virus-specific CD8 T-cell responses had significantly declined, except those directed at the pools of Pol and Gag peptides (Figure 3.2).

Daily injections of a ‘supra-physiological’ dose (4mg/day) of rhGH for 12 weeks, in addition to HAART, resulted in significant increases in both proliferative CD4 and IFN-γ-producing CD8 HIV-1-specific T-cell responses. This increase was predominantly seen for HIV-1 Gag-specific T-cell responses. Subsequent randomisation into different dosing regimens lead to a decline in HIV-1-specific proliferative CD4 T-cell responses in patients receiving less frequent dosing of rhGH, while virus-specific IFN-γ-producing CD8 T-cell responses were maintained for longer periods of time.

Table 3.1 Patient characteristics at baseline (week 0), and weeks 12, 24 and 48 of the study.

		Baseline				Week 12			Week 24			Week 48		
Patient[a]		Age (years)	CD4 T-cell count (cells/µl blood)	CD8 T-cell count (cells/µl blood)	HIV-1 RNA (copies/ ml plasma)	CD4 T-cell count (cells/µl blood)	CD8 T-cell count (cells/µl blood)	HIV-1 RNA (copies/ ml plasma)	CD4 T-cell count (cells/µl blood)	CD8 T-cell count (cells/µl blood)	HIV-1 RNA (copies/ ml plasma)	CD4 T-cell count (cells/µl blood)	CD8 T-cell count (cells/µl blood)	HIV-1 RNA (copies/ ml plasma)
1 B	●	40	823	1239	<50	714	1218	<50	688	1027	<50	745	956	<50
2 C	●	32	436	555	<50	422	735	<50	456	667	<50	418	567	<50
3 C	▲	38	636	1235	1555	616	1209	5017	676	1179	2027	518	737	2788
4 B	▲	39	604	1613	<50	425	1137	<50	371	899	<50	415	980	<50
5 A	●	45	667	553	<50	697	680	<50	647	559	<50	540	557	<50
6 A	▲	58	320	421	<50	431	837	<50	393	917	<50	364	750	<50
7 C	■	41	257	884	<50	269	747	<50	375	986	<50	443	954	<50
8 B	■	40	622	1602	17089	435	776	<50	609	1202	<50	577	942	<50
9 B	◆	53	211	1074	<50	217	1336	<50	844	1369	<50	241	884	<50
10 C	◆	40	293	715	<50	262	791	<50	286	708	<50	ND	ND	ND
11 A	■	52	374	1288	<50	563	1624	<50	713	2357	<50	599	1364	<50
12 A	◆	43	498	1061	<50	375	980	<50	421	908	<50	341	709	<50
Median		41	467	1068		428	909		533	952		443	884	
IQR		40-47	313-626	675-1251		349-576	769-1211		389-679	851-1185		390-559	723-955	

[a] Randomisation group at week 12 to 24: A – placebo recipients; B – alternate day dosing; and C – twice weekly dosing. Changes in CD4 and CD8 T-cell numbers and viral load between different time points are all non-significant. <50 = undetectable. ND – Not Done.

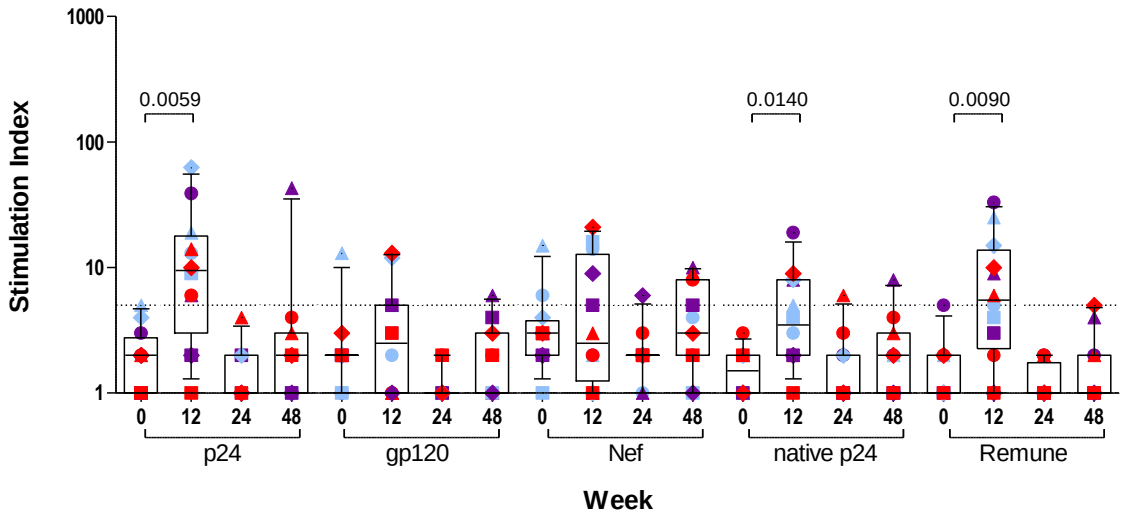


Figure 3.1 Proliferative CD4 T-cell responses to HIV-1 antigens in ART-treated HIV-1-infected patients before and after rhGH immunotherapy.
PBMC from 12 HIV-1-infected patients were cultured in the presence of various HIV-1 antigens in triplicate for 6 days and ^{3}H-thymidine incorporation was measured. Patient visits are depicted at baseline, weeks 12, 24 and 48. Results are expressed as the mean stimulation index of triplicate cultures with percentage error of the mean <15%. The positive threshold of an SI ≥ 5 is indicated. Box plots show the median and IQR, and whiskers represent the 10th and 90th percentiles. Symbols are specific to each patient according to Table 3.1. Randomisation into three groups at week 12 is represented by different colours. Group A (red) received placebo, group B (purple) received alternate day dosing of rhGH and group C (blue) received twice weekly dosing of rhGH. Significant p values of ≤0.05 are shown.

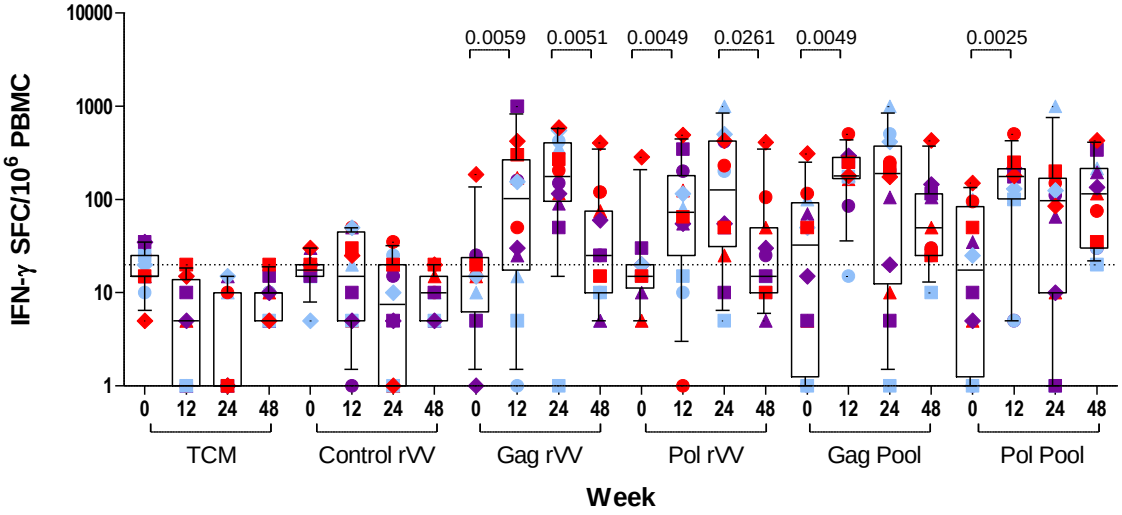


Figure 3.2 IFN-γ production by CD8 T cells in response to rVV HIV-1 constructs and peptide pools in ART-treated HIV-1^{+} patients before and after rhGH therapy.
Patient visits are depicted at baseline and at weeks 12, 24 and 48. Results are expressed as the mean number of SFC per 10^{6} PBMC of duplicate cultures with <10% variation among duplicates. The positive threshold of ≥20 SFC per 10^{6} PBMC is indicated. Box plots show the median and IQR, and whiskers represent the 10th and 90th percentiles. Symbols are specific to each patient according to Table 3.1. Randomisation into three groups performed at week 12 is represented by different colours. Group A (red) received placebo, group B (purple) received alternate day dosing of rhGH and group C (blue) received twice weekly dosing of rhGH. Significant p values of ≤0.05 are shown.

3.2 Long-term, low-dose rhGH in treated HIV-1 infection

HIV-1-specific T-cell responses were investigated from a study in which a low-dose of rhGH (0.7mg) was administered daily for 40 weeks in chronically infected individuals on ART.

Samples were obtained from a randomised, placebo-controlled, double-blind, clinical, single-centre study (carried out at the Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre, Denmark). Caucasian, male subjects on effective ART received either 0.7mg/day rhGH (n = 21) or placebo (n = 15) subcutaneously for 40 weeks (Genotropin or placebo, obtained from Pfizer A/S, DK-2750 Ballerup, Denmark). Subjects were eligible to participate in the study if they were male, Caucasian, aged between 21 and 60 years, on a HAART regimen for at least 12 months, with HIV-1-associated lipodystrophy syndrome (HALS) or not (classification according to the clinical definition applied in The Lipodystrophy Definition Case Study [Carr *et al*., 2003]), <1000 copies/ml plasma HIV-1 RNA and >200 CD4 T cells/µl blood [Hansen *et al*., 2009].

IFN-γ ELISpot assays were carried out on cryopreserved PBMC from baseline (week 0) and week 40, stimulated with peptide pools of Gag 20mers and Gag 9mers (section 2.17, Table 2.5 and Table 2.6, respectively) to assess the virus-specific CD4 and CD8 T cells recognising antigenic peptides in the context of MHC class II or class I, respectively. Flow cytometric, phenotypic analysis was carried out on T cells from the most robust responders (n = 4) in the IFN-γ ELISpot assays. The panel included markers used to determine maturation/differentiation (CCR7, CD45RA, CD27, CD28), activation (CD25, HLA-DR, CD38), exhaustion (PD-1, PD-L1), senescence (CD57) and apoptosis (CD95), as well as assessing markers associated with regulatory T cells ($CD4^{+}CD45RO^{+}CD25^{high}$), panel as per section 2.12 and Table 2.1.

Flow cytometry data was analysed using FACS DIVA™ software and statistical analysis carried out using GraphPad Prism. For analysis of data between patient groups at baseline, the Mann-Whitney U test was used and for intra-group analysis, comparing the paired samples at baseline (week 0) and week 40, the Wilcoxon signed rank test was performed. Results are presented as p values and considered significant where $p \leq 0.05$.

3.2.1 Patient characteristics

Subjects were well matched between the two groups with a median age of 44 years (interquartile range, IQR, 41-51 years) and median CD4 T-cell count of 633 cells/µl blood (IQR 493-902 cells/µl blood) for the entire study group (Table 3.2). Median duration of HIV-1 infection for the whole study population was 14 years (IQR 9-19 years) and median ART duration was 8 years (IQR 6-8 years). Table 3.2 shows patient characteristics with median and IQR calculated for each treatment group (rhGH or placebo) and for the entire data set. For sample identification, 'H' and 'NH' refer to HALS (HIV-1-associated lipodystrophy syndrome) and non HALS, respectively. The patients depicted with an open symbol in Table 3.2 (H17, H20, H22 and H25) indicate individuals who showed the most robust responses at week 40 following daily administration of rhGH and were further characterised phenotypically.

Table 3.2 Baseline characteristics for patients on the low-dose rhGH study.
rhGH- (n=21) and placebo- (n=15) treated patients are shown here in the upper and lower part of the table respectively, with the median and IQR for each group shown below the appropriate dataset, and with the median and IQR for the entire dataset at the bottom of the table. For the sample ID, 'H' and 'NH' refer to HALS and non HALS, respectively. Symbols depict patients that were further analysed phenotypically.

Treatment	Sample ID		Age (years)	Viral Load (copies/ml)	CD4 T-cell count (cells/µl)	CD8 T-cell count (cells/µl)	HIV-1 duration (years)	ART duration (years)
rhGH	H01/0		53	<40	924	730	13	8
	H03/0		58	<40	1213	3088	12	8
	H05/0		55	<40	413	1022	14	8
	H07/0		44	<40	740	1531	13	8
	H09/0		46	<40	619	929	19	6
	H10/0		59	<40	672	710	8	8
	H11/0		46	43	507	681	7	7
	H13/0		37	<40	1296	1399	14	8
	H15/0		41	<40	779	1335	19	8
	H17/0	□	41	<40	276	1771	20	9
	H20/0	▽	50	<40	552	1355	10	5
	H21/0		48	<40	519	1037	20	7
	H22/0	△	61	<40	347	469	19	10
	H25/0	◇	59	<40	589	498	14	11
	NH04/0		43	102	654	2623	3	3
	NH07/0		38	40	843	1265	10	7
	NH08/0		58	<40	1556	1282	22	8
	NH09/0		42	<40	527	1060	21	10
	NH16/0		44	<40	1355	1396	11	9
	NH17/0		42	<40	1113	1254	7	7
	NH21/0		29	<40	345	953	2	2
Median			46		654	1254	13	8
IQR			42-55		519-924	929-1396	10-19	7-8
Placebo	H02/0		55	<40	941	1141	6	6
	H04/0		38	<40	1108	918	11	7
	H06/0		41	<40	761	1131	16	7
	H08/0		49	<40	404	951	16	8
	H12/0		41	<40	894	1253	9	8
	H14/0		39	<40	618	995	3	2
	H18/0		55	<40	208	709	8	8
	H19/0		46	<40	524	1550	21	6
	H24/0		46	<40	698	1101	16	9
	NH03/0		28	<40	401	952	1	1
	NH06/0		37	<40	647	1175	18	9
	NH10/0		50	<40	547	1207	20	5
	NH11/0		42	<40	449	837	20	3
	NH18/0		43	<40	216	800	20	10
	NH20/0		37	<40	1018	1657	19	8
Median			42		618	1101	16	7
IQR			39-47		427-828	935-1191	9-19	6-8
Overall Median			44		633	1116	14	8
Overall IQR			41-51		493-902	926-1340	9-19	6-8

3.2.2 IFN-γ production in response to peptide stimulation

At baseline (week 0), there was no statistically significant difference in IFN-γ production in response to peptide pools of overlapping HIV-1 Gag 20mers or Gag 9mers between the rhGH-treated and the placebo-treated groups ($p = 0.6188$ and $p = 0.6512$, respectively). At week 40, patients who received placebo showed no significant difference in their response to Gag 20mers or Gag 9mers compared to baseline (Figure 3.3B and 3.3D, respectively). However, patients that received 0.7mg rhGH/day showed increased responses to both pools of peptides at week 40, compared to baseline. Responses to the MHC class II restricted peptides increased but did not reach statistical significant ($p = 0.0559$; Figure 3.3A), whilst responses to MHC class I restricted peptide pools increased significantly ($p = 0.0355$; Figure 3.3C).

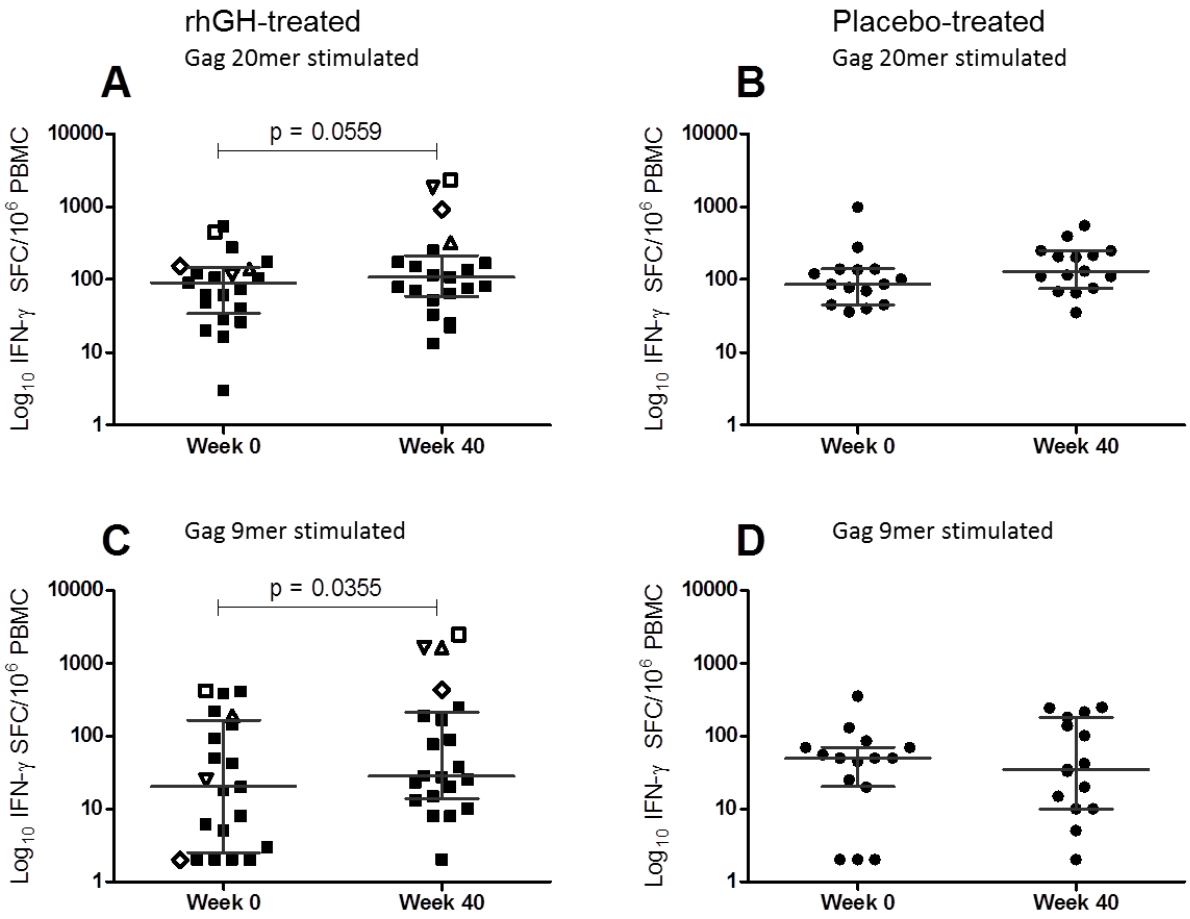


Figure 3.3 IFN-γ production by T cells in response to Gag 20mer and Gag 9mer peptide stimulation in patients receiving low-dose rhGH or placebo.
IFN-γ production at baseline and at week 40 was assessed in ART-treated patients receiving daily administration of rhGH (n = 21), shown here in solid black squares (■) and open shapes, or placebo (n = 15), shown here in solid black circles (●). PBMC were stimulated with pools of overlapping Gag 20mer (A and B) or Gag 9mer (C and D) peptides overnight and IFN-γ production determined. The robust responders at week 40 (n = 4), from which PBMC were further characterised phenotypically at baseline and at week 40, are depicted with the open shapes: □ H17, ▽ H20, △ H22, ◇ H25. Results are expressed on a log transformed scale as mean spot forming cells (SFC) per million PBMC of duplicate cultures with <10% variation among duplicates. Individual data points, the median and interquartile range are illustrated for each graph. Statistical analysis was carried out in GraphPad Prism 5.0 and changes from baseline were determined using the Wilcoxon signed rank test, with significant p values (≤ 0.05) and those approaching statistical significance ($0.05<p<0.1$) shown.

3.2.3 Phenotypic characterisation of the strongest responders

CD4 and CD8 T-cell phenotypes of the most robust responders in terms of IFN-γ production at week 40 (n = 4) were characterised (these patients are depicted with open symbols in Table 3.2 and Figures 3.3A and 3.3C). Statistical analyses were not performed on these results due to the small sample number. Immunophenotyping, performed at baseline and at week 40 on these samples, demonstrated a trend toward a reduction in the percentage expression of the activation markers HLA-DR and CD38 within both T-cell subsets following administration of low-dose rhGH for 40 weeks (Figure 3.4). This reduction was more pronounced when looking at CD38 expression, for both percentage expression and mean fluorescence intensity (MFI; Figure 3.4C and Figure 3.4E, respectively). The MFI of the apoptotic marker CD95 was also reduced on CD4 and CD8 T cells following 40 weeks of rhGH (Figure 3.5E) whereas there were no other apparent trends in the expression of senescent (CD57) or apoptotic markers (CD95; Figure 3.5).

Markers associated with a regulatory T-cell phenotype ($CD4^{+}CD45RO^{+}CD25^{high}$) were assessed at baseline and week 40 and did not reveal any distinct trends (two patients showed an increase in the percentage expression of these markers, and two patients showed a decrease; Figure 3.6A). For all four patients, however, there was a decrease in the expression of CD25, on both T-cell subsets, in terms of both percentage expression and MFI (Figure 3.6B and Figure 3.6C, respectively).

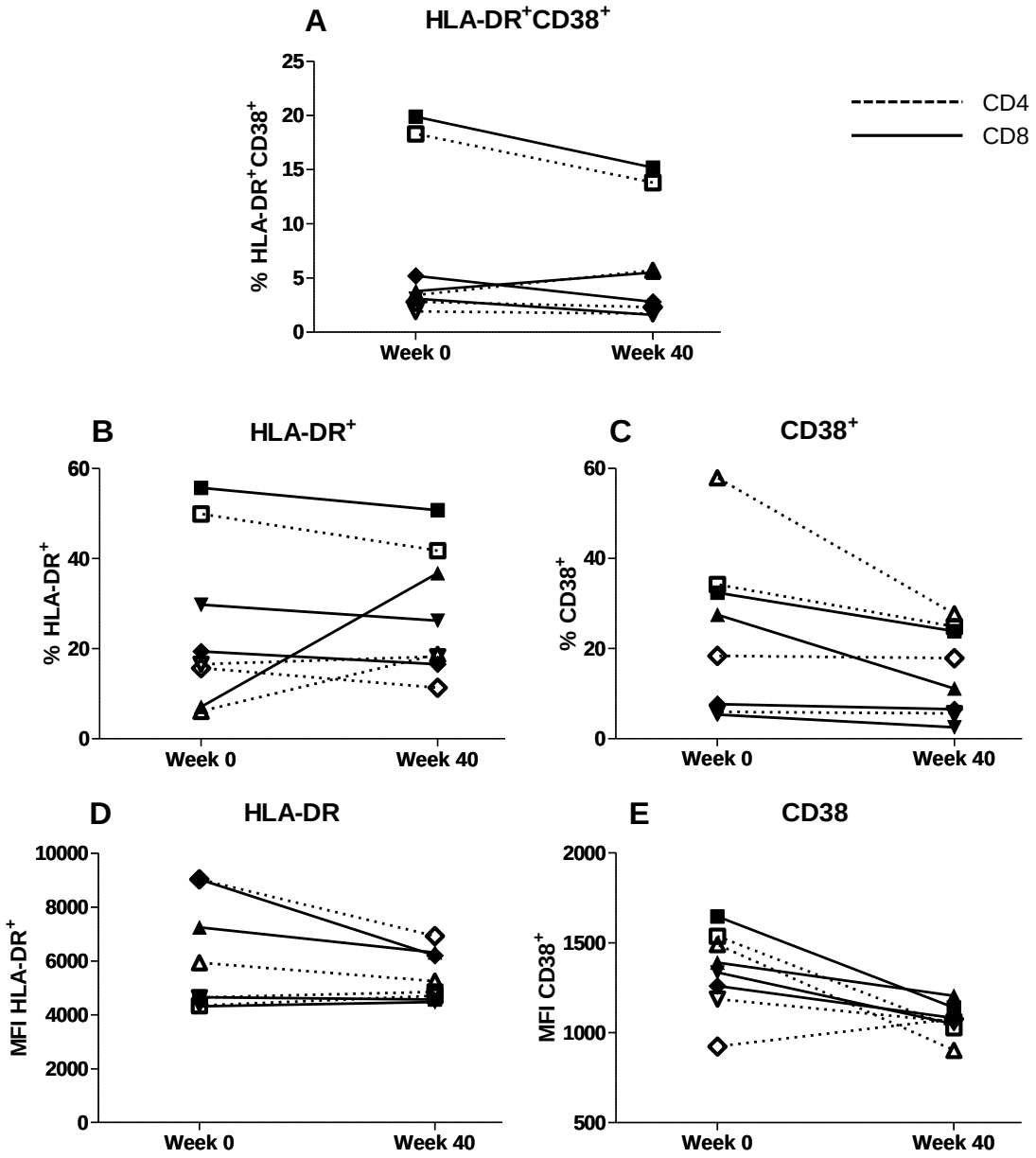


Figure 3.4 The effect of low-dose rhGH on the expression of activation markers.
Flow cytometric analysis was carried out at baseline (week 0) and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of HLA-DR and CD38 (A), HLA-DR (B), CD38 (C) and the MFI of both of these markers (D and E, respectively).

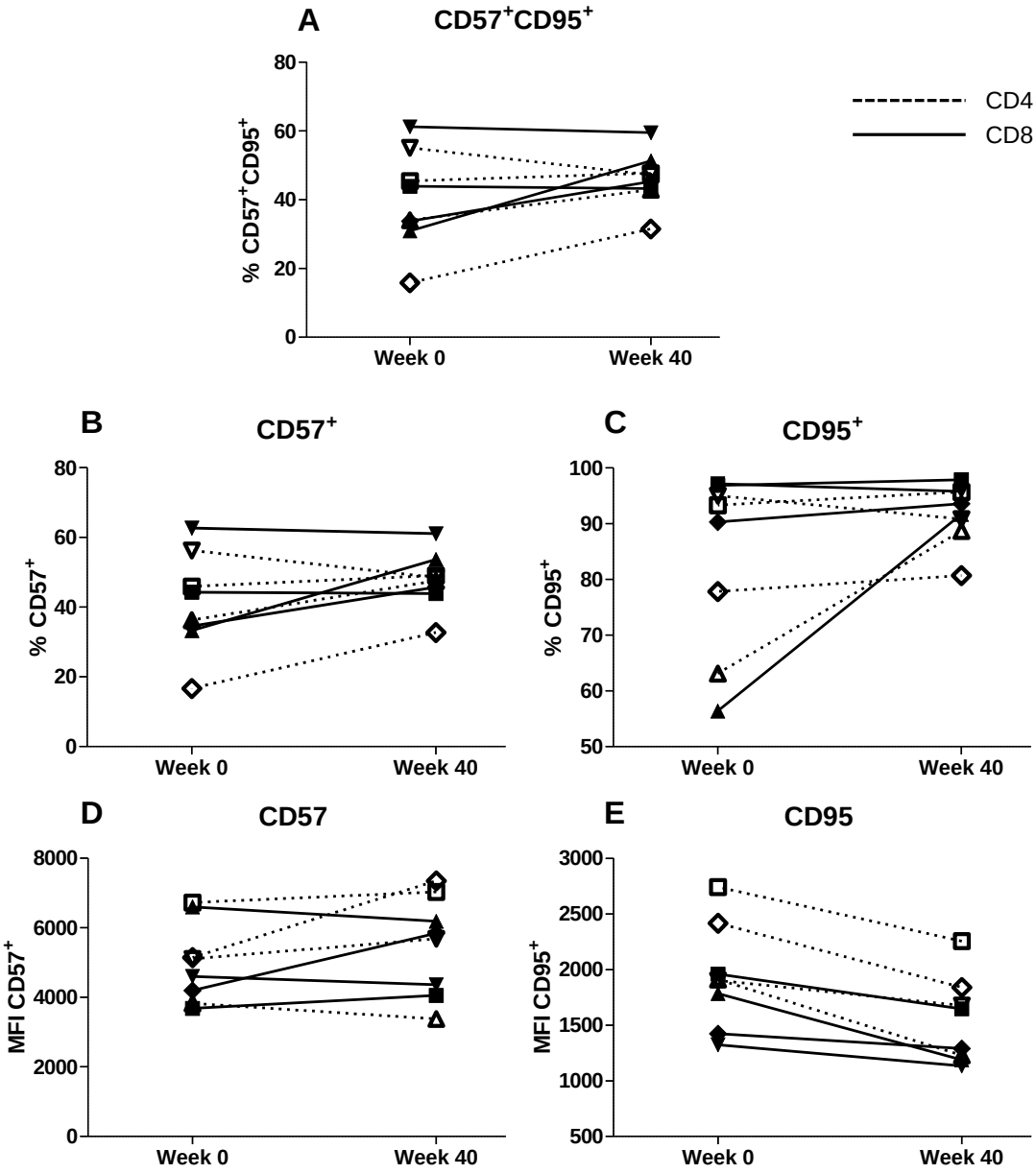


Figure 3.5 The effect of low-dose rhGH on the expression of markers of senescence and apoptosis.
Flow cytometric analysis was carried out at baseline and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of CD57 and CD95 (A), CD57 (B), CD95 (C) and the MFI of both of these markers (D and E, respectively).

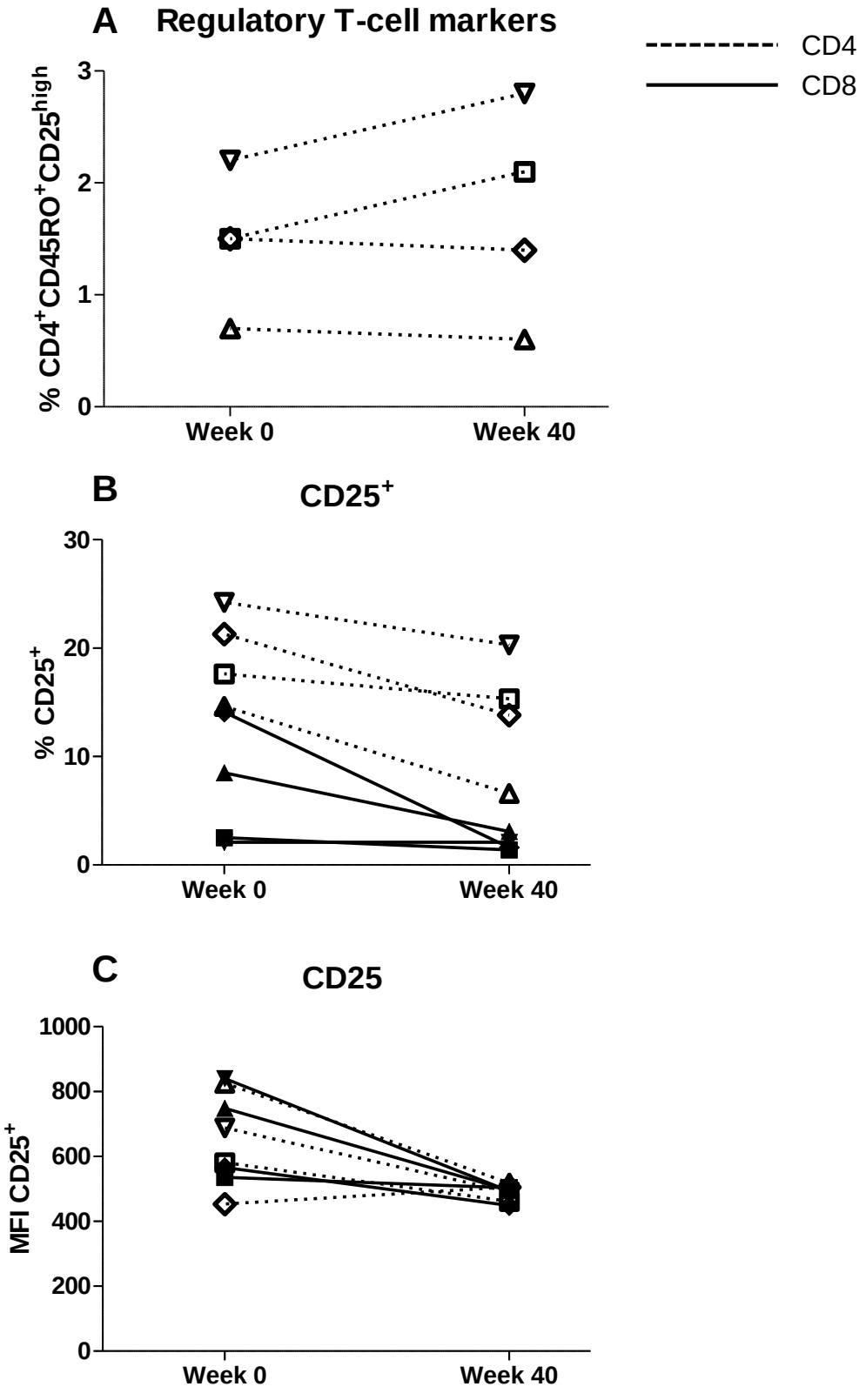


Figure 3.6 Regulatory T-cell markers following long-term, low-dose rhGH.
Flow cytometric analysis was carried out at baseline and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of markers associated with a regulatory T-cell phenotype ($CD4^+CD45RO^+CD25^{high}$; (A), and CD25 expression both in terms of percentage expression (B) and MFI (C).

Administration of low-dose rhGH for 40 weeks resulted in a shift from an early T-cell phenotype to an intermediate and late phenotype, as determined by the expression of CD27 and CD28 (Figure 3.7) for both T-cell subsets. When differentiation status was assessed using CCR7 and CD45RA expression, the trend was similar, with a reduction in naive CD4 and CD8 T cells following rhGH therapy (Figure 3.8A) and an increase in CD8 T_{CM} (Figure 3.8B) and CD4 T_{EM} (Figure 3.8C). There was no apparent effect on the markers associated with a T_{EMRA} phenotype (Figure 3.8D). Following 40 weeks of low-dose rhGH, no apparent trends in the percentage expression of PD-1 or PD-L1 were observed on the differentiation/maturation subsets of CD4 and CD8 T cells (Figure 3.9 and Figure 3.10, respectively). Similarly, the MFI of PD-1 and PD-L1 on both T-cell subsets did not appear to be altered by rhGH therapy (Figure 3.11A and 3.11B, respectively). However, there was a trend towards a reduction in the percentage expression of PD-L1 on total lymphocytes following daily administration of 0.7mg rhGH for 40 weeks (Figure 3.11C).

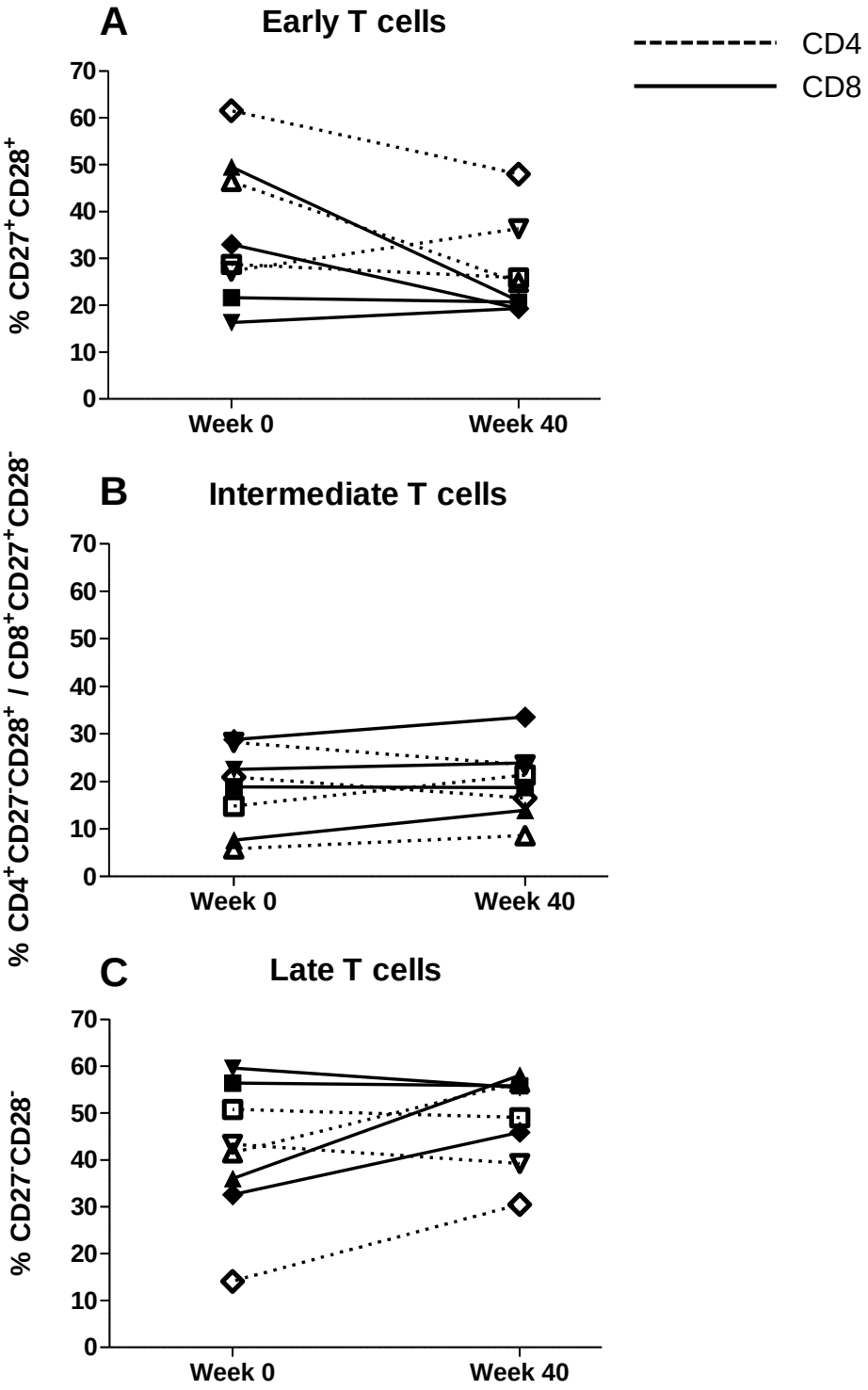


Figure 3.7 Early, intermediate and late T cells as determined by expression of CD27 and CD28.
Flow cytometric analysis was carried out at baseline and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of markers associated with early ($CD27^+CD28^+$) T cells (A), intermediate ($CD4^+CD27^-CD28^+$ or $CD8^+CD27^+CD28^-$) T cells (B) or late ($CD27^-CD28^-$) T cells (C).

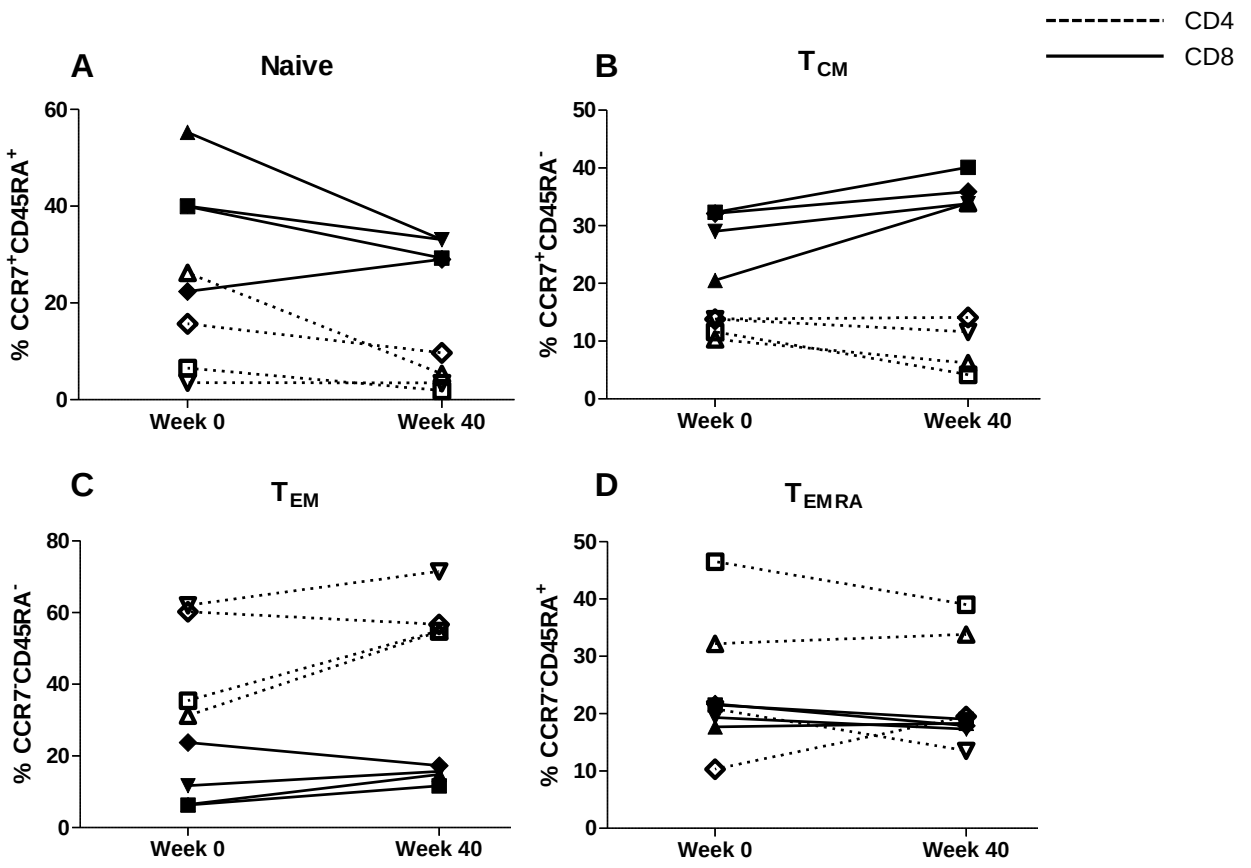


Figure 3.8 CD4 and CD8 T-cell differentiation defined by expression of CCR7 and CD45RA.
Flow cytometric analysis was carried out at baseline (week 0) and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of markers associated with a naive ($CCR7^+CD4RA^+$; A), T_{CM} ($CCR7^+CD45RA^-$; B), T_{EM} ($CCR7^-CD45RA^-$; C) and a T_{EMRA} ($CCR7^-CD45RA^+$; D) phenotype.

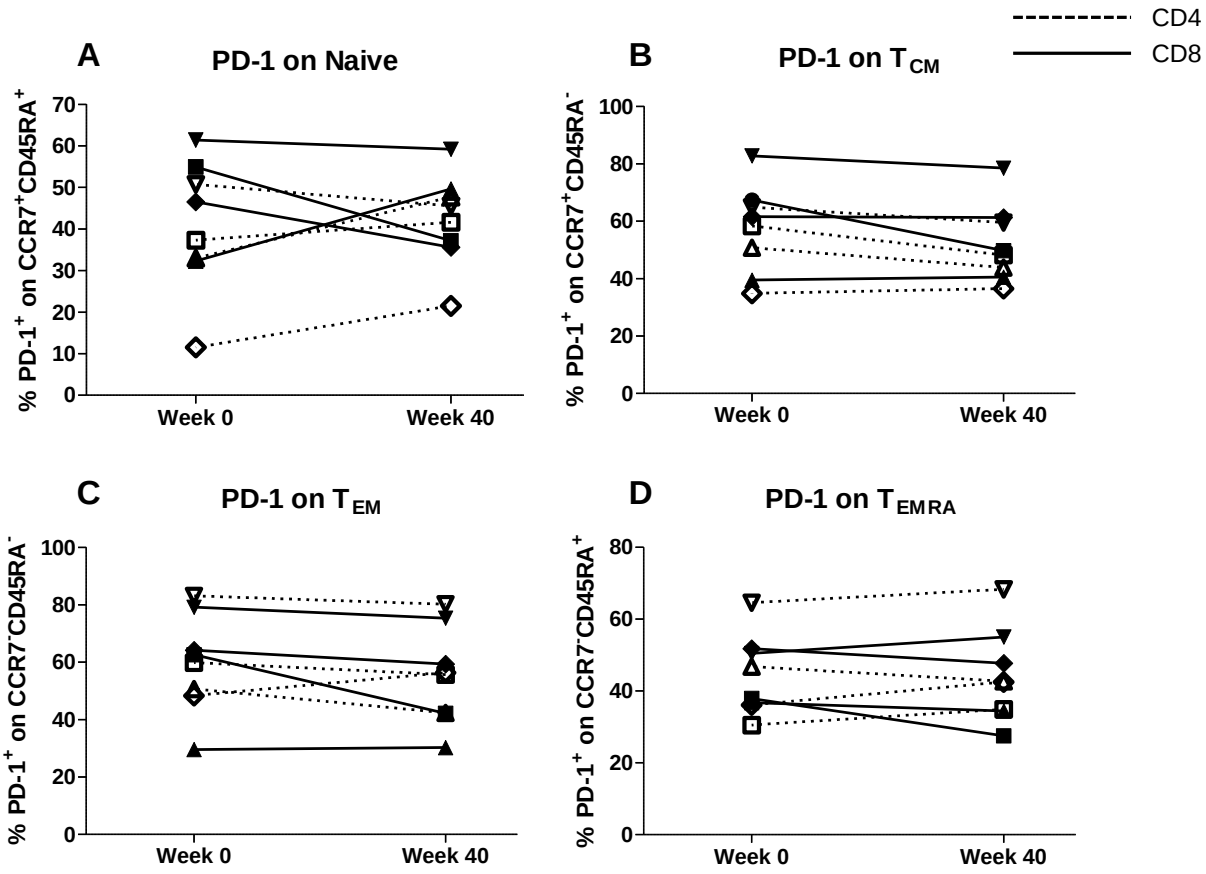


Figure 3.9 PD-1 expression on differentiated subsets of CD4 and CD8 T cells following low-dose rhGH.
Flow cytometric analysis was carried out at baseline (week 0) and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of PD-1 on naive (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) subsets, defined as previously described by the expression of CCR7 and CD45RA.

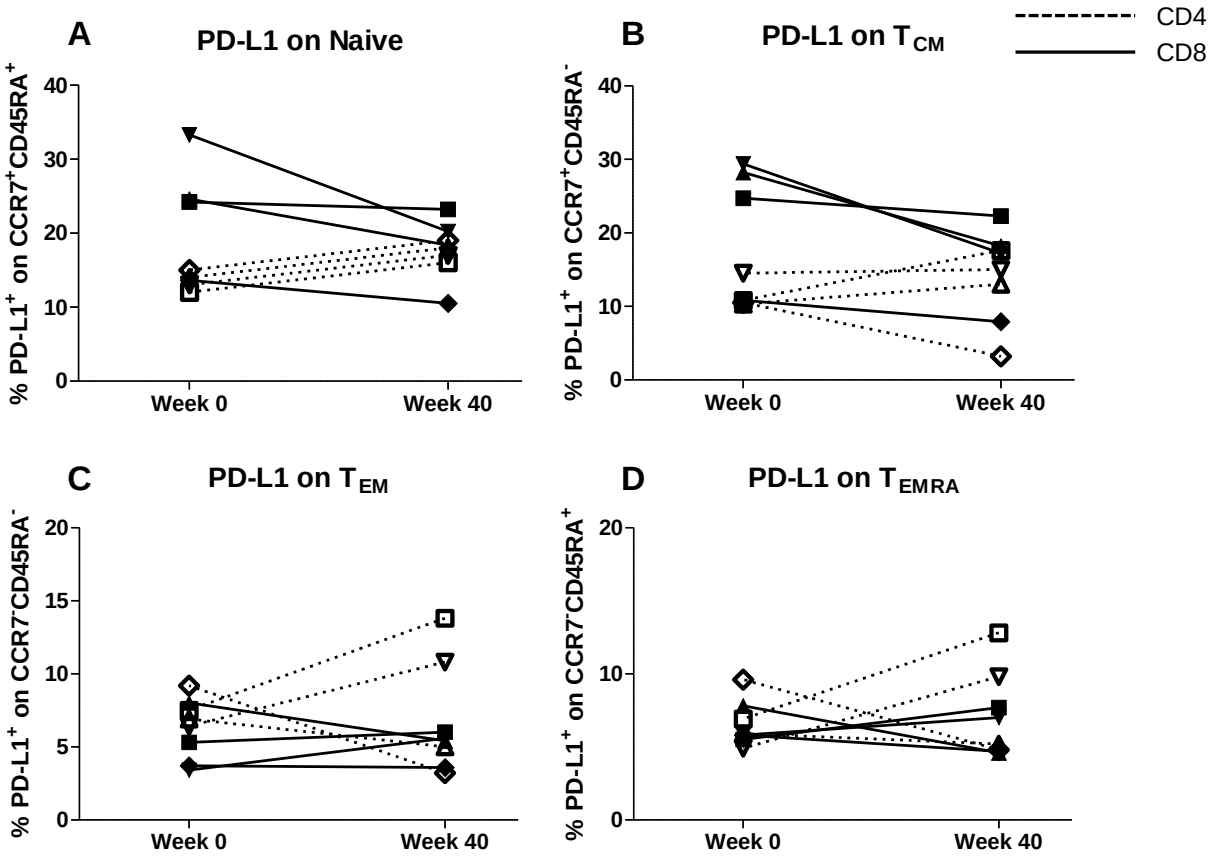


Figure 3.10 PD-L1 expression on differentiated subsets of CD4 and CD8 T cells following low-dose rhGH.
Flow cytometric analysis was carried out at baseline (week 0) and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the percentage expression of PD-L1 on naive (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) subsets, defined as previously described by the expression of CCR7 and CD45RA.

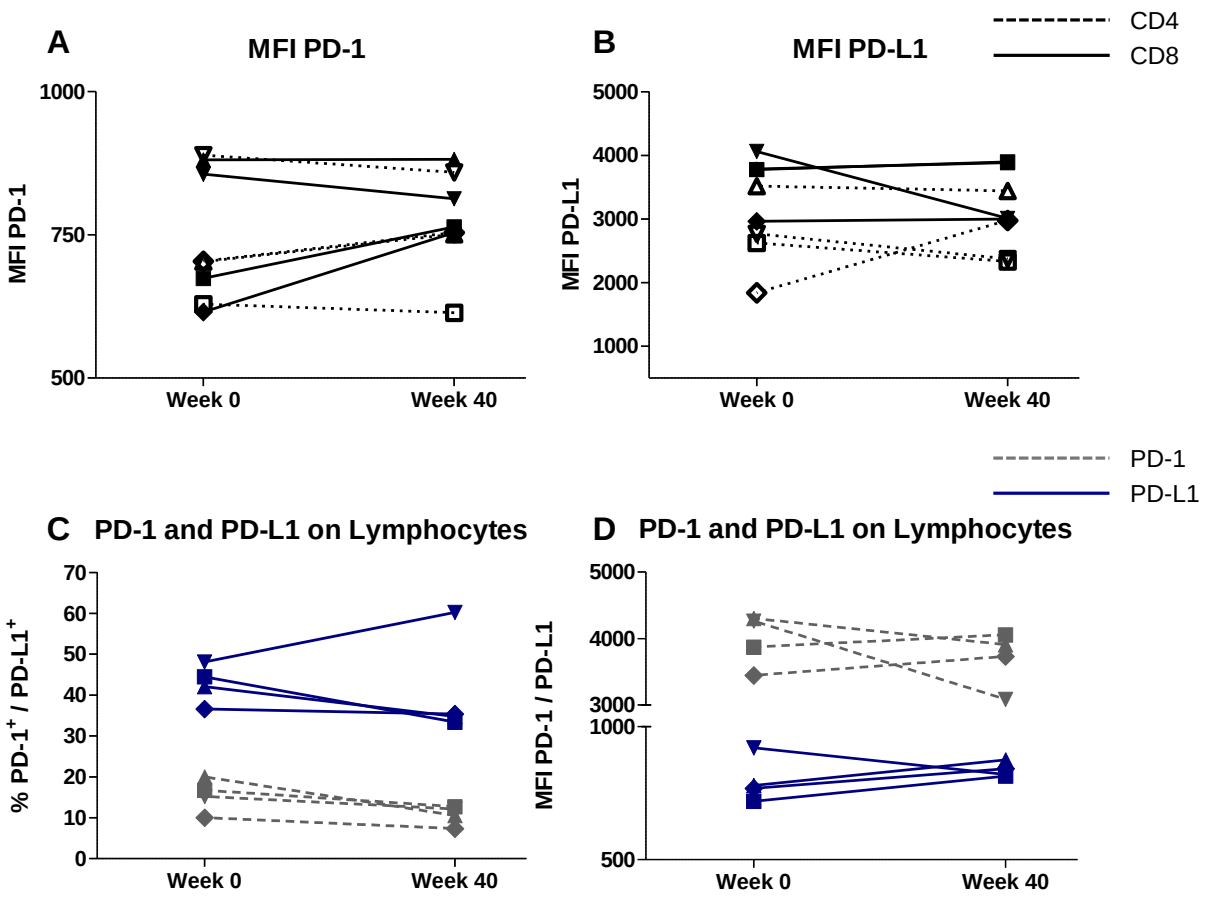


Figure 3.11 Expression of PD-1 and PD-L1 on CD4 and CD8 T cells and on total lymphocyte subset.
Flow cytometric analysis was carried out at baseline (week 0) and at week 40, following daily administration of 0.7mg rhGH, for the most robust responders in the IFN-γ ELISpot assays at week 40 (n = 4; symbols for each patient correspond to the symbols used in Figure 3.3). CD4 and CD8 T cells (open symbols and dotted lines, and closed symbols and solid lines, respectively) were assessed for the expression in terms of MFI of PD-1 (A) and PD-L1 (B). The percentage expression of PD-1 (C; blue symbols and solid blue lines) and PD-L1 (grey symbols and dotted lines) and the MFI (D) were also determined.

Daily administration of low-dose rhGH (0.7mg/day) in HIV-1^+ subjects on HAART for 40 weeks resulted in increased IFN-γ production in response to overlapping peptide pools of HIV-1 Gag 20mers and Gag 9mers; the increase in response to the latter being significant. There were no significant changes in IFN-γ production in response to these peptide pools for patients who had received placebo for 40 weeks. Phenotypic characterisation of CD4 and CD8 T cells from the four rhGH-treated patients with the most robust responses (in terms of IFN-γ production) at week 40 showed a decrease in markers associated with chronic immune activation at this time point, compared to baseline.

3.3 rhGH *in vitro* culture studies

To elucidate further the impact of rhGH on T-cell phenotype, PBMC from HIV-1-infected individuals (n =10) and from healthy donors (n = 6) were cultured with rhGH at a concentration of 1µg/ml (corresponding to the pharmacological dose) for 72 hours (as described in section 2.14). Immunophenotyping was carried out at baseline and following *in vitro* culture to assess the effect of rhGH on the surface markers of T cells using the panel of markers described in section 2.12, Table 2.1. The Wilcoxon signed rank test was used for statistical analysis. P values are shown where there is a significant difference between the means ($p \leq 0.05$). As a control, cells from each donor were also cultured with AB media alone (no rhGH) for 72 hours and phenotyped.

3.3.1 Patient characteristics

HIV-1^+ donors were all male, had a median age of 46 years (IQR 43 – 51 years), median CD4 T-cell count of 621 cells/µl blood (IQR 533-658 cells/µl blood) and HIV-1 RNA was <50 copies/ml plasma in 9/10 individuals. The duration of infection, duration of ART, treatment regimen and nadir CD4 T-cell count are detailed in Table 3.3. The healthy control samples had a median age of 29 years (IQR 27-32 years) and included three male and three female donors.

Table 3.3 Patient characteristics for HIV-1^+ samples and seronegative controls used in rhGH culture experiments. NA – not applicable; patient was not on HAART.

Patient short code	Age (years)	Gender	Viral load (copies/ml)	ART regimen	Duration of ART (months)	CD4 T-cell count (cells/µl)	Nadir CD4 T-cell count (cells/µl)	Duration of HIV-1 infection (months)
Healthy controls (n = 6)	Median 29 IQR: 27-32	3M, 3F						
B732	51	M	<50	NA	NA	554	418	36.66
W693	37	M	<50	TFV+FTC+EFV	29.84	526	203	49.77
K560	51	M	<50	ABC+3TC+SQV+RTV	128.33	617	113	135.77
N374	45	M	81	TFV+3TC+NVP	17.15	1039	733	229.28
Z171	38	M	<50	TFV+FTC+EFV	64.66	641	182	65.41
J567	57	M	<50	TFV+FTC+RTV+LOP	152.56	625	27	153.21
L061	44	M	<50	FTC+TFV+ATZ+RTV	130.03	218	48	164.07
C828	43	M	<50	FTC+TFV+RTV+ATZ	50.33	664	235	80.49
E760	46	M	<50	TFV+FTC+EFV	87.34	513	64	87.61
G201	51	M	<50	ABC+3TC+NVP	144.56	975	7	222.16
Median	46				87.34	621	148	111.69
IQR	43-51				50.33-130.03	533-658	52-227	69.18-161.35

3.3.2 Immunophenotyping before and after culture with rhGH

PBMC from seronegative healthy donors (n = 6) generally showed no significant difference in the percentage expression of surface markers before and after culture with either rhGH or AB TCM alone. Figure 3.12 illustrates percentage co-expression of the activation markers HLA-DR and CD38 on CD4 and CD8 T cells (Figure 3.12 A and B, respectively). Only a statistically significant reduction in the percentage expression of CD38 on CD4 T cells was seen following 72-hour culture either with rhGH or TCM alone (Figure 3.12 E). Statistically significant reductions in the percentage expression of senescence and apoptosis markers on CD8 T cells was seen following 72-hour culture with TCM (Figures 3.13 B, C and D). The phenotypic changes for all other markers investigated on the panel (Table 2.1) were minimal (data not shown).

Following 72-hour culture with rhGH, a significant reduction in activation, senescence and apoptosis markers was seen compared to baseline in HIV-1^+ donors (n = 10). Figure 3.14 shows representative flow cytometry plots for one individual, before and after culture with rhGH. However, when a control was added, and cells were cultured in parallel in the presence of TCM alone for 72 hours, these markers were similarly reduced. This was true for markers of immune activation (Figure 3.15), markers of senescence and apoptosis (Figure 3.16) and other markers in the panel, and this down-regulation was also seen on PBMC from HIV-1^+ patients cultured with varying concentrations of rhIGF-1 (data not shown). The Wilcoxon signed rank test was used as data sets were not normally distributed and changes following culture were being observed (within patient changes).

T-cell surface markers were significantly down-regulated following 72-hour culture of PBMC from HIV-1^+ individuals, and this occurred both in the presence and absence of rhGH. This decreased surface marker expression was not seen following culture of PBMC from healthy donors in the same way.

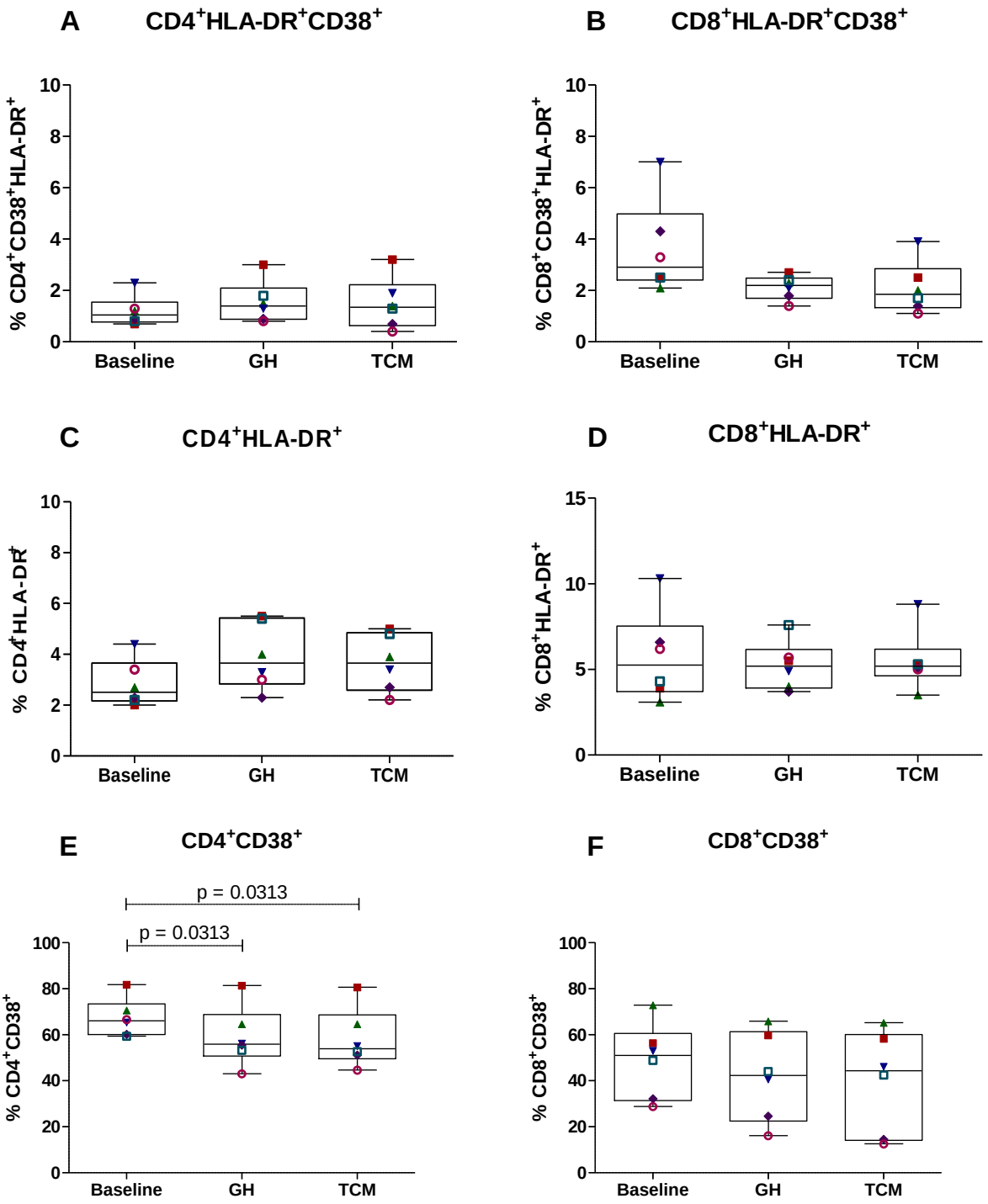


Figure 3.12 Changes in the expression of activation markers in healthy donors following culture with rhGH and TCM. Percentage co-expression of HLA-DR and CD38 was assessed on CD4 (A) and CD8 (B) T cells from healthy control individuals (n = 6). Percentage expression of HLA-DR and CD38 was assessed on both T-cell subsets (C and E show expression on CD4 T cells, and D and E show expression on CD8 T cells). Symbols represent results from individual donors and are consistent throughout the plots. Box plots show the median and IQR and whiskers represent the 10th and 90th percentiles.

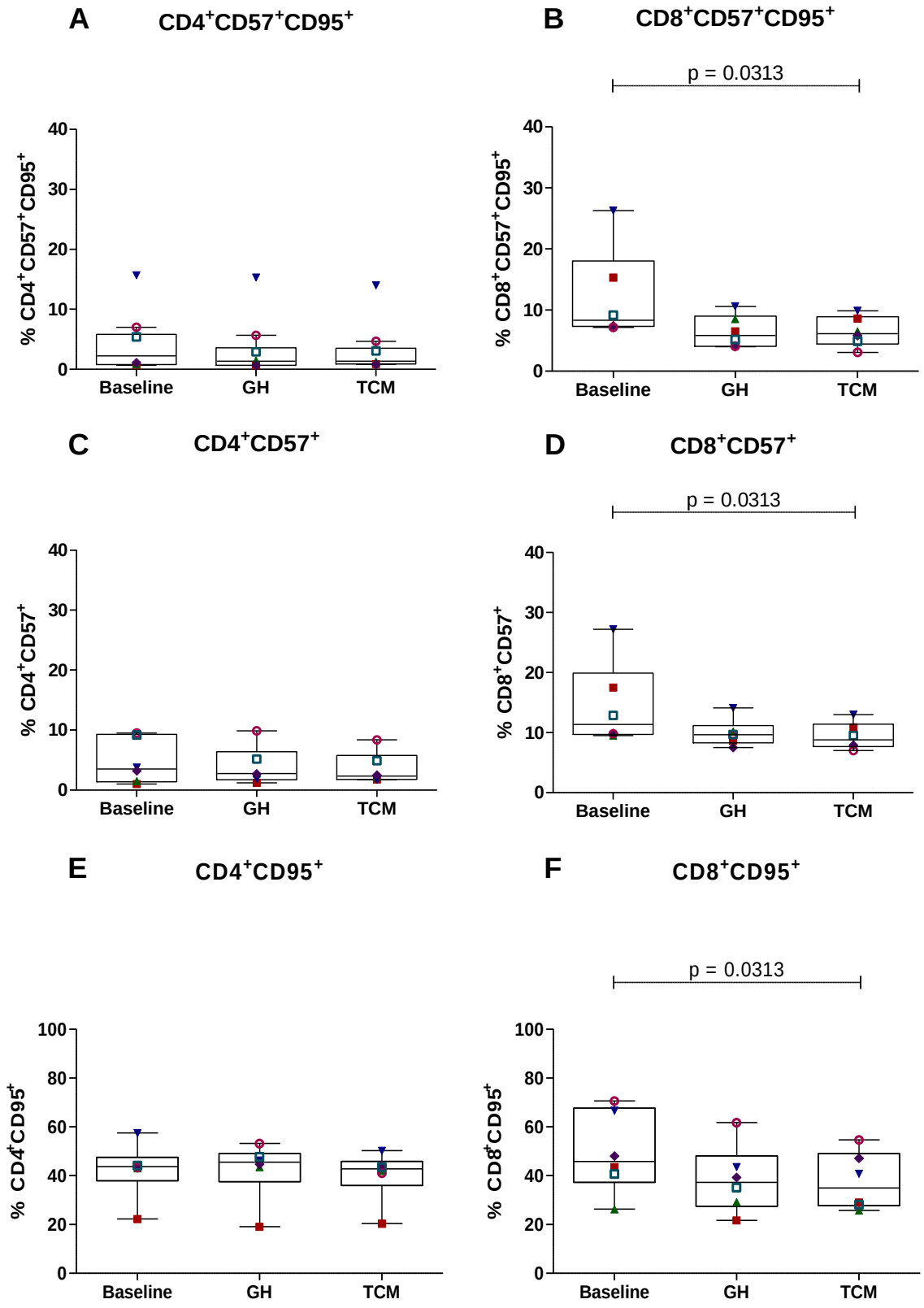


Figure 3.13 Changes in the expression of senescence and apoptosis markers in healthy donors following culture with rhGH and TCM.
Percentage co-expression of CD57 and CD95 was assessed on CD4 (A) and CD8 (B) T cells from healthy control individuals (n = 6). Percentage expression of CD57 and CD95 was assessed on both T-cell subsets (C and E show expression on CD4 T cells, and D and E show expression on CD8 T cells). Symbols represent results from individual donors and are consistent throughout the plots. Box plots show the median and IQR and whiskers represent the 10th and 90th percentiles.

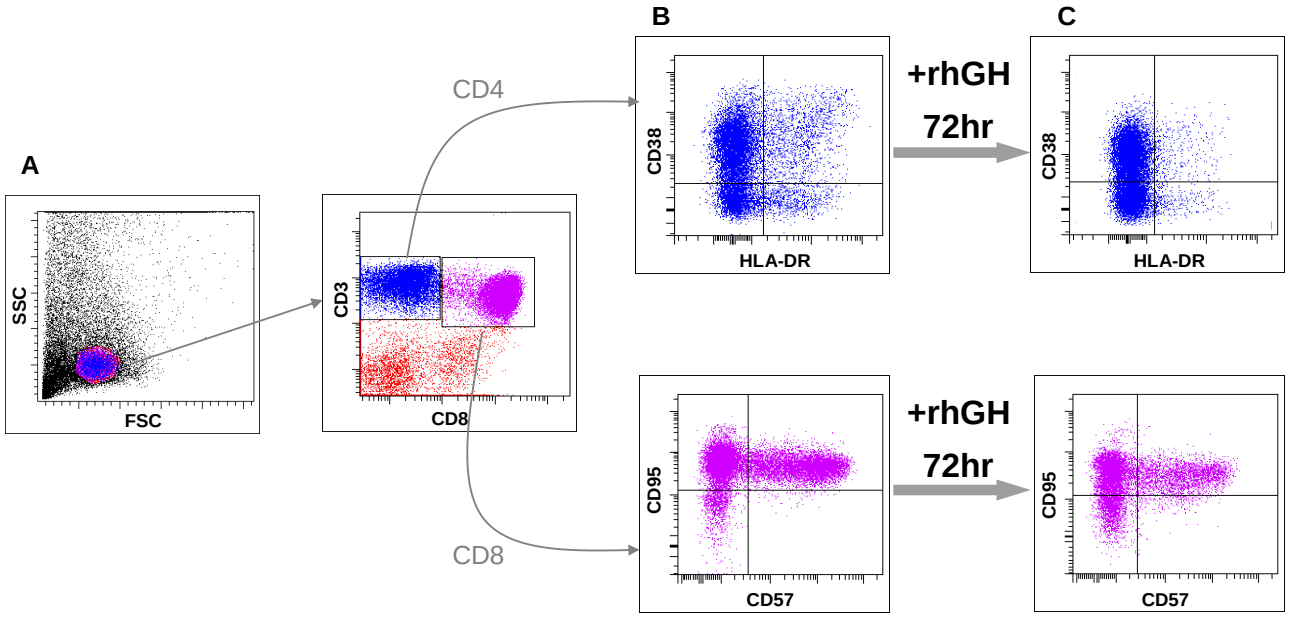


Figure 3.14 Representative example of changes in extracellular surface staining following culture with rhGH.
The lymphocyte population was determined (A) as described previously and in this example, markers of activation (CD38 and HLA-DR; top panel) and markers of senescence and apoptosis (CD57 and CD95, lower panel) were assessed in an HIV-1-infected donor (K087) both before (B) and after (C) 72-hour culture with rhGH.

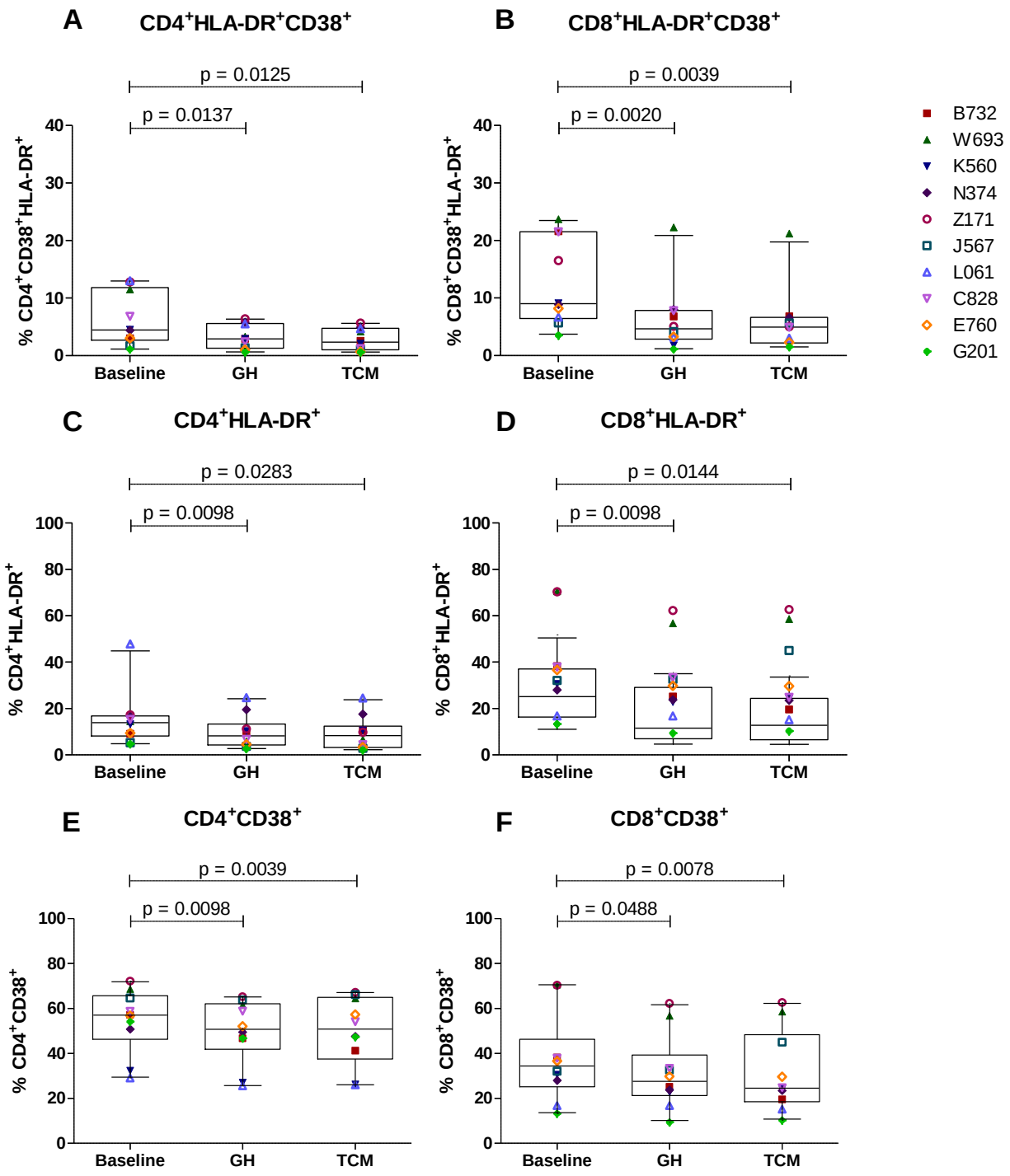


Figure 3.15 Changes in the expression of activation markers on PBMC from HIV-1$^+$ donors following culture with rhGH and TCM.
Percentage co-expression of HLA-DR and CD38 was assessed on CD4 (A) and CD8 (B) T cells from HIV-1-infected individuals (n =10). Percentage expression of HLA-DR and CD38 was assessed on both T-cell subsets (C and E show expression on CD4 T cells, and D and E show expression on CD8 T cells). Symbols represent results from individual donors and are consistent throughout the plots. Box plots show the median and IQR and whiskers represent the 10th and 90th percentiles.

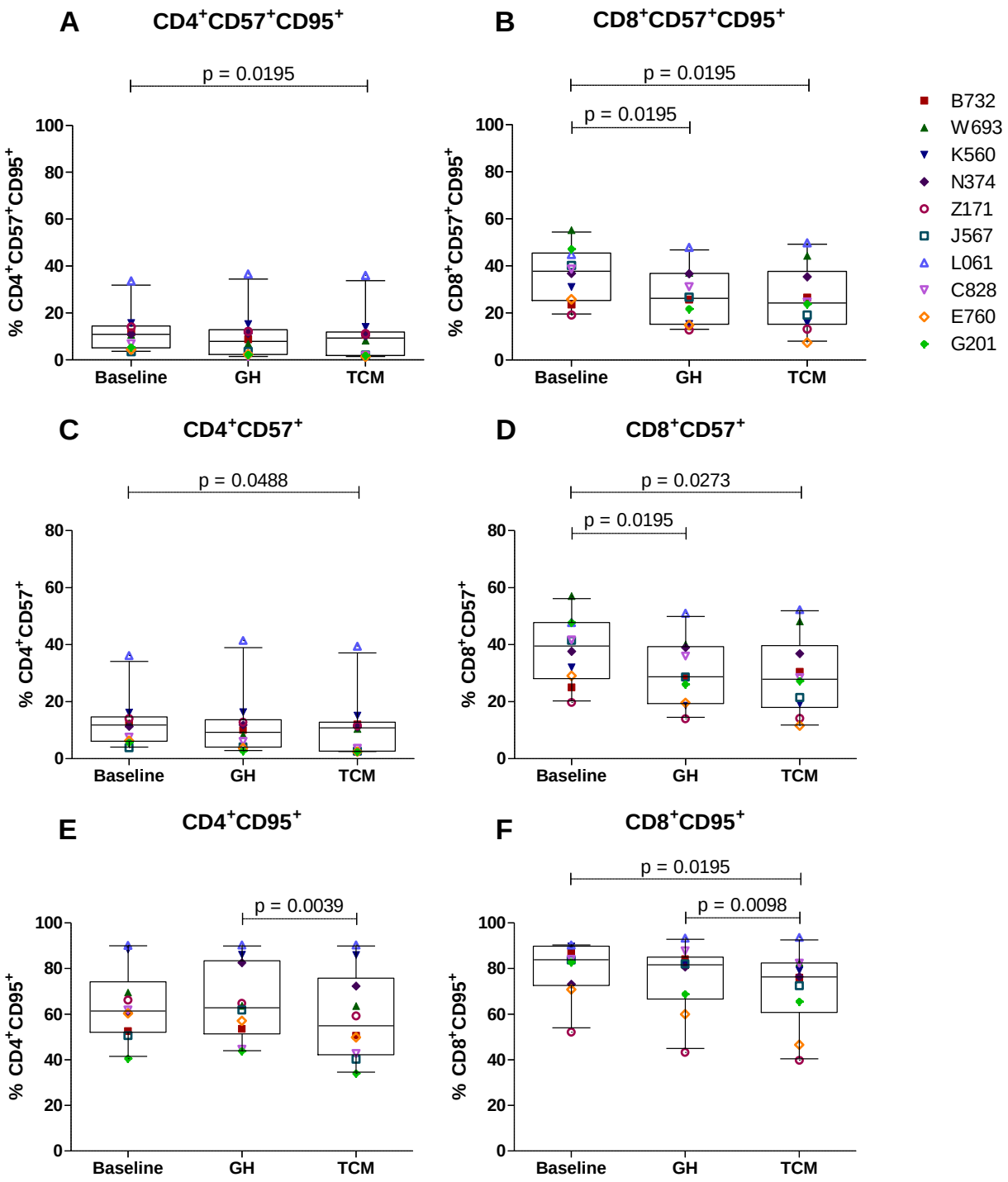


Figure 3.16 Changes in the expression of senescence and apoptosis markers on PBMC from HIV-1^{+} donors following culture with rhGH and TCM.
Percentage co-expression of CD57 and CD95 was assessed on CD4 (A) and CD8 (B) T cells from HIV-1-infected individuals (n =10). Percentage expression of CD57 and CD95 was assessed on both T-cell subsets (C and E show expression on CD4 T cells, and D and E show expression on CD8 T cells). Symbols represent results from individual donors and are consistent throughout the plots. Box plots show the median and IQR and whiskers represent the 10th and 90th percentiles.

3.4 Growth hormone receptor expression on CD4 and CD8 T cells

In order to elucidate further the impact of GH in the context of HIV-1 infection, characterisation of the growth hormone receptor (GHR) on CD4 and CD8 T cells was carried out using PBMC from HIV-1 infected individuals and healthy control subjects. The FITC-conjugated GHR antibodies, and appropriate isotype-matched control, were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) as detailed in Table 2.4. A panel was set up as detailed in Table 2.3 with all three GHR antibodies (which differed by clone) tested. Flow cytometric analysis was carried out and 100,000 gated lymphocytes were acquired in order to detect the GHR expression. The panel was designed to determine GHR expression on differentiation/maturation subsets of both T-cell subsets using the markers CCR7 and CD45RA. Although all three GHR antibodies gave similar results in terms of percentage expression of the GHR on CD4 and CD8 T cells, the MAB1 clone produced more consistent results in preliminary experiments and was used for all subsequent experiments which are described in this section. PBMC from eight ART-treated HIV-1^{+} individuals (patient characteristics for these individuals can be found in Table 5.2, and their short codes are as follows: F810, G739, M160, O523, P087, R771, S648, W097), and four healthy seronegative donors were assessed. GHR expression was found to be significantly lower in HIV-1^{+} subjects compared to healthy controls on both CD4 and CD8 T cells (Figure 3.17). There was no significant difference between the MFI of GHR on either T-cell subset between HIV-1^{+} and healthy persons and expression of the GHR on CD4 T cells did not differ significantly from expression on CD8 T cells in either group (data not shown).

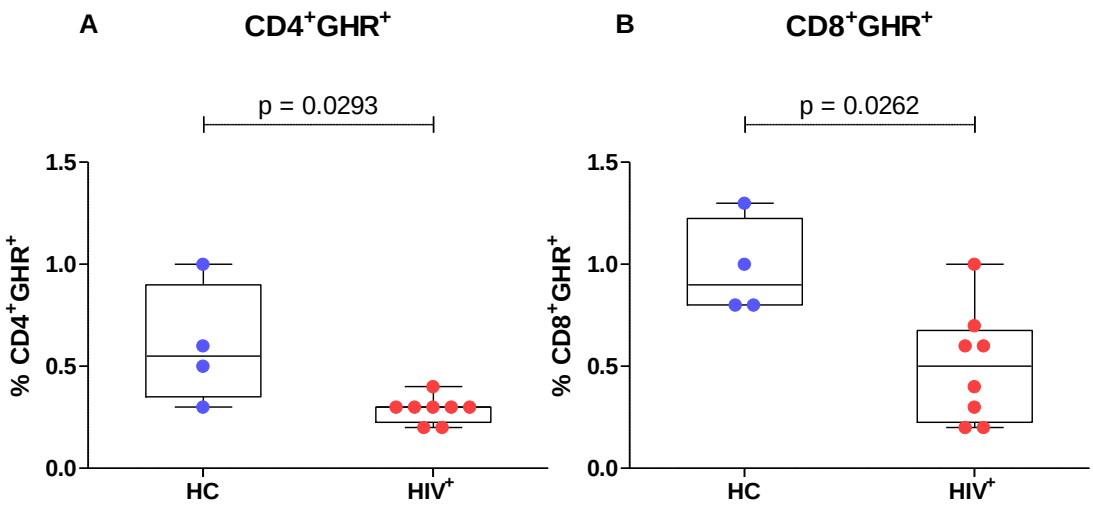


Figure 3.17 Growth hormone receptor (GHR) expression on CD4 and CD8 T cells.
Percentage expression of GHR on both CD4 (A) and CD8 (B) T cells was determined using PBMC from seronegative healthy controls (shown here in blue circles, n = 4) and HIV-1^{+} patients (shown here in red circles, n = 8). Box plots show the median and IQR while whiskers represent the 10^{th} and 90^{th} percentiles. Statistical analysis was carried out using the Mann-Whitney U Test and P values are shown where there is a statistically significant difference between the means ($p \leq 0.05$).

3.5 Discussion

In order to determine the biological significance of GH in the immune system, it is important to consider which cells produce GH and which cells express GHR. GH, identical to pituitary GH, has been found to be synthesised and secreted by lymphocytes [Varma *et al*., 1993; Hattori *et al*., 2001]. PBMC have also been shown to bear GHR with almost all monocytes and close to 90% of B cells positive for GHR [Bresson *et al*., 1999]. Studies using flow cytometric analysis of healthy control PBMC have described percentage expression of GHR on peripheral T cells to range from 2-6% [Bresson *et al*., 1999] and from 2-20% [Rapaport *et al*., 1995]; with less than 5% of activated tonsillar T-cell sub-populations (CD4 and CD8 T cells) possessing GHR [Thellin *et al*., 1998]. An extensive literature search revealed no previous characterisation of GHR expression on T cells in the context of HIV-1 infection. Here CD4 and CD8 T-cell subsets from healthy control individuals showed higher expression of GHR compared to HIV-1-infected persons. No significant differences were seen in GHR expression in terms of MFI for either T-cell subset between the two groups and CD4 and CD8 T cells did not express significantly different amounts of GHR when looking at the

study groups separately, and this concurs with a previous report which found no difference in expression between CD4 and CD8 tonsillar T-cell populations [Thellin *et al*., 1998]. GHR expression levels in both subject groups were low and ranged from 0.2 – 1.3% which did not agree with previous reports. These found GHR expression to be higher on peripheral T cells than found here at 5% [Bresson *et al*., 1999] and between 2-20% [Rapaport *et al*., 1995]. Further elucidation of the percentage expression on T-cell maturation/differentiation subsets was not carried out as percentage expression on the T cells was so low. The supplier of the GHR antibodies was contacted for information on positive and negative control staining (using these antibodies on human cells), and references reporting on the use of these antibodies to characterise GHR expression in flow cytometric analysis, however, this could not be provided. As the validity of these antibodies was questioned, further experiments using these reagents were not carried out.

Using the methodology described here, further experiments to characterise GHR expression on T cells in the context of HIV-1 infection are warranted. In one study, GHR expression on PBMC from female individuals was found to be lower than age-matched males, and expression differed between different age groups [Bresson *et al*., 1999]. It would be of interest to investigate GHR expression on T cells from HIV-1^{+} persons and to look at the effect of ART, and perhaps even in the context of immune-based therapy, such as before and after administration of rhGH. Characterisation of the prolactin receptor could yield important findings as this receptor is found on all GHR positive peripheral T cells [Welniak *et al*., 2002], and prolactin has been shown to promote T-cell function *in vitro* and *in vivo* [Clevenger *et al*., 1990; Welniak *et al*., 2002]. The expression of GHR on T-cell differentiation/maturation (naïve vs memory) subsets has yet to be addressed and it is not known whether GH-mediated effects, following administration of rhGH for example, are due to GH binding directly to T cells or as a result of indirect stimulation of monocytes and APC [Welniak *et al*., 2002]. Future experiments could employ the use of a GHR antibody clone (and conjugate) that has been previously published and validated [Rapaport *et al*., 1995; Bresson *et al*., 1999]. Alternatively, GHR expression on T cells could be assessed using RT-PCR [Hattori *et al*., 2001].

Administration of rhGH in treated HIV-1 infection has been shown to significantly reduce the expression of markers associated with immune activation (HLA-DR and CD38 on CD4 and CD8 T cells) and apoptosis [Napolitano *et al*., 2008], with effects on memory T-cell subsets [Pires *et al*., 2004b].To ascertain the direct effect of rhGH on peripheral T cells, cultures were set up with PBMC from healthy controls and HIV-1-infected individuals and incubated for 72 hours with rhGH. Cultures with PBMC in TCM alone were set up as negative controls. Preliminary lymphoproliferation assays showed that rhGH did not result in the proliferation of T-lymphocytes. Phenotypic analysis was carried out before and after culture with rhGH and markers of activation, exhaustion, senescence, apoptosis and differentiation/maturation assessed. PBMC from healthy donors showed little change in terms of percentage expression of these markers whether cultured with rhGH or TCM alone. Preliminary experiments using PBMC from HIV-1-infected individuals, however, showed promising results, with a down-regulation in markers associated with immune activation (HLA-DR and CD38), senescence (CD57) and apoptosis (CD95). When negative control wells were run in parallel, these also produced a down-regulation of the same markers, suggesting that this decrease was an effect of culture. The provider of the rhGH for the clinical trial (Merck Serono Ltd, Feltham, UK) was contacted in order to use the same rhGH given to patients in this culture system, however this was unavailable. These rhGH culture experiments may be optimised by ensuring the use of carrier/adjuvant-free rhGH and by resting the cells overnight prior to 72-hour culture which might improve cell viability. IL-2 was not added to these cultures as an observation of the direct effect of rhGH on T cells was the main objective and IL-2 is known to induce proliferation *in vitro* [Mookerjee *et al*., 1989; Besser *et al*., 2009], and may alter the expression of surface markers.

Daily administration of a ‘supra-physiological’ dose of rhGH (4mg/day) for 12 weeks in HAART-treated HIV-1-infected individuals resulted in significant increases in HIV-1-specific CD4 and CD8 T-cell responses. At week 24, when patients had received less frequent dosing of rhGH, a decline was observed in HIV-1-specific CD4 T-cell responses (this was the case for all patients and also

noted at week 48). The increased HIV-1-specific CD8 T-cell responses measured at week 12 were maintained at week 24 (regardless of randomisation group) and despite a lack of virus-specific CD4 T-cell responses. At the week 48 follow-up visit, when patients had received HAART alone for a further 24 weeks, only HIV-1-specific CD8 T-cell responses to Gag and Pol remained whereas responses to other HIV-1 antigens had significantly declined and virus-specific CD4 T-cell responses remained undetectable. These findings support previously published data investigating the role of rhGH in HIV-1 infection, not only for the treatment of lipodystrophy [Lo *et al*., 2001; Sivakumar *et al*., 2011] but as an immunomodulator to reverse T-cell dysfunction in combination with effective HAART. Administration of rhGH in treated HIV-1-infected adults has been shown to increase thymic density [Napolitano *et al*., 2002; Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010], increase numbers of circulating naïve CD4 T cells [Napolitano *et al*., 2002; Pires *et al*., 2004b; Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010], increase TREC frequency [Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010], and reduce expression of markers associated with immune activation (HLA-DR and CD38) and senescence (Fas) [Napolitano *et al*., 2008], whereas it has not been shown to have a discernible effect on viral load. A report by Smith *et al*., investigating rhGH administration in treated HIV-1^{+} patients did not find improvements in HIV-1-specific responses (in terms of lymphoproliferative responses to recall, neo or HIV-1 antigens) [Smith *et al*., 2010]. This could be due to the lower dose of rhGH used in their study (1.5mg/3.0mg). Additionally, their patient demographics differed with a median baseline CD4 T-cell count of 219 cells/µl blood, compared to 467 cells/µl blood in this study, although duration of ART was similar for both (>4 years). Differences in nadir CD4 T-cell counts between the two study groups might also explain the variation, in terms of recovery of virus-specific responses, although these data were not available for comparison. The decrease of the CD4 T-cell response following withdrawal of rhGH suggests that cells may require a stronger or more sustained signal from rhGH to recover and provide help to the CD8 T cells. CD8 T-cell responses have been shown to be maintained for a limited amount of time without help from CD4 T cells [Williams and Bevan,

2007], but eventually diminished following discontinuation of rhGH therapy. The responses at week 48; the absence of HIV-1-specific CD4 T-cell responses and decline in virus-specific CD8 T-cell responses may reflect exhaustion or a lack of CD4 T-cell help to facilitate the complete function of effective CD8 T cells [Shedlock & Shen, 2003; Sun & Bevan, 2003; Janssen *et al*., 2003]. This immunomodulatory therapy, however, has provided adequate help to HIV-1-specific CD4 T cells which have in turn induced the appropriate effective anti-HIV-1 CD8 T cells [Herasimtschuk *et al*., 2008] and these findings suggest that stronger or prolonged treatment with rhGH may be needed to further maintain these virus-specific responses.

Low 'physiological' dosing of rhGH provided findings that were consistent with short-term high-dose studies. The robust and durable responses seen following 40 weeks of rhGH suggest that this treatment may improve key defects in both MHC Class I and II presentation and appropriate T-cell function, and show a reversal of T-cell anergy that is greater than seen with ART alone. Clinical observations showed long-term, low-dose rhGH to be well tolerated and to significantly increase thymic density and thymic index, as well as significantly increasing the number of total CD8 T cells with a trend towards an increase in both the number of total CD4 T cells and the number of naïve T cells (in the rhGH group compared to the placebo) [Hansen *et al*., 2009]. Few studies have investigated such a low dose of rhGH in the context of treated HIV-1 infection. A study by Lo *et al*., investigated long-term (18 months), low-dose rhGH (average dose 0.33mg/day) in GH-deficient treated HIV-1^{+} individuals and did not find an increase in CD4 T-cell numbers following rhGH administration. However, patient demographics differed considerably to this study in terms of baseline CD4 T-cell counts, plasma HIV-1 RNA, length of time on ART and duration of infection, and the effect on the thymus was not determined [Lo *et al*., 2008]. Further studies into the phenotype of both total peripheral and HIV-1-specific T cells following treatment with rhGH are warranted. High levels of chronic immune activation have been associated with HIV-1 disease progression [Hazenberg *et al*., 2003] and although these levels are lowered with effective ART [Bartovska *et al*., 2011], they are not normalised. These experiments provide additional evidence

that even at a low dose rhGH may provide a means of further lowering the levels of expression of activation and apoptotic markers which concurs with other (high-dose) studies [Napolitano *et al*., 2008]. Additionally, a shift towards a central memory phenotype in the CD8 T-cell compartment following rhGH administration may be beneficial as an accumulation of effector memory CD8 T cells has been described in chronic HIV-1 infection [Downey & Imami, 2010]. Interestingly, the four samples that showed the most robust IFN-γ ELISpot responses at week 40, and were characterised phenotypically, were all from patients presenting with HIV-1-associated lipodystrophy syndrome (HALS). This is perhaps not unexpected as patients with HIV-1 lipodystrophy have been found to have abnormalities in GH secretion that are strongly associated with body composition and metabolic irregularities [Stanley & Grinspoon, 2009], and thus these patients may show the most benefit not only clinically (in terms of reduced visceral fat and lipid parameters) but also immunologically. Similarly, the study by Smith *et al*., found that subjects with low baseline TREC had the greatest increase in TREC, suggesting rhGH had the most benefit for those with poor thymopoiesis at baseline [Franco *et al*., 2002; Smith *et al*., 2010]. The down-regulation of surface markers seen in the low-dose rhGH study may be an effect of an additional 40 weeks of ART. Phenotypic characterisation of PBMC samples from placebo-treated individuals and non HALS rhGH-treated patients would give a clearer indication of the effect of rhGH on immunophenotype in HIV-1 infection. Long-term, low-dose rhGH also has the added benefit of being associated with fewer treatment-related adverse events such as peripheral edema, arthralgias and increased blood glucose [Kotler *et al*., 2004; Grunfeld *et al*., 2007], although it should be noted that this was not an issue in the high-dose rhGH study presented here. Withdrawal of low-dose rhGH in the study by Lo *et al*., saw a rapid rebound in visceral adipose tissue (VAT) to a level significantly above initial baseline values within 6 months of crossover to placebo [Lo *et al*., 2010] however, immunological parameters were not ascertained and again, the population studied presented with abdominal fat accumulation and relative GH deficiency. It would be of interest to assess the phenotype of the non-HALS individuals from this study but samples were limited.

IGF-1 treatment in HIV-1 infection was not shown to have any impact on visceral adiposity [Rao *et al*., 2010]. However, immunological parameters were not investigated. Immunological changes following administration of a molecule upstream of GH in treated HIV-1^+ individuals has not been investigated. The growth hormone releasing factor analogue, tesamorelin (hypothesised to induce endogenous GH secretion), was found to significantly reduce visceral adipose tissue in ART-treated HIV-1^+ patients with excess fat accumulation [Falutz *et al*., 2010], and the function of this molecule on the function and phenotype of HIV-1-specific T cells has yet to be discerned.

In summary, the results from the two clinical trials presented here, demonstrate the ability of rhGH at a high- or low- dose to induce HIV-1-specific T-cell responses in chronically infected patients on effective HAART, with the potential to improve the dysfunctional immunophenotype observed in chronic HIV-1 infection. These findings clearly illustrate the importance of the development and further study of new and current immune-based therapeutic approaches in the context of HAART-treated chronic HIV-1 infection.

Chapter 4 Novel Interventions in HIV-1 Infection: IMIRC 1003 Clinical Trial

The development of new, and modification of existing, immune-based therapeutic approaches in the context of treated HIV-1 infection are needed. Effective HAART controls HIV-1 infection and improves morbidity and mortality in infected patients [Montaner *et al*., 2006], however, it cannot eradicate HIV-1 and does not improve the immune system's ability to control the virus [Pires *et al*., 2004a; Imami *et al*., 2007]. ART-treated HIV-1-infected persons are at a higher than normal risk for certain age-associated diseases, which may be due in part to irreversible virus-associated immunological damage [Deeks, 2011]. Studies have sought to optimise the timing and duration of ART; initiation of ART in early HIV-1 infection has been shown to result in lower residual viral reservoirs [Pires *et al*., 2004a; Cellerai *et al*., 2011], lower levels of immune activation and improved polyfunctionality of HIV-1-specific T-cell responses [Cellerai *et al*., 2011]. Although it appears that a very small number of individuals, treated at the time of primary HIV-1 infection, maintain immunovirological control following treatment interruption [Hocqueloux *et al*., 2010], for the majority of individuals, cessation of ART results in viral rebound [Burton *et al*., 2005; Hocqueloux *et al*., 2010] and treatment interruptions are discouraged [El-Sadr *et al*., 2006]. Novel therapeutic interventions may provide a means of further depleting viral reservoirs, improving cellular immune responses and providing full and prolonged control of viral replication in the absence of ART.

The design of this clinical trial, the immunomodulatory agents used, and the timing of their administration have been carefully based upon findings from previous studies and preliminary data. Therapeutic vaccination in conjunction with effective HAART may lead to a reversal of anergy by selective activation of the immune system against specific viral proteins, such that specific memory responses may be induced or augmented. Administration of IL-2, GM-CSF and rhGH may lead to the *in vivo* expansion of useful T-cell subsets [Imami *et al*., 2007]. A brief summary of the rationale

for the study is shown in Table 4.1, with more detailed information on each of the treatments described in detail in the introduction of this thesis.

Table 4.1 Rationale for the treatments given in the IMIRC 1003 clinical trial.

Treatment	**Immunological effect**
Therapeutic vaccination	Induce/boost HIV-1-specific (new and memory) responses in functional cells [Imami *et al*., 2007]
IL-2	Growth, survival, differentiation/maturation; de-anergise; increase in frequency and function of effector T cells and regulatory T cells (particularly HIV-1-specific CD4 T cells) [Imami *et al*., 1999; Imami *et al*. 2001; Levy *et al*., 2003]
GM-CSF	De-anergise; increase effector T cells; increase HIV-1-specific CD4 and CD8 T-cell, APC and NK cell function [Imami *et al*., 1999; Pires *et al*., 2005; Brown & Angel, 2005]
rhGH	Increase thymic activity and differentiation/maturation of T cells, increased NK cell function [Pires *et al*., 2004b; Goodier *et al*., 2003]

Approaches using therapeutic vaccination or immunotherapy with cytokines appear to result in greater benefits, than seen with ART alone, when viral load is undetectable (when patients are on effective HAART) [Pires *et al*., 2004b; Imami *et al*., 2007]. The duration of ART-induced viral control and nadir CD4 T-cell counts have been shown to be important factors for CD4 T-cell recovery [Koegl *et al*., 2009], thus patient eligibility criteria were strictly defined. Enhanced responses were predicted through the induction/re-introduction of specific cellular immune responses by priming with therapeutic immunisation, sustaining these responses through the administration of cytokines/hormones and boosting memory responses with further immunisations [Imami *et al*., 2007]. Previous animal models have shown that administration of IL-2 during the antigen-specific T-cell contraction phase of the immune response, approximately 8-15 days following vaccination, may result in the preservation and maintenance of clinically relevant responses [Blattman *et al*., 2003; Nacsa *et al*., 2005]. IL-2 administration pre-vaccination in HIV-1^{+} HAART-treated patients did not result in an increase in specific T-cell proliferation [Levy *et al*., 2003, Valdez *et al*., 2003]. However, IL-2 administered following tetanus vaccination in HIV-1-infected persons was found to better sustain the tetanus-specific responses than IL-2 administered together with or before vaccination [Hardy *et al*., 2004]. Further immune reconstitution may occur

in the periphery, via enhanced antigen presentation to fully functional HIV-1-specific CD4 and CD8 T cells as a result of GM-CSF administration [Pires *et al*., 2005; Brown & Angel, 2005], and via the thymus, following treatment with rhGH [Pires *et al*., 2004b; Napolitano *et al*., 2002; Napolitano *et al*., 2008; Smith *et al*., 2010].

To this effect, patients were recruited with CD4 T-cell counts >400 cells/µl blood, nadir CD4 T-cell counts >200 cells/µl blood and HIV-1 RNA <50 copies/ml plasma. They had to be on suppressive HAART for at least 6 months prior to study initiation and could not be receiving or have received immunomodulatory drugs.

4.1 Patient characteristics and HLA-typing

The baseline characteristics of the twelve patients enrolled on the trial are detailed in Table 4.2. Of these individuals, eleven were male, infected with HIV-1 clade B, and one was female, infected with HIV-1 clade C. The median age of those enrolled was 48 years (IQR 42-51 years), with a median CD4 T-cell count at baseline of 757 cells/µl blood (IQR 567-886 cells/µl blood). The majority of patients had nadir CD4 T-cell counts >200 cells/µl blood (median 219; IQR 177-289 cells/µl blood) and for all patients HIV-1 RNA at baseline was <50 copies/ml plasma. Median duration of HIV-1 infection was 142.92 months (IQR 89.04-186.98 months) and median duration of ART was 93.07 months (IQR 58.63-143.89 months). Three individuals were randomised into group 1, four into group 2 and five into group 3 (Table 4.2).

The HLA-type and ethnicity of the twelve enrolled patients is given in Table 4.3. The majority of individuals were of White/Caucasian ethnicity and there was no observable difference in protective or non-protective HLA Class I allele requencies between individuals in each group.

Table 4.2 Characteristics of the twelve patients enrolled on the IMIRC 1003 clinical trial.
IQR – interquartile range; NK – not known.

Patient short code	Group	Graph symbol	Date of birth	Age (years)	Gender	Clade of infection	Duration of HIV-1 infection (months)	Duration of ART (months)	ART regimen	CD4 T-cell count (cells/µl blood)		Plasma viral load at baseline (copies/ml)
										Nadir	Baseline	
R771	1	●	20/03/1946	64	M	B	160.13	139.05	FTC+TFV+EFV	391	884	<50
B784	1	●	30/05/1970	40	M	B	99.25	98.95	FTC+TFV+EFV	80	1332	<50
G739	1	●	28/06/1967	43	M	B	141.61	113.15	FTC+TFV+EFV	227	534	<50
P087	2	■	02/09/1959	50	M	B	172.95	162.75	FTC+TFV+EFV	210	731	<50
C789	2	■	07/01/1981	29	M	B	20.75	12.20	FTC+TFV+EFV	309	535	<50
L043	2	■	27/10/1963	47	M	B	90.69	45.87	FTC+TFV+EFV	303	782	<50
C319	2	■	04/09/1962	48	M	B	229.05	80.03	FTC+TFV+ETV	93	1077	<50
F810	3	▲	07/08/1959	50	M	B	233.41	193.54	FTC+TFV+NVP	166	582	<50
O523	3	▲	31/05/1956	53	F	C	84.10	62.89	FTC+TFV+ETR	284	892	<50
S648	3	▲	18/01/1957	52	M	B	300.39	158.43	TFV+DRV+RTV	227	578	<50
C241	3	▲	07/02/1963	47	M	B	144.23	87.18	FTC+TFV+DRV+RTV	180	466	<50
P054	3	▲	17/04/1978	33	M	B	40.80	28.80	FTC+TFV+EFV	200	840	<50
Median				48			142.92	93.07		219	757	
IQR				42-51			89.04-186.98	58.63-143.89		177-289	567-886	

Table 4.3 Ethnicity and HLA-type of the individuals on the IMIRC 1003 clinical trial.
Patient are grouped and colour-coded according to randomisation arm: group 1 (blue), group 2 (green) and group 3 (red). *New HLA-type identified which is most similar to B*37:01 except that position 454 is C instead of G.

Patient short code	Ethnicity	HLA-A	HLA-B	HLA-Cw	HLA-DR
R771	White/Caucasian	0201, 2902	3924, 4403	0701, 1601	0701, 1102
B784	White/Caucasian	1101, 6802	1402, 1501	0102, 0802	0101, 1301
G739	Asian	0201, 3201	4402, 5101	0501, 1502	0407, 1201
P087	White/Caucasian	0201/09, 6801	4001, 0702	0304, 0702	0404, 1501
C789	White/Caucasian	0101/0201	3503, 3701*	0602, 1203	0701, 1502
L043	White/Caucasian	0205, 2601	5801, 5802	0602, 0701	0401, 1302
C319	White/Caucasian	0301, 3201	0702, 1401	0702, 0802	0103, 0701
F810	White/Caucasian	0101, 2402	0801, 2705	0102, 0701	0404, 1501
O523	White/Caucasian	0101, 3201	0702, 4402	0501, 0702	0407, 1301
S648	White/Caucasian	3101, 2402/09N	0801, 0702	0701, 0702	0301, 0404
C241	Hispanic	0101, 2402	3905, 8102	0702, 1801/02	0301, 1402
P054	White/Caucasian	0201, 2601	0702, 4001	0304, 0702	1302

4.2 Lymphocyte subsets and plasma viral load

The results presented in this chapter show changes from baseline in all clinical and immunological parameters as described in section 2.23, thus all baseline values are tabulated in Appendix 1. For the graphs presented herein, a statistically significant difference compared to baseline can be observed easily where the 95% CI does not cross the line of origin (the point where the X axis crosses the Y axis at zero); where it is above, a statistically significant increase can be inferred and where it is below, a statistically significant decrease. Where 95% CI from the groups do not overlap for an individual time point, there is a statistically significant difference between the means of the groups. All mean changes from baseline, 95% CI values and p values can be found in Appendix 2.

Administration of IL-2 and GM-CSF for five days resulted in a mean change from baseline to week 2 of 1307 cells/µl and 1511 cells/µl blood in groups 1 and 3, respectively (95% CI 1104-1511 cells/µl blood, p <0.0001; and 95% CI 1353-1688 cells/µl blood, p <0.0001, respectively), and this was significantly higher than for group 2 (in which patients received vaccine alone; Figure 4.1A). At week 6, CD4 T-cell counts were elevated compared to baseline in both groups that received

cytokines and hormone and the mean increase was 413 cells/µl and 239 cells/µl blood in groups 1 and 3, respectively (95% CI 210-617 cells/µl blood, p = 0.0001; and 95% CI 82-397 cells/µl blood, p = 0.0039, respectively), whereas for group 2 there was a marginal but statistically significant decrease in the mean CD4 T-cell count compared to baseline at week 6 of -191 CD4 T cells (95% CI -367 – -15, p = 0.0369). At the final study time point, week 48, only patients in group 1 (that had received therapeutic vaccination and cytokines and hormone) demonstrated an increase in mean CD4 T-cell count compared to baseline of 281 cells/µl blood (95% CI 78-484 cells/µl blood, p = 0.0083; Figure 4.1A).

A similar, though less pronounced pattern was seen when looking at the change from baseline in CD4 T-cell percentage. Following cytokine administration in treatment groups 1 and 3, a statistically significant increase in CD4 T-cell percentage was observed at week 2, with a mean change from baseline to week 2 of 8.3% and 8.2%, respectively (95% CI 4.5-12.1%, p <0.0001; 95% CI 5.2-11.2%, p <0.0001, respectively; Figure 4.1B). For group 3, statistically significant increases in CD4 T-cell percentage from baseline values were seen again at weeks 4 and 8 with mean increases of 3.1% and 3.7% (95% CI 0.1-6.0%, p = 0.0453; 95% CI 0.8-6.8%, p = 0.0156, respectively), with a trend toward a statistically significant increase at week 48 (the mean increase was 2.9%, 95% CI -0.1-5.9%, p = 0.0590). Patients in group 1, however, had CD4 T-cell percentages which remained elevated at each time point for the remainder of the study, up until week 48, where the mean increase from baseline was 5% (95% CI 1.2-8.8%, p = 0.0124). Throughout the course of the study, the CD4 T-cell percentage did not deviate significantly from baseline in patients that received vaccine alone (group 2; Figure 4.1B).

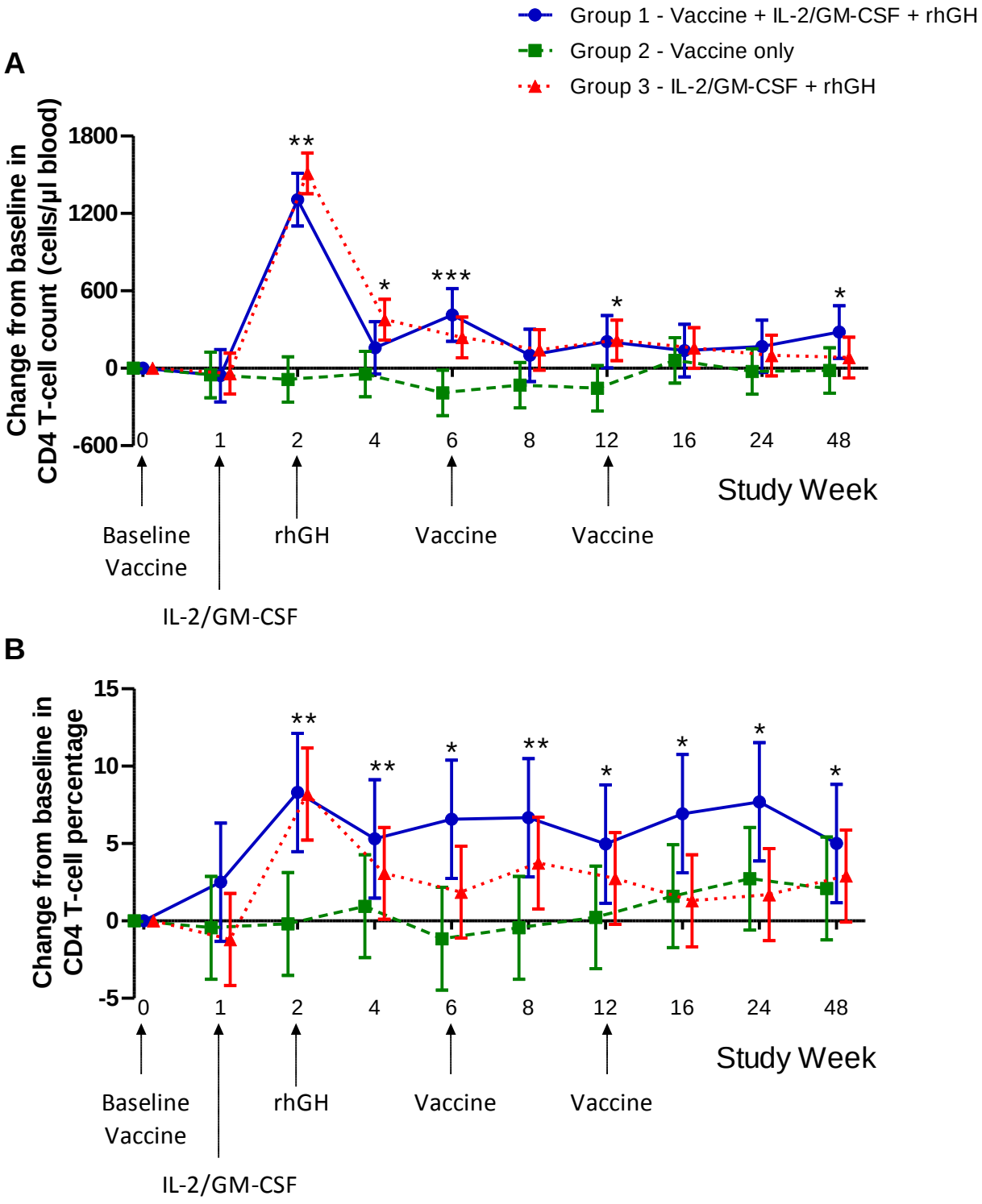


Figure 4.1 CD4 T-cell changes from baseline to each of the study time points.
CD4 T-cell count (A) and CD4 T-cell percentage (B) are depicted for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

Throughout the course of the 48 weeks, the CD8 T-cell counts followed a similar pattern to the CD4 T-cell counts. Patients in groups 1 and 3 demonstrated statistically significant increases in their

CD8 T-cell counts at week 2, following administration of the cytokines, IL-2 and GM-CSF, compared to baseline and compared to group 2. The mean increase from baseline at week 2 was 634 cells/µl and 999 cells/µl blood, respectively (95% CI 377-891 cells/µl blood, p <0.0001; and 95% 800-1199 cells/µl blood, p <0.0001, respectively; Figure 4.2A). The increase was more prominent for group 3 for which CD8 T-cell counts remained elevated at weeks 4 and 6, with a mean increase from baseline of 248 cells/µl and 220 cells/µl blood, respectively (95% CI 49-448 cells/µl blood, p = 0.0168; and 95% CI 21-420 cells/µl blood, p = 0.0330, respectively). Patients in group 2 experienced a marginal but significant reduction in the numbers of CD8 T cells at weeks 6 and 12 with a mean decrease of -283 cells/µl and -239 cells/µl blood, respectively (95% CI -505 – -60 cells/µl blood, p = 0.0151; and 95% CI -462 – -16 cells/µl blood, p = 0.0387, respectively). By week 48, there was no difference from baseline in CD8 T-cell counts for any of the groups (Figure 4.2A).

CD8 T-cell percentages were significantly reduced compared to baseline at weeks 2 (p = 0.0008), 6 (p = 0.0387), 16 (p = 0.0169), 24 (p = 0.0003) and 48 (p = 0.0453) for individuals in group 1, whereas significant decreases in CD8 T-cell percentage were seen at weeks 2 (p <0.0001) and 4 (p = 0.0421) in group 3 and only at week 48 for group 2 (p = 0.0472; Figure 4.2B).

Marked increases in CD3 T-cell counts (compared to baseline and compared to group 2) were observed at week 2 following cytokine administration in patients in groups 1 and 3 with mean increases from baseline at week 2 of 2014 cells/µl and 2510 cells/µl blood (95% CI -589-208 cells/µl blood, p <0.0001; and 95% CI 2154-2867 cells/µl blood, p <0.0001, respectively). A less pronounced increase was found at week 6 (p = 0.0096 for group 1, and p = 0.0100 for group 3) following growth hormone treatment in these groups. At week 48, there was no significant difference from baseline in CD3 T-cell count for any of the treatment groups (Figure 4.3A).

In terms of the percentage of CD3 T cells, significant increases were seen for individuals in groups 1 and 3 at week 2 with mean increase from baseline of 4.2% and 3.9% (95% CI 1.2-7.2%, p = 0.0075; and 1.6-6.2%, p = 0.0014, respectively). CD3 T-cell percentages remained elevated for

individuals in group 1 but by week 48, there was no statistically significant difference from baseline in CD3 T-cell counts for any of the groups (Figure 4.3B).

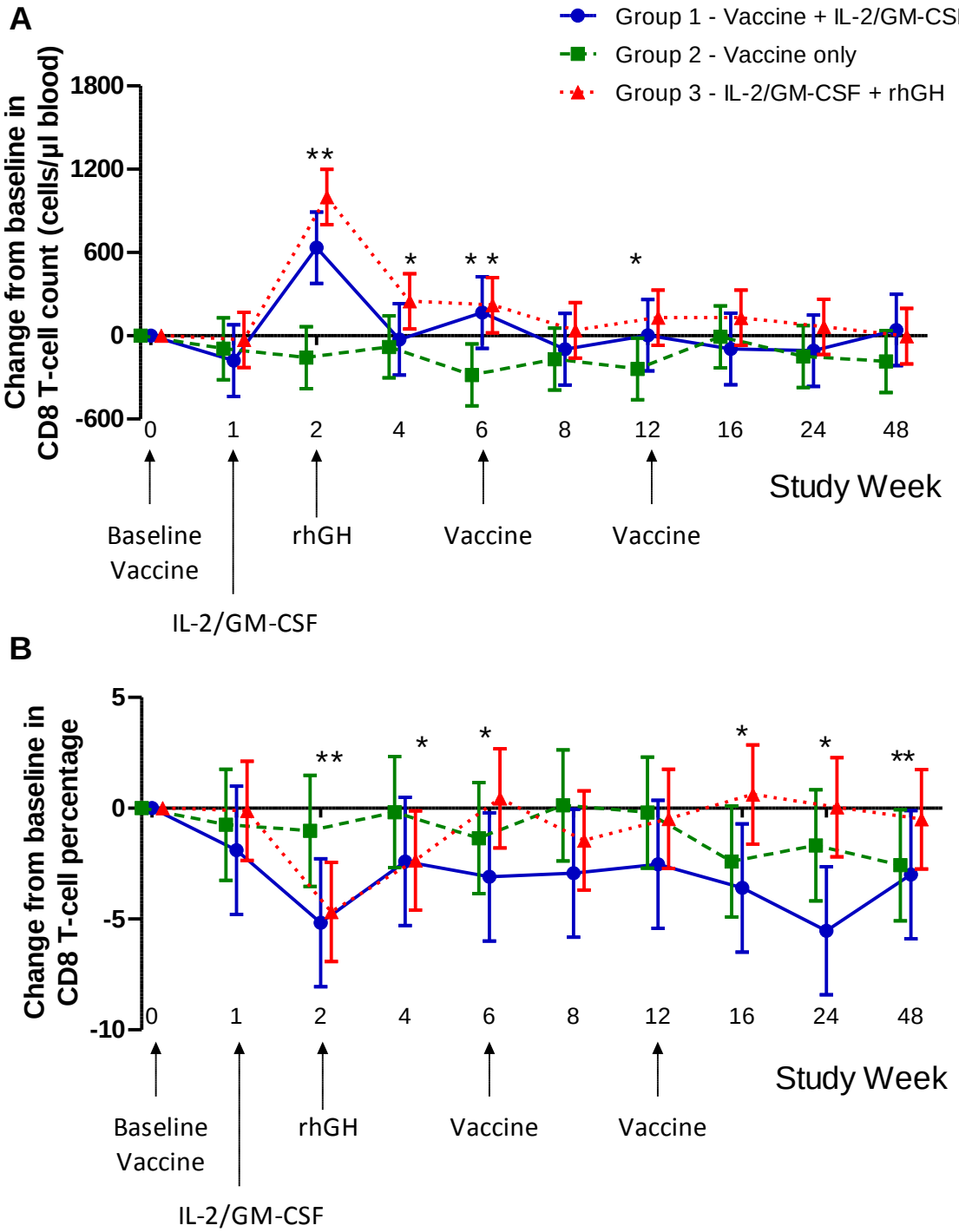


Figure 4.2 CD8 T-cell changes from baseline to each of the study time points.
CD8 T-cell count (A) and CD8 T-cell percentage (B) are depicted for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

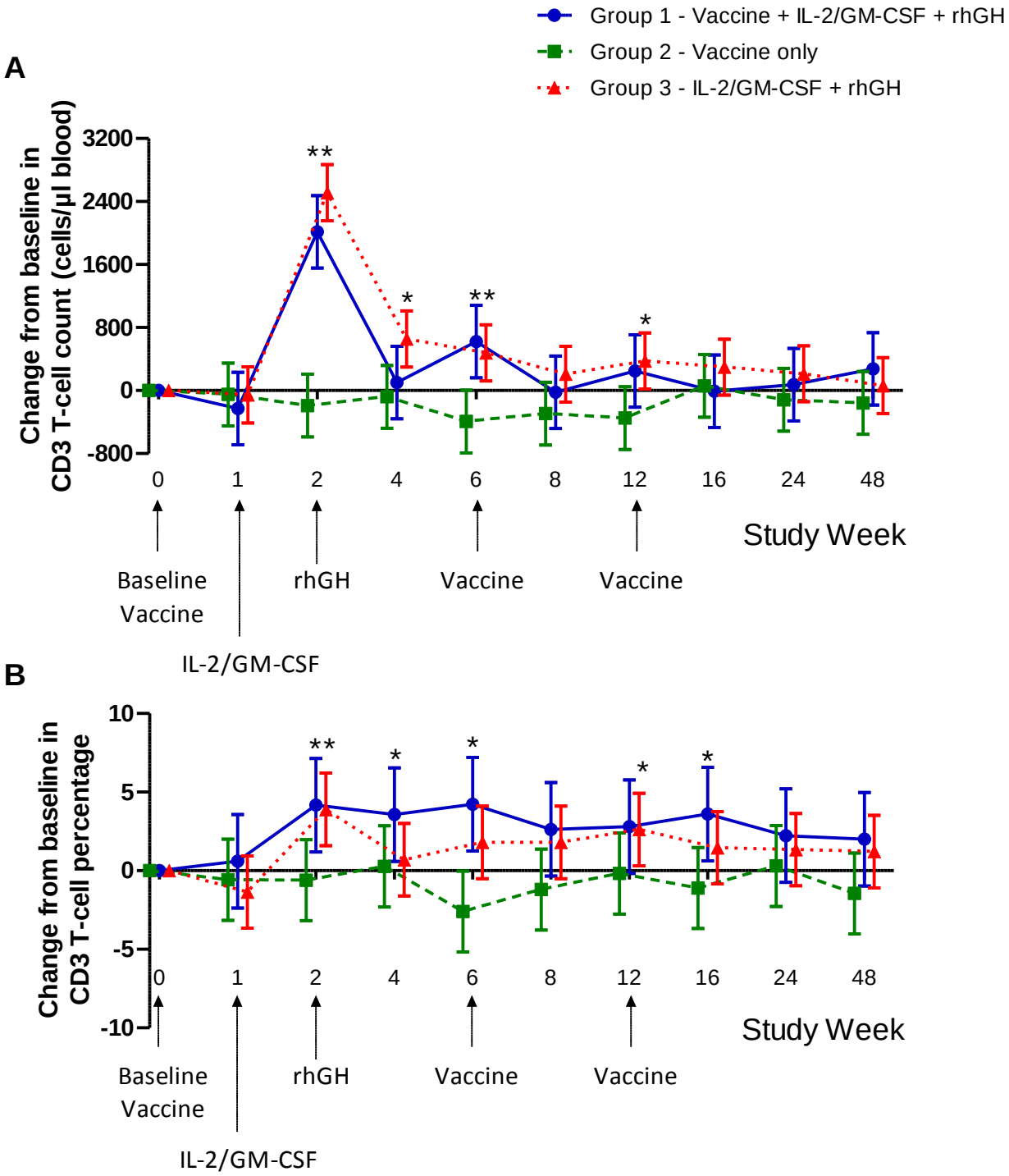


Figure 4.3 CD3 T-cell changes from baseline to each of the study time points.
CD3 T-cell count (A) and CD3 T-cell percentage (B) are depicted here for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

In terms of CD19 B-cell counts (Figure 4.4A), individuals that received cytokines and hormone showed a mean decrease from baseline to week 4 of -131 cells/µl and -77 cells/µl blood, for group 1 and group 3, respectively (95% CI -204 – -58 cells/µl blood, p = 0.0007; and 95% CI -134 – -21 cells/µl blood, p = 0.0085, respectively). Both groups also showed a mean decrease from baseline to week 8 of the study of -78 cells/µl and -63 cells/µl blood, in groups 1 and 3 respectively (95% CI -151 – -6.0 cells/µl blood, p = 0.0376; and 95% CI -119 – -6.3 cells/µl blood, p = 0.0321, respectively). Again, by week 48, there were no statistically significant differences from baseline in CD19 B-cell counts in any of the three randomisation groups (Figure 4.4A).

The profile of CD19 B-cell percentage change from baseline over the time course of the trial was somewhat different. There was a mean decrease from baseline at week 2 of -6.3% and -5.5% in groups 1 and 3, respectively (95% CI -8.3 – -4.4%, p <0.0001; and 95% CI -7.1 – 4.0%, p <0.0001, respectively). This CD19 B-cell percentage remained statistically lower than baseline for these two groups until week 16 but by week 48, there was no statistically significant difference in CD19 B-cell percentage in any of the treatment groups compared to baseline (Figure 4.4B).

Looking at CD56 NK-cell count, there was a mean increase from baseline to week 2 of 235 cells/µl and 511 cells/µl blood in groups 1 and 3, respectively (95% CI 82.7-387 cells/µl blood, p = 0.0033; and 95% CI 393-629 cells/µl blood, p <0.0001, respectively). The more pronounced increase for group 3 in CD56 NK-cell declined at the following study time points but remained significantly elevated compared to baseline until week 8. By week 48 of the study, no statistically significant differences in CD56 NK-cell count were seen regardless of treatment group (Figure 4.5A). In terms of C56 NK-cell percentage, the mean change from baseline to each of the study time points did not reveal any statistically significant findings regardless of randomisation group (Figure 4.5B).

Changes in ISUM (total number of lymphocytes; CD3 T cells, CD19 B cells, and CD56 NK cells) and CD4/CD8 T-cell ratios are shown in Figure 4.6. There is a statistically significant increase in ISUM for patients in groups 1 and 3 with a mean increase from baseline to week 2 of 2254 cells/µl and 3063 cells/µl blood, respectively (95% CI 1665-2843 cells/µl blood, p <0.0001; and 95% CI

2606-3516 cells/µl blood, p <0.0001, respectively). At week 6, there was a statistically significant increase in ISUM for these two groups compared to baseline although it was less prominent and by week 48, no statistically significant changes from baseline were seen in either group (Figure 4.6A). CD4/CD8 T-cell ratios were elevated at week 2 for individuals in groups 1 and group 3 with a mean increase from baseline of 0.43 and 0.34, respectively (95% CI 0.26-0.61, p <0.0001; and 0.21-0.47, p <0.0001, respectively). This increased CD4/CD8 T-cell ratio was maintained for all of the other time points for patients randomised to group 1 with a mean increase from baseline to week 48 of 0.27 (95% CI 0.09-0.44, p = 0.0033; Figure 4.6B).

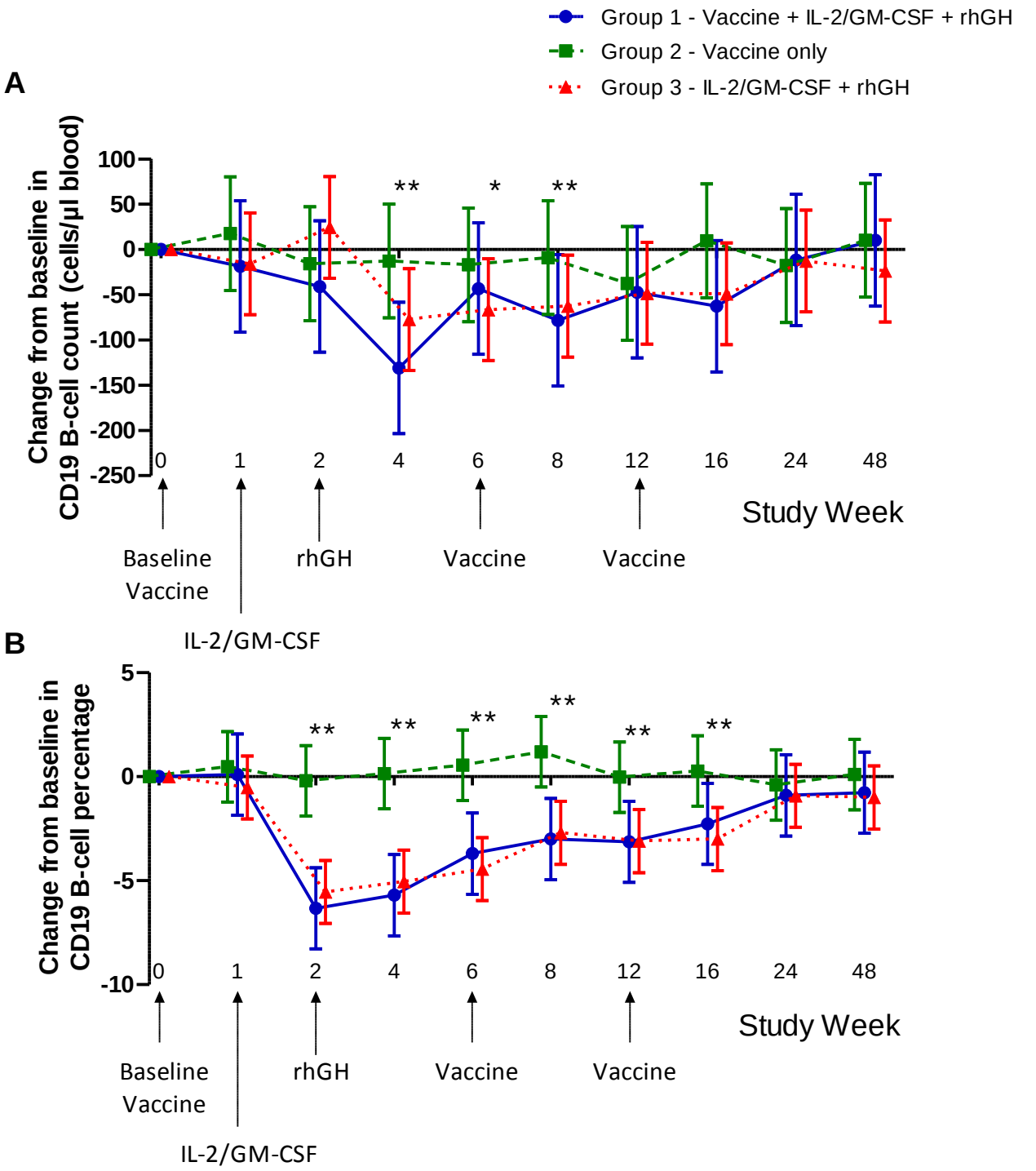


Figure 4.4 CD19 B-cell changes from baseline to each of the study time points.
CD19 cell count (A) and CD19 cell percentage (B) are shown here for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

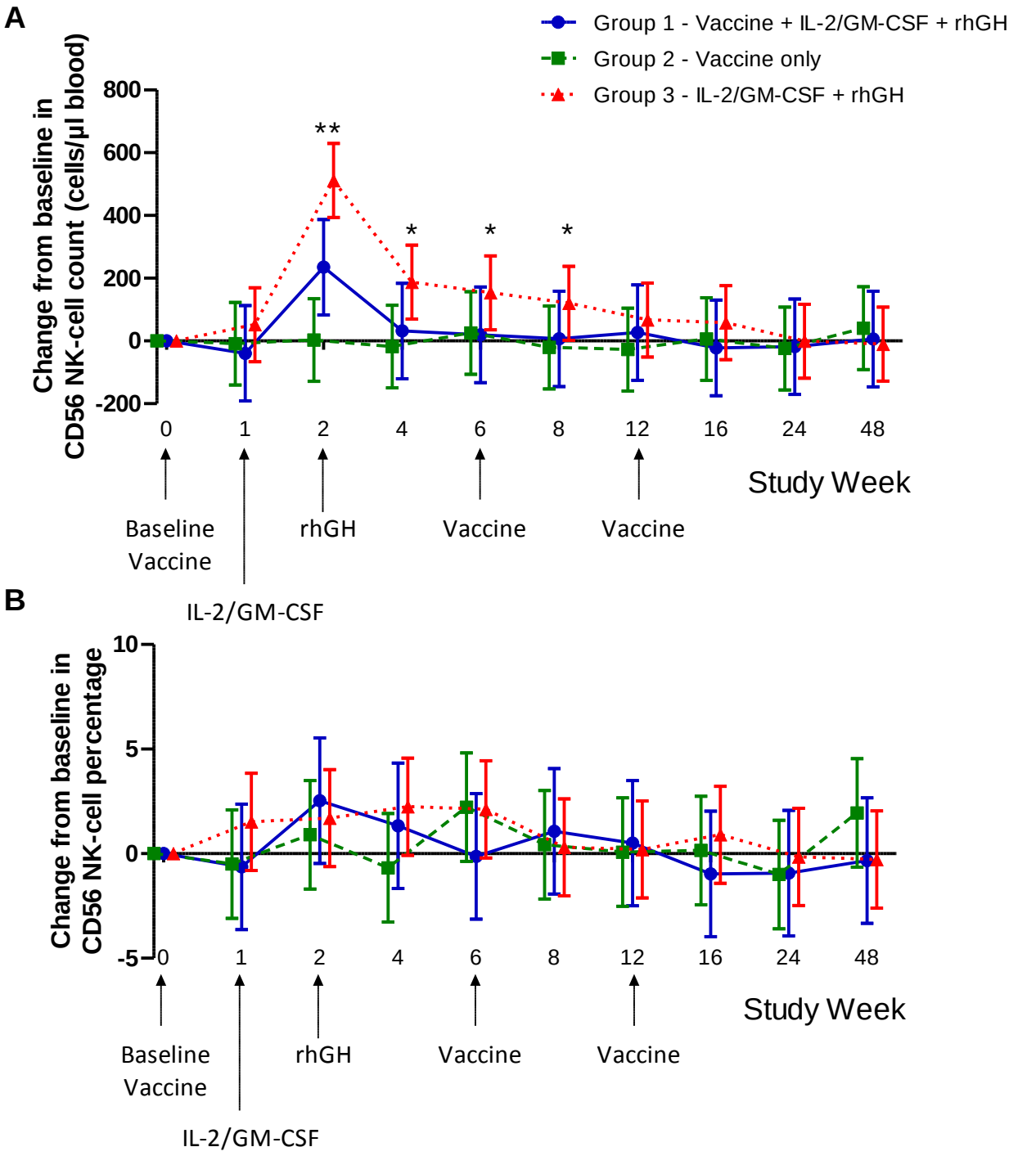


Figure 4.5 CD56 NK-cell changes from baseline to each of the study time points.
CD56 cell count (A) and CD56 cell percentage (B) are shown here for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

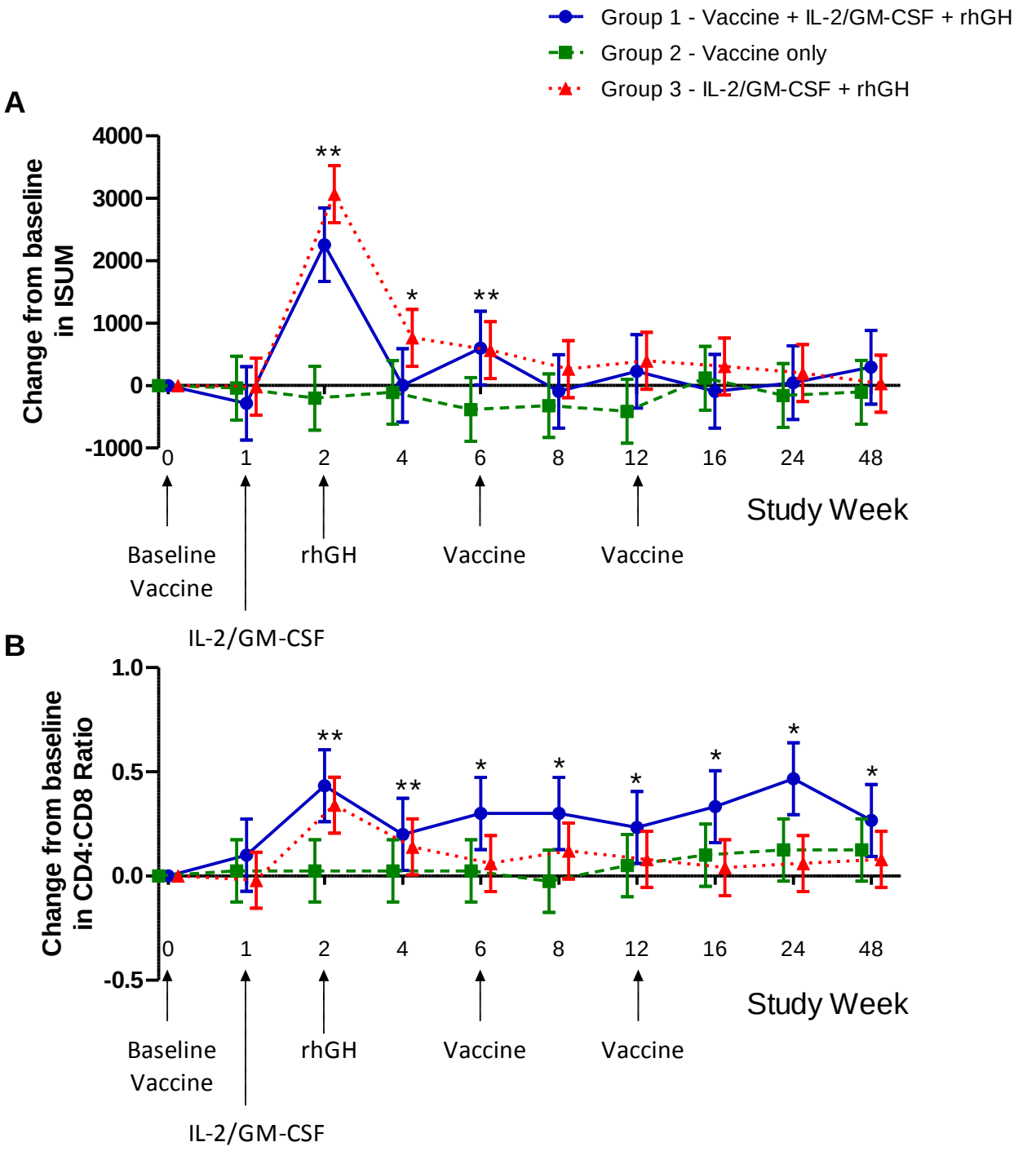


Figure 4.6 ISUM and CD4/CD8 ratio changes from baseline to each of the study time points.
ISUM (A) and CD4/CD8 ratio (B) are shown here for each of the patient groups: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Mean change from baseline to the study time point (week) is plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical significance, where $p \leq 0.05$.

Plasma viral load was <50 copies/ml for all individuals at both screen visits and at baseline (Table 4.4). Patients from each treatment group experienced detectable viraemia throughout the course of the study and this was not attributable to a particular treatment or time point, although one individual in group 1 and one individual in group 3 demonstrated the most pronounced increases in plasma viral load (G739 at week 2, and O523 at week 8, with viral loads of 345 and 588 copies/ml plasma, respectively).

Table 4.4 Plasma viral load (in copies/ml) for all twelve patients, for all study time points.
ND – not done. Values in bold indicate where plasma viral load was detectable.

Study visit (week)	Group 1			Group 2				Group 3				
	R771	B784	G739	P087	C789	L043	C319	F810	O523	S648	C241	P054
-4 (Screen)	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
-2 (Screen)	<50	<50	<50	<50	<50	<50	ND	<50	<50	<50	<50	ND
0 (Baseline)	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
1	<50	<50	**69**	<50	<50	<50	<50	<50	<50	<50	<50	<50
2	<50	<50	**345**	<50	<50	<50	<50	<50	<50	<50	<50	<50
4	<50	<50	<50	<50	**88**	<50	**53**	<50	<50	<50	<50	<50
6	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
8	<50	<50	<50	<50	<50	<50	<50	<50	**588**	<50	<50	<50
12	**80**	<50	**62**	<50	<50	<50	<50	**77**	<50	<50	<50	<50
16	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
24	<50	<50	<50	<50	**57**	<50	<50	<50	<50	<50	<50	<50
48	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

4.3 Toxicology and patient compliance

In terms of clinical chemistry, very few values were outside the normal limits throughout the course of the study. The majority of patients had high triglyceride levels at one of the screen visits or at the baseline visit (9/12). After week 2, plasma chloride levels were higher for patients in group 1 (who received therapeutic immunisation and IL-2, GM-CSF and rhGH). Following cytokine administration, patient C241 showed an acute peak in ALT and AST at week 2. Throughout the course of the study, patient G739 showed a degree of hypophosphataemia, patients B784 and S648

had consistently raised ALT levels, and patient F810 had raised alkaline phosphatase (toxicology data not shown).

The adverse events documented were mostly mild and included erythema at the injection site and slightly more severe reactions in patients that failed to take ibuprofen and paracetamol prophylaxis the evening prior to IL-2 and GM-CSF administration. The protocol deviations that arose were discussed with the principle investigator, study clinician and on-site statistician and the majority deemed minor: five study visits were performed outside of the schedule (the trial team decided that these were within a timeframe that would not impact the immunological relevance of the subsequent time points); subject O523 did not administer rhGH on day 16 but took it on day 19 instead; F810 did not receive rhGH day 8 dose due to problems with reconstitution; and for P087, the on-site clinical team were only able to administer 8 x 0.1 ml instead of 10 x 0.1 ml of the vaccine on day one (they confirmed that the vial was not dropped or drawn up incorrectly). There was only one major protocol deviation which occurred for patient C241; this subject was admitted to the hospital with headaches following the first administration of rhGH on day 14. It was agreed that due to this serious adverse event (SAE) the individual should receive no further rhGH but continue on the study on an observational basis. A more comprehensive look at the adverse events can be found in Appendix 4 (Appendix Tables 37 and 38).

4.4 IFN-γ, IL-2, IL-4 and perforin production in response to peptide stimulation

Preliminary ELISpot assays were run using PBMC from seronegative donors and from HIV-1-infected individuals (prior to initiation of the study) in order to confirm that the aliquoted NIBSC and FIT Biotech peptides were of a high quality and not cross-reactive. Details of the results from these assays can be found in Appendix 3. The baseline responses to each of the peptide pools and to TCM and PHA can be found in Appendix 1. Graphs presented here are as previously for the clinical data and mean changes from baseline are presented with 95% CI and significant p values ($p \leq 0.05$) shown. Changes from baseline in IFN-γ production in response to stimulation with peptide pools from two sources (NIBSC and FIT Biotech) are shown in Figure 4.7 and Figure 4.8 respectively.

Following treatment with IL-2 and GM-CSF (in the absence of therapeutic immunisation), group 3 individuals showed a statistically significant reduction in IFN-γ production in response to peptide pools of Gag p17 and Gag p 24 from NIBSC (Figures 4.7A and B, respectively) and a reduction was seen in response to the Gag p17/p24 sub-pool from FIT Biotech (Figure 4.8A). The mean decrease from baseline to week 2 was -148 SFC/10^6 PBMC (95% CI -242 – -54, p = 0.0027) for Gag p17 NIBSC, -260 SFC/10^6 PBMC (95% CI -419 – -100 SFC/10^6 PBMC, p = 0.0020) for Gag p24 NIBSC, and -285 SFC/10^6 PBMC (95% CI -470 – -100 SFC/10^6 PBMC, p = 0.0034) for Gag p17/p24 FIT Biotech sub-pool (Figures 4.7A and B, and Figure 4.8A, respectively). This reduction in IFN-γ production was also seen at week 24 for individuals randomised to group 3 to all three Gag peptide pools although by week 48, there was no significant difference from baseline in IFN-γ production for this group. Group 2 (vaccine only) showed moderate increases in IFN-γ response to the NIBSC Gag p17 peptide pool at weeks 12 and 24, but these were not maintained by week 48 (Figure 4.7A). Patients in group 1 (who received vaccine in addition to IL-2, GM-CSF and rhGH), however, showed a statistically significant increase in IFN-γ production in response to the Gag p17/p24 peptide pool from FIT Biotech at week 48. The mean increase from baseline to week 48 was 312 SFC/10^6 PBMC (95% CI 73-551 SFC/10^6 PBMC, p = 0.0122; Figure 4.8A). This was reflected in the group 1 response to Gag p24 from NIBSC at week 48, although statistical significance was not reached (p = 0.0589). IFN-γ production in response to peptide pools of Nef from the two sources revealed a reduction in responsiveness from patients in group 3 at week 2 (following administration of cytokines). The mean decrease from baseline was -114 SFC/10^6 PBMC for NIBSC Nef and -107 SFC/10^6 PBMC for the FIT Biotech sub-pool (95% CI -211 – -17 SFC/10^6 PBMC, p = 0.0244; and -205 – -9 SFC/10^6 PBMC, p = 0.0353; Figures 4.7C and 4.8B, respectively). At week 12, patients in group 2 (vaccine alone) showed a statistically significant increase in IFN-γ production in response to the NIBSC peptide pool of Nef (Figure 4.7C). The mean increase from baseline to week 12 was 123 SFC/10^6 PBMC (95% CI 14-232 SFC/10^6 PBMC, p = 0.0297). By week 48, no statistically significant differences were observed in any of the three

groups compared to baseline in terms of IFN-γ response to peptide pools of Nef from either source. IFN-γ responses to Tat stimulation were consistent between the two peptide sources for individuals in group 2 which show no statistically significant difference from baseline to any of the study time points. Responses from individuals in group 3 demonstrated significantly reduced responses from baseline at weeks 2, 6, 8, 12, 16 and 24 of the study in response to the NIBSC peptide pool (Figure 4.7D) whereas no significant differences from baseline were seen in response to the sub-pool from FIT Biotech (Figure 4.8C). Responses to Tat from both peptide sources also differed for patients randomised into group 1 and a significant increase in IFN-γ production was seen at week 48 compared to baseline in response to NIBSC Tat (Figure 4.7D) whereas a significant decrease was seen in response to the Tat peptide pool from FIT Biotech (Figure 4.8C). IFN-γ production in response to peptide pools of Rev from both sources was very similar across the board. For individuals that received therapeutic immunisation only (group 2) or therapeutic immunisation in conjunction with cytokines and hormone (group 1), no significant differences were detected in IFN-γ production at any of the study time points compared to baseline for either NIBSC or FIT Biotech peptide pools (Figures 4.7E and 4.8D, respectively). Statistically significant reductions in IFN-γ production in response to Rev were seen for both peptide providers, at week 2 compared to baseline (Figures 4.7E and 4.8D) for individuals in group 3 (who received cytokine and hormone only with no vaccine). This was the case at week 24 for both peptide sources but only maintained statistical significance for the FIT Biotech peptide pool, which saw a significant reduction in IFN-γ production in response to the Rev sub-pool at week 48. Mean decrease from baseline to week 48 was -41 SFC/10^6 PBMC (95% CI -70 – -13, p = 0.0059; Figure 4.8D). Responses to the CTL sub-pool from FIT Biotech showed reduced IFN-γ production in response to peptide stimulation at week 12 compared to baseline and this significant reduction was maintained until week 48 (Figure 4.8E).

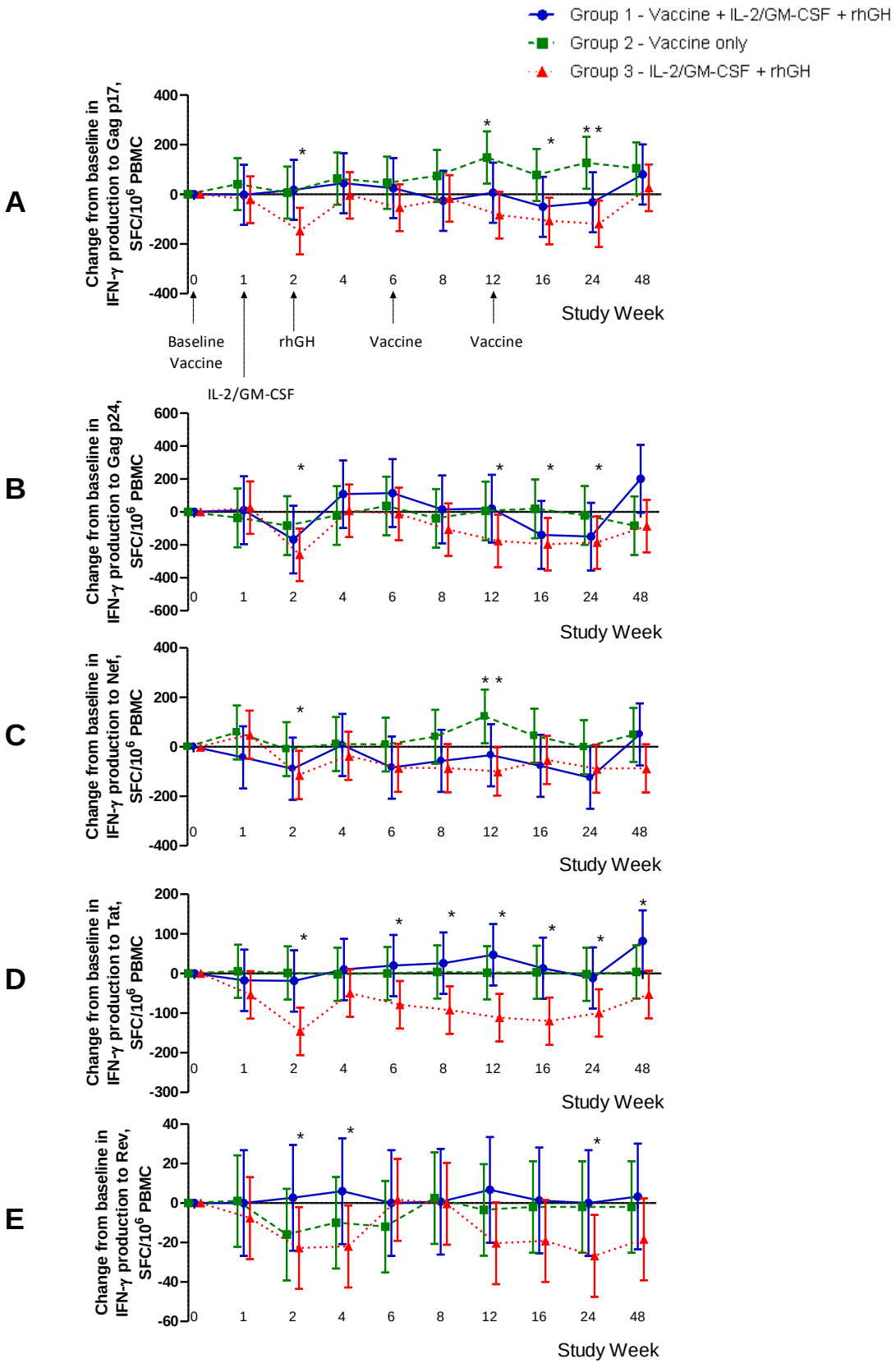


Figure 4.7 Changes from baseline to each of the study time points in IFN-γ production in response to NIBSC peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from NIBSC: Gag p17 (A), Gag p24 (B), Nef (C), Tat (D) and Rev (E), and IFN-γ production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

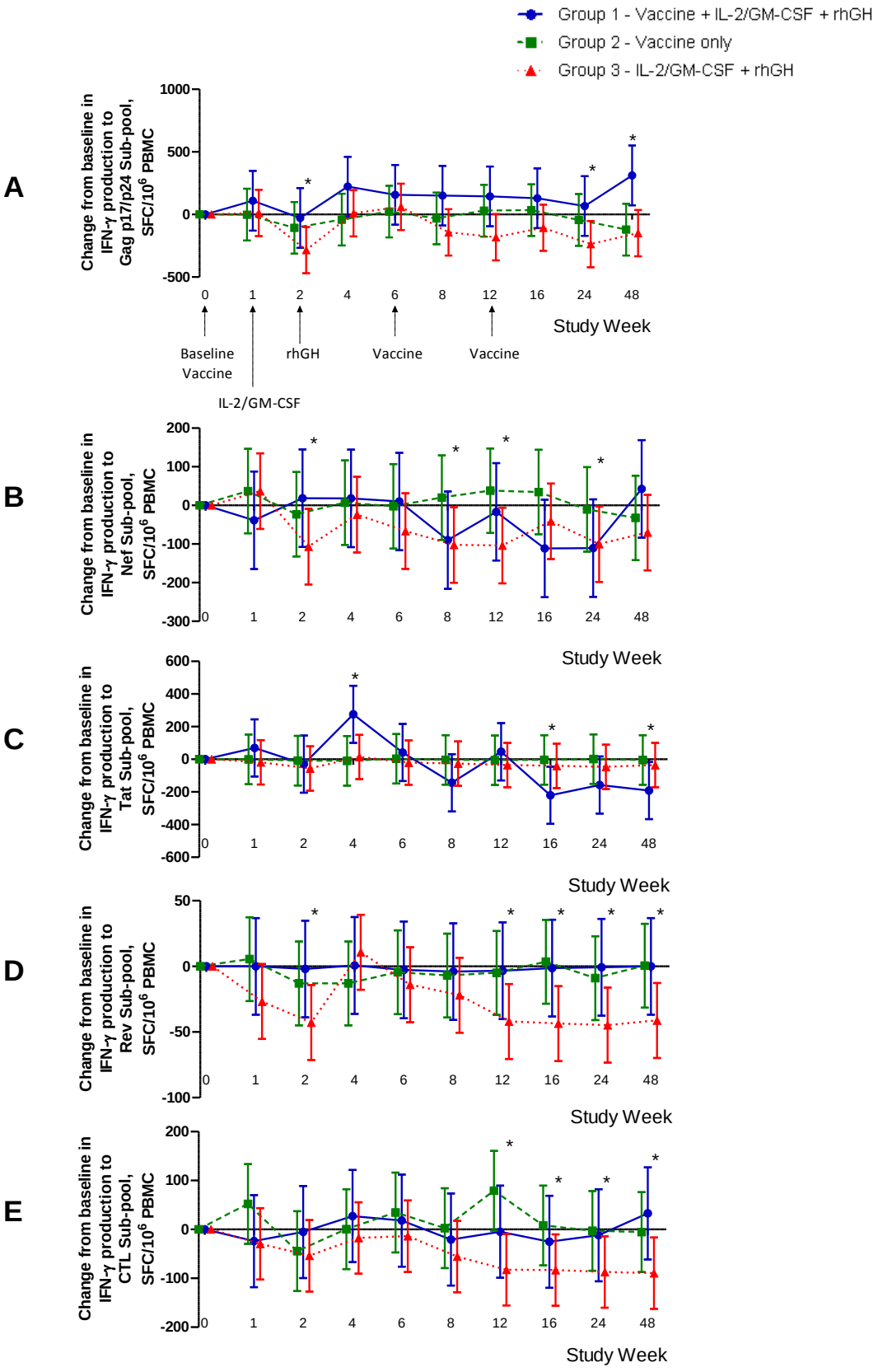


Figure 4.8 Changes from baseline to each of the study time points in IFN-γ production in response to FIT Biotech peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from FIT Biotech: Gag p17/p24 (A), Nef (B), Tat (C), Rev (D) and CTL (E) and IFN-γ production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

IL-2 production in response to peptide pools of Gag p17 and p24 from NIBSC showed a trend towards an increase compared to baseline at week 48 for patients in group 1 (Figures 4.9A and 4.9B, respectively). The mean increase from baseline to week 48 was 115 SFC/10^6 PBMC (95% CI -4 – 235 SFC/10^6 PBMC, p = 0.0621) and 42 SFC/10^6 PBMC (95% CI -5 – 89 SFC/10^6 PBMC, p = 0.0817), respectively. Responses to the FIT Biotech sub-pool of Gag p17/p24 at week 48 for individuals that received therapeutic vaccine in addition to cytokines and hormone were significantly increased compared to baseline (Figure 4.10A). The mean increase from baseline to week 48 was 79 SFC/10^6 PBMC (95% CI 31-127 SFC/10^6 PBMC, p = 0.0017; Figure 4.10A). At week 2, following administration of IL-2 and GM-CSF, patients in group 3 showed significantly increased responses to peptide pools of Nef from both sources, compared to baseline. In response to NIBSC Nef, mean change from baseline to week 2 was 31 SFC/10^6 PBMC (95% CI 11-51 SFC/10^6 PBMC, p = 0.0030) and in response to FIT Biotech Nef, mean change from baseline to week 2 was 32 SFC/10^6 PBMC (95% CI 12-52 SFC/10^6 PBMC, p = 0.0022; Figures 4.9C and 4.10B, respectively). IL-2 production in response to peptide pools of Nef remained statistically unchanged compared to baseline for all remaining time points for group 3 and no changes were seen at any of the study time points for individuals in groups 1 and 2 (Figures 4.9C and 4.10B, respectively). Stimulation with peptide pools of Tat from both sources resulted in significantly increased responses at week 2 compared to baseline for individuals in group 3. Mean increase from baseline to week 2 in response to NIBSC Tat was 35 SFC/10^6 PBMC (95% CI 18-53 SFC/10^6 PBMC, p = 0.0002) and mean increase in response to FIT Biotech Tat was also 35 SFC/10^6 PBMC (95% CI 17-53, p = 0.0003; Figures 4.9D and 4.10C, respectively). No other statistically significant changes were seen in terms of IL-2 production in response to Tat for any of the other study groups at any of the study time points. Patients that received IL-2 and GM-CSF, both with and without vaccine, showed increased IL-2 responses following stimulation with NIBSC Rev at week 2 compared to baseline (Figure 4.9E). Mean change from baseline to week 2 was 30 SFC/10^6 PBMC for patients in group 1 (95% CI 7-53, p = 0.0130) and it was 36 SFC/10^6 PBMC for individuals in group 3 (95%

CI 18-57 SFC/10^6 PBMC, p = 0.0001; Figure 4.9E). For the FIT Biotech sub-pool, only patients in group 3 showed a significantly increased response to the sub-pool of Rev at week 2. The mean change from baseline to week 2 was 35 SFC/10^6 PBMC (95% CI 18-52 SFC/10^6 PBMC, p = 0.0001; Figure 4.10D). Patients that received IL-2 and GM-CSF showed increased IL-2 production in response to the CTL sub-pool from FIT Biotech, with the mean change from baseline to week 2 of the study 34 SFC/10^6 PBMC (95% CI 16-52 SFC/10^6 PBMC, p = 0.0003; Figure 4.10E). There were no significant changes for individuals in groups 1 and 2 at any of the study time points compared to baseline, and IL-2 responses to the CTL sub-pool was statistically unchanged compared to baseline for patients in group 3 at all other time points (Figure 4.10E).

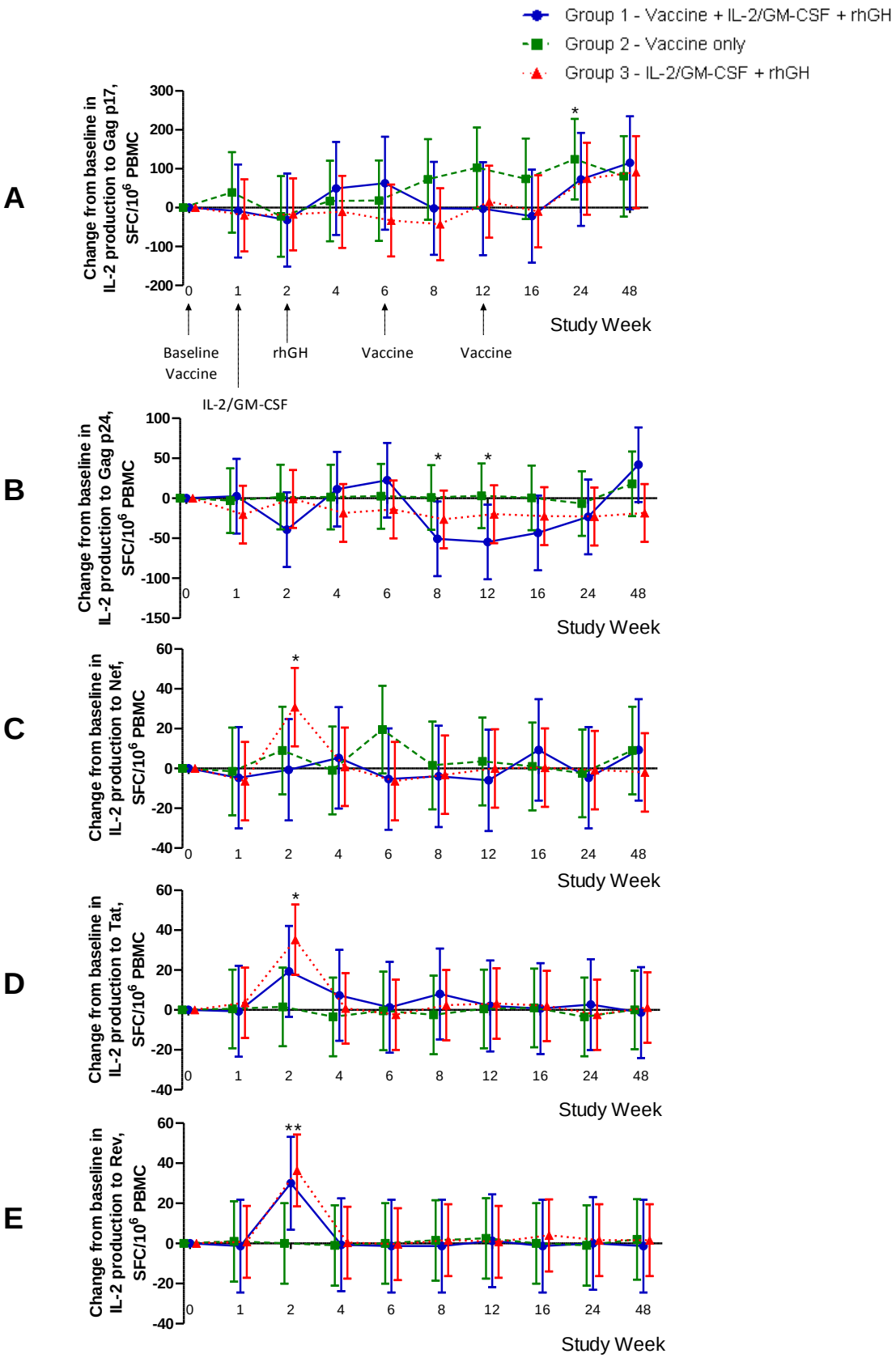


Figure 4.9 Changes from baseline to each of the study time points in IL-2 production in response to NIBSC peptide stimulation.

Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from NIBSC: Gag p17 (A), Gag p24 (B), Nef (C), Tat (D) and Rev (E), and IL-2 production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asteriks indicate statistical significance, where $p \leq 0.05$.

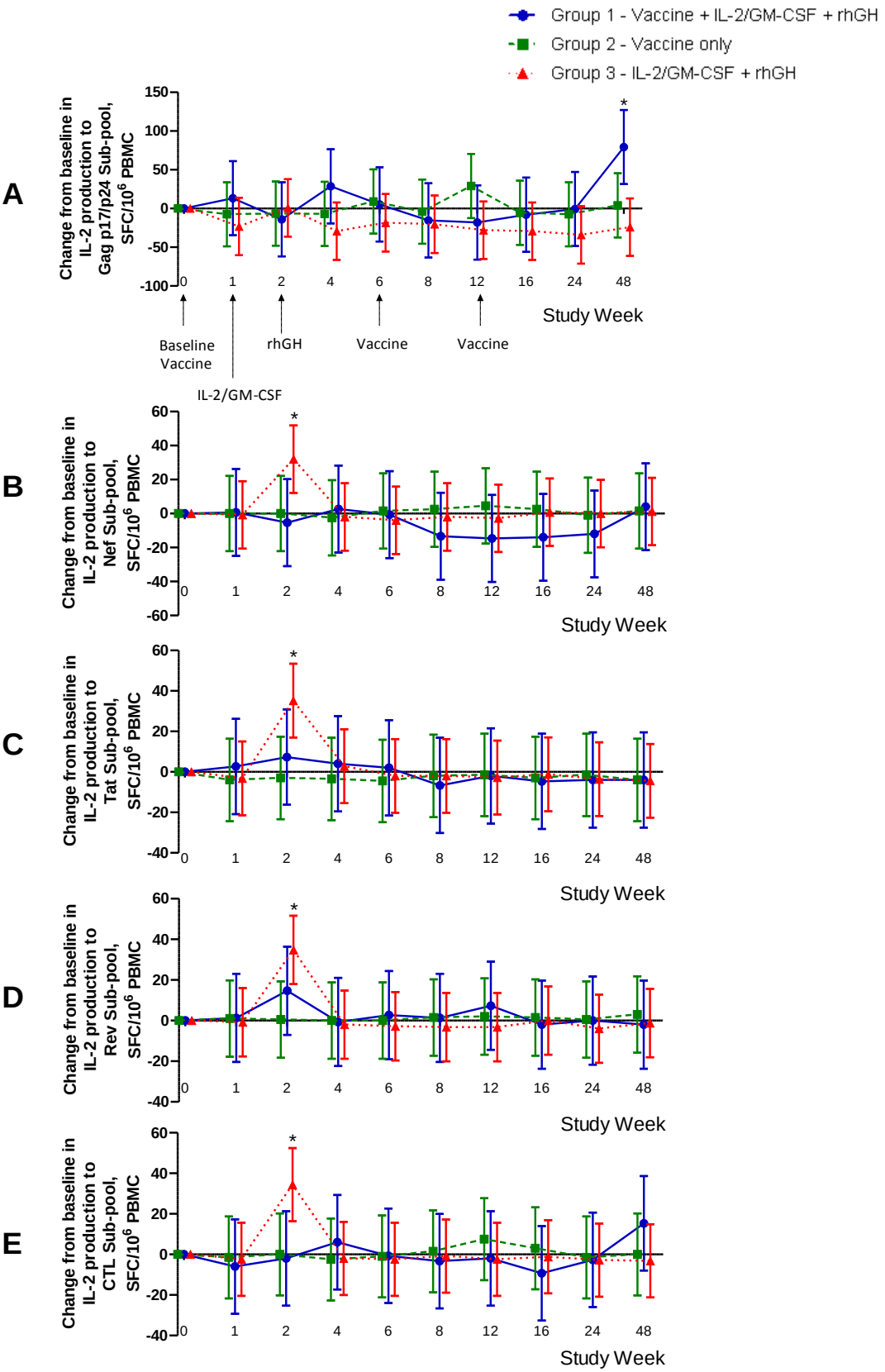


Figure 4.10 Changes from baseline to each of the study time points in IL-2 production in response to FIT Biotech peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from FIT Biotech: Gag p17/p24 (A), Nef (B), Tat (C), Rev (D) and CTL (E), and IL-2 production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

IL-4 production in response to Gag p17 from NIBSC was significantly increased at week 12 and at week 24 compared to baseline for patients that received vaccine alone (group 2; Figure 4.11A). At week 24, patients in group 3 also had a significantly increased IL-4 response following stimulation with NIBSC Gag p17 but by week 48, these responses were statistically unchanged compared to baseline. It was only individuals in group 1 that received cytokine and hormone in addition to therapeutic vaccine that showed increased IL-4 production in response to NIBSC Gag p17 at week 48 compared to baseline. Mean change from baseline to week 48 was 111 SFC/10^6 PBMC (95% CI 16-206 SFC/10^6 PBMC, p = 0.0240; Figure 4.11A). In response to NIBSC Gag p24, patients that received vaccine in addition to cytokines and hormone (group 1) and those that received vaccine alone (group 2) showed increased IL-4 responses at week 48 compared to baseline. Mean increase from baseline to week 48 was 11 SFC/10^6 PBMC for patients in group 1 (95% CI 0.3-21 SFC/10^6 PBMC, p = 0.0475) and 11 SFC/10^6 PBMC for individuals in group 2 (95% CI 2-19 SFC/10^6 PBMC, p = 0.0248; Figure 4.11B). In response to Gag p17/p24 from FIT Biotech, only patients in group 2 showed a significant increase at week 48 compared to baseline; mean increase from baseline to week 48 was 38 SFC/10^6 PBMC (95% CI 18-58 SFC/10^6 PBMC, p = 0.0003; Figure 4.12A). Looking at IL-4 production in response to the Nef sub-pool from FIT Biotech, there are no significant differences from baseline to any of the study visit time points for any of the treatment groups (Figure 4.12B). However, stimulation with the NIBSC Nef peptide pool resulted in a significant reduction in IL-4 production at week 1 and week 16 compared to baseline for individuals in group 1 and significant reductions at weeks 1 to 48 for individuals in group 3 (Figure 4.11C). IL-4 production in response to the Tat peptide pool from NIBSC was increased at week 2 for patients that received vaccine, IL-2 and GM-CSF (group 1); mean change from baseline to week 2 was 78 SFC/10^6 PBMC (95% CI 44-112 SFC/10^6 PBMC, p <0.0001; Figure 4.11D). The production of IL-4 in response to FIT Biotech Tat only showed a significant increase at week 48 for individuals that received vaccine alone (group 2) with a mean increase from baseline to week 48 of 9 SFC/10^6 PBMC (95% CI 2-16 SFC/10^6 PBMC, p = 0.0164; Figure 4.12C). PBMC stimulation with the

overlapping peptide pool of Rev from NIBSC showed significant reductions compared to baseline for all study time points from week 1 to week 48 for patients in group 1. Mean decrease in IL-4 production at week 48 compared to baseline was -19 SFC/10^6 PBMC (95% CI -28 – -9 SFC/10^6 PBMC, p = 0.0002; Figure 4.11E). IL-4 production in response to the Rev peptide pool from FIT Biotech for individuals in group 1 showed a trend towards a reduction at week 48 compared to baseline with a mean decrease from baseline to week 48 of -7 SFC/10^6 PBMC (95% CI -14 – 0.8, p = 0.0839; Figure 4.12D). The increase in IL-4 production in response to Rev from FIT Biotech at week 48 compared to baseline for individuals in group 2 (who received vaccine alone) did not reach statistical significance (Figure 4.12D), however, responses to NIBSC Rev for this treatment group were significantly elevated at week 48 compared to baseline. The mean increase from baseline to week 48 was 10 SFC/10^6 PBMC (95% CI 2-18 SFC/10^6 PBMC, p = 0.0185; Figure 4.11E). Only patients in group 2 showed significantly increased responses to the CTL sub-pool from FIT Biotech at week 48 compared to baseline with a mean increase at week 48 of 7.5 SFC/10^6 PBMC (95% CI 2-13 SFC/10^6 PBMC, p = 0.0089). All other IL-4 responses to this overlapping CTL peptide pool remained statistically unchanged compared to baseline for all other study time points and for all other treatment groups (Figure 4.12E).

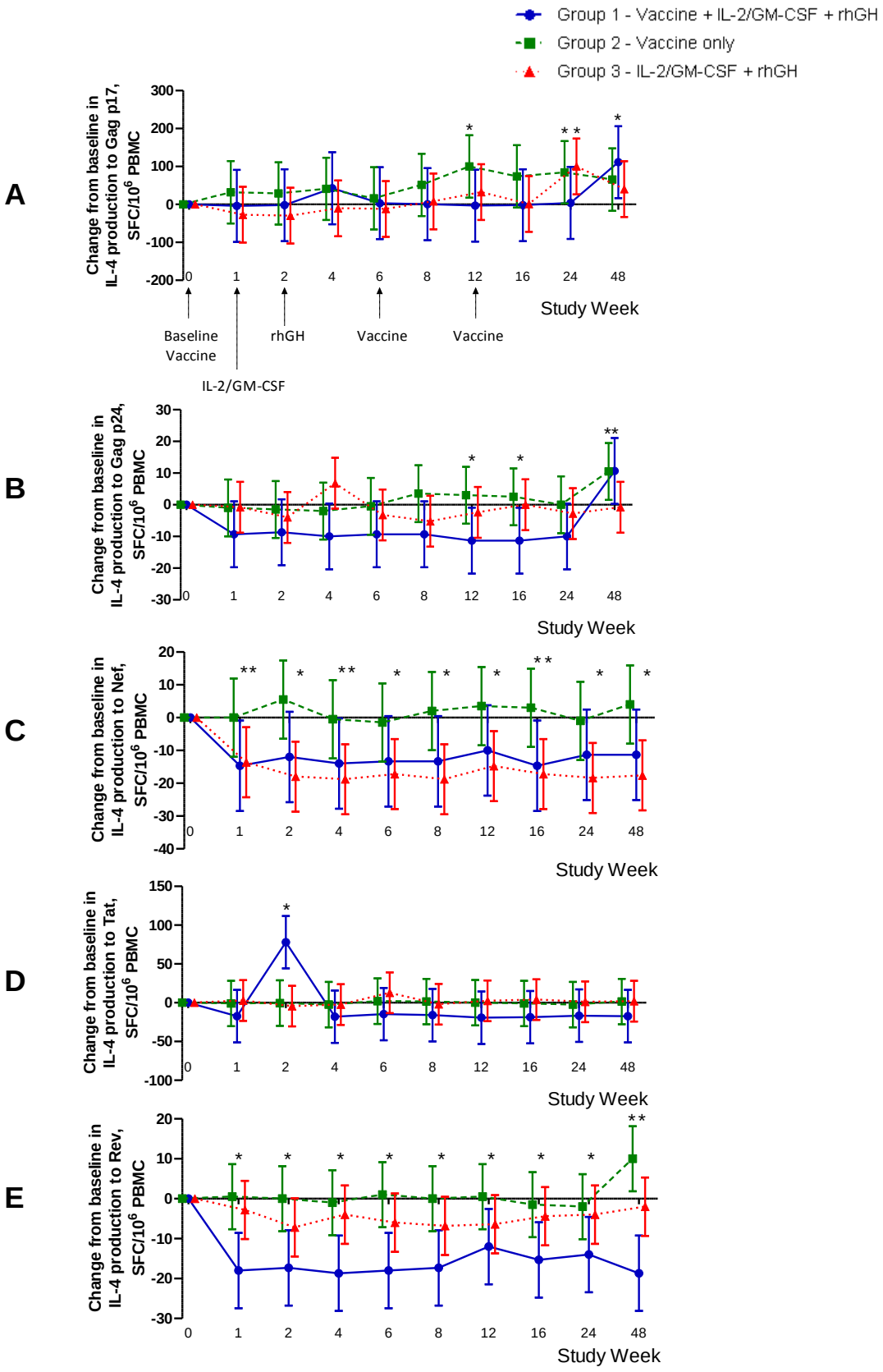


Figure 4.11 Changes from baseline to each of the study time points in IL-4 production in response to NIBSC peptide stimulation.

Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from NIBSC: Gag p17 (A), Gag p24 (B), Nef (C), Tat (D) and Rev (E), and IL-4 production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

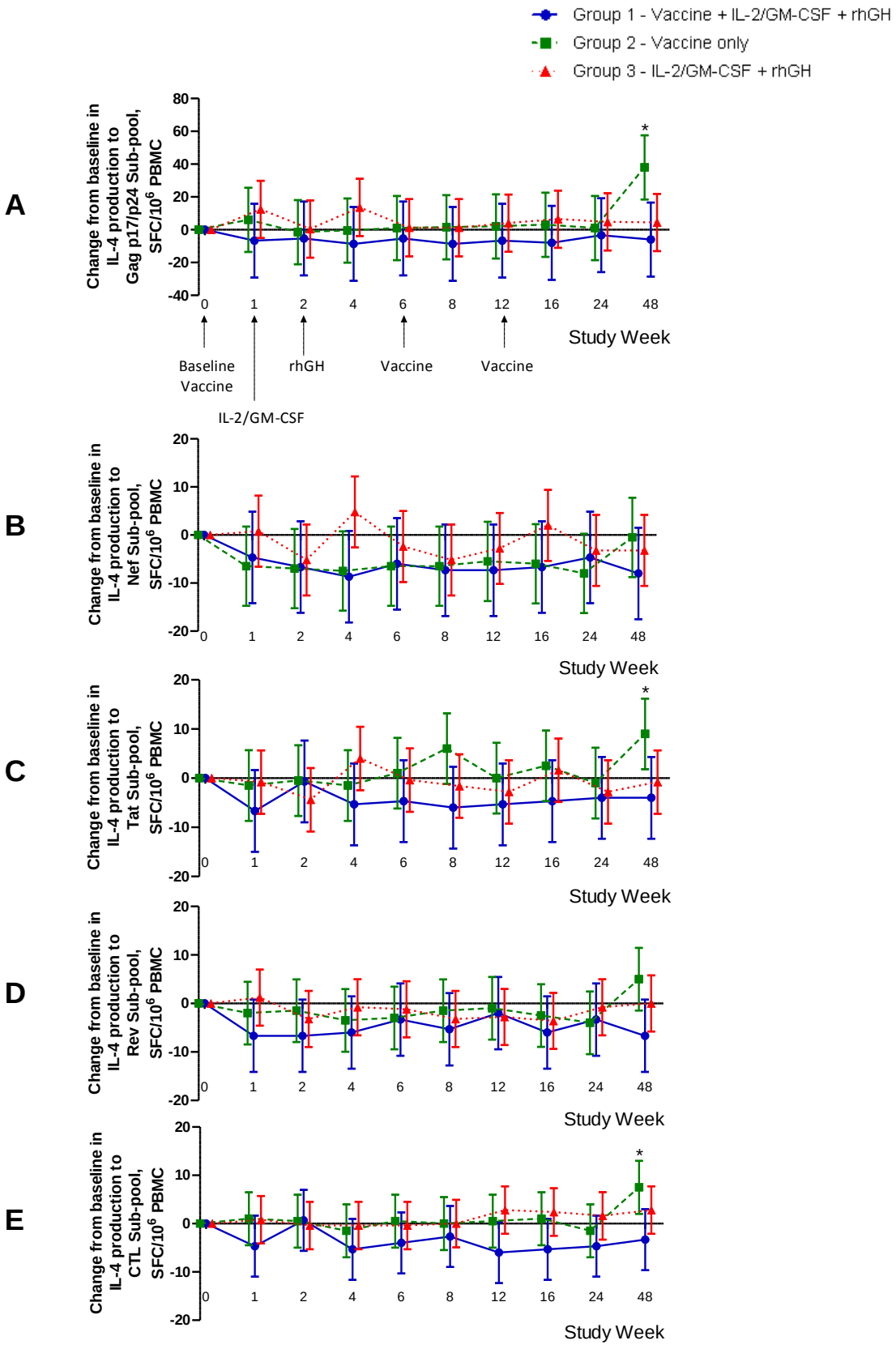


Figure 4.12 Changes from baseline to each of the study time points in IL-4 production in response to FIT Biotech peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from FIT Biotech: Gag p17/p24 (A), Nef (B), Tat (C), Rev (D) and CTL (E), and IL-4 production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Stimulation with overlapping Gag p17 peptides from NIBSC resulted in a significant increase in perforin production at week 4 compared to baseline for patients in group 1; mean increase at week 4 compared to baseline was 271 SFC/10^6 PBMC (95% CI 54-488 SFC/10^6 PBMC, p = 0.0166; Figure 4.13A). Individuals in group 3, however, demonstrated a modest but significant increase in their perforin response to Gag p17 at week 24 with mean change at this time point compared to baseline of 172 SFC/10^6 PBMC (95% CI 4-340 SFC/10^6 PBMC, p = 0.0487; also Figure 4.13A). Only individuals in group 3 showed a marked increase in perforin production at week 8 in response to Gag p24 stimulation compared to baseline. Mean change in perforin production compared to baseline was 276 SFC/10^6 PBMC (95% CI 156-395 SFC/10^6 PBMC, p <0.0001; Figure 4.13B). For the FIT Biotech Gag p17/p24 peptide pool, only patients in group 1, who received vaccine in addition to cytokines and hormone, showed an increase in perforin production at week 48 compared to baseline; with a mean change of 95 SFC/10^6 PBMC (95% CI 11-179 SFC/10^6 PBMC, p = 0.0287; Figure 4.14A). Perforin production in response to Nef, Tat and Rev from NIBSC was significantly increased at week 8 compared to baseline for individuals in group 3 but these responses were only transient (Figures 4.13C, 4.13D and 4.13E, respectively). Perforin production in response to Tat and CTL sub-pools from FIT Biotech showed increased responses at week 8 compared to baseline for patients in group 3 (Figures 4.14C and 4.14E, respectively). Mean increase from baseline was 164 SFC/10^6 PBMC (95% 39-288 SFC/10^6 PBMC, p = 0.0120) and 136 SFC/10^6 PBMC (95% CI 20-252 SFC/10^6 PBMC, p = 0.0241; Figures 4.14C and 4.14E, respectively). No changes were observed for any of the treatment groups between baseline and any of the study time points in terms of perforin production in response to the Rev sub-pool from FIT Biotech (Figure 4.14D). Responsiveness to the Nef sub-pool decreased slightly but significantly at week 24 compared to baseline for patients in group 3 (mean change from baseline -85 SFC/10^6 PBMC; 95% CI -169 – -2 SFC/10^6 PBMC, p = 0.0488) but by week 48 there was no significant change in perforin production in response to this peptide pool compared to baseline (Figure 4.14B).

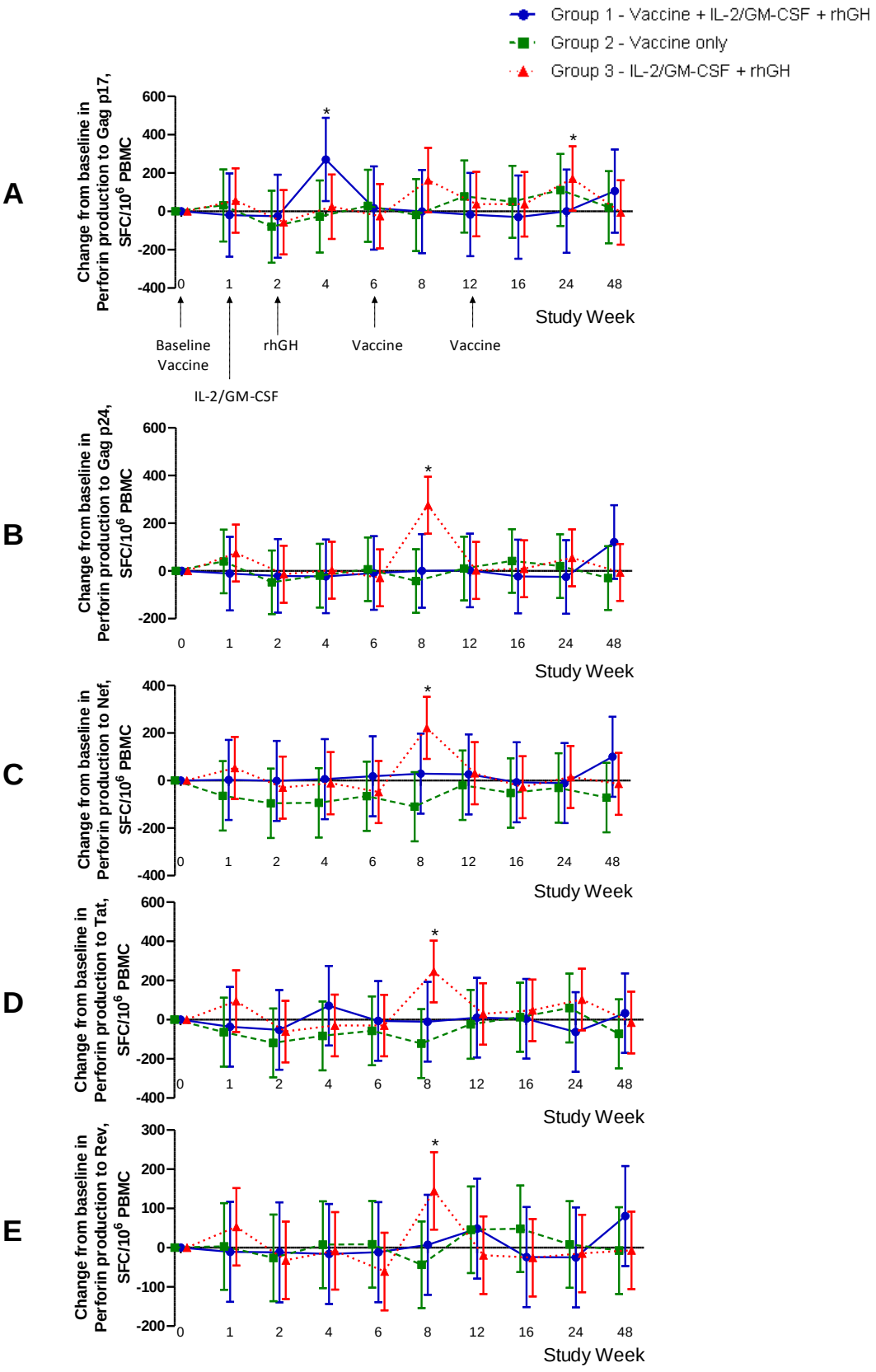


Figure 4.13 Changes from baseline to each of the study time points in perforin production in response to NIBSC peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from NIBSC: Gag p17 (A), Gag p24 (B), Nef (C), Tat (D) and Rev (e), and perforin production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

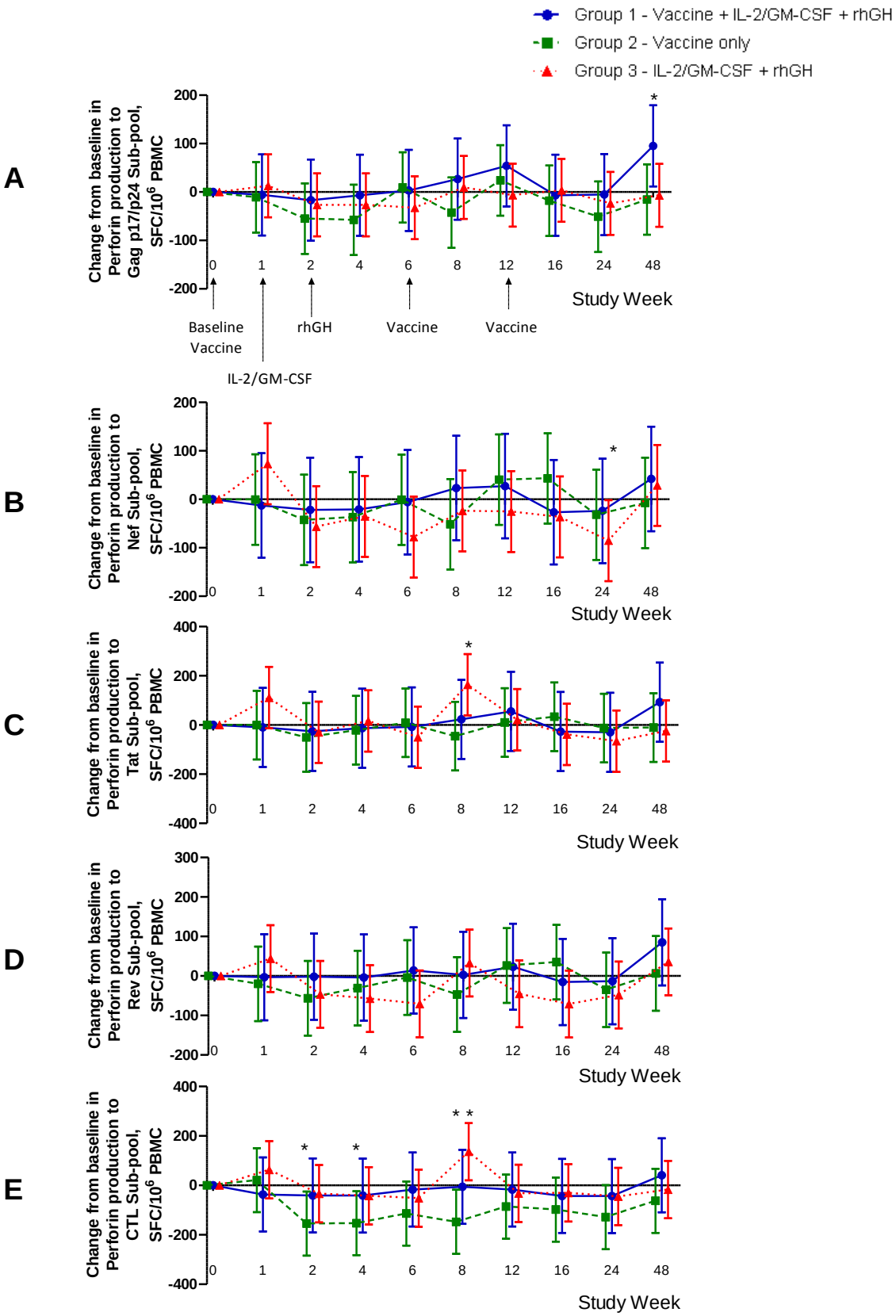


Figure 4.14 Changes from baseline to each of the study time points in perforin production in response to FIT Biotech peptide stimulation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were stimulated with peptides from FIT Biotech: Gag p17/p24 (A) ,Nef (B), Tat (C), Rev (D) and CTL (E), and perforin production determined by calculating the number of SFC per million PBMC. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

4.5 Phenotypic characterisation of CD4 and CD8 T cells

Following administration of IL-2 and GM-CSF, there was a marked increase in the percentage co-expression of the activation markers CD38 and HLA-DR on CD4 T cells. The mean increase from baseline to week 2 was 18.3% (95% CI 15.2-21.3%, p <0.0001) and 18.1% (95% CI 15.7-20.4%, p <0.0001) for patients in groups 1 and 3 respectively (Figure 4.15A). The co-expression of these two markers was also significantly increased at week 2, but to a lesser extent, for individuals that received vaccine alone (group 2) with a mean increase from baseline of 4.9% (95% CI 2.3-7.5%, p = 0.0005; Figure 4.15A). CD38 and HLA-DR co-expression on CD8 T cells at week 2 was also significantly higher in all groups, with the greatest increase noted for individuals in group 1, with a mean increase from baseline to week 2 of 30.9% (95% CI 26.1-35.7%, p <0.0001; Figure 4.16A). By week 48, co-expression of these activation markers had normalised and there were no statistically significant differences compared to baseline for either T-cell subset (Figures 4.15A and 4.16A). Percentage expression and MFI of HLA-DR on CD4 and CD8 T cells from the trial participants followed a similar pattern, with notable increases in the HLA-DR expression at week 2 which was normalised by week 48 of the study (Figures 4.15B and 4.15D show percentage expression and MFI on CD4 T cells and Figures 4.16B and 4.16D depict percentage expression and MFI on CD8 T cells). Elevated expression of HLA-DR (in terms of MFI) was only seen for individuals in group 2 at week 48, with a marginal yet significant increase from baseline of 3407 (95% CI 446-6368, p = 0.0268; Figure 4.15D). CD38 percentage expression was also increased at week 2 for all study groups on both T-cell subsets, with more pronounced increases for individuals that received IL-2 and GM-CSF (Figures 4.15C and 4.16C). By week 4, CD38 expression on the CD4 T cells had normalised for patients in all study groups and by week 6, there was a significant reduction in the percentage expression of CD38 on CD4 T cells for individuals that received cytokine and hormone and this decline was maintained up to and including the final study time point (week 48). At week 48, the mean decrease from baseline was -8.3% (95% CI -15.1 – 1.5%, p = 0.0194) and -6.1% (95% CI -11.4 – -0.9%, p = 0.0254; Figure 4.15C). At week 24, individuals in

groups 1 and 3 showed a significant reduction in the percentage expression of CD38 on their CD8 T cells, and this was only maintained for individuals in group 3, with a mean decrease from baseline to week 48 of -8.8% (95% CI -15.6 – -1.9%, p = 0.0139), although there was a trend towards a decrease in this markers for individuals in group 1 with a mean decline from baseline of -8.4% (95% CI -17.3 – 0.4%, p = 0.0651; Figure 4.16C). In terms of CD38 MFI, only CD4 T cells from patients in groups 1 and 3 showed marginal but significant reductions at week 48 compared to baseline with mean decreases of -468 (95% CI -905 – -31, p = 0.0387) and -346 (95% CI -684 – -8, p = 0.0484) for groups 1 and 3 respectively (Figure 4.15E).

CD69 expression was also assessed on both CD4 and CD8 T lymphocytes and no significant changes from baseline were observed in percentage expression of this marker in any of the study groups. MFI of CD69 on CD4 T cells revealed significantly increased expression at weeks 16 and 24 for patients in groups 1 and 3, with elevated levels at week 48 for individuals in group 3. CD8 T-cell expression of CD69 in terms of MFI showed similar elevations at weeks 16 and 24 for patients in groups 1 and 3 but these values had normalised by week 48, with increased levels measured on CD8 T cells from patients in group 2 at the final study time point (graphs not shown but mean change from baseline, 95% CI and p values are detailed in Appendix 2).

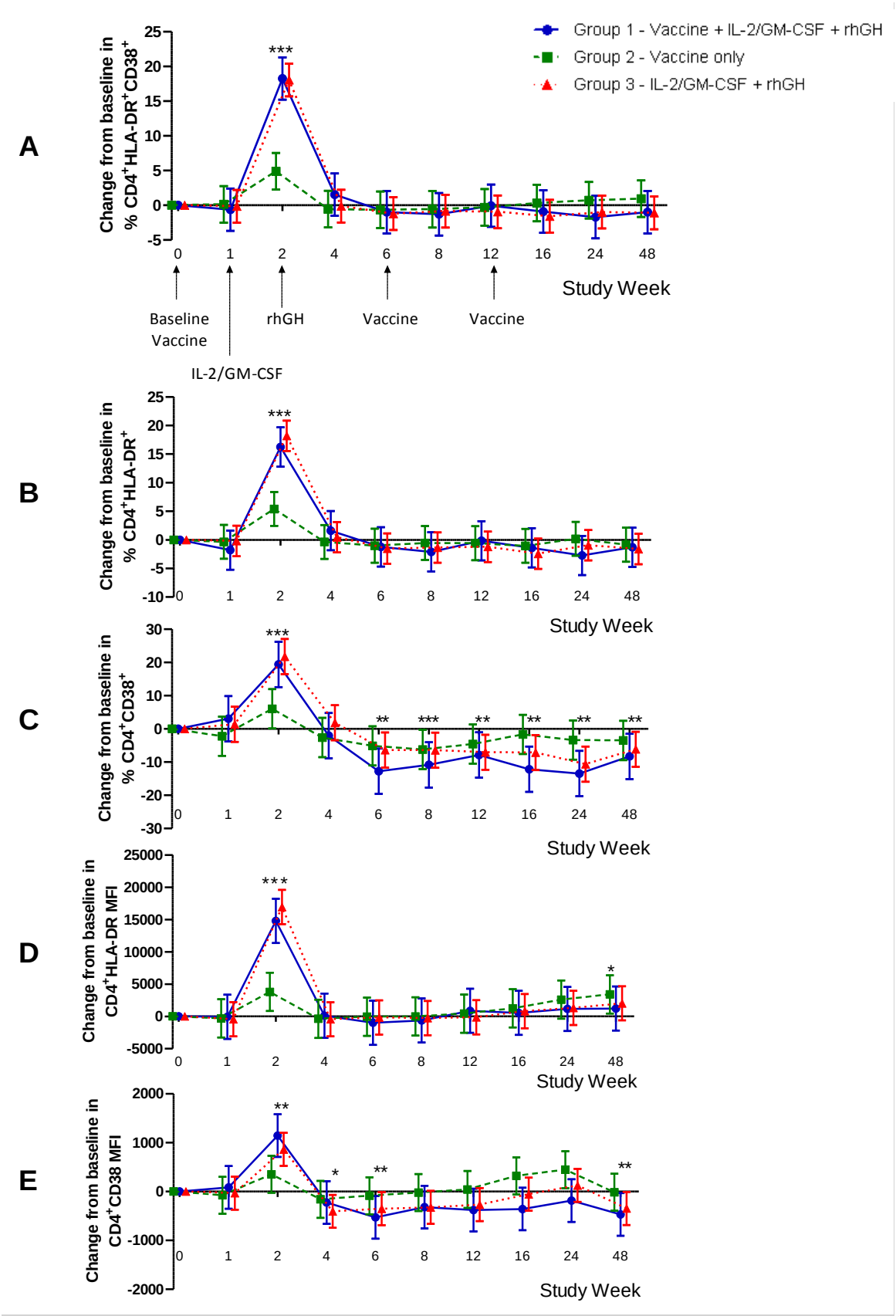


Figure 4.15 Changes from baseline to each of the study time points in CD4 T-cell activation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for surface expression of markers associated with immune activation: shown here are percentage co-expression of HLA-DR and CD38 (A), and HLA-DR (B) and CD38 (C) individually. The MFI of both of these markers was also determined (D and E, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

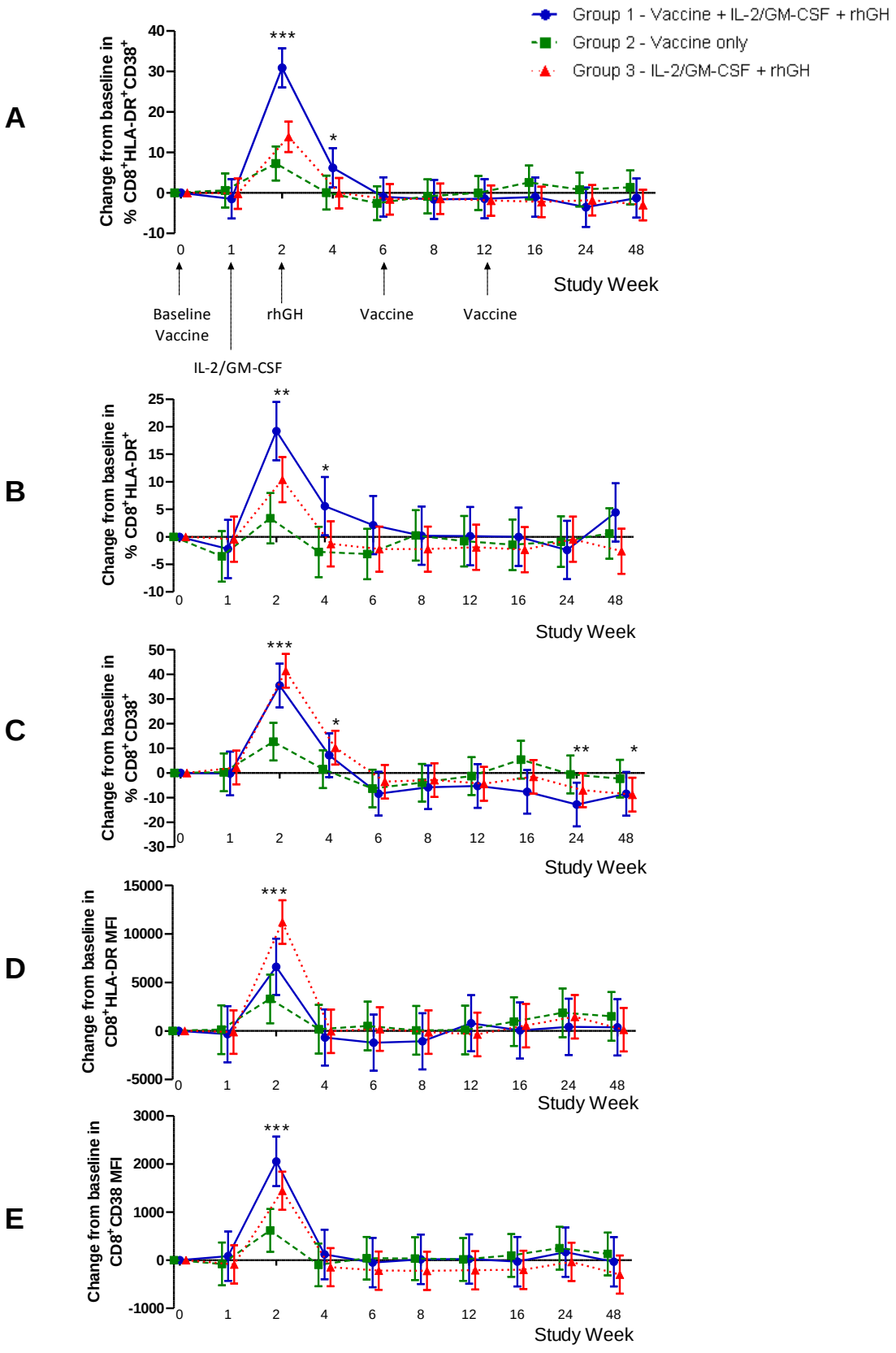


Figure 4.16 Changes from baseline to each of the study time points in CD8 T-cell activation.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for surface expression of markers associated with immune activation: shown here are percentage co-expression of HLA-DR and CD38 (A), and HLA-DR (B) and CD38 (C) individually. The MFI of both of these markers was also determined (D and E, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Percentage co-expression of markers of immune senescence (CD57) and apoptosis (CD95) showed a significant decrease on CD4 T cells at week 4 for individuals that received cytokines and hormone. Mean reduction from baseline to week 4 was -3.5% (95% CI -6.6 – 0.5, p = 0.0261) and -3.2% (95% CI -5.6 – 0.8%, p = 0.0097) for groups 1 and 3 respectively (Figure 4.17A). Co-expression of these markers was reduced for patients in groups 2 and 3 by week 48, with a trend towards a decrease for individuals in group 1 (Figure 4.17A). Expression of CD57 on CD4 T cells followed a similar pattern (Figure 4.17B). At week 2, all study groups showed a significant increase in the percentage expression of CD95 compared to baseline but this was more pronounced for individuals that received IL-2 and GM-CSF, and expression of the apoptotic marker had normalised by week 48 (Figure 4.17C). CD4 T-cell MFI of CD95, however, was significantly reduced by week 48, despite a significant increase at week 2, and mean decrease from baseline was -1562 (95% CI -2682 – -442, p = 0.0077) and -1675 (95% CI -2542 – -808, p = 0.0003) for groups 1 and 3 respectively (Figure 4.17E). CD95 expression in terms of MFI followed the same pattern on the CD8 T cells, with a significant increase at week 2 compared to baseline for individuals that received IL-2 and GM-CSF and a significant reduction by week 48 for patients in group 3, with a trend towards a reduction for those in group 1 (Figure 4.18E). Co-expression of CD57 and CD95 on CD8 T cells was significantly reduced at weeks 2, 8 and 48 for patients in group 3 with a mean reduction from baseline of -10.5% (95% CI -16.2 – -4.8, p = 0.0005; Figure 4.18A). A similar pattern emerged when looking at the single expression of CD57 on the CD8 T cells but by week 48, patients in group 2 also demonstrated a reduction in this marker although this was less pronounced (Figure 4.18B). MFI of CD57 on the CD8 T-cell subset was significantly reduced by week 48 for patients that received cytokine and hormone, although more so for those in group 3 (Figure 4.18D). Percentage expression of CD95 did not deviate significantly from baseline for any group at any study time point except for a marginal but significant increase at week 24 for patients in group 3 with a mean increase from baseline of 5.0% (95% CI 0.2-9.8%, p = 0.0460; Figure 4.18C).

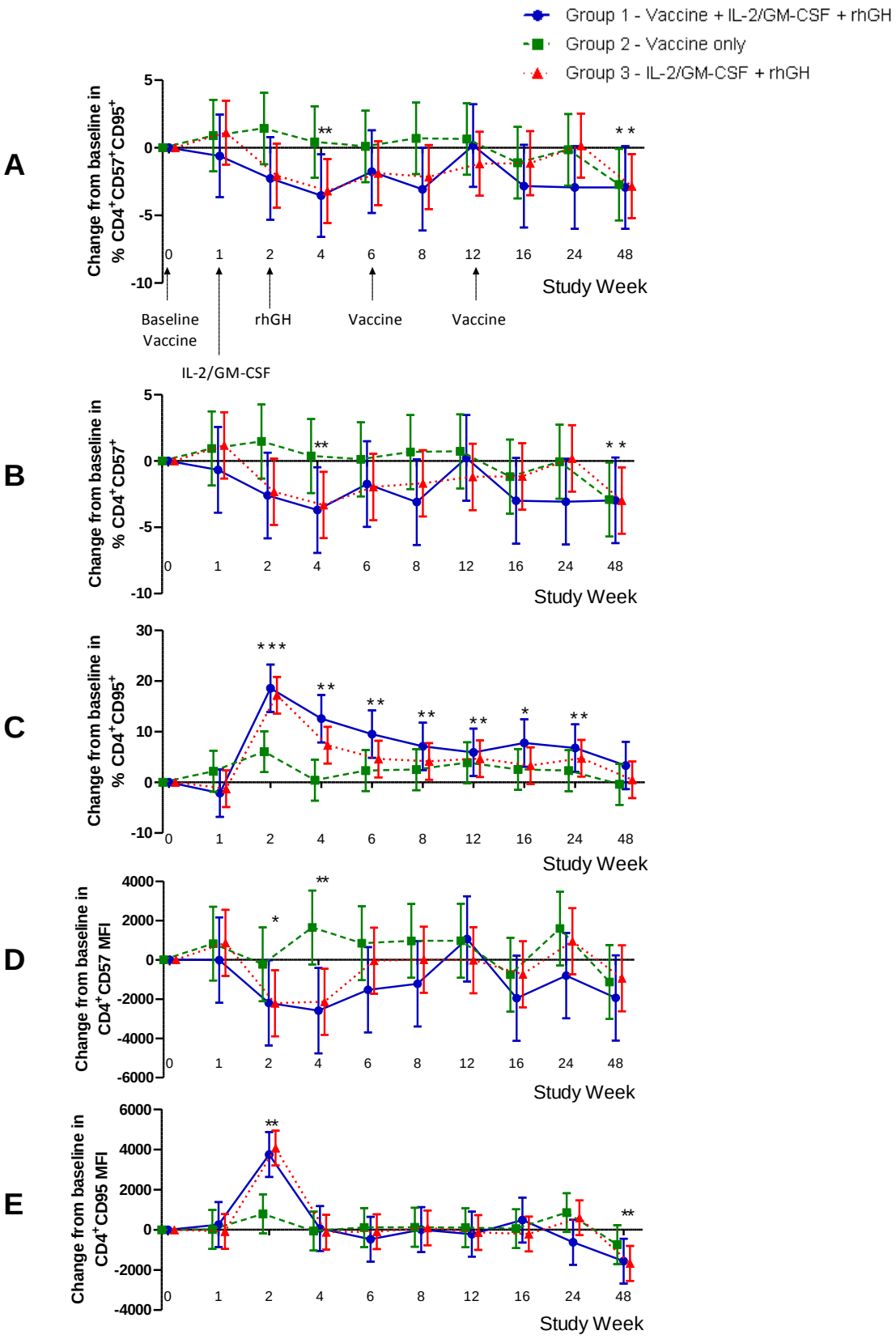


Figure 4.17 Changes from baseline to each of the study time points in CD4 T-cell senescence and apoptosis.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for surface expression of markers associated with senescence and apoptosis: shown here are percentage co-expression of CD57 and CD95 (A), and CD57 (B) and CD95 (C) individually. The MFI of both of these markers was also determined (D and E, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

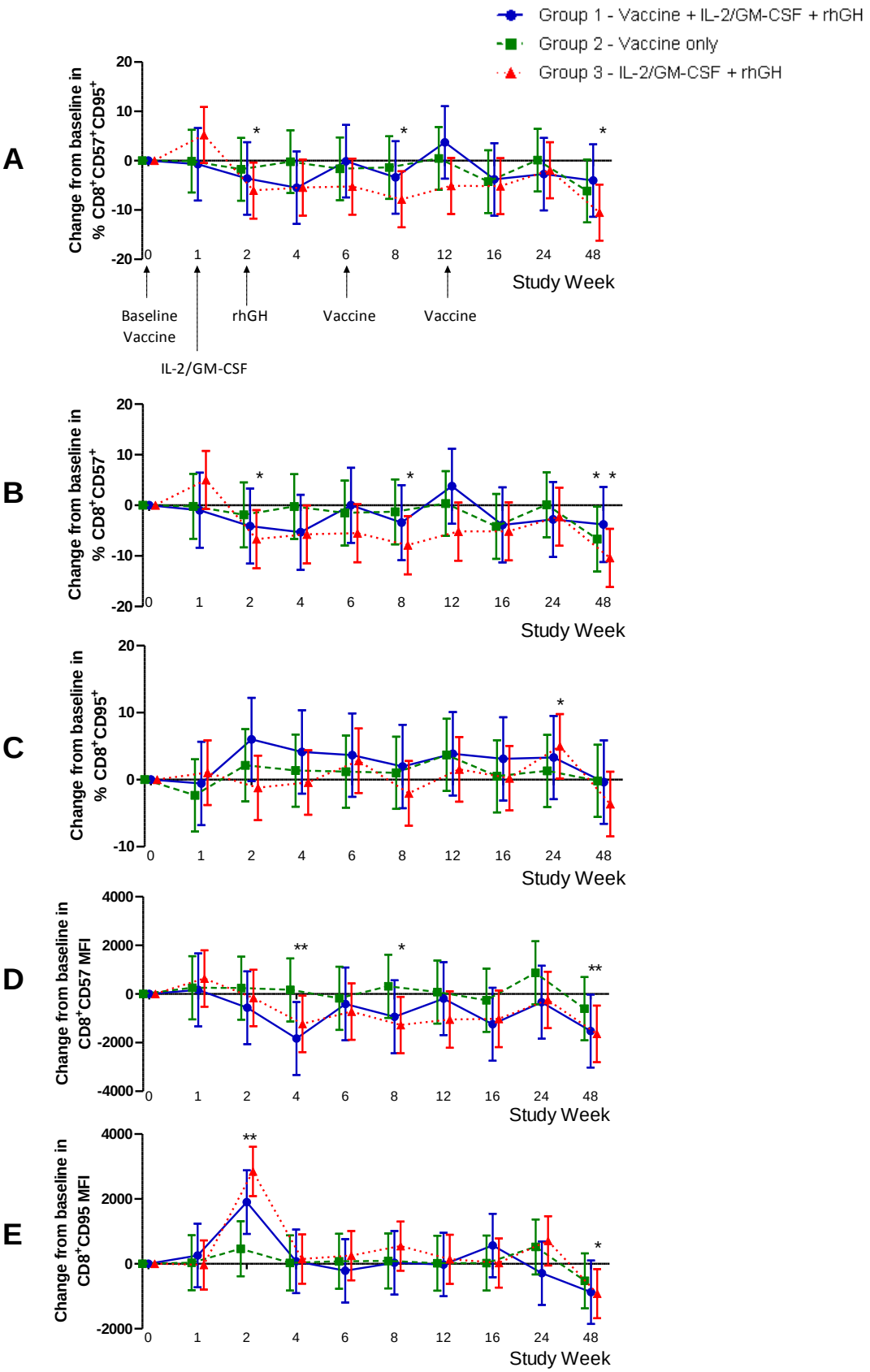


Figure 4.18 Changes from baseline to each of the study time points in CD8 T-cell senescence and apoptosis.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for surface expression of markers associated with senescence and apoptosis: shown here are percentage co-expression of CD57 and CD95 (A), and CD57 (B) and CD95 (C) individually. The MFI of both of these markers was also determined (D and E, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

At week 6, markers associated with an early T-cell phenotype ($CD27^{+}CD28^{+}$) were elevated moderately on CD4 T cells for individuals in group 3, but by week 48 no significant changes compared to baseline were observed for any of the treatment groups (Figure 4.19A). The intermediate phenotype ($CD27^{-}CD28^{+}$) was significantly increased at week 2 for patients that received IL-2 and GM-CSF, and this was more pronounced for patients that received vaccine in addition to the cytokines (Figure 4.19B). Mean increase from baseline to week 2 was 5.3% (95% CI 3.8-6.8%, $p < 0.0001$) and 2.2% (95% CI 1.0-3.4%, $p = 0.0004$) for groups 1 and 3 respectively. These increases were maintained, but to a lesser degree, at week 4 for both groups and only significantly elevated for patients in group 1 by week 6, and by week 48, no significant differences were seen compared to baseline for any of the treatment groups (Figure 4.19B). CD4 T-cell expression of markers associated with a late differentiation state were significantly reduced at weeks 2 and 6 for patients in group 3 and at week 24 for patients in group 1 but no significant changes compared to baseline were observed at week 48 (Figure 4.19C). Co-expression of CD27 and CD28 on CD8 T cells was significantly increased for patients in group 2 at weeks 4, 8 and 24 whereas patients in group 3 showed an initial decrease in these early T-cell markers at week 1 followed by significant increases at weeks 6, 12 and 48 (Figure 4.20A). Mean increase from baseline to week 48 was 6.7% (95% CI 1.2-12.3%, $p = 0.0196$) for patients in group 3. The percentage expression of markers associated with an intermediate CD8 T-cell phenotype were reduced at weeks 1, 2, 4, 8, 12, 16, 24 and 48 of the study for patients that received vaccine alone (Figure 4.20B). Markers associated with a late CD8 T-cell differentiation state were found to be significantly reduced at weeks 2, 6, 8 and 12 for individuals in group 3 with a trend towards a reduction at week 48 for these patients that received cytokine and hormone only (Figure 4.20C).

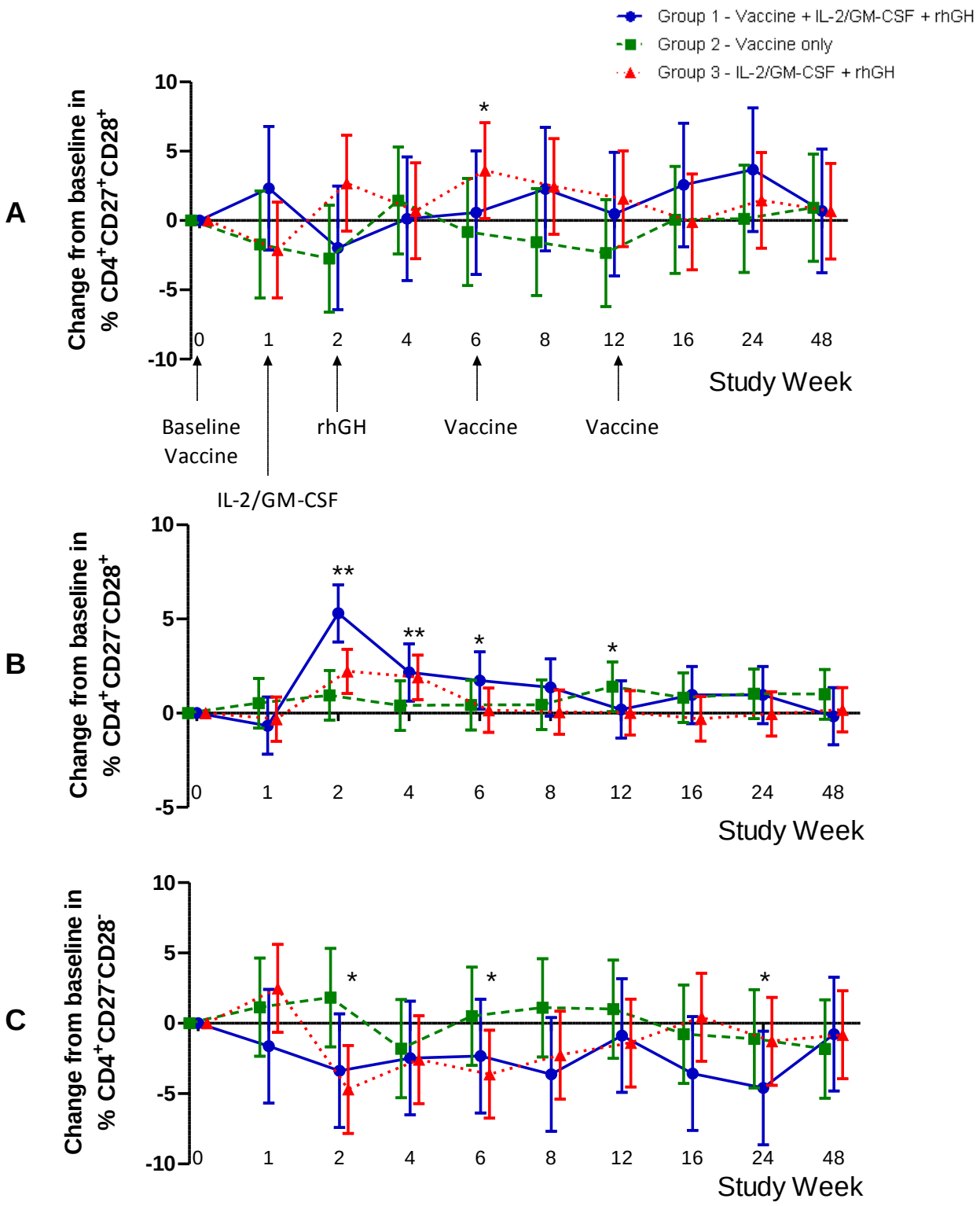


Figure 4.19 Changes from baseline to each of the study time points in early, intermediate and late CD4 T cells. Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage expression of CD27 and CD28 and early ($CD27^+CD28^+$; A), intermediate ($CD27^-CD28^+$; B) and late ($CD27^-CD28^-$; C) CD4 T cells were determined. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

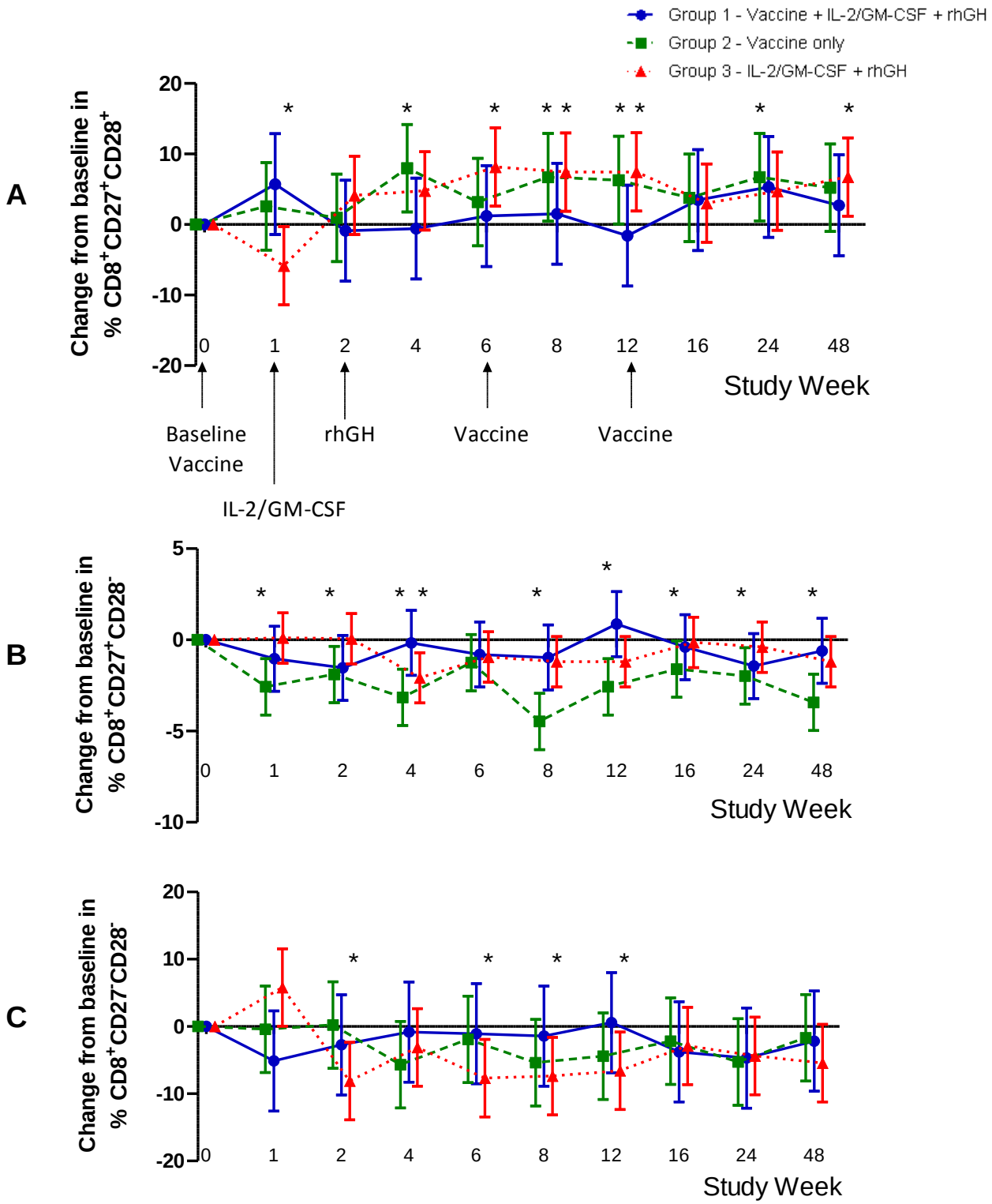


Figure 4.20 Changes from baseline to each of the study time points in early, intermediate and late CD8 T cells. Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage expression of CD27 and CD28 and early ($CD27^+CD28^+$; A), intermediate ($CD27^+CD28^-$; B) and late ($CD27^-CD28^-$; C) CD8 T cells were determined. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Characterisation of the expression of markers associated with a regulatory T-cell phenotype revealed a significant increase at week 2 following administration of IL-2 and GM-CSF for patients in groups 1 and 3, with a more pronounced increase for patients in group 3 (Figure 4.21A). The mean increase from baseline to week 2 was 7.0% (95% CI 4.4-9.6%, p <0.0001) and 11.0% (95% CI 9.0-13.0%, p <0.0001) for groups 1 and 3 respectively, and by week 48, expression of these markers had normalised and no significant changes compared to baseline were found for any of the treatment groups (Figure 4.21A). A similar increase at week 2 was seen when looking at the $CD45RO^{+}CD25^{+}$ CD4 T cells for patients that received IL-2 and GM-CSF, however by week 48, all study treatment groups demonstrated elevated co-expression of these markers (Figure 4.21B). Single expression of these markers followed the same pattern with marked increases at week 2 for those that received IL-2 and GM-CSF, but by week 48 only patients in group 2 showed a significant increase in CD25 expression with a mean increase from baseline of 8.3% (95% CI 1.5-15.2%, p = 0.0198; Figure 4.21C). Individuals in groups 1 and 3 showed elevated levels of CD45RO at week 48 with mean increases from baseline of 9.0% (95% CI 3.3-14.6%, p = 0.0025) and 6.6% (95% CI 2.3-11.0%, p = 0.0038; Figure 4.21D).

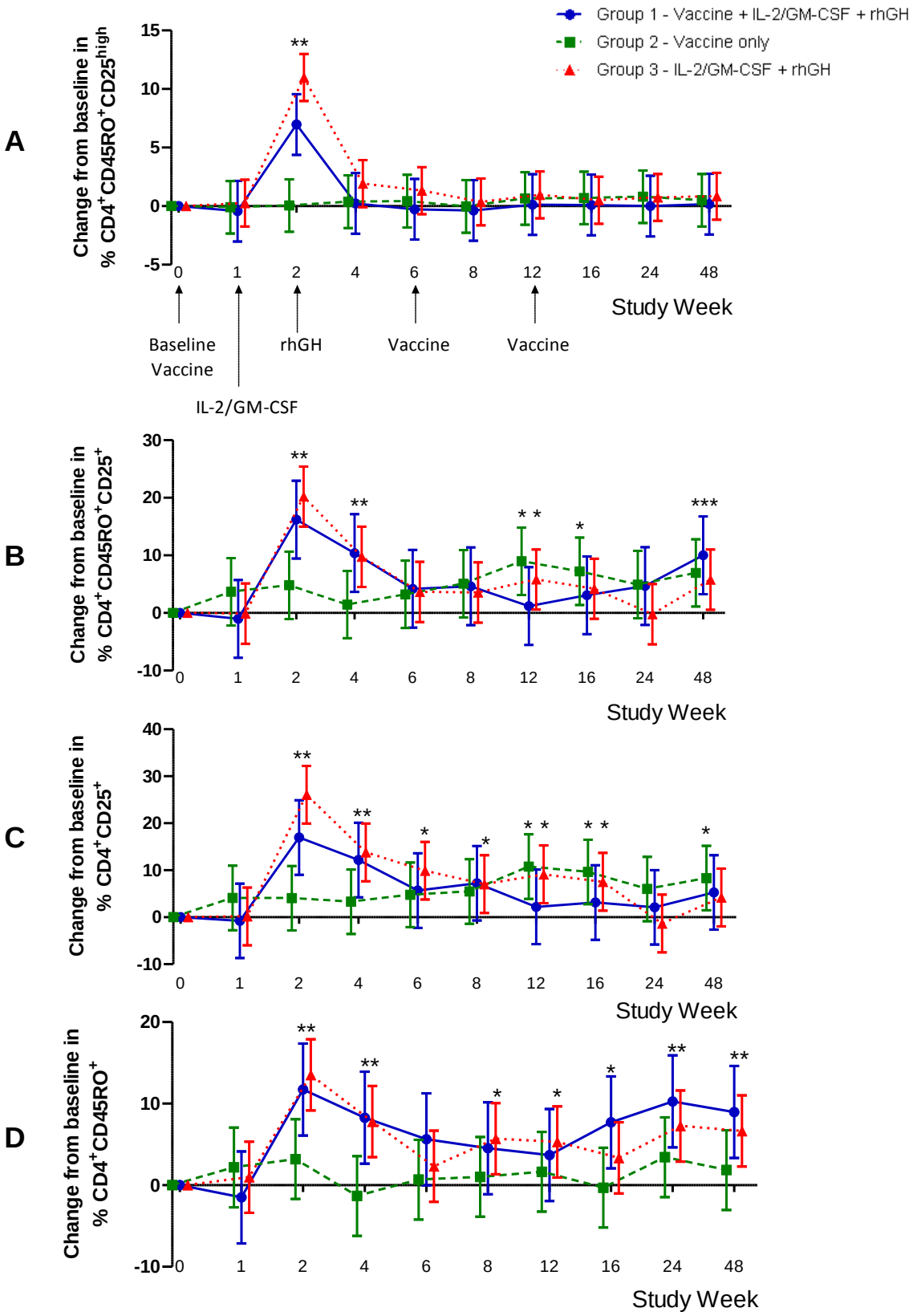


Figure 4.21 Changes from baseline to each of the study time points in markers associated with a regulatory T-cell phenotype.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage expression of markers associated with a regulatory T-cell phenotype ($CD4^+CD45RO^+CD25^{high}$; A), as well as looking at co-expression of CD45RO and CD25 (B) and expression of CD25 (C) and CD45RO (D). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Using CCR7 and CD45RA to determine T-lymphocyte subsets, decreased expression of markers associated with a naïve CD4 T-cell phenotype were observed at weeks 2 and 4 for patients that received IL-2 and GM-CSF but by week 48, only patients in group 1 demonstrated reduced percentage expression of naïve T-cell markers. Mean decrease from baseline to week 48 was -5.8% (95% CI -10.7 – 0.8%, p = 0.0242; Figure 4.22A).In terms of CD4 T_{CM}, patients that received vaccine alone showed significant increases in this population at weeks 2 and 48 of the study, with individuals in group 1 showing a trend towards an increase by week 48 (Figure 4.22B). Patients that received cytokines demonstrated a significant increase in CD4 T_{EM} at week 2 with mean increases from baseline of 8.7% (95% CI 3.3-14.1%, p = 0.0023) and 4.7% (95% CI 0.5-8.9%, p = 0.00306) for groups 1 and 3 respectively (Figure 4.22C). The elevation in CD4 T_{EM} was maintained for patients in group 1 until week 6 but by week 48, there were no significant changes compared to baseline for any of the treatment groups (Figure 4.22C). Despite moderate fluctuations in the percentage expression of markers associated with a differentiated T-cell phenotype, there were no significant changes in CD4 T_{EMRA} for any of the study time points for any of the study groups (Figure 4.22D). The expression of the differentiation markers on the CD8 T-cell subsets showed a very different profile change over the course of the study (Figure 4.23) with patients in group 3 demonstrating significantly elevated levels of markers associated with a naïve T-cell phenotype from week 1 and this was maintained until week 48 of the study. The mean increase in naïve CD8 T cell markers was 10.6% (95% CI 3.2-11.1%, p = 0.0065; Figure 4.23A) and this was reflected by a significant reduction in T_{EMRA} markers from week 2 until week 48 of the study, with a mean decrease from baseline to week 48 of -10.6% (95% CI -19.0 – -2.2%, p = 0.0158; Figure 4.23D). For group 3 individuals, increases in T_{EM} were reflected by decreases in T_{CM} at various study time points but these changes rarely reached statistical significance when compared to baseline (Figures 4.23B and 4.23C, respectively). Patients in group 2 that received vaccine alone showed significant reductions in the expression of T_{EM} markers at weeks 2, 12, 16 and 24 but by week 48, there were no significant changes from baseline in T_{EM} cells for any of the treatment groups (Figure 4.23C).

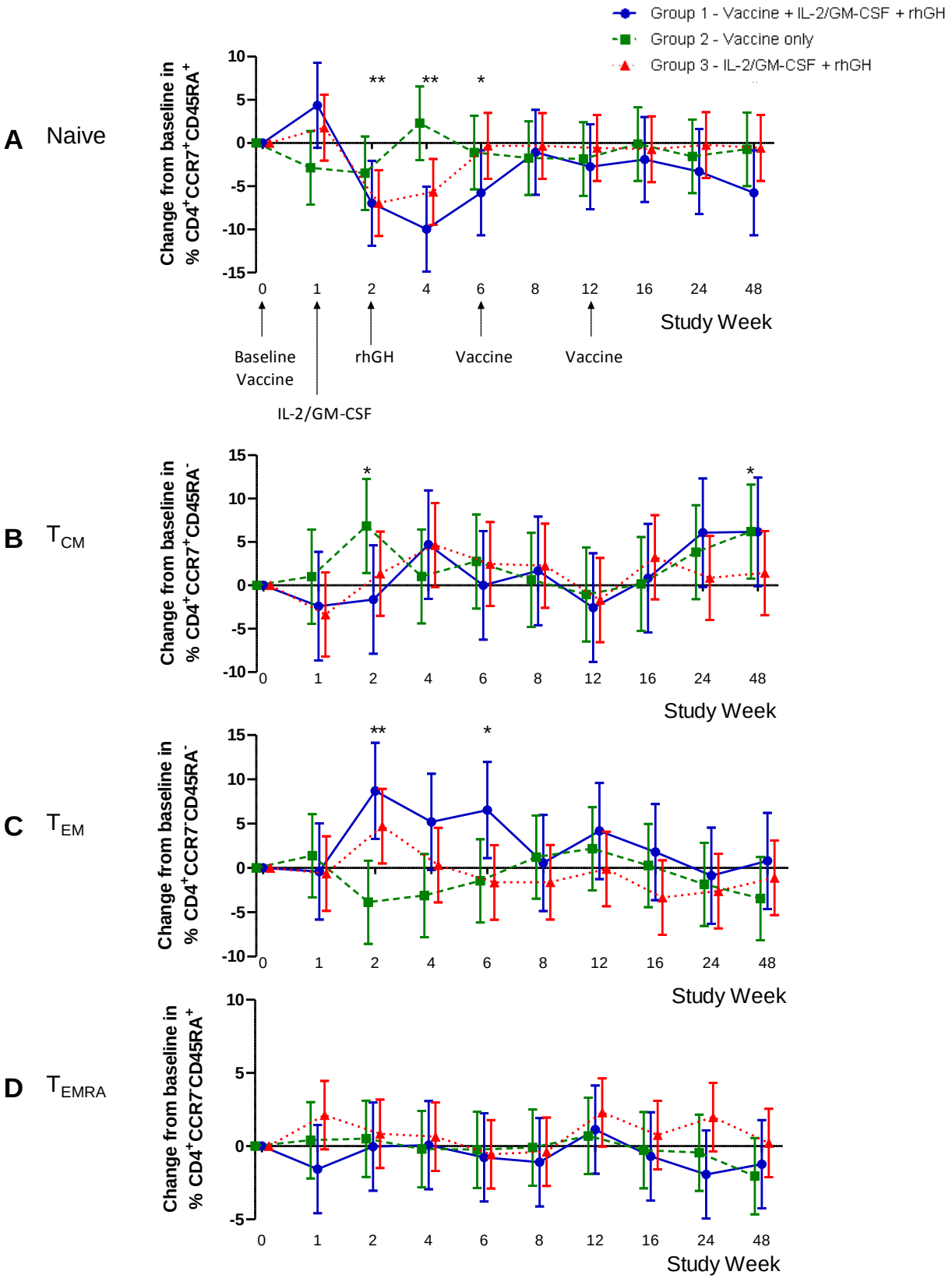


Figure 4.22 Changes from baseline to each of the study time points in CD4 T-cell differentiation, as determined by CCR7 and CD45RA expression.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage expression of markers associated with a naive profile ($CCR7^+CD45RA^+$; A), a T_{CM} profile ($CCR7^+CD45RA^-$; B), a T_{EM} phenotype ($CCR7^-CD45RA^-$; C) and a T_{EMRA} profile ($CCR7^-CD45RA^+$; D). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

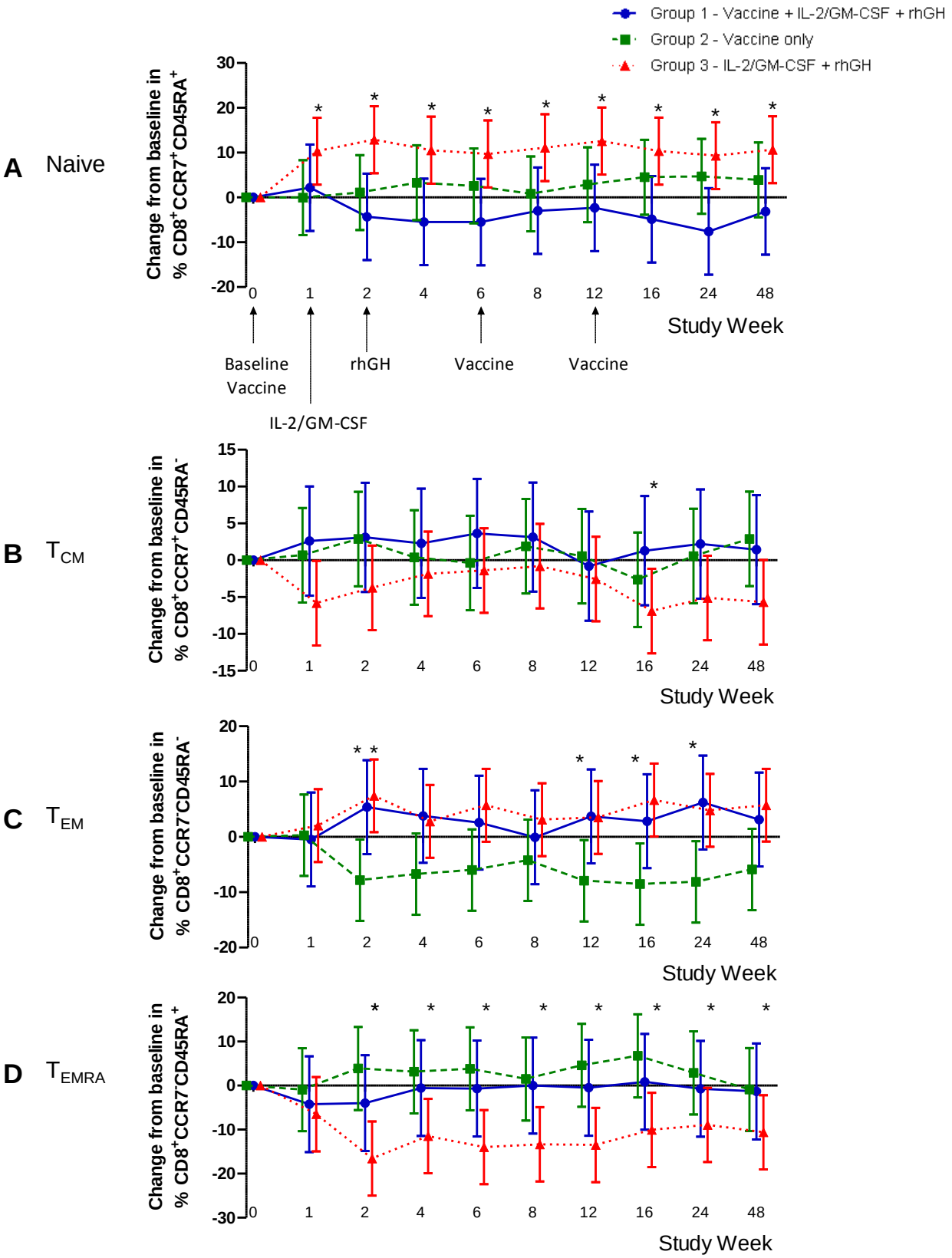


Figure 4.23 Changes from baseline to each of the study time points in CD8 T-cell differentiation, as determined by CCR7 and CD45RA expression.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage expression of markers associated with a naive profile ($CCR7^+CD45RA^+$; A), a T_{CM} profile ($CCR7^+CD45RA^-$; B), a T_{EM} phenotype ($CCR7^-CD45RA^-$; C) and a T_{EMRA} profile ($CCR7^-CD45RA^+$; D). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

PD-1 expression was investigated on each of the CD4 T-cell subsets as determined by CCR7 and CD45RA surface markers. Individuals in group 1 showed a significant reduction in percentage PD-1 expression at week 6 compared to baseline for all CD4 T-cell differentiation states: naïve, T_{CM}, T_{EM} and T_{EMRA} (Figures 4.24A, 4.24B, 4.24C and 4.24D, respectively). By week 48, all study groups, regardless of randomisation and subsequent treatment schedule, showed a significant decrease in the percentage of PD-1 expressed on each of the CD4 T-cell differentiation/maturation subsets (Figure 4.24). Assessment of PD-1 expression on the CD8 T-cell subsets revealed an early reduction of PD-1 on CD8 T_{EM} cells (Figure 4.25C) but by week 48, all patient groups showed a significant decline in the percentage expression of PD-1, regardless of treatment group for each of the differentiation states: naïve, T_{CM}, T_{EM} and T_{EMRA} (Figures 4.25A, 4.25B, 4.25C and 4.25D, respectively).

PD-L1 expression on naïve CD4 T cells for patients in group 2 increased significantly at week 2 with a mean increase from baseline of 6.0% (95% CI 0.8-11.2%, p = 0.0266; Figure 4.26A) with a trend towards an increase at week 2 on CD8 T_{CM} and T_{EMRA} (Figures 4.26B and 4.26D, respectively). Individuals that received cytokine and hormone alone (group 3) showed a significant reduction in percentage PD-L1 expression at week 16 for all CD4 T-cell differentiation states and by week 48, no significant changes compared to baseline were observed for any of the treatment groups (Figure 4.26A-D). PD-L1 expression on the CD8 T-cell subsets for the individuals in group 3 demonstrated a significant increase at week 2 for all subsets but this was normalised by week 48 (Figure 4.27A-D). CD8 T_{EM} for patients in group showed elevated levels of PD-L1 at week 1 following administration of the therapeutic immunisation with a mean increase from baseline of 12.5% (95% CI 1.6-23.4%, p = 0.0270) and this remained increased (but not significantly at week 2) but PD-L1 expression on T_{EM} for group 1 individuals was not significantly different from baseline by week 48 (Figure 4.27C).

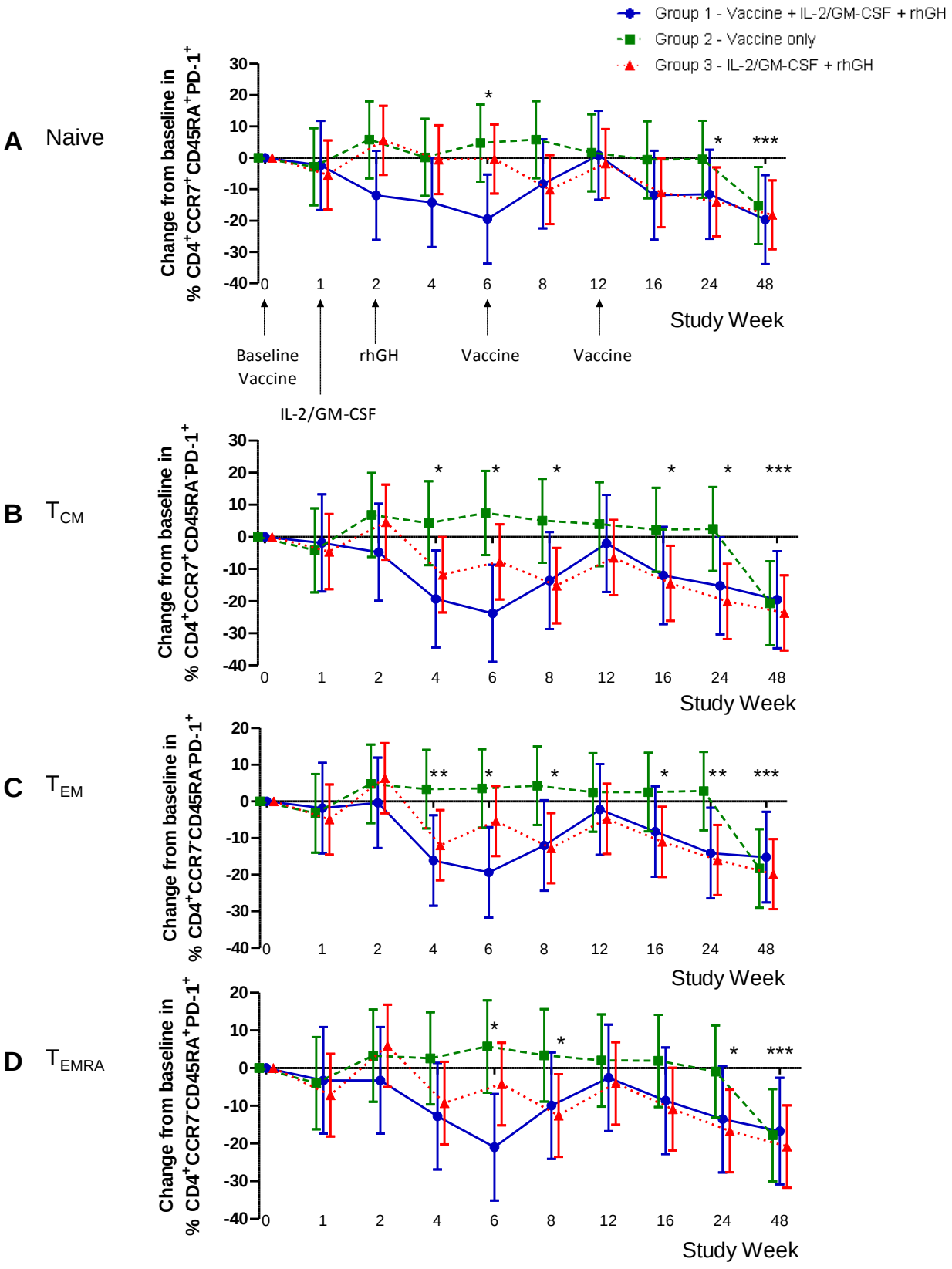


Figure 4.24 Changes from baseline to each of the study time points in PD-1 expression on differentiated CD4 T-cell populations.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage PD-1 expression on naive ($CCR7^+CD45RA^+$; A), T_{CM} ($CCR7^+CD45RA^-$; B), T_{EM} ($CCR7^-CD45RA^-$; C) and T_{EMRA} ($CCR7^-CD45RA^+$; D) subsets. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

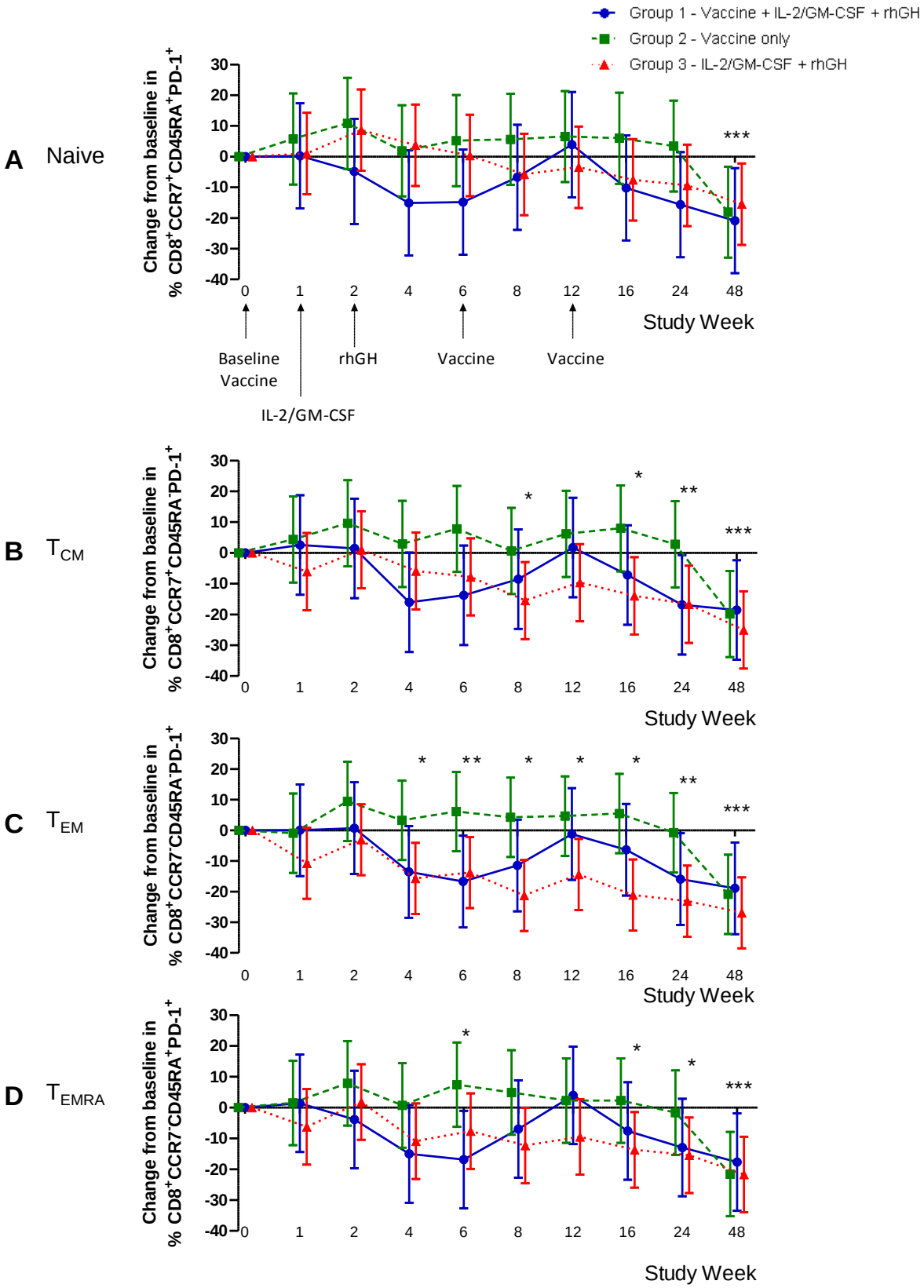


Figure 4.25 Changes from baseline to each of the study time points in PD-1 expression on differentiated CD8 T-cell populations.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage PD-1 expression on naive ($CCR7^+CD45RA^+$; A), T_{CM} ($CCR7^+CD45RA^-$; B), T_{EM} ($CCR7^-CD45RA^-$; C) and T_{EMRA} ($CCR7^-CD45RA^+$; D) subsets. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

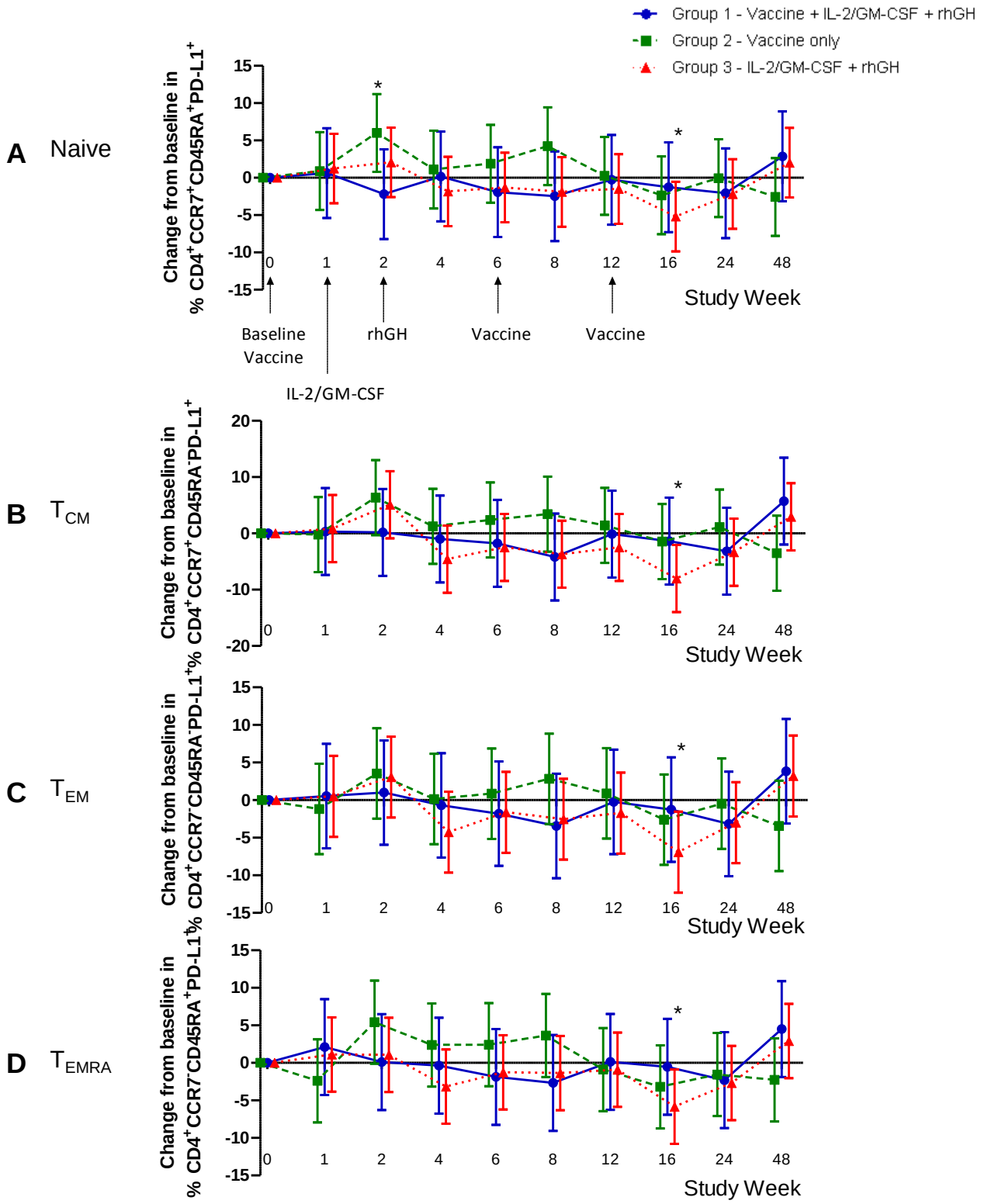


Figure 4.26 Changes from baseline to each of the study time points in PD-L1 expression on differentiated CD4 T-cell populations.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage PD-L1 expression on naive ($CCR7^+CD45RA^+$; A), T_{CM} ($CCR7^+CD45RA^-$; B), T_{EM} ($CCR7^-CD45RA^-$; C) and T_{EMRA} ($CCR7^-CD45RA^+$; D) subsets. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

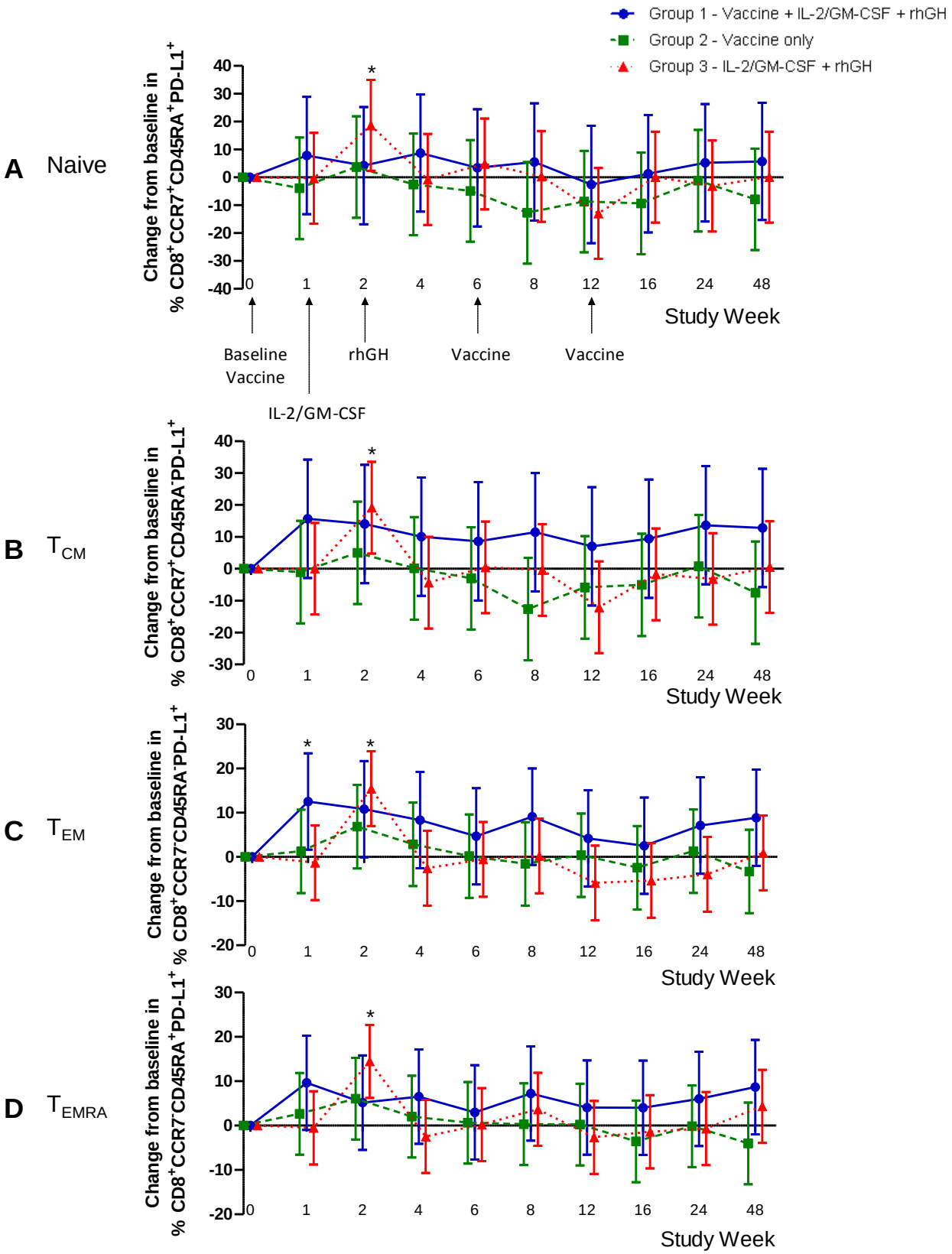


Figure 4.27 Changes from baseline to each of the study time points in PD-L1 expression on differentiated CD8 T-cell populations.
Plots show mean change from baseline with error bars representing a 95% confidence interval. PBMC were assessed for percentage PD-L1 expression on naive ($CCR7^+CD45RA^+$; A), T_{CM} ($CCR7^+CD45RA^-$; B), T_{EM} ($CCR7^-CD45RA^-$; C) and T_{EMRA} ($CCR7^-CD45RA^+$; D) subsets. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

PD-1 expression on the entire CD4 T-lymphocyte population revealed a similar pattern to that observed looking at the individual subsets, with a significant reduction in percentage PD-1 expression observed at week 48 for all treatment groups (Figure 4.28A). PD-L1 expression on the CD4 T cells showed a significant decrease in percentage expression at week 16 for individuals in group 3 with a mean reduction from baseline of -6.6% (95% CI -12.0 – -1.2%, p = 0.0192; Figure 4.28B). The MFI of PD-1 and PD-L1 showed significant increases at week 2 for patients in group 3 following cytokine administration, and significant elevations of PD-L1 at week 16 and week 24 for individuals in groups 1 and 3 (Figures 4.28C and 4.28D, respectively). By week 48, the MFI of PD-1 and PD-L1 were not significantly different to baseline however patients in group 2 showed elevated levels of both the negative regulatory molecule and its ligand. Mean increase from baseline to week 48 in MFI was 123 (95% CI 40-205, p = 0.0046) and 876 (95% CI 349-1403, p = 0.0017) for PD-1 and PD-L1 respectively (Figures 4.28C and 4.28D, respectively).

Percentage PD-1 expression on the whole CD8 T-lymphocyte population was significantly reduced compared to baseline at week 48 for all treatment groups regardless of randomisation (Figure 4.29A). PD-L1 increased in terms of percentage expression at week 2 for patients in group 3 with a mean change from baseline of 18.9% (95% CI 7.8-30.1%, p = 0.0013; Figure 4.29B). The MFI of both PD-1 and PD-L1 was significantly higher than the baseline value for patients in group 2 with a mean increase from baseline of 126 (95% CI 17-235, p = 0.0265) and 833 (95% CI 100-1565, p = 0.0286; Figures 4.29C and 4.29D, respectively).

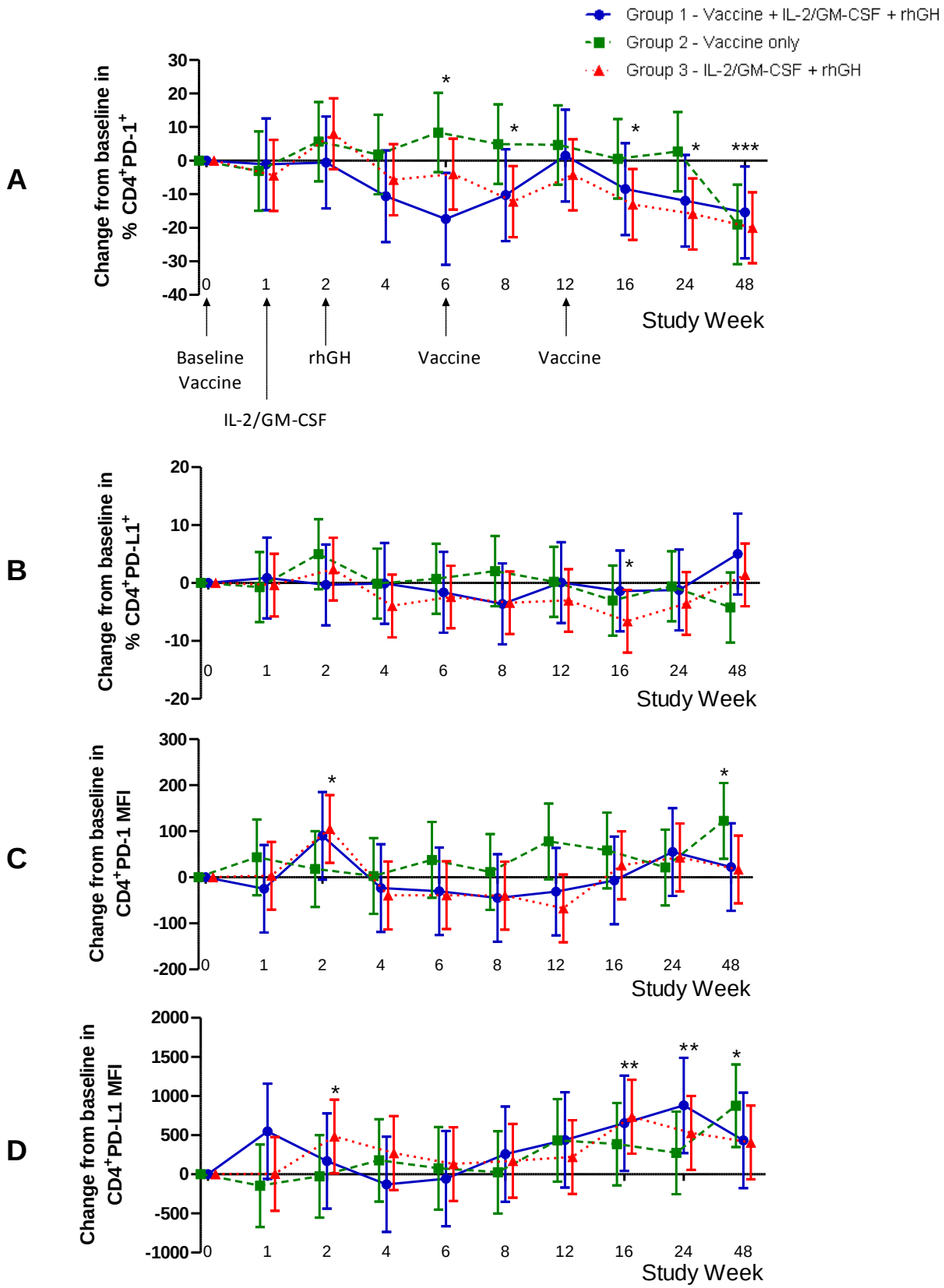


Figure 4.28 Changes from baseline to each of the study time points in PD-1 and PD-L1 expression on CD4 T cells. Plots show mean change from baseline with error bars representing a 95% confidence interval. CD4 T cells were assessed for percentage PD-1 expression (A) as well as MFI (C), and percentage expression and MFI of PD-L1 was also assessed (B and D, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

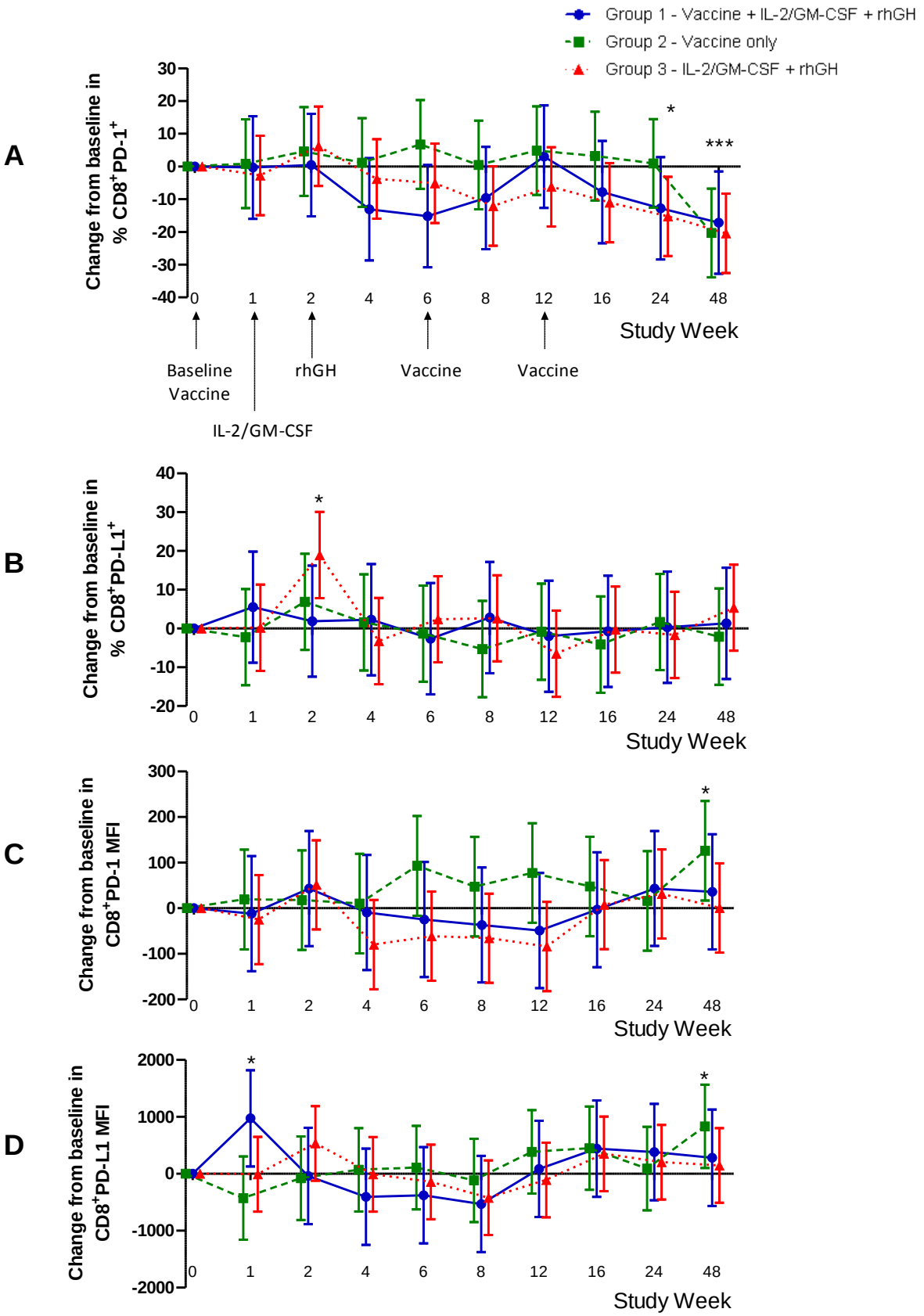


Figure 4.29 Changes from baseline to each of the study time points in PD-1 and PD-L1 expression on CD8 T cells. Plots show mean change from baseline with error bars representing a 95% confidence interval. CD4 T cells were assessed for percentage PD-1 expression (A) as well as MFI (C), and percentage expression and MFI of PD-L1 was also assessed (B and D, respectively). This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Percentage co-expression of CTLA-4 and TIM-3 on CD4 T cells was significantly increased for patients in group 1 at weeks 2, 4 and 6, and reduced for patients in group 2 at weeks 8, 16 and 24. It was only patients in group 3 that showed a significant reduction in the co-expression of these markers at week 48 with a mean decrease from baseline of -0.06% (95% CI -0.1 – -0.01%, p = 0.0153; Figure 4.30A). At week 48, patients that received cytokine and hormone immunotherapy demonstrated significant reductions in the co-expression of these markers on CD8 T lymphocytes at week 48 with mean decreases from baseline of -0.1 (95% CI -0.2 – -0.05%, p = 0.0015) and -0.08% (95% CI -0.1 – -0.02%, p = 0.0126; Figure 4.30B).

Looking at single percentage expression of CTLA-4, there was no change from baseline regardless of randomisation group at week 48 on CD4 or CD8 T lymphocytes, and also for the MFI of CTLA-4 on CD8 T cells at week 48. The MFI of CTLA-4 on the CD4 T cells, however, was significantly reduced compared to baseline for all three groups at week 12 and at the final study time point (week 48). TIM-3 percentage expression at week 48 did not differ significantly from baseline for any of the study groups in the CD4 T-cell compartment but the CD8 T cells showed a trend towards a reduction in the expression of this marker at week 48 for patients in group 3. The MFI of TIM-3 on both CD4 and CD8 T cells was significantly reduced for individuals in group 2 at week 48, with a trend towards a reduction for patients in group 1 at this study time point (graphs not shown but mean change from baseline, 95% CI and p values are given in Appendix 2).

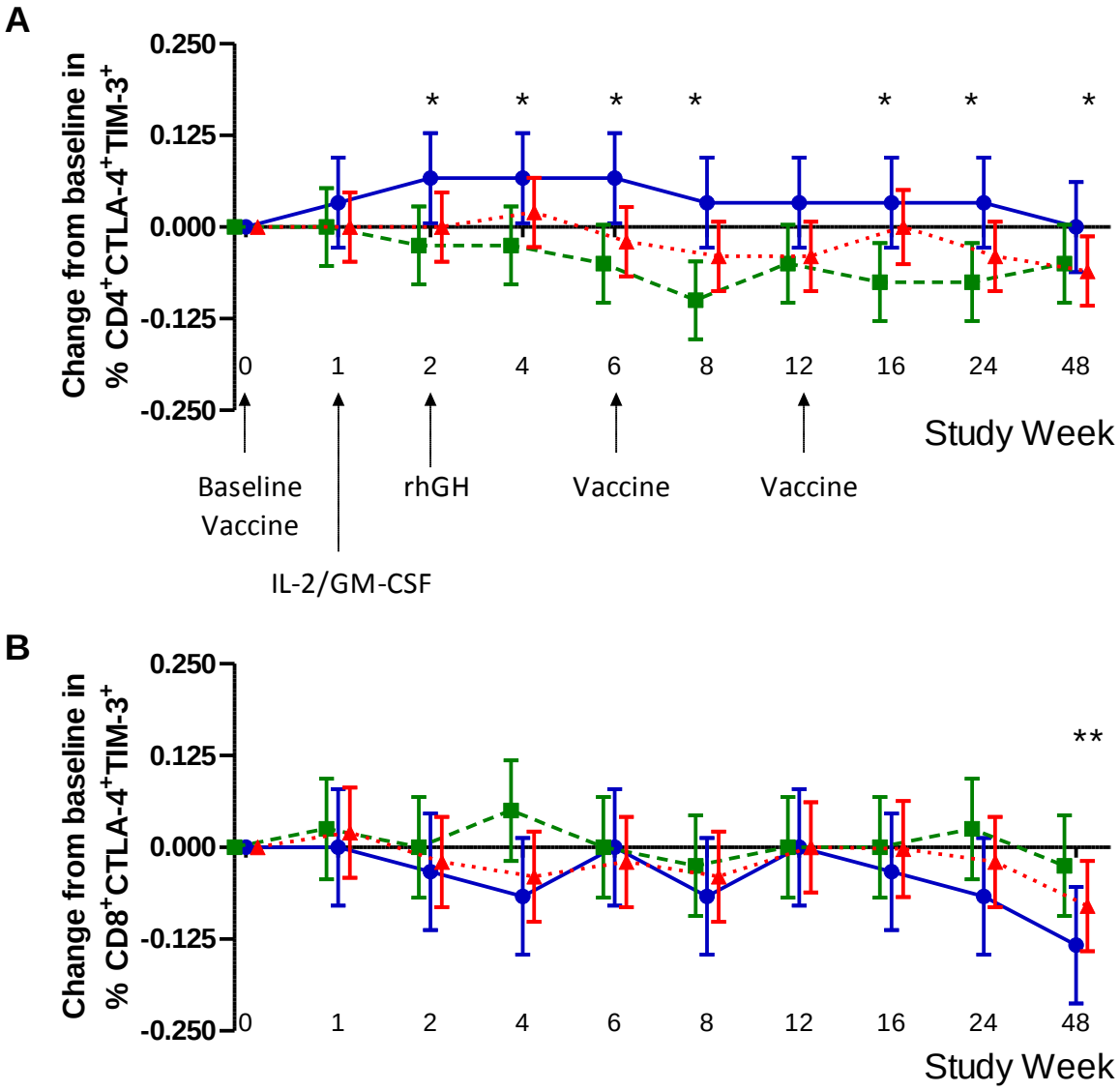


Figure 4.30 Changes from baseline in co-expression of CTLA-4 and TIM-3 on CD4 and CD8 T cells.
Plots show mean change from baseline with error bars representing a 95% confidence interval. CD4 (A) and CD8 (B) T cells were assessed for percentage co-expression of CTLA-4 and TIM-3. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

Following cytokine administration, the percentage co-expression of CD45RA and CD31 on CD4 T cells was significantly reduced at week 2 compared to baseline for patients in groups 1 and 3 (Figure 4.31A). By week 48, only patients in group 1 showed a significant decrease in the expression of these markers on CD4 T cells with a mean change from baseline of -4.7% (95% CI -8.0 – -1.4%, p = 0.0060; Figure 4.31A). Percentage expression of CD31 on CD4 T lymphocytes was significantly reduced for patients in groups 1 and 3 at weeks 2, 6 and 48, while expression for

study participants in group 2 was significantly elevated at weeks 8 and 12 with a mean increase from baseline of 6.5% (95% CI 0.3-12.8%, p = 0.0437) and 10.1% (95% CI 3.9-16.3%, p = 0.0021) for week 8 and week 12, respectively (Figure 4.31B). CD31 percentage expression on CD8 T cells increased significantly at week 2 for patients in group 3 with a mean change from baseline of 10.8% (95% CI 6.1-15.4%, p <0.0001) and decreased marginally but significantly at week 16, but by week 48 there were no changes from baseline in CD31 expression for any of the treatment groups (Figure 4.31C). MFI of CD31 on both CD4 and CD8 T cells from patients in groups 1 and 3 showed significant increases at week 2 following administration of IL-2 and GM-CSF but these were normalised by week 48 (graphs not shown but mean changes from baseline, CI and p values are detailed in Appendix 2). PTK-7 percentage expression on CD4 T cells was significantly reduced at weeks 2, 4, 6, 12, 16, 24 and 48 for patients in group 1, and significantly increased on CD8 T cells at weeks 2 and 6 for patients in the same treatment group. MFI of PTK-7 on CD4 T cells was significantly reduced at week 48 compared to baseline for patients in groups 1 and 3, while there was no change compared to baseline in PTK-7 expression (MFI) on CD8 T cells for patients in any of the treatment groups, although patients in group 3 showed significant increases compared to baseline at weeks 2 and 8 (graphs not shown but mean changes from baseline, CI and p values are detailed in Appendix 2).

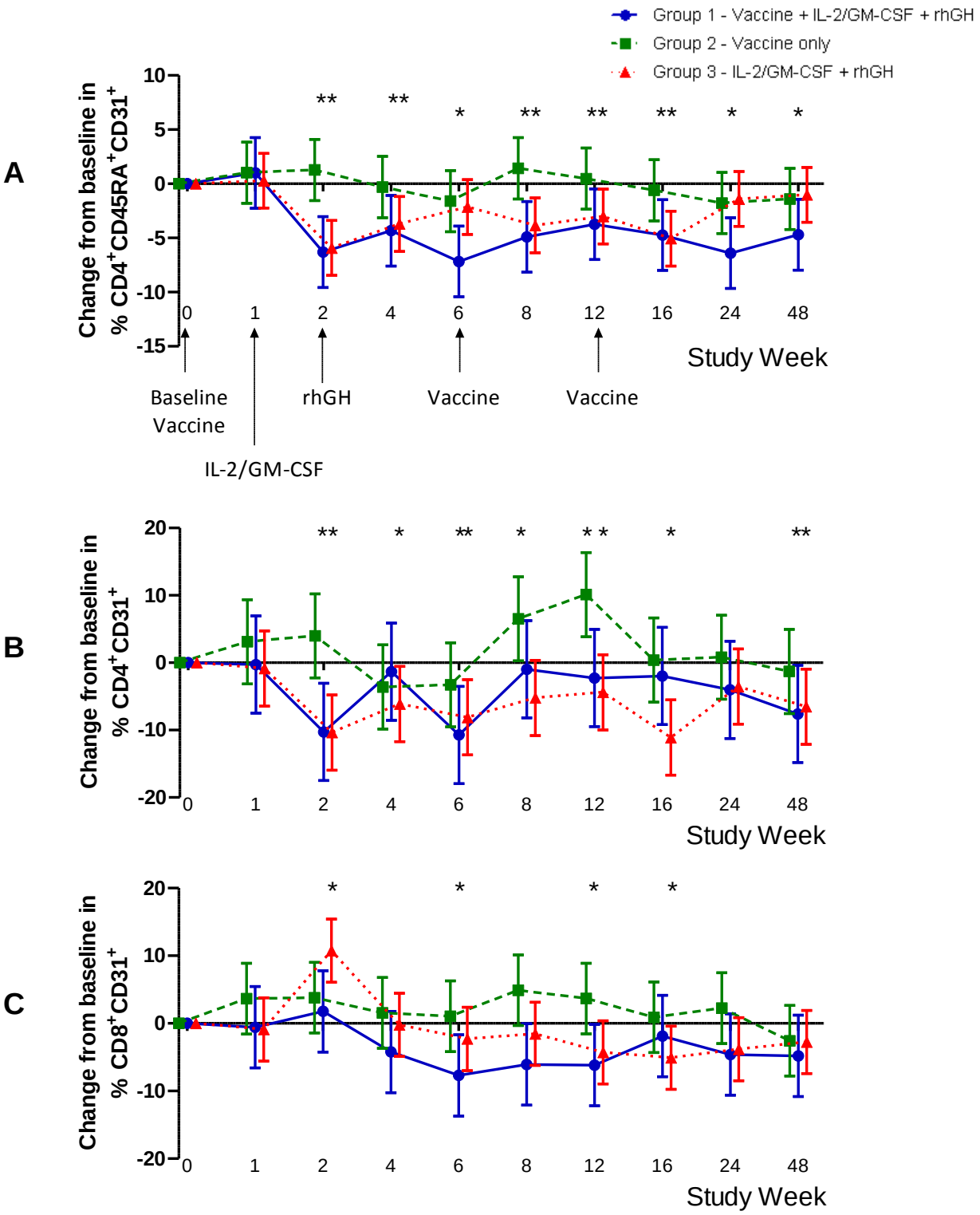


Figure 4.31 Changes from baseline to each of the study time points in CD31 expression.
Plots show mean change from baseline with error bars representing a 95% confidence interval. CD4 T cells were assessed for percentage co-expression of CD45RA and CD31 (A), and percentage expression of CD31 measured on CD4 (B) and CD8 (C) T cells. This was done for each study time point and the treatment groups are as previous: group 1 in blue circles, group 2 in green squares and group 3 in red triangles. Asterisks indicate statistical significance, where $p \leq 0.05$.

4.6 Discussion

It had been intended to recruit 30 patients in total for this study; ten per randomisation arm. However, out of 93 patient referrals and 21 screens, only 12 individuals were enrolled. This discrepancy (between the number screened and number enrolled, and why so few referred patients were screened) was primarily due to individuals not meeting the required nadir CD4 T-cell count of 200 cells/µl blood. In actuality four out of the twelve patients had nadir CD4 T-cell counts of less than the requirement. Two of these were only moderately below (F810 had a nadir CD4 T-cell count of 166 cells/µl blood and C241 had 180 cells/µl blood); these individuals were approved by the recruiting clinician as these were determined to be 'blips' in otherwise stable and high CD4 T-lymphocyte numbers. The other two patients were recruited onto the trial once the enrolment criteria had been amended and nadir CD4 T-cell requirements removed; these were B784 (group 1) and C319 (group 2) with 80 cells/µl and 93 cells/µl blood respectively. All patients on the study met the requirement of greater than 400 cells/µl blood at baseline, and all were aviraemic at baseline. These carefully selected characteristics are of utmost importance in the context of assessing the impact of immune-based therapies in treated HIV-1-infection. CD4 T-cell recovery has been shown to be influenced by the duration of ART-induced aviraemia and nadir CD4 T-lymphocyte counts [Koegl *et al*., 2009], Moreover, nadir CD4 T-cell counts have been found to inversely correlate with levels of HIV-1 proviral DNA in the circulating CD4 T cells of ART-treated HIV-1-infected individuals [Boulassel *et al*., 2012], which implies that the timing of ART initiation and its duration have an effect on immune reconstitution [Cellerai *et al*., 2011]. Earlier initiation of ART and higher nadir CD4 T-cell counts have also been shown to be associated with functional responses to immunisation [Lange *et al*., 2002]. In a pilot study investigating IL-2 administration and immunisation with the therapeutic HIV-1 vaccine Remune™, functional responses were not boosted in ART-treated HIV-1-infected patients that had low pre-ART CD4 T-cell counts [Hardy *et al*., 2007]. This lack of immune recovery was postulated to be due to protracted immunosuppression and/or clonal dysfunction [Hardy *et al*., 2007] and differed from previous Remune™ studies which

demonstrated proliferative responses in patients that had higher pre-ART CD4 T-cell counts [Robbins *et al*., 2003].

In terms of functional responses following administration of the therapeutic DNA vaccine, no apparent increases in functionality were seen at the time points immediately following any of the three immunisations. Administration of IL-2 and GM-CSF following the first immunisation for individuals in group 1 did not boost these responses, to either the NIBSC or FIT Biotech peptide pools. The timing of the immunotherapy was designed based on previous reports which found IL-2 administered following immunisation to be associated with improved antigen-specific responses [Imami *et al*., 2001; Hardy *et al*., 2004]. The clinical parameters for patients in group 2, who received the DNA vaccine alone, did not show any striking changes at any of the study time points. There was a moderate but significant decrease in the percentage of CD8 T cells at week 48, which was also observable for patients in group 1 (who received cytokine and hormone in addition to immunisation).

The delivery route of the immunisation may influence the resulting antigen-specific responses. The FIT Biotech DNA vaccine was delivered via ten intradermal injections (five per arm), whereas another study reported enhanced HIV-1-specific CD8 T-cell responses following immunisation on the skin with DNA plasmids (although this was not associated with viral control during treatment interruption) [Gudmundsdotter *et al*., 2011]. *In vivo* electroporation (EP) delivery of a DNA vaccine was found to significantly improve cellular immune responses in terms of rate, magnitude, duration and breadth of response to a number of antigens [Vasan *et al*., 2011] which concurred with results from animal models that demonstrated improved responses with EP [Hallengard *et al*., 2011].

Patients that received IL-2 and GM-CSF showed elevated levels of CD4 T cells at week two, but only patients who also received vaccine (group 1) demonstrated CD4 T-lymphocyte numbers that were significantly higher than baseline values at week 48, and increased CD4/CD8 T-cell ratios at this time point (these ratios were raised from week 2 of the study). These individuals also demonstrated increased IFN-γ, IL-2 and perforin production in response to the FIT Bitoech peptide

pools at the final study time point (week 48). Increased functionality, and specifically polyfunctionality, is a characteristic attributed to LTNP or HIV controllers [Makedonas and Betts, 2011] and the induction of these responses suggests that immunotherapy may serve as a means of reversing T-cell anergy, in addition to HAART. The increased CD4/CD8 T-cell ratios may reflect improved immune reconstitution, and be influenced in part by the increased CD4 T-lymphocyte numbers in these patients, but studies have suggested that CD4/CD8 T-cell ratios in patients on suppressive ART negatively correlate with HIV-1 proviral DNA levels [Chun *et al*., 2002; Boulassel *et al*., 2012] which proposes a role for immunotherapy in the depletion of the viral reservoir. It has been suggested that ART leads to a reduction in the latent viral reservoir, and in one study, decreased levels of HIV-1 proviral DNA were seen in HAART-treated individuals (these were still higher than, but not significantly different from, levels seen in elite controllers) [Julg *et al*., 2010]. In another study, early initiation of ART resulted in levels of proviral DNA similar to those found in LTNP [Pires *et al*., 2004a]. Thus, for future work, it would be of added benefit to carry out HIV-1 proviral DNA analysis on cryopreserved samples from these twelve individuals, at baseline and at week 48, to determine the impact of immunotherapy on this parameter.

The expression of markers associated with a regulatory T-cell phenotype and those linked to immune activation were both significantly increased at week 2 for patients that had received IL-2 and GM-CSF. The administration of IL-2 to patients on ART has been shown to induce regulatory T-cell populations [Ndhlovu *et al*., 2010]. A previous study suggested a role for T-regulatory cells in controlling residual immune activation and/or spontaneous T-cell proliferation *in vivo* for patients on suppressive ART [Weiss *et al*., 2010], which may explain why both of these T-cell subsets were found to be increased at this time point and why there was no increase in plasma viraemia. Only by taking samples at more time points subsequent to cytokine administration would it be possible to elucidate whether an initial rise in T-cell activation was followed by this rise in Treg. Reports on this particular T-cell subset in the context of HIV-1 infection have been varied; the levels of regulatory T cells have been found to be unaffected, elevated and decreased with regard to disease

progression [Weiss *et al*., 2010], although a study which found Treg in the periperhal blood of elite suppressors to be preserved indicates a beneficial role for Treg in HIV-1^{+} patients that control viral replication [Chase *et al*., 2008]. It is important to note that in these studies, markers used to assess the Treg population have differed and although intracellular FoxP3 staining was not carried out, the $CD3^{+}CD4^{+}CD45RO^{+}CD25^{high}$ phenotype has been found to correlate with FoxP3 expression in HIV-1-infected individuals and in healthy seronegative donors [Burton *et al*., 2011]. Chronic immune activation has been associated with disease progression and although the expression of HLA-DR and CD38 was lowered with ART (compared to untreated HIV-1 infection) [Bartovska *et al*., 2011], abnormal T-cell activation persists even in patients on suppressive ART [Hunt *et al*., 2003; Hunt *et al*., 2008]. In the present study, treatment with IL-2, GM-CSF and rhGH (with or without therapeutic immunisation) significantly reduced the expression of CD38 on CD4 T cells (both in terms of percentage expression and MFI) at week 48 compared to baseline, and at this time point, patients in group 3 (that received cytokine and hormone therapy alone) showed a reduction in the percentage expression of CD38 on CD8 T cells, with a trend towards a reduction for patients that also received the DNA vaccine. These findings suggest that immune-based therapy in the context of treated HIV-1 infection may serve to further reverse T-cell dysfunction, and to this effect, GH therapy in addition to ART has been shown to reduce T-cell activation [Napolitano *et al*., 2008]. Treatment with valganciclovir was found to reduce CD8 T-cell activation in patients with CMV co-infection and incomplete CD4 T-cell recovery on ART [Hunt *et al*., 2011]. Coupled with this decrease in immune activation, reductions in markers associated with immune senescence and apoptosis were seen at the final study time point (week 48), particularly for patients that received IL-2, GM-CSF and rhGH alone. One study found that treated HIV-1-infected adults had a $CD8^{+}CD57^{+}CD28^{-}$ phenotype that was similar to much older uninfected controls [Desai & Landay, 2010], suggesting a role for immunotherapy in the reversal of immune senescence and the premature aging that is seen in chronic, treated HIV-1 infection [Deeks, 2011].

The expression of PD-1 on both CD4 and CD8 T cells was found to be reduced at week 48 compared to baseline for all treatment groups regardless of randomisation, which may be an effect of prolonged HAART. A study by Kassu *et al*., found the percentage of HIV-1-specific $CD4^{+}IFN\text{-}\gamma^{+}PD\text{-}1^{+}$ T cells to be significantly lower in ART treated patients compared to untreated viraemic subjects [Kassu *et al*., 2010] and PD-1 has even been proposed as a marker to identify individuals on long-term suppressive ART for whom immune reconstitution is incomplete [Grabmeier-Pfisterhammer *et al*., 2011]. Co-expression of CTLA-4 and TIM-3 was significantly reduced at week 48 compared to baseline for patients that had received IL-2, GM-CSF and rhGH, suggesting a benefit of immunotherapy for treated HIV-1-infected patients over the reduction seen in these markers with suppressive ART alone [Kassu *et al*., 2010].

Assessment of T-cell differentiation/maturation showed that patients in group 1 (who received IL-2, GM-CSF and rhGH in addition to therapeutic immunisation) had fewer naïve CD4 T cells at week 48 (as measured by the co-expression of CCR7 and CD45RA, and single expression of CD31) compared to baseline, but that the number of T_{CM} at this time point was increased (although not significantly). Chronic progressive HIV-1 infection has been shown to result in depletion of the naïve and T_{CM} cells and accumulation of T_{EM} and T_{EMRA} (for the CD8 T-cell compartment) [Ladell *et al*., 2008]. To this effect, patients in group 3 (that received cytokine and hormone alone), demonstrated a significant reduction in CD8 T_{EMRA} from week 2 of the study, and a significant increase in the expression of markers associated with a naïve CD8 T-cell phenotype from week 1, and both of these phenotypic changes were maintained at week 48 of the study.

The primary endpoint of this phase I study was to determine the safety profile of this treatment schedule and overall, it was found to be safe and well-tolerated. The majority of beneficial clinical and immunological parameters were observed in patients that received IL-2, GM-CSF and rhGH, with or without therapeutic immunisation. Due to the complexities of the treatment schedule, there was no placebo group but the changes from baseline give a clear representation of the benefits of immunotherapy in addition to suppressive ART. rhGH has been shown to induce T-cell

thymopoeisis and improve T-cell functionalitiy [Napolitano *et al*., 2008; Herasimtschuk *et al*., 2008] but here, the beneficial effects of rhGH were either masked due to prior administration of IL-2 and GM-CSF, or it is possible that a longer duration of rhGH treatment is needed for immunological improvements to be seen. The results from the SILCAAT and ESPRIT studies found no clinical benefit when IL-2 was administered in addition to ART in HIV-1-infected patients, despite substantial and maintained increases in CD4 T-lymphocyte counts [Abrams *et al*., 2009], while another study described no effect on the proportion of CD4 T cells in the gut mucosa of patients treated with IL-2 in addition to ART compared to ART alone [Read *et al*., 2011]. However, this does not rule out IL-2 as a candidate molecule to be used in conjunction with other immunomodulators. Combination IL-2 and GM-CSF immunotherapy was associated with improved antigen-specific T-cell responses and clinical recovery in patients with advanced HIV-1 infection presenting with mycobacterial immune reconstitution inflammatory syndrome [Pires *et al*., 2005].

The focus of future work investigating immune-based therapeutic approaches in treated HIV-1 infection will be to find the optimal therapeutic strategy that will not only induce, but maintain, increased T-cell functionality, improve immunophenotypes and result in beneficial clinical characteristics.

Chapter 5 Functional and phenotypic assessment of T-cell anergy in HIV-1 infection

T-cell dysfunction is a well-known characteristic of HIV-1 infection. Markers of chronic immune activation, exhaustion and senescence are up-regulated and differentiation/maturation pathways are skewed, particularly in acute infection [Papagno *et al*., 2004; Day *et al*., 2006; Rosignoli *et al*., 2009; Jones *et al*., 2008; Garber *et al*., 2004, Appay *et al*., 2008]. Treatment with ART has been found to reduce the expression of markers associated with immune activation [Shou *et al*., 2011] and reverse disrupted maturation states, especially when initiated in early HIV-1 infection [Bisset et al., 1998], as well as lowering the expression of co-inhibitory and exhaustion markers [Kassu *et al*., 2010]. However, other reports suggest that levels of peripheral T-cell activation are not normalised with ART [Rueda *et al*., 2012], nor are the expression levels of markers such as PD-L1 [Rosignoli *et al*., 2007] and CTLA-4 [Rueda *et al*., 2012]. The functionality of PBMC from HIV-1^{+} patients on ART is important to consider in addition to immunophenotype. Increased polyfunctionality has been described in LNTP; they demonstrated a greater proportion of HIV-1-specific CD8 T cells that were able to perform five functions simultaneously (including the secretion of IL-2, IFN-γ, TNF-α, CD107a and MIP-1β) compared to chronically infected individuals [Betts *et al*., 2006]. Furthermore, HIV-1-specific CD8 T cells from LTNP were found to be more cytotoxic against autologous HIV-1-infected CD4 T cells, and to have a greater proliferative capacity than CD8 T lymphocytes from chronic progressors [Migueles *et al*., 2008; Horton *et al*., 2006].

It is important to consider the frequency with which the protective MHC class I alleles (HLA-B*27, B*57, B*5801) are represented in these LTNP populations and how this might influence the quality of the CD8 T-cell response; these HLA types have been associated with robust HIV-1-specific CD8 T-lymphocyte responses and viral control [Migueles *et al*., 2000; O'Brien *et al*., 2001; Kiepiela *et al*., 2004; Tang *et al*., 2010; Xie *et al*., 2010]. Investigations into CD8 T-cell antigen specificity have revealed Gag-specific T cells to be more protective [Kiepiela *et al*., 2007; van Baalen *et al*.,

1997; Lichterfeld *et al.*, 2004] with higher cell avidity, polyfunctionality and clonal turnover described for Gag-specific CD8 T cells from HLA-B*27^{+} individuals [Almeida *et al*., 2007]. Whereas HLA-B*27 only presents an immunodominant epitope in Gag, but not Nef, to CD8 T cells, HLA-B*57/5801 present a number of Gag and Nef epitopes [Altfeld *et al*., 2006; Bailey *et al*., 2009]. In a study by Xie *et al*., IFN-γ-producing Gag-specific CD8 T cells from HLA-B*57/5801^{+} patients had a higher proportion of CD27^{+} central memory cells compared to Nef-specific CD8 T lymphocytes from the same individuals [Xie *et al*., 2010]. Moreover, it was only the Gag-specific CD27^{+} central memory cells from these individuals with the protective HLA allele that showed a negative correlation with cell-associated HIV-1 DNA, suggesting that better control can be conferred by Gag-specific T cells [Xie *et al*., 2010]. Another group reported HIV-1 controllers to have higher functional avidities of their Gag-specific and HLA-B-restricted responses, compared to non-controllers; and these responses were not restored following the initiation of ART in HIV-1 non-controllers [Berger *et al*., 2011]. Other studies have reinforced the idea that Gag-specific responses are better able to control viral replication [Kiepiela *et al*., 2007; Mendiratta *et al*., 2011] but Nef-specifity has been shown to be maintained in HLA-B*57/B*5801 non progressors [Navis *et al*., 2008], and a population of Nef-specific effector CD8 T cells (CD8^{+}CD45RA^{+}IFN-γ^{-}MIP-1β^{+}) was shown to be associated with non-progressive HIV-1 infection [Dembek *et al*., 2010].

This chapter investigates the immunophenotypic differences between PBMC from HIV-1-infected individuals and healthy seronegative donors. From a subset of HIV-1^{+} patients with carefully matched characteristics (undetectable plasma viraemia and high CD4 T-cell counts), the phenotype of functionally defined Gag- and/or Nef- specific responders is characterised. Again, comparisons are made to samples from healthy, uninfected controls. Finally, using data from all of the patients described in this chapter, correlations are drawn between CD4 T-cell count and the surface expression of markers associated with immune dysregulation, as well as investigating other parameters, such as the influence of the nadir CD4 T-lymphocyte count and the length of time on ART on discordant immunophenotypes.

5.1 T-cell phenotype in HIV-1$^+$ persons compared to seronegative donors

The phenotype of CD4 and CD8 T cells from HIV-1$^+$ (n = 24) and HIV-1$^-$ (n = 13) donors was compared using the panel of antibodies described in section 2.12, Table 2.1 and patient characteristics are displayed in Table 5.1. Due to limitations with blood volumes, not all subjects were analysed using all antibody panels, and the number of samples run for each experiment can be found in the figure legends. Statistical analysis was carried out using the Mann-Whitney U Test and p values are shown where there was a statistically significant difference between the medians of the two groups ($p \leq 0.05$).

5.1.1 Patient characteristics

The majority of patients were male (23/24) with a median age of 47 years (IQR 41-51 years), a median CD4 T-cell count of 434 cells/µl blood (IQR 247-619 cells/µl blood) and a median nadir CD4 T-lymphocyte count of 122 cells/µl blood (IQR 53-211 cells/µl blood; Table 5.1). Only three subjects had detectable viraemia (N374, B887 and S803 with 81, 106 and 2734 HIV-1 RNA copies/ml plasma, respectively) and the rest had HIV-1 RNA of <50 copies/ml plasma. The median duration of ART was 50.33 months (IQR 7.97-110.10 months) and the median duration of HIV-1 infection was 153.21 months (IQR 72.95-171.64 months). Only three of the twenty four patients were not on ART therapy (two with HIV-1 RNA <50 copies/ml plasma) and they are indicated with an asterisk in Table 5.1. Of the subjects in the table, PBMC from 23 were run for each of the three phenotypic panels and all were the same between panels except for the two patients at the end; J565 was used for assessment of T-cell differentiation subsets using CCR7 and CD45RA and W309 was used for panels looking at activation, senescence and apoptosis, as well as CD27 and CD28 expression (as per panel in section 2.12, Table 2.1). This was due to limitations in the blood volumes available from these particular patients.

The healthy control subjects had a median age of 29 years (IQR 26-32 years), with near even numbers of males and females (n = 7 and n = 6, respectively). The number of these donors tested for each of the phenotype panels is indicated in the figure legends and range from 9-13.

Table 5.1 HIV-1^+ patient characteristics.
Patients are indicated by their short code, and all patients were on ART except those marked with an asterisk (*). The top panel shows information for healthy seronegative control subjects. NA – not applicable, ND – not done, NK – not known.

Patient short code	Age (years)	Gender	Viral load (copies/ml)	Duration of ART (months)	ART regimen	CD4 T-cell count (cells/µl blood)	Nadir CD4 T-cell count (cells/µl blood)	Duration of HIV-1 infection (months)
Healthy donors (n = 13)	Median: 29 IQR: 26-32	6F 7M	NA	NA	NA	ND	NA	NA
B732*	51	M	<50	NA	NA	554	418	36.66
C806	48	M	<50	159.84	ABC+3TC+TFV+NVP	301	3	179.21
W693	37	M	<50	29.84	FTC+TFV+EFV	526	203	49.77
K560	51	M	<50	128.33	ABC+3TC+SQV+RTV	617	113	135.77
N374	45	M	81	17.15	TFV+3TC+NVP	1039	733	229.28
Z171	38	M	<50	64.66	FTC+TFV+EFV	641	182	65.41
J567	57	M	<50	152.56	FTC+TFV+RTV+LOP+RTV	625	27	153.21
L061	44	M	<50	130.03	FTC+TFV+ATZ+RTV	218	48	164.07
W080	41	M	<50	7.97	FTC+TFV+NVP	250	133	NK
C828	43	M	<50	50.33	FTC+TFV+ATZ+RTV	664	235	80.49
E760	46	M	<50	87.61	FTC+TFV+RFV	513	64	87.34
G201	51	M	<50	144.56	ABC+3TC+NVP	975	7	222.16
B671	47	F	<50	4.66	ABC+3TC+EFV	150	58	115.34
B887	33	M	106	0.75	FTC+TFV+EFV	474	55	21.93
H202	41	M	<50	37.11	FTC+TFV+DRV+RTV	144	120	217.48
H850	45	M	<50	60.98	FTC+TFV+DRV+RTV	206	22	163.21
K087	28	M	<50	4.30	FTC+TFV+DRV+RTV	367	235	NK
M569	48	M	<50	15.02	FTC+TFV+EFV	400	306	45.05
S819	59	M	<50	5.08	FTC+TFV+EFV	303	19	NK
S881	53	M	<50	0.10	NK	583	233	163.80
T215	62	M	<50	76.56	TFV+3TC+EFV	433	123	246.82
S803*	39	M	2734	NA	NA	238	156	NK
J565	49	M	<50	110.10	NVP+ABC+3TC	1489	160	164.07
W309*	62	M	<50	NA	NA	228	110	NK
Median	47			50.33		434	122	153.21
IQR	41-51			7.97-110.10		247-619	53-211	72.95-171.64

5.1.2 Immunophenotype

Percentage co-expression of the activation markers HLA-DR and CD38 on CD4 and CD8 T cells was significantly higher in HIV-1^+ individuals compared to seronegative donors ($p < 0.0001$ in both cases; Figures 5.1A and 5.1B, respectively). Looking at percentage of single positive cells with these markers, HLA-DR was significantly higher in HIV-1-infected persons compared to healthy controls on both T-cell subsets ($p < 0.0001$ for both; Figure 5.1C and 5.1D, respectively) whereas no statistically significant difference was seen in percentage expression of CD38 (Figures 5.1E and 5.1F).

Evaluation of the senescence marker CD57 and the apoptotic marker CD95 on CD4 and CD8 T cells from HIV-1^+ patients and seronegative donors showed significantly higher percentage expression of double positive ($CD57^+CD95^+$) CD4 and CD8 T cells in HIV-1-infected individuals compared to healthy donors ($p = 0.0003$ and $p = 0.0001$ for CD4 and CD8 T lymphocytes; Figures 5.2A and 5.2B, respectively). For single expression of CD57, by both T-cell subsets, levels are significantly higher on PBMC from HIV-1^+ individuals compared to healthy controls ($p = 0.0006$ and $p = 0.0001$ for CD4 and CD8 T cells; Figures 5.2C and 5.2D, respectively). Single expression of CD95 was also significantly elevated in HIV-1^+ patients compared to seronegative donors for both T-lymphocyte subsets ($p = 0.0013$ and $p<0.0001$; Figures 5.2E and 5.2F for CD4 and CD8 T cells, respectively).

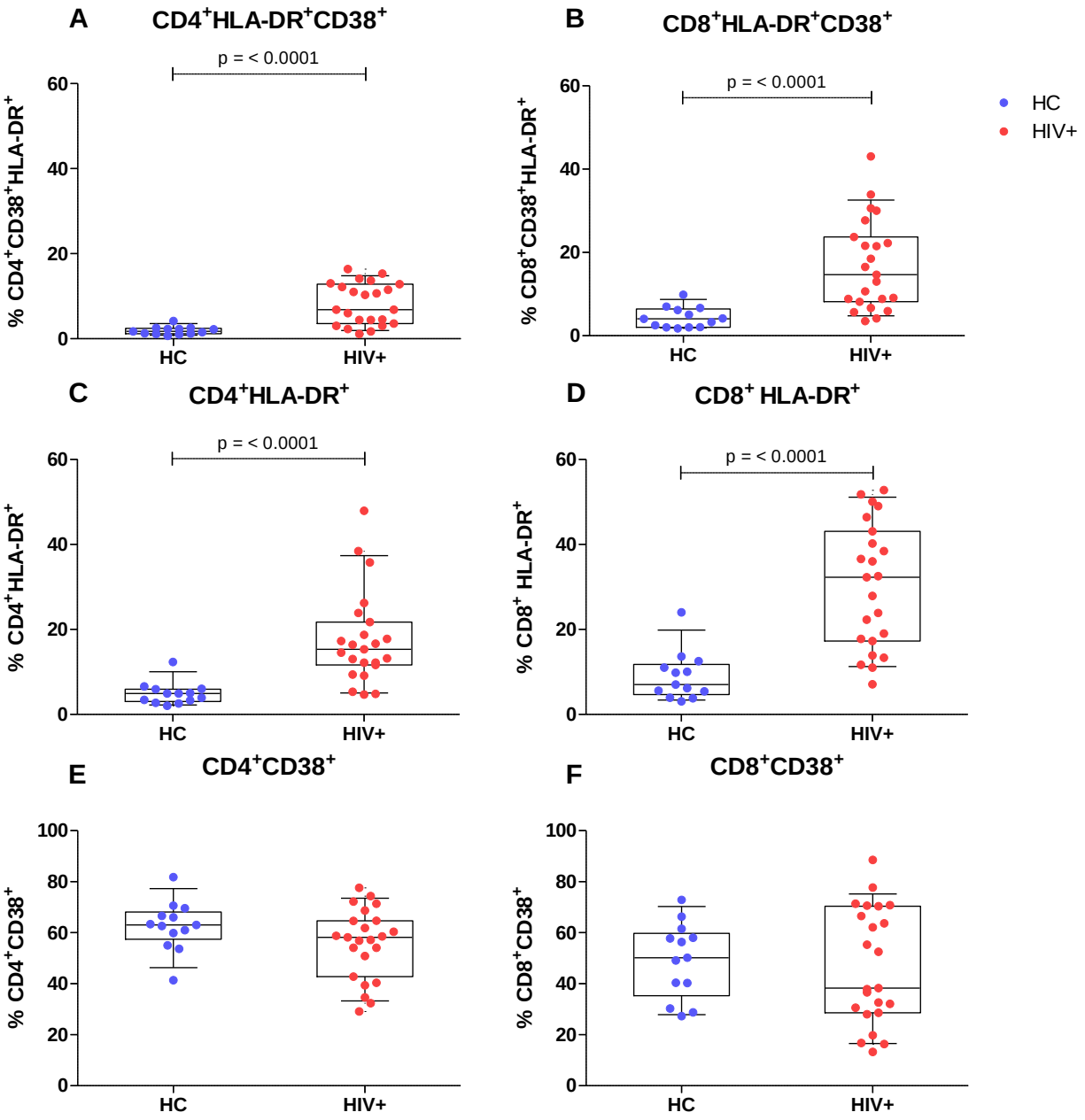


Figure 5.1 CD4 and CD8 T-cell activation in HIV-1$^+$ individuals and healthy donors.
Percentage co-expression of HLA-DR and CD38 was determined on CD4 and CD8 T cells (A and B, respectively) from seronegative controls (n = 13; depicted with blue circles) and HIV-1-infected persons (n = 23; depicted with red circles). Percentage expression of HLA-DR on CD4 and CD8 T cells was also assessed (C and D) as was the percentage expression of CD38 on both subsets (E and F). All individual data points are shown with box plots representing the median and IQR, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the groups.

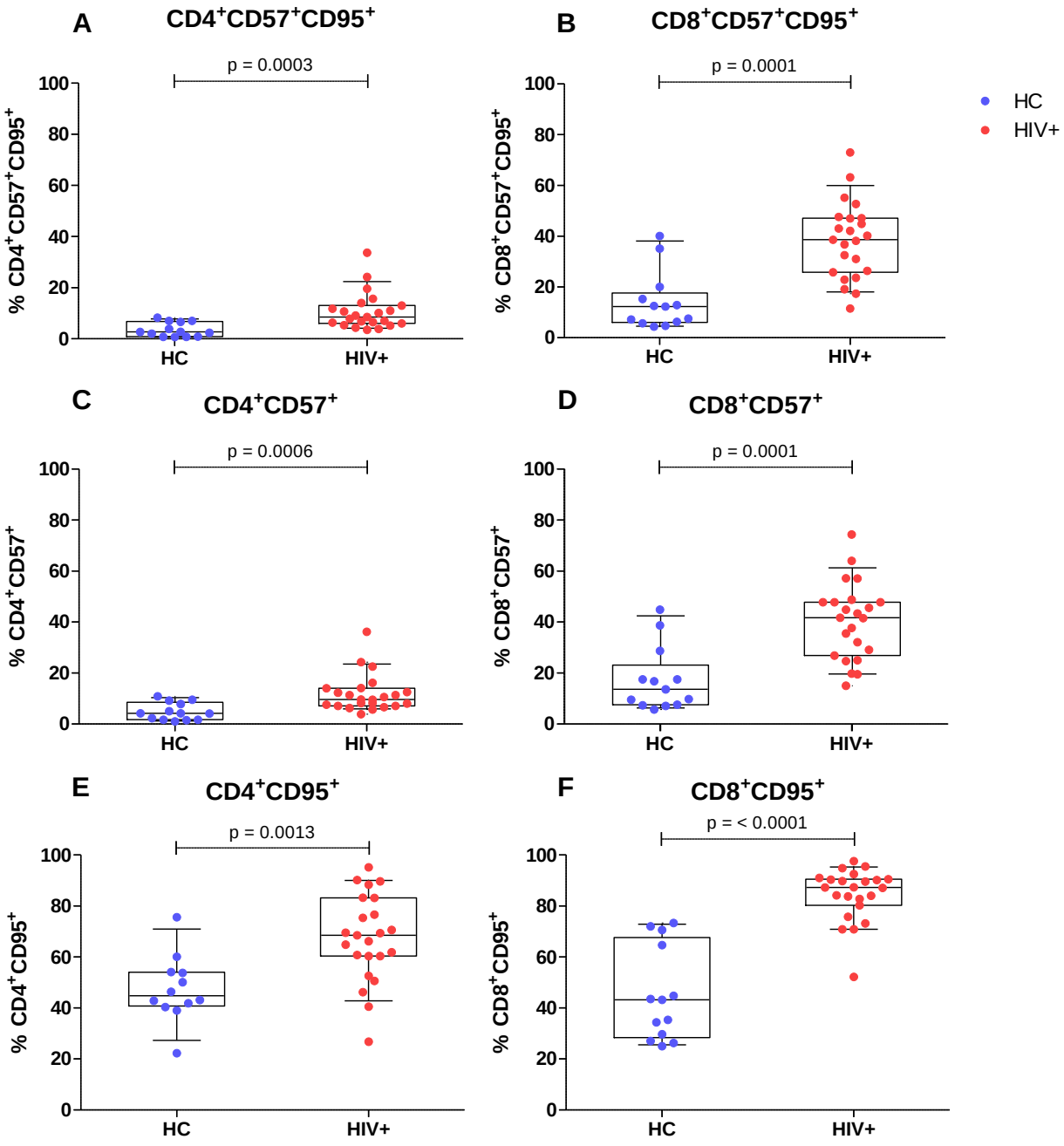


Figure 5.2 CD4 and CD8 T-cell senescence and apoptosis in HIV-1^{+} individuals and healthy donors.
Percentage co-expression of CD57 and CD95 was determined on CD4 and CD8 T cells (A and B, respectively) from seronegative controls (n = 13; depicted with blue circles) and HIV-1-infected persons (n = 23; depicted with red circles). Percentage expression of CD57 on CD4 and CD8 T cells was also assessed (C and D) as was the percentage expression of CD95 on both subsets (E and F). All individual data points are shown with box plots representing the median and IQR, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the groups.

Early, intermediate and late differentiation states of CD4 and CD8 T cells were determined using percentage expression of CD27 and CD28, with early T cells defined as double positives ($CD27^+CD28^+$), intermediate T cells as $CD4^+CD27^-CD28^+$ or $CD8^+CD27^+CD28^-$, and late as double negatives ($CD27^-CD28^-$). For the CD4 T-lymphocyte compartment, HIV-1^+ persons had significantly lower expression of markers associated with an early T-cell phenotype and significantly higher expression of markers associated with intermediate and late differentiation states ($p = 0.0159$, $p = 0.0022$ and $p = 0.0105$, respectively; Figure 5.3A). CD8 T cells showed significantly higher percentage expression of phenotypically defined late T cells in HIV-1-infected patients compared to healthy controls ($p = 0.0159$; Figure 5.3B).

CD4 and CD8 T-cell differentiation/maturation was also assessed using the expression of CCR7 and CD45RA. CD4 and CD8 T cells from HIV-1^+ patients had significantly lower expression of markers associated with naïve T cells ($p = 0.0057$ and $p = 0.0005$; Figures 5.4A and 5.4B, respectively). Similarly, for both T-cell subsets, HIV-1-infected persons had higher expression of markers associated with terminally differentiated phenotype (T_{EMRA}; $p = 0.0420$ and $p = 0.0011$; Figures 5.4A and 5.4B, respectively).

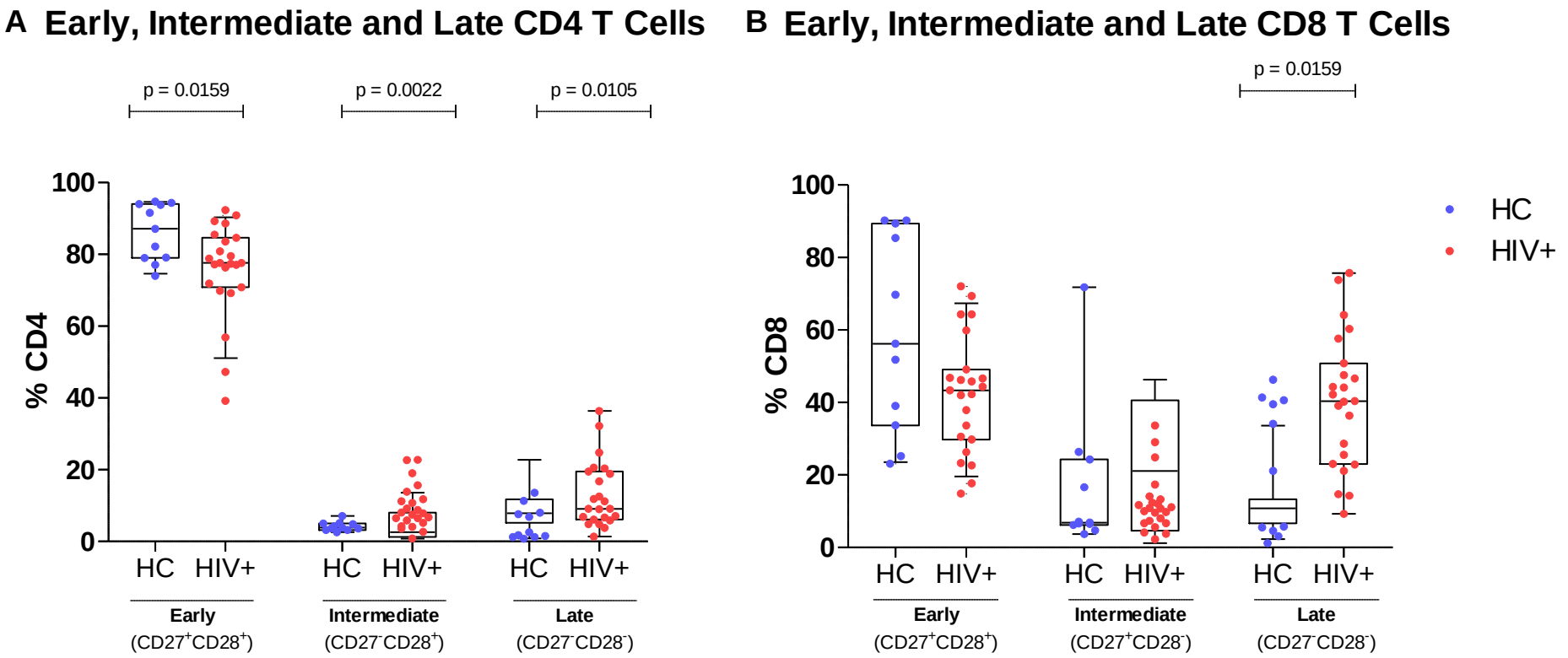


Figure 5.3 Early, intermediate and late CD4 and CD8 T-cell subsets in healthy control and HIV-1-infected individuals.
Expression of CD27 and CD28 was used to determine early ($CD27^+CD28^+$), intermediate ($CD4^+CD27^-CD28^+$ or $CD8^+CD27^+CD28^-$) and late ($CD27^-CD28^-$) CD4 (A) and CD8 (B) T cells in healthy control individuals (n = 11; depicted with blue circles) and HIV-1^+ individuals (n = 23; red circles). Box plots show the median and IQR and whiskers represent the 10^{th} and 90^{th} percentiles. P values are shown where there is a statistically significant difference between the two groups.

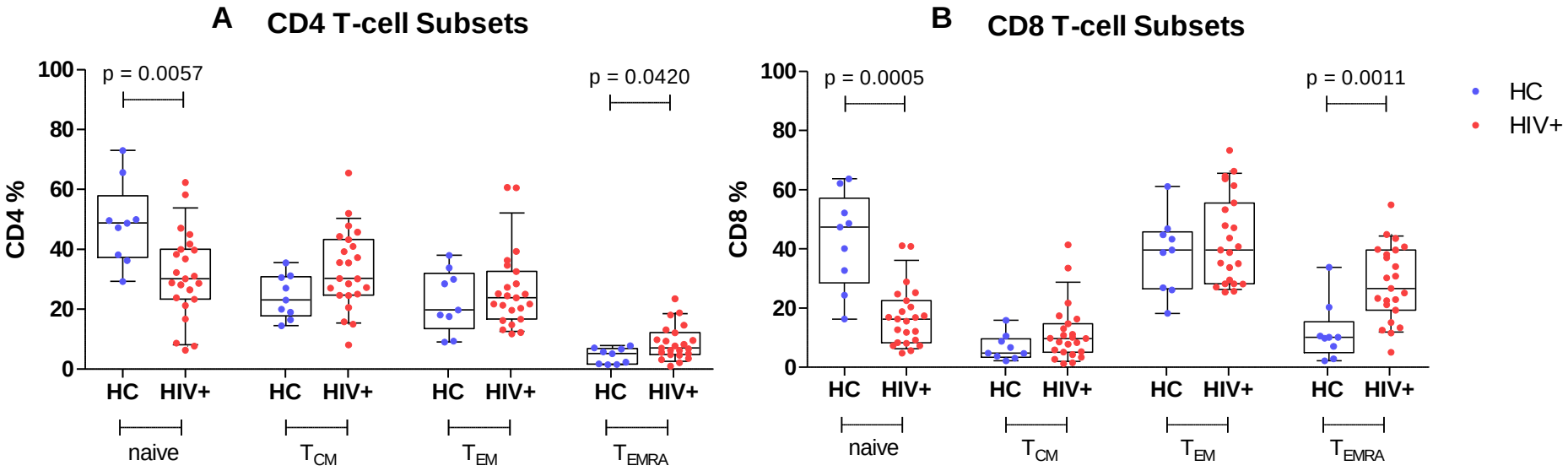


Figure 5.4 CD4 and CD8 T-cell differentiation in healthy donors and HIV-1^+ individuals.
Subsets of CD4 (A) and CD8 (B) T-cell differentiation were determined using surface expression of CCR7 and CD45RA. Naive ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$) T_{EM} ($CCR7^-CD45RA^-$) and T_{EMRA} ($CCR7^-CD45RA^+$) were defined in healthy control individuals (n = 9; shown here with blue circles) and HIV-1-infected patients (n = 23; red circles). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there is a statistically significant difference between the two groups.

5.2 Distinct HIV-1 Gag- or Nef- specific responses and their correlations with altered immunophenotypic profiles

5.2.1 Patient characteristics

The clinical characteristics of HIV-1-infected individuals (n = 17) and healthy controls (n = 11) used in this section are shown in Table 5.2. The majority of the HIV-1^+ subjects were male (16/17) with a median age of 47 years (IQR 37-50 years), a median CD4 T-cell count of 731 cells/µl blood (IQR 534-884 cells/µl blood), a median nadir CD4 T-lymphocyte count of 210 cells/µl blood (IQR 180-284 cells/µl blood) and all patients had <50 copies/ml plasma HIV-1 RNA. The median duration of HIV-1 infection was 142.92 months (IQR 90.40-197.48 months) and all individuals had been on ART for a median duration of 84.79 months (IQR 45.87-139.05 months). Table 5.2 also details the responder statuses of the subjects tested, the classification of which is described in section 5.2.2, and the symbols used for figures in this chapter. No statistically significant differences were found in the characteristics (age, CD4 T-cell count, nadir CD4 T-cell count, duration of HIV-1 infection and duration of HAART) between individuals that were responders to Gag, Nef, Gag and Nef, or low responders; the Kruskal-Wallis test was used to determine if there were significant differences between the medians (data not shown). The healthy seronegative donors were mostly male (7/11) with a median age of 35 years (IQR 28-44 years; Table 5.2).

Table 5.2 Clinical characteristics of HIV-1$^+$ patient and control cohorts.
F: female, M: male; NA: not applicable; ND: not done; NK: not known

Patient short code	Responder status	Graph Symbol	Age (years)	Gender	Duration of HIV-1 infection (months)	Duration of ART (months)	ART regimen	CD4 T-cell count (cells/µl)	Nadir CD4 T-cell count (cells/µl)	Plasma viral load (copies/ml)
Healthy controls (n = 11)		●	Median: 35 IQR: 28-44	4 F 7 M	NA	NA	NA	ND	NA	NA
R771	Gag	▲	64	M	160.13	139.05	FTC+TFV+EFV	884	391	<50
B784	Gag & Nef	■	40	M	99.25	98.95	FTC+TFV+EFV	1332	80	<50
G739	Nef	◆	43	M	141.61	113.15	FTC+TFV+EFV	534	227	<50
P087	Low	●	50	M	172.95	162.75	FTC+TFV+EFV	731	210	<50
C789	Low	●	29	M	20.75	12.20	FTC+TFV+EFV	535	309	<50
L043	Gag	▲	47	M	90.69	45.87	FTC+TFV+EFV	782	303	<50
C319	Low	●	48	M	229.05	80.03	FTC+TFV+ETV	1079	93	<50
F810	Gag	▲	50	M	233.41	193.54	FTC+TFV+NVP	582	166	<50
O523	Nef	◆	53	F	84.10	62.89	FTC+TFV+ETR	892	284	<50
S648	Gag	▲	52	M	300.39	158.43	TFV+DRV+RTV	616	227	<50
C241	Gag	▲	47	M	144.23	87.18	FTC+TFV+DRV+RTV	307	180	<50
P054	Gag & Nef	■	33	M	40.80	28.80	FTC+TFV+EFV	840	200	<50
M160	Nef	◆	35	M	120.43	36.43	FTC+TFV+DRV+RTV	397	200	<50
C766	Low	●	37	M	89.54	84.79	RTV+3TC+LOP+RTV	755	136	<50
W097	Low	●	30	M	NK	19.41	FTC+TFV+RTV+ATZ	462	201	<50
L765	Gag & Nef	■	48	M	186.95	178.95	ABC+3TC+EFV	529	275	<50
B548	Nef	◆	42	M	252.72	58.03	FTC+TFV+DRV+RTV	1213	444	<50
Median			47		142.92	84.79		731	210	
IQR			37-50		90.40-197.48	45.87-139.05		534-884	180-284	

5.2.2 Defining HIV-1 Gag and Nef responders

IFN-γ production in response to peptide stimulation was used to determine HIV-1 Gag-and/or Nef-specific responders. Figure 5.5 shows the responses to HIV-1 Gag and Nef overlapping peptide pools from two sources (the overlapping peptide pools and their sources are detailed in Tables 2.7 and 2.8 in section 2.16). The threshold for positivity in the ELISpot assays is indicated in Figure 5.5 and Figure 5.6: a positive response is defined as >50 SFC/10^6 PBMC and at least three times the background (cells cultured in TCM without peptides, the background value was always less than 25 SFC/10^6 PBMC for these assays; definitions on thresholds and cut offs as per Burton *et al*., 2006). Individuals with responses to the overlapping peptide pools between 51 and 250 SFC/10^6 PBMC were considered 'low responders', and individuals with responses >250 SFC/10^6 PBMC were termed 'responders'. Responses to the mitogen control PHA were >250 SFC/10^6 PBMC for all IFN-γ ELISpot assays (median 1440, range 282-1958 SFC/10^6 PBMC) and >130 SFC/10^6 PBMC for all IL-2 ELISpot assays (median 400, range 136-1600 SFC/10^6 PBMC). The lower responses to PHA in the IL-2 ELISpot assays were expected as the ability to produce IL-2 has been found to be impaired in chronic HIV-1 infection [Younes *et al*., 2003; Iyasere *et al*., 2003] and studies have shown that even prolonged ART is only associated with a partial recovery of HIV-1-specific T cells that secrete IL-2 [Day & Walker, 2003]. This dysfunction has been attributed to defects in the JAK/STAT signalling pathway and unrelated to expression level of the IL-2 receptor [Kryworuchko *et al*., 2004].

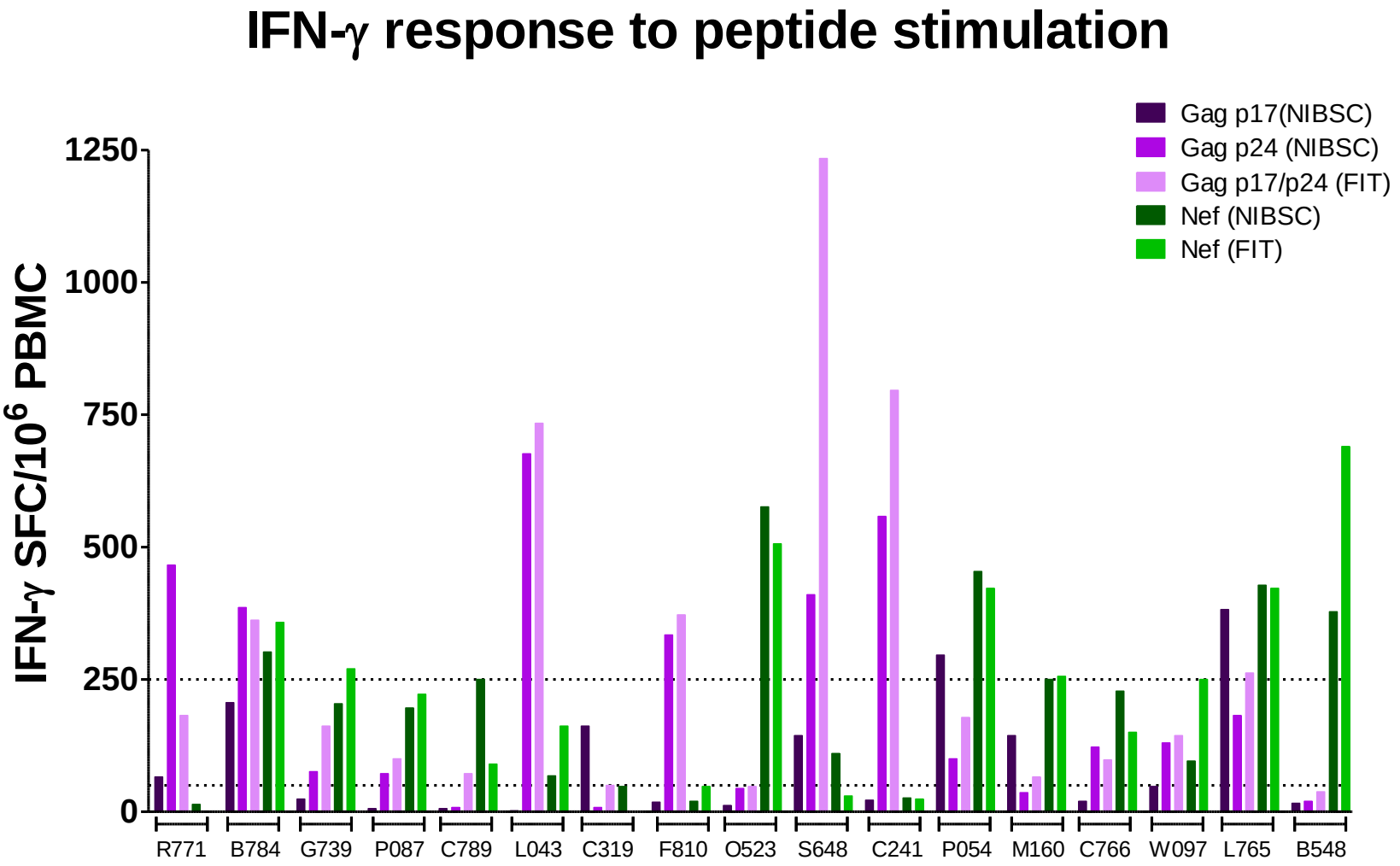


Figure 5.5 Defining distinct HIV-1 Gag- and Nef- specific responses.
IFN-γ responses to overlapping peptide pools of Gag (purple bars) and Nef (green bars) from two sources (NIBSC and FIT Biotech; the peptide source and corresponding bar colour is detailed in the figure legend). A threshold of >50 SFC/10^6 PBMC was used to determine a positive response (with the cut off line indicated on the graph). Responses between 51-250 SFC/10^6 PBMC were considered 'low responders' and individuals with responses >250 SFC/10^6 PBMC were termed 'responders'. Patient short codes are given on the X axis and n = 17.

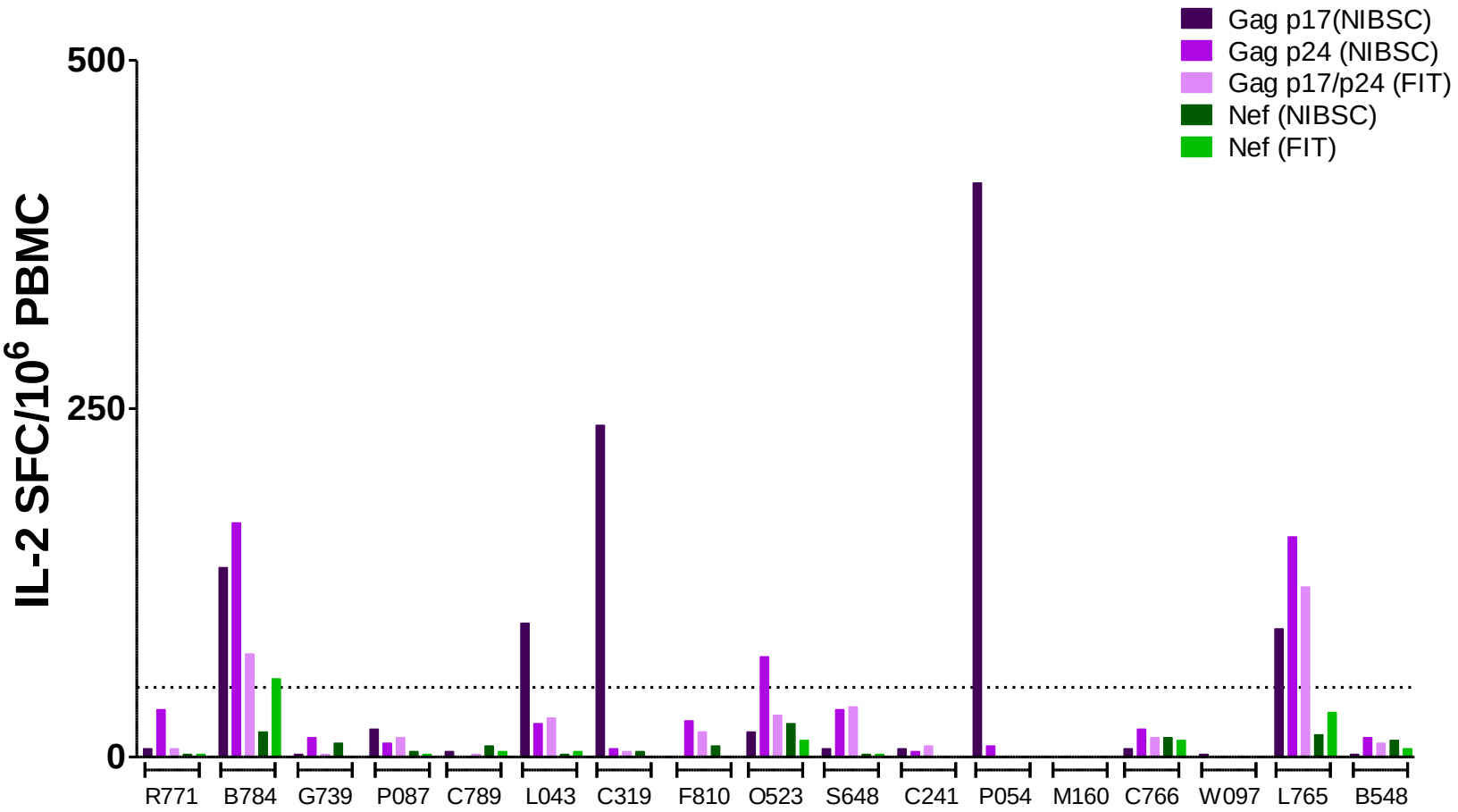


Figure 5.6 HIV-1 Gag- and Nef- specific responses in terms of IL-2 production.
IL-2 responses to peptide pools of Gag (purple bars) and Nef (green bars) from two sources (NIBSC and FIT Biotech; the peptide source and corresponding bar colour is detailed in the figure legend). The cut off line shown here illustrates the threshold for positivity in the ELISpot assays where responses >50 SFC/10^6 PBMC are considered positive. Patient short codes are given on the X axis and n = 17.

Using the aforementioned threshold for positivity and cut offs to determine low responders and responders to HIV-1 Gag, Nef, or both (Figure 5.5) five individuals were classified as 'low responders' (P087, C789, C319, C766 and W097), five as responders to Gag (R771, L043, F810, S648 and C241), four as responders to Nef (G739, O523, M160 and B548) and three as responders to both Gag and Nef (B784, P054 and L765), as shown in Table 5.2. Following characterisation of the patients in this manner, the corresponding phenotype at the same time point was assessed in order to determine if distinct functional responses are associated with a particular phenotype.

5.2.3 Phenotypic profiles of Gag and Nef responders

Phenotypic characterisation of the patient PBMC was carried out and the graphs depicting the phenotypic profiles of these individuals highlight particular types of responder. Low responders are shown with red circles, Gag responders with purple triangles, Nef responders with green rhombi and responders to both Gag and Nef with orange squares (as detailed in Table 5.2). The immunophenotypic profiles of healthy control individuals were also assessed and they are shown on the graphs with blue circles. The Kruskal-Wallis test was first used to determine if there were any significant differences between the medians of the phenotypic properties for the patients classified according to their Gag and Nef responses. No statistically significant differences between the groups were found for the parameters assessed, and this warranted grouping all HIV-1^+ patients together. Thus, the Mann-Whitney U test was used for the statistical analysis of data between HIV-1^+ patients and healthy control individuals with all statistically significant differences (and corresponding p values) shown on the graph, and any trends, particularly with regard to Gag/Nef responder status, described in the text.

The markers HLA-DR and CD38 were used to assess the immune activation status of CD4 and CD8 T cells (Figure 5.7 and Figure 5.8, respectively) in treated HIV-1-infected persons compared to healthy control individuals. Specific responses to Gag and/or Nef were also considered in these investigations. On both T-cell subsets, the percentage co-expression of HLA-DR and CD38 was significantly higher in HIV-1-infected individuals compared to healthy donors ($p < 0.0001$ for both;

Figure 5.7A and Figure 5.8A, respectively). In terms of single expression of these markers, looking at both percentage expression and MFI, CD4 and CD8 T cells of HIV-1-infected patients had significantly higher expression of HLA-DR compared to healthy controls ($p = 0.0002$ and $p < 0.0001$, respectively; Figure 5.7B and Figure 5.8B). Although no apparent differences could be distinguished in the phenotypes of the differently classified responders to Gag and Nef peptides when looking at CD8 T cells, the CD4 T-cell profile depicted a tendency for Gag responders to have higher percentage co-expression of HLA-DR and CD38 and the 'low-responders' to have a lower expression of these two markers (Figure 5.7A). The percentage expression of CD38 appeared higher on the Gag responders, whereas the Nef responders tended to have lower percentage expression of this marker on their CD4 T cells (Figure 5.7C).

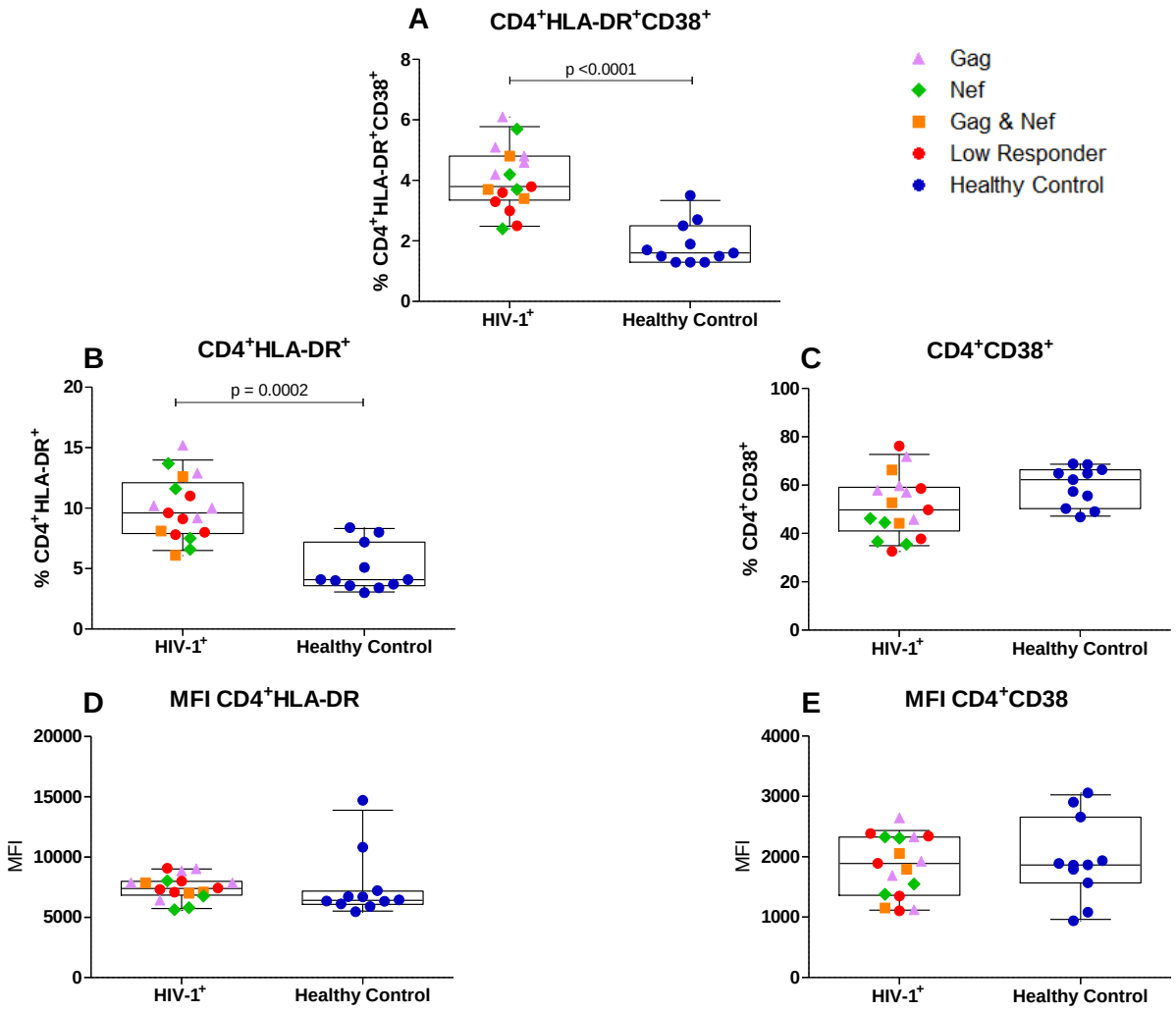


Figure 5.7 CD4 T-cell activation in HIV-1$^+$ patients and healthy control individuals.
Percentage co-expression of HLA-DR and CD38 was determined on CD4 T cells from HIV-1-infected individuals (n = 17) and healthy controls (n = 11; A). Percentage expression and MFI were also assessed for HLA-DR (B and D, respectively) and CD38 (C and E, respectively). For all graphs, HIV-1$^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the two groups.

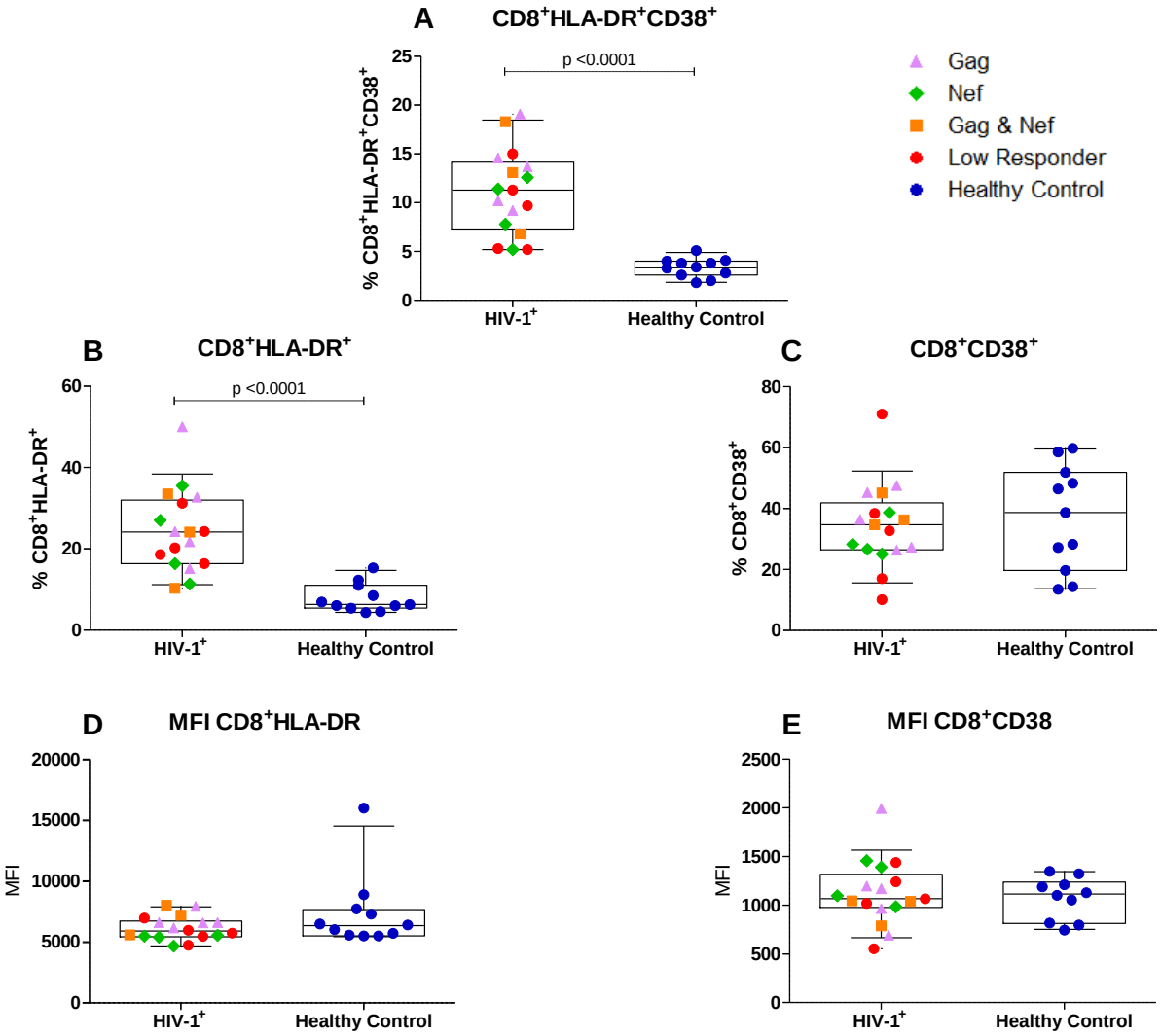


Figure 5.8 CD8 T-cell activation in HIV-1^+ patients and healthy control individuals.
Percentage co-expression of HLA-DR and CD38 was determined on CD8 T cells from HIV-1-infected individuals (n = 17) and healthy controls (n = 11; A). Percentage expression and MFI were also assessed for HLA-DR (B and D, respectively) and CD38 (C and E, respectively). For all graphs, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

The percentage co-expression of markers associated with immune senescence (CD57) and apoptosis (CD95) were significantly higher in HIV-1^+ patients compared to healthy controls on both CD4 and CD8 T cells ($p = 0.0030$ and $p = 0.0111$, respectively; Figure 5.9A and Figure 5.10A). Percentage expression of CD57 was found to be higher for both subsets in HIV-1-infected persons compared to healthy donors ($p = 0.0118$ and $p = 0.0144$, respectively; Figure 5.9B and Figure 5.10B) and the same was found for CD95 percentage expression on CD4 and CD8 T cells ($p = 0.0064$ and $p = 0.0004$, respectively; Figure 5.9C and Figure 5.10C). The MFI of CD57 on the CD4 T cell population was significantly higher in HIV-1^+ persons compared to seronegative individuals ($p = 0.0022$; Figure 5.9D). No clear pattern emerged looking at the graphs divided into type of responder, however, Nef responders tended to have higher percentage expression of CD95 on CD4 T cells (Figure 5.9C) and low responders had a lower percentage expression of CD57 on their CD4 T cells (Figure 5.9B). Responders to both Gag and Nef, tended to have higher co-expression of CD57 and CD95 on the CD8 T-cell population (Figure 5.10A) and higher percentage expression of CD57, again on CD8 T cells (Figure 5.10B).

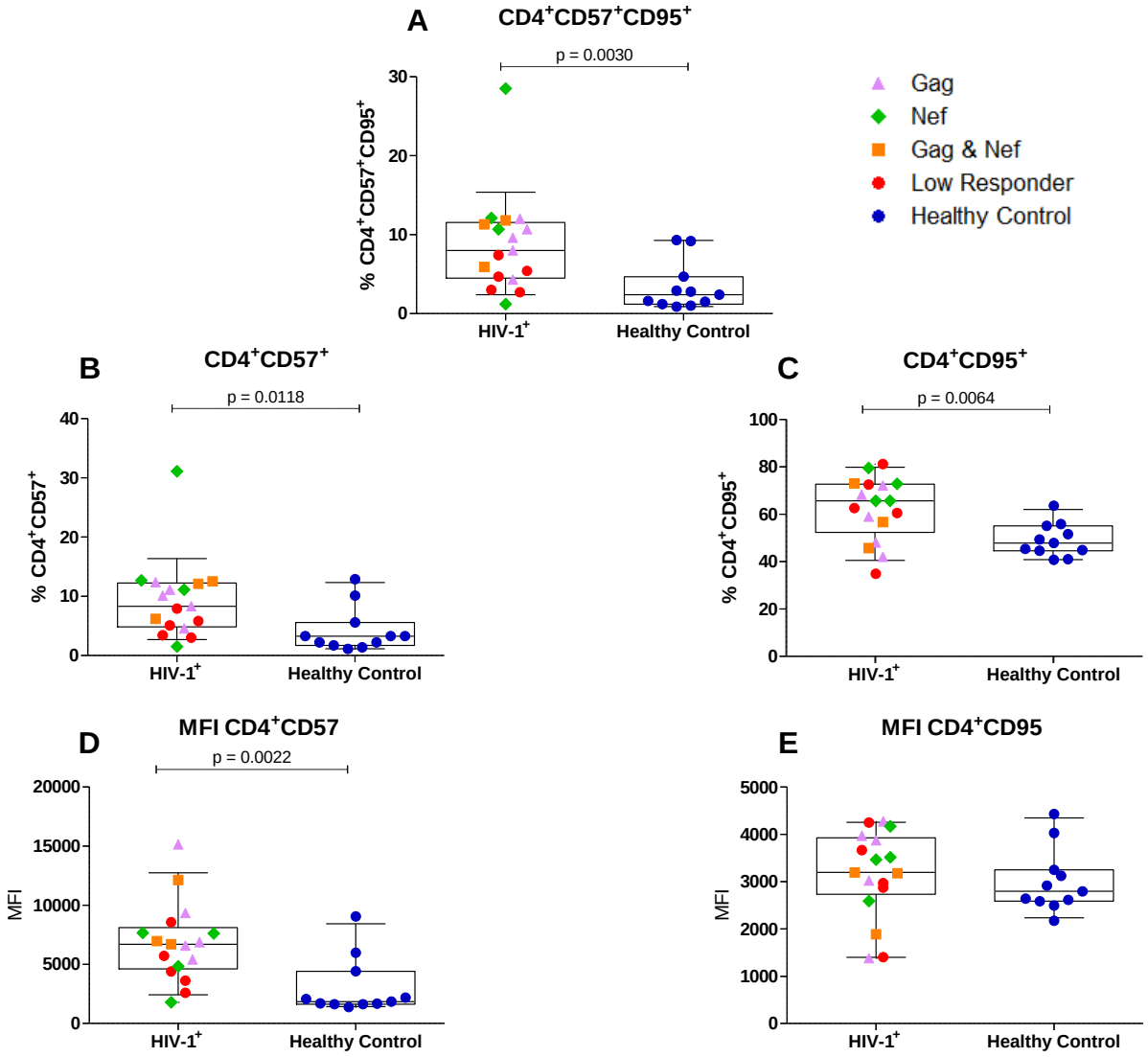


Figure 5.9 CD4 T-cell senescence and apoptosis in HIV-1$^+$ patients and healthy control individuals.
Percentage co-expression of CD57 and CD95 was determined on CD4 T cells from HIV-1-infected individuals (n = 17) and healthy controls (n = 11; A). Percentage expression and MFI were also assessed for CD57 (B and D, respectively) and CD95 (C and E, respectively). For all graphs, HIV-1$^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the two groups.

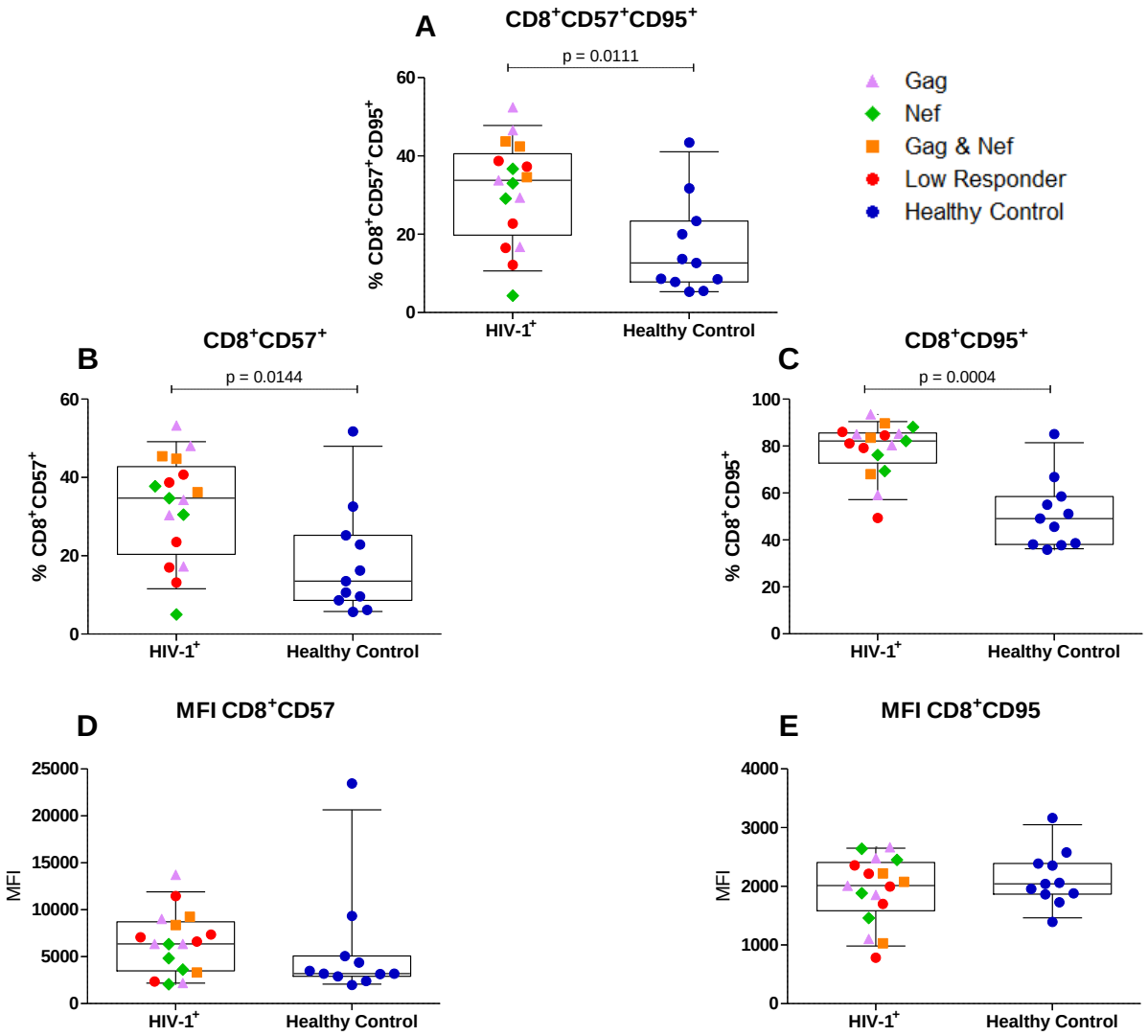


Figure 5.10 CD8 T-cell senescence and apoptosis in HIV-1$^+$ patients and healthy control individuals.
Percentage co-expression of CD57 and CD95 was determined on CD8 T cells from HIV-1-infected individuals (n = 17) and healthy controls (n = 11; A). Percentage expression and MFI were also assessed for CD57 (B and D, respectively) and CD95 (C and E, respectively). For all graphs, HIV-1$^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the two groups.

The differentiation status of both CD4 and CD8 T cells was assessed using two panels of markers. The first used the expression of CD27 and CD28 to determine early, intermediate, and late CD4 and CD8 T cells (Figure 5.11A and Figure 5.11B, respectively). For both subsets, the double positives ($CD27^{+}CD28^{+}$) are the early T cells, intermediate T cells are characterised by the loss of CD27 on the CD4 T cells ($CD27^{-}CD28^{+}$), and by the loss of CD28 expression on the CD8 T cells ($CD27^{+}CD28^{-}$). Cells which are double negative for both markers ($CD27^{-}CD28^{-}$) are classed as being in a late differentiation state. Healthy control individuals had significantly more early CD4 and CD8 T cells as determined by surface expression of these two markers (Figure 5.11A, $p = 0.0073$ and Figure 5.11B, $p = 0.0019$, respectively). For both T-cell subsets, the HIV-1-infected individuals showed profiles shifted to a late differentiation status (Figure 5.11A, $p = 0.0030$ and Figure 5.11B, = 0.0035). Nef responders in particular appeared shifted toward a later differentiation state with noticeably lower expression of markers associated with an early phenotype and higher expression of markers associated with a late maturation profile on the CD4 T cells (Figure 5.11A). A similar pattern was seen on CD8 T cells from Gag and Nef responders, with fewer early, and more late, CD8 T cells (Figure 5.11B).

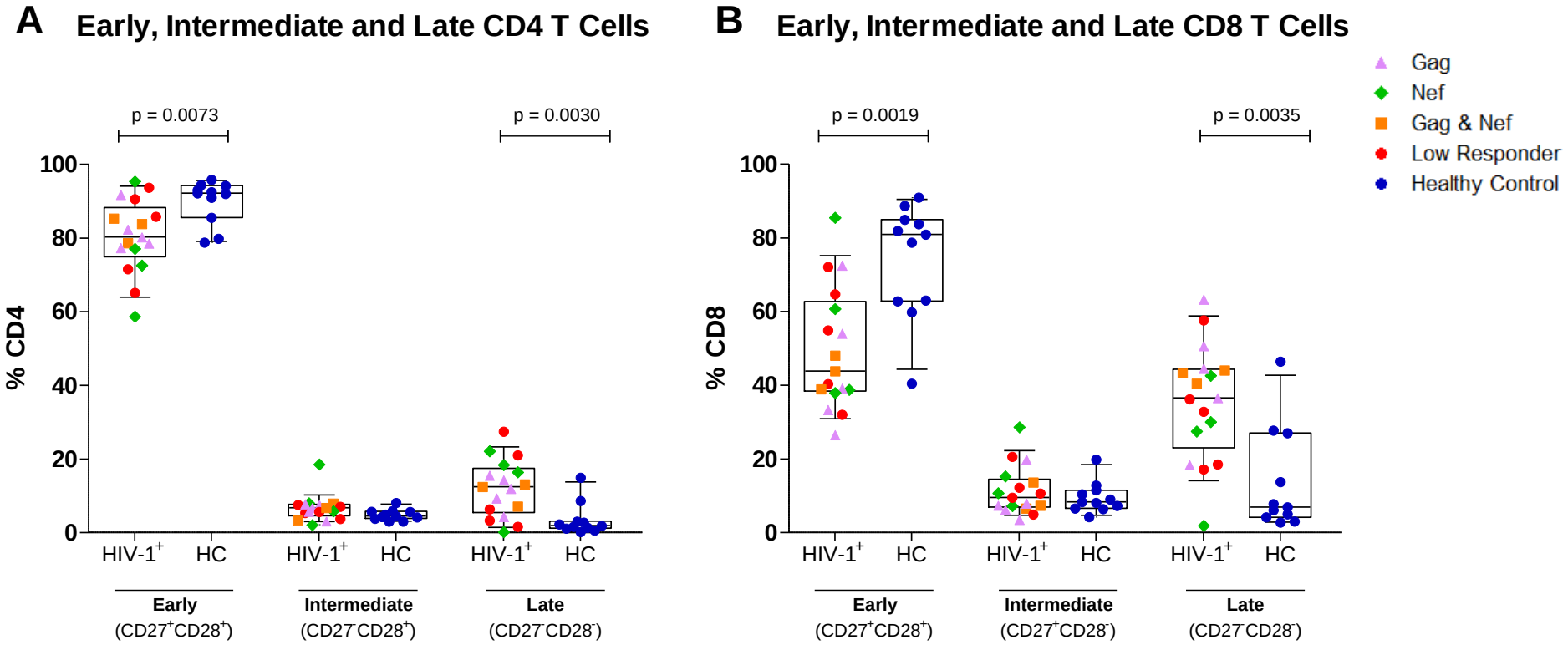


Figure 5.11 Early, intermediate and late CD4 and CD8 T-cell subsets.
Expression of CD27 and CD28 was used to determine early ($CD27^+CD28^+$), intermediate ($CD4^+CD27^-CD28^+$ or $CD8^+CD27^+CD28^-$) and late ($CD27^-CD28^-$) CD4 (A) and CD8 (B) T cells in HIV-1^+ individuals (n = 17) and healthy controls (n = 11). For each differentiation state (early, intermediate or late) shown, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

The percentage expression of CCR7 and CD45RA was also used to determine the maturation/differentiation profiles of both T-cell subsets (Figure 5.12A and Figure 5.12B, respectively). Naïve ($CCR7^{+}CD45RA^{+}$), central memory (T_{CM}; $CCR7^{+}CD45RA^{-}$), effector memory (T_{EM}; $CCR7^{-}CD45RA^{-}$) and terminally differentiated (T_{EMRA}; $CCR7^{-}CD45RA^{+}$) CD4 and CD8 T cells from HIV-1-infected individuals were characterised and compared to healthy donors. Healthy, seronegative donors had significantly higher percentage expression of naïve CD4 and CD8 T cells (Figure 5.12A, $p = 0.0431$, and Figure 5.12B, $p = 0.0016$, respectively) whereas HIV-1-infected patients demonstrated significantly higher expression of T_{EMRA} cells in both T-cell subsets. In the CD4 T-cell compartment, the Nef responders tended to have fewer naïve cells and more T_{EM} cells (Figure 5.12A), and when looking at the CD8 T cells, the Gag and Nef responders tended to have lower expression of markers associated with a T_{CM} phenotype and higher expression of those associated with a T_{EMRA} profile (Figure 5.12B). Interestingly, the low responders tended to have higher expression of markers associated with a T_{EM} phenotype and lower expression of markers associated with a T_{EMRA} phenotype (Figure 5.12B).

Regardless of the combination of markers used to determine differentiation status, healthy control individuals had significantly more early/naïve CD4 and CD8 T cells and HIV-1-infected individuals had more late/T_{EMRA} CD4 and CD8 T cells. Parallels can also be seen in the phenotype of the Gag and Nef responders with Nef responders having a shift in CD4 T-cell differentiation/maturation from an early/naïve phenotype to a late, or T_{EM} phenotype, and Gag and Nef responders having a shift from an early/T_{CM} to a late/T_{EMRA} profile in the CD8 T-cell compartment.

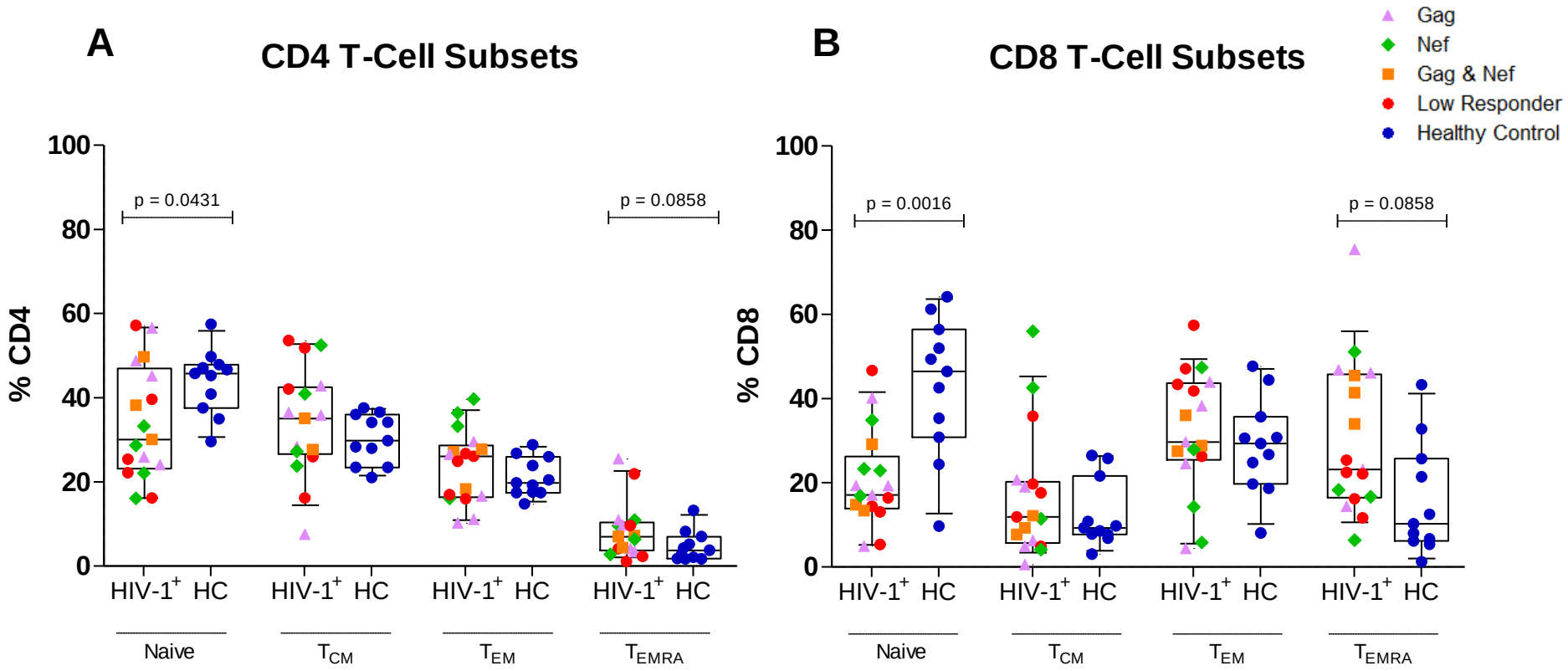


Figure 5.12 CD4 and CD8 T-cell differentiation.
Subsets of CD4 (A) and CD8 (B) T-cell differentiation were determined using surface expression of CCR7 and CD45RA. Naive ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$) T_{EM} ($CCR7^-CD45RA^-$) and T_{EMRA} ($CCR7^-CD45RA^+$) were defined in HIV-1-infected patients (n = 17) and healthy control individuals (n = 11). For each differentiation state (Naïve, T_{CM}, T_{EM} or T_{EMRA}) depicted, $HIV\text{-}1^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

In terms of PD-1 and PD-L1 expression on the differentiation/maturation subsets, according to expression of CCR7 and CD45RA, there were no significant differences seen in either the CD4 or CD8 T-cell subsets from HIV-1-infected individuals or healthy controls (Figure 5.13 and Figure 5.14, respectively). The Nef responders tended to have higher percentage expression of PD-L1 on all of the subsets of CD4 T cells (Figure 5.13B) and PD-L1 expression on the naïve and T_{CM} CD8 T cells was lower for Gag and Nef responders and tended to be higher for low responders (Figure 5.14B).

PD-1 and PD-L1 expression did not differ significantly between the CD4 and CD8 T cells of HIV-1-infected individuals and healthy donors (Figure 5.15 and Figure 5.16, respectively). The low responders appeared to express a higher percentage of PD-L1 on their CD8 T cells compared to the other types of responder (Figure 5.16B).

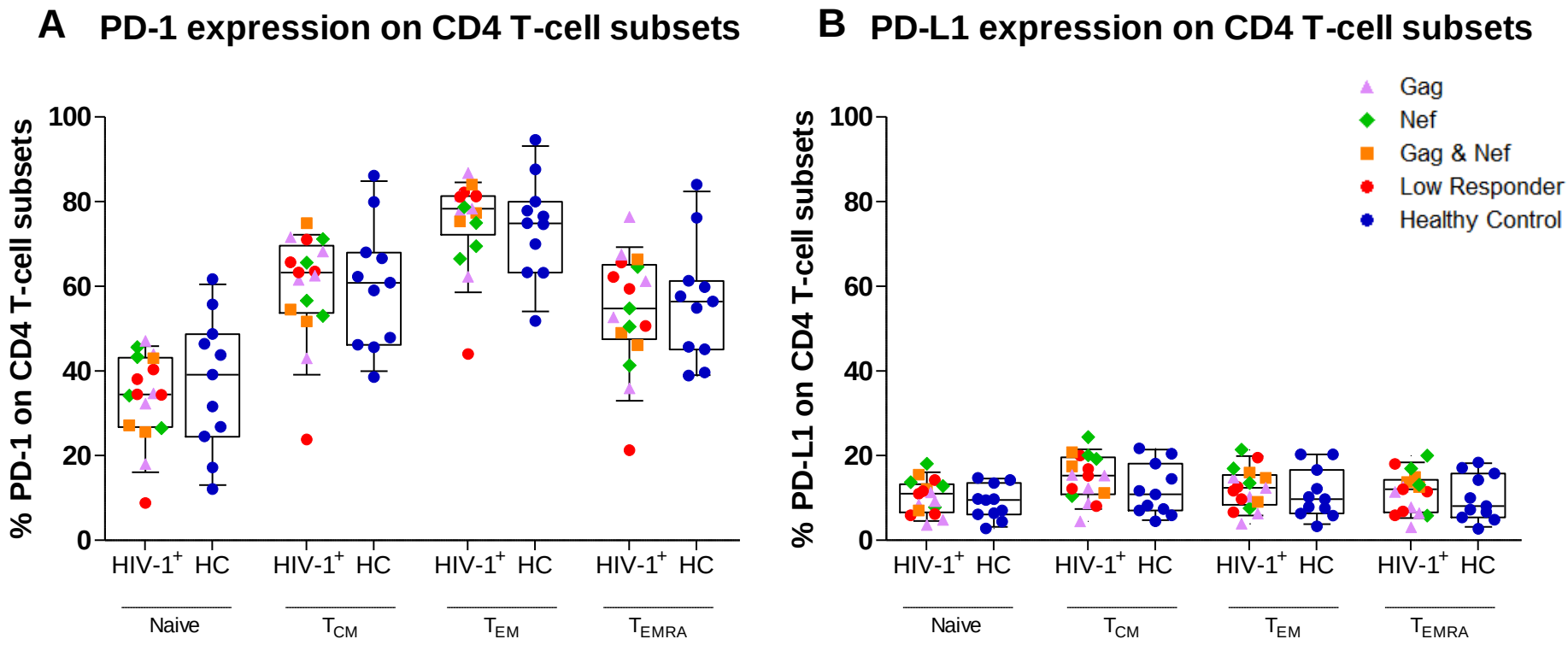


Figure 5.13 PD-1 and PD-L1 expression on CD4 T-cell subsets.
PD-1 (A) and PD-L1 (B) expression on CD4 T-cell differentiation/maturation subsets (according to CCR7 and CD45RA expression, as described previously) was assessed in HIV-1^+ patients (n = 17) and healthy control individuals (n = 11). For each differentiation state (Naïve, T_{CM}, T_{EM} or T_{EMRA}) depicted, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

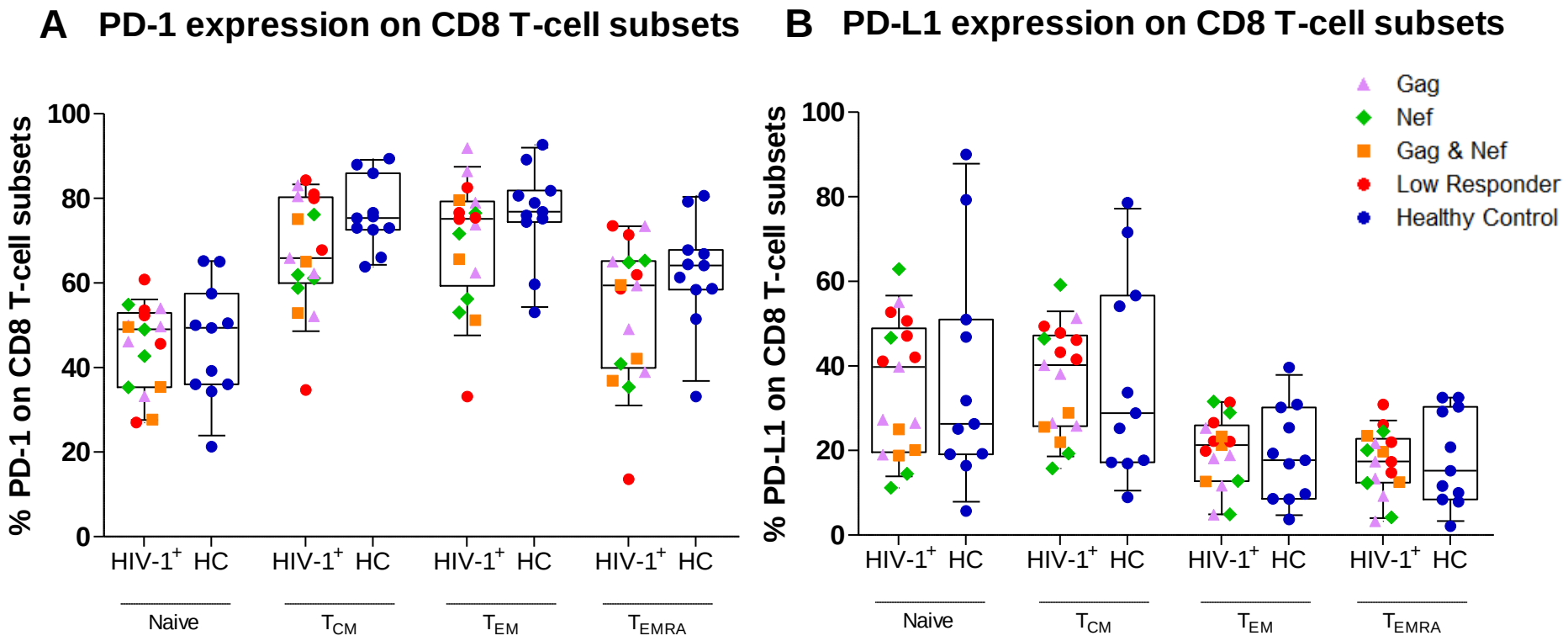


Figure 5.14 PD-1 and PD-L1 expression on CD8 T-cell subsets.
PD-1 (A) and PD-L1 (B) expression on CD8 T-cell differentiation/maturation subsets (according to CCR7 and CD45RA expression, as described previously) was assessed in HIV-1^+ patients (n = 17) and healthy control individuals (n = 11). For each differentiation state (Naïve, T_{CM}, T_{EM} or T_{EMRA}) depicted, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

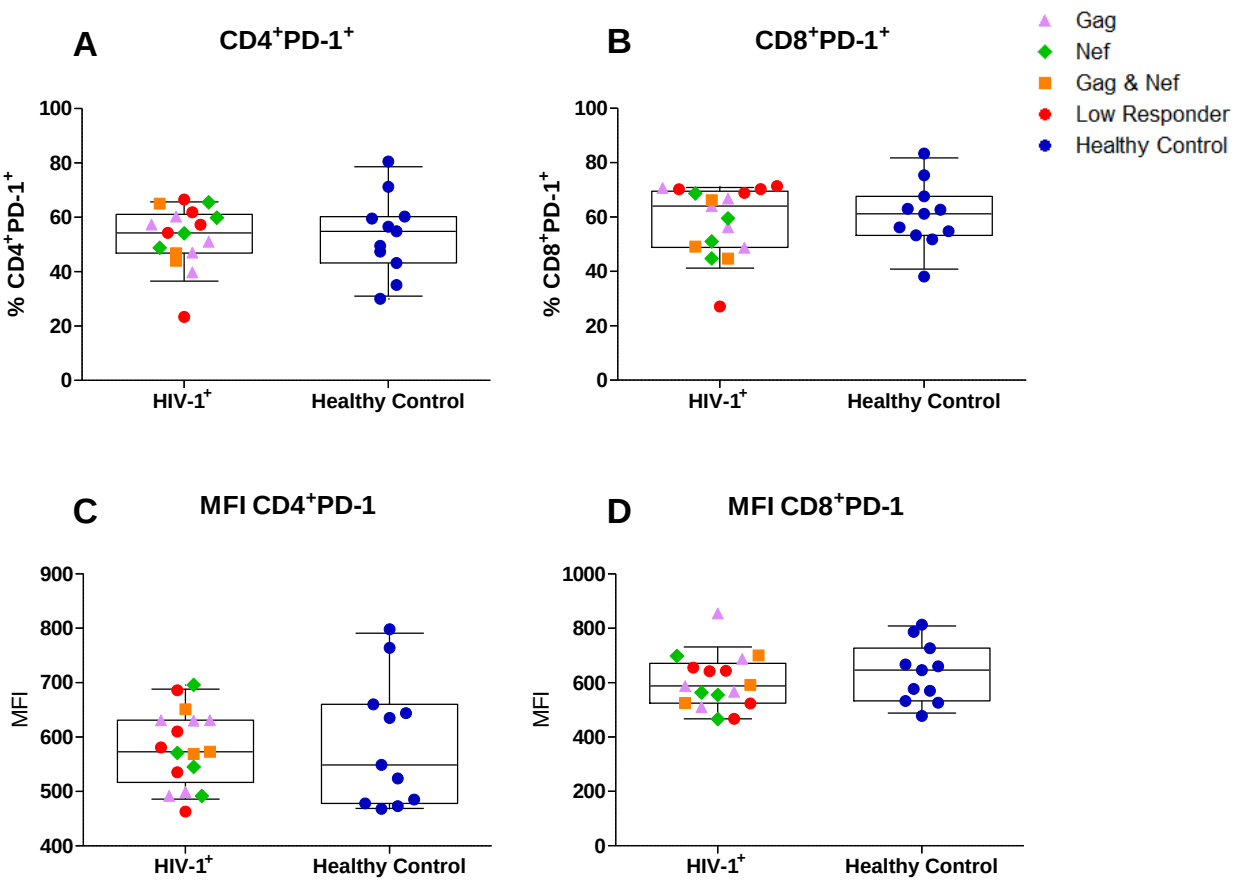


Figure 5.15 PD-1 expression on CD4 and CD8 T cells.
Percentage expression and MFI of PD-1 on CD4 (A and C, respectively) and CD8 (B and D, respectively) T cells from HIV-1^+ patients (n = 17) and healthy controls (n = 11). For all graphs, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

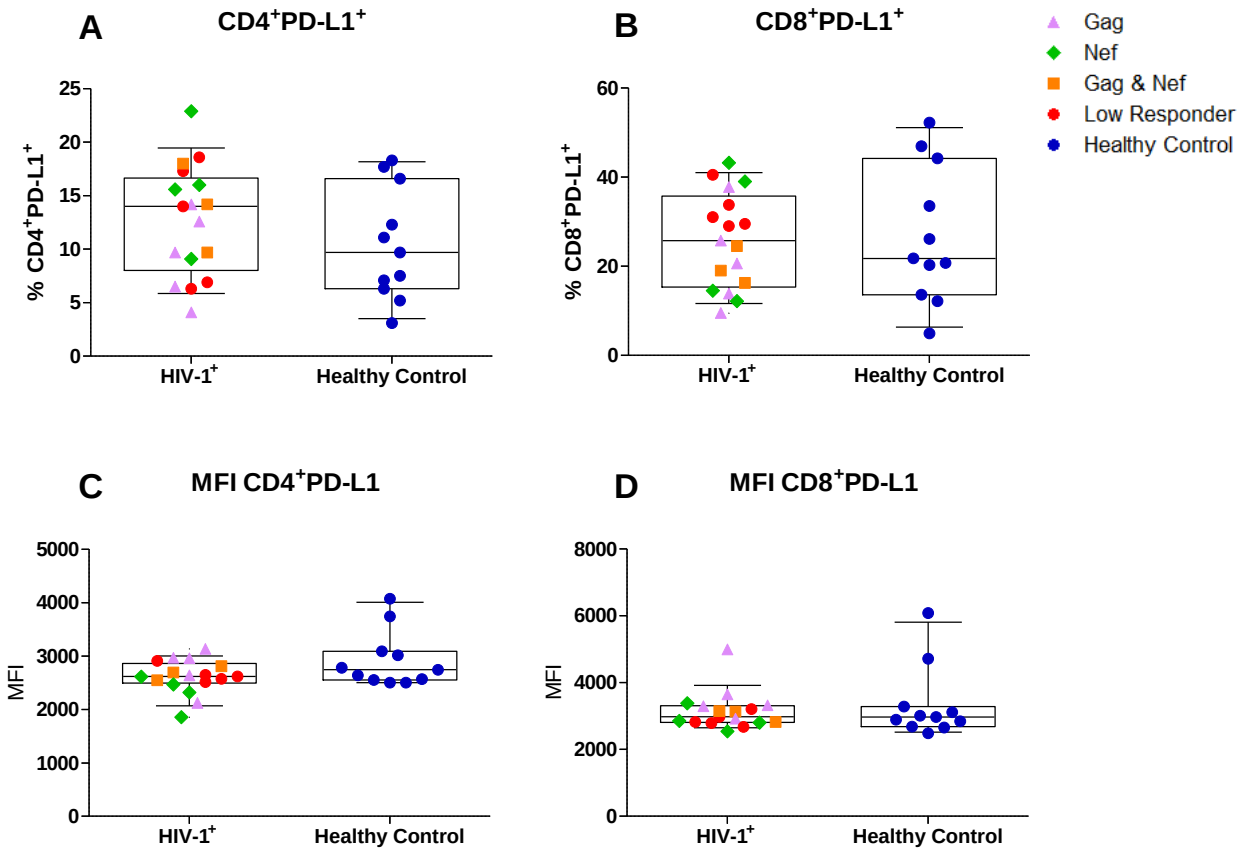


Figure 5.16 PD-L1 expression on CD4 and CD8 T cells.
Percentage expression and MFI of PD-L1 on CD4 (A and C, respectively) and CD8 (B and D, respectively) T cells from HIV-1^+ patients (n = 17) and healthy controls (n = 11). For all graphs, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

Co-expression of the exhaustion markers TIM-3 and CTLA-4 did not differ significantly on the CD4 and CD8 T cells from HIV-1^+ patients and healthy individuals (Figure 5.17 and Figure 5.18, respectively). TIM-3 expression on the CD4 T cells was significantly higher in HIV-1-infected persons compared to healthy donors (Figure 5.17B, $p = 0.0077$) and there was a trend toward a higher MFI in the HIV-1^+ group (Figure 5.17D, $p = 0.0997$). The MFI of TIM-3 on the CD4 T cells appeared elevated in the low responders and lower for patients who responded to both Gag and Nef peptides (Figure 5.17D). For the CD8 T-cell compartment, only when looking at the MFI of TIM-3 was there a statistically significant difference between the HIV-1^+ patients and the seronegative controls (Figure 5.18D, $p = 0.0212$), and again here, the low responders appeared to have higher expression of TIM-3 in terms of MFI and the Gag and Nef responders had lower expression of TIM-3 and CTLA-4 (Figure 5.18D and Figure 5.18E, respectively).

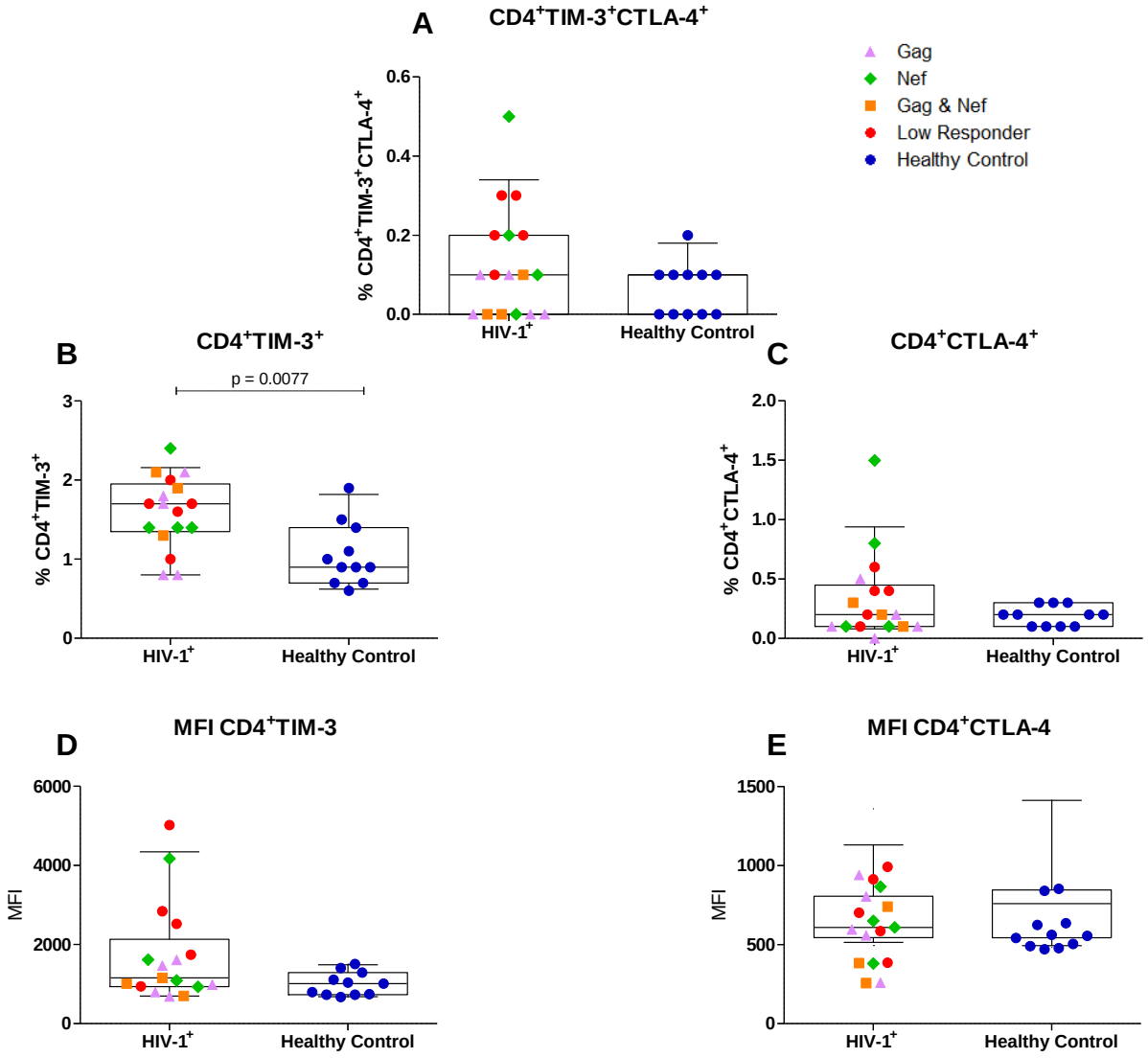


Figure 5.17 CD4 T-cell expression of the exhaustion marker TIM-3 and the co-inhibitory marker CTLA-4. Percentage co-expression of TIM-3 and CTLA-4 (A), percentage expression and MFI of TIM-3 (B and D, respectively) and CTLA-4 (C and E, respectively) on $CD4^+$ T cells from $HIV\text{-}1^+$ patients (n = 17) and healthy controls (n = 11). For all graphs, $HIV\text{-}1^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

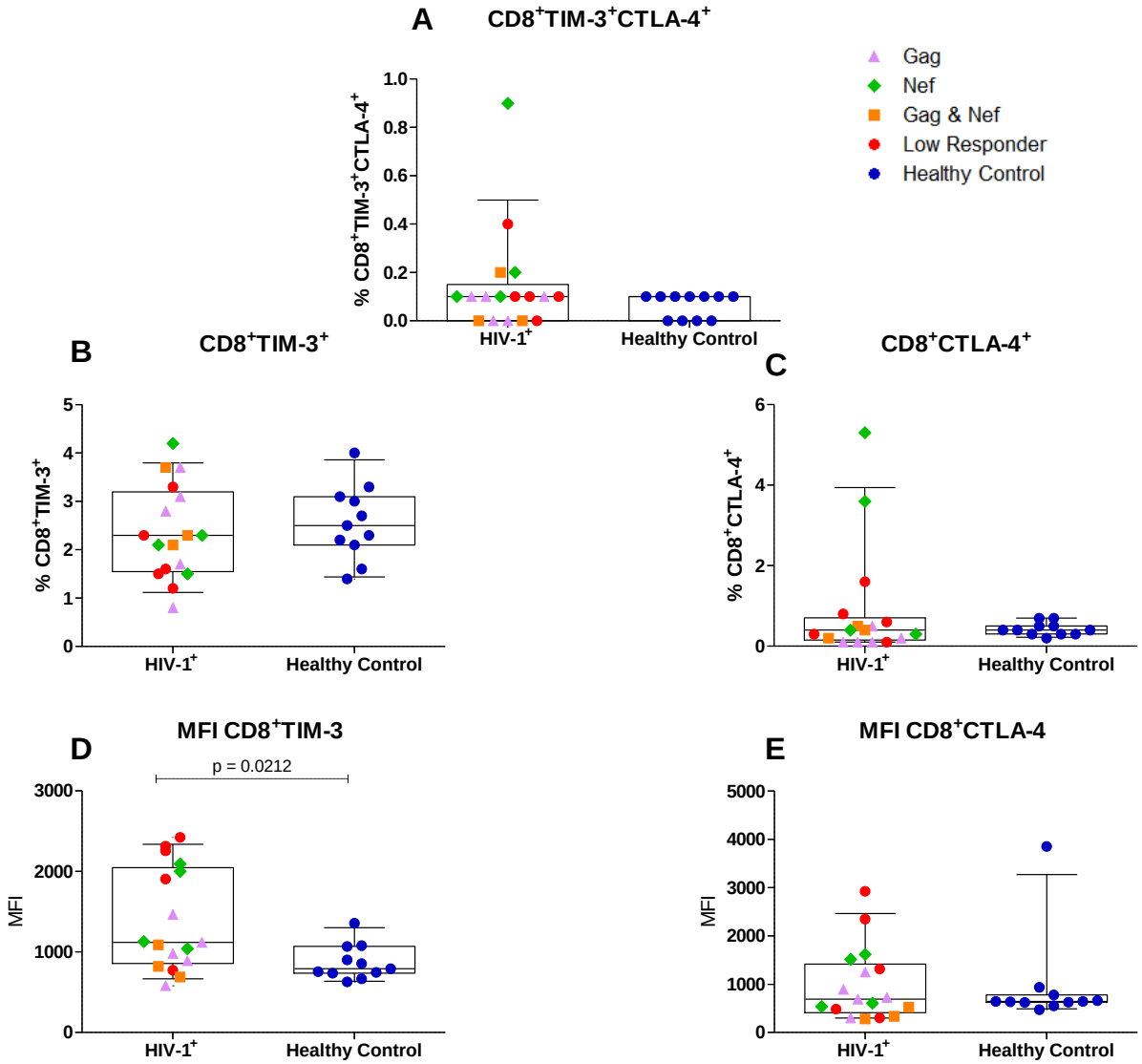


Figure 5.18 CD8 T-cell expression of the exhaustion marker TIM-3 and the co-inhibitory marker CTLA-4. Percentage co-expression of TIM-3 and CTLA-4 (A), percentage expression and MFI of TIM-3 (B and D, respectively) and CTLA-4 (C and E, respectively) on $CD8^+$ T cells from HIV-1^+ patients (n = 17) and healthy controls (n = 11). For all graphs, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

Markers associated with a regulatory T-cell phenotype ($CD4^+CD45RO^+CD25^{high}$) did not differ significantly between $HIV\text{-}1^+$ patients and healthy controls (Figure 5.19A), although the Gag and Nef responders tended to have a higher percentage expression of this combination of markers compared to the other types of responder. Only when looking at the MFI of CD25 on the CD4 T cells was a significant difference seen between HIV-1-infected persons and healthy controls (Figure 5.19D, p = 0.0482).

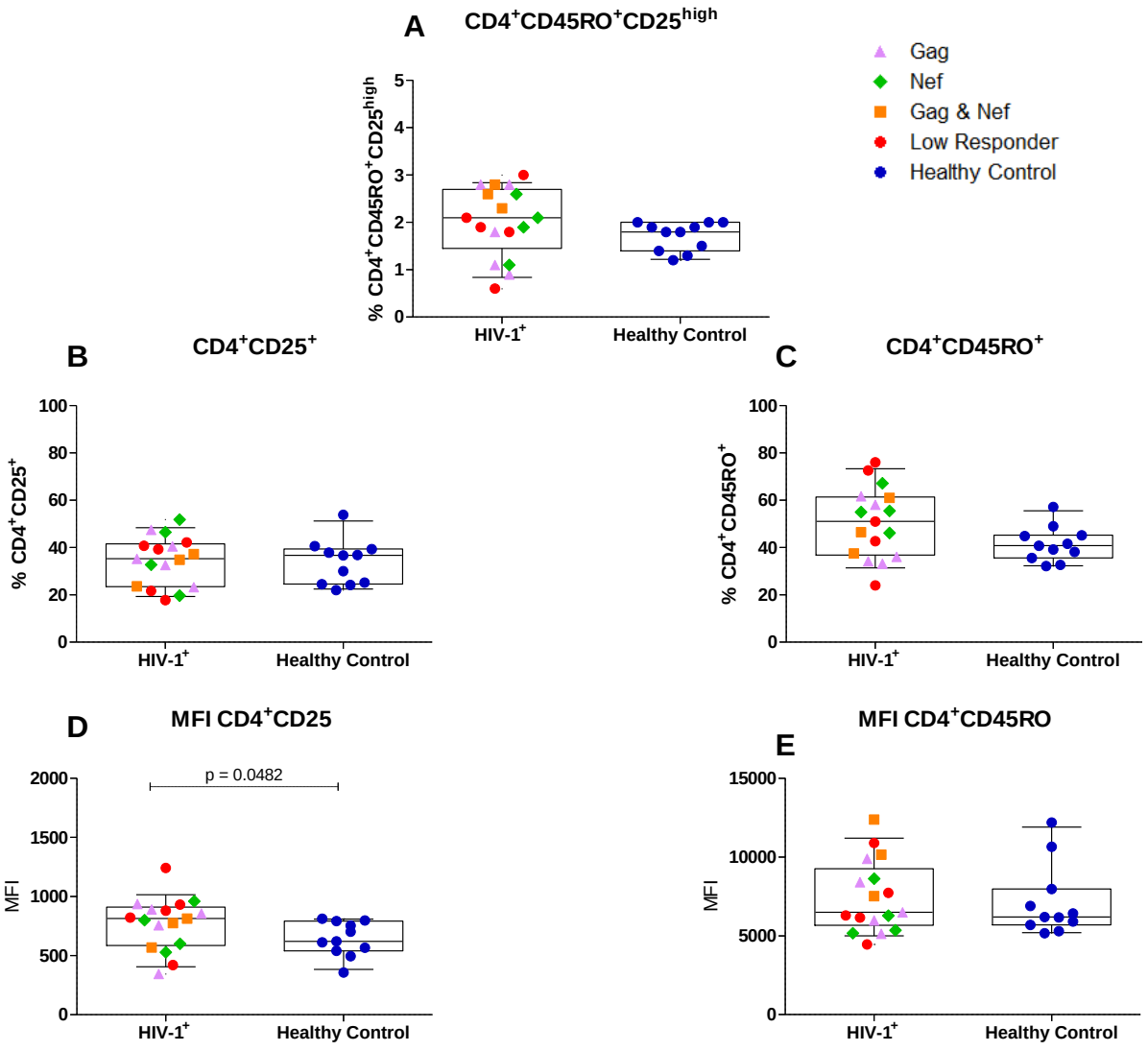


Figure 5.19 Markers associated with a regulatory T-cell phenotype.
$CD4^+CD45RO^+CD25^{high}$ T cells from HIV-1 infected individuals (n =17) and healthy controls (n = 11) were compared (A) as was percentage expression and MFI of CD25 and CD45RO on this subset (B and D, and C and E, respectively). For all graphs, $HIV\text{-}1^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

The expression of CD45RA, CD31 and PTK-7 were used to determine recent thymic emigrants. Co-expression of CD45RA and CD31 on CD4 T cells did not differ significantly between HIV-1^+ persons and healthy donors (Figure 5.20A), nor did single expression of PTK-7 in terms of percentage expression (Figure 5.20C) and MFI (Figure 5.20E). However, healthy control individuals had significantly higher percentage expression of CD31 compared to HIV-1-infected patients (Figure 5.20C, $p = 0.0033$) with a trend toward an increased MFI of CD31 in the HIV-1^+ group (Figure 5.20D, $p = 0.0666$). The MFI of CD31 on CD8 T cells was significantly higher in HIV-1-infected persons compared to healthy controls (Figure 5.21C, $p = 0.0482$). The Nef responders tended to have lower percentage co-expression of CD45RA and CD31 on the CD4 T cells (Figure 5.20A) but higher expression of PTK-7 on the CD8 T cells (Figure 5.21B).

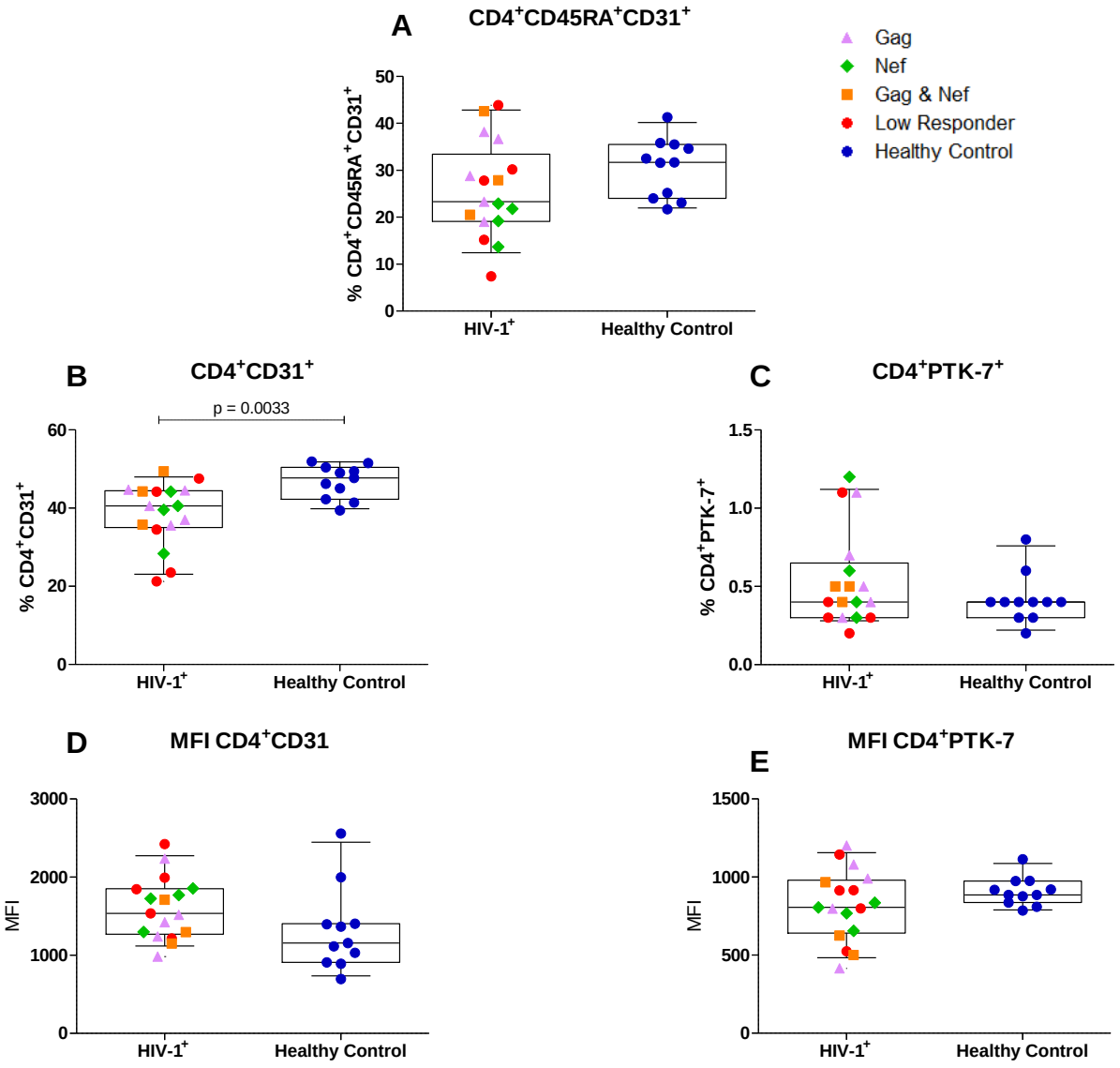


Figure 5.20 Markers associated with recent thymic emigrants on CD4 T cells.
Co-expression of CD31 and CD45RA (A) was assessed on CD4 T cells from HIV-1$^+$ individuals (n = 17) and healthy controls (n = 11). Percentage expression and MFI of CD31 (B and D, respectively) and PTK-7 (C and E, respectively) were also determined for the CD4 T-cell population. For all graphs, HIV-1$^+$ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10th and 90th percentiles. P values are shown where there are statistically significant differences between the two groups.

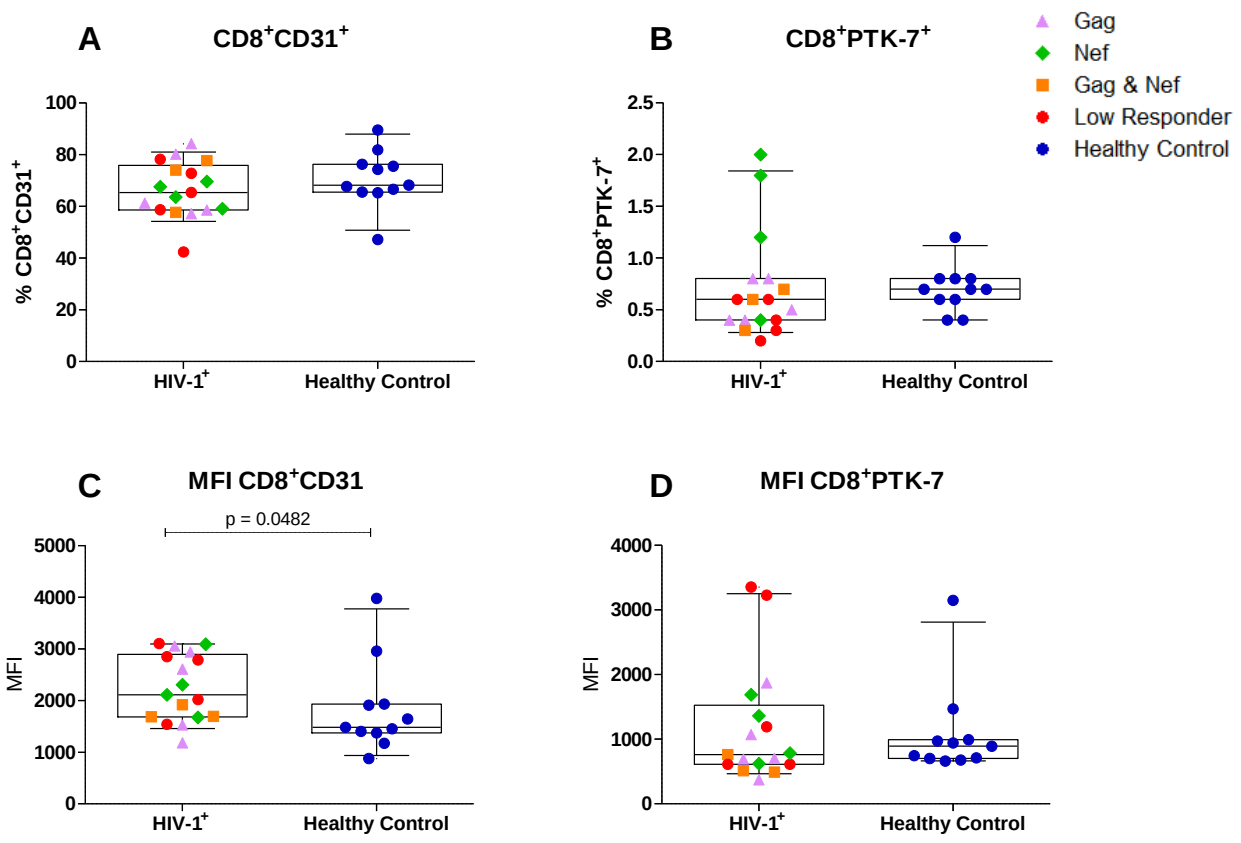


Figure 5.21 Markers of recent thymic emigrants on CD8 T cells.
Percentage expression and MFI of CD31 (A and C, respectively) and PTK-7 (B and D, respectively) on CD8 T cells were determined for HIV-1-infected patients and healthy control subjects. For all graphs, HIV-1^+ patients are shown in the left hand plot, with Gag (▲), Nef (◆), Gag and Nef (■), or low responder (●) status indicated. Healthy control individuals are depicted with blue circles (●). All individual data points are shown with box plots representing the median and interquartile range, and whiskers representing the 10^{th} and 90^{th} percentiles. P values are shown where there are statistically significant differences between the two groups.

5.3 Correlations between clinical parameters and immunophenotype

Correlations between clinical parameters (CD4 T-cell count, nadir CD4 T-lymphocyte count, duration of HIV-1 infection and duration of ART) and CD4 and CD8 T-cell phenotype were carried out for the 17 patients that were classified according to their IFN-γ ELISpot responses to peptide pools of Gag and Nef (Table 5.2). The non-parametric Spearman correlation coefficient was the statistical test used as the data were not normally distributed. Graphs display the correlation coefficient (r_s), with negative values representing a negative correlation, and p value, with p values ≤0.05 considered to be significant. For the seventeen patients, there were no significant correlations in terms of CD4 T-cell count and percentage expression of the activation markers HLA-DR and

CD38 on the CD4 and CD8 T lymphocytes, or with the expression of CD57 and CD95, or with PD-1 (data not shown). There were however, significant negative correlations between CD4 T-cell count and percentage expression of TIM-3 on CD4 and CD8 T cells (r_s = 0.6671, p = 0.0034; and r_s = -0.5674, p = 0.0175; Figures 5.22A and 5.22B, respectively).

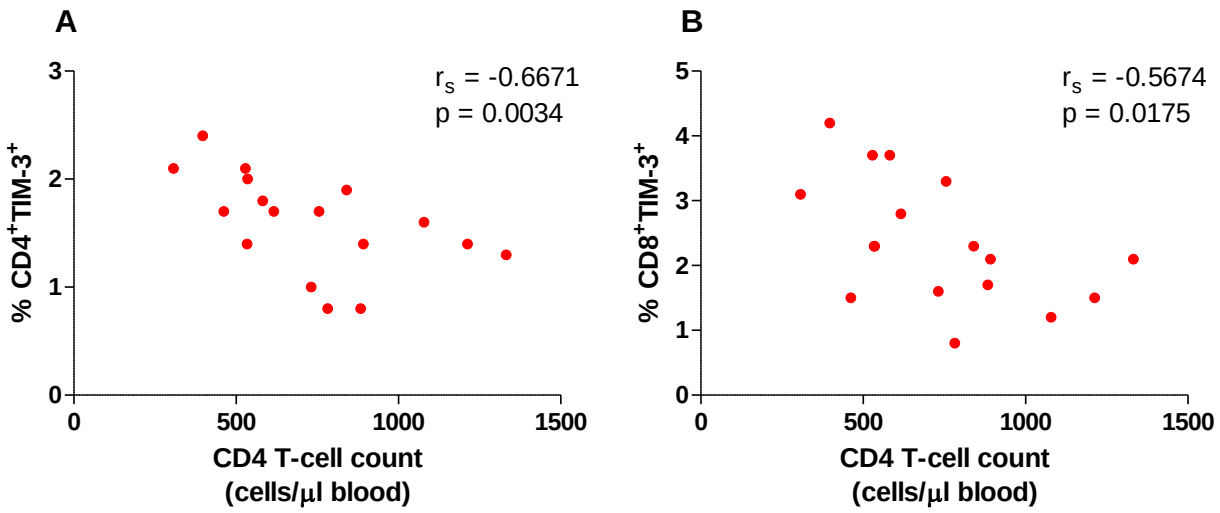


Figure 5.22 Correlation between CD4 T-cell count and expression of TIM-3 on CD4 and CD8 T lymphocytes.
A significant negative correlation was seen between CD4 T-cell count and percentage expression of TIM-3 on CD4 (A) and CD8 (B) T cells; all subjects were HIV-1-infected and on HAART (n = 17). The correlation coefficient (r_s) and p value are shown for each plot.

These patients were well-matched according to CD4 T-cell count and nadir CD4 T-cell count (Table 5.2), thus in order to diversify the group and increase numbers for the correlation analyses, the twenty four patients phenotyped in this chapter (Table 5.1) were added to the analysis. Between the two groups of patients, there was no significant difference in age or duration of HIV-1 infection but the seventeen patients that were also assessed for functionality (Table 5.2) had significantly greater numbers of peripheral CD4 T cells (p = 0.0031), higher nadir CD4 T-lymphocyte counts (p = 0.0124) and had been on ART for longer (p = 0.0253) than the HIV-1-infected individuals in Table 5.1 (the Mann-Whitney U test was used for statistical analysis). Once the patients were grouped together, analysis revealed a significant negative correlation between the CD4 T-cell count and the percentage of CD4 and CD8 T cells co-expressing the activation markers HLA-DR and

CD38 (r_s = -0.5941, p <0.0001; and r_s = -0.3634, p = 0.0212; Figures 5.23A and 5.23B for CD4 and CD8 T cells, respectively). A significant negative correlation was also seen between CD4 T-lymphocyte count and single expression of HLA-DR on CD4 T cells (r_s = -0.4831, p = 0.0016; Figure 5.23C). Moreover, the duration of ART correlated negatively with the percentage co-expression of the two aforementioned activation markers, but only on the CD8 T-cell population, and this correlation approached statistical significance (r_s = -0.3090, p = 0.0524; Figure 5.23D).

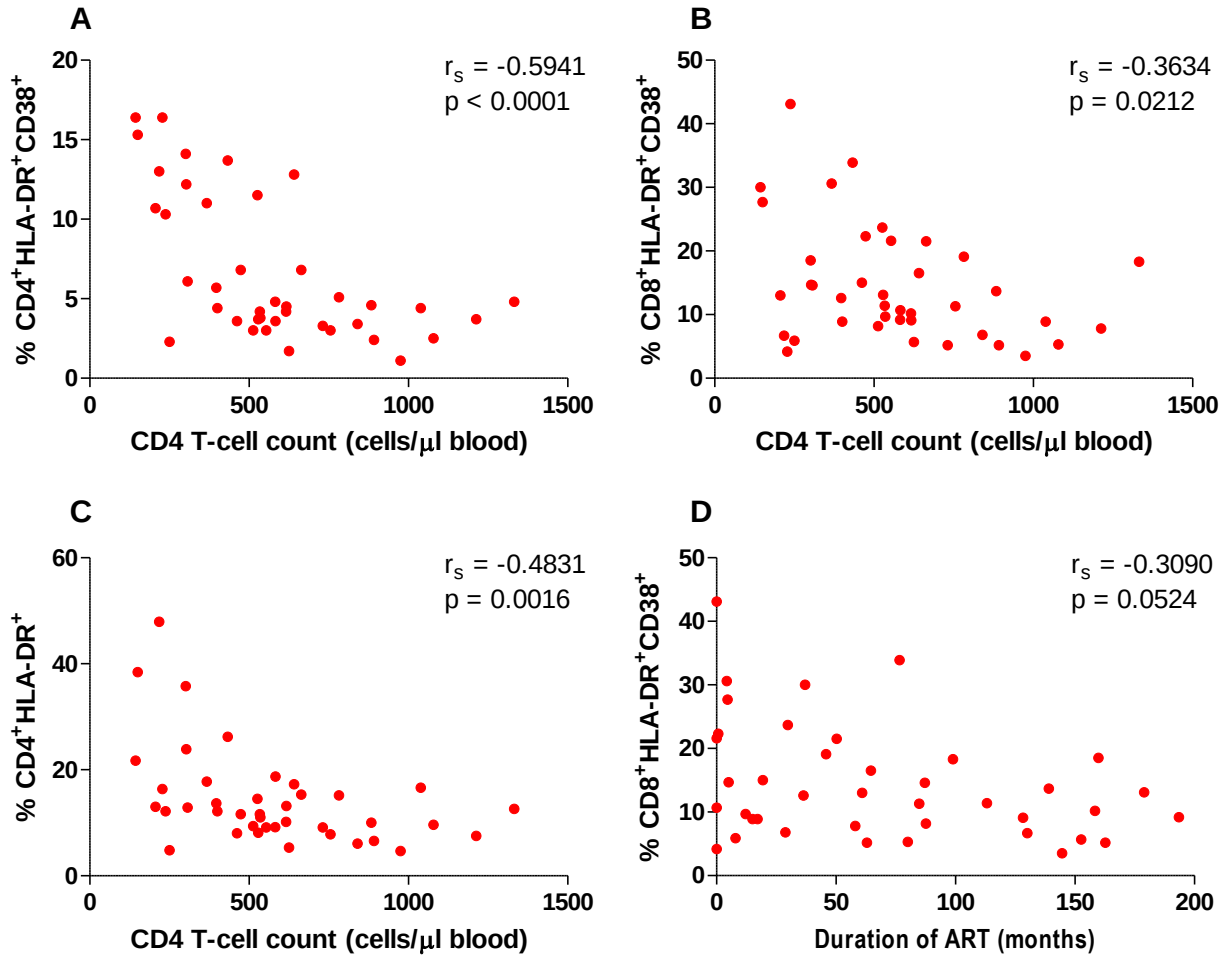


Figure 5.23 Correlations between clinical parameters and immune activation.
The correlation between CD4 T-cell count and percentage co-expression of HLA-DR and CD38 on both CD4 (A) and CD8 (B) T cells is shown (n = 41), as well as single expression of HLA-DR on CD4 T cells (C; n =41). The way in which duration of ART correlates with CD8 T-cell activation is also depicted (D; n= 40). The correlation coefficient (r_s) and p value are shown for each plot.

Significant negative correlations were found between the CD4 T-cell count and both the percentage expression of markers associated with a terminally differentiated T-cell phenotype (r_s = -0.3896, p = 0.0130; Figure 5.24A) and the expression level of the apoptosis marker CD95 (r_s = -0.4090, p =

0.0088; Figure 5.24B) on CD4 T lymphocytes. Investigation of the clinical parameters and patient characteristics revealed significant positive correlations between CD4 T-cell count and both nadir CD4 T lymphocyte count (r_s = 0.3680, p = 0.0179; Figure 5.25A) and duration of ART (r_s = 0.3476, p = 0.0259; Figure 5.25B).

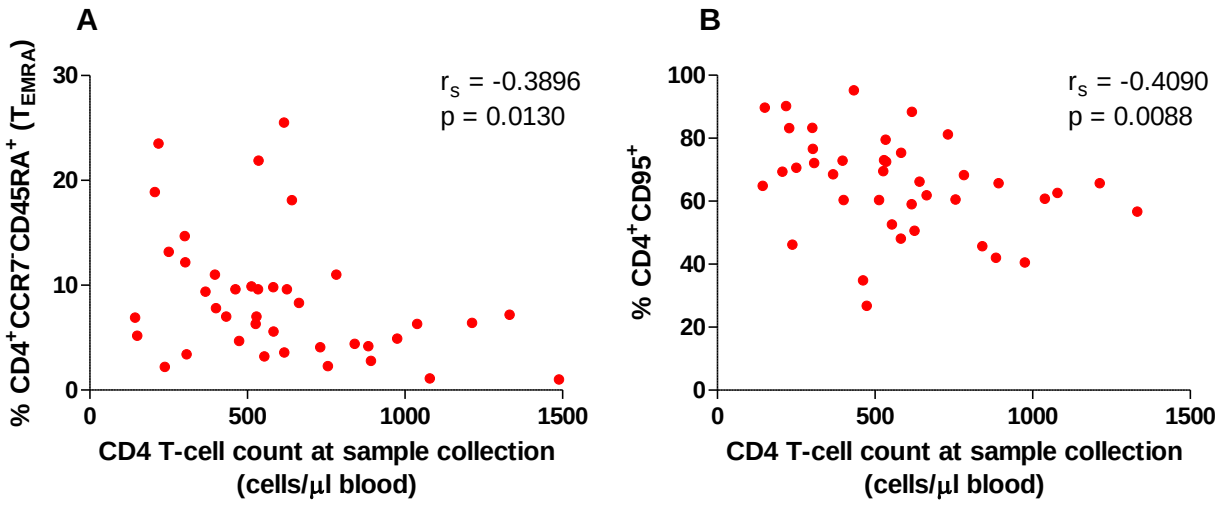


Figure 5.24 Correlation between CD4 T-cell count and markers of terminal differentiation and apoptosis.
The correlation between CD4 T-cell count and markers associated with a terminally differentiated T-cell phenotype ($CCR7^+CD45RA^+$; T_{EMRA}) is shown (A; n = 40). The correlation between peripheral CD4 T-lymphocyte numbers and the apoptotic marker CD95 is also depicted (B; n = 40). The correlation coefficient (r_s) and p value are shown for each plot.

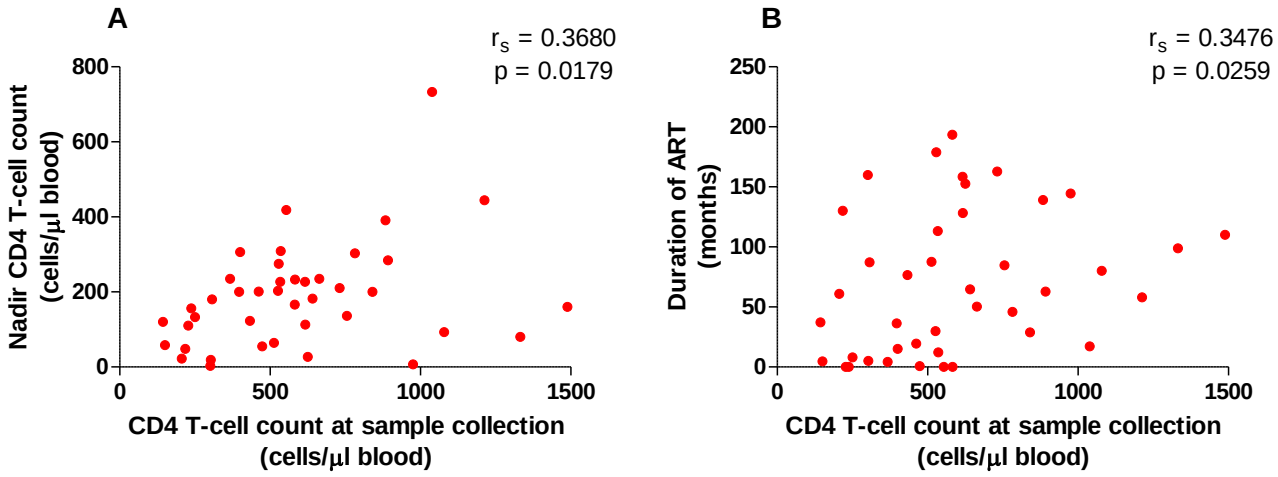


Figure 5.25 Duration of ART and nadir CD4 T-cell count correlate with peripheral CD4 T-lymphocyte numbers.
The correlation between CD4 T-cell count and nadir CD4 T-cell count was assessed (A; n = 40), as was the correlation between CD4 T-lymphocyte number and duration of ART (B; n = 40). The correlation coefficient (r_s) and p value are shown for each plot.

5.4 Discussion

No differences were observed in CD38 expression on CD4 or CD8 T lymphocytes between HIV-1-infected patients and healthy control subjects for either study group described in this chapter. This agrees with findings from other studies in which CD38 expression is elevated in acute HIV-1 infection but normalises following treatment with ART [Papagno *et al*., 2004; Shou *et al*., 2011]. It has been suggested that the mean density of CD38 per cell (MFI) rather than the proportion of cells that express CD38 might provide a better indicator of activation [Liu *et al*., 1996; Deeks *et al*., 2004]. However, even when comparing the MFI of CD38 on PBMC from HIV-1 infected individuals from both groups of patients and healthy controls, no significant differences were found. The increased co-expression of HLA-DR and CD38 on both T-cell subsets from HIV-1^+ persons, and increased expression of HLA-DR, compared to healthy seronegative donors was significant even when the population of HIV-1^+ individuals was more carefully regulated and the subjects had significantly higher baseline CD4 T-cell counts, higher nadir CD4 T-lymphocyte counts and had been on ART for a longer duration. This suggests that chronic immune activation persists even with suppressive ART, which agrees with previous findings in terms of percentage co-expression of both markers on CD4 and CD8 T-cell subsets [Hunt *et al*., 2003], although reports from other groups describe a reduction in HLA-DR expression on CD8 T cells to levels approaching those found in healthy donors following 7 years of ART [Shou *et al*., 2011]. It should also be noted that the median co-expression of these markers was lower for the seventeen patients assessed for Gag- and Nef- specific responses compared to the 23 patients originally phenotyped in this chapter. This could be due to significantly longer treatment with ART for these individuals or explained by higher peripheral CD4 T-lymphocyte counts and higher nadir CD4 T-cell counts; suggesting better immune reconstitution in these patients.

T-cell immune activation has been linked to declines in CD4 T-lymphocyte numbers and disease progression [Hunt *et al*., 2008; Hazenberg *et al*., 2003] and a recent study found CD38 to be expressed at similar levels in LTNP and patients treated with ART in early HIV-1 infection for a

prolonged period of time [Cellerai *et al*., 2011]. Furthermore, atypical activation profiles have been described (in terms of CD38 and HLA-DR expression) on HIV-1-specific CD8 T cells from HIV-1 controllers; these cells express low levels of CD38 (similar to levels observed in the total CD8 T-lymphocyte population) but unusually high levels of HLA-DR (higher than levels observed among total CD8 T cells), thus this $CD38^{low}$/$HLA\text{-}DR^{high}$ phenotype of HIV-1-specific CD8 T cells may characterise cells with an ability to proliferate upon antigenic stimulation and exert effector function [Saez-Cirion *et al*., 2007].

HIV-1-infected patients from both groups had significantly greater co-expression of the senescence marker CD57 and the apoptosis marker CD95 on both CD4 and CD8 T lymphocytes compared to healthy control individuals and this was also true for single positive expression of both of these markers on both T-cell subsets. These findings agree with previously published data; the expression of CD57 on peripheral T cells was found to be significantly higher on PBMC from HIV-1-infected persons compared to healthy seronegative donors and its expression associated with a loss of proliferative capacity, increased apoptosis and defective cytokine production [Papagno *et al*., 2004; Brenchley *et al*., 2003; Palmer *et al*., 2005]. HIV-1-specific CD8 T cells have been described as having an increased sensitivity to CD95/Fas-induced death, particularly in the $CD45RA^{-}CD62L^{-}$ effector memory compartment [Mueller *et al*., 2001] and the expression of CD95, similar to CD57, has been associated with reduced *ex vivo* survival [Mueller *et al*., 2001] and impaired cytokine production [Betts *et al*., 2006]. CD8 T cells that are sensitive to CD95/Fas-induced apoptosis may be unable to exert a cytotoxic response against HIV-1-infected macrophages and CD4 T cells expressing CD95 ligand (CD95L) [Tateyama *et al*., 2000]. HIV-1 has been found to induce an up-regulation of CD95L in macrophages, T cells and DC and a role for Nef in the increased expression of this ligand on T cells and macrophages has been proposed [Strauss *et al*., 2009]. It was suggested that this up-regulation on APC following infection with HIV-1 may inhibit T-cell activation and proliferation and that this may allow HIV-1 to evade the immune response [Strauss *et al*., 2009]. Further understanding and characterisation of these markers in HIV-1 infection is of the utmost

importance and perhaps therapies targeting these molecules might help to improve the survival and differentiation of HIV-1-specific T cells, thereby enhancing viral clearance.

CD4 and CD8 T-cell differentiation was characterised based on the expression of the co-stimulatory molecules CD27 and CD28 and for both groups of HIV-1^+ patients, the percentage of expression of markers associated with an early T-cell phenotype ($CD27^+CD28^+$) were significantly reduced, and the percentage expression of those defining a late differentiation state ($CD27^-CD28^-$) were significantly increased, when compared to PBMC from healthy uninfected donors. It is interesting to note that the expression of the late phenotype is particularly elevated in the first 23 patients investigated; those with lower CD4 T-cell count, lower nadir CD4 T-lymphocyte counts and less time on ART. These findings fit with other reports in which chronic progressive HIV-1-infection is associated with a CD8 T-cell differentiation that is skewed toward a $CD28^-$ TEM population [Ellefsen *et al*., 2002; Downey and Imami, 2010], and this population has been associated with a lack of proliferative capacity [Appay *et al.*, 2008]. Similarly, examining CD4 and CD8 T-cell differentiation in terms of CCR7 and CD45RA expression, markers associated with a naïve T-cell phenotype were significantly reduced and markers associated with a terminally differentiated (T_{EMRA}) phenotype were significantly increased in HIV-1^+ persons compared to healthy controls. This partially correlates with previous reports, although these also describe a reduction in the central memory subset and an increase in the effector memory subset for CD8 T cells [Ladell *et al*., 2008; Downey and Imami, 2010]. There were no significant differences found in the expression of PD-1 and PD-L1 on CD4 or CD8 T lymphocytes (or their differentiation subsets) between HIV-1-infected persons and healthy control individuals. PD-1 expression has been found to correlate with plasma viraemia and disease progression [Kaufmann and Walker, 2009; Day *et al*., 2006] and initiation of ART and subsequent control of plasma HIV-1 RNA has been shown to reduce the expression of PD-1 on HIV-1-specific CD8 CTL and PD-L1 on PBMC [Kaufmann and Walker, 2009]. However others reported that PD-L1 levels may not be normalised on ART [Rosignoli *et al*., 2007]. It is important to consider that the patients involved in these studies may differ in their

clinical characteristics and duration of ART, and that these factors may result in discrepancies in such findings.

There were no significant differences found in CTLA-4 expression on PBMC from HIV-1^+ persons compared to healthy controls. CTLA-4 increases during chronic HIV-1 infection [Leng *et al*., 2002], although up-regulation only occurs on CD4 T lymphocytes and has not been described at high levels on CTL in HIV-1 infection [Steiner *et al*., 1999]. Of note, PD-1 is expressed by the majority of CD4 T cells that express CTLA-4 [Downey and Imami, 2010] in the context of HIV-1 infection, and suppressive ART has been found to reduce the expression of this molecule [Kaufmann *et al*., 2007] but levels remained high. Another study found decreased expression of PD-1, TIM-3 and CTLA-4 on virus-specific CD4 T cells in patients receiving effective ART but they did not look at expression on total peripheral PBMC, nor did they include analysis of healthy donors [Kassu *et al*., 2010]. Percentage expression of TIM-3 was significantly elevated on CD4 T cells from HIV-1 infected individuals compared to healthy donors and an increased MFI was observed on the CD8 T-lymphocyte population from these same subjects. TIM-3 levels have been found to be increased in HIV-1-infected patients with acute or chronic progressing HIV-1 infection and this is on both virus-specific and non-specific CD4 and CD8 T cells with impaired responses to stimuli; expression was found to be reduced in some, but not all, patients following initiation of ART [Golden-Mason *et al*., 2009; Jones *et al*., 2008; Downey and Imami, 2010]. Of note, the levels of TIM-3 expressed on T lymphocytes from HIV-1-infected patients and healthy donors were very low compared to previous publications.

There were no significant differences in the expression of markers associated with a regulatory T-cell phenotype in HIV-1^+ subjects compared to healthy controls and this agrees with another report of largely normalized T-regulatory cell frequencies with ART [Presicce *et al*., 2011].

A significantly diminished expression of CD31 was found on CD4 T cells from HIV-1^+ subjects compared to healthy donors. In one study, the proportion of $CD4^+CD45RA^+CD31^+$ T cells declined with age in both ART-treated HIV-1 infected individuals and in healthy control subjects

[Tanaskovic *et al*., 2010] and no differences were seen in the co-expression of these two markers. Higher single expression of CD31 on CD4 T cells from the healthy donors might be in part explained by the younger median age of this control group compared to the HIV-1-infected group; ideally the two groups would be age-matched to account for phenotypic differences that are seen with age [Appay and Sauce, 2008; Tenorio *et al*., 2009; Cao *et al*., 2009]. PTK-7, a marker to measure circulating $CD4^+$ recent thymic emigrants, was found to be expressed at very low levels on both T-cell subsets with no significant differences between HIV-1-infected individuals and healthy control subjects. The frequency of PTK-7 has been found to be age-dependent with approximately 10% of $CD45RA^{high}CD45RO^{low}PTK\text{-}7^+$ described in the CD4 T-cell compartment [Haines *et al*., 2009] which is considerably more than the percentage expression found in these experiments.

These results indicate that treatment with ART only serves to partially restore immunophenotypic dysfunction seen in chronic HIV-1-infection and that recovery of these parameters is perhaps in part better for individuals that have been on ART for longer, with higher CD4 T-cell counts and higher nadir CD4 T-lymphocyte counts.

There were no significant differences found in the immunophenotype of patients classified as Gag- and/or Nef-specific responders but trends were noted, such as a tendency for the Nef-specific responders to express lower levels of CD38 on CD4 T cells. Increased numbers of patient samples, for individuals with closely matched characteristics, defined according to their Gag- or Nef-specificity might help to further determine any correlations between functional responses and immunophenotype. Understanding the impact of antigen specificity on markers associated with T-cell dysfunction might provide further insight into targets for immune-based therapies.

Significant negative correlations were found between CD4 T-cell count and parameters associated with chronic immune activation, differentiation, exhaustion and apoptosis, and significant positive correlations were found between CD4 T-cell count and nadir CD4 T-lymphocyte count, and between CD4 T-cell count and duration of ART. Nadir CD4 T-cell numbers and the duration of ART-induced aviraemia have been shown to be important factors for CD4 T-cell recovery [Cellerai

et al., 2011]. This emphasises the importance of the timing of ART initiation and duration, particularly in the context of immune reconstitution [Koegl *et al*., 2009] and may provide insights into the timing and duration of immune-based therapies in the context of treated HIV-1 infection.

Chapter 6 General Discussion

The work presented in this thesis centres around the investigation of novel immune-based therapeutic approaches in treated chronic HIV-1 infection, and further assessment of known immunomodulators. There are three clinical trials which are described and which formulate the main findings of this thesis. Although there are a number of studies in which rhGH has been administered to HIV-1-infected patients on ART, rarely do these studies assess HIV-1-specific functional T-cell responses. Most other rhGH trials investigate the impact on the thymus (thymic density, TRECs, naïve CD4 T-cell counts), other clinical parameters, and even immune activation, highlighting a major strength of the experiments carried out here. 4mg/day rhGH for 12 weeks was found to increase proliferative CD4 and IFN-γ-producing CD8 HIV-1-specific T-cell responses, although these responses declined with less frequent dosing. The low-dose rhGH study is the first study to describe the immunological impact of low-dose, long-term rhGH in treated HIV-1 infection. Administration of 0.7mg rhGH daily for 40 weeks resulted in increased IFN-γ production in response to peptide pools of HIV-1 Gag (both 9mers and 20mers), with a trend toward a reduction in activation markers at week 40 compared to baseline for the most robust responder in the ELISpot assays. The IMIRC 1003 clinical trial incorporated known immunomodulators (therapeutic immunisation, IL-2, GM-CSF and rhGH) in a novel treatment schedule in order to induce and sustain desired immunological responses. In this study, the most promising immune changes were seen following administration of cytokine and hormone in addition to therapeutic immunisation, with increased total CD4 T-cell counts, improved CD4/CD8 T-cell ratios and reduced immune activation seen at week 48 compared to baseline. Limitations of this particular study were due to problems finding patients who fit the strict enrolment criteria, and 12 were enrolled rather than an anticipated 30. The findings described in this thesis could have been improved with further investigations which include but are not limited to: HIV-1 proviral DNA assessment for the IMIRC 1003 clinical trial samples (to elucidate the effect on the viral reservoir),

pentamer/tetramer staining (using HLA-types of the twelve patients; to allow for HIV-1-specific T cells to be phenotyped in addition to peripheral T-cell analysis), FoxP3 staining (to further determine/confirm the impact of this treatment schedule on regulatory T cells), and greater numbers of patients defined as Gag and/or Nef responders (to allow for more accurate correlations of this functional parameter with immunophenotype to be made).

The findings shown here highlight the importance of the development of new, and/or modification of existing, immune-based therapeutic approaches in the context of treated chronic HIV-1 infection. Even for a carefully defined subset of aviraemic HAART-treated patients, with high CD4 T-cell counts and high nadir CD4 T-lymphocyte counts, immune defects were seen to persist for both CD4 and CD8 T cells. Expression levels of activation markers (HLA-DR, CD38), senescence (CD57), co-inhibition (CTLA-4), exhaustion (TIM-3), apoptosis (CD95), and a late ($CD27^{-}CD28^{-}$) or terminally differentiated ($CCR7^{-}CD45RA^{+}$) state were found to be significantly higher in HIV-1-infected patients compared to healthy seronegative controls. Most HIV-1^{+} persons on ART achieve and maintain viral suppression to less than 50 copies/ml plasma HIV-1 RNA [Palmer *et al.*, 2008], however abnormally high ($CD8^{+}HLA\text{-}DR^{+}CD38^{+}$) T-cell activation levels persist, despite successfully suppressive antiretroviral therapy, compared to healthy controls [Hunt *et al*., 2003; Hunt *et al*., 2008, Robbins *et al*., 2009]. The increased translocation of microbial products as a result of the compromised gastrointestinal mucosal barrier has been proposed to be a continuous trigger of immune activation in chronic HIV-1 infection [Brenchley *et al*., 2006; Marchetti *et al*., 2008]. Microbial translocation has been found to correlate positively with immune activation and inversely with immune reconstitution, and although levels (in terms of plasma LPS) are reduced with ART, they are not normalised [Brenchley *et al*., 2006; Jiang *et al*., 2009; Brenchley & Douek, 2012]. This hyper-activation of the immune system may be associated with, and causally related to, the higher than expected risk of premature heart disease, kidney disease, liver disease, bone disease, cancer and neurocognitive dysfunction in ART-treated HIV-1-infected persons compared to healthy controls [Volberding & Deeks, 2010]. Interestingly, sooty mangabeys, natural hosts for SIV

infection, do not develop AIDS despite high levels of viral replication (and limited virus-specific CD8 T-cell responses), which has been attributed to lower levels of immune activation and senescence than that observed in pathogenic SIV or HIV infections [Silvestri *et al*., 2003; Pandrea *et al*., 2008]. Immune activation is reportedly lower in HIV-1-infected patients that are able to suppress viraemia in the absence of ART (elite controllers) when compared to non-controllers (patients not on ART with detectable plasma HIV-1 RNA) [Hunt *et al*., 2008; Owen *et al*., 2010], but this reduced activation is still higher than in seronegative controls [Hunt *et al*., 2008]. In one study, HIV-1 controllers were found to have higher levels of activated CD8 T cells (as measured by co-expression of HLA-DR and CD38) compared to ART-treated aviraemic patients [Hunt *et al*., 2008], while another reported no difference in activation between these groups, although it is important to note that this second study calculated expression of $CD8^{+}IFN\text{-}\gamma^{+}HLA\text{-}DR^{+}CD38^{+}$ T cells following stimulation with HIV p55 [Owen *et al*., 2010]. They also reported fewer recently proliferated HIV-1-specific CD4 T cells from elite controllers compared to patients on suppressive ART, as measured by expression of CD4, IFN-γ and Ki-67 subsequent to stimulation with HIV p55 [Owen *et al*., 2010]. Elite controllers are unable to completely clear HIV-1 [Hatano *et al*., 2009], and it has been suggested that these individuals, as well as those identified as LTNP, will eventually progress to AIDS [Deeks & Walker, 2007; Hunt *et al*., 2008; Mandalia *et al*., 2012]. Thus, there is evidence that even with viral control (both in the presence and absence of ART) a means of reducing immune activation in HIV-1 infection is required.

Attempts have been made using ART intensification to reduce immune activation and low-level viraemia in ART-treated HIV-1-infected patients; however this approach did not decrease levels of plasma HIV-1 RNA (as measured using a single copy sensitivity assay) for efavirenz, lopinavir/ritonavir, or atazanavir/ritonavir intensified regimens [Dinoso *et al*., 2009], or following raltegravir intensification [Gandhi *et al*., 2010]. Reductions in immune activation were not observed when comparisons were made between patients that had received raltegravir intensification versus those that didn't [Buzon *et al*., 2010]. Further, enfuviritide therapy (two reverse transcriptase

inhibitors and a ritonavir-boosted protease inhibitor) did not result in a depletion of the latent viral reservoir, nor did it result in a reduction in immune activation [Gandhi *et al*., 2010]. One study, however, did report a decrease in HIV-1 RNA in the ileum of subjects receiving a raltegravir-containing intensification (with no impact on HIV-1 RNA from plasma, PBMC, duodenum, colon or rectum), as well as trends toward a reduction in CD4 and CD8 T-cell activation at these sites (although these were not statistically significant) [Yukl *et al*., 2010]. Optimisation of ART has included use of the CCR5-antagonist Maraviroc; in a phase II randomised clinical trial (MOTIVATE); significant increases in CD4 T-cell counts and reduced plasma HIV-1 RNA were observed in pre-treated patients that received Maraviroc in addition to ART [Gulick *et al*., 2008], and Maraviroc has been associated with decreased immune activation [Wilkin *et al*., CROI, 2010; Wilkin & Gulick, 2012].

Therapeutic approaches targeting the gut-associated lymphoid tissue (GALT), where CD4 T-cell numbers and T_H17 cells are not restored with ART, have been proposed as a means of reversing the damage to the gut mucosa and minimising the translocation of microbial products, factors thought to play a role in the persistent inflammation and immune activation in chronic HIV-1 infection [Hunt, 2010]. Pigtail macaques infected with SIV were found to have increased reconstitution of CD4 T cells, increased polyfunctionality, and reduced T-cell turnover (as measured by Ki-67 expression on CD4 memory T cells) in the colon when probiotics were given in combination with antiretroviral treatment [Klatt *et al*., CROI, 2012]. A phase II trial is currently recruiting ART-treated patients with incomplete CD4 T-cell recovery in order to determine the effects of the antibiotic rifaximin on microbial translocation and systemic immune activation (clinicaltrials.gov, NCT01466595). Evidence from pigtail macaques infected with SIV that received rifaximin and (an anti-inflammatory drug) sulfasalazine, demonstrated that together these agents reduced microbial translocation (as measured by sCD14), reduced viral load, lowered levels of immune activation ($CD8^+HLA\text{-}DR^+Ki\text{-}67^+$) and inflammation (IL-6 and RANTES), and decreased mucosal CD4 T-cell depletion during acute SIV infection (compared to untreated controls) [Pandrea *et al*., CROI, 2012].

Patients with HIV-1 and CMV co-infection treated with valganciclovir showed undetectable levels of CMV DNA following eight weeks of treatment, and significantly greater reductions in CD8 T-cell activation (in terms of percentage HLA-DR and CD38 co-expression) compared to placebo-treated patients, although these individuals presented with incomplete CD4 T-cell recovery on ART and immune activation was thought to arise as a result of CMV replication [Hunt *et al*., 2011]. Administration of hydroxychloroquine to patients defined as immunologic non-responders (CD4 T-cell counts <200 cells/μl blood, CD4 T-cell increase <5% in 12 months prior to study initiation) despite suppressive ART, resulted in reduced CD4 T-cell proliferation (Ki-67), decreased CD8 T-cell activation ($HLA\text{-}DR^+$, $CD38^+$), lower levels of plasma LPS and a decline in pro-inflammatory cytokines (IL-6 and TNF-α), as well as increased CD4 T-cell percentages [Piconi *et al*., 2011]. In another study, chloroquine in untreated subjects resulted in reduced CD4 and CD8 T-cell proliferation (as measured by Ki-67) and reduced CD8 T-cell activation (again, as determined by co-expression of HLA-DR and CD38) [Murray *et al*., 2010], although it is thought to be more promising as an adjunctive therapy to ART [Ries *et al*., 2012]. Chloroquine interferes with endosomal acidification, and in cell culture it was found to reduce IFN-α production by HIV-1 and HIV-1-infected cells, lower the expression of CD38 on CD8 T cells, and block IDO and PD-L1, two negative regulators of T-cell responses [Martinson *et al*., 2010].

The patients described here who received 40 weeks of low-dose rhGH showed a trend toward a reduction in the expression of HLA-DR and CD38 (both in terms of co-expression and single expression, looking at percentage expression and MFI) on both CD4 and CD8 T cells compared to baseline. In another study in which rhGH was administered daily to ART-treated patients for one year (3.0mg daily for 6 months and 1.5mg/day for 6 months), significant reductions in the percentage co-expression of HLA-DR and CD38 on both CD4 and CD8 T lymphocytes were seen compared to placebo-treated controls [Napolitano *et al*., 2008]. Individuals in the IMIRC 1003 trial who received IL-2, GM-CSF and rhGH (with or without immunisation) had marked increases in the percentage co-expression of HLA-DR and CD38 at week 2 following administration of the

cytokines. However, by week 48 these individuals showed significant reductions in the percentage expression of CD38 on both CD4 and CD8 T cells (and a decrease in CD38 MFI on CD4 T lymphocytes) compared to baseline. These results provide further evidence that immune-based therapy in the context of treated HIV-1 infection may serve to reduce immune activation further to the effects of ART alone.

Although there was no marked impact of rhGH administration to patients in the IMIRC trial treatment schedule, particularly looking at the analysis of markers associated with recent thymic emigration (CD31 and PTK-7), it cannot be said that this immunomodulator did not contribute to the reduced expression of activation and senescence markers seen at week 48 in patients that received cytokine and hormone therapy. It is possible that the duration of rhGH treatment (five days) was not long enough to induce significant HIV-1-specific responses, whereas the two rhGH trials described in this thesis showed improved virus-specific functionality following 12 and 40 weeks of rhGH therapy. The incomplete restoration of the immune system with ART has been partly attributed to thymic dysfunction and an inability to produce new CD4 T cells [Valdez, 2002; Douek *et al*., 1998]. rhGH treatment in addition to ART has been shown to increase thymic density [Napolitano *et al*., 2002; Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010], elevate the frequency of circulating TREC within PBMC [Napolitano *et al*., 2008; Hansen *et al*., 2009; Smith *et al*., 2010] and increase the number of naïve CD4 T cells [Pires *et al*., 2004b; Napolitano *et al*., 2008]. Following 12 weeks of rhGH, there were no significant changes in TREC levels; this might be reflective of unaltered thymic output or it may be due to an increased division rate of the naïve T-cell pool [Herasimtschuk *et al*., 2008]. Thymic density, TREC levels and naïve T-cell numbers were not assessed in the IMIRC 1003 trial following rhGH administration; however, assessment of naïve T cells based on their immunophenotpye (using CCR7 and CD45RA co-expression) did not show any impact at week 4. The duration of rhGH treatment may not have been enough to elicit an increase in the number of naïve T cells, or transient increases may have occurred which were then absent at week 4 (samples were not taken at week 3). Another attractive adjuvant

therapy in treated HIV-1 infection is recombinant human interleukin-7 (rhIL-7). IL-7 responsiveness is largely dependent upon the presence or absence of the IL-7 receptor (IL-7R) that is found on most mature T cells, and it plays a crucial role in T-cell homeostasis [Park *et al*., 1990], which includes promoting the survival of naïve T cells [Schluns *et al*., 2000]. HIV-1-infected individuals have higher levels of IL-7 and reduced levels of IL-7R compared to healthy controls [Llano *et al*., 2001; Rethi *et al*., 2005], and these levels are not restored even after seven years of ART [Shou *et al*., 2011]. Phase I and II studies using rhIL-7 in HIV-1-infected patients described it to be well-tolerated but caused a transient increase in viral replication; an effect that is not seen when it is used in conjunction with ART [Sereti *et al*., 2009; Levy *et al*., 2009]. rhIL-7 administration was found to significantly increase both naïve and central memory CD4 T-cell counts, in a dose-dependent manner, elevate levels of RTE, increase numbers of TREC, and increase naïve CD4 T-cell counts, an indication of enhanced thymopoiesis [Sereti *et al*., 2009; Levy *et al*., 2009; Sportes *et al*., 2008; Rosenberg *et al*., 2006; Okamoto *et al*., 2002]. Preliminary data from the INSPIRE 2 study indicate that IL-7 administration may increase the expression of gut-homing receptors (α4β7 integrin) and decrease the expression of PD-1 on peripheral CD4 and CD8 T cells [Sereti *et al*., CROI, 2011]. The increased homing potential may account for the induced expansion of CD4 T cells in the gut mucosa following treatment with IL-7, and these CD4 T lymphocytes were predominantly of a central memory phenotype ($CD45RO^{+}CD27^{+}$) [Sereti *et al*., CROI 2012].

Patients that received both the pharmacological dose and the longer term physiological dose of rhGH demonstrated increased HIV-1-specific T-cell responses whereas patients on the IMIRC 1003 trial only showed occasional alterations in T-cell functionality throughout the course of the study. The production of IL-2 and IL-15 is defective in HIV-1-infected patients [Sirskyj *et al*., 2008], and PBMC from immunologic non-responders (where CD4 T-cell recovery does not occur with ART) stimulated with PHA have been shown to produce less IL-2 than PBMC from HIV-1^{+} subjects with higher CD4 T-lymphocyte counts [Erikstrup *et al*., 2010]. Data from one group found that LTNP

had a greater recovery of CD8 T-cell polyfunctionality, proliferation and cytotoxicity when compared to aviraemic subjects in which ART had been initiated in the chronic stage of infection [Migueles *et al*., 2009]. Another study found that patients treated with ART in early HIV-1 infection had strong polyfunctional CD4 and CD8 T-cell responses with low perforin expression in virus-specific CD8 T cells, similar to responses from patients in their LTNP cohort; however, a higher breadth of response and a trend towards a higher magnitude of response was seen particularly for Gag-specific CD8 T cells from LTNP [Cellerai *et al*., 2011]. Slow, or non-progressive HIV-1 infection has been found to be associated with polyfunctional HIV-1-specific T-cell responses [Betts *et al*., 2006; Zimmerli *et al*., 2005; Almeida *et al*., 2007; Daucher *et al*., 2008], although the role of this polyfunctionality in viral control has been the subject of much debate [Cellerai *et al*., 2011]. HIV-2-infected patients, who have an overall better prognosis than subjects with HIV-1, have been shown to possess polyfunctional T-cell profiles [Duvall *et al*., 2008], lending weight to the idea that polyfunctionality is indeed imperative. A recent re-evaluation of the cellular correlates of protection [Makedonas & Betts, 2011], particularly in terms of the design of a prophylactic HIV-1 vaccine, and taking into consideration the results from the Merck STEP trial [Buchbinder *et al*., 2008] and the Thai RV144 trial [Rerks-Ngarm *et al*., 2009], has speculated on the parameters of CD8 T-cell phenotype and function that need to be considered. The authors highlight the necessity for, at the very least, a high frequency of effector CD8 T cells present at the site of infection (the mucosa) with moderate cytolytic ability, and they propose a panel of T-cell markers that may have a role, either alone or in combination, in defining a correlate of protection against HIV-1, and that may be used to assess potential HIV-1 vaccine candidates (Table 6.1) [Makedonas & Betts, 2011].

Table 6.1 T-cell markers that may potentially correlate with protection against HIV-1 infection. (Taken from Makedonas & Betts, 2011).

T-cell aspect	Markers
Memory phenotype	CCR7, CD27, CD28, CD45RA/RO, CD57, CD62L
Cytokine production and Cytotoxicity	IFN-γ, TNF-α, IL-2, IL-4, IL-10, 1L-12, IL-17, IL-23 Perforin (new and granule-associated) Granzyme A/B/K, Granulysin
Miscellaneous	Degranulation (CD107a exposure) MIP-1α/β, proliferation (CFSE dilution)
Transcriptional control	T-bet, Eomesodermin, BLIMP-1, GATA, ROR-γT
Activation status	HLA-DR, Ki-67, bcl-2, CD38, CD69, CD95
Exhaustion	PD-1, CD160, Lag-3, 2B4, TIM-3
Tissue trafficking	α4β7, CCR5, CCR9, CCR10, CD161
Regulatory function	CTLA-4, FoxP3, GITR, CD25, CD39, CD73, TGF-β

Analysis of frozen PBMC from the IMIRC trial could further elucidate the impact of the immunotherapy treatment schedule, particularly on CD8 T-cell responses. A six-colour panel was designed and samples run on an LSR II for all phenotypic experiments described in this thesis. Another laser has since been added to the flow cytometer, allowing for the design of ten colour panels. Assessing multiple parameters by increasing the number of antibodies per tube allows for the distinctive co-expressions of markers to be determined, for example, one study compared the expression of CTLA-4, PD-1 and TIM-3 on HIV-1-specific T cells from treated and untreated HIV-1-infected patients, and further determined the IFN-γ and IL-2-producing capability of these cells (these were significantly lower for ART-treated patients compared to viraemic untreated individuals) [Kassu *et al*., 2010]. Another group investigated the co-expression of CD38 and PD-1 on virus-specific CD8 T cells (and found levels to be higher in HIV-1 progressors compared to HIV-1 controllers, and that levels decreased following initiation of ART) [Vollbrecht *et al*., 2010]. Non-progressive HIV-1 infection has been found to be associated with Nef-specific CD8 T cells that are $CD45RA^{+}$ and secrete MIP-1β but not IFN-γ [Dembek *et al*., 2010]. Future work on the IMIRC 1003 cryopreserved PBMC samples could thus include the evaluation of HIV-1-specific T-cell responses and an expanded antibody panel which includes intracellular markers in addition to the surface markers. HLA-typing has been performed for all twelve individuals enrolled on the trial

(the results of which are presented in Chapter 4), and pentamer/tetramer staining would allow for more concise evaluation of the HIV-1-specific T cells. A long-lived memory T-cell population with an enhanced capacity for self-renewal and the ability to become central memory, effector memory and effector T cells was recently described, and these were termed memory stem T cells (T_{SCM}) [Gattinoni *et al*., 2011]. These T_{SCM} cells had a phenotype characteristic of naïve T cells ($CD45RO^-$ $CCR7^+CD45RA^+CD62L^+CD27^+CD28^+IL\text{-}7R\alpha^+$), but displayed functional attributes of memory cells (they expressed large amounts of CD95, IL-2Rβ, CXCR3 and LFA-1) and compared with known memory populations, they had increased proliferative capacity and more efficiently reconstituted immunodeficient hosts, however, this profile remains to be elucidated in HIV-1 infection and may be relevant in terms of vaccine design and T-cell therapies [Gattinoni *et al*., 2011]. One emerging technology that allows a very large number of parameters to be simultaneously measured at a single cell level, thus making it possible to more easily evaluate these complex T-cell profiles, is mass cytometry. Instead of fluorescent dyes, mass cytometry joins rare-earth metals to antibodies and eliminates the need for the calculation of spectral overlap (compensation) and has no issues with cell-dependent background signal [Bendall *et al*., 2011].

The viral inhibition assay, which measures CD8 T-cell-mediated inhibition of HIV-1 replication using autologous T cells and replication competent viruses (including primary isolates and lab-adapted strains) and does not require knowledge of HLA-type, has been proposed as an immunogenicity assay to test potential HIV-1 vaccine candidates [Spentzou *et al*., 2010]. This cell-mediated inhibition of HIV-1 was found to correlate with CD107a or MIP-1β expression from HIV-1-specific T cells from chronically infected virus controllers [Freel *et al*., 2010]. The development and optimisation of assays that identify antiviral CD8 T-cell responses, is key, not only in terms of assessing HIV-1 prophylactic vaccines, but also in the evaluation of immune-based therapies. The results of such experiments on samples from ART-treated chronically HIV-1-infected persons have been mixed; one study found that CD8 T cells from these individuals were able to efficiently inhibit

viral replication [Freel *et al*., 2010], while another study reported that this CD8 T-cell-mediated viral inhibition was not preserved in treated aviraemic subjects [Spentzou *et al*., 2010].

Immune-based therapeutic approaches in treated, chronic HIV-1 infection have a number of targets, some of which have been discussed: improvement in the integrity of the gut mucosa and GALT, enhanced thymic output or reversal of thymic involution, increased production of naïve CD4 T cells, increased T-cell polyfunctionality, and reduced immune activation, immune senescence and exhaustion. A crucial parameter that must be considered is the impact of such immunotherapies on the latent HIV-1 reservoir. Studies of ART intensification found that it was unable to eradicate HIV-1 from the stable reservoir [Gandhi *et al*., 2010], and administration of rhGH in treated HIV-1 infection had no impact on the levels of proviral DNA [Herasimtschuk *et al*., 2008]. HIV-1^{+} patients on suppressive ART have detectable levels of HIV-1 DNA in PBMC, as well as measurable levels of HIV-1 DNA and HIV-1 RNA in the rectal mucosa-associated lymphoid tissue [Anton *et al*., 2003]. One study comparing HIV-1 RNA and DNA levels in the GALT found HIV-1 controllers to have lower levels than HIV-1 non-controllers, but similar levels to subjects on suppressive ART, suggesting that lymphoid tissues are an active viral reservoir even for patients that are able to maintain low plasma viraemia in the absence of ART [Yukl *et al*., CROI, 2012]. Initiation of ART during the early stages of HIV-1 has been associated with a decrease in the residual viral reservoir, comparable to levels found in LTNP, an effect that is not seen when ART is initiated during chronic HIV-1 infection [Pires *et al*., 2004a]. The major cellular reservoirs for HIV-1 have been found to be the central memory (T_{CM}; $CD45RA^{-}CCR7^{+}CD27^{+}$) and the transitional memory (TTM; $CD45RA^{-}CCR7^{-}CD27^{+}$) CD4 T cells [Chomont *et al*., 2009]. The T_{CM} cells were shown to primarily harbour this reservoir for ART-treated HIV-1^{+} patients with higher CD4 T-cell counts, whereas for aviraemic patients on ART with poor CD4 T-lymphocyte reconstitution, HIV-1 proviral DNA resided mainly in the T_{TM} cells, which persisted through IL-7-mediated homeostatic proliferation [Chomont *et al*., 2009]. LTNP individuals with the protective HLA-B*27/B*57 alleles have recently been described as having lower levels of cell-associated HIV-1 DNA in their CD4

T_{CM} cells compared to LTNPs without these alleles [Descours *et al*., 2012]. This reduced viral reservoir was found to correlate with preserved CD4 T_{CM} cell numbers, the magnitude of HIV-1 Gag-specific CD8 T cells, and lower levels of activation on memory CD4 T cells [Descours *et al*., 2012]. Thus it would be of considerable interest to elucidate the impact of immune-based therapy on the viral reservoir in this particular subset; to determine if there is the potential to boost the immune system in chronic HIV-1 infection to more closely resemble that of a LTNP.

For patients enrolled on the IMIRC 1003 study who received IL-2, GM-CSF and rhGH in addition to therapeutic immunisation, significantly elevated CD4/CD8 T-cell ratios were seen at week 48 compared to baseline. CD4/CD8 T-lymphocyte ratios in patients on suppressive ART have been reported to inversely correlate with levels of HIV-1 proviral DNA [Boulassel *et al*., 2012], suggesting that this combination of immunomodulatory agents in addition to ART may have reduced the latent viral reservoir in these patients. Future work would include assessment of proviral DNA levels at baseline and at week 24 (and/or at week 48) of the study, using cryopreserved PBMC samples, in order to determine the impact of this treatment schedule on the HIV-1 latent reservoir, expansion of the phenotypic panel to include markers to assess T_{SCM} and T_{TM} cells in addition to T_{CM} and T_{EM} profiles. To this effect, analysis of plasma samples could be measured for LPS or sCD14 in order to determine the effect of these immunotherapies on microbial translocation, especially as the levels of circulating LPS in the first years of chronic HIV-1 infection have been found to be a strong indicator of disease progression [Marchetti *et al*., 2011].

One compound that has been described as able to activate the latent viral reservoirs in HIV-1 infection, without global T-cell activation, is the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA, vorinostat); a drug approved for the treatment of cutaneous T-cell lymphoma in patients with progressive, persistent, or recurrent disease [Edelstein *et al*., 2009]. Patients on fully suppressive ART that received a single dose of vorinostat demonstrated a marked increase in HIV-1 RNA expression in highly purified resting $CD4^+$ T cells [Archin *et al*., CROI, 2012]. *In vitro* studies have reported that stimulation of HIV-1-specific CTL

may be essential for latent viral eradication, as resting CD4 T cells (in which latency had been reversed with SAHA), were not killed by viral cytopathic effects, nor by the host CTL response following viral reactivation [Shan *et al*., CROI, 2012]. Following the case of Timothy Ray Brown, the leukaemia patient who was the first person to ever be cured of HIV-1 infection [Allers *et al*., 2011], new lines of investigation have opened up in search of a cure for HIV-1, and although a number of methodologies are being tested, it is generally believed that a combinatorial approach, particularly one that targets the viral reservoir, is most likely to be successful [Cairns, 2011; Cairns, 2012]. The potential to induce a functional cure is currently being explored by treating patients in very early acute HIV-1 infection with a combination of three or five antiretroviral drugs for two years, followed by ART cessation; with the aim to induce remission at acute infection [Lafeuillade, 2012].

Continued investigations into immunomodulatory agents that might purge the HIV-1 reservoir are warranted. The development and testing of new therapies, or the modification of existing ones to reduce chronic immune activation and/or inflammation, to decrease immunosenescence, and to reverse HIV-1-specific T-cell dysfunction have the potential to improve quality of life and delay the time to progression in chronic treated HIV-1 infection [Richman *et al*., 2009]. Such studies will form an essential foundation for large phase II/III immunotherapy trials with analytical treatment interruption, in the UK and elsewhere in the world. There is the potential for successful immunotherapy to be followed by the removal of ART with long periods of self-sustained low viraemia, and such strategies have a clear patient benefit.

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Appendix

Appendix 1 Baseline values for IMIRC 1003 clinical trial

The statistical analysis for Chapter 4 (the IMIRC 1003 trial) looked at changes from baseline. Thus, here the baseline values are documented, and these include baseline patient characteristics, baseline ELISpot responses and baseline phenotypic values (both percentage expression and MFI).

Appendix Table 1 Baseline patient characteristics for the twelve IMIRC 1003 study participants.
Cell counts are in cells per µl blood and HIV-1 RNA is per ml plasma.

Short code	Group	CD3 count	CD3 %	CD19 count	CD19 %	CD4 count	CD4 %	CD8 count	CD8 %	CD56 count	CD56 %	ISUM	CD4: CD8 Ratio	HIV-1 RNA
R771	1	1652	79.7	304	14.1	884	44.4	659	33.1	88	4.1	2044	1.3	<50
B784	1	2887	84	304	8.8	1332	39	1471	43.1	161	4.6	3352	0.9	<50
G739	1	1107	81.6	145	10.5	534	40.1	496	37.2	86	6.3	1338	1.1	<50
P087	2	1862	81.5	169	7.6	731	31.4	1158	49.8	225	10.1	2256	0.6	<50
C789	2	1356	74.8	229	12.6	535	29.8	642	35.7	108	5.9	1693	0.8	<50
L043	2	2204	91.1	76	3.2	782	31.8	1416	57.6	107	4.5	2387	0.6	<50
C319	2	2430	80.2	375	12.9	1077	34.2	1543	49	188	6.4	2993	0.7	<50
F810	3	1255	87.8	65	4.5	582	40.6	710	49.5	87	6.1	1407	0.8	<50
O523	3	1514	82.4	187	10.3	892	47.6	673	35.9	116	6.4	1817	1.3	<50
S648	3	1664	62.8	268	9.9	578	22.4	962	37.2	704	25.9	2636	0.6	<50
C241	3	957	67.7	158	11.3	466	32.5	505	35.2	258	18.5	1373	0.9	<50
P054	3	2066	78.6	500	18.6	840	32.8	1168	45.6	73	2.7	2639	0.7	<50

Appendix Table 2 Baseline ELISpot responses to peptide pools from NIBSC.
IFN-γ, IL-2, IL-4 and perforin baseline values are shown here as spot forming cells per million PBMC, and include responses to TCM and PHA.

Short code	Group	IFNg_TCM	IFNg_p17	IFNg_p24	IFNg_Nef	IFNg_Tat	IFNg_Rev	IFNg_PHA
R771	1	0	66	466	14	52	0	1584
B784	1	2	206	386	302	22	0	1726
G739	1	2	24	76	204	12	2	1410
P087	2	14	6	72	196	0	14	420
C789	2	14	6	8	250	20	22	1600
L043	2	0	2	676	68	0	36	414
C319	2	0	6	22	66	0	2	792
F810	3	12	18	334	20	0	2	1704
O523	3	4	12	44	576	62	30	282
S648	3	44	358	382	262	154	0	1568
C241	3	164	162	916	166	544	98	1740
P054	3	0	296	100	454	42	34	1762

Short code	Group	IL-2_TCM	IL-2_p17	IL-2_p24	IL-2_Nef	IL-2_Tat	IL-2_Rev	IL-2_PHA
R771	1	0	6	34	2	0	2	418
B784	1	2	136	168	18	2	2	438
G739	1	4	2	14	10	2	0	212
P087	2	6	20	10	4	4	2	200
C789	2	2	4	0	8	2	2	408
L043	2	0	96	24	2	0	0	254
C319	2	0	50	0	0	10	0	24
F810	3	2	0	26	8	0	0	412
O523	3	6	18	72	24	6	2	1600
S648	3	0	6	54	10	10	0	506
C241	3	0	4	30	8	0	4	1652
P054	3	0	412	8	0	0	0	212

Short code	Group	IL-4_TCM	IL-4_p17	IL-4_p24	IL-4_Nef	IL-4_Tat	IL-4_Rev	IL-4_PHA
R771	1	0	0	26	28	18	48	212
B784	1	6	12	0	4	6	0	394
G739	1	0	0	10	12	34	8	770
P087	2	8	2	2	4	10	4	374
C789	2	0	6	4	4	6	4	418
L043	2	0	0	2	0	0	0	56
C319	2	0	16	0	0	0	0	2
F810	3	0	14	14	12	8	14	462
O523	3	10	10	12	94	4	14	904
S648	3	0	2	0	0	2	0	232
C241	3	0	0	2	0	0	0	1186
P054	3	0	210	0	0	10	10	60

Short code	Group	PER_TCM	PER_p17	PER_p24	PER_Nef	PER_Tat	PER_Rev	PER_PHA
R771	1	22	82	74	20	66	72	236
B784	1	8	12	6	6	22	2	210
G739	1	4	12	2	8	106	4	50
P087	2	6	12	10	6	300	12	6
C789	2	200	390	210	546	232	148	414
L043	2	0	16	38	12	24	54	2
C319	2	0	22	0	40	40	26	0
F810	3	56	46	74	58	48	108	850
O523	3	12	4	14	0	32	10	6
S648	3	122	118	34	188	158	20	24
C241	3	60	222	104	16	226	130	186
P054	3	152	256	156	208	172	228	220

Appendix Table 3 Baseline ELISpot responses to peptide pools from FIT Biotech.
IFN-γ, IL-2, IL-4 and perforin baseline values are shown here as spot forming cells per million PBMC.

Short code	Group	IFNg_RevSub	IFNg_NefSub	IFNg_TatSub	IFNg_p17p24sub	IFNg_CTLsub
R771	1	0	0	4	182	2
B784	1	2	358	724	362	120
G739	1	16	270	12	162	10
P087	2	6	222	6	100	122
C789	2	54	90	24	72	164
L043	2	8	162	10	734	0
C319	2	0	0	2	44	4
F810	3	14	48	8	372	72
O523	3	16	506	28	48	6
S648	3	68	200	240	932	160
C241	3	154	152	116	1044	392
P054	3	10	422	6	178	24

Short code	Group	IL-2_RevSub	IL-2_NefSub	IL-2_TatSub	IL-2_p17p24sub	IL-2_CTLsub
R771	1	0	2	8	6	4
B784	1	2	56	14	74	26
G739	1	4	0	2	2	2
P087	2	2	2	18	14	6
C789	2	0	4	0	2	0
L043	2	0	4	2	28	4
C319	2	0	0	0	0	0
F810	3	0	0	4	18	0
O523	3	14	12	10	30	8
S648	3	2	6	10	134	6
C241	3	4	12	0	10	6
P054	3	0	0	0	0	0

Short code	Group	IL-4_RevSub	IL-4_NefSub	IL-2_TatSub	IL-4_p17p24sub	IL-4_CTLsub
R771	1	2	0	0	2	0
B784	1	2	14	6	10	14
G739	1	16	14	14	18	4
P087	2	2	14	2	2	0
C789	2	14	18	0	4	6
L043	2	0	0	0	0	0
C319	2	0	0	4	2	2
F810	3	20	28	20	0	2
O523	3	2	2	2	2	0
S648	3	0	2	2	0	0
C241	3	2	2	0	0	8
P054	3	0	0	0	2	0

Short code	Group	PER_RevSub	PER_NefSub	PER_TatSub	PER_p17p24sub	PER_CTLsub
R771	1	34	68	76	48	136
B784	1	6	16	10	2	4
G739	1	8	4	10	2	0
P087	2	52	24	18	26	8
C789	2	170	244	240	192	640
L043	2	46	44	40	56	50
C319	2	14	12	0	10	16
F810	3	68	70	38	40	58
O523	3	10	2	2	12	2
S648	3	40	26	40	4	80
C241	3	224	202	248	14	48
P054	3	236	266	196	280	258

Appendix Table 4 Baseline phenotype in terms of percentage expression.

Short code	Group	CD4+ HLADR+ CD38+	CD4+ HLADR+	CD4+ CD38+	CD4+ CD57+CD95+	CD4+ CD57+	CD4+ CD95+
R771	1	4.6	10	71.8		4.6	42
B784	1	4.8	12.6	52.7	11.8	12.5	56.7
G739	1	4.2	11.6	36.6	12.1	12.7	79.5
P087	2	3.3	9.1	32.6	4.7	5.1	81.2
C789	2	3.8	11	49.8	3	3.4	72.5
L043	2	5.1	15.2	45.8	12	12.4	68.3
C319	2	2.6	11.9	36.9	10.6	11.5	63
F810	3	4.8	9.2	59.7	10.7	11.1	48.1
O523	3	2.4	6.6	46.2	1.2	1.5	65.7
S648	3	4.4	10.5	52.7	11.4	11.9	61.5
C241	3	3.8	11	49.8	3	3.4	72.5
P054	3	3.4	6.1	66.3	11.3	12.1	45.7

Short code	Group	CD4+ CD27+CD28+	CD4+ CD27- CD28+	CD4+ CD27- CD28-	CD4+ CD27+	CD4+ CD28+	CD4+ CD45RO+CD25+
R771	1	91.8	3.1	4.3	92.7	94.8	18.6
B784	1	78.6	7.9	13.1	79	86.5	23.8
G739	1	72.6	8	18.4	73.6	80.6	42.5
P087	2	85.8	7.5	6.3	86.2	93.3	33.4
C789	2	65.1	5.3	27.4	67.4	70.5	13.3
L043	2	77.3	6.7	14.2	79	84.1	20.6
C319	2	80	3.3	16.3	80.3	83.3	41
F810	3	78.5	5.7	15.5	78.9	84.2	20.1
O523	3	95.4	2.1	0.2	97.7	97.4	32.3
S648	3	76.4	9	13.4	77.6	85.4	20
C241	3	85.3	7.9	6.5	85.5	93.3	38.3
P054	3	83.8	3.3	12.4	84.3	87.2	14

Short code	Group	CD4+ CD45RO+ CD25high	CD4+ CD25+	CD4+ CD45RO+	CD4+ CCR7+CD45RA+	CD4+ CCR7+CD45RA-	CD4+ CCR7-CD45RA-
R771	1	0.9	35.3	33.2	48.8	35.9	11.1
B784	1	2.8	34.9	46.5	38.2	27.4	27.2
G739	1	2.6	51.9	67.2	16.1	41	33.3
P087	2	2.1	39.3	72.6	16.2	53.6	26.1
C789	2	0.6	17.8	42.7	25.4	26	26.7
L043	2	2.8	23.3	58.1	25.9	36.5	26.6
C319	2	2	51.1	65.9	23.9	46.6	27.7
F810	3	1.1	32.6	36.1	45.2	28.4	16.7
O523	3	1.1	46.5	55.5	28.7	52.5	16.1
S648	3	1.5	37.7	29.4	47.8	9.9	15.9
C241	3	2.7	50.7	61.6	22.6	49.1	26.3
P054	3	2.6	23.6	37.5	49.7	27.7	18.3

Short code	Group	CD4+ CCR7- CD45RA+	CD4+ PD1+	CD4+PDL1+	CD4+ CCR7+CD45RA+ PD1+	CD4+ CCR7+CD45RA- PD1+	CD4+ CCR7-CD45RA- PD1+
R771	1	4.2	57.4	4.1	44	68.2	86.8
B784	1	7.2	46.8	14.2	25.6	51.7	77.3
G739	1	9.6	54.1	9.1	34.2	56.6	66.5
P087	2	4.1	66.5	18.6	38.1	65.7	82.2
C789	2	21.9	61.9	17.3	34.5	63.5	81.2
L043	2	11	39.8	14.2	18	43	62.3
C319	2	1.9	25.3	8.9	8.8	22.8	42.5
F810	3	9.8	51	6.5	34.7	61.5	77.8
O523	3	2.8	59.9	22.9	45.6	65.6	75

Appendix Table 4 continued

S648	3	26.4	43.2	7.1	31.8	57.7	72.7
C241	3	2	59.5	11.6	30.1	61.8	81.2
P054	3	4.4	44	18	27.1	54.5	75.3

Short code		CD4+ CCR7- CD45RA+ PD1+	CD4+ CCR7- CD45RA- PDL1+	CD4+ CCR7- CD45RA+ PDL1+	CD8+ HLADR+CD38+	CD8+ HLADR+	CD8+ CD38+
R771	1	76.4	3.9	3.1	13.7	32.7	47.6
B784	1	46.1	14.7	13.7	18.3	33.5	45.1
G739	1	41.3	7.6	5.8	11.4	35.5	26.6
P087	2	62.2	19.5	18	5.2	16.4	10.1
C789	2	65.6	12.6	11.5	9.7	18.6	38.4
L043	2	35.9	14.8	12.6	19.1	50	27.4
C319	2	25.2	7	8.2	5	30.8	14.7
F810	3	52.7	6.3	6.4	9.2	15.2	26.4
O523	3	64.6	21.4	20	5.2	11.4	25.1
S648	3	46.9	6.1	5.3	8.6	17.7	36.3
C241	3	65.7	9.7	5.4	9.7	18.6	38.4
P054	3	49	16	14.9	6.8	10.3	34.7

Short code	Group	CD8+ CD57+ CD95+	CD8+ CD57+	CD8+ CD95+	CD8+ CD27+CD28+	CD8+ CD27+CD28-	CD8+ CD27-CD28-
R771	1	16.8	17.3	59.2	72.5	6.2	18.3
B784	1	34.6	36.2	83.5	43.8	6.5	43.2
G739	1	36.7	37.8	88.1	38.8	15.3	42.6
P087	2	12.2	13.2	86	64.7	12.2	18.5
C789	2	16.5	17	79.2	40.3	20.6	36.2
L043	2	29.4	30.4	93.5	33.3	19.8	44.5
C319	2	32	34	84.2	47.2	4.8	41.9
F810	3	46.6	48.1	80.4	26.5	7.9	63.3
O523	3	4.3	5	76.2	85.5	10.7	1.9
S648	3	58.1	59.4	88	36.5	6.7	53.7
C241	3	16.5	17	79.2	67.1	3.3	22.8
P054	3	42.4	44.8	68	48	7.3	40.4

Short code	Group	CD8+ CD27+	CD8+ CD28+	CD8+ CD25+	CD8+ CD45RO+	CD8+ CCR7+CD45RA+	CD8+ CCR7+CD45RA-
R771	1	78.8	75.6	6.6	34.3	40.2	20.7
B784	1	50.4	50.2	9.2	28.9	14.8	7.7
G739	1	54	41.3	5.4	40.8	34.9	42.6
P087	2	77	68.7	11.1	48.1	5.3	35.8
C789	2	60.5	43.7	1	31.6	14.4	11.9
L043	2	53.3	35.8	1.9	27.2	5	5
C319	2	52.1	53.3	12	53.1	15.2	23.6
F810	3	34.6	28.8	6.7	19.6	17.1	6.3
O523	3	96.2	87.4	33.5	38.7	23.3	56
S648	3	43.3	39.8	2.7	18	18.3	1.2
C241	3	70.5	73.8	31.1	30.3	20	20.2
P054	3	55.5	52.3	2.6	16.5	29.2	9.3

Short code	Group	CD8+ CCR7- CD45RA-	CD8+ CCR7- CD45RA+	CD8+ PD1+	CD8+ PDL1+	CD8+ CCR7+CD45RA+ PD1+	CD8+ CCR7+CD45RA- PD1+
R771	1	24.6	14.4	70.7	37.8	54	83.1
B784	1	36	41.4	49.1	19	27.7	52.9
G739	1	5.8	16.7	51	14.5	42.7	61.1
P087	2	47.1	11.7	70.3	40.5	52.3	67.8
C789	2	57.4	16.2	71.4	31	45.6	81
L043	2	43.9	46.1	48.7	13.9	33.2	52.1
C319	2	40.1	21.2	22.9	21.9	15.6	28.5
F810	3	29.7	46.8	56.3	25.8	46.1	65.9

Appendix Table 4 continued

O523	3	14.3	6.4	59.5	43.2	49	58.8
S648	3	6	74.5	55	9.3	41.6	89.7
C241	3	45.1	14.7	62.9	24.1	43.3	63.8
P054	3	27.5	34	44.7	16.3	35.4	65

Short code	Group	CD8+ CCR7- CD45RA- PD1+	CD8+ CCR7- CD45RA+ PD1+	CD8+ CCR7+CD45RA+ PDL1+	CD8+ CCR7+CD45RA- PDL1+	CD8+ CCR7-CD45RA- PDL1+	CD8+ CCR7-CD45RA+ PDL1+
R771	1	86.4	73.4	39.8	26.5	4.8	3.3
B784	1	65.6	42.1	20.1	25.6	21.3	19.7
G739	1	53	35.4	14.5	15.8	4.9	4.2
P087	2	76.6	61.9	52.7	49.4	31.4	30.9
C789	2	75.1	73.5	41.1	46.1	26.6	26.1
L043	2	62.4	38.9	27.3	38.1	11.7	9.2
C319	2	25.5	11.9	33	31.4	17	14
F810	3	79	49.1	55.1	51.3	25.3	13.4
O523	3	71.7	65.3	46.7	46.5	31.6	24.5
S648	3	86.3	56.5	14.8	23.6	8.7	7.6
C241	3	72.8	60.6	26.1	32.8	22.1	16.9
P054	3	51.2	36.9	18.8	22	12.7	12.5

Short code	Group	CD4+ CD45RA+ CD31+	CD4+ CD31+	CD4+ PTK7+	CD4+ CTLA4+ TIM3+	CD4+ CD69+	CD4+ CTLA4+	CD4+ TIM3+
R771	1	36.7	44.5	0.5	0	1	0	0.8
B784	1	27.9	35.8	0.5	0.1	2.3	0.1	1.3
G739	1	13.7	40.6	1.2	0	1.2	0.1	1.4
P087	2	7.4	21.3	0.4	0.1	1.1	0.1	1
C789	2	30.2	44.2	0.3	0.2	2.1	0.2	2
L043	2	23.3	37	0.7	0.1	2.2	0.5	0.8
C319	2	15.8	27.8	0.4	0.1	1.7	0.5	1.9
F810	3	28.8	35.6	0.3	0.1	3.8	0.1	1.8
O523	3	19.2	44.2	0.6	0.1	0.7	0.1	1.4
S648	3	34.9	37.2	0.8	0.1	2.2	0.6	1.7
C241	3	14.2	42.9	0.5	0.1	1.3	0.2	1.5
P054	3	42.6	49.4	0.5	0	0.8	0.2	1.9

Short code	Group	CD8+ CD31+	CD8+ PTK7+	CD8+ CTLA4+ TIM3+	CD8+ CD69+	CD8+ CTLA4+	CD8+ TIM3+
R771	1	84.3	0.5	0.1	2.7	0.1	1.7
B784	1	77.7	0.6	0.2	4	0.5	2.1
G739	1	63.6	2	0.1	1.4	0.3	2.3
P087	2	65.4	0.3	0.1	2.5	1.6	1.6
C789	2	58.7	0.2	0	2.3	0.3	2.3
L043	2	80.2	0.8	0	7.6	0.5	0.8
C319	2	50.9	0.4	0	1.7	0.2	1.6
F810	3	58.6	0.4	0	6.5	0.1	3.7
O523	3	69.6	1.2	0.2	2.2	5.3	2.1
S648	3	52.8	0.6	0.1	2.6	0.3	3.2
C241	3	65.1	0.5	0.1	2.2	0.3	1.8
P054	3	57.7	0.3	0	0.7	0.4	2.3

Appendix Table 5 Baseline phenotype in terms of mean fluorescence intensity (MFI).

Short code	Group	CD4+ HLADR	CD4+ CD38	CD4+ CD57	CD4+ CD95	CD4+ CD45RO	CD4+ CD25	CD4+ CD28	CD4+ CD27	CD4+ PD1	CD4+ PDL1
R771	1	8833	2329	6571	3021	8407	857	4696	3956	492	2639
B784	1	7081	2054	6954	3177	9399	814	4871	3411	651	2693
G739	1	5780	1547	7608	3464	6278	601	6055	3291	571	2615
P087	2	7969	1349	3603	2873	6291	932	5107	2493	610	2618
C789	2	7066	1893	5710	3665	7732	1242	4936	3349	686	2648
L043	2	6393	1122	5416	1383	9891	345	2617	2268	499	2122
C319	2	7134	1135	8650	1254	12402	589	2603	2618	546	3075
F810	3	7842	1689	9336	3967	5133	938	4765	3901	631	2961
O523	3	8009	2327	1792	3516	5355	800	4740	3732	545	2470
S648	3	7291	2234	7940	3829	4660	783	5392	2808	604	2748
C241	3	7066	1893	5710	3665	4916	648	5186	2713	645	2403
P054	3	6974	1153	12132	1886	10157	569	2560	2962	573	2546

Short code	Group	CD8+ HLADR	CD8+ CD38	CD8+ CD57	CD8+ CD95	CD8+ CD45RO	CD8+ CD25	CD8+ CD28	CD8+ CD27	CD8+ PD1	CD8+ PDL1
R771	1	7910	966	6374	2013	7179	519	3181	3558	567	3321
B784	1	7172	1045	3329	2079	9260	651	4859	2498	700	3136
G739	1	5373	985	4850	1885	6414	493	5814	2434	564	3382
P087	2	5942	1439	2367	1998	7075	768	4386	2114	655	3208
C789	2	6936	1017	7361	2357	5553	1029	4724	2454	644	2826
L043	2	6571	693	2189	1106	8347	326	2808	1568	510	2926
C319	2	5621	550	4873	774	7917	413	2919	2033	555	3483
F810	3	6567	1993	6371	1856	5835	834	3356	2807	855	3647
O523	3	5461	1457	2081	2642	4287	716	4000	3113	555	2859
S648	3	6042	984	10049	2377	5891	457	6228	2181	710	4237
C241	3	6936	1017	7361	2357	5271	573	6429	2169	674	2589
P054	3	7993	789	9262	1031	9773	369	2728	2290	525	3149

Short code	Group	CD4+ CD31	CD4+ PTK7	CD4+ CD69	CD4+ CTLA4	CD4+ TIM3	CD8+ CD31	CD8+ PTK7	CD8+ CD69	CD8+ CTLA4	CD8+ TIM3
R771	1	1423	1202	4947	941	1613	2940	1870	4963	894	1465
B784	1	1710	967	5462	741	1157	1917	760	5845	337	1088
G739	1	1299	768	5851	609	934	1673	620	10650	541	1128
P087	2	1993	799	6357	992	2522	2019	3355	8119	2347	2424
C789	2	2423	914	8709	702	2843	2846	3227	9700	2924	2257
L043	2	984	416	6917	259	688	1181	373	7546	301	580
C319	2	1525	382	7937	250	569	1688	397	13581	333	637
F810	3	1518	991	6027	804	1469	2610	1070	7198	1251	1119
O523	3	1855	655	6079	867	1617	2305	1689	6348	1514	2000
S648	3	1810	927	4648	523	970	2404	816	7006	656	797
C241	3	1087	810	7450	596	905	1574	937	10743	1041	943
P054	3	1151	500	6555	257	702	1694	511	8161	282	688

Appendix 2 Detailed statistical analysis tables for IMIRC 1003 clinical trial

The following tables show the statistical analysis output for the IMIRC 1003 trial, evaluating changes from baseline to each of the study time points. For each randomisation group, mean change from baseline is shown, as well as the lower and upper confidence intervals. All p values are shown, in red where they are significant, and in blue where they are approaching significance.

Appendix Table 6 Changes from baseline in patient characteristics for each group.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
CD3 count	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-227	-687	233	0.3355	-50	-448	348	0.8063	-54	-410	302	0.7663
Week 2	2014	1554	2474	<.0001	-190	-589	208	0.352	2510	2154	2867	<.0001
Week 4	102	-358	562	0.666	-80	-478	319	0.6958	654	298	1011	0.0005
Week 6	623	163	1083	0.0096	-393	-791	6	0.0568	480	123	836	0.01
Week 8	-22	-482	438	0.9255	-293	-692	105	0.1529	207	-149	563	0.2581
Week 12	248	-212	708	0.2937	-349	-747	50	0.0902	375	19	732	0.0421
Week 16	-7	-467	453	0.9763	59	-340	457	0.7742	297	-59	653	0.1064
Week 24	74	-386	534	0.7522	-117	-516	281	0.5656	213	-143	569	0.2443
Week 48	276	-184	736	0.243	-158	-556	240	0.4391	63	-293	419	0.729
CD3%												
Week 1	0.60	-2.38	3.58	0.6939	-0.58	-3.15	2.00	0.6632	-1.36	-3.67	0.95	0.2512
Week 2	4.17	1.19	7.14	0.0075	-0.60	-3.18	1.98	0.6496	3.90	1.59	6.21	0.0014
Week 4	3.57	0.59	6.54	0.0213	0.28	-2.30	2.85	0.835	0.70	-1.61	3.01	0.5536
Week 6	4.23	1.26	7.21	0.0066	-2.60	-5.18	-0.02	0.0515	1.80	-0.51	4.11	0.13
Week 8	2.63	-0.34	5.61	0.0868	-1.20	-3.78	1.38	0.3644	1.80	-0.51	4.11	0.13
Week 12	2.80	-0.18	5.78	0.069	-0.18	-2.75	2.40	0.8945	2.62	0.31	4.93	0.0288
Week 16	3.60	0.62	6.58	0.0202	-1.10	-3.68	1.48	0.4056	1.46	-0.85	3.77	0.2183
Week 24	2.23	-0.74	5.21	0.1454	0.30	-2.28	2.88	0.8202	1.34	-0.97	3.65	0.2582
Week 48	2.00	-0.98	4.98	0.1917	-1.45	-4.03	1.13	0.2737	1.22	-1.09	3.53	0.3029
CD4 count												
Week 1	-58	-261	145	0.578	-52	-228	124	0.5646	-41	-198	117	0.615
Week 2	1307	1104	1510	<.0001	-87	-263	90	0.3389	1511	1353	1668	<.0001
Week 4	158	-45	362	0.1312	-46	-222	131	0.6142	377	219	534	<.0001
Week 6	413	210	617	0.0001	-191	-367	-15	0.0369	239	82	397	0.0039
Week 8	100	-103	304	0.3367	-131	-307	45	0.1498	142	-16	300	0.0812
Week 12	206	3	409	0.0506	-155	-331	22	0.0896	216	59	374	0.0087
Week 16	137	-66	341	0.1896	62	-115	238	0.4959	157	-1	314	0.055
Week 24	170	-34	373	0.1061	-26	-202	151	0.7774	99	-59	256	0.2228
Week 48	281	78	484	0.0083	-16	-192	160	0.857	83	-75	240	0.3074
CD4%												
Week 1	2.50	-1.33	6.33	0.2047	-0.45	-3.77	2.87	0.7911	-1.20	-4.17	1.77	0.4305
Week 2	8.30	4.47	12.13	<.0001	-0.20	-3.52	3.12	0.9063	8.20	5.23	11.17	<.0001
Week 4	5.30	1.47	9.13	0.0082	0.95	-2.37	4.27	0.5763	3.08	0.11	6.05	0.0453
Week 6	6.57	2.73	10.40	0.0012	-1.15	-4.47	2.17	0.499	1.86	-1.11	4.83	0.2229
Week 8	6.67	2.83	10.50	0.001	-0.45	-3.77	2.87	0.7911	3.74	0.77	6.71	0.0156
Week 12	4.97	1.13	8.80	0.013	0.23	-3.09	3.54	0.8946	2.74	-0.23	5.71	0.0741
Week 16	6.93	3.10	10.77	0.0007	1.60	-1.72	4.92	0.3475	1.30	-1.67	4.27	0.3932
Week 24	7.70	3.87	11.53	0.0002	2.73	-0.59	6.04	0.1114	1.70	-1.27	4.67	0.265

Appendix Table 6 continued

Week 48	5.00	1.17	8.83	0.0124	2.10	-1.22	5.42	0.2185	2.90	-0.07	5.87	0.059
CD8 count												
Week 1	-179	-436	79	0.1774	-94	-316	129	0.4134	-30	-229	170	0.7718
Week 2	634	377	891	<.0001	-158	-381	65	0.1692	999	800	1199	<.0001
Week 4	-26	-284	231	0.8416	-81	-304	142	0.4797	248	49	448	0.0168
Week 6	167	-90	424	0.2071	-283	-505	-60	0.0151	221	21	420	0.033
Week 8	-97	-355	160	0.4607	-168	-391	55	0.1429	39	-160	238	0.7024
Week 12	4	-254	261	0.9778	-239	-462	-16	0.0387	130	-69	330	0.2042
Week 16	-95	-352	162	0.4715	-7	-230	216	0.9493	130	-70	329	0.2063
Week 24	-108	-365	149	0.4133	-149	-372	74	0.1931	64	-135	264	0.5284
Week 48	42	-216	299	0.7518	-186	-408	37	0.1067	-3	-202	197	0.9781
CD8%												
Week 1	-1.90	-4.79	0.99	0.2014	-0.75	-3.25	1.75	0.5588	-0.12	-2.36	2.12	0.9166
Week 2	-5.17	-8.06	-2.28	0.0008	-1.03	-3.53	1.48	0.4247	-4.68	-6.92	-2.44	<.0001
Week 4	-2.40	-5.29	0.49	0.1077	-0.18	-2.68	2.33	0.8914	-2.36	-4.60	-0.12	0.0421
Week 6	-3.10	-5.99	-0.21	0.0387	-1.35	-3.85	1.15	0.2938	0.44	-1.80	2.68	0.7012
Week 8	-2.93	-5.82	-0.04	0.0502	0.13	-2.38	2.63	0.9223	-1.46	-3.70	0.78	0.205
Week 12	-2.53	-5.42	0.36	0.0898	-0.20	-2.70	2.30	0.876	-0.48	-2.72	1.76	0.6756
Week 16	-3.60	-6.49	-0.71	0.0169	-2.40	-4.90	0.10	0.0639	0.62	-1.62	2.86	0.5889
Week 24	-5.53	-8.42	-2.64	0.0003	-1.68	-4.18	0.83	0.1936	0.04	-2.20	2.28	0.9722
Week 48	-3.00	-5.89	-0.11	0.0453	-2.58	-5.08	-0.07	0.0472	-0.50	-2.74	1.74	0.6629

Appendix Table 7 Changes from baseline in patient characteristics for each group.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
CD19 count	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-19	-91	54	0.6158	18	-45	80	0.5871	-16	-72	40	0.5788
Week 2	-41	-114	32	0.2719	-16	-79	47	0.6249	24	-32	81	0.3978
Week 4	-131	-204	-58	0.0007	-13	-76	50	0.6922	-77	-134	-21	0.0085
Week 6	-43	-116	29	0.2457	-17	-80	46	0.5978	-67	-123	-10	0.0229
Week 8	-78	-151	-6	0.0376	-9	-72	54	0.7799	-63	-119	-6	0.0321
Week 12	-47	-120	25	0.2052	-38	-100	25	0.2461	-48	-105	8	0.0956
Week 16	-63	-135	10	0.0947	10	-53	72	0.768	-49	-105	7	0.0917
Week 24	-12	-84	61	0.7537	-18	-81	45	0.5818	-13	-69	43	0.6569
Week 48	10	-63	83	0.788	10	-53	73	0.7503	-24	-80	32	0.4095
CD19%												
Week 1	0.10	-1.85	2.05	0.9203	0.48	-1.22	2.17	0.5837	-0.52	-2.03	0.99	0.5025
Week 2	-6.33	-8.29	-4.38	<.0001	-0.20	-1.89	1.49	0.8174	-5.54	-7.05	-4.03	<.0001
Week 4	-5.70	-7.65	-3.75	<.0001	0.15	-1.54	1.84	0.8625	-5.04	-6.55	-3.53	<.0001
Week 6	-3.70	-5.65	-1.75	0.0004	0.55	-1.14	2.24	0.5258	-4.44	-5.95	-2.93	<.0001
Week 8	-3.00	-4.95	-1.05	0.0035	1.20	-0.49	2.89	0.1683	-2.70	-4.21	-1.19	0.0008
Week 12	-3.13	-5.09	-1.18	0.0023	-0.03	-1.72	1.67	0.977	-3.10	-4.61	-1.59	0.0001
Week 16	-2.27	-4.22	-0.31	0.0256	0.28	-1.42	1.97	0.7509	-3.00	-4.51	-1.49	0.0002
Week 24	-0.90	-2.85	1.05	0.3692	-0.40	-2.09	1.29	0.6443	-0.92	-2.43	0.59	0.2369
Week 48	-0.77	-2.72	1.19	0.444	0.10	-1.59	1.79	0.9081	-1.00	-2.51	0.51	0.1989
CD56 count												
Week 1	-39	-192	113	0.6141	-9	-141	123	0.8969	52	-66	170	0.392
Week 2	235	83	387	0.0033	3	-129	135	0.9646	511	393	629	<.0001
Week 4	32	-121	184	0.6847	-18	-150	114	0.7926	187	69	305	0.0026
Week 6	20	-133	172	0.8008	25	-107	157	0.7085	153	35	271	0.0127
Week 8	7	-146	159	0.9318	-21	-153	111	0.7586	120	2	238	0.0496
Week 12	27	-126	179	0.7324	-28	-159	104	0.6839	67	-51	185	0.2689
Week 16	-22	-174	130	0.7778	6	-126	138	0.9262	58	-60	176	0.3381
Week 24	-18	-171	134	0.8141	-24	-156	108	0.7223	-1	-119	117	0.9921
Week 48	6	-146	159	0.9352	41	-91	173	0.5465	-10	-128	108	0.8659
CD56%												
Week 1	-0.63	-3.63	2.37	0.6801	-0.50	-3.10	2.10	0.7069	1.52	-0.80	3.84	0.2034

Appendix Table 7 continued

Week 2	2.53	-0.47	5.53	0.1017	0.90	-1.70	3.50	0.499	1.70	-0.62	4.02	0.1554
Week 4	1.33	-1.67	4.33	0.3862	-0.68	-3.27	1.92	0.6119	2.24	-0.08	4.56	0.0624
Week 6	-0.13	-3.13	2.87	0.9308	2.23	-0.37	4.82	0.097	2.12	-0.20	4.44	0.0774
Week 8	1.07	-1.93	4.07	0.4878	0.43	-2.17	3.02	0.7493	0.30	-2.02	2.62	0.8008
Week 12	0.50	-2.50	3.50	0.7447	0.08	-2.52	2.67	0.955	0.20	-2.12	2.52	0.8664
Week 16	-0.97	-3.97	2.03	0.5294	0.15	-2.45	2.75	0.9102	0.90	-1.42	3.22	0.4499
Week 24	-0.93	-3.93	2.07	0.5436	-1.00	-3.60	1.60	0.4527	-0.16	-2.48	2.16	0.893
Week 48	-0.33	-3.33	2.67	0.8281	1.95	-0.65	4.55	0.145	-0.28	-2.60	2.04	0.8139
ISUM												
Week 1	-285	-874	304	0.3453	-41	-551	469	0.8745	-18	-475	438	0.9372
Week 2	2254	1665	2843	<.0001	-203	-713	307	0.4378	3063	2606	3519	<.0001
Week 4	2	-587	591	0.9938	-110	-620	400	0.673	764	308	1221	0.0015
Week 6	599	10	1188	0.0496	-385	-895	126	0.1435	566	110	1023	0.0172
Week 8	-94	-683	495	0.7561	-323	-833	187	0.2182	264	-192	721	0.2595
Week 12	227	-362	816	0.4516	-414	-924	97	0.1161	394	-62	850	0.0944
Week 16	-92	-681	497	0.7612	118	-393	628	0.6529	306	-151	762	0.1927
Week 24	44	-545	633	0.8831	-159	-669	351	0.543	200	-257	656	0.3933
Week 48	292	-297	881	0.3337	-107	-617	403	0.6821	29	-427	486	0.9005
CD4/CD8 ratio												
Week 1	0.10	-0.07	0.27	0.2603	0.03	-0.12	0.17	0.7443	-0.02	-0.15	0.11	0.7705
Week 2	0.43	0.26	0.61	<.0001	0.03	-0.12	0.17	0.7443	0.34	0.21	0.47	<.0001
Week 4	0.20	0.03	0.37	0.026	0.03	-0.12	0.17	0.7443	0.14	0.01	0.27	0.0437
Week 6	0.30	0.13	0.47	0.001	0.03	-0.12	0.17	0.7443	0.06	-0.07	0.19	0.3825
Week 8	0.30	0.13	0.47	0.001	-0.03	-0.17	0.12	0.7443	0.12	-0.01	0.25	0.0828
Week 12	0.23	0.06	0.41	0.0098	0.05	-0.10	0.20	0.5146	0.08	-0.05	0.21	0.2451
Week 16	0.33	0.16	0.51	0.0003	0.10	-0.05	0.25	0.1942	0.04	-0.09	0.17	0.5599
Week 24	0.47	0.29	0.64	<.0001	0.13	-0.02	0.27	0.1056	0.06	-0.07	0.19	0.3825
Week 48	0.27	0.09	0.44	0.0033	0.13	-0.02	0.27	0.1056	0.08	-0.05	0.21	0.2451

Appendix Table 8 Changes from baseline in IFN-γ ELISpot responses to peptide pools from NIBSC.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IFN-γ	Group 1				Group 2				Group 3			
p17	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-2	-123	119	0.9743	41	-64	146	0.4459	-22	-115	72	0.6531
Week 2	18	-103	139	0.7716	7	-98	112	0.8963	-148	-242	-54	0.0027
Week 4	45	-76	166	0.4719	64	-41	168	0.2389	-4	-98	90	0.9336
Week 6	25	-96	146	0.683	47	-58	151	0.3875	-54	-147	40	0.2662
Week 8	-26	-147	95	0.6751	74	-31	179	0.1706	-16	-110	77	0.7328
Week 12	7	-114	128	0.9144	149	44	253	0.0069	-84	-177	10	0.0846
Week 16	-50	-171	71	0.4209	78	-27	183	0.1489	-107	-201	-13	0.0279
Week 24	-32	-153	89	0.606	127	22	232	0.02	-119	-213	-25	0.0152
Week 48	80	-41	201	0.1992	105	0	210	0.0532	26	-67	120	0.5828
p24												
Week 1	11	-195	217	0.9194	-36	-214	142	0.6934	26	-133	186	0.7465
Week 2	-168	-374	38	0.1137	-83	-261	95	0.3644	-260	-419	-100	0.002
Week 4	108	-98	314	0.3071	-22	-200	157	0.8138	7	-153	166	0.9336
Week 6	115	-91	321	0.2784	37	-142	215	0.6894	-12	-171	148	0.887
Week 8	15	-191	221	0.8843	-39	-217	140	0.6733	-108	-267	52	0.1899
Week 12	20	-186	226	0.8495	6	-172	184	0.9476	-176	-336	-17	0.0331
Week 16	-139	-345	67	0.1885	19	-159	197	0.8351	-196	-356	-37	0.0181
Week 24	-150	-356	56	0.1572	-21	-199	157	0.8181	-186	-346	-26	0.0249
Week 48	201	-5	407	0.0589	-84	-262	95	0.3615	-86	-246	73	0.2916
Nef												
Week 1	-43	-168	83	0.5081	58	-51	167	0.2998	49	-48	147	0.3253
Week 2	-89	-214	37	0.1709	-10	-118	99	0.8647	-114	-211	-17	0.0244
Week 4	7	-118	133	0.9093	12	-97	120	0.8366	-36	-134	61	0.4662
Week 6	-84	-210	42	0.1943	9	-100	118	0.8718	-85	-182	13	0.0919
Week 8	-57	-182	69	0.3799	41	-68	149	0.4683	-86	-184	11	0.086
Week 12	-34	-160	92	0.5977	123	14	232	0.0297	-100	-197	-2	0.0485
Week 16	-77	-202	49	0.2357	45	-64	153	0.4257	-53	-151	44	0.2877
Week 24	-125	-250	1	0.0556	-2	-110	107	0.9785	-88	-186	9	0.0791
Week 48	50	-76	176	0.4382	48	-61	157	0.3904	-87	-184	11	0.0846
Tat												
Week 1	-17	-95	60	0.6619	6	-62	73	0.8726	-54	-114	6	0.0813
Week 2	-19	-96	59	0.6377	2	-66	69	0.9651	-146	-206	-86	<.0001
Week 4	10	-67	87	0.8007	-2	-69	65	0.9535	-50	-110	10	0.1088
Week 6	20	-57	97	0.6139	0	-67	67	1	-79	-139	-19	0.0118
Week 8	26	-51	103	0.5122	4	-63	71	0.9072	-92	-152	-32	0.0034
Week 12	47	-30	125	0.2342	2	-65	69	0.9535	-112	-172	-52	0.0005
Week 16	13	-64	91	0.7365	3	-64	70	0.9303	-120	-180	-60	0.0002
Week 24	-11	-89	66	0.7749	-2	-69	65	0.9535	-100	-160	-40	0.0017
Week 48	82	5	159	0.041	4	-63	71	0.9072	-53	-113	7	0.0858
Rev												
Week 1	0	-27	27	1	1	-22	24	0.9329	-8	-28	13	0.475
Week 2	3	-24	29	0.8458	-16	-39	7	0.1803	-23	-44	-2	0.0343
Week 4	6	-21	33	0.6619	-10	-33	13	0.4008	-22	-43	-1	0.0409
Week 6	0	-27	27	1	-12	-35	11	0.3138	2	-19	22	0.8803
Week 8	1	-26	27	0.9612	3	-21	26	0.8333	0	-21	20	0.97
Week 12	7	-20	33	0.6271	-4	-27	20	0.7683	-20	-41	0	0.0575
Week 16	1	-25	28	0.9225	-2	-25	21	0.8663	-19	-40	2	0.0735
Week 24	0	-27	27	1	-2	-25	21	0.8663	-27	-48	-6	0.0133
Week 48	3	-23	30	0.808	-2	-25	21	0.8663	-18	-39	2	0.0861

Appendix Table 9 Changes from baseline in IL-2 ELISpot responses to peptide pools from NIBSC.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IL-2	Group 1				Group 2				Group 3			
p17	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-9	-128	111	0.8873	39	-65	143	0.4623	-20	-113	73	0.6731
Week 2	-32	-152	88	0.6012	-23	-126	81	0.6712	-17	-110	75	0.7167
Week 4	49	-70	169	0.4208	17	-87	121	0.7483	-11	-103	82	0.8197
Week 6	63	-57	182	0.3071	18	-86	122	0.7341	-33	-126	59	0.4841
Week 8	-2	-122	118	0.9739	73	-31	176	0.1736	-43	-135	50	0.3675
Week 12	-3	-122	117	0.9652	103	-1	206	0.0557	15	-77	108	0.7484
Week 16	-22	-142	98	0.7192	74	-30	178	0.1649	-10	-102	83	0.8394
Week 24	73	-47	192	0.2368	125	21	228	0.0208	74	-18	167	0.1191
Week 48	115	-4	235	0.0621	81	-23	184	0.1313	91	-1	184	0.057
p24												
Week 1	3	-44	49	0.9112	-3	-43	37	0.8848	-20	-57	16	0.2722
Week 2	-39	-86	7	0.1026	2	-39	42	0.9422	-1	-37	35	0.9655
Week 4	11	-35	58	0.6356	2	-39	42	0.9422	-18	-55	18	0.3217
Week 6	23	-24	69	0.3442	3	-38	43	0.9039	-14	-50	22	0.4503
Week 8	-51	-97	-4	0.0365	1	-39	41	0.9615	-26	-63	10	0.1564
Week 12	-55	-101	-8	0.0243	3	-37	43	0.8848	-20	-56	16	0.2817
Week 16	-43	-90	3	0.0726	1	-40	41	0.9807	-22	-59	14	0.2283
Week 24	-23	-70	23	0.3303	-7	-47	34	0.7535	-23	-59	13	0.2202
Week 48	42	-5	89	0.0817	18	-22	58	0.3856	-18	-55	18	0.3217
Nef												
Week 1	-5	-30	21	0.7202	-2	-24	21	0.8942	-6	-26	13	0.5264
Week 2	-1	-26	25	0.9592	9	-13	31	0.4259	31	11	51	0.003
Week 4	5	-20	31	0.6824	-1	-23	21	0.9294	1	-19	21	0.9368
Week 6	-5	-31	20	0.6824	20	-3	42	0.0867	-6	-26	13	0.5264
Week 8	-4	-29	21	0.7588	2	-21	24	0.8942	-3	-23	17	0.7512
Week 12	-6	-31	19	0.6453	4	-19	26	0.7564	0	-20	20	1
Week 16	9	-16	35	0.4743	1	-21	23	0.9294	0	-19	20	0.9684
Week 24	-5	-30	21	0.7202	-3	-25	20	0.8246	-1	-21	19	0.9368
Week 48	9	-16	35	0.4743	9	-13	31	0.4259	-2	-22	18	0.8429
Tat												
Week 1	-1	-23	22	0.9544	1	-19	20	0.9605	4	-14	21	0.6902
Week 2	19	-3	42	0.1	2	-18	21	0.8819	35	18	53	0.0002
Week 4	7	-15	30	0.5298	-4	-23	16	0.7289	1	-17	18	0.9294
Week 6	1	-21	24	0.9089	-1	-20	19	0.9605	-2	-20	15	0.7904
Week 8	8	-15	31	0.4931	-3	-22	17	0.8044	2	-15	20	0.7904
Week 12	2	-21	25	0.8638	1	-19	20	0.9605	3	-14	21	0.7231
Week 16	1	-22	23	0.9544	1	-19	21	0.9211	2	-16	20	0.8247
Week 24	3	-20	25	0.8191	-4	-23	16	0.7289	-2	-20	15	0.7904
Week 48	-1	-24	21	0.9089	0	-20	20	1	1	-16	19	0.8943
Rev												
Week 1	-1	-24	22	0.9104	1	-19	21	0.9223	1	-17	19	0.9305
Week 2	30	7	53	0.013	0	-20	20	1	36	18	54	0.0001
Week 4	-1	-24	22	0.9551	-1	-21	19	0.9223	0	-18	18	0.9652
Week 6	-1	-24	22	0.9104	0	-20	20	1	0	-18	18	0.9652
Week 8	-1	-24	22	0.9104	2	-19	22	0.8837	2	-16	20	0.8616
Week 12	1	-22	24	0.9104	3	-18	23	0.8075	1	-17	19	0.9305
Week 16	-1	-24	22	0.9104	0	-20	20	1	4	-14	22	0.663
Week 24	0	-23	23	1	-1	-21	19	0.9223	2	-16	20	0.8616
Week 48	-1	-24	22	0.9104	2	-18	22	0.8454	2	-16	20	0.8616

Appendix Table 10 Changes from baseline in IL-4 ELISpot responses to peptide pools from NIBSC.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IL-4	Group 1				Group 2				Group 3			
p17	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-4	-99	91	0.9343	32	-50	114	0.4475	-27	-101	46	0.4703
Week 2	-2	-97	93	0.9671	29	-53	111	0.4911	-30	-103	44	0.4322
Week 4	43	-52	138	0.3807	41	-41	123	0.331	-10	-84	63	0.7822
Week 6	3	-92	98	0.9453	16	-66	98	0.7037	-12	-85	61	0.7498
Week 8	1	-94	96	0.989	52	-31	134	0.2228	8	-65	81	0.8316
Week 12	-3	-98	92	0.9453	100	18	182	0.0194	32	-41	106	0.3901
Week 16	-2	-97	93	0.9671	74	-8	156	0.0813	1	-72	75	0.9745
Week 24	4	-91	99	0.9343	85	2	167	0.0472	100	27	173	0.0092
Week 48	111	16	206	0.024	66	-17	148	0.1221	40	-33	114	0.2845
p24												
Week 1	-9	-20	1	0.082	-1	-10	8	0.8281	-1	-9	7	0.846
Week 2	-9	-19	2	0.1059	-2	-10	7	0.7447	-4	-12	4	0.3328
Week 4	-10	-20	0	0.0628	-2	-11	7	0.6642	7	-1	15	0.1015
Week 6	-9	-20	1	0.082	-1	-9	8	0.9135	-3	-11	5	0.438
Week 8	-9	-20	1	0.082	4	-5	12	0.4479	-5	-13	3	0.2089
Week 12	-11	-22	-1	0.0355	3	-6	12	0.5152	-2	-10	6	0.5604
Week 16	-11	-22	-1	0.0355	3	-6	11	0.5875	0	-8	8	1
Week 24	-10	-20	0	0.0628	0	-9	9	1	-3	-11	5	0.4972
Week 48	11	0	21	0.0475	11	2	19	0.0248	-1	-9	7	0.846
Nef												
Week 1	-15	-28	-1	0.0403	0	-12	12	1	-14	-24	-3	0.0146
Week 2	-12	-26	2	0.092	6	-6	17	0.3695	-18	-29	-7	0.0014
Week 4	-14	-28	0	0.05	-1	-12	11	0.9348	-19	-29	-8	0.0009
Week 6	-13	-27	0	0.0617	-2	-13	10	0.8062	-17	-28	-7	0.0023
Week 8	-13	-27	0	0.0617	2	-10	14	0.7436	-19	-29	-8	0.0009
Week 12	-10	-24	4	0.1592	4	-8	15	0.5674	-15	-25	-4	0.0081
Week 16	-15	-28	-1	0.0403	3	-9	15	0.6239	-17	-28	-7	0.0023
Week 24	-11	-25	2	0.1112	-1	-13	11	0.8701	-18	-29	-8	0.0011
Week 48	-11	-25	2	0.1112	4	-8	16	0.5135	-18	-28	-7	0.0018
Tat												
Week 1	-17	-51	16	0.3182	-1	-30	28	0.9468	3	-23	29	0.8346
Week 2	78	44	112	<.0001	-1	-30	29	0.9734	-4	-31	22	0.7429
Week 4	-18	-52	16	0.3001	-3	-32	27	0.8676	-2	-29	24	0.858
Week 6	-15	-48	19	0.3979	2	-27	31	0.8939	13	-13	39	0.3412
Week 8	-16	-50	18	0.3567	2	-28	31	0.9203	-2	-28	24	0.8815
Week 12	-19	-53	14	0.266	0	-29	29	1	2	-24	29	0.858
Week 16	-19	-52	15	0.2827	-1	-30	28	0.9468	4	-22	30	0.7656
Week 24	-17	-50	17	0.3371	-3	-32	27	0.8676	1	-25	27	0.9287
Week 48	-17	-51	16	0.3182	2	-28	31	0.9203	2	-24	28	0.8815
Rev												
Week 1	-18	-27	-9	0.0003	1	-8	9	0.9047	-3	-10	4	0.454
Week 2	-17	-27	-8	0.0005	0	-8	8	1	-7	-14	0	0.0565
Week 4	-19	-28	-9	0.0002	-1	-9	7	0.8107	-4	-11	3	0.2857
Week 6	-18	-27	-9	0.0003	1	-7	9	0.8107	-6	-13	1	0.1108
Week 8	-17	-27	-8	0.0005	0	-8	8	1	-7	-14	0	0.0714
Week 12	-12	-21	-3	0.0145	1	-8	9	0.9047	-6	-14	1	0.0893
Week 16	-15	-25	-6	0.002	-2	-10	7	0.7194	-4	-12	3	0.2406
Week 24	-14	-23	-5	0.0046	-2	-10	6	0.6321	-4	-11	3	0.2857
Week 48	-19	-28	-9	0.0002	10	2	18	0.0185	-2	-9	5	0.5925

Appendix Table 11 Changes from baseline in perforin ELISpot responses to peptide pools from NIBSC.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

Perforin	Group 1				Group 2				Group 3			
p17	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-19	-236	198	0.8618	31	-60	62	0.7472	56	-110	111	0.5125
Week 2	-25	-242	192	0.8195	-80	158	-156	0.4064	-56	111	-110	0.5125
Week 4	271	54	488	0.0166	-27	53	-53	0.7789	24	-47	49	0.7767
Week 6	17	-200	234	0.8759	29	-56	58	0.763	-25	49	-48	0.7731
Week 8	-1	-218	216	0.9952	-19	38	-36	0.8434	163	-319	321	0.0605
Week 12	-17	-234	200	0.8807	78	-151	153	0.4211	38	-74	75	0.6588
Week 16	-30	-247	187	0.787	50	-97	99	0.6033	37	-72	74	0.6655
Week 24	1	-216	218	0.9952	112	-218	220	0.2481	172	-336	337	0.0487
Week 48	106	-111	323	0.3411	22	-42	42	0.8231	-6	12	-10	0.9481
p24												
Week 1	-11	-165	143	0.8858	40	-94	173	0.5635	75	-44	195	0.2206
Week 2	-21	-175	133	0.7934	-48	-181	86	0.4875	-14	-133	105	0.8188
Week 4	-23	-177	131	0.7739	-20	-153	113	0.7698	3	-117	122	0.9635
Week 6	-9	-163	145	0.9125	7	-126	140	0.9184	-29	-148	91	0.6376
Week 8	0	-154	154	1	-43	-176	91	0.5344	276	156	395	<.0001
Week 12	2	-152	156	0.9798	10	-123	143	0.8836	2	-117	121	0.9739
Week 16	-23	-177	131	0.7675	42	-92	175	0.544	9	-110	129	0.8803
Week 24	-25	-179	129	0.7482	20	-113	153	0.7698	55	-64	175	0.3675
Week 48	121	-33	275	0.1268	-30	-163	103	0.6608	-6	-126	113	0.9166
Nef												
Week 1	3	-166	171	0.9753	-65	-210	81	0.3891	53	-78	183	0.4304
Week 2	-1	-170	167	0.9877	-96	-241	50	0.2035	-30	-160	101	0.658
Week 4	6	-163	175	0.9446	-94	-239	52	0.213	-11	-141	120	0.8716
Week 6	18	-151	187	0.8348	-66	-212	80	0.3782	-48	-179	82	0.4696
Week 8	29	-139	198	0.7339	-110	-256	36	0.1436	222	91	352	0.0013
Week 12	26	-143	195	0.7632	-20	-165	126	0.7941	31	-99	162	0.6408
Week 16	-7	-176	161	0.9323	-53	-198	93	0.4829	-28	-159	103	0.6754
Week 24	-11	-179	158	0.9016	-31	-177	115	0.6784	15	-116	145	0.8248
Week 48	100	-69	269	0.2484	-72	-217	74	0.34	-14	-145	117	0.8341
Tat												
Week 1	-36	-239	167	0.7293	-64	-240	112	0.4779	95	-63	252	0.2412
Week 2	-52	-255	151	0.6173	-119	-295	57	0.1887	-61	-218	97	0.4511
Week 4	71	-132	275	0.4933	-83	-259	93	0.3579	-30	-187	128	0.7134
Week 6	-6	-209	197	0.954	-57	-233	119	0.5272	-30	-187	127	0.7097
Week 8	-11	-214	193	0.9183	-122	-298	54	0.1779	246	89	404	0.0029
Week 12	11	-193	214	0.9183	-24	-200	152	0.7899	29	-128	187	0.7171
Week 16	5	-199	208	0.9642	13	-163	188	0.8896	48	-110	205	0.5549
Week 24	-63	-266	141	0.5472	60	-116	235	0.5093	103	-55	260	0.2041
Week 48	33	-170	237	0.7486	-73	-248	103	0.4217	-15	-172	143	0.8542
Rev												
Week 1	-11	-138	117	0.8702	3	-107	113	0.9577	53	-46	152	0.2943
Week 2	-12	-140	116	0.8541	-26	-136	84	0.6458	-32	-131	66	0.5222
Week 4	-16	-144	112	0.8064	8	-103	118	0.8945	-8	-107	91	0.8743
Week 6	-11	-139	116	0.8622	9	-102	119	0.8805	-61	-160	38	0.2312
Week 8	7	-120	135	0.9105	-44	-154	67	0.4424	145	46	244	0.0052
Week 12	49	-79	176	0.4567	46	-65	156	0.4218	-19	-118	80	0.7043
Week 16	-24	-152	104	0.7132	48	-62	158	0.3969	-26	-124	73	0.6129
Week 24	-25	-152	103	0.7056	8	-102	118	0.8875	-15	-114	84	0.7698
Week 48	81	-47	208	0.2187	-8	-118	103	0.8945	-7	-106	92	0.893

Appendix Table 12 Changes from baseline in IFN-γ ELISpot responses to peptide pools from FIT Biotech.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IFN-γ	Group 1				Group 2				Group 3			
p17/p24	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	109	-129	348	0.3717	-2	-208	205	0.9887	12	-173	196	0.9024
Week 2	-28	-267	211	0.8186	-107	-314	100	0.313	-285	-470	-100	0.0034
Week 4	223	-16	461	0.071	-42	-248	165	0.6948	9	-176	194	0.9225
Week 6	157	-82	395	0.2017	23	-184	230	0.8278	60	-124	245	0.5235
Week 8	150	-89	389	0.2213	-32	-239	175	0.7622	-143	-328	42	0.1327
Week 12	144	-95	383	0.2402	30	-177	237	0.7767	-182	-367	3	0.057
Week 16	129	-109	368	0.2911	35	-172	241	0.7443	-106	-291	78	0.2624
Week 24	67	-171	306	0.5816	-44	-251	163	0.6775	-237	-422	-52	0.0138
Week 48	312	73	551	0.0122	-121	-328	86	0.2543	-150	-334	35	0.1164
Nef												
Week 1	-39	-165	88	0.55	37	-72	146	0.509	37	-61	135	0.4629
Week 2	19	-108	145	0.7727	-23	-132	86	0.6812	-107	-205	-9	0.0353
Week 4	18	-108	144	0.7806	7	-102	116	0.9004	-24	-122	74	0.6318
Week 6	10	-116	136	0.877	-3	-112	107	0.9644	-66	-164	31	0.1869
Week 8	-90	-216	36	0.1661	20	-89	129	0.7209	-102	-200	-5	0.0433
Week 12	-17	-143	110	0.7965	38	-71	147	0.4976	-104	-202	-6	0.0403
Week 16	-111	-238	15	0.0877	35	-75	144	0.538	-41	-139	57	0.4113
Week 24	-111	-237	16	0.0896	-11	-120	99	0.8512	-100	-198	-3	0.0475
Week 48	43	-84	169	0.5096	-33	-142	77	0.5617	-70	-168	27	0.162
Tat												
Week 1	69	-106	245	0.4406	-1	-152	151	0.9949	-19	-155	117	0.7868
Week 2	-29	-205	146	0.7438	-9	-160	143	0.9129	-56	-192	79	0.4181
Week 4	275	100	451	0.0028	-10	-161	142	0.9027	14	-122	150	0.8404
Week 6	41	-134	217	0.6453	3	-149	155	0.9692	-21	-157	115	0.7648
Week 8	-144	-319	31	0.1113	-4	-156	148	0.9589	-27	-163	109	0.6999
Week 12	46	-129	221	0.6085	-6	-158	146	0.9385	-36	-171	100	0.6088
Week 16	-221	-396	-45	0.0157	-4	-156	148	0.9589	-40	-176	95	0.5615
Week 24	-157	-333	18	0.0824	1	-151	153	0.9897	-47	-183	89	0.5013
Week 48	-191	-367	-16	0.0355	-5	-156	147	0.9538	-36	-171	100	0.6088
Rev												
Week 1	0	-37	37	1	6	-26	37	0.7365	-27	-55	2	0.0695
Week 2	-2	-39	35	0.9156	-13	-45	19	0.4272	-43	-71	-14	0.0043
Week 4	1	-36	38	0.9718	-13	-45	19	0.4272	11	-18	39	0.4607
Week 6	-3	-40	34	0.8876	-5	-36	27	0.7831	-14	-43	15	0.3395
Week 8	-4	-41	33	0.8321	-7	-39	25	0.6685	-22	-51	7	0.135
Week 12	-3	-40	34	0.8598	-5	-37	27	0.7597	-42	-71	-13	0.005
Week 16	-1	-38	36	0.9437	4	-28	35	0.8304	-44	-72	-15	0.0037
Week 24	-1	-38	36	0.9718	-9	-41	23	0.5821	-45	-73	-16	0.0029
Week 48	0	-37	37	1	1	-31	32	0.9756	-41	-70	-13	0.0059
CTL												
Week 1	-24	-118	70	0.6192	52	-30	134	0.2156	-30	-103	43	0.4293
Week 2	-5	-100	89	0.912	-45	-126	37	0.2887	-54	-127	19	0.1512
Week 4	27	-67	122	0.5715	1	-81	82	0.9905	-18	-91	55	0.638
Week 6	18	-76	112	0.7093	35	-47	116	0.4101	-14	-87	59	0.7081
Week 8	-21	-115	74	0.6686	3	-79	84	0.9523	-56	-129	17	0.1396
Week 12	-5	-99	90	0.923	79	-3	161	0.0615	-82	-155	-9	0.0298
Week 16	-25	-120	69	0.5999	8	-74	90	0.8482	-83	-156	-10	0.0283
Week 24	-12	-106	82	0.8037	-3	-85	79	0.9428	-87	-160	-14	0.0217
Week 48	33	-62	127	0.4991	-6	-87	76	0.8953	-90	-163	-17	0.0185

Appendix Table 13 Changes from baseline in IL-2 ELISpot responses to peptide pools from FIT Biotech.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IL-2	Group 1				Group 2				Group 3			
p17/p24	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	13	-35	61	0.5866	-8	-49	34	0.7238	-23	-60	14	0.2235
Week 2	-14	-62	34	0.568	-7	-48	35	0.7594	1	-36	38	0.9664
Week 4	29	-19	77	0.2439	-7	-48	34	0.7415	-29	-66	8	0.1265
Week 6	5	-43	53	0.8277	9	-32	50	0.6715	-18	-55	19	0.3336
Week 8	-15	-63	33	0.5318	-4	-45	37	0.8505	-20	-57	17	0.284
Week 12	-18	-66	30	0.4632	29	-12	70	0.1741	-28	-65	9	0.1427
Week 16	-8	-56	40	0.744	-6	-47	36	0.7955	-29	-66	8	0.1265
Week 24	-1	-49	47	0.9783	-8	-49	34	0.7238	-34	-71	3	0.076
Week 48	79	31	127	0.0017	4	-37	45	0.8505	-24	-61	13	0.2081
Nef												
Week 1	1	-25	26	0.9594	0	-22	22	1	-1	-21	19	0.9371
Week 2	-5	-31	20	0.6838	0	-22	22	1	32	12	52	0.0022
Week 4	3	-23	28	0.8386	-3	-25	20	0.8254	-2	-22	18	0.8436
Week 6	-1	-26	25	0.9594	2	-21	24	0.8947	-4	-24	16	0.6933
Week 8	-13	-39	12	0.3098	3	-20	25	0.8254	-2	-22	18	0.8436
Week 12	-15	-40	11	0.2642	5	-18	27	0.6915	-3	-23	17	0.7824
Week 16	-14	-40	12	0.2864	3	-20	25	0.8254	1	-19	21	0.9371
Week 24	-12	-38	14	0.3604	-1	-23	21	0.9297	0	-20	20	1
Week 48	4	-22	30	0.7599	2	-21	24	0.8947	1	-19	21	0.9058
Tat												
Week 1	3	-21	26	0.8248	-4	-24	16	0.7014	-3	-21	15	0.7316
Week 2	7	-16	31	0.543	-3	-23	17	0.7737	35	17	53	0.0003
Week 4	4	-20	28	0.7398	-4	-24	17	0.7372	3	-15	21	0.7641
Week 6	2	-22	26	0.8681	-5	-25	16	0.6663	-2	-20	16	0.8302
Week 8	-7	-30	17	0.5802	-2	-22	18	0.8479	-2	-20	16	0.8302
Week 12	-2	-26	22	0.8681	-2	-22	19	0.8856	-3	-21	15	0.7641
Week 16	-5	-28	19	0.6985	-3	-23	17	0.7737	-1	-19	17	0.8976
Week 24	-4	-28	20	0.7398	-2	-22	19	0.8856	-4	-22	15	0.6997
Week 48	-4	-28	20	0.7398	-4	-24	16	0.7014	-4	-23	14	0.6374
Rev												
Week 1	1	-20	23	0.9045	1	-18	20	0.9173	-1	-18	16	0.926
Week 2	15	-7	36	0.1893	1	-18	19	0.9586	35	18	52	0.0001
Week 4	-1	-22	21	0.9522	0	-19	19	1	-2	-19	15	0.8163
Week 6	3	-19	24	0.8104	0	-19	19	1	-3	-20	14	0.7451
Week 8	1	-20	23	0.9045	2	-17	20	0.8762	-3	-20	14	0.7102
Week 12	7	-14	29	0.5099	2	-17	21	0.8354	-3	-20	14	0.7102
Week 16	-2	-24	20	0.8572	2	-17	20	0.8762	0	-17	17	1
Week 24	0	-22	22	1	1	-18	19	0.9586	-4	-21	13	0.6424
Week 48	-2	-24	20	0.8572	3	-16	22	0.7553	-1	-18	16	0.8891
CTL												
Week 1	-6	-29	17	0.6151	-2	-22	19	0.8845	-2	-20	16	0.795
Week 2	-2	-25	21	0.8668	0	-20	20	1	34	16	52	0.0003
Week 4	6	-17	29	0.6151	-3	-23	18	0.8087	-2	-20	16	0.8286
Week 6	-1	-24	23	0.9554	-1	-21	19	0.9229	-2	-20	16	0.795
Week 8	-3	-27	20	0.7799	2	-19	22	0.8845	-1	-19	17	0.931
Week 12	-2	-25	21	0.8668	8	-13	28	0.4684	-2	-20	16	0.795
Week 16	-9	-33	14	0.4346	3	-17	23	0.7715	-1	-19	17	0.8966
Week 24	-3	-26	21	0.8231	-2	-22	19	0.8845	-3	-21	15	0.7618
Week 48	15	-8	39	0.2007	0	-20	20	1	-3	-21	15	0.7291

Appendix Table 14 Changes from baseline in IL-4 ELISpot responses to peptide pools from FIT Biotech.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

IL-4	Group 1				Group 2				Group 3			
p17/p24	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-7	-29	16	0.5634	6	-14	26	0.5482	12	-5	30	0.1674
Week 2	-5	-28	17	0.6438	-2	-21	18	0.8806	0	-17	18	0.9643
Week 4	-9	-31	14	0.4529	-1	-20	19	0.96	14	-4	31	0.1304
Week 6	-5	-28	17	0.6438	1	-19	21	0.9202	1	-16	19	0.8931
Week 8	-9	-31	14	0.4529	2	-18	21	0.8806	1	-16	19	0.8931
Week 12	-7	-29	16	0.5634	2	-18	22	0.8412	4	-13	21	0.6543
Week 16	-8	-31	15	0.4883	3	-17	23	0.7638	6	-11	24	0.4741
Week 24	-3	-26	19	0.7725	1	-19	21	0.9202	5	-13	22	0.5911
Week 48	-6	-29	17	0.603	38	18	58	0.0003	4	-13	22	0.6224
Nef												
Week 1	-5	-14	5	0.3398	-7	-15	2	0.1264	1	-7	8	0.8322
Week 2	-7	-16	3	0.1739	-7	-15	1	0.1001	-5	-13	2	0.171
Week 4	-9	-18	1	0.0783	-8	-16	1	0.0785	5	-3	12	0.2059
Week 6	-6	-16	4	0.2206	-7	-15	2	0.1264	-2	-10	5	0.5256
Week 8	-7	-17	2	0.1352	-7	-15	2	0.1264	-5	-13	2	0.171
Week 12	-7	-17	2	0.1352	-6	-14	3	0.195	-3	-10	5	0.4591
Week 16	-7	-16	3	0.1739	-6	-14	2	0.1578	2	-5	9	0.5967
Week 24	-5	-14	5	0.3398	-8	-16	0	0.0609	-3	-11	4	0.3978
Week 48	-8	-18	2	0.1036	-1	-9	8	0.9057	-3	-11	4	0.3978
Tat												
Week 1	-7	-15	2	0.1199	-2	-9	6	0.6841	-1	-7	6	0.8082
Week 2	-1	-9	8	0.8755	-1	-8	7	0.8921	-4	-11	2	0.1842
Week 4	-5	-14	3	0.2122	-2	-9	6	0.6841	4	-2	10	0.2269
Week 6	-5	-13	4	0.2745	1	-6	8	0.7861	0	-7	6	0.9034
Week 8	-6	-14	2	0.161	6	-1	13	0.1062	-2	-8	5	0.6276
Week 12	-5	-14	3	0.2122	0	-7	7	1	-3	-9	4	0.3966
Week 16	-5	-13	4	0.2745	3	-5	10	0.4981	2	-5	8	0.6276
Week 24	-4	-12	4	0.3484	-1	-8	6	0.7861	-3	-9	4	0.3966
Week 48	-4	-12	4	0.3484	9	2	16	0.0164	-1	-7	6	0.8082
Rev												
Week 1	-7	-14	1	0.0839	-2	-8	4	0.5461	1	-5	7	0.6853
Week 2	-7	-14	1	0.0839	-2	-8	5	0.6506	-3	-9	3	0.2814
Week 4	-6	-13	1	0.1191	-4	-10	3	0.2919	-1	-7	5	0.787
Week 6	-3	-11	4	0.3841	-3	-9	3	0.3659	-1	-7	5	0.6853
Week 8	-5	-13	2	0.1653	-2	-8	5	0.6506	-3	-9	3	0.2814
Week 12	-2	-9	5	0.601	-1	-7	5	0.7626	-3	-9	3	0.3455
Week 16	-6	-13	1	0.1191	-3	-9	4	0.4508	-4	-9	2	0.226
Week 24	-3	-11	4	0.3841	-4	-10	2	0.2289	-1	-7	5	0.787
Week 48	-7	-14	1	0.0839	5	-1	11	0.1335	0	-6	6	1
CTL												
Week 1	-5	-11	2	0.1525	1	-4	6	0.7217	1	-4	6	0.7501
Week 2	1	-6	7	0.837	1	-5	6	0.8586	0	-5	5	0.8734
Week 4	-5	-12	1	0.1027	-2	-7	4	0.5934	0	-5	5	0.8734
Week 6	-4	-10	2	0.2193	1	-5	6	0.8586	0	-5	5	0.8734
Week 8	-3	-9	4	0.4116	0	-5	5	1	0	-5	5	1
Week 12	-6	-12	0	0.0669	1	-5	6	0.8586	3	-2	8	0.2665
Week 16	-5	-12	1	0.1027	1	-4	6	0.7217	2	-3	7	0.3404
Week 24	-5	-11	2	0.1525	-2	-7	4	0.5934	2	-3	7	0.5244
Week 48	-3	-10	3	0.3053	8	2	13	0.0089	3	-2	8	0.2665

Appendix Table 15 Changes from baseline in perforin ELISpot responses to peptide pools from FIT Biotech.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

Perforin	Group 1				Group 2				Group 3			
p17/p24	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-6	-90	78	0.8889	-11	-84	62	0.7675	13	-52	78	0.7005
Week 2	-17	-101	67	0.6981	-55	-128	18	0.1418	-26	-91	39	0.4283
Week 4	-7	-91	77	0.8766	-58	-130	15	0.1248	-26	-91	39	0.4283
Week 6	3	-81	87	0.9381	10	-63	82	0.7984	-32	-97	33	0.3315
Week 8	27	-57	111	0.5351	-43	-115	30	0.255	10	-55	75	0.7729
Week 12	54	-30	138	0.2108	24	-49	97	0.5192	-6	-71	59	0.8474
Week 16	-7	-91	77	0.8766	-18	-91	55	0.6286	4	-61	69	0.9138
Week 24	-5	-89	79	0.9012	-51	-124	22	0.1727	-24	-89	41	0.4787
Week 48	95	11	179	0.0287	-16	-88	57	0.677	-7	-72	58	0.838
Nef												
Week 1	-13	-120	95	0.8184	-1	-94	93	0.9916	73	-10	157	0.0895
Week 2	-22	-130	86	0.6901	-43	-136	51	0.3747	-56	-140	27	0.1891
Week 4	-21	-128	87	0.708	-37	-130	56	0.4394	-35	-119	48	0.4109
Week 6	-6	-114	102	0.9134	-1	-94	92	0.9833	-78	-161	5	0.0707
Week 8	23	-84	131	0.6724	-52	-145	42	0.2826	-24	-107	59	0.5746
Week 12	27	-80	135	0.6204	41	-53	134	0.3975	-25	-109	58	0.5557
Week 16	-27	-134	81	0.629	43	-50	136	0.3692	-36	-120	47	0.3952
Week 24	-24	-132	84	0.6636	-32	-125	61	0.5035	-85	-169	-2	0.0488
Week 48	42	-66	150	0.4471	-8	-101	86	0.8752	29	-55	112	0.5008
Tat												
Week 1	-10	-171	151	0.9035	-1	-140	139	0.9944	111	-14	236	0.0846
Week 2	-26	-187	135	0.7527	-51	-190	89	0.4802	-30	-154	95	0.6434
Week 4	-13	-174	148	0.8716	-22	-161	118	0.7635	16	-108	141	0.7974
Week 6	-8	-169	153	0.9227	9	-131	149	0.8997	-50	-175	75	0.4347
Week 8	23	-138	184	0.7835	-46	-185	94	0.5246	164	39	288	0.012
Week 12	55	-106	216	0.5029	10	-130	150	0.8887	21	-104	146	0.7401
Week 16	-27	-188	134	0.7465	34	-106	173	0.6393	-38	-163	86	0.5482
Week 24	-30	-191	131	0.7162	-13	-152	127	0.8611	-66	-191	59	0.3032
Week 48	93	-68	254	0.2631	-11	-150	129	0.8831	-24	-149	100	0.7026
Rev												
Week 1	-3	-112	106	0.9524	-20	-115	75	0.6795	44	-41	129	0.3108
Week 2	-2	-111	107	0.9714	-57	-151	38	0.2449	-46	-131	38	0.2853
Week 4	-4	-113	105	0.9429	-31	-125	64	0.5289	-57	-142	27	0.1886
Week 6	14	-95	123	0.8022	-4	-99	91	0.9341	-71	-155	14	0.1046
Week 8	3	-106	112	0.9619	-47	-142	48	0.3327	33	-52	117	0.4493
Week 12	23	-86	132	0.6764	27	-68	121	0.5842	-45	-130	39	0.2979
Week 16	-15	-124	94	0.7838	35	-60	130	0.4701	-71	-156	13	0.1027
Week 24	-13	-122	96	0.8114	-35	-130	60	0.4701	-48	-133	37	0.2691
Week 48	85	-24	194	0.1294	7	-88	101	0.8931	36	-49	120	0.4117
CTL												
Week 1	-37	-186	113	0.6324	21	-109	151	0.7517	63	-53	179	0.2885
Week 2	-41	-190	109	0.5958	-155	-284	-25	0.022	-34	-150	82	0.5716
Week 4	-41	-191	108	0.5898	-153	-283	-23	0.0232	-42	-158	74	0.4756
Week 6	-17	-166	133	0.8278	-114	-244	16	0.0886	-52	-168	64	0.382
Week 8	-6	-156	144	0.9376	-148	-277	-18	0.0285	136	20	252	0.0241
Week 12	-17	-166	133	0.8278	-86	-216	44	0.1972	-32	-148	84	0.5854
Week 16	-43	-192	107	0.5779	-98	-228	32	0.1423	-30	-146	86	0.6087
Week 24	-43	-193	106	0.572	-129	-258	1	0.0555	-45	-161	71	0.447
Week 48	41	-109	190	0.5958	-63	-192	67	0.3475	-17	-133	99	0.7771

Appendix Table 16 Changes from baseline in markers associated with CD4 T-cell immune activation.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+HLA-DR+CD38+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-0.63	-3.69	2.42	0.6853	0.13	-2.52	2.77	0.9264	-0.14	-2.50	2.22	0.9079
Week 2	18.27	15.21	21.32	<.0001	4.90	2.26	7.54	0.0005	18.06	15.70	20.42	<.0001
Week 4	1.53	-1.52	4.59	0.3277	-0.55	-3.19	2.09	0.6845	-0.12	-2.48	2.24	0.921
Week 6	-1.00	-4.05	2.05	0.5226	-0.65	-3.29	1.99	0.6311	-1.20	-3.56	1.16	0.3228
Week 8	-1.30	-4.35	1.75	0.4063	-0.58	-3.22	2.07	0.671	-0.84	-3.20	1.52	0.4882
Week 12	-0.07	-3.12	2.99	0.966	-0.30	-2.94	2.34	0.8245	-0.94	-3.30	1.42	0.4381
Week 16	-0.90	-3.95	2.15	0.5649	0.33	-2.32	2.97	0.8102	-1.58	-3.94	0.78	0.194
Week 24	-1.70	-4.75	1.35	0.2782	0.73	-1.92	3.37	0.5923	-0.98	-3.34	1.38	0.4189
Week 48	-1.00	-4.05	2.05	0.5226	0.95	-1.69	3.59	0.4832	-1.10	-3.46	1.26	0.3645
%CD4+HLA-DR+												
Week 1	-1.80	-5.23	1.63	0.3068	-0.35	-3.32	2.62	0.818	-0.20	-2.86	2.46	0.8831
Week 2	16.23	12.80	19.66	<.0001	5.40	2.43	8.37	0.0006	18.20	15.54	20.86	<.0001
Week 4	1.60	-1.83	5.03	0.3633	-0.38	-3.35	2.60	0.8052	0.46	-2.20	3.12	0.7352
Week 6	-1.23	-4.66	2.20	0.483	-1.03	-4.00	1.95	0.5008	-1.52	-4.18	1.14	0.2655
Week 8	-2.10	-5.53	1.33	0.2337	-0.55	-3.52	2.42	0.7176	-1.36	-4.02	1.30	0.3187
Week 12	-0.17	-3.60	3.26	0.9244	-0.60	-3.57	2.37	0.6932	-1.22	-3.88	1.44	0.3708
Week 16	-1.40	-4.83	2.03	0.4261	-1.05	-4.02	1.92	0.4904	-2.42	-5.08	0.24	0.078
Week 24	-2.73	-6.16	0.70	0.1222	0.18	-2.80	3.15	0.9084	-0.94	-3.60	1.72	0.49
Week 48	-1.30	-4.73	2.13	0.4597	-0.85	-3.82	2.12	0.5765	-1.60	-4.26	1.06	0.2413
%CD4+CD38+												
Week 1	3.07	-3.75	9.89	0.3807	-2.23	-8.13	3.68	0.4624	1.38	-3.90	6.66	0.61
Week 2	19.40	12.58	26.22	<.0001	6.05	0.14	11.96	0.048	21.78	16.50	27.06	<.0001
Week 4	-2.03	-8.85	4.79	0.5606	-2.60	-8.51	3.31	0.3908	1.84	-3.44	7.12	0.4967
Week 6	-12.77	-19.59	-5.95	0.0004	-5.13	-11.03	0.78	0.0928	-6.38	-11.66	-1.10	0.0203
Week 8	-10.83	-17.65	-4.01	0.0026	-6.20	-12.11	-0.29	0.0429	-6.42	-11.70	-1.14	0.0196
Week 12	-7.87	-14.69	-1.05	0.0264	-4.58	-10.48	1.33	0.1328	-7.04	-12.32	-1.76	0.0107
Week 16	-12.17	-18.99	-5.35	0.0008	-1.68	-7.58	4.23	0.5798	-7.14	-12.42	-1.86	0.0097
Week 24	-13.47	-20.29	-6.65	0.0002	-3.38	-9.28	2.53	0.266	-10.62	-15.90	-5.34	0.0002
Week 48	-8.30	-15.12	-1.48	0.0194	-3.50	-9.41	2.41	0.2488	-6.14	-11.42	-0.86	0.0254
CD4+HLA-DR MFI												
Week 1	-58	-3476	3361	0.9737	-306	-3267	2655	0.8401	-443	-3091	2205	0.7437
Week 2	14785	11366	18204	<.0001	3793	832	6753	0.014	16939	14291	19587	<.0001
Week 4	107	-3312	3526	0.9512	-366	-3327	2595	0.8093	-448	-3096	2200	0.741
Week 6	-987	-4406	2431	0.5729	-46	-3007	2915	0.9757	-165	-2813	2483	0.903
Week 8	-628	-4047	2790	0.7196	-7	-2967	2954	0.9966	-257	-2905	2392	0.8498
Week 12	873	-2546	4292	0.6181	433	-2528	3393	0.7754	-137	-2785	2511	0.9195
Week 16	545	-2873	3964	0.7554	1249	-1712	4209	0.411	810	-1838	3458	0.5506
Week 24	1153	-2266	4572	0.5105	2597	-364	5558	0.0894	1318	-1330	3966	0.3321
Week 48	1223	-2196	4642	0.4852	3407	446	6368	0.0268	2023	-625	4671	0.1382
CD4+CD38 MFI												
Week 1	85	-351	522	0.7026	-75	-453	303	0.6975	-33	-371	305	0.8479
Week 2	1144	707	1581	<.0001	353	-25	731	0.0711	862	524	1200	<.0001
Week 4	-225	-662	211	0.3147	-160	-538	218	0.4093	-405	-743	-67	0.0213
Week 6	-528	-965	-92	0.0201	-86	-464	292	0.656	-351	-689	-13	0.0452
Week 8	-320	-756	117	0.1551	-21	-399	357	0.9126	-324	-662	15	0.0643
Week 12	-377	-814	60	0.0944	43	-335	421	0.8252	-267	-605	71	0.1254
Week 16	-357	-793	80	0.1132	321	-58	699	0.1005	-54	-392	284	0.7542
Week 24	-184	-621	253	0.4112	448	70	826	0.0227	125	-213	463	0.4701
Week 48	-468	-905	-31	0.0387	-14	-392	364	0.9423	-346	-684	-8	0.0484

Appendix Table 17 Changes from baseline in markers associated with CD8 T-cell immune activation.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+HLA-DR+CD38+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-1.47	-6.32	3.38	0.555	0.60	-3.60	4.80	0.7802	-0.20	-3.96	3.56	0.9171
Week 2	30.90	26.05	35.75	<.0001	7.28	3.08	11.47	0.0011	13.86	10.10	17.62	<.0001
Week 4	6.20	1.35	11.05	0.0142	0.08	-4.12	4.27	0.9722	-0.06	-3.82	3.70	0.9751
Week 6	-1.00	-5.85	3.85	0.6871	-2.55	-6.75	1.65	0.2375	-1.58	-5.34	2.18	0.4121
Week 8	-1.60	-6.45	3.25	0.5196	-0.85	-5.05	3.35	0.6926	-1.44	-5.20	2.32	0.4546
Week 12	-1.43	-6.28	3.42	0.564	-0.03	-4.22	4.17	0.9907	-1.90	-5.66	1.86	0.3244
Week 16	-1.03	-5.88	3.82	0.6773	2.58	-1.62	6.77	0.2329	-2.24	-6.00	1.52	0.2459
Week 24	-3.53	-8.38	1.32	0.1571	0.83	-3.37	5.02	0.7012	-1.80	-5.56	1.96	0.3504
Week 48	-1.27	-6.12	3.58	0.6101	1.35	-2.85	5.55	0.5304	-3.00	-6.76	0.76	0.1214
%CD8+HLA-DR+												
Week 1	-2.20	-7.49	3.09	0.4177	-3.55	-8.13	1.03	0.133	-0.44	-4.54	3.66	0.8339
Week 2	19.20	13.91	24.49	<.0001	3.38	-1.21	7.96	0.1529	10.38	6.28	14.48	<.0001
Week 4	5.57	0.27	10.86	0.0425	-2.75	-7.33	1.83	0.2431	-1.28	-5.38	2.82	0.5423
Week 6	2.13	-3.16	7.43	0.4319	-3.13	-7.71	1.46	0.1853	-2.24	-6.34	1.86	0.2875
Week 8	0.20	-5.09	5.49	0.9412	0.25	-4.33	4.83	0.9151	-2.24	-6.34	1.86	0.2875
Week 12	0.13	-5.16	5.43	0.9607	-0.80	-5.38	3.78	0.7332	-1.90	-6.00	2.20	0.3665
Week 16	0.00	-5.29	5.29	1	-1.45	-6.03	3.13	0.537	-2.34	-6.44	1.76	0.2666
Week 24	-2.40	-7.69	2.89	0.3768	-0.88	-5.46	3.71	0.7093	-0.44	-4.54	3.66	0.8339
Week 48	4.43	-0.86	9.73	0.1046	0.60	-3.98	5.18	0.7982	-2.62	-6.72	1.48	0.214
%CD8+CD38+												
Week 1	-0.17	-9.01	8.67	0.9706	0.30	-7.36	7.96	0.939	2.26	-4.59	9.11	0.5195
Week 2	35.50	26.66	44.34	<.0001	12.73	5.07	20.38	0.0016	41.46	34.61	48.31	<.0001
Week 4	7.23	-1.61	16.07	0.1127	1.58	-6.08	9.23	0.6878	10.30	3.45	17.15	0.0042
Week 6	-8.37	-17.21	0.47	0.0672	-6.28	-13.93	1.38	0.1121	-3.52	-10.37	3.33	0.3167
Week 8	-5.77	-14.61	3.07	0.2047	-3.93	-11.58	3.73	0.318	-2.86	-9.71	3.99	0.4154
Week 12	-5.23	-14.07	3.61	0.2493	-1.25	-8.91	6.41	0.7498	-4.36	-11.21	2.49	0.2156
Week 16	-7.60	-16.44	1.24	0.0958	5.43	-2.23	13.08	0.1687	-1.52	-8.37	5.33	0.6647
Week 24	-12.77	-21.61	-3.93	0.0059	-0.55	-8.21	7.11	0.8884	-6.96	-13.81	-0.11	0.0497
Week 48	-8.43	-17.27	0.41	0.0651	-2.28	-9.93	5.38	0.5619	-8.78	-15.63	-1.93	0.0139
CD8+HLA-DR MFI												
Week 1	-332	-3228	2564	0.8226	140	-2368	2648	0.9133	-115	-2358	2129	0.9205
Week 2	6605	3709	9501	<.0001	3305	797	5813	0.0116	11214	8971	13457	<.0001
Week 4	-668	-3564	2228	0.6524	183	-2325	2691	0.8868	-28	-2271	2215	0.9805
Week 6	-1201	-4097	1695	0.4187	516	-1992	3024	0.688	197	-2046	2440	0.8636
Week 8	-1064	-3960	1832	0.4737	64	-2445	2572	0.9605	-120	-2363	2123	0.9166
Week 12	798	-2098	3694	0.5908	95	-2413	2603	0.9408	-359	-2603	1884	0.7543
Week 16	50	-2846	2946	0.9731	966	-1542	3474	0.4525	556	-1687	2799	0.6285
Week 24	429	-2467	3325	0.7723	1873	-635	4381	0.1471	1464	-780	3707	0.2046
Week 48	377	-2519	3273	0.7993	1503	-1005	4011	0.2435	130	-2113	2374	0.9096
CD8+CD38 MFI												
Week 1	84	-429	598	0.7485	-77	-522	368	0.7345	-88	-486	310	0.6673
Week 2	2056	1543	2570	<.0001	620	175	1064	0.0078	1445	1047	1843	<.0001
Week 4	120	-393	634	0.6474	-96	-541	349	0.6735	-145	-543	253	0.4778
Week 6	-49	-563	465	0.8522	42	-403	487	0.8545	-216	-614	182	0.2896
Week 8	20	-494	533	0.9404	34	-411	478	0.883	-220	-618	178	0.2809
Week 12	25	-488	539	0.9232	18	-427	463	0.937	-210	-608	188	0.305
Week 16	-30	-543	484	0.9102	99	-346	544	0.6639	-199	-597	199	0.3294
Week 24	169	-345	683	0.5209	250	-195	695	0.274	-35	-433	363	0.8651
Week 48	-31	-544	483	0.9071	130	-315	575	0.5684	-299	-697	99	0.1452

Appendix Table 18 Changes from baseline in markers associated with CD4 T-cell immune senescence and apoptosis. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+CD57+ CD95+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-0.60	-3.65	2.45	0.7013	0.90	-1.75	3.55	0.5068	1.12	-1.25	3.49	0.3563
Week 2	-2.27	-5.32	0.79	0.1497	1.43	-1.22	4.07	0.2942	-2.06	-4.43	0.31	0.0918
Week 4	-3.53	-6.59	-0.48	0.0261	0.43	-2.22	3.07	0.7537	-3.20	-5.57	-0.83	0.0097
Week 6	-1.77	-4.82	1.29	0.2603	0.10	-2.55	2.75	0.9411	-1.88	-4.25	0.49	0.1233
Week 8	-3.07	-6.12	-0.01	0.0525	0.70	-1.95	3.35	0.6054	-2.16	-4.53	0.21	0.0773
Week 12	0.17	-2.89	3.22	0.9151	0.65	-2.00	3.30	0.6314	-1.18	-3.55	1.19	0.3313
Week 16	-2.83	-5.89	0.22	0.0728	-1.10	-3.75	1.55	0.4175	-1.14	-3.51	1.23	0.3478
Week 24	-2.93	-5.99	0.12	0.0634	-0.15	-2.80	2.50	0.9118	0.16	-2.21	2.53	0.8949
Week 48	-2.93	-5.99	0.12	0.0634	-2.73	-5.37	-0.08	0.0468	-2.84	-5.21	-0.47	0.0211
%CD4+CD57+												
Week 1	-0.67	-3.90	2.57	0.6871	0.95	-1.85	3.75	0.5079	1.18	-1.32	3.68	0.3584
Week 2	-2.60	-5.83	0.63	0.1188	1.48	-1.32	4.27	0.3048	-2.32	-4.82	0.18	0.0731
Week 4	-3.70	-6.93	-0.47	0.0276	0.38	-2.42	3.17	0.7936	-3.32	-5.82	-0.82	0.0111
Week 6	-1.73	-4.97	1.50	0.2964	0.13	-2.67	2.92	0.9305	-1.96	-4.46	0.54	0.1289
Week 8	-3.10	-6.33	0.13	0.0638	0.68	-2.12	3.47	0.6378	-1.68	-4.18	0.82	0.1922
Week 12	0.23	-3.00	3.47	0.8878	0.73	-2.07	3.52	0.6131	-1.20	-3.70	1.30	0.3504
Week 16	-3.00	-6.23	0.23	0.0726	-1.18	-3.97	1.62	0.4131	-1.16	-3.66	1.34	0.3666
Week 24	-3.07	-6.30	0.17	0.0666	-0.05	-2.85	2.75	0.9722	0.20	-2.30	2.70	0.876
Week 48	-2.97	-6.20	0.27	0.0758	-2.90	-5.70	-0.10	0.0456	-2.98	-5.48	-0.48	0.0221
%CD4+CD95+												
Week 1	-2.13	-6.80	2.53	0.373	2.20	-1.84	6.24	0.2893	-1.28	-4.90	2.34	0.4898
Week 2	18.57	13.90	23.23	<.0001	6.05	2.01	10.09	0.0044	17.20	13.58	20.82	<.0001
Week 4	12.57	7.90	17.23	<.0001	0.43	-3.62	4.47	0.8373	7.32	3.70	10.94	0.0002
Week 6	9.53	4.87	14.20	0.0001	2.33	-1.72	6.37	0.263	4.60	0.98	8.22	0.0147
Week 8	7.10	2.43	11.77	0.0038	2.50	-1.54	6.54	0.229	4.14	0.52	7.76	0.0275
Week 12	5.93	1.27	10.60	0.0148	3.88	-0.17	7.92	0.0639	4.68	1.06	8.30	0.0131
Week 16	7.77	3.10	12.43	0.0016	2.55	-1.49	6.59	0.2199	3.30	-0.32	6.92	0.0774
Week 24	6.77	2.10	11.43	0.0057	2.30	-1.74	6.34	0.2681	4.76	1.14	8.38	0.0117
Week 48	3.33	-1.33	8.00	0.1654	-0.40	-4.44	3.64	0.8467	0.50	-3.12	4.12	0.7871
CD4+CD57 MFI												
Week 1	-12	-2184	2161	0.9916	824	-1058	2706	0.3933	866	-817	2549	0.3164
Week 2	-2193	-4366	-21	0.0513	-230	-2111	1652	0.8115	-2216	-3899	-533	0.0117
Week 4	-2587	-4760	-415	0.0221	1646	-236	3527	0.0903	-2136	-3819	-453	0.0149
Week 6	-1527	-3699	646	0.1723	847	-1035	2728	0.3804	-49	-1732	1634	0.9546
Week 8	-1222	-3394	951	0.2737	966	-915	2848	0.3172	3	-1680	1686	0.9974
Week 12	1065	-1108	3237	0.3397	970	-912	2852	0.3153	-22	-1705	1661	0.9796
Week 16	-1956	-4129	216	0.0814	-757	-2638	1125	0.4329	-738	-2421	945	0.3926
Week 24	-804	-2977	1368	0.4702	1593	-288	3475	0.1009	949	-734	2632	0.2722
Week 48	-1940	-4113	232	0.0838	-1130	-3011	752	0.2428	-942	-2625	741	0.2761
CD4+CD95 MFI												
Week 1	257	-862	1377	0.6536	15	-955	985	0.9759	-91	-958	777	0.8379
Week 2	3750	2630	4869	<.0001	790	-179	1760	0.1141	4068	3200	4935	<.0001
Week 4	63	-1057	1183	0.9125	-62	-1032	907	0.9002	-121	-989	746	0.7849
Week 6	-471	-1591	648	0.4118	109	-861	1078	0.827	-96	-963	771	0.8288
Week 8	7	-1113	1127	0.9903	123	-846	1093	0.8039	97	-770	964	0.8271
Week 12	-219	-1339	901	0.7025	103	-867	1072	0.836	-135	-1002	733	0.7618
Week 16	486	-634	1605	0.3978	55	-914	1025	0.9114	-214	-1081	654	0.6306
Week 24	-623	-1743	497	0.2787	855	-115	1824	0.0879	594	-274	1461	0.1836
Week 48	-1562	-2682	-442	0.0077	-739	-1709	230	0.139	-1675	-2542	-808	0.0003

Appendix Table 19 Changes from baseline in markers associated with CD8 T-cell immune senescence and apoptosis. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+CD57+ CD95+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-0.73	-8.07	6.60	0.8451	-0.10	-6.45	6.25	0.9755	5.20	-0.48	10.88	0.0766
Week 2	-3.60	-10.94	3.74	0.3389	-1.75	-8.10	4.60	0.5907	-6.06	-11.74	-0.38	0.0397
Week 4	-5.47	-12.80	1.87	0.148	-0.20	-6.55	6.15	0.9509	-5.46	-11.14	0.22	0.0632
Week 6	-0.10	-7.44	7.24	0.9787	-1.65	-8.00	4.70	0.6121	-5.26	-10.94	0.42	0.0733
Week 8	-3.40	-10.74	3.94	0.3663	-1.40	-7.75	4.95	0.6669	-7.84	-13.52	-2.16	0.0083
Week 12	3.70	-3.64	11.04	0.3258	0.45	-5.90	6.80	0.8899	-5.12	-10.80	0.56	0.0811
Week 16	-3.80	-11.14	3.54	0.313	-4.25	-10.60	2.10	0.1935	-5.18	-10.86	0.50	0.0777
Week 24	-2.73	-10.07	4.60	0.4673	0.10	-6.25	6.45	0.9755	-1.94	-7.62	3.74	0.5053
Week 48	-4.03	-11.37	3.30	0.2844	-6.15	-12.50	0.20	0.0613	-10.52	-16.20	-4.84	0.0005
%CD8+CD57+												
Week 1	-0.97	-8.37	6.44	0.7987	-0.20	-6.61	6.21	0.9514	5.00	-0.73	10.73	0.0913
Week 2	-4.10	-11.50	3.30	0.281	-1.90	-8.31	4.51	0.563	-6.70	-12.43	-0.97	0.0246
Week 4	-5.33	-12.74	2.07	0.1618	-0.25	-6.66	6.16	0.9393	-5.76	-11.49	-0.03	0.0524
Week 6	0.00	-7.40	7.40	1	-1.53	-7.94	4.89	0.6424	-5.52	-11.25	0.21	0.0628
Week 8	-3.43	-10.84	3.97	0.3661	-1.30	-7.71	5.11	0.6921	-7.90	-13.63	-2.17	0.0084
Week 12	3.77	-3.64	11.17	0.3217	0.35	-6.06	6.76	0.9151	-5.20	-10.93	0.53	0.0793
Week 16	-3.87	-11.27	3.54	0.3091	-4.18	-10.59	2.24	0.2055	-5.14	-10.87	0.59	0.0828
Week 24	-2.80	-10.20	4.60	0.4607	0.10	-6.31	6.51	0.9757	-2.24	-7.97	3.49	0.4462
Week 48	-3.80	-11.20	3.60	0.3174	-6.68	-13.09	-0.26	0.0446	-10.40	-16.13	-4.67	0.0006
%CD8+CD95+												
Week 1	-0.57	-6.78	5.65	0.8587	-2.35	-7.74	3.04	0.3949	1.04	-3.78	5.86	0.6733
Week 2	6.00	-0.22	12.22	0.0622	2.15	-3.24	7.54	0.4362	-1.22	-6.04	3.60	0.6209
Week 4	4.13	-2.08	10.35	0.1963	1.35	-4.04	6.74	0.6245	-0.42	-5.24	4.40	0.8647
Week 6	3.67	-2.55	9.88	0.2512	1.20	-4.19	6.59	0.6634	2.82	-2.00	7.64	0.2545
Week 8	1.97	-4.25	8.18	0.5371	1.03	-4.36	6.41	0.7101	-2.04	-6.86	2.78	0.4089
Week 12	3.87	-2.35	10.08	0.2265	3.70	-1.69	9.09	0.1818	1.54	-3.28	6.36	0.5326
Week 16	3.10	-3.12	9.32	0.3314	0.50	-4.89	5.89	0.856	0.22	-4.60	5.04	0.9289
Week 24	3.30	-2.92	9.52	0.3014	1.30	-4.09	6.69	0.6374	4.98	0.16	9.80	0.046
Week 48	-0.37	-6.58	5.85	0.9083	-0.18	-5.56	5.21	0.9494	-3.64	-8.46	1.18	0.1424
CD8+CD57 MFI												
Week 1	171	-1328	1669	0.824	258	-1040	1556	0.6981	637	-524	1798	0.2855
Week 2	-566	-2065	933	0.4613	241	-1057	1539	0.7166	-163	-1324	998	0.7841
Week 4	-1831	-3329	-332	0.019	169	-1129	1466	0.7998	-1229	-2390	-68	0.0412
Week 6	-407	-1905	1092	0.5963	-176	-1473	1122	0.7917	-724	-1885	437	0.2249
Week 8	-936	-2434	563	0.2246	316	-982	1614	0.6343	-1275	-2436	-114	0.0343
Week 12	-189	-1687	1310	0.8057	79	-1219	1377	0.9053	-1049	-2210	112	0.0803
Week 16	-1244	-2743	254	0.1076	-256	-1553	1042	0.7006	-1020	-2181	141	0.0888
Week 24	-338	-1837	1161	0.6597	872	-426	2170	0.1916	-242	-1403	919	0.6839
Week 48	-1524	-3023	-25	0.0496	-604	-1902	694	0.3646	-1640	-2801	-479	0.007
CD8+CD95 MFI												
Week 1	258	-721	1237	0.6069	35	-813	882	0.9366	-35	-793	724	0.9286
Week 2	1903	924	2882	0.0003	463	-385	1311	0.2876	2848	2089	3606	<.0001
Week 4	79	-900	1058	0.8752	27	-821	875	0.9499	148	-611	906	0.7034
Week 6	-215	-1194	764	0.6685	79	-769	927	0.856	252	-507	1010	0.517
Week 8	32	-947	1011	0.9485	90	-758	937	0.8366	545	-213	1304	0.1626
Week 12	-21	-1000	958	0.9671	19	-829	867	0.9646	141	-617	900	0.7161
Week 16	566	-413	1545	0.2608	24	-824	872	0.9559	25	-733	784	0.9482
Week 24	-290	-1269	689	0.5636	520	-328	1368	0.2326	707	-51	1466	0.0712
Week 48	-874	-1853	105	0.0838	-525	-1373	323	0.2286	-919	-1677	-160	0.0199

Appendix Table 20 Changes from baseline in markers associated with early, intermediate and late CD4 T- cells. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+CD27+ CD28+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	2.33	-2.12	6.79	0.3079	-1.73	-5.58	2.13	0.3837	-2.12	-5.57	1.33	0.2323
Week 2	-1.97	-6.42	2.49	0.3897	-2.75	-6.61	1.11	0.1664	2.70	-0.75	6.15	0.1292
Week 4	0.13	-4.32	4.59	0.9534	1.45	-2.41	5.31	0.4637	0.70	-2.75	4.15	0.6921
Week 6	0.57	-3.89	5.02	0.8038	-0.83	-4.68	3.03	0.6764	3.60	0.15	7.05	0.0442
Week 8	2.27	-2.19	6.72	0.3218	-1.55	-5.41	2.31	0.4335	2.46	-0.99	5.91	0.1664
Week 12	0.47	-3.99	4.92	0.8379	-2.35	-6.21	1.51	0.2362	1.58	-1.87	5.03	0.3724
Week 16	2.57	-1.89	7.02	0.2624	0.05	-3.81	3.91	0.9798	-0.10	-3.55	3.35	0.9549
Week 24	3.67	-0.79	8.12	0.1108	0.13	-3.73	3.98	0.9495	1.46	-1.99	4.91	0.4096
Week 48	0.70	-3.76	5.16	0.759	0.93	-2.93	4.78	0.6398	0.68	-2.77	4.13	0.7005
%CD4+CD27-CD28+												
Week 1	-0.67	-2.19	0.85	0.3921	0.53	-0.79	1.84	0.4363	-0.32	-1.50	0.86	0.5954
Week 2	5.30	3.78	6.82	<.0001	0.95	-0.37	2.27	0.1607	2.22	1.04	3.40	0.0004
Week 4	2.17	0.65	3.69	0.0065	0.40	-0.92	1.72	0.5528	1.90	0.72	3.08	0.0022
Week 6	1.73	0.21	3.25	0.028	0.43	-0.89	1.74	0.5283	0.16	-1.02	1.34	0.7905
Week 8	1.37	-0.15	2.89	0.0816	0.45	-0.87	1.77	0.5044	0.06	-1.12	1.24	0.9206
Week 12	0.20	-1.32	1.72	0.797	1.40	0.08	2.72	0.0401	0.02	-1.16	1.20	0.9735
Week 16	0.97	-0.55	2.49	0.2158	0.83	-0.49	2.14	0.2225	-0.30	-1.48	0.88	0.6186
Week 24	0.97	-0.55	2.49	0.2158	1.03	-0.29	2.34	0.1306	-0.04	-1.22	1.14	0.947
Week 48	-0.17	-1.69	1.35	0.8302	1.00	-0.32	2.32	0.1401	0.18	-1.00	1.36	0.765
%CD4+CD27-CD28-												
Week 1	-1.63	-5.67	2.40	0.4298	1.15	-2.34	4.64	0.5207	2.48	-0.64	5.60	0.1237
Week 2	-3.37	-7.40	0.67	0.1058	1.83	-1.67	5.32	0.309	-4.70	-7.82	-1.58	0.0042
Week 4	-2.47	-6.50	1.57	0.2343	-1.80	-5.29	1.69	0.3156	-2.58	-5.70	0.54	0.1095
Week 6	-2.33	-6.37	1.70	0.2603	0.50	-2.99	3.99	0.7798	-3.62	-6.74	-0.50	0.0258
Week 8	-3.63	-7.67	0.40	0.0813	1.10	-2.39	4.59	0.5389	-2.26	-5.38	0.86	0.1602
Week 12	-0.87	-4.90	3.17	0.6748	1.00	-2.49	4.49	0.5763	-1.40	-4.52	1.72	0.3825
Week 16	-3.57	-7.60	0.47	0.0869	-0.78	-4.27	2.72	0.6649	0.44	-2.68	3.56	0.7833
Week 24	-4.60	-8.63	-0.57	0.0282	-1.10	-4.59	2.39	0.5389	-1.28	-4.40	1.84	0.4244
Week 48	-0.77	-4.80	3.27	0.7105	-1.83	-5.32	1.67	0.309	-0.82	-3.94	2.30	0.6084
CD4+CD27 MFI												
Week 1	182	-333	698	0.49	30	-417	476	0.8972	29	-370	428	0.8871
Week 2	168	-347	684	0.5238	-137	-583	310	0.5498	743	344	1143	0.0005
Week 4	-309	-824	206	0.2433	-17	-463	430	0.9424	-341	-740	58	0.0979
Week 6	-583	-1099	-68	0.0293	-35	-481	411	0.8782	-85	-484	314	0.6775
Week 8	-449	-964	66	0.0915	-216	-662	230	0.3456	-285	-684	114	0.1652
Week 12	-85	-601	430	0.7463	-411	-857	35	0.0748	-307	-706	92	0.1358
Week 16	-273	-788	242	0.3022	-21	-468	425	0.9259	115	-284	514	0.5732
Week 24	-80	-596	435	0.7607	-86	-533	360	0.7058	206	-194	605	0.3157
Week 48	-349	-865	166	0.1877	34	-412	480	0.8817	-255	-654	144	0.2138
CD4+CD28 MFI												
Week 1	229	-974	1433	0.7098	-128	-1170	915	0.8111	262	-671	1194	0.5836
Week 2	498	-706	1701	0.4201	281	-762	1323	0.599	914	-18	1846	0.0582
Week 4	-274	-1478	929	0.6563	-149	-1191	894	0.7804	35	-897	968	0.9412
Week 6	-980	-2183	224	0.1145	518	-525	1560	0.3332	92	-841	1024	0.8474
Week 8	-251	-1455	953	0.6838	-151	-1194	891	0.7768	445	-488	1377	0.3527
Week 12	-510	-1713	694	0.409	-68	-1110	975	0.8993	354	-578	1286	0.4589
Week 16	722	-482	1925	0.2434	162	-881	1204	0.7622	854	-79	1786	0.0765
Week 24	-1084	-2288	119	0.0812	228	-815	1270	0.6696	2092	1160	3025	<.0001
Week 48	-2341	-3544	-1137	0.0003	-1126	-2168	-83	0.0373	-1876	-2808	-943	0.0002

Appendix Table 21 Changes from baseline in markers associated with early, intermediate and late CD8 T- cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+CD27+ CD28+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	5.73	-1.43	12.89	0.1205	2.58	-3.63	8.78	0.4181	-5.84	-11.39	-0.29	0.0423
Week 2	-0.87	-8.03	6.29	0.8131	0.95	-5.25	7.15	0.7648	4.12	-1.43	9.67	0.1493
Week 4	-0.57	-7.73	6.59	0.8771	7.98	1.77	14.18	0.0137	4.78	-0.77	10.33	0.0951
Week 6	1.20	-5.96	8.36	0.7434	3.18	-3.03	9.38	0.3186	8.16	2.61	13.71	0.005
Week 8	1.53	-5.63	8.69	0.6758	6.70	0.50	12.90	0.0373	7.44	1.89	12.99	0.0102
Week 12	-1.57	-8.73	5.59	0.6692	6.30	0.10	12.50	0.0498	7.46	1.91	13.01	0.0101
Week 16	3.47	-3.69	10.63	0.3455	3.78	-2.43	9.98	0.2363	3.02	-2.53	8.57	0.2891
Week 24	5.33	-1.83	12.49	0.1482	6.73	0.52	12.93	0.0366	4.72	-0.83	10.27	0.0992
Week 48	2.73	-4.43	9.89	0.4566	5.23	-0.98	11.43	0.1025	6.74	1.19	12.29	0.0196
%CD8+CD27+ CD28-												
Week 1	-1.03	-2.81	0.75	0.2587	-2.58	-4.12	-1.03	0.0016	0.10	-1.28	1.48	0.8874
Week 2	-1.53	-3.31	0.25	0.0953	-1.90	-3.44	-0.36	0.018	0.06	-1.32	1.44	0.9323
Week 4	-0.17	-1.95	1.61	0.8549	-3.15	-4.69	-1.61	0.0001	-2.08	-3.46	-0.70	0.0041
Week 6	-0.80	-2.58	0.98	0.3812	-1.25	-2.79	0.29	0.116	-0.94	-2.32	0.44	0.1854
Week 8	-0.97	-2.75	0.81	0.2905	-4.48	-6.02	-2.93	<.0001	-1.20	-2.58	0.18	0.092
Week 12	0.87	-0.91	2.65	0.343	-2.58	-4.12	-1.03	0.0016	-1.20	-2.58	0.18	0.092
Week 16	-0.40	-2.18	1.38	0.6609	-1.60	-3.14	-0.06	0.0453	-0.14	-1.52	1.24	0.8428
Week 24	-1.43	-3.21	0.35	0.1185	-1.98	-3.52	-0.43	0.0141	-0.40	-1.78	0.98	0.5713
Week 48	-0.60	-2.38	1.18	0.5109	-3.43	-4.97	-1.88	<.0001	-1.20	-2.58	0.18	0.092
%CD8+CD27- CD28-												
Week 1	-5.13	-12.57	2.30	0.1799	-0.43	-6.87	6.02	0.8974	5.80	0.04	11.56	0.0519
Week 2	-2.73	-10.17	4.70	0.4734	0.23	-6.22	6.67	0.9456	-8.12	-13.88	-2.36	0.0071
Week 4	-0.83	-8.27	6.60	0.8267	-5.68	-12.12	0.77	0.088	-3.12	-8.88	2.64	0.2916
Week 6	-1.07	-8.50	6.37	0.7794	-1.93	-8.37	4.52	0.5597	-7.68	-13.44	-1.92	0.0107
Week 8	-1.43	-8.87	6.00	0.7066	-5.38	-11.82	1.07	0.1058	-7.38	-13.14	-1.62	0.014
Week 12	0.57	-6.87	8.00	0.8817	-4.43	-10.87	2.02	0.1819	-6.58	-12.34	-0.82	0.0279
Week 16	-3.77	-11.20	3.67	0.3238	-2.18	-8.62	4.27	0.5099	-2.90	-8.66	2.86	0.3268
Week 24	-4.70	-12.14	2.74	0.2191	-5.28	-11.72	1.17	0.1123	-4.38	-10.14	1.38	0.1401
Week 48	-2.17	-9.60	5.27	0.5696	-1.68	-8.12	4.77	0.6116	-5.46	-11.22	0.30	0.0669
CD8+CD27 MFI												
Week 1	221	-190	633	0.2951	-3	-359	354	0.988	21	-298	339	0.8996
Week 2	47	-365	459	0.8235	-62	-418	295	0.7351	437	118	756	0.0088
Week 4	-270	-682	141	0.2017	67	-290	424	0.7136	-235	-554	84	0.1528
Week 6	-372	-784	40	0.0803	-19	-376	338	0.9171	-109	-428	209	0.5032
Week 8	-251	-662	161	0.2362	-81	-438	275	0.6563	-250	-569	69	0.1286
Week 12	-61	-472	351	0.7734	-289	-645	68	0.1166	-174	-493	145	0.2885
Week 16	-102	-514	310	0.6285	49	-308	406	0.7883	107	-212	426	0.5126
Week 24	70	-342	482	0.7398	3	-354	360	0.9869	83	-236	402	0.6113
Week 48	-202	-614	209	0.3382	145	-211	502	0.4269	-76	-394	243	0.6434
CD8+CD28 MFI												
Week 1	256	-1197	1709	0.7307	-471	-1729	788	0.4655	237	-888	1362	0.6809
Week 2	155	-1298	1608	0.8352	-280	-1538	979	0.6642	631	-494	1757	0.2749
Week 4	99	-1354	1552	0.8941	-419	-1677	839	0.5158	206	-919	1332	0.7204
Week 6	-302	-1755	1151	0.6845	24	-1234	1282	0.9703	333	-793	1458	0.564
Week 8	-20	-1473	1433	0.9785	-394	-1652	864	0.5411	490	-635	1616	0.3956
Week 12	431	-1022	1884	0.5623	344	-915	1602	0.5941	1057	-68	2183	0.0693
Week 16	2038	585	3491	0.0074	995	-264	2253	0.1252	1881	756	3007	0.0015
Week 24	73	-1380	1526	0.9214	670	-589	1928	0.2999	3432	2307	4558	<.0001
Week 48	-1723	-3176	-270	0.0226	-783	-2041	476	0.2264	-1456	-2581	-330	0.0132

Appendix Table 22 Changes from baseline in markers associated with a regulatory T-cell phenotype.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+CD45RO+ CD25high	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-0.43	-3.02	2.16	0.7437	-0.10	-2.34	2.14	0.9306	0.26	-1.75	2.27	0.8
Week 2	6.97	4.38	9.56	<.0001	0.05	-2.19	2.29	0.9652	10.98	8.97	12.99	<.0001
Week 4	0.23	-2.36	2.82	0.8602	0.38	-1.87	2.62	0.7439	1.92	-0.09	3.93	0.0642
Week 6	-0.27	-2.86	2.32	0.8405	0.43	-1.82	2.67	0.7112	1.32	-0.69	3.33	0.2007
Week 8	-0.37	-2.96	2.22	0.782	-0.03	-2.27	2.22	0.9826	0.36	-1.65	2.37	0.7259
Week 12	0.13	-2.46	2.72	0.9198	0.65	-1.59	2.89	0.5714	0.96	-1.05	2.97	0.3509
Week 16	0.10	-2.49	2.69	0.9398	0.70	-1.54	2.94	0.5423	0.50	-1.51	2.51	0.6264
Week 24	0.00	-2.59	2.59	1	0.80	-1.44	3.04	0.4863	0.74	-1.27	2.75	0.4716
Week 48	0.17	-2.42	2.76	0.8999	0.50	-1.74	2.74	0.6632	0.84	-1.17	2.85	0.4141
%CD4+CD45RO+ CD25+												
Week 1	-1.03	-7.79	5.72	0.765	3.68	-2.17	9.52	0.2217	-0.12	-5.35	5.11	0.9643
Week 2	16.20	9.45	22.95	<.0001	4.80	-1.05	10.65	0.1116	20.20	14.97	25.43	<.0001
Week 4	10.40	3.65	17.15	0.0034	1.45	-4.40	7.30	0.6284	9.76	4.53	14.99	0.0005
Week 6	4.20	-2.55	10.95	0.2264	3.23	-2.62	9.07	0.283	3.64	-1.59	8.87	0.1764
Week 8	4.63	-2.12	11.39	0.1825	5.08	-0.77	10.92	0.0929	3.54	-1.69	8.77	0.1885
Week 12	1.20	-5.55	7.95	0.7286	8.98	3.13	14.82	0.0035	5.80	0.57	11.03	0.0327
Week 16	3.07	-3.69	9.82	0.3761	7.23	1.38	13.07	0.0177	4.20	-1.03	9.43	0.1195
Week 24	4.67	-2.09	11.42	0.1794	4.93	-0.92	10.77	0.1027	-0.22	-5.45	5.01	0.9345
Week 48	10.00	3.25	16.75	0.0048	6.95	1.10	12.80	0.0224	5.78	0.55	11.01	0.0333
%CD4+CD25+												
Week 1	-0.77	-8.69	7.16	0.8501	4.10	-2.76	10.96	0.2451	0.18	-5.96	6.32	0.9543
Week 2	16.97	9.04	24.89	<.0001	4.05	-2.81	10.91	0.2509	26.06	19.92	32.20	<.0001
Week 4	12.17	4.24	20.09	0.0035	3.30	-3.56	10.16	0.3488	13.80	7.66	19.94	<.0001
Week 6	5.67	-2.26	13.59	0.1649	4.78	-2.09	11.64	0.1765	9.90	3.76	16.04	0.0022
Week 8	7.23	-0.69	15.16	0.0774	5.48	-1.39	12.34	0.1219	7.04	0.90	13.18	0.0273
Week 12	2.20	-5.73	10.13	0.5879	10.78	3.91	17.64	0.0029	9.12	2.98	15.26	0.0046
Week 16	3.13	-4.79	11.06	0.4407	9.63	2.76	16.49	0.0074	7.54	1.40	13.68	0.0184
Week 24	2.10	-5.83	10.03	0.605	6.00	-0.86	12.86	0.0905	-1.34	-7.48	4.80	0.6699
Week 48	5.27	-2.66	13.19	0.1965	8.33	1.46	15.19	0.0198	4.20	-1.94	10.34	0.1837
%CD4+CD45RO+												
Week 1	-1.50	-7.13	4.13	0.6033	2.18	-2.70	7.05	0.3849	0.98	-3.38	5.34	0.661
Week 2	11.73	6.10	17.37	0.0001	3.20	-1.68	8.08	0.2023	13.52	9.16	17.88	<.0001
Week 4	8.27	2.63	13.90	0.0052	-1.33	-6.20	3.55	0.596	7.80	3.44	12.16	0.0008
Week 6	5.63	0.00	11.27	0.0535	0.68	-4.20	5.55	0.787	2.32	-2.04	6.68	0.3006
Week 8	4.53	-1.10	10.17	0.1187	1.03	-3.85	5.90	0.6816	5.70	1.34	10.06	0.0123
Week 12	3.70	-1.93	9.33	0.2017	1.65	-3.23	6.53	0.5094	5.30	0.94	9.66	0.0197
Week 16	7.70	2.07	13.33	0.009	-0.30	-5.18	4.58	0.9044	3.34	-1.02	7.70	0.1375
Week 24	10.27	4.63	15.90	0.0006	3.43	-1.45	8.30	0.1727	7.26	2.90	11.62	0.0016
Week 48	8.97	3.33	14.60	0.0025	1.85	-3.03	6.73	0.4596	6.64	2.28	11.00	0.0038
CD4+CD25 MFI												
Week 1	25	-280	330	0.8711	-137	-401	128	0.3143	14	-222	251	0.9066
Week 2	1197	892	1502	<.0001	-83	-347	181	0.5411	1404	1168	1641	<.0001
Week 4	211	-94	516	0.1798	-174	-438	90	0.1999	221	-16	457	0.0708
Week 6	20	-285	325	0.8981	-141	-405	123	0.2988	76	-160	313	0.5282
Week 8	5	-300	310	0.9728	-147	-411	117	0.2788	-7	-243	229	0.9539
Week 12	-103	-408	202	0.5101	-156	-420	108	0.2499	-71	-308	165	0.5554
Week 16	-92	-397	213	0.5562	-155	-419	109	0.2529	-31	-267	206	0.7991
Week 24	-115	-420	190	0.4609	-196	-460	68	0.1499	-97	-333	140	0.4254
Week 48	-94	-399	211	0.5477	-252	-516	12	0.0652	-99	-336	137	0.4131
CD4+ CD45RO MFI												
Week 1	-487	-4554	3581	0.8152	-411	-3933	3112	0.8198	980	-2171	4130	0.5439
Week 2	1341	-2727	5408	0.5201	178	-3345	3700	0.9216	3493	343	6643	0.0327
Week 4	-675	-4743	3392	0.7457	229	-3294	3751	0.8991	327	-2824	3477	0.8395

Appendix Table 22 continued

Week 6	-2999	-7066	1069	0.1523	1273	-2249	4796	0.4807	-230	-3380	2921	0.8868
Week 8	-527	-4595	3540	0.8	-38	-3561	3484	0.9831	2122	-1029	5272	0.1905
Week 12	1234	-2834	5301	0.5538	409	-3114	3931	0.8208	972	-2178	4123	0.547
Week 16	1845	-2222	5912	0.3766	3098	-425	6620	0.0886	2593	-557	5744	0.1105
Week 24	2460	-1607	6528	0.2392	2760	-762	6283	0.1284	4792	1642	7942	0.0038
Week 48	3244	-823	7311	0.1219	3042	-481	6564	0.0944	4194	1043	7344	0.0108

Appendix Table 23 Changes from baseline in markers associated with CD4 T-cell differentiation/maturation.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4 Naïve	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	4.37	-0.55	9.29	0.0857	-2.88	-7.14	1.39	0.1897	1.78	-2.03	5.59	0.3627
Week 2	-6.97	-11.89	-2.05	0.0068	-3.50	-7.76	0.76	0.1113	-6.94	-10.75	-3.13	0.0006
Week 4	-9.97	-14.89	-5.05	0.0002	2.30	-1.96	6.56	0.2932	-5.66	-9.47	-1.85	0.0047
Week 6	-5.77	-10.69	-0.85	0.0242	-1.10	-5.36	3.16	0.6142	-0.32	-4.13	3.49	0.8697
Week 8	-1.07	-5.99	3.85	0.672	-1.75	-6.01	2.51	0.4232	-0.34	-4.15	3.47	0.8616
Week 12	-2.73	-7.65	2.19	0.2794	-1.85	-6.11	2.41	0.3973	-0.58	-4.39	3.23	0.7662
Week 16	-1.90	-6.82	3.02	0.4513	-0.13	-4.39	4.14	0.9543	-0.72	-4.53	3.09	0.7121
Week 24	-3.30	-8.22	1.62	0.1924	-1.55	-5.81	2.71	0.4779	-0.24	-4.05	3.57	0.9021
Week 48	-5.77	-10.69	-0.85	0.0242	-0.73	-4.99	3.54	0.7396	-0.58	-4.39	3.23	0.7662
%CD4 TCM												
Week 1	-2.40	-8.66	3.86	0.4544	1.00	-4.42	6.42	0.7186	-3.36	-8.21	1.49	0.1781
Week 2	-1.63	-7.89	4.62	0.6104	6.85	1.43	12.27	0.0153	1.34	-3.51	6.19	0.5895
Week 4	4.70	-1.56	10.96	0.1449	1.03	-4.39	6.44	0.7118	4.64	-0.21	9.49	0.0643
Week 6	0.00	-6.26	6.26	1	2.75	-2.67	8.17	0.3229	2.46	-2.39	7.31	0.3229
Week 8	1.67	-4.59	7.92	0.6031	0.63	-4.79	6.04	0.8218	2.26	-2.59	7.11	0.3636
Week 12	-2.57	-8.82	3.69	0.4238	-1.05	-6.47	4.37	0.7052	-1.70	-6.55	3.15	0.4938
Week 16	0.83	-5.42	7.09	0.7948	0.15	-5.27	5.57	0.9569	3.24	-1.61	8.09	0.1939
Week 24	6.07	-0.19	12.32	0.061	3.83	-1.59	9.24	0.1704	0.86	-3.99	5.71	0.729
Week 48	6.17	-0.09	12.42	0.0569	6.20	0.78	11.62	0.0277	1.42	-3.43	6.27	0.5675
%CD4 TEM												
Week 1	-0.40	-5.83	5.03	0.8855	1.38	-3.32	6.07	0.5679	-0.64	-4.84	3.56	0.7661
Week 2	8.70	3.27	14.13	0.0023	-3.88	-8.57	0.82	0.11	4.72	0.52	8.92	0.0306
Week 4	5.20	-0.23	10.63	0.064	-3.13	-7.82	1.57	0.1962	0.32	-3.88	4.52	0.8818
Week 6	6.53	1.11	11.96	0.0207	-1.45	-6.15	3.25	0.5471	-1.64	-5.84	2.56	0.4467
Week 8	0.57	-4.86	5.99	0.8383	1.23	-3.47	5.92	0.6108	-1.62	-5.82	2.58	0.4522
Week 12	4.17	-1.26	9.59	0.1362	2.18	-2.52	6.87	0.3671	-0.12	-4.32	4.08	0.9555
Week 16	1.80	-3.63	7.23	0.5175	0.28	-4.42	4.97	0.909	-3.34	-7.54	0.86	0.1233
Week 24	-0.87	-6.29	4.56	0.7551	-1.85	-6.55	2.85	0.4426	-2.62	-6.82	1.58	0.2254
Week 48	0.80	-4.63	6.23	0.7734	-3.45	-8.15	1.25	0.1541	-1.12	-5.32	3.08	0.6029
%CD4 TEMRA												
Week 1	-1.57	-4.58	1.45	0.3115	0.40	-2.21	3.01	0.7647	2.12	-0.22	4.46	0.079
Week 2	-0.03	-3.05	2.98	0.9828	0.50	-2.11	3.11	0.7084	0.84	-1.50	3.18	0.4828
Week 4	0.07	-2.95	3.08	0.9655	-0.20	-2.81	2.41	0.881	0.64	-1.70	2.98	0.5926
Week 6	-0.77	-3.78	2.25	0.6195	-0.25	-2.86	2.36	0.8516	-0.56	-2.90	1.78	0.6396
Week 8	-1.10	-4.11	1.91	0.4766	-0.10	-2.71	2.51	0.9403	-0.38	-2.72	1.96	0.7506
Week 12	1.13	-1.88	4.15	0.4634	0.70	-1.91	3.31	0.6007	2.30	-0.04	4.64	0.0571
Week 16	-0.70	-3.71	2.31	0.6503	-0.28	-2.89	2.34	0.837	0.76	-1.58	3.10	0.5254
Week 24	-1.93	-4.95	1.08	0.2124	-0.45	-3.06	2.16	0.7364	1.98	-0.36	4.32	0.1004
Week 48	-1.23	-4.25	1.78	0.425	-2.05	-4.66	0.56	0.1277	0.22	-2.12	2.56	0.854

Appendix Table 24 Changes from baseline in markers associated with CD8 T-cell differentiation/maturation.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8 Naïve	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	2.17	-7.48	11.81	0.6609	-0.05	-8.40	8.30	0.9907	10.32	2.85	17.79	0.0083
Week 2	-4.33	-13.98	5.31	0.3812	1.10	-7.25	9.45	0.797	12.88	5.41	20.35	0.0011
Week 4	-5.47	-15.11	4.18	0.2699	3.28	-5.08	11.63	0.4445	10.54	3.07	18.01	0.007
Week 6	-5.50	-15.15	4.15	0.267	2.58	-5.78	10.93	0.5474	9.70	2.23	17.17	0.0128
Week 8	-2.97	-12.61	6.68	0.5483	0.80	-7.55	9.15	0.8516	11.10	3.63	18.57	0.0046
Week 12	-2.33	-11.98	7.31	0.6367	2.85	-5.50	11.20	0.5056	12.58	5.11	20.05	0.0014
Week 16	-4.87	-14.51	4.78	0.3257	4.50	-3.85	12.85	0.2942	10.34	2.87	17.81	0.0082
Week 24	-7.60	-17.25	2.05	0.1264	4.70	-3.65	13.05	0.2734	9.32	1.85	16.79	0.0167
Week 48	-3.13	-12.78	6.51	0.5261	3.90	-4.45	12.25	0.3629	10.64	3.17	18.11	0.0065
%CD8 TCM												
Week 1	2.60	-4.81	10.01	0.4934	0.68	-5.74	7.09	0.8371	-5.82	-11.56	-0.08	0.0501
Week 2	3.10	-4.31	10.51	0.4144	2.88	-3.54	9.29	0.3823	-3.74	-9.48	2.00	0.205
Week 4	2.30	-5.11	9.71	0.5444	0.38	-6.04	6.79	0.9091	-1.86	-7.60	3.88	0.5269
Week 6	3.63	-3.77	11.04	0.3392	-0.38	-6.79	6.04	0.9091	-1.40	-7.14	4.34	0.6337
Week 8	3.13	-4.27	10.54	0.4094	1.90	-4.51	8.31	0.5631	-0.80	-6.54	4.94	0.7853
Week 12	-0.80	-8.21	6.61	0.8329	0.55	-5.86	6.96	0.8669	-2.56	-8.30	3.18	0.3844
Week 16	1.30	-6.11	8.71	0.7317	-2.65	-9.06	3.76	0.4204	-6.90	-12.64	-1.16	0.0208
Week 24	2.20	-5.21	9.61	0.562	0.58	-5.84	6.99	0.861	-5.12	-10.86	0.62	0.084
Week 48	1.43	-5.97	8.84	0.7054	2.90	-3.51	9.31	0.3781	-5.70	-11.44	0.04	0.055
%CD8 TEM												
Week 1	-0.47	-8.96	8.03	0.9145	0.30	-7.06	7.66	0.9365	2.04	-4.54	8.62	0.5451
Week 2	5.37	-3.13	13.86	0.2191	-7.83	-15.18	-0.47	0.0402	7.42	0.84	14.00	0.0299
Week 4	3.80	-4.69	12.29	0.3831	-6.73	-14.08	0.63	0.0769	2.80	-3.78	9.38	0.4066
Week 6	2.57	-5.93	11.06	0.5553	-6.00	-13.36	1.36	0.1138	5.70	-0.88	12.28	0.0933
Week 8	-0.07	-8.56	8.43	0.9878	-4.23	-11.58	3.13	0.2636	3.10	-3.48	9.68	0.3585
Week 12	3.70	-4.79	12.19	0.3957	-7.93	-15.28	-0.57	0.0378	3.50	-3.08	10.08	0.3002
Week 16	2.83	-5.66	11.33	0.5151	-8.53	-15.88	-1.17	0.0258	6.66	0.08	13.24	0.0506
Week 24	6.20	-2.29	14.69	0.1564	-8.13	-15.48	-0.77	0.0333	4.80	-1.78	11.38	0.1566
Week 48	3.13	-5.36	11.63	0.4717	-5.90	-13.26	1.46	0.1198	5.72	-0.86	12.30	0.0922
%CD8 TEMRA												
Week 1	-4.23	-15.11	6.65	0.4478	-0.93	-10.35	8.50	0.8479	-6.50	-14.93	1.93	0.1344
Week 2	-3.97	-14.85	6.91	0.4769	3.88	-5.55	13.30	0.4225	-16.56	-24.99	-8.13	0.0002
Week 4	-0.57	-11.45	10.31	0.9189	3.13	-6.30	12.55	0.5174	-11.46	-19.89	-3.03	0.0093
Week 6	-0.67	-11.55	10.21	0.9047	3.83	-5.60	13.25	0.4285	-13.98	-22.41	-5.55	0.0017
Week 8	0.00	-10.88	10.88	1	1.50	-7.92	10.92	0.7558	-13.34	-21.77	-4.91	0.0026
Week 12	-0.50	-11.38	10.38	0.9284	4.60	-4.82	14.02	0.3414	-13.50	-21.93	-5.07	0.0024
Week 16	0.87	-10.01	11.75	0.8763	6.75	-2.67	16.17	0.164	-10.08	-18.51	-1.65	0.0215
Week 24	-0.73	-11.61	10.15	0.8952	2.88	-6.55	12.30	0.5514	-8.94	-17.37	-0.51	0.0407
Week 48	-1.33	-12.21	9.55	0.8108	-0.90	-10.32	8.52	0.8519	-10.60	-19.03	-2.17	0.0158

Appendix Table 25 Changes from baseline in PD-1 expression on CD4 T-cell differentiation/maturation subsets. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4 Naïve PD-1	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-2.43	-16.61	11.74	0.7374	-2.85	-15.13	9.43	0.6503	-5.46	-16.44	5.52	0.3326
Week 2	-11.97	-26.14	2.21	0.1019	5.75	-6.53	18.03	0.3613	5.56	-5.42	16.54	0.3239
Week 4	-14.23	-28.41	-0.06	0.0525	0.13	-12.15	12.40	0.9841	-0.60	-11.58	10.38	0.915
Week 6	-19.50	-33.68	-5.32	0.0085	4.70	-7.58	16.98	0.4552	-0.38	-11.36	10.60	0.9461
Week 8	-8.30	-22.48	5.88	0.2545	5.78	-6.50	18.05	0.3593	-10.14	-21.12	0.84	0.074
Week 12	0.80	-13.38	14.98	0.9122	1.60	-10.68	13.88	0.799	-1.82	-12.80	9.16	0.7461
Week 16	-11.90	-26.08	2.28	0.1038	-0.60	-12.88	11.68	0.9239	-11.12	-22.10	-0.14	0.0505
Week 24	-11.60	-25.78	2.58	0.1126	-0.45	-12.73	11.83	0.9429	-14.00	-24.98	-3.02	0.0145
Week 48	-19.67	-33.84	-5.49	0.008	-15.20	-27.48	-2.92	0.0175	-18.14	-29.12	-7.16	0.0017
%CD4 TCM PD-1												
Week 1	-1.87	-16.97	13.24	0.8092	-4.20	-17.28	8.88	0.5309	-4.60	-16.30	7.10	0.4432
Week 2	-4.80	-19.91	10.31	0.5351	6.80	-6.28	19.88	0.3113	4.58	-7.12	16.28	0.4452
Week 4	-19.33	-34.44	-4.23	0.0141	4.25	-8.83	17.33	0.5261	-11.80	-23.50	-0.10	0.0515
Week 6	-23.80	-38.91	-8.69	0.0028	7.43	-5.66	20.51	0.2692	-7.80	-19.50	3.90	0.195
Week 8	-13.60	-28.71	1.51	0.0814	5.00	-8.08	18.08	0.4559	-15.18	-26.88	-3.48	0.0129
Week 12	-2.07	-17.17	13.04	0.7893	3.95	-9.13	17.03	0.5556	-6.48	-18.18	5.22	0.2809
Week 16	-12.03	-27.14	3.07	0.1223	2.20	-10.88	15.28	0.7425	-14.46	-26.16	-2.76	0.0177
Week 24	-15.23	-30.34	-0.13	0.0515	2.43	-10.66	15.51	0.7173	-20.10	-31.80	-8.40	0.0012
Week 48	-19.57	-34.67	-4.46	0.013	-20.68	-33.76	-7.59	0.0027	-23.66	-35.36	-11.96	0.0002
%CD4 TEM PD-1												
Week 1	-1.83	-14.19	10.52	0.7719	-3.25	-13.95	7.45	0.5533	-4.94	-14.51	4.63	0.3147
Week 2	-0.37	-12.72	11.99	0.9538	4.80	-5.90	15.50	0.3819	6.36	-3.21	15.93	0.1964
Week 4	-16.13	-28.49	-3.78	0.0124	3.35	-7.35	14.05	0.5412	-11.96	-21.53	-2.39	0.0165
Week 6	-19.37	-31.72	-7.01	0.0029	3.55	-7.15	14.25	0.5174	-5.36	-14.93	4.21	0.2756
Week 8	-12.03	-24.39	0.32	0.0598	4.30	-6.40	15.00	0.4332	-12.76	-22.33	-3.19	0.0107
Week 12	-2.20	-14.56	10.16	0.728	2.45	-8.25	13.15	0.6548	-4.76	-14.33	4.81	0.3326
Week 16	-8.23	-20.59	4.12	0.1952	2.55	-8.15	13.25	0.6417	-11.04	-20.61	-1.47	0.0264
Week 24	-14.10	-26.46	-1.74	0.0281	2.83	-7.88	13.53	0.6062	-16.02	-25.59	-6.45	0.0015
Week 48	-15.20	-27.56	-2.84	0.0182	-18.30	-29.00	-7.60	0.0012	-19.86	-29.43	-10.29	0.0001
%CD4 TEMRA PD-1												
Week 1	-3.27	-17.40	10.86	0.6517	-4.00	-16.24	8.24	0.5236	-7.16	-18.11	3.79	0.2035
Week 2	-3.27	-17.40	10.86	0.6517	3.30	-8.94	15.54	0.5986	5.90	-5.05	16.85	0.2939
Week 4	-12.77	-26.90	1.36	0.0804	2.60	-9.64	14.84	0.6782	-9.26	-20.21	1.69	0.1012
Week 6	-21.00	-35.13	-6.87	0.0046	5.73	-6.51	17.96	0.3619	-4.24	-15.19	6.71	0.4499
Week 8	-9.93	-24.06	4.20	0.1721	3.38	-8.86	15.61	0.5903	-12.56	-23.51	-1.61	0.0272
Week 12	-2.60	-16.73	11.53	0.7193	2.00	-10.24	14.24	0.7495	-4.06	-15.01	6.89	0.4693
Week 16	-8.63	-22.76	5.50	0.2346	1.90	-10.34	14.14	0.7617	-10.86	-21.81	0.09	0.0553
Week 24	-13.53	-27.66	0.60	0.0641	-0.90	-13.14	11.34	0.8857	-16.66	-27.61	-5.71	0.0038
Week 48	-16.70	-30.83	-2.57	0.0231	-17.80	-30.04	-5.56	0.0055	-20.80	-31.75	-9.85	0.0004

Appendix Table 26 Changes from baseline in PD-1 expression on CD8 T-cell differentiation/maturation subsets. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8 Naïve PD-1	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.23	-16.90	17.36	0.9788	5.75	-9.09	20.59	0.4497	1.00	-12.27	14.27	0.8829
Week 2	-4.83	-21.96	12.30	0.5818	10.83	-4.01	25.66	0.1565	8.62	-4.65	21.89	0.2066
Week 4	-15.10	-32.23	2.03	0.0879	1.88	-12.96	16.71	0.805	3.70	-9.57	16.97	0.5862
Week 6	-14.83	-31.96	2.30	0.0935	5.20	-9.64	20.04	0.494	0.36	-12.91	13.63	0.9577
Week 8	-6.73	-23.86	10.40	0.4433	5.60	-9.24	20.44	0.4615	-5.80	-19.07	7.47	0.3941
Week 12	3.90	-13.23	21.03	0.6566	6.53	-8.31	21.36	0.3912	-3.46	-16.73	9.81	0.6107
Week 16	-10.20	-27.33	6.93	0.2466	5.98	-8.86	20.81	0.4322	-7.58	-20.85	5.69	0.2662
Week 24	-15.67	-32.80	1.46	0.0768	3.43	-11.41	18.26	0.6521	-9.40	-22.67	3.87	0.1688
Week 48	-20.87	-38.00	-3.74	0.0193	-18.10	-32.94	-3.26	0.0191	-15.50	-28.77	-2.23	0.0247
%CD8 TCM PD-1												
Week 1	2.53	-13.63	18.70	0.7595	4.38	-9.62	18.37	0.5419	-6.08	-18.60	6.44	0.344
Week 2	1.43	-14.73	17.60	0.8624	9.63	-4.37	23.62	0.1815	1.00	-11.52	13.52	0.876
Week 4	-16.07	-32.23	0.10	0.0548	2.90	-11.10	16.90	0.6858	-5.88	-18.40	6.64	0.36
Week 6	-13.80	-29.96	2.36	0.0981	7.78	-6.22	21.77	0.2795	-7.82	-20.34	4.70	0.2244
Week 8	-8.53	-24.70	7.63	0.3038	0.63	-13.37	14.62	0.9305	-15.50	-28.02	-2.98	0.0175
Week 12	1.73	-14.43	17.90	0.834	6.18	-7.82	20.17	0.3898	-9.66	-22.18	2.86	0.1344
Week 16	-7.20	-23.36	8.96	0.3852	7.95	-6.05	21.95	0.2689	-13.96	-26.48	-1.44	0.0317
Week 24	-16.87	-33.03	-0.70	0.0441	2.78	-11.22	16.77	0.6986	-16.70	-29.22	-4.18	0.0107
Week 48	-18.57	-34.73	-2.40	0.0271	-19.88	-33.87	-5.88	0.0067	-25.04	-37.56	-12.52	0.0002
%CD8 TEM PD-1												
Week 1	0.03	-14.93	15.00	0.9965	-0.93	-13.88	12.03	0.8891	-10.76	-22.35	0.83	0.0725
Week 2	0.70	-14.26	15.66	0.9272	9.45	-3.51	22.41	0.1567	-3.06	-14.65	8.53	0.6062
Week 4	-13.57	-28.53	1.40	0.0793	3.25	-9.71	16.21	0.6243	-15.70	-27.29	-4.11	0.0095
Week 6	-16.70	-31.66	-1.74	0.0316	6.10	-6.86	19.06	0.3589	-13.80	-25.39	-2.21	0.0221
Week 8	-11.47	-26.43	3.50	0.137	4.25	-8.71	17.21	0.5221	-21.26	-32.85	-9.67	0.0006
Week 12	-1.23	-16.20	13.73	0.8721	4.63	-8.33	17.58	0.4862	-14.42	-26.01	-2.83	0.0169
Week 16	-6.37	-21.33	8.60	0.4067	5.48	-7.48	18.43	0.41	-21.14	-32.73	-9.55	0.0006
Week 24	-15.93	-30.90	-0.97	0.04	-0.80	-13.76	12.16	0.904	-23.12	-34.71	-11.53	0.0002
Week 48	-18.93	-33.90	-3.97	0.0152	-20.88	-33.83	-7.92	0.0022	-26.92	-38.51	-15.33	<.0001
%CD8 TEMRA PD-1												
Week 1	1.40	-14.42	17.22	0.8627	1.50	-12.20	15.20	0.8306	-6.28	-18.53	5.97	0.318
Week 2	-3.87	-19.68	11.95	0.6331	7.88	-5.82	21.57	0.2631	1.82	-10.43	14.07	0.7716
Week 4	-15.03	-30.85	0.78	0.0661	0.68	-13.02	14.37	0.9233	-10.94	-23.19	1.31	0.0838
Week 6	-16.87	-32.68	-1.05	0.0397	7.43	-6.27	21.12	0.2911	-7.66	-19.91	4.59	0.2239
Week 8	-6.97	-22.78	8.85	0.3905	4.88	-8.82	18.57	0.4874	-12.34	-24.59	-0.09	0.0518
Week 12	3.93	-11.88	19.75	0.6272	2.28	-11.42	15.97	0.7456	-9.54	-21.79	2.71	0.1308
Week 16	-7.60	-23.42	8.22	0.3491	2.25	-11.45	15.95	0.7483	-13.70	-25.95	-1.45	0.0313
Week 24	-12.97	-28.78	2.85	0.1119	-1.65	-15.35	12.05	0.8139	-15.46	-27.71	-3.21	0.0155
Week 48	-17.70	-33.52	-1.88	0.0311	-21.58	-35.27	-7.88	0.0028	-21.72	-33.97	-9.47	0.0008

Appendix Table 27 Changes from baseline in PD-L1 expression on CD4 T-cell differentiation/maturation subsets. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4 Naïve PD-L1	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.63	-5.38	6.65	0.837	0.90	-4.31	6.11	0.7357	1.24	-3.42	5.90	0.6033
Week 2	-2.20	-8.21	3.81	0.4755	6.00	0.79	11.21	0.0266	2.06	-2.60	6.72	0.3887
Week 4	0.17	-5.85	6.18	0.9568	1.10	-4.11	6.31	0.68	-1.84	-6.50	2.82	0.4411
Week 6	-1.93	-7.95	4.08	0.5304	1.88	-3.33	7.08	0.4825	-1.30	-5.96	3.36	0.5859
Week 8	-2.47	-8.48	3.55	0.4238	4.23	-0.98	9.43	0.1157	-1.90	-6.56	2.76	0.4264
Week 12	-0.27	-6.28	5.75	0.931	0.25	-4.96	5.46	0.9253	-1.50	-6.16	3.16	0.5298
Week 16	-1.27	-7.28	4.75	0.6808	-2.35	-7.56	2.86	0.3791	-5.20	-9.86	-0.54	0.0316
Week 24	-2.07	-8.08	3.95	0.5025	-0.05	-5.26	5.16	0.985	-2.18	-6.84	2.48	0.3618
Week 48	2.87	-3.15	8.88	0.353	-2.58	-7.78	2.63	0.3354	2.02	-2.64	6.68	0.3979
%CD4 TCM PD-L1												
Week 1	0.33	-7.37	8.03	0.9326	-0.23	-6.89	6.44	0.9474	0.86	-5.10	6.82	0.7782
Week 2	0.17	-7.53	7.87	0.9663	6.35	-0.32	13.02	0.0656	5.08	-0.88	11.04	0.0989
Week 4	-1.00	-8.70	6.70	0.7997	1.25	-5.42	7.92	0.7143	-4.58	-10.54	1.38	0.1362
Week 6	-1.77	-9.47	5.93	0.6541	2.38	-4.29	9.04	0.4871	-2.50	-8.46	3.46	0.4138
Week 8	-4.20	-11.90	3.50	0.2882	3.43	-3.24	10.09	0.3171	-3.70	-9.66	2.26	0.2276
Week 12	-0.13	-7.83	7.57	0.973	1.43	-5.24	8.09	0.6764	-2.50	-8.46	3.46	0.4138
Week 16	-1.37	-9.07	6.33	0.7288	-1.45	-8.12	5.22	0.6711	-8.02	-13.98	-2.06	0.0101
Week 24	-3.17	-10.87	4.53	0.4226	1.13	-5.54	7.79	0.7418	-3.36	-9.32	2.60	0.2728
Week 48	5.73	-1.97	13.43	0.1483	-3.53	-10.19	3.14	0.3033	2.96	-3.00	8.92	0.3336
%CD4 TEM PD-L1												
Week 1	0.53	-6.41	7.48	0.8808	-1.18	-7.19	4.84	0.7029	0.50	-4.88	5.88	0.856
Week 2	1.00	-5.95	7.95	0.7786	3.55	-2.47	9.57	0.2509	3.06	-2.32	8.44	0.2684
Week 4	-0.70	-7.65	6.25	0.844	0.15	-5.87	6.17	0.9612	-4.26	-9.64	1.12	0.1247
Week 6	-1.80	-8.75	5.15	0.613	0.85	-5.17	6.87	0.7826	-1.62	-7.00	3.76	0.5569
Week 8	-3.43	-10.38	3.51	0.3357	2.83	-3.19	8.84	0.3602	-2.54	-7.92	2.84	0.3577
Week 12	-0.23	-7.18	6.71	0.9477	0.90	-5.12	6.92	0.7702	-1.72	-7.10	3.66	0.5328
Week 16	-1.27	-8.21	5.68	0.7218	-2.60	-8.62	3.42	0.3995	-6.92	-12.30	-1.54	0.0137
Week 24	-3.17	-10.11	3.78	0.3743	-0.48	-6.49	5.54	0.8774	-3.00	-8.38	2.38	0.2778
Week 48	3.83	-3.11	10.78	0.2827	-3.43	-9.44	2.59	0.2679	3.20	-2.18	8.58	0.2473
%CD4 TEMRA PD-L1												
Week 1	2.10	-4.29	8.49	0.5212	-2.40	-7.93	3.13	0.3977	1.12	-3.83	6.07	0.6585
Week 2	0.10	-6.29	6.49	0.9756	5.43	-0.11	10.96	0.0581	1.06	-3.89	6.01	0.6757
Week 4	-0.37	-6.76	6.02	0.9107	2.38	-3.16	7.91	0.4026	-3.16	-8.11	1.79	0.2143
Week 6	-1.87	-8.26	4.52	0.5684	2.43	-3.11	7.96	0.3928	-1.26	-6.21	3.69	0.6191
Week 8	-2.67	-9.06	3.72	0.4157	3.65	-1.88	9.18	0.1997	-1.36	-6.31	3.59	0.5916
Week 12	0.13	-6.26	6.52	0.9675	-0.90	-6.43	4.63	0.7507	-0.92	-5.87	4.03	0.7165
Week 16	-0.53	-6.92	5.86	0.8704	-3.20	-8.73	2.33	0.2603	-5.84	-10.79	-0.89	0.0233
Week 24	-2.30	-8.69	4.09	0.4824	-1.55	-7.08	3.98	0.5844	-2.68	-7.63	2.27	0.2916
Week 48	4.50	-1.89	10.89	0.1712	-2.28	-7.81	3.26	0.4226	2.92	-2.03	7.87	0.2508

Appendix Table 28 Changes from baseline in PD-1 expression on CD8 T-cell differentiation/maturation subsets. Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8 Naïve PD-L1	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	7.83	-13.22	28.89	0.4679	-3.93	-22.16	14.31	0.6742	-0.34	-16.65	15.97	0.9675
Week 2	4.20	-16.85	25.25	0.6968	3.70	-14.53	21.93	0.6919	18.66	2.35	34.97	0.0276
Week 4	8.73	-12.32	29.79	0.4186	-2.55	-20.78	15.68	0.7847	-0.76	-17.07	15.55	0.9274
Week 6	3.40	-17.65	24.45	0.7524	-4.88	-23.11	13.36	0.6017	4.82	-11.49	21.13	0.564
Week 8	5.53	-15.52	26.59	0.6079	-12.73	-30.96	5.51	0.1751	0.32	-15.99	16.63	0.9694
Week 12	-2.57	-23.62	18.49	0.8117	-8.70	-26.93	9.53	0.3524	-12.94	-29.25	3.37	0.1238
Week 16	1.30	-19.75	22.35	0.904	-9.35	-27.58	8.88	0.3178	0.08	-16.23	16.39	0.9924
Week 24	5.23	-15.82	26.29	0.6274	-1.18	-19.41	17.06	0.8998	-3.10	-19.41	13.21	0.7104
Week 48	5.73	-15.32	26.79	0.595	-7.90	-26.13	10.33	0.3982	0.08	-16.23	16.39	0.9924
%CD8 TCM PD-L1												
Week 1	15.67	-2.88	34.21	0.1016	-1.08	-17.13	14.98	0.8959	0.02	-14.34	14.38	0.9978
Week 2	14.00	-4.54	32.54	0.1428	4.98	-11.08	21.03	0.5454	19.12	4.76	33.48	0.0108
Week 4	10.07	-8.48	28.61	0.2904	0.10	-15.96	16.16	0.9903	-4.38	-18.74	9.98	0.5517
Week 6	8.57	-9.98	27.11	0.3679	-3.03	-19.08	13.03	0.7129	0.42	-13.94	14.78	0.9544
Week 8	11.47	-7.08	30.01	0.229	-12.65	-28.71	3.41	0.1265	-0.40	-14.76	13.96	0.9566
Week 12	7.03	-11.51	25.58	0.4594	-5.85	-21.91	10.21	0.4773	-12.10	-26.46	2.26	0.1026
Week 16	9.40	-9.14	27.94	0.3234	-5.08	-21.13	10.98	0.5374	-1.76	-16.12	12.60	0.8108
Week 24	13.63	-4.91	32.18	0.1534	0.80	-15.26	16.86	0.9225	-3.20	-17.56	11.16	0.6635
Week 48	12.80	-5.74	31.34	0.1798	-7.53	-23.58	8.53	0.3611	0.54	-13.82	14.90	0.9414
%CD8 TEM PD-L1												
Week 1	12.53	1.63	23.44	0.027	1.25	-8.20	10.70	0.796	-1.32	-9.77	7.13	0.7602
Week 2	10.80	-0.11	21.71	0.0558	6.85	-2.60	16.30	0.1591	15.44	6.99	23.89	0.0006
Week 4	8.33	-2.57	19.24	0.1382	2.85	-6.60	12.30	0.556	-2.56	-11.01	5.89	0.5543
Week 6	4.67	-6.24	15.57	0.4042	0.18	-9.27	9.62	0.9711	-0.56	-9.01	7.89	0.897
Week 8	9.10	-1.81	20.01	0.1059	-1.58	-11.02	7.87	0.7447	0.22	-8.23	8.67	0.9594
Week 12	4.20	-6.71	15.11	0.4526	0.38	-9.07	9.82	0.9382	-5.90	-14.35	2.55	0.1749
Week 16	2.53	-8.37	13.44	0.6502	-2.45	-11.90	7.00	0.6126	-5.34	-13.79	3.11	0.219
Week 24	7.10	-3.81	18.01	0.2057	1.30	-8.15	10.75	0.7881	-3.94	-12.39	4.51	0.3635
Week 48	8.87	-2.04	19.77	0.115	-3.30	-12.75	6.15	0.4955	0.90	-7.55	9.35	0.8352
%CD8 TEMRA PD-L1												
Week 1	9.63	-1.00	20.26	0.0795	2.65	-6.56	11.86	0.5742	-0.52	-8.75	7.71	0.9018
Week 2	5.17	-5.46	15.80	0.3436	6.05	-3.16	15.26	0.2014	14.46	6.23	22.69	0.0009
Week 4	6.53	-4.10	17.16	0.2319	2.03	-7.18	11.23	0.6675	-2.46	-10.69	5.77	0.5598
Week 6	2.97	-7.66	13.60	0.5859	0.63	-8.58	9.83	0.8945	0.22	-8.01	8.45	0.9584
Week 8	7.23	-3.40	17.86	0.186	0.30	-8.91	9.51	0.9492	3.66	-4.57	11.89	0.3862
Week 12	4.07	-6.56	14.70	0.4555	0.23	-8.98	9.43	0.9619	-2.66	-10.89	5.57	0.5284
Week 16	4.03	-6.60	14.66	0.4592	-3.58	-12.78	5.63	0.4488	-1.40	-9.63	6.83	0.7398
Week 24	6.03	-4.60	16.66	0.2692	-0.15	-9.36	9.06	0.9746	-0.68	-8.91	7.55	0.8718
Week 48	8.67	-1.96	19.30	0.1139	-4.03	-13.23	5.18	0.394	4.34	-3.89	12.57	0.3046

Appendix Table 29 Changes from baseline in CD27, CD28, PD-1 and PD-L1 expression on CD4 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+CD27+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	2.23	-2.13	6.60	0.319	-1.63	-5.41	2.16	0.4021	-2.18	-5.56	1.20	0.2101
Week 2	-1.97	-6.33	2.40	0.3799	-2.75	-6.53	1.03	0.1579	2.50	-0.88	5.88	0.1512
Week 4	0.33	-4.03	4.70	0.8814	1.53	-2.26	5.31	0.4315	0.76	-2.62	4.14	0.6608
Week 6	0.67	-3.70	5.03	0.7655	-0.90	-4.68	2.88	0.6421	3.48	0.10	6.86	0.047
Week 8	2.23	-2.13	6.60	0.319	-1.45	-5.23	2.33	0.4545	2.28	-1.10	5.66	0.1901
Week 12	0.70	-3.67	5.07	0.7541	-2.30	-6.08	1.48	0.2366	1.44	-1.94	4.82	0.4064
Week 16	2.60	-1.77	6.97	0.2466	0.03	-3.76	3.81	0.9897	-0.10	-3.48	3.28	0.9539
Week 24	3.60	-0.77	7.97	0.11	0.05	-3.73	3.83	0.9794	1.32	-2.06	4.70	0.4465
Week 48	0.93	-3.43	5.30	0.6763	0.85	-2.93	4.63	0.6607	0.66	-2.72	4.04	0.7031
%CD4+CD28+												
Week 1	1.70	-2.41	5.81	0.4203	-1.23	-4.79	2.34	0.5023	-2.44	-5.63	0.75	0.1373
Week 2	3.37	-0.75	7.48	0.1126	-1.85	-5.41	1.71	0.3118	4.88	1.69	8.07	0.0036
Week 4	2.33	-1.78	6.45	0.2695	1.78	-1.79	5.34	0.3317	2.56	-0.63	5.75	0.1192
Week 6	2.43	-1.68	6.55	0.2497	-0.43	-3.99	3.14	0.8157	3.74	0.55	6.93	0.024
Week 8	3.70	-0.41	7.81	0.0817	-1.15	-4.71	2.41	0.5287	2.48	-0.71	5.67	0.131
Week 12	0.70	-3.41	4.81	0.7396	-1.00	-4.56	2.56	0.5837	1.62	-1.57	4.81	0.322
Week 16	3.60	-0.51	7.71	0.0901	0.85	-2.71	4.41	0.6413	-0.40	-3.59	2.79	0.8063
Week 24	4.73	0.62	8.85	0.0268	1.10	-2.46	4.66	0.5467	1.46	-1.73	4.65	0.3718
Week 48	0.57	-3.55	4.68	0.7878	1.85	-1.71	5.41	0.3118	0.80	-2.39	3.99	0.624
%CD4+PD-1+												
Week 1	-1.13	-14.80	12.53	0.8712	-3.13	-14.96	8.71	0.6061	-4.44	-15.02	6.14	0.4133
Week 2	-0.57	-14.23	13.10	0.9354	5.63	-6.21	17.46	0.3542	7.98	-2.60	18.56	0.1433
Week 4	-10.63	-24.30	3.03	0.131	1.80	-10.03	13.63	0.7663	-5.70	-16.28	4.88	0.2942
Week 6	-17.37	-31.03	-3.70	0.0148	8.35	-3.48	20.18	0.1704	-4.04	-14.62	6.54	0.4565
Week 8	-10.30	-23.96	3.36	0.1434	4.90	-6.93	16.73	0.4193	-12.22	-22.80	-1.64	0.0263
Week 12	1.47	-12.20	15.13	0.8339	4.63	-7.21	16.46	0.4458	-4.26	-14.84	6.32	0.4324
Week 16	-8.50	-22.16	5.16	0.2262	0.50	-11.33	12.33	0.9342	-13.08	-23.66	-2.50	0.0176
Week 24	-11.97	-25.63	1.70	0.0898	2.65	-9.18	14.48	0.6618	-15.90	-26.48	-5.32	0.0042
Week 48	-15.43	-29.10	-1.77	0.0296	-19.03	-30.86	-7.19	0.0023	-20.02	-30.60	-9.44	0.0004
%CD4+PD-L1												
Week 1	0.87	-6.12	7.85	0.8086	-0.70	-6.75	5.35	0.8212	-0.36	-5.77	5.05	0.8966
Week 2	-0.33	-7.32	6.65	0.9257	4.98	-1.08	11.03	0.111	2.40	-3.01	7.81	0.3874
Week 4	-0.07	-7.05	6.92	0.9851	-0.10	-6.15	5.95	0.9742	-3.96	-9.37	1.45	0.1555
Week 6	-1.60	-8.59	5.39	0.6548	0.73	-5.33	6.78	0.815	-2.42	-7.83	2.99	0.3835
Week 8	-3.60	-10.59	3.39	0.3156	2.05	-4.00	8.10	0.5086	-3.42	-8.83	1.99	0.2192
Week 12	0.07	-6.92	7.05	0.9851	0.20	-5.85	6.25	0.9485	-3.02	-8.43	2.39	0.2774
Week 16	-1.37	-8.35	5.62	0.7025	-3.03	-9.08	3.03	0.3302	-6.60	-12.01	-1.19	0.0192
Week 24	-1.20	-8.19	5.79	0.7373	-0.58	-6.63	5.48	0.8527	-3.54	-8.95	1.87	0.2036
Week 48	5.00	-1.99	11.99	0.1646	-4.23	-10.28	1.83	0.175	1.40	-4.01	6.81	0.6136
CD4+PD-1 MFI												
Week 1	-25	-120	70	0.6124	44	-39	126	0.3034	3	-70	77	0.9281
Week 2	90	-5	185	0.0671	18	-64	100	0.6693	105	31	179	0.0065
Week 4	-23	-118	72	0.6317	3	-80	85	0.948	-39	-113	34	0.2973
Week 6	-30	-125	65	0.5334	38	-45	120	0.3714	-39	-112	35	0.3047
Week 8	-45	-140	50	0.3597	12	-71	94	0.7849	-40	-113	34	0.2925
Week 12	-31	-126	64	0.5245	78	-4	160	0.0669	-67	-141	6	0.0765
Week 16	-7	-102	88	0.891	59	-24	141	0.1674	26	-48	100	0.4908
Week 24	55	-40	150	0.2601	21	-61	104	0.6142	43	-30	117	0.2535
Week 48	22	-73	117	0.6464	123	40	205	0.0046	17	-57	91	0.6521
CD4+PD-L1 MFI												
Week 1	550	-59	1159	0.0806	-146	-673	382	0.5901	6	-466	477	0.9815
Week 2	170	-439	779	0.5865	-27	-554	501	0.9218	483	11	954	0.0482
Week 4	-128	-737	481	0.6822	178	-350	705	0.5113	272	-200	743	0.2624
Week 6	-56	-665	553	0.8574	77	-451	604	0.7769	130	-341	602	0.59

Appendix Table 29 continued

Week 8	258	-351	867	0.4081	25	-502	552	0.9262	172	-299	644	0.4758
Week 12	440	-169	1049	0.1608	433	-94	960	0.1114	221	-251	692	0.3616
Week 16	653	44	1262	0.0388	385	-142	912	0.1563	736	264	1208	0.003
Week 24	881	272	1490	0.0058	275	-253	802	0.3106	529	57	1001	0.0308
Week 48	434	-175	1043	0.1666	876	349	1403	0.0017	407	-64	879	0.0943

Appendix Table 30 Changes from baseline in CD27, CD28, PD-1 and PD-L1 expression on CD8 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+CD27+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	4.63	-2.29	11.56	0.1935	0.05	-5.95	6.05	0.987	-5.74	-11.11	-0.37	0.0391
Week 2	-2.50	-9.43	4.43	0.4813	-0.80	-6.80	5.20	0.7945	4.10	-1.27	9.47	0.1381
Week 4	-0.63	-7.56	6.29	0.8582	4.78	-1.22	10.77	0.1226	2.66	-2.71	8.03	0.3341
Week 6	0.40	-6.53	7.33	0.9102	1.98	-4.02	7.97	0.5206	7.20	1.83	12.57	0.0102
Week 8	0.60	-6.33	7.53	0.8656	2.23	-3.77	8.22	0.4693	6.16	0.79	11.53	0.0271
Week 12	-0.67	-7.59	6.26	0.8508	3.75	-2.25	9.75	0.224	6.14	0.77	11.51	0.0276
Week 16	3.07	-3.86	9.99	0.3881	2.28	-3.72	8.27	0.4594	2.80	-2.57	8.17	0.3094
Week 24	3.90	-3.03	10.83	0.2731	4.85	-1.15	10.85	0.1169	4.26	-1.11	9.63	0.1236
Week 48	2.07	-4.86	8.99	0.5603	1.85	-4.15	7.85	0.5472	5.40	0.03	10.77	0.052
%CD8+CD28+												
Week 1	6.13	-1.65	13.92	0.1264	3.20	-3.54	9.94	0.3549	-5.94	-11.97	0.09	0.057
Week 2	4.50	-3.28	12.28	0.2605	1.90	-4.84	8.64	0.5822	8.08	2.05	14.11	0.0103
Week 4	1.37	-6.42	9.15	0.7316	8.90	2.16	15.64	0.0114	5.16	-0.87	11.19	0.0973
Week 6	2.03	-5.75	9.82	0.61	3.10	-3.64	9.84	0.3701	8.68	2.65	14.71	0.006
Week 8	3.00	-4.78	10.78	0.4522	9.70	2.96	16.44	0.006	8.74	2.71	14.77	0.0057
Week 12	-1.20	-8.98	6.58	0.7633	6.90	0.16	13.64	0.0482	7.68	1.65	13.71	0.0146
Week 16	4.40	-3.38	12.18	0.2712	3.85	-2.89	10.59	0.2663	3.08	-2.95	9.11	0.3197
Week 24	6.50	-1.28	14.28	0.1056	7.38	0.63	14.12	0.035	4.74	-1.29	10.77	0.1272
Week 48	2.97	-4.82	10.75	0.4572	5.13	-1.62	11.87	0.1401	6.62	0.59	12.65	0.0344
%CD8+PD-1+												
Week 1	-0.33	-15.98	15.31	0.9668	0.83	-12.72	14.37	0.9053	-2.76	-14.88	9.36	0.6565
Week 2	0.43	-15.21	16.08	0.9568	4.58	-8.97	18.12	0.51	6.18	-5.94	18.30	0.3205
Week 4	-13.07	-28.71	2.58	0.1055	1.18	-12.37	14.72	0.8655	-3.80	-15.92	8.32	0.5405
Week 6	-15.17	-30.81	0.48	0.061	6.73	-6.82	20.27	0.3335	-5.14	-17.26	6.98	0.4082
Week 8	-9.63	-25.28	6.01	0.231	0.45	-13.10	14.00	0.9483	-12.10	-24.22	0.02	0.0538
Week 12	3.00	-12.65	18.65	0.708	4.83	-8.72	18.37	0.4872	-6.22	-18.34	5.90	0.3174
Week 16	-7.83	-23.48	7.81	0.3293	3.18	-10.37	16.72	0.6473	-11.06	-23.18	1.06	0.0774
Week 24	-12.80	-28.45	2.85	0.1127	0.93	-12.62	14.47	0.8939	-15.26	-27.38	-3.14	0.0157
Week 48	-17.17	-32.81	-1.52	0.0345	-20.33	-33.87	-6.78	0.0043	-20.44	-32.56	-8.32	0.0014
%CD8+PD-L1												
Week 1	5.50	-8.84	19.84	0.4545	-2.23	-14.65	10.20	0.7264	0.20	-10.91	11.31	0.9719
Week 2	1.90	-12.44	16.24	0.7958	6.88	-5.55	19.30	0.2812	18.94	7.83	30.05	0.0013
Week 4	2.27	-12.08	16.61	0.7576	1.58	-10.85	14.00	0.8044	-3.24	-14.35	7.87	0.5692
Week 6	-2.63	-16.98	11.71	0.7199	-1.33	-13.75	11.10	0.8349	2.38	-8.73	13.49	0.6757
Week 8	2.83	-11.51	17.18	0.6996	-5.30	-17.72	7.12	0.4055	2.62	-8.49	13.73	0.6452
Week 12	-2.00	-16.34	12.34	0.7853	-0.83	-13.25	11.60	0.8967	-6.50	-17.61	4.61	0.2549
Week 16	-0.73	-15.08	13.61	0.9204	-4.15	-16.57	8.27	0.5144	-0.28	-11.39	10.83	0.9607
Week 24	0.33	-14.01	14.68	0.9638	1.68	-10.75	14.10	0.7922	-1.66	-12.77	9.45	0.7704
Week 48	1.33	-13.01	15.68	0.8559	-2.10	-14.52	10.32	0.7412	5.38	-5.73	16.49	0.3454
CD8+PD-1 MFI												
Week 1	-12	-138	114	0.8526	19	-90	129	0.7308	-25	-123	73	0.6176
Week 2	43	-83	169	0.5062	18	-92	127	0.7511	51	-46	149	0.3058
Week 4	-9	-136	117	0.8851	10	-99	120	0.8546	-80	-178	18	0.1135
Week 6	-25	-151	102	0.7027	93	-16	202	0.0992	-61	-159	36	0.2219
Week 8	-37	-163	90	0.5707	47	-62	157	0.3993	-66	-164	32	0.1908
Week 12	-49	-175	77	0.4489	77	-32	187	0.1698	-84	-182	14	0.096

Appendix Table 30 continued

Week 16	-3	-130	123	0.9588	48	-62	157	0.3944	8	-90	106	0.8761
Week 24	43	-83	170	0.5029	16	-93	125	0.7749	31	-66	129	0.5308
Week 48	36	-90	162	0.5777	126	17	235	0.0265	1	-97	98	0.9904
CD8+PD-L1 MFI												
Week 1	974	128	1819	0.0267	-427	-1160	305	0.2563	-8	-663	647	0.98
Week 2	-39	-884	807	0.9288	-79	-811	654	0.8336	535	-120	1190	0.1135
Week 4	-404	-1249	442	0.3523	70	-662	802	0.8519	-9	-664	646	0.9795
Week 6	-377	-1222	469	0.3853	108	-624	841	0.7728	-143	-798	512	0.6699
Week 8	-531	-1376	315	0.2223	-116	-848	616	0.757	-421	-1076	234	0.2119
Week 12	86	-760	932	0.8425	385	-348	1117	0.3066	-110	-765	545	0.7438
Week 16	442	-403	1288	0.3084	449	-283	1182	0.2328	351	-304	1006	0.2965
Week 24	382	-463	1228	0.3782	92	-640	824	0.8061	204	-451	859	0.5441
Week 48	282	-564	1127	0.5157	833	100	1565	0.0286	147	-508	802	0.6621

Appendix Table 31 Changes from baseline in CD25 and CD45RO expression on CD8 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+CD25+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.67	-5.50	6.83	0.8327	2.65	-2.69	7.99	0.3336	0.30	-4.48	5.08	0.9023
Week 2	2.27	-3.90	8.43	0.4732	2.18	-3.16	7.51	0.427	13.56	8.78	18.34	<.0001
Week 4	2.07	-4.10	8.23	0.513	3.80	-1.54	9.14	0.1669	10.94	6.16	15.72	<.0001
Week 6	2.40	-3.77	8.57	0.4477	2.55	-2.79	7.89	0.352	12.30	7.52	17.08	<.0001
Week 8	2.03	-4.13	8.20	0.5198	4.65	-0.69	9.99	0.0917	10.46	5.68	15.24	<.0001
Week 12	-0.03	-6.20	6.13	0.9916	3.60	-1.74	8.94	0.1901	11.28	6.50	16.06	<.0001
Week 16	2.60	-3.57	8.77	0.4109	4.13	-1.21	9.46	0.1339	9.20	4.42	13.98	0.0003
Week 24	1.30	-4.87	7.47	0.6805	2.05	-3.29	7.39	0.4539	5.28	0.50	10.06	0.0332
Week 48	2.73	-3.43	8.90	0.3875	1.98	-3.36	7.31	0.4706	7.86	3.08	12.64	0.0018
%CD8+CD45RO+												
Week 1	-0.10	-8.18	7.98	0.9807	2.18	-4.83	9.18	0.5443	1.76	-4.50	8.02	0.5832
Week 2	8.17	0.08	16.25	0.0511	-1.43	-8.43	5.58	0.691	11.36	5.10	17.62	0.0006
Week 4	4.57	-3.52	12.65	0.2715	3.53	-3.48	10.53	0.3266	4.06	-2.20	10.32	0.2074
Week 6	-1.00	-9.08	7.08	0.809	1.28	-5.73	8.28	0.722	3.96	-2.30	10.22	0.2187
Week 8	1.47	-6.62	9.55	0.7231	3.28	-3.73	10.28	0.3619	7.02	0.76	13.28	0.0308
Week 12	0.13	-7.95	8.22	0.9743	4.68	-2.33	11.68	0.1943	8.32	2.06	14.58	0.0109
Week 16	3.37	-4.72	11.45	0.4167	3.43	-3.58	10.43	0.3405	6.56	0.30	12.82	0.0433
Week 24	5.83	-2.25	13.92	0.1611	9.63	2.62	16.63	0.0086	10.50	4.24	16.76	0.0015
Week 48	5.77	-2.32	13.85	0.1659	5.03	-1.98	12.03	0.1633	10.72	4.46	16.98	0.0012
CD8+CD25 MFI												
Week 1	31	-185	247	0.7792	-119	-306	68	0.217	25	-143	192	0.7722
Week 2	392	176	608	0.0006	-88	-275	99	0.3579	523	355	690	<.0001
Week 4	165	-51	381	0.139	-177	-364	10	0.0669	181	13	348	0.0375
Week 6	-14	-230	202	0.8968	-162	-349	26	0.0945	33	-135	200	0.7036
Week 8	33	-183	249	0.7677	-195	-382	-8	0.044	-1	-168	167	0.9925
Week 12	-72	-288	144	0.5154	-154	-341	33	0.11	-7	-174	160	0.9349
Week 16	-62	-278	154	0.5732	-143	-330	44	0.1373	21	-146	188	0.8063
Week 24	-31	-247	185	0.7769	-167	-354	20	0.0835	-109	-276	59	0.2061
Week 48	-46	-262	170	0.6753	-198	-385	-10	0.0417	-102	-269	65	0.2356
CD8+CD45RO MFI												
Week 1	-358	-3763	3046	0.8371	-145	-3093	2804	0.9237	907	-1731	3544	0.5023
Week 2	1530	-1875	4934	0.3812	381	-2567	3330	0.8006	1992	-646	4629	0.1427
Week 4	-347	-3752	3058	0.8422	-450	-3399	2499	0.7656	-126	-2763	2511	0.9256
Week 6	-2720	-6125	685	0.1213	687	-2261	3636	0.649	-792	-3429	1846	0.5579
Week 8	-510	-3915	2895	0.7698	-527	-3475	2422	0.7273	737	-1900	3375	0.5853
Week 12	2196	-1209	5600	0.2099	-154	-3102	2795	0.9188	189	-2449	2826	0.8888
Week 16	1834	-1570	5239	0.2941	474	-2475	3422	0.7536	1892	-745	4529	0.1635
Week 24	1191	-2214	4595	0.495	356	-2593	3304	0.8137	4207	1570	6845	0.0025
Week 48	2652	-753	6057	0.1307	1148	-1800	4097	0.4475	2560	-77	5197	0.0607

Appendix Table 32 Changes from baseline in markers associated with recent thymic emigrants on CD4 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+CD45RA+ CD31+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	1.00	-2.27	4.27	0.55	1.03	-1.80	3.85	0.4795	0.28	-2.25	2.81	0.8288
Week 2	-6.30	-9.57	-3.03	0.0003	1.28	-1.55	4.10	0.3795	-5.92	-8.45	-3.39	<.0001
Week 4	-4.33	-7.60	-1.07	0.0111	-0.30	-3.13	2.53	0.8358	-3.70	-6.23	-1.17	0.0053
Week 6	-7.17	-10.43	-3.90	<.0001	-1.60	-4.43	1.23	0.2708	-2.14	-4.67	0.39	0.1011
Week 8	-4.90	-8.17	-1.63	0.0043	1.43	-1.40	4.25	0.3263	-3.84	-6.37	-1.31	0.0039
Week 12	-3.73	-7.00	-0.47	0.0278	0.48	-2.35	3.30	0.7429	-3.02	-5.55	-0.49	0.0217
Week 16	-4.73	-8.00	-1.47	0.0057	-0.60	-3.43	2.23	0.6786	-5.06	-7.59	-2.53	0.0002
Week 24	-6.40	-9.67	-3.13	0.0002	-1.78	-4.60	1.05	0.2222	-1.40	-3.93	1.13	0.2812
Week 48	-4.70	-7.97	-1.43	0.006	-1.40	-4.23	1.43	0.3348	-1.02	-3.55	1.51	0.4316
%CD4+CD31+												
Week 1	-0.27	-7.47	6.94	0.9424	3.10	-3.14	9.34	0.3332	-0.86	-6.44	4.72	0.7635
Week 2	-10.27	-17.47	-3.06	0.0065	3.98	-2.27	10.22	0.2155	-10.36	-15.94	-4.78	0.0005
Week 4	-1.33	-8.54	5.87	0.7178	-3.60	-9.84	2.64	0.2616	-6.12	-11.70	-0.54	0.0346
Week 6	-10.73	-17.94	-3.53	0.0045	-3.28	-9.52	2.97	0.3068	-8.10	-13.68	-2.52	0.0056
Week 8	-0.97	-8.17	6.24	0.7933	6.53	0.28	12.77	0.0437	-5.22	-10.80	0.36	0.0705
Week 12	-2.27	-9.47	4.94	0.5393	10.10	3.86	16.34	0.0021	-4.40	-9.98	1.18	0.1263
Week 16	-1.97	-9.17	5.24	0.5942	0.40	-5.84	6.64	0.9003	-11.10	-16.68	-5.52	0.0002
Week 24	-4.03	-11.24	3.17	0.2759	0.83	-5.42	7.07	0.7962	-3.54	-9.12	2.04	0.2175
Week 48	-7.60	-14.81	-0.39	0.0419	-1.30	-7.54	4.94	0.6842	-6.52	-12.10	-0.94	0.0247
%CD4+PTK-7+												
Week 1	-0.20	-0.47	0.07	0.1536	0.00	-0.24	0.24	1	-0.06	-0.27	0.15	0.5784
Week 2	-0.33	-0.61	-0.06	0.0186	0.05	-0.19	0.29	0.6786	-0.08	-0.29	0.13	0.4591
Week 4	-0.47	-0.74	-0.19	0.0012	0.08	-0.16	0.31	0.5345	-0.10	-0.31	0.11	0.3552
Week 6	-0.27	-0.54	0.01	0.0583	-0.03	-0.26	0.21	0.8358	-0.04	-0.25	0.17	0.7109
Week 8	-0.07	-0.34	0.21	0.6324	0.03	-0.21	0.26	0.8358	0.10	-0.11	0.31	0.3552
Week 12	-0.33	-0.61	-0.06	0.0186	0.03	-0.21	0.26	0.8358	0.00	-0.21	0.21	1
Week 16	-0.33	-0.61	-0.06	0.0186	-0.03	-0.26	0.21	0.8358	0.10	-0.11	0.31	0.3552
Week 24	-0.33	-0.61	-0.06	0.0186	0.10	-0.14	0.34	0.408	0.20	-0.01	0.41	0.0665
Week 48	-0.37	-0.64	-0.09	0.0099	-0.08	-0.31	0.16	0.5345	-0.04	-0.25	0.17	0.7109
CD4+CD31 MFI												
Week 1	204	-250	657	0.3812	-271	-663	122	0.1803	147	-205	498	0.4157
Week 2	572	119	1025	0.0155	-306	-698	87	0.1309	415	64	767	0.023
Week 4	200	-253	653	0.3898	-597	-989	-204	0.0038	-283	-634	69	0.1187
Week 6	62	-391	516	0.7883	-366	-758	27	0.0729	165	-187	516	0.361
Week 8	140	-313	594	0.5458	-364	-757	29	0.0729	-11	-363	340	0.9494
Week 12	119	-334	573	0.6074	-679	-1071	-286	0.0011	28	-323	379	0.8771
Week 16	70	-383	523	0.763	-103	-496	289	0.6077	74	-277	425	0.6799
Week 24	318	-135	771	0.173	-177	-570	215	0.3789	215	-136	567	0.2328
Week 48	-183	-636	271	0.4321	-532	-924	-139	0.0096	-105	-457	246	0.558
CD4+PTK-7 MFI												
Week 1	-5	-279	269	0.9715	93	-144	330	0.4442	114	-98	326	0.296
Week 2	-38	-312	235	0.7844	48	-189	285	0.6925	-8	-220	204	0.9398
Week 4	33	-241	307	0.8138	70	-167	307	0.563	5	-207	217	0.9662
Week 6	20	-253	294	0.8846	-50	-287	188	0.6835	17	-195	229	0.8755
Week 8	-118	-392	155	0.3994	142	-96	379	0.2455	167	-45	379	0.1266
Week 12	-184	-458	89	0.1906	6	-231	243	0.9606	-91	-303	121	0.4017
Week 16	-150	-424	124	0.2861	116	-122	353	0.3425	-65	-277	147	0.5497
Week 24	-238	-511	36	0.0927	-114	-351	123	0.3477	56	-156	268	0.6062
Week 48	-357	-631	-83	0.0125	-151	-388	87	0.217	-327	-539	-115	0.0034

Appendix Table 33 Changes from baseline in markers associated with recent thymic emigrants on CD8 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD8+CD31+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	-0.57	-6.60	5.46	0.8543	3.65	-1.57	8.87	0.1745	-0.90	-5.57	3.77	0.7067
Week 2	1.77	-4.26	7.80	0.5674	3.80	-1.42	9.02	0.1576	10.76	6.09	15.43	<.0001
Week 4	-4.23	-10.26	1.80	0.1726	1.55	-3.67	6.77	0.5623	-0.22	-4.89	4.45	0.9267
Week 6	-7.70	-13.73	-1.67	0.0143	1.05	-4.17	6.27	0.6945	-2.30	-6.97	2.37	0.3373
Week 8	-6.07	-12.10	-0.04	0.052	4.90	-0.32	10.12	0.0696	-1.56	-6.23	3.11	0.5146
Week 12	-6.17	-12.20	-0.14	0.0484	3.68	-1.55	8.90	0.1716	-4.28	-8.95	0.39	0.0762
Week 16	-1.87	-7.90	4.16	0.5457	0.90	-4.32	6.12	0.7364	-5.08	-9.75	-0.41	0.0361
Week 24	-4.60	-10.63	1.43	0.1387	2.25	-2.97	7.47	0.4009	-3.82	-8.49	0.85	0.1128
Week 48	-4.80	-10.83	1.23	0.1226	-2.58	-7.80	2.65	0.3367	-2.76	-7.43	1.91	0.2502
%CD8+PTK-7+												
Week 1	0.43	-0.60	1.47	0.4147	0.28	-0.62	1.17	0.5497	0.74	-0.06	1.54	0.0744
Week 2	1.13	0.10	2.17	0.035	0.38	-0.52	1.27	0.4151	0.58	-0.22	1.38	0.1604
Week 4	-0.23	-1.27	0.80	0.6601	0.40	-0.50	1.30	0.3848	0.16	-0.64	0.96	0.697
Week 6	1.80	0.76	2.84	0.001	1.05	0.15	1.95	0.0244	0.16	-0.64	0.96	0.697
Week 8	0.57	-0.47	1.60	0.2869	1.05	0.15	1.95	0.0244	0.04	-0.76	0.84	0.9224
Week 12	-0.23	-1.27	0.80	0.6601	0.30	-0.60	1.20	0.5141	-0.06	-0.86	0.74	0.8839
Week 16	0.10	-0.94	1.14	0.8504	0.23	-0.67	1.12	0.6244	0.32	-0.48	1.12	0.4368
Week 24	-0.53	-1.57	0.50	0.316	0.23	-0.67	1.12	0.6244	0.02	-0.78	0.82	0.9612
Week 48	-0.47	-1.50	0.57	0.3799	0.05	-0.85	0.95	0.9133	0.04	-0.76	0.84	0.9224
CD8+CD31 MFI	543	-379	1464	0.2517	303	-495	1101	0.4592	489	-224	1203	0.1827
Week 1	1476	554	2397	0.0024	-170	-968	628	0.6769	2054	1340	2768	<.0001
Week 2	266	-656	1187	0.5735	-485	-1282	313	0.2375	-303	-1016	411	0.4081
Week 4	25	-896	946	0.9577	-330	-1128	468	0.42	263	-451	977	0.4722
Week 6	62	-859	983	0.8954	-348	-1146	450	0.3952	-259	-972	455	0.4796
Week 8	-114	-1035	807	0.809	-615	-1413	183	0.1348	-211	-924	503	0.5646
Week 12	-46	-967	875	0.9223	54	-744	852	0.8948	630	-84	1343	0.0876
Week 16	285	-636	1206	0.546	-81	-878	717	0.8437	-60	-773	654	0.8704
Week 24	-307	-1228	614	0.5155	-319	-1116	479	0.4363	-312	-1025	402	0.3943
Week 48												
CD8+PTK-7 MFI												
Week 1	-423	-2278	1432	0.9715	-869	-2475	738	0.4442	-73	-1510	1364	0.296
Week 2	16	-1839	1871	0.7844	-903	-2509	704	0.6925	3450	2013	4887	0.9398
Week 4	-248	-2103	1607	0.8138	-1104	-2711	503	0.563	88	-1349	1525	0.9662
Week 6	272	-1583	2127	0.8846	-1239	-2845	368	0.6835	78	-1359	1515	0.8755
Week 8	-336	-2191	1519	0.3994	301	-1306	1908	0.2455	1698	261	3135	0.1266
Week 12	-593	-2448	1262	0.1906	-1246	-2853	360	0.9606	-372	-1809	1065	0.4017
Week 16	-525	-2380	1330	0.2861	-1067	-2674	540	0.3425	-147	-1584	1290	0.5497
Week 24	-450	-2305	1405	0.0927	-1331	-2937	276	0.3477	-88	-1525	1349	0.6062
Week 48	-593	-2448	1262	0.0125	-1250	-2856	357	0.217	-552	-1989	885	0.0034

Appendix Table 34 Changes from baseline in expression of the negative inhibitory marker CTLA-4 and the exhaustion marker TIM-3, on CD4 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

	Group 1				Group 2				Group 3			
%CD4+ TIM-3+CTLA-4+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.03	-0.03	0.09	0.2895	0.00	-0.05	0.05	1	0.00	-0.05	0.05	1
Week 2	0.07	0.01	0.13	0.036	-0.03	-0.08	0.03	0.3585	0.00	-0.05	0.05	1
Week 4	0.07	0.01	0.13	0.036	-0.03	-0.08	0.03	0.3585	0.02	-0.03	0.07	0.4113
Week 6	0.07	0.01	0.13	0.036	-0.05	-0.10	0.00	0.0684	-0.02	-0.07	0.03	0.4113
Week 8	0.03	-0.03	0.09	0.2895	-0.10	-0.15	-0.05	0.0004	-0.04	-0.09	0.01	0.1025
Week 12	0.03	-0.03	0.09	0.2895	-0.05	-0.10	0.00	0.0684	-0.04	-0.09	0.01	0.1025
Week 16	0.03	-0.03	0.09	0.2895	-0.08	-0.13	-0.02	0.007	0.00	-0.05	0.05	0.9985
Week 24	0.03	-0.03	0.09	0.2895	-0.08	-0.13	-0.02	0.007	-0.04	-0.09	0.01	0.1025
Week 48	0.00	-0.06	0.06	1	-0.05	-0.10	0.00	0.0684	-0.06	-0.11	-0.01	0.0153
%CD4+TIM-3+												
Week 1	0.07	-0.67	0.81	0.8605	0.33	-0.32	0.97	0.3239	0.12	-0.45	0.69	0.6831
Week 2	1.07	0.33	1.81	0.006	0.20	-0.44	0.84	0.5431	1.40	0.83	1.97	<.0001
Week 4	-0.13	-0.87	0.61	0.7253	0.08	-0.57	0.72	0.8194	-0.22	-0.79	0.35	0.4548
Week 6	0.07	-0.67	0.81	0.8605	-0.13	-0.77	0.52	0.7037	-0.24	-0.81	0.33	0.415
Week 8	-0.07	-0.81	0.67	0.8605	-0.20	-0.84	0.44	0.5431	0.02	-0.55	0.59	0.9457
Week 12	0.17	-0.57	0.91	0.6605	1.05	0.41	1.69	0.0019	-0.02	-0.59	0.55	0.9457
Week 16	0.10	-0.64	0.84	0.7921	0.28	-0.37	0.92	0.4035	0.18	-0.43	0.79	0.5622
Week 24	-0.23	-0.97	0.51	0.5389	0.28	-0.37	0.92	0.4035	-0.16	-0.73	0.41	0.5864
Week 48	0.30	-0.44	1.04	0.4299	-0.05	-0.69	0.59	0.879	-0.22	-0.79	0.35	0.4548
%CD4+CTLA-4+												
Week 1	0.07	-0.19	0.32	0.6071	-0.03	-0.24	0.19	0.8237	0.16	-0.04	0.36	0.1136
Week 2	0.13	-0.12	0.39	0.3049	-0.05	-0.27	0.17	0.656	0.04	-0.16	0.24	0.6903
Week 4	0.10	-0.15	0.35	0.441	0.05	-0.17	0.27	0.656	0.04	-0.16	0.24	0.6903
Week 6	0.20	-0.05	0.45	0.1254	0.25	0.03	0.47	0.0282	0.04	-0.16	0.24	0.6903
Week 8	0.13	-0.12	0.39	0.3049	-0.03	-0.24	0.19	0.8237	-0.02	-0.22	0.18	0.842
Week 12	0.23	-0.02	0.49	0.0745	0.28	0.06	0.49	0.0161	0.06	-0.14	0.26	0.5503
Week 16	0.23	-0.02	0.49	0.0745	-0.10	-0.32	0.12	0.3739	0.18	-0.02	0.39	0.0871
Week 24	0.13	-0.12	0.39	0.3049	-0.10	-0.32	0.12	0.3739	0.10	-0.10	0.30	0.3204
Week 48	0.10	-0.15	0.35	0.441	0.08	-0.14	0.29	0.5044	-0.06	-0.26	0.14	0.5503
CD4+TIM-3 MFI												
Week 1	146	-449	741	0.6326	-264	-779	251	0.3177	186	-275	646	0.4322
Week 2	317	-278	912	0.2989	-206	-721	309	0.4349	360	-101	820	0.1301
Week 4	263	-332	858	0.3888	-252	-767	263	0.3406	188	-273	648	0.4272
Week 6	127	-468	722	0.6767	-531	-1046	-15	0.0469	-64	-524	397	0.7874
Week 8	-111	-706	484	0.7163	-596	-1111	-81	0.0261	-183	-644	277	0.4376
Week 12	-150	-745	445	0.6217	-897	-1412	-382	0.001	-272	-733	189	0.2504
Week 16	-152	-747	443	0.6179	-785	-1300	-269	0.0038	-221	-713	270	0.3804
Week 24	-37	-632	558	0.9033	-753	-1268	-238	0.0053	-41	-502	420	0.862
Week 48	-458	-1053	137	0.1352	-812	-1327	-297	0.0028	-396	-857	64	0.0957
CD4+CTLA-4 MFI												
Week 1	-13	-252	225	0.913	103	-103	310	0.3299	61	-124	245	0.5205
Week 2	-117	-355	122	0.3403	-79	-285	128	0.4569	-72	-257	113	0.447
Week 4	-76	-314	163	0.5356	21	-186	227	0.8462	37	-148	222	0.6955
Week 6	-176	-414	63	0.1525	-17	-223	190	0.8759	-45	-230	139	0.6327
Week 8	-184	-422	54	0.1342	-44	-250	163	0.6807	-67	-252	117	0.4777
Week 12	-307	-545	-68	0.0137	-229	-435	-22	0.033	-189	-374	-4	0.0482
Week 16	-265	-504	-27	0.0321	94	-113	300	0.3761	-127	-324	70	0.2097
Week 24	-247	-485	-9	0.0456	-187	-393	20	0.0804	-121	-306	63	0.202
Week 48	-431	-669	-193	0.0007	-238	-444	-32	0.0266	-295	-480	-110	0.0024

Appendix Table 35 Changes from baseline in expression of the negative inhibitory marker CTLA-4 and the exhaustion marker TIM-3, on CD8 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

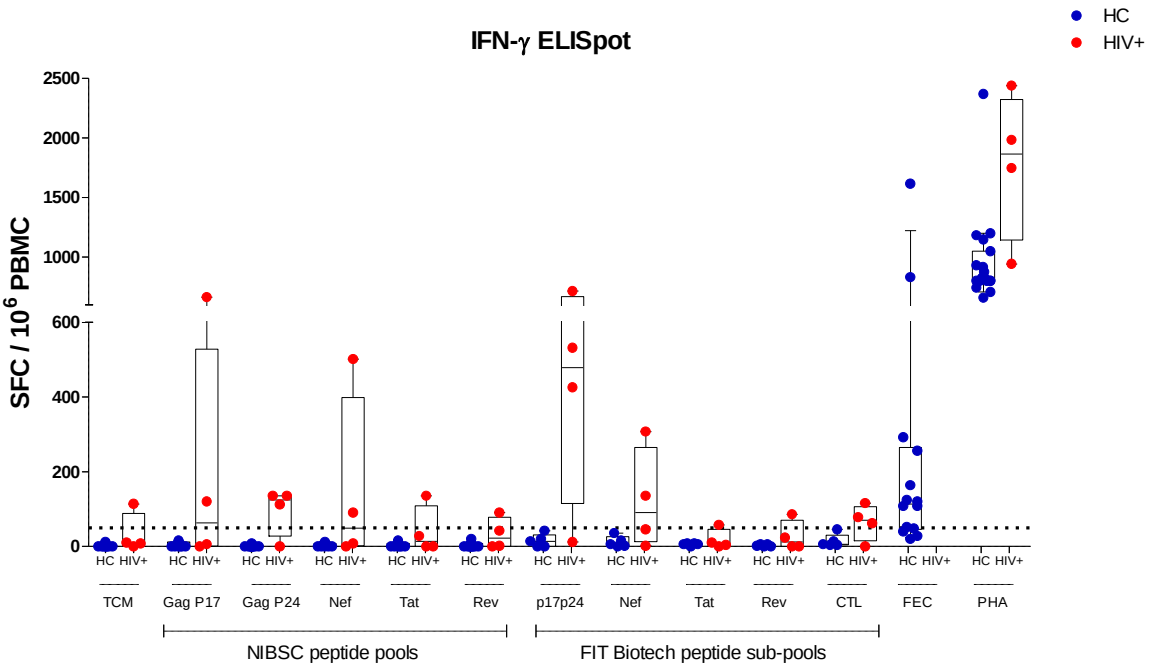
	Group 1				Group 2				Group 3			
%CD8+ TIM-3+CTLA-4+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.00	-0.08	0.08	1	0.03	-0.04	0.09	0.4778	0.02	-0.04	0.08	0.5254
Week 2	-0.03	-0.11	0.05	0.4127	0.00	-0.07	0.07	1	-0.02	-0.08	0.04	0.5254
Week 4	-0.07	-0.15	0.01	0.1035	0.05	-0.02	0.12	0.1577	-0.04	-0.10	0.02	0.2057
Week 6	0.00	-0.08	0.08	1	0.00	-0.07	0.07	1	-0.02	-0.08	0.04	0.5254
Week 8	-0.07	-0.15	0.01	0.1035	-0.03	-0.09	0.04	0.4778	-0.04	-0.10	0.02	0.2057
Week 12	0.00	-0.08	0.08	1	0.00	-0.07	0.07	1	0.00	-0.06	0.06	1
Week 16	-0.03	-0.11	0.05	0.4127	0.00	-0.07	0.07	1	0.00	-0.07	0.06	0.9485
Week 24	-0.07	-0.15	0.01	0.1035	0.03	-0.04	0.09	0.4778	-0.02	-0.08	0.04	0.5254
Week 48	-0.13	-0.21	-0.05	0.0015	-0.03	-0.09	0.04	0.4778	-0.08	-0.14	-0.02	0.0126
%CD8+TIM-3+												
Week 1	1.27	0.29	2.24	0.0127	0.35	-0.49	1.19	0.4186	-0.32	-1.07	0.43	0.4084
Week 2	2.23	1.26	3.21	<.0001	0.33	-0.52	1.17	0.4524	0.56	-0.19	1.31	0.1497
Week 4	0.10	-0.87	1.07	0.8411	0.28	-0.57	1.12	0.5247	-0.62	-1.37	0.13	0.1113
Week 6	0.03	-0.94	1.01	0.9467	0.10	-0.74	0.94	0.8169	-0.60	-1.35	0.15	0.1231
Week 8	-0.37	-1.34	0.61	0.4629	1.10	0.26	1.94	0.0125	0.20	-0.55	0.95	0.6049
Week 12	0.03	-0.94	1.01	0.9467	1.23	0.38	2.07	0.0056	-0.10	-0.85	0.65	0.7957
Week 16	0.27	-0.71	1.24	0.5931	0.65	-0.19	1.49	0.135	0.11	-0.70	0.91	0.7982
Week 24	0.40	-0.57	1.37	0.4233	0.45	-0.39	1.29	0.299	-0.44	-1.19	0.31	0.2565
Week 48	0.50	-0.47	1.47	0.3175	0.50	-0.34	1.34	0.2489	-0.74	-1.49	0.01	0.0582
%CD8+CTLA-4+												
Week 1	0.63	-0.28	1.55	0.1791	0.13	-0.67	0.92	0.7582	0.06	-0.65	0.77	0.8687
Week 2	0.53	-0.38	1.45	0.2571	0.08	-0.72	0.87	0.8534	-0.88	-1.59	-0.17	0.0173
Week 4	-0.07	-0.98	0.85	0.8869	-0.23	-1.02	0.57	0.5797	-0.80	-1.51	-0.09	0.0299
Week 6	0.07	-0.85	0.98	0.8869	0.15	-0.64	0.94	0.7118	-0.96	-1.67	-0.25	0.0096
Week 8	0.03	-0.88	0.95	0.9433	0.18	-0.62	0.97	0.6665	-0.76	-1.47	-0.05	0.0389
Week 12	0.27	-0.65	1.18	0.5698	-0.10	-0.89	0.69	0.8054	-0.86	-1.57	-0.15	0.0199
Week 16	0.07	-0.85	0.98	0.8869	-0.33	-1.12	0.47	0.4242	-0.87	-1.63	-0.12	0.0261
Week 24	0.03	-0.88	0.95	0.9433	-0.38	-1.17	0.42	0.3568	-0.88	-1.59	-0.17	0.0173
Week 48	-0.17	-1.08	0.75	0.7222	-0.28	-1.07	0.52	0.4987	-1.02	-1.73	-0.31	0.0061
CD8+TIM-3 MFI												
Week 1	-167	-684	350	0.5275	-76	-524	371	0.7393	214	-186	614	0.298
Week 2	2	-515	519	0.995	-164	-612	283	0.4741	399	-1	800	0.0542
Week 4	188	-329	705	0.4788	-319	-766	129	0.167	164	-236	565	0.4239
Week 6	39	-478	556	0.8838	-499	-946	-51	0.0319	-23	-423	378	0.9114
Week 8	-15	-532	502	0.9538	-674	-1122	-226	0.0042	-149	-549	252	0.4691
Week 12	-79	-596	438	0.7643	-788	-1236	-341	0.0009	-202	-603	198	0.3252
Week 16	-220	-737	297	0.4073	-683	-1130	-235	0.0037	-175	-602	252	0.4246
Week 24	-228	-745	289	0.3898	-600	-1048	-153	0.0103	-48	-448	353	0.8156
Week 48	-485	-1002	32	0.0698	-769	-1216	-321	0.0012	-339	-740	61	0.1007
CD8+CTLA-4 MFI												
Week 1	-18	-2050	2014	0.9859	-298	-2058	1462	0.7411	-38	-1612	1536	0.9628
Week 2	252	-1780	2284	0.8083	-390	-2149	1370	0.6656	3546	1972	5120	<.0001
Week 4	249	-1783	2281	0.8111	-997	-2906	913	0.3095	-8	-1582	1566	0.9919
Week 6	84	-1948	2116	0.9354	-494	-2254	1266	0.5839	-196	-1770	1378	0.8076
Week 8	524	-1508	2556	0.6149	622	-1138	2381	0.4908	1396	-178	2970	0.0861
Week 12	-91	-2123	1941	0.9303	-935	-2695	825	0.301	-373	-1947	1201	0.6436
Week 16	-66	-2098	1966	0.9497	-976	-2735	784	0.2806	-422	-2098	1253	0.6226
Week 24	-95	-2127	1937	0.9272	-1036	-2795	724	0.2523	-338	-1912	1236	0.6752
Week 48	-183	-2215	1849	0.8603	-1107	-2867	653	0.2212	-588	-2162	986	0.4661

Appendix Table 36 Changes from baseline in CD69 expression on CD4 and CD8 T cells.
Mean change (mean Δ) from baseline to each of the study time points is shown, with lower and upper confidence intervals (CI) and the corresponding p value. P values in red are significant (where $p \leq 0.05$) and p values in blue are approaching significance ($0.05 < p \leq 0.1$).

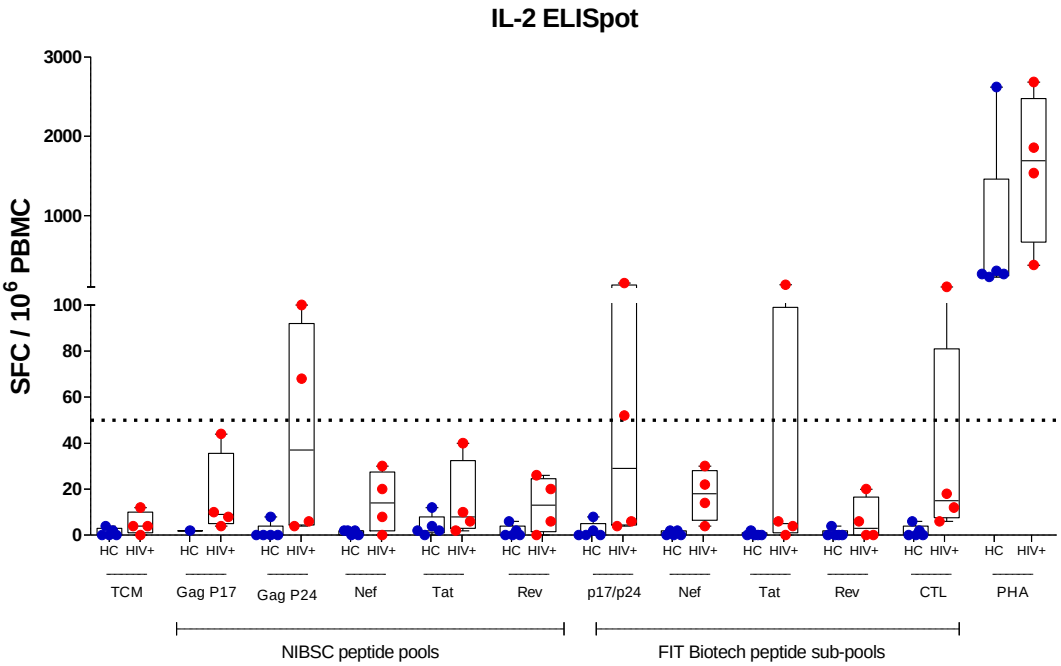
	Group 1				Group 2				Group 3			
%CD4+CD69+	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value	Mean Δ	Lower CI	Upper CI	P value
Week 1	0.20	-0.62	1.02	0.6339	0.00	-0.71	0.71	1	0.18	-0.46	0.82	0.5802
Week 2	0.03	-0.79	0.85	0.9367	0.18	-0.54	0.89	0.6304	-0.04	-0.68	0.60	0.9021
Week 4	0.07	-0.75	0.89	0.8738	-0.13	-0.84	0.59	0.731	-0.52	-1.16	0.12	0.1125
Week 6	0.27	-0.55	1.09	0.5257	0.08	-0.64	0.79	0.8365	-0.78	-1.42	-0.14	0.0184
Week 8	-0.07	-0.89	0.75	0.8738	0.50	-0.21	1.21	0.1714	-0.42	-1.06	0.22	0.1987
Week 12	-0.13	-0.95	0.69	0.7508	-0.13	-0.84	0.59	0.731	-0.64	-1.28	0.00	0.0517
Week 16	-0.30	-1.12	0.52	0.4754	0.28	-0.44	0.99	0.4501	-0.32	-1.00	0.36	0.3596
Week 24	-0.27	-1.09	0.55	0.5257	0.23	-0.49	0.94	0.5364	-0.36	-1.00	0.28	0.27
Week 48	-0.17	-0.99	0.65	0.6914	0.23	-0.49	0.94	0.5364	-0.28	-0.92	0.36	0.3902
%CD8+CD69+												
Week 1	2.33	0.39	4.27	0.0208	0.80	-0.88	2.48	0.3533	0.56	-0.94	2.06	0.4671
Week 2	-0.07	-2.01	1.87	0.9464	0.35	-1.33	2.03	0.684	-0.74	-2.24	0.76	0.3372
Week 4	0.37	-1.57	2.31	0.7119	0.88	-0.80	2.55	0.3102	-0.12	-1.62	1.38	0.876
Week 6	0.50	-1.44	2.44	0.6147	0.48	-1.20	2.15	0.5809	-0.88	-2.38	0.62	0.2543
Week 8	-0.83	-2.77	1.11	0.4022	1.08	-0.60	2.75	0.2133	-0.90	-2.40	0.60	0.2437
Week 12	-0.07	-2.01	1.87	0.9464	-0.93	-2.60	0.75	0.2836	-1.10	-2.60	0.40	0.1551
Week 16	-0.50	-2.44	1.44	0.6147	0.23	-1.45	1.90	0.7935	-1.46	-3.06	0.14	0.0781
Week 24	-0.63	-2.57	1.31	0.5239	-1.55	-3.23	0.13	0.0742	-1.00	-2.50	0.50	0.1957
Week 48	-0.87	-2.81	1.07	0.3837	-1.43	-3.10	0.25	0.1002	-1.16	-2.66	0.34	0.1341
CD4+CD69 MFI												
Week 1	-93	-2515	2329	0.9402	-317	-2415	1780	0.7676	1483	-393	3359	0.1252
Week 2	764	-1658	3185	0.5383	-40	-2137	2057	0.9703	1525	-351	3401	0.115
Week 4	365	-2056	2787	0.7682	-256	-2353	1842	0.8117	499	-1377	2374	0.6038
Week 6	66	-2356	2488	0.9575	-614	-2711	1484	0.5679	-49	-1924	1827	0.9596
Week 8	494	-1927	2916	0.6902	-580	-2678	1517	0.5891	687	-1189	2563	0.4748
Week 12	1961	-461	4383	0.1164	263	-1835	2360	0.8067	333	-1542	2209	0.7285
Week 16	3062	641	5484	0.0153	191	-1906	2289	0.8586	4833	2833	6832	<.0001
Week 24	2603	182	5025	0.0383	-139	-2236	1959	0.8973	2785	909	4661	0.0047
Week 48	3069	648	5491	0.0151	1952	-146	4049	0.0719	1329	-547	3204	0.1689
CD8+CD69 MFI												
Week 1	-300	-3666	3065	0.8616	-1533	-4448	1382	0.3057	993	-1614	3600	0.4576
Week 2	1028	-2337	4394	0.551	-313	-3228	2602	0.8338	3644	1037	6251	0.0076
Week 4	165	-3201	3530	0.9238	-1596	-4510	1319	0.2866	-738	-3345	1869	0.5808
Week 6	-171	-3537	3195	0.9209	-2230	-5145	685	0.1376	-684	-3291	1923	0.6083
Week 8	1402	-1964	4768	0.4167	-1388	-4302	1527	0.3536	530	-2077	3137	0.6911
Week 12	1403	-1963	4768	0.4164	901	-2014	3816	0.5464	50	-2557	2657	0.9703
Week 16	3779	413	7144	0.0307	398	-2517	3313	0.7895	6678	3897	9458	<.0001
Week 24	3550	184	6915	0.042	2764	-151	5679	0.0667	3670	1063	6277	0.0072
Week 48	3208	-158	6574	0.0654	6478	3563	9392	<.0001	2281	-326	4888	0.0902

Appendix 3 Preparation and validation of procedures and materials for the IMIRC 1003 clinical trial – testing of peptides for ELISpot assays

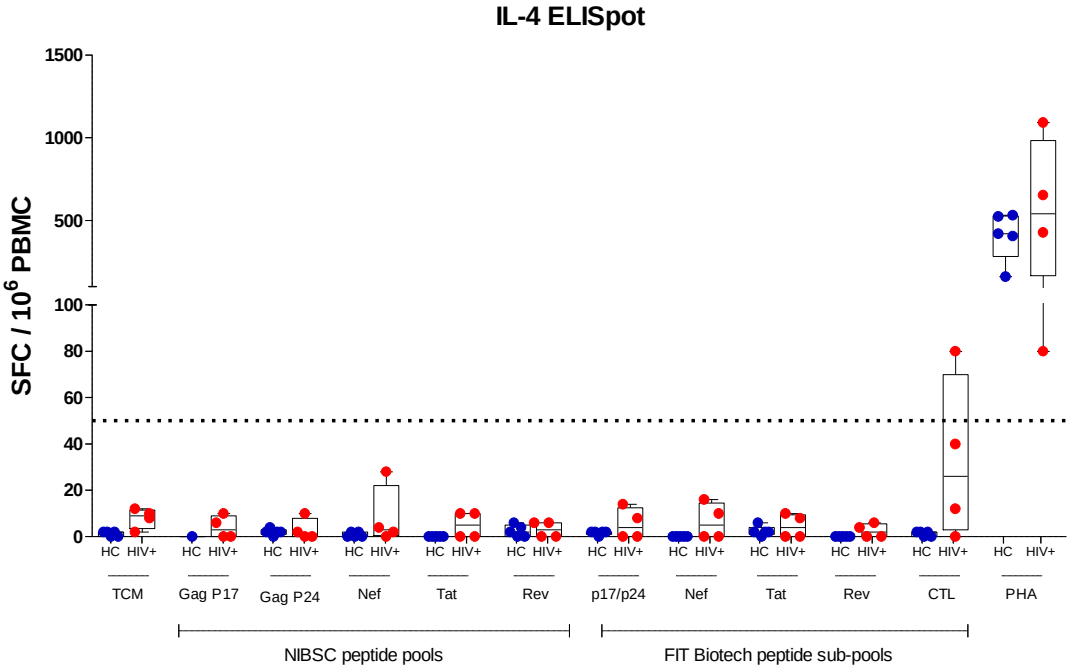
The preparation of peptide antigens from NIBSC and FIT Biotech was carried out as described in the Materials & Methods section 2.16.3. The pooled peptides were run in ELISpot assays (detailed in section 2.17) on PBMC from both seronegative donors and from HIV-1-infected individuals.



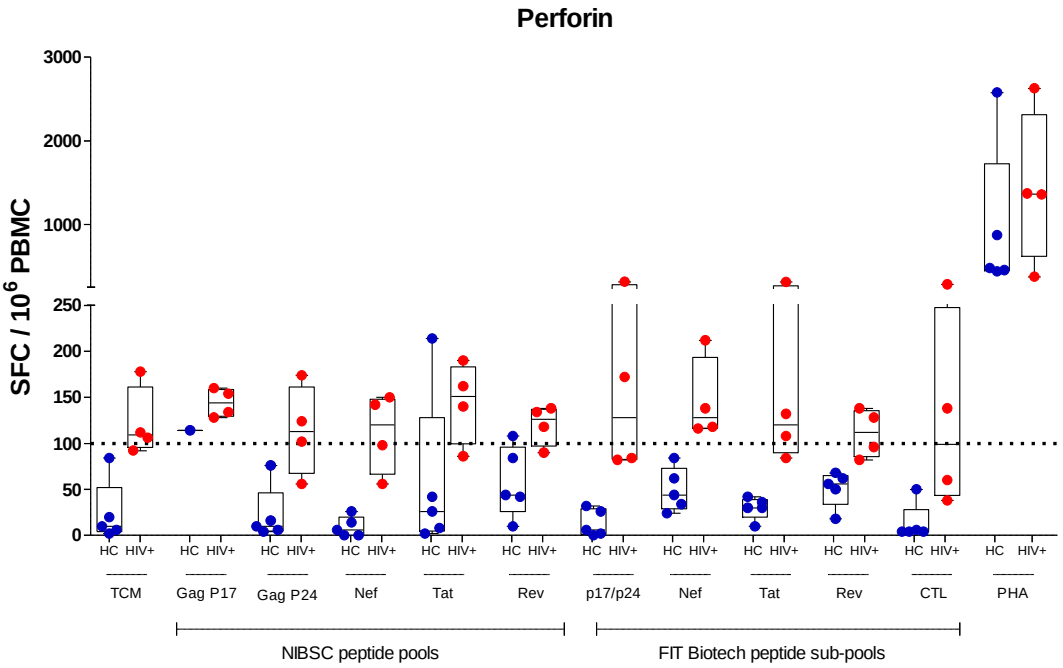
Appendix Figure 1 IFN-γ production in response to HIV-1 peptides in HIV-1^+ persons and healthy donors.
IFN-γ response to overlapping peptide pools of Gag p17, Gag p24, Nef, Tat, Rev (from NIBSC) and sub-pools of Gag p17/p24, Nef, Tat, Rev and CTL, in addition to FEC (healthy donors only), PHA and TCM (negative control). HIV-1^+ individuals are represented with red circles (●) and healthy controls with blue circles (●). Data represent mean values of duplicate wells with <10% variation among duplicates. The threshold of 50 SFC/10^6 PBMC is marked with a dotted line. Box plots show the median and IQR while whiskers represent the 10^{th} and 90^{th} percentiles.



Appendix Figure 2 IL-2 production in response to HIV-1 peptides in HIV-1^+ persons and healthy donors.
IL-2 response to overlapping peptide pools of Gag p17, Gag p24, Nef, Tat, Rev (from NIBSC) and sub-pools of Gag p17/p24, Nef, Tat, Rev and CTL, in addition to FEC (healthy donors only), PHA and TCM (negative control). HIV-1^+ individuals are represented with red circles (●) and healthy controls with blue circles (●). Data represent mean values of duplicate wells with <10% variation among duplicates. The threshold of 50 SFC/10^6 PBMC is marked with a dotted line. Box plots show the median and IQR while whiskers represent the 10^{th} and 90^{th} percentiles.



Appendix Figure 3 IL-4 production in response to HIV-1 peptides in HIV-1^+ persons and healthy donors.
IL-4 response to overlapping peptide pools of Gag p17, Gag p24, Nef, Tat, Rev (from NIBSC) and sub-pools of Gag p17/p24, Nef, Tat, Rev and CTL, in addition to FEC (healthy donors only), PHA and TCM (negative control). HIV-1^+ individuals are represented with red circles (●) and healthy controls with blue circles (●). Data represent mean values of duplicate wells with <10% variation among duplicates. The threshold of 50 SFC/10^6 PBMC is marked with a dotted line. Box plots show the median and IQR while whiskers represent the 10^{th} and 90^{th} percentiles.



Appendix Figure 4 Perforin production in response to HIV-1 peptides in HIV-1^+ persons and healthy donors.
Perforin response to overlapping peptide pools of Gag p17, Gag p24, Nef, Tat, Rev (from NIBSC) and sub-pools of Gag p17/p24, Nef, Tat, Rev and CTL, in addition to FEC (healthy donors only), PHA and TCM (negative control). HIV-1^+ individuals are represented with red circles (●) and healthy controls with blue circles (●). Data represent mean values of duplicate wells with <10% variation among duplicates. The threshold of 50 SFC/10^6 PBMC is marked with a dotted line. Box plots show the median and IQR while whiskers represent the 10^{th} and 90^{th} percentiles.

Appendix 4 Adverse events for IMIRC 1003 clinical trial

The adverse events (AE) reported for patients enrolled on the IMIRC 1003 clinical trial are summarised here. Appendix Table 37 gives an overview of the adverse events per treatment group, defined by grade and the likelihood of their relation to the study drugs. Appendix Table 38 gives a more detailed outline of the types of adverse events reported for patients in each of the three treatment groups. All patients, apart from one (P054, in group 3), reported at least one AE during the course of the study, however there was only one serious adverse event (SAE), and this was for patient C241 (who had been randomised to group 3). This individual received one dose of rhGH and was admitted to the hospital with headaches. The subject received no further rhGH but remained in the study on an observational basis.

Appendix Table 37 Adverse events for the IMIRC 1003 clinical trial.

Adverse Events	Group 1 (n = 3) Vaccine + IL-2/GM-CSF + rhGH	Group 2 (n = 4) Vaccine	Group 3 (n = 5) IL-2/GM-CSF + rhGH
Total AEs	31	24	64
Patients reporting no AEs	0	0	0
SAE	0	0	1
Grade 3	0	0	5
Definitely	0	0	1
Probably	0	0	0
Possibly	0	0	2
Unlikely/not related	0	0	2
Grade 2 and 1	30	22	53
Definitely	9	0	13
Probably	15	0	9
Possibly	2	3	18
Unlikely/not related	3	19	13
Ungraded	1	2	6

Appendix Table 38 Adverse events in detail for the IMIRC 1003 trial.

AE reported	Group 1 (n = 3) Vaccine + IL-2/GM-CSF + rhGH	Group 2 (n = 4) Vaccine	Group 3 (n = 5) IL-2/GM-CSF + rhGH
Total Grade 3 AE definitely, probably or possibly attributed to study	0	0	3
Fatigue	0	0	1
Headache	0	0	1
Transaminitis	0	0	1
Total Grade 2 and 1 definitely, probably or possibly attributed to study	27	3	39
Erythema at injection site; lumps at injection site; blisters	5	0	5
Generalised erythamous rash; rash	1	0	3
Itching	2	0	3
Nausea; vomitting	1	0	4
Diarrhoea; abdominal cramps; loose stools; blood in stool	2	0	5
Muscle pains; joint aches	2	0	1
General malaise; fatigue; lethargy; loss of appetite; fevers	3	1	4
Headache	1	0	0
Dizziness	0	0	1
Sore throat; nasal congestion; congestion; earache	4	0	4
Dry mouth; dry skin; dry lips	3	0	3
Rigors	2	0	2
Insomnia	1	0	1
Myalgia right and left arm	0	1	0
Blurred vision	0	1	0
Hiccups	0	0	1
Swelling of ankles	0	0	1
Photophobia	0	0	1